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ORIGINAL RESEARCH

Neuron-Specific Gene Family Member 1 is a Potential New Therapeutic Target Associated with Immune Cell Infiltration for Breast Cancer

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Background: Breast cancer (BC) is the most common cancer and is highly morphologically and molecularly heterogeneous. Neuron-specific gene family member 1 (NSG1) is a small single-channel transmembrane protein that consists of 185 amino acids and has been reported in a variety of tumours in recent years. However, the role of NSG1 in BC is unclear.

Objective: This study aimed to explore the role of NSG1 in the pathogenesis and development of BC and its potential as a prognostic marker for BC.

Methods: This study analysed data from The Cancer Genome Atlas database and the Gene Expression Omnibus database to determine the expression level and prognostic value of NSG1 messenger ribonucleic acid in BC. Using this data, we constructed a clinical risk model. Immunohistochemistry was performed in combination with a clinical cohort of 192 patients with BC to explore the NSG1 protein expression in BC. Enrichment analysis was used to predict the biological function of NSG1 in BC. To analyse the correlation between NSG1 and the BC immune microenvironment, a single-cell analysis of NSG1 expression and cells in BC was performed. Kaplan–Meier curves and Cox regression analysis were utilised to identify the relationship between the expression of NSG1 protein and clinicopathological features and prognosis.

Results: Neuron-specific gene family member 1 is highly expressed in patients with early BC, and its expression suggests a good prognosis for patients with BC. Neuron-specific gene family member 1 is involved in the T-cell receptor complex in BC and is associated with CD8 T cells in the BC immune microenvironment and may induce M1 polarisation of macrophages.

Conclusion: Neuron-specific gene family member 1 is a biomarker of good prognosis in BC. It is associated with the immune microenvironment of BC and may be a potential therapeutic target.

Keywords: neuron-specific gene family member 1, prognosis, immune microenvironment, single-cell ribonucleic acid sequencing, breast cancer

Introduction

Breast cancer (BC) is the most common cancer, with high morbidity and mortality rates and a significant economic impact on society, and it is the second leading cause of cancer deaths in women.[1] According to the National Cancer Database, 287,000 people have already been diagnosed with BC in 2022.[2] Breast cancer is a pathological state with a high degree of morphological and molecular heterogeneity, not only in BC cells but also in its tumour microenvironment, which consists of multiple cell types and states that influence its development.[3] Despite advances in early detection and treatment, patients with BC presenting with distant metastases are faring poorly, and the global burden has increased significantly. This trend is driven by factors such as late diagnosis, limited options for BC subtype therapy and drug resistance, which are the leading causes of BC-related deaths.[4] Breast cancer has different clinical manifestations due to the diversity of its mechanisms of action, which poses many challenges in selecting the appropriate treatment.[5]

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Neuron-specific gene family member 1 (NSG1) is a small single-channel transmembrane protein consisting of 185 amino acids. It has a molecular weight of 21 kDa and is localised on human chromosome 4p16.3; the protein is also known as D4S234E and NEEP21 because of its specificity for neuronal cells.[6,7] Neuron-specific gene family member 1 can play a complex biological function in the transport of β-amyloid precursors, α-amino-3-hydroxy-5-methyl-4-iso-xazolepropionic acid (AMPA)-type glutamate receptors GluR1 and GluR2, neurotensin receptors and adhesion proteins.[8–11] Initially, most studies on NSG1 were limited to neurological development, whereas in recent years, studies on the association between NSG1 and malignant tumours have gradually increased. The presence of abnormally upregulated NSG1 antibodies in early-stage oesophageal squamous cell carcinoma and colon cancer is considered an early diagnostic biomarker,[12–14] and similarly, NSG1 expression is much higher in stage I lung adenocarcinoma than in advanced stages.[15] Neuron-specific gene family member 1 can activate the extracellular signal-regulated kinase signalling pathway to promote epithelial-mesenchymal transition in oesophageal squamous cell carcinoma, leading to tumour progression.[16]

However, the relationship between NSG1 and the mammary gland has not been determined. In this study, we explore the role of NSG1 in the pathogenesis and development of BC and its potential as a prognostic marker for BC. We investigate the expression of NSG1 in BC using an online database and demonstrate NSG1 as a prognostic factor that is correlated with immune infiltrating in BC. We also explore the NSG1 expression in BC in conjunction with clinical cohort information, indicating that NSG1 is a diagnostic and potential therapeutic target in BC.

Methods

Bioinformatics Data Acquisition

Ribonucleic acid sequencing (RNA-seq) data and relevant clinical information for patients with BC were obtained from The Cancer Genome Atlas (TCGA) database (https://www.cancer.gov/tcga). Gene microarray data of patients with BC were obtained from the GSE139038 and GSE15852 datasets in the Gene Expression Omnibus (GEO) database (http://ww.ncbinlm.nih.gov/geo/).[17] All data were processed using R software (V4.3.0).

Analysis of Single-Cell Ribonucleic Acid Sequencing Data

The single-cell RNA-seq dataset GSE176078 of BC was obtained from the GEO website for subsequent analysis. Single-cell data were processed using an R package repair pull (21) workflow to normalise the data, and data quality was inferred by filtering single cells expressing <300 genes and cells with >25% of mitochondrial genes for unique molecular identifier and gene correlation analysis. Next, highly variable gene expression matrices were downscaled using principal component analysis, and then the JackStrawPlot and ElbowPlot functions were used to determine which principal components were selected for downstream analysis. A graph-clustering algorithm was used to visualise the clustering results in two dimensions using the uniform manifold approximation and projection algorithm. Cell clusters were annotated using the R "SingleR" package.

Analysis of the Differential Expression of Neuron-Specific Gene Family Member 1 Messenger Ribonucleic Acid in Breast Cancer

Based on the dataset obtained from the TCGA database, the expression levels of NSG1 mRNA in BC versus normal breast tissue were analysed by the *t*-test. Validation was also performed using the online database Gene Expression Profiling Interactive Analysis 2 (GEPIA2) database (http://gepia2.cancer-pku.cn/).

Analysis of the Prognostic Value of Neuron-Specific Gene Family Member 1 Messenger Ribonucleic Acid in Breast Cancer

Utilising the GEPIA2 database, overall survival (OS) and disease-free survival curves for NSG1 mRNA in BC were generated. Based on the Kaplan–Meier Plotter (http://kmplot.com/) databases, OS and recurrence-free survival (RFS) curves for NSG1 mRNA in BC were generated.[18] Using the TCGA database sequencing data, the NSG1 mRNA expression levels and clinicopathological characteristics were analysed using the chi-squared test or Wilcoxon signed-

rank test, and an assessment was made of whether NSG1 was a prognostic factor for BC using univariate and multi-factorial Cox regression. Receiver operating characteristic (ROC) curves were plotted based on the GEO database gene chip data. The analysis was performed using the R packages "survminer", "timeROC", "ggpubr" and "ComplexHeatmap".

Development and Evaluation of Column Line Diagrams

A prognostic model was developed to predict the OS of patients with BC, consisting of an NSG1 nomogram and a survival calibration curve. Additionally, a nomogram was constructed based on Cox regression analysis to predict the prognosis of patients with BC at 1, 3 and 5 years. This nomogram included prognostic factors such as gender, age and grading and was created using the R software package. To assess the predictive performance of the nomogram, calibration plots were used to evaluate its calibration, and a consistency index was employed to quantify its discriminatory power. These analyses were carried out to ensure the accuracy and reliability of the nomogram as a prognostic tool for patients with BC. The analysis was performed using the R packages "survival", "regplot", "rms" and "survcomp".

Analysis of Differential Genes, Pathways and Biological Functions of Neuron-Specific Gene Family Member 1 in Breast Cancer

The "Limma" R package generated a list of differential genes in low-expression and high-expression NSG1. The adjusted *p*-value of <0.05 and $|\log_2(\text{fold change})| > 1$ were set as the thresholds to determine differential expression genes. Additionally, the R packages "clusterProfiler", "org.Hs.eg.db", "enrichplot" and "ggplot2" were applied to Gene Ontology (GO) and Kyoto Encyclopedia of Gene Genomes (KEGG) pathway enrichment analysis of differential expression genes. The GO enrichment analysis included the biological process, cellular component and molecular function categories, with the top 20 terms visualised in a bubble plot. The KEGG enrichment analysis was conducted ($p < 0.05$), and gene set enrichment analysis (GSEA) was performed with 1000 gene set permutations, considering terms with false discovery rate < 0.001 and *p*-value < 0.25. These analyses aimed to uncover molecular mechanisms between the two risk groups.

Analysis of Neuron-Specific Gene Family Member 1 and Breast Cancer Immune Microenvironment Correlation

The immune microenvironment of BC was investigated by analysing transcriptomic data with the CIBERSORT algorithm,[19] which can accurately characterise the relative scores of different cell subsets in tissue by analysing the gene expression profile of the tissue. This allowed the quantification of the proportions of 20 immune cell types and a comparison between the two groups based on the median values of the NSG1 gene. Additionally, the relationship between NSG1 gene expression and immune cells, as well as immune checkpoints, was explored. To provide a comprehensive evaluation of immune cell infiltration in BC, data mining of the Tumor Immune Estimation Resource v2.0 (TIMER2.0) employs a pathological examination-validated statistical methodology to determine the abundance of tumour-infiltrating immune cells. Therefore, TIMER2.0 was used to study the association between NSG1 expression and the abundance of tumour-infiltrating immune cells in BC. The Tumor-Immune System Interactions database (TISIDB), which pre-calculates the association between any gene and immune characteristics through literature mining and high-throughput data analysis, was used to assess the relevance of NSG1 to the abundance of tumour-infiltrating immune cells in BC comprehensively. These analyses shed light on the immune response in BC and its potential connection with NSG1 expression.

Breast Cancer Tissue Samples and Clinical Cohort

A total of 192 BC tissue samples and 10 BC peritumoural tissue samples were collected in this study, all from patients who underwent surgical treatment for BC at the Affiliated Hospital of Hebei Engineering University between January 2014 and January 2016. The inclusion criteria were as follows: (1) patients with BC whose surgery was able to completely remove the primary tumour lesion and metastatic lymph nodes, (2) all patients were pathologically

diagnosed as invasive ductal carcinoma of any subtype after surgery, (3) all patients had received standardised adjuvant therapy after surgery. According to the patient's condition, different adjuvant treatments are selected, mainly including chemotherapy, radiotherapy, endocrine therapy and targeted therapy, (4) all patients had not received any neoadjuvant therapy and (5) all patients had complete clinical information and follow-up data for 5 years after surgery. The exclusion criteria were as follows: (1) stage IV patients with BC, (2) combination with other malignant tumours, (3) combination with severe mental illness, (4) combination with autoimmune diseases. The study and experimental methods were approved by the Ethics Committee of the Affiliated Hospital of Hebei Engineering University (Approval Number: No.2021[K]047), and written informed consent was signed by all participants in this study in accordance with the Declaration of Helsinki.

The RFS was defined as the duration from the date of the initial pathological diagnosis to the occurrence of a BC-related event, the last follow-up visit or death. Breast cancer-related events encompassed local recurrence, distant metastases, the development of new primary breast tumours and death resulting from BC. The OS was defined as the duration from the initial diagnosis of the tumour to either death from any cause or the last follow-up visit.

Immunohistochemical Analysis

In this study, immunohistochemical (IHC) analysis was performed to investigate the expression levels of NSG1 protein in BC tissue samples. Briefly, paraffin-embedded tissue sections were deparaffinised and rehydrated, followed by antigen retrieval in a citrate buffer solution. Sections were incubated in 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity and in 5% bovine serum albumin for 30 min to block nonspecific binding. Sections were then incubated overnight at 4°C with a primary antibody against NSG1 protein (1:150) (Affinity, DF4242), incubated with a secondary antibody and detected with a Dako REALTM EnVisionTM Detection System (ZSGB-BIO, ZLI-9017). Counterstaining was performed using haematoxylin. The negative control consisted of replacing the primary antibody with a nonimmune serum. Immunostaining results were assessed by two independent pathologists in a blinded manner and graded according to the intensity of staining (0 [no staining]; 1 [weak yellow]; 2 [brownish yellow]; 3 [tan]) and according to the percentage of positive tumour cells (0 [<5%; 1 [5%–25%]; 2 [26%–50%); 3 [51%–75%]; 4 [>75%]). The final score is the product of the staining intensity score and the percentage of positive tumour cells score. A final score of 0–3 was defined as a "0 score", 3–6 as a "1 score", 6–9 as a "2 score" and 9–12 as a "3 score". For the analysis, "0 score" and "1 score" were considered negative expressions, and "2 score" and "3 score" were considered positive expressions.

The BC molecular typing approach was categorised with reference to the 2013 St. Gallen International Expert Consensus.[20]

Statistical Methods

The clinical data were analysed using SPSS 24.0 (IBM, Chicago, USA). For count data, one-way logistic regression analysis was conducted. The prognostic value of NSG1 was assessed by Kaplan–Meier curves and Log rank tests. Univariate Cox regression analysis was performed to identify statistically significant factors, and these factors were included in the multivariate analysis using the entry method. Hazard ratios (HRs) and their corresponding 95% confidence intervals (CIs) were calculated using Cox regression analysis. A significance level of $p < 0.05$ was used for the two-tailed test.

Results

Expression Levels of Neuron-Specific Gene Family Member 1 Messenger Ribonucleic Acid in Breast Cancer

The analysis of the NSG1 mRNA expression levels in 1093 BC samples and 112 normal breast samples using the TIMER database showed that NSG1 mRNA expression was significantly downregulated in breast cancer ($p < 0.001$, Figure 1A). The TCGA database demonstrated the same results, with NSG1 mRNA expression being abnormally downregulated in BC, much less than in peritumoural tissues ($p < 0.001$, Figure 1B). To verify the diagnostic performance of NSG1 mRNA in BC, ROC curves were constructed from two datasets of the GEO database, and the

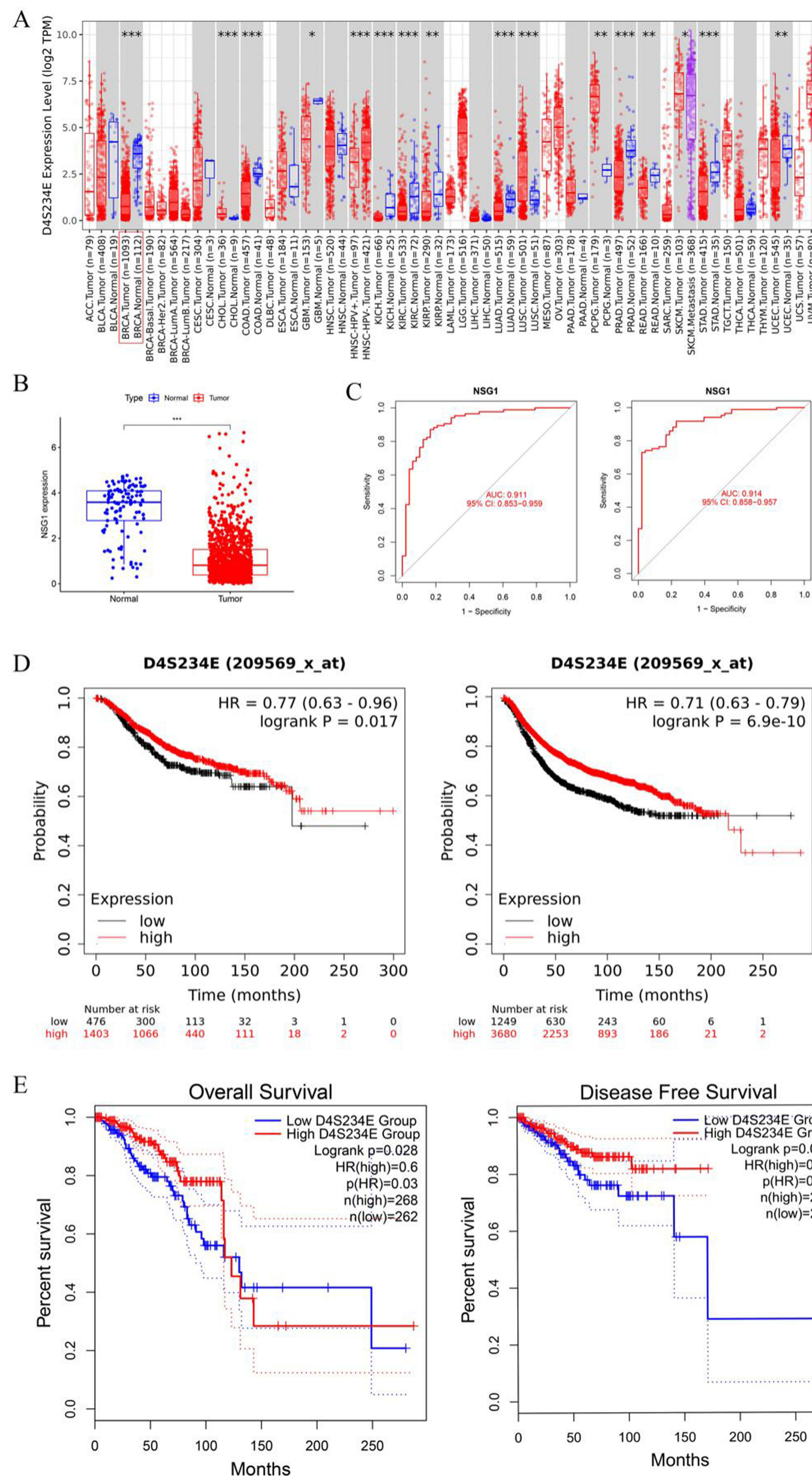

Figure 1 Expression value of neuron-specific gene family member 1 (NSG1) mRNA in breast cancer (BC). (**A**) Analysis of NSG1 mRNA expression level based on TIMER database (*indicates p<0.05;**indicates p<0.01;***indicates p<0.001). (**B**) Analysis of NSG1 mRNA expression level based on the Cancer Genome Atlas (TCGA) database. (**C**) Construction of receiver operating characteristic (ROC) curve of NSG1 in BC based on the Gene Expression Omnibus (GEO) database. (**D**) Plotting based on Kaplan-Meier Plotter NSG1 survival curves in BC. (**E**) Plotting NSG1 survival curves in BC based on Gene Expression Profiling Interactive Analysis 2(GEPIA2).

results showed that NSG1 had a significant diagnostic value in BC, with AUC1 = 0.911 (95% CI: 0.853–0.959) and AUC2 = 0.914 (95% CI: 0.858–0.957) (Figure 1C). The survival curves of NSG1 in BC were then plotted using the Kaplan–Meier Plotter database and showed that patients with BC in the NSG1 high-expression group had better OS and RFS results ($p < 0.05$, Figure 1D). Survival analysis of the GEPIA2 database showed the same results (Figure 1E). This evidence suggests that NSG1 is a potential biomarker of good prognosis in BC.

Neuron-Specific Gene Family Member 1 Messenger Ribonucleic Acid Expression, Breast Cancer Clinical Analysis and Clinical Risk Model Construction

Based on the TCGA database, an analysis was conducted of the association between NSG1 mRNA expression levels and various clinicopathological characteristics of BC, including age, gender, T stage, N stage and molecular classification (Figure 2A). The findings revealed that NSG1 mRNA expression levels were significantly higher in patients aged <65 years with BC. Additionally, patients with stage 1 BC exhibited elevated levels of NSG1 expression compared with those with stage 4 ($p < 0.05$).

The heatmap depicted a notable correlation between NSG1 mRNA expression and the age, gender, T stage and molecular classification of patients with BC ($p < 0.05$, Figure 2B). Specifically, the heatmap illustrated a higher frequency of patients aged ≤65 years with stage 1 BC in the NSG1 high expression group.

One-way Cox regression (Figure 2C) showed that NSG1 was a favourable prognostic indicator of BC ($p < 0.001$, HR = 0.623), and a subsequent analysis using multiway Cox regression (Figure 2D) also showed that NSG1 was a protective factor for BC ($p < 0.005$, HR = 0.668). Subsequently, a clinical risk model was developed via a nomogram. By integrating NSG1 expression with the clinical parameters of patients with BC, a nomogram was constructed to predict the probability of survival at 1, 3 and 5 years for the entire cohort of patients with BC (Figure 2E), with a C-index of 0.843 for the column line graph and nomogram area under the curve (AUC) predictions of 0.991, 0.950 and 0.904 for 1-, 3- and 5-year OS, respectively. Additionally, the calibration plots showed good agreement with the predicted and observed values for the probability of survival at 1, 3 and 5 years (Figure 2F). In summary, these findings suggest that the column line plots accurately predicted 1-, 3- and 5-year survival in patients with BC.

Expression Value of Neuron-Specific Gene Family Member 1 Protein in Breast Cancer

A total of 192 breast invasive ductal carcinoma samples and 10 cases of peritumoural tissue (Table 1) were included in this study for IHC staining. Neuron-specific gene family member 1 protein was mainly expressed in the cytoplasm of BC samples (Supplementary Figure 1A), with 90 positive expressions and 102 negative expressions, and the positivity rate was 46.88%. Neuron-specific gene family member 1 protein expression was detected in all 10 cases of peritumoural tissues (Supplementary Figure 1B), with a positive rate of 100%.

The correlation between NSG1 and the clinicopathological characteristics of patients with BC was analysed using one-way logistic regression (Table 2), and the results showed that the expression level of NSG1 was negatively correlated with the pathological tumour-node-metastasis (pTNM) stage, T category, HER2+ and triple-negative breast cancer (TNBC) subtype ($B < 0$, $p < 0.05$) and positively correlated with the ER status ($B > 0$, $p < 0.05$) of patients with BC. Subsequently, the survival curves of NSG1 protein were constructed based on the follow-up information from the clinical cohort patients, and the results showed that patients with BC with positive NSG1 protein expression had better OS (HR = 0.490 [0.250–0.961], $p = 0.046$) (Supplementary Figure 1C) and RFS (HR = 0.438 [0.254–0.754], $p = 0.005$) (Supplementary Figure 1D). The Cox regression analysis, as shown in Table 3, identified several factors influencing RFS and OS in BC. For RFS, the grade, pTNM stage, T category, LN metastasis and TNBC subtype were identified as risk factors, whereas PR status and NSG1 status emerged as protective factors. Regarding OS, risk factors included the pTNM stage, T category and TNBC subtype, whereas PR status and NSG1 status were found to be protective factors.

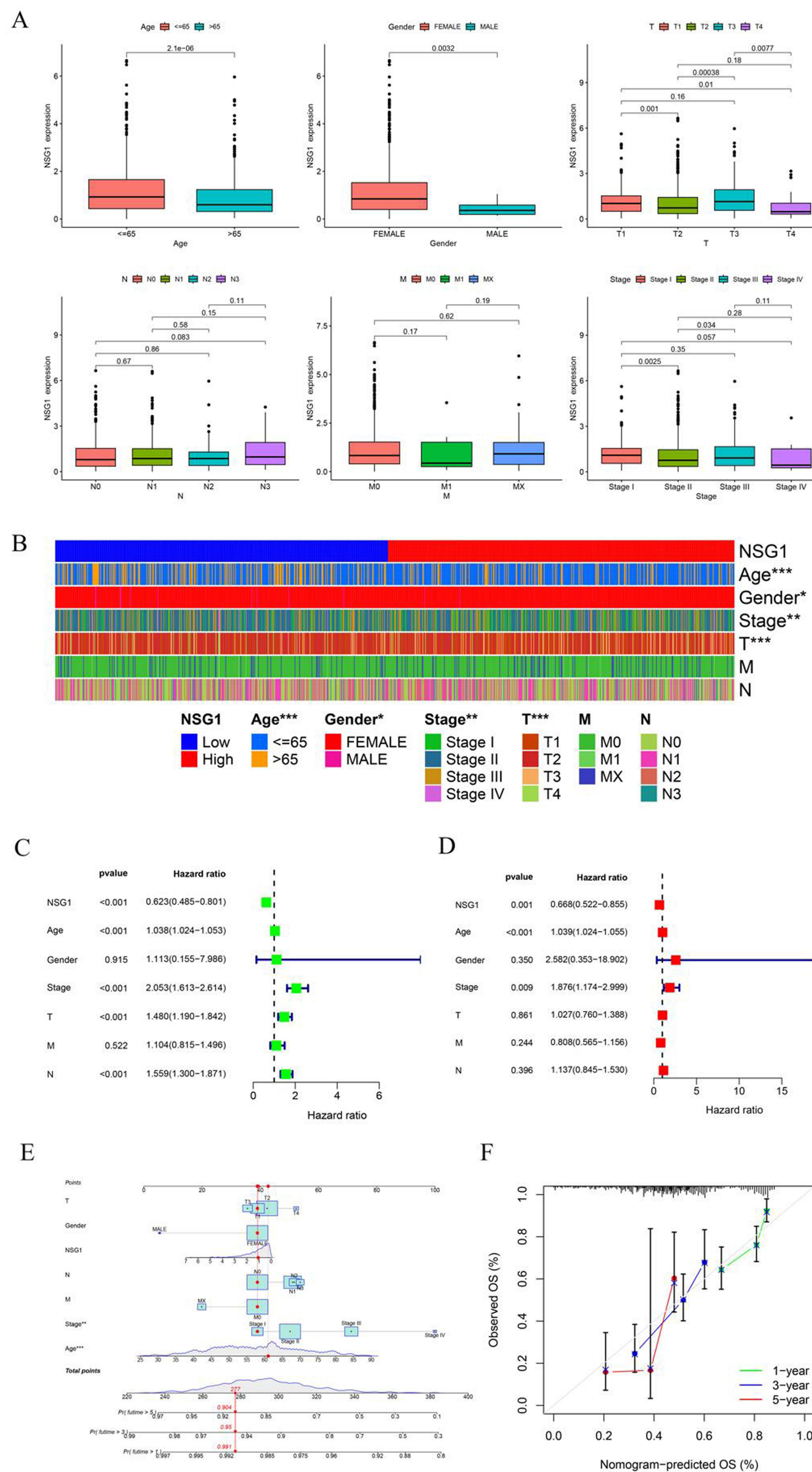

Figure 2 Neuron-specific gene family member 1 (NSG1) mRNA and breast cancer (BC) clinical analysis. (**A**) The box plot illustrates the association between NSG1 mRNA expression and the age, gender, T, N and M categories and molecular classification of BC patients. (**B**) The heat map demonstrates the correlation between NSG1 mRNA expression and the clinical characteristics of BC (* indicates p<0.05; ** indicates p<0.01; *** indicates p<0.001). (**C**) Single-factor COX regression analysis. (**D**) Multi-factor COX regression. (**E**) Construction of clinical risk model by nomogram. (**F**) Construction of calibration chart based on nomogram.

Table 1 NSG1 Protein Expression Profile and BC Clinicopathological Features (N=192)

Clinicopathologic Characteristics	N	NSG1(+)	NSG1(-)
Race			
asian	192(100%)	90(46.9%)	102(53.1%)
Age			
>60	79(41.1%)	37(19.3%)	42(21.9%)
<60	113(58.9%)	53(27.6%)	60(31.3%)
Grading			
1	28(14.6%)	13(6.8%)	15(7.8%)
2	62(32.3%)	30(15.6%)	32(16.7%)
3	102(53.1%)	47(24.5%)	55(28.6%)
pTNM stage			
I	54(28.1%)	34(17.7%)	20(10.4%)
II	105(54.7%)	47(24.5%)	58(30.2%)
III	33(17.2%)	9(4.7%)	24(12.5%)
Category T			
T1	69(35.9%)	42(21.9%)	27(14.1%)
T2	96(50.0%)	40(20.8%)	56(29.2%)
T3	20(10.4%)	7(3.6%)	13(6.8%)
T4	7(3.6%)	1(0.5%)	6(3.1%)
LN metastasis			
0	108(56.3%)	57(29.7%)	51(26.6%)
1	84(43.8%)	33(17.2%)	51(26.6%)
Subtype			
LA	65(33.9%)	40(20.8%)	25(13.0%)
LB	53(27.6%)	24(12.5%)	29(15.1%)
HER2+	42(21.9%)	14(7.3%)	28(14.6%)
TN	32(16.7%)	12(6.3%)	20(10.4%)
ER status			
Positive	114(59.4%)	61(31.8%)	53(27.6%)
Negative	78(40.6%)	29(15.1%)	49(25.5%)
PR status			
Positive	104(54.2%)	55(28.6%)	49(25.5%)
Negative	88(45.8%)	35(18.2%)	53(27.6%)
HER2 status			
Positive	95(49.5%)	38(19.8%)	57(29.7%)
Negative	97(50.5%)	52(27.1%)	45(23.4%)

Abbrevations: pTNM stage, refer to AJCC 8th edition; T, tumor size; LN, lymph node involvement; ER, estrogen receptor; PR, progesterone receptor; HER2, Human epidermal growth factor receptor 2; LA, Luminal A; LB, Luminal B (include hormone-positive and/or HER2-positive cases); HER2+, HER2 over expression; TN, Triple-negative.

Biological Functions of Neuron-Specific Gene Family Member 1 in Breast Cancer

The genes were grouped according to the median value of NSG1 expression in the TCGA database, and the top 50 genes with the most significant variations were identified to display in the heatmap (Supplementary Figure 2A).

Subsequently, based on the relevant differentially expressed genes, GO analysis was performed (Supplementary Figure 2B), which showed that NSG1 is mainly involved in leukocyte migration (GO:0050900), the immune response-regulating cell surface receptor signalling pathway (GO:0002768), the T-cell receptor complex (GO:0042101), the plasma membrane signalling receptor complex (GO:0098802), receptor–ligand activity (GO. 0048018) and the structural constituents of the skin epidermis (GO:0030280).

In addition, to further explore the function of NSG1 in BC, GSEA enrichment analysis was performed. The GSEA GO analysis (Supplementary Figure 2C) showed that NSG1 is mainly involved in the T-cell receptor complex and

Table 2 One-Way Logistic Regression Analysis of Correlation Between NSG1 Protein Expression and Pathological Characteristics of BC (N=192)

Clinicopathologic Characteristics	B	P	HR	95% CI
Year	−0.003	0.993	0.997	0.561–1.774
Grading		0.958		
2	0.079	0.863	1.082	0.442–2.645
3	−0.014	0.974	0.986	0.426–2.281
pTNM stage		0.006		
II	−0.741	0.031	0.477	0.243–0.934
III	−1.511	0.002	0.221	0.086–0.567
T category		0.020		
T2	−0.778	0.016	0.459	0.244–0.863
T3	−1.061	0.045	0.346	0.123–0.978
T4	−2.234	0.044	0.107	0.012–0.940
LN metastasis	−0.547	0.064	0.579	0.325–1.032
Subtype		0.022		
LB	−0.659	0.079	0.517	0.248–1.080
HER2+	−1.163	0.005	0.313	0.139–0.705
TN	−0.981	0.028	0.375	0.157–0.898
ER status	0.665	0.027	1.945	1.080–3.503
PR status	0.530	0.071	1.700	0.957–3.020
HER2 status	−0.550	0.060	0.577	0.325–1.023

Abbrevations: pTNM stage, refer to AJCC 8th edition; T, tumer size; LN, lymph node involvement; ER, estrogen receptor; PR, progesterone receptor; HER2, Human epidermal growth factor receptor 2; LB, Luminal B (include hormone-positive and/or HER2-positive cases); HER2+, HER2 over expression; TN, Triple-negative.

Table 3 Univariate COX Regression Analysis of RFS, OS and Clinicopathological Characteristics of NSG1 (N=192)

Factor	Recurrence Free Survival				Overall Survival			
	B	P	HR	95% CI	B	P	HR	95% CI
Year	−0.227	0.430	0.797	0.453–1.401	−0.402	0.273	0.669	0.326–1.372
Grading		0.010				0.315		
2	1.185	0.119	3.270	0.738–14.493	9.927	0.885	–	–
3	1.828	0.012	6.222	1.499–25.822	10.515	0.878	–	–
pTNM stage		0.000				0.003		
II	0.891	0.049	2.438	1.003–5.923	0.613	0.229	1.845	0.681–5.002
III	2.095	0.000	8.129	3.258–20.278	1.602	0.003	4.963	1.747–14.098
T stage		0.000				0.021		
T2	1.114	0.005	3.046	1.396–6.646	0.890	0.057	2.436	0.973–6.101
T3	1.529	0.002	4.612	1.730–12.295	1.466	0.011	4.332	1.396–13.439
T4	2.541	0.000	12.686	4.389–36.671	1.896	0.007	6.658	1.664–26.646
LN metastasis	0.972	0.001	2.643	1.502–4.651	0.504	0.193	1.655	0.775–3.536
Subtype		0.191				0.174		
LB	0.197	0.609	1.218	0.572–2.591	0.509	0.313	1.663	0.619–4.465
HER2+	0.388	0.324	1.475	0.682–3.189	0.751	0.136	2.120	0.789–5.692
TN	0.807	0.036	2.242	1.053–4.771	1.093	0.030	2.982	1.110–8.009
ER status	−0.391	0.159	0.677	0.393–1.166	−0.557	0.105	0.573	0.292–1.124

(*Continued*)

Table 3 (Continued).

Factor	Recurrence Free Survival				Overall Survival			
	B	P	HR	95% CI	B	P	HR	95% CI
PR status	−0.689	0.015	0.502	0.289–0.874	−0.999	0.006	0.368	0.179–0.755
HER2 status	−0.026	0.926	0.975	0.566–1.679	0.154	0.654	1.167	0.595–2.288
NSG1 status	−0.827	0.006	0.437	0.243–0.788	−0.713	0.052	0.490	0.239–1.006

Abbrevations: pTNM stage, refer to AJCC 8th edition; T, tumer size; LN, lymph node involvement; ER, estrogen receptor; PR, progesterone receptor; HER2, Human epidermal growth factor receptor 2; LA, Luminal A; LB, Luminal B (include hormone-positive and/or HER2-positive cases); HER2+, HER2 over expression; TN, Triple-negative; NSG1, Neuron-specific gene family member 1.

mitochondrial translation processes. The GSEA KEGG analysis (Supplementary Figure 2D) revealed that NSG1 is mainly involved in oxidative phosphorylation and the spliceosome complex.

Neuron-Specific Gene Family Member 1 and the Breast Cancer Immune Microenvironment

Based on the median grouping of NSG1 expression and immune score, the results showed that the stromal score, estimate score and immune score were higher in the NSG1 high-expression group than in the NSG1 low-expression group ($p < 0.05$, Figure 3A). In addition, the differential levels of 20 immune cell types were compared between the NSG1 high- and low-expression groups (Figure 3B). Further analysis of the correlation between NSG1 expression levels and immune cell infiltration showed that NSG1 expression was positively correlated with naïve B cells, plasma cells, CD8 T cells and M1 macrophages and was negatively correlated with M2 macrophages, M0 macrophages and natural killer (NK) resting cells (Figure 3C). Subsequently, the correlation between NSG1 and immune checkpoints was analysed, and the results showed that NSG1 was associated with immune checkpoints, such as CD40, CD28 and TNFSF14 (Figure 3D).

Neuron-Specific Gene Family Member 1 Expression and Cells in Breast Cancer Were Examined by Single-Cell Analysis

An analysis was performed on RNA-seq data from the GSE176078 dataset, and 2000 highly variable genes were screened based on quality control criteria (Figure 4A). The cell clusters were clustered into six groups and manually annotated by corresponding markers (Figure 4B), and the six clusters were merged into five cell groups according to marker gene expression (Figure 4C) for CD8 T cells, Treg cells, macrophages, NK cells and dendritic cells. In addition, the correlation of NSG1 with related representative genes was analysed (Figure 4D), and the results showed that NSG1 was most closely associated with CD3D and CD3E.

Discussion

To our knowledge, the present study is the first report on the NSG1 expression level in BC. In this study, we analysed several dimensions, including NSG1 mRNA expression, protein expression, clinical analysis, downstream function prediction, immune microenvironment and single-cell data, and the results showed that NSG1 is a marker of good BC prognosis and is associated with the immune microenvironment of BC.

In this study, we analysed the expression level and prognostic value of NSG1 mRNA in BC through a large sample analysis in the database. The results showed that NSG1 mRNA has good predictive value for the OS and RFS of patients with BC. We observed that the expression level of NSG1 mRNA is associated with the age and tumour stage of patients with BC. Both univariate and multivariate Cox regression analyses showed that NSG1 mRNA is a protective factor for patients with BC. Considering the potential differences between gene transcription levels and protein levels, we combined clinical cohorts to detect the expression of NSG1 protein in patients with BC through immunohistochemistry. Similarly, our results showed a negative correlation between NSG1 protein and tumour stage in the clinical cohort of patients with BC. Previous studies have shown that, except for steroids, most substances required for growth,

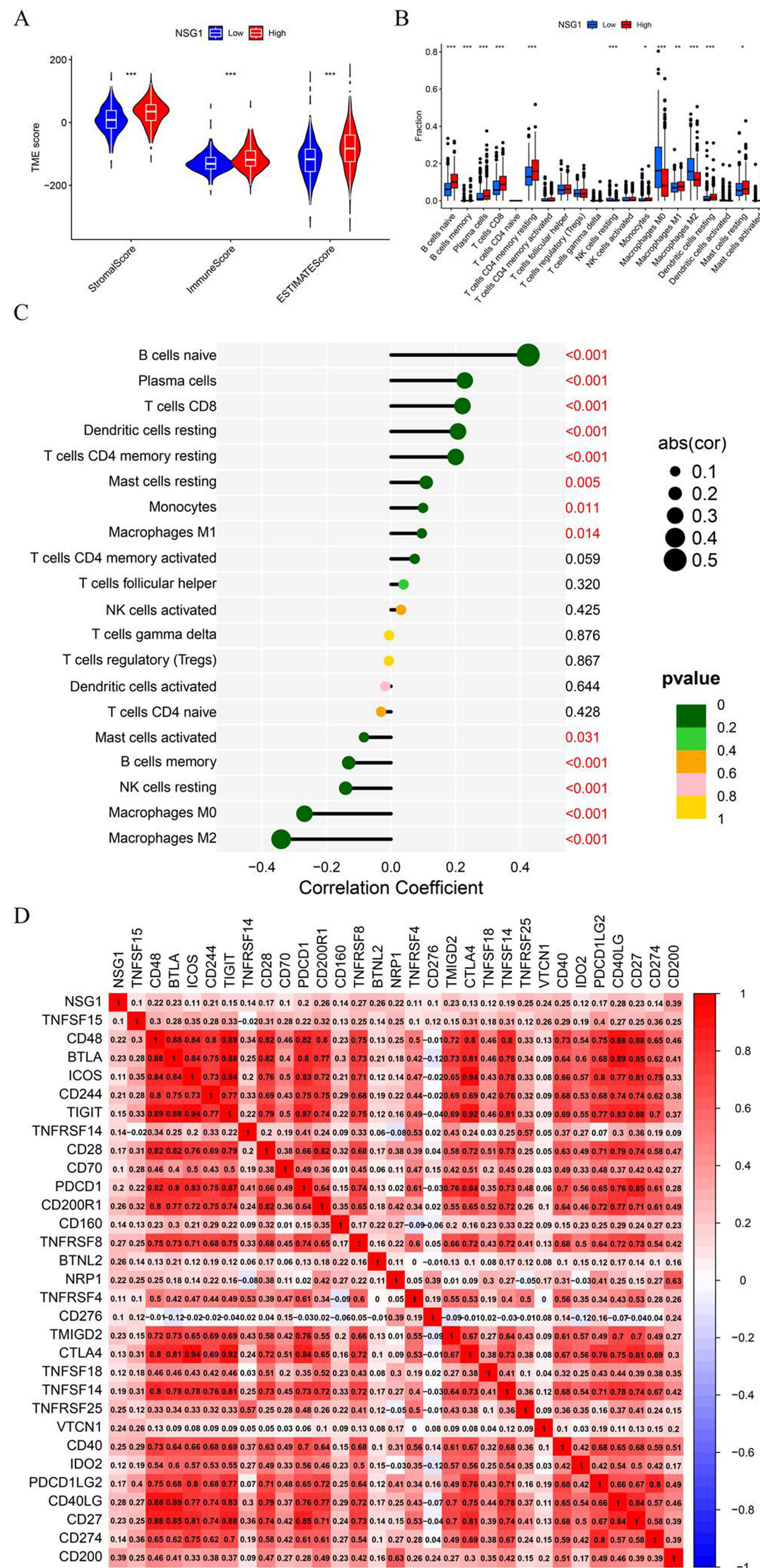

Figure 3 Analysis of neuron-specific gene family member 1 (NSG1) and the immune microenvironment in breast cancer (BC). (**A**) Immunoscore of high and low levels of NGS1 expression (***indicates p<0.001). (**B**) Differences in immune cell composition between high and low levels of NGS1 expression for 20 immune cell types (*indicates p<0.05; **indicates p<0.01; ***indicates p<0.001). (**C**) Correlation between NSG1 and tumor immune cell infiltration. D. Correlation between NSG1 and immune checkpoint expression.

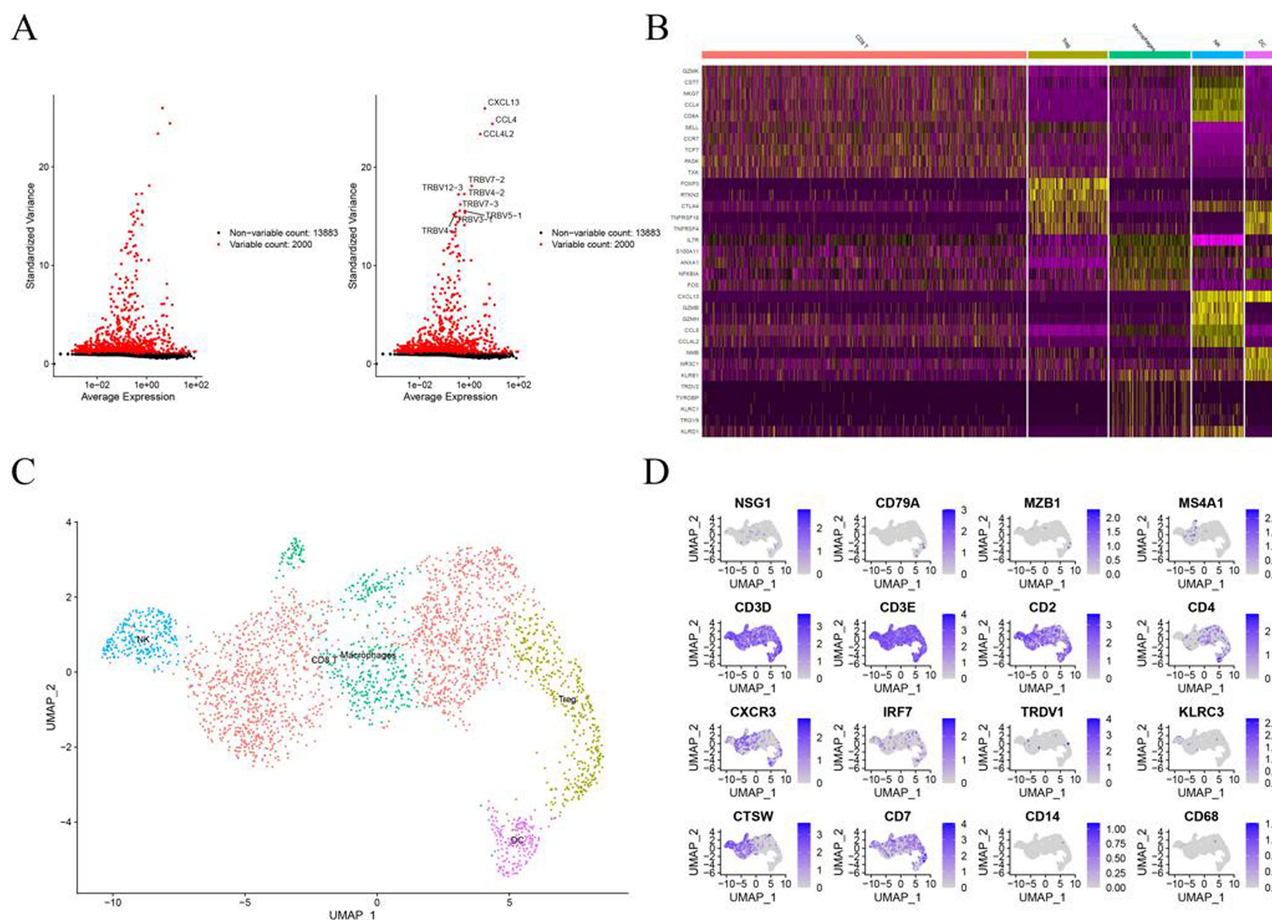

Figure 4 Neuron-specific gene family member 1 (NSG1) single cell analysis. (**A**) Variable gene screening and labeling. (**B**) Heatmap showing the relationship between immune cell markers and 6 groups of cell populations. (**C**) Spatial distribution of 5 cell populations. (**D**) Feature map showing the expression levels and distribution of representative marker genes for each cell population on the UMAP map.

proliferation and signal transduction cannot penetrate hydrophobic membranes. Endocytosis is a prerequisite for all cell signalling, and NSG1 can participate in regulating cell growth and development through endocytosis, and its expression has certain temporal dynamics.[21,22] Similarly, higher expression levels of NSG1 have also been observed in early-stage lung adenocarcinoma and hypopharyngeal squamous cell carcinoma cancer.[15,23] This suggests that NSG1 may contribute to the regulation of tumour development stages.

In the clinical cohort, we observed that the expression level of NSG1 is related to the oestrogen receptor (ER) status in patients with BC. Furthermore, NSG1 can interact directly with glutamate receptor-interacting protein 1 (GRIP1) to regulate the intracellular trafficking of AMPA-type glutamate receptors.[24] Interestingly, GRIP1 is an important regulatory factor for the activity of ER alpha, and it also has certain anti-proliferative functions in BC cells.[25] Neuron-specific gene family member 1 regulates the sorting and recycling of membrane receptors by forming complexes with transferrin or neurotensin receptor 1 (NTSR-1) and neurotensin receptor 2. Overexpression of NSG1 can alter the transport of NTSR-1 from non-transcriptional levels, while low expression of NSG1 significantly reduces NTSR-1 levels.[26] Neurotensin receptor 1 can inhibit the invasive and migratory capabilities of tumour cells.[27] Additionally, NSG1 is one of the ligands of the family with sequence similarity 171 member A1 and can participate in regulating cell shape and invasiveness.[28] Neuron-specific gene family member 1 could be a novel candidate participating in organelle sensor-initiated apoptosis. Genotoxic stress also induces secondary endoplasmic reticulum stress and eventually induces apoptosis.[29] Kudoh et al found knockdown of NSG1 diminished DNA damage-induced CHOP expression, suggesting the possibility that NSG1 is, at least in part, involved in endoplasmic reticulum stress.[30] A recent report demonstrated that NSG1 localises in the

early endosome of the neuronal cells. Neuron-specific gene family member 1 facilitates vesicular transport of AMPA receptor subunit GluR2 and mediates GluR2 surface expression by cooperating with GRIP1.[24] These findings indicate that NSG1 functions as a carrier protein. Based on these findings, we suspect that NSG1 transports apoptosis signals to several intracellular organelles as an adaptor protein via sorting and transporting apoptosis-related molecules to the endoplasmic reticulum in response to DNA damage. A study reported that mutant p53 enhanced the recycling of epidermal growth factor receptor (EGFR) and integrin, leading to constitutive activation of EGFR/integrin signalling.[31] Therefore, p53 may regulate endocytosis of EGFR and integrin via the transcriptional activation of NSG1, resulting in the degradation of these oncogenic receptors. If this is true, NSG1 is a novel p53 target that mediates p53-dependent endocytic degradation of oncogenic receptors, representing a new mechanism for p53-regulated tumour suppression. Further investigation on the role of NSG1 in p53-regulated endocytosis is urgently required.

Tumour protein p53 is a tumour suppressor gene commonly mutated in various malignancies, including BC. Mutations in the gene lead to changes in the expression of various genes directly or indirectly controlled by p53 transcription, resulting in faulty DNA damage repair pathways, cell cycle arrest, chromatin remodelling and apoptosis.[32] Neuron-specific gene family member 1 is reported to be a transcriptional target of p53 based on the observation that some non-neuronogenous cancer cells can express NSG1 in a p53-dependent manner under the effect of hydrogen peroxide, doxorubicin, ultraviolet and γ-ray.[33] The enforced expression of NSG1 suppresses colony formation of p53-deficient cancer cell lines by inducing apoptosis.[33] Furthermore, p53 can bind to the NSG1 promoter, regulate its expression after DNA damage and inhibit apoptosis.[30] These results suggest that NSG1 plays a critical role in apoptosis as a novel transcriptional target of tumour suppressor p53. Therefore, treatment methods such as doxorubicin chemotherapy and γ-ray radiotherapy can promote NSG1 expression and further induce apoptosis of BC tumour cells.

To delve deeper into the potential biological functions of NSG1 in BC, this study identified through GO and GSEA GO analysis that NSG1 primarily participates in substance delivery and immune response in BC. Consequently, we proceeded to investigate the relationship between NSG1 and the immune microenvironment of BC. The immune score in the NSG1 high-expression group was higher than that in the NSG1 low-expression group in BC, implying that NSG1 may be closely associated with the BC immune microenvironment. In addition, we observed that NSG1 expression was positively correlated with CD8+ T cells and M1 macrophages and negatively correlated with M2 macrophages. The accumulation of CD8 T cells, which are killer cells that destroy tumour cells, in tumour immune infiltration is associated with a good prognosis.[34] In the BC immune microenvironment, macrophage polarisation patterns can be divided into M1 activation and M2 activation; an increase in M1 macrophages is associated with reduced tumour aggressiveness, whereas an increase in M2 macrophages stimulates tumour growth.[35] In the analysis of NSG1 and associated immune checkpoints, we observed a positive association of NSG1 expression with CD28. In contrast, CD28 is an oncogene and a major costimulatory molecule of T cells that enhances the activation and proliferation of T cells.[36] Since BC is highly heterogeneous, we further explored the association of NSG1 with cells by single-cell analysis. In the BC dataset of NSG1 expression, we observed substantial expression of CD8 T cells consistent with immune infiltration cell analysis. From our results, a plausible explanation is that NSG1 can inhibit BC progression by affecting CD8 T-cell expression in the BC immune microenvironment and by inducing M1 directional polarisation of macrophages. Based on our research results and theoretical support, we speculate that NSG1 may play a role as a "tumour suppressor gene" in BC. Several previous studies have explored the value of NSG1 in predicting the response to immunotherapy in cancer patients.[37,38] Zhu Y et al constructed an NSG1-based model to predict the response to immunotherapy in ovarian cancer patients.[37] Du Y et al used the NSG1 gene to construct a predictive model for evaluating the response to immunotherapy and antineoplastic therapy in metastatic uveal melanoma.[38] Therefore, future studies could construct genetic models associated with CD8+ T lymphocytes to identify patients with BC who can benefit from immunotherapy and predict their prognosis.

However, our study also has certain limitations. It is a retrospective study and may have potential selection bias. Therefore, it may be necessary to include samples from multiple centres for validation. Additionally, further experimental evidence is needed to confirm the biological function of NSG1 in BC. Finally, future studies are expected to incorporate more information related to survival to further confirm the impact of NSG1 on BC prognosis. Neuron-specific gene family member 1 is a highly promising potential prognostic factor in BC and may be involved in regulating the immune microenvironment of BC.

Conclusion

Neuron-specific gene family member 1 is a biomarker of good prognosis in BC. It is associated with the immune microenvironment of BC and is a potential biomarker or therapeutic target for BC. Neuron-specific gene family member 1 is associated with the expression of CD8 T cells and may induce M1 polarisation in macrophages, thus exerting an anti-cancer effect.

Data Sharing Statement

The data that support the findings of this study are openly available on the TCGA database and the GEO database.

Ethics Approval and Consent to Participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Affiliated Hospital of Hebei Engineering University (Approval Number: No.2021[K]047), and written informed consent was signed by all participants in this study.

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Disclosure

The authors report no conflicts of interest in this work.

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