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ORIGINAL ARTICLE

Significance of Siglec-15 expression in colorectal cancer: association with advanced disease stage and fewer tumor-infiltrating lymphocytes

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Abstract

Siglec-15, a novel immune suppressor, is upregulated in many human cancers. The aim of this study was to explore the expression of Siglec-15 in colorectal cancer (CRC), and investigate whether Siglec-15 could be a potential target for cancer immunotherapy in patients with CRC. We performed immunohistochemical analyses of Siglec-15 on a cohort of 805 patients with CRC and made comparisons between clinicopathological characteristics, PD-L1 expression, CD3, CD8, CD45RO tumor-infiltrating lymphocytes (TILs), and prognosis. We found that Siglec-15 expression was commonly detected in tumor cells (48.3%) and tumor-associated stromal cells (33.4%), and was more frequently observed than PD-L1 expression in tumor cells. In contrast, Siglec-15 expression was weakly and scarcely found in normal mucosa (13%). Siglec-15 overexpression in tumor cells was associated with advanced TNM stage ($p = 0.020$). Co-expression of Siglec-15 and PD-L1 in tumor cells was found in 14.4% of patients, and Siglec-15 expression was detected in almost half of PD-L1 negative cases. Elevated Siglec-15 expression in tumor and stromal cells was associated with sparser CD45RO and CD8 TILs ($p = 0.035$ and $p = 0.004$, respectively). The expression of Siglec-15 did not have prognostic significance. In summary, compared to PD-L1, Siglec-15 protein expression is more prevalent in CRC and is associated with advanced disease stage and fewer TILs. These findings support Siglec-15 as a potential cancer immunotherapy target, in addition to PD-1/PD-L1 inhibitors, in patients with CRC.

Keywords: Siglec-15; PD-L1; colorectal cancer; tumor-infiltrating lymphocytes

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Introduction

The incidence of colorectal cancer (CRC) is increasing in China and the West [1,2]. Although CRC screening is carried out, about 25% of patients are diagnosed with metastatic disease and approximately 50% patients suffer from distant metastases during long-term follow-up [3,4]. For these individuals, the prognosis remains poor, with limited ability to prolong their survival. Therefore, the exploration of more effective therapies for these patients is urgently warranted.

Cancer immunotherapy suppresses immune checkpoints to selectively normalize immunity in the tumor microenvironment and has shown remarkable effectiveness against a broad spectrum of refractory malignancies, including metastatic CRC (mCRC) [5–7]. At present, nivolumab and pembrolizumab are approved

to treat mCRC due to their promising efficacy. However, only patients with deficient mismatch repair (dMMR) can benefit from immunotherapy [5,6]. Unfortunately, the prevalence of dMMR is very low at approximately 5% [6]. Therefore, further exploration of other therapeutic strategies is necessary.

Siglec-15 was originally characterized in 2007 as an osteoclast modulator and was recently found to have a continuous effect on inhibiting T-cell activity [8–13]. Unlike the majority of the Siglecs, Siglec-15 displays only one IgV and one IgC2 domain, exhibiting high homology with B7 family members. Also, Wang *et al* [12] have demonstrated that Siglec-15 overexpression is detected in various tumors and tumor-associated immune cells, with rare expression in normal tissues and a mutually exclusive expression with PD-L1, supporting Siglec-15 as a novel immune evasion mechanism. Meanwhile, Siglec-15 can significantly inhibit the function of antigen-specific T cells *in vitro* and *in vivo*, and leads to immune suppression and tumor growth. Importantly, the phase I study of NC318 (NCT03665285) has demonstrated promising efficacy in lung cancer patients treated with Siglec-15 inhibitors. Recently, several studies have demonstrated Siglec-15 overexpression in pancreatic ductal, lung, gastric, nasopharyngeal, breast, and bladder cancers [14–19]. Moreover, Siglec-15 expression is associated with prognosis and immunosuppression [14,17,20]. All these findings indicate that Siglec-15 may be a new and promising target for cancer immunotherapy.

The current study used tissue microarray (TMA) analysis, including 805 patients with CRC, to comprehensively investigate the relationships between Siglec-15 expression and patient characteristics, and the prognostic significance of Siglec-15 as a biomarker. The correlations between Siglec-15 and PD-L1 expression, or CD3, CD8, and CD45RO tumor-infiltrating lymphocytes (TILs), were further explored.

Materials and methods

TMAs cohort

We obtained 805 primary CRC specimens at the Department of Colorectal Surgery, Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, as reported previously [21]. In brief, unselected and nonconsecutive CRC patients from 2010 to 2014 were included in this study. All participants were pathologically diagnosed with colorectal adenocarcinoma, did not receive neoadjuvant treatment, and had completed radical dissection of both primary and metastatic tumors. Patients with other malignancies or recurrence or multiple primary tumors or without complete follow-up data or enough tissue to construct the TMAs were excluded from this study. Written or oral consent was obtained from all participants. Approval from the Institutional Review Board Committee was obtained to perform this study.

The patients' baseline characteristics, including age, sex, tumor location, tumor differentiation, TNM stage, mismatch repair (MMR) status, and *KRAS* mutation, were collected retrospectively. After radical surgery, all stage III and IV, and some stage II patients with high risk factors (such as T4, poor differentiation, lymphovascular invasion, etc) received adjuvant treatment. Generally, the XELOX regimen was used in colon cancer, and long-course chemoradiotherapy plus the XELOX regimen was used in rectal cancer. In total, 563 (69.9%) patients received adjuvant treatment. After radical surgery, patients were followed up regularly, as described previously [22]. At last follow-up (1 May 2019), 286 and 251 patients were confirmed to have experienced recurrences and deaths, respectively.

Representative areas of primary tumors and matched normal mucosal tissues were selected from formalin-fixed and paraffin-embedded tissue blocks. Then, the 1.5-mm diameter punched samples were embedded in recipient paraffin blocks using an automatic tissue arrayer (AutoTiss 10C, EverBio, Taiwan, ROC).

Immunohistochemistry and evaluation system

To perform immunohistochemistry (IHC), rabbit polyclonal antibody for Siglec-15 (PA5-72765, 1:100 dilution, Thermo Fisher Scientific, Shanghai, PR China) was used. All TMA slides were cut into 4-μm sections. IHC was conducted using an automated immunostainer (BenchMark ULTRA, Ventana Medical Systems, Inc., Shanghai, PR China) following the manufacturer's protocol. Then, the whole slides were scanned by a digital scanner (KFBIO, Ningbo, PR China), and separately assessed by two experienced pathologists (SZ and BW) who were blinded to patient data. For discrepant results, the particular tumor spots were reviewed, and a consensus was achieved. Immunohistochemical analyses were performed for PD-L1, CD3, CD8, and CD45RO, as previously described [21].

The staining of Siglec-15 in tumor cells and normal mucosal tissue was scored only for intensity, and patients with weak, moderate, or strong staining were considered to exhibit positive expression. We also

evaluated Siglec-15 expression in stromal cells in the tumor area, and immunoreactivity score for Siglec-15 in stromal cells was calculated according to the corresponding fraction of immunoreactive stromal cells (0–100%), regardless of the staining intensity. Given that TMAs comprise a small section of the tumor, a few positive cells in the core can indicate a positive tumor. Therefore, Siglec-15 staining on ≥5% stromal cells was defined as positive in our study.

Statistical analysis

Categorical data are presented as numbers (percent values) and compared using the Chi-square analysis. Continuous variables are presented as mean ± standard deviation and compared using *t*-tests or Mann–Whitney *U* tests. Disease-free survival (DFS) and overall survival (OS) rates are summarized using Kaplan–Meier methods and between-group comparisons were carried out using the log-rank test. The Cox proportional hazard model was used to investigate independent factors associated with DFS and OS. A two-sided $p < 0.05$ was deemed significantly different. All statistical analyses were accomplished using SPSS software (Version 25.0; IBM Corp., New York, USA) and GraphPad Prism software (Version 6; La Jolla, CA, USA).

Results

Expression of Siglec-15 in CRC

Patient characteristics are summarized in Table 1. We examined expression levels of the Siglec-15 protein in 805 cases of CRC and, as shown in Figure 1A–D, different staining intensities of Siglec-15 were detected in CRC TMAs, which showed mainly cytoplasmic/membrane staining. Siglec-15 expression was observed in tumor cells and tumor-associated stromal cells (Figure 1E,F), with scarce and weak expression in normal mucosal tissues (13%) (Figure 1G,H). According to the expression scores, 389 (48.3%) and 416 (51.7%) of patients were defined as positive and negative for Siglec-15 expression in tumor cells, respectively. The proportions of positive and negative Siglec-15 expression in stromal cells were 33.4 and 66.6%, respectively.

Co-expression of Siglec-15 and PD-L1 in tumor cells was relatively low (14.4%, 116/805). For PD-L1 negative cases, 46.2% (273/591) and 34.2% (202/591) of patients exhibited elevated Siglec-15 expression in tumor cells and stromal cells, respectively.

Table 1. Clinicopathological characteristics of patients

Characteristics	*n* (%)
Sex	
Male	446 (55.4%)
Female	359 (44.6%)
Age (years), mean	58.3
Tumor location	
Rectum	417 (51.8%)
Left colon	217 (27%)
Right colon	171 (21.2%)
Tumor differentiation	
Well-moderate	736 (91.4%)
Poor	69 (8.6%)
T stage	
T1	24 (3%)
T2	106 (13.2%)
T3	521 (64.7%)
T4	154 (19.1%)
N stage	
N0	371 (46.1%)
N1	259 (32.2%)
N2	175 (21.7%)
TNM stage	
I	101 (12.5%)
II	241 (29.9%)
III	319 (39.6%)
IV	144 (17.9%)
MMR status	
dMMR	68 (8.4%)
pMMR	737 (91.6%)
Adjuvant treatment	
No	242 (30.1%)
Yes	563 (69.9%)
KRAS mutation	
Yes	129 (16%)
No	181 (22.5%)
Unknown	495 (61.5%)

pMMR, proficient mismatch repair.

Correlations between Siglec-15 and clinicopathological data

As summarized in Table 2, we investigated the correlations between Siglec-15 and clinicopathological characteristics. This revealed that 43.6% of patients with stage I–II disease and 51.8% of those with stage III–IV disease showed positive Siglec-15 expression in tumor cells ($p = 0.020$). However, no significant relationships were found between tumor Siglec-15 expression and age, sex, tumor location, tumor differentiation, MMR status, or *KRAS* mutation. All these findings suggest that tumor Siglec-15 expression correlates with advanced disease stage. However, we found no significant correlation between Siglec-15

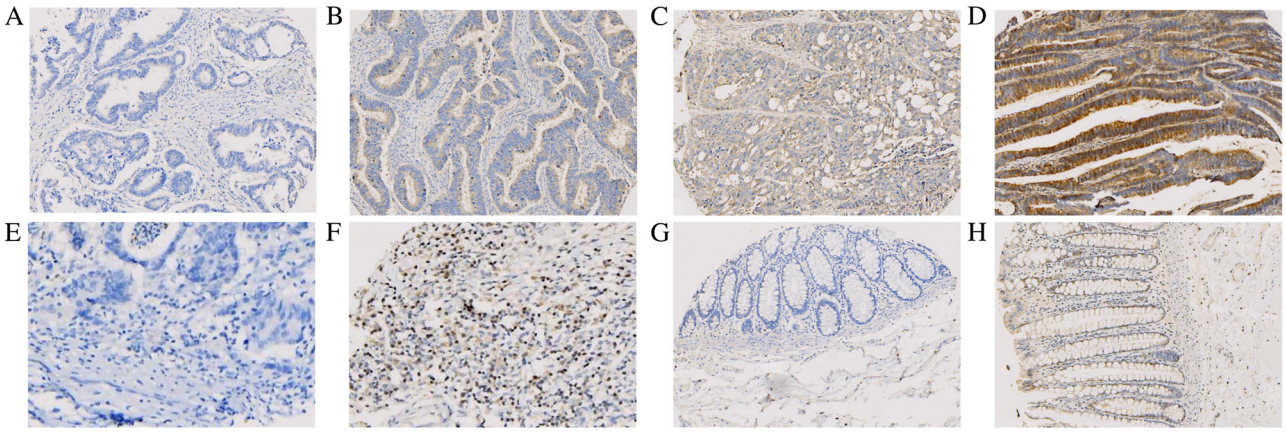

Figure 1. Representative images of Siglec-15 expression in colorectal cancer. (A–D) Negative to strong expression of Siglec-15 in tumor cells; (E and F) negative and positive expression of Siglec-15 in tumor-associated stromal cells; (G and H) negative and positive expression of Siglec-15 in normal mucosa.

expression in stromal cells and any clinicopathological characteristics.

Prognostic significance of Siglec-15 expression in CRC

As shown in Figure 2, Kaplan–Meier outcomes revealed that only positive Siglec-15 expression in stromal cells was significantly correlated with decreased DFS. However, after multivariate analyses, there was no significant association between Siglec-15 and survival. The prognosis of patients with CRC was associated with tumor differentiation, TNM stage, and MMR status (Table 3).

TILs and their associations with Siglec-15 expression

The densities of CD3, CD8, and CD45RO TILs, which represent total T cells, cytotoxic T cells, and memory

Table 2. Association between clinicopathological characteristics with Siglec-15 expression

Characteristics	Siglec-15 expression in TC			Siglec-15 expression in SC		
	Positive	Negative	*P* value	Positive	Negative	*P* value
All cases	389	416		269	536	
Sex			0.433			0.876
Male	210 (47.1%)	236 (52.9%)		148 (33.2%)	298 (66.8%)	
Female	179 (49.9%)	180 (50.1%)		121 (33.7%)	238 (66.3%)	
Age (years)			0.168			0.806
<60	199 (46.1%)	233 (53.9%)		146 (33.8%)	286 (66.2%)	
≥60	190 (50.9%)	183 (49.1%)		123 (33%)	250 (67%)	
Tumor location			0.252			0.664
Rectum	190 (45.6%)	227 (54.4%)		140 (33.6%)	277 (66.4%)	
Left colon	113 (52.1%)	104 (47.9%)		68 (31.3%)	149 (68.7%)	
Right colon	86 (50.3%)	85 (49.7%)		61 (35.7%)	110 (64.3%)	
Tumor differentiation			0.555			0.988
Well-moderate	358 (48.6%)	378 (51.4%)		246 (33.4%)	490 (66.6%)	
Poor	31 (44.9%)	38 (55.1%)		23 (33.3%)	46 (66.7%)	
TNM stage			*0.020*			0.120
I–II	149 (43.6%)	193 (56.4%)		104 (30.4%)	238 (69.6%)	
III–IV	240 (51.8%)	223 (48.2%)		165 (35.6%)	298 (64.4%)	
MMR status			0.137			0.464
dMMR	27 (39.7%)	41 (60.3%)		20 (29.4%)	48 (70.6%)	
pMMR	362 (49.1%)	375 (50.9%)		249 (33.8%)	488 (66.2%)	
KRAS mutation			0.730			0.198
Yes	61 (47.3%)	68 (52.7%)		38 (29.5%)	91 (70.5%)	
No	82 (45.3%)	99 (54.7%)		66 (36.5%)	115 (63.5%)	

Italics indicates statistical significance; *KRAS* mutation was available for 310 cases.
pMMR, proficient mismatch repair; SC, stromal cell; TC, tumor cell.

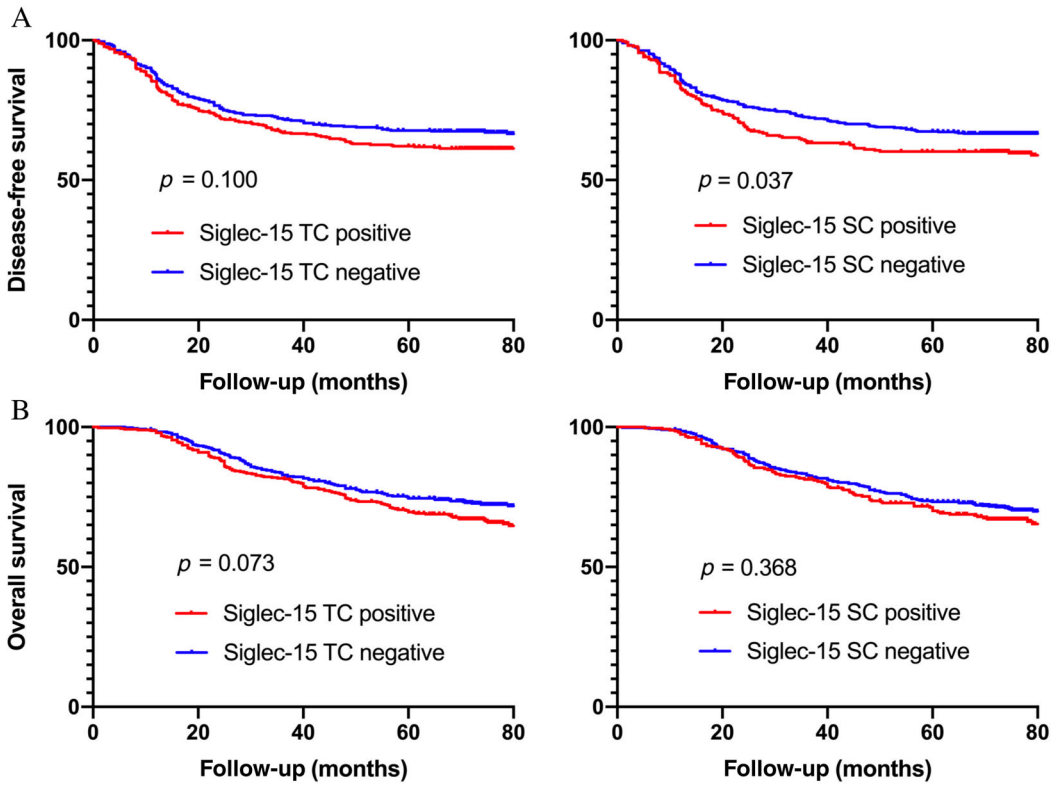

Figure 2. Kaplan–Meier survival curves according to Siglec-15 expression. Statistical analyses associated with (A) DFS and (B) OS were performed. SC, stromal cell; TC, tumor cell.

T cells, respectively, were calculated. As shown in Figure 3, Siglec-15 positive tumor cells were inversely related to CD45RO TILs ($p = 0.035$). The counts of CD3 and CD8 TILs were not significantly related to Siglec-15 expression in tumor cells. Intriguingly, Siglec-15 expression in stromal cells was associated with lower CD8 TIL counts ($p = 0.004$), but not with CD3 and CD45RO TIL counts. These findings indicate that differential immune checkpoint expression at different sites plays a diverse role in regulating the immune response.

Table 3. Univariate and multivariate analyses of factors associated with survival

Variables	DFS				OS			
	Univariate analysis	Multivariate analysis			Univariate analysis	Multivariate analysis		
	P value	HR	95% CI	*P* value	*P* value	HR	95% CI	*P* value
Sex (male versus female)	0.111				0.057			
Age, years (<60 versus ≥60)	0.973				0.244			
Tumor location (rectum versus colon)	0.459				0.782			
Tumor differentiation (poor versus well-moderate)	*<0.001*	2.678	1.922–3.732	*<0.001*	*<0.001*	2.928	2.084–4.112	*<0.001*
TNM stage (III–IV versus I–II)	*<0.001*	3.288	2.345–4.609	*<0.001*	*<0.001*	3.108	2.177–4.437	*<0.001*
Adjuvant treatment (yes versus no)	*<0.001*	0.872	0.608–1.250	0.455	*<0.001*	0.967	0.667–1.403	0.861
MMR status (pMMR versus dMMR)	*0.017*	2.334	1.357–4.013	*0.002*	*0.018*	2.639	1.446–4.818	*0.002*
Siglec-15 expression in SC (positive versus negative)	*0.037*	0.803	0.632–1.021	0.073	0.368			
Siglec-15 expression in TC (positive versus negative)	0.100				0.073			

Italics indicates statistical significance.
CI, confidence interval; HR, hazard ratio; pMMR, proficient mismatch repair; SC, stromal cell; TC, tumor cell.

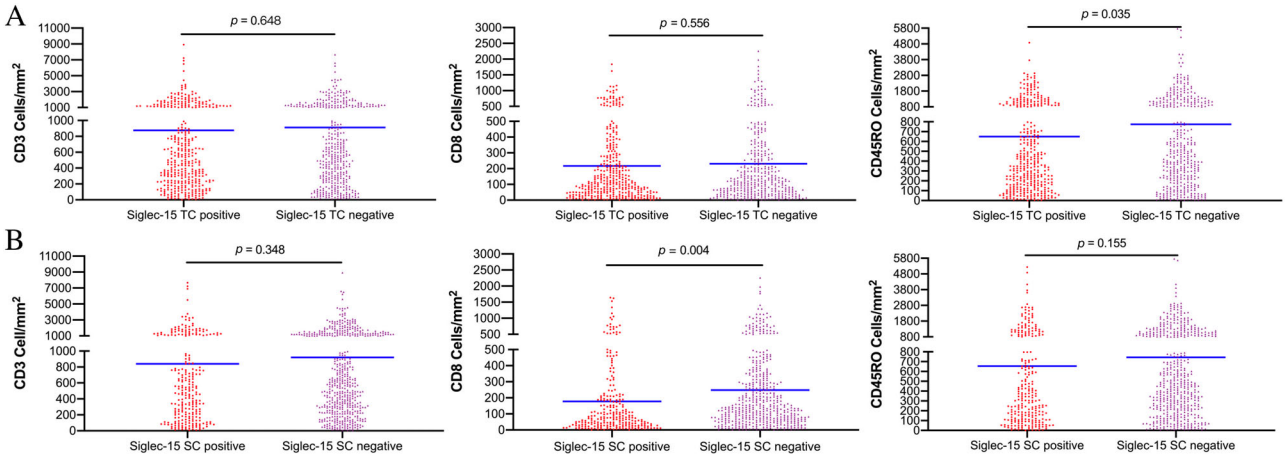

Figure 3. Correlation between Siglec-15 expression and TILs. (A) Positive Siglec-15 expression in tumor cells was significantly associated with fewer CD45RO T cells; (B) positive Siglec-15 expression in stromal cells was significantly associated with fewer CD8 T cells. The blue dashes indicate the mean values. SC, stromal cell; TC, tumor cell.

Discussion

In the current study, we comprehensively investigated Siglec-15 expression in a large cohort of patients with CRC. Compared with PD-L1 expression, Siglec-15 expression was more frequent in tumor cells and tumor-associated stromal cells. Furthermore, co-expression of Siglec-15 and PD-L1 in tumor cells was not prevalent, and Siglec-15 expression was detected in almost half of PD-L1 negative cases. In addition, the presence of Siglec-15 expression was associated with advanced disease stage, and Siglec-15 positivity correlated with sparser CD45RO and CD8 TILs.

To fully understand Siglec-15 expression in cancers, TCGA database analyses conducted by Li *et al* showed that Siglec-15 was overexpressed in a variety of human cancers – including colon cancer – and rarely expressed in normal tissues [23]. We have performed IHC to explore Siglec-15 expression in CRC at the protein level, and found that Siglec-15 protein expression was common in tumor cells, but rare in normal mucosal tissues. This finding suggests that anti-Siglec-15 may not cause adverse effects. Meanwhile, Siglec-15 expression was also detected in tumor-associated stromal cells, which was similar to Wang *et al*'s study [12]. Moreover, co-expression of Siglec-15 and PD-L1 was infrequent, which might explain why only a few patients with CRC were responsive to PD-1/PD-L1 therapy. This further indicated that anti-Siglec-15 alone might be another choice for those who were resistant to PD-1/PD-L1 therapy or show a combined effect with PD-1/PD-L1 inhibitors.

Inspiringly, Chen has developed a Siglec-15 antibody, NC318. During the Society for Immunotherapy of Cancer 2019 Annual Meeting, the results of the phase I study of NC318 (NCT03665285) were discussed [24]. This study recruited 49 cancer patients, and found that 2 patients with non-small lung cancer exhibited complete and partial responses, respectively. Other patients experienced stable disease – lasting at least half a year – with tolerable side effects. Based on these encouraging preliminary results, the phase II study is ongoing, and will be completed in 2023.

Cell immunity induced by T cells plays a vital antitumor role; therefore, current immunotherapy has focused on normalizing T-cell function [25,26]. Among the differential T-cell subsets, CD8 T cells efficiently restrain and destroy tumor cells through the production of cytotoxic molecules and cytokines [27]. Wang *et al* demonstrated that Siglec-15 on tumor-associated macrophages could directly suppress T-cell activity and immune response both *in vitro* and *in vivo*. Conversely, in Siglec-15 knockout mice, it resulted in significant improvement in CD8 T-cell infiltration and the production of cytokines such as IFN-γ [12]. Similarly, we found that Siglec-15 expression in tumor-associated stromal cells correlated with sparser CD8 TILs. This suggested that Siglec-15 might inhibit CD8 T-cell activity via its influences on T-cell densities, implicating Siglec-15 as a potential immune regulator in CRC. However, we did not confirm Siglec-15 expression in macrophages. Multiplexed staining of Siglec-15, and CD68 is warranted to verify Wang *et al*'s findings in a lung cancer model [12]. We also found

that Siglec-15 positive tumors were associated with lower CD45RO TIL counts. CD45RO TILs, which represent memory T cells, suppress tumor dissemination of CRC [28]. This suggests that tumor Siglec-15 expression may influence CD45RO T-cell density and promote tumor metastasis. Taken together, Siglec-15 expression at different sites may play a diverse role in regulating the immune response.

In this study, we found that Siglec-15 overexpression in tumor cells was significantly associated with advanced TNM stage, indicating that anti-Siglec-15 might prolong the survival of patients with mCRC; however, we did not observe a correlation between Siglec-15 expression and prognosis, and more clinical trials should be conducted to explore the significance of anti-Siglec-15 treatment in CRC.

Our results revealed a positive correlation between tumor PD-L1 expression and dMMR status. Consistent with our findings, previous studies also found that dMMR status was associated with a higher rate of tumor PD-L1 expression than MMR proficient cancers [5,29]. Patients with dMMR status are considered good responders to PD-1/PD-L1 inhibitors and exhibit a higher rate of tumor mutations and more T-cell infiltration. However, we did not observe a significant association between Siglec-15 expression and MMR status. Considering the small proportion of dMMR patients in this study, other studies including more dMMR cases should be conducted to investigate whether anti-Siglec-15 might be applicable to more patients with CRC, and not only those with dMMR cancers.

Although this is the first large-cohort study to explore Siglec-15 expression in patients with CRC, several limitations warrant consideration. First, this was a retrospective study from a single institution in China, which might have produced selection bias. Also, Siglec-15 expression in different ethnic groups needs to be investigated. Second, TMAs were used to explore Siglec-15 expression. This might have caused potential misclassification due to tumor heterogeneity. However, a large number of samples were selected randomly and should therefore make our results more generalizable. Third, it was difficult to compare our findings with others because there are so few published studies on this topic. Fourth, only IHC was used in this study, and *in vitro*/*in vivo* experiments, and clinical trials, should be conducted to investigate the significance of Siglec-15 in CRC further.

In summary, compared with PD-L1 expression in CRC, Siglec-15 expression was more frequent in tumor cells and tumor-associated stromal cells. Siglec-15 overexpression was significantly related to advanced disease stage and fewer TILs. This indicates that Siglec-15 may be a potential cancer immunotherapy target – next to PD-1/PD-L1 inhibitors – in patients with CRC.

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Author contributions statement

ZL contributed to data analysis and manuscript drafting. PC, FH and JL offered technical support. BW and SZ assessed the pathological slides and evaluated the staining scores. CP and ZZ contributed to the design of the study and supervision. All authors read and approved the final manuscript.

Data availability statement

The data sets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

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