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Original Article

Predictive value of immunoscore in patients with colorectal cancer

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Abstract: As increasing evidences suggest that tumor-infiltrating immune cells do influence tumor development. In this study, the expression of intratumoral neutrophils, CD3+ T cells and CD8+ T cells were assessed by immunohistochemistry from 207 specimens from patients with CRC following resection. Prognostic values for immunoscore were evaluated. The results showed decreased CD3+ T cells, CD8+ T cells, immunoscore, and increased neutrophils were all significantly associated with poor disease-free survival (DFS) and overall survival (OS) ($P < 0.05$). In multivariate analysis, immunoscore was not only an independent prognostic factor for DFS and OS, but also more advantageous over the use of current tumor staging (AJCC/UICC-TNM classification) for DFS (HR, 3.394; 95% CI, 2.209 to 5.213; $P < 0.001$ and HR, 1.275; 95% CI, 1.048 to 1.550; $P = 0.015$) and OS (HR, 3.904; 95% CI, 2.492 to 6.117; $P < 0.001$ and HR, 1.734; 95% CI, 1.423 to 2.114; $P < 0.001$). The receiver operating characteristic (ROC) curve analysis revealed the immunoscore to be the better predictor to TNM stages. Our data indicate that immunoscore is a strong independent prognostic factor, which may better predict the outcome for CRC patients.

Keywords: Immunoscore, Neutrophils, CD3+ T cells, CD8+ T cells, colorectal cancer

Introduction

Colorectal cancer (CRC) is a frequent cancer worldwide. There are almost 40 thousands new cases of CRC and half of deaths attributable to this disease per year in China [1]. Current clinical risk predictions in CRC are based on histopathology of the tumor tissue obtained during surgery. Despite the great advancement in diagnosis and treatment modalities, the outcome remains challenging due to frequent recurrence [2]. The American Joint Committee on Cancer/International Union Against Cancer (AJCC/UICC) TNM staging system provides the most reliable guidelines for the routine prognostication and treatment of CRC [3]. Despite the prognostic power of this traditional staging system, determining the outcome for patients is imprecise. Clinical outcome always vary among patients within the same stage [4]. Therefore, new prognostic markers of CRC are urgently needed.

Recently, the tumor microenvironment has become the focus of intense research. Primary tumors develop and metastasis within a microenvironment. There is clear evidence in support of the conclusion that the innate immune system does influence tumor development and inflammatory mediators promote tumor development [5, 6]. The host immune reaction is an important player in the tumor microenvironment, represented by tumor-infiltrating immune/inflammatory cells [7].

Inflammation has emerged as the seventh hallmark of cancer [8]. Neutrophils are the most abundant subpopulation of leucocytes, and initially recognized as short-lived effector cells providing the first line of defense against invading microorganisms [9]. Recent reports revealed

Table 1. Correlations of immunoscore with clinicopathologic features

Variable	Total (N = 207)	Immunoscore (Cut-off 50%)		
		Low	High	*p*
Gender				
Female	83 (39.7%)	43	40	0.668
Male	124 (59.3%)	68	56	
Age (years)				
≥ 56	116 (56%)	59	57	0.368
< 56	91 (44%)	52	39	
Tumor location				
Colon	120 (57.4%)	63	57	0.704
Rectum	87 (41.6%)	48	39	
Differentiation				
Well	69 (33.3%)	29	40	0.002
Moderate	102 (49.3%)	54	48	
Poor	36 (17.4%)	28	8	
T stage				
T1	10 (4.8%)	4	6	0.018
T2	18 (8.7%)	6	12	
T3	147 (71.0%)	77	70	
T4	32 (15.5%)	24	8	
N stage				
N0	123 (59.4%)	54	69	0.001
N1+N2	84 (32.4%)	57	27	
TNM stage				
I	23 (11.1%)	7	16	0.001
II	95 (45.9%)	43	52	
III	66 (31.9%)	43	23	
IV	23 (11.1%)	18	5	

that elevated blood neutrophils play an important role in cancer progression and prognosis [10, 11]. Meanwhile, tumor-associated neutrophils have also been found and were independent prognostic factors in various cancer types [12, 13].

Primary tumors are commonly infiltrated by immune cells. Data from previous studies proved tumor-infiltrating immune cells have been associated with improved prognosis in patients with several carcinomas [14, 15]. High expression of the tumor-infiltrating lymphocytes, first observed in melanoma [16], is often of good prognosis. Mice with elimination of T cells tend to develop malignant [17]. In a study of CRC showed that the location, type and density of tumor-infiltrating immune cells in the local microenvironment are essential for prediction of the clinical outcome [15].

The immune score (immunoscore) is first described in patients with early-stage CRC, based on the combined evaluation of memory and cytotoxicity T cells for the prediction of tumor recurrence and survival [14]. As a large number of translational research studies have revealed an association of tumor-associated neutrophils with poor clinical outcome in various cancers, tumor-associated neutrophils were added to immunoscore in our research.

In this study, we performed immunohistochemistry to detect intratumoral CD3+ T cells, CD8+ T cells, neutrophils and examined their clinicopathological influences. Furthermore, we put forward an immunoscore based on these immune/inflammation cells and further demonstrate the predictive value of the immunoscore.

Materials and methods

Patients and specimens

In our study, a total of 207 patients diagnosed with CRC received resection in the Department of Gastrointestinal Surgery of the First Affiliated Hospital of Anhui Medical University (Hefei, China) between 2005 and 2008. Tumor samples were collected from these patients who were pathologically diagnosed with CRC. None of the patients received anticancer therapy before samples were obtained. Clinical stages were classified according to the guidelines of the American Joint Committee on Cancer (AJCC, 7th edition). All the clinicopathological data are shown in **Table 1**. The study was approved by the local ethics committee.

Immunohistochemistry

Formalin-fixed and paraffin-embedded surgical tissue specimens were used for the immunohistochemical analyses. The presence of available tumors was confirmed on hematoxylin and eosin staining. The specimens were cut into 4-μm sections, which were then processed for immunohistochemistry as described [18]. Primary antibodies were against CD66b (clone G10F5, diluted at 1:400, BD Biosciences, San Jose, CA, USA), CD3 (polyclonal rabbit, diluted at 1:400, Abcam, Cambridge, UK) and CD8

(polyclonal rabbit, diluted at 1:400, Abcam, Cambridge, UK). Horseradish peroxidase (HRP) and diaminobenzidine (DAB) were used according to the instructions (Dako Cytomation). Finally, all sections were stained with hematoxylin and mounted.

Evaluation of immunostaining

The CD66b/CD3/CD8 staining was evaluated by two independent pathologists blinded to all the patients' clinicopathological parameters at 200× magnification light microscopy (Leica DM IRB, Germany). The number of intratumoral immune cells per fields (×200) was estimated using a stereological counting technique (CAST software). The sections were screened at five most representative fields in intratumoral areas for CD66b/CD3/CD8 were counted. Areas of artifacts and necrosis were omitted. A mean of the 5 fields of visions were assessed for each section. Median values were used as a cut-off to categorize into high or low groups for intratumoral immune cells in subsequent analyses unless specified.

Immunoscore calculation

We proposed to classify the patients with CRC on the basis of immunoscore for CD66b/CD3/CD8 in each intratumor [19, 20]. There were some modifications. CD66b was added to immunoscore. The immunoscore defined four groups (I0, I1, I2, I3). Patients with high densities of CD66b and low densities of both CD8 and CD3 were defined I0. Patients with one high density of CD8 and CD3 or low density of CD66b were defined I1. Patients with one high density of CD8 and CD3 and low density of CD66b or high density of both CD8 and CD3 and high density of CD66b were defined I2. Patients with low densities of CD66b and high densities of both CD8 and CD3 were defined I3.

Statistical analysis

SPSS statistical software (version 19.0, IBM) was used for all the statistical analysis. Correlations between clinicopathologic features and immunostaining parameters were analyzed by the chi-square test (for categorical variables) and the t test (for continuous variables). Associations between continuous variables were examined by calculating Pearson's correlation coefficient. Kaplan-Meier method estimates of survival were used to illustrate the survival curves, and survival was measured in months from the resection to either recurrence or the last review. Univariate and multivariate analysis of the prognostic factors for disease-free survival (DFS) and overall survival (OS) were performed using the Cox proportional hazards model. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the predictive value of the obtained model. All tests were two-sided, and $P < 0.05$ were considered statistically significant.

Results

Patient characteristics

The clinical characteristics of patients are listed in **Table 1**. The estimated 5-year DFS (except for stage IV) and OS rates were 46.2% and 56.5%, respectively. Median time to recurrence was 7 months (range, 2 to 95 months). At the last follow-up, 109 (52.7%) patients were deceased, at a median of 28 months (range, 4 to 104 months). For the 98 patients still alive, the median follow-up was 90 months (range, 58 to 123 months).

Immune cells

The following antigens as markers were used for detecting different cell types: CD66b for neutrophils, CD3 and CD8 for subsets of T cells. The median densities of neutrophils, CD3+ T cells, and CD8+ T cells were 138 (range, 0 to 487 cells/field), 188 (range, 22 to 598 cells/field), and 152 (range, 4 to 551 cells/field) (**Figure 1**). Median values were used as a cut-off to categorize into high or low groups for in subsequent analysis unless specified.

CD8+ T cells showed a significant positive correlation with CD3+ T cells ($r = 0.406$, $P < 0.001$), but negative correlation with neutrophils ($r = -0.259$, $P < 0.001$). In addition, no significant correlation was evident between CD3+ T cells and neutrophils ($r = -0.020$, $P = 0.770$).

Correlation of these immune cells with clinicopathological variables

We analyzed the correlations between intratumoral neutrophils, CD3+ T cells and CD8+ T cells with clinicopathological variables of CRC (data not showed). The results demonstrated

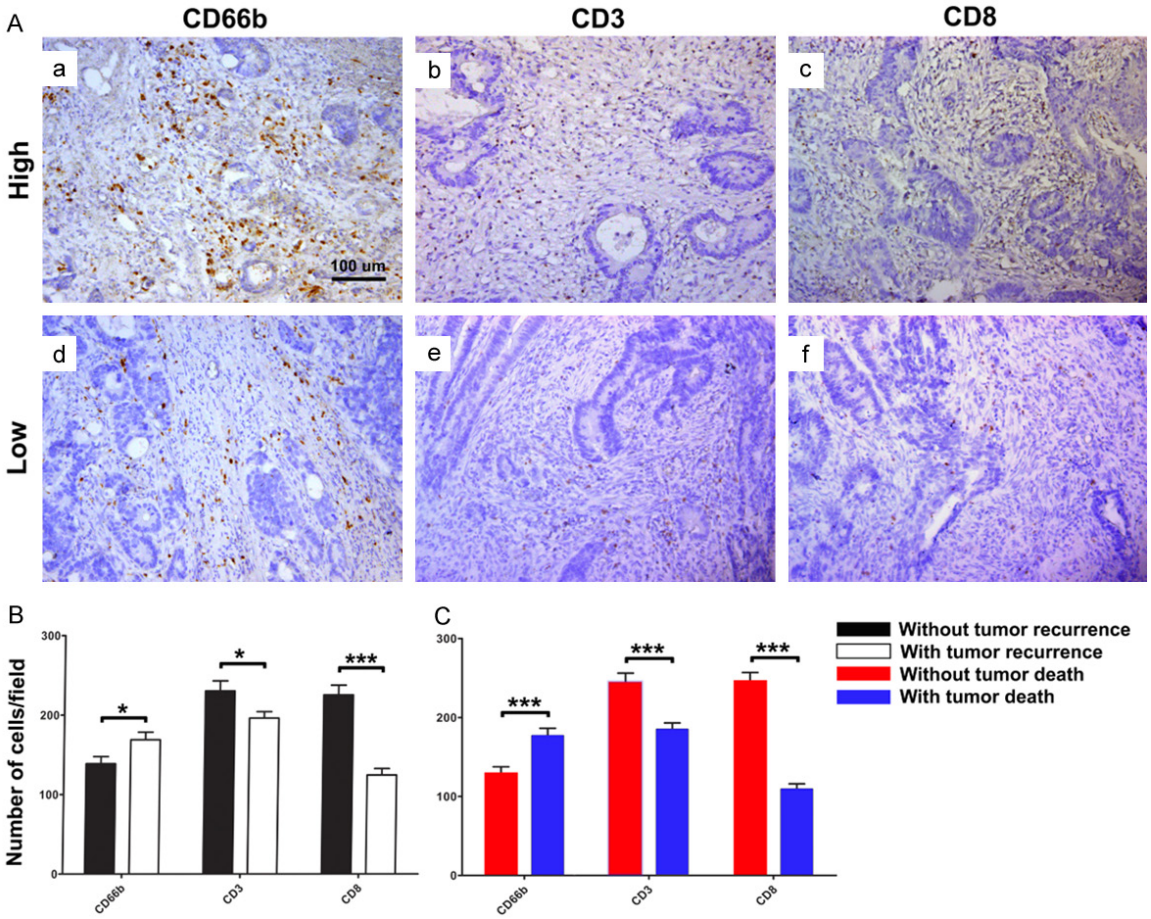

Figure 1. Representative photos of CD66b/CD3/CD8 immunostaining (200×) in CRC samples. A. Representative immunohistochemical staining for CD66b (a, d), CD3 (b, e), CD8 (c, f). Different levels of CD66b/CD3/CD8 expression can be observed in the intratumoral regions: a/b/c, low density; d/e/f, high density. B. Comparison of the immune cell densities in patients with (White bars) or without (Black bars) tumor recurrence. C. Comparison of the immune cell densities in patients with (Blue bars) or without (Red bars) tumor death. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

the neutrophils had no association with any of clinicopathological parameters. The expression of CD3/CD8 T cells above median was significantly associated with depth of invasion, lymph node metastasis and advanced pathological TNM stages ($P < 0.05$). Furthermore, the increased intratumoral CD8+ T cells were correlated with advanced differential grade ($P < 0.001$).

Immunoscore

The cohort of patients was classified I0, I1, I2 and I3. Patients with the low immunoscore (I0 and I1) presented with DFS rates and OS at 5 years of 21.4% and 35.0%. Meanwhile, patients with the high immunoscore (I2 and I3) presented with DFS rates and OS at 5 years of 60.6% and 77.9%. The presence of immunoscore was statistically significantly associated with differential grade ($P = 0.002$), depth of invasion ($P = 0.018$), lymph node metastasis ($P = 0.001$), and TNM stages ($P = 0.001$) respectively (**Table 1**). In univariate analysis, low immunoscore was significantly with short DFS and OS (**Table 2**).

Survival analysis

Kaplan-Meier analysis was used in order to clarify more precisely the prognostic characteristics of intratumoral immune cells. We found that higher densities of CD3+ T cells, CD8+ T cells, and lower neutrophils were significantly associated with both longer OS and DFS in the patients with CRC (data not showed). Kaplan-Meier survival analysis for DFS and OS revealed

Table 2. Univariate analyses of prognostic factors in CRC

Variable	OS			DFS		
	HR	95% CI	*P*	HR	95% CI	*P*
Age (years) (≥ 55 vs. < 55)	1.211	0.827-1.773	0.326	1.217	0.826-1.794	0.320
Gender (female vs. male)	0.774	0.523-1.145	0.200	0.887	0.600-1.312	0.549
Tumor location (colon vs. rectum)	0.986	0.675-1.440	0.940	0.987	0.671-1.451	0.948
Differentiation (poor vs. well+moderate)	1.821	1.471-2.254	0.000	1.591	1.255-2.018	0.000
N stage (N1+N2 vs. N0)	2.919	1.991-4.279	0.000	2.000	1.362-2.937	0.000
T stage (T3+4 vs. T1+2)	2.329	1.177-4.611	0.015	1.831	0.980-3.420	0.058
TNM stage (III+IV vs. I+II)	1.861	1.532-2.262	0.000	1.414	1.167-1.714	0.000
CD66b express (low vs. high)	0.534	0.364-0.785	0.001	0.541	0.368-0.796	0.002
CD3 express (high vs. low)	0.559	0.382-0.818	0.003	0.682	0.466-1.000	0.050
CD8 express (high vs. low)	0.158	0.100-0.248	0.000	0.223	0.147-0.341	0.000
Immunoscore (I2+I3 vs. I0+I1)	0.479	0.384-0.597	0.000	0.522	0.422-0.644	0.000

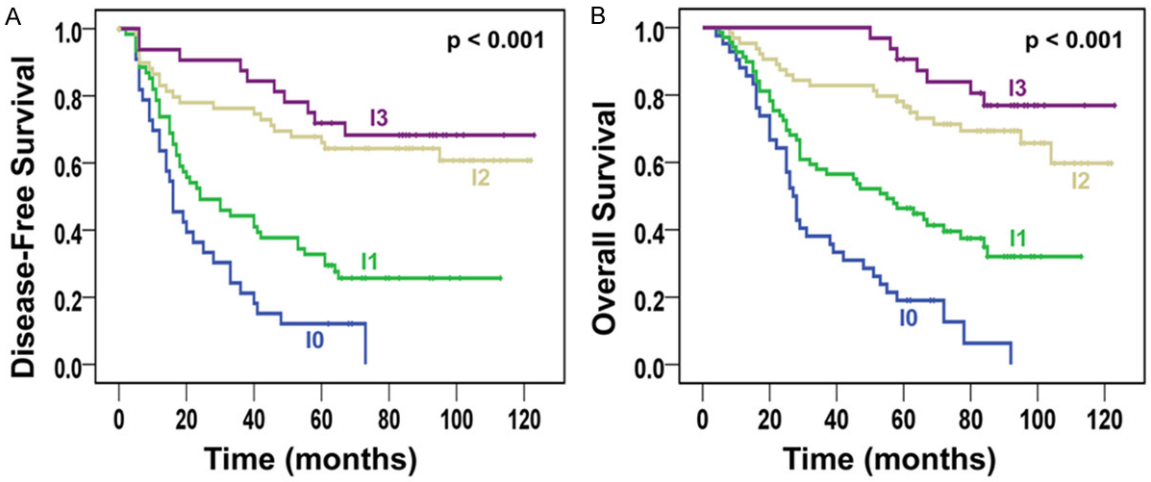

Figure 2. Kaplan-Meier analysis of immune score in CRC patients. A and B. Increased immune score was significantly associated with prolonged DFS and OS. The OS and DFS time were calculated by the Kaplan-Meier method and analyzed by the log-rank test.

Table 3. Multivariate analyses of prognostic factors in CRC

Variable	OS			DFS		
	HR	95% CI	*P*	HR	95% CI	*P*
Immunoscore (I0+1 vs. I2+3)	3.904	2.492-6.117	0.000	3.394	2.209-5.213	0.000
TNM Stage (III+IV vs. I+II)	1.734	1.423-2.114	0.000	1.275	1.048-1.550	0.015

differences according to the immunoscore (**Figure 2**).

Clinicopathological and immunohistochemical characters for prediction of CRC were further investigated by Cox's univariate and multivariate hazard regression model. As exhibited in **Table 2**, in univariate analysis, clinical factors statistically significantly associated with short DFS and OS were poor differentiation (low), deeper invasion (T3/T4), lymph node metastasis (N1/2), further stages (III+IV) ($P < 0.05$ for the above clinical factors), except for depth of invasion for predicting for DFS ($P > 0.05$). Among the immunohistochemical characteristics, all of intratumoral neutrophils, CD3+ T cells, CD8+ T cells and immunoscore were significantly associated with DFS and OS ($P < 0.05$ for all), except CD3+ T cells was not associated with DFS ($P = 0.05$).

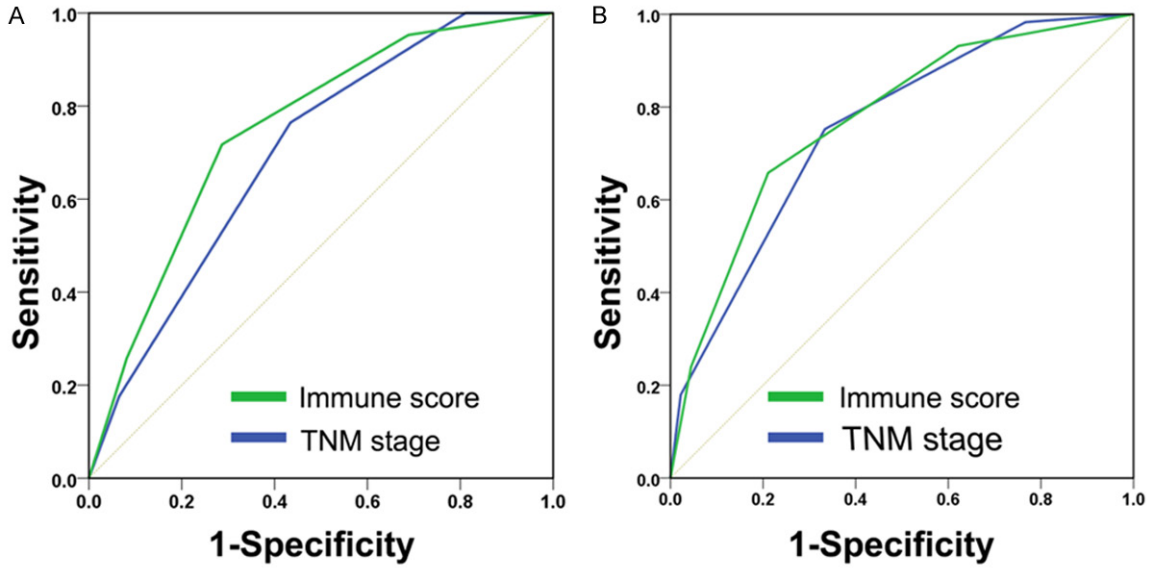

Figure 3. Receiver operating characteristic (ROC) curves of DFS (A) and OS (B) at 5 years in the validation cohort using immune score and TNM stage. The c-index of immune score was significantly higher than TNM stage for both DFS (0.750 vs. 0.701; $P < 0.001$) and OS (0.772 vs. 0.754; $P < 0.001$).

We then performed Cox multivariate regression analysis by adding immunoscore and TNM stages into a model. The result showed that immunoscore (I0+1 vs. I2+3), and Stage (III+IV vs. I+II), both of them significantly associated with DFS and OS. Moreover, immunoscore was an independent prognostic factor with a higher hazard ratio (**Table 3**).

The predicting values of the immunoscore and the TNM stages were further evaluated using ROC analysis. The areas under curve of the immunoscore and the TNM stages were 0.750 (95% CI, 0.630-0.771) versus 0.701 (95% CI, 0.684-0.817) for 5-year DFS and 0.772 (95% CI, 0.707-0.836) versus 0.754 (95% CI, 0.688-0.821) for 5-year OS, respectively (**Figure 3**). The result revealed that immunoscore to be better predictor than the TNM stages in predicting DFS and OS ($P < 0.001$ for both).

Discussion

CRC remain the main cause of cancer-related death worldwide. Conventional clinical and pathological risk prediction provides limited information for prognosis since cancer outcome can significantly vary among patients within the same histological tumor stage [21]. Therefore, effective prognostic markers are needed urgently to predict the survival, which may provide information to avoid unreasonable treatment in CRC patients.

Here, we evaluated the densities of intratumoral neutrophils, CD3+ T cells and CD8+ T cells using 207 cases of CRC. We found that decreased intratumoral neutrophils, the increased CD3+ T cells and CD8+ T cells were equally significantly associated with better DFS and OS.

It is generally accepted that the host immune system plays a key role in tumor development [22]. The immune pattern has prognostic discriminatory power that is superior to the classical TNM classification for survival [23]. In this study, we found the patients whose tumors have high densities of CD8+ T cells and CD3+ T cells are negative correlated with lower recurrence. Our results is consistent with others reports that CD8+ T cells and CD3+ T cells is used as prognostic factors in a variety of tumors [20, 24, 25].

Intratumoral neutrophils has been identified as a poor prognostic factor in hepatocellular carcinoma [26], esophageal squamous cell carcinoma [27] and renal cell carcinoma [28]. However, the functional of neutrophils can be anti-tumorigenic or pro-tumorigenic, depending on the milieu of TGF-β expression [29].

Recently, Immunoscore have been illustrated to have a prognostic value that can be superior to the TNM classification [19]. The predictive value of immunoscore has been reported in

several tumor types [20, 30]. Unlike other studies mentioned above, we combined the markers of neutrophils, CD3+ T cells and CD8+ T cells into a simple scoring method (Immunoscore), as profiles of prognostic marker in CRC. Our results demonstrated that the 4 immunoscore groups were associated with obviously differences in DFS and OS ($P < 0.001$) (**Figure 2**). Patients with higher immunoscore significantly associated with good prognosis. Furthermore, to further verify the prognostic power of immunoscore, we built a multivariate analysis model combining immunoscore and TNM stages. The results revealed that immunoscore was an independent prognostic factor that superior to the TNM stages in predicting outcome. Meanwhile, we used ROC curve analysis of DFS and OS at 5 years in the cohort containing the TNM stages and immunoscore. Both of them had c-indices of more than 0.70, but immunoscore was more reliable than the TNM stages.

Immunoscore as a prognostic marker is simple and feasible in routine settings. The immunoscore model is not only providing a tool for the prediction of prognosis, but also identifying patients at high risk of tumor recurrence. Nonetheless, there are limitations to our study. First, the expressions of peritumoral immune cells were not examined. Second, we were unable to perform the mechanism of different immunoscore in tumor tissues. Third, external verification by other centers are needed.

In conclusion, our findings confirm and extend to patients with CRC immunoscore as a poor prognostic marker in predicting patient survival. We further found that immunoscore is a prognostic factor superior to TNM stages in the Cox multivariate analysis model and ROC curve analysis. However, the immunoscore is not restricted to CRC, further assessment of immunoscore in other tumors is encouraged.

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Disclosure of conflict of interest

None.

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