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Review

Immunohistochemical evaluation of mismatch repair proteins in colorectal carcinoma: the AIFEG/GIPAD proposal

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Key words

Colorectal adenocarcinoma • Mismatch repair proteins • Immunohistochemistry • Biomarkers • Lynch syndrome

Summary

Microsatellite instability (MSI) is a hypermutable phenotype that usually arises from either a germline mutation in components of the mismatch repair (MMR) machinery (i.e. hMLH1, MSH2, MSH6 and PMS2) in patients with Lynch syndrome (LS) or somatic hypermethylation of the hMLH1 promoter in sporadic carcinomas. In all colorectal cancers (CRC) is possible to identify the MMR deficiency through protein expression by immunohistochemistry (IHC). Recently, the predictive role of MMR deficiency in reduced chemotherapy benefit and the introduction of universal screening for Lynch syndrome suggest to include MMR testing into routine clinical practice. In this scenario is mandatory to update the minimal requirements for MMR IHC standardization and evaluation. According to international guidelines, these are the GIPAD and AIFEG suggestions for MMR IHC testing.

Introduction

Microsatellite instability (MSI) is a hypermutable phenotype that usually arises from either a germline mutation in components of the mismatch repair (MMR) machinery (i.e. *MLH1*, *MSH2*, *MSH6*, and *PMS2*) in patients with Lynch syndrome (LS) or somatic hypermethylation of the *MLH1* promoter in sporadic adenocarcinomas [1 2].
LS is an autosomal dominant genetic condition that has a high risk of colorectal cancer (CRC) as well as other cancers including endometrial cancer, ovary, stomach, small intestine, hepatobiliary tract, upper urinary tract, brain, and skin (3-5). Screening of CRCs for MSI and expression of DNA MMR proteins is an effective strategy to facilitate identification of patients with LS and, thus, has significant implications for cancer risk assessment and surveillance of affected patients and their at-risk relatives [3].
Nearly 15% of sporadic CRCs show MSI. MMR-deficient (MMRd) tumors have been demonstrated to be characterized by: i) a significantly improved prognosis in comparison to MMR-proficient (MMRp) cases; ii) a decreased response to 5-fluorouracil-based (5-FU) adjuvant chemotherapeutic regimens [6 7]. Recently, MSI status has been associated to a better response to anti-PD1 inhibitors in patients who failed conventional therapy [8 9].
Both LS screening and the widespread oncologists' request of MMR status for predictive evaluation of benefit from adjuvant chemotherapy has forced the introduction of MMR proteins immunohistochemical (IHC) evaluation into clinical practice. However, MMR IHC testing suffers from technical and staining interpretation issues [10]. Therefore, shared standardized protocols are mandatory.

The DNA mismatch repair complex

The DNA MMR complex maintains the genomic integrity by correcting base substitution mismatches and

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small insertion-deletion incongruities that are generated by errors in base-pairing during DNA replication [1 2 11]. Four of the MMR proteins form two functional key heterodimers consisting of MSH2/MSH6 and MLH1/PMS2. MSH2 may also form a heterodimer with MSH3. MMR machinery deficiency results in repair complex failure and the accumulation of numerous DNA replication errors (i.e., mutator phenotype), of which MSI is a marker.

Microsatellites are repeats (usually 10-60 times) of the same base or sequence of bases, with a unit length ranging from one to six bases, distributed throughout coding and noncoding regions of the genome. The human genome comprehends at least 500,000 microsatellites, and in normal conditions the number of repeats obtained in any microsatellite is the same in every cell of the body. This is not true for MMRd tumors, which are characterized by MSI that is the presence of widespread insertion or deletion mutations within microsatellite loci.

Lynch syndrome and MMR testing

CRC represents the most common neoplasm occurring in LS patients, and almost 3% of all CRCs arise in the scenario of a LS. The recent comprehensive characterization of the molecular landscapes occurring in familial CRC has pinpointed a broad spectrum of different molecular alterations, even harbouring clinical features similar to that observed in LS. Thus, the historical definition of "hereditary non-polyposis colorectal cancer" (HNPCC), which was previously interchangeably used with LS, now refers to different disorders that can mimic some clinical features of LS, but which are characterized by a different molecular background [3].

LS patients usually carry a germline mutation in one allele of a MMR gene; the second allele is subsequently inactivated by mutation, loss of heterozigosity or epigenetic silencing. More recently, also bi-allelic germline mutations in MMR genes have been described, and this peculiar condition has been called "constitutional mismatches repairs deficiency syndrome" (CMMRD). About 2-3% of LS results from germline deletion in the *EPCAM* gene, which is located immediately upstream of MSH2 [12 13]. A larger group, representing as 60-70% of cases in which LS is clinically suspected, and defined as "Lynch-like syndrome", is not characterized by an identified pathogenetic germline mutation but present a MMRd status. In the 70% of these "Lynch-like syndrome" cases, a somatic biallelic inactivation of MMR genes has been described (i.e. biallelic mutations, or mutation in one allele and LOH of the second one) [14-16]. Of note, a small part of these biallelic MMR genes inactivations have been identified in germline *MUTYH* gene mutated patients [17].

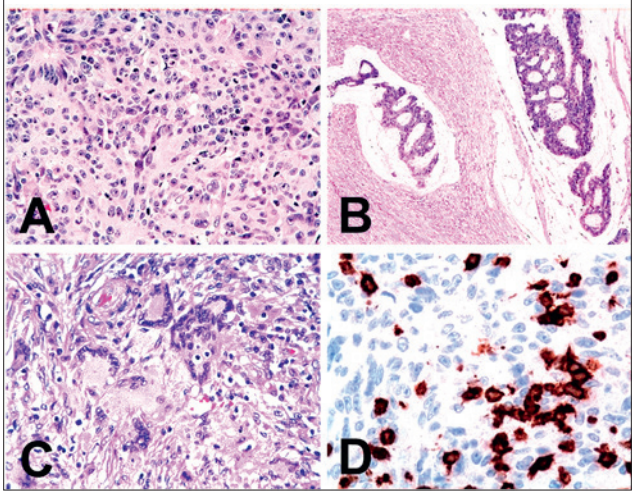
Screening for LS within the general population has unquestionable advantages for the definition of adequate secondary prevention strategies and personalization of medical care for the affected individual and family members [18]. Universal LS testing on incidental CCR patients is recommended by the most important international gastroenterological and oncological societies [19-23], and is likely to become the future standard of care in US and in several European nations. Two main approaches have been proposed: i) the IHC evaluation of MMR proteins followed by *BRAF* mutational analysis when MLH1 protein is absent, or ii) MSI phenotype testing. *BRAF*^{wt} MHL1/PMS2 negative, or MSH2/MSH6 negative tumors, should be referred to genetic counselling for consideration for germline testing.

To significantly reduce the costs and workload of the surgical pathology units, more focused LS screening approaches have been proposed: (a) screening all patients with CRC diagnosed in patients younger than 70 years; and (b) a selective approach of screening all patients with CRC diagnosed in patients younger than 70 years and in those above 70 years fulfilling the revised Bethesda guidelines [24 25].

The histopathology landscape of MMR-deficient colorectal cancer

Peculiar clinico-pathological features of MMRd tumors are: i) proximal colon location; ii) poor differentiation (i.e. G3); iii) presence of mucinous and signet ring cells differentiation; iv) medullary growth pattern; v) presence of tumor intraepithelial lymphocytes; vi) Crohn's-like lymphocytic reaction (Fig. 1) [10 26-31]. Also a significant intra-tumor phenotypic heterogeneity, expanding pattern of growth and lack of tumor budding have been associated with MSI [10]. Thus, histologic evaluation by expert gastrointestinal pathologists may improve the efficacy in detecting MMR deficiency [32]. However, a significant fraction of MMRd tumors do not present such MSI-specific phenotypes [33].

Fig. 1. CRC morphological characteristics suggesting a MSI status. A) Poor differentiation (G3). B) Mucinous histotype. C) Presence of giant cells around tumor cells (i.e., Crohn-like reaction). D) Evident intratumoral T cell infiltration (CD3 positive T lymphocytes are shown). Original magnifications 20x.



By the pathogenic point of view, LS carcinomas may develop from adenomas, may have abnormalities in *APC*, *CTNNB1* or *KRAS* genes, but *BRAF* mutations are extremely rare. On the other hand, sporadic MMRd tumors are not following the conventional adenoma-carcinoma sequence, but are frequently characterized by *BRAF* mutation, a CpG island methylator phenotype (CIMP) and originate from serrated polyps [34].

DNA mismatch repair machinery status testing

It is possible to identify MMR defective CRCs through molecular tests evaluating MSI or by IHC analysis of MMR proteins expression.

For MSI testing of CRC, a panel of five microsatellites (D2S123, D5S346, D17S250, BAT26 and BAT25) has been validated and implemented into clinical practice [35]. MSI has an overall reported sensitivity and specificity > 90% for LS [20 25]. According to this panel, CRCs are subclassified as MSI-High frequency (MSI-H), if two or more of the five microsatellite markers show instability, low frequency MSI (MSI-L) if only one of the five markers shows instability, and microsatellite stable (MSS) if none of the markers show instability. This panel remains the most commonly used among laboratories, although a variety of different panels or methods (such as the Pentaplex PCR) have been recently introduced.

As for MSI, MMR proteins IHC has an overall reported sensitivity and specificity >90% for LS [19 21 25]. An important advantage of IHC testing is that lack of a specific MMR protein can direct germline testing to that specific gene. A major disadvantage is that IHC accuracy is operator-dependent and is significantly affected by the experience and skill of the laboratory performing the testing.

In vitro studies demonstrated that MLH1 and MSH2 proteins are obligatory partners of the heterodimers. Usually, anomalies in MLH1/MSH2 result in proteolytic degradation of the heterodimers and consequent loss of both obligatory and secondary proteins. Of note, the isolated loss of PMS2 could be related to a germline mutation in the *hMLH1* gene [36 37]. On the other hand, when mutations occur in genes of the secondary proteins (i.e., PMS2 and MSH6), the heterodimers may retain stable and there is no concurrent loss of the obligatory partner proteins. This is because the function of the secondary proteins may be compensated by other proteins, such as MSH3 (instead of MSH6), MLH3 and PMS1 (instead of PMS2) [10]. Consequently, the PMS2 antibody detect all cases that harbour either MLH1 or PMS2 abnormalities [37]; and the MSH6 antibody detect all cases that harboured either MSH2 or MSH6 abnormality. MLH1 and MSH2 alone don't recognize cases that have PMS2 or MSH6 abnormalities. Some laboratories are performing initial screening using a limited two-antibody panel including only PMS2 and MSH6 to reduce the cost of the screening [10]. However, most institutions prefer to use the four-antibody IHC assay as MSH6 and PMS2 are technically more challenging and more frequently difficult to interpret.

Tumors showing loss of MLH1 expression should be further investigated for *BRAF* exon 15 mutational status and hypermethylation of *hMLH1* gene promoter to exclude sporadic cases and select the patients with suspected LS for genetic counselling and germline testing.

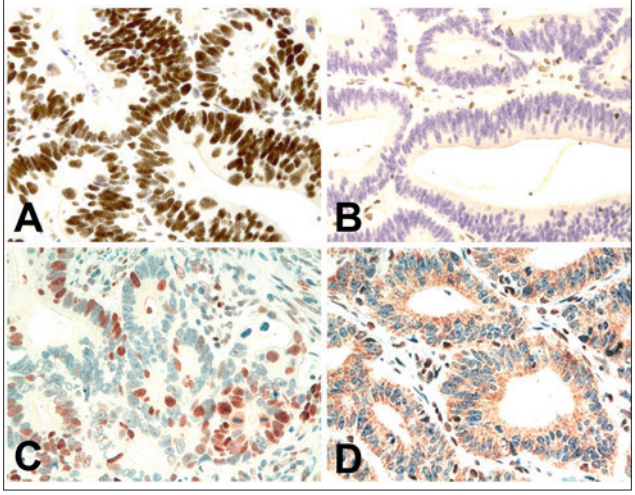
Evaluation of MMR proteins immunohistochemistry

Normal expression of MMR consists of neoplastic cells with strong nuclear immunoreactivity for all four markers. Note that DNA repair machinery is upregulated in cells with increased turnover, and so a strong expression is expected. IHC positive stain is defined as an unequivocal nuclear labelling in tumor cells with staining intensity comparable to that of internal control. A weak positive case is defined when IHC staining is visible as nuclear labelling in tumor cells, but its intensity is weaker that the internal control or only comparable to the intensity of the inert stromal cells.

Loss of protein expression is defined as complete absence of nuclear staining within tumor cells with concurrent positive labelling in internal non-neoplastic tissue (Fig. 2) [38]. A good gradient of expression should be demonstrated in normal crypts, fading out towards the lumen. Other internal controls are represented by positive stromal and lymphoid cells.

An important point to be considered in the optimization of IHC staining, is to use normal colon or appendix as positive controls and not lymphoid tissue (such as lymph nodes or tonsil). In fact, lymphoid follicles usually show a higher MMR proteins expression than colonic crypts. Hence, adequate staining intensity in tonsil may correspond to a weak staining of colonic mucosal cells.

Fig. 2. Representative MMR immunohistochemical expression patterns. A) Positive immunoexpression for MSH2. B) Negative expression for MLH1 (positive T lymphocites as internal control). C) Heterogeneity of PMS2 expression whitin carcinoma cells. D) Loss of nuclear MSH2 expression with a gain of cytoplasmatic staining. In C and D, the complementary heterodimer protein were negative, suggesting a MMR deficiency. Original magnifications 20x and 40x.



Tab. I. Immunoistochemical interpretation of mismatch repair proteins (MMR) expression.

MLH1	PMS2	MSH2	MSH6	Comment suggested to report in diagnosis
++ / +-	++	++ / +-	++	IHC staining suggests a MSS status
++	++/+-	++	++ / +-	
--	--	++	++	IHC staining suggest MSI status. *BRAF* exon 15 mutational analysis should be performed to exclude sporadic cases.
--	++ / +-	++	++	
++ / +-	--	++	++	
++	++	--	--	IHC staining suggest MSI status and patient should be referred to genetic counseling for Lynch syndrome.
++	++	--	++ / +-	
++	++	++ / +-	--	
+-	+-	++	++	IHC staining support MSI that should be confirmed by MSI molecular testing.
++	++	+-	+-	
+-	+-	+-	+-	IHC staining support MSI that should be confirmed by MSI molecular testing (For excluding technical artifacts IHC should be repeated to confirm the result before MSI analysis).
--	++	--	++	Biologically unlikely. For excluding technical artifacts IHC should be repeated and/or sent to MSI analysis.
--	++	++	--	
++	--	--	++	
++	--	++	--	
Normal expression= ++; "patchy"/weak expression = +/- loss expression = --				

In some instances, an abnormal MMR expression could be represented by a patchy and/or weak expression consisting of a nuclear loss associated with a gain in cytoplasmatic staining or a heterogeneous expression within adjacent tumor areas. Loss of nuclear expression or patchy expression in tumor cell nuclei, in the presence of a strong internal positive control in benign surrounding tissue, strongly supports the presence of a MMR gene mutation [39].

Other aberrant staining patterns, such as cytoplasmic staining, peri-nuclear staining, dot-like nuclear staining, and extremely patchy staining significantly affect IHC interpretation. In most cases, these features are related to technical problems (prolonged ischemia, delayed fixation, type and length of fixation, decalcification), and further underlay the importance of performing continuous quality control. Among the others, patchy MSH6 staining has been observed in tumors post neoadjuvant chemo or chemoradiation, due to tumor hypoxia or oxidative stress (10). Thus, for LS screening pre-treatment CRC biopsy samples should be preferred for IHC analysis.

Few studies report MMR status analysis among primary and metastatic samples. A MMR IHC concordance of 100% between MMRs primary CRC and their corresponding regional LN or distant metastasis has been demonstrated. The tumor cells do not change their MMR state from deficient to proficient not even if sporadically hypermethylated and even though some cases had their metastasectomy months or years after the primary CRC diagnosis [40].

MMR IHC in extra colonic tissue tends to be less robust than in colonic tissue; the internal control can appear weak because many extracolonic tissues are less proliferative than colonic tissue. Notably, not all extra-colonic tumors in LS will show microsatellite instability on PCR testing even when there is evidence of MMR protein loss. Thus, a negative MSI test on an extra-colonic tumor sample does not necessarily rule out the possibility of MMR deficiency [41].

Some laboratories are actually performing the limited PMS2/MSH6 IHC testing. However, to exclude fixation/tissue processing technical artefacts in "patchy/weak" cases, staining distribution of all four MMR proteins in both tumor and normal colon mucosa should be performed. In the case that both proteins of a heterodimer are patchy/weak is strongly recommended to test MSI. When all MMR markers show a patchy/weak immunoreaction is better to exclude a technical artefact and repeat the IHC staining before submitting the case for MSI testing (Tab. I).

Clinical aspects

Routine MMR IHC of CRC specimen is a reliable method for LS screening, which can be easily available in most Italian institutions. The management of mutation carriers allows for cancer risk reduction by intensified screening or prophylactic surgery which extend to at-risk family members. In addition, MMR status is another molecular marker to be introduced into the systematic approach of the clinical-therapeutic decision process (Tab. II).

Tab. II. Clinical impact of MMR on prognosis and management of patients affected by colorectal carcinoma.

MSI testing	MMR STATUS	*BRAF* mutation testing	Biological aspect	Clinical practice
MSS (85%)	MMR proficient	Mutant	Likely sporadic CRC, poor prognosis	i) if metastatic test *RAS* status (low probability of *RAS* mutation). Likely, will be use other targeted therapy than cetuximab
	MMR proficient	Wild type	Likely sporadic CRC	i) if metastatic test *RAS* status for using cetuximab
MSI-H (15%) (Sporadic and Lynch)	MMR deficient MLH1/PMS2	Mutant	Likely sporadic "serrated" CRC, poor prognosis	i) reduced 5-FU benefit
		Wild type	Suspected for Lynch syndrome, good prognosis	i) referring to genetic counselling for Lynch syndrome, ii) reduced 5-FU benefit, iii) if metastatic test *RAS* status
	MMR deficient MSH2/MSH6	To not perform	Suspected for Lynch syndrome, good prognosis	i) referring to genetic counselling for Lynch syndrome, ii) reduced 5-FU benefit

Final remarks and management of challenging cases

- All colorectal carcinoma regardless of patient's age should be tested for MMR IHC (Universal screening Lynch syndrome).
- Use of 4 IHC panel is recommended over the use of alternative limited IHC panels to overcome interpretation biases (The use of automated staining platforms is highly suggested).
- In the selection of the specimens to be analysed, prefer tumor samples with adjacent normal mucosa.
- Use appendix or normal colon mucosa instead of lymphoid tissues as IHC control samples.
- Avoid the selection of poorly-fixed central parts of the tumors.
- In patients underwent neoadjuvant therapy (*i.e.*, rectal carcinoma) IHC should be performed on pre-therapy biopsies.
- In cases with inconspicuous amount of neoplastic tissue, IHC testing should be performed only after multidisciplinary discussion (i.e., if M1 could be preferred testing *RAS* status).
- Metastatic lesions can be tested for MMR when primary CRC tissue is not available.
- Extracolonic tumors should not be test for MMR when primary CRC tissue is not available; It's to be preferred reclaiming colon tissue.
- IHC reports should be concluded with a statement on: i) marker expression results; ii) IHC interpretation; iii) further analyses to be performed (i.e. *BRAF* mutational analysis, *hMLH1* methylation status, MSI); iv) referral (or not) to genetic counselling (see Tables).
- When available, molecular tests evaluating MSI could be considered as alternative to MMR IHC analysis. Some MMR IHC expression profiles require the integration with MSI molecular tests to be informative on MSI status (see Tab. I).

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