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MSI testing and its role in the management of colorectal cancer

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Opinion Statement

TNM stage remains the key determinant of patient prognosis after surgical resection of colorectal cancer (CRC), and informs treatment decisions. However, there is considerable stage-independent variability in clinical outcome that is likely due to molecular heterogeneity. This variability underscores the need for robust prognostic and predictive biomarkers to guide therapeutic decision-making including the use of adjuvant chemotherapy. Although the majority of CRCs develop via a chromosomal instability pathway, approximately 12-15% have deficient DNA mismatch repair (dMMR) which is characterized in the tumor by microsatellite instability (MSI). Tumors with the dMMR/MSI develop from a germline mutation in an MMR gene (MLH1, MSH2, MSH6, PMS2), i.e., Lynch syndrome, or more commonly from epigenetic inactivation of *MLH1* MMR gene. CRCs with dMMR/MSI status have a distinct phenotype that includes predilection for the proximal colon, poor differentiation, and abundant tumor infiltrating lymphocytes. Consistent data indicate that these tumors have a better stage-adjusted survival compared to proficient MMR or microsatellite stable (MSS) tumors, and may respond differently to 5-fluorouracil-based adjuvant chemotherapy. To increase the identification of dMMR/MSI patients in clinical practice that includes those with Lynch Syndrome, it is recommended that all resected CRCs to be analyzed for MMR status. Available data indicate that patients with stage II dMMR CRCs have an excellent prognosis and do not benefit from 5-FU-based adjuvant chemotherapy which supports their recommended management by surgery alone. In contrast, the benefit of standard adjuvant chemotherapy with the FOLFOX regiment in stage III dMMR CRC patients awaits further study and therefore, all patients should be treated with standard adjuvant FOLFOX.

Keywords

colorectal cancer; DNA mismatch repair; microsatellite instability; adjuvant chemotherapy

Introduction

TNM stage remains the gold standard for informing patient prognosis and guiding management after resection for non-metastatic colorectal cancer (CRC). Despite the same disease stage, however, CRC patients exhibit considerable variability in clinical outcome that is likely related to molecular tumor heterogeneity. Therefore, the molecular

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Conflict of Interest

The authors declare no conflict of interest.

classification of CRC may identify patient subgroups at varying risk of recurrence and death and for who personalized approaches to therapy may beneficial. The majority of CRCs develop via the chromosomal instability pathway (CIN), whereas 12-15% arise from the microsatellite instability (MSI) pathway that is a consequence of deficient (d) DNA mismatch repair (MMR). Deficient MMR can develop from an inherited germline mutation in a MMR gene (MLH1, MSH2, MSH6, PMS2) i.e., Lynch Syndrome, or more commonly due to epigenetic inactivation of the *MLH1* gene and the CpG island methylator phenotype (CIMP). These sporadic dMMR tumors carry somatic mutations in the *BRAF* oncogene in approximately half of cases. Studies have shown that dMMR tumors have phenotypic features including poor differentiation, proximal colon location, and abundant tumor-infiltrating lymphocytes. Furthermore, dMMR tumors have been consistently associated with a better stage-adjusted survival compared to proficient MMR (pMMR) tumors.

Among early stage CRCs, the survival advantage of dMMR status appears to be greater among stage II compared to stage III patients. In patients with stage II colon cancers and dMMR, studies demonstrate a lack of benefit of adjuvant 5-FU-based chemotherapy. Among patients with stage III disease, the predictive impact of MMR status for adjuvant chemotherapy remains controversial. Multiple prior studies have demonstrating a lack of benefit for 5-fluorouracil (FU) as adjuvant chemotherapy, although only limited data exist for patients with stage III dMMR CRCs treated with standard the adjuvant FOLFOX regimen. In contrast to 5-FU, *in vitro* data indicate that dMMR/MSI CRC cell lines display sensitivity to oxaliplatin and accordingly, this agent may provide benefit in patients with dMMR CRCs.

DNA Mismatch Repair and Microsatellite Instability

The Cancer Genome Atlas (TCGA) Research Network has revealed a comprehensive characterization of the genomes of 224 cancerous colorectal tumors and normal pairs (1). Among CRCs studied, 16% of were found to be hypermutated, and 77% of these tumors displayed high frequency microsatellite instability (MSI-H) that was generally associated with hypermethylation and *MLH1* gene. The remaining hypermutated tumors were primarily characterized by having mutations in somatic MMR pathways and in polymerase epsilon (POLE)[1].

The DNA MMR system repairs base-base mispairs introduced into microsatellites during DNA synthesis to maintain genomic stability (2). Microsatellites are short, tandemly-repeated sequences that occur throughout the genome and are used as markers of deficient (d) MMR. The DNA MMR system is composed of 4 MMR genes and their encoded proteins (MLH1, MSH2, MSH6, PMS2). Inactivation of MLH1 and MSH2 account for over 90% of dMMR cases. Deficiency of MMR results in production of a truncated, nonfunctional protein or loss of a protein that causes MSI. Therefore, dMMR is frequently analyzed by testing for loss of an MMR protein or for MSI using a PCR-based assay.

MSI testing

MSI testing can be performed on fresh, frozen or paraffin-embedded tumor tissue using a PCR-based assay for detection of instability (3, 4).

- The National Cancer Institute Workshop agreed on five microsatellite markers necessary to determine MSI (5) that include two mononucleotide – BAT25/26 and three dinucleotide markers – D2S123, D5S346, and D17S250. Interpretation of the profiles requires a comparison with normal DNA from each patient. An alternative molecular method based exclusively on quasi-monomorphic mononucleotide markers was developed to avoid the analysis of matching normal DNA. This method has been proven to be more specific and sensitive than the original NCI panel (5).
- On the basis of the MSI status, CRCs can be classified into three groups: MSI-H, if two or more of the five microsatellite markers show instability; MSI-L (low-frequency MSI), if only one of five markers shows instability; and microsatellite stable (MSS) if none of the markers show instability (6).

MMR Protein Expression: Immunohistochemistry (IHC)

- Analysis of MMR protein expression by IHC is an alternative test that is widely available with the advantage of not requiring a molecular laboratory, and the ability to identify the affected gene by detecting loss of its protein product.
- Another advantage of IHC testing is that loss of a specific mismatch gene product (MLH1, MSH2, MSH6, and PMS2) can direct germline testing to that specific gene, and assists in the identification of patients with LS (4).
- MSI testing and IHC are complimentary, and loss of MMR protein expression by IHC has been shown to be highly concordant with DNA-based MSI testing with a good sensitivity (>90%) and a excellent specificity (100%) (4).
- Only loss of hMLH1 protein expression has been described in sporadic CRCs (7). MLH1 and PMS2 proteins are often lost together, which indicates loss of MLH1 function generally due to epigenetic silencing or germline mutation. Isolated loss of PMS2 protein generally indicates an underlying germline *PMS2* mutation.
- In CRCs with loss of MLH1 protein expression, testing for a mutation in the *BRAF* oncogene is the most cost-effective approach to confirm a sporadic case and generally exclude LS which support the use of this strategy for LS screening (8). Patients with non-mutated *BRAF* would then have germline testing for a mutation in the presumed altered *MLH1* gene (Figure 1).
- MSH2 and MSH6 proteins are often lost concurrently. Isolated loss of MSH2 or MSH6 proteins on IHC testing has high specificity for a germline mutation of these genes leading to the diagnosis of LS (Figure 1). Also, loss of the MSH2 protein can be caused by germline mutation in the *EPCAM* gene rather than *MSH2* gene.
- Tumors displaying loss of an MMR protein can be collectively referred to as dMMR and are considered to be MSI-H, whereas those with intact MMR proteins can be classified as pMMR are are expected to be microsatellite stable (MSS) or MSI-low (MSI-L).

➢ **Lynch Syndrome (LS)**

- LS accounts for approximately 3-4% of all CRCs and one third of all dMMR/MSI-associated CRCs. LS is inherited in an autosomal dominant manner and results from a germline loss-of-function mutation (9) that occurs more commonly in *MLH1* or *MSH2*, and infrequently in *MSH6* or *PMS2* (10). A germline mutation in an *MMR* gene followed by a second hit (somatic event) to the wild-type copy is needed to produce LS, and can occur due to point mutation, loss of heterozygosity or methylation.
- Patients with LS develop early age at onset of CRCs, and rates of synchronous CRCs increase with age.
- Patients with LS are at highest risk of developing CRC followed in frequency by endometrial carcinoma. Patients are also at increased risk of cancers of the stomach, ovary, urinary tract, small intestine, and prostate (11). The estimated cumulative risk of CRC by age 70 years for LS patients was approximately 50% in case of *MLH1* or *MSH2* mutations (12) (Figure 1).
- CRCs from LS patients are significantly less likely to carry *KRAS* mutations compared to pMMR/MSS cancers and importantly, $BRAF^{V600E}$ mutations are lacking in these patients. Among dMMR/MSI CRCs, $BRAF^{V600E}$ mutation testing can be performed to distinguish LS cases from sporadic dMMR tumors (13) (Figure 1).
- Bethesda criteria were revised in 2004 to guide selection of patients for LS testing (14). The guideline indicated that tumors should be tested for MSI in the following clinical situations:

    1. CRC diagnosed in a patient who is less than 50 years of age.
    2. Presence of synchronous, metachronous colorectal, or other LS-associated tumors, regardless of age.
    3. CRC with the MSI-H histology diagnosed in a patient who is less than 60 years of age.
    4. CRC diagnosed in one or more first-degree relatives with an LS-related tumor, with one of the cancers being diagnosed under age 50 years.
    5. CRC diagnosed in two or more first- or second-degree relatives with LS-related tumors, regardless of age.

- Patients with suspected hereditary CRC should be referred for genetic counseling, where the identification of germline mutations and evaluation/screening of family members can be appropriately addressed.
- Families that meet the Amsterdam Criteria (15) but who lack a germline mutation in an *MMR* gene and an MSI-H tumor, have been termed familial CRC, type × (16).

➢ **Sporadic dMMR CRC**

- Approximately 12-15% of all CRCs have an MSI-H phenotype and about two-thirds of these MSI-H tumors are sporadics.
- Sporadic MSI-H CRCs show loss of *MLH1* that generally occurs in a background of the CpG island methylator phenotype (CIMP) (17, 18). CIMP represents dense promoter hypermethylation of cancer-specific genes. CIMP-related silencing of the *MLH1* gene is responsible for about 80% of cases in which MLH1/PMS2 protein expression are lost (7).
- Approximately 50% of sporadic dMMR cases harbor *BRAF (V600E)* mutations (19, 20) that indicate a sporadic origin and thereby distinguish them from LS cases (13).
- Patients with MSI-H sporadic CRCs share most of the clinicopathological features with LS cases, yet have distinct epidemiological features including older age at diagnosis, predominance of female gender and increased rate of cigarette smoking (21).

Phenotypic features of deficient MMR CRCs

- CRC patients with dMMR tumors have distinct clinical and pathologic features compared with their proficient MMR (pMMR) counterparts, including proximal colon predominance, poor differentiation and/or mucinous histology, increased numbers of tumor infiltrating lymphocytes, and diploid DNA content (2).
- CRC with dMMR is more frequent in stage II (almost 20%) compared to stage III (12%), and are relatively uncommon among metastatic tumors (4%) (22). This highlights the importance of MSI testing in early stage disease where patients can be potentially cured by surgery alone or combined with adjuvant chemotherapy.

Prognostic value of MMR status

- Multiple retrospective and population-based studies have shown that patients with dMMR CRCs have a more favorable stage-adjusted prognosis than those with pMMR tumors (23-27).
- A meta-analysis from 32 studies with 1,277 MSI/dMMR cases included 7,642 patients with stage I-IV CRC. A better prognosis was found for patients with MSI/dMMR than those with MSS, MSI-L/pMMR tumors (28) among patients that were untreated or treated with 5-fluorouracil (5-FU)-based adjuvant chemotherapy. The hazard ratio (HR) for overall survival (OS) was 0.65 [95 % confidence interval (CI), 0.59-0.71] in favor of dMMR CRC patients. Results were confirmed when the analyses was restricted to patients with stage II or III CRC participating in clinical trials (28).
- Important in the biology of CRC are somatic mutations in the *KRAS* and *BRAF* oncogenes and the status of the DNA MMR system. Recently, a couple of studies examined the utility of combining these molecular markers to subtype of CRC for prognosis (29, 30). One study analyzed stage III colon cancer patients from an adjuvant trial of FOLFOX-based chemotherapy (29). The study found patients with

proficient (p) MMR tumors without *BRAF* or *KRAS* mutations had similar 5-year DFS rates as did dMMR sporadic or familial subtypes. In contrast, those patients whose tumors had mutated *KRAS* or *BRAF* and were pMMR exhibited poor 5-year DFS rates. Similar results were found in another study that investigated the association between CRC subtypes and survival in a large population-based registry cohort of patients that including analysis of the CpG island methylator phenotype (CIMP) (30).

- Studies indicate that the better prognosis of dMMR CRCs is more apparent in earlier stage tumors (31).

Treatment

5-FU based adjuvant chemotherapy

- A fluoropyrimidine (5-FU or capecitabine) combined with leucovorin is considered as standard care for patients with stage II CRC. Data indicate that 5-FU–based adjuvant chemotherapy is ineffective in stage II CRC patients with dMMR (32), consistent with the preclinical data showing that dMMR is associated with 5-FU resistance in CRC cells (33-38).
- The prognostic/predictive value of dMMR was investigated in 457 stage II and stage III CRC patients from five randomized trials evaluating 5-FU + levamisole or leucovorin as adjuvant chemotherapy *vs* surgical treatment alone (39). Overall, patients with dMMR *vs* pMMR cancers had significantly better survival, yet dMMR patients with stage II and stage III tumors did not benefit from 5-FU-based adjuvant therapy. These findings were maintained in a pooled analysis (Table 1) that combined the cases above with those from a prior study from the same group (40). In the combined dataset of 1027 CRC patients, those with dMMR showed with more favorable outcome compared to pMMR cancers (DFS: HR, 0.51; 95% CI, 0.29 to 0.89; $P = .009$; OS: HR, 0.47; 95% CI, 0.26 to 0.83; $P = .004$)
- In stage II CRC patients treated with 5-FU-based adjuvant chemotherapy *vs* surgery alone in the Quick and Simple and Reliable (QUASAR) trial, the recurrence rate for dMMR tumors was half that for pMMR tumors [11% (25/218) *vs* 26% (438/1,695); RR, 0.53; 95% CI, 0.40 to 0.70; $P < .001$) (41). Of note, the reduced risk of recurrence with chemotherapy did not differ significantly by MMR status (42).
- In a study that included 2,141 stage II and stage III colon cancers from randomized trials of 5-FU-based adjuvant therapy, patients with dMMR tumors was associated with reduced rates of tumor recurrence, delayed TTR, and improved survival rates compared with patient with pMMR cancers (43).
- A pooled data analysis from the ACCENT database involving 7,803 stage II and III CRC cases revealed that among stage II patients who received surgery alone, dMMR was strongly associated with delayed TTR (HR = 0.27; 95% CI, 0.10 to 0.75; $P = 0.01$) and improved OS (HR = 0.27; 95% CI, 0.10 to 0.74; $P = 0.01$) compared to pMMR, but not in stage III CRC patients (HR = 0.59; 95% CI, 0.28 –

1.23; *P* = 0.162) (44) (Table 1). In stage II CRCs treated with 5-FU adjuvant chemotherapy, TTR or OS did not differ between dMMR and pMMR (TTR, HR = 0.81, 95% CI, 0.55 to 1.19; *P* = 0.29; OS, HR = 0.87; 95% CI, 0.61 to 1.26; *P* = 0.47). In stage III CRC, however, patients with dMMR cancers treated with adjuvant 5-FU had better outcome compared to pMMR tumors (TTR, HR = 0.80, 95% CI, 0.66 to 0.97; *P* = 0.025; OS, HR = 0.79; 95% CI, 0.65 to 0.97; *P* = 0.023).

- The favorable prognosis and the evidence of lack of benefit from 5-FU based adjuvant chemotherapy in stage II CRC patients with dMMR indicate that these patients should not receive adjuvant chemotherapy.

5-FU plus Oxaliplatin Adjuvant Therapy

- In stage III colon cancer patients, oxaliplatin combined with 5-FU is the current standard of care for adjuvant chemotherapy (45-47).
- In contrast to 5-FU, sensitivity to oxaliplatin was independent of the MMR system in CRC cell lines(48). Retrospective analyses of stage III colon cancer patients who received adjuvant FOLFOX suggest that dMMR CRCs maybe sensitive to oxaliplatin (49, 50); however, only limited data from prospective clinical trials evaluating oxaliplatin-based treatment are available (51, 50, 52).
- An analysis of 2,299 stage II and stage III CRC patients from National Surgical Adjuvant Breast and Bowel Project (NSABP) C-07 [5-FU plus leucovolin (LV) ±oxaliplatin] and C-08 [FOLFOX ± bevacizumab] adjuvant studies revealed that dMMR was prognostic for recurrence in patients treated with FOLFOX (TTR, HR = 0.58; 95 % CI, 0.35 to 0.96; *P* =0.03) (53, 54), but not predictive of oxaliplatin efficacy since the interaction test between MMR status and treatment was not statistically significant (54) (Table 1).
- In an analysis of the Multicenter International Study of Oxaliplatin/5-Fluorouracil/ Leucovorin in the Adjuvant Treatment of Colon Cancer (MOSAIC) (45), MMR status was evaluated in 986 of the 2,240 patients enrolled. In a modest number of patients with dMMR colon cancers, a DFS benefit from FOLFOX compared with 5-FU alone was observed (55) (Table 1).
- In stage III colon cancers, the addition of the anti-EGFR antibody cetuximab to FOLFOX as adjuvant chemotherapy did not improve outcome compared to FOLFOX alone in patients with wild-type *KRAS* tumors (North Central Cancer Treatment Group (NCCTG) N0147 trial) (56). In prospectively collected tumor samples (57-59), dMMR was detected in 314 (12 %) of 2,580 stage III tumors and was prognostic overall for TTR, but not for DFS or OS (57) (Updated data in Table 1, ref 60). However, dMMR *vs* pMMR was associated with significantly better outcome among tumors in the proximal colon (HR= 0.71; 95 % CI, 0.53-0.94; p=0.018), but not in the distal after adjustment for *KRAS* and $BRAF^{V600E}$ mutations and relevant covariates (57).
- Available data in stage III CRC patients does not change the current approach of treating these patients, irrespective of MMR status, with adjuvant FOLFOX.

5-FU Plus Irinotecan-Based Adjuvant Therapy

- Irinotecan is commonly used for the treatment of advanced CRC, however, it was ineffective in the adjuvant setting based on the several randomized phase III studies [Cancer and Leukemia Group B (CALGB) 89803 (60), FNCLCC Accord02/FFCD9802 (61), and Pan-European Trials in Alimentary Tract Cancers 3 (PETACC-3) trials (62)]. Accordingly, irinotecan is not used in the adjuvant setting in CRC patients.
- In a retrospective analysis of the CALGB 89803 trial where patients with stage III colon cancer were randomly assigned to weekly bolus 5-FU/leucovorin (LV) or weekly bolus irinotecan, 5-FU, and LV (IFL), IFL-treated patients with dMMR/MSI-H tumors showed improved 5-year DFS as compared to pMMR tumors (HR=0.76; 95% CI, 0.64 to 0.88 *vs.* 0.59; 95% CI, 0.53 to 0.64; $P = .03$), which was not observed among patients treated with 5-FU/LV (63). However, data from the PETACC-3 study (64) failed to show a benefit for irinotecan in patients with dMMR colon cancers.

Bevacizumab in Adjuvant Setting

- The National Surgical Adjuvant Breast and Bowel Project protocol C-08 (NSABP C-08) trial failed to show the benefit of adding 1 year of bevacizumab to standard FOLFOX in the treatment of stage II/III colon cancer (65). However, post hoc analyses found that patients with dMMR tumors derived a statistically significant survival benefit from the addition of bevacizumab (HR = 0.52; 95% CI = 0.29 to 0.94; P = .02), in contrast with no benefit in patients with pMMR tumors (HR = 1.03; 95% CI = 0.84 to 1.27; p = .78; P interaction = .04)(66). An explanation for this finding awaits further study and moreover, these preliminary results await confirmation.

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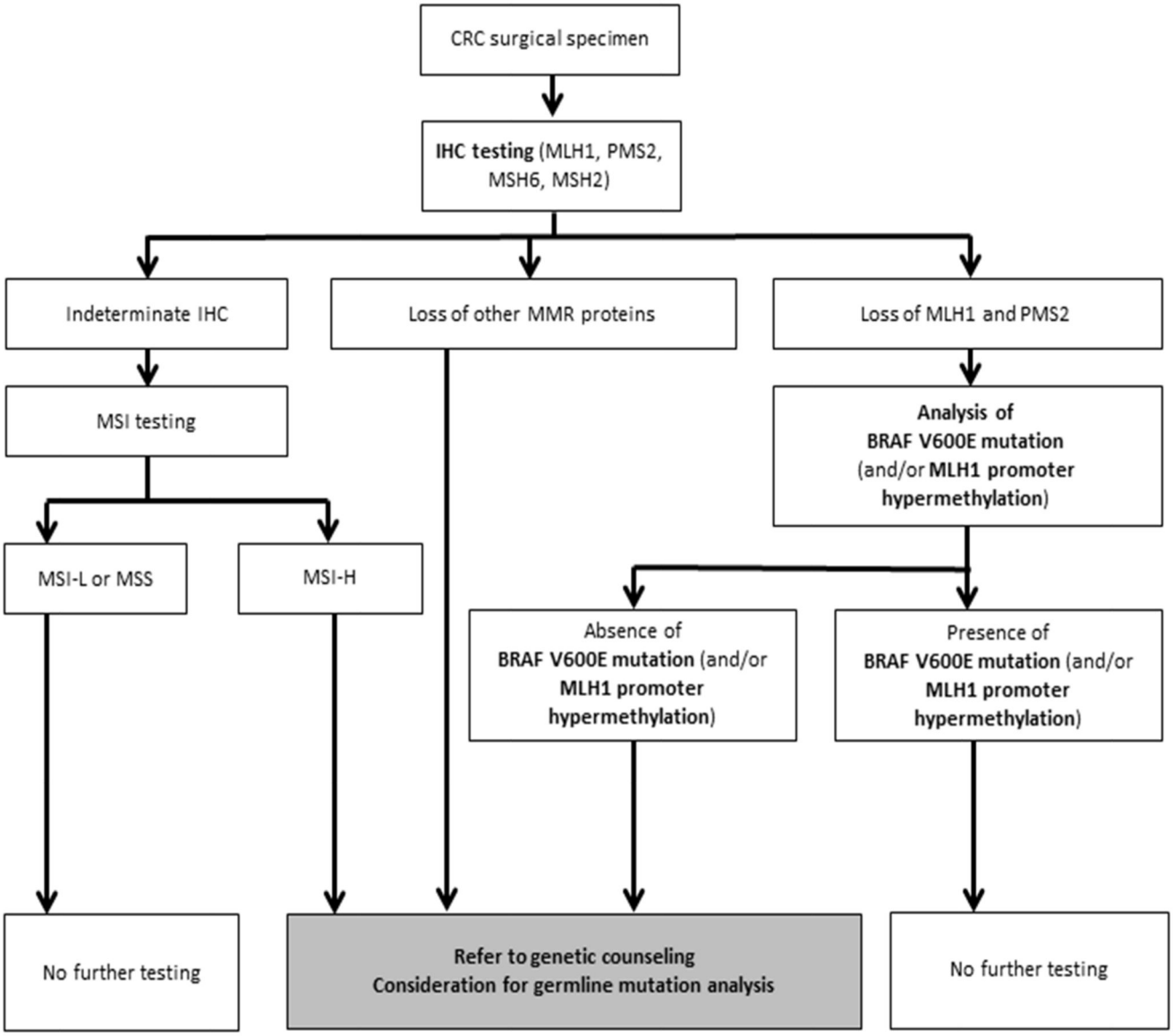

Figure 1.

Algorithm for systematic evaluation for Lynch Syndrome in patients with colorectal cancer. CRC, colorectal cancer; IHC, immunohistochemistry.

Table 1

randomized trials evaluating the prognosis/predictive impact of the mismatch repair status in stage II and III colorectal cancer

References	Tumor stage	Treatment	MMR status	Number of patients	HR for DFS/TTR/RFS (95% CI)	P value	HR for OS (95% CI)	P value
5-FU-based adjuvant chemotherapy								
Sargent et al. (39) (pooled analysis)	II & III	Surgery alone 5-FU	pMMR	436 426	0.69 (0.55-0.86) DFS	0.001	0.73 (0.58-0.91)	0.006
		Surgery alone 5-FU	dMMR	79 86	1.61 (0.84-3.10) DFS	0.15	1.58 (0.81-3.09)	0.18
Sargent et al. (44) (from ACCENT)	II	Surgery alone	pMMR dMMR	244 63	0.27 (0.10-0.75) RFS	0.012	0.27 (0.10-0.74)	0.011
		5-FU	pMMR dMMR	920 235	0.81 (0.55-1.19) RFS	0.285	0.87 (0.61-1.26)	0.469
	III	Surgery alone	pMMR dMMR	277 37	0.59 (0.28-1.23) RFS	0.162	0.69 (0.35-1.36)	0.283
		5-FU	pMMR dMMR	2333 390	0.80 (0.66-0.97) RFS	0.025	0.79 (0.65-0.97)	0.023
Oxaliplatin-based adjuvant chemotherapy								
Gavin et al. (54) (from the NSABP C-07 study)	II & III	5-FU 5-FU+oxaliplatin	pMMR	635 675	0.82 (0.67-1.00) TTR	0.054	NA	
		5-FU 5-FU+oxaliplatin	dMMR	86 85	1.01 (0.45-2.25) TTR	0.98	NA	
Gavin et al. (54) (from the NSABP C-07 and C-08 studies)	II & III	5-FU+oxaliplatin	pMMR dMMR	806 102	0.58 (0.35-0.96) TTR	0.03	NA	
Flejou et al. (45) (from MOSAIC)	II & III	5-FU 5-FU+oxaliplatin	dMMR	50 40	0.52 (0.24-1.14) DFS	NA	0.45 (0.19-1.05)	NA
	III	5-FU 5-FU+oxaliplatin	dMMR	28 17	0.51 (0.18-1.41) DFS	NA	0.44 (0.15-1.34)	NA
Sinicrope et al. (67) (from N0147)	III	5-FU+oxaiplatin	pMMR dMMR	2575 329	0.82 (0.64-1.04) DFS	0.106	0.83 (0.63-1.09)	0.182
		5-FU+oxaiplatin	pMMR dMMR	2575 329	0.71 (0.55-0.93) TTR	0.014		

DFS, disease free survival; RFS, recurrence free survival; TTR, time to recurrence; 5-FU, 5-fuluorouracil; HR, hazard ratio; CI, confidence interval; OS, overall survival; MMR, mismatch repair; pMMR, proficient MMR; dMMR, dMMR, deficient MMR; NA, not available