

Suppression of $\alpha\beta6$ downregulates P-glycoprotein and sensitizes multidrug-resistant breast cancer cells to anticancer drugs

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Multidrug resistance (MDR) in breast cancer treatment is the major cause leading to the failure of chemotherapy. P-glycoprotein (P-gp), the product of the human MDR1 gene, plays a key role in resistance to chemotherapy and confers cross-resistance to many structurally unrelated anticancer drugs. We have previously reported that integrin $\alpha\beta6$ plays a critical role in breast cancer invasion and metastasis. However, whether and how $\alpha\beta6$ is associated with P-gp and regulated by potential genetic mechanisms in breast cancer remains unclear. In the present study, we further investigated the reversal effect and underlying mechanisms of MDR in breast cancer. Two small interfering RNA constructs (pSUPER- $\beta6$ shRNAs) targeting two different regions of the $\beta6$ gene have been designed to inhibit $\alpha\beta6$ expression by transfecting them into adriamycin-resistant MCF-7/ADR cell lines. Suppression of $\alpha\beta6$ dramatically downregulated the levels of MDR1 gene mRNA and P-gp. In particular, $\beta6$ shRNA-mediated silencing of $\alpha\beta6$ gene increased significantly the cellular accumulation of Rhodamine 123 and markedly decreased drug efflux ability, suggesting that $\beta6$ shRNAs indeed inhibit P-gp mediated drug efflux and effectively overcome drug resistance. In addition, inhibition of integrin $\alpha\beta6$ suppressed the expression of ERK1/2. Interestingly, our data demonstrate that suppression of integrin $\alpha\beta6$ caused significant downregulation of Bcl-2, Bcl-xL and upregulation of caspase 3, Bad, accompanied by increasing activity of cytochrome C. A possible connection between $\alpha\beta6$ and P-gp in drug resistance biology is suggested. Taken together, $\beta6$ shRNA could efficiently inhibit $\alpha\beta6$ and MDR1 expression in vitro and these findings may offer specifically useful means to reverse MDR in breast cancer therapy.

Key words: multidrug resistance, $\alpha\beta6$ gene, RNA interference, MDR1, P-glycoprotein, breast cancer

Breast cancer is now the most frequently diagnosed cancer and also the leading cause of cancer death among females [1]. Clinical multidrug resistance (MDR) of malignancies to chemotherapeutic agents, a common phenomenon in cancer patients, is eventually the major obstacle to successful treatment in breast cancer management [2]. The most important mechanism conferring the MDR phenotype in cancer cells is an overexpression of the P-glycoprotein (P-gp), encoded by the MDR1 gene, which is a member of the adenosine triphosphate (ATP)-binding cassette (ABC) membrane transporter family [3]. P-gp functions as an energy-dependent efflux pump and exports chemotherapeutic agents from cancer cells, thereby resulting in reduced intracellular accumulation and decreased cytotoxicity of anticancer drugs [4, 5].

A member of the $\alpha\beta$ integrin subfamily, $\alpha\beta6$, is mostly undetectable in the normal epithelium; however, it becomes highly expressed during morphogenesis, epithelial repair and tumorigenesis [6]. Integrin $\alpha\beta6$ is only restrictedly distrib-

uted to epithelial cells and particularly localized at the infiltrating edge of tumor islands [7]. Experimental evidence has suggested that integrin might be involved in chemotherapeutic drug resistance in cancer because of enhanced expression of certain integrin subunits found in different drug-resistant cells, such as $\beta1$, $\alpha5\beta1$, $\alpha6\beta1$, $\alpha4$, $\alpha\beta3$ and $\alpha\beta5$. Moreover, integrins have also been shown to participate in regulating the chemotherapy-induced apoptosis of cancer cells. For instance, $\beta1$ integrin-mediated drug resistance protected human leukemia K562 cells from the apoptotic effects of chemotherapeutic drugs by modulating the expression of the pro-apoptotic Bcl-2 family protein Bim [8–9]. Further studies are needed to determine the role of integrins in the mechanisms of chemotherapy resistance and apoptosis of cancers.

To date, little is known about the relationship and the potential molecular mechanisms between $\alpha\beta6$ expression and chemotherapy resistance in human breast adenocarci-

noma. Herein, the aim of this study was to ascertain whether and how $\alpha\text{v}\beta 6$ modulates chemoresistance in breast adenocarcinoma. In the present study, we employed both chemically synthesized siRNA oligonucleotides and short hairpin RNAs (shRNAs) targeting $\beta 6$ gene and constructed recombinant plasmids by using a retroviral vector, in order to further investigate the MDR reversal effect and underlying mechanisms involved and to explore a novel therapeutic strategy to improve chemosensitivity in breast adenocarcinoma.

Materials and methods

Reagents. The monoclonal antibody against integrin $\alpha\text{v}\beta 6$ (R6G9, 10D5) was obtained from Chemicon (Temecula, CA, USA). The anti-P-gp/MDR1 monoclonal antibody C219 and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody were purchased from Abcam (Cambridge, UK). Antibodies against phosphorylated and total ERK1/2 (p-ERK1/2, t-ERK1/2) were from Cell Signaling Technology (Danvers, USA). The antibodies against Bcl-2, Bcl-xL and Bad were purchased from Cell Signaling Technology (Beverly, MA, USA). The horseradish peroxidase (HRP) or fluorescein (FITC) conjugated secondary antibody goat anti-mouse IgG was from Amersham Biosciences (Buckinghamshire, UK). Caspase-3 activity assay kit, cytochrome C releasing apoptosis assay kit and Annexin V-FITC/PI cell apoptosis detection kit were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Rhodamine 123 were purchased from Sigma (St. Louis, MO, USA).

Cell lines and culture conditions. The human breast adenocarcinoma cell line MCF-7/ADR, resistant to adriamycin (doxorubicin), expresses P-gp and its MDR phenotype is stable in an adriamycin-free medium for at least 4 months. It was maintained in RPMI-1640 culture medium containing 10% (v/v) FBS at 37°C in a humidified atmosphere containing 5% CO₂ and subcultured weekly using trypsin-EDTA (0.05%, 0.53 mM) solution. Stable transfections of MCF-7/ADR cells with pSUPER- $\beta 6$ shRNAs (MA/ $\alpha\text{v}\beta 6$) were cultured in RPMI-1640 containing the above components and subsequently selected by geneticin (G418, 400 μM).

Design of shRNAs targeting $\beta 6$ gene and construction of siRNA vectors. The siRNA sequences targeting $\beta 6$ were selected according to the previous [10]. The selected sequences were screened against the human genome sequence databases (NCBI Unigene and EST libraries) by using 15a high stringency BLAST search to avoid unintentional silencing and ensure that no other human gene was targeted. The two sequences were individually incorporated into a pair of complementary oligonucleotides, which were composed of a 19-nucleotide target sequence as sense strand. The nucleotides were then directionally subcloned into the BglII and HindIII sites of pSUPER to generate pSUPER- $\beta 6$ shRNA, named as pSUPER- $\beta 6$ shRNA1 and pSUPER- $\beta 6$ shRNA2, respectively.

Transfection. Human breast adenocarcinoma cells MCF-7/ADR were transfected with recombinant plasmid pSUPER- $\beta 6$ shRNAs or control empty vector pSUPER using Lipofectamine™ 2000 according to the manufacturer's instructions, to establish MA/ $\alpha\text{v}\beta 6$ -1, MA/ $\alpha\text{v}\beta 6$ -2 and MA/CON cells.

Detection mRNA expression of $\alpha\text{v}\beta 6$ and MDR1. Total RNA was prepared and subsequent RT-PCR detection of MDR1 mRNA or $\alpha\text{v}\beta 6$ mRNA expression was performed as described previously [11–12]. The purified RNA samples were reverse-transcribed using avian myeloblastosis virus reverse transcriptase (AMV RTase) and random primers, and the resultant single-strand cDNAs were subsequently PCR-amplified with Taq DNA polymerase by 35 PCR cycles. A set of specific primers for $\beta 6$ (forward 5'-AGGATAGTTCTGTTTCCTGC-3' and reverse 5'-ATCATAGGAATATTTGGAGG-3'), MDR1 (forward 5'-AACGGAAGCCAGAA-CATTCC-3' and reverse 5'-AGGCTTCCTGTGGCAAGAG-3') were used to amplify a 141-bp fragment of $\beta 6$ gene and a 180-bp fragment of MDR1 gene, respectively. As an internal control, amplification of GAPDH mRNA (551 bp fragment) was also carried out with the following primers (sense 5'-ATCACCATCTTCCAGGAGCGA-3' and antisense 5'-GCTTCACCACCTTCTTGATGT-3'). The RT-PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide. Each resulting band was quantified using a gel documentation system (Gel Doc 2000, Bio-Rad, Hercules, Germany). The ratio of $\beta 6$ /GAPDH or MDR1/GAPDH by scanning densitometry was named $\alpha\text{v}\beta 6$ index or MDR1 index, respectively.

Western blot analysis. Briefly, untransfected and stably transfected MCF-7/ADR cells were collected and lysed in a lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Triton X-100, 2 mM EDTA (pH 8.0), 5mM dithiothreitol (DTT), 0.1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 0.2% sodium azide, 0.5% sodium deoxycholate and 10 $\mu\text{g}/\text{ml}$ aprotinin] for 30 min on ice, respectively. The mixture was centrifuged at 11,000 $\times g$ for 15 min at 4°C, and then the supernatants were collected. The protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as a standard. Protein samples were boiled at 100°C for 5 min and then electrophoresed using SDS-PAGE. After blocking with 5% skimmed milk powder in TBST for 2 h at room temperature, filters were incubated with primary antibodies specific for the following proteins: integrin $\alpha\text{v}\beta 6$, P-gp, ERK1/2, phospho-ERK1/2, Bcl-2, Bcl-xL, Bad, caspase-3 and cytochrome C overnight at 4°C. As an internal control for equivalent protein loading and analysis, the filters were simultaneously incubated with goat monoclonal antibody against human GAPDH (1:5000). Immunoreactive bands were visualized using DAB method, and the optical density was analyzed with the Scion image software.

Rhodamine 123 efflux assay to analyze the function of P-gp. The efflux assays were performed based on the previ-

ously described protocol with minor modifications [11, 20]. Rhodamine 123 was used as a fluorescence tracer since Rhodamine 123 accumulation is sensitive and specific for indicating the transport function of P-gp [13]. Briefly, one million cells were seeded in 6-well plates and cultured for 12 h. After washing, the cells were incubated with 0.25 $\mu\text{g}/\text{ml}$ Rhodamine 123 for 1 h at 37°C to allow uptake and harvested to detect Rhodamine 123 efflux. Then, the cells were centrifuged, washed twice with ice-cold phosphate-buffered saline (PBS), and resuspended in fresh medium at 37°C for 30 min. Each sample was subjected to flow cytometry for measurement of mean fluorescence intensity, indicating cellular Rhodamine 123 accumulation. All analyses were performed in triplicate in three separate experiments and the results were expressed as the mean fluorescence intensity, which reflected cellular content of the dye retained.

Cytotoxicity assay. MTT assay was adopted to assess the silencing effect of $\alpha v\beta 6$ on the chemosensitivity of MCF-7/ADR cells to anticancer drugs. Cells were plated in 96-well plates at a density of 5×10^4 cells per well and incubated for 24 h. Afterward, the cells were incubated with various concentrations of the anticancer drug (adriamycin or paclitaxel) for another 48 h, and then were stained with 15 μl MTT solution (5 mg/ml) for further 4 h at 37°C. Subsequently, the culture medium was removed, and 150 μl of dimethyl sulfoxide (DMSO) was added to dissolve the resulting MTT formazan crystals and thoroughly mixed for 10 min. Spectrometric absorbance at a wavelength of 570 nm was measured against background subtraction at 630 nm on a microplate reader (Bio-Rad, Coda, Richmond, CA). The absorbance from the cells treated with adriamycin or paclitaxel was corrected against the absorbance from the untreated control cells. Relative drug resistance was evaluated by comparing the IC_{50} , which was defined as the concentration of drugs

causing 50% inhibition of cell growth from dose-response curves. All analyses were performed in triplicate in three independent experiments.

Flow cytometric analysis of apoptosis. Cell apoptosis profiles were analyzed by flow cytometry. Cells were placed in a 12-well plate at the density of 1.5×10^5 cells/well, and then adriamycin was added to the culture medium for 48 h. Cells (1×10^6) cells were harvested, washed with cold PBS and resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl_2). Then, cells were fixed with ice-cold 70% ethanol and incubated at 4°C for 1 h with 0.1% RNase A. Annexin V-FITC and PI at concentrations of 1 $\mu\text{g}/\text{ml}$ each were added into the cell suspension, and the mixture was incubated for 30 min in the dark at room temperature. The stained cells were analyzed by flow cytometry.

Statistical analysis. The unpaired Student's t-test and one-way analysis of variance (ANOVA) were used to evaluate the statistical significance of differences in two groups and multiple groups, respectively. A p-value <0.05 was considered statistically significant. Data were reported as the mean \pm SD and were representative of at least three independent experiments.

Results

Knockdown of $\alpha v\beta 6$ mRNA and protein levels in MCF-7/ADR cells by $\beta 6$ shRNAs. As shown in Figure 1A, $\beta 6$ shRNA1 and $\beta 6$ shRNA2 markedly reduced $\alpha v\beta 6$ mRNA level to 6.2% and 14.9% in MA/ $\alpha v\beta 6$ -1 and MA/ $\alpha v\beta 6$ -2 cells, respectively, while the control vector did not suppress mRNA expression of $\alpha v\beta 6$. In other words, the expression inhibition rates of $\alpha v\beta 6$ mRNA in MA/ $\alpha v\beta 6$ -1 and MA/ $\alpha v\beta 6$ -2 cells were 93.8% and 85.1%, respectively. Moreover, the $\alpha v\beta 6$ protein levels in MA/ $\alpha v\beta 6$ -1 and MA/ $\alpha v\beta 6$ -2 cells also decreased by 91.4 and 85.7%, respectively (Figure 1B), which was evidently lower than that of untreated control cells. In MCF-7/ADR cells, plasmid vector based $\beta 6$ shRNA effectively downregulated integrin $\alpha v\beta 6$ mRNA and protein expression. However, GAPDH expression was not affected by the same experimental conditions.

MDR1 mRNA and P-gp expression efficiently suppressed by pSUPER- $\beta 6$ shRNAs in MCF-7/ADR cells. RT-PCR and western blot analysis of cell extracts were also carried out to determine whether the inhibition of $\alpha v\beta 6$ mRNA by $\beta 6$ shRNA expressing plasmid influences MDR1 mRNA and P-gp expression. Similar trends were observed in MA/ $\alpha v\beta 6$ -1 and MA/ $\alpha v\beta 6$ -2 cells (Figures 1C, 1D). Compared with the high expression of MCF-7/ADR control cells, the relative levels of MDR1 mRNA were significantly reduced to 10.9%, 17.6%, and P-gp decreased by 87.3 and 74.1% in MA/ $\alpha v\beta 6$ -1 and MA/ $\alpha v\beta 6$ -2 cells, respectively (Figures 1C and 1D), obviously lower than that of the controls. Not unexpectedly, there was no difference in the mRNA and protein expression level between MA/CON and untreated MCF-7/ADR cells. In

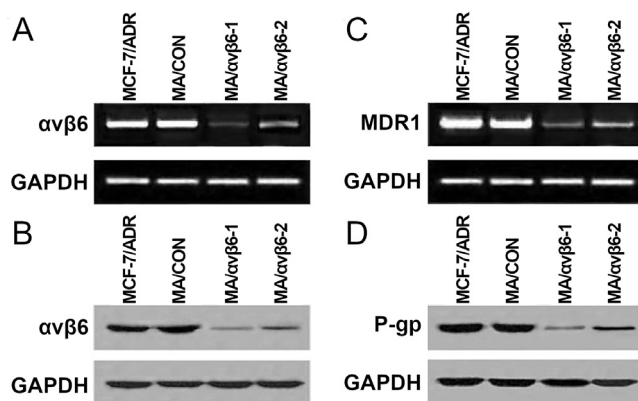


Figure 1. Analysis of the expression of $\alpha v\beta 6$ (A, B) and MDR1 (C, D) gene in $\beta 6$ shRNA-transfected cells. A) The expression of $\alpha v\beta 6$ mRNA detected by RT-PCR $\alpha v\beta 6$ protein (B) detected by western blot. C) The expression of MDR1 mRNA and P-gp protein (D). Values are expressed as a fold of the loading control, respectively. ** $p < 0.01$ and *** $p < 0.001$, as compared with MCF-7/ADR cells.

addition, no effects of RNAi were observed on the expression of GAPDH, which was used as an internal control. This change of MDR1 mRNA and P-gp expression is in agreement with that of $\alpha\beta 6$ gene.

Enhancing the accumulation of Rhodamine 123 and alteration of P-gp function in $\beta 6$ shRNA transfected breast cancer cells. Rhodamine 123, known as a high-affinity substrate of P-gp, was utilized to be a model substrate for detecting the transport activity of P-gp. To further evaluate whether the function of P-gp mediated drug efflux is affected by $\beta 6$ shRNA, the intracellular accumulation and retention of Rhodamine 123 were quantitated by flow cytometric analysis. As shown in Figure 2, the relative fluorescence intensities of Rhodamine 123 were $72.9 \pm 3.0\%$ (MA/ $\alpha\beta 6$ -1) and $61.4 \pm 2.7\%$ (MA/ $\alpha\beta 6$ -2 cells), which are significantly higher than in MCF-7/ADR control cells ($15.8 \pm 1.3\%$). In other words, the fluorescence intensity of Rhodamine 123 in MCF-7/ADR was set at 100% in this study. After 30 min

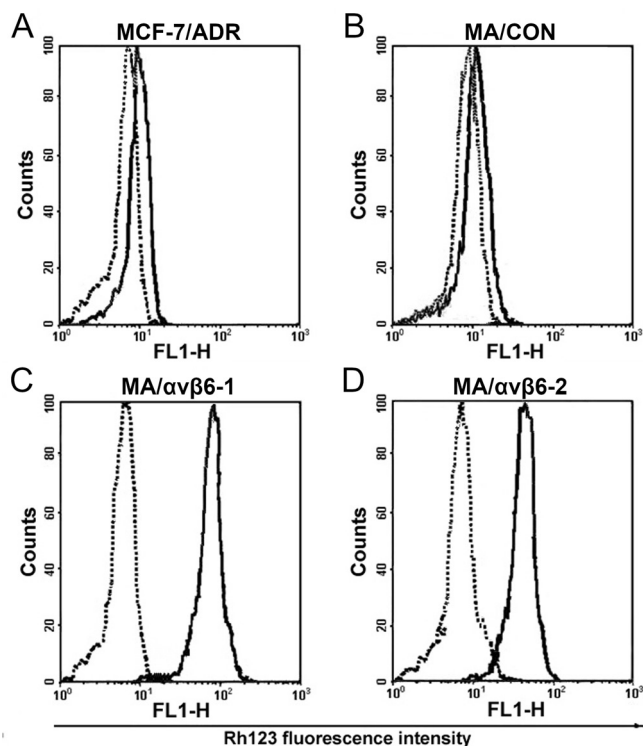


Figure 2. Effects of $\beta 6$ shRNA-mediated decrease of $\alpha\beta 6$ protein content on the intracellular accumulation and retention of Rhodamine 123 in $\beta 6$ shRNA-transfected cells. The fluorescent intensity of Rhodamine 123 in MCF-7/ADR (A), MA/CON (B), MA/ $\alpha\beta 6$ -1 (C) and MA/ $\alpha\beta 6$ -2 (D) cells. Relative fluorescence intensity of Rh123 was used to assess the function of P-gp. The lines stand for the fluorescence intensity of Rhodamine 123 in untreated MCF-7/ADR cells and treated cells (MA/CON, MA/ $\alpha\beta 6$ -1 and MA/ $\alpha\beta 6$ -2 cells) for 30 min efflux. A-D) dotted lines untreated MCF-7/ADR control cell, (A) solid lines untreated MCF-7/ADR cell, (B-D) solid lines transfected cells (MA/CON, MA/ $\alpha\beta 6$ -1 and MA/ $\alpha\beta 6$ -2 cells). Under each set of experimental conditions, 20,000 events were analyzed. All analyses were performed in triplicate in three separate experiments and representative results are shown.

of incubation in Rhodamine 123-free culture medium, a rapid increase of intracellular Rhodamine 123 was observed in MA/ $\alpha\beta 6$ -1 and MA/ $\alpha\beta 6$ -2 cells, by approximately 4.6-fold and 3.9-fold, respectively, as compared with MCF-7/ADR cells. These findings are in agreement with the change in MDR1 mRNA and P-gp levels (Figures 1C and 1D). As shown in Table 1, the efflux activity of MCF-7/ADR cells was set as 100% in this study. The results revealed that the efflux activity of Rhodamine 123 was reduced by 87.5% and 71.9% in MA/ $\alpha\beta 6$ -1 ($12.5 \pm 1.9\%$) and MA/ $\alpha\beta 6$ -2 ($28.1 \pm 2.7\%$), significantly lower than that of MCF-7/ADR control cells. As for MA/CON cells, no significant changes were observed in intracellular Rhodamine 123 accumulation and efflux activity, when compared with MCF-7/ADR cells. Taken together, such data indicate that $\beta 6$ shRNAs obviously inhibited the cellular efflux capacity of P-gp, and thus may be able to enhance the intracellular uptake of anticancer drugs and therefore reverse P-gp mediated resistance.

Evaluation of chemosensitivity in transfected cells. To investigate whether inhibition of $\alpha\beta 6$ by RNAi is able to affect the chemosensitivity of anticancer drugs, we compared the MA/ $\alpha\beta 6$ and control cells after the treatment with adriamycin or paclitaxel. The reversal of the multidrug-resistant phenotype was evaluated by comparing IC_{50} values determined by MTT assay. As shown in Table 2, the relative resistance to adriamycin dropped from 201.3 to 10.6 and 20.8, equivalent to a 19.0-fold and 9.7-fold reduction of resistance for adriamycin in MA/ $\alpha\beta 6$ -1 and MA/ $\alpha\beta 6$ -2 cells, respec-

Table 1 Effects of $\beta 6$ -shRNAs on P-gp mediated Rhodamine 123 efflux activity.

Cell lines	Efflux activity (%)
MCF-7/ADR	100
MA/CON	97.4 ± 5.3
MA/ $\alpha\beta 6$ -1	$12.5 \pm 1.9^{**}$
MA/ $\alpha\beta 6$ -2	$28.1 \pm 2.7^*$

Rhodamine 123 efflux assay. P-gp mediated Rhodamine 123 efflux assay was determined by flow cytometry. The value of MCF-7/ADR cells was set as 100%. * $p < 0.05$, ** $p < 0.01$ compared with the MCF-7/ADR control cells.

Table 2. Evaluation of chemosensitivity in cells treated with $\beta 6$ -shRNAs.

Group	IC_{50} (μM) for adriamycin	Relative drug resistance (reversal fold)	IC_{50} (μM) for paclitaxel	Relative drug resistance (reversal fold)
MCF-7	1.2 ± 0.1	1.00	2.3 ± 1.8	1.00
MCF-7/ADR	241.6 ± 5.3	201.3	565.1 ± 13.9	245.7
MA/CON	236.0 ± 4.9	196.7	543.9 ± 15.2	236.5
MA/ $\alpha\beta 6$ -1	12.7 ± 2.1	10.6**	37.1 ± 3.6	16.1#
MA/ $\alpha\beta 6$ -2	24.9 ± 2.5	20.8**	63.0 ± 5.2	27.4#

The value of relative drug resistance was the IC_{50} of the treated cells relative to MCF-7. The value of relative drug resistance of the sensitive MCF-7 untreated control cells was set as 1. The reversal factor was calculated by comparing the IC_{50} of treated cells for anticancer agents (adriamycin or paclitaxel) with that of MCF-7 cells. Results represent mean values \pm SD from triplicate determinations. ** $p < 0.01$ versus MCF-7 cells.

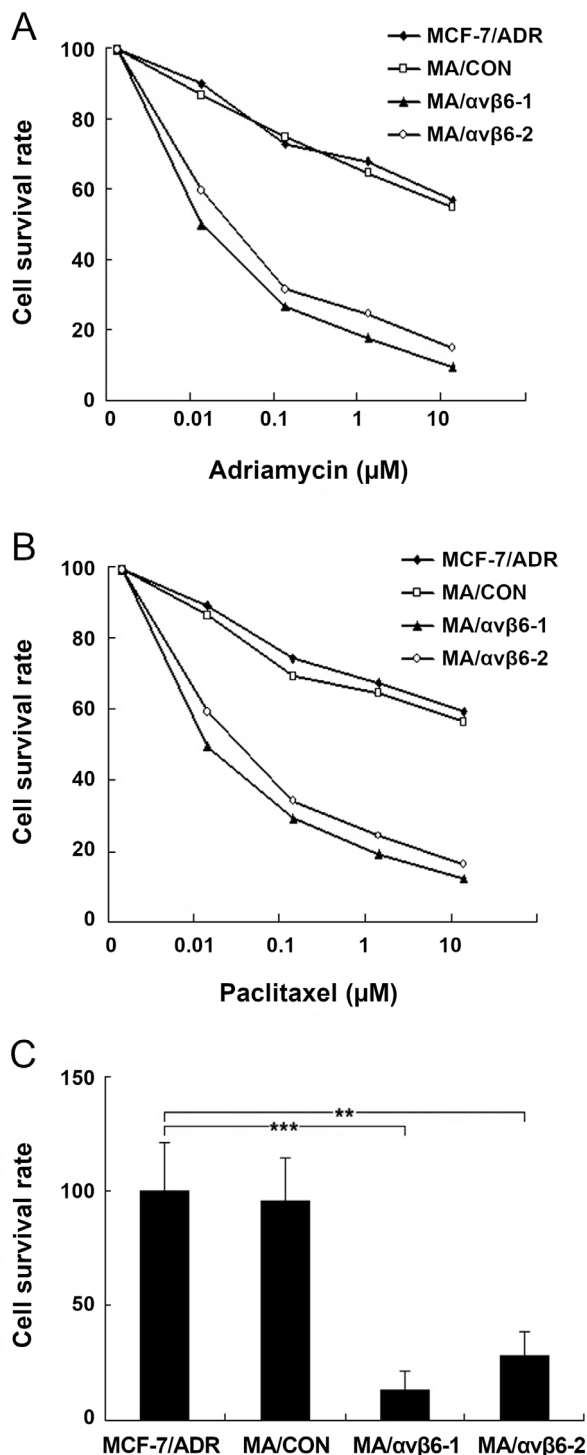


Figure 3. The survival rate and apoptosis rate in $\beta 6$ shRNA-transfected cells. Reversal effects of $\beta 6$ shRNAs on chemosensitivity in untreated MCF-7/ADR cells and transfected cells (MA/CON, MA/ $\alpha\text{v}\beta 6$ -1 and MA/ $\alpha\text{v}\beta 6$ -2 cells) to adriamycin (A) and paclitaxel (B). C) The survival rate in $\beta 6$ shRNA-transfected cells compared to the untransfected MCF-7/ADR control cells in all dose points. Values are expressed as a percentage of each corresponding control. Cytotoxicity was assessed by using MTT assay. The mean and standard deviation of three independent experiments are shown. ** $p < 0.01$ and *** $p < 0.001$ in comparison with control cells.

tively. Moreover, the relative resistance to paclitaxel was significantly decreased from 245.7 to 16.1 and 27.4, equivalent to a 15.3-fold and 9.0-fold reduction of that in MA/ $\alpha\text{v}\beta 6$ -1 and MA/ $\alpha\text{v}\beta 6$ -2 cells, respectively. However, there was no significant difference in both MCF-7/ADR and MA/CON cells.

Furthermore, cytotoxicity was shown as growth inhibition rate and cell survival rate compared with untransfected MCF-7/ADR control cells. As shown in Figure 3A and 3B, MA/ $\alpha\text{v}\beta 6$ -1 and MA/ $\alpha\text{v}\beta 6$ -2 cells transfected with recombinant plasmid pSUPER- $\beta 6$ shRNAs showed obviously higher chemosensitivity to adriamycin and paclitaxel than MCF-7/ADR and MA/CON cells. Moreover, the survival rate was significantly lower in $\beta 6$ shRNA-transfected cells, compared to the untransfected MCF-7/ADR control cells in all dose points (Figure 3C). In other words, suppression of $\alpha\text{v}\beta 6$ by siRNA led to a significant decrease in survival rates of MCF-7/ADR and MA/CON cells.

In contrast, MA/CON cells transfected with control empty vector pSUPER did not affect the cytotoxicity of adriamycin and paclitaxel as compared with untransfected MCF-7/ADR cells (Figures 3A and 3B). Therefore, these findings suggest that $\beta 6$ shRNA may be able to reverse the multidrug resistance in MCF-7/ADR cells by downregulating $\alpha\text{v}\beta 6$ expression, and further support our hypothesis that the knockdown of $\alpha\text{v}\beta 6$ expression by siRNA results in the sensitivity of cells to anticancer drugs.

$\alpha\text{v}\beta 6$ protected MCF-7/ADR breast adenocarcinoma cells from adriamycin-induced apoptosis. Apoptosis was detected as the second marker of drug resistance to chemotherapy. To quantify the rate of apoptosis, Annexin V-FITC/PI cell apoptosis assay and flow cytometry were used. Firstly, cells were exposed to adriamycin (10 $\mu\text{g}/\text{l}$) for 48 h. As shown in Figure 4A, in the absence of adriamycin treatment, the apoptosis rate of MCF-7/ADR, MA/CON, MA/ $\alpha\text{v}\beta 6$ -1 and MA/ $\alpha\text{v}\beta 6$ -2 cells were $2.7 \pm 0.5\%$, $1.9 \pm 0.4\%$, $51.8 \pm 2.6\%$ and $44.1 \pm 2.3\%$, respectively. There were significant differences in the apoptosis rate of the untransfected and $\beta 6$ shRNA-transfected cells. As shown in Figure 4B, in the presence of adriamycin treatment, the apoptosis rates of MCF-7/ADR, MA/CON, MA/ $\alpha\text{v}\beta 6$ -1 and MA/ $\alpha\text{v}\beta 6$ -2 cells were $19.4 \pm 1.2\%$, $23.1 \pm 1.5\%$, $76.3 \pm 2.4\%$ and $65.2 \pm 1.9\%$, respectively. The difference was significant. In MA/ $\alpha\text{v}\beta 6$ -1 and MA/ $\alpha\text{v}\beta 6$ -2 cells, more apoptotic cells occurred when $\alpha\text{v}\beta 6$ was inhibited. In other words, cells transfected with $\beta 6$ shRNAs showed a significantly higher apoptosis rate than control cells. Taken together, these data further indicate that suppression of $\alpha\text{v}\beta 6$ -integrin enhanced the adriamycin-induced apoptosis.

Downregulation of Bcl-2 and Bcl-xL while upregulation of caspase-3 and Bad protein expression by $\beta 6$ shRNAi constructs. Members of the Bcl-2 family have a crucial role in modulating programmed cell death and have a significant impact on chemosensitivity [14]. In order to ascertain whether Bcl-2 family proteins are involved in $\alpha\text{v}\beta 6$ -mediated

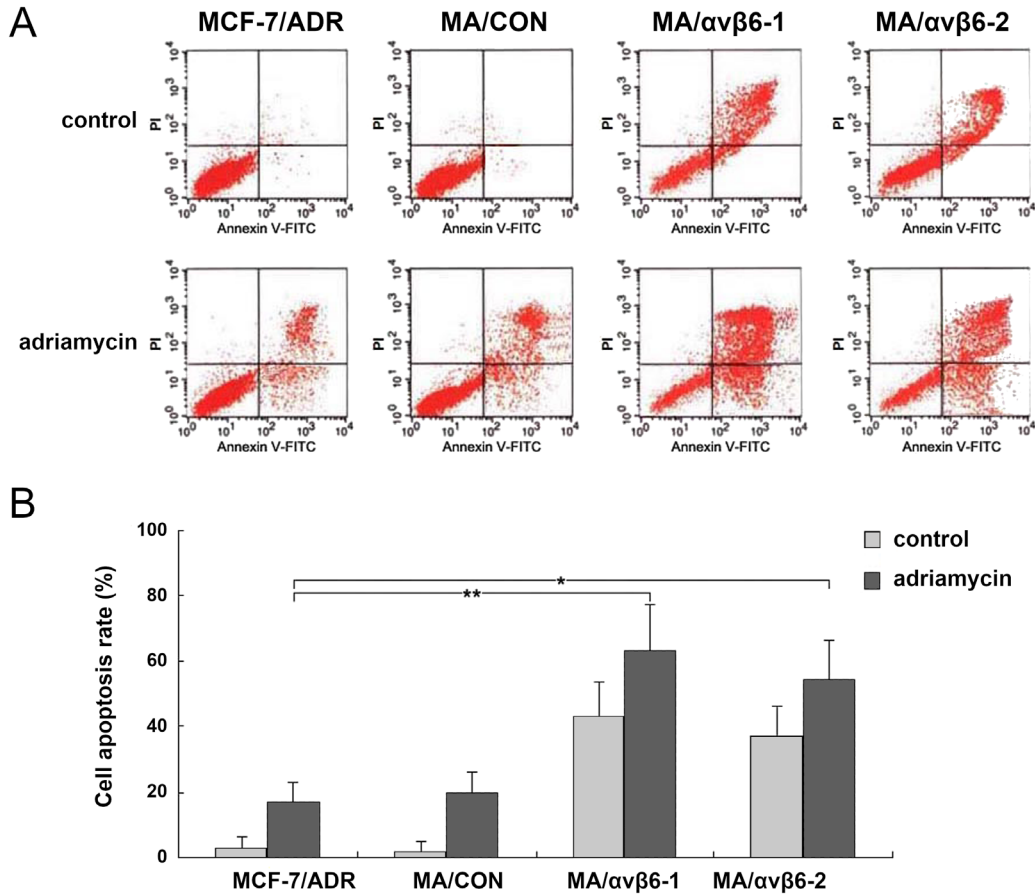


Figure 4. The apoptosis rate in $\beta 6$ shRNA-transfected cells. A) The representative dot plots of Annexin V-FITC/PI stained untransfected MCF-7/ADR control and $\beta 6$ shRNA-transfected cells. B) The apoptosis rate in $\beta 6$ shRNA-transfected cells compared to the untransfected MCF-7/ADR control cells. Annexin V-FITC/PI assay analysis showed suppression of integrin $\alpha\beta 6$ induced the apoptosis of MCF-7/ADR breast cancer cells. The mean and standard deviation of three independent experiments are shown. * $p < 0.05$ and ** $p < 0.01$ in comparison with control cells.

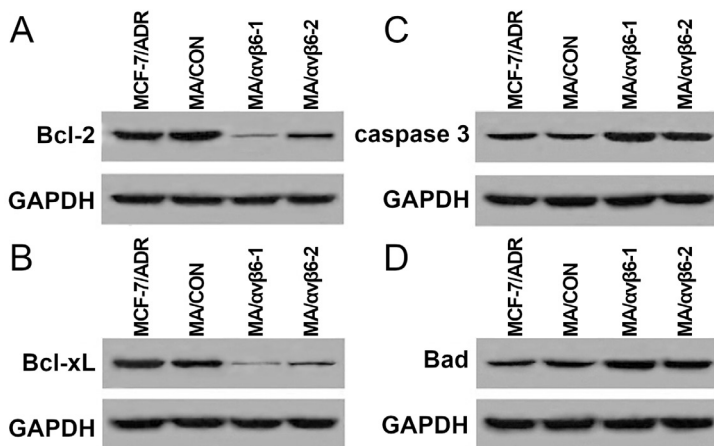


Figure 5. Effect of $\beta 6$ shRNA on Bcl-2 (A), Bcl-xL (B), caspase-3 (C) and Bad (D) protein expression in $\beta 6$ shRNA-transfected and control MCF-7/ADR cells. Western blot analysis (Figure S1) showed downregulation of Bcl-2 and Bcl-xL and upregulation of caspase-3 and Bad in MA/ $\alpha\beta 6-1$ and MA/ $\alpha\beta 6-2$ cells. ** $p < 0.01$ and *** $p < 0.001$ versus MCF-7/ADR cells.

adriamycin resistance, we detected anti-apoptotic proteins Bcl-2 and Bcl-xL, pro-apoptotic proteins caspase-3 and Bad by western blot analysis. As shown in Figure 5, in MA/ $\alpha\beta 6-1$ and MA/ $\alpha\beta 6-2$ $\beta 6$ shRNAs-transfected cells, the expressions of Bcl-2 and Bcl-xL were significantly lower, while caspase-3 and Bad were dramatically higher compared with those in MCF-7/ADR wild type cells ($p < 0.01$ vs the control). However, there were no obvious changes in those of MCF-7/ADR and MA/CON cells ($p > 0.05$). That is to say, anti-apoptotic proteins Bcl-2 and Bcl-xL, pro-apoptotic proteins caspase-3 and Bad, almost remained unchanged in the control cells. The findings demonstrate that suppressed $\alpha\beta 6$ expression permits cells to dramatically maintain high levels of pro-apoptotic proteins caspase-3 and Bad, low levels of anti-apoptotic proteins Bcl-2 and Bcl-xL for progression of apoptosis.

Suppression of integrin $\alpha\text{v}\beta\text{6}$ induced mitochondrial release of cytochrome C and activated caspase-3 by $\beta\text{6shRNAi}$. In order to examine whether $\alpha\text{v}\beta\text{6}$ -mediated adriamycin resistance in MCF-7/ADR breast adenocarcinoma cells was involved in the apoptosis pathway, we detected the level of cytochrome C and the activity of caspase-3. As shown in Figure 6A, western blot analysis indicated that the expression of cytosolic cytochrome C was high in MA/ $\alpha\text{v}\beta\text{6-1}$ and MA/ $\alpha\text{v}\beta\text{6-2}$ cells ($p < 0.01$ vs the control), while no significant changes were observed among MCF-7/ADR and MA/CON cells ($p > 0.05$). It was significantly higher in $\beta\text{6shRNAs}$ -transfected cells compared with the control cells. As shown in Figure 5C, the $\beta\text{6shRNAs}$ -transfected cells have higher caspase-3 activity as expected from cytochrome C release. In MA/ $\alpha\text{v}\beta\text{6-1}$ and MA/ $\alpha\text{v}\beta\text{6-2}$ cells, suppression of $\alpha\text{v}\beta\text{6}$ by $\beta\text{6shRNAs}$ induced high expression of cytochrome C and high caspase-3 activity leading to rapid apoptosis. **Downregulation of $\alpha\text{v}\beta\text{6}$ inhibits ERK1/2 expression.** As shown in Figure 6B and 6C, the expression of ERK1/2 markedly decreased by $\beta\text{6shRNAs}$ in the MA/ $\alpha\text{v}\beta\text{6-1}$ and MA/ $\alpha\text{v}\beta\text{6-2}$ cells, compared with those in the control group, the difference is significant ($p < 0.01$). These results indicate downregulation of integrin $\alpha\text{v}\beta\text{6}$ by $\beta\text{6shRNAs}$ inhibited the ERK1/2 level in the MA/ $\alpha\text{v}\beta\text{6-1}$ and MA/ $\alpha\text{v}\beta\text{6-2}$ cells. That is to say, it showed that decreased $\alpha\text{v}\beta\text{6}$ has the inhibitory effect on the ERK1/2 level in the MA/ $\alpha\text{v}\beta\text{6-1}$ and MA/ $\alpha\text{v}\beta\text{6-2}$ cells.

Discussion

As is known to all, multidrug resistance (MDR) to chemotherapeutic agents is a leading obstacle to successful treatment in breast cancer. In particular, P-gp, the product of the MDR1 gene, plays a major role in chemotherapeutic drug resistance [15]. Recently, more studies pay attention to integrin $\alpha\text{v}\beta\text{6}$, which is particularly interesting for the fact that it is not expressed in the normal epithelium, but highly expressed in the process of tumorigenesis [16]. The integrin $\alpha\text{v}\beta\text{6}$ may serve to identify patients that are at higher risk of developing metastatic disease and emerged as an attractive new candidate as a potential novel therapeutic target for anticancer therapy. However, it is not clear whether and how $\alpha\text{v}\beta\text{6}$ is related to the modulation of MDR in breast adenocarcinoma cells, or whether there is any potential relationship between $\alpha\text{v}\beta\text{6}$ and apoptosis or ECM degradation in breast adenocarcinoma cells. The aim of this current study was to investigate the effect and possible underlying molecular regulatory mechanism of the suppression of $\alpha\text{v}\beta\text{6}$ expression targeting the β6 gene by RNAi on MDR in breast cancer cells.

In this study, our results demonstrated that the levels of $\alpha\text{v}\beta\text{6}$ and MDR1 mRNA and protein in successfully established MA/ $\alpha\text{v}\beta\text{6-1}$ and MA/ $\alpha\text{v}\beta\text{6-2}$ cells were significantly lower than those in the control group. In addition to reducing the expression of $\alpha\text{v}\beta\text{6}$, especially these cells also confirmed a marked decrease in the expression levels of MDR1 and P-gp.

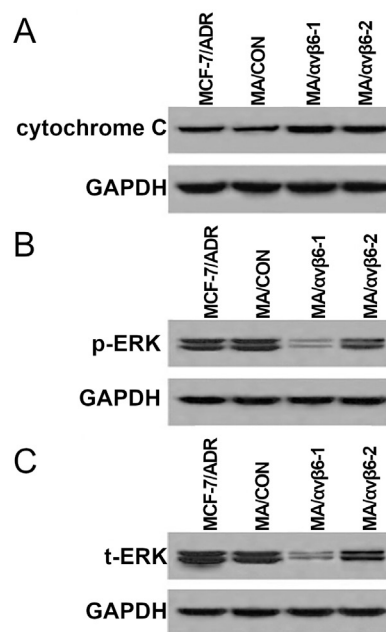


Figure 6. Effect of β6shRNA on the expression of cytochrome C (A), p-ERK (B) and t-ERK (C) in β6shRNA -transfected cells. Analysis of western blots is shown in Figure S1.

The reduction in drug efflux subsequently led to an abnormally high intracellular accumulation of Rh 123 due to the decrease of P-gp. Our data thus demonstrated that β6shRNA was able to efficiently downregulate the expression of $\alpha\text{v}\beta\text{6}$ and MDR1, thereby reversing adriamycin-mediated MDR. Furthermore, our study revealed that inhibition of $\alpha\text{v}\beta\text{6}$ expression allowed cells to dramatically maintain high levels of pro-apoptotic proteins caspase-3 and Bad, and low levels of anti-apoptotic proteins Bcl-2 and Bcl-xL contributing to the induction of apoptosis, which were accompanied by changes in cytochrome C released from the mitochondria, as well as changes in the caspase-3 activity. Moreover, our findings also indicated that the reduced $\alpha\text{v}\beta\text{6}$ expression resulted in the inactivation of ERK/MAP kinase, and blocking the pathway can effectively reverse adriamycin resistance of breast cancer cells. An important finding of this study was that inhibition of the expression of $\alpha\text{v}\beta\text{6}$ obviously suppressed MDR1 and P-gp expression. To the best of our knowledge, this is the first report to demonstrate that knockdown of integrin $\alpha\text{v}\beta\text{6}$ by RNAi led to reduction of P-gp expression to reverse adriamycin-mediated MDR in breast carcinoma cells, which might be involved in dramatically reduced Bcl-2 and Bcl-xL, enhanced Bax, meanwhile increased release of cytochrome C and activated caspase-3 resulting in apoptosis through the ERK/MAP kinase pathway. Taken together, our data confirmed that β6shRNA can effectively downregulate the expression of $\alpha\text{v}\beta\text{6}$ and MDR1, thereby reversing MDR, and thus implicated the potential for breast cancer gene therapy using $\alpha\text{v}\beta\text{6}$ as an attractive candidate target.

Integrin, as the most critical mediator of cell adhesion and the surrounding environment, is not just a simple attachment molecule, but mediate bidirectional signal transduction across the cell membrane. More studies suggested that integrins play an important role and influence the tumor chemoresistance [17]. Several different molecular mechanisms had been suggested, including one proposing that certain integrin subtypes can inhibit apoptosis induced by chemotherapeutic drugs. For instance, it has been reported that $\beta 1$ -integrin suppressed chemotherapy-induced apoptosis in hematologic tumors [18]. Besides, integrin $\alpha 5$ and $\beta 7$ have also been found to be associated with drug resistance [19, 20]. Noteworthy, a more recent study has found that in breast cancer cell lines MDA-MB-231 and MDA-MB-435 the ligation of $\beta 1$ integrins inhibited apoptosis induced by paclitaxel and vincristine, two microtubule-directed chemotherapeutic agents widely used in the therapy of breast cancer [21]. However, the underlying relationship between $\alpha \beta 6$ and tumor chemoresistance remains unclear. In the present study, we have constructed permanent cell lines expressing the $\beta 6$ gene-specific siRNA and investigated whether multidrug resistance in those cells was able to be reversed. It is noteworthy that suppression of integrin $\alpha \beta 6$ by RNAi dramatically downregulated the expression levels of MDR1 gene mRNA and P-gp, thereby to reverse adriamycin-mediated MDR in breast cancer cells. The most important and meaningful finding was that inhibition of the expression of $\alpha \beta 6$ dramatically suppressed MDR1 and P-gp expression.

The potential molecular mechanism of this inhibitory effect still remains unclear. Further studies have extended the role of integrins in chemoresistance to other cancer cell types and several different classes of chemotherapy drugs [22–24]. Of note, recent research has found that the protective effect of $\beta 1$ integrin in breast cancer cells was mediated via activation of the PI 3-kinase/AKT pathway, which prevented the downregulation of the Bcl-2 protein level and inhibited drug-induced cytochrome C release upon drug treatment [25]. Thus, the discovery of significant inhibition of MDR1 expression may be due to the decreased expression of $\alpha \beta 6$. Overall, our findings further indicate that $\alpha \beta 6$ plays a key role in MDR functional phenotype. Decreased expression of $\alpha \beta 6$ was connected with an evident reversal of chemoresistance, which was confirmed by MTT assay as the IC50 value and relative drug resistance decreased significantly. Most notably, inhibition of integrin $\alpha \beta 6$ by RNAi targeting $\beta 6$ gene significantly downregulated the expression levels of MDR1 gene mRNA and P-gp, thereby reversing adriamycin-mediated chemoresistance in breast cancer cells.

However, it is unclear why a decrease in the expression of $\alpha \beta 6$ led to the reversal of MDR. A prevailing hypothesis is that the resistance of cancer cells to apoptosis contributes to the development of drug resistance, which is a critical factor in clinical recurrence or relapse of cancer patients treated with chemotherapeutic drugs. It was supported by increased activity of caspase-3 and Bad, decreased levels of Bcl-2 and

Bcl-xL in our transfected breast cancer cells, accompanied by changes in cytochrome C release from the mitochondria. Mitochondrial cell death is regulated by a balance between pro-apoptotic and anti-apoptotic Bcl-2 family proteins. Apoptotic stimuli that activate the mitochondrial death pathway result in the activation of Bcl-2 pro-apoptotic proteins and inactivation of the Bcl-2 anti-apoptotic proteins. Consequently, the activation of pro-apoptotic Bcl-2 family proteins, such as Bax/Bak, causes mitochondrial permeabilization, which in turn leads to the release of apoptotic factors from mitochondria, among which is cytochrome C [26–27]. In the present study, we found that the mitochondrial apoptosis pathway is also involved in $\alpha \beta 6$ -mediated adriamycin resistance of MCF-7 breast adenocarcinoma cells. Considering that the mitochondrial apoptosis pathway is the preferred mechanism for most chemotherapeutic agents, we suspect that it is also the primary mechanism of $\alpha \beta 6$ -mediated chemoresistance. However, at least in MCF-7 breast cancer cells, it can be speculated that $\alpha \beta 6$ may play a role in adriamycin resistance by indirectly or directly modulating the protein expression of Bcl-2/Bax. In this study, data indicated that $\beta 6$ gene-specific siRNA could activate the pro-apoptotic proteins caspase-3 and Bad, while inhibiting the anti-apoptotic proteins Bcl-2 and Bcl-xL in transfected MCF-7/ADR breast cancer cells, accompanied by changes in cytochrome C released from mitochondria. Thus, the reduced ability of $\alpha \beta 6$ could promote cytotoxicity through the mitochondrial apoptotic pathway and Bcl-2 family proteins, eventually leading to the reversal of drug resistance.

The ERK/MAPK pathway plays a critical role in modulating many fundamental processes, involved in cell proliferation, differentiation, migration and apoptosis. In the present study, we found that suppression of $\alpha \beta 6$ led to the inactivation of ERK/MAP kinase and blocking the pathway effectively reversed integrin $\alpha \beta 6$ -mediated chemoresistance to adriamycin in breast cancer cells. Considering that ERK inhibition itself affects chemosensitivity of cells and integrin $\alpha \beta 6$ may act through multiple mechanisms, the extent of abrogation elimination of chemoresistance indicated that integrin $\alpha \beta 6$ -mediated adriamycin resistance in MCF-7 breast cancer cells was at least partly dependent on the ERK/MAP kinase pathway. Evidence suggests that Bcl-2 and Bax act as modulators of this pathway by affecting the release of cytochrome C and subsequent activation of caspase-9 and caspase-3 [28]. Thus, our study here extends these findings and demonstrates that knockdown of the $\beta 6$ gene downregulates Bcl-2 and Bcl-xL, and upregulates caspase-3 and Bad expression by decreasing the activity of ERK/MAP kinase, thus inhibiting the mitochondrial apoptotic pathway, and eventually reversing the adriamycin-mediated chemoresistance in breast cancer cells.

Taken together, this data suggested an efficient RNAi-based method that specifically inhibits $\alpha \beta 6$ and MDR1 genes *in vitro*, thereby reversing MDR in breast adenocarcinoma. Our research, therefore, provides a particularly useful

strategy to improve the chemosensitivity. Our findings thus revealed that integrin $\alpha v \beta 6$ contributing to adriamycin resistance in breast adenocarcinoma cells is dependent on the ERK/MAP kinase pathway, which is involved in changes in mitochondrial pathway and expression of Bcl-2/Bcl-xL and caspase-3/Bad. Most notably, an important potential consequence of our work is that integrin $\alpha v \beta 6$ must be an attractive therapeutic candidate for breast cancer. Considering the close relationship between $\alpha v \beta 6$ and breast adenocarcinoma, the specific strategy targeting $\beta 6$ gene can improve the therapeutic effect of breast adenocarcinoma.

Supplementary information is available in the online version of the paper.

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