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Original Article

Epirubicin inhibits proliferation of breast cancer cells through upregulating $p21^{cip1}$ expression

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Abstract: Increasing evidence suggested that epirubicin possess antitumor effect, and could be used in cancer chemotherapy. However, the role of epirubicin in breast cancer remains unclear. Therefore, in this context, the present study investigated the effects of epirubicin on breast cancer cell lines (MCF-7 and T47D) proliferation, apoptosis, as well as the underlying mechanism. Our results showed that epirubicin inhibited the proliferation of both MCF-7 and T47D breast cancer cells, with time- and concentration-dependent manners. Specifically, we observed that MCF-7cell lines treatment with epirubicin had an obvious cell-cycle arrest at the G_0/G_1 phase ($P < 0.01$), although it has no effect on apoptosis. Furthermore, we investigated the effect of epirubicin on $p21^{cip1}$ expressionin MCF-7 and T47D cells, and found that epirubicin increased the expression of $p21^{cip1}$ at both mRNA and protein levels. Moreover, we demonstrated that knockdown of $p21^{cip1}$ significantly reversed epirubicin-inhibited cell proliferation in breast cancer cells, as compared with the control group, and also reversed epirubicin-inhibited cell cycle arrest the G_0/G_1 phase in breast cancer cell lines. Taken together, it is, therefore, concluded that epirubicin inhibits proliferation of breast cancer cells through upregulating $p21^{cip1}$ expression. Therefore, epirubicin may represent a chemopreventive and/or therapeutic agent in the prevention of breast cancer. However, further more studies need to be carried out to verify this point.

Keywords: Epirubicin, breast cancer cells, cell cycle arrest, $p21^{cip1}$

Introduction

Although it's necessary for most tumors to achieve full malignancy by breaching various physiological barriers, cancer is increasingly viewed as a cell cycle disease [1]. This view reflects the evidence that the vast majority of tumors have suffered defects that derail the cell cycle machinery, which lead to uncontrolled cell proliferation. Consequently, the pathways regulating the cell cycle incorporate both oncogenes and tumor suppressors and are frequently dysregulated in human cancers [2]. Unraveling the underlying regulatory mechanism would provide insight into the balance of normal and cancerous cell proliferation and is central for the exploration of novel anticancer strategies.

$p21^{cip1}$ is the first cyclin-dependent kinase inhibitor(CKI) to be identified and it's known to be induced by both p53-dependent and-independent mechanisms following stress, and induction of $p21^{cip1}$ might cause cell cycle arrest [3]. As a proliferation inhibitor, $p21^{cip1}$ is poised to play an important role in preventing tumor development [4]. This notion has been supported by data indicating that p21-null mice was more prone to spontaneous and induced tumorigenesis, and in vitro the ectopic expression of $p21^{cip1}$ protected against rumor progression in mice. On the other hand, the reduced levels of $p21^{cip1}$ transcript found in some malignant cells with absence of functional p53, which suggested that low levels of p21 might constitute an advantage in tumor growth [5]. Furthermore, it has previously reported that p21 selectively inhibited genes involved in cell-cycle progression, cell division and DNA repair. Much more interesting, some p21-inhibited genes contain E2F sites or SP1 sites in their promoters, suggesting a highly selective nature of $p21^{cip1}$-involved inhibition partly mediated through transcriptional factors [6].

Increasing evidence suggested that epirubicin possess antitumor effect, and could be used in cancer chemotherapy. However, the role of epirubicin in breast cancer remains unknown. In this context, the present study investigated the effects of epirubicin on breast cancer cell proliferation, apoptosis, and cell cycle progression, as well as the underlying mechanism. Firstly, we investigated the effect of epirubicin on p21^{cip1} expressionin MCF-7 and T47D cells, and found that epirubicin increased the expression of p21^{cip1} at both mRNA and protein levels. Next, we demonstrated that knockdown of p21^{cip1} significantly reversed epirubicin-inhibited cell proliferation in breast cancer cells, as compared with the control group, and also reversed epirubicin-inhibited cell cycle arrest the G_0/G_1 phase in breast cancer cell lines. This is the first study report on the inhibition mechanism of epirubicin regulating p21^{cip1} in breast cancer cells.

Materials and methods

Materials and reagents

Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS) and G418 were obtained from Gibco BRL. Epirubicin and propidium iodide (PI) were obtained from Sigma Chemical Company. Lipofectamine 2000, RIzol reagent and Hoechst 33342 were purchased from Invitrogen Company. ReverTra Ace-a-First Strand cDNA Synthesis Kit was purchased from TOYOBO Co. (Osaka, Japan). PGEM-T-easy Vector System was purchased from Promega Company. Real-time fluorescence quantitative PCR detection kit for GAPDH gene was purchased from ShenYou Biotechnology Co. (Shanghai, China). SyberGreen I and LightCycler analyzer were supplied by Roche Diagnostics.

Epirubicin treatment

MCF7 cell line, a human breast cancer cell line was maintained in DMEM with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Cultures were performed at 37°C in a 5% CO_2 atmosphere and passaged every 3 days. For drug treatment, Exponentially growing cell was treated with 0.2 μg/mL (final concentration) epirubicin at the indicated time (0 h, 8 h, 12 h, 24 h, 48 h), then harvested for total RNA extraction.

Cell viability assays

MCF7 cells at logarithmic stage was taken, made into cell suspension of 2×10^4/mL and seeded onto 96-well plats, with each well containing 200 μL of cell suspension and each group having 6 wells. From the first to the fifth day, the cells were stained to detect the cell growth of MCF7 and examine the inhibitory effect epirubicin alone or adriamycin plus p21 transfection on the cell proliferation of MCF7 cells. Before the termination of the test, 20 μL MTT was added to each well and 4 hours later the media were discarded. Then 150 μL DMSO was added to dissolving formazan crystals, and the samples were read on a plate reader at a wavelength of 570 nm to determine their absorbency (A_{570}). All the experiments were repeated three times. Since the initial cell concentration of samples of the transfection group might differ, in this study, the A_{570} value measured at each time point was divided by the corresponding initial A_{570} value and the ratios were used for plotting the curve of cell growth trend over time (day) after the transfection. The cell inhibition rate (IR) was calculated by the following formula: IR = (1-Average A value of the experimental group/A value of the control group)×100%. The inhibition curve over time (day) was prepared.

Real time quantitative PCR detection

The primers of p21^{cip1} were designed and synthesized by Takara life Technique Company, then purified byPAGE. Real-time quantitative PCR, utilizing 2 μl cDNA, The transcription level of GAPDH gene was confirmed as a housekeeping target. The sequences of primers were used as follows: P21^{cip1} forward primer, 5'-GGGAGG AGGGAAGTGTTTTT-3'; and reverse primer, 5'-ACAACTACTCACACCTCA ACT-3'. β-actin forward primer, 5'-GCACCACACCTTCTACAATG-3'; and reverse primer, 5'-TGCTTGCTGATCCACATCTG-3'.

Real-time PCR was performed on Lightcycler real-time PCR system. The reaction mixture contained 1 μl upper primer and lower primer (10 pmol/μl), 0.4 μl dNTP mixture (10 mmol/L), 2 μl 10× buffer, 3.2 μl $MgCl_2$ (25 mmol/L), 1 μl SyberGreen I (20×), 2 μl cDNA template, 1 μl Taq polymerase (1 U) and 8.4 μl ddH_2O. The amplification conditions were as follows: predenatured at 94°C for 2 min, followed by 40 cycles(denatured at 94°C for 5 s, annealed at

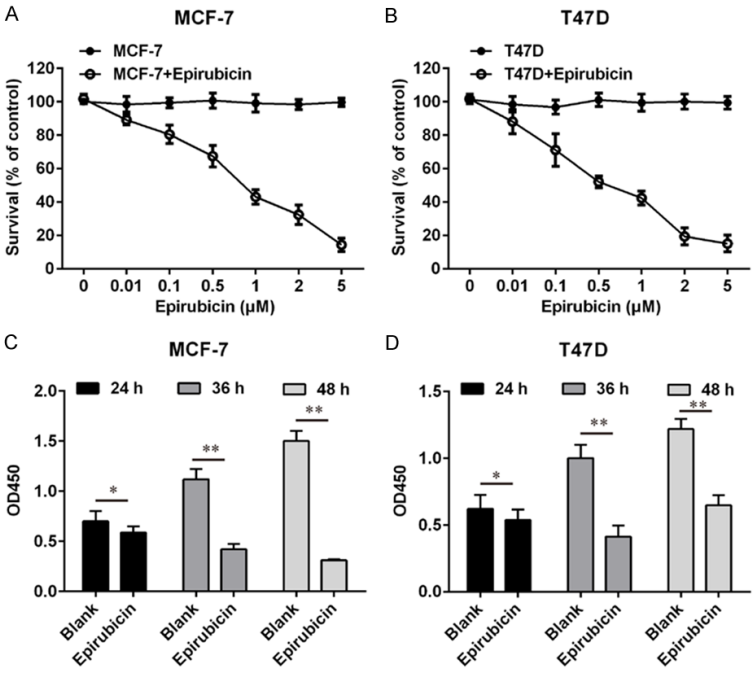

Figure 1. Effects of epirubicin on breast cancer cell proliferation. Cells were treated with various concentrations of Epirubicin for 24, 36 and 48 h; cell proliferation was determined by CCK-8 kit assay. A: Epirubicin inhibited proliferation of MCF-7 cells in a concentration-dependent manner. B: Epirubicin inhibited proliferation of T47D in a concentration-dependent manner. C and D: MCF-7 and T47D cells were treated with epirubicin for 24, 36 and 48 h, the absorbance at 450 nm was measured. Data are mean ± SD. All experiments were repeated at least three times. *P < 0.05 compared with control group.

different temperatures as below for 15 s, extended at 72°C for 15 s and measured fluorescence intensity at 85°C for 3 s), then cooled at 40°C for 30 s. Additionally, melting curve analysis was performed verify the specificity of all PCR product.

Standard curves analysis and results expression

In this study, plasmid pEGFP-C2-p21cip1, pGEM-T-easy-p53, pGEM-T-easy-survivin were used as standards respectively. pGEM-T vector consisted of p53 or survivin gene PCR product was prepared by T-A clone as described in the pGEM-T-easy manual (Promega). The standard concentration was quantitated with ultraviolet spectrometry.

The amount of the selected genes mRNA was determined by comparison with the generated standard curve and normalized with housekeeping gene GAPDH of the same sample, respectively. For each sample, the corrected gene mRNA concentration was described as follows, the level of corrected gene mRNA = $10^4 \times (C)_{analyzed\text{-}gene} / (C)_{GAPDH}$. By the way, this normalization against the housekeeping gene was feasible if both PCR presented the same efficiency. This latter would be judged by the slope of the standard curve for each PCR run, i.e. Efficiency = $[10^{(1/slope)}] - 1$.

Flow cytometry assay

Cell (1×10^6/mL) was harvested by trypsin treatment, washed with cold PBS, and fixed in 70% ethanol at -20°C overnight. The next day cell was also washed twice in cold PBS, then stained with PI solution (50 mg/mL PI, 100 mg/mL RNase, and 0.1% Triton X-100 in PBS) for 30 min at 4°C. The stained cell was analyzed for DNA histograms and cell cycle phase distribution by flow cytometry (FACSCalibur, BD Bioscience). G_0/G_1, S and G_2/M phase cell populations were measured according to the program CellQuest.

Apoptosis assessment by Hoechst 33342 staining

Hoechst 33342 is a cell-permeable DNA stain that is excited by ultraviolet light and emits blue fluorescence at 460 to 490 nm. MCF7 cells (2×10^5 cells/well in a six-well plate) were treated for 0, 8, 16 and 24 h. After trypsinization, cells were washed with 1X PBS and stained with 3 μg/ml of Hoechst 33342 for 15 min. Stained cells were examined using fluorescence microscope (Olympus, Japan) with an ultraviolet filter.

Western blotting analysis

Total cell protein was abstracted from cells after transfection. The concentration of the protein was measured by BCA protein assay kit

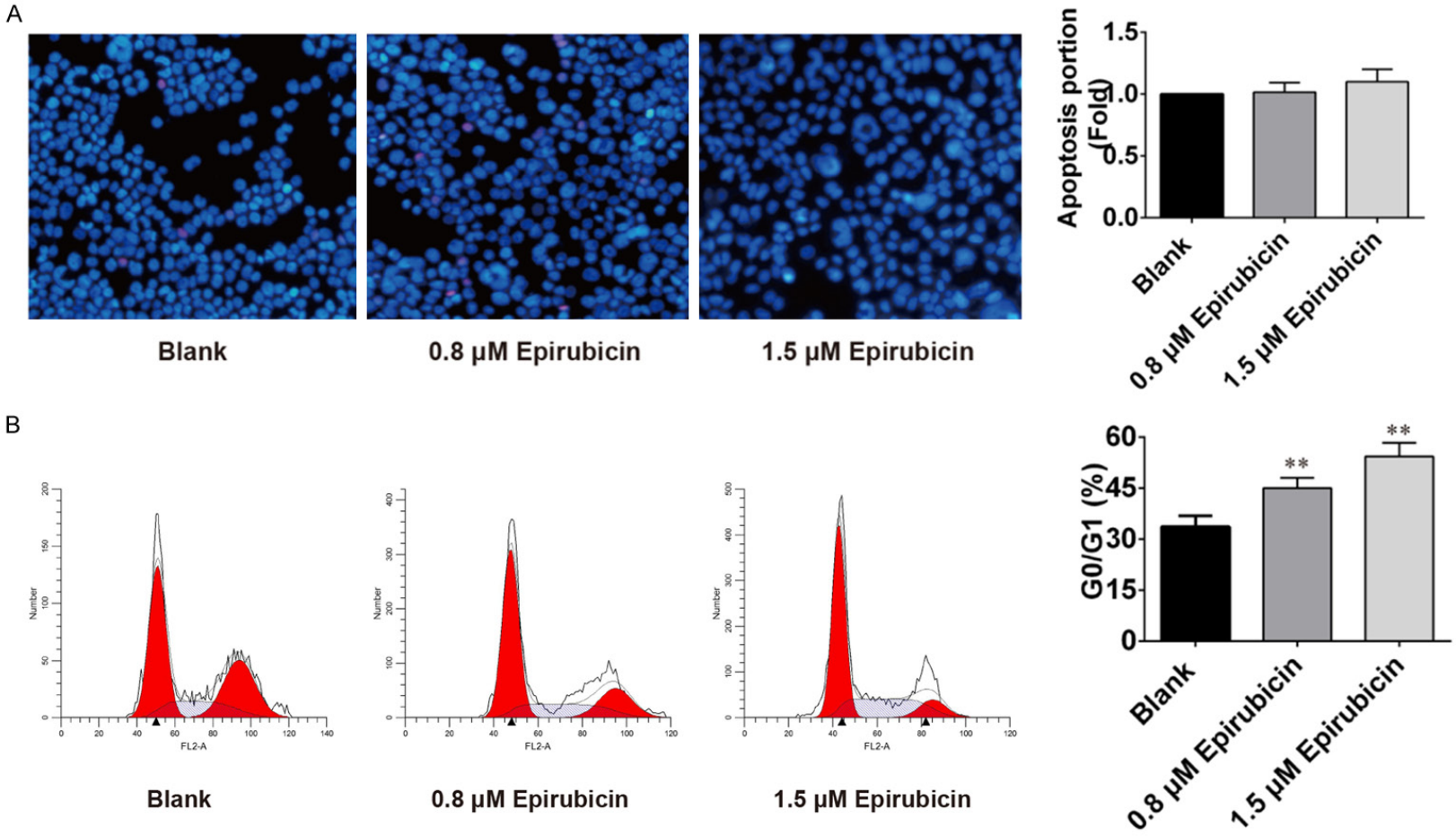

Figure 2. Effect of epirubicin on apoptosis and cell cycle. MCF-7 Cells were treated with epirubicin for 48 h, with concentrations at 0.8 μM, and 1.5 μM, respectively. The apoptosis were detected by Hoechst 33342 staining, while cell cycle was detected by flow cytometry. A: Epirubicin has no significant increase in apoptotic rate in MCF-7 cells. B: MCF-7cell lines treatment with epirubicin had an obvious cell-cycle arrest at the G_0/G_1 phase. **: $P < 0.01$.

(Beyotime, Shanghai, China), following manufacturer's instruction. Samples were electrophoresed by using 10% SDS-PAGE. The protein was then transferred onto a PVDF (polyvinylidene fluoride) membrane (Bio-Rad, USA). After blocking in skim milk, the membranes were incubated with specific antibodies. Autoradiograms were quantified by densitometry (Quantity One software; Bio-Rad). β-actin was used as internal reference; goat anti-p21^{cip1} (1:1000) was purchased from Sigma.

Plasmid construction and transfection

The open reading frame of P21^{cip} cDNA was cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) to construct the recombinant pcDNA3.1-P21^{cip} expression vector. Cells were transfected with siRNA-P21^{cip} using LipofectamineTM2000 (Invitrogen, USA), according to the manufacturer's protocols. The transfection efficiency was examined by Real-Time PCR.

Statistical analysis

The software SPSS for Windows version 17.0 was used for statistical analysis and each experiment was repeated at least three times. The values were expressed as the *mean* ± *S.D*, and then statistical analysis of the data was performed using *Student-Newman-Keuls*-test and *Dunnett's T3*-test. Values with $P < 0.05$ were considered statistically significant.

Results

Epirubicin inhibits proliferation of breast cancer cells

In the present work, firstly, we investigated the effect of epirubicin on breast cancer cell proliferation by determining the percentage of cell survival in MCF-7 and T47D breast cancer cells at concentrations ranging from 0 to 5 μm. As shown in **Figure 1A**, epirubicin dramatically inhibited the proliferation of MCF-7 cells in a concentration-dependent manner. Likewise, it is also observed that epirubicin significantly suppressed T47D cells proliferation in a concentration-dependent manner (**Figure 1B**). Furthermore, after treatment with epirubicin for 24 h, 36, and 48h, the proliferation of MCF-7 and T47D cells was significantly decreased in a time-dependent manner, respectively (**Figure 1C** and **1D**).

Effect of epirubicin on apoptosis and cell cycle arrest

We next examined the effect of epirubicin on apoptosis of breast cancer cells. Specifically, MCF-7 cells were treated with 0, 0.8 and 1.5μmepirubicin for 36 h, respectively. The apoptosis was quantified by using Hoechst 33342 staining. As shown in **Figure 2A**, there was no significant increase in apoptotic rate in MCF-7 cells following treatment with 0.8 and 1.5 μm of epirubicin, respectively. It suggested that epirubicin may have no effect on the apoptosis of breast cancer cells.

In addition, to further examine whether the effect of epirubicin on proliferation of breast cancer cells reflected cell-cycle arrest, cell-cycle progression was analyzed by flow cytometric analysis. The results revealed that MCF-7cell lines treatment with epirubicin had an obvious cell-cycle arrest at the G_0/G_1 phase (**Figure 2B**).

Effect of epirubicin on p21^{cip1} expression in MCF-7 and T47D cells

It is well known that p21^{cip1} is related to cell cycle arrest, herein, we examined the effect of epirubicin on p21^{cip1} expression in MCF-7 cells. As shown in **Figure 3A**, epirubicin significantly increased the expression of p21^{cip1} mRNA in MCF-7 cells in a time-dependent manner, as compared with the control group. Similarly, the expression of p21^{cip1} protein was also enhanced by epirubicin treatment (**Figure 3B**).

Knockdown of p21^{cip1} relieved epirubicin-induced proliferation and cell cycle arrest

Furthermore, to examine whether p21^{cip1} is implicated in epirubicin-inhibited cell proliferation and cell cycle arrest, breast cancer cells were transfected with siRNA-P21^{cip1} and the expressions of p21^{cip1} was analyzed. It is observed that p21^{cip1} mRNA expression level was significantly repressed in cells transfected with siRNA-P21^{cip1} compared with that in Blank and cells transfected with mock (**Figure 4A**).

Then, we examined whether down-regulation of p21^{cip1} could reverse epirubicin-inhibited cell proliferation and cell cycle arrest. Knockdown of p21^{cip1} significantly reversed epirubicin-inhib-

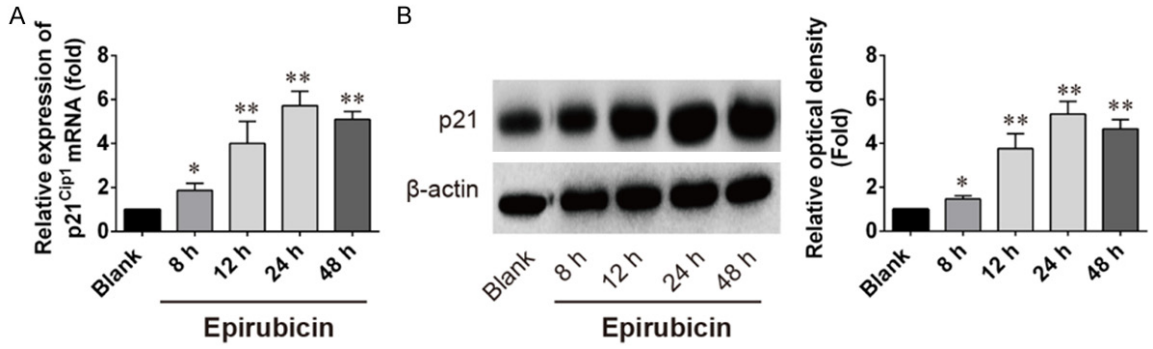

Figure 3. Expression of p21cip1 at both protein and mRNA levels in breast cancer cell lines treatment with epirubicin. A: The relative level of p21cip1 mRNA was determined by qPCR. B: The expression of p21cip1 protein was analyzed by Western blotting. β-actin was used as control. *P < 0.05; **P < 0.01.

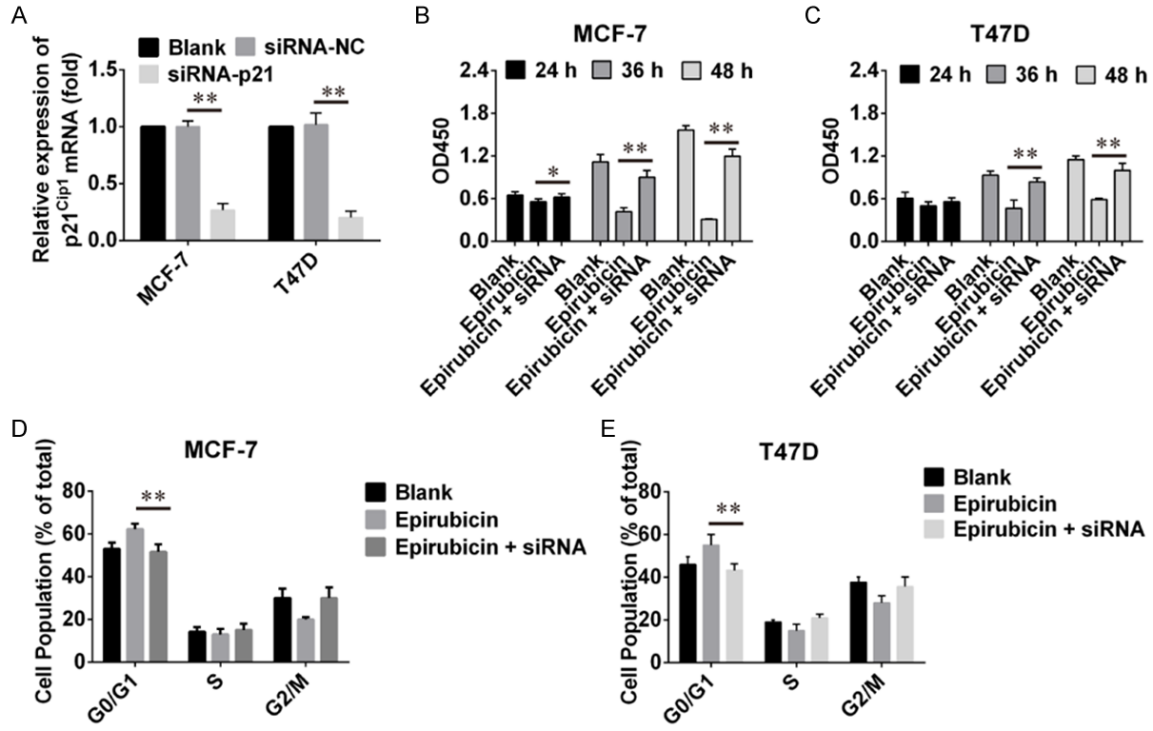

Figure 4. Knockdown of p21cip1 reversed Epirubicin-induced proliferation and cell cycle arrest. (A) The expression of p21cip1 mRNA in siRNA-P21cip1, siRNA-NC and Blank cells, respectively. (B, C) Knockdown of p21cip1 markedly reversed epirubicin-inhibited cell proliferation in MCF-7 (B) and T47D (C) cells, respectively, and the reverse contention is dependent on the time-manner. (D, E) The bar chart represents the percentage of cells in G_0/G_1, S, or G_2/M phase, as indicated. Knockdown of p21cip1 markedly relieved epirubicin-inhibited cell cycle arrest in MCF-7 (D) and T47D (E) cells, respectively. Data are mean ± SD. All experiments were repeated at least three times. **P < 0.01 compared with control group.

ited cell proliferation in breast cancer cells, as compared with the mock group (**Figure 4B** and **4C**). And also, knockdown of p21cip1 significantly reversed epirubicin-inhibited cell cycle arrest the G_0/G_1 phase in breast cancer cell lines (**Figure 4D** and **4E**).

Discussion

Epirubicin is an inhibitor of topo-isomerases I and II. It can induce DNA damage, activate P53-P21 pathway, down-regulate phosphorylation of RB proteins, thereby causing cell cycle

arrest at G_1 phase. It can also induce cell cycle arrest at G_2 phase by down-regulating the expression of Cyclin B1, and inhibiting the phosphorylation of CDC2 and histone H3, thereby inhibiting the growth of tumor cells [7, 8].

In this study, the human breast carcinoma cell line MCF7 was treated with epirubicin, the most efficient chemotherapy agents in clinical practice of breast cancer [9], and it was found that it inhibit the growth of MCF7 cells, to some extent. 24 h and 48 h after the treatment, the expression of survivin mRNA was significantly lower than that of untreated group but no significant differences were found between the two groups at two time points. Furthermore, the level of p21 mRNA increased gradually and reached the highest at 24 h after treating with epirubicin. Much more interesting, when the p21 mRNA levels almost restored to basal levels at 48 h after treating with epirubicin, in accompanying with survivin mRNA levels restored partially (about 54.87%), which indicating that: (1) p21 inhibited the survivin expression at transcription level directly or indirectly; (2) epirubicin may be involved in inhibiting survivin gene expression at transcription level simultaneously. Genotoxic agents, such as adriamycin, can specifically inactivate survivin by activating P53 signal pathway, thereby, inhibiting its expression. Hoffman*et al* [10], by analyzing the start codon of survivin, found that inhibition of survivin transcription was achieved by the interaction of down-stream target molecules of wild-type p53. The p21 protein, a cyclin-dependent protein kinase inhibitor, is an important tumor suppressor gene of downstream target molecules of wild-type p53 and the biological functions of p53 requires the help of p21.

In the departed research, p21 gene is highly conservative, rarely mutates in the tumorigenesis. As a down-streammolecules, the broad-spectrum mutation of p53 gene may lead to $p21^{cip1}$ reduced expression or absence of the expression, thereby inhibit its functions and loss of p21 functions was highly associated with some pre-cancerous changes, suggesting that p21 might outperforms p53 as a tumor suppressor gene [11]. In this research, we investigate whether $p21^{cip1}$ are involved in inhibiting the survivin expression at transcription level directly or indirectly, an extrinsic $p21^{cip1}$ gene was transfected into breast cancer cells MCF7, which contain wild-type p53 and the normal biological functions of p53. After transfection treatment, MCF7 cells which induced its overexpression and the expression levels of p21 mRNA in MCF7, MCF7-C2 and MCF7-p21 groups were 12.41±3.14 copies/ml, 26.57±7.95 copies/ml, and 26062.35± 18861.58 copies/ml respectively, whereas the level of survivin mRNA inMCF7, MCF7-C2 and MCF7-p21 groups was 116.71±62.02, 109.32±19.56 and 0.64±0.14 respectively. The results indicated that p21 overexpression could inhibit the expression of survivin at transcriptional level. In addition, flow cytometric analysis clearly revealed that the amounts of cells in G_0/G_1 remarkably increased accompanying with that of S or G_2/M decreased due to p21 overexpression, but its overexpression did not induce apoptosis in MCF7 cells by Hoechst 33342 staining. It suggested that survivin could be characterized by cell cycle dependent expression, which mainly expressed in G_2/M phase therefore we speculated that p21 might negatively regulate the expression of survivin gene through cell cycle arrest in G_0/G_1 phase to some extent.

Because p21 might negatively regulate the expression of survivin gene at transcription level through cell cycle arrest in G_0/G_1 phase, we thought part of transcription factors at the promoter region of survivin gene were inhibited under overexpression of p21. Several transcription factor sites have been found at the promoter region of survivin gene, for example, Sp1 sites, E2F sites and NF-κB [12]. In the one hand, with RT-PCR, we found that p21 overexpression could decrease the level of E2F-1 expression, one of E2F family transcription factors; in the other hand, p21 overexpression could decrease the level of SP1 expression, one of the most important transcription factor for survivin [12]. Therefore, p21 might negatively regulate the expression of survivin gene at transcription level by inhibiting E2F-1 and SP1 expression partially [13, 14].

Recent studies showed that the acetylation state of histones has a major influence on transcriptional activity of survivin gene, and decreased histone acetylase activity is frequently involved in transcriptional repression

[15]. Our studies found that p21 overexpression could also inhibit the activity of histone acetyltransferase (HAT) p300/CBP by suppressing its expression at transcription levels. p300/CBP can transfer an acetyl group to the e-amino group of a lysine residue in the NH2-termination of histone H3, and in this respect lead to the alteration of chromatin structure and affect access of transcription factors to nucleosomal DNA [16]. Additionally, p300/CBP can be recruited to promoter of survivin gene by a large number of DNA-binding proteins and stimulate gene expression either through their inherent HAT activity or through its ability to interact with other coactivators and components of the basal transcriptional machinery [17]. Zhu Net al [18] reported that repression of survivin gene expression could result from suppression of histone acetylation of the promoter in the wild-type p53 dependent ways, which are in accordance with our conclusion. Thus, it appears conceivable that p21^{cip1} may negatively regulate the expression of survivin gene partially through inhibiting the e of HAT.

In this study, we have shown that epirubicin could inhibit proliferation of breast cancer cells. Furthermore, our results demonstrated that epirubicin up-regulated the expression of p21^{cip1} in breast cancer cells; in addition, knockdown of p21^{cip1} reversedepirubicin-inhibited proliferation and cell cycle arrest in breast cancer cells. It is therefore, suggested that epirubicin inhibit cell proliferation by upregulating p21^{cip1} in breast cancer cells.

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Disclosure of conflict of interest

None.

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