NTN4 as a prognostic marker and associated with immune infiltrates in breast cancer

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Abstract

Netrin-4 (NTN4), a member of neurite guidance factor family, can promote neurite growth and elongation. NTN4 participates in breast cancer, however, whether NTN4 correlates with prognosis and immune infiltration in breast cancer remain unclear. This study aims to investigate prognostic landscape of NTN4 in breast cancer and relationship of NTN4 expression with immune infiltration. Breast carcinoma tissue were used to IHC. Expression pattern and prognostic value of NTN4 were explored in breast cancer using multiple databases, including UALCAN, TIMER, Kaplan-Meier Plotter and Prognoscan. Using TIMER database, relationships of NTN4 expression with tumor immune invasion and immune cell surface markers were evaluated. Transcription and survival of NTN4 were investigated in breast cancer based on cBioPortal database. STRING database was explored to identify molecular functions and signaling pathways downstream of NTN4. NTN4 expression was significantly lower in invasive breast carcinoma compared with adjacent non-malignant tissues. Promoter methylation of NTN4 in breast cancer exhibited different patterns. Low NTN4 expression was associated with poorer survival. NTN4 expression was significantly positively related to infiltration of $CD8^+$ T cells, macrophages and neutrophils, whereas significantly negatively related to B cells and tumor purity. Association patterns were different from different subtypes. Different patterns of association between NTN4 expression and immune cell surface markers were revealed. Different subtypes of breast cancer carried different gene alterations. NTN4 was involved in mediating multiple biological processes including morphogenesis and migration.

1. Introduction

Netrins are a conserved laminin-like secreted protein family originally identified as axon-guiding molecules [1]. Netrins are expressed ectopic nervous system and involved in a variety of biological processes, including tissue morphogenesis [2], angiogenesis [3], lymphangiogenesis [4], tumorigenesis [5], migration [6], invasion [7], adhesion [8], apoptosis [9] and inflammatory regulation [10]. Netrins are highly conserved during evolution. Netrin1 (NTN1), netrin3 (NTN3) and netrin4 (NTN4) have been identified in mammals. Netrin-4 (NTN4, also known as β-netrin) is a new member of Netrins family in vertebrates, localized to basement membrane surrounding lobular structures in blood vessels, kidneys, breast and ovaries [11]. NTN4 is secreted by breast epithelial cells and sequestered by basement membrane. NTN4 may participate in development and progression of a variety of cancers, NTN4 serve as a prognostic biomarker for breast cancer has been reported [12–14]. Whether NTN4 levels are associated with tumor immune infiltrates and clinical outcome have not been evaluated in breast cancer.

In early 2021, the World Health Organization (WHO) International Agency for Research on Cancer agency (IARC) published 2020's global cancer data (https://www.iarc.fr/faq/latest-global-cancer-data-2020-qa/). Breast Cancer (BC) has replaced lung cancer as the most common malignancy globally, with estimated annual cases reaching 2.26 million worldwide, ranking the first in both morbidity and mortality among women. Breast Cancer is a serious threat to human health. Continuous development of molecular markers to specific cell subsets and targeted therapies [15] will become an important research direction in the future. In recent decades, mRNA expression as well as prognostic predictive value has become

increasingly attractive. Transcriptome (including mRNA) of primary breast tumors can help predict intrinsic subtypes, tumor grade, drug responsiveness, risk of recurrence, as well as prognostic biomarker [16–18].

In this study, association between NTN4 mRNA and breast cancer prognosis was evaluated using public databases such as Kaplan-Meier plotter and PrognoScan. In addition, associations of NTN4 mRNA levels with clinicopathological characteristics and tumor-infiltrating immune cells were investigated in breast cancer. Meanwhile, gene alterations of NTN4, NTN4 molecular functions and regulation pathways were explored. Our findings shed light on NTN4 in breast cancer and provided potential interaction between NTN4 and tumor-immune.

2. Materials And Methods

2.1. Tissue Samples

Breast invasive ductal carcinoma tissue from patients were analyzed. The criteria for tissue included an original histological diagnosis of invasive breast carcinoma, and the efficiency of clinical pathological data. Specimens were frozen in liquid nitrogen (-80°C) for analysis. The study are conducted in accordance with the Declaration of Helsinki and was approved by The Ethical Committee of Liaocheng People's Hospital and each patient provided informed consent.

2.2. TIMER database analysis

We analyzed relationship of NTN4 expression with respective abundance of infiltrating immune cells (B cells, $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, macrophages, and dendritic cells) in breast cancer patients using The Tumor IMmune Estimation Resource (TIMER) algorithm database (https://cistrome.shinyapps.io/timer/) [19]. Tumor purity is a vital factor that influences immune infiltration in tumor samples by genomic approaches.

2.3. RNA-sequencing data and bioinformatics analysis

We used TCGA database to collect RNA-seq data and clinical information from 1222 cases of breast cancer. Original format of downloaded data was level 3 HTSeq-fragments per kilobase per million (FPKM) and converted into transcripts per million (TPM) for subsequent analysis. Paired and unpaired tests were performed to compare expression patterns of NTN4. Area under curve (AUC) of NTN4 was analyzed, to determine whether NTN4 can be used as a biomarker to distinguish between tumor and adjacent tissues. These analysis was based on R software.

2.4. UALCAN database analysis

The UALCAN database (ualcan.path.uab.edu/index.html) was applied to analyze relationships of NTN4 mRNA expression or NTN4 promoter methylation levels with clinicopathological characteristics [20].

2.5. Survival Analysis using PrognoScan and Kaplan-Meier Plotter

To investigate prognostic value of NTN4 mRNA in breast cancer, Kaplan-Meier Plotter (http://www.kmplot.com, P-value < 0.05) [21] and PrognoScan database [22] (http://dna00.bio.kyutech.ac.jp/PrognoScan/, adjust the threshold Cox P-value < 0.05) were applied. Specifically, NTN4 expression level was searched in all available microarray datasets of PrognoScan to determine its relationship with prognosis. The threshold was set as a Cox P-value < 0.05.

2.6. Gene alterations of NTN4 in breast cancer using cBioPortal

Gene alteration of NTN4 were explored using cBioPortal (http://www.cbioportal.org) regarding BC. OncoPrint was constructed in cBioPortal (TCGA provisional) to directly reflect all types of changes in NTN4 gene amplification, deep deletion, mRNA upregulation, and mRNA downregulation in patients with BC. In addition, potential effects of NTN4 gene alterations on survival of BC patients were estimated using Kaplan-Meier survival curves in cBioPortal.

2.7. STRING database

For an in-depth exploration of relationship, STRING database version 11.0 was applied [23]. STRING contains both known and predicted protein–protein associations based on information sources, including curated databases, experimental/biochemical data, PubMed abstracts, and others. Using NTN4 as an input parameter, proteins that might interact with NTN4 were searched. The default scoring threshold of interaction was 0.4, and a subnetwork constructed with genes that might interact with NTN4 was extracted. NTN4 driving genes and interactive genes were constructed into a network. Then, STRING database was used to conduct gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of all selected genes.

2.8. Immunohistochemistry (IHC)

Tissue paraffin sections were dewaxed in xylene (Yantai fast eastern fine chemical CO., LTD) for 3 times, each for 10 min. Sections were followed by serial rehydration in graded ethanol (Yantai fast eastern fine chemical CO., LTD) from 100% ethanol followed by 95%, 90%, 80%, 70% and 60% ethanol, and finally in distilled water. Heat-mediated antigen retrieval was conducted in EDTA buffer (pH 9.0) (MVS-0098; MXB) using a microwave pressure cooker for 10 min. They were blocked with 5% BSA for 30 min in 37℃, followed a mouse anti-NTN4 monoclonal antibody (sc-365280; Santa Cruz Biotechnology) at 1:100 dilution for the night in 4℃. Sections were washed 3 times by PBS7.2 for 5 min. Binding of the anti-NTN4 antibody was detected using Biotin-conjugated secondary anti-mouse antibody from BOSTER detection system (SA1051) for 30 min in 37℃, followed washed 3 times by PBS7.2 for 5 min. Next, Sections were incubated with SABC-AP (SA1051; BOSTER) for 30 min in 37℃, followed washed 4 times by 0.01M TBS

(pH 9.0-9.5) for 5min. And they developed with BCIP/NBT as the chromogen for 30 min. The sections were counterstained with Nuclear fast red (SA1051; BOSTER) for 5 min.

2.9. Statistical Analysis

Wilcoxon test was used to compare different expression levels of NTN4 in different cancers. Kaplan-Meier plot was used to estimate survival curve. In order to describe survival curve more accurately, log rank test was used to calculate log rank P value. Univariate Cox regression model was applied to calculate HR, 95% CI and Cox P values in PrognoScan. Spearman's coefficient was used to analyze correlation of gene expression. Using receiver operating characteristic (ROC) curve of NTN4, optimal cut-off point was calculated to distinguish “high” from “low” expression, and a ROC was generated with MedCalc. In the absence of special circumstances, a $P < 0.05$ was considered statistically significant.

3. Results

3.1. The mRNA expression level of NTN4

To evaluate NTN4 expression in pan-cancer, RNA sequencing data in TCGA was examined using TIMER. The differential NTN4 expression patterns between tumorous and adjacent tissues were summarized in Fig. 1A. NTN4 expression was significantly lower in invasive breast carcinoma (BRCA), as well as in basal, Her2+, and luminal breast cancer subtypes, compared with adjacent tissues. Meanwhile, expression of NTN4 in tumor was significantly lower than those in adjacent tissue in unpaired (Fig. 1B) and paired samples (Fig. 1C). In addition, receiver operating characteristic (ROC) curve was used to analyze effectiveness of NTN4 expression level on distinguishing breast cancer tissues from non-tumor tissues. The area under curve (AUC) of NTN4 was 0.764, suggesting that NTN4 could serve a biomarker to distinguish BC from non-tumor tissue (Fig. 1D). NTN4 expression was verified by IHC in adjacent tissue (Fig. 1E.G) and tumor tissue (Fig. 1F.H).

Demographic and clinical characteristics of patients were summarized in Table 1, in which 1083 primary breast cancer cases were collected from TCGA database. According to relative NTN4 levels, breast cancer patients were divided into low (n = 541) and high (n = 542) expression groups. The associations between NTN4 expression levels and clinicopathological characteristics were evaluated. Chi-square tests revealed that NTN4 expression was associated with T stage ($P < 0.001$), Histological type ($P < 0.001$), Pathologic stage ($P = 0.004$), PR and ER status ($P < 0.001$). No significant correlation was observed between NTN4 expression and age ($P = 0.035$), M stage ($P = 1.000$), menopausal status ($P = 0.916$) or HER2 status ($P = 0.438$).

Table 1
The Relationship Between the Expression of NTN4 and Clinicopathological Data

Characteristic	NTN4 mRNA		P
	Low(n = 541)	High(n = 542)	
Age (years)			**0.035**
Median (IQR)	57 (48, 66)	60 (49, 68)	0.012
<=60	318 (29.4%)	283 (26.1%)	
> 60	223 (20.6%)	259 (23.9%)	
T stage, n (%)			**< 0.001**
T1	113 (10.5%)	164 (15.2%)	
T2	349 (32.3%)	280 (25.9%)	
T3	60 (5.6%)	79 (7.3%)	
T4	17 (1.6%)	18 (1.7%)	
N stage, n (%)			**0.653**
N0	258 (24.2%)	256 (24.1%)	
N1	179 (16.8%)	179 (16.8%)	
N2	61 (5.7%)	55 (5.2%)	
N3	33 (3.1%)	43 (4%)	
M stage, n (%)			**1.000**
M0	452 (49%)	450 (48.8%)	
M1	10 (1.1%)	10 (1.1%)	
Menopause status, n (%)			**0.916**
Pre	117 (12%)	112 (11.5%)	
Peri	20 (2.1%)	20 (2.1%)	
Post	348 (35.8%)	355 (36.5%)	
Histological type, n (%)			**< 0.001**
Infiltrating Ductal Carcinoma	440 (45%)	332 (34%)	
Infiltrating Lobular Carcinoma	52 (5.3%)	153 (15.7%)	

Abbreviations: IQR = interquartile range; ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor.

Characteristic	NTN4 mRNA		P
	Low(n = 541)	High(n = 542)	
Pathologic stage, n (%)			**0.004**
Stage I	71 (6.7%)	110 (10.4%)	
Stage II	335 (31.6%)	284 (26.8%)	
Stage III	117 (11%)	125 (11.8%)	
Stage IV	8 (0.8%)	10 (0.9%)	
PR status, n (%)			**< 0.001**
Negative	267 (25.8%)	75 (7.3%)	
Indeterminate	3 (0.3%)	1 (0.1%)	
Positive	245 (23.7%)	443 (42.8%)	
ER status, n (%)			**< 0.001**
Negative	202 (19.5%)	38 (3.7%)	
Indeterminate	1 (0.1%)	1 (0.1%)	
Positive	313 (30.2%)	480 (46.4%)	
HER2 status, n (%)			**0.438**
Negative	264 (36.3%)	294 (40.4%)	
Indeterminate	7 (1%)	5 (0.7%)	
Positive	82 (11.3%)	75 (10.3%)	

Abbreviations: IQR = interquartile range; ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor.

3.2. Relationships between NTN4 expression and clinicopathological characteristics

NTN4 mRNA expression level in breast cancer was explored using UALCAN database. Consistently, NTN4 mRNA expression level in BC tumor was significantly higher than in normal tissue ($P < 0.001$, Fig. 2A). In different molecular subtypes, luminal had higher NTN4 mRNA expression than HER2 positive and triple negative BC ($P < 0.001$, Fig. 2B), and HER2 positive had higer than triple negative BC ($P < 0.05$, Fig. 2B). Based on clinical stages, normal tissues had higher NTN4 mRNA expression than stage 1, stage 2, stage 3 or stage 4 BC (Fig. 2C). At last, four different stages of lymph node involvement had lower NTN4 mRNA expression than normal tissue (Fig. 2D).

3.3. Relationships between NTN4 promoter methylation and clinicopathological characteristics

Using UALCAN database, we explored if promoter methylation of NTN4 was related to clinicopathological characteristics of breast cancer patients. NTN4 promoter methylation level was significantly higher in primary tumor than in normal tissue ($P < 0.001$, Fig. 3A). Based on molecular subtypes, luminal and triple negative BC had higher level of NTN4 promoter methylation ($P < 0.001$, Fig. 3B). Based on clinical stage, stage 2 and stage 3 had higher level of NTN4 promoter methylation than stage 4 (Fig. 3C). At last, based on lymph node status, N0 and N1 had higher level of NTN4 promoter methylation than N3 (Fig. 3D). Thus, NTN4 promoter methylation may contribute to breast cancer development and progression.

3.4. NTN4 mRNA level predicts prognosis in breast cancer

Survival analysis of NTN4 expression was evaluated using PrognoScan (Supplementary Table 1). Among four cohorts (GSE6532-GPL570, GSE1379, GSE3494-GPL97, GSE4922-GPL97) including different stages of breast cancer, high NTN4 expression was associated with favorable prognosis (Table 2). Similarly trend was observed, in Kaplan-Meier plotter database, based on Affymetrix microarrays (Fig. 4A-C, OS: HR (95% CI) : 0.68 (0.52–0.89), P = 0.0047; RFS: HR (95% CI) : 0.7 (0.67–0.82), P = 3.9e-06; DMFS: HR (95% CI) : 0.68 (0.52–0.89), P = 0.0046). Therefore, it is conceivable that low NTN4 expression could be a risk factor for a poor prognosis in breast cancer patients.

Table 2
Survival analysis of NTN4 mRNA in breast cancer patients (the Prognoscan)

Dataset	Endpoint	Number	$\ln(HR_{high}/HR_{low})$	COX P-value	ln(HR)	HR[95% CI^{low} CI^{upp}]
GSE6532-GPL570	Relapse Free Survival	87	-1.54	0.008389	-0.23	0.79[0.66–0.94]
	Distant Metastasis Free Survival	87	-1.54	0.008389	-0.23	0.79[0.66–0.94]
GSE1379	Relapse Free Survival	60	-1.64	0.033113	-0.27	0.76[0.59–0.98]
GSE3494-GPL97	Distant Specific Survival	236	-1.15	0.004196	-0.38	0.68[0.52–0.89]
GSE4922-GPL97	Distant Free Survival	249	-0.97	0.043828	-0.21	0.81[0.66–0.99]

Abbreviations: HR = hazard ratio; CI = confidence interval of the estimated HR; COX = cox proportional-hazards model.

3.5. Correlation between NTN4 expression and 6 types of infiltrating immune cells

Immune cells in tumor microenvironment (TME) can affect patient's survival, Hence, it would be meaningful to explore association between immune infiltration and NTN4 expression. We determined whether NTN4 expression was related to immune infiltration in different cancers by calculating coefficient index of NTN4 expression with immune infiltration in breast cancer using TIMER. Six types of infiltrating immune cells (B cells, $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, macrophages, and dendritic cells) were explored. NTN4 expression was positively associated with $CD8^+$ T cells (r = 0.117, P = 2.64e-04), macrophages (r = 0.247, P = 3.82e-15) and neutrophils (r = 0.07, P = 3.09e-02) in breast cancer whereas negatively with B cells (r=-0.064, P = 4.62e-02) and tumor purity (r=-0.187, P = 2.53e-09), but not dendritic cells (r = 0.004, P = 9.04e-01) (Fig. 5A). In different breast cancer subtypes, associations differed (Fig. 5B-5D). In base-like subtype, NTN4 expression was not related to tumor purity (r=-0.168, P = 5.71e-02), whereas related to macrophages only (r = 0.201, P = 2.38e-02). In HER2 + breast cancer, NTN4 expression level was not related to tumor purity (r=-0.097, P = 1.67e-01), whereas only negatively related to $CD8^+$ T cells (r=-0.352, P = 7.27e-03). In luminal subtype, NTN4 expression level was negatively associated with tumor purity (r=-0.258, P = 9.80e-10), whereas positively associated with B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells.

3.6. Correlation of NTN4 with markers of immune cells

Potential relationship of NTN4 with infiltrating immune cells was explored using TIMER. Cellular markers characterized immune cells, including B cells, $CD8^+$ T cells, M1/M2 macrophages, tumor-associated macrophages, monocytes, NK, neutrophils, and dendritic cells. Different functional T cells such as Tfh, Th1, Th2, Th9, Th17, Th22, Treg, and exhausted T cells were analyzed (Table 3). In TIMER, NTN4 expression levels was significantly correlated with 33 out of 45 immune cell markers after adjustment for tumor purity.

Table 3
Correlations between NTN4 and gene markers of immune cells

Cell type	Gene marker	None		Purity	
		Cor	P	Cor	P
B cell	CD19	-0.076	1.17e-02*	-0.195	5.45e-10***
	CD20	0.011	7.05e-01	-0.096	2.56e-03**
	CD38	-0.152	4.25e-07***	-0.262	3.98e-17***
$CD8^+$ T cell	CD8A	0	1e + 00	-0.122	1.08e-04***
	CD8B	-0.04	1.87e-01	-0.153	1.27e-06***
Tfh cell	CXCR5	0.013	6.7e-01	-0.095	2.73e-03**
	ICOS	-0.109	3.12e-04***	-0.226	5.61e-13***
	BCL-6	0.322	5.55e-28***	0.309	2.31e-23***
Th1 cell	IL12RB2	-0.235	2.98e-15***	-0.292	4.65e-21***
	WSX-1	0.027	3.67e-01	-0.049	1.26e-01
	T-BET	-0.05	9.77e-02	-0.189	1.81e-09***
Th2 cell	CCR3	0.058	5.32e-02	0.004	9.05e-01
	STAT6	0.355	4.21e-34***	0.333	3.18e-27***
	GATA3	0.332	1.12e-29***	0.396	1.14e-38***
Th9 cell	TGFBR2	0.388	9e-41***	0.351	3.97e-30***
	IRF4	-0.032	2.86e-01	-0.16	4.30e-07***
	PU.1	0.033	2.71e-01	-0.06	3.86e-02*
Th17 cell	IL-21R	0.001	9.67e-01	-0.114	3.16e-04***
	IL-23R	0.051	9.21e-02	-0.012	7.08e-01
	STAT3	0.436	3.96e-52***	0.415	1.10e-42***
Th22 cell	CCR10	0.001	9.69e-01	-0.051	1.09e-01
	AHR	0.475	4.58e-63***	0.432	1.93e-46***
Treg cell	FOXP3	-0.085	4.97e-03**	-0.179	1.38e-08***
	CCR8	-0.069	2.12e-02*	-0.135	1.88e-05***
	CD25	-0.165	3.8e-08***	-0.273	1.86e-18***

Abbreviations: TAM = tumor associated macrophage; NK = natural killer cell; DC = dendritic cell.

T cell exhaustion	PD-1	-0.122	5.08e-05***	-0.261	6.15e-17***
	CTLA4	-0.16	9.97e-08***	-0.283	8.12e-20***
Macrophage	CD68	0.03	3.22e-01	-0.044	1.70e-01
	CD11b	0.255	7.54e-18***	0.191	1.28e-09***
M1	NOS2	-0.013	6.69e-01	-0.014	6.68e-01
	ROS1	-0.068	2.34e-02*	-0.084	8.35e-03**
M2	ARG1	0.016	6.02e-01	-0.005	8.80e-01
	MRC1	0.067	2.68e-02*	-0.023	4.71e-01
TAM	HLA-G	-0.036	2.27e-01	-0.103	1.09e-03**
	CD80	-0.025	4.16e-01	-0.093	3.43e-03**
Monocyte	CD14	-0.001	9.63e-01	-0.079	1.32e-02*
	CD16	0.103	6.21e-04***	0.045	1.53e-01
NK	XCL1	-0.021	6.86e-01	-0.12	1.46e-04***
	KIR3DL1	-0.052	8.35e-02	-0.13	4.11e-05***
	CD7	-0.127	2.36e-05***	-0.27	4.58e-18***
Neutrophil	CD15	0.069	2.24e-02*	-0.012	6.98e-01
	MPO	0.023	4.38e-01	-0.023	4.75e-01
DC	CD1C	0.235	3.29e-15***	0.16	4.03e-07***
	CD141	0.415	4e-47***	0.381	1.21e-35***

Abbreviations: TAM = tumor associated macrophage; NK = natural killer cell; DC = dendritic cell.

3.7. Gene alterations in NTN4 in breast cancer tissue from cBioPortal

Gene alterations in NTN4 were harbored in 1.1% of sequenced cases from OncoPrint schematic of cBioPortal (Fig. 6A). Among Breast Invasive Carcinoma, no alteration in NTN4 was identified. Among Breast Invasive Ductal Carcinoma, amplification in NTN4 was common. Mutations and deep deletion occurred with equal frequency. Among Breast Invasive Lobular Carcinoma, amplification and mutation of NTN4 occurred with equal frequency as well. Amplification of NTN4 occurred in Breast Invasive Mixed Mucinous Carcinoma (Fig. 6B). All mutations of NTN4 in breast cancer were described in Fig. 6C: NTN4 harbored one truncating mutation and three missense mutations. Furthermore, correlation between NTN4 gene changes and breast cancer patient survival was assessed. However, there was no significant

relationship between overall survival (OS), disease specific survival (DSS), disease free survival (DFS) and progress free survival (PFS) of breast cancer patients and gene alterations in NTN4 (Fig. 6D-G).

3.8. Exploration of NTN4 molecular functions and regulation pathways based on bioinformation tools

NTN4 molecular function and regulation pathway were preliminarily explored to demonstrate potential mechanism underlying how NTN4 regulates biological behaviors of breast cancer. First, STRING database was searched for genes that possibly interact with NTN4 (Fig. 7A). These selected genes were subjected to GO analysis to identify cellular component (CC) (Fig. 7B), biological process (BP) (Fig. 7C) and molecular function (MF) (Fig. 7D) in which NTN4 interacted genes were involved. Based on CC, differentially expressed proteins were extrinsic components of membrane. According to BP, differentially expressed proteins were mainly involved in morphogenesis and motility. Based on MF, differentially expressed proteins functioned mainly for signaling receptor binding. KEGG pathway analysis was performed to identify molecular pathways in which NTN4 interacted genes were involved. The top 20 pathway enrichments, such as ECM-receptor interaction, adhesion and extracellular part, were presented in Fig. 7E.

4. Discussion

Based on data from public database, NTN4 correlates with breast cancer prognosis and immune infiltration. NTN4 mRNA expression was significantly lower in breast invasive carcinoma compared with adjacent tissues, while increasing NTN4 mRNA levels are related to favorable prognosis in breast cancer patients.

NTN4 has been established as a prognostic marker. In breast invasive carcinoma, NTN4 expression is associated with longer DFS and OS, as an independent prognostic factor affecting OS [13]. In addition, NTN4 has been identified as a potential mediator of breast cancer risk [24]. For example, rs61938093 CCV in this region is located in enhancer that interacts with NTN4 promoter. This risk allele reduces activity of NTN4 promoter. Knockout of NTN4 in breast cells increased cell proliferation *in vitro* and tumor growth *in vivo*, suggesting that low expression of NTN4 promoted breast cancer development. And NTN4 is associated with breast cancer cell migration and invasion via regulation of epithelial mesenchymal transition (EMT)-related biomarkers [25].

Another important aspect of this study is that NTN4 correlates with diverse immune infiltration (Fig. 5). NTN4 mRNA level may reflect lymphocyte infiltration of breast cancer. In the era of precision medicine [26], immunological biomarkers are important [27], for patient subpopulation selection. Immune biomarkers are numerous [28], and immune checkpoint inhibitors (ICIs) and tumor mutation burden (TMB) hold promise as such biomarkers [29, 30].

In addition, frequency of NTN4 gene alteration was low (1.1%), with patterns stratified by molecular subtypes of breast cancer. NTN4 gene alterations include missense mutation and truncating mutation. However, genetic variation may not affect patient's survival. Finally, in NTN4 interaction gene signaling pathway cluster analysis, signaling pathways were mostly enriched in cell morphogenesis and cell motility, which may explain potential involvement of NTN4 in tumor development and progression of breast cancer.

As our findings were obtained from public databases, there are some limitations in our study. With the update of databases, relationship of NTN4 with prognosis will change accordingly. Similarly, relationship of NTN4 mRNA level with different immune cell types and markers based on sequencing data from public databases will also change. On the other hand, with accumulation of resources, data stratification will become more refined so that reliability of results may increase. However, further experimental verification is required to validate our findings.

5. Conclusion

The current research expored NTN4 and its prognostic significance in breast cancer. In all, NTN4 is downregulated in breast cancer tissues. Besides, NTN4 is associated with immune infiltrates and survival in breast cancer. Collectively, these data suggest that NTN4 is worthy of further study in breast cancer, and it may be a potential biomarker, which can be used to guide the prognosis of breast cancer patients.

Declarations

Author Contributions

LY conceived this study and wrote the manuscript. YL analyzed the partial raw data. FY and CT proformed the experimental verification and processed the partial raw data. JC drafted the part of manuscript. MG provided the theoretical guidance and revised the manuscript.

Data Availability Statement

The datasets of the study have mainly been collected, obtained, and analyzed from corresponding online databases, and other data generated or analyzed are available from the corresponding authors upon reasonable request.

Competing interests

All authors declare that they have no conflict of interest.

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Supplementary materials

Supplementary Table 1 Survival analysis of NTN4 mRNA in PrognoScan.

Click here for additional data file.

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Figures

Figure 1

NTN4 expression and clinicopathological features of breast cancer. (A) NTN4 expression levels in different tumor types from TCGA database were determined by TIMER (*P<0.05, **P<0.01, ***P<0.001). (B) Wilcoxon rank sum test was used to analyze differential expression of NTN4 between BC tumor and adjacent tissue. (C) Wilcoxon signed rank sum test was used to detect the differential expression of NTN4 between BC tumor and adjacent tissue. (D) ROC curve established effciency of NTN4 expression level on distinguishing BC tumor from non-tumor tissue. X-axis represents false positive rate, and Y-axis represents true positive rate. (E) and (G): Representative negative NTN4 immunohistochemical staining results in the adjacent tissue. (E): ×100 magnification; (G): ×200 magnification. (F) and (H): Representative positive NTN4 immunohistochemical staining in the breast cancer specimens. (F): ×100 magnification; (G): ×200 magnification. Scale bar for (E.F): 100 μm; Scale bar for (G.H): 200 μm.

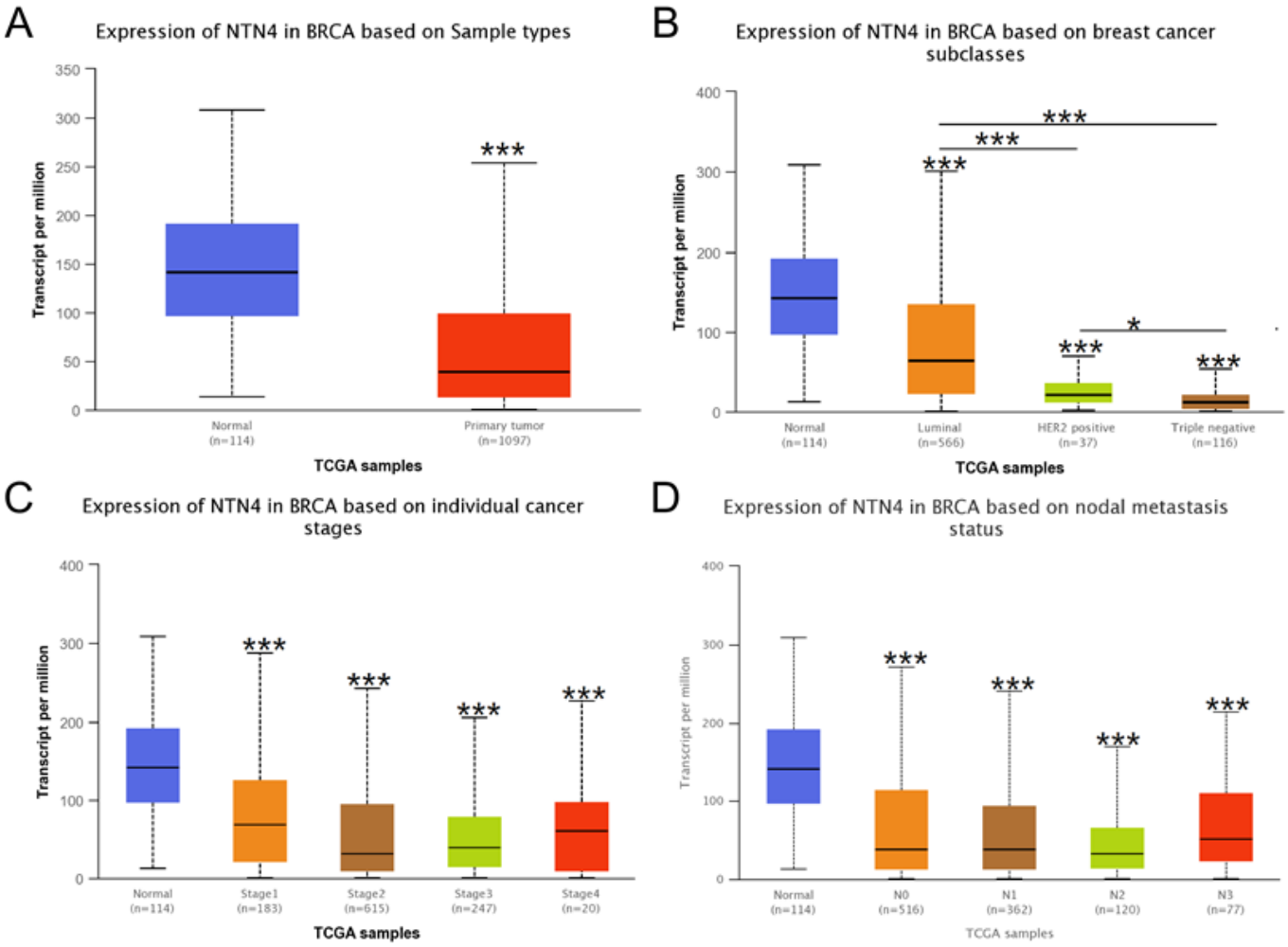

Figure 2

NTN4 mRNA expression in breast cancer based on UALCAN. Expression of NTN4 in breast cancer based on different (A) sample types, (B) molecular subtypes (in particular TNBC), (C) individual clinical stage, (D) lymph nodal status.

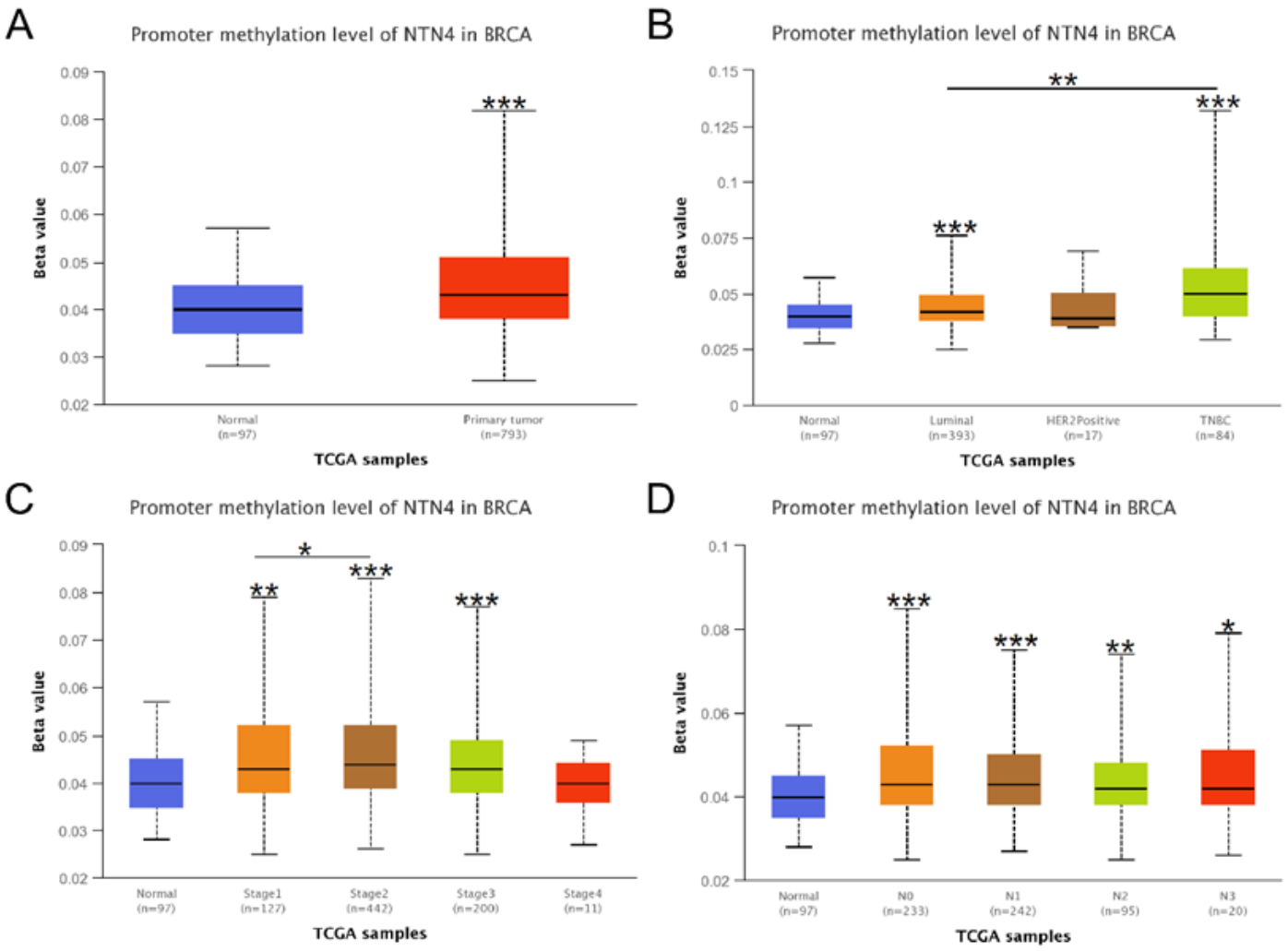

Figure 3

UALCAN analysis of NTN4 promoter methylation in breast cancer. NTN4 promoter methylation levels in breast cancer were compared based on different (A) sample types, (B) molecular subtypes (TNBC), (C) individual cancer stages, (D) lymph nodal status.

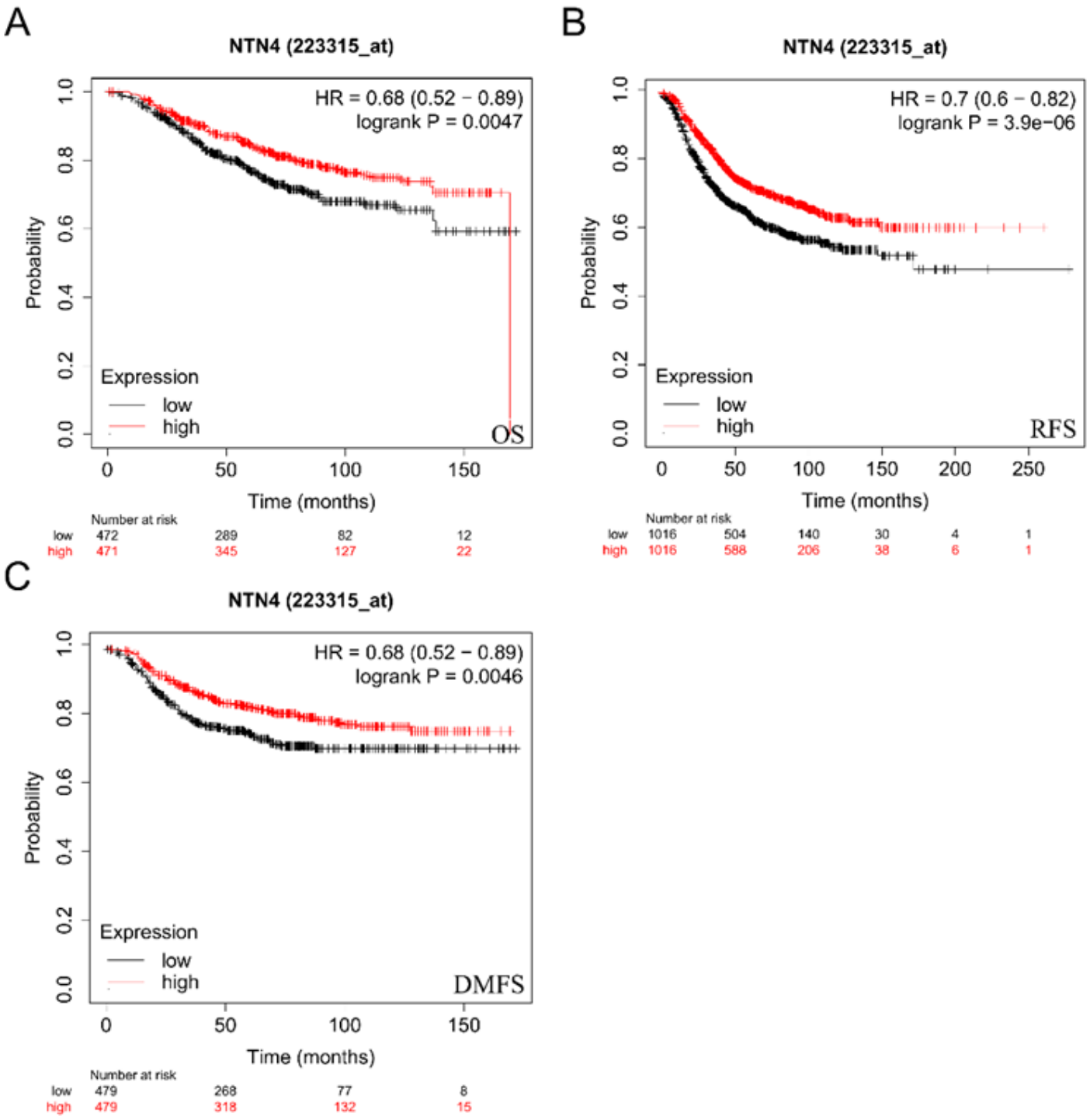

Figure 4

Kaplan-Meier survival curves compare high vs. low expression of NTN4 in breast cancer. High expression of NTN4 was associated with good survival. (A) OS: HR (95% CI) : 0.68 (0.52-0.89), P=0.0047. (B) RFS: HR (95% CI) : 0.7 (0.67-0.82), P=3.9e-06. (C) DMFS: HR (95% CI) : 0.68 (0.52-0.89), P=0.0046. RFS: Relapse-free survival; DMFS: Distant metastasis-free survival; OS: Overall survival.

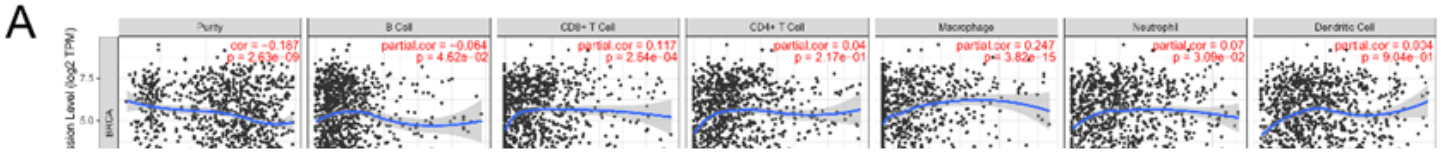

Figure 5

Correlation of NTN4 expression with immune infiltration level using TIMER database. (A) NTN4 expression was associated with infiltration of $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, or dendritic cells in breast cancer. (B) Basal subtype, (C) Her2+ subtype, and (D) luminal subtype.

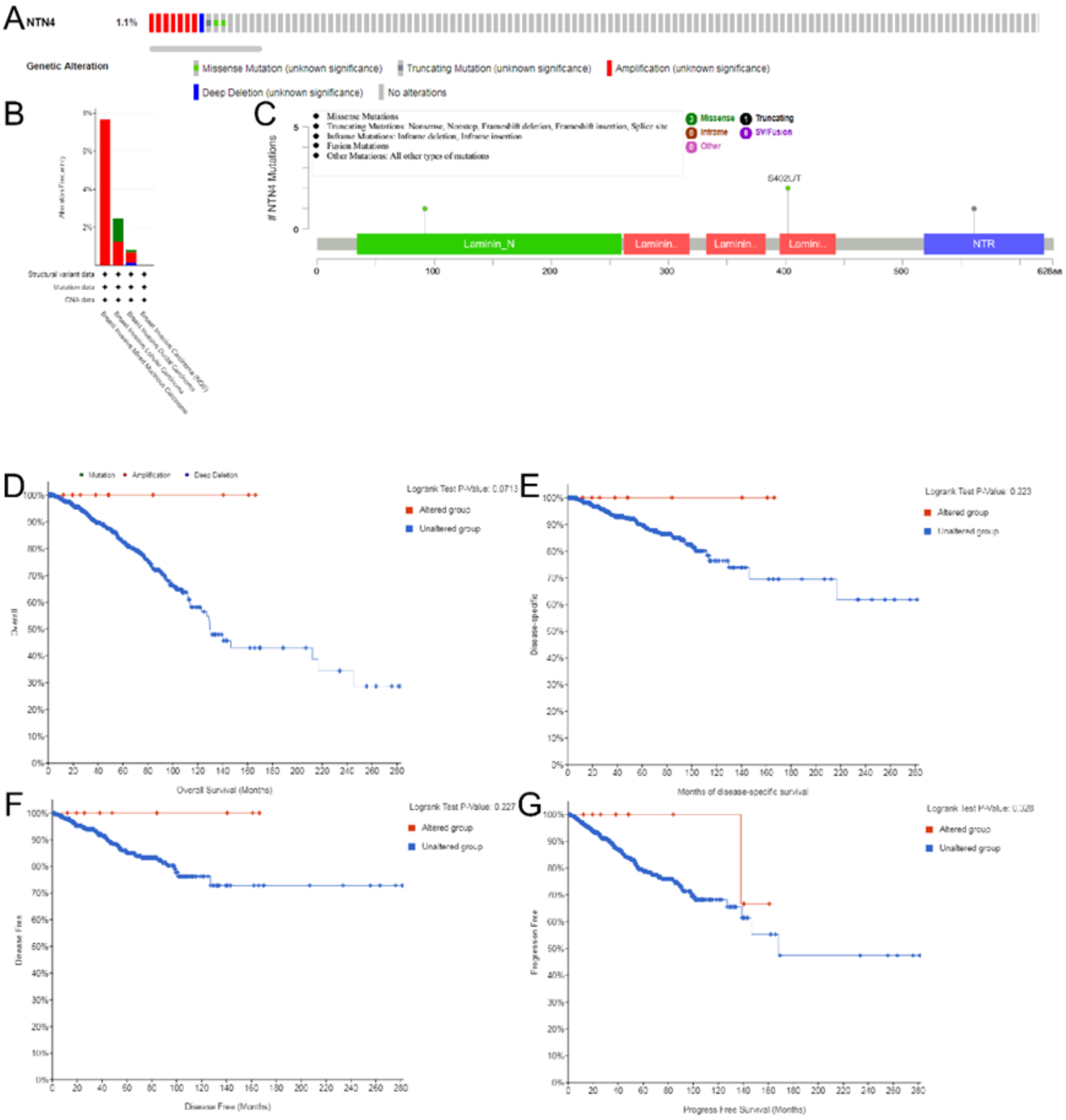

Figure 6

Gene alterations in NTN4 in breast cancer tissue. (A) Mutations in NTN4 in breast cancer tissues are shown. (B) Frequency of gene alterations in NTN4 in different types of breast cancer. (C) All mutations in breast cancer. (D) Kaplan-Meier survival curves analyzed relationship of gene alterations in NTN4 with overall survival, (E) the disease specific survival, (F) diease free survival or (G) progress free survival of breast cancer patients.

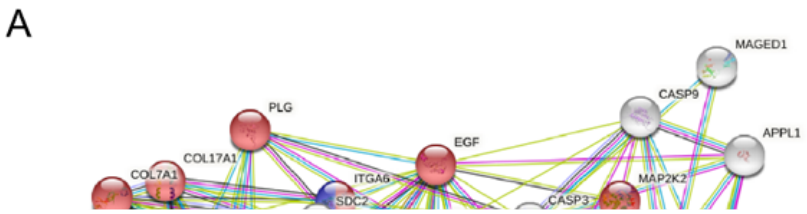

Figure 7

Exploration of NTN4 molecular functions and regulation pathways based on bioinformation tools. (A) Interaction network of NTN4 based on STRING database. (B) Cellular component. (C) GO biological process. (D) Molecular function analysis. (E) Pathway enrichment based on KEGG

Supplementary Files

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