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Six1 mediates resistance to paclitaxel in breast cancer cells

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ABSTRACT

Paclitaxel resistance remains a major challenge in the treatment of breast cancer. Six1 is a homeodomain-containing transcription factor invloved in the initiation, progression and metastasis of breast cancer. We herein investigate the relationship between Six1 and resistance of paclitaxel in this study. The results indicate that six1 is a mediator of the paclitaxel resistance in breast cancer. The expression level of Six1 in breast cancer cells correlates with their resistance to paclitaxel. On the one hand, forced overexpression of Six1 in Six1-low/paclitaxel-sensitive MCF-7 or HS578T breast cancer cells induce their resistance to paclitaxel treatment directly; On the other hand, knockdown of endogenous Six1 in Six1-high/drug-resistant BT-474 breast cancer cells sensitized these cells to paclitaxel treatment. Besides, Six1 overexpression confers resistance to paclitaxel-mediated apoptosis in breast cancer cells. Furthermore, clinical data and the publicly available breast cancer gene expression datasets display that the association of Six1 expression with paclitaxel sensitivity is clinically relevant. In conclusion, these data suggest that Six1 may function as an important modifier of the paclitaxel response in breast cancer cells, and serve as a potential target for overcoming paclitaxel resistance in breast cancer.

1. Introduction

Breast cancer is the most common cancer in women worldwide [1]. Paclitaxel, as one of the most effective chemotherapeutic drugs for cancer, has been successfully used in therapy of breast cancer [2]. However, its effectiveness has been seriously limited by the acquired resistance of cancer cells [3]. There is therefore an urgent need to explore mechanisms of paclitaxel resistance so as to improve response rates and potentially extend survival in these patients.

Six1, highly conserved from Drosophila to humans, was first identified as a mammalian homolog of the Drosophila sine oculis(so) gene [4–7]. It is expressed widely in many tissues during the early development, while low or absent in most adult tissues [8,9]. Aberrant overexpression of Six1 is observed in numerous human cancers, where it leads to increased proliferation, survival, and metastasis [4,8,10,11]. In human breast cancer, gene amplification of Six1 is detected in 5% of these subjects [12]. Six1 overexpression induces malignant transformation of immortalized, nontumorigenic cancer cells [13], and its ectopic expression leads to increased proliferation by transcriptional activation of cyclin A1 in breast cancer [14]. What's more, the level of Six1 correlates closely with poor prognosis in breast cancer and many other tumors [15,16]. To sum up, these data suggest that Six1 plays an important role in tumorigenesis as well as in metastasis. However, until renently, the mechanisms of Six1 participate in the tumorgenesis and metastasis of breast cancer is not entirely clear.

Recent studies have revealed that Six1 overexpression facilitated breast cancer metastasis through TGF-β signaling, epithelial-mesenchymal transition, and inducing lymphangiogenesis via upregulation of VEGF-C [10,11,17,18]. Moreover, its overexpression causes marked resistance to TRAIL-induced apoptosis in ovarian cancer cells, demonstrating a prosurvival role for Six1 [19,20]. We speculate that Six1 might play a part in acquired paclitaxel resistance in breast cancer.

In this study, we demonstrate that Six1 regulates paclitaxel sensitivity in breast cancer cells by modulating the effects of paclitaxel on apoptosis, and the association of Six1 expression with paclitaxel sensitivity is clinically relevant.

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2. Materials and methods

2.1. Cell culture and transfection

All breast cancer cell lines (MCF-7, HS578T, MDA-MB-231, ZR-751, T47D and BT-474) were obtained from American Type Culture Collection ATCC (Rockville, MD, USA). All cell lines were cultured in RPMI1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen, California, USA) in 5% CO_2 atmosphere at 37 °C. Construction of plasmid for overexpression of Six1 (pcDNA4/TO–Six1) was as described previously [21]. MCF-7 and HS578T cells were seeded in six-well plate and transfected with vector control pcDNA4/TO or pcDNA4/TO-Six1 by using Lipofectamine 2000 reagent as recommended by the manufacturer (Invitrogen, California, USA). Twenty-four hours after transfections the cells were passaged and selected using 100 μg/ml Zeocin for 2 weeks, and then got the pcDNA4/TO-Six1 stable cell lines. Small interfering RNA (siRNA) oligonucleotides targeting Six1 (5′ -AGAACGAGAGCGUACUCAA-3′ or 5′-GGGAGAAC ACCGAAAACAA-3′) were synthesized by RiboBio (Guangzhou, China) and transfected to BT474 cells with the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

2.2. Tissue Samples

Tumor specimens were obtained from 12 patients with breast cancer who received paclitaxel-containing neoadjuvant chemotherapy and then underwent surgical resection. The paired samples of breast cancer tissue including pre-neoadjuvant chemotherapy, post-neoadjuvant (operative) and recurrence tissue were taken in pairs from every patient. Written informed consents were obtained from all patients and the collection of tissue samples was approved and supervised by the Research Ethics Committee of Zhengzhou University (Zhengzhou, China).

2.3. RNA isolation and quantitative real-time RT-PCR

Total RNA was isolated from patient specimens by the RNeasy mini kit according to the manufacturer's instructions (Qiagen, Germany). Quantitative Real-time RT-PCR analysis was done as described [21]. Primer sets used were as follows: for gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-GGAGCG AGATCCCTCCAAAAT-3′ and 5′-GGCTGTTGTCATACTTCTCAT GG-3′; for Six1, 5′-AAGGAGAAGTCGAGGGG TGT-3′ and 5′-TGCTTGTTG GAGGAGGAGTT-3′.

2.4. Analysis of microarray data

Gene expression data were obtained from NCBI Gene Expression Omnibus (GEO) database (Accession no: GSE22513; http://www.ncbi.nlm.nih.gov/geo) [22]. We analyzed the Six1 expression in patients that achieved a pathologic complete response (pCR) and those with residual disease (non-pCR). Expression data for Six1 were log-transformed, median centered per array, and the standard deviation was normalized to one per array.

2.5. Western blot analysis

Western blot analysis was performed as we previously described [21]. Briefly, cells were lysed in cold lysis buffer contaning protease inhibitor mixture. Proteins (10–25 μg) were resolved on SDS–PAGE, transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). The membrane was blocked in TBS-T buffer containing 5% (w/v) non-fat milk at room temperature for 1 h and then probed with antibodies for Six1, GAPDH, cleaved Caspase3 and cleaved PARP (all from Santa Cruz Biotech, Santa Cruz, CA, USA) at 4 °C overnight. Detection was performed with the SuperSignal West Femto Maximum Sensitivity Substrate Trial Kit (Pierce, Rockford, IL, USA). The band images were digitally captured and quantified with a FluorChem FC2 imaging system (Alpha Innotech, San Leandro, CA, USA).

2.6. Cell viability assay

Cells were seeded into 96-well culture plates and incubated at 37 °C. Paclitaxel was purchased from Sigma (St. Louis, MO) and dissolved in DMSO. After treatment with different concentrations of paclitaxel for 72 h, the 20 μL of tetrazolium bromide (5 mg/mL, GE Healthcare) was added to each well and incubated for 4 h at 37 °C. The culture medium was removed and 150 μL of DMSO was added to solubilize the crystals for 20 min at room temperature and the absorbance at 570 nm was read by an ELISA plate reader (Model 680, Bio-Rad, CA). Each paclitaxel concentration was tested in triplicate in 96-well plates, and experiments were repeated independently at least three times. The 50% inhibitory concentration (IC50) was calculated with GraphPad Prism software using the sigmoidal dose-response function.

2.7. Assessment of cell death

MCF-7 cells stably transfected with vector control and Six1 were treated with paclitaxel (10 nM) for 48 h, and then apoptosis was determined by the Sub-G1 and Annexin V/PI fow cytometry assays as described previously [23].

2.8. Statistical analysis

All data were expressed as mean ± s.e.m. Between groups and among groups comparisons were conducted with Student t test and ANOVA, respectively. Mann–Whitney U test is used for non-parametric variables. The Spearman rank correlation test was assessed to verify the association between expression levels of Six1 in breast cancer cells and their resistance to paclitaxel (IC50). Statistical analysis was performed using GraphPad Prism software version 4.0 (PRISM4) (GraphPad Software Inc, LaJolla, CA), and $P < 0.05$ was considered significant.

3. Results

3.1. Expression of Six1 in breast cancer cells correlates with their resistance to paclitaxel

To determine whether Six1 expression is associated with chemoresistance of breast cancer cells, we examined the expression of Six1 in several breast cancer cell lines by Western blot analysis. As shown in Fig. 1A, the level of Six1 was low in HS578T, MCF-7 and T47D cells, and high in MDA-MB-231, ZR-751 and BT-474 cells. Subsequently, we examined the responses of these cells to increasing concentrations of paclitaxel. The IC50 values of paclitaxel, which stand for the concentration of paclitaxel needed for preventing cell proliferation by 50%, in these cell lines were then determined. It was found that cells with high expression of Six1 (ex., MDA-MB-231, ZR-751 and BT-474) had much higher IC50 values than other cell lines (Fig. 1B). To define the relationship between Six1 and paclitaxel sensitivity, the correlation between the IC50 values and the relative Six1 expression levels in these breast cancer cell lines was analyzed. We found that the expression level of Six1 significantly correlated with the IC50 values of paclitaxel in these cells ($r = 0.886$, $p = 0.033$). These results suggest that the higher

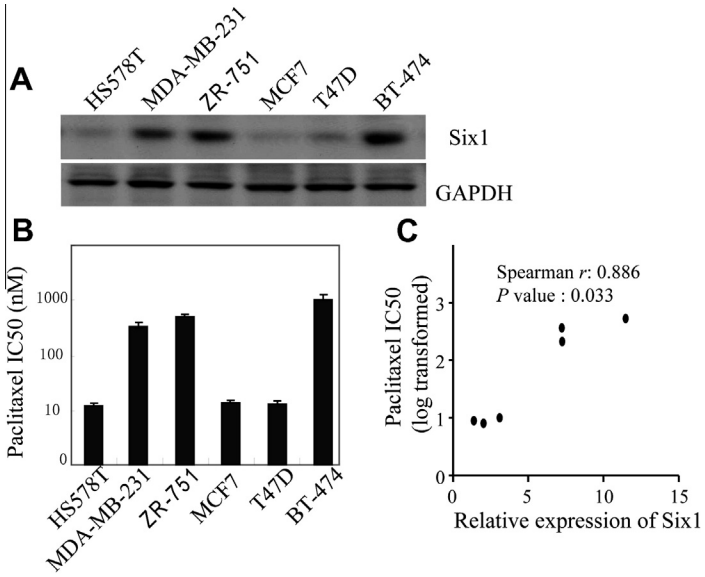

Fig. 1. Correlation between paclitaxel resistance and the level of Six1 expression in breast cancer cells. (A) Western blot analysis showed the expression of Six1 in breast cancer cells. GAPDH was used as an internal control. (B) Breast cancer cells were treated with gradient concentrations of paclitaxel for 72 h, and the drug concentrations needed for preventing cell proliferation by 50% (IC50) were then determined by in vitro cell proliferation assay. (C) The correlation between the IC50 values and the relative Six1 expression levels in breast cancer cells was quantified by Spearman's rank correlation. All experiments were performed in triplicate; $^{*}P<0.05$.

level of Six1 in breast cancer cells correlates with their resistance to paclitaxel.

3.2. Six1 confers resistance to paclitaxel in breast cancer cells

To confirm that overexpression of Six1 is directly responsible for Paclitaxel resistance in breast cancer cells, we carried out the following experiments. First, we overexpressed Six1 in Six1-low/drug-sensitive MCF-7 and HS578T cells. Cells were stably transfected with either pcDNA4/TO-Six1 or control plasmid. The western blot analysis confirmed that the expression of Six1 increased in both MCF-7 and HS578T cells transfected with pcDNA4/TO-Six1, in comparison with those transfected with control plasmid (Fig. 2A). It showed that overexpression of Six1 rendered MCF-7 cells more resistant to paclitaxel. MCF-7 control cells had an IC50 value of 13.5 nM, whereas the IC50 value of MCF-7 cells overexpressing Six1 was 34.6 nM (Fig. 2B). Similarly, HS578T cells transfected with Six1 were more resistant to paclitaxel than the control. ($IC50_{Control}$ vs. $IC50_{Six1}$, 11.5 nM vs. 32.9 nM; Fig. 2C). These results indicate that overexpression of Six1 enhances resistance of breast cancer cells to paclitaxel. Second, we knocked down Six1 in Six1-high/drug-resistant BT-474 by using siRNAs (Fig. 2A). Significantly, knockdown of Six1 by both siRNAs in BT-474 cells sensitizes their response to paclitaxel treatments ($IC50_{siRNA\ control}$: 672.0 nM; $IC50_{siRNA\text{-}Six1\text{-}1}$: 234.7 nM; $IC50_{siRNA\text{-}Six1\text{-}2}$: 238.0 nM; $P < 0.05$, Fig. 2D). In summary, our findings strongly suggest that Six1 overexpression confers resistance to paclitaxel in breast cancer cells.

3.3. Six1 overexpression inhibits paclitaxel-induced apoptosis

To determine the mechanisms by which Six1 enhances resistance of breast cancer cells to paclitaxel, we analyzed the effect of Six1 overexpression on paclitaxel-induced apoptosis. MCF-7 cells stably expressing Six1 were exposed to 10 nM paclitaxel for 48 h, and then apoptosis was determined by the Sub-G1 and Annexin V/PI fow cytometry assays. The Sub-G1 assay showed that MCF-7 cells with stable Six1 expression exhibited a significantly reduced level of paclitaxel-induced apoptosis compared to the control cells, resulting in a reduction from 25.6% to 15.8% ($^{*}P < 0.05$, Fig. 3A). Similarly, the Annexin V/PI assay showed that paclitaxel-mediated apoptosis in Six1 overexpression cells was significantly inhibited when compared to the control, with a decrease of apoptosis from 47.3% to 27.6% ($^{*}P < 0.05$, Fig. 3B). Moreover, these findings were further confirmed by the subsequent protein changes shown in Fig. 3C. After exposure to paclitaxel, expression of both cleaved Caspase3 and cleaved PAPR were increased in MCF-7 control cells, and significantly decreased after forced overexpression of Six1 (Fig. 3C). Taken together, these data suggest that Six1 overexpression confers resistance to paclitaxel-mediated apoptosis in breast cancer cells.

3.4. The association of Six1 expression with paclitaxel sensitivity is clinically relevant

To determine whether the association of Six1 expression with paclitaxel sensitivity is clinically relevant, we examined the expression of Six1 gene in tumor tissues from patients who received neoadjuvant chemotherapy containing paclitaxel. Six1 was measured in 12 paired samples of breast cancer tissue including pre-neoadjuvant chemotherapy, post-neoadjuvant, and relapse. It revealed that Six1 expression was dramatically elevated in post-neoadjuvant or local recurrent breast cancer tissues compared to primary tissues ($^{*}P < 0.05$, Fig. 4A). These results suggest that the breast cancer cells with low Six1 levels were more sensitive and prone to die, whereas cells with high expression of Six1 were more resistant to the paclitaxel-containing chemotherapy. Furthermore, to address whether Six1 is related with the pathological response of breast cancer to paclitaxel-containing chemotherapy, we utilized publicly available breast tumor gene expression datasets that were categorized based on pathologic response to neoadjuvant paclitaxel treatment (GSE22513) [22]. We compared the Six1 expression in patients that achieved a pathologic complete response (pCR) and those with residual disease (non-pCR). The result showed that Six1 expression in tumors of subjects with non-pCR was higher than in those who achieved a pCR ($^{*}P < 0.05$, Fig. 4B). Altogether, these results indicate that Six1 is of clinical significance as a mediator of paclitaxel resistance.

4. Discussion

One of the major obstacles for the effective treatment of breast cancers is the acquired resistance of cancer cells to chemotherapy, even though that it can sometimes be suppressed by therapeutic drugs [24–28]. Therefore, the identification of proteins responsible for drug resistance is critical for successful breast cancer treatment. In this study, we report for the first time that Six1 as a novel gene is responsible for paclitaxel resistance in breast cancer. Moreover, elevated expression of Six1 is associated with relapse of breast cancer treated with paclitaxel-containing chemotherapy.

Six1 belongs to a subfamily of the Six family of homeodomain-containing transcription factors, and its overexpression is likely to be related to progression in breast cancer [8]. To explore the relationship between dysregulation of Six1 and the chemoresistance in breast cancer cells, we first examined the level of Six1 expression in several breast cancer cell lines and their response to paclitaxel, a chemotherapeutic drug commonly used for the treatment of breast cancer [2]. Our analysis showed that levels of Six1 in breast cancer cells correlate with their resistance to paclitaxel. Also, we have shown that enhanced levels of Six1 can directly cause resistance of breast cancer cells to paclitaxel. On the contrary, knockdown of endogenous Six1 sensitized breast cancer cells to paclitaxel.

The important cellular events after paclitaxel treatment are mitotic arrest and the following apoptosis [29,30]. Previous studies have shown that Six1 overexpression in ovarian carcinoma causes resistance to TRAIL-mediated apoptosis [19]. Therefore, we further explore the possible mechanisms involved in the Six1-mediated resistance to paclitaxel by evaluating the effect of forced Six1 overexpression on paclitaxel-induced apoptosis. Our data showed that forced overxpression of Six1 significantly suppressed paclitaxel-induced apoptosis in MCF-7 cells. Six1 may regulate paclitaxel resisitance by modulating the effects of paclitaxel on apoptosis in breast cancer cells. However, whether Six1 affects the ability of paclitaxel to cause mitotic arrest needs to be further investigated. In addition, both our clinical data and the publicly available breast tumor gene expression datasets indicated that the expression of Six1 and paclitaxel sensitivity is clinically relevant. It is coincident with previous studies showing that overexpression of Six1 correlates with poor prognosis in breast cancer [15].

In conclusion, our findings suggest that Six1 may function as an important modifier of the response of breast cancer cells to paclitaxel. Six1 may provide a novel therapeutic target for treatment of paclitaxel-resistant breast cancers.

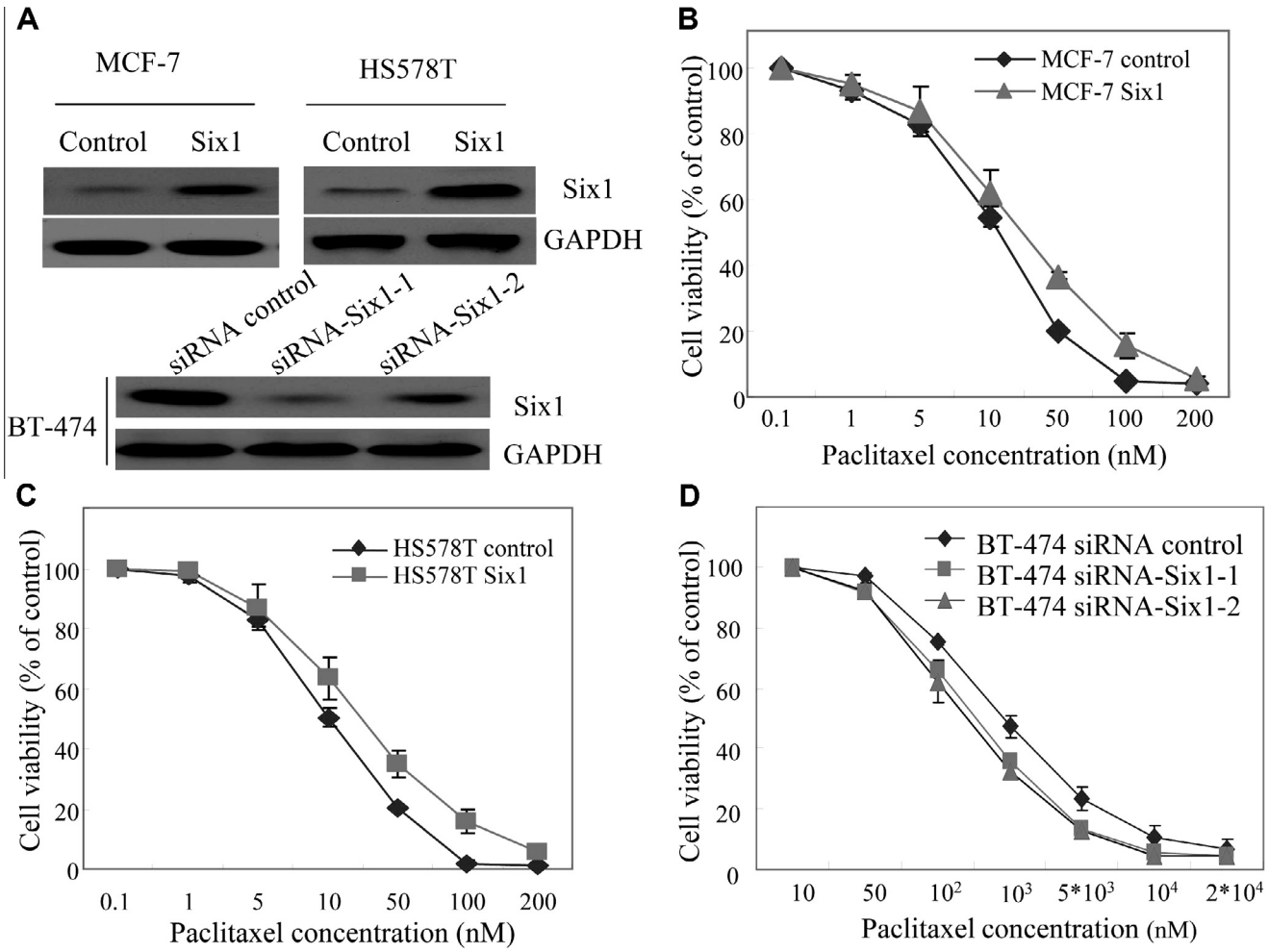

Fig. 2. Effects of Six1 overexpression or knockdown on paclitaxel sensitivity in breast cancer cells. (A) The expression of Six1 in MCF-7, HS578T and BT474 cells was determined by Western blot analyses. GAPDH was used as an internal control. (B) and (C), MCF-7 and HS578T cells transfected with vector control (pcDNA4/TO) or pcDNA4/TO-Six1 were treated with gradient concentrations of paclitaxel for 72 h. The percentage of cell survival as a function of drug concentration was plotted. (D) BT474 cells transfected with siRNA control or Six1 siRNAs were treated with gradient concentrations of paclitaxel for 72 h. The percentage of cell survival as a function of drug concentration was plotted. All experiments were performed in triplicate; $^{*}P<0.05$.

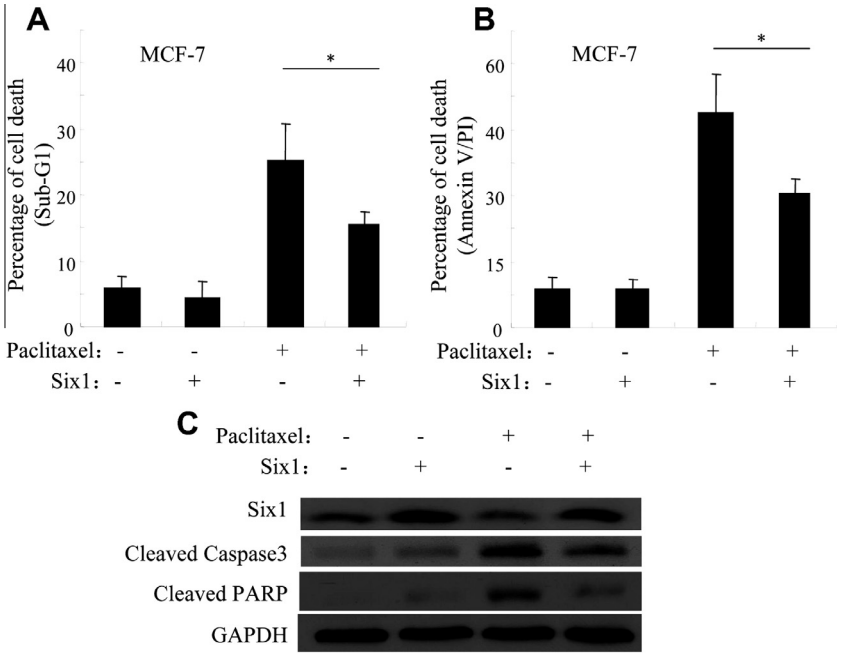

Fig. 3. Six1 overexpression inhibits paclitaxel-induced apoptosis. A and B, MCF-7 cells stably transfected with vector control and Six1 were treated with paclitaxel (10 nM) for 48 h, and then apoptosis was determined by the Sub-G1 (A) and Annexin V/PI (B) fow cytometry assays. (C) Western blot analysis shows the subsequent protein changes such as cleaved Caspase3 and cleaved PAPR. GAPDH was used as an internal control. All data are expressed as the mean of triplicate experiments ± SEM. $^{*}P < 0.05$.

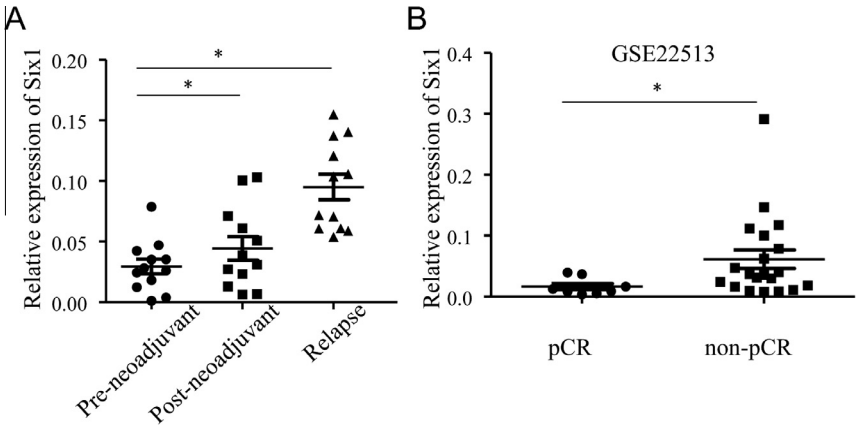

Fig. 4. The association of Six1 expression with paclitaxel sensitivity is clinically relevant. (A) The relative mRNA expression level of Six1 was determined by quantitative Real-time RT-PCR in 12 paired samples of breast cancer tissue including pre-neoadjuvant chemotherapy, post-neoadjuvant (operative), and relapse. (B) The publicly available breast tumor gene expression datasets (GSE22513) were analyzed to determine the Six1 expression in patients that achieved a pathologic complete response (pCR) and those with residual disease (non-pCR). All experiments were performed in triplicate; $^{*}P < 0.05$.

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