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TP53AIP1 induce autophagy via the AKT/mTOR signaling pathway in the breast cancer cells

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ABSTRACT

Breast cancer ranks the first in the incidence of female cancer and is the most common cancer threatening the life and health of women worldwide.Tumor protein p53-regulated apoptosis-inducing protein 1 (TP53AIP1) is a pro-apoptotic gene downstream of p53. However, the role of TP53AIP1 in BC needs to be investigated. In vitro and in vivo experiments were conducted to assess the biological functions and associated mechanisms. Several bioinformatics analyses were made, CCK8 assay, wound healing, transwell assays, colony formation assay, EDU, flow cytometry, Immunofluorescence, qRT-PCR and Western-blotting were performed. In our study, we discovered that BC samples had low levels of TP53AIP1 expression, which correlated with a lower survival rate in BC patients. When TP53AIP1 was up-regulated, it caused a decrease in cell proliferation, migration, and invasion. It also induced epithelial-to-mesenchymal transition (EMT) and protective autophagy. Furthermore, the over-expression of TP53AIP1 suppressed tumor growth when tested in vivo. We also noticed that TP53AIP1 up-regulation resulted in decreased levels of phosphorylation in AKT and mTOR, suggesting a mechanistic role. In addition, we performed functional rescue experiments where the activation of AKT was able to counteract the impact of TP53AIP1 on the survival and autophagy in breast cancer cell lines. This suggests that TP53AIP1 acts as an oncogene by controlling the AKT/mTOR pathway. These findings reveal TP53AIP1 as a gene that suppresses tumor growth and triggers autophagy through the AKT/mTOR pathway in breast cancer cells. As a result, TP53AIP1 presents itself as a potential target for novel therapeutic approaches in treating breast cancer.

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KEYWORDS
TP53AIP1; breast cancer; autophagy; mTOR

Introduction

According to the data provided by the International Agency for Research on Cancer, female breast cancer has now surpassed lung cancer as the leading cause of global cancer incidence in 2020.[1] The incidence of breast cancer (BC) rises with age until menopause, and BC is more aggressive in younger female.[2] BC becomes the main reason threatening women's health. Despite significant improvements in treatment strategies for BC, patients continue to experience a number of adverse effects, such as poor prognosis and drug resistance. Even though there have been significant advancements in the ways we treat BC, patients still face a range of negative impacts, including poor prognosis and resistance to medication.[3] In order to effectively address BC, it is crucial to thoroughly investigate pathogenesis of the disease and discover fresh and potent targets for treatment.

Tumor protein p53-regulated apoptosis-inducing protein 1 (TP53AIP1) is a proapoptotic gene downstream of p53 identified in 2000.[4] TP53AIP1 is located in the mitochondrial membrane and only wild-type p53 protein phosphorylated at Ser46 was transcriptionally competent to induce TP53AIP1 protein.[4] Upon severe DNA damage, TP53AIP1 is phosphorylated by Ser46 of p53 and thus activated, followed by depolarization of mitochondrial membranes and release of cytochrome c and apoptosis-inducing factors to induce apoptosis.[5] It is crucial to observe that the abnormal expression of TP53AIP1 is strongly linked to various types of cancer. For instance, according to Jiang et al., TP53AIP1 overexpression can induce apoptosis and cell cycle arrest in liver hepatocellular carcinoma cells.[6] In addition, mutations in TP53AIP1 in prostate cancer, resulting in its low TP53AIP1 expression[7] and patients with non-small cell lung cancer who have a decreased expression of TP53AIP1 gene are correlated with a reduced rate of overall survival.[8] Previous study has demonstrated over-expression of TP53AIP1 promotes apoptosis in breast cancer.[9] We successfully utilize transmission electron microscopy to show that the overexpression of TP53AIP1 triggers the generation of autophagosomes in breast cancer cells MCF-7 and MDA-MB-231, marking a significant breakthrough.

Autophagy is a lysosome-dependent catabolic pathway by which damaged or senescent organelles are removed. Autophagy plays an important role in the regulation of cancer

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progression and in determining the response of tumor cells to stress induced by chemotherapy. However, the role of autophagy in cancer therapy is multifaceted, depending on the cell type, microenvironment and the stage of tumor development.[10–12] Several studies have suggested that autophagy could have both positive and negative effects on tumor development. For example, autophagy can increase tumor cell tolerance to stressors, promoting cancer cell survival in unfavorable environments. Autophagy can also inhibit tumor occurrence and metastasis, promoting tumor cell death through apoptotic pathways.[13,14] It is unknown, though, whether TP53AIP1 is involved in the induction of autophagy or even the prevention of breast cancer.

In the current study, cell lines and BC were examined for the expression of TP53AIP1. In vitro, TP53AIP1 overexpression inhibited breast cancer cell growth and motility, as well as induced apoptosis and autophagy. In vivo, TP53AIP1 decreased breast carcinoma xenograft growth. Next, we investigated if TP53AIP1 controls the AKT/mTOR pathway, thereby influencing the progression of BC. This study has the potential to reveal the molecular mechanism of TP53AIP1 in BC and present a novel therapeutic target for the treatment of BC.

Results

TP53AIP1 is down-regulated in breast cancer and correlated with a poor prognosis

To explore the role of TP53AIP1 in human breast cancer, seventy-seven pairs of cancer and adjacent normal tissues from the tissue bank were subjected to mRNA extraction and qRT-PCR quantification. The result showed that TP53AIP1 levels in cancer tissues was significantly lower than that in adjacent tissues (Figure 1a). Next, We also analyzed the expression of TP53AIP1 in normal mammary epithelial cells lines (MCF-10A, HMEC) and breast cancer cells lines (BT-549, MDA-MB-231, MDA-MB-468, YCCB1, SK-BR3, MCF-7, T47D, ZR-75-1) by qRT-PCR. The results showed that the mRNA expression of TP53AIP1 was significantly down-regulated in the breast cancer cells, especially in MCF-7, YCCB1 and MDA-MB-231 cells (Figure 1b). Furthermore, the analysis of the TCGA dataset revealed a significant decrease in the expression of TP53AIP1 in breast cancer patients. Survival curve analysis, also based on TCGA data, demonstrated a strong correlation between low expression of TP53AIP1 and a poor prognosis (Figure 1c,d). These findings collectively point toward the down-regulation of TP53AIP1

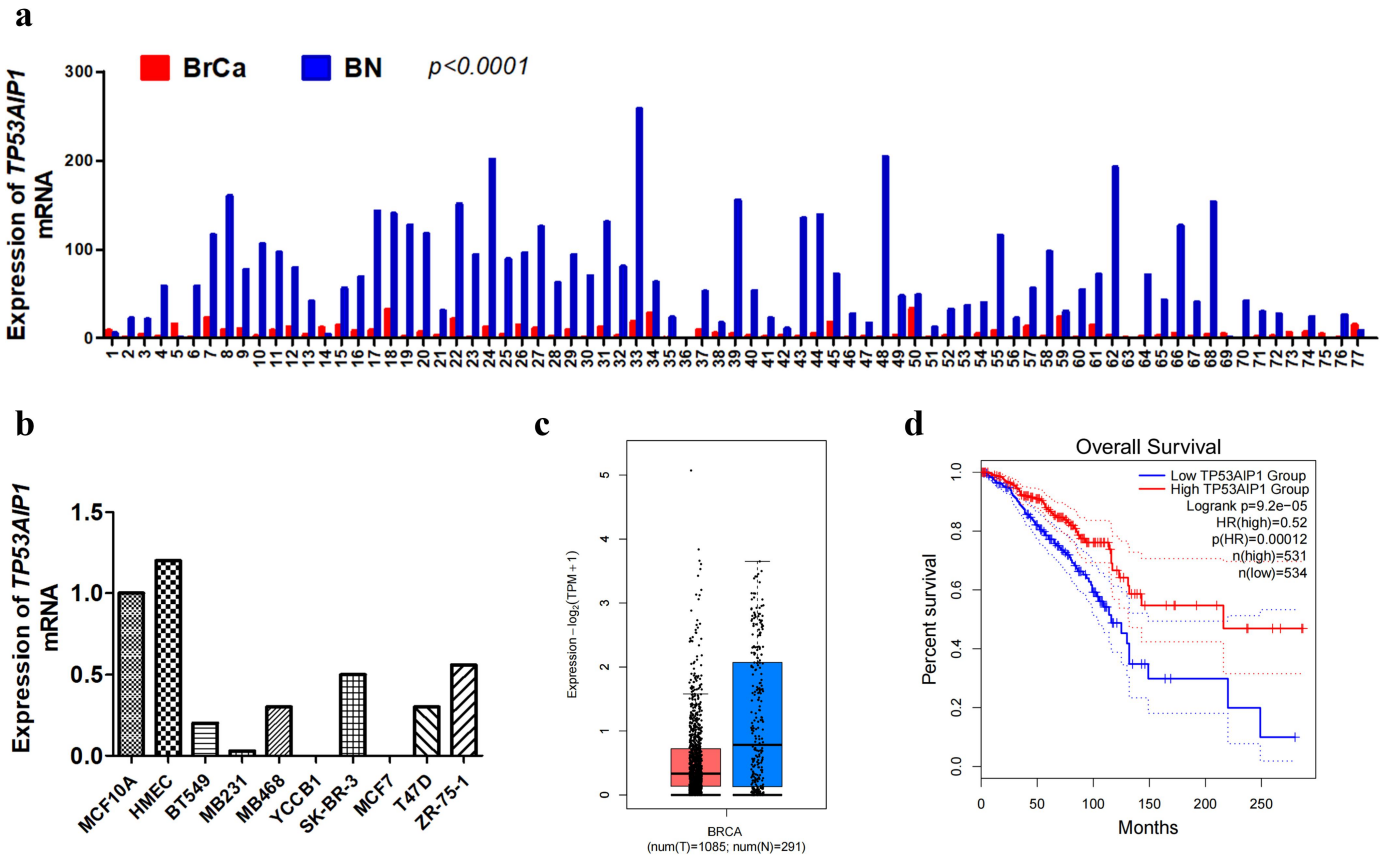

Figure 1. TP53AIP1 is down-regulated in breast cancer and correlated with a poor prognosis. (a) TP53AIP1 expression was analyzed in both tumor tissues and normal tissues using RT-qPCR (n = 77); (b) The expression of TP53AIP1 was measured in both normal mammary epithelial cells and breast cancer cells using RT-qPCR (n = 10); (c) Analysis of the TCGA database revealed that TP53AIP1 was found to be down-regulated in breast tumor tissues. Normal tissues (n = 291) are represented by the color blue, while tumor tissues (n = 1085) are indicated in red; (d) The TCGA database indicated that decreased expression of TP53AIP1 was associated with an unfavorable prognosis.

in BC, which is associated with an unfavorable prognosis for BC patients.

Over-expression of TP53AIP1 suppresses the proliferation in MCF-7 and MDA-MB-231 cells

As seen in Figure 2a,b, the transfection with pcDNA3.1-TP53AIP1 significantly increased the TP53AIP1 expression in MCF-7 and MDA-MB-231. The CCK-8 results revealed that the proliferation of cells decreased markedly after over-expression of TP53AIP1 (Figure 2c). In addition, the EdU positive cell rate was significantly decreased in MCF-7 and MDA-MB-231 cells after up-regulating TP53AIP1 (Figure 2d,e). The inhibitory effect on cell growth was further convinced by colony formation assay, in which TP53AIP1 decreased the number and size of colonies in MCF-7 and MDA-MB-231 compared with the vector cells (Figure 2f). Collectively, these results confirmed that up-regulating TP53AIP1 may suppress the proliferation in MCF-7 and MDA-MB-231 cells.

Over-expression of TP53AIP1 suppresses the migration and invasion and promotes apoptosis in MCF-7 and MDA-MB-231 cells

We then investigated whether there were other underlying mechanisms about the ability of TP53AIP1 for growth inhibition in breast cancer cells. Figure 3a,b demonstrate that the wound healing and transwell assays revealed a significant decrease in invasion and migration of MCF-7 and MDA-MB -231 cells upon over-expression of TP53AIP1. Furthermore, flow cytometry analyses were conducted to examine cell cycle and apoptosis, which showed an increased cell

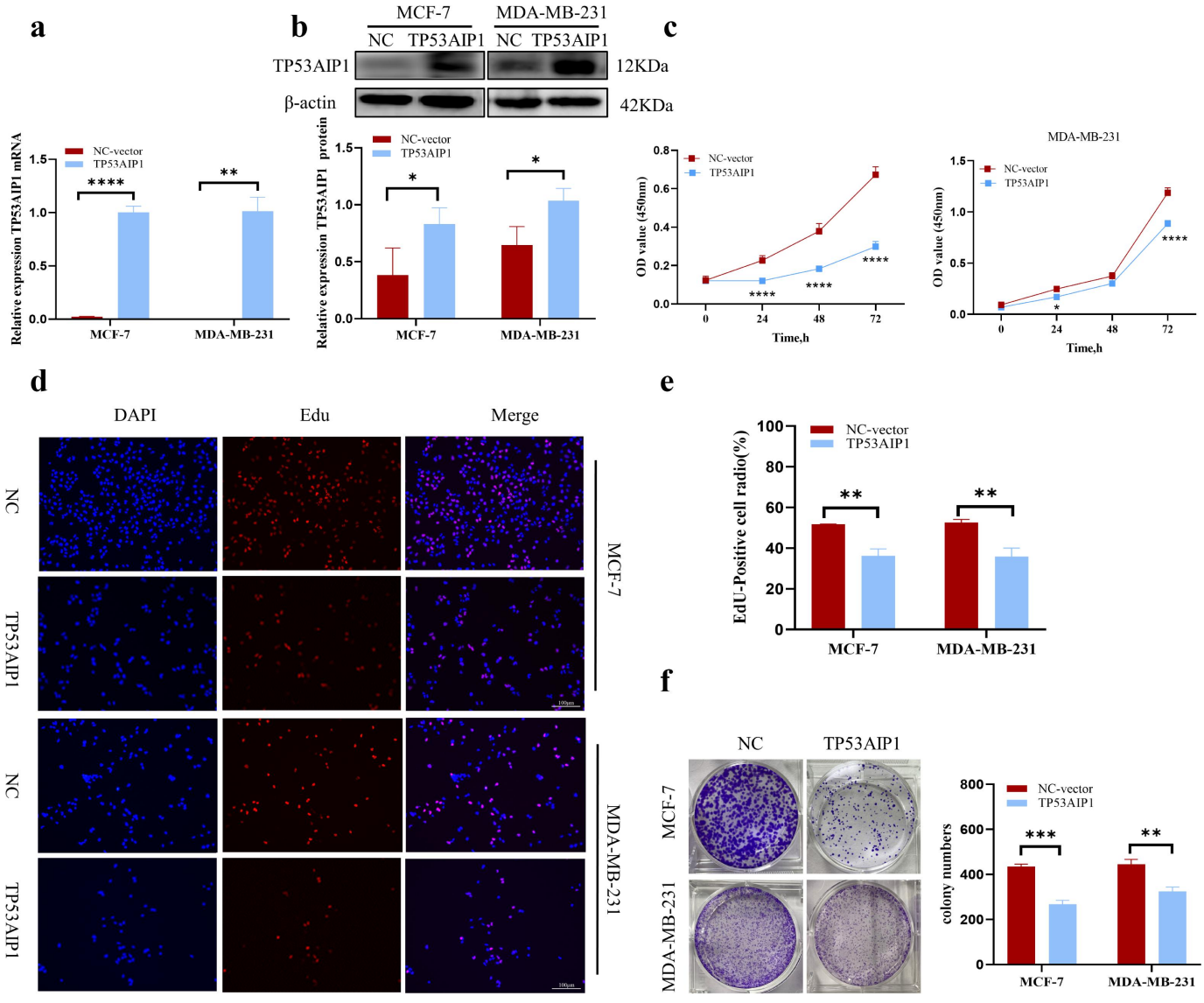

Figure 2. Over-expression of TP53AIP1 suppresses the proliferation in MCF-7 and MDA-MB-231 cells. (a, b) TP53AIP1 levels in breast cancer cells were assessed using RT-qPCR and western blot after transfection with pcDNA3.1-TP53AIP1 ($n = 3$); (c) The CCK-8 assay measured the growth of MCF-7 and MDA-MB-231 cells at 0, 24, 48, and 72 hours, respectively,($n = 3$); (d, e) The EdU assay was utilized to evaluate the proliferation of the transfected MCF-7 and MDA-MB-231 cells (Scale bar: 100μm). The percentage of EdU positive cells was quantified ($n = 3$); (f) The colony formation assay was performed to determine the number of colonies formed by the transfected MCF-7 and MDA-MB-231 cells ($n = 3$). Data are means±s.d. *, $p<.05$; **, $p<.01$; ***, $p<.001$; ****, $p<.0001$. Statistical significance was determined by comparing the data with their corresponding control groups.

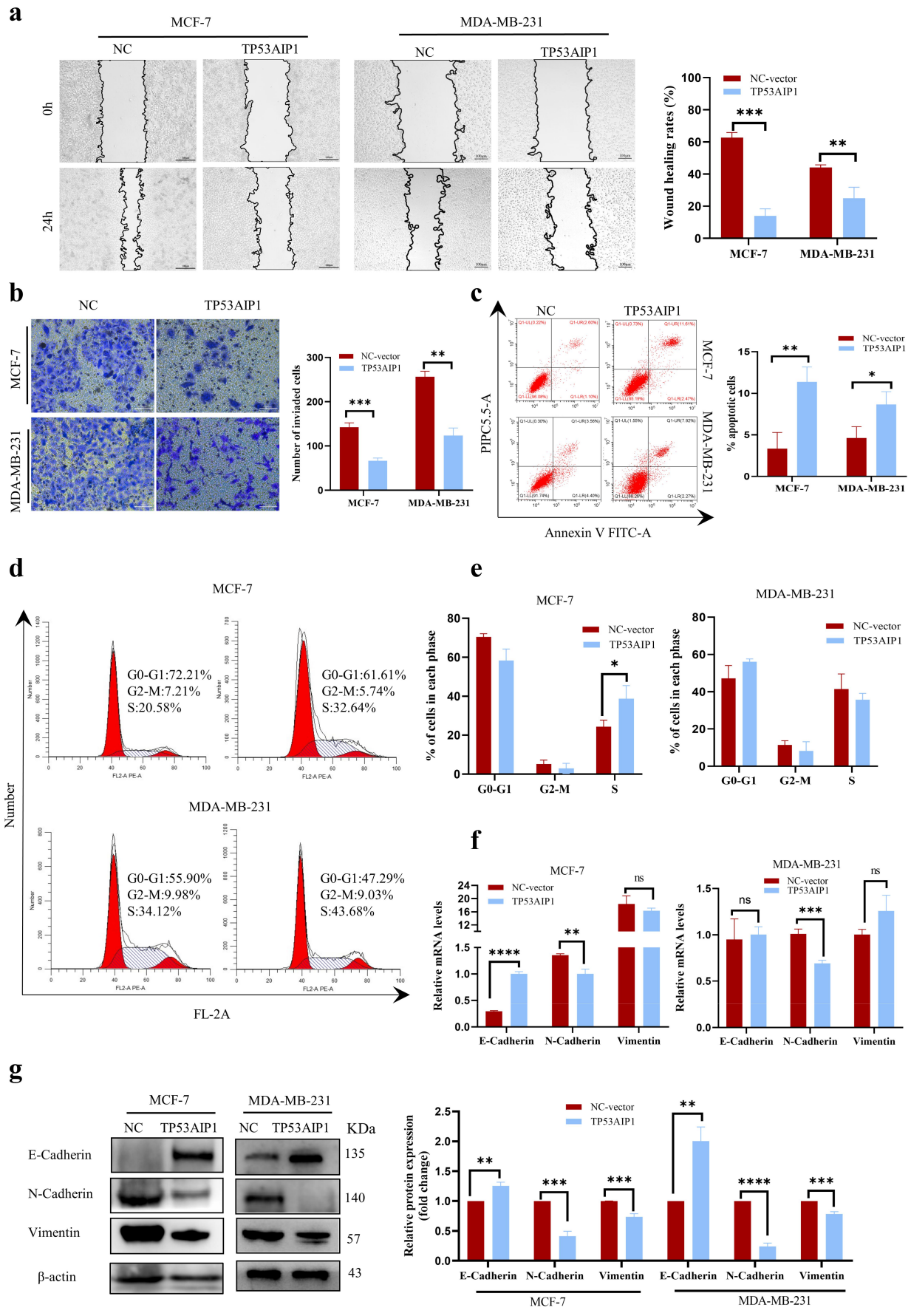

Figure 3. Over-expression of TP53AIP1 suppresses the migration and invasion and promotes apoptosis in MCF-7 and MDA-MB-231 cells. (a) The migratory abilities of MCF-7 and MDA-MB-231 cells were assessed by wound healing assay ($n = 3$); (b) The invasive abilities of MCF-7 and MDA-MB-231 cells were assessed by Transwell assay ($n = 3$); (c) Flow cytometry analysis of apoptotic cell population by Annexin V/PI staining; (d, e) Flow cytometry analysis of cell cycle of MCF-7 and MDA-MB-231 cells which was over-expression pcDNA 3.1 or pcDNA3.1-TP53AIP1 ($n = 3$); (f) The mRNA expression levels of EMT-associated transcription factors and markers in stably

apoptosis rate when TP53AIP1 was over-expressed compared to the respective controls (Figure 3c). TP53AIP1 up-regulation in MCF-7 cells caused cell cycle arrest in the S phase, resulting in a decrease in the proportion of cells in the G0/G1 phase without any significant difference in the G2/M phase. Similarly, in MDA-MB-231 cells, there was a slight increase in the proportion of cells in the G0/G1 phase and a decrease in the proportion of cells in the G2/M and S phases, but these changes were not statistically significant. To investigate the impact of TP53AIP1 on epithelial-mesenchymal transition (EMT), we examined the expression of EMT-related mRNA and proteins using qRT-PCR and western blot analyses. Our results showed that TP53AIP1 over-expression led to an increase in the levels of the epithelial marker E-cadherin and a decrease in the expression of key EMT-related transcription factors, as well as the interstitial markers N-cadherin and Vimentin. These findings suggest that up-regulating TP53AIP1 may inhibit migration and invasion, while promoting apoptosis in both MCF-7 and MDA-MB-231 cells.

Over-expression of TP53AIP1 promotes autophagy in MCF-7 and MDA-MB-231 cells

The association between autophagy and apoptosis has been long established and is dependent on the context. Our study examined whether TP53AIP1 triggers autophagy in breast cancer cells. A electron microscopic image revealed that TP53AIP1 resulted in the formation of double membrane structures, known as autophagosomes, in breast cancer cells (Figure 4a). Subsequently, we conducted a western blot analysis to assess the protein expression levels of LC3B and SQSTM1/p62. We observed a significant increase in the LC3-II/I ratio and a clear decrease in the protein expression of SQSTM1/p62 in MCF-7 and MDA-MB-231 cells over-expressing TP53AIP1 (Figure 4b). Additionally, when we introduced the RFP-GFP-LC3B plasmid through transient transfection, we observed a significant increase in the number of red fluorescent autophagosomes in TP53AIP1-treated cells (Figure 4c,d). It is worth noting that the accumulation of LC3-II can occur either due to enhanced autophagosome formation or due to a disruption in the fusion process between autophagosomes and lysosomes.[15] To distinguish between these two possibilities, we assessed the levels of LC3-II and P62 in the presence of chloroquine (CQ), a substance that hinders the fusion between autophagosomes and lysosomes, leading to the buildup of autophagosomes.[16]

In MCF-7 and MDA-MB-231 cells, the presence of TP53AIP1 resulted in increased accumulation of autophagic vesicles (LC3 puncta) compared to the control group. When TP53AIP1 was combined with CQ, the accumulation of LC3 spots was even greater (Figure 4e,f and S1a,b). This suggests that TP53AIP1 promotes the formation of autophagosomes in MCF-7 and MDA-MB-231 cells. Furthermore, when treated with TP53AIP1 and CQ, the ratio of LC3-II/I was higher compared to cells treated with TP53AIP1 alone (Figure 4g and S1c). These findings indicate that TP53AIP1 plays a role in the early stages of autophagy.

Autophagy attenuates TP53AIP1-induced anti-cancer effects

The role of autophagy in cancer is quite complex; autophagy has a bi-directional effect on cancer cells and may be cytoprotective or cytotoxic. Autophagy has a tumor-suppressive effect during cancer development, however, it contributes to the survival of tumor cells during cancer progression.[17] In addition, tumor cells can use autophagy to resist various anticancer therapies.[18] Therefore, we further investigated the biological function of TP53AIP1-induced autophagy in breast cancer cells. We conducted an experiment on breast cancer cells by using an autophagy inhibitor, CQ. When compared to the effects of TP53AIP1 alone, the co-administration of CQ resulted in a decrease in cell viability of breast cancer cells (Figure 5a). Furthermore, a significant reduction in cell proliferation was observed, as confirmed by EdU labeling (Figure 5b, c). Overall, the data clearly indicates that TP53AIP1-induced autophagy plays a cytoprotective role, while autophagy dampens the anti-cancer properties of TP53AIP1 in breast cancer.

AKT/mTOR signaling pathway involves in TP53AIP1 modulated the proliferation and autophagy in MCF-7 and MDA-MB-231 cells

In order to investigate the impact of TP53AIP1 on the AKT/mTOR signaling pathway in MCF-7 and MDA-MB-231 cells, we conducted Western blot analysis to measure the relevant proteins of this pathway. The results, as depicted in Figure 6a, demonstrated that the overexpression of TP53AIP1 led to a significant decrease in the levels of AKT and mTOR in both MCF-7 and MDA-MB-231 cells compared to the control groups. These findings suggest that the up-regulation of TP53AIP1 may effectively inhibit the activation of the AKT/mTOR signaling pathway in MCF-7 and MDA-MB-231 cells. In order to further confirm that TP53AIP1 inhibits the malignant behavior of breast cancer cells by interfering with the AKT/mTOR pathway, we conducted functional rescue experiments. The cells were transfected with a plasmid called CA-AKT, which continually activates AKT. As anticipated, the inclusion of CA-AKT reversed the inhibitory effects of TP53AIP1 on proteins related to the AKT/mTOR pathway (Figure 6b). The outcomes from the CCK8 and colony formation assay revealed that the proliferation of MCF-7 and MDA-MB-231 cells in the CA-AKT group was significantly higher compared to the TP53AIP1 group (Figure 6c,d). Furthermore,

transfected MCF-7 and MDA-MB-231 cells were measured by qRT-PCR (n = 3); (g) The protein expression levels of epithelial and mesenchymal markers in MCF-7 and MDA-MB-231 cells after TP53AIP1 over-expression were detected by western blot. β-actin was used as loading control, the relative intensity of blots was determined using ImageJ software (n=3). Data are means±s.d. ns: not significant; *, p<.05; **, p<.01; ***, p<.001; ****, p<.0001. Statistical significance was determined by comparing the data with their corresponding control groups.

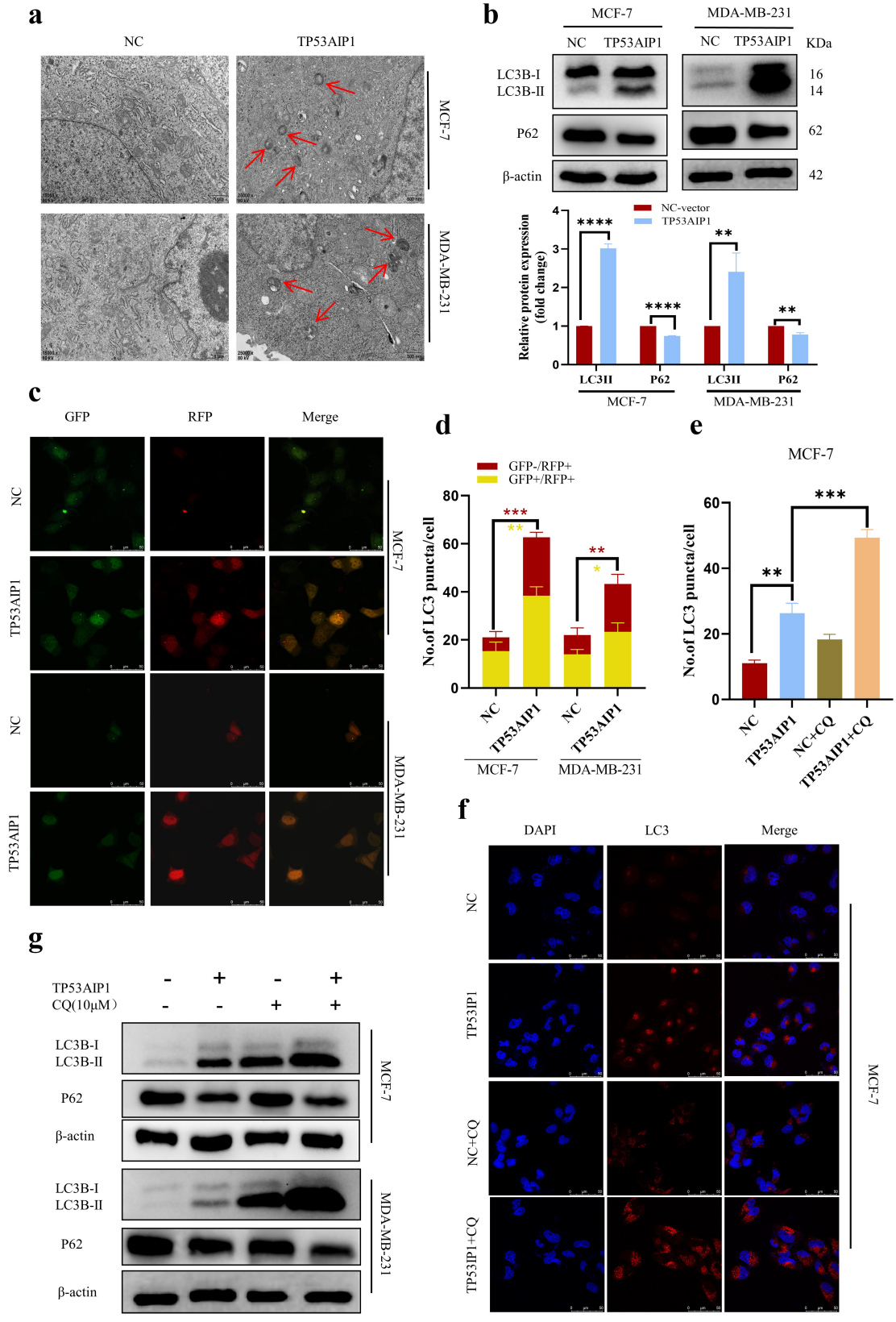

Figure 4. Over-expression of TP53AIP1 promotes autophagy in MCF-7 and MDA-MB-231 cells. (a) Transmission electron microscope was performed to observe the autophagic vesicles in MCF-7 and MDA-MB-231 cells with or without TP53AIP1 (NC:15,000x, Scale bar: 1 μm, TP53AIP1: 25000x, Scale bar: 500 nm) ($n = 3$); (b) Western blot analysis of LC3 and p62 expression in MCF-7 and MDA-MB-231 cells transfected of pcDNA3.1-TP53AIP1, the relative intensity of blots was determined using ImageJ software ($n = 3$); (c, d) Cells stably transfected with RFP-GFP-LC3B were additionally transiently transfected. Images were captured using confocal microscopy with a scale bar of 50 μm. The analysis involved quantifying the ratio of red puncta representing autolysosomes (GFP/RFP+) to yellow puncta representing autophagosomes (GFP+/RFP+), the relative fluorescence intensity was determined by ImageJ software ($n = 3$); (e, f) Immunofluorescence analysis was performed to observe the development of natural LC3B puncta in MCF-7 cells after treatment with TP53AIP1, CQ (10 μM), or a combination of both for 24 hours (Scale bar: 50 μm), LC3 fluorescent spots of different treatment groups was determined ($n = 5$); (g) Cells that had been stably transfected were treated to 10 μM of chloroquine (CQ) for a period of 24 hours. The levels of p62 and LC3 were then analyzed using western blotting. β-actin was used as loading control, the relative intensity of blots was determined using ImageJ software ($n = 3$). Data are means ± s.d. **, $p < .01$; ***, $p < .001$; ****, $p < .0001$. Statistical significance was determined by comparing the data with their corresponding control groups.

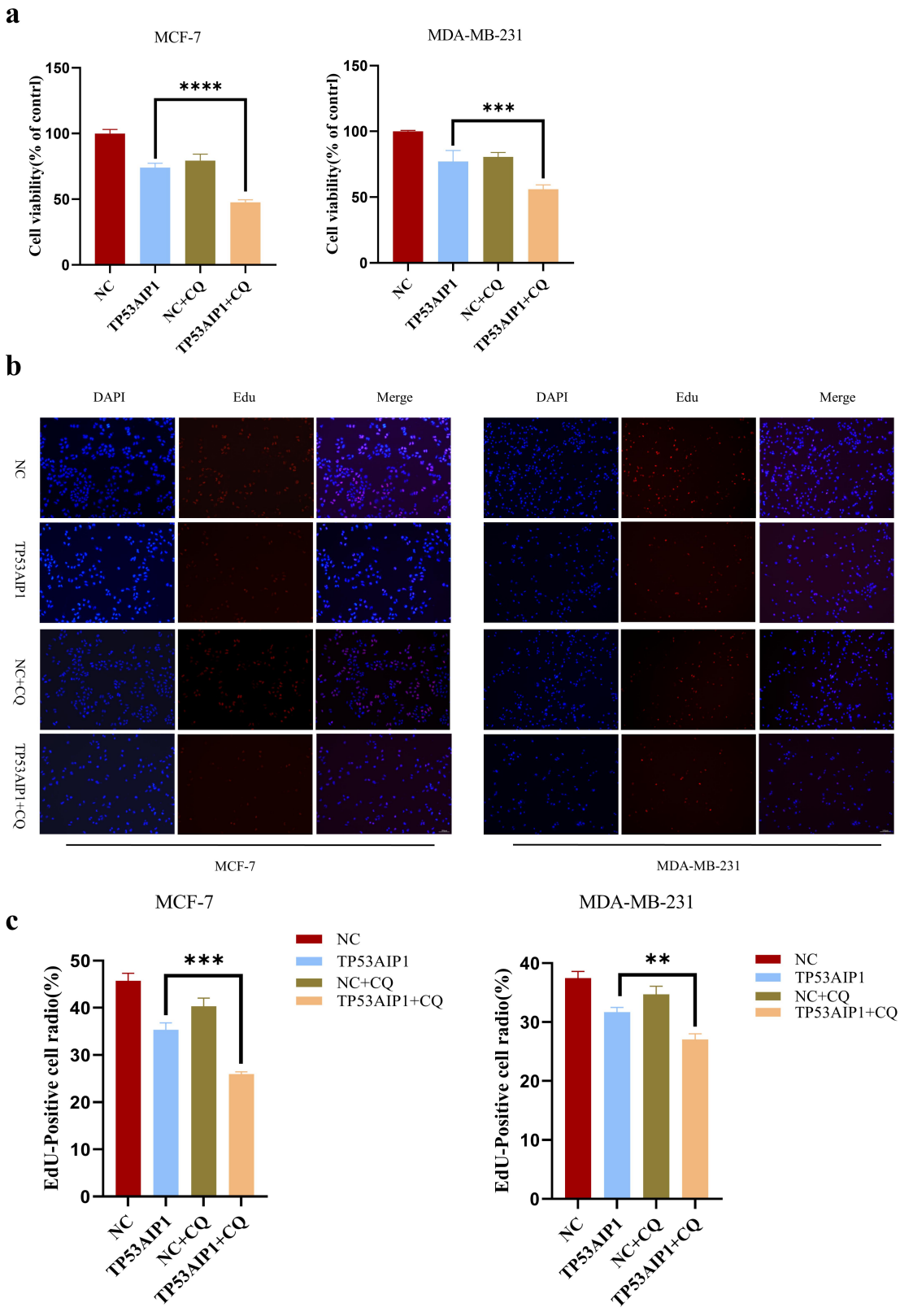

Figure 5. Autophagy attenuates TP53AIP1-induced anti-cancer effects. (a) Cell viability of MCF-7 and MDA-MB-231 cells treated with 10μM CQ was assessed using the CCK8 assay, both with and without transfection of pcDNA3.1-TP53AIP1 (n = 3); (b, c) The EdU assay was used to measure the proliferation of MCF-7 and MDA-MB-231 cells treated with 10μM CQ with or without TP53AIP1 present (Scale bar, 100μm). The percentage of EdU positive cells was quantified (n=3). Data are means±s.d. **, p<.01; ***, p<.001; ****, p<.0001. Statistical significance was determined by comparing the data with their corresponding control groups.

the findings from Immunofluorescence analysis and Western blot experiments supported the observation that the expression of LC3 in MCF-7 and MDA-MB-231 cells was noticeably reduced in the CA-AKT group when compared to

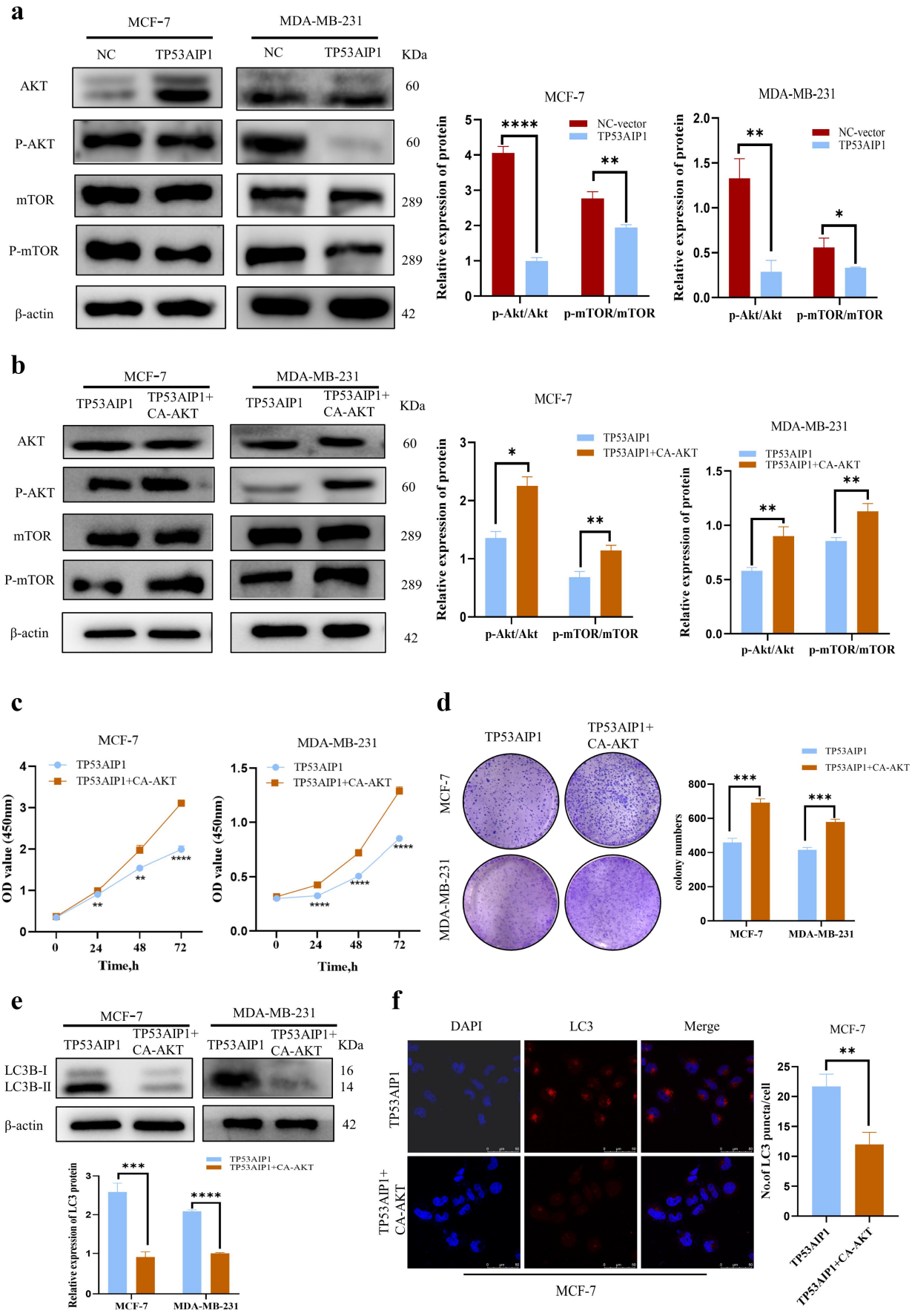

Figure 6. AKT/mTOR signaling pathway involves in TP53AIP1 modulated the proliferation and autophagy in MCF-7 and MDA-MB-231 cells. (a) MCF-7 and MDA-MB-231 cells were stable transfected with TP53AIP1. The western blot technique was used to analyze the total and phosphorylated levels of Akt and mTOR. β-actin was included as a loading control, the relative intensity of blots was determined using ImageJ software (n=3);(b) The stable transfected cells were transfected with CA-AKT for a period of 24 hours. The total and phosphorylation levels of Akt and mTOR were detected by western blot. β-actin was used as loading control, the relative intensity

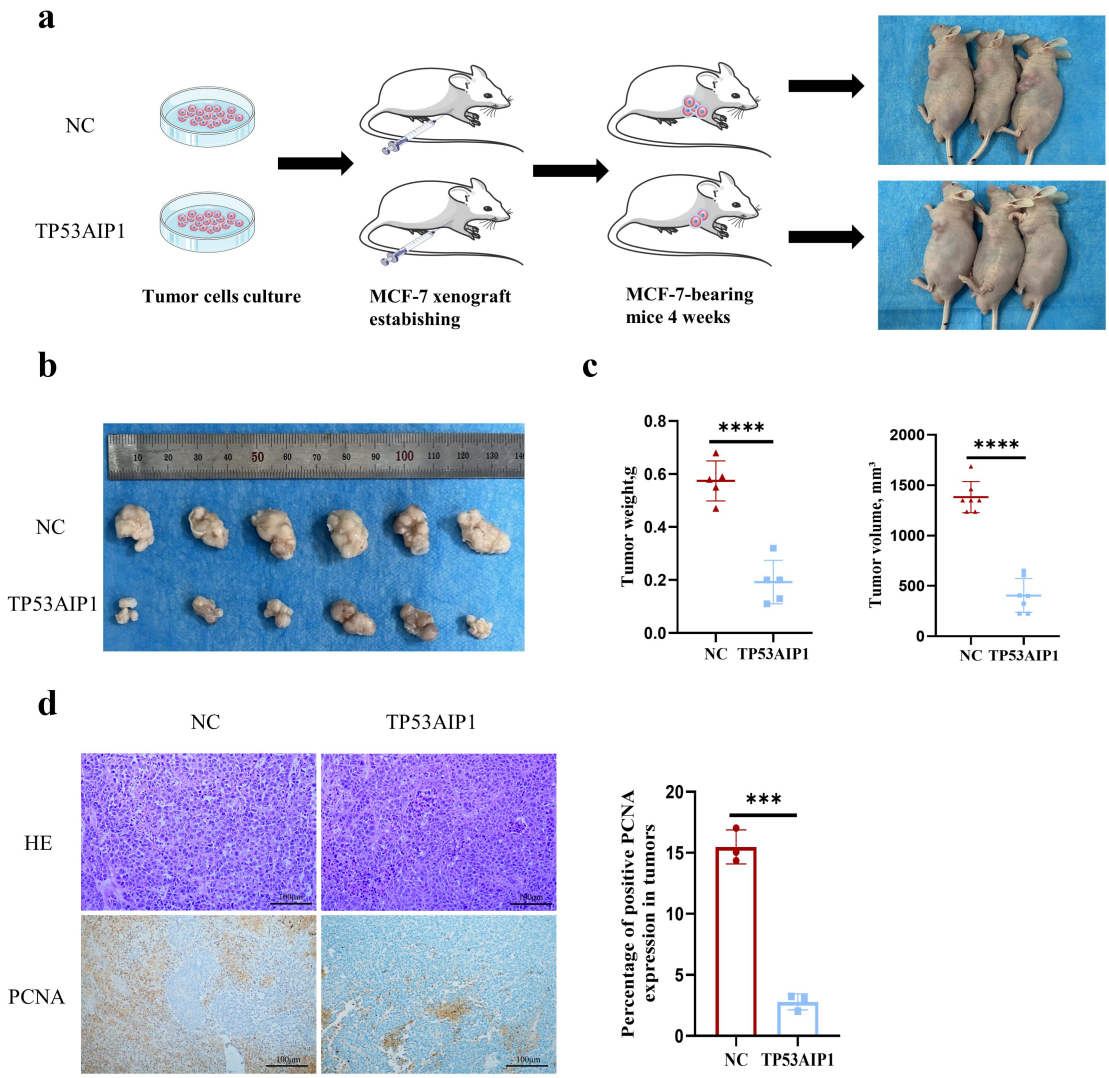

Figure 7. Over-expression of TP53AIP1 inhibits breast cancer cell growth in vivo. (a) Procedure and animal grouping in the in vivo experiment (n=40);(b, c) Tumors collected from nude mice, measurement results of Tumors'volume and weight mass on the 29th day (n=6);(d) Representative images of H&E staining, PCNA expression (Scale bar: 100μm). The relative expression of PCNA were determined by ImageJ software (n=3). Data are means±s.d. ***, p<.001; ****, p<.0001. Statistical significance compared with respective control groups.

the TP53AIP1 group (Figure 6e,f and S1d). In combination, these results provide confirmation that the AKT/mTOR signaling pathway might play a role in TP53AIP1's regulation of cell proliferation and autophagy in MCF-7 and MDA-MB-231 cells.

Over-expression of TP53AIP1 inhibits breast cancer cell growth in vivo

In order to evaluate the effect of TP53AIP1 on breast cancer in live organisms, a tumor xenograft model was established. MCF-7 cells were genetically modified to express TP53AIP1 and then injected into nude mice. The size and weight of the resulting tumors were measured. The introduction of TP53AIP1 led to a decrease in tumor volume and mass (Figure 7a–c). Further investigation was conducted through histological analysis using staining techniques such as H&E and PCNA. Comparing TP53AIP1 ×enografts to control xenografts revealed a more consistent and uniform morphology through HE staining. Immunohistochemical staining using a monoclonal antibody against PCNA demonstrated that TP53AIP1 expression inhibited the proliferation of breast cancer xenografts, as evidenced by a lower proliferative index compared to the control group (Figure 7d).

of blots was determined using ImageJ software (n=3);(c, d) CCK-8 and colony formation assay was executed to detect the proliferation of MCF-7 and MDA-MB-231 cells (n=3);(e) Transfected cells were stably transfected with CA-AKT for 24 hours, and western blot analysis was conducted to detect p62 and LC3. β-actin was used as loading control, the relative intensity of blots was determined using ImageJ software (n=3);(f) Immunofluorescence was used to examine the distribution of endogenous LC3B in MCF-7 cells following treatment with TP53AIP1 alone or in combination with CA-AKT for 24hours (Scale bar, 50μm). LC3 fluorescent spots of different treatment groups was determined (n=5). Data are means±s.d. *, p<.05; **, p<.01; ***, p<.001; ****, p<.0001. Statistical significance was determined by comparing the data with their corresponding control groups.

Discussion

BC is the most common malignancy among women. Breast cancer (BC) is a multifaceted and diverse illness.[19] Aberrations in gene expression are linked to BC pathogenesis. At present, the main treatment strategy for BC is surgery supplemented by chemotherapy.[20] The rising occurrence of BC and its resistance to drugs have intensified the challenges faced in clinical management. Therefore, the identification of potential targets in cancer progression may help develop promising diagnostic and therapeutic management strategies. Within this research, we focused on the involvement of TP53AIP1 in breast cancer and unraveled its potential role in signaling pathway.We found that TP53AIP1 was markedly reduced in BC, besides, patients diagnosed with breast cancer who had lower levels of TP53AIP1 exhibited decreased rates of survival compared to those with higher levels of TP53AIP1. The results obtained from our testing confirmed that the increased expression of TP53AIP1 hindered the growth, movement, invasion, and EMT of breast cancer cells. Additionally, it promoted protective mechanism of autophagy. Furthermore, experiments conducted on living organisms showed that the increased levels of TP53AIP1 inhibited tumor growth. Subsequently, further investigations revealed that TP53AIP1 controlled the progression of cancer and autophagy through the AKT/mTOR pathway.

The TP53AIP1 has potent pro-apoptotic effects on a range of p53-resistant tumor cells and can suppress tumor development and induce tumor cell apoptosis.[21] TP53AIP1 may become a new option in tumor gene therapy and may bring new survival hope to patients with p53-resistant tumors. Still, more investigation and study are required to fully understand the TP53AIP1 gene's potential for treating breast cancer.In this work, MCF-7 and MDA-MB-231 breast cancer cells were transiently transfected with the eukaryotic expression vector pcDNA3.1-TP53AIP1 using Lipofectamine 3000. In breast cancer cells, the TP53AIP1 gene was substantially expressed. Additionally, the effects on MCF-7 and MDA-MB-231 human breast cancer cells' migration, invasion, cell cycle, apoptosis, and proliferation were noted. Using the CCK-8 test, EDU assay, and plate cloning, TP53AIP1 suppressed the growth of MCF-7 and MDA-MB-231 cells. According to flow cytometry, TP53AIP1 promoted breast cancer cells to undergo apoptosis. Furthermore, the Transwell experiment demonstrated that TP53AIP1 might prevent breast cancer cells from migrating and invading.

EMT characteristics, including invasion, dissemination, and metastasis, are altered during cancer progression.[22,23] It has been previously reported that drug resistance in various types of cancer is closely related to the activation of EMT.[24,25] Numerous studies have investigated the effects of chemotherapy on breast cancer patients and have discovered a strong connection between an elevated gene profile associated with EMT and resistance to treatment. The increased expression level of these genes is partially attributed to the activation of EMT in cancer cells.[26] The markers E-cadherin, N-cadherin and Vimentin play a crucial role in the cancer metastasis process. The findings of this study demonstrate that TP53AIP1 has the capability to influence the expression levels of these markers.

Autophagy is an evolutionarily conserved cellular catabolic process that delivers intracellular components such as cytoplasmic macromolecules and organelles to lysosomes for degradation.[27,28] Autophagy has significant implications in human cancer, neurodegenerative disorders, and infectious diseases. Additionally, it plays a role in several physiological pathways, including cellular apoptosis.[29–31] Autophagy can be triggered as a response to unfavorable environmental conditions such as lack of nutrients, exposure to harmful substances, and various stress signals.[32–34] This process acts as a survival mechanism to uphold cellular functions. To detect autophagy,[35] commonly used methods including western blot analysis, immunofluorescence, and TEM were utilized. These techniques revealed that the autophagy level significantly increased when TP53AIP1 was over-expressed. Next, we investigated the role of TP53AIP1-induced autophagy on the proliferation of breast cancer cells. We observed that the use of autophagy inhibitors, such as CQ, greatly enhanced the inhibitory impact of TP53AIP1 on the proliferation of breast cancer cells. These findings suggest TP53AIP1-induced autophagy plays a cytoprotective role, autophagy attenuates TP53AIP1-induced anti-cancer effects. Additionally, our investigation revealed that TP53AIP1 induces autophagy through the AKT/mTOR pathway.

Studies have reported that the AKT/mTOR pathway plays a crucial role in essential cellular activities, such as cell proliferation, growth, and metabolism and found that it is commonly activated in human cancers.[35] BC tumorigenesis is strongly linked to the AKT/mTOR pathway.[36] mTOR, especially the mTOR complex 1, plays a crucial role in controlling autophagy and cell growth. Studies have indicated that the increased presence of TP53AIP1 prevents cell proliferation and disrupts the progression of the cell cycle in breast cancer cells. This action is accomplished through the regulation of the PI3K/AKT pathway, leading to an increase in apoptosis.[9] In addition, many of the efforts toward the inhibition of PI3K/AKT signaling involve its indirect inhibition by targeting mTOR. In the present study, it was demonstrated that alternations to AKT, an upstream modulator, were consistent with the variation tendency of mTOR. The over-expression of TP53AIP1 suppressed AKT/mTOR pathway activity in breast cancer cells, suggesting that TP53AIP1 may interact with this pathway. To confirm our findings, we conducted feedback experiments by introducing CA-AKT, a plasmid that continuously activates AKT, to MCF-7 and MDA-MB-231 cells. These experiments demonstrated that CA-AKT reversed the upregulating effects of TP53AIP1 on cell viability and autophagy levels in breast cancer cells. Therefore, we concluded that TP53AIP1-induced autophagy is mediated through the AKT/mTOR signaling pathway.

In this study, we revealed for the first time the pro-autophagy role of TP53AIP1 in breast cancer. According to our research, the expression of TP53AIP1 is at a low level in breast cancer and is linked to a negative prognosis. Furthermore, we discovered a new molecular interaction wherein increasing the expression of TP53AIP1 hindered the progression of Breast Cancer by deactivating the AKT/mTOR pathway.

Materials and methods

Cells lines, tumor samples and normal tissues

MCF-7 and MDA-MB-231 cells, were purchased from American Type Culture Collection (ATCC, Manassas, USA) and cultured in Dulbecco's modified Eagle's medium (Hyclone™; Thermo Fisher Scientific, Waltham, USA) with 10% fetal bovine serum (FBS, Gibco, Germany), 100 U/mL penicillin (Gibco, Germany) and 100 mg/mL streptomycin (Gibco, Germany). MCF-7 and MDA-MB-231 cells were cultured in an incubator at 37°C with 5% CO2 at humid temperature. The Second Affiliated Hospital of Chongqing Medical University provided all the breast cancer clinical samples, which consisted of primary tumors and normal breast tissue. This study was granted by the Ethics Committee of Chongqing Medical University. Informed consent was obtained from all individual participants included in the study.

Bioinformatics analysis

The Cancer Genome Atlas (TCGA) data portal (https://tcga-data.nci.nih.gov/tcga/) was used to detect TP53AIP1 mRNA expression between breast tumor tissues and normal tissues. GEPIA online database (http://gepia2.cancer-pku.cn/#index) was used to analyze survival analysis associated with TP53AIP1 in breast cancer. Kaplan – Meier and logarithmic rank analyses were used to evaluate prognostic factors.

Cell transfection

The TP53AIP1 group, which consisted of the pcDNA3.1-TP53AIP1 plasmid obtained from GenePharma in Shanghai, China, and the negative control [NC]-vector group, consisting of the empty vector pcDNA3.1 also obtained from GenePharma, were introduced into MCF-7 and MDA-MB-231 cells using Lipofectamine 3000, a transfection reagent from Invitrogen in Carlsbad, USA. The transfection efficiency was determined by examining the levels of TP53AIP1 through RT-qPCR and Western Blot analysis.

Colony formation assay

Colony formation in MCF-7 and MDA-MB-231 cells were assessed through a colony formation assay. In this experiment, MCF-7 and MDA-MB-231 cells were transfected with pcDNA3.1-TP53AIP1 and the empty vector pcDNA3.1, respectively, with 500 cells per well. These cells were then plated in 6-well plates and cultured in Dulbecco's modified Eagle's medium at 37°C in a 5% CO2 incubator for a period of two weeks. Subsequently, the cells were washed with PBS three times, fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet dye from Beyotime, based in Shanghai, China. Finally, the number of colonies was determined using a light microscope.

Cell viability assay

To evaluate the impact of TP53AIP1 over-expression on the survival of breast cancer cells, the Cell Counting Kit-8 (CCK-8) assay acquired from MedChemExpress in Monmouth Junction, USA was utilized. In this experiment, cells from both the NC-vector and TP53AIP1 groups were cultured in 96-well plates, with a seeding density of 2,000 cells per well, after incubation at 37°C for different time intervals (0, 24, 48, and 72 hours). Following this, 10 μL of the CCK-8 reagent was added to the various cell samples and incubated for a duration of 1 hour. Lastly, the optical density values were measured at 450 nm using a microplate reader.

Wound healing assay

The MCF-7 and MDA-MB-231 cells, which had been transfected, were placed in a 6-well plate at a density of 1×10^5 cells/well. When the cells upon reaching 100% confluence, a sterile pipette tip was used to create a vertical scratch across the cell monolayer. After removing any remaining debris, the cells were cultured in medium without serum for 24 hours. Finally, the scratch wounds were examined and photographed using an inverted microscope.

Transwell assay for cell invasion

To evaluate the invasive properties of MCF-7 and MDA-MB -231 cells, a transwell chamber (Corning, Corning, NY, USA) was used. The transfected cells were suspended and placed in the upper chamber coated with Matrigel (Millipore, Billerica, USA) to conduct the invasion assay. Subsequently, 600 μL of complete medium was added to the lower chamber. After incubation at 37°C for 48 hours, the cells that migrated to the lower surface of the membrane were fixed with 4% paraformaldehyde for 10 minutes and stained with a 0.1% crystal violet solution for 10 minutes. Finally, the invaded cells were observed and captured using an inverted microscope.

EdU assay in vitro

To detect EdU, the BeyoClickTM EdU Cell Proliferation Kit with Alexa Fluor 568 (Beyotime Biotechnology,Haimen, China) was used as instructed by the manufacturer. In this experiment, breast cancer cells were cultured in 96-well plates, with a seeding density of 4,000 cells per well, after incubation at 37°C for 72 hours. Next, breast cancer cells were exposed to 10 μM EdU for 2 hours at 37°C. Subsequently, the cells were fixed using 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 15 minutes at room temperature. After removing the fixatives, the cells were washed with PBS containing 1% BSA. Lastly, the cells were incubated in Click Additive Solution, protected from light, for 30 minutes and then stained with Hoechst to label the nucleus. Microscopic images were captured to observe the EdU detection samples. The proliferation of cells was further assessed by calculating the ratio of EdU-positive cells to the overall cell count.

Flow cytometry (FCM)

To analyze the cell cycle, cells that had been stably transfected were collected using trypsin and then washed twice with PBS.

After that, they were suspended in ice-cold 75% ethanol and left to be fixed overnight. The following day, RNA enzyme was added to the cells and they were incubated at 37°C for 30 minutes. Then, they were stained with propidium iodide (PI) in the dark for 30 minutes to determine their cell cycle distribution. The Annexin V-FITC apoptosis detection kit (Thermo Fisher Scientific) was used to assess the apoptosis of MCF-7 and MDA-MB-231 cells, following the manufacturer's instructions.

Immunofluorescence

Place coverslips in 24-well plates, breast cancer cells were inoculated in 24-well plates at 1×10^4 cells per well. After the cells were incubated for 24 hours at 37°C, cells grown on coverslips were transfected with TP53AIP1 for 24 hours. After that, they were fixed with 4% paraformaldehyde (Sigma) for 30 minutes and washed three times with PBS. Subsequently, the fixed cells were permeabilized using 0.5% Triton 100 for 20 minutes, followed by blocking with 1% BSA for 2 hours at 37°C. To stain the cells, primary antibodies were incubated with them at 4°C for 12 hours, followed by a 1-hour incubation with secondary antibodies (Thermo Scientific) at 37°C. For nucleus staining, DAPI was applied for 10 minutes. For autophagy flux studies, cells were initially transfected with GFP-RFP-LC3 for 24 hours and then underwent an additional transfection with TP53AIP1 for another 24 hours. The images were captured using a Leica confocal microscopy.

Animal experiments

Fourty BALB/c nude mice (4 ~ 6 weeks old, weighing 18 ~ 22 g) were sourced from Chongqing Ensiweier Biotechnology Co. Ltd. MCF-7 cells (5×10^6) were transfected with pcDNA3.1-TP53AIP1 or NC and then injected into individual mice with no immune response. The measurements of the largest and smallest dimensions of the tumors were taken using vernier calipers, and the volume of the tumors (in cubic millimeters) was calculated as follows: volume = length multiplied by the square of width, and then the product is multiplied by 0.52. On the 29th day after injection, all the mice were humanely killed, and their tumors were gathered and weighed. The experimental procedures were approved by the Research Ethics Committee of Chongqing Medical University (No. IACUC-CQMU-2023–0460).

H&E staining

After removing the wax, the tissue sections were stained using hematoxylin (Beyotime), followed by differentiation using a mixture of 1% hydrochloric acid and alcohol for a duration of 3 seconds. Next, they were stained with 1% eosin for 1 minute. Afterward, the sections underwent dehydration, clearing, sealing, and were observed under an optical microscope.

Immunohistochemistry

First, the paraffin sections underwent a treatment to eliminate wax and were subsequently rinsed twice in absolute ethanol. The protocol provided by ZSGB-BIO, PV-9000 was used for the process of hydration and antigen retrieval. Next, the slides were exposed to reagent 1 at room temperature for a duration of 10 minutes in order to deactivate the endogenous peroxidase. Subsequently, they were washed three times in PBS for 3 minutes each. Following this, the sections were incubated in blocking solution (Reagent 2) for 15 minutes, and then left overnight at a temperature of 4°C with the PCNA antibody inside an immunohistochemical wet box.

On the next day, the sections were washed three times with PBS. Subsequently, they were incubated with reagent 3 at a temperature of 37°C for a period of 30 minutes inside a wet box. Following this, another round of washing with PBS was performed, and the sections were then incubated with reagent 4 at 37°C for 30 minutes. After being washed three more times with PBS, immunolabeling was carried out using 1× diaminobenzidine (DAB, ZSGB-BIO). Finally, the sections were counterstained with hematoxylin (BL702B, Biosharp, China).

Transmission electron microscopy

The transmission electron microscopy analysis was used to observe the autophagic vesicles. To prepare the samples, MCF-7 and MDA-MB-231 cells were fixed in glutaraldehyde from Sigma and then sliced into ultrathin sections using a sorvall MT5000 microtome after being transfected with pcDNA3.1-TP53AIP1 for a period of 24 hours. The sections were later stained with lead citrate and/or 1% uranyl acetate and examined using Philips EM420 electron microscopy.

RNA extraction and quantitative real time-PCR

The Vazyme reagent (Vazyme, Nanjing, China) was used to isolate total RNA from tissues and cells, and its quality was assessed using a microplate reader. The Reverse Transcription PrimeScript 1st Strand cDNA Synthesis kit (TaKaRa, Otsu, Japan) was used for reverse transcription. For quantification, RT-qPCR was conducted following the manufacturer's instructions using the SYBR PremixEx TaqTM quantitative PCR reagents (TaKaRa). The levels of GAPDH, which served as an internal control for target genes, were measured, and fold-changes were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primer sequences for the RT-qPCR analysis can be found in Supplementary Table S1A.

Western blot analysis

The cells were lysed on ice for 30 minutes using RIPA lysis buffer (Boster, Wuhan, China). The proteins were then quantified using BCA (Pierce, Boston, USA). After that, the proteins were separated using SDS-PAGE and

transferred to PVDF membranes. The membranes were then blocked with 5% nonfat milk and incubated with primary antibodies overnight at 4°C. Following this, the membranes were incubated with secondary antibodies at room temperature for 2 hours. The enhanced chemiluminescence detection reagents (biosharp) were used to visualize the proteins, and the Bio-Rad ChemiDoc XRS densitometry was used to analyze them. To ensure that the proteins were loaded equally, β-actin was used as a loading control. The list of antibodies used can be found in Supplementary Table S1B.

Statistical analysis

Data were expressed as means ± s.d. All experiments were performed at least three times. Statistical analysis was performed with GraphPad Prism 8.0 statistical software. The statistical differences between groups were determined using the two-tailed Student's t-test. Significance was indicated in the following manner: *, $p < .05$, **, $p < .01$, ***, $p < .001$, ****, $p < .0001$. Statistical significance compared with respective control groups.

Abbreviations

BC	Breast Cancer
TP53AIP1	Tumor protein p53-regulated apoptosis-inducing protein 1
TCGA	The Cancer Genome Atlas
NC	negative control
EdU	5-ethynyl-2′ -deoxyuridine
EMT	Epithelial mesenchymal transition
CQ	Chloroquine
LC3I	The nonlipidated form of LC3B
LC3II	The lipidated form of LC3B
p62	SQSTM1/p62
qRT-PCR	quantitative reverse transcription-PCR
CA-AKT	Continuously activating AKT plasmid

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71(3):209–249. doi:10.3322/caac.21660.
2. Sáez-Freire MDM, Blanco-Gómez A, Castillo-Lluva S, Gómez-Vecino A, Galvis-Jiménez JM, Martín-Seisdedos C, Isidoro-García M, Hontecillas-Prieto L, García-Cenador MB, García-Criado FJ, et al. The biological age linked to oxidative stress modifies breast cancer aggressiveness. Free Radic Biol Med. 2018;120:133–146. doi:10.1016/j.freeradbiomed.2018.03.012.
3. Fahad Ullah M. Breast cancer: current perspectives on the disease status. Adv Exp Med Biol. 2019;1152:51–64.
4. Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T, Nishimori H, Tamai K, Tokino T, Nakamura Y, et al. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell. 2000;102(6):849–862. doi:10.1016/S0092-8674(00)00073-8.
5. Matsuda K, Yoshida K, Taya Y, Nakamura K, Nakamura Y, Arakawa H. p53AIP1 regulates the mitochondrial apoptotic pathway. Cancer Res. 2002;62(10):2883–2889.
6. Jiang Y, Chen H, Jia H, Xu Y, Liu G, Wang Y, Yang X, Lu Y. Adenovirus Ad-p53AIP1-mediated gene therapy and its regulation of p53-MDM2 interactions. Exp Ther Med. 2010;1(2):363–368. doi:10.3892/etm_00000057.
7. Luedeke M, Coinac I, Linnert CM, Bogdanova N, Rinckleb AE, Schrader M, Vogel W, Hoegel J, Meyer A, Dörk T, et al. Prostate cancer risk is not altered by TP53AIP1 germline mutations in a German case-control series. PLOS ONE. 2012;7(3):e34128. doi:10.1371/journal.pone.0034128.
8. Yamashita SI, Masuda Y, Yoshida N, Matsuzaki H, Kurizaki T, Haga Y, Ikei S, Miyawaki M, Kawano Y, Chujyo M, et al. p53AIP1 expression can be a prognostic marker in non-small cell lung cancer. Clin Oncol (R Coll Radiol). 2008;20(2):148–151. doi:10.1016/j.clon.2007.08.006.
9. Liang Y, Wang S, Liu J. Overexpression of tumor protein p53-regulated apoptosis-inducing protein 1 regulates proliferation and apoptosis of breast cancer cells through the PI3K/Akt pathway. J Breast Cancer. 2019;22(2):172–184. doi:10.4048/jbc.2019.22.e21.
10. Folkerts H, Hilgendorf S, Vellenga E, Bremer E, Wiersma VR. The multifaceted role of autophagy in cancer and the microenvironment. Med Res Rev. 2019;39(2):517–560. doi:10.1002/med.21531.
11. Kondo Y, Kanzawa T, Sawaya R, Kondo S. The role of autophagy in cancer development and response to therapy. Nat Rev Cancer. 2005;5(9):726–734. doi:10.1038/nrc1692.
12. Wirawan E, Vanden Berghe T, Lippens S, Agostinis P, Vandenabeele P. Autophagy: for better or for worse. Cell Res. 2012;22(1):43–61. doi:10.1038/cr.2011.152.
13. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol. 2007;8(9):741–752. doi:10.1038/nrm2239.
14. Eisenberg-Lerner A, Kimchi A. The paradox of autophagy and its implication in cancer etiology and therapy. Apoptosis. 2009;14(4):376–391. doi:10.1007/s10495-008-0307-5.
15. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, Adachi H, Adams CM, Adams PD, Adeli K,

et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy. 2016;12(1):1–222. doi:10.1080/15548627.2015.1100356.
16. Levy JMM, Towers CG, Thorburn A. Targeting autophagy in cancer. Nat Rev Cancer. 2017;17(9):528–542. doi:10.1038/nrc.2017.53.
17. Rouschop KMA, Wouters BG. Regulation of autophagy through multiple independent hypoxic signaling pathways. Curr Mol Med. 2009;9(4):417–424. doi:10.2174/156652409788167131.
18. Chen N, Karantza-Wadsworth V. Role and regulation of autophagy in cancer. Biochim Biophys Acta. 2009;1793(9):1516–1523. doi:10.1016/j.bbamcr.2008.12.013.
19. Nagini S. Breast cancer: current molecular therapeutic targets and new players. Anticancer Agents Med Chem. 2017;17(2):152–163. doi:10.2174/1871520616666160502122724.
20. Jatoi I, Kemp Z. Surgery for breast cancer prevention. JAMA. 2021;325(17):1804. doi:10.1001/jama.2021.1647.
21. Yoshida K, Monden M, Nakamura Y, Arakawa H. Adenovirus-mediated p53AIP1 gene transfer as a new strategy for treatment of p53-resistant tumors. Cancer Sci. 2004;95(1):91–97. doi:10.1111/j.1349-7006.2004.tb03176.x.
22. Hugo H, Ackland ML, Blick T, Lawrence MG, Clements JA, Williams ED, Thompson EW. Epithelial—mesenchymal and mesenchymal—epithelial transitions in carcinoma progression. J Cell Physiol. 2007;213(2):374–383. doi:10.1002/jcp.21223.
23. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. Cell. 2011;147(2):275–292. doi:10.1016/j.cell.2011.09.024.
24. Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene. 2010;29(34):4741–4751. doi:10.1038/onc.2010.215.
25. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm [J]. Nat Rev Cancer. 2013;13(10):714–726. doi:10.1038/nrc3599.
26. Farmer P, Bonnefoi H, Anderle P, Cameron D, Wirapati P, Becette V, André S, Piccart M, Campone M, Brain E, et al. A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer. Nat Med. 2009;15(1):68–74. doi:10.1038/nm.1908.
27. Macintosh RL, Ryan KM. Autophagy in tumour cell death. Semin Cancer Biol. 2013;23(5):344–351. doi:10.1016/j.semcancer.2013.05.006.
28. Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. Science. 2000;290(5497):1717–1721. doi:10.1126/science.290.5497.1717.
29. Yang Y, Klionsky DJ. Autophagy and disease: unanswered questions. Cell Death Differ. 2020;27(3):858–871. doi:10.1038/s41418-019-0480-9.
30. Klionsky DJ, Petroni G, Amaravadi RK, Baehrecke EH, Ballabio A, Boya P, Bravo-San Pedro JM, Cadwell K, Cecconi F, Choi AMK, et al. Autophagy in major human diseases. Embo J. 2021;40(19):e108863. doi:10.15252/embj.2021108863.
31. Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, Baehrecke EH, Lenardo MJ. Regulation of an ATG7 - beclin 1 program of autophagic cell death by caspase-8. Science. 2004;304(5676):1500–1502. doi:10.1126/science.1096645.
32. Kim I, Lemasters JJ. Mitochondrial degradation by autophagy (mitophagy) in GFP-LC3 transgenic hepatocytes during nutrient deprivation. Am J Physiol Cell Physiol. 2011;300(2):C308–317. doi:10.1152/ajpcell.00056.2010.
33. Lee J-A. Neuronal autophagy: a housekeeper or a fighter in neuronal cell survival? Exp Neurobiol. 2012;21(1):1–8. doi:10.5607/en.2012.21.1.1.
34. Shen H-M, Codogno P. Autophagy is a survival force via suppression of necrotic cell death. Exp Cell Res. 2012;318(11):1304–1308. doi:10.1016/j.yexcr.2012.02.006.
35. Klionsky DJ, Abdel-Aziz AK, Abdelfatah S, Abdellatif M, Abdoli A, Abel S, Abeliovich H, Abildgaard MH, Abudu YP, Acevedo-Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (4th edition) 1. Autophagy. 2021;17(1):1–382. doi:10.1080/15548627.2020.1797280.
36. Sharma VR, Gupta GK, Sharma AK, Batra N, Sharma DK, Joshi A, Sharma AK. PI3K/Akt/mTOR intracellular pathway and breast cancer: factors, mechanism and regulation. Curr Pharm Des. 2017;23(11):1633–1638. doi:10.2174/1381612823666161116125218.