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Comprehensive molecular analysis of mismatch repair gene defects in suspected Lynch Syndrome (Hereditary Non-polyposis Colorectal Cancer) cases

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Abstract

An accurate algorithm is essential for effective molecular diagnosis of hereditary colorectal cancer. Here we have extended the analysis of 71 colorectal cancer cases suspected to be Lynch Syndrome cases for *MSH2, MLH1, MSH6* and *PMS2* gene defects. All cases were screened for mutations in *MSH2, MLH1* and *MSH6* and all cases where tumors were available were screened for microsatellite instability and expression of MSH2 and MLH1. Subsequently, mutation negative cases were screened for *MLH1* methylation and mutations in *PMS2*. Of the MSI-H cases, 96% had a mismatch repair gene defect, mostly involving *MSH2* or *MLH1*; 1 *PMS2* mutation, 1 *MLH1* epimutation, and no *MSH6* mutations were found. Four of the 28 MSI-H cases, including 1 Amsterdam criteria case, had biallelic tumor *MLH1* methylation indicating that sporadic cases can be admixed in with Lynch Syndrome cases even those meeting the strongest criteria for Lynch Syndrome. Mismatch repair gene defects were found in similar frequency in cases where tumors were and were not available. One *MLH1* and 1 *MSH2* deletion mutation were found in MSI-S/L cases indicating that microsatellite instability testing can exclude cases with pathogenic mutations. Our analysis support a diagnostic algorithm where cases are selected for analysis based on clinical criteria or prediction models; isolated sporadic young-onset cases can be pre-screened by tumor testing whereas familial cases may be directly subjected to molecular analysis for mutations in mismatch repair genes followed by microsatellite instability, protein expression and DNA methylation analysis to aid in the resolution of mutation negative cases.

Introduction

Lynch Syndrome, also called hereditary non-polyposis colon cancer (HNPCC), is an autosomal dominant inherited cancer predisposition syndrome characterized by predisposition to develop a number of cancers at an early age and high penetrance with mutation carriers having a significantly increased lifetime risk of developing colorectal (CRC) and other forms of cancer (1–3). Inherited defects in genes encoding components of the major post replication DNA

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Conflicts of Interest

R. Kolodner is an inventor on patents owned by the Dana-Farber Cancer Institute covering sequences of the *MSH2, MLH1* and *PMS2* genes, and reagents related to these genes including antibodies for detecting the proteins encoded by these genes. S. Syngal has in the past served in an advisory role to Myriad Genetics, Inc.

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mismatch repair (MMR) system have been found to underlie many cases of Lynch Syndrome with most of the genetic defects identified being attributable to mutations in two genes, *MSH2* and MLH1 (4–6). A small proportion of cases have been shown to be caused by germline mutations in two other MMR genes, *MSH6* and *PMS2*; however, *PMS2* mutations and *MSH6* mutations are often associated with weaker family histories, later ages of diagnosis and potentially a different cancer spectrum (7–13). The range of mutations identified in *MSH2* and *MLH1* includes missense, nonsense, frameshift, splice site mutations and deletion mutations as well as the more recently appreciated rare epimutations (4–6,14–17). In addition, there are apparently polymorphisms in MMR genes that may cause increased risk of developing cancer (18–20). The complete loss of mismatch repair function in tumors leads to increased mutations at microsatellite sequences resulting in the microsatellite instability high (MSI-H) phenotype, although numerous studies report MSI-H Lynch Syndrome cases that lack mutations in known MMR genes; examples include (4,5,21).

Early detection of a germline alteration in an MMR gene definitively diagnoses Lynch Syndrome within a family, allowing for monitoring and early treatment for appropriate family members, which leads to a reduction in morbidity and mortality of mutation carriers (22,23). Conversely, unaffected individuals can be spared unnecessary screening procedures. To achieve these benefits, it is important to have appropriate, simple criteria for identifying individuals who should receive genetic testing and efficient, accurate genetic testing methods. An essential step in the clinical diagnostic setting is to identify all cases that will prove to have a causal mutation while including as few cases as possible that lack a mutation in order to provide definitive diagnosis to as many relevant cases as possible while keeping costly uninformative genetic testing to a minimum. Therefore, efforts have been made to find the most sensitive clinical criteria, based on family history, to be used to select families for mutation detection analyses (24–27). Studies of families meeting the most restrictive criteria for Lynch Syndrome, the Amsterdam criteria, have identified germline *MSH2* and *MLH1* mutations with a relatively high sensitivity [~60%] and specificity [~70%] (28). In contrast, germline *MSH2* and *MLH1* mutations were found with a higher sensitivity [~94%] and a lower specificity [~50%] when families meeting the least restrictive criteria, the Bethesda criteria, were studied (28). Both the original and revised Bethesda criteria appear to be equally effective for selecting CRC cases with weak or no known family history of Lynch Syndrome associated cancers that are associated with MMR defects (29,30). More recently, new patient selection algorithms have been developed that have the potential to improve the sensitivity and specificity of mutation detection (31). However, there has been little evaluation of potential criteria for identifying isolated individuals with Lynch Syndrome associated cancers other than colorectal or endometrial cancer for genetic testing. Thus, the use of restrictive criteria improves the likelihood of finding a germline mutation at the cost of excluding cases that have a germline mutation, whereas less restrictive criteria can in principal lead to the identification of most germline mutations at a cost of analyzing many cases without a germline mutation. Given this need to include many individuals in genetic studies who may not have a germline MMR defect, development of accurate and efficient genetic testing strategies is important.

There are many published studies using a diversity of genetic testing methods and strategies describing genetic defects in MMR genes in different cancers. However, there does not appear to be a generally accepted strategy for detecting genetic defects in MMR genes, possibly because a considerable amount of genetic testing is done at local research and clinical sites. Most present large-scale studies screen cases selected on the basis of family history for tumor MSI and MMR gene expression to identify cases for subsequent molecular analysis for MMR gene defects. Such strategies may not be applicable to cancer predisposition clinic based testing where it is important to find all pathogenic mutations because of reports of MSI-L/S cases with MMR gene mutations and observations that not all missense mutations result in loss of protein (4,5,7,32–34); indeed a recent functional bases study of missense mutation in the *MSH2* gene

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found that two thirds of mutations causing an MMR defect did not significantly reduce MSH2 protein levels (35). We have previously studied 71 cases of familial colorectal cancer for the presence of germline mutations in MMR genes by analyzing MSI, expression of MLH1 and MSH2 proteins and by direct sequencing of genomic DNA to detect mutations in *MLH1*, *MSH2* and *MSH6* (10,14,28,33). The suspected Lynch Syndrome cases were selected as meeting at least one of several established Lynch Syndrome criteria including the Amsterdam, modified Amsterdam, Bethesda or Lynch Syndrome-like criteria (24–27). In the present study, we have extended the prior analysis by including additional methods for detecting MMR gene defects. Of the 71 total cases analyzed, an MMR gene defect was implicated in 38 (54%) of the cases, and of the 28 MSI-H cases studied, an MMR gene defect was implicated in 27 (96%) cases. Because the majority of the genetic and molecular analysis was performed in parallel rather than sequentially, the results reported can be used to guide the development of efficient screening strategies in both the near-term as well as in the future when better prescreening strategies become widely available.

Methods

Patients

DNA samples from the Lynch Syndrome families analyzed here and Caucasian control samples have been previously described (28,36). These Lynch Syndrome patients were collected as a clinic based series of patients meeting one of the criteria for suspect Lynch Syndrome cases including Amsterdam, Modified-Amsterdam, HNPCC-like and Bethesda criteria; the distribution of the patients among these different criteria and descriptions of the different criteria are summarized in Table 1. Previous results obtained by screening these samples by DNA sequencing for mutations in *MSH2*, *MSH6* and *MLH1*, screening for germline methylation of *MLH1* and tumor MSI and expression of MSH2 and MLH1 proteins have also been described (10,14,28,33,36).

MLPA

Deletion analysis was performed with both the MLPA P003 *MLH1/MSH2* and MLPA P008 Lynch Syndrome-2 Exon Deletion Test Kit's (MRC-Holland) according to the manufacturer's protocol. A 5µl aliquot of 100 ng genomic DNA was heated to 98˚C for 5 min and cooled to room temperature. A 3 µl aliquot of SALSA probe and MLPA buffer was added to the DNA and heated to 95˚C for 1 min and then incubated at 60˚C for 16 hrs to hybridize the probes and DNA. Ligase-65 and Ligase-65 buffers were then added yielding a final volume of 40 µl which was then incubated at 54˚C for 15 min. The ligated products were then heated to 98˚C for 5 min and kept at 4˚C until the initiation of PCR using the manufacture's PCR Protocol 2. The PCR product was mixed with formamide and ROX-500 standard. Subsequent electrophoresis was performed on an ABI 3730 sequencer. Genescan and Genotyper software (Applied Biosystems) was used to collect and export the peak areas to an Excel spreadsheet. Individual peak areas were divided by the sum of that samples total peak areas for a relative peak area. A ratio of each probe's relative peak area and the average relative peak areas of the controls corresponded to the number of copies for that individual probe. A ratio of 1.0 corresponded to two copies of that particular probe and a ratio of 0.5 corresponded to a single copy.

Primer Design

Regions of Alu repeats were found with RepeatMasker (http://repeatmasker.org/cgi-bin/WEBRepeatMasker) and excluded as areas for primer design. Primers for breakpoint and promoter PCR and DNA sequencing were chosen from genomic DNA sequence using the Primer3 web interface (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The *PMS2* exon PCR and sequencing primers were previously published as follows: Exons 1, 2, 6–9 (37); Exons 3–5,

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10, 12–15 (38); and exon 11a, 11b (two separate PCR reactions) (39). The primers used for sequencing *PMS2* and the *MSH2* and *MLH1* promoters are listed in Table 2 and Table 3.

Breakpoint PCR and Sequencing

Intronic primers distal to the deleted exons were used to amplify the breakpoints of those samples with deletions limited to exons not including the most 5' and 3' most exons of the gene of interest. For the SKOV3 cell line, which is hemizygous for an *MLH1* deletion (40), primer pairs comprising 200–500 bp amplicons were designed and tested every 5–10 kb downstream of the 3′ end of the gene until a PCR product was observed. Once the breakpoint was localized at 5 to 10 kb resolution, this mapping procedure was repeated with more closely spaced amplicons until it was possible to locate the breakpoint precisely enough to then amplify across it. The Expand Long Template PCR System (Roche) was used to PCR amplify across the breakpoints. PCR reactions of 25 µl were performed using final concentrations of 1x Buffer 2, 500 µM dNTPs, 300 nM primers (each), 2U polymerase, and 50–100 ng genomic DNA. Cycling conditions consisted of: 93˚C for 2 min; followed by 10 cycles of 93˚C for 10 sec, 55˚C for 30 sec, 68˚C for 45 sec up to 8 min depending on the experiment; followed by 20 cycles of 93˚C for 10 sec, 55˚C for 30sec, 68˚C for 45 sec to 8 min +20 sec per cycle; one cycle of 68˚C for 7 min; and finally hold at 4˚C. The PCR products were analyzed on 1% agarose gels run in TBE. Pre-sequence clean-up of the PCR template was performed in 20 µl reactions with 2U SAP and 10U EXO1 (US Biochemicals) and then sequencing was performed on an ABI 3730 using procedures provided by the manufacturer.

Sequencing-based mutation screening of *MLH1/MSH2* promoters and *PMS2* exons

The *MLH1* promoter was amplified with the primers *MLH1* promoter F1 and *MLH1* promoter R1 yielding a 1393 bp PCR product that was then sequenced with the same primers as well as with *MLH1* promoter F2 and *MLH1* promoter R2. The *MSH2* promoter was amplified with the primers *MSH2* promoter F1 and *MSH2* promoter R1 yielding a 1450 bp PCR product and sequenced with the same primers as well as with *MSH2* promoter F2 and *MSH2* promoter R2 (see Table 3). The promoter regions of *MLH1* and *MSH2* were amplified in 25 µl volumes consisting of 1x PCII buffer, 150 µM dNTP's, 200 nM primers (each), 5U Klentaq, and 25–50 ng genomic DNA. The cycling conditions were as follows: 94˚C for 3 min; 8 cycles of 94˚C for 30 sec, 63˚C (-1˚C/cycle) for 30sec, 72˚C for 1 min; then 32 cycles of 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 1 min; and finally hold at 4˚C. The PMS2 exons were amplified with the same reaction and cycling conditions as above except for exons 1 and 12. For these latter exons, the reaction conditions consisted of 1x PCR buffer II (Roche), 1.5 mM $MgCl_2$, 100 µM dNTP's, 200 nM primers (each), 1U Amplitaq (Roche), and 25–50 ng genomic DNA. Exon 12 PCR used the same cycling conditions as above. Exon 1 PCR used the following cycling conditions: 94˚C for 3 min; 35 cycles of 94˚C for 30 sec, 52˚C for 30 sec, 72˚C for 1 min; and finally hold at 4˚C. Pre-sequence clean-up and sequencing were performed as previously described (10).

MLH1 methylation

Methylation of the *MLH1* promoter in tumor and blood DNA samples was analyzed by both methylation specific PCR and bisulfite DNA sequencing as previously described (14). Samples scored as somatic methylation did not have methylated species present in DNA from blood and appeared to have only methylated DNA species present in tumor DNA, and hence likely showed biallelic methylation (41–43).

Results

We have been exhaustively analyzing a series of 71 colorectal cancer cases suspected of being Lynch Syndrome cases by different clinical criteria (Table 1) for germline defects in MMR genes. Through several previously published studies and the work described here, these cases

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have been analyzed by the following strategy (10,14,28,33,36). First, the coding region and intron-exon junctions of the *MSH2*, *MSH6* (including the *MSH6* promoter) and *MLH1* genes were sequenced in DNA from blood of all cases. Tumors, when available, were screened for MSI using 5 and 10 microsatellite marker panels recommended by the NCI consensus groups (44,45) and were screened for expression of Msh2 and Mlh1 protein by immunohistochemistry. All tests were performed on all samples independent of any results obtained. Second, for all cases where mutations were not initially found, DNA from blood was screened for deletion mutations in *MSH2* and *MLH1* and all mutation negative MSI-H cases were also screened for germline and tumor *MLH1* methylation. Third, all cases in which an alteration in *MSH2*, *MSH6* or *MLH1* or MLH1 methylation was not found were then screened for deletions in *PMS2, MSH6, MUTYH, MLH3* and *TACSTD1* including the region between *MSH2* and *TACSTD1*. Finally, the coding region, intron-exon junctions and promoter regions of the *PMS2* gene and the promoters of *MSH2* and *MLH1* were sequenced in DNA from blood of all MSI-H cases where mutations had not been found. The results from this analysis, some of which has been published and discussed previously (10,14,28,33,36,46), are summarized in Table 1 and Figure 1.

Using MLPA, 4 of the 71 families were found to have a deletion covering all or part of the *MSH2* gene and 5 of the 71 families were found to have a deletion covering part of the *MLH1* gene (Table 1). To identify additional deletions, we subsequently screened an additional 39 families obtained from another study and identified two additional *MSH2* deletions and one additional *MLH1* deletion. In addition, analysis by MLPA confirmed the *MSH2* and *MLH1* deletions we previously reported in the LoVo and SKOV3 tumor cell lines (40). By using a large selection of primer pairs designed to potentially amplify across deletion breakpoint junctions, we were ultimately able to amplify and sequence 4 *MLH1* and 3 *MSH2* deletion breakpoint junctions and then confirm each breakpoint junction by amplification with an optimized pair of PCR primers followed by sequencing (Table 4). This analysis showed that 3 of the 7 deletion events involved Alu elements flanking both sides of the deleted region and 6 of the deletion events involved flanking microhomologies ranging from 1 to 21 base pairs in length. One of the deletions (Cases 1120 and 2738, Exon 12) had the same breakpoint as that of a previously published deletion (47). None of the remaining deletions appeared to correspond to previously described deletions identified by either sequencing deletion breakpoint junctions or through identification of primer pairs for amplifying breakpoint junctions even though in several cases the same combinations of exons were found to be deleted (17,48–50).

Of the initial 28 MSI-H cases analyzed, 8 did not have a mutation in *MSH2, MSH6* or *MLH1*. Subsequent analysis found that one of these cases had a germline missense variant in *PMS2* that was not found in 184 normal control DNAs. The resulting *PMS2* amino acid substitution did not affect a conserved amino acid and is in a region that is homologous to a region of bacterial MutL for which no structural information is available and which can tolerate different deletions without effecting MMR (51). One of the 8 cases showed apparent germline methylation of *MLH1* with associated LOH of the non-methylated allele in the tumor (14). Four of the cases showed apparent bi-allelic methylation of the *MLH1* promoter in the tumor consistent with the idea that these cases might represent sporadic cancers (14,32,42,43,52). One of the cases had no detectable alteration in any gene analyzed but showed loss of *MSH2* expression in the tumor; this case is a candidate to have a mutation in a region that was not sequenced such as an intron or is a candidate for germline or somatic methylation of *MSH2* (16), although this sample did not appear to have a deletion eliminating the *TACSTD1* termination codon associated with germline *MSH2* methylation (16). In total, only 1 out of 28 MSI-H cases could not be linked to loss of function or an alteration in a known MMR gene. One *MLH1* and 1 *MSH2* deletion, and no other mutations were found among the 20 MSI-L/S cases (10% mutation frequency). The yield and distribution of mutations in cases without available tumors was essentially the same as that in cases where a tumor was available.

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Discussion

In the present study, we have extended our prior analysis of 71 suspected Lynch Syndrome cases as defined by a diversity of clinical criteria for MMR defects using methods that had not been previously applied to these cases. This extensive analysis has yielded a number of key results. First, among the 28 clearly MMR defective cases as evidenced by their MSI-H signature, it was possible to link 27 of the cases (96%) to a defect in a known MMR gene and in most of these cases it was possible to identify the underlying mutation at the DNA level. This is arguably the highest or among the highest frequencies reported (4,5,21). Second, consistent with previous experience, the vast majority of mutations detected (97 %) were in the *MSH2* or *MLH1* genes with only 1 mutation in *PMS2* and no mutations in *MSH6* detected (4–6,21). Third, we detected mutations in cases where tumor samples were not available at the same frequency as in cases where tumor samples were available. Fourth, our ability to detect mutations and provide insight into the genetics of a high proportion of cases did not require the use of complex techniques like "conversion of diploidy to haploidy" (5). Fifth, of the 7 MSI-H cases where no mutation was found at the DNA sequence level, 4 had somatic silencing of *MLH1* and were likely sporadic cases (14,32,42,43,52), 1 had an *MLH1* epimutaton (14, 15), 1 lacked expression of MSH2 protein and in 1 case we were not able to develop any insight into the underlying MMR defect. And finally 1 *MSH2* mutation and 1 *MLH1* mutation were detected among 20 MSI-S, L cases indicating that recommended criteria for MSI testing may misclassify some MMR defective tumors. In sum, these results suggest that it is possible to detect the genetic basis for MMR defects in virtually every suspected Lynch Syndrome case that actually is associated with a MMR defect and, as discussed below, provide a guide to efficient mutation detection strategies.

After analysis for mutations in *MSH2* and *MLH1*, there were 8 MSI-H cases that did not have mutations in these two genes; 1 met Amsterdam criteria, 1 met modified Amsterdam criteria, 3 met the even weaker Lynch Syndrome-like criteria and 3 were isolated early onset cases without a significant associated family history. Extensive analysis of these cases revealed insights into the nature of their underlying MMR defects in all but one case and several of these cases deserve further comment. We were not able to find any molecular basis for a MMR defect in an individual diagnosed with CRC before 45 years of age and having no family history of CRC; we did not have immunohistochemistry data for this case that might have provided further insight into the nature of the MMR defect. One case meeting modified Amsterdam criteria did not have a mutation in *MSH2* or in the *TACSTD1* transcription termination region but the tumor from this case did not express MSH2 protein; potentially this case has an MSH2 epimutation (14–16), although we lacked sufficient tumor DNA for analysis. One case meeting modified Amsterdam criteria had a *PMS2* mutation. An *MLH1* epimutation was found in an individual diagnosed with CRC before 45 years of age and having no family history of CRC. And, finally, in 4 cases we found somatic methylation of *MLH1* indicating they were sporadic cases (14,32,42,43,52). Three of these cases had weak family histories of CRC or were isolated CRC cases diagnosed before 45 years so it is not surprising that they might be sporadic CRC cases. However, 1 of these latter cases met Amsterdam criteria suggesting it was a sporadic case within a potential Lynch Syndrome family. These results indicate both that exhaustive analysis of the ~25% of MSI-H cases initially lacking mutations in *MSH2* or *MLH1* can ultimately implicate known MMR genes in virtually all of the cases and that one should not ignore the possibility that some of the cases are sporadic, particularly when analyzing cases meeting some of the weaker Bethesda criteria for Lynch Syndrome. A further implication of these results is that it is possible that some suspected Lynch Syndrome cases that are MSI-S, L or for which no tumor is available but lack a mutation in *MSH2* or *MLH1* may be sporadic cases and that in families with a strong family history of appropriate cancers a second individual should be analyzed.

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We observed 1 *MSH2* and 1 *MLH1* deletion mutation among 20 MSI-S, L cases, which would be predicted to not be MMR defective (40,45,53) and hence not associated with defects in either *MSH2* or *MLH1*. Other studies have also observed mutations in *MSH2* and *MLH1* in MSI-S/L Lynch Syndrome cases, although most of the mutations observed could not be definitively classified as pathogenic mutations (4). It is possible that MSI testing misclassifies some fraction of true MSI-H cases as MSI-S, L. It is known that no individual microsatellite is unstable in 100% of MSI-H tumors (45). We have calculated for MSI-H being defined as 4 out of 10 microsatellites being unstable, 17, 6 and 1% of true MSI-H cases would be misclassified as MSI-L if on average each microsatellite was unstable in 50, 60 and 70% of MMR defective tumors, respectively; use of larger numbers of microsatellites will reduce misclassification and using fewer will increase misclassification. As a consequence, MSI status should probably not be used to eliminate suspected Lynch Syndrome cases from testing for mutations in *MSH2* or *MLH1* by direct DNA sequencing and deletion screening, particularly in cases where family history makes a strong prediction of Lynch Syndrome.

The suspected Lynch Syndrome cases described here were predominantly analyzed by different methods in parallel without using any one set of results to stratify samples for subsequent analysis. Because we were able to link 96% of the obviously MMR defective MSI-H cases to a specific gene, our results can be used to guide efficient screening of suspected Lynch Syndrome cases for MMR defects. Based on our results, we recommend that in cases where clinical criteria suggest genetic risk for Lynch Syndrome, initial screening for mutations in MMR genes should involve analyzing genomic DNA for mutations in *MSH2* and *MLH1* by direct DNA sequencing and MLPA. This will detect essentially all mutations in *MSH2* and *MLH1* and the vast majority of all mutations in MMR genes. Mutation negative cases should then be analyzed for MSI status and for protein expression by IHC, which will identify those cases for which additional, more detailed analysis is warranted. The most useful subsequent analysis would be screening for mutations in *PMS2*, methylation (silencing) of *MLH1*, methylation (silencing) of *MSH2* and potentially "Conversion Analysis" (5) to resolve difficult cases. Prescreening our suspected Lynch Syndrome cases for MSI status and for protein expression by IHC would have reduced the yield of mutations by misclassifying MSI-H cases as MSI-L cases and because not all mutations in *MSH2* and *MLH1* alter protein expression levels (4,5,7,33,34); of the 28 cases where a tumor sample was available where we identified an MMR defect, 2 would have been omitted by MSI prescreening and 6 would have been omitted by prescreening for expression of MSH2 and MLH1 (see data of Ref. 34 for protein expression data). In contrast, screening sporadic CRC cases for MSI status and for protein expression by IHC would be highly effective because silencing of *MLH1* that underlies these cases results in significant MMR defects and substantive loss of MLH1 protein expression; such data could possibly be used in making treatment choices for sporadic CRCs as MMR status is thought to predict the sensitivity to some commonly used therapeutic agents (54,55). Because mutation screening in cases where tumors are not available, and particularly mutation screening in MSI-S, L cases, will increase the number of cases screened where a mutation will not be found relative to just screening MSI-H cases, the use of newer patient selection algorithms might be considered to provide greater sensitivity and specificity in mutation detection compared to using the Bethesda criteria for patient selection (31).

In considering the choice of testing methods, its should be noted that high-quality DNA sequencing for mutation testing is readily available and has become relatively inexpensive, MLPA analysis for genome rearrangements is also inexpensive and can easily be performed by any facility offering capillary based sequencing using a commercially available kit, and both MSI and IHC testing are readily available in many clinical testing laboratories. Commercially available testing at a number of clinical labs now includes both complete gene sequencing and testing for genome rearrangements. In contrast, breakpoint sequencing is a research problem not a routine test, and does not provide information that is generally useful

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for diagnostic purposes. Testing for gene specific DNA methylation does not appear to be widely available at clinical testing sites; however, there are many different platforms for methylation analysis available to the research community.

We did not find *MSH6* mutations in our suspected Lynch Syndrome cases but have found *MSH6* mutations in familial cases not meeting any Lynch Syndrome criteria (10). Others have found *MSH6* mutations at low frequency in Lynch Syndrome cases including those with atypical family histories (8,11–13,56). *MSH6* mutations have also been associated with inherited predisposition to endometrial cancer and have been found in Lynch Syndrome families with endometrial cancer (8,11,56). These results suggest that a critical evaluation of family history might be used to identify suspected Lynch Syndrome cases for analysis for *MSH6* mutations, and certainly only cases lacking defects in *MSH2* or *MLH1* should be considered for analysis of *MSH6*.

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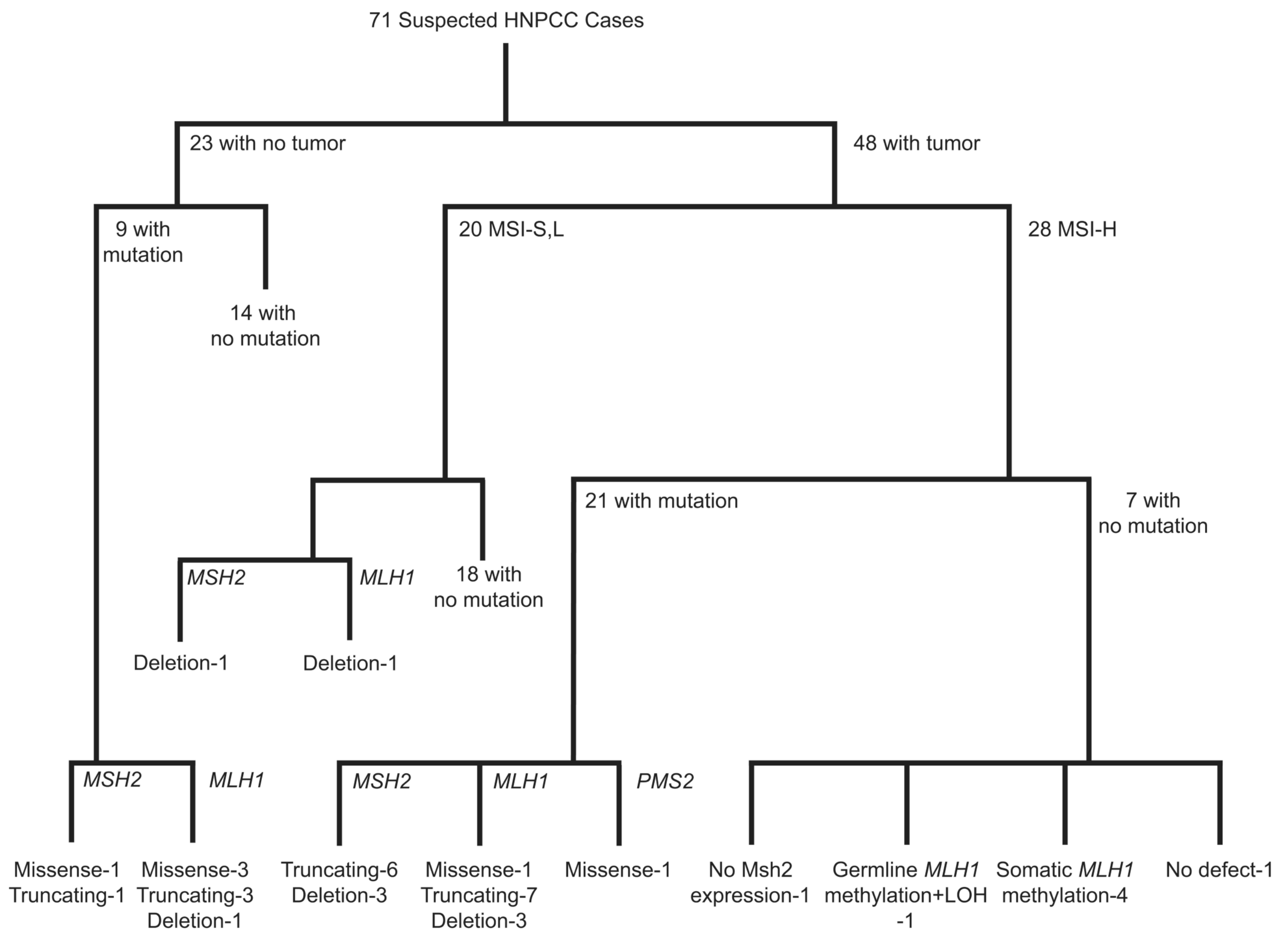

Figure 1.

Flow diagram summarizing the results of mutation detection analyses. The mutation analysis strategy and order of mutation testing is described under "Results".

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Table 1

Summary of clinical, genetic and microsatellite instability data.

Case	Clinical Criteria				MMR Gene Defect	Other	MSI
	AMS	M-AMS	HNPCC-like	Bethesda		Variant	
Tumor Available							
1755	X	X		1,2,3,4,7	MLH1 IVS7-2A>G Splice	MLH1 c.2147G>A V716M	H
2675	X	X		2,3,4,7	MLH1 c.677G>A Splice		H
2906	X	X		2,3,4	MLH1 c.676C>T R226X		H
357				4	MLH1 IVS9-1G>T Splice		H
2722	X	X		3,4	MLH1 c.1810A>T K604X		H
397	X	X		2,3,4	MLH1 IVS16+1G>A Splice		H
232	X	X		3,4	MLH1 c.2265G>C R755S		H
1448	X	X		2,3	MLH1 c.2104-2105ΔAG FS	MSH2 c.965G>A G322D	H
4103		X		2,3,4	MSH2 c.704-705ΔAA FS		H
1251				2,3,4	MSH2 IVS5+3A>T Splice		H
1025				7	MSH2 IVS5+3A>T Splice		H
1851		X		3	MSH2 IVS5+3A>T Splice		H
1754				2,3,4	MSH2 c.1352-1353ΔAG FS		H
241	X	X		3	MSH2 c.1786ΔAAT N596Δ		H
257	X	X		3,4	MLH1 Δ exons 1–13		H
1642	X	X		3,4,7	MSH2 Δ exons 5, 6		H
2956	X	X		3,4	MSH2 Δ exons 1-16		H
1103				4	MLH1 germline methylation/LOH		H
3055	X	X		3	MLH1 somatic methylation		H
1446		X			MSH2 no expression		H
1769			X		PMS2 c.1211C>G P404R		H
2496			X		MLH1 somatic methylation		H
245				4	None		H
629	X	X		2	MSH2 Δ exon 7		H
1120	X	X		3,4	MLH1 Δ exon 12		H
1102				4	MLH1 somatic methylation		H
2214			X		MLH1 somatic methylation		H
2738	X	X		3,4	MLH1 Δ exon 12		H

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Case	Clinical Criteria				MMR Gene Defect	Other	MSI
	AMS	M-AMS	HNPCC-like	Bethesda		Variant	
1524	X	X		3,4	None		L
261			X		None		L
2911				2	None		L
230				2,4	None		L
1252	X	X			None		L
3045		X		2,3,4	None		L
2825	X	X			None		L
1239				4	None		L
1648				3,4	None		L
2228		X			None		L
1657		X		2	None		S
1264				4	MSH2 Δ exon 7		S
1372				4	None		S
2848		X			None		S
2703		X			None		S
2248				3,4	None		S
2642				3,4	None		S
362	X	X		3,4,7	MLH1 Δ exons 16-19		S
2851			X		None		L
487		X			None		S
No Tumor Available							
5	X	X		1,2,3,4	MLH1 c.245C>T T82I		
260	X	X		1,2,3,4	MLH1 c.1517T>C V506A	MLH1 c.1853AA>GC K618A	
3002			X	3	MSH2 c.944G>T G315V		
336	X	X		1,3,4	MLH1 c.1852ΔAAG K618Δ		
171	X	X		1,3,4	MLH1 c.2250C>G Y750X		
1846	X	X		1,3,4	MLH1 c.1381A>T K461X		
2913	X	X		1,3,4	MLH1 c.2198insAACA		
951			X	1,2,3,4	MSH2 c.704ΔAA		
597	X	X		1,2,3,4	MLH1 Δ exon 6	MSH2 c.815C>T A272V	
1370				4	None	MSH2 c.965G>A G322D	

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Case	Clinical Criteria				MMR Gene Defect	Other Variant	MSI
	AMS	M-AMS	HNPCC-like	Bethesda			
170	X	X		1,4	None		
2763	X	X		7	None		
3012	X	X		1,3	None		
419		X			None		
1373		X		3,4	None		
352		X		1,2	None		
2915		X			None		
2224		X		4	None		
1253			X	4	None		
1525				4	None		
104				4	None		
1114				4	None		
349				4	None		

Clinical criteria, MSH2 and MLH1 mutations detected by DNA sequencing, analysis in normal controls and MSI status have been described (Refs. 10,14,24–28,32,35). HNPCC-like; family history suggestive of Lynch Syndrome, but not fulfilling Amsterdam or Modified Amsterdam criteria. The original Bethesda Criteria were used in this study: 1 - Individuals with cancer in families that fulfill the Amsterdam criteria; 2 - Individuals with 2 Lynch Syndrome related cancers, including synchronous and metachronous CRCs or associated extracolonic cancer; 3 - Individuals with CRC and a first degree relative with CRC and/or Lynch Syndrome related extracolonic cancer and/or colorectal adenoma with one of the cancers diagnosed at < 45 years and the adenoma diagnosed at < 40 years; 4 - Individuals with CRC or endometrial cancer diagnosed at < 45 years; 5 - Individuals with right-sided CRC with an undifferentiated pattern (solid/cribform) on histopathology diagnosed at < 45 years; 6 - Individuals with signet-ring-cell type CRC diagnosed at < 45 years; 7 - Individuals with adenomas diagnosed at <40 years.

Abbreviations and definitions: AMS, Amsterdam; M-AMS, Modified Amsterdam.

Other Variant: Known polymorphisms or variants found in normal controls (MSH2 G322D, MLH1 K618A), variants found in normal controls and in conjunction with a more definitive pathogenic mutation (MLH1 V716M) or variants not found in normal controls but found in conjunction with a more definitive pathogenic mutation (MSH2 A272V).

PMS2 c.1211C>G P404R was not found in 184 normal control DNAs.

Screening of an additional 39 suspected Lynch Syndrome cases revealed three additional deletions: case 3919 with deletion of *MLH1* exon 19, case 3769 with deletion of *MSH2* exons 1 – 7 and case 3173 with deletion of *MSH2* exons 3 – 6.

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Table 2

PMS2 sequencing primers

Gene	Exon	Forward	Reverse	Size(bp)	Source
PMS2	1	5′-ggtcacgacggagaccg-3′	5′ ccatgttccccccatttcc-3′	400	36
PMS2	2	5′-tgtttcttgtaactgatttctc-3′	5′ cttaactacaacaacattcacag-3′	233	36
PMS2	3	5′-actgatagcatgggtccg-3′	5′ caaaattctgagacatgtga-3′	201	37
PMS2	4	5′-acactgtcttgggaaatg-3′	5′ attaattttcagagaggtttc-3′	278	37
PMS2	5	5′-ctcaaccatttagatcttga-3′	5′ aataaagcatttctcaataat-3′	451	37
PMS2	6	5′-acttgagctgtgtaattcc-3′	5′ cccgctataatcactagagc-3′	289	36
PMS2	7	5′-gtccactgtgtctttattag-3′	5′ agctctcaggataaaatgttc-3′	204	36
PMS2	8	5′-taatccctttcactctgg-3′	5′ ccataaactgcctattatcag-3′	237	36
PMS2	9	5′-ggggctgggaacatttgtc-3′	5′ atagcagagctgtagaatttc-3′	215	36
PMS2	10	5′-attaagcccttccgtattt-3′	5′ ggaaacacattagctaaaagc-3′	742	37
PMS2	11a	5′-gtcctctcaccatttcagg-3′	5′ agtttggctgaggcaaaactc-3′	621	38
PMS2	11b	5′-actgcagcagcgaatatgc-3′	5′ aaaaaagaaaattttagataaaaagag-3′	519	38
PMS2	12	5′-gtgtacaggtctgaaaacttg-3′	5′ cgcctggccaactagata-3′	1000	37
PMS2	13	5′-cacttagctgagtagtgttgttattt-3′	5′ tgaacacctgaaagagaggaaac-3′	704	37
PMS2	14	5′-tccaaaaagcattttgtgagtt-3′	5′ gagttcaaggtcacagagaacg-3′	780	37
PMS2	15	5′-aactactaaaacgttgaacc-3′	5′ tttttgagacacagtcttgt-3′	726	37

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Table 3

MLH1 and *MSH2* promotor primers

MLH1 Promoter Primer		Coordinate[†]
F1	5′-AACCCTTTCACCATGCTCTG-3′	−1469
F2	5′-TACATGCTCGGGCAGTACCT-3′	−881
R2	5′-TGAAGAGAGAGCTGCTCGTG-3′	−749
R1	5′-GCTCACGTTCTTCCTTCAGC-3′	−77
MSH2 Promoter Primer		Coordinate
F1	5′-CACCTCCCAGGTTCAAGAGA-3′	−1491
F2	5′-GCCTCAGCCCTGCTAATATC-3′	−930
R2	5′-CGGTAGCTCACGCCTGTAAT-3′	−795
R1	5′-CCCACACCCACTAAGCTGTT-3′	−42

[†] The coordinate given is the nucleotide position of the 5′ nucleotide of the primer.

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Table 4

MSH2 and *MLH1* Deletion Breakpoints

Deleted Region	Breakpoint	Sequence[†]
MLH1 exon6[#]	5′ w.t. (c.453+769_799)	GACCAGCCTGACCAACATGGaGAAACcCCAT
	Case 597	GACCAGCCTGACCAACATGGCGAAACGCCAT
	3′ w.t. (c.546-483_513)	tACCAGCCTGgCCAACATGGCGAAACGCCAT
MLH1 exon12	5′ w.t. (c.1039–2315_2345)	TCCAATTTAATTCCAAcaCtGtctacttgga
	Cases 1120 & 2738	TCCAATTTAATTCCAAAGCAGGAATAATAAT
	3′ w.t. (c.1409+812_842)	aaagctggagaaaaggAGCAGGAATAATAAT
MSH2 exon5–6[#]	5′ w.t. (c.793-397_427)	TCTAACCTCACAAGGTtgaAagggcctAatT
	Case 1642	TCTAACCTCACAAGGTCTGACCTTGAGATCT
	3′ w.t. (c.1077-491_521)	ggcgggtctcaAActTCTGACCTTGAGATCT
MSH2 exon3–6[#]	5′ w.t. (c.367-267_297)	CCCCATCTCTACTAAAAATACAAAAAttaGc
	Case 3173	CCCCATCTCTACTAAAAATACAAAAAAATGA
	3′ w.t. (c.1077–2095_2125)	CCCCgTCTCTACTAAAAATACAAAAAAATGA
MSH2 exon3–8[#]	5′ w.t. (c.367–446_476)	CCCAAAGTGTTGGGATTACAggcgTGAGCCA
	Cell line LoVo	CCCAAAGTGTTGGGATTACAAGTATGAGCCA
	3′ w.t. (c.1387-692_722)	CCCAAAGTGcTGGGATTACAAGTATGAGCCA
MLH1 exon4–19	5′ w.t. (c.360_c.380+10)	TGATGGAAAGTGTGCATacAggtataGTGct
	Cell line SKOV3	TGATGGAAAGTGTGCATCGACTCCAGGTGGC
	3′ w.t. (*93,487_93,517)	gcccctcAtagcTaCATCGACTCCAGGTGGC

[†] The sequence following the case number is the breakpoint junction and flanking sequence, with the junction sequence underlined. The nucleotide coordinates in () are the coordinates of the upstream and downstream target sequences.

[#] Breakpoints occur in Alu repeat region