Original Article

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TRPC6 expression is negatively correlated with breast cancer progression

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SUMMARY: The transient receptor potential channel 6 (TRPC6), a Ca^{2+}-permeable, non-selective cation channel, is a potential drug target widely expressed in most human tissues. However, the role of TRPC6 in breast cancer remains largely unknown. We comprehensively investigated the relationships between TRPC6 and breast cancer using online databases, including TIMER, cBioPortal, UALCAN, the Kaplan-Meier plotter, the Human Protein Atlas, STRING, and the LinkedOmics, to evaluate the prognostic value of TRPC6. We also tentatively explored the effects of TRPC6 modulation on proliferation and metastasis potential in several breast cancer cell lines by MTT assay, colony formation assay, flow cytometry, and scratch wound healing assay. The TCGA data showed lower *TRPC6* gene expression in breast tumours *versus* normal breast tissues. The Human Protein Atlas revealed that the TRPC6 protein was generally expressed at low levels in both normal and breast carcinoma tissue. The Kaplan-Meier plotter showed that the low TRPC6 level is correlated with worse relapse-free survival probability in breast cancer. Moreover, the *TRPC6* gene was linked to immune infiltration in breast cancer, and this relationship affected the prognosis of breast cancer. In contrast to published researches, we found that TRPC6 expression was negatively correlated with breast cancer cell proliferation and metastasis abilities, suggesting TRPC6 as a promising prognostic biomarker for breast cancer, where low expression of TRPC6 was related to worse prognoses. This research raises the necessity of rethinking the mechanism perspective and targetability of TRPC6 in breast cancer, which warrants further investigation.

Keywords: transient receptor potential channel, biomarker, metastasis

1. Introduction

The transient receptor potential canonical (TRPC) channels are nonselective cation channels belonging to the TRP family expressed in various tissues. The TRPC subfamily comprises seven transmembrane proteins (TRPC1–7). TRPC6 is a crucial membrane protein composed of multiple subunits that assemble to form a functional channel pore, which allows Ca^{2+} and Na^{+} ions to enter the cells between the fifth and sixth transmembrane domains. Among all TRPCs, TRPC6 deficiency or increased activity has been associated with many diseases (*1*). Aberrant overexpression of TRPC6 has been documented across multiple tumour types compared to normal tissues, including different types of cancers such as glioma (*2*), salivary gland tumours (*3*), renal cancer (*4*), prostate cancer (*5*), ovarian cancer (*6*), cervical cancer (*7*), and breast cancer (*8*).

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer-related deaths in women, accounting for one-quarter of all new female cancer cases diagnosed worldwide (*9,10*). Progress in clinical management strategies and earlier detection through increased awareness and use of mammography has improved survival for breast cancer patients, with 5-year relative survival rates of 89% (*11*). However, for metastatic breast cancer, the 5-year relative survival rate remains low at 27% (*12*). There is an urgent need for novel prognostic biomarkers and a deeper understanding of molecular mechanisms to drive innovations in more effective treatment options, especially for those more aggressive and metastatic breast cancer cases. Early studies have shown that TRPC6 mRNA and protein levels are elevated in breast carcinoma specimens compared to normal breast tissue, with functional channels present in MCF-7 and primary cultures (*8,13*). TRPC6 is the predominant amongst

all TRPCs in the samples of human breast ductal adenocarcinomas and breast cancer cell lines, such as MCF-7 and MDA-MB-231 (*13*). The specific activator of TRPC6, hyperforin, significantly reduced the growth and viability of the breast cancer cell lines but did not affect the non-cancerous breast cell line. Silencing of TRPC6 in MDA-MB-231 cells resulted in a significant reduction in cell growth but not viability (*13*). More recent research confirms that TRPC6 is overexpressed in ER-positive and triple-negative breast cancer cell lines (*14*).

While the relevance of TRPC6 in breast cancer have been proposed in several studies, its histological detection in breast tumour tissues, as well as its role in breast cancer progression, lacks systematic study. Studying TRPC6 in breast cancer could lead to new therapeutic options. The main focus of this study is to systematically explore the role of TRPC6 as a prognostic biomarker in breast cancer and the correlation of TRPC6 expression with breast cancer progression.

2. Materials and Methods

2.1. Gene expression databases, survival analysis

TIMER (*https://cistrome.shinyapps.io/timer/*) was used for analyzing *TRPC6* gene expression across all TCGA tumours and the correlations between TRPC6 expression and abundance of multiple immune infiltrate cells as well as tumour purity in BRCA. "Survival" module of TIMER was applied to analyse the clinical relevance of different tumour immune subsets.

The cBioPortal for Cancer Genomics (*http://cbioportal.org*) was used for exploring, visualizing, and analysing the genetic alterations or changes in gene expression of TRPC6 in the TCGA breast cancer cases. The cancer study "Breast Invasive Carcinoma (TCGA, PanCancer Atlas, 1084 total samples)" was selected for genetic alterations or changes in gene expression analysis (mutations, structural variants, putative copy-number alterations, mRNA expression, and protein/phosphoprotein level) in breast cancer. In addition, clinical patient data in UALCAN (*http://ualcan.path.uab.edu/analysis.html*) was used to classify primary tumour samples and generate box plots for each gene expression level in each subgroup.

The Kaplan-Meier plotter (KM plotter, *http://kmplot.com/analysis/*) was applied to analyse the prognostic value of TRPC6 in breast cancer patients using the chip data. The parameter settings include "split patients by median", "only JetSet best probe set", and "follow up threshold = 120 months". The web tool *TNMplot.com* in KM plotter was applied for the comparison of *TRPC6* gene expression in normal, tumour and metastatic tissues, and in selected tissues at different stages.

2.2. TRPC6 expression analysis from Human Protein Atlas

Direct comparisons of TRPC6 protein expression between normal and breast cancer tissues were based on the immunohistochemical analysis data from the Human Protein Atlas (HPA, *https://www.proteinatlas.org*).

2.3. Cell culture and cell transfection

Human breast cancer cell lines (MCF-7, MDA-MB-231, and MDA-MB-468) were purchased from the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% FBS (Procell, Wuhan, China) at 37°C in a humid atmosphere with 5% CO_2-95% air, routinely confirmed negative for mycoplasma and bacteria contamination.

The human TRPC6 expression plasmid (pcDNA3.1-hTRPC6-YFP) (*15*) was obtained from Addgene (Addgene plasmid # 21084, Massachusetts, USA). Plasmid transient transfections were carried out with jetPRIME transfection reagent (Polyplus, Illkirch, France). The TRPC6 knock-down transfection was carried out at 25 nM final concentration of siRNA with siRNA-Mate transfection reagent (Genepharma, Shanghai, China). The TRPC6 overexpression (vector *vs* hTRPC6 group) and knock-down (ncRNA *vs* siTRPC6 group) efficiency were verified by qPCR.

2.4. Cell proliferation by MTT and colony formation assay

For MTT assay, cells were collected and seeded in 96-well plates (3×10^3/100 µL/well). After adherence, the cells were transfected for TRPC6 overexpression/knock-down or treated with different concentrations of hyperforin (HY-116330A, MCE, Shanghai, China). Cells were then incubated for 48 h. MTT reagent (Solarbio, Beijing, China) was added after treatment (5 mg/mL in PBS, 10 µL/well), and then the cells were incubated at 37°C for 2 h. The medium was removed after centrifugation. DMSO was added (150 µL/well) to dissolve the formazan by shaking on an orbital shaker for 5 minutes. Light absorbance of the solution was measured at 570 nm on the SpectraMax iD3 plate reader (Molecular Devices, San Jose, CA). Cell viability was calculated as a percentage of the mean of the respective control group (vector/ncRNA or vehicle group).

For the colony formation assay, 500 cells were plated into 6-well plates and continuously cultured for ~ 7 days. After fixation with 4% paraformaldehyde for 15 min, staining with 0.2% crystal violet for 10 min, and washing, visible colonies were imaged and counted.

2.5. Scratch wound healing assay

Cells (2×10^5 cells/well) were grown in 24-well plates

overnight and then serum-starved for 24 h. Then, the cells were scratched using 200 µL pipette tips, immediately rinsed twice with PBS, and subsequently cultured in serum-free medium. Photos were taken under a microscope at 0 h, 12 h, 24 h, and 48 h after the scratches were made. The wound area was measured using Fiji/ImageJ.

2.6. Transwell invasion assay

Cells from each group were serum-starved for 24 h. Then, the cells were harvested and suspended in serum-free DMEM with 0.1% BSA. The cells were loaded into the upper chambers (1×10^5 cells in 0.1 mL/insert) embedded with Matrigel and incubated for ~48 h. After incubation, the cells inside the inserts were gently removed using wet cotton swabs; the filter membrane was washed with PBS, fixed with 70% ethanol, and stained with 0.2% crystal violet. The number of invading cells was counted manually under a light microscope.

2.7. Flow cytometry analysis of cell apoptosis

Cells seeded in 6-well plates (2×10^5 per well) were transfected with plasmid or siRNA for TRPC6 overexpression or knockdown. For cell apoptosis analysis, cells were harvested and washed with cold PBS 48 h after transfection. Then cell surface of phosphatidylserine in apoptotic cells was quantitatively estimated by using Annexin-V/FITC and PI apoptosis detection kit (E-CK-A211, Elabscience, Wuhan, China). Stained cells were subsequently analysed using a FACScan flow cytometer (Becton-Dickinson, New Jersey, USA) for cell apoptosis.

2.8. Interaction networks and enrichment analysis

STRING (*https://string-db.org/*), containing massive amounts of data on protein–protein interactions, was applied to construct TRPC6 interaction network. LinkedOmics (*http://linke domics.org*) includes 32 TCGA cancer-associated multi-dimensional datasets. The LinkFinder module in LinkedOmics was used to research differentially expressed genes associated with TRPC6 levels in the TCGA BRCA cohort (n = 1093). The results were analyzed using Pearson's correlation coefficient. All results are graphically represented in a volcano, heat map. We signed and sorted the LinkFinder results and used GSEA to analyze GO terminology (biological process) and KEGG pathway.

2.9. Statistical analysis

All experimental data were expressed as the mean ± standard error of the mean (SEM) of at least three independent assays unless otherwise specified. The data were analysed using Prism 9 (GraphPad Software Version 9.0.0, USA). Student's t-test was applied for comparing two-group experiments. ANOVA analysis, followed by Tukey's multiple comparisons test, was used when comparing more than two groups. The significance level was considered as below 0.05 in all experiments.

3. Results

3.1. TRPC6 expression in breast cancer patients

To understand the basic expression information of TRPC6 in breast cancer, TIMER was applied to identify *TRPC6* gene expression at the transcriptional level across all TCGA tumours compared with the respective normal tissues. In breast carcinoma (BRCA) patients, the *TRPC6* gene level in tumour tissues is significantly lower compared with the normal breast tissues (Figure 1A, $p < 0.001$). The lower TRPC6 in breast cancer tissues was further validated by ULCAN and KM plotter analysis (Figures 1B and 1C). In ULCAN analysis, there was no significant difference among the individual cancer stages (Figure 1E) or different nodal metastasis (Figure 1F), but in TNMplot analysis, TRPC6 expression level in stages II, III and IV was significantly lower than stage I (Figure 1D). Moreover, the subtype analysis in ULCAN showed that TRPC6 expression in more aggressive HER2 and triple negative subtypes was notably lower than that in the luminal subtype tumour (Figure 1G). The data suggest that TRPC6 downregulation may be correlated with more aggressive subtypes and higher metastatic potential, and *TRPC6* might act as a tumour suppressor gene in breast cancer.

3.2. Genetic alteration in *TRPC6* gene and prognostic value of TRPC6 in BRCA patients and TRPC6 protein expression analysis in breast cancer tissues

The cBioPortal platform was applied to determine the genetic alterations or gene expression changes of the *TRPC6* gene, which indicates that 4% (45 out of 1084, Figure 2A) of the sequenced BRCA cases/patients have genetic alterations or changes in gene expression in the *TRPC6* gene. We further investigated the prognostic value of TRPC6 in breast cancer patients using the Kaplan-Meier plotter. As shown in Figure 2B, although there is no significant difference in the overall survival (OS, $p > 0.05$), high *TRPC6* gene expression is correlated with better relapse-free survival (RFS, HR = 0.8 (0.72-0.88), p = 21.5e-05, follow-up time = 120 months) (Figure 2C). The median RFS for breast cancer patients with high TRPC6 expression was 59 months, and 42 months for patients with low TRPC6 expression. These findings suggested that the changes of the *TRPC6* gene could have an important clinical impact, although without statistical significance in a subset of breast cancer patients.

We next explored the TRPC6 protein expression in

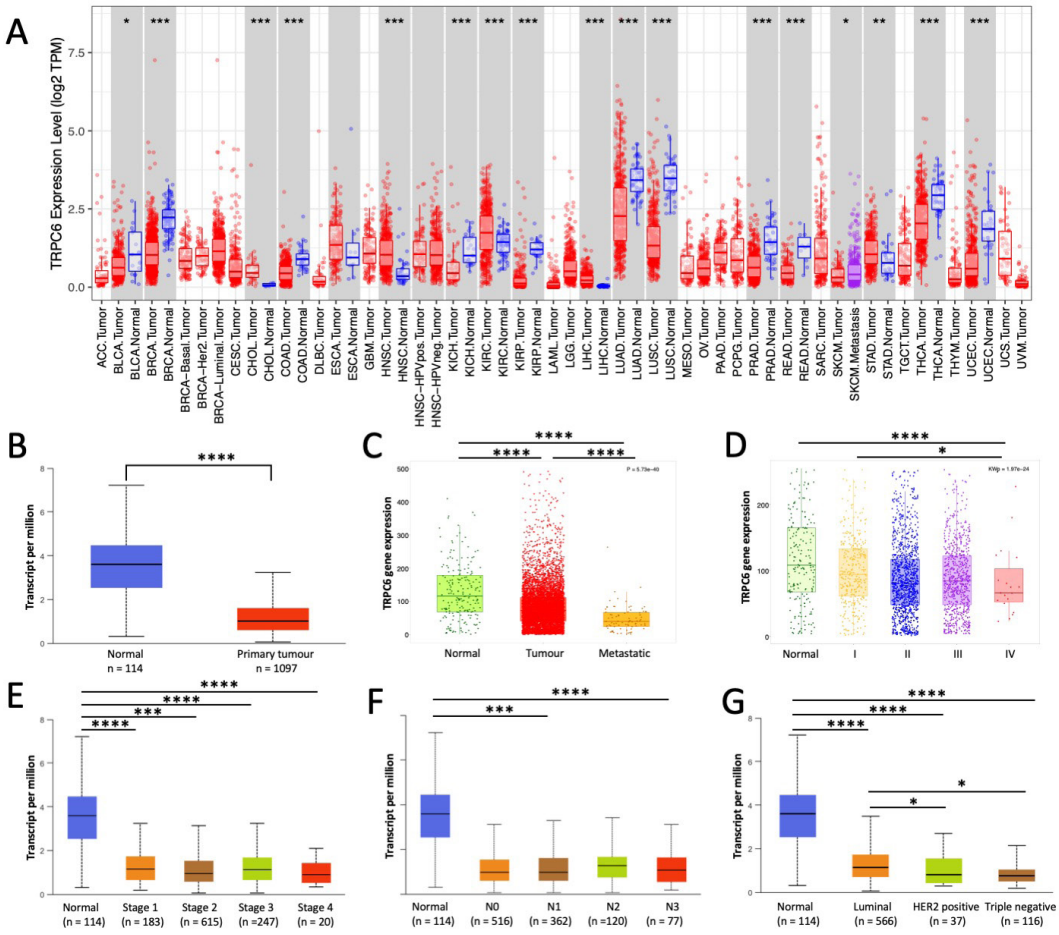

Figure 1. Analysis of TRPC6 in normal and cancerous breast tissues. A. RNA-Seq expression data of TRPC6 in tumour and normal tissues from each cancer type obtained from TIMER. Distributions of gene expression levels are displayed using box plots, with statistical significance of differential expression evaluated using the Wilcoxon test. **B.** Validation of TRPC6 downregulation in breast primary tumours from the UALCAN cohort analysis. **C.** Comparison of TRPC6 expression in normal, tumour, and metastatic tissues in TNMplot using gene chip based data. **D.** Comparison of TRPC6 expression in normal and BRCA tumours of different stages by the stage comparison tool in TNMplot using gene array data. **E-G.** Differential expression of TRPC6 among breast cancer patients with different molecular subtypes, individual cancer stages, and nodal metastasis status from the UALCAN analysis. ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

breast tumour tissues according to IHC staining from the HPA database, and the results are shown in Figures 2D and 2E. We noticed that the TRPC6 protein was generally lowly expressed in both normal breast tissues and breast carcinoma tissues. TRPC6 protein expression was detected in both normal and cancerous breast tissues, but not enhanced in either tissue type. In normal breast tissues, TRPC6 is present in glandular cells and myoepithelial cells, not in adipocytes (Figure 2D). In breast cancer tissues, TRPC6 is not detected in 7 out of 11 tissues (63.6%), low-stained in 2 out of 11 tissues (18.2%), and medium-stained in 2 out of 11 tissues (18.2%), respectively (Figure 2E).

3.3. Correlation of the *TRPC6* gene with immune infiltration in BRCA patients

The correlation of the *TRPC6* gene with immune infiltration in the breast tumour microenvironment was then analysed using TIMER. As shown in Figure 3, there is a positive relationship between TRPC6 expression level and infiltration level of $CD8^+$ T cells, $CD4^+$ T cells, macrophage cells, neutrophil cells, and dendritic cells ($r > 0.1$, $p < 0.01$) (Figure 3A). These results indicate that the *TRPC6* gene is involved in the immune infiltration of breast cancer. Boxplots comparing immune cell levels across copy number states of TRPC6 in Figure 3B showed that TRPC6 deletion correlates with lower immune cell levels, which suggests that the decreased copy number of TRPC6 in tumour tissues suppresses immune infiltration. The prognostic value of immune cell infiltration in BRCA was also analysed. Only B cell abundance was significantly related to better cumulative survival in BRCA patients, especially in the HER2 subtype (Figure 3C). These data suggest that TRPC6 might play a role in recruiting or supporting immune cells within the tumour microenvironment.

3.4. Effects of TRPC6 expression regulation on cell proliferation and cell migration

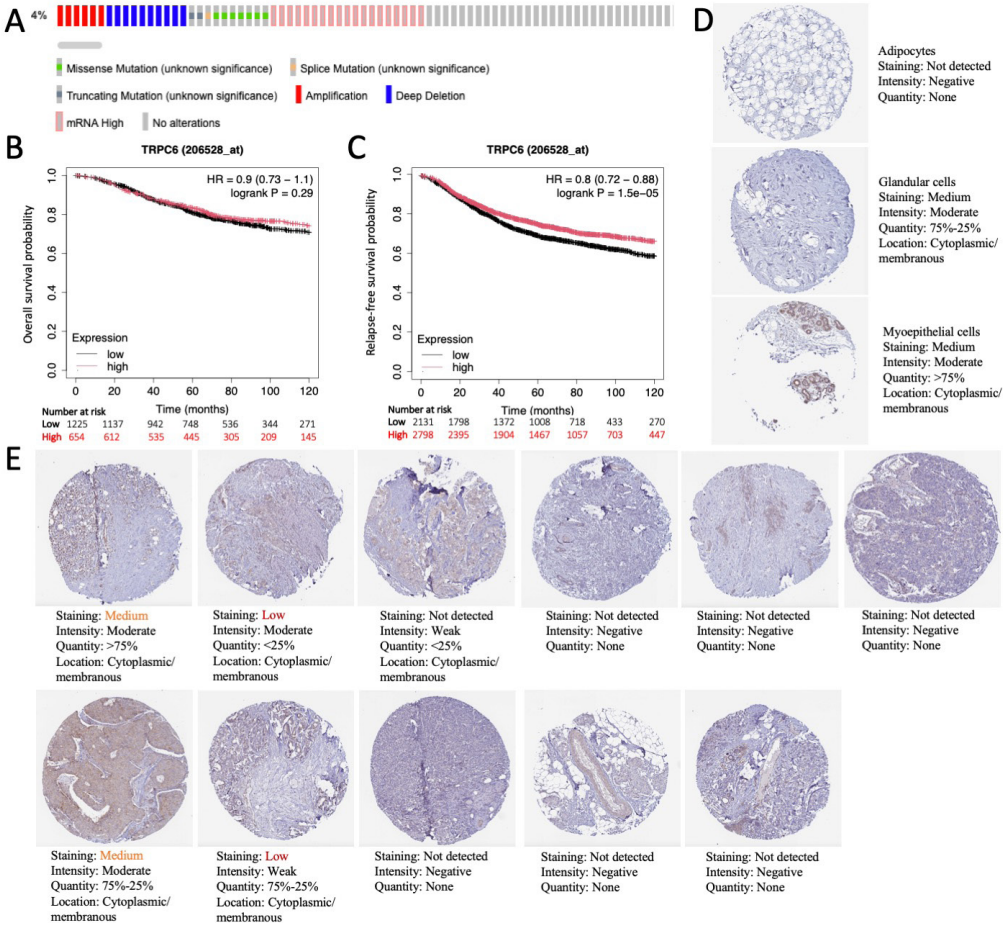

Figure 2. Genetic alteration in *TRPC6* gene, prognostic value of TRPC6 in BRCA patients, and TRPC6 protein expression analysis in breast cancer tissues. A. Genetic alterations and gene expression changes of TRPC6 in breast cancer. **B&C.** The effects of TRPC6 expression level on overall survival (B) and relapse-free survival (C) of breast cancer patients. Auto select best cut-off. Follow-up time = 120 months. **D&E.** TRPC6 protein expression in normal (D) and cancerous (E) breast tissues. TRPC6 protein is lowly expressed in both cancerous and normal breast tissues (HPA data, antibody: HPA045098), but not enhanced in either tissue type.

Since TRPC6 was lower in breast tumour tissues than normal breast tissues, we verified the molecular effects of TRPC6 expression on different breast cancer cell lines, including MCF-7, MDA-MB-231, and MDA-MB-468. Surprisingly, compared to previously published researches, we observed opposite effects of TRPC6 overexpression on breast cancer cell proliferation and migration abilities in breast cancer cells.

As shown in Figures 4A and 4B, TRPC6 overexpression correlated with delayed cell proliferation ability in all cell lines. On the other hand, TRPC6 knockdown by siRNA transfection significantly increased the cell proliferation abilities of all three cell lines above, as evidenced by MTT and colony formation assays (Figures 4A and 4B). We also tested the effects of hyperforin, a natural compound known as a TRPC6 agonist, on the proliferation of these three cell lines. We found that hyperforin could significantly inhibit the proliferation of MCF-7, MDA-MB-231, and MDA-MB-468 cell lines, with IC_{50} of 3.76 μM, 14.47 μM, and 9.96 μM, respectively. Furthermore, we observed that changes in TRPC6 expression did not induce significant cell apoptosis in the MDA-MB-231 and MDA-MB-468 cell lines, as detected by flow cytometry (Figure 4C), suggesting that TRPC6 affects only cell proliferation, but not cell viability.

We then explored the effects of TRPC6 expression regulation on cell migration and invasion ability using scratch wound healing assays and transwell invasion assays in the more metastatic triple-negative MDA-MB-231 and MDA-MB-468 cell lines. Again, we observed that TRPC6 expression was negatively correlated with cell migration ability (Figure 5A) and invasion ability (Figure 5B) of the aggressive TNBC cell lines MDA-MB-231 and MDA-MB-468 cells (Figure 5), which is contrary to previous reports. The observations of TRPC6 level being negatively related to cell proliferation and metastasis abilities raise the necessity of rethinking the mechanism perspective and targetability of TRPC6 in breast cancer.

3.5. Signalling pathway interaction networks analysis

The biological effects of a gene are shaped by the

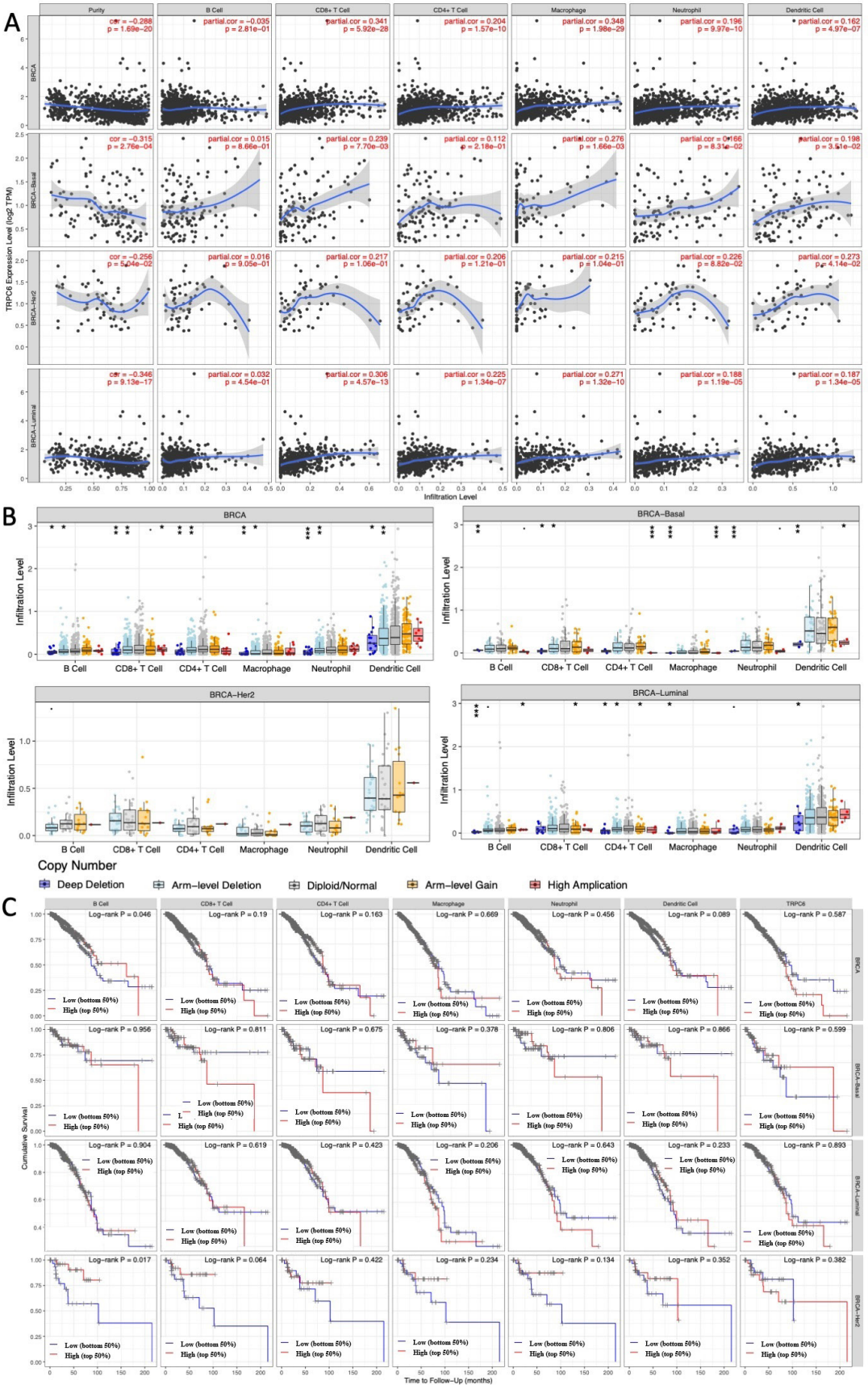

Figure 3. Correlation of the TRPC6 gene with the immune infiltration level in breast tumours. A. The expression of TRPC6 is positively associated with the infiltration abundance of $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells. **B.** The comparison of tumour infiltration levels among tumours with different somatic copy number alterations for the TRPC6 gene in BRCA patients. Box plots are presented to show the distributions of each immune subset at each copy number status in BRCA patients (Deep Deletion, Shallow Deletion, Diploid, Gain, Amplification). **C.** The clinical relevance of different tumour immune subsets.

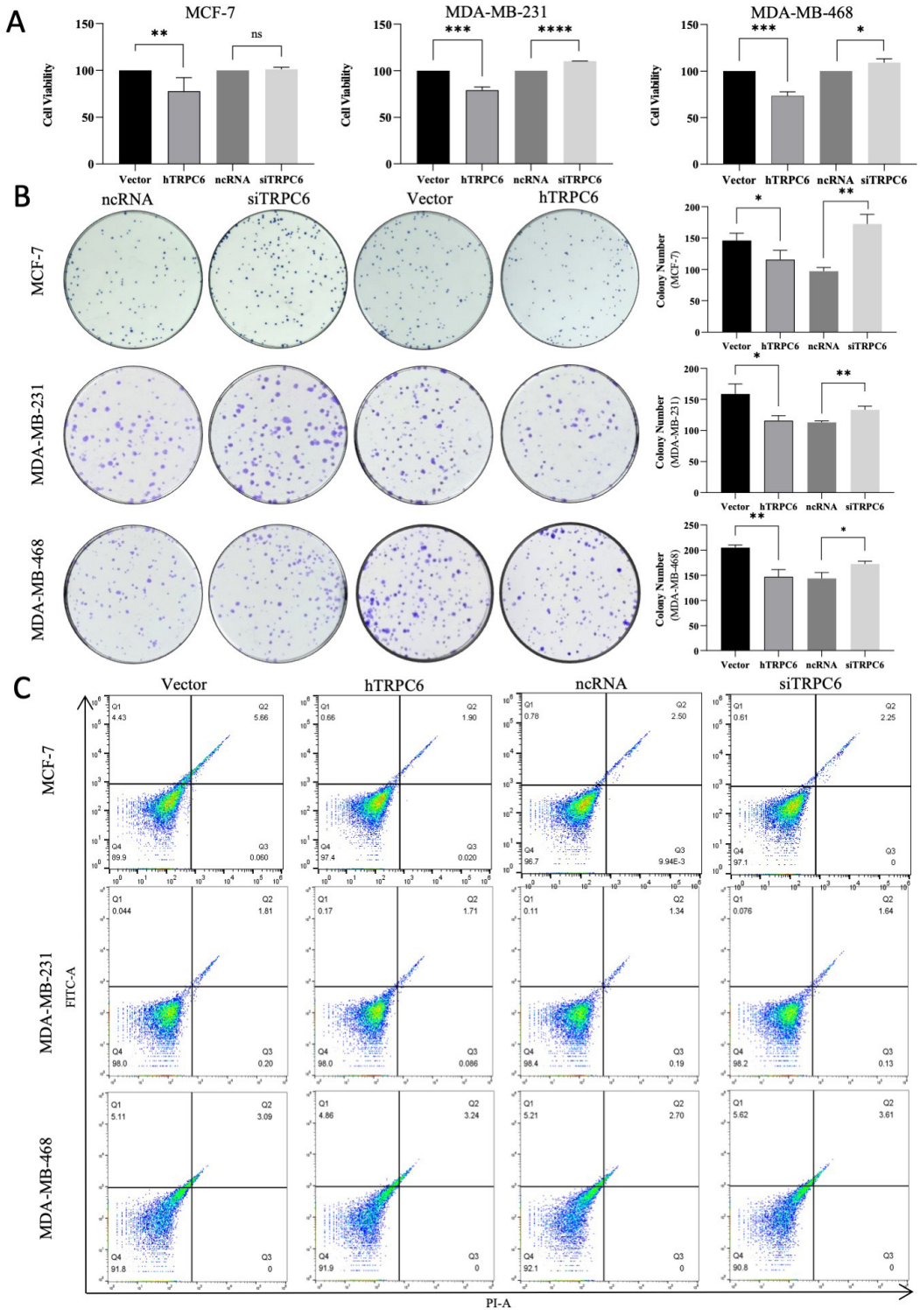

Figure 4. Effects of TRPC6 expression on the proliferation of breast cancer cell lines *in vitro*. MTT assay (**A**) and colony formation assay (**B**) assessing effects of TRPC6 expression modulation on breast cancer proliferation. **C.** Effects of TRPC6 expression on cell apoptosis detected by flow cytometry apoptosis assay. Vector, cells transfected with empty vector plasmid; hTRPC6, cells transfected with pcDNA3.1-hTRPC6-YFP plasmid for TRPC6 overexpression; ncRNA, cells transfected with scrambled RNA; siTRPC6, cells transfected with TRPC6-targeted siRNA for TRPC6 knock-down.

proteins or genes it interacts with. Understanding the interaction network for the *TRPC6* gene is crucial in biology and cancer research. Figure 6A shows the PPI (protein-protein interaction) network mapped by STRING, with the top 20 proteins highly functionally associated with or interacting with TRPC6. The Function module of LinkedOmics was further used to analyze mRNA sequencing data from 1,093 patients with BRCA in TCGA. As shown in the volcano chart (Figure 6B) and heat map (Figures 6C and 6D), 50 gene sets were

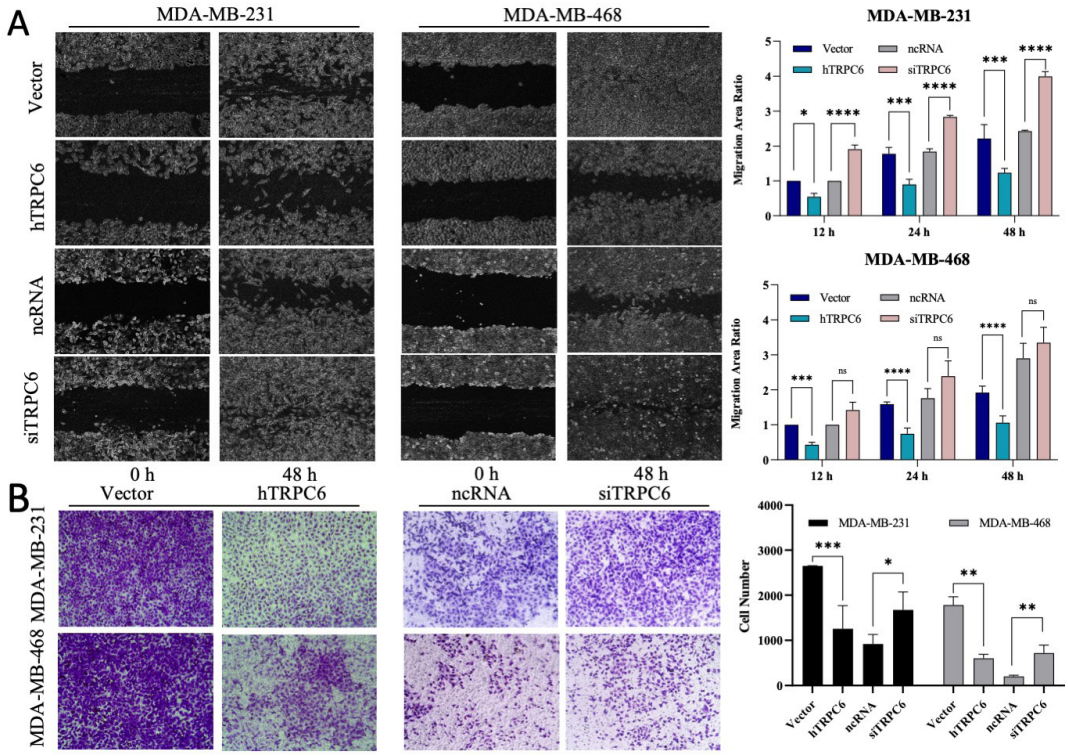

Figure 5. Effects of TRPC6 expression on migration and invasion abilities of breast cancer cell lines *in vitro*. A. Representative picture of scratch wound healing assay taken at 0 h and 48 h. The bar chart showed the cell migration ratio of different groupss at 12 h, 24 h, and 48 h. Migration area ratio = (scratch area at 0 h of each group – scratch area at 12/24/48 h of each group)/(scratch area at 0 h of control group – scratch area at 12 h of control group). **B.** Representative images of transwell invasion assay testing the effects of TRPC6 expression on cell invasion ability. The bar chart showed the number of cells invaded to the bottom of the inserts in each group. Vector, cells transfected with empty vector plasmid; hTRPC6, cells transfected with pcDNA3.1-hTRPC6-YFP plasmid for TRPC6 overexpression; ncRNA, cells transfected with scrambled RNA; siTRPC6, cells transfected with TRPC6-targeted siRNA for TRPC6 knock-down. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

substantially positively or negatively correlated with TRPC6. The results suggest that the TRPC6 expression level has a substantial effect on the transcriptome in breast cancer cells. TRPC6 expression showed a strong positive association with expression of CDH6 (Pearson correlation coefficient = 0.6782, p = 4.104e−148), GPR116 (Pearson correlation coefficient = 0.6772, p = 1.456e−147), and ELTD1 (Pearson correlation coefficient = 0.6453, p = 9.155e−130) (Figures 6E–6G). GO terms identified in gene set enrichment analysis (GSEA) revealed that differentially expressed genes correlated with TRPC6 were mainly involved in casculogenesis (Figure 6H). KEGG pathway analysis showed enrichment of ECM-receptor interaction, which regulates cancer-related signalling pathways (Figure 6I).

4. Discussion

Numerous studies have reported the involvement of TRP channels in cancer (*16*). However, limited studies have thoroughly analysed the expression pattern of TRPC6 or its possible role in the development of breast cancer. No previous studies have systematically investigated the TRPC6 expression in breast cancer according to the published sequencing datasets with a large sample size. We comprehensively explore the potential role of TRPC6 in breast cancer from multiple aspects in this study, based on bioinformatic analysis and preliminary experimental data from our laboratory.

Previous researches have reported the overexpression of TRPC6 in breast cancer tissues compared to normal or adjacent tissues (*8,13,17*). However, our exploration presented herein shows a different observation of TRPC6 expression pattern in breast tissues. The ULCAN and TNMplot analyses based on multiple clinicopathological features in breast cancer samples in TCGA, GEO, GTex, TCGA, and TARGET databases consistently indicated lower expression of the TRPC6 gene in breast cancer tumour tissues than in normal controls. Sub-group analyses using TNMplot showed that TRPC6 expression level in stage I tumours was significantly higher than in stages II-IV (Figure 1D). The subtype analysis in ULCAN showed that TRPC6 expression in luminal subtype tumours was notably higher than the more aggressive HER2 and triple negative subtypes, which is also contrary to a previous study showing that TRPC6 is expressed substantially more in TNBC than in non-TNBC (ER^+) tumour specimens (*18*). Moreover, high *TRPC6* gene expression is correlated with better RFS (Figure 2C). We thus speculate that the lower TRPC6

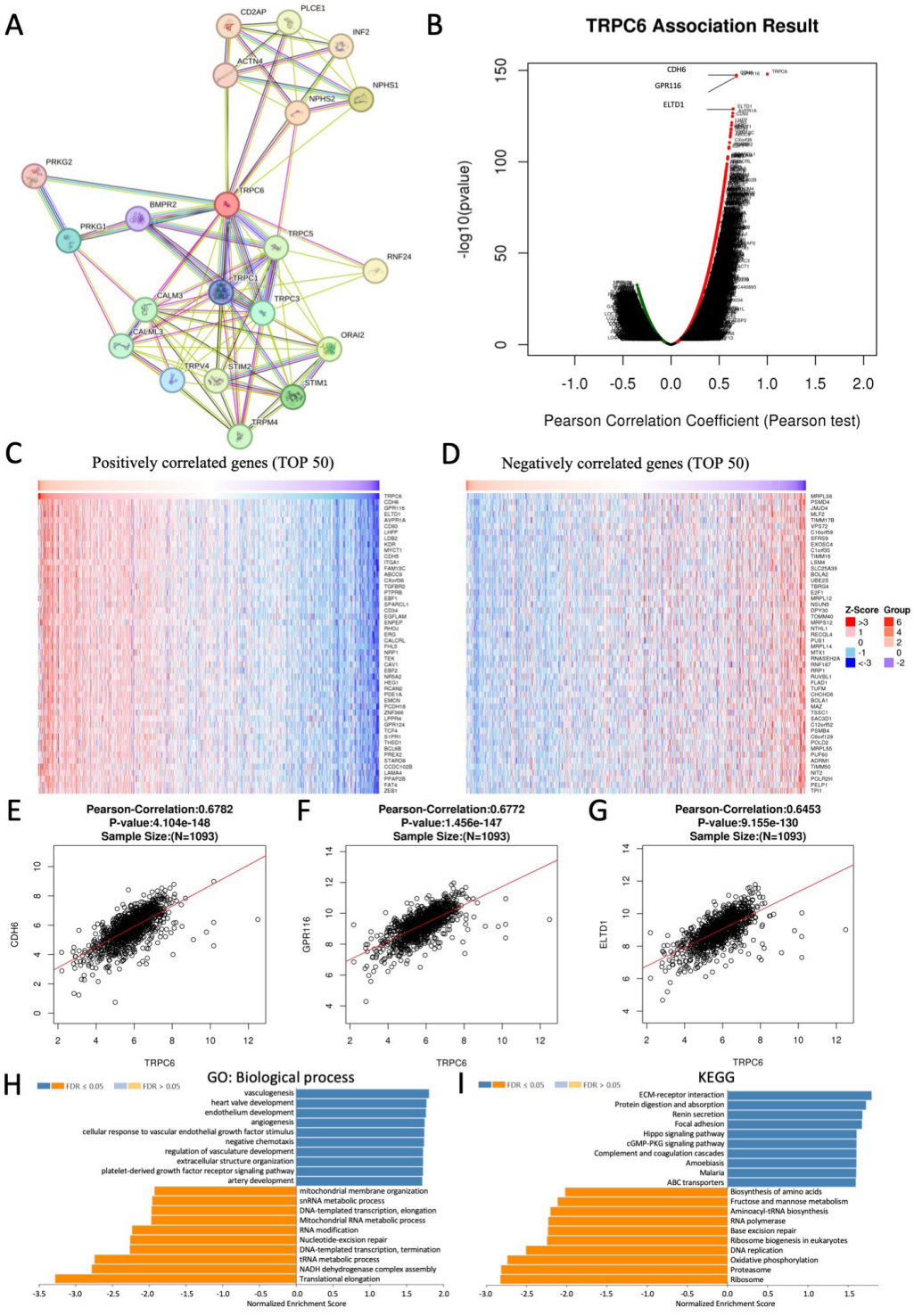

Figure 6. Interaction networks, differentially expressed genes correlated with TRPC6 in breast cancer, and GO, KEGG enrichment analysis. A. PPI network of TRPC6. **B.** Pearson correlation coefficients of relationships between TRPC6 and differentially expressed genes in BRCA. **C&D.** Heat maps showing genes positively and negatively correlated with TRPC6 in breast cancer (TOP 50). Red indicates positively correlated genes and green indicates negatively correlated genes. **E–G.** Scatter plot showing Pearson correlation coefficients for the relationship between TRPC6 expression and CDH6 (E), GPR116 (F), and ELTD1 (G). **H&I.** Significantly enriched GO annotations (H) and KEGG pathways (I) of TRPC6 in the BRCA cohort.

expression in BRCA tissues may be correlated with higher metastatic potential and more aggressive subtypes, and TRPC6 might be a novel prognostic marker of invasive and metastatic breast cancer.

Even so, gene expression may not always be in accordance with the protein level. The Human Protein Atlas database was further applied for TRPC6 protein expression. We noticed that TRPC6 protein expression was detected in both normal and cancerous breast tissues, but not enhanced in either tissue type (Figures

2D and 2E). There were only 11 breast tumours and 3 normal breast tissues in the HPA database for TRPC6 protein analysis. The TRPC6 protein analysis with a larger sample size is warranted with reliable antibodies, not only for quantification but also for its localisation.

The tumour microenvironment consists of all non-cancerous host cells and non-cellular components of the tumour. One of the most challenging questions is how the immune system affects cancer development and progression. Analysing immune infiltration within human tumours has become one of the most pivotal aspects of cancer research and precision oncology. Immune infiltrate characterisation using TIMER helped identify informative and reliable characteristics representative of the local immune tumour microenvironment related to TRPC6 expression, which could serve as independent predictors of cancer survival and guide the management of breast cancer patients. In this study, we noticed a positive relationship between TRPC6 expression level and infiltration level of $CD8^+$ T cells, $CD4^+$ T cells, macrophage cells, neutrophil cells, and dendritic cells, and TRPC6 deletion correlates with lower immune cell levels (Figures 3A and 3B). These data suggest that TRPC6 might play a role in recruiting or supporting immune cells within the tumour microenvironment. Loss of TRPC6 may promote tumour progression in BRCA by suppressing immune infiltration. Therefore, TRPC6 may play an important role in immune cell infiltration and may serve as a biomarker for guiding immunotherapy and prognosis in patients with breast cancer.

Multiple studies have revealed that TRPC6 downregulation or inhibition by small molecules may contribute to cancer inhibition (*1,13,19,20*). In the field of breast cancer research, silencing TRPC6 significantly reduced the growth but not the viability of the MDA-MB-231 cells (*13*). A recent research study showed that TRPC6 knockdown impaired cell proliferation, migration and invasion in both MCF7 and MDA-MB-231 cell lines (*14*). Nanoparticles carrying TRPC6-targeting siRNA also showed inhibition effects on the growth and survival of breast cancer MCF-7 cells *in vitro* and 4T1 cells in the mouse model *in vivo* (*21*). TRPC6 could contribute to TNBC chemotherapy resistance, dependent on TRPC6-mediated Myc suppression (*18*). While most studies suggest that TRPC6 promotes cancer progression by enhancing proliferation, migration, invasion, chemoresistance, and shaping an immunosuppressive microenvironment, in this study, we surprisingly observed that TRPC6 expression was negatively correlated with proliferation and metastasis abilities, which were contradictory observations of the expression and role of TRPC6 in breast cancer compared to previously published researches. Our observations highlight the necessity of rethinking and evaluating the functional mechanism and targetability of TRPC6 in breast cancer. The contradictory observations may be due to different experimental environments and setups. The disadvantage of our preliminary data is that we did not test whether the TRPC6 is functional or not.

On the other hand, the role of TRPC6 in cancer can be context- and activation-level dependent. TRPC6 may also have tumour-suppressive roles under certain conditions. This is not an isolated case where TRP channels serve as a tumour-suppressive factor. A recent study demonstrates that TRPA1 (transient receptor potential ankyrin 1) suppressed colorectal carcinogenesis through its immunomodulatory functions within the colitis-cancer transformation axis (*22*). TRPC6 may still serve as a therapeutic target for inducing cancer cell death under certain conditions. TRPC6-targeted compounds may have a future role as therapeutic agents for a subset of breast cancer patients. Hyperforin, a compound from *Hypericum perforatum* (St John's wort), is a well-known TRPC6 agonist/activator (*23*). Hyperforin has been shown to inhibit proliferation, induce apoptosis, and reduce invasiveness across various cancer cell lines (*e.g.*, glioblastoma, breast, colorectal, and melanoma). We confirmed that hyperforin could significantly inhibit the proliferation of MCF-7 (IC_{50} = 3.76 μM), MDA-MB-231 (IC_{50} = 14.47 μM), and MDA-MB-468 (IC_{50} = 9.96 μM) cells.

One of the key mechanisms of hyperforin-induced cancer inhibition can be attributed to calcium overload, triggering endoplasmic reticulum stress, disrupting mitochondrial membrane potential, and initiating intrinsic and extrinsic apoptotic pathways (*24,25*). The exact role of TRPC6 in breast cancer progression warrants further investigation. Therefore, the conflicting results regarding the role of TRPC6 in modulating breast cancer cell growth can be partially explained by differences in the status of TRPC6 activation. This does not contradict the role of TRPC6 as an oncogenic factor in many settings where its overexpression and moderate activity promote cancer cell growth, but it highlights its biphasic role in modulating cancer cell growth.

Under physiological or moderately elevated activity, TRPC6 promotes cancer cell growth and survival; while at hyperactivation conditions, TRPC6 can lead to intracellular calcium overload and trigger apoptotic pathways to induce cancer cell death. On the other hand, certain breast cancer cells may have dysregulated or deficient calcium influx pathways, making them less reliant on TRP channels or altering their ability to utilise calcium for signalling. For example, we previously found that the classical oxytocin receptor-mediated G_q pathway is compromised in the MDA-MB-231 cells (*26*). Understanding the interaction network of TRPC6 in breast cancer can reveal potential therapeutic targets and biomarkers for disease progression and treatment response. However, the exact role or TRPC6 in modulating cancer cell growth and strategies of targeting TRPC6 using small molecules for breast cancer management warrant further study both *in vitro* and *in vivo*.

5. Conclusion

In conclusion, this study demonstrates the potential role and function of TRPC6 in breast cancer from multidimensional aspects. TRPC6 is generally lowly expressed in both normal and breast carcinoma tissue, the elevated expression of TRPC6 may be a biomarker of a good prognosis for breast cancer patients. The limitation of the current study is that we did not test whether the TRPC6 is functional or not in the breast cancer cell lines, and the currently known TRPC6-targeted compounds were not tested in these cell lines for cancer inhibition or signalling pathway exploration. Further study of TRPC6 in breast cancer is warranted to provide new mechanistic insights and opportunities for more treatment options for breast cancer management.

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