Immunoscore is a superior prognostic tool in stage II/III colorectal cancer and significantly correlated to PD-L1 expression on tumor-infiltrating mononuclear cells.

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Running Head: PD-L1 expression and Immunoscore in CRC

Key words: PD-L1, Immunoscore, tumor-infiltrating lymphocytes, colorectal cancer,
Immunoscore was an independent prognostic indicator in CRC. Strong expression of PD-L1 on interstitial tumor-infiltrating mononuclear cells (TIMCs) showed a good prognosis and correlated significantly with Immunoscore. We suggested that PD-L1 positive TIMCs may have M2 type macrophages.
Abstract

Background: In colorectal cancer (CRC), the indication of immune checkpoint inhibitors is determined by the tumors’ microsatellite instability status. However, an optimal biomarker for their indication has not yet been fully identified. We aimed to establish the clinicopathological importance of Immunoscore (IS) in CRC, and clarify the relationships between IS, PD-L1 expression, and tumor-associated macrophages.

Methods: A total of 132 cases were diagnosed with CRC and surgically treated in our department from 2009 to 2010. Immunohistochemical staining using primary antibodies PD-L1, CD3, CD8, CD68, and CD163 was performed. The IS was determined according to the proposal of an international task force. Statistical analyses were performed to investigate the correlation between IS, clinicopathological variables, and the expression of immune checkpoint molecules.

Results: The overall survival (OS) and relapse-free survival (RFS) of the high IS group (I3-4) were significantly better than those of the low IS group (I0-2) (OS: $P=0.0420$, RFS: $P=0.0226$). The positivity rate of PD-L1 on tumor cells was only 0.8% (tPD-L1), while that of PD-L1 on interstitial tumor-infiltrating mononuclear cells (iPD-L1) was
18.2%. The iPD-L1 positive group showed significantly better survival both in terms of OS and RFS than the iPD-L1 negative group (OS: P=0.0278, RFS: P=0.0253). IS and iPD-L1 expression were significantly correlated (P<0.0001).

**Conclusions:** We found that high IS was a good indicator of better prognosis and significantly correlated to the iPD-L1 expression in CRC.

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**Introduction**

Colorectal cancer (CRC) is one of the most common malignant diseases worldwide, and is the second leading cause of cancer-related death in Japan. Although a variety of anticancer drugs have been developed, the number of CRC-related deaths has not been significantly reduced. Host immune response, including tumor-infiltrating lymphocytes (TILs), plays an important role in CRC prognosis and other malignant diseases, and interest in the use of immune checkpoint inhibitors as part of a new therapeutic strategy has increased.

The mechanism behind immune checkpoint inhibition is the blockade of the
programmed death-1 (PD-1) / PD-ligand 1 (PD-L1) pathway. PD-1 is strongly expressed on activated lymphocytes, particularly on TILs, while PD-L1 is expressed not only on antigen-presenting cells but also on tumor cell surfaces. The binding of both molecules causes the suppression of T cells’ immune response and results in immune tolerance. The immune checkpoint inhibitors Nivolumab and Pembrolizumab have both been reported effective in malignant melanoma, non small-cell lung cancer, renal cell carcinoma, and malignant lymphoma. Galon et al. first proposed the use of the Immunoscore (IS) system, which is defined by the evaluation of TILs, reporting that IS was a significantly better prognostic indicator in CRC. However, the use of IS alone for determining the use of immune checkpoint inhibitors needs to be verified. PD-L1 expression is observed not only in tumor cells (TCs) but also in tumor-infiltrating mononuclear cells (TIMCs). The expression of PD-L1 on TCs correlates with poor prognosis in various malignancies, however, few studies have investigated the association between PD-L1 expression in TIMCs and the prognosis of CRC patients. The presence of macrophages in the interstitium of PD-L1-expressing tumors has previously been reported; studies have also reported that
tumor-associated macrophages (TAMs), particularly M2-type macrophages, form an
environment favorable for tumor growth.\textsuperscript{23,24}

Therefore, the primary purpose of this study was to establish the clinicopathological
importance of IS in CRC, and the secondary purpose was to clarify the relationships
between IS, PD-L1 expression, and TAMs.

\section*{Materials and methods}

\textbf{Patients and samples}

A total of 192 patients were diagnosed with CRC and surgically treated in our
department from 2009 to 2010. After obtaining written informed consent,
formalin-fixed paraffin-embedded tissue specimens were obtained from each patient.
Cases with neoadjuvant chemotherapy or neoadjuvant chemoradiotherapy and multiple
cancer cases were excluded; 132 patients were enrolled in this study.
Clinicopathological features of the patients are shown in Supplementary Table S1.
Post-operative pathological staging was determined according to the seventh edition of
the UICC-TNM classification of malignant tumors. Clinical outcome records and
pathological reports were reviewed retrospectively.

This study was conducted in accordance with the provisions of the Declaration of Helsinki and approved by the Institutional Review Board of Kurume University Hospital (No. 300).

**Immunohistochemistry**

Paraffin-embedded tissue samples were cut at a thickness of 4 μm and spread on coated slide glasses. The slide glasses were labeled with the following antibodies using BenchMark ULTRA (Ventana Automated Systems, Inc., Tucson, AZ, USA) and Bond-Max autostainer (Leica Microsystems, Newcastle, UK). The primary antibodies (with dilutions) used were: CD3 (×300, clone LN10, Leica Microsystems, Newcastle, UK), CD8 (×200, clone 4B11, Leica Microsystems, Newcastle, UK), CD68 (×1200, clone KP1, DakoCytomation, Glostrup, Denmark), CD163 (×100, clone 10D6, Leica Microsystems, Newcastle, UK), and PD-L1 (×100, clone E1L3N, Cell Signaling Technology, Inc., Danvers, MA, USA).

Immunostaining with CD3, CD8, CD68, and PD-L1 was performed by the fully automated Bond-III system (Leica Microsystems, Newcastle, UK). Antigen retrieval
was performed using onboard heat-induced retrieval with epitope retrieval solution 2 (ER2, EDTA-based buffer, pH9.0, Leica Microsystems, Newcastle, UK) for 10 min at 99 °C. Slide glasses were incubated with each antibody for 30 min at room temperature. A refine polymer detection system (Leica Microsystems, Newcastle, UK) was used, and slide glasses were incubated with secondary antibody for 30 min at room temperature. All slides were visualized using diaminobenzidine (DAB).

BenchMark ULTRA was used to stain CD163. Each slide was heat-treated using Ventana’s ULTRA cell conditioning 1 (CC1, Ventana Automated Systems, Inc., Tucson, AZ, USA) retrieval solution for 30 min at 95 °C, and incubated with the CD163 antibody for 30 min at 37 °C. This automated system used the streptavidin biotin complex method with 3,3’ DAB as the chromogen (Ventana UltraVIEW DAB detection kit).

Image analysis and evaluation of PD-L1 expression

All stained slides were scanned and digitized using NanoZoomer2.0-HT: C9600-13 (Hamamatsu Photonics KK, Shizuoka, Japan). The scanned images were analyzed using NDP.view2: U12388-01 software (Hamamatsu Photonics KK, Shizuoka, Japan), and
five points of the center of tumor (CT) and invasive margin (IM) each were captured and stored as JPEG images with a x200 field of view. The captured images were processed and quantified using image-processing software, Image J 1.50i. For evaluation, the primary deconvolution of the image was performed, followed by the selection of the red image, and creation of the binary image. The color density threshold was set to be constant, and the auto counting of positive cells was performed using particle count.

The median value was calculated from the measured values of the five points measured at the CT and IM. The cutoff value of PD-L1 expression was determined using receiver operating characteristic curves. PD-L1 expression was evaluated by distinguishing between TC expression (tPD-L1) and TIMC expression (iPD-L1).

**Evaluation of IS and TAMs**

The IS was quantified according to the protocol proposed by the international task force, and classified into five stages according to the density of CD3 and CD8 positive lymphocytes in the CT and IM; specifically, we classified the IS from I0 to I4.26,27 Similar to the evaluation procedure for PD-L1 expression, the expression of CD3 and
CD8 was measured by the CT and IM at five points respectively, and the median of each was taken as the cutoff value. The evaluation of CD68 and CD163 was performed according to the procedure used for PD-L1, CD3, and CD8 evaluations.

Statistical analysis

The correlations between PD-L1 expression, IS, and the clinicopathological characteristics of patients were analyzed using a Chi square test. Survival curves were estimated by the Kaplan-Meier method and statistical significance was evaluated using a log-rank test. Overall survival (OS) and relapse-free survival (RFS) were defined as the time from surgery to death or disease recurrence, respectively. Univariate and multivariate analyses were performed using the Cox hazards model. All statistical analyses were conducted using JMP software version 12.0 (SAS Institute Inc., Cary, NC, USA), and a p-value less than 0.05 was considered statistically significant.

Results

Staining results of each marker

Representative PD-L1 stained images are shown in Figs. 1a-1c. PD-L1 was expressed
on TCs and TIMCs. Fig. 1a shows a PD-L1 negative case, Fig. 1b shows a tPD-L1 positive case, and Fig. 1c shows an iPD-L1 positive case. Only 1 case (0.8%) was positive for tPD-L1, and 24 cases (18.2%) were positive for iPD-L1. There were no PD-L1 positive cases in both tPD-L1 and iPD-L1. Representative examples of CD3 and CD8 positivity in the CT and IM are shown in Figs. 1d-1g.

**IS and clinicopathological variables**

We first performed IS scoring in the lesion. The IS was classified into five stages, I0 to I4, according to the evaluation of CD3 and CD8 in the CT and IM [I0: 35 cases (26.5%), I1: 21 cases (15.9%), I2: 21 cases (15.9%), I3: 20 cases (15.2%), and I4: 35 cases (26.5%)]. The IS was divided into two groups: a high score group (I3-4) and a low score group (I0-2), and each of these were analyzed. There was no significant association between the IS and each clinicopathological feature (data not shown).

**IS and survival analysis**

In the I3-4 group, the OS and RFS ratio was significantly higher than that of the I0-2 group (OS: P=0.0420, RFS: P=0.0226) (Fig. 2a, 2b). Further examination by TNM stage revealed that there was no significant difference between the OS and RFS in the
case of stage I and stage IV disease. In stage III cases, those in the I3-4 group had significantly improved prognoses compared to those in the I0-2 group (OS: P=0.0390, RFS: P=0.0125). Even in Stage II cases, the I3-4 group tended to show a good prognosis (OS: P=0.2138, RFS: P=0.0792) (Fig. 2c-2f, Supplementary Fig. S1).

To analyze the effects of clinicopathological variables and IS on OS and RFS in stage II/III cases, univariate and multivariate analyses were performed (Table 1). In the univariate analysis of OS, there was a significant difference only in the IS (P=0.0116). In the multivariate analysis, only IS was an independent prognostic factor (hazard ratio [HR]: 2.71, 95% confidence interval [CI]: 2.72-2.87, P=0.0026). In the univariate analysis of RFS, there was a significant difference between IS and N stage (P=0.0019 and P=0.0208, respectively), and in the multivariate analysis, IS and N stage were extracted as independent prognostic factors (HR: 11.7, 95% CI: 2.38-210, P=0.0006, and HR: 3.32, 95% CI: 1.17-11.8, P=0.0229, respectively).

Correlation between iPD-L1 expression, IS, and clinicopathological characteristics

The relationships between iPD-L1 expression, IS, and clinicopathological features are shown in Table 2. In the iPD-L1 positive group, the proportion of right-sided, early
TNM stage, T1-2, N0 cases was significantly higher (P=0.0178, P=0.0026, P=0.0035, P=0.0145, respectively). The iPD-L1 positive and I3-4 groups were significantly correlated (P < 0.0001).

Localization of PD-L1 expression and survival analysis

We used CD68 as a marker to assess M1 macrophage distribution and CD163 as a marker of M2 macrophage. The results of the immunohistochemical staining of CD68 and CD163 performed to clarify the localization of PD-L1 expression are shown in Fig. 3a-3f. PD-L1 positive TIMCs were positive for both CD68 and CD163, suggesting the possibility of the presence of macrophages, especially M2-type macrophages. The OS and RFS were significantly better in the iPD-L1 positive cases than in the iPD-L1 negative cases (P=0.0278 and P=0.0253, respectively) (Fig. 3g, 3h).

【Discussion】

Our study showed that IS is an independent prognostic factor for OS/RFS in stage II/III CRCs. We observed an almost complete lack of tPD-L1 expression, and that the iPD-L1 positive cases and IS were significantly correlated. Furthermore, iPD-L1 expression was
associated with good prognosis, suggesting that PD-L1 may be expressed in M2-type macrophages.

IS is determined by the density of CD3 and CD8 in the CT and IM, and is an excellent prognostic factor for CRC. Typically, post-operative adjuvant chemotherapy is recommended for stage II high-risk cases and stage III cases. As per regular clinicopathological analysis, stage II low-risk cases are excluded from adjuvant therapy. However, our results suggest that IS can identify truly high-risk cases that could not have been identified by traditional risk analysis. We also compared the significance of IS by stage I and stage IV cases, but could not identify the influence of IS on prognosis in each stage. This could be attributed to the fact that in stage I cases, radical resection is possible and the involvement of immune response should be relatively low. In stage IV cases, even though the effect of pre-surgical factor would be excluded, most cases receive a variety of post-operative combination chemotherapy, possibly causing the true benefit of immune status for OS/RFS to be canceled out.

PD-L1 is expressed not only on TCs but also on TIMCs, and tumor PD-L1 expression is correlated to poor prognosis in various carcinomas. However, analysis by MSI
status is advanced in CRCs, and the clinicopathological evaluation of the expression site of PD-L1 has not been determined. Lee et al. showed that PD-L1 expression in TCs correlated with poor prognosis in mismatch-repair deficient CRCs.\textsuperscript{31} In contrast, Li et al. reported that PD-L1 expression in TCs is associated with better prognosis in CRCs.\textsuperscript{32} However, few studies have examined the relationship between PD-L1 expression in TIMCs and prognosis in CRC cases. The relationships between PD-L1 in TIMCs expression and clinicopathological features have been reported in other carcinomas. Some reports state that PD-L1 in TIMCs expression correlates with poor prognosis in esophageal squamous cell carcinoma, gastric cancer, and uterine cervix adenocarcinoma;\textsuperscript{33–35} and favorable prognosis in urothelial carcinoma and head and neck cancer.\textsuperscript{36,37} Koganemaru et al. reported that PD-L1 tumor expression is associated with poor prognosis, while high PD-L1 in TIMCs expression is related to better prognosis in stage III CRCs.\textsuperscript{38} In addition, Lee et al. reported that PD-L1 in tumor-infiltrating immune cells expression correlated with good prognosis in CRCs.\textsuperscript{39} In our study, high iPD-L1 expression correlated significantly to OS improvement, supporting the results of the above-mentioned studies. As for the analysis of PD-L1, in
addition to the implication of its expression on tumors or their marginal interstitium, the
results may vary depending on the diagnostic reagents used. Therefore, to interpret all
analysis results in a similar manner, it is necessary to unify these reagents and make a
common diagnosis.

TAMs are important to the formation of a tumor microenvironment. When macrophages
are activated in the tumor microenvironment, they are polarized into M1 and M2 types;
M2 macrophages produce angiogenic factors and cell growth factors and form an
environment favorable for cancer growth. In this study, the immunohistochemical
staining of CD68, which is characteristic of common macrophages, and CD163, which
is characteristic of M2 macrophages, suggested that PD-L1 positive TIMCs may have
M2-type macrophages. The presence of M2 macrophages is correlated to poor
prognosis in various carcinomas. However, Edin et al. showed that the high
infiltration of M1 and M2 macrophages correlates with good prognosis in CRCs, and
that the local role of TAM is controversial. While it has been established that the
blockade of the PD-1 / PD-L1 pathway activates T cells, little is known of the role of
this pathway in TAMs, and further investigation is warranted.
Based on these results, we made the following hypothesis. M2 macrophages may be exhausted and may not be able to fulfill the tumor growth function, just like the lymphocytes may be exhausted and unable to attack the tumor cells. As a result of the exhaustion, there is the possibility that PD-L1 is expressed on macrophages. However, to prove this hypothesis, it will be necessary to investigate the functional mechanism by examining IFN$\gamma$, a regulator of PD-L1 expression, and M2 macrophage secretion factors.

Our study has several limitations. First, as it was performed in a single center, generalizability of the results may be low. Second, there is a possibility of patient selection bias due to the nature of the retrospective study. Third, the PD-L1 expression on TCs was weak; however, it has been suggested that the antibody we used (E1L3N) in this study, is comparable with other antibodies used in other studies. In CRCs, PD-L1 expression on TCs is observed in approximately 12-30% MSI-high cases; the corresponding value is very low in microsatellite stable (MSS) cases. Although the prevalence of MSI-high is an estimated 10-15% worldwide, it is as low as 3-7% in Japan; thus, it is possible that that there was almost no PD-L1 expression on TCs.
Fourth, as a result of the low proportion of MSI-high cases in Japan, we did not investigate the MSI status as a routine exam. We plan to study the role of MSI status in IS in future studies.

**Conclusions**

Our findings suggest that IS is a good indicator of better prognosis and is significantly correlated with iPD-L1 expression in CRC. Therefore, the evaluation of PD-L1 expression should distinguish between TIMCs and TCs. Further research is necessary to clarify the significance of PD-L1 expression on TIMCs, especially M2 type macrophages.

**Acknowledgements:** None

**References**


expression patterns in squamous cell carcinoma and adenocarcinoma of the cervix.


40. Tiainen S, Tumelius R, Rilla K, et al. High numbers of macrophages, especially M2-like (CD163-positive), correlate with hyaluronan accumulation and poor


46. Kim JH, Park HE, Cho NY, Lee HS, Kang GH. Characterisation of


Figure legends

Fig 1

Fig 1a-c: Immunohistochemical staining of representative PD-L1 expression (×50 and ×200). PD-L1 expression negativity (a). PD-L1 expression positivity on tumor cells (b). PD-L1 expression positivity on tumor-infiltrating mononuclear cells (c).

Fig 1d-g: Immunohistochemical staining of representative CD3 and CD8 (×50 and
CD3 positive at the center of the tumor (d). CD3 positive at the invasive margin (e). CD8 positive at the center of the tumor (f). CD8 positive at the invasive margin (g).

Fig 2

Fig 2a, 2b: Kaplan-Meier curves of overall survival (a) and relapse-free survival (b) according to the Immunoscore (IS) in patients with colorectal cancer.

Fig 2c-2f: Kaplan-Meier curves of RFS according to the IS by each TNM stage. The solid line represents the group with a high score (I3-4) and the dashed line represents the group with a low score (I0-2).

Fig 3

Fig 3a-3f: Immunohistochemical staining of CD68 and CD163 in tumor-infiltrating mononuclear cells with positive PD-L1 expression (iPD-L1). iPD-L1 positive ×25 (a) and ×200 (b). CD68 expression at the same position ×25 (c) and ×200 (d). CD163 expression at the same position ×25 (e) and ×200 (f).

Fig 3g, 3h: Kaplan-Meier curves of overall survival (g) and relapse-free survival (h)
according to the expression of PD-L1 on the tumor-infiltrating mononuclear cells (iPD-L1) in patients with colorectal cancer.
Table 1. Univariate and multivariate analysis of overall survival and relapse-free survival in stage II/III colorectal cancer

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^aHR, hazard ratio; ^bCI, confidence interval
Table 2. Relationship between PD-L1 expression on tumor-infiltrating mononuclear cells (iPD-L1), clinicopathological features, and Immunoscore in colorectal cancer

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<td>83 (84.7)</td>
<td>15 (15.3)</td>
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<tr>
<td><strong>Recurrence</strong></td>
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<tr>
<td>Negative</td>
<td>112</td>
<td>89 (79.5)</td>
<td>23 (20.5)</td>
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<tr>
<td>Positive</td>
<td>17</td>
<td>16 (94.1)</td>
<td>1 (5.9)</td>
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<td><strong>Immunoscore</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>I 0-2</td>
<td>77</td>
<td>73 (94.8)</td>
<td>4 (5.2)</td>
<td></td>
</tr>
<tr>
<td>I 3-4</td>
<td>55</td>
<td>35 (63.6)</td>
<td>20 (36.4)</td>
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Fig 1

<table>
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<tr>
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<th>PD-L1 -</th>
<th>tPD-L1 +</th>
<th>iPD-L1 +</th>
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<tr>
<td></td>
<td>CD3 CT+</td>
<td>CD3 IM+</td>
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<tr>
<td>x50</td>
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<td></td>
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<tr>
<td>x200</td>
<td>CD8 CT+</td>
<td>CD8 IM+</td>
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</table>

[Images a-g showing different tissue samples with varying immunostaining for PD-L1, CD3, and CD8 under different magnifications (x50, x200).]
Fig 1a-c: Immunohistochemical staining of representative PD-L1 expression (×50 and ×200). PD-L1 expression negativity (a). PD-L1 expression positivity on tumor cells (b). PD-L1 expression positivity on tumor-infiltrating mononuclear cells (c).

Fig 1d-g: Immunohistochemical staining of representative CD3 and CD8 (×50 and ×200). CD3 positive at the center of the tumor (d). CD3 positive at the invasive margin (e). CD8 positive at the center of the tumor (f). CD8 positive at the invasive margin (g).
Fig 2:

Fig 2a, 2b: Kaplan-Meier curves of overall survival (a) and relapse-free survival (b) according to the Immunoscore (IS) in patients with colorectal cancer.

Fig 2c-2f: Kaplan-Meier curves of RFS according to the IS by each TNM stage. The solid line represents the group with a high score (I3-4) and the dashed line represents the group with a low score (I0-2).
Figure 3

a. iPD-L1

b.

c. CD68
d.

e. CD163
f.

g. Overall survival probability

\[ p = 0.0278 \]

h. Relapse-free survival probability

\[ p = 0.0253 \]
Fig 3a-3f: Immunohistochemical staining of CD68 and CD163 in tumor-infiltrating mononuclear cells with positive PD-L1 expression (iPD-L1). iPD-L1 positive ×25 (a) and ×200 (b). CD68 expression at the same position ×25 (c) and ×200 (d). CD163 expression at the same position ×25 (e) and ×200 (f).

Fig 3g, 3h: Kaplan-Meier curves of overall survival (g) and relapse-free survival (h) according to the expression of PD-L1 on the tumor-infiltrating mononuclear cells (iPD-L1) in patients with colorectal cancer.