Biomarkers for Immune Therapy in Gastrointestinal Cancers

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Abstract: Immunotherapy with checkpoint blockade of programmed death 1 (PD-1) and cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) has substantially increased the number of anticancer agents in our arsenal. However, these therapies are not effective in all cancer types, benefitting only a subset of patients with susceptible, immunogenic cancers. This problem is especially significant in gastrointestinal malignancies, which infrequently respond to immunotherapy. Although we clearly need more accurate biomarkers to predict response to immune checkpoint inhibition in gastrointestinal cancers, the established markers of mismatch repair deficiency, microsatellite instability, programmed death ligand 1 (PD-L1) expression, and tumor mutational burden are good starting points to identify patients who may benefit. Tumor-infiltrating lymphocytes, Epstein-Barr virus, and the stool microbiome are candidates for future immuno-oncology biomarkers in gastrointestinal malignancies. The availability of better biomarkers will improve patient selection for immunotherapy; it will also improve the design of clinical trials of agents intended for this population of patients, who require more effective treatment options.

Introduction

Immune checkpoint inhibitors have revolutionized the field of oncology, providing new therapeutic options for patients with melanoma,1 lung cancer,2-4 kidney cancer,5 bladder cancer,6 Merkel cell carcinoma,7,8 Hodgkin lymphoma,9 head and neck cancer,10 cutaneous squamous cell carcinoma,11 gastric cancer,12 hepatocellular carcinoma (HCC),13 anal squamous cell cancer,14,15 and cancers with high microsatellite instability (MSI-H) or mismatch repair deficiency (MMR-D).16,17 However, little progress has been made in the utilization of immunotherapy for other gastrointestinal cancers, including biliary tract cancers, pancreatic cancer, and colorectal cancer (CRC) that is microsatellite stable (MSS).16,18 In addition, although immune checkpoint inhibitors can be very effective for patients with some cancers, not all patients who have these cancers benefit from immune checkpoint blockade. Therefore, it is crucial to identify biomarkers that effectively predict response to immunotherapy across all cancers, especially gastrointestinal cancer.
cancers, which collectively have demonstrated low rates of response to immune checkpoint blockade. This article reviews MSI-H, MMR-D, programmed death ligand 1 (PD-L1), tumor mutational burden (TMB), and other novel immunotherapy biomarkers throughout the landscape of gastrointestinal cancers.

Definitions of MMR-D and MSI-H, and Methods of Measurement

Microsatellites are short, tandem sequences of mononucleotide, dinucleotide, or higher-order nucleotide repeats that are scattered throughout the human genome. These sites are prone to DNA replication errors as a result of DNA polymerase slippage, leading to mismatched DNA strands. Each time a cell divides, approximately 100,000 polymerase errors occur, and polymerase attempts to correct them through its proofreading activity. Nonetheless, some errors escape proofreading and are corrected through the MMR system, which is responsible for surveillance and repair, and recombination. MLH1, MSH2, MSH6, and PMS2 are the main proteins involved in the MMR system. Loss of function of any of these proteins leads to a state of MMR-D and high instability in microsatellite repeats (MSI-H).

Polymerase chain reaction (PCR) and immunohistochemistry (IHC) are 2 molecular biology methods that are in routine use for clinical MMR testing. MSI PCR analysis is used to detect MSI, whereas MMR IHC is used to detect the lack of expression of one or more MMR proteins. MSI is detected by the PCR amplification of specific microsatellite repeats, and their size is assessed by capillary electrophoresis.

The National Cancer Institute (NCI) has recommended a panel (known as the NCI or Bethesda panel) of 5 microsatellite loci for testing: BAT-25, BAT-26, D2S123, D5S346, and D17S250. On the basis of this panel, 3 categories of MSI have been established: MSI-H, indicating a shift in the size of at least 2 of the 5 microsatellite loci in a tumor in comparison with normal tissue; MSS, indicating no loci with instability; and MSI-low (MSI-L), indicating a shift in the size of 1 locus. Another panel has been developed by the Promega Corporation, and this MSI Analysis System uses a fluorescent multiplex assay for the detection of 5 mononucleotide microsatellite markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27). In a comparison study, the Promega system was superior to the Bethesda panel; complete concordance was observed in all MSI-H cases, and all MSI-L cases were appropriately reclassified as MSS.

Next-generation sequencing (NGS) with targeted gene sequencing or whole-exome/whole-genome sequencing has emerged as a new tool for identifying MSI-H tumors. NGS can be used to identify MSI by comparing sequences around microsatellite regions in a tumor and a matched normal control genome. NGS can readily be used to analyze the formalin-fixed and paraffin-embedded (FFPE) tissue that is routinely prepared in pathology departments. MSI testing by NGS with circulating tumor DNA (ctDNA) or cell-free DNA (cfDNA) is also available, potentially obviating the need for invasive testing and allowing the serial monitoring of response to immunotherapy.

Relevance to Lynch Syndrome

Hereditary CRC syndromes are more common among younger patients. The most frequent hereditary CRC syndrome is hereditary nonpolyposis colorectal cancer, also known as Lynch syndrome (LS). LS, a disorder with autosomal-dominant inheritance, is caused by germline mutations in MMR genes (MLH1, MSH2, MSH6, and PMS2) or germline deletions in the EPCAM gene (resulting in loss of expression of the MSH2 protein). These genetic alterations result in MSI-H tumors. MSI-H CRC can develop in older persons owing to acquired MLH1 methylation, often seen with a co-occurring BRAFV600E mutation. The presence of MLH1 methylation and/or BRAFV600E tumor mutations does not suggest LS.

Comparison of MLH1, MSH2, MSH6, and PMS2 Alterations

The prevalence of MMR gene alterations differs among tumor types. MSH2 and MSH6 are more frequently altered in CRC than in endometrial cancers. The risk for CRC is higher in patients with germline MLH1 or MSH2 mutations than in those with MSH6 or PMS2 mutations. The TMB associated with MSH2 and MSH6 alterations is significantly higher than the TMB associated with MLH1 and PMS2 alterations across several cancer types. The rate of PD-L1 overexpression is significantly higher in tumors with MSH2 (23%) mutations than in those with MSH6 (16%), MLH1 (16%), or PMS2 (14%) alterations across tumor types. Therefore, although we tend to think of MMR-D tumors as a singular group, considerable heterogeneity exists depending on which MMR gene is altered. Although we presume that the rates of response to immunotherapy in patients with MSH2 and MSH6 alterations will be higher than those in patients with MLH1 and PMS2 alterations owing to a higher TMB and rate of PD-L1 expression in the former, this hypothesis needs to be clinically validated.

Tumor Mutational Burden

TMB is calculated according to the number of nonsynonymous missense mutations not previously described as...
germline alterations per megabase sequenced with NGS. Le and colleagues found that MMR-D tumors have higher TMBs, which correlate with response to immune checkpoint inhibition\(^{16,17}\); in addition, Yarchoan and colleagues showed that across 27 tumor types, response to programmed death 1 (PD-1) inhibition was linearly correlated with TMB.\(^{30}\) This relationship is better established in relatively immunogenic cancers, such as non–small cell lung cancer, in which progression-free survival in patients who have a high TMB is longer with immunotherapy than with chemotherapy owing to the production of clonal neoantigens that elicit T-cell responses.\(^{37}\)

Unfortunately, no standard definition of high vs low TMB is available. A cutoff of 17 or more mutations per megabase correlates with MSI-H status in CRC,\(^{38}\) but other thresholds have been used throughout the literature.\(^{39,40}\)

Why does TMB matter in gastrointestinal cancers? High TMB may detect up to 3% of patients who have CRC with MSS and may still respond to immune checkpoint blockade.\(^{39}\) These patients have higher rates of MSH2, MSH6, and POLE mutations. POLE mutations affect polymerase function, which can cause a hyper-mutated state without a high level of MSI. Endometrial carcinomas with POLE mutations have been shown to respond to checkpoint blockade.\(^{41-43}\) High TMB correlates with longer overall survival in patients who have metastatic CRC (mCRC) with MSS, but the power of TMB to predict response to immunotherapy in CRC requires further investigation.\(^{44}\)

**PD-L1 Positivity, Scoring System, and Antibody Staining**

PD-1 is an inhibitory receptor expressed on several immune cells, particularly cytotoxic T cells. PD-1 interacts with 2 ligands: PD-L1 and PD-L2. PD-L2 is expressed primarily on macrophages and dendritic cells, whereas PD-L1 is expressed on tumor cells and immune cells. The interaction of PD-1 with PD-L1 inhibits T-cell activation and cytokine production, which is critically important in maintaining homeostasis of the immune response and preventing autoimmunity. However, their interaction within the tumor microenvironment provides an immune escape pathway for tumor cells by turning off cytotoxic T cells. Thus, blocking these interactions may subject tumor cells to attack by cytotoxic T cells.

PD-L1 expression is measured most commonly by IHC, which is performed on FFPE sections on glass slides. Slides are stained with automated techniques per the manufacturer’s instructions and optimized and validated per Clinical Laboratory Improvement Amendments/College of American Pathologists (CLIA/CAP) and International Organization for Standardization (ISO) requirements. Staining is scored for intensity (0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining) and percentage (0%-100%). Results are categorized as positive or negative by thresholds specific to each marker, defined on the basis of published clinical literature that associates biomarker status with patient response to therapeutic agents. Alternative methods of measurement use the combined positive score (CPS), which equals the number of cells staining for PD-L1 cells (tumor cells, lymphocytes, and macrophages) divided by the total number of evaluated tumor cells, multiplied by 100.\(^{45}\) A variety of PD-L1 antibody stains can be used depending on the tumor type and anti–PD-1/PD-L1 treatment, which unfortunately makes it difficult to compare PD-L1 positivity across stains and tumor histologies.\(^{46}\) As discussed below, PD-L1 status is of vital importance as a biomarker predictive of response to anti–PD-1 therapy in gastric and gastroesophageal adenocarcinoma, whereas it does not reliably predict response in HCC or CRC. Although PD-L1 staining is generally similar across antibodies and between paired primary and metastatic lesions, discrepancies may occur and cause false-negative results.\(^{47,48}\) Therefore, PD-L1 positivity must always be interpreted within the context of the tumor type, treatment, antibody stain, and scoring.

**FDA Approval for Pembrolizumab and Nivolumab in MSI-H/MMR-D Tumors**

In a phase 2 study, Le and colleagues showed that patients with MMR-D tumors benefited from immune checkpoint blockade with pembrolizumab (Keytruda, Merck).\(^{16}\) This study enrolled patients who had progressive metastatic carcinoma with or without MMR-D. The primary endpoints of immune-related objective response rate (ORR) and immune-related progression-free survival rate at 20 weeks were 40% and 78%, respectively, for MMR-D CRC and were 0% and 11%, respectively, for MMR-proficient (MMR-P) CRC. The responses of patients with MMR-D tumors that were not CRC were similar to those of patients with MMR-D CRC. Whole-exome sequencing revealed a mean of 1782 somatic mutations in MMR-D tumors, and high somatic mutation loads were associated with prolonged progression-free survival.\(^{16}\) Further work by Goodman and colleagues demonstrated that PD-1 blockade with pembrolizumab is effective across 12 different MMR-D tumor types.\(^{17}\) Therefore, tumors with a large number of somatic mutations due to MMR-D are susceptible to immune checkpoint blockade.

In metastatic MSI-H cancers, PD-1 checkpoint blockade provides a survival benefit, likely owing to the presence of more mutation-associated neoantigens and tumor-infiltrating lymphocytes (TILs) in the tumor.
Pembrolizumab and nivolumab (Opdivo, Bristol-Myers Squibb) are approved for the treatment of adult and pediatric patients who have unresectable or metastatic MSI-H or MMR-D solid tumors that have progressed following prior treatment and who have no satisfactory alternative treatment options, or who have mCRC that has progressed following treatment with a fluoropyrimidine, oxaliplatin, and irinotecan. In addition, the combination of nivolumab with the anti–cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) monoclonal antibody ipilimumab (Yervoy, Bristol-Myers Squibb) is approved for MSI-H/MMR-D mCRC on the basis of CheckMate 142 (An Investigational Immunotherapy Study of Nivolumab, and Nivolumab in Combination With Other Anti-cancer Drugs, in Colon Cancer That Has Come Back or Has Spread).49

Although disease control is achieved in most patients with MSI-H/MMR-D cancers who are on anti–PD-1 therapy (approximately 77% have a response or stable disease), disease progression occurs in a substantial subgroup of patients. Mutations in B2M, affecting the β2-microglobulin protein required for antigen presentation, are implicated in acquired resistance to anti–PD-1 monoclonal antibodies.17

**Gastric and Gastroesophageal Junction Cancers**

Patients with advanced gastric or gastroesophageal junction adenocarcinoma have generally poor outcomes and limited therapeutic options. In the initial phase 1b KEYNOTE-012 study (Study of Pembrolizumab in Participants With Advanced Solid Tumors), patients with PD-L1–positive tumors received pembrolizumab at 10 mg/kg intravenously (IV) every 2 weeks. A 22C3 antibody was used to define PD-L1 positivity as at least 1% membranous staining on tumor cells and contiguous immune cells.30 Of 36 evaluable patients, 22% had a partial response, and the treatment was well tolerated (see Table). In the phase 2 KEYNOTE-059 study (A Study of Pembrolizumab in Participants With Recurrent or Metastatic Gastric or Gastroesophageal Junction Adenocarcinoma), among 259 patients who received pembrolizumab at 200 mg IV every 3 weeks after disease progression following 2 or more lines of therapy, the ORR was 11.6% in all patients, 15.5% (23/148) in patients with PD-L1–positive tumors, and 6.4% (7/109) in patients with PD-L1–negative tumors.12 In the phase 3 KEYNOTE-061 trial (A Study of Pembrolizumab Versus Paclitaxel for Participants With Advanced Gastric/Gastroesophageal Junction Adenocarcinoma That Progressed After Therapy With Platinum and Fluoropyrimidine), 592 patients with disease progression during first-line platinum plus fluoropyrimidine were randomized in a 1:1 ratio to receive pembrolizumab at 200 mg IV every 3 weeks or paclitaxel at 80 mg/m² IV on days 1, 8, and 15 of a 28-day cycle.51 The safety profile of pembrolizumab was better than that of paclitaxel; however, no overall survival benefit was noted in the intention-to-treat population with a CPS of at least 1 (median overall survival, 9.1 vs 8.3 months with paclitaxel; hazard ratio [HR], 0.82; 95% CI, 0.66-1.03; P=0.421). Patients who derived significantly greater benefit from pembrolizumab than from paclitaxel had better Eastern Cooperative Oncology Group (ECOG) performance status (0) or a CPS of at least 10. Finally, the phase 3 KEYNOTE-181 trial (Study of Pembrolizumab Versus Investigator’s Choice Standard Therapy for Participants With Advanced Esophageal/Esophagogastric Junction Carcinoma That Progressed After First-Line Therapy) of second-line pembrolizumab at 200 mg IV every 3 weeks vs investigator’s choice of paclitaxel, docetaxel, or irinotecan (1:1 randomization) recently met its primary endpoint, improving overall survival in patients with a PD-L1 CPS of at least 10 (9.3 vs 6.7 months; HR, 0.69; 95% CI, 0.52-0.93; P=.0074).32

Pembrolizumab is approved by the US Food and Drug Administration (FDA) for the treatment of patients with recurrent locally advanced or metastatic gastric or gastroesophageal junction adenocarcinoma whose tumors express PD-L1 (CPS ≥1) following disease progression during or after 2 or more prior lines of therapy, including fluoropyrimidine- and platinum-containing chemotherapy and, if appropriate, HER2/neu-targeted therapy.39 A CPS of less than 1 is a strongly predictive biomarker for lack of benefit from pembrolizumab.

Nivolumab and ipilimumab have also been studied in gastric and gastroesophageal junction adenocarcinoma. ATTRACTION-2 (Study of ONO-4538 in Unresectable Advanced or Recurrent Gastric Cancer), conducted in Asia, randomly assigned in a 2:1 ratio 493 patients who had previously received at least 2 lines of chemotherapy to receive nivolumab at 3 mg/kg IV or placebo every 2 weeks.54 Median overall survival was 5.26 months with nivolumab vs 4.14 months with placebo (HR, 0.63; 95% CI, 0.51-0.78; P <.0001). Although only 40% of patients had tumors evaluable for PD-L1 expression, trends existed toward overall survival benefit from nivolumab regardless of PD-L1 status (≥1% [N=26]: 5.22 vs 3.83 months; HR, 0.51; 95% CI, 0.21-1.25; <1% [N=166]: 0.65 vs 4.19 months; HR, 0.72; 95% CI, 0.49-1.05). The CheckMate 032 trial (A Study of Nivolumab by Itself or Nivolumab Combined With Ipilimumab in Patients With Advanced or Metastatic Solid Tumors) randomly assigned in a 1:1:1 ratio 160 patients who had received at least 1 prior line of chemotherapy to nivolumab at 3 mg/kg IV every 2 weeks (NIVO 3); nivolumab at 1 mg/kg IV plus ipilimumab at 3 mg/kg IV every 3 weeks for 4 cycles followed by
nivolumab at 3 mg/kg IV every 2 weeks (NIVO1 + IPI3); or nivolumab at 3 mg/kg IV plus ipilimumab at 1 mg/kg IV every 3 weeks for 4 cycles followed by nivolumab at 3 mg/kg IV every 2 weeks (NIVO3 + IPI1). The highest ORR (centrally reviewed) was seen in the NIVO1 + IPI3 arm (20%). The next highest ORR (centrally reviewed) was seen in the NIVO3 arm (7%), and the lowest was seen in the NIVO3 + IPI1 arm (4%; Figure 1). The same pattern was observed for median overall survival: 6.9 vs 6.2 vs 4.8 months. Therefore, the NIVO1 + IPI3 arm had the best efficacy signal, and most of the responses occurred in subgroups of patients with PD-L1–positive or MSI-H tumors (Table). Nivolumab is not yet FDA-approved for use in gastric or gastroesophageal junction cancers.

Other Gastrointestinal Cancers

Small-bowel adenocarcinoma is thought to be susceptible to immune checkpoint inhibition owing to the frequency of MSI-H tumors (12%-28%). Single-agent pembrolizumab is currently being evaluated in a multicenter, randomized phase 2 clinical trial for patients who have disease progression on chemotherapy (NCT02949219).

Hepatocellular Carcinoma

For patients with advanced HCC, sorafenib (Nexavar, Bayer) was the only available therapy for many years. The phase 1/2 CheckMate 040 study (An Immunotherapy Study to Evaluate the Effectiveness, Safety and Tolerability of Nivolumab or Nivolumab in Combination With Other Agents in Patients With Advanced Liver Cancer) assessed the safety and efficacy of nivolumab in patients who had advanced HCC with or without chronic viral hepatitis. Patients in the dose expansion cohort (nivolumab at 3 mg/kg IV every 2 weeks) had a 20% ORR. Efficacy was seen in patients with or without prior hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, and PD-L1 status did not seem to have an effect in the small subset of patients analyzed. Nivolumab was granted accelerated FDA approval for patients previously treated with sorafenib. First-line trials evaluating sorafenib vs nivolumab (CheckMate 459, NCT02576509) and the combination of sorafenib plus nivolumab (NCT03439891) are ongoing.

Pancreatic adenocarcinoma has been notoriously resistant to immune checkpoint blockade, owing to low TMB and an immunosuppressive tumor microenvironment. A small percentage of pancreatic adenocarcinomas—approximately 1%—are MSI-H. However, the tide may finally be turning, as some early data suggest...
### Table. Clinical Responses Related to Immune Checkpoint Inhibition and Biomarkers of Response in Gastrointestinal Cancers

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<td>Pembro 3 mg/kg q2wk</td>
<td>3+</td>
<td>PD-L1+ (±1%)</td>
<td>16</td>
<td></td>
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<td>G/GEJ</td>
<td>ATTRACTION-2 54</td>
<td>Pembro 3 mg/kg q2wk</td>
<td>3+</td>
<td>PD-L1− (±1%)</td>
<td>114</td>
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<tr>
<td>G/GEJ</td>
<td>JAVELIN 71,72</td>
<td>Avelumab 10 mg/kg q2wk</td>
<td>3</td>
<td>All</td>
<td>185</td>
<td>2%</td>
<td>22%</td>
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<td>JAVELIN 71,72</td>
<td>Avelumab 10 mg/kg q2wk</td>
<td>3</td>
<td>PD-L1+ (±1%)</td>
<td>46</td>
<td>4%</td>
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*Table continued on next page*
that nivolumab may be effective in combination with the colony-stimulating factor 1 receptor (CSF1R) antibody cabiralizumab, which depletes tumor-associated macrophages responsible for local immunosuppression. Of 31 patients in a phase 1 trial, 4 (13%) had a durable clinical response (all with MSS and low TMB), which is a vast improvement over the lack of clinical response seen with single-agent checkpoint inhibitor therapy. Indeed, high levels of CSF1R expression in pancreatic adenocarcinoma are associated with inferior overall survival but also susceptibility to CSF1R inhibition. The combination of nivolumab plus cabiralizumab is being actively evaluated in a randomized phase 2 trial with various chemotherapy combinations (NCT03336216) to confirm this early efficacy signal.

**Overlap of MMR-D/MSI-H, PD-L1, and TMB**

Although significant overlap exists among MMR-D/MSI-H, PD-L1, and TMB, significant differences also exist depending on the tumor type (Figure 2). In a study of 4125 gastrointestinal tumors, 7.1% had high PD-L1 expression with low TMB and MSS, and 4.3% had low PD-L1 expression but high TMB and/or high MSI. The largest discrepancies were seen in anal squamous cell carcinoma and esophageal squamous cell carcinoma.

---

<table>
<thead>
<tr>
<th>Disease</th>
<th>Study</th>
<th>Agent</th>
<th>Line</th>
<th>Subgroup</th>
<th>N</th>
<th>ORR</th>
<th>DCR</th>
<th>mPFS, mo</th>
<th>mOS, mo</th>
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<td>G/GJE JAVELIN</td>
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<td>3</td>
<td>PD-L1+ (≥1%)</td>
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<td>2%</td>
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<td>Nivo 3 mg/kg q2wk</td>
<td>3+</td>
<td>All</td>
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<td>17%</td>
<td>42%</td>
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<td>MMR-D</td>
<td>10</td>
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<td>90%</td>
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<td>MMRp</td>
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<td>0%</td>
<td>11%</td>
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<td>52%</td>
<td>82%</td>
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<td>All</td>
<td>214</td>
<td>20%</td>
<td>64%</td>
<td>4</td>
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<td>Pembrolizumab 3 mg/kg q2wk</td>
<td>1+</td>
<td>Uninfected Untreated/ intolerant</td>
<td>56</td>
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<td>Uninfected progressor</td>
<td>57</td>
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<td>61%</td>
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<td>HCV infected</td>
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<td>20%</td>
<td>66%</td>
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<td>NR</td>
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<tr>
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<td>Pembrolizumab 3 mg/kg q2wk</td>
<td>2+</td>
<td>HBV infected</td>
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<td>14%</td>
<td>55%</td>
<td>4</td>
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<td>All</td>
<td>104</td>
<td>17%</td>
<td>62%</td>
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<tr>
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<td>2+</td>
<td>PD-L1+ (CPS ≥1%)</td>
<td>22</td>
<td>32%</td>
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<td>HCC KN-224</td>
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<td>2+</td>
<td>PD-L1+ (CPS &lt;1%)</td>
<td>30</td>
<td>20%</td>
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<tr>
<td>HCC KN-224</td>
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<td>PD-L1+ (TPS ≥1%)</td>
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<td>Pembrolizumab 200 mg q3wk</td>
<td>2+</td>
<td>PD-L1+ (TPS &lt;1%)</td>
<td>45</td>
<td>22%</td>
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<td>Anal SCC NCI967314</td>
<td>Pembrolizumab 3 mg/kg q2wk</td>
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<td>All</td>
<td>37</td>
<td>24%</td>
<td>72%</td>
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<td>All</td>
<td>24</td>
<td>17%</td>
<td>58%</td>
<td>3</td>
<td>9.3</td>
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</tbody>
</table>

*Clinical data from patients who had gastrointestinal cancers treated with immune checkpoint inhibitors. PD-L1 testing varies between trials; objective response rates according to central review are included where available.*

CM, CHECKMATE; CPS, combined positive score; CRC, colorectal cancer; DCR, disease control rate (complete response, partial response, and stable disease); Eso, esophageal; G/GJE, gastric and gastroesophageal cancer; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; Ipi, ipilimumab; KN, KEYNOTE; MMRp, mismatch repair proficient; MMR-D, mismatch repair deficient; mOS, median overall survival; mPFS, median progression-free survival; MSI-H, microsatellite instability-high; N, number of evaluable patients; Nivo, nivolumab; NR, not reached; ORR, objective response rate; PD-L1, programmed death ligand 1; Pembro, pembrolizumab; q2wk, every 2 weeks; SCC, squamous cell carcinoma; TPS, tumor proportional score.
Approximately 8.3% of anal squamous cell cancers had high TMB and MSS, suggesting that human papillomavirus (HPV) infection could be driving the rate of TMB in this MMR-P population. Similarly, smoking may account for the 3.5% of esophageal squamous cell cancers with high TMB and MSS. Rates of PD-L1 positivity were higher in both these cancers than in other gastrointestinal cancers.

### Other Biomarkers for Immune Checkpoint Inhibition

Cancers with an underlying viral etiology appear to be relatively immunogenic. This finding holds for HPV-associated anal squamous cell cancers and Epstein-Barr virus (EBV)–associated gastric cancers. Nivolumab is effective in advanced anal squamous cell carcinoma, with an ORR of 24%. Likewise, pembrolizumab had a 17% response rate in patients with PD-L1–positive (CPS ≥1%) advanced anal squamous cell cancer. More than 80% of anal squamous cell cancers are associated with HPV. The HPV E7 oncoprotein elicits an interferon-γ response in T cells, causing increases in TILs and PD-L1 expression. EBV-positive gastric cancers are associated with amplification of PD-L1 and PD-L2. In a study of 61 patients who had advanced gastric cancer treated with pembrolizumab, an objective response occurred in all 6 EBV-positive patients despite their having MSS tumors. Therefore, EBV-positive gastric tumors are very responsive to checkpoint inhibitors and have strong PD-L1 positivity but are not MSI-H.

TILs are another potential positive predictive biomarker for immunotherapy. The Immunoscore, which is calculated from CD3+ and CD8+ T-cell densities in the tumor microenvironment, reliably estimates the risk for recurrence in patients with resected stages I to III CRC. Moreover, the Immunoscore is a better prognostic biomarker than MSI status and can separate MSS and MSI...
subgroups according to low vs high recurrence risk. These findings could possibly be taken a step further to predict response to immune checkpoint blockade, identifying more patients with MSS who might benefit owing to higher numbers of TILs. Likewise, the broader use of consensus molecular profiling for mCRC could identify more patients with the CMS1 phenotype, associated with MSI-H status, hypermutation, and immune activation, seen in 14% of patients with CRC. However, consensus molecular subtype profiling is not yet done routinely in clinical practice, but it may be integrated into future commercial molecular profiling panels.

Gene expression profiles (GEPs) may ultimately prove to be the preferred biomarker for immunotherapy. Cristescu and colleagues pooled samples from 315 patients across multiple pembrolizumab trials and performed RNA profiling on 18 inflammatory genes: CCL5, CD27, CD274 (PD-L1), CD276 (B7-H3), CD8A, CMKLR1, CXCL9, CXCR6, HLA-DQA1, HLA-DRB1, HLA-E, IDO1, LAG3, NKG7, PDCD1LG2 (PD-L2), PSMB10, STAT1, and TIGIT. Remarkably, high TMB or high GEP was associated with tumor response to pembrolizumab, and the correlation between high-TMB and high-GEP tumors was low. MSI-H CRC was an exception; nearly all tumors had high TMB, but only approximately 50% had high GEP. Importantly, approximately 25% of gastric adenocarcinomas and 50% of HCCs had low TMB and low GEP, correlating with a lack of benefit from pembrolizumab in these subgroups. Thus, high GEP may identify more patients with MSS tumors that have low TMB, and patients with these tumors might benefit from immune checkpoint inhibition, although the associations need to be validated in prospective clinical trials.

Finally, the colonic microbiome is thought to influence the response to immune checkpoint inhibition in patients with melanoma. Thus, patients in whom certain gut bacteria (Bifidobacterium longum, Collinsella aerofaciens, and Enterococcus faecium) are relatively numerous have shown better responses to checkpoint inhibitors. Fascinatingly, Fusobacterium nucleatum not only is carcinogenic for a small subset of CRCs but also is associated with decreased TILs in MSI-H CRC, suggesting that the presence of this bacterium may limit response to immunotherapy. These findings need to be evaluated in patients with gastrointestinal cancers, but they do suggest the possibility of a biomarker that could be modified with probiotics and antibiotics.

**Conclusion**

Superior biomarkers are clearly needed to identify more accurately those patients with gastrointestinal cancers who will benefit from immune checkpoint inhibition. MMR-D/MSI-H, TMB, and PD-L1 are helpful, but they are merely pieces of a larger, more complicated puzzle. The relative rates and overlap of the presence of MSI-H, TMB, and PD-L1 vary significantly across tumor types. The clinical relevance of PD-L1 positivity is high for gastric and gastroesophageal cancers but low for HCC. Viral etiologies, TILs, GEP, and the gut microbiome are important additions to our predictive arsenal for immunotherapy response; however, more work needs to be done to validate their utility prospectively. Molecular profiling companies should strongly consider adding TILs, GEP, and eventually stool microbiome to their future platforms.

**Disclosures**

The authors have no relevant conflicts of interest to disclose.

**Acknowledgements**

The authors thank Marion Hartley, PhD, Science Writer for Clinical Research at The Ruesch Center for the Care of Gastrointestinal Cancers, Georgetown Lombardi Comprehensive Cancer Center, for her edits and suggestions during the composition of this manuscript.

**References**


