Impact of Overexpression of Immune Suppressor Siglec-15 on The Prognosis of Clear Renal Cell Carcinoma and Fibrosis Level

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Abstract

Background The expression of Siglec-15, as a critical immune suppressor, in renal clear cell carcinoma (ccRCC) was few evaluated and remains unclear, especially in protein level. As previous studies reported, tumor fibrosis plays an essential role in assessing the prognosis of ccRCC, but the exact mechanism is not precise. This study evaluated the expression of Siglec-15, its role in prognosis, and the association with tumor fibrosis in ccRCC.

Methods: Immunohistochemistry was used to analyze the Siglec-15 expression in one tissue microarray (cohort A, tumor: n=134, adjacent normal tissues: n=29). Subsequently, the mRNA expression of Siglec-15 and its clinical significance in ccRCC were analyzed using The Cancer Genome Atlas database (TCGA, cohort B, n = 534) and samples. Spearman correlation coefficients were calculated for correlation analysis of correlated expression genes of Siglec-15, and then functional annotation analysis was obtained with correlated expression genes. We detected the tumor fibrosis grade in cohort C (n=32) via second harmonic generation/two-photon excitation fluorescence.

Results: Siglec-15 was overexpressed in tumor tissues compared with adjacent normal tissues in both cohort A (n=29, $p<0.001$) and cohort C (n=25, $p<0.001$). However, there was no significant difference in mRNA expression of Siglec-15 between tumor and adjacent normal tissues in cohort B ($p>0.05$). Moreover, over-expression of Siglec-15 is associated with higher Fuhrman grade in cohort A□C (n=166, p=0.001, OR=3.132, 1.563-6.275), cohort B (n=534, p=0.008, OR=1.606, 1.138-2.267). Univariate Kaplan-Meir survival analysis showed that patients with high Siglec-15 mRNA expression had shorter survival periods without significance in cohort B (p=0.073). Multivariate analysis employing the Siglec-15 regression model revealed that AJCC and Fuhrman grade was the only significant independent prognostic indicators. Besides, an inverse correlation was found between Siglec-15 protein expression and the tumor's fibrosis level (p = 0.02).

Conclusions: Siglec-15 expression increases in ccRCC compared with adjacent normal tissues. Siglec-15 was frequently expressed and positively associated with pathology grade in ccRCC. This study indicated a significant role of Siglec-15 in the prognosis and immunotherapy target of ccRCC. This study also found an inverse correlation between Siglec-15 protein expression and the fibrosis level of the tumor.

Background

Renal clear cell carcinoma (ccRCC) is the most common subtype of renal cell carcinoma, accounting for about 80% of cases[1]. Although most patients with early ccRCC can be cured by surgery, ccRCC is often occult in the early stage, and thus more than 30% of the patients were found with distant metastases at the time of diagnosis. For these ccRCC patients with distant metastases, 5-year survival was only 12%[2]. Immunotherapy is of great significance to advanced ccRCC because it becomes resistant to Antiangiogenic agents after a period of good results[3]. The anti-PD therapy selectively corrected the

defective immunity in the tumor microenvironment (TME), fully vindicating the importance of normalization cancer immunotherapy.

Nevertheless, only a small percentage of patients respond to current immunotherapy. Therefore, it is essential to continue to look for other targets for immunotherapy. Siglec-15, structured with sialic acid-binding immunoglobulin-type lectin, is one of the Siglec gene family members and identified as a critical immune suppressor. Siglec-15 has attracted much interest in more recent years as a potential new target for immunotherapy. Siglec-15 is expressed in myeloid cells' membrane in the tumor microenvironment, but the mRNA was upregulated in some human cancer cells, such as colon cancer and thyroid cancer. However, the expression of Siglec-15 in ccRCC was few evaluated and remains unclear, especially in protein level. Consequently, this study aims to examine the expression in both the protein and transcription level of immune suppressor Siglec-15 in ccRCC, its role in clinicopathological prognostic parameters.

As previous studies reported, intratumor fibrosis plays an essential role in assessing the prognosis of ccRCC, although the exact mechanism of this phenomenon is not clear[4]. The fibrosis of the tumor microenvironment may result from the interaction between the immune system and the tumor[5]. Therefore, we put forward the hypothesis that Siglec-15 as an immunosuppressive factor may be related to the fibrosis of the tumor in ccRCC. In this study, we revealed for the first time the inverse correlation between Siglec15 and the degree of fibrosis in ccRCC.

Materials And Methods

Tissue microarrays, samples, and immunohistochemistry analysis

The study was approved by the Ethics Committee of the Peking University People's Hospital. As Fig. 1 showed, a tissue microarray (TMA) from 134 ccRCC samples and 29 cases of adjacent normal tissues (Cohort A) consisted of PD-L1, and BCL-2 score was obtained from Shanghai Outdo Biotech (Shanghai, China). Besides, 32 ccRCC samples and 25 adjacent normal renal tissues (Cohort B) were collected from Peking University Peoples Hospital, of which fibrosis level was quantified by second harmonic generation/two-photon excitation fluorescence (SHG/TPEF) as the previous studies[4]. The polyclonal antibody (1:400, #ab174732, Abcam, Cambridge, UK) to Siglec-15 was used in IHC to quantitative evaluated the protein expression of Siglec-15. The procedures for IHC staining and calculation score of Siglec-15 staining have been described previously. The IHC intensity score was also scaled as 0 for no IHC signal, 1 for weak, 2 for moderate, and 3 for strong IHC signals. The extent score was scaled as the percentage of positively stained cells. Then, the final score was obtained by multiplying the extent score and intensity score.

TCGA RNA-Seq and matching clinical data of ccRCC (Cohort B) were collected from the GDC portal website (https://portal.gdc.cancer.gov/). RNA-Seq analysis was carried out with the TCGA data of 538

ccRCC and 72 adjacent normal renal tissues, and 4 cases were excluded for missing Siglec-15 information. The cut-off point value of Siglec-15 was determined by the receiver operating characteristic curve (ROC) of Siglec-15 and pathological grade, which was then divided into the high-expression group (HEG) and the low-expression group (LEG). The expression pattern of individual genes in normal human tissues and the tumor tissues were analyzed in BioGPS using TCGA databases normalized by the cancer browser (https://www.cbioportal.org/study/summary?id=kirc_tcga).

Statistical analyses

SPSS 22.0 (SPSS Inc., Chicago, IL, USA), R software (R 4.0.3), and GraphPad Prism 8.4 was used for statistical analyses. Continuous data were presented as the mean ± standard error. The deference of Siglec-15 expression detected by immunohistochemistry between tumor tissues and adjacent normal tissues was analyzed with a paired Student t-test in Cohort A and Cohort C, with limma package in Cohort B. The deference of pathological grade in OEG and LEG was evaluated with a Chi-square test in Cohort A, Cohort B, and Cohort C. Spearman correlation coefficients were calculated for correlation analysis of correlated expression genes of Siglec-15 in Cohort B. Then functional annotation analysis was performed by Metascape, a simple and powerful online function annotation analysis tool (http://metascape.org/gp/). Bivariate correlation and linear regression were used to correlate the mean IHC score of Siglec-15 to PD-L1 and BCL-2 value. $P < .05$ was deemed statistically significant. The Kaplan-Meier method and log-rank test were performed to show survival differences, respectively, according to Siglec-15 expression. The time for overall survival was calculated from the time of surgery until the occurrence of death.

Results

3.1 | Clinical characteristics of the patients and immunohistochemistry

The clinicopathological characteristics of cohort A, cohort B, and cohort C were separately presented in Table 1, Table 2, and Table 3. Siglec-15 was frequently expressed in ccRCC. Siglec-15 was detected in 119/134 and 32/34 cases by IHC in cohort A and cohort C separately and was exclusively localized in the cytomembrane of malignant cells of ccRCC (Fig. 2). As Fig. 3 exhibited, Siglec-15 in protein level was overexpressed in tumor tissues compared with paired adjacent normal tissues in cohort A ($n = 29$, 66.90 ± 50.77 *vs.* 15.17 ± 18.35, $p < 0.001$) and cohort C ($n = 25$, 78.20 ± 66.35 *vs.* 24.60 ± 46.16, $p < 0.001$). However, there was no significant difference in mRNA expression of Siglec-15 between tumor and adjacent normal tissues in cohort B ($p > 0.05$). These results indicate that Siglec-15, translationally upregulated in ccRCC. Siglec-15 does not correlate with PD-L1 and BCL-2 levels ($p > 0.05$).

Table 1
Associations of Siglec15 expression with clinicopathological factors in cohort A

	Number of patients	Siglec15		χ2	*P*
		High expression (%)	Low expression (%)		
Gender				0.00	0.998
Male	101	49	52		
Female	33	16	17		
Age				0.002	1.00
≤ 65	107	52	55		
> 65	27	13	14		
AJCC TNM Stage				0.708	0.400
I + II	123	61	62		
III + IV	11	4	7		
Grade				12.633	0.001
Grade 1 + 2	90	34	56		
Grade 3 + 4	44	31	13		
Distant metastasis				1.335	0.248
M0	133	65	68		
M1	1	0	1		

Table 2
Associations of Siglec15 expression with clinicopathological factors in cohort B

	Number of patients	Siglec15		χ2	*P*
		High expression (%)	Low expression (%)		
Gender				6.254	0.012
Male	346	210(69.3)	136(58.9)		
Female	188	93(30.7)	95(41.1)		
Age				1.386	0.239
≤ 65	350	205(67.7)	145(62.8)		
> 65	184	98(32.3)	86(37.2)		
AJCC TNM Stage				1.203	0.273
I + II	328	180(59.4)	148(64.1)		
III + IV	206	123(40.6)	83(35.9)		
Grade				7.284	0.007
Grade 1 + 2	251	127(41.9)	124(53.7)		
Grade 3 + 4	283	176(58.1)	107(46.3)		
Distant metastasis				0.548	0.459
M0	453	254(83.8)	199(86.1)		
M1	81	49(16.2)	32(13.9)		

Table 3
Associations of Siglec15 expression with clinicopathological factors in cohort C

	Number of patients	Siglec15		χ2	*P*
		High expression (%)	Low expression (%)		
Gender				0.000	1.000
Male	25	13(81.3)	12(75.0)		
Female	7	3(18.8)	4(25.0)		
Age				0.205	0.651
≤ 65	26	12(75.0)	14(87.5)		
> 65	6	4(25.0)	2(12.5)		
AJCC TNM Stage				0.502	0.479
I + II	17	7(43.8)	10(62.5)		
III + IV	15	9(56.3)	6(37.5)		
Grade				0.000	1.000
Grade 1 + 2	23	12(75.0)	11(68.8)		
Grade 3 + 4	9	4(25.0)	5(31.3)		
Distant metastasis				0.000	1.000
M0	29	15(93.8)	14(87.5)		
M1	3	1(6.3)	2(12.5)		

3.2 | The role of Siglec-15 in ccRCC prognosis and clinical characteristics

As the Fig. 4 showed, over-expression of Siglec-15 is associated with higher Fuhrman grade in cohort A□C (n = 166, p = 0.001, OR = 3.132, 1.563–6.275), cohort B (n = 534, p = 0.008, OR = 1.606, 1.138–2.267). Translational over-expression of Siglec-15 is associated with higher Fuhrman grade. However, there were no significant correlations between Siglec-15 and age, tumor stage in ccRCC. Univariate Kaplan-Meir survival analysis showed that patients with high Siglec-15 expression had shorter survival periods without significance in cohort B (p = 0.073). Multivariate analysis employing the Siglec-15 regression model revealed that the AJCC stage and Fuhrman grades were the only significant independent prognostic indicators. Univariate Kaplan-Meir survival analysis (Fig. 5) showed that patients with high Siglec-15 expression had shorter survival periods without significance in cohort B (p = 0.073). Multivariate analysis employing the Siglec-15 regression model revealed that AJCC was the only significant independent prognostic indicator.

3.3 |Genes associated with Siglec-15 expression and functional enrichment analysis

There were 117 genes associated with the expression of Siglec-15 in ccRCC in TCGA cohorts ($p < 0.05$). We further enriched the functions of the 117 genes (Fig. 6). As the functional annotation analysis indicated, Siglec-15 may be related to the occurrence of renal cancer, abnormality of the erythrocyte sedimentation rate, EGFR tyrosine kinase inhibitor resistance, and cisplatin resistance.

3.4 |Siglec-15 is associated with tumor fibrosis

To further explore whether Siglec15 is related to tumor fibrosis, 32 ccRCC samples in cohort C were randomly selected, and SHG was used to measure the degree of tumor fibrosis and immunohistochemical staining of Siglec15. The relative degree of tumor fibrosis quantified by SHG/TPEF was separately 4.77 ± 5.13 and 1.52 ± 1.51 in LSG ($n = 16$) and HSG ($n = 16$) group. As Fig. 7 indicated, Siglec-15 protein was inversely associated with the tumor's fibrosis level ($p = 0.02$).

Discussion

Like PD-L1 in protein sequence, Siglec-15 is involved in osteoclast differentiation and is considered a potential therapeutic target for osteoporosis[6]. More recent studies indicated that Siglec-15 plays an essential role in inhibiting tumor immune response in tumor microenvironment[7, 8]. Previous studies showed Siglec-15 was not only expressed in the myeloid cell membrane, and the transcription level in kidney cancer cells also have greatly improved[7]. This study found that the Siglec-15 protein was frequently expressed in ccRCC, and compared with normal adjacent tissues, the Siglec-15 protein expression in tumor tissue was significantly raised, which indicated Siglec-15 as one potential immunotherapy target in ccRCC. This study also showed that the Siglec-15 expression level was positively associated with pathology grade in ccRCC. This study confirmed that the Siglec-15 expression level was not significantly correlated with the PD-L1 level ($p>0.05$), suggesting that it is a potential immunotherapy target independent of PD-L1 and likely to expand the immunotherapy benefit patient cohorts of ccRCC.

Compared with the adjacent normal tissues, Siglec-15 in tumor tissues were overexpressed at the protein level, but there was no significant difference at the mRNA level in the TCGA cohort ($p>0.05$), which may be because proteins can be regulated not only at the transcriptional level but also at the translation level and turnover levels, such as NFkB, EcR, and P53. However, further research is needed to confirm the regulation post-translationally of Siglec-15.

The changes in the morphology and quantity of collagen fibers contribute to the formation of tumor fibrotic microenvironment, stimulate tumor cell proliferation, change cell polarity, and promote tumor progression and metastasis [9]. It has been reported that intratumor fibrosis plays an essential role in tumor progression in ccRCC. Although the fibrogenic response in cancer is currently thought to be due in part to activated myofibroblasts or fibroblasts in the extracellular matrix production with tumor immunopolarization, the exact mechanism is not precise. For the first time, this study revealed a

significant correlation between the expression of Siglec15 on the ccRCC cell membrane and tumor fibrosis. The study indicated that Siglec15 also played a vital role in the tumor fibrosis and suppressed the tumor fibrosis in ccRCC. In breast cancer, intra-tumor fibrosis has been associated positively with larger tumor size, vascular invasion, and HER-2+ with poor prognosis and low lymphocyte infiltration[10]. Previous studies indicated that a high fibrosis level inside the tumor was associated with poor prognosis in ccRCC[4]. This study illuminated that tumor fibrosis in ccRCC may be the result of immunologic action. In the future, it is necessary to expand the sample size further to explore the relationship between Siglec-15 and false capsule invasion of renal cancer and tumor cell envelope invasion. The limitations of this study are as follows: 1. This study has no biological experiments performed on cells grown in vitro or animal experiments on the role of Siglec-15 in ccRCC. However, previous studies in vitro and in vivo have demonstrated genetic ablation or antibody blockade of Siglec-15 amplifies anti-tumor immunity and inhibits tumor growth in non–small cell lung cancer and brain tumors[11]. This study identified that Siglec-15 protein was frequently expressed in ccRCC, and Siglec-15 expression increases in tumors compared with adjacent normal tissues, emphasized the necessity of further investigation on Siglec-15 in ccRCC. 2. The sample size of IHC and SHG/TPEF is relatively small, but the IHC sample is randomly extracted, and the samples of IHC come from different centers, so the result is universal.

Conclusion

In conclusion, Siglec-15 protein was frequently expressed in ccRCC and Siglec-15 expression increases in tumors compared with adjacent normal tissues. Furthermore, Siglec-15 expression level was positively associated with pathology grade in ccRCC. These findings indicated a significant role of Siglec-15 in the prognosis and immunotherapy target of ccRCC. This study is the first to found an inverse correlation between Siglec-15 protein expression and the fibrosis level of the tumor, which provides an essential reference for the formation mechanism of pseudo-capsule and tumor fibrosis. In terms of protein level, Siglec-15 does not correlate with PD-L1 level and is an independent target, which may be of great significance for patients with the effectless PD-L1 targeted immunotherapy and the combination of drugs to increase the prognosis of ccRCC.

Abbreviations

ccRCC: clear cell renal cell carcinoma; TME: tumor microenvironment; TMA: tissue microarray; SHG/TPEF: second harmonic generation/two-photon excitation fluorescence; HEG: high-expression group; LEG: low-expression group.

Declarations

Ethics approval and consent to participate

The study was approved by the Institutional Ethics Committee Board of Peking University peoples' hospital. Consent to participate was deemed to be not required.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YWB, QCP and conceived and designed the study, YWB, DYQ, and HSC performed data curation. QCP and DYQ performed formal analysis. YWB and DYQ conducted methodology. XT performed supervision. YWB and QCP wrote the paper. DYQ, BWJ and XT reviewed and edited the manuscript. All authors read and approved the manuscript.

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Figures

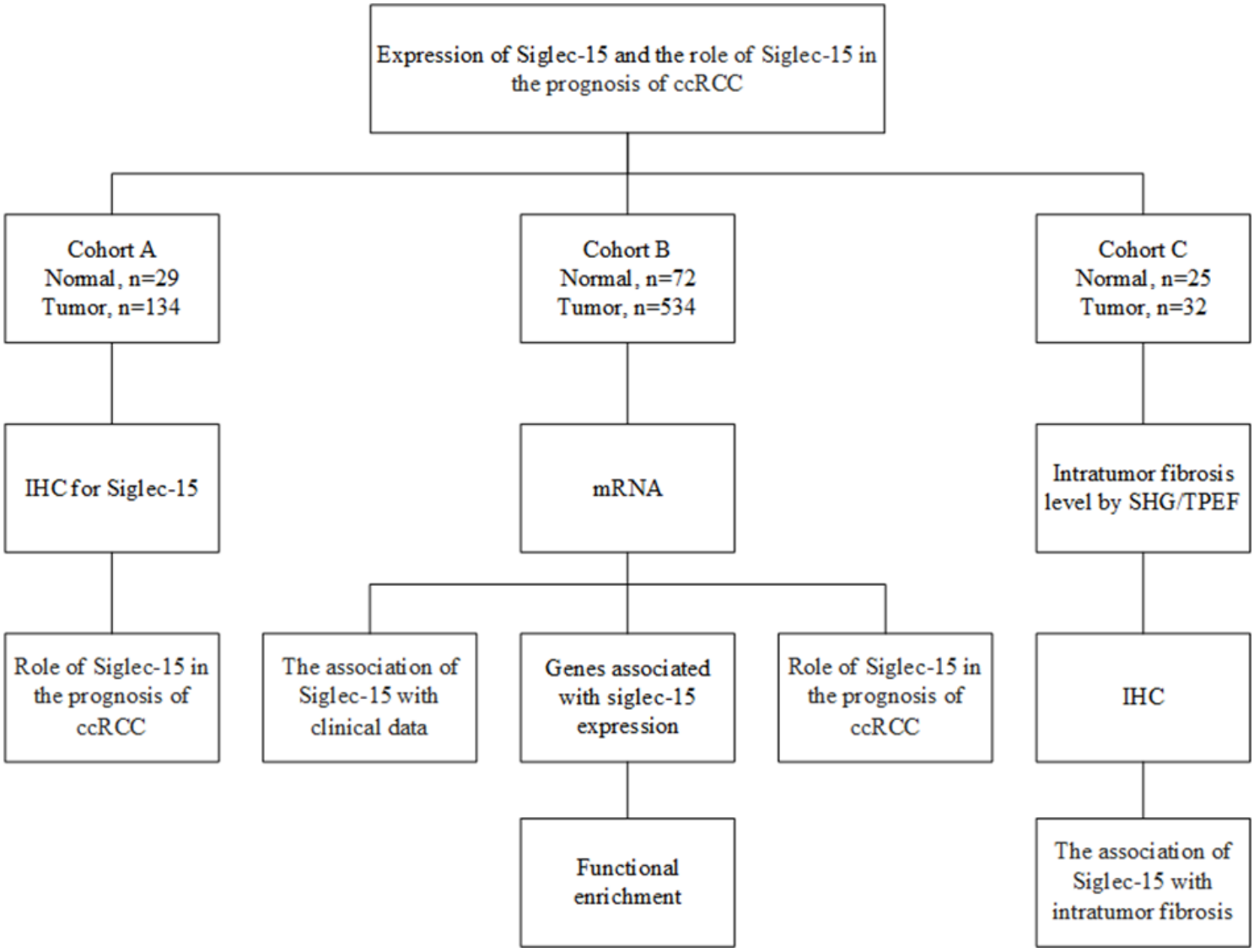


Figure 1

Flowchart of the study.

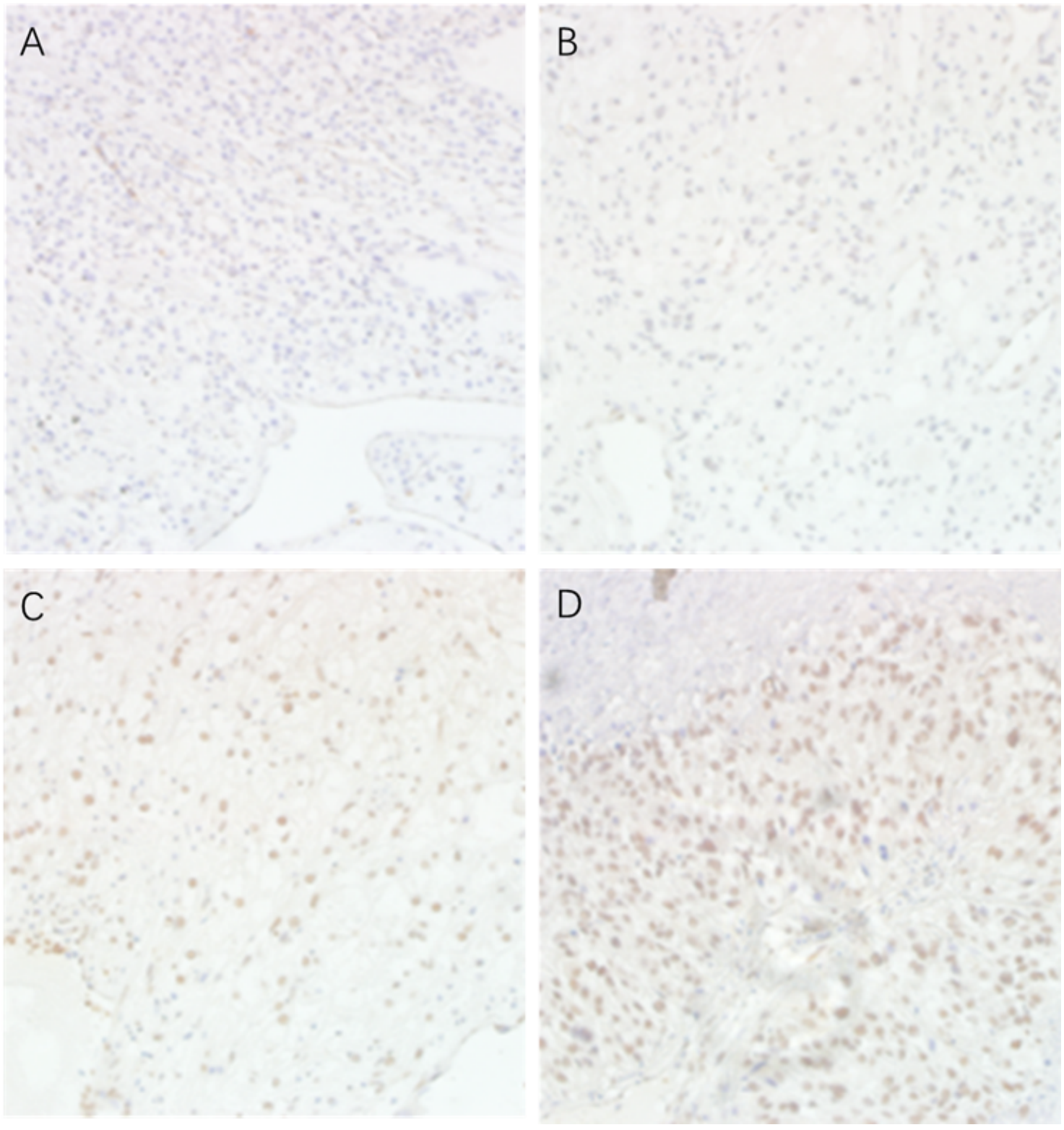


Figure 2

The expression of Siglec-15 in ccRCC. Siglec-15 was frequently expressed in ccRCC. Different patterns of immunohistochemical Siglec-15 expression in A) ccRCC (none cytomembrane positivity), B) ccRCC (weak cytomembrane positivity), C) ccRCC (moderate cytomembrane positivity), and D) ccRCC (marked cytomembrane expression).

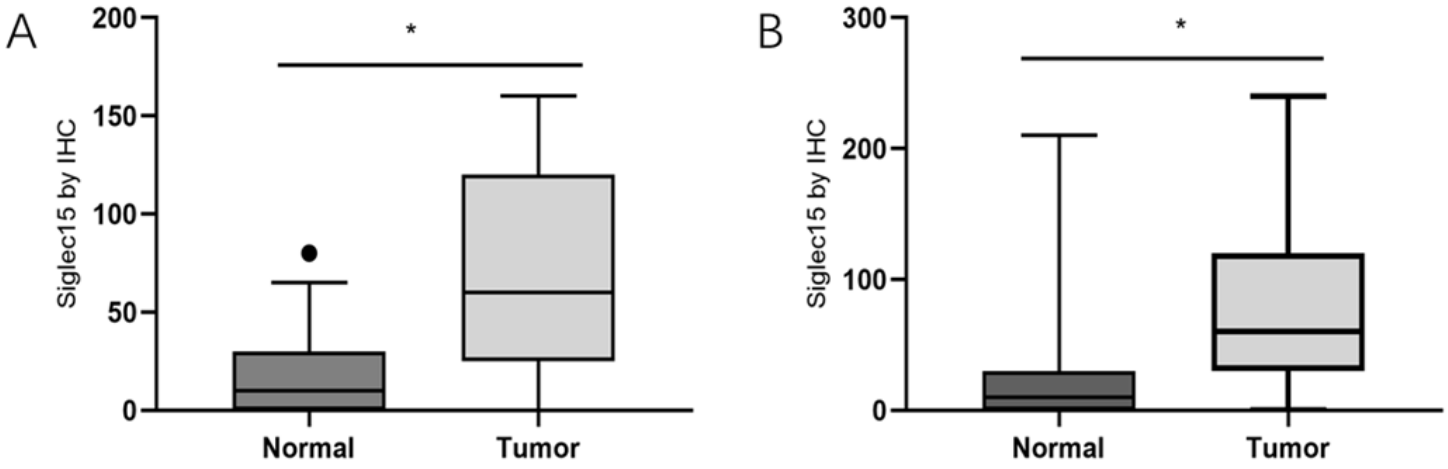


Figure 3

Different immunohistochemical Siglec-15 expression between tumor tissues and adjacent normal tissues in ccRCC. Siglec-15 was overexpressed in carcinoma compared with paired adjacent normal tissues in ccRCC of cohort A (n=29, p<0.001) in A) and of cohort C (n=25, p<0.001) in B).

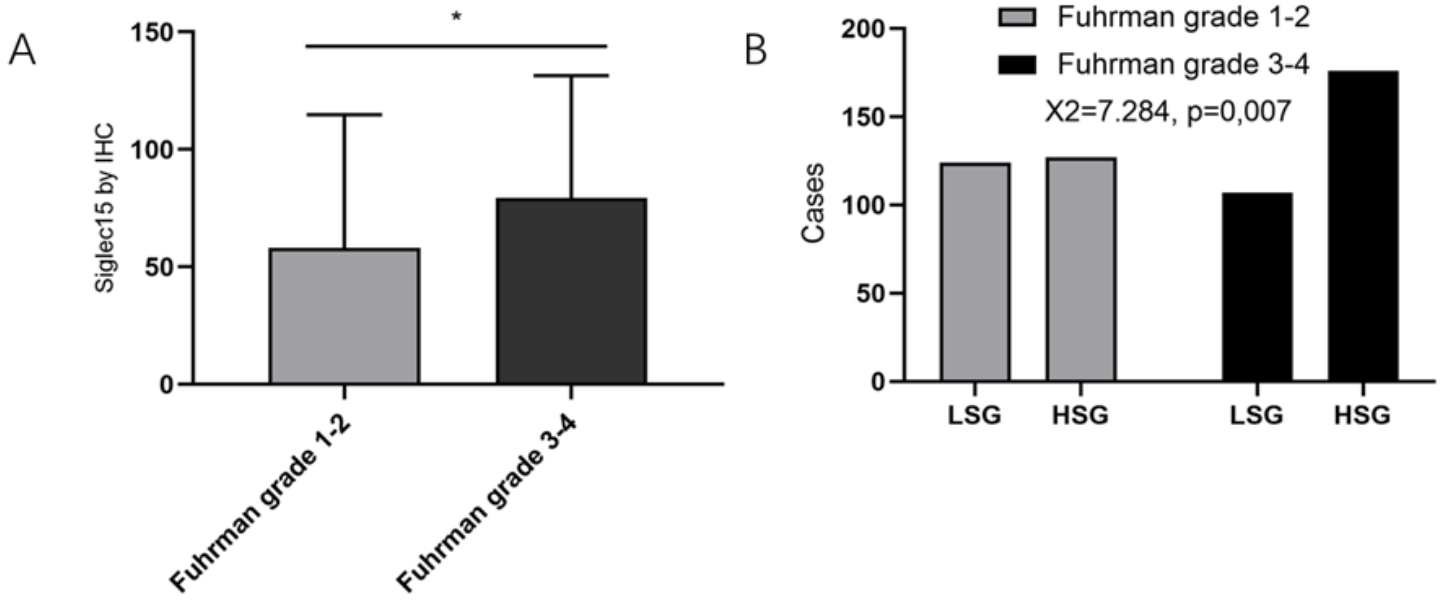


Figure 4

Over-expression of Siglec-15 is associated with higher Fuhrman grade in corhort A C (n=166, p=0.001, OR=3.132, 1.563-6.275) in A), corhort B (n=534, p=0.008, OR=1.606, 1.138-2.267) in B).

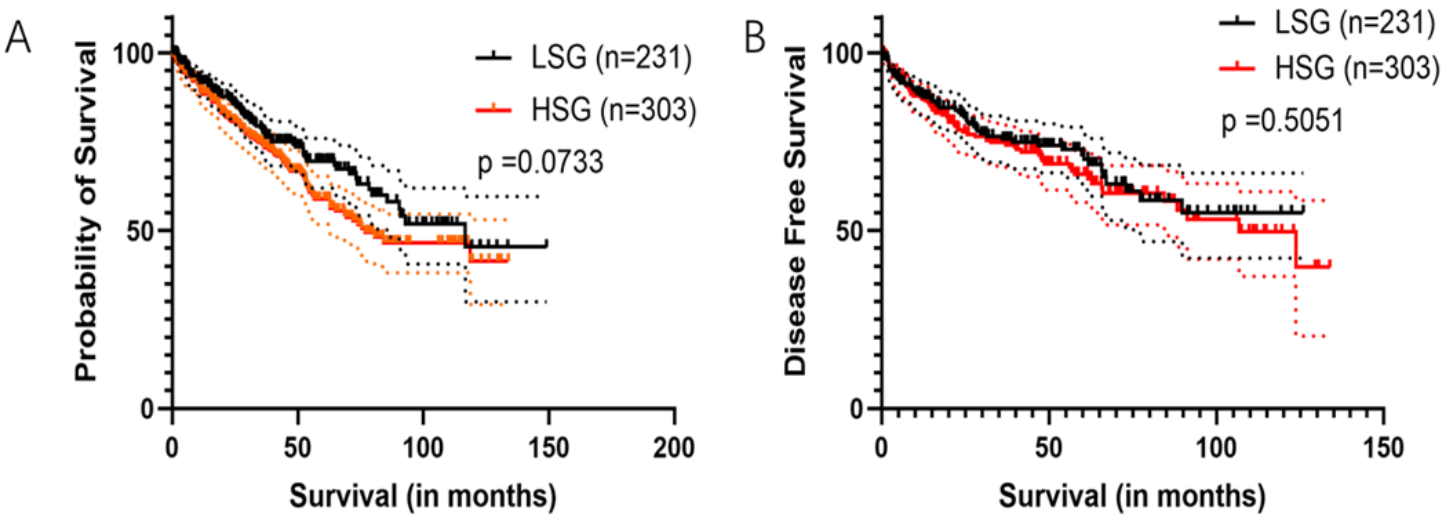


Figure 5

Kaplan-Meier curves for overall survival (A) and disease-free survival (B) functions for Siglec-15 expression in ccRCC.

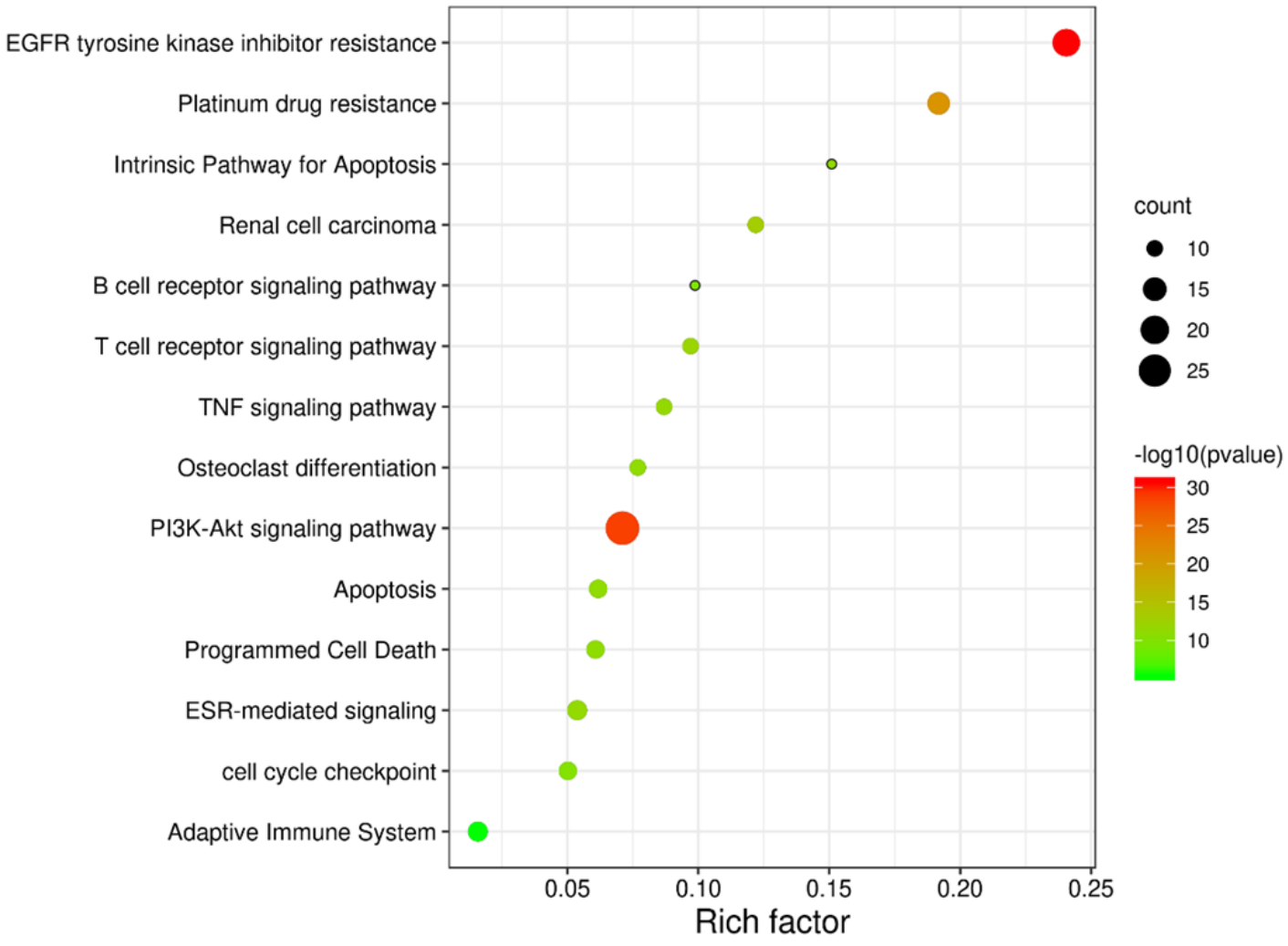
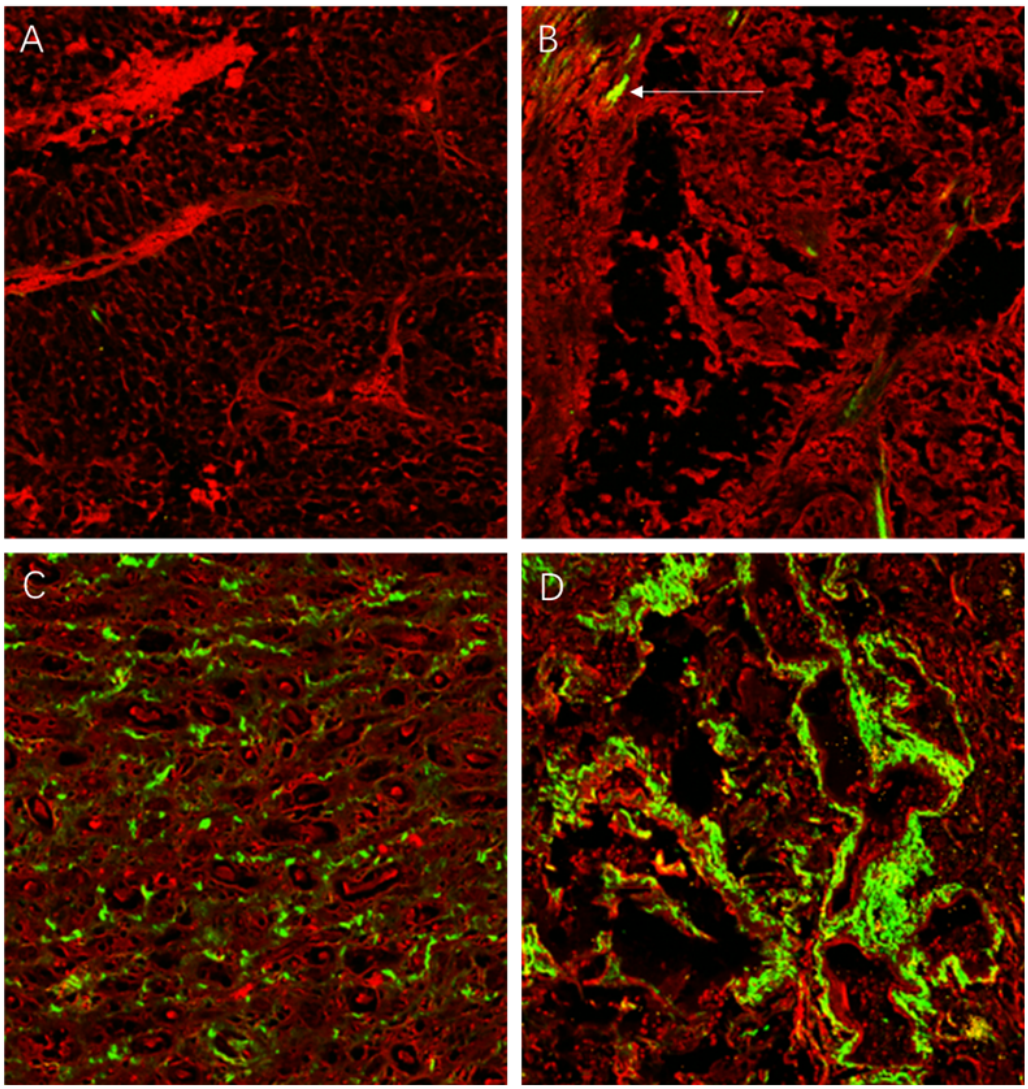


Figure 6

Functional annotation analysis. Functional enrichment was performed on 117 genes related to siglec15 expression. Siglec15 is related to the immune system and osteoclast differentiation and may be related to the occurrence of renal cancer, abnormal erythrocyte deposition rate, EGFR-targeted drug resistance, cisplatin resistance.



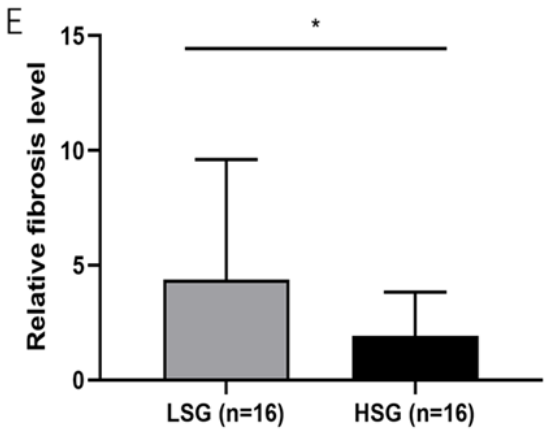


Figure 7

Different fibrosis grade of tumor quantified by SHG/TPEF in A) ccRCC (meager Siglec-15 expression in IHC score), B) ccRCC (slightly lower IHC score of Siglec-15 expression), C) ccRCC (moderate Siglec-15 expression in IHC score), and D) ccRCC (the highest IHC score of Siglec-15 expression). Arrows indicate collagen fibers (the green light in SHG/TPEF). E) Siglec-15 protein was inversely associated with the tumor's fibrosis level ($p<0.05$).