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REVIEW ARTICLE

Controversies in Pathology

Detecting mismatch repair deficiency in solid neoplasms: immunohistochemistry, microsatellite instability, or both?

Chiyun Wang[1], Liying Zhang[2], Efsevia Vakiani[1] and Jinru Shia[1]✉

In managing patients with solid tumors, the value of detecting the status of tumor DNA mismatch repair function is widely recognized. Mismatch repair protein immunohistochemistry and molecular microsatellite instability testing constitute the two major test modalities currently in use, yet each is associated with caveats and limitations that can be consequential. Most notably, the traditional approach of defining mismatch repair protein immunohistochemistry abnormality by complete loss of staining in all tumor cells is evolving. Partial or clonal loss is becoming recognized as a manifestation of gene abnormality; in some cases, such clonal loss is associated with germline pathogenic variants. The current criteria and cutoff values for defining microsatellite instability-high are developed primarily according to colorectal tumors. Non-colorectal cases, and occasionally even colorectal tumors, that are mismatch repair-deficient by immunohistochemistry but not microsatellite instability-high by current standards are being recognized. Emerging data suggest that these immunohistochemistry abnormal / non-microsatellite instability-high cases warrant further genetic workup for Lynch syndrome detection. Whether these tumors respond to immunotherapy is a question still to be addressed. It is imperative that pathologists as well as clinicians and investigators be aware of such intricacies regarding routine immunohistochemistry and microsatellite instability testing and the results they generate. This review summarizes our current understanding of the advantages and limitations of these tests and offer our view on what constitutes the most optimal strategy in test selection and how best to utilize case context to enhance the interpretation of the test results.

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INTRODUCTION

In human cells, the process of DNA mismatch repair (MMR) deficiency starts when a major MMR gene (*MLH1*, *MSH2*, *MSH6*, or *PMS2*) is inactivated via germline and/or somatic mutations or epigenetic silencing. This is followed by loss of the MMR protein which then leads to loss of MMR function and subsequent accumulation of non-repaired mismatch mistakes (base-base mismatches and insertion-deletion [indel] errors) in the repetitive DNA sequences known as microsatellites. When such altered or "unstable" microsatellites involve the coding or other functionally important sequences of cancer-associated genes, tumor initiation or accelerated progression ensues[1–5]. These mutations, particularly the indels, can also cause exonic sequence frameshifting and generate unique neopeptides, that in turn elicit robust T cell infiltration in the tumor, bringing about a heightened tumor immune microenvironment[6,7].

There are two major categories of tissue-based testing currently in use for the detection of the status of MMR[8–14]. One is MMR immunohistochemistry (IHC) which detects the MMR proteins in the cells (in this review, MMRd and MMRp specifically refer to MMR protein deficient or proficient as detected by IHC). The other is microsatellite instability (MSI) testing which detects the unstable microsatellites (in this review, MSI-H, MSI-L, MSI-I, and MSS refer to microsatellite instability-high, low, indeterminate, and microsatellite stable, respectively, as detected by MSI testing). Various PCR- and next-generation sequencing (NGS)-based MSI testing platforms have been developed; the classic assay that was standardized early on is the PCR-MSI[1] utilizing a National Cancer Institute (NCI)-endorsed panel of five microsatellite markers.

MMR IHC and MSI testing are garnering growing attention owing to the significant implications of the test results (MMR loss signifying increased risk of Lynch syndrome [LS] and likelihood of responding to immunotherapy) on the one hand, and the persistence of certain limitations to the testing methodologies on the other. As technologies evolve, various test-related questions remain open, such as "is one test sufficient", "if both are needed, what would the best strategy be to incorporate them into routine clinical practice", and "how best to deal with discrepant results from the two types of tests".

This review provides an updated summary of our current understanding of the advantages and limitations of these two tests and offers our view on what constitutes best strategies in selecting tests and interpreting results. Special emphasis is placed on (1) the changing IHC definition of MMRd, and (2) the potential clinical implication of cases with "MMRd/non-MSI-H".

[1]Department of Pathology and Laboratory Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, USA. [2]Department of Pathology and Laboratory Medicine, University of California, Los Angeles (UCLA), Los Angeles, CA, USA. ✉email: Shiaj@MSKCC.org

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MMR IMMUNOHISTOCHEMISTRY

MMR IHC has been in use for more than two decades[15]. For the vast majority of the cases, the test is easy and yields straightforward results. Most tumors show the presence of nuclear staining for all four proteins, indicative of MMRp. The MMRd cases typically manifest loss of MLH1 and PMS2 (associated with *MLH1* inactivation), loss of MSH2 and MSH6 (associated with *MSH2* inactivation), loss of MSH6 alone (associated with *MSH6* inactivation), or loss of PMS2 alone (associated with *PMS2* inactivation). In these MMRd cases, the loss of staining is frequently uniform throughout the tumor.

Advantages

A number of salient features of MMR IHC are generally accepted as advantageous. They include:

- Wide availability in routine diagnostic laboratories.
- Fast turn-around time.
- Relatively low cost.
- Feasibility in samples with <20% tumor content.
- Amenability to IHC external quality assurance measures. Proficiency tests and recommended protocols offered by organizations such as the College of American Pathologists (CAP) and Nordic immunohistochemical Quality Control (NordiQC) serve to promote test quality across laboratories.
- Direct visualization of the stained cells and correlation with morphology.
- Identification of the specific defective protein, thus allowing inference of the affected gene; in certain situations, this also helps the determination of the pathogenicity of MMR gene variants of uncertain significance.
- Detection of cases that have MMR deficiency but are not MSI-H.
- Ability to suggest the possibility of Constitutional MMR Deficiency (CMMRD) when properly processed stains fail to show unequivocal nuclear staining in both the neoplastic and non-neoplastic cells, especially in pediatric patients or young adults; these cases can be missed by MSI testing.

A further added value of routine use of MMR IHC is the continued accumulation of IHC data that can facilitate the profiling of various atypical staining patterns, leading to continued refinement of the definition of MMRd and consequently the detection of cases that would have been missed otherwise. For example, IHC MMR clonal loss (with part of the tumor still having retained MMR) has gradually been recognized as a likely form of MMRd that can be associated with germline defect when occurring in certain case scenarios (see below).

Caveats and limitations

MMR IHC is subject to all the caveats and limitations inherent to the IHC technique in general as well as those that are more specific to the testing of the MMR proteins specifically[15–22].

- Pre-analytic (e.g., tissue ischemia, fixation, decalcification, age of the blocks) and analytic factors (e.g., tissue or staining artifacts like tissue tearing or folding, fixation gradients, edge effects, DAB trapping, antibody specificity, etc.) can affect IHC performance. Thus, stringent IHC quality assurance and control procedures are paramount, and lab participation in proficiency testing (as offered by CAP and other organizations such as NordiQC) is necessary.
- Poor fixation in particular can result in reduced staining intensity to various degrees and could lead to an erroneous interpretation of MMR protein loss.
- The type of fixative warrants attention. In general, MMR IHC does not work well with non-formalin-based fixatives.
- The performance of various antibody clones and associated detection systems may vary (commonly used MMR antibody clones are listed in Supplementary Table 1).
- MLH1 IHC alone can miss some MLH1 deficient cases, possibly related to *MLH1* missense or in-frame indel variants (that can produce a functionally inactive but antigenically intact mutant protein causing false normal staining on IHC) or technical issues (including poor sensitivity of the MLH1 IHC antibody)[15–19]. Adding PMS2 IHC increases the sensitivity in detecting these cases[23].
- While a two-antibody approach (PMS2 + MSH6), first proposed in 2009[20], detects almost all MMRd cases that are detectable by the full four-antibody panel, it can still miss cases. Thus, the most optimal approach calls for the use of all four MMR antibodies.
- IHC staining patterns and staining intensity can have both inter- and intra-tumor variability[15,21,22], making concrete scoring recommendations or guidelines difficult and causing interpretation inconsistency among observers.
- The current IHC protocols, mostly optimized according to colorectal samples (which is highly proliferative and therefore has high expression of MMR proteins), may not be optimal for non-colorectal tissues that are less proliferative and possess lower expression of MMR proteins.
- IHC staining, particularly the staining for MSH6, can be significantly reduced or abolished altogether in carcinomas that have been subjected to chemo-radiation therapy via as-yet undefined mechanisms but typically not related to germline or somatic MMR gene alteration or MSI-H, making post-treatment specimens unreliable for interpretation[24,25].
- IHC can miss cases in which the MMR deficiency is due to inactivation of genes other than the four tested.

Additional comments on pattern and intensity of MMR IHC and the changing definition of MMRd

It has long been recognized that MMR IHC staining can be "heterogeneous". Focal and weak staining, as well as punctate nuclear staining, frequently associated with MLH1, could potentially be related to the presence of a mutant protein or the lack of specificity of the MLH1 antibody (which is developed against a full length protein), and can be remedied by adding PMS2 to the IHC panel[15,23,26] (Table 1, Fig. 1).

Another source of "heterogeneity" is poor tissue fixation or poor performance of the staining procedure in regions of the tumor or parts of the section, and this can be remedied by vigilance in detecting the presence or absence of acceptable internal positive control (staining in lymphocytes, stromal cells, and normal epithelium in the vicinity of the tumor). ***Internal positive controls are mandatory for interpretation of results.*** Regions of poor tumor cell staining without acceptable internal positive control should be regarded as not interpretable.

Edge effect may also give rise to observed "heterogeneity". This typically affects biopsy samples and the MSH6 stain[27], with some nuclei staining towards the periphery while the remaining tumor shows loss of staining. Often these tumors have a true MSH6 protein abnormality.

An as-yet under-recognized issue related to staining heterogeneity pertains to regional loss of tumor cell staining in a properly stained section with consistent internal positive control[18,19]. In this review, we regard distinct regional loss of staining as "clonal loss". The extent of the "clone" with loss of staining could range from minimal to almost the entire tumor (on individual sections, it could be 1% to 100%).

Traditionally, the definition of MMRd is "no staining in the tumor anywhere" (i.e., "***all absent = abnormal***"), and as such, tumors with clonal loss have been regarded as MMRp because parts of the tumor still have retained staining with an intensity stronger than the surrounding stroma. This is in fact still the definition currently recommended by the College of American Pathologists[28]. It is indicated that any positive reaction in the nuclei of tumor cells is considered as intact expression (normal),

Table 1. Atypical mismatch repair protein immunohistochemistry staining patterns.

Tumor staining	Internal control	Interpretation	Technical/biological explanation
Equivocal throughout	Weak or none	Staining not working, repeat test on same or different block	Typically due to poor fixation
Focally weak or lost	Also weak or none in these foci	Regard these foci as non-interpretable, rely on the remaining interpretable regions for results (Fig. 4A, B)	Typically due to regional poor fixation, tissue degeneration, or poor exposure to antibody/reagents during staining
Weaker than internal control	Present and optimal	Correlate with staining of its partner protein as follows:	
		MLH1 weak/PMS2 normal (Fig. 1C, D): - Report both as normal	
		MLH1 weak/PMS2 abnormal (Fig. 1A, B): - Report both as abnormal	
		MLH1 normal/PMS2 weak (unlikely scenario): - Report PMS2 as equivocal	
		MLH1 abnormal/PMS2 weak (unlikely scenario): - Report both as abnormal	
		MSH2 weak (or lost)/MSH6 normal (unlikely scenario): - Report MSH2 as equivocal	Have been observed in *POLE*-mutated cases, mechanism unclear
		MSH2 weak/MSH6 abnormal (Fig. 2): - Report both as abnormal	
		MSH2 normal/MSH6 weak: - Report MSH6 as abnormal	
		MSH2 abnormal/MSH6 weak (Fig. 4D, E): - Report both as abnormal	
Distinct clonal loss	Present and optimal	Report as abnormal:	
		Clonal loss of MLH1 and PMS2 (Fig. 3D–F)	Typically associated with clonal *MLH1* methylation (maybe mutation as well, see below)
		Clonal loss of MSH6 in MLH1/PMS2-deficient tumors	Typically associated with secondary mutation of coding microsatellites in *MSH6* in the tumor
		Clonal loss of MLH1/PMS2, MSH2/MSH6 (Fig. 3A–C), PMS2 alone, or MSH6 alone	Could potentially be associated with germline mutation, suggest genetic workup
Cytoplasmic staining		- Mostly aberrant, regard as non-interpretable; rely on nuclear staining status for result interpretation - When occurring with MSH2, and accompanied by loss of nuclear staining, it could reflect EPCAM/MSH2 abnormality	In some EPCAM-Lynch syndrome cases, cytoplasmic localization of EPCAM-MSH2 fusion proteins can result in cytoplasmic MSH2 staining[21,22]

General Comment:
Interpretation of the test results need to be correlated with clinical findings. If there exists a strong family/clinical history suggestive of Lynch or related syndromes, referral to clinical genetics service should be considered despite a normal immunohistochemistry (or microsatellite instability-PCR) result.

and it is common for intact staining to be somewhat patchy. The VENTANA MMR RxDx Panel (Roche Diagnostics, Rotkreuz, Switzerland) that was recently approved by the U.S. Food and Drug Administration (FDA) as a companion diagnostic to identify MMRd solid tumor patients eligible for anti-PD1 immunotherapy took this approach as well[29,30]. Its scoring algorithm indicates "focal weak equivocal nuclear staining in the viable tumor cells in the presence of internal positive controls should be given a Clinical Status of Loss. On the other hand, focal strong unequivocal nuclear staining in the viable tumor cells in the presence of internal positive controls should be given a Clinical Status of *Intact*."

However, emerging literature[18,19,31–34] and personal experience have indicated that in some cases with focal staining, the area of staining loss represents distinct clonal loss (Figs. 2 and 3), and such clonal loss is frequently associated with MMR gene alterations as can be seen in the following scenarios:

- Clonal *MLH1* promoter methylation. This results in clonal loss of MLH1 and PMS2 on IHC.
- Clonal somatic mutation in one or more MMR genes in a tumor that is already MMRd/MSI-H. Most major MMR genes harbor coding microsatellites. Thus, in MLH1 and/or PMS2 deficient tumors, secondary mutation in the *MSH6* coding microsatellites can lead to the emergence of a tumor clone that has loss of MSH6, resulting in an IHC phenotype of concurrent loss of MLH1/PMS2 and MSH6 in this clone[24]. Sometimes, such secondary clones can overtake the entire tumor. In MSH6 deficient cancers, secondary mutation could occur in the coding microsatellites of *MSH3* in a clonal fashion leading to clonal loss of MSH3; as MSH3 is the major alternative partner for MSH2, its loss (in the absence of MSH6) could lead to MSH2 proteolytic degradation and a phenotype of concurrent clonal MSH2 loss and complete MSH6 loss (in some situations, the MSH6 loss can also be "heterogenous" due to edge artifact or other reasons).
- *POLE*-mutated tumors. The ultra-mutating phenotype may secondarily involve MMR genes.
- *De novo* clonal somatic mutation in an MMR gene is speculated to occur as well. These cases do not have other MMR gene deficiency.
- Germline MMR pathogenic variants. It has been suggested in the literature and we have also observed in our practice that

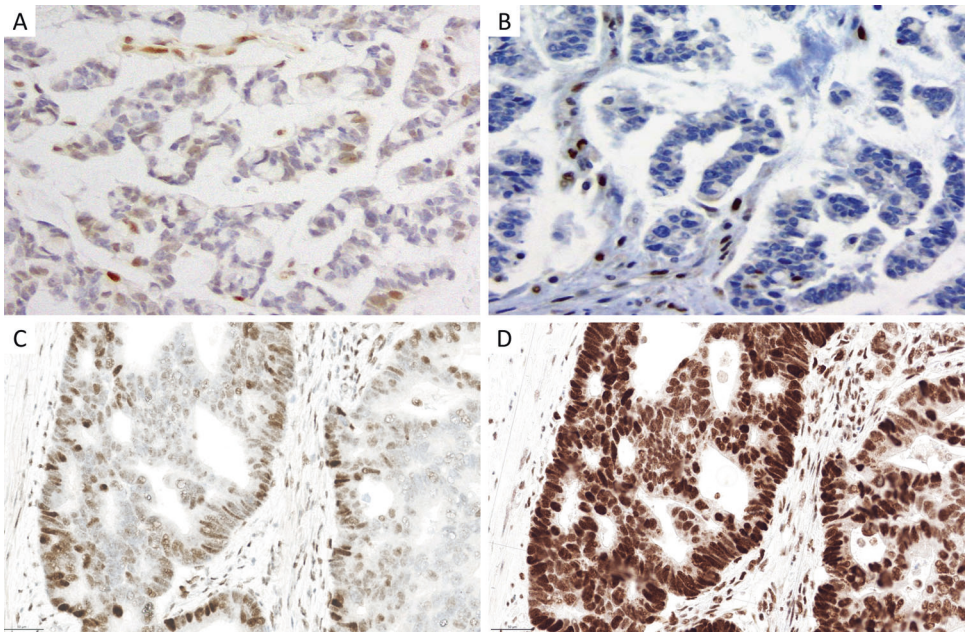

Fig. 1 Interpretation of atypical MLH1 immunohistochemistry can be facilitated by the addition of PMS2 immunohistochemistry. In case 1 (**A**, **B**), a right colon adenocarcinoma, the staining for MLH1 (**A**) appears variable with most tumor cells having a staining intensity weaker than the internal control or no staining, whereas the staining for PMS2 (**B**) is lost. In this scenario, the loss of PMS2 serves as a surrogate marker for *MLH1* abnormality. This patient was indeed found to carry a deleterious germline *MLH1* mutation. In case 2 (**C**, **D**), another colonic adenocarcinoma, the variable staining of MLH1 (**C**) is accompanied by normal staining of PMS2 (**D**) as well as normal staining of MSH2 and MSH6 (not shown). The normal PMS2 staining suggests that the variable MLH1 staining is likely due to technical or other clinically inconsequential etiologies. This tumor was microsatellite stable.

LS cases with defined germline MMR pathogenic variants can manifest clonal loss (rather than complete loss) of the corresponding MMR protein. This is presumably a reflection of the varied carcinogenesis pathways in LS-associated tumors. The frequency of this phenomenon is still to be determined. Our experience suggests that it is more likely to involve *MSH6* or *PMS2*.

These observations have thus led investigators to propose that clonal loss also be regarded as "MMR IHC abnormal". According to this proposal, only when nuclear staining is uniformly present in all tumor cells can we regard the stain as normal. In other words, "***all present = normal***" (Table 2). It has been emphasized[19,35] that normal MMR IHC staining should consist of staining throughout the tumor that is clearly stronger in intensity than that of the internal control (ideally carried out on well-fixed biopsy tissue); any deviation from this potentially constitutes an abnormal pattern. This is indeed what the British Association of Gynaecological Pathologists (BAGP) recommends for endometrial carcinomas; in this recommendation, a 10% cutoff is suggested for the lower limit of the tumor proportion with loss of staining for the tumor to be categorized as MMRd[18,19,36].

This approach of "***all present = normal***" will undoubtedly capture certain MMRd cases that could have been missed by the "***all absent = abnormal***" approach. Caution is needed, however, to avoid over-interpretation of MMRd when applying this approach to routine practice. One pitfall, for example, is the phenomenon of intra-tumor gland staining heterogeneity that is known to occur, particularly with MSH6 staining. The pattern of staining loss in such intra-tumor gland heterogeneity is typically indistinct, with cells losing staining intimately juxtaposed to cells with retained staining. However, the number of tumor cells without staining (or with only weak staining) may still amount to 10% or more of the tumor and would therefore fulfill the 10% cutoff. Yet these cases have not been documented to be associated with gene abnormality thus far. Some examples are illustrated in Fig. 4.

Our own experience suggests that true clonal MMR protein loss on IHC reflective of gene alteration has a distinct pattern; the MMRd regions are typically sharply demarcated from the MMRp regions (Figs. 2 and 3); the relative proportions or percentages of the two regions can vary widely across sections or cases.

The clinical implication of such genuine clonal MMRd is twofold. From the perspective of detecting LS, it is the distinct clonal loss that cannot be explained by secondary somatic alteration (e.g., focal *MLH1* methylation, or *MSH6* mutation in tumors that are already MLH1/PMS2 deficient) that will warrant further genetic workup. From the perspective of predicting response to immunotherapy, the meaning of clonal loss remains to be defined. A noteworthy phenomenon is that the metastasis from these rare tumors with clonal loss of MMR could be either MMRd or MMRp (MSS), as has been documented in the literature[33]. When the metastasis is MMRp/MSS, it may not respond to immunotherapy.

Summary

The easy availability of MMR IHC, along with its other advantages, has allowed its widespread use among clinical laboratories. However, to ensure test accuracy, pathologists need to be aware of its various caveats and limitations. Vigilance towards the existence of varied staining patterns is of particular importance. Additionally, the definition of what constitutes abnormal protein expression is still evolving. The traditional definition considers MMRd as complete loss of staining in the tumor (all absent = abnormal), whereas more recent observations have indicated the association of clonal (i.e., partial) loss of staining with gene abnormality in some cases and suggest that only when staining is present throughout the tumor can the tumor be regarded as MMR normal (all present = normal).

Table 1 summarizes our approach in dealing with the various atypical staining patterns. With regard to the interpretation of clonal loss, while awaiting more definitive guidelines, we suggest that its presence be documented in the pathology report; a comment can be used to indicate that "this pattern is likely

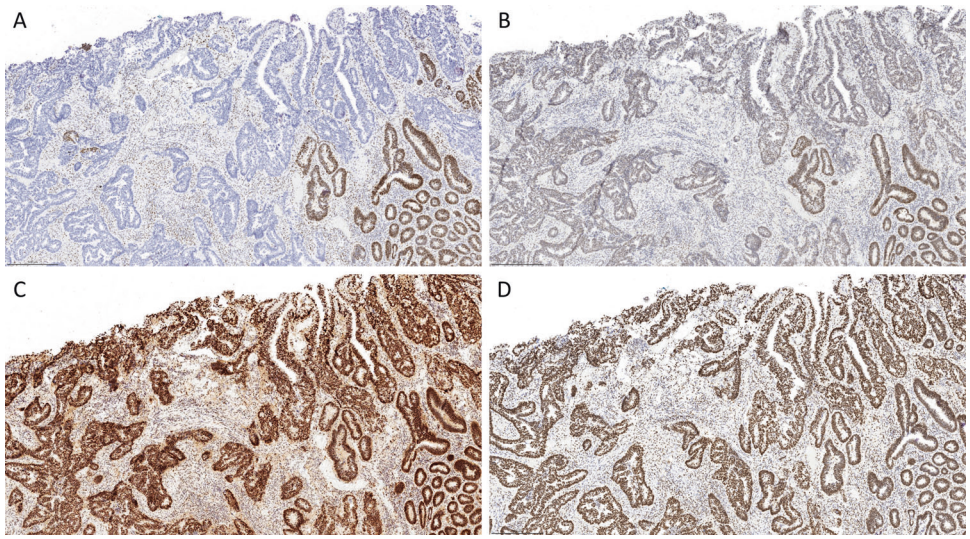

Fig. 2 A colorectal adenocarcinoma with distinct MSH6 clonal loss by immunohistochemistry in a patient who was found to have a germline pathogenic variant in *MSH6*. The MSH6 immunohistochemical stain (**A**) shows regions with unequivocal positive labeling and juxtaposed regions with distinct staining loss. In the regions with MSH6 loss, the staining for MSH2 (**B**) is reduced in intensity. The reduced MSH2 staining intensity does not appear to be related to secondary loss of MSH3 as the MSH3 staining (**C**) is diffuse and strong. The staining for MLH1 (not shown) and PMS2 (**D**) is retained throughout the tumor.

sporadic in nature (particularly when involving both MLH1 and PMS2) but could also be due to Lynch or related syndromes". Caution is needed in distinguishing true clonal loss from clinically inconsequential staining variability that is typically caused by technical issues such as poor fixation. True clonal loss should have a distinct pattern, with the regions of staining loss sharply demarcated from the adjacent regions with retained staining; the presence of a consistent reliable internal positive control is mandatory.

MSI TESTING

MSI is defined as "a change of any length due to either insertion or deletion of repeating units, in a microsatellite within a tumor when compared to normal tissue"[1]. It is a molecular phenotype of MMR loss and characterizes a hypermutable state of cells. It is the hypermutability, as well as the nature of the mutations (frequently frameshift), that renders tumors with this phenotype rich in neoantigens leading to the development of a hyper-intense immune microenvironment[37]. The immune checkpoint inhibitors —pembrolizumab, nivolumab, and ipilimumab, all approved by the Food and Drug Administration in the treatment of MMRd or MSI-H metastatic colorectal cancer (CRC) or in patients with MMRd or MSI-H CRC who have previously received chemotherapy—achieve their therapeutic efficacy by blocking tumor specific checkpoints and enabling T cells to exert their cytotoxic effect. Detection of MSI-H status (as well as MMRd) is thus paramount in selecting patients for this treatment.

The classic MSI detection method is a PCR-MSI test using an NCI-endorsed marker panel consisting of two [A/T]n mononucleotide repeats (BAT-26 and BAT-25) and three [GT/CA]n dinucleotide repeats (D5S346, D2S123, and D17S250) commonly referred to as the Bethesda Panel[1]. Recent years have seen the emergence of various modified and improved MSI testing platforms with more sensitive marker amplification[38–42] (multiplex instead of simplex, fluorescent primers instead of radio-labeled primers) and more effective read-out strategies[43,44] (capillary electrophoresis or other methods such as denaturing high performance liquid chromatography and high resolution melting analysis instead of gel electrophoresis). The marker panels have also been improved[45,46], incorporating more mononucleotide markers at the growing appreciation that such markers are quasi-monomorphic (nearly all individuals have the same number of repeats at these loci) and sensitive in detecting MSI[47]. Some examples of such improved MSI platforms are the Pentaplex MSI-PCR (Fig. 5), MSI-PCR incorporating long mononucleotide repeats[48], HSP110 (T17) PCR[49], and the Idylla™ MSI Assay (Table 3, Supplementary Table 2). The Idylla assay is fully-automated with fast turnaround time and no need for matched normal tissue[50,51]. At the current time, the pentaplex PCR-based methods are the standard test for detecting MSI, and are recommended by major organizations such as the European Society for Medical Oncology (ESMO) as the preferred MSI testing modality in the context of immunotherapy[52,53].

NGS-based MSI-detection is another testing modality that has emerged in recent years and infer the status of tumor microsatellites from tumor or tumor/normal genome sequencing data. By utilizing computational software tools, these methodologies allow automatic detection of somatic microsatellite changes. MSIsensor, for example, is a program first proposed by Niu et al.[54] that assesses the number and length of homopolymers/microsatellites within the targeted regions of tumor-normal sample pairs. Other programs that similarly perform tumor-normal comparisons of repeat length distribution of microsatellites include MSIseq[55], MOSAIC[56], MANTIS[57] (5), a model by Cortes-Ciriano et al.[58], MSIsensor-ct[59], and MiMSI[60]. As paired tumor-normal sequencing may not be feasible for a lot of academic institutions and commercial labs, researchers have developed MSI prediction tools that do not rely on paired tumor-normal data, such as mSINGS[61], MIRMMR[62], MSI-pred[63], MSI-ColonCore[64], MIAmS[65], a model by Pang et al.[66] and MSIsensor-pro[67]. A detailed summary of these NGS-based MSI detection methods is presented in Table 4.

These programs can then be incorporated into the clinical NGS platforms and, at some institutions, serve as an alternative test modality for MSI in solid tumors. MSK MSIsensor for example applies the MSIsensor program[54] to all available genomic microsatellites covered by MSK-IMPACT (a custom targeted sequencing platform in clinical use at Memorial Sloan Kettering Cancer Center) within tumor samples against the matched normal DNA. The result is a continuous rather than categorical MSI score assignment for the tumor sample. Loci are considered unstable if *k*-mer distributions are significantly different between

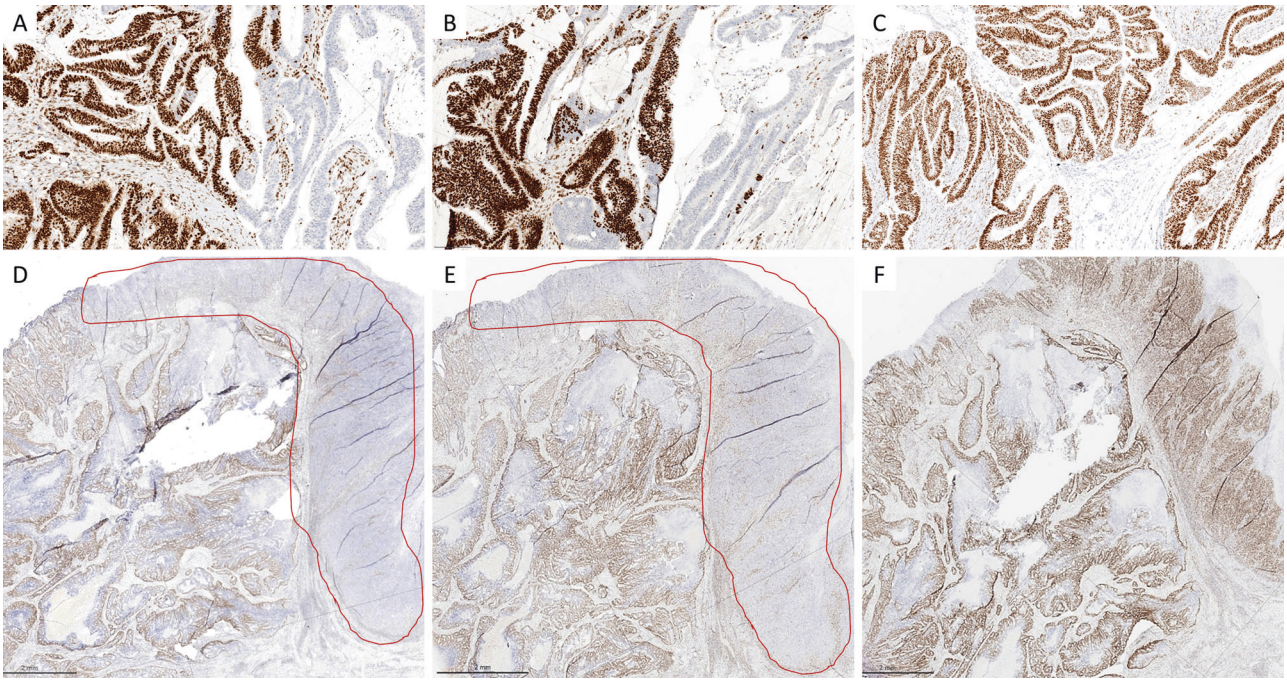

Fig. 3 Two colorectal adenocarcinomas showing distinct clonal loss of specific mismatch repair proteins by immunohistochemistry. In patient 1 (**A**–**C**), the clonal loss involves both MSH2 (**A**) and MSH6 (**B**), while the staining for MLH1 (not shown) and PMS2 (**C**) is normal. Genetic testing in this patient has not been performed. In patient 2 (**D**–**F**), the clonal loss involves both MLH1 (**D**) and PMS2 (**E**), while the staining for MSH2 (not shown) and MSH6 (**F**) is normal. This patient was negative for germline pathogenic mutation, the clonal MLH1/PMS2 loss is likely due to clonal *MLH1* promoter methylation.

Table 2. Definitions for immunohistochemistry of mismatch repair protein deficiency (MMRd).

Traditional definition	MMRd = Complete loss of nuclear staining in the tumor ***All absent = abnormal*** (implying: Partially present = normal)
Alternative definition	MMRd = Either complete loss or distinct clonal loss of nuclear staining in the tumor All absent or partially absent = abnormal (implying: ***All present = normal***)

the tumor and matched normal using a standard multiple testing correction of χ^2 *p*-values. The percentage fraction of unstable sites is reported as the MSIsensor score and based on validation data, the assay uses a MSIsensor score threshold of 10 or greater to define MSI-H[68].

Advantages

A common advantage of all MSI testing modalities is their ability to detect the functional status of the MMR system. They inform whether the system is dysfunctional and are not limited to protein expression. Cases in which the MMR deficiency is due to inactivation of genes other than the four major ones tested by MMR IHC can be picked up by these modalities.

For PCR-based MSI testing, a few salient features are generally accepted as advantageous. They include:

- Fast turnaround time. Some newer platforms now have a turnaround time comparable to MMR IHC.
- Decreasing cost. The cost of some platforms is now comparable to MMR IHC.
- Ability to detect MSI-H and separate that from MSI-L and MSS.
- Amenability to external proficiency testing.
- In general high reproducibility.

For NGS-based MSI detection programs, commonly accepted advantages include:

- Multi-functionality. It allows simultaneous detection of tumor mutation burden (TMB), and additional gene alterations that may be clinically actionable. The detection of somatic alterations in the MMR genes and/or in *BRAF* can inform LS diagnostics in certain scenarios.
- "Quantification" of the degree of MSI. The MSIsensor via MSK-IMPACT[16], for example, calculates a numeric score (>10 = MSI-H, 3–10 = MSI-I, < 3 = MSS). Although not backed by direct data, some cases in the MSI-I category may overlap with MSI-L by PCR methods. This carries implications in clinical application (see below).
- Amenability to enlarged microsatellite panels including microsatellites other than mononucleotide repeats that may help discover novel DNA repair mechanism failures beyond the current understanding of MMR loss.
- As NGS-based mutational testing has become a standard clinical practice for cancer patient management, especially for late-stage cancers where standard therapeutic approaches are not available, determining MSI status using the methods in Table 4 does not increase the wet-lab cost and MSI detection can be easily implemented in the routine bioinformatics pipeline. Therefore, detection and reporting MSI status by NGS testing can provide guidance on immunotherapy and identify potential LS patients.

Caveats and limitations

Both the PCR-based and the NGS-based MSI detection methods have issues that warrant attention.

- One size may not fit all. Currently, all tumor types are subject to the same marker panel and cutoff values for any given MSI testing platform, typically calibrated according to CRC

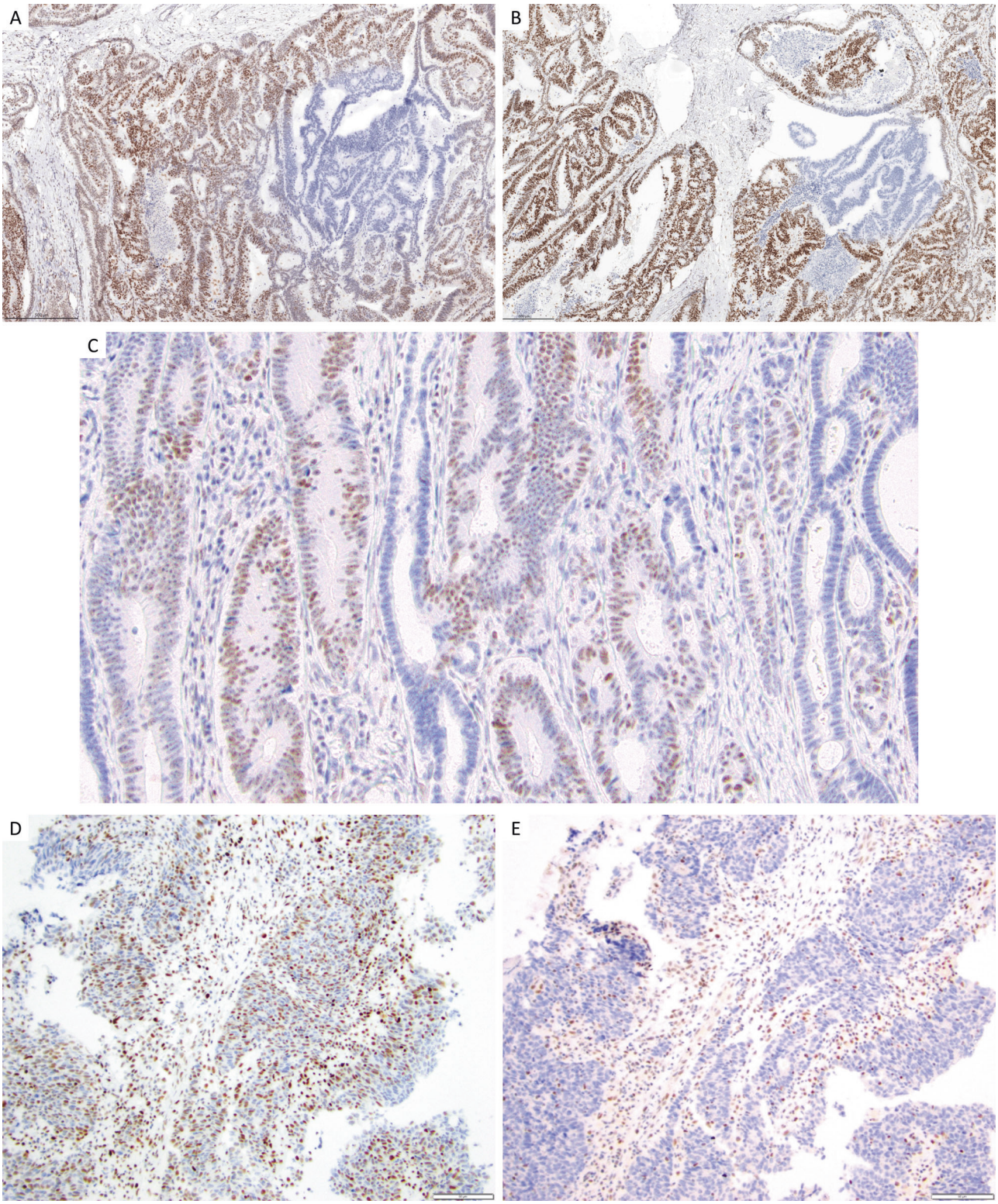

Fig. 4 Examples of mismatch repair protein immunohistochemical staining variability. In case 1 (**A**, **B**), the region without staining is likely due to poor tissue fixation (or other technical issues) as indicated by the lack of staining not only in tumor cells but also in internal control cells. In this case, the phenomenon affected all four proteins (shown here are MSH6 in **A** and PMS2 in **B**). This tumor was microsatellite stable. In case 2 (**C** MLH1), scattered individual glands or stretches of cells within individual glands show lack of staining, resulting in an indistinct pattern of staining heterogeneity. It is unclear what biological implications this phenomenon carries. This particular tumor was microsatellite stable and there was no evidence of germline mutation. In case 3 (**D**, **E**), the MSH6 staining (**D**) appears heterogeneous, with some tumor cells showing loss of staining while other tumor cells have retained staining with good intensity, but the MSH2 staining is lost (**E**). The etiology of the heterogenous MSH6 staining is unclear, but the phenomenon underscores the importance of using the four-antibody panel (as opposed to the two-antibody panel) in tumor testing.

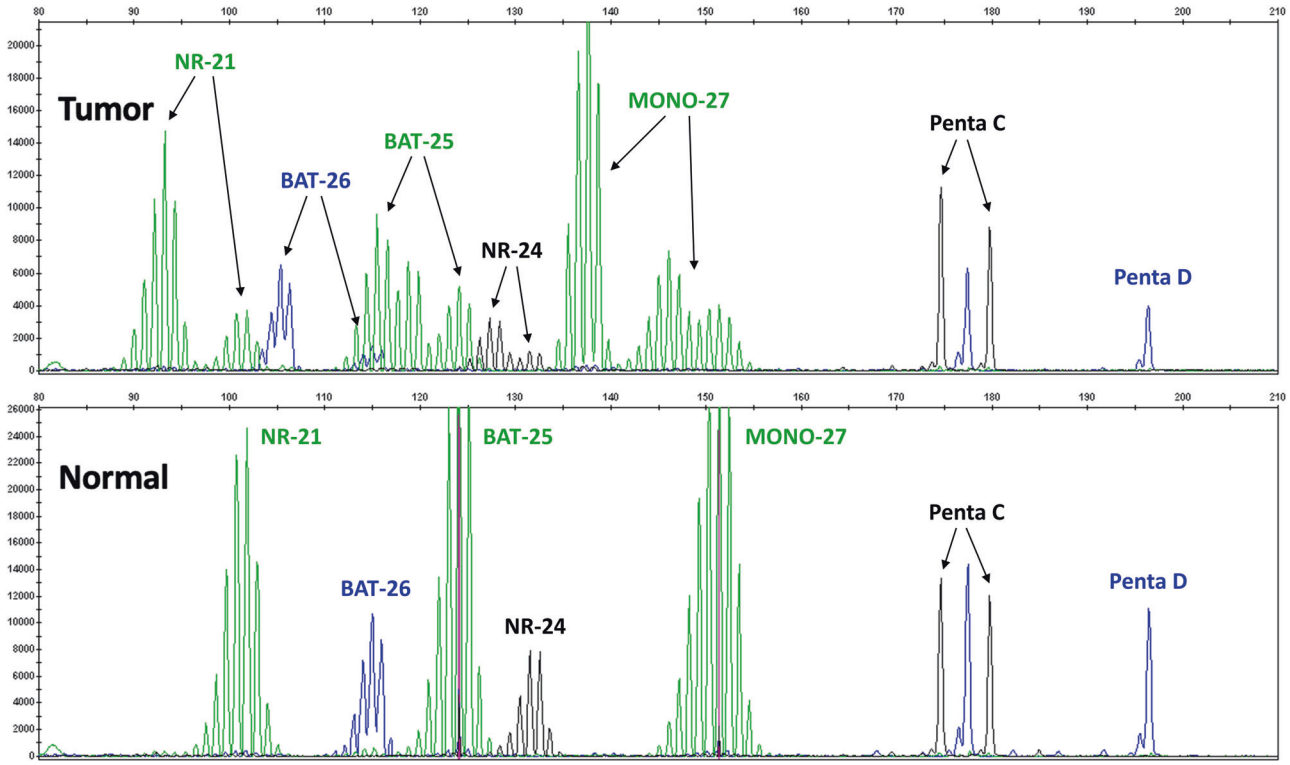

Fig. 5 Representative electropherograms of the Promega MSI Analysis System showing MSI-high by PCR and fragment analysis. Top panel: tumor tissue; bottom panel: matched normal tissue. The shifted alleles are indicated by arrows. Green: electropherogram showing the peaks of 2′,7′-dimethoxy-4,5-dichloro-6-carboxyfluorescein (JOE)-labeled loci, NR-21, BAT-25, and MONO-27. Blue: electropherogram showing peaks of the fluorescein-labeled loci, BAT-26 and Penta D. Black: electropherogram showing the peaks of tetramethyl rhodamine (TMR)-labeled loci, NR-24 and Penta C.

samples. This may not be the most optimal because differences exist in the degree of MSI between CRC and other tumor types. This may have resulted in the low sensitivity in detecting MSI in non-colorectal neoplasms by some or all MSI testing platforms.

- Related to the above, the specific MSI profiles may also differ across tumor types. Endometrial cancers, for example, harbor smaller size deletions or insertions in microsatellite regions than CRC[69]. This results in a smaller number of additional peaks on post-PCR fragment length analysis, or subtle alternations on the integrative genomics viewer. These minimal shifts, therefore, call for extra vigilance in the interpretation of these results[12].
- MSI detection assays in general require a minimum of 20% tumor content; non-colorectal samples may require even higher tumor cellularity[12].
- Some assays require matched normal DNA.
- MSI detection assays do not have tissue correlation. In cases with clonal MMR loss, the MSI results may be equivocal depending on the relative proportion of the MMRd and MMRp components captured in the sample being tested.
- MSI detection cannot imply the defective MMR gene.
- The multitude of assay platforms, while serving to foster continued improvement of test accuracy, can cause difficulties in standardization and cross-sectional comparison.
- All tests require well-established molecular facility and expertise, including sophisticated bioinformatics protocols in the case of NGS-based assays. The NGS platform also has a slow turnaround time.

Summary

MSI testing allows the detection of the functionality of the MMR system, and can identify functional deficiencies beyond the presence or absence of the four major MMR proteins. However, limitations exist. As MSI-H/MMRd has become a tissue-agnostic indication for immunotherapy, MSI testing is expanding increasingly beyond its traditional use in CRC. In this context, one current issue that particularly deserves attention is the presence of MSI heterogeneity across tumor types. This heterogeneity can manifest in the degree and/or the specific profiles of MSI. Many of the existing test platforms are developed and validated primarily for CRC. Simple adoption of these tests to other tumor types may result in under-detection of certain unique forms of MMR abnormality in unique tumor types.

SHOULD IT BE MMR IHC, MSI TESTING, OR BOTH?

Comparisons of MMR IHC vs. MSI testing

In detecting MMR loss in solid tumors, be it for LS screening or immunotherapy eligibility, a persistent question is what constitutes the best testing strategy.

Numerous studies in the literature have compared the concordance between MMR IHC and MSI testing, but many of these studies have limitations. Common issues are inconsistent test methodologies, small case numbers including a lack of large population-based cases with known germline or other molecular data for the evaluation of true test specificity, and significantly, over-representation of CRC (often enriched for typical LS tumors or *MLH1*-methylated cases) in analyses that aim to address MSI pan-cancer. Nonetheless, the available data do suggest a high sensitivity and specificity for both test modalities in detecting MMR loss (the sensitivity in predicting LS is estimated at 94% and 85% for MMR IHC and PCR-MSI, respectively, with the specificity at >90% for both)[15,70–72], and a high concordance rate between the two tests, at about 95%[73]. At the same time, discordances occur, and each of the two test modalities will miss

Table 3. Summary of PCR-based MSI testing systems.

Method	Microsatellite markers	Paired normal	Marker amplification	Readout strategies	TAT	Advantages	Limitations
NCI/Bethesda PCR-MSI testing[1,5]	BAT-25, BAT-26, D5S346, D2S123, D17S250	Yes	Fluorescent multiplex PCR	CE	~10 h	- Well-studied	- Established for CRC - Lower sensitivity compared to newer panels
OncoMate™ MSI Dx Analysis System (Promega*)[50]	BAT-25, BAT-26, MONO-27, NR-21, NR-24; Penta C and Penta D (for sample authentication)	Yes	Fluorescent multiplex PCR	CE	10 h	- Quasi-monomorphic markers - Better performance than NCI/Bethesda panel and considered a "gold standard"	- LOD 15%, FFPE tissue volume: 0.1–2.0 mm^3 - Intended for CRC
Idylla™ MSI Assay (Biocartis†)[72]	ACVR2A, BTBD7, DIDO1, MRE11, RYR3, SEC31A, SULF2	No	Real-time (RT) PCR	HRM	2.5 h	- Cross-ethnicity monomorphic markers - High concordance with IHC and other MSI tests for CRC - Automated from loading of FFPE to reporting, hands on time <2 min	- LOD 10%, FFPE tissue volume: 0.25–3.0 mm^3 - Intended for CRC - Lower sensitivity for non-CRC tumor types (e.g., endometrial cancers showing MSH6d[8])

CE capillary electrophoresis, *CRC* colorectal cancer, *FFPE* formalin-fixed paraffin-embedded, *HRM* high resolution melting analysis, *IHC* immunohistochemistry, *LOD* limit of detection, *MSH6d* MSH6 deficient, *MSI* microsatellite instability, *NCI* National Cancer Institute, *PCR* polymerase chain reaction, *TAT* turnaround time.
*OncoMate™ MSI Dx Analysis System, formerly MSI Analysis System (Promega, Madison, WI, USA).
†Idylla™ MSI Assay (Biocartis, Mechelen, Belgium).

a small but not insignificant number of cases (estimated to be 5–10%)[16,36,73].

Two major discordant patterns are (1) MMRp/MSI-H, and (2) MMRd/non-MSI-H.

MMRp/MSI-H is commonly a result of "false normal IHC" due to misinterpretation or biological factors such as germline and/or somatic mutations in MMR genes that lead to loss of MMR function but do not affect the antigenicity of the protein, or MMR loss caused by genes other than the four tested by IHC.

MMRd/non-MSI-H could also be related to technical or interpretational issues, i.e., false IHC or MSI test results. However, a biological etiology also exists[74–77].

It has long been recognized that certain non-colorectal/non-endometrial LS-associated tumors (typically less proliferative tumors) may be MMRd—losing the syndrome-defining MMR protein on IHC, but not always exhibiting detectable MSI-H[76]. A recent analysis of 15,045 tumor samples encompassing >50 tumor types[77] showed that about 30% of the non-colorectal and non-endometrial LS-associated tumors do not have MSI-H (by the MSK-IMPACT MSIsensor program[68]), instead they have "MSI-I" (MSK-IMPACT MSIsensor score in the range of 3–10). In fact, in this study, up to 29% of the LS-associated endometrial cancers also had only MSI-I. In contrast, of all the LS-associated CRCs tested, 96% were MSI-H and only 4% were MSI-I. By IHC, all of the tested MSI-I LS-associated tumors, including non-canonical tumors, exhibited loss of MMR protein, indicating that these tumors are indeed MMRd. These MMRd/non-MSI-H tumors have two important clinical implications.

First, for the purpose of LS detection, tumors found to exhibit MSI-I may warrant further IHC testing irrespective of tumor type, and if IHC shows MMRd, the patient should undergo LS germline testing despite the non-MSI-H result.

Second, for the purpose of predicting response to immunotherapy with checkpoint inhibitors, the implication of MSI-I (or MSI-L) is still to be defined. Literature data exist[78] that the lower degree of MSI in MSI-I tumors (typically accompanied by a lower TMB) may not be sufficient to render the tumor responsive to anti-PD1 treatment, even though the tumor is MMRd by IHC; only fully developed MSI-H (with accompanying high TMB) may confer tumor sensitivity to such treatment. Currently, the regulatory approvals for use of anti-PD1 therapy in advanced solid tumors apply to tumors that are either MSI-H or MMRd[53]; the inclusion of these MMRd/non-MSI-H tumors could therefore account for the lack of response in some patients. This calls for further investigation and clarification.

Recommendations

At present, the National Comprehensive Cancer Network (NCCN) recommends screening for MMR status either by IHC or by MSI-PCR[79,80], whereas the ESMO consensus recommendation (in the context of immunotherapy)[52] calls for MMR IHC to be the first-line test and molecular testing be performed when IHC is doubtful.

Given the intricacies related to MMR IHC versus MSI testing and the clinical implications of the different patterns of results, we regard both test modalities as essential, and think both should be maintained in the clinical and molecular diagnostic laboratories (hospital-based or reference laboratories). When dealing with patients who are at high risk for LS, both tests may be necessary (i.e., if one test shows normal results, the other should also be performed). In most other scenarios, the two tests can be used sequentially as recommended by ESMO (Fig. 6). MMR IHC has sufficient advantages to be used as the first-line method, and MSI testing can follow if the IHC is inconclusive or if the IHC is normal but there is clinical or pathological concern (e.g., positive family history concerning for Lynch syndrome, or tumor morphology suggestive of MMRd/MSI-H). However, the decision about first-line test also hinges on local resources and expertise. In places where MSI testing is well-established, MSI should yield similar efficacy as

Table 4. Summary of NGS-based MSI detection methods.

Method	Year	Description	Input	Output	Statistical method	Additional information
MSIsensor[54]	2014	Homopolymers of at least 5-bp length and microsatellites of maximum repeat unit length 5 from the reference genome are recorded. A site with at least 20 reads in both the tumor and normal samples is tagged as somatic if the distribution of repeat lengths is significantly different. The percentage of somatic sites out of the total sites with sufficient read depth is the MSIsensor score.	T-N	Percentage of unstable sites	Chi-square	Good correlation between MSIsensor score and the experimental measurements of MSI in 242 endometrial tumor-normal pairs
mSINGS[61]	2014	For each mononucleotide microsatellite locus, the number of differently sized repeats in experimental samples are quantified and compared to a population of normal controls. Loci are considered unstable if the experimental number of repeats is statistically greater than in the control population. MSI status is determined by the fraction of unstable microsatellite loci.	T	Percentage of unstable sites	Z-score	Performance evaluated on three panels compared to PCR-based MSI are reported to have good sensitivity (96.4% to 100%) and specificity (97.2% to 100%)
MSIseq[55]	2015	Decision tree analysis depends only on the number of somatic length-change mutations in simple repeats per Mb; tumors with >0.395 unstable sites are classified as MSI-H.	T-N	Percentage of unstable sites	Machine learning frameworks	Decision tree had 98.6% concordance with PCR-based MSI tests in 163 tumors (gastric, colon, rectal and endometrial cancers)
MOSAIC[56]	2016	Uses only the top 100 most significantly unstable microsatellites based on mSINGS to predict clinical MSI-H or MSS diagnosis. The power of each microsatellite locus to differentiate between MSI-H and MSS tumors is calculated using Fisher's exact tests.	T-N	The top most unstable sites	Fisher's exact tests, decision tree model	96.6% accuracy (gastric, colon, rectal and endometrial cancers)
MANTIS[57]	2017	Each microsatellite locus is evaluated separately, with the normal and tumor read distributions normalized to a fraction of each one's total reads, to account for any differences in sequencing depth and coverage. Once the scores for each locus are assigned, the average of all the locus instability scores is calculated, to provide a single numerical value representing the average aggregate instability present in the sample.	T-N	Aggregate score	Average distance	- Higher sensitivity/specificity compared to MSIsensor and mSINGS - Saves space/memory - Faster
MIRMMR[62]	2017	MIRMMR predicts microsatellite instability status in cancer samples using methylation and mutation information. MIRMMR performs full logistic regression model building for the purpose of MSI status prediction via binary classification. Building a prediction model highlights genes contributing to the MSI phenotype, and users can set intuitive classification thresholds based on probabilities.	T	Binary classification	Logistic regression model	- A cutoff score of 0.1922 gives good sensitivity (91.9%) and specificity (94.2%) - Similar AUC for MIRMMR (0.9727), mSINGS (0.9799), and MSIsensor (0.9977)
Model by Cortes-Ciriano et al.[58]	2017	This study analyzed the extent and characteristics of MSI in 8,000 exomes and 1,000 whole genomes spanning 23 tumor types, utilizing data from The Cancer Genome Atlas (TCGA). Random forest models were used to build binary classifiers for the prediction of MSI status. Each tumor was encoded with a vector recording the number of MSI events and the presence or absence of MSI events in 7,863 genes targeted by MSI in at least one sample.	T-N	Binary classification	Random forest models	The number of MSI events (i.e., MSI detection sensitivity) recovered decreases substantially when the coverage is reduced to 20–30x

Table 4. continued

Method	Year	Description	Input	Output	Statistical method	Additional information
MSI-pred[63]	2018	Python package for automatic MSI classification using a machine learning technology – support vector machine (SVM) based on data in mutation annotation format (MAF). The classifier has a radial basis function kernel for MSI classification using aforementioned 22 features of all tumors from the training set.	T	SVM classification	SVM	Overall accuracy of ≥98% and AUC of 0.967
MSI-ColonCore[64]	2018	Read-count-distribution-based approach that uses the coverage ratio of a specific set of repeat lengths as the main characteristic of each microsatellite locus, and categorizes a locus as unstable if the coverage ratio is less than a given threshold. The MSI status of a sample is determined by the percentage of unstable loci in the given sample.	T	Percentage of unstable sites	Read-count-distribution-based approach	97.9% sensitivity and 100% specificity compared with PCR-MSI
MIAmS[65]	2019	Designed to tag MSI status from Amplicon NGS of tumor samples using two independent classifiers (mSINGS and SVM). The five key features include: (i) log management, (ii) error recovery, (iii) distribution on cluster, (iv) analysis based on two distinct methods, and (v) a user-friendly interface to evaluate the results.	T	Binary classification	Z-score and SVM	Robust on two datasets with accuracy of 99% (ERP110591, CRC dataset) and 100% (ERP114314, data from two centers including CRC, endometrial, stomach and other cancers)
Model by Pang et al.[66]	2020	Microsatellite regions across the genome identified using the RepeatFinder tool and Python-based scikit-learn package with default parameters used for decision tree classifier generation. The number of altered microsatellite loci is used as a discriminating feature.	T	Number of unstable sites	Decision tree classifier	100% sensitivity and specificity
MSIsensor-pro[67]	2020	Introduces a multinomial distribution model to quantify polymerase slippages for each tumor sample and a discriminative site selection method to enable MSI detection without matched normal samples.	T	Fraction of unstable sites	Multinomial distribution model	More accurate than MANTIS, MSIsensor and mSINGS
MSIsensor-ct[59]	2021	For each sample, MSIsensor-ct reports the distributions of 1476 classifiable sites and binary classifiers are then applied to classify their stabilities. The percentage of unstable sites is the MSIscore.	T-N	Binary classification	Chi-square	100% accuracy within the limit of detection (LOD) of 0.05% ctDNA content (<3000x coverage)
MiMSI[60]	2022	MSI classifier based on deep neural networks and trained using a dataset that included low tumor purity MSI cases in a multiple instance learning framework. MiMSI's architecture can be separated into two main components. The first portion of the model creates an instance-level feature embedding for every microsatellite site, and the second calculates a bag-level embedding on which we calculate our final MSI prediction.	T-N	Binary classification	Deep convolutional neural network	MiMSI showed higher sensitivity (94%) and AUC (0.988) than MSIsensor

AUC area under the ROC (receiver operating characteristic) curve, *CRC* colorectal cancer, *T* tumor, *T-N* tumor-normal.

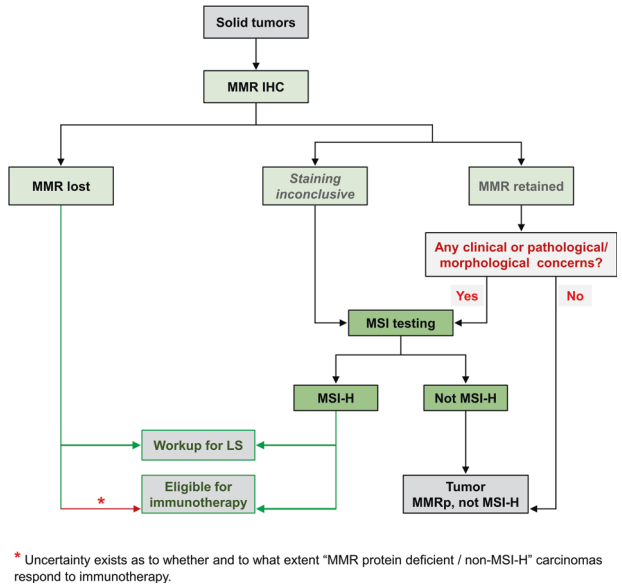

Fig. 6 A stepwise approach for detecting loss of mismatch repair (MMR) in solid neoplasms. IHC, immunohistochemistry; LS, Lynch syndrome; MMRp, mismatch repair-proficient; MSI-H, microsatellite instability-high.

IHC as the front-line test. Additionally, at institutions where validated NGS platforms are in use, NGS-based MSI testing could be the primary modality for cases where there is no clinical urgency for MMR results at the time of diagnosis (e.g., not needed to inform the immediate next-step treatment); this is particularly applicable to tumor types with low frequency of MMR abnormality and are therefore not otherwise routinely tested for MMR.

CONCLUDING REMARKS

The important clinical value of detecting the status of MMR in solid tumors is widely recognized. The current IHC and MSI testing methodologies have served to accurately detect this status in the vast majority of the cases. However, much remains to be improved. It is imperative that pathologists, and clinicians and investigators alike, keep abreast of the most up-to-date understanding of these tests and the caveats and limitations they bear. A recent report of an almost 10% false positive MMRd or MSI-H rate by local laboratories in metastatic colorectal cancer patients enrolled in immunotherapy trials[81] serves to further enforce this need. In these trials, the false results were associated with therapy failure.

Towards achieving the most accurate MMR IHC interpretation, some key points worthy of attention are as follows:

- A reliable internal positive control is mandatory for result interpretation.
- Consistent weak staining in the tumor cells, at an intensity lower than that of the surrounding stroma, should prompt additional testing (MSI or genetic testing). This is particularly true with MSH2, MSH6, and PMS2 IHC (Table 1).
- The old concept of "all absent = abnormal" is being challenged. Partial or clonal loss (especially when occurring in a distinct pattern) often also reflects gene abnormality.
- While awaiting standard guidelines, we suggest documentation of distinct clonal loss in routine pathology reports.

MSI testing, PCR- or NGS-based, is being continuously refined. Established methods have predominantly focused on and been optimized for CRC; constructing MSI markers or sequence panels that are tumor type-specific is challenging but warranted.

The best strategy in the utilization of MMR IHC vs. MSI testing will depend, in large part, on local resources and expertise. In general, the two test modalities should be regarded as complementary.

Particular attention is warranted towards the tumor variant that harbors MMRd but not MSI-H by current methodologies. These cases should be followed up with further workup to assess the possibility of LS if clinically indicated. Whether and how these MMRd/non-MSI-H tumors respond to immunotherapy remains to be clarified.

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JS conceived the review manuscript. All authors participated in reviewing the literature and drafting the manuscript. JS and CW revised the final manuscript which was approved by all authors.

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Correspondence and requests for materials should be addressed to Jinru Shia.

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