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Reticulon 4 in Breast Cancer: A Comprehensive Analysis of its Biomarker Potential for Prognosis, Immunology, and Drug Sensitivity

Tuğcan Korak[1*]
https://orcid.org/0000-0003-4902-4022

Murat Kasap[1]
https://orcid.org/0000-0001-8527-2096

[1]Kocaeli University, Faculty of Medicine, Department of Medical Biology, Kocaeli, Turkey.

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*Correspondence: tugcan.korak@kocaeli.edu.tr; Tel.: +90 262 303 75 40 (T.K.).

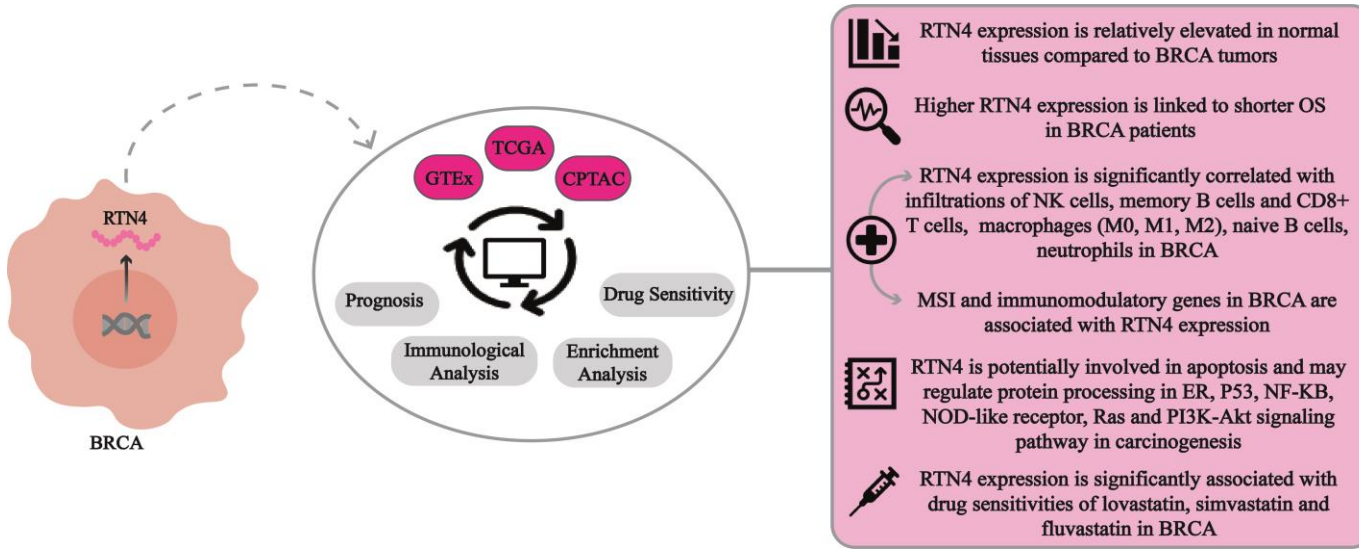
HIGHLIGHTS

- Higher RTN4 expression was linked to shorter overall survival in BC patients
- RTN4 expression was correlated with nine immune cell infiltrations
- MSI and immunomodulatory genes in BC were associated with RTN4 expression
- RTN4 expression may correlate with three statin drug sensitivities in BC

Abstract: Breast cancer (BC), the most prevalent cancer among women, necessitates the identification of effective early detection biomarkers. Reticulon 4 (RTN4), a neurite growth inhibitor primarily expressed in the central nervous system, has recently been implicated in cancer development. This study aimed to comprehensively examine the expression level, prognostic and immunological value, function and drug sensitivity of RTN4 in BC. Expression and survival analyses were performed using HPA, TIMER, GEPIA, UALCAN, and PROGgeneV2; immune-related features were explored via TISIDB, TIMER, and Sangerbox, while drug sensitivity analysis was conducted using the CellMiner database. RTN4 expression was observed significantly lower in BC compared to the normal tissues ($p<0.05$). Elevated mRNA expression levels of RTN4 were significantly associated with shorter overall survival in BC patients ($p<0.05$). Analysis of the tumor microenvironment (TME) revealed significant correlations between RTN4 and immune cell infiltration, immune and molecular subtypes, and stromal components ($p<0.05$). Furthermore, microsatellite instability, most immunomodulatory genes, and nearly half of immune checkpoints in BC showed significant associations with RTN4 expression ($p<0.05$). In addition, its expression showed significant correlations with the drug sensitivities of lovastatin, simvastatin, and fluvastatin ($p<0.05$). Considering its differential expression and significant correlation with BC prognosis, TME, and immune-related genes, RTN4 shows promise as a potential biomarker candidate, offering guidance for innovative treatment approaches for BC.

Keywords: Reticulon 4; breast cancer; prognosis; biomarker; oncology.

GRAPHICAL ABSTRACT



INTRODUCTION

Breast cancer, also known as BC, is one of the most common, complex, and aggressive malignant tumors among women, with a remarkably high incidence rate and mortality [1,2]. In recent decades, notable advancements have been achieved in the treatment of BC, including radiotherapy, chemotherapy, immunotherapies, and so on. However, BC prognosis and mortality rate persists, primarily owing to its complicated pathogenesis and progression. Hence, there is a significant requirement to investigate novel and efficient biomarkers, and therapeutic targets in order to enhance the prognosis and quality of life for individuals with BC [1,3,4].

Reticulon 4 (RTN4, also known as Nogo) is a neurite growth inhibitor predominantly expressed in the central nervous system (CNS). It has three isoforms: Nogo-A, Nogo-B, and Nogo-C. Nogo-A exerts a potent inhibitory effect on both neurite growth and cell migration, whereas the functions of Nogo-B and Nogo-C remain largely unexplored. Studies have predominantly focused on Nogo-A and its associated receptor, Nogo-receptor 1 (NgR1) [5–8]. The Nogo-A signaling pathway regulates the actin cytoskeletal dynamics in neurons through RhoA/ROCK cascade [9]. Recently, NgR1 has been detected in several types of immune cells such as natural killer (NK) cells and has been shown to modulate the adhesion of immune cells. Within the available literature, there is only one study that investigate the connection between Nogo-A and NK cell cytotoxicity, uncovering the presence of Nogo-A expression in various mouse and human cancer cell lines. The absence of NgR1 or its blockade was revealed to enhance NK cell-mediated tumor control. This enhancement occurs by increasing the stability of NK-target cell interactions during immunological synapse (IS) formation, enabling NK cells to recognize and eliminate cancer cells. Subsequently, NgR1, which acts as an inhibitory receptor of NK cells, has been defined as an immune checkpoint in IS formation. This evidence supported immunomodulatory function of the interaction between and Nogo-A and NgR1, in addition to their well-established roles in the CNS [10]. Furthermore, several studies focusing on different types of cancer have provided evidence suggesting that Nogo-A may play significant roles in cell cycle regulation, cell survival, and carcinogenesis [11,12]. In addition, RTN4 was considered to play a role in the malignant advancement of gastric cancers and holds prognostic value [13].

While the roles of RTN4 in the nervous system are well-documented, its involvement in cellular and molecular pathways within BC have not yet been elucidated. In the current study, RTN4 expression patterns, prognostic significance, associations with the tumor microenvironment (TME) and immune-related marker genes, impact on signaling pathways and influence on drug sensitivity were analyzed using various publicly available databases within the context of BC. As a result of investigating the potential roles of RTN4 in cancer-related mechanisms, this study offers novel perspectives and a theoretical basis for the exploration of new tumor biomarkers and potential therapeutic targets.

MATERIAL AND METHODS

mRNA and protein expression profiles of RTN4

In normal human tissues, various cancer types, and cell lines, the expression levels of RTN4 at the RNA and protein levels were recruited from the Genotype-Tissue Expression (GTEx), The Cancer Genome Atlas (TCGA), and the HPA databases [14]. RTN4 RNA expression data were shown as normalized transcript per million (nTPM) values for cell lines and consensus dataset.

Differential expression analysis of RTN4

The Gene_DE module of TIMER2.0 database was utilized to visualize differential mRNA expression between tumor and adjacent normal tissues for RTN4 across all TCGA tumors [15]. GEPIA web tool was used to examine the variation in RTN4 mRNA expression levels between BC tissues and normal tissues, based on data obtained from the TCGA and the GTEx [16]. We also confirmed the protein-level expression of RTN4 using the UALCAN [17].

Survival analysis of RTN4 in BC

The survival analysis of RTN4 in BC was assessed using the KM plotter at the mRNA level within 2976 samples [18]. Subsequently, PROGgeneV2 (pan cancer prognostics database) tool was used to analyze prognostic implications of RTN4 expression in BC [19]. Finally, forest map for Cox proportional hazard regression test were performed by Sangerbox 3.0 mapping tool with OS module by longrank test in statistical analysis [20].

Correlation of RTN4 expression and TME

TIMER2.0 database was used to analyze correlation between RTN4 expression levels and the several immune infiltrating cells which are NK cells, CD8+ T cells, CD4+ T cells, naive B cells, memory B cells, macrophages (M0, M1, M2) and neutrophils based on the CIBERSORT algorithm in BC. CIBERSORT was utilized for immune infiltration analysis due to its superior accuracy in resolving closely related cell types and handling complex tissue mixtures. It has been extensively validated and widely used in cancer research for characterizing tumor-infiltrating lymphocytes (TILs) [21] .The interaction between BC type tumor and the immune system was created by TISIDB which incorporates comprehensive data from immunotherapy patient cohorts and TCGA to thoroughly analyze tumor-immune system interactions [22]. The association between RTN4 expression and immune subtypes (C1, wound healing; C2, IFN-γ dominant; C3, inflammatory; C4, lymphocyte depleted; C5, immunologically quiet; C6, TGF-β dominant) and molecular subtypes in BC was analyzed using TISIDB. In order to investigate the association between TME and the levels of RTN4 gene expression, we computed stromal and immune cell scores in BC using the ESTIMATE algorithm in Sangerbox. The association were evaluated using Pearson's correlation coefficients.

Association of RTN4 expression with TMB, MSI and immune-related markers

The relationship between RTN4 expression and TMB, MSI, immunomodulatory genes (150 marker genes) and immune checkpoint pathway genes (60 genes) were analyzed using Sangerbox. The unified and standardized pan-cancer data set from the UCSC and Pearson correlation method were used for all analysis. $p<0.05$ indicates significant difference.

Construction of RTN4 PPI network and Functional Enrichment Analysis

PPI network of RTN4 was constructed with Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database in Cytoscape v3.10.1 to predict potential interactions of RTN4 at the protein level. We then utilized CytoHubba, a Cytoscape plugin employing the Maximal Clique Centrality (MCC) algorithm, to identify core genes within the PPI network. This method has proven highly effective in accurately predicting key proteins in the network [23].

To determine whether RTN4 has a functional annotation with cancer, all proteins in the constructed RTN4-targeted PPI network were selected, and functional enrichment was performed in different knowledgebase of Cytoscape software. The redundancy cutoff was set as 0.5. Annotations with the potential relevance to cancer were individually selected by searching the literature and assigned to the PPI network. Neural pathways associated annotations were eliminated. To support and expand the enrichment analysis obtained in Cytoscape, KEGG pathway enrichment analysis was performed using the ShinyGo v0.77 [24].

This analysis was conducted using 26 proteins (ENSG00000142192, ENSG00000170540, ENSG00000144746, ENSG00000198513, ENSG00000119787, ENSG00000171791, ENSG00000171552, ENSG00000127022, ENSG00000136986, ENSG00000072849, ENSG00000111711, ENSG00000105695, ENSG00000064300, ENSG00000126861, ENSG00000105426, ENSG00000129625, ENSG00000067560, ENSG00000125744, ENSG00000133318, ENSG00000115310, ENSG00000040608, ENSG00000185924, ENSG00000186907, ENSG00000028528, ENSG00000110025, ENSG00000127863) involved in cancer-related annotations in Cytoscape. For both enrichment analyses, significance was considered when the false discovery rate (FDR) was < 0.05.

Drug sensitivity and RTN4 expression

The CellMiner database was used to analyze correlation between RTN4 expression and drug sensitivity [25]. RTN4 mRNA expression levels for different cancers and NCI-60 average z-scores data for FDA approved drugs exhibiting significant drug activity patterns were retrieved from the database and Pearson's correlation analysis was performed to analyze the relationship between the expression levels of RTN4 and drug scores. Moreover, correlation analysis was performed using the RTN4 mRNA expression levels specifically from BC cell lines (MCF7, MDA-MB-231, HS578T, BT-549, T-47D) available in the NCI-60 database. Data processing and visualization were carried out in the IBM SPSS 20.0 (IBM Corp., Armonk, NY, USA).

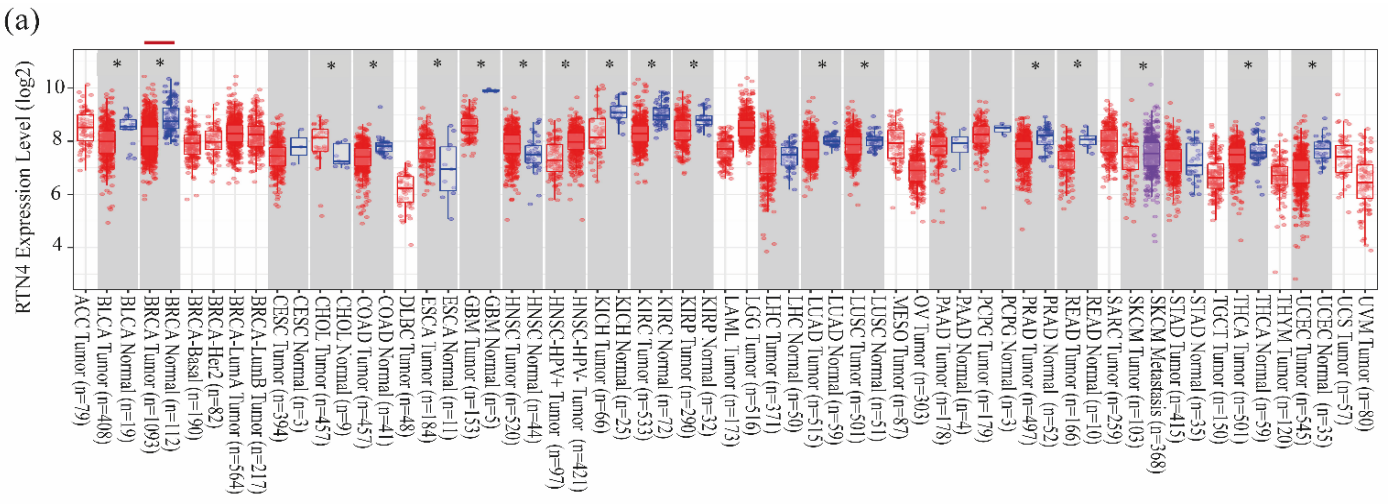
RESULTS

Expression profile of RTN4 in normal and cancer tissues

Through an analysis of the RTN4 expression profile in Human Protein Atlas (HPA), we observed that the expression of RTN4 was more frequently distributed in brain-associated tissues such as cerebral cortex, spinal cord and midbrain (Supplementary Figure 1a). Similarly, RTN4 protein expression was predominantly detected at high and moderate levels in brain-associated tissues, including the cerebellum, caudate, cerebral cortex, and hippocampus, as well. (Supplementary Figure 1b). RTN4 mRNA expression was highest in glioma, renal, breast, and urothelial cancers, with median Fragments Per Kilobase of exon per Million reads (FPKM) values of 71.9, 57.4, 51.1, and 50.7, respectively (Supplementary Figure 1c). In a similar line, RTN4 protein expression in glioma was observed at the highest levels compared to other cancers (Supplementary Figure 1d). Predominant RNA expression was observed in the Hs578T (invasive breast carcinoma), CAL-120 (metastatic breast adenocarcinoma), and CAL-85-1 (invasive galactophoric breast adenocarcinoma) cell lines (Supplementary Figure 1e).

The Expression level of RTN4 in BC and normal tissues

The results from the Tumor Immune Evaluation Resource (TIMER) database indicated a significant alteration in the mRNA expression of RTN4 across 18 cancer types, including BC. In both TIMER and Gene Expression Profiling Interactive Analysis (GEPIA) database, the mRNA expression of RTN4 in the healthy group was significantly higher than that in the tumor group (Figure 1a and b). These findings were verified through University of Alabama at Birmingham Cancer data analysis (UALCAN) portal at the protein expression level (Figure 1c).



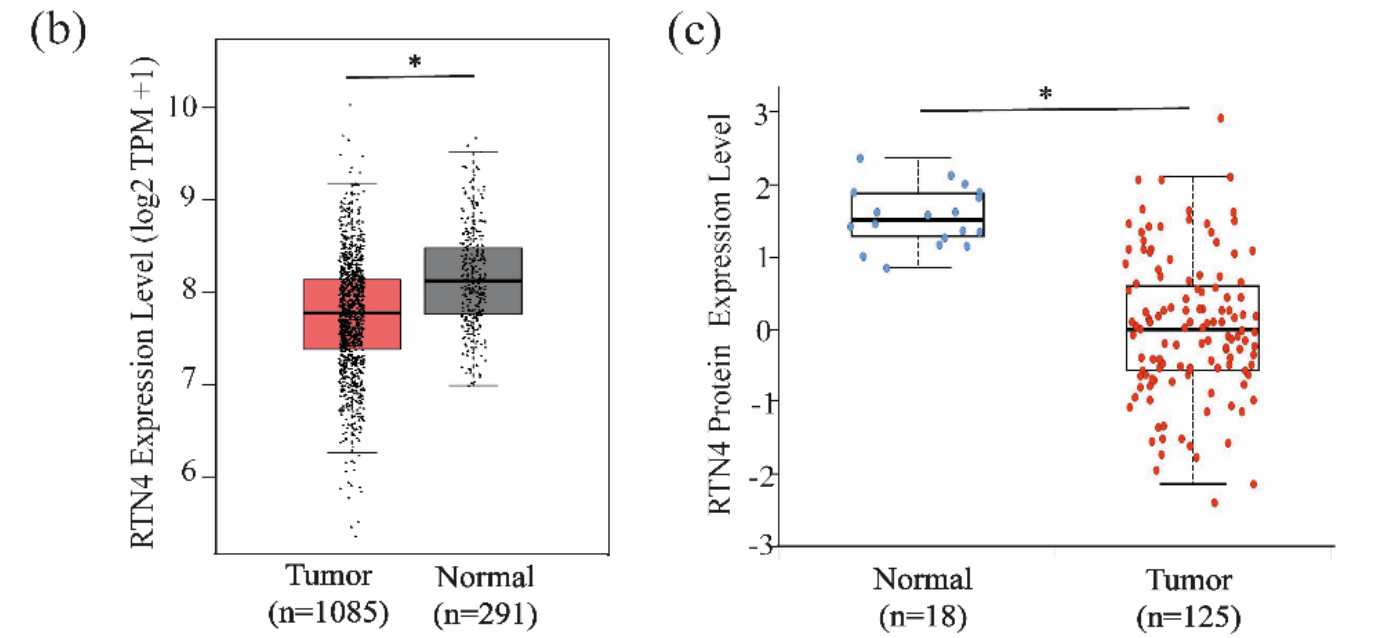
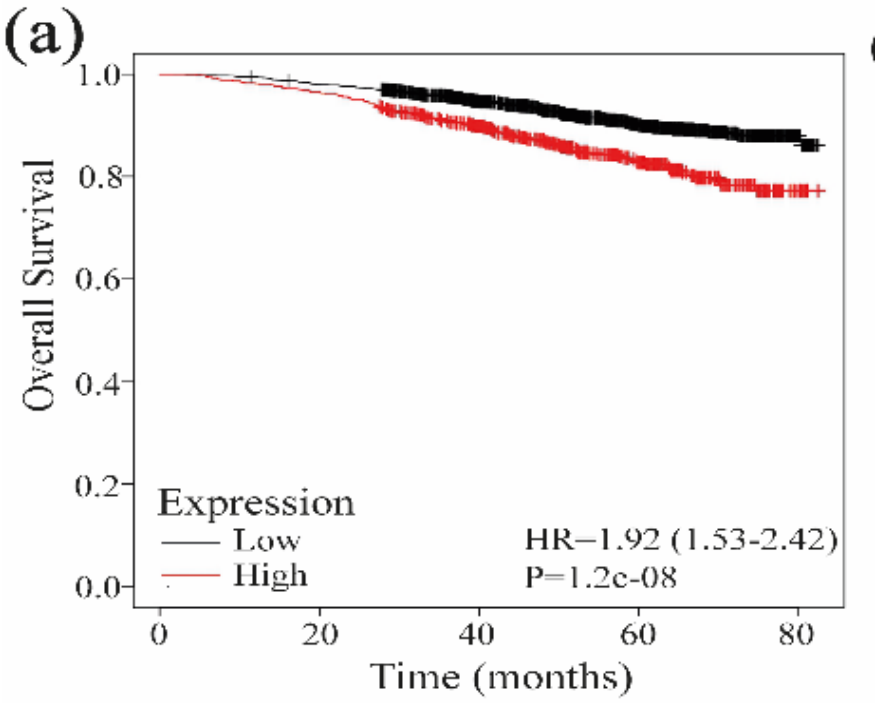
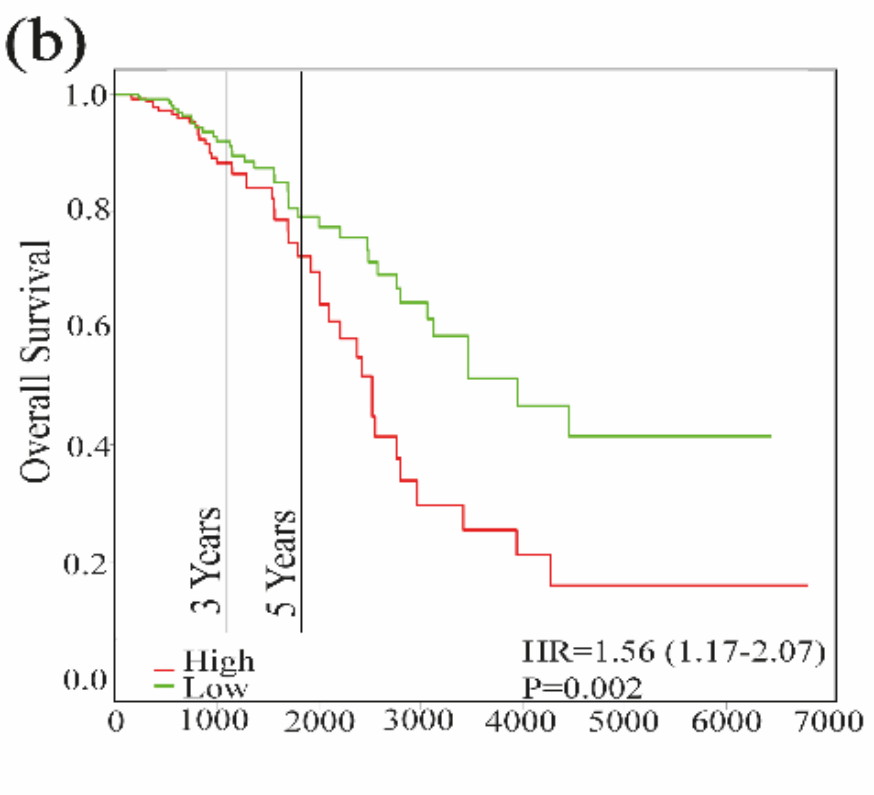


Figure 1. RTN4 expression level in BC and normal tissues; (a) TIMER2.0: Differential RTN4 expression between tumor and normal tissues derived from the TIMER2.0 database (*p<0.05; Wilcoxon test); (b) GEPIA: Comparison of RTN4 mRNA expression levels in BC and normal breast tissues based on GEPIA analysis (*p<0.05; Student's *t*-test); (c) UALCAN: Protein-level expression of RTN4 in breast tumor and normal tissues obtained from the UALCAN database (*p<0.05; Student's *t*-test). Red line indicates BRCA: Breast Cancer (BC). Full names of TCGA cancer names are as listed in Supplementary Table 1.

Prognostic value of RTN4 expression in BC

Kaplan-Meier (KM) plots represent overall survival (OS) of the patients and prognostic value of RTN4 in mRNA and protein level. The results showed that higher mRNA expression level of RTN4 was significantly associated with shorter OS for BC patients (HR = 1.93, 95% CI: 1.53-2.42, p<0.05) (Figure 2a). It was also validated by the survival plot obtained from the PROGgeneV2 (HR = 1.56, 95% CI: 1.17-2.07, p<0.05) (Figure 2b). The results of Cox analysis showed that high RTN4 expression level was significantly associated with shorter OS times in several cancers including BC, (p=0.002) (Figure 2c).




(b)
Overall Survival
1.0
0.8
0.6
0.4
0.2
0.0
3 Years
5 Years
High
Low
HR=1.56 (1.17-2.07)
P=0.002
0
1000
2000
3000
4000
5000
6000
7000

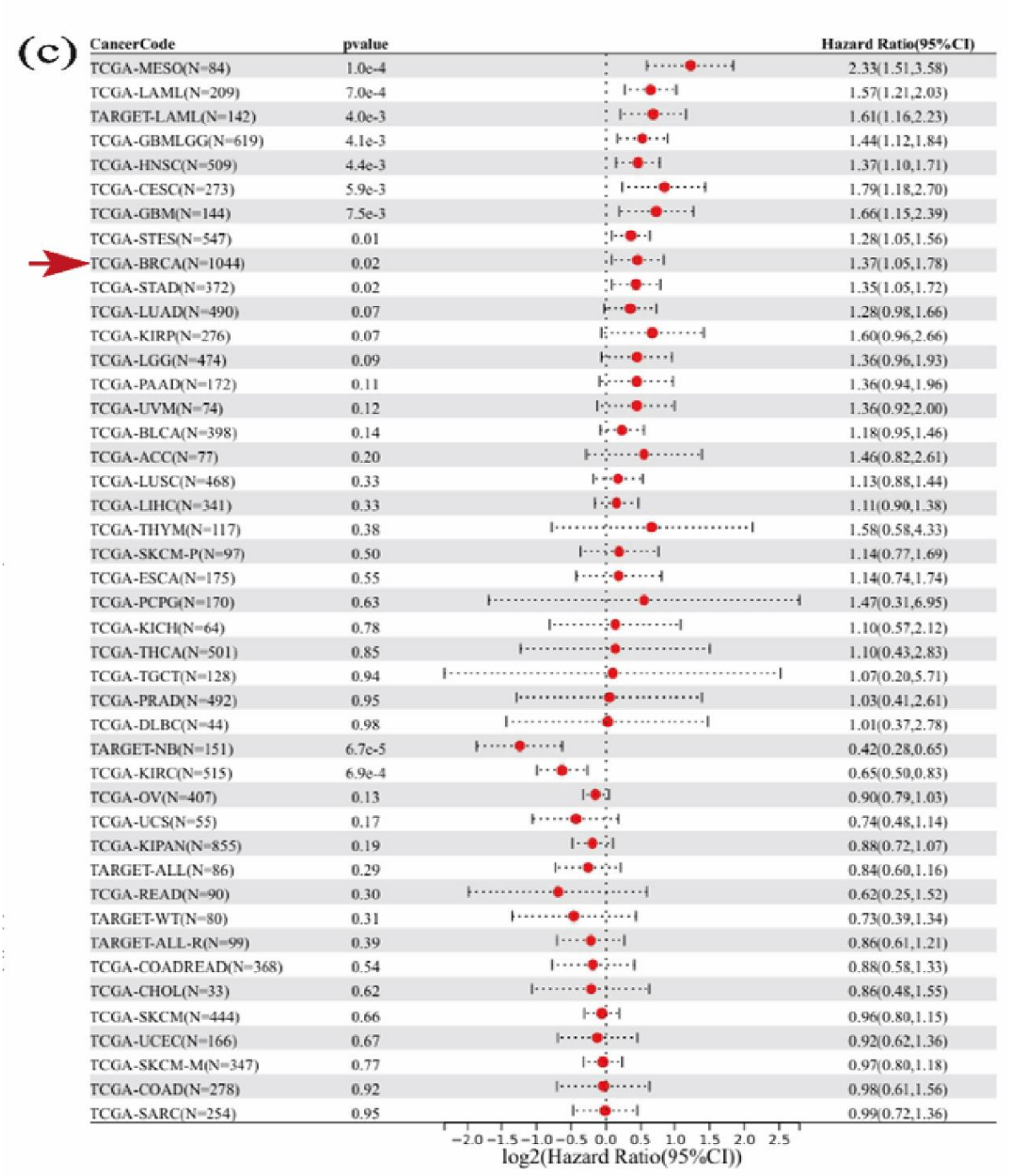


Figure 2. Survival analysis of RTN4 expression in (a) Kaplan–Meier database and (b) PROGgeneV2. Patients whose expression levels are above the median are represented by the red line, while patients with expressions below the median are depicted by the black and yellow line; (c) The forest plots represent the results of Cox regression analysis for OS, where the vertical line positioned in the middle of the plot represents the null line. An odds ratio (OR) of 1 indicating no statistically significant relationship between RTN4 expression and the outcome. The middle horizontal line within the forest plot depicts the 95% confidence interval (CI) of the OR. BRCA: Breast Cancer (BC). Arrow indicates BC. HR: hazard ratio. p<0.05 indicates significant difference. Full names of TCGA cancer names are as listed in Supplementary Table 1.

Association between RTN4 expression and the immune cell infiltration degree, immune subtypes and molecular subtypes of BC

The correlation analysis between RTN4 expression and immune cell infiltration in BC revealed significant negative associations with the levels of NK cells, memory B cells, CD8+ T cells, and macrophages M0 and M1 (p<0.05). Conversely, significant positive correlations were observed between RTN4 expression and the levels of naive B cells, macrophages M2, and neutrophils (p<0.05). There was no significant association

between CD4+ T cells, tumor purity and RTN4 expression (Figure 3a). Based on the analysis performed on the tumor-immune system interactions database (TISIDB), it was observed that RTN4 expression levels exhibited significant relationship with the five immune subtypes in BC (Figure 3b). Furthermore, molecular subtypes of BC also displayed significant associations with RTN4 expression (Figure 3c). In light of these results, it can be concluded that RTN4 expression varies significantly among both the immune subtypes and molecular subtypes in BC. Furthermore, relationship between RTN4 expression and immune scores and stromal scores were analyzed in BC. Statistically significant negative correlation with immune scores were obtained, while there was a statistically significant positive association with stromal scores ($p<0.01$) (Figure 3d and Figure 3e).

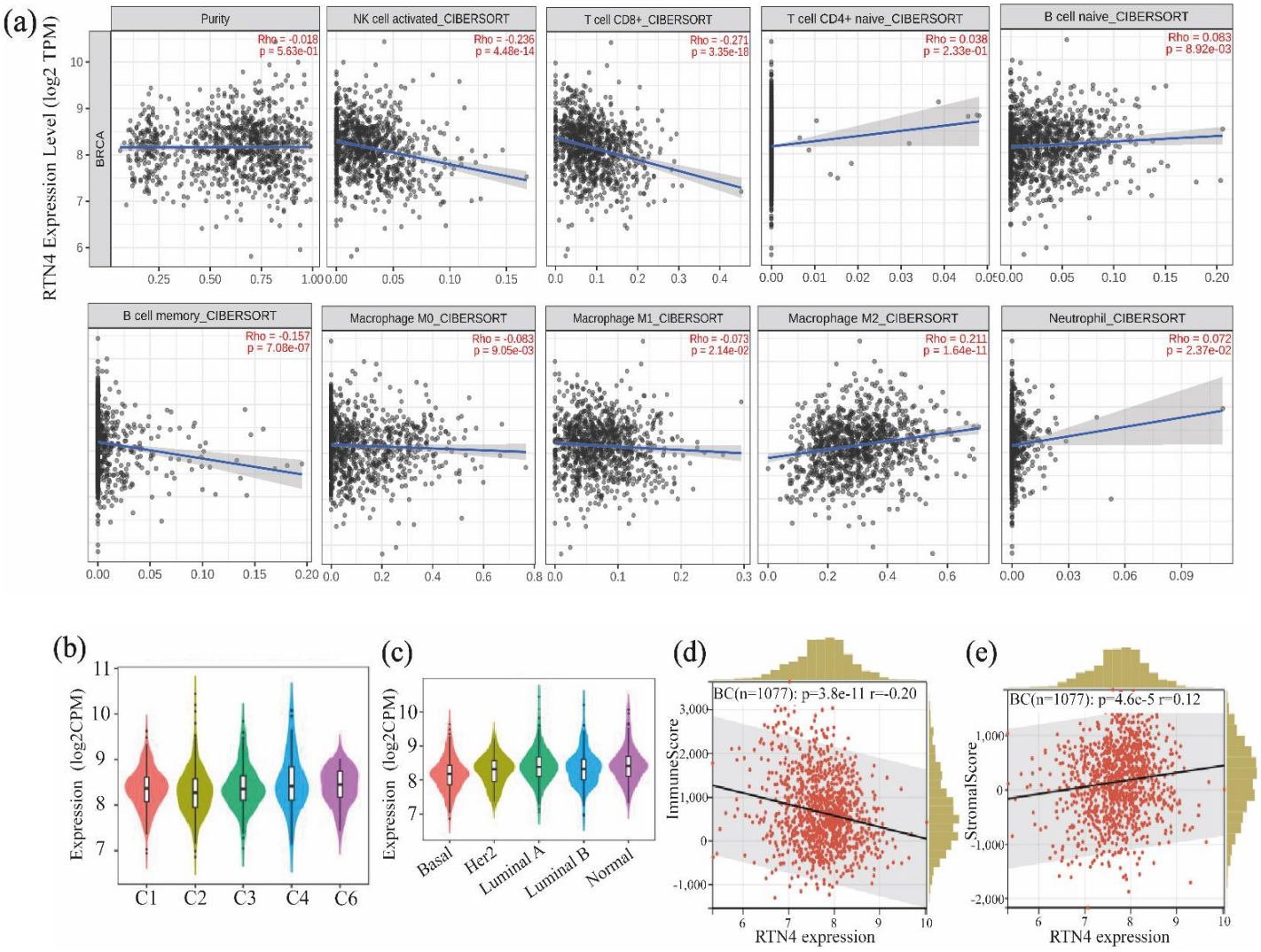
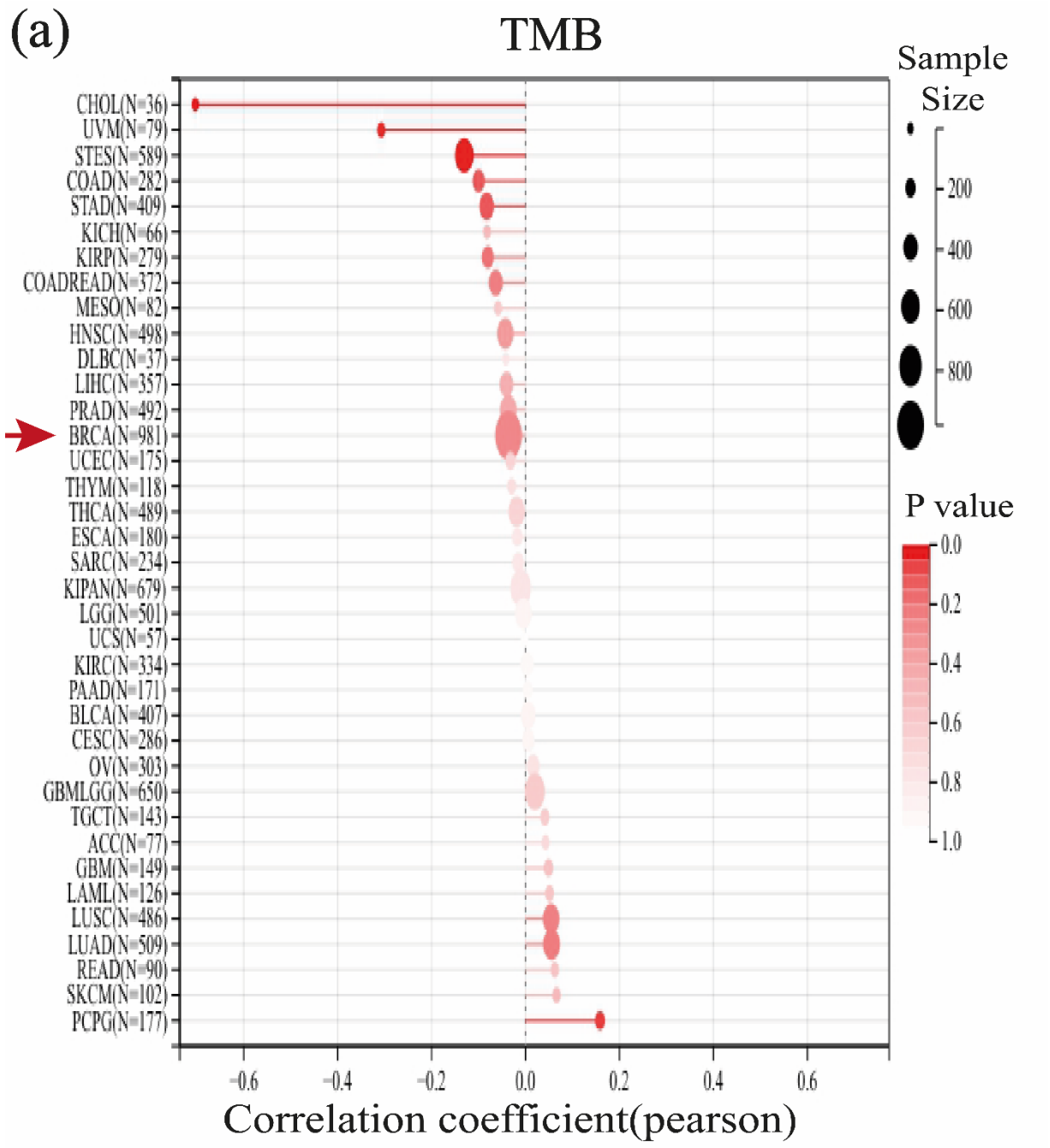


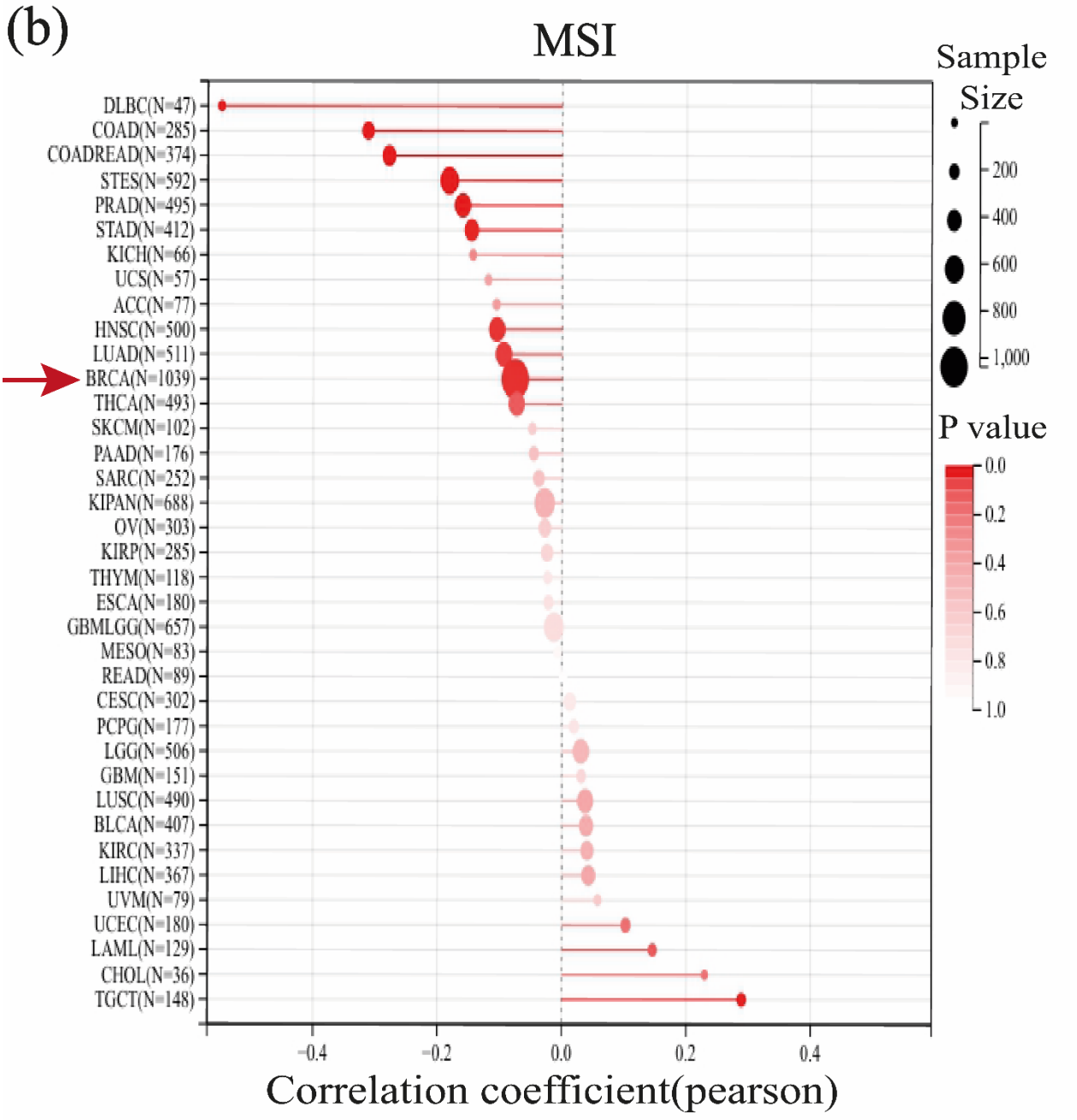
Figure 3. Relationship between RTN4 expression and the level of immune cell infiltration in BC; (a) Immune cell infiltration and tumor purity: Scatterplots show the correlation between RTN4 expression and tumor purity, as well as infiltration levels of various immune cells in BC, based on TIMER algorithm analysis; (b) Immune subtypes: Violin plots represent differences in RTN4 expression across six immune subtypes in BC: C1 (wound healing), C2 (IFN-γ dominant), C3 (inflammatory), C4 (lymphocyte depleted), and C6 (TGF-β dominant). Note: C5 was excluded due to minimal representation in the dataset; (c) Molecular subtypes: RTN4 expression across different molecular subtypes of BC is presented using violin plots; (d-e) Immune and stromal scores: RTN4 expression is correlated with (d) immune score and (e) stromal score in BC. $p<0.05$ indicates significant difference (CPM: counts per million, TPM: transcript per million).

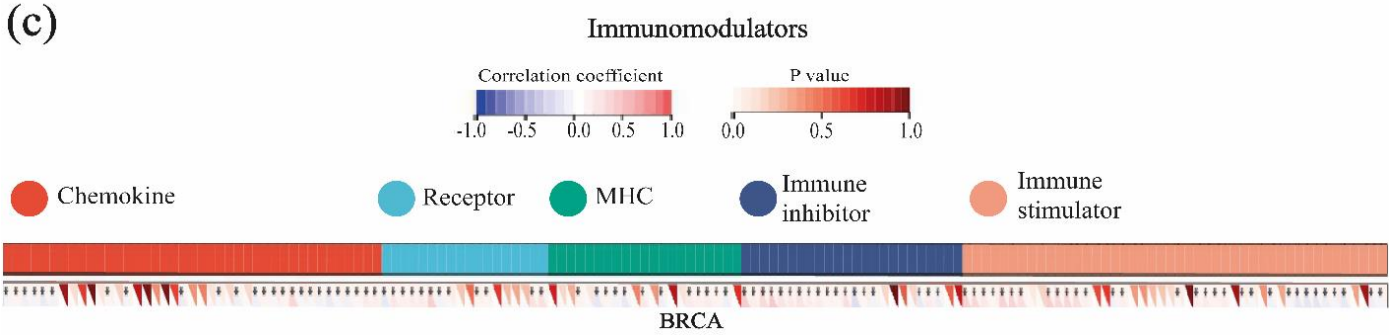
Correlation of RTN4 expression with TMB, MSI and immunotherapy biomarkers

RTN4 expression was significantly positive associated with tumor mutational burden (TMB) in pheochromocytoma and paraganglioma (PCPG, $p<0.05$), while it was significantly negatively correlated with esophageal carcinoma (STES, $p<0.05$), uveal melanoma (UVM, $p<0.05$), cholangiocarcinoma (CHOL, $p<0.05$). There was no statistical significance for BC (Figure 4a).

In the context of RTN4 expression, a significant association was obtained in microsatellite instability (MSI) across 10 different types of tumors, among which BC displayed a negative correlation with MSI ($p<0.05$) (Figure 4b). A significant positive and negative correlation of RTN4 expression and immunomodulatory and immune checkpoint-related genes obtained for different cancers including BC were shown in Figure 4c and Figure 4d. In general aspects, positive correlation between RTN4 expression and immunomodulatory genes was obtained for the most types of cancers including BC. In BC, the expression of RTN4 demonstrated significant correlations with 98 out of 150 immunomodulatory genes and with 35 out of 60 immune checkpoint genes.




(b)
MSI
Sample Size
200
400
600
800
1,000
P value
0.0
0.2
0.4
0.6
0.8
1.0
DLBC(N=47)
COAD(N=285)
COADREAD(N=374)
STES(N=592)
PRAD(N=495)
STAD(N=412)
KICH(N=66)
UCS(N=57)
ACC(N=77)
HNSC(N=500)
LUAD(N=511)
BRCA(N=1039)
THCA(N=493)
SKCM(N=102)
PAAD(N=176)
SARC(N=252)
KIPAN(N=688)
OV(N=303)
KIRP(N=285)
THYM(N=118)
ESCA(N=180)
GBMLGG(N=657)
MESO(N=83)
READ(N=89)
CESC(N=302)
PCPG(N=177)
LGG(N=506)
GBM(N=151)
LUSC(N=490)
BLCA(N=407)
KIRC(N=337)
LIHC(N=367)
UVM(N=79)
UCEC(N=180)
LAML(N=129)
CHOL(N=36)
TGCT(N=148)
-0.4
-0.2
0.0
0.2
0.4
Correlation coefficient(pearson)


(c)
Immunomodulators
Correlation coefficient
-1.0 -0.5 0.0 0.5 1.0
P value
0.0 0.5 1.0
Chemokine
Receptor
MHC
Immune inhibitor
Immune stimulator
BRCA

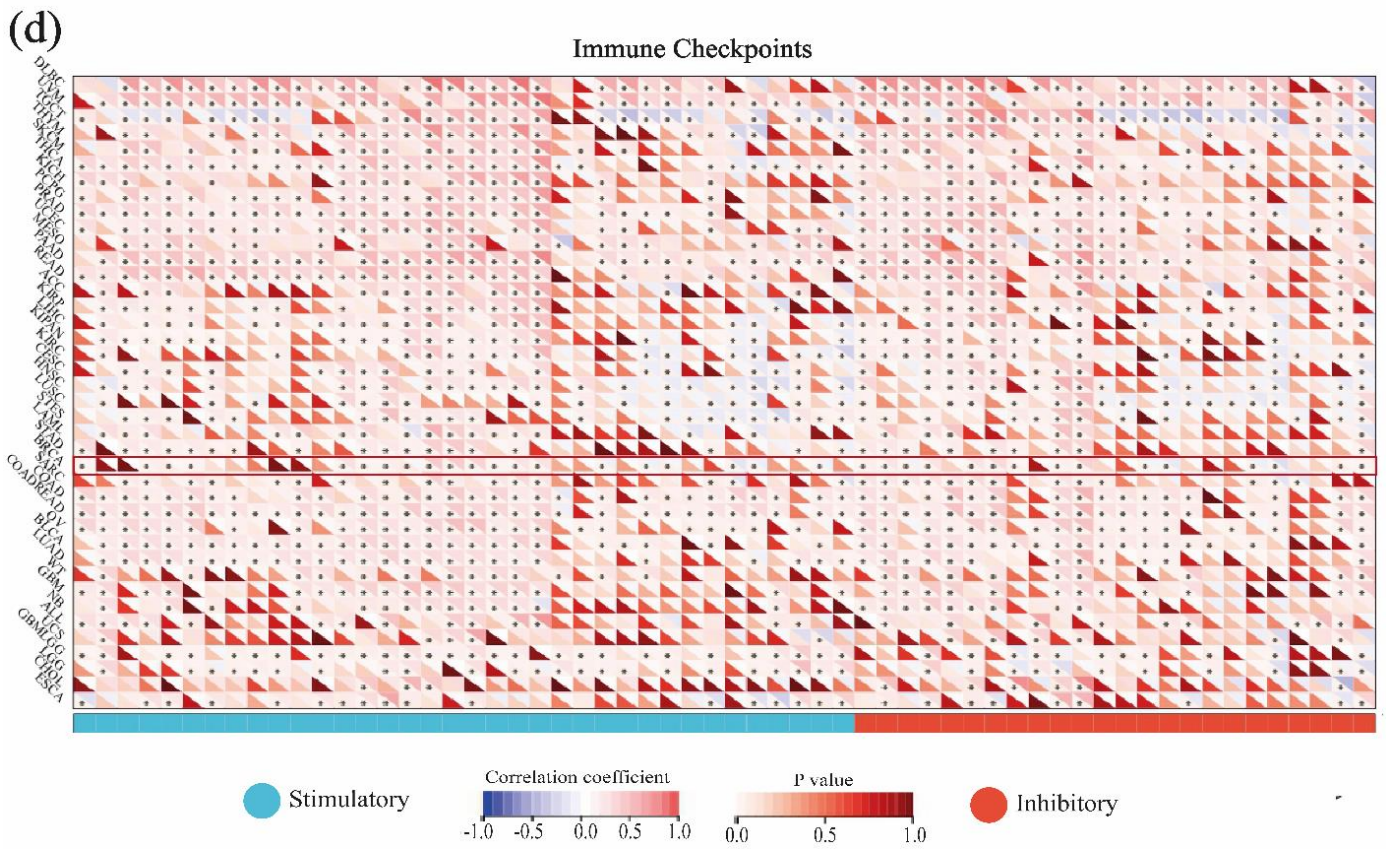
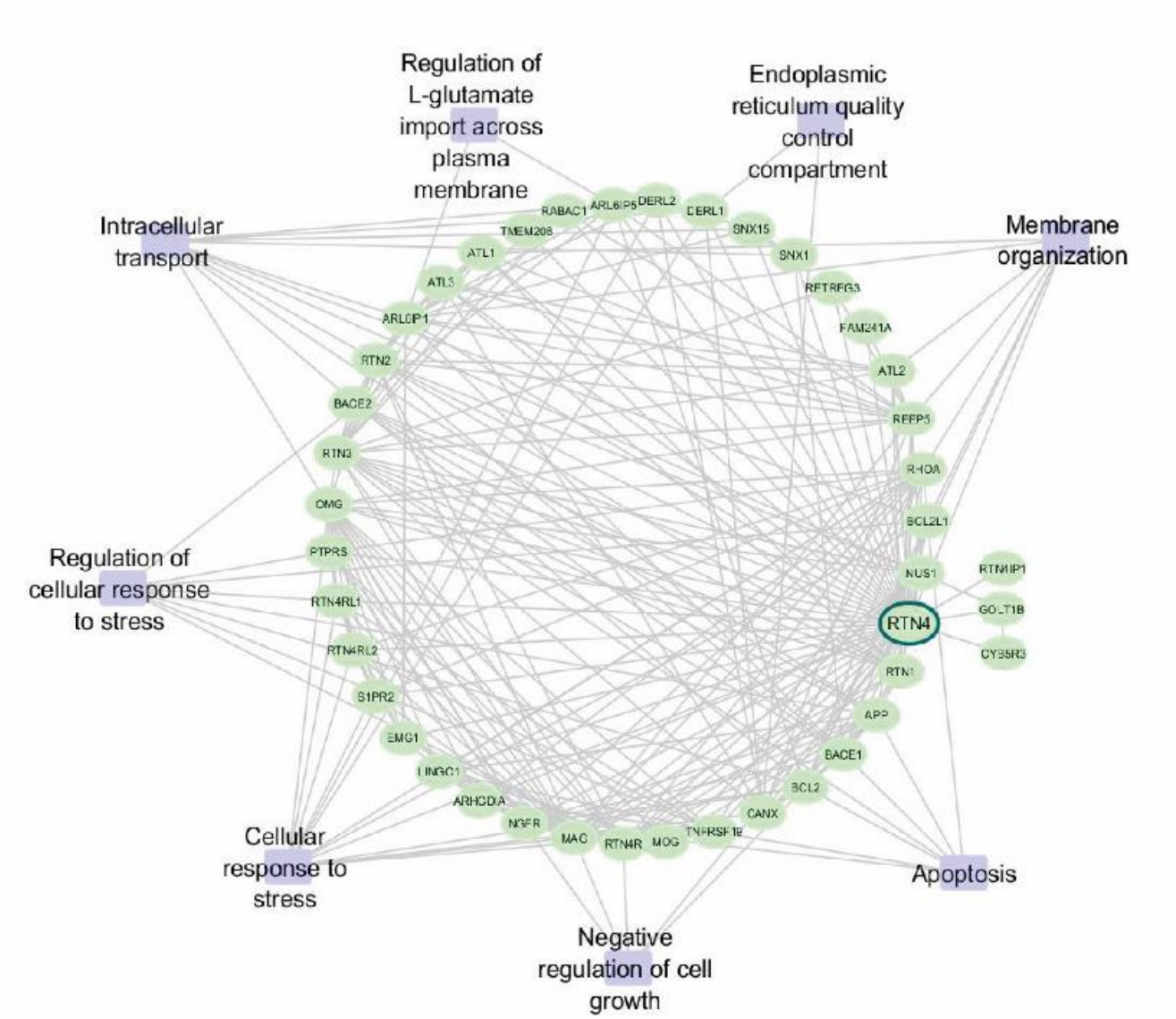


Figure 4. Correlation of RTN4 expression with TMB, MSI and immune-related markers. (a-b) Tumor mutational burden (TMB) and microsatellite instability (MSI): Lollipop plots display correlations between RTN4 expression and (a) TMB and (b) MSI across BC samples. The x-axis represents Pearson's correlation coefficients. (c-d) Immune-related genes: Heatmaps show the correlation between RTN4 expression and (c) immunomodulatory genes and (d) immune checkpoint-associated genes. *p<0.05 indicates significant difference. BC was indicated by bold letters or rectangular. BRCA: Breast Cancer (BC). Full names of TCGA cancer names are as listed in Supplementary Table 1.

Potential interactors of RTN4 and functional enrichment analysis

Potential interacting partners of RTN4 were represented in protein-protein interaction (PPI) network. (Figure 5a). The core 10 central genes of the PPI network were sequentially ordered as follows: RTN4, MAG, OMG, RTN4R, LINGO1, NGFR, TNFRSF19, RHOA, ARHGDIA, RTN3 (Figure 5b). After the functional enrichment analysis were performed, eight potential cancer associated pathways were represented in the network. The seven of them were in Gene Ontology (GO) knowledgebase and related to negative regulation of cell growth, cellular response to stress, regulation of cellular response to stress, intracellular transport, regulation of L-glutamate import across plasma membrane, endoplasmic reticulum (ER) quality control compartment and membrane organization. The one of them, apoptosis, retrieved from UniProt database (Figure 5a). The KEGG pathway analysis of the interactor proteins belonging to these eight cellular events revealed 20 potential pathways is implicated in Figure 5c. The KEGG functional pathways exhibiting significant enrichment in cancer associated pathways including apoptosis, protein processing in ER, P53, NF-KB, NOD-like receptor, Ras and PI3K-Akt signaling pathway.

(a)


Regulation of L-glutamate import across plasma membrane
Endoplasmic reticulum quality control compartment
Intracellular transport
Membrane organization
Regulation of cellular response to stress
Cellular response to stress
Apoptosis
Negative regulation of cell growth
RABAC1
ARL6IP5
DERL2
DERL1
TMEM208
SNX15
ATL1
SNX1
ATL3
RFTRFG3
ARL6IP1
FAM241A
RTN2
ATL2
BACE2
REEP5
RTN3
RHOA
OMG
BCL2L1
PTPRS
NUS1
RTN4IP1
RTN4RL1
GOLT1B
RTN4
CYB5R3
RTN4RL2
RTN1
S1PR2
APP
EMG1
BACE1
LINGO1
BCL2
ARHGDIA
CANX
NGFR
MAG
RTN4R
MOG
TNFRSF19

(b)

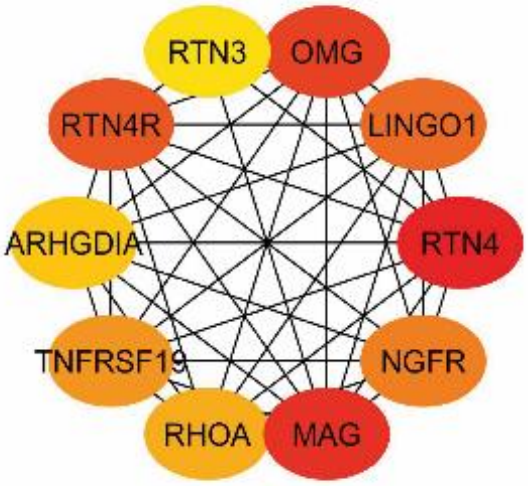

RTN3
OMG
RTN4R
LINGO1
ARHGDIA
RTN4
TNFRSF19
NGFR
RHOA
MAG

Figure 5. PPI network and functional enrichment analysis of RTN4. (a) RTN4 PPI network constructed using the STRING database and visualized in Cytoscape. Green nodes represent RTN4 protein interactors, while purple nodes indicate associated cancer-related cellular processes. (b) The core network of the top 10 key proteins within the RTN4 PPI network, identified using the MCC algorithm. Node color intensity reflects the ranking of each protein: darker red indicates higher centrality and greater potential functional importance, while yellow represents lower-ranked proteins. (c) KEGG pathway enrichment analysis of RTN4-associated targets. Red dots highlight the most statistically significant pathways, while blue indicates lower significance based on log10(FDR) values. Larger dots correspond to pathways involving a greater number of genes. Pathways with FDR<0.05 were considered statistically significant, and the top 20 most significant pathways are presented.

Correlation between RTN4 Expression and BC Drug Sensitivity

The expression levels of RTN4 in all cancer cells within the NCI-60 dataset were found to have a significant correlation with the sensitivity to nine different FDA-approved anti-cancer drugs (Figure 6). RTN4 expression levels exhibited a negative correlation with the sensitivity of tamoxifen ($r=-0.444$, $p<0.001$), estramustine ($r=-0.305$, $p=0.019$), artesunate ($r=-0.269$, $p=0.047$), and raloxifene ($r=-0.311$, $p=0.016$), while the expression levels displayed a positive correlation with the sensitivity to bleomycin ($r=0.346$, $p=0.007$), lovastatin ($r=0.405$, $p=0.001$), simvastatin ($r=0.468$, $p<0.001$), fluvastatin ($r=0.462$, $p<0.001$), and BLU-667 ($r=0.280$, $p=0.033$). In the analysis conducted only on BC cell lines, a significant positive relationship was found between the expression of RTN4 and the sensitivities of lovastatin ($r=0.988$, $p=0.002$), simvastatin ($r=0.987$, $p=0.002$), and fluvastatin ($r=0.986$, $p=0.002$).

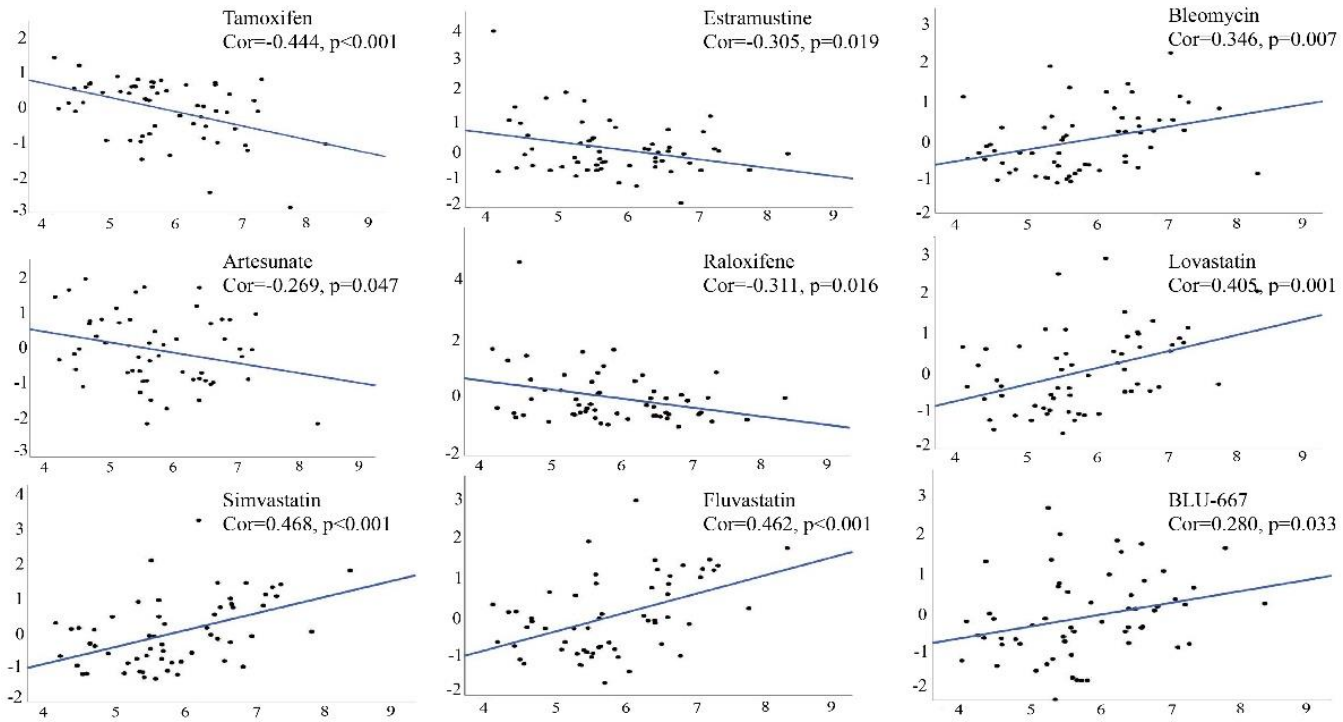


Figure 6. Association between RTN4 gene expression and sensitivity to anti-cancer drugs. The x-axis displays RNA-Seq–based gene expression levels in log2(FPKM + 1) units. The y-axis represents the drug response activity, expressed as normalized z-scores. A positive correlation was defined as a Pearson correlation coefficient (Cor) greater than 0, and results with $p<0.05$ were considered statistically significant. Positive correlations indicate that higher RTN4 expression is associated with greater drug sensitivity, while negative correlations suggest reduced sensitivity.

DISCUSSION

BC stands as the primary contributor of cancer-related deaths in women globally and ranks as the second most prevalent cancer overall [3]. It is a heterogeneous disease at both clinical and molecular levels. In the era of personalized medicine, conventional prognostic indicators like lymph node metastasis, tumor size, and histological tumor grade are no longer adequate for guiding the care of early-diagnosed BC. The identification of molecular biomarkers with potential prognostic and predictive utility significantly enhances the optimization of treatment strategies. Although several biomarkers for BC have improved the effectiveness of treatment, there is still an urgent demand for the identification of highly responsive new prognostic biomarkers and therapeutic targets for BC [4].

Considering the newly emerging focus on RTN4 and its roles in cancer and immunology, we evaluated the potential of RTN4 to serve as a biomarker in BC using multiple databases and analyzing tools. Initially, we assessed the mRNA and protein expression profiles of RTN4 in normal and cancer human tissues, and BC cell lines, as well. RTN4 expression was primarily detected in normal tissues associated with the brain, including the cerebral cortex, as well as in brain-related cancer types such as glioma. This outcome was anticipated as the primary role of RTN-4 is linked to the neurite outgrowth and axon growth [26]. Among the BC cell lines, it was frequently observed in metastatic and invasive cell lines, indicating its potential effectiveness in metastatic processes.

RTN4 expression was found to be relatively higher in normal tissues compared to BC tumors; however, elevated expression levels were associated with shorter overall survival OS in BC patients. This paradoxical finding may be explained by several potential mechanisms. Firstly, RTN4 may have dual functions, where they can be beneficial in normal tissues but detrimental in tumor tissues due to context-dependent roles. In normal tissues, the gene may be involved in essential functions, but in tumors, its overexpression might support tumor progression [27]. Secondly, tumor cells often employ various strategies to evade the immune system and promote their growth [28]. High RTN4 expression in tumors might indicate an immune escape mechanism, where the gene helps the tumor in evading immune surveillance [28], despite its normal tissue function. Subsequently, this evasion may contribute to poorer OS. This suggestion is consistent with findings indicating that NgR1, an inhibitory receptor on NK cells, interacts with its ligand Nogo-A (an RTN4 isoform) on cancer cells, thereby suppressing NK cell cytotoxicity [10]. Moreover, RTN4 may play a crucial role in maintaining normal tissue functions, and its overexpression in tumor tissues could indicate that the tumor cells are attempting to mimic normal tissue behavior. This could also mean that the gene is dysregulated in

tumor cells and contributing to tumor progression [29]. All in all, elevated RTN4 expression in tumors could serve as a prognostic marker for poor OS in BC patients. It might point out its role in tumor aggressiveness, metastasis, or resistance to therapy. Additionally, our data support that the expression pattern of RTN4 could be a valuable biomarker, helping to predict patient outcomes and guide treatment decisions.

TME consists of various components such as tumor cells, immune cells that envelop tumor cells, and stromal cells. It plays a crucial role in supporting the survival and advancement of tumor cells. The balance between these different cell types within the microenvironment significantly affects the rate of tumor cell proliferation [30]. Investigating the roles of RTN4 in the TME, its interactions with immune-related factors, and its potential roles in cancer can provide insights into the paradoxical findings observed. Tumor infiltrating cells have a significant impact on tumor development and can either inhibit or facilitate the formation of tumors [31]. In the current study, RTN4 expression associated significantly and negatively with the level of immune cell infiltration including NK cells, memory B cells and CD8+ T cells, macrophages (M0 and M1) in BC. This suggests that RTN4 may contribute to an immunosuppressive TME by limiting the presence of these cytotoxic cell types [32,33]. Conversely, RTN4 expression showed positive correlations with naive B cells, M2 macrophages, and neutrophils, indicating a potential role in promoting immune cell populations associated with tumor progression or immune evasion [34]. Therefore, RTN4 may modulate the immune landscape in BC by both suppressing anti-tumor immune responses and promoting the presence of specific immune cell types, underscoring its potential as a novel immunotherapeutic target.

In the past, BC was considered poorly immunogenic, with low levels of inflammation in the TME. However, different molecular subtypes are now known to have greater immunogenicity, TMB and immuno-related molecule expression [35]. Numerous studies have shown that diverse immune and molecular subtypes can impact the outcome of immunotherapy for BC and other cancers, as well [35,36]. The immune subtypes (C1-C6) represent specific immune features and mechanisms, offering a classification of BC cases according to the nature of the immune response. The present study demonstrated a significant correlation between RTN4 expression and five immune subtypes in BC, with higher expression levels observed in C4 and lower levels in C2. The RTN4 expression levels in these immune subtypes might be indicative of favorable or unfavorable response to specific treatments or OS [37]. Additionally, Luminal A subtype tends to have higher RTN4 expression, while the basal subtype typically exhibits lower RTN4 expression. The both molecular and immune subtype analyses suggested that RTN4 might be involved in immune regulation, potentially serving as a diagnostic marker for subtype classification. This could lead to enhance immunotherapy options for BC patients.

TME also comprises both immune and non-immune stromal elements, and studies have emphasized their significant connection to the development of cancer, the aggressive characteristics of tumors and drug resistance [38,39]. Immune scores and stromal scores are employed for predicting the degree of infiltration by immune and stromal cells, respectively [40]. Existing evidence suggests that a high immune score, signifying increased activity of immune cells within solid tumors, offers prognostic advantages including better differentiation, reduced transition to invasive cancer, a higher rate of complete response to neoadjuvant treatment and improved OS. Moreover, growing evidence has emphasized the vital roles of stromal cells in malignant tumors, such as their significance in BC alongside chemotherapy and endocrine therapy [39,41]. Our findings showed that RTN4 expression in BC was significantly associated with lower immune scores and higher stromal scores, suggesting a potential role in shaping an immunosuppressive TME. These results, including its known interaction with the inhibitory NK cell receptor NgR1, highlight RTN4’s possible contribution to immune evasion [10]. On the other hand, significant correlation between RTN4 expression and both immune and stromal scores in BC underscore the importance of exploring the interplay between RTN4 expression and the immune microenvironment, offering new perspectives to enhance therapeutic approaches.

TMB refers to the count of somatic nonsynonymous mutations within a specific genomic region. It can indirectly indicate a tumor's ability to generate neoantigens and predict the effectiveness of immunotherapy for various types of cancer. On the other hand, MSI is the occurrence of additional microsatellite alleles at a particular microsatellite location in tumors compared to normal tissues. This occurs due to the insertion or deletion of repeat units. TMB and MSI, as potential indicators for predicting the effectiveness of immune checkpoint inhibitors [30], have also been linked to antitumor immunity in previous studies [42]. In BC, while the results of our study did not show a significant relationship between TMB and RTN4 expression, a statistically significant negative relationship was obtained with MSI. The occurrence of high levels of MSI in BC is exceedingly rare, falling within the range of 0% to 1.5% [43,44]. It has been demonstrated that the presence of MSI can be regulated by genes. For instance, research has demonstrated that in BC, the expression of BC1 can influence MSI by modulating the silencing mechanism of satellite DNA within the

chromatin structure [45,46]. In this context, higher RTN4 expression might be associated with mechanisms that help maintain genomic stability, leading to lower MSI levels. This relationship might also imply that RTN4 could potentially serve as a biomarker for assessing MSI levels or susceptibility to MSI-related conditions.

The regulation of an immune response is heavily dependent on the expression levels and interactions of immunomodulatory proteins. Dysregulation of the immune response is linked to the process of oncogenesis [47] Immune checkpoints, which serve as essential regulatory molecules in immune activation, play a critical role in maintaining the immune system's proper function. Cancer cells often overactivate immune checkpoints to evade detection by the immune system. The significant correlation detected between RTN4 expression and majority of immunomodulatory genes suggests that RTN4 is associated with the regulation of the immune system within the BC microenvironment. Alterations in the expression of RTN4 may be linked to changes in the activity of these immunomodulatory genes such as tumor necrosis factor (TNF) receptor superfamily (TNFRSF)-related genes, MICB, PVR and TGFB1. Furthermore, the significant correlation observed between RTN4 and more than half of immune checkpoint genes (i.e., TNFSF4, TNFRSF9, VEGFA, VEGFB, IL10) could suggest that RTN4 has the potential to impact the ability of immune checkpoints to regulate the immune response against BC, which plays a pivotal role in cancer progression and treatment [48]. Collectively, the data above indicate that RTN4 may contribute to tumor immunotherapy by means of these targets.

The pathway analysis conducted to determine through which signaling pathways RTN4 affects cancer-related cellular events. PPI network of RTN4 is associated with various cancer-associated cellular processes including apoptosis, cell growth, cellular response to stress, intercellular transport, regulation of L-glutamate import, endoplasmic reticulum quality control and membrane organization, as well [49–53]. In our Cytoscape and, specifically, KEGG analyses, RTN4 has notably indicated its involvement in apoptosis mechanisms. Likewise, the impact of RTN4 on apoptosis has been supported by several recent studies [54–56]. Upon investigating the central interactors of RTN4, TNFRSF19 was recognized within these interactors. Along similar lines, during analyses of immunomodulators and immune checkpoints, TNFRSF-related genes exhibited a significant correlation with RTN4 expression. Given that TNFRSF is involved in crucial functions such as cell proliferation, cell death, immune regulation, and morphogenesis, it has been the focus of extensive research for the treatment of cancers, including BC [57]. In addition, certain members of the TNFRSFs have demonstrated prognostic significance in BC [58]. Hence, the interplay between this crucial protein superfamily and RTN4 may contribute to the progression of BC.

Increasing the effectiveness of drugs and uncovering possible combination treatments for cancer necessitates an examination of how different genes influence drug activity within a comprehensive biological context. This can assist to define key factors that may be secondary targets. An approach to achieve this goal involves identifying genes associated with drug response through the screening of cell lines [59]. RTN4 expression levels were observed to have correlations with the sensitivity of a variety of FDA-approved drugs, including tamoxifen, estramustine, artesunate, raloxifene, bleomycin, lovastatin, simvastatin, fluvastatin, and BLU-667. In BC, RTN4 expression was significantly associated with only the sensitivity of statins (lovastatin, simvastatin, and fluvastatin) in a conspicuously noteworthy manner. It implies that there may be a notable relationship between RTN4 and these specific statins in the context of BC. The mechanism of action of these three drugs is regulation of cell cycle by blocking CDK2/Cyclin E-mediated G1/S transition. Statins act to inhibit the proliferation of cancer cells by causing cell cycle arrest, blocking signals that promote cell growth, and triggering apoptosis in cancer cells. Additionally, they have the capacity to suppress the metastasis process in BC. RTN4 might be involved in the regulation of these cellular processes and could be a contributing factor to the mechanisms of action of these drugs in BC [60]. Taken together, RTN4 may be an essential factor in cancer cell chemosensitivity and drug resistance, making it a promising target for combating drug resistance in different cancers. Furthermore, the current data contributes to advancing personalized treatment strategies and identifying predictive biomarkers for a patient's response to a particular drug [61].

Beyond BC, RTN4 has been implicated in the development and progression of several other malignancies, further supporting its potential as a broad-spectrum oncogenic modulator. In glioma, RTN4—particularly the Nogo-A isoform—has been shown to influence tumor cell migration and invasion, potentially by altering the TME [62]. In non-small cell lung cancer, genetic variations in the RTN4 gene and reduced expression of Nogo-B and its receptor NgBR have been linked to tumor size, lymph node involvement, and advanced disease stage [63]. Similarly, in colorectal cancer, knockdown of RTN4-C isoform suppresses cell proliferation and promotes apoptosis, while novel ligands targeting RTN4 may attenuate tumorigenic activity [56,64]. In hepatocellular carcinoma, RTN4 has been associated with cell growth and apoptotic signaling, and in nasopharyngeal carcinoma, elevated Nogo-B levels correlate with metastasis and poor prognosis

[65,66]. Therefore, in addition to its relevance in BC, RTN4 may also represent a promising target for investigation in other cancer types.

Despite improving our understanding of the biomarker potential of RTN4 and its associations with various processes, this study has certain limitations. Firstly, our findings lack verification through *in vivo*/*in vitro* experiments, and there is a deficiency of clinical data to validate our results. Secondly, in the analysis of multiple datasets and tools, we were unable to remove or minimize unwanted batch effects. Thirdly, given the dynamic and complex nature of cellular events, PPI networks may not fully reflect the dynamic interaction flow and interplay between different molecular types, such as DNA and RNA. Validation of these interactions may therefore be essential to ensure their accuracy and relevance.

CONCLUSION

To our knowledge, prognostic significance of RTN4, as well as its potential signaling network associated with cancer, have not been previously reported. The existing study provided support for the idea that RTN4 could serve as a prognostic indicator for BC and might offer new insights for BC immunotherapy. Although the focus is on BC, our study presents the results on RTN4 (i.e., cox regression analysis, correlation with immunotherapy biomarkers, and drug sensitivity), which also encompass various other cancers, opening up avenues for future research on them. RTN4 is highly expressed in normal tissues than in BC tumors; however, its elevated levels are associated with poorer OS, indicating the need for further experimental studies to clarify its functional role in BC progression. Moreover, elucidating the mechanisms that drive the accumulation of specific immune cells within the TME may clarify their roles in immune regulation. As the present study is based entirely on computational analyses, future *in vitro* and *in vivo* experiments will be essential to functionally validate these findings and to support their potential translational relevance in BC. Such studies will contribute to a more precise understanding of the observed correlations and their relevance to BC progression.

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Data Availability Statement: Data are available in the figshare repository (https://doi.org/10.6084/m9.figshare.29134622.v1). mRNA and protein expression overview of RTN4; (a) RTN4 mRNA expression profiles from consensus dataset, created by combination of GTEx and HPA datasets in normal human tissues; (b) RTN4 protein expression data from HPA in normal human tissues; (c) RTN4 mRNA expression profiles from TCGA dataset in human tumor tissues; (d) RTN4 protein expression profiles from HPA 023977 dataset in human tumor tissues; (e) mRNA expression data from HPA in breast cancer cell lines. Arrows and line indicated breast and BC. The Cancer Genome Atlas (TCGA) abbreviations were used for cancer types description.

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