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Survivin transcription is associated with P-glycoprotein/MDR1 overexpression in the multidrug resistance of MCF-7 breast cancer cells

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Abstract. Breast carcinoma is the most common malignancy in women. The progression of tumor is associated with over-expression of inhibitors of apoptosis proteins (IAPs) such as survivin and multidrug resistant P-glycoprotein (P-gp). PI3K/Akt pathway is involved in cell cycle progression, apoptosis and neoplastic transformation. PI3K/Akt has been shown to regulate survivin in breast cancer. The aim of this study was to investigate the expression of survivin and P-gp, the modulation of survivin by P-gp in PI3K/Akt during the progression of drug resistance (MDR) in MCF-7 breast cancer cells and adriamycin (ADR)-resistant MCF-7/ADR cells. The expression of survivin and P-gp in MCF-7/ADR cells were higher than that of the MCF-7 cells using RT-PCR and Western blot analysis. Survivin transcription was associated with P-glycoprotein/MDR1 overexpression using promoter activity analysis. LY294002, specific inhibitor of PI3K could suppress survivin and P-gp expression, decreased survivin promoter activity and enhanced cell sensitivity to drugs. This study shows survivin transcription was associated with P-glycoprotein/MDR1 overexpression, PI3k/Akt pathway was involved in P-glycoprotein/MDR1 associated survivin transcription activity in the multidrug resistant MCF-7 breast cancer cells.

Introduction

Breast carcinoma is the most common malignancy in women and is the most frequent cause of cancer death. Although chemotherapy plays an important role in the management of patients with breast carcinoma, the tumors subsequently are unresponsive to chemotherapy and result in death of patients (1,2). The progression of a tumor to an untreatable form is suggested to be associated with several genetic, morphological and phenotypical changes, which may involve the increased expression of inhibitors of apoptosis proteins (IAPs) such as survivin (3-5) and a dramatic increased expression of multidrug resistant P-glycoprotein (P-gp) (6-8).

Survivin is a structurally unique member of the inhibitor of apoptosis (IAP), which blocks apoptosis induced by a variety of apoptosis triggers (9,10). Survivin is known to directly or indirectly bind and inhibit the terminal effector cell death protease cascades, caspase-3 and -7, as well as to inhibit the activation of caspase-9 (11-13). Normally, survivin expression is undetectable or at very low levels in normal adult tissues and is overexpressed in most human tumors (14,15). Patients who had tumors with high survivin mRNA expression revealed shorter survival (16), increased rate of recurrence (17), and resistance to therapy (5,18). It has been reported that survivin may serve as a radio- and chemo-resistance factor (19). Thus, survivin potentially plays a key role in resistance to anticancer drugs.

MDR in tumor treatment is characterized by resistance to a broad spectrum of structurally unrelated cytotoxic drugs and is one of the most formidable challenges in the field of cancer therapy (20). One of the mechanisms of MDR is the over-expression of the mdr1 gene (encoding P-gp) (21,22). Recently, resistance to chemotherapy has been associated with decreased apoptosis, which has been a good marker of the chemo-resistance of tumor cells. Both survivin and P-gp associate with drug resistance.

PI3K/Akt has been shown to regulate survivin in breast cancer (23). Activation of Akt has been associated with the increased chemoresistance. Sustained activation of Akt result in a large increase in survivin levels. There is evidence to suggest that Akt-mediated survival pathway may inhibit apoptosis by stimulating survivin synthesis in various cancer cell lines. It has been reported that the PI3K signaling pathway is involved in the regulation of P-gp (24).

In this study, we reported that survivin was regulated in the transcriptional level at the fragment -1054 to 1 site of promoter by P-glycoprotein (MDR1) involvement in PI3k/Akt

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pathway in the multidrug resistant MCF-7 breast cancer cells. This will help in understanding the molecular mechanism of P-gp, survivin and PI3k/Akt signaling pathway in the progression of drug resistance and provide a promising approach for combating drug resistance of chemotherapy.

Materials and methods

Chemicals and reagents. Cytotoxic drugs adriamycin (ADR) and mitoxantrone (MIT) were purchased from Wanle (Main Luck Pharmaceuticals Inc., China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Life Technologies, USA). Penicillin and streptomycin were obtained from Amresco (USA). 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) and verapamil (V106) were purchased from Sigma Aldrich (USA). PI3K/Akt inhibitor, LY294002 was purchased from Beyotime Biotechnology (China). Anti-survivin and anti-P-gp antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ß-actin antibody was purchased from Beyotime Biotechnology (China). Horseradish peroxidase-conjugated secondary antibodies were purchased from KPL (USA).

Cell culture. Human breast adenocarcinoma cell line MCF-7 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Its ADR resistant cell line MCF-7/ADR propagated in 0.2 μg/ml ADR was derived from the MCF-7 cell. Briefly, MCF-7 cells were exposed to increasing doses of ADR by step-wise selection to develop MCF-7/ADR cells. Cells were routinely maintained in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37˚C.

Plasmid construction and transfection. The shRNA targeting survivin sequence (nucleotides 486-504) was inserted into *Apa*I/*Hin*dIII sites of pCMV5 to generate the vector psh1/survivin. The plasmid encoding survivin was generated by reverse transcription PCR using forward primer (5'-CTCGG ATCCATCCATGGCAGCCAGCTG-3') and reverse primer (5'-GCTGAATTCGCCACCATGGGTGCCCCGACG TTG-3'). The PCR product was inserted into *Bam*HI/*Eco*RI sites of pEGFP-N3 to generate the plasmid pEGFP/survivin. Both of the constructs were confirmed by sequence analysis. Transfection by Lipofectamine 2000 (Invitrogen, USA) was carried out according to the methods outlined in the manufacturer's instructions.

Generation of survivin promoter-luciferase constructs. To make the luciferase expression plasmid under the control of the survivin promoter, we generated 2082,1054 and 441 bp fragments of the human survivin gene promoter by PCR of human MCF-7 genomic DNA as a template. The sequences of the primers were: forward 2082 bp, 5'-GTCAGATCTA GTGAAAAGGAGTTGTTCCTTTCCTCCCTC-3'; 1054 bp, 5'-GTCAGATCTAAAGACAGTGGAGGCACCAGGC-3'; 441 bp, 5'-GTCAGATCTTTGGGATTACAGGCATGCAC CAC-3' and the same reverse primer, 5'-GTCAAGCTTGC CGCCGCCGCCACCTC-3'. After digestion and purification, the fragments were inserted into the luciferase vector pGL410 (Promega, USA) at the *Bgl*II and *Hin*dIII sites. These plasmids were confirmed by sequence analysis.

Table I. The sequences of primers.

Primers	Sequences (5' to 3')
Pgp-F	AAAGCGACTGAATGTTCAGTGG
Pgp-R	AATAGATGCCTTTCTGTGCCAG
Survivin-F	TGGCTGCCATGGATTGAG
Survivin-R	TCTGAGGAGGCACAGGTGTG
GAPDH-F	CAACGTGTCAGTGGTGGACCTG
GAPDH-R	TTACTCCTTGGAGGCCATGTGG

Transient transfection and survivin promoter activity assay. To normalize transfection efficiency, the Dual Luciferase reporter assay system was used according to the manufacturer's instructions (Promega). Cells ($5x10^4$) and normal control were plated in 24-well plates with the suggested medium until 60-80% confluent. Then, the cells were transiently transfected with 400 ng plasmid DNA along with 10 ng of pRL-TK using 1.2 μl of Lipofectamine 2000 (Invitrogen) per well for 4-6 h. Cell lysates and dual luciferase assay were prepared 24 h after transfection following the manufacturer's instructions. The dual luciferase ratio was defined as the luciferase activity of the tested plasmids divided by the luciferase activity of pRL-TK.

RNA extraction and real-time PCR. Total RNA was isolated using TRIzol reagent (Invitrogen) as recommended by the supplier. Total 1 μg purified RNA was reverse transcribed by ImProm-II™ Reverse Transcription System (Promega, USA). The real-time PCR amplification was carried out in ABI7500 Real-time PCR system (Applied Biosystems, USA) with the primers listed in Table I and SYBR Premix Ex Taq reagent (Takara, Japan). Cycling parameters were followed as the protocol described. Glutaraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Western blot analysis. Treated cells were washed with PBS and lysed in buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 2 μg/ml leupeptin). Aliquots were kept on ice for 30 min and centrifuged at 10000 x g for 15 min and supernates were used for assay. Equal amounts of protein extracts were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking in 5% non-fat dried milk at room temperature for 1 h, the membrane was incubated with primary antibodies against P-gp, survivin and ß-actin for 1 h at room temperature. Then horseradish peroxidase conjugated secondary antibodies were used to incubate with membrane for 1 h at room temperature. The signals were visualized using enhanced chemiluminescence detection (Pierce).

Cell proliferation assays. Cells were plated in 96-well plates at a density of $2x10^4$ cells/well. After treatment, 10 μl of 5 mg/

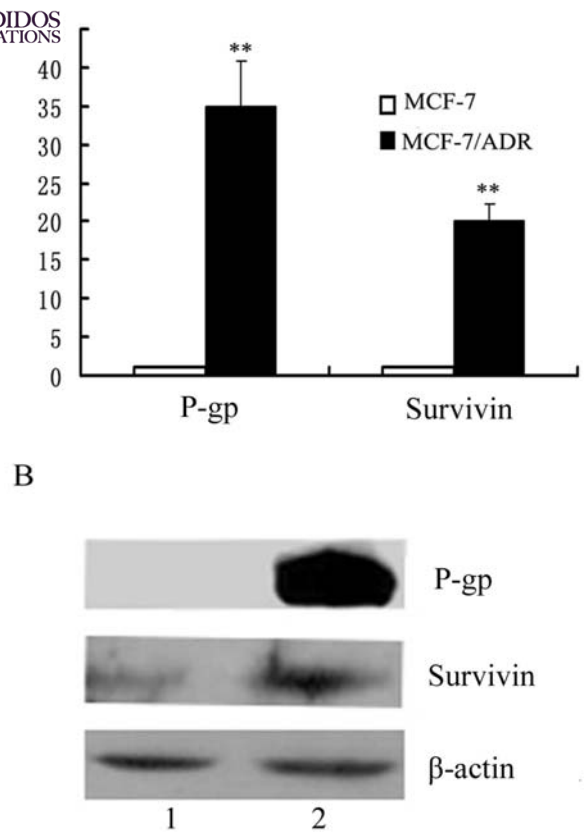

Figure 1. mRNA and protein expressions of the survivin and P-gp in MCF-7 and MCF-7/ADR cells. (A) mRNA expression of survivin and P-gp were detected with real-time PCR as described in Materials and methods; **p<0.01. (B) Protein expression of survivin and P-gp. Lane 1, MCF-7; lane 2, MCF-7/ADR.

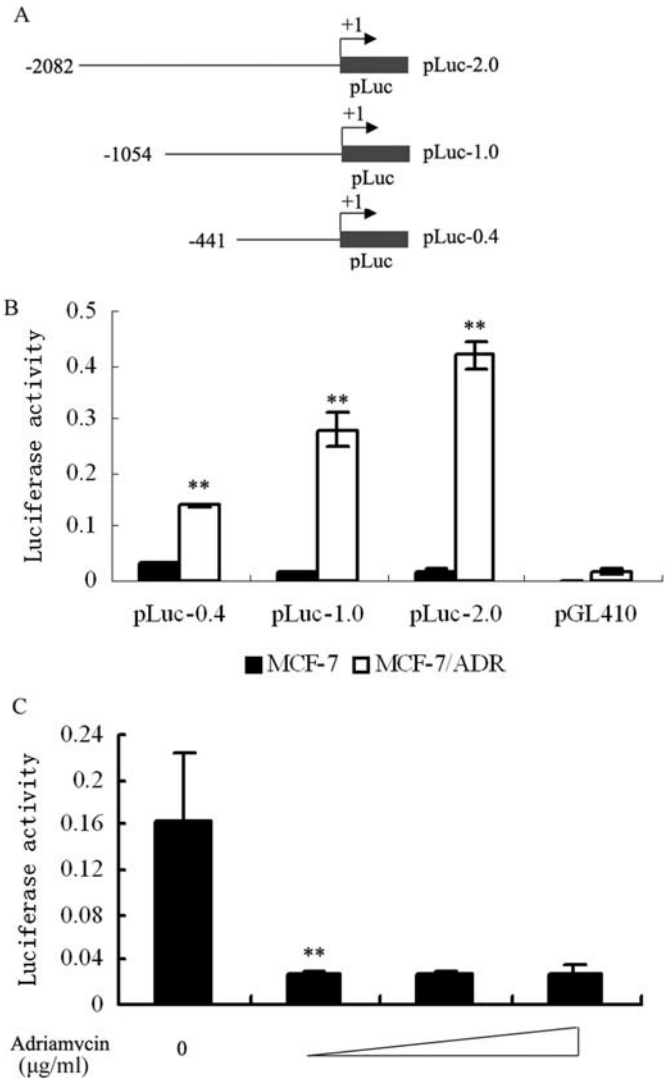

Figure 2. Survivin promoter transcription activity assay. (A) A series of survivin promoter luciferase constructs were generated as described in Materials and methods. (B) The survivin promoter transcription activity in MCF-7 and MCF-7/ADR cells. (C) The pLuc-2.0 luciferase reporter construct transcription activity in MCF-7 cells treated with increased concentration of adriamycin (0.5, 1.0, 2.0 μg/ml). The level of luciferase activity was determined in cell lysates 24 h after transfection. Values were normalized relative to the value of pRL-TK. The error bars indicate standard deviation from triplicate measurements and the data are representative of three independent transfections; **p<0.01.

ml MTT was added to each well, and incubated for another 4 h. The formazan dissolved in DMSO was measured at 490 nm using a DTX 880 multimode detector (Beckman Coulter, USA). The relative percentage of survival was calculated by dividing the absorbance of treated cells by that of the control in each experiment. The IC_{50} was determined as the concentration of drug that produced a 50% reduction of absorbance at 490 nm.

Measurement of ADR accumulation. Control and treated cells were incubated with 25 μM of ADR for 2 h. At the end of incubation, cells were washed 3 times with PBS and observed under a fluorescence microscope with x100 magnification (Olympus IX51, Olympus Corporation, Japan).

Results

Survivin expression was higher in MCF-7/ADR cells than that of MCF-7 cells. Compared with MCF-7 cells, both mRNA and protein expression of survivin and P-gp increased markedly in MCF-7/ADR cells (Fig. 1A and B). The MTT assay was performed with MCF-7, MCF-7/ADR and MCF-7 transfected with pEGFP/survivin. As shown in a previous study (25), the IC_{50} of MCF-7/ADR cells was significant higher than that of MCF-7 when the cells were exposed to ADR and MIT for 48 h. At the same time, compared with the control, the intracellular accumulation of ADR increased in MCF-7/ADR cells transfected with the survivin targeting RNAi construct psh1/survivin (25). These results showed that survivin could participate in the drug resistance in the presence of P-gp in MCF-7 cells and was involved in the efflux of drug out of cells.

Analysis of survivin promoter activity in vitro. The survivin promoter activity was determined in different conditions of transiently transfected cells. The survivin promoter activity was remarkably higher in MCF-7/ADR cells than the MCF-7 cells. It was about 4 to 26-fold higher than that observed in sensitive cells from pLuc-0.4 to pLuc-2.0 (Fig. 2B). For the promoter fragment from pLuc-0.4 to pLuc-2.0, the longer the fragment, the higher the activity. However in MCF-7 cells,

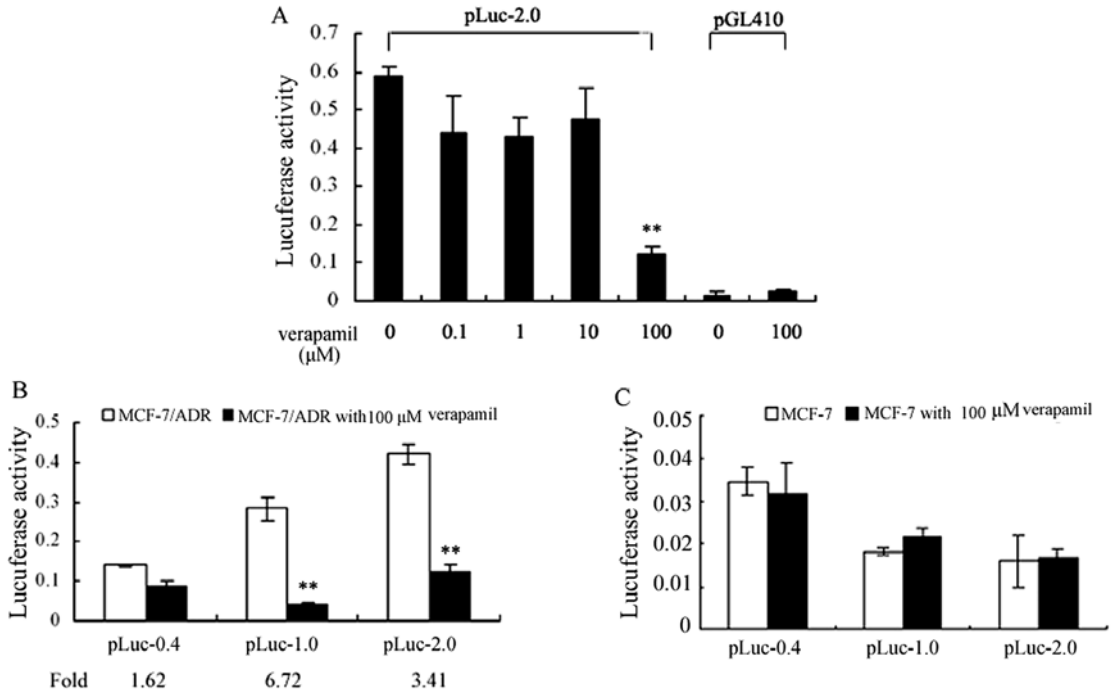

Figure 3. Verapamil represses survivin promoter activity. (A) The pLuc-2.0 luciferase reporter construct was transfected into MCF-7/ADR cells and treated with increased concentration of verapamil (0, 0.1, 1, 10, 100 μM) for 24 h. The empty luciferase reporter vector pGL410 was the control. (B and C) Three different survivin promoter constructs were transfected into MCF-7/ADR and MCF-7 cells in the presence of 100 μM verapamil. The level of luciferase activity was determined in cell lysates for 24 h after transfection. Values were normalized relative to the value of pRL-TK. Values are mean ± SD of at least three independent experiments; **p<0.01.

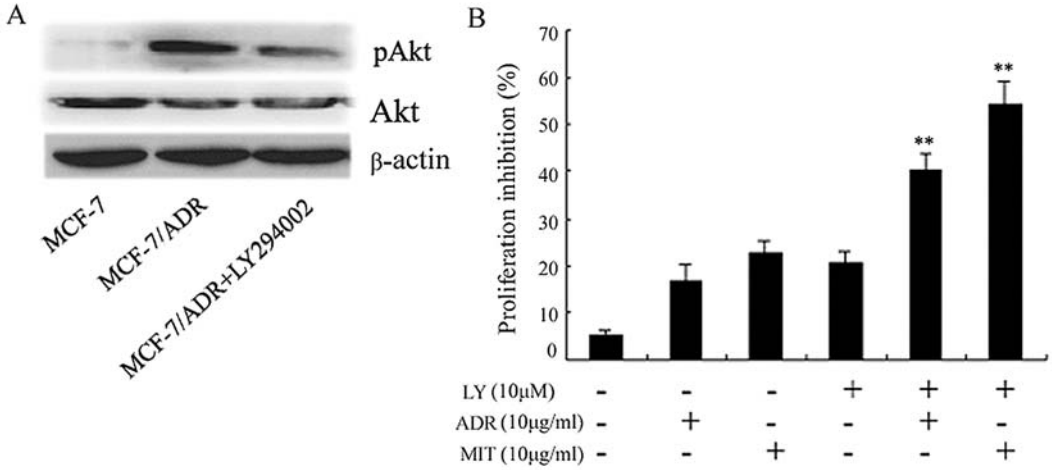

Figure 4. Inhibition of PI3K/Akt pathway by LY294002 enhanced cellular chemosensitivity to drugs. (A) Western blot analysis was performed to detect the pAkt expression in MCF-7 and MCF-7/ADR cells. The data are representative of three independent experiments. (B) LY294002 enhanced chemosensitivity of cells to ADR and MIT. After the cells were pretreated with LY294002 for 12 h, the cells proliferation inhibition rate were assayed with MTT in the presence of ADR or MIT for 24 h. Values are mean ± SD of at least three independent experiments; **p<0.01.

the survivin promoter activity decreased dramatically after ADR treatment. As shown in Fig. 2C, 0.5 μg/ml ADR abolished the survivin promoter activity. The experiments suggested that the survivin promoter was more active in the drug resistant MCF-7/ADR cancer cell line.

Participation of P-gp in survivin gene transcription. Along with the decreased expression of P-gp in MCF-7/ADR cells treated with P-gp-specific inhibitor verapamil, the mRNA expression level of survivin was also suppressed significantly in a previous study (25). The survivin gene could not even be detected after 36 h of verapamil treatment. Then the survivin promoter activity was analyzed with the verapamil treatment. As shown in Fig. 3A, the luciferase activity driven by survivin promoter decreased in MCF-7/ADR cells after incubated with verapamil at different concentrations for 24 h. The inhibition of survivin promoter acticity by verapamil depended on the length of promoter fragment in MCF-7/ADR cells, it was about a 1.62-, 6.72- and 3.41-fold reduction for pLuc-0.4, pLuc-1.0 and pLuc-2.0, respectively (Fig. 3B). However, there was no

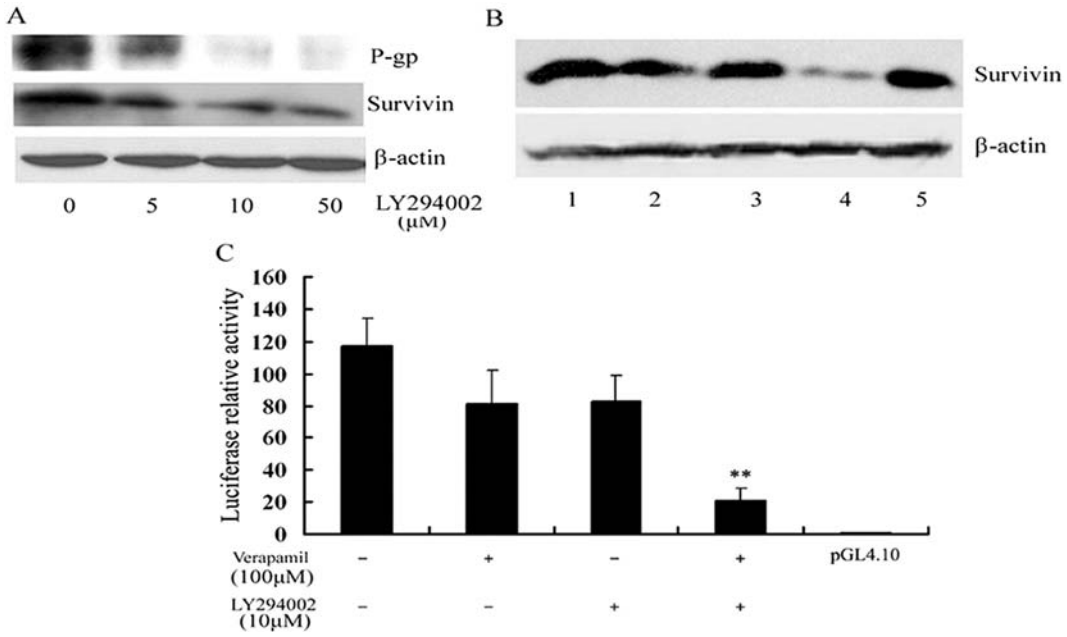

Figure 5. PI3K/Akt pathway involved in P-gp mediated survivin expression. (A) Western blot analysis was performed to detect survivin and P-gp expression with an increased concentration of LY294002 (0, 5, 10, 50 μM) for 24 h. The data are representative of three independent experiments. (B) Western blot analysis was performed to detect the survivin expression in MCF-7/ADR cells. Lane 1, control; 2, cells were treated with 100 μM verapamil for 24 h; 3, cells were treated with 10 μM LY294002 for 24 h; 4, cells were treated with 10 μM LY294002 and 100 μM verapamil for 24 h; 5, DMSO control. (C) Survivin promoter activity analysis. The level of luciferase activity was determined in cell lysates for 24 h after treatment with verapamil or LY294002. Values were normalized relative to the value of pRL-TK. Vaules are mean ± SD of at least three independent experiments; **p<0.01.

obvious influence of verapamil on promoter activity in MCF-7 cells (Fig. 3C). It demonstrated that verapamil, a specific inhibitor of P-gp, could reduce survivin gene transcription.

PI3K/Akt is involved in regulation of survivin in the transcriptional level by P-gp in the drug resistance of breast cancer cells. As shown in Fig. 4A, activated PI3K/Akt was overexpressed in MCF-7/ADR cells, and LY294002 could repress the PI3K/Akt activation level significantly. Then, the chemosensitivity of cells to ADR and MIT increased distinctively when the PI3K/Akt pathway was inhibited by 10 μM LY294002 (Fig. 4B). The results showed that PI3K/Akt participated in the chemoresistance in MCF-7/ADR cells.

As shown in Fig. 5A, the expression of P-gp was clearly repressed in MCF-7/ADR cells treated with LY294002 at the concentration of 10 μM, the expression of survivin was also suppressed. The expression of survivin was suppressed remarkably when the MCF-7/ADR cells were treated with LY294002 and verapamil (Fig. 5B). Compared with control, the luciferase relative activity was almost the same when MCF-7/ADR cells were treated with verapamil or LY294002 only, but it decreased dramatically when treated with both verapamil and LY294002 (Fig. 5C). Therefore, the PI3K/Akt pathway is involved in the regulation of survivin in the transcription level at the fragment -1054 to 1 site of promoter in the presence of P-gp during the progression of drug resistance.

Discussion

The overexpression of ATP-binding cassette (ABC) transporters, such as P-gp (26) is a common mechanism for MDR. Besides the membrane transporter, apoptosis inhibition is also an important mechanism for drug resistance. Survivin, one of the IAP family has been investigated in recent years not only on the anti-apoptosis effect but also the chemoresistance of cancer treatment.

In this study, survivin and P-gp expression were much higher in MCF-7/ADR cells than in the MCF-7 cells. Overexpression of survivin enhanced cellular resistance to ADR and MIT distinctively. Further results suggested that downregulation of survivin by transfection with psh1/survivin increased the drug accumulation. As ADR and MIT were the substrates of Pgp, drug transportation was mediated by P-gp, these results indicated that the stability and activity of P-gp was influenced by the expression level of survivin.

As survivin transcripts were the most frequently overexpressed transcript in common human cancers relative to levels in normal cells, more and more studies believe that the survivin promoter specificity and overexpression in many cancer types make it an excellent candidate for gene therapy (27-29). In this study, it was demonstrated that the survivin promoters were activated higher in MCF-7/ADR cells than in MCF-7 cells, which suggested that the overexpression of survivin in MCF-7/ADR cells was due to the over transcriptional regulation of survivin gene. It is consistent with another study that transcriptional regulation of survivin changes the cell sensitivity to drugs (30).

Our previous study showed that survivin plays a role in the chemoresistance mediated by Pgp in cancer cells (25). However, the mechanism of modulation of survivin by P-gp is unclear. In this study, we found that verapamil, a specific inhibitor of P-gp, could abrogate survivin promoter activity in MCF-7/ADR cells. The 1054 bp promoter activity was mostly suppressed by verapamil. Another study has

characterized that the survivin promoter lacks a typical TATA or CCAAT box and contains several putative Sp1/Sp3 transcription factor-binding sites extending up to -254 nt (31). Another group showed that the Sp1 cooperate with Sp3 to regulate survivin promoter activity (32). In addition, a C to G mutation in the promoter region of the human survivin gene at -31 is correlated to overexpression of survivin in cancer cells (33). Our survivin promoter constructs contain all of these regions, the difference of survivin promoter activity at different lengths of suppression by verapamil is not due to the function of these regions. The survivin was regulated by P-gp at the transcriptional level.

Abundant evidence suggests an important role of the PI3K/Akt survival pathway in several solid tumor types as well as breast cancer cell lines (23,34-36). Also, in several cancer cells a parallel expression of P-gp and activation and/or expression of Akt kinase was found. Moreover, the PI3K/Akt pathway has been reported to modulate the expression or activity of P-gp in some multidrug resistant cell lines (24). It was also found that blocking this pathway using specific inhibitor (LY294002) reduced basal levels of P-gp and antagonized the multidrug resistance (37). In this study, the expression of both P-gp and survivin were inhibited by PI3K specific inhibitor, LY294002. The cell sensitivity to anticancer drugs increased when MCF-7/ADR cells were pretreated with LY294002. The expression of survivin was blocked, and the survivin promoter activity decreased dramatically when MCF-7/ADR cells were treated with both LY294002 and verapamil compared with treatment with LY294002 or verapamil only. It showed that PI3K/Akt pathway is involved in the regulation of survivin by P-gp at the transcriptional level during the progression of drug resistance in MCF-7/ADR cells.

It is known that P-gp serves as a substrate for several protein kinases, and the ability of modulation of protein phosphorylation/dephosphorylation can control the P-gp mediated multidrug resistance (38). Although a previous study showed different results (39), the present study demonstrated that survivin and P-gp played important roles in multidrug resistance in breast cancer MCF-7 cells. The expression and transcription of survivin were regulated by P-gp to result in drug resistance. The 1054 bp fragment of survivin promoter was responsible for its transcription activity. PI3K/Akt pathway participate in P-gp mediated survivin expression and drug resistance. This helps in the understanding of the molecular mechanism of P-gp, survivin and PI3k/Akt pathway in the progression of drug resistance and reveal a strategy for evaluating drug resistance of clinical chemotherapy.

Acknowledgements

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