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Review Article

Clinical Aspects of Microsatellite Instability Testing in Colorectal Cancer

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Abstract

Microsatellite instability (MSI) is a molecular hallmark for some colorectal cancers (CRCs) in which short tandem repeats are prone to mutations along with DNA sequences. It is due to DNA-mismatch-repair system deficiency because of a germline/somatic mutation in mismatch-repair (MMR) genes. The germline mutations lead to Lynch syndrome (LS) while epigenetic gene silencing results in sporadic CRC tumors. We discuss in our paper the most important clinical aspects of MSI testing in CRCs. We reviewed the most reliable relevant studies and clinical trials according to their high-quality methods, particularly within two recent decades. MSI testing is used to classify CRC tumors as MSI-high (MSI-H), MSI-low, and microsatellite stable tumors. MSI-H or MMR deficient tumors have shown the best prognosis among all CRCs, so MSI testing is considered as a good prognostic marker. Moreover, it is used to identify LS among familial CRC patients. There is a diagnostic mutation in *BRAF* gene (V600E) by which sporadic CRCs could be distinguished from LS associated CRCs, due to its concordance with sporadic CRCs not LS. Although, some previous studies had demonstrated a predictive role for MSI testing in chemotherapy process, emerging some controversial findings in recent studies has not convinced many authors to recommend it as a routine examination to evaluate therapeutic response. Though emerging new molecular findings have opened novel windows to develop clinical management of CRC, MSI testing has remained as an excellent prognostic and diagnostic tool for CRC tumors.

Keywords: *Colorectal cancer, Lynch syndrome, microsatellite instability, microsatellite instability testing, mismatch-repair*

What are Molecular Pathways Behind Colorectal Cancer?

Overall, there are at least three main molecular pathways underlying the development of colorectal cancer (CRC): Chromosomal instability (CIN) pathway, microsatellite instability (MSI) pathway, and CpG island methylator phenotype (CIMP) pathway.[1-3] CIN is the most prevalent molecular cause of genomic instability in CRC, so it is an original genetic basis of about 65%–70% of all sporadic CRC tumors.[4] CIN is characterized with an imbalance in number of chromosomes (aneuploidy), chromosomal amplification, and a high frequency of loss of heterozygosity resulting in some deleterious mutations in tumor suppressor genes such as *APC* and *TP53*, and oncogenes including *KRAS*.[5,6]

The second molecular pathway is MSI including about 8%–20% of all CRC tumors which is more common in stage II (20%) than stage III (12%) and stage IV (4%).[7,8] This genetic change is a molecular fingerprinting for DNA-mutation in mismatch repair (MMR) system deficiency because of germline mutations or epigenetic changes in MMR genes about which we discuss more in the article.[2,9]

The last pathway is epigenetic molecular changes leading to alteration in gene expression or gene function without any change in its DNA sequence.[10] For instance, CIMP within specific sites of promoter could lead to silencing of some vital tumor suppressor genes concluding tumor development which is found in about 35% of CRC tumors.[11,12]

What is Microsatellite Instability?

MSI is a particular molecular change as a hallmark of averagely 15% of CRCs.[3,9] At first, these molecular changes were named "dispersed somatic mutations" in simple tandem repeats[13] or a replication error phenotype (RER).[14] Due to a defect in DNA-mismatch repair (MMR) system, microsatellites or short tandem repeats, repetitive sequences containing 1–6 nucleotide

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units up to 100 times, are prone to accumulation of mutations. It is mainly attributed to a failure inefficient DNA polymerases attachment to these repetitive sequences during DNA replication.[14] The most common microsatellite-associated errors are base-base mismatches which escape from internal proofreading function of DNA polymerases resulting in DNA hairpins.[15] DNA-MMR system is responsible for proofreading of replication errors in microsatellites, including four well-known proteins: MLH1, MSH2, PMS2, and MSH6 interacting with each other as heterodimer complexes.[13] In MMR-deficient cells, genes including microsatellites in their coding regions, like transforming growth factor (*TGF*)-βRII gene, are more susceptible to frame shift mutations.[16] Among MMR genes, two genes including *MSH3* and *MSH6* contain coding microsatellite regions which are prone to mutation in MSI-high (MSI-H) cancers.[11]

MSI is a molecular change in some different tumors such as colorectal, stomach, endometrium, ovarian, sebaceous carcinoma, glioblastomas, and lymphomas.[17] Most of MSI CRC tumors are sporadic usually due to epigenetic silencing of *MLH1* promoter because of somatic hypermethylation.[18] These contain about 12%–15% of all CRCs in which lack of MLH1 function could lead to fast accumulation of mutations in other genes like *TGF*-β and *BAX* resulting in tumor development.[12] Meanwhile, a somatic hereditable hypermethylation of *MSH2* gene promoter has been also recently reported which is rarely occurred by some large deletion mutations in last exon of *EPCAM*, a gene located next to *MSH2*, or *EPCAM-MSH2* locus.[19] A few of MSI-CRC tumors including about 2%–3% of the all CRC tumors is related to Lynch syndrome (LS), a hereditary predisposing cancer syndrome, which is mainly due to a germline mutation in one of the four DNA-MMR genes: *MLH1, MSH2, PMS2*, and *MSH6*.[20,21]

How is Microsatellite Instability Testing Done?

MSI testing is performed by polymerase chain reaction (PCR)-based amplification of microsatellite repeats and comparing their size along with DNA in normal cells versus tumor cells. Currently, it is prepared through fluorescent primers and capillary electrophoresis.[22] National Cancer Institute has recommended a diagnostic panel for MSI testing in which two mononucleotide markers named BAT-25 and BAT-26, and three dinucleotide markers named D2S123, D5S346, and D17S250 are used. With this panel, MSI-H is defined when at least two markers out of five markers in tumor cells show variability in their size compared to normal cells. Meanwhile, if only one marker present instability in tumor cells, the molecular phenotype is classified as MSI-low (MSI-L). In microsatellite stable (MSS) status, there is no unstable marker in DNA of the tumor cells.[23]

Afterward, some studies showed an upper specificity and equal or upper sensitivity for mononucleotide markers than dinucleotide markers in MSI testing, a fact according which some commercial kits were developed. These mononucleotide repeats are quasimonomorphic; hence nearly all people are homozygote for every common allele of a provided marker. Using the monomorphic markers simplifies interpretation of the data.[24,25] Rather than mononucleotide markers, pentanucleotide markers were also included in a commercial diagnostic MSI kit, Promega MSI analysis system, to identify tissue mix-up.[24,25] It uses a multiplex fluorescent survey in which PCR of the all five mononucleotide markers and two pentanucleotide markers is done in just a single reaction. The length of the amplified products is easily observable via a capillary electrophoresis method by which the cost of MSI testing has been significantly reduced.[26,27] Some studies have shown that in MSI-L tumors, instability is usually observed just in dinucleotide markers. Therefore, if MSI analysis is only limited to dinucleotide markers, it might overestimate wrongly MSI-L or MSS tumors as MSI-H tumors.[28]

Naturally, surgical resected tumors and their adjacent healthy tissues are the best sources to provide samples for MSI testing. Meanwhile, for rectal cancers which are being treated with neoadjuvant therapy to shrink the residual tumor, a presurgical biopsy usually prepares a better sample for MSI testing than surgical tumor [Figure 1].[11]

What are Clinical Applications of Microsatellite Instability Testing in Colorectal Cancer?

Historically, at least three clinical applications could be considered for MSI testing in CRC: Prognostic, diagnostic, or predictive applications. So, the most important questions according which we designed this article are as following:

1. Can we use MSI testing as a prognostic marker in CRC patients?
2. What is the diagnostic usage of MSI testing in CRC?
3. Can we use MSI testing as a predictive marker to treat CRC patients with different chemotherapy regimens?

Microsatellite instability as a tumor prognostic marker

MSI CRC tumors have presented a better prognosis and a less metastasis compared to MSS-CRC tumors according to different studies.[13,29,30] The prognostic value of MSI status in stage II CRC patients has been more than in patients with stage III tumors.[29,31] MSI-CRC tumors contain numerous active, cytotoxic tumor-infiltrating lymphocytes, a reaction independently associated with a better survival.[32,33] In a meta-analysis included 1277 CRC patients, MSI-CRC tumors had a better survival compared to MSS-CRC tumors.[7]

Different studies have presented a lower recurrence rate in MSI tumors in comparison to MSS tumors.[30,34] In a large series on 2141 CRC patients in II or III pathologic stages, lower recurrence rate, delayed time to recurrence, and better survival were reported in MMR deficient patients compared to MMR proficient patients.[30] Other studies also demonstrated a better prognosis and lower recurrence risk

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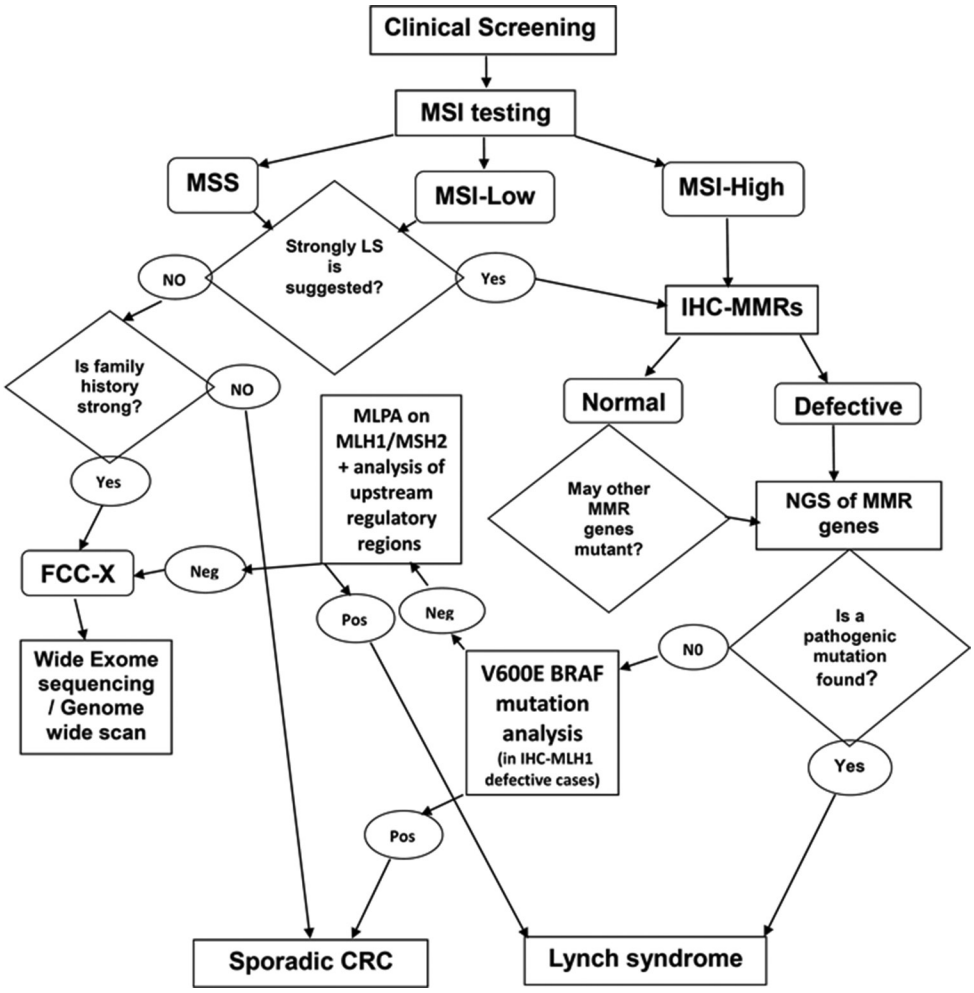

Figure 1: The proposal algorithm for clinical and molecular screening of hereditary non-polyposis colorectal cancer. MSI: Microsatellite instability, MSS: Microsatellite stable, LS: Lynch syndrome, Pos: Positive, Neg: Negative, FCC: Familial colorectal cancer, IHC: Immunohistochemistry

in MSI-H CRC patients with II pathologic stage tumors compared to MSS/MSI-L CRC patients.[35-37] Moreover, some other studies have reported reduced incidence rate of MSI-H tumors in pathologic stages of III or IV than early stages, indicating a less possibility of metastasis in these cases.[38,39] Meanwhile in a recent study, it was, interestingly, determined that MSI-H CRCs in stage II had a poorer prognosis and 3-year survival in comparison to MSS and MSI-L CRCs. Multivariate analysis of the results confirmed MSI-H phenotype as a poor independent prognostic marker [Table 1].[40]

Genetic diagnosis of Lynch syndrome

DNA-MSI is a molecular demonstration of MMR deficient tumors through which human MMR genes and their important role in pathogenesis of LS was explored.[9,31,41] Since this achievement in 1993, MSI testing has remained as a main method in research and clinical interventions associated to hereditary nonpolyposis CRC (HNPCC).[42]

There are some clinical criteria for primary screening of patients at risk for HNPCC. Amsterdam I and II criteria, and revised Bethesda guidelines, historically, have been used to clinical selection of these patients. Sensitivity of these triple criteria increases, respectively, so the revised Bethesda guidelines has the most sensitivity among them.[43-45] After clinical screening of at risk patients, some molecular approaches are considered to evaluate what proportion of them are affected to LS.

Although both techniques including MSI testing and immunohistochemistry (IHC) have high efficacy in screening of at risk patients for genetic testing of germline mutations in MMR genes, given the some properties of MSI testing which in IHC are not seen, MSI testing is considered as an excellent and accessible method to identify LS patients.[26,41,46] There are usually some deleterious mutations in at least half of the microsatellites or more in MMR deficient tumor cells, leading to instability of their sequences. Therefore, MSI status provides an outstanding and accessible marker to evaluate MMR deficiency. Since that both MSI status and LS are made by MMR deficiencies, MSI testing can be used to identify LS as a surrogate marker for MMR deficiency.[26]

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Table 1: Some important published studies within 1993-2014 according which MMR deficiency has been proposed as a positive prognostic marker in colorectal cancer patients

Publication year	Author/s	Trial groups		The group with increased survival	*P*
		Group 1	Group 2		
2002	Liang JT, *et al.*	MSI-H(+) HDFL(+), *n*=35; MSI-H(-) HDFL(+), *n*=134	MSI-H(+) HDFL(-), *n*=17; MSI-H(-) HDFL(-), *n*=58	MSI-H(+) HDFL(+)	0.0001
2003	Brueckl WM, *et al.*	MSI-H(+) HDFU(+) , *n*=7	MSI-H(-) HDFU(+) , *n*=36	MSI-H(+) HDFU(+)	0.021
2003	Ribic CM, *et al.*	MSI-H(+) HDFU(-), MSI-H(-) HDFU(-), *n*=287	MSI-H(+) HDFU(+), MSI-H(-) HDFU(+), *n*=283	MSI-H(+) HDFU(-)	0.004
2004	Carethers JM, *et al.*	MSI-H(+) HDFU(-), MSI-H(-) HDFU(-), *n*=138	MSI-H(+) HDFU(+), MSI-H(-) HDFU(+), *n*=66	MSI-H(-) HDFU(+)	<0.05
2008	Muller CI, *et al.*	MSI-H(+) FUFOX(+), MSI-H(-) FUFOX(+), *n*=474	MSI-H(+) CAPOX(+), MSI-H(-) CAPOX(+), *n*=474	MSI-H(-) FUFOX(+)	0.02
2009	Bertagnolli MM, *et al.*	MSI-H(+) IFL(+), MSI-H(-) IFL(-), *n*=629	MSI-H(+) FU/LV(+), MSI-H(-) FU/LV(-), *n*=635	MSI-H(+) IFL(+)	0.03
2010	Sargent DJ, *et al.*	MSI-H(+) HDFU(-), MSI-H(-) HDFU(-), *n*=228	MSI-H(+) HDFU(+), MSI-H(-) HDFU(+), *n*=229	MSI-H(-) HDFU(+)	0.02
2010	Kim ST, *et al.*	MSI-H(+) FUFOX(+), MSI-H(-) FUFOX(+), *n*=75	MSI-H(+) CAPOX(+), MSI-H(-) CAPOX(+), *n*=96	none	0.95
2016	Tougeron D, *et al.*	MSI-H(+) surgury alone, *n*=263	MSI-H(+) surgury/FUFOX, *n*=119; MSI-H(+) surgury/FU, *n*=51	MSI-H(+) surgury/FUFOX	<0.001

MMR: Mismatch-repair

MSI testing has some advantages compared to IHC in LS diagnosis, including convenience of doing and interpreting the results, high rate of reproducibility, identification of nontruncating missense mutations in normal IHC, and also sufficiency of just one tumor section for MSI testing instead four sections in IHC. Meanwhile, MSI testing has also some limitations in LS diagnosis. MSI is not LS-specific and it is demonstrated in about 10%–15% of the sporadic CRCs too. In these cases, MMR deficiency is almost due to CpG island hypermethylation of the *MLH1* promoter and associated gene silencing.[18,47] Although *MLH1* hypermethylation could be rarely observed with germline MMR genes mutations, it can also be considered as a second hit to inactivate of the *MLH1* wild allele in LS tumors.[48,49]

Given the concordance of V600E mutation of *BRAF* with sporadic MSI-CRC tumors which is associated with *MLH1* hypermethylation and lack of this mutation in LS tumors, according to many studies,[18,50-52] *BRAF* V600E mutation could be used as a surrogate marker to distinguish of sporadic MSI-CRC tumors from LS tumors [Figure 1].[48,53]

Some different studies have presented that CRC tumors due to a germline mutation of *MSH6* could demonstrate MSI-L instead MSI-H phenotype. It means that some *MSH6* mutant CRC tumors did not show MSI-H status.[54,55] According to these studies, *MSH2/MSH3* protein dimer remains active in *MSH6* mutant cells and MSI may be limited only to mononucleotide repeats.[55] It seems more *MSH6* mutant tumors would be detectable with MSI-H phenotype instead MSS or MSI-L if enough mononucleotide markers are used for MSI testing.[26]

Predicting response to chemotherapy

Although some previous studies had indicated improved response of MSI tumors to chemotherapy with 5-fluouracil (5-FU),[56-58] later studies showed a weak response of locally advanced MSI-H CRC tumors to 5-FU-based regimens in adjuvant therapy,[59,60] indicating no benefit from single-agent fluoropyrimidine therapy in MMR-deficient CRC tumors.[36,61-63] Further, some studies indicate not only resistance of MSI-H CRC patients to treatment with 5-FU, but also lower survival of them after receiving 5-FU in comparison with the patients who did not receive 5-FU.[59,63] Ignoring lack of enough samples and some methodological problems as significant limitations to interpret the results, next studies demonstrated improved response of MSI-CRC tumors to combination chemotherapy with oxaliplatin and irinotecan in comparison to 5-FU based agents.[37,64-66] Also in a recent retrospective multicenter study on 433 MMR deficient CRC patients, more disease-free survival was observed using adjuvant oxaliplatin-based chemotherapy in comparison to adjuvant fluoropyrimidine alone.[67] Apparently, MSI-H CRC cells have been more sensitive to irinotecan, a topoisomerase inhibitor, compared to MSS CRC cells. So, complete response rate to neoadjuvant therapy with irinotecan was about 60% in MSI-H CRC patients versus 20% in MSS CRC patients.[68] Simultaneously, in a meta-analysis study including 964 metastatic CRC patients, 91 patients with MSI-H tumors presented no distinct benefit from chemotherapy.[69] Some clinical trials such as the Quick and Simple and Reliable suggest that MSI status cannot predict who may benefit from chemotherapy [Table 2].[70]

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Table 2: Some important clinical trials within 2002-2016 evaluating predictive value of microsatellite instability state in different chemotherapy regimens for colorectal cancer patients

Publication year	Author/s	Type of study	Number of cases		The group with increased survival	*P*
			MMR deficient	MMR proficient		
1993	Thibodeau B, *et al.*	Case series	25	65	MMR deficient	0.02
2005	Popat S, *et al.*	Meta-analysis	1277	6365	MMR deficient	0.16
2005	Benatti P, *et al.*	Case series	256	1007	MMR deficient	<0.01
2009	Ligtenberg K, *et al.*	Case series	344	1797	MMR deficient	0.035
2009	Koopman M, *et al.*	Case series	18	532	MMR deficient	<0.001
2010	Guastadisegni C, *et al.*	Meta-analysis	1278	7965	MMR deficient	<0.0001
2011	Sinicrope FA, *et al.*	Case series	344	1797	MMR deficient	<0.001
2013	Phipps AI, *et al.*	Cohort	460	2774	MMR deficient	<0.001
2014	Inamura K, *et al.*	Case series	190	1021	MMR deficient	<0.001

MSI-H: Microsatellite instability-high, HDFL: High-dose 5-fluorouracil plus leucovorin chemotherapy, HDFU: High-dose fluorouracil-based chemotherapy, FUFOX: Fluorouracil plus oxaliplatin chemotherapy, CAPOX: Capecitabine plus oxaliplatin chemotherapy, IFL: Irinotecan/fluorouracil/levoleucovorin, FU/LV: 5-fluorouracil/leucovorin

Due to some controversial findings in the studies related to predicting role of MSI status in chemotherapy response, recently European Society for Medical Oncology has not considered MSI as a predictive marker for chemotherapy.[71] Anyway, the association between MSI status and response to chemotherapy has been still remained as an active area in clinical and molecular cancer research.

Summary

Despite of so much molecular findings about CRC tumorigenesis, MSI testing is being still used as an excellent accessible prognostic and diagnostic marker in CRC patients. According to MSI analysis, CRC tumors are classified to MSI-H, MSI-L, and MSS tumors. MSI-H CRC tumors have shown the best prognosis and a better survival in comparison with the two others. MSI testing has been also used to identify MMR deficiency in CRC tumors due to a germline mutation in MMR genes, leading to LS, or epigenetic gene silencing leading to sporadic CRC tumors. Since *BRAF* mutation is observed with CIMP in CRC tumors without existing in LS CRC tumors, it can be used to distinguish sporadic CRC tumors from hereditary ones. Although there are some evidences for poor response of MMR deficient CRC tumors to chemotherapy with 5-FU based regimens, recent studies have explored some different features. Therefore, application of MSI testing for predicting response to chemotherapy has remained ambiguous as an active field for more investigation.

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Conflicts of interest

There are no conflicts of interest.

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