RESEARCH ARTICLE

Involvement of GRP78 in the Resistance of Ovarian Carcinoma Cells to Paclitaxel

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Abstract

Background: Glucose regulated protein 78 (GRP78) is a type of molecular chaperone. It is a possible candidate protein that contributes to development of drug resistance. We first examined the involvement of GRP78 in chemotherapy-resistance in human ovarian cancer cell. Materials and Methods: The expression of GRP78 mRNA and protein were examined by RT-PCR and western blotting, respectively, in human ovarian cancer cells line (HO-8910). Sensitivity of HO-8910 to paclitaxel was determined with methyl thiazolyl tetrazolium (MTT). Suppression of GRP78 expression was performed using specific small-interfering RNA (siRNA) in HO-8910 cells, and cell apoptosis was assessed by flow cytometry. Statistical analysis was performed using the SPSS 15.0 statistical package. Results: HO-8910 cells, with high basal levels of GRP78, exhibited low sensitivity to paclitaxel. The mRNA and protein levels of GRP78 were dramatically decreased at 24h, 48h and 72h after transfection and the sensitivity to paclitaxel was increased when the GRP78 gene was disturbed by specific siRNA transfection. Conclusions: The results suggested that high GRP78 expression might be one of the molecular mechanisms causing resistance to paclitaxel, and therefore siRNA of GRP78 may be useful in tumor-specific gene therapy for ovarian cancer.

Keywords: Glucose regulated protein 78 - paclitaxel-resistance - human ovarian cancer cell - siRNA

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Introduction

Ovarian carcinoma (OC) is the most lethal of the gynaecological malignancies and it is the fourth most common cause of cancer-related death in women (Sevim et al., 2014). Ovarian carcinoma was predominant in the western countries originally and it is increased gradually in the developing countries. Epithelial ovarian carcinoma (EOC) is moderately frequent throughout mainland South-East Asia (Saranya et al., 2014). It is estimated that in 2013 nearly 22 thousand new cases of ovarian cancer will be diagnosed and about 14 thousand deaths will occur in the United States (Siegel et al., 2013). With a 5-year survival rate of only 30% EOC is poorly diagnosed because diagnosis at its early stages is often difficult (approximately 70% of cases are diagnosed at advanced stage) due to vague initial signs and symptoms (Vincenza et al., 2014). However, the current situation could be changed by a better knowledge of its molecular pathogenesis, which may surely contribute both to the identification of new biomarkers useful for its early detection, and to the development of personalized therapies.

The glucose regulated proteins (GRPs) were first described as a set of proteins whose expressions were enhanced when mammalian cells were deprived of glucose (Shiu et al., 1979). Because GRPs are involved in protein folding, the GRPs are referred to as molecular chaperons (Gething et al., 1992). GRP78, a 78,000 dalton protein residing in the endoplasmic reticulum (ER), participates in protein folding, transportation and degradation. In addition, it is involved in cell survival during calcium and other physiological stress (Li et al., 1992). It is reported that GRP78 can be induced rapidly and its function will be enhanced by stress (Zhang et al., 2010) and its expression is up-regulated by ER stress conditions, such as low glucose levels, low pH, viral infection, hypoxia and the expression of mutated proteins (Lee, 2001). Enhancement of GRPs appears to protect tissue culture cells against pathophysiological conditions (Li et al., 1992).

Chemotherapy plays a very important role in the treatment of epithelial ovarian cancer. But the drug-resistance seriously influences the effect of treatment and the survival rate. Current approaches to the treatment of ovarian cancer are limited because of the development of the resistance to chemotherapy (Gregory-Bass et al., 2008). Silencing of GRP78 by small interfering RNA (siRNA) could induce cell apoptosis (Martin et al., 2010). It is not well understood that whether and how GRP is involved in tumor proliferation and confer resistance to anti-cancer treatment. GRP78 is a possible candidate protein that contributes to development of drug resistance, which

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could be targeted in neoplastic cells. To address this issue, our study was designed to determine the role of GRP78 in regulating cellular apoptosis in ovarian cancer cells. Furthermore, silencing of the GRP78 gene expression may prove to be a valuable therapeutic approach for chemoresistant cancer by increasing sensitivity of cancer cells to apoptosis.

In this study, we used ovarian cancer cell line (HO-8910) to determine the levels of GRP78 and their relationship to paclitaxel-resistance. Furthermore, we also examined whether decrease in GRP78 protein level by the specific small-interfering RNA (siRNA) would lead to a reduction in paclitaxel-resistance.

Materials and Methods

HO-8910 cell lines were obtained from Scientific Research Foundation of Second Affiliated Hospital of Harbin Medical University.

We detected the basal levels of GRP78 mRNA of the HO-8910 cell lines by RT-PCR. Total RNA was isolated from the cell lines. β-action DNA amplification was used as the internal PCR control. The sequences of GRP78 and β-action were as follows: GRP78:5'TGGGTCGACTCG AATTCCAAAG3'and5'GTCAGGCGATTCTGGTCAT TGG3' (513bp); β-actin:5'CTGCAATCCGAAAGAAG CTG3'and 5'ATCTTCAAACCTCCATGATG3' (320bp). The reactive mixture was subjected to 30 cycles of PCR amplification. Each cycle included denaturation at 94°C for 30s, annealing at 55°C for 30s, and primer extension at 72°C for 30s. The last extension cycle was kept at 72°C for 10 min.

We detected the basal levels of GRP78 protein by western blotting assay in the HO-8910 cell lines. Equal amounts of total protein (20μg) were loaded into 10% SDS-PAGE gel and transferred to PVDF membrane. The membrane was rinsed in TBS-T solution and incubated in blocking buffer at room temperature for 2h. Then the filter was incubated with GRP78 antibody (Santa Cruz, C-19) at room temperature for 1h. Then after washing with TBS-T, the blot followed by incubation with a secondary antibody (Sigma), and the immunoreactive bands were visualized using 3, 30-diaminobenzidene-4 HCl (DAB).

We detected the sensitivity to paclitaxel of HO-8910 by MTT assay. The HO-8910 cell lines were seeded into 96-well culture plates and treated with different concentrations of paclitaxel for 72h. The absorbance values for each sample were measured at 546nm using a microplate reader (Bio-Rad Laboratories).The sensitivity to paclitaxel of HO-8910 cell, psiSTRIKETM/GRP78 HO-8910 cell and psiSTRIKETM/NC-GRP78 HO-8910 cell was also analyzed by MTT assay.

The small interfering RNA GRP78 mRNA (siGRP78) was prepared according to the requirement of siRNA expression vector siSTRIKETMU6. The human GRP78 gene coding sequence was chosen in GeneBank as analysed sequence. The sense (top) and antisense (bottom) sequesces of the GRP78 siRNA duplex were as follows:

5'-ACCGCAAGAATTGAAATTGAGTTTCAAG
AGAACTCAATTTCAATTCTTGCTTTTTC -3'
5'-TGCAGAAAAAGCAAGAATTGAAATTG
AGTTCTCTTGAAACTCAATTTCAATTCTTG -3'

As a nonspecific control, a NC (nonspecific control) siRNA duplex with random sequences was designed as follows:

5'-ACCGAGAACATTAGATGAGATTTTCAAG
AGAACTCAATTTCAATTCTTGCTTTTTC -3'
5'-TGCAGAAAAAGAGAACATTAGATGAG
ATTTTCTCTTGAAACTCAATTTCAATTCTTG -3

Then after Oligonucleotide dilution and anneal, we connect oligonucleotide and psiSTRIKETMU6 vector to form the recombination vector according to the general method. The HO-8910 cells were trypsinized, suspended in fresh medium ($2.5x10^6$cells/ml), and transferred to 6 well plates (2ml per well). After 24 hours, 4.0ug of the recombination vector was mixed with 10ul of Lipofectamine 2000, and applied to the cells. The cells were harvested at 24h, 48h, 72h, respectively, after transfection. The test group was transfected by siGRP78, and the vehicle group was transfacted by the nonspecific siRNA. As to the blank group, nothing was added. Then, we detected the expression of GRP78 mRNA by RT-PCR and GRP78 protein by western blotting assay in the different time respectively. And cell apoptosis assess and MTT analysis were also performed. The methods were described as above.

Cell apoptosis assess was performed using flow cytometry by Annexin-V-FITC-PI kit (BD, USA). We detected the apoptosis assess in the HO-8910 cells, NC-HO-8910 cells and siRNA-transfected HO-8910 cells after transfection at 24h, 48h and 72h respectively. The left of upper picture represents systematic error; the right of upper picture represents late apoptosis cells or necrotic cells; the left of lower picture represents normal live cell and the right of lower picture represents early apoptosis cells.

Statistical analysis was performed using SPSS 15.0(IBM) statistical package. One-way ANOVA was used to determine whether there were significant differences within the groups, followed by Student-Newman-Kuels test to determine which groups were significantly different. The value of $p<0.05$ was considered as statistical significance.

Results

PsiSTRIKETMU6 vector contains a pstIenzyme site and the recombination vector will form two pstIenzyme sites. After the digest of restriction pstI incision enzyme, the successful recombination vector will form two DNA fragments, while the vector without oligonucleotide insertion will have only one linearity fragment in the agarose gel electrophoresis. In the agarose gel electrophoresis, two DNA fragments presented, which indicated the vector construction was successful (Figure 1).

To deregulate the basal GRP78 mRNA expression, siRNA targeted to GRP78 was designed and transfected into HO-8910 cells (psiSTRIKETM/GRP78) and nonspecific siRNA was also designed (psiSTRIKETM/NC-GRP78) and used for observing the non-specific effect of siRNA transfection. Figure 2A showed that

GRP78 mRNA expression was significant degraded after siRNA-transfection, especially in 48h and 72h, while the expression of GRP78 mRNA was not degraded significantly in cells with the treatment of NC siRNA. The specific value was showed in Figure 2B. The β-actin

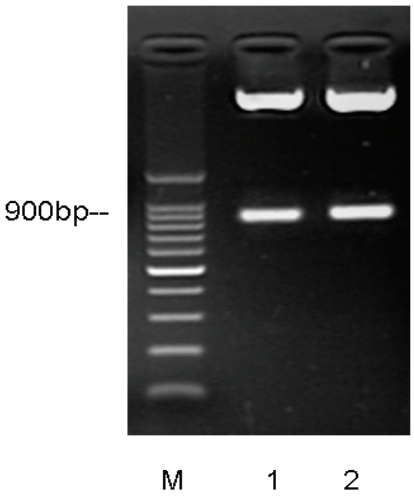

Figure 1. GRP78 Recombination Vector Assessment. M: 100bp marker, 1: psiSTRIKETM/GRP78 recombination vector, 2: psiSTRIKETM/NC-GRP78 recombination vector

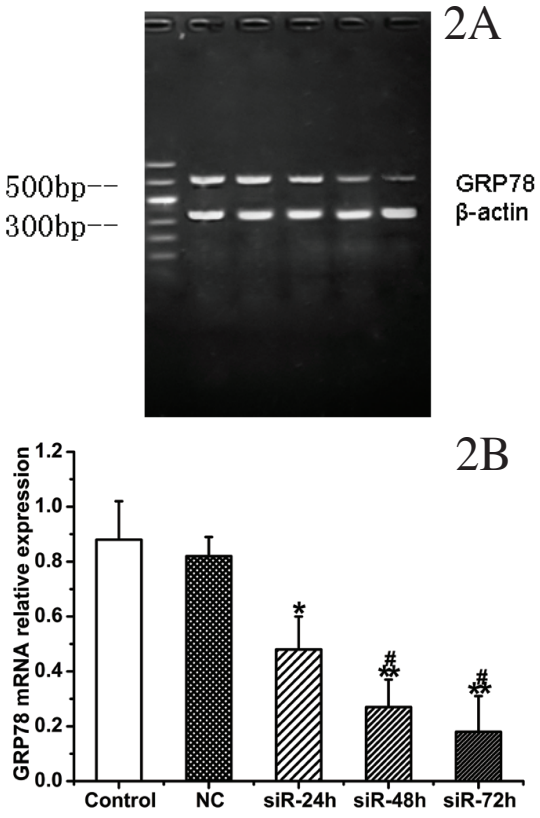

Figure 2. GRP78 mRNA expression in HO-8910 cells and siRNA-transfected HO-8910. GRP78 mRNA expression was decreased in siR-24h, siR-48h and siR-72h (A). The quantitative analysis of the GRP78 mRNA expression in different groups (B). All of the values are denoted as means±SEM; *p<0.05 *vs* NC; ** p< 0.01 *vs* NC; # p<0.05 vs siR-24h. Control indicates HO-8910 cells without treatment; NC, NC siRNA-transfected HO-8910 cells; siR-24h (48h, 72h), siRNA-transfected HO-8910 24h (48h, 72h)

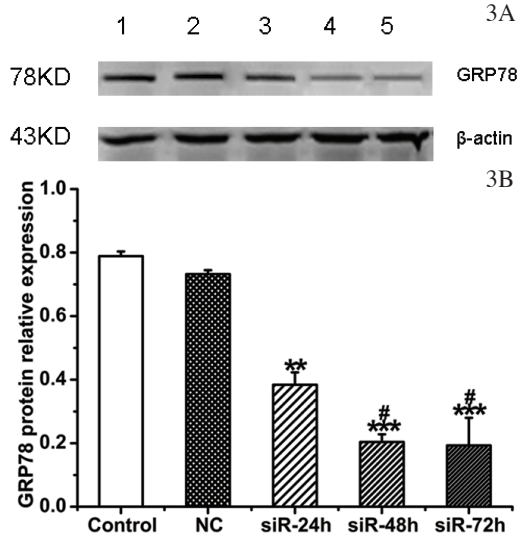

Figure 3. GRP78 Protein Expression in HO-8910 cells and siRNA-Transfected HO-8910. GRP78 protein expression was decreased in siR-24h, siR-48h and siR-72h A). The quantitative analysis of the GRP78 mRNA expression in different groups B). All of the values are denoted as means±SEM; ** p<0.01 *s* NC; ***p<0.001 *vs* NC; # p<0.05 vs siR-24h. Control indicates HO-8910 cells without treatment; NC, NC siRNA-transfected HO-8910 cells; siR-24h, 48h and 72h, siRNA-transfected HO-8910 24h, 48h and 72h

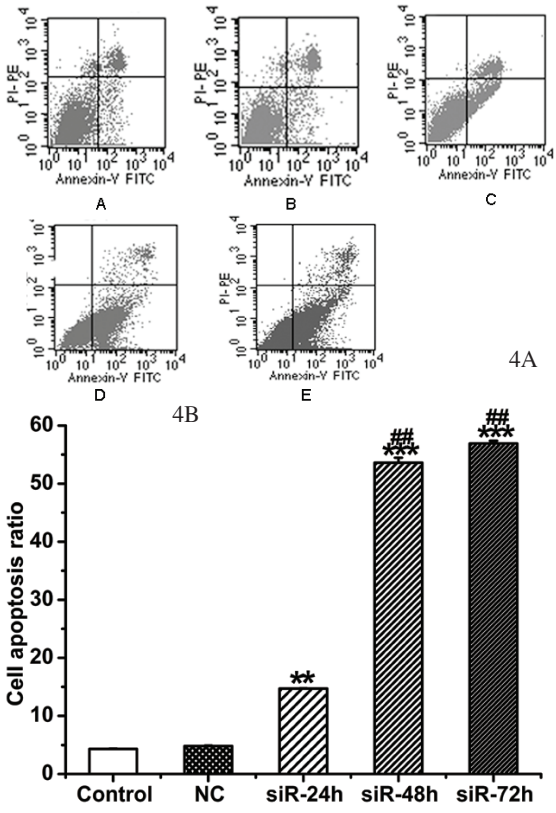

Figure 4. The representative pictures of cell apoptosis (A) and apoptosis ratio analysis (B). All of the values are denoted as means±SEM; ** p<0.01 *vs* NC; *** p<0.001 *vs* NC; ## p<0.01 *vs* siR-24h. Control indicates HO-8910 cells without treatment; NC, NC siRNA-transfected HO-8910 cells; siR-24h, 48h and 72h, siRNA-transfected HO-8910 24h, 48h and 72h

Table 1. Comparison of Cell Viability (%) among HO-8910, NC-HO-8910 and Si-HO-8910 Cells Exposed to Paclitaxel till 72h

Group	Paclitaxel concentration (PPC)					
	0	0.1	0.2	1	5	10
HO-8910	100	92.8	83.2	60.3	24.8	17.7
NC-HO-8910	100	91.5	79.3	56.4	22.6	18.5
Si-HO-8910	100	63.4*	50.1*	41.5*	15.8*	15.3*

Cell viability (%)=A546(experimental group)/A546(blank group) x 100%; *p<0.05

mRNA expression was invariable before and after siRNA-transfection.

The results of protein expression were similar to that of mRNA expression. Figure 3A showed that the expressions of HO-8910 cells and psiSTRIKETM/NC-GRP78 HO-8910 cells were high and the expression was gradually decreased in 24h, 48h and 72h after siRNA-transfection, while the β-actin protein expression was invariable. The difference was significant which can be seen in Figure 3B.

The sensitivities to paclitaxel of HO-8910 cell, psiSTRIKETM/GRP78 HO-8910 cell and psiSTRIKETM/NC-GRP78 HO-8910 cell were analyzed by methyl thiazolyl tetrazolium (MTT) assay. The results of MTT indicated that survival rates of the three groups were gradually decreased with the gradually increased of the paclitaxel concentration, but the degree of decrease was different, the psiSTRIKETM/GRP78 HO-8910 cell decreased significantly (Table 1). With the same paclitaxel concentration, the survival rate of psiSTRIKETM/GRP78 HO-8910 cell was lower than HO-8910 cell and psiSTRIKETM/NC-GRP78 HO-8910 cell significantly (p<0.05). The results indicated that psiSTRIKETM/GRP78 HO-8910 cell had higher sensitivity to paclitaxel than the other two groups. The sensitivity to paclitaxel of HO-8910 cell before and after siRNA transfection was changed dramatically.

In the psiSTRIKETM/GRP78 HO-8910 cells, the apoptosis ratio was gradually increased with transfection and the apoptosis ratio in 72h after transfection was the highest reaching to 56.92±0.46%. The change of apoptosis ratio in psiSTRIKETM/NC-GRP78 group and blank group was not obvious. The difference between psiSTRIKETM/GRP78 HO-8910 group and psiSTRIKETM/NC-GRP78 group, blank group had statistical significance respectively (p < 0.01), while the difference between psiSTRIKETM/NC-GRP78 group and blank group had no statistical significance (p>0.05). GRP78 siRNA can induce apoptosis obviously (Figure 4A and 4B).

Discussion

In the present study, we have performed an analysis of involvement of GRP78 in the resistance of ovarian carcinoma cell line to paclitaxel. Here, our results showed that *i)* we recombined GRP78 vector which is assessed successfully; *ii)* Sensitivity to paclitaxel was increased in GRP78 siRNA-transfected HO-8910 cells; *iii)* GRP78 siRNA can induce apoptosis obviously.

The glucose-regulated protein (GRP) system in mammalian cells is induced by glucose deprivation, anoxia, the calcium ionophore A23187, and 2-deoxyglucose (Shen et al., 1987). The mammalian stress response is an evolutionarily conserved mechanism, which can induce cells to respond to stimulus from adverse environmental or metabolic conditions (Foti et al., 1999). This response is clarified at the molecular level by the induced synthesis of specific sets of cellular protective proteins. The glucose-regulated stress response in mammalian cells is characterized by the increased synthesis of GRPs. A glucose-regulated response is caused by hypoxia and nutrient deprivation that occur naturally in solid tumors, and then the resistance occurs in some solid tumors, which is an obstacle to chemotherapy (Tomida et al., 1996).

GRP78 is one of the most important members of GRPs. In a variety of cancer cell lines, solid tumors and human cancer biopsies, the levels of GRP78 are elevated, correlating with malignancy. GRP78 expression is known to be up-regulated by viral infections and other ER stresses (Kozutsumi et al., 1988). It has also been reported that GRP78 was an effective protein in vaccination against cancer cell proliferation (Srivastava et al., 1986; Srivastava.,1993). GRP78 has also been shown to be over-expressed in some cancer cell lines and human tumor samples, and to be correlated with malignancy (Gazit et al., 1999; Somersan et al., 2001). In addition, GRP78 overexpression could be closely related to increased tumor cell survival resulting in resistance to cancer treatment. Some reports suggested that resistance to hyperoxgen - or thermo-therapy and chemotherapy might be related to GRP78.

The induction of GRP by a variety of specific inducers leads to an increase in resistance to Adriamycin (Shen et al., 1987). According to the recent researches, overexpression of GRP78 was associated with many malignance tumors, such as lung cancer, oesophagus cancer and so on. Wang Q's research indicated that GRP78 was related with the differentiation and progression of the lung cancer and the expression in mRNA and protein level may be valuable in evaluating the grade of differentiation and clinical stage of human lung cancer (Wang et al., 2005). Another research also showed that decreased GRP78 protein expression is a potential prognostic marker of oral squamous cell carcinoma in Taiwan (Huang et al., 2010). The 78-kD glucose-regulated protein (GRP78) is a potent anti-apoptotic factor, conferring drug resistance. Recently, a study reported that high GRP78 expression in breast cancer specimens predict a shorter recurrence-free survival in patients who received doxorubicin-based adjuvant chemotherapy. This provides further evidence that GRP78 is a potential independent predictor for response to taxane-based adjuvant chemotherapy in breast cancer (Lee et al., 2011). GRP78 promoted the invasion

of hepatocellular carcinoma both *in vitro* and *in vivo*. Overexpression of GRP78 in hepatocellular carcinoma cells enhanced the activation and activity of FAK which negatively regulated Rock kinase activity by promoting the phosphorylation of p190RhoGAP (Su et al., 2010). Another research also showed that in human renal cell carcinoma (RCC) the high levels of GRP78 mRNA and protein expression were related to the large tumor size and high clinical stage (Fu et al., 2010). As to ovarian cancer, there is no systemical research about the expression of GRP78 and the relationship of drug- resistance by far.

In our study, we have found that basal expression of GRP78 mRNA and protein existed in the human ovarian HO-8910 cell lines. And high basal levels of GRP78 expression were related with an obvious decrease in sensitivity to paclitxel -chemotherapy. In addition, when GRP78 expression was inhibited specifically by siRNA in the HO-8910 cell line, sensitivity to paclitaxel -chemotherapy was increased dramatically. This result suggests a strong correlation between GRP78 expression levels and resistance to paclitaxel -chemotherapy. It is reported that the transcription of a family of GRP genes encodes endoplasmic reticulum (ER) and are invoved in ER stress response (Roy et al., 1999). The abnormal elevation could be attributed to glucose deprivation, oxidative stress, and hypoxia in ovarian cancer cells, which can induce ER stress. So it is possible that the microenvironment of ovarian cancer would cause GRP78 produced. The present study is the first to report the expression of GRP78 and that it is possibly involved in resistance to paclitaxel -chemotherapy in ovarian cancer.

As a therapy target, GRP78 has been used to treat many cancers in past years. In one study of breast carcinomas, GRP78 has been shown to associate with and stabilize p185/erbB2, which is commonly overexpressed and is associated with the poor prognosis. Antisense knockdown strategies could suppress the expression levels of GRP78, which results in enhanced sensitivity to etoposide-induced cell death (Belfi et al., 1999). The enhanced sensitivity could trigger proteolytic cleavage of GRP78 by calpain, which also cleaves Bcl-xl during apoptosis, therefore turning an anti-apoptotic protein into a pro-apoptotic molecule (Nakagawa et al., 2000). GRP78 could also be used in immunotherapy (Manjili et al., 2002). In a recent study indicated that GRP-based immunotherapy and radiation therapy may be a potentially effective strategy for cancer treatment (Liu et al., 2005). And our results showed that the siRNA of the GRP78 reversed chemotherapy sensitivity *in vitro* thus providing further evidence for the notion that GRP78 specific RNA interference might be a viable approach for ovarian cancer chemotherapy treatment. Moreover, ovarian cancers with higher levels of GRP78 mRNA and protein, treated with siRNA, could be treated with lower chemotherapy dose to reducing the adverse reaction of drugs. We further suggest that it may be a possible therapeutic agent to improve clinical outcome of ovarian cancer. The success of such an approach, however, still awaits the development of an efficient delivery system that will affect most of the tumor cells *in vitro*.

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