RESEARCH ARTICLE

Modulation of Drug Resistance in Ovarian Cancer Cells by Inhibition of Protein Kinase C-alpha (PKC-α) with Small Interference RNA (siRNA) Agents

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Abstract

Objective: To determine whether silence of PKC-α expression by small interference RNA (siRNA) might regulate MDR1 expression and reverse chemoresistance of ovarian cancer. Methods: We measured gene and protein expression of MDR1 and PKC-α in ovarian cancer cells and assessed their correlation with cell drug resistance. We also examined whether blocking PKC-α by RNA interference (RNAi) affected MDR1 expression and reversed drug resistance in drug sensitivity tests. Results: The drug resistance cell lines, OV1228/DDP and OV1228/Taxol, had higher gene and protein expression of MDR1 and PKC-α than their counterpart sensitive cell line, OV1228. SiRNA depressed PKC-α gene protein expression, as well as MDR1 and protein expression and improved the drug sensitivity in OV1228/DDP and OV1228/Taxol cells. Conclusion: These results indicated that decreasing PKC-α expression with siRNA might be an effective method to improve drug sensitivity in drug resistant cells with elevated levels of PKC-α and MDR1. A new siRNA-based therapeutic strategy targeting PKC-α gene could be designed to overcome the chemoresistance of ovarian cancer.

Keywords: Ovarian cancer - drug-resistance - MDR1 - PKC-α - siRNA

Asian Pacific J Cancer Prev, **13**, 3631-3636

Introduction

Ovarian carcinomas (OC) are the leading cause of death from gynecologic neoplasia (Ozols, 2002). Up to 13,850 of women with OC had died in United State in 2010 (Jemal et al., 2010). For ovarian cancer patients, chemotherapy is adjective cure after surgery. Unfortunately, the efficacy of chemotherapy is hampered by multidrug resistance (MDR), which is either present initially or is induced during treatment (Persidis, 1999). One of the major causes of MDR is the over-expression of P-glycoprotein (P-gp), 170KDa plasma membrane glycoprotein (P-gp170), which function as an ATP-dependent efflux pump for structurally different drugs (Rao et al., 2010). P-gp is coded by MDR1 gene, high which has been detected in many human malignancies (Mayur et al., 2009).

Protein kinase C (PKC) is a phospholipid-dependent, cytoplasmic, serine/threonine kinase that is involved in intracellular signal transduction (Nishizuka, 1988; Lahn et al., 2004). PKC is a family of isoenzymes that are being phosphorylated, or activated, in response to growth factors, hormones and neurotransmitters (Nishizuka, 1988; Lahn et al., 2006). Among them, PKC-α plays a major role in the balance of proliferation and apoptosis (Ghoul et al., 2005), and its protein is widely expressed in various tissues. Abnormal level of PKC-α has been found in many transformed cell lines and in several human tumors (Gescher, 1992). PKC-α expression was associated with changes in the invasion capacity of cancer cells (Gill et al., 2001). Some studies indicated that PKC-α was a central regulating protein for MDR, and regarded as a target for cancer therapy (Mackay et al., 2003). Over-expression of PKC-α has been associated with increased expression of the MDR phenotype (Gravitt et al., 1994). Activation of PKC-α results in phosphorylation of P-gp170 and a decrease intracellular drug accumulation, while inhibition of PKC can partially reverse the MDR phenotype (Chakrabarty et al., 1996; Masanek et al., 2002). Moreover, a stable transfection with PKC-α increase resistance to doxorubicin and vinblastine in MCF-7 breast cancer cells (Yu et al., 1991). However, few studies were concentrated on PKC-α and MDR1 reversing chemoresistance in ovarian cancer.

In our previous studies, we had established two kinds of drug-resistant human ovarian cell lines, OV1228/DDP and OV1228/Taxol, respectively, and found that the expression of MDR1 gene and protein was accompanied with the expression of the PKC-α gene and protein in

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drug resistance ovarian cancer cells (Wang et al., 2010). We supposed that PKC-α might regulate the MDR1 gene and protein expression, and suppression of PKC-α might down-regulate the expression of MDR1 and revise the drug resistance. In the present study, we used technology of siRNA to determine whether PKC-α could regulate MDR1 expression, and whether silence of PKC-α expression could reverse the drug resistance in ovarian cancer.

Materials and Methods

Cells and medium

The OV1228 human ovarian carcinoma cell line was obtained by primary culture. Based on the OV1228, the OV1228/DDP and OV1228/Taxol human ovarian carcinoma drug-resistant cell lines were established through intermission inducement method, as described previously (Wang et al., 2010). The cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. Carcinoma cell lines were maintained in RPMI-1640 media (Gibco, Invitrogen Corporation, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone Corporation, USA). The culture medium was changed two or three times weekly.

PKC-α siRNA sequence design and optimization

We got the mRNA sequence of PKC-α from Genebank (NM_002737). According to the rules suggested by Reynolds et al. (2004), we chose the targeted sequences as follows: 5'-492 AAAGGCTGAGGTTGCTGAT-3', 5'-746 GAACAACAAGGAATGACTT-3', 5'-1114 AAGGATGTGGTGATTCAGGAT-3', 5'-1676 AATCCTTGTCCAAGGAGGCTG-3'. The PKC-α siRNA interference plasmid was constructed by Applied Biological Materials (Canada) as Figure 1.

Choice of gene delivery vector

To obtain the best transfection, three different transfection reagents, Lipofectamine 2000 (Invitrogen Co.) and FuGENE 6 (Roche Co.), as well as EnoGeneFec 2000 (EnoGeneCo.), were used to test the transfection efficiency. Briefly, 2.5-10×10^4 carcinoma cells which had grown to 8--90% in 24-well plates (Shengyou Bitotechnology Co., Ltd) were transfected with the PKC-α siRNA plasmid by using different transfection reagents according to the protocols supplied by each manufacturer. Mock-transfected cells transfected with pEGFP-iLenti vector (Applied Biological Materials, Canada) were included as controls. The cells were split by trypsinization into 24-well plates with 5×10^4 cells/well. After incubation at 37 °C for 24 hours, the wells were washed with phosphate-buffered saline (PBS) and 900 μl fresh medium containing 10% FBS was added to each well. The moderate plasmid and transfection reagents were mixed to 100 μl free-sera and free-antibiotic medium for 15 min. The mixtures were put in wells respectively. After incubation at 37 °C for two days, the plates were examined under a fluorescence microscope.

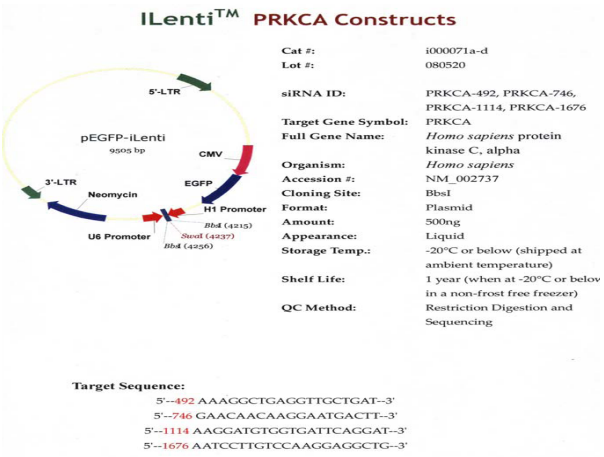

Figure 1. PKC-α siRNA Sequences were Constructed into pEGFP-iLenti. Four PKC-α siRNA sequences were designed including 5'-492 AAAGGCTGAGGTTGCTGAT-3', 5'-746 GAACAACAAGGAATGACTT-3', 5'-1114 AAGGATGTGGTGATTCAGGAT-3', 5'-1676 AATCCTTGTCCAAGGAGGCTG-3', respectively

RT-PCR

Total RNA was extracted from cells using an RNeasy Mini Kit (Invitrogen) according to the manufacturer's guidelines. The practical aspects of real-time quantitative reverse transcription-PCR (RT-PCR) using the ABI Prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems) have been described in detail elsewhere (Parekh et al., 2000). Primers sets used were: for PKC-α (310bp), (5'-CGTAGGAGTGTCCGTGGA-3') and (5'-TCGGAAAACCATGTATCG-3'); for MDR1 (378bp), (5' CGTAGGAGTGTCCGTGGA 3') and (5'-TCGGAAAACCATGTATCG-3'); for GADPH (358bp), (5'-CGGGAAGCTTGTCATCAATGG-3') and (5'-GGCAGTGATGGCATGGACTG-3'). The thermal cycling condition consisted of pre-heating (90 sec at 94 °C) and 30 cycles of denaturation (15 sec at 94 °C), annealing (20 sec at 55 °C, 58 °C, 55 °C for GAPDH, MDRI, PKC-α, respectively) and elongation (30 sec at 72 °C), a final extension (10 min at 72 °C). Each mRNA level was normalized with the internal control GAPDH mRNA level.

Western blot analysis

Cells were lysed in buffer containing 10 mmol/L Tris-Cl (pH 7.6), 50 mmol/L NaCl, 5 mmol/L EDTA, 2% Triton X-100, 30 mmol/L sodium pyrophosphate, 0.2 mmol/L sodium vanadate, 50 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 0.01 mg/mL Leupeptin. Equal amounts of protein (50 μg /lane) were separated to SDS-PAGE separated by SDS-PAGE on 10% polyacrylamide, and were transferred onto a PVDF membrane (Bio-Rad, USA) using a mini-Trans Blot Transfer cell (Bio-Rad) in a transfer buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol). Then the membrane was blocked with 5% milk in PBS at 4 °C for 16 h, and incubated with primary antibodies at 37 °C for 2 hrs, respectively. The following primary antibodies were used: PKC-α antibody (at 1:500 dilution), MDR antibody (at 1:500 dilution) (Santcruz Co.), beta-actin antibody (at 1:2000 dilution) (EnoGene Co.). After five washes in PBST, the membranes were incubated with

the horseradish peroxidase (HRP) conjugated secondary antibody (at 1:2000 dilution) (EnoGene Co) at 37 °C for 1 h. Bands were visualized by using the enhanced chemiluminescence western blotting detection system. Densitometric analysis was performed under conditions that yielded a linear response.

Cell cytotoxicity assay

After we have determined PKC-α mRNA and protein expressing in the OV1228/DDP and OV1228/Taxol human ovarian carcinoma drug-resistant cell lines transfected with pEGFP-iLenti-PKC-α 492 siRNA interference plasmid, the effect of DDP (QiLu pharmaceutical factory, China) and Taxol (HaiKou pharmaceutical factory, China) on transfected cells proliferation was performed. Cell viability was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (BIOSHARP). The conversion of yellow water-soluble tetrazolium MTT into purple insoluble formazan was catalyzed by mitochondrial dehydrogenases (Lage, 2006) and was used to estimate the number of viable cells. In brief, cell transfection was performed using EnoGeneFec 2000 (EnoGene Co.) as previously described. After transfected for 72 hrs, cells were seeded in 96-well culture plates at a density of 5×10^5 (100 μl) per well, then incubated for 24 hrs at 37 °C. After incubation, according to effective concentration, DDP or Taxol were serially twofold diluted in complete medium from 500 μg/ml or 10 μg/ml, respectively. Then, 100 μl corresponding drugs were mixed with cells at an equal volume. After incubation for 72 hrs at 37 °C, cells were incubated with 20 μl MTT (5 mg/mL) for 4 hrs at 37 °C. Then, the supernatant was discarded, insoluble formazan precipitates were dissolved in 0.1 mL DMSO, and the absorbance was measured at 490 nm by use of a microplate reader (Thermo). Wells with untreated cells or with drug containing medium without cells were used as positive and negative controls, respectively. Growth inhibition curves were plotted as a percentage of untreated control cells.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS 16.0) software. Differences between each group were analyzed by using two-sided t-test for unpaired samples and $p < 0.05$ was considered statistically significant.

Results

The effect of different transfection reagents on cells transfected with the PKC-α siRNA interference plasmid The uptake of PKC-α siRNA interference plasmid transfected with Lipofectamine 2000, FuGENE 6, EnoGeneFec 2000 were shown in Supplemental Figure 2. Transfection efficiencies for different transfection reagents were determined by manual counting. We found that there are significant differences in transfection efficiencies using different transfection reagents. Both OV1228/DDP and OV1228/Taxol cells transfected with EnoGeneFec 2000 had the strongest fluorescence signals. Therefore, we selected the EnoGeneFec 2000 as the transfection tool in our studies.

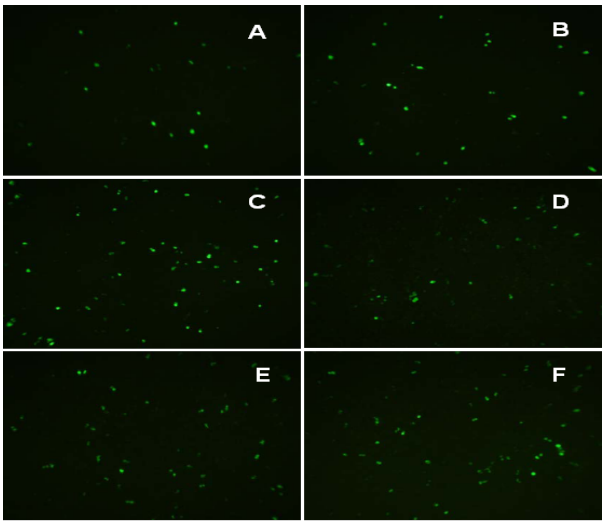

Figure 2. The IFA Reactions of the Difference in Transfection Effects on OV1228/DDP Cells and OV1228/Taxol Cells Transfected by Different Transfection Reagents. A, B, C the OV1228/DDP cells transfected by Lipofectamine 2000, FuGENE 6, EnoGeneFec 2000, respectively. D, E, F the OV1228/Taxol cells transfected by Lipofectamine 2000, FuGENE 6, EnoGeneFec 2000, respectively

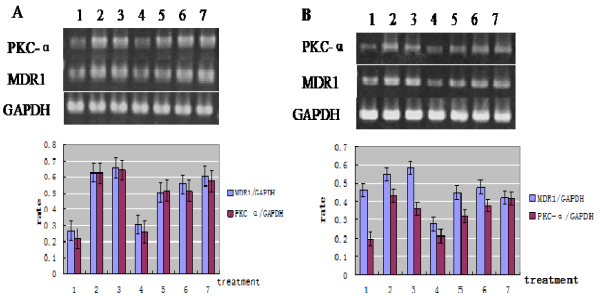

Figure 3. The Expression of PKC-α and MDR1 Gene in OV1228/DDP and OV1228/Taxol Cells Transfected with pEGFP-iLenti-PKC-α siRNA Interference Plasmid. The expression of PKC-α gene in OV1228/DDP and OV1228/ Taxol cell lines were detected after siRNA interference, respectively. 1: OV1228 cell line. 2: OV1228/DDP cell line (A); OV1228/Taxol cell line (B). 3: pEGFP-iLenti blank plasmid, 2μg, 6 well plates, 72hrs. 4: pEGFP-iLenti-PKC-α 492, 2μg, 6 well plates, 72hrs. 5: pEGFP-iLenti-PKC-α 746, 2μg, 6 well plates, 72hrs. 6: pEGFP-iLenti-PKC-α 1114, 2μg, 6 well plates, 72hrs. 7: pEGFP-iLenti-PKC-α 1676, 2μg, 6 well plates, 72hrs. The gene expression of PKC-α and MDR1 in drug resistant cells was higher than in OV1228 cells ($P<0.05$). Compared with pEGFP-iLenti-PKC-α 746, 1114 and 1676, pEGFP-iLenti-PKC-α 492 inhabited the gene expression obviously ($P < 0.05$)

The effect of RNA interference on the expression of PKC-α and MDR1 genes

The expression of PKC-α and MDR1 genes in OV1228, OV1228/DDP and OV1228/Taxol cells were shown in Figure 3. The expression of PKC-α and MDR-1 in OV1228/DDP or OV1228/Taxol cells was higher than OV1228 cells, respectively. The pEGFP-iLenti-PKC-α 492, 746, 1114 and 1676 were used to reverse the chemoresistance of OV1228/DDP or OV1228/ Taxol cells. After both chemoresistance cells were transfected with pEGFP-iLenti-PKC-α, the expression of PKC-α

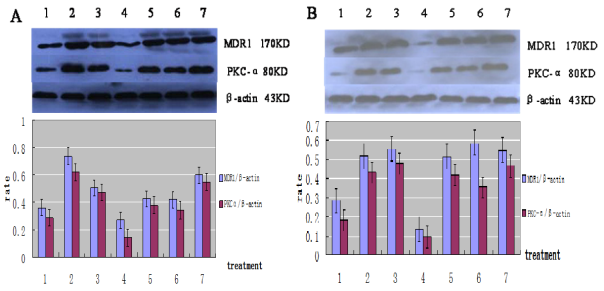

Figure 4. The Expression of PKC-α and MDR1 Protein in OV1228/DDP and OV1228/Taxol Cells Transfected with pEGFP-iLenti-PKC-α siRNA Interference Plasmid. The expression of PKC-α gene in OV1228, OV1228/DDP and OV1228/Taxol cell lines were detected after siRNA interference, respectively. 1: OV1228 cell line. 2: OV1228/DDP cell line (A); OV1228/Taxol cell line (B). 3: pEGFP-iLenti blank plasmid, 2μg, 6 well plates, 72hrs. 4: pEGFP-iLenti-PKC-α 492, 2μg, 6 well plates, 72hrs. 5: pEGFP-iLenti-PKC-α 746, 2μg, 6 well plates, 72hrs. 6: pEGFP-iLenti-PKC-α 1114, 2μg, 6 well plates, 72hrs. 7: pEGFP-iLenti-PKC-α 1676, 2μg, 6 well plates, 72hrs. The protein expression of PKC-α and MDR1 in drug resistant cells was higher than in OV1228 cells ($P < 0.05$). Compared with pEGFP-iLenti-PKC-α 746, 1114 and 1676, pEGFP-iLenti-PKC-α 492 inhabited the protein expression obviously ($P < 0.05$)

Table 1. Drug-fast Index of OV1228/DDP or OV1228/Taxol Cells before and after RNA Interference

	OV1228 (μg/ml) (IC_{50})	OV1228 /DDP (μg/ml) (IC_{50})	Drug-fast index	OV1228 (μg/ml) (IC_{50})	OV1228 /Taxol (μg/ml) (IC_{50})	Drug -fast index
Pre-RNAi	3.56	22.19	6.23	1.26	42.36	33.59
Post-NAi	3.52	7.96	2.26	1.52	4.66	3.06

OV1228/DDP and OV1228/Taxol cells were transfected with pEGFP-iLenti-PKC-α 492 siRNA interference plasmid; IC_{50}: drug concentration when 50% cells were inhibited

gene and MDR1 gene was lower than corresponding original cell lines. The pEGFP-iLenti-PKC-α 492 effectively reduced the expression of PKC-α and MDR1 in both chemoresistance cells, compared to the level of OV1228/DDP or OV1228/Taxol cells transfected with pEGFP-iLenti-PKC-α 746, 1114 and 1676 ($p < 0.05$). In addition, the expression of PKC-α and MDR1 in both chemoresistance cells transfecte with pEGFP-iLenti-PKC-α 492 was closed to OV1228 cell line. This data suggested that siRNA interference targeting at PKC-α 492 might reverse the chemoresistance of OV1228/DDP or OV1228/Taxol cells.

The influences of PKC-α siRNA interference on the expression of PKC-α and MDR1 proteins were detected by western blotting

As shown in Figure 4, compared to OV1228 cells, the expression of PKC-α and MDR1 protein was significantly higher in OV1228/DDP or OV1228/Taxol cells ($p < 0.05$). This indicates that PKC-α and MDR1 play important roles in drug resistance in OV1228/DDP or OV1228/Taxol cells. Compared to original cells, OV1228/DDP or OV1228/Taxol cells, the PKC-α rate (PKC-α/β-actin) and MDR1 rate (MDR1/β-actin) in cells transfected with pEGFP-iLenti-PKC-α 492 were obviously lower ($p < 0.05$). However, the expression of PKC-α and MDR1 protein was not significantly reduced in cells transfected with pEGFP-iLenti-PKC-α 746, 1114 and 1676. This data showed that the pEGFP-iLenti-PKC-α 492 could effectively reduce the expression of PKC-α together with MDR1 protein in OV1228/DDP cells or OV1228/Taxol cells. The expression of PKC-α protein and MDR1 protein in both chemoresistance cells transfected with pEGFP-iLenti-PKC-α 492 was similar to proteins expression of OV1228 cells. This indicated that PKC-α 492 siRNA interference might improve the drug sensitivity of OV1228/Taxol cells or OV1228/DDP cells.

Drug-fast index of OV1228/Taxol or OV1228/DDP before and after RNA interference

The results of gene and protein expression indicated pEGFP-iLenti-PKC-α 492 siRNA inhabited the PKC-α and MDR1 expression effectively. We performed drug sensitivity in the cells transfected with pEGFP-iLenti-PKC-α 492 siRNA by MTT assay. The inhibition ratios against DDP or Taxol of OV1228/DDP or OV1228/Taxol cells before and after RNA interference were presented in Table 1. It indicated that the OV1228/DDP or OV1228/Taxol cells after RNA interference appeared to become sensitive to DDP and Taxol, respectively. The drug-fast indexes of OV1228/DDP and OV1228/Taxol were 2.26 and 3.06 after the interference of pEGFP-iLenti-PKC-α 492 siRNA, which were significant lower than the original drug-fast indexes (6.23 and 33.59) before RNA interference, respectively. It suggested RNA interference targeting PKC-α gene could reverse the drug resistance of the OV1228/DDP and OV1228/Taxol cells.

Discussion

Ovarian cancer (OC) is the third most common malignant tumor of female reproductive system, however, it is the leading cause of death from gynecologic cancer for women in industrialized countries. The overall 5-year survival is below 20% of patients with advanced ovarian cancer, although more than 70% patients respond to primary platinum based chemotherapy (Guppy et al., 2005). The development of tumor resistance to chemotherapy (chemoresistance) presents a major hurdle in cancer therapy. Multidrug resistance, the principal mechanism by which many cancers develop resistance to chemotherapy drugs, is a major factor in the failure of many forms of chemotherapy. It affects patients with a variety of blood cancers and solid tumors, including breast, ovarian, lung, and lower gastrointestinal tract cancers (Higgins, 2007). Tumor cells exposed to a single cytotoxic drug could develop to resistant to structurally and functionally unrelated drugs, and P-glycoprotein (P-gp) coded by MDR1 gene, is largely responsible for this MDR (Li et al., 2011). MDR resulting from the over-expression of P-gp has been reported in different types of soft tissue sarcomas and hematologic malignancies (Shih et al., 1999). In this study, we used two chemoresistance cell lines resisted DDP and Taxol, named with OV1228/DDP and OV1228/Taxol, respectively. The results illuminated that the expression of MDR1 gene and protein

in the OV1228/DDP and OV1228/Taxol cells was higher than OV1228. Over-expression of the MDR1 gene within drug-sensitive cells could confer resistance to the agents described.

PKC is an important element in cellular signal transduction pathways that regulate cell growth, survival, differentiation and malignant transformation (Liu, 1996). Previous studies have shown that PKC activity is elevated in some human tumors when compared with that in adjacent normal tissues (O'Brian et al., 1989) and that elevated PKC activity is associated with increased metastatic or invasive potential in some human carcinoma cells (Parekh et al., 2000). Among PKC family of isoenzymes, more recent studies indicated that PKC-α plays an important role in promotion of tumor invasion, migration, and development of the MDR phenotype (Gill et al., 2001). In our study, the expression of PKC-α and MDR1 in chemoresistance cell lines was higher than that in the OV1228 sensitive cells. The possible reason is that PKC-α protein was related to chemoresistance in ovarian cancer. According to previous reports, over-expression of PKC has been found in many disorders, these kinases have become major targets for therapeutic intervention in a wide range of diseases, including cancers (Hofmann, 2001), and sensitization of tumors to radiotherapy and chemotherapy has been achieved with PKC inhibitors (Zaugg, 2001). In the present study, there was similarity and concertedness in the expression of PKC-α and MDR1 gene or protein, which suggested the expression of PKC-α and MDR1 gene or protein were related each other.

In vitro and in vivo studies strongly suggested that over-expression of P-gp, encoded by MDR1, might be the single most important cause for multidrug resistance in some tumors and for failure of chemotherapy (Goldstein, 1996; Mahadevan et al., 2005). In some recent studies, RNA interference (RNAi) was used to suppress MDR1 expression in tumor cells, effectively reversing multidrug resistance in an array of tumor cells (Nieth, 2003; Li, 2006). In the present study, the pEGFP-iLenti-PKC-α siRNA plasmids were transfected into the chemoresistance cell lines, OV1228/DDP and OV1228/Taxol cells. It is appeared that the expression of PKC-α and MDR1 genes or proteins were inhibited after RNA interference, especially using pEGFP-iLenti-PKC-α 492 siRNA plasmids. The expression of PKC-α and MDR1 gene or protein could be downregulated in vitro, with different silence efficiency in different pEGFP-iLenti-PKC-α siRNA plasmids. However, the OV1228/DDP and OV1228/Taxol cells transfected with pEGFP-iLenti-PKC-α 492 plasmids showed significantly downregulated PKC-α and MDR1 expression. After transfected with pEGFP-iLenti-PKC-α 492 plasmids, the OV1228/DDP or OV1228/Taxol cells were re-sensitized to DDP or Taxol, as demonstrated by the results of MTT assay, respectively. Compared with drug sensitive cells OV1228, the expression of PKC-α and MDR1 gene or protein in the OV1228/DDP and OV1228/Taxol cells was significantly upregulated. However, the expression of PKC-α and MDR1 protein in the OV1228/DDP and OV1228/Taxol cells after transfection with pEGFP-iLenti-PKC-α 492 was lower than drug sensitive cells OV1228. The DDP or Taxol resistant cell strains, OV1228/DDP or OV1228/Taxol, that we established, displayed a tendency of reduced DDP or Taxol resistance after RNA interference, which was consistent with the tendency of down-regulated of PKC-α and MDR1 protein, suggesting an important role of PKC-α and MDR1 over-expression in ovarian cancer drug resistance. According to MTT result, drug-fast index of OV1228/Taxol or OV1228/DDP after RNA interference was decreased, compared to before RNA interference.

The present study demonstrated that PKC-α siRNA plasmids transfected into chemoresistance cell lines, OV1228/DDP and OV1228/Taxol, not only significantly down-regulated PKC-α gene and protein, but also MDR1 expressions, and effectively reversing drug resistance in ovarian cancer cell lines. This result suggests that PKC-α siRNA therapy shows promise in reducing the drug resistance to DDP or Taxol which are frequently encountered in the clinical cases.

Acknowledgements

This work was supported by grants from Jiangsu Government Six Major Talent Fund (2008-D-02), Jiangsu Government "333" Plan, and Jiangsu Key Medical Personnel (RC2011091).

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