

CLEC10A can serve as a potential therapeutic target and its level correlates with immune infiltration in breast cancer

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Abstract. Breast cancer (BC) is one of the most common malignant cancers in females worldwide and greatly threatens women's health. The C-type lectin domain family 10 member A (CLEC10A) is a member of the C-type lectin receptor family that has been previously reported to promote the antitumor activity of immune cells. In the present study, the potential prognostic value of CLEC10A expression in BC was assessed using data from The Cancer Genome Atlas online database. Differences in the mRNA expression levels of CLEC10A between BC and normal tissues were then analyzed using the Tumor Immune Estimation Resource (TIMER) platform and the University of Alabama at Birmingham Cancer data analysis portal. Reverse transcription-quantitative PCR was performed to validate the results of this analysis. The Kaplan-Meier plotter database was used to evaluate the association between the mRNA expression levels of CLEC10A and clinical prognosis of BC. Based on the association between the mRNA expression levels of CLEC10A and the tumor immune microenvironment, the TIMER platform and the Tumor and Immune System Interaction Database website were utilized to assess the correlation between CLEC10A expression and the degree of tumor immune cell infiltration. The present study

revealed that CLEC10A expression was significantly lower in BC tissues compared with that in normal tissues, which was in turn associated with poorer clinical outcomes. This suggested that lower CLEC10A expression levels were associated with unfavorable prognosis in BC. In addition, the expression level of CLEC10A was found to be positively associated with the level of different tumor-infiltrating immune cells in BC, including CD8 T cells, B cells, macrophages and NK cells which, was in turn closely correlated with some gene markers such as CD19, CD8A, KIR2DS4 and PTGS2. These results suggest that the relationship between lower CLEC10A expression level and poor prognosis in BC may be due to the role of CLEC10A in the tumor immune microenvironment. In conclusion, CLEC10A may be a potential biomarker that can be used to efficiently predict prognosis in patients with BC.

Introduction

Breast cancer (BC) is the most common malignant tumor and ranks as the second leading cause of cancer-related mortality in women, which poses a considerable threat to their health globally (1,2). BC can be subdivided into four molecular subtypes: i) Luminal A, defined as estrogen receptor (ER) and/or progesterone receptor (PR) positive (+) and human epidermal growth factor receptor-2 (HER2) negative (-); ii) luminal B (ER+ and/or PR+ and HER2+); iii) basal-like (ER-, PR-, HER2-, cytokeratin 5/6+ and EGFR+); and iv) HER2-overexpressing subtypes (ER-, PR- and HER2+) (3). The early treatment methods for BC are surgery, chemotherapy, endocrine therapy and radiation therapy, which have improved the outcome of this disease (4,5). The classical clinical prognostic biomarkers, including the ER, PR and HER2 status, continue to serve a significant role in the identification of patients who may benefit from endocrine or targeted therapy (5). Although the management strategies of BC have improved rapidly over the past few decades, including the early diagnostic and effective therapeutic approaches, metastasis remains a major cause of poor prognosis in patients with BC (6). Due to tumor heterogeneity, available biomarkers that can be used to accurately predict BC prognosis remain limited. Therefore, there is a demand for the exploration of novel effective biomarkers as prognostic indicators and for designing individualized treatments.

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Abbreviations: CLEC10A, C-type lectin domain family 10 member A; BC, breast cancer; BRCA, breast invasive carcinoma; GBM, glioblastoma multiforme; LUAD, lung adenocarcinoma; SKCM, skin cutaneous melanoma; UCEC, uterine corpus; TIMER, Tumor Immune Estimation Resource; TCGA, The Cancer Genome Atlas; ROC, receiver operating characteristic; AUC, area under curve; OS, overall survival; DSS, disease-specific survival; PFI, progress-free interval

Key words: C-type lectin domain family 10 member A, breast cancer, overall survival, prognosis, biomarkers, immune cell

C-type lectin domain family 10 member A (CLEC10A) is a member of the C-type lectin receptor (CLR) family and is also named macrophage galactose type C-type lectin (7). Similar to other members of the CLR family, CLEC10A has been reported to be involved in increasing antigen-specific CD8 T-cell activation (7). In addition, galactose/N-acetylgalactosamine (Tn antigen) was recognized by the carbohydrate recognition domain of CLEC10A, which is associated with tumors, rendering it one of the most effective antigen presentation proteins expressed on CD4 T cells to facilitate immune responses and significantly promote antigen-specific CD8 T-cell activation (7,8). Tumor-specific CD8 and CD4 T cells are required for effective tumor eradication (9). The ability of CLEC10A in promoting the antitumor activity of immune cells has been garnering attention (10,11), where it was proposed to be a target for cancer immunotherapy (8). Lower expression levels of CLEC10A in lung cancer have been reported to be associated with poorer clinical prognosis (12). Furthermore, a previous study based on artificial intelligence algorithms revealed that CLEC10A can be applied as prognostic biomarkers for BC (13). However, the clinical significance and biological function of CLEC10A in BC remain unclear.

In the present study, a comprehensive analysis of the association between CLEC10A expression and the risk of BC progression was performed using The Cancer Genome Atlas (TCGA) database. Reverse transcription-quantitative (RT-q) PCR was also performed to validate the results of this analysis. Furthermore, the association between CLEC10A expression levels and alterations in the tumor immune microenvironment was assessed.

Materials and methods

RNA-sequencing data and bioinformatics analysis. Normalized RNA-seq data and corresponding clinical characteristics were collected from the TCGA-breast invasive carcinoma (TCGA-BRCA) datasets (<https://tcga.xenahubs.net>). A total of 1,109 BC tissues and 113 healthy breast tissues were obtained. The downloaded data format was level 3 HTSeq-fragments per kilobase per million (FPKM) for subsequent analysis. The XIANTAO platform (www.xiantao.love) was used to conduct a paired comparison between CLEC10A expression in BC and case-matched adjacent benign tissues obtained from the TCGA database.

Tumor Immune Estimation Resource database analysis. The TIMER database (TIMER 2.0; <https://cistrome.shinyapps.io/timer/>) is a comprehensive resource for the analysis of gene expression and tumor-infiltrating immune cells across multiple cancer types (14). The TIMER website was utilized to analyze the differential expression levels of CLEC10A in tumor and normal tissues. In addition, the association between CLEC10A and the infiltration of various immune cell types into tumors and the expression of molecular markers of immune cell types was investigated using TIMER.

University of Alabama at Birmingham Cancer (UALCAN) data analysis portal. The UALCAN data analysis portal (<http://ualcan.path.uab.edu/index.html>) is available for the online analysis of the differential gene expression in cancer

and normal tissues based on the TCGA-RNA sequencing datasets and clinical datasets (15). In addition, this website provides survival prognostic data based on the gene expression differences in 31 cancer types (15). The present study used the UALCAN database to validate the results obtained from analysis using the XIANTAO database and to verify the possible association between CLEC10A gene expression and clinical features, including T stage, N stage, age, pathological stage, ER status and HER2 status.

Clinical significance of CLEC10A expression in BC. CLEC10A expression was compared between BC tumor and para-cancerous tissues by receiver operating characteristic (ROC) analysis to test the predictive value of CLEC10A expression for BC diagnosis. The Kaplan-Meier (KM) plotter (<http://kmplot.com/analysis/>) is an open, intuitive portal tool for prognostic analysis that can be used to assess the relationship between CLEC10A expression levels and patient survival (16). The patients with BC from the TCGA-BRCA dataset were divided into the high and low CLEC10A expression groups according to the median value. One-way Cox survival analysis was performed and the results were visualized as Forest plots using the 'Forest plot' R package (17). A prognostic analysis was performed based on the CLEC10A expression levels in the relevant immune cell subgroups using the TIMER database, where the hazard ratios, 95% confidence intervals, $P < 0.05$ and \log fold-change (FC) > 1 were set as the threshold values.

Immune infiltration analysis. The levels of tumor immune infiltration was determined using the single sample gene set enrichment analysis (ssGSEA) method with the 'GSVA' R package based on the TCGA-BRCA datasets (18). Correlation analysis between CLEC10A and infiltration by the immune cell types was conducted by Spearman's rank correlation coefficient analysis. Graphs and figures were generated using the 'ggplot2' R package (19). The Tumor and Immune System Interaction Database website (TISIDB; <http://cis.hku.hk/TISIDB/>) was utilized to calculate the correlation between CLEC10A expression and the relative number of tumor-infiltrating lymphocytes (20). P-values were determined by Spearman and Wilcoxon rank sum tests.

Cell lines and culture. Human BC cell lines MCF-7, SKBR-3 and MDA-MB-231 and the normal breast epithelial cell line MCF10A were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. SKBR-3, MCF-7 and MDA-MB-231 cells were routinely cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and a 1% (v/v) penicillin and streptomycin solution (Beyotime Institute of Biotechnology). MCF10A cells were cultured in mammary epithelial cell medium (Procell Life Science & Technology Co., Ltd.) supplemented with 10% horse serum, EGF, hydrocortisone, insulin and 1% penicillin-streptomycin. All cell lines were cultured in an incubator at 37°C with 5% CO₂ and passaged using standard cell culture techniques.

RT-qPCR. Total RNA of SKBR-3, MCF-7, MDA-MB-231 and MCF10A cells was isolated using the RNAiso Plus Kit

(cat. no. 9109; Takara Biotechnology Co., Ltd.) according to the manufacturer's protocols. A total of 1,000 ng mRNA was reverse transcribed into cDNA using the PrimeScript™ RT Reagent Kit with the genomic DNA Eraser (cat. no. RR047; Takara Biotechnology Co., Ltd.) according to the manufacturer's protocols. Quantitative PCR (qPCR) analysis was performed in the LightCycler® 96 Instrument (Roche Diagnostics) using the TB Green Premix Ex Taq (cat. no. RR420; Takara Biotechnology Co., Ltd.) to detect the expression of each of the target genes. The following primer pairs were used for qPCR: CLEC10A forward, 5'-GCCAGG TGGCTACTCTCAAC-3' and reverse, 5'-TTCTGCTCCTCC CTGGAGTT-3'; and GAPDH forward, 5'-CATTGACCTCAA CTACATGGTTT-3' and reverse, 5'-GAAGATGGTGATGGG ATTTCC-3'. The following thermocycling conditions were used for qPCR: 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. The relative cycle threshold of the housekeeping gene GAPDH was measured as an endogenous control and the relative mRNA expression levels were normalized to those of GAPDH using the standard $2^{-\Delta\Delta Cq}$ method (21). The experiment was performed in triplicate.

Statistical analysis. Bioinformatics analysis was performed in R version 3.6.3 (<http://www.r-project.org/>). The association between clinical features and CLEC10A expression was analyzed using the U Mann Whitney test, Wilcoxon signed-rank test, Fisher's exact test, χ^2 test and logistic regression. A paired t-test was used to compare the differential expression levels of CLEC10A between the BC tissues and the paired normal tissues from TCGA database. Comparisons of CLEC10A expression levels between normal breast epithelial cells and BC cells were performed using one-way ANOVA followed by Dunnett's (comparing all experimental groups with control group) post hoc test. ROC curve analysis was applied, with the area under curve (AUC) used as the index of diagnostic accuracy. Survival curves were generated using the Kaplan-Meier plotter database. Spearman's correlation analysis was utilized to evaluate the correlation between gene expression and immune cell infiltration in the TIMER. The RT-qPCR data are presented as the mean \pm standard deviation, and each experiment was repeated three times. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Aberrant downregulation of CLEC10A in BC. To determine the overall expression levels of CLEC10A in different malignancies, the CLEC10A expression profiles were first analyzed among the different cancer types using the TIMER database. The analysis indicated that the expression level of CLEC10A was significantly downregulated in the majority of cancer tissues, including BRCA, colon adenocarcinoma, rectum adenocarcinoma, bladder urothelial carcinoma, head and neck squamous cell carcinoma and kidney chromophobe, compared with that in their corresponding normal tissues (Fig. 1A). To determine the expression levels of CLEC10A in BC and normal tissues in further detail, RNA-seq data and clinical information from 1109 BC tissues and 113 normal breast tissues were collected from the TCGA-BRCA datasets. The expression levels of CLEC10A were found to be significantly

lower in BC tissues compared with those in the normal tissues ($P < 0.001$; Fig. 1B). Subsequently, the CLEC10A expression levels in 112 BC tissues and their matched adjacent normal tissues were also measured, which showed that BC tissues expressed significantly lower levels of CLEC10A compared with those of the normal tissues ($P < 0.001$; Fig. 1C). According to RT-qPCR analysis, the expression level of CLEC10A in all three types of BC cells tested was significantly lower (SKBR-3, MCF-7, MDA-MB-231) compared with that of MCF10A cells (Fig. 1D), which is consistent with the aforementioned results.

In addition, the association between the clinicopathological characteristics of BC and the expression of CLEC10A was then analyzed using the Wilcoxon rank sum test. CLEC10A expression level was higher for patients with T2&T3 ($P < 0.01$; Fig. 1E), pathological stage I ($P < 0.01$; Fig. 1G) and negative HER2 status ($P < 0.001$; Fig. 1I) than in patients with T4, pathologic stage II and positive HER2 status, respectively. However, there was no significant association with N stage or ER status (Fig. 1F and H). This indicates that the expression of CLEC10A is associated with disease malignancy degree in BC.

Predictive value of CLEC10A for BC prognosis and in other cancers. To explore the potential clinical applicability of CLEC10A expression, ROC curve was used to assess its value in discriminating BC during diagnosis. With an AUC of 0.779, CLEC10A showed marked median sensitivity and specificity for BC diagnosis (Fig. 2A). KM analyses were also performed to verify the predictive value of CLEC10A regarding clinical outcomes. The results showed that the patients with BC and higher CLEC10A expression levels tended to have more favorable overall survival (OS; $P = 0.031$; Fig. 2B), disease-specific survival (DSS; $P = 0.063$; Fig. 2C) and progression-free survival (PFS, $P = 0.024$; Fig. 2D) rates.

Furthermore, the possible association between CLEC10A expression and OS and PFS in 30 TCGA tumor types was also investigated using univariate survival analysis. CLEC10A expression was found to be associated with the OS of patients with lung adenocarcinoma (LUAD; $P = 0.001$), uterine corpus (UCEC; $P = 0.035$), glioblastoma multiforme (GBM; $P = 0.024$), skin cutaneous melanoma (SKCM; $P = 0.001$) and uveal melanoma (UVM; $P = 0.027$) in addition to BRCA (Fig. 3A). CLEC10A expression also correlated with the PFS of patients with LUAD ($P = 0.004$), adrenocortical carcinoma ($P = 0.009$), cervical and endocervical cancer ($P = 0.009$), liver hepatocellular carcinoma ($P = 0.04$), UCEC ($P = 0.038$), GBM ($P = 0.024$) and SKCM ($P = 0.002$; Fig. 3B). These results suggest the value of CLEC10A in predicting prognosis in several cancers and that CLEC10A may be a potential prognostic biomarker for BC.

CLEC10A expression is associated with immune infiltration in BC. Tumor infiltrating lymphocytes have been previously identified to be an independent factor for predicting sentinel lymph node status and prognosis in various cancers (9). Therefore, the present study next used the TISIDB database to further analyze the potential correlation between expression levels of CLEC10A and tumor immune infiltrating cells. CLEC10A expression was correlated with all immune cell subtypes in BC (Fig. 4A; Table I). Correlation analysis between CLEC10A expression and immune cell subsets in BC using ssGSEA with

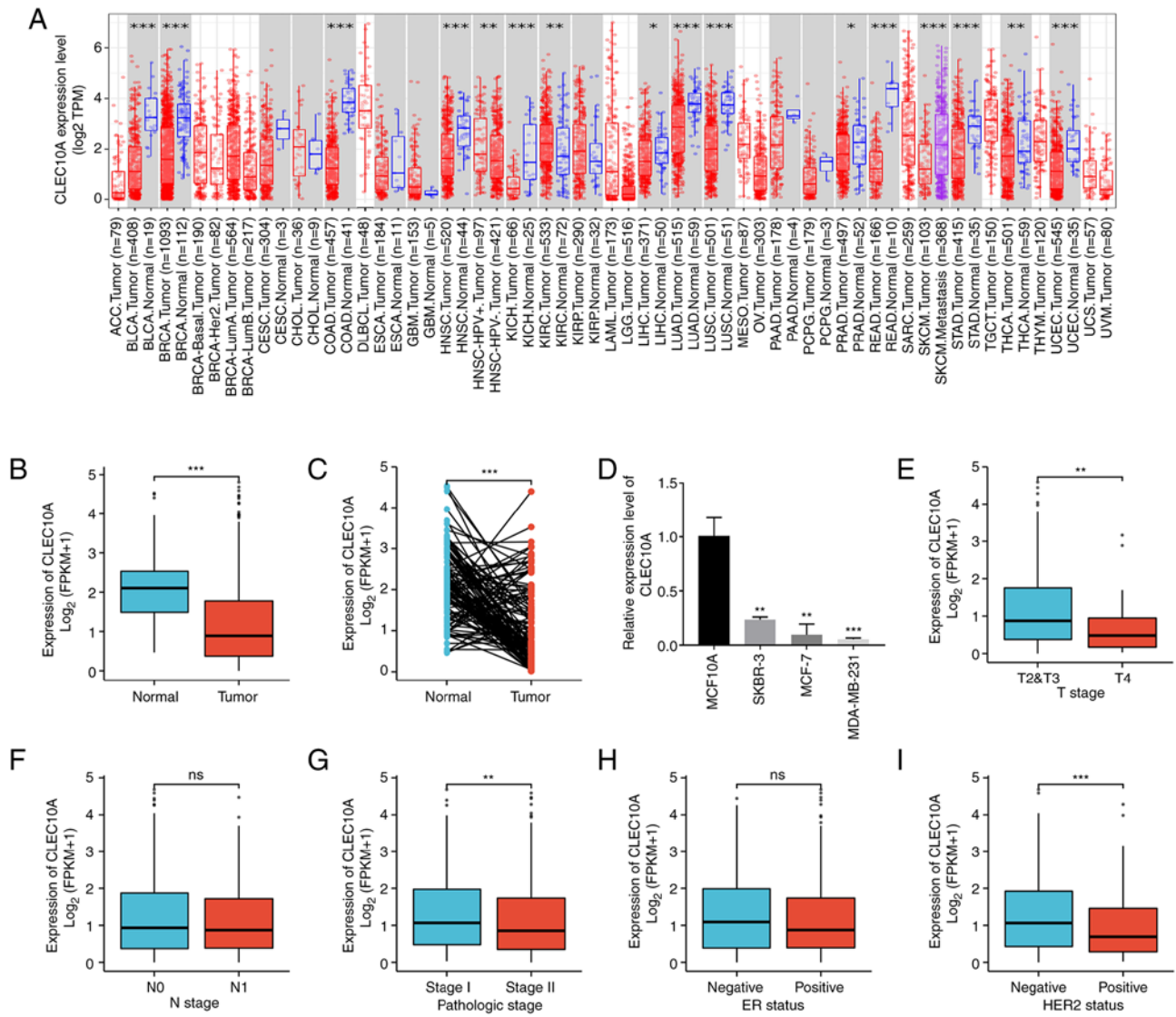


Figure 1. Expression levels of the CLEC10A gene in different cancers and their association with individual clinical parameters of BC. (A) CLEC10A gene expression levels in different cancer tissues compared with normal tissues in the Tumor Immune Estimation Resource database. (B) Difference in the CLEC10A gene expression levels between primary BCs and unmatched normal samples from the TCGA-BRCA data sets using Mann-Whitney U test. (C) Difference in the CLEC10A gene expression levels between primary BCs and matched normal samples from the TCGA-BRCA data sets using Wilcoxon signed-rank test. (D) Reverse transcription-quantitative PCR analysis of the mRNA expression levels of CLEC10A in normal breast epithelial and breast cancer cell lines. Mann Whitney U test of the association between CLEC10A gene expression from the TCGA-BRCA datasets and (E) clinical T, (F) clinical N and (G) pathological stages, (H) ER and (I) HER2 statuses. *P<0.05, **P<0.01, ***P<0.001. ns, not significant; CLEC10A, C-type lectin domain family 10 member A; BC, breast cancer; COAD, colon adenocarcinoma; ACC, adrenocortical carcinoma; READ, rectum adenocarcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical and endocervical cancer; CHOL, cholangiocarcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; UVM, uveal melanoma; SKCM, skin cutaneous melanoma; LUSC, lung squamous cell carcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; STAD, stomach adenocarcinoma; THCA, thyroid carcinoma; UCEC, uterine corpus; DLBCL, diffuse large B cell lymphoma; LAML, acute myeloid leukemia; LGG, low-grade glioma; MESO, mesothelioma; OV, ovarian cancer; PAAD, pancreatic adenocarcinoma; SARC, sarcoma; TGCT, tenosynovial giant cell tumor; THYM, thymoma; UCS, uterine carcinosarcoma; FPKM, fragments per kilobase of exon per million mapped fragments; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2.

Spearman's correlation coefficient indicated that CLEC10A expression significantly correlated with immune infiltration by B cells ($r=0.682$; $P<0.001$), CD8 T cells ($r=0.615$; $P<0.001$), macrophages ($r=0.404$; $P<0.001$), dendritic cells (DCs; $r=0.755$; $P<0.001$), natural killer (NK) cells ($r=0.267$; $P<0.001$), central memory T cells ($r=0.236$; $P<0.001$), Th1 cells ($r=0.558$; $P<0.001$), Th17 cells ($r=0.107$; $P<0.001$) and neutrophils ($r=0.426$; $P<0.001$) in BC positively (Fig. 4B-J). These results suggest that CLEC10A can potentially serve as a major tumor immune infiltration regulator in BC.

CLEC10A expression is associated with the expression of immune cell type markers. The possible association between CLEC10A expression and the expression levels of tumor-infiltrating immune cell markers in BC tissues were analyzed using the TIMER database. The expression levels of CLEC10A in BC tissues were significantly correlated with immune markers of B cells, CD8 T cells, M2 macrophages, monocytes, NK cells, T cells (general), DCs, T helper cells, Tregs, and exhausted T cells (Table I). The expression levels of CLEC10A were significantly correlated with the levels of various

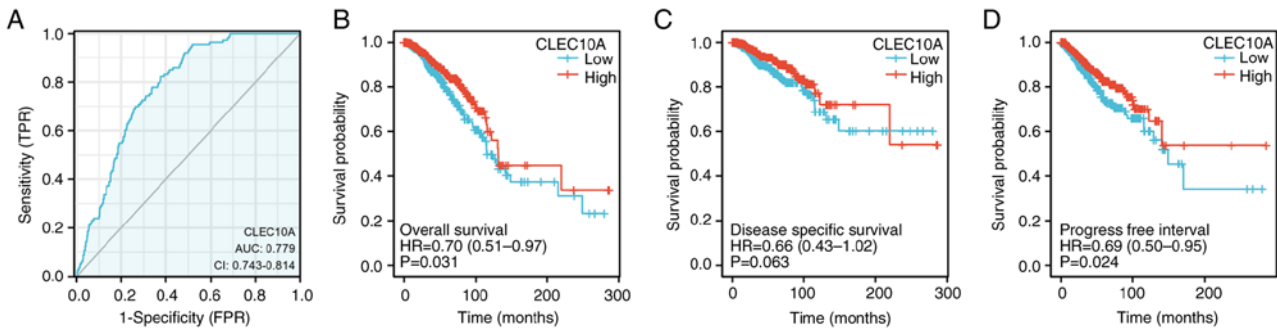


Figure 2. Predictive value of the CLEC10A gene expression levels for the diagnosis and prognosis in patients with BC. (A) Receiver operating characteristic curve analysis evaluating the performance of CLEC10A gene expression for BC diagnosis. (B) Kaplan-Meier analysis comparing the overall survival of patients in the high and low CLEC10A gene expression groups. (C) Kaplan-Meier analysis comparing the disease-specific survival of patients in the high and low CLEC10A gene expression groups. (D) Kaplan-Meier analysis comparing the progression-free interval of patients in the high and low CLEC10A gene expression groups. BC, breast cancer; CLEC10A, C-type lectin domain family 10 member A; HR, hazard ratio; FPR, false-positive rate; TPR, true-positive rate.

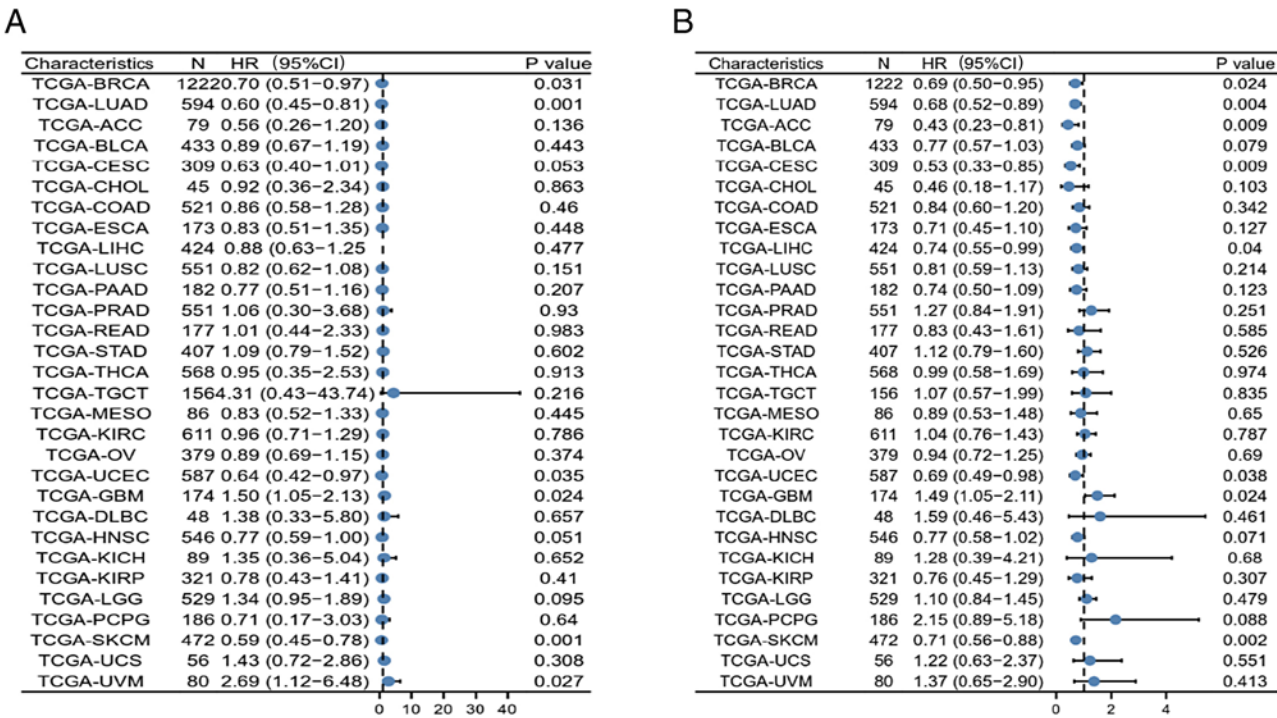


Figure 3. Forest plot of the prognostic value of C-type lectin domain family 10 member A gene expression in 30 The Cancer Genome Atlas tumors. (A) Overall survival HR. (B) Progression-free survival HR. HR, hazard ratio.

subtypes of T cell markers, including CD8 T cell markers, CD8A and CD8B, T cell (general) markers, CD3D, CD3E, and CD2, exhausted T cell markers cytotoxic T-lymphocyte antigen 4, hepatitis A virus cellular receptor 2, granzyme B, lymphocyte activating 3 and programmed cell death protein 1, Th2 markers, Th17 markers STAT6 and 5A and IL17A, Treg markers forkhead box P3, C-C motif chemokine receptor (CXCR) type 8, STAT5B, and TGF-β1, Tfh marker Bcl-6 and neutrophil markers integrin subunit α M and C-C motif chemokine receptor type 7 (all P<0.001; Table II). The expression levels of CLEC10A were also significantly correlated with macrophage markers [M2 macrophage markers membrane spanning 4-domains A4A, V-set and immunoglobulin domain containing 4 and CD163; monocyte markers colony stimulating factor 1 receptor and CD86; tumor-associated macrophage

markers CD68, IL21, IL10, BCL-6 (P<0.01) and C-C motif chemokine ligand 2; all P<0.001 unless stated otherwise; Table II] and B cell markers CD19 and CD79A (all P<0.001; Table II). However, the expression levels of CLEC10A were not significantly correlated with the DC marker CD1C, M1 macrophage marker nitric oxide synthase 2, neutrophil marker carcinoembryonic antigen-related cell adhesion molecule 8 or Th2 marker GATA binding protein 3 in BC (Table II). These results suggest that CLEC10A is associated with the expression levels of infiltrating immune cell markers in BC tissues and may be involved in the regulation of tumor immune infiltration in BC.

Prognostic analysis of CLEC10A expression in BC based on immune cell types. The present study found that the expression

Table I. Correlation between C-type lectin domain family 10 member A expression and tumor lymphocyte infiltration in breast cancer^a.

Cell type	Rho-value	P-value
Activated CD8 T cell	0.59	<2.2x10 ⁻¹⁶
Central memory CD8 T	0.134	8.57x10 ⁻⁰⁶
Effector memory CD8 T	0.701	<2.2x10 ⁻¹⁶
Activated CD4 T	0.382	<2.2x10 ⁻¹⁶
Central memory CD4 T	0.296	7.30x10 ⁻²⁴
Effector memory CD4 T	0.392	<2.2x10 ⁻¹⁶
T follicular helper	0.607	<2.2x10 ⁻¹⁶
Gamma delta T	0.434	<2.2x10 ⁻¹⁶
Type 1 T helper	0.69	<2.2x10 ⁻¹⁶
Type 17 T helper	0.526	<2.2x10 ⁻¹⁶
Type 2 T helper	0.315	<2.2x10 ⁻¹⁶
Regulatory T	0.504	<2.2x10 ⁻¹⁶
Activated B	0.782	<2.2x10 ⁻¹⁶
Immature B	0.688	<2.2x10 ⁻¹⁶
Memory B	0.381	<2.2x10 ⁻¹⁶
natural killer	0.464	<2.2x10 ⁻¹⁶
CD56bright natural killer	0.388	<2.2x10 ⁻¹⁶
CD56dim natural killer	0.209	3.02x10 ⁻¹⁶
Myeloid derived suppressor	0.566	<2.2x10 ⁻¹⁶
Natural killer T	0.576	<2.2x10 ⁻¹⁶
Activated dendritic	0.326	<2.2x10 ⁻¹⁶
Plasmacytoid dendritic	0.353	<2.2x10 ⁻¹⁶
Immature dendritic	0.098	0.00111
Macrophage	0.585	<2.2x10 ⁻¹⁶
Eosinophil	0.601	<2.2x10 ⁻¹⁶
Mast	0.685	<2.2x10 ⁻¹⁶
Monocyte	0.483	<2.2x10 ⁻¹⁶
Neutrophil	0.359	<2.2x10 ⁻¹⁶

^aCorrelation analysis was performed using the Tumor and Immune System Interaction database using Spearman's rank correlation coefficient analysis.

levels of CLEC10A were associated with immune infiltration in BC. Additionally, downregulation of CLEC10A expression was associated with unfavorable prognosis in patients with BC. Therefore, it was next hypothesized that CLEC10A may affect the prognosis of patients with BC through immune infiltration. A prognostic analysis based on CLEC10A expression levels in enriched immune cell subgroups was performed between the expression levels of CLEC10A in BC and the infiltration levels of CD4 T cells, macrophages, neutrophils, NK cells, Th2 cells and mesenchymal stem cells through the KM Plotter. High CLEC10A levels were found to be associated with a favourable prognosis in patients with BC with enriched CD4 T cells (P=0.04), neutrophils (P=0.032), macrophages (P=0.002), NK cells (P=0.017) and Th2 cells (P=0.0018) (Fig. 5A-E). However, there was no significant association in mesenchymal stem cells (P=0.06; Fig. 5F). The present results supported the hypothesis that the higher CLEC10A expression levels may

improve the prognosis of patients with BC by altering immune infiltration levels.

Discussion

BC has an intrinsically complex tumor microenvironment (TME), which plays a central role in the pathogenesis of BC (22). The TME can be divided into two parts: Tumor cells and the surrounding extracellular matrix (ECM) (23). The ECM is a complex network consisting of collagenous and non-collagenous components, both of which are key regulators of interstitial transport (24). The ECM is mainly comprised of collagen I, collagen IV, fibronectin and laminin, which provide biochemical scaffold and structural support for the tumor cells (24). Furthermore, immune cells of the TME can influence the course of tumor progression and become key to the overall efficacy (25).

CLEC10A has been previously reported to be associated with improved immune responses of immune cells (7). CLEC10A recognizes tumor-associated Tn antigens and can efficiently present antigens to CD4 T cells (8,10,11). It has been found to significantly increase the activation of antigen-specific CD8 T cells by binding with tumor-associated antigens carrying α -N-acetylgalactosamine (8). The ability of CLEC10A to influence the antitumor immune response has been garnering attention, leading to previous reports proposing that CLEC10A can serve as a potential therapeutic target for tumor therapies, including those for colorectal and ovarian cancer (7,8,26). In addition, a number of reviews have also discussed its value in breast and lung cancer based on the immune response (27,28). Based on these previous findings, the present study therefore investigated the potential association between CLEC10A and immune cell infiltration in BC cells, in addition to its expression and prognostic significance.

In the present study, it was found that CLEC10A expression is lower in some cancer tissues, including BC tissues, compared with that in the normal tissues. Furthermore, the expression levels of CLEC10A were associated with tumor pathological stage, histological type, T stage, and HER2 status in BC. In particular, lower expression levels of CLEC10A were associated with T4 stage (Fig. 1E). Therefore, the present results suggest that lower expression of CLEC10A may serve a role in the progression of tumors, including BC.

A previous study on 146 patients with early invasive ductal breast carcinoma revealed that positive CLEC10A expression is significantly associated with increased disease-free survival and OS (11). The present study was performed using data from 1,222 patients with BC, which also included clinicopathological data, from the TCGA database. Lower CLEC10A expression was found to be associated with poorer prognosis. Some similar findings were reported in lung carcinoma, in a bioinformatics analysis on LUAD data downloaded from TCGA and Gene Expression Omnibus (12). Therefore, CLEC10A may be applied as a potentially powerful prognostic biomarker in BC clinical management.

Subsequently, the present study demonstrated that CLEC10A expression is associated with some infiltrating immune cell types in BC, in particular CD8 T cells, macrophages, DC and NK cells (Fig. 4). Efficient CLEC10A binding

Table II. Correlation analysis between the expression of C-type lectin domain family 10 member A and related immune cell markers using the Tumor IMMune Estimation Resource online database.

Description	Gene markers	None		Purity	
		Rho value	P-value	Rho value	P-value
B cell	CD19	0.655	9.19x10 ⁻¹³⁶	0.532	7.48x10 ⁻⁷⁴
	CD79A	0.650	4.69x10 ⁻¹³³	0.511	3.23x10 ⁻⁶⁷
CD8 T Cell	CD8A	0.726	7.88x10 ⁻¹³	0.507	7.73x10 ⁻¹⁰⁹
	CD8B	0.677	1.31x10 ⁻¹⁴⁶	0.565	2.54x10 ⁻⁸³
Dendritic cell	ITGAX	0.492	7.06x10 ⁻⁶⁷	0.343	2.95x10 ⁻²⁸
	NRP1	0.273	2.35x10 ⁻¹⁹	0.121	3.05x10 ⁻⁴
	CD1C	0.876	0.923	0.819	5.21x10 ⁻²⁴⁰
	HLA-DPA1	0.642	1.96x10 ⁻¹²⁷	0.511	3.52x10 ⁻⁶⁶
	HLA-DRA	0.648	2.70x10 ⁻¹³⁰	0.514	2.41x10 ⁻⁶⁷
	HLA-DQB1	0.535	7.90x10 ⁻⁸¹	0.411	1.34x10 ⁻⁴⁰
	HLA-DPB1	0.715	5.86x10 ⁻¹⁷¹	0.586	2.54x10 ⁻⁹¹
M1 Macrophage	PTGS2	0.415	2.19x10 ⁻⁴⁵	0.284	1.26x10 ⁻¹⁸
	IRF5	0.307	1.05x10 ⁻²⁴	0.226	2.12x10 ⁻¹²
	NOS2	0.011	0.847	-0.022	0.563
M2 Macrophage	MS4A4A	0.554	7.70x10 ⁻⁸⁸	0.430	2.94x10 ⁻⁴⁵
	VSIG4	0.399	8.77x10 ⁻⁴³	0.272	7.93x10 ⁻¹⁸
	CD163	0.440	1.34x10 ⁻⁵²	0.327	1.31x10 ⁻²⁵
Monocyte	CSF1R	0.549	1.12x10 ⁻⁸⁶	0.405	5.48x10 ⁻⁴⁰
	CD86	0.475	2.08x10 ⁻⁶²	0.338	1.21x10 ⁻²⁷
Natural killer cell	KIR2DS4	0.332	4.69x10 ⁻²⁸	0.234	3.15x10 ⁻¹²
	KIR3DL3	0.235	1.15x10 ⁻¹³	0.181	1.97x10 ⁻⁷
	KIR3DL2	0.469	1.23x10 ⁻⁵⁹	0.356	2.16x10 ⁻²⁹
	KIR3DL1	0.432	9.59x10 ⁻⁵⁰	0.340	1.24x10 ⁻²⁶
	KIR2DL4	0.388	2.51x10 ⁻³⁹	0.279	6.63x10 ⁻¹⁸
	KIR2DL3	0.356	1.17x10 ⁻³²	0.266	2.93x10 ⁻¹⁶
	KIR2DL1	0.340	1.50x10 ⁻²⁹	0.255	1.40x10 ⁻¹⁴
Neutrophils	CCR7	0.792	1.06x10 ⁻²³⁵	0.704	2.44x10 ⁻¹⁴⁸
	ITGAM	0.392	4.19x10 ⁻⁴¹	0.259	2.18x10 ⁻¹⁶
	CEACAM8	0.041	0.178	0.005	0.886
T cell general	CD3D	0.771	7.67x10 ⁻²¹⁶	0.671	2.02x10 ⁻¹²⁹
	CD3E	0.782	5.90x10 ⁻⁴⁵	0.686	8.12x10 ⁻¹³⁸
	CD2	0.735	3.30x10 ⁻¹⁸⁴	0.628	1.463x10 ⁻¹⁰⁸
T cell exhaustion	CTLA4	0.570	2.14x10 ⁻⁹⁴	0.440	2.63x10 ⁻⁴⁷
	LAG3	0.387	7.68x10 ⁻⁴⁰	0.286	2.37x10 ⁻¹⁹
	HAVCR2	0.393	2.38x10 ⁻⁴¹	0.252	1.80x10 ⁻¹⁵
	GZMB	0.625	8.536x10 ⁻¹¹⁹	0.508	1.02x10 ⁻⁶⁴
	PDCD1	0.675	2.22x10 ⁻⁴⁵	0.552	1.06x10 ⁻⁷⁸
Tumor-associated macrophages	CCL2	0.472	1.21x10 ⁻⁶⁰	0.338	4.71x10 ⁻²⁷
	IL10	0.455	6.35x10 ⁻⁵⁶	0.334	1.38x10 ⁻²⁶
	CD68	0.420	2.05x10 ⁻¹⁷	0.288	1.11x10 ⁻¹⁹
	BCL6	0.126	1.48x10 ⁻⁴	0.083	0.030
	IL21	0.367	8.59x10 ⁻³⁵	0.279	3.60x10 ⁻¹⁸
	TBx21	0.745	7.90x10 ⁻¹⁹⁴	0.643	1.538x10 ⁻¹¹⁵
Th1	STAT4	0.686	3.40x10 ⁻¹⁵²	0.554	2.21x10 ⁻⁷⁹
	STAT1	0.255	3.93x10 ⁻¹⁷	0.184	1.79x10 ⁻⁸
	IFNG	0.520	9.83x10 ⁻⁷⁶	0.404	1.30x10 ⁻³⁸
	IL13	0.273	4.06x10 ⁻¹⁹	0.216	6.44x10 ⁻¹¹
	GATA3	-0.198	1.03x10 ⁻¹⁰	-0.061	0.075
Th2	STAT6	0.249	9.69x10 ⁻¹⁶	0.223	4.08x10 ⁻¹¹
	STAT5A	0.424	3.19x10 ⁻¹⁶	0.310	6.09x10 ⁻²³

Table II. Continued.

Description	Gene markers	None		Purity	
		Rho value	P-value	Rho value	P-value
Th17	STAT3	0.083	0.011	0.038	0.229
	IL17A	0.203	1.36x10 ⁻¹⁰	0.102	0.006
Treg	FOXP3	0.525	8.75x10 ⁻⁷⁸	0.384	2.19x10 ⁻³⁹
	CCR8	0.420	9.70x10 ⁻⁴⁸	0.333	1.41x10 ⁻²⁶
	STAT5B	0.234	3.30x10 ⁻¹⁴	0.207	2.08x10 ⁻¹⁰
	TGFB1	0.411	5.85x10 ⁻⁴⁵	0.263	2.02x10 ⁻¹⁶

None, correlation without adjustment; Purity, correlation adjusted by purity; ITGAX, integrin subunit alpha X; NRP1, neuropilin 1; MHC, major histocompatibility complex; HLA-DAP1, MHC class II DP α 1; HLA-DRA, MHC, class II, DR α ; HLA-DQB1, MHC, class II, DQ β 1; HLA-DPB1, MHC, class II, DP β 1; PTGS2, prostaglandin-endoperoxide synthase 2; IRF5, interferon regulatory factor 5; NOS2, nitric oxide synthase 2; MS4A4A, membrane spanning 4-domains A4A; Ig, immunoglobulin; VSIG4, V-set and Ig domain containing 4; CSF1R, colony stimulating factor 1 receptor; KIR, killer cell Ig like receptor; KIR2DS4, KIR two Ig domains and short cytoplasmic tail 4; KIR3DL3, KIR Three Ig domains and long cytoplasmic tail 3; KIR3DL2, KIR three Ig domains and long cytoplasmic tail 2; KIR3DL1, KIR three Ig domains and long cytoplasmic tail 1; KIR2DL4, KIR two Ig domains and long cytoplasmic tail 4; KIR2DL3, KIR two Ig domains and long cytoplasmic tail 3; KIR2DL1, KIR two Ig domains and long cytoplasmic tail 1; CCR, C-C chemokine receptor; ITGAM, integrin subunit α M; CEACAM8, carcinoembryonic antigen-related cell adhesion molecule 8; CTLA4, cytotoxic T-lymphocyte antigen 4; LAG3, lymphocyte activating 3; HAVCR2, hepatitis A virus cellular receptor 2; GZMB, granzyme B; PDCD1, programmed cell death protein 1; CCL2, C-C motif chemokine ligand 2; TBX21, T-Box transcription factor 21; STAT, signal transducer and activator of transcription; IFNG, IFN gamma; GATA3, GATA binding protein 3; FOXP3, FOX P3; TGFB1, TGF- β 1; BCL6, B-cell lymphoma 6.

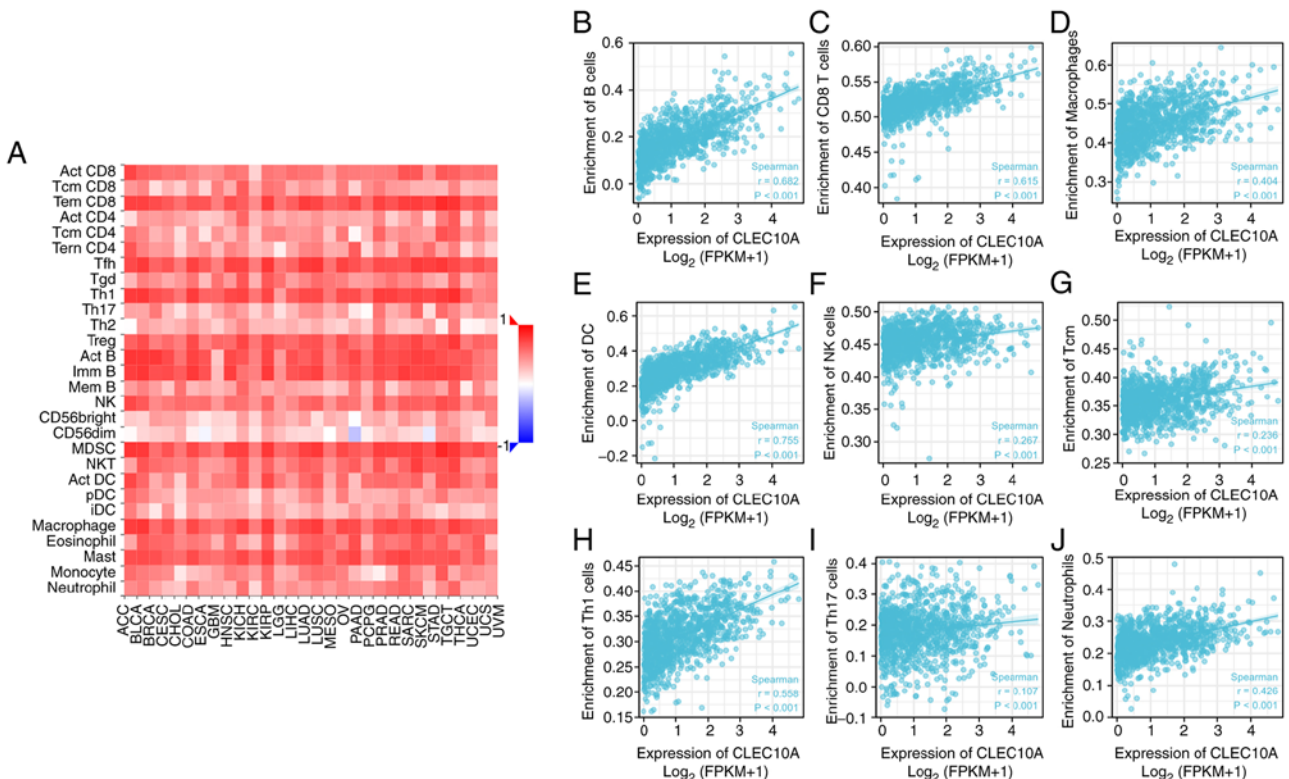


Figure 4. Association analysis of CLEC10A gene expression and immune infiltration using the Tumor And Immune System Interaction Database by Spearman's correlation analysis. (A) Relationships between the gene expression of CLEC10A and 28 types of tumor-infiltrating lymphocytes among a panel of heterogeneous human cancers. Correlation of CLEC10A gene expression with the degree of infiltration by (B) B, (C) CD8+ T, (D) macrophages, (E) dendritic, (F) NK, (G) Tcm, (H) Th1, (I) Th17 cells and (J) neutrophils. CLEC10A, C-type lectin domain family 10 member A; Tcm, T central memory; NK, natural killer; DC, dendritic cells; FPKM, fragments per kilobase per million.

requires multivalent ligands and a corresponding multivalent binder, such as a fragment of the mucin 1 protein, to provide

greater affinity to the receptor (27). In addition, the structure of the ligand can determine the cellular response, with large

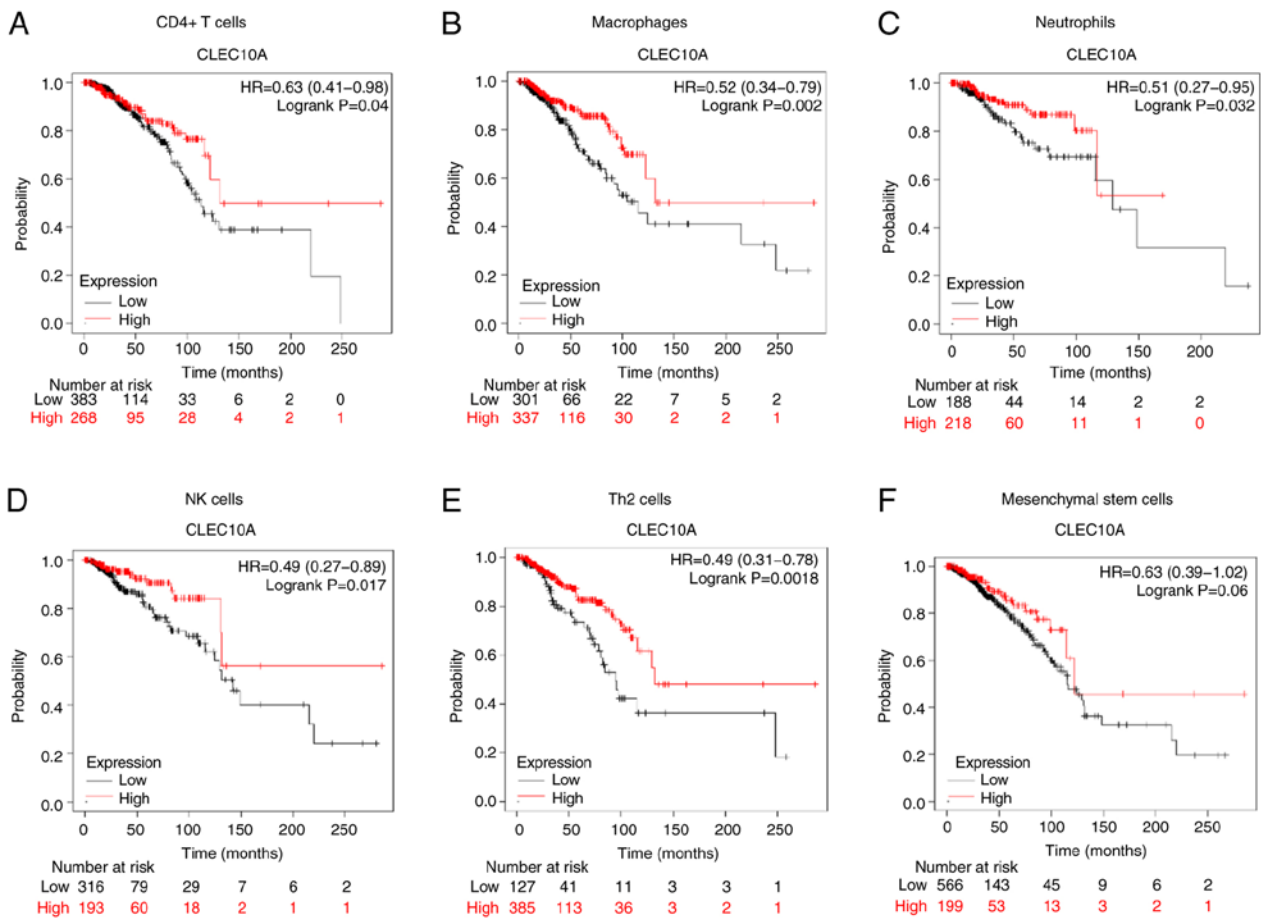


Figure 5. Comparison of Kaplan-Meier survival curves of the expression of CLEC10A in patients with breast cancer based on the immune cell subgroups. (A) CD4+ T cells. (B) Macrophages. (C) Neutrophils. (D) NK cells. (E) Th2 cells. (F) Mesenchymal stem cells. CLEC10A, C-type lectin domain family 10 member A; NK, natural killer; HR, hazard ratio.

Tn-bearing glycoproteins typically trapped in the endolysosomal compartment, whilst smaller glycopeptides tend to be processed further in the human leukocyte antigen (HLA) I/HLA II compartments (29). CLEC10A can bind to the Tn ligand to induce the maturation of CD4 and CD8 T cells to combat tumor progression (11,30).

Expression of markers of the immune system in BC were then analyzed. After cell purity correction, CLEC10A was found to be positively correlated with a wide range of immune cell markers in BC (Table II). These results implied further that CLEC10A is associated with immune infiltration in BC. Notably, increased CLEC10A expression level was positively correlated with Treg and exhausted T cell markers. There was also a significant correlation between CLEC10A expression levels and Th1 and Th2 markers in BC. These correlations may reflect the underlying mechanisms by which CLEC10A regulates T cell function in BC. Therefore, CLEC10A may confer favorable prognosis in patients with BC by recruiting and regulating immune cells.

The results of the KM plotter database analysis indicated that CD4 T cells, neutrophils, macrophages, NK cells Th2 cells infiltration and CLEC10A expression were significantly correlated with BC prognosis (Fig. 5). Tregs can suppress antitumor responses, leading to tumor immune escape. DCs can promote tumor metastasis by increasing the Treg cell population whilst decreasing the cytotoxicity of CD8 T cells (31-34). Previous

studies have also shown that the proportion of macrophages, CD8 T cells, Tregs and myeloid-derived suppressor cells in patients with BC is associated with poor prognosis (34). These results may explain why lower expression of CLEC10A can affect the prognosis of patients with BC through immune infiltration.

In summary, the present study found that lower expression levels of CLEC10A were significantly associated with poorer survival and immune infiltration in patients with BC through bioinformatics analysis. CLEC10A may be a potential novel biomarker for predicting prognosis in BC. Furthermore, the present study provides novel and promising insights for the further elucidation of significant clinicopathological factors and molecular pathogenesis mechanisms of BC. However, the mechanism by which CLEC10A regulates the TME and prognosis in BC remains unclear. Further *in vivo* and clinical studies are necessary to comprehensively elucidate the biological impact of CLEC10A in BC.

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Availability of data and materials

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

Authors' contributions

FC designed and managed the entire study. ST downloaded and analyzed the data and wrote the main manuscript. YZ, XL and HW helped with the statistical analysis. LY and HZ performed the RT-qPCR. FC and ST confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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