Original Article
Glycosylation of Siglec15 promotes immunoscape and tumor growth

Yun-Long Wang1,2*, Ming-Biao Wei1,2, Wan-Wen Zhao1, Li-Li Feng1, Xin-Ke Yin1, Shao-Mei Bai1,2, Xiang-Bo Wan1,2, Mien-Chie Hung4,5,6, Andrew Z Zou7, Michael H Wang8, Jian Zheng1,2, Caolitao Qin1,2, Xin-Juan Fan1,3

1Guangdong Provincial Key Laboratory of Colorectal and Pelvic Floor Diseases, Guangdong Institute of Gastroenterology, The Sixth Affiliated Hospital of Sun Yat-sen University, Guangzhou 510655, Guangdong, China; 2Department of Radiation Oncology, The Sixth Affiliated Hospital of Sun Yat-sen University, Guangzhou 510655, China; 3Department of Pathology, The Sixth Affiliated Hospital of Sun Yat-sen University, Guangzhou 510655, China; 4Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas 77030, USA; 5Graduate Institute of Biomedical Sciences and Research Centers for Cancer Biology and Molecular Medicine, China Medical University, Taichung 404, Taiwan; 6Department of Biotechnology, Asia University, Taichung 413, Taiwan; 7American International School of Guangzhou, Guangzhou 510663, China; 8Bloomington High School South, Bloomington, Indiana 47401, USA. *Equal contributors.

Received December 31, 2020; Accepted March 3, 2021; Epub May 15, 2021; Published May 30, 2021

Abstract: Siglec15 is a recently characterized immunosuppressive transmembrane protein, which expresses in various types of solid tumors and promotes cancer development. Several studies reported that Siglec15 is a prognostic biomarker of cancer patients, and targeting Siglec15 may be a promising strategy for cancer therapy. However, the regulation of Siglec15 function remains unclear. Here we show that the immunosuppression activity of Siglec15 is largely modulated by N-glycosylation. Through mass spectrum and site mutation analysis, we identified that Siglec15 was extensively glycosylated at N172 (N173 for mouse) in cancer cells. Meanwhile, Siglec15 N172Q had a similar molecular weight with PNGase-F-treated Siglec15, suggesting N172 as the only one glycosylation residue. In xenograft model, glycosylation deficiency of Siglec15 reduced tumor growth in C57BL/6 mice, but had no impact in nude mice, indicating the requirement of N-glycosylation for immunosuppressive function of Siglec15. Furthermore, colorectal cancer patients with high Siglec15 expression had a poor response to neoadjuvant chemo-radiotherapy and short survival time. Interestingly, removal of N-glycosylation enhances the detection of Siglec15, which may be employed in the prediction of immunotherapy response. Together, our results disclose a pivotal role of glycosylated Siglec15 in tumor immune escape, which may be a therapeutic target for cancer immunotherapy.

Keywords: N-glycosylation, Siglec15, immune escape, colorectal cancer, immunotherapy

Introduction

As a new milestone, immunotherapy has achieved a big success in cancer treatment and led to conceptual revolution in the management of many tumor types [1, 2]. As the most widely used immune checkpoint blockade therapy, anti-(programmed cell death) PD-1/PD-L1 therapy has shown encouraging outcome in many types of cancer, including blood, skin, lung, liver, bladder and kidney cancers [3-5]. However, the most challenging issue of this treatment is the low response rate in most cancer types [6-8]. In reports of the effects of PD-1 blockade in human tumors, less than 5.3% of all patients with colorectal cancer had a response to this treatment [9, 10]. Consistent with the low response rate of anti-PD-1/PD-L1 therapy, several other immune suppressive receptors/ligands have been characterized, including but not limited to CTLA-4, LAG3, TIM-3, TIGIT, BTLA, TIA-1 [11-13] and sialic acid-binding ig-like lectin 15 (Siglec15) [14], which are referred as immune checkpoints. High expression of these membrane proteins on tumor cells may be the reason of anti-PD-1/PD-L1 therapy failure [13, 15]. The discovery of regulatory mechanism of these immune checkpoints may provide new strategies for improving immunotherapy.
Glycosylation of Siglec15 promotes tumor immunoescape

Siglecs constitute a family of cell surface proteins playing important roles in the regulation of immune homeostasis [16]. The majority of Siglecs contain immunoreceptor tyrosine-based inhibitory motifs (ITIM) and/or ITIM-like domains in their intracellular domain, and recognize different kinds of sialic acids to distinguish self and non-self [17]. As the most conserved Siglecs in vertebrates, Siglec15 was found to be highly structural homologous with PD-L1, and the sequence of its extracellular domain showed 20%-30% identity to B7 family, suggesting a potential cancer immune regulatory function similar to B7 family [18]. Indeed, using a genome-scale T cell activity array, Lieping Chen’s lab identified Siglec15 as a critical immune suppressor [19]. Siglec15 was broadly upregulated on human cancer cells [20] and blocking Siglec15 via mAbs sufficiently suppressed tumor growth in mice [18]. Meanwhile, an anti-Siglec15 mAb, name NC318, is being evaluated in a phase I clinical trial and showing an encouraging result in preliminary data [21]. Despite the important function of Siglec15 in tumor immunosuppression, little is known about how it is regulated.

N-linked glycosylation (N-glycosylation) is one of the most abundant posttranslational modifications of membrane-bound proteins in eukaryotes [22, 23]. Evidences showing that N-glycosylation plays pivotal roles in the regulation of cancer immunity, especially PD-1/PD-L1 immune checkpoints [24, 25]. N-glycosylation in PD-L1 increases its protein stability and enhances the PD-1/PD-L1 interaction [26], which could be a biomarker for immunotherapy response [27, 28]. As an immune regulatory transmembrane protein that showed highly structural homologous with PD-L1, it remains unclear that whether Siglec15 was glycosylated. In this study, we identified that Siglec15 was N-glycosylated at N172, and it was required for the immunosuppressive function of Siglec15, which may be a potential target for improving cancer immunotherapy.

Materials and methods

Cell culture

HEK293T cells and U87 cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM, Hyclone) supplemented with 10% Fetal bovine serum (FBS, Gibco). MC38 cells and KM12 were cultured with Roswell Park Memorial Institute 1640 (RPMI 1640, Hyclone) supplemented with 10% FBS. All cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C.

Plasmid construction

For transient ectopic expression, the cDNA of Human or Mouse Siglec15 was cloned, fused with Flag tag at N-terminal and inserted into pCDNA3.0 vector. For stable expressing cell line construction, Mouse Siglec15 cDNA was subcloned into the pCDH-puro vector. N172Q (Human) or N173Q (Mouse) site mutation plasmids were constructed using a site-directed mutagenesis kit (Toyobo) as described by the manufacturer. Lentivirus was produced by HEK293T cells using the envelope plasmid pMD2. G and packing plasmid psPAX2. Cells were cultured in antibiotic-free DMEM for 15 hours and then infected with lentivirus in the presence of polybrene for 24 hours. The virus was removed and transfected cells were selected with 2 μg/ml puromycin for 14 days.

Cell transfection

For plasmid transfections, cells were placed on a 12-well plate one day before transfected with Lipofectamine 2000 Reagent (Invitrogen). Cells were cultured for additional 24 hours before analysis.

For siRNA transfections, cells were transfected with 25 μM siRNA duplex using Lipofectamine RNAiMAX (Invitrogen) and cultured for 48 hours before analysis. siRNAs were designed and synthesized by GenePharma. siSiglec15-1 (Sense: GCGACGUCCAGACGCUAdTdT; Antisense: UAGCGGCAUGGACGUCGAdTdT); siSiglec15-2 (Sense: CCGGCAUCGUAACACUAdTdT; Antisense: AGAUGUGACGACGCUAdTdT); siSiglec15-3 (Sense: GCAACAGCUUGGCAGCGUdTdT; Antisense: AGCGGUUGCAGCACCGGdTdT); siNC (Sense: UUCUCGGACGUCGUGGAdTdT; Antisense: UUGCUGACGUGAUGAdTdT). For siRNA transfections, cells were transfected with 25 μM siRNA duplex using Lipofectamine RNAiMAX (Invitrogen) and cultured for 48 hours before analysis. siRNAs were designed and synthesized by GenePharma.

Materials and methods

Cell culture

HEK293T cells and U87 cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM, Hyclone) supplemented with 10% Fetal bovine serum (FBS, Gibco). MC38 cells and KM12 were cultured with Roswell Park Memorial Institute 1640 (RPMI 1640, Hyclone) supplemented with 10% FBS. All cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C.

Plasmid construction

For transient ectopic expression, the cDNA of Human or Mouse Siglec15 was cloned, fused with Flag tag at N-terminal and inserted into pCDNA3.0 vector. For stable expressing cell line construction, Mouse Siglec15 cDNA was subcloned into the pCDH-puro vector. N172Q (Human) or N173Q (Mouse) site mutation plasmids were constructed using a site-directed mutagenesis kit (Toyobo) as described by the manufacturer. Lentivirus was produced by HEK293T cells using the envelope plasmid pMD2. G and packing plasmid psPAX2. Cells were cultured in antibiotic-free DMEM for 15 hours and then infected with lentivirus in the presence of polybrene for 24 hours. The virus was removed and transfected cells were selected with 2 μg/ml puromycin for 14 days.

Cell transfection

For plasmid transfections, cells were placed on a 12-well plate one day before transfected with Lipofectamine 2000 Reagent (Invitrogen). Cells were cultured for additional 24 hours before analysis.

For siRNA transfections, cells were transfected with 25 μM siRNA duplex using Lipofectamine RNAiMAX (Invitrogen) and cultured for 48 hours before analysis. siRNAs were designed and synthesized by GenePharma. siSiglec15-1 (Sense: GCGACGUCCAGACGCUAdTdT; Antisense: UAGCGGCAUGGACGUCGAdTdT); siSiglec15-2 (Sense: CCGGCAUCGUAACACUAdTdT; Antisense: AGAUGUGACGACGCUAdTdT); siSiglec15-3 (Sense: GCAACAGCUUGGCAGCGUdTdT; Antisense: AGCGGUUGCAGCACCGGdTdT); siNC (Sense: UUCUCGGACGUCGUGGAdTdT; Antisense: UUGCUGACGUGAUGAdTdT). For siRNA transfections, cells were transfected with 25 μM siRNA duplex using Lipofectamine RNAiMAX (Invitrogen) and cultured for 48 hours before analysis. siRNAs were designed and synthesized by GenePharma. siSiglec15-1 (Sense: GCGACGUCCAGACGCUAdTdT; Antisense: UAGCGGCAUGGACGUCGAdTdT); siSiglec15-2 (Sense: CCGGCAUCGUAACACUAdTdT; Antisense: AGAUGUGACGACGCUAdTdT); siSiglec15-3 (Sense: GCAACAGCUUGGCAGCGUdTdT; Antisense: AGCGGUUGCAGCACCGGdTdT); siNC (Sense: UUCUCGGACGUCGUGGAdTdT; Antisense: UUGCUGACGUGAUGAdTdT).
Glycosylation of Siglec15 promotes tumor immunoescape

For cell lysate, 10 μg of cell lysate was incubated with 1 μl Glycoprotein Denaturing Buffer (10 ×) and H2O (if necessary) to make a 10 μl total reaction volume. Denature glycoprotein by heating reaction at 100°C for 10 minutes. Then 2 μl GlycoBuffer 2 (10 ×), 2 μl 10% NP-40, 1 μl PNGase-F (PNG-F) and 6 μl H2O were added to the chilled system. Incubate the reaction at 37°C for 1 hour.

For colorectal cancer (CRC) tissue sections, formalin-fixed paraffin-embedded (FFPE) tissue sections were incubated at 58-65°C for 3 hours, deparaffinized with xylene and ethanol, and hydrated in distilled water. Antigen retrieval was performed with 10 mM citric acid (pH 6.0) in the microwave for 10 minutes (1000 W for 2 minutes and 200 W for 8 minutes) and cooled at room temperature for 60 minutes. After washing twice with 1 × PBS, tissue sections were incubated with 1 × Glycoprotein Denaturing Buffer at room temperature for 3 hours, washed with 1 × PBS four times, treated without or with PNG-F (5%) containing 1 × PBS at 37°C overnight (12-18 hours), and subjected to IHC staining.

Western blotting

Cellular proteins were extracted with RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate) supplemented with protease inhibitors (Roche). The protein expression level was analyzed by Western blotting using following antibodies: anti-Siglec15 antibody (1:1000, SAB3500654, Sigma), anti-α-Tubulin (1:5000, 10094-1-AP, Proteintech) and anti-GAPDH (1:12000, 60004-1-lg, Proteintech). α-Tubulin or GAPDH was used as internal control.

qRT-PCR

RNA was extracted using TRIZol reagent (Invitrogen). cDNA was synthesized using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO). qRT-PCR was performed with SYBR Green Realtime PCR Master Mix (TOYOBO) using the following PCR primers in LightCycler480 instrument (Roche): mSiglec15 (Forward: GCTTGCGATTCTGGGAGC; Reverse: TTCTCCACGCAAACACGTG); mGapdh (Forward: AAGACCCCTTATTGACCT; Reverse: ATGTAGTGCGGTCTGCCTG). mGapdh was used as internal control.

Animal studies

C57BL/6 and Nude mice were purchased from the animal center of the Sixth Affiliated Hospital, Sun Yat-sen University. The animal study protocol was approved by the Central Ethics Committee of The Sixth Affiliated Hospital, Sun Yat-sen University. In xenograft study, 2 × 10^6 MC38 cells suspended in 100 μl 1 × PBS was subcutaneously injected into the right or left posterior flank of 6-week-old male C57BL/6 or Nude mice. Tumor length and width were measured using calipers at indicated time, and tumor volume was calculated using the following formula: 0.5 × length × width^2.

Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded (FFPE) tissue sections were incubated at 4°C overnight and then at 58-65°C for 3 hours, deparaffinized with xylene and ethanol, and hydrated in distilled water. Antigen retrieval was performed with 10 mM citric acid (pH 6.0) in the microwave for 10 minutes (1000 W for 2 minutes and 200 W for 8 minutes) and cooled at room temperature for 60 minutes. Sections were then blocked with 3% H2O2 for 10 minutes at room temperature, washed with 1 × PBS for three times and incubated with primary Siglec15 antibody (SAB3500654, 1:100) overnight at 4°C. Then, the sections were incubated with a secondary biotinylated immunoglobulin G antibody. At last, after being washed three times with phosphate buffer saline, the sections were incubated with DAB solution for a proper time. Siglec15 expression level on tumor region were scored according to Siglec15 signal intensity. All tested patients were subgrouped into low (37 patients) or high (26 patients) Siglec15 expression group.

Extraction of exosomes

Serum from CRC patients were centrifuged at 12,000 × g for 30 minutes at 4°C, ultrafiltered with a 0.22 μm filter, and ultracentrifuged at 110,000 × g for 2 hours at 4°C. Extracellular vesicle pellet was washed in 1 × PBS and ultracentrifuged at 110,000 × g for 2 hours at 4°C. The pelletted exosomes were resuspended in 1 × PBS and kept at -80°C for a long-term storage.
Glycosylation of Siglec15 promotes tumor immunoescape

**Statistical analysis**

Overall survival was calculated using the Kaplan-Meier method with the log-rank test. The survival time of patients was calculated from the date of the diagnostic biopsy to the date of last follow-up or death. The associations between Siglec15 pression and Tumor Regression Grading (TRG) were evaluated with chi-square test. Statistical analysis was performed with the SPSS software package. All tests were two-sided except as indicated, and $P$ values of <0.05 were considered significant.

**Result**

**Siglec15 is extensively N-glycosylated on tumor cells**

The predicted molecular weight of Siglec15 is 35 kDa, which is slightly smaller than that detected in immunoblotting assay (about 37 kDa, Figure 1A). N-glycosylation plays an important role in determining protein structure and function, and often results in a heterogeneous pattern on protein immunoblotting. To examine whether Siglec15 was glycosylated in cells, cell lysates of U87 and KM12 were treated with PNG-F, an effective enzymatic method for removing almost all N-linked oligosaccharides from glycoproteins, and analyzed with immunoblotting. PNG-F treatment reduced the molecular weight of Siglec15 in U87 cells. Cells were treated with 1 μg/ml TM for 15 hours before Western blotting analysis. D. Western blotting analysis of ectopic expressed Flag-Siglec15 in HEK293T cells treated with PNG-F or TM. E. Flag-Siglec15-expressing HEK293T cells were treated with indicated dose of TM for 15 hours before Western blotting analysis.
Glycosylation of Siglec15 promotes tumor immunoescape

N173Q mutation reduced the molecular weight of mSiglec15, indicating that N173Q was glycosylated (Figure 3B). Meanwhile, similar to human Siglec15, N173Q mutation increased the protein level of mSiglec15 (Figure 3B), with no impact on the mRNA level (Figure 3C), suggesting that glycosylation increased the stability of mSiglec15 protein. Siglec15 was reported to enhance immune escape of tumor cells. To explore the impact of N-glycosylation on Siglec15 function, mSiglec15 WT or N173Q expressing MC38 cells, a mouse CRC cell line without endogenous mSiglec15 expression, were used to construct xenograft model. In agreement with the previous report [1], overexpression of mSiglec15 promoted the xenografts growth in C57BL/6 mice (Figure 3D), but had no impact on the xenograft growth in nude mice (Figure 3E), indicating an immunosuppressive role of mSiglec15. Interestingly, glycosylation-deficient mSiglec15 (N173Q)-expressing MC38 cells-derived xenografts grew much slower than that of mSiglec15 WT in C57BL/6 mice (Figure 3D), whereas in nude mice, there is no difference between two groups (Figure 3E). These results suggest that glycosylation at N173 is essential for the immunosuppressive function of mSiglec15, even though N-glycosylation reduced the stability of mSiglec15.

Siglec15 expression is correlated with the poor response of neoadjuvant chemo-radiotherapy and the short survival time of CRC patients

To analyze the association between Siglec15 expression and clinical characteristics of CRC patients, the Siglec15 protein level in CRC tissues was evaluated with IHC assay. CRC tissues were obtained from 63 CRC patients (44 males and 19 females, age ranges from 18 to 81) who received neoadjuvant chemo-radiotherapy before surgery. Positive staining of Siglec15 protein was mainly observed in the cytoplasm of cancer cells. The expression of Siglec15 was scored and tissues were divided to high or low Siglec15 expression group (Figure 4A). As shown in Figure 4B, high expression of Siglec15 was correlated with the poor response of neoadjuvant chemo-radiotherapy (P = 0.001). Consistently, CRC patients with high Siglec15 expression have a shorter survival time, including overall survival, local recurrence-free survival, distant metastasis-free survival.

The glycosylation site of Siglec15 is highly conserved between human and mouse (Figure 3A). N-glycosylation is essential for the immunosuppressive function of Siglec15

To determine the glycosylation site of Siglec15, ectopically expressed Flag-Siglec15 in HEK293T cells was purified by immunoprecipitation and subjected to tandem mass spectrometry (MS), which revealed that N172 may be the glycosylated residue of Siglec15 (Figure 2A). To verify the results of MS, a Siglec15 mutant, N172Q (replacing 172 asparagine with glutamine), was constructed and expressed in HEK293T cells (Figure 2B). Consistent with the MS results, N172Q mutant reduced the molecular weight of Siglec15 in immunoblotting (Figure 2B), indicating that N172 of Siglec15 was glycosylated. Moreover, Siglec15 N172Q had the same molecular weight with PNG-F treated Siglec15 (Figure 2C), suggesting N172 as the only glycosylated residue in Siglec15. As the protein level of Siglec15 N172Q is higher than wildtype (WT), we thought that glycosylation may regulate the stability of Siglec15. Interestingly, N172Q mutated Siglec15 was more stable than Siglec15 WT (Figure 2D), suggesting that glycosylation decreased the stability of Siglec15 protein. Furthermore, blocking of lysosomal pathway with inhibitor Bafilomycin A1 significantly upregulated Siglec15 in U87 cells, whereas diminishing ubiquitin-proteasome pathway via inhibitor MG132 has no impact (Figure 2E). Additionally, Bafilomycin A1 treatment increased the protein level of Siglec15-WT, but had not influence on Siglec15-N172Q expression (Figure 2F). These data suggested that glycosylation may accelerate lysosomal pathway-mediated Siglec15 degradation.

N-glycosylation is essential for the immunosuppressive function of Siglec15

Reduced the molecular weight of Siglec15 in U87 cells (Figure 1C). Furthermore, both PNG-F and TM treatment reduced the molecular weight of ectopically expressed Flag-Siglec15 in HEK293T cells (Figure 1D, 1E), a cell line that the endogenous Siglec15 was undetectable. These results suggest that Siglec15 is extensively N-glycosylated in tumor cells, and removal of N-glycosylation enhances its’ recognition by antibodies.

Siglec15 is glycosylated at N172 and destabilized by glycosylation

To analyze the association between Siglec15 expression and clinical characteristics of CRC patients, the Siglec15 protein level in CRC tissues was evaluated with IHC assay. CRC tissues were obtained from 63 CRC patients (44 males and 19 females, age ranges from 18 to 81) who received neoadjuvant chemo-radiotherapy before surgery. Positive staining of Siglec15 protein was mainly observed in the cytoplasm of cancer cells. The expression of Siglec15 was scored and tissues were divided to high or low Siglec15 expression group (Figure 4A). As shown in Figure 4B, high expression of Siglec15 was correlated with the poor response of neoadjuvant chemo-radiotherapy (P = 0.001). Consistently, CRC patients with high Siglec15 expression have a shorter survival time, including overall survival, local recurrence-free survival, distant metastasis-free survival.
Figure 2. Siglec15 is glycosylated at N172 and destabilized by glycosylation. (A) Tandem mass spectrometry shows that Siglec15 is glycosylated at N172. (B) N172Q mutation disrupted the glycosylation of Siglec15. Flag-Siglec15 WT or Flag-Siglec15 N172Q were transfected into HEK293T cells for 48 hours. (C) Flag-Siglec15 N172Q had a similar
Glycosylation of Siglec15 promotes tumor immune escape

molecular weight with PNG-F-treated Flag-Siglec15. (D) Siglec15 N172Q protein was more stable than Siglec15. HEK293 cells were transfected with Siglec15 WT or Siglec15 N172Q plasmid and treated with 20 mM CHX for indicated time. (E) BafA1 increased Siglec15 expression in U87 cells. (F) Glycosylation at N172 enhanced lysosomal pathway-mediated Siglec15 degradation. For (E, F), U87 or HEK293T cells were treated with BafA1 (50 ng/ml) or MG132 (10 ng/ml) for 12 hours before analysis.

survival and disease-free survival time (Figure 4C-F). Furthermore, Consistent with the result that removal of N-glycosylation increased Siglec15 signal in immunoblotting assay, we found this treatment enhanced Siglec15 IHC staining signal of rectum specimens (Figure 4G), which may be employed in immunotherapy response prediction like PD-L1 [27].

Taken together, our study reveals that Siglec15 is glycosylated at the residue of N172 (N173 in mouse), which is essential for its immunosuppressive function in tumor cells. For patients with locally advanced CRC, higher expression of Siglec15 indicates a poorer neoadjuvant chemo-radiotherapy response and shorter survival time.

Discussion

In this study, we characterized N-glycosylation as an essential regulator of Siglec15 immunosuppression activity. In agreement with our findings, N-glycosylation has been reported as an important regulator of immune response and tumor immune escape [29]. Actually, almost all of the key molecules involved in the innate and adaptive immune response are glycoproteins. For tumor immunosuppression, the well-known immune checkpoint ligand PD-L1 was found to be N-glycosylated at four asparagine residues, which enhances protein stability, promotes the
Glycosylation of Siglec15 promotes tumor immunoescape

binding between PD-L1 and PD-1 and increases its translocation onto membrane. Usually, glycosylation results in increased stability of protein due to its higher resistance to protease in cells, like immunoglobulin G [30], TCR subunits, MHC I and II complex [31] and FNDC5 [32]. However, we found that while Siglec15 glycosylation at N172 was required for its immunosuppression function, the glycosylated Siglec15 was less stable than non-glycosylated one. Previous study reported that the mechanism underlying glycosylation-induced protein destabilization may be ascribed to a conformational distortion of the protein caused by the interaction of the monosaccharide with the protein surface [33]. The authors thought it might be a feedback mechanism to prevent the over-suppression of immune response.

Siglec15 was upregulated across most types of cancer, such as breast invasive carcinoma, cholangiocarcinoma, esophageal carcinoma, glioblastoma multiforme, head and neck squamous cell carcinoma, etc., and Siglec15 expression varied significantly in different clinical stages of these cancers. However, high Siglec15 level may be associated with a favorable or unfavorable outcome of patients depending on different cancer type [20]. In our study, we found that CRC patients with high Siglec15 expression trend to have a bad OS and a higher risk of tumor recurrence and metastasis. Interestingly, we found that high Siglec15 level was correlated with a poor response to neoadjuvant chemo-radiotherapy for CRC patients, and this result was similar with the analysis on PD-L1, suggesting that chemo-radiotherapy-activated anti-tumor immune response had a great contribution to

Figure 4. Siglec15 expression is correlated with the poor response of neoadjuvant chemo-radiotherapy and short survival time of CRC patients. Scale bar, 20 μm. A. Immunohistochemical staining of Siglec15 on CRC specimens. B. High level of Siglec15 was correlated with high TRG of CRC patients. C. Kaplan-Meier curve analysis of overall survival for CRC patients according to Siglec15 expression. D. Kaplan-Meier curve analysis of local recurrence-free survival for CRC patients according to Siglec15 expression. E. Kaplan-Meier curve analysis of distant metastasis-free survival for CRC patients according to Siglec15 expression. F. Kaplan-Meier curve analysis of disease-free survival for CRC patients according to Siglec15 expression. G. Removal of N-glycosylation with PNG-F increased Siglec15 IHC staining signal in rectum specimens. Scale bar, 100 μm.
Glycosylation of Siglec15 promotes tumor immunoecape

In our manuscript, we further verified the importance of glycosylation for the function of Siglec15 with xenograft model, which showed that glycosylation at N172 residue was required for the immunosuppressive function of Siglec15. Additionally, we analyzed the correlation between the expression of Siglec15 and prognosis of CRC patients; high level of Siglec15 was correlated with a bad response to neo-adjuvant chemo-radiotherapy and a short survival time of CRC patient. It has been well-characterized that in addition to the direct toxicity to cancer cells, chemo-radiotherapy could stimulate the anti-tumor immune response by triggering the release of pro-inflammatory mediator and neo-antigen of tumor, to further kill cancer cells [37, 38]. Furthermore, chemo- or radio-therapy combining immune-checkpoint inhibitors has shown a great improvement in the treatment of several types of cancer [39, 40]. This promotes us to investigate whether chemo- or radio-therapy influenced the level of Siglec15. Interestingly, although radiation or 5-FU treatment has no impact on the expression of Siglec15 in tumor cells (Figure S1A, S1B), the examination of exosomes extracted from the serum of neo-adjuvant chemo-radiotherapy-treated CRC patients showed that Siglec15 existed in exosomes and exosomal Siglec15 was increased by chemo-radiotherapy (Figure S1C). Several studies have reported that chemo- or radio-therapy increases exosomal PD-L1 level in the serum of tumor patients, which prevents tumor cells from the attack of T cells. Similar to exosomal PD-L1 [41], exosomal Siglec15 may contribute to tumor immune escape and chemo- or radiotherapy induced exosomal Siglec15 may increase the survival of tumor cells. Moreover, the influence of cytokines in tumor microenvironment, including IFN-γ, TGF-β, IGF-1, EGF, and HGF were investigated, and only TGF-β and HGF negatively regulated Siglec15 expression (Figure S1D), suggesting that these cytokines in tumor microenvironment were not the drivers of high Siglec15 expression in tumor cells. Radiation enteritis is a most frequent and inde-terminism complication in pelvic floor radiation therapy, 5%-20% of CRC patients suffers a recurrent intestinal injury [42]. Interestingly, we found that Siglec15-positive immune cells in the radiation enteritis tissues were significantly enriched than paired-normal colon tissues (Figure S2), indicating that Siglec15 may play an important role in the development of radiation enteritis.

Taken together, our results identified that Siglec15 was N-glycosylated at N172 in tumor cells. N-glycosylation is required for the Siglec15 function in immunosuppression, but reduced its protein stability. High expression of Siglec15 was correlated with a short survival time of CRC patients. These results suggest a pivotal role of N-glycosylated Siglec15, which may be a promising target for cancer immunotherapy.

Acknowledgements

This work was funded by the Natural Science Foundation of China (No. 81872188 to X.B. Wan, No. 81903152 to Y.L. Wang), Guangdong Science and Technology Project (No. 61123-1078086 to X.B. Wan, No. 2019B030316003, 2019A1515010901 to X.J. Fan and No. 2020-A1515010314 to Y.L. Wang), Fundamental Research Funds for the Central Universities (No. 20ykpy11 to Y.L. Wang) and China Postdoctoral Science Foundation (No. 2019M66-3301 to Y.L. Wang).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xin-Juan Fan and Caolitao Qin, Guangdong Provincial Key Laboratory of Colorectal and Pelvic Floor Disease, The Sixth Affiliated Hospital of Sun Yat-sen University, #26 Yuancun Erheng Road, Guangzhou 510655, Guangdong, China. E-mail: fanxjuan@mail.sysu.edu.cn (XJF); qincit@mail.sysu.edu.cn (CQ); Dr. Jian Zheng, Department of Radiation Oncology, The Sixth
Glycosylation of Siglec15 promotes tumor immunoescape

Affiliated Hospital, Sun Yat-sen University, 26 Yuancun Erheng Road, Guangzhou 510065, China. E-mail: zhengj48@mail.sysu.edu.cn

References


Glycosylation of Siglec15 promotes tumor immunoescape


Glycosylation of Siglec15 promotes tumor immunoescape


Glycosylation of Siglec15 promotes tumor immunoescape

Figure S1. A, B. Radiation or 5-FU had no influence on Siglec15 expression. Cells were treated with 10 Gy X-ray or 20 μg/ml 5-FU for indicated time. C. Neoadjuvant Chemo-radiotherapy increased exosomal Siglec15 in the serum of CRC patients. The serum was collected from CRC patient at different time among neoadjuvant Chemo-radiotherapy progress. Exosomes extracted from the same volume of serum were analysis. D. U87 cells treated with IFN-γ (100 ng/mL), TGF-β (100 ng/ml), IGF-1 (100 ng/mL), EGF (100 ng/ml) and HGF (100 ng/ml) in serum free medium for 24 hours before Western blotting analysis.

Figure S2. Siglec15 protein of radiation enteritis and paired normal tissues were analysed with IHC staining. The Siglec15-positive monocytes in submucosa were counted under a microscope (200 ×). ***, P<0.001.