

PKIB regulates tamoxifen resistance in estrogen receptor-positive breast cancer through inhibition of autophagy

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Abstract

Tamoxifen is a first-line adjuvant endocrine drug used in the treatment of patients with estrogen receptor (ER)-positive breast cancer. The drug's therapeutic benefit is limited by the development of resistance due to alterations in autophagy function. In this study, the role of the cAMP-dependent protein kinase inhibitor- β (PKIB) in autophagy and the development of tamoxifen-resistant breast cancer were evaluated. Expression of PKIB was assessed by RTq-PCR, Western blot analysis, and immunohistochemistry (IHC) in tamoxifen-resistant and sensitive breast cancer cell lines and clinical samples. Knockdown and overexpression of PKIB were used to determine the sensitivity to tamoxifen in vitro and the status of autophagy. Further, immunofluorescence and Western blot analysis were used to explore the tamoxifen resistance mechanism of PKIB. CREB/ATG7 signaling activation was evaluated after knocking down of PKIB in MCF7 and T47D cells. The level of PKIB expression was upregulated in the tamoxifen-sensitive breast cancer cell lines (MCF7 and T47D) and in primary tumor tissues. Knock down of PKIB decreased the sensitivity to tamoxifen both in vitro and in vivo, and significantly enhanced the autophagy level in the tamoxifen-sensitive cell lines. In contrast, overexpression of PKIB inhibited autophagy and restore tamoxifen sensitivity in tamoxifen-resistant cells. Moreover, an increase in p-CREB and ATG7 protein expression levels were observed in MCF7/si2-PKIB and T47D/si2-PKIB cells by Western blot. Finally, Kaplan-Meier curve analysis also indicated that the high level of PKIB predicts a good prognosis in breast cancer. Overall, our findings demonstrated that PKIB suppresses CREB/ATG7 activation and subsequent autophagy which could contribute to tamoxifen resistance in breast cancer.

Introduction

Breast cancer is the most prevalent and malignant tumor and endangers the health and lives of women in the worldwide, resulting in high morbidity and mortality. Data released by the World Health Organization (WHO) illustrates that there are 2.3 million estimated new cases of breast cancer each year worldwide, representing 11.7% of all cancer cases (1). Breast cancers can be classified into several subtypes, including Luminal A or B, Her2 positive and triple negative (2, 3). In clinical settings, approximately 75% of breast tumors are estrogen receptor- α (ER α) positive (4). Tamoxifen, which functions as a selective estrogen receptor modulator, can inhibit proliferation of ER positive breast cancer cells by competitively binding to ER α , and is recommended as a first-line endocrine treatment (5, 6). However, it is reported that relapse was occurred in one third of patients after 5 years of treatment with tamoxifen within 15 years (7), presenting a significant clinical challenge.

Autophagy is an evolutionarily conserved cellular process used for the degradation of cytoplasmic protein and malfunctioning organelles (8). Under severe conditions, autophagy may exert the prosurvival roles in cancer cells. Antineoplastic treatment such as chemotherapy agents or endocrine drugs like tamoxifen could induce cell protective autophagy as a prosurvival mechanism (9). Meanwhile, the induction of autophagy in breast cancer cells is related to the development of therapeutic resistance (9–12). Consequently, the inhibition of autophagy could promote the therapeutic efficacy of antitumor drugs. Lysosome-associated membrane protein (LAMP) serves as a crucial mediator in the process of

autophagy and lysosome fusion. It was reported that once autophagy was suppressed, the cells were re-sensitized to tamoxifen after LAMP3 knockdown (13). Compared with tamoxifen-sensitive cells, tamoxifen-resistant cells possess a higher level of autophagy, and thus inhibition of autophagy will enhance the therapeutic effect of tamoxifen (14). The silencing of autophagy related genes such as ATG5, ATG7, and Beclin1, could restore tamoxifen sensitivity. In support, treatment with the autophagy inhibitors, 3MA, LY294002 and hydroxychloroquine (HCQ), increases the death of tamoxifen-resistant cells and makes these resistant cells susceptible to tamoxifen again (11, 15–17). However, the underlying mechanism by which autophagy mediates tamoxifen resistance in breast cancer remains to be elucidated. Exploring the detailed mechanisms by which autophagy is associated with tamoxifen resistance could provide new insights in seeking proper prognostic or predictive biomarkers for the prospect of tamoxifen resistance, as well as promote the design of new strategies for the re-sensitization of tamoxifen-resistant breast cancer cells. Altogether, this indicates that antitumor drugs, for example, tamoxifen, could induce cell protective autophagy as a survival mechanism.

PKIB, the cAMP-dependent protein kinase inhibitor beta, also known as PRKACN2, a member of the protein kinase inhibitors (PKIs) (18, 19), serves as an inhibitor of protein kinase A (PKA). It is thought to be the significant regulatory factor controlling the PKA signaling (20). PKA signaling axis activation is fundamental for cancer cell resistance to various kinds of severe stressful conditions like glucose starvation, hypoxia, etc. Enhanced activity of PKA signaling pathway can be found in tamoxifen-resistant breast tumors, which indicated that the activation of PKA signaling pathway could contribute to tamoxifen resistance (21). However, the molecular functions of PKIB in tamoxifen resistance was unclear.

Interestingly, tamoxifen and 4-OH-tamoxifen treatment could increase the intracellular level of cyclic adenosine monophosphate (cAMP), which serves as a key factor involved in the signal transduction pathways in tumors (22). Whereas accumulated cAMP level could in turn activate protein kinase A(PKA). cAMP-responsive element-binding protein (CREB) is a cellular ubiquitous transcription factor that enhances the transcriptional activity of target promoters at its binding site (23). Moreover, CREB phosphorylation at ser133 activates its transcriptional activity, thereby inducing the expression of target genes that are associated with different cellular functions. Autophagy related gene 7, ATG7, acts as a crucial autophagy gene, and could restore tamoxifen sensitivity in breast cancer cells by silencing of ATG7 (24). Therefore, we hypothesized that tamoxifen could cause an increase in intracellular cAMP levels, which in turn cascade to activate PKA/CREB signaling axis, thereby inducing autophagy. However, this hypothesis needs to be verified by subsequent experiments.

Here, we identified that higher PKIB expression was markedly upregulated in tamoxifen-sensitive cells and primary tumor tissues. It was also determined that PKIB could inhibit tamoxifen resistance in ER-positive breast cancer cells by negatively regulating the CREB/ATG7 signaling axis, suggesting an underlying molecular mechanism for this effect of PKIB.

Materials And Methods

Bioinformatic methods and data analysis. Data derived from 1,211 breast cancer samples (including 114 normal breast tissues and 1097 cases of primary breast tumor) and 833 breast cancer samples (which divided into four groups based on their histological subtype) were downloaded from The Cancer Genome Atlas (TCGA) database (<https://cancergenome.nih.gov>). Correlation analysis was conducted through Gene Expression Profiling Interactive Online Analysis (<http://gepia.cancer-pku.cn/index.html>) in TCGA breast cancer datasets by Pearson's correlation analysis method (Pearson's $r = 0.5$; $P = 0$).

Cell culture

ER-positive human breast cancer cell lines (MCF7 and T47D), and MCF7 and T47D derived tamoxifen-resistant cell lines (MCF7R, T47DR), were grown in RPMI 1640 medium containing 1% double antibody with 10% fetal bovine serum and cultured at 37°C in a standard incubator with 5% CO₂.

Plasmids and siRNAs. Synthetic small interfering RNA (siRNA) was used to temporarily knock down PKIB (RiboBio, Guangzhou, China). Short hairpin RNA (shRNA) oligonucleotides (GenePharma, Shanghai, China) were used to generate stable PKIB knockout cells via lentivirus-mediated transduction according to the manufacturer's protocol.

Antibodies and reagents. The antibodies used for Western blots in this study included: anti-PKIB (ab233521, Abcam, 1:1000), anti-ATG7 (67341-1-Ig, ProteinTech, 1:100), anti-pCREB (9198S, Cell Signaling Technology, 1:1000), anti-CREB (9197T, Cell Signaling Technology, 1:1000) LC3I II (9116, Cell Signaling Technology, 1:1000), anti-Lamin-B1 (17416S, Cell Signaling Technology, 1:1000), anti-β-actin (66009-1-Ig, ProteinTech, 1:1000), anti-SQSTM1 (66184-1-Ig, ProteinTech, 1:1000). Autophagy inhibitors and autophagy agonists were purchased from MCE Biosciences Inc. Tamoxifen was from Sigma.

Cell lines and transfection. Tamoxifen-resistant cell lines were constructed using a low-concentration continuous induction method according to the literature (25, 26). MCF7 and T47D were purchased from ATCC. siRNA transfection was performed using Lipofectamine 3000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Stable clones were selected and maintained in 2 mg/mL of puromycin (Sigma) for PKIB knock down of shRNAs.

Quantitative RT-PCR. Total cellular RNA was extracted by the Trizol method and cDNA was formed using the Reverse Transcription Kit(Thermo Fisher Scientific). The expression of genes was calculated according to the method mentioned in the literature.(15).

Western blot analysis. Cells were washed twice with sterile pre-cooled PBS before harvested, added cell lysis solution (Beyotime, China) and protease inhibitor. Centrifuged at 12,000rpm for 30min at 4°, remove supernatant. Protein concentration were assessed via BCA method. Protein samples were subjected to 12.5% SDS-PAGE and transferred to PVDF membranes (Bio-Rad, USA). The membranes were then blocked with 5% skim milk in 0.1% TBST buffer at 4°C overnight. The membranes were subsequently incubated with PKIB (ab233521, Abcam, 1:1000), ATG7 (67341-Ig, ProteinTech, 1:100), p-CREB1 (9198S, Cell Signaling Technology, 1:1000), CREB (9197T, Cell Signaling Technology, 1:1000), LC3I II (9116, Cell

Signaling Technology, 1:1000), Lamin-B1 (17416S, Cell Signaling Technology, 1:1000), β -Actin (66009-1-Ig, ProteinTech, 1:1000), and SQSTM1 (66184-1-Ig, ProteinTech, 1:1000). Protein-antibody complexes were detected using HRP-conjugated secondary antibodies and enhanced chemiluminescence (Pierce). Band intensities were quantified using ImageJ software (NIH, Bethesda).

Immunofluorescence and confocal microscopy. The cells were seeded onto coated cover slips, fixed with 4% paraformaldehyde for 15 min at room temperature, treated with 0.1% Triton X-100, and blocked with 5% normal goat serum. They were then incubated with primary antibodies overnight at 4°C, washed thrice in phosphate-buffered saline (PBS), and stained with an FITC-labeled goat anti-rabbit secondary antibody (1:200; Zhongshan Golden Bridge); DAPI was used as the nuclear stain. Immunofluorescence images were taken using a confocal microscopy (Carl Zeiss, Zeiss LSM510 Meta). The LC3B-puncta in each cell were counted (10 fields per sample are indicated). Data obtained from 1 of 3 independent experiments with similar results were presented

Small interfering RNA studies. The cells were planted in serum-free medium without antibiotics at 2×10^4 per well on a 24-well plate. They were then transfected with 50 nmol/L negative control small interfering RNA (siRNA), or 50 nmol.L⁻¹ PKIB siRNA (RiboBio, China) using Lipofectamine® RNAi MAX Transfection Reagent (Life Technologies, USA). The medium was changed after 6 h and every other day thereafter until the end of the experiment.

Transmission electron microscopy. For transmission electron microscopy assay, cells samples were prepared as previously described(29) ,and then were examined with an electron microscope (JEOL, JEM-1010).

Animal experiment. All in vivo experiments were conducted using 4–6 week old female nude mice. All animal experiments were approved by the Laboratory Animal Care and Use Committee of Chongqing Medical University. The 4–5 week old female Balb/c nude mice were pre-treated with subcutaneous estrogen injections one week prior to inoculation of tumour cells. The amount of tumour cells inoculated was 1×10^7 , (including MCF7/sh-PKIB cells or MCF7/sh-NC cells) and sterile PBS solution and stromal gel were prepared in a 1:1 ratio to make a 100ul solution to fully resuspend the tumour cells. After inoculation, tumour formation in nude mice was regularly observed, and when tumours were palpable, tumour size was regularly measured and tumour volume was calculated using the following formula: tumour volume (mm³) = length x width² x 0.5. When tumour volume reached or exceeded 100 mm³, MCF7/sh-PKIB or MCF7/sh-NC xenograft mice were divided into three groups (5 mice per group) as follows. The details are as follows. (1) control group (PBS), (2) tamoxifen group (ip, 4 mg/kg, twice weekly), and (3) tamoxifen + HCQ group (Tam, ip, 4 mg/kg, twice weekly; HCQ, ip, 5 mg/kg). Tumour volumes were continuously measured until 2 weeks after the complement of treatment. Mice were humanely executed and tumour tissues were collected for immunohistochemical staining.

Immunohistochemistry. Paraffin blocks of prepared mouse tumour tissue were cut into thin slices of 4 μ m thickness. Antigen retrieve was performed in 0.01 M citrate buffer (pH 6.0) for 30 minutes in an autoclave,

followed by treatment with 3% hydrogen peroxide for 15 minutes. Specimens were closed with 10% goat serum for 1 hour at room temperature. Samples were incubated overnight at 4°C with antibodies specific for PKIB, ATG7 and pCREB1. Immunostaining was performed using DAB (Dako) according to the manufacturer's instructions. Immunohistochemical sections were placed under a 200X microscope with 5 randomly selected fields of view, images were saved and the mean expression intensity was calculated using Image J software 6.0. Two independent pathologists in our hospital analyzed the staining results

Cell viability. Cell proliferation was detected using the Cell Counting Kit-8 (Boster Biological Technology co.ltd), according to the manufacturer's instructions. 4000 cells/well were seeded into a 96-well plates, then were incubated at 37°C. 10 µL of CCK-8 solution was added and the cells incubated at 37°C for 2 h before the absorbance was determined by Multiskan Spectrum 1500 (Thermo Scientific, PA) at 450 nm.

Sample collection. Twenty paired human primary breast cancer specimens and recurrent tumor tissues were surgically obtained from the Department of Endocrine Breast Surgery, at the First Affiliated Hospital of Chongqing Medical University, and frozen at -80°C. Written informed consent was obtained from each patient. The Ethics Committee of the First Affiliated Hospital of Chongqing Medical University approved this study.

Statistical analysis. Survival curve analysis was carried out through Kaplan-Meier plotter (<http://kmplot.com/>), an online survival analysis platform. All experiments were performed independently at least three times. Statistical analysis was conducted using the SP2222.0 standard software package (Chicago, USA) and in vitro experiments were reread three times and the results expressed as mean ± SD. Data between two independent sample groups were analysed using t-tests, data between multiple samples were analysed using ANOVA tests, and data on categorical variables were analysed using chi-square or fisher's exact probability tests, and were considered statistically different when $p < 0.05$.

Results

PKIB expression correlates with ER-positive breast cancer tamoxifen resistance.

To evaluate PKIB expression in breast cancer, the bioinformatic analysis was first performed. The TCGA dataset indicated that PKIB expression is higher in breast cancer than in normal controls (Fig. 1A, $P=0.005$). Meanwhile, PKIB expression was higher in TAM-sensitive luminal breast cancer subtype, and lower in TAM-insensitive HER2 overexpression subtype and triple negative breast cancer (TNBC) subtype (Fig. 1B, $P=0.005$). This suggests that PKIB may play an important role in ER positive breast cancer. Next, to initially clarify the relationship between PKIB expression and tamoxifen resistance, tamoxifen-resistant breast cancer cell lines were established from MCF7 and T47D as described before (25, 26). Western blot and RT-qPCR analyses confirmed that the protein (Fig. 1C) and mRNA levels (Fig. 1D, $P=0.005$) of PKIB in MCF7 cells were significantly higher than those in MCF7R cell line, similar results were obtained in T47D parental cells and derived tamoxifen resistant cells (T47DR) (Fig. 1C and 1D). We also evaluated the expression of PKIB with IHC in 20 paired human primary breast cancer specimens and recurrent tumor

tissues. It was observed that PKIB was indeed highly expressed in recurrent breast cancer specimens (Fig. 1E, $P < 0.05$, 1F). To examine the clinical significance of the upregulated expression level of PKIB, prognostic values were analyzed using Kaplan-Meier analysis (<http://kmplot.com/>). We found that higher expression of PKIB was associated with increased overall survival (OS) in breast cancer patients (Fig. 1G, $P < 0.01$). PKA, which is reported to be associated with resistance to endocrine therapy using tamoxifen and is an important prognosis factor in breast cancer (21). PKIB serve as a kinase inhibitor of PKA, is differentially expressed between tamoxifen resistant expression We therefore presumed that PKIB may be involved in tamoxifen resistance.

Downregulated PKIB promotes cell proliferation, increases breast cancer cell resistant to tamoxifen, and induces autophagy. Previous investigations indicated that tamoxifen could induce cell protective autophagy, and enhanced autophagy level further promotes tamoxifen resistance in breast cancer cells (9, 11). In addition, recent research has also demonstrated that the MCF7R cell line confers a higher autophagy level than wild type (14). To determine the role of PKIB in tamoxifen resistance, specific siRNAs targeting PKIB were designed to reduce the expression of endogenous PKIB in MCF7 and T47D cells (Fig. 2A-D). Figure 2A and 2B exhibited a decrease in the protein and mRNA expression level in MCF7/si2PKIB cells compared to that in MCF7/siNC cells (Fig. 2B, $P < 0.01$), similar results were obtained in T47D cancer cells (Fig. 2C and Fig. 2D, $P < 0.01$). The cell growth and tamoxifen sensitivity of MCF7 cells were then evaluated using CCK8 assay. We found that reduced PKIB promoted the growth of MCF7 cells (Fig. 2E, $P < 0.01$). The CCK-8 assay indicated that MCF7/si2-PKIB cells showed a greater resistance than MCF7 control cells under the 10 μ M tamoxifen treatment (Fig. 2F, $P < 0.01$). Consistently, similar cell viability results were obtained in T47D cells (Fig. 2G, and 2H, $P < 0.01$). This result indicates that knockdown of PKIB enhanced tamoxifen resistance in MCF7 and T47D cells. Previous research indicated that autophagy activity was enhanced in the tamoxifen-resistant breast cancer cell lines and that this was associated with tamoxifen resistance. Next, we examined whether knockdown of PKIB could affect autophagy level in MCF7 cells. Thus, Western blot analyses were conducted to determine the relationship between PKIB and autophagy. It was found that PKIB knockdown enhanced the LC3B levels compared with the control group in MCF7 and T47D cells (Figs. 2I and 2J). Immunofluorescence assay of LC3B revealed that the downregulated PKIB expression level promoted the fluorescence intensity of LC3B (Figs. 2K and 2L, $P < 0.01$). Furthermore, typical double membrane autophagic vesicles were detected in MCF7/T47D cells by knocking down PKIB when their ultrastructure was examined using transmission electron microscopy (Fig. 2M). Together, these results showed that the downregulated expression of PKIB could overcome the growth-inhibitory effect of tamoxifen in tamoxifen-sensitive breast cancer cells.

PKIB reverses ER-positive breast cancer Tamoxifen resistance by inhibiting autophagy. To further clarify the function of PKIB in tamoxifen resistance, we established MCF7R sublines that expressed PKIB using the plasmids and verified its overexpression efficiency (Figs. 3A and 3B, $P < 0.005$). First, we found that the PKIB-overexpressing cells exhibited decreased cell viability (Figs. 3C and 3D, $P < 0.01$) and increased tamoxifen sensitivity (Figs. 3E and 3F, $P < 0.01$). In addition, the number of autophagic puncta decreased in the PKIB-overexpressing breast cancer cells (Fig. 3G and 3H, $P < 0.01$). Moreover, transmission electron microscopy was conducted to identify autophagosomes (Fig. 3I). These results further support the

contention that decreased expression of PKIB plays a role in developing tamoxifen resistance as well as in enhancing the autophagy level. To examine whether the PKIB-negatively regulated autophagy could hamper tamoxifen sensitivity, cells were treated with LY294002 (10 μ M) combined with tamoxifen (5 μ M) for 24h in the PKIB-overexpressing cells. The combined treatment had little effect on the growth of the control cells but induced significant growth inhibition in the PKIB-overexpressing cells (Fig. 3E and 3F, $P < 0.01$). Together these data indicate that PKIB reverses ER-positive breast cancer tamoxifen resistance by inhibiting autophagy.

PKIB inhibits autophagy by inhibiting the PKA-CREB signaling pathway.

It has been reported that tamoxifen could activate the cAMP/PKA/CREB axis (27). Recent research has discovered that CREB could transcriptional upregulation of autophagy gene ATG7 (28). Lee et al (29) also demonstrated that knockdown of ATG7 gene could restored sensitivity to 4OHT in both the MCF7/MTA1 and tamoxifen-resistant cells. To determine the mechanism by which PKIB regulates autophagy through PKA/CREB axis, we hypothesized that the autophagy-related gene ATG7 might be potential downstream target of CREB. Bioinformatics analysis using Gene Expression Profiling Interactive Online Analysis (<http://gepia.cancer-pku.cn/index.html>) indicated that ATG7 exhibited a positive correlation with CREB in TCGA breast cancer datasets (Fig. 4A, $R = 0.5$). Next, we examined the expression levels of ATG7 and CREB in TAM-sensitive cells MCF7 and T47D with downregulated PKIB expression. The protein expression level of ATG7 increased consistently with PKIB knockdown, while the CREB level was unchanged and phosphorylated CREB (pCREB) protein level was increased after knocking down of PKIB in MCF7 and T47D cells (Fig. 4B). Silencing PKIB expression promoted the immunofluorescence intensity of pCREB (Fig. 4C and 4D, $P < 0.01$). To confirm the capacity of transcriptional factor CREB to regulate ATG7 in breast cancer cells, we visualized pCREB and found it translocated from the cell membrane and cytoplasm into the nuclei when PKIB knocked down in MCF7 and T47D cells (Fig. 4E).

Our data manifested that CREB transcriptionally regulates ATG7, which consequently increases ATG7-mediated autophagy. These results indicate that knocking down PKIB promotes transcription factor CREB binding and activation of ATG7 to increase the transcription of ATG7 in breast cancer cells. (Fig. 4F).

Autophagy inhibitor restores the sensitivity to tamoxifen in xenografts of tamoxifen-resistant tumor in vivo. To validate these results in vivo, we inoculated MCF7/shNC and MCF7/shPKIB cells into the mammary fat pads of female Balb/c nude mice. When tumors were palpable, and tumor volume reached at least 100 mm³, they were treated with PBS control solution, tamoxifen alone, or tamoxifen combined with HCQ for 4 weeks. Tamoxifen treatment significantly shrank the MCF7/sh-NC tumors, while having no obvious effect on the MCF7/sh-PKIB tumors, indicating the endocrine resistance of the latter. Furthermore, treatment with HCQ and tamoxifen significantly decreased the size of the MCF7/sh-PKIB tumors compared with the treatment with tamoxifen alone, suggesting that the autophagy inhibitor restores the sensitivity to tamoxifen in MCF7/sh-PKIB tumors (Fig. 5A and 5B). Immunohistochemical (IHC) staining showed that the expression of ATG7 was markedly higher in MCF7/sh-PKIB tumors than in MCF7/sh-NC tumors and was decreased with HCQ treatment. (Fig. 5C). In conclusion, PKIB plays a

crucial role in tamoxifen sensitivity in MCF7 xenografts model and treatment of autophagy inhibitor counteract PKIB's effecting.

Discussion

Tamoxifen is a selective estrogen receptor (ER) modulator initially developed in the 1960s, and is the most firmly established adjuvant endocrine therapy in premenopausal estrogen receptor-positive breast cancer patients. However, one third of breast cancer patients still relapse and show metastasis due to de novo (primary) or acquired tamoxifen resistance, despite receiving 5-years of tamoxifen treatment. This remains a significant clinical challenge for the therapy of ER positive breast cancer (7, 30). Hence further exploration of tamoxifen resistance mechanisms remains quite urgent. In this study, we explored a novel model in which downregulated PKIB expression enhances autophagy via upregulation of the ATG7 transcription level, resulting in tamoxifen resistance (Fig. 4J).

PKIB, the cAMP-dependent protein kinase inhibitor beta, a potent inhibitor of PKA catalytic activity, is aberrantly expressed in breast cancer. However, little is known about the mechanisms by which PKIB regulates tamoxifen resistance in breast cancer. PKIB has been identified as a member of the protein kinase inhibitor peptide family, which is a class of endogenous thermostable peptide (19). It participates in many oncogenic processes, such as tumor cell proliferation and metastasis. Chung et al. reported that upregulated PKIB contributes to the prostate cancer cell aggressive phenotype via an enhanced Akt phosphorylation level (31). Additionally, PKIB promotes cell proliferation and tumorigenesis by activating the PI3K/Akt pathway in non-small cell lung cancer (NSCLC), implying that this is an important underlying mechanism that affects the progression of NSCLC (32). In this study, we revealed that the expression of PKIB is substantially downregulated in tamoxifen-resistant breast cancer cell lines and tumor tissues, and that overexpressing PKIB sensitizes MCF7R cells to tamoxifen treatment in vitro. Kaplan-Meier curve analysis also indicated that the high level of PKIB predicts a good prognosis in breast cancer.

Autophagy plays a complex role in cancer, and can confer a different fate on tumor cells depending on the cellular context (33). On one hand, autophagy allows tumor cells to survive from extreme environmental stress such as nutrient deprivation, hypoxia, and therapeutic drugs, which induces resistance to antineoplastic therapies. On the other hand, tumor cells can undergo autophagic cell death through self-phagocytosis, after which the cytoskeleton is mostly preserved (11, 34). Recent studies have implied that antitumor therapies could induce autophagy, using the enhancement of autophagy as a protective mechanism for cell survival that is initiated in response to metabolic or therapeutic stress. Tamoxifen has been demonstrated to induce cytoprotective autophagy and exert a prosurvival mechanism in human breast cancer cell lines (35). Moreover, the usage of tamoxifen enhances autophagy, leading to drug resistance (36). It is reported that the use of tamoxifen could be related to the energy metabolism of cells; the ATP level of breast cancer cells decreased after tamoxifen treatment. The administration of tamoxifen could lead to the upregulation of the expression of MTA1, which further destroys mitochondrial function, while tumor cells meet their energy needs through enhanced autophagy (29, 37). The enhancement of autophagy may be the result of the increased energy demand and the anti-

stress response of tumor cells. In fact, inhibition of autophagy could restore drug sensitivity. In this study, we elucidated how PKIB regulates autophagy in the setting of tamoxifen-resistant breast cancer cells. We found overexpression of PKIB in breast cancer cells reduced the number of autophagic puncta (Fig. 3C and 3D), and autophagy inhibitor LY294002 combined with tamoxifen showed a markedly growth suppression (Fig. 3E and 3F).

Current studies have implied that PKA is a key regulator of autophagy (38). PKA signaling axis activation is fundamental for cancer cell resistance to harsh tumor microenvironment like antitumor drug therapy, glucose deprivation, hypoxia etc. Indeed, the inhibition of PKA-mediated autophagy increased the level of cell death. Besides, PKA could induce resistance to tamoxifen (39–45). PKIB often exerts its molecular biological functions by inhibiting PKA activity, which is presumed to be one of the regulatory factors controlling the PKA signaling pathway. In this study, we demonstrated that PKIB negatively inhibits PKA signaling-mediated autophagy and tamoxifen resistance. We also demonstrated that the expression level of ATG7 increased significantly by silencing PKIB and decreased by overexpressing PKIB. In the PKIB knock down group combined with autophagy inhibitor treatment, a recovery of autophagy level was observed compared with the autophagy level in the PKIB knockdown group. Therefore, we propose a novel mechanism by which PKIB negatively regulates PKA/CREB/ATG7 signaling, thus inducing autophagy.

Conclusions

In summary, we demonstrated that downregulation of PKIB expression enhances autophagy level, which ultimately leads to tamoxifen resistance in ER-positive breast cancer cells by increasing ATG7 transcription level via the PKA/CREB/ATG7 signaling axis. We propose that PKIB can serve as a potential therapeutic target for the treatment of patients with ER-positive breast cancer.

Declarations

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Not applicable

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Ethics approval and consent to participate

This study was performed according to the Guidelines on Laboratory Animals of Chongqing Medical University and were approved by the Institute Research Ethics Committee at Chongqing Medical University.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LS and SL designed the research study. LS, BZ, YQ, FL and TJ performed the experiments. LS, BZ, FL, YW, YZ and YJ analyzed the data. LS and SL wrote the paper. All authors read and approved the final manuscript.

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Figures

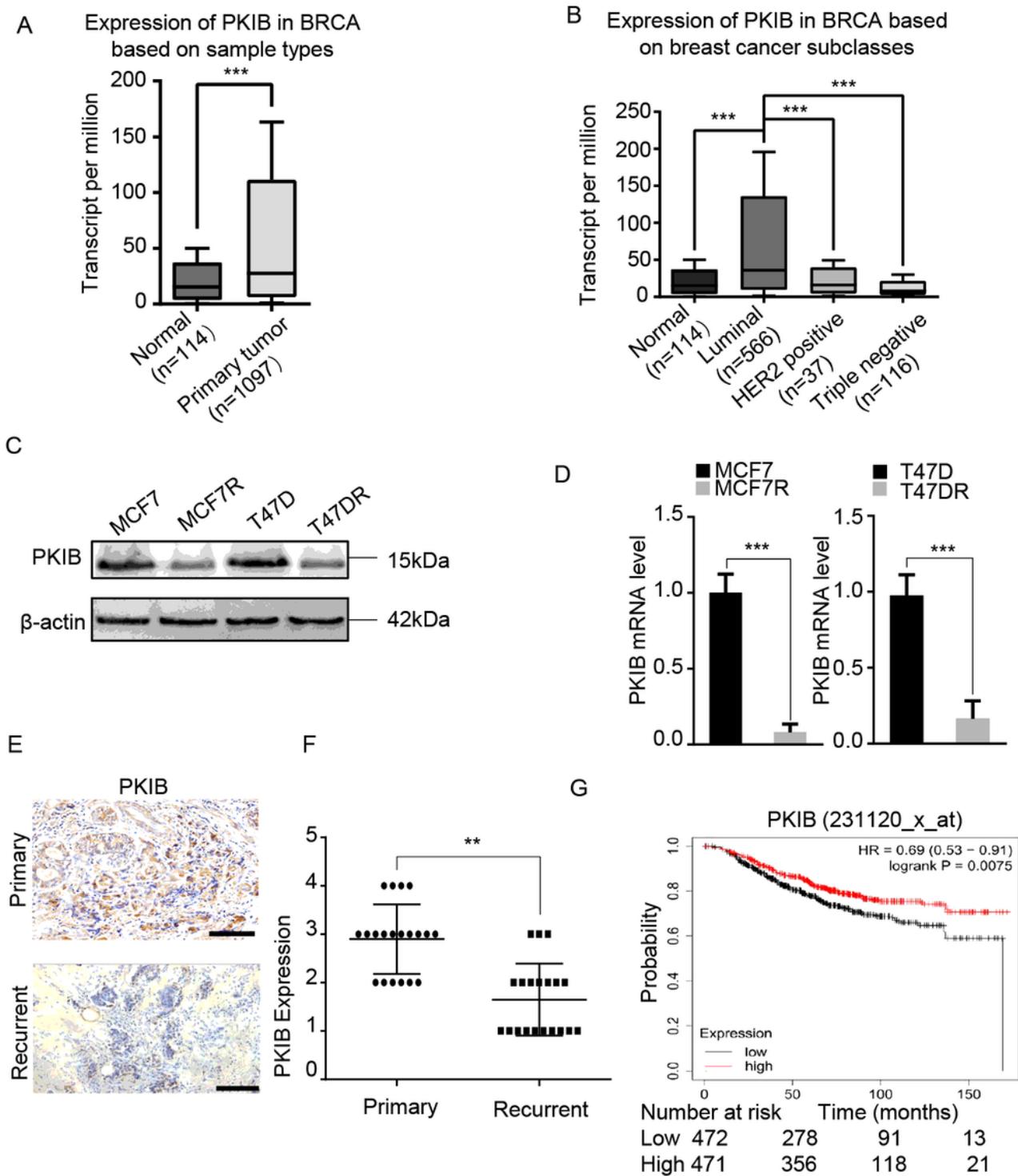


Figure 1

PKIB expression correlates with ER positive breast cancer tamoxifen resistance. (A) TCGA dataset indicates the PKIB expression is higher in breast tumor tissues(n=1097) than normal control group(n=114), $p < 0.001$. (B)TCGA datasets also exhibited PKIB expression is higher in luminal breast cancer subtype(n=566) compared with normal control group[(n=114), $p < 0.001$], HER2 positive subtype[(n=37) , $p < 0.001$], and Triple negative subtype [(n=116), $p < 0.001$] respectively. (C)The protein

and mRNA (D) expression level of PKIB in MCF7/T47D and MCF7R/T47DR breast cancer cells. (F) Representative pictures and quantification (G) of IHC staining of PKIB in paired primary and Recurrent breast cancer tissues. (H) Kaplan–Meier survival curves of breast cancer patients with low (black) and high (red) PKIB expression. MCF7R, MCF7-derived tamoxifen resistant breast cancer cell lines; T47DR, T47D-derived tamoxifen resistant breast cancer cell line. In A,B,D,F, data show means \pm SD (n = 3) **p < 0.01; ***p < 0.001, Student's t test was used for statistical analysis.

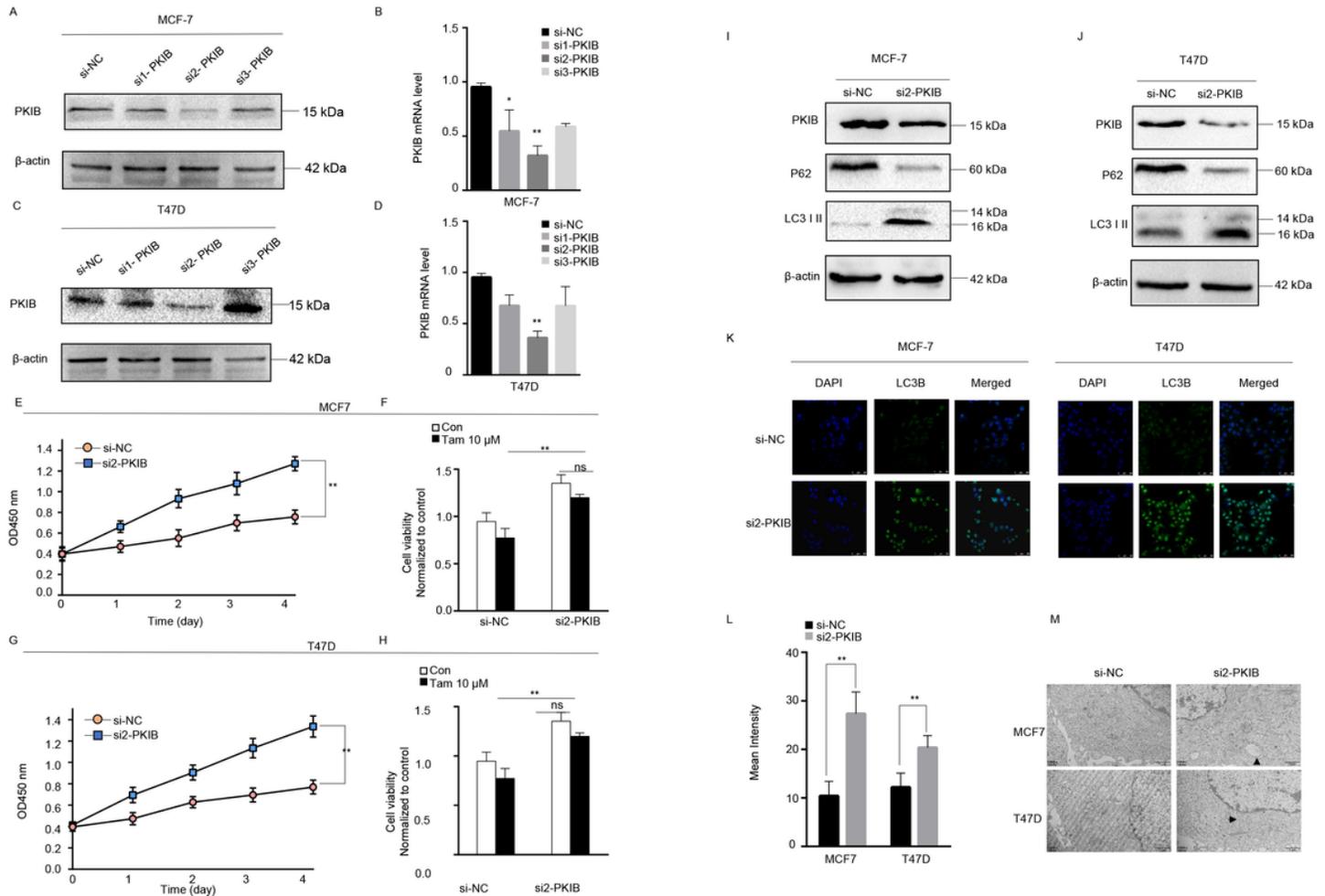


Figure 2

Downregulated PKIB promotes cell proliferation, confers tamoxifen resistance, and induces autophagy.

The MCF7 (A,B) and T47D (C,D) breast cancer cells were transfected with siRNA of PKIB—the knock down efficiency of PKIB were analyzed by western blot and RT-qPCR. (E, G) Cell viability assay indicating that downregulated expression of PKIB increases cell proliferation. (F, H) Cell viability assay showing the effect of 10 μ m tamoxifen treatment on MCF7/T47D cells when transfected with negative control siRNA (si-NC), PKIB siRNA(Si2-PKIB). (I,J)Western blot indicated autophagy level in MCF7 and T47D cancer cells. (K)Representative immunofluorescence images of LC3B in MCF7/T47D cells transfected with control siRNA (si-NC) and si2-RNA of PKIB. LC3B puncta was examined by confocal microscopy. (L)The LC3B-puncta in each cell were counted (10 fields per sample are indicated). Data obtained from 1 of 3 independent experiments with similar results were presented. Data are presented as the means \pm SEM.**

$P < 0.01$ (bottom). (M) Transmission electron microscopy images of MCF7 and T47D breast cancer cells transfected with si2-RNA of PKIB. Black arrowheads indicate autophagic vacuoles. Scale bar 1 μm . si-NC, siRNA negative control; si-PKIB, small interfering RNA-PKIB; ns, no significance; RT-qPCR, reverse transcription-quantitative PCR.

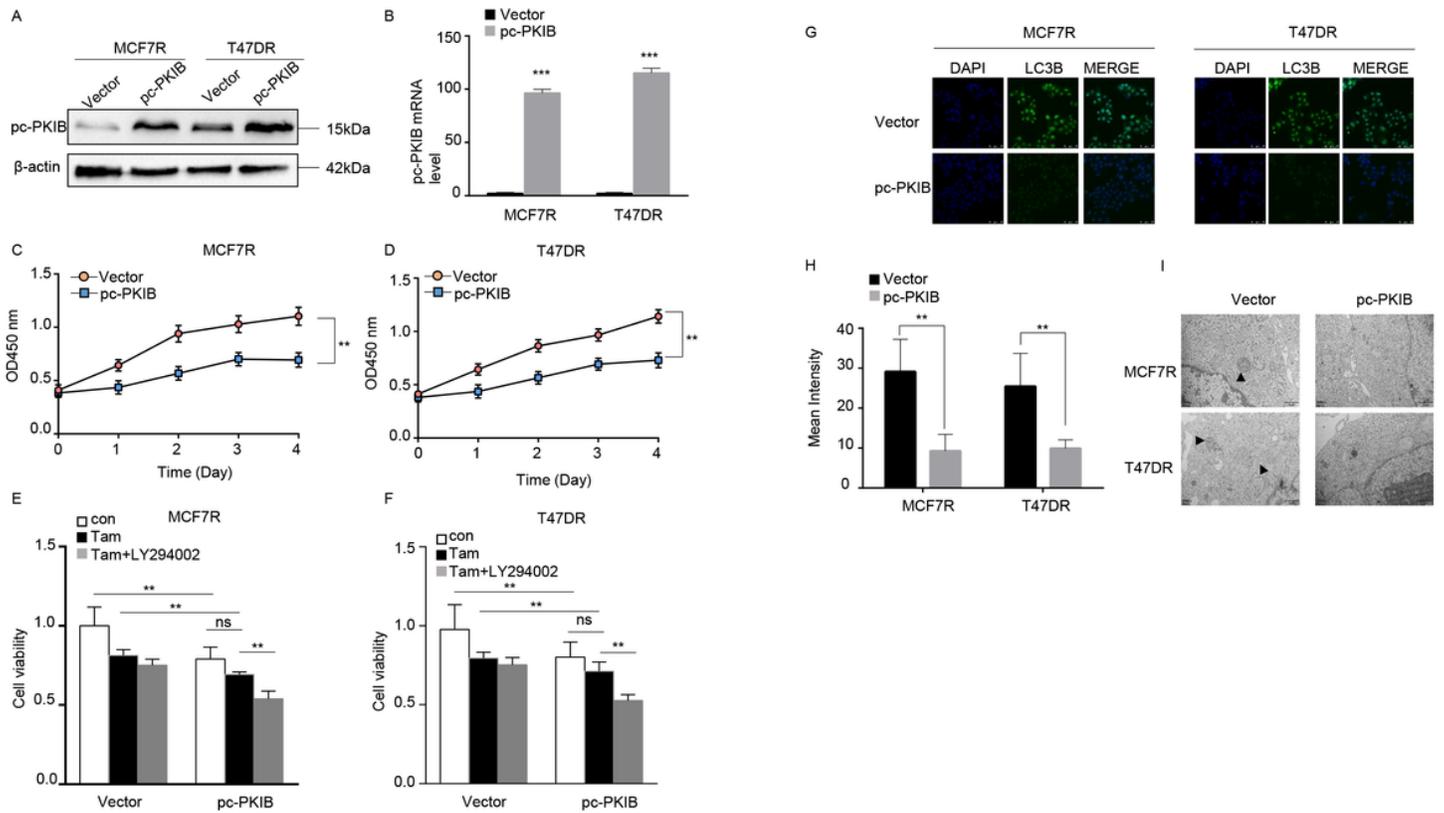


Figure 3

PKIB reverses ER-positive breast cancer tamoxifen resistance by inhibiting autophagy. (A-B) The MCF7R and T47DR breast cancer cells were transfected with plasmids of PKIB; the overexpression efficiency of PKIB were analyzed by RT-qPCR (B) and western blot (A). (C-D) Cell viability assay indicated that upregulated PKIB expression could inhibit the cell proliferation in MCF7R cells (C) and T47D cells (D). (E-F) Cell viability assay exhibited the effect of tamoxifen treatment alone or cotreatment with autophagy inhibitor LY294002 on MCF7R cells (E) and T47DR cells (F) when transfected with empty plasmid control (vector), plasmids of PKIB. (G) Representative immunofluorescence images of LC3B in TAM resistant cells MCF7R cells (left) and T47DR cells (right) transfected with plasmid of control vector and PKIB. LC3B puncta was examined by confocal microscopy. (H) The bar graphs show the quantified data of fluorescence LC3B puncta, and the data were taken from the mean \pm SD of three replicate experiments compared with control group, ** $P < 0.01$ (bottom). (I) Transmission electron microscopy images of MCF7R and T47DR breast cancer cells transfected with empty plasmid control (vector) and plasmids of PKIB. Black arrowheads indicate autophagic vacuoles. Scale bar 1 μm . In B-F, data were taken from the mean \pm SD of three replicate experiments, compared with control by Student's t test. Vector, empty plasmid

control; pc-PKIB, PKIB overexpression plasmid; ns, no significance; RT-qPCR, reverse transcription-quantitative PCR.

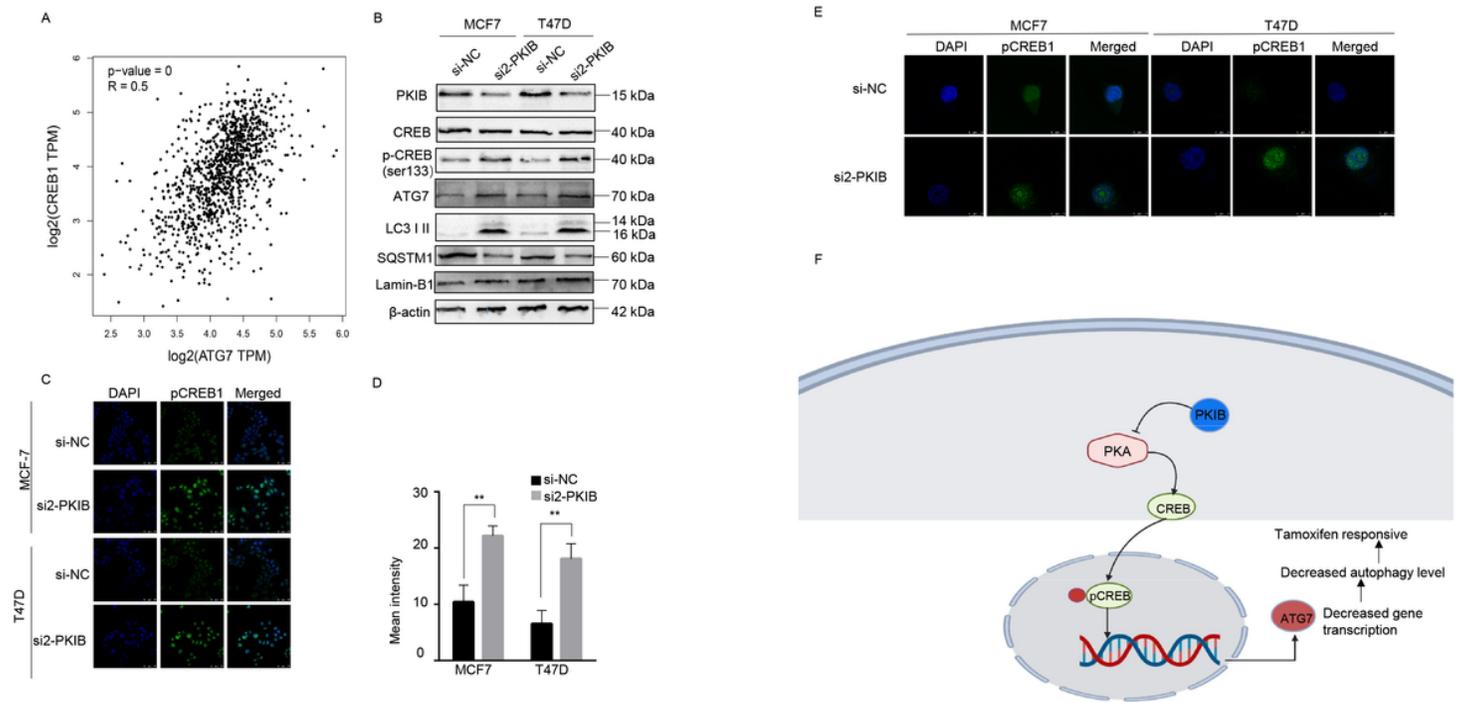


Figure 4

PKIB inhibits autophagy by inhibiting the PKA-CREB signaling pathway. (A) Online correlation analysis(<http://gepia.cancer-pku.cn/index.html>) indicated the positive correlation between transcription factor CREB and ATG7 (Pearson's $r=0.5$; $P=0$). (B) Downregulated expression of PKIB in MCF7 and T47D cells promoted the p-CREB1 and ATG7 expression and enhanced autophagy. (C) Representative immunofluorescence confocal images of p-CREB in MCF7 cells (Upper) and T47D cells (lower) transfected with si-NC and si2-PKIB, scale bar 50 μ m. (D) The bar graphs show the quantified data of fluorescence intensity of p-CREB, and the data were taken from the mean \pm SD of three replicate experiments, compared with control group. ** $P<0.01$ (bottom). (E) Representative immunofluorescence confocal images of p-CREB nuclear localization in MCF7 cells (left) and T47D cells (right) transfected with si-NC and si2-PKIB Scale bar, 10 μ m. (F) A working model for PKIB-CREB-ATG7 axis mediated autophagy in Tamoxifen resistance. In MCF7/T47D breast cancer cells, knocking down of PKIB induces the nuclear translocation of CREB to regulate the transcriptional activity of pCREB to increase ATG7 expression. Elevated ATG7 enhances the autophagy in breast cancer cells thus contributes to tamoxifen resistance.

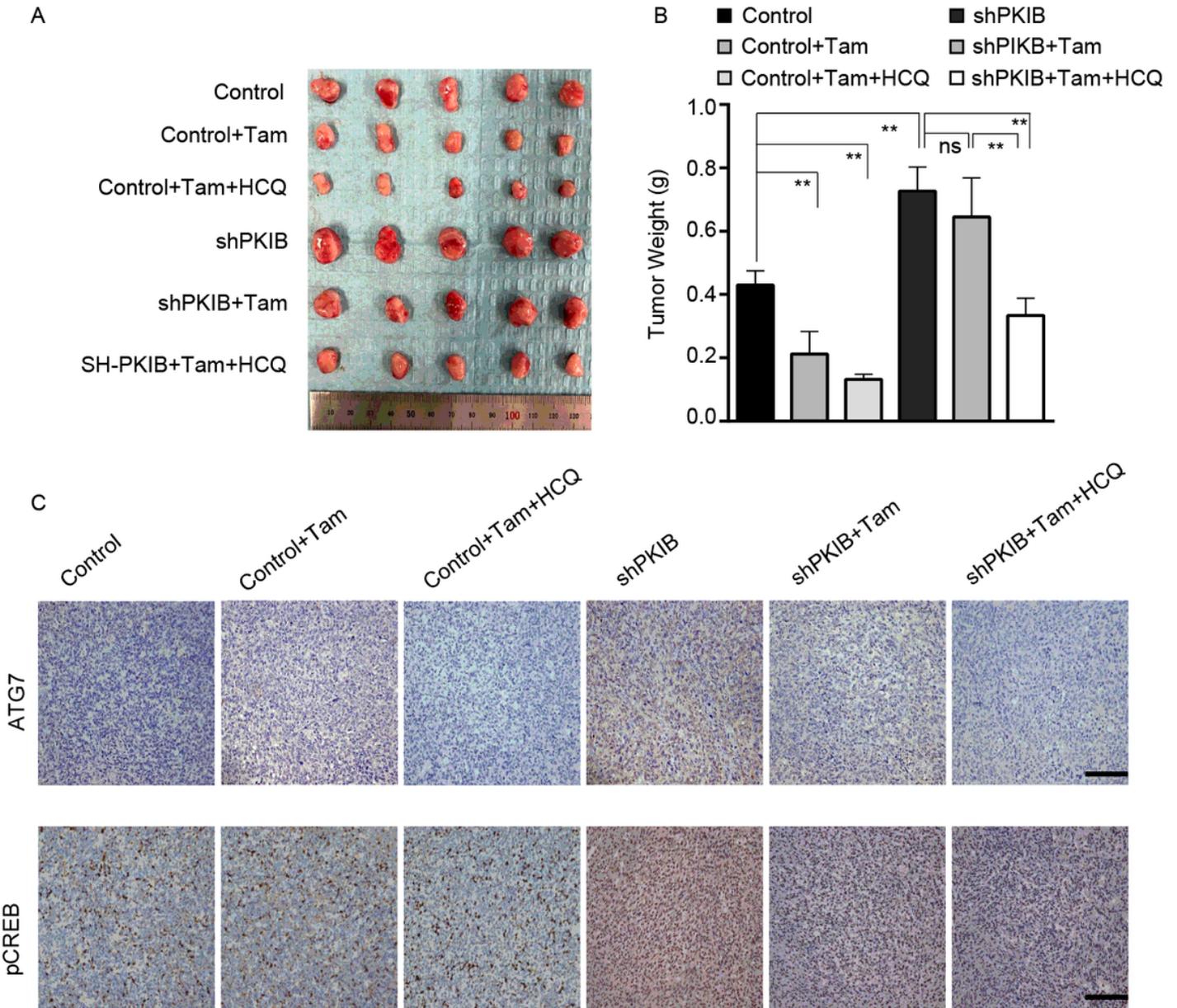


Figure 5

MCF7/sh-NC and MCF7/sh-PKIB cells were inoculated into the mammary fat pads of Balb/c nude mice. When tumors became palpable, tumors were treated with PBS control, tamoxifen, or tamoxifen with HCQ. (A) The picture of xenograft tumor. (B) Tumor weight from indicated treatment, data show means \pm SD ($n = 5$), $**p < 0.01$ by Student's t test. (C) Immunohistochemical staining of ATG7 of xenografts derived from MCF7/sh-NC and MCF7/sh-PKIB cells with indicated treatment. Scale bar indicated 200 μ m.