

Article

Evaluation of Predictive Markers for Immunotherapy in Colorectal Cancer: Concordance Between MMR Protein Expression and Microsatellite Instability in a Retrospective Series

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Abstract

Background/Objectives: In metastatic colorectal cancer (mCRC) the evaluation of mismatch repair (MMR) and microsatellite instability (MSI) status is essential to identify patients eligible for treatment with immune-checkpoint inhibitors (ICI). This study aims to evaluate the potential utility of Comprehensive Genomic Profiling (CGP) in assessing MSI status, in addition to other immunotherapy-predictive biomarkers such as high tumor molecular burden (TMB) and the *POLE* and *POLD1* mutations. **Methods:** A total of 138 mCRC tumor samples underwent a first-level molecular test (MMR status by immunohistochemistry, MSI by a melting-based PCR approach and *RAS/BRAF* mutational status by a small next-generation sequencing (NGS) panel) and second-level CGP analysis by the FoundationOne CDx assay. The prevalence of dMMR and MSI tumors was reported. Moreover, the concordance between the MMR and MSI status was determined, and discordant cases were discussed. **Results:** Twelve cases (8.7%) were MMR-deficient (dMMR); 10 showed high MSI and TMB (>10 mut/Mb). MSI status assessed by CGP and PCR was concordant in all cases except one MSH6-deficient tumor. Two dMMR cases were stable with low TMB. Moreover, in two MLH1/PMS2-deficient cases CGP revealed pathogenic alterations in the *MSH2* and *MSH6* genes; in both cases, the *MLH1* promoter was hypermethylated. A high TMB was the only positive biomarker in 11 cases with a proficient MMR system and no MSI. **Conclusions:** MSI assessment by CGP analysis showed high concordance (98%) with MMR and was helpful in evaluating ICI eligibility in three out of twelve dMMR cases. Overall, compared to standard methods, analyzing a broader range of microsatellite loci and the simultaneous assessment of multiple predictive biomarkers by CGP may increase diagnostic accuracy and improve therapeutic assessment.

Keywords: colorectal cancer; microsatellite instability; comprehensive genomic profiling

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1. Introduction

Colorectal cancer (CRC) is the second-leading cause of cancer death worldwide, accounting for approximately 1.1 million new cases each year [1,2].

Approximately 20% of patients present with metastatic disease at diagnosis, while 50% of patients with initially localized disease will develop metastases later [3].

Despite the growing incidence in recent years, which is partly linked to the increasing frequency of CRC in younger patients (early onset CRCs [EOCRC]) [4], the prognosis has significantly improved, thanks to more effective therapeutic approaches, including more aggressive surgery for metastases, chemotherapy combinations, new targeted therapies and immunotherapy [5,6].

Microsatellite instability (MSI) is reported in about 15–20% of all CRCs and 3–5% of metastatic CRC and is due to defects in the DNA mismatch repair (MMR) system that lead to abnormal accumulations of genetic mutations in vulnerable coding and non-coding nucleotide repeat sequences (microsatellites). The MMR system consists of four main proteins: MLH1 (MutL Homolog 1), MSH2 (MutS Homolog 2), MSH6 (MutS Homolog 6) and PMS2 (Postmeiotic Segregation Increased 2 Protein). These proteins identify and correct base-pairing errors caused by polymerase during DNA replication, which are more frequent in repetitive sequences such as microsatellites. The major MMR proteins function in pairs as heterodimers: MLH1 with PMS2, and MSH2 with MSH6, forming the MutL α and MutS α complexes, respectively. MLH1 and MSH2 are obligatory partners for PMS2 and MSH6; therefore, when a mutation occurs in the *MLH1* and *MSH2* genes, the mutated protein and its secondary partner (PMS2 or MSH6) are rapidly degraded. On the other hand, MLH1 and MSH2 can also interact with other proteins involved in the DNA repair system, such as MSH3 (mutS homologue 3), MLH3 (mutL homologue 3) and PMS1 (ostmeiotic segregation increased 1 protein) (Figure 1) [7].

Most tumors with a high microsatellite instability (MSI-H) phenotype are sporadic (75%), and the MMR system deficiency (dMMR) is usually caused by somatic alterations in genes encoding for MMR proteins or by the hypermethylation of the *MLH1* gene promoter. A smaller percentage of cases (25%) is due to germline mutations in MMR genes in the context of Lynch syndrome (LS), a condition that increases the risk of developing CRC and other gastrointestinal and non-gastrointestinal tumors [8,9].

In routine clinical practice, MSI and MMR status profiling has, historically, primarily been performed for the identification of LS [10]. More recently, however, the assessment of these molecular features has become an integral component of therapeutic decision-making in patients with CRC. In this context, current ESMO guidelines recommend that clinically relevant biomarkers predictive of response or resistance to targeted therapies, such as *KRAS*, *NRAS* and *BRAF* mutations and *ERBB2* amplification, be evaluated as early as possible in order to optimize treatment selection and improve outcomes, particularly in patients with metastatic disease [3]. Accordingly, determination of dMMR and MSI status at the time of diagnosis is considered essential, given the established predictive value for response to immune checkpoint inhibitors (ICIs).

Moreover, in patients with resectable CRC, the risk of recurrence is determined by integrating the clinicopathological features and the MMR/MSI status. The latter is currently the only validated molecular marker for adjuvant decision-making in stage II CRC. As reported by Ribic et al. in 2002 [11] and confirmed by Sargent et al. in 2010 [12], patients with stage II dMMR CRC have a better prognosis, compared with those with MMR proficient (pMMR) CRC, and do not benefit from adjuvant 5-Fluorouracil (5-FU). Indeed, ESMO (European Society for Medical Oncology) guidelines do not recommend adjuvant chemotherapy in dMMR stage II CRC [3,13–15].

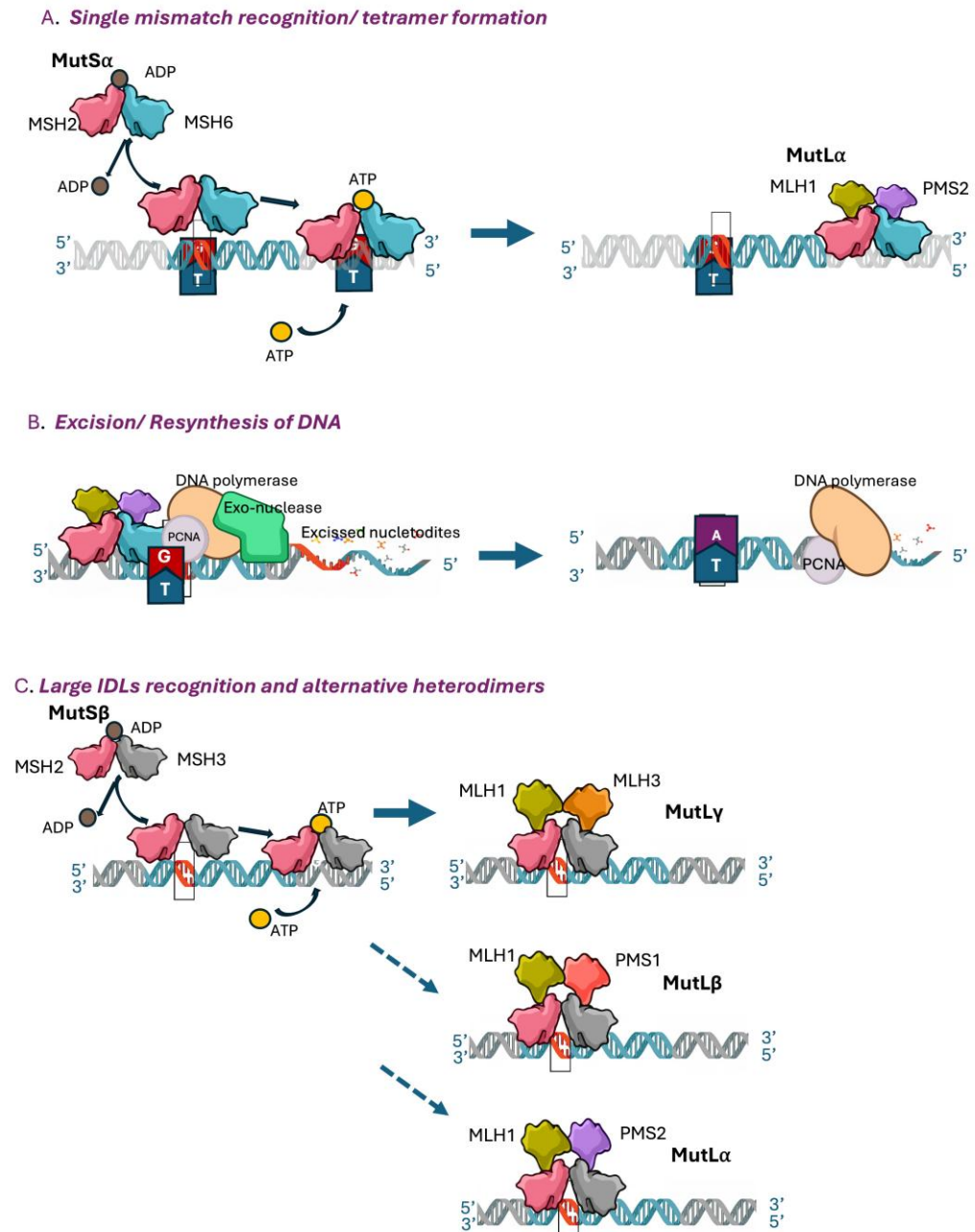


Figure 1. MMR system: (A). The MutS α complex recognizes a single mismatch and creates a sliding clamp around the DNA by exchanging ATP for ADP, which causes the complex to shift away from the mismatch site. At this point, MutL α binds to MutS α , forming the complete tetramer. (B). The tetramer then interacts with different enzymes, such as PCNA, exonuclease, and DNA polymerase, and excision of the strands containing the incorrect nucleotide begins. Once the error is corrected, DNA resynthesis occurs through the action of DNA polymerase. (C). While MSH2-MSH6 primarily recognizes base–base mismatches and small insertion–deletion loops (IDLs), the MSH2-MSH3 heterodimer (MutS β) expands its recognition to larger IDLs. Additionally, MLH1 forms alternative heterodimers with PMS2, PMS1, or MLH3. ADP = adenosine diphosphate; ATP = adenosine triphosphate; PCNA = proliferating cell nuclear antigen; MSH2 = MutS Homolog 2; MSH6 = MutS Homolog 5; MLH1 = MutL Homolog 1; PMS2 = Postmeiotic Segregation Increased 2 Protein; MSH3 = MutS homologue 3; MLH3 = MutL homologue 3; PMS1 = postmeiotic segregation increased 1 protein; IDLs = insertion/deletion loops.

In relation to predictive biomarkers for ICIs, it is also worthwhile to mention high (h) tumor mutational burden (TMB) and the presence of pathogenic mutations in genes encoding for DNA polymerase ϵ (*POLE*) and polymerase δ 1 (*POLD1*) [16].

The threshold defining high tumor mutational burden (hTMB) is usually set at ≥ 10 mutations per megabase. High TMB reflects the accumulation of somatic mutations; these turn into a higher number of new antigens that foster immune activation and, finally, the response to immunotherapy. Together with convincing data from the KEYNOTE-158 trial, this provided the rationale for the Food and Drug Administration (FDA) approval of pembrolizumab for the treatment of advanced solid tumors with hTMB that are refractory to conventional treatment [17].

Similarly, specific mutations in the *POLE* and *POLD1* genes cause errors during DNA replication and lead to a hypermutated phenotype linked to high tumor immunogenicity. These mutations are commonly found in MSI-H tumors and correlate with hTMB, which is often higher than that detected in dMMR tumors [18–20]. *POLE* mutations occur in approximately 1% of CRC cases and are associated with distinctive clinical features, including younger age at diagnosis, male sex, earlier disease stages, and a lower risk of recurrence, particularly in stage II disease [21]. Moreover, CRC patients carrying *POLE* mutations have been reported to show improved overall survival (OS) compared with wild-type patients, regardless of MSI status [22]. Most importantly, the *POLE* and *POLD* pathogenic mutations are associated with clinical benefit from ICIs.

Next-generation sequencing (NGS) is the preferred method for evaluating gene alterations like single nucleotide variants, gene amplifications and fusions. Two complementary approaches are currently recommended to assess dMMR/MSI status. The first is screening for loss of MMR protein expression (dMMR), using immunohistochemistry (IHC) on tumor tissue. The second is testing for MSI through molecular testing on DNA extracted from tumor cells, using techniques based primarily on multiplex real-time Polymerase Chain Reaction (PCR) or NGS [6].

Based on the current evidence, discordances between the IHC tests for MMR proteins and molecular tests for microsatellite instability in CRC range from 1% to 10%. For example, Jaffrelot et al. reported a discordance of 1.1% between the PCR pentaplex panel, which includes 5 single-nucleotide quasi-monomorphic microsatellite loci (BAT-25, BAT-26, NR-21, NR-24, and NR-27), and IHC. In contrast, Cohen et al. found a higher discordance of 9.8%, using the same methods [23]. The main causes of discordance may include technical artifacts, errors and, most importantly, misinterpretation of IHC by pathologists. These studies also highlighted that the most frequent discordances are related to the loss of expression of the MSH6 protein. However, in most cases, MSI status was determined by PCR-based approaches. Data comparing MMR status by IHC, and MSI status evaluated by NGS, which allows the analysis of a greater number of loci in comparison to PCR-based approaches, are still limited.

Furthermore, recent studies have supported the use of a multi-marker testing approach by NGS to broaden the potential benefit of immunotherapy [22,24].

In this study, we aimed to describe the potential utility of Comprehensive Genomic Profiling (CGP) in improving the assessment of eligibility for ICIs. We evaluated discordance between MMR and MSI status and analyzed discordant cases, considering the additional information provided by CGP compared with routine standard diagnostic tests. These elements include the presence or absence of MMR gene pathogenic alterations and the TMB, *POLE* and *POLD1* status. Descriptive clinical insights were also provided

This study was conducted on a single-center, retrospective case series of metastatic CRCs.

2. Materials and Methods

2.1. Patients

A total of 138 patients with metastatic CRC diagnosed at the Unit of Pathological Anatomy—University Hospital of Pisa between 2020 and 2024 were retrospectively and consecutively enrolled in this study. All considered cases underwent a first-level molecular analysis to assess the status of approved CRC predictive biomarkers according to routine clinical practice, followed by a second-level analysis (CGP).

Demographic and clinical information, such as age, sex, date of metastatic presentation, therapeutic approaches, and survival indicators, were retrieved from the databases of the Oncology Unit of the University Hospital of Pisa and are reported in Table 1.

Table 1. Patients’ clinical and pathological features.

Clinical and Pathological Features		All Patients n = 138	RAS/BRAF WT n = 77	RAS/BRAF MUT n = 61
Age at diagnosis		59,41	59,84	58,88
Sex				
	Females	67	37	30
	Males	71	40	31
Primitive tumor site				
	Right colon	43	19	24
	Left colon	95	58	37
Histology				
	Classic	103	62	41
	Mucinous features	14	7	7
	Mucinous	11	4	7
	Mucinous + signet ring cells	7	2	5
	Mucinous features + signet ring cells	1	1	0
	Signet ring cells	1	1	0
	Adenosquamous	1	0	1

WT = wild type; MUT = mutated.

This study was conducted in accordance with the principles of the 1975 Helsinki Declaration and was approved by the local Ethics Committee. Written informed consent was obtained from patients before molecular analyses. All cases were anonymized for this study, and no sensitive data were used. This study did not interfere with routine clinical practice.

2.2. Tumor Samples

Tumor samples included both biopsy material and surgical specimens from primary or metastatic tumors. All samples were fixed in 10% buffered formalin (4% formaldehyde) for 6–48 h, depending on their type and size, and then embedded in paraffin (formalin-fixed paraffin-embedded, FFPE). Sections of 4–5 µm thickness were used for hematoxylin-eosin (HE) staining and immunohistochemical staining, while 10 µm sections were used for nucleic acid purification for molecular analyses.

2.3. First-Level Analysis

Histological diagnoses were performed by skilled pathologists at the Pathological Anatomy Unit 3 of the University Hospital of Pisa, according to the 5th edition of the World Health Organization (WHO) classification [25], and following the reporting

standards recommended by the College of American Pathologists (CAP) and the 8th edition of the American Joint Committee on Cancer (AJCC) TNM staging system [26].

According to ESMO recommendations for mCRC, first-level tests included the assessment of MMR/MSI status, *KRAS* and *NRAS* (hot-spot mutations within exons 2, 3, 4) and *BRAF* p.(V600E) mutation status, *ERBB2* amplification, and *NTRK* gene (*NTRK1*, *NTRK2* and *NTRK3*) fusions [3].

First-level mutational analysis was performed using a 17-gene amplicon-based NGS panel, the Myriapod® NGS Cancer panel DNA kit (Diatech Pharmacogenetics, Jesi, Italy) on the MiSeq platform (Illumina, San Diego, CA, USA).

Briefly, for each FFPE sample, DNA was purified from three 10 µm unstained sections after standard deparaffinization in xylene and rehydration in graded ethanol solutions.

All samples were enriched for cancer cells by manual microdissection; DNA was then purified using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA concentration and fragmentation were assessed using a real-time PCR kit (Diatech Pharmacogenetics, Jesi, Italy). A total of 25–50 ng of DNA was used for the NGS test according to the manufacturer's instructions. NGS data analysis was performed using Myriapod NGS Data Analysis Software (Diatech Pharmacogenetics) v.5.0.11 (Minimum accepted median coverage: 500x, Variant allele frequency (VAF) > 5%).

The clinical–therapeutic significance of variants was based on ESMO Scale for Clinical Actionability of molecular Targets (ESCAT). This NGS panel allows the analysis of the main hotspot alterations in 17 genes (*BRAF*, *HER-2*, *HRAS*, *KRAS*, *NRAS*, *IDH2*, *PIK3CA*, *RET*, *ALK*, *EGFR*, *FGFR3*, *IDH1*, *KIT*, *MET*, *PDGFRA*, *POLE*, *ROS1*). It does not include MSI status evaluation.

Immunohistochemistry was used to assess HER-2 (rabbit monoclonal clone 4B5, Ventana Medical system) expression and as screening for *NTRK* gene fusions (pan-TRK EPR117341 rabbit monoclonal antibody, Ventana medical system).

MMR status was evaluated by IHC using the automatic stainer BenchMark Ultra (Ventana Medical Systems, Roche Diagnostics Division, Hoffmann-La Roche Ltd., Basel, Switzerland), and the antibody–antigen reaction was visualized with the OptiView DAB IHC Detection Kit for MLH1, MSH2, MSH6, and with the OptiView DAB IHC Detection Kit with OptiView Amplification Kit for PMS2 (Ventana Medical Systems). We used MLH1-Ventana anti-MLH1 (M1) Mouse Monoclonal Primary Antibody; PMS2-Ventana anti-PMS2 (A16-4) Mouse Monoclonal Primary Antibody; MSH2-Ventana anti-MSH2 (G219-1129) Mouse Monoclonal Primary Antibody; and MSH6-Ventana anti-MSH6 (SP93) Rabbit Monoclonal Primary Antibody. The technical validation of IHC adequacy was performed using an internal control of normal colonic mucosa and tumor-associated stroma, lymphocytes and fibroblasts.

Each case was classified as pMMR, dMMR, indeterminate, or inadequate. pMMR was the definition when nuclear staining in the tumor was comparable to the internal control (lymphocytes, fibroblasts, and normal mucosal epithelium), whereas dMMR was defined as a complete loss of nuclear expression in tumor cells with preserved expression in internal control nuclei. Staining was considered indeterminate if tumor nuclei showed focal (<10% of the area) expression that was weaker than in the controls. Staining was considered inadequate if no nuclear staining was observed in the tumor or control nuclei, if the cellular elements of interest were poorly visualized, if fewer than 50 viable cells were present, or if background interfered with interpretation. In such cases, staining was repeated [27,28]. A tumor was defined as deficient if at least one of the four main MMR proteins was not expressed.

Microsatellite instability was assessed as part of first-line analyses in samples reported as dMMR by IHC or showing equivocal IHC results. DNA was purified from FFPE

tumor tissue as previously described and the EasyPGX Ready-MSI Real-Time PCR Kit (Diatech Pharmacogenetics) was used according to the manufacturer's protocol. Briefly, the kit required 8 assays per sample, analyzing the 5 loci of the pentaplex panel (BAT25, BAT26, NR21, NR24 and NR27) and 3 additional mononucleotide loci (NR22, CAT25 and MONO27). A total of 100 ng of DNA was used for each PCR assay. PCR was performed on the EasyPgx qPCR instrument (Diatech) and data were analyzed using EasyPGX Analysis Software v4.0.17 (Diatech). This melting-based analysis determines instability by comparing each sample's melting profile with a reference control across all loci (Figure 2). Tumors were classified as microsatellite stable (MSS) if no unstable markers or only one unstable marker were detected, and as MSI-H if two or more unstable markers were present [29].

Figure 2 shows the evaluation of microsatellite instability status by PCR-based melting analysis.

A. Example of a stable microsatellite locus (BAT25);

B. Example of an unstable microsatellite locus (BAT25).

The red melting curves are clinical samples. The blue melting curves are the reference control.

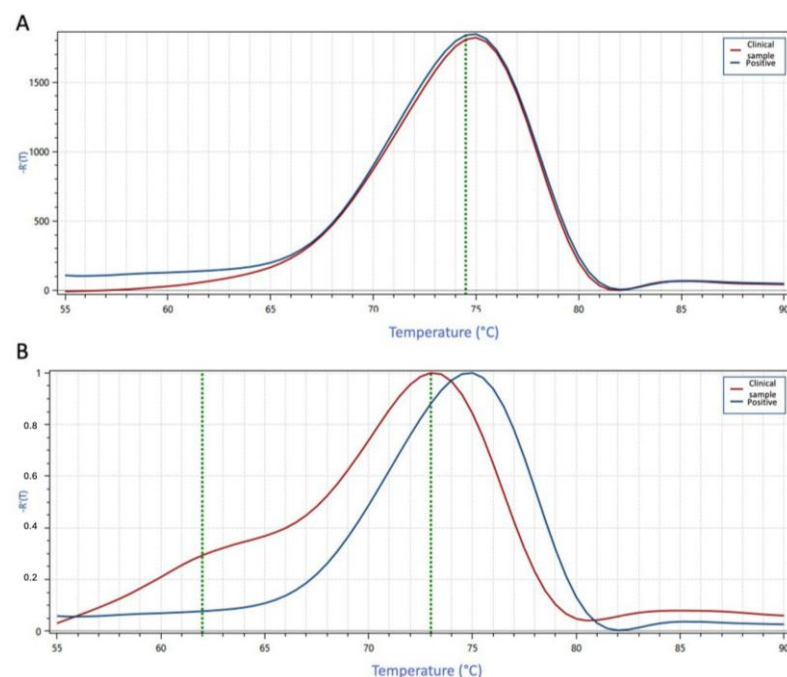


Figure 2. MSI test utilizing real-time PCR melting curves.

2.4. Second-Level Analysis

Second-level test evaluation or CGP was performed by the NGS panel FoundationOne CDx on the Illumina platform. This panel includes targeted regions within 324 genes and also allows researchers to evaluate genomic signatures like TMB and MSI status “<https://www.foundationmedicine.com/genomic-testing/foundation-one-cdx>” (accessed on 22nd January 2026)” [29].

2.5. Data Analysis

The prevalence of patients with dMMR, MSI, high TMB, and *POLE/POLD1* mutations was reported, and the concordance between MMR status by IHC and MSI status by CGP was determined. Cases showing pMMR by IHC and MSI-H by CGP, or dMMR by IHC and MSS by CGP, were considered discordant and are described in this paper

Although CGP cannot be considered the reference diagnostic method, discordant cases were discussed, including TMB (all MSI-H cases showed hTMB), and the presence or absence of MMR gene alterations.

3. Results

In our cohort of 138 tumors, at first-level molecular evaluation, 77 did not harbor *RAS/BRAF* alterations: 58 were left-sided colon/rectal cancer, and 19 were right-sided colon cancer. Conversely, 61 tumors were *RAS/BRAF* mutated: 37 were left-sided and 24 were right-sided.

Regarding MMR status, 126 of 138 patients were pMMR (91,3%) while 12 were dMMR (8,7%) at the first-level IHC test.

The dMMR cohort consisted of 3 *RAS/BRAF* wild type tumors and 9 *RAS/BRAF* mutated tumors (7 with *KRAS* mutations and 2 with *BRAF* mutations). Nine were right-sided colon cancer, and three were left-sided colon or rectal cancer. Regarding histological characteristics, five tumors had a conventional histology, two were poorly differentiated, five showed mucinous CRC subtype, and two had mucinous features.

3.1. Comparison of MMR Status and MSI

Five of twelve dMMR tumors showed loss of MLH1/PMS2, five showed loss of MSH2/MSH6, and two had isolated loss of MSH6 staining.

Second-level CGP analysis revealed an MSI-H status in 10 of the 12 dMMR tumors. Additionally, one pMMR case was MSI-H by CGP. Overall agreement between MMR status by IHC and MSI-H status by CGP, considering both pMMR and dMMR, was 98%.

Six cases showed discrepancies between MMR status by IHC, microsatellite instability determined at first-level test by PCR and at the second level by CGP, and the presence of pathogenic MMR gene alterations (Table 2).

Table 2. Cases with discordant MMR and MSI status.

ID	Specimen Type	Histological Subtype	Site	First-Level Analysis			Second-Level Analysis (CGP)			1st Line Therapy	OS (Months)	PFS (Months)
				MMR *	IHC (Protein Lost)	PCR MSI Status **	MSI Status *	TMB Status	Pathogenic MMR Gene Variants			
ID 1	TR	Mucinous	RC	dMMR	MSH6	MSS	MSI-High	68.09	<i>MSH6</i> p.(R1172fs*5)/p.(R240 *)	NA	NA	NA
ID 2	TB	Conventional	RC	pMMR	-	MSI-High	MSI-High	42.87	<i>MSH2</i> p.(E749K)	Folfoxiri + Cetuximab + Avelumab	12,69	4,11
ID 3	TR	Poorly differentiated	RC	dMMR	MLH1, PMS2	MSS	MSS	3.78	-	Capecitabine + Bevacizumab	67,56	21,99
ID 4	TR	Mucinous + signet ring cells features	RC	dMMR	MLH1 (10%), PMS2	NA	MSS	2.52	-	Capecitabine + Bevacizumab	24,13	5,46
ID 5	TR	Conventional	LC	dMMR	MLH1, PMS2	MSI-High	MSI-High	116	<i>MSH2</i> p.(A230fs*16)	Folfoxiri + Bevacizumab	67,33	14,63
ID 6	TR	Mucinous features	RC	dMMR	MLH1, PMS2	MSI-High	MSI-High	113.5	<i>MSH6</i> p.(R361H)	Capecitabine + Bevacizumab	35,67	9,28

MMR = mismatch repair; pMMR = MMR-proficient; dMMR = MMR-deficient; MSS = microsatellite stable; MSI = microsatellite instability; TMB = Tumor Mutational Burden; CGP = Comprehensive Genomic Profiling; NA = Not Available; TR = Tumor Resection; TB = Tumor Biopsy; RC = Right Colon; LC = Left Colon. * MMR by IHC and CGP tests were performed for all cases included in this study as first- and second-level tests, respectively. ** PCR melting-based MSI testing was performed for all dMMR cases before CGP test, except for case ID4, which directly underwent CGP. For case ID2, PCR was executed after CGP due to discordant results between IHC and CGP.

In Table 2, only cases with discordant MMR status by IHC and MSI status by PCR-based assay and/or CGP are reported.

The MMR IHC of all the discordant cases was reviewed by gastrointestinal pathologists.

The first discordant case was MSH6 deficient, stable by PCR, but unstable by CGP, which also revealed two pathogenic mutations in the *MSH6* gene. This case involved a 78-year-old male patient with mucinous right-sided colorectal cancer (Figure 3 Case ID1; Table 2). He underwent hemicolectomy and developed a liver metastasis one year later, which was surgically removed. The patient was not treated with ICIs and, after four years of follow-up, showed no signs of disease relapse.

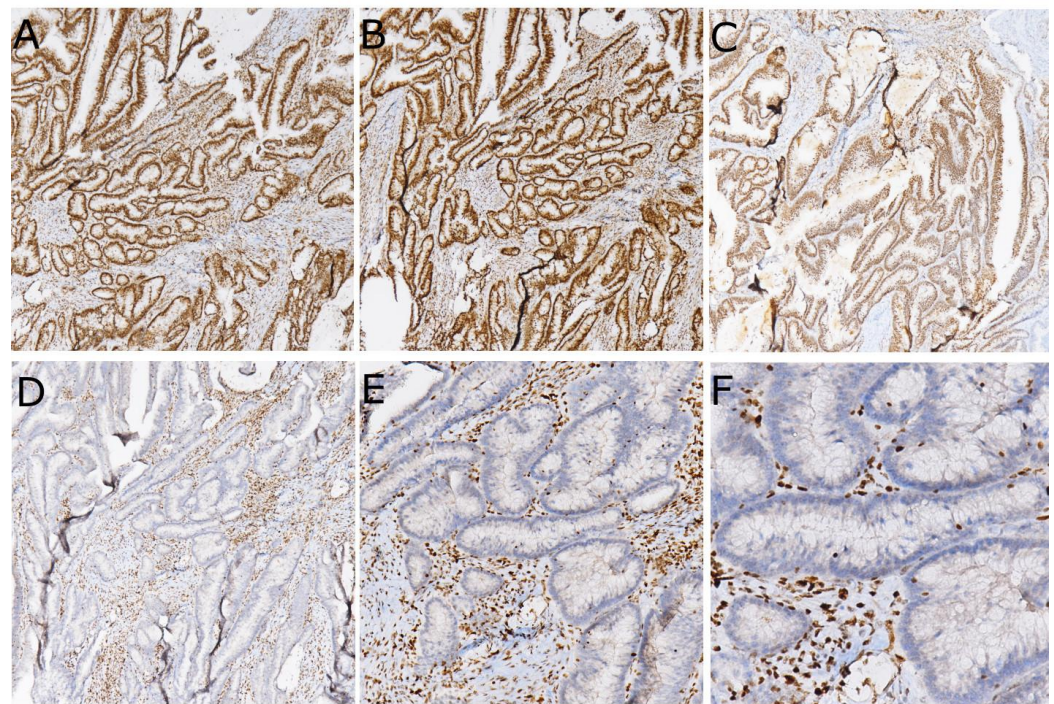


Figure 3. IHC staining of case ID1 [Table 2] showing a dMMR pattern characterized by an isolated and complete loss of the MSH6 protein. (A). Retained nuclear expression of MSH2, 4x magnification; (B). Retained nuclear expression of MLH1, 4x magnification; (C). Retained nuclear expression of PMS2, 4x magnification; complete loss of MSH6 protein in the presence of a preserved strong and specific control staining (stromal cells and lymphocytes) at 4x (D), 10x (E); 20x (F) magnification.

The second discordant case (ID2) retained expression of all MMR proteins and was classified as pMMR, but CGP analysis revealed MSI-H status and a pathogenic *MSH2* missense mutation p.(E749K). The case was carefully reviewed, and retention of all four MMR proteins was confirmed. This patient was a 52-year-old woman with a mucinous right-sided colon tumor and three synchronous metastases at diagnosis in the lungs, peritoneum and lymph nodes. She was treated for 4 months with a combination of immunotherapy and chemotherapy, specifically FOLFOXIRI plus cetuximab and avelumab, achieving a progression-free survival equal to 4.1 months and an OS of 12.7 months (Figure 4 Case ID2; Table 2).

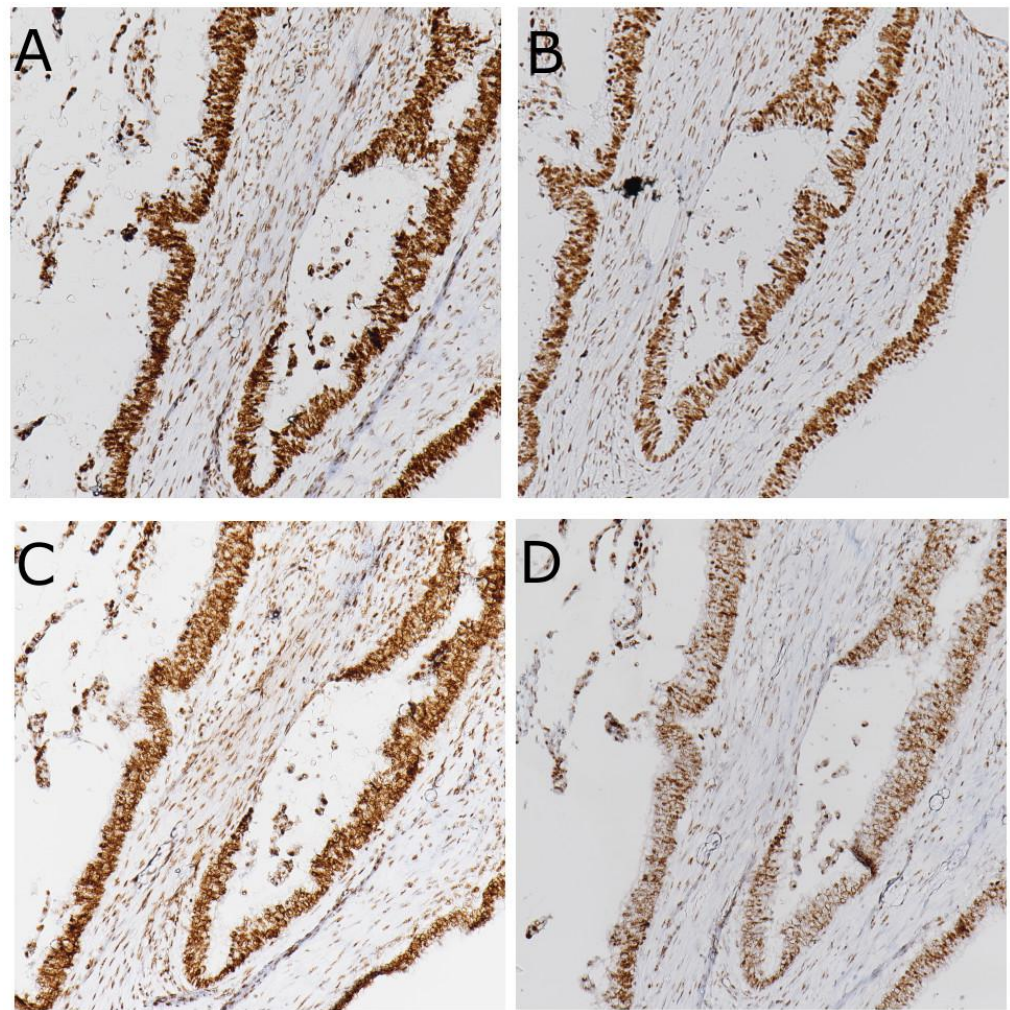


Figure 4. IHC staining of case ID2 [Table 2], showing a pMMR pattern. All MMR proteins are retained. The nuclear staining is strong, homogeneously diffused, and comparable to those of the control (stromal cells and lymphocytes). (A–D) Intact nuclear expression of MSH2 (A), MSH6 (B), MLH1 (C), and PMS2 (D) at 10x magnification.

The third (ID3) and fourth (ID4) discordant cases were both deficient for MLH1 and PMS2 but were MSS with low TMB by CGP. IHC revision in both cases revealed indeterminate and heterogenous MLH1 and PMS2 expression with suboptimal staining, making interpretation challenging for the pathologist (Figure 5; Table 2 ID3). Case ID3 involved an 80-year-old man with poorly differentiated left-sided primary cancer, which had metastasized to the lung at diagnosis. He partially responded to initial therapy with capecitabine and bevacizumab, achieving a progression-free survival of about 18 months. He then underwent radiotherapy for lung oligoprogression, showing stable disease for four months without treatment. After disease recurrence, he received second-line irinotecan, achieving stable disease as the best response. Due to his age, he discontinued therapy and died approximately six years after diagnosis. Case ID4 involved an 85-year-old woman with a mucinous right-sided CRC with signet ring cell and synchronous peritoneal metastases. She did not receive immunotherapy but was treated with capecitabine and bevacizumab, showing disease progression at the first follow up visit. She died two years after the diagnosis.

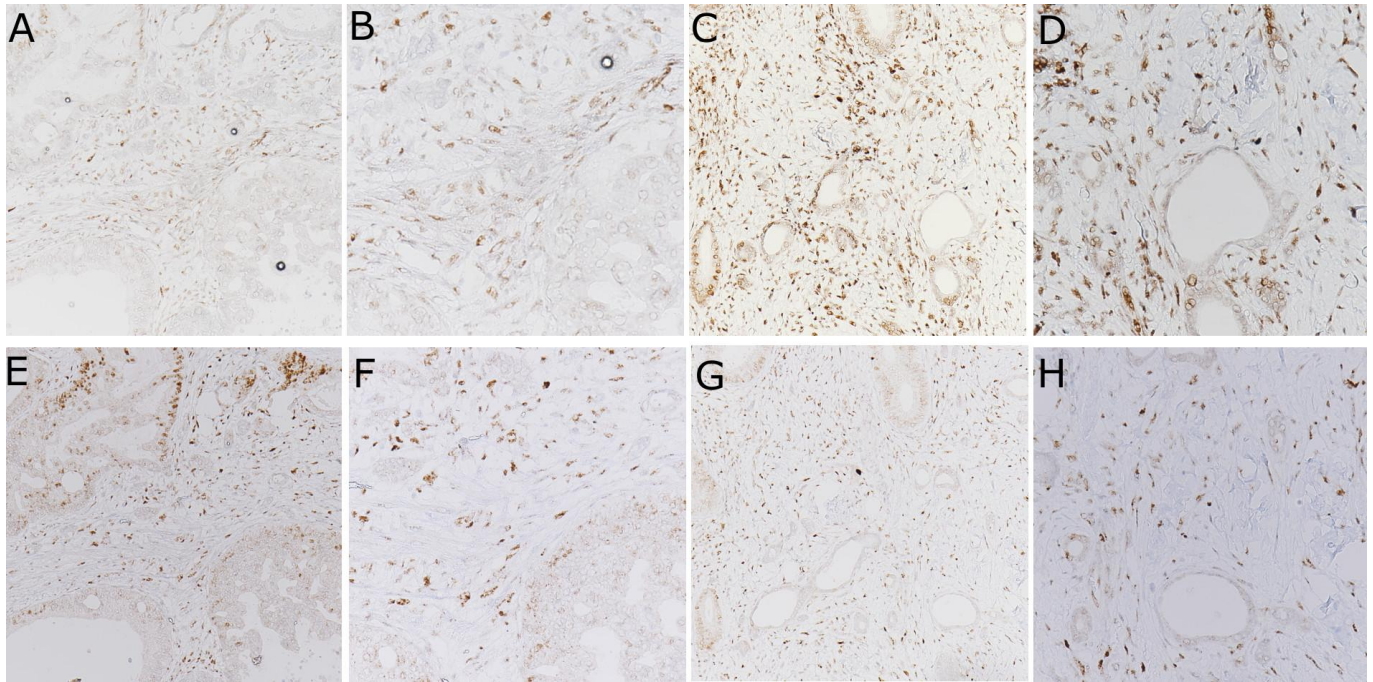


Figure 5. IHC staining of case ID3 [Table 2] showing MLH1 and PMS2 deficiency with an heterogeneous pattern and difficult interpretation in presence of a faint internal control staining (A,B) Loss of MLH1 in the more differentiated component of the tumor, at 10x (A) and at 20x (B) magnification. (C, D) Loss of MLH1 in the poorly differentiated component of the tumor, at 10x (C) and at 20x (D) magnification; (E,F) Loss of PMS2 in the more differentiated component of the tumor, at 10x (E) and at 20x (F) magnification; (G,H) Loss of MLH1 in the poorly differentiated component of the tumor, at 10x (G) and at 20x (H) magnification.

The last two cases showed discordance between the deficient proteins revealed by IHC and the mutated MMR gene detected by CGP (Table 2, ID 5–6).

The fifth patient (ID5) was a 48-year-old female with conventional left-sided CRC diagnosed at stage IV. She was treated with FOLFOXIRI plus Bevacizumab, with a progression-free survival of 14,63 and an OS of 67,33 months (Table 2, ID-5).

The sixth case (ID6) involved a 70-year-old man with mucinous right-sided CRC who developed lung metastases. He initially received monotherapy plus Bevacizumab, followed by second-line pembrolizumab, achieving a progression-free survival of 9,28 and an OS equal to 5,69 months. Review of his tumor sections, which were MLH1- and PMS2-deficient with a *MSH6* pathogenic mutation, highlighted an unusual MMR IHC staining pattern. Specifically, IHC showed loss of MLH1 and PMS2 staining and concomitant clonal loss of MSH6 and MSH2 with retention of staining in interstitial control cells (Figure 6; Table 2 ID6).

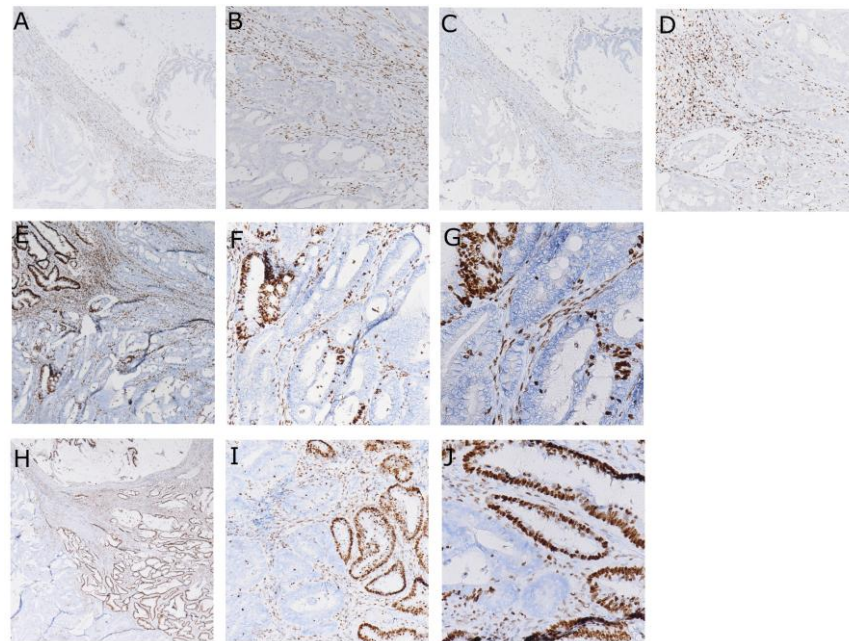


Figure 6. Immunohistochemical (IHC) staining of the ID6 case [Table 2], showing MLH1 and PMS2 deficiency with MSH2 and MSH6 proficiency, with the presence of adequate internal control staining. (A,B) Loss of MLH1 expression in both the mucinous and non-mucinous tumor components, at 10x (A) and 20x (B) magnification. (C,D) Loss of PMS2 expression in both the mucinous and non-mucinous tumor components, at 10x (C) and 20x (D) magnification. (E–G) Preserved MSH2 expression in the presence of completely negative tumor clones, with adequate internal control staining, at 4x (E), 10x (F) and 20x (G) magnification. (H–J) Preserved MSH6 expression in the presence of completely negative tumor clones, with adequate internal control staining, at 2x (H), 10x (I) and 20x (J) magnification.

In both cases, loss of expression of MLH1 was due to gene promoter hypermethylation, as determined by using the EasyPGX readyMLH1 (Diatech Pharmacogenetics) real-time PCR kit according to the manufacturer's protocol.

3.2. TMB and POLE or POLD1 Alterations

In our case series, tumors with high microsatellite instability (10 cases) and 11 pMMR/MSS tumors had hTMB (Table 2).

Only one MSI-H case had a concomitant activating mutation in *POLD1*, whereas none of the 138 patients had mutations in *POLE*.

4. Discussion

MSI and dMMR are established markers for predicting response to ICIs in CRC. The KEYNOTE-177 study led to the approval of pembrolizumab as first-line therapy for patients with stage IV CRC exhibiting dMMR proteins and/or MSI [30]. Similarly, the CheckMate-142 and CheckMate-8HW studies demonstrated the efficacy of nivolumab and ipilimumab for patients with stage IV MSI and/or dMMR CRC [31]. ICI efficacy has also been observed in localized stages of dMMR CRC. The NICHE-2 study reported a high pathological response rate to ICIs in patients with localized colon cancer [32], while Cercek et al. reported a 100% clinical complete response rate with dostarlimab in locally advanced dMMR rectal cancer [33]. The predictive value of MSI and MMR for ICI response in CRC is well-established in clinical practice. However, discrepancies between MMR status and MSI status can hamper the appropriate selection of patients for ICI therapy.

In fact, the available studies have shown that variable discordance can exist between MMR status by IHC and MSI evaluated by PCR or NGS [34,35].

In this study, CGP was performed as an externalized test, and analytical thresholds and calling criteria were not fully specified by the manufacturer, which constitutes a limitation. However, we observed a good concordance (98%) between MSI evaluation using NGS and MMR assessment by IHC. These findings are encouraging, considering that current ESMO guidelines primarily recommend immunohistochemical tests to identify patients eligible for immunotherapy and for LS screening, and that some centers often only have access to IHC [3,36,37].

Although based on a small number of discordant cases, our results highlight some important aspects that can affect the evaluation of both MMR and MSI.

IHC evaluation of MMR protein expression is the simplest and most cost-effective method. However, it can be challenging or misleading because it depends not only on biological tumor characteristics but also on technical issues that arise in the pre-analytical phase (e.g., grossing procedure, fixation timing), analytical phase (e.g., staining procedures that vary across laboratories, including antigen unmasking, antibody dilution, incubation time), and post-analytical phase. Moreover, IHC misinterpretation is one of the main causes of MMR status discrepancies [38,39].

Regarding the accuracy of IHC interpretation for MMR proteins, a study by McCarthy et al. reported sensitivity values between 77% and 100% and specificity values between 89% and 99%, with higher sensitivity observed among experienced pathologists [40]. In our cohort, the two dMMR cases were MSS by NGS, and one of these was also MSS by PCR. These patients would have been considered candidates for immunotherapy, based solely on IHC results. Given the absence of microsatellite instability, IHC slides were reviewed by two expert independent pathologists. In the first case, deficiency for MLH1 and PMS2, staining was indeterminate on review (with staining in <10% of tumor cells) and therefore further molecular investigations were required. The second case, initially showing borderline MLH1 and PMS2 expression, was determined on review to be highly heterogeneous (see case ID3, Table 2; Figure 5).

These findings are in line with well-known recommendations, endorsing that the interpretation of IHC for MMR proteins should be limited to experienced pathologists in specialized settings and that molecular analysis of MSI status should be performed in all doubtful or ambiguous cases [40–42].

Discordance can also be explained by isolated loss of MSH6 or PMS2, which does not necessarily imply MMR system deficiency and therefore may not result into an MSI-H status [10,43]. In fact, functional redundancy exists among MMR proteins (such as PMS2 and PMS1, MSH6 and MSH3). For this reason, even when loss of MSH6 or PMS2 expression is detected, an MSS status may be maintained [40,44].

In our study, discordance between PCR and NGS was reported in only one patient. This case showed isolated loss of MSH6 expression by IHC. MSI-H status by CGP was associated with a double mutation of the *MSH6* gene, while MSS was detected by PCR. MSH6-mutated CRCs might display a relatively lower degree of MSI, which could explain discrepancies between the PCR melting-based approach and NGS. Overall, NGS/CGP methods, compared with PCR melting-based tests, allow fragment length analysis of a larger number of microsatellite loci and can enable detection of lower instability levels [45].

IHC may also yield false positive results. Some mutations, although responsible for MMR deficiency, do not affect protein expression. As a result, nonfunctional MMR proteins may still be detected by IHC [46]. In this context, we reported a clear pMMR case showing a high microsatellite instability status as determined by both first-level PCR and NGS. CGP identified the responsible genetic alteration: a missense *MSH2* mutation

p.(E749K) (see case ID2, Table 2, Figure 1). Several studies, both in vitro using human CCR cell lines (LoVo) and on FFPE samples from various solid tumors, have identified pathogenic *MSH2* mutations, like p.(E749K), that cause functional alteration without loss of protein expression [47,48].

Microsatellite instability is considered an early event in CRC carcinogenesis; however, recent studies have shown that this instability is not always homogeneous within a tumor, particularly in sporadic CRC. While intratumoral heterogeneity of mutations in genes such as *KRAS*, *TP53*, and *PIK3CA* in mCRC is well-documented and associated with resistance or the reduced efficacy of anti-EGFR therapies, limited data are available on the impact of heterogeneity on MSI or MMR testing [49,50].

In contrast to LS, in sporadic dMMR/MSI CCR, microsatellite instability may be an initial event, whereas MMR defects can occur later during the tumor progression, leading to intra- or inter-tumoral heterogeneity. Some studies have reported atypical MMR IHC staining patterns in sporadic tumors, with loss of expression limited to small tumor areas, likely reflecting intra-tumoral heterogeneity [51,52].

Although rare, in cases of discordant results (dMMR/MSS), it may be useful to test different areas of the primary tumor or metastases to assess the degree of heterogeneity. In this context, analysis of microsatellite status by NGS on circulating tumor DNA may be helpful [51,53]. Liquid biopsy-based testing offers several advantages. It is a minimally invasive and rapid approach that demonstrates a high concordance with tissue-based assays and exhibits strong specificity for gene mutation detection. However, regarding MSI testing, the diagnostic accuracy and sensitivity of liquid biopsy are still under evaluation, with few validated NGS panels available for the clinical practice [54–56].

In our study, we identified two cases showing MLH1 and PMS2 deficiency by IHC but harboring exclusive *MSH2* and *MSH6* gene alterations by NGS (see Table 2; cases ID5 and ID6). Careful re-examination of the specimens from one of these cases revealed some tumor areas negative for MSH6 and MSH2 expression, as well as areas, although limited to <10%, that were positive for MLH1 and PMS2 (Figure 6). These findings suggest that tumor subclones probably coexist with different genotypes within the same tumor mass. In these cases, MLH1 deficiency was associated with promoter hypermethylation, a frequent sporadic event in CRC [57,58]. In this context, identification of pathogenic *MSH2* and *MSH6* alterations was crucial for appropriately referring patients to genetic counseling.

Here, we also evaluated other predictive biomarkers for ICIs, such as TMB and pathogenic mutations in *POLD1* and *POLE*. However, in current CRC clinical practice, neither TMB nor *POLD1/POLE* mutations are well-established predictive biomarkers.

Notably, we have identified a high TMB in 11 (8.0%) MSS tumors with a proficient MMR system.

In the KEYNOTE-158 trial, response to pembrolizumab was correlated to TMB in patients with a variety of advanced solid tumors, but not in those with colon cancer. Because responses were enriched in patients with high TMB (≥ 10 mut/Mb), the FDA approved pembrolizumab in 2020 for patients with unresectable or metastatic solid tumors with high TMB.

In the KEYNOTE-177 trial, although TMB was shown to impact on survival in CCR, limitations were observed relevant to its use as a predictor of response to anti-Programmed Death 1 (PD1) immunotherapy, mainly due to challenges in defining an optimal TMB cut-off value [59–61].

Overall, although evidence suggests that TMB cannot be used as an independent predictive biomarker for immunotherapy in colon cancer, it may still be informative for predicting treatment efficacy, especially when elevated.

In our study, only one patient harbored a pathogenic *POLD1* mutation, specifically, p.(D402N). This case was *RAS/BRAF* wild-type, dMMR by IHC, and MSI-H by both PCR and CGP analysis, and it carried a pathogenic *MSH2* mutation.

Mutations in *POLE* and *POLD1* genes lead to the accumulation of DNA replication errors and are typically associated with increased neoantigen load and enhanced immune cell infiltration. Two ongoing clinical trials, NCT05103969 and NCT03810339, are currently evaluating the predictive value of pathogenic *POLE/POLD1* alterations [62,63]. Strong biological and clinical evidence supports the role of *POLE* and *POLD1* as predictive biomarkers for ICI response in CRC. However, the rarity of these mutations hampers large-scale clinical trials and limits definitive validation, despite the highly promising results reported by Rosseau et al. and Ambrosini et al. [19,20].

The primary aim of this study was to evaluate technical aspects related to the analysis of predictive biomarkers for immunotherapy, particularly MMR and MSI status. Nevertheless, patients' selection for ICIs, beyond CGP, could be further improved through transcriptomic, epigenomic and proteomic approaches, which may better capture tumor genetic complexity and its immune microenvironment [64–66].

Overall, in our study, MMR and MSI showed a good concordance rate; however, the simultaneous assessment of both markers may further refine patients' selection for immunotherapy [67,68]. Moreover, compared with first-level analysis, CGP offers meaningful advantages in expanding molecular characterization of CRC for the detection of both predictive biomarkers and germline variants and hereditary syndromes (i.e., identification of pathogenic alterations within MMR genes). This approach, however, may require a well-structured genetic counseling process.

Although the clinical use of CGP is still limited by costs, technical and ethical complexity, and longer turnaround time, it should be considered, at least in selected cases appropriately chosen based on clinical pathological characteristics [69,70].

5. Conclusions

Despite the retrospective and descriptive nature of this study, our results highlight the potential role of CGP in clinical practice. MSI assessment by CGP showed a high concordance (98%) with MMR status determined by IHC. Moreover, beyond microsatellite status evaluation, CGP enables detection of pathogenic variants in MMR genes, thereby facilitating interpretation of discordant cases. Specifically, in three of twelve dMMR cases the simultaneous assessment of MSI status and MMR gene mutational status was helpful. In this study, cases discordant as to MMR status by IHC and MSI by CGP were likely attributable to MMR protein redundancy, tumor heterogeneity, or challenges in IHC interpretation. In this context, evaluating both MSI and MMR status can increase diagnostic accuracy.

Regarding MSI analysis, although currently available PCR-based assays have been specifically optimized for CRC, CGP allows investigation of a broader number of microsatellite loci. In addition, CGP enables the simultaneous evaluation of other genomic alterations and signatures with predictive value, such as TMB.

Furthermore, the concurrent evaluation of microsatellite instability and pathogenic mutations in MMR genes may improve LS screening by facilitating timely confirmatory genetic testing and appropriate follow-up.

These exploratory findings warrant the undertaking of prospective studies and appropriate patient follow-up to better define the clinical utility and cost-effectiveness of CGP in refining patient selection for immunotherapy.

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Abbreviations

The following abbreviations are used in this manuscript:

CRC	Colorectal cancer
EOCRC	Early-onset CRCs
MSI	Microsatellite Instability
MMR	Mismatch Repair
MLH1	MutL Homolog 1
MSH2	MutS Homolog 2
MSH6	MutS Homolog 6
PMS2	Postmeiotic Segregation Increased 2 Protein
MSH3	MutS Homolog 3
MLH3	MutL Homolog 3
PMS1	Postmeiotic Segregation Increased 1Protein
MSI-H	High microsatellite instability
dMMR	MMR System Deficiency
LS	Lynch Syndrome
5-FU	5-Fluorouracil
pMMR	MMR System Proficiency
ESMO	European Society for Medical Oncology
mCRC	Metastatic CRC
ICIs	Immune Checkpoint Inhibitors
IHC	Immunohistochemistry
PCR	Polymerase Chain Reaction
NGS	Next-Generation Sequencing
TMB	Tumor Mutational Burden
hTMB	High Tumor Mutational Burden
POLE	DNA polymerase ϵ
POLD1	DNA polymerase δ 1
FDA	Food and Drug Administration
OS	Overall Survival
CGP	Comprehensive Genomic Profiling
WT	Wild Type
MUT	Mutated
FFPE	Formalin-Fixed Paraffin-Embedded

HE	Hematoxylin and Eosin
WHO	World Health Organization
CAP	College of American Pathologists
AJCC	American Joint Committee on Cancer
MSS	Microsatellite Stable
CR	Complete Response
PD1	Programmed Death 1
NA	Not Available
TR	Tumor Resection
TB	Tumor Biopsy
RC	Right Colon
LC	Left Colon

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