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A Highly Sensitive Pan-Cancer Test for Microsatellite Instability

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Microsatellite instability (MSI) is an evolving biomarker for cancer detection and treatment. MSI was first used to identify patients with Lynch syndrome, a hereditary form of colorectal cancer (CRC), but has recently become indispensable in predicting patient response to immunotherapy. To address the need for pan-cancer MSI detection, a new multiplex assay was developed that uses novel long mononucleotide repeat (LMR) markers to improve sensitivity. A total of 469 tumor samples from 20 different cancer types, including 319 from patients with Lynch syndrome, were tested for MSI using the new LMR MSI Analysis System. Results were validated by using deficient mismatch repair (dMMR) status according to immunohistochemistry as the reference standard and compared versus the Promega pentaplex MSI panel. The sensitivity of the LMR panel for detection of dMMR status by immunohistochemistry was 99% for CRC and 96% for non-CRC. The overall percent agreement between the LMR and Promega pentaplex panels was 99% for CRC and 89% for non-CRC tumors. An increased number of unstable markers and the larger size shifts observed in dMMR tumors using the LMR panel increased confidence in MSI determinations. The LMR MSI Analysis System expands the spectrum of cancer types in which MSI can be accurately detected. *(J Mol Diagn 2023, 25: 806–826; https://doi.org/10.1016/j.jmoldx.2023.07.003)*

Microsatellite Instability

Microsatellites are 1 to 6 bp short tandem DNA repeats constituting approximately 3% of the human genome.[1] Microsatellites are prone to DNA replication errors resulting from polymerase slippage, which are effectively corrected by the DNA mismatch repair (MMR) system.[2] Inactivation of any of the MMR genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2)* results in hypermutability of these microsatellite repeats, a condition referred to as microsatellite instability (MSI).[3–6] Individuals carrying a germline pathogenic variant in one copy of an MMR gene are said to have

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Lynch syndrome and have up to an 80% lifetime risk of developing cancer of the colon, endometrium, stomach, ovary, small intestine, hepatobiliary tract, urinary tract, pancreas, prostate, or brain or developing sebaceous skin tumors.[7] Tumors with an MSI phenotype can arise from loss of both alleles of an MMR gene, either via somatic loss of the second MMR allele in an individual with Lynch syndrome or by other mechanisms, including somatic biallelic MMR gene mutation or somatic biallelic hypermethylation of the *MLH1* gene causing nonhereditary sporadic MSI tumors.[8–11]

MSI-High/Deficient Mismatch Repair Detection

Detection of MMR deficiency is determined by assessing MMR protein levels by immunohistochemistry (IHC) or functionally by MSI testing, with proficient mismatch repair (pMMR) tumors exhibiting normal MMR protein levels and lack of MSI, and deficient mismatch repair (dMMR) tumors exhibiting loss of one or more MMR proteins and presence of MSI.[12,13] Both MSI and IHC assays are sensitive tests for detection of loss of MMR activity, and results from the two tests are usually highly concordant and complementary. A recent comparison of the PCR-based MSI Analysis System, version 1.2 (Promega Corporation, Madison, WI; also known as the Promega pentaplex panel), with IHC in a large population-based study of colorectal cancer (CRC) found that concordance between the two methods was approximately 97%.[14] The concordance between MSI-PCR and IHC testing for endometrial cancers is also high but is dependent on MSI-PCR analysis methods and the microsatellite marker panels used.[15–18] Diagnosis of Lynch syndrome is a multistep process that begins with MSI-PCR or IHC screening for detection of MMR deficiency. Subsequently, dMMR tumors are tested for the presence of *MLH1* promoter methylation or the *BRAF* V600E mutation in CRC to differentiate from Lynch syndrome tumors, which rarely exhibit these molecular features. Germline sequencing for pathogenic variants in any of the four MMR genes and *EPCAM* (deletions in the 3′ end of the *EPCAM* gene can cause methylation-induced transcriptional silencing of *MSH2*) is then performed on suspected cases of Lynch syndrome to confirm diagnosis.[19]

The guidelines from the 1998 National Cancer Institute workshop referred to as the Bethesda guidelines recommended a reference panel of five microsatellite markers consisting of two mononucleotide repeats (*BAT-25* and *BAT-26*) and three dinucleotide repeats (*D2S123*, *D5S346*, and *D17S250*) for MSI-PCR testing.[20] Instability in two or more of these markers classifies an individual as MSI-High (MSI-H), one marker as MSI-Low, and zero markers as MSI stable (MSS). In 2004, the Bethesda guidelines were revised to recommend the use of a panel consisting entirely of mononucleotide repeats to further increase sensitivity and specificity for detecting MSI.[13,21]

Discordances of 3% to 5% between MSI-PCR and MMR IHC assays can occur via different mechanisms.[14,22–26] False-negative IHC results can occur in MSI-H tumors expressing a nonfunctional protein with retained antigenicity.[27–31] False-positive IHC test results have been reported in MSS tumors with loss of MSH6 expression after neoadjuvant therapy.[32] Interpretation of IHC staining results can be challenging because tumors rarely exhibit loss of an MMR protein throughout the entire sample, and the definition of what constitutes abnormal MMR expression is still evolving.[25,33] This intratumor heterogeneity contributes to variable interpretation by observers, as does the experience of the pathologist and the guidelines being followed.[12,29,34,35] Conversely, current MSI-PCR tests are reportedly less sensitive for certain types of cancers, especially tumors with MSH6 loss, or in samples with low tumor cellularity.[17,18] MSI-PCR sensitivity can vary depending on which MSI marker panel is used and the method of analysis. The use of outdated or poorly designed microsatellite marker panels and interpretation methods probably contributes to the reported lower sensitivity of MSI compared with IHC in some cancer types.[17,18,21,25]

Clinical Significance of MSI

Determination of MSI status in cancers is of clinical importance because of its diagnostic, prognostic, and therapeutic significance. Universal MSI testing for all CRC and endometrial cancers is now recommended, regardless of age at diagnosis or family history, by many professional organizations and guidelines, including the National Comprehensive Cancer Network.[36] MSI-High cancers are more common in early-stage cancers and are therefore associated with a more favorable prognosis. For example, about 20% of stage I to II, 12% of stage III, and 4% to 5% of stage IV metastatic CRC are MSI-H.[37] In recent years, MSI has gained considerable attention because of its role in predicting patient response to immune checkpoint inhibitor therapy across multiple tumor types.[38,39] Tumors with MMR deficiency elicit a positive immune response due to the expression of neoantigens by the tumor cells.[40–42] MMR-deficient cancer cells producing neoantigens may evade the immune system by up-regulation of inhibitory pathways, including the programmed cell death 1/programmed death ligand-1 immune checkpoint, and blockade of this inhibitory pathway with monoclonal antibodies permits an antitumor immune response. The first clinical trial for immune checkpoint blockade with pembrolizumab (Merck, Kenilworth, NJ), a programmed cell death 1 immune checkpoint inhibitor, found that the objective response rate was 40% for patients with MSI-High CRC and 0% with MSS CRC.[38] In the follow-up trial on 12 different cancer types with evidence of MMR deficiency, the objective response rate was 53%, and complete responses were achieved in 21% of patients.[39] Based on results of these clinical trials, the US Food and Drug Administration approved the use of pembrolizumab for previously treated patients with

MSI-H/dMMR advanced or metastatic solid tumors in 2017. More recently, first-line treatment with pembrolizumab monotherapy was found to provide significant and clinically meaningful improvements in progression-free survival compared with standard of care with chemotherapy as first-line treatment in patients with MSI-H/dMMR metastatic CRC.[43] Other immune checkpoint blockade therapies are being evaluated for a variety of cancer types, and predictive biomarkers of immunotherapy such as MSI status are urgently needed to identify individuals who are likely to respond.

Differences in the Prevalence and Intensity of MSI Between Cancer Types

MSI has been identified in most cancer types, with varying prevalence ranging from a high of approximately 30% in endometrial cancers to a low of approximately 0.1% in melanoma.[44–50] Using next-generation sequencing (NGS) of microsatellite repeats, the overall frequency of MSI-H tumors across all cancer types was estimated to be approximately 4% of 12,019 cases from 32 cancer types by Le et al.[39] A similar MSI-H frequency of 3.7% of 26,464 cases from 43 cancer types was obtained by using MSI-PCR.[50]

Studies of microsatellite alterations in Lynch syndrome–associated cancers report differing patterns of MSI, including the size of deletions and number of affected markers.[17,51] Blake et al[52] speculated that this varying mutational burden between cancer types could be explained by different time intervals since loss of MMR, with longer intervals resulting in larger deletions in a greater number of microsatellites. This hypothesis is supported by Mandal et al,[53] who studied newly generated dMMR cell lines serially passaged over 1 or 4 months producing differing levels of MSI in the genome. The MSI-H cell line cultured for 4 months exhibited both a higher proportion of unstable microsatellites and higher tumor mutational burden compared with the MSI intermediate cell line cultured for only 1 month. Similar results were observed in studies of 4- and 12-month–old *MLH1*-deficient mice, in which single-molecule MSI analysis revealed that deletions in mononucleotide repeats were larger and occurred more frequently in the intestinal cells of the older mice.[54] This hypothesis is further supported by studies showing accumulation of larger mononucleotide repeat deletions in more advanced neoplasms compared with precancerous and early-stage tumors. For example, pediatric constitutional dMMR cancers caused by biallelic germline pathogenic variants in MMR genes exhibit mostly 1 bp deletions.[55] Similarly, precancerous colon polyps often present with smaller changes in microsatellite repeat length compared with more advanced adenocarcinomas.[56,57]

Another factor influencing the overall size of microsatellite deletions in dMMR tumors is the length of the repeat. Two models have been proposed for accumulation of deletions in mononucleotide repeats: a stepwise model in which only one repeat motif is altered per mutational event, and a two-phase model in which either a single repeat motif or multiple motifs are altered per mutational event.[55] In dMMR tumors, the stepwise model best described mutations observed in short mononucleotide repeats (SMRs), and the two-phase model fit mutations in longer mononucleotide repeats. Similar results have been reported in a dMMR mouse model study in which most mutations in mononucleotide repeats involved losses of single repeat units.[58] At mononucleotide repeats >15 bp, a few cases of deletions involving multiple repeat units were observed and, although rare, indicate that a two-phase mutational process may be operating at longer repeat tracts.

Advances in MSI Testing

A key advancement in MSI testing was the adoption of marker panels exclusively containing mononucleotide repeats. This change improved the sensitivity and specificity of MSI testing over the Bethesda reference panel, which includes dinucleotide repeats that exhibit lower sensitivity and specificity for detection of dMMR, especially for *MSH6*-deficient tumors.[21,57] In contrast, a panel of mononucleotide repeats correctly identified 100% of *MSH6*-deficient cancers.[59] Another advance in MSI testing is the lack of requirement for use of matching normal samples. This has been achieved with a panel of monomorphic microsatellite markers, using the Idylla (Biocartis, Mechelen, Belgium) PCR-based assay that utilizes high-resolution melt analysis for MSI determination, as well as some of the MSI-NGS systems that do not require the use of matching normal samples.[50,60–62] However, sensitivity in non-CRCs may be reduced without a normal sample comparator.[62–64]

Non-CRC tumors often exhibit a less pronounced MSI phenotype; that is, they have smaller alterations to microsatellites and can be more challenging to detect by MSI-PCR.[17,18] Multiple strategies to improve pan-cancer MSI detection are being explored, including: i) the use of more microsatellite markers, ii) selection of microsatellite markers for specific cancer types, and iii) selection of microsatellite markers with a generalized higher sensitivity across all cancer types.

Several groups have used larger marker panels for MSI-PCR testing, with mixed results. Cicek et al[65] used a 10-locus panel consisting of four mononucleotide and six dinucleotide markers and achieved a sensitivity of 97% for detecting MSI-H/dMMR tumors using just the four mononucleotide markers in the panel. Bai et al[66] used a 24-locus panel consisting of six mononucleotide markers and 18 dinucleotide markers; the highest sensitivity of any mononucleotide or dinucleotide marker was 96% and 50%, respectively. In that study, the percent agreement with IHC as the reference standard was 87% for the five markers in the Bethesda panel compared with 56% for the entire 24-locus panel. Thus, increasing the number of markers used

for MSI-PCR did not improve detection of MSI and resulted in reduced accuracy of MSI determination.

MSI detection using NGS of microsatellite repeats has emerged as an alternative to standard MSI-PCR testing, and the technology lends itself to simultaneous evaluation of a large number of microsatellite markers.[44,47,67] However, using more markers does not necessarily translate into better results. Typically, the microsatellite markers used in NGS assays are incidentally included because they are in intronic regions of the target gene panels. The use of unselected microsatellite markers not specifically chosen for their ability to detect MSI-H/dMMR results in a wide range of individual marker sensitivities.[68] Other limitations with current NGS technologies are the high error rate for sequencing long homopolymer runs, limitation of low tumor cell content, high cost, and the lack of standardization.[69,70] Recent guidelines from the College of American Pathologists conclude there is currently insufficient evidence to support broad-based MSI testing by NGS.[71]

The strategy of using large numbers of unselected microsatellite markers for MSI determination can be illustrated by the Memorial Sloan Kettering−Integrated Mutation Profiling of Actionable Cancer Targets NGS platform, which contains >2000 mononucleotide repeats used to assess MSI status in >15,000 tumors from >50 cancer types.[47] A total of 103 Lynch syndrome cases were identified with germline MMR pathogenic variants; 51% were MSI-H, 13% MSI-Indeterminate, and 36% MSS. A follow-up study on 1100 endometrial cancers found 25 cases with germline MMR gene pathogenic variants, of which 83% with *MSH6* pathogenic variants and 31% with *MLH1*, *PMS2*, or *MSH2* pathogenic variants were classified as MSS or MSI-Indeterminate.[72] Thus, NGS assays using large numbers of microsatellite markers for MSI determination may not increase sensitivity and can identify a substantial number of MSI-Indeterminate cases of uncertain clinical significance.

Use of markers specific to cancer type is another approach that has been investigated to improve detection of dMMR tumors. Long et al[73] examined 9438 tumor-normal exome pairs and 901 whole-genome sequence pairs from 32 different cancer types for MSI by NGS. The MSI status of the top 2000 microsatellite markers most strongly associated with MSI-H status across tissue types were examined. Cancer-specific microsatellite panels of fewer than seven markers were found to be sufficient to attain ≥95% sensitivity and specificity for 11 of 15 cancer types examined. Thus, only a small number of markers were needed to provide accurate detection of MSI in most cancer types. However, marker panels selected for specific cancers were not generally applicable across cancers.

The approach taken in development of the LMR MSI Analysis System to increase MSI sensitivity across all cancer types was to use new markers with a generalized higher MSI sensitivity for detection of all dMMR tumors. It has been shown that sequence instability in microsatellites increases exponentially with increasing repeat length.[74−78] Based on the observation of increasing instability with increasing homopolymer length, we hypothesized that use of long mononucleotide repeat (LMR) markers for MSI analysis would improve the sensitivity of MSI detection. This hypothesis was confirmed in previous studies using LMR markers.[15,57,77] In the current study, we significantly expand on our previous work and assessed the accuracy of dMMR detection using the LMR MSI Analysis System on a pan-cancer cohort of Lynch syndrome and sporadic tumors from the Colon Cancer Family Registry (CCFR).

Materials and Methods

Study Population

Patient selection for this study included individuals having any type of cancer exhibiting loss of MMR protein expression by IHC and/or a pathogenic germline variant in *MLH1*, *MSH2*, *MSH6*, or *PMS2* genes. In addition, patients with sporadic MSI-H/dMMR CRC were included if tumors exhibited loss of MLH1 expression according to IHC and either *MLH1* promoter methylation or absence of germline MMR pathogenic variants. Patients with sporadic MSS/pMMR CRC were included if tumors had normal MMR expression and absence of germline MMR pathogenic variants. Tumor DNA from a total of 469 patients with cancer was obtained from the CCFR, including 149 Lynch syndrome CRCs, 170 non-CRC Lynch syndrome cancers, 71 sporadic MSI-H CRCs, and 79 sporadic MSS CRCs. The number and type of cancer samples in this study are provided in Table 1. Data on the MMR protein expression by IHC, *MLH1* promoter methylation, *BRAF* V600E mutations, and germline MMR mutations were provided by the participating CCFR sites (Mount Sinai Hospital, University of Melbourne, and the Mayo Clinic).[65,79,80] Tumor and patient information from the pathology reports was provided to the CCFR by the treating institutions. All participants gave informed consent for the study, which was approved by the Institutional Review Board at each CCFR site.

IHC and Germline MMR DNA Sequencing

IHC analysis of MLH1, PMS2, MSH2, and MSH6 protein expression was previously performed on formalin-fixed, paraffin-embedded tumor samples at CCFR centers.[81] The interpretation of IHC slides was performed by a pathologist without knowledge of the tumor MSI status. Germline sequencing of *MLH1*, *MSH2*, *MSH6*, and *PMS2* genes was performed on the Lynch syndrome samples used in this study by the participating CCFR institutions as described previously.[65,79,81]

MSI Testing

DNA from paired blood and formalin-fixed, paraffin-embedded tumor samples was tested for MSI by using the

Table 1 Tumor Characteristics and MMR Status of Study Cohort

Organ system	Cancer: tissue type	No. of tumor samples	MMR germline pathogenic variant	pMMR-IHC status	dMMR-IHC status	Mean age at diagnosis, y	Male	Female
Digestive	Stomach	7	7	1	6	62	5	2
	Duodenum	4	4	0	3	52	3	1
	Small intestine	6	6	0	5	58	4	2
	Ileum	1	1	0	1	36	1	0
	Colon (Lynch syndrome)	127	127	1	126	47	62	65
	Colon (sporadic dMMR)	71	1	0	71	59	28	43
	Colon (sporadic pMMR)	79	0	79	0	52	39	40
	Rectum	22	22	1	20	51	11	11
	Gallbladder	1	1	0	1	67	0	1
	Pancreas	1	1	0	1	46	1	0
	Bile duct	2	2	0	2	56	1	1
Urinary	Kidney	11	11	3	8	58	5	6
	Ureter	15	15	1	14	60	9	6
	Bladder	4	4	1	3	66	2	2
Reproductive	Prostate	12	12	5	7	61	12	0
	Endometrium	54	54	2	49	49	0	54
	Breast	28	28	11	17	53	1	27
	Ovary	17	17	0	17	48	0	17
Respiratory	Lung	2	2	1	1	51	0	2
Endocrine	Thyroid gland	1	1	1	0	32	0	1
Integumentary	Skin	3	3	0	3	51	3	0
Nervous	Brain	1	1	0	1	73	1	0
	Total combined	469	320	107	356	52	188	281

dMMR, deficient mismatch repair; IHC, immunohistochemistry; MMR, mismatch repair; pMMR, proficient mismatch repair.

LMR MSI Analysis System, which contains four SMRs (*NR-21*, *BAT-25*, *BAT-26*, and *MONO-27*) and four LMRs (*BAT-52*, *BAT-56*, *BAT-59*, and *BAT-60*) (Table 2). Confirmation of matching normal/tumor sample pairs was achieved by comparing allelic profiles for the polymorphic LMR markers, replacing the need for use of polymorphic pentanucleotide repeats. The LMR MSI Analysis System results were compared versus those of the Promega pentaplex panel, which consists of five mononucleotide repeat markers (*NR-21*, *NR-24*, *BAT-25*, *BAT-26*, and *MONO-27*)

Table 2 Microsatellite Markers Included in the LMR and Promega Pentaplex Panels

Panel	Marker	Marker class*	Repeat structure	Size range (bp)†	Detection channel	Chromosome
LMR	*NR-21*	SMR	A (21)	82–103	Blue	14
	BAT-25	SMR	A (25)	72–101	Green	4
	BAT-26	SMR	A (26)	79–102	Black	2
	MONO-27	SMR	A (27)	99–126	Red	2
	BAT-52	LMR	A (52)	126–177	Red	X
	BAT-56	LMR	A (56)	114–189	Black	X
	BAT-59	LMR	A (59)	102–172	Green	12
Pentaplex	*NR-21*	SMR	A (21)	83–108	Green	14
	NR-24	SMR	A (24)	103–138	Black	2
	BAT-25	SMR	A (25)	110–132	Green	4
	BAT-26	SMR	A (26)	83–121	Blue	2
	MONO-27	SMR	A (27)	134–168	Green	2
	PENTA C	Penta	AAAAC (4–17)	140–228	Black	9
	PENTA D	Penta	AAAGA (2–17)	123–253	Blue	21

*The SMR markers in the LMR and pentaplex panels are quasi-monomorphic, and most individuals in the population have the same size allele. LMR markers *BAT-52*, *BAT-56*, *BAT-59*, and *BAT-60* in the LMR MSI Analysis System are polymorphic, and repeat number varies between individuals. The largest known repeat size for LMR markers is listed.

†Allele sizes may vary when using different polymers, instruments, or instrument configurations.

LMR, long mononucleotide repeat; SMR, short mononucleotide repeat.

for MSI analysis and two polymorphic pentanucleotide repeat markers (*Penta C* and *Penta D*) for sample identification.[82] PCR amplification products from both the MSI assays were analyzed with an ABI 3500xL capillary electrophoresis instrument (Applied Biosystems, Foster City, CA) using a 36 cm capillary array and POP-4 polymer, and data were analyzed with GeneMapper software version 6.0 (Thermo Fisher Scientific, Waltham, MA). A microsatellite marker was called unstable if one or more tumor alleles were shifted by at least 2 bp from the germline allele or exhibited other subtle forms of instability described later in this section. The tumor MSI status is based on the number of unstable markers. For both panels, a tumor was designated as MSI-H if two or more mononucleotide markers were unstable and MSS if one or no mononucleotide markers were unstable in the tumor sample. MSI results were reported for tumors in cases in which at least two markers were scored for MSI-H calls and at least four markers were scored for MSS calls using the Promega pentaplex panel. MSI results were reported for the LMR MSI panel when at least two markers were scored for MSI-H calls and at least seven markers were scored for MSS calls.

Differences in the extent of MSI among tumors has been investigated because this variation may affect a patient's response to immunotherapy.[53] The extent, or intensity, of the MSI phenotype for a tumor is reported here as an MSI Intensity Score. MSI Intensity Scores were calculated by using the formula:

$$\text{MSI Intensity Score} = \sum_{\text{Locus}=NR-21}^{\text{Locus}=BAT-60} \left(\frac{|\,\text{Observed size shift}\,|}{\text{Maximum observed size shift}} \right) \div \text{Number loci} \times 100 \quad (1)$$

Locus equals *NR-21*, *BAT-25*, *BAT-26*, *MONO-27*, *BAT-52*, *BAT-56*, *BAT-59*, and *BAT-60*. Observed size shift is the absolute value of the deletion or insertion in base pairs for a given locus for a given sample. The Maximum observed size shift is the largest size shifts observed for a locus across all samples in this study (ie, *NR-21* = 14 bp, *BAT-25* = 13 bp, *BAT-26* = 15 bp, *MONO-27* = 19 bp, *BAT-52* = 32 bp, *BAT-56* = 41 bp, *BAT-59* = 43 bp, *BAT-60* = 35 bp). Number loci is the total number of loci in the MSI panel with results for a given sample. If all loci in a sample have size shifts as large as the Maximum observed size shift at each corresponding locus, then it would have an MSI Intensity Score of 100.

Interpretation of MSI using the LMR MSI Analysis System has been described previously.[15] Briefly, the allelic pattern of mononucleotide microsatellites in the electropherograms includes multiple peaks due to PCR slippage events in the homopolymer sequences (Supplemental Figure S1). These artifact peaks are referred to as stutter peaks. The tallest peak of each allele is referred to as the modal peak and represents the true DNA fragment length. New alleles in dMMR tumors result in a multimodal distribution of electropherogram peaks at one or more mononucleotide repeat markers. Most microsatellite alterations in dMMR tumors are deletions of one or more repeat units resulting in a decrease in the PCR fragment length size compared with the normal germline allele. When the shift is less than three to four bases, the shifted tumor peaks may overlap with germline stutter peaks, resulting in a "shoulder" pattern without a new modal peak. Low tumor cellularity in combination with small size shifts can complicate the interpretation of shoulders, and therefore $\geq$20% neoplastic cell content is generally recommended for robust MSI testing.[83] A marker is called unstable if there is a shift of at least 2 bp (rounding up from 1.5 bp) between the tallest peaks in paired normal and tumor samples, or if the shoulder pattern extends the range of the smallest stutter peak in the tumor sample by at least 2 bp. An expansion of microsatellite length caused by insertion of repeat units, while rare in the Promega pentaplex panel markers and occurring in only a few percent of LMR markers in MSI-H/dMMR tumors, was considered in MSI determinations.

The recommended method for MSI analysis is to compare microsatellite profiles of a tumor sample with those of a matching normal sample.[20] However, in some cases, a matching normal sample is not available. To address this issue, Suraweera et al[84] proposed using microsatellite markers that are monomorphic in the population, allowing the use of a standard reference normal sample in place of matching normal samples for MSI analysis. The markers in the Promega pentaplex panel are quasi-monomorphic, and it has been shown that the MSI status of CRC can be accurately determined in most cases without comparisons versus a matching normal sample.[61,85] To account for slight variation in allele sizes in a population, the quasi-monomorphic variation range (QMVR) of pooled normal samples is used.[50,61,85] QMVR values were calculated for each of the SMR markers in the LMR and Promega pentaplex panels by taking the average size of alleles $\pm$2.5 bp from all normal samples in our study cohort.[85] The LMR markers *BAT-52*, *BAT-56*, *BAT-59*, and *BAT-60* are polymorphic, and QMVR values are not applicable; modified rules were therefore applied for tumor-only MSI determinations with the LMR panel. All LMR markers with three or more alleles or an "obvious" shoulder pattern (ie, a clear visual difference in the pattern of stutter peaks between matching normal and tumor alleles) (Supplemental Figure S1) were considered unstable. For the X-linked LMR markers *BAT-52* and *BAT-56*, the presence of two or more alleles in a tumor from male patients was considered unstable. Any SMR marker in the LMR panel with alleles falling outside its respective QMVR size range was scored as unstable, or if an allele exhibited an obvious shoulder pattern. If a tumor exhibited instability in two or more markers it was classified as MSI-H, the same cutoff used when a matching normal sample is included.

Receiver-operating characteristic (ROC) curves were generated in R version 4.3 using the ROCR and ggplot2 packages (*https://cran.r-project.org/web/packages/available_packages_by_name.html*) to determine the optimal cutoff for

number of unstable markers used for tumor MSI classification. To determine the effect of marker number on MSI assessment, the overall assay sensitivity for panel sizes ranging from 3 to 50 markers was calculated. For each iteration tested, all markers were assigned the same individual sensitivity value ranging from 40% to 90% and a cutoff value for MSI-H classification between 20% and 40% unstable markers. Each marker in a panel was assumed to be an independent Bernoulli trial, and the probability of having at least the number of successes required by the percent unstable marker cutoff to call a patient MSI-H was calculated by using the binomial distribution.

The Fisher exact test, *t*-test, one-way analysis of variance, and Dunn's method for pairwise comparisons were performed to calculate *P* values. Sensitivity, specificity, and accuracy of the MSI-PCR tests for determination of tumor MSI status were determined with IHC as the reference standard using formulas given in Supplemental Table S1. The 95% confidence intervals were calculated by using the Clopper-Pearson exact binomial interval. Specimens that were MSI-H according to PCR and dMMR according to IHC were classified as true positives, or false positives if a tumor was MSI-H and pMMR. Specimens that were MSS according to PCR and pMMR according to IHC were classified as true negatives, or false negatives if a tumor was MSS and dMMR. Although MMR IHC was defined as the reference standard for the purposes of this study, the MMR status as determined by IHC may not always be correct because the test is not 100% accurate.[14,27,34]

Results

Characterization of the Study Cohort

A total of 469 DNA samples from 20 different cancer types were obtained from the CCFR; they included 319 (149 CRC and 170 non-CRC) cancers from patients with Lynch syndrome and 150 sporadic or nonheritable CRC cases. The characteristics of the study population are summarized in Table 1. The average age at diagnosis for Lynch syndrome MSI-H/dMMR CRC was 47.3 years, 51.7 years for sporadic MSS/pMMR CRC, and 59.2 years for sporadic MSI-H/dMMR CRC (Lynch versus sporadic MSS, $P = 0.037$; Lynch versus sporadic MSI-H, $P < 0.001$; sporadic MSS versus sporadic MSI-H, $P < 0.001$). The age at diagnosis for individuals with various MMR gene deficiencies across all cancer types was not significantly different ($P = 0.218$). There were 188 male subjects and 281 female subjects included in the study. Lynch syndrome colon tumors were more often right-sided (ie, proximal to the splenic flexure, excluding rectum) [77% (98 of 127); $P < 0.001$], which is consistent with previous studies.[86]

MMR protein expression by IHC was available for 463 of the 469 study cases. Of the dMMR cancers, 161 displayed loss of expression of MLH1 or MLH1/PMS2, 136 loss of MSH2 or MSH2/MSH6, 31 loss of MSH6 only, and 24 loss of PMS2 only. Germline sequencing data on the MMR genes were available for 467 of 469 samples. Of the 319 cases classified as Lynch syndrome by the CCFR, germline MMR pathogenic variants were found in 150 *MSH2*, 103 *MLH1*, 39 *MSH6,* 26 *PMS2*, and 1 *EPCAM*. As previously reported, *MLH1* promoter methylation [1.7% (2 of 118)] and *BRAF* V600E mutations [2.5% (5 of 202)] were uncommon across all MSI-H/dMMR Lynch syndrome cancers as well in sporadic MSS/pMMR CRC [2.5% (1 of 40) and 5.6% (4 of 72), respectively].[42] In contrast, 57.2% (36 of 63) of sporadic MSI-H/dMMR CRCs tested had *MLH1* promoter methylation and 60.2% (41 of 68) a *BRAF* V600E mutation.

Performance of the LMR MSI Analysis System

To assess the performance of the LMR MSI Analysis System, comparisons were made with the current standard MSI and IHC tests. For MSI analysis, the Promega pentaplex panel was used as the standard for this study. The LMR MSI Analysis System is a newly developed pan-cancer test for MSI that contains four of the five SMR markers contained in the Promega pentaplex panel plus four LMRs for improved pan-cancer MSI detection (Table 2). The microsatellite markers in both panels consist of adenine mononucleotide repeats ranging from 21 to 27 repeats in the SMR markers and 52 to 60 repeats in the LMR markers. LMR markers are polymorphic in the population, however, and the number of repeats at a given locus can vary among individuals. Amplified PCR products generated with the MSI kits were sized by using capillary electrophoresis and the data analyzed using GeneMapper software to determine the size differences between the paired normal and tumor samples. Interpretation of electropherograms is illustrated in Supplemental Figure S1. Representative electropherograms of MLH1-deficient colon and endometrial cancer specimens using the LMR panel are shown in Figures 1 and 2 and *MSH6*-deficient colon and endometrial cancers in Supplemental Figures S2 and S3.

The tumor MSI status using the LMR and the Promega pentaplex panels and the MMR status using IHC across all cancers is summarized in Table 3 and detailed in Supplemental Table S2. The accuracy (using IHC as the reference standard) across all cancers was significantly higher with the LMR panel than with the Promega pentaplex panel (96.5% versus 92.6%; $P = 0.009$) (Table 4). The increased accuracy of the LMR MSI panel was primarily due to the higher sensitivity of LMR compared with the Promega pentaplex panel (97.5% versus 91.8%; $P < 0.001$), as there was no statistically significant difference in specificity (93.5% versus 95.34%; $P = 0.7678$). In the CRC cohort, the sensitivity of the LMR and the Promega pentaplex panels was 98.6% and 96.8%, and the specificity was 98.8% and 98.8%, respectively. Neither sensitivity nor specificity was significantly different for CRC ($P = 0.338$ and $P = 1.000$). In the non-CRC tumors, sensitivity was greater with LMR (95.7% versus 83.9%; $P = 0.001$), but

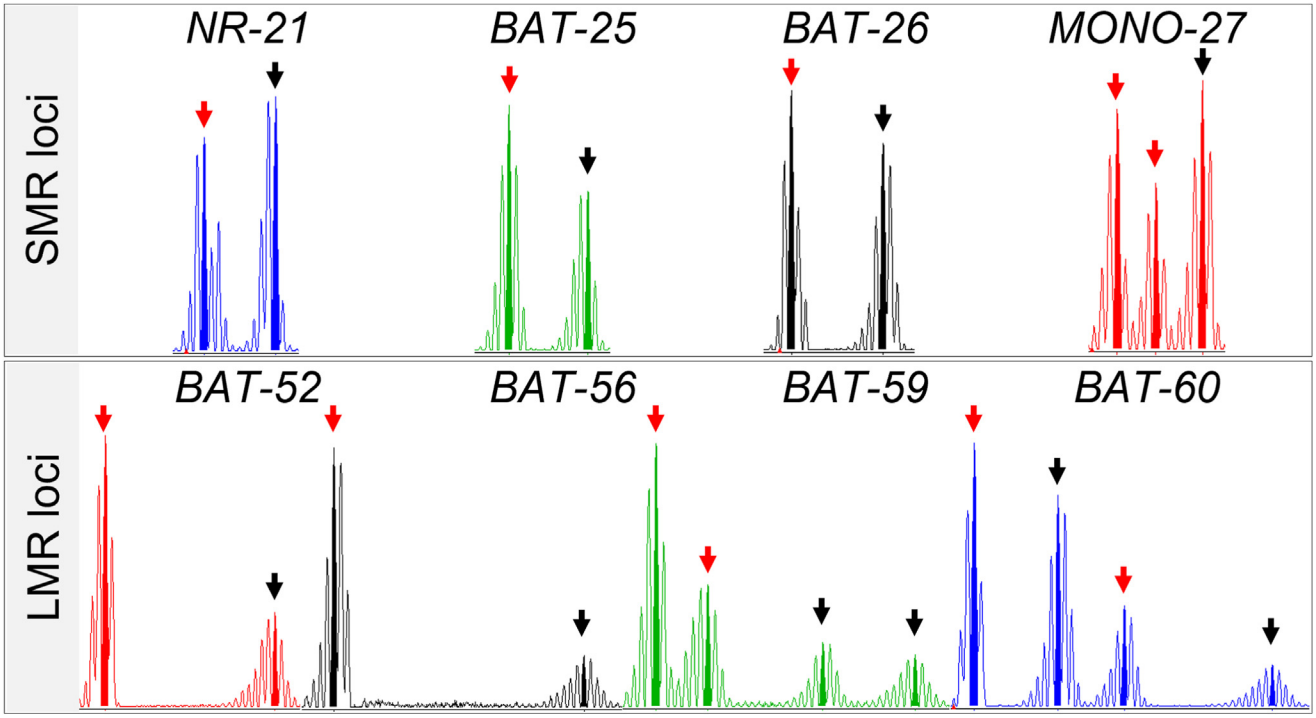

Figure 1 Electropherogram showing a representative *MLH1*-deficient colorectal cancer specimen using the LMR MSI Analysis System. The four short mononucleotide repeat (SMR) markers are shown in the **top panel** and the four long mononucleotide repeat (LMR) markers in the **bottom panel**. **Black arrows** denote germline alleles and **red arrows** novel tumor alleles. Both normal and tumors samples were run, but only the tumor sample is displayed for simplicity.

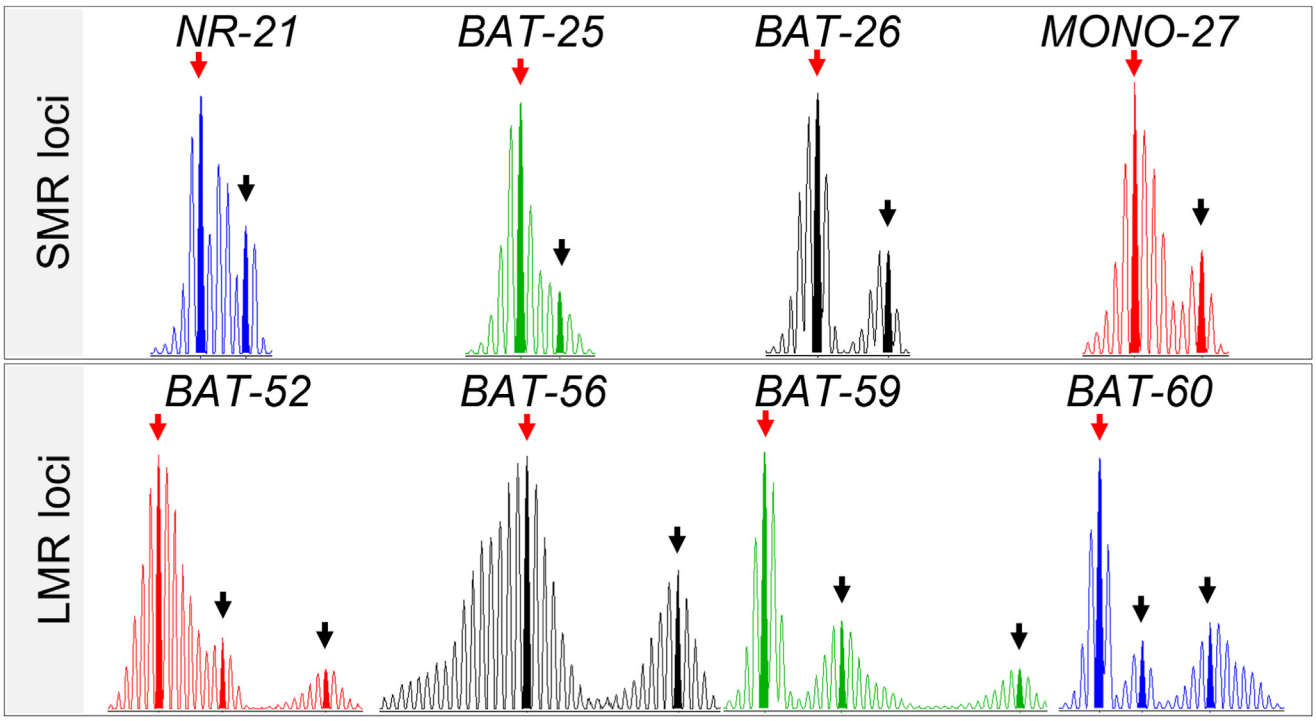

Figure 2 Electropherogram showing a representative *MLH1*-deficient endometrial cancer specimen using the LMR MSI Analysis System. The four short mononucleotide repeat (SMR) markers are shown in the **top panel** and the four long mononucleotide repeat (LMR) markers in the **bottom panel**. **Black arrows** denote germline alleles and **red arrows** novel tumor alleles. Both normal and tumors samples were run, but only the tumor sample is displayed for simplicity.

Table 3 MSI Test Results for the LMR and Promega Pentaplex Panels for all Cancers

			Pentaplex panel			LMR panel		
Organ system	Cancer tissue type	IHC	*n*	MSS	MSI-H	*n*	MSS	MSI-H
Digestive	Stomach	Normal	1	0	1	1	0	1
		Abnormal	6	0	6	6	0	6
Digestive	Small intestine	Normal	0	0	0	0	0	0
		Abnormal	9	0	9	9	0	9
Digestive	Colorectal (Lynch syndrome)	Normal	2	1	1	2	1	1
		Abnormal	146	3	143	145	1	144
Digestive	Colorectal (sporadic)	Normal	79	79	0	79	79	0
		Abnormal	71	4	67	71	2	69
Digestive	Gallbladder	Normal	0	0	0	0	0	0
		Abnormal	1	1	0	1	0	1
Digestive	Pancreas	Normal	0	0	0	0	0	0
		Abnormal	1	0	1	1	0	1
Digestive	Bile duct	Normal	0	0	0	0	0	0
		Abnormal	2	1	1	2	0	2
Urinary	Kidney	Normal	3	2	1	3	1	2
		Abnormal	8	4	4	8	3	5
Urinary	Ureter	Normal	1	0	1	1	0	1
		Abnormal	13	0	13	14	0	14
Urinary	Bladder	Normal	1	1	0	1	1	0
		Abnormal	3	0	3	3	0	3
Reproductive	Prostate	Normal	5	5	0	5	5	0
		Abnormal	7	3	4	7	0	7
Reproductive	Endometrial	Normal	2	1	1	2	1	1
		Abnormal	48	9	39	49	2	47
Reproductive	Breast	Normal	11	11	0	11	11	0
		Abnormal	17	4	13	17	1	16
Reproductive	Ovary	Normal	0	0	0	0	0	0
		Abnormal	17	0	17	17	0	17
Respiratory	Lung	Normal	1	1	0	1	0	1
		Abnormal	1	0	1	1	0	1
Endocrine	Thyroid gland	Normal	1	1	0	1	1	0
		Abnormal	0	0	0	0	0	0
Integumentary	Skin	Normal	0	0	0	0	0	0
		Abnormal	3	0	3	3	0	3
Nervous	Brain	Normal	0	0	0	0	0	0
		Abnormal	1	0	1	1	0	1
	Total combined	Normal	107	102	5	107	100	7
		Abnormal	354	29	325	355	9	346

IHC, immunohistochemistry; LMR, long mononucleotide repeat; MSI, microsatellite instability; MSI-H, microsatellite instability high (tumors with 2 or more unstable markers); MSS, microsatellite stable (tumors with 0 or 1 unstable markers).

there was no significant difference in specificity (76.9% versus 84.6%; $P = 0.727$). The lower-than-expected specificity value for non-CRC samples is likely due to the small number of pMMR samples ($n = 26$) tested in this study, including six with normal MMR IHC results that had germline MMR pathogenic variants. Discordant samples from the inter-test MSI comparisons in Table 4 are described in Supplemental Table S3 (between MSI-PCR and IHC) and Supplemental Table S4 (between LMR and the Promega pentaplex panels).

Next, all cancer types were examined to determine whether the discrepancy in accuracy between panels was related to cancer types. The overall percent agreement between the LMR and the Promega pentaplex panels for CRC was 98.7% and 89.3% for non-CRC tumors ($P < 0.001$), indicating that most of the discrepancy was related to the non-CRC tumors. Indeed, for CRC tumors, there was no significant difference in the accuracy of the LMR and Promega pentaplex panels (98.7% versus 97.3%; $P = 0.383$) compared with IHC. However, in the non-CRC cohort, the accuracy was significantly higher for the LMR panel than for the Promega pentaplex panel (92.7% versus 84.0%; $P = 0.016$). For endometrial cancers specifically, which have the highest percentage of MSI-H cases by

Table 4 Interassay Comparison: Performance of the LMR MSI Analysis System

Test	Reference	Cancer group	True positive	False negative	False positive	True negative	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Accuracy, % (95% CI)
LMR	IHC	CRC	213	3	1	80	98.6 (96.0–99.7)	98.8 (93.3–100)	98.7 (96.6–99.6)
LMR	IHC	Non-CRC	133	6	6	20	95.7 (90.8–98.4)	76.9 (56.4–90.8)	92.7 (87.6–96.1)
LMR	IHC	ALL	346	9	7	100	97.5 (95.2–98.8)	93.5 (87–97.3)	96.5 (94.4–98)
Pentaplex	IHC	CRC	210	7	1	80	96.8 (93.5–98.7)	98.8 (93.3–100)	97.3 (94.8–98.8)
Pentaplex	IHC	Non-CRC	115	22	4	22	83.9 (76.7–89.7)	84.6 (65.1–95.5)	84 (77.5–89.2)
Pentaplex	IHC	ALL	325	29	5	102	91.8 (88.4–94.4)	95.3 (89.4–98.4)	92.6 (89.8–94.8)
LMR	Pentaplex	CRC	211	0	4	83	100 (98.3–100)	95.4 (88.6–98.7)	98.7 (96.6–99.6)
LMR	Pentaplex	Non-CRC	122	0	18	28	100 (97.0–100)	60.9 (45.4–74.9)	89.3 (83.6–93.5)
LMR	Pentaplex	ALL	333	0	22	111	100 (98.9–100)	83.5 (76–89.2)	95.3 (92.9–97)

ALL, acute lymphoid leukemia; CRC, colorectal cancer; IHC, immunohistochemistry; LMR, long mononucleotide repeat; MSI, microsatellite instability.

cancer type, the sensitivity of the LMR panel for detection of dMMR tumors was 95.9% (47 of 49). In addition, there was a pMMR endometrial cancer sample by IHC that was MSI-H and had a germline pathogenic variant in *MSH6*. Overall, the LMR panel showed significantly greater accuracy for detection of non-CRC tumors and equivalent accuracy in CRC tumors compared with the Promega pentaplex panel.

Although MSI and MMR IHC results are typically highly concordant, in cases in which differences arise, a third orthogonal test (typically MMR gene sequencing) may help to resolve the differences. In this study, there were nine MSS/dMMR and five MSI-H/pMMR discordant cases between LMR and IHC (Supplemental Table S3). Of these, seven of nine MSS/dMMR cases had germline MMR pathogenic variants, indicating these may be false-negative MSI results, and five of five MSI-H/pMMR cases had germline MMR pathogenic variants, indicating these may be false-negative IHC results. For the Promega pentaplex panel discordant cases, 25 of 29 MSS/dMMR cases had germline MMR pathogenic variants, indicating these may be false-negative MSI results, and all four of the MSI-H/pMMR cases had germline MMR pathogenic variants, indicating these may be false-negative IHC results. The result from orthogonal testing indicates that dual testing of MSI and IHC would yield the greatest overall accuracy.

Discordant cases between the LMR and the Promega pentaplex panels are shown in Supplemental Table S4. There were 21 discordant cases involving all MMR genes (with slightly more occurring in *MSH6*, which is consistent with the occurrence of milder MSI phenotypes in tumors with *MSH6* loss). The major characteristic associated with discordant cases is that most are non-CRCs, and this is illustrated by the difference in sensitivity between the LMR and the Promega pentaplex panels for non-CRC (Table 4). There was also a significant difference in the average MSI Intensity Scores between the discordant cases compared with all dMMR cases (9.1 versus 38.56; $P < 0.001$). Lower scores are expected for non-CRCs, which typically have fewer unstable markers and exhibit smaller size shifts, and this translates into lower MSI Intensity Scores.

Characterization of Individual Markers

Microsatellite markers are not equally sensitive and specific for detection of dMMR and pMMR tumors, and considerable effort has gone into identifying the best markers for MSI testing.[21,82,87] The relative performance of the individual markers in the LMR MSI Analysis System was assessed to determine how often each marker was stable or unstable in pMMR and dMMR tumors, and what was the magnitude of the change. Other marker characteristics, including allele frequencies, percent heterozygosity, and inter-assay variation between SMR markers in the LMR and the Promega pentaplex panels, were also assessed.

An evaluation of the sensitivity and specificity of the individual markers within the LMR panel is summarized in Figure 3. As a group, the average sensitivity for the four LMR markers was significantly higher than that for the four SMR markers in non-CRC tumors (88.3 versus 73.7; $P < 0.001$) but not in CRC (96.4 versus 95.0; $P = 0.191$). The average specificity of the LMR and SMR markers was significantly different for CRC (95.7 versus 98.5; $P = 0.038$) but not for non-CRC (81.7 versus 88.5; $P = 0.242$). The LMR markers *BAT-52* and *BAT-56* are located on the X chromosome, and therefore male subjects always appear homozygous at these loci. Because there is only a single copy of *BAT-52* and *BAT-56* in male cells that can potentially be mutated, the question arises as to whether these markers may be less sensitive to MSI in tumors occurring in male subjects compared with female subjects. For any given LMR marker, there were no significant difference in the sensitivity between male subjects and female subjects ($P > 0.1$) (Supplemental Figure S4). In summary, the sensitivity of all four LMR markers was higher than the best SMR marker for non-CRC, but for CRC, all markers exhibited comparably high levels of sensitivity.

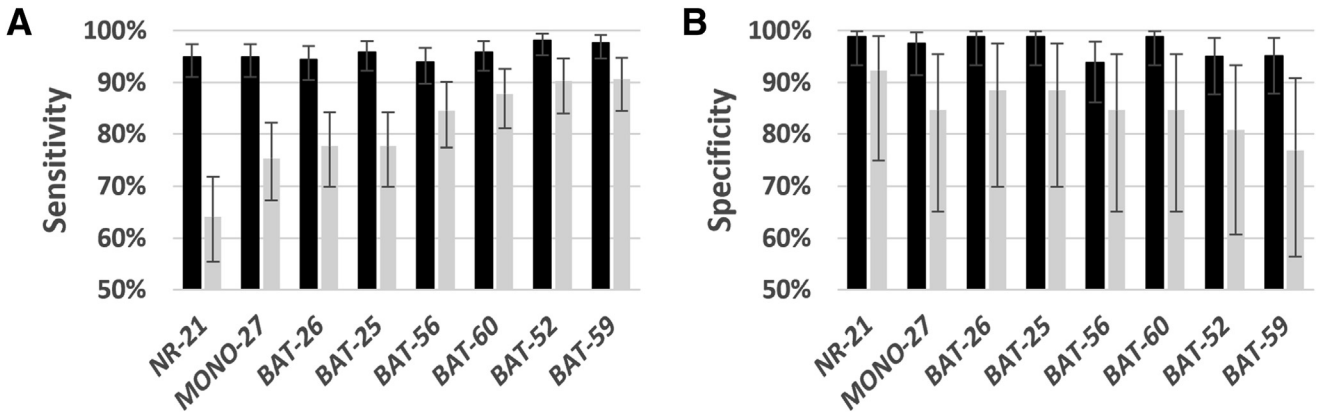

Figure 3 Sensitivity and specificity of markers in the LMR MSI Analysis System. The sensitivity (**A**) and specificity (**B**) with the 95% confidence intervals were determined for all markers in the LMR MSI Analysis System panel using immunohistochemistry as a reference standard. **Black bars** indicate colorectal cancer and **gray bars** non—colorectal cancer.

The size of insertion/deletion mutations in microsatellite sequences varied widely among markers (Figure 4). The average size shifts were larger for all four LMR markers compared with the SMR markers (14.9 bp versus 5.8 bp; $P < 0.001$). Most mutations were deletions, but there were two insertions in 318 MSI-H tumors detected with the Promega pentaplex panel markers and 23 insertions in 349 MSI-H tumors detected with the LMR panel markers (21 in LMRs and 2 in SMRs). A minimum size shift of 2 bp (rounded up from $\geq$1.5 bp) was required to classify a marker as unstable. Size shifts <1.5 bp were commonly observed in MSI-H/dMMR tumors, but they were also observed in 80% (85 of 106) of pMMR tumor in one or more markers. Therefore, markers with <1.5 bp shifts cannot reliably be considered unstable. The average marker size shift varied widely among cancer types, with small shift sizes commonly observed in non-gastrointestinal tract cancers such as endometrial, breast, and prostate (Figure 5).

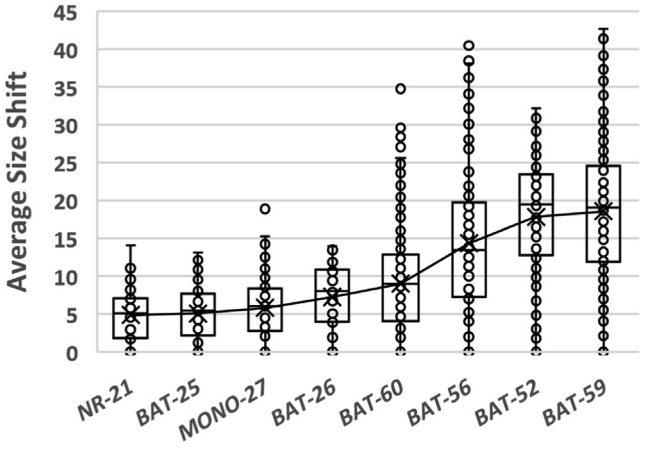

Figure 4 Size shift by marker in microsatellite instability—high/deficient mismatch repair tumors using the LMR MSI Analysis System markers. The size shifts for each long mononucleotide repeat marker (average is indicated by an X) for all microsatellite instability—high/deficient mismatch repair tumors is shown. Each **dot** is a value that may represent multiple data points.

The allele frequency and percent heterozygosity for the markers in the LMR MSI Analysis System were determined for the 469 matching normal samples in the study cohort (Supplemental Figures S5 and S6). The SMR markers all exhibited very low levels of variability as measured by total number of alleles (five to nine alleles per marker) and percent heterozygosity (0% to 3% per marker), in agreement with previous reports.[82] In contrast, the four LMR markers exhibited a broad range of allele sizes (on average 40 alleles per marker) and much higher heterozygosity levels. This difference between SMR and LMR loci is largely attributable to the near monomorphic nature of the SMR loci, which have a common germline allele as opposed to the LMR loci, which are polymorphic and do not have a common germline allele. Heterozygosity for the autosomal LMR markers *BAT-59* and *BAT-60* was around 70% and lower for X-linked markers *BAT-52* and *BAT-56* as expected because these markers can only be heterozygous in XX female subjects.

In MSI-H/dMMR tumors, mutant alleles seem to have been created predominantly by small deletions in the germline microsatellite allele. This is illustrated by the distribution of mutant alleles in non-gastrointestinal MSI-H/dMMR tumors, in which the modal peak for the most common size shift is approximately 2 bp (Supplemental Figure S7). In contrast, CRCs exhibited right shifted modal peaks with deletions >2 bp, presumably due to the accumulation of multiple mutational events from a high number of replication cycles after loss of MMR function in rapidly dividing colon cells. This pattern is consistent with the stepwise deletion model in which a single repeat unit is altered per mutational event, and larger deletions are created through accumulation of multiple smaller events moving toward relative stability at a minimum repeat number of

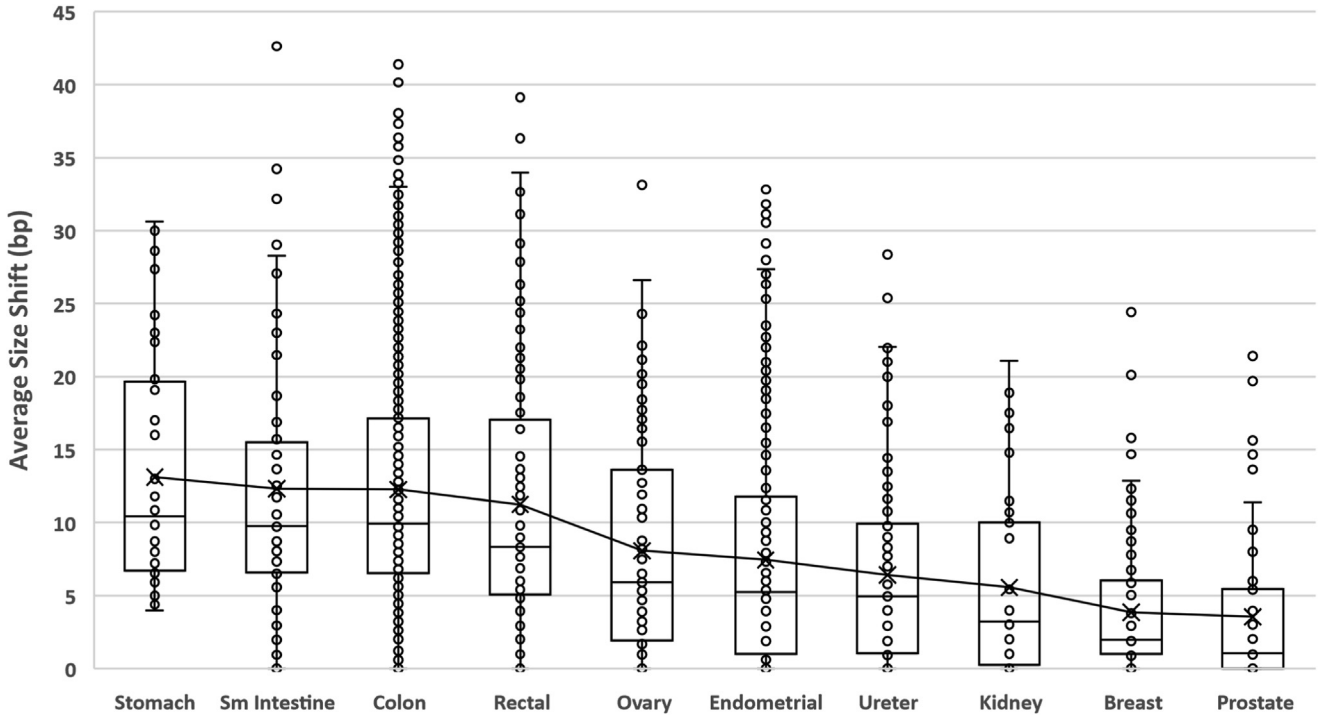

Figure 5 Size shift by cancer type for microsatellite instability—high/deficient mismatch repair tumors using the LMR MSI Analysis System. The size shifts for all long mononucleotide repeat markers (average is indicated by an X) are shown for each cancer type with five or more samples. Each **dot** is a value that may represent multiple data points. Sm, small.

approximately 15 bp.[55] The minimum estimated number of repeats observed in SMR loci ranged from 7 to 12 repeats, as opposed to 15 to 25 repeats for the LMR loci, which require more stepwise mutational events before reaching a minimum repeat number. Deletions in LMR loci involving loss of multiple repeat units in a single event may have also occurred but at a lower frequency. This is shown by the pattern of spontaneous mutant alleles in the four LMR loci from 107 MSS/pMMR tumors in which 1 repeat unit deletions accounted for 92% (54 of 59) of mutational events and larger deletions of 9 to 26 repeat units occurred in only 8%.

Interassay comparison of SMR marker alterations contained in both the LMR MSI Analysis System and the Promega pentaplex panel in MSI-H cancers is shown in Supplemental Table S5. Discordant alterations in *NR-21*, *BAT-25*, *BAT-26*, and *MONO-27* markers were observed in 4.3% (80 of 1856) of marker comparisons. Using only these four markers for MSI classification, the discordant calls changed the overall tumor MSI status determination in 1.1% (5 of 464) of cases. Differences in the MSI calls between panels were often due to small 1 to 2 bp changes in the size of deletions or the presence or absence of a subtle shoulder pattern that resulted in a change in marker classification. Amplicon sizes for the SMR loci are smaller in the LMR panel and therefore tend to amplify DNA from formalin-fixed, paraffin-embedded samples more robustly, which may account for some of the observed variation.

Cutoffs and Optimal Number of Markers for MSI Classification

The Bethesda guidelines established a reference set of five microsatellite markers (mononucleotide and dinucleotide repeats) and a method for classifying MSI status based on the number of unstable markers observed in a tumor.[20] These guidelines were later revised, and a panel of five mononucleotide repeat markers replaced the original panel, although the total number of markers and the cutoffs remains the same.[21] Guidelines for MSI tumor classification using the LMR MSI Analysis System have been provided by Promega.[88] A tumor is considered as MSI-H if two or more markers are unstable and MSS if 0 or 1 marker is unstable. The guidelines do not provide cutoff criteria for classification of an MSI-Low group. In contrast, a recent publication on the validation of the LMR MSI kit used a different cutoff of three or more unstable markers as the MSI-H and one or two unstable markers as MSI-Low.[15] ROC analysis was therefore performed to determine the optimal cutoff value for the accurate classification of pan-cancer MSI status (Supplemental Figure S8). The optimal cutoff for MSI-H tumor classification based on ROC analysis was two or more unstable markers with an AUC value of 0.949. There were seven samples with two unstable markers of the eight markers tested. All but one exhibited loss of MMR expression by IHC and had germline MMR pathogenic variants. There were 14 samples with 3 unstable markers of 8 markers tested. All these samples showed loss

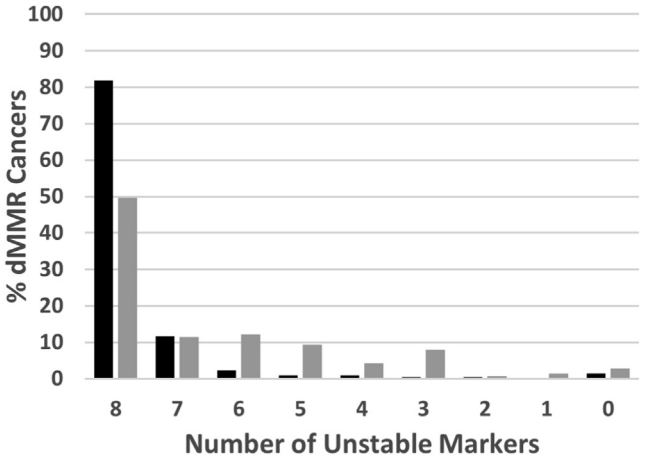

Figure 6 Percent of unstable markers in deficient mismatch repair (dMMR) colorectal cancer (CRC) and non-CRC samples using the LMR MSI Analysis System. Percentage of the dMMR tumors determined by immunohistochemistry with zero to eight unstable markers is shown for CRC (**black bars**) and non-CRC (**gray bars**) samples.

of MMR expression by IHC, and 11 had germline MMR pathogenic variants. Thus, the cutoff of two or more unstable markers for classification of MSI-H tumors determined by ROC analysis is supported by both IHC and sequencing data.

The average percentage of unstable LMR panel markers in CRC and non-CRC samples is shown in Figure 6. More than 93% of MSI-H/dMMR CRC exhibited instability in at least seven of eight markers compared with 61% in non-CRC samples ($P = 0.013$). The number of unstable markers per dMMR tumor was more evenly distributed in non-CRC samples. This raised the question of whether the use of more markers would increase detection of MSI for non-CRC. *In silico* analysis for the overall sensitivity of panels of markers for MSI detection with varying levels of marker sensitivity ranging from 40% to 90% was calculated by using panels ranging in size from 3 to 50 markers with cutoffs of 20% to 40% unstable markers (Supplemental Figure S9). These results indicate that increasing the number of markers in the LMR panel, which in this study have a sensitivity of 98.6% for CRC and 95.7% for non-CRC samples with a cutoff of 25% unstable markers, would not have significantly improved overall sensitivity for MSI detection.

Differences in MSI Intensity

The extent or intensity of MSI can vary between dMMR tumors in terms of the percent unstable microsatellite markers and in the number of repeat units inserted or deleted. Differences in the intensity of MSI among tumors was investigated as this variation may be important for predicting a patient's response to immunotherapy.[53] Different cancer types exhibited different levels of MSI as assessed by their MSI Intensity Scores (Supplemental Figure S10). Cancers of the gastrointestinal tract, including the stomach, small intestine, colon, and rectum, had significantly higher MSI Intensity Scores than non-gastrointestinal cancer types ($P < 0.001$). Furthermore, wide variation in MSI Intensity Scores among tumors of the same cancer type was observed. This variation in MSI intensity may be due in part to differences in the size of a tumor because MSI is believed to be a progressive phenomenon that increases in intensity over time after loss of MMR as the tumor develops.[52] Tumor size of MSI-H/dMMR colon cancers was found to be positively associated with MSI intensity ($P < 0.001$).

Next, the effect of deficiencies in the four MMR genes on MSI intensity as well as the epigenetic loss of *MLH1* in sporadic CRCs were assessed. MSI Intensity Scores for CRCs that were MSI-H with the LMR MSI Analysis System and dMMR with IHC were determined (Supplemental Figure S10). Sporadic MSI-H/dMMR CRCs with epigenetic loss of *MLH1* had the highest average MSI Intensity Score, followed in decreasing order by MMR gene deficiencies in *MLH1*, *MSH2, PMS2*, and *MSH6* (sporadic MSI-H, *MLH1* and *MSH2* scores were significantly different from *MSH6*; $P < 0.001$). There was only one case in the study with an *EPCAM* pathogenic variant. This case was MSI-H, exhibited loss of MSH2 expression by IHC, and had an MSI Intensity Score of 49.2, which is very close to the mean score for cases with germline *MSH2* pathogenic variants. In addition to the gene effects, there was variation in MSI intensity between cancer types with the same MMR gene deficiency.

Finally, the association between MSI intensity and tumor immune response was examined by assessing the presence of tumor-infiltrating lymphocytes (TILs). A high level of TILs in the tumor indicates that the body has initiated an immune response against the tumor. MSI-H CRC exhibited a high level of TILs ($\geq$1:10 lymphocyte to epithelial nuclei ratio) in 72.9% (124 of 170) of tumors compared with 3.9% (3 of 76) in MSS CRC ($P < 0.001$). Overall, MSI Intensity Scores were found to vary between cancer types, among cancers of the same type, by tumor size, by MMR gene deficiency, and between pMMR and dMMR tumors. Further investigation into the utility of MSI intensity as a biomarker for personalizing disease management is needed to confirm these observations.

MSI Testing with and without Matching Normal Sample

The Bethesda guidelines for MSI testing recommend testing paired normal and tumor samples because some of the markers in the original Bethesda panel are polymorphic, and to identify a new allele in the tumor sample the germline genotype must be known.[20] The markers *NR-21, NR-24, BAT-25, BAT-26,* and *MONO-27* in the Promega pentaplex panel are quasi-monomorphic, meaning that most individuals in the population have the same size allele, and therefore a common population reference standard can be used in place of a paired normal sample in most cases.[50,82] For example, a panel of quasi-monomorphic

mononucleotide-repeat markers (*NR-21, NR-24, BAT-25, BAT-26, MONO-27*) (FALCO biosystems, Kyoto, Japan) was approved as a companion diagnostic for pembrolizumab for the treatment of MSI-H solid tumors without requiring the use of matching normal samples. In contrast, the four LMR markers in the LMR MSI Analysis System are polymorphic and require matching normal samples to achieve the most accurate MSI test results (Supplemental Figures S5 and S6).

Matching normal samples are not always available; therefore, the effectiveness of testing only the tumor samples with the LMR MSI kit was investigated. The analysis criteria used for MSI testing of only tumor samples is described in detail in *Materials and Methods*. Briefly, the average normal allele size and QMVR values were calculated for the four SMR markers in the LMR panel using the matching normal samples from the CCFR cohort (Table 5). QMVR values were not calculated for the four LMR markers because they are polymorphic. The SMR markers were scored as unstable if a tumor allele was outside the respective normal QMVR size range or exhibited an obvious shoulder pattern. For the LMR markers, which lacked QMVR values, tumors with three or more alleles per marker or an obvious shoulder pattern were scored as unstable. In male subjects, X-linked hemizygous markers *BAT-52* and *BAT-56* were scored as unstable when two alleles for a given marker were observed. For the LMR MSI Analysis System, the percent agreement for determining tumor MSI status with and without matching normal sample was 97.6% for CRC samples and 90.9% for non-CRC samples ($P = 0.002$) (Table 6). For CRC samples, there were five false-negatives and two false-positive samples of a total of 288 tumors. For non-CRC samples, there were 15 false-negative findings and no false-positive findings of a total of 164 tumors. False-negative results were mainly due to small allele size shifts resulting in new alleles that were still within the normal QMVR range or subtle shoulders that could not reliably be called without a matching normal sample. The two false-positive sample calls were due to germline heterozygosity in the SMR marker in which one allele fell outside of the normal QMVR size range. Overall, the data indicate that testing on samples using the LMR MSI Analysis System is feasible if matching normal sample is not available, but sensitivity may be reduced, especially for non-CRC samples.

The ability to correctly identify MSI status using only tumor samples was also assessed for the Promega pentaplex panel and compared versus the four SMR markers (*NR-21, BAT-25, BAT-26*, and *MONO-27*) also contained in the LMR panel (Table 6). For the Promega pentaplex panel, the percent agreement for determining tumor MSI status with and without matching normal sample was 99% for CRC samples and 86.8% for non-CRC samples ($P < 0.001$). Similar results were observed using just the four SMR markers from the LMR panel (for CRC and non-CRC, the percent agreement was 98% and 82.8%; $P < 0.001$). There were no significant differences in MSI calls using the SMR markers versus the Promega pentaplex panel for either CRC or non-CRC [CRC agreement was 98% versus 99% ($P = 0.504$); non-CRC agreement was 82.8% versus 86.8% ($P = 0.363$)]. Thus, MSI testing with the Promega pentaplex panel using only CRC tumor samples resulted in a nonsignificant 1% loss of sensitivity compared with tests using both tumor and normal samples, whereas there was a 13.2% loss for non-CRC samples.

Discussion

LMR MSI Analysis System Performance

This study evaluated the performance of the LMR MSI Analysis System for detection of MSI in a pan-cancer cohort of 469 individuals enriched for Lynch syndrome. For CRC, the sensitivity and specificity of the LMR and pentaplex panels for correctly identifying the underlying MMR status of a tumor using MMR IHC as the reference were not significantly different (sensitivity was 99% versus 97% and specificity 99% versus 99%, respectively) (Table 4). For non-CRC, the sensitivity of the LMR MSI panel was significantly greater than the Promega pentaplex panel (96% versus 84%), whereas the specificity for detecting pMMR non-CRC tumors was not significantly different. Thus, the major performance benefit for the LMR panel was increased sensitivity for detecting dMMR in non-CRC.

The results from the current study are consistent with a previously published validation study by Lin et al[15] that compared the LMR MSI Analysis System with the Promega pentaplex panel. The reported sensitivity and specificity of both panels were 100% for CRC. For endometrial cancers, sensitivity was 98% and 88% for the LMR and pentaplex panels, and specificity was 100% for both panels. An earlier study by Bacher et al[57] using a panel of five LMR markers, including *BAT-52, BAT-56*, and *BAT-59* from the current LMR MSI Analysis System, reported increased sensitivity of the LMR marker panel compared with the Promega pentaplex panel for detection of dMMR colon polyps. The sensitivity and specificity for detection of dMMR polyps were 100% and 96% for the LMR panel and 67% and 100% for the Promega pentaplex panel using IHC as the reference.

In addition to the increased sensitivity of LMR markers in dMMR cancers, the other notable performance advantage is the larger allele size shifts of the LMR markers compared with SMR markers, providing greater confidence in calling variants (Figure 4). For example, the allele size shifts for MSI-H/dMMR cancers were larger for the four LMR markers (mean, 14.9 bp; range, 0 to 43 bp) compared with that of the four SMR markers (mean, 5.9 bp; range, 0 to 19 bp) in the LMR MSI panel ($P < 0.001$). Size shifts varied across cancer types, with tumors of the gastrointestinal tract exhibiting the largest average size shifts (mean, 12.2 bp), and breast (mean, 3.9 bp) and prostate (mean, 3.5 bp) cancers the smallest (Figure 5). The average size shifts generally correlated with ability to detect the dMMR tumor

Table 5 QMVR for SMR Markers in Promega Pentaplex Panel and the LMR MSI Analysis System in CCFR Samples

	SMR				Pentaplex				
Measure	*NR-21*	*BAT-25*	*BAT-26*	*MONO-27*	*NR-21*	*NR-24*	*BAT-25*	*BAT-26*	*MONO-27*
Mean allele size*	90.5	90.5	94	114.4	96.7	131.4	119.9	111.6	149.1
Standard deviation	0.76	0.81	0.64	0.47	0.91	0.55	0.82	0.60	0.43
Minimum	85.6	77.9	91.8	106.8	90.7	130	107.7	109.8	142.8
Maximum	91.8	93.1	95.6	116.8	100.2	132.9	122	112.7	151.2
No. of alleles	948	948	948	946	944	944	944	944	944
QMVR (lower)†	88	88	91.5	111.9	94.2	128.9	117.4	109.1	146.6

*Allele sizes (bp) may vary when using different polymers, instruments, or instrument configurations.

†QMVR equals the mean allele size for pooled normal samples ±2.5 bp for a given locus following the method described by Patil et al.[85] Only the lower QMVR values are shown.

CCFR, Colon Cancer Family Registry; QMVR, quasi-monomorphic variability range; SMR, short mononucleotide repeat.

phenotype, with smaller size shifts being associated with decreased sensitivity. This is due in part to the challenge of discriminating small, shifted tumor peaks from the germline stutter peaks, which can lead to misinterpretation of microsatellite patterns. The larger size shifts in LMR markers simplify analysis and increase confidence in MSI calls because novel tumor alleles are generally easily resolved from germline alleles.

Another performance advantage associated with the larger size shifts in LMR markers was the improved detection of MSI in *MSH6*-deficient tumors, for which other MSI assays have been reported to be suboptimal.[13] The average size shift for *MSH6*-deficient CRC in this study was about 9 bp with the LMR MSI panel, which is easily resolved by capillary electrophoresis. It has been shown that MSI sensitivity can vary depending on MSI marker panels and the method of analysis used.[17,18] The use of outdated microsatellite marker panels and interpretation methods likely accounts in part for the reported lower sensitivity for *MSH6*-deficient tumors. For example, the Bethesda panel contains dinucleotide repeats that exhibit lower sensitivity for detection of *MSH6*-deficient tumors.[56,59] In the current study, analysis using the LMR MSI panel of mononucleotide repeats correctly classified 97% of *MSH6*-deficient tumors as MSI-H.

Cutoffs and Number of Markers for Optimal MSI Classification

Improved sensitivity of the LMR MSI Analysis System was achieved by optimizing the cutoffs, the number of markers, and the type of markers used. The Bethesda guidelines established the cutoff for MSI-H at two or more unstable markers or 40% of the five-marker Bethesda panel.[20] Promega also recommends a cutoff of two or more unstable makers for MSI-H for both the LMR and pentaplex panels, although the percentage of unstable markers is different (Promega Technical Manual 649: LMR MSI Analysis System; and Promega Technical Manual 255; MSI Analysis System, Version 1.2.). The lower percentage cutoff of 25% for the LMR panel effectively increases sensitivity because alterations in any two of eight markers is considered MSI-H compared with two of five markers for the Promega pentaplex and Bethesda panels. ROC analysis of

Table 6 MSI Analysis with and without Matching Normal Using the QMVR Method

Cancer	Comparison	% Agreement with and without normal* (*n/N*)	% Agreement with IHC with normal† (*n/N*)	% Agreement with IHC without normal‡ (*n/N*)	*P* value§
CRC	LMR panel¶	97.6 (281/288)	97.9 (282/288)	96.9 (278/287)	0.448
	SMR‖	98.0 (288/294)	96.9 (284/293)	95.6 (282/295)	0.516
	Pentaplex	99.0 (289/292)	97.3 (287/295)	96.6 (282/292)	0.641
Non-CRC	LMR panel¶	90.9 (149/164)	92.0 (150/163)	85.7 (138/161)	0.079
	SMR‖	82.8 (140/169)	82.5 (137/166)	67.5 (112/166)	0.002
	Pentaplex	86.8 (144/166)	84.2 (138/164)	71.8 (117/163)	0.008

*Percent agreement between MSI analysis with and without matching normal sample.

†Percent agreement with IHC as reference using tumor and matching normal samples.

‡Percent agreement with IHC as reference using only tumor samples.

§*P* value for the Fisher exact test differences in number of correct calls with and without normal sample using IHC as the reference.

¶The LMR panel consists of four long (LMR) and four short (SMR) microsatellite repeats.

‖SMR markers are four SMRs markers contained in both the LMR and pentaplex panels.

CRC, colorectal cancer; IHC, immunohistochemistry; LMR, long mononucleotide repeat; MSI, microsatellite instability; QMVR, quasi-monomorphic variability rang; SMR, short mononucleotide repeat.

the LMR panel confirmed an optimal cutoff for MSI-H tumor classification at two or more unstable markers with an area under the curve value of 0.949 (Supplemental Figure S9). This cutoff was further supported by orthogonal IHC and MMR sequencing data.

The number of markers used for MSI determination is also an important consideration. To determine the optimal number of markers for the LMR MSI panel, the overall sensitivity for panels of varying sizes was calculated by using markers with various levels of sensitivity (Supplemental Figure S9). The results indicate that increasing the number of markers above the eight included in the LMR panel would not substantially improve the performance of the assay. The LMR MSI Analysis System contains eight markers compared with five for the Promega pentaplex panel and uses the same cutoff of two or more markers, instead of a percentage, effectively increasing the sensitivity of the LMR assay.

Finally, the repeat motif and variation in performance among markers with the same type of repeat motif can have a profound effect on the overall accuracy of the MSI test. The LMR panel consists of four mononucleotide markers shared with the Promega pentaplex panel, which have a proven performance record over decades of use for MSI testing, as well as four new LMRs. The inclusion of LMR markers was based on the finding that instability in microsatellites increases exponentially with increasing repeat length, and therefore longer repeats tend to be more sensitive.[74–78] This observation has been confirmed in the current study and agrees with previous studies with LMR markers.[57,77]

MSI Testing with Tumor Sample Only

The Bethesda guidelines for MSI testing recommend testing paired normal and tumor samples.[20] The reason for this requirement is that most of the markers in the original Bethesda panel are polymorphic, and to identify a new allele in the tumor sample, the germline genotype must be known. The same is also true for the LMR markers in the LMR MSI Analysis System. Matching normal samples are not always available for MSI testing, and therefore the effectiveness of testing only the tumor samples was investigated. The LMR MSI Analysis System consists of four SMR markers that are quasi-monomorphic and four LMR markers that are polymorphic. Because of this difference, two different sets of criteria were used for MSI classification, as described previously. For the LMR MSI Analysis System, the estimated percent agreement for determining tumor MSI status with and without matching normal sample was 98% for CRC and 91% for non-CRC (Table 6). The ability to correctly identify MSI status using only tumor samples was also evaluated for the Promega pentaplex panel, which contains all quasi-monomorphic SMR markers. The estimated percent agreement for determining tumor MSI status with and without matching normal sample was 99% for CRC and 87% for non-CRC. Thus, the data indicate that it is feasible to conduct MSI testing using the LMR MSI Analysis System and the Promega pentaplex panel if matching normal sample is not available with minimal loss of sensitivity for CRC. However, this approach can result in decreased sensitivity for non-CRC samples.

MSI Intensity

Analysis of MSI by NGS can report a quantitative measurement of MSI intensity and has revealed that MSI levels in dMMR tumors are a continuous scale.[68] Traditionally, MSI-PCR tests return a yes or no answer for the presence or absence of MSI. However, quantitative MSI measurement is possible with MSI-PCR. A favorable response of patients with MSI-H tumors to immune checkpoint inhibitor therapy has been attributed to the high mutation rate in dMMR tumors, which produces neoantigens recognized by the immune system as foreign and elicits a positive immune response.[40,41] Quantitative measures of MSI may be important as variation in the MSI intensity has been shown to influence response to immunotherapy in dMMR tumors. For example, Mandal et al[53] investigated genomic MSI levels from tumor exomes of pMMR and dMMR gastrointestinal tumors from patients receiving anti–programmed cell death 1 therapy. They found that clinical responders were associated with higher intensities of MSI and, conversely, patients with progressive disease had the lowest levels. In the current study, variation in MSI intensity in different cancer types was observed and reported as a semiquantitative MSI Intensity Score (Supplemental Figure S10). The cancer types with the highest MSI scores (such as colorectal, small intestine, and gastric cancers) typically respond well to immune checkpoint inhibitor therapy,[89] whereas those cancer types with lower MSI scores (such as prostate and breast cancers) are notoriously resistant to immune checkpoint blockade.[90–92] MSI Intensity Scores between patients with Lynch syndrome and sporadic MSI-H cancer were not significantly different (Supplemental Figure S10), in agreement with reports showing no difference in the immune checkpoint inhibitor therapy response rates between Lynch and sporadic MSI cancer patients.[38,93]

Differential effects of MMR gene deficiencies on MSI intensity were also observed in this study, with *MSH6* inactivation resulting in the lowest MSI Intensity Scores (Supplemental Figure S10). Despite lower intensity, the LMR MSI Analysis System still detected 97% of the *MSH6*-deficient tumors across all cancer types. Importantly, the body's immune response to a tumor, as measured by the level of TILs, was associated with the MSI-H phenotype (ie, tumors exhibiting a high level of TILs were found to have a higher MSI Intensity Score). It is important to note that MSI Intensity Scores varied widely between individuals, even in those with the same type of cancer or MMR gene deficiency (Figure 7 and

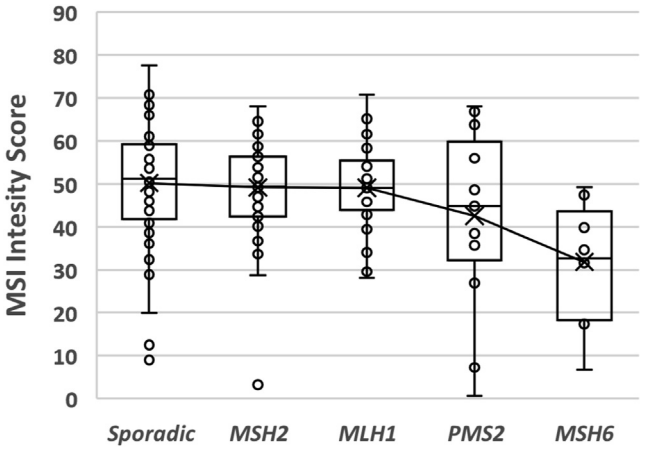

Figure 7 Effect of MMR gene deficiency on microsatellite instability (MSI) Intensity Scores for MSI-high/deficient mismatch repair (dMMR) colorectal cancer (CRC) samples. MSI Intensity Scores are plotted for 144 MSI-H/dMMR CRC samples from Lynch syndrome patients with germline mutations in one of the four MMR genes and 69 MSI-H/dMMR sporadic CRC cases with somatic *MLH1* inactivation. Each **dot** is a value that may represent multiple data points.

Supplemental Figure S10). Thus, MSI intensity as a biomarker may have utility for personalizing immunotherapy. Response to immune checkpoint inhibitors is complex and likely involves many variables. More research is needed to clarify the role of MSI intensity in predicting response to immune checkpoint inhibitor therapy.

Strengths and Limitations of This Study

Strengths of the current study include the comprehensive MSI evaluation of a large number of dMMR tumors from 20 cancer types using both the new LMR MSI Analysis System and the current gold standard MSI-PCR assay. The availability of orthogonal tests for dMMR status (ie, MMR IHC, germline MMR mutation analysis, tumor *MLH1* methylation, family history) for most samples allowed for accurate determination of the performance of the LMR test. A limitation of this study was that the samples were all obtained through the CCFR and by design were primarily derived from patients with Lynch syndrome. Therefore, there were only a small number of cases for cancer types with low prevalence in Lynch syndrome, and there was a limited number of MSS cancers. The expected low number of MSS CRCs in the CCFR Lynch syndrome cohort was compensated for by inclusion of sporadic MSS CRCs. However, similar controls were unavailable for non-CRCs, which limits the reliability of specificity values calculated for non-CRCs. Undetected carriers of MMR germline pathogenic variants may exist in the study population because germline MMR sequencing for *MSH6* and *PMS2* mutations was not available for all cases. In addition, IHC results for MSH6 and PMS2 were not available for all cases.

Conclusions

MSI as a biomarker has evolved over the years since its discovery in 1992.[3,4] Originally, MSI testing was used to identify individuals with Lynch syndrome, but the demand for MSI testing has exploded with the discovery that the MSI tumor status predicts response to certain types of immunotherapy.[13] The introduction of the Bethesda microsatellite marker panel in 1998 helped to standardize MSI testing.[20] This panel has been largely displaced by the next key advancement in MSI testing that occurred in 2004, the development of a panel of all mononucleotide repeat markers, which improved sensitivity and specificity of detecting dMMR tumors.[82] The next major improvement in MSI-PCR testing came with the introduction of "long mononucleotide repeats" in the LMR MSI Analysis System in 2021.[15,57]

In the current study, we show that the sensitivity and specificity of the LMR MSI Analysis System for detection of dMMR CRC were 99% and 96%, respectively. Importantly, the sensitivity of the LMR MSI Analysis System for detection of dMMR in non-CRCs was similar at 96%. Specificity was lower in non-CRCs than previously reported, likely due to small sample size and potential false-negative IHC results. The overall percent agreement between the LMR and pentaplex panels was high for CRC (99%) but lower for non-CRC (85%) tumors. Thus, the LMR MSI panel showed high concordance in CRC and greater sensitivity in non-CRC compared with the Promega pentaplex panel. The LMR MSI Analysis System also correctly identified 97% of challenging *MSH6*-deficient tumors from eight different cancer types, including all CRCs and all but one endometrial cancer. An increased number of unstable markers and the larger size shifts observed in dMMR tumors using the LMR panel reduce ambiguity in MSI calling and the necessity to repeat runs. Thus, the introduction of the LMR MSI Analysis System takes another leap forward in the evolution of MSI testing and expands the spectrum of cancer types in which MSI can be accurately detected.

Disclosure Statement

J.W.B., E.B.U., E.E.S., I.V., and D.R.S. are research scientists employed by Promega Corporation. J.R.E. has received grant funding from Promega Corporation for an independent validation study of the LMR MSI Analysis System. R.B.H. has received grant funding from Promega Corporation for research on detection of microsatellite instability in colon polyps and the utility of the LMR MSI Analysis System for predicting response to immunotherapy.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2023.07.003*.

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