

# Association between Twist and multidrug resistance gene-associated proteins in Taxol<sup>®</sup>-resistant MCF-7 cells and a 293 cell model of Twist overexpression

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**Abstract.** Multidrug resistance (MDR) severely limits the effectiveness of chemotherapy. Previous studies have identified Twist as a key factor of acquired MDR in breast, gastric and prostate cancer. However, the underlying mechanisms of action of Twist in MDR remain unclear. In the present study, the expression levels of MDR-associated proteins, including lung resistance-related protein (LRP), topoisomerase II $\alpha$  (TOPO II $\alpha$ ), MDR-associated protein (MRP) and P-glycoprotein (P-gp), and the expression of Twist in cancerous tissues and pericancerous tissues of human breast cancer, were examined. In order to simulate Taxol<sup>®</sup> resistance in cells, a Taxol<sup>®</sup>-resistant human mammary adenocarcinoma cell subline (MCF-7/Taxol<sup>®</sup>) was established by repeatedly exposing MCF-7 cells to high concentrations of Taxol<sup>®</sup> (up to 15  $\mu$ g/ml). Twist was also overexpressed in 293 cells by transfecting this cell line with pcDNA5/FRT/TO vector containing full-length hTwist cDNA to explore the dynamic association between Twist and MDR gene-associated proteins. It was identified that the expression levels of Twist, TOPO II $\alpha$ , MRP and P-gp were upregulated and LRP was downregulated in human breast cancer tissues, which was consistent with the expression of these proteins in the Taxol<sup>®</sup>-resistant MCF-7 cell model. Notably, the overexpression of Twist in 293 cells increased the resistance to Taxol<sup>®</sup>, Trichostatin A and 5-fluorouracil, and also upregulated the expression of MRP and P-gp. Taken together, these data demonstrated that Twist may promote drug resistance in cells and cancer tissues through regulating the expression of MDR gene-associated proteins,

which may assist in understanding the mechanisms of action of Twist in drug resistance.

## Introduction

Breast cancer, with increasing rates of incidence and mortality worldwide, is a major cause of mortality within female malignancies (1,2). Due to its late disease presentation, breast cancer exhibits poor prognosis and frequently presents with distant metastases (3). Clinically, it is difficult to treat advanced breast cancer due to the inability to completely resect the diffused tumor cells and to overcome the chemoresistance of cancer cells to chemotherapy. Paclitaxel (Taxol<sup>®</sup>) is a widely used chemotherapeutic drug for the treatment of breast cancer, and acts through the induction of proapoptotic signaling, blocking of the cell cycle in the G<sub>2</sub>-M phases and stabilization of the microtubule (4,5). Although breast cancer cells demonstrate high sensitivity to Taxol<sup>®</sup>, the prognosis of patients with advanced disease remains poor due to chemoresistance to Taxol<sup>®</sup>. Therefore, it is important to study the underlying mechanisms involved in the development of Taxol<sup>®</sup> resistance, to improve the effectiveness of chemotherapy.

Twist is a member of the basic helix-loop-helix (bHLH) transcription factor family. It includes a bHLH domain that mediates heterodimerization or homodimerization and a DNA binding domain, which combines with DNA sequences (6). Functionally, Twist was primitively identified as a potential oncogene (6,7), and previous studies have identified that Twist also contributed to acquired Taxol<sup>®</sup> resistance (8) and metastasis in cancer (9). In addition, a previous study indicated that the upregulation of Twist was positively associated with the level of disease aggression and poor survival rate (10), suggesting Twist may be a potential target for cancer therapy. Although elevated expression of Twist was revealed to be associated with Taxol<sup>®</sup> resistance, the molecular mechanism remains unclear. Notably, a series of studies demonstrated that multidrug resistance (MDR)-associated proteins served a critical role in chemical resistance, such as Taxol<sup>®</sup> resistance (11,12). Previously, lung resistance-related protein (LRP), topoisomerase II $\alpha$  (TOPO II $\alpha$ ), MDR-associated protein (MRP) and

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P-glycoprotein (P-gp) have attracted attention for their functions as MDR-associated proteins (13), which may induce MDR in chemotherapy through increasing or decreasing drug efflux, inactivation of drug and alteration of drug targets (13). LRP, a major vault protein, pumps drugs away from intracellular targets to trigger drug resistance (14,15). TOPO II $\alpha$ , a nuclear enzyme, regulates the topology of DNA and maintains genomic integrity (16). A previous study suggested that the overexpression of TOPO II $\alpha$  is markedly associated with alterations in tumor behavior and chemotherapeutic resistance via the inhibition of apoptosis (17). MRP and P-gp, two important adenosine 5-triphosphate (ATP)-binding cassette transporter proteins, mediated intracellular drug influx or efflux to alter the concentration of drugs, which increased chemoresistance to therapeutic agents, including Taxol<sup>®</sup> and anthracyclines (18). Although Twist also affects drug resistance, no studies have explored the association between Twist and MDR proteins. Therefore, the present study aimed to examine the association between Twist and MDR proteins in order to identify a novel mechanism of chemoresistance.

The present study was performed to investigate the association between Twist and MDR-associated proteins. In order to identify how Twist increases drug resistance in cancer, a MCF-7 cell model of Taxol<sup>®</sup> resistance was generated by repeatedly exposing MCF-7 cells to Taxol<sup>®</sup>, and a 293-cell model of Twist overexpression was created through transfecting pcDNA5/FRT/TO-Twist vectors into 293 cells. Concurrently, the expression of Twist, LRP, MRP, TOPO II $\alpha$  and P-gp were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) or immunohistochemistry in these two cell models to determine the clear association between Twist and these 4 types of MDR-associated proteins, and to uncover the underlying molecular mechanisms.

## Materials and methods

**Cell culture.** The MCF-7 cell line was a gift from the Infection and Immunology Laboratory of Southwest Medical University (Luzhou, China). MCF-7 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum (FCS; Gibco; Thermo Fisher Scientific, Inc.), 1x10<sup>5</sup> U/l penicillin and 100 mg/l streptomycin at 37°C with 5% CO<sub>2</sub>. The 293 cell line was also a gift from the Genetic Laboratory of Southwest Medical University. 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FCS, 1x10<sup>5</sup> U/l penicillin and 100 mg/l streptomycin at 37°C with 5% CO<sub>2</sub>. The morphology of 293 cell was observed using a light microscope (Olympus Corporation, Tokyo, Japan) at x400 magnification, and the evaluation of shape and size of 293-Twist and 293-vector were based on microscopic examination.

**Construction of MCF-7 cell model of Taxol<sup>®</sup> resistance.** The Taxol<sup>®</sup>-resistant breast cancer cell line was established by exposing the Taxol<sup>®</sup>-sensitive mammary cell line MCF-7 to 15  $\mu$ g/ml Taxol<sup>®</sup> (Taiji Industry (Group) Co., Ltd., Chongqing, China), as follows: MCF-7 cells were cultured in RPMI-1640 medium with 15  $\mu$ g/ml Taxol<sup>®</sup> for 24 h at 37°C with 5% CO<sub>2</sub>, followed by washing with warm phosphate-buffered saline

(PBS) for 2 min three times and culturing in RPMI-1640 medium without Taxol<sup>®</sup> at 37°C with 5% CO<sub>2</sub> until cells grew normally. These cells were labeled MCF-7/Taxol<sup>®</sup> I. Subsequent to culturing in RPMI-1640 medium without Taxol<sup>®</sup> for 10 days at 37°C, the MCF-7/Taxol<sup>®</sup> I cells were cultured with 15  $\mu$ g/ml Taxol<sup>®</sup> for 24 h at 37°C, followed by washing with warm PBS for 2 min three times and culturing in RPMI-1640 medium without Taxol<sup>®</sup> at 37°C until the cells grew normally, these cells were labeled MCF-7/Taxol<sup>®</sup> II. Cells from step 2 (MCF-7/Taxol<sup>®</sup> II cells) were cultured with 15  $\mu$ g/ml Taxol<sup>®</sup> for 24 h at 37°C after 10 days of normal culture without Taxol<sup>®</sup>, followed by washing with warm PBS for 2 min three times and culturing in RPMI-1640 medium without Taxol<sup>®</sup> at 37°C until cells grew normally, these cells were labeled to MCF-7/Taxol<sup>®</sup> III. MCF-7/Taxol<sup>®</sup> IV cells were generated by exposing MCF-7/Taxol<sup>®</sup> III to Taxol<sup>®</sup> as aforementioned (15  $\mu$ g/ml Taxol<sup>®</sup> for 24 h at 37°C). The half maximal inhibitory concentration (IC<sub>50</sub>) and Taxol<sup>®</sup> resistance index (RI) of MCF-7 and MCF-7/Taxol<sup>®</sup> cell lines were calculated based on an MTT assay as follows: Cell growth inhibition ratio (%)=[optical density (OD)<sub>control</sub>-OD<sub>experiment</sub>]/OD<sub>control</sub> x 100%; and RI=resistance cell IC<sub>50</sub>/parent cell IC<sub>50</sub>. Cells in 96-well plates were treated with 0.5% MTT (Affymetrix; Thermo Fisher Scientific, Inc.) for 4 h at 37°C, followed by the addition of 150  $\mu$ l dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) to dissolve the purple formazan. Cells were then agitated for 10 min at room temperature and the absorbance at 570 nm was read using a reference filter of 630 nm. MCF-7/Taxol<sup>®</sup> IV and MCF-7 cells were treated with vincristine (0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 15  $\mu$ g/ml) (Guangdong Lingnan Pharmaceutical Co., Ltd., Guangzhou, China), cisplatin (data not shown; Sigma-Aldrich; Merck KGaA), fluorouracil (0, 0.625, 1.25, 2.5, 5, 10, 20 and 40  $\mu$ g/ml) (5-FU; data not shown; Xian Haixin Pharmaceutical Co., Ltd., Xi'an, China) and mitomycin (data not shown; Sigma-Aldrich; Merck KGaA) to determine the ability of these cells to acquire multidrug resistance.

**Overexpression of Twist in 293 cells.** The plasmid pcDNA5/FRT/TO (1  $\mu$ g; Invitrogen; Thermo Fisher Scientific, Inc.) was digested with 10 U restriction enzymes *NotI* and *BamHI* (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 6 h to harvest the vector backbone. Concurrently, 1  $\mu$ g plasmid pcDNA3-Twist (a gift from the Faculty of Medical Molecular Biology, Southwest Medical University, Sichuan, China) was digested with *NotI* and *BamHI* to obtain the hTwist fragment. These two fragments were ligated to pcDNA5/FRT/TO-Twist by 1 U T4 ligase (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. Following this, 7.5  $\mu$ g pOG44 (Invitrogen; Thermo Fisher Scientific, Inc.) and 0.75  $\mu$ g pcDNA5/FRT/TO-Twist were co-transfected into Flp-In-T-Rex-293 cells by using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 24 h to generate the 293-Twist cells, and 7.5  $\mu$ g pOG44 and 0.75  $\mu$ g pcDNA5/FRT/TO were also co-transfected into Flp-In-T-Rex-293 cells to serve as negative control. Following transfection for 24 h, the medium containing 5  $\mu$ g/ml blastidcin (Invitrogen; Thermo Fisher Scientific, Inc.) was changed and the cells were cultured for 48 h at 37°C. Subsequently, 200  $\mu$ g/ml Hygromycin B (Invitrogen; Thermo Fisher

Scientific, Inc.) was added to the medium following the transfer of the cells to a new plate at 25% confluence for 3-h incubation. Once the anti-Hygromycin B cell clones had grown, they were seeded onto a 24-well plate in DMEM medium with 200  $\mu\text{g/ml}$  Hygromycin B, 5  $\mu\text{g/ml}$  blasticidin and 10% FCS at 37°C for expansion. Finally, the 293-Twist cells and the 293-vector cells were successfully constructed. Prior to performing western blot analysis and immunohistochemistry, 293-Twist and 293-vector cells were cultured with 0.1  $\mu\text{g/ml}$  tetracycline. 293-Twist and 293-vector cells were treated with Trichostatin A (TSA; 1, 4 or 8  $\mu\text{mol/l}$ ; Beyotime Institute of Biotechnology, Haimen, China), 5-FU (2.5, 12, 62.5 or 312.5  $\mu\text{mol/l}$ ) and Taxol (0.276, 1.38, 6.9 or 34.5  $\mu\text{mol/l}$ ) to test the multidrug resistance of these cells.

**Immunohistochemistry.** Human breast cancer tissues were obtained from the Affiliated Hospital of Southwest Medical University between Jan 2016 and Apr 2016. A total of 32 patients, aged between 37 and 69 years (48.32±8.41), who had not received preoperative radiation therapy and chemotherapy prior to breast cancer surgery were included in the present study. The present study obtained ethical approval from the Ethics Committee of Southwest Medical University and written informed consent was obtained from all participants. All human breast cancer tissues were fixed in 10% formalin (Chengdu KeLong Chemical Co., Ltd., Chengdu, China) at room temperature overnight and embedded in paraffin. The paraffin-embedded tissues were sliced into 4- $\mu\text{m}$  thick sections. Subsequent to dewaxing with 100% xylene for 10 min three times, the section was rehydrated with gradient (100, 90, 80 and 70%) ethanol for 1 min. Antigen retrieval was performed by boiling at 98°C for 10 min in 10 mM citric acid solution (pH 6.0). For cell samples, cells were cultured on glass coverslips and fixed with 4% paraformaldehyde for 10 min at room temperature followed by permeabilization with 0.25% Triton X-100 for 10 min at room temperature. Immunohistochemistry was conducted with an ABC staining kit (OriGene Technologies, Inc., Beijing, China) according to the manufacturer's protocol. Briefly, following antigen retrieval or Triton X-100 treatment, sections or cells were blocked at room temperature with 10% goat serum (cat. no. 16210072; Gibco; Thermo Fisher Scientific, Inc.) for 30 min and incubated with the indicated primary antibodies at the indicated dilution rate overnight at 4°C. Subsequent to washing with PBS twice for 5 min, biotinylated goat anti-mouse (dilution, 1:150; cat. no. ZB-2055; OriGene Technologies, Inc., Beijing, China) or biotinylated goat anti-rabbit (dilution, 1:150; cat. no. ZB-2011; OriGene Technologies, Inc.) secondary antibodies were applied to recognize the primary antibody at 37°C for 30 min. Then, the sections were washed again with PBS twice prior to being incubated with Vectastain ABC reagent (dilution, 1:50; Vector Laboratories, Inc., CA, USA) at 37°C for 20 min. Chromogenesis was performed with DAB for 10-15 min. The primary antibodies used in the present study included: Mouse anti-TOPO II $\alpha$  antibody (dilution, 1:200; cat. no. ZM-0245; OriGene Technologies, Inc.); mouse anti-MRP antibody (dilution, 1:200; cat. no. ZM-0345; OriGene Technologies, Inc.); rabbit anti-P-gp antibody (dilution, 1:500; cat. no. ab103477; Abcam, Cambridge, UK); mouse anti-LRP antibody (dilution, 1:200; cat. no. ZM-0325; OriGene Technologies, Inc.); and

rabbit anti-Twist antibody (dilution, 1:500; cat. no. ab49254; Abcam). Images from immunohistochemistry were captured using a Nikon Eclipse 50i microscope (Nikon Corporation, Tokyo, Japan) at x400 magnification.

**RT-qPCR.** Total RNA was extracted from the MCF-7 and 293 cells using an RNeasy Pure Cell/Bacteria kit (Qiagen Biotech Co., Ltd., Beijing, China). cDNA was synthesized from total RNA using a PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). Amplification of harvested cDNA (1  $\mu\text{g}$ ) was performed in real time within a 20- $\mu\text{l}$  reaction system consisting of SYBR Green PCR Master mix (Toyobo Life Science, Osaka, Japan) at 99°C for 10 min, followed by 40 cycles of: 30 sec denaturation at 94°C, 30 sec annealing at 58°C and 30 sec extension at 72°C. Primers used in this reaction were as follows: Twist, forward 5'-GTGCGCAGTCTTACGAGGAG-3' and reverse 5'-GCTTGAGGGTCA GAATCTTGCT-3'; and GAPDH, forward 5'-ATGCTGGCGCTGAGTACGTC-3' and reverse 5'-GGTCATGAGTCCTTCACGATA-3'. The results were analyzed using the  $2^{-\Delta\Delta C_q}$  method (19).

**Western blot analysis.** Western blot analysis was performed as previously described (20). The primary antibodies used in the present study were rabbit anti-Twist antibody (dilution, 1:1,000; cat. no. ab49254; Abcam) and rabbit anti- $\beta$ -actin antibody (dilution, 1:3,000; cat. no. PR-0255; OriGene Technologies, Inc.), and the secondary antibodies were horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (dilution, 1:5,000; cat. no. ZB-2301; OriGene Technologies, Inc.). The relative protein level of Twist was normalized to  $\beta$ -actin.

**Statistical analysis.** All data were presented as the mean  $\pm$  standard deviation and all results were analyzed using SPSS statistical software (version 19.0; IBM Corp., Armonk, NY, USA). Multivariate comparisons of the means were performed using one-way analysis of variance followed by the Student-Newman-Keuls post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Expression of Twist and MDR-associated proteins in human breast cancer.** Immunohistochemical analysis was performed to detect the expression levels of Twist and MDR-associated proteins in human breast cancer samples. It was identified that the expression of Twist was largely increased in the cancerous tissues of human breast cancer compared with the pericancerous tissues. Notably, the expression levels of TOPO II $\alpha$ , MRP and P-gp in cancerous tissues were concomitantly increased with the expression of Twist (Fig. 1). However, the expression of LRP was reduced in cancerous tissues compared with the pericancerous tissues.

**Establishment of Taxol®-resistant MCF-7 cell lines.** To simulate Taxol® resistance in cells, MCF-7 cells were repeatedly exposed to high concentrations of Taxol® to establish the Taxol®-resistant MCF-7 cells. Notably, the four types of Taxol®-resistant MCF-7 cells (MCF-7/Taxol® I-IV) exhibited a

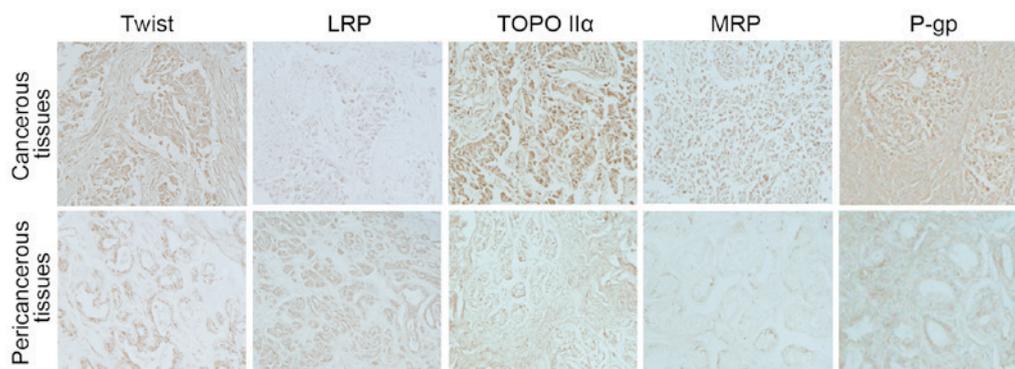


Figure 1. Expression of Twist and drug resistance-associated proteins in human breast cancer. The immunohistochemistry results (magnification, x400) demonstrated that, compared with the pericancerous tissue, the expression of Twist, TOPO II $\alpha$ , MRP and P-gp were upregulated in cancerous tissues, whereas the expression of LRP was downregulated in cancerous tissues. TOPO II $\alpha$ , topoisomerase II $\alpha$ ; MRP, multidrug resistance-associated protein; P-gp, P-glycoprotein.

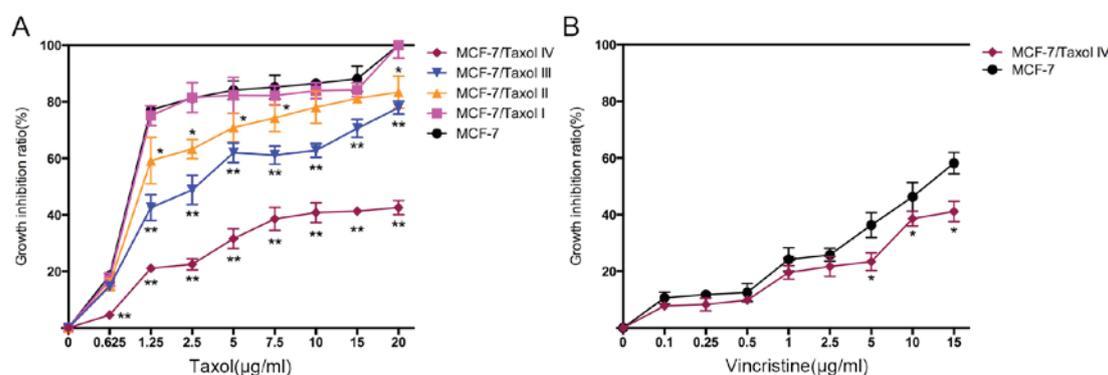


Figure 2. MCF-7/Taxol<sup>®</sup> cells exhibit Taxol<sup>®</sup> and vincristine resistance. (A) Four levels of Taxol<sup>®</sup>-resistant cells were established by repeatedly exposing cells to 15  $\mu\text{g/ml}$  Taxol<sup>®</sup>. Cell growth inhibition ratios of these cells from different concentrations of Taxol<sup>®</sup> (0, 0.625, 1.25, 2.5, 5, 7.5, 10, 15 and 20  $\mu\text{g/ml}$ ) were calculated based on MTT assay data. Compared with MCF-7 cells, MCF-7/Taxol<sup>®</sup> IV cells demonstrated a much lower cell growth inhibition ratio to Taxol. The cell growth inhibition ratio (%) =  $(\text{OD}_{\text{control}} - \text{OD}_{\text{experiment}}) / \text{OD}_{\text{control}} \times 100\%$ . (B) Cell growth inhibition ratios of various concentrations of vincristine (0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 15  $\mu\text{g/ml}$ ). The results were from MCF-7 and MCF-7/Taxol<sup>®</sup> IV cells. MCF-7/Taxol<sup>®</sup> IV cells exhibited lower growth inhibition ratios following vincristine treatment compared with MCF-7 cells. OD, optical density. \* $P < 0.05$  and \*\* $P < 0.01$  vs. MCF-7 cells.

gradually reduced growth inhibition ratio to Taxol<sup>®</sup> (Fig. 2A), and an increased  $\text{IC}_{50}$  value and RI (Table I), which suggests that the Taxol<sup>®</sup>-resistant cell models were successfully established. Notably, compared with the MCF-7 cells, MCF-7/Taxol<sup>®</sup> IV cells also exhibited lower growth inhibition ratios to other chemotherapy medications, including vincristine (Fig. 2B and Table II), cis-platinum (data not shown), 5-FU (data not shown) and mitomycin (data not shown), suggesting that Taxol<sup>®</sup>-resistant cells may have generated MDR.

**Expression of Twist and MDR-associated proteins in Taxol<sup>®</sup>-resistant cells.** To investigate the expression of Twist in Taxol<sup>®</sup>-resistant cells, RT-qPCR was firstly performed to examine the mRNA expression levels of Twist in MCF-7 cells and MCF-7/Taxol<sup>®</sup> I-IV cells. Compared with the MCF-7 cells, the mRNA expression of Twist was significantly increased in the second, third and fourth levels of Taxol<sup>®</sup>-resistant cells (MCF-7/Taxol<sup>®</sup> II-IV; Fig. 3). Notably, compared with the MCF-7 cells, the greatest increase in the mRNA expression level of Twist was observed in the fourth level of Taxol<sup>®</sup>-resistant cells (MCF-7/Taxol<sup>®</sup> IV), with the expression level being upregulated by >60-fold compared with the level in the MCF-7 cells (Fig. 3). This may partially suggest that Twist is associated with Taxol<sup>®</sup>

resistance. Furthermore, immunohistochemistry was performed to investigate the expression of MDR-associated proteins in each level of Taxol<sup>®</sup>-resistant cells (Fig. 4). According to the results of immunohistochemistry, it was identified that the expression of Twist, TOPO II $\alpha$ , MRP and P-gp were increased in Taxol<sup>®</sup>-resistant cells, particularly in MCF-7/Taxol<sup>®</sup> IV cells (Table III). Conversely, the expression of LRP was reduced in Taxol<sup>®</sup>-resistant cells (Table III). These results were consistent with the expression of Twist, LRP, TOPO II $\alpha$ , MRP and P-gp in cancerous tissues of breast cancer.

**Overexpression of Twist in 293 cells.** Although the expression of Twist was increased in Taxol<sup>®</sup>-resistant cells, it was unclear if overexpression of Twist was responsible for the drug resistance. Therefore, a Twist-overexpressing cell line, 293-Twist, was constructed by transfecting a pcDNA5/FRT/TO-Twist vector into Flp-In-T-Rex-293 cells; a negative control cell line, 293-vector, was also constructed. No differences in the morphology of 293-vector and 293-Twist cells were observed (Fig. 5A). However, the results of western blotting demonstrated that the expression of Twist was gradually upregulated by treatment with tetracycline for 0-6 h, and then gradually downregulated from 6-12 h (Fig. 5B and C). Concurrently,

Table I. IC<sub>50</sub> and RI of MCF-7 and MCF-7/Taxol® cells treated with Taxol®.

Cell population	IC <sub>50</sub>	RI
MCF-7	3.83±0.04	1.00
MCF-7/Taxol® I	3.76±0.07	0.94±0.04
MCF-7/Taxol® II	10.41±0.27 <sup>a</sup>	2.36±0.33 <sup>a</sup>
MCF-7/Taxol® III	24.82±0.32 <sup>b</sup>	7.51±1.23 <sup>b</sup>
MCF-7/Taxol® IV	107.05±1.79 <sup>b</sup>	28.83±1.05 <sup>b</sup>

<sup>a</sup>P<0.05 and <sup>b</sup>P<0.01 vs. parent MCF-7 cells. IC<sub>50</sub>, half maximal inhibitory concentration; RI, resistance index.

Table II. IC<sub>50</sub> and RI of MCF-7 and MCF-7/Taxol® IV cells treated with vincristine.

Cell population	IC <sub>50</sub>	RI
MCF-7	12.86±0.34	1.00
MCF-7/Taxol® IV	22.06±1.12 <sup>a</sup>	1.71±0.13 <sup>a</sup>

<sup>a</sup>P<0.01 vs. parent MCF-7 cells. IC<sub>50</sub>, half maximal inhibitory concentration; RI, resistance index.

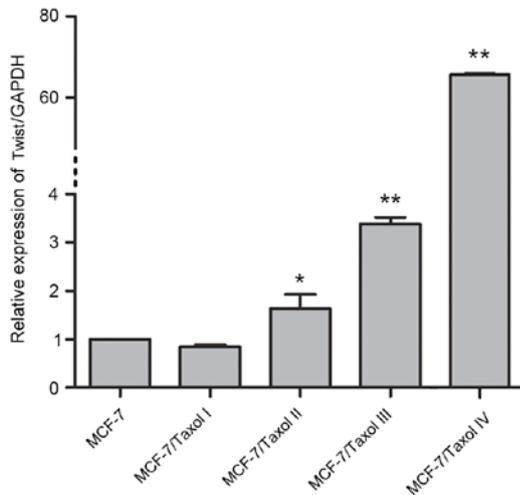


Figure 3. Taxol® resistance increases the mRNA expression of Twist in MCF-7/Taxol® cells. Reverse transcription-quantitative polymerase chain reaction was performed to measure the mRNA expression level of Twist in MCF-7 cells and in four types of Taxol®-resistant cells. Compared with MCF-7 cells, mRNA expression of Twist was significantly upregulated in Taxol®-resistant cells. \*P<0.05 and \*\*P<0.01 vs. MCF-7 cells.

the growth curves of 293-vector and 293-Twist cells were not different (Fig. 5D), suggesting that transfection with the pcDNA5/FRT/TO-Twist vector may increase the expression of Twist in 293 cells and not lead to cell damage in the Twist-overexpressing cells.

*Overexpression of Twist leads to MDR in 293-Twist cells.* MDR was measured by exposing 293-Twist cells to different concentrations of TSA, 5-FU and Taxol®. TSA is an anticancer

Table III. Immunohistochemistry analysis of LRP, TOPO IIα, MRP, P-gp and Twist in Taxol-resistant breast cancer cell lines.

Cell populations	Proteins				
	LRP	TOPO IIα	MRP	P-gp	Twist
MCF-7	44,541.00±2,778.42	26,378.60±1,574.08	34,513.80±4,343.94	33,550.20±5,850.43	11,911.20±3,679.47
MCF-7/Taxol I	38,349.80±6,402.81	34,582.40±4,560.87 <sup>a</sup>	68,355.60±6,911.49 <sup>a</sup>	35,952.80±4,961.75	20,463.50±6,344.64
MCF-7/Taxol II	32,210.80±6,751.98 <sup>a</sup>	36,871.60±3,966.03 <sup>a</sup>	64,004.40±11,964.28 <sup>b</sup>	46,370.60±18,817.21	25,191.40±6,876.17 <sup>b</sup>
MCF-7/Taxol III	28,835.00±4,177.66 <sup>a</sup>	37,207.60±3,915.78 <sup>a</sup>	116,419.00±18,992.03 <sup>a</sup>	50,992.40±13,135.89	28,575.40±6,421.50 <sup>a</sup>
MCF-7/Taxol IV	15,225.80±2,219.39 <sup>a</sup>	49,597.20±3,256.65 <sup>a</sup>	151,732.40±50,276.11 <sup>a</sup>	344,788.00±71,465.02 <sup>a</sup>	88,988.20±7,431.32 <sup>a</sup>

Data are presented as the mean ± standard deviation of the integrated optical density values. <sup>a</sup>P<0.01 and <sup>b</sup>P<0.05 vs. parent MCF-7 cells. TOPO IIα, topoisomerase IIα; MRP, multidrug resistant-associated protein; P-gp, P-glycoprotein; LRP, lung resistance-related protein.

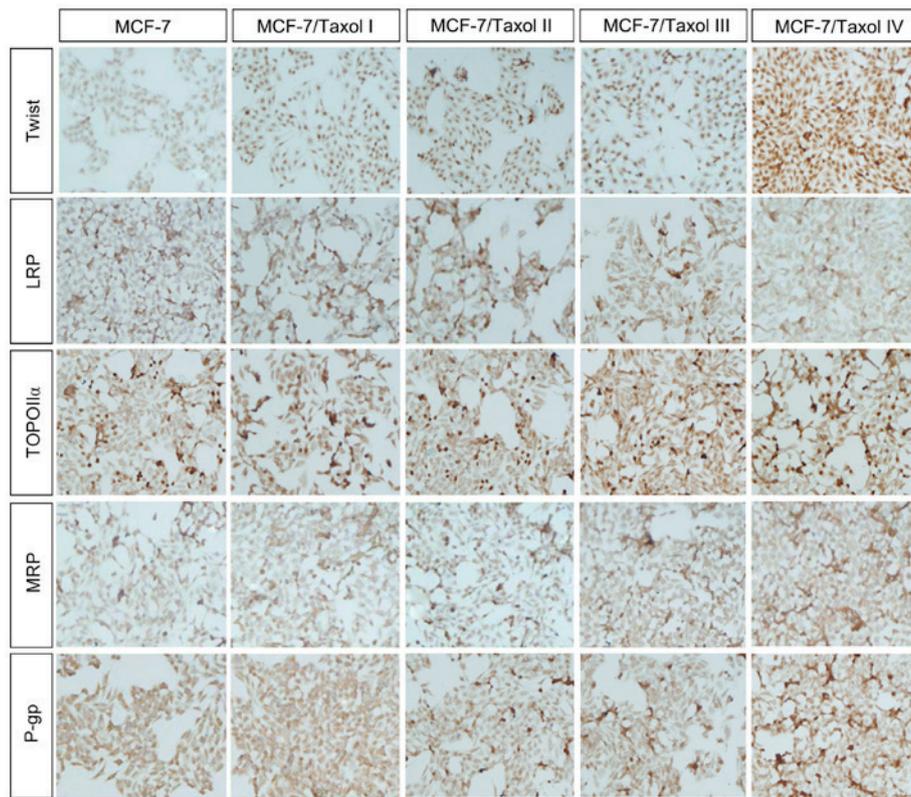


Figure 4. Taxol<sup>®</sup> resistance increases the protein expression of Twist, TOPO II $\alpha$ , MRP and P-gp in MCF-7/Taxol<sup>®</sup> cells. Immunohistochemistry results (magnification, x400) demonstrated that the protein expression of Twist, TOPO II $\alpha$ , MRP and P-gp were increased in Taxol<sup>®</sup>-resistant cells, whereas the protein expression of LRP was reduced. TOPO II $\alpha$ , topoisomerase II $\alpha$ ; MRP, multidrug resistant-associated protein; P-gp, P-glycoprotein; LRP, lung resistance-related protein.

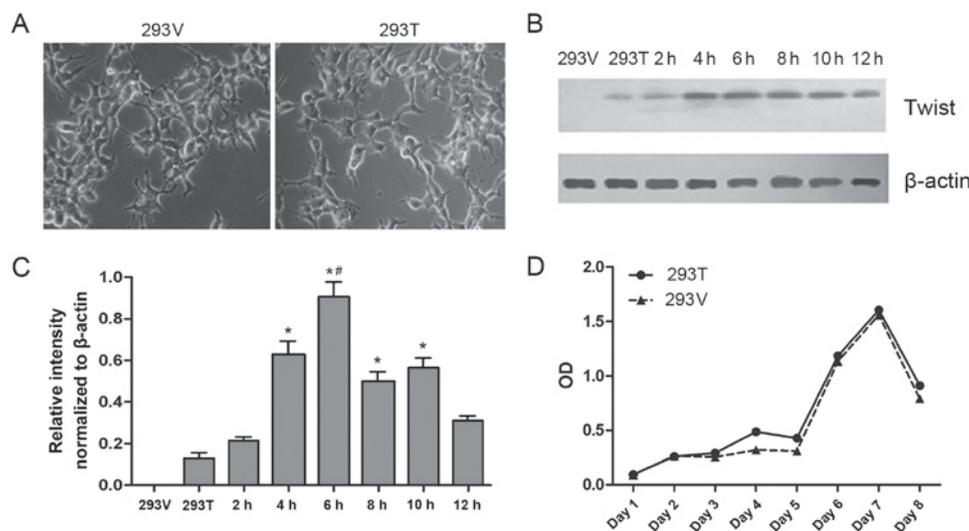


Figure 5. Overexpression of Twist in 293 cells. (A) Morphology of 293 cells transfected with pcDNA5/FRT/TO vector (293V) and pcDNA5/FRT/TO-Twist vector (293T). No difference was observed. (magnification, x400) (B) Western blot analysis of Twist expression subsequent to the addition of 0.1  $\mu$ g/ml tetracycline for 2, 4, 6, 8, 10 and 12 h in 293T and 293V cells. (C) Quantification of Twist protein expression levels, normalized to  $\beta$ -actin. (D) Growth curve of 293T and 293V cells in 8 days. These data demonstrated that overexpression of Twist did not affect cell growth in 293 cells. \* $P$ <0.05 vs. 293T cells; # $P$ <0.05 vs. 293T cells subsequent to the addition of tetracycline for 4 and 8 h. OD, optical density; 293V, 293-vector; 293T, 293-Twist.

drug, which promotes the expression of apoptosis-associated genes, resulting in a decrease in the survival rates of the cancerous cells (21). 5-FU is also widely used as an anticancer drug (22). 293-Twist cells were exposed to 1, 4 and 8  $\mu$ mol/l TSA; 0.276, 1.38, 6.9 and 34.5  $\mu$ g/ml Taxol<sup>®</sup>; and 2.5, 12.5, 62.5

and 312.5  $\mu$ g/ml 5-FU. The results indicated that the growth inhibition ratios of TAS, Taxol<sup>®</sup> and 5-FU were significantly decreased in 293-Twist cells compared with that in 293-vector cells (Fig. 6). These data suggest that the overexpression of Twist increased the level of MDR in 293 cells.

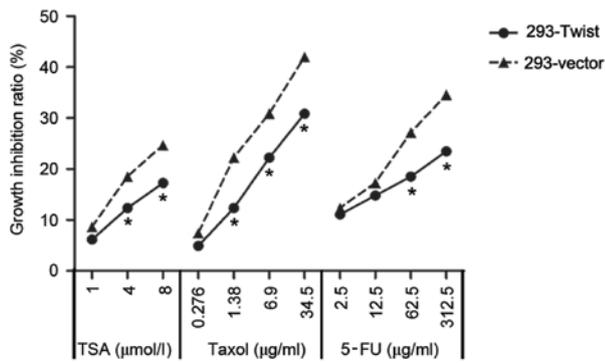


Figure 6. Overexpression of Twist leads to multidrug resistance in 293 cells. Overexpression of Twist in 293 cell reduces the cell growth inhibition ratio of TSA, Taxol<sup>®</sup> and 5-FU, suggesting that Twist serves a key role in acquired multidrug resistance. \*P<0.05 vs. 293-vector under the same concentration. TSA, Trichostatin A; 5-FU, 5-fluorouracil.

**Expression of drug resistance-related proteins in 293-vector and 293-Twist cells.** To demonstrate the association between Twist overexpression and the expression of MDR-associated proteins, immunohistochemistry was performed in 293-vector and 293-Twist cells (Fig. 7). The results indicated that, compared with the 293-vector cells, expression levels of MRP and P-gp were upregulated in 293-Twist cells, which was consistent with the expression of these proteins in Taxol<sup>®</sup>-resistant MCF-7 cells and breast cancer tissues; however, the expression of TOPO II $\alpha$  and LRP were equivalent in 293-vector and 293-Twist cells. These data suggest that the overexpression of Twist may lead to MDR through regulating the expression of MDR-associated proteins.

## Discussion

In this present study, Taxol<sup>®</sup>-resistant and Twist overexpression cell models were established to investigate the role of Twist in drug resistance in cancer therapy. It was identified that the expression of Twist was significantly amplified in Taxol<sup>®</sup>-resistant cells, accompanied by increased expression of TOPO II $\alpha$ , MRP and P-gp, and a decreased expression of LRP. Concurrently, vincristine resistance was also identified in these cells. Conversely, overexpression of Twist augmented Taxol<sup>®</sup>, TSA and 5-FU resistance in 293 cells, and upregulated the expression of MDR-associated proteins, such as MRP and P-gp. All results from the present study suggested that Twist increased drug resistance, and may be associated with the alteration of expression of MDR-associated proteins.

Drug resistance is a major limitation of anticancer therapy, including in breast cancer treatment. Unfortunately, poor prognoses are often observed in patients with advanced breast cancer, due to severe drug resistance to chemotherapeutics (23). Therefore, it is important to reveal the mechanisms of drug resistance in order to identify potential targets to limit the increasing chemoresistance observed in cancer therapy. According to the features of drug resistance in cancer, there are two types of drug resistance: Primary drug resistance and MDR (24). MDR in cancer therapy refers to cancer cells that are resistant to certain types of chemotherapy drug, and that also exhibit cross-resistance to other drugs that have different

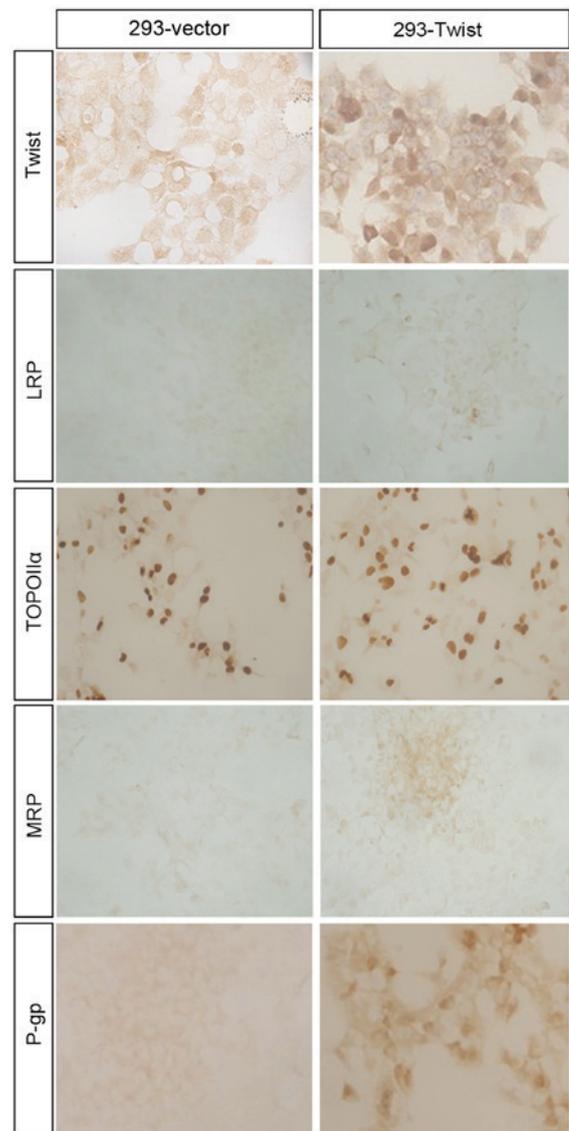


Figure 7. Overexpression of Twist regulates the expression of multidrug resistance-related proteins in 293 cells. The immunohistochemistry results (magnification, x400) indicated that the protein expression of Twist and P-gp were elevated in 293-Twist cells. However, no difference in protein expression of LRP, TOPO II $\alpha$  and MRP were observed in 293-Twist cells and 293-vector cells. TOPO II $\alpha$ , topoisomerase II $\alpha$ ; MRP, multidrug resistant-associated protein; P-gp, P-glycoprotein; LRP, lung resistance-related protein.

mechanisms of pharmacology (11,25). Technically, MDR has been divided into four types according to the different intracellular targets of chemotherapeutic drugs: Type 1, classical MDR, mediated by P-gp; type 2, non-P-glycoprotein MDR, mediated by MDR-associated proteins; type 3, atypical MDR, mediated by TOPO II; and type 4, mediated by LRP (26). Although a previous study demonstrated that acquired Taxol<sup>®</sup> resistance is associated with a high expression of P-gp in breast cancer (27), the overall interactions between drug resistance and MDR-associated proteins have not been clearly demonstrated. Notably, a previous study indicated that Twist, a transcription factor, was identified to promote Taxol<sup>®</sup> resistance in the nasopharyngeal carcinoma HNE1-T3 cell line by performing comparative genomic hybridization analysis (8). Furthermore, it was also revealed that overexpression of Twist elevated Taxol<sup>®</sup> resistance in gallbladder carcinoma, ovarian

cancer and prostate cancer cells, suggesting that the ectopic expression of Twist may lead to drug resistance in different cell lines (8). An additional study indicated that Twist reduced the cell apoptotic rate and increased Taxol<sup>®</sup> resistance by upregulating the expression of RAC- $\beta$  serine/threonine-protein kinase in the breast cancer MCF-7 cell line (28); this was also observed in a nasopharyngeal carcinoma cell line (29). In addition, Twist-1, as a downstream target gene of nuclear factor (NF)- $\kappa$ B, may block programmed cell death induced by daunorubicin or tumor necrosis factor when NF- $\kappa$ B is deficient (30), and RNA interference of Twist-1 may improve chemotherapeutic effectiveness in breast cancer (31). Indeed, these data demonstrate that Twist may lead to MDR in numerous types of cancer; however, whether the augmentation of drug resistance by Twist is associated with the expression level of MDR-associated proteins remains unclear, and requires additional examination.

In the present study, Taxol<sup>®</sup> resistance in breast cancer was simulated by repeatedly exposing MCF-7 cells to Taxol<sup>®</sup>; this resulted in an increase in the expression levels of Twist, P-gp, MRP and TOPO II $\alpha$  and a decrease in the expression level of LRP in MCF-7/Taxol<sup>®</sup> cells. Notably, the different expression pattern of LRP compared with the other three MDR-associated proteins may due to the following reasons: i) Although LRP protein is an efflux protein, it does not belong to the ATP binding cassette transporter family; or ii) the mechanism of MDR mediated by LRP is different from those mediated by P-gp and MRP. However, the overexpression of Twist identified in Taxol<sup>®</sup>-resistant cells did not sufficiently explain the association between Twist and MDR-associated proteins. Therefore, an inverse cell model was established through transfecting a pcDNA5/FRT/TO-Twist vector into Flp-In-T-Rex-293 cells, a transfection-sensitive cell line, to generate a Twist overexpression cell line, which was used to examine the potential drug resistance and altered expression of MDR-associated proteins. In this cell model, elevated drug resistance to Taxol, TSA and 5-FU, and an increased expression of MRP and P-gp, was detected. Nevertheless, the expression levels of LRP and TOPO II $\alpha$  in 293-Twist cells were not different to those in 293-vector cells, which may be due to the fact that the transfected 293 cell was not a cancer cell line. However, this result may suggest that the overexpression of Twist in 293 cells increases drug resistance through altering the expression of MRP and P-gp. Notably, the results of the immunohistochemistry analysis in the human breast cancer tissues demonstrated a lower expression of LRP compared with the pericancerous tissue, suggesting that an increase in the level of malignancy of the tumor tissue corresponded to a notable downregulation of LRP expression.

In summary, the data from the present study indicated that with the gradual formation of Taxol<sup>®</sup> resistance, the expression levels of Twist and MDR-associated proteins gradually increased in Taxol<sup>®</sup>-resistant cells, suggesting that Twist serves a crucial role in the process of acquiring Taxol<sup>®</sup> resistance in breast cancer cells. Notably, the overexpression of Twist in 293 cells also resulted in MDR, and increased the expression of MDR-associated proteins. The present study may assist in understanding the association between Twist and MDR-associated proteins, and contribute to identifying novel chemotherapeutic targets.

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