

Clinical Relevance and Prognostic Value of the Neuronal Protein Neuroligin 2 in Breast Cancer

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Research

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Abstract

Background: Neuroligin 2 (NLGN2) is a well-recognized transmembrane scaffolding protein that functions in synapse development and neuronal signal transduction. Interestingly, NLGN2 has recently been implicated involved in multiple diseases of peripheral ectodermal origin. However, its potential roles in tumor genesis and progression remain ill-defined. The aim of this study was to determine the clinical relevance and prognostic value of NLGN2 in breast cancer.

Methods: Breast cancer datasets were extracted from TCGA, THPA and other public databases, and survival analyses were performed using the Kaplan-Meier method. The immunological relevance of NLGN2 and its subcellular localization were also investigated by cross-analyses. The results of the *in silico* outcome were further validated by immunohistochemistry analyses using in-house tumor tissue specimens.

Results: NLGN2 was identified as a prognostic factor in breast cancer subtypes, and its high expression levels were correlated to a favorable survival outcome. Moreover, NLGN2 overexpression in the breast cancer was significantly associated with larger size, lymph node metastasis, late TNM stage and high histological grade. Interestingly, there was a significant correlation between the expression level of NLGN2 and the immunomodulatory molecules, as well as increased interstitial infiltration of lymphocytes. Additionally, NLGN2 was observed predominantly localized in the mitochondria of breast cancer cells.

Conclusions: NLGN2 has a prognostic role and immunoregulatory potential in breast cancer, and its functions likely have a mitochondrial basis. It is a promising therapeutic target in breast cancer and should be explored further.

Background

The incidence of breast cancer has increased steadily from 2005 to 2016 due to a combination of lifestyle-related factors and genetic predisposition [1, 2]. It currently accounts for 30% of all newly diagnosed cancers, and is the leading cause of cancer-related mortality among females [3]. Notwithstanding, recent advances in early diagnosis and treatment strategies have improved the 5-year survival rate of breast cancer patients to 89% [4]. In addition, molecular typing of breast tumor cells is increasingly being used to predict the clinical characteristics and prognostic outcomes of each subtype of breast cancer [5]. However, the current typing approaches and treatment strategies have been largely ineffective against advanced stage IV breast cancer as well as the highly invasive clinicopathological subtypes, such as the inflammatory type, with high mortality and fatality rates. Therefore, it is crucial to identify novel diagnostic and prognostic molecular markers for refractory breast cancer in order to predict patient prognosis with greater precision, accuracy and reproducibility, and improve the clinical outcome [6, 7].

Neuroligin 2 (NLGN2) is one of the most important post-synaptic neural cell adhesion proteins and is associated with the formation, maturation and function of synapses [8]. The neurological functions of

NLGN2 are well documented, and mainly depend on its interactions with the members of the neurexin (NRXN) family [9–13]. Moreover, mutations or genetic variations in NLGN2 have been reported in several neurocognitive diseases, such as motor incoordination, social impairment, aggression, schizophrenia, anxiety, depression and intellectual disability [9, 14–16]. Interestingly, there is emerging evidence of non-neurological functions of NLGN2 in the peripheral tissues. For instance, Zhang et al reported that the NLGN2 expressed on pancreatic beta-cells promotes normal insulin secretion through transcellular interactions [17]. In addition, Yang et al. found that down-regulation of NLGN2 in the ganglion colon segment is associated with excessive intestinal contraction and increased risk of Hirschsprung disease [18]. However, the potential involvement of NLGN2 in peripheral tumorigenesis and progression remains poorly identified so far.

In this study, we analyzed the potential role of NLGN2 in breast cancer, which is under partial neuroendocrine neoplastic growth. We assessed the expression levels and prognostic values of NLGN2 in the genome and proteome datasets of breast cancer, and validated the *in silico* bioinformatics results with in-house patient tissue samples. In brief, we investigated the relevance of NLGN2 to overall survival of patients in different breast cancer subtypes, its correlation with local immunomodulatory molecules and cells, as well as its intracellular localization.

Methods

Patient tissue sample collection

Fifty paraffin-embedded breast tumor specimens were collected from patients that underwent surgery at the High-tech district of the First Affiliated Hospital of Anhui Medical University (Hefei, Anhui, China) between 2017 and 2020. Each patient provided written informed consent, and the study was approved by the institutional review board.

Kaplan-Meier survival analysis

The prognostic values of NLGN2 at mRNA level in breast cancer were analyzed using the Kaplan-Meier Plotter (KM Plotter, <http://kmplot.com/analysis>) [19], an online database of gene expression profiles and overall survival (OS) data of cancer patients. The respective patient cohorts, including all BRCA cases available on KM Plotter, were divided into the NLGN2^{hi} and NLGN2^{low} groups based on the median mRNA expression levels, and their survival rates and duration were analyzed using the Kaplan-Meier survival plots. Hazard ratio (HR), 95% confidence interval (95% CI), and log-rank *p* value were calculated, and *p* < 0.05 was considered statistically significant.

Gene expression profiling interactive analysis

The correlation between NLGN2 expression and multiple immune signatures were assessed by Gene Expression Profiling Interactive Analysis (GEPIA, <http://gepia2.cancer-pku.cn>, version 2) [20]. The raw RNA-Seq data downloaded from TCGA and GTEx databases were processed with the UCSC Xena project

following a standard analysis pipeline to avoid data imbalance and ineffective differential analyses. The pair-wise correlations were calculated by Spearman analysis, and $p < 0.05$ was considered statistically significant.

Immunohistochemistry analysis

The in situ expression of NLGN2, CD3 and CD8 in paraffin-embedded breast tumor tissue sections were examined by immunohistochemistry as previously described [21], using rabbit polyclonal antibodies against NLGN2 (1:200, bs-11098R, Bioss), CD3 (1:1000, 60181-1-Ig, Proteintech) and CD8 (1:1000, 66868-1-Ig, Proteintech) respectively. Five random fields were viewed per slide under high power. The NLGN2 staining intensity in the tumor cells (graded 0–3) and the percentage of stained cells (0 - no tumor cells positive; 1 - 10%–25% positive cells, 2 - 25%–50%, and 3 > 50%) were recorded, and multiplied to obtain the staining index ranging from 0 to 9 [22]. Samples with staining indices 0-3 and >3 were respectively designated as NLGN2^{low} and NLGN2^{hi}. The number of infiltrating CD3 and CD8 positive cells in the tumor stroma were scored (1 - 0 ~ 25, 2 - 26 ~ 50, 3 - 51 ~ 75, and 4 > 75) [23], and the samples were classified as low expressing and high expressing based on positive cell count of 1 and 2 ~ 4 respectively.

Subcellular location analysis by THPA

The NLGN2 immunofluorescence images of multiple cell lines were acquired from The Human Protein Atlas (THPA, <http://www.proteinatlas.org>) [24], after authorization by the HPA team for the use for specific and scientific publication.

Statistical analysis

SPSS22.0 was used for data analysis. Chi-square test was used to compare variables, and the correlation between factors was assessed by the Spearman method. $P < 0.05$ was considered statistically significant.

Results

NLGN2 overexpression is favorable for the survival of breast cancer patients

To determine the prognostic relevance of NLGN2 in breast cancer, we analyzed the survival patterns of the NLGN2^{hi} and NLGN2^{low} groups for each subtype. As shown in Fig. 1A, the NLGN2^{hi} patients had significantly longer overall survival compared to the NLGN2^{low} group (HR, 0.59; 95%CI, 0.5 to 0.69; $p < 0.05$), including those post-treated (HR, 0.51; 95%CI, 0.41 to 0.64; $p < 0.05$) (Fig. 1B). The favorable prognostic function of NLGN2 was also confirmed for the basal (HR, 0.72; 95%CI, 0.52 to 1; $p < 0.05$) (Fig. 1C), luminal A (HR, 0.65; 95%CI, 0.5 to 0.83; $p < 0.05$) (Fig. 1D), and luminal B (HR, 0.54; 95%CI, 0.39 to 0.73; $p < 0.05$) (Fig. 1E) subtypes. In contrast, higher expression of NLGN2 was not conducive to the survival of the HER2 + breast cancer patients (HR, 0.59; 95%CI, 0.5 to 0.69; $p > 0.05$) (Fig. 1F). Taken

together, elevated NLGN2 indicates favorable prognosis for breast cancer patients with the basal, luminal A, and luminal B phenotypes.

The prognostic value of NLGN2 for specific molecular subtypes of breast cancer

To further determine the clinical pertinence of NLGN2 in breast cancer, we assessed its prognostic performance in different intrinsic subtypes with or without the estrogen receptor (ER), progesterone receptor (PR) and Erb-B2 receptor tyrosine kinase 2 (HER2) expression. NLGN2 overexpression was not associated with prognosis in the ER negative (-) patients (HR, 0.97; 95%CI, 0.7 to 1.36; $p > 0.05$) (Fig. 2A) and PR- patients (HR, 1.07; 95%CI, 0.75 to 1.53; $p > 0.05$) (Fig. 2B), but associated with favorable prognosis in the HER2- patients (HR, 0.7; 95%CI, 0.52 to 0.95; $p < 0.05$) (Fig. 2C) and not associated with the unfavorable prognosis in ER-/PR-/HER2- patients (HR, 1.18; 95%CI, 0.68 to 2.04; $p > 0.05$) (Fig. 2D). In addition, there was no significant correlation between NLGN2 and OS in patients with wild type tumor protein p53 (TP53) (HR, 1.46; 95%CI, 0.63 to 3.42; $p > 0.05$) (Fig. 2E) or mutated TP53 (HR, 0.87; 95%CI, 0.48 to 1.58; $p > 0.05$) (Fig. 2F). Thus, upregulation of NLGN2 is associated with better prognosis in HER2-breast cancer as opposed to other molecular subtypes.

NLGN2 is favorable in breast cancer patients without lymph node metastasis

Given the relevance of clinical staging and pathological grading in the survival of breast cancer patients, we next analyzed the relationship between NLGN2 expression and the clinicopathological grades. NLGN2 was identified as a favorable prognostic factor in the lymph node non-metastatic (-) patients (HR, 0.65; 95%CI, 0.44 to 0.97; $p < 0.05$) (Fig. 3A), but not in the lymph node metastatic (+) patients (HR, 0.92; 95%CI, 0.72 to 1.19; $p > 0.05$) (Fig. 3B). Surprisingly, the expression of NLGN2 was not correlated with the survival of patients diagnosed as Grade 1 (HR, 0.73; 95%CI, 0.25 to 2.1; $p > 0.05$) (Fig. 3C), Grade 2 (HR, 1.18; 95%CI, 0.71 to 1.97; $p > 0.05$) (Fig. 3D), and Grade 3 (HR, 0.81; 95%CI, 0.6 to 1.11; $p > 0.05$) (Fig. 3E). Taken together, NLGN2 correlates to the clinical staging of breast cancer as opposed to pathological grading.

NLGN2 positively correlates with the immunomodulatory signature in breast cancer

Since immune regulation is a key factor in cancer progression, we also analyzed the potential influence of NLGN2 on breast cancer immunity to better understand its prognostic role. Intriguingly, NLGN2 expression was closely related to levels of critical immune effector molecules, including IFNG (Fig. 4A) and GZMB (Fig. 4B). At the cellular landscape, NLGN2 correlated significantly with signatures of crucial subpopulations of tumor-infiltrating lymphocytes [25], including but not limited to cytotoxic T cells (CD3/CD8) (Fig. 4C), helper T cells (CD3/CD4) (Fig. 4D), B cells (CD19/CD20) (Fig. 4E), macrophages (CD14/CD11b/HLA-DR) (Fig. 4F), NK cells (CD16/CD56/NKG2D) (Fig. 4G), and dendritic cells (CD135/Flt3/CD117/CD26/CD103) (Fig. 4H). These findings strongly indicate an immune system-dependent role of NLGN2 in breast cancer.

NLGN2 is associated with clinicopathological features and tumor infiltrating CD3 + and CD8 + T lymphocytes in breast cancer

To validate the *in silico* prognostic data of NLGN2 in breast cancer, we firstly analyzed its in-situ expression levels in fifty in-house patient tissue samples. NLGN2 was highly expressed in 80% (40/50) of the tumor samples, and was significantly associated with larger size, lymph node metastasis, late TNM stage and high histological grade (all $p < 0.05$), but not with patient age or the expression levels of ER, PR and HER2 (all $p > 0.05$) (Table 1). Moreover, 84% (42/50) of the samples exhibited high CD3 + T lymphocyte infiltration and 74% (37/50) displayed high CD8 + T lymphocyte infiltration. Furthermore, NLGN2 expression was significantly correlated with the interstitial infiltration of both CD3 + and CD8 + T lymphocytes (both $p < 0.01$) (Table 2). The representative images of NLGN2, CD3 and CD8 immunohistochemical staining were shown in Fig. 5. These findings further underscore the close association between NLGN2 expression in breast cancer and the clinicopathological features as well as lymphocytes infiltration.

Table 1
The relationship between NLGN2 expression and the clinicopathological features of breast cancer

Parameter	n	NLGN2		P value
		Low expression	High expression	
Age (years)				0.84
< 50	17	4(23.5%)	13(76.5%)	
≥ 50	33	6(18.2%)	27(81.8%)	
Tumor size (cm)				0.03
< 5	42	10(23.8%)	32(76.2%)	
≥ 5	8	0(0%)	8(100%)	
Lymph node metastasis				0.03
+	25	2(8%)	23(92%)	
-	25	8(32%)	17(68%)	
Grade				< 0.01
I or II	26	8(30.8%)	18(69.2%)	
III	24	2(8.3%)	22(91.7%)	
Stage				< 0.01
I or II	40	4(10%)	36(90%)	
III	10	6(60%)	4(40%)	
ER				0.07
+	38	9(23.7%)	29(76.3%)	
-	12	1(8.3%)	11(91.7%)	
PR				0.32
+	35	8(22.9%)	27(77.1%)	
-	15	2(13.3%)	13(86.7%)	
HER2				0.32
0 ~ 1 +	20	5(25%)	15(75%)	
2+ ~ 3 +	30	5(16.7%)	25(83.3%)	
ER, PR, and HER2 data were obtained from patients' pathology records.				

Table 2
The relationship between NLGN2 expression and CD3 + and CD8 + tumor infiltrating lymphocytes

	n	Tumor infiltrating		<i>P</i> value	Tumor infiltrating		<i>P</i> value
		CD3 + lymphocyte			CD8 + lymphocyte		
		Low level	High level		Low level	High level	
NLGN2				< 0.01			< 0.01
Low expression	10	5(50%)	5(50%)		8(80%)	2(20%)	
High expression	40	3(7.5%)	37(92.5%)		5(12.5%)	35(87.5%)	

NLGN2 is located in the mitochondria of peripheral breast cancer cells

As shown in Fig. 5, the CD3 + and CD8 + lymphocytes were mainly distributed in the interstitial tissue, but intriguingly, NLGN2 was primarily localized to the cytoplasm rather than the membrane of normal and malignant breast epithelial cells. Given the spatial distribution largely dictates the precise function and mechanism of proteins, more detailed position of NLGN2 was further investigated in breast cancer cells. Interestingly, NLGN2 expression was predominantly localized in the mitochondria of the MCF7 breast cancer cell line (Figure S1A). Moreover, the unusual positioning of NLGN2 in mitochondria was also confirmed in several additional tumor and normal cell lines, including U2OS (Figure S1B), U251MG (Figure S1C), and NIH3T3 (Figure S1D) cell lines. Mitochondrion is a major determinant of cancer cell growth and patient survival due to its pivotal roles in metabolite transport, energy production, apoptosis induction, and the immune stimulation [26]. We therefore hypothesize that the mitochondrial localization of NLGN2 is instrumental to its prognostic role in breast cancer, and should be explored further.

Discussion

Although neurological involvement in cancer development is widely accepted, the underlying molecular mechanisms remain to be elucidated. Studies show that neural cell adhesion molecules that mediate signaling transduction in the nervous system are also associated with the genesis and progression of multiple cancer types, not merely limited to cancers in nervous system. For instance, the L1 cell adhesion molecule (L1CAM), an axonal glycoprotein involved in neuronal migration and differentiation, is an oncogene that is overexpressed in colon and ovarian cancers, and associated with increased invasion and poor prognosis [27–29]. We found that the neuronal protein NLGN2 was upregulated in breast cancer samples and correlated to higher survival rates, likely due to its correlation with a favorable immune signature. We also analyzed the prognostic relevance of NLGN2 with respect to other classification criteria (data not shown), but did not observe any significant correlation.

The mitochondrion is increasingly being recognized as a key player in cancer cell metabolism, differentiation, proliferation and survival [30–33]. Mitochondrial dysfunction alters energy metabolism, blocks apoptosis and increases production of reactive oxygen species (ROS), and thus is closely associated with tumor initiation and progression. In addition, mitochondria are also involved in anti-cancer immune surveillance and immunotherapeutic response. It is rational to surmise therefore that the mitochondrial location of NLGN2 is critical for its prognostic role in breast cancer. Taken together, the close association between NLGN2, mitochondria and immune signatures in breast cancer indicates that NLGN2 may play an immunoregulatory role by reversing mitochondrial dysfunction. To summarize, this study reveals the prognostic function of NLGN2 in breast cancer as well as its immunological relevance and mitochondrial location. NLGN2 may contribute to the therapeutic efficacy of breast cancer treatment in a mitochondria-mediated immune system-dependent manner. Nevertheless, in addition to an observational study, specific mechanism remains to be defined in order to consider NLGN2-targeted therapy against breast cancer in the foreseeable future.

Conclusions

To our knowledge, this is the first study to prospectively evaluate the prognostic value of NLGN2 in breast cancer. NLGN2 expression level in breast tumors is associated with molecular subtypes, metastatic status, immunomodulatory signature, and lymphocyte infiltration. The prognostic role of NLGN2 can be attributed to its mitochondrial location, and the mechanism warrants further investigation.

Abbreviations

NLGN2: neuroligin 2; NRXN: neurexin; KM Plotter: Kaplan-Meier Plotter; OS: overall survival; HR: hazard ratio; GEPIA: Gene Expression Profiling Interactive Analysis; THPA: The Human Protein Atlas; ER: estrogen receptor; PR: progesterone receptor; HER2: Erb-B2 receptor tyrosine kinase 2; TP53: tumor protein p53; L1CAM: L1 cell adhesion molecule.

Declarations

Ethics approval and consent to participate

The acquisition of patient tissue samples and all procedures used in this study were approved by the Ethics Committee of Anhui Medical University. Informed consent was obtained from each patient, and the specimen usage was in line with the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

The results shown in this study are based on TCGA Research Network (<https://www.cancer.gov/tcga>), THPA (v18.1.proteinatlas.org), and IHC staining. All datasets analyzed during the current study are available in TCGA (<http://cancergenome.nih.gov>) and THPA (<http://www.proteinatlas.org>). All other data generated during the current study are included in this published article. Further information is available from the corresponding authors upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

X.H. conceived this study. X.H., G.Z. and Y.S. collected the data. X.H. and Z.W. analyzed and interpreted the data. X.H. and G.Z. wrote and revised the manuscript. Y.S. and Z.W. discussed the manuscript and provided inputs. All authors have read and approved the final version. G.Z. and Y.S. contributed equally to the study. X.H. and Z.W. supervised the study and share the senior authorship.

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Figures

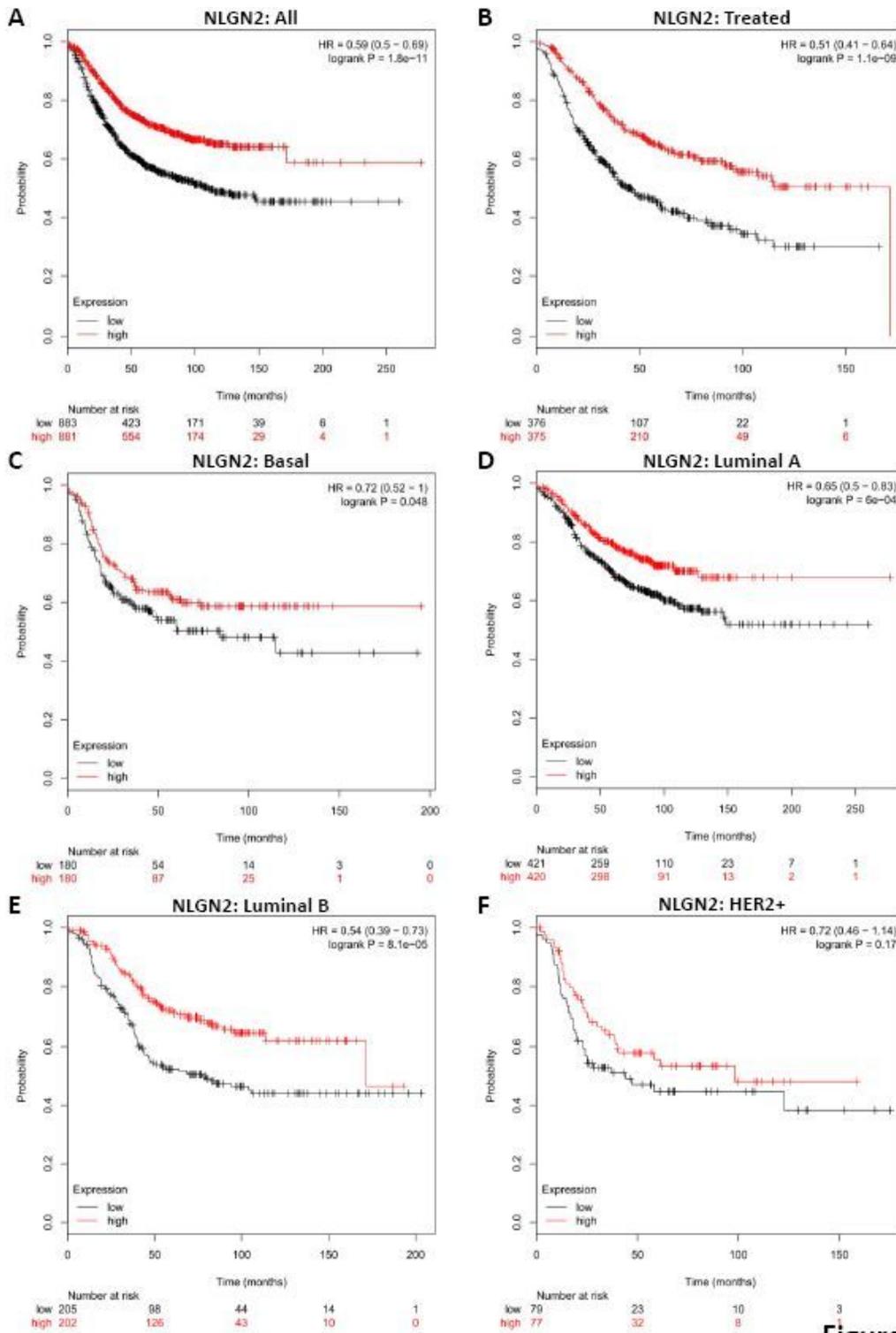


Figure 1

Figure 1

NLGN2 is a prognostic factor of breast cancer. (A) Prognostic analysis of NLGN2 in breast cancer. (B) Prognostic analysis of NLGN2 in post-treated breast cancer. (C) Prognostic analysis of NLGN2 in basal breast cancer. (D) Prognostic analysis of NLGN2 in luminal A breast cancer. (E) Prognostic analysis of NLGN2 in luminal B breast cancer. (F) Prognostic analysis of NLGN2 in HER2+ breast cancer. The HR and log rank p value are indicated in each panel, and $p < 0.05$ is statistically significant.

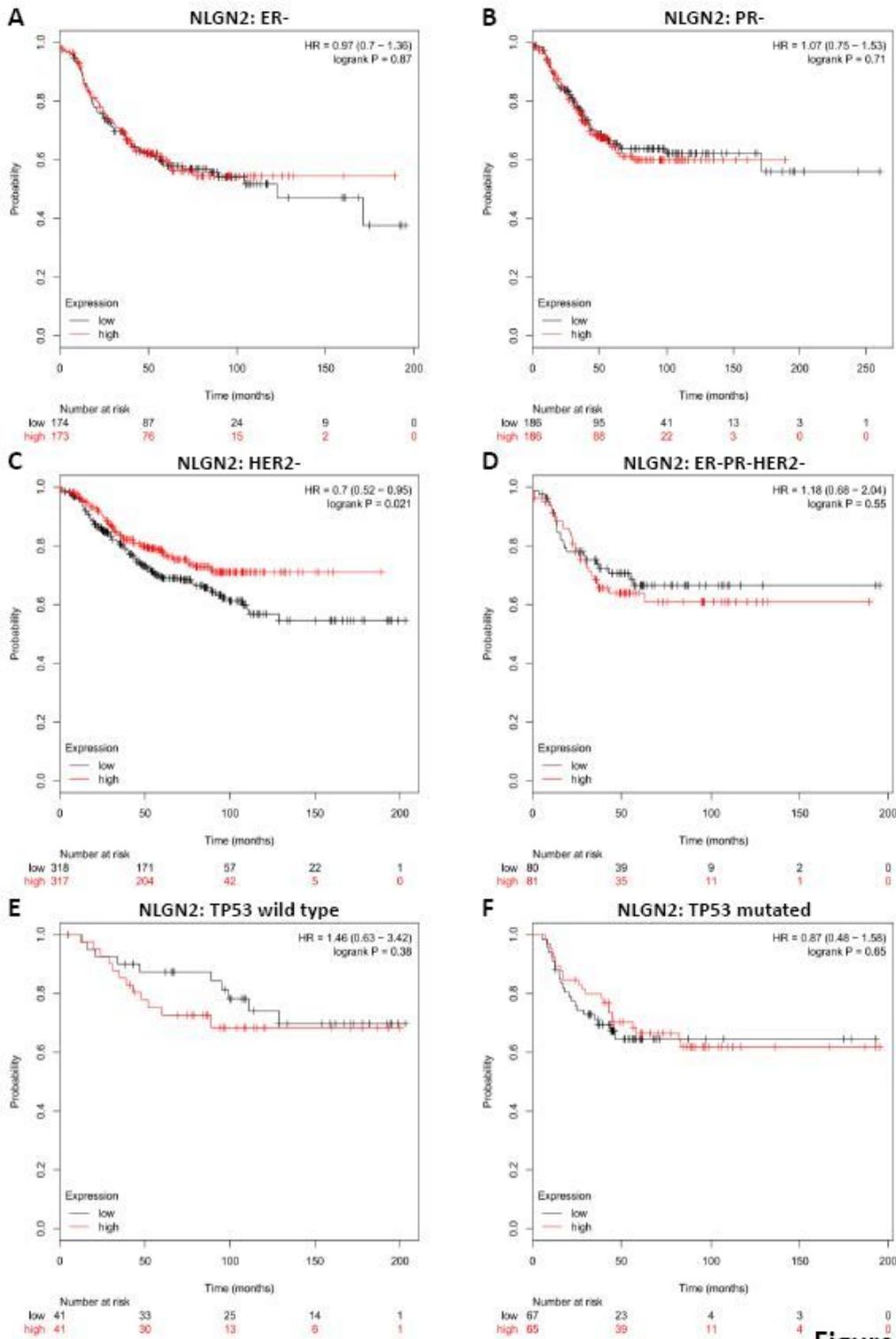


Figure 2

Figure 2

NLGN2 is favorable for HER- breast cancer patients. (A) Prognostic analysis of NLGN2 in ER- breast cancer. (B) Prognostic analysis of NLGN2 in PR- breast cancer. (C) Prognostic analysis of NLGN2 in HER2- breast cancer. (D) Prognostic analysis of NLGN2 in ER-/PR-/HER2- breast cancer. (E) Prognostic analysis of NLGN2 in TP53 wild type breast cancer. (F) Prognostic analysis of NLGN2 in TP53 mutated

breast cancer. The HR and log rank p value are indicated in each panel, and $p < 0.05$ is statistically significant.

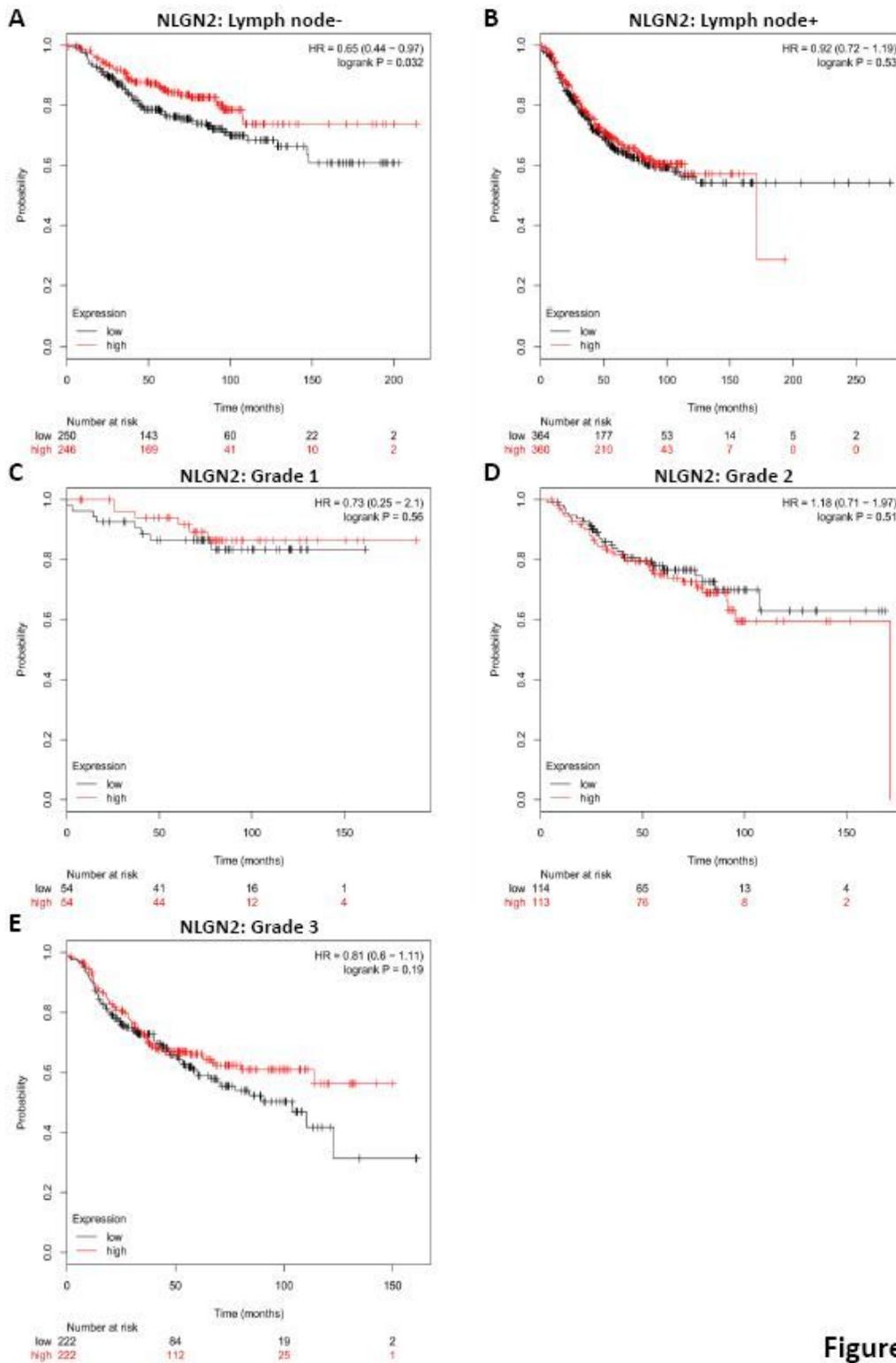


Figure 3

Figure 3

NLGN2 is favorable in lymph node non-metastatic breast cancer patients. (A) Prognostic analysis of NLGN2 in lymph node- breast cancer. (B) Prognostic analysis of NLGN2 in lymph node+ breast cancer. (C) Prognostic analysis of NLGN2 in Grade 1 breast cancer. (D) Prognostic analysis of NLGN2 in Grade 2

breast cancer. (E) Prognostic analysis of NLGN2 in Grade 3 breast cancer. The HR and log rank p value are indicated in each panel, and $p < 0.05$ is statistically significant.

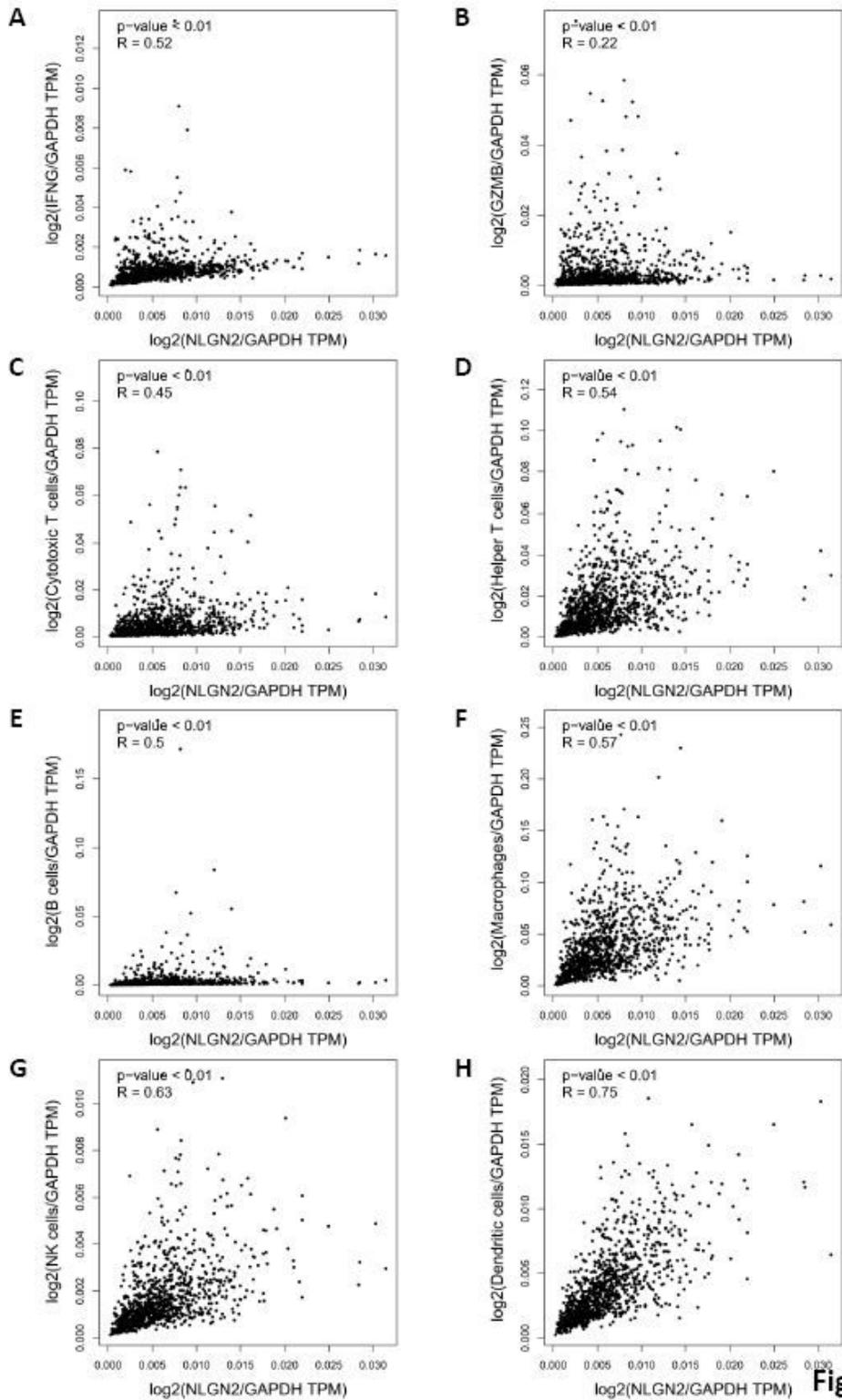


Figure 4

Figure 4

NLGN2 correlates with immune signatures in breast tumor. (A) Correlation analysis of NLGN2 and IFNG in BRCA. (B) Correlation analysis of NLGN2 and GZMB in BRCA. (C) Correlation analysis of NLGN2 and Cytotoxic T cell signatures in BRCA. (D) Correlation analysis of NLGN2 and Helper T cell signatures in

BRCA. (E) Correlation analysis of NLGN2 and B cell signatures in BRCA. (F) Correlation analysis of NLGN2 and Macrophage cell signatures in BRCA. (G) Correlation analysis of NLGN2 and NK cell signatures in BRCA. (H) Correlation analysis of NLGN2 and Dendritic cell signatures in BRCA. The p value and R coefficient are indicated in each panel, and $p < 0.05$ is statistically significant.

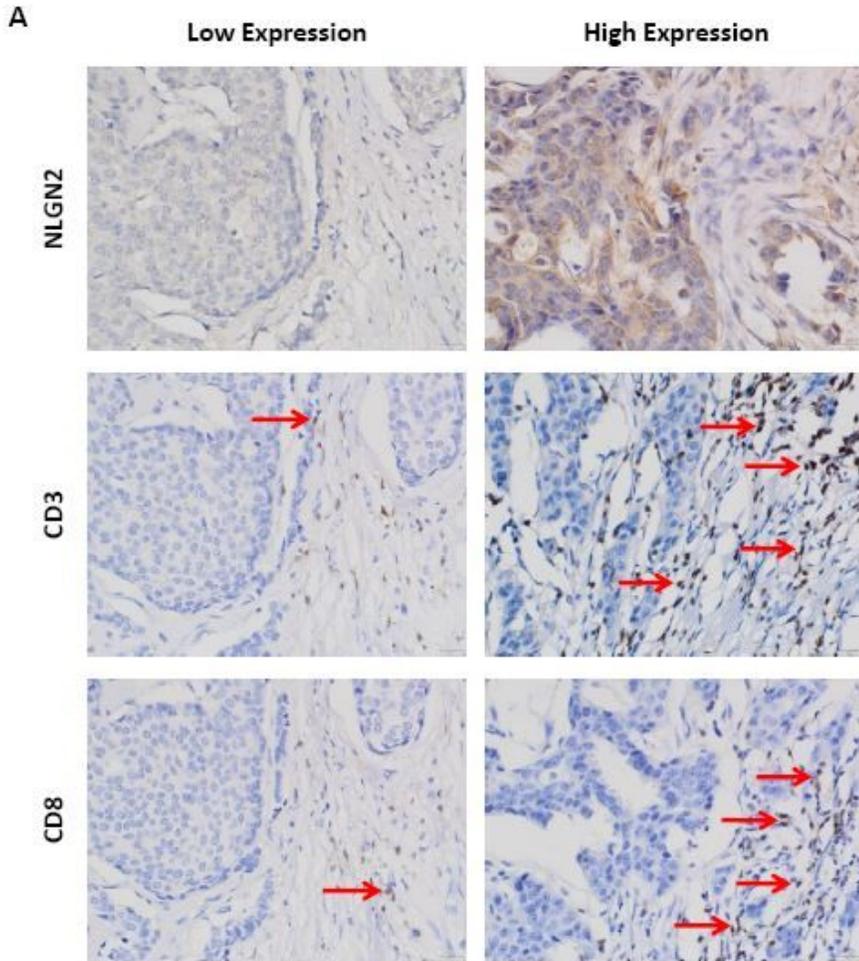


Figure 5

Figure 5

Association of the expression levels of NLGN2, CD3 and CD8 in breast cancer. (A) Representative images of immunohistochemical staining showing in situ expression of NLGN2, CD3 and CD8 in breast cancer tissue specimens. Left panels, low expression of NLGN2 (+) in breast tumor tissue, and CD3+ and CD8+ T cells in the same tissue. Right panels, high expression of NLGN2 (+++) in breast tumor tissue, and CD3+ and CD8+ T cells in the same tissue. The arrows point to CD3+ or CD8+ lymphocytes. All photos are at ×400 original magnification.

Supplementary Files

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- [FigureS1.JPG](#)