High expression of transmembrane P24 trafficking protein 9 predicts poor prognosis in breast carcinoma

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\textbf{ABSTRACT}

Over the years, molecular subtypes based on estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2) status have been observed to effectively guide decision-making for the optimal treatment of patients with breast carcinoma (BRCA). However, despite this progress, there are still more than 41,000 BRCA-related fatalities each year in the United States. Moreover, effective drug targets for triple-negative breast carcinoma (TNBC) are still lacking. Given its high mortality rate, it is necessary to investigate more biomarkers with prognostic and pathological relevance in BRCA. In our study, we examined the expression patterns and prognostic implications of transmembrane P24 trafficking protein 9 (TMED9) in BRCA using multiple public cohorts and BRCA specimens collected from Shanghai General Hospital. In addition to this, in vitro experiments were also performed to evaluate the effects of TMED9 expression in BRCA cell proliferation and migration. Our results have demonstrated that a high expression of TMED9 promoted BRCA cell proliferation and migration and predicted poor prognosis in patients with BRCA. In conclusion, TMED9 is a potential prognostic indicator and a possible drug target of BRCA.

\textbf{INTRODUCTION}

Breast carcinoma (BRCA) is the most common type of cancer in females, accounting for 30% of total cases in females, with approximately 49,000 new cases every year in the United States [1]. Although most patients with BRCA have a good prognostic performance, more than 41,000 patients die from BRCA each year in the United States [1,2]. Globally, BRCA is still considered a huge burden with almost two million cases and approximately 612,000 deaths every year, and is ranked as the fifth leading cause of cancer-related deaths in 2017 [3].

The treatment response and prognosis of BRCA rely on molecular characteristics that have been well established, and molecular subtypes based on ER, PR, and HER-2 status have been shown to effectively guide clinicians in selecting the optimal treatment for BRCA [4,5]. With the development of modern genomic and transcriptomic technologies, numerous gene markers are being identified to predict the treatment response and prognosis of many different types of cancer [6,7]. Feng and his colleagues detailed the risk factors and signaling pathways that were correlated with the progression and prognosis of BRCA, including molecular subtypes, mutation status of \textit{BRCA1} and \textit{BRCA2}, and WNT/\textbeta-catenin signaling pathway [8]. Recently, researchers suggested that tumor-infiltrating lymphocytes (TIL) in BRCA such as cytotoxic T cells, B cells, macrophages, and dendritic cells may predict the prognosis and response to chemotherapy [9]. In addition, adipocytes in BRCA may also drive tumor progression and metastasis via their secreted factors.
In this study, we propose that TMED9 may have prognostic implications in BRCA.

TMED9 belongs to the transmembrane emp24 domain-containing protein (TMED)/p24 family that is involved in the innate immune and protein transport via the ER-Golgi cargo pathway [12,13]. Previous studies have reported that high TMED9 expression promoted hepatocellular carcinoma (HCC) cell proliferation, migration, and invasion. Similarly, it predicted poor prognosis in patients with HCC by enhancing the expression of β-Catenin, GLI1 [14]. In a study by Algera et al., it was proposed that TMED9 may be a possible target of miR-802 and that overexpression of TMED9 increased β-Catenin expression and TCF/LEF activity in human intestinal epithelial cells [15]. In contrast, Sonakshi et al. demonstrated that TMED9 promoted colon cancer metastasis by driving the CNIH4/TGFα/GLI signaling pathway while opposing the TMED3-WNT-TCF pathway [16]. At present, there is little evidence supporting the correlation between TMED9 and cancers, including BRCA.

In this study, we aimed to evaluate the expression patterns and prognostic implications of TMED9 in BRCA using multiple public cohorts, both at the transcriptional and protein levels. Additionally, we aimed to validate these results through immunohistochemistry (IHC) staining for TMED9 in 78 BRCA samples, along with 63 paired adjacent normal breast specimens collected from the Shanghai General Hospital. Lastly, we aimed to evaluate and validate the functional status such as proliferation, migration and drug resistance of TMED9 in BRCA cell lines.

**Methods and materials**

**Data acquisition**

4 BRCA related Gene Expression Omnibus (GEO) cohorts [GSE15852 [17], GSE24124 [18], GSE33447 [19], GSE53752 [20]] were downloaded from GEO website (https://www.ncbi.nlm.nih.gov/geo) for validating the mRNA expression of TMED9 between BRCA and normal breast tissues.

**Gene Expression Profiling Interactive Analysis (GEPIA) 2.0 database**

GEPIA 2.0 (http://gepia2.cancer-pku.cn/#index) database [21] was utilized to evaluate the mRNA expression of TMED9 between tumor tissues and normal tissues based on their respective transcriptional profiles from the cancer genome atlas (TCGA). Besides, GEPIA 2.0 was also utilized to evaluate the prognostic implications of TMED9 in various tumors.

**Human protein atlas (HPA) database**

HPA (https://www.proteinatlas.org/) database [22,23] was utilized to validate the protein expression of TMED9 between BRCA and normal breast tissues, assessed through immunohistochemistry (IHC) staining.

**UALCAN database**

UALCAN (http://ualcan.path.uab.edu/) database [24] was also utilized to validate the protein expression of TMED9 in tissue samples using the data from Clinical Proteomic Tumor Analysis Consortium (CPTAC). A list of genes that are positively correlated (Pearson-CC ≥ 0.3) with TMED9 in BRCA was generated from the database using the TCGA data and GEPIA 2.0 was used to evaluate the prognostic implications of TMED9 in various tumors.

**PrognoScan database**

PrognoScan (http://dna00.bio.kyutech.ac.jp/PrognoScan/index.html) database [25] was utilized to validate the prognostic implication of TMED9 in BRCA.

**Construction of a TMED protein-protein interaction (PPI) network**

Genes that are positively correlated with TMED9 in BRCA, referred to as TMED9 co-expressed genes, were determined to construct a PPI network using the STRING database (https://www.string-db.org/) [26] and Cytoscape 3.8.2 software [27].
Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of TMED9 co-expressed genes

Aside from the construction of a PPI network, co-expressed genes were also utilized for the GO and KEGG analysis in R software, specifically using its ‘clusterProfiler’ package [28]. Terms with a false discovery rate (FDR) < 0.05 were illustrated.

Analysis of TMED9 and co-expressed genes in BRCA tumor tissues

The Gene Set Cancer Analysis (GSCA) database (http://bioinfo.life.hust.edu.cn/GSCA/#!/drug) [29] was utilized to evaluate the Pearson correlation between gene expression and drug sensitivity using the data from the Genomics of Drug Sensitivity in Cancer (GDSC) and Genomics of Therapeutics Response Portal (CTRP). In this study, the mRNA expression of TMED9 and some of its co-expressed genes were analyzed in relation to drug sensitivity. A positive correlation coefficient ($r > 0$) implies high drug resistance in a cell line with high gene expression. Drugs were ranked based on the integrated levels of the correlation coefficients and the FDRs of the searched genes. The top 30 ranked drugs were plotted.

The dependency map portal database

The Dependency Map Portal database (https://depmap.org/portal/) [30] was utilized to evaluate the probabilities of dependency of TMED9 in 38 BRCA cell lines using CERES scores from the CRISPR cohort (DepMap 21Q2 Public, CERES).

Collection of human BRCA specimens

Seventy-eight (78) BRCA samples, along with 63 paired adjacent normal breast specimens, were collected from the Shanghai General Hospital. All experimental protocols were approved by the Ethics Committee of Shanghai General Hospital, Shanghai Jiaotong University School of Medicine. All participants provided written informed consent prior to harvesting tissue samples.

Cell culture maintenance

T47D, BT474, MCF-7, BT549, and MDA-MB-231 cells were cultivated in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco) with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco) in a 37°C incubator with 5% CO₂.

Western blotting

Cellular proteins were extracted using RIPA buffer (Beyotime) containing 1X protease inhibitor mixture (Millipore) at 4°C for 30 minutes and were quantified through a Pierce™ BCA protein assay (Thermo Scientific). The separation of the protein lysate was performed through SDS-PAGE. Afterward, the lysate was transferred onto a nitrocellulose filter membrane (Millipore), blocked, and incubated with primary antibodies specific to TMED9 (Proteintech, 21,620-1-AP, 1:2000). This is followed by incubation with an HRP-conjugated secondary antibody (Sigma). Lastly, the setup was exposed with enhanced chemiluminescence for visualization. β-Actin (Proteintech, 20,536-1-AP, 1:5000) was used as an internal control.

TMED9 knockdown by lentivirus infection

TMED9-knockdown shRNAs were inserted into a pLKO.1 plasmid. The shTMED9 expression plasmids were then transfected into HEK293T cells together with the psPAX and pMD2.0 G vectors. Next, the lentiviruses were collected and used to infect MDA-MB-231 and BT549 cells. The primer sequences used are shown in Table 1.

Immunohistochemistry staining for TMED9

IHC staining (Proteintech, 21,620-1-AP, 1:200) was performed following a standard IHC protocol as described previously [31]. The staining index (0–12) was determined by multiplying the staining intensity score with the score for positive cell frequency. The staining intensity scores were defined as: 0 = negative; 1 = weak; 2 = moderate; and 3 = strong. On the other hand, frequency of positive cells was defined as: <5% = 0; 5%-25% = 1; 26%-50% = 2; 51%-75% = 3; >75% = 4 [32].
**Cell proliferation and cytotoxicity assay**

Cells were seeded into 96-well plates and cultured in DMEM with 10% fetal bovine serum (2000 cells per well, 3 parallel wells). Then, the cells were collected at different points in time, and cell counting was done using the Counting Kit-8 (CCK-8) reagent. For the cytotoxicity assay, the cells were treated with different concentrations of gemcitabine, ranging between 0 and 2.5 μmol, 24 hours after the plating of cells. Treatment was performed for 48 hours, and the number of viable cells was quantified through absorbance measurements at 450 nm.

**Colony-forming assay**

Cells were seeded in triplicates at a density of 100 cells per well in a 6-well plate, followed by three weeks of cell cultivation in DMEM with 10% fetal bovine serum. Afterward, the resulting colonies were fixed using 10% formalin and stained with 0.1% crystal violet.

**Transwell assay**

Cells were seeded in the upper chamber of a Transwell chamber (24-well, 8 μm pore, Corning) in 200 μL of serum-free DMEM (1 x 10^5 cells per well, 3 parallel wells). The lower chamber was added with 500 μL of DMEM with 10% fetal bovine serum, and the Transwell was incubated at 37°C for 24 hours. The cells were then harvested and filtered from the upper surface of the membrane and were consequently fixed with 4% paraformaldehyde, followed by staining.
with 0.1% crystal violet solution. The cells were visualized using an inverted optics microscope.

**Wound healing assay**

Cells were seeded in triplicates using 6-well plates and were incubated for 24 hours to reach approximately 80% confluency. The cell monolayers were scratched using a sterile 100-μL pipette tip. Afterward, the cells were treated with serum-free medium. Photographs of the cell cultures were taken before and after 36 hours of incubation, and the cell migration distance was calculated using ImageJ software. The migratory ratio was expressed as the ratio of the width of the wound after 36 hours divided by the width of the wound before incubation.

**Statistical analysis**

Student’s t-test was performed to analyze differences between two groups. P-values of < 0.05 are considered statistically significant.

**Results**

It has been established that TMED9 affected the progression and prognosis of HCC and colon cancer. Similarly, TMED9 may also served as a biomarker of BRCA. Through the analysis of multiple public datasets and *in vitro* experiments, we found that TMED9 played a critical role in the progression and prognosis of BRCA. With this, we proposed that TMED9 might serve as a prognostic predictor and drug target for BRCA.

![Figure 2. TMED9 protein expression profiles in BRCA and normal breast samples. Expression profiles based on the (a) HPA database and (b) CPTAC cohort. HPA, human protein atlas; CPTAC, Clinical proteomic tumor analysis consortium. ****p < 0.0001.](image)
TMED9 expression is higher in BRCA compared to normal breast tissues

Evaluating TMED9 mRNA expression in different cancer tissues revealed that, compared with paired normal samples, the mRNA expression of TMED9 was higher in most types of cancer, including BRCA, colon adenocarcinoma, lymphoid neoplasms, diffuse large B-cell lymphoma, glioblastoma multiforme, brain lower grade glioma, liver hepatocellular carcinoma, prostate adenocarcinoma, rectum adenocarcinoma, skin cutaneous melanoma, testicular germ cell tumors, thymoma, uterine corpus endometrial carcinoma, and uterine carcinosarcoma (Figure 1a). Using data from four publicly available GEO cohorts confirmed that the mRNA expression of TMED9 was indeed higher in BRCA tissues than in normal breast samples (Figure 1B-1E). All tumor samples in the GSE53752 cohort belonged to TNBC, while tumor samples in the GSE24124 cohort belonged to ER+ BRCA. These samples indicated that TMED9 expression might be higher in breast tumor samples regardless of their molecular subtype. Additionally, the protein expression of TMED9 was also found to be higher in BRCA

Figure 3. High TMED9 expression predicts poor prognosis in BRCA. (a) High expression of TMED9 predicted poor prognosis in BRCA, LGG, and LiHC as per the GEPIA database. (b-l) The correlations between the TMED9 expression and the survival of patients with BRCA were evaluated using data from different cohorts. The given Kaplan-Meier curves illustrate the probability of (b-c) overall survival (OS), using data from the (b) TCGA-BRCA and (c) GSE1453 cohort; (d) disease-free survival (DFS), based on data from the GSE4922 cohort; (e-f) disease-specific survival (DSS) based on the (e) GSE1456 cohort and the (f) GSE3494 cohort; (g-k) relapse-free survival (RFS) based on the (g) GSE1456, (h) GSE6532, (i) GSE12276, (j) GSE7390, and (k) GSE9195 cohorts; and (l) distant metastasis-free survival (DMFS) based on data from the GSE2034 cohort.
tissues than in normal breast tissues (Figure 2A, 2B). With these findings, we proposed that TMED9 was a potential diagnostic biomarker in BRCA.

**TMED9 is a prognostic indicator of BRCA**

In evaluating the prognostic implications of TMED9 in various cancer types, it was demonstrated that TMED9 was a significant risk factor in the prognosis of BRCA, brain lower grade glioma, and liver hepatocellular carcinoma (Figure 3A, 3B). Furthermore, our findings also revealed that a high expression of TMED9 predicted poor overall survival (Figure 3A-3C), disease-free survival (Figure 3d), disease-specific survival (Figure 3E, 3F), relapse-free survival (Figure 3G-3K), and distant-metastasis-free survival (Figure 3l) in BRCA. Hence, we inferred that TMED9 might serve as a potential prognostic biomarker in BRCA.

**IHC staining validates the expression patterns and prognostic implications in BRCA**

To validate the link between TMED9 and clinical character in BRCA patients, IHC assay was conducted in BRCA tissue microarray. The results have shown that TMED9 protein expression level was higher in BRCA samples than in normal breast samples dramatically (Figure 4A, 4B) and that high TMED9 expression predicted poor prognosis in BRCA (Figure 4c). These results generated were consistent with that in public databases.

![Figure 4](image_url)

**Figure 4.** Validation of the expression patterns and prognostic implications of TMED9 in BRCA. (a) IHC staining for TMED9 in two representative BRCA cases. (b) Scatter plot of TMED IHC scores between BRCA and adjacent breast samples. (c) Kaplan-Meier analysis was utilized to compare the disease-free survival between BRCA patients with a high and low expression of TMED9. IHC, immunohistochemistry; ***P < 0.001.
**TMED9 and its co-expressed genes may be involved in cancer development and progression**

Through the construction of a PPI network combining TMED9 and its co-expressed genes, it was suggested that TMED9 might be capable of directly interacting with COPI coat complex subunit epsilon (COPE), transmembrane p24 trafficking protein 1 (TMED1), BCL2 interacting protein 1 (BNIP1), peptidylprolyl isomerase B (PPIB), prolyl 4-hydroxylase subunit beta (P4HB), myeloid derived growth factor (MYDGF), and beta-1,4-galactosyltransferase 7 (B4GALT7) (Figure 5a). Results of the functional enrichment analysis implied that TMED9 and its co-expressed genes, particularly those involved in biological processes, might play critical roles in cancer development and progression, such as retrograde vesicle-mediated transport, Golgi to ER, endoplasmic reticulum lumen, coated vesicle, and protein processing in the endoplasmic reticulum (Figure 5B, 5C).

Pan-cancer analysis has shown that the correlations between TMED9, TMED1, PPIB, P4HB, and B4GALT7 mRNA expression and the top 30 drugs were consistent, both in the GDSC platform and the CTRP platform (Figure 6a, 6b). With these findings, we proposed that these five genes might be critical players in the occurrence and development of BRCA.

**The TMED9 gene is essential in BRCA cell lines**

The CERES scores of the given cell lines ranged from −0.09 to −0.52 (from MFM223 to HCC1954), with a mean value of −0.32 (Figure 7). As a consensus, a low score implied that a gene was more likely to be essential in a given cell line, and a negative score implied that the downregulation of a gene might inhibit the proliferation of a given cell line. Since the CERES scores generally had negative values, it was inferred that TMED9 might be essential in these BRCA cell lines.

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**Figure 5.** PPI network and functional annotation of TMED9 and its co-expressed genes. (a) PPI network combining TMED9 and its co-expressed genes. (b) GO and (c) KEGG analysis of the co-expressed genes. PPI, protein-protein network; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.
**TMED9 knockdown suppresses the proliferative, drug resistant and migratory capacities of BT549 and MDA-MB-231 cells**

In evaluating the expression of TMED9 in five BRCA cell lines, we found that TMED9 expression was higher in both the BT549 and MDA-MB-231 cell lines than that in the T47D, BT474, and MCF-7 cell lines (Figure 8a). ShRNAs specifically targeting TMED9 was used to constructed the TMED9-knockdown MDA-MB-231 and BT549 stable expression cell lines (Figure 8b). Moreover, it was observed that TMED9 knockdown effectively suppressed the proliferative (Figure 8c) and colony-forming (Figure 8d) capacities of BRCA cells, and that TMED9-knockdown BT549 and MDA-MB-231 cells were more sensitive to gemcitabine (Figure 8e). Lastly, TMED9 knockdown was also observed to suppress the migratory ability of BT549 and MDA-MB-231 cells, as shown in the transwell assay (Figure 9A, 9B) and wound healing assay (Figure 9C, 9D). With these results, it was therefore suggested that TMED9 affected the proliferative, drug resistant and migratory capacities of BRCA cell lines.

**Discussion**

Endocrine therapy based on ER, PR, and targeting therapy based on HER2 status (tamoxifen treatment and trastuzumab treatment) [33,34] has
Figure 7. Probability of TMED9 dependency in BRCA cell lines.
exhibited a high performance as BRCA treatments, although they are quite ineffective for triple-negative breast cancer (TNBC). Numerous molecular markers are being utilized for the clinical diagnosis and treatment of BRCA. These markers include BRCA1 and BRCA2 for examining BRCA genetic susceptibility [35], and PAI-1, Ki67, cyclin D, cyclin E, p27, p21 for the assessment of BRCA prognosis [36]. Moreover, several multi-gene signatures that could predict BRCA prognosis were approved by the US Food and Drug Administration. These include Oncotype DX, MammaPrint, and Rotterdam Signature, among many others [37]. Currently, molecular markers that guide immune therapies (e.g., tumor-associated antigen [38,39] and the immune checkpoint molecule PD-L1) are attracting more attention [40].

It has been reported that TMED9 promoted metastasis and predicted poor prognosis in HCC and colon cancer [14,16]. However, the correlation between TMED9 and BRCA is still unclear. Previous studies have shown that TMED9 interacted with ARFGAP1 and BPIFB3 and downregulated the degradation of cytoplasmic proteins or organelles in the non-canonical autophagy pathway by promoting lysosomal degradation [41]. This evidence suggested that the high expression of TMED9 may promote the proliferation of cancer cells by inhibiting autophagy.

In our study, we examined the expression patterns and prognostic implications of TMED9 in BRCA using data from multiple public cohorts and BRCA specimens. Our results demonstrated that knockdown of TMED9 suppressed the proliferative, drug resistant and migratory abilities of BRCA cell lines. We also found that TMED9 directly interacted with the COPE, TMED1, BNIP1, PPIB, P4HB, MYDGF, and B4GALT7 genes. Previous studies have reported that TMED1 participated in interleukin-13 signaling [42,43] and in the RNF26-related complex, which modulated innate immune signaling [44]. Hence, the involvement of TMED9 in anti-tumor immunity needs further investigation. Numerous studies have reported that BNIP1, a pro-apoptotic protein in the Bcl-2 family [45], promoted the apoptosis of cancer cells [46,47], including cervical cancer cells [48]. On the other hand, PPIB, a peptidylpropyl isomerase, participated in colorectal cancer progression and chemoresistance [49]. Studies have also suggested that P4HB may extensively regulate tumor progression and prognosis of patients with cancer [50–54], including BRCA [55,56]. In BRCA, TMED9 may affect cancer progression and prognosis by interacting with these proteins.
Despite being an extensive analysis, there are still several limitations in our study. Firstly, this study does not cover the elucidation of molecular mechanisms on how TMED9 specifically affects the proliferation, drug resistance and migration of BRCA cells. Secondly, in vivo experiments are not conducted to consolidate the results generated. Despite these limitations, our study is still the first to report that TMED9 may predict BRCA prognosis and serves as a drug target for treating BRCA.

**Conclusion**

TMED9 is a potential prognostic indicator and drug target for BRCA treatment.
Research highlights

1. TMED9 expression was higher in BRCA samples than in normal breast samples.
2. High TMED9 expression predicted poor prognosis in patients with BRCA.
3. TMED9 knockdown suppressed MDA-MB-231 and BT549 cells proliferation and migration in vitro.

Abbreviations

TMED9, transmembrane P24 trafficking protein 9; TCGA, the cancer genome atlas; GEO, Gene Expression Omnibus; BRCA, breast carcinoma; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor-2; TNBC, triple negative breast carcinoma; IHC, immunohistochemistry; GEPIA, Gene Expression Profiling Interactive Immunohistochemistry; gesterone-231, hormone associated with breast cancer risk; MYDGF, myeloid derived growth factor; P4HB, peptidylprolyl isomerase B; PPIB, peptidylprolyl 4-hydroxylase subunit beta; MYDGF, myeloid derived growth factor; B4GALT7, beta-1,4-galactosyltransferase 7.

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