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IMMUNOPATHOLOGIC STRATIFICATION OF COLORECTAL CANCER FOR CHECKPOINT BLOCKADE IMMUNOTHERAPY

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Conflict of interest:

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Abstract

Mismatch-repair deficiency in solid tumors predict their response to PD-1 blockade. Based on this principle, pembrolizumab is approved as standard of care for patients with unresectable or metastatic microsatellite instability–high (MSI-H) cancer. Despite this success, a large majority of metastatic colorectal cancer (mCRC) patients are not MSI-H and do not benefit from checkpoint blockade treatment. Predictive biomarkers to develop personalized medicines and guide clinical trials are needed for these patients. We, therefore, asked whether immunohistological stratification of mCRC based on primary tumor PD-L1 expression associated with the presence or absence of extracellular mucin defines a subset of mCRC patients who exhibit a pre-existing antitumor immune response and who could potentially benefit from the checkpoint blockade. To address this, we studied 26 advanced mCRC patients treated with pembrolizumab (). To stratify patients, incorporation of histopathological characteristics (% extracellular mucin) and PD-L1 expression at the invasive front were used to generate a composite score, the CPM score (composite PD-L1 and mucin), which discriminated patients who exhibited clinical benefit (complete, partial, or stable disease) from those patients with progressive disease. When validated in larger cohorts, the CPM score in combination with MSI testing may guide immunotherapy interventions for CRC patient treatment.

Keywords

extracellular mucin; PD-L1; biomarker; immunotherapy; checkpoint blockade

Introduction

Oncologic precision medicine involves screening for and selecting therapies based on, an individual's tumor-specific biomarkers to optimize clinical outcomes and minimize adverse events. The use of mismatch-repair deficiency as a predictive biomarker of colorectal cancer (CRC) response to PD-1 blockade was first reported by Le et al. in 2015 (1) and confirmed in 2017 (2). Mismatch-repair deficiency, leading to accumulation of non-synonymous mutations, predicts the response of solid tumors to PD-1 blockade, and based on this principle, the food and drug administration (FDA) granted accelerated approval to pembrolizumab for adult and pediatric patients with unresectable or metastatic microsatellite instability–high (MSI-H) tumors. This is the first example of a tissue-agnostic FDA approval of a treatment based on a patient's tumor biomarker status, rather than on tumor histology. Despite this success, a large majority of patients do not benefit from checkpoint inhibitors (3). Multiple genomic and immunological factors may potentially contribute to anti–PD-1's efficacy in subsets of patients with melanoma or NSCLC, among other cancers (4). Therefore, predictive biomarkers to develop personalized medicines and guide clinical trial development are an urgent unmet need.

Four elements have taken the limelight in the search for biomarkers: (i) PD-L1 expression in the tumor microenvironment; (ii) the presence of abundant T-cell infiltrates and surrogate transcriptional signatures of IFNγ function; (iii) estimations of tumor mutational burden (TMB); and (iv) studies on the composition of the gut microbiome, all contributing in identifying baseline (pre-treatment) immune-related biomarkers to predict clinical outcome

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of immunotherapy (5-8). Integration of PD-L1 expression and TMB was proposed to better identify patients who will benefit from checkpoint inhibition (9,10). However, each biomarker by itself may not be able to accurately delineate patients who benefit from immunotherapy (11). We, therefore, focused our study on the tumor immune microenvironment (TiME) of metastatic (m)CRC using primary colon tumor specimens from mCRC patients treated with pembrolizumab (trial) and compared tumor specimens of patients who exhibited clinical benefit [CB: complete response (CR), partial response (PR), and stable disease (SD)] with patients developing progressive disease (PD). The objective was to understand the nature of the immunohistopathological components of the TiME that associated with the CB of these patients and ultimately delineate a population of immuno-reactive CRC potentially suitable for immune interventions.

Materials and Methods

Clinical trial and patient selection.

Patients with previously treated mCRC were selected from six centers (Johns Hopkins University, Providence Portland Medical Center, Stanford University, Ohio State University, Abramson Cancer Center at University of Pennsylvania, National Cancer Institute) for this phase II study (clinicaltrial.gov;) using pembrolizumab (anti–PD-1). To be eligible for participation in this study, patients had to be at least 18 years of age and have histologically confirmed evidence of previously treated, progressive carcinoma. All patients underwent MMR status testing prior to enrollment. All patients had at least one measurable lesion as defined by the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1, an Eastern Cooperative Oncology Group (ECOG) performance-status score of 0 or 1, and adequate hematologic, hepatic, and renal function. Eligible patients with CRC must have received at least 2 prior cancer therapies, and patients with other cancer types must have received at least 1 prior cancer therapy. Patients with untreated brain metastases, history of HIV, hepatitis B, hepatitis C, clinically significant ascites/effusions, or autoimmune disease were excluded. A total of 86 patients with treatment-refractory progressive, advanced, mismatch repair-deficient cancers were recruited in three cohorts, cohort A for the MSI^+ mCRC, cohort B for the MSS mCRC, and cohort C for the MSI^+ non-CRC (1). Additional longitudinal data from eleven CRC and seven non-CRC patients with mismatch repair-deficient cancers from our previous report were included (1,2).

For study enrollment, mismatch repair deficiency was determined at each participating institution by immunohistochemistry for mismatch repair proteins or by PCR-based tests for microsatellite instability. When sufficient tissue was available, microsatellite instability in DNA purified from the tumor was assessed with an MSI Analysis System (Promega). Our analysis utilized samples obtained from cohorts A and B of the trial, CRC-only cases, and specimens included in this manuscript were those with sufficient material available and had corresponding clinically annotated data. We segregated patients according to their clinical benefit to checkpoint inhibition. Groups were composed of CR/PR/SD patients who were deemed to have CB versus the PD patients. This study was approved by the Institutional Review Board of Johns Hopkins University and was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization Good Clinical

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Practice guidelines. The patients described in this study provided written informed consent, and tumor colon tissues were obtained in accordance with the Health Insurance and Accountability Act [detailed in ref (1,2)].

Histopathology, immunohistochemistry, and image analysis.

Formalin-fixed paraffin embedded (FFPE) tissue sections from resected colon tumors obtained at the diagnosis were stained with a hematoxylin and eosin (H&E) combination. Extracellular mucin pools were defined as the collection of mucin not associated with malignant epithelial cells and quantified as the percentage of the tumor surface area replaced by extracellular mucin pools(12). Digital quantification of tumor and mucin areas was performed utilizing the HALO™ image analysis platform from Indica labs (Supplementary Fig. S1). FFPE colon tumor tissue sections were also stained for CD8 (clone C8144B, Cell Marque, Rocklin, CA) and PD-L1 (clone 5H1) as previously reported (13,14). For $CD8^+$ T-cell density quantification, 90% of the tumor cellular area was annotated, and we selectively included the tumor area that contained malignant epithelial cells and excluded extracellular mucinous areas. Digital quantification was performed utilizing the HALO™ image analysis. PD-L1 was scored at the invasive front, which is the region where the tumor tissue juxtaposes the normal colonic tissue (13). We assessed inter-observer agreement by using independent readings from two pathologists (RAA and ET) blinded to the outcomes of patients. Correlation of the scoring between the two pathologists was tested by determining the Spearman's rank correlation coefficients. Statistical comparisons of percentage of mucin detected or PD-L1 expression between patients who had CB and PD were performed using the non-parametric Mann-Whitney test. All the analyses were performed using the software R version 3.5.1 (The R Foundation for Statistical Computing).

Composite PD-L1 mucin (CPM) score.

Logistic regression was applied to build a composite score combining PD-L1 and mucin (CPM score) to distinguish patients who did and did not benefit from pembrolizumab treatment based on the data from 26 patients (16 CR/PR/SD and 10 PD). According to this model, we calculated the CPM score as the average of the percentage of mucin detected and PD-L1 expression (CPM = [% PD-L1 + % extracellular mucin]/2). The performance of the CPM score in distinguishing CB vs. PD was assessed using receiver operating characteristic (ROC) analysis. The area under the ROC curve (AUC) for CPM score, a measure of how well it distinguishes the two groups, was compared with PD-L1 alone and mucin alone using DeLong's test. The classification tree method, based on recursive partitioning that minimizes the misclassification error, was used to determine the cutoff threshold of CPM score in classifying patients into CB vs. PD. All the analyses were performed using the software R version 3.5.1 (The R Foundation for Statistical Computing).

Results

We first sought to compare the baseline histopathological characteristics of mCRC patients with CB to checkpoint inhibition (CR/PR/SD) with the features of patients with PD. Demographics of patients used in this study are described in Supplementary Table S1. With this approach, the two main distinctive features noted in the analyzed pre-treatment colon

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specimens of patients were the presence of a mucinous component and PD-L1 positivity at the invasive front (Fig. 1, Supplementary Fig. S1). Overall, mCRC patients who exhibited CB had tumors that contained higher percentages of mucin covering the tumor area and PD-L1 expression at the invasive front (Fig. 2). In contrast, patients who experienced PD neither developed mucinous features nor exhibited high percentages of PD-L1 staining (Fig. 2). These data suggested that large mucinous areas could result from tumor tissue destruction by an advancing field of immune cells, leaving behind extracellular mucin pools.

This phenomenon has been observed in the setting of chemotherapy, and the current recommendation by the College of American Pathologists is to regard extracellular mucin as a type of treatment response and not as residual tumor (12,15,16). However, we could not rule out the possibility of the tumor tissue actively secreting mucin. We, therefore, devised a composite PD-L1/mucin (CPM) score that integrated expression of PD-L1 at the invasive front with the detection of extracellular mucinous areas. The weighting factors for mucin and PD-L1 were almost identical (0.106 and 0.130, respectively), according to the logistic model estimates. Therefore, we calculated the CPM score as the average of the percentage of mucin detected and PD-L1 expression to predict clinical benefit (Fig. 3, Supplementary Table S2). With this approach, the AUC for the CPM score was 0.994, and therefore higher than the AUC for the PD-L1 alone and mucin alone [AUC=0.787 (PD-L1 versus CPM, p=0.018) and 0.882 (mucin versus CPM, p=0.057), respectively], indicating the good performance of the CPM score in distinguishing clinical benefit vs. progressive disease (Fig. 4).

The cut-off value of CPM score was determined to be 14% (see materials and methods section). Among the 16 patients who achieved CB, 15 (94%) had CPM scored greater than or equal to 14%; all PD patients had CPM score lower than 14% (Fig.3). Note that due to a limited set of data available extracted from the clinical trial, any level between 10.5 and 17.5 as cut-off threshold would lead to the same classification accuracy.

In our analysis, two blinded pathologists analyzed biopsy slides from 16 patients with CB and 10 PD patients. Scoring between the two pathologists correlated, with r values of 0.99 and 0.97, respectively (Supplementary Fig. S2). Although significantly different between CB and PD patients, mucin and PD-L1 scores, individually (p=0.0004 and 0.0120, respectively, CB versus PD patients), did not clearly segregate CB and PD patients (Fig.2). On the contrary, we demonstrated that 15 out of 16 CB patients had a CPM score >14% (CB versus PD, p<0.0001; Fig.3). We also found that two mismatch repair–proficient (MMRp) mCRC with SD (patients 1-010 and 1-040) had a CPM score >14% (Fig. 3). None of the PD patients had a CPM score >14% (Fig.3).

Although prior observations indicate that the presence of $CD8^+$ tumor-infiltrating lymphocytes (TILs) in tumor biopsy samples is associated with improved survival in CRC patients (17), in our study, $CD8^+$ T-cell densities in mCRC patients with clinical benefit and PD were not statistically different, and our analysis did not separate patients according to their response pattern once an outlier patient was removed from the dataset (patient 1-052 with intratumoral $CD8^+$ T-cell density=3025 cells/mm^2 versus an average of 282 $CD8^+$ cells/mm^2 for the rest of the cohort; Supplementary Table S3, Supplementary Fig. S3-S4),

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demonstrating that $CD8^+$ T cells are often excluded from tumor areas where large extracellular mucin areas replace tumor tissue. We, therefore, propose that $CD8^+$ T-cell counts in these tissue areas may underestimate the extent of the endogenous intratumoral immune response. Spearman correlations between individual components of our composite score (% extracellular mucin and % PD-L1 expression) and their respective correlation with the corresponding intratumoral $CD8^+$ T-cell densities did not show an association, suggesting that the selected immunopathologic features, extracellular mucin and PD-L1, are largely independent, thus complementary, to each other (Supplementary Fig. S5-S6).

Discussion

We herein proposed a complementary score that integrates the expression of PD-L1 at the invasive front region of tumors in combination with the detection of extracellular mucinous areas. With this approach, we demonstrated that mismatch repair deficient (MMRd) and MMRp mCRC patients with CB from checkpoint blockade exhibited significantly higher CPM scores than mCRC patients with PD. Given that pre-existing immunological features of both the host and the tumor may contribute to how patients will respond with immunotherapy (5,10), we believe that reporting mucinous features along with PD-L1 expression in routine pathology practices may delineate a subset of mCRC patients who might benefit from checkpoint blockade-based immunotherapies. The role of the adaptive immune response in controlling the growth and recurrence of human tumors has now been documented in the setting of multiple cancers (18). Thus, identifying baseline immune-related biomarkers to select patients and predict clinical outcome to immunotherapy is of the essence.

Although immune checkpoint blockade, which activates the endogenous immune system against cancer, has led to breakthroughs for a variety of malignancies, clinical responses is limited to a subgroup of patients (~12%)(3). The type, density, and location of immune cells within colorectal tumor samples have been found to be better at predicting survival of patients over the histopathological methods currently used to stage colorectal cancer (17). Adaptive immune cell infiltration was observed to have a prognostic value superior to the classical extension and invasion tumor criteria (19). The “Immunoscore” quantifying the density of $CD3^+$ and $CD8^+$ T cells in the tumor center and its invasive margin was proposed as a novel immune classification in colorectal tumors (17). Accumulating exceptions (such as lack of response to treatment in some patients, the incomplete correlation between PD-L1 expression and clinical effectiveness of PD-1 blockade (5,20,21), and the counter examples in renal cell carcinoma in which the presence of T cells is generally associated with poor outcome (22)) indicate that a more comprehensive profiling of local immune cells and their function is warranted. In our study, by pairing the clinical response data with an interrogation of the TiME of samples served as an inestimable window into the TiME of mCRC patients, which is critically important to identify relevant biomarkers independent of the MSI status. We conclude that mCRC patients who exhibited CB to anti–PD-1 treatment displayed consistent immunopathological features that could be quantified; i.e. the percent of PD-L1 expression at the tumor invasive front and the corresponding amount of extracellular mucin present in the specimens. This composite score can be readily calculated

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using existing FDA-approved IHC tests for PD-L1 expression, which use different anti–PD-L1 clones (4), and H&E staining to estimate extracellular mucin content.

Both parameters used in this study were valid, regardless of the MSI status of patients. Two MMRp mCRC patients, who observed long term SD, had a CPM score >14%. Mucinous adenocarcinoma is part of the WHO classification of colorectal carcinoma. Traditionally, the grading of mucinous and signet ring carcinomas, which were previously invariably graded as G3/high-grade, is dependent on the MSI status (23). Interestingly, a previous article reported an inverse association of tumor CD274 (PD-L1) expression with tumor MSI status and the extent of extracellular mucin (24). The focus of our study was to justify that the CPM combination score was better than mucin or PD-L1 alone for discriminating response vs. no response in CRC patients. Although $CD8^+$ T-cell infiltration has been clearly established as a prognostic factor in the case of CRC and is associated with better PFS and OS (17), it has not been shown to be a predictive marker that can guide patient selection to receive immuno-oncology therapy. Our observations on a limited number of patients enrolled in the clinical trial will be prospectively validated in our follow-up clinical trial, in which two cohorts of MMRp mCRC patients are enrolled based on their positive or negative CPM score and subsequently assigned to receive combination immunotherapy (). Combined with MSI testing to select MMRd CRC for checkpoint blockade treatment, this approach has the potential to open up immunotherapy to a broader population of CRC cancer patients by including microsatellite stable (MSS) CRC patients who are currently not captured by current molecular biomarkers. Such is the case of the outlier patient (#1-010) characterized by a CPM score >14% and who exhibited SD for more than 3 years after initiation of anti–PD-1 therapy (25). MSS stage IV CRC patients and their caregivers have a sense of urgency that is not currently reflected in clinical trials, and we believe that our data could contribute to addressing this unmet need.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments.

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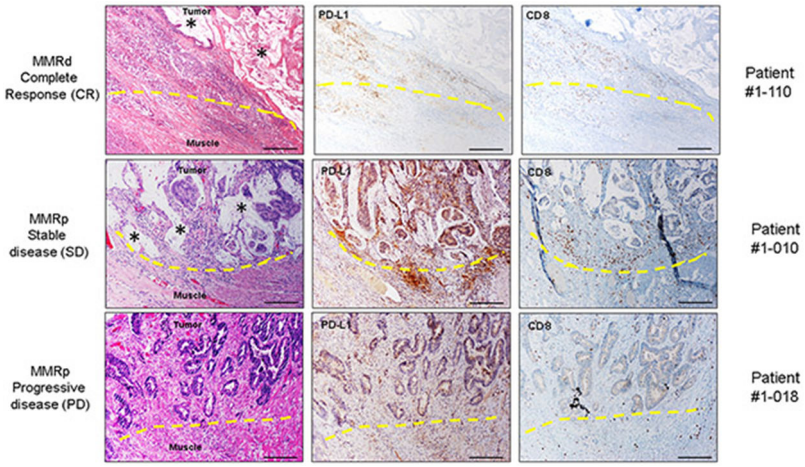

Figure 1. Representative H&E, PD-L1 IHC, and CD8$^+$ T-cell IHC of baseline colon tumor specimens of patients treated with pembrolizumab (anti–PD-1).

Characteristic patterns of mucinous component, PD-L1 expression at the invasive front (IF), and CD8$^+$ T-cell infiltration in samples from patients exhibiting CB (CR/PR/SD) versus PD. Patient #1-110, MMRd with CR; patient #1-010, MMRp with SD; and patient #1-018, MMRp with PD. Asterisks represent extracellular mucin pools in tumor area and dashed yellow line marks IF region. Images at 10x magnification; Scale bar:1 millimeter.

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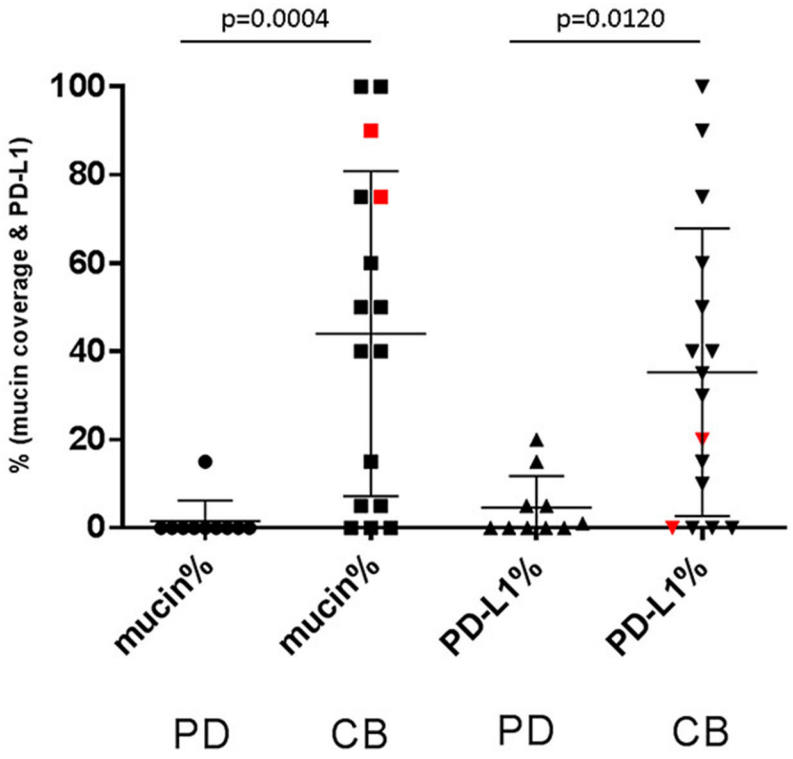

Figure 2. Individual extracellular mucin quantification and PD-L1 scoring at IF of pre-treatment resected colon tumor specimens.

Invasive front (IF), Progressive disease (PD; n=10), clinical benefit (CB: CR/PR/SD; n=16). Two MMRp CRC patients with SD are indicated in red. Mean+standard deviation; Two-sided non-parametric Mann-Withney test; statistical significance when $p<0.05$.

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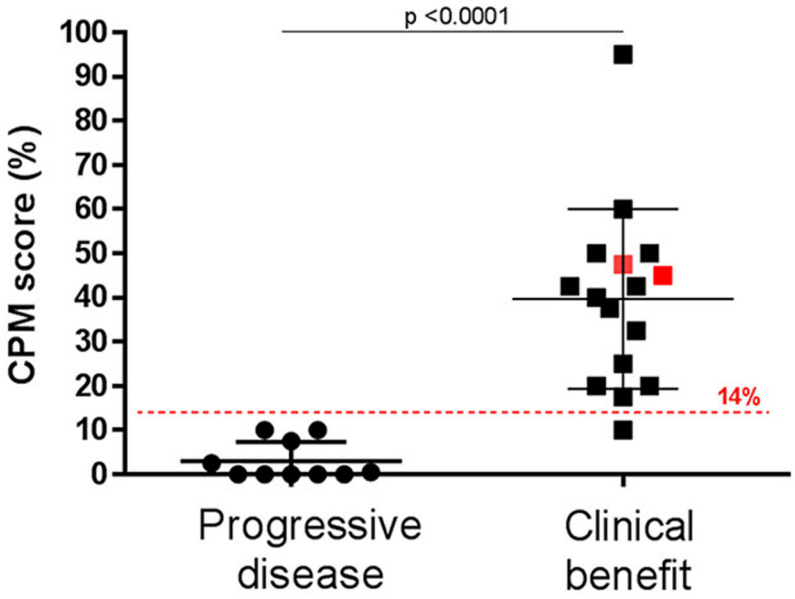

Figure 3. Composite PD-L1/mucin (CPM) score segregates CB from PD patients.

The CPM score integrates expression of PD-L1 at the invasive front and the detection of acellular mucinous areas in colon tumor specimens. The cut-off value of CPM score was determined to be 14% (red dashed line). Two MMRp CRC patients with SD are indicated in red. Progressive disease (PD; n=10), clinical benefit (CB, CR/PR/SD; n=16). Mean +standard deviation; Two-sided non-parametric Mann-Whitney test; statistical significance when $p<0.05$.

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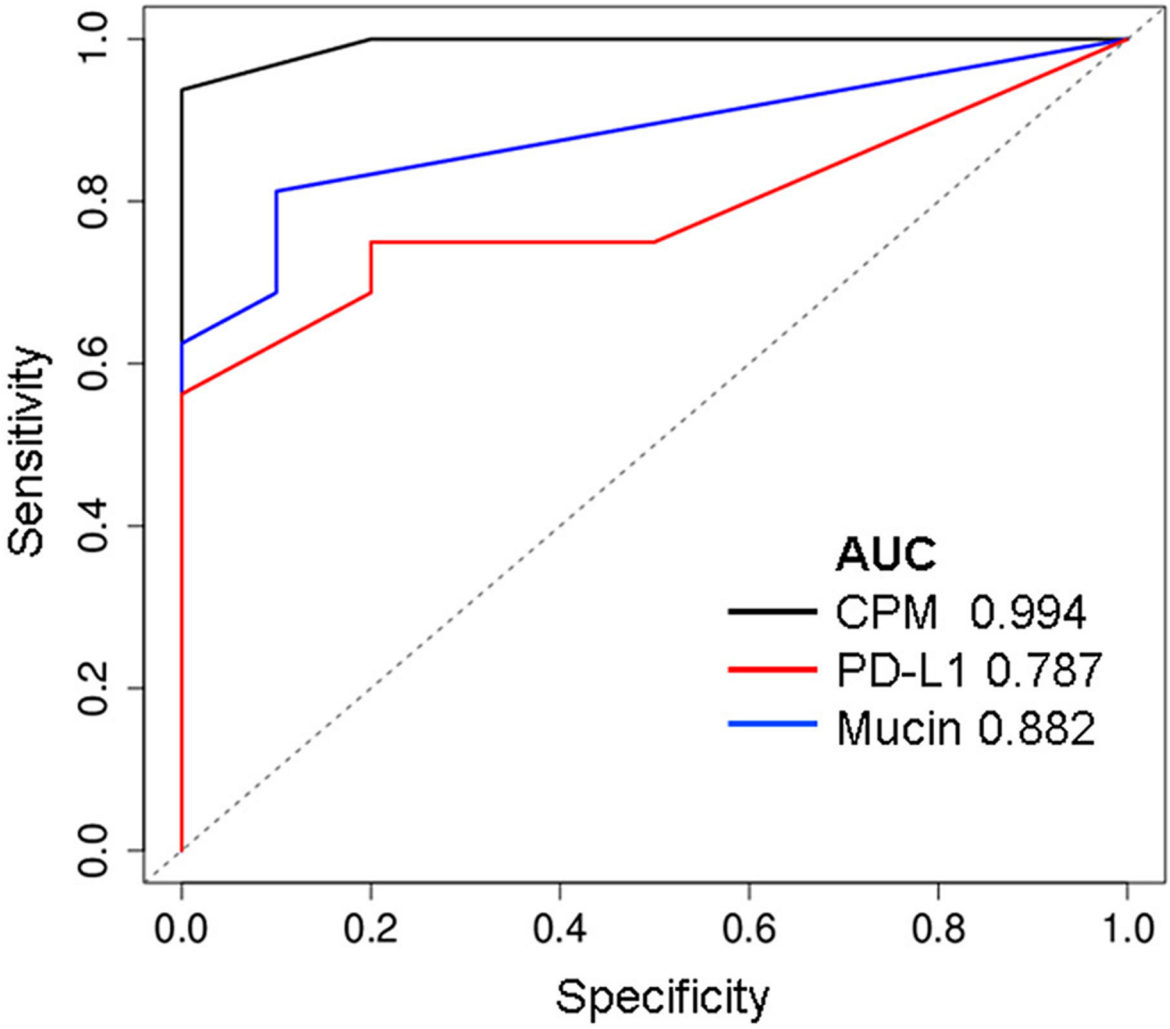

Figure 4. Receiving operating characteristic (ROC) curves demonstrates the performance of the CPM score to stratify CB versus PD patients.

Area under the curve (AUC) is shown for the CPM score, % PD-L1 expression, and % mucin area. DeLong's test was used to compare PD-L1 alone vs. CPM (p=0.018) and mucin alone vs. CPM (p=0.057). Progressive disease (PD), clinical benefit (CB; CR/PR/SD). The dotted diagonal line represents a ROC curve of a classifier based on pure chance.