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Glucose-Related Protein 78 Expression and Its Effects on Cisplatin-Resistance in Cervical Cancer

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Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: GRP78, the 78-kDa glucose-regulated protein, occupies a significant position in endoplasmic reticulum stress. Emerging evidences have shown that GRP78 induces chemoresistance in several tumors; however, the role of GRP78 in cervical cancer (CVC) still needs to be elucidated clearly.

Material/Methods: In the present study, we evaluated the expression levels of GRP78 in CVC tissues collected from patients through immunocytochemistry, western blot, and real-time PCR. To explore the exact role of GRP78 in CVC cells in the presence of cisplatin, we generated GRP78 knockdown CVC cells through small interfering RNA. After transfection, the apoptosis rate was assessed by flow cytometry. Then the expression levels of caspase-3, CHOP, and Bcl-2 in GRP78 knockdown cells were determined by western blot.

Results: The GRP78 levels in CVC tissues were increased significantly. Three types of CVC cells HeLa, SiHa, and C33A were treated with different concentrations of cisplatin and cultured for 12 hours, 24 hours, and 48 hours respectively. And SiHa cells exhibited the highest resistance to cisplatin at all time. Specifically, after 25 μM cisplatin treatment, more than 80% of C33A cells underwent apoptosis, whereas the apoptotic rate of SiHa cells was only 30–40%. Data suggested that GRP78 silencing increased chemo-sensitivity and improved the effects of cisplatin-induced apoptosis in SiHa cells. Moreover, inhibition of GRP78 could upregulate caspase-3 and CHOP expression and downregulate Bcl-2 expression.

Conclusions: GRP78 may represent a key bio-marker of CVC and silencing GRP78 may strengthen the resistance against cisplatin. GRP78 may be a potential molecular target for CVC therapies in future.

MeSH Keywords: Cisplatin • Drug Resistance • Endoplasmic Reticulum Stress • Uterine Cervical Neoplasms

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Background

Cervical cancer (CVC) is one of women's most prevailing malignant diseases worldwide [1]. Despite advances in screening, diagnosis, prevention, and therapies in CVC, this disease is still ranked as the third main reason of cancer death in women [2]. It occupied approximately 4% of all recently diagnosed cancers and 4% of all cancer deaths as stated by GLOBOCAN in 2012 [3]. The exact pathogenesis of CVC has been widely studied by researchers. Human papillomavirus is considered to be a major etiological agent in the development of CVC, whose gene products could interfere with cell cycle control giving rise to secondary accumulation of genetic dysfunctions. In some patients, however, secondary pathogenetic factors like smoking and immune response are also obviously crucial [4].

Clinically, CVC is presently divided into three stages according to the International Federation of Gynecology and Obstetrics (FIGO) guidelines: 1) early stage, which ranges from micro-invasive disease IA1 and IA2 to macroscopic disease confined to the cervix and measuring <4 cm, IB1; 2) locally advanced FIGO stages IB2-IVA, and 3) IVB and recurrent disease [5]. Patients with advanced/recurrent CVC especially have poor prognosis and their one-year survival rate is merely 10–20% [6]. At present, the standard therapy for these patients is chemotherapy. Cisplatin, a chemotherapeutic agent, has been regarded as one of the most efficacious anti-carcinogen therapies against CVC. But the development of resistance to cisplatin is a serious problem during CVC treatment, which has largely restricted the application and effectiveness of cisplatin in the clinic setting. The fundamental cellular and molecular mechanisms of cisplatin resistance (CPR) are complicated and usually connected with alteration in DNA methylation, microRNA profile, cancer stem cell characteristics, and expression of stress-response chaperones [7]. Exploring the underlying mechanisms of CPR may contribute to providing newly effective therapy for patients.

Chemotherapeutic agents usually tend to trigger stress response when controlling cell growth [8]. Playing a pivotal role in cellular response to environmental agents, endoplasmic reticulum (ER) stress can be induced by changes in tumor microenvironment or antitumor medicine. Endoplasmic reticulum (ER) stress may cause ER folding error, unfolded proteins accumulation and calcium ion disbalance in cells. GRP78, also referred to Bip or HSPA5, plays crucial roles in a number of cellular events, including facilitating proteins folding and assembly in a proper manner, preventing aggregating and targeting misfolded proteins for proteasome-mediated degradation. It also acts as calcium balance regulator and ER stress signaler.

Studies have disclosed that GRP78 is over-expressed at the genome as well as protein level in several kinds of human tumors such as breast cancer [9], prostate cancer [10], leukemia [11], and hepatocarcinoma [12]. It seems that GRP78 is a positive inducer for chemoresistance acquisition. However, in the research field of cervical cancer, little was known about the expression level of GRP78 and its effects on cisplatin resistance. Therefore, in the current study, we explored the expression patterns of GRP78 in CVC tissues both at gene and protein level. In addition, we screened a relatively stable cisplatin-resistant cell line and adopted siRNA knockdown transfection technology to detect the influence of GRP78 on cellular events. Finally, the expression levels of downstream proteins were assayed by western blot to assess the effects of GRP78 silencing. We believe that the data acquired in our study can contribute to better understanding of the biological role of GRP78 in CVC and help to solve the tough problems of drug resistance during the course of CVC therapy.

Material and Methods

Patients and tissue samples

The present research was approved by The First Affiliated Hospital with Nanjing Medical University. In this study, 50 tissue samples were collected in patients during operation or cervical biopsy from 2010 to 2011. Tissues adjacent to cervical lesion or the pericarcinomatous tissues were used as normal control. All patients signed the written informed consent before treatment and experiment. Among all 50 patients aged from 32 years to 60 years old, there were 41 cases of squamous carcinoma and nine cases of adenocarcinoma. According to the FIGO classification of cervical cancer, one patient was categorized as stage IA, 39 patients were categorized as stage IB and the other 10 patients were categorized as stage IIA. Referring to the pathological classification, five patients were classified as cell grade I, 29 patients were classified as cell grade II, 16 patients were classified as cell grade III. Of all the 50 patients, nine cases exhibited lymphatic metastasis, and 13 cases were diagnosed with lymphovascular space invasion (LVSI). According to the tumor infiltrating degree, 11 cases were infiltrated less than half of the cervical full-thickness, and 39 cases were equal or greater than half of the cervical full-thickness.

Cells and cell culture

In the present study, we used three human CVC cell lines HeLa, SiHa, and C33A, all of which were obtained from Nanjing KeyGen Biotech Co., Ltd., (Nanjing, China). HeLa and SiHa cells were cultivated in minimum essential medium (MEM; Gibco Co., USA) supplemented with 10% fetal calf serum (FBS; Gibco Co., USA). C33A cells were cultured in Dulbecco's Modified Eagles Medium (DMEM; Gibco Co., USA). All the cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

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Histology and Immunohistochemical analysis

All the specimens were fixed in 4% paraformaldehyde and paraffin-embedded. The tissue samples were serially cut into 5 μm sections by histotome. For histologic examination, the sections were routinely stained using hematoxylin and eosin (H&E). The stained sections were classified according to WHO histological criteria. GRP78 immunohistochemical analysis was performed on the paraffin-embedded tissue sections using a standard method. In brief, after dewaxed and rehydrated, antigen retrieval was performed using heat-induced method. Slides were washed with PBS three times and block with 10% normal serum. Then the primary antibody (GRP78, 1: 100 dilution) was applied to the sections and the incubation was maintained at 4°C overnight. After washing by PBS, the secondary antibody was added. Diaminobenzidine (DAB) was used as a choromogenic agent. The slices were counter stained with hematoxylin. Images were captured with Olympus systems. The evaluation of GRP78 immunohistochemical expression was performed as follows. All the immuno-positive cells were counted in 10 consecutive fields (400×). The scoring criterion was established according to the percentage of positive cells and stain intensity: 0% of positive cells received a score of 0; 1% to 25% of positive cells received a score of 1; 26% to 50% of positive cells received a score of 2; 51% to 75% of positive cells received a score of 3, 76% to 100% of positive cells received a score of 4. As to the stain intensity, negative staining received a score of 0, faint yellow staining received a score of 1, claybank staining received a score of 2, dark brown staining received a score of 3. Adding two types of scores together, the final score was used to represent the expression level of GRP78. Score of 0–1 represents low expression (–); 2–3 represents middle expression (+); 4–5 (++) and 5–6 (+++) represents high expression.

Cisplatin chemosensitivity assay

Cisplatin was obtained from Sigma and dissolved in 0.9% NaCl solution to make a 0.001 M stock solution. Then proper amounts of cisplatin stock solution were added to HeLa, SiHa, and C33A cells to reach terminal concentrations of 6.25, 12.5, 25, 50, and 100 μM. Then every cell line was divided into two groups, which were incubated in a normal circumstance of 5% CO_2 and in hypoxia circumstance, respectively. The cell viability was assayed using CCK-8 kit (Dojindo, Japan) at 12 hours, 24 hours and 48 hours respectively.

Western blot

For tissue samples, we first prepared the lysate using an ice-cold lysis buffer and then the lysates were centrifuged for 30 minutes at 1,000 rpm at 4°C. The supernatant was obtained and preserved at –80°C. For cells lysate, we used an ice-cold lysis buffer and constant agitation was maintained for 30 minutes at 4°C. The lysate was then clarified by a microcentrifuge at 12,000 rpm for 10 minutes at 4°C. Protein was quantified using a protein assay kit (Beyotime, China). We loaded equal amount of protein (80 μg per lane) into the wells of the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, then transferred the protein to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories). We blocked the membranes for one hour with TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.15% Tween-20) containing 5% (w/v) nonfat milk powder. Then we incubated the membrane with anti-GRP78 (Cell Sign Tech, USA) and anti-GAPDH (Bio Tech, USA) at 4°C overnight. After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for one hour. The signals were detected by ChemiDoc XRS$^+$ System (Bio-Rad Laboratories) using the ECL plus kit (Beyotime, China).

Real-time quantitative PCR (RT-qPCR)

TRIzol reagent (Generay, Shanghai, China) was used to isolate the total RNA samples according to the manufacturer's protocol. Yield and purity were assessed by measuring A260 and A280. A RevertAid First Strand cDNA synthesis Kit was obtained from Fermentas (Lithuania) and used for reverse transcription. GRP78 and GAPDH primer sequences were as follows:
GRP78, forward, 5'-GGCCACTAATGGAGATACTCA-3';
reverse, 5'-CCGTTTGGCCTTTTCTACCTC-3';
GAPDH, forward, 5'-GGCCACTAATGGAGATACTCA-3';
reverse, 5'-CCGTTTGGCCTTTTCTACCTC-3'.

The 10 μL IQ SYBR Green Supermix (TIANGEN, China), 1 μL forward primer, 1 μL reverse primer and 8 μL cDNA were contained in PCR reaction system. PCR conditions were as follows: 50°C for three minutes, 95°C for 15 minutes, 95°C for 10 seconds, 60°C for 20 seconds, 72°C for 15 seconds, GOTO line 3 for 40 times, melt curve 70°C to 95°C, increment 0.5°C for 0.05 seconds, and plate read. GAPDH has been applied as the internal control.

Small interfering RNA (siRNA) transfection

For cell transfection, Lipofectamine2000 transfection reagent (Invitrogen) was used. The siRNA for GRP78 was synthesized and chemically modified by Hibio Technologies (Hibio, Hangzhou, China). Three siRNA were designed and utilized to optimize the best silencing effect. The transfection efficacy was evaluated through RT-PCR. The target sequences were as follows: GAPDH positive control sense: GUAUGACAACAGCCUCAAGTT; GAPDH positive control antisense: CUUGAGGCUGUUGUCAUACTT; negative control sense: UUCUCCGAACGUGUCACGUTT; negative control antisense: ACGUGACACGUUCGGAGAATT; GRP78-homo-753 sense: GGUUACCCAUGCAGUUGUUTT; GRP78-homo-753

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antisense: AACAACUGCAUGGGUAACCTT; GRP78-homo-1929 sense: GGAGCGCAUUGAUACUAGATT; GRP78-homo-1929 antisense: UCUAGUAUCAAUGCGCUCCTT; GRP78-homo-1416 sense: GUGGCAUAAACCCAGAUGATT; GRP78-homo-1416 antisense: UCAUCUGGGUUUAUGCCACTT.

Flow cytometry

Annexin V/PI staining method was performed to examine cell apoptosis. 24 hours after SiHa cells transfection, cells were subsequently treated with cisplatin for 24 hours or thapsigargin for 32 hours. Then cells were labeled with Annexin V-FITC and propidium iodide for cell apoptotic rate detection according to the manufacturer's instructions. The apoptotic rates were analyzed by flow cytometry (Becton Dickinson, NJ, USA). Each experiment was independently repeated three times.

Analysis of caspase-3, CHOP, and Bcl-2

The activity of caspase-3, CHOP, and Bcl-2 was analyzed by western blotting. Following cells transfection and subsequent treatment with cisplatin or thapsigargin, cells were harvested and resuspended in IP lysis buffer (Beyotime, China), incubated at 4°C for 30 minutes with occasional vortexing. Subsequently, cells were centrifuged at 12,000 rpm for 10 minutes at 4°C. The following western blot assay was performed according to aforementioned protocol. The primary antibodies were anti-caspase-3 (1: 1,000, Bioworld Technology, USA), anti-Bcl-2 (1: 1,000, Abcam, UK), anti-CHOP (1: 1,000, Santa Cruz Biotechnology) and anti-GAPDH (1: 10,000, Bioworld Technology, USA).

Statistical analysis

All the statistical analyses were performed by SPSS19.0. Data were presented as mean ± standard deviation from a representative experiment performed in triplicate. Correlation between the expression levels of GRP78 expression and clinico-pathological characteristics in CVC patients was assessed using the χ^2 test and Pearson's χ^2 test. Statistical significance between two groups of data sets was compared by Student's *t*-test; $p<0.05$ was considered as statistical significance.

Results

Pathological assessments and GRP78 expression in cervical tissues

In our current study, we performed H&E staining on clinical obtained CVC tissues to provide precise pathological analysis. According to H&E staining, the CVC tissues were divided into cervical squamous carcinoma and cervical adenocarcinoma. Representative figures of each kind of CVC lesion were presented in Figure 1. To further explore the role of GRP78 in cervical cancer progression, cervical tissues were analyzed through immunohistochemical approach. The representative figures of low expression (–), medium expression (+), and high expression (++/+++) are shown in Figure 2. Compared with normal cervical tissues, GRP78 expression level was significantly increased in CVC tissues with 68% of samples presenting high expression ($p<0.001$) (Table 1). Correlation between GRP78 expression and main clinico-pathological characteristics of patients were analyzed and are summarized in Table 2. Obviously, high expression of GRP78 tends to be strongly correlated with FIGO classification and the pathologic grade of cervical cancer. For FIGO classification, the high expression of GRP78 was 64.1% (25/39) in IB stage and 90.0% (9/10) in IIA stage. Similarly, for pathologic grade of cervical cancer, high expression level of GRP78 occupied 65.5% (19/29) in cell grade II and 87.5% (14/16) in cell grade III, which was significantly higher than

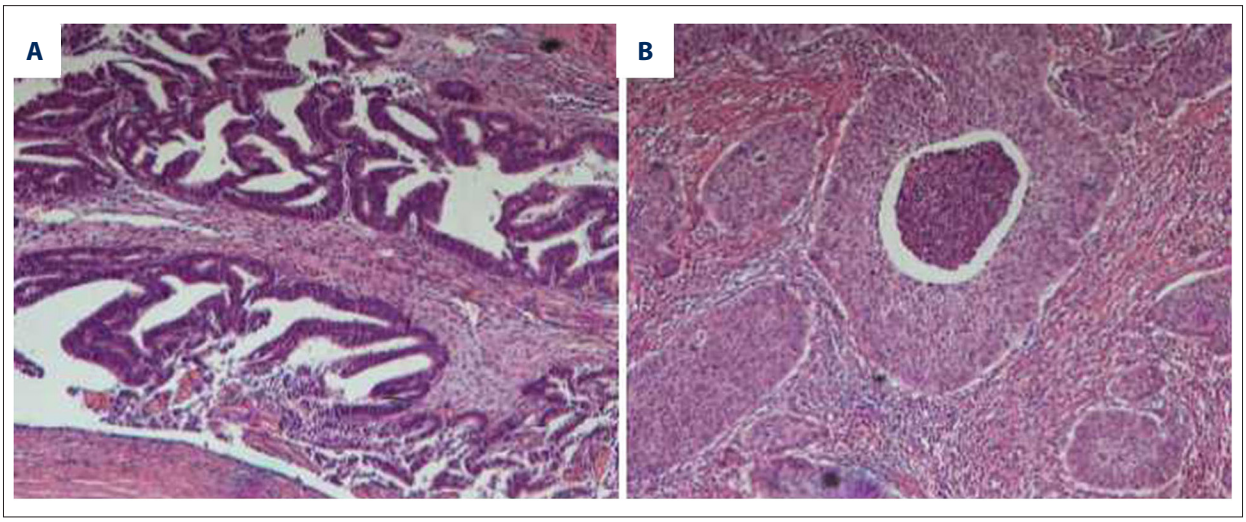

Figure 1. Representative figures of H&E staining in cervical adenocarcinoma and cervical squamous carcinoma (H&E, 100×).

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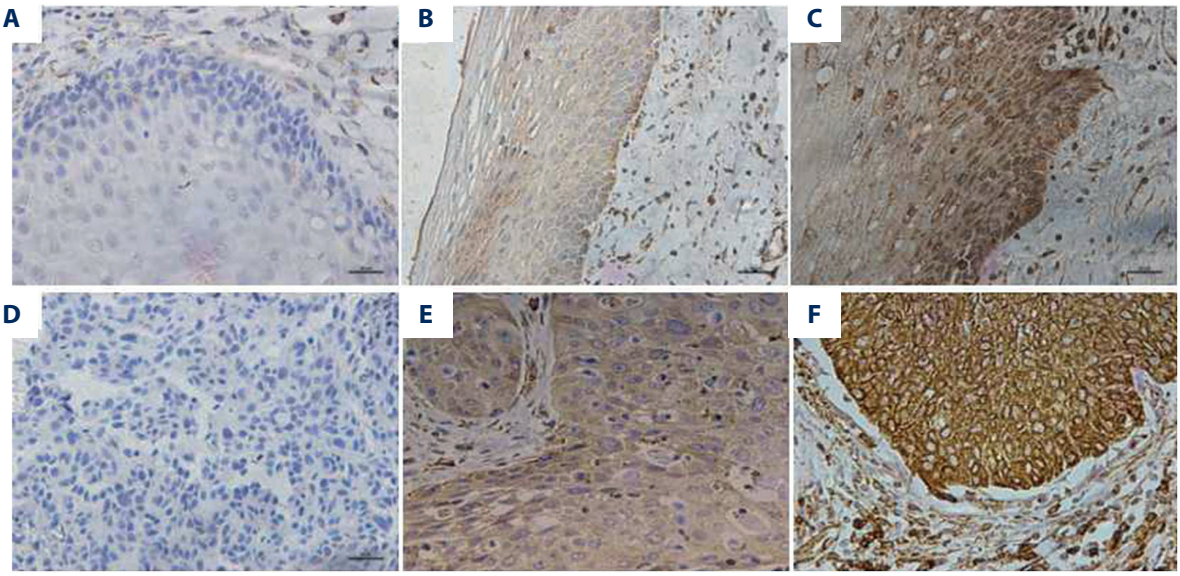

Figure 2. Representative images of different immunohistochemical staining categories of GRP78 in normal cervix and cervical cancer tissues (immunohistochemical staining, 400×).

Table 1. The comparison of GRP78 expression level between normal cervical tissues and cervical cancer tissues quantified by immunohistochemical analysis.

Expression level of GRP78	Normal cervical tissue (n=50)	Cervical cancer tissue (n=50)	*P* value
Low expression	2 (4.0%)	37 (74%)	<0.001***
Medium expression	14 (28.0%)	10 (20.0%)	
High expression	34 (68.0%)	3 (6%)	

*** $p<0.001$, significant difference.

cell grade I (20.0%, 1/5). Then we used western blot and RT-PCR to further study the expression level of GRP78 in CVC. As shown in Figure 3, GRP78 was significantly elevated in CVC tissues both in protein and gene levels. The results were consistent with the aforementioned immunohistochemical analysis.

The establishment of cisplatin-resistant CVC cells

To quantify the anti-proliferation ability of cisplatin in CVC cells and screen a stable cell line with high resistance to cisplatin, different concentrations of cisplatin were added into the incubation systems of SiHa, HeLa, and C33A cells. The cell viability was assayed using CCK-8 method after incubation for 12 hours, 24 hours and 48 hours. A dose-dependent relationship between cisplatin and cell viability was displayed in Figure 4A. In comparison, C33A cells presented higher chemosensitivity to cisplatin treatment than HeLa and SiHa cells. Specifically, when with 25 μM cisplatin treatment for 12 hours and 24 hours, inhibition rate occurred in over 80% of C33A cells compared with only 30–40% of SiHa cells under the same experimental conditions. Furthermore, the half maximal inhibitory concentration (IC50) decreased gradually along with incubation time. The IC50 of C33A, HeLa, and SiHa cells after 24 hours cisplatin treatment under normal incubation circumstance were 6.48 μM, 8.36 μM, and 38.78 μM, respectively, which indicated that SiHa cells were more resistant to cisplatin. Due to the stable high resistance to cisplatin, SiHa cells were chosen for the next study.

GRP78, as a stress protein, is induced by hypoxia in tumor cells [13]. To test the effects of hypoxia on drug-resistance in cells, SiHa cells were normal or hypoxic incubated. The mRNA and protein level of GRP78 were analyzed after 25 μM cisplatin treatment for 24 hours and 48 hours. Following cisplatin treatment, GRP78 levels in hypoxic and normal incubated group

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Table 2. Association of GRP78 expression with various clinic pathological features of patients with cervical cancer.

	n=50	GRP78 expression level			*P* value
		Low expression	Medium expression	High expression	
Age (years)					0.902
<45	24	1 (4.2%)	6 (25.0%)	17 (70.8%)	
≥45	26	1 (3.9%)	8 (30.8%)	17 (65.4%)	
Histological type					0.323
Squamous carcinoma	41	2 (4.9%)	13 (31.7%)	26 (63.4%)	
Adenocarcinoma	9	0 (0.0%)	1 (11.1%)	8 (88.9%)	
FIGO classification					0.001**
I a	1	1 (100%)	0 (0.0%)	0 (0.0%)	
I b	39	1 (2.6%)	13 (33.3%)	25 (64.1%)	
II a	10	0 (0.0%)	1 (10.0%)	9 (90.0%)	
Pathologic cell grade					0.016**
I	5	1 (20.0%)	3 (60.0%)	1 (20.0%)	
II	29	0 (0.0%)0	10 (34.5%)	19 (65.5%)	
III	16	1 (6.3%)	1 (6.3%)	14 (87.5%)	
Lymphatic metastasis					0.696
Positive	9	0 (0.0%)	2 (22.2%)	7 (77.8%)	
Negative	41	2 (4.9%)	12 (29.3%)	27 (65.9%)	
LVSI					0.108
Positive	13	0 (0.0%)	6 (46.2%)	7 (53.8%)	
Negative	37	5 (5.4%)	8 (21.6%)	27 (73.0%)	
Depth of invasion					0.440
<1/2 full-thickness	11	1 (9.1%)	4 (36.4%)	6 (54.5%)	
≥1/2 full-thickness	39	1 (2.6%)	10 (25.6%)	28 (71.8%)	

showed no significant difference (Figure 4B, 4C). Therefore, SiHa cells were normal incubated for the next study.

Silencing of GRP78 by siRNA

To further explore the exact role of GRP78 in CVC, we adopted siRNA technology to block GRP78 expression in SiHa cells. Three siRNAs were used to optimize the transfection experiment and obtain a best silencing effect. GRP78 silencing was confirmed through RT-PCR, which is displayed in Figure 5. Compared with unaffected cells (NC group), cells transfected with siRNA1929 showed the lowest level of GRP78 mRNA (Figure 6A, $p<0.05$). The mRNA level of GRP78 was decreased over 50% in siRNA1929 group. The results of western blot also presented the same trend (Figure 6B and 6C, $p<0.05$). Consequently, siRNA1929 successfully silenced the expression level of GRP78 in SiHa cells.

Silencing of GRP78 induces SiHa cell apoptosis

Next, we evaluated the role of GRP78 in apoptosis using flow cytometry. As shown in Figure 6, the apoptotic rates were remarkably higher in GRP78-silencing groups than it was in the negative control group (NC group). In addition, the percentage of apoptotic cells was significantly enhanced in the group of GRP78 silencing combination with cisplatin treatment.

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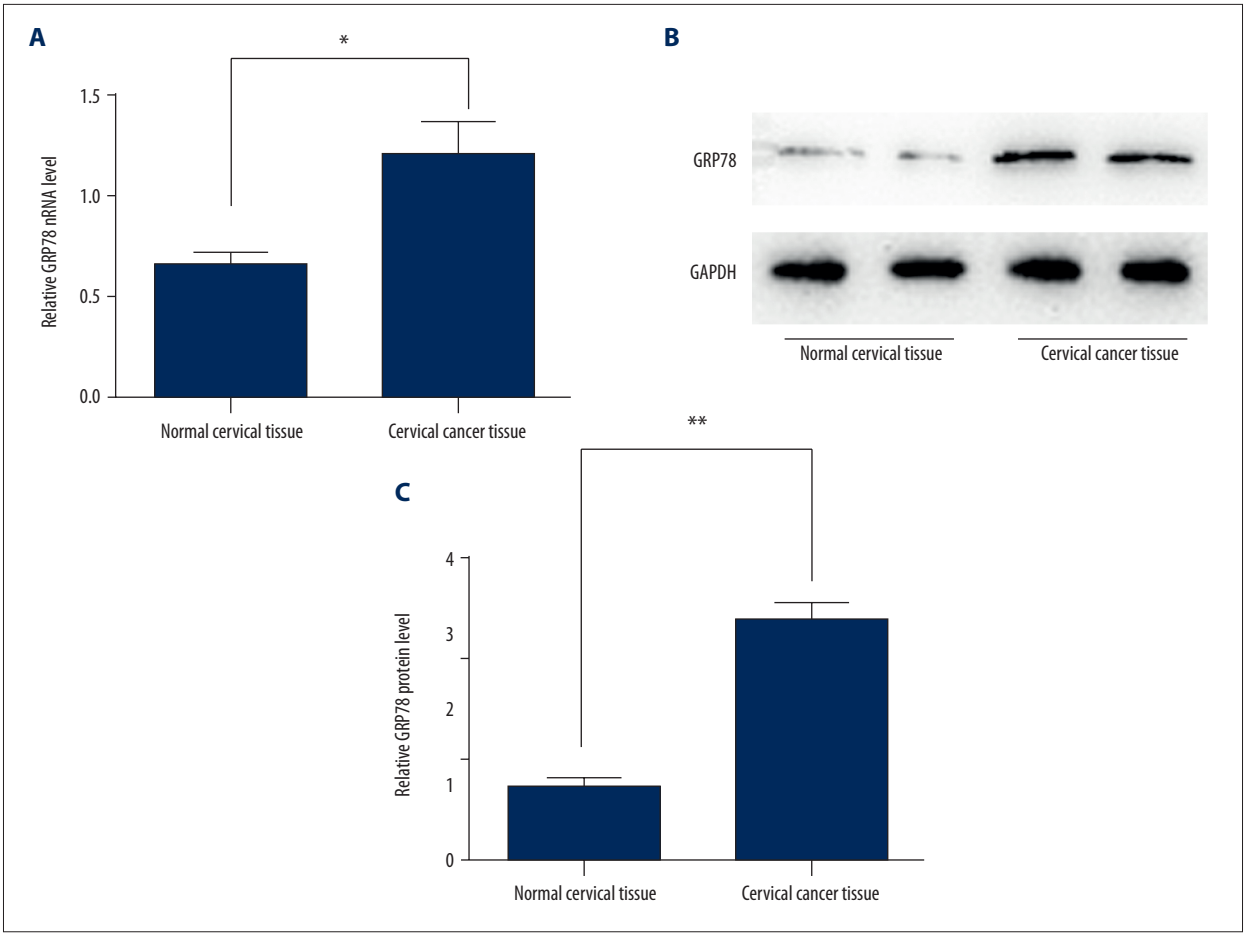

Figure 3. The comparison of GRP78 expression level between normal cervix and cervical cancer tissues: (**A**) comparison of relative GRP78 mRNA level; (**B**) western blot assay of GRP78 in normal cervix and CVC tissues; (**C**) comparison of relative GRP78 protein level. GAPDH was used as reference. Error bars indicate standard deviation; * $p<0.05$; ** $p<0.01$.

GRP78 silencing alters caspase-3, CHOP, and Bcl-2 protein levels

To investigate the potential molecular mechanism underlying the effect of GRP78 on cisplatin resistance in CVC, we assessed the apoptosis-associated proteins expression after transfection and cisplatin treatment. As shown in Figure 7, the levels of caspase-3 and CHOP were higher in GRP78-silencing cells than the NC group. But the level of Bcl-2 decreased in GRP78-silencing cells compared with the NC group. In addition, the caspase-3 and CHOP levels in GRP78-silencing cells treated with cisplatin were decreased but Bcl-2 level was increased. The data indicated that the altered activation of apoptosis-associated proteins may account for the chemoresistance to cisplatin in GRP78-silencing cells.

Discussion

CVC continues to be one of women's greatest killers worldwide. Patients with advanced or recurrent CVC exhibit poor prognosis with only 10–20% of one-year survival rate. Even if notable progresses in CVC therapy have been made in the past decades, tumors eventually presented resistant to chemotherapy after an initial response, which accounts for the death of most cancer patients [8]. Cisplatin has been successfully used to treat various tumors for many years. However, resistance to cisplatin treatment of cancer cells is becoming more common and has largely restricted the use of cisplatin in clinic. Thus for researchers, great efforts should be made to explore the underlying mechanism and key molecules of CPR which may help to promote therapeutic strategies in CVC.

GRP78 is a member of HSP70 protein family, which plays a significant role in the progress of oncogenesis. Traditionally, in vital organs of adults including brain, heart, and lung, GRP78 expression is maintained at a low basal level. However, it is

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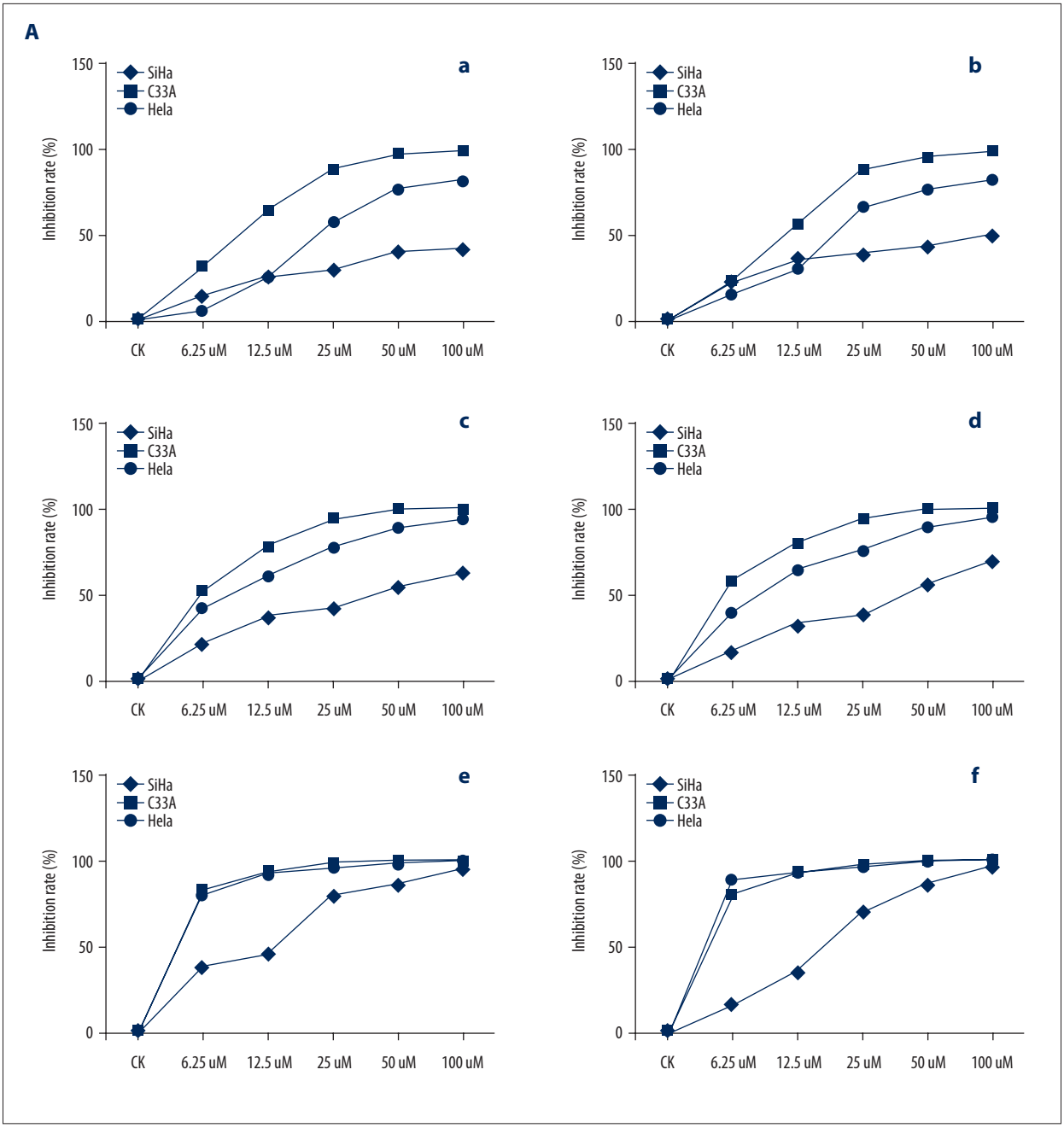

Figure 4. Effect of cisplatin on cell viability and GRP78 expression level in cervical carcinoma cells: (**A**) dose-dependent cytotoxic effects for cisplatin in cervical carcinoma cells: (**a**) normal incubation for 12 hours; (**b**) hypoxic incubation for 12 hours; (**c**) normal incubation for 24 hours; (**d**) hypoxic incubation for 24 hours; (**e**) normal incubation for 48 hours; (**f**) hypoxic incubation for 48 hours; (**B**) the mRNA (**a**) and protein (**b, c**) expression level of GRP78 after receiving 25 μM cisplatin treatment for 24 hours; (**C**) the mRNA (**a**) and protein (**b, c**) expression level of GRP78 after receiving 25 μM cisplatin treatment for 48 hours. CK – normal control; CT – cisplatin treatment.

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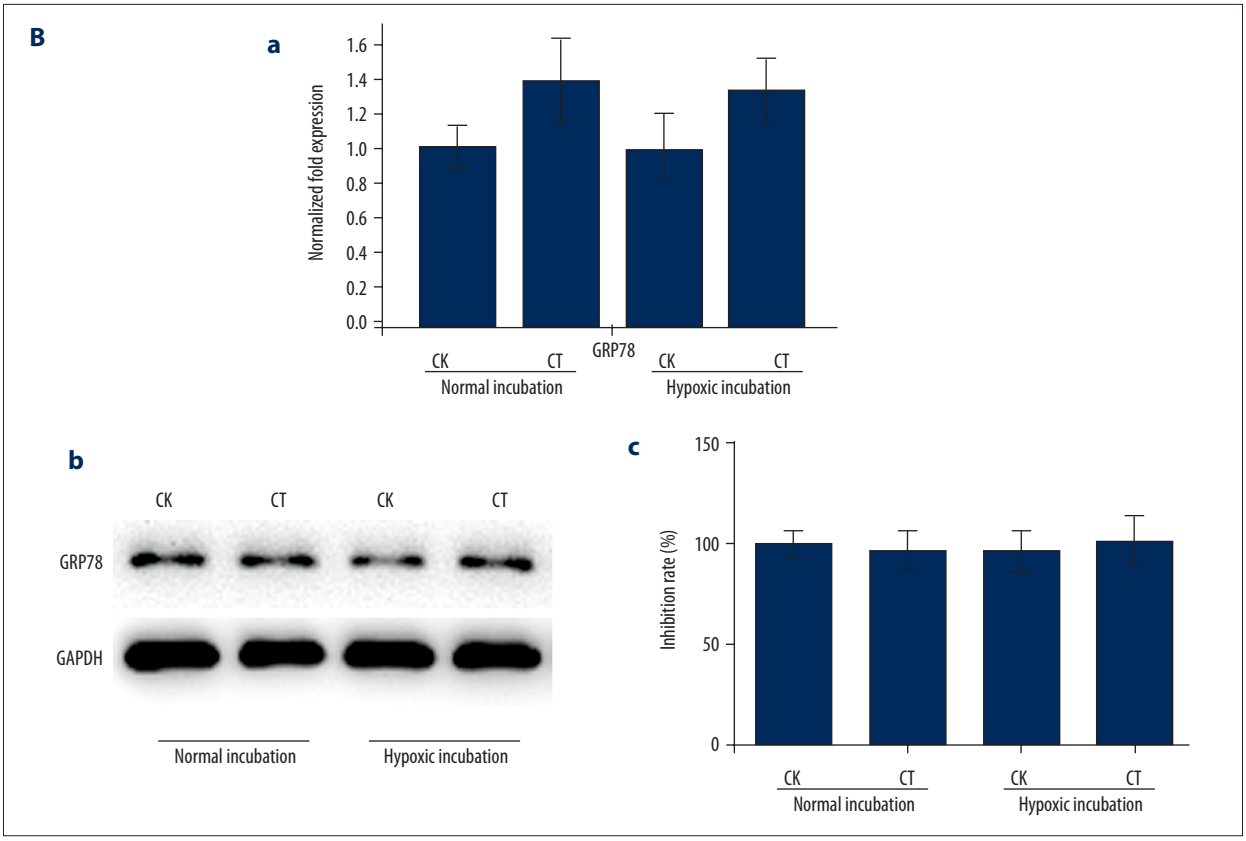

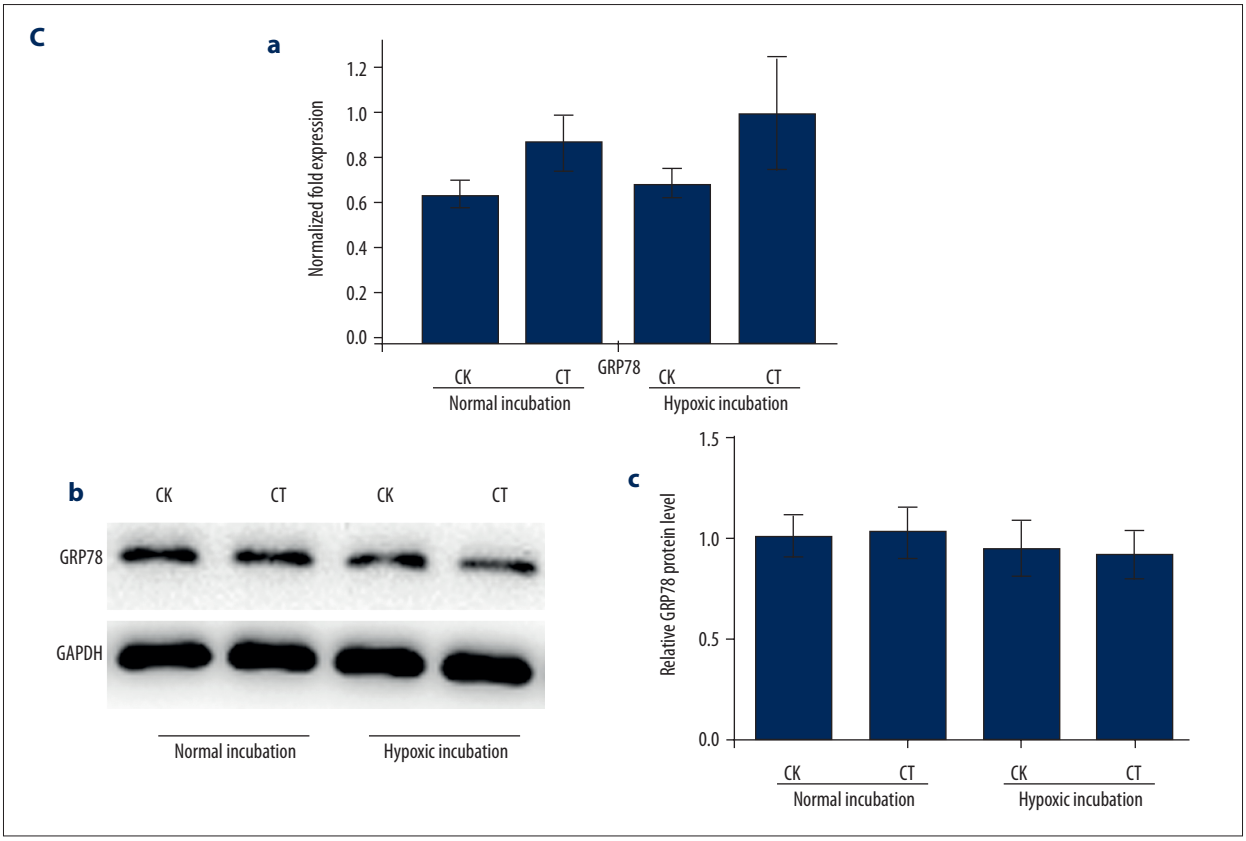

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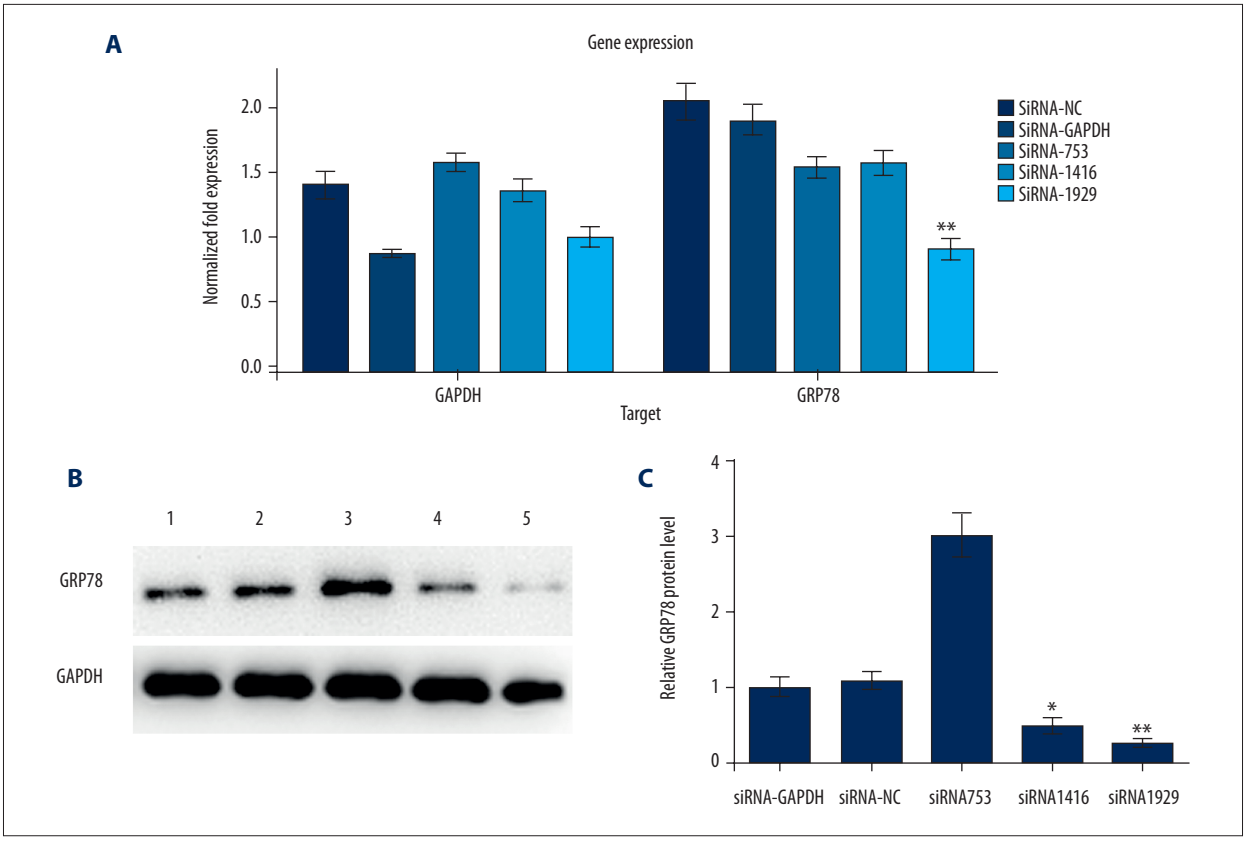

Figure 5. Assessment of transfection efficiency: (**A**) the mRNA level of GRP78; (**B**) western blot assay of GRP78; (**C**) comparison of relative GRP78 protein level in different transfected groups; * $p<0.05$; ** $p<0.01$. 1 – siRNA-GAPDH; 2 – siRNA-NC; 3 – siRNA753; 4 – siRNA1416; 5 – siRNA1929.

dramatically increased in tumors [14]. GRP78, which has the ability to accelerate tumor cells proliferation, survival, metastasis, and resistance to various therapeutic approaches, can be induced to be expressed by cancer cells to accommodate themselves to chronic stress in the tumor micro-environment [15]. In this study, GRP78 expression level was significantly elevated in CVC tissues both at the protein and gene level. High expression of GRP78 tends to be strongly correlated with FIGO classification and pathologic grade of CVC. These results suggested that GRP78 has the potential to become a biomarker for CVC behavior.

The literature has reported that GRP78 is over-expressed in several kinds of human tumors including leukemia [11], glioma [16], prostate cancer [17], and breast cancer [18]. Cancer cells are inherently heterogeneous and different tumors can use diverse signaling and defense mechanisms to acquire resistance to specific drugs [19]. In order to precisely and definitely elucidate the role of GRP78 on CPR, we quantified the anti-proliferation activity of cisplatin in cervical carcinoma cells and screened a stable cell line with high resistance to cisplatin. Consequently, SiHa cells were found to be highly resistant to cisplatin. These observations implied that GRP78 may act as a specific biomarker to predict CPR in CVC.

RNA interference refers to the target gene silencing at the post-transcriptional level in cellular events, which is induced by degradation of the target mRNAs complementary to the antisense strands of double-stranded, short interfering RNAs [20]. This evolutionary regulatory technology has emerged to be an important new therapeutic approach to intractable diseases including cancer and some metabolic and infectious diseases both in the laboratory and in the clinic. In the present study, we employed GRP78 siRNA technology to further explore the role of GRP78 in resistance ability towards cisplatin in SiHa cells. The results showed GRP78 silencing significantly enhanced SiHa cells apoptosis, which suggested GRP78 may serve as a promoter in the survival of CVC cells. The observations in our researches were consistent with previous reports [21–24]. In addition, the combination of GRP78 silencing and cisplatin treatment significantly enhanced the cell apoptosis rate, thus indicating GRP78 is a positive modifier for CPR acquisition in CVC.

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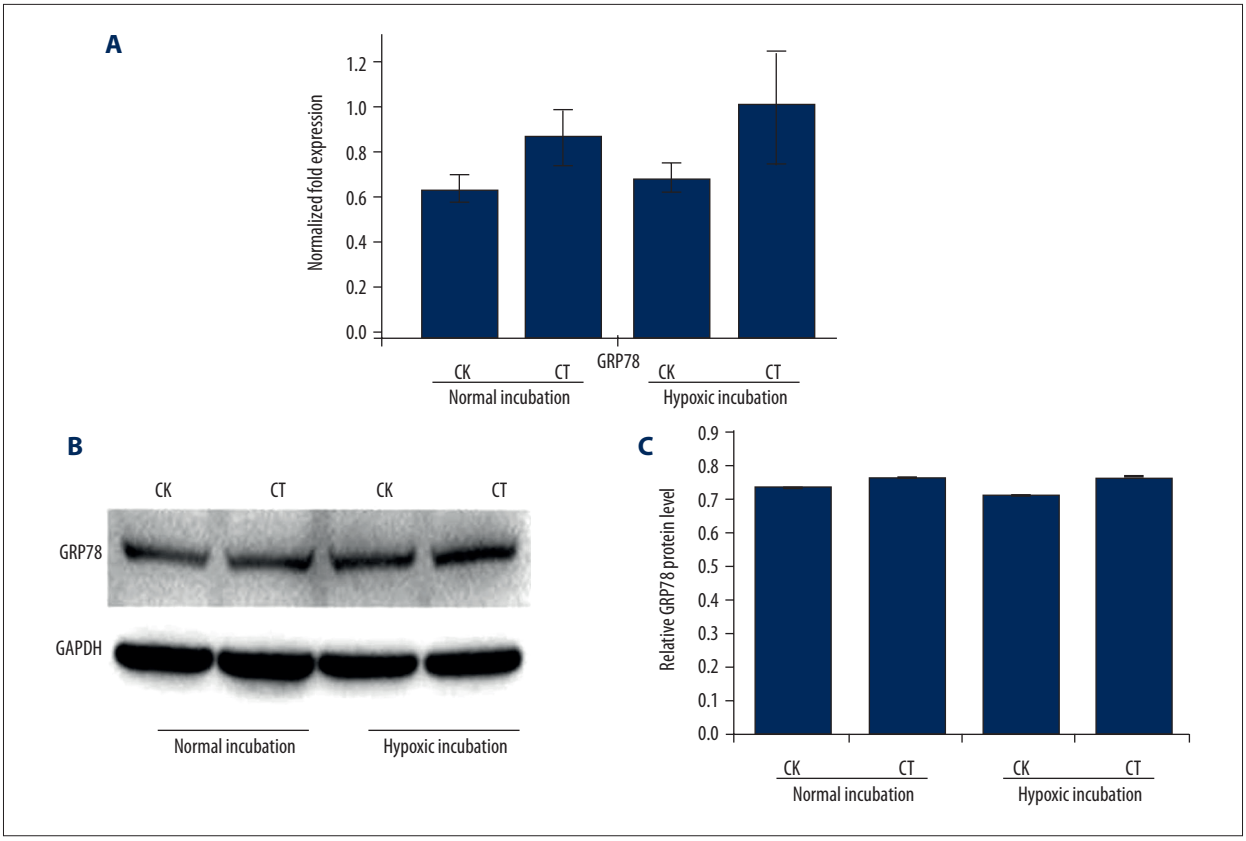

Figure 6. The role of GRP78 on apoptosis in SiHa cells measured by flow cytometry: (**A**) representative images of apoptosis in in different groups; (**B**) the apoptosis rate in different groups. NC – siRNA negative control; SiRNA1929 – cells transfected by siRNA1929; SiRNA1929+cisplatin – siRNA1929 transfected cells treated with cisplatin; ** $p<0.01$ versus NC group, ## $p<0.01$ versus SiRNA1929 group.

Recent studies have shown that CVC may be associated with the mTOR pathway [25], the NF-κB pathway [26] and inflammatory reaction [27]. In this study, we focused on cell apoptosis in CVC. Apoptosis is the death of a cell mediated by a highly regulated intracellular program. It plays a fundamental role in keeping homeostasis in multicellular organisms. In tumorigenesis, apoptosis can protect against cancer development by eliminating genetically altered hyperproliferative cells. Thus, defects in apoptosis signaling pathways contribute to carcinogenesis and chemoresistance [28]. Many proteins are involved in apoptosis processes. Caspases play the role of initiators and executioners in programmed death. Caspase-3 is directly responsible for cell demise [29]. In addition, B-cell lymphoma/leukemia-2 (Bcl-2) family proteins are known as the key modulators of apoptosis process. CHOP is a central pro-apoptotic transcription factor which is closely associated with ER stress-mediated apoptosis. Usually the downregulation of Bcl-2 is considered to contribute to CHOP mediated apoptosis [30]. ER stress is a response to protect against cell death, but it will induce apoptosis in case of severe injury and intracellular environment alteration. In general, severe ER stress results in CHOP elevation and the caspase family activation to trigger apoptosis. And the increasing GRP78 expression is an important indication of ER stress [31]. Thus we examined the expression levels of caspase-3, Bcl-2, and CHOP to search for the proper molecular mechanism of GRP78-mediated apoptosis and its effects on cisplatin resistance in CVC. The data demonstrated that GRP78 silencing lead to a reduction of Bcl-2 expression and elevation of caspase-3 and CHOP expression. In addition, knockdown of GRP78 induced lower levels of caspase-3 and CHOP but higher level of Bcl-2 proteins. As a result, the data strongly suggested that GRP78-knockdown cells are more resistant to cisplatin treatment owing to the alteration of caspase-3, CHOP, and Bcl-2 proteins activity.

Conclusions

In the present research, we described the expression pattern of GRP78 in human cervical tissues and cervical carcinoma cells. And the relationship between GRP78 expression level and main clinico-pathological characteristics of patients was analyzed. We speculated that high expression level of GRP78 is an important marker in the acquisition of an aggressive

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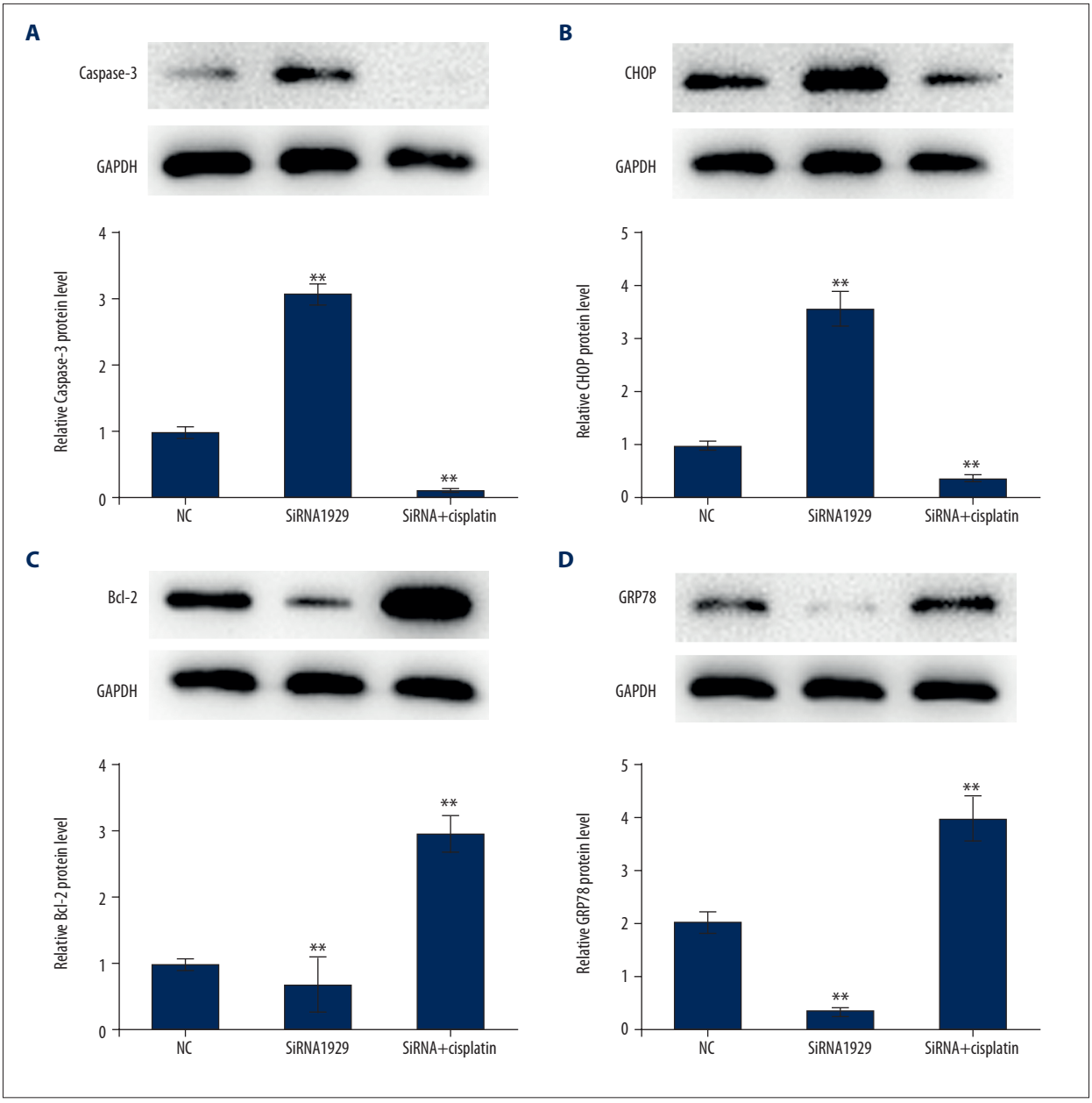

Figure 7. GRP78 silencing alters caspase-3, CHOP, and Bcl-2 protein levels: (**A**) western blot assay of caspase-3 in different groups; (**B**) western blot assay of CHOP in different groups; (**C**) western blot assay of Bcl-2 in different groups; (**D**) western blot assay of GRP78 in different groups. NC – siRNA negative control; SiRNA1929 – cells transfected by siRNA1929; Si1929+cisplatin – siRNA1929 transfected cells treated with cisplatin; ** $p<0.01$.

phenotype and/or a poor therapeutic response of cervical tumor. Furthermore, functional studies of GRP78 silencing suggested that GRP78 knockdown plays a critical role in sensitizing cervical cancer cells to cisplatin. As a consequence, we speculated that GRP78 may be a potential therapeutic target for CVC in future.

Conflicts of interest

None.

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