RESEARCH ARTICLE

Establishment of Paclitaxel-resistant Breast Cancer Cell Line and Nude Mice Models, and Underlying Multidrug Resistance Mechanisms *in Vitro* and *in Vivo*

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Abstract

Background: Breast cancer is a common malignant tumor which affects health of women and multidrug resistance (MDR) is one of the main factors leading to failure of chemotherapy. This study was conducted to establish paclitaxel-resistant breast cancer cell line and nude mice models to explore underlying mechanisms of MDR. Methods: The breast cancer drug-sensitive cell line MCF-7 (MCF-7/S) was exposed in stepwise escalating paclitaxel (TAX) to induce a resistant cell line MCF-7/TAX. Cell sensitivity to drugs and growth curves were measured by MTT assay. Changes of cell morphology and ultrastructure were examined by optical and electron microscopy. The cell cycle distribution was determined by flow cytometry. Furthermore, expression of proteins related to breast cancer occurrence and MDR was tested by immunocytochemistry. *In Vivo*, nude mice were injected with MCF-7/S and MCF-7/TAX cells and weights and tumor sizes were observed after paclitaxel treatment. In addition, proteins involved breast cancer and MDR were detected by immunohistochemistry. Results: Compared to MCF-7/S, MCF-7/TAX cells had a higher resistance to paclitaxel, cross-resistance and prolonged doubling time. Moreover, MCF-7/TAX showed obvious alterations of ultrastructure. Estrogen receptor (ER) expression was low in drug resistant cells and tumors while expression of human epidermal growth factor receptor 2 (HER2) and Ki-67 was up-regulated. P-glycoprotein (P-gp), lung resistance-related protein (LRP) and glutathione-S-transferase-π (GST-π) involved in the MDR phenotype of resistant cells and tumors were all overexpressed. Conclusion: The underlying MDR mechanism of breast cancer may involve increased expression of P-gp, LRP and GST-π.

Keywords: Breast cancer - paclitaxel - chemotherapy - multidrug resistance

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Introduction

Breast cancer is the most common disease among women, its incidence and mortality continues to rise rapidly in Asian countries (Siegel et al., 2012; Bhoo-Pathy et al., 2013), in China, the incidence rate has increased by up to 30% over the last 10 years (Coughlin and Ekwueme, 2009). The chemotherapy is one of the major treatments for breast cancer cases in clinic, however, the resistance to chemotherapeutic drug in many patients has gradually emerged following the drug widespread use. The resistance to chemotherapeutics, especially multidrug resistance (MDR), remains the leading cause of chemotherapy failed. The MDR mechanism is extremely complicated, it involves drug efflux increase, drug metabolic biotransformation and alteration of repair ability for anticancer drugs-induced DNA damage (Fromm, 2002). Mutations of apoptosis-related genes and cytokinetic factors also induce resistance to antitumor drugs (Baguley, 2010). Consequently, it is a critical factor in treatment that exploring the mechanism of MDR and finding molecular targets for drug resistance.

Paclitaxel, as the standardized first-line chemotherapeutics with platinum and anthracycline compounds, is bind to β-tubulin to reinforce the microtubule stabilization (Bedard et al., 2010). Unfortunately, the use of paclitaxel in breast cancer chemotherapy appears a major obstacle with the development of drug resistance (Murray et al., 2012). Although some researches of paclitaxel resistance have been made, such as βIII-tubulin, Tau protein and glutathione S-transferase P1 expression are involved in taxanes resistance (Arai et al., 2008; Smoter et

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al., 2011; Kaira et al., 2013), the mechanism of paclitaxel resistance is not completely understood. In this study, to explore the underlying mechanism of MDR resistance, we established paclitaxel-resistant breast cancer cell line and nude mice models. The biological characteristics of resistant cell line and potential mechanisms related to MDR were verified.

Materials and Methods

Cell line and cell culture

The human breast cancer cell line MCF-7 was obtained from the Chinese Academy of Science (Shanghai, China) and cultured in RPMI-1640 medium (Gibco, USA), supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin, at 37 °C in a humidified atmosphere containing 5% CO_2.

Establishment of the paclitaxel-resistant breast cancer cell line MCF-7/TAX

Paclitaxel-resistant MCF-7/TAX cell line was established from its drug sensitive cell line MCF-7 (MCF-7/S) with a stepwise increase of paclitaxel (Nanjing Sike Pharmaceutical, China) concentration. The culture condition was the same as the MCF-7 cell line. Drug concentration of paclitaxel was increased by 1.5 fold increase at each step of resistance, from 2 nM up to 30 nM. Cells were cultured in paclitaxel of each step for at least 2 weeks with medium exchange every 2 days. The resistant cell line was established after an induction of 12 months.

Chemosensitivity assay

The sensitivity of the MCF-7/TAX cells (MCF-7/S cells as a control) to paclitaxel was detected using MTT assay (Sigma, USA). Briefly, cells at the exponential growth phase were seeded at a density of 5×10^4 cells per well in a volume of 100 μL in 96-well plate for 24 h. The culture medium was replaced with fresh medium containing paclitaxel at different concentrations for a period of 72 h. Then, the culture medium was replaced with 180 μL RPMI-1640 and 20 μL MTT (5 mg MTT/mL) for additional 4 h incubation. The supernatant was discarded and the formazan crystals were solubilized with 150 μL of DMSO on a shaker for 10 min. The absorbance was measured at 490 nm on a microplate reader (Bio-Rad 550, USA). The IC_{50} value was estimated by GraphPad Prism 5 software. Resistance Factor (RF) was calculated by the ratio of the IC_{50} values of MCF-7/TAX to MCF-7 cells.

MDR detection

The sensitivity, IC_{50} and RF values of MCF-7/S and MCF-7/TAX cells to Adriamycin (Shanxi Pude Pharmaceutical, China), Methotraxate (Shanghai Xinyi Pharmaceutical, China), 5-Fluorouracil (Shanghai Xudonghaijin Pharmaceutical, China), Pingyangmycin (Zhejing Haizheng Pharmaceutical, China) and Lobaplatin (Shandong Qilu Pharmaceutical, China), were measured by MTT assay as described above.

Cell growth and population doubling time assay

We performed MTT assay to observe cells proliferation in the MCF-7/S and MCF-7/TAX cell lines. Cells were adjusted to the density of 8×10^3 cells/mL, and seeded in a 96-well plate. The numbers of cells were counted every 24 h for 7 days. Cell doubling time (Td) was calculated using the formula: $Td = T \times \log 2/(\log N_t - \log N_o)$, where N_o and N_t represent the number of cells at the beginning and the end of culture during time T, respectively.

Morphological observations

MCF-7/TAX and MCF-7/S cells in exponential phase growth were observed under the inverted light microscope (Olympus, Japan). For transmission electron microscopy, cells were digested with 0.25% trypsin, fixed with 3% glutaraldehyde, and post-fixed with 2% osmium tetroxide. Cells were then dehydrated with increasing concentrations of ethanol from 50% to 100% and embedded in epoxy resin, cut, stained with uranyl acetate and lead citrate. Ultimately, the samples were observed using H-7650 transmission electron microscope (Hitachi, Japan).

Analysis of cell cycle distribution

MCF-7/TAX and MCF-7/S cells were harvested with trypsin, pelleted by centrifugation, washed twice with cold PBS and adjusted density to 1×10^6 cells/ml. Then cells were fixed by 70% ethanol at 4 °C overnight, washed and re-suspended by 500 μL PBS, subsequently, cells were incubated with 59 μL RNase (1 mg/mL, Sigma) at 37 °C and 30 μL propidium iodide (1 mg/mL, Sigma) at 4 °C in the dark for 30 min. The cell cycle was detected using a flow cytometer (FACSCantoTMII, BD Biosciences, San Jose, CA, USA).

Immunocytochemistry analysis

MCF-7/TAX and MCF-7/S cells were cultured overnight on glass cover slips in six-well plates, washed and fixed with 4% paraformaldehyde for 20 min, after washing with PBS for 5 min three times, cells were incubated with 3% hydrogen peroxide for 20 min and 1.5% blocking serum for 10 min. Afterwards, the cells were incubated with primary antibody against ER (1:200 dilution, Epitomics, Inc.), HER2 (1:400 dilution, Epitomics, Inc.), Ki-67 (1:100 dilution, Epitomics, Inc.), P-gp (1:100 dilution, Epitomics, Inc.), LRP (1:200 dilution, Epitomics, Inc.) and GST-π (1:400 dilution, Epitomics, Inc.) at 4 °C overnight, the control group was incubated with serum, washed and incubated with the anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (1:1000 dilution, Zhongshan Goldenbrdige, Beijing, China) for 30 min. The result was visualized according to protocol of horseradish peroxidase-diaminobenzidine (HRP-DAB) staining kit (Abcam Inc.). After staining, the cells were examined using a microscope (OLYMPUS, Japan). Images were analyzed using a quantitative analysis system (Image-Pro Plus).

In vivo tumor model and immunohistochemistry

Athymic nude mice (BALB/c, females, 4–6 weeks of age) were purchased from Shanghai Laboratory Animal

Table 1. IC_{50} Values (ng/mL) for selected Anticancer Drugs (mean±SD)

Drugs	MCF-7/S	MCF-7/TAX	RF	*P*
Adriamycin	47±5.00	130±10	2.77	<0.01
Methotrexate	20±1.00	720±10	36	<0.01
Lobaplatin	1880±30	4890±20	2.60	<0.01
5-Fluorouracil	790±30	900±10	1.14	>0.05
Pingyangmycin	2900±400	3550±300	1.34	>0.05

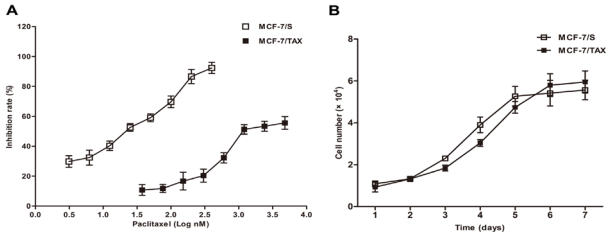

Figure 1. The Paclitaxel Inhibition and Growth Curves of MCF-7/S and MCF-7/TAX Cells. (A) MCF-7/TAX cell line is resistant to paclitaxel. MCF-7/S and MCF-7/TAX cells were performed with the indicated increasing concentrations of paclitaxel for 72 h. Dose-inhibition rate was detected by MTT assay. Each point represents mean ± SD of four independent measurements. (B) Cell growth curves showed the proliferation rate of MCF-7/TAX cells was slower than that of MCF-7/S cells, this was detected in three independent experiments

center of the Chinese Academy of Sciences and maintained under specific bacteria-free and ventilated conditions for the studies. All animal protocols were approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University. MCF-7/S and MCF-7/TAX cells re-suspended in 5% saline were used to establish tumor models in mice by subcutaneous injection, respectively. Tumor volumes were measured on alternate day using a vernier caliper and calculated using an equation: V (cm^3) = $a \times b^2/2$ (a is the largest diameter and b is the smallest diameter), meanwhile, mice weights were monitored three times per week. After growing for approximately 14 days, the volumes of tumor size were allowed to reach no less than 0.1 cm^3 and these mice were randomly assigned to control and treatment group, each experimental group consisted of eight mice. Mice were injected intraperitoneally with paclitaxel diluted in 0.2 mL of normal saline at doses of 10 mg/kg (1/3 LD10) (Lloyd, 1974), whereas control group was injected with equivalent volumes of normal saline. After 21 days of initial treatment, mice were sacrificed and tumor tissues were excised, weighed and fixed in 4% paraformaldehyde for immunohistochemical analysis. Samples were embedded into paraffin and paraffin sections were used for immunostaining with antibody including ER (1:200 dilution), HER2 (1:200 dilution), Ki-67 (1:100 dilution), P-gp (1:100 dilution), LRP (1:200 dilution) and GST-π (1:200 dilution), and control group with serum. Images from 6 random fields of each group were used for quantitative analysis (Image-Pro Plus).

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Student's t-test was used to compare the statistical difference between two groups. $P < 0.05$ was considered statistically significant.

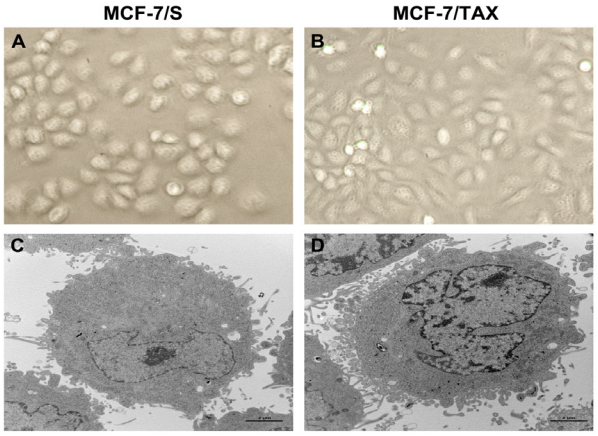

Figure 2. Morphological Observation. MCF-7/S cells (A) and MCF-7/TAX cells (B) were observed with inverted microscope (original magnification ×200). Compared with MCF-7/S cells, MCF-7/TAX cells tended to grow in clusters and spindle-shaped morphology. MCF-7/S cells (C) and MCF-7/TAX cells (D) were viewed under the transmission electron microscopy. In contrast with parental cells, MCF-7/TAX cells had more microvilli and protuberance on cell surface with malformed cell nuclei, mitochondrias, endoplasmic reticulums and Golgi apparatus also increased in resistant cells

Results

Establishment and MDR phenotype of MCF-7/TAX cell line

The MCF-7/TAX cell line was established after a continuous induction from 2 nM to 30 nM paclitaxel in a stepwise increasing concentration manner. The IC_{50} values of paclitaxel for MCF-7/S and MCF-7/TAX cells were 20 ± 0.85 nM and 2291 ± 125 nM, respectively, the RF was 115 (Figure 1A). MCF-7/TAX cells demonstrated a typical MDR phenotype compared to its parental cell line MCF-7/S. Both cell lines were treated with different concentrations of drugs, IC_{50} and RF values were summarized in Table 1. Results showed that MCF-7/TAX cells exhibited cross-resistance to adriamycin, methotraxate and lobaplatin, but not to 5-fluorouracil and pingyangmycin, which would have a potential MDR phenotype.

Cell growth and doubling time

The growth curves between MCF-7/TAX and MCF-7/S cells were shown in Figure 1B. Compared with parental cells, MCF-7/TAX cells indicated slower proliferation rates. The population doubling time for MCF-7/TAX and MCF-7/S cells were 44.60 ± 1.0 h and 45.42 ± 1.9 h, respectively ($P > 0.05$).

Cell morphology observation

The MCF-7/TAX cells were distinct from their parental cell line under the microscopic observation. MCF-7/S cells were relatively consistent in size and shape in monolayer proliferation (Figure 2A) and the resistant cells had spindle-shaped morphology, and tended to grow in clusters (Figure 2B). As ultrastructural measure via transmission electron microscopy, MCF-7/S cells exhibited relatively inerratic and oval form with a few microvilli and protuberance on cell surface, big and irregular nuclei was comprised of

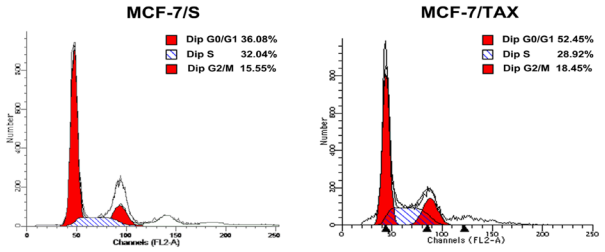

Figure 3. Cell Cycle Analysis of MCF-7/S and MCF-7/TAX Cells. The cells were detected the DNA content by flow cytometry. Data were represented as proportion of cells in G0/G1, S and G2/M phases of the cell cycle. Results were the representive images of three independent experiments

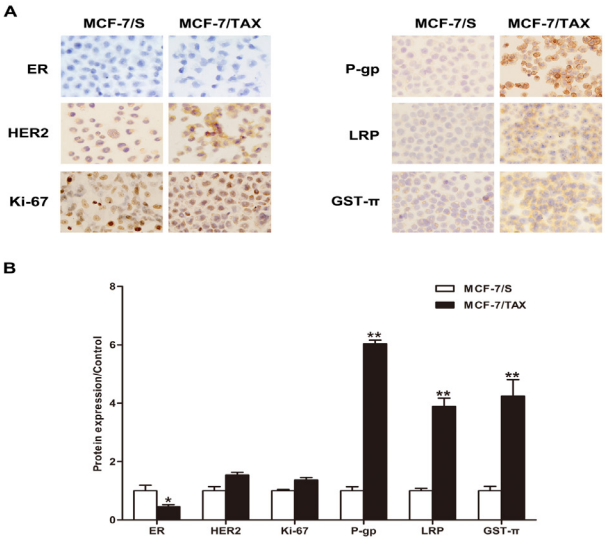

Figure 4. Immunocytochemistry of ER, HER2, Ki-67, P-gp, LRP and GST-π in MCF-7/S and MCF-7/TAX Cells. (A) The immunostaining images of different protein expressions from two cell lines were detected by immunocytochemistry analysis (original magnification ×200). (B) These proteins expression from three random fields of each cell were quantified by image analysis system. Data was shown as mean ± SD, MCF-7/TAX cells compared with parental cell line MCF-7/S (*$P < 0.05$, **$P < 0.01$)

more nucleolus, however, small mitochondrias were also found in MCF-7/S cells (Figure 2C). MCF-7/TAX cells emerged more microvilli and protuberance on cell surface with malformed cell nuclei. Compared with parental cells, the resistant cells had more mitochondrias, endoplasmic reticulums and Golgi apparatus (Figure 2D).

Cell cycle distribution

In order to investigate the effect of paclitaxel on MCF-7/S cells, cell cycle assay was performed by flow cytometry. The result demonstrated that the proportion of MCF-7/TAX cells in G0/G1 and G2/M phases significantly increased from 36.08% to 52.45% and 15.55% to 18.45% respectively, on the contrary, the S phase decreased from 32.04% to 28.92% (Figure 3). This indicated that paclitaxel hampered G0/G1 phase cell cycle progression.

Evaluation of the expression of proteins putatively related to breast cancer and MDR phenotype

Immunocytochemistry were performed to detect the

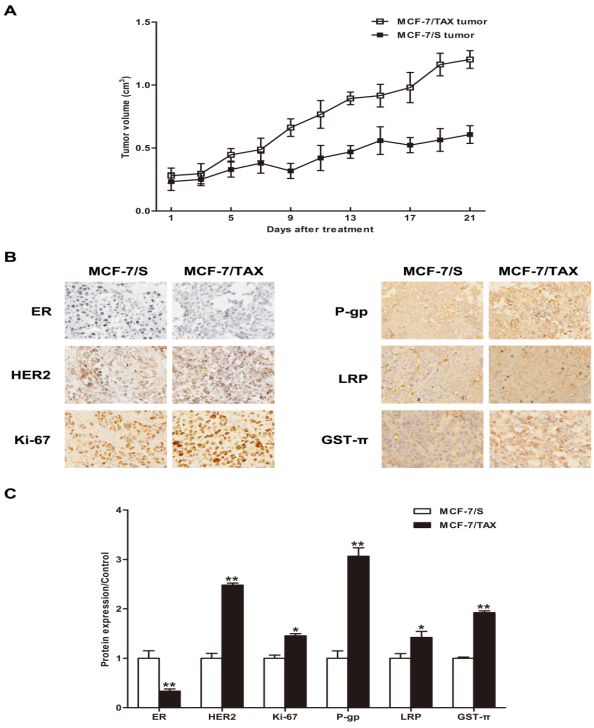

Figure 5. The Effects of Chemotherapeutic Drug Paclitaxel on the MCF-7/S and MCF-7/TAX Cells Xenograft Tumor in Mice. (A) The alteration of tumor volumes was measured under the treatment. The tumor growth of drug sensitive group was significantly inhibited by paclitaxel treatment compared with the drug resistant group ($P < 0.05$). (B) Immunohistochemical analysis of ER, HER2, Ki-67, P-gp, LRP and GST-π expression in tumor tissues of two treated groups (original magnification ×200). (C) The positive indices of these proteins expression from six random fields of each group were quantified by image analysis system. Data was shown as mean ± SD, drug resistant group compared with drug sensitive group (*$P < 0.05$, **$P < 0.01$)

expressions of proteins potentially involved in breast cancer and MDR phenotype of MCF-7/TAX cells. The results were shown in Figure 4 that ER was expressed in the cell nucleus of MCF-7/S cell and less expressed in MCF-7/TAX cells. HER2 and Ki-67 were also positively expressed in the sensitive and resistant cells, respectively; however, there were no significant differences between these two cell lines. Nevertheless, three proteins related to MDR, P-gp and LRP were both expressed on the cell membrance, GST-π was also positively expressed in the cytosol, and they were all significantly overexpressed in the MCF-7/TAX cell line compared to its parental cells.

MDR phenotype of mouse model

To assess whether our findings *in vitro* experiments would extend to an animal model, we established MCF-7/S and MCF-7/TAX xenograft athymic mice models with paclitaxel treatment to investigate the expressions of proteins related to breast cancer and MDR *in vivo*. Compared with drug resistant group, tumor growth of sensitive group was significantly inhibited with the drug treatment (Figure 5A), the tumor inhibition rate of MCF-7/S and MCF-7/TAX athymic mice were 52.46% and 14.58%, respectively. Moreover, it is observed that nude mice body weights were not obviously decreased

during the period of experiment. We further detected the expressions of ER, HER2, Ki-67, P-gp, LRP and GST-π in tumor tissues of nude mice by immunohistochemical analysis (Figure 5B). The expression of ER was downregulated in the drug resistant group, while HER2 and Ki-67 were apparently overexpression. P-gp, LRP and GST-π expression levels were significantly increased in the drug resistant group (Figure 5C), it indicated that these proteins had remarkable correlation with MDR.

Discussion

Breast cancer is the second most common malignancy among women, as the significant morbidity and mortality, it accounts for 30% incidence rate in the new female cancers (Siegel et al., 2012). Although the prognosis and treatment of breast cancer continue to evolve, MDR in cancer is still frequently occurred and becomes the crucial obstacle to limit the effectiveness of clinical antitumor drug treatment (Saeki et al., 2005; Dizdarevic and Peters, 2011). It has been well known that the mechanisms and signal pathways of MDR is complicated and multifactorial, it potentially involved alterations in the expression of various proteins. The regulation of factors play an important effect in MDR occurrence including transporters belonged to drug efflux, apoptosis-related targets, cytokinetic factors, tumor heterogeneity, host immune responses, and so forth (Baguley, 2010). Therefore, overcome the generation of MDR could improve the efficacy of chemotherapy on tumor patients. However, paclitaxel is one of first-line chemotherapy drugs for breast cancer treatment-constituting a part of many regular regimens, such as TAC (paclitaxel/adriamycin/cyclophosphamide), it has a mechanism of action with promoting tubulin polymerization and maintaining it stabilization by inhibiting depolymerization of tubulin and cell mitosis (Saloustros et al., 2008). Furthermore, its efficacy is usually limited by emergence of MDR with continuous application of paclitaxel for treatment of wide range of solid tumors (Podolski-Renić et al., 2011).

In this study, a paclitaxel resistant MCF-7 cell line was established to investigate the acquired MDR mechanism in-vitro. We found the RF value of MCF-7/TAX cell line by progressively increasing drug concentration was 115, reached a high degree of resistance, in general, resistant sublines exhibited high resistance (RF > 20×), moderate resistance (RF 5-15×) and low or no resistance (RF < 5×) (Snow and Judd, 1991). In addition, MCF-7/TAX cells exhibited stable biological characteristics and cross-resistance to certain widespread used chemotherapeutic drugs including adriamycin, methotraxate and lobaplatin, whereas it was not suggested to apply 5-fluorouracil and pingyangmycin to treat breast cancer when cells have been resistant to paclitaxel. Compared to parental cell line, MCF-7/TAX cells had a slower proliferation and prolonged doubling time, the alterations of cells ultrastructure were significant, these features were consistent with other resistant cell lines, BEL-7402/5-FU and MCF-7/Adr (Gu et al., 2012; Lu et al., 2012). Simultaneously, as the long-term stimulation of paclitaxel, the resistant cells demonstrated a significant increase in the G0/G1 phase of cell cycle and influence the synthesis of S phase cells. These properties showed that MCF-7/TAX cell line had varying degrees resistance.

The further study analyzed expressions of ER, HER2 and Ki-67 associated with hormone levels, tumorigenesis and cells proliferation in resistant cell line, similarly, in-vivo experiment which nude mice models of MDR phenotype was performed to measure these factors in tumor tissues. ER is an important biomarker for breast cancer which plays a key role in the process of the formation and development of breast cancer. In our experiment, the expression of ER was downregulated in resistant cells and tumor tissues compared with sensitive group, loss of estrogen-dependent led to poor efficiency of endocrine treatment, this may be associated with the occurrence of MDR. On the contrary, HER2 and Ki-67 were upregulated in MCF-7/TAX cells and tumor tissues. HER2, as a oncogene, its overexpression was related to poor prognosis and chemoresistance in breast cancer and enhanced cells proliferation leading to tumor formation and growth acceleration (Slamon et al., 2001; Arteaga et al., 2002; Zhang et al., 2011). Ki-67 was a common indicator of cells proliferation, in particular, used to reflect the proliferation ability of malignant cells, high expression of Ki-67 was also a sign of poor prognosis associated with clinical response to chemotherapy (Urruticoechea et al., 2005). In brief, abnormal expressions of these factors were all shown varying degrees of resistant characteristics.

It is well known that chemotherapy plays an important role in the treatment of breast cancer, but MDR has become a serious obstacle to chemotherapy response. In this study, adenosine triphosphate (ATP) binding cassette (ABC) transporters P-gp and LRP were significantly high expression in the MCF-7/TAX cells and tumor tissues with medication. Overexpression of ABC transporters in maligant cells involved in pumping out chemotherapeutic drugs from the cytosol and reducing their intracellular accumulation. P-gp was also upregulated in certain resistant cell lines including doxorubicin resistant human bladder cancer cell line BIU-87/ADM and human MDR gastric cancer cells SGC7901/VCR (Zhao et al., 2011; Zhou et al., 2013), however, P-gp played a limited role in the MDR hepatocellular carcinoma cell line such as BEL-7402/5-FU (Gu et al., 2012). Similarly, the overexpression of LRP was associated with MDR and used as a prognostic marker for advanced ovarian serous carcinoma (Kerr et al., 2013). The obviously enhanced efflux pump activity in the resistant cells was attributed to abnormal expression of functional MDR proteins. Although we identified that P-gp and LRP were both upregulated in resistant cells and tissues, the mediated MDR mechanisms were demanded to be further investigated.

Gultathione S transferases (GSTs), the isozymes with detoxification function, are key cellular enzymes of glutathione reaction and non-transporter-based MDR; they can usually change the metabolism or target of the anticancer agents. GST-π belongs to this family, studies show that glutathione (GSH) can be catalyzed by GST-π to combine with chemotherapeutics to prevent cytotoxic drugs attack the tumor cells, leading to the occurrence of drug resistance (Ballerini et al., 2003). We found that

GST-π was apparently over-expressed in the MCF-7/TAX cell line which was associated with paclitaxel resistance, simultaneously, in in-vivo experiment, the expression of GST-π was also upregulated in tumor tissues of MDR nude mice model, these demonstrated that the paclitaxel-induced MDR was mediated by GST-π. In adriamycin resistant human myelogenous leukemia (K562/A02) cells and MCF-7 cells (MCF-7/adr), the MDR was both modulated by the alteration of GST-π and activity of GST-related enzymes as well (Yu et al., 2009; Song et al., 2011). In brief, it illustrated that the overexpression of GST-π was related to MDR in breast tumors and indicated poor prognosis to chemotherapy.

In summary, the paclitaxel resistant MCF-7 cell line, MCF-7/S and MCF-7/TAX cells nude mice models were successfully established to investigate the MDR phenotype in breast cancer. The data in our study indicated that increased expressions of P-gp, LRP and GST-π proteins could arouse the occurrence of MDR. They may be molecular targets to treat chemotherapy induced MDR in breast tumors. Further research is required to elucidate the detailed mechanism and screen the effective agents to reverse drug resistance.

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