**Opinion**

Mismatch Repair Status Characterization in Oncologic Pathology: Taking Stock of the Real-World Possibilities

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**Abstract:** The mismatch repair (MMR) system has a key role in supporting the DNA polymerase proofreading function and in maintaining genome stability. Alterations in the MMR genes are driving events of tumorigenesis, tumor progression, and resistance to therapy. These genetic scars may occur in either hereditary or sporadic settings, with different frequencies across tumor types. Appropriate characterization of the MMR status is a crucial task in oncologic pathology because it allows for both the tailored clinical management of cancer patients and surveillance of individuals at risk. The currently available MMR testing methods have specific strengths and weaknesses, and their application across different tumor types would require a tailored approach. This article highlights the indications and challenges in MMR status assessment for molecular pathologists, focusing on the possible strategies to overcome analytical and pre-analytical issues.

**Keywords:** mismatch repair system; microsatellite instability; testing methods; immunohistochemistry; molecular testing; NGS

1. Introduction

DNA mismatch repair (MMR) is a highly conserved system aimed at recognizing and repairing single-base mismatches that evaded polymerase proofreading activity [1]. Given its active role in ensuring DNA stability, this system is essential for cell homeostasis [2]. Four key proteins belong to the MMR, namely mutL homolog 1 (MLH1), mutS homolog 2 (MSH2), mutS homolog 6 (MSH6), and postmeiotic segregation increased 2 (PMS2). They are arranged in heterodimers, namely MutLα (MLH1-PMS2), MutSα (MSH2-MSH6), and, when indels involve > 2 nucleotides, MutSβ (MSH2-MSH3) [3]. MutSα mediates the initial identification of mismatched bases, while MutLα, by interacting with MutSα, stimulates the endonuclease activity and initiates re-synthesis [4].

Mutations in the MMR-related genes may lead to a functional impairment of the entire MMR system, leading to tumorigenesis, tumor progression, and therapy resistance [5]. These alterations can be observed not only in the DNA sequence, but also at the proteomic (i.e., loss of nuclear expression of the MMR proteins) and/or epigenetic (i.e., hypermethylation of the MMR gene promoters) levels [5]. Neoplasms with MMR dysfunctions are prone to have a hypermutator phenotype that frequently results in microsatellite instability (MSI) [6]. This condition is defined by the detection of alternate-sized microsatellite tandem repeats, which are small DNA motifs that are distributed throughout the genome [7–9].

Several combinations of microsatellite loci can be targeted for MSI testing; if variations in more than two of these markers are observed, the status of the tumor is classified as MSI high (MSI-H) [5,10,11]. Of note, MMR deficiency/MSI may also be caused by germline mutations in the MMR genes, this hereditary condition is referred to as Lynch syndrome (LS) or MMR-deficient (dMMR) hereditary nonpolyposis colorectal cancer [10]. Germline mutations in MLH1 and MSH2 are responsible for 80–90% of LS tumors [11]. Notably, a very
small subset of LS patients is characterized by the presence of constitutional epimutations of MLH1 [12,13].

Taken together, approximately 4% of solid tumors display an MSI-H phenotype [14]. However, both genetic characteristics and frequency distribution are heterogeneous across different tumor types [14–16]. For example, MSI is particularly frequent in endometrial and colorectal cancers, but exceedingly rare in breast, prostate, and ovarian cancer [17–21]. For this reason, the harmonization of MMR clinical testing strategies is a goal to be achieved for next-generation pathologists [22–24]. Hence, MMR profiling has been historically standardized in tumors where MMR deficiency is a rather common event, such as those related to LS (i.e., endometrial and colorectal cancer) [25,26]. In recent years, however, MMR/MSI routine testing has also been proposed in other cancer types (e.g., gastroesophageal cancers, ovarian cancer, breast cancer, and glioblastoma) for prognostication and immunotherapy patient selection, and not necessarily for LS genetic screening [27,28]. Regrettably, there are no tumor-specific biomarkers and guidelines available to-date for this analysis in non-endometrial and non-colorectal cancers [29]. Completion of strict laboratory procedures, regular quality controls, cutting-edge infrastructure maintenance, and periodic training programs are therefore required.

2. Testing Strategies

Current reference methods for MMR profiling depend on immunohistochemistry (IHC) for the four MMR proteins and sequencing assays directed against selected microsatellite markers (e.g., Bethesda panel and MSI Analysis System) [30,31]. Despite their reliability, these diagnostic strategies have several limitations, including the relatively low sensitivity in cancers not belonging to the LS spectrum and/or showing heterogeneous expression of the MMR proteins [18,32,33]. To overcome these issues, new molecular-based methods, such as novel real-time PCR (RT-PCR) panels, droplet digital PCR (ddPCR)-based assays, and next-generation sequencing (NGS) are emerging (Table 1) [27].

| Attribute                                      | IHC        | RT-PCR    | NGS       |
|-----------------------------------------------|------------|-----------|-----------|
| Cost effective                               | Yes        | Yes       | Yes (*)   |
| Widely available                              | Yes        | Yes       | Not yet   |
| Multi-target                                  | Yes (#)    | Yes (##)  | Yes       |
| Discrimination LS vs. sporadic                | No         | Yes       | Yes       |
| Intra-tumor heterogeneity identification      | Yes        | No        | Yes (**)  |
| Required amount of material                   | Low        | High      | Low (*)   |
| Standardized guidelines in all tumor types    | No         | No        | No        |

IHC, immunohistochemistry; RT-PCR, real-time quantitative polymerase chain reaction; NGS, next-generation sequencing; LS, Lynch syndrome. (*) Providing optimization of the lab workflow and (**) bioinformatics infrastructures. (#) Only proteins. (##) Low throughput.

2.1. Immunohistochemistry

Pathogenic mutations in MMR genes lead to the proteolytic degradation of the heterodimers and consequent loss of MMR protein expression in the cell nucleus [34]. Given the reliability and cost effectiveness of IHC, this method is widely considered as a pillar of first-line diagnostic tests [5]. Hence, antibodies against MLH1, MSH2, MSH6, and PMS2 are commonly available in pathology laboratories across the globe [35]. The loss of nuclear staining of at least one of the MMR proteins in all of the neoplastic cells defines the dMMR status. Conversely, the retained expression of these proteins is usually considered diagnostic of an MMR-proficient (pMMR) status. Of note, the irregular loss of immunoeexpression, both in terms of intra-tumor and staining intensity heterogeneity, albeit prognostically relevant, does not suffice for qualifying a tumor as dMMR [20,36]. A major problem of this analysis is represented by the substantial lack of specific recommendations on cold ischemia time, fixation protocols, primary antibody clones, concentrations, and platforms, as well as detailed diagnostic guidelines [37].
2.2. PCR-Based MSI Testing

MSI analysis has been initially performed by RT-PCR for five microsatellite markers, consisting of three dinucleotides (i.e., D2S123, D5S346, and D17S250) and two mononucleotide (i.e., BAT-25 and BAT-26) repeats, as recommended by the revised Bethesda Guidelines [30,31]. Comparing the tumor with the matched non-neoplastic tissue, instability of at least two markers identifies the MSI-H status, whereas, in MSI-low (MSI-L) tumors, only one locus is unstable [38,39]. Recent lines of evidence, however, suggest that mononucleotide markers are more (or at least as) specific than dinucleotides for MSI testing [40,41]. For this reason, other PCR panels (e.g., MSI Analysis System, Promega®, Madison, WI, USA) targeting five quasimonomorphic mononucleotide repeats (e.g., BAT-25, BAT-26, NR-21, NR-24, and NR-27) have been proposed as reliable alternative options to the traditional one [42]. Lately, new high-performance assays have been proposed as viable and complementary options to IHC and standard RT-PCR panels. In this regard, PlentiPlex™ MSI (Pentabase, Odense, Denmark), OncoMate™ (Promega), Idylla™ MSI Test (Biocartis, Mechelen, Belgium), TrueMark (Thermofisher, Waltham, MA, USA), and Bio-Rad ddPCR showed a short runtime and high levels of sensitivity and specificity when compared to standard MSI/MMR detection methods [43].

In particular, the PlentiPlex™ MSI assay evaluates MSI by using PentaBase (BAT-25, BAT-26, MONO-27, NR-22, and NR-24 loci) or mono- and dinucleotide Bethesda panels. MSI evaluation is provided by comparing capillary electrophoresis gel migration charts of the tumor samples with reference DNA samples [44]. The OncoMate™ MSI Dx Analysis System (Promega) is a PCR-based test used to determine MSI and MMR status in solid tumors. This assay has less than three hours of running time and can be performed on DNA purified from ≤1 formalin-fixed, paraffin-embedded (FFPE) section sample with ≥20% tumor content. Five mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) are targeted, and tumor samples are then matched with reference DNA for quality control and sample authentication. MSI status is determined by comparing the allelic profiles after size separating the amplified markers using capillary electrophoresis. OncoMate™ MSI Dx shows high concordance with immunohistochemistry results for MMR status evaluation, and is approved for the identification of patients that may benefit from further diagnostic testing [45]. The Idylla system is a fully-automated RT-PCR platform set to perform the detection of MSI directly from FFPE tissues [43]. Unlike the traditional systems, the Idylla system does not require normal tissues for comparison. This system amplifies and screens seven regions (i.e., ACVR2A, BTBD7, DIDO1, MRE11, RYR3, SEC31A, and SULF2) in an ~150 min run [43]. The MSI score is thus generated for each biomarker, and ranges from 0 to 1 with a set cutoff of ≥0.5 for positive results. Tumors are defined MSI-H if at least two of the seven MSI markers are positive, and MSS if these criteria are not fulfilled [38,43]. TrueMark is a fast low-input RT-PCR-based assay that shows high reliability compared to standard MSI RT-PCR testing. This assay has been assessed to test MSI in Lynch syndrome-associated cancers, and can be performed even on small amounts, for example 2 ng, of FFPE-isolated DNA. TrueMark is composed of 13 MSI markers, including the five Bethesda loci and eight additional homopolymers, increasing in this way the range of sequences that can be used to determine the MSI state [46]. The PlentiPlex™ MSI assay evaluates MSI by using PentaBase (BAT-25, BAT-26, MONO-27, NR-22, and NR-24 loci) or mono- and dinucleotide Bethesda panels. MSI evaluation is provided by comparing capillary electrophoresis gel migration charts of the tumor samples with reference DNA samples [44]. Finally, the Bio-Rad droplet digital PCR (ddPCR) MSI assay is based on the analysis of five markers (i.e., BAT25, BAT26, NR21, NR24, and Mono27). This assay can be used on either tumor tissue (FFPE or fresh) or plasma cell-free (cf)DNA. It works through the generation of about 15,000 droplets used to perform a competitive-probe drop-off assay after thermal cycling amplification [38]. In this system, two probes are competing for the same target sequence, and, depending on mutation level/microsatellite length, one of the two probes cannot find the binding site.
Microsatellite stability is set whenever both of the probes bind to the target sequence, and, conversely, MSI is evidenced [38,47].

The major limitation of MSI molecular assays is that insufficient tumor content may not allow the detection of MSI instability. Usually, 10–20% tumor cells on the whole tissue are required for the analysis. This evaluation precedes macrodissection, and constitutes important admitting criteria [48]. Although IHC and MSI RT-PCR are routinely used in the clinical settings, a recent report on immunotherapy in metastatic colorectal cancer has shown that approximately 10% of the patients enrolled in immunotherapy trials experienced failure in the therapy due to false-positive dMMR or MSI RT-PCR results assessed by local laboratories [49]. Furthermore, in tumors with low MSI/dMMR frequency, such as breast cancer, few data are available, and the exploitation of IHC and MSI RT-PCR protocols is highly questioned [20,50].

2.3. NGS-Based Approaches

Lately, NGS has emerged as a sensitive and accurate method to characterize MSI and MMR status in tumors, showing several advantages over traditional assays [14,51]. Thereby, NGS-based methods demonstrated higher performances when compared to previous technologies and are potentially useful to expand MSI testing, particularly in those cancers characterized by lower MSI-H/dMRR frequencies [52]. NGS panels, indeed, can screen a larger number of microsatellite loci compared to RT-PCR [14]. This allows parallel high-throughput analysis of both microsatellites and genes and leads to the simultaneous identification of other actionable alterations. Interestingly, MSI testing performed using NGS can be easily integrated with other relevant biomarkers as tumor mutational burden (TMB), using targeted specific panels and avoiding the costs of whole-exome or whole-genome sequencing.

The estimation of TMB from comprehensive genomic profiling is a candidate biomarker with available specific NGS panels and is correlated to immuno-checkpoint inhibitors’ response in several types of cancer [53]. TMB is calculated by counting the number of synonymous and non-synonymous mutations across a region spanning 315 genes. The result is reported as the number of mutations per megabases (mut/Mb), thus patients with ≥10 mut/Mb are classified TMB-high. To date, several NGS panels as FoundationOne CDx and MSK-IMPACT have been approved for TMB evaluation after accuracy validation against whole-exome sequencing [54,55].

NGS-based panels, such as MSIplus and ColoSeq, and software including MSIsensor and MANTIS combine sequencing with biostatistics to address MSI in tumor samples [15,17,56]. MSIplus assay has been optimized for colorectal cancer and screens microsatellites in 16 loci located along driver oncogenes, such as KRAS, NRAS, and BRAF [57]. ColoSeq assay detects mutations, deletions, or complex structural rearrangements in seven genes involved in DNA repair (MLH1, MSH2, MSH6, PMS2, EPCAM, APC, and MUTYH) and associated with MSI [58]. MSIsensor [52] and MANTIS (Microsatellite Analysis for Normal Tumor InStability) are customized software for the automatic detection of somatic microsatellite changes. They operate by computing length distributions of microsatellites per site in paired tumor and normal sequence data. Therefore, these data are processed to statistically compare observed distributions in both samples and result in a specific scoring for MSI [59,60].

3. Conclusions

Although RT-PCR and IHC are interchangeable analyses in most tumor types for MMR status profiling, each of these methods provides different information [61]. Compared with RT-PCR and NGS, IHC is cost-effective and more reliable but operative limitations such as false negative results should not be underestimated [20,56]. IHC is widely available in most pathology laboratories and the majority of cases show straightforward interpretation without requiring high expertise [62]. However, IHC shows remarkable variability due to the heterogeneous expression of MMR proteins within the tumor and to the fixation process. [20]. This last factor indeed could remarkably affect the result of the entire analysis.
as well as the fixatives, the time in formalin before embedding, and the intrinsic uniformity of the fixation [63]. While RT-PCR provides molecular information about the loss of MMR function, it is not indicative of the specific MMR protein that is not expressed and does not inform on whether the dMMR/MSI tumor has sporadic or germline origin [64]. PCR analysis is performed on genetic material isolated from tissue blocks containing an adequate amount of tumor sample (the tumor must be at least 20% of the entire tissue). Usually, biopsies do not provide enough material for successful RT-PCR testing, whereas most resections are sufficient. In contrast, IHC can be performed, within 48 h, on both biopsy and resection specimens and does not require a large amount of tissue. MSI RT-PCR shows a higher turn-around time compared to IHC, but the main disadvantage of this approach remains the lack of translation across different tumor types due to the limited number of loci that are evaluated [62,65]. Notably, Bethesda and pentaplex mononucleotides loci may be inadequate for pan-cancer MSI evaluation. This could potentially change the MSI testing approach [20].

Nevertheless, MSI and MMR deficiency have proven to be clinically important biomarkers for predicting response to immunotherapy and the outcome of the disease [65]. These events have been observed across a wide variety of cancer types, but a pan-cancer scope of testing is urgently required. MSI RT-PCR only tests five to seven loci and IHC is only indicative at the proteomic level. Currently, NGS represents the most promising tool to test MSI and MMR among all cancer types. These new approaches, indeed, demonstrate superior performance to previous technologies and testing can be easily integrated into other sequencing assays for more comprehensive genomic analysis. NGS-based methods permit to test of a great variety of loci leading forward to more-thorough assessment and genomic profiling of the tumor. On the other hand, NGS is expensive and requires expertise and facilities which are not available in the majority of the laboratories. NGS approach additionally, could provide important information on cancers not belonging to the LS spectrum. These cancers are usually characterized by low MSI/dMMR frequencies accompanied by poor IHC/MSI RT-PCR available data and represent a dramatic grey area in MSI/dMMR cancer assessment.

Despite NGS-based testing are still far to be a reality in MSI and MMR status clinical assessment, many steps forward have done in recent years. The large quantity of free available data provided from tumor genome sequencing projects as the Cancer Genome Atlas is widely used for research. The development of customized algorithms for MSI detection such as MSIsensor and MANTIS allows discriminating between MSI-H and other hypermutation signatures leading the way for the identification of MSI-H/dMMR in cancers with lower mutation rates. These factors accompanied by the progressive reduction of sequencing cost will boost in the next few years NGS applications both in research and clinical settings, leading the way to the landing of this technology in diagnostic and even more personalized medicine.

Author Contributions: Conceptualization, N.F.; resources, N.F.; data curation, R.P.; writing—original draft preparation, R.P.; writing—review and editing, K.V., E.S., N.F.; supervision, N.F.; project administration, N.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: N.F. has received honoraria for consulting, advisory role, and/or speaker bureau from Merck Sharp & Dohme (MSD), Boehringer Ingelheim, and Novartis. These companies had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and/or in the decision to publish the results. All other authors declare no conflicts of interest.
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