

• Basic Research •

Multidrug resistance in breast cancer cells during epithelial-mesenchymal transition is modulated by breast cancer resistant protein

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[Abstract] Background and Objective: Epithelial-mesenchymal transition (EMT) not only initiates invasion and metastasis of tumors, but also induces multidrug resistance in tumor cells. Our experiment analyzed the dependability between breast cancer resistant protein (BCRP) and EMT in breast cancer to explore the effect of EMT on BCRP-mediated multidrug resistance. **Methods:** The expressions of BCRP and transcription inhibitor Snail (Snail) in breast cancer were detected by immunohistochemistry. The eukaryotic expression vector pCDNA3.1-Snail was constructed and then transfected into human breast cancer cell line MCF-7. Snail, epithelial marker gene E-cadherin, interstitial marker gene Vimentin, multidrug resistance protein BCRP, and relative drug resistance were measured by immunofluorescence, Western blot, real-time polymerase chain reaction (PCR), and MTT assay. **Results:** Immunohistochemistry showed that Snail was highly correlated with BCRP in breast cancer. Immunofluorescence, Western blot, real-time PCR revealed that compared with parent cell MCF-7, after transfected with Snail, the expression of E-cadherin in MCF-7 decreased, but Snail, Vimentin, and BCRP increased. MTT displayed that the relative drug resistance increased to 9.93. **Conclusion:** After transfected with eukaryotic expression vector pCDNA3.1-Snail, breast cancer cells MCF-7 showed EMT with BCRP-mediated multidrug resistance.

Key words: Breast cancer, multidrug resistance, epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a process where epithelial cells lose polarity, undergo a restructuring of the cytoskeleton, and assume the mesenchymal phenotype with migratory abilities. It has been found that EMT exists in a variety of malignant tumors of epithelial origin and is closely correlated with invasion and distant metastasis of tumor cells¹.

Breast cancer resistant protein (BCRP) is a member of the adenosine-triphosphate binding cassette (ABC) family, whose overexpression leads to multidrug resistance in tumor cells. A number of studies have shown that the invasive and metastatic abilities of multidrug-resistant breast cancer cell lines were higher than those in parent cells². Both EMT and multidrug resistance would result in changes in cell morphology and the invasive and

metastatic behaviors of the cell lines would increase³. To investigate the relationship between EMT and multidrug resistance in breast cancer cells, experiments were designed to observe the correlation between the expression of BCRP and Snail (Snail) in breast cancer tissue and the changes of the expression of BCRP and the cell resistance index in MCF-7/Snail cells after transfection of the Snail gene.

Materials and Methods

Materials

Surgical specimens were obtained from 80 patients with breast cancer between June 2007 and May 2008 in the Department of Pathology, Weifang People's Hospital. No patient received chemotherapy or radiotherapy, and all specimens derived from women. The age of the patients ranged from 32 to 69 years, and the median age was 49 years. The clinical TNM staging included 9 in stage I, 36 in stage II, and 35 in stage III, 43 with lymph-node metastasis, and 37 without lymph-node metastasis.

Main reagents

The primary antibodies for Snail, BCRP, E-cadherin, and

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Vimentin, fluorescent antibodies, and a streptavidin-biotin complex (SABC) universal kit were purchased from Beijing Zhongshan Biotechnology Co., Ltd. The TRIzol reagent for RNA extraction was purchased from Beijing Solar Biotechnology Co., Ltd. The first strand cDNA synthesis kit, polymerase chain reaction (PCR) kit, restriction enzymes, real-time PCR kits, and Pfu DNA polymerase were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. The TA Cloning kit was purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. Eukaryotic expression vector pCDNA3.1(-) and Lipofectamine™ 2000 reagent were purchased from Invitrogen Co., Ltd. Total protein extraction kit was purchased from BestBio Co., Ltd.

Immunohistochemical staining

After antigen retrieval in a citrate buffer, immunohistochemical SABC methods and 3,3'-diaminobenzidine (DAB) staining were conducted. Instead of the primary antibody, phosphate-buffered saline (PBS) was defined as the negative control. Five visual fields were observed randomly, with 500 cells counted. When the percent of well-defined positive cells in the 5 visual fields was 1%–25%, it was scored 1; 26%–50% scored 2; 51%–75% scored 3; ≥ 75% scored 4; when no staining was observed, it was scored 0, light yellow staining scored 1, yellow scored 2, brown scored 3. The total score equals the product of the positive range score and the positive extent score: ≤ 3 was defined as (-); 4–6 was defined as (+); 7–9 was defined as (++), and 10–12 was defined as (+++).

Constructed pCDNA3.1-Snail eukaryotic expression vectors

RNA was extracted from the tumor tissue using TRIzol. A total of 2 µg of RNA was used to synthesize the cDNA and taken as a template. The Snail gene was then amplified with Pfu (NM_005985.2). The primer pairs were: 5'-CCACTATGC CGCGCTCTTT-3' (forward) and 5'-TCAGCGGGGACATCCT GAGCA-3' (reverse). After A was attached to the end of the PCR product, it was ligated with a T vector to construct the pUCm-T-Snail vector. After the ligated product was transformed into *E. coli*, blue-white screening was conducted. White colonies were isolated and then confirmed by *Not* I/*Bam* H I digestion. Positive clones were sequenced and confirmed by *Not* I/*Bam* H I digestion. The digestion product was ligated to the pCDNA3.1 (-) to construct the pCDNA3.1-Snail eukaryotic expression vector and confirmed by enzyme digestion sequencing.

Cell culture and transfection

Human breast cancer MCF-7 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum at 37°C under 5% CO₂ in a humidified atmosphere. When reaching 80% confluence, the cells were transfected with pCDNA-Snail and control plasmid pCDNA3.1(-) using liposome transfection methods and named MCF-7/Snail and MCF-7/pCDNA, respectively. A total of 48 h later, rapid screening was done with 500 µg/mL G418. Media was exchanged every 3–4 days. When cell death discontinued and a few cells underwent division and proliferation began to increase, 200 µg/mL G418 was used to maintain screening (about 12–14 days). Cells were digested in the original

cell culture bottles when positive cell clones were grown to a certain number. Cells were processed after reaching 100% confluence. One week later, the cells were used in the experiment.

Cytotoxicity tests

Exponentially growing cells (1×10^4 /mL) were suspended and then placed in 96-well plates. After 24 h, the cell media was supplemented with 1, 4, 16, 64, and 254 ng/mL of mitoxantrone added to each well separately. When cultured for 48–72 h, the supernatant fluid was decanted and MTT was added to each well, followed by incubation of the plates for 4 h. Afterwards, dimethyl sulfoxide (DMSO) was added and the optical density (OD) at 540 nm was measured. Survival rate was calculated according to the absorbance ratio of the dosing group and the control group and multiplied by 100%. Each experiment was performed 3 times and the mean value was graphed to get the 50% inhibition concentration (IC₅₀).

Cell immunofluorescence

Cells were seeded onto glass cover slips, washed in PBS, then fixed with paraformaldehyde for 20 min, washed twice in PBS for 5 minutes, permeabilized with Triton × 100 for 30 min, washed in PBS with 1% BSA twice, and incubated overnight at 4°C with the primary antibody. Cells were washed twice in PBS with 1% BSA followed by incubating for 45 min with fluorescein isothiocyanate (FITC) (1:100) or CY3 (1:100)-conjugated secondary antibody at 37°C. Washed in PBS twice, the cells were observed with a fluorescence microscope. The parent cells were used as controls.

Western blot

Total protein was extracted with an extraction reagent and quantified by Coomassie Brilliant Blue staining. 100-µg proteins were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Blotting was performed with a semi-dry transfer, blocked with 5% nonfat milk for 1 h, incubated with the primary antibody for 2 h at 37°C, washed in Tris-buffered saline tween-20 (TBS-T) then incubated in a horseradish peroxidase (HRP)-conjugated second antibody for 1 h at 37°C. Membranes were then washed in TBS-T three times for 10 min each; an electrochemical luminescence system was used. β-actin was assumed to be the internal reference. Quantity One analysis software was used to calculate the integral gray value of target proteins and β-actin in each group.

Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted with TRIzol. After reverse transcription of 2 µg RNA to cDNA with Moloney murine leukemia virus (MMLV) reverse transcriptase, real-time PCR was performed. PCR was performed following manufacturer instructions. PCR primers (synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.), sequences, and reaction conditions are shown in Table 1.

Statistical analysis

SPSS version 17.0 was used for statistical analysis. Comparisons between the two groups used a *t* test. Correlations between the two groups were done by χ^2 test. The results were

Table 1 The primer and reaction conditions of real-time PCR

| Gene | Primer | Annealing temperature (°C) | Production (bp) |
|----------------|----------------------------------|----------------------------|-----------------|
| E-cadherin | FR: 5'CGGTGGTCAAAGAGCCCTTACT3' | 58 | 171 |
| | RR: 5'TGAGGGTTGGTGCAACGTCGTTA3' | | |
| vimentin | FR: 5'CGC TTC GCC AAC TAC AT3' | 58 | 690 |
| | RR: 5'AGG GCA TCC ACT TCA CAG13' | | |
| snail | FR: 5'CCACTATGCCGCGCTCTTT3' | 58 | 799 |
| | RR: 5'TCAGCGGGGACATCCTGAGCA3' | | |
| BCRP | FR: 5'CTGAGATCCTGAGCCTTTGG3' | 58 | 441 |
| | RR: 5'TGCCCATCACAACATCATCT3' | | |
| β -actin | FR: 5'CACCCCACTTCTCTAAGG3' | 58 | 126 |
| | RR: 5'AAAAGTATTAAGGCGAAGAT3' | | |

expressed as mean \pm standard deviation (SD). Statistical significance was assumed when $P < 0.05$.

Results

The expression of BCRP and Snail in breast tissue

Snail was mainly located in the nucleus, and the positive rate in breast tissue was 71% (57/80). While BCRP was mainly located in the cytomembrane, the positive rate in breast tissue was 52% (42/80) (Figure 1). There was a correlation between the expression of BCRP and Snail through correlation analysis ($\chi^2 = 30.013$, $P < 0.05$, $r = 0.5225$)

pCDNA3.1-Snail eukaryotic expression vector and transfection

Snail-specific gene fragments were obtained by RT-PCR, 799 bp in length (Figure 2). The fragments were gel-purified, ligated to a T-vector, and confirmed by restriction-enzyme digestion after transformation. Specific fragments were observed at about 800 bp. After sequencing, the selected sequence was compared on the Blast and was consistent with the original sequence. It was then ligated to pCDNA3.1(−) plasmid, which had been digested with enzymes to construct the pCDNA-Snail eukaryotic expression vector (Figure 3).

Cell toxicity test

The results of the MTT assay showed that the relative resistance index of MCF/Snail to mitoxantrone was 9.93 and IC_{50} was 0.129 ± 0.006 , which increased 9-fold compared with the MCF-7 group, and the difference was significant ($P < 0.05$) (Table 2). The results showed that cells transfected with the pCDNA3.1-Snail eukaryotic expression vector were resistant to mitoxantrone.

Figure 4 shows that the survival rate of MCF-7/Snail cells in media supplemented with mitoxantrone was higher than that of other cells.

Results of cell immunofluorescence

Compared with the parent cells, the expression of Snail and BCRP in MCF-7/Snail cells was significantly higher and the increased amount of BCRP was consistent with that of Snail, suggesting that the expression of BCRP showed an increased

tendency accompanied by an increased expression of Snail (Figure 5).

Results of Western blot

Compared with MCF-7/Snail cells, there was no significant difference in the protein expression of Snail, BCRP, E-cadherin, or Vimentin in the parent cells. Compared with parent cells and MCF-7/Snail cells, the expression of E-cadherin in MCF-7/Snail cells decreased and the expression of Vimentin, Snail, and BCRP increased ($P < 0.05$) (Table 3, Figure 6).

The mRNA expression of Snail, BCRP, E-cadherin, and Vimentin

The ratio between the four kinds of mRNA mentioned above and the β -actin mRNA in the parent cells was considered to be 1. The relative ratios in MCF-7/pCDNA cells were 0.96 ± 0.09 , 1.03 ± 0.21 , 0.97 ± 0.01 , and 1.04 ± 0.17 , respectively, with no significant difference compared with the parent cells. The relative ratios in MCF-7/Snail were 6.51 ± 0.68 , 2.24 ± 0.18 , 0.25 ± 0.02 , and 4.54 ± 0.48 , respectively. Compared with parent cells, the mRNA expression of Snail, BCRP, and Vimentin significantly increased, while mRNA expression of E-cadherin significantly decreased ($P < 0.05$). Therefore, it could be concluded that the increased expression of BCRP mRNA was consistent with that of Snail mRNA (Figure 7).

Discussion

Multidrug resistance is a major reason for the failure of chemotherapy in the treatment of patients with cancer, in which the ABC transporter family plays an important role. P-glycoprotein (P-gp) and BCRP, belong to common members of the ABC membrane transporter superfamily, can pump drugs such as doxorubicin and mitoxantrone out of cells, and result in drug resistance. In recent years, studies have found that the invasive and metastatic behavior of the drug-resistant breast cancer cell line MCF-7/ADR with high expressions of P-gp significantly increased compared with parent MCF-7 cells².

Increased invasive and metastatic behavior in tumors is one characteristic of EMT. EMT is a process where epithelial cells lose their original cell polarity and intercellular connections and

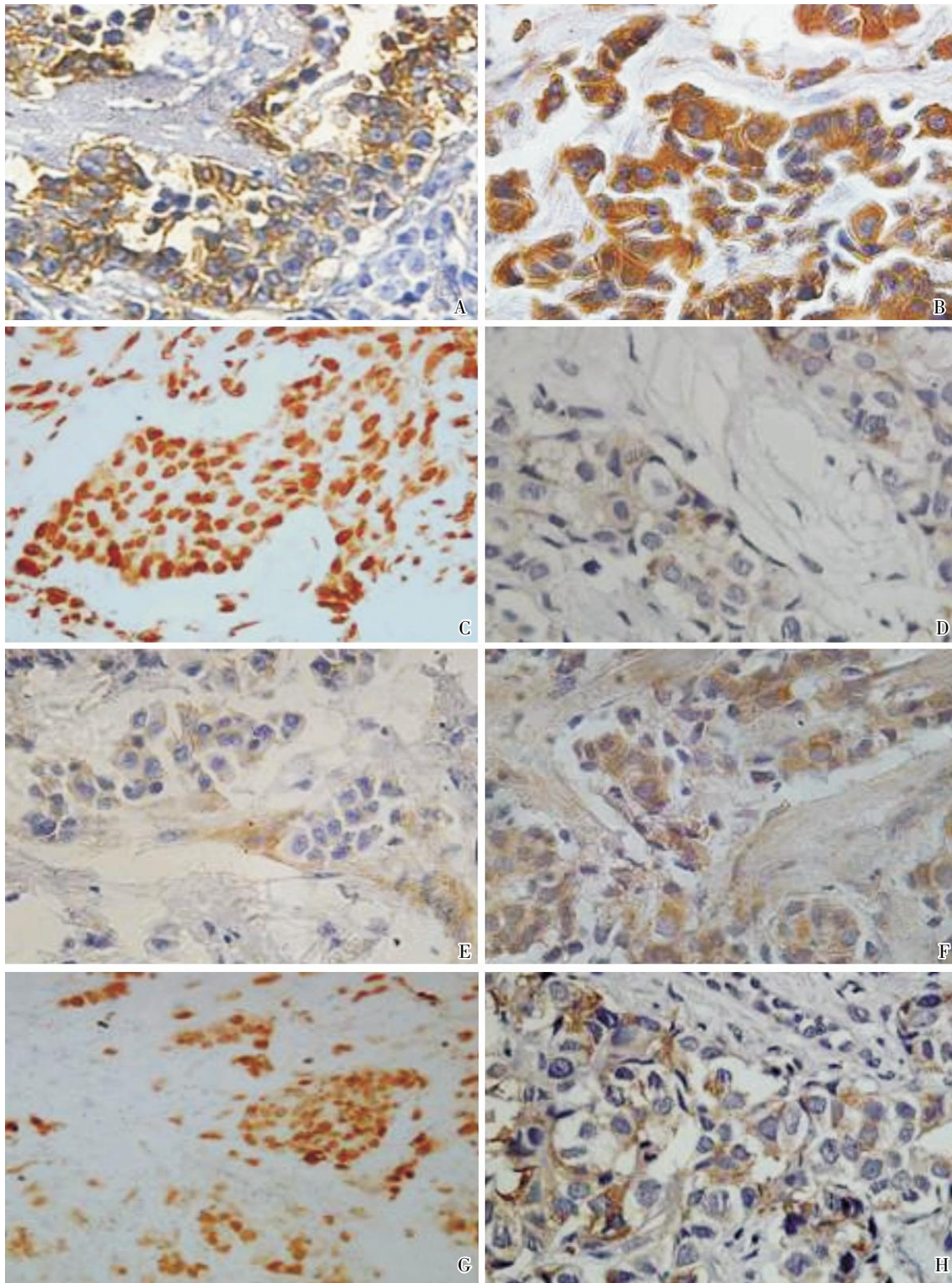


Figure 1 The results of immunohistochemical (SP \times 400) staining in metastatic and nonmetastatic breast cancer cells
A, BCRP is strongly expressed (metastatic); B: Vimentin is strongly expressed (metastatic); C, Snail is strongly expressed (metastatic); D, E-cadherin is weakly expressed (metastatic); E, BCRP is weakly expressed (nonmetastatic); F, Vimentin is weakly expressed (nonmetastatic); G, Snail is weakly expressed (nonmetastatic); H, E-cadherin is strongly expressed (nonmetastatic). As shown in the figure, after Snail expression increased, the expression of BCRP and Vimentin increased, and E-cadherin expression decreased. For a decrease in Snail expression, the opposite is true.

acquire some characteristics of mesenchymal cells, such as a loss of expression of E-cadherin and an increased expression of Vimentin. The locomotory capacity of cells that underwent EMT

significantly increased¹. Li *et al.*³ showed that Adriamycin could induce apoptosis and EMT simultaneously in breast cancer cells, depending on the phases of the cell cycle in vitro, and only tumor

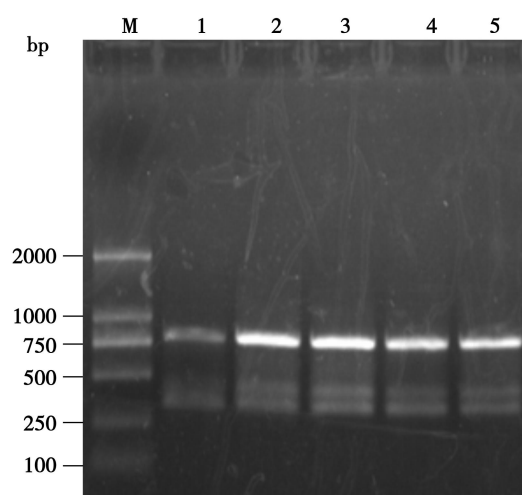


Figure 2 Electrophoresis for Snail amplified by real-time PCR
Line M for marker, lines 1–5 for Snail amplified by real-time PCR, the size is 799 bp.

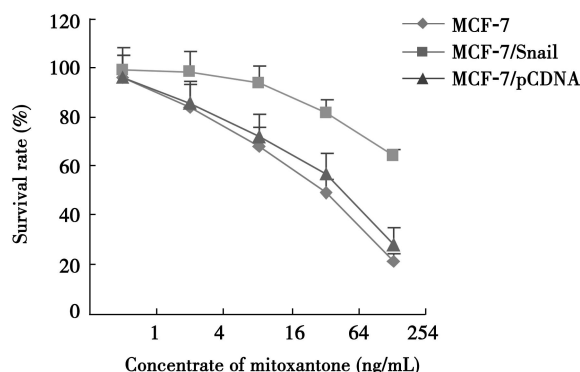


Figure 4 Survival rates of cells to mitoxantone

From the graph we know the survival rate of MCF-7/Snail cells in media added mitoxantone at a higher concentration than MCF-7/ADM.

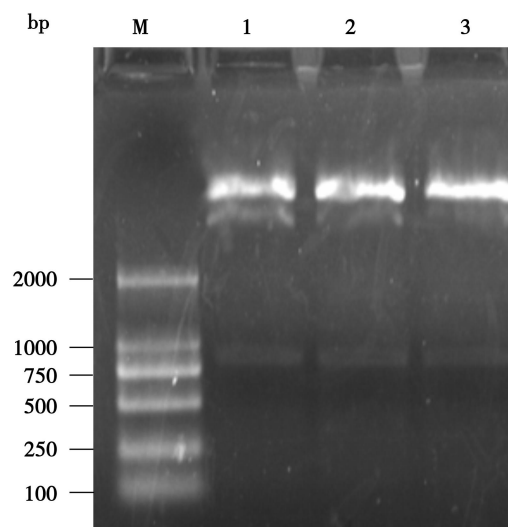


Figure 3 Enzyme digestion of eukaryotic expression vector pCDNA3.1-Snail

Line M for marker, lines 1–3 for the enzyme digestion of the plasmid, 3000 bp for linear plasmid pCDNA3.1(–) after digestion, 800 bp for Snail inserted in pCDNA3.1

Table 2 IC₅₀ values of cells with or without being transfected with pCDNA3.1-Snail construct

| Cell line | IC ₅₀ (μg/mL) | RR |
|------------|--------------------------|-------------------|
| MCF-7 | 0.013 ± 0.0006 | 1 |
| MCF/Snail | 0.129 ± 0.0060 | 9.93 ^a |
| MCF/ pCDNA | 0.014 ± 0.0007 | 1.07 |

^a $P < 0.05$, compared with MCF-7 group, RR for relative drug resistance.

Table 3 Expression of gray values of Snail, BCRP, E-cadherin, Vimentin proteins in each cell line

| Cell line | E-cadherin | BCRP | Snail | Vimentin |
|-------------|----------------------------|----------------------------|----------------------------|----------------------------|
| MCF-7 | 0.589 ± 0.067 | 0.127 ± 0.016 | 0.101 ± 0.021 | 0.135 ± 0.013 |
| MCF-7/Snail | 0.107 ± 0.019 ^a | 0.283 ± 0.033 ^a | 0.785 ± 0.096 ^a | 0.427 ± 0.096 ^a |
| MCF-7/pCDNA | 0.547 ± 0.077 | 0.119 ± 0.022 | 0.111 ± 0.021 | 0.149 ± 0.044 |

^a $P < 0.05$, compared with the MCF-7 group.

cells that underwent EMT had enhanced invasive and metastatic behaviors and multidrug resistance. Arumugam *et al.*⁵ cultured 9 kinds of pancreatic cancer cells with Gemcitabine, 5-FU, and cisplatinum in vitro, and divided the cells into two types: drug-resistant and non-drug-resistant, in which the drug-resistant cell lines were associated with a number of specific gene expressions including characteristics of EMT. The experimental results above showed that the occurrence of multidrug resistance was closely correlated with EMT. Using immunohistochemical methods to detect paraffin-embedded tissue sections of breast cancer, we found that there was a significant correlation between

the expression of Snail and BCRP in tumor tissue ($r = 0.5225$).

To prove that the occurrence of EMT can lead to and promote BCRP-mediated multidrug resistance, cells obtained EMT-like histologic features by the transfection of the Snail expression vector⁶ in the experiment, and immunofluorescence, real-time PCR, and cell toxicity tests were used to detect changes in EMT and BCRP-mediated resistance. The results showed that, after the Snail expression vector was transfected into the MCF-7 cells, the expression of E-cadherin in MCF-7/Snail cells decreased while the expression of Vimentin increased, which showed that cells underwent EMT to some degree⁷. Snail, a zinc finger protein

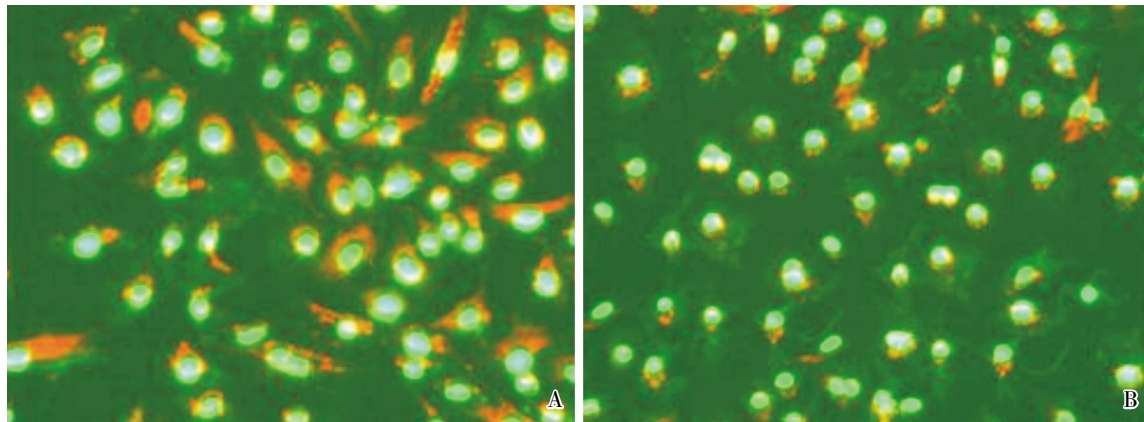


Figure 5 Results of cell immunofluorescence

A shows the expression of BCRP and Snail in MCF-7/Snail cells (red fluorescence for Snail, green fluorescence for BCRP). B shows the expression of BCRP and Snail in MCF-7 cells. In A, the expression of Snail and BCRP have increased. In B, the expression of Snail is lower than in A, and for BCRP this is also true.

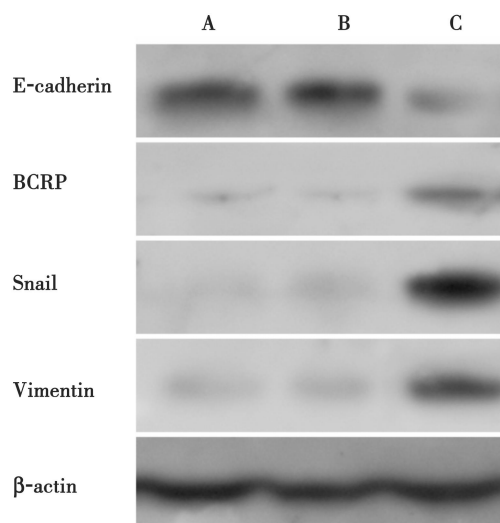


Figure 6 Results of Western blot

A represents the group of MCF-7, B of MCF-7/pCDNA, and C of MCF-7/Snail. In the MCF-7/Snail cell lines, the expression of BCRP, Snail, and Vimentin are significantly higher than in other groups, and the expression of E-cadherin is the opposite.

transcription factor, can inhibit the expression of E-cadherin. Therefore, Snail is closely related to tumorigenesis and tumor progression. Overexpression of Snail can lead to poor prognosis⁸. These studies have shown that Snail, which reflects the invasive ability of cancer cells to a certain extent, is an important regulating factor of EMT in epithelial tumors⁹.

In the present study, MCF-7 cells were transfected with snail expression vectors, and immunofluorescent microscopy revealed the elevated expression of BCRP in the cells undergoing EMT. In addition, the resistance index to mitoxantrone increased to 9.93, which is consistent with the results that chemotherapy for patients who develop metastasis is less effective and patients who experience chemotherapeutic failure are more prone to clinical metastasis. RT-PCR and Western blot showed the same results.

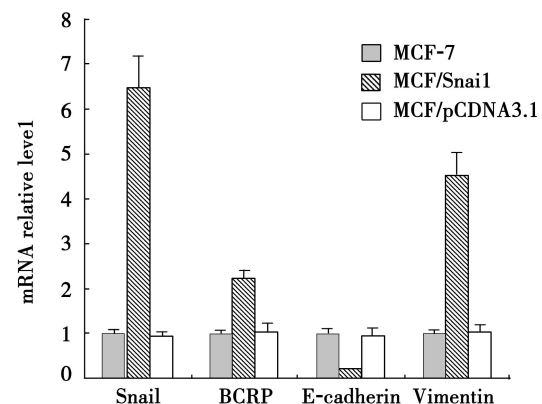


Figure 7 The relative levels of mRNA

After transfecting with expression vector pCDNA-Snail, the relative mRNA levels of Snail, BCRP, and Vimentin in MCF-7/Snail cell lines increased to 6.51, 2.24, and 4.54, respectively, relative to the mRNA level of E-cadherin, which reduced to 0.25.

Positive correlations between Snail and BCRP were proved in the experiment, which further demonstrated an interaction between EMT and multidrug resistance. Previous studies have found that TGF-β can lead to EMT by activating the mitogen-activated protein kinase (MAPK) pathway¹⁰, while activation of the MAPK pathway may also induce multidrug resistance by increasing the expression of BCRP¹¹. Jechlinger *et al.*¹² also found that MAPK inducing EMT was accompanied by increased expression of multidrug resistance protein 2 (MDR2). The fact that the PI3 kinase/Akt pathway was involved in the occurrence of EMT in hepatocellular carcinoma cells and that the activation of this pathway could lead to the high expression of BCRP in epithelial cells further demonstrates that there may exist co-activating mechanisms between EMT and MDR. Of course, these mechanisms and the relationships between them are complex, and there may exist similar signaling pathways between tumor

drug resistance and metastasis, so further study is required.

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