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Siglec-15 promotes progression of clear renal cell carcinoma

Wen-Bo Yang, Cai-Peng Qin, Yi-Qing Du, Song-Chen Han, Tao Xu

Department of Urology, Peking University People's Hospital, Beijing 100044, China.

Clear cell renal cell carcinoma (ccRCC) is the most common subtype of renal cell carcinoma, accounting for approximately 80% of cases. Although most patients in the early stages of ccRCC can be cured by surgery, ccRCC is often in the advanced stage when patients are diagnosed, with >30% of the patients presenting with distant metastases at the time of diagnosis. For ccRCC patients with distant metastases, the 5-year survival is only 12%. Immunotherapy selectively corrects tumor microenvironment (TME) immunity, which is of great significance for patients with advanced ccRCC.

Nevertheless, only a small minority respond well to current immunotherapy treatments. Therefore, it is essential to continue to look for other targets for immunotherapy. Siglec-15, which contains a sialic acid-binding immunoglobulin lectin, has attracted much interest in more recent years as a potential new target for immunotherapy, and drugs targeting Siglec-15 are under development. With systemic or lineage-specific gene ablation in mice, previous studies reported Siglec-15 was mutually exclusive to the B7-H1/PD-1 pathway.[1] However, the expression of Siglec-15 in ccRCC has rarely been evaluated, especially at the translation level. Therefore, this study aimed to explore Siglec-15 expression at both the transcriptional and translational levels and its role in clinicopathological prognostic parameters in ccRCC.

This study was approved by the Ethics Committee of Peking University People's Hospital (No. 2018PHC039). Informed consents to participate and for publication were all obtained from all individual participants included in the study. A specimen microarray was constructed with 150 ccRCC samples and 30 paired adjacent normal tissues. All patients had associated programmed death ligand-1 (PD-L1) and B-cell lymphoma-2 (BCL-2) immunohistochemistry (IHC) staining scores, and all information was obtained from Shanghai Outdo Biotech (Shanghai, China). Besides, 32 ccRCC samples and 25 adjacent normal renal tissues were obtained from our center, and fibrosis levels were quantified with second harmonic generation/two-photon excitation fluorescence (SHG/TPEF), as outlined in our previous studies.[2] The strength of the SHG signal positively correlates with the collagen in biological tissues, which specifically indicates the abnormal deposition of collagen in tissues.

The polyclonal antibody to Siglec-15 (1:400, #ab174732, Abcam, Cambridge, UK) was used in IHC to evaluate the expression of Siglec-15. The procedures for IHC staining and the calculation score for Siglec-15 staining have been described previously.[3] The formula for the total staining score was as follows: total staining score of Siglec-15 = positive staining rate score × staining intensity score. The positive staining rate was scored according to the percentage of positive cells: 0 (negative), 1 (1%–25%), 2 (26%–50%), and 3 (51%–100%). The staining intensity score of Siglec-15 was evaluated as 0 (negative), 1 (weak), 2 (medium), and 3 (strong) by two independent pathologists.

RNA sequencing and clinical information of patients with ccRCC were obtained from The Cancer Genome Atlas (TCGA) website. RNA sequencing analysis was carried out with the TCGA data of 537 ccRCC and 72 adjacent normal tissues, and 4 cases were excluded because Siglec-15 information was missing. The cutoff value of Siglec-15 was set at the median value for division into a high-expression group and a low-expression group.

To understand the relationship between Siglec-15 and immune cell infiltration in ccRCC, we used the xCell method[4] to analyze the transcriptome profile of 534 tumors and deconvoluted immune, stromal, and microenvironmental cell gene characteristics. Higher immune scores and lower stromal scores were characterized by a high degree of CD8+ T cell infiltration and increased expression of the immune therapy markers PD1, PD-L1, PD-L2, and CTLA4.

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Correspondence to: Dr. Tao Xu, Department of Urology, Peking University People's Hospital, Beijing 100044, China
E-Mail: xutao@pkuph.edu.cn

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SPSS 22.0 (SPSS Inc., Chicago, IL, USA), R software (R 4.0.3), and GraphPad Prism 8.4 were used for statistical analyses. Continuous data were presented as the mean ± standard error. The difference of Siglec-15 between tumor tissues and adjacent normal tissues was analyzed by a paired *t* test. The association of pathological grade with Siglec-15 expression was evaluated by a chi-square test. Genes coexpressed with Siglec-15 in TCGA were analyzed with the Limma package. Then, functional annotation analysis was performed to identify the role of Siglec-15 in ccRCC progression on the Metascape website, an online function annotation analysis tool (http://metascape.org/gp/). Bivariate correlation and linear regression were used to correlate the IHC score of Siglec-15 with PD-L1 and BCL-2 expression. The Kaplan-Meier method was used to show survival differences. The time for overall survival was calculated from the time of surgery until death. $P < 0.050$ was considered statistically significant.

The clinicopathological characteristics were separately presented in Supplementary Tables 1–3, http://links.lww.com/CM9/A766. Notably, Siglec-15 was frequently expressed in ccRCC. In addition, Siglec-15 was localized in the ccRCC and the stromal cells' membranes [Supplementary Figure 1A, http://links.lww.com/CM9/A762].

As Supplementary Figure 1B, http://links.lww.com/CM9/A762 showed, Siglec-15 was translationally overexpressed in tumor tissues compared with paired adjacent normal tissues in the tissue microarray ($n = 29$, 0.83 ± 0.70 *vs.* 3.38 ± 2.11, $P < 0.001$), and the same results were found in our centers' cohort ($n = 25$, 1.44 ± 1.72 *vs.* 4.00 ± 2.41, $P < 0.001$). Notably, Siglec-15 was not significantly correlated with PD-L1 ($n = 150$, $P = 0.082$). Moreover, Siglec-15 overexpression was not statistically associated with BCL-2 overexpression ($P = 0.078$).

The high expression of Siglec-15 was associated with a higher Fuhrman grade at the protein level ($n = 150$, $P = 0.001$, odds ratio [OR] = 4.292, 2.021–9.113) and the transcript level ($n = 534$, $P = 0.008$, OR = 1.606, 1.138–2.267). However, there were no significant correlations between Siglec-15 and sex, age, or tumor stage in ccRCC ($P > 0.050$). The summary of the correlation of Siglec-15 overexpression with clinicopathological variables is depicted in Supplementary Tables 1–3, http://links.lww.com/CM9/A766. Multivariate analysis indicated that overexpression of Si glec-15 was an independent risk factor for higher Fuhrman grades ($P < 0.001$, OR = 4.937, 2.101–11.602). Kaplan-Meier survival analysis [Supplementary Figure 2, http://links.lww.com/CM9/A763] showed that patients with a higher Siglec-15 expression had shorter overall survival periods without statistical significance at the transcript level ($P = 0.073$). Figure 1 shows a statistically significant decrease in overall survival for patients with high expression of Siglec-15 at the protein level compared with patients with a lower expression of Siglec-15 ($n = 150$, $P = 0.007$).

Overexpression of Siglec-15 at the transcript level was positively associated with a higher immune score (OR = 2.577, 1.794–3.702, $P < 0.001$) and microenvironment score (OR = 1.816, 1.271–2.596, $P = 0.001$). In contrast,

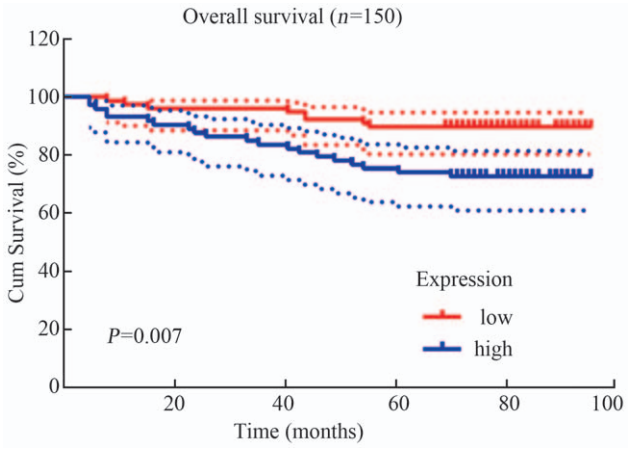

Figure 1: Kaplan-Meier curves for overall survival functions for Siglec-15 expression in tissue microarray cohort.

overexpression of Siglec-15 was negatively associated with a higher stromal score (OR = 0.597, 0.418–0.853, $P = 0.004$). These results indicate that the TME of patients with overexpression of Siglec-15 was significantly immunosuppressed compared with that of patients with low expression and that the development of immunotherapy for these patients can be expected to be more effective. To clarify the role of Siglec-15 in the TME, we further evaluated the relationship between Siglec-15 on carcinoma-associated fibroblasts and tumor-associated macrophages in the ccRCC microenvironment. With the xCell method to analyze the transcriptome profile of 534 tumors, our study showed that the expression of Siglec-15 was significantly and positively correlated with the infiltration of tumor-associated macrophages and tumor-associated fibroblasts in most malignant tumors, including ccRCC ($P < 0.050$).

To further explore whether Siglec-15 is associated with tumor fibrosis, 32 ccRCC samples in our center cohort were included, and SHG/TPEF was used to quantify the degree of tumor fibrosis [Supplementary Figure 3, http://links.lww.com/CM9/A764]. Furthermore, Siglec-15 was stained by IHC. The relative tumor fibrosis was 4.77 ± 5.13 and 1.52 ± 1.51 in the low Siglec-15 group ($n = 16$) and the high Siglec-15 group ($n = 16$) groups, respectively. Siglec-15 was inversely associated with the tumor fibrosis level ($P = 0.020$).

As the Gene Ontology analysis and Kyoto Encyclopedia of Genes and Genomes Pathway analysis [Supplementary Figure 4, http://links.lww.com/CM9/A765] indicated that Siglec-15 may take part in the myeloid leukocyte activation, cell activation involved in immune response, regulation of cell adhesion, extracellular matrix organization, and regulation of supramolecular fiber organization ($P < 0.050$).

With a protein sequence similar to that of PD-L1, Siglec-15 is involved in osteoclast differentiation and was previously identified as a potential therapeutic target for osteoporosis. Recent studies indicated that Siglec-15 also played an

essential role in inhibiting the tumor immune response *in vitro* and *in vivo*, by binding with receptors on T cells and inhibiting the T cell response.[1] Previous *in vitro* and *in vivo* studies have demonstrated that genetic ablation or antibody blockade of Siglec-15 amplifies anti-tumor immunity and inhibits tumor growth in non-small-cell lung cancer, fully demonstrating the mechanism by which Siglec-15 inhibits tumor immunity. However, more interestingly, relevant studies have shown that Siglec-15 is likely to be the main immunosuppressive factor for the vast majority of patients with PD-L1-negative tumors in lung cancer, ovarian cancer, and head and neck cancer. When PD-1/PD-L1 treatment is ineffective, Siglec-15 may be a potential immunotherapy choice. The present study showed that the Siglec-15 protein was frequently expressed in ccRCC and that Siglec-15 expression in tumor tissue was significantly increased compared with that in normal adjacent tissues, which indicated Siglec-15 as a potential immunotherapy target in ccRCC.

Interestingly, this study also found that Siglec-15 was positively associated with pathology grade and a poorer prognosis in ccRCC, which had not been reported. Through a pan-cancer mRNA analysis, Li *et al*[5] found that the effect of Siglec-15 on the prognosis of tumors varied favorably or unfavorably with different types of tumors. Therefore, therapies targeting Siglec-15 may be more effective in ccRCC than that in other malignant tumors. In the present study, Kaplan-Meier survival analysis also showed that patients with a higher expression of Siglec-15 had a significantly poorer prognosis.

Consistent with previous studies, the present study confirmed that the Siglec-15 expression level was also not significantly correlated with the PD-L1 level in ccRCC ($P > 0.050$), suggesting that it is a potential immunotherapy target and may expand the immunotherapy benefit cohorts of ccRCC.

Previous studies based on proteomics identified ccRCC with a higher immune score and lower interstitial score as the CD8 + flamed subtype, in which a higher expression of immunosuppressive molecules has been reported. The present study indicated that the high expression of Siglec-15 was associated with a higher immune score and a lower stromal score. In a further evaluation of the effect of Siglec-15 on the TME of ccRCC, this study illustrated that the expression of Siglec-15 was significantly and positively correlated with the infiltration of tumor-associated macrophages and tumor-associated fibroblasts ($P < 0.050$). Interaction between the immune system and tumors may promote fibrosis of the TME. Besides, previous studies indicated intra-tumor fibrosis was associated with poor prognosis in ccRCC.[2] Moreover, we rarely elaborated, that Siglec-15 plays an essential role in the tumor fibrosis process.

The limitations of this study are as follows. This study presents no biological experiments on the role of Siglec-15 in ccRCC *in vivo* or *in vitro*. Second, no commercial monoclonal antibody against Siglec-15 was available when the study was initiated. Additionally, the sample sizes of IHC and SHG/TPEF were relatively small. However, the samples of IHC were derived from different centers; therefore, the results are highly reliable.

Availability of data and materials

The data sets of Peking University People's Hospital and specimen microarray analyzed during the current study are available from the corresponding author on reasonable request. Other datasets generated during and/or analyses during the current study are available on the TCGA website.

Code availability

The software application or custom code used analyzed during the current study is available from the corresponding author on reasonable request.

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Conflicts of interest

None.

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