UBE2C affects breast cancer proliferation through the AKT/mTOR signaling pathway

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Background: Ubiquitin-conjugating enzyme E2C (UBE2C) has been shown to be associated with the occurrence of various cancers and involved in many tumorigenic processes. This study aimed to investigate the specific molecular mechanism through which UBE2C affects breast cancer (BC) proliferation.

Methods: BC-related datasets were screened according to filter criteria in the Gene Expression Omnibus (GEO) database and The Cancer Genome Atlas (TCGA) database. Then differentially expressed genes (DEGs) were identified using Venn diagram analysis. By using DEGs, we conducted the following analyses including Gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), protein–protein interaction (PPI), and survival analysis, and then validated the function of the hub gene *UBE2C* using quantitative reverse transcription-polymerase chain reaction (RT-qPCR), cell counting kit-8 (CCK-8) assay, transwell assay, and Western blot assay.

Results: In total, 151 DEGs were identified from the GEO and TCGA databases. The results of GO analysis demonstrated that the DEGs were significantly enriched with mitotic nuclear division, lipid droplet, and organic acid-binding. KEGG analysis showed that the peroxisome proliferators-activated receptor (PPAR) signaling pathway, regulation of lipolysis in adipocytes, and proximal tubule bicarbonate reclamation were significantly enriched in the signal transduction pathway category. The top three hub genes that resulted from the PPI network were *FOXM1*, *UBE2C*, and *CDKN3*. The results of survival analysis showed a close relationship between UBE2C and BC. The results of CCK-8 and transwell assays suggested that the proliferation and invasion of *UBE2C* knockdown cells were significantly inhibited ($P < 0.050$). The results of Western blot assay showed that the level of phosphorylated phosphatase and tensin homology deleted on chromosome 10 (p-PTEN) was obviously increased ($P < 0.050$), whereas the levels of phosphorylated protein kinase B (p-AKT), phosphorylated mammalian target of rapamycin (p-mTOR), and hypoxia-inducible factor-1 alpha (HIF-1α) were dramatically decreased ($P < 0.050$) in the *UBE2C* knockdown cell.

Conclusion: UBE2C can promote BC proliferation by activating the AKT/mTOR signaling pathway.

Keywords: AKT/mTOR; Breast cancer; Invasion; Proliferation; Ubiquitin-conjugating enzyme E2C

Introduction

Breast cancer (BC) is one of the primary malignant tumors in women worldwide.[1] The incidence of BC varies greatly from country to country due to different geographical environmental conditions and living habits. The etiology of BC is multifaceted and includes genetic factors, environmental pollution, and dietary habits, among which BC occurrence is closely associated with gene mutation.[2,3] Many functional studies have shown that genomic modifications are related to a malignant phenotype.[4,5] In addition, the occurrence and growth of tumors depend on the surrounding microenvironment.[6-8] Many studies have reported numerous tumor-suppressor genes and oncogenes for the past few years. However, further researches are still needed to search for new therapeutic targets for BC.

In the last few years, more and more studies have used high-throughput sequencing technology and gene chip technology, so we have a large accumulation of genetic data. High-throughput sequencing technology has been used to detect genetic alterations in carcinogenesis and the discovery of biomarkers for many diseases.[9] However, by collecting multifold microarray datasets, adequate samples can be received and more credible results can be obtained. The Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (NCBI) is the largest public resource of gene expression databases, containing hundreds of millions of resources. These databases are useful for gaining a better understanding of several

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diseases, such as BC. Therefore, we carried out a systematic analysis based on gene expression profiles from the GEO and The Cancer Genome Atlas (TCGA) databases. If we screen and predict the genetic characteristics and significance connected with the origin, progression, and prognosis of BC, then we can generate new ideas for BC research. In the current research, the differentially expressed genes (DEGs) were first identified by integrating GEO and TCGA databases. Then, we detected that ubiquitin-conjugating enzyme E2C (UBE2C) is related to the diagnosis, progression, and proliferation of BC. Finally, we conducted Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of DEGs and generated protein–protein interaction (PPI) networks to elucidate the underlying mechanisms of UBE2C involving in tumor progression.

UBE2C (also known as UBCH10) is a member of the ubiquitin-conjugating enzyme complex family. It is encoded on chromosome 20q13 and has a molecular weight of 19.65 kDa.[10] As a ubiquitin ligase of anaphase-promoting complex/cyclosome (APC/C), UBE2C participates in the ubiquitination modification of cyclin and mitosis-related factors, thus playing a role in promoting the process of cell mitosis.[11] Over the past few years, there is mounting evidence that upregulation of *UBE2C* gene expression is closely associated with tumorigenesis and tumor progression in multiple human malignancies, including gastric cancers, lung cancers, hepatocellular carcinomas, brain cancers, ovarian cancers, and colorectal cancers.[12-17] Thus, this study aims to elucidate the potential mechanism of UBE2C affecting BC proliferation and provides a target for BC treatment.

Methods

Data collection

GEO and TCGA databases were used to download the original data for this study. In the GEO database, the inclusion criteria were that a dataset of BC tissues and normal adjacent control tissues were available. And the exclusion criteria were as follows: dataset of too few samples and no normal control were included. In the TCGA database, all data were normalized and processed with the TCGA biolinks pipeline. The parameters set for differential expression analysis were FDR <0.05 with $|Log_2FC|>2$.

Screening DEGs

The BC-related datasets (GSE21422, GSE45827, and GSE70947) were downloaded from the GEO database. The GEO2R analysis platform was used to extract DEGs information from the expression profile data, including gene ID, gene symbol, gene title, *P*-value, adjusted *P*-value (adj. *P*-value), and differential multiple (Log_2FC), whereas ambiguous gene expression results were deleted. To select the different gene files from the three datasets and further screen "$|Log_2FC|>2$ and $P < 0.050$" genes, the datasets were further screened by Venn diagram analysis. Then, the genes from the GEO and the TCGA databases were intergrated.

Functional annotation and pathway enrichment analysis of DEGs

We used the R3.4.1 with the clusterProfiler package to obtain GO terms and KEGG pathways to explore the biological functions of the DEGs correlated with BC development. The DEGs were analyzed with respect to the biological process (BP), cell component (CC), and molecular function (MF) categories.

Construction of a PPI network

To further assess the interactions and functions of the DEGs, the online tool STRING was used to evaluate their functional associations and to construct a PPI network. Then, the hub genes were further screened using Cytoscape. According to the size of the degree value, the top three genes were selected as hub genes.

Survival analysis of DEGs

The Kaplan-Meier (K-M) Plotter Online Tool is free to access and enables to comprehensively analyze the impact of hub genes on survival time in patients with BC. Using this tool, we evaluated the total survival of hub genes in patients with BC based on relapse-free survival (RFS), which is determined by the hazard ratio (HR) and log-rank *P*-value.

Cell culture

This study selected human BC cell lines MCF-7 and MDA-MB-231 (Procell Life Science & Technology, China) as *in vitro* models. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with 10% fetal bovine serum (FBS; Bioind, Israel) at 37 °C in a humidified cell culture incubator under an atmosphere with 5% CO_2 and were passaged when reaching 85% to 95% confluency.

Cell transfection

The two cell lines were transferred in 6-well plates before transfection. When the tumor cells reached the required confluency, we treated them with small interfering RNA (siRNA) (Ribobio, Guangzhou, China) mixed with Lipofectamine 2000 transfection reagent (Invitrogen, CA, USA). The cells transfected with validated siRNAs were used as the experimental group; whereas the cells transfected with nontarget control siRNAs were used as the control group. After incubation for 4 h with the siRNAs, the transfected cells were placed in a complete medium to continue culturing, and after culturing for 48 h, the transfected cells were used for further experiments described in the following sections.

Quantitative reverse transcription PCR (RT-qPCR) assay

The total RNA of MCF-7 and MDA-MB-231 was isolated by using TRIzol reagent (TaKaRa, Japan). The extracted RNA was used for reverse transcription and the following PCR experiment. RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA), which was then used for RT-qPCR. The PCR system included the following: 10 μL of SYBR Premix Ex Taq™ II (TaKaRa, Japan), 0.2 μL of forward primers, 0.2 μL of

reverse primers, 2 μL of cDNA, with double distilled water (ddH_2O) added to a final volume of 20 μL. In this study, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the internal control. The sequences of the primers used to assess *UBE2C* expression were as follows: *UBE2C* forward primer (5′-CTGCCTTCCCTGAATCAGACAACC-3′), UBE2C reverse primer (5′-TCGGCAGCATGTGTGTTCAAGG-3′), *GAPDH* forward primer (5′-TGACTTCAACAGCGACACCCA-3′), and *GAPDH* reverse primer (5′-CACCCTGTTGCTGTAGCCAAA-3′). The first step is to conduct pre-denaturation at 95°C for 30 s, and the second step is to conduct PCR reaction at 95°C for 3 s and 60°C for 30 s for 40 cycles, and then the separation stage.

CCK-8 assay

The transfected and untransfected cells were inoculated into 96-well plates at a density of 8×10^3 cells/well. The cells were cultured in a cell culture incubator and cell viability was assessed at 12 h, 24 h, 48 h, and 72 h time points using a CCK-8 kit (Dojindo, Japan). At each time point, 10 μL of CCK-8 solution was added to each well. Then the cells were cultured in an incubator for 1 h, and a microplate reader was used to measure the optical density (OD) value at 450 nm. The cell proliferation rate was calculated as follows: cell proliferation rate = (control well OD − experience well OD)/(control well OD − blank OD) × 100%.

Transwell assay

For the invasion assay, 24-well transwell chambers were used and the upper chamber was precoated with Matrigel (BD Bioscience, CA, USA). Cell invasion ability was measured by adding each group cell suspension to the chamber precoated with Matrigel. The serum concentration in the lower chamber is higher than that in the upper chamber. Subsequently, the cells were incubated in the cell culture incubator for 48 h. Then, the invaded cells on the underside of the membrane were fixed with 4% paraformaldehyde and stained with a crystal violet staining solution (0.1%) before being observed under a microscope.

Western blot analysis

The cells were collected after transfection, and total protein was extracted from two kinds of cells using radioimmunoprecipitation assay (RIPA) buffer (Beyotime, China). The protein concentration was estimated using the bicinchoninic acid (BCA) protein assay (Boster, China). Equal amounts of prepared protein were loaded onto sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels for electrophoresis and then transferred onto polyvinylidene fluoride membranes. Then, we used 5% nonfat milk (Solarbio, China) to block the membranes at room temperature for 1 h. Subsequently, the membranes were incubated with anti-UBE2C (1:2000; ab125002), anti-GAPDH (1:600; CST5174), anti-p-PTEN (1:2000; CST9551), anti-p-AKT (1:1000; CST4060), anti-AKT (1:1000; CST4691), anti-p-mTOR (1:1000; CST5536), anti-mTOR (1:1000; CST2972), or anti-HIF-1α (1:1000; CST36169) at 4°C overnight. Subsequently, the membranes were briefly rinsed with tris buffered saline with tween (TBST) three times (10 min each), incubated with an anti-rabbit secondary antibody for 1 h (1:2000, CST7074), and then washed with TBST three times (10 min each). Finally, the protein bands were visualized using an electrochemiluminescence (ECL) kit (Beyotime, China).

Statistical analysis

SPSS 18.0 (IBM Corp., Armonk, NY, USA) was used for statistical analyses. We used one-way analysis of variance (ANOVA) to analyze the difference between groups, and differences were considered significant at $P < 0.050$.

Results

DEG analysis

A total of 292 tumor samples and 164 normal adjacent tissue samples were collected from the GEO database. Finally, the GSE21422, GSE45827, and GSE70947 datasets were screened, and information of these three datasets are presented in Table 1. A total of 161 genes were selected from the three datasets, including 65 upregulated and 96 downregulated genes. The number of common and unique mRNAs across the different datasets is shown in the Venn diagram [Figure 1A]. A total of 1834 genes were obtained from TCGA database, including 710 upregulated and 1124 downregulated genes. The final number of DEGs obtained was 151, including 55 upregulated and 96 downregulated genes [Figure 1B].

Analysis of gene function enrichment

To further research the functions and mechanisms of the identified DEGs, all DEGs were assessed by GO term and

Table 1: Three mRNA expression datasets in the GEO datase.

Dataset	Author	Year	Platform	Samples (N:P)
GSE21422	Schaefer C*	2011	GPL570[HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	5:14
GSE45827	Gruosso T†	2016	GPL570[HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	11:130
GSE70947	Quigley DA‡	2016	GPL13607 Agilent-028004 SurePrint G3 Human GE 8x60K Microarray (Feature Number version)	148:148

GEO: Gene Expression Omnibus. * https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21422. † https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE45827. ‡ https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70947.

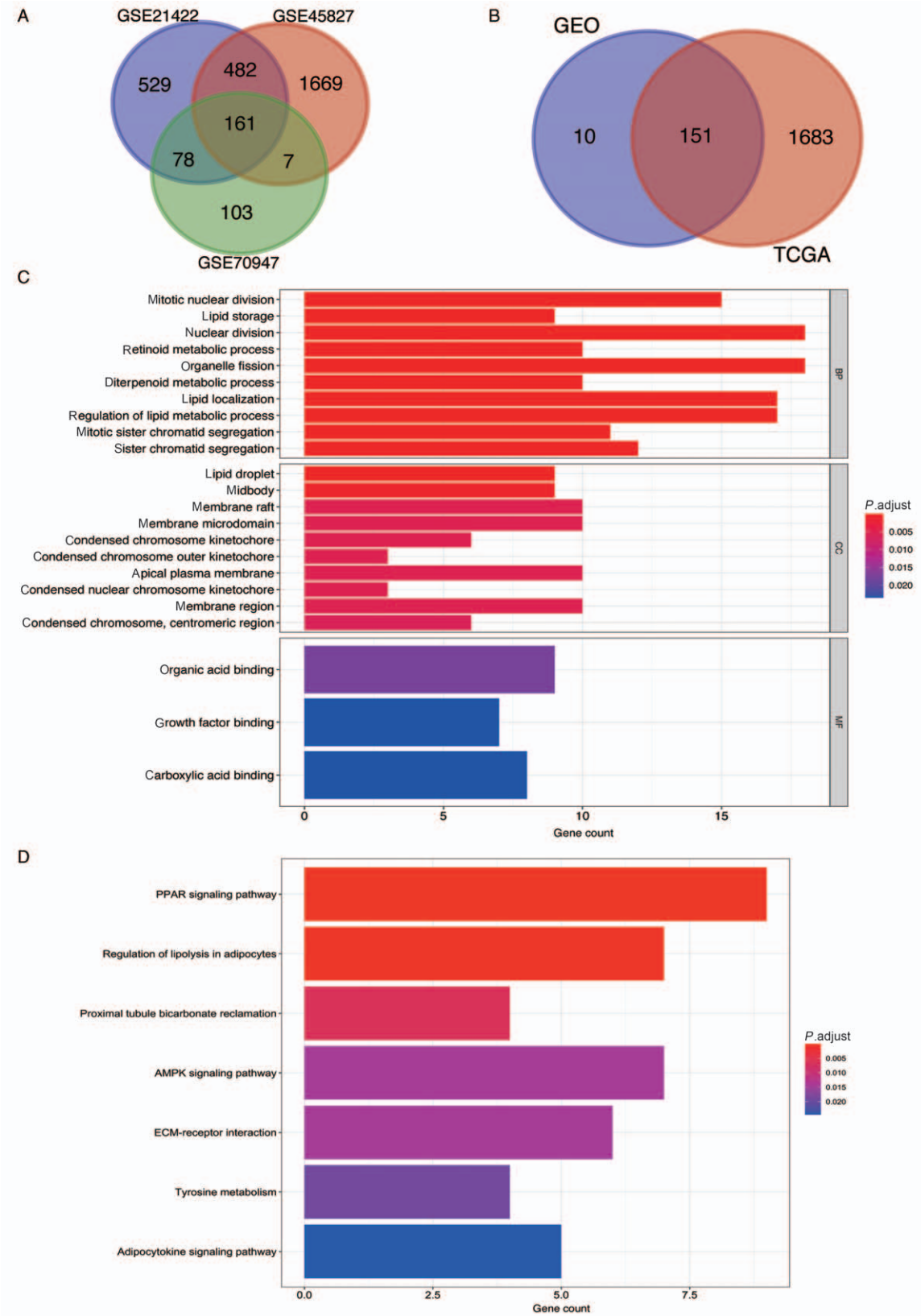

Figure 1: The DEGs identified and their function. (A) Venn diagram of DEGs in the GEO database (GSE21422, GSE45827, and GSE70947); (B) Venn diagram of 162 DEGs in the GEO database and 1824 genes in the TCGA database; (C) BP, CC, and MF in GO analysis; (D) The enriched KEGG pathways of DEGs. BP: Biological process; CC: Cell component; DEGs: Differentially expressed genes; GEO: Gene Expression Omnibus; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; MF: Molecular function; TCGA: The Cancer Genome Atlas.

KEGG analyses. The results of GO analysis demonstrated that the DEGs were significantly enriched with mitotic nuclear division, lipid storage, and nuclear division in the BP category. Moreover, in the CC analysis, the DEGs were predominantly involved in lipid droplet, midbody, and membrane raft. In addition, organic acid-binding, growth factor binding, and carboxylic acid binding were remarkably enriched in the molecule function category. Furthermore, the PPAR signaling pathway, regulation of lipolysis in adipocytes, and proximal tubule bicarbonate reclamation were significantly enriched in the signal transduction pathway category. The results of the GO and KEGG analyses are shown in Figure 1C and D.

PPI network and hub genes

Subsequently, the STRING database was used to identify the interrelationships between the 151 DEGs (55 upregulated and 96 downregulated DEGs according to Log_2FC) by constructing PPI networks. The network of PPI between DEGs is shown in Figure 2A. Moreover, three hub genes were chosen based on the top three degree values. The final hub genes were *FOXM1*, *UBE2C*, and *CDKN3*, and all of them were upregulated genes.

Survival analysis of hub genes

K-M plot analysis was performed to illustrate the influence of the hub genes on RFS. The results demonstrated that the overall survival rate of BC patients with lower UBE2C expression levels was significantly better than that of patients with higher UBE2C [Figure 2C] expression levels; similar results can be observed in FOXM1 [Figure 2B] and CDKN3 [Figure 2D]. The HR of UBE2C was 1.83, which was closely associated with survival. To assess the relationship between UBE2C and BC, we evaluated its functions *in vitro* subsequently.

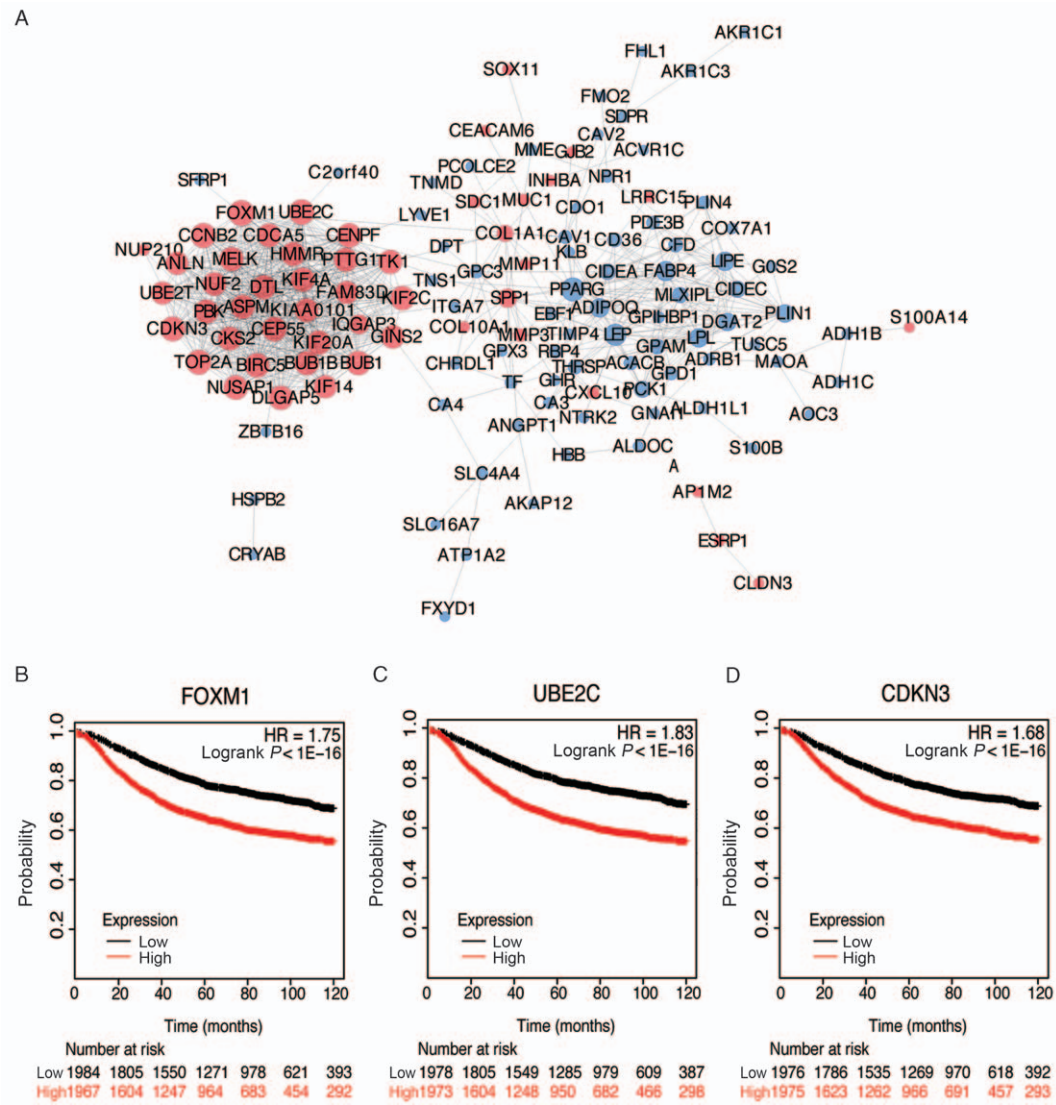

Figure 2: The PPI network and K-M curves for survival analysis of DEGs. (A) The PPI network of 151 DEGs. The size of a node is proportional to the degree of the node. Upregulated genes are indicated in red, and downregulated genes are indicated in blue. (B) K-M survival curve of FOXM1; (C) K-M survival curve of UBE2C; (D) K-M survival curve of CDKN3. DEGs: Differentially expressed genes; K-M: Kaplan-Meier; PPI: Protein-protein interaction; UBE2C: Ubiquitin-conjugating enzyme E2C.

Knockdown of UBE2C affected cell proliferation and invasion

To investigate the function of UBE2C in BC progression and development, siRNA (siUBE2C-1 and siUBE2C-2) knockdown of *UBE2C* in MCF-7 and MDA-MB-231 cells was performed. The results of RT-qPCR and Western blot assay revealed that the mRNA and protein expression levels of UBE2C were significantly reduced in both MDA-MB-231 ($P < 0.001$, $P < 0.050$) and MCF-7 ($P < 0.010$, $P < 0.050$) *UBE2C* knockdown cells [Figure 3A]. CCK-8 assays showed that the proliferation of BC cells in the knockdown groups was significantly lower than that observed in the control groups after 72 h for both cell types ($P < 0.001$, $P < 0.010$, $P < 0.050$; Figure 3B). What's more, after inhibiting the expression of UBE2C, the appreciation rate of cells decreases over time. Then, we performed transwell assays to assess the invasiveness of the different groups of MDA-MB-231 and MCF-7 cells. As shown in Figure 3C, notably more MDA-MB-231 cells passed through the transwell chamber in the control group than that observed in the siUBE2C-1 and siUBE2C-2 groups ($P < 0.001$). The same results were observed in MCF-7 cells ($P < 0.010$). These results demonstrated that downregulation of UBE2C expression apparently inhibits the invasion ability of both MCF-7 and MDA-MB-231 cells. Furthermore, no significant difference was observed between the control and the non-transfected groups, indicating that the vector had no impact on the proliferation and invasion of two cells.

Downregulation of UBE2C suppresses BC cell growth via inactivation of the AKT/mTOR signaling pathway

To assess the mRNA and protein expression levels of UBE2C in different human BC cells, we performed RT-qPCR and Western blot analyses, respectively. The results proved that the expression of UBE2C in MDA-MB-231 cells was higher than that observed in MCF-7 cells ($P < 0.050$, $P < 0.010$; Figure 4A). To elucidate the related mechanisms of UBE2C affecting BC proliferation, we evaluated the phosphorylation status of crucial proteins in the proliferation-related signaling pathways in BC cells. Among these proteins, the level of phosphorylated PTEN (p-PTEN) was notably increased ($P < 0.050$); although the levels of expression of phosphorylated AKT (p-AKT), phosphorylated mTOR (p-mTOR), and hypoxia-inducible factor-1 alpha (HIF-1α) were dramatically decreased in the *UBE2C* knockdown cells ($P < 0.050$), total AKT (pan-AKT) and total mTOR (pan-mTOR) levels remained unchanged [Figure 4B]. Above all, the results demonstrated that inhibiting the expression of UBE2C can suppress the growth of BC cells by inhibiting the AKT/mTOR signaling pathway.

Discussion

In recent years, with the continuous advancements made in the early diagnosis and treatment of BC, the survival rate and quality of life of patients with BC have significantly improved. However, the incidence of this disease is rising in most countries and is projected to further increase over the next 20 years.[3] Thus, it is of great importance to elucidate the pathogenesis of BC and to identify associated molecular biomarkers with high sensitivity and specificity. This study aimed to elucidate the functions and molecular mechanisms of UBE2C in BC. By searching the GEO and TCGA databases, hub genes of BC were identified and determined. Subsequently *in vitro* assays showed that UBE2C can affect the proliferation and invasion of BC. At the protein level, we proved that UBE2C affects the proliferation of BC through the AKT/mTOR signaling pathway.

In the present study, the genes from three GEO datasets and TCGA database were integrated, resulting in the identification of 55 upregulated and 96 downregulated DEGs. The results of GO analysis revealed that the DEGs were primarily involved in the mitotic nuclear division, lipid storage, and nuclear division. The previous study indicated that the cell cycle is controlled by various mechanisms, and the loss of normal cell cycle control is associated with the occurrence of cancer.[18] The proliferative response in normal cells is constrained, while this inhibition in tumors is perturbed, including external mitogenic signals.[19] Through PPI analysis, hub genes can be identified according to the degree values, resulting in the identification of three hub genes, including *FOXM1*, *UBE2C*, *CDKN3*. The survival analysis showed that the *UBE2C* gene is highly correlated with the risk of death in BC.

UBE2C has been previously reported to be highly expressed in various tumors and can affect the cell cycle, drug resistance, radiosensitivity, and other biological processes.[20-22] UBE2C played a crucial role in mitotic progression in various eukaryotes.[23] Zeng *et al*[24] revealed that *UBE2C* can exhibit oncogenic activities via interaction with Derlin-1. *In vitro* studies showed that knockdown of UBE2C can markedly inhibit the Derlin-1-induced phosphorylation of ERK and AKT and the subsequent cell proliferation and migration in BC cells. The results suggested that the molecules interacting with UBUE2C deserve further studying. In this study, functional studies have shown that inhibition of UBE2C expression reduced the proliferation rate and invasion ability of two BC cell lines, confirming that UBE2C plays an important role in the carcinogenesis of BC. This result is consistent with the previously reported results of this protein in other cancer types.[25-27] To further explore the mechanism of UBE2C promoting BC proliferation, we focused on the AKT/mTOR signaling pathway.

To further investigate the underlying mechanism of UBE2C function in human BC, we used MCF-7 and MDA-MB-231 cells to evaluate its function. Our results indicated that among several assayed signaling pathways, AKT signaling pathway was observed to be activated by UBE2C. Many studies have indicated that changes in the AKT/mTOR signaling pathway are associated with multiple human malignancies, including BC.[28-33] The mutation and inactivation of PTEN enable to upregulate AKT/mTOR signaling pathway. Besides, the changes in this signaling pathway are also manifested in somatic mutations of AKT and overexpression or aberrant phosphorylation of AKT, mTOR, and their downstream targets.[34,35] Our results suggest that knockdown of *UBE2C* can increase the phosphorylated level of PTEN. In

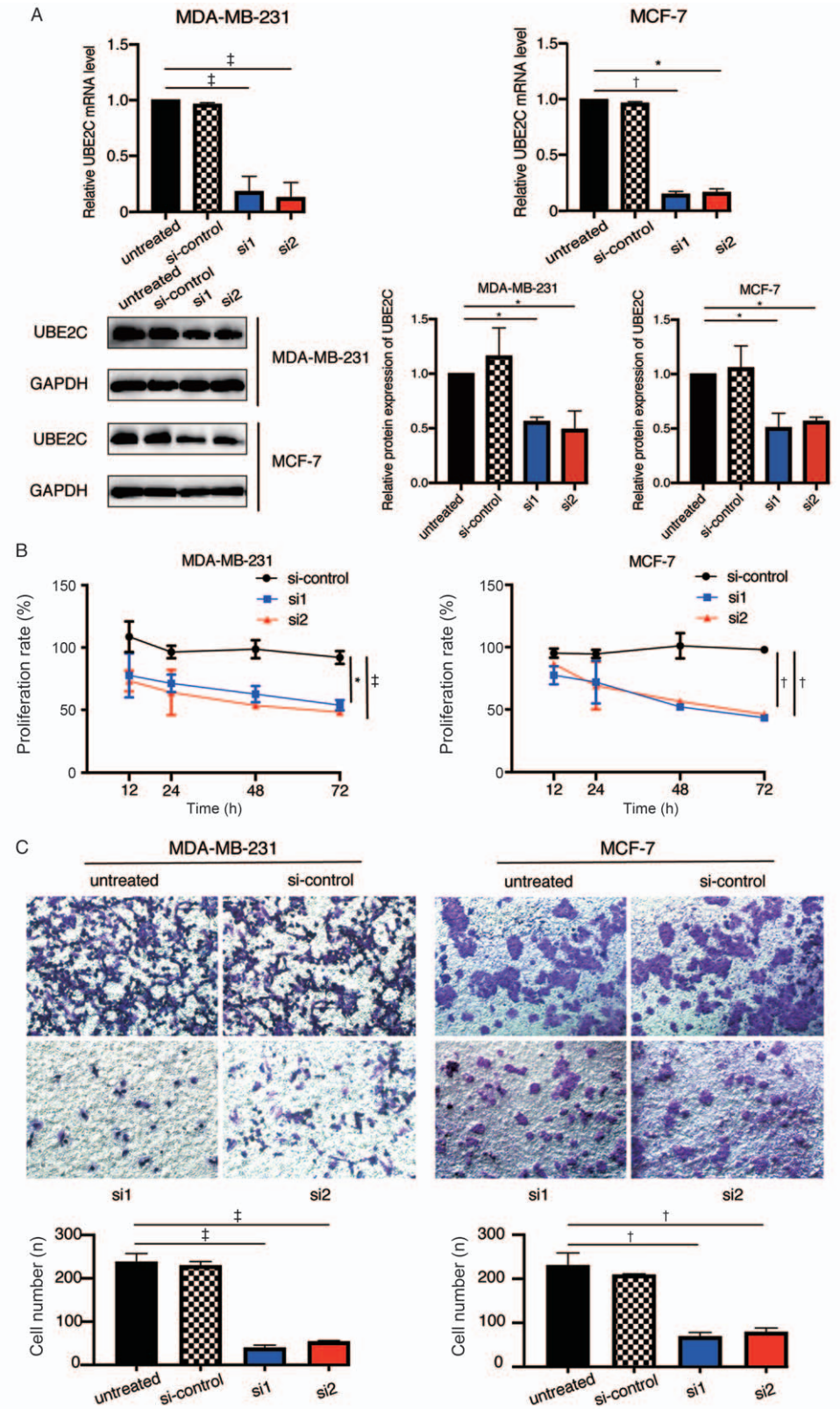

Figure 3: Suppressing UBE2C expression affects BC cell proliferation and invasion. (A) mRNA and protein expression levels of UBE2C detected after siRNA transfection in MDA-MB-231 and MCF-7 cells; (B) CCK-8 assay was performed to assess cell proliferation in MDA-MB-231 and MCF-7 cells transfected with UBE2C siRNA (siUBE2C-1 and siUBE2C-2) and control siRNA. $^{*}P < 0.050$; (C) Knockdown of *UBE2C* significantly decreased the invasion of two cell lines (original magnification × 400). $^{\dagger}P < 0.010$, $^{\ddagger}P < 0.001$. BC: Breast cancer; UBE2C: Ubiquitin-conjugating enzyme E2C.

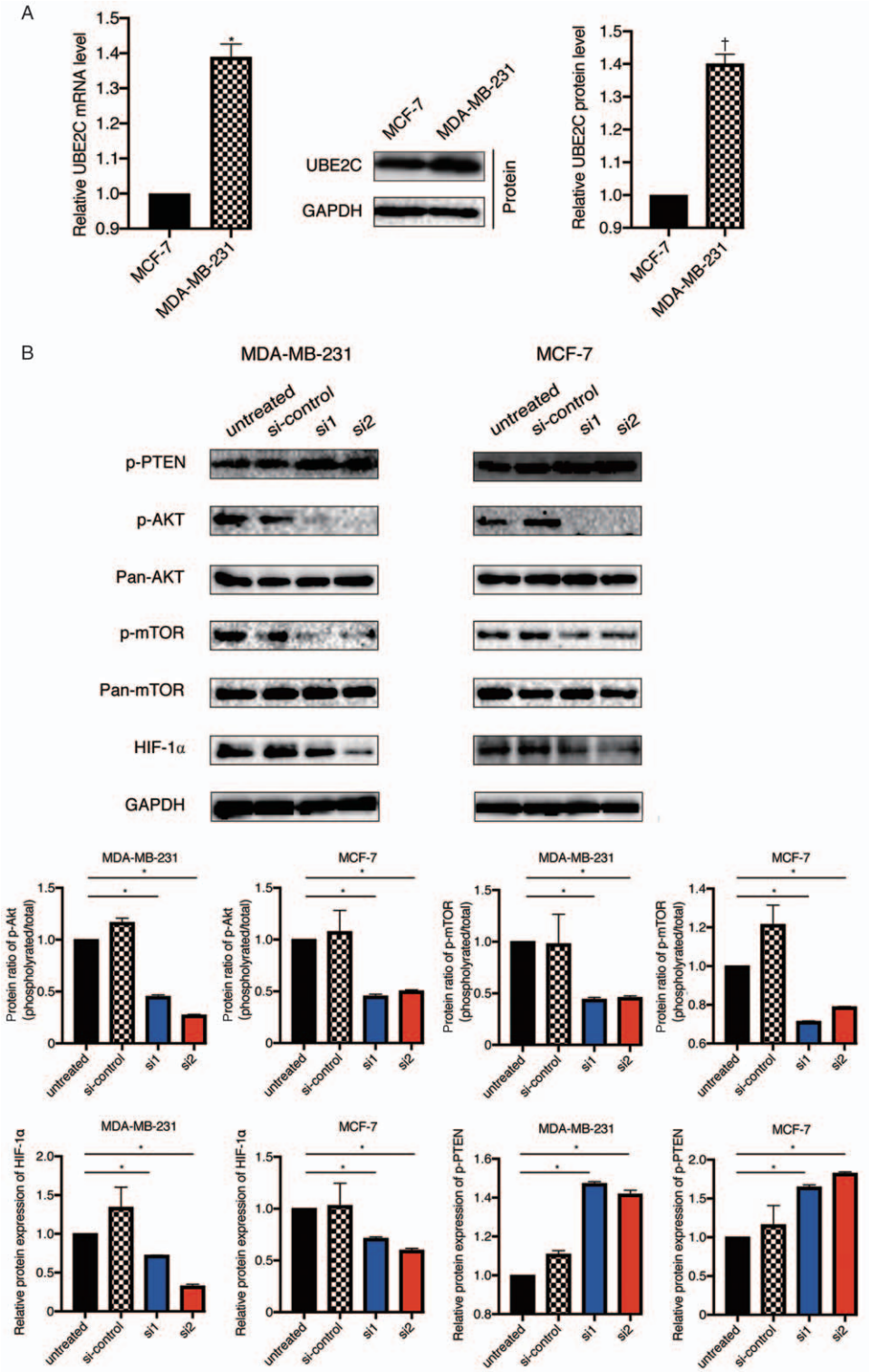

Figure 4: Knockdown of *UBE2C* affects the AKT/mTOR signaling pathway. (A) mRNA and protein expression levels of endogenous UBE2C in MDA-MB-231 and MCF-7 cells. (B) Western blot analysis of the expression of AKT/mTOR signaling pathway-related proteins. $^{*}P < 0.050$, $^{\dagger}P < 0.010$. p-AKT: Phosphorylated AKT; p-mTOR: Phosphorylated mTOR; p-PTEN: Phosphorylated PTEN; UBE2C: Ubiquitin-conjugating enzyme E2C.

the future, the biological mechanism of UBE2C activation of PTEN and inhibition of the AKT/mTOR singling pathway should be further studied. In recent years, inhibitors against AKT/mTOR signaling pathways have also been studied, with the goal of identifying better treatments for patients with BC.[36,37] HIF-1α is an important transcriptional regulator during hypoxia in the body. In tumor cells, rapid cell proliferation leads to the increased oxygen consumption of the cells, which makes the cells hypoxic. There is a large amount of clinical and experimental data to prove that tumor cells can activate HIF-1α and HIF-2α to promote cell proliferation,

invasion, and metastasis processes.[38] The results of this study show that downregulation of UBE2C can inhibit the expression of HIF-1α, which suggests that UBE2C may affect the occurrence and development of BC by regulating different signaling pathways. In recent years, HIF-1α has been proposed as a new therapeutic target for tumors.[39] Although the specific mechanism of UBE2C action still requires further research, our current results suggest that UBE2C can be used as a new target for clinical treatment to inhibit the growth of BC.

In summary, we proved that UBE2C is associated with the occurrence and development of BC and further confirmed that it can promote BC proliferation by affecting the AKT/mTOR signaling pathway. Identification of the target of UBE2C will promote the diagnosis and clinical treatment of BC.

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Conflicts of interest

None.

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