Review

Clinical Cancer Research

Microsatellite Instability as a Biomarker for PD-1 Blockade

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Abstract

Initial results by Le and colleagues, which were published in the June 25, 2015 issue of the *New England Journal of Medicine*, report significant responses of cancers with microsatellite instability (MSI) to anti–PD-1 inhibitors in patients who failed conventional therapy. This finding fits into a broader body of research associating somatic hypermutation and neoepitope formation with response to immunotherapy, with the added benefit of relying on a simple, widely used diagnostic test. This review surveys the pathogenesis and prognostic value of MSI, diagnostic guidelines for detecting it, and the frequency of MSI across tumors, with the goal of providing a reference for its use as a biomarker for PD-1 blockade. MSI usually arises from either germline mutations in components of the mismatch repair (MMR) machinery (MSH2, MSH6, MLH1, PMS2) in patients with Lynch syndrome or somatic hypermethylation of the *MLH1* promoter. The result is a cancer with a 10- to 100-fold increase in mutations, associated in the colon with poor differentiation, an intense lymphocytic infiltrate, and a superior prognosis. Diagnostic approaches have evolved since the early 1990s, from relying exclusively on clinical criteria to incorporating pathologic features, PCR-based MSI testing, and immunohistochemistry for loss of MMR component expression. Tumor types can be grouped into categories based on the frequency of MSI, from colorectal (20%) and endometrial (22%–33%) to cervical (8%) and esophageal (7%) to skin and breast cancers (0%–2%). If initial results are validated, MSI testing could have an expanded role as a tool in the armamentarium of precision medicine. *Clin Cancer Res; 22(4); 813–20. ©2016 AACR.*

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Learning Objectives

Upon completion of this activity, the participant should have a better understanding of the pathogenesis and clinicopathologic associations of microsatellite instability, approaches to diagnosing it, its frequency across tumor types, and its potential value as a biomarker for PD-1 blockade immunotherapy.

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Introduction

Recent work has highlighted the importance of the immunologic response to cancer for therapy. Interaction of the PD-1 T-cell coreceptor and its ligand B7-H1/PD-L1 promotes an immunosuppressive tumor microenvironment, and antibodies to either molecule have shown promise for inducing durable tumor responses, even in late-stage patients who have failed multiple previous lines of therapy (1–3). Despite this success, only a minority of patients respond, and it has been unclear which patients and which tumors are the best candidates for this therapy.

At the same time, immunologic factors have shown promise as prognostic tools. Characterizing the lymphocytic response to tumors can be more prognostically valuable than traditional histopathologic parameters. In colorectal cancer, Galon and colleagues showed that tumors with a high infiltrate of $CD8^+$ CTL and T helper 1 (Th1)-type cells had a superior prognosis to those with a low infiltrate, with the degree of infiltrate surpassing

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tumor–node–metastasis staging in prognostic value (4, 5). Although there is evidence that such infiltrate suppresses tumor growth and metastasis (5), it has been unclear why some tumors elicit it and others do not and how tumors with such a response are able to survive it.

Recent publications have clarified the answers to these questions. By analyzing the genetics and immune microenvironment of colorectal cancer specimens in parallel, Llosa and colleagues demonstrated, consistent with previous studies, that tumors with a high Th1/CTL infiltrate had defects in mismatch repair (MMR), resulting in microsatellite instability (MSI; ref. 6). Following previous studies (7), the authors suggested that the increased mutational burden in such tumors created neoepitopes responsible for the immune response. Most significantly, they found that tumors with MSI had significant upregulation of immune checkpoint proteins, including PD-1 and PD-L1, enabling them to survive. In MSI colorectal cancer, the PD-L1 expression appears not to be on tumor cells, but rather on tumor-infiltrating lymphocytes and/or myeloid cells. This model is presented in Fig. 1.

A follow-up clinical trial demonstrated the utility of MSI status as a predictive marker for response to PD-1 blockade in stage IV cancer patients. The article reported on 11 patients with MMR-deficient (dMMR) colorectal cancer, 21 with MMR-proficient colorectal cancer, and 9 with dMMR noncolorectal cancer (4 ampullary or cholangiocarcinomas, 2 endometrial carcinomas, 2 small bowel carcinomas, and 1 gastric carcinoma). Patients were stage IV and had failed multiple other chemotherapies. They were treated with pembrolizumab, an anti–PD-1 antibody. MSI was a significant predictor of the immune-related objective response rate (40% in dMMR colorectal cancer, 71% in dMMR noncolorectal cancer, 0% in MMR-proficient colorectal cancer) and also the immune-related progression-free survival rate (78%, 67%, and 11%, respectively). Whole-exome sequencing of tumor tissue revealed an average of 1,782 somatic mutations in cancers with MSI (578 were predicted to result in neoantigens) versus 73 somatic mutations in cancers without MSI (21 of which were predicted to result in neoantigens; ref. 8).

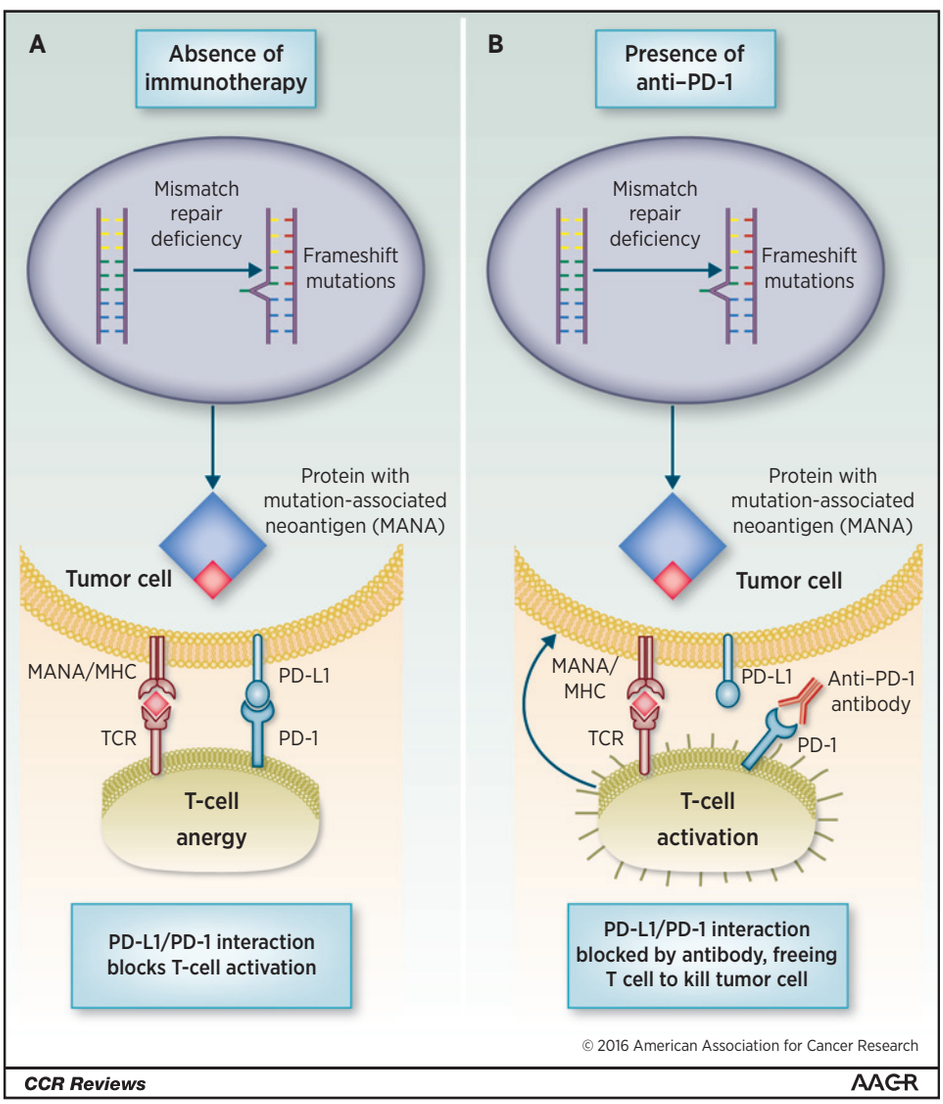

Figure 1.
Proposed relationship between MSI status and immunologic response. Tumor cell (top) and T cell (bottom) in the absence (A) or presence (B) of immunotherapy. For simplicity, the tumor cell is drawn as the source for the PD-L1; however, in some tumors, such as MSI colorectal cancer, the dominant source may be macrophages or other tumor-infiltrating lymphocytes and myeloid cells (6). In the absence of functional MMR, nascent strand slippage (green base) goes unrepaired, producing frameshift mutations (red bases) and resulting in proteins (blue diamond) containing a mutation-associated neoantigen (MANA, red square). MANA-MHC complexes are presented to T cells. MANAs can also arise from missense mutations (not shown). MHC, major histocompatibility complex; TCR, T-cell receptor.

These results have the potential to significantly improve therapeutic options. Immune checkpoint blockade inhibition is less toxic than chemotherapeutic regimens and has potential for durable responses in advanced cancer patients who would otherwise live a few months. About 4% of advanced colorectal, 18% of advanced endometrial, and 11% of advanced ovarian cancers have been estimated to harbor MMR deficiency (9). Additional studies are under way to test PD-1 inhibition in early-stage and chemotherapy-naïve patients. Positive results in these groups could potentially spare these patients' chemotherapy and expand the number of patients who could benefit. As noted below, MSI is a common phenomenon across a significant fraction of tumor types, reaching 10% to 30% in frequency.

Many causes of somatic hypermutation exist other than MSI: mutations in the DNA polymerases encoded by *POLE* or *POLD1*, exposure to external mutagens (cigarette smoke, UV radiation), and endogenous mutagens (reactive oxygen species; refs. 10, 11). It can be predicted that tumors with hypermutation caused by these alternative mechanisms would also have enhanced sensitivity to checkpoint blockade. Indeed, Rizvi and colleagues demonstrated a strong correlation between response to PD-1 blockade and burden of nonsynonymous mutations in non–small cell lung cancer (12). Snyder and colleagues examined cytotoxic T-lymphocyte antigen 4 (CTLA-4) blockade in melanoma, finding a correlation between mutational load and clinical benefit. More significantly, they identified a set of putative neoepitopes able to predict clinical benefit in an independent patient cohort (13). These approaches, and others not yet utilized in clinical settings (14), have significant promise, but they rely on whole-exome sequencing of tumor samples followed by extensive bioinformatic analysis, an approach not yet practical for routine diagnostic use.

MSI testing, by contrast, is routinely performed in most diagnostic laboratories, increasing its potential as an immediately useful approach to predict immunotherapeutic response in patients who have failed conventional therapy. The purpose of this review is to examine current knowledge of MSI and the clinical assays used to detect it, with a focus on its potential role as a predictive biomarker for immune checkpoint blockade.

Pathogenesis of MSI in Colorectal Cancers

Microsatellites or "short-tandem repeats" are defined as repeats (usually 10–60 times) of the same base or sequence of bases, with a unit length ranging from one to six bases. They are scattered throughout coding and noncoding regions of the genome. DNA polymerases are more prone to make mistakes in these regions, by either inserting additional bases when slippage occurs on the synthesized strand or removing them when slippage occurs on the template strand, leading to mismatched DNA strands. MSI cancers are associated with 100- to 1,000-fold increased mutation rates of frameshift and missense mutations (experimentally measured; refs. 15–17), which result from defects in the DNA MMR system (18–23). Following DNA replication, the MMR machinery slides along the DNA and targets mismatches for correction when it encounters them. Its core components are two heterodimers consisting of MSH2/MSH6 and MLH1/PMS2 (MSH2 may also form a heterodimer with MSH3). Patients with a defect in any one of these components, or, in a gene upstream of *MSH2* that encodes EPCAM that results in loss of MSH2 expression when mutated (24), develop frameshift mutations leading to truncated protein products. Genes most commonly affected include *TGFBRII*, *ACVR2A*, *TCF4*, *IGF2R*, *BAX*, *MSH6*, and *MSH3* (25–28). About 15% of all colorectal cancers have MSI, with about 2.5% resulting from genetic inheritance and the remaining 12.5% being sporadic (29).

Patients with MSI due to germline mutations in one of the MMR genes are defined as having Lynch syndrome. Lynch syndrome is an autosomal dominant condition characterized by an elevated risk for cancers of the ovaries, kidneys, bladder, stomach, small bowel, bile ducts, and brain, with the biggest increase in risk for endometrial cancer (60% of females) and colorectal cancer (80% of patients; ref. 30). Sporadic MSI usually arises from epigenetic silencing of the *MLH1* promoter, often from a global increase in CpG island methylation, and is associated with a somatic *BRAF* p.V600E mutation (29, 31–33). Less commonly, it may arise from biallelic somatic inactivation of the genes encoding a MMR component (34). The manifestation of Lynch syndrome reflects the specific mutation and genetic background of the patient. Compared with patients with an *MLH1* mutation, for example, those with an *MSH2* mutation have a higher risk of a noncolorectal malignancy and those with an *MSH6* mutation have a higher risk of endometrial cancer (35, 36). Patients with an *MLH1* mutation, in turn, are more likely to develop colorectal cancer at a young age in the presence of specific risk alleles (37).

Whether hereditary or sporadic, MSI colorectal cancers have unique clinicopathologic associations. Whereas microsatellite stable (MSS) cancers tend to arise from adenomatous polyps, distributed anywhere in the colon, MSI malignancies tend to arise from sessile serrated adenomas in the proximal colon (38). MSS cancers are associated with chromosomal instability, whereas MSI cancers are typically diploid, with preserved chromosomal architecture (39). Under the microscope, MSS colorectal cancers typically show infiltrating glands with dirty necrosis, whereas MSI tumors classically show tumor-infiltrating lymphocytes and the Crohn-like lymphocytic reaction, poor differentiation with pushing margins, and mucinous differentiation (40).

Prognostication and Prediction

Even apart from its potential value as a biomarker for PD-1 blockade response, MSI status has been used to predict outcomes. MSI colorectal cancer is associated with a lower stage at diagnosis and improved stage-specific prognosis (though conflicting results have been observed in stage IV patients; refs. 41, 42), the likely result of a significant immunologic response elicited by neoepitopes. Assessing for somatic mutations in *BRAF* in conjunction with MSI status is also prognostically valuable (43, 44). The p.V600E mutation renders BRAF constitutively active, resulting in a worse prognosis. A recent study stratified colorectal cancer patients based on MSI and *BRAF* status into three prognostic groups: MSI/*BRAF*-wild type or mutant (best prognosis), MSS/*BRAF*-wild type (intermediate prognosis), and MSS/*BRAF* mutant (worst prognosis; ref. 43), though other studies have reached conflicting results (45), and no consensus exists to date on the best prognostic subgroupings.

The role of MSI as a predictive marker for therapy has been controversial. A 2003 study in stage II or III colorectal cancer patients examined the relationship between MSI status and response to 5-fluorouracil (5-FU)–based adjuvant chemotherapy. Although MSS patients showed a benefit from 5-FU treatment, MSI patients did not (46), a finding confirmed in smaller follow-up

studies (47, 48). Laboratory-based investigations have suggested that components of the MMR machinery bind to 5-FU incorporated DNA, raising the possibility that they mediate the observed cytotoxic response (49, 50). Further clinical studies have generally substantiated the finding that MSI patients do not benefit from 5-FU therapy, with some exceptions (51). A 2011 study of 2,141 colorectal cancer patients suggested the benefits of 5-FU therapy were restricted to stage III MSI patients with germline (versus sporadic) mutations (52). More recently, a 2015 meta-analysis of 14 studies concluded that there is a trend for lack of benefit of 5-FU in MSI cancers ($P = 0.11$), which did not reach statistical significance (53). Further studies are needed to reconcile these findings.

Diagnosis

Diagnostic guidelines relating to MSI status have evolved over the past two-and-a-half decades, from using clinical criteria to identify colorectal cancer patients with Lynch syndrome to using clinical, histologic, and genetic criteria to identify all patients with Lynch syndrome (including those with noncolorectal tumors) and distinguish sporadic and inherited cases of MSI.

The Amsterdam criteria were set forth in 1991, before the discovery of the genes responsible for Lynch syndrome. At least three relatives had to be diagnosed with histologically confirmed colorectal cancer, with the following additional criteria met: One patient had to be a first-degree relative of the other two; one relative had to have been diagnosed with colorectal cancer before the age of 50 years; and the colorectal cancer had to involve at least two generations (54).

The Bethesda guidelines, first proposed in 1997 and then revised in 2003, expanded the scope of testing, adding criteria for testing when patients do not fulfill the Amsterdam requirements (55, 56). These guidelines are especially applicable when there is no known family history of cancer. Only one of the following criteria need to be met in the revised guidelines: colorectal cancer in a patient under the age of 50 years, presence of other Lynch syndrome–related tumors (including synchronous or metachronous colorectal cancers), typical MSI histology in a patient under the age of 60 years, a colorectal cancer patient with a first-degree relative under the age of 50 years with a Lynch syndrome–related cancer, or a colorectal cancer patient with at least two first- or second-degree relatives with Lynch syndrome–related cancers (56). Patients who fulfill the Amsterdam criteria are also recommended to undergo further testing.

The National Comprehensive Cancer Network (NCCN) has issued the most recent guidance. The 2015 NCCN guidelines provide criteria for testing based on clinical or pathologic parameters. The clinical testing criteria recommend further evaluation of patients who meet the Amsterdam criteria or revised Bethesda guidelines, in addition to patients under the age of 50 years with endometrial cancer, patients with known Lynch syndrome in the family, and patients with a 5% or higher likelihood of having a germline mutation (as assessed by risk-prediction modeling). The pathologic testing criteria focus on patients with colorectal cancer, recommending that all patients be tested for MSI regardless of whether or not they meet other criteria or, at a minimum, testing all colorectal cancer patients under the age of 70 years and those over the age of 70 years who meet the revised Bethesda guidelines. A 2012 study of the performance characteristics of different screening approaches for detecting Lynch syndrome in 10,206 colorectal cancer patients found the revised Bethesda guidelines to have a sensitivity of 87.8% and specificity of 97.5%, the strategy endorsed by the NCCN a sensitivity of 95.1% and specificity of 95.5%, and universal tumor testing a sensitivity of 100% and specificity of 93% (57).

Although the criteria for deciding which patients to test have developed, the methods of testing have also evolved. As the human genome has several hundred thousand microsatellites, it is only practical to examine representatives. To ensure reproducibility and standardization, the 1997 Bethesda guidelines recommended a reference panel of five microsatellites (the "Bethesda panel") for testing: two mononucleotide loci (BAT-25 and BAT-26) and three dinucleotide loci (D2S123, D5S346, and D17S250). These regions are amplified within both tumor and normal tissue via fluorescent multiplex PCR and their size assessed by capillary electrophoresis (58). A shift (usually downward) in the size of at least two of the five microsatellite loci in tumor relative to normal is defined as the MSI-high (MSI-H) phenotype, or of 30% or more loci when larger panels are tested. A shift in the size of one locus constitutes the MSI-low (MSI-L) phenotype, which behaves in a similar manner to MSS tumors (55). The Promega Corporation has developed a widely used alternative to the Bethesda panel, called the MSI Analysis System, replacing the dinucleotide markers with mononucleotide markers (NR-21, NR-24, and MONO-27; refs. 56, 59, 60). The resulting panel has several practical advantages, though recent work has suggested that a panel targeting longer loci may have improved sensitivity, especially for endometrial cancer (61). Genome- or exome-wide sequencing of matched tumor and normal tissue has also been used to characterize MSI (12, 13, 62). This approach is useful in a research setting to identify affected genes, but it is expensive and technically challenging due to the repetitive nature of microsatellites (62).

Immunohistochemistry for MMR proteins has comparable performance characteristics to MSI testing and a high concordance rate (29). MLH1 and MSH2 are stable without their dimer partners (PMS2 and MSH6, respectively), but the reverse is generally not true. As a result, tumors with an *MLH1* mutation will show loss of both MLH1 and PMS2 on immunohistochemistry and tumors with an *MSH2* mutation will show loss of MSH2 and MSH6. Mutations in *PMS2* or *MSH6*, by contrast, lead to loss of only the affected protein (an exception is that MSH2 may be lost when *both* of its binding partners, MSH6 and MSH3, are lost). These features allow one to determine which of the MMR genes is likely mutated (or methylated) by immunohistochemistry, an advantage not present with MSI testing. An additional advantage of immunohistochemistry is its ability to work with paucicellular specimens. However, about 5% to 11% of MSI cases will not show MMR protein loss, likely due to retained antigenicity in an otherwise nonfunctional protein. Given its focus on representative microsatellites, MSI panel testing itself is an imperfect marker for Lynch syndrome, missing an estimated 0.3% to 10% of cases (29). The sensitivity of both tests varies based on which MMR gene is mutated (63).

These limitations have led the Association of Molecular Pathology to recommend all new colorectal cancer cases to be subjected to concurrent MSI analysis, immunohistochemistry for MMR proteins, and *BRAF* mutation screening (Fig. 2; refs. 29). This approach would allow almost all cases to be categorized and managed appropriately: Lynch syndrome patients would need screening for other Lynch syndrome–associated malignancies and counseling of family members; patients with sporadic MSI would

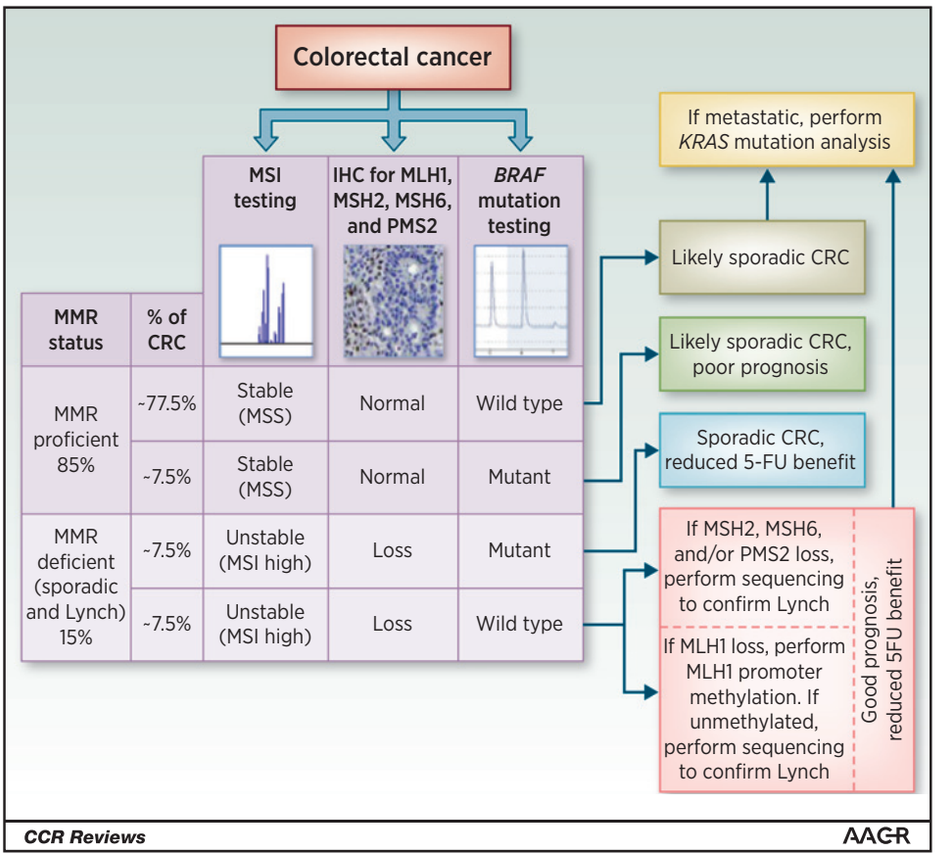

Figure 2.
MSI testing algorithm proposed in 2012 by the Association for Molecular Pathology. Reprinted with permission from ref. 29. This figure was published in *The Journal of Molecular Diagnostics*, Vol. 14, Funkhouser WK, Jr, Lubin IM, Monzon FA, Zehnbauer BA, Evans JP, Ogino S, et al. Relevance, pathogenesis, and testing algorithm for mismatch repair-defective colorectal carcinomas: a report of the Association for Molecular Pathology, 91–103. Copyright Elsevier 2012.

be apprised of their superior prognosis and potential lack of response to 5-FU chemotherapy; and MSS cases could plan for a poorer prognosis but could also potentially take advantage of 5-FU (29). Whether this is the most cost-effective approach however, is debatable. Two cost-effectiveness analyses evaluating money spent per life-year gained have favored screening patients with immunohistochemistry (64, 65). A more recent cost-effectiveness study favored PCR-based MSI testing as an initial measure (66). In either case, the biggest impact on cost saved per life-year gained came from identifying and properly managing affected relatives.

Frequency of MSI Across Human Cancers

The inference that a hypermutation syndrome such as MSI may serve as a biomarker for checkpoint blockade response across tumor types is supported by the efficacy of such blockade in other tumors with high mutational burdens. Further, of the noncolorectal tumors for which the information is available, endometrial, gastric, esophageal, and sebaceous tumors with MSI have been found to have an elevated rate of lymphocytic infiltration relative to those without MSI (67–69). Further investigation will benefit from understanding the frequency of MSI in different tumors so they can be appropriately tested.

Tables 1–3 compare the frequency of MSI-H across tumor types. MSI-H is here defined, unless otherwise noted, as size variation in at least 2/5 or 30% of tested microsatellite loci. In constructing the tables, preference was given to studies published after the revised Bethesda guidelines were released, studies with larger cohorts, and studies with unselected or minimally selected cohorts. Because the frequencies given here

Table 1. Cancers with an MSI-H frequency greater than 10%

Tumor type	Frequency, % (*n*)	Study
Colorectal cancer	13% (1066)	Hampel et al. (72)
Endometrial	22% (543), 33% (446)	Zighelboim et al. (73), Hampel et al. (74)
Gastric	22% (295)	TCGA (75)
Hepatocellular carcinoma	16% (37)[a]	Chiappini et al. (76)
Ampullary carcinoma	10% (144)	Ruemmele et al. (77)
Thyroid	63% (30)[a]	Mitmaker et al. (78)
Skin (sebaceous tumors)	35% (20)[a], 60% (25)[a]	Cesinaro et al. (79), Kruse et al (80)
Skin (melanoma)	11% (56)[a]	Palmieri et al. (81)

[a]Studies of less than 100 patients.

Table 2. Cancers with an MSI-H frequency between 2% and 10%

Tumor type	Frequency, % (*n*)	Study
Ovarian	10% (1234)	Murphy and Wentzensen (82)
Cervical	8% (344)[a]	Lazo (83)
Esophageal adenocarcinoma	7% (76)	Farris et al. (84)
Soft-tissue sarcoma	5% (40)	Kawaguchi et al. (85)
Head and neck SCC	3% (153)[b]	Glavac et al. (86)
Renal cell carcinoma	2% (152)	Hammerschmied et al. (87)
Ewing sarcoma	2% (55)	Alldinger et al. (88)

Abbreviation: SCC, squamous cell carcinoma.
[a]This number represents an aggregate of studies with different definitions of MSI-H, not all of which meet the Bethesda guidelines.
[b]MSI-H was defined as positivity in at least 2/8 markers.

Table 3. Cancers with an MSI-H frequency less than 2%

Tumor type	Frequency, % (*n*)	Study
Skin (squamous cell)	0% (30), 0% (56)	Reuschenbach et al. (89)[a]
Skin (basal cell)	0% (53), 2% (104)	Reuschenbach et al. (89)[a]
Prostate	1% (79)	Burger et al. (90)
Lung	0% (80), 2% (55)	Okuda et al. (91), Ninomiya et al. (92)
Osteosarcoma	0% (68)	Entz-Werle et al. (93)
Glioblastoma	0% (109)	Martinez et al. (94)
Pancreatic ductal adenocarcinoma	0%–2% (338)	Laghi et al (95)
Breast	0% (267), 0% (34), 0% (52), 1% (100)	Anbazhagan et al. (96), Adem et al. (97), Kuligina et al. (98), Toyama et al. (99)
Bladder	1% (84)	Catto et al. (100)
Testicular germ cell	0% (100)	Mayer et al. (70)

[a]The first percentage given resulted from looking at a series of cases with three mononucleotide markers (BAT25, BAT26, and CAT25).The second number was derived by the authors by summing the results of several other studies, not all of which defined MSI-H in a manner consistent with the Bethesda guidelines.

are for unselected groups, they will be underestimates for the frequency of MSI in tumors of patients already known to have Lynch syndrome.

Several points regarding Tables 1, 2, and 3 deserve mentioning. Despite selecting the largest studies found for each tumor category, many of these studies still included relatively few patients, highlighting the need for further investigation to better characterize the frequency and characteristics of MSI in many tumor types. Cancers that have a very low proportion in unselected groups, potentially making standard MSI testing impractical, may have a significantly higher proportion in specific subgroups. A study of testicular germ cell tumors, for example, found no cases of MSI in an unselected series of 100 tumors but 5 cases in a series of 11 cisplatin-resistant tumors ($P = 0.001$; ref. 70). By contrast, a study of hepatocellular carcinoma in 55 Japanese patients failed to reveal a single case of MSI, despite its relatively high frequency in other cohorts (71). These examples illustrate that the optimal approach to MSI testing will be based not only on tumor type but also on specific clinicopathologic characteristics.

Conclusions

Further studies are needed to assess the potential for MSI status to guide immunotherapy across tumor types. If initial results are validated, however, it would represent a significant advance in precision medicine. If the mechanism proposed for the efficacy of MSI-guided immunotherapy is correct (Fig. 1), the ultimate biomarker for immunotherapeutic response is not MSI or even the mutational burden but the presence of immunogenic neoepitopes. This could potentially be assessed more directly with future assays, leading to more precise guidance of immunotherapy, but at present, MSI status is a practical surrogate.

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