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ORIGINAL ARTICLE

QUALITATIVE AND QUANTITATIVE ASPECTS OF DISCREPANCIES BETWEEN VARIOUS METHODS FOR MICROSATELLITE INSTABILITY DETECTION

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ABSTRACT

The discordance rate of the results between immunohistochemistry (IHC) and molecular microsatellite instability (MSI-PCR) tests, the most commonly used methods for qualitative deficient mismatch repair (dMMR) testing, is 1-10%, highlighting the need for a more precise testing strategy. The next-generation sequencing (NGS) offers a more sensitive and effective dMMR analysis (MSI-NGS), which also provides quantitative data. The aim of the study was to evaluate the qualitative and quantitative aspects of IHC and MSI-PCR testing compared to MSI-NGS in detecting dMMR in patients with Lynch Syndrome (LS)-associated and sporadic colorectal (CRC) and endometrial cancers (EC). Our results demonstrate both qualitative and quantitative discrepancies in the results obtained with different methods. Regarding qualitative differences, dMMR was inadequately interpreted only in LS when relying solely on IHC or MSI-PCR testing. This was primarily due to the specific mutational profile in our population, indicating the need for the implementation of a specific strategy that combines both methods. Concerning the quantitative differences, we detected great variability in the MSI levels, which was partly attributed to the tissue type or to the type of mutation in LS patients. Our results suggest that MSI-NGS level could be used as a potential surrogate marker for neoantigen levels and provide more precise predictive information for immunotherapy in patients with dMMR deficiency.

KEY POINTS

- There are both qualitative and quantitative discrepancies in the results for dMMR testing using IHC or molecular MSI, determined both by PCR or NGS, in tumors of patients with Lynch Syndrome-associated and sporadic colorectal and endometrial cancers.
- dMMR was inadequately interpreted in 26.9% and 7.7% of our cohort of Lynch Syndrome cases when relying solely on IHC or molecular MSI-PCR testing, respectively, primarily due to specific mutational profiles.
- A great variability in the MSI levels was observed, which was partly attributed to the tissue type in sporadic cancers and/or to the type of mutation in Lynch Syndrome patients.
- The specific strategy that combines IHC and molecular MSI-PCR is needed for initial dMMR assessment with MSI-NGS emerging as an alternative that provides additional quantitative data potentially useful as a surrogate predictive marker for immunotherapy response.

INTRODUCTION

DNA mismatch repair (MMR) deficiency arises from defects in the MMR machinery, impairing cells' ability to correct replication errors within repetitive DNA sequences,

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known as microsatellites. This deficiency manifests in expansion or contraction of mono or dinucleotide repeated sequences and is defined as microsatellite instability (MSI). MSI is observed in nearly 3-5% of newly diagnosed cancer cases, with particularly high prevalence in endometrial (EC) (17-39%) and colorectal cancers (CRC) (15-17%)[1,2]. Deficient MMR tumors (dMMR) are predominantly present in sporadic cases and a small proportion of cases with a familial history of cancer. The dMMR phenotype in these tumors has a different molecular pattern of development, where sporadic tumors primarily develop due to epigenetic silencing of the *MLH1* gene promotor, while hereditary tumors due to a pathogenic germline variant in one of the four MMR genes (*MLH1, MSH2, MSH6 or PMS2*), a condition known as the Lynch Syndrome (LS) [3]. MMR deficiency has been used as a predictive marker for many years, initially for treatment with 5-fluorouracil (5-FU) in CRC patients. Since dMMR tumors are characterized by a high mutation load, a presence of activated tumor-infiltrating lymphocytes, and a production of numerous neoantigens that trigger a strong immune response, it has recently been used as an agnostic predictive biomarker for response to treatment of various types of tumors with immune checkpoint inhibitors (ICI) [4,5].

In clinical practice, MMR deficiency testing is typically performed using two common approaches: immunohistochemistry (IHC) and molecular MSI-polymerase chain reaction (PCR) testing. The IHC analysis is based on the qualitative detection of the loss of expression of at least one of the MMR proteins, which can be complex (MLH1/PMS2 or MSH2/MSH6 when there is a mutation in the *MLH1* or *MSH2* genes, respectively) or isolated (MSH6 or PMS2 when there is a mutation in either of these two genes). The alternative MSI-PCR testing allows the detection of MSI by evaluating at least five microsatellite loci using one of the two common testing panels: the Bethesda panel (consisting of two mono and three dinucleotide markers) or the Pentaplex assay (consisting of five quasi-monomorphic mononucleotide markers) [6]. It has been shown that the IHC testing has a lower sensitivity compared to MSI-PCR (IHC sensitivity: ~92-100%; specificity: ~88-100% and MSI-PCR sensitivity 95-98%; specificity 98-100% if using 5 mononucleotide markers), but this technique is still preferred by many laboratories due to its simplicity and availability [7]. The estimated discordance rate of the results between IHC and MSI-PCR is 1-10% [8-14]. Consequently, nearly 10% of the patients selected for immunotherapy may experience treatment failure due to incorrect testing, highlighting the need for a more precise testing strategy.

The advent of next-generation sequencing (NGS) is yielding promising results as a more sensitive and effective method for dMMR testing (molecular MSI-NGS), which also allows for simultaneous detection of the LS [15]. This approach assesses MSI by detecting microsatellite instability across a large set of microsatellite loci (hundreds or thousands) through a comparison of the microsatellite length distributions between tumor and normal/MSI-negative samples using various computational algorithms. Although this approach has not yet been implemented in clinical practice due to its high cost and long turnaround time, it holds promise to replace current methods in the near future [16,17].

The primary objective of this study was to assess the efficacy of IHC and molecular MSI-PCR testing in detecting mismatch repair deficiency compared to next-generation sequencing (MSI-NGS) in patients with Lynch Syndrome-associated and sporadic colorectal and endometrial cancers. Additionally, this study aimed to address discrepancies in the results obtained by various methods, with the goal of providing a more accurate and streamlined approach for detecting MMR deficiency in patients with different clinical characteristics who are eligible for ICI treatment.

MATERIALS AND METHODS

Sample Collection and Selection

MMR deficiency was assessed in a total of 44 patients, of which 26 patients with LS and 18 patients with sporadic dMMR CRC or EC. All patients were preselected during routine testing, and their MSI status was evaluated using both IHC and molecular MSI-PCR testing. Subsequently, MSI-NGS was applied to all patients with discordant results and to half of the remaining cases.

A total of 44 formalin-fixed paraffin-embedded (FFPE) specimens with a minimum of 50% tumor cells, along with 3 mL of peripheral blood, were collected between 2016 and 2022 in collaboration with the University Clinic of Radiotherapy and Oncology and the Institute of Pathology, Faculty of Medicine, and the Clinical Hospital Acibadem Sistina in Skopje, North Macedonia. The inclusion criteria for hereditary tumors included positive family history and molecularly confirmed LS, with 23 out of 26 LS patients having a known or novel germline pathogenic variant, while three patients had a rare variant of uncertain significance (VUS) in one of the MMR genes, as previously reported [18]. Sporadic cases were selected based on the absence of a positive family history and the presence of the *MLH1* promoter methylation status.

DNA Extraction

DNA was extracted from all samples using the MagCore HF16 Plus automated nucleic acid extractor (RBC Bioscience, New Taipei City,Taiwan) with the MagCore Genomic DNA FFPE Kit for the FFPE samples and MagCore Genomic DNA Whole Blood Kit for peripheral blood samples, following the manufacturer's protocol instructions.

Immunohistochemical analysis

Immunohistochemical staining for the expression of the four MMR proteins (MLH1, MSH2, MSH6, and PMS2) were done using 3-5 μm-thick sections from FFPE blocks obtained from patient tumor specimens and mounted on poly L-lysine coated slides. Heat deparaffinization was conducted at 58-60ºC, followed by PT-Link (Agilent, Santa Clara, CA, USA) pretreatment. Corresponding primary antibodies from DAKO (Agilent, Santa Clara, CA, USA) were used in dilutions of 1:50 for MLH1 (Clone ES05), MSH2 (Clone FE11), and MSH6 (Clone EP49), and 1:40 for PMS2 (Clone EP51). The EnVision FLEX (Agilent, Santa Clara, CA, USA) visualization system was employed using a Labophot-2 EFD3-Fluorescence light microscope (Nikon, Minato City, Tokyo, Japan).

Molecular MSI-PCR Analysis

Pared tumor and germline DNA were analyzed for MSI using multiplex fluorescent PCR with the Bethesda panel, as defined by the National Cancer Institute, supplemented with four additional mononucleotide markers: BAT40, NR21, NR24, and MONO-27, as recommended [19-21]. PCR products were detected and analyzed by capillary electrophoresis on an Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific, Boston, MA, USA). Samples were classified as MSI-H (MSI-High) if instability was observed at more than 30% of the loci screened, MSI-L (MSI-Low) if at least one but fewer than 30% of the loci showed instability, or MSS (microsatellite stable) if all loci were stable.

Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MLPA)

The detection of aberrant CpG island methylation in the MMR genes was performed using the SALSA MS-MLPA kit ME011 according to the manufacturer's instructions (MRC Holland, Amsterdam, The Netherlands). The obtained amplicons were separated by capillary electrophoresis on Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific, Boston, MA, USA) and the methylation ratio was analyzed using Coffalyser software (MRC Holland, Amsterdam, The Netherlands).

MLH1 promoter methylation analysis by PCR

The presence of the MLH1 promoter methylation was also analyzed by PCR method using 1μg of FFPE DNA converted by bisulfite modification using EZ DNA Methylation-Lightning Kit (Zymo Research, Irvine, CA, USA), following the procedure recommended by the manufacturer. A total of 50-100 ng of converted DNA was subjected to qPCR analysis using primers and fluorescent probes specific for methylated DNA in the MLH1 promoter on Stratagene Mx3005P real time PCR system (Agilent, Santa Clara, CA, USA) [22]. For efficacy of the bisulfite modification, a control reaction was run with primers and a probe located in the *ACTB* gene that does not contain CpG islands and is not subjected to methylation.

Molecular MSI-NGS analysis

Molecular MSI-NGS was performed on the NovaSeq 6000 platform (Illumina, San Diego, CA, USA) using the Twist Human Core Exome + RefSeq + Mitochondrial Panel (Twist Bioscience, San Francisco, CA, USA) for library preparation, covering more than 99% of protein-coding genes (Whole Exome Sequencing, WES). Each reaction used 50ng of tumor DNA. Following initial alignment against the genome reference sequence hg19, MSI evaluation was conducted using the FDA-approved computational algorithm MSIsensor [23], which employs a binary MSI/MSS classifier for MSI detection (sensitivity 97-100%; specificity 97-100% when applied to data with >100-150x average coverage and with >200 reads per microsatellite site). The MSI levels were calculated as the ratio of unstable sites among ~2000 sites with a read-depth of at least 200 reads/site and a cut-off value of 20% for MSI classification for each site. MSI calling for each sample was based on a threshold of 3.5% unstable loci for MSI.

All analyses were performed at the Center for Biomolecular Pharmaceutical Analyses at the UKIM-Faculty of Pharmacy in Skopje, with the exception of the immunohistochemical analyses which were performed at the Institute of Pathology, Faculty of Medicine and the Clinical Hospital Acibadem - Sistina in Skopje, and the NGS exome sequencing analyses which were performed at the Research Center for Genetic Engineering and Biotechnology at the Macedonian Academy of Sciences and Arts in Skopje.

RESULTS

Classical MMR deficiency, characterized by MSI-H and loss of MMR proteins (either complex or isolated), was detected by both MSI-PCR and IHC in 34 of 44 (77.3%) patients analyzed. Discordant results between MSI-PCR and IHC were defined as unusual MMR deficiency. This trait was present in 9 of 44 (20.5%) patients, all with Lynch Syndrome. Additionally, one patient with clinically diagnosed LS and a pathogenic mutation in the *MSH6* gene had MSS status and normal expression of all four MMR proteins. All results are summarized in Table 1.

All patients with sporadic colorectal/endometrial cancer enrolled in the study (N=18) manifested the classical dMMR phenotype, showing MSI-H and MLH1/PMS2 complex loss. Of the patients with Lynch Syndrome, 16

Table 1. Clinical and molecular data of the patients included in the study

Cancer type	MMR deficiency type	MSI/IHC status	ID number	Sex	Age	Tumor localization*	Affected gene	DNA (protein) change**	Molecular defect	MLH1 methylation	MSI-PCR	IHC				MSI-NGS
												MLH1	MSH2	MSH6	PMS2	
Hereditary	Classical MMR deficiency (n=16)	MSI+ IHC+	H1	F	43	proximal CRC	MLH1	c.896_897insC (p.Pro300SerfsTer7)	deletion out-of-frame	-	+	X			X	
			H2	F	60	proximal CRC	MLH1	c.392C>G (p.Ser131Ter)	nonsense mutation	-	+	X			X	
			H3	F	57	proximal CRC	MLH1	c.683T>C (p.Leu228Pro)	missense mutation	-	+	X			X	49,3
			H4	F	41	proximal CRC	MLH1	c.1667+1del (p.?)	splice site mutation	-	+	X			X	74
			H5	M	15	proximal CRC	MSH2	c.2211-2A>C (p.?)	splice site mutation	-	+		X	X		
			H6	M	50	proximal CRC	MSH2	c.2211-2A>C (p.?)	splice site mutation	-	+		X	X		
			H7	F	63	proximal CRC	MSH2	c.1786_1788delAAT (p.Asn596del)	deletion in-frame	-	+		X	X		
			H8	M	46	distal CRC	MSH2	c.1786_1788delAAT (p.Asn596del)	deletion in-frame	-	+		X	X		
			H9	M	41	distal CRC	MSH2	c.209_211+11del (p.?)	deletion in-frame	-	+		X	X		
			H10	F	27	proximal CRC	MSH2	c.1012 G>A (p.Gly338Arg)	missense mutation	-	+		X	X		56,5
			H11	F	52	distal CRC	MSH6	c.3991C>T, (p.Arg1331Ter)	nonsense mutation	-	+			X		50
			H12	F	48	endometrial	MSH6	:c.3172G>C (p.Asp1058His)	missense mutation	-	+			X		36
			H13	M	40	distal CRC	MSH6	c.3263delT (p.Phe1088SerfsTer2)	deletion out-of-frame	-	+			X		42,9
			H14	M	41	proximal CRC	PMS2	g.(5984924_5987848)_ (6015520_?)del (p.?)	large deletion	-	+				X	
			H15	M	68	distal CRC	PMS2	c.2192_2196delTAACT (p.Leu731CysfsTer3)	deletion out-of-frame	-	+				X	73,5
			H16	M	65	proximal CRC	PMS2	c.1321_1322delA (p.Arg443GlufsTer5)	deletion out-of-frame	+	+	X			X	
	Unusual MMR deficiency (n=9)	TypeI MSI+IHC-	H17	M	55	proximal CRC	MLH1	c.244A>G (p.Thr82Ala)	missense mutation	-	+					54
			H18	M	51	proximal CRC	MLH1	c.244A>G (p.Thr82Ala)	missense mutation	-	+					53,8
			H19	M	38	proximal CRC	MLH1	c.62C>T (p.Ala21Val)	missense mutation	-	+					43
			H20	F	37	distal CRC	MSH6	c.2927G>C (p.Arg976Pro)	missense mutation	-	+					40
		TypeII MSI-IHC+	H21	F	52	endometrial	MSH6	c.900_901insTC (p.Lys301SerfsTer5)	insertion out of frame	+	-	/ X	/ X	X	/ X	12
			H22	F	41	endometrial	MSH6	c.3514dupA (p.Arg1172LysfsTer5)	deletion out-of-frame	-	-			X		4,3
			H23	F	45	distal CRC	PMS2	c.2437C>T (p.Arg813Trp)	missense mutation -VUS	-	-				X	1,7
			H24	F	44	distal CRC	MSH6	c.2384T>C (p.Ile795Thr)	missense mutation -VUS	-	-			X		1,16
			H25	M	51	proximal CRC	MSH6	c.1151_1156dupGGAGGC (p.Arg384_385dup)	Insertion in-frame - VUS	-	-			X		2
	pMMR (n=1)	MSI-IHC-	H26	F	44	distal CRC	MSH6	c.457+1G>T (p?)	splice site mutation	-	-					3,12
Sporadic	Classical MMR deficiency (n=18)	MSI+ IHC+	S1	M	54	proximal CRC	MLH1	NA	promoter methylation	+	+	X			X	79,5
			S2	M	39	distal CRC	MLH1	NA	promoter methylation	+	+	X			X	73,7
			S3	M	72	distal CRC	MLH1	NA	promoter methylation	+	+	X			X	
			S4	F	57	endometrial	MLH1	NA	promoter methylation	+	+	X			X	56,5
			S5	F	57	distal CRC	MLH1	NA	promoter methylation	+	+	X			X	47,9
			S6	M	73	distal CRC	MLH1	NA	promoter methylation	+	+	X			X	76,1
			S7	F	75	endometrial	MLH1	NA	promoter methylation	+	+	X			X	
			S8	F	73	endometrial	MLH1	NA	promoter methylation	+	+	X			X	19,9
			S9	F	53	endometrial	MLH1	NA	promoter methylation	+	+	X			X	19,2
			S10	M	57	distal CRC	MLH1	NA	promoter methylation	+	+	X			X	9,1
			S11	M	68	distal CRC	MLH1	NA	promoter methylation	+	+	X			X	4,4
			S12	F	60	distal CRC	MLH1	NA	promoter methylation	+	+	X			X	41,1
			S13	F	58	proximal CRC	MLH1	NA	promoter methylation	+	+	X			X	77,9
			S14	M	67	proximal CRC	MLH1	NA	promoter methylation	+	+	X			X	76,7
			S15	M	72	proximal CRC	MLH1	NA	promoter methylation	+	+	X			X	48,9
			S16	M	77	proximal CRC	MLH1	NA	promoter methylation	+	+	X			X	64,3
			S17	F	68	proximal CRC	MLH1	NA	promoter methylation	+	+	X			X	52,8
			S18	F	78	distal CRC	MLH1	NA	promoter methylation	+	+	X			X	

* Proximal CRC = cancer in the caecum, colon ascendens, colon transversum and flexura lienalis; Distal CRC= cancer in colon descendens, colon sygmoideum and rectum
** Referent sequences: MLH1= NM_000249.3; MSH2= NM_000251.2; MSH6= NM_000179.2; PMS2= NM_000535.6.

out of 26 (61.5%) patients showed classical MMR deficiency. Among these, 31.3% (5/16) had MSI-H and MLH1/PMS2 complex loss due to a germline pathogenic variant in the *MLH1* gene (4 patients) or a pathogenic variant in the *PMS2* gene accompanied by promoter methylation of the *MLH1* gene (1 patient); 37.5% (6/16) had MSI-H and MSH2/MSH6 complex loss, all of whom were carriers of a pathogenic germline variant in the *MSH2* gene; 18.7% (3/16) had MSI-H and isolated MSH6 loss; and 12.5% (2/16) had MSI-H with isolated PMS2 loss. (Table 1)

Unusual MMR deficiency was detected only in 9 patients with the LS, which were further divided into two groups: type 1 (4 patients with MSI-H tumors with intact MMR protein expression) and type 2 (5 patients with MSS tumors and isolated loss of PMS2 or MSH6 proteins). Unusual MMR deficiency type 1 patients had a missense pathogenic variant in one of the MMR genes and tumors predominantly located in the proximal colon. The detected variants were located in the *MLH1* and *MSH6* genes in three and one patient, respectively. Type 2 unusual MMR deficiency was present in two patients with endometrial cancer who had a pathogenic variant in the *MSH6* gene, and 3 patients with a VUS in the *MSH6* or *PMS2* genes. Patients with VUS had CRC in the distal colon and isolated loss of the affected protein. One of the patients with type 2 deficiency and *MSH6* gene mutation had additional partial loss of the remaining three proteins due to sporadic hypermethylation in the tumor (Table 1).

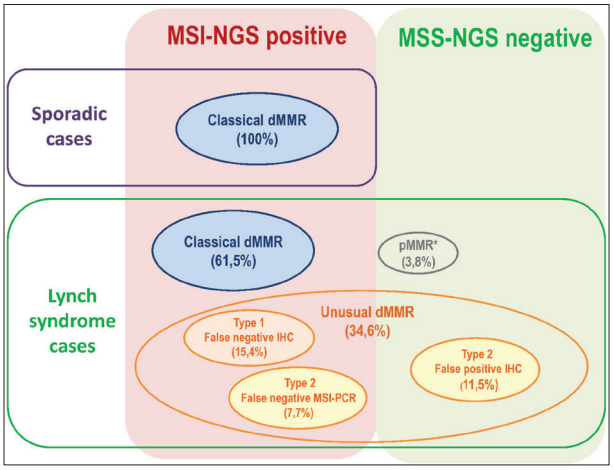

Figure 1. Distribution of MSI patients by various methods for MSI detection. Classical MMR deficiency = detected by both MSI-PCR and IHC (MSI-H and loss of MMR proteins). Unusual MMR deficiency = detected by one method, MSI-PCR or IHC: type 1 (MSI-H with intact MMR protein expression) and type 2 (MSS and isolated loss of PMS2 or MSH6). *Clinically and molecularly diagnosed Lynch syndrome (pathogenic mutation in the MSH6) with MSS status, normal expression of all four MMR proteins and negative MSI-NGS result. MSI, microsatellite instability, MSS, microsatellite stability, NGS, next-generation sequencing, dMMR, deficient mismatch repair, pMMR, proficient mismatch repair, IHC, immunohistochemistry, PCR, polymerase chain reaction

The results from the NGS-MSI analysis confirmed the MMR deficiency in all patients with classical MMR deficiency and in 6 out of 9 (66.7%) patients with unusual MMR deficiency. All three patients harboring VUS variants had negative NGS-MSI results since their MSI levels were below the 3.5 threshold. (Figure 1) The MSI levels were variable in the group of patients with sporadic CRC, with lower MSI levels (4-20%) detected in 4 patients and higher MSI levels (42-80%) in 11 patients (Figure 2). The patients with unusual MMR deficiency type 1 showed a higher level of MSI (>40%) compared to the patients with unusual MMR deficiency type 2 (12% and 4.3% of

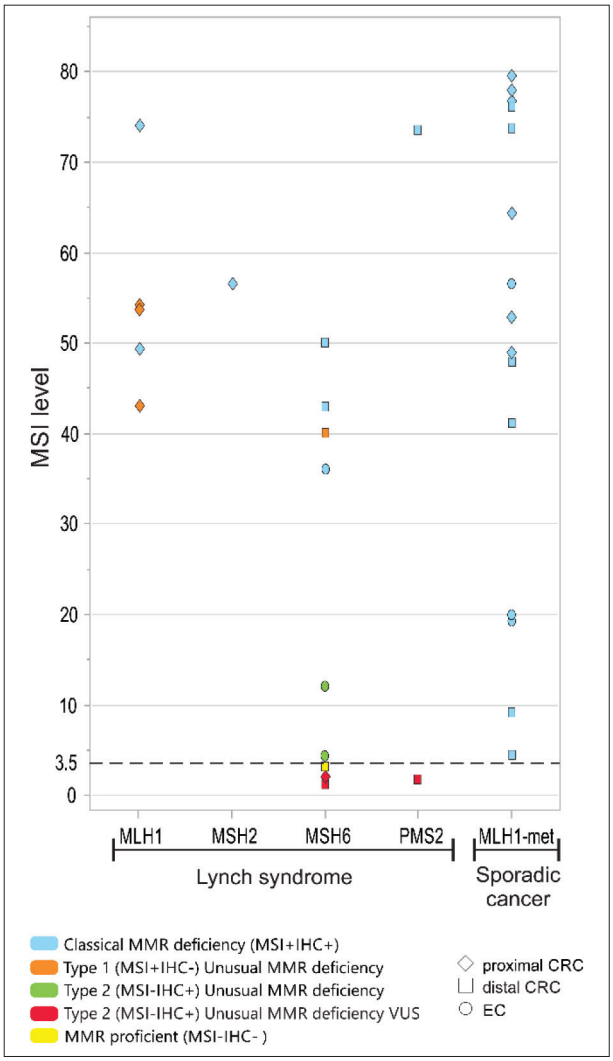

Figure 2. Quantitative MSI-NGS levels of patients with sporadic and Lynch Syndrome-associated CRC or endometrial cancers in relation to different types of MMR deficiencies, affected genes, and localization.
The threshold level for NGS-MSI positivity is set at 3.5%.
MSI, microsatellite instability, MMR, mismatch repair, IHC, immunohistochemistry, VUS, variant of uncertain significance, CRC, colorectal cancer, EC, endometrial cancer

unstable loci). Additionally, a borderline negative MSI with 3.12% of unstable loci was detected in the patient with clinically and molecularly diagnosed LS who had negative IHC and MSI-PCR results. This patient had a pathogenic splice site variant in the *MSH6* gene, resulting in exon 3 skipping and production of an in-frame aberrant transcript that lacks exon 3 (unpublished data).

Based on the MSI-NGS, as the most accurate method for detecting true positives, the sensitivity of IHC and molecular MSI-PCR tests in our series was 90% and 95%, respectively. Regarding true negatives, the IHC method was inferior, identifying only 1 of the 4 negative patients, whereas the MSI-PCR method accurately detected all pMMR patients. Overall, the observed concordance was 92.3% for IHC and 95% for MSI-PCR. The largest discrepancy between these two methods was observed in patients with missense pathogenic variants in the N-terminal part of *MLH1* gene and in patients with variants in the *MSH6* or *PMS2* genes.

DISCUSSION

Our results demonstrate that there are both qualitative and quantitative discrepancies in the results obtained with different methods for MSI testing in patients with CRC and EC. Concerning the qualitative differences, our data indicate that mismatch repair deficiency can be overlooked or inadequately interpreted in a substantial proportion of Lynch Syndrome cases i.e. 26.9% and 7.7% when relying solely on IHC or molecular MSI-PCR testing, respectively. Regarding IHC, MMR deficiency was not correctly detected in 15.4% and 11.5% of Lynch Syndrome patients due to false negative or false positive results, respectively (Figure 1). False negative IHC results were observed in four patients harboring pathogenic missense variants in the *MLH1* and *MSH6* genes. It has been previously demonstrated that pathogenic missense variants may result in the production of a stable protein with impaired function that can still be detected by IHC [24]. The *MLH1* variants found in three LS patients with false negative results were located in the N-terminal domain of the MLH1 protein, suggesting that the location of these variants may not be part of the epitope where the antibody for MLH1 protein detection binds. This suggests that in patients having clinically suspected LS and normal MMR protein expression, molecular MSI testing should be performed. In contrast, false positive IHC results, seen as an isolated loss of the PMS2 and MSH6 proteins in three patients, were observed in carriers of rare variants of uncertain significance. These variants were classified as VUS due to their low frequency and lack of evidence of their functional activity, according to classification guidelines (ACMG, InSight, and ClinVar). We hypothesize that these variants are likely rare polymorphisms that do not affect MMR activity but may potentially alter the protein conformation and disrupt the antibody binding site, resulting in false protein loss. Therefore, in patients with isolated loss of MMR proteins, the MSI phenotype should also be confirmed with subsequent MSI-PCR testing [25].

False positive results were not obtained using the MSI-PCR method. However, this method failed to detect MMR deficiency in two LS patients, both with endometrial cancer and a pathogenic variant in the *MSH6* gene (Figure 1). Previously published data suggested that patients with germline *MSH6* mutations develop tumors that display lower levels of MSI which could be missed if only MSI-PCR is used [26,27]. The MSH6 deficient tumors tend to show only mononucleotide MSI pattern, due to compensation in repair of the larger insertion-deletion loops (dinucleotide/tetranucleotide) with *MSH3* [28]. However, the panel used for MSI detection in this study employs 6 mononucleotide markers (out of ten), thus the partial redundancy of the function of the MSH6 and MSH3 proteins could not completely explain the false negative results seen for these patients. It is worth noting that both samples are of endometrial origin and had lower MSI-NGS levels (12% and 4.7%) compared to colorectal cancer samples obtained from other LS patients. Similar results were observed in the group of patients with sporadic cancer, where lower MSI levels (4-20%) were detected in patients with EC compared to patients with CRC (40-80%) (Figure 2). Given that endometrial tumors show subtler and less frequent microsatellite shifts and exhibit lower detectable MSI levels, especially if isolated MSH6 loss is involved, we suggest that tailored detection methods need to be validated for these cases.

Based on the discrepancies observed in this study, we propose a cost-effective strategy of combining IHC and MSI-PCR methods to provide more precise qualitative detection of MMR deficiency (Figure 3). The proposed strategy suggests that IHC could be used as the initial method for MMR deficiency detection due to its simplicity, high-speed, and accessibility. However, the analysis should include subsequent MSI-PCR testing in patients with ambiguous IHC results, i.e. isolated loss of MMR proteins or negative IHC results in patients with clear LS clinical findings. This strategy is in line with previously published data indicating that IHC is more sensitive than MSI-PCR in detecting MMR deficiency induced by pathogenic *MSH6* variants in Lynch Syndrome patients with endometrial cancer, while MSI-PCR is more sensitive or equivalent to MSI-NGS in cases of MMR deficiency induced by pathogenic missense variants in the other three MMR genes [29-32]. This suggestion further refines the current guidelines from the National Comprehensive Cancer Network (NCCN) which recommends that PCR-based

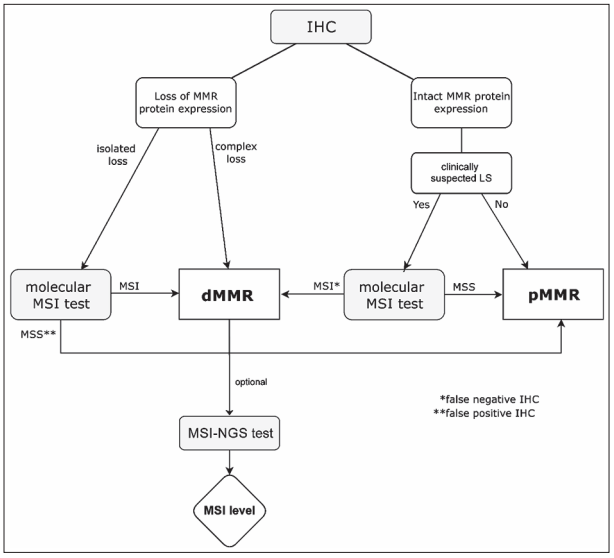

Figure 3. Proposed strategy for more accurate cost-effective evaluation of MMR deficiency and potential subsequent NGS-MSI testing for quantitative assessment.
IHC, immunohistochemistry, LS, Lynch syndrome, dMMR, deficient mismatch repair, pMMR, proficient mismatch repair, MSI, microsatellite instability, MSS, microsatellite stability, NGS, next-generation sequencing

confirmation of the dMMR result on IHC is obligatory, and the American Society of Clinical Oncology (ASCO) which suggests that MSI-PCR or MSI-NGS should be considered only if the results from IHC are doubtful [17,33,34].

Concerning the quantitative differences, our results indicate that there is a great variability in the levels of MSI, both in patients with LS and in patients with sporadic cancer. This variability is primarily due to the tissue type (CRC or EC) or due to the type of mutation in LS patients, although variability within each subgroup was also observed (Figure 2). Since most patients presented in this study were diagnosed with early-stage disease and hence were not treated with ICI therapy, we cannot comment on whether the quantification of the MSI level is clinically meaningful and can be used as a predictive marker for response. Although high response rates (30% to over 50%, depending on the treatment regimen) have been observed in MSI-H patients, nearly 30% of patients with MSI-H CRC exhibit primary resistance to ICIs, and some develop resistance during the course of the disease [35-37]. According to current data, the variability in immunotherapy response across dMMR tumors can be attributed to several underlying molecular mechanisms, such as variability in the tumor mutational burden and neoantigen load, antigen presentation and processing, immune checkpoint expression, tumor heterogeneity and evolution, and the tumor microenvironment [37,38]. This study provides clinically relevant insights by comprehensively evaluating discrepancies between IHC, MSI-PCR and MSI-NGS methods, underscoring the need for integrated testing strategy including MSI quantification to improve diagnostic accuracy and optimize immunotherapy selection in dMMR EC/CRC cancers. However, more comprehensive studies addressing this issue are needed, which should also determine the exact quantitative MSI threshold for the definition of the dMMR phenotype due to the variability of the machine learning pipelines [39]. Alternatively, the development of cellular MSI-NGS testing should be considered, which could also address the intertumoral heterogeneity of this phenotype, further refining the predictive value of this marker [40].

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DECLARATION OF INTEREST

The authors report no conflicts of interest.

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