

An Efficient Prognostic Immune Scoring System For Colorectal Cancer Patients With Peritoneal Metastasis

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ABSTRACT

Immunoscore can accurately predict the prognosis of patients with stage I–III colorectal cancer. However, whether it can be used to predict the prognosis of colorectal cancer peritoneal metastases (CRCPM) remains to be validated. We analyzed peritoneal and ovarian metastases in 68 patients with CRCPM. The immunoscore (IS) was based on the infiltration level of CD3+ and CD8+ T cells, whereas the TBM score was derived from the infiltration level of CD3+, CD8+, CD20+ and CD163+ cells to tumor microenvironment (TME). The predictive value of IS and TBM scores for relapse-free survival (RFS) and overall survival (OS) of patients with CRCPM was analyzed using Kaplan Meier curve and Cox multivariate models. Significant difference in the infiltration levels of different immune cell subtypes in primary lesions, peritoneal metastasis and ovarian metastasis were compared using t-test. CRCPM patients with high IS (>1), high TBM1 score (≥2) or high TBM2 score (≥2) had a significantly longer OS (IS: median OS, not reached vs 23 months, $p = .0078$; TBM1: not reached vs 21.5 months, $p = .013$; TBM2: 39.3 months vs 15.2 months, $p = .001$). On the other hand, patients with high IS had a trend of improved RFS (13.4 months vs 11.0 months, $p = .067$). However, TBM1 and TBM2 score has no predictive utility for RFS. Multivariate analysis revealed that IS, TBM1 and TBM2 can accurately predict OS, but not RFS. Finally, the infiltration level of CD3+ T cells, CD8+ T cells, CD20+ B cells, and CD68+ macrophage was significantly higher in peritoneal metastatic tissue and ovarian metastatic tissue, relative to primary tumor tissues. The IS and TBM score of peritoneal metastases could effectively predict OS of patients with CRCPM. Peritoneal metastasis of colorectal cancer decreased the infiltration level of T and B cells.

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Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors, and the third leading cause of cancer-related morbidity and mortality worldwide.¹ Besides, peritoneal metastasis (PM) exacerbates the CRC-related deaths.² Previously, CRCPM was thought to be a terminal and an incurable cancer stage, requiring only palliative treatment.³ Due to its unique biological properties, PM is associated with very poor prognosis, relative to other organ metastases, such as liver or lung metastasis.⁴ However, recent advances in surgical technology and other treatment interventions have improved the overall survival (OS) of patients with M1c CRC.^{5,6} The advent of monoclonal antibody therapies (Programmed Cell Death 1, PD-1; Programmed Cell Death 1 Ligand 1, PDL-1; Cytotoxic T Lymphocyte Antigen 4, CTLA-4) that kill cancer cells by activating the adaptive immune system,⁷ have revolutionized cancer treatment. However, this treatment module is only effective in few patients with high mutation burden, characterized by under-expression of DNA mismatch repair proteins (dMMR) and microsatellite instability-high (MSI-high).⁸ On the other hand, tumors with low mutation

burden or lower infiltration of immune cells usually exhibit immune resistance.^{9,10}

Tumor microenvironment (TME) comprises of malignant and nonmalignant cells (immune cells, tumor blood vessels, lymphatic vessels, fibroblasts, adipocytes, vascular endothelial cells, etc.). The immune cells perform different function at various stages of tumor development and progression, resulting into a dynamic immune system.¹¹ Infiltration of CD8+ T lymphocytes to TME can directly mount an immune response against the tumor. Other critical anti-tumor cells include tumor associated macrophages (TAMs) and B lymphocytes.^{11,12} Presence of tumor infiltrating lymphocytes (TILs) in TME influence the prognosis of head, neck, lung, kidney, ovarian, breast, colorectal and pancreatic cancer.^{13–19} Infiltration pattern of TILs, particularly CD8+ and CD3+ T cells both in early and advanced stage CRC strongly and independently predicts the prognosis of CRC.^{18,20} In addition, high-density B lymphocyte infiltration also indicates improved prognosis of CRC.²¹ Given their plasticity, of TAMs are polarized into different subtypes.²² Research shows that pan-TAMs are associated with a poor prognosis of most solid tumors.²³

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However, the prognostic value of TAMs in CRC remains controversial.^{23,24}

The immunoscore based on the infiltration density of CD3+ and CD8+ TILs is used independently in predicting the prognosis of patients with stage II and III colon cancer.¹⁸ Based on this traditional immune scoring system, the prognostic value of TB scoring system (CD8+ T cells/CD20+ B cells) has also been validated for advanced CRC (liver metastasis, lung metastasis).²¹ However, very few studies have explored the immune cell infiltration profile of CRCPM. Here, we derived an accurate immune scoring system for predicting the prognosis of CRCPM. Thus we provide a theoretical basis for screening patients with CRCPM who are more suitable for immunotherapy. We analyzed the subtypes and infiltrating density of the immune cells in TME of primary CRC and PM. We also analyzed the immunological changes in TME following metastasis.

Methods and materials

Research participants

This study involved patients with CRCPM, who underwent abdominal metastasis resection at the Second Affiliated Hospital, Zhejiang University School of Medicine from January 2011 to December 2018. We included patients with pathologically diagnosed with CRCPM, fit for tumor cytoreductive surgery as a treatment option as well as those with sufficient pathological data and specimens of PM for analysis. Patients who underwent preoperative radiotherapy for abdominal tumors and those with autoimmune diseases were excluded from the study. The protocol for this study was approved by Institutional Review Board (IRB). All study participants consented to this research in writing.

Data collection

Patients were followed up to October 2019. The survival as well as recurrence status of patients were obtained using an outpatient system, inpatient records or telephone follow-up. Clinical and pathological data of those patients were collected and recorded. The data included pathological and histological type, T stage of the primary tumor, number of lymph node metastases, neurovascular invasion, among others. Clinical information, such as the anatomical site of the primary tumor, whether of the patients received hyperthermic intraperitoneal chemotherapy (HIPEC), presence of ascites, peritoneal cancer index (PCI) and completeness of cytoreduction (CC) were also recorded.

Immunohistochemistry

Paraffin-embedded slides of peritoneal metastatic lesion specimens were stained and labeled using anti CD3+ T cells (BOSTER, No. PB0112), CD8+ T cells (BOSTER, No. PB0235) and CD20+ B cells (BOSTER, No. PB0028) and CD68+ (BOSTER, No.M00602) as well as CD163+ macrophages (BOSTER, No.BA13856) using specific antibodies. The slides were stained using hematoxylin and eosin (HE).

The CD3+, CD8+, CD20+, CD68+ and CD163+ cells were identified by a qualified pathologist. Two pathologists blinded to patient's clinical information divided the immunohistochemical section into invasive margin (IM) or central area of tumor (CT). To confirm the location of the CT/IM, the CT area was defined to be at least a distance of 20x fields from the border of normal mucosa. The IM area was defined as a 20x field within the most distal tumor cells.^{25,26} The hot spots with positive staining in the two regions were obtained. Computer-assisted calculations of density of positively stained immune cells in both the CT and the IM of the primary focus of CRC were performed using Image J software (National Institute of Health, Bethesda, MD, USA). The same method was used in counting the positively stained immunocytes in the CT area of the PM and ovarian metastasis.

Image acquisition and quantification

The tissue sites with the highest density of tumor infiltrating immune cells in the CT and IM images were captured under high-power magnification (20x) using a Leica dm6000 B molecular microscope. A high-density image of positive cell was captured in the CT area and IM of primary focus, while for noninvasive margin staining sections, only the CT image of high-density infiltrating positive cells was taken. Computer assisted image analysis was performed using ImageJ software V1.48. Five built-in functions of ImageJ were used to calculate the number of positively stained cells per high power field of view (HP). The cutoff value of the infiltrating density for each tumor-associated immune cell subtype was calculated using the X-tile software V. 3.6.1. For relapse-free survival (RFS), the best cutoff values for high infiltration of CD3+, CD8+, CD20+, CD68+ and CD163+ immune cells were 110, 117, 143, 268 and 99 cells/HP, respectively. On the other hand, for OS, the best cutoff points for high infiltration of CD3+, CD8+, CD20+, CD68+ and CD163+ immune cells were 117.5, 88, 92, 158 and 40 cells/HP, respectively. Each immune cell subtype was assigned a dichotomous score (high infiltration density of CD3+, CD8+, CD20+ cells were scored 1, low density was scored 0; low infiltration density of CD163+ was scored 1, high infiltration density was scored 0) based on the set cutoff point. The traditional Immunoscore was derived using the infiltration density of CD3+ and CD8+ T cells. Patients with an Immunoscore = 2 were classified in to high-score group (IS-high), whereas those with an Immunoscore <2 were classified into a low-score group (IS-low). We also established a TBM scoring system 1 (TBM1), based on the infiltration density of CD8+, CD20+ and CD163+ immune cells. Patients with TBM1 ≥ 2 were classified in to high-score group (TBM1-high), TBM1 < 2 was defined as low-score group (TBM1-low). TBM scoring system 2 (TBM2) was established according to infiltration density of CD3+, CD8+, CD20+ and CD163+ immune cells. Patients with TBM2 ≥ 2 were classified into the high-score group (TBM2-high), whereas those with TBM2 < 2 were classified into the low-score group (TBM2-low).

Statistical analysis

The example of computer-assisted analysis of the densities of the positively stained cells is provided in Figure 1a to 1d (a→b→c→d). This method has been used in previous studies.^{20,27}

RFS was defined as the period from the date of peritoneal metastasectomy to the date of first relapse at any site, or death due to any cause instead of relapse. OS was calculated from the peritoneal metastasectomy to death due to any cause. The prognostic utility of the Immuno-score, TBM immune score and various clinicopathological factors was analyzed using Kaplan Meier curve and log rank test. Cox multivariate models were used for multivariate analysis to determine independent prognostic factors affecting the survival of patients with CRCPM. T-test was used to analyze the difference in infiltration density of the tumor-associated immune cells in primary tissue, peritoneal metastatic tissue and ovarian metastatic tissue of CRC. All statistical examinations were conducted using GraphPad Prism (version 8.0.1) and IBM SPSS software (version 22). $P < .05$ was considered statistically significant.

Results

Basic characteristics of patients with CRCPM

Overall, we recruited 68 patients with a mean age of 55 years. Of these, 38 (55.88%) were female whereas 30 (44.12%) were male. All the patients underwent cytoreductive surgery, in

which 51.5% of the patients presented with primary lesions of CRC. There were 36 cases (52.9%) of right colon cancer and 22 cases (32.4%) of left colon cancer, 10 (14.7%) cases of rectal cancer. Thirteen patients (19.1%) had ovarian metastasis. Moreover, 50% of the CRCPM patients presented with histologically confirmed for mucinous adenocarcinoma or signet ring cell carcinoma, whereas the rest was presented with non-mucinous adenocarcinoma/signet ring cell carcinoma. The G1 histological grade had 4 cases (5.9%), G2 had 23 cases (33.8%), while G3 had 41 cases (60.3%). Intraoperative ascites was identified in 41 patients (60.3%). However, metastatic lesions for 11 patients were not quantitatively analyzed for having less tumor cells or poor staining. The median infiltrating density of CD3+ cells in CT was 205/HP (Interquartile Range, IQR, 152.-0-295.8/HP), whereas that of CD8+ T cells was 58.5/HP (IQR, 29.0-140.5/HP). The median infiltrating density of CD20+ B cells was 55/HP (IQR, 33-130.3/HP), that of CD68+ macrophages was 148/HP (IQR, 72.8-215.0/HP), while that of CD163+ macrophages was 45/HP (IQR, 25.8-88.5/HP). These clinical characteristics are summarized in Table 1.

Comparison of immune status of primary and metastatic focus in patients with CRCPM

The comparative infiltration densities of the immune cells in four patients with primary lesions, PM and ovarian metastasis are shown in Figure 2a-2e. The ovarian metastasis showed an

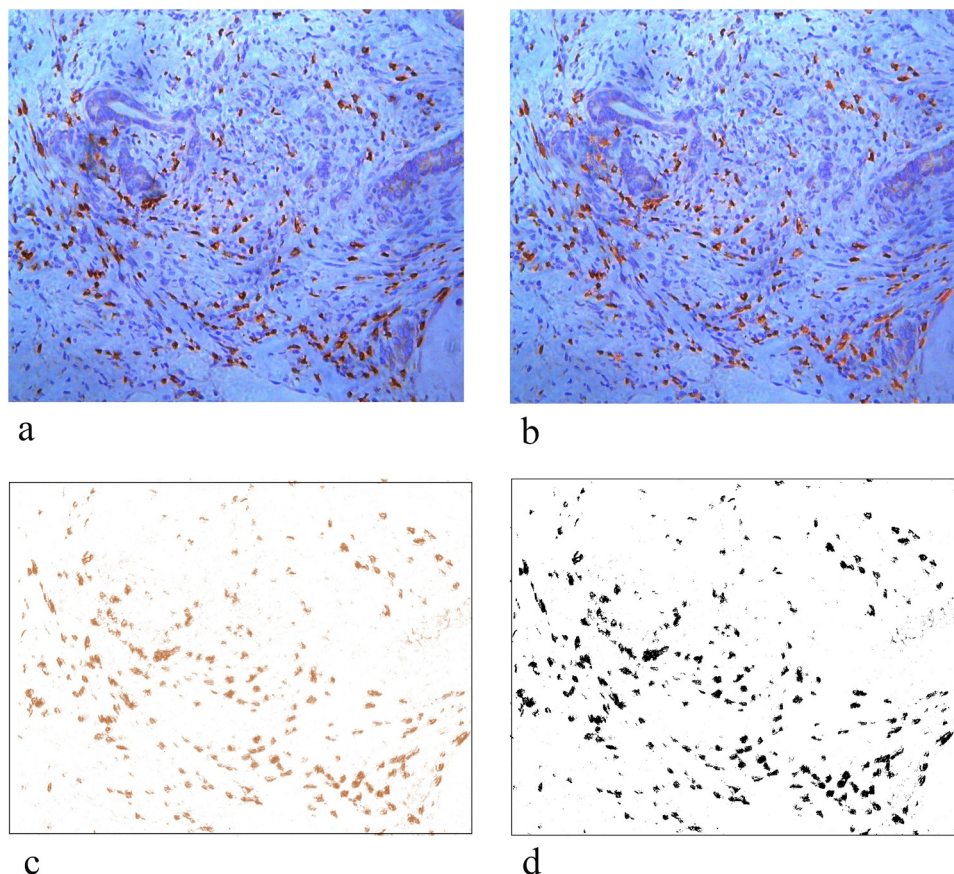


Figure 1. Example of computer-assisted analysis of the densities of the positively stained cells. (a) Image of a peritoneal metastatic tumor center with CD3+ staining. (b) Image after conducting rolling-ball method for background subtraction. (c) Image after performing color deconvolution for separating DAB (brown) and hematoxylin. (d) Image after thresholding to obtain a binary image.

Table 1. Clinical characteristics of patients with colorectal cancer peritoneal metastasis.

Variables	N (%)
Age (mean)	55 (25–87)
Sex	
Female	38 (55.9)
Male	30 (44.1)
PCI	
PCI<10	42 (61.8)
PCI≥10	26 (38.2)
Primary cancer	
Right colon cancer	36 (52.9)
Left colon cancer	22 (32.4)
Rectal cancer	10 (14.7)
Pathological type	
Non-Adenocarcinoma/Signet-ring cell cancer	34 (50.0)
Adenocarcinoma/Signet-ring cell cancer	34 (50.0)
Grade	
G1-2	27 (39.7)
G3	41 (60.3)
T stage	
T1-3	48 (70.6)
T4	20 (29.4)
N stage	
unknown	15 (22.1)
N0	17 (25.0)
N1-2	36 (52.9)
Ovarian metastasis	
yes	13 (19.1)
no	55 (80.9)
CC	
CCR0-1	55 (80.9)
CCR2-3	13 (19.1)
HIPEC	
Yes	61 (89.7)
No	7 (11.5)
Ascites	
Yes	41 (60.3)
No	27 (39.7)
Recurrence	
Yes	56 (82.4)
No	12 (17.6)
Survival status	
Yes	39 (57.4)
No	29 (42.6)

immune desert state, extremely deficient in each subtype immune cells. Compared with the primary lesions, the infiltration of CD3+ T cells, CD8+ T cells and CD20+ B cells, associated with better prognosis, were substantially lower both in peritoneal and ovarian metastases. In addition, the infiltration of immune cells in ovarian metastases were substantially low, relative to peritoneal metastases.

The infiltration of CD3+, CD8+, CD20+, CD68+ and CD163+ immune cells in the IM area was substantially higher than that of CT area in 34 CRCPM patients (CD3: $p = .002$, CD8: $p = .007$, CD20: $p = .005$, CD68: $p = .01$, CD163: $p = .008$) (Figure 3a–3e). On the other hand, the infiltrating density of CD8 + T cells and CD20 + B cells in 27 patients with CRCPM was significantly lower than in the corresponding primary lesions (CD8: $p = .0061$; CD20: $p = .0036$) (Figure 4a–4e).

The infiltrating density of CD3+ T cells, CD8+ T cells, CD20 + B cells and CD68+ macrophages in PM or ovarian metastasis was significantly lower compared to the primary lesions (peritoneal metastasis vs primary lesion: CD3, $p = .0004$; CD8, $p = .01$; CD20, $p < .0001$; CD68, $p = .0015$; ovarian metastasis vs primary lesion: CD3, $p = .0001$; CD8, $p = .0117$; CD20, $p = .011$; CD68, $p = .0245$). However, there was no difference

in the expression level of CD163+ macrophages in PM or ovarian metastases and primary focus (peritoneal metastasis vs primary lesion: CD163, $p = .65$; ovarian metastasis vs primary lesion: CD163, $p = .86$). The immune status of the lesions with multiple PMs was similar, and infiltrating density of the immune cells in each subtype showed no statistical difference (second metastasis vs first metastasis: CD3, $p = .69$; CD8, $p = .69$; CD20, $p = .16$; CD68, $p = .86$; CD163, $p = .67$). Our data showed that the density of CD3+ T cells and CD20+ B cells in PM was significantly different from that of ovarian metastasis, but the infiltrating density of other subtype immune cells was similar (ovarian metastasis vs peritoneal metastasis: CD3, $p = .0003$; CD8, $p = .21$; CD20, $p = .002$; CD68, $p = .76$; CD163, $p = .60$) (Figure 5a–5e).

The infiltrating level of immune cell of different histological types was shown in Figure S1. We only observed a differential infiltration of T cells (CD3+ T cell, CD8+ T cell) between mucinous adenocarcinoma/signet ring cell carcinoma and non-mucinous adenocarcinoma/signet ring cell carcinoma.

Prognostic value of the different immune scoring systems in PM

Out of the 57 CRCPM patients who were followed up to October 2019, 84.2% had tumor recurrence while 56.1% patients died. The median follow-up time was 24 months (IQR, 13.2–36.9 months). All three immune scoring systems predicted 3-year OS (66% IS-high vs 27% IS-low, $p = .0078$; 62% TBM1-high vs 32% TBM1-low, $p = .013$; 55% TBM2-high vs 12% TBM2-low, $p = .001$). However, the immune scoring system did not demonstrate good predictive values for 3-year RFS (33% IS-high vs 7% IS-low, $p = .067$; 28% TBM1-high vs 12% TBM1-low, $p = .30$; 16% TBM2-high vs 0% TBM2-low, $p = .75$).

Univariate and multivariate analysis of prognostic factors

Univariate analysis of the various clinicopathological factors and subsets of tumor-infiltrating immune cells revealed that ascites (non-existent vs existent, HR, 0.35, 95% CI (0.16–0.75), $p = .0017$), PCI (<10 vs > 10, HR, 0.43, 95% CI (0.20–0.94), $P = .033$), CD3+ T cell infiltrating density (high vs low, HR, 0.44, 95% CI (0.15–1.34), $p = .05$), CD8+ T cell infiltrating density (high vs low, HR, 0.27, 95% CI (0.14–0.55), $p = .0009$), IS (IS-high vs IS-low, HR, 0.36, 95% CI (0.18–0.72), $p = .008$), TBM1 score (TBM1-high vs TBM1-low, HR, 0.36, 95% CI (0.18–0.73) and TBM2 score (TBM2-high vs TBM2-low, HR, 0.33, 95% CI (0.14–0.81), $p = .001$) had prognostic value. There was no difference in the prediction of OS using the other univariate factors, such as age, gender, location of primary tumor, histological grade, pathological type, T stage of primary tumor, infiltrating density of CD20+ B cells, infiltrating density of CD68+ macrophages, and infiltrating density of CD163 + macrophages. Furthermore, data from the univariate survival analysis indicated that only the T stage of primary tumor (T2-3 vs T4, HR, 0.48, 95% CI (0.27–0.87) $p = .03$), ascites (non-existent vs existent, HR, 0.44, 95% CI (0.33–0.84), $p = .002$), PCI (<10 vs ≥10, HR, 0.57, 95% CI (0.32–1.04), $p = .04$) and

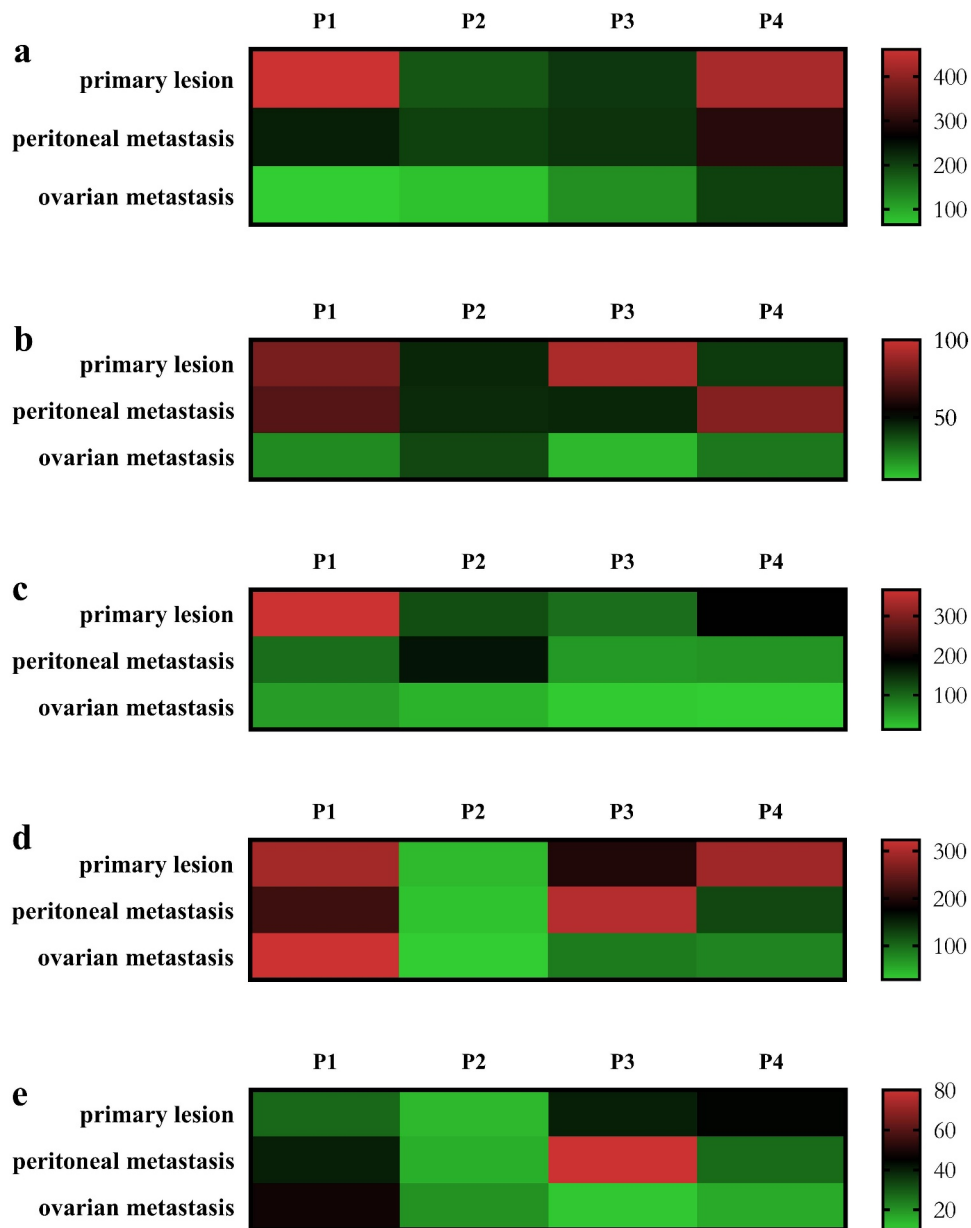


Figure 2. Infiltrating densities (cells/HP) of CD3 (a), CD8 (b), CD20 (c), CD68 (d) and CD163 (e) were quantified using hotspot in the center of tumor. Immune densities were scaled from low (green) to high (red) in four patients with primary tumor, peritoneal metastasis and ovarian metastasis.

infiltration density of CD8+ T cell (high vs low, HR, 0.52, 95% CI (0.29–0.94), $p = .04$) had predictive value in RFS. The prognostic value of other factors was unsatisfactory (Table 2, OS: Figure 6a–6c; RFS: Figure 6d–6e).

As such only T stage of the primary tumor, presence of ascites and PCI were included in the multivariable analysis. We combined IS and TBM1 score or TBM2 score to establish three risk proportion models. Immunoscore (IS-high vs IS-low, HR, 0.35, 95% CI (0.15–0.80), $p = .013$), TBM1 score (TBM1-high vs TBM1-low, HR, 0.307, 95% CI (0.14–0.67), $p = .003$) and TBM2 score (TBM2-high vs TBM2-low, HR, 0.29, 95% CI (0.12–0.69), $p = .005$) had independent predictive value for OS. In addition, existence of ascites was an independent risk factor in the first and third multivariate risk proportion models. However, none of the three immune scoring systems indicated independent predictive value for RFS. In the three risk

proportion models, only T stage of primary tumor and presence of ascites could independently predict RFS (Table 3).

Discussion

In this study, immunoscore and TBM score were used to evaluate peritoneal metastasis in patients with CRC. Our data demonstrated that TBM scoring system could accurately predict the prognosis of patients with CRCPM after cytoreductive surgery. We successfully established a traditional IS scoring model based on the infiltration density of CD3+ T cells and CD8+ T cells in PM, TBM1 scoring system based on the infiltration density of CD8+ T cells, CD20+ B cells and CD163+ macrophages, as well as TBM2 scoring system based on infiltration density of CD3+ T Cells, CD8+ T cells, CD20+ B

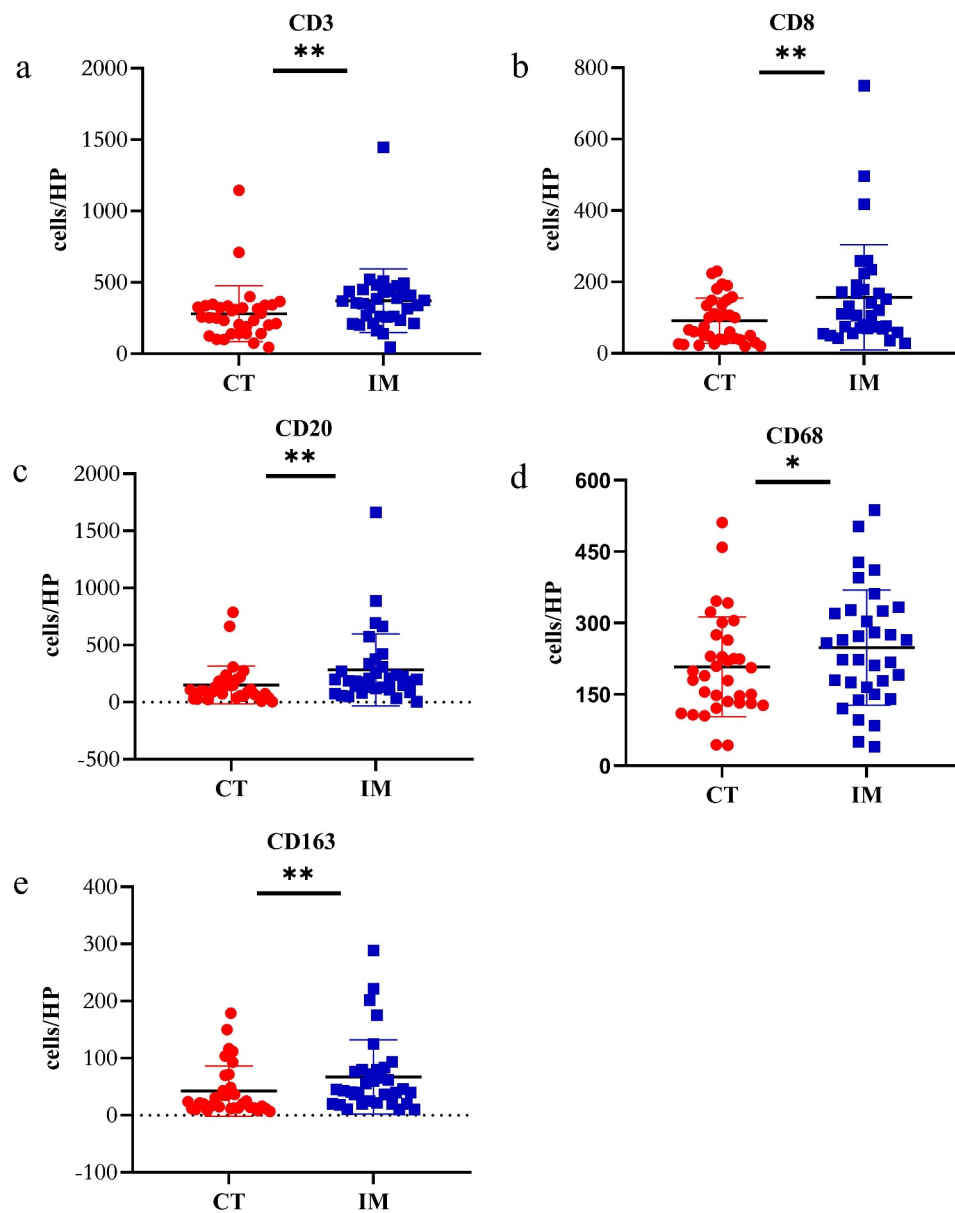


Figure 3. The density was calculated as the number of positive cells/HP (Y-axis), X-axis represents different infiltrating sites of primary lesions. A two-sided paired t test was applied to compare CT with IM. a: CD3 + T cell, b: CD8 + T cell, c: CD20 + B cell, d: CD68+ macrophage, e: CD163+ macrophage. CT: central area of tumor, IM: invasive margin, HP: high power field of view. (*represents $P < .05$, ** represents $P < .005$, ns: no statistical difference).

cells and CD163+ macrophages. The three scoring systems were associated with good prognostic value for OS. Although the three scoring systems did not display statistical significance in predicting RFS, it was observed that the IS-high or TBM-high group had improved RFS.

Compared with other related studies, we used infiltration of multiple immune cell subtypes in TME to predict the prognosis of CRCPM. Comparatively, the predictive value of TBM score was superior to that of clinical factors-based model. In this study, Image J was used to quantify the status of immune cell infiltration in tumor tissue. Compared with other methods, this software had simple operation steps and strong practicability. However, the methodology and calculations were not fully automated, which consumed a lot of time and was easy to mix in subjective factors.

Many studies have reported that the density of T-cell subtypes and the ratio of each subtype in the primary lesions strongly predict the OS and recurrence of CRC.^{9,28–33} Besides, the influence of T cell infiltration density or ratio of each subtype on prognosis has also been reported in many CRC studies with liver metastasis.^{34–36} Recent studies have quantitatively analyzed immune status of primary lesions or liver metastasis to predict prognosis of early CRC and liver metastasis. The results showed that high immune scores are effective index to predict neoplastic outcome.^{18,20,21,37} For instance, Mlecnik *et al.* reported that traditional high immunoscore can predict disease-free survival and OS of CRC with liver and lung metastases, and the established TB scoring system in this study can improve DFS and OS (TB3-4 vs TB0-2; 5-year OS, 63.7% vs 21.4%, $p < .001$; 5-year DFS,

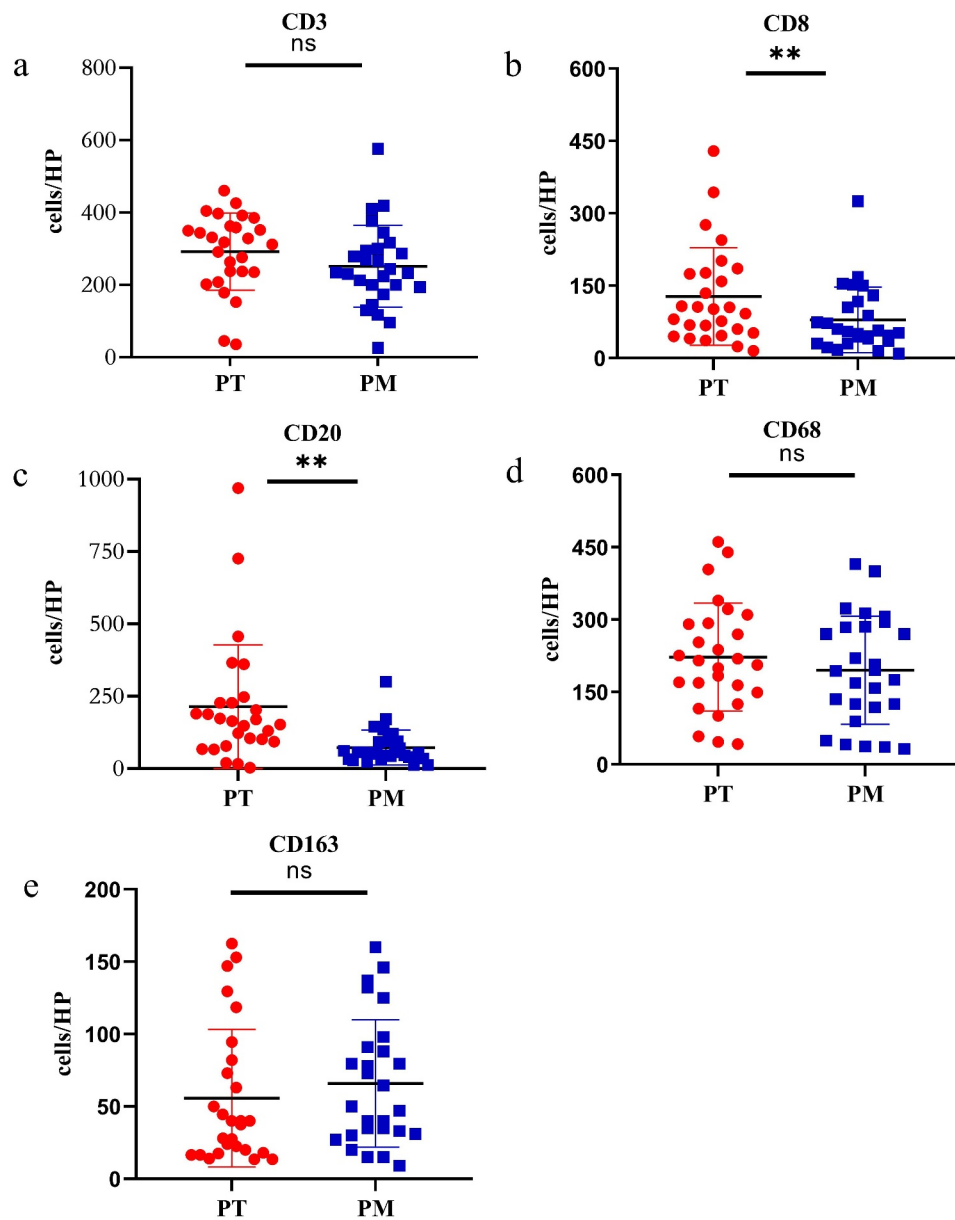


Figure 4. The density was calculated as the number of positive cells/HP (Y-axis), X-axis represents the lesions in different parts. A two-sided paired t test was applied to compare the infiltrating density of each subtype immunocyte in PM with the corresponding primary lesions. a: CD3 + T cell, b: CD8 + T cell, c: CD20 + B cell, d: CD68 + macrophage, e: CD163+ macrophage. PT: primary tumor (red), PM: peritoneal metastasis (blue), HP: high power field of view, (*represents $P < .05$, ** represents $P < .005$, ns: no statistical difference).

25.7% vs 5.0%, $p < .001$) (13–4). The TB scoring system combines both the infiltrating densities of B cells and CD8 + T cells in the liver and lung metastases.²¹ Kwak et al. used the M2 phenotype tumor associated macrophages to conduct an immunoquantitative analysis of advanced CRC, using only CD163+ macrophages in the primary lesions. The results showed that high-density infiltration of CD163+ macrophages was associated with poor prognosis. Besides, they observed that IS-ma scoring system based on the infiltration density of CD3 + T cells and CD8+ T cells in metastases, and the infiltration density of CD163+ macrophages in the primary lesions could also predict prognosis (OS: high IS-ma vs low IS-ma, $p = .005$).²⁶ However, only few studies have explored the value of immune

cell infiltrating status in predicting prognosis of peritoneal metastases in patients with CRCPM. Here, we evaluated the tumor infiltrating T lymphocytes (CD3+ T cells, CD8+ T cells), tumor infiltrating B lymphocytes (CD20+ B cells) and TAMs (CD163+ macrophages) in peritoneal metastases of CRC. We then established a novel immune scoring system – TBM scoring system, and proved its prognostic value in patients with CRCPM. It was equivalent to the prognostic value of a single immune marker (especially CD20, CD68, CD163), and the immune score composed of multiple immune indexes had more independent prognostic value. For patients with CRC who could not obtain or resect the primary tumor, the analysis of the immune status of CRCPM could help to predict the

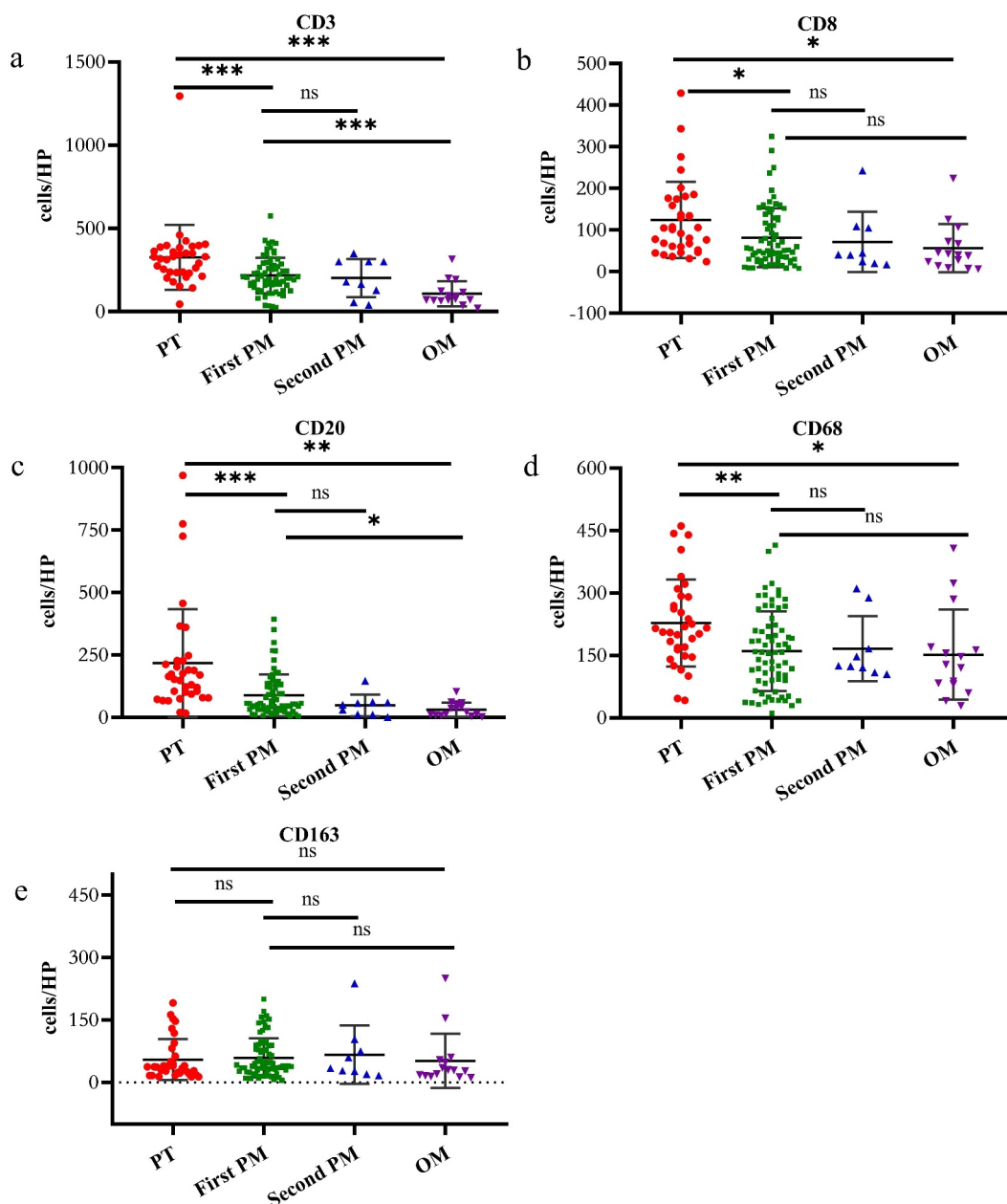


Figure 5. The density was calculated as the number of positive cells/HP (Y-axis), X-axis represents the lesions in different parts. A two-sided paired t test was applied to compare PT with first PM, PT with OM, first PM with second PM, first PM with OM, respectively. a: CD3 + T cell, b: CD8 + T cell, c: CD20 + B cell, d: CD68+ macrophage, e: CD163+ macrophage. PT: primary tumor (red), first PM: first peritoneal metastasis (green), second PM: second peritoneal metastasis (blue), OM: ovarian metastasis (purple), HP: high power field of view, (*represents $P < .05$, ** represents $P < .005$, ns: no statistical difference).

prognosis of these patients. However, one of the shortcomings of this study was that the short follow-up period dilutes the credibility of this study.

CD3+ T cells and CD8+ T cells are important immune components in TME of CRC and determine the anti-tumor immune response.^{18,20,38} Higher infiltration of CD8+ T cells in the epithelium and stroma of tumor tissue is indicative of an effective immune response and indicates improved survival.³⁹ Previous meta-analysis indicated that high-density infiltrating CD3+ T cells and CD8+ T cells in the CT of CRC do not project improved OS and DFS. However, in the IM, the high-

density infiltration of CD8+ T cells was associated with improved OS. In addition, high-density CD3+ T cells predicted the prolongation of OS and DFS in the IM.⁴⁰ Contrarily, a recent meta-analysis showed that high-density infiltration of CD8+ T cells in CT depicts improved OS.⁴¹ Our study also demonstrated that the infiltration density of T and B lymphocytes as well as TAMs was higher in IM CT in primary CRC.

In previous studies, CD68 and CD163 were found to be independent prognostic markers in patients with solid tumors. These markers hold great potential to be therapeutic targets for

Table 2. Univariate analysis of the prognosis of patients with colorectal cancer peritoneal metastasis.

Variables	OS		RFS	
	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P Value	HR (95% CI)	P Values
Age (year)				
>65 vs ≤65	0.64 (0.28–1.46)	<i>P</i> = .23	1.02 (0.54–1.93)	<i>P</i> = .95
Sex				
Female vs male	1.22 (0.61–2.46)	<i>P</i> = .58	0.94 (0.54–1.66)	<i>P</i> = .83
Primary site				
Right colon vs left colon	0.85 (0.42–1.72)	<i>P</i> = .65	1.41 (0.80–2.48)	<i>P</i> = .24
Grade				
G1-2 vs G3	0.98 (0.47–2.08)	<i>P</i> = .97	0.72 (0.40–1.28)	<i>P</i> = .28
Pathological type				
Non-mucinous vs mucinous/signet-ring cancer	0.68 (0.34–1.36)	<i>P</i> = .28	0.87 (0.50–1.54)	<i>P</i> = .63
T stage				
T2-3 vs T4	0.59 (0.28–1.24)	<i>P</i> = .2	0.48 (0.27–0.87)	<i>P</i> = .03
Ascites				
no vs yes	0.35 (0.16–0.75)	<i>P</i> = .0017	0.44 (0.33–0.84)	<i>P</i> = .002
PCI				
<10 vs ≥10	0.43 (0.20–0.94)	<i>P</i> = .033	0.57 (0.32–1.04)	<i>P</i> = .04
CD3+ cell (/HP)				
High infiltration vs low infiltration	0.44(0.15–1.34)	<i>P</i> = .05	0.52 (0.18–1.46)	<i>P</i> = .09
CD8+ cell (/HP)				
High infiltration vs low infiltration	0.27(0.14–0.55)	<i>P</i> = .0009	0.52 (0.29–0.94)	<i>P</i> = .04
CD20+ cell (/HP)				
High infiltration vs low infiltration	0.68 (0.33–1.40)	<i>P</i> = .31	0.59 (0.33–1.05)	<i>P</i> = .09
CD68+ cell (/HP)				
High infiltration vs low infiltration	1.61 (0.8–3.23)	<i>P</i> = .18	0.68 (0.34–1.33)	<i>P</i> = .31
CD163+ cell (/HP)				
High infiltration vs low infiltration	1.59 (0.79–3.19)	<i>P</i> = .21	0.47 (0.24–0.91)	<i>P</i> = .07
IS				
High vs low	0.36 (0.18–0.72)	<i>P</i> = .008	0.56 (0.31–0.99)	<i>P</i> = .067
TBM1				
High vs low	0.36 (0.18–0.73)	<i>P</i> = .01	0.71 (0.39–1.31)	<i>P</i> = .3
TBM2				
High vs low	0.33 (0.14–0.81)	<i>P</i> = .001	0.87 (0.35–2.15)	<i>P</i> = .75

the treatment of solid tumors.^{42–44} For intestinal tumors, infiltration level of CD163+ macrophages is associated with perineural invasion, MSI and TIL densities. Stromal CD68+ and CD163+ macrophage infiltration levels are significantly related to the infiltration level of CD3+ and CD8+ T cells.⁴³ During tumorigenesis and development, the polarization of TAM subtypes affects disease prognosis. Previous studies have confirmed that the TME of most solid tumors is acidified, a condition detected by macrophage G Protein-Coupled Receptor (GPCR), resulting in the expression of macrophage transcription factor-ICE (inducible cyclic adenosine monophosphate (cAMP) early repressor). Acidified TME triggers polarization of macrophages to non-inflammatory subtypes, leading to immune evasion. Unlike melanoma and lung cancer with strong acidic TME, the microenvironment of colon cancer is less acidic.⁴⁵ This might explain why the polarization of the pan-macrophages to non-inflammatory macrophages in the less acidic intestinal tumors is weaker than in other solid tumors. Thus, high infiltrating pan-macrophages predict good OS in CRC,^{23,46} and high-density TAMs infiltration correlate with high density CD8+ T cell infiltration, lack of distant and lymph node metastasis, high microsatellite instability, and non-mucinous adenocarcinoma.⁴⁶ Although CD68+ macrophages could not predict the OS of CRCPM (*p* = .18), the low-density CD68+ macrophages infiltration group showed an improved OS. This contradicted with the results from

a recent meta-analysis which showed that the high-density infiltration of pan-macrophages in primary lesions of CRC was associated with prolonged OS. This may be due to the fact that CRCPM does not exhibit the same low acid TME as the primary tumor. Although low-density infiltration CD163+ macrophage group did not significantly influence the prognosis, it tended to prolong the OS (*p* = .21). This matched with a previous observation that overexpression of CD163+ macrophages in tumor tissues is associated with poor prognosis.^{47,48}

TIL subpopulations in human solid tumors contain different proportions of infiltrating B cells. B cells are considered to be the main effector cells of humoral immunity. They secrete immunoglobulins, promote T cell responses or directly kill tumor cells.⁴⁹ Tumor infiltrating B lymphocytes (TIBs) exist in many solid tumors, but the prognostic value of TIBs in malignant tumors is controversial. High-density TIBs/tumor infiltrating plasma cells in TME of some malignant solid tumors, such as CRC or lung cancer has been associated with good prognosis.^{50,51} On the contrary, high-density TIBs is associated with high recurrence risk or poor prognosis in prostate and ovarian cancer.⁵² Existing evidence shows that TIBs control tumor progression by secreting immunoglobulins, promoting T cell responses, or directly killing tumor cells.⁵³ In addition, TIBs act as antigen presenting cells and promotes immune response of T cells in TME. For example, in non-small cell lung cancer and metastatic CRC, T cells with

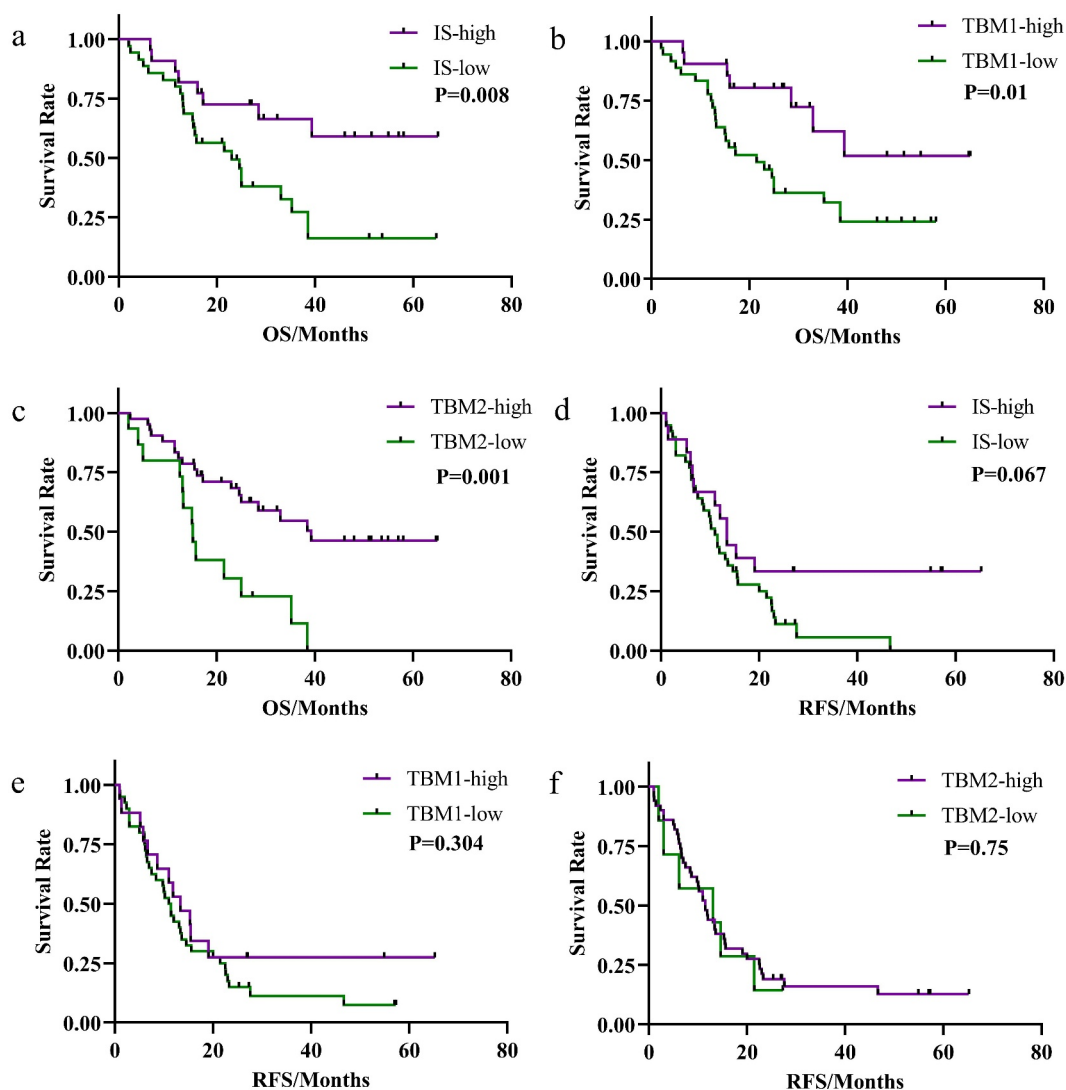


Figure 6. Kaplan-Meier estimates and log-rank test OS according to the IS (a), TBM1 score (b) and TBM2 score (c); Kaplan-Meier estimates and log-rank test RFS according to the IS (d), TBM1 score (e) and TBM2 score (f). The number of patients at risk for each group is shown. (a, d) patients with IS = 2 (IS-high, purple) are grouped. Similarly, patients with IS 0 and IS 1 are grouped (IS-low, green). (b, e) patients with TBM1 ≥ 2 and TBM1 < 2 are shown in purple and green, respectively. (c, f) patients with TBM1 ≥ 2 and TBM1 < 2 are shown in blue and black, respectively. IS = immunoscore.

high infiltration density were located in the high-density B cell infiltration area, and the high-immunoscore based on infiltrating density of TILs and B cells predicted good prognosis.^{14,21} Another subtype called regulatory B cell (Bregs) in the TME of solid tumors enhances tumor activity through immunosuppressive factors such as IL-10, TGF- β , and inhibits CTL response by inducing polarization of TAMs and differentiation of regulatory T cells (Tregs).^{54–57} Our findings demonstrate that the infiltrating level of B cells in peritoneal metastases does not affect prognosis of tumors. The impact of different subtypes of B cells on the prognosis should be further investigated.

In addition, our results showed that, compared to primary tumors, the tumor infiltrating T or B cells are down-regulated in peritoneal or ovarian metastasis. This state of immune cell desertification may explain why patients with CRCPM are prone to relapse and metastasis, thus poor disease prognosis. Among the clinical factors, PCI and

ascites are independent prognostic factors. Similar to previous studies, we demonstrate that high-PCI is associated with poor prognosis in advanced cancer patients.^{6,58–60} Therefore, quantification of tumor burden coupled with the quantification of immune effector and suppressor cells in TME may be more accurate in predicting the long-term prognosis of cancer patients.

Conclusion

The microenvironment of peritoneal and ovarian metastases usually exhibits low expression and desertification of immune cells compared to the primary lesions. Here, we demonstrate that the IS scoring system constructed by combining the infiltration density of CD3+ and CD8+ T cells in CRCPM, and TBM scoring system constructed by combining CD3+ T cells,

Table 3. Multivariate analyses of prognostic factors of patients with CRCPM. Bold value indicates $P < .05$, HR hazard ratio, CI confidence interval, T stage of primary tumor, ascites, PCI score and Immunoscore were included in the Multivariate analysis 1; T stage of primary tumor, ascites, PCI score and TBM1 were included in the Multivariate analysis 2; T stage of primary tumor, ascites, PCI score and TBM2 were included in the Multivariate analysis 3.

OS	Multivariate analysis 1		Multivariate analysis 2		Multivariate analysis 3	
	HR (95% CI)	<i>P</i> Value	HR (95% CI)	<i>P</i> Value	HR (95% CI)	<i>P</i> Value
T Stage						
T4 vs T2-3	1.72 (0.72–4.10)	<i>P</i> = .219	2.09 (0.86–5.07)	<i>P</i> = .103	2.07 (0.86–4.98)	<i>P</i> = .106
Ascites						
Yes vs no	2.63 (1.28–5.40)	<i>P</i> = .009	1.96 (0.91–4.19)	<i>P</i> = .085	2.52 (1.21–5.25)	<i>P</i> = .014
PCI						
≥10 vs <10	1.694 (0.81–3.56)	<i>P</i> = .163	1.91 (0.89–4.13)	<i>P</i> = .099	1.97 (0.92–4.22)	<i>P</i> = .079
IS						
High vs low	0.35 (0.15–0.80)	<i>P</i> = .013				
TBM1						
High vs low			0.307 (0.14–0.67)	<i>P</i> = .003		
TBM2						
High vs low					0.29 (0.12–0.69)	<i>P</i> = .005
RFS	Multivariate analysis 1		Multivariate analysis 2		Multivariate analysis 3	
T Stage						
T4 vs T2-3	2.13 (1.03–4.41)	<i>P</i> = .041	2.20 (1.06–4.55)	<i>P</i> = .034	2.30 (1.12–4.70)	<i>P</i> = .023
Ascites						
Yes vs no	2.17 (1.17–4.01)	<i>P</i> = .014	2.19 (1.18–4.06)	<i>P</i> = .013	2.20 (1.18–4.08)	<i>P</i> = .013
PCI						
≥10 vs <10	1.68 (0.91–3.08)	<i>P</i> = .095	1.78 (0.96–3.30)	<i>P</i> = .068	1.72 (0.93–3.19)	<i>P</i> = .085
IS						
High vs low	0.69 (0.35–1.35)	<i>P</i> = .28				
TBM1						
High vs low			0.80 (0.40–1.58)	<i>P</i> = .521		
TBM2						
High vs low					0.93 (0.39–2.23)	<i>P</i> = .87

CD8+ T cells, CD20+ B cells and CD163+ macrophages, could successfully predict the OS of patients with CRCPM. In addition, clinical factors such as high PCI and ascites are independent risk factors.

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Ethics approval and consent to participate

This study was approved by the local institutional ethical committee of the Second Affiliated Hospital, Zhejiang University School of Medicine

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