Original Article

Short Hairpin RNA-mediated *MDR1* Gene Silencing Increases Apoptosis of Human Ovarian Cancer Cell Line A2780/Taxol

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ABSTRACT

Objective: Recurrent ovarian cancer is often resistant to drugs such as paclitaxel. Short hairpin RNA (shRNA) targeting *MDR1*, a gene involved in the process of drug resistance, may be a promising strategy to overcome drug resistance.

Methods: Construction and identification of eukaryotic expression plasmid of shRNA targeting on *MDR1* gene. The plasmid was transiently transfected into human ovarian cancer cell line A2780/Taxol. Apoptosis was determined by flow cytometry using annexin V-FITC/PI double labeling. Expression of *MDR1* mRNA was detected by quantitative polymerase chain reaction (qPCR) and P-glycoprotein expression was detected using Western blot.

Results: The IC₅₀ of paclitaxel in *MDR1* shRNA-transfected group was significantly reduced (1.986 ± 0.153) $\mu\text{mol/ml}$ as compared with that in negative control (5.246 ± 0.107) $\mu\text{mol/ml}$ and empty vector-transfected group (5.212 ± 0.075) $\mu\text{mol/ml}$ ($P < 0.05$). The percent of the relative reverse sensitivity to paclitaxel on A2780/Taxol cells was 67.1%, and the apoptotic rate was significantly increased [$(6.977 \pm 0.333)\%$] compared with control [$(1.637 \pm 0.111)\%$] and empty vector-transfected group [$(1.663 \pm 0.114)\%$] ($P < 0.05$). Expressions of *MDR1* mRNA and P-glycoprotein were significantly reduced compared with control ($P < 0.05$).

Conclusion: The present study demonstrated that the eukaryotic expression plasmid of shRNA targeting on *MDR1* inhibited the expression of *MDR1* effectively, thus enhance the sensitivity of A2780/Taxol cells to paclitaxel.

Key words: Ovarian cancer, Short hairpin RNA, Paclitaxel

INTRODUCTION

Ovarian cancer is the fourth leading cause of cancer death in women and the most lethal among gynecological malignancies. In the United States, it is estimated that 21,880 new cases were diagnosed and 13,850 women died of ovarian cancer in 2010. The high rate of mortality attributable to late diagnosis may be largely due to the subtle symptom as well as lack of reliable screening methods. Early detection of ovarian cancer represents the best hope for mortality reduction and long-term disease control^[1]. However, surgical treatment may be only sufficient for malignant tumors well-differentiated and confined to the ovary. For most of the ovarian cancers, chemotherapy is an indispensable part of standard care.

Although chemotherapy is relatively effective for

patients with ovarian cancer, its long-term effect remains unsatisfactory. It is reported that chemo-resistance to regimens such as taxanes and carboplatin is quite common in recurrent ovarian cancer^[2]. The response rate may drop from up to 76% in the first-line chemotherapy to merely 20% as a second-line. Drug resistance is a complex process with multiple genes involved, among which multidrug resistance gene *MDR1* is most widely recognized. *MDR1* encodes P-glycoprotein (P-gp) and P-gp can pump out drugs thus increases the efflux of the drugs^[3]. The membrane-associated protein encoded by this gene is a member of the superfamily of ATP-binding cassette (ABC) transporters, which transport various molecules across extra- and intra-cellular membranes. It is responsible for decreased drug accumulation in multidrug-resistant cells and often mediates the development of resistance to anticancer drugs.

Various strategies have been developed to overcome multidrug resistance. Micro (mi) RNAs are

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small noncoding RNAs that negatively regulate protein expression^[4,5,6], which can cause sequence-specific gene silencing. RNA interference (RNAi) has been cultivated as a means to manipulate gene expression in experiments^[7]. The purpose of this study was to explore the effect of short hairpin RNA (shRNA)-mediated *MDR1* gene silencing on the growth of human ovarian cancer cell line A2780/Taxol. In the present study, PGCsi3.0 plasmid was used to construct shRNA vector containing *MDR1*.

MATERIALS AND METHODS

Cell Culture

Human ovarian cancer cell line A2780 (Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China) and drug-resistant ovarian cancer cell line A2780/Taxol (Cancer Hospital of Guangxi Medical University, China) were cultured at 37°C, 5% CO₂ atmosphere and 90% humidity, in RPMI 1640 medium (Gibco, USA) with 10% fetal bovine serum (FBS) (Hangzhou sijiqing Company, China). The cells were passaged every 2 to 3 days using 0.25% trypsin (Sigma, Germany). The log-phase cells were collected for further experiment.

Cell Transfection

The log-phase cells of A2780/Taxol were placed on the cell culture plate (96-well) in RPMI 1640 medium with 10% FBS for 24 h. The transfection process was performed when cell fusion rate reached 90% by using Lipofectamine 2000 (Invitrogen, USA) following the operation instruction. There were three groups in the study, experimental group (shRNA-*MDR1*), control group (shRNA-control) and untransfection group. The amount of the shRNA vector being used is 8 µg and the knockdown is performed for 48 h.

MTT Assay

The log-phase cells 5×10⁴/ml (0.1 ml/well) were placed on 96-well plate with different concentrations of Taxol, then cultured at 37°C, 5% CO₂ atmosphere and 90% humidity in RPMI 1640 medium with 10% FBS. After incubation for 48 h, 20 µl MTT solution (5 mg/ml) (Sigma, Germany) was added into each well and incubated at 37°C in the dark for 4 h. After removal of the supernatant fluid, 150 µl dimethyl sulfoxide, (DMSO, Sigma, Germany) was added in each well with vibration for 10 minutes. The reduction of MTT was measured by absorbance at 570 nm using a plate reader (Model 550; Bio-tek instrument; USA). The survival rate of cells was determined as follows: (1–A of tests cells/A of black control)×100%. Each assay was repeated at least three times. The concentration-effect curve with drug concentration on the horizontal axis and cell survival rate on the vertical axis was drawn to obtain

regression equation and determine the half inhibitory concentration (IC₅₀). The resistance index (RI) was determined as follows: experimental IC₅₀/parent cells IC₅₀. The relative reversal efficiency was got by the formula: (IC_{50A}–IC_{50B})/(IC_{50A}–IC_{50C}), in which IC_{50A} means IC₅₀ of A2780/Taxol cells, IC_{50B} means IC₅₀ of cells with plasmid transfection (with or without shRNA-*MDR1*), and IC_{50C} means IC₅₀ of A2780 cells. The shRNA interference plasmid targeting *MDR1* was constructed with assistance of Shanghai Genechem Co., Ltd.

Flow Cytometry

Cells of each group were plated in 6-well plates separately, and each well added Taxol 0.5 µg/ml for 48 h. Get 1×10⁶/ml single cell suspension after digested by conventional trypsin. These cells were rinsed 2 times by cold phosphate-buffered saline (PBS), centrifugated at 1,000 r/min for 5 min, then added 100 µl binding buffer, stained in the dark for 15 min by propidium iodide (PI) and Calcium-dependent phospholipid binding protein (Annexin V) in 4°C, at last added 600 µl PBS for resuspension and detected by flow cytometry.

Quantitative Polymerase Chain Reaction (qPCR)

After RNA extraction of all cells in each group by Trizol reagent (Invitrogen, USA), cDNA was synthesized by reverse transcriptase kit (PCR Amplification Kit, TaKaRa, China). The primers were synthesized by Sangon Company (China) with β-actin mRNA as the internal reference (Beijing Genomics Institute, China). The sequence of *MDR1* upstream primer was 5'-CCCATCATGTGCAATAGCAGG-3', and the downstream primer was 5'-GTTCAAACCTTCTGCT CCTGA-3'. The amplified production was 157 bp. The sequence of β-actin upstream primer was 5'-CTTCTACAATGAGCTGCGTG-3', and its downstream primer was 5'-TCATGAGGTAGTCAGTCAGG-3'. The amplified production was 305 bp. The newly synthesized cDNA was amplified by PCR (TaKaRa, China). The PCR conditions were 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 30 s, and 60°C for 1 min. Control amplifications were conducted either without reverse transcription (RT) or without RNA. Following PCR amplification, the reaction products were electrophoresed at 100 V on 2% agarose gels with 0.5 µg/ml ethidium bromide (Sigma Aldrich, USA) and PCR fragments were visualized by UV illumination (GDS7500 Gel; UVP Inc., Upland, CA, USA). Densitometric analysis was performed by using the electrophoresis image analysis system Smart View 2000 software (Furi, Shanghai, China).

Western Blot

Cells of each group were collected after transfection for 72 h, washed with PBS and lysed in 100 µl lyses

buffer, then collected by cell scraping at 4°C, and centrifugated at 25,000 r/min for 25 min to save the supernatant, and the total protein concentration was determined by bicinchoninic acid (BCA). A total of 60 µg protein was electrophoresed in polyacrylamide gel, transferred onto polyvinylidene fluoride (PVDF) membrane. The non-specific antigens were closed by using 5% skim milk powder, and then 1:1,000 rabbit-anti-human P-gp monoclonal antibody (Santa Cruz, USA) was added. The membrane was incubated at 4°C overnight, washed by Tris-buffered saline with Tween (TBST) with 0.1% Tween-20. Goat-anti-rabbit antibody conjugated with Horseradish peroxidase (HRP) was added for 2 h at room temperature. ECL Plus was used as the chemiluminescent substrate after washing the membrane, and then GIS Image system was used to analyze the results.

Statistical Analysis

All data was expressed as $\bar{x} \pm s$. All analyses were performed with Statistical Package for Social Science (SPSS 10.0; SPSS Inc., Chicago, IL, USA). Differences of relative reversal rate, apoptosis rate, *MDR1* gene expression and the expression of P-gp between each group, were evaluated using Student's *t*-test or paired

t-test and $P < 0.05$ was considered statistically significant.

RESULTS

Changes of Sensitivity of Cancer Cell Lines to Paclitaxel

The IC₅₀ of Taxol in *MDR1* shRNA-transfected group was significantly reduced compared with the non-transfection group with a relative reversal rate of 67.1%. There was no significant difference between shRNA-transfected group and shRNA-control group with a relative reversal rate of 0.7% (Table 1). RI of A2780/Taxol cells in untransfected group was 10.206, 3.864 in cells of shRNA-*MDR1* group which indicated that the plasmid of shRNA targeting on *MDR1* after transfection enhanced the sensitivity of A2780/Taxol cells to paclitaxel.

Change of Apoptosis Rate

The apoptosis rate was (1.637±0.111)% in A2780/Taxol cells, and (6.977±0.333)% in shRNA-*MDR1* cells ($P < 0.05$). There was no significant difference in the apoptosis rate of cells between shRNA-control group and A2780/Taxol cells [(1.663±0.114)%, $P > 0.05$] (Figure 1).

Table 1. Sensitivities to Taxol of each group

Groups	IC ₅₀ (µmol/ml)	RI	Relative reversal
A2780/Taxol	0.384 ± 0.099		
Untransfected	5.246 ± 0.107	10.206	
shRNA- <i>MDR1</i>	1.986 ± 0.153*	3.864	67.1
shRNA-control	5.212 ± 0.075**	10.140	0.7

Compared with A2780/Taxol group, * $P < 0.05$, ** $P > 0.05$.

Figure 1. Apoptosis rate of each group tested by flow cytometry. **A:** Changes of apoptosis rate of each group; **B:** Relative apoptosis rate of each group (compared with untransfected group, * $P < 0.05$, # $P > 0.05$).

Changes in *MDR1* Gene Expression

Value of relative quantitation (RQ) of cells in shRNA-*MDR1* group was 333.03±67.62, which was significantly reduced compared with RQ of A2780/Taxol cells (1,297.00±160.04, $P < 0.05$) (Figure 2). There

was no significant difference between shRNA-control and untransfected group.

Detection of P-gp by Western Blot

The expression of P-gp in cells of shRNA-*MDR1*

groups was significantly lower than that of A2780/Taxol group, while there was no difference when compared with shRNA-control group. It

indicated that to some extent, the plasmid of shRNA targeting on *MDR1* after transfection reduced the expression of P-gp in A2780/Taxol cells (Figure 3).

Figure 2. Expressions of *MDR1*-mRNA in cells of each group. **A:** Gel electrophoresis of *MDR1*-mRNA (1. A2780/Taxol group; 2. shRNA-*MDR1* group; 3. shRNA-control group); **B:** Relative expressions of *MDR1*-mRNA (compared with the normal group, * $P < 0.05$, # $P > 0.05$).

Figure 3. Expression of P-gp in each group.

DISCUSSION

P-gp, the protein encoded by *MDR1* gene, is an energy dependent drug pump, which can pump chemicals out of cells, decrease the intracellular drug concentration, and cause cross-resistance to lipid hydrophobic pro-drugs of different structures^[8]. In many *in vitro* experiments, many small molecule drugs such as verapamil, cyclosporin A, and tetrandrine have been proved the reversal function of multidrug resistance, but the application is limited for serious side effects due to large dosage in clinic application^[9]. Thus, targeted gene therapy for cancer now becomes a new mode of cancer therapy aimed at drug resistance caused by P-gp and other factors.

In 1998, Andrew found that something can block the expression of gene efficiently and particularly *in vitro*, they called it RNAi. In nematode experiments, researchers found that the first step of RNAi progress was the production of sequence-specific small interfering RNA (siRNA) induced by siRNA in mammalian cells^[10], and expanded this technology

rapidly to the mammalian animals^[11]. RNAi can produce protein complex in cells, bind and lyse specific target mRNA, and inhibit the protein expression^[12]. Multidrug resistance is a major challenge in the treatment of ovarian cancer. Its reversal can be a new direction as a target gene therapy, which can block the expression of *MDR1*, and finally solve drug resistance.

siRNA fragments of ovarian cancer gene *MDR1* were designed and synthesized by RNAi technology in this study, which reversed the multidrug resistance phenomenon through inhibiting the expression of *MDR1* gene and P-gp, enhanced the sensitivity to chemotherapeutic drugs, so as to achieve the purpose of improving the efficacy of chemotherapy. It showed that the IC₅₀ of A2780/Taxol resistant cells was decreased after transfection by shRNA interference plasmid targeting *MDR1* ($P < 0.05$), the RI was significantly reduced, apoptosis rate was increased, and the transcription level of *MDR1* mRNA and the expression of P-gp were lower ($P < 0.05$). It was found that it specifically inhibited the activity of transcriptase of *MDR1* and reduced the expression of membrane protein P-gp on the resistant cells' surface. And to some extent, RNAi can reverse the drug resistance of ovarian cancer A2780/Taxol cells. In this study, specific shRNA-mediated RNAi system provided important experimental basis for its clinical application to reverse drug resistance induced by *MDR1* in the treatment of ovarian cancer.

Although currently gene therapy has already been proved promising, this experiment also proved the excellence of RNAi, still there are some problems to be addressed in the future. For example, transient transfection of siRNA can only inhibit the expression of gene temporarily^[13]; double-stranded RNA (dsRNA) or

shRNA synthesized *in vitro* which can be used in human is not efficient^[11]; different sequences of dsRNA have different inhibition effects, which is not clear yet; different target sites of mRNA in RNAi have different sensitivities, which is also not clear yet. Based on RT-PCR and Western blot data (Figures 2 and 3), there is still certain amount of *MDR1* mRNA and protein left after knockdown. And this might partially explain why A2780 apoptosis rate does not change significantly after shRNA treatment (Figure 1). The knockdown procedure needs to be optimized in our future research.

In all, the preliminary result demonstrated that the expression of *MDR1* gene mRNA and P-gp can be inhibited by shRNA *in vivo* in this study. The next step should be taken to construct a long-term and stable vector expressing *MDR1*-siRNA *in vivo*, which may lead to its final clinical application in the future.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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