High Siglec-15 Expression in Triple-negative Breast Cancer Predicts Poor Prognosis

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Research Article

Keywords: Breast cancer, Sialic acid binding Ig-like lectin 15 (Siglec-15), Gene expression, M1 macrophages, Dendritic cells, Cancer survival

Posted Date: December 13th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1018792/v2>

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Abstract

Introduction:

Normalization cancer immunotherapy is a new strategy to treat breast cancer. Sialic acid binding Ig-like lectin 15 (Siglec-15) is a new potential target for normalization cancer immunotherapy. In this study, we evaluated the role of Siglec-15 in breast cancer and investigated the influence of Siglec-15 on the microenvironment of infiltrating immune cells in cancer.

Methods

We performed immunohistochemical staining to analyse Siglec-15 expression in primary invasive breast cancer tissue microarrays. The tissue specimens were from 90 patients. Furthermore, the relationship between Siglec-15 and clinicopathological features was analysed with logistic regression and the Wilcoxon signed-rank test. The association between clinical characteristics and overall survival in The Cancer Genome Atlas (TCGA) and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) patients was assessed.

Results

Immunohistochemical staining of tissue microarrays showed that Siglec-15 had higher expression in breast cancer tissues than in adjacent normal tissues. Breast cancer tissues had higher Siglec-15 expression than normal tissues did. Kaplan–Meier survival analysis suggested that triple-negative breast cancer with high Siglec-15 expression had poorer survival than those with lower Siglec-15 expression ($p = 0.042$). Furthermore, the high Siglec-15 expression group had low activated dendritic cells, follicular helper T cells, and M1 macrophages.

Conclusions

Siglec-15 had a high expression in breast cancer tissues. High Siglec-15 expression is associated with low activated dendritic cell, follicular helper T cell, and M1 macrophage proportions in breast cancer tissue and predicts poor prognosis in triple-negative breast cancer. Siglec-15 expression may be a potential prognostic molecular marker of poor survival in breast cancer.

Introduction

Breast cancer is one of the most common cancers in women. [1–3] Women who have localized stage cancer have the best prognosis, with a 5-year relative survival rate of 99%. However, patients with distant stage cancers have a much worse prognosis, with a 5-year relative survival rate of only 27%. [3] The main factors that influence the risk of breast cancer include ageing, pregnancy history, overweight, lack of

exercise, genetics, and cigarette smoking. [4] Triple-negative breast cancer (TNBC) has increased aggressiveness, higher histologic grade, and higher risks of local recurrence and visceral metastasis.[5]

Normalization cancer immunotherapy is a new strategy to treat cancer that can normalize compromised immunity without disproportionate side effects. In recent years, immunotherapy has attracted great interest with its promise to treat breast cancer. The programmed death ligand 1 (PD-L1) checkpoint protein helps keep immune cells from attacking normal cells. Blocking PD-L1 can inactivate T cells through both programmed death 1 (PD-1) and B7 and can prevent cancer cells from being killed by CD8 + T cells. The Food and Drug Administration has approved atezolizumab to treat PD-L1-positive unresectable locally advanced or metastatic triple-negative breast cancer (TNBC).[6] Furthermore, some clinical trials have been performed to investigate other medicines that target immune pathways in breast cancer. Tremelimumab, a cytotoxic T lymphocyte antigen-4 antibody, has been used to treat metastatic hormone-responsive breast cancer.[7] Lymphocyte activation gene-3 antibody has been used to treat metastatic breast cancer.[8] Pembrolizumab, an anti-PD-1 antibody, has been used to treat metastatic triple-negative breast cancer.[9] However, only 20–30% of solid tumour patients respond to anti-PD-1/PD-L1 treatment[10, 11]. It is necessary to find new checkpoint targets to restore defective immune responses in the tumour microenvironment as a principle for normalizing cancer immunotherapy.

Sialic acid binding Ig-like lectin 15 (Siglec-15) is a new potential target for normalization cancer immunotherapy. It is an immunoreceptor that was originally categorized into the immunoglobulin superfamily and the Siglec family. A recent study found that Siglec-15 can suppress CD8 + T cell responses. Suppression of Siglec-15 inhibits tumour growth in animal models.[12] However, the expression of Siglec-15 and its effects on breast cancer have not been well investigated.

The aim of the current study was to evaluate the prognostic value of Siglec-15 expression in human breast cancer. First, we performed immunohistochemical staining to analyse Siglec-15 expression in primary invasive breast cancer tissue microarrays. Furthermore, we used the data obtained from The Cancer Genome Atlas (TCGA) and the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) to investigate the association of Siglec-15 expression and breast cancer. Moreover, we illustrated the atlas of the influence of Siglec-15 on the immune cell microenvironment of breast cancer with the CIBERSORT algorithm[13], which is a method to estimate the immune cell composition in a mixed cell population from cell gene expression profiles.

Materials And Methods

Tissue microarrays

The study received approval from the Institutional Review Board (approval document number: 050432-4-1911D, Supplementary Fig. 2). Informed consent was obtained from all subjects in the study. The tissue microarray (TMA) chips were obtained from Shanghai Zhuoli Biotechnology Company Ltd. Breast cancer tissues and matched adjacent tissues were obtained from 90 patients. Representative tumour areas were

carefully selected. Formalin-fixed, paraffin-embedded core cylinders were punched and deposited into a recipient paraffin block. Ninety pairs (180 points) of 4- μ m-thick sections were cut and placed on charged poly-L-lysine-coated slides using a tissue-arraying instrument (Beecher Instruments®, Silver Spring, MD, USA).

Immunohistochemistry

Immunohistochemistry analyses were performed as we previously described.[14] In short, the tissue microarray was incubated overnight in a 59°C oven for 60 minutes, dewaxed in xylene 3 times for 10 minutes each, and dehydrated in a series of graded alcohols. To exhaust endogenous peroxidase activity, the chips were treated with 3% hydrogen peroxide and NaN_3 for 10 min. To unmask the epitopes, the chips were microwaved in 10 mM citrate buffer pH 6.0 for 1 minute. Rabbit polyclonal anti-human Siglec-15 antibody (Abcam, # ab198684), the primary antibody, was diluted 1:50 (Supplementary Fig. 1) in phosphate-buffered saline (PBS). The chips were incubated with diluted primary antibody overnight at 4°C. After washing with PBS, the chips were incubated with anti-rabbit IgG secondary antibody at 37°C for 35 minutes. Horseradish peroxidase (HRP) conjugate was then applied to the chips. After washing, the chips were incubated with the peroxidase substrate diaminobenzidine for 2 min and counterstained with haematoxylin.

Evaluation Of Immunohistochemical Staining

The immunohistochemical staining scoring of Siglec-15 expression was defined as follows: 0, no staining in cells; 1+, weak and incomplete cytoplasmic staining in cells; 2+, an intermediate between 1 + and 3 + in cells; and 3+, diffuse cytoplasmic staining in more than 80% of cells. The percentage of positively stained cells (0–100%) was also calculated. Normal ducts and lobules were evaluated in adjacent normal tissue.

Data Acquisition And Bioinformatics Analysis

The clinical information and gene expression data of breast cancer patients and normal controls were downloaded from the official TCGA and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) websites. The control samples were taken from breast cancer patients' normal tissues near the tumours. Boxplots were used to visualize expression differences for discrete variables. The RNA-Seq gene expression level 3 HTSeq count data of breast cancer patients with breast invasive carcinoma (BRCA) and clinical data were retained and further analysed.

The gene expression profiles of normal controls and breast cancer patients were downloaded from the official TCGA and METABRIC websites in September 2021. The expression profiles of genes in breast invasive carcinoma (BRCA) were analysed. Clinical characteristics from the corresponding patients were downloaded.

Evaluation Of Tumour-infiltrating Immune Cells (Tiic)

We used the CIBERSORT algorithm (<http://cibersort.stanford.edu/>) to calculate the tumour-infiltrating immune cell composition. [15] TIIC immune cells include B cells, plasma cells, T cells, NK cells, macrophages, monocytes, mast cells, dendritic cells, neutrophils and eosinophils. Perl (The Perl Programming Language, version 5.28.1, <http://www.perl.org>) was used to convert IDs and group samples. The limma package of R (The R Project for Statistical Computing, version 3.5.3, <https://www.r-project.org>) was used to normalize the gene expression data. TIIC, P value, root mean square error and Pearson's correlation coefficient were quantified for each sample. Furthermore, we applied CIBERSORT to calculate the P value for the deconvolution.

Statistical analysis

All statistical analyses were accomplished using R software (version 3.6.0, R Foundation). The expression of Siglec-15 in the patient dataset was evaluated. The chi-square test and Fisher's exact test were applied to identify correlations between Siglec-15 mRNA expression and the clinical features of breast cancer. The relationship between clinicopathological features and Siglec-15 expression was analysed with the Wilcoxon signed-rank test and logistic regression. The associations of clinical and pathological characteristics with overall survival in patients were assessed using Cox regression and the Kaplan–Meier method. The cut-off value of Siglec-15 expression was determined by its median value. The correlations between TNM stage and immune cells were measured by the Wilcoxon test. The associations between clinical follow-ups, immune cells and survival curves were evaluated by the log-rank test and Kaplan–Meier analysis. Student's t test was performed to examine the statistical relevance between two groups. P values < 0.05 were considered statistically significant.

Results

Microarray immunohistochemistry showed that breast cancer tissue had high Siglec-15 protein expression

The expression level and positive rates of Siglec-15 were compared between breast cancer tissue and adjacent normal tissue samples by immunohistochemistry on a microarray. The samples in odd columns are from breast cancer tissues (Fig. 1A, E). The samples in even columns are from adjacent normal tissues (Fig. 1A, E). Figure 1B, C and D shows a classic pair of samples. Breast cancer tissue had higher positive staining (Fig. 1C) than adjacent tissue (Fig. 1D). Breast cancer tissues had a higher Siglec-15-positive staining rate ($70.79 \pm 6.95\%$) than adjacent normal tissues ($46.78 \pm 7.05\%$, $p < 0.001$, Fig. 1F).

Databases Showed That Breast Cancer Tissue Had High Siglec-15 Mrna Expression

From the Cancer Genome Atlas database, we obtained 1,091 breast cancer samples and 113 control samples. In the breast cancer samples, 844 cases contained both clinical and Siglec-15 gene expression data. The detailed clinical characteristics are listed in Table 1.

Table 1
Clinical characteristics of breast cancer patients who had Siglec-15 and tumor-infiltrating immune cells data

Clinical Characters		Case, N	%
Tumor size (TNM-T)	T1	215	25.47
	T2	493	58.41
	T2	106	12.56
	T4	28	3.32
	NA	2	0.24
(TNM-N)	N0	395	46.80
	N1	297	35.19
	N2	98	11.61
	N3	64	7.58
	NA	11	1.30
(TNM-M)	M0	709	84.00
	M1	16	1.90
	NA	119	14.10
Stage	I	137	16.23
	II	475	56.28
	III	199	23.58
	IV	15	1.78
	NA	16	1.90
Total		844	100.00
NA: not available; Siglec-15: Sialic acid binding Ig-like lectin 15			

We compared Siglec-15 expression in breast cancer and normal tissues. The results indicated that Siglec-15 expression was higher in breast cancer tissues than in normal controls (Fig. 2A, 2.10 vs. 1.34. $p = 0.000326$).

TNM-T1 had higher expression of Siglec-15 than the control groups (Fig. 2B, $p = 0.002$). However, no significant difference was found between them (Normal vs. T2: $p = 0.262$, Normal vs. T3: $p = 0.150$, Normal vs. T4: $p = 0.289$, Table 2). Moreover, TNM-N0 and N1 had higher expression of Siglec-15 than the control groups (Fig. 2C). TNM-M0 had higher expression of Siglec-15 than the control groups ($p = 0.062$, Fig. 2D). Furthermore, stage I had higher expression of Siglec-15 than the control groups ($p = 0.005$, Fig. 2E). However, the expression of Siglec-15 did not differ among other TNM-Ns, TNM-Ms and stages (Table 2).

Table 2. Siglec15 mRNA expression in the Cancer Genome Atlas database

		Mean	Standard Error	p valule
Normal		1.34	0.16	
	T			
	T1	2.91	0.35	Normal vs. T1: $p=0.002$
	T2	1.69	0.14	Normal vs. T2: $p=0.262$
	T3	1.95	0.40	Normal vs. T3: $p=0.150$
N	T4	1.81	0.58	Normal vs. T4: $p=0.289$
	N0	2.08	0.19	Normal vs. N0: $p=0.049$
	N1	2.15	0.23	Normal vs. N1: $p=0.035$
	N2	1.66	0.48	Normal vs. N2: $p=0.508$
	N3	2.03	0.37	Normal vs. N3: $p=0.050$
M	M0	2.03	0.14	Normal vs. M0: $p=0.062$
	M1	1.68	0.69	Normal vs. M1: $p=0.501$
Stage	I	2.75	0.43	Normal vs. SI: $p=0.005$
	II	1.83	0.15	Normal vs. SII: $p=0.122$
	III	1.98	0.31	Normal vs. SIII: $p=0.127$
	IV	1.29	0.60	Normal vs. SIV: $p=0.914$

Tnbc Patients With High Siglec-15 Expression Had Poorer Survival

Kaplan–Meier survival analysis suggested that triple-negative breast cancer patients with high Siglec-15 expression had a poorer prognosis than those with low Siglec-15 expression ($p = 0.042$, Fig. 3A). However, an interesting finding is that low Siglec-15 expression had a poorer prognosis in luminal A subtypes ($p = 0.029$, Fig. 3B). Siglec-15 expression did not influence survival in the luminal B ($p = 0.51$, Fig. 3C) and HER2+ ($p = 0.71$, Fig. 3D) subtypes. The Kaplan–Meier Plotter database (<https://kmplot.com>) was used to validate the survival analysis and calculate the hazard ratio. The findings confirmed that triple-negative

breast cancer patients with high Siglec-15 expression had a poorer prognosis than those with low Siglec-15 expression (HR = 1.59, 95% confidence interval 1.11–2.29. $p = 0.011$, Supplementary Fig. 3).

Composition Of Tumour-infiltrating Immune Cells

We performed CIBERSORT to investigate the fractions of infiltrated immune cells between high Siglec-15 breast tumour tissue, low Siglec-15 breast tumour tissue and adjacent nontumour tissues. A bar plot was drawn to visualize the proportions of infiltrating immune cells (Fig. 4A).

The breast cancer tissues with high Siglec-15 expression had low fractions of activated dendritic cells ($p < 0.001$), follicular helper T cells ($p < 0.001$), activated memory CD4 T cells ($p < 0.001$) and M1 macrophages ($p = 0.008$, Fig. 4B, C).

The breast cancer tissues with high Siglec-15 expression had high fractions of resting memory CD4 T cells ($p < 0.001$), resting dendritic cells ($p < 0.001$), and resting mast cells ($p < 0.001$, Fig. 4B, C).

Discussion

In this study, our microarray immunohistochemistry showed that breast cancer tissue had high Siglec-15 protein expression. TCGA also showed that breast cancer had high Siglec-15 mRNA expression. High Siglec-15 expression was associated with poor prognosis in triple-negative breast cancer. Furthermore, breast cancer tissues with high Siglec-15 expression had low fractions of activated dendritic cells, follicular helper T cells, activated memory CD4 T cells and M1 macrophages.

Tumour immune escape involves tumour expression of inhibitory immune checkpoints such as programmed cell death ligand 1 (PD-L1).[16] However, anti-PD-1/PD-L1 treatment is only feasible for some cancer patients. [17] Therefore, it is necessary to identify other immune checkpoints.

Siglecs, members of the immunoglobulin gene superfamily, are cell-surface proteins that bind sialic acid. Recent studies utilizing high-throughput screening identified that Siglec-15 has therapeutic potency in human cancers.[12] Siglec-15 antibody improves the response rate to cancer immunotherapies, especially those refractory to PD-1 blockade.[12]

In the present study, we used a tissue microarray to study the protein level of Siglec-15, while public databases were used to provide data on the mRNA expression of Siglec-15. We found that Siglec-15 had both high protein and mRNA expression levels in breast cancer tissues. Siglec-15 is broadly upregulated in cancer cells and tumour-infiltrating myeloid cells.[18] Li et al. reported that breast invasive carcinoma had higher Siglec-15 mRNA expression in the UALCAN database.[19] However, no previous studies have investigated the protein expression of Siglec-15 in breast cancer patients. Our microarray immunohistochemistry confirmed that breast cancer tissue had higher Siglec-15 protein levels than controls.

High Siglec-15 expression was associated with poor prognosis in triple-negative breast cancer in this study. This may partially explain the poor prognosis of triple-negative breast cancer. [20] However, Li et al. reported that a higher level of Siglec-15 expression in breast cancer indicates better overall survival.[19] This may be because different subtypes have different immune environments. [21] Siglec-15 is an immune suppressor, and high expression of Siglec-15 leads to suppression of the breast cancer immune microenvironment. [12] Triple-negative breast cancer is more sensitive to immunotherapy than other subtypes. [4, 22, 23] Previous studies also suggested that Siglec-15 is an immune suppressor of T cells. [12] Therefore, high expression of Siglec-15 leads to poor survival in triple-negative breast cancer.

Our tumour-infiltrating immune cell study may help to explain why high Siglec-15 expression is associated with poor prognosis. We found that breast cancer tissues with high Siglec-15 expression had low fractions of activated dendritic cells, follicular helper T cells, activated memory CD4 T cells and M1 macrophages. First, activated dendritic cells can present antigens to appropriate T cell receptors. [24] A low fraction of activated dendritic cells leads to low activation of the immune system. Second, CD4 + T cells play a major role in mediating the immune response.[25] Low fractions of follicular helper T cells and memory CD4 T cells lead to a suppressed immune microenvironment. Furthermore, M1 macrophages have anticancer and proinflammatory functions. A low fraction of M1 macrophages was associated with poor breast cancer survival. [26] Therefore, the immune microenvironment is generally suppressed in breast cancer tissue with high Siglec-15 expression.

In conclusion, Siglec-15 had high expression in breast cancer tissues. High Siglec-15 expression is associated with low activated dendritic cell, follicular helper T cell, and M1 macrophage proportions in breast cancer tissue and predicts poor prognosis in triple-negative breast cancer. Siglec-15 expression may be a potential prognostic molecular marker of poor survival in breast cancer.

Conclusions

In conclusion, Siglet-15 had high expression in breast cancer tissues. High Siglec15 expression is associated with low activated dendritic cells, follicular helper T cells, and M1 macrophages proportions in breast cancer tissue, and predicts poor prognosis in triple negative breast cancer. Siglec15 expression may be a potential prognostic molecular marker of poor survival in breast cancer.

Abbreviations

Siglec-15: Sialic acid binding Ig-like lectin 15

TCGA: the Cancer Genome Atlas

METABRIC: Molecular Taxonomy of Breast Cancer International Consortium

TNBC: Triple-negative breast cancer

PD-L1: programmed death ligand 1

PD-1: programmed death 1

TMA: tissue microarray

PBS: phosphate-buffered saline

HRP: horseradish peroxidase

BRCA: breast invasive carcinoma

TIIC: tumor-infiltrating immune cells

TNM: the size of the cancer, lymph nodes and metastasis

Declarations

Ethics approval and consent to participate

This was a retrospective study conducted with the approval of the ethics committee of Fudan University Cancer Hospital (No. 2019171) and operated in compliance with the Helsinki Declaration. Informed consent was obtained.

Consent for publication

Not applicable.

Availability of data and material

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

Competing interests

The authors report no conflict of interest.

Funding

Sponsored by Natural Science Foundation of Shanghai, Grant No. 20ZR1412400

Authors' contributions

LF and XYS contributed to the conception of the work, data analysis, interpretation of imaging data for the work and writing the manuscript. YSY analyzed and interpreted imaging data for the work. XYL

contributed to the acquisition of MRI data. PZ and ZMS contributed to revising the manuscript. The author(s) read and approved the final manuscript.

Acknowledgements

The results shown here are in whole or part based upon data generated by the TCGA Research Network and the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC).

Preprint statement

A preprint has previously been published.[27]

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Figures

Figure 1

Figure 1

Breast cancer microarray immunohistochemistry.

Scanning of the breast cancer microarray after Siglec-15 immunohistochemistry staining (A). A classic pair of samples (B, C and D). The left sample is breast cancer tissue. The right sample is adjacent normal tissue. Breast cancer tissue had higher Siglec-15-positive staining than adjacent normal tissue. The scatter bubble graph shows the Siglec-15 positive rates of each sample in Fig. A (E). The violin graph shows the general Siglec-15-positive rates in breast cancer tissues and adjacent normal tissues (F).

Figure 2

Figure 2

Association of Siglec-15 expression with clinicopathological characteristics.

Siglec-15 expression was higher in breast cancer subtype than in normal controls (A). TNM-T1 had higher expression of Siglec-15 than the other T groups (B). However, the expression of Siglec-15 did not differ among TNM-N (C), TNM-M (D) and TNM-M stage (E).

Siglec-15: Sialic acid binding Ig-like lectin 15; **TNM-T:** tumour size; **TNM-N:** lymph node; **TNM-M:** metastasis.

Figure 3

Figure 3

High Siglec-15 expression was associated with poorer survival in triple-negative breast cancer

Triple-negative breast cancer patients with high Siglec-15 expression had a poorer prognosis than those with low Siglec-15 expression (A). However, luminalA patients with high Siglec-15 expression had a better prognosis (B). Siglec-15 expression did not influence survival in the luminal B (C) and HER2+ (D) subtypes.

Figure 4

Figure 4

Proportions of each immune cell type in normal, low PD-L1 and high PD-L1 breast cancer tissues.

Heatmap of immune cell proportions of breast cancer and normal samples. The blue horizontal bar indicates the normal control, while the red horizontal bar represents breast cancer samples (A). Violin plot of the proportions of each immune cell in normal, low and high Siglec-15 breast cancer tissues. Blue

represents normal samples, green indicates low Siglec-15 breast cancer samples, and red indicates high Siglec-15 breast cancer samples (B). The breast cancer tissues with high Siglec-15 expression had low fractions of activated dendritic cells, follicular helper T cells, activated CD4 memory T cells, and M1 macrophages and high fractions of resting CD4 memory T cells ($p < 0.001$), resting dendritic cells, and resting mast cells (C).

Supplementary Files

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