Matjaž Vogelsang *Editor*

DNA Alterations in Lynch Syndrome

Advances in molecular diagnosis and genetic counselling

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Preface

In 1913 Aldred Scott Warthin, a pathologist at the University of Michigan, described a family with distinct susceptibility to colon, gastric and uterine cancers, and referred to it as "cancer fraternity". Today, one hundred years later, this same cancer family syndrome which is associated with greatly increased risks for developing colorectal cancer and endometrial cancer, is recognized as Lynch syndrome (LS), acknowledging the role of Henry T. Lynch who described the cardinal features of this predisposition. Our understanding and molecular diagnosis of LS has rapidly improved in the early 1990s, following the elucidation of the molecular mechanisms which cause the syndrome. Colorectal cancer is currently the fourth most frequent malignant disease. The estimated annual worldwide incidence of colorectal cancer (CRC) is 1,235,108, resulting in an estimated 609,051 deaths each year and about 3 % of all diagnosed colorectal cancer cases are attributed to Lynch syndrome (~37,000 cases per year) [1]. These statistics are important, knowing that identified at-risk relatives of each LS case could benefit from genetic counseling, DNA testing, surveillance and targeted management, possibly resulting in reduced CRC incidence and related mortality [2]. However, according to Lynch et al. (see Chap. 1) a great deal of work still needs to be done in order to realize the potential of translation of LS knowledge to clinical use.

One of the important remaining challenges is the ability of the medical community to recognize Lynch syndrome. The first step towards recognizing any hereditary syndrome, including Lynch syndrome, is the acquisition of a patient's family history. Therefore, acquiring a nuclear pedigree of cancer patients has to be an essential part of clinical evaluation in order to identify Lynch syndrome families [3].

Moreover, current data shows that most LS patients and their relatives lack the relevant knowledge about LS and cancer risk, which results in low participation rate for predictive genetic screening. Close communication between physicians and LS families, with the assistance of genetic counselors, is required to provide information to patients and family members and help improve the participation rate [4].

The book in front of you assembled a panel of leading experts to critically review current advances and discuss future improvements in the multidisciplinary field of Lynch syndrome. This book aims to provide an important basis for the medical and

scientific community, in order to improve current understanding and clinical awareness of this frequently occurring predisposition. Chapter 1 presents a comprehensive history of LS. Molecular aspects of mismatch repair (MMR) genes and advances in the field are reviewed in Chap. 2. In Chaps. 3 and 4, current trends in Lynch syndrome diagnosis and clinical management of patients are revised. Chapter 5 explains the importance of functional studies in the case of unclassified variants being identified in cancer patients. In Chap. 6, the role of epigenetic-based dysregulation of MMR genes in LS-related cancers is discussed, whereas Chap. 7 provides a review of mutations in non-MMR genes that modify or mimic the Lynch syndrome phenotype. Challenges in genetic counselling of LS families and ethical aspects are addressed in Chap. 8.

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Contents

Abbreviations

5-FU	fluorouracil
ABC	ATP binding cassette
AC	Amsterdam Criteria
A-GVGD	Align GVGD
AID	activation-induced deaminase
CAPPII	Colorectal Adenoma/carcinoma Prevention Programme
CIMP	CpG island methylator phenotype
CNV	copy number variations
COBRA	combined bisulphite and restriction analysis
CpG	cytosine-guanine
CRC	colorectal cancer
CYP	cytochrome P450
DHPLC	denaturing high-performance liquid chromatography
DTC	direct-to-consumer genetic testing
EGAPP	Evaluation of Genomic Applications in Practice and Prevention Working Group
EPCAM	epithelial cell adhesion molecule
FAP	familial adenomatous polyposis
FH	family history
FIS	Family Information Service
GST	glutathione-S-transferase
GWAS	Genome-Wide Association Study
HNPCC	Hereditary nonpolyposis colorectal cancer
HR	hazard ratio
HRM	high-resolution melting
HVP	Human Variome Project
ICF	instability-facial anomalies
ICG-HNPCC	International Collaborative Group on HNPCC
IDL	insertion-deletion loop
Ig	immunoglobulin
IHC	immunohistochemistry

InSiGHT	International Society for Gastrointestinal Hereditary Tumours
LOH	loss of heterozygosity
LS	Lynch syndrome
MLPA	multiplex ligation-dependent probe amplification
MMR	mismatch repair
MNNG	N-methyl-N′-nitro-N-nitrosoguanidine
MNU	N-methyl-N-nitrosourea
MS	methylation-specific
MSI	microsatellite instability
MSI-H	high level microsatellite instability
MSI-L	low level microsatellite instability
MS-MLPA	methylation-specific multiplex ligation-dependent probe amplification
MSP	methylation-specific PCR
MSS	microsatellite stable
MTS	Muir-Torre syndrome
MTSST	Muir-Torre spectrum skin tumors
NAT	N-acetyltransferase
NCCN	National Comprehensive Cancer Network
NCI	National Cancer Institute
NSGC	National Society of Genetic Counselors
O^6meG	O^6-methyl-G residues
OR	odds ratio
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PGD	preimplantation genetic diagnosis
PND	prenatal diagnosis
PolyPhen	Polymorphism Phenotyping
PON-MMR	Pathogenic-or-Not mismatch repair
PT	predictive genetic testing
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SIFT	sorting intolerant from tolerant
SNP	single nucleotide polymorphism
SNV	nucleotide variant
SSB	single strand DNA binding protein
SSCP	single-stranded conformation polymorphism analysis
UV	unclassified variant
VUS	variants of uncertain significance

Chapter 1
Historical Development of Lynch Syndrome

Henry T. Lynch, Stephen J. Lanspa, Carrie L. Snyder, Trudy G. Shaw, and Patrick M. Lynch

Abstract One hundred years have passed since Aldred Warthin published the first report of a family with the combination of nonpolyposis colorectal cancer and extracolonic cancers that is now recognized as Lynch syndrome. His work from 1913 was rediscovered in the 1960s, when more families with the syndrome, then called "cancer family syndrome," were recognized. In the 1990s, causal mutations were identified in mismatch repair genes; this led to greater scientific acceptance of the disorder, as well as to the ability to determine which family members were at increased cancer risk and thereby in need of rigorous surveillance and management strategies. Since that time, our knowledge of the syndrome, albeit still incomplete, has continued to increase. However, a great deal of work still needs to be done in order to realize the potential of translation of this knowledge to clinical use.

Keywords Lynch syndrome • Hereditary nonpolyposis colorectal cancer • Hereditary cancer • Colorectal cancer • Endometrial cancer

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1.1 Introduction

Nearly eight decades of clinical reports described families with colorectal cancer (CRC) in the absence of adenomatous polyposis before the 1993 discovery of linkage between specific genetic loci and CRC susceptibility [1–6]. Notwithstanding the generally early age at onset of CRC and the potentially associated spectrum of extracolonic tumors, the highly variable onset of cancer and the absence of any one characteristic feature left some unconvinced that any inherited predisposition existed.

Prior to the discovery of the mismatch repair (MMR) genes responsible for this condition, diagnosis was dependent upon a detailed family history depicting a pattern of cancers with these relatively peculiar features: (a) multiple primary cancers; (b) early age of onset; (c) right-sided predominance of CRCs; (d) clustering of certain extracolonic tumors, most commonly endometrial.

1.1.1 Terminology

Since the earliest report by Warthin [7] of a colon cancer-prone family, a host of case reports loosely described families with the features noted above. In the 1960s when Lynch and Krush began systematically collecting such families, the term "Cancer Family Syndrome" was adopted. This worked well enough for a time, but other "Cancer Family Syndromes" such as that of Li and Fraumeni were described, and with similar terminology which led to some confusion. An international workgroup that developed in the late 1980s chose to implement the somewhat more descriptive term "Hereditary Nonpolyposis Colorectal Cancer" or HNPCC, to emphasize heritability, absence of polyposis, and predominance of CRC. In more recent years this term, too, has been criticized as failing to capture the extracolonic tumor spectrum, as well as simply being too wordy. Boland [8] recommended the term Lynch syndrome (LS) in deference to the early work of Henry Lynch. This term has enjoyed an increased usage, though many still prefer HNPCC and others would like a term that emphasizes the centrality of the MMR family of genes that are responsible for it. Examples of terminology evolution in the molecular era have emerged. Families that meet Amsterdam Criteria but with no evidence of MSI and no germline MMR mutations have been labeled as "Familial CRC type X" [9]. Not surprisingly, average ages of onset are not as low as in LS and less evidence of extracolonic tumor spectra are seen when more in-depth family studies are conducted. Others have decided to retain the term HNPCC when there is evidence of nonsporadic MSI (non epigenetic, with no *MLH1* methylation or *BRAF* mutation) but when no germline mutation is detected, while reserving the term LS for those otherwise identical cases but in which a germline MMR mutation is detected. It seems to us that the distinction between "HNPCC" and "LS", when based on this dichotomy, has only to do with the ability to do predictive genetic testing, rather

than any evidence that a different genetic basis exists. This is in stark distinction from the situation with the so-called type X, in which a different genetic basis is highly likely.

Other chapters in this book will delve more deeply into the intricacies of genetic diagnosis. Others will explore the role of testing tumors for the presence of microsatellite instability (MSI), the key feature of LS tumors. This will include comments regarding the role of MSI and a host of other acquired genetic alterations in framing categories of tumors that vary in their prognosis as well as their sensitivity to chemotherapeutic agents [10, 11].

1.1.2 Family History

Ideally, a comprehensive family history (FH) will encompass several generations, will list tumors of all anatomic sites, and will include pathology verification. That said, as genetic diagnosis becomes more routinely available to clinicians in the community, such comprehensive histories will be an elusive goal. It is now clearly established that the Amsterdam Criteria are often absent in a patient found to have a MMR mutation. While a striking family history should easily lead to a diagnosis of LS, there appears to be a trend toward greater reliance on tumor testing for microsatellite instability (MSI) and/or immunohistochemistry (IHC), including proposals for universal testing by authors of population studies of LS frequency, a position endorsed by the Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group [12]. This practice has already been adopted at some centers. MSI and IHC testing, however, are performed on tumor tissue of individuals with CRC. Family histories are still valuable for their ability to recognize a pattern of cancer in the family before the proband or another available family member has had a syndrome cancer.

Recording cancer of all anatomic sites is mandatory when developing the family pedigree. Indeed, this extra effort will frequently be rewarded by allowing the recognition of specific patterns of tumor combinations that could be crucial in hereditary cancer syndromes' diagnosis.

1.1.3 Lynch Syndrome

Approximately 3 % of all CRCs, are caused by LS [13, 14]. However, because of the lack of a recognizable pre-cancerous phenotype, such as the multiple colonic adenomas found in familial adenomatous polyposis (FAP), LS was much more diagnostically challenging and it took longer to comprehend its medical genetic importance. This was evidenced by a large number of steps which were necessary to understand its cardinal clinical, pathology, genetic, and molecular genetics features:

1.1.3.1 Cardinal Features of Lynch Syndrome

- Autosomal dominant inheritance pattern.
- Earlier average age of CRC onset than in the general population:

  average age of 45 years in Lynch syndrome vs. 63 years in the general population

- Proximal (right-sided) colonic cancer predilection:

  70–85 % of Lynch syndrome CRCs are proximal to the splenic flexure.

- Accelerated carcinogenesis: (tiny adenomas can develop into carcinomas more quickly):

  within 2–3 years in Lynch syndrome vs. 8–10 years in the general population

- High risk of additional CRCs:

  25–30 % of patients having surgery for a Lynch syndrome-associated CRC will have a second primary CRC within 10 years of surgical resection if the surgery was less than a subtotal colectomy.

- Increased risk for malignancy at certain extracolonic sites:
  - endometrium (40–60 % lifetime risk for female mutation carriers)
  - ovary (12–15 % lifetime risk for female mutation carriers)
  - stomach (higher risk in Asia)
  - small bowel
  - hepatobiliary tract (nevertheless rare, with no screening recommendations)
  - pancreas (sufficiently rare that no screening recommendations have been made)
  - upper uro-epithelial tract (transitional cell carcinoma of the ureter and renal pelvis)
  - prostate cancer (remains controversial)
  - breast cancer
  - adrenal cortical carcinomas (rare, unclear if truly associated)
  - hepatocellular carcinoma (rare, unclear if truly associated)
  - brain (glioblastomas)
  - sebaceous adenomas, sebaceous carcinomas, and multiple keratoacanthomas in the Muir-Torre syndrome variant of Lynch syndrome
- Pathology of CRCs is more often poorly differentiated, with an excess of mucinous and signet-ring cell features, a Crohn's-like reaction, and a significant excess of infiltrating lymphocytes within the tumor.
- Increased survival from CRC.
- The *sine qua non* for diagnosis of LS is the identification of a germline mutation in a MMR gene: *MLH1*, *MSH2*, *MSH6*, *PMS2*. Operationally, we will include for

completeness the *EPCAM* gene which is not, strictly speaking, a MMR gene, but has been repeatedly found to have mutations that are inherited and which cause inactivation of *MSH2*.

1.2 History of Lynch Syndrome

1.2.1 Warthin's Family G and Cancer Family Syndrome: Historical Perspective

The first family study of what is now known as LS was published in 1913 by Aldred Warthin, M.D., (Fig. 1.1) of the University of Michigan. In 1895, his seamstress informed him of an excess of cancer in her family, and told him that she believed she would die of cancer of the uterus, colon, or stomach because "… everyone in the family dies of these cancers." Warthin recognized that, given the enormity of the cancer problem, the family manifested a familial cancer cluster. (The seamstress subsequently died of endometrial cancer.) Warthin began studying this family in 1895 and published his first report on it in 1913 [7], documenting a pattern of endometrial and gastrointestinal cancers, particularly stomach and colon (see Fig. 1.2). He also reported other examples of "cancerous fraternities" and in a later report, he noted that most of the cancers involved the stomach, colon, and uterus [15]. The seamstress's family (Family G) was subsequently updated by Lynch and Krush [16]. A germline mismatch repair (MMR) gene mutation involving *MSH2* was eventually identified [17, 18].

Fig. 1.1 Photograph of Aldred Warthin, M.D.

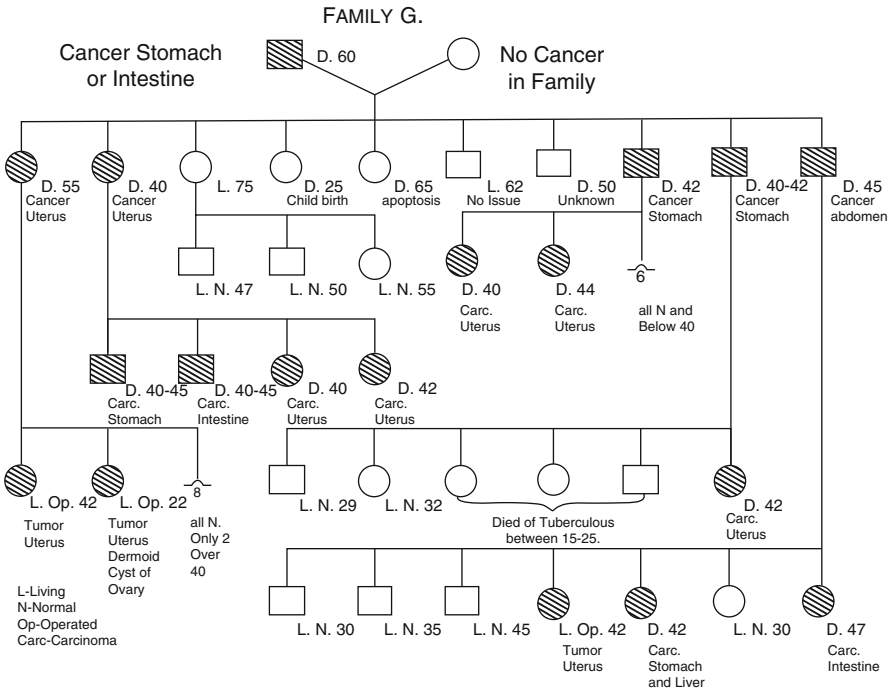

Fig. 1.2 Warthin's pedigree of "Family G"

1.2.2 Families N and M

A similar family was identified in 1962 (Fig. 1.3), when a gastroenterologist, knowing of Lynch's background in human genetics, asked for advice regarding a patient with a strong family history of CRC. Initially thought to be a variant of FAP, which at the time was the only known CRC-predisposing hereditary condition, the cases in the family were notable for an absence of multiple colon polyps, somewhat like Family G. As with Family G, there was a high frequency of extracolonic cancers, especially of the endometrium. The findings from this family were presented at a meeting of the American Society of Human Genetics in 1964. Marjorie Shaw, M.D., a geneticist at the University of Michigan at Ann Arbor, was in attendance, and during the discussion period she stated that the report reminded her of a similar family she had studied. A collaboration was immediately established between Lynch and Shaw, and the findings on these two large families, that is, Family N (Nebraska) and Family M (Michigan) were published in 1966. Importantly, the pedigrees of the two families were considered consistent with an autosomal dominant inheritance pattern of cancers involving the colon, endometrium, ovary, stomach, and other anatomic sites [19].

Dr. Warthin's successor at the University of Michigan, A. James French, M.D., heard about Families N and M, and was reminded of Warthin's early work. He gave

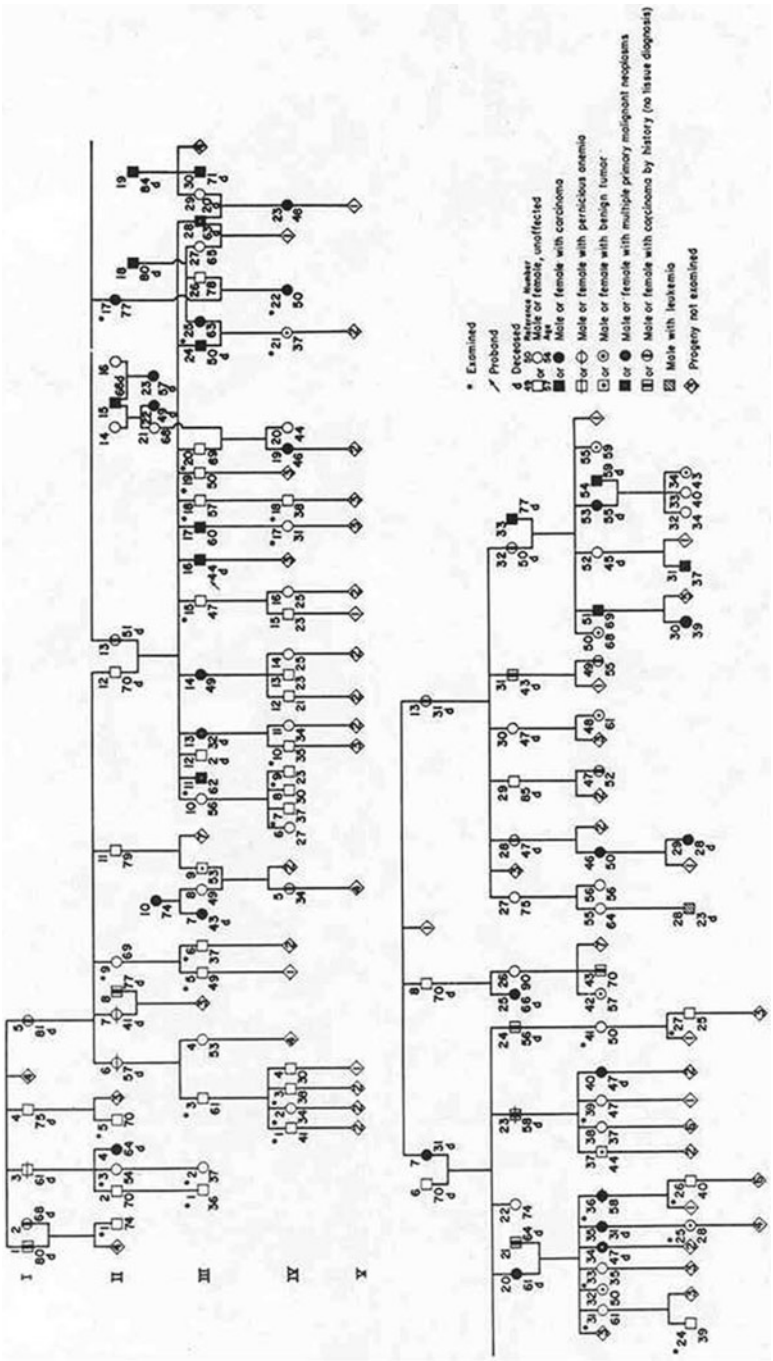

Fig. 1.3 Pedigree of "Family N"

Dr. Lynch access to Warthin's records and pathology specimens. Lynch, working with his associate Ann Krush, continued study of Family G, as stated above, and published an updated description in 1971 [16]; in 2005, a further update of the family was published by Douglas et al. [18].

Lynch and colleagues continued to identify many hundreds of additional families with a strikingly similar pattern of cancers [20–24]. The phenomenon was subsequently labeled "Cancer Family Syndrome."

1.2.3 Limited Belief

At the time of Lynch and Krush's early work, there were few other reports of similar families, so their frequency was considered low. Indeed, it was speculated that such aggregations could occur by chance alone. However, additional reports accumulated in the literature. Indeed, by the late 1980s there was sufficient worldwide interest in nonpolyposis familial colorectal cancer clustering that several freestanding meetings were held. After some discussion, it was felt appropriate to rename the "cancer family syndrome" with the somewhat more descriptive term "Hereditary Nonpolyposis Colorectal Cancer" or HNPCC. During this same period, the informal gathering of international investigators decided that a more formal working group should be established, and the International Collaborative Group for HNPCC was formed, the ICG-HNPCC. While many of the clinical investigators were active in the management of FAP, it was felt that a level of independence and separate identity was important.

The first meeting was held in Jerusalem in 1989, hosted by Dr. Paul Rozen. An excellent history of the development of the ICG has been provided by Dr. Hans Vasen [25], who hosted an early meeting of the ICG, at which stringent clinical criteria for HNPCC were adopted, the so-called "Amsterdam Criteria". These required, in a given family, the presence of three or more cases of colorectal cancer, occurring over two or more generations, with at least one case diagnosed before age 50, and no evidence supporting a diagnosis of FAP. On this latter point, recall that at this time, around 1990, the *APC* gene had only recently been identified and the contours of attenuated FAP were not well-defined, much less established to be an FAP variant caused by *APC* mutations.

Once the MMR germline mutations were recognized in the mid-1990s [1–3], much more nuanced approaches to clinical management became possible. We simply list some of the categories of investigations that have emerged: studies of genotype-phenotype relationships, estimates of mutation frequency, expanded role for microsatellite testing of tumor tissue in a step-wise approach to genetic characterization of individual patients, evaluation of screening regimens based on a more homogeneous grouping of patients known to be at risk (mutation carriers), an improvement over earlier, more empiric investigations. These issues are discussed in considerable detail in other chapters and are beyond the scope of this historical sweep.

1.2.4 Molecular Verification

We would like to think that the efforts of the ICG-HNPCC in bringing investigators together played at least a small part in the 1993 identification of genetic linkage between patterns of cancer in several large families and a locus on chromosome 2. The establishment of the Amsterdam Criteria forced a certain degree of stringency in considering affectedness in the subjects investigated. Certainly a good deal of fanfare was associated with the presentation of these data to the annual meeting of the ICG later in that same year.

In 1993, a large international collaboration used linkage analysis methods to identify the locus of a Lynch syndrome gene in two large LS families [1]. Causal mutations were identified subsequently in MMR genes (*MSH2* [2, 3], *MLH1* [1], *MSH6* [4, 5], *PMS2* [6]). The function of these MMR proteins is to maintain genetic fidelity by repairing DNA errors. MMR mutations do not permit adequate repair processes to occur, allowing mutations to accumulate in genes that contain repetitive elements (microsatellites) within their coding regions. A high level of this microsatellite instability (MSI) is found in LS, although it is also found in approximately 15 % of sporadic CRCs. There is commonly a host lymphoid response, either in the form of lymphoid aggregates at the edge of the tumor or lymphocytes infiltrating the tumor. The latter have been found to be the single best pathologic marker of MSI-status [26]. Another way of identifying LS is through immunohistochemistry (IHC), which can determine a lack of one of the proteins synthesized by the MMR genes, thus pointing toward the gene most likely to be mutated.

1.2.5 Amsterdam Criteria and HNPCC

In order to distinguish the term Cancer Family Syndrome from FAP, it was subsequently given the name hereditary nonpolyposis colorectal cancer (HNPCC). The Amsterdam Criteria [25, 27] were subsequently developed to enable researchers to better garner a uniform definition of the syndrome when accruing these families; their aim was to identify "reagent grade" families for linkage analysis studies. The AC-I [25] were focused solely on CRC and were found to be too stringent to be used as clinical guidelines. The AC-II [27] subsequently added the syndrome's integral extracolonic cancers, thereby enhancing the diagnostic criteria. Further diagnostic refinement for LS was achieved by creation of the Bethesda Guidelines [28, 29] (discussed below).

1.2.6 Recognition of Clinical/Pathology Characteristics and Expansion of Tumor Spectrum

Several of the characteristics of Lynch syndrome were quite well worked out during the era before the discovery of MMR genes, and carried with them clinical implications that continue to be important.

CRC localization, rapid growth: Early onset of CRC was, of course, the key feature that led to suspicion that a given family might be expressing a familial/genetic predisposition, particularly if several relatives had early-onset disease. But further refinements were made following the discovery of MMR genes. It was observed that approximately 70 % of CRCs in members of these families occurred in the proximal colon [30, 31]. Up to this time, colonoscopy screening was not very prevalent in average risk patients. But because of the recognition of a tendency toward right colon involvement in HNPCC, recommendations for full colonoscopy existed for this patient population well before its widespread use in average risk population. This recommendation appeared somewhat more controversial at the time, as it was accompanied by recommendation that the practice be initiated at a much earlier age than would otherwise be considered. Finally, because of the common, albeit anecdotal, observation of CRCs developing at short intervals following screening, recommendation for more frequent surveillance was made. It must be conceded that these recommendations were not widely adopted before it became possible to routinely specify mutation carrier status. Nevertheless, several early studies established that colorectal surveillance in first-degree relatives of cancer patients from affected kindreds resulted in lower rates of cancer and lower cancer stage than in those not so surveilled, presumably due in part to the removal of precancerous adenomas [32, 33].

Sebaceous skin tumors and the Muir-Torre syndrome (*MTS*): The association between rare skin appendage tumors (sebaceous adenomas and carcinomas) and visceral malignancy had been the subject of historical case reports. However, upon noticing several such cases in HNPCC families from the Creighton registry, a systematic review of families from that registry yielded a number of additional cases. These were sufficient enough in number and with multiple cases in related individuals as to allow the conclusion that such Muir-Torre spectrum skin tumors (MTSST) were very likely a feature, albeit uncommonly, of HNPCC. Even before the discovery of MMR genes, the recognition of even one case of MTS supported a more detailed exploration of family history [34–36]. Of course, now it is possible to perform MSI testing on series of otherwise unselected MTSSTs and to perform mutation analysis on informative cases. Though not worked out to a level of certainty, there now appear to be clinical features of MTSSTs that are part of HNPCC that would allow the dermatologist to select lesions more likely to be informative upon MSI testing. As with other tumors that comprise the spectrum of tumors seen in HNPCC, clinical selection of cases for MSI testing continues to compete with arguments in favor of "universal testing," that is, subjecting all sebaceous adenomas, for example, to MSI testing and/or to IHC for MMR protein expression, issues discussed in greater detail in other chapters.

Extracolonic tumor spectrum: Watson and Lynch [37] added to LS's extracolonic spectrum, which has now included carcinoma of the pancreas, hepatobiliary system, small bowel, ovary, brain (as in Turcot syndrome variant [38, 39]), adrenal cortical carcinoma [19, 40, 41], transitional cell carcinoma of the upper uro-epithelial tract, and the mentioned cutaneous lesions of the Muir-Torre syndrome; this study was updated by Watson et al. [42] in 2008. There is now strong evidence that breast [43, 44] and prostate [45] cancer are also integral to the syndrome. It is believed that the

complete tumor complement for LS has not yet been elucidated, and other tumors will eventually be associated with LS as new data are collected. Indeed, MMR mutations in LS appear to have veritably unlimited potential for predisposing to an increasingly wide spectrum of such syndrome integral cancers. A recently suggested association is that of hepatocellular carcinoma, investigated in a MTS family by Morando et al. [46]. In large part due to the presence of multiple extracolonic cancers, the term hereditary nonpolyposis colorectal cancer (HNPCC) came to appear inappropriate inasmuch as it inferred a near exclusive importance of CRC. In part for this reason, Boland [8], as previously mentioned, proposed that the term Lynch syndrome be adopted. Whether or not this term is appropriate remains for others to determine. Some have proposed that the term Lynch syndrome be reserved for patients/families in which an MMR mutation has been identified, while reserving the "older" term HNPCC for patients/families in which no mutation is detected. There are patients and families that clearly meet clinical criteria and whose tumors show consistent loss of, for example, MSH2 protein, but in whom no pathogenic MMR mutation is identified. They certainly have the same "condition," whatever it may be called, and the failure to detect a mutation does not seem to alter this fact. The only difference is the ability to perform predictive testing when a mutation is found, an ability that is not possible when no mutation is found. However, it seems to us that the similarities of these circumstances outweigh any differences, and to use different terms for the same condition can only foster confusion.

Figure 1.4 depicts the pedigree of a family manifesting the cutaneous lesions of the Muir-Torre syndrome as well as a wide variety of cancers integral to Lynch syndrome.

1.2.7 MSI/IHC Screening Benefits

The National Cancer Institute hosted an international workshop in 1996 [28], which led to the development of the Bethesda Guidelines for the identification of individuals at high risk for Lynch syndrome who should be tested for MSI. To consider revision and improvement of the Bethesda Guidelines, another workshop was subsequently held at the National Cancer Institute in Bethesda, MD, in 2002 [29].

Schofield et al. [47] evaluated the utility of routine MSI/IHC screening among previously undiagnosed patients and families with LS in a cohort from western Australia. This involved CRC at less than 60 years of age. The tumors were then tested for MSI and for expression of MLH1, PMS2, MSH2, and MSH6 employing IHC. Those showing MSI and/or loss of staining expression on IHC were tested for *BRAF* V600E mutation. Of 70 cases with MSI, 25 were excluded as possible LS because of *BRAF* V600E mutation, leaving 45 cases eligible for germline testing. Findings disclosed that among 31 cases tested to date, 15 germline mutations were identified. Furthermore, 13 were from individuals "…not previously recognized as LS and two were untested members from known LS families. Extrapolation of the mutation incidence (15/31, 48 %) to all red flag cases (n=45) suggests that approximately 22 mutation carriers exist in this cohort…" The authors concluded that routine MSI and IHC testing in western Australia indicate that the majority of LS cases are being identified.

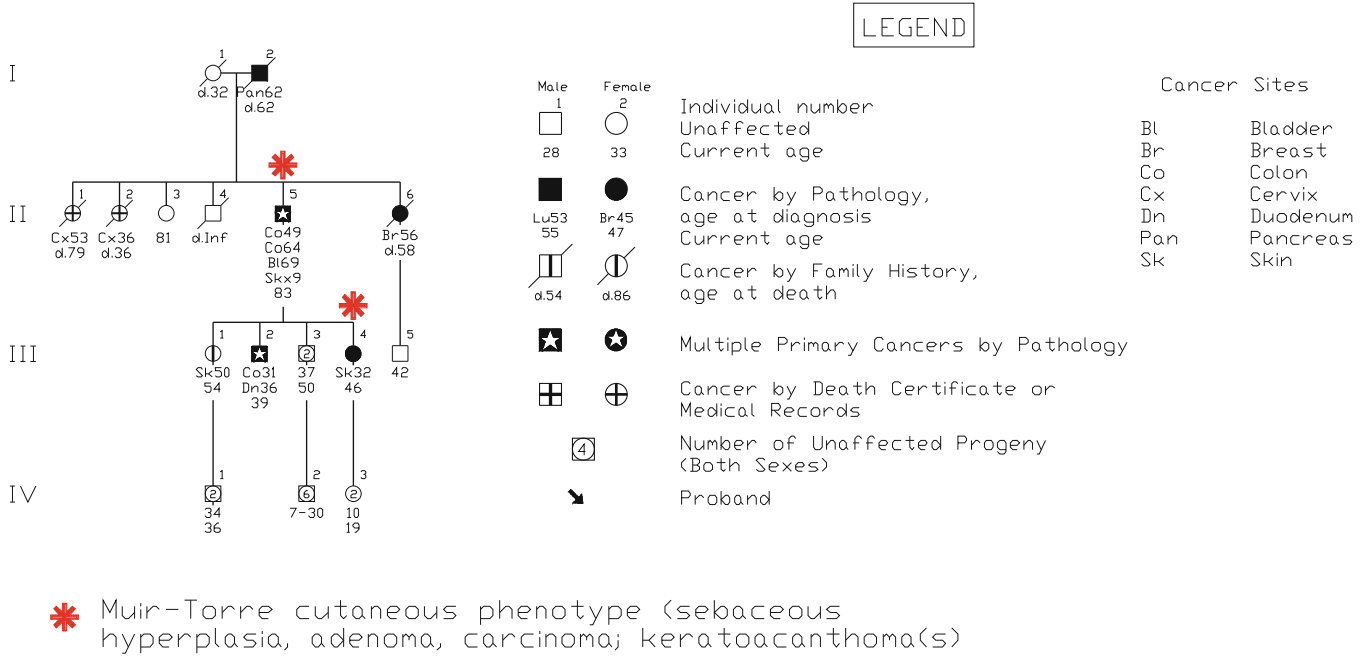

Fig. 1.4 Pedigree showing large Lynch syndrome family manifesting numerous extracolonic cancers, including cutaneous lesions of the Muir-Torre syndrome

However, Hampel et al. [14] performed DNA testing on 500 consecutive CRC patients, wherein they found that only 72 % (13/18) of those with LS MMR mutations met the revised Bethesda Guidelines that had been developed to identify individuals who should receive MSI testing. The investigators concluded that, since MSI and IHC testing makes it possible to cost-effectively test all patients diagnosed with CRC, such testing should be done in order to not miss the approximately 28 % of MMR mutation carriers who do not fulfill the Bethesda Guidelines.

The Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group [48] also supported the Hampel et al. findings [14] as being cost effective. They concluded that using genetic testing strategies to reduce morbidity and mortality in CRC probands and their relatives at high risk for and/or affected by LS is a sound and prudent measure. Using IHC as the preliminary diagnostic test was shown to be the most efficient screening method, since it reduces the need for sequencing all four of the genes normally tested for the diagnosis of LS, to sequencing of only one or two of these genes.

1.3 Recent Molecular Genetic Discoveries

Gene sequencing was able to identify many of the mutations in the MMR genes. However, more mutations have been identified as techniques that are more advanced become available. For example, in 2000, the mutation in Warthin's Family G was finally identified as a T to G transversion in *MSH2*, using conversion of diploidy to haploidy [17].

1.3.1 American Founder Mutation

In 2002, a previously unidentified large deletion in the *MSH2* gene was found in a family through the use of a method employing inverse polymerase chain reaction and monoallelic expression analysis [49]. Through use of Creighton University's hereditary colorectal cancer family resource, this specific mutation was found to be widespread in the United States. It has been termed the American Founder Mutation [50, 51]. Further research determined that over 32,000 individuals carry this mutation in the United States, constituting approximately 1–2 % of all CRC cases [51]. Therefore, testing for it has become an accepted part of genetic screening for families with excess CRC in the United States.

1.3.2 EPCAM

In 2009 it was found that a deletion in the epithelial cell adhesion molecule (*EPCAM*) gene could silence the downstream *MSH2* gene [52, 53]. This provided an

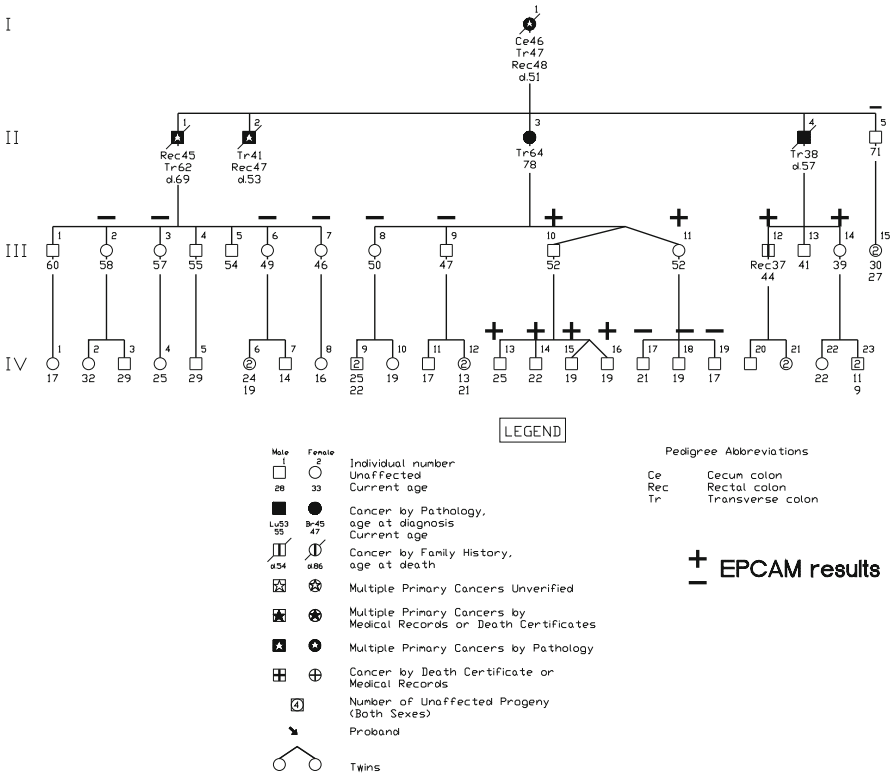

Fig. 1.5 Pedigree of family with site-specific CRC and *EPCAM* deletion (Republished with permission from H. Lynch et al. [54])

explanation for a subset of families showing a variant Lynch syndrome phenotype, namely site-specific CRC, but having no evidence of a mutation in an MMR gene [54].

A highly-extended kindred with this phenotype, had been followed by Lynch et al. for more than 35 years (Fig. 1.5) [55]. A deletion confined to *EPCAM* was identified in this kindred, which contained more than 700 individuals wherein 50 individuals manifested CRC. The breadth and size of this *EPCAM*-prone family is testament to the potential impact of a single mutation event. Even though no MMR mutation had been found in this family, members had complied over the years with colonoscopy recommendations; this compliance led to the diagnosis of CRCs at an early, curable stage in many of these patients [54].

1.3.3 Epigenetics

Epigenetics is defined as "heritable changes in gene expression that are not due to any alteration in the DNA sequence" [56, 57]. Epigenetic modifications serve as a secondary code, providing functional instructions on whether a gene should be

active or suppressed. Recent evidence has emerged that shows constitutional *MLH1* epimutations exist in two distinct forms: those that arise in the context of a normal gene sequence and are reversible between generations, and those that are linked to an underlying genetic defect and demonstrate classic autosomal dominant inheritance. A full understanding of epigenetics and its role in CRC carcinogenesis, particularly its role in heritable cancers, is a challenge which will likely provide researchers and clinicians with insight into how best to identify and manage familial CRC cases [58, 59].

1.3.4 Familial CRC Type X

A weakness of the AC-I is that they fail to identify many Lynch syndrome families. It has now been found that the AC-I also encompass a large number of families which do not have a Lynch syndrome mutation. Approximately 60 % of families fulfilling AC-1 show molecular genetic evidence of LS. A subset of the 40 % lacking LS molecular evidence has been referred to as Familial CRC Type X; families with this type of CRC show cancer at a later age than Lynch syndrome, and typically show a modest excess of only CRC (often less than its LS counterpart), rather than including the extracolonic cancers integral to Lynch syndrome [9, 60].

1.4 Surveillance and Management: Historical Perspective

1.4.1 Colonoscopic Surveillance

The story of colonoscopic surveillance in LS is one of data confused by case-finding challenges and evolving understanding of the molecular pathways of CRC development (including eventual discernment of "accelerated carcinogenesis"). In only the last few years have data been reported that strongly support the cancer prevention potential of colonoscopy in LS. Future research involves improved endoscopic detection, and case-finding using molecular studies of presumed sporadic adenomas.

Before the 1980s, it was unclear whether CRC in LS arose from colonic adenomas. The high cancer burden in the absence of florid polyposis suggested that cancer occurred *de novo*. Various histochemical analyses of normal colonic mucosa from at-risk patients were unsuccessful in predicting cancer risk. Colonoscopy was intended to find cancers at an early stage. Nevertheless, Lanspa et al. [61], in studying an at-risk cohort, showed that the short-term risk of CRC was low in those that had negative colonoscopy, as was the risk in those that had all adenomas removed. The study found one adenocarcinoma arising in a tubulovillous adenoma.

In 1995, Järvinen et al. [32] studied screening in at-risk LS family members and showed a 3-year interval more than halves the risk of CRC, and Vasen et al. [62] showed improved 5-year survival with surveillance.

Love and Morrisey [63] performed colonoscopies in asymptomatic members of LS kindreds and reported an adenoma prevalence of 17 % with two polyps containing invasive carcinoma. In 1986, Love reported [64] cancers arising in colonic adenomas in LS patients. By 1990, it was accepted that adenomas were, indeed, the premalignant lesion in LS [65]. But the high rate of synchronous and metachronous CRCs suggested a pathological process different from sporadic adenomas. In 1994, a report on interval CRCs by Lanspa et al. [66] supported an accelerated adenoma-carcinoma sequence and showed a 5-year screening interval would not prevent CRC. By 1997, the consensus was for aggressive colonoscopy surveillance at 1–3-year intervals [67]. Recently, Edelstein et al. [68] reported the rapid development of colon neoplasia in patients with LS, recommending annual colonoscopy.

In addition to the observed accelerated carcinogenesis, adenomas from LS patients may have unique phenotypic characteristics such as an increase in adenoma-infiltrating lymphocytes [69], MSI-H, and loss of MMR protein expression [70].

In the last decade, with genetic testing and large registries, several authors have demonstrated the cancer control efficacy of colonoscopic surveillance [33, 71–73]. Narrow band imaging, high magnification, and chromoendoscopy are techniques that may further improve our efforts [74].

1.4.2 Prophylactic Surgery and Risk Reduction for Endometrial and Ovarian Cancer in LS

Schmeler et al. [75] have shown significant risk reduction for carcinoma of the endometrium and ovary through prophylactic surgery in women who are MMR germline mutation carriers who have completed their families, have had genetic counseling relevant to the pros and cons of this surgery, and have signed informed consent. It is extremely important that these patients fully understand the reduced penetrance of carcinoma of the endometrium and ovary in LS, as well as the limitations of screening, particularly in ovarian carcinoma. In the Schmeler et al. study, those high-risk women who had undergone prophylactic surgery had no manifestations of endometrial, ovarian, or primary peritoneal cancer. On the other hand, among the control group for endometrial cancer, 69 women (33 %) had been diagnosed with this cancer before the end of the study; among the control group for ovarian cancer, 12 women (5 %) were diagnosed with ovarian cancer during that time. These findings suggest that prophylactic hysterectomy with bilateral salpingo-oophorectomy is effective in preventing endometrial and ovarian cancer in women carrying a mutation for Lynch syndrome. Historically, the discovery of MMR gene mutations and the resulting ability to determine who is vs who is not a mutation carrier has made prophylactic surgery a more targeted approach.

1.4.3 MSI, Differences in Prognosis, Response to Chemotherapy (5-FU) for CRCs

Ribic et al. [76] found survival to be better in MSI patients who have not undergone treatment when compared with microsatellite stable (MSS) and MSI-Low patients. Data were pooled and they showed an absence of benefit or perhaps even worse outcome in those MSI-bearing patients undergoing adjuvant therapy when compared with no treatment in distinction from those with MSS or MSI-Low who benefitted.

In trials containing untreated placebo arms, MSI patients did better than those with MSS tumors. Generally, patients with resected stage III MSI tumors when treated with 5-FU fared significantly worse than their MSS counterparts.

MSI can occur in LS or sporadically (hypermethylation of the MLH1 promoter CIMP as one pathway). The question is "Are there differences between these two groups with respect to prognosis and therapy?"

Sinicrope et al. [77] made an evaluation of the role of 5-FU and identified a decrease in distant recurrences in patients with stage III MSI tumors, though this was not seen in stage II patients. Furthermore, treatment benefit was seen only in those likely LS but not in those considered sporadic. It was concluded that MSI patients with stage II do not benefit from adjuvant therapy and should not receive such therapy.

Boland and Goel [78] suggested that current guidelines do not recommend using MSI status to determine whether or not to use chemotherapy. However, this recommendation merits a second look, given the wealth of data showing the inadequacy of 5-FU for CRC with MSI. Such a study should be performed only in the context of a randomized clinical trial.

1.4.4 Aspirin and Long-Term Colonic Polyp Reduction in LS Patients

Burn et al. studied the effects of aspirin for up to 4 years and found that it had no effect on colorectal adenomas or CRC in LS [79]. In a follow-up study, 600 mg of aspirin had been administered for 55.7 months wherein it substantially reduced cancer incidence in carriers of hereditary CRC germline mutations [80]. The lesson learned was that in chemoprevention studies of cancer, the time from administration of the chemopreventive agent to effect must be carefully considered.

1.5 Genetic Counseling

The clinical discipline of genetic counseling for hereditary cancer disorders, which has been emerging over the past several decades, has been a true blessing to the medical geneticist, cancer specialist, family practice physician, and genetic counselor.

It has changed many of the responsibilities inherent in hereditary cancer syndrome discovery, surveillance, management, and even decisions regarding surgical prophylaxis [81–83]. This significantly benefits our high-risk patients, as it fosters the integration of molecular genetics at the level of the patient and family, wherein the counselor's expertise can signal who needs DNA testing vs. who does not [84].

As mentioned earlier, the physician's time is frequently too limited to give appropriate attention to the labor intensive tasks of collecting and documenting the family history, meeting with family members, and other aspects of genetic counseling. As more is learned about cancer genetics, the field also becomes increasingly more complex, to the point that a primary care physician cannot be expected to keep up with all the developments that would affect the diagnosis, surveillance, and management of hereditary cancer syndromes. One response to this situation is an increasing use of genetic counselors, particularly those specializing in cancer genetics. Centers of hereditary cancer expertise are becoming more available for referral.

1.5.1 Family Information Service (FIS)

How can a genetic counseling team efficiently reach a large number of high-risk family members in a single setting? An FIS [85], as developed over the years at Creighton, is an excellent method. It is a cost-effective, highly efficient way of educating and counseling all available and interested family members from a geographic catchment area during a single setting. It makes the best use of the physician's and genetic counselor's time and effort, has group therapy potential, and patients welcome it. It has become an extremely valuable application of an expanded genetic counseling model thereby enabling effective communication with many family members who have gathered together as a group. It is particularly helpful once a deleterious germline mutation has been identified in the family [86]. In preparation for the FIS, key family members will often volunteer to help inform their relatives about the objectives of the FIS and encourage their attendance. These same individuals may also be instrumental in spreading knowledge to family members who did not attend the FIS, regarding the family's high cancer risk status and the availability of genetic counseling and testing.

1.6 Remaining Concerns

1.6.1 Physicians' Insufficient Approach to Identification of Lynch Syndrome

Rubin et al. [87] investigated the awareness of CRC-affected patients regarding the increased cancer risk to their close relatives. Their findings, based upon 253 CRC

patients, showed that only 120 (47.7 %) knew that their first-degree relatives were at increased risk for CRC. A mailed educational brochure on the subject did not improve their knowledge. It was concluded that most patients lacked knowledge about their family members' risk and required more effective educational tools. We believe that personal interaction with the family in the FIS setting is an optimal way of providing so-called 'hands on' education.

Another study pertaining to cancer risk among probands' relatives, by van Dijk et al. [88], involved 244 patients from the Netherlands who satisfied at least one of the Bethesda Guidelines. A complete family history was recorded for only 38 of the 244 patients (16 %). Those patients with a more complete family history were more likely to be referred to the clinical genetic center than those with an incomplete or absent family history (53 % vs 13 % and 4 %, respectively; $P = 0.0001$) and were more likely to be analyzed for microsatellite instability (MSI) (34 % vs 6 % and 1 % respectively; $P < 0.001$). These authors concluded that the family history is neglected in the majority of patients with CRC. Furthermore, MSI is pursued in only a small proportion of patients who meet the guidelines for this testing.

Tranø [89] studied medical records of CRC patients in Norway and found a striking lack of attention to family history. Sixty-nine percent of patients had no family history recorded on the medical chart. The family history information of the 31.0 % who did have it included in their medical record had not been clinically assessed, adding up to 0.0 % whose records had been beneficially utilized.

This is not a new observation. Thirty years ago, a study was published in *JAMA* [90] that looked at outpatient cancer clinic records of oncology patients and found that, "In most cases, the family history of cancer had been either omitted altogether, reported as negative despite substantial evidence to the contrary, or, if noted as positive, not pursued or acted on" [90]. This 1979 study found, as did Tranø's 2009 study, that interviews with the patients whose records had been studied elicited enough evidence of positive family histories to be of real concern, as did investigations during the intervening years [91–93].

1.6.2 Direct-to-consumer (DTC) Genetic Testing

The genetic revolution has made patients increasingly more aware of genetic testing and its benefits. However, unfortunately, many physicians and patients are being misled by DTC test offerings of questionable accuracy and utility [94]. This problem has been addressed by the U.S. General Accounting Office which concluded that such DTC tests were "misleading and of little or no practical use" [95]. Risk profiles provided by such testing companies have been described as having "no predictive value" and may falsely alarm or reassure consumers [96].

Lovett et al. [97] discussed a survey in which online information would be sought in place of medical advice by approximately 50 % of respondents [98], in the face of studies which have highlighted fraudulent claims by online DTC medical product monitoring wherein dangers have been part and parcel of a variety of disease-based DTC screening strategies [99–101]. Such online DTC screening tests include a wide

spectrum of tumor markers constituting an extensive variety of cancers. Therein, DTC marketing of cancer screening constitutes a panoply of products which pose a significant threat to patient safety and public health.

1.7 Future Directions

One hundred years after the publication of Warthin's original paper [7], the primary challenge remains a lack of recognition of Lynch syndrome among many in the medical community [89, 102]. As evidence has mounted regarding the benefit of early surveillance and management among germline mutation carriers [71, 75, 103], it becomes even more crucial that physicians, with the assistance of genetic counselors and centers of hereditary cancer expertise, determine those patients who would benefit from molecular genetic testing and entry into targeted surveillance and management programs.

Future research will be needed to more fully elucidate the impact of MMR mutations and MSI-H tumors on treatment of CRC patients, particularly the use of 5-FU and FOLFOX chemotherapy regimens. Promising results with the use of aspirin as a chemopreventive agent in germline mutation carriers also call for further investigation [80].

As has always been true with this complex syndrome, future advances are most likely to come from observations that will lead research in as yet unexpected directions.

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Chapter 2
Molecular Mechanisms and Functions of DNA Mismatch Repair

Hui Geng and Peggy Hsieh

Abstract DNA mismatch repair (MMR) is an evolutionarily conserved DNA repair pathway that plays an essential role in maintaining genomic fidelity and stability. MMR targets errors generated during DNA replication, contributing 100–1,000-fold to the overall fidelity of DNA replication. Inactivating mutations in highly conserved MMR genes greatly increase the spontaneous mutation rate, and loss of MMR predisposes individuals to hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome. Loss of MMR activity due to epigenetic silencing of MMR genes or somatic mutation is associated with a variety of sporadic tumors. Proteins involved in MMR also participate in DNA damage signaling inducing cell cycle arrest and apoptosis in response to certain DNA alkylating agents and other DNA-damaging agents or base analogs. This review summarizes our current understanding of the MMR pathway and its roles in cancer avoidance.

Keywords Mismatch repair • Colon cancer • MutSα • MutLα • Apoptosis • DNA damage

2.1 Introduction

Virtually all organisms possess DNA repair pathways that protect the integrity of the genome and repair DNA damage caused by both exogenous and endogenous sources. The MMR pathway, conserved from bacteria to humans, corrects mismatches that occur during DNA replication or homologous recombination and as

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a consequence of certain types of DNA damage. Mismatches fall into two general categories, base-base mispairs arising from incorrect nucleotide insertion opposite the templating base by DNA polymerases that escape their proofreading function, and insertion-deletion loops (IDLs) resulting from strand slippage during replication through short repetitive tracts that can give rise to frame-shift mutations, duplications or deletions [1–5]. Nucleotide selection at the base incorporation step and exonuclease proofreading by the replicative DNA polymerases lead to an error rate of roughly 10^{-7} per base pair per cell division. The actual mutation rate is much lower, approximately 1 in 10^9–10^{10} base pairs per cell division, reflecting the contribution of the MMR system, which contributes an additional 50–1,000 fold to overall fidelity [6]. Therefore, MMR inactivation confers a strong mutator phenotype, and in many cases, microsatellite repeat instability. Loss of MMR is the largest contributor to a genetic predisposition to colorectal cancer, e.g. Lynch syndrome or hereditary nonpolyposis colorectal cancer, and inactivation of MMR through epigenetic silencing or, more rarely, somatic mutation is associated with a large subset of sporadic cancers [7, 8]. Interestingly, MMR proteins are involved in several other fundamental processes, including the cellular response to DNA damage, the modulation of homologous recombination between divergent sequences, chromosome disjunction in meiosis, trinucleotide repeat expansion and antibody diversity and somatic hypermutation [1, 4, 9, 10].

2.2 MMR Deficiency and HNPCC

The link between MMR defects and human cancer was discovered almost two decades ago. In 1993, several groups reported that tumor tissue derived from HNPCC patients showed high levels of microsatellite instability [11–13]. In fact, more than 90 % of HNPCC tumor cells exhibit varying levels of MSI [14]. Microsatellite regions are short, repetitive DNA sequences 1–4 nucleotides in length. These regions can misalign or undergo “slippage” during replication leading to loops of unpaired bases or IDLs. When MMR is inactive, IDLs that arise in regions of mono- and di-nucleotide repeats and escape detection at the proofreading step give rise to microsatellite repeat instability that is a hallmark of Lynch syndrome tumor cells. The observation that similar MSI phenotypes are observed in budding yeast harboring mutations in *MSH2*, *MLH1* and *PMS1* (equivalent to *PMS2* in eukaryotes) provided an important clue in the identification of inactivation of MMR as the underlying defect in HNPCC [15]. Indeed, mutations in human *MSH2* and *MLH1* have been identified as the underlying genetic defect in the majority of Lynch syndrome/HNPCC population [16–19]. In addition, rare mutations in human *MSH6* and *PMS2* are also detected [20, 21, 22, 23]. Several databases have been developed that catalog MMR gene variants,

some of which are possibly associated with Lynch syndrome (http://www.insight-group.org/mutations/, http://www.hgmd.org/,http://www.mmrmissense.net/, http://www.med.mun.ca/mmrvariants/).

Biochemical studies supply corroborative support for the association between MMR defects and HNPCC. In general, strand-specific MMR is impaired in cell lines from HNPCC patients and sporadic tumors with MSI [24, 25]. The introduction of purified hMutSα or hMutLα proteins can restore the MMR function in the nuclear extracts derived from these colorectal tumor cell lines *in vitro* [26, 27]. Similar evidence is also achieved from chromosome or gene transfer experiments to support the concept that MMR genes are essential to genomic stability. Human chromosome 3 containing a wild type copy of hMSH1 can reconstitute MMR in an hMLH1-deficient colorectal tumor cell line [28]. Complementation of MMR defects in cell lines deficient in hMSH2, hMSH6 or hPMS2 has also been demonstrated *in vitro* [29–31].

HNPCC mutations in hMutSα have been mapped onto the three-dimensional structure (Fig. 2.1; [32]). These hMutSα mutations (http://www.insight-group.org/) reside in all domains of both subunits and can be classified into six broad groups: interference with DNA binding, loss of ATPase activity, loss of allosteric communication between DNA and ATP binding sites, loss of allosteric communication between DNA and ATP binding sites, loss of protein-protein interactions with downstream effectors, loss of MSH2-MSH6 interaction, and general loss of protein stability. Remarkably, mutations are distributed throughout the whole protein structure.

Epigenetic modification can also contribute to MMR deficiency. Several groups reported that different mechanisms might exist to cause MMR defects, as no significant mutations in the known MMR genes were identified in a great number of MSI positive spontaneous colorectal cancers [33–35]. In 1997, Kane and colleagues found that hypermethylation of the h*MLH1* promoter is associated with a lack of hMLH1 expression in several sporadic colon tumors and cell lines without mutations in the h*MLH1* gene, indicating a general role of hypermethylation on MMR gene inactivation [36]. Later studies showed that epigenetic silencing of *MLH1* is responsible for about 15 % of spontaneous colorectal cancers displaying MSI [37, 38]. The demethylating agent 5-aza-doxycytidine can restore hMLH1 protein expression in such tumor cells, thus restoring strand-specific MMR [39, 40]. In contrast, no hypermethylation of the h*MSH2* gene has been discovered in these tumors [41].

Mueller, et al. screened 71 Lynch syndrome cases for *MSH2, MLH1, MSH6* and *PMS2* gene defects [42]. Their analysis supports a diagnostic algorithm where suspected Lynch syndrome cases identified on the basis of clinical criteria should be subjected to direct DNA sequencing and multiplex ligase-mediated probe amplification to detect deletions of *MSH2* and *MLH1* MMR genes followed by MSI, protein expression and DNA methylation analyses. Prescreening utilizing MSI status as has routinely been done would, based on their analysis, miss some cases. The advent of faster and cheaper DNA sequencing makes this approach feasible for at-risk individuals.

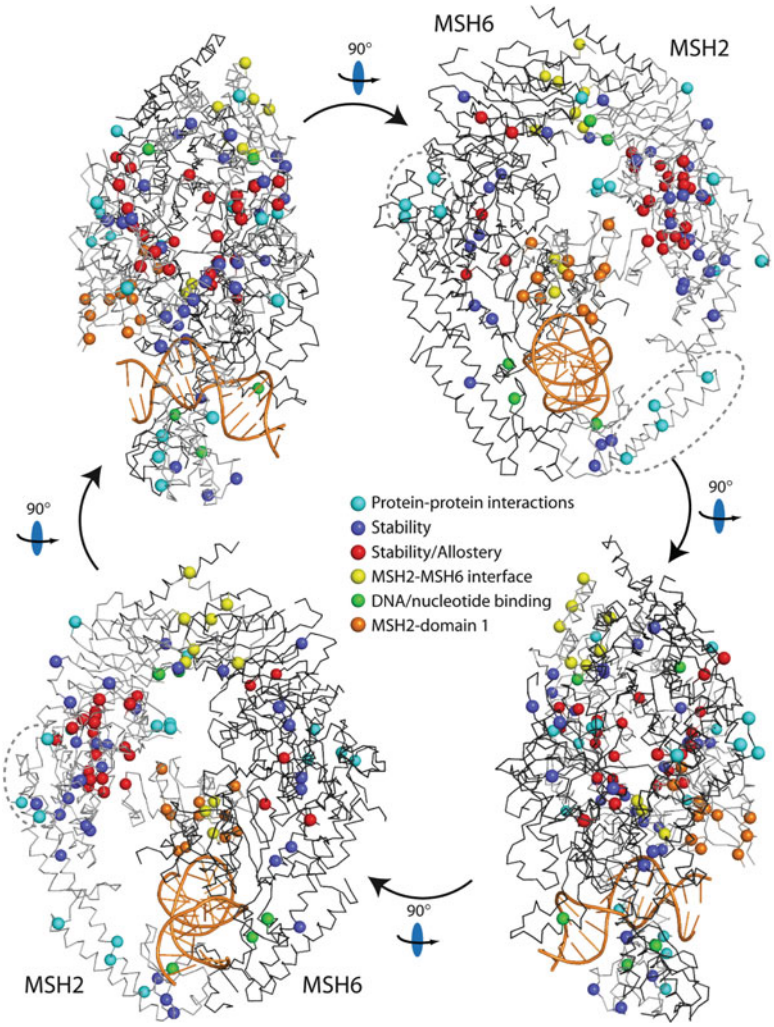

Fig. 2.1 Structural model for human MutSα with HNPCC mutations. Four views of MutSα related by 90° rotations as indicated, with positions of HNPCC missense mutations indicated by *spheres*. Hypothetical functional classification of mutations is indicated by *sphere color* (see legend). MSH2 and MSH6 are shown as *light* and *dark gray* Cα chain traces, respectively, and the DNA is *colored orange*. Three clusters of surface mutations, which may correspond to sites of protein-protein interactions are indicated with *dashed ovals* (Reproduced with permission from Warren et al. [32])

2.3 Animal Models of MMR Defects

Mouse lines with targeted inactivating mutations in MMR genes have been generated for *MSH2*, *MSH6*, *MSH3*, *MSH4*, *MSH5*, *MLH1*, *PMS2*, *PMS1*, *MLH3*, and *EXO1* [43, 44]. To a first approximation, those mutant mice missing MMR proteins that

have been implicated in MMR have characteristics consistent with a predisposition to cancer resulting from loss of mismatch repair. In contrast to human Lynch syndrome patients, heterozygous mice are largely unaffected and do not develop early onset tumors. This is thought to reflect the substantially reduced probability of loss of the wild-type allele, LOH, in mice due to their relatively short lifespan compared to humans and their small size. Mice harboring homozygous inactivating mutations in *MSH2, MSH3, MSH6, MLH1, PMS2* and *EXO1* exhibit the expected mutator phenotype as evidenced by the greatly increased incidence of MSI. They have shortened lifespan and are cancer-prone though they fail to develop colorectal cancer; instead they develop gastrointestinal and skin cancer and succumb largely to lymphomas [43].

Msh2$^{-/-}$ mice are fertile, develop T cell lymphomas, or gastrointestinal, skin or other tumors within 12 months of age, and have a shortened lifespan [45–47]. Cells lacking MSH2 are MSI-positive, display defective mismatch binding activity *in vitro* and are tolerant of the cytotoxic effects associated with some DNA damaging reagents. *Msh6*$^{-/-}$ mice develop tumors at later ages, and they are MSI-negative at mononucleotide runs but have low but detectable MSI at dinucleotide repeats [48]. *Msh3*$^{-/-}$ mice do not have an obvious phenotype and are substantially tumor-free [49, 50]. Remarkably, the double knockout *Msh3*$^{-/-}$*Msh6*$^{-/-}$ mice present significant phenotypes, similar to those of mice lacking MSH2 [49, 50], implying that MSH3 and MSH6 cooperate with each other in tumor suppression.

Loss of some MutL homologs confers sterility possibly reflecting the loss of functional MMR, but more relevant, the disruption of meiosis and homologous chromosome pairing in germ line cells [1]. Sterility is a particular characteristic of *Mlh1* and *Mlh3* mutant mice. Both male and female *Mlh1*$^{-/-}$ mice are sterile. They share a similar tumor spectrum, genomic instability and shortened lifespan to *Msh2*$^{-/-}$ mice [51, 52]. Fibroblasts lacking MLH1 are MSI-positive and fail to carry out MMR in vitro. In *Pms2*$^{-/-}$ mice, only the males are sterile; all of the *Pms2*$^{-/-}$ mice develop lymphomas and sarcomas with MSI at early ages [53]. *Exo1*$^{-/-}$ mice have reduced survival, enhanced susceptibility to lymphoma and are sterile [54].

Can a single amino acid substitution in a MMR protein affect cancer susceptibility? To address this question, Edelmann and co-workers have generated four knock-in mouse strains harboring a single amino acid substitution in either MSH2, MSH6, MLH1 or PMS2 [55–58]. The MSH2^{G674A}, MSH6^{T1217D} and MLH1^{G67R} mutant mice display a mutator phenotype, develop cancer, and lack MMR function, but retain the apoptotic response to DNA damaging agents [55–57]. These studies provide strong support for the notion that loss of MMR and the resulting genomic instability contribute directly to increased cancer predisposition in mammals. They also suggest that these three mutations are separation-of-function alleles in the MMR signaling and DNA damage response though more detailed studies of these mutant proteins are required to understand the molecular basis of their respective defects (see below). Gly674 and Gly67 are located in the ATP binding motifs of MSH2 and MLH1, respectively. Thr1217 resides at the MSH2-MSH6 interface, adjacent to the ABC transporter ATPase signature domain that is essential for ATP hydrolysis. The PMS2^{E702K} mouse harbors an inactivating mutation in an active site Glu residue essential for the endonuclease function of PMS2. This mouse exhibits elevated

genomic mutation rates and greatly increased predisposition to cancer. Thus, the endonuclease function of PMS2 plays an important role in genome maintenance and tumor suppression reflecting its essential role in initiating MMR. In contrast, the endonuclease activity of PMS2 appears to be dispensable for spermatogenesis as $PMS2^{E702K}$ males are fertile [58].

2.4 Mismatch Repair in Eukaryotes

DNA mismatch repair is present from bacteria to humans, and many key features of MMR in bacteria have been preserved in eukaryotes. The methyl-directed MMR system in *Escherichia coli* has been extensive studied, and the MMR proteins involved in this pathway are well characterized. MutS, a highly conserved MMR protein, initiates DNA repair by binding directly to DNA mismatches. MutL, another highly conserved MMR protein, is recruited to the heteroduplex DNA in a MutS- and ATP-dependent manner. The assembly of the MutS-MutL-DNA ternary complex activates the endonucleolytic cleavage activity of MutH, an endonuclease that binds in a sequence-specific fashion to hemimethylated dGATC sequences, sites of *dam* methylation in *E. coli*. In newly replicated DNA, the daughter strand is transiently unmethylated leading to MutH-directed nicks specifically on the unmethylated stand at the nearest GATC sequence either at 5′ or 3′ of the mismatch. This methyl-directed stand break is the actual signal that targets repair exclusively to the newly synthesized DNA strand containing the error. With the help of MutL, this nick in the nascent stand serves as an entry point for helicase II that separates the two strands [59, 60]. The nicked strand is cleaved by one of four single-strand exonucleases – ExoI, ExoVII, ExoX or RecJ [61], resulting in the generation of a gapped DNA intermediate. The single-strand gap is bound by single strand DNA binding protein (SSB). DNA polymerase III then completes the gap filling step, and DNA ligase seals the nick restoring the duplex to the parental configuration [1, 2, 4, 6].

MMR in eukaryotes (Fig. 2.2) retains many of the key features of the *E. coli* methyl-directed MMR pathway, including substrate specificity, bidirectionality of the excision process and nick-directed strand specificity, but bears some notable differences [4, 62]. Bacterial MutS and MutL proteins are homodimers, while their eukaryotic homologues function as heterodimers comprised of two related but distinct protein subunits. There are several MutS and MutL homologues in eukaryotes, and the different combinations of subunit partner dictate their substrate specificity and cellular function (Table 2.1). MutSα, a heterodimer of MSH2 and MSH6, targets both base mispairs and +1 and much less frequently, +2 IDLs; MutSβ, a heterodimer of MSH2 and MSH3, directs the repair of IDLs [63, 64]. MutLα, a heterodimer of MLH1 and PMS2, is the chief MutL homolog in human cells and supports repair initiated by either MutSα or MutSβ with MutLβ, MLH1-MLH3, playing a lesser role. A minimal human system has been

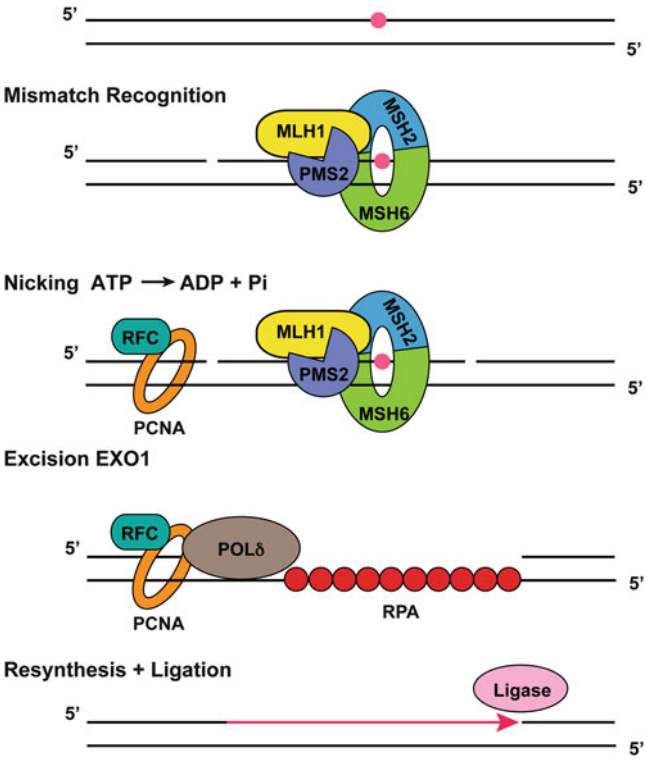

Fig. 2.2 Schematic representation of 5′-directed eukaryotic MMR. Mismatch recognition of by a MutSα (MSH2-MSH6)-MutLα (MLH1-PMS2) complex leads to the formation of a ternary complex in an ATP-dependent manner. PCNA and RFC loaded onto the newly replicated DNA helps trigger the endonuclease function of PMS2, and the recruitment of EXO1 resulting in the generation of a gapped DNA. The RPA-stabilized single strand DNA is then resynthesised by high fidelity Polδ and sealed by DNA ligase I

Table 2.1 Functions of human MMR proteins involved in replication correction

MMR proteins (*Homo sapiens*)	Molecular function
MutSα (MSH2-MSH6)	Recognizes base-base and 1-2 base IDL mismatches
MutSβ (MSH2-MSH3)	Recognizes IDL mismatches
MutLα (MLH1-PMS2)	Matchmaker, participates in multiple steps of MMR PMS2 endonuclease activity
RFC complex	Loads PCNA and helps direct excision polarity
PCNA	Recruits MMR proteins to mismatches, directs strand excision Facilitates excision and DNA repair synthesis
EXO1	5′-3′ ssDNA exonuclease
DNA POLδ	High fidelity repair synthesis
RPA	Binds to ssDNA during excision and DNA synthesis
DNA ligase	Seals nicks after DNA synthesis

reconstituted with purified proteins. These *in vitro* reconstitution studies indicate that a 5′-directed G:T mismatch repair reaction requires the following protein components: MutSα, MutLα, single strand DNA binding protein RPA, exonuclease EXOI, PCNA replication clamp, the RFC clamp-loader complex, HMGB1, DNA polymerase δ, and DNA ligase I [65–68].

2.4.1 Mismatch Detection by MutS Homologues

Recent studies have revealed the crystal structures of human MutSα bound to several mismatched DNAs and human MutSβ bound to unpaired DNA loops [32, 69, 70]. Overall, these structures closely resemble those obtained earlier for bacterial MutS complexes, but there are some notable differences [71, 72]. In contrast to homodimeric bacterial MutS proteins, the structure of human MutSα is derived from a heterodimer between full-length MSH2 and a truncated MSH6 lacking 340 amino acids at the N-terminal, a region that is not present in bacterial MutS proteins. In the complex with a series of DNA substrates, this MutSα heterodimer forms an asymmetric *θ*-shaped clamp. Each subunit retains a five-domain configuration: mismatch binding domain, connector domain, long α-helical lever domain, clamp domain, and a composite ATPase domain found in the ABC transporter protein superfamily. The DNA is sharply kinked at the mismatch but only MSH6 makes specific binding with the mispaired bases. Two composite ABC transporter ATPase domains are connected to the distant mismatch-binding site via long α-helical levers. The structure of human MutSβ utilized full-length MSH2 and trimmed MSH3 missing the N-terminal 210 amino acids. The overall shape of the MutSβ-IDL complexes is similar to that of human MutSα-DNA complex, with five structural domains in both MSH2 and truncated MSH3 subunits. In marked contrast to MutSα, the mismatched IDLs associate mainly with MSH3 but also interact with MSH2 though to a lesser extent.

The MutS proteins belong to the ABC (ATP binding cassette) ATPase transporter superfamily, and their ability to bind and hydrolyze ATP is essential for MMR function [2, 4, 6]. Each dimeric MutS subunit contains two composite ATPase sites located at the C-termini but opposite from the mismatch binding sites. Both MutSα and MutSβ show asymmetric ATP binding and hydrolysis in the two subunits, with the site in MSH6 or MSH3 bearing higher affinity for ATP and the site in MSH2 having higher affinity for ADP [73–77]. Several groups have reported that MutSα acts as a "molecular switch" involving communication between the mismatch recognition and the nucleotide association sites [78, 79]. MutSα recognizes mismatches during DNA scanning and can do so in the presence of ADP or absence of nucleotide [80, 81]. When bound to a mismatch via interactions involving the DNA binding domain of MSH6 first targets the lesion site in an ADP dependent-manner, which inhibits its ATPase activity but promotes MSH2 ATP-binding ability. Consequently, ATP can be transferred to the MSH6 subunit, leading to the release of MutSα from mispaired DNA. Therefore, mismatch recognition

by MutSα induces a rapid exchange of ADP for ATP, which in turn mediates the conformational changes that alter MutSα associations with DNA. *In vitro*, MutSα forms a clamp like structure that diffuses along the DNA. ATP binding but not ATP hydrolysis is essential for this procedure [78, 79, 82]. And the MSH2 subunit plays a key role in modulating all aspects of the ADP/ATP cycle, thus regulating MutSα mismatch recognition activity [83, 84]. Recent studies confirm that MutS undergoes both large and more subtle conformational changes during MMR initiation [85, 86]. ATP binding by MutSα is also required for its interaction with MutLα, leading to the excision process [6, 62]. The same mismatch recognition mechanism may hold true for MutSβ activity [70].

2.4.2 Multitasking by MutL Homologues

The MutL proteins function as "matchmarkers" to stimulate the activities of other MMR partners. Four MutL homologues, MLH1, MLH3, PMS1, and PMS2, have been identified in human cells [18, 19, 87, 88]. hMutLα, a heterodimer between MLH1 and PMS2, is critical for post-replication repair. hMutLβ, a heterodimer of MLH1 and MLH3 may function as a "backup" for MutLα in DNA metabolism. hMutLγ, composed of MLH1 and PMS1, participates in meiosis [2, 3]. hMutLα binds nonspecifically to DNA and is a weak ATPase, possessing asymmetric nucleotide binding sites, one in each subunit. Whereas MLH1 possesses a high affinity ATP binding site, PMS2 has lower affinity for ATP [89–91]. ATP binding and hydrolysis is essential for MutLα-mediated repair excision and re-synthesis. In a reconstituted human MMR system from purified proteins, hMutLα controls the termination of mismatch-provoked excision [67]. The C-terminal regions of hPMS2 and hMLH3 subunits of MutLα and MutLγ, respectively, possess a cryptic endonuclease activity that can introduce additional single-strand breaks into the pre-nicked strand [92, 93]. Recent structural studies on the endonuclease domain of bacterial MutL from *Bacillus subtilis* provide a plausible molecular model by which MutL licenses its endonuclease activity in a MutS-dependent manner [94]. The endonuclease activity utilizes a conserved $DQHA(X)_2E(X)_4E$ catalytic motif along with three shorter motifs that together constitute the active site for endonuclease activity. A regulatory domain with a cluster of negative charges is proposed to limit access to DNA to prevent inadvertent strand cleavage. A regulatory Zn^{2+} ion bound to a metal binding site modulates the relative orientations of the regulatory and catalytic sites. The activation of MutLα endonuclease depends on its interaction with PCNA and RFC, thus providing a loading site for MutSα-activated EXO1 [92, 95]. The extensive role of PCNA in supporting multiple steps of MMR reflects the physical association of the MMR machinery with the replication machinery or replisome. This has been observed for both bacterial and eukaryotic MMR systems and underscores the need to better understand how the replisome and MMR machinery coordinate their activities [96, 97].

2.4.3 Strand Excision and Gap Filling

The excision step of eukaryotic MMR is mainly performed by 5′-3′ single-strand exonuclease EXO1, which supports both 5′ and 3′ nick-directed MMR [98]. *In vitro* studies reveal that Human EXO1 can interact with MSH2 and MLH1 [99, 100]. In a 5′-directed mismatch excision, EXO1 can be activated in the presence of MutSα or MutSβ and RPA; in a 3′-directed MMR reaction, the activity of EXO1 requires the MutLα endonuclease, which is mediated by PCNA and RFC [66, 92, 101]. The structure of hEXO1 N-terminal catalytic domain suggests that the turnover of EXO1 activity depends on MutSα [102].

Replication processivity factor PCNA is an important player in the initiation and DNA resynthesis steps of MMR [103–105]. PCNA is a homotrimer that, with the aid of RFC, a clamp loader, forms a sliding clamp that encircles DNA. During DNA replication, PCNA functions as a matchmaker that interacts with multiple MMR proteins, including MutSα, MutLα, RFC, EXO1 and DNA polymerase [2, 3]. In the present of a clamp loader RFC, the PCNA sliding clamp can be loaded onto the 3′ terminus of an Okazaki fragment, or onto the 3′ end of the leading strand that has a nick, thus allowing its association with the replicating DNA polymerase [4]. Previous studies suggest that PCNA may also contribute to localize MutSα and MutSβ to mispairs in the newly synthesized DNA strand [106, 107].

Single-strand DNA-binding protein RPA is another essential MMR partner, which seems to be involved in all stages of repair. RPA binds to the nicked heteroduplex DNA before MutSα, MutLα, DNA complex formation, triggers mismatch-provoked excision, protects the single-strand DNA gap generated during excision, and facilitates DNA resynthesis [66, 67, 108, 109]. In canonical MMR, the gap filling step is catalyzed by the replicative DNA polymerases δ and ε. Recent work in *S. cerevisiae* has shown that Polδ and Polα preferentially carry out lagging strand synthesis while Polε carries out leading strand synthesis. MMR harnesses these polymerases to ensure high fidelity repair that is its hallmark [110].

Interestingly, it has been appreciated for some time that MMR proteins participate in somatic hypermutation at immunoglobulin (Ig) loci in activated B cells. In mice missing MSH2, MSH6 or EXO1, levels of mutations in Ig variable regions drop precipitously giving rise to a limited repertoire of antigenic diversity. Somatic hypermutation begins with the deamination of deoxycytosines to deoxyuracil catalyzed by activation-induced deaminase (AID). The creation of G/U mispairs creates a target for MutSα and MutLα resulting in the recruitment of an error-prone DNA polymerase eta (Pol-η) in a process mediated by mono-ubiquitinated PCNA [10]. Remarkably, it has been recently shown that a related, highly mutagenic pathway exists in non-lymphoid cells that are exposed to certain DNA modifying agents including S_N1 DNA alkylators that give rise to O^6-methyl-G residues (O^6meG) [111]. MutSα and MutLα target the resulting O^6meG/T mispairs and utilize Pol-η to complete gap repair resulting in a highly mutagenic process. This so called "non-canonical" MMR pathway may be an underappreciated factor contributing to carcinogenesis in somatic cells.

2.5 MMR and DNA Damage Signaling

MutS proteins can also identify mispairs containing damaged or modified bases that arise from cellular responses to certain types of DNA damaging agents, including O^6meG resulting from S_N1 DNA methylators, 8-oxoguanine, 6-thioguanine, 5-fluoro-deoxyuridine, cisplatin adducts, and several environmental carcinogens [1, 3, 5, 112]. O^6-methyl-guanine (O^6meG) is the most relevant lesion produced by S_N1 alkylators, such as N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) or N-methyl-N-nitrosoures (MNU) [113, 114]. The association of MutSα with O^6meG mispairs has been observed in several *in vitro* studies and requires the ATPase activity of MutSα [115].

S_N1 DNA methylators modify all four DNA bases producing targets for correction by the base excision repair pathway, but it is O^6meG that is largely responsible for the cytotoxicity of this class of DNA alkylators that are commonly used in chemotherapy regimens. The resulting cell cycle checkpoint arrest and apoptosis in cells treated with MNNG or MNU or the antimetabolite 6-thioguanine requires functional MutSα and MutLα MMR proteins. Thus, MMR-deficient cells are tolerant of the cytotoxic effects of these drugs, reflecting a critical role of the MMR system in DNA damage signaling, cell cycle checkpoint arrest and apoptosis [3, 116].

In the alkylation-directed DNA damage signaling, hMutSα and hMutLα can trigger many downstream targets, such as ATR, ATM, Chk1, Chk2, p53 and p73 [5]. A number of reports indicated that MutSα and MutLα could physically associate with ATR, ATM, Chk1 and p73 in response to several DNA damaging agents [115, 117–119]. MutLα and MutSα contribute to recruit PCNA, ATR and Chk1 to chromatin after MNNG treatment [120].

The precise mechanism by which cells initiate a MMR-dependent DNA damage response is not fully understood yet. Any model must accommodate the fact that MMR, by directing excision exclusively to the newly synthesized strand, must leave the O^6meG lesion in the intact strand. Furthermore, *in vitro* DNA binding studies clearly establish that MutSα recognizes O^6meG/T mispairs, but not O^6meG/C [115, 121]. Finally, the S_N1 DNA methylators-induced checkpoint response takes place in the second cell cycle after exposure to drugs [122, 123]. Two models that have received some attention are futile cycle and direct signaling. The “futile cycle” model posits that an error-prone DNA polymerase can bypass the alkylated base leaving O^6meG in the parental strand. Since DNA polymerase will, with low frequency, misincorporate dT opposite O^6meG, subsequent rounds of replication will regenerate the original O^6meG/T mispair triggering a second round of MMR. As MMR proteins can only target the newly synthesized DNA strand, the O^6meG will never be removed, thus inducing the repeated loading of the mismatch binding proteins. The iterative rounds of futile MMR eventually lead to DNA breaks and cell death [124, 125]. In support of this scheme, York and Modrich have showed repeated rounds of excision repair of O^6meG lesions *in vitro* using mammalian proteins [121]. However, separation-of-function alleles in mouse *MSH2* and *MSH6* in which MMR is abolished but the apoptotic response to DNA damaging agents is retained

suggest that futile cycles of MMR cannot be the only pathway [55, 56]. Jiricny and co-workers have observed the accumulation of single-strand DNA in response to MNNG treatment in mammalian cells. These single-strand DNA regions coated with RPA may activate the checkpoint machinery via interaction with ATR without resorting to multiple rounds of MMR [123, 126]. The "direct signaling" model suggests that at the DNA damage site MMR proteins serve as a scaffold to recruit ATR-ATRIP complex and activate the subsequent downstream damage response [127–129]. Indeed, ATR and ATRIP localize to the O^6meG:T mismatches in the presence of MutSα and MutLα *in vitro* [115]. Co-IP experiments reveal that MutSα and MutLα function as a sensor to recruit the checkpoint proteins ATR, TopBP1, and Chk1 after MNNG treatment [120]. Cisplatin treatment triggers MSH2-deficient recruitment and activation of ATR [130]. The role of MMR proteins in the DNA damage response remains an area of active study.

2.6 Future Directions

Many important problems remain to be solved to better understand the role of MMR in cancer avoidance. Obtaining a detailed understanding of the molecular mechanisms underlying post-replication MMR remains a goal. *In vitro* studies of MMR proteins harboring single amino acid changes found in HNPCC patients can provide new insights. A number of studies have focused on such mutant proteins from budding yeast and human counterparts. Two recent examples include studies of hMSH2(M688R), hMSH2(G674A) and hMSH6(T1219D) mutant proteins that provide insights into discrete steps of MMR and the resulting mutator phenotype [131, 132]. How is chromatin structure modulated in the context of post-replication MMR? Recent work has established that histone nucleosomes, the building block of chromatin, can inhibit mismatch recognition and the subsequent excision step in some, but not all cases [133–135]. In turn, MMR can inhibit nucleosome deposition in a process that may be regulated by physical interactions between MutSα and CAF-1 a chromatin remodeling protein [136]. How do microRNAs regulate MMR protein levels and what are the consequences of misregulation of these microRNAs? Recently, microRNA-21 whose overexpression has been linked to colorectal cancer among others, appears to down-regulate hMSH2 and hMSH6 [137, 138]. How does aspirin and other nonsteroidal anti-inflammatories modulate cancer risk in HNPCC/Lynch syndrome carriers? The Colorectal Adenoma/carcinoma Prevention Programme (CAPPII), a genetically targeted chemoprevention trial of 937 Lynch syndrome patients followed over 6 years, examined the long-term effect of aspirin on cancer risk in HNPCC carriers and concluded that "substantial protection" against colorectal cancer was afforded by aspirin [139]. Earlier studies showed that aspirin could suppress MSI in nonapoptotic colorectal cancer cells missing MMR proteins whereas apopototic cells retained high levels of MSI; aspirin and related nitric oxide-donating aspirin could also increase the lifespan of a Lynch syndrome mouse model by roughly

20 % [140, 141]. Perhaps aspirin has a pro-apoptotic function in MMR-deficient cells that are undergoing oxidative stress. Understanding how MMR is modulated by many factors remains an important goal.

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Chapter 3
New Insights into Lynch Syndrome Diagnosis

Emile Coetzee, Ursula Algar, and Paul Goldberg

Abstract Colorectal cancer (CRC) is the third most common cancer worldwide. Lynch syndrome accounts for 1–3 % of patients developing colorectal cancer. This autosomal dominant disorder is caused by germline mutations in the DNA mismatch repair (MMR) genes MLH1, MSH2, MSH6 and PMS2. A mutation in one of these genes is characterised by the development of CRC and various other associated cancers at an early age. The diagnosis of Lynch syndrome has evolved over the last two decades to include family history, tumour histopathological characteristics, immunohistochemistry, testing for microsatellite instability as well as germline genetic testing as modalities for making the diagnosis. By identifying families and individuals with Lynch syndrome, individuals can be enrolled in focussed screening programmes that have been shown to decrease mortality from colorectal cancer. In this chapter we define the terms "HNPCC", "Lynch syndrome" and "Familial colorectal cancer syndrome X" and discuss the different diagnostic modalities. We propose a logical and cost-effective algorithm to diagnose Lynch syndrome, by appropriately using all the diagnostic modalities in the at-risk individual.

Keywords Lynch Syndrome • HNPCC • Diagnosis • Mismatch repair • Immunohistochemistry • Genetic testing

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3.1 Introduction

Colorectal cancer (CRC) is the third most common cancer and cause of cancer-related death worldwide [1]. Although the majority of individuals who develop CRC have sporadic disease, up to 20 % may have inherited a predisposition to develop it [2]. Lynch syndrome is the most common form of hereditary CRC and accounts for between 1 and 3 % of patients with these tumours [3]. This autosomal dominant disorder is due to germline mutations in DNA mismatch repair (MMR) genes. It is characterised by the development of colorectal cancer as well as endometrial cancer and various other cancers at a young age [4]. MMR genes implicated in Lynch syndrome include MLH1, MSH2, MSH6 and PMS2 [3, 5]. Abnormalities in the function of these MMR genes lead to errors during DNA replication, in particular microsatellite instability (MSI) [6]. MSI can be found in over 90 % of tumours in individuals with Lynch syndrome, but can also be found in approximately 15 % of sporadic cases of CRC [5]. In almost all sporadic cases of MSI, a mutation in the MLH1 gene due to hypermethylation in the MLH1 promoter is present, and is not related to any inherited factor. Patients with a germ line mutation should be distinguished from those with a sporadic form of CRC. Methods to identify patients with a germ line mutation will be discussed in this chapter.

It is important to identify individuals with germ line mutations as these individuals can be enrolled in screening programmes to allow for polyp and early cancer detection. Intensive screening for colorectal cancer by colonoscopy as well as prophylactic gynaecological surgery reduces the incidence of Lynch syndrome related tumours and mortality [7, 8]. Family history and clinical criteria suggest Lynch syndrome the definitive diagnosis requires confirmation with germ line testing [9].

3.2 Nomenclature

The terms HNPCC, Lynch syndrome and Familial CRC Type X are often confused and used inappropriately in the literature.

Hereditary non-polyposis colorectal cancer (HNPCC) was originally defined by the Amsterdam 1 criteria to distinguish this form of inherited CRC from familial adenomatous polyposis (FAP) [10]. The term HNPCC was therefore used before the molecular aetiology of this disease was discovered.

Germline mutations are identified in only about 50 % of individuals who meet the Amsterdam criteria and many of the tumours from individuals who meet the Amsterdam criteria also do not show features of MSI [11–13].

The term Lynch syndrome is reserved for individuals with a known mutation in one of the MMR genes. Familial Colorectal Cancer Type X should be the term used to refer to those families who meet the Amsterdam Criteria, but do not have MSI-H tumours [14]. HNPCC should be used as an umbrella term including both these groups, although calls have been made to retire the term [15].

3.3 Detection Methods

3.3.1 Methods Based on Family History

3.3.1.1 Amsterdam Criteria

In 1990, the International Collaborative Group on HNPCC (ICG-HNPCC), comprising 30 experts from eight countries, met in Amsterdam and proposed a set of criteria to identify families who were likely to have an autosomal dominant inherited colon cancer predisposition [16]. The criteria generated (known as the Amsterdam criteria) were not intended for diagnosis, but rather to identify families that should be referred for mutation analysis, thereby allocating resources to an appropriate at-risk population. The criteria are listed in Table 3.1.

These criteria provided a basis for uniformity in collaborative studies and most investigators made use of them. The criteria were criticized for excluding extra-colonic Lynch syndrome associated cancers, therefore excluding many families from being offered genetic testing.

These shortcomings were recognised and a new set of criteria were drawn up at a meeting held in Coimbra, Portugal, in 1998. This resulted in a definition of HNPCC (Table 3.2) and the revised criteria listed in Table 3.3 [10].

When setting the new criteria, the ICG-HNPCC decided the criteria should be simple and not differ too much from the original criteria. The criteria should be clinical and accurate to ensure that families meeting these criteria would have a high likelihood of having HNPCC. The main difference between the new criteria (known as the Amsterdam II Criteria) and the Amsterdam I criteria was the inclusion of extra-colonic HNPCC-related tumours. Among all these tumours, cancer of the endometrium, ureter and renal pelvis and small bowel cancers have the highest relative risk [10].

3.3.1.2 Bethesda Criteria and Revised Bethesda Criteria

The use of the Amsterdam II criteria to identify patients with HNPCC is limited because the sensitivity is 78 % [12]. Improved understanding of the clinical and histological manifestations led the National Cancer Institute (NCI) to hold an

Table 3.1 Amsterdam criteria

At least three relatives with colorectal cancer, one of whom should be a first degree relative to the other two
At least two successive generations should be involved
At least one colorectal cancer should be diagnosed before the age of 50
FAP should be excluded
Tumours should be verified by pathological examination

Table 3.2 Definition of HNPCC (Lynch syndrome)

Familial clustering of colorectal and/or endometrial cancer
Associated cancers: Cancer of the stomach, ovary, ureter/renal pelvis, brain, small bowel, hepatobiliary tract and skin (sebaceous tumours)
Development of cancer at an early age
Development of multiple cancers
Features of colorectal cancers:
1. Predilection for proximal colon
2. Improved survival
3. Multiple colorectal cancers
4. Increased portion of mucinous tumours
5. Poorly differentiated tumours
6. Tumours with marked host-lymphocytic infiltration and lymphoid aggregation at the tumour margin
Features of colorectal adenoma
1. Numbers vary from one to few
2. Increased proportion of adenomas with villous growth pattern
3. High degree of dysplasia
4. Rapid progression from adenoma to carcinoma
High frequency of MSI
Immunohistochemical loss of MLH1, MSH2 or MSH6 protein expression
Germline mutation in MMR genes (MSH2, MLH1, MSH6, PMS1, PMS2)

Table 3.3 Amsterdam 2 criteria

At least three relatives with HNPCC-related cancer (CRC, cancer of the endometrium, small bowel, ureter or renal pelvis)
One should be a first degree relative of the other two
At least two successive generation should be affected
At least one should be diagnosed before the age of 50
Familial adenomatous polyposis should be excluded
Tumours should be verified by pathological examination

international workshop on HNPCC in 1996. The aim of this meeting was to clarify the role of genetics in the pathology of HNPCC and to develop a set of criteria for the identification of colorectal tumours that should be tested for the presence of microsatellite instability (MSI) [17].

A set of guidelines, called the Bethesda Guidelines (Table 3.4) were proposed [18]. It was estimated that the guidelines would potentially apply to 15–20 % of all colorectal cancers. Elements of the Bethesda Guidelines included both criteria for assessing tumours in families meeting the Amsterdam Criteria as well as other characteristics. The criteria would provide a sensitive set of guidelines that would include almost all HNPCC-associated colorectal cancers as well as many sporadic cancers. MSI-testing would then be used to exclude the individuals lacking microsatellite instability, who are highly unlikely to have Lynch syndrome. Tumours testing MSI-high (MSH-H) could then be further tested with immunohistochemistry and the patients with tumour that displayed loss of one of the MMR proteins should then be offered genetic testing.

Table 3.4 Bethesda guidelines

1. Individuals with cancer in families that meet the Amsterdam criteria
2. Individuals with two HNPCC-related cancers, including synchronous and metachronous colorectal cancers or associated cancers
3. Individuals with colorectal cancer and a first degree relative with colorectal cancer and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma; one of the cancers diagnosed at the age <45 years, and the adenoma diagnosed <40 years.
4. Individuals with colorectal cancer or endometrial cancer diagnosed at the age <45 years
5. Individuals with right-sided colorectal cancer with an undifferentiated pattern (solid/cribiform) on histopathology diagnosed at age <45 years
6. Individuals with signet-ring-cell-type colorectal cancer diagnosed at age <45
7. Individuals with adenomas diagnosed at age <40

Table 3.5 Revised Bethesda guidelines [19]

Tumours from individuals should be tested for MSI in the following situations:
1. Colorectal cancer diagnosed in a patient less than 50 years of age
2. Presence of synchronous or metachronous colorectal, or other HNPCC-associated tumours, regardless of age
3. Colorectal cancer with MSI-H histology, diagnosed in a patient less than 60
4. Colorectal cancer diagnosed in one or more first-degree relatives with an HNPCC-related tumour, with one of the cancers being diagnosed under the age of 50
5. Colorectal cancer diagnosed in two or more first- or second-degree relatives with HNPCC-related tumours, regardless of age

Resources would therefore be allocated to test only tumours with a high likelihood of having MSI.

Even though the Bethesda guidelines are used to identify tumours to be tested for MSI, the goal is to identify patients with Lynch syndrome [19]. It was therefore important to test how well the Bethesda guidelines did in identifying patients with MLH1 and MSH2 mutations. Data from these trials suggested that the criteria needed to be updated [12]. For this reason the Revised Bethesda Guidelines (Table 3.5) were drawn up, after a meeting held in Bethesda in 2002.

In a multicentre prospective study the revised Bethesda guidelines were shown to identify patients at risk for HNPCC with a sensitivity of 81 %, specificity of 98 % and positive predictive value of 29 %. It would, therefore, be reasonable to use these criteria to identify those patients who should have MSI tested [20].

3.4 Laboratory Methods

3.4.1 Histopathological Identification

Studies have suggested that MSI-H tumours may share morphologic characteristics that differ from non-MSI-H tumours [21, 22]. These tumours are more

likely to be mucinous type or have a signet ring cell component, have a solid cribiform growth pattern, and show increased tumour-infiltrating lymphocytes [2, 17, 22].

The features on histology should alert both the pathologist and clinician to test the tumour for MSI. The pathological features of undifferentiated (solid/cribiform) growth pattern and signet-ring type have been included in the Bethesda criteria. However, the exact role of histopathology for screening for MSI-H tumours remains unclear. The sensitivity for signet-ring type histology or an undifferentiated (solid/cribiform) growth pattern is low for patients over the age of 45 [10, 17]. Sensitivities of features, such as increased tumour-infiltrating lymphocytes, proved promising in some studies [22, 23], but not in others [21]. Currently, histopathology is used as adjunct to the Bethesda criteria to select those tumours to be tested for MSI.

3.4.2 Immunohistochemistry (IHC)

In Lynch syndrome, there is an inherited mutation in the gene coding for one of the mismatch repair (MMR) genes MLH1, MSH2, MSH6 and PMS2. Tumours of patients with these mutations have a functional loss of one of these mismatch repair proteins or gene products, and more than 90 % of these tumours will lack the expression of the involved protein [24–26].

Monoclonal antibodies to mismatch repair proteins are commercially available. When the specific gene product is expressed, the stain is positive and the MMR gene is present. When the protein is not expressed, the stain will be negative, indicating a mutation in the MMR gene. If all the MMR gene products are present on IHC, the patient is very unlikely to have Lynch syndrome and further genetic testing should not be offered.

IHC is less labor intensive, does not require a skilled molecular geneticist and has a shorter turnaround time than MSI testing. IHC can also aid in identifying the specific mismatch repair gene that is not expressed, therefore directing genetic testing. Studies validating IHC however, were performed with different patient populations, and with different aims. There are also numerous other biological, technical and clinical factors that affect the interpretation of these studies, all influencing validation of IHC as a universally accepted test.

In a review of the published literature on the sensitivity and specificity of IHC for MLH1 and MSH2 in predicting MSI in general, the sensitivity of IHC in predicting MSI was 90 % and the specificity greater than 99 % [27]. When the accuracy of IHC from different laboratories was tested, most laboratories produced similar results when staining for MSH2, but there was significant inter-laboratory variability when staining for MLH1. Factors influencing the accuracy and reproducibility of IHC include biological as well as technical factors.

3.4.2.1 Biological Factors

Gene mutations Different gene mutations have different variations of protein expression. For example, most MSH2 mutations are protein truncating; therefore most tumours from patients with MSH2 germline mutations will have absence of MSH2 protein when stained for by IHC. Conversely, approximately half of the mutations in MHL1 genes are missense mutations, resulting in expression of proteins that are catalytically inactive but antigenically intact [26, 28, 29]. These findings may explain the lower sensitivity in predicting germline mutations in MLH1 as well as the focal weak staining that is sometimes observed [30].

Antibodies MLH1 and MSH2 antibodies are most commonly used for IHC staining. Although more infrequently, Lynch syndrome is caused by mutations in the mismatch repair genes MSH6 or PMS2. These mutations would therefore be missed if IHC using antibodies from MLH1 and MSH2 are used [27].

MMR Protein interactions Interactions among mismatch repair proteins can affect the sensitivity of IHC. MMR proteins are only stable when they are in heterodimer pairs. It has been observed that MSH2 forms heterodimers with MSH3 and MSH6, forming a functional complex MutSα [31]. MSH6 however, can only dimerize with MSH2. The result of this pairing is that if there is a mutation in MSH2, MSH6 has no heterodimer partner and that tumour will stain negative for both MSH2 and MSH6 proteins. If there is a mutation in the MSH6 MMR, MSH2 can still dimerize with MSH3 and is therefore stable. Tumour tissue, in this instance, will stain positive for MSH2, but MSH6 will be absent [32]. MLH1 dimerizes with PMS2 and forms a functional complex MutLα [33–35]. MLH1 can also pair with PMS1 and MLH3. PMS2, however, can only pair with MLH1. If there is a germline mutation in the MLH1 gene, PMS is unstable and both MLH1 and PMS2 will be absent on staining. When there is a mutation in PMS2, the MLH1 will pair with PMS1 or MSH3 and will therefore be stable. In such a tumour PMS2 will stain absent, but MLH1 will stain positive [32].

In some MHL1 mutations, missense mutations results in proteins that is catalytically inactive but antigenically intact. The IHC staining for MLH1 in these tumours will also show presence of the MLH1 protein, but PMS2 staining will be absent. Additional staining with MSH6 and PMS2 antibodies therefore increases sensitivity of IHC.

3.4.2.2 Technical Factors

Protocols The importance of standardized IHC laboratory protocols has been evaluated in studies, and such protocols should be implemented at all laboratories offering IHC testing [36].

Antibody Clones Accurate and successful staining depends on the specific clone for a particular protein that is used. Differences in reproducibility and sensitivity of different clones were shown by some authors [37].

Adequate tissue sampling Adequate tissue sampling cannot be overemphasized. Identical samples will yield similar results. Discordant samples often yield inaccurate results. For example, frozen tissue used for MSI testing and paraffin block of different tumour areas used for IHC has been shown to yield different results. The most accurate results between IHC and MSI testing have been demonstrated using identical tissue samples for both methods using microdissection techniques [38].

3.4.3 MSI Testing

Microsatellite instability is the responsible carcinogenic pathway in approximately 15 % of all sporadic cases of colorectal cancer and all of the cancers associated with Lynch syndrome [39]. Microsatellites are repetitive short DNA sequences occurring throughout the genome. The majority of these sequences are non-coding. The even spacing between microsatellites is a sign of a healthy genome.

MSI is defined as altered lengths between microsatellites due to deletions or insertions and is associated with heterozygosity of loss of mismatch repair genes [40]. Tumour tissue is tested for MSI with a PCR-based test and this is compared to normal tissue from the same patient. Extra-colonic tumours associated with Lynch syndrome can also be tested for MSI [41]. MSI is reported as either MSI-high (MSI-H) or MSI-low (MSI-L). MSI-H is defined as instability of more than 30 % of the loci compared to that of normal tissue [42]. If there is no MSI in tumour tissue, it is referred to as microsatellite stable (MS-S). Tumours with MSI-L behave very similar to MS-S tumours [43]. For individuals with a tumour that is either MS-S or MSI-L, the likelihood of having a mutation in a MMR gene is very low and these individuals should not be offered germline testing [41, 44].

MSI determination is the current "gold standard" for testing tumour tissue for mismatch repair competency and is sensitive and highly reproducible [45]. The test is labour intensive, time consuming and requires a skilled molecular geneticist [43]. The result of the MSI test can be important in surgical decision making, because it might influence the extent of colonic resection. Unfortunately, the MSI test results are often not available at the time of surgical resection.

When tumour tissue tests MSI-H, IHC, will also be required to identify the specific mismatch repair gene mutation that is involved. For these reasons, several studies have compared MSI testing to IHC [3, 20, 28, 43, 46–54]. Shia comprehensively reviewed these studies [46]. The studies were divided into 2 groups: the first group included studies assessing IHC testing for MLH1 and MSH2 (with or without MSH6), whereas the second group included studies assessing IHC for MLH1, MSH2, MSH6 and PMS2.

The sensitivity for IHC with MLH1 and MSH2 antibodies was 85 %, compared to the sensitivity of 93 % of MSI testing. The low sensitivity of IHC in this group was largely attributed to the low rate of MLH1 mutation detection of 74 %. When

all four antibodies were used in IHC (group 2) the sensitivity of IHC was 92 %, equivalent to MSI testing. This improvement in sensitivity is due to the ability of PMS2 in predicting MLH1 missense mutations [46].

MSI is currently still regarded as the gold standard screening test for MMR mutations. When comparing MSI versus IHC as screening of Lynch syndrome, IHC is a feasible tool to use, but only if all four proteins are stained for. The advantages of being a simpler, more inexpensive test with shorter turn-around times makes IHC an attractive option. It also helps to identify the mutated gene, therefore directing genetic testing [46].

3.4.4 Genetic Testing

Ten to fifteen percent of sporadic colorectal cancers express MSI, and therefore MSI is not specific for Lynch syndrome [45, 55]. The deficient MMR in sporadic CRC cases is almost always due to MLH1 deficiency secondary to hepermethylation of the 5′ CpG Island in the MLH1 promoter, leading to transcriptional silencing [56]. The V600E mutation in BRAF is associated with MSI-H colorectal cancers but not associated with Lynch syndrome [57–60]. The presence of BRAF V600E mutation therefore excludes Lynch syndrome in a patient with a tumour that has loss of MLH1 on IHC with specificity of ~100 % [45].

When MSI and/or IHC suggests that there is a mutation in one of the mismatch repair genes and BRAF mutation testing is negative for MLH1 deficient tumours, the patient should be offered genetic testing on a blood sample [55, 60]. Mutations in MLH1 and MSH2 account for 70–90 % of all cases of Lynch syndrome. MLH1 is situated on chromosome 3p21 and MSH2 on 2p22. The genes for MLH6 and PMS2 are situated on chromosomes 2p16 and 7q11 respectively. Commercial testing is available for MLH1, MSH2, MSH6 and PMS2 and is done by gene sequencing or by in vitro synthesized protein assays [61]. These tests are very expensive and should therefore be done only on individuals where there is strong suspicion of a germline mutation. Genetic testing for the first member of the family is known as mutation detection and costs around $1,500.

When testing for MMR genes in the index patient, it should include full gene sequencing and large re-arrangement testing. Large re-arrangements account for 20 % of the known mutations [32]. Identification of a germline mutation is diagnostic for Lynch syndrome. Once a mutation is identified in a family, the other members of the family can be tested only for that mutation. Testing for only one mutation is much more straight forward and less expensive.

Hundreds of different mutations in the MMR genes in Lynch syndrome have been reported, with the incidence of different mutations varying in different populations. MLH1 is by far the most common MMR gene involved, followed by MSH2. MLH6 and PMS2 mutations are rarer [4, 44, 62].

IHC helps to direct germline testing. Loss of MSH6 or PMS2 on IHC suggests a mutation in the corresponding gene and testing for the specific gene should be

undertaken [32, 63]. With the loss of MLH1 on IHC, the decision making is more complex. Genetic testing is preceded by testing for the BRAF mutation. If the BRAF mutation is not present, genetic testing for MLH1 is pursued. In this situation, even if no MLH1 germline mutation can be found, the diagnosis of Lynch syndrome due to an undetectable mutation can be made. When IHC demonstrates absence of both MLH1 and PMS2 gene product, genetic testing for PMS2 is not indicated [14].

If IHC shows absence of MSH2 and MSH6, genetic testing should be started by analyzing the MSH2 gene, as Lynch syndrome is more frequently associated with a mutation in the MSH2 gene. Another germline mutation in a gene called the EpCAM gene, has recently been identified in a subset of families with Lynch syndrome with loss of MSH2 on IHC [64]. The incidence of an EpCAM mutation may be as high as 30 % when IHC shows absence of MSH2 [65]. Many laboratories now include EpCAM testing as part of the analysis for MLH2. If a mutation in the MSH2 gene is not identified, the MSH6 gene should be analyzed. Loss of MSH2/MSH6 on IHC is strongly associated with a germline defect of the MMR. If genetic testing does not detect a germline mutation in MSH2, EpCAM or MSH6 in an individual with loss of MSH2/MSH6 on IHC, the diagnosis of Lynch syndrome due to an undetectable mutation should be made [14].

Germline gene testing should only be done after adequate genetic counseling in a multidisciplinary environment. Genetic counseling is discussed in detail elsewhere in this book. If a member of a family test positive for the identified mutation, that member should be enrolled into a surveillance program. Family members who do not have the mutation can be discharged from further surveillance.

In cases where genetic testing is negative for a mutation or a mutation of undetermined significance is detected, genetic tests are considered indeterminate or uninformative [66]. Families with a history in keeping with Lynch syndrome, but without a detected mutation are at a lower risk for development of colorectal cancer than individuals with Lynch syndrome [67].

3.5 Familial Colorectal Cancer Syndrome X

Families who meet the Amsterdam II criteria with no identifiable deficiency in one of the mismatch repair genes should be considered to have Familial colorectal cancer syndrome X. The risk for developing colorectal cancer in these individuals are lower than in families diagnosed with Lynch syndrome, and they are not at increased risk for extra-colonic malignancies [68].

The gene(s) responsible in these families have not been identified yet, but seems to be inherited in an autosomal dominant fashion. Because the gene(s) are not identified, clinical testing is not available and all family members should be subjected to surveillance [32].

3.6 Algorithm for the Diagnosis of Lynch Syndrome

As part of the work-up for any patient with malignancy, a detailed family history should be obtained [4]. The clinician dealing with colorectal cancer should also enquire about family history of Lynch syndrome-associated malignancies. It is the duty of the treating physician to draw up pedigrees, thereby identifying at-risk first- and second degree relatives. This information must be clearly documented and all tumours from patients from families meeting the Amsterdam II and Revised Bethesda criteria should be further investigated. Genetic counsellors and nurses are often better equipped than surgeons to identify and contact relatives who are at risk. In certain centres, genetic counsellors and nurses receive in-depth training in genetics and are trained to help people at-risk or affected by diseases with a genetic component. Genetic nurses can:

- Perform risk assessment
- Analyse the genetic contribution to disease risk
- Discuss the impact of risk on health care management
- Provide genetic education
- Nursing care to patients and families and
- Conduct research in genetics

Genetic nurse counselors are a cost effective alternative to clinical geneticists [69]. They add another dimension of skills, but also a level of empathy and understanding, often lacking in a busy surgical out-patient department. If available, the genetic counsellor or genetic nurse counsellor should be involved very early in the management of a patient suspected to have Lynch syndrome. The formulation and maintenance of family trees would be a start of a cancer registry.

Tumour histopathology from an individual without a significant family history that meets the Revised Bethesda criteria should be subjected to further testing. MSI or IHC testing, using all four MMR proteins, can be used as first line screening test to diagnose Lynch syndrome. It seems reasonable and cost effective to use IHC as initial screening. If MSI is used as initial screening test, all tumours that are reported as MSI-H should undergo IHC testing to help identify the MMR that may be responsible.

Patients with tumours that show absence of one of the MMR proteins on IHC should be offered germline genetic testing. Genetic testing should be tailored to the IHC results (see Fig. 3.1).

If a germline mutation is identified on genetic testing, the diagnosis of Lynch syndrome is confirmed. Family members of such an individual should be offered genetic counselling and testing for that specific mutation. If a family member tests positive for the mutation, that individual should be enrolled in a surveillance program. Family members without the mutation can be discharged from surveillance. A co-ordinated predictive testing and colonoscopic surveillance program has been shown to extend the life of a compliant individual with Lynch syndrome, by about 20 years [7].

If no germline mutation is identified, the diagnosis of Familial Colorectal Cancer Syndrome X should be entertained. All at-risk family members of such an individual should be enrolled in a surveillance programme.

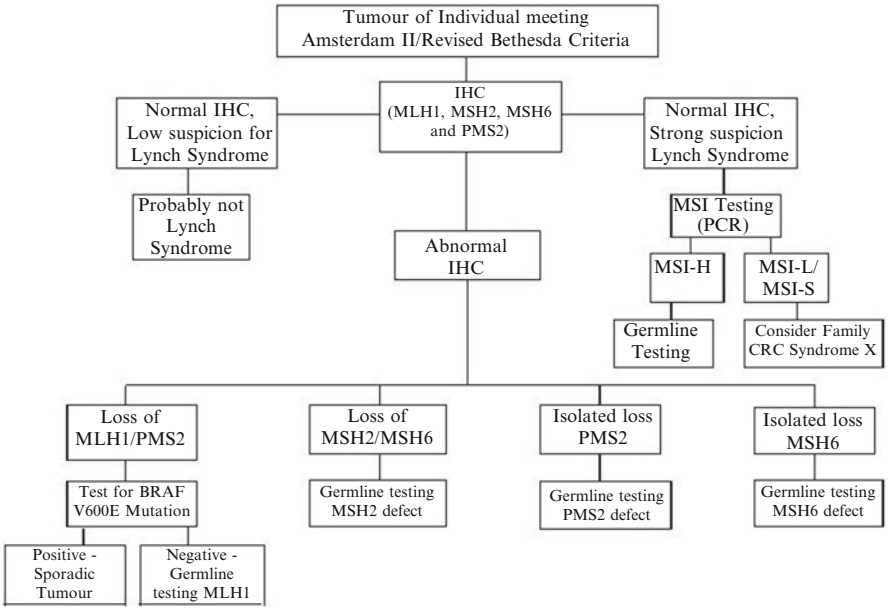

Fig. 3.1 Algorithm for the diagnosis of Lynch syndrome [40, 70]

3.7 Summary and Future Directions

The integration of molecular biology in the diagnosis of inherited colorectal cancer has become part of everyday practice when managing patients with a familial predisposition to colorectal cancer. Identifying patients with mutations in MMR genes has resulted in directed surveillance programmes that include prophylactic surgery when indicated, resulting in improved outcomes for patients with Lynch syndrome. A simple and cost-effective algorithm for the diagnosis of Lynch syndrome is the cornerstone in identifying patients with this inherited disorder. The future in the diagnosis of Lynch syndrome will be directed in point-of-care tests identifying MSI or IHC, thereby influencing surgical decision-making and family surveillance strategies in a cost-effective manner.

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Chapter 4
Genetic Testing, an Optimal Strategy for Lynch Syndrome Identification

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Abstract During the last few decades, cancer genomics has facilitated the discovery of underlying causes implicated in the development of hereditary colorectal syndromes, such as Lynch syndrome. This heterogeneous disease usually arises as the consequence of germline mutations in DNA mismatch repair (MMR) genes. The most prominent feature of defective MMR system is genomic instability, which is manifested as microsatellite instability (MSI) in more than 90 % of Lynch-syndrome associated cancers. Molecular characterization of MSI by fragment analysis and absence of MMR proteins by immunohistochemistry (IHC) in tumor tissues is useful for selecting at-risk patients who might be carriers of mutations in MMR genes or EPCAM. EPCAM is epithelial cell adhesion molecule gene, which, when mutated, affects the translation of MMR gene MSH2. The diagnosis in these cases is confirmed by identifying the mutation utilizing sequencing or other sequence detection methods or testing for large deletions and rearrangements of MMR genes. Clinical management of confirmed Lynch syndrome patients differs from those with sporadic colorectal cancers and also offers an opportunity to provide predictive screening for family members to determine mutation carriers. Susceptible members are offered regular clinical surveillance, which is beneficial for early diagnosis of colorectal and endometrial cancers and in reducing cancer morbidity. Despite the complexity of molecular diagnostic procedure, it is the most accurate diagnostic method for identifying patients with Lynch syndrome. In the future, the advances in next-generation sequencing methods and instrumentation could provide even more accurate and straightforward diagnostic modality due to simultaneous detection of mutations, large deletions, chromosome rearrangements and copy number variations using different library preparation methods and sequencing approaches.

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4.1 Introduction

The genomic era has provided a rapid expansion of our understanding of molecular mechanisms responsible for tumor initiation and progression. Genetic research identified an enormous number of molecules, which could be useful as biomarkers in genetic tests. However, the progress in this direction for most of the complex diseases, such as different types of cancer, has been slow. Only a few markers have been integrated into standard clinical practice so far [1]. The molecular complexity and heterogeneity of tumors attributes to low specificity and poor sensitivity of currently used markers, they are often detected only in the late stages of the disease and very few of them are specific for the particular disease [1–4]. However, for some of the inherited diseases, genetic testing also enabled identifying the family members, who inherited pathogenic gene alteration(s), and have thus greater risk for the development of the disease. Lynch syndrome is an example of a model disease, where genetic testing for cancer predisposition has become a standard component of clinical practice over the last few years [5]. Traditionally, since the first recognition of hereditary factors involved in certain types of colorectal cancers in 1913 by Warthin and later by Lynch et al. in 1966, the diagnosis was based on family history, early age of cancer onset, presence of multiple primary tumors, proximal colon involvement, accelerated carcinogenesis (i.e. small adenomas progress to carcinoma within 2–3 years, as opposed to 8–10 years in population with sporadic colorectal cancer), and certain histopathological features, such as poor differentiation, predominantly mucinous or signet-type differentiation, and tumor lymphocyte infiltration [6–8].

In 1991 a set of diagnostic criteria for identification of patients with Lynch syndrome was published, which was formulated at a meeting of The international collaborative group on HNPCC (ICG-HNPCC). This set became known as Amsterdam Criteria I [9]. These were later broadened in Amsterdam Criteria II to include the diagnostic role of extra-colonic tumors [10]. At this meeting the genetic role of mutations in mismatch repair (MMR) system and microsatellite instability (MSI) was recognized, and genetic testing was recommended for relatives of patients with Lynch syndrome. Around the same time, the new Bethesda Guidelines were established, which helped the clinicians in selection of patients and family members to receive the genetic testing [11].

Several other algorithms and cost-effective approaches have been simultaneously developed, mainly due to the cost and complexity of genetic tests available [4, 12, 13]. However, the advances in molecular methods reduced and are still reducing the cost of molecular testing, and new high-throughput methods are promising to reduce the complexity, effort, and time needed to screen all MMR genes for mutations.

4.2 Lynch Syndrome

Lynch syndrome (previously called hereditary non-polyposis colorectal cancer or HNPCC) is an autosomal dominantly inherited disorder of cancer predisposition, characterized by early onset, predominantly colorectal neoplasia as well as extracolonic cancers including cancer of the endometrium, ovary, stomach, small intestine, hepatobiliary tract, pancreas, upper urinary tract, brain, and skin [13–15]. It is the most prevalent hereditary colorectal cancer syndrome accounting for approximately 2–3 % of all colorectal cancer cases [16].

Lynch syndrome results from heterozygous germline mutations in mismatch repair (MMR) genes, which are involved in recognizing and repairing erroneous insertions, deletions and miss-incorporation of bases that can arise during DNA replication and recombination or due to chemical or physical DNA damage [16–21]. Approximately 97 % of all reported inherited mutations have been found in three different MMR genes, MLH1, MSH2 and MSH6 [22]. The remaining 3 % of pathogenic genetic changes associated with Lynch syndrome have been found in other genes, such as PMS2 (MMR gene) and EPCAM [16, 23–25]. EPCAM is located directly upstream of MSH2, coding epithelial cell adhesion molecule, and interestingly, various types of mutations in EPCAM gene can lead to dysfunctional translation of MSH2 affecting the function of MMR [23]. Functional impairment of MMR system results in the accumulation of spontaneous mutations (a mutator phenotype) that are thought to drive the neoplastic transformation process [26]. Most notably, MMR defects lead to microsatellite instability (MSI) detected in as numerous insertion or deletion mutations in short repetitive sequences (microsatellite sequences) [14]. Genes, containing these sequences are susceptible to frameshift mutations due to replication slippage, which lead to loss of function. Tumor suppressors TGFbRII, BAX, IGFRII, MSH3 and MSH6 are most commonly affected [27].

High level MSI (MSI-H) is characteristic for more than 90 % of Lynch syndrome-associated colorectal cancers [28]. MSI testing has been proven to be 90–95 % sensitive for diagnosing Lynch syndrome, however, mutations in MSH6 and MSH2 can give rise to a few microsatellite stable Lynch syndrome colorectal cancers (along with typical MSI-H Lynch syndrome colorectal cancers) [23]. Furthermore, MSI is not specific for Lynch syndrome as it occurs in 15 % of sporadic colorectal tumors and other tumors [29].

4.3 Genetic Testing

Genetic testing within medicine is becoming a valuable tool in diagnostics, screening and evaluating response to therapy. Traditionally, genetic analyses were used to diagnose the presence of monogenetic diseases, such as cystic fibrosis or

thalassemia. Advances in the research of diseases and evolving nature of genetic methods and technologies, enabled the development of several genetic tests, which could improve diagnosis, prognosis, treatment, screening, risk assessment, and prediction of treatment response or adverse effects of drugs [30]. Genetic testing is also a key to personalized medicine and can shift the health care from reactive to proactive or preventive in the case of inherited diseases [31]. According to the report of the Secretary's Advisory Committee on Genetics, Health, and Society (Department of Health and Human Services USA) the most appropriate (and widely accepted) definition of genetic test is:

> A genetic test involves the analysis of chromosomes, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), genes, or gene products (e.g., enzymes and other proteins) to detect heritable or somatic mutations, genotypes, or phenotypes related to disease and health. The purpose of genetic tests includes predicting risk of disease, screening newborns, directing clinical management, identifying carriers, and establishing prenatal or clinical diagnoses or prognoses in individuals, families, or populations. Whether a laboratory method is considered a genetic test also depends on the intended use, claim or purpose of a test [31].

The term genetic test encompasses different biochemical, cytogenetic, and molecular approaches or a combination of these methods to analyze DNA, RNA, chromosomes, proteins, and certain metabolites [31]. Biochemical assays are used for identifying the amount or activity of hormones or enzymes, and the presence of different metabolites in clinical samples and do not directly evaluate DNA. Cytogenetic testing refers to chromosomal studies detecting normal and abnormal chromosomes [32]. Molecular genetic methods examine DNA or RNA to identify alterations, such as mutations, polymorphisms, deletions, insertions, aberrant methylation patterns, etc. [31]. From the historical perspective, the first genetic testing began in the early twentieth century, with A. E. Garrod's description of inherited alkaptonuria and testing for homogentisic acids in urine, and T. Boveri's (and M. Boveri's, his wife) description of atypical chromosomes in cancer cells as the basis of their aberrant behavior [33–37]. In the last few decades, advances in genetic methodologies have enabled that genetic testing is becoming an integral part of clinical diagnostics. Emerging technologies, such as new high-throughput proteomic methods, hold promise to further expand our understanding of diseases and could be of use in simultaneous identifying of aberrant proteins [38, 39].

However, detailed genetic information of an individual and his/her family members also raised a few issues, concerning medical, psychological, social, ethical, and legal implications. For example, detecting germline mutations in Lynch syndrome provides predictive information about the risk of developing the disease in the next decades of individual's life. Knowledge of carrying a mutation that could cause cancer may cause psychological distress [40–42]. Furthermore, even negative result could be uninformative and could cause a false sense of security in an individual.

4.4 Current Guidelines for Selection of Candidates for Mutational Testing for Cancer Susceptibility

The primary goal of mutation testing for cancer susceptibility is to lower the cancer burden in the population by early detection of the disease. Screening for hereditary mutations in family members of an affected individual can identify at-risk carriers of mutation, who are offered surveillance. Regular surveillance in individuals carrying mutations in genes associated with Lynch syndrome has been shown to reduce risk of colorectal cancer development and may also be of benefit in the early diagnosis of endometrial cancer, which is also associated with Lynch syndrome [4, 13, 43]. Life-time risk of colorectal cancer development in individuals with MMR mutations approaches 85 % and 30–50 % for developing endometrial cancer depending on penetrability of mutated genotype [29, 44]. A large study, using data from literature and publicly available data sets of American population showed that the identification of mutation carriers by genetic testing could reduce colorectal and endometrial cancer incidence by approximately 44 % and 40 %, respectively [44]. In parallel, in a simulated population of 100,000 individuals, it was estimated that the treatment costs for these patients would be reduced by at least 39 % and absolute life-expectancy would be increased by 4.07 years.

Several different algorithms have been developed to identify the patients who should be tested for possible Lynch syndrome. In the beginning the Amsterdam Criteria I and II were usually used, however, with advances in genetic research it was established that these criteria are too stringent and miss as many as 68 % of patients with Lynch syndrome [10, 45]. A few years later, classical and revised Bethesda guidelines were introduced, which more accurately selected patients for genetic testing [11, 46, 47]. Several professional organizations established clinical practice guidelines for risk assessment, genetic testing, genetic counseling, and clinical management for patients and their families [13]. Among the most detailed and comprehensive are the criteria from National Comprehensive Cancer Network (NCCN), American Cancer Society, American Society of Colorectal Surgeons, Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group, and others [13, 48–50]. In Europe the biggest difficulty is to reach a consensus about how to establish uniform clinical guidelines and synchronize quality assurance of genetic tests, given the diversity and number of different countries. The largest depositories of information on genetic testing are maintained by European Society of Human Genetics – ESHG, EuroGenTest, The European Directory of DNA Diagnostic Laboratories – EDDNAL etc. The clinical guidelines for genetic testing in Europe are mostly set up in each member country separately in clinical laboratories performing mutational analyses and usually adopt established recommendations from Amsterdam Criteria II, revised Bethesda guidelines, NCCN guidelines and others.

Furthermore, computer algorithms and open source software for assessing the likelihood that the patient carries a mutation in MMR gene have been developed [45, 51–54]. The advantage of these customized models is identifying patients at risk for Lynch syndrome and for selecting patients for molecular investigation on the basis of family history. They may be particular useful if there is no tumor or insufficient tumor available for MSI or immunohistochemical (IHC) testing (NNCN Guidelines Version 2.2012, Colorectal Cancer Screening).

With advances in molecular methods and cost-reduction associated with establishing necessary infrastructure for performing genetic tests many laboratories now perform MSI and IHC testing as routine practice for all newly diagnosed patients with colorectal cancer (and endometrial cancers) regardless of family history. This strategy proved to identify more patients, who should benefit from further, more detailed genetic tests searching for mutations in MMR genes [48, 49, 55].

4.5 Methods Used in Clinical Setting for Diagnosing Lynch Syndrome

Recognizing genetic susceptibility in colorectal cancer patients is critical for patients with well-defined hereditary syndromes, such as Lynch syndrome. The clinical management of these patients differs from that of patients with sporadic type of colorectal cancer and furthermore, they tend to have a significantly better clinical outcome [56–59]. Since the integration of genetic testing into clinical setting in the last few years, it has been recommended that family members of the proband should be offered genetic counseling and subsequent DNA testing for identifying carriers of MMR mutations, followed by regular clinical screening for colorectal cancer and Lynch syndrome-associated cancers [60]. It was estimated that colonoscopic surveillance could decrease colorectal cancer incidence by 58 % and mortality by 78 % [61]. Removing colorectal adenomas during colonoscopy, which in patients with Lynch syndrome usually undergo a fast neoplastic transformation, successfully prevents development of cancer.

Any strategy for genetic testing in suspected Lynch syndrome involves several phases and it is usually performed in a stepwise manner [44, 61, 62] (Fig. 4.1). Initially, the proband with suspected Lynch syndrome, according to revised Bethesda criteria or other algorithm used in clinics, is usually tested for the presence of MMR genes by immunohistochemistry (IHC) and/or for MSI in tissues obtained during colonoscopy [13, 48, 55, 57, 61]. Some laboratories perform IHC in two phases: the first strategy is analyzing the expression of MLH1 and if negative, they follow with testing for the specific BRAF mutation (V600E). If that mutation is not found, they continue with MMR testing, however, if the BRAF mutation is present, the testing for Lynch syndrome is not necessary, as virtually 100 % of Lynch syndrome patients do not carry this mutation [27, 48, 63]. The second IHC strategy utilized is identifying the presence of MLH1 and

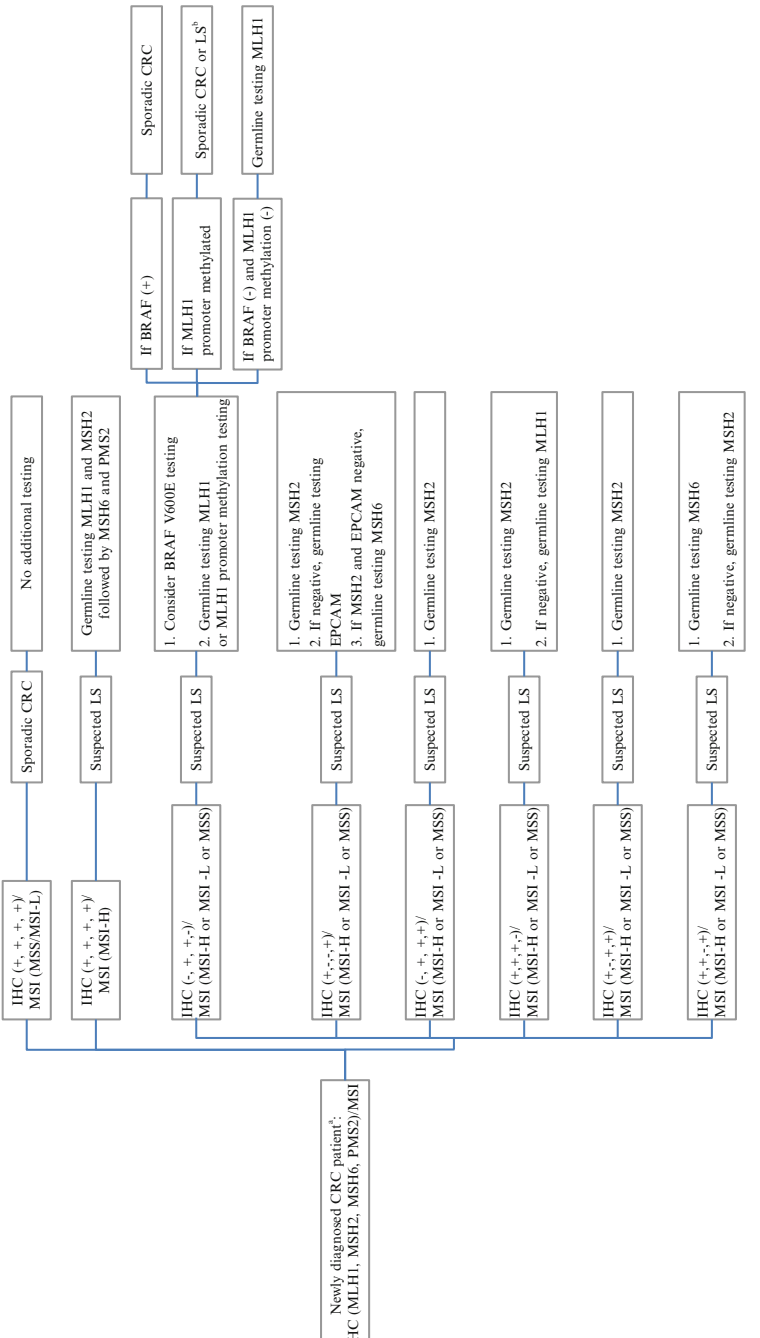

Fig. 4.1 A strategy for Lynch syndrome genetic testing in newly diagnosed CRC or endometrial cancer patient. Note: recommended combined IHC/MSI pre-screening approach was considered and that tissue samples are available. If tissue samples are not available, direct screening for MMR mutations should be considered (*LS* Lynch syndrome, *CRC* colorectal cancer)

[a]or endometrial cancer patient

[b]consider MMR mutation testing, if young age of cancer onset or significant family history

MSH2 in tissues, and if the assay is normal, then testing for other two MMR genes, MSH6 and PMS2, is employed [49]. However, a majority of clinical diagnostic laboratories perform testing for all MMR genes.

A combination of MSI and IHC screening testing was found to be the most cost-effective in selecting patients for MMR mutation testing, due to the fact that using either MSI or IHC testing alone resulted in false-negative results [46, 64]. Individuals with diminished expression of any of the MMR gene and showing MSI are usually further selected for mutation screening, the last phase of genetic testing for Lynch syndrome [50]. Mutation screening for germline mutations can be performed by obtaining DNA from blood. In addition, some centers perform analysis of biallelic hypermethylation of MLH1, before proceeding with mutation screening [5, 27, 65, 66]. Biallelic hypermethylation of CpG islands in MLH1 promoter is implicated in development of sporadic colorectal cancers.

4.5.1 MSI

The testing for MSI in Lynch syndrome patients began in the early 1990s of the previous century and one of the first strategies for defining MSI using microsatellite markers was discussed in 1997 at the National Cancer Institute (NIH) Workshop on HNPCC (later renamed into Lynch syndrome) [47, 67–70].

For the purposes of clinical diagnostics a marker panel (Bethesda panel) of two mononucleotide and three dinucleotide repeats (BAT-25, BAT-26, D5S346, D2S123, and D17S250) was initially recommended [11, 71]. MSI-H was defined as instability at two loci. Instability at one locus was defined as low level MSI (MSI-L) and tumors showing no instability at examined loci were defined as microsatellite stable (MSS). However, the distinction between these three features proved to be very difficult, therefore it was recommended to test additional markers. When greater than five loci were studied, MSI-H was defined as instability at more than 30 % of loci tested and MSI-L as instability at less than 30 % of loci tested. However, extensive research showed that mononucleotide repeats are more reliable in distinguishing MSI-H, MSI-L, and MSS tumors, and a panel of five mononucleotide repeats (BAT-25, BAT-26, NR-21, NR-24 and NR-27) is currently recommended for determining the MSI status of colorectal tumors [11, 28, 55, 62, 72–74]. Furthermore, this panel overcame one of disadvantages of the Bethesda recommended panel, that is, distinguishing MSS-L and MSI-H tumors, in cases where only dinucleotide markers were altered [28, 74–76]. Research showed that the instability of dinucleotide repeats was not associated with MMR-deficient tumors [73]. In addition, interpretation of allelic profiles using dinucleotide repeats is complicated by the presence of stutter, which is a PCR artifact produced by DNA polymerase slippage during amplification of regions containing microsatellites [73].

The sensitivity for the individual markers from this new panel ranged from 86 to 98 % with high specificity from 96 to 100 %, while the sensitivity from the original Bethesda panel was up to 94 %, however, the overall specificity of this panel

was only modest 25 % [28, 73, 77]. The new mononucleotide panel is currently recommended as a standard for MSI testing in Lynch syndrome and many laboratories have already integrated the methodology in routine clinical diagnostics [48]. Overall, the sensitivity of MSI testing using the new panel for identifying the individuals with Lynch syndrome has been estimated to range from 90 to 95 % [23]. However, several studies showed that tumors with mutations in MSH6 often do not show MSI [78–80]. MSI testing alone therefore is not sufficient, and combination of different approaches, such as IHC and MSI testing, followed by mutation screening when appropriate, is currently recommended [81].

The most common method to detect MSI is to measure the length of amplified DNA fragment, containing the microsatellite region [82]. The DNA fragments are amplified by polymerase chain reaction (PCR) using fluorescent-labeled primers, followed by separation of fragments with capillary electrophoresis on automated DNA sequencers and analysis of their length with appropriate software [82].

The drawbacks of this method are: (1) it is considered a pre-screening method, as it detects MSI, which is also present in sporadic colorectal cancers, (2) the detection using the first Bethesda panel of microsatellite markers requires comparison between tumor and normal tissues, (3) the tumor sample must contain a certain percentage of cells exhibiting MSI in order for reliable detection, (4) the method must be performed by a trained specialist on relatively expensive equipment, (5) it does not reveal the nature and location of MMR gene mutation, (6) false negative results due to large deletions, that include examined microsatellite loci (for example, deletions of MSH2 gene often include BAT-26 marker), (7) it is unable to identify MSH6-deficient colorectal cancers, showing no MSI, (8) stutter peaks, which are the result of DNA polymerase slippage during amplification, and (9) in some tumors, such as extremely mucinous tumors, it cannot be detected [27, 28, 62, 83].

The advantages are that after the initial cost of equipment and training of personnel, the assay is relatively cheap, sensitive, if the quality of samples is good, and can be performed on fresh, frozen or even formalin-fixed paraffin-embedded tissues. Goel et al., Patil et al., Suraweera et al., and other researchers improved the method using five quasi-monomorphic markers (NR-21, BAT-25, NR-27, NR-24, and BAT-26) by establishing the quasi-monomorphic variation range of each marker on a test group of normal individuals, thus omitting the use of paired tumor-normal tissues [28, 74, 84]. Their analyses showed that the five quasi-monomorphic markers detected MSI-H in cancerous tissues with a sensitivity ranging from about 86 to 95 % without the need for testing paired normal tissues, and a specificity in the range from 96 to 100 % [28].

Additional approaches of separating amplified products have also been employed, such as different heteroduplex analyses, using denaturing high-performance liquid chromatography (DHPLC) or polyacrylamide gels, however, mostly because of greater complexity and difficult optimization, capillary electrophoresis is preferred by most clinical laboratories. Several groups also tested alternative methods for detecting MSI with varying results. Janavicius et al. compared high-resolution melting (HRM) detection of two MSI markers, BAT-25 and BT-26, with standard fragment analysis on automated DNA sequencer [85]. Their analyses showed that HRM

could detect MSI-H with up to 99 % specificity. The method is attractive, because it is performed immediately after PCR, without the need of further processing the samples; therefore, it is less possible to contaminate the PCR products, and additionally, the time needed to process the samples is reduced.

4.5.2 Immunohistochemistry (IHC)

Immunostaining for the presence of MMR proteins is an inexpensive technique that is one of the most established techniques in routine clinical and pathology laboratories [49, 86–88]. The basis of the method is detection of MMR proteins in cells of a tissue section using specific monoclonal antibodies, which specifically bind to antigens, present in cells. The classical approach involves detecting one protein in one tissue sample on a glass slide. The newer technique, tissue microarray IHC, allows detection of targeted proteins on a large number of patient samples in an efficient and cost-effective manner.

The sensitivity and specificity of IHC is analogous to those of MSI analysis. Its sensitivity was estimated to be in the range from 83 to 94 % and the specificity around 89 % [48–50, 89, 90].

IHC can be performed only in tumor tissues, because absence of expression indicates a mutation or epimutation is present in affected MMR gene. IHC has an additional advantage compared to MSI analysis, namely, based on lack of expression of MMR gene, it indicates which gene could be affected [50]. This usually simplifies further analyses for determining the mutation in MMR genes, since the assessment which gene to test first can be made [50]. IHC is a valuable tool for detecting mutations, which are associated with loss of functional protein, such as nonsense and frameshift mutations, mutations that affect splicing sites or other important splicing regulatory sites, and large deletions, resulting in loss of certain MMR genes. One of the disadvantages of this method is that it cannot detect missense mutations or mutations, resulting in production of non-functional MMR protein [50]. Therefore, a positive expression of MMR proteins detected by IHC is not conclusive for elimination of Lynch syndrome diagnosis in patient. Another drawback is that the interpretation of results can be somewhat difficult, and experienced laboratory personnel is necessary [27]. The power of IHC for detecting loss of MSH2 and MSH6 is high, while on the other hand, the sensitivity for detecting MLH1 and PMS2 loss is lower [91]. Furthermore, loss of MSH2 or MSH6 expression indicates carriers of germline mutations in any of those two genes, but patients whose tumors show loss of MLH1 may either have hereditary or sporadic disease [92]. In the majority of sporadic tumors the loss of MLH1 expression is the result of promoter hypermethylation in CpG islands leading to somatic inactivation. The detection of MLH1 loss is therefore not specific for Lynch syndrome; however, a simple PCR-based method for detecting methylation of its promoter can guide further analyses. If methylation is present, further screening for mutations is usually not necessary, because inactivation of

MLH1 by methylation has been reported to be a rare cause of Lynch syndrome [93]. In addition, there is a strong relationship between the BRAF V600E mutation and hypermethylation of the MLH1 MMR gene [49, 94, 95]. Researchers have hypothesized that performing cost-effective BRAF testing on tumors with absent MLH1 staining might identify a group that is nearly entirely composed of sporadic colorectal cancers that would not benefit from MLH1 sequencing [49, 96]. However, a few studies showed that BRAF V600E mutation might coexist in MMR mutation carriers, but nevertheless, due to the lack of larger evaluation studies, it is still accepted that BRAF V600E activating mutation eliminates Lynch syndrome diagnosis [64, 95–100].

Traditionally, all four MRR proteins, MLH1, MSH2, MSH6, and PMS2 are assayed by IHC [101]. However, since PMS2 and MSH6 are obligatory dimerization partners with MLH1 and MSH2, it was hypothesized that testing for PMS2 and MSH6 expression would capture all mutations in MLH1 and MSH2, along with mutations in PMS2 and MSH6 [102, 103]. Despite the cost reduction of this approach, it is not widely used due to more complex interpretation of results [101].

4.5.3 Mutation Screening in Suspected Lynch Syndrome Patient

The last phase in diagnosis of Lynch syndrome is identifying the mutation in affected MMR gene. The demonstration of germline mutation in a patient is "the gold standard" for the diagnosis of this disease [27]. Although it is the most expensive and labor-intensive method, it is useful, because it allows for mutation-specific inexpensive germline testing in family members, thus enabling early detection of the neoplastic processes, removal of polyps, and reducing the cost of clinical management of these patients [27]. Another advantage is that it is performed on DNA isolated from peripheral blood, the most easily obtainable clinical sample.

Germline testing is usually performed by direct sequencing for detecting small intragenic deletions/insertions and missense, nonsense, and splice site mutations; typically, exonic or whole-gene deletions and duplications are not detected. Quantitative PCR, long-range PCR, multiplex ligation-dependent probe amplification (MLPA), high-resolution melting (HRM), and copy number variations (CNV) approaches utilizing different technologies, such as whole-genome sequencing or oligonucleotide arrays, are used for identifying large deletions and chromosome rearrangements [99, 104–108]. Several other indirect approaches, such as single-stranded conformation polymorphism analysis (SSCP), denaturing gradient gel electrophoresis or DHPLC, can be used to detect aberrant heteroduplexes, which are subsequently sequenced to confirm the nature of variation [109–116].

The most important advantage of these methods is accurate determination of mutation in MMR gene with sensitivity, reaching almost 100 % in the case of direct sequencing [27]. However, due to the hundreds of described mutations in MMR genes, the performance of other above mentioned methods is still below 100 %.

The drawbacks of these methods are complexity, cost, and subsequent interpretation of biological relevance of mutations, which have not yet been described in the literature.

4.5.4 Interpretation of Biological Data

Finally, the last step in deciphering the underlying cause of the development of Lynch syndrome is interpretation of the biological effect of the mutation or alteration found in MMR genes. MMR gene truncating mutations considered to be pathogenic have been identified in approximately 50 % of Lynch syndrome patients [14, 98]. A considerable number of other variations, such as missense substitutions, small in-frame deletions, and alterations of or near splicing sites, exist, which do not lead to premature termination of translation and have uncertain biological effect on pathogenesis of the disease [14, 117]. These variations represent a difficult problem for clinicians and genetic counselors, who must manage the patients and family members [118]. Furthermore, mutation screening with high-throughput next-generation sequencing methods leads to detection of an increasing number of novel missense, silent, and intronic variations [119].

These so called unclassified variants (UVs) could be associated with the disease phenotype or merely represent rare variants [117, 120]. Their biological effect is usually determined by (1) phenotype-genotype segregation studies, (2) the incidence of variations in population must be less than 1 %, (3) absence of variations in healthy control individuals, and (4) determining that they are associated with tumors exhibiting hallmarks of MMR deficiency, such as MSI or loss of MMR proteins [117, 118].

However, due to the inadequate amounts of clinical samples, small family sizes, insufficient analysis of clinico-pathological features, molecular parameters, and high variability in phenotypes among mutation carriers due to low penetrance of non-truncating mutations, the pathogenicity and biological effect of the inherited alterations is not clear [117]. Rasmussen et al. proposed that in this case the diagnostic procedure continues with a number of functional assays to determine their effect [26]. A number of molecular assays and in silico-based approaches have been employed by different research groups with varying success [14, 26, 118–125]. Furthermore, almost none of these tests is currently applicable in clinical setting, either due to the requirement of specialized molecular laboratory or due to the complexity of interpretation of the results. In addition, majority of VUs demonstrated intermediate activity, thus one could conclude that VUs alone could not be responsible for initiating and driving the neoplastic process [118, 126]. Further analyses are needed to evaluate the combined effects of other genetic variations in different genes, associated with genome integrity, and possible environmental contribution. Studies of different combinations of polymorphisms and mutations in yeast identified weak MMR alleles and MMR gene polymorphisms that are capable of interacting with other weak alleles of MMR genes to produce strong polygenic MMR defects [127].

4.6 Future Prospects

The advances in next-generation sequencing methods have greatly increased throughput and identified a vast number of novel variations associated with Lynch syndrome [119, 128, 129]. Furthermore, the cost per nucleotide sequenced is considerably lower compared to Sanger sequencing, although the number of samples and the read-depth of whole-genome sequencing usually increase the final cost [129]. Pritchard et al. developed directed approach for screening genes, implicated in Lynch syndrome and polyposis syndrome development, using massively parallel sequencing, called ColoSeq [129]. The first step is targeted capture of MMR genes, EPCAM, MUTYH and APC from isolated DNA, followed by labeling the library fragments of captured genes with specific bar-codes utilizing PCR-based approach, The 96 bar-coded libraries, representing 96 patient cases were then pooled and sequenced on next-generation sequencer. They detected a number of different mutations and variations with almost 100 % specificity. Promising approaches are also proteomics-based technologies, which could aid in discovery of relevant biomarkers in feces, blood or tissue biopsies [39, 130]. The integration of these novel genomic and proteomic methods could be helpful in identification of potential candidate minor variations, which could affect the development of tumors, identification of novel drug targets, and for establishing cost-effective screening methods.

4.7 Conclusion

Recent workshops on Lynch syndrome recommend genetic testing for MSI for all newly diagnosed patients with colorectal cancer, regardless of family history and application of Amsterdam or Bethesda criteria, since they are not reliable [48, 82, 131]. Several studies showed that combined approaches including IHC and MSI testing as pre-screening method applied to all newly diagnosed colorectal cancers significantly improved identifying patients with Lynch syndrome [27, 132]. Detecting MSI in colorectal cancer patients has prognostic implications, since it has been established that they might not benefit from fluoro-uracil based chemotherapy [133, 134]. However, in the last few years, different algorithms and strategies have been developed in order to aid the healthcare personnel in selecting the patients for genetic testing of MMR mutations, which is becoming more and more available [23, 135]. From the technical viewpoint, the Sanger sequencing is still the most accurate approach for detecting mutations in MMR genes (except large deletions and chromosome rearrangements), however, the next-generation sequencing instruments are also slowly paving their way into clinical setting. The later technology with its larger productivity and ability to process many samples could enhance the detection of Lynch syndrome patients. Furthermore, selected platforms have also the ability to detect not only single-nucleotide polymorphisms and mutations, but also larger aberrations [136]. In addition, whole-genome approach will greatly aid in deciphering the biological effect and penetrance of combinations of different alleles, and could thus open new avenues for personalized medicine.

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Chapter 5
Functional Analyses Help to Assess the Pathogenicity of MMR Gene Variants of Uncertain Significance

Minna Nyström and Minttu Kansikas

Abstract Knowing that inherited defects in mismatch repair (MMR) genes predispose to Lynch syndrome (LS), the identification of these mutations in suspected LS families is of prime importance. However, a major problem in the diagnosis and management of LS is the frequent occurrence of variants of uncertain significance (VUS) in the MMR genes. The consequence of a non-truncating mutation can vary from none to complete dysfunction of the protein. Thus, functional assessment by investigating how a non-truncating mutation affects the quantity and biochemical behaviour of the protein variant as compared to the wild-type protein has been shown to be an efficient manner to determine the pathogenicity of MMR gene variations. Furthermore, a stepwise assessment model emphasizing the use of family history and tumour pathological data to guide during the assessment process has been applied. Overall, the model utilizes data from incompletely validated assays supplemented with data derived from other sources such as from *in silico* analyses to classify VUS for clinical purposes.

Keywords Functional analysis • Lynch syndrome • Mismatch repair • Variant of uncertain significance

5.1 Introduction

Lynch syndrome (LS, often referred to as hereditary non-polyposis colorectal cancer syndrome; HNPCC; MIM# 120435) is highly associated with autosomal dominant inheritance of mutations in genes fundamental to the DNA mismatch repair (MMR) mechanism. The susceptibility genes include the most frequently affected

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MLH1 (MIM# 120436, RefSeq NM_000249.3), *MSH2* (MIM# 609309, RefSeq NM_000251.1) and *MSH6* (MIM# 600678, RefSeq NM_000179.2), and less frequently affected *PMS2* (MIM# 600259, RefSeq NM_000535) and *MLH3* (MIM# 604395, RefSeq NM_001040108.1). Discovery of an inherited deleterious mutation in a cancer patient permits predictive gene testing in the family and enables targeted cancer surveillance. Once a predisposing mutation is found in a family, frequent surveillance of the mutation carriers has been shown to significantly reduce mortality [1–3].

However, a major problem in the diagnosis and management of LS is the frequent occurrence of non-truncating mutations. As in all genes, a point mutation changing one amino acid in the polypeptide chain may have either harmful or no consequences in the protein function and is often difficult to distinguish from harmless polymorphisms. These missense mutations already account for 35 % of *MLH1*, 29 % of *MSH2*, 38 % of *MSH6*, 59 % of *PMS2* and 74 % of *MLH3* unique variations reported in the LOVD database (http://www.insight-group.org/; http://www.lovd.nl/). Many of such alterations are referred to as variants of uncertain significance (VUS) [4] due to the uncharacterized effect of the variation on the function of the polypeptide.

Several clinical guidelines have been established to distinguish the alteration underlying tumourigenesis. In fact, the clinical diagnosis of LS greatly relies on the Amsterdam criteria (AC) [5, 6] or the revised Bethesda guidelines [7], which take into account the age of cancer onset, the number and segregation of affected individuals in a family, and the level of microsatellite instability (MSI). The pathogenicity status of a variation is classically determined based on the conservation status and biochemical significance of the amino acid alteration, segregation of the mutation with the cancer phenotype in a family and on the MSI and MMR proteins´ immunohistochemical (IHC) statuses in the tumours of the mutation carriers [8, 9]. However, many colorectal cancer families do not fit these clinical criteria and segregation and tumour data is not always available to confirm LS diagnosis. Thus, where a VUS is identified, the characterization of its effect on the function of the polypeptide remains a challenge and calls for a unified assessment model to clarify its association with the observed disease phenotype in a family.

5.2 Functional Analysis of MMR Gene Variants

Functional assays aim to investigate how a non-truncating mutation affects the biological and biochemical behaviour of the protein variant as compared to the wild-type. Accordingly, in this chapter we are solely concentrating on assays, which study the functionality of the MMR protein variants *in vitro* or *in vivo*, and not on *in silico* alignment analyses used for pathogenicity prediction or steps preceding the protein production such as mRNA splicing or gene regulation. MMR specific functional assays can be differentiated into two groups where either the MMR ability of the variant protein is evaluated, mainly *in vivo* in yeast or *in vitro* with mammalian

cell extracts, or where a more specific feature and function such as stability, interaction with other MMR components, subcellular localization, or mismatch binding/release capability is assayed. The two types of assays complement each other as the MMR efficiency of the variant reveals its ability to repair mismatches without being functionally descriptive, whereas the more specific assays can help to further detail the cause of pathogenicity. This is clearly depicted by the variant data achieved through functional assays collected in the LOVD database (http://www.lovd.nl/).

5.2.1 *In Vivo MMR Assays in Yeast*

Yeast has proven to be an optimal model for studying the MMR mechanism and the functionality of the MMR protein variants *in vivo* due to the feasibility of yeast experiments. However, the conservation between human and yeast MMR mechanisms is limited and hence a fundamental restriction is that many of these assays rely on the homology between human and yeast proteins. Moreover, the use of a heterologous system may increase false positive or negative interpretations.

Several approaches using either a haploid or a diploid yeast strain as a host have been developed. Some of the first assays using *Saccharomyces cerevisiae* were based on interference of the human MMR polypeptide with the MMR system of yeast [10]. Since the natural MMR activity of a haploid strain can be rendered dysfunctional by introducing a functional human MMR protein into it, pathogenic human MMR protein variants can be detected by their inability to inactivate the yeast MMR [10, 11]. Alternatively, MMR deficiency can be revealed based on the phenotype of yeast strains expressing mutated yeast MMR genes but in that case allowing only conserved amino acids to be tested [12, 13]. The pathogenicity of MMR gene variations can also be studied by replacing the yeast orthologs of endogenous MMR genes by both human heterodimer partners, like *MLH1* and *PMS2* in a haploid strain and hence co-expressing them under a yeast promoter [14]. So far, the yeast approaches have been mainly used to assess variations found in *MSH2* and *MLH1* genes [10–14]. Furthermore, to overcome the limitations associated with the use of a heterologous system, an assay using yeast-human hybrid MMR proteins in a yeast context has been developed and thus far applied to *MLH1* variations and particularly those affecting the ATPase domain [15, 16].

5.2.2 *In Vitro MMR Assays in Cell Extracts*

In vitro MMR assays study the repair capability of human variant proteins in a homologous human MMR system and thus all variations, irrespective of their evolutionary conservation status, can be studied in these experiments, revealing information regarding the proteins ability to perform the multistep process of nuclear MMR. The limitations lie in the decreased ability to assess steps prior to the repair

e.g. protein expression and subcellular localization since the variant protein is constructed *in vitro* and its functionality assayed in ready-made extracts.

In vitro MMR assays are based on the ability of the human cell extract, which is complemented with a wild type or a variant MMR protein, to repair DNA mismatches. The variant or wild-type protein produced *in vitro* can be introduced into the nuclear or cytoplasmic extract deficient in the protein in question [17] or the human cell line deficient in the protein can be transfected with the protein cDNA prior to cell lysate preparation [18]. The error introduced in the DNA heteroduplex substrate is commonly a single nucleotide mismatch or an insertion/deletion loop of one or more nucleotides. Where detection of the repair is based on the restoration of a unique restriction site [19], the MMR efficiency is analysed by enzyme digestion and separation of different fragments by gel electrophoresis or in combination with fragment analysis where the repair efficiency measurements focus on the fluorescent signal peaks [20]. Another approach to detect repair is based on a construct where the reading frame of the *E. coli LacZ* gene is altered [21]. After repair, the substrate is transfected into MMR deficient bacterial cells and repair is determined through the changes in the ratio of white/blue plaques.

These human homologous *in vitro* MMR assays can be applied to most MMR genes, and thus far, assays using nuclear protein extracts complemented with the recombinant protein have been used to demonstrate MMR deficiencies in *MLH1*, *MSH2*, *MSH6*, and *MLH3* [17, 20, 22–25]. Moreover, the ability to assess *MSH3* variations has also been demonstrated, yet no pathogenic variations have been functionally assessed [26]. Transfecting a MMR deficient cell line with either wild-type or variant cDNA has been used to assess multiple *MLH1* variations [18, 27].

Furthermore, an approach assessing MMR protein function inside the mammalian cell has been demonstrated with some *MSH2* variations [28]. Here, cDNA is transfected together with a heteroduplex substrate into MMR deficient cells. Upon MMR restoration, expression of the EGFP reporter gene integrated into the substrate results in fluorescence detected by flow cytometry.

5.2.3 Protein Expression Analyses

Protein expression analysis is a method frequently used to indicate protein instability. Expression analyses can be carried out in various types of cells and are particularly useful in cases where a potentially pathogenic variant appears functional in an *in vitro* MMR assay when tested with a protein expressed under an efficient promoter. As MMR proteins function as heterodimers, recombinant MMR proteins are expressed with their native heterodimerisation partner in a cell of choice, e.g. *Spodoptera frugiperda 9* insect cells, which are favoured due to their highly conserved post translational modification systems. Alternatively, human cells deficient in the gene of interest can be co-infected with recombinant viruses or transfected with the recombinant cDNAs for protein production. The protein

content of the cell extracts can be visualized using conventional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting methods where expression differences between recombinant and wild-type proteins can be detected.

So far, decreased levels of variant proteins have been demonstrated in multiple LS-associated *MLH1*, *MSH2* and some *MLH3* variations [22, 23, 29, 30]. Furthermore, it has been suggested that of LS-associated *MSH2* variations, up to half could be linked to the disease phenotype through reduced protein expression [30].

5.2.4 Protein Interaction Analyses

The ability of the MMR proteins to interact with their native interaction partners is a particularly suitable functional analysis for human MMR proteins which function as heterodimers e.g. MutSα (MSH2 + MSH6), MutSβ (MSH2 + MSH3), MutLα (MLH1 + PMS2) and MutLγ (MLH1 + MLH3). For example, glutathione-S-transferase (GST) pull-down, co-immunoprecipitation and yeast two-hybrid assays all utilize one of the two heterodimerisation partners to detect interaction between the subunits. Even though interaction assays examine only a specific function of the variant protein they can be helpful to further characterize the reason for MMR deficiency found by another method. Furthermore, interaction assays have been particularly useful in characterizing functional domains of key MMR proteins.

The GST pull-down method assays the physical interaction between two proteins. Interaction is detected when the *in vitro* translated and GST-tagged protein immobilized on glutathione beads interacts with its radioactively labelled interaction partner. This method has been applied to study the interaction efficiency of native heterodimerisation partners as well as of other key MMR components. For example, variations in *MLH1* have been shown to cause deficient interactions between MLH1 and its counterparts PMS2 [31, 32] and EXO1 [33, 34], and variations in *MSH2* to interfere interactions between MSH2 and its counterparts MSH6 and MSH3 [35].

Co-immunoprecipitation is a similar commonly applied method used to study protein interactions in a manner where one MMR heterodimerisation subunit is pulled down with agarose beads coated with a specific antibody. After washing, only proteins immobilized to the agarose beads should remain and hence the presence of the interacting partner can be detected using conventional SDS-PAGE methods. Co-immunoprecipitation has been described for assaying deficiency of recombinant MLH1 and PMS2 protein interaction in MutLα [17, 22], recombinant MSH2 and MSH6 protein interaction in MutSα [29, 36, 37] and recombinant MLH1 and MLH3 protein interaction in MutLγ [23].

The yeast two-hybrid assay is an alternative *in vivo* approach to study protein-protein interactions. The protein of interest and its interaction partner are fused to the DNA binding domain and DNA activation domain of a reporter gene,

respectively. When interacting, the domains are brought together and the reporter gene is activated allowing the yeast cells to grow in a selective media [32, 38]. So far, this method has been applied to investigate the disability of MSH2 to interact with MSH3 and MSH6 [30] and MLH1 to interact with PMS2 and EXO1 [32].

5.2.5 Subcellular Localization Experiments

To study the subcellular localization of variant MMR proteins whose primary role is in the nucleus is essential when the variant shows functionality in the *in vitro* MMR assay, in which the variant protein is added to the ready-made extracts. Nevertheless, as has been indicated in the case of MLH3, the nuclear localization can be conditional [39] which complicates the interpretation of localization. Using a reporter gene such as EGFP integrated into the plasmid used to transfect the cells of interest, allows for the localization of the protein to be visualized. Several such experiments have demonstrated problems in nuclear transfer of LS-associated MLH1 and MSH2 variants, which have not only helped to determine the pathogenicity of the variants assayed but have also served to characterize the functional domains of the protein area the variations fall in [22, 34, 40].

5.2.6 DNA Binding and Release Experiments

DNA binding and release of the MutSα and MutSβ subunits is also a concern when looking at more specific assays to further detail the cause of pathogenicity. Worth noticing is that unlike expression and localization analyses, DNA binding and release experiments as well as those assaying protein interactions, characterize the type of pathogenicity revealed also by the *in vitro* MMR assays. As the MutS heterodimer complexes are responsible for initiating the MMR mechanism by recognizing and binding of mispair, DNA binding capability of variants can be assayed by incubating error substrates together with purified proteins. Efficient binding can be visualized through a label incorporated into the substrate or by electrophoretic mobility shift assay using a native polyacrylamide gel. Here the bound substrate will travel differently from the unbound substrate as MutS heterodimers are required to undergo a conformational change upon mismatch recognition and subsequent ATP-ADP exchange before releasing the site of mismatch. Interestingly, the steady state ATP hydrolysis and binding, ATP-ADP exchange as well as ATP-dependent disassociation from the mismatch have been shown to be affected by several *MSH2* variations, hence affecting the functions of MutSα in human [41, 42] and both MutSα and MutSβ in yeast [11, 12] MMR.

5.3 Assessing the Pathogenicity of MMR Gene Variants

As is evident, the methods to study MMR protein function are diverse and thorough, hence together they can be combined to form powerful tools for assessing the pathogenicity of MMR gene variants. As variants are subjected to different assays, more information becomes available to help characterize the given alteration. Recently, all data derived from published functional assays on MMR gene variations has been collected and listed. In 2007, an international multidiscipline scientific organization with a mission to improve the quality of care of patients and their families with any condition resulting in hereditary gastrointestinal tumours, The International Society for Gastrointestinal Hereditary Tumours (InSiGHT) (http://www.insight-group.org/), established a program with the Human Variome Project (HVP) aiming to collect information on all inherited variations affecting colon cancer genes. Subsequently, several of the gene mutation/variation repositories were brought together, forming the InSiGHT Colon Cancer Gene Variant Databases hosted on the Leiden Open Variation Database (www.lovd.nl/insight). This effort has resulted in a dramatic increase of variants listed and the process to handle the huge amount of data to help in pathogenicity assessments is still underway.

5.3.1 VUS Classification

The ultimate aim of the international collaborative working group on Lynch syndrome is to obtain a classification of the VUS based on the probability of being pathogenic using classes ranging from definitely pathogenic to not pathogenic or of no clinical significance according to published guidelines [43]. The InSiGHT Mutation Interpretation Committee has worked towards establishing rules for VUS classification with the use of a multifactorial likelihood model [43, 44] applied to MMR gene variants [45, 46]. Here, among clinical and tumour pathological data, results of functional assays help to differentiate between classes when submitting variant data into the LOVD database.

Currently, a varying amount of research is done on different variations across the different genes. Nevertheless, efforts have been made to clarify whether findings reported in the database indicate pathogenicity or not. Reported findings are categorized into five groups; ‘pathogenic’, ‘probably pathogenic’, ‘no known pathogenicity’, ‘probably no pathogenicity’ or ‘effect unknown’. In many cases, several entries including results of *in silico* predictions for a single variation exist and more commonly these are of different pathogenicity class pinpointing the need for individual variant evaluation in order to obtain the concluded pathogenicity per variation. Here, in Fig. 5.1 we try to summarize all reported pathogenicity classifications of unique single nucleotide substitutions of the five MMR genes, *MSH2*, *MSH6*, *MLH1*, *PMS2* and *MLH3*, across their functional domains as currently listed in the LOVD database. It is notable that the situation is, as expected, very different for the most commonly LS-associated genes *MLH1* and *MSH2* when compared to the other MMR genes

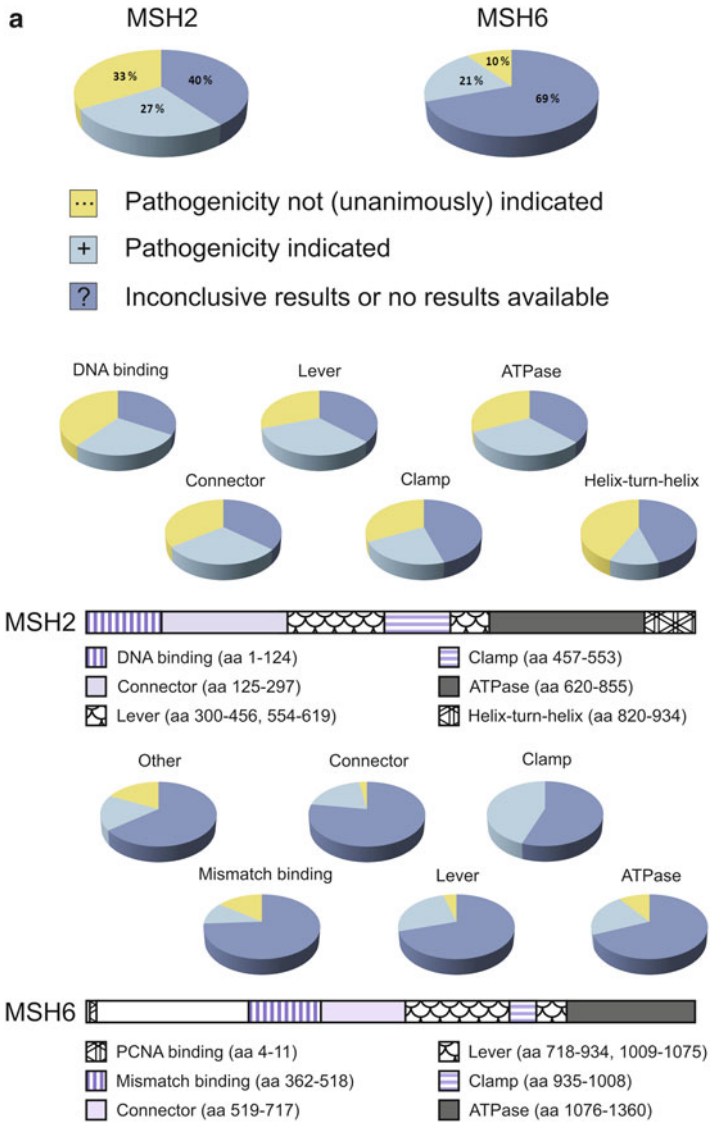

Fig. 5.1 A schematic representation of the information reported in the LOVD database regarding the pathogenicity of unique single nucleotide substitutions affecting (**a**) the MutSα and (**b**) the MutLα and MutLγ heterodimerisation proteins, and across their different functional domains: MSH2, n = 396 (DNA binding domain, n = 51; connector domain, n = 72; lever domain, n = 103; clamp domain, n = 31; ATPase domain, n = 97; Helix-turn-helix, n = 42); MSH6, n = 255 (PCNA binding domain, n = 0; mismatch binding domain, n = 27; connector domain, n = 40; lever domain, n = 47; clamp domain, n = 9; ATPase domain, n = 70; other regions, n = 62); MLH1, n = 469 (ATP binding and hydrolysis domains, n = 38; interaction domains, n = 128; other regions, n = 303); PMS2, n = 149 (ATPase domain, n = 7; interaction domain, n = 17; other regions, n = 125) and; MLH3, n = 55 (putative ATP-binding and hydrolysis domain, n = 0; interaction domain, n = 0; other regions, n = 55)

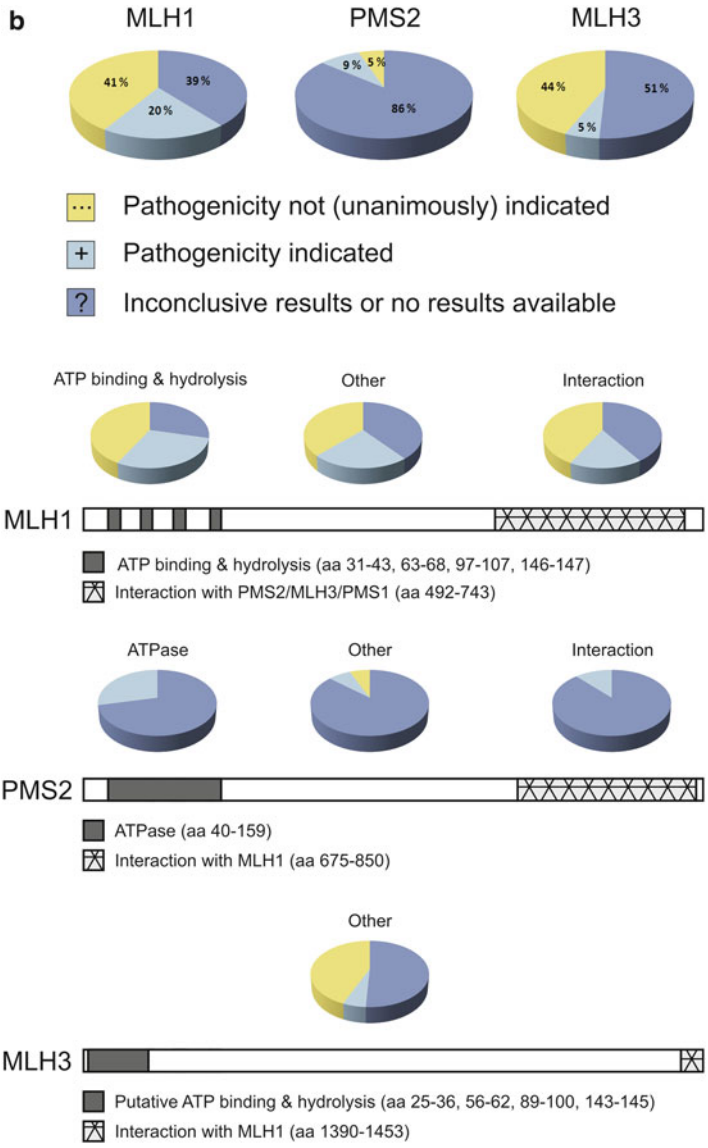

Fig. 5.1 (continued)

listed. In fact, pathogenicity or probable pathogenicity (+) is reported for 20 % of *MLH1* and 27 % of *MSH2* single nucleotide substitutions with only 39–40 % of the variations listed as 'effect unknown' (?). In the case of *MSH6*, a majority of the variations (69 %) have been reported as 'effect unknown' (?), whilst the amount of pathogenic or probably pathogenic (+) variations also represents approximately a fifth of the single nucleotide substitutions listed. As expected, the less studied *PMS2* and

MLH3 genes have many (86 % and 51 %, respectively) variations with 'effect unknown' (?). The pathogenicity information represented across the functional domains of the five genes is based on the amino acid boundaries of domains as previously summarized [23, 47, 48]. Although not obvious, functionally important domains, such as ATPase domains, do tend to have a higher amount of variations clearly indicated as pathogenic (+). Nevertheless, it is obvious that many of these variations, particularly ones reported with 'no known pathogenicity' only or with entries also indicating pathogenicity (…), are inaccurately categorized due to insufficient clinical, tumour pathological or functional data.

5.3.2 Assessment Model

To overcome the limitations of available functional analyses and due to the evident need to develop systematic methods that resolve the clinical significance of VUS, an assessment model for MMR gene variants has been described [49]. The model acknowledges the importance of appropriate VUS identification by emphasizing the use of family history, MSI and IHC data to ultimately identify the VUS by genetic testing in STEP1. For genetic sequencing dictated by the IHC and MSI results, a strategy taking into account the four MMR genes (*MLH1*, *MSH2*, *MSH6* and *PMS2*) together with the potential hypermethylation of the *MLH1* promoter region has been proposed [50]. In the case of a variation with a known effect, LS can either be confirmed as likely, or in contrast, ruled out in the absence of MMR gene variations. In the case of a VUS, however, further analysis is required to determine the effect of the variation on the function of the protein. After the identification of the VUS, the second step of the model suggests to combine data derived from an *in vitro* MMR assay together with data from an *in silico* analysis. Variations indicating MMR deficiency in these assays indicate LS, whereas variations with no apparent MMR deficiencies require a selection of biochemical assays for further characterization of the effect of the variation on the protein expression or other function (STEP3) that may have been missed at prior steps.

This kind of stepwise functional assessment of VUS has been shown to be an appropriate and efficient manner to determine the pathogenicity of MMR gene variations [48]. In order to distinguish pathogenic MMR variations from variants of uncertain significance the first two steps utilizing clinical as well as the laboratory and *in silico* data, seem to be sufficient for the majority of *MSH2* and *MLH1* variations, as STEP3 provides no imperative information concerning the variant pathogenicity. However, the importance of STEP3 assays are seen in the assessment of MMR proficient variations showing discrepant *in silico* results as their pathogenicity cannot be confirmed or ruled out after STEP2 only. *MSH6* variations may be applicable to the model if appropriate selection in terms of ruling out *MLH1* and *MSH2* variations and *MLH1* promoter hypermethylation is ensured prior to the completion of STEP2 [48]. So far, there are no reports of the assessments of *PMS2* and *MLH3* variants through this kind of a multistep model.

Since the three step assessment model seems to be a valuable tool for correctly identifying pathogenic LS susceptibility mutations, which in terms of clinical relevance mirror the efficiency and appropriate delivery of treatment, counselling and patient follow up, it is important to highlight some specific features arising from it. The absence of an MMR protein in IHC gives a good but not absolute indication of the causative gene responsible for the MSI phenotype and subsequent tumourigenesis. Thus, although pathogenic *MSH2* mutations are shown to be highly associated with the lack of protein expression in IHC analyses [42, 51], which is also frequently characterized by the absence of MSH6 [52], it has been shown, that the lack of MSH2 may also be caused by alterations in EPCAM, a gene not directly associated with the MMR mechanism [53]. Similarly, results demonstrating the lack of MLH1 expression may be misleading as the MLH1 protein, especially in sporadic cancers is often lost due to the hypermethylation of its promoter region [54], a phenomenon that is found to be associated with the p.Val600Glu alteration in protein kinase BRAF [55]. Remarkably, the sensitivity of IHC in predicting pathogenic *MSH6* mutations has been said to be as high as 90 % [56]. In addition, the presence of a protein cannot be implied to indicate its functionality as pathogenicity can be caused by functional problems not affecting the stability of the protein [22, 51]. Nevertheless, as indicated by the verification of the proposed model [48], of all STEP3 analyses applied, pathogenicity indicated by expression analysis was usually detected alone or in combination with another STEP3 assay result and only in a few cases was pathogenicity indicated by another STEP3 assay but not the expression analysis. For *MSH2* and *MLH1* variants, pathogenicity is reliably indicated by the STEP2 *in vitro* MMR assay as supported by other functional assays in STEP3. Nonetheless, when no indication of pathogenicity is seen in the *in vitro* MMR assay the importance of computational methods become apparent. *In silico* methods have been shown to have a high predictive value (88.1 %) when four different methods sorting intolerant from tolerant (SIFT), PolyPhen, A-GVGD, and BLOSUM62 matrix, are all in agreement [57] and when alignments are manually revised [44]. Subsequently developed *in silico* method, Pathogenic-or-Not Pipeline Prediction for mismatch repair system protein mismatch variants (PON-MMR) has been introduced to improve the consensus-based prediction accuracy [58].

5.4 How a Stepwise Assessment Model Can Guide a Clinician

Even though functional assays cannot be taken into routine clinical use without appropriate validation, they continue to be of great help in assessing variant pathogenicity and classifying VUS. The three step model described in the previous section [49] is a general model to assess the pathogenicity of different kind of VUS. However, dependent on the results from tumour pathological data in STEP1, a clinician may follow different strategies to continue the assessment process in an efficient manner. Here, we demonstrate how the IHC and MSI statuses may guide a clinician

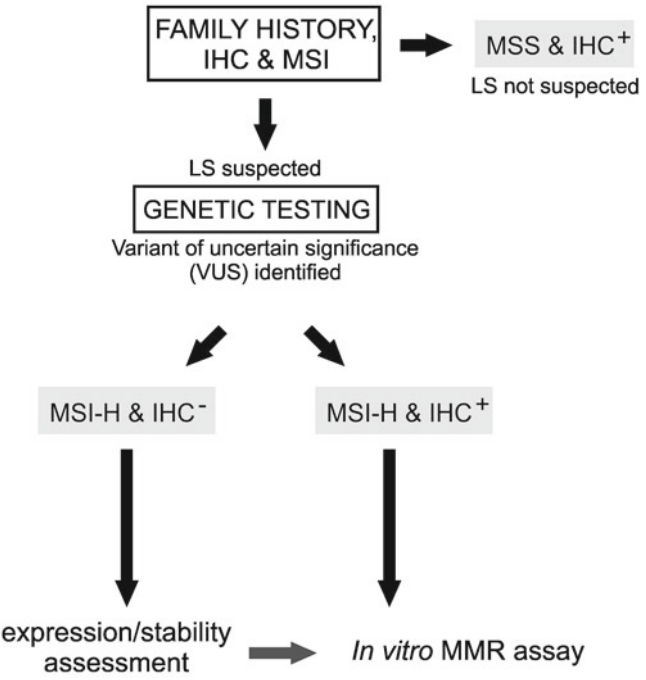

Fig. 5.2 A work flow for assessing the pathogenicity of variants of uncertain significance in MMR genes based on the information gained from MSI and IHC analyses. Where microsatellite instability is not detected Lynch syndrome is not suspected. In the case of MSI-H and the identification of VUS, the continuation of the work flow depends on the IHC status of tumour cells. Where IHC indicates the loss of protein expression in nuclei the cause is likely due to protein instability, whereas when IHC indicates protein stability, the most direct route to assaying the pathogenicity of the VUS is by the *in vitro* MMR assay that tests the proteins overall ability to function in the MMR process

during the assessment process (Fig. 5.2). Firstly, microsatellite stability (MSS) and expression of all MMR proteins in the tumour tissue are not indicative of LS. Where MSI is evident (MSI-H), the IHC results should continue to guide the decision making process after genetic testing and identification of a VUS. The commonly found reason for protein loss detected by IHC is protein instability caused by the variant. This can be assessed with a relatively simple protein expression analysis where the differences in protein quantities between variant and wild type proteins are detected. However, when IHC results indicate that all MMR proteins are present in nuclei of the tumour tissue, the reason for MSI-H can only associate with non-functionality of the expressed protein, which can be broadly analysed by the *in vitro* MMR assay. And only when IHC results show an abnormal subcellular localization of the protein, which is functional in the *in vitro* MMR assay, its ability to go to the nucleus should be studied. Thereafter, pathogenicity can be further characterized by more specific biochemical assays.

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Chapter 6
The Role of Epimutations of the Mismatch Repair Genes in the Development of Lynch Syndrome Related Cancers

Megan P. Hitchins

Abstract Lynch syndrome, characterised by an autosomal dominant predisposition to the development of young-onset colorectal, endometrial and additional cancers exhibiting the microsatellite instability phenotype, is typically caused by heterozygous germline mutations within one of the DNA mismatch repair genes. The *MLH1* and *MSH2* genes are the most frequently mutated of the mismatch repair genes. Until the turn of this century, no pathogenic mutation could be identified in up to one third of cases with a strong clinical suspicion of Lynch syndrome, but screens for disease-causing mutations within alternative genes were largely fruitless. In the past decade, an alternative aetiological mechanism for the development Lynch syndrome-related cancers emerged, which involved epigenetic-based dysregulation of the two key mismatch repair genes *MLH1* and *MSH2*. Termed a constitutional epimutation, this type of defect manifests as methylation of a single allele of the CpG island promoter accompanied by transcriptional inactivation of the affected allele within normal somatic tissues, in the context of a normal gene sequence. *MSH2* epimutations are caused by linked germline deletions of the neighbouring *EPCAM* gene, although *MSH2* itself remains intact, and demonstrate classic dominant inheritance. However *MLH1* epimutations can arise spontaneously in the carrier and are reversible between generations, resulting in unpredictable non-Mendelian patterns of inheritance, or display dominant inheritance with particular genetic alleles due to the presence of a linked genetic alteration within or nearby *MLH1*. Constitutional epimutations are thus associated with complex underlying mechanisms, and furthermore, give rise to atypical cases of Lynch syndrome both in terms of phenotypic heterogeneity and the risk of intergenerational transmission. This confounds molecular diagnosis, clinical management and genetic counselling of carriers and their families. As more information emerges from clinical and molecular

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research in this nascent field, tailored management strategies need to be devised so patients can realise the benefits of these discoveries.

Keywords Mismatch repair • Cancer susceptibility • Constitutional epimutation • Epigenetic silencing

Glossary of Terms

Epigenetics The stable changes in gene expression that occur independent of (but can be affected by) changes in the primary DNA sequence [1]. These changes are brought about by the attachment of various biochemical modifications to the DNA sequence, which include DNA methylation, and other chromatin modifications. This chapter will refer only to methylation.

Methylation A reversible biochemical modification to the cytosine nucleotide within the DNA sequence, which is universal to vertebrates. Methyl groups can be enzymatically added to or removed from cytosine bases in the genetic code, but occur primarily at cytosine-guanine (CpG) dinucleotides in mammals and are associated with transcriptional silencing of the DNA sequence.

Monoallelic methylation Methylation affecting a single allele/copy of a gene, as detected by linking CpG methylation to a single allele of a polymorphism or other genetic variant for which the subject is heterozygous.

Hemiallelic methylation Methylation of half of alleles, but not linked to a particular allele eg if the subject is uninformative for a polymorphism such that the two genetic alleles cannot be distinguished.

Epimutation An epigenetic aberration that results in a change in the transcriptional state of a gene. This can take the form of transcriptional silencing of a gene that is normally active, or conversely, reactivation of a gene that is normally silent [2].

Primary Epimutation An epimutation that has arisen in the absence of any alteration to the DNA sequence in the locality of the epigenetic aberration.

Secondary Epimutation/genetically-facilitated epimutation An epimutation that has arisen as a consequence of (or is accompanied by) a genetic alteration on the affected allele.

Germline epimutation Origination in, or transmission through, the germline of an epigenetically intact epimutation (with epigenetic modifications remaining attached to the affected DNA sequence) [3].

Constitutional epimutation An epigenetic aberration present within normal somatic cells that causes/predisposes to disease, but neither precludes nor dictates that its origin is in the germline, or that it is distributed evenly throughout somatic tissues [4, 5].

Allelic epigenetic mosaicism Variation in the epigenetic state (methylation status or levels) of a particular allele within a particular cell type or organism.

Haplotype A combination of alleles at multiple loci that are transmitted together on the same chromosome.

De novo Spontaneously arising; not inherited from a parent.
Allelic expression imbalance Relative loss or reduction in expression of one allele of a gene as compared to the other allele.

6.1 Introduction

Constitutional epimutation represents an alternative causative mechanism for genetic disease. A constitutional epimutation is an epigenetic aberration that results in the abnormal expression of a disease-causing gene within normal tissues. This can result in either the inactivation of a gene that would normally be expressed, or the re-activation of a gene that should normally be silent, within an individual or a particular normal tissue type. In the case of tumour suppressor or DNA mismatch repair genes that are associated with a particular cancer predisposition syndrome, constitutional epimutations have manifested as aberrant methylation of a single allele of the CpG island promoter that drives gene expression, accompanied by transcriptional silencing of the affected allele. A small number of index cases with a young onset or familial cancer syndrome that would usually be caused by a germline mutation within the DNA sequence of the established cancer-associated gene, have instead, been identified with a constitutional epimutation affecting the same gene.

Just as autosomal dominant familial cancer syndromes are caused by heterozygous loss-of-function germline mutations, constitutional epimutations also affect a single copy of the gene. While genetic mutations in these syndromes typically occur within the coding region and result in loss of the encoded protein function, a constitutional epimutation of the same gene results in loss of transcription and hence an absence of the protein product derived from the affected allele. However, both distinctive mechanisms similarly predispose the carrier to cancer development. In both cases, cancer onset is attributable to the somatic loss of function of the remaining normal allele. However, whilst inherited germline mutations are present at conception, hence are contained within every somatic cell of the carrier, constitutional epimutations can sometimes be “mosaic” and thus only present within a proportion of cells, or only manifest within a specific tissue type. This can give rise to altered expressivity of the disease observed as a variation on the classic genetic phenotype. Furthermore, the origins of constitutional epimutations are likely to vary considerably. Some are caused by an underlying genetic alteration within the vicinity of the affected gene, and these give rise to inheritance patterns following classic Mendelian patterns of genetic-based inheritance. Other types of epimutation have no apparent genetic basis and can be reversed between generations. Hence unravelling the underlying biological mechanisms and inheritance patterns associated with various forms of epimutation remains a significant challenge, since these factors will inform appropriate clinical management and genetic counselling advice provided to carriers and their families. This is a major focus of current clinical research efforts in this field and is discussed in detail herein.

6.1.1 *Constitutional Epimutation of the Mismatch Repair Genes as an Alternative Cause for Lynch Syndrome*

Constitutional epimutation of the DNA mismatch repair genes *MLH1* and *MSH2* have been identified as an alternative cause for Lynch syndrome [6, 7]. This is the most common familial cancer syndrome characterised by the development of colorectal, endometrial and additional types of cancer at a young age of onset, usually below 50 years of age [8]. Lynch syndrome is typically caused by heterozygous loss-of-function germline mutations within one of the four main DNA mismatch repair genes, most commonly *MLH1* and *MSH2*, followed by *MSH6* and *PMS2* [9]. Tumourigenesis is initiated following the acquired loss of the remaining normal allele of the affected gene within somatic cells and the consequent accumulation of genetic mutations throughout the genome during DNA replication due to impaired DNA mismatch repair activity [10, 11]. Lynch syndrome-related tumours classically exhibit microsatellite instability (MSI) as a marker of DNA mismatch repair deficiency, since microsatellite repeat sequences are particularly vulnerable to contraction or expansion [11]. For a significant proportion of cases with a strong clinical suspicion of Lynch syndrome (25–30 %), no genetic mutation of the key mismatch repair genes is identified by standard genetic screening for point mutations or larger genetic alterations, and so the aetiological basis for their disease remains undefined [9]. Some of these cases are likely to have cryptic genetic mutations within one of the four main genes, which require additional molecular testing for diagnosis, since they are unidentifiable by existing routine screening protocols. However, a number of such cases without an apparent pathogenic mutation within a mismatch repair gene have now been identified as carriers of a constitutional epimutation affecting either *MLH1* or *MSH2* [4]. This chapter provides a detailed review of the role of these two alternative epigenetic causes for developing Lynch syndrome-related cancers, as well as the distinctions in terms of molecular diagnosis, clinical presentation and family history between epimutation carriers and their counterparts with genetic mutations. Confounding factors that remain to be addressed, in particular how diagnostic screening for these alternative causes of Lynch syndrome may be implemented in routine clinical practice and the complexities of genetic counseling, are also discussed.

6.2 Constitutional Epimutation of *MLH1* as an Alternative Cause for Lynch Syndrome-Related Cancers Demonstrating MLH1 Loss

The first case to be identified as a carrier of the defect now referred to as a constitutional *MLH1* epimutation was reported by Gazzoli and colleagues in 2002 [6]. This case was a female who had developed an early-onset colorectal cancer

exhibiting MSI and loss of MLH1 expression in the absence of a family history or mismatch repair mutation. Instead, extensive methylation affecting a single allele of the *MLH1* CpG island promoter was found in the peripheral blood DNA. Deletion of the unmethylated allele in the tumour was detected as loss-of-heterozygosity (LOH), strongly implicating the constitutional methylation as the "first hit" in cancer susceptibility [6]. Constitutional epimutations of the *MLH1* gene have now been identified in over 50 index cases, comprising a small subset of patients presenting with a phenotype synonymous with Lynch syndrome, but in whom no pathogenic germline sequence mutation of the mismatch repair genes was found. Through the study of individual carriers and their families, the molecular and clinical manifestations, as well as the inheritance patterns associated with this epigenetic defect have, and continue to be, elucidated.

6.2.1 Molecular Characteristics of MLH1 Epimutations

Constitutional *MLH1* epimutations are characterised by soma-wide methylation confined to a single allele of the CpG island promoter accompanied by loss of expression from the methylated allele (Fig. 6.1) [12]. Monoallelic methylation was proven by the demonstration that CpG sites flanking a promoter SNP, such as the common c.-93G>A SNP, within the *MLH1* promoter are linked to one particular allele in heterozygous patients [3, 6]. However, some degree of allelic methylation mosaicism is observed in most patients, whereby some copies of the affected genetic allele are completely methylated, whilst other copies of the same allele are unmethylated, consistent with epigenetic variation between different cells [13–15]. Allelic loss or reduction of expression of the affected allele has also been demonstrated by using expressible SNP sites located within an *MLH1* exon in heterozygous patients, for example at the common benign c.655A>G SNP within exon 8, to trace the allelic origin of transcripts [12, 16]. Where an epimutation has resulted in complete allelic loss of expression, only one allele is observed in the mRNA, despite the presence of both alleles in the genomic DNA of the heterozygous carrier (Fig. 6.1). Partial losses of expression are sometimes detected in patients with a mosaic *MLH1* epimutation using precise allele quantification assays, whereby a significant reduction in the levels of transcripts derived from one allele is observed compared to the other allele at the designated exonic SNP site [17, 18].

The allelic methylation in carriers of a constitutional *MLH1* epimutation is relatively evenly distributed throughout the somatic tissues and present in tissue types derived from all three embryonic germ cell lineages, which separate from the inner cell mass of the embryo around gastrulation; normal colorectal mucosa, buccal mucosa and saliva (endoderm), peripheral blood lymphocytes (mesoderm), and hair follicles (ectoderm) [3]. Allelic loss of expression has also been typically demonstrated in mRNA from peripheral blood lymphocytes or derived

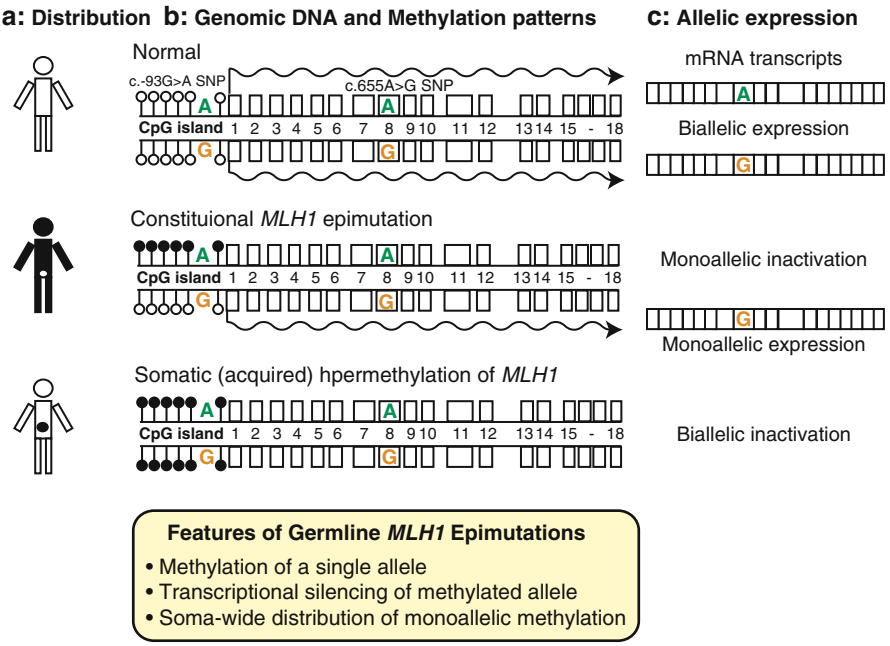

Fig. 6.1 Characteristics of a constitutional *MLH1* epimutation. The molecular characteristics of a constitutional *MLH1* epimutation are shown compared to the normal gene state and to the common mechanism of somatic hypermethylation of both *MLH1* alleles observed in most sporadic colorectal cancers demonstrating microsatellite instability. The bodily distribution of these mechanisms is shown, where *black* shading indicates the presence of methylation. In the case of an *MLH1* epimutation, this is soma-wide, but eradicated in the germline. Aberrant methylation of the CpG island promoter is shown as *black lollipops*. *Waved arrows* denote transcriptional activity. Exons in the gene and transcript are shown as boxes and the positions of two common single nucleotide polymorphisms within the promoter and exon 8, which are frequently used to assess allelic methylation and expression patterns respectively, are included

EBV-transformed lymphoblastoid cells [12, 16]. These findings provide strong evidence that *MLH1* epimutations, or their underlying mechanism, have already arisen by early embryogenesis. The finding that the soma-wide methylation is monoallelic points towards the origin of *MLH1* epimutations in the germline, or post-fertilization within the zygote prior to fusion of the male and female pronuclei. Thus constitutional *MLH1* epimutations were originally thought to be present within the germline and were initially coined a "germline epimutation" [3]. However, as discussed below, it is clear that *MLH1* epimutations are cleared in the male (paternal) germline, since they are absent in the spermatozoa of male carriers [16, 19]. However, it is possible that they may arise in the oocyte, and indeed there is evidence pointing towards the origination of most *MLH1* epimutations in the female (maternal) germline, in which case they may indeed represent a "germline epimutation" [4].

6.2.2 Clinical Characteristics of Cases with a Constitutional *MLH1* Epimutation

A constitutional *MLH1* epimutation has been identified as the cause for cancer susceptibility in over 50 index cases, with patients presenting primarily with colorectal cancers, as well as endometrial cancers in women. The majority of cases met at least one of the criteria listed in the revised Bethesda Guidelines [20] for the identification of Lynch syndrome, usually the development of a MSI colorectal cancer below the age of 50 years. A small number met stricter Amsterdam criteria [21, 22] due to the presence of Lynch syndrome-related cancer in at least one first-degree relative. Approximately half of the cases have presented with multiple primary tumours within the Lynch syndrome spectrum, which have included metachronous colorectal, endometrial, gastrointestinal, urological and skin cancers [3, 13, 14, 23]. However, a few cancers have been reported among epimutation carriers that are relatively infrequent in Lynch syndrome, including haematological malignancy and breast cancer in addition to typical Lynch syndrome-type cancers [3, 24]. One East Asian case with a mosaic *MLH1* epimutation presented with gastric cancer as the first cancer, which is not uncommon among Lynch syndrome carriers of germline sequence mutations of East Asian heritage [25].

Clearly case reports are likely to be the subject of significant ascertainment bias and therefore do not allow firm conclusions regarding the average age of onset of cancer in epimutation carriers. Having said this, it appears that cancer occurs at a young age in this group of patients, with a mean age of onset of the first diagnosis of colorectal cancer approximately 5 years younger (~39 years) than in cases with sequence mutations of *MLH1* (~44 years) [26, 27]. Thus, constitutional *MLH1* epimutations appear to confer a severe Lynch syndrome-related cancer phenotype. However, additional carriers of an *MLH1* epimutation have also been reported – first-degree relatives of probands – who have been asymptomatic at ages reaching 50 years [16, 18, 28]. Nevertheless, on the basis of the case histories reported, these individuals have a significant risk of developing Lynch syndrome-type tumours in their lifetime.

Interestingly, case reports of carriers of an *MLH1* epimutation have revealed this aetiological mechanism has arisen in individuals from a diverse range of racial groups. Patients of Caucasian (from Northern Europe, the Mediterranean, Australia, USA), Japanese, Chinese, Sri Lankan, Indian, Filipino and African descent have been reported [3, 13, 14, 23, 24, 29–33]. Screening for this defect should thus be implemented on the basis of clinical history and phenotypic presentation, as described below, irrespective of ethnicity. Furthermore, this argues against the involvement of a common environmental factor or singular genetic mutation as the mechanistic basis for this defect.

The salient difference in carriers of an *MLH1* epimutation and their counterparts with sequence mutations relates to the risk of cancer in their family members. *MLH1* epimutations appear to be dichotomised into two categories: those that are reversible between generations and rarely transmitted to the next generation, and

those that conform to the classic autosomal dominant inheritance pattern seen in mutation-carriers due to an underlying *cis*-genetic cause. This is discussed at length below. Nevertheless, this means selection of patients warranting screening for a constitutional *MLH1* epimutation must be irrespective of any family history.

6.2.3 Tumour Development and Features in MLH1 Epimutation Cases

The molecular profile of tumours from individuals with a constitutional *MLH1* epimutation are similar to those found in individuals with conventional sequence mutations of *MLH1*. In epimutation carriers the tumours have almost all demonstrated MSI and immunohistochemical loss of the MLH1 protein [4]. The 'second hit' affecting the unmethylated allele has also been identified in a proportion of these tumours, further indicating that the epimutation confers the 'first-hit' that predisposes to the development of Lynch syndrome-related cancers. In most cases the second hit has constituted an acquired somatic loss-of-heterozygosity of the unmethylated allele, or a *de novo* point mutation, consistent with the genetic based losses that typify Lynch syndrome-associated cancers [6, 12, 14, 24]. No cases have yet been reported where somatic methylation of the second allele has been definitively detected as the second hit. Whatever the molecular basis for establishing the epimutation in the germline or early embryo, this does not appear to confer a propensity for subsequent somatic methylation of the second allele.

In the small proportion of tumours tested, common hotspot mutations of *KRAS*, which are regularly found in Lynch syndrome-related tumours, have been identified in a few. Interestingly, the oncogenic BRAF V600E mutation has been found in 2–3 cases among epimutation carriers [14, 34]. This mutation is rarely found in the context of Lynch syndrome and so this finding was unexpected. However, one of these was a CRC that had developed in a female aged 60 years and was also positive for the CpG island methylator phenotype (CIMP), in which numerous genes scattered through the genome are simultaneously methylated [35]. The CIMP phenotype, BRAF V600E mutation and MSI are found in close correlation in a subset of sporadically arising CRCs, suggesting this particular tumour may have arisen independently of the *MLH1* epimutation and instead represents a classic case of "sporadic MSI CRC".

6.2.4 Distinction Between a Constitutional MLH1 Epimutation and Somatic MLH1 Hypermethylation in Sporadic Colorectal Cancers Exhibiting Microsatellite Instability (MSI)

Approximately 15 % of all CRCs demonstrate the MSI phenotype. While these include cases associated with Lynch syndrome, the majority arise sporadically in older patients over 65 years of age, with a 2:1 predominance in females [36, 37].

Most sporadic CRCs exhibiting MSI are caused by the acquired somatic hypermethylation of both alleles of *MLH1* and neighbouring genes within the tumour, and are closely associated with the BRAF V600E mutation and CIMP [38, 39]. Constitutional *MLH1* epimutations are distinct from the somatically acquired hypermethylation of *MLH1* in two key ways (Fig. 6.1). Firstly, *MLH1* epimutations affect a single allele of the gene resulting in loss of expression from that allele, whereas sporadic MSI cancers have been shown to be biallelically methylated, resulting complete loss of *MLH1* expression within the neoplastic cells. Secondly, in the case of constitutional *MLH1* epimutations, methylation is typically soma-wide and thus detectable in multiple normal somatic tissues, whereas in cases with a sporadic MSI cancer, the methylation is confined to the tumour tissue [39]. Nevertheless, when presented with CRC on a population basis, distinguishing between sporadic MSI cases and those associated with Lynch syndrome presents a challenge. Lynch syndrome-related CRCs are typically BRAF wild-type and the average age of onset of is more than 10 years younger than sporadic cases with a MSI CRC. These features have been used in clinical practice to identify cases with a suspicion of Lynch syndrome from among those presenting with a MSI CRC. However, the presence of *MLH1* promoter methylation is a feature common to Lynch syndrome-related tumours that are predisposed by a constitutional *MLH1* epimutation as well as most sporadic MSI CRCs. Of the few tumours from among epimutation carriers that have been studied, a proportion has also been BRAF V600E mutant. Therefore, the key means by which to distinguish any CRC arising as a consequence of a constitutional *MLH1* epimutation (from a common case of sporadic MSI CRC) would be to test for the presence of *MLH1* methylation in DNA derived from a normal tissue, such as a sample of normal colonic epithelium removed alongside the tumour during surgery or peripheral blood. Very few cases with a sporadic MSI CRC have been found to carry methylation within their normal colorectal mucosa, and this has been detected at trace levels only by highly sensitive techniques [39]. Certainly additional methylation testing of a normal (non-neoplastic) sample is recommended upon the identification of a MSI CRC demonstrating *MLH1* methylation that has arisen at a comparatively young age of onset.

6.2.5 *Molecular Diagnosis of Constitutional MLH1 Epimutations*

There is presently no ‘gold standard’ test for the molecular diagnosis of a constitutional *MLH1* epimutation, however, the most definitive proof comes with the detection of allelic methylation at the *MLH1* promoter in genomic DNA derived from a source of normal (non-neoplastic) tissue. Peripheral blood lymphocytes or normal colorectal mucosa from patients who have undergone surgical resection of a CRC, are the most readily available sources of constitutional genomic DNA. For the initial detection of constitutional methylation, two main considerations need to be taken into account. Firstly, each technique has its limitations and so the application of a second screening

method is ideal. Certainly the finding of a positive result in constitutional DNA with an initial assay would require confirmation by a second method. Secondly, the assay employed should target the "Deng-C" or "Deng D" regions of the *MLH1* promoter between the transcriptional start site and 250 bp upstream of it, since these have been correlated most closely with the loss of transcription and are less susceptible to age-related or other non-specific methylation [40].

Most techniques currently employed to detect the presence of methylation (in any gene) are based on the treatment of genomic DNA with sodium bisulphite, which then serves as the template for subsequent PCR-based assays. Sodium bisulphite converts unmethylated cytosines to uracils, and thence to thymine during PCR amplification, whereas methylated cytosines remain inert and unconverted. Thus, methylation is detected by the retention of cytosines at CpG dinucleotides within the CpG island promoter, as opposed to thymines at the corresponding CpG sites when the promoter is unmethylated. PCR-based assays for the detection of methylation are designed to exploit this sequence difference. These include traditional combined bisulphite and restriction analysis (COBRA), which employs restriction endonucleases that contain a differential CpG within their cleavage recognition site and thus only digest samples that were methylated in the original genomic DNA [12, 41]. Methylation-specific PCR (MSP) and MethyLight make use of allele-specific oligonucleotides designed to specifically amplify templates that have retained the cytosines at CpG sites [42]. These two similar methods have the advantage of being highly sensitive and semi-quantitative when performed with fluorescent labels against a standard curve by real-time PCR, and are thus capable of detecting mosaic forms of epimutation [15, 33]. For this reason, real-time MSP was used to demonstrate clearage of the *MLH1* methylation from spermatozoa samples [16, 19]. However, these methods can detect methylation levels so low their biological significance is questionable, confounding the interpretation of meaningful results [33, 39, 43]. Furthermore, MSP is prone to false positive results unless the given assay is carefully optimised. With real-time MSP this caveat can be overcome by applying a temperature gradient denaturation curve of the amplicons once PCR cycling is complete to demonstrate that the products have the anticipated melting temperature (thereby ruling out non-specific amplification and other false-positives) [33]. CpG pyrosequencing measures the relative levels of cytosine and thymine bases at a small number of designated CpG sites within a short stretch of sequence [44]. This method has the advantage of being quantitative, so the relative levels of methylated to unmethylated CpGs can be directly determined, and as applied to *MLH1*, is sensitive to methylation levels of 5–10 % [14, 15]. Each of these techniques uses sodium bisulphite treated genomic DNA as the template. The disadvantage of these PCR based techniques is that they may produce false negative results in cases where methylation may be accompanied by, or even caused by, a structural genetic change or other sequence error affecting either primer-binding site, resulting in a failure to amplify the affected allele and hence a normal-appearing result.

A modern technique that may be applied for epimutation detection and does not require prior conversion of the DNA with sodium bisulphite is methylation-specific

multiplex ligation-dependent probe amplification (MS-MLPA) [45]. This technique has been successfully implemented for the identification of *MLH1* epimutations, including one epimutation that accompanied a deletion of the first two exons of *MLH1* [32]. MS-MLPA makes use of methylation-sensitive restriction endonucleases such as *Hha*I that fail to cleave the (CpG-containing) recognition site if it is methylated or hemi-methylated and uses genomic DNA as the template. In MS-MLPA, probes are designed to encompass such a restriction site and anneal to the denatured genomic DNA. Unmethylated DNA is digested and thus fails to amplify, whereas the hemi-methylated DNA-probe hybrids remain undigested and thus produce an amplification product. The relative level of methylation is detected in the digested DNA versus an undigested sample. MS-MLPA provides a reliable semi-quantitative technique suitable for a first-pass screen for *MLH1* epimutations with a sensitivity to detect methylation levels of 10 %. This technique is especially suitable for laboratories practiced in traditional MLPA but unfamiliar with sodium bisulphite-based methods. Furthermore, it has the advantage of incorporating multiple probes from one or more genes in a multiplex reaction, allowing for the identification of epimutations that are associated with localised structural rearrangements. The ME011 MS-MLPA kit (MRC Holland, Amsterdam, the Netherlands) interrogates both *MLH1* and *MSH2* for the presence of methylation concurrently, and has been used on both constitutional genomic DNA to detect epimutations of either of these genes, as well as on tumour tissue to detect somatically-acquired methylation [32, 46, 47]. This kit contains five probe pairs targeting separate CpG sites within the *MLH1* CpG island that are each contained within a H*ha*I recognition site. These sites are located at −638, −402, −241/−245, −8 and +220 relative to the translation start site, and in methylation-positive samples, produce peaks of height/area relative to the level of methylation. No peaks are produced if the sample is either unmethylated, or the sequence interrogated is deleted. This method has been successfully applied to the identification of a patient who was methylation-positive but carried an interstitial deletion on the methylated allele, where other methods had failed to positively identify this case. The probes situated within the genetically intact region gave a methylation-positive signal, whilst the probes contained within the deletion gave no signal peaks [32].

Each of the above techniques, as applied to the *MLH1* gene, can readily detect the presence of 10–50 % allelic methylation in constitutional DNA and thus may be applied to the initial detection of a constitutional *MLH1* epimutation. To confirm this molecular diagnosis ideally a second DNA sample from a distinct tissue source should be tested and a second method which is quantitative, or allelic bisulphite sequencing, should be applied. Allelic bisulphite sequencing or demonstration of allelic transcriptional down-regulation at an informative exonic SNP site provides clear confirmation of the presence or effect of a constitutional *MLH1* epimutation and can also provide information as to which allele may be affected in family members of the proband. Conversely, in cases where a significant allelic imbalance in expression levels has been identified at an exonic SNP site, methylation testing for the presence of a constitutional epimutation is indicated as a potential cause [32].

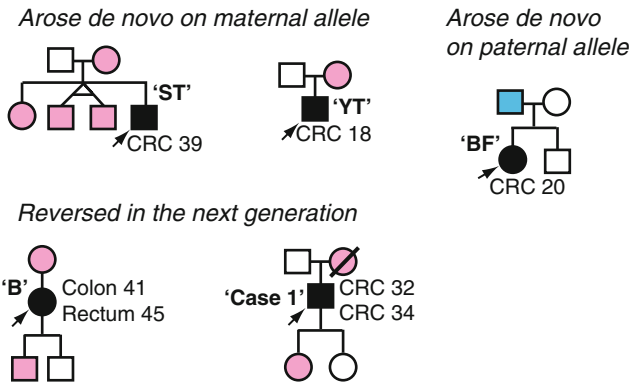

Fig. 6.2 Sporadic Lynch syndrome-like cases with a *de novo* constitutional *MLH1* epimutation that is 'reversible' in the germline. Pedigrees are shown for five cancer-affected probands (*black shaded* and indicated by an *arrow*) with a confirmed *MLH1* epimutation. The age of onset of colorectal cancer (CRC) or other cancer is given. Where the epimutation arose on the maternally derived allele (probands ST [12], YT [14], B [16], and Case 1 [30]) and the same genetic allele is carried by other members of the family, these are shaded in *pink*. Where the epimutation arose on the paternally derived allele (proband BF [14]), and the same genetic allele is carried by other members of the family, these are shaded in *blue*. *Circles*, females; *squares*, males; *diagonal line*, deceased

6.2.6 *Inheritance Patterns Associated with Constitutional MLH1 Epimutations*

6.2.6.1 Sporadic Cases due to De Novo Occurrence of MLH1 Epimutation

The majority of index cases identified with a constitutional *MLH1* epimutation have a personal history of Lynch syndrome-related cancer, but have little if any relevant family history. These sporadic cases have been ascertained either on a clinical basis, having developed a Lynch syndrome-related cancer (meeting at least one of the revised Bethesda criteria) which could not be attributed to a germline mismatch repair mutation [6, 12], or on the basis of having developed a colorectal cancer demonstrating MSI and *MLH1* methylation below the age of 50 years [30, 34]. The fundamental reason for the sporadic occurrence of Lynch syndrome in *MLH1* epimutation carriers is that these epimutations have a tendency to arise *de novo* in the carrier (as opposed to inherited from a parent). The first clear demonstration of this came with the identification of sporadic male proband 'ST' and study of the methylation status and inheritance patterns of the *MLH1* alleles in him and his family (Fig. 6.2) [12]. ST developed a CRC demonstrating MSI and MLH1 loss at the age of 39 years and had no family history of cancer or mismatch repair mutation, but had soma-wide *MLH1* methylation. Both his parents were negative for *MLH1* methylation in their normal tissues, indicating that neither were carriers of the epimutation. Allelic expression analysis in ST showed that the genetic allele subject to

transcriptional silencing as a consequence of the epimutation was the one he had inherited from his mother. Interestingly, all three of his siblings had also inherited the very same maternal allele of *MLH1*, but none of them carried any methylation. Collectively, these findings provided strong evidence that the *MLH1* epimutation arose spontaneously on the maternal allele in ST, hence its absence in his mother and siblings.

With one exception, in all of the sporadic patients for whom parental samples were studied, their *MLH1* epimutation had arisen *de novo* on the maternal allele [12–14, 16, 30]. This is entirely consistent with their lack of a family history of cancer. Interestingly, the predominance in occurrence of *MLH1* epimutations on the maternally derived allele may provide insight into the origins of this defect. The finding that the maternal allele is typically affected suggests that *MLH1* epimutations tend to arise in the oocyte and are propagated relatively stably through embryogenesis and into adulthood.

To date, there has been one exception to this generalisation. A single sporadic case, female proband BF, whose *MLH1* epimutation was shown to have arisen *de novo* on the paternally derived allele, has been reported (Fig. 6.2) [14]. Interestingly, the epimutation in proband BF, demonstrated significant mosaicism with methylation levels ranging from 10 % in her peripheral blood to 40 % in her hair follicles. This led to the suggestion that the epimutation in proband BF may have arisen on the paternal allele post-fertilization, or failed to withstand the preferential demethylation of the paternal genome within the male pronucleus that occurs in the zygote [48], or in demethylation events during early embryogenesis.

6.2.6.2 Reversal of MLH1 Epimutations Between Generations due to Their Erasure in the Germline

Unlike germline sequence mutations, which are stably transmitted through the germline giving rise to predictable inheritance patterns, constitutional *MLH1* epimutations are unstable in the germline. Where methylation testing and haplotyping to determine the transmission of *MLH1* alleles in the offspring of *MLH1* epimutation carriers has been feasible, reversal of the epimutation between generations has been demonstrated in some families. For example, study of members from three generations in the family of sporadic female patient “B”, including her mother and her two sons, revealed that the *MLH1* epimutation carried by patient B arose *de novo* on her maternally derived genetic allele (Fig. 6.2) [16]. This was evidenced by the loss of expression from the maternal *MLH1* allele as a consequence of promoter methylation in her normal somatic tissues, but a complete absence of methylation of this allele in her mother. The very same allele was then transmitted to her eldest son, but in him, the allele lost all trace of methylation and resumed normal expression (Fig. 6.2). Thus, this genetic allele was passed through three generations, but was only methylated in patient B in the second generation [16]. This was consistent with the erasure of the soma-wide epimutation in the germline of patient B.

Indeed evidence for the complete eradication of *MLH1* promoter methylation and an associated re-expression of the somatically-inactivated allele has now been clearly demonstrated in the spermatozoa from four male carriers of an *MLH1* epimutation [16, 18, 19, 30]. This was demonstrated by applying sensitive techniques to detect any *MLH1* methylation that may have been present in the DNA extracted from pure spermatozoa cells from these four male epimutation carriers. (Although Suter et al. initially claimed that *MLH1* methylation and hence the epimutation was retained in about 1 % of the spermatozoa from male carrier, patient "TT" [3], a subsequent addendum and accompanying correspondence to this report showed this finding was an artefact due to the presence of low-level contamination of the spermatozoa DNA sample with somatically-derived DNA [19]. Analysis of a second pure spermatozoa sample from patient TT found no trace of methylation, confirming erasure of methylation in his germline) [19]. In two of the carriers who were also informative for an expressed SNP within an *MLH1* exon, equal levels of expression from both alleles of *MLH1* were observed in their spermatozoa, even though each had shown a loss of expression of one allele in their somatic cells [16, 18]. These experiments clearly demonstrated that *MLH1* epimutations are efficiently eradicated in the male germline, consistent with the lack of transmission of the *MLH1* epimutation from parent to offspring in some cases. This was confirmed in one sporadic male proband, designated "Case 1", who had developed metachronous CRCs at the age of 32 and 34 years and carried soma-wide monoallelic methylation of his *MLH1* promoter (Fig. 6.2) [30]. His spermatozoa showed an absence of *MLH1* methylation. The "epimutant" genetic allele was passed from the proband (Case 1) to his eldest daughter, but was completely unmethylated in her, consistent with the permanent reversion of the epimutation in his germline (Fig. 6.2) [30]. However, this does not necessarily equate to a lack of potential for transmission of the epimutation to successive generations, since reversal of the epimutation in the germline may only be transient and it can be reinstated in the next generation. This has been demonstrated in one case described later, whereby a dominantly inherited form of *MLH1* epimutation was re-established in the next generation, despite its apparent erasure in the paternal spermatozoa [18].

6.2.6.3 Non-Mendelian Trans-Generational Inheritance

A single family has been described by Hitchins et al. in which intergenerational inheritance of a 'reversible' constitutional *MLH1* epimutation occurred, but in a non-Mendelian pattern (Fig. 6.3) [16]. Proband "A" had been affected by multiple Lynch syndrome-related cancers demonstrating immunohistochemical loss of MLH1 and MSI, but had no family history of cancer and was negative for a pathogenic germline mutation of the mismatch repair genes. She was identified as carrying a soma-wide constitutional *MLH1* epimutation affecting a single allele of the *MLH1* promoter, as detected at the c.-93G>A SNP. This was accompanied by monoallelic transcription at the expressed exon 8 c.655A>G SNP, as detected by the absence of one allele in her mRNA. Her older sister carried the same genetic

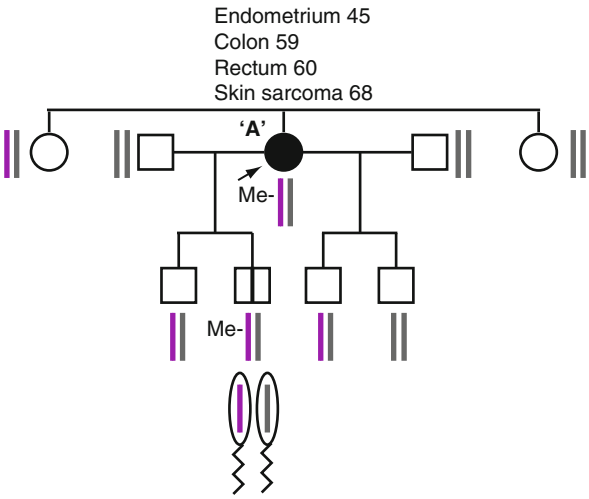

Fig. 6.3 Non-Mendelian *MLH1* epimutation in Family "A". The pedigree of Family A is shown, with the cancer-affected proband shaded in *black*. Alleles are shown as *lines*. *Grey* indicates an unmethylated *MLH1* allele. *Purple* lines denote the epimutant *MLH1* allele, with the presence of methylation indicated by Me. Methylation was only detected in the somatic tissues of the proband and her second son (*vertical line* in *square*). No methylation was detected in the proband's sister and her first and third son who each carried the identical genetic allele. No *MLH1* methylation of this allele was found in the spermatozoa of the second son (shown as *ovals*) due to germline erasure of the epimutation

haplotype to which the epimutation was linked in patient A, but had no trace of methylation, thus it is likely the epimutation arose *de novo* in patient A. Therefore patient A represented a classic sporadic case with a spontaneously arising *MLH1* epimutation. Interestingly, however, methylation analyses in her four sons revealed that one of them, her second son, also carried a soma-wide *MLH1* epimutation. This was evidenced by constitutional methylation and loss of expression from his maternally derived allele, confirming he had inherited the epimutation directly from his mother. Of further interest, haplotyping of her four sons to determine allele sharing revealed that her first three sons had each inherited the identical maternal allele on which the epimutation was present in the mother and second son. Thus, patient A had transmitted the epimutant genetic allele to her first three sons, yet only the second son inherited the epimutation itself. In the first and third son, erasure of the epimutation from this allele was evidenced by the absence of methylation and the resumption of normal (biallelic) *MLH1* expression. This family demonstrated that *MLH1* epimutations which are 'reversible' in the germ line can nevertheless be transmitted to a proportion of offspring. Whether this occurred with the epigenetic error still attached to the allele through the maternal germline, due to a failure to eradicate the epimutation in a proportion of her germ cells, or whether the epimutation was transiently erased in the germline and subsequently reinstated in just one

of her offspring, remains a mystery. Analysis of the spermatozoa from the second son, who carried the epimutation throughout his somatic tissues, found no trace of methylation in his spermatozoa and the allele was reactivated again [18]. Thus *MLH1* epimutations are efficiently cleared in the male germline, though as discussed below, this may only be temporarily. The question remains of whether erasure occurs with the same efficiency in the female germline. Given that the majority of *de novo MLH1* epimutations have arisen on the maternal allele, it seems the maternal germline is more vulnerable to the acquisition of, or transmission of, altered epigenetic states than the paternal germline.

6.2.6.4 Mendelian (Autosomal Dominant) Inheritance of MLH1 Epimutations Caused by a Linked Genetic Alteration

It is now apparent that constitutional *MLH1* epimutations are in some cases, heritable in a classic autosomal dominant manner due to an underlying genetic aberration on the affected allele. Through the recent identification of a handful of familial cases whose members from two or more generations consented to be studied, the presence of soma-wide methylation was found to pass from one generation to the next segregating with, or directly linked to, particular genetic haplotypes, strongly implicating a Mendelian genetic basis to their epimutation.

The first case report of constitutional *MLH1* methylation in association with a linked genetic defect was in the female proband from Finnish family "36" (Fig. 6.4a) [32]. F36 was diagnosed with two primary colorectal cancers at the ages of 22 and 45 years and endometrial cancer at 40 years. A complete loss of expression of one allele of *MLH1* had been observed at the common c.655A>G SNP in her mRNA for which she was heterozygous in her genomic DNA [49]. The proband was subsequently found to carry a 6.5 kb deletion extending from c.-69 within the *MLH1* promoter to intron 2, with methylation of the promoter identified upstream of the proximal deletion breakpoint. The presence of these concomitant defects was initially identified by MS-MLPA, using the ME011 kit. In patient F36, DNA from her normal tissues displayed a 0.5 methylation dosage ratio (indicating 50 % methylation) by MS-MLPA at the three upstream CpG sites interrogated by the five probes, but no signal at the last two further downstream. Long-range PCR mapped the breakpoints and found this MS-MLPA pattern to be consistent with monoallelic methylation at the first three loci and deletion of the last two [32]. The proband's deceased father had an extensive Lynch syndrome phenotype, having been affected by multiple primary colorectal cancers at the age of 41, 49 and 69 years, and skin cancer at 56 years [49]. Although no samples were available from him to confirm he was a carrier of the combined deletion and epimutation, dominant inheritance is probable. How the epimutation became manifest on the deleted allele is not clear, though it is likely that the loss of regulatory elements preventing transcription resulted in methylation as a secondary consequence of this. This case provided the first evidence that genetic disruption at the start of the *MLH1* gene could concomitantly give rise to an altered epigenetic state. This epimutation may thus be regarded as a "secondary" or "genetically-facilitated" epimutation.

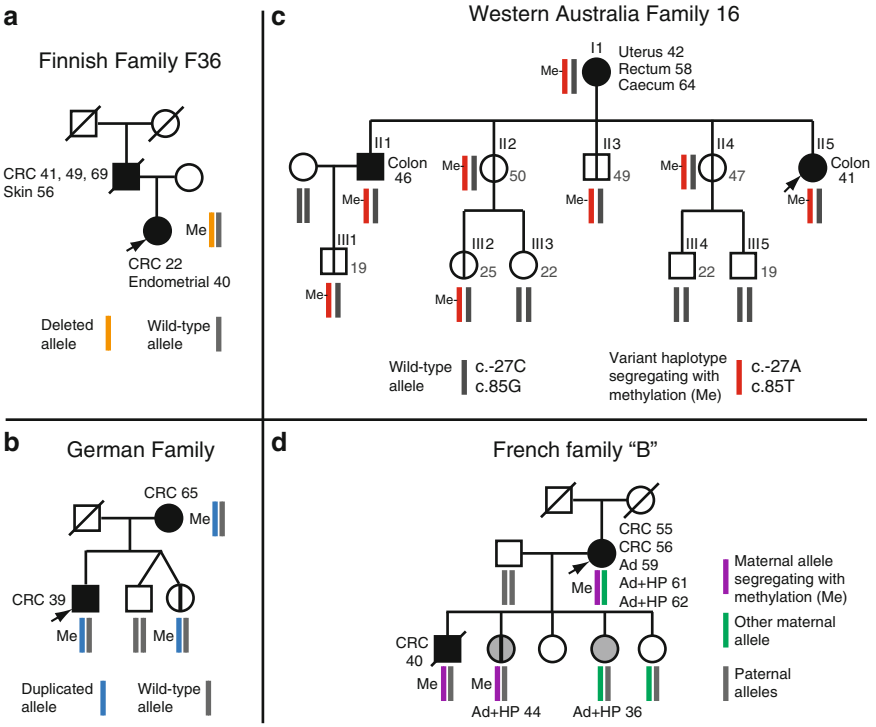

Fig. 6.4 Familial cases with autosomal dominant inheritance of an *MLH1* epimutation. Pedigrees of four families with presumed or confirmed autosomal dominant inheritance of a constitutional *MLH1* epimutation. (**a**) Finnish family F36 whose methylated *MLH1* allele (*orange*) bore a linked deletion from c.-69 to intron 2 [32]. (**b**) German family whose methylated allele (*blue*) bore a duplication encompassing *MLH1* and additional neighbouring genes [28]. (**c**) Caucasian family 16 from Western Australia whose methylated allele (*red*) bore two single nucleotide variants, c.-27C>A and c.85G>T, which was transmitted with the epimutation through three generations [18]. (**d**) French family of patient "B" whose epimutation segregated with a particular genetic allele (*purple*), although no causative cis-genetic alteration on this allele has yet been identified [56]. Probands are indicated by an *arrow* and the age of cancer onset is given. *Circles*, females; *squares* males; *black shading*, affected by colorectal cancer (CRC) or other cancer as listed; *grey shading*, affected by a colorectal adenoma (Ad) or hyperplastic polyp; *vertical line*, asymptomatic carrier of *MLH1* epimutation

The presence of a further local *cis*-acting structural defect as the mechanistic basis for a dominantly inherited *MLH1* epimutation was reported by Morak et al. [28]. The male proband (designated patient 4) presented with CRC at the age of 39 years. His peripheral blood showed partial methylation at a level of 10 %. His mother, who at 64 years was unaffected by cancer at the time of the initial study, also exhibited 8 % methylation in her peripheral blood lymphocytes [13]. Follow up work on this family subsequently showed that both mother and son, and an additional unaffected daughter, harboured a large duplication of size ranging from 280–375 kb, which included the entire *MLH1* gene as well as four additional flanking genes [28]. The two siblings who had inherited the duplicated maternal allele

similarly exhibited a mosaic pattern of methylation associated with this allele in their somatic cells, whereas no methylation was found in the third sibling, who did not carry the duplication (Fig. 6.4b). The identification of this genetic anomaly segregating with the *MLH1* epimutation prompted surveillance colonoscopy in family members, which led to the identification of colorectal cancer in the mother at the age of 65 years, but the carrier sister remained unaffected by cancer at the age of 44 years [28]. One again, precisely how the duplication encompassing *MLH1* and neighbouring genes resulted in the partial methylation of the *MLH1* promoter remains unknown, but this case nevertheless provides further evidence that a loss of genomic integrity in the vicinity of *MLH1* can result in the altered epigenetic state of this locus.

Clear evidence for an autosomal dominant form of *MLH1* epimutation came with the identification by Hitchins et al. of a three-generation Caucasian family from Western Australia, designated "Family 16", in which the epimutation was found in members from each generation segregating with a particular *MLH1* haplotype (Fig. 6.4c) [18]. The mother and two siblings (the female proband and her eldest brother) were affected by Lynch syndrome-related cancers demonstrating MSI and loss of MLH1 activity, but germline screening did not identify a clearly pathogenic mismatch repair mutation. Methylation testing revealed multiple family members to be positive for soma-wide methylation, including the affected mother, both affected siblings, as well as five other family members spanning all three generations. The members of the third generation were still in their early twenties or younger and thus had not reached the average age of onset for Lynch syndrome cancers. Haplotyping in the family revealed that the soma-wide methylation segregated perfectly with a particular genetic haplotype of *MLH1*, which bore two single nucleotide variants (SNVs) in tandem: a c.-27C>A within the promoter and 5′UTR and a c.85G>T within exon 1. Allelic methylation studies confirmed that the *MLH1* methylation specifically affected this variant c.-27A/c.85T *MLH1* haplotype and was present soma-wide in all haplotype carriers in the family, but with high levels of allelic methylation mosaicism among them. This was accompanied by a variable degree of transcriptional loss from the variant haplotype, ranging from complete to partial silencing in different tissues from within family members, as well as between different family members, further indicative of mosaicism. The dominant inheritance of this epimutation strongly implicates a genetic-based cause located on this affected haplotype, the c.-27C>A SNV being the prime candidate. Although various functional studies were performed in this study to determine if either of the SNVs borne on this haplotype was responsible for inducing the epimutation, these were not conclusive. Nevertheless, luciferase promoter reporter assays designed to examine the effect of the SNVs on transcriptional activity, revealed that the c.-27C>A variant alone resulted in reduced transcription levels, whereas the c.85G>T variant had no detrimental effect on transcriptional activity. Hence, the c.-27C>A SNV remains the most likely candidate underlying this dominant form of *MLH1* epimutation [18].

Interestingly, although the affected male sibling in Family 16 transmitted the variant c.-27A/c.85T haplotype and linked *MLH1* epimutation to his son, the epimutation itself was completely erased in his spermatozoa. This reversal was evidenced by

the absence of *MLH1* methylation in the spermatozoa, as well as the re-expression of the somatically-silenced allele at levels equivalent to the wild-type allele [18]. This indicates the epimutation was temporarily reversed in the paternal germline, but subsequently reset in his son, presumably post-fertilization in the somatic cells during early embryogenesis. Since the epimutant haplotype was reactivated to normal expression levels in the spermatozoa upon eradication of the methylation, this finding indicates that the genetic change underlying this epimutation does not by itself cause transcriptional silencing. Rather, it is likely that the causative sequence change interacts with particular nuclear factors to bring about epigenetic modification of the allele, which in turn, results in transcriptional repression.

Two other Caucasian index cases with Lynch syndrome have also been reported as carriers of the c.-27C>A/c.85G>T haplotype, whose pedigrees do not appear to be related to Family 16 from Western Australia [33, 50]. No methylation or allelic expression studies were conducted in the first reported case, and this study focussed on the potential role of the c.85G>T SNP, which encodes a conservative A29S amino acid change [50]. However, functional studies of this protein variant showed it had no detrimental effect on protein location or stability, or on mismatch repair ability, and so was deemed benign [50]. The third carrier of this haplotype was also shown to carry constitutional methylation linked specifically to the c.-27A/c.85T variant haplotype, but no samples were available from family members to study the inheritance in this family [33]. It is likely that these families share a common ancestral haplotype that causes cancer susceptibility via epigenetic silencing of the allele. Interestingly, a number of other germline *MLH1* promoter sequence variants have previously been reported in Lynch syndrome cases, but in most cases, their pathogenic significance and mechanism of action has remained uninterpreted [51–55]. It will be interesting to determine if any of these act via the induction of an *MLH1* epimutation. In one study in which screening for *MLH1* epimutations and promoter variants was performed in parallel, the c.-42C>T and c.-11C>T variants, as well as a microdeletion within the *MLH1* promoter, were identified and shown to reduce transcriptional activity, but were not associated with the presence of constitutional methylation [33]. Thus it appears that some promoter sequence changes may act via epigenetic mechanisms, whilst others act independently of these.

In a fourth case of a dominantly inherited *MLH1* epimutation reported in a French family by Crépin et al., mosaic constitutional methylation was transmitted from the mother to the first two of her five children (Fig. 6.4d) [56]. The mother, "patient B", had an extensive Lynch syndrome phenotype having developed multiple colorectal carcinoma and adenomas, as well as hyperplastic polyps, from the age of 55 years. Her son (the eldest) developed CRC demonstrating MSI and MLH1 loss at the age of 40 years, and died of metastatic disease. Her eldest daughter also developed an adenoma and hyperplastic poly aged 44 years. Mother, son and daughter each harboured mosaic *MLH1* promoter methylation at levels of 15–25 % within their normal tissues and carried the same genetic haplotype. Three younger daughters were unmethylated and did not carry this haplotype. Thus the methylation state in this family segregated with a particular genetic haplotype, but could not be directly linked to this allele due to the lack of

heterozygosity for any polymorphism within *MLH1* itself. Nevertheless, the findings in this family implicate a cis-genetic basis to their dominantly inherited epimutation, although this remains to be defined and no candidates have been identified so far [56]. Interestingly, one of the daughters who did not harbour methylation or the allele segregating with it also developed an adenoma and a hyperplastic polyp at the age of 36 years, which may have arisen sporadically, but also suggests this family may harbour another independent disease-susceptibility allele that has influenced their phenotype.

Collectively, these families provide strong evidence that a proportion of cases identified with a constitutional *MLH1* epimutation may have a dominantly heritable form of epimutation attributable to an underlying genetic defect present on the epimutant allele, presumably in the vicinity of the *MLH1* gene.

6.2.6.5 Dominant Versus 'Reversible' Forms of MLH1 Epimutation

Through the study of the families of epimutation carriers, there is now evidence for two distinct forms of *MLH1* epimutation; a reversible type that is unstable in the germline and occasionally transmitted to successive generations in an unpredictable manner, and a dominant form that is faithfully transmitted to the next generation linked to particular genetic alleles bearing causative cis-acting genetic aberrations. In the latter form, since genetic defects are stably inherited through the germline, the faithfully segregating epimutation is transmitted with it. Yet, both forms of epimutation have been shown to be erased in the male germline. Whether the epimutation is transiently erased in the germline appears to have no bearing on its heritability to successive generations, since as demonstrated in one male member of the Western Australian Family 16, the epimutation was transmitted to his son despite its erasure in the paternal spermatozoa [18]. Presumably the *MLH1* epimutation in this family was re-established post-fertilization in the somatic cells of each new generation upon inheritance of the defective genetic allele. Thus, irrespective of the temporary erasure of the epigenetic marks in the parental germline, epimutations can be inherited. Future genetic and epigenetic diagnostic practices will need to distinguish between cases with a 'dominant' *versus* a 'reversible' *MLH1* epimutation if appropriate clinical management and genetic counselling guidance is to be provided to families. This represents a major challenge for ongoing clinical research and molecular diagnosis in this field, since the genetic or other mechanistic basis for epimutations in most cases remain to be defined. Clearly, the two forms of epimutation are caused by distinct mechanisms. Dominant cases implicate a cis-genetic error on the affected allele, which induces the epimutation as a secondary event and may thus be termed "genetically facilitated" or "secondary" epimutations. Even if the primary genetic defect on the disease-susceptibility allele remains unidentified, a cis-acting aberration may be presumed. Therefore, the carrier status of the risk allele, and/or methylation status, could be identified in family members to define their risk of cancer development, until such a time as the nature of the underlying defect is defined. These types of epimutation

would be associated with a 50 % risk of transmission. In contrast, 'reversible' or 'primary' *MLH1* epimutations are unlikely to be caused by a cis-genetic defect, given that the allele can revert from an abnormal state back to the normal state from one generation to the next, and their inheritance currently appears to be arbitrary. However, this type of epimutation may involve *trans*-acting factors that are co-inherited, for example in a digenic model, allowing for transmission of the epimutation to a proportion of offspring. This is speculative at present. However, until the mechanistic basis for these reversible epimutations is defined, family members of probands should be offered methylation testing to determine their own carrier status.

6.2.7 *Frequency of MLH1 Epimutations in the Lynch Syndrome Population*

Approximately 50 index cases have been identified as carriers of a constitutional *MLH1* epimutation. The incidence of this defect in the Lynch syndrome and general population remains to be determined, but appears to be rare. The frequency of identification of *MLH1* epimutations among those tested has depended largely on ascertainment criteria of the study group. In single centre studies, where screening was performed on cases with suspected Lynch syndrome due to the development of a young onset CRC, who had tested negative for a germline mismatch repair mutation of the three most frequently affected mismatch repair genes (*MLH1*, *MSH2* and *MSH6*), but the MSI and mismatch repair status of the tumour had not been investigated, the detection rate for *MLH1* epimutations ranged from 0 to 1.6 % [12, 15, 24, 56]. In single centre studies in which suspected Lynch syndrome patients had been selected for *MLH1* epimutation testing on the basis of having developed a young onset MSI CRC or endometrial cancer that also demonstrated immunohistochemical loss of MLH1, the detection rate has ranged from 3 to 8 % [12, 16, 32, 46]. In a multicentre study comprising of 416 CRC cases with tumours demonstrating either or both MSI/MLH1 loss from the Colon Cancer Family Registry, nine cases (2 %) were confirmed as carriers of a constitutional *MLH1* epimutation, of which two were linked to alleles bearing a single nucleotide sequence change [33].

In a population-based study of CRC, no constitutional *MLH1* epimutations were identified in 104 cases with a sporadic MSI CRC (tested irrespective of age) [39]. However, in a study by van Roon et al., patients were selected for epimutation screening on the basis of having developed a CRC that demonstrated MSI and *MLH1* promoter methylation, with an enrichment of patients aged under 50 years [34]. They tested the normal colonic epithelium and/or peripheral blood DNA that was available for seven patients with an age of CRC onset below 50 years, and 13 patients with an age of onset above 50 years, for the presence of constitutional *MLH1* methylation. One female patient who had developed CRC at 33 years followed by endometrial cancer at 52 years, and had a sister who had developed endometrial cancer aged 37 years, was identified with a constitutional *MLH1*

epimutation. This provided a comparatively high frequency, 14 %, for the identification of an *MLH1* epimutation in patients with a MSI, *MLH1*-methylated CRC at an age of onset below 50 years [34]. The second case was a female who had developed CRC at 60 years of age and a microsatellite stable pancreatic cancer at 62 years of age. However, her CRC was also CIMP+ and BRAF V600E mutant [34], and so may have developed sporadically, independent of the *MLH1* epimutation she carried.

6.3 Implementation of Routine Molecular Diagnosis, Genetic Counselling and Clinical Surveillance of Individuals with a Constitutional *MLH1* Epimutation

Given that constitutional *MLH1* epimutation represents an uncommon cause for Lynch syndrome and the precise risk of cancer onset and inheritance cannot presently be accurately quantified, the circumstances under which cases warrant screening for the presence of this aetiological defect continue to be debated. Nevertheless, identification of an *MLH1* epimutation has profound clinical significance for carriers and their families. First of all, instead of merely receiving an uninformative genetic test result due to the lack of detection of a germline mismatch repair mutation, patients can receive a positive molecular diagnosis for their cancer phenotype. The available evidence from among the 50 or more confirmed cases identified to date is that carriage of a constitutional *MLH1* epimutation confers a phenotype closely reminiscent of Lynch syndrome; development of young onset (<60 years) CRC or endometrial cancer, or metachronous Lynch syndrome-related cancers, demonstrating MSI and MLH1 loss. Therefore, the current recommendation for confirmed carriers is that they be advised their risk of developing Lynch syndrome-related tumours is equivalent to carriers of a germline sequence mutation within *MLH1*. Until formal guidelines for the clinical care of carriers of an *MLH1* epimutation are developed, carriers should thus be advised to undergo the same clinical surveillance regimen for Lynch syndrome as their counterparts with a germline mismatch repair mutation. In practical terms this means 1–2 yearly colonoscopies from age 25 years and the consideration of subtotal colectomy in selected patients [57].

In terms of a molecular diagnosis, the first major consideration is the selection of cases warranting methylation testing. Patient ascertainment, criteria for referral for germline screening, pathology and genetic testing practices can all vary between countries, family cancer clinics and service providers, making a “one-size-fits-all” standardised algorithm for triaging of patients for epimutation testing difficult to formulate. As per germline screening to identify pathogenic mismatch repair mutations, a priori knowledge that the tumour is MSI and which mismatch repair protein is lacking by immunohistochemistry, can greatly expedite mutation detection by prioritising screening of the implicated mismatch repair gene. Testing for an

MLH1 epimutation in patients who have raised a clinical suspicion of Lynch syndrome should be considered after loss of expression of MLH1 has been demonstrated in a tumour and routine screening for a germline mutation of *MLH1* (by exonic sequencing and MLPA) has returned an uninformative (negative) result. These recommendations are proffered due to the comparative rarity of *MLH1* epimutations and thus their anticipated low detection rate among the Lynch syndrome population. A positive result may be expected in 2–12 % of cases selected on the basis of the above criteria. Since we now know that *MLH1* epimutations can arise *de novo* or be inherited, screening should be undertaken irrespective of the presence or absence of a family history. The second major consideration is which molecular diagnostic test(s) to employ. This has been described in detail above. In summary, until such a time as a gold standard test is recommended, laboratories should test constitutional DNA for the presence of methylation within the "Deng C" region of the *MLH1* promoter using at least one technique that can detect methylation levels to a sensitivity of 5–10 %, such as MS-MLPA, pyrosequencing or other reliable assay.

The different risks and patterns of inheritance of constitutional *MLH1* epimutations, presumably caused by their distinct mechanistic causes, are a major confounding factor in providing informed genetic counselling advice. In cases where an *MLH1* epimutation can be linked to an underlying promoter sequence change or structural alteration linked to the same allele, genetic counselling advice can follow existing guidelines for families with a confirmed germline mismatch repair mutations. To date, the phenotypes seen in familial *MLH1* epimutation cases is similar to Lynch syndrome, though more work is needed to more precisely identify the associated cancer risks in cases with a genetically-facilitated epimutation. Relatives who do not carry the genetic allele to which a dominant epimutation is linked can be reassured that their cancer risks may not be different from the general population. Those who do carry the epimutant genetic allele warrant close follow-up and frequent communication as knowledge of this field matures. However, for those with *MLH1* mono- or hemi-allelic methylation but no identified underlying DNA sequence change, the clinical and familial risks remain poorly defined. Relatives of a confirmed carrier should be offered *MLH1* methylation testing of their constitutional DNA to determine their own carrier status. Those who manifest methylation may optimally be followed up under a research protocol, as if they have risks akin to those reported in Lynch syndrome. Furthermore, it is not clear if those family members who do not manifest *MLH1* methylation in their blood, but who carry the same "epimutant" genetic allele as an affected family member who does carry an epimutation, are at an increased risk of cancer development. It remains to be determined whether they may develop methylation as they age, or whether the methylation may be mosaic and or present only in particular tissues. Multicentre collaborations to generate larger numbers of cases will be necessary to more fully understand the clinical ramifications and risk of transmission of *MLH1* epimutations.

6.4 Constitutional Epimutation of *MSH2* as an Alternative Cause for Lynch Syndrome-Related Cancers Demonstrating MSH2 Deficiency

The first case of a constitutional epimutation affecting the *MSH2* gene to be described was in a three-generation Chinese Lynch syndrome family from Hong Kong (designated HK-family A) by Chan et al. in 2006 [7]. In this family, three siblings had developed young onset CRC or endometrial cancer all demonstrating MSI and immunohistochemical loss of MSH2, and the mother had developed multiple colorectal adenomas, any of which may have progressed to CRC without clinical intervention. However, no germline sequence mutation could be identified within *MSH2*, or any other mismatch repair gene in this family. Methylation testing using sensitive methylation specific PCR (MSP) revealed the presence of methylation within the *MSH2* CpG island promoter in somatic tissues from each of the affected individuals and additional as yet unaffected relatives in the family spanning three generations. Haplotyping revealed that all carriers of methylation had inherited the same genetic haplotype of *MSH2*, indicating faithful segregation of the methylation with this particular allele. By exploiting two SNPs within the *MSH2* promoter for which some individuals in the family were heterozygous, allelic methylation analyses showed the methylation was linked specifically to the one haplotype [7]. Therefore, this epimutation was transmitted faithfully with a particular genetic allele in a classic autosomal dominant inheritance pattern, strongly implicating an underlying *cis*-acting genetic basis, which was subsequently identified. This case represented the first case in which dominant inheritance of an epimutation within any mismatch repair gene was identified.

Interestingly, the *MSH2* epimutation in this family demonstrated significant mosaicism between different tissues [7]. All carriers had high levels of methylation reaching 40 % (amounting to 80 % of the affected alleles) in their normal colonic epithelium and lower levels in their buccal mucosa and endometrial aspirates, ranging from 5 to 14 %. The levels detected in blood were less than 5 %, but nevertheless detectable by MSP, and just distinguishable from healthy control samples using CpG pyrosequencing. The reason for this highly tissue-specific type mosaicism subsequently became apparent when the genetic basis for *MSH2* epimutations was defined.

6.4.1 MSH2 Epimutations Are Caused by Terminal Deletions of the Neighbouring EPCAM Gene

Follow-up linkage analysis and long-range PCR plus sequencing in HK-family A led to the identification of a ~23 kb deletion extending from intron 5 of the *EPCAM* (formerly *TACSTD1*) gene, located immediately upstream of *MSH2*, to 2.4 kb upstream of *MSH2*, leaving the entire *MSH2* gene and promoter region intact [58]. Concurrently, during the routine screening of Dutch Lynch syndrome families for

structural alterations of the *MSH6* gene by MLPA, for which a dosage-control probe was located within *EPCAM*, a reduced signal of this *ECPAM* probe was observed in four families. Like HK-family A, members of these families had developed MSI colorectal tumours demonstrating dual loss of MSH2 and MSH6, and presence of the deletion segregated with disease. More detailed MLPA analysis and long-range PCR revealed the same 5 kb deletion in these four families, which encompassed the final two exons of *EPCAM*, but ended several kb upstream of *MSH2*, once again leaving the mismatch repair gene intact [58]. Methylation testing of the *MSH2* promoter in all families revealed methylation to be present on the deleted allele, thus linking epimutations of *MSH2* to deletions of the terminal portion of the adjacent *EPCAM* gene located 17 kb upstream [58].

These terminal deletions of *EPCAM* resulted in the loss of its polyadenylation (transcription termination) signal and consequently in a failure to terminate *EPCAM* transcription in EPCAM-expressing cell types. Analysis of RNA in these families showed the interstitial deletions indeed led to transcriptional "read-through" from *EPCAM* into *MSH2*, resulting in *EPCAM-MSH2* fusion transcripts. For example, in HK-family A, exon 5 of *EPCAM*, the final intact exon, was spliced onto exon 2 of *MSH2*. Thus *EPCAM* transcription presumably proceeded through the intact *MSH2* promoter, over-riding its own activity, and subsequent splicing events formed the fusion transcripts observed. EPCAM, the epithelial cell adhesion molecule, is only expressed in epithelial tissues. Correspondingly, the levels of the *EPCAM-MSH2* fusion transcripts were considerably higher in epithelial tissues. This correlated closely with the degree of methylation observed in the various tissues examined from members of HK-family A and thus explained the tissue-specific methylation mosaicism; the highest levels of methylation in colorectal epithelium matched the highest levels of the fusion transcripts, whereas 100-fold lower levels of the fusion transcript were found in the less methylated peripheral blood [58].

The mechanistic basis for *MSH2* epimutation was thus revealed by Ligtenberg et al. to be germline deletions encompassing the 3′ end of the *EPCAM* gene immediately upstream of *MSH2*, which via a failure in transcription termination of *EPCAM*, lead to epigenetic inactivation of the *MSH2* promoter specifically in EPCAM-expressing epithelial cells [58]. In cell types that do not express EPCAM, the *MSH2* promoter presumably retains its own activity on the deleted allele and is normally expressed, since it is not over-ridden by the *EPCAM* promoter in these tissues. These cells are presumably less predisposed to the development of MSH2-deficient cancers. The clear genetic basis for *MSH2* epimutations explains the classic autosomal dominant inheritance pattern observed in families. However, it is the consequent transcriptional dysregulation of the *MSH2* mismatch repair gene incurred by the *EPCAM* deletion that predisposes to the development of Lynch syndrome-related cancers (given that the sequence within the entire *MSH2* gene and its promoter are normal in these cases). Furthermore, it also later became apparent that the mosaic manifestations of this defect modify the Lynch syndrome phenotype. Carriers of an *MSH2* epimutation have a higher risk of developing cancers in those tissues containing the highest levels of EPCAM expression, also marked by the highest levels of *MSH2* promoter methylation.

6.4.2 Prevalence of EPCAM Deletions in the Lynch Syndrome Population

In subsequent screens of various Lynch syndrome populations worldwide, additional cases with deletions of the last exons that included the polyadenylation signal of *EPCAM* were identified among patients and families with MSH2 loss in their tumours that had remained unaccounted for by the lack of *MSH2* germline mutations. Transcriptional read-through from *EPCAM* across the *MSH2* promoter and into *MSH2* with resultant *EPCAM-MSH2* fusion transcripts and methylation of the *MSH2* promoter in epithelial and tumour tissues were a consistent finding [47, 59–61]. Another consistent feature of the *EPCAM* deletions was their origination through mal-recombination events between linked unidirectional *Alu* repeat elements, which are plentiful in the genomic region spanning *EPCAM* and *MSH2* [47, 58, 59].

In multicentre studies aimed to estimate the frequency of this alternative causative mechanism in the Lynch syndrome population found *EPCAM* deletions among Germans comprised 1.1 % of all explained Lynch syndrome cases (those in whom a definitive pathogenic mutation had been identified), contributing to 2.3 % of all explained families with MSH2-deficient tumours [47]. In the Dutch Lynch syndrome population, *EPCAM* deletions accounted for 2.8 % of all explained Lynch syndrome families, amounting to 6.9 % of all explained families with MSH2-deficient tumours [47]. Interestingly, among these Dutch families, a significant fraction was accounted for by a particular founder deletion (c.859–1462_*1999 del), proven through shared haplotyping in the genomic region around the deletion in seemingly unrelated families. This may have elevated the frequency of *EPCAM* deletions in this population [46, 47, 58]. Single and multicentre studies combined showed that *EPCAM* deletions accounted for about one quarter of all cases with tumours demonstrating MSH2-loss that had not previously been accounted for by germline mutations of *MSH2* [62, 63]. This represents a substantive aetiological contribution to Lynch syndrome, warranting routine screening for this defect in molecular diagnostics.

6.4.3 Restricted Lynch Syndrome Phenotype Associated with EPCAM Deletions

An analysis of cancer incidence and estimated risk in a cohort of pooled cases, comprising 194 carriers of an *EPCAM* deletion from 41 families, showed that carriers of this defect have a high risk of developing CRC (75 % cumulative risk before the age of 70 years), comparable to that of their counterparts with a germline mutation of *MSH2* (77 % cumulative risk). However, *EPCAM* deletion carriers had a significantly reduced risk of developing endometrial cancer (12 % cumulative risk) as compared to carriers of a germline *MSH2* mutation (51–55 % cumulative risk)

[64]. This difference in risk for endometrial cancer was striking, given the high rate of extracolonic cancers in *MSH2* mutation carriers. While 93 of the 194 cases had developed a CRC, just three of 92 female *EPCAM* deletion carriers were diagnosed with endometrial cancer. Interestingly, these three cases were from families that carried the largest *EPCAM* deletions, which extended closest to the *MSH2* promoter (within 3 kb upstream) [64]. These findings were further substantiated in two of the largest Lynch syndrome pedigrees ever recorded in which the c.-859–1462_*1999 del Dutch founder mutation was identified. No incidence of endometrial cancer was identified in any of the confirmed or obligate female carriers of this more confined deletion from these two families, although nearly half of all carriers over 20 years of age had developed CRC [65].

6.4.4 Molecular Diagnosis of MSH2 Epimutations/ EPCAM Deletions

Since the link between germline *EPCAM* deletion and *MSH2* epimutation has been well established, molecular diagnosis of this defect has relied primarily on detection of the deletion by dosage analysis screening of constitutional DNA using MLPA. The current P072 version 6 MLPA kit produced by MRC Holland, Amsterdam, the Netherlands, has probes that interrogate *EPCAM* exons 3, 8 and 9 (the final exon), plus two probes within the intervening sequence between *EPCAM* and *MSH2*, one 3 kb downstream of *EPCAM* and another 2.5 kb upstream of *MSH2*, as well as one within *MSH2* exon 1, allowing for the initial detection and limited definition of the extent of the deletion. This assay has been successfully applied as the first-pass screen for the detection of numerous cases with an *EPCAM* deletion [62, 65].

Screening for *MSH2* methylation as a primary means of detection is likely to be highly unreliable given the extensive methylation mosaicism, especially if genomic DNA from peripheral blood lymphocytes is the sole source of clinical material available. While low-level methylation has been detected in the peripheral blood of some patients using sensitive techniques such as MSP, methylation-specific (MS) MLPA has failed to detect *MSH2* methylation in the blood DNA of confirmed carriers of an *EPCAM* deletion [46]. However, if tumour and accompanying normal colonic epithelium tissue are available, detection of *MSH2* methylation using MSP [7], CpG pyrosequencing [7], or MS-MLPA [47], would be suitable as a confirmatory test, given that methylation levels are typically high in these tissues. The SALSA MS-MLPA ME011 mismatch repair genes kit from MRC Holland contains three probes interrogating the methylation status at distinct *Hha*I CpG sites within the *MSH2* CpG island, including positions −269, −193 and +124 within exon 1. This allows for the concurrent testing for the presence of methylation in *MLH1* and *MSH2* in a single assay.

Interestingly, one proband with an MSH2-deficient tumour positive for *MSH2* methylation has been reported in whom no *EPCAM* deletion was detected. This case

may have a small deletion not identified by the MLPA probes, or a point mutation within the polyadenylation signal that similarly abrogates transcription termination [62]. Nevertheless, this case suggests that *EPCAM* deletion screening by MLPA may fail to identify a small number of cases with an *MSH2* epimutation as the cause for their phenotype. Thus *MSH2* methylation testing in an appropriate tissue source would serve as a complementary test for comprehensive screening, but due to the above caveats, should not serve as the primary test.

6.5 Implementation of Routine Screening for *EPCAM* Deletions/*MSH2* Epimutations and Genetic Counselling

6.5.1 Patient Selection for Molecular Diagnosis

EPCAM deletions leading to the indirect inactivation of *MSH2* account for a major fraction of those patients with a clinical diagnosis of Lynch syndrome whose tumours have shown loss of MSH2 activity, but have no germline mutation within *MSH2* itself. Yet, given that this defect is less frequent than germline point mutations or structural rearrangements within *MSH2*, screening for *EPCAM* deletions would most efficiently and cost-effectively be performed following standard screening for *MSH2* mutations by exonic sequencing and MLPA in cases presenting with a MSH2-deficient tumour. This is the triaged algorithm that has been utilized in the research setting for the identification of *EPCAM* deletions. An MLPA kit that combines screening for structural alterations within *MSH2* and *EPCAM* deletions concurrently would be ideal for screening those cases in whom *a priori* loss of MSH2 has been observed in their tumours, but is not currently available.

The *MSH2* methylation mosaicism observed in the different tissues among *EPCAM* deletion carriers has now been found to reflect the spectrum of tumour types observed. Endometrial tumours are rarer than in *MSH2* mutation carriers, and these have been confined to those patients with large *EPCAM* deletions extending close to the *MSH2* promoter, which may thus abrogate *MSH2* activity in additional non-epithelial tissues that do not express *EPCAM* [64]. This presents with a genetic counselling conundrum. If further studies confirm these findings among larger cohorts of patients, then this would allow genetic counselling advice and clinical management of *EPCAM* deletion carriers to be tailored accordingly, and the extent of their deletion could be predictive of their risk profile for cancer development. For example, female carriers of a small *EPCAM* deletion may be spared clinical surveillance for endometrial cancer, as well as prophylactic hysterectomy upon completion of child-bearing to prevent the future development of endometrial cancer [65]. Precision molecular diagnostics may be implemented in future, accompanied by mutation-specific cancer risk advice and clinical management.

6.6 Conclusion

While the potential involvement of subsidiary components of the mismatch repair pathway in the aetiology of Lynch syndrome to account for those cases without an apparent germline mutation within one of the four key mismatch repair genes continues to be debated, no substantive role for these additional genes has transpired. Constitutional epimutations illustrate that distinct mechanisms affecting the two major mismatch repair genes can give rise to a Lynch syndrome-related phenotype in a subset of these "mutation-negative" cases. This mechanism causes an epigenetic, as opposed to a direct genetic, disruption of gene activity. The discovery of these novel mechanisms within the last decade, and the recognition that they elicit a phenotype strongly reminiscent of Lynch syndrome, has provided a molecular diagnosis to a number of patients and families who until recently received an uninformative genetic test result. Nevertheless, the complexities that beset this novel epigenetic disease-causing mechanism mean that strategies for molecular diagnosis on a routine clinical basis and accurate genetic counselling advice have yet to be formulated. Further research is required to fully elucidate the molecular basis for epimutations, where this has yet to be defined, as well as the risks and patterns of transmission to successive generations, before informed genetic counselling and the full clinical translational benefits of these recent research findings are realised. Moving ahead, the assessment of cancer predisposition syndromes, including Lynch syndrome and others, must now take into account constitutional epigenetic changes, as well as genetic mutations.

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Chapter 7
Mutations in Non-MMR Genes Modifying or Mimicking Lynch Syndrome Phenotype

Mala Pande, Jinyun Chen, Chongjuan Wei, Yu-Jing Huang, and Marsha L. Frazier

Abstract Lynch syndrome is an inherited cancer predisposition syndrome associated with an increased risk of colorectal, endometrial and many other cancers, typically with an earlier age of cancer onset. The underlying cause of Lynch syndrome is a defect in DNA mismatch repair (MMR) due to mutations in one of the DNA MMR genes: *MLH1*, *MSH2*, *MSH6* or *PMS2*. It has been observed that cancer risk and cancer age of onset vary significantly among MMR gene mutation carriers suggesting that other non-MMR genes may modify the Lynch syndrome phenotype. Studies examining the association of genetic variation in non-MMR genes and colorectal cancer (CRC) risk in MMR mutation carriers have found an increased risk or an earlier age of cancer onset for polymorphisms in genes in pathways such as cell cycle, DNA repair and methylation, carcinogen metabolism, and others. Some common genetic variants associated with sporadic CRC risk in genome wide association studies have also been implicated in modifying CRC risk in Lynch syndrome suggesting a global role of these variants in influencing CRC risk. The Lynch syndrome cancer phenotype is also manifested indirectly through epimutations outside the MMR genes that influence expression of these genes. For example, deletions in the *EPCAM* gene upstream of the *MSH2* promoter region lead to hypermethylation of the *MSH2* promoter, resulting in loss of MSH2 expression and predisposing people with *EPCAM* deletions to a cancer spectrum that mimics Lynch syndrome. The chapter provides a review of mutations in non-MMR genes that modify or mimic the Lynch syndrome phenotype.

Keywords Lynch syndrome • Colorectal cancer • DNA mismatch repair • Modifier genes • Hereditary • Early onset • Polymorphism

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7.1 Introduction

It is well established that patients with Lynch syndrome (also commonly known as hereditary non-polyposis colorectal cancer – HNPCC) are susceptible to many types of early onset cancers. However, relatives sharing the same genetic predisposition display heterogeneity in cancer expression, particularly time to cancer onset. This variation in phenotypic expression suggests that in addition to susceptibility due to mutations in cancer predisposing DNA mismatch repair (MMR) genes, these patients' cancer risk may also be modified by other genetic and environmental factors. Considerable effort has been devoted to investigating alterations in genes involved in the cell cycle, DNA repair, carcinogen metabolism, and more recently, hits from genome-wide association studies as modifiers of cancer risk in patients with Lynch syndrome. A review of studies that examine mutations in non-MMR genes that modify or mimic the Lynch syndrome phenotype follows.

7.2 Mutations in Non-MMR Genes Modifying Lynch Syndrome Phenotype

7.2.1 Cell-Cycle Gene Modifiers

Cell cycle checkpoints play a key role in the cellular response to DNA damage by arresting the cell cycle to provide more time for repair before the critical phases of DNA replication [1]. Checkpoint loss can lead to genomic instability, a common cause and hallmark of cancer. Therefore, genetic polymorphisms in cell cycle genes have been investigated for their modifying effect on cancer risk. Previous studies indicate that polymorphisms in the cell cycle genes *CCND1*, *TP53*, *IGF1*, and *AURKA* are associated with earlier age of onset of colorectal cancer (CRC) in Lynch Syndrome [2–5].

7.2.1.1 *CCND1* (Cyclin D1)

CCND1 regulates the cell cycle by controlling the transition from the G1 to the S phase, which is regulated by cyclin-dependent kinases [6, 7]. The most highly investigated polymorphism in *CCND1* is the G-to-A substitution in codon 242 (rs603965). *CCND1* mRNA exhibits alternative splicing, giving rise to two different transcripts (transcripts a and b). The polymorphism at the boundary of exon 4 and intron 4 affects alternative splicing and results in variant transcript for *CCND1* (transcript b) [8]. Kong et al. analyzed 86 MMR mutation carriers in *MSH2* or *MLH1* with Lynch syndrome, 49 of whom had CRC and 37 of whom were unaffected [4]. They found that subjects with the AA and AG *CCND1* genotypes developed CRC on average 11 years earlier and were 2.46 times more likely to develop cancer during any interval than were those with the GG genotype (hazard ratio = 2.46;

95 % confidence interval [CI] = 1.16–5.21; P = 0.019) [4]. The median ages of onset for the genotypes GG, AG, and AA were 48, 38.5, and 37 years, respectively. This was the first report indicating that the *CCND1* polymorphism influences the age of onset of cancer. In contrast, a Finnish study did not observe a correlation between *CCND1* genotype and age-associated CRC risk in 146 Finnish affected carriers, most of whom (n = 138, 94.5 %) carried a single truncating mutation in *MLH1* [9]. Similarly, no association was seen in the German Lynch syndrome patients [10]. However, an Australian study found an association between polymorphism and the age of CRC onset in patients harboring *MSH2* mutation [11]. The discrepancy in these studies might result from the genetic heterogeneity between the different populations analyzed and the predominance of *MSH2* mutation carriers in one population over *MLH1* carriers in the other.

7.2.1.2 *TP53* (Tumor Protein 53)

TP53 is expressed at low levels in the cell, but it can be upregulated by stimuli such as DNA damage, hypoxia, or dysregulated cell-cycle progression [12]. A G-to-C polymorphism (rs1042522) in codon 72 of exon4 of *TP53* results in an arginine-to-proline amino acid substitution. Jones et al. suggested that the common polymorphism was associated with an earlier age of CRC onset in 92 confirmed MMR mutation carriers from 47 Caucasian families with Lynch syndrome [3]. The patients who were heterozygous for the *TP53* polymorphism developed CRC 13 years earlier, with a 40-year median onset age, than did the patients who were homozygous for the wild-type allele, with a 53-year median onset age. The hazard ratio from this analysis indicated that individuals with heterozygous *TP53* were 1.94 times more likely to get CRC during any age interval than those with homozygous wild-type *TP53* (P = 0.04; 95 % CI = 1.03–3.63). Kruger et al. reported a similar result from a study of a German population of 167 unrelated patients with germline mutations in *MSH2* or *MLH1* and colorectal carcinoma as their first tumor [13]. The median ages at cancer onset were 41, 36, and 32 years for patients with the GG, GC, and CC *TP53* genotypes, respectively. The age of onset was significantly different between the three genotypes in a global comparison (log-rank P = 0.0001). They further confirmed their results in another study of 246 unrelated Lynch syndrome patients with mutations either in *MSH2* or *MLH1* [14]. Furthermore, they found the additive effect of TP53 Arg72Pro and RNASEL, which encodes ubiquitously expressed endoribonuclease acting in the interferon regulated 2′-5′-linked oligoadenylates (2-5A) system, Arg462Gln genotypes on age of onset in Lynch syndrome patients. Their group also found the *RNASEL* polymorphism was significantly associated with an earlier age of CRC in a dose-dependent manner in a study with 251 unrelated Lynch syndrome patients [15]. However, a study of Lynch syndrome patients from Finland failed to show association of the *TP53* polymorphism with age of CRC onset in 193 MMR mutation carriers [16]. Similarly, there was no evidence of an association between the *TP53* polymorphism and age at diagnosis of CRC in 84 Australian and 134 Polish Lynch syndrome patients with MMR mutations [17].

7.2.1.3 *MDM2* (p53 E3 Ubiquitin Protein Ligase Homolog (Mouse))

The main function of MDM2 is to negatively regulate TP53 tumor suppressor activity [18]. A single nucleotide polymorphism (SNP) in the promoter of *MDM2* (SNP309T→G) (rs2279744) upregulates the gene, thereby enhancing subsequent attenuation of the TP53 pathway [19]. The SNP was reported to be associated with a significantly earlier age of onset (9 or more years earlier) for all tumor types and an increased occurrence of multiple primary tumors in patients with Li-Fraumeni syndrome [20]. However, SNP309 was reported to have had no effect on age of onset of CRC in Lynch syndrome patients alone or in combination with the *TP53* R72P polymorphism [16, 20].

7.2.1.4 *IGF1* (Insulin-Like Growth Factor 1)

Insulin-like growth factor 1 (IGF1) is a polypeptide that plays an important role in regulating cellular proliferation and apoptosis, and high levels of circulating IGF-1 are associated with increased risk of several common cancers [21]. A CA dinucleotide repeat polymorphism located in the untranslated region 969 bp upstream of the initiation start site of *IGF1* is thought to alter promoter activity and, thus, influence the transcription rate of *IGF1* [22]. Zecevic et al. found there was a statistically significant association between CA dinucleotide-repeat polymorphism and age of onset of CRC in 121 individuals (from 59 families) with confirmed germline mutations in *MLH1* or *MSH2* (for every decrease by one CA-repeat length, hazard ratio = 1.17; 95 % CI = 1.05–1.31; P = 0.006). Patients carrying an allele with 17 CA repeats had a significantly higher CRC risk (hazard ratio = 2.36; 95 % CI = 1.28–4.36; P = 0.006) and were younger at cancer onset (44 years versus 56.5 years; P = 0.023) than all other patients. These findings indicate a significant inverse association between *IGF1* CA-repeat length and risk for CRC in Lynch syndrome [5]. Reeves et al. confirmed that the *IGF1* polymorphism is an important modifier of disease onset in Lynch syndrome in both 220 Australian and 223 Polish *MLH1* or *MSH2* mutation carriers [23, 24]. Houlle et al. did not reproduce these results in 748 French *MLH1*, *MSH2*, or *MSH6* mutation carriers. However, the study included *MSH6* mutation carriers in the analysis and they observed a significant difference in the CRC-free survival time between *MSH2*/*MLH1* and *MSH6* mutation carriers [25]. Including *MSH6* mutation carriers in the analysis may have attenuated their results because of the later age of onset associated with *MSH6* mutation carriers. The other studies did not have *MHSH6* mutation carriers in their analysis.

7.2.1.5 *AURKA* (Aurora Kinase A)

AURKA positively regulates the G2-to-M phase of the cell cycle. DNA damage inhibits the activation of AURKA in late G2 [26]. A common T-to-A SNP (rs2273535) in the *AURKA* gene results in a change in the protein sequence from

phenylalanine to isoleucine. Chen et al. found that the median age of onset in subjects who were homozygous for the wild-type allele (TT) was 51 years, which was 7 years earlier than the median age of onset (58 years) in subjects who were homozygous (AA) or heterozygous (TA) for the polymorphic allele in 125 Caucasians with germ-line mutations in *MLH1* or *MSH2* from 60 families [2]. This result was not replicated by a Lynch syndrome study with the sample population consisting of 312 participants from Australia and Poland [11]. However, another study by Chen et al. found this SNP worked together with other SNPs in cell cycle-related genes to modify the age at the onset of CRC in 220 MMR gene mutation carriers from 129 families [27].

7.2.1.6 Classification and Regression Analysis

Cancer is a multifactorial complex human disease and even though many genetic risk factors are known to play a role in the etiology of cancer, individually these factors may not be sufficient to predict the risk for cancer. A multigenic pathways-based approach, which assesses the combined effects of a comprehensive panel of genetic factors that interact in same pathways, may amplify the effects of individual factors and enhance predictive power. Chen et al. evaluated polymorphisms in a panel of cell cycle-related genes in 220 MMR gene mutation carriers from 129 families. They applied a novel statistical approach, tree modeling (classification and regression tree), to the analysis of data to identify individuals with a higher probability of developing CRC at an early age and explore the gene-gene interactions between polymorphisms in cell cycle genes. They found that the subgroup with *CDKN2A* C580T wild-type genotype, *IGF1* CA-repeats >19, *E2F2* variant genotype, *AURKA* wild-type genotype, and *CCND1* variant genotype had the youngest age of onset, with a 45-year median onset age, while the subgroup with *CDKN2A* C580T wild-type genotype, *IGF1* CA-repeats >19, *E2F2* wild-type genotype, and *AURKA* variant genotype had the latest median age of onset, which was 70 years. Furthermore, they found evidence of a possible gene-gene interaction between *E2F2* and *AURKA* genes related to CRC age of onset (Fig. 7.1) [27]. This study provided evidence that polymorphisms in the cell cycle-related genes work together to modify the age at the onset of CRC in patients with Lynch syndrome. Future studies should be directed toward confirming these findings in a larger sample population.

7.2.2 Modifier Genes Involved in Metabolism

Xenobiotic metabolizing genes are involved in metabolizing and detoxifying carcinogens in the body. Genetic variation in these genes can alter the carcinogen metabolizing and detoxifying capacity of the body and potentially influence cancer risk. Polymorphisms in genes that encode enzymes involved in the metabolism of

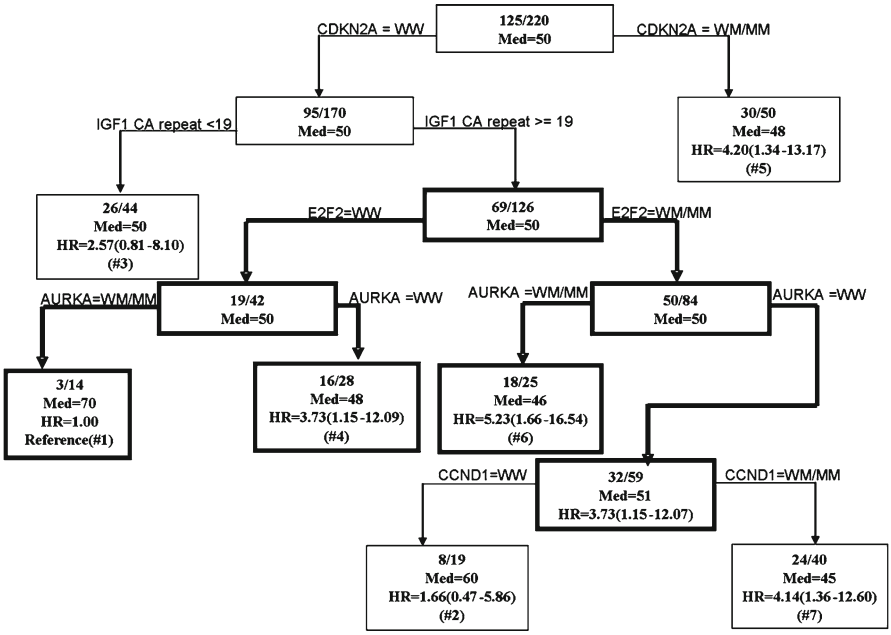

Fig. 7.1 Time-to-onset tree for age of onset of CRC. Inside each node is the number of affected subjects/the total number of subjects. *Med*, median age of onset. The hazard ratios (*HRs*) are calculated for all terminal nodes such that terminal node 1 is the reference group. *WW* wild type, *WM* heterozygote, *MM* homozygous polymorphism. The nodes giving rise to gene–gene interaction are shown in *bold* (This figure is reproduced from Chen et al. [27]. Figure copyright Springer 2009. With kind permission from Springer)

carcinogens result in varying activity levels of these enzymes, which can then influence xenobiotic clearance. Some exogenous chemicals such as paracyclic aromatic hydrocarbons are ubiquitous environmental, dietary and tobacco carcinogens. Cancer risk resulting from exposure to these and other such xenobiotics may vary according to the ability to clear these from the body. Although MMR mutation carriers are at increased cancer risk because of deficient DNA mismatch repair, their risk may be further influenced by variation in their ability to process exposure to carcinogens. Therefore, gene polymorphisms in the xenobiotic metabolism genes could influence the cancer phenotype and age of onset of cancer in MMR mutation carriers. The influence of genes metabolizing carcinogens on cancer risk has been widely studied in sporadic CRC (reviewed in [28]). Some of the frequently studied xenobiotic metabolizing genes include the cytochrome P450 group (*CYP*s), glutathione S-transferase group (*GST*s) and N-acetyltransferase group (*NAT*s). Other metabolic genes that have been studied as modifiers of risk include genes influencing endogenous hormone metabolism and genes involved in the folate metabolism pathway. Polymorphisms in genes involved in metabolism have also been investigated as modifiers of CRC age of onset in MMR mutation carriers but the results have not been consistent as evident from the review of studies that follows.

7.2.2.1 *NAT2* (N-Acetyl Transferase 2)

One of the earliest and most commonly studied xenobiotic metabolizing genes, *NAT2* catalyzes the metabolism of chemical carcinogens by transferring an acetyl group to these agents, rendering them water soluble so that they are easily excreted from the body. Based on allelic variants of this gene, individuals can be classified as rapid or slow acetylators. In one of the first *NAT2* studies among 78 MMR mutation carriers from 21 Swiss Lynch syndrome families, Heinemann et al. found a higher prevalence of slow acetylators among cancer-affected MMR mutation carriers ($P < 0.03$) but did not find a difference in the mean age at cancer onset between rapid and slow acetylators [29]. Thereafter, in a study of 86 MMR mutation carriers from 43 families, there was no significant difference in the time to CRC onset between rapid (*NAT2*4*) and slow (*NAT2*5*, *NAT2*6*, and *NAT2*7*) acetylators [30]. However, on stratifying by *NAT2* polymorphisms, heterozygous carriers of *NAT2*7* had a significantly higher risk of CRC than homozygous wild-type *NAT2*7* allele carriers, after adjusting for *NAT2*5* and *NAT2*6* (HR = 2.96, 95 % CI = 1.28–6.85) [30]. These results were not replicated by two subsequent studies that reported no association between *NAT2* and CRC risk or age at CRC onset [31, 32].

7.2.2.2 *NAT1* (N-Acetyl Transferase 1), *GSTM1*, *GSTT1*, and *GSTP1* (Glutathione S-Transferase Mu 1, Theta 1 and Pi 1)

Polymorphisms in *NAT1*, *GSTM1*, and *GSTT1* were investigated as modifiers of risk in a Finnish population of 182 MMR mutation carriers. Study subjects were subdivided into two groups (n = 150 and 32), each group carrying one ancestral *MLH1* founder mutation. *NAT1* was binary according to presence or absence of allele 10, and *GSTM1* and *GSTT1* were defined as either present or deleted. The authors reported a 2.5 years earlier median age of CRC onset in group 1, and 5 years earlier median age of CRC onset in group 2, associated with the presence of *NAT1*10*. *GSTM1* and *GSTT1* null (deleted) were each independently associated with a 6-years-earlier median age of CRC onset in group 1 but with no observable difference in group 2 [33]. Another study among a Swiss group of 78 MMR mutation carriers reported no association between *GSTM1* and *GSTT1* polymorphisms and age of CRC onset [29]. Null results were also reported for *GSTM1* and age of CRC onset in a US study of 86 MMR mutation carriers [34]. However, authors of a later study in a South African cohort of 129 individuals from 13 families, all carrying an identical mutation in the *MLH1* gene, reported a threefold increased risk for earlier onset of CRC among males if they were carriers of null alleles of both *GSTM1* and *GSTT1* compared with men who had neither null allele but this was the only study that reported such a large effect size [35]. Two subsequent studies on polymorphisms in *GSTM1* and *GSTT1*, one in a combined Australian (n = 86) and Polish (n = 134) cohort of mutation carriers [32] and another an expanded US cohort of 257 mutation carriers from 130 families (that included the subjects in [34]), found no association between *GSTM1* or *GSTT1* polymorphisms and age-associated risk of

CRC [36]. The latter two studies also examined a non-synonymous I105V SNP in *GSTP1* but the results were non-significant [32, 36].

7.2.2.3 *CYP1A1* (Cytochrome P450, Family 1, Subfamily A, Polypeptide 1) and *EPHX1* (Microsomal Epoxide Hydrolase 1)

Evidence of increased risk of CRC by *CYP1A1* genotypes in MMR mutation carriers was first reported by Talseth et al. who found that patients with CRC harbored more of the mutant allele of *CYP1A1* T3810C than did patients who did not have CRC (odds ratio [OR] = 0.39, 95 % CI = 0.17–0.88), although there was no difference in the age of CRC onset by *CYP1A1* genotypes [32]. In another study, two *CYP1A1* SNPs, I462V and Msp1 (g.6235T>C), were associated with an increased risk for developing CRC and median age of onset of CRC was 4 years earlier for those heterozygous for the I462V mutant allele (log-rank test $P = 0.016$), suggesting that genetic variation in *CYP1A1* may be an additional susceptibility factor for CRC development in people with Lynch syndrome [36]. However, the results have to be viewed with caution because the mutant allele frequency for the variant allele was low and the results have not been further validated. The study also analyzed the Y113H SNP in *EPHX1* but found no association with CRC age of onset. Interestingly there was suggestion of a multiplicative interaction between *CYP1A1* I462V and *EPHX1* Y113H such that patients carrying any mutant allele of *CYP1A1* I462V in the presence of any mutant allele of *EPHX1* Y113H had a median age of CRC onset of 37 years, compared to 42 years for those carrying the homozygous wild-type allele (log-rank test $P = 0.002$) [36].

7.2.2.4 *CYP17A1* (Cytochrome P450, Family 17, Subfamily A, Polypeptide 1) and *COMT* (Catechol O-Methyltransferase)

Two polymorphisms that influence metabolism of endogenous hormones were genotyped in 146 Caucasian Lynch syndrome mutation carriers, and it was observed that homozygous carriers of the variant allele of *CYP17A1* were diagnosed with CRC on average 18 years earlier than carriers of the homozygous wild-type allele and that there was an increasing trend in risk associated with the increasing number of adverse alleles of the *CYP17A1* polymorphism [37]. The polymorphism in *COMT* did not influence age at diagnosis [37].

7.2.2.5 *MTHFR* (Methylenetetrahydrofolate Reductase)

MTHFR plays a key role in folate metabolism, which in turn influences DNA synthesis and repair and DNA methylation. In view of the potential for alterations in these processes to influence carcinogenesis, 2 polymorphisms in the *MTHFR* gene

were examined as risk modifiers in 185 Caucasian Lynch syndrome mutation carriers from 81 families. Of these, the C677T polymorphism was reported to positively influence the age at CRC onset: carriers of any variant allele had a median age of onset 4 years later than homozygous carriers of the wild-type allele, and a significantly reduced age-associated CRC risk (HR = 0.55, 95 % CI = 0.36–0.85) [38]. Another MTHFR polymorphism, A1298C, was not significantly associated with CRC onset age or risk [38].

7.2.3 Modifier Genes Involved in DNA Repair and DNA Methylation

DNA repair plays an essential role in maintaining genomic integrity. There are at least 130 genes involved in a variety of different DNA repair pathways, including base excision repair, nucleotide excision repair, double-strand break repair, and DNA mismatch repair [39, 40]. Polymorphisms within genes that are involved in these processes have been extensively reported to be associated with susceptibility to various types of cancer, including CRC. Since Lynch syndrome is caused by germline mutations in DNA mismatch repair gene, there are some attempts to assess whether genetic polymorphisms in other DNA repair genes will modify the age-associated risk for CRC in the presence of inherited mutation in the MMR genes in Lynch syndrome. In addition, genes involved in methylation also play an important role in DNA repair and genome stability [41].

7.2.3.1 *ATM* (Ataxia Telangiectasia Mutated)

The protein encoded by this gene is an important cell cycle checkpoint kinase and also functions as the master controller of cellular response to DNA double-strand breaks and for genome stability [42]. Germline mutations in *ATM* lead to an autosomal recessive disorder ataxiatelangiectasia. Two studies of *ATM* rs1801516 (D1853N) polymorphism in Lynch syndrome provided contradictory results. In the first study, 167 Swiss individuals from 20 Lynch syndrome families were evaluated. Among the 67 individuals with *MLH1* or *MSH2* mutations, the carriers of mutant *ATM* allele had an eight-times-higher risk of developing Lynch syndrome or Lynch syndrome-related cancer (OR = 8.9; P = 0.02) compared with the carriers of the *ATM* wild-type allele [43], suggesting that the *ATM* D1853N polymorphism modulates the penetrance of *MLH1* and *MSH2* germline mutations. However, in the second study of 109 individuals from 53 American Lynch syndrome families with MMR mutations, the *ATM* polymorphism D1853N did not show a significant influence on the age of onset or the overall risk of CRC [44]. Although the modifying effects of *ATM* D1853N are not consistent, future studies should consider the influence of the differences in study populations and possible environmental and other genetic factors.

7.2.3.2 *OGG1* (8-Oxoguanine DNA Glycosylase)

This gene encodes a member of the base-excision repair family. The OGG1 protein is responsible for the excision of 8-oxoguanine, a mutagenic base byproduct that occurs as a result of exposure to reactive oxygen. The action of this enzyme includes lyase activity for chain cleavage. Polymorphic variation in *OGG1* has been associated with cancer susceptibility [45–47]. Among those, rs56053615, also known as *OGG1* R154H, has been identified as a cancer-related mutation [45, 46]. Kim et al. conducted analysis of the R154H mutation in samples from 625 patients (20 with familial adenomatous polyposis, 19 with HNPCC, 86 with suspected HNPCC, 500 with sporadic CRC) and 527 normal controls [47]. The R154H polymorphism was not correlated with Lynch syndrome but associated with sporadic CRC (OR = 3.586), with a *P*-value of borderline significance ($P = 0.053$). The SNP, rs1052133, also known as S326C in *OGG1*, has been reported to be associated with increased risk for bladder cancer [48], gallbladder cancer [48], and lung cancer [49]. More recently, Reeves et al. [50] conducted a study on a cohort of Australian (n = 220) and Polish (n = 204) MMR mutation carriers to assess the association of rs1052133 with the age of CRC onset; the data were analyzed by combining two ethnic groups together and also by individual ethnic groups, but the results were not significant. Together, these data suggest that these 2 *OGG1* SNPs did not modify CRC risk in the context of Lynch Syndrome.

7.2.3.3 *XRCC1* (X-Ray Repair Cross-Complementing 1)

The protein encoded by *XRCC1* also belongs to the base-excision repair family. It interacts with DNA ligase III, polymerase beta and poly (ADP-ribose) polymerase to efficiently repair DNA single-strand breaks formed by exposure to ionizing radiation and alkylating agents. A polymorphism, rs25487 in *XRCC1* R399Q, has been reported to be associated with increased risk of lung cancer [51], glioma [52] and cervical carcinoma [53], but a decreased risk of bladder cancer [48]. In a study conducted by Reeves et al. [50], rs25487 in *XRCC1* was not found to contribute to CRC risk in Lynch syndrome.

7.2.3.4 *XRCC2* (X-Ray Repair Complementing Defective Repair in Chinese Hamster Cells 2) and *XRCC3* (X-Ray Repair Complementing Defective Repair in Chinese Hamster Cells 3)

These two genes encode members of the RecA/Rad51-related protein family that participate in homologous recombination to maintain chromosome stability and repair DNA damage. XRCC2 and XRCC3 are two key mediators in homologous recombination that repair double-strand DNA. Polymorphisms in *XRCC2* and *XRCC3* have been linked to a risk of CRC [54], breast cancer [55], and endometrial cancer [55]. Polymorphisms in *XRCC2* (rs3218536) and *XRCC3* (rs861539) were investigated in a cohort including Australian (n = 220) and Polish (n = 204) MMR mutation carriers by Reeves et al. [50]. No significant association of these DNA repair SNPs were found in an overall analysis of all 424 participants or in each ethnic group separately.

7.2.3.5 *MSH3* (DNA Mismatch Repair Protein Msh3)

The protein encoded by *MSH3* belongs to the mismatch repair family. MSH3 heterodimerizes with MSH2 to form MutS beta, part of the post-replicative DNA mismatch repair system, to initiate mismatch repair by binding to a mismatch and then forming a complex with MutL alpha heterodimer. In addition to MSH2 [56–59], MSH3 has been shown to interact with proliferating cell nuclear antigen (PCNA) [60–62] and BRCA1 [63, 64]. A non-synonymous polymorphism A1045T, also known as *MSH3* rs26279 was associated with esophageal cancer [65] and CRC [66]. Most recently, Reeves et al. [50] examine the same polymorphism in a cohort including Australian (n = 220) and Polish (n = 204) MMR mutation carriers. No significant association was observed in an overall analysis of those 424 participants or either of the two ethnic groups. However, in the Polish population, it did display a trend in age of onset in the presence of the SNP.

7.2.3.6 *DNMT3B* (DNA (Cytosine-5)-Methyltransferase 3 Beta)

This gene encodes a DNA (cytosine-5)-methyltransferase (DNMT) which is thought to function in *de novo* methylation. Mutations in this gene cause the immunodeficiency-centromeric instability-facial anomalies (ICF) syndrome. DNMT methylates newly replicated mammalian DNA through binding PCNA that is an auxiliary factor for DNA replication and repair [67]. A C-to-T polymorphism (rs2424913) in *DNMT3B* promoter region, −149 bp from the transcription start site, was associated with an increased risk of lung cancer [68], prostate cancer [69], and colorectal polyps [70]. Most recently, this polymorphism was identified as a risk factor for Late Onset Alzheimer's Disease [71]. The polymorphism is thought to significantly increase *DNTM3B* promoter activity, resulting in increased aberrant *de novo* methylation of CpG islands and thus transcriptional repression of some tumor suppressor genes [72, 73]. Jones et al. [74] examined the same polymorphism in 146 individuals with MMR mutations from 72 families with Lynch syndrome. Patients carrying mutated *DNMT3B* allele developed CRC significantly earlier than the patients with homozygous wild-type allele ($P = 0.021$). In addition, patients carrying the mutated *DNMT3B* allele were 2.03 times more likely to develop CRC than patients with homozygous wild-type allele ($P < 0.03$). The median age of onset by genotype was 71 years for wild-type homozygotes, 57 years for heterozygotes, and 51 years for SNP homozygotes.

The recognition that many polymorphisms in DNA repair genes have been associated with cancer susceptibility in the general population led to the investigations on whether altered repair function may explain some of the phenotypic differences such as age of onset observed in Lynch syndrome. However, definite conclusions cannot be drawn about the role of DNA repair genes in modifying the variability of age of onset in Lynch Syndrome. Those genes probably contribute subtly in the presence of MMR gene mutations. In addition, the observation of MMR proteins hMSH2, hMSH6 and hMLH1 coupled together with BRCA1, ATM, hRAD50 and hMRE11 has suggested MMR proteins response to other forms of DNA damage

such as double-strand breaks in addition to mismatch [75]. Recently, MSH6 was identified as a novel interacting protein with Ku that plays an essential role in repair of DNA double-strand breaks [76]. Altogether, it raises the possibility that MMR proteins play a unique role as "damage sensors" in the DNA repair signaling pathway, and the effect of the MMR mutation may outweigh the effects conferred by other more subtle alterations in DNA repair. Since the replication of genetic and epigenetic information is directly coupled, the ability of restoration of methylation of CpG islands during DNA repair may also play an important role in the age-associated cancer risk. Therefore, further studies in this area are needed.

7.3 Genome-Wide Association Studies (GWASs) Identified CRC Susceptibility Loci

Recent GWASs have identified a number of common genetic susceptibility loci associated with CRC risk in chromosome regions of 1q41 [77], 3q26.2 [77], 6p21 [78], 8q23.3 [79], 8q24.21 [80, 81], 9p24 [81], 10p14 [79], 11q13.4 [78], 11q23.1 [82], 12q13.13 [77], 14q22.2 [83], 15q13.3 [84], 16q22.1 [83], 18q21.1 [85], 19q13.11 [83], 20p12.3 [83], 20q13.33 [77], and Xp22.2 [78]. Some of these loci have been successfully replicated in different populations (summarized in Table 7.1).

The first GWAS-identified risk variants (SNPs) were rs6983267 and rs10505477 (which were in strong linkage disequilibrium with each other) at chromosome 8q24.21 which is located close to *POUF1P1*/*MYC* [80, 81]. The findings have been independently validated across multiple studies [79, 83, 90, 92–96, 98, 99]. These variants have also been associated with elevated risk of other types of cancer, including prostate [105–107], breast [108]and ovarian cancer [109]. A second locus was identified at the 18q21.1 region, with three SNPs (rs4939827, rs12953717 and rs4464148) that map to an intronic sequence of *SMAD7* [85], and then Tenesa et al. further replicated previous findings in another GWAS consisting of 16,759 cases and 15,545 controls [82]. Subsequently, other loci were identified at 15q13.3 (rs4779584 and rs10318; *GREM1*/*SCG5*), 8q23.3 (rs16892766; *EIF3H*), 10p14 (rs10795668, *FLJ3802842*), and 11q23.1 (rs3802842; *C11orf53*/*FLJ45803*/*LOC12 0376*/*POU2AF1*) [79, 82, 84]. Overall, these above-mentioned genetic variants have relatively modest effects on CRC risk (allelic or genotypic OR of about 1.2).

Four additional loci, 14q22.2 (rs4444235; *BMP4*), 16q22.1 (rs9929218; *CDH1*), 19q13.1 (rs10411210; *RHPN2*) and 20p12.3 (rs961253; *BMP2*), were detected in a meta-analysis by pooling data from two previous GWASs and conducting replication analyses in multiple independent case-controls series from various populations [83]. However, no interaction between the identified loci was observed in the study, and the common variants identified were estimated to collectively contribute to a small proportion of the excess risk for CRC. Most recently, a meta-analysis was conducted by pooling data from 5 GWASs with extensive replication analyses identified three new CRC susceptibility loci at 6p21 (rs1321311; *CDKN1A*), 11q13.4 (rs3824999; *POLD3*) and Xp22.2 (rs5934683; *SHROOM2*) for the first time [78].

Table 7.1 Summary of the CRC susceptibility loci identified through GWAS

Chromosome	Gene/locus	SNP ID	MAF[a]	GWAS	Sample size (case/control)		OR (95 % CI)[b]	*P* value[c]	Replications
					GWAS	Replication			
1q41	*DUSP10*	rs6691170	T, 0.26	[77]	3,334/4,628	18,095/20,197	1.06 (1.03–1.09)	9.6×10^{-10}	
3q26.2	*MYNN*	rs10936599	T, 0.30	[77]	3,334/4,628	18,095/20,197	0.93 (0.91–0.96)	3.4×10^{-8}	
6p21	*CDKN1A*	rs1321311	A, 0.25	[78]	8,682/9,649	21,096/19,555	1.10 (1.07–1.13)	1.1×10^{-10}	
8q23.3	*EIF3H*	rs16892766	C, 0.07	[79]	922/927	2,854/2,822	1.25 (1.19–1.32)	3.3×10^{-18}	[83, 86–89]
8q24.21	*POU5F1P1/ DQ515897/MYC*	rs6983267 (or rs10505477)[d]	T, 0.44	[80, 81]	930/960	7,334/5,246	1.21 (1.15–1.27)	1.3×10^{-14}	[79, 83, 86, 90–99]
					1,257/1,336	7,480/7,779	1.17 (1.12–1.23)	3.2×10^{-11}	
9p24	*Intergenic*	rs719725[e]	C, 0.33	[81]	1,257/1,336	7,480/7,779	1.08 (1.01–1.15)	0.023	[97, 100]
10p14	*FLJ3802842*	rs10795668	A, 0.27	[79]	922/927	2,854/2,822	0.89 (1.05–1.16)	2.5×10^{-13}	[83]
11q13.4	*POLD3*	rs3824999	G, 0.38	[78]	8,682/9,649	21,096/19,555	1.08 (1.05–1.10)	3.7×10^{-10}	
11q23.1	*C11orf53/FLJ45803/ LOC120376/POU2AF1*	rs3802842	C, 0.31	[82]	3,004/3,094	14,453/13,259	1.11 (1.08–1.15)	5.8×10^{-10}	[83, 87–89]
12q13.13	*ATF1/ DIP2B*	rs11169552	T, 0.24	[77]	3,334/4,628	18,095/20,197	0.92 (0.90–0.95)	1.9×10^{-10}	
14q22.2	*BMP4*	rs4444235	C, 0.43	[83]	6,780/6,843	13,406/14,012	1.11 (1.08–1.15)	8.1×10^{-10}	[101]
15q13.3	*GREM1/SCG5*	rs4779584	T, 0.46	[84]	718/960	7,922/6,741	1.26 (1.19–1.34)	4.4×10^{-14}	[79, 83, 86, 102]
15q13.3	*GREM1/SCG5*	rs10318	T, 0.24	[84]	718/960	7,922/6,741	1.19 (1.12–1.26)	7.9×10^{-9}	
16q22.1	*CDH1*	rs9929218	A, 0.25	[83]	6,780/6,843	13,406/14,012	0.91 (0.89–0.94)	1.2×10^{-8}	[86]
18q21.1	*SMAD7*	rs4939827	T, 0.38	[85]	620/960	7,377/5,867	0.85 (0.81–0.89)	1.0×10^{-12}	[79, 82, 83, 86, 92, 103] only in women [104]
19q13.1	*RHPN2*	rs1041210	T, 0.21	[83]	6,780/6,843	13,406/14,012	0.87 (0.83–0.91)	4.6×10^{-9}	
20p12.3	*BMP2*	rs961253	A, 0.29	[83]	6,780/6,843	13,406/14,012	1.12 (1.08–1.16)	2.0×10^{-10}	[86, 101]
20q13.33	*LAMA5*	rs4925386	T, 0.41	[77]	3,334/4,628	18,095/20,197	0.93 (0.91–0.95)	1.9×10^{-10}	
Xp22.2	*SHROOM2*	rs5934683	C, 0.38	[78]	8,682/9,649	21,096/19,555	1.07 (1.04–1.10)	7.3×10^{-10}	

[a]MAF, minor allele frequency reported in the NCBI dbSNP database

[b]OR (95 % CI): odds ratio with its 95 % confidence interval from the first listed GWAS reference

[c]*P*-value from first listed GWAS reference

[d]rs6983267 and rs10505477 are in high linkage disequilibrium

[e]Having combined OR for stages 1–4 of 1.14 ($P=1.32\times10^{-5}$), but it was not validated in all replication phases (all 7 cohorts)

7.4 Genetic Variation in the Transforming Growth Factor-β (TGFβ) Pathway to CRC Susceptibility

Because a majority of these identified variants were not mapped within or near a coding region, and most variants identified so far have no predicted functional relevance that leads to CRC [110, 111], it is believed that the actual causal variants underlying these findings have not yet been identified. Interestingly, although the GWAS studies are hypothesis-free and interrogate genes across the entire genome, five susceptibility loci identified are linked to genes involved in the TGFβ superfamily signaling pathway, including *SMAD7*, *GREM1*, *BMP2*, *BMP4*, and *RHPN2* [110, 112]. TGFβ superfamily proteins are known to play an important part in developmental biology, cell proliferation, differentiation, and migration. Previous studies have shown that genes in the TGFβ pathway are commonly mutated in human cancers [113, 114]. The pathway signals through the TGFβ receptors and downstream intercellular proteins of the SMAD transcription factor family [115] to inhibit cell proliferation and induce apoptosis, which leads to unchecked proliferation and tumor progression [116, 117]. Moreover, TGF-β also plays a crucial role in the cell cycle regulation [118]. In carcinoma cells, TGFβ inhibits progression of the cell cycle at the G1 stage through the induction of CDKIs, InK4B (CDKN2B), and p21 [119, 120]. The overrepresentation of TGFβ-related loci in the GWASs reinforces the hypothesis that genetic variation in the TGFβ-related genes may cause perturbations in this signaling pathway and therefore confer an inherited predisposition to CRC risk [110, 113].

7.5 GWAS-Identified Variants and CRC Risk in the Enriched Population

To increase the statistical power of GWASs, a number of researchers have investigated the relationship between susceptibility loci and CRC risk in populations enriched for early onset or family history of CRC. For example, the stronger effects of SNPs rs10505477 (8q24.21) and rs16892766 (8q23.3) on CRC risk have been observed among younger patients (defined as individuals under the age of 50 or 60 years, respectively) [79, 93]. Similarly, Middeldorp et al. demonstrated that individuals with early-onset CRC (≤50 years) carried a significantly higher number of risk alleles identified through GWASs (8q23.3, 8q24.21, 10p14, 11q23.1, 15q13.3, and 18q21.1), compared with those with late-onset disease (>50 years) [102]. In a case-unaffected sibling control design of population- and clinic-based discordant sibships (at least one affected sibling and one unaffected sibling), Poynter et al. [97] observed a stronger association between two SNPs in the 8q24 region (rs10505477 and rs6983627) and CRC risk in microsatellite instability (MSI)-high tumors versus MSI-low and microsatellite stable tumors [97].

In the absence of any GWAS on Lynch syndrome to date, it is of great interest to determine if the genetic variants implicated in sporadic CRC also modify age-associated cancer risk in MMR gene mutation carriers. A study of 675 subjects from

127 Dutch Lynch syndrome families demonstrated that SNP rs16892766 (8q23.3) was significantly associated with an elevated CRC risk, and the risk allele C was shown to act independently in a dose-dependent manner. Specifically, the homozygous variant carriers of the SNP rs16892766 experienced a 2.16-fold higher CRC risk than heterozygotes. For rs3802842 (11q23.1), a statistically significant effect on CRC risk was only observed among female carriers of the rs3802842 C-allele (additive model: hazard ratio = 1.64; 95 % CI = 1.07–2.49; P = 0.022), whereas the risk was not significant in male carriers (hazard ratio = 0.94; 95 % CI = 0.68–1.30; P = 0.717) [89]. Talseth-Palmer et al. replicated the association between the same loci (8q23.3 and 11q23.1) and the risk of developing CRC and age of onset in Lynch syndrome, but the association was only observed in *MLH1* mutation carriers. Moreover, the highest risk of developing CRC was observed among *MLH1* mutation-positive females who are homozygous variant carriers of the SNP rs3802842 [88].

In a pilot study to examine the influence of the GWAS-identified variants on age-associated CRC risk in 267 Lynch syndrome patients [121], Pande et al. genotyped two risk variants from one of the most studied loci: 8q24 (rs10505477: T>C and rs6983627: T>G), and a potential susceptibility allele on 9p24 (rs719725: A>C), which was reported in a previous study [97]. However, none of the 3 SNPs analyzed in this study were found to be associated with CRC risk. The lack of association observed in the study is possibly because the study may have been underpowered to detect the small to modest effects of these variants on CRC risk. Further studies with a larger sample size and other ethnic populations are required to validate the results.

7.6 Mutations in Non-MMR Genes Mimicking Lynch Syndrome Phenotype

Recently, alternate mechanisms of silencing single copies of *MLH1* or *MSH2* have been identified as a result of epimutations. An epimutation mimics a somatic or germline mutation and can alter gene expression that does not affect the actual base pair sequence of DNA of the genes. CpG island methylation in the promoter region of these genes is a type of epimutation that causes a phenotype that is typically seen in patients with pathologic germline mutations in these genes.

7.6.1 MSH2 Epimutations

EPCAM (epithelial cell adhesion molecule), also known as *TACSTD1* (tumor-associated calcium signal transducer 1) [122, 123] is a pan-epithelial differentiation antigen that is expressed in most carcinomas. The gene maps to chromosome 2p21 and is located approximately 17 Kb upstream of the *MSH2* gene promoter on chromosome 2 [124]. Deletion of the 3′ exons of *EPCAM* result in a translational read-through into *MSH2*. By unknown mechanisms, this causes methylation in the *MSH2* promoter. Patients with these mutations are predisposed to cancers seen in Lynch syndrome, with CRC and endometrial cancer being the most common [125].

Chan et al. [126] first identified a family transmitting an epimutation displaying hypermethylation of the promoter region of *MSH2* through three successive generations [126]. Of the seven siblings in the second generation of this Chinese family, six carried the epimutation, and of those, three carrying the epimutation had developed early onset cancers displaying microsatellite instability. Two of these developed CRC and the other developed endometrial cancer. The epimutation segregated within the family in an autosomal dominant pattern of inheritance. There was no evidence for germline mutations in their *MSH2* gene. Different levels of methylation were observed in different somatic tissues displaying a mosaic state of methylation. The study provided compelling evidence for a heritable germline epimutation predisposing to cancer.

Two studies, one conducted on Dutch and the other on Hungarian families suspected to be having Lynch syndrome, elucidated a mechanism by which the *MSH2* promoter can become methylated [127, 128]. One of the Dutch families displayed MSI-high CRC with loss of staining for MSH2 and MSH6. No germline mutations were detected in the coding region of *MSH2*, *MLH1*, or *MLH6*. Multiplex ligation-dependent probe amplification (MLPA) analysis using probes between *EPCAM* exon 3 and *MSH2* exon 1 identified a deletion that encompassed the two most 3′ exons of *EPCAM* while leaving the promoter region of *MSH2* intact. As a result of the deletions, transcription of the gene extends into the *MSH2* gene, causing the promoter region of *MSH2* to become methylated. This epigenetic event results in inactivation of *MSH2* gene expression. The same exact deletion was later found in three additional subjects from unrelated Dutch families. Further studies using haplotype analysis suggested that this was a common founder mutation.

Kovacs et al. reported very similar results in Hungarian families, finding large germline deletions of the last exons of the *EPCAM* gene, upstream of *MSH2*, which co-segregated with the Lynch syndrome phenotype in 5 of the 27 families tested [127]. They showed that the *EPCAM* deletions result in loss of the 3′ region of the gene, including the polyadenylation signal, which abolishes transcription termination and leads to transcriptional read-through into the downstream gene (*MSH2*), resulting in an EPCAM/MSH2 fusion transcript.

A more recent screening study of a Dutch cohort of unexplained Lynch syndrome-like families for the presence of *EPCAM* deletions identified 27 independent MSH2-deficient families, all with varying deletions in the 3′ end of *EPCAM*, accounting for 2.8 % of the confirmed Lynch syndrome families in The Netherlands [129]. The authors also reported that among the 45 families studied, 19 different *EPCAM* deletions were found, all covering the last 2 exons and the transcription termination signal of *EPCAM*. The deletions appeared to originate from areas of Alu-repeat mediated recombination events, and regions of microhomology around the breakpoints were suggestive of a nonallelic homologous recombination as the likely mechanism [129].

A deletion was also identified in a Chinese family that extended from intron 5 of *TACSTD1* to approximately 2.4 kb upstream of *MSH2*, leaving the promoter intact. This 22.8-kb deletion co-segregated with the disease and the same identical mutation was identified in another Chinese family [128]. However, it was determined that it was not a founder mutation based on haplotype analysis. *EPCAM* germline

deletions as a cause of Lynch syndrome was also identified in a cohort of Spanish families suspected of having Lynch syndrome [130].

These epimutations do not occur in all cells of the body, but may be confined to only those cells that normally express EPCAM [124]. EPCAM is expressed in colon and endometrial epithelium, the two most common Lynch syndrome-related cancers.

7.6.2 MLH1 Epimutations

Crepin et al. [131] evaluated constitutional epimutations of the *MLH1* and *MSH2* genes in a cohort of 134 unrelated patients suspected of having Lynch syndrome, but without detectable germline mutations in MMR genes. Patients were screened for constitutional epimutations of *MLH1* and *MSH2*. In addition, the patients were also screened for *EPCAM* deletions. They identified constitutional *MLH1* epimutations in two of the patients. One of these patients transmitted the epimutation to two children, who developed early onset CRC. This was the first report providing evidence that the epimutation could be transmitted to the offspring. The frequency of constitutional MMR epimutations was 1.5 % in this series of patients.

A report on two individuals with no family history of CRC who developed CRC at 18 and 20 years of age showed that both cases arose as a result of a de novo constitutional *MLH1* epimutation and somatic loss-of-heterozygosity of the functional allele in the tumors [132]. The authors reported for the first time that the epimutation in one of the cases arose from the paternally inherited allele. Further analysis of 13 tumors from seven individuals with constitutional *MLH1* epimutation showed that eight tumors had lost the second *MLH1* allele, two tumors had pathogenic missense mutations in the other allele, and three retained hetrozygosity. It is clear that the second hit in carriers of *MLH1* epimutation-associated tumors typically has a genetic, not epigenetic, hit. The constitutional epimutations of MLH1 were characterized by soma-wide methylation of a single allele of the promoter and allelic transcriptional silencing, in a subset of patients displaying characteristics of Lynch syndrome but lacking a pathogenic mutation in *MLH1* [132].

The absence of *MLH1* methylation in the spermatozoa of two male carriers of a soma-wide constitutional *MLH1* epimutation suggests that this epigenetic defect is not transmitted through the male germline with its epigenetic modifications intact [133, 134]. However, the potential for paternal transmission remains if epimutations are genetically predetermined, as the epimutation could be reinstated in the somatic cells of offspring post fertilization [135].

More recently, epigenetic silencing of the *MLH1* gene was associated with a G-to-A SNP located in the CpG island of the gene at −93 bases from the translation start site in patients with endometrial and CRCs. To understand the mechanism by which this SNP influenced epigenetic silencing a luciferase reporter assay and electrophoretic mobility shift assay were performed in order to determine whether the −93 SNP affected MLH1 protein expression. The findings suggested that the −93 A

allele affected *MLH1* gene expression by altering protein binding to the promoter of the *MLH1* gene [136, 137]. The epigenetic silencing of the *MLH1* gene was found in patients with CRC and endometrial cancer in this study.

Hitchins et al. provide further evidence that the epimutation can be passed on to the next generation [133]. Two women, Patient A and B, carrying *MLH1* epimutations, had two male offspring each. The authors determined that the allele with the epimutation was transmitted from one of the mothers to her son, but the epimutation was erased in his spermatozoa. The affected maternal allele was inherited by the three other siblings from the two families, but for those offspring, the allele had reverted to the normal active state. These findings demonstrate a novel pattern of inheritance of cancer susceptibility [133, 138]. Here the epimutation was transmitted from the mother to one of her three sons, and interestingly, the other two sons inherited the same maternal allele, but the epimutation was erased. The mechanism for this is unknown. The *MLH1* epimutation tends to arise spontaneously on the maternally inherited allele or have been inherited through the maternal germline [133, 138, 139]. Therefore, there appears to be a maternal bias in the origin of *MLH1* epimutations.

Epigenetic inactivation of the *MLH1* gene is seen in 15 % of sporadic CRCs due to methylation of the 5′ promoter region of the gene [140, 141]. This trait is known as the CpG island methylator phenotype, CIMP [142]. The finding of *MLH1* promoter hypermethylation can help eliminate a diagnosis of Lynch syndrome. Somatic *BRAF* V600E mutation can also help rule out Lynch syndrome, as this mutation is present in sporadic but negative in Lynch syndrome tumors [143, 144].

Gazzoli et al. provided further evidence that heritable *MLH1* CpG island methylation exists [145]. In their study, genetic testing of 14 patients suspected of having Lynch syndrome with MSI-high CRCs failed to reveal a germline mutation in *MLH1*, *MSH2* or *MSH6*. However, in one of the cases, DNA hypermethylation of one of the *MLH1* alleles was detected in DNA isolated from the patient's blood. Analysis of the colorectal tumor DNA revealed that one of the alleles of the *MLH1* gene was also inactivated by hypermethylation. The other unmethylated allele displayed loss of heterozygosity. The findings suggested the possibility that methylation of the *MLH1* resulting in silencing of *MLH1* could be inherited and could predispose a person to Lynch syndrome, indicating that the methylation could represent a germline alteration.

Frazier et al. examined the association of DNA methylation and family history of cancer. Using methylation-specific PCR [146], the DNA methylation status was assessed in CpG islands of the *p16*, *Mint1*, *Mint2*, *Mint31*, and *hMLH1* genes. Four of the loci that were studied (*p16*, *Mint1*, *Mint31*, *and MLH1*) were methylated more frequently in CRC cases with a family history of cancer than in those without [147]. Methylation at the *MLH1* locus occurred exclusively in adenocarcinomas of patients with a family history of cancer. Patients with methylation at all four loci were 14 times more likely to have a family history of cancer than patients with methylation at none of the four loci.

Roughly 15 % of sporadic CRCs display methylation of the *MLH1* promoter on both alleles [147, 148]. However, hypermethylation of *MLH1* can arise in germ cells resulting in hypermethylation in a single allele of *MLH1* in somatic cells [145, 149].

A summary of the genes and polymorphisms discussed in the preceding sections is presented in Table 7.2.

Table 7.2 Genes and polymorphisms modifying Lynch syndrome phenotype

Group/Gene	Polymorphism	Effect	Authors
Cell cycle			
CCND1	G to A at codon 242	AA or AG associated with median age of onset 11 years earlier than GG (HR = 2.46; 95 % CI = 1.16–5.21; $P = 0.019$)	Kong et al. [4]
		Onset age difference between GG and GA/AA in patients harboring *MSH2* mutation	Talseth et al. [11]
		No correlation between *CCND1* genotypes and age of onset	Bala et al. [9]
		No correlation between genotype and age of onset	Kruger et al. [10]
TP53	G to C at codon 72	GC associated with median onset 13 years earlier than GG (HR = 1.94; 95 % CI = 1.03–3.63; $P = 0.04$)	Jones et al. [3]
		Median age of onset 41, 36, and 32 years with GG, GC, and CC genotypes, respectively (log-rank $P = 0.0001$)	Kruger et al. [13]
		No correlation between genotype and age of onset	Sotamaa et al. [16]
		No correlation between genotype and age of onset	Talseth et al. [20]
MDM2	T to G at 309 in the promoter	No correlation between genotype and age of onset	Sotamaa et al. [16]
		No correlation between genotype and age of onset	Talseth et al. [20]
IGF1	CA repeat in 5′ UTR	≤17 CA repeats associated with onset 12.5 years earlier than all others (HR = 2.36; 95 % CI = 1.28–4.36; $P = 0.006$)	Zecevic et al. [5]
		≤17 CA repeats associated with onset 15 years earlier than all others (HR = 1.5; 95 % CI = 1.02–2.16; $P = 0.044$)	Reeves et al. [23]
		≤17 CA repeats associated with onset 12 years earlier than all others (HR = 1.70; 95 % CI = 1.25–2.31; $P = 0.030$)	Reeves et al. [24]
		No correlation between genotype and age of onset	Houlle et al. [25]

(continued)

Table 7.2 (continued)

Group/Gene	Polymorphism	Effect	Authors
AURKA	T to A at codon 31	TT associated with onset 7 years earlier than TA or AA (HR = 0.53; 95 % CI = 0.30–0.96; $P = 0.036$)	Chen et al [2]
		No correlation between genotype and age of onset	Talseth et al. [11]
DNA repair			
ATM	G to A at codon 1853	AA or AG associated with higher risk of developing Lynch syndrome or Lynch syndrome-related cancer (OR: 8.9; $P = 0.02$)	Maillet et al. [43]
	rs1801516	No correlation between genotype and age of onset or overall risk	Jones et al. [44]
DNMT3B	C to T–149 bp from transcription start site rs2424913	TT or TC associated with onset 21 years earlier than CC (log-rank $P = 0.021$)	Jones et al. [74]
OGG1	G to A at codon 154 rs56053615	R154H polymorphism not correlated with HNPCC but associated with sporadic colorectal cancer (OR: 3.586; $P = 0.053$)	Kim et al. [47]
	C to G at codon 326 rs1052133	No correlation between genotype and age of onset	Reeves et al. [50]
XRCC1	G to A at codon 399 rs25487	No correlation between genotype and age of onset	Reeves et al. [50]
XRCC2	C to T in 3′UTR rs3218536	No correlation between genotype and age of onset	Reeves et al. [50]
XRCC3	C to T at codon 241 rs861539	No correlation between genotype and age of onset	Reeves et al. [50]
MSH3	G to A at codon 1045 rs26279	No significant association between genotype and age of onset	Reeves et al. [50]

Carcinogen metabolism			
NAT1	Allele 10 present or absent	Median age of onset 2.5 and 5 years earlier in two groups with *NAT1*10* present (no test of statistical significance reported)	Moisio et al. [33]
NAT2	Rapid (*NAT2**4) and slow (*NAT2**5, *NAT2**6 and *NAT2**7) acetylators	No correlation between genotype and age of onset, but slow acetylators were more prevalent among cancer-affected individuals with MMR mutations ($P < 0.03$), suggesting protective effect of rapid acetylator phenotype on cancer development	Heinimann et al. [29]
		No difference in age of onset between rapid and slow acetylators, but *NAT2**7 heterozygotes (after adjusting for *NAT2**5 and *NAT2**6) had significantly higher risk of colorectal cancer (HR = 2.96; $P = 0.012$)	Frazier et al. [30]
		No correlation between genotype and age of onset	Pistorius et al. [31]
GSTM1	Alleles present or absent	Median age of onset 6 years earlier in one group for *GSTM1* absent; no difference in a second group (no test of statistical significance reported)	Moisio et al. [33]
		Males who were null for *GSTM1* had a median age at diagnosis 12 years earlier than *GSTM1* present.	Felix et al. [35]
		No correlation between genotype and age of onset	Heinimann et al. [29]
		No correlation between genotype and age of onset	Jones et al. [34]
GSTT1	Alleles present or absent	Median age of onset 6 years earlier in one group for *GSTT1* absent; no difference in a second group (no test of statistical significance reported)	Moisio et al. [33]
		In males, median age at diagnosis was 39 years for *GSTT1* null compared to 54 years for non-null.	Felix et al. [35]
		No correlation between genotype and age of onset	Heinimann et al. [29]

(continued)

Table 7.2 (continued)

Group/Gene	Polymorphism	Effect	Authors
GSTP1	rs1695:c.330A>G p.I105V	No correlation between genotype and age of onset	Talseth et al. [32] Pande et al. [36]
	rs1138272:c.343C>T p.A114V	No correlation between genotype and age of onset	Pande et al. [36]
CYP1A1	rs4646903:g.6235T>C	Higher frequency of mutant allele carriers among the CRC cases as compared to those without CRC (OR = 0.39, 95 % CI = 0.17–0.88)	Talseth et al. [32]
	MspI	Heterozygous carriers of the variant allele were at increased CRC risk (HR = 1.53, 95 % CI = 1.06–2.22)	Pande et al. [36]
	rs1048903:c.1384A>G p.I462V	Increased risk of CRC in carriers of 1 or 2 copies of the variant allele (HR = 1.63, 95 % CI = 1.08–2.44)	Pande et al. [36]
EPHX1	rs1051740:c.339T>C p.Y113H	No correlation between genotype and age associated risk. Interaction effect with *CYP1A1*I462V. Carriers of any mutant allele of *CYP1A1*I462V and any mutant allele of *EPHX1* Y113H had a median age of CRC onset of 37 years compared to 42 years for those carrying the homozygous wild-type alleles (log-rank test $P = 0.002$)	Pande et al. [36]
	rs2234922:c.418A>G p.H139R	No correlation between genotype and age associated risk	Pande et al. [36]

Endogenous hormone metabolism			
CYP17A1	c.-34T>C	Homozygous carriers of the variant C allele were diagnosed with CRC 18 years earlier than carriers of the homozygous wild-type allele	Campbell et al. [37]
COMT	c.472G>A	No significant correlation between genotype and age of onset	Campbell et al. [37]
Folate metabolism			
MTHFR	rs1801131:c.-62A>C p.A1298C	No correlation between genotype and age associated risk	Pande et al. [38]
	rs1801133:c.79C>T p.C677T	4 year earlier median age at onset and reduced age-associated risk of CRC for carriers of 1 or 2 copies of the variant allele	Pande et al. [38]
GWAS-identified			
EIF3H	*Intergenic*	The homozygous variant carriers of the SNP rs16892766 in 8q23.3 experienced a 2.16-fold higher CRC risk, and the risk allele was shown to act independently in a dose-dependent manner	Wijnen et al. [89]
	rs16892766	The SNP is associated with age of onset only in *MLH1* mutation carriers	Talseth-Palmer et al. [88]
C11orf53/FLJ45803/LOC120376/POU2AF1	*Intergenic*	In female subjects, the heterozygote and homozygous variant carriers of the risk allele of the SNP rs3802842 (11q23.1) had an increased CRC risk by 1.49- and 3.08-fold, respectively	Wijnen et al. [89]
	rs3802842	The SNP was associated with age of onset only in *MLH1* mutation carriers	Talseth-Palmer et al. [88]

HR hazard ratio, *OR* odds ratio, *UTR* untranslated region, *CRC* colorectal cancer

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Chapter 8
Lynch Syndrome: Genetic Counselling of At-Risk Individuals and Families

Zandrè Bruwer and Raj Ramesar

Abstract Predictive genetic testing for Lynch syndrome can identify whether or not an individual has inherited a mutation predisposing to a high-risk of cancer. Individuals faced with the decision to test for a future health-related risk require knowledge, not just information, on the genetic contribution to disease in their family. Promoting predictive genetic testing within a framework of genetic counselling allows for the interpretation of complex genetic information so as to facilitate decision-making, enhance coping mechanisms and promote preventative measures. Individuals who are identified to be mutation-positive can be enrolled into a targeted screening programme to detect colorectal cancer at an early and potentially curable stage while those individuals testing mutation-negative can be released from intensive surveillance. The process of genetic counselling ensures that the psychological meaning of the condition together with the potential impact of the genetic test result is wholly explored ensuring that the individual is capable of coping with a favourable or unfavourable result. This chapter provides an overview of the current literature on predictive testing with regards to theoretical developments in the context of Lynch syndrome. The pre- and post-test counselling sessions are discussed in detail with specific focus on the importance of informed consent, safeguarding an autonomous decision-making process. An update on barriers to uptake of genetic testing, potential emotional and psychological disruption subsequent to testing, and avenues open for future improvement in the profession are addressed. The communication of the test result and implications for the family members, along with the ethical aspects centred on non-disclosure of genetic information, to those at-risk, are also revised.

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8.1 Genetic Counselling

Since the first introduction of the term 'genetic counselling', by Sheldon Reed in 1974, many and varied definitions have been used to describe the profession [1–6]. One of the most widely published, is that from the American Society of Human Genetics (1975), which describes genetic counselling as a communication process around the occurrence or risk of occurrence of a genetic disorder in a family [1]. In the three decades since the definition was proposed, genetic counselling has expanded beyond its traditional borders and importantly placed emphasis on the therapeutic relationship and need for emotional support [7–11]. The definition accepted by the National Society of Genetic Counselors (NSGC) enumerates three core aspects which are commonly integrated into the process of genetic counselling. These include interpretation, education and counselling:

- Assessing the chance of disease occurrence or recurrence based on the family and medical history;
- Facilitating patient education in terms of the genetics, testing options, management, prevention, ongoing research and available resources; and
- Counselling of clients to enable an informed decision concerning their choices and adaptation to the risk or condition [9].

Essentially, genetic counselling involves the interpretation of complex genetic data into information that is easily understood by the client and has the potential to help the client make and cope with the decisions relating to genetic diagnoses and results of genetic testing [12, 13].

A number of elements outline a genetic counselling session. The first, and perhaps most integral part, includes taking a medical and family history, usually recorded in the format of a three-generation pedigree [14–16]. Verification of medical records such as pathology reports can provide clues when assessing hereditary cancer susceptibility as certain types of tumours are more likely to be associated with a genetic cause [17]. For example colorectal tumours with microsatellite instability may be suggestive of Lynch syndrome. The counselling session also includes educational aspects, whereby the client is provided with information on the genetic condition including the prognosis, management and treatment options. An assessment of reproductive or personal health including the hereditary aspects of the genetic condition are also discussed [7, 9, 18, 19].

If appropriate, informed decisions regarding genetic testing are made by the counsellee. A major tenet in the decision-making process is not to persuade the counsellees to make certain decisions, but rather to assist them in making the best decision for themselves, taking into account their beliefs, values and circumstances [15, 20–22]. Traditionally, genetic counselling aimed to uphold this non-directive

standard described as helping the clients reach a decision based on their personal perspectives without any particular guidance towards a decision [8, 21, 22]. Elwyn et al. (2000), however, argue that the counsellor should contribute his or her personal views to the counsellee if there is a medical benefit to a particular course of action. In the model, the counsellee's values are still respected, but cognisance is also taken of the opinion of the medical expert [23]. The concept, 'shared decision-making', can be applicable in situations such as those in Lynch syndrome where the individual clearly benefits from medical management.

The most powerful part of the genetic counselling session is the emotional support and psychological counselling which can help the counsellee prepare and cope with their genetic concerns [15]. Should genetic testing be available and appropriate, informed consent and the discussion of other ethical and or legal issues are addressed during the consultations.

8.2 Predictive Testing for Lynch Syndrome

In Lynch syndrome, genetic testing starts with an affected individual. If a mutation is identified in a mismatch repair (MMR) gene, predictive genetic testing (PT) is offered to the individual's family members, as they are at-risk of carrying the mutation. A negative test result for a known mutation indicates that the individual is not at an increased cancer risk. However, if genetic testing does not identify a mutation in an affected individual, the results are uninformative and all members of such families are advised to adhere to high-risk surveillance recommendations. For these families, genetic testing does not help determine which relatives may or may not be at an increased risk for developing Lynch syndrome-related cancers [17, 24].

PT aims to provide future health-related information to the unaffected individual at a suspected high-risk of developing Lynch syndrome [25, 26]. Ideally this information can lead to the timely identification and knowledge of their mutation status enabling targeted screening to detect colorectal cancer (CRC) at an early and potentially curable stage [26, 27]. The idea of PT and its possible implications, namely, to identify individuals who are predisposed to a disease that has not yet developed, is a difficult concept. Furthermore the uncertainty about whether or not the condition will develop, when it will appear and how severely it will manifest itself adds to the complexity of PT [28, 29]. There is thus a strong consensus that PT should be conducted within a framework of genetic counselling as the process of considering, arranging and interpreting such a test is not uncomplicated [20].

8.2.1 Predictive Testing and Counselling Protocol

PT protocols were originally developed in the context of Huntington disease, an incurable, usually late-onset, autosomal dominant neurodegenerative disorder

[19, 30]. The PT protocol was developed, not only to protect the test applicant, but also to assist healthcare professionals in dealing with the difficulties that may arise from the application of the genetic test, and, even more specifically, the test result. This extended protocol was implemented to facilitate reflection around the consequences of genetic testing in light of a condition for which there is no cure or preventative management. Numerous authors have highlighted that such extensive discussion and reflection may not be required by individuals contemplating PT for Lynch syndrome, as it is known to have reduced penetrance and that preventative management is available [28, 31–35]. These studies give cognisance to the opinion that most individuals at a high risk for a cancer syndrome have been satisfied with a single pre-test genetic counselling session and that no long-term psychological distress has been reported by shortening the protocol. Moreover, when the psychological and decision-making outcomes were compared between 26 individuals attending either a shortened (single pre-test counselling session and test results delivered at second session 2 weeks later) or extended counselling protocol (two sessions prior to receiving test results 6 weeks later) for Lynch syndrome, no evidence of harm was identified [25]. The authors however, did suggest that in light of a shortened protocol, participants would benefit from having information on what to expect, suggesting that a preparatory leaflet or telephone call outlining the session could be provided.

8.2.2 Pre-test Counselling

The pre-test counselling session provides the client with extensive information on Lynch syndrome and the process of PT. The inheritance and clinical features of the condition are discussed together with information on cancer surveillance. A well-recognised barrier to the transmission of information is the emotional state of the individual [36] and, therefore, the psychological meaning of the disease and the potential impact of the test result are extensively explored. The aim of the pre-test session is to aid individuals in developing a sense of how they will cope with a favourable or unfavourable result [20, 24, 36]. It has been suggested that clients have an expectation of their test result attributing to preconceived notions, which in turn may influence their reactions when the test result is delivered [17, 30, 37]. Counselling is thus used to explore all the pros and cons of testing, motivations for testing and elucidates the counsellee's expectations, identifying and explaining any unrealistic views that they may have [24].

8.2.3 Risk Perception

Many clients enter into PT with a sense of confusion around genetic risk. This is understandable if one thinks about the various categories of probabilities: the chance

of inheriting the pathogenic mutation, the chance of developing CRC if a mutation is found, the chance of developing other specific cancers, the chance that cancer may develop at a specific age and the chance that other family members (such as children) may inherit the predisposing gene [36]. Despite being able to review and explain these specific risk figures in counselling, risk perception is often based on more abstract factors such as the emotional and psychological aspects shaped by the client's experiences of cancer [30]. Appreciating the psychosocial side can aid the counsellor in relaying information in a more understandable and sensitive manner to the client [17, 38].

8.2.4 Informed Consent

Once a decision is made for an individual to embark on the PT process, there is a mandate for informed consent to take place prior to any blood being drawn to safeguard an autonomous decision-making process [17]. Informed consent is largely defined by the notion that decisions are made in a collaborative manner, between the physician/counsellor and the competent patient, whereby the patient provides authorisation for the procedure in a voluntary manner based on a substantial understanding of the information [39]. The components of informed consent have been comprehensively reviewed by the American Society of Clinical Oncology (1996) and Geller et al. (1997) and it is imperative that certain issues are discussed before and after genetic testing is offered [38, 40]. The aspects relating to the PT protocol and consent include:

- **A discussion of the genetic test**. This includes information on the type of information that the test may be able to elicit, what it might not be able to show and the subsequent health risks and medical management [38, 40].
- **Implications of the test result**. A PT provides a positive or negative test result, indicating an increased or lowered cancer risk for the individual undergoing testing. The health-related risks associated with a positive test as well as the risks, even after a negative test result, must be elucidated [37]. It is imperative that the individual understands that the identification of a pathogenic mutation does not equate to having cancer nor is it a certainty that cancer will imminently develop [38].
- **Options for risk management without testing**. Should an individual not want to know his or her genetic status, intensive colonic surveillance, as recommended to a mutation-positive individual, should be encouraged.
- **Risk of passing on a mutation**. Individuals who are identified as mutation-positive have a 50 % risk of passing on the mutation to each of their offspring, while those individuals, without the mutation, do not pass on the risk. The offspring (of the individual declining testing) should also be made aware of their likelihood of a risk and their parent, informed that, should their child be tested and a mutation identified, by way of implication, their result (the parent) will be

known. Testing of minors (individuals under the age of 18 years) is largely dissuaded as a result of the potential emotional and psychological harm that may result [40, 41]. The appropriate age for PT is assessed on the age of expression of the disease. If medical benefits of testing are not apparent in childhood, testing is postponed until such an age when the child reaches adulthood and is able to make an informed decision for her/himself. In the context of Lynch syndrome, PT is usually only offered to individuals over the age of 18 years [14, 17, 40].

- **Technical aspects of the genetic test**. This includes information on the detection rate, sensitivity and specificity of molecular genetic testing.
- **Risks of genetic discrimination**. Should genetic testing limit coverage in obtaining life or health insurance, it is advised that the individual reviews policies prior to testing. Other legal and ethical complications may include the possibility of employment discrimination. The law governing the prohibition of discrimination of healthy individuals based on genetic test results is prohibited [37, 42] and may be beneficial to divulge during the discussion with the individual.
- **Risk of psychological distress**. Participants should be informed of the potential adverse psychological reactions that may result such as anxiety, depression or family dysfunctioning. Dorval et al. (2000) identified that failure to anticipate the reaction to the result has the potential to lead to an increased emotional distress [43]. Even if an individual is not found to carry the mutation, aspects of psychological and emotional disruption such as regret for making major life decisions, prior to knowledge of the test result or even survivor's guilt, could transpire. Notably, guilt could also be experienced if there is a possibility of passing the mutation on to an offspring [38, 40]. Additional elements that need to be addressed are the timing and readiness for testing, family concerns and the preparation for the result session [37].
- **Confidentiality**. The individual should be informed of the effort made to maintain their confidentiality and to keep the genetic information secure. It is also important that the individual is aware of other persons with access to the information. For instance, other medical professionals involved in their management or the referring physician [37, 38]. A potential ethical dilemma may evolve in maintaining the confidentiality of a client when seen at the same clinic that a family member is attending, especially when communication about the result has purposefully been restricted within the family [17].
- **Medical surveillance and screening (options and limitations**). Medical management following a positive test result can reduce the risk of developing CRC [27, 44, 45]. Information on surveillance, optimal frequency of attendance and the limitations of the screening approaches should be provided. Recommendations for screening, even if the test result is negative, as per the general population requirements, must also be discussed.
- **Storage and reuse of genetic material**. This is of particular relevance in the research setting where a portion of the individual's blood sample may be kept for possible re-analysis for the benefit of other family members or for research purposes.

The informed consent process attempts to empower the client, through the provision of extensive information, to facilitate a more thoroughly considered, educated and informed decision about genetic testing [36]. The PT protocol (including the informed consent document) is based on the general principles of medical ethics and includes:

- **Autonomy** – the principle of the right to choose whether or not to proceed with testing. This requires sufficient information to be given to the patient to allow for an informed independent decision;
- **Beneficence and nonmaleficence** – the principles of doing good and not harm requiring the informed consent to disclose all benefits, limitations as well as possible risks of the testing; and
- **Justice and confidentiality** – the assurance that the genetic information will not be disclosed to third parties such as other family members, insurance companies and employers [20, 24, 46, 47].

Implicit in the whole process is the right of the individual to decline testing at any stage without affecting their or their family member's future medical management [20, 40].

8.2.5 Post-test Counselling

Trepanier et al. (2004) maintain that the result-disclosure session should take place during a face-to-face session with the client [37]. Should the individual consent to being informed of their results, the disclosure and implications thereof are discussed at this stage, with special attention given to the possible emotional impact [7, 14, 48, 49]. Peters and Biesecker (1997) state that the psychological reactions may need to be addressed in an ongoing manner, and follow-up sessions with the counsellor or a referral to a mental health professional may be required [36]. The counselling of the post-test session is further dedicated to reviewing and co-ordinating the medical management and compliance with the screening protocol if needed [36].

Aronson (2009) advises that the matter of disclosing the genetic result should be raised during the pre-test session to ensure that the client has already considered the issues of disclosure prior to testing [14]. Another means to assisting the dissemination of information may be through the provision of written documentation [50].

Individuals who refrain from having biological children due to their concern over passing on a genetic risk can be enlightened to the possibilities of adoption, in vitro fertilisation with either sperm donation (if the father is mutation-positive) or ovum donation (if the mother is mutation-positive) and prenatal diagnosis (PND), including chorionic villi sampling, amniocentesis and cordocentesis. Should PND identify a mutation-positive fetus, selective termination of pregnancy is optional. Preimplantation genetic diagnosis (PGD), whereby the embryo's that are selected and implanted are screened to ensure that they do not carry the familial mutation is additionally available. However, some controversy about offering PND and PGD

for late-onset cancer syndromes has been raised. Concerns relate mainly to the reduced penetrance of the condition, the onset of cancer in adulthood (excluding Familial adenomatous polyposis, which manifests in childhood), and the effectiveness of surveillance. In an extensive review of the literature by Offit et al. (2006), no cases of PND could be identified for Lynch syndrome, whilst more than a dozen cases utilising PGD, have been reported [51].

Genetic counselling and PT should, where possible, be undertaken by those with experience to ensure that the issues of confidentiality and information provision are explained within the consent process [14].

8.3 Psychological Impact of Genetic Testing on the Individual

Knowing whether or not an individual has inherited a predisposition to a Lynch syndrome-associated cancer is beneficial due to the availability of effective surveillance. However, psychological distress could be anticipated subsequent to disclosure of a mutation-positive test result [52]. Despite these concerns, research has shown that individuals undergoing PT for Lynch syndrome do not demonstrate excessive levels of anxiety and depression, with the exception of a short-term increase in individuals testing positive [28, 31, 53]. Similar results for long-term psychological distress have also been reported [32].

Key predictors of distress, in healthy individuals undergoing PT, include a history of depression, lower quality of life, social support, complicated grief and the number of affected first-degree relatives [54–56]. Findings from a longitudinal prospective survey highlighted that distress can be anticipated in cases where an extended family history of CRC or loss related to CRC are present [57].

The psychological impact of a positive genetic test result among cancer patients has been a relatively neglected topic in the literature [58, 59]. In individuals with CRC a positive test result indicates a risk of developing a second cancer and may therefore result in the individual being vulnerable to distress. Gritz et al. (2005) measured psychological distress among 126 CRC patients and identified a subgroup of distressed individuals, whereby race and education were significantly associated with increased distress. Non-whites had higher mean scores than whites and individuals with lower education levels had higher scores than those with higher education levels [60]. In a larger study of 200 patients within the same population group, lower education, a poor support structure, being of a younger age and non-white race aswell as being female was associated with greater psychological distress [61]. These findings suggest that the emotional reaction to a positive test result in a CRC patient should not be underestimated. Bonadona et al. (2002), further propose that one cannot assume that the patient who has already had the diagnosis of cancer will consequently expect a positive test result. The authors identified more than a third of their patients having stated that the disadvantages of knowing their genetic test result outweighed the advantages [62]. A favourable outcome, in this group of individuals, was identified by Esplen and colleagues (2007) who reported

that distress levels were lower in individuals found to be mutation-positive, following a previous cancer diagnosis than those mutation-positive individuals (unaffected with cancer) [59].

8.3.1 Uptake of Predictive Testing

PT uptake rates vary extensively. Studies preceding the availability of genetic testing for Lynch syndrome have shown that the majority (83 %) of relatives would request genetic testing if available [63]. A large study undertaken in America, selected both unaffected and affected family members eligible for genetic testing. Among these individuals, only 43 % elected to have a genetic test. Noteworthy, of the 84 participants who provided a blood sample for genetic testing, eight declined to receive their test result when available to them [64]. More recently, two American studies, exploring interest in genetic testing, documented rates of 51 and 64 %, respectively [65, 66].

The documented uptake rate reported by Hadley et al. (2003) and Lerman et al. (1999) was significantly lower than predicted by Croyle (1993) [63–65]. However figures from a study of the Finnish population and that of a South-African population greatly exceeded those from the American studies, suggesting that interest in genetic testing for a predisposition to CRC may be high [65–68]. Aktan-Collan et al. (2000) invited 446 at-risk unaffected individuals to participate in their study. The research was based on questionnaires, which were completed three times during the study period (before the initial counselling session, 1 month after and 1 year after test disclosure). Of the 446 eligible participants, 381 consented to the study and 88 % (334/381) requested PT [67]. The South-African study focused on the uptake rate of genetic testing among nuclear family members of 80 individuals with Lynch syndrome, including all siblings and eligible children (over the age of 18 years). The reported figures were 97 % (siblings) and 73.6 % (children) [68].

Countries such as Finland and South-Africa are largely managed by the public health-care system as opposed to private health-care. Decreased uptake rates may occur in countries with a national health system, where PT and counselling is not only expensive but has the potential to increase medical insurance policies [67, 68].

8.3.2 Barriers and Facilitators to Predictive Testing

Several sociodemographic and psychological reasons for not participating in PT have been highlighted in the literature. Among individuals undergoing genetic testing, higher education, being employed, higher pre-test risk perception, and more frequent thoughts about cancer are commonly identified in acceptors of PT as compared to decliners [34, 64, 65, 67, 69]. Hadley et al. (2003) also identified that individuals with a personal history of cancer or who are unaffected, but have a greater

number of affected relatives with CRC, accepted testing more often [65]. Individuals concerned about their ability to handle the emotional effect and the psychosocial effects on their family, pursued testing less frequently, while the presence of depression has been identified to significantly reduce uptake rates [34, 64, 65, 70]. Studies conducted in America have highlighted that insurance coverage and concern over possible discrimination may also impede the pursuit of genetic testing [65, 71].

Key motivational factors driving the pursuit of genetic testing for Lynch syndrome include: early detection of cancer, obtaining knowledge of the offspring's risk, the opportunity to reduce uncertainty as well as obtaining information that may reduce screening frequencies [33, 53, 59, 65, 68].

8.4 Impact of Lynch Syndrome on the Family

8.4.1 Communication of the Genetic Information to the Family

Genetic information is not only restricted to the individual receiving a mutation-positive test result, it also has important implications for the proband's biological family. Informing relatives about a familial risk for cancer allows unaffected family members the opportunity to ascertain their genetic status and determine if high-risk cancer screening is required [72]. Current standards of practice dictate that the responsibility of disclosing the genetic information to the at-risk family members lies with the individual [73, 74]. Consequently, the dissemination of cancer-risk information and subsequent access to genetic counselling and testing services among relatives depends, partly, on whether or not the proband discusses the test result with family members. Family communication and timely disclosure of the health information is thus vital to ensure that at-risk family members are informed and understand the genetic information. Previous research has found that a high proportion of mutation–positive individuals do disclose their genetic test result to their family. For example 81–85 % of individuals, selected from a cancer registry, discussed their BRCA1/2 test result with a family member and disclosure usually took place in a timely manner (95 % of those who discussed their test result did this within a week) [75, 76]. Comparable figures have been published in clinic-based studies determining attitudes towards informing relatives about genetic testing for breast cancer [77, 78]. In the context of Lynch syndrome, an American study identified that 98 % of individuals undergoing genetic testing informed their first-degree relatives of their test result [72].

Rates of communicating genetic test results are significantly lower if relatives beyond the nuclear family are considered [53, 62, 79–81]. Stoffel et al. (2008) identified a 23 % decline in the rate of communication to second- or third-degree relatives [72]. Indeed, information about genetic testing is most often disclosed to partners and/or siblings and less often to children and parents [62, 66, 68].

Non-disclosure has been attributed to perceiving the information as potentially disturbing to the relative; if prior conflict or a lack of cohesion exists among family

members; or if there is an unwillingness to cause concern [72, 78, 79, 81 82–84]. Additional barriers to communication, as far as breast cancer is concerned, include: adoption, divorce, remarriage, and a large age gap between siblings, while patients already affected with a cancer are more likely to disclose genetic information to their families [78, 85].

Communication of a genetic test result can also be influenced by the mutation-status of the individual [86]. Hughes et al. (1999) identified that women receiving a mutation-positive BRCA1/2 result were more likely to convey the information to their family than those with a negative result [75]. In a later study by the same author, a similar pattern of disclosure was described for sister pairs with a definitive result compared to those with an inconclusive result [76]. Disclosure was additionally less likely to occur when an individual had younger children, as telling children about their genetic risk occurred around key life decisions, at a specific life stage or when they were old enough to understand [79, 81, 84]. Motives for informing family members are largely to obtain emotional support, to encourage genetic testing and to promote the provision of risk information to relatives [68, 72, 76, 81, 85].

Women have been described to play a greater role in communicating at-risk information when compared to their male counterparts, and female relatives, rather than male relatives, are more likely to be informed about genetic testing, particularly in breast cancer [75, 83, 87]. In the context of Lynch syndrome, where both males and females have a high risk of developing cancer, the impact of gender has not been as easily ascertained as that identified from the extensive literature available on breast cancer. However, in an Australian study investigating this phenomenon in Lynch syndrome patients, it was tentatively suggested that males may find the process of informing the at-risk relatives less natural than females [88]. The authors further suggested that men, especially, may therefore benefit from professional support during the period of communicating genetic test results to the family. Patients have previously expressed difficulties with being the person responsible for transmitting the results to their family, however individuals do not advocate for this role to be taken on by anyone else [56, 62, 79, 84, 85]. In contrast to this, Bruwer (2011) identified that 56.3 % of individuals with Lynch syndrome, from a rural area, approved of the idea of having a healthcare provider inform their family about the genetic risk [68].

Family communication remains a complex issue. Simply telling patients to inform their at-risk relatives about the implications of their genetic test result is insufficient. Even though the large majority of individuals are willing to share information about the presence of a gene mutation in the family and it has been reported that individuals do not deliberately withhold their test result from family members, passive failure to disclose the result to the at-risk family does occur [72, 81, 88]. This is of concern as the information can have life-saving implications.

It has been suggested that a detailed letter containing all relevant information around testing and especially which family members should be informed about genetic testing may be of benefit in facilitating family communication [66, 68, 72]. Further support and strategies to augment communication may include genetic counselling, information pamphlets and regular contact by health professionals [62, 66, 85, 88].

8.4.2 *Ethical Aspects: Familial Nature of Lynch Syndrome*

When non-disclosure occurs, maintaining confidentiality must be weighed up against the right of the at-risk relative to be informed about their susceptibility to a high cancer risk [89]. An extensive review on the ethical guidelines and policies addressing the communication of genetic information in families was conducted by Forrest et al. (2007). The general recommendations arising from this review are that the health professional, at the very least, informs the patient about the implications of the genetic information in light of its relevance for family members [74].

Certain guidelines permit disclosure when attempts to encourage the patient to disclose the genetic information have failed. Confidentiality may be breached and the genetic information released if the following criteria are met: "… (a) reasonable efforts to elicit voluntary consent to disclosure have failed; (b) there is a high probability both that harm will occur if the information is withheld and that the disclosed information will actually be used to avert harm; (c) the harm that identifiable individuals would suffer if the information is not disclosed would be serious; and (d) appropriate precautions are taken to ensure that only the genetic information needed for diagnosis and/or treatment of the disease in question is disclosed" [47]. In the United States, the healthcare professional is not required by law to warn the at-risk family members, while the European guidelines take a stronger stance recommending that genetic healthcare professionals actively encourage disclosure [1, 90]. In contrast the German and French authorities strongly advocate against disclosure if the patient does not inform the family of the genetic concerns, giving priority to the patient's privacy [91, 92]. There is no South-African law applicable to breaching confidentiality or towards warning an endangered third party [93].

Individuals with Lynch syndrome will mostly understand the implications of a mutation-positive test result and inform their at-risk relatives of the implications of the information, including the cancer–related risks and screening options. Refusal to inform at-risk family members has however been reported [78, 94–96]. In a survey of patients from a Canadian Colon Cancer Registry, only 73.5 % of individuals were willing to give the health care professional permission to inform their at-risk relatives if they could or would not inform them [84]. Suthers et al. (2006), in an attempt to increase awareness among at-risk family members about the availability of genetic testing for a familial condition, sent out letters to at-risk relatives with the permission of the proband. The result was an uptake of genetic testing, among at-risk relatives, from 23 to 40 % [97]. Based on similar research conducted in Finland, 92 % (n = 236) of at-risk relatives approved of this form of direct contact [98]. The direct approach may work well in countries where registries are available (Finland, Denmark and to an extent South-Africa), whereby direct recruitment can be facilitated. The model may not be as effective in countries without comprehensive registries and mailed letters may not be effective where the population is of a low functional literacy level.

Importantly, it must be considered that at-risk relatives may not want to be informed about a genetic condition for which they are at-risk. On the contrary, they may consider the contact an invasion of their privacy, capable of causing financial and emotional harm [15, 97].

8.5 Satisfaction and Potential for Improvement in the Field of Genetic Counselling

Patient satisfaction is an important measure for assessing the quality of a health care service, as it reflects on the experience of care received from the patient's perspective [99]. Given the importance of patient satisfaction in genetic counselling and the role it plays in the continual advancement of the profession, several scales for assessing satisfaction have already been developed. These scales, available in quantitative and qualitative formats, evaluate different components of the patient's genetic counselling experience [100–102]. Typically, three dimensions are usually assessed and include: (1) competence of the health care profession; (2) the health care professional's affective behaviour towards the patient or client and (3) satisfaction with the administrative procedures including costs and convenience of the service [101]. Such evaluations facilitate the further exploration of counsellee needs from the service, identifying aspects where improvements can be implemented [48, 100].

Much of the research on patient satisfaction suggests that the majority of patients are pleased with the genetic counselling that they receive [99, 103–108]. In a study by Stadler and Mulvihill (1998) conducted in America, the level of satisfaction among 51 self-referred patients seen for breast cancer genetic counselling was reported to be "high" amongst a significant proportion of women [109]. Overall, the patients considered that the consultation was worth their time and money. Similarly, Nordin et al. (2002) described Swedish patients referred for genetic counselling at an oncogenetic clinic (breast, ovarian and colorectal cancer referrals) as being "highly satisfied" [110]. These and other findings have led to the belief that most patients view the genetic counselling session as helpful, valuable, informative and capable of addressing concerns adequately. Additionally, counsellee expectations of genetic counselling are often exceeded [99].

One possible explanation for the high levels of satisfaction may be the lack of awareness of what genetic counselling entails among counsellees [106, 111]. Bernhardt et al. (2000) point out that educating counsellees about the process of genetic counselling, prior to the session, may be one way of promoting realistic expectations [112]. Furthermore, Michie et al. (1997) and Shiloh et al. (1990), found that satisfaction is determined by the fulfilment of patient expectations, whereby patient satisfaction increases when expectations are in line with what is received from the counselling session [101, 113]. Perhaps most importantly, counsellors should be aware that their agendas may be very different from those of their clients.

Dissatisfaction with genetic counselling does, however, occur. For example, individuals receiving information on genetic testing that may be negative or inconclusive in nature, may assess genetic counselling as less satisfying, while higher education, younger age, cancer-specific distress (prior to the genetic counselling session), pessimism and poor family functioning is negatively associated with satisfaction [101, 102, 105]. Asking too many medical questions and not receiving

enough medical information during the counselling session is also associated with counsellees who are less satisfied [114]. Bleiker et al. (1997) conducted a pilot study on individuals with a family history of cancer attending a familial cancer clinic in the Netherlands. The authors identified several areas where dissatisfaction was expressed by the 36 counsellees. Receiving particular attention was the perceived lack of communication between the counsellor and other health care professionals, the limited involvement of the family doctor, inadequate information on the possible consequences of daily life functioning and a greater need for psychosocial support during and after the genetic counselling session [103]. Further suggestions, for improvement offered by patients, included more frequent outreach visits, and information of updates on ongoing trials [68, 113]. With regard to breast cancer patients, Bober et al. (2007), identified that women who receive more complex information are likely to report lower levels of satisfaction [115].

Greater satisfaction may be achieved if the information, given to the patient, is adapted to their coping style. Nordin et al. (2002) identified that "monitors" (individuals who seek more information on a particular health threat) are not only less distressed, but are more satisfied with information provision. "Blunters" (individuals who avoid information on a particular health threat) on the other hand, show the opposite, namely, that less information reduces psychological distress and increases satisfaction [110].

8.6 Conclusion

The field of cancer genetic counselling is rapidly evolving, reflecting the increase in knowledge of the genetic basis of cancer, scientific and technological advances and media publicity promoting greater public awareness. As a result, cancer counselling has grown to become a major area of specialization within the genetic counselling field. In the 2006 NSGC Professional status survey, 39 % of genetic counsellors were practicing in the field of cancer genetics, the second largest contributor, followed only by the prenatal sector. Interestingly, the cancer field was the only sector to illustrate growth over the 6-year period captured during the survey (34–39 %) [116].

Although cancer counselling builds on the general principles of genetic counselling, it has many unique aspects with regards to complex risk factors, concepts and preventative measures. Furthermore, the counselling sessions often raise substantial psychological, ethical and social considerations and consequently involve greater provider time than most clinical services. Given these differences, together with the need for a more directive approach due to the ability to offer preventative screening for mutation-positive individuals, cancer genetic counselling is very likely to develop into a unique branch of the counselling profession. Common principles and practices can be built on upon and augmented for a greater suitability to cancer counselling and counsellors will be able to play an increasingly important role in defining this framework through research and clinical experience.

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