Molecular Pathways: Microsatellite Instability in Colorectal Cancer: Prognostic, Predictive and Therapeutic Implications

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Microsatellite instability (MSI) is the molecular fingerprint of the deficient mismatch repair (MMR) system that characterizes approximately 15% of colorectal cancers (CRCs). MSI develops due to germline mutations in MMR genes or more commonly, from epigenetic silencing of MLH1 in sporadic tumors that occurs in a background of methylation of CpG islands in gene promoter regions and in tumors that frequently show hotspot mutations in the BRAF oncogene. MSI tumors have distinct phenotypic features and have been consistently associated with a better stage-adjusted prognosis compared to microsatellite stable tumors. MSI negatively predicts response to 5-fluorouracil and may also determine responsiveness to other drugs used in CRC treatment. Recent data expand the molecular heterogeneity of MSI tumors that may contribute to the understanding of differential chemosensitivity. Identifying deficient MMR has important implications for patient management and its exploitation holds promise for improving patient outcomes and for the development of novel therapeutics.
BACKGROUND

The future of personalized oncology centers around identifying molecular subtypes of cancer with distinct clinical behavior for which targeted therapeutic agents can be developed and selectively utilized. Studies have identified two molecularly distinct pathways of colorectal tumorigenesis which are chromosomal instability and microsatellite instability (MSI) (1). The majority of colorectal cancers (CRCs) show chromosome instability characterized by loss or gain of chromosome arms, chromosomal translocations or gene amplifications (1). In contrast, inactivation of a DNA mismatch repair (MMR) gene (MLH1, MSH2, MSH6 or PMS2) by mutation or transcriptional silencing results in deficient function of the MMR system with an accumulation of errors in DNA within microsatellites that is termed MSI (2, 3). Microsatellites are short, repetitive DNA sequences found throughout the tumor genome that are prone to mutations. The most frequent errors associated with microsatellites are base–base mismatches, and insertions/deletions in DNA coding regions produce frameshift mutations that can lead to protein truncations. The MMR system functions to correct errors introduced in microsatellites through a series of steps involving the interaction of MMR proteins as heterodimers with a complex formed by a MutS and a MutL (4) (Figure 1). When a mismatch is detected, MSH2 associates with either MSH6 or MSH3 to form MutSα or MutSβ complexes, respectively. Furthermore, MLH1 interacts with PMS2, PMS1 or MLH3 to form MutLα, MutLβ or MutLγ complexes, respectively. The MSH3 gene has been shown to encode another MutS homologue that dimerizes with MSH2 (5). Excision of the mismatch is performed by proteins such as exonuclease 1 and proliferating-cell-nuclear antigen, and is subsequently followed by re-synthesis and re-ligation of the DNA strand (4). In addition to MMR, DNA repair systems also include base excision repair that removes small, non-helix-distorting base lesions from the
genome, and the related nucleotide excision repair pathway repairs bulky, helix-distorting lesions. PARP has emerged as an important therapeutic target given that its inhibition leads to blockade of base excision repair and is especially toxic in BRCA-deficient tumor cells that are more dependent upon PARP and homologous recombination to repair DNA damage (6). Nuclear PARP1 is activated by DNA strand breaks and is involved in DNA repair.

MSI tumors carry mutations in mononucleotide tracts in the coding regions of several genes, including BRAFV600E, TGFβRII, BAX, and IGFIIR (1). Deficient MMR and MSI arise due either to germline mutations in MMR genes or more commonly, from somatic hypermethylation of CpG islands surrounding the promoter region of MLH1 and other genes that is known as the CpG island methylator phenotype (CIMP) (7, 8) (Figure 2). CIMP tumors comprise the majority of sporadic MSI CRCs (9). A heritable somatic methylation of MSH2 has been reported that is caused by a deletion of the last exon of EPCAM that is adjacent to MSH2 (10). Germline MMR mutations give rise to Lynch Syndrome, also known as hereditary nonpolyposis CRC, an autosomal dominant disorder that accounts for ~3% of all CRCs (11).

While MSI testing and/or analysis of MMR protein expression by immunohistochemistry are routinely performed to identify patients with suspected Lynch syndrome (11), their use outside of this indication has yet to gain widespread acceptance.

CRCs with MSI have distinct pathological features that include proximal colon predominance, poor differentiation and/or mucinous histology, and increased numbers of tumor-infiltrating lymphocytes (12, 13). In population-based studies, the prevalence of
MSI among CRCs is approximately 15% (9, 14) and is more common among stage II compared to lymph node-positive or stage III CRCs. MSI is relatively uncommon among stage IV or metastatic CRCs (~4%) (15). MSI is more frequent in women, especially older women, compared to men given differences in MLH1 methylation frequencies (9). Both observational studies as well as data from patients enrolled on randomized clinical trials have consistently shown that MSI or deficient MMR is independently associated with improved survival compared with individuals with MSS tumors (13, 14, 16), and include a meta-analysis (17). Patients with deficient vs proficient MMR tumors also have significantly reduced rates of tumor recurrence (18, 19). Confirmatory prognostic data were recently reported from large clinical trials [Quick and Simple and Reliable (QUASAR) trial (19) and Pan-European Trials in Alimentary Tract Cancers (PETACC-3) (20, 21) in patients with stage II or III colon carcinomas. MSI CRCs typically have diploid DNA content and in a prior study, the prognostic impact of deficient MMR was no longer evident when ploidy was taken into account (22). Despite the aforementioned data, testing for MSI or MMR proteins has not been routinely incorporated into clinical practice to inform patients about their prognosis or to guide management. To address the lack of prospective data, an ongoing trial in stage II colon cancers categorizes patients into high and low risk groups based upon MSI status and allelic loss at 18q (ECOG-E5202). Low risk tumors are defined as having MSI and intact 18q and are assigned to no postoperative treatment, whereas high risk patients receive the standard adjuvant regimen for stage III CRC which is 5-fluorouracil (5-FU) and oxaliplatin. Since the MSI tumors are not treated with 5-FU, the study will not provide predictive information regarding 5-FU-based treatment.
CLINICAL-TRANSLATIONAL ADVANCES

Emerging evidence indicates that certain microRNAs can regulate MMR expression to influence genomic stability in CRC. Overexpression of miR-155 and miR-21 were independently shown to downregulate MMR proteins and to induce MSI in CRC cells (23, 24). In human CRCs, overexpression of miR-155 or miR-21 was inversely related to the level of hMLH1 and/or hMSH2 MMR expression (23, 24). Furthermore, miR-155 overexpression was found in a tumor subset with an unknown cause of MMR inactivation (23). In a CRC xenograft model, miR-21 overexpression was shown to markedly reduce the efficacy of 5-FU that was associated with downregulation of hMSH2 (24). Together, these preliminary data suggest a potential role for these miRNAs in the pathogenesis of CRC and as a potential indicator of therapeutic efficacy. Recent data expand the molecular heterogeneity found within MSI CRCs. A mutation in the gene encoding heat shock protein (HSP)110 was found in MSI cell lines and human CRCs (25). The HSP110 truncated protein lacked chaperone activities or anti-apoptotic properties typical of HSPs. This mutation was shown to sensitize MSI CRC cells to treatment with 5-FU and oxaliplatin, and while very preliminary, there was a suggestion of survival benefit in two small retrospective cohorts of MSI CRCs treated with adjuvant 5-FU with or without oxaliplatin (25). In prior studies, MSI tumors were associated with higher rates of inactivation of the PTEN tumor suppressor gene by mutation or hypermethylation compared to MSS tumors (26, 27).

Sporadic MSI colon cancers with epigenetic inactivation of hMLH1 show frequent (~50%) co-occurrence of BRAFV600E mutations compared to an overall BRAF mutation frequency of 8-11% among CRCs (21, 28). BRAF encodes a serine/threonine kinase that is an essential component of the RAF/MEK/ERK/MAPK signaling cascade (29, 30). In CRCs, BRAF mutations are located in a hotspot in exon 15 that leads to a V600E
single-amino-acid substitution (29). \textit{BRAF} mutations are mutually exclusive with \textit{KRAS} mutations that are more commonly associated with microsatellite stable (MSS) tumors (30). The presence of a \textit{BRAF} mutation indicates a sporadic MSI CRC and essentially excludes a diagnosis of Lynch syndrome (31). Within CRCs, \textit{BRAFV600E} mutations have been associated with a worse prognosis across tumor stage (28, 32), although conflicting data have recently been reported in patients with stage II/III colon cancers participating in adjuvant chemotherapy trials. Specifically, \textit{BRAF} mutation was not prognostic in stage II tumors in the QUASAR study (19), but was associated with OS, but not RFS, in stage II/III tumors in the PETACC-3 trial (21). An unanswered question is whether \textit{BRAF} mutations can confer prognostic information within the subgroup of MSI tumors (33). Recent findings from a large study in independent cohorts, suggests that CIMP-high is associated with a favorable prognosis in CRC patients that was independent of MSI and \textit{BRAF} mutation status (32). Limited data exist with regard to the prognostic or predictive impact of CIMP. Studies examining the predictive utility of CIMP for 5-FU-based therapy have been inconclusive (34, 35). More recently, analysis of a population-based cohort of patients with stage II and III colon cancers found that CIMP-positive tumors did not benefit from adjuvant 5-FU, whereas patients with CIMP-negative tumors treated with 5-FU showed improved survival (36). Importantly, discrepant results among studies may be related to different methylation markers used as well as definitions of CIMP(8, 32, 37). An unanswered question is whether differences in chemosensitivity exist between MSI tumors of sporadic origin vs germline cases. In a recent study, tumor metastases were reduced by 5-FU-based adjuvant treatment in stage III colon cancers with deficient or proficient MMR, and a subset analysis of deficient MMR cases suggested that any treatment benefit was restricted to suspected germline tumors (38). While provocative, these data await confirmation in an independent dataset where genotyping for \textit{MMR} genes has been performed.
Studies have shown that MSI is a negative predictive marker of response to 5-FU. Using MLH1-deficient HCT116 colon cancer cells that display resistance to 5-FU, transfer of chromosome 3 introduces a functional MLH1 gene that restores sensitivity to 5-FU (39). Similarly, re-expression of hMLH1 by the demethylating drug, 5-azacytidine, was shown to restore 5-FU sensitivity (40). Tumor xenograft studies also demonstrate that MSI is associated with resistance to 5-FU (41). Most, but not all (42, 43) studies of patients with MSI tumors show a lack of survival benefit from 5-FU as adjuvant treatment of colon cancer. Studies include randomized clinical trials (13, 16), retrospective case series (44, 45) and a meta-analysis (17). Relevant to this issue are data in CRCs showing that MSI status and thymidylate synthase expression are unrelated (18). Predictive marker analyses are ideally conducted in studies that incorporate untreated control arms. While most studies do not meet this criterion, data from stage II/III colon cancer patients treated with 5-FU-based adjuvant therapy vs observation have confirmed a lack of benefit for 5-FU in deficient vs proficient MMR tumors (16). Accordingly, it has been recommended that patients with stage II colon cancer showing MSI not receive 5-FU as adjuvant therapy given their favorable prognosis and lack of benefit from 5-FU (16, 46).

Preclinical studies suggest that MSI colon cancer cells and tumor xenografts are more sensitive to irinotecan compared with microsatellite stable (MSS) cells (47-49), although the molecular mechanisms are only partially defined. Irinotecan is a camptothecin analog and a potent inhibitor of the topoisomerase I enzyme that results in the inhibition of DNA replication. MSI CRC cell lines and human tumors carry frequent mutations in the MRE11A and hRAD50 genes that control repair of DNA double-strand breaks (47). Mutations in these genes were shown to confer sensitivity to camptothecins.
compared to cells with intact expression (47). These data suggest that secondary mutations in genes regulating DNA double-strand breaks rather than MSI itself, may be responsible for increased sensitivity to camptothecins. Of note, \textit{MRE11A} mutations are found in 70-85\% of MSI colon cancers (50, 51). The predictive utility of MSI for the efficacy of irinotecan has been studied in the colon cancer patients. In an adjuvant trial (CALGB 89803), a statistically significant improvement in disease-free survival (DFS) was seen in MSI vs MSS tumors for the addition of irinotecan to 5-FU/LV (52). However, this finding was not supported by another adjuvant trial in stage II and III colon cancer patients (PETACC-3 trial) that compared infusional 5-FU/LV with or without irinotecan and where MSI cases treated with irinotecan did not show improved survival (20). Therefore, the issue of irinotecan benefit in MSI colon cancers is unresolved and awaits further study.

Studies have shown that MMR deficient cells are resistant to cisplatin and carboplatin, whereas MMR proteins do not recognize oxaliplatin-related adducts since oxaliplatin contains a bulky moiety that becomes incorporated into DNA via cytotoxic intra- and interstrand adducts (53). Accordingly, oxaliplatin chemosensitivity is independent of the MMR system (54). Recent data indicate that suppression of the \textit{MSH3} MMR gene can sensitize CRC cells to both cisplatin and oxaliplatin at clinically relevant dose (55). \textit{MSH3}, together with \textit{MSH2}, forms the MutS\beta heteroduplex which interacts with interstrand cross-links induced by platinum-based anti-cancer drugs (55). The \textit{MSH3} gene frequently undergoes somatic mutation in MMR-deficient CRCs (56) (57). To date, only limited data exist in MSI tumors from patients treated with oxaliplatin combined with 5-FU, and predictive information is lacking. A retrospective single-arm study in 5-FU plus oxaliplatin-treated stage III colon cancer patients found that the 3-year DFS rate was significantly higher in patients with deficient vs proficient MMR
tumors (58), suggesting that the prognostic impact of MMR status is maintained since oxaliplatin is expected to provide equivalent benefit irrespective of MMR status.

In addition to utilizing MMR status to guide treatment decisions, an important goal is to develop molecularly targeted agents to exploit the specific mechanisms of MSI cancers. Evaluation of the predictive impact of MSI is limited by its 4% prevalence in metastatic CRCs (15). Data also suggest the utility of PARP inhibitors in MSI tumors deficient in homologous recombination due to mutations in the coding microsatellites of *MRE11A* and *hRAD50* genes involved in double-strand DNA repair (51). Preferential cytotoxicity to the PARP-1 inhibitor, ABT-888, was seen in those MSI cell lines containing mutant copies of *MRE11A* compared with wild-type or MSS cells (59). Furthermore, the observed ability of *MSH3* to protect against double-strand breaks was exploited by the combination of oxaliplatin and a PARP inhibitor that produced a synergistic cytotoxic effect against CRC cells (55). These data suggest that synthetic lethality can potentially be exploited in MSI cancers. A screen to identify drugs inducing death in *MSH2*-deficient tumor cells identified methotrexate, which is supported by the finding that suppression of dihydrofolate reductase led to increased death in these same cells (60). An ongoing, single arm phase II study is evaluating methotrexate in *MSH2*-deficient advanced CRCs. Using high-throughput array technology for the molecular profiling of tumor tissue, drugs targeting the PI3K/AKT/mTOR pathway have been identified and shown to selectively inhibit MSI cancer cell lines, suggesting the relevance of this pathway in this tumor subtype (61). Given that chromosomal instability is associated with taxane resistance, it has been hypothesized that MSI tumors may exhibit greater sensitivity to taxane therapy. To test this hypothesis, a clinical trial is underway evaluating patupilone, a microtubule stabilizing drug, in patients with MSI tumors (62). Another potential strategy is the use of demethylating agents, such as Decitabine, which
can demethylate \textit{MLH1} but lacks selectivity. Given that \textit{BRAF} mutations are common in sporadic MSI CRCs (31, 32) selective inhibitors of \textit{BRAF} such as PLX-4032 are of therapeutic interest in these tumors. Given that deficient MMR represents a relatively small molecular subtype, multi-institutional studies will be needed to recruit sufficient numbers of patients to conduct adequately powered clinical trials. Going forward, RNA interference screening studies may identify new genetic targets within MSI tumors that are important for drug development.

**CONCLUSIONS**

The recognition of molecular subtypes of human cancers represents the future of personalized oncology and will guide drug development strategies. CRCs with MSI have distinct phenotypic features and a molecular etiology that includes epigenetic inactivation of \textit{MLH1} with frequent evidence of CIMP and somatic \textit{BRAF} mutations \textit{versus} germline mutations in \textit{MMR} genes conferring Lynch Syndrome. There is increasing evidence of further molecular heterogeneity within MSI tumors with respect to secondary mutations in genes regulating diverse biological processes. Evidence suggests that genetic and epigenetic alterations in MSI tumors can influence their clinical behavior and/or response to anti-cancer drugs. MSI tumors have been consistently associated with a favorable prognosis and an apparent resistance to treatment with 5-FU, although the predictive impact of MMR for oxaliplatin or irinotecan in MSI tumors awaits further study. The interpretation of these data from patient studies is complicated by the combination of drugs with 5-FU. Well-designed clinical trials based upon molecular classification are needed to further address treatment response issues. Given increasing evidence for the molecular heterogeneity of MSI tumors, the use of high-throughput technology for molecular profiling and screening for novel drug targets holds promise for the development of novel therapeutic strategies.
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Figure Legends

Fig. 1. The MMR system functions to correct errors introduced in microsatellites through a series of steps involving the interaction of MMR proteins as heterodimers. (A) MSH2–MSH6 (MutSα) recognizes single base-pair mismatches, shown by the incorrect base (G) matched with T on the template, and creates a sliding clamp around the DNA. This step requires the exchange of adenosine triphosphatase (ATP) for adenosine diphosphate (ADP). The MutSα complex is then bound by the MLH1-PMS2 (MutLα) complex. (B) Excision of the mismatch occurs when the DNA MMR protein sliding clamp interacts with exonuclease-1, proliferating cell nuclear antigen (PCNA), and DNA polymerase. This complex excises the daughter strand back to the site of the mismatch. The complex comes off the DNA and resynthesis then occurs with correction of the error. (C) MSH2–MSH3 (MutSβ) can recognize larger insertion deletion loops (IDLs) that can complement the function of MSH2–MSH6 that recognizes single pair mismatches and small IDLs. Potential interactions with other MutL dimers are shown, as. MLH1 can dimerize with PMS2, PMS1, or MLH3. (Reprinted with permission from Boland CR, Goel A. Microsatellite instability in colorectal cancer. Gastroenterology. 2010;138:2073-87.)

Fig. 2. Two molecular pathways can lead to CRCs with MSI. These include germline mutations in a MMR gene followed by a second hit to the wild-type copy that may occur due to point mutation, LOH or methylation. Germline MMR mutations lead to Lynch Syndrome (LS) that represents approximately one-fifth of all MSI CRCs. The more common, non familial form of MSI is due to epigenetic inactivation of MLH1 that occurs in a background of CIMP that results in hypermethylation of the promoters of multiple genes. These sporadic tumors show CIMP and BRAFV600E hotspot mutations that serve to distinguish them from LS cases.
A **Single mismatch**

MutSα ADP

MSH2

ADP

MSH6

ATP

MutLα

MLH1

PMS2

ADP

Daughter strand

Template strand

B **Exonuclease complex and resynthesis**

Exonuclease

DNA polymerase

Excised nucleotides

PCNA

DNA polymerase

C **Insertion/deletion loop and variations in MutL complexes**

MutSβ ADP

MSH2

ADP

MSH3

ATP

MLH1

MLH3

MutLγ

MLH1

PMS1

MutLβ

MLH1

PMS2

MutLα
Pathways to mismatch repair deficiency in colorectal cancer

- Germline mutation (MLH1, MSH2, MSH6, PMS2)
  - Lynch syndrome (~3%)
- Biallelic MLH1 methylation
  - CIMP+
  - Sporadic (~12%)

Second hit (mutation, LOH, methylation)

Deficient MMR repair

Microsatellite instability (MSI)

- Frameshift mutations in genes with coding microsatellites
- Other mutations

BRAFV600E mutation

Colorectal cancer
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