High Siglec15 Expression in Triple Negative Breast Cancer Predicts Poor Prognosis

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Research Article

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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 Siglec15 in breast cancer and investigated the influence of Siglec15 on the microenvironment of infiltrating immune cells in the cancer.

Methods: We performed immunhistochemical staining to analyze Siglet-15 expression in primary invasive breast cancer tissue microarrays. The tissue specimens were from 90 patients. Furthermore, The relationship between Siglec15 and clinicopathological features was analyzed 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: Immunhistochemical staining of tissue microarrays showed that Siglet-15 had higher expression in breast cancer tissues than those in adject normal tissues. Breast cancer tissues had higher Siglec15 expression than normal tissues. Kaplan-Meier survival analysis suggested that triple negative breast cancer with high Siglec15 expression had poorer survival than those with lower Siglec15 expression (p=0.042). Furthermore, high Siglec15 expression group had low activated dendritic cells, follicular helper T cells, and M1 macrophages.

Conclusions: 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.

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 aging, pregnancy history, overweight, lack of exercise, genetics, and smoking cigarettes. [4] Triple-negative breast cancer (TNBC) have an increased aggressiveness, higher histologic grade, and higher risk of local recurrence and visceral metastasis.[5]

Normalization cancer immunotherapy is a new strategy to treat cancer, which can normalize the compromised immunity without disproportionate side-effects. In recent years, immunotherapy has become of great interest in 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.[6] Furthermore, some clinical

trials have been performed to investigate the other medicines that target immune pathways in breast cancer. The 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 tumor patients respond to anti-PD-1/PD-L1 treatment.[10, 11].It's necessary to find new checkpoint targets to restore the defective immune responses in the tumor microenvironment as a principle for normalization cancer immunotherapy.

Sialic acid binding Ig-like lectin 15 (Siglec-15) is a new potential target for normalization cancer immunotherapy. It is animmunoreceptor that originally categorized into the immunoglobulin superfamily and Siglec family. Recent study found that Siglec-15 can suppress CD8 + T cell responses. Suppression of Siglec-15 inhibits tumor growth in animal models.[12]However, the expression of Siglec-15 and its effects on breast cancer has 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 immunhistochemical staining to analyze Siglet-15 expression in primary invasive breast cancer tissue microarrays. Furthermore, we used the data obtained from The Cancer Genome Atlas (TCGA and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) to investigate the association of Siglec15 expression and breast cancer. Moreover, we illustrated the atlas of the influence of Siglec-15 on immune cell microenvironment of breast cancer with the CIBERSORT algorithm[13], 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 has attained the approval of the Institutional Review Board. The informed consents were 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 tumor areas were carefully selected. Formalin-fixed, paraffin-embedded core cylinders were punched and deposited into a recipient paraffin block. Ninety pairs (180 points) 4 μm-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 10 minutes a time for 3 times, and dehydrated in a series of graded alcohols. To exhaust the 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 Siglec15 antibody (Abcam, # ab198684), the primary antibody, was diluted 1:50 in phosphate-buffered saline (PBS). The chips were incubated with diluted primary antibody overnight at 4°C. After PBS wash, 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 peroxidase substrate diaminobenzidine for 2 min and counterstained with hematoxylin.

Evaluation of immunohistochemical staining

The immunohistochemical staining scoring of Siglec-15 expression was defined as: 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% cells. The percentage of positive staining cells (0% to 100%) were also calculated.

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. Boxplots were used to visualize expression differences for discrete variables. The RNA-Seq gene expression level 3 HTSeq-Counts data of breast cancer patients with breast invasive carcinoma (BRCA) and clinical data were retained and further analyzed.

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 of genes in breast invasive carcinoma (BRCA) were analyzed. Clinical characteristics from the corresponding patients were downloaded.

Evaluation of tumor-infiltrating immune cells (TIIC)

We used the CIBERSORT algorithm (http://cibersort.stanford.edu/) to calculate the tumor-infiltrating immune cell composition. [15] The TIIC immune cells included 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 conducted 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 Siglec15 in patients dataset was evaluated. The chi-square test and Fisher’s exact test were applied to identify correlations between Siglec15 mRNA expression and the clinical features of breast

cancer. The relationship between clinicopathological features and Siglec15 expression was analyzed 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 Siglec15 expression was determined by its median value. The correlations between TNM stage and immune cells were measured by the Wilcoxon test. The association between clinical follow-ups and immune cells and survival curves were evaluated by 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.

Result

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

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

Databases showed breast cancer tissue had high Siglec15 mRNA expression

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

We compared the Siglec15 expression in breast cancer and normal tissues. The results indicated that Siglec15 expression was higher in breast cancer tissues than them in normal controls (Fig 2A, 2.10 V.S. 1.34. p=0.000326).

TNM-T1 has higher expression of Siglec15 than control groups (Fig 2B, p=0.011). However, the expressions of Siglec15 do not have differences among TNM-N (Fig 2C, p=0.749), TNM-M (Fig 2D, p=0.799) and stages (Fig 2E, p=0.119).

High Siglec15 expression TNBC patients had a poorer survival

Kaplan-Meier survival analysis suggested that Triple-Negative breast cancer patients with high Siglec15 expression had a poorer prognosis than those with low Siglec15 expression (p = 0.042, Fig. 3A). However, an interesting finding is that low Siglec15 expression had a poorer prognosis in Luminal A subtypes. Siglec15 expressions do not influence the survival in Luminal B (p = 0.51, Fig. 2B), and HER2+ (p = 0.71, Fig. 2D) subtypes.

Composition of tumor-infiltrating immune cells

We performed CIBERSORT to investigate the fractions of infiltrated immune cells between high Siglec15 breast tumor tissue, low Siglec15 breast tumor tissue and adjacent non-tumor tissues. A bar plot was drawn to visualize the proportions of infiltrating immune cells. (Fig 4A)

The high Siglec15 expression breast cancer tissues had a low fraction of activated dendritic cells ($p<0.001$), follicular helper T cells ($p<0.001$), activated CD4 memory T cells ($p<0.001$) and M1 Macrophages ($p=0.008$, Fig 4B, C).

The high Siglec15 expression breast cancer tissues had a high fraction of resting CD4 memory 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 Siglec15 mRNA expression. High Siglec15 expression was associated with poor prognosis in triple negative breast cancer. Furthermore, high Siglec15 expression breast cancer tissues had a low fraction of activated dendritic cells, follicular helper T cells, activated CD4 memory T cells and M1 Macrophages.

Siglecs, members of the immunoglobulin gene super-family, are cell-surface proteins that bind sialic acid. Recent studies utilizing a high-throughput screening identified that Siglec15 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 found that Siglec15 had both high protein and mRNA expressions in breast cancer tissues. Li et. al reported that breast invasive carcinoma has higher Siglec15 mRNA expression in UALCAN database.[16] However, no previous studies investigated the protein expression of Siglec15 in breast cancer patients. Our microarray immunohistochemistry confirmed the breast cancer tissue had higher protein lever of Siglec15 than controls.

High Siglec15 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. [17] However, Li et. al reported that higher level of Siglec-15 expression in breast cancer indicates better overall survival. [16] This may due to that different subtypes have different immune environment. [18] Siglec-15 as an immune suppressor. High expression of Siglec-15 leads to suppression of breast cancer immune microenvironment. [12] Triple-negative breast cancer is more sensitive to immunotherapy than other subtypes. [4, 19, 20] Therefore, high expression of Siglec-15 leads to poor survival in triple-negative breast cancer.

Our tumor-infiltrating immune cells study may help to explain why the high Siglec15 expression associated with poor prognosis. We found that high Siglec15 expression breast cancer tissues had a low fraction of activated dendritic cells, follicular helper T cells, activated CD4 memory T cells and M1

Macrophages. First, activated dendritic cells can present antigen to appropriate T cell receptors. [21] Low fraction of activated dendritic cells leads to low activation of immune system. Second, CD4+ T cells play a major role in mediating immune response.[22] Low fraction of follicular helper T cells and CD4 memory T cells lead to suppressed immune microenvironment. Furthermore, M1 macrophages have anticancer and proinflammatory functions. A low fraction of M1 macrophages was associated with poor breast cancer survival. [23] Therefore, the immune microenvironment is generally suppressed in high Siglec15 expression breast cancer tissue.

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.

Conclusion

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.

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Authors' contributions

PZ 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. ZMS and LF contributed to revising the manuscript. The author(s) read and approved the final manuscript.

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Tables

Table 1 is not available with this version.

Figures

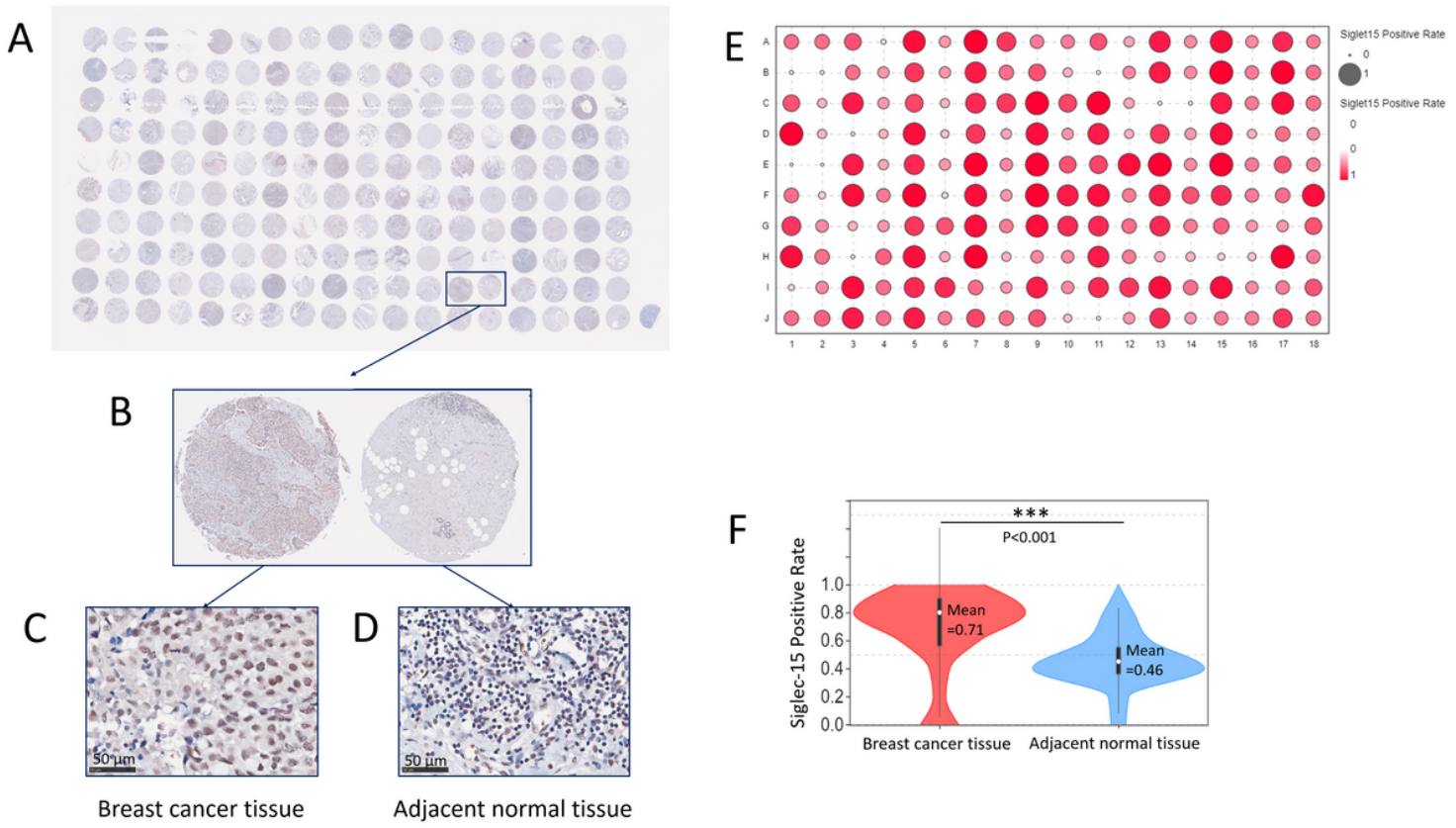

Figure 1

Figure 1

Breast cancer microarray immunohistochemistry. The scan of breast cancer microarray after Siglec-15 immunohistochemistry staining (A). A classic pair of samples (B, C and D.). Left sample is breast cancer tissue. Right sample is adjacent normal tissue. Breast cancer tissue has higher Siglec-15 positive staining than adjacent normal tissue. Scatter bubble graph shows the Siglec-15 positive rates of each sample in Fig. A (E). Violin graph shows the general Siglec-15 positive rates in breast cancer tissues and adjacent normal tissues (F).

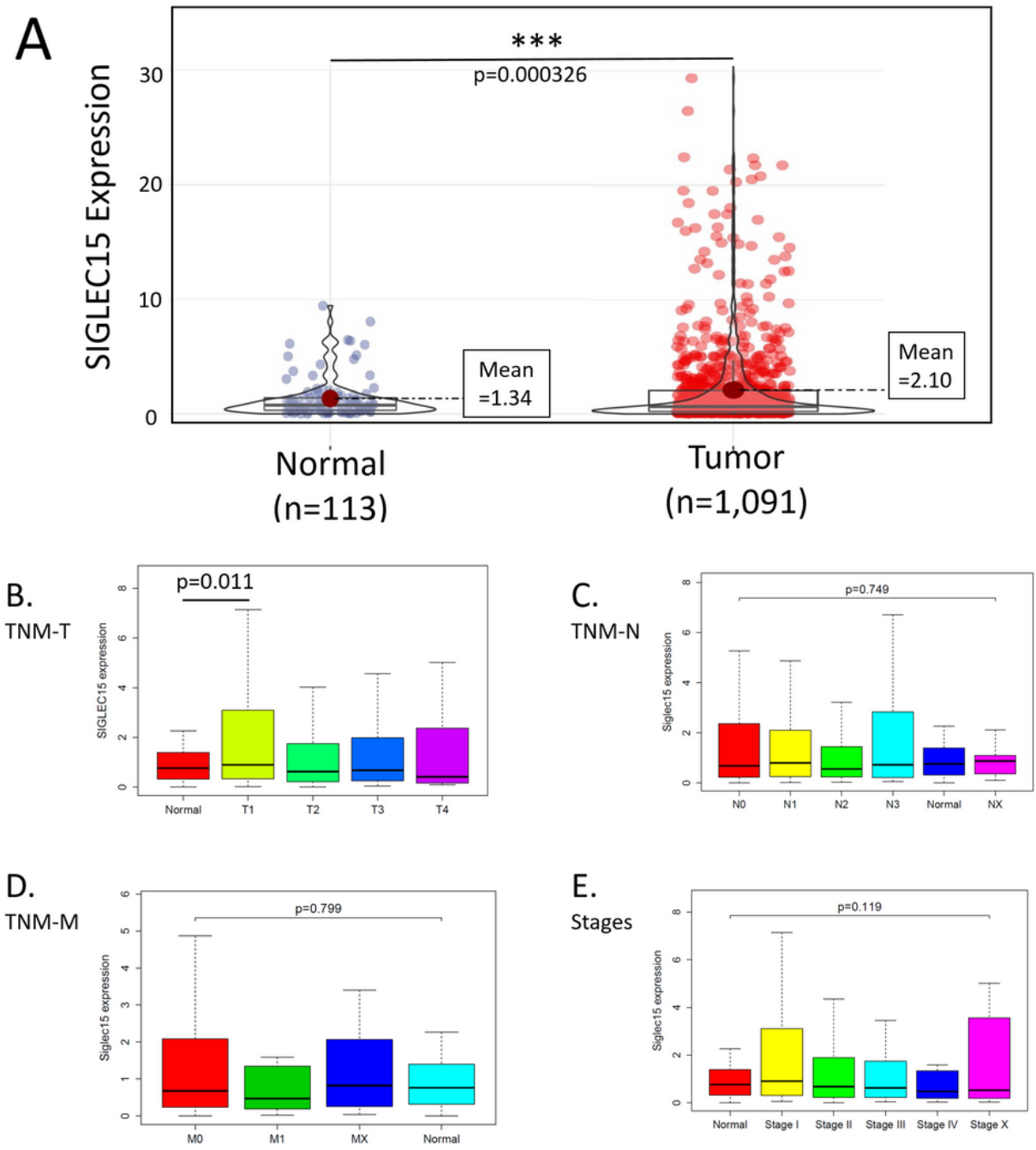

Figure 2

Figure 2

Association of Siglec15 expression with clinicopathological characteristics. Siglec15 expression is higher in breast cancer subtype than them in normal controls (A). TNM-T1 has higher expression of Siglec15 than other T groups (B). However, the expressions of Siglec15 do not have differences among TNM-N (C), TNM-M (D) and stages (E). Siglec15: Sialic acid binding Ig-like lectin 15;TNM-T: tumor size; TNM-N: lymph node;TNM-M:metastasis.

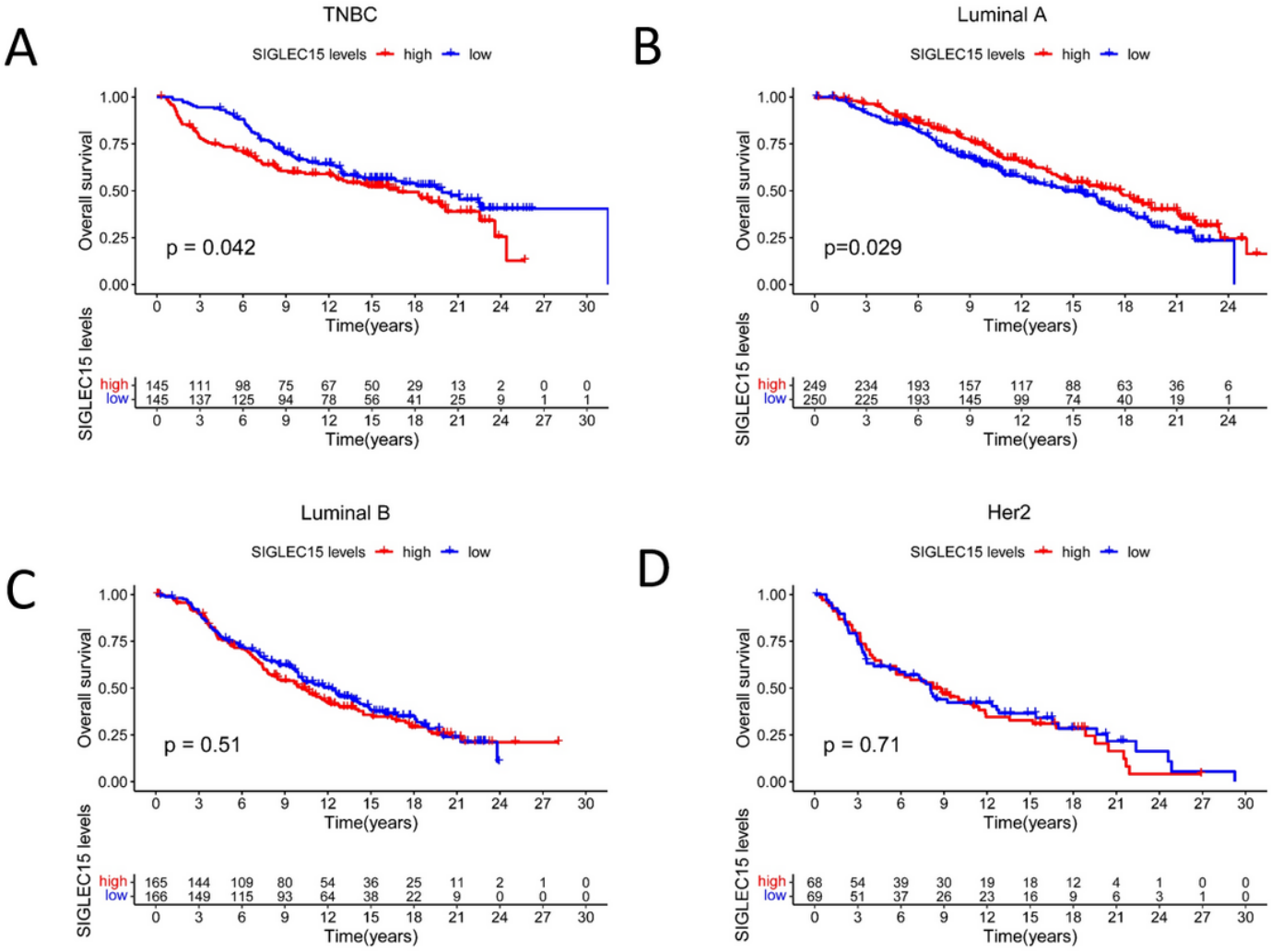

Figure 3

Figure 3

High Siglec15 expression had a poorer survival in Triple-negative breast cancer Triple-negative breast cancer patients with high Siglec15 expression had a poorer prognosis than those with low Siglec15 expression (A). However, Luminal A patients with high Siglec15 expression had a better prognosis. Siglec15 expressions do not influence the survival in Luminal B (C) and HER2+ (D) subtypes.

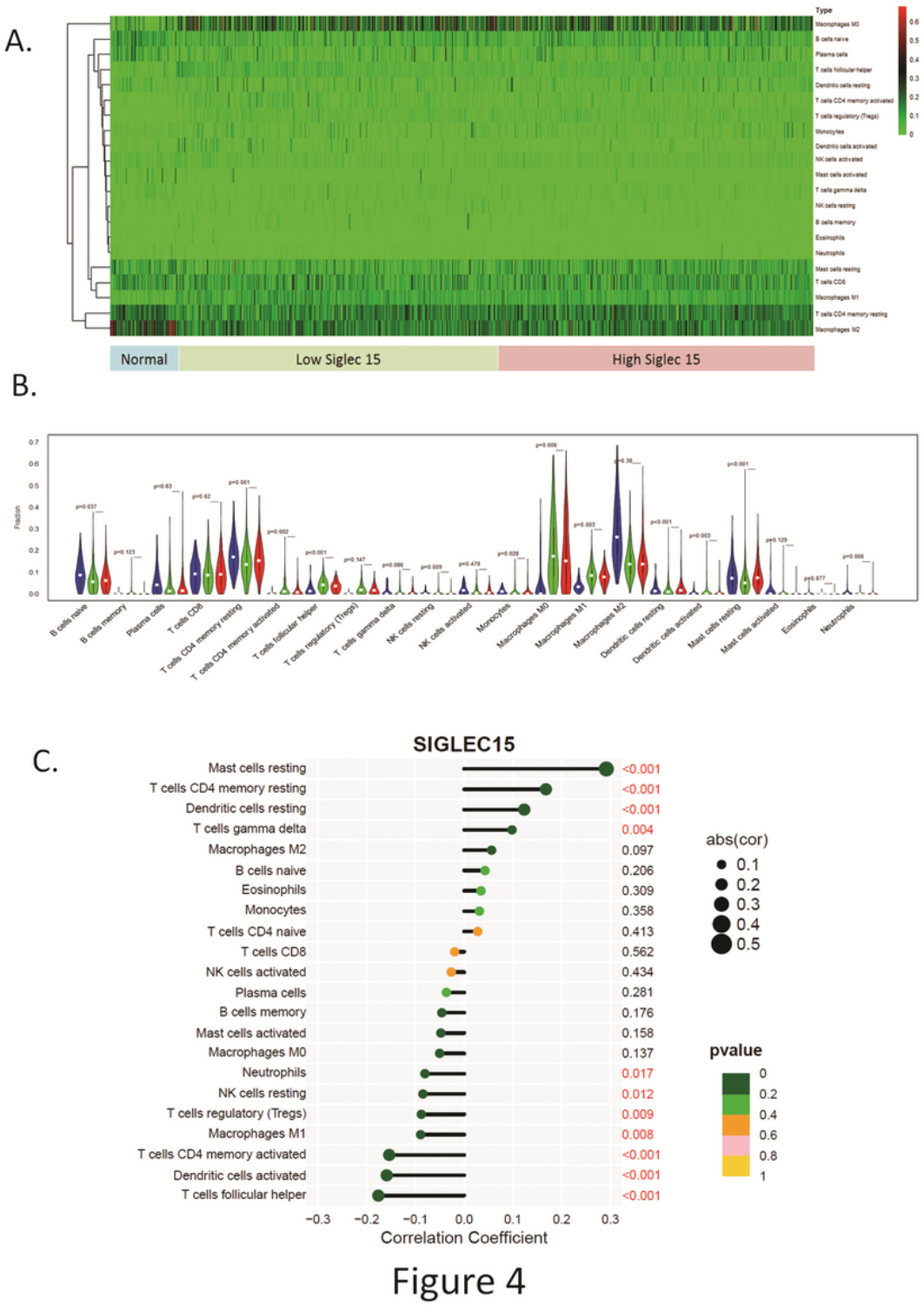

Figure 4

Figure 4

The proportion of each immune cells in normal, low PD-L1 and high PD-L1 breast cancer tissues. Heat map 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). The violin plot of the proportion 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 high Siglec15 expression breast cancer tissues had a low fraction of activated dendritic cells, follicular helper T cells, activated CD4 memory T cells M1. Macrophages, and a high fraction of resting CD4 memory T cells ($p<0.001$), resting dendritic cells, and resting Mast cells (C).