Article

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Wild-Type p53 Regulates Apoptosis of Human Breast Cancer Cells

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Background: The tumor suppressor wild-type p53 is known for its role in inducing apoptosis in tumor cells. This study investigated the relationship between wild-type p53 and protein phosphatase 1 (PP1) and caspase in promoting apoptosis of breast cancer cells.

Methods: Human breast cancer cell lines MCF-7 and MDA-MB-231 obtained from the American Type Culture Collection were used in this study. Small interference RNAs (Si-RNA) and plasmids were used to regulate wild-type p53 expression in these two tumor cell lines through liposome-mediated transfection. GSK-2830371 (PP1 inhibitor) and zVAD (Caspase inhibitor) were employed to further verify the PP1 activating function of wild-type p53 in Caspase-dependent MCF-7 and MDA-MB-231 apoptosis. PP1 activity was quantitatively detected by phosphorus colorimetric assay. Co-immunoprecipitation (Co-IP), flow cytometry assay, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, Western blot, the real-time reverse transcriptase-polymerase chain reaction (RT-qPCR), and immunofluorescence staining were used to analyze cell apoptosis degree and marker protein expression.

Results: The expression level of PP1 in the breast cancer cells was successfully regulated by cell transfection. The phosphatase activity was increased, and obvious apoptotic cytological characteristics were observed in p53-overexpressed breast cancer cells. p53 knockdown/overexpression increased/decreased the level of B cell lymphoma 2 (Bcl-2), and decreased/increased levels of Caspase-3, cleaved Caspase-3, cleaved Caspase-8, Cytochrome C (Cyt-C), Truncated BID (tBid), Bcl-2-associated X (Bax), and cell apoptosis ($p < 0.01$). The promotion of proteins and apoptosis induced by p53 overexpression was reversed by GSK-2830371 or zVAD.

Conclusion: Wild-type p53 might promote Caspase-dependent apoptosis of human breast cancer cells through PP1 activation.

Keywords: wild-type p53; protein phosphatase 1; Caspase-dependent apoptosis; human breast cancer

Introduction

Breast cancer is the most common cancer type in females, and mainly occurs in middle-aged and older women [1]. The American Cancer Society estimated that roughly 43,200 women would die from breast cancer in the United States in 2023. Breast cancer has been ranked as the malignancy with the highest incidence worldwide since 2020 and has the highest mortality among cancers in women [2–5]. With the help of the optimized breast cancer screening strategy and the enhanced awareness of early detection and early treatment, the prognosis and survival rate of breast cancer patients have been gradually improved, but this trend has slowed down in recent years [6]. Therefore, there is an urgent need for new and effective therapeutic strategies or targets for breast cancer treatment.

In recent years, one of the research hotspots in tumor treatment has been cell apoptosis [7–9]. Many tumor suppressor genes suppress tumor growth by inducing tumor cell apoptosis [10,11]. Moreover, the primary mechanisms of some chemotherapeutic medicines involve promotion of apoptosis in tumor cells as well [12,13]. Apoptosis is a cell death pathway that operates under physiological and pathological conditions. Wild-type p53 is the most common tumor suppressor gene in human malignant tumors [14]. p53 is a transcription factor that is affected by stress signals in cells. It is activated in response to DNA damage [15,16], hypoxia [17,18], nutritional deprivation [19], oncogene activation [20], etc. The protein products of p53 target genes have been shown to be involved in the process of cell cycle arrest [21], apoptosis [22], senescence [23], autophagy [24], and cell metabolic adaptation [25]. p53, along with its regulated signaling pathways, has been shown to play an important role in cancer treatment [26–28]. Protein phosphatase 1 (PP1), a protein expressed by a target gene regulated by p53, has been found to affect the apoptosis of breast cancer

cells [29,30]. Other studies [31–33] have shown that p53 could participate in Caspase-dependent apoptosis during tumor progression. The deletion or mutation of p53 can promote tumorigenesis both *in vitro* and *in vivo* [34,35]. Mutations in p53 have been widely observed in cancers, and such mutations are linked to function defects and carcinogenesis [14]. Thus, p53 remains an attractive target for cancer therapy. Therapeutic strategies based on p53 have been established, including restoration of p53 or elimination of mutant p53 [14,36,37], inhibition of p53-related interaction [38], and p53-based vaccines [39].

In this study, we explored the effects of p53 on PP1 activation, as well as effects of PP1 activation on tumor apoptosis in breast cancer cells.

Materials and Methods

Cell Culture

Human breast cancer cell lines MDA-MB-231 (HTB-26) and MCF-7 (HTB-22) were obtained from the American Type Culture Collection (Manassas, VA, USA), and were cultured in McCoy's 5A medium (SK-BR-3, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; C0235, Beyotime, Shanghai, China) at 37 °C and 5% CO_2. Cell culture medium was collected and a PCR mycoplasma test kit (K0103, HuaAn Biotech, Hangzhou, China) was use to verify that MDA-MB-231 and MCF-7 cells were mycoplasma free. Short tandem repeat (STR) profiling was performed to authenticate MDA-MB-231 and MCF-7 cell lines.

Cell Transfection

Small interference RNAs (Si-RNA) against p53 (Si-p53) or scrambled negative control (Si-NC) were transfected into cells according to the manufacture's protocols for p53 downregulation. p53 si-RNA was designed and synthesized by GeneChem Inc. (Daejeon, Korea; Gene ID: GCD950481; sequence, 5′-GCAUGAACCGGAGGCCCAU-3′) and the control siRNA (cat no. D6145) was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Plasmids encoding p53 overexpression-RNA (OE-p53) and blank plasmids (OE-NC) were transfected in cells for p53 upregulation. p53 overexpression plasmid (Catalog: 24859) and the control plasmids (Catalog: 24860) were obtained from Addgene, Watertown, MA, USA.

Western Blot Assay

Total proteins were extracted from cells using Radio Immunoprecipitation Assay (RIPA) (P0013, Beyotime, Shanghai, China). After protein concentration was determined and proteins were separated, they were transferred to membranes. Then, membranes containing proteins were incubated with primary antibodies at 4 °C. Primary antibodies included: p53 (1:1000 dilution; 10442-1-AP, Proteintech, Hubei, China), anti-PP1 (1:1000 dilution; ab308389, Abcam, Cambridge, MA, USA), cleaved Caspase-3 (1:1000 dilution; A16793, ABclonal, Wuhan, China), cleaved Caspase-8 (1:1000 dilution; A11324, ABclonal, Wuhan, China), Cytochrome C (Cyt-C) (1:1000 dilution; A13430, ABclonal, Wuhan, China), Truncated BID (tBid) (1:1000 dilution; ab10640, Abcam, Cambridge, MA, USA), B cell lymphoma 2 (Bcl-2) (1:1000 dilution; A19693, ABclonal, Wuhan, China), Bcl-2-associated X (Bax) (1:1000 dilution; A19684, ABclonal, Wuhan, China), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000; A19056, ABclonal, Wuhan, China). Membranes were subsequently incubated with the horseradish-peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:1000 dilution; A0208, Beyotime, Shanghai, China) at room temperature. Finally, the bands were measured using an enhanced chemiluminescence kit (ab133406, Abcam, Cambridge, MA, USA). ImageJ (1.48, National Institutes of Health, Rockville, MD, USA) was used to quantify the intensity of the protein bands and mean gray value was recorded as the raw value. Background signal from each band was subtracted to correct for nonspecific staining. The intensity of each protein band was normalized to a loading control (GAPDH) to correct for variations in protein loading or transfer efficiency.

Phosphate Colorimetric Assay

The cells were diluted to a concentration of 5×10^6 for use, washed and mixed with reagent A, and then centrifuged at 4 °C, 300 ×g, for 5 min. After discarding the supernatant, 500 µL of Reagent B was added and mixed. Cells were incubated in an ice tank for 30 min, and then centrifuged at 4 °C, 16,000 ×g, for 5 min. Then, 2 µL was collected for quantitative determination of protein. Reagent E and Reagent I were added to the solution and mixed. 40 µL of the sample to be tested was transferred to a new cuvette, and 70 µL of buffer Reagent C and 10 µL of inhibitor Reagent D were added. After incubation at 30 °C for 2 min, 20 µL of the reaction solution Reagent F was added and incubated at 30 °C for 10 min. 60 µL of stop solution Reagent G was added and mixed well. Finally, 800 µL of Reagent H was added and mixed well. The absorbance reading at 660 nm was measured using a phosphate colorimetric assay purchased from Sigma (MAK030, Sigma-Aldrich, Burlington, MA, USA). The phosphorus concentration (micromolar/liter) corresponding to the sample activity was obtained according to the standard curve, and the sample activity was calculated according to the formula ($S_a/S_v = C$, S_a = amount of phosphate in the unknown sample well (nmol) from standard curve; S_v = sample volume (mL) added to reaction well; C = concentration of phosphate in sample).

Cell Morphology Examination

Cell morphology was observed under a phase contrast microscope (CKX53, Olympus, Tokyo, Japan). After

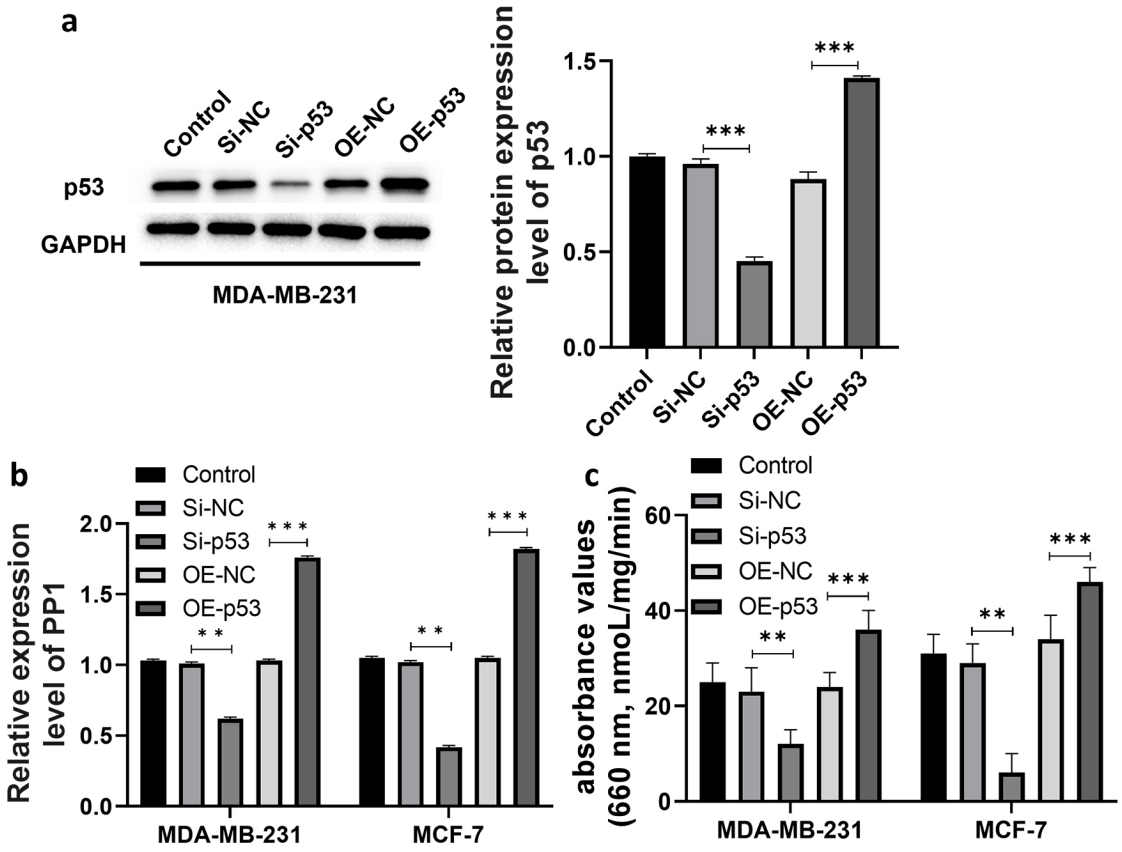

Fig. 1. Wild type p53 induces the production and activation of protein phosphatase 1 (PP1). (a) The protein expression of p53 after silencing p53 or overexpressing p53. (b) The mRNA expression of *PP1* in each group. (c) *In vitro* detection of PP1 activity. N = 5. $^{**}p < 0.01$ $^{***}p < 0.001$. NC, negative control.

4',6-diamidino-2-phenylindole (DAPI) staining, morphological observation was performed under a fluorescence microscope (BX63, Olympus, Tokyo, Japan) and the typical characteristics of apoptotic cells were marked.

Apoptosis Assay

Cells were digested by protease K (39450-01-6, Roche, Basel, Switzerland) and incubated with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reagent (11684817910, Roche, Basel, Switzerland) overnight at 4 °C. After cells were stained by DAPI (C1002, Beyotime, Shanghai, China), they were observed under the fluorescence microscope (CKX53, Olympus, Tokyo, Japan).

Flow Cytometry Detection (Annexin V/PI Staining)

After harvesting, cells were divided into four groups: an untreated control group, a single-stained Annexin-V group, a single-stained propidium iodide (PI) group, and a double-stained PI and Annexin-V group. Under subdued light conditions, appropriate dyes from Annexin-V-FITC Apoptosis Detection Kit (C1062S, Beyotime, Shanghai, China) were added to corresponding groups. Cells were incubated in subdued light at room temperature for 15 min. Then, cells were analyzed using a flow cytometer (FC500 MLP, Beckman Coulter Inc., Brea, CA, USA) within 1 h.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from cells using TRIZOL Reagent (12183555, Thermo Fisher Scientific, Wilmington, NC, USA) and cDNA was prepared using FastQuant cDNA first-strand Synthesis kit according to the relevant protocol (K1622, Thermo Fisher Scientific, Wilmington, NC, USA). The specific amplification primers were designed based on the target gene sequence (Table 1). Real-time reverse transcriptase-polymerase chain reaction (RT-qPCR) was performed with Fast SYBR Green QPCR master mix (4385612, Thermo Fisher Scientific, Wilmington, NC, USA).

Co-Immunoprecipitation (Co-IP) Assay

Immunoprecipitation (IP) lysis buffer (20 mM Tris pH7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 0.1% NP40, 1% pro-tease inhibitor, P0013, Beyotime, Shanghai, China) was used for cell lysis. Then cell lysates

Table 1. The target gene sequence.

Primer name	Primer sequences (5′-3′)
Protein phosphatase 1-F	ATGCTGGGGGGGGGTCAC
Protein phosphatase 1-R	CCTTTATTCAAGAGACCAGATGGG
GAPDH-F	GGACTCATGACCACAGTCCAT
GAPDH-R	TTCCAGTAGGGACTCGACTTG
p53-F	CCTCAGCATCTTATCCGAGTGG
p53-R	TGGATGGTGGTACAGTCAGAGC

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

were precleared with Protein A+G Agarose (P2055, Beyotime, Shanghai, China), followed by one hour of incubation with p53 (P8999, Sigma-Aldrich, Burlington, MA, USA), PP1 (ab308389, Abcam, Cambridge, MA, USA), or control IgG (B900620, Proteintech, Hubei, China) at 4 °C. The immunoprecipitations were captured on Protein A+G Agarose and analysed by Western blot with antibodies against p53 or PP1, respectively.

Statistical Analysis

Statistical analysis was performed by GraphPad Prism 8.0.2 (GraphPad Software, La Jolla, CA, USA). Data were presented as mean ± standard deviation (SD). Group differences were tested using independent samples, 2-tailed Student's *t* tests or one-way analysis of variance with Tukey's post hoc test. p values < 0.05 were considered statistically significant.

Results

Wild Type p53 Induces the Production and Activation of PP1

Fig. 1a shows that after silencing p53, the expression level of p53 decreases significantly ($p < 0.001$), while after overexpression, the expression level of p53 increases significantly ($p < 0.001$). The mRNA levels of *PP1* were diminished in MDA-MB-231 and MCF-7 cells transfected with Si-p53 compared to those transfected with Si-NC, indicating a suppressive effect of p53 silencing on PP1 expression. Conversely, *PP1* mRNA was elevated in cells transfected with OE-p53 relative to the OE-NC group, suggesting that p53 overexpression promotes PP1 synthesis (Fig. 1b, $p < 0.001$). PP1 activity assay showed that the Si-p53 group displayed decreased PP1 activity in both breast cancer cell groups, whereas the OE-p53 group displayed increased PP1 activity in both cell groups (Fig. 1c, $p < 0.05$, $p < 0.01$). Subsequently, the efficiency of p53 silencing and overexpression was verified.

Wild-Type p53 Promotes Caspase-Dependent Apoptosis

As shown in Fig. 2a, the degree of apoptosis was higher in the OE-p53 group than in the OE-NC group ($p < 0.001$). The degree of apoptosis was reduced in the Si-p53 group compared with Si-NC group ($p < 0.001$). No significant differences in cell apoptosis were observed among the control group, the Si-NC group, and the OE-NC group. The results of Western blot are shown in Fig. 2b,c. The expression levels of cleaved Caspase-3, cleaved Caspase-8, Cyt-C, tBid, and Bax were significantly increased in OE-p53-transfected MDA-MB-231 cells ($p < 0.001$), whereas they were significantly decreased in Si-p53-transfected MDA-MB-231 cells ($p < 0.001$). On the other hand, the Bcl-2 level was significantly decreased in OE-p53-transfected MDA-MB-231 cells ($p < 0.001$), whereas it was significantly increased in Si-p53-transfected MDA-MB-231 cells ($p < 0.001$). In Fig. 2d, flow cytometry detection of cell apoptosis showed the same pattern of differences. When wild-type p53 was decreased in the Si-p53 group, fewer apoptotic cancer cells were detected compared with the Si-NC group (20.22% vs 32.2%) ($p < 0.001$). In contrast, overexpression of wild-type p53 promoted greater cell apoptosis in the OE-p53 group compared with the OE-NC group (56.4% vs 30.37%) ($p < 0.001$).

p53 Regulates Cell Apoptosis of Breast Cancer by Activating PP1

The results of Western blot are shown in Fig. 3a. The PP1 level was decreased by GSK-2830371 (PP1 inhibitor) or zVAD (Caspase inhibitor) in the OE-p53+zVAD and OE-p53+GSK-2830371 groups compared with the OE-p53 group ($p < 0.001$). The expression levels of cleaved Caspase-3 and cleaved Caspase-8 were lower in both OE-p53+GSK-2830371 and OE-p53+zVAD groups compared with the OE-p53 group ($p < 0.001$). There were no significant differences between the Control group and the OE-NC group. Moreover, TUNEL results showed that the apoptosis rate of the OE-p53+GSK-2830371 group and the OE-p53+zVAD group was lower than that of the OE-p53 group (Fig. 3b). In Fig. 3c, flow cytometry detection of cell apoptosis shows the same pattern as the TUNEL results. Compared to the OE-p53+GSK-2830371 and OE-p53+zVAD groups, an increased percentage of Annexin V-positive and PI-positive cells was observed in the OE-p53 group (76.1% vs 61.5% and 76.1% vs 61.4%, respectively). There were no significant differences between the Control group and the OE-NC group ($p < 0.001$). To further verify the regulatory function of p53 on PP1, Co-IP assay was used. Results in Fig. 3 showed that PP1 and P53 can be co-precipitated. These results confirmed the binding status of p53 and PP1 in MDA-MB-231 cells.

Discussion

p53 is responsible for the regulation of transcriptional homeostasis under the stimulation of carcinogenic factors in human body. Loss of p53 function is considered a common feature of cancers, including breast cancer [40]. Therefore, activating wild-type p53 has become a potential can-

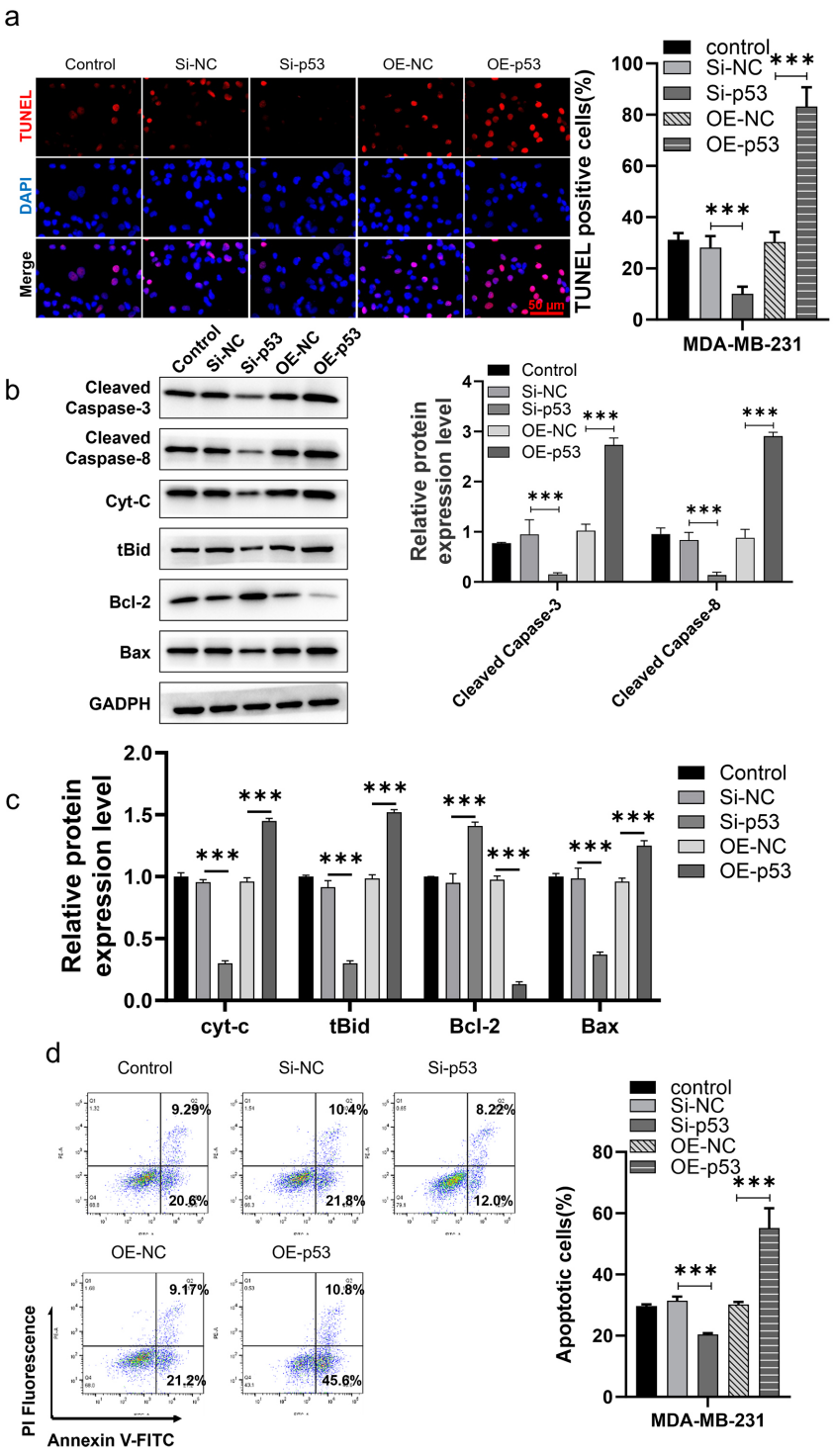

Fig. 2. Wild-type p53 promotes Caspase-dependent apoptosis of breast cancer cells. (a) TUNEL method was used to detect the degree of cell apoptosis. Scare bar = 50 μm. (b,c) Western blot results showed the content of Caspase protein and apoptosis marker protein. (d) Flow cytometry detection (Annexin-V/PI staining) was used to detect the degree of cell apoptosis. N = 5, ***$p < 0.01$. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; Cyt-C, Cytochrome C; tBid, Truncated BID; Bcl-2, B cell lymphoma 2; Bax, Bcl-2-associated X.

cer treatment strategy. In mammalian tumor cells, apoptosis could be induced by two distinct yet interrelated pathways: the BCL-2 regulated pathway, also referred to as intrinsic or mitochondrial pathway, and the death receptor pathway, also known as the extrinsic pathway. The BCL-2-regulated pathway is triggered by various stress condi-

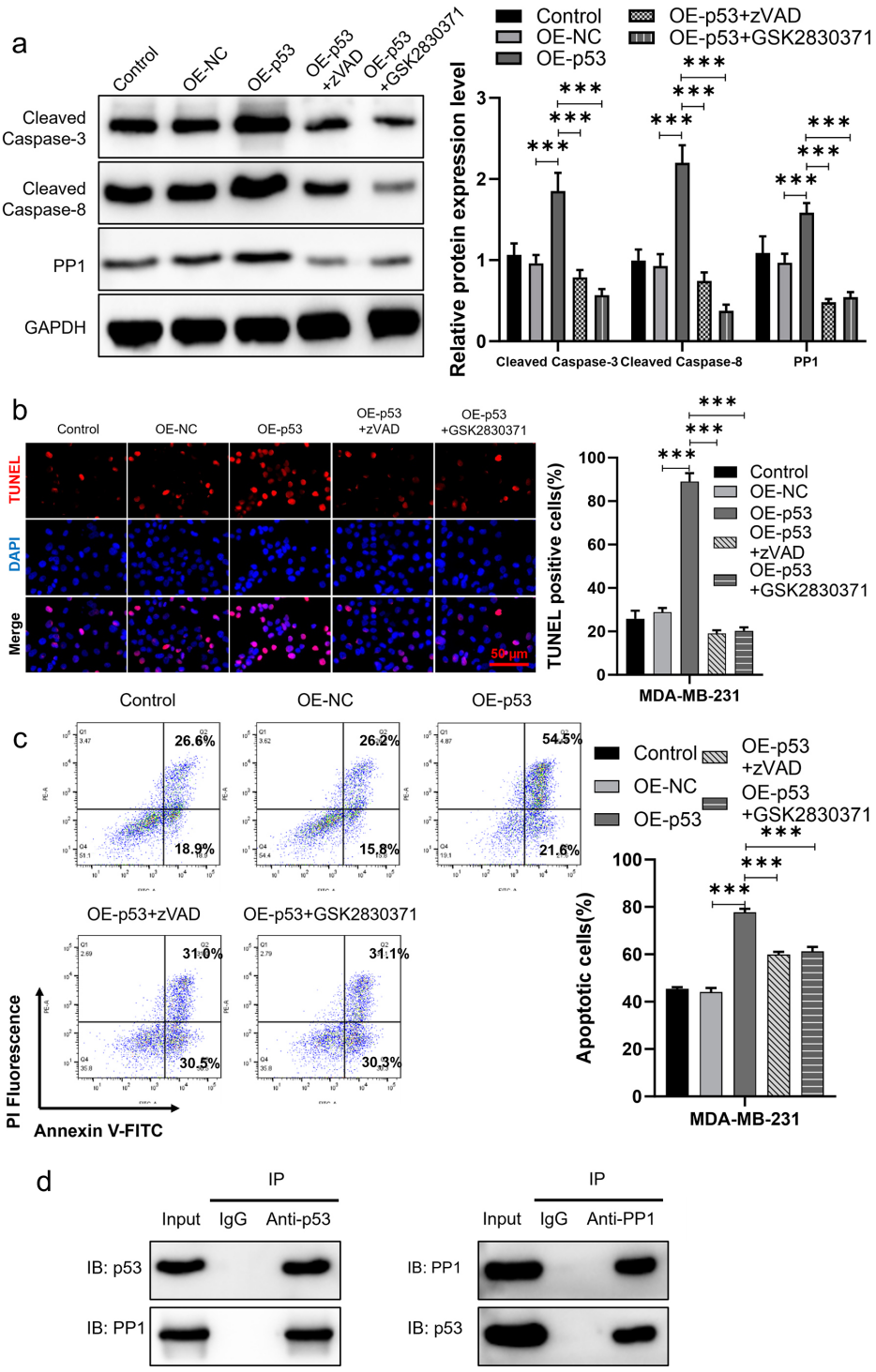

Fig. 3. p53 regulates Caspase-dependent apoptosis of breast cancer cells by activating PP1. (a) The levels of Caspase protein and PP1 were detected by Western blot. (b) TUNEL method was used to detect the degree of cell apoptosis. Scare bar = 50 μm. N = 5. (c) Flow cytometry detection (Annexin V/PI staining) was used to detect the degree of cell apoptosis. (d) Co-immunoprecipitation (Co-IP) was performed to confirm the binding of p53 and PP1. GSK-2830371 (PP1 inhibitor); zVAD (Caspase inhibitor). ***$p < 0.01$.

tions, including cytokine deprivation, endoplasmic reticulum (ER) stress, or DNA damage. Conversely, the death receptor pathway is initiated upon the binding of ligands to members of the tumor necrosis factor receptor (TNFR) family, which possess intracellular death domains. The activation of caspase-3 is the important initiating marker of cell apoptosis in this process [41]. One study has shown that p53 may be involved in Caspase-dependent cell death through

two mechanisms [33]. One suggests that the transcriptional activity of p53 up-regulates the expression of multiple potential death genes. The second mechanism is independent of active transcription. Methods that inactivate mutant p53 or activate wild type p53 have been developed as potential cancer treatments. Therefore, our study investigated the apoptosis of breast cancer cells under the influence of p53, and associated mechanisms, from the perspective of *in vitro* and *in vivo* experiments.

We found that the expression of PP1 in the Si-p53 group was significantly down-regulated, and the activity of PP1 was also decreased. The expression level and activity of PP1 in the OE-p53 group were significantly increased. These results indicated that wild-type p53 might induce PP1 activation. Some previous studies have demonstrated the regulation of PP1 in breast cancer cells by interfering with p53 *in vitro* [42]. However, it has also been reported [43] that wild-type p53-induced PP1 plays a key role in the dephosphorylation and inactivation of p53 and some ataxia-telangiectasia mutated (ATM)/RAD3-related (ATR) target proteins. In the present study, apoptosis and the expression of cleaved Caspase-3/8 were increased in the OE-p53 group. This is also consistent with the research results of Han-Fei Ding *et al*. [33]. Our results indicate that the regulation of p53 expression in breast cancer might affect the level of Caspase protein and corresponding cleavage substrate in cells. In addition, we found that the expression of other apoptosis-related proteins changed in the OE-p53 and Si-p53 groups. p53 upregulation promoted expression of tBid and Bax and inhibited expression of Bcl-2. These results suggest that up-regulation of p53 expression can promote the apoptosis of breast cancer cells by a mechanism involving regulation of tBid, Bax, Bcl-2, and Cyt-C by the Caspase protein. Cyt-C is a protein encoded by a nuclear gene [44] and is the carrier for electron transfer in the mitochondrial respiratory chain. The deletion of Cyt-C leads to abnormal function of the mitochondrial respiratory chain, which leads to the loss of ATP in cells. Under normal circumstances, Cyt-C exists in the gap between the inner and outer membranes of mitochondria. The stimulation of apoptotic signals releases it from mitochondria to the cytoplasm and mediates apoptosis [45]. We also found that the expression of Cyt-C and apoptosis were all increased when p53 was upregulated.

Subsequently, we used PP1 inhibitors and Caspase inhibitors to confirm the specific pathway mechanism of p53-induced apoptosis in breast cancer cells. From the analysis of the results, the use of any inhibitor weakened the promotion of apoptosis in the OE-p53 group of breast cancer cells. Together, these results indicate that increasing the expression of p53 in breast cancer cells might promotes Caspase-dependent apoptosis through PP1 activation.

Conclusion

For the last decade, different candidate drugs against wild-type p53 tumors, such as atezolizumab, AMG-232, and paclitaxel, have been studied in early-phase clinical trials including breast cancer patients [46,47]. However, the results were disappointing due to modest efficacy and serious adverse events. Our identification of the p53-PP1 pathway as a potential therapeutic target for breast cancer presents promising avenues for clinical translation. While our study highlights its significance, there remains a crucial gap between theoretical fundamental researches and concrete clinical practice strategies. Moving forward, one promising direction lies in the exploration of high-throughput drug screening approaches specifically targeting the p53-PP1 axis. Additionally, gene therapy such as viral vector-mediated delivery of p53 or PP1 modulators could be investigated for their efficacy in restoring tumor suppressor function or inducing apoptosis in breast cancer cells. However, it is imperative to acknowledge the inherent limitations associated with these approaches. These may include off-target effects, dose-dependent toxicity, and the development of resistance mechanisms. Therefore, future research efforts should focus on refining these strategies, optimizing delivery methods, and elucidating mechanisms to overcome resistance, ultimately paving the way for the clinical translation of p53-PP1 targeted therapies in breast cancer treatment.

Availability of Data and Materials

Data to support the findings of this study are available on reasonable request from the corresponding author.

Author Contributions

XLZ and GZY designed the research study. DX and MJ performed the research. LC and LXC provided help and advice on the experiments and analyzed the data. XLZ and LXC have written the first draft. All authors contributed significantly to editorial changes of important content. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

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