BAI1 Acts as a Tumor Suppressor in Triple-Negative Breast Cancer via Modulating p73 Transactivation

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Research

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Abstract

Background: Brain-specific angiogenesis inhibitor 1 (BAI1) which belongs to putative G-protein-coupled receptors (GPCRs), has been found down-expressed in various cancers and involved in cancer pathogenesis. However, the role and underlying mechanisms of BAI1 in triple negative breast cancer (TNBC) are still unclear.

Methods: The expression levels of BAI1 in TNBC samples and cell lines were examined by immunohistochemistry (IHC), quantitative real-time polymerase chain reaction (qRT-PCR), and western blotting (WB). The functional effects of BAI1 on biological behaviors of TNBC cells were detected using plasmid and siRNA for BAI1 overexpression and knockdown, and the underlying mechanisms were investigated by Immunoprecipitation (IP), immunofluorescence (IF) and luciferase reporter assay.

Results: BAI1 was downregulated in TNBC tissues and was significantly associated with poor disease-free survival. Functional experiments indicated that BAI1 inhibited cell proliferation and induced cell apoptosis and cell cycle arrest. Additionally, BAI1 overexpressed cells were more sensitive to cisplatin. Mechanistically, BAI1 interacted with MDM2, thereby enhanced p73 transcriptional activity, then promoted p21and BAX mRNA and protein expression. Overexpression of p73 abolished the BAI1 knockdown induced cell proliferation and the G2 phase cell population of TNBC, the sensitivity to cisplatin also rescued by overregulating p73 in BAI1 knockdown TNBC cells.

Conclusions: Our results indicate that BAI1 is a promising prognostic factor in TNBC, and the expression of BAI1 inhibits cell proliferation and induces cell apoptosis and cell cycle arrest; Meanwhile, BAI1 increases the sensitivity of TNBC to cisplatin. For the underlying mechanism, BAI1 specifically binds to MDM2, and exerts its anti-tumor function by affecting the transcriptional activity of p73 protein, then inhibits the malignant progression of TNBC. The BAI1/MDM2/p73 axis may represent a potential target in the future research for TNBC.

Introduction

Breast cancer is the most commonly cancer and the leading cause of cancer death among females[1]. Triple-negative breast cancer (TNBC) is a breast cancer subtype that lacks estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression, and accounts for 15–20% of all breast cancers[2]. Compared to other subtypes, TNBC patients usually have poorer survival due to lack of effective targeted therapeutics. Thus, further exploring the potential molecular mechanisms of TNBC is critical to improving clinical outcomes.

BAI1, an orphan G protein-coupled receptor-type seven transmembrane protein, was initially identified in a screen for genes regulated by the tumor suppressor p53[3]. Structurally, The C-terminus of BAI1 contains a QTEV motif that mediates binding to PDZ domain–containing proteins which associates with a number of scaffolding proteins involved in intracellular signaling[4]. The N-terminal of BAI1 can be cleaved at the GPS site and releases soluble 40 and 120 kDa fragments known as vasculostatins, which suppress angiogenesis in vitro and inhibit tumor growth in vivo[5, 6]. Reduced BAI1 expression has been observed in several tumor types, including glioblastoma, Pulmonary adenocarcinomas, gastric and colorectal cancer[7–10]. Previous study

showed that significant downregulation of BAI1 in primary breast cancers and was associated with unfavorable patient outcomes[11]. Currently, an emerging and promising therapeutic modality called oncolytic viral (OV) therapy has been introduced in several malignant tumors[12, 13]. A recent study showed that delivering Vstat120 (extracellular fragment of BAI1) to highly malignant breast cancer cells using oncolytic virus (34.5ENVE) transport system has showed promising results to selectively kill these tumor cells which may help provide promising future prospect for use of BAI1 as human diagnostic and therapeutic tool[11]. Given the functional importance of BAI1 in tumorigenesis and malignance, and its clinical translational value, understanding the underlying mechanism of BAI1 regulation in TNBC should facilitate the development of new therapeutic agents for these patients.

The well-defined p53 tumor suppressor protein, which plays an important role in preventing tumor development, has been found occurs somatic mutation in approximately 60% of TNBCs[14]. Whereas p73, a member of the p53 family, shares remarkable homology in DNA sequence and protein structure with p53, and transactivates most p53 transcriptional target genes by recruiting to p53-response elements within gene promoters, like p21, Bax, thereby induces cell cycle arrest and apoptosis in tumor cells[15–17]. Unlike p53, which is widely mutated in human cancers, mutations of p73 is rare in human cancers[18–20]. Furthermore, p73 forms an interaction with mouse double minute 2 (MDM2), an E3 ubiquitin ligase, which binds to the p53 N-terminal transactivation domain[21], inhibiting its transcriptional activity [22] and triggering its degradation via ubiquitin-proteasome pathways [23, 24]. However, the interaction between MDM2 and p73 negatively regulates the transcriptional activity of p73 but slightly impacts its degradation which distinct from the mechanism used for p53 regulation [25, 26]. Previous studies found that MDM2 deletion increased levels of p73, then induced apoptosis and G2 cell-cycle arrest, which compensates for the loss of p53 in p53-deficient cancers[27]. Therefore, p73 provides a legitimate and attractive target in cells lacking functional p53 and provides a promising strategy for cancer therapy.

Previous studies indicated that BAI1 prevents PSD95 and p53 polyubiquitination and degradation through an interaction with MDM2 which regulates synaptic plasticity and suppress medulloblastoma formation respectively[28, 29]. Meanwhile, a great number of studies showed that MDM2 interacts with p73 and inhibits its transcriptional activity in p53 mutated tumors. Whether BAI1 excises its anti-tumor function by binding to MDM2 and thereby enhances the transcriptional activity of p73 in TNBC cells still needs study. Meanwhile, few studies focus on the chemosensitivity influence of BAI1 in malignant cancers. Therefore, we examined the potential role of BAI1 in chemosensitivity of TNBC and evaluated whether the anti-tumor activity of BAI1 is modulated through interaction with the MDM2/p73 pathway.

In the present study, we investigated BAI1 expression and correlation with clinicopathologic features and prognosis in TNBC patients. Then exploited BAI1 function and its interaction with p73-MDM2 pathway.

Materials And Methods

Patients and samples

This study was approved by the Institutional Review Board of the Fudan University Shanghai Cancer Center, and informed consent was obtained from all patients. The expression of BAI1 in TNBC patients was detected

using immunohistochemistry (IHC), quantitative real-time polymerase chain reaction (qRT-PCR), and western blotting (WB). A tissue microarray (TMA) with 120 paraffin-embedded TNBC samples were performed to identify the expression levels of BAI1 in TNBC patients. qRT-PCR was performed to examine BAI1 mRNA expression in 159 breast cancer with different subtypes, 63 paired TNBC tumor samples and adjacent normal tissues (ANTs). Western blotting was performed to examine BAI1 protein expression in eight paired TNBC tumor samples and ANTs.

Analysis of Publicly Available Datasets

To analyze mRNA expression of BAI1 in whole breast cancer samples and different subtypes, and the effect of BAI1 expression on the prognosis of patients with breast cancer, the Gene Expression Profiling Interactive Analysis (GEPIA) online tool (http://gepia.cancer-pku.cn) were used to analyze mRNA expression of BAI1 and prognostic value in BC samples from TCGA provisional dataset.

Cell culture and reagents

Human triple negative breast cancer cell lines (MDA-MB- 231, MDA- MB- 453, MDA- MB- 468, Hs578T, BT549 and BT20), and human embryonic kidney 293T (HEK293T) cell line were kindly provided by Dr. Daqiang Li and were maintained according to standard American Type Culture Collection (ATCC) protocols. All cell lines were cultured in high- glucose DMEM medium containing 10% FBS and 1% penicillin/streptomycin in a 37°C humidified incubator with 5% CO2.

Cisplatin (purchased from MCE) was suspended in dimethyl sulfoxide (DMSO). Cells were seeded in 96-well plates with 100 μl of medium at a concentration of 4000 cells/well. DMSO was used as the negative control. The cells were then cultured for 48h and then tested using a CCK-8 kit to measure the half maximal inhibitory concentration (IC50).

The antibody for BAI1 (ab135907) and antibodies targeting GAPDH (ab181602) were purchased from Abcam. Antibodies targeting MDM2 (Cell Signaling Technology, CST#86934), p73 (CST#14620), p21 (CST#8831), Cyclin A2 (CST#4656), Cyclin B1 (CST#4138), BCL2 (CST#15071), BAX (CST#5023), Flag (CST#14793) and IgG (CST#5415) were purchased from CST. The antibodies used in this article are listed in (Additional file 1: Table S1).

Immunohistochemistry (IHC)

Slides were dewaxed at 60 °C for 2 h followed by three washes with xylene and rehydrated with different concentration gradients of ethanol, then washed with distilled water for 10 min. For antigen retrieval, slides were put into the ethylene diamine tetraacetic acid buffer (EDTA, pH 6.0) for 4 min when the buffer was heated to 100 °C. Then inhibited the endogenous peroxidase activity by 3% hydrogen peroxide for 30 min. After that, the slides were incubated with anti-BAI1 antibody (1:100 dilution; Abcam, Shanghai, China) overnight at 4 °C. The slices were incubated with a biotin-labeled secondary antibody for 30 min, then followed by 3, 3'-diaminobenzidine (DAB; Zhongshan biotech) substrate.

For each sample, IHC staining was scored by two pathologists using the immunoreactive score (IRS), which is based on the staining intensity and the percentage of positive cells. The staining intensity was scored 0–3 (0 =

negative; 1 = weak; 2 = moderate; 3 = strong), the percentage of positive stained cells was also scored into four categories: 1 (0–25%), 2 (26–50%), 3 (51–75%) and 4 (76–100%), then staining pattern was defined as negative (IRS: 0), weak (IRS: 1–3), moderate (IRS: 4–6), and strong (IRS: 8–12).

RNA extraction and quantitative real-time PCR

Cells were collected, and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription (RT) was conducted using PrimeScript RT Master Mix (Takara, Dalian, China) to synthesize cDNA. qRT–PCR was performed with SYBR Green PCR Master Mix (Takara, Dalian, China) on an ABI 7900HT (PE Applied Biosystems) qPCR machine. For relative quantification, target gene mRNA expression was normalized to GAPDH expression. The primers used in the qRT-PCR assays are listed in (Additional file 1: Table S2).

Western blotting

Cells were collected and lysed with RIPA buffer (Thermo Scientific) supplement with a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Cell lysates was centrifugated at 12,000 g for 15 min at 4 °C, the protein supernatants were collected and quantified using a bicinchoninic acid assay (Thermo Scientific) according to the manufacturers' instruction, then proteins were subjected to SDS-PAGE, and transferred onto PVDF membranes (Millipore, Billerica, USA). after incubated with blocking buffer (5% non-fat dry milk in TBST) for 1 h, PVDF membranes were cultured in indicated primary antibodies overnight at 4℃. After three washes with TBST and incubated with HRP-conjugated secondary antibodies for 1 h, antibody detection was conducted using an enhanced chemiluminescent substrate kit (Yeasen).

RNA interference, plasmid transfection, and lentivirus transduction

BAI1 siRNA sequences and negative controls were designed by GenePharma (Shanghai, China) and used to transfect cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. The target sequences of BAI1 siRNAs are listed in (Additional file 1: Table S3). For overexpression, full-length BAI1 and p73 were identified and cloned to generate Flag-BAI1 and His-p73 constructs. To construct stable BAI1 overexpression cells, lentivirus was constructed and used to infect MDA-MB-231 and MDAMB-468 cells, which were then selected with puromycin.

Cell viability and colony-formation assay

Cells were seeded in 96-well plates and cell viability was assessed using a Cell Counting Kit-8 (CCK8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. For colony-formation assays, 1000 viable cells were seeded in 6-well plates and cultured for 2 weeks. Colonies were fixed with ethanol and stained with 1% crystal violet before being counted.

Flow cytometry analysis

Cell cycle and cell apoptosis analyses were performed via flow cytometry on a FACS can instrument (Beckman Coulter, Brea, CA, USA). For the cell cycle analysis, cells were harvested, and fixed with 70% ethanol at 4 °C overnight, and stained with propidium iodide (50 μg/ml), containing 100 μg/ml RNase A for 15 min. For

apoptosis analysis, cells were harvested, washed with PBS, and incubated with Annexin V-PE and 7-AAD (BD Biosciences, San Diego, CA, USA).

Nuclear cytoplasmic fractionation

The extraction and isolation of nuclear and cytoplasmic protein were performed by using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions.

Immunofluorescence (IF)

Cells were washed 3 times in PBS and then fixed with 4% paraformaldehyde for 15 min, permeabilized in 0.3% Triton X-100 for 15 min and blocked with 10% goat serum for 1 h at room temperature. Then, cells were incubated with primary antibodies overnight at 4 °C, washed 3 times in PBST, and incubated with appropriate secondary antibody conjugated with Alexa 555 (red) or Alexa 488 (green) (Cell Signaling Technology), respectively. DAPI (Abcam, Shanghai, China) was used to stain nuclei and mount cells. A Leica SP5 confocal Laser Scanning Microscope (Leica Microsystems, Buffalo Grove, USA) was used to capture and analyze images.

Immunoprecipitation (IP)

MDA-MB-231 and MDA-MB-468 cells infected with Flag-BAI1 were lysed on ice in NP-40 lysis buffer (50 mM Tris-HCl, pH 8, 150mM NaCl, 0.5% NP- 40, 10% glycerol, 2mM MgCl2, and 1mM EDTA) supplemented with containing 1× protease inhibitor cocktail (Selleck, B14001) and 1× phosphatase inhibitor cocktail (Selleck, B15001). Cell lysates were incubated with 3 μg anti-BAI1 or IgG antibody (Cell Signaling Technology) overnight at 4 °C, then added 50μl protein A/G Sepharose beads (CST#70024) in the cell lysates and incubated for 4h at 4 °C. Immuno-complexes were washed with lysis buffer three times before being resolved by SDS-PAGE and detected by western blot using the indicated antibodies.

Luciferase reporter assays

Cells were co-transfected with 1μg of the indicated firefly luciferase reporter plasmid (p21 or BAX) plus the renilla luciferase control vector, with or without 1μg of the indicated expression plasmid (p73) in the presence or absence of BAI1. Cell lysates were prepared and quantified using Firefly & Renilla Dual luciferase assay (Yeasen) after transfection (48h later) according to the manufacturer's recommendations.

Statistics

Statistical analyses were performed using IBM SPSS 20.1 and GraphPad Prism v 8.0 (GraphPad Software, San Diego, CA, USA). The results are presented as the mean ± standard deviation from at least three independent experiments. Two-tailed Student t tests or a one-way ANOVA was applied to assess difference between or among different groups. The chi-square test was used to analyze the relationship between BAI1 protein expression and clinicopathologic parameters. The Kaplan– Meier method and log-rank test were used for survival analysis. Spearman rank correlation test was used to calculate correlation coefficients, and $p < 0.05$ was considered to indicate statistical significance.

Results

BAI1 suppresses breast cancer progression and correlates with better prognosis

BAI1 mRNA expression in TNBC samples was examined by specific database and qRT-PCR, and the protein expression of BAI1 in TNBC samples was examined by IHC, and western blot. BAI1 mRNA was downregulated in BC samples compared with that in normal tissues (Fig.1a) and was significantly downregulated in TNBC samples compared with that in luminal subtype BC (Fig.1b) in TCGA breast cancer dataset. Importantly, BAI1 low expression was significantly correlated with poor OS in TCGA database (P= 0.025, Fig.1c). similarly, BAI1 mRNA was significantly downregulated in TNBC samples compared with other subtypes (P< 0.001, Fig1d) and that in the paired ANTs (P< 0.001, Fig.1e) in our cohort. Furthermore, we found that BAI1 protein expression was also downregulated in eight TNBC tumor samples compared with that in the paired ANTs (Fig.1f). The IHC results of 120 TNBC samples showed that BAI1 downexpression was associated with higher tumor burden, higher Ki67 index and increased risk of local recurrence (Additional file 2: Table 1). Kaplan-Meier survival analysis showed that BAI1 low staining were correlated with worse DFS and OS in TNBC (p = 0.008, p = 0.037, Fig. 1g,h). Moreover, BAI1 low expression was also an independent prognostic factor that indicated poor DFS (HR = 0.47, 95%CI 0.36–0.68, p = 0.038), but not poor OS (HR = 0.95, 95%CI 0.81–1.42, p = 0.104) in TNBC (Additional file 2: Table 2). Taken together, downregulated of BAI1 is related to poor prognosis in TNBC patients. Representative BAI1 IHC stanning were showed in Fig. 1i.

BAI1 inhibits TNBC cell proliferation and induces cell apoptosis

To evaluate the potential effect of BAI1 on the biological behaviors of TNBC cells, basal BAI1 mRNA and protein expression were evaluated in six TNBC cell lines (Fig. 2 a, b). BAI1 expression was relatively low in MDA-MB-231 and MDA-MB468 cells which were selected for overexpression, while Hs-578t and BT549 cells with relatively high basal BAI1 expression were selected for knockdown. qRT-PCR and western blotting analyses were used to confirm the efficiencies of overexpression and knockdown (Fig. 2 c, d). Both CCK8 (Fig. 2e) and colony formation (Fig. 2f-i) assays revealed that overexpression of BAI1 in MDA-MB-231 and MDA-MB-468 cells significantly suppressed cell proliferation, while BAI1 knockdown in Hs-578t and BT549 cells promoted cell proliferation. Regarding to cell apoptosis, ectopic BAI1 expression induced apoptosis in MDA-MB-231 and MDA-MB-468 cells, while BAI1 knockdown inhibited apoptosis in Hs-578t and BT549 cells (Fig. 3a, b). Next, we examined the effect of BAI1 on cell-cycle progression by flow cytometry. Overexpression of BAI1 increased the S-phase cell distribution and decreased the cell distribution at G2-phase, whereas BAI1 knockdown decreased the S-phase cell population and increased the G2-phase cell population, indicating that BAI1 promotes the S/G2 cell arrest (Fig. 3c, d). Taken together, these results suggest that BAI1 plays an important anti-tumorigenic role in suppressing TNBC cell growth.

BAI1 overexpression results in enhanced tumor sensitivity to cisplatin

The expression of BAI1 is significantly associated with the cell cycle and cell death progression. BAI1 overexpression MDA-MB-231 and MDA-MB-468 cells were significantly more sensitive to cisplatin than the negative control cells (Fig. 4a). Consistently, BAI1 knockdown Hs578T and BT549 cells were more resistant to cisplatin than the vector control cells (Fig. 4a). Both colony formation (Fig. 4b, c) and CCK8 (Fig. 4d) assays indicated that BAI1 overexpression promoted the efficacy of cisplatin, while BAI1 knockdown decreased the

efficacy of cisplatin in TNBC cells. These data suggest that BAI1 plays an important role in cisplatin sensitivity of TNBC.

BAI1 interacts with MDM2 and regulates the transcriptional activity of p73

We further examined whether MDM2/p73 axis plays a role in the molecular regulation by which BAI1 alters cell cycle and cell death progression. First, Western blotting revealed that key mediators of the cell cycle and apoptosis, namely Bax and p21 were positively regulated, while Cyclin A2, Cyclin B1, Bcl2 were negatively regulated by BAI1 (Fig. 5a). However, the mRNA and protein expression of p73 did not significantly affected by BAI1 in TNBC cells (Fig. 5b, c), Then we validated the interaction of BAI1 and MDM2 in TNBC cells. IP analysis with an anti-Flag antibody showed that exogenously expressed Flag-BAI1 specifically interacted with endogenous MDM2 (Fig. 5d). Consistently, immunofluorescence (IF) staining experiments showed that BAI1 reduces the co-localization of MDM2 and p73 (Fig. 5e). Besides, cytoplasmic and nuclear fractions analysis showed that BAI1 expression increased the expression of p73 and reduced the content of MDM2 in the nucleus (Fig. 5f). These data confirmed that BAI1 interacts with MDM2 in TNBC and affect the interaction between MDM2 and p73. Luciferase assay was performed to evaluate to further investigate whether BAI1 regulates the transcriptional activity of p73 protein through interact with MDM2. The results showed that BAI1 overexpressing in MDA-MB-231 cells significantly induced the occupancy of p73 on the promoters of p21 and BAX (Fig. 5g). Furthermore, qRT-PCR was used to validate mRNA expression of the p73 target genes p21, Bax were upregulated or downregulated by overexpression or knockdown of BAI1 without affecting the expression of p73 in TNBC cells (Fig. 5h). Collectively, we demonstrate that BAI1 could interact with MDM2 and increases p73 transcriptional activity in TNBC.

BAI1 inhibits TNBC tumor proliferation in a MDM2/p73-mediated manner

To further elucidate whether BAI1 regulated TNBC cell-cycle and apoptosis progression and sensitivity to cisplatin by increasing p73 transcriptional activity, we transfected His-p73 plasmids into Hs578T and BT549 cells with BAI1 downexpression. We found that overexpression of p73 significantly abolished the positive effects of BAI1 knockdown on cell proliferation (Fig. 6 a-c). Apoptosis assay indicated that p73 overexpression reversed the inhibitory effects of BAI1 knockdown on cell apoptosis (Fig. 6d,e). Cell cycle analysis indicated that overexpression of p73 significantly abolished the negative effects of BAI1 downexpression on cell-cycle arrest (Fig. 6f,g). The resistance to cisplatin induced by BAI1 knockdowning in Hs578T and BT549 cells was also significantly abolished by p73 overexpression (Fig. 6h). Furthermore, the increased expression of Cyclin A2, Cyclin B1, Bcl2, and decreased expression of Bax, p21 induced by BAI1 downexpression was partly blocked by incubation with p73 overexpressing plasmids (Fig. 6i). These results provide evidence that the anti-tumorigenesis role of BAI1 is mediated through the regulation of p73 transcriptional activity via interaction with MDM2.

Discussion

In this study, we found that BAI1 expression was downregulated in TNBC, which correlated with poor DFS. Furthermore, we demonstrated that BAI1 induced cell apoptosis and suppressed tumor growth by increasing p73 transcriptional activity via interaction with MDM2 (Fig. 6j). In addition, BAI1 overexpressing increased the sensitivity to cisplatin in TNBC cells. Notably, there is an established body of research in several types of

cancer showing that BAI1 is involved in tumorigenesis and patient prognosis. Reduced BAI1 expression has been observed in several tumor types, including glioblastoma, Pulmonary adenocarcinomas, bladder transitional cell carcinoma, gastric and colorectal cancer[7–10, 30–32]. Immunohistochemistry staining indicated that lower levels of BAI1 contributed to poor prognosis of lung cancer patients[33]. Strikingly, previous study showed that significant downregulation of BAI1 in primary breast cancers and was associated with unfavorable patient outcomes[11]. Accordingly, our data suggested that BAI1 downexpression is associated with worse patient clinical outcomes, which is consistent with the previous study.

The investigation of the biological function of BAI1 in TNBC suggested that BAI1 suppressed TNBC cells proliferation. Meanwhile, cell apoptosis and cell cycle analysis also indicated that BAI1 could induce cell apoptosis and S/G2 cell cycle arrest. Ectopic BAI1 expression decreased Cyclin A2, Cyclin B1, and Bcl2 expression, but increased P21 and Bax expression. A recent research suggestted that BAI1 involved into the antitumorigenic effect of EPZ-6438 (Tazemetostat, an enhancer of zeste homolog 2 inhibitor), blocked medulloblastoma cell growth in vitro and in vivo, and prolonged survival in orthotopic xenograft models[34]. Moreover, BAI1 has also been found play an important role in the antitumorigenic action of HspB2 (Heat shock protein B2), which effectively inhibited pancreatic cancer cell proliferation[35]. Lei Liu et al found that overexpressed of BAI1 dramatically inhibited lung cancer cell proliferation, migration, invasion, colony formation, and in vivo metastasis by inducing metabolic reprograming[33]. According to our data, which suggested that BAI1 acts as a tumor suppressor and involves in tumor growth of TNBC.

BAI1 was found involved into the inhibition effect of decylubiquinone on breast cancer growth and metastasis[36]. Increasing evidence showed that p73 is an important determinant of chemosensitivity in TNBC via activated cell death signaling pathways[37, 38], and a large variety of chemotherapeutic agents, such as camptothecin, etoposide, and cisplatin, can upregulate p73 expression[39]. Cisplatin is a chemotherapy agent that has been widely used in clinical trials targeting breast cancers. Here, we found that BAI1 expression is related to sensitivity to cisplatin of TNBC. Our findings showed that BAI1 overexpression increased the sensitivity of MDA-MB-231 and MDA-MB-468 cells to cisplatin, while BAI1 knockdown in Hs578T and BT549 cells decreased the sensitivity to cisplatin. Taken together, these data suggest that BAI1 expression may be used to stratify TNBC patients for cisplatin therapy to enable more effective tailoring of chemotherapy.

Further investigations about the molecular mechanisms of BAI1 in malignant tumors confirmed the interaction between BAI1 and MDM2[29]. Previous studies found that MDM2 deletion increased levels of p73 and induced apoptosis and G2 cell-cycle arrest, which compensates for loss of p53 in p53-deficient cancers[25, 26, 40, 41]. Reports have indicated that p73 plays a key role in cancer growth and progression via MDM2 regulation in p53 mutated breast cancers [27, 42, 43]. We observed that BAI1 had no significant influence on the mRNA and protein expression of p73. The IP experiment confirmed the interaction between BAI1 and MDM2, and the IF analysis validated the decreasing interaction of p73 and MDM2 when overexpressing BAI1. As shown in our results, overexpressing of BAI1 could lead to increased expression of p73 target genes related to cell cycle progression and cell death signaling pathway. The luciferase assay confirmed that BAI1 overexpressing significantly induces the occupancy of p73 on the promoters of p21 and BAX, which supports our speculation that BAI1 can positively regulate p73 transcriptional activity by interacting with MDM2. To determine whether MDM2/p73 axis is a downstream target involved in BAI1-reduced TNBC growth and apoptosis suppression, we knocked in

p73 in BAI1 downexpressing cells. The BAI1 downexpression-mediated induction of cell growth and reduction of apoptosis was dramatically inhibited when p73 was knocked in. These data suggest that p73 is a crucial downstream target of BAI1 that mediates BAI1-reduced cell growth and BAI1-induced cell apoptosis in TNBC cells.

In summary, our study indicated that BAI1 was downregulated in TNBC tissues and BAI1 downexpression was associated with high risk of local recurrence and poorer clinical outcomes in TNBC patients. BAI1 suppressed TNBC cells growth by inhibiting cell cycle progression and inducing cell apoptosis. Moreover, BAI1 overexpressing increased TNBC cell sensitivity to cisplatin. Furthermore, BAI1 interacts with MDM2, then increases p73 transcriptional activity. It is important to further elucidate the interaction of BAI1 and MDM2 and test the combination of BAI1 inhibition and cisplatin to achieve an optimal effect in TNBC. Overall, our data suggested that BAI1 functioned as an anti-tumorigenesis protein in TNBC by interacting with MDM2 and increasing p73 transcriptional activity. Thus, BAI1 may represent a potential therapeutic target for the clinical intervention of TNBC in the future.

Abbreviations

ANTs: Adjacent noncancerous tissues; ATCC: American Type Culture Collection; BRCA: Breast cancer-associated gene; CCK8: Cell Counting Kit-8; DFS: Disease free survival; DMSO: Dimethyl sulfoxide; IF: Immunofluorescence; IHC: Immunohistochemistry; IP: Immunoprecipitation; MDM2: mouse double minute 2; OS: Overall survival; PCR: Polymerase chain reaction; qRT–PCR: Quantitative real-time PCR; BAI1: Brain-specific angiogenesis inhibitor 1; TCGA: The Cancer Genome Atlas; TMA: Tissue microarray; TNBC: Triple-negative breast cancer

Declarations

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were existed.

Authors' Contributions

Conception and design: All Authors conducted the study conception and design. X.Y carried out the assays and collected the samples. X.Y and P.W performed and interpreted the statistical analysis. X.Y and P.W written and reviewed the manuscript. R.S revised the manuscript and supervised this study. All authors reviewed and approved the final manuscript.

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Ethics approval and consent to participate

The study was approved by Ethics Institutional Review Board of Fudan University Shanghai Cancer Center. Written informed consent was obtained from all patients of the study.

Consent for publication

Informed consent was obtained from all individual participants included in the study.

References

1. BRAY F, FERLAY J, SOERJOMATARAM I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries [J]. CA Cancer J Clin. 2018;68(6):394–424.
2. BOSE S. Triple-negative Breast Carcinoma: Morphologic and Molecular Subtypes [J]. Adv Anat Pathol, 2015, 22(5): 306–13.
3. NISHIMORI H, SHIRATSUCHI T, URANO T, et al. A novel brain-specific p53-target gene, BAI1, containing thrombospondin type 1 repeats inhibits experimental angiogenesis [J]. Oncogene. 1997;15(18):2145–50.
4. CORK SM, VAN MEIR E G. Emerging roles for the BAI1 protein family in the regulation of phagocytosis, synaptogenesis, neurovasculature, and tumor development [J]. J Mol Med (Berl). 2011;89(8):743–52.
5. KAUR B, BRAT D J, DEVI N S, et al. Vasculostatin, a proteolytic fragment of brain angiogenesis inhibitor 1, is an antiangiogenic and antitumorigenic factor [J]. Oncogene. 2005;24(22):3632–42.
6. KAUR, B, CORK S M, SANDBERG E M, et al. Vasculostatin inhibits intracranial glioma growth and negatively regulates in vivo angiogenesis through a CD36-dependent mechanism [J]. Cancer Res. 2009;69(3):1212–20.
7. YOSHIDA Y, OSHIKA Y, FUKUSHIMA Y, et al. Expression of angiostatic factors in colorectal cancer [J]. Int J Oncol. 1999;15(6):1221–5.
8. HATANAKA H, OSHIKA Y, ABE Y, et al. Vascularization is decreased in pulmonary adenocarcinoma expressing brain-specific angiogenesis inhibitor 1 (BAI1) [J]. Int J Mol Med. 2000;5(2):181–3.
9. KAUR B, BRAT D J, CALKINS C C, et al. Brain angiogenesis inhibitor 1 is differentially expressed in normal brain and glioblastoma independently of p53 expression [J]. Am J Pathol. 2003;162(1):19–27.
10. LEE JH, KOH J T, SHIN B A, et al. Comparative study of angiostatic and anti-invasive gene expressions as prognostic factors in gastric cancer [J]. Int J Oncol. 2001;18(2):355–61.
11. MEISEN W H, DUBIN S, SIZEMORE S T, et al. Changes in BAI1 and nestin expression are prognostic indicators for survival and metastases in breast cancer and provide opportunities for dual targeted therapies [J]. Mol Cancer Ther. 2015;14(1):307–14.
12. HARDCASTLE J, KUROZUMI K, DMITRIEVA N, et al. Enhanced antitumor efficacy of vasculostatin (Vstat120) expressing oncolytic HSV-1 [J]. Mol Ther. 2010;18(2):285–94.
13. FUJII K, KUROZUMI K, ICHIKAWA T, et al. The integrin inhibitor cilengitide enhances the anti-glioma efficacy of vasculostatin-expressing oncolytic virus [J]. Cancer Gene Ther. 2013;20(8):437–44.

14. SHAH SP, GOYA ROTHA. R, et al. The clonal and mutational evolution spectrum of primary triple-negative breast cancers [J]. Nature. 2012;486(7403):395–9.
15. JOST C A, MARIN M C, KAELIN W G JR. p73 is a simian [correction of human] p53-related protein that can induce apoptosis [J]. Nature. 1997;389(6647):191–4.
16. KAGHAD M, BONNET H, YANG A, et al. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers [J]. Cell. 1997;90(4):809–19.
17. ZHU J, JIANG J, ZHOU W, et al. The potential tumor suppressor p73 differentially regulates cellular p53 target genes [J]. Cancer Res. 1998;58(22):5061–5.
18. MAI M, YOKOMIZO A, QIAN C, et al. Activation of p73 silent allele in lung cancer [J]. Cancer Res. 1998;58(11):2347–9.
19. SUNAHARA M, ICHIMIYA S, NIMURA Y, et al. Mutational analysis of the p73 gene localized at chromosome 1p36.3 in colorectal carcinomas [J]. Int J Oncol. 1998;13(2):319–23.
20. TAKAHASHI H, ICHIMIYA S, NIMURA Y, et al. Mutation, allelotyping, and transcription analyses of the p73 gene in prostatic carcinoma [J]. Cancer Res. 1998;58(10):2076–7.
21. OLINER JD, PIETENPOL J A, THIAGALINGAM S, et al. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53 [J]. Nature. 1993;362(6423):857–60.
22. MOMAND J, ZAMBETTI G P, OLSON D C, et al. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation [J]. Cell. 1992;69(7):1237–45.
23. HAUPT Y, MAYA R, KAZAZ A, et al. Mdm2 promotes the rapid degradation of p53 [J]. Nature. 1997;387(6630):296–9.
24. KUBBUTAT M H, JONES S N, VOUSDEN K H. Regulation of p53 stability by Mdm2 [J]. Nature. 1997;387(6630):299–303.
25. BALINT E, BATES S, VOUSDEN K H. Mdm2 binds p73 alpha without targeting degradation [J]. Oncogene. 1999;18(27):3923–9.
26. DOBBELSTEIN M, WIENZEK S, KONIG C, et al. Inactivation of the p53-homologue p73 by the mdm2-oncoprotein [J]. Oncogene. 1999;18(12):2101–6.
27. FEELEY K P, ADAMS C M, MITRA R, et al. Mdm2 Is Required for Survival and Growth of p53-Deficient Cancer Cells [J]. Cancer Res. 2017;77(14):3823–33.
28. ZHU D, LI C, SWANSON A M, et al. BAI1 regulates spatial learning and synaptic plasticity in the hippocampus [J]. J Clin Invest. 2015;125(4):1497–508.
29. ZHU D, OSUKA S, ZHANG Z, et al. BAI1 Suppresses Medulloblastoma Formation by Protecting p53 from Mdm2-Mediated Degradation [J]. Cancer Cell, 2018, 33(6): 1004–16 e5.
30. TIAN D, HU H, SUN Y, et al. Expression of brainspecific angiogenesis inhibitor1 and association with p53, microvessel density and vascular endothelial growth factor in the tissue of human bladder transitional cell carcinoma [J]. Mol Med Rep. 2015;12(3):4522–9.
31. CHOI Y J, LEE S H, KIM M S, et al. Whole-exome sequencing identified the genetic origin of a mucinous neoplasm in a mature cystic teratoma [J]. Pathology. 2016;48(4):372–6.
32. LIU H, LI F, ZHU Y, et al. Whole-exome sequencing to identify somatic mutations in peritoneal metastatic gastric adenocarcinoma: A preliminary study [J]. Oncotarget. 2016;7(28):43894–906.

33. LIU L, CHAI L, RAN J, et al. BAI1 acts as a tumor suppressor in lung cancer A549 cells by inducing metabolic reprogramming via the SCD1/HMGCR module [J]. Carcinogenesis, 2020.
34. ZHANG H, ZHU D, ZHANG Z, et al. EZH2 targeting reduces medulloblastoma growth through epigenetic reactivation of the BAI1/p53 tumor suppressor pathway [J]. Oncogene. 2020;39(5):1041–8.
35. YU Z, WANG H, FANG Y, et al. Molecular chaperone HspB2 inhibited pancreatic cancer cell proliferation via activating p53 downstream gene RPRM, BAI1, and TSAP6 [J]. J Cell Biochem. 2020;121(3):2318–29.
36. CAO J, LIU X, YANG Y, et al. Decylubiquinone suppresses breast cancer growth and metastasis by inhibiting angiogenesis via the ROS/p53/ BAI1 signaling pathway [J]. Angiogenesis, 2020, undefined.
37. LUNGHI P, COSTANZO A. MAZZERA L, et al. The p53 family protein p73 provides new insights into cancer chemosensitivity and targeting [J]. Clin Cancer Res. 2009;15(21):6495–502.
38. TONSING-CARTER E, BAILEY B J, SAADATZADEH M R, et al. Potentiation of Carboplatin-Mediated DNA Damage by the Mdm2 Modulator Nutlin-3a in a Humanized Orthotopic Breast-to-Lung Metastatic Model [J]. Mol Cancer Ther. 2015;14(12):2850–63.
39. IRWIN MS, KONDO K, MARIN MC, et al. Chemosensitivity linked to p73 function [J]. Cancer Cell. 2003;3(4):403–10.
40. ZENG X, CHEN L, JOST C A, et al. MDM2 suppresses p73 function without promoting p73 degradation [J]. Mol Cell Biol. 1999;19(5):3257–66.
41. WANG X Q, ONGKEKO W M, LAU A W, et al. A possible role of p73 on the modulation of p53 level through MDM2 [J]. Cancer Res. 2001;61(4):1598–603.
42. WU H. LENG R P. MDM2 mediates p73 ubiquitination: a new molecular mechanism for suppression of p73 function [J]. Oncotarget. 2015;6(25):21479–92.
43. KUBO N, OKOSHI R, NAKASHIMA K, et al. MDM2 promotes the proteasomal degradation of p73 through the interaction with Itch in HeLa cells [J]. Biochem Biophys Res Commun, 2010, 403(3–4): 405 – 11.

Tables

Table 1
clinicopathological characteristics of patient samples and expression of BAI1 in TNBCs

Variables		No. of patients (%)	BAI1 expression		*P* value
			Low (N = 84)	High (N = 36)	
Age(y)	50	71(59.2)	45 (37.5)	26 (21.7)	0.079
	≥ 50	49(40.8)	39 (32.5)	10 (8.3)	
Tumor size	≤ 2 cm	82(68.3)	64 (53.3)	18 (15.0)	0.018
	2-5cm	17(14.2)	9 (7.5)	8 (6.7)	
	5 cm	21(17.5)	11 (9.2)	10 (8.3)	
Historical grade	I-II	12(10.0)	7 (5.8)	5 (4.2)	0.378
	III	100(83.3)	70 (58.4)	30 (25.0)	
	Unclassified	8(6.7)	7 (5.8)	1 (0.8)	
Ki67 index (%)	≤ 30	21(17.5)	19 (15.8)	2 (1.7)	0.034
	30	99(82.5)	65 (54.2)	34 (28.3)	
node status	N0	54(45.0)	40 (33.3)	14 (11.7)	0.451
	N1	50(41.7)	35 (29.2)	15 (12.5)	
	N2	9(7.5)	5 (4.2)	4 (3.3)	
	N3	7 (5.8)	4 (3.3)	3 (2.5)	
Lymphovascular invasion	Negative	77(64.2)	59 (49.2)	18 (15.0)	0.067
	Positive	43(35.8)	25 (20.8)	18 (15.0)	
Disease recurrence	No	91(75.8)	71 (59.2)	20 (16.6)	0.010
	Yes	29(24.2)	13 (10.8)	16 (13.4)	
Distance metastasis	No	102(85.0)	77 (64.2)	25 (20.8)	0.067
	Yes	18(15.0)	7 (5.8)	11 (9.2)	

Table 2
Correlations between BAI1 with DFS and OS in TNBCs

Variables		Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
		Disease-free survival				Overall survival			
Age (Y)	< 50	-	-	-	-	-	-	-	-
	≥ 50	0.67 (0.50–1.77)	0.715			0.75 (0.63–3.11)	0.721		
Tumor size	≤ 2 cm	-	-	-	-	-	-	-	-
	2-5cm	1.96 (1.27–5.47)	0.007	1.54 (1.12–3.38)	0.068	1.31 (1.04–5.32)	0.004	2.14 (1.52–8.72)	0.047
	5 cm	2.2 (1.39–6.58)	< 0.001	2.28 (1.02–5.45)	0.030	1.49 (1.11–5.64)	< 0.001	2.74 (1.16–9.58)	0.026
Histologic grade	I-II	-	-	-	-	-	-	-	-
	III	0.78 (0.42–1.47)	0.446			0.56 (0.11–3.07)	0.443		
	Unclassified	0.63 (0.36–2.77)	0.822			0.62 (0.12–3.44)	0.508		
Ki67 index (%)	≤ 30	-	-	-	-	-	-	-	-
	> 30	0.54 (0.44–4.67)	0.548			0.88 (0.33–2.31)	0.792		
Node status	N0	-	-	-	-	-	-	-	-
	N1	0.92 (0.16–3.54)	0.180	0.87 (0.49–1.56)	0.644	0.71 (0.88–4.29)	0.230	0.65 (0.38–2.72)	0.253
	N2	0.98 (0.77–1.84)	0.090	1.01 (0.71–2.21)	0.285	0.85 (0.64–3.13)	0.070	1.39 (0.83–2.33)	0.126
	N3	1.22 (1.03–3.15)	0.023	1.47 (0.94–2.30)	0.088	1.36 (1.12–4.24)	0.001	1.52 (1.08–3.28)	0.027

Variables		Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Lympho-vascular invasion	Negative	-	-	-	-	-	-	-	-
	Positive	8.23 (3.20-21.09)	< 0.001	2.68 (1.19–8.53)	0.012	0.75 (0.40–1.31)	0.237		
BAI1 expression	Low	-	-	-	-	-	-	-	-
	High	0.55 (0.26–0.87)	0.002	0.47 (0.36–0.98)	0.038	0.95 (0.81–1.42)	0.104		

Figures

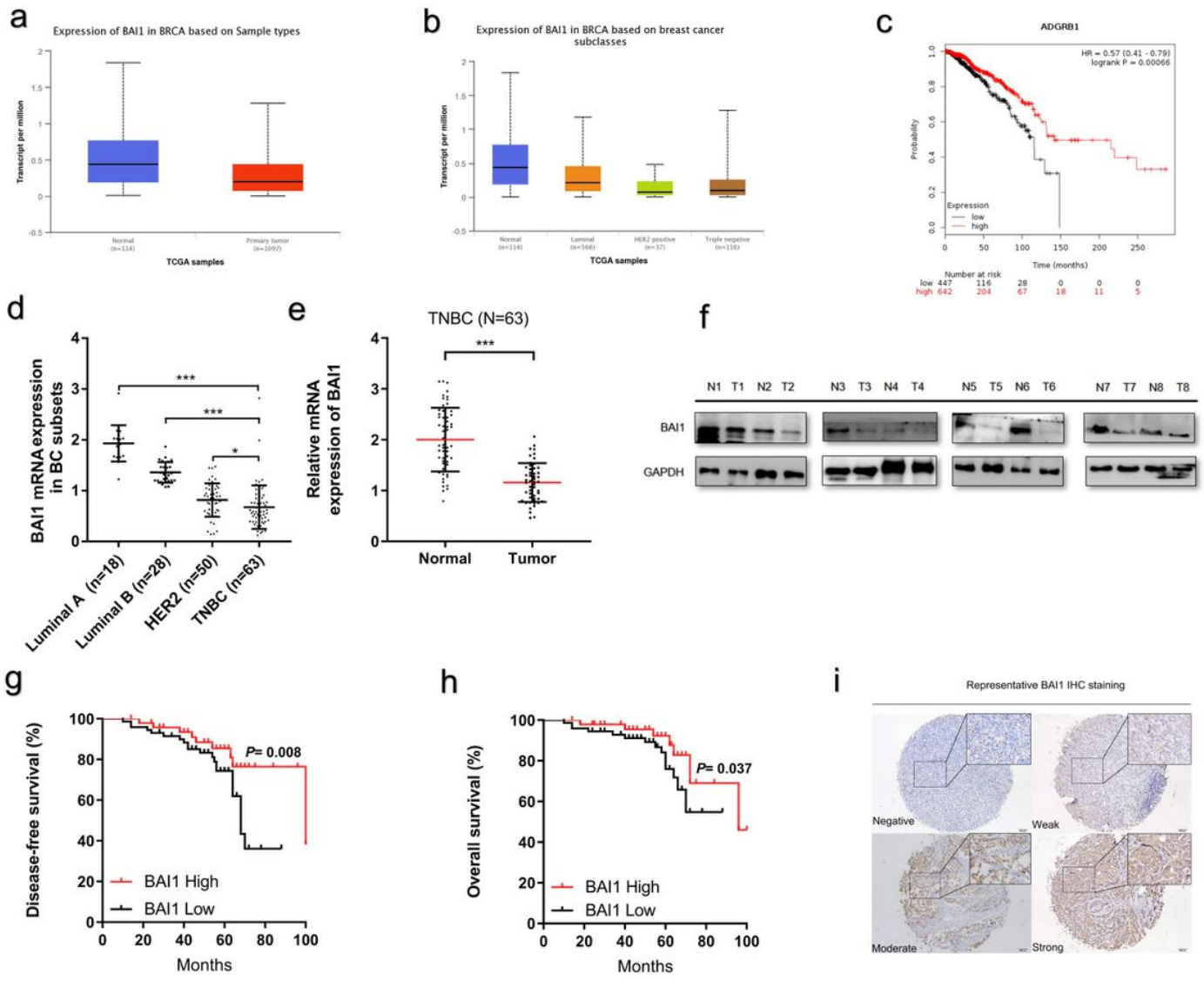

Figure 1

Downregulated of BAI1 promotes TNBC progression and related to poor prognosis. BAI1 mRNA levels in tumor tissues versus normal tissues (a) and in different molecular subtypes of breast cancer (b) in TCGA breast cancer mRNA dataset. Kaplan–Meier curve of OS for BC patients with BAI1 high-expression versus BAI1 down-expression group in TCGA breast cancer mRNA dataset(c). BAI1 mRNA levels in different molecular subtypes of breast cancer (n=159) (d) and in paired tumor tissues versus non-tumor tissues in TNBC samples (n=63) (e) in our cohort. Protein expression of BAI1 in paired TNBC versus non-tumor tissues in our cohort (n=8) (f). Kaplan–Meier curve of DFS (g) and OS (h) in TNBC patients with BAI1 protein high-expression versus BAI1 down-expression group in our cohort. Representative IHC image of BAI1 expression in TNBC samples(i). All *p<0.05, **p<0.01, ***p<0.001, n.s. not significant

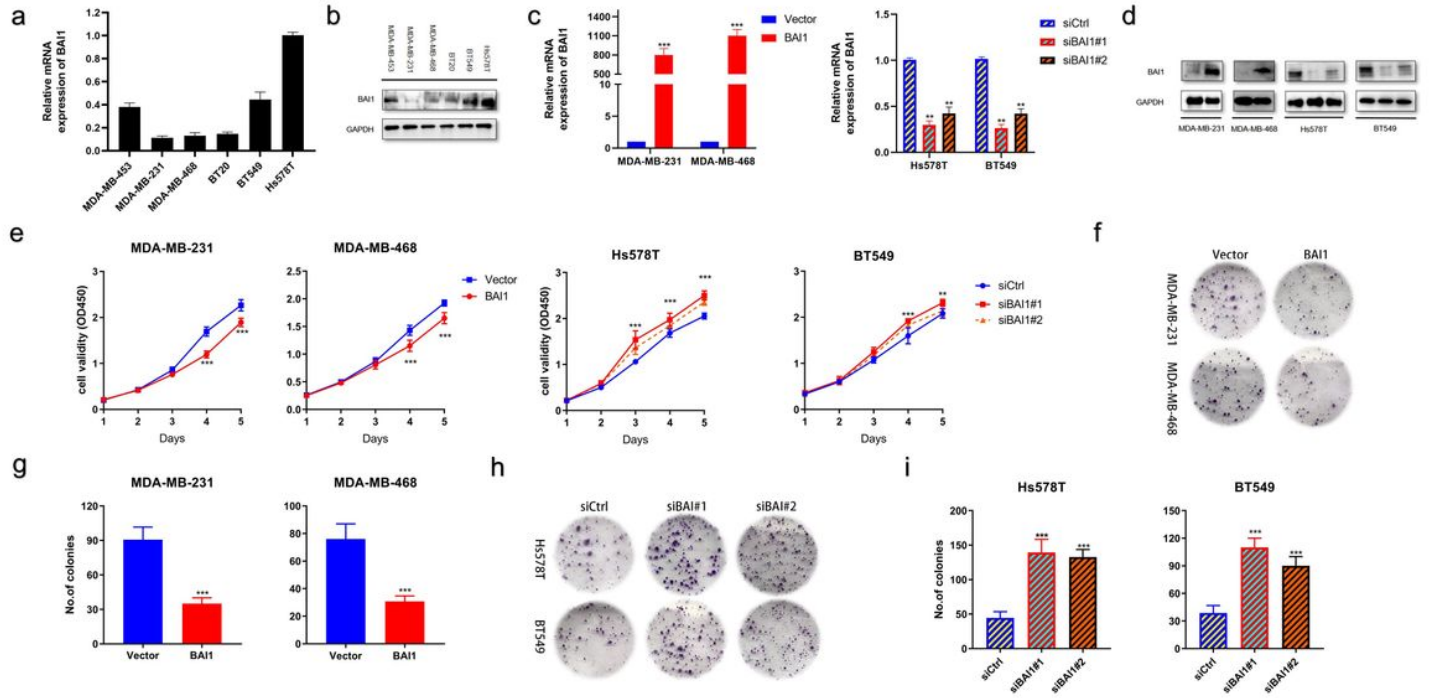

Figure 2

Downregulated of BAI1 promotes the proliferation of TNBC. mRNA (a) and protein expression (b) levels of BAI1 in six TNBC cell lines. RT-qPCR (c) and Western blot (d) results showed the efficiencies of BAI1 overexpression in MDA-MB-231 and MDA-MB-468 cells and BAI1 knockdown in Hs-578t and BT549 cells. CCK-8 assays (e) and colony-forming (f-i) assays showed that overexpression of BAI1 suppress cell proliferation in MDA-MB-231 and MDA-MB-468 cells, while knockdown of BAI1 promote cell proliferation in Hs-578t and BT549 cells. All *p<0.05, **p<0.01, ***p<0.001, n.s. not significant

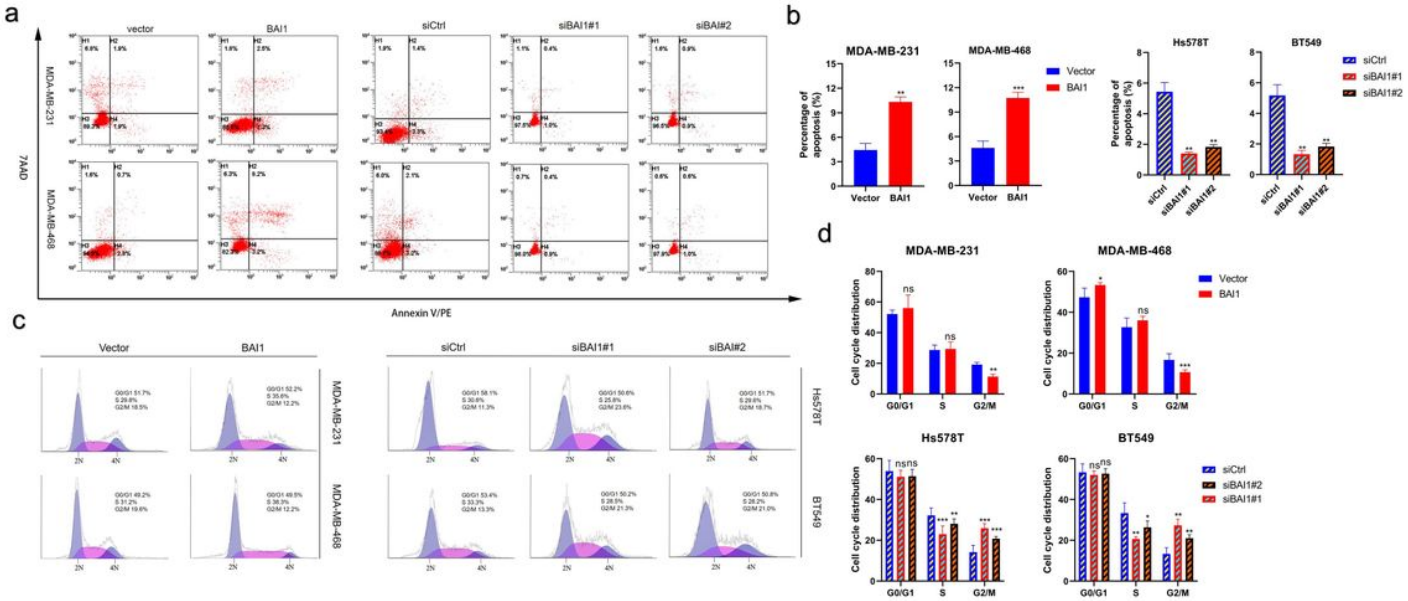

Figure 3

BAI1 expression induces cell apoptosis and promotes cell cycle arrest of TNBC. (a-b) Flow cytometry showed that overexpression of BAI1 increase cell apoptosis in MDA-MB-231 and MDA-MB-468 cells, while knockdown of BAI1 decrease cell apoptosis in Hs-578t and BT549 cells. (c-d) Flow cytometry indicated that overexpression of BAI1 inhibit S to G2 transition in MDA-MB-231 and MDA-MB-468 cells, while knockdown of BAI1promote S to G2 transition in Hs-578t and BT549 cells. All *p<0.05, **p<0.01, ***p<0.001, n.s. not significant

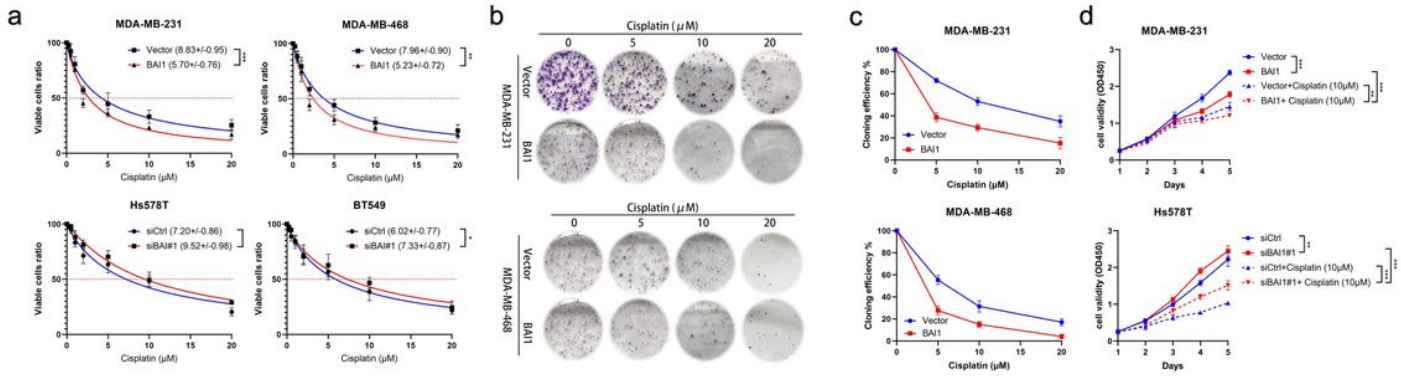

Figure 4

BAI1 enhances cisplatin sensitivity of TNBC. (a) CCK8 assays showed IC50 values for cisplatin in BAI1 overexpression MDA-MB-231 and MDA-MB-468 cells and BAI1 knockdown Hs-578t and BT549 cells. (b-c) Colony-forming assays indicated that overexpression of BAI1 promote the efficacy of cisplatin in MDA-MB-231 and MDA-MB-468 cells. (d) CCK-8 assays showed that overexpression of BAI1 increase the efficacy of cisplatin in MDA-MB-231 cells, while knockdown of BAI1 decrease the efficacy of cisplatin in Hs-578t cells. All *p<0.05, **p<0.01, ***p<0.001, n.s. not significant

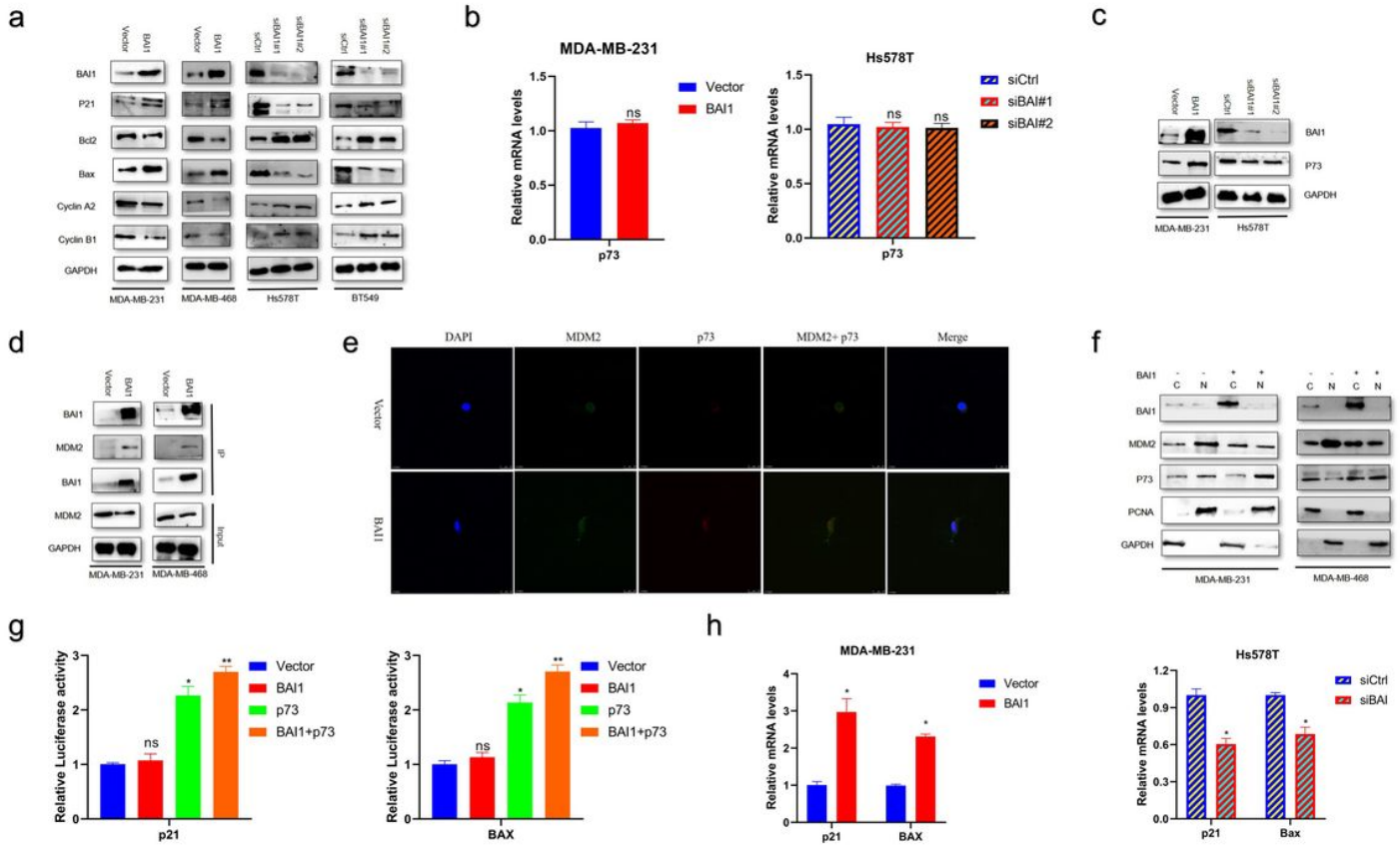

Figure 5

BAI1 increases p73 expression and transcriptional activity through interaction with MDM2. (a) Western blot of BAI1, P21, Bcl2, Bax, Cyclin A2 and Cyclin B1 in MDA-MB-231 and MDA-MB-468 cells treated with BAI1 overexpression and Hs-578t and BT549 cells treated with BAI1 siRNAs. (b) RT-qPCR results suggested that the mRNA level of p73 was not related to BAI1 in BAI1-overexpressing MDA-MB-231 and BAI1-knockdown Hs-578t cells. (c) Western blot results showed that the protein level of p73 in BAI1-overexpressing MDA-MB-231 and BAI1-knockdown Hs-578t cells. (d) IP assays indicated that exogenously express Flag-BAI1 specifically interact with endogenous MDM2 in MDA-MB-231 and MDA-MB-468 cells. (e) IF assays suggested that BAI1 reduce the co-localization of MDM2 and p73 in MDA-MB-231 cells. (f) cytoplasmic and nuclear fractions analysis showed that BAI1 increase the expression of p73 and reduce the content of MDM2 in the nucleus. (g) Luciferase assay results showed that BAI1 overexpressing in MDA-MB-231 cells induce the occupancy of p73 on the promoters of p21 and BAX. (h) RT-qPCR results suggested that mRNA expression of p21, Bax were upregulated or downregulated by overexpression or knockdown of BAI1. All *p<0.05, **p<0.01, ***p<0.001, n.s. not significant

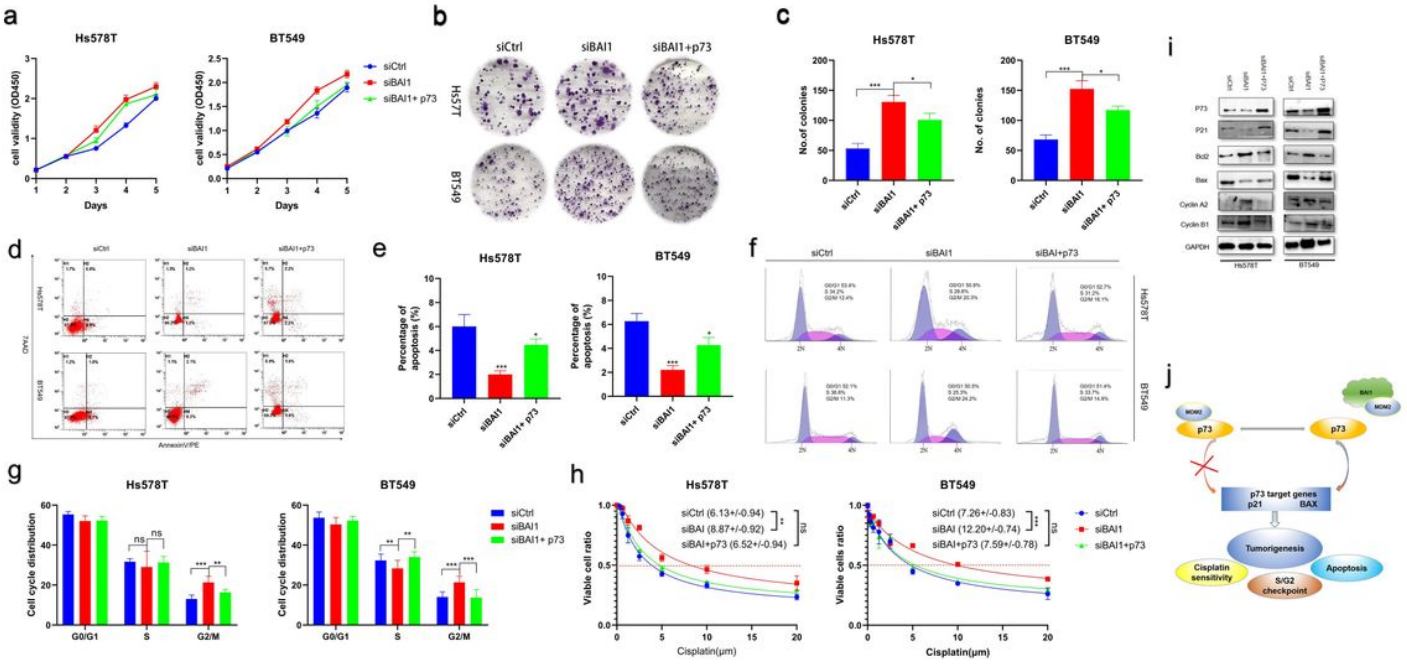

Figure 6

p73 is partially involved in BAI1 regulated breast cancer cell growth. CCK-8 (a) and colony-forming (b,c) assays showed that overexpression of p73 partially attenuate the increased cell proliferation induced by knockdown of BAI1 in Hs578t and Bt549 cells. (d,e) Flow cytometry indicated that overexpression of p73 partially reverse the reduced cell apoptosis induced by knockdown of BAI1 in Hs578t and Bt549 cells. (f,g) Flow cytometry indicated that overexpression of p73 partially reverse the enhanced S to G2 transition induced by knockdown of BAI1 in Hs578t and Bt549 cells. (h) CCK8 assays showed IC50 values for cisplatin in BAI1 knockdown Hs578t and Bt549 cells transfected with p73 plasmid. (i) Western blot of p73, P21, Bcl2, Bax, Cyclin A2 and Cyclin B1 in BAI1 knockdown Hs578t and Bt549 cells treated with p73 plasmid. (j) Model showed the effect of BAI1/MDM2/p73 axis on breast tumor growth. All *p<0.05, **p<0.01, ***p<0.001, n.s. not significant