Article

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AEBP1 Contributes to Breast Cancer Progression by Facilitating Cell Proliferation, Migration, Invasion, and Blocking Apoptosis

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Background: The aberrant expression of adipocyte enhancer binding protein 1 (AEBP1) has been observed in many cancers and it seems to be involved in the tumorigenesis, progression, and metastasis in numerous tumor types. However, the contribution of AEBP1 in breast cancer (BCa) remains inexplicable.

Methods: Information related to the diagnostic significance and expression of AEBP1 in BCa was obtained from the public dataset Kaplan–Meier Plotter (http://kmplot.com/analysis/) and the dataset UALCAN (https://ualcan.path.uab.edu/index.html). The MTT (methyl thiazolyl tetrazolium) assay, colony formation assay, Transwell® assay, and FACS (fluorescence-activated cell sorting) assay were used to detect the proliferation, invasive and apoptotic ability of cells before and after treatment. In addition, we constructed an AEBP1 overexpression vector and silenced AEBP1, combined with Real-Time Quantitative Reverse Transcription PCR (qRT-PCR), western blot, immunohistochemistry and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) assay to investigate the prognostic significance, biological functions and potential mechanisms of AEBP1 in BCa.

Results: Higher expression of AEBP1 mRNA (message RNA) was observed in BCa patients with later-stage, who obtained poorer overall survival. Meanwhile, compared with adjacent noncancerous tissues, AEBP1 protein expression was dramatically upregulated in the BCa ones. Furthermore, overexpressed AEBP1 enhanced cell proliferation, migration, invasion, and blocked cell apoptosis in BCa cells. Moreover, the research certificated that AEBP1 upregulated the expression of MMP (matrix metalloproteinase)-2, 9, vimentin, N-cadherin (neural-cadherin), phosphorylation of ERK (extracellular signal-regulated kinase), Smad2/3 (Abbreviated from Sma for nematode and Mad for Drosophila) and AKT (V-akt murine thymoma viral oncogene homolog), while down-regulated the expression of E-cadherin (epithelial cadherin) and PTEN (phosphatase and tensin homolog deleted on chromosome 10). To inhibit cell apoptosis, enforced expression of AEBP1 effectively blocked the cleavage of caspase 9 and p53 (protein 53) and promoted the expression of anti-apoptotic protein Bcl-2 (B-cell lymphoma-2). Finally, AEBP1 accelerated subcutaneously transplanted tumor growth in nude mice by increasing the expression of the cell proliferation biomarker ki67, the phosphorylation of AKT, and blocked apoptosis *in vivo*.

Conclusions: In summary, these data suggested the important role of AEBP1 in the BCa progression, which could be used as a potential biomarker for prognostic hallmark and a novel therapeutic strategy.

Keywords: breast cancer (BCa); adipocyte enhancer binding protein 1 (AEBP1); cancer progression; epithelial-mesenchymal transition (EMT); apoptosis; signaling pathways

Introduction

After outnumbering lung cancer, breast cancer (BCa) becomes the most diagnosed cancer in females worldwide [1]. In China, BCa also has a higher rate of incidence and mortality [2]. Due to its rapid progression, the treatments are still unsatisfactory. Therefore, it is urgent to excavate the underlying mechanisms of BCa progression and discover novel and effective therapeutic strategies to combat the disease.

A transcriptional suppresser, the adipocyte enhancer-binding protein 1 (AEBP1), was found to promote cell proliferation and diminishes differentiation of adipocytes by binding to adipocyte enhancer 1 to manage the activity of mitogen-activated protein kinase (MAPK) [3]. Additionally, some studies have elucidated that AEBP1 expression

was highly induced in macrophages to stimulate inflammatory responsiveness by generating multitudinous inflammatory mediators, including tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) [4–6]. Moreover, AEBP1 participates in the progression of several kinds of diseases, for instance, Alzheimer's Disease, Ehlers-Danlos syndrome and abdominal aortic aneurysm [7–10]. Similarly, previous studies have found that AEBP1 may play key roles in tumorigenesis and the development of multiple cancer types, such as colon adenocarcinoma, glioma, melanoma, gastric cancer, and ovarian cancer [11–15]. However, the biological function and underlying mechanisms of AEBP1 on BCa progression remain to be further elucidated.

In this study, data from the Kaplan–Meier Plotter database (http://kmplot.com/analysis/) showed that AEBP1 mRNA expression levels are negatively correlated with the overall survival of BCa sufferers. The UALCAN database (https://ualcan.path.uab.edu/index.html) showed higher AEBP1 mRNA expressed in later-stage in BCa patients. Meanwhile, we found that AEBP1 was dramatically up-regulated in BCa. Furthermore, the AEBP1 overexpression enhanced BCa cell proliferation, colony formation, and cell motility and blocked cell apoptosis. Moreover, we illustrated that AEBP1 functions as a regulator of BCa progression by activating various signal pathways including AKT (V-akt murine thymoma viral oncogene homolog), NF-κB (nuclear factor kappa-B), TGF-β (transforming growth factor-β), and MAPK/ERK (extracellular signal-regulated kinase).

Materials and Methods

Dataset Analysis

In the present research, the prognostic significance of AEBP1 in BCa was analyzed using the public dataset Kaplan–Meier Plotter (http://kmplot.com/analysis/). The expression profiling of AEBP1 in BCa with different stages was obtained from the dataset UALCAN (http://ualcan.path.uab.edu/).

Immunohistochemistry (IHC) of Clinical Specimens

Sixteen pairs of BCa biopsy specimens were obtained at the Taizhou Central Hospital. For immunohistochemistry, after tumor sections were deparaffinized and rehydrated, 3% H_2O_2 was used to quench the activity of endogenous peroxidase. A 95 °C steamer was used for heat-induced antigen retrieval (HIAR) in 0.01 M and pH 6.0 citrate buffer. The primary antibody (AEBP1, Novus, Port Orchard, CO, USA, NO. 912-1013) was diluted at 1:100 in phosphate-buffered saline (PBS) and incubated at 4 °C overnight. Then, the HRP (horseradish peroxidase)-labeled secondary antibodies were incubated for 30 min at room temperature, following incubation of peroxidase substrate for 10 min. The sections were counterstained with haematoxylin, followed by dehydration, the addition of a coverslip and visualized.

Cell Culture

The human BCa cell lines including MDA-MB-231 (ZK1423(XR)), MCF-7 (ZK0333(XR)), MDA-MB-468 (ZK0340(XR)), MDA-MB-453 (ZKCC-X1637), SK-BR-3 (ZKCC-X1639), and T-47D (ZKCC-X1639), as well as normal human mammary epithelial cell line MCF-10A, were obtained from ATCC. The cell lines above were all cultured in a 5% (v/v) CO_2-included humidified incubator at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA, 02-5062EJ) supplemented with 10% FBS (fatal bovine serun), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Invitrogen, Waltham, CA, USA, NO. 15140122). All the cell lines used tested for mycoplasma.

MTT Assay

Cell proliferation of BCa was detected using MTT assays. In brief, 5000 cells were cultured into each well of 96-well plates and allowed to incubate for 24, 48, or 72 h. After incubation at each point, the MTT (Sigma-Aldrich, St. Louis, MO, USA, NO. M2128) was added to the cells and incubated for 3 h at 37 °C. The purple crystal was absolutely dissolved by DMSO by shaking. The absorbance at 570 nm was read using a microplate reader (Molecular Device, Thermo Scientific, Waltham, MA, USA, NO. 51119570).

Western Blotting

Cells were lysed with RIPA (Radio Immunoprecipitation Assay) lysis buffer, including proteinase inhibitors (Bimake, Houston, TX, USA, NO. B14002). A BCA assay was performed to determine the concentrations of protein. Proteins were separated by electrophoresis on SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels (8%, 10%, or 15%), shifted to PVDF (polyvinylidene fluoride) membranes, and the membranes were incubated for 2 h with 5% skimmed milk at room temperature. After the membranes were incubated with the primary antibodies overnight at 4 °C, the corresponding HRP (horseradish peroxidase)-conjugated secondary antibody was added and incubated for another 2 h. The signals were obtained subsequent to the reaction with the HRP substrate. The information for all antibodies is shown in Table 1.

Plasmid Construction and Transfection

The cDNA (complementary DNA) sequence of AEBP1 was synthesized and subcloned into the pCDH vector (Invitrogen, Waltham, CA, USA, JLC5460) to yield pCDH-AEBP1, the overexpression plasmid. The empty pCDH vector was used as a control.

The plasmid and siRNA were allowed to form complexes with Lipofectamine^{TM} 2000 Transfection Reagent (Invitrogen, Waltham, CA, USA, NO. 11668-019) in a serum-free medium for 20 min at 37 °C. The transfection

Table 1. Primary antibodies used in this study.

Name	Brand	NO.	Dilution rate
β-actin	CST	4970	1:5000
AEBP1	Novus	912-1013	1:1000
Caspase 9	CST	9502	1:1000
p53	CST	2524	1:1000
Bcl-2	CST	4223	1:1000
E-cadherin	Proteitech	20874-1-AP	1:1000
N-cadherin	CST	13116	1:1000
Vimentin	CST	5741	1:1000
MMP9	Abcam	Ab76003	1:1000
p-Smad2	CST	3108	1:1000
p-Smad3	CST	9520	1:1000
Smad2/3	CST	8685	1:1000
PTEN	CST	9551	1:1000
p-AKT	CST	4060	1:1000
AKT	CST	4685	1:1000
p-NF-κB	CST	3033	1:1000
NF-κB	CST	8242	1:1000
p-ERK	CST	4370	1:1000
ERK	CST	4695	1:1000

was performed by adding the complex into the cell culture drop by drop gently. Puromycin was used to screen AEBP1 stably expressed BCa cells. The siRNA against AEBP1 and negative control (NC) were obtained from GemmaPharma (Shanghai, China). The sequence for the AEBP-siRNA was: 5′-GCG AGC GGC UAG UAU CCU A-3′.

RNA Extraction and qRT-PCR

TRIzol (Invitrogen, Waltham, CA, USA, NO. 15596026) was used to extract total RNA from the cell samples. Then, cDNA synthesis was performed by using a cDNA Synthesis Kit (Vazyme Biotech, Nanjing, China, NO. R111-01). Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) was implemented using SYBR Real-time PCR I kit (Takara, Kyoto, Japan, NO. RR420L) on the machine of ABI-7500. The qRT-PCR reaction system is shown in Table 2.

Table 2. Information of the qRT-PCR reaction system.

Reagents	Volume
cDNA	8 μL
SYBR Green I	10 μL
Forward primer	1 μL
Reverse primer	1 μL

The thermal cycle conditions used are the following: 5 min at 95 °C followed by 40 cycles at 95 °C for 5 s and 52 °C for 34 s. The relative mRNA expression was calculated by the relative Standard Curve Method ($2^{-\Delta\Delta Ct}$) using β-actin as the reference. The sequence of primers used in this study were the following: AEBP1 forward, 5′-GAG CAA CCC ACA CTG GAC T-3′; AEBP1 reverse, 5′-CGG AGG AGG CCC AAA GTA ATA G-3′; β-actin forward, 5′-ACA CCC CAG CCA TGT ACG TT-3′; β-actin reverse: 5′-TCA CCG GAG TCC ATC ACG AT-3′.

Colony Formation Assay

Briefly, 500 cells of BCa were transfected with the pCDH and pCDH-AEBP1 and were cultured in a 6-well plate individually for 14 days. After being washed with PBS, the colonies were fixed with methanol for 20 min, at room temperature. Then, staining was performed using a crystal violet (Aladdin, Beijing, China, NO. C110703) solution. Finally, a microscope was employed to count the number of colonies (>50 cells).

Transwell® Assay

The Transwell® (Corning, Corning, NY, USA, NO. 3422) coated with or without Matrigel (BD, New York, NJ, USA, NO. 356234) was used to verify the migration or invasion of BCa cells transfected with the pCDH and pCDH-AEBP1 overexpression plasmids. The cells were placed into the upper chambers with serum-free DMEM medium. DMEM containing 10% FBS was then added into the lower ones used as an enticement. After 48 h incubation, a cotton swap was used to remove the upper chamber cells. Cells were stained with 0.5% crystal violet and the cells were counted under a microscope (Olympus Corp., Tokyo, Japan, CKX53).

FACS Assay for Apoptosis Detection

FITC (fluorescein isothiocyanate)-labeled annexin-V and propidium iodide (PI) double-staining experiments were performed to examine apoptosis via flow cytometry. Briefly, after BCa cells were transfected with the pCDH and pCDH-AEBP1 plasmids for 48 h, the cells were stained with Annexin V-FITC (Sigma-Aldrich, MO, USA, NO. APOAF) and PI (Sigma-Aldrich, St. Louis, MO, USA, NO. P4170) at room temperature and in dark for 20 min. Flow cytometry (BD, New York, NJ, USA, FACSCanto II) was used to detect the fluorescence signal in the cells.

Xenograft Mouse Model

Four weeks old, 20 g weight Balb/c female nude mice were obtained from Vital River Laboratories (China). AEBP1 stably expressed MDA-MB-468 cells-derived xenografts were established by subcutaneously injecting 1×10^7 cells into the right flank. Tumor size was obtained by a micrometer caliper. Tumor volume (mm^3) was calculated as $V = (a \times b^2)/2$. After the largest tumors reached a volume of about 100 mm^3, tumor size was measured every five days. Twenty-five days after inoculation, mice were killed and tumors were excised and weighed.

IHC and TUNEL Assays

For immunohistochemistry (IHC), a 95 °C steamer was used for antigen retrieval. The sections were deparaffinized, rehydrated, and then immersed in citrate buffer (0.01 M, pH 6.0). Subsequently, sections were incubated with the Ki67 (Cell Signaling Technology, Danvers, MA, USA, NO. 62548) or p-AKT (Cell Signaling Technology, Danvers, MA, USA, NO. 4060) primary antibodies (both diluted at 1:50 in PBS) at 4 °C overnight. After washing with PBS, corresponding secondary antibodies, with HRP conjugated (Goat anti-Rabbit Secondary Antibody, NO. 31461; Goat anti-Mouse Secondary Antibody, NO. 31436, Invitrogen, Waltham, CA, USA) (both diluted at 1:500 in PBS) were co-incubated for another 2 h at room temperature. Slides were incubated with diaminobenzidine substrate for color development and counterstained with hematoxylin.

For the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) assay, a commercial TUNEL assay kit-FITC was applied (Abcam, Cambridge, UK, NO. ab66108) according to the manufacturer's protocol. The slides were counterstained with DAPI (4,6-diamino-2-phenyl indole). Apoptotic cells were counted.

Statistical Analysis

Data in this study are all shown as mean ± standard deviation (SD) from three replicate experiments at least. GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA) software packages were used to perform data analyses. A two-sided Student's *t*-test was applied to determine statistical significance, $p < 0.05$, *means a significant difference.

Results

The Prognostic Significance of AEBP1 in BCa

To explore the clinical significance of AEBP1 mRNA in BCa, the KM (Kaplan–Meier) plotter database was used, and the data found those patients with higher AEBP1 mRNA levels displayed shorter overall survival rate (Fig. 1A). In addition, the expression profiling of AEBP1 mRNA in BCa was analyzed by the UALCAN dataset and the results indicated that AEBP1 expression was positively associated with the stage of BCa (Fig. 1B). To further confirm the expression of AEBP1 protein in BCa, an IHC staining assay was subsequently performed within clinical specimens, and our data confirmed that the AEBP1 protein was evidently heightened in BCa tissues in contrast to the normal ones (Fig. 1C). All this data suggests dysregulation of AEBP1 which seems to be drawn into the BCa progression.

AEBP1 Enhanced Cell Proliferation and Colony Formation in BCa

To further evaluate the biological roles of AEBP1 in BCa, we inspected the background expression of AEBP1 in 6 human BCa cell lines and the normal mammary cell line MCF-10A. As illustrated in Fig. 2A,B, AEBP1 expression was higher in MDA-MB-231 and MCF-7, comparatively lower in MDA-MB-468, SKBR3, and MCF-10A cells. Therefore, we chose MDA-MB-468 and SKBR3 cells to build the AEBP1-overexpressing models for studying the biological roles of AEBP1.

Initially, qRT-PCR and western blot experiments were performed to determine the efficiency of transfection. The results showed that both mRNA and protein expression levels of AEBP1 were markedly elevated in both SKBR3 and MDA-MB-468 cells transfected with the AEBP1 overexpression plasmid (Fig. 2C–E). First, the upregulation of AEBP1 substantially quickened the cell growth rates of SKBR3 and MDA-MB-468 cells (Fig. 2F). We also examined the colony formation, and the data revealed that the overexpression of AEBP1 augmented the colony formation efficiency in SKBR3 and MDA-MB-468 cells (Fig. 2G,H). On the other hand, the functions of AEBP1 silencing in MDA-MB-231 and MCF-7 cells were also further confirmed. Using siRNA, we effectively knocked down the expression of AEBP1 in two cell lines (Fig. 2I,J). Our data demonstrate that the silencing of AEBP1 effectively impaired cell proliferation (Fig. 2K), and significantly suppressed the colony formation (Fig. 2L,M) in two cell lines. Therefore, these data explained that AEBP1 exerted a stimulative effect on BCa cell growth.

AEBP1 Blocked Cell Apoptosis by Inhibiting Caspase 9 Cleavage and p53 Expression and Promoted Bcl-2 Expression in BCa Cells

To elucidate the mechanism of how AEBP1 accelerates BCa cell growth, a flow cytometry assay was performed to compare apoptosis on overexpressed AEBP1 in SKBR3 and MDA-MB-468 cells and the control ones. As shown in Fig. 3A, AEBP1 effectively inhibited apoptosis in MDA-MB-468 and SKBR3 cells. Subsequently and interestingly, decreased cleavage of caspase 9 protein was noticed in the SKBR3 and MDA-MB-468 cells after overexpression of AEBP1 (Fig. 3B). Bcl-2 (B-cell lymphoma-2) protein, an important anti-apoptotic gene, is one of the key regulators on the intrinsic apoptosis pathway [16]. Our results also demonstrated that overexpression of AEBP1 selectively increased the Bcl-2 protein levels in SKBR3 and MDA-MB-468 cells, while it decreased the protein levels of p53 (protein 53) (Fig. 3B). These data implied that AEBP1 suppressed apoptosis in BCa cells by restraining caspase 9 cleavage and p53 expression, and elevating Bcl-2 expression.

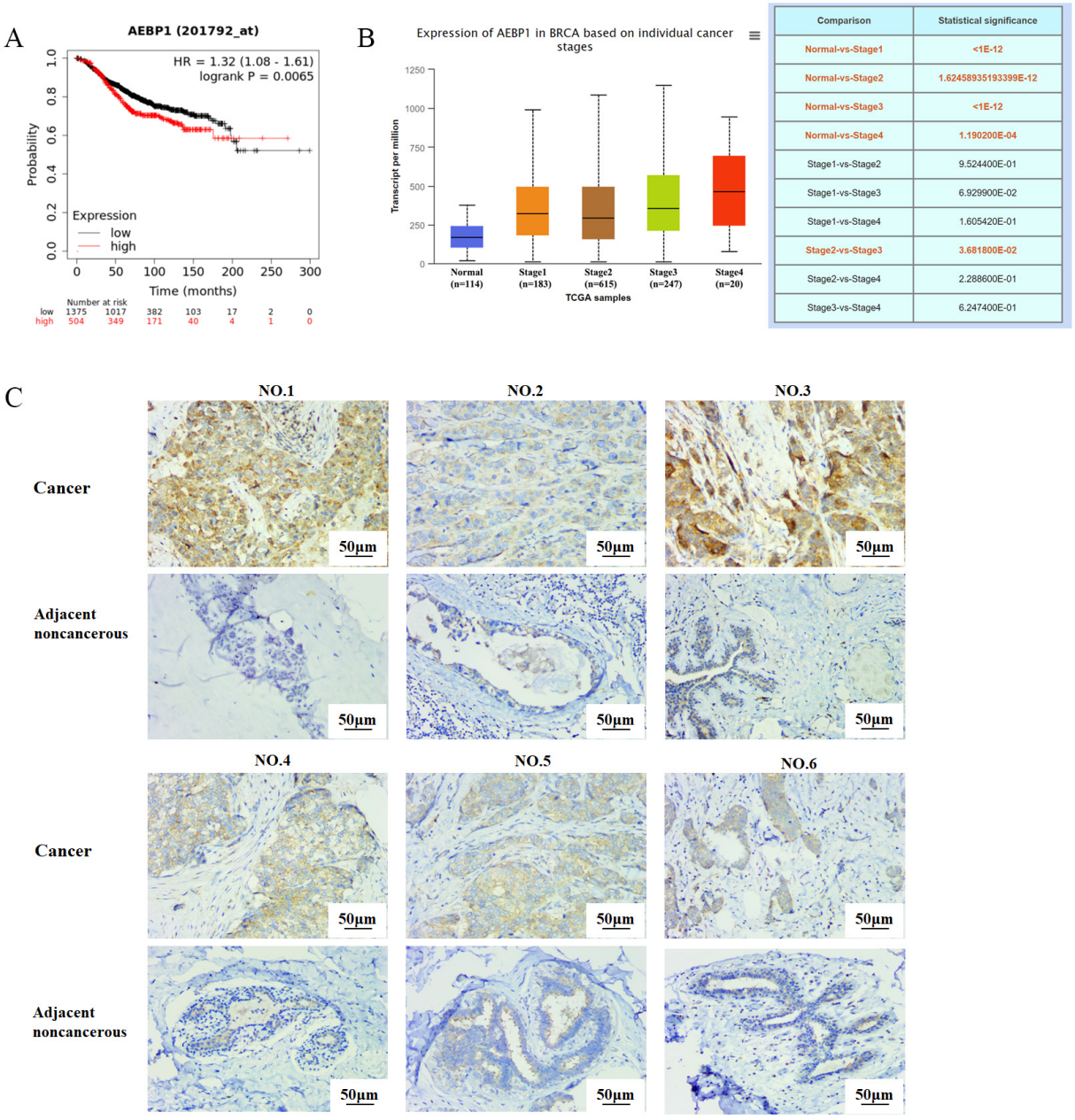

Fig. 1. AEBP1 expression in human BCa tissues. (A) Overall survival curves according to the expression levels of AEBP1 in patients with breast cancer generated by KM plotter. (B) Graph showing the expression of AEBP1 in different stages of BCa, obtained from the UALCAN database. (C) Representative IHC staining images for AEBP1 in human BCa tissues and matched adjacent noncancerous. The brown color indicates AEBP1 protein levels. Scale bar: 50 μm.

Overexpression of AEBP1 Accelerated Cell Migration and Invasion Potentials of BCa via Promoting Epithelial-Mesenchymal Transition (EMT)

To elucidate the effects of AEBP1 on BCa metastasis, AEBP1-overexpressed and control cells were used to perform Transwell® assays to detect cell migration and invasion. Initially, the overexpression of AEBP1 remarkably heightened the migrated cells at the bottom of the insert area compared with the control group in SKBR3 and MDA-MB-468 cells (Fig. 4A). Similarly, the overexpression of AEBP1 dramatically invaded cells into the bottom of the insert area coated with Matrigel compared with the control group in SKBR3 and MDA-MB-468 cells (Fig. 4A). Moreover, we also tested whether AEBP1 promoted BCa cells migration and invasion by promoting epithelial-mesenchymal transition (EMT). The data indicated that AEBP1 overexpression induced EMT in SKBR3 and MDA-MB-468 cells, with loss expression of E-cadherin and gain expression of Vimentin, N-cadherin, and MMP9 (Matrix metalloproteinase-9) (Fig. 4B). There-

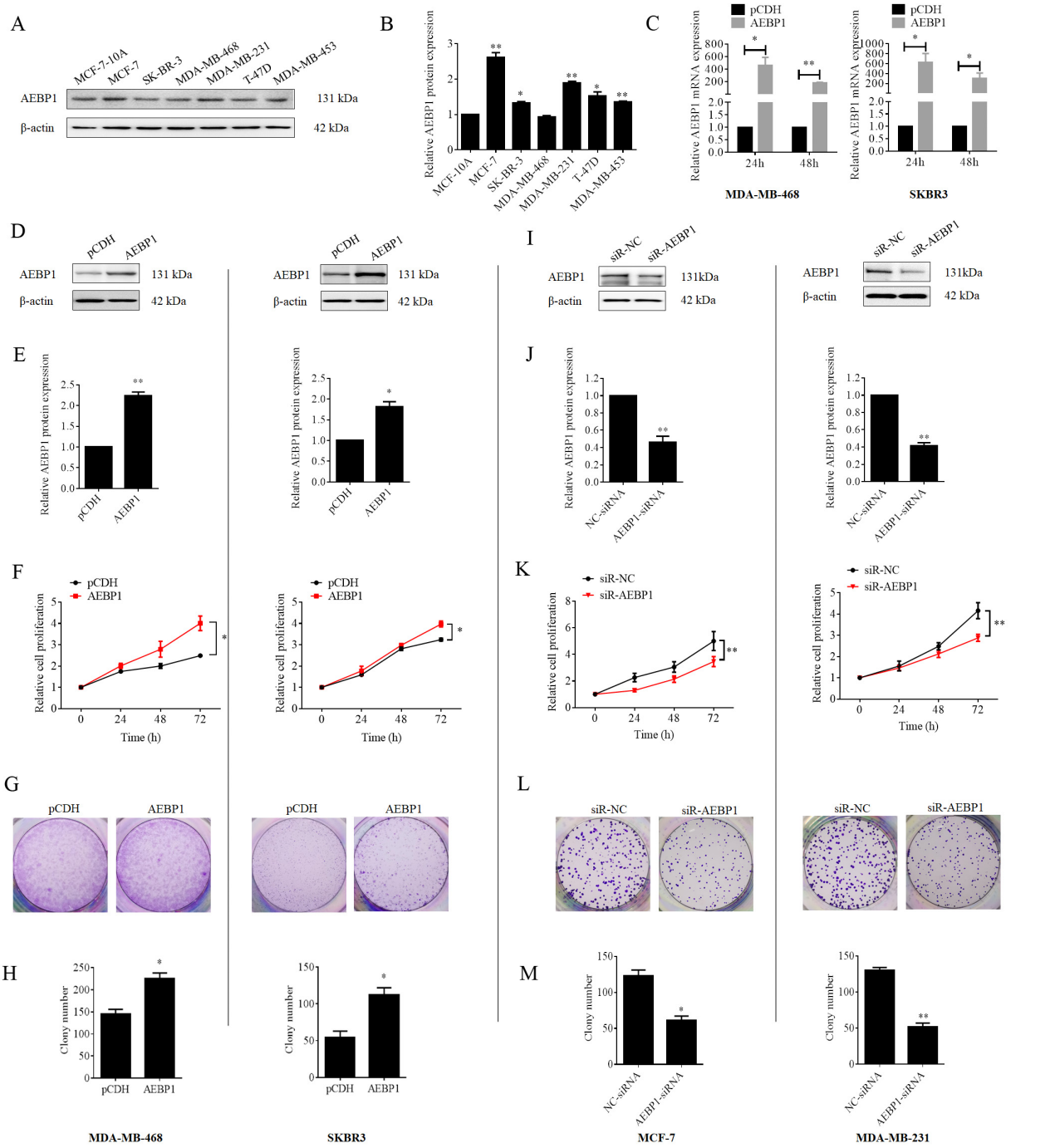

Fig. 2. AEBP1 enhanced cell proliferation and colony formation in BCa. (A) AEBP1 levels expressed in six BCa cell lines and normal mammary cells MCF7-10A. (B) Quantitative analysis of AEBP1 protein expression. (C) qRT-PCR was performed to detect the mRNA levels of AEBP1 in MDA-MB-468 and SKBR3 cells transfected with pCDH or pCDH-AEBP1 plasmids for 24 h and 48 h. (D) Western blot was used to detect the overexpression of AEBP1 in MDA-MB-468 and SKBR3 cells transfected with pCDH or pCDH-AEBP1 plasmids for 24 h. (E) Quantitative analysis of AEBP1 expression in D was shown. (F) The effect of AEBP1 overexpression on the proliferation of MDA-MB-468 and SKBR3 was determined by MTT assay. (G) Representative photographs of AEBP1 overexpression on colony formation. (H) Quantitative analysis of the number of colonies. (I) Western blot was used to detect the silencing of AEBP1 in MDA-MB-231 and MCF-7 cells transfected with NC-siRNA or AEBP1-siRNA plasmids for 24 h. (J) Quantitative analysis of AEBP1 expression in (I). (K) The effect of AEBP1 silencing on the proliferation of MDA-MB-231 and MCF-7 was determined by MTT assay. (L) Representative photographs of AEBP1 silencing on colony formation. (M) Quantitative analysis of the number of colonies. $^{*}p < 0.05$; $^{**}p < 0.01$.

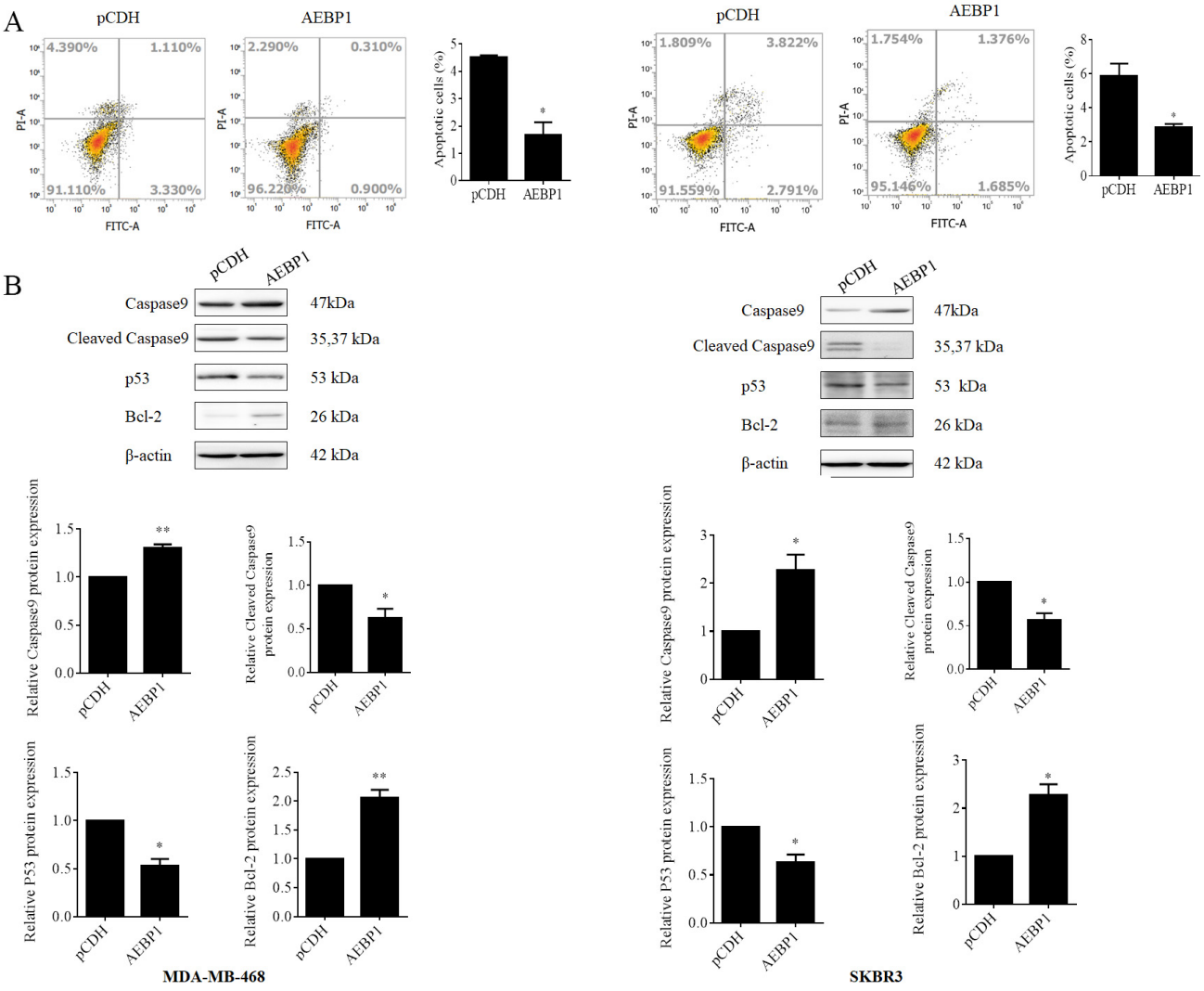

Fig. 3. AEBP1 blocked cell apoptosis by inhibiting caspase 9 cleavage and p53 expression and promoted Bcl-2 expression. (A) The effect of AEBP1 overexpression on the apoptosis of MDA-MB-468 and SKBR3 cells was determined by flow cytometry. Apoptosis was quantified in the histogram. (B) MDA-MB-468 and SKBR3 cells were transfected with pCDH and pCDH-AEBP1 plasmids for 24 h. Then, the cells were analyzed by western blot for the detection of the protein caspase 9, cleaved caspase 9, p53 and Bcl-2. Quantitative analysis of protein expression is shown in the histogram. Data are presented as mean ± SD for three separate experiments. $^{*}p < 0.05$; $^{**}p < 0.01$.

fore, AEBP1 advanced cell migration and invasion in BCa by upregulating EMT at least partially.

AEBP1 Promoted BCa Progression by Activating Multiple Oncogenic Signaling Pathways

To resolve which tumor-related signaling pathway played a key role by AEBP1 in BCa progression, the UniProt dataset was utilized and it was found that AEBP1 can interact with NF-κB, MAPK1/3 and PTEN (phosphatase and tensin homolog deleted on chromosome 10), which is also supported by the previous publications [17–19]. On the other hand, the TGF-β signaling pathway plays an indispensable role in the progression of BCa [20]. Western blotting was performed to measure the protein levels of p-Smad2, p-Smad3, Smad2/3, PTEN, p-AKT, AKT, p-NF-κB, NF-κB, p-ERK and ERK. As illustrated in Fig. 5A–D, after AEBP1 overexpression in SKBR3 and MDA-MB-468 cells, the phosphorylation levels of Smad2, Smad3, AKT, NF-κB, ERK were observably enhanced, but the protein levels of PTEN were significantly lessened while the total protein levels of AKT, NF-κB, ERK did not change compared to the pCDH control. The results manifested that AEBP1 may take part in the BCa progression by activating multiple tumor-promoting signaling pathways.

AEBP1 Facilitated BCa Xenograft Growth

To further resolve the oncogenic role of AEBP1 in BCa *in vivo*, we implemented experiments with xenograft

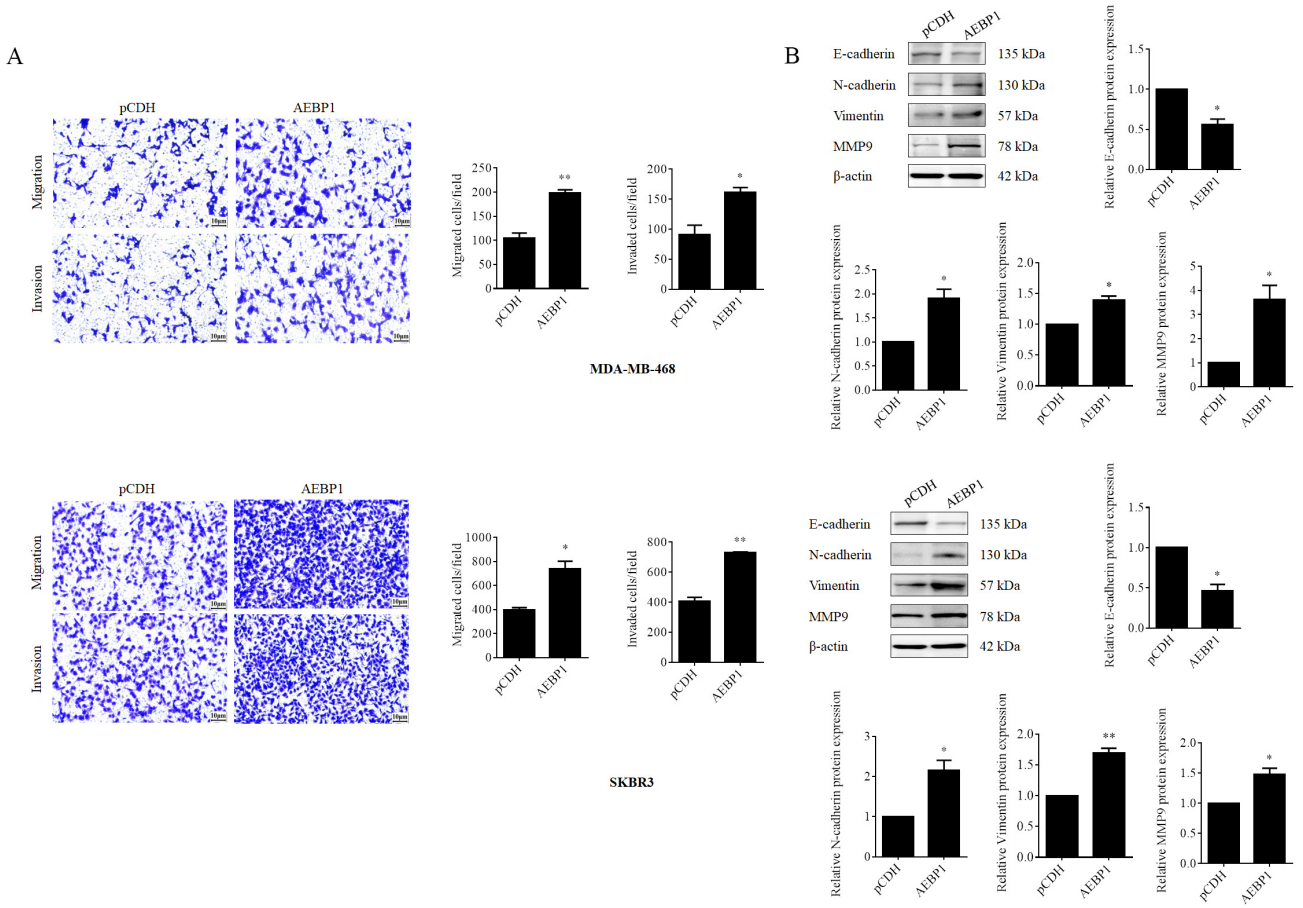

Fig. 4. AEBP1 overexpression heightened cell migration and invasion of BCa cells. (A) Representative images of pCDH and pCDH-AEBP1 plasmids on the cell migration and invasion abilities of MDA-MB-468 and SKBR3 cells were determined by Transwell® assays. Quantitative analysis is shown in the histogram. (B) MDA-MB-468 and SKBR3 cells were transfected with the pCDH and pCDH-AEBP1 plasmids for 24 h. Then, a western blot was used for the detection of the EMT-related proteins and MMP9. Quantitative analysis expression of proteins is shown. Data are presented as mean ± SD for three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$. Scale bar: 10 μm.

models using AEBP1 stably-overexpressed MDA-MB-468 cells. The AEBP1 overexpression significantly quickened tumor growth with extraordinarily strengthened tumor volumes (Fig. 6A). The reduction of TUNEL, a marker of apoptosis, was also conducted along with the overexpression of AEBP1 (Fig. 6B). Moreover, the expression of Ki67, a biomarker of cell proliferation, was also increased along with the overexpression of AEBP1 (Fig. 6C). Furthermore, the data testified that p-AKT expression was markedly elevated in the AEBP1 overexpression group contrasted with the pCDH control group (Fig. 6C). The results suggested that AEBP1 facilitated BCa tumor growth and inhibited cell apoptosis by activating p-AKT *in vivo* at least partially.

Discussion

Previous studies have investigated authenticating tumor suppressor genes or oncogenes that play a crucial role in accelerating or restraining BCa progression [21–23]. In this study, we found that AEBP1 was higher expressed in BCa than paracancerous by dataset and IHC analysis, and also certified a prominent positive correlation between the mRNA expression levels of AEBP1 and different stages of BCa. Moreover, patients with higher levels of AEBP1 had worse overall survival (OS) rates. These findings indicate that AEBP1 may be abnormally expressed in BCa and is related to BCa progression.

Recent studies have published that abnormal expression of AEBP1 was also related to clinical characteristics of other cancers. For example, higher expression of AEBP1 was interrelated to larger tumor size, lower levels of histological differentiation, more metastasis in the lymph node, and higher cancer stage in patients with colon adenocarcinoma and glioblastoma [19,24,25]. Similarly, overexpression of AEBP1 may be a distinguished factor in the progression of malignant BCa via bone differentiation and matrix remodeling [26–28]. In the present study, we discussed

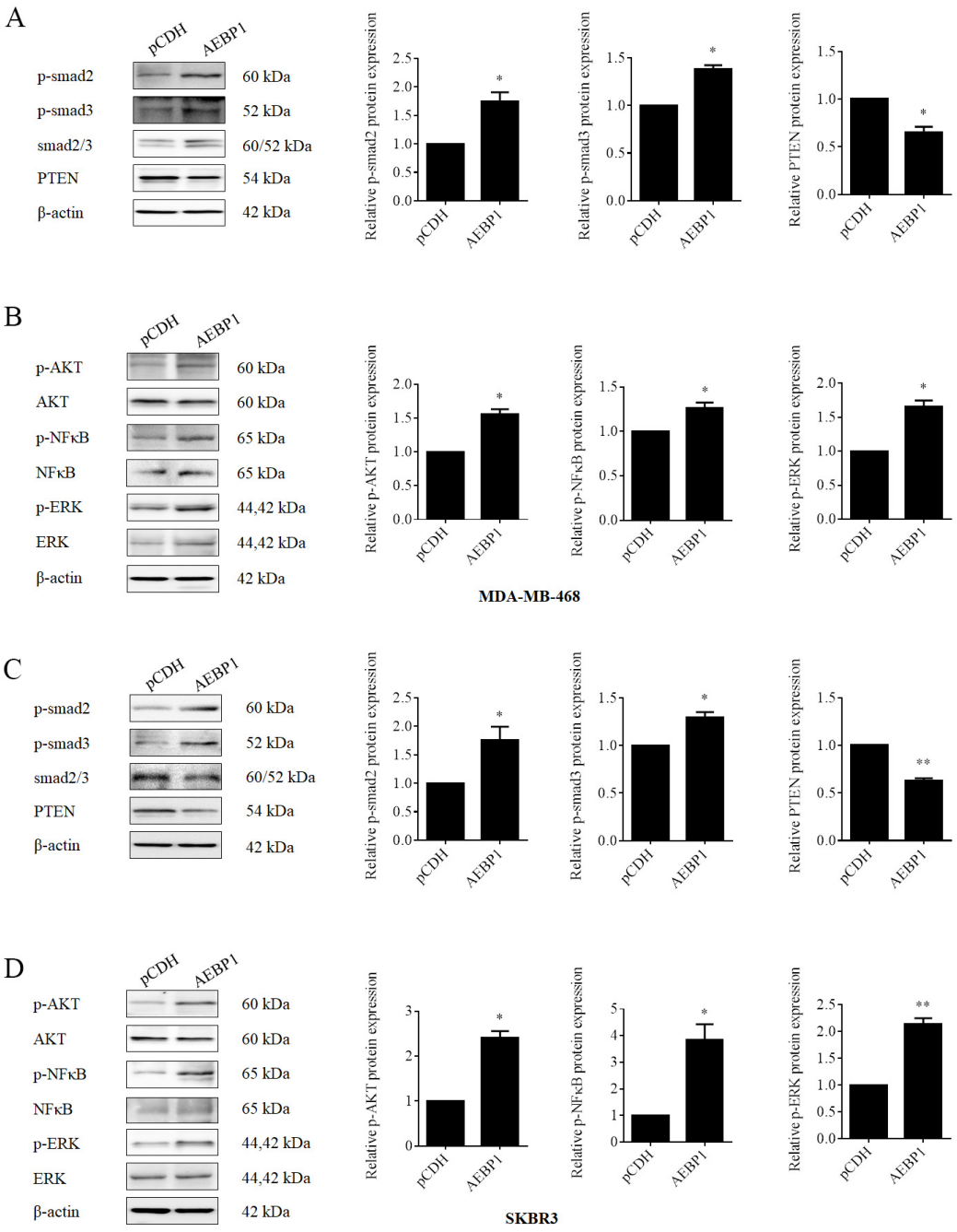

Fig. 5. AEBP1 overexpression activates the AKT/NF-κB, and Smad2/3 signaling pathway. (A,B) MDA-MB-468 cells were transfected with the pCDH and pCDH-AEBP1 plasmids for 24 h. Then, the cells were analyzed by western blot for the detection of p-Smad2, p-Smad3, Smad2/3, PTEN, p-AKT, AKT, p-NF-κB, NF-κB, p-ERK and ERK. Quantitative analysis expression of proteins is shown in the histogram. (C,D) SKBR3 cells were transfected with the pCDH and pCDH-AEBP1 plasmids for 24 h. Then, the cells were analyzed by western blot for the detection of p-Smad2, p-Smad3, Smad2/3, PTEN, p-AKT, AKT, p-NF-κB, NF-κB, p-ERK and ERK. Quantitative analysis expression of proteins is shown in the histogram. Data are presented as mean ± SD for three separate experiments. $^{*}p < 0.05$; $^{**}p < 0.01$.

the roles of AEBP1 in BCa progression through a sequence of cell functional assays. First, we chose the representative BCa cell lines SKBR3 and MDA-MB-468, which displayed considerably low expression levels of AEBP1, to construct overexpression cell models. Our data illustrated that the ectopic expression of AEBP1 prominently promoted cell proliferation, migration, and invasion abilities of BCa cells and significantly inhibited cell apoptosis. Furthermore, the data from *in vivo* experiments revealed that AEBP1 overexpression increased the subcutaneous tumors growth, and de-

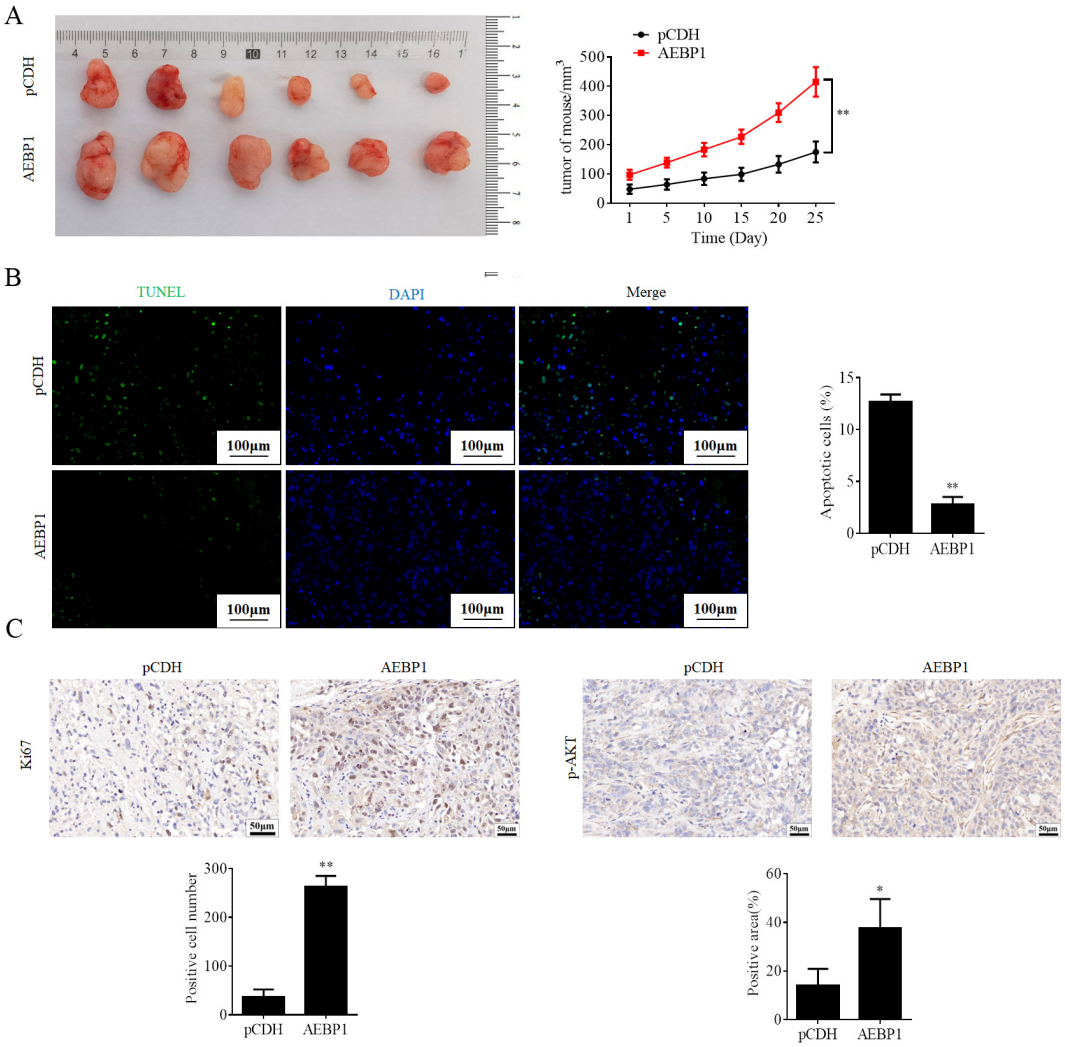

Fig. 6. AEBP1 overexpression facilitated BCa xenograft growth. (A) Tumor xenograft models were constructed with stable AEBP1-overexpression (n = 6) BCa cells MDA-MB-468 cells and corresponding negative control cells. Tumor sizes were recorded consecutively to constitute tumor growth curves. (B) Representative TUNEL images of the two groups. The apoptotic cells were counted and analyzed as shown in the histogram. Scale bar: 100 μm. (C) Representative IHC-Ki67 and IHC-p-AKT images of the tumor with AEBP1-overexpression and corresponding control groups. Scale bar: 50 μm. Data are expressed as mean $\pm$ SD of three separate experiments, $^{*}p < 0.05$; $^{**}p < 0.01$.

creased tumor cell apoptosis. In MDA-MB-231 and MCF-7 cells, the role of AEBP1 silencing on cell proliferation and colony formation was also detected. Opposite roles of AEBP1 silencing were observed in breast cancer cell lines compared with cells with AEBP1 overexpression.

EMT, a hallmark of cancer, is related to the aggressive characteristics of BCa [29]. EMT in the beginning was thought to be a growing procedure during which epithelial cells exhibit a migratory and invasive mesenchymal phenotype [30]. Consequently, epithelial markers such as E-cadherin are reduced, and mesenchymal markers such as N-cadherin, Vimentin, Snail, and MMP2/9 are increased [31]. Our data authenticated that AEBP1 overexpression strengthened the expression of mesenchymal markers, including N-cadherin, Vimentin, and MMP9, and attenuated the expression of the epithelial marker E-cadherin. This data elucidated that AEBP1 enhances the invasion and migration of BCa by heightening EMT.

PI3K/AKT signaling, as the most crucial signaling pathway in tumorigenesis and progression, regulates cancer cells by activating the metabolic enzymes and transporters to accelerate malignant biological behaviors [32]. PTEN, a tumor suppressor, is involved in the regulation of tumor cell proliferation and metastasis [33]. Due to the activity of lipid phosphatase, PTEN represses cell proliferation and motility by inhibiting PI3K-Akt signaling. As a result, mutated PTEN was vigorously related to cancer development and progression. In our study, the results corroborated that AEBP1 elevated BCa cell proliferation and motility by suppressing PTEN, while phosphorylating AKT. Furthermore,

our data also confirmed that other classical oncogenic signaling pathways such as TGF-β/SMAD, MAPK/ERK and NF-κB pathways were also activated by AEBP1 overexpression in BCa cells.

Conclusions

In summary, these data illustrated the role of AEBP1 in BCa progression, which could be used as a potential prognostic hallmark and a novel therapeutic target.

Abbreviations

AEBP1, adipocyte enhancer binding protein 1; BCa, breast cancer; qRT-PCR, quantitative real-time polymerase chain reaction; MMP9, matrix metalloproteinase-9; N-cadherin, neural-cadherin; E-cadherin, epithelial cadherin; ERK, extracellular signal-regulated kinase; AKT, V-akt murine thymoma viral oncogene homolog; PTEN, phosphatase and tensin homolog deleted on chromosome 10; Bcl-2, B-cell lymphoma-2; p53, protein 53; EMT, epithelial-mesenchymal transition; MAPK, mitogen-activated protein kinase; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; TGF-β, transforming growth factor-β; NF-κB, nuclear factor kappa-B; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; IHC, immunohistochemistry; HIAR, heat-induced antigen retrieval; NC, negative control; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay; SD, standard deviation.

Availability of Data and Materials

The original data in this study is included in the article. Further inquiries can be directed to the corresponding author.

Author Contributions

JL and YR—contributed to conception and design of the study, and wrote the first draft of the manuscript; CZ, YP, BL and QC—contributed to data analysis and manuscript revision; ZZ—contributed to manuscript revision and project management. All authors approved the submitted version.

Ethics Approval and Consent to Participate

Before sample clustering, informed consent was procured from the patients and the project was approved by the Ethics Committee of Taizhou Central Hospital (Taizhou, PRC; No. 2022-SC-015).

All animal studies were executed according to the regulations and guidelines of Taizhou University institutional animal care (No. TZYXY2022-532) and conducted according to the AAALAC and the IACUC guidelines.

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Conflict of Interest

The authors declare no conflict of interest.

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