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Effect of GREM 1 gene on chemoradiotherapy sensitivity of cervical squamous carcinoma cells

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Abstract. – OBJECTIVE: To investigate the effect of GREM1 on the sensitivity of cervical squamous carcinoma cells to radiotherapy and chemotherapy.

PATIENTS AND METHODS: The resected cancer tissues and paracancerous tissues of patients with cervical carcinoma were collected. The expressions of GREM1 protein and mRNA in SiHa cell line of cervical squamous carcinoma were tested by Western blot and reverse transcription-polymerase chain reaction (qRT-PCR). The effect of GREM1 on chemotherapy sensitivity of SiHa cells was tested by MTT assay. SiHa cells were irradiated with different doses of X-ray, and the changing trend of GREM1 in cell lines was observed.

RESULTS: Compared with paracancerous tissues, the expression level of GREM1 in cervical squamous carcinoma was significantly higher than that in paracancerous tissues ($p<0.05$). After adding different concentrations of chemotherapeutic drugs, the relative survival rate of SiHa cells overexpressing GREM1 group was significantly increased. After high-energy X-ray irradiation with different radiation doses of SiHa cells, the expression levels of GREM1mRNA and protein in SiHa cell lines showed a significant downward trend. GREM1 was highly expressed in cervical squamous carcinoma.

CONCLUSIONS: Down-regulating GREM1 expression can increase the chemotherapy sensitivity of SiHa cells. GREM1 may be related to the radiotherapy sensitivity of cervical squamous carcinoma.

Key Words
GREM1 gene, Cervical squamous carcinoma, Tumor, Sensitivity of radiotherapy and chemotherapy.

Introduction

Cervical carcinoma is one of the most common malignant tumors endangering women's health in the world. There are about 527,600 new cases of cervical carcinoma every year, and China accounts for 19% in the world[1] and 70% in Asia[2]. Squamous carcinoma is the most common pathological type of cervical carcinoma. Surgery can be considered in the early stage of cervical carcinoma, and radiotherapy and chemotherapy are the first choice for locally advanced cervical carcinoma[3], which can be also suitable for the radical treatment of early cervical carcinoma without retaining functions of the ovary and achieve the same therapeutic effect as the surgery. It is clearly recommended in the National Comprehensive Cancer Network (NCCN) guidelines[4]. Most cervical carcinoma reacted well to radiotherapy and chemotherapy, but there are still about 40% of the patients with radiotherapy resistance after treatment, and the prognosis was poor[5,6]. Therefore, it is of great significance to study the responsiveness of cervical carcinoma to radiotherapy and chemotherapy. GREM1 gene is an antagonist of bone morphogenetic protein. Some reports[7,8] have shown that GREM1 is involved in the occurrence and development of a variety of tumors. So far, there have been few reports on the occurrence, development, and therapeutic reactivity of GREM1 gene and cervical carcinoma. In this study, the relationship between GREM1 expression in SiHa cells of cervical squamous carcinoma and chemoradiotherapy sensitivity of cervical carcinoma was investigated by interfering with the expression of GREM1 in SiHa cells of cervical squamous carcinoma to provide new prognostic indicators for treatment.

Patients and Methods

Patients

72 patients with cervical carcinoma treated in the Xuzhou Cancer Hospital from January 2015 to May 2018 were selected. All patients had complete clinicopathological data. They did not receive special treatment such as chemotherapy and radiotherapy before surgery, aged 24-71 years old, with an average age of 44.1 years. Inclusion

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criteria: all patients must have histopathological results. The diagnostic criteria of cervical carcinoma were confirmed by pathology according to the 2009 International Federation of Gynecology and Obstetrics Staging Criteria of the International Union of Obstetrics and Gynecology[9]. Patients undergoing radical hysterectomy were confirmed by pathology after surgery. Exclusion criteria: patients with incomplete clinicopathological data, patients with congenital genital tract acute inflammation, genetic diseases, and serious medical diseases, and pregnant women. Patients with corresponding paracancerous tissue (tissue 2 cm from the cancer tissue confirmed by pathological examination as normal) were divided into the normal control group. All patients signed informed consent forms and this investigation was approved by the Ethics Committee of Xuzhou Cancer Hospital.

Cell Grouping and Transfection

Human normal immortalized epithelial cell line HCerEPic and cervical carcinoma SiHa cell line (Jiangsu Ruijie Life Technology Co., Ltd., Suzhou, Jiangsu, China) were cultured in RPMI-1640 Roswell Park Memorial Institute-1640 (RPMI-1640) medium (10% FSB, 1% penicillin-streptomycin solution), in the incubator with 5% CO_2 at 37°C. To observe the effect of GREM1 gene on the biological behavior of cervical carcinoma cells, we divided the cells into four groups: NC group (negative control), siRNA-GREM1 group (transfected siRNA-GREM1 sequence), Vector group (transgenic empty vector sequence), overexpression group (GREM1 group). We first replaced the complete medium of 12-well plate with serum-free medium, and then transfected pCMV6-Entry vector, pCMV6-Entry-GREM1 plasmid, NC siRNA and siRNA GREM1 into SiHa cells with cell abundance of about 40% in 12-well plate. Lipofectamine 2000 transfection reagent (11668-019, Invitrogen, Carlsbad, CA, USA) was used. The plasmid was transfected with 500 ng per well, and the final concentration of siRNA was 50 nM per well. After cultured for 8 h, the complete medium was changed, and the cells were collected and preserved after cultured for 48 h.

QRT-PCR Test

100 mg cancer tissue and paracancerous tissue were ground by adding proper amount of liquid nitrogen into the grinder, transferred into 1.5 ml RNase-free centrifuge tube, or collected cells were added appropriate amount of TRIzol (Bao Bioengineering Co., Ltd., Dalian, Liaoning, China) to extract mRNA (the steps referred to the instructions). The concentration and purity of RNA were tested, and the sample RNA was reversely transcribed into cDNA according to the instructions of reverse transcription kit (Fermentas K1621, Hangzhou Zhunuo Biotechnology Co., Ltd., Hangzhou, Zhejiang, China). GREM1 and GAPDH primers were designed and synthesized by Suzhou Jinweizhi Biotechnology Co., Ltd., Suzhou, Jiangsu, China (Table I). TransStart Green qPCR SuperMix kit (AQ 101, TransGene Biotech, Beijing, China) was used, and the fluorescence quantitative PCR reaction was carried out using 20 μL reaction system and ABI 7500 PCR amplifier. The conditions were as follows: pre-denatured for 30 s at 95°C, denatured for 10 s at 95°C, annealed for 15 s at 60°C, extended for 15 s at 72°C, and cycled 40 times. Meanwhile, GAPDH was used as an internal reference, and the reactions in each group were repeated 3 times. (This method can also be suitable for cell experiments).

Western Blot Test

The protein concentration of cancer tissue, paracancerous tissue or cell was tested by bicinchoninic acid (BCA) kit. Then, 5 × sample buffer was added, boiled for 5 min at 95°C, and put in refrigerator at –80°C for later use. The sample size of each sample was 30 μg and then transferred to polyvinylidene difluoride (PVDF) membrane. 5% skimmed milk powder was blocked for 1 h at room temperature and incubated with rabbit monoclonal antibody GREM1 (Abcam China, Shanghai, China; # 4383, 1:1000, Cell Signaling Technology) and β-Actin (ab6276, 1:8000, Abcam China, Shanghai, China) at 4°C for a night. Next, the membrane was washed with Tris-Buffered Saline and Tween (TBST) for 3 times, 15 min each time. Goat anti-rabbit (ab6721, 1:5000) or goat anti-mouse (ab6789, 1:5000) was incubated at room temperature for 1 h and membrane was washed

Table I. RT-PCR primer sequences.

Target gene	Primer sequence (5'-3')
GREM1	F: TAACACTGCCACAAGAATGCAA R: GCAAGACTGTGGTACAAGCTCCTAA
GAPDH	F: TGTGGGCATCAATGGATTTGG R: ACACCATGTATTCCGGGTCAAT

for 3 times with 10 min each time. The A solution and B solution of enhanced chemiluminescence (ECL) in the luminous kit were mixed with 0.5 ml equally and stayed for 1 min respectively. Then, they were uniformly dispersed on the PVDF film and placed in the dark chamber. X-ray film was used to sense light and the film was scanned by gel imaging system for gray analysis. Each group of experiments was repeated 3 times. (This method can also be applied to tissue experiments).

MTT Assay to Determine the Effect of Down-regulated GREM1 on Chemotherapy Sensitivity of SiHa Cells

Inoculated cells: the logarithmic growth phase cells were collected, the concentration of cell suspension was adjusted to 4 × 104 cells/ml, inoculated 96 well plate, and l00 μl cell suspension was added to each well, i.e., 4000 cells/hole. Each cell has 5 compound pores per drug concentration gradient. Only the same amount of medium was added to the zero hole without cells, the control hole was only supplemented with medium and cells without chemotherapy, and the marginal hole was filled with aseptic PBS. The 96-well plate was put into the cell culture box with 5% CO_2 at 37°C to incubate overnight to make the cells adhere to the wall. Dosage: the concentration gradients of various chemotherapeutic drugs are as follows: didecyl phthalate (DDP): 0, 125, 250, 500, 1000, 2000 μg/ml, Paclitaxel: 0, 6. 25, 12. 5, 25, 50, 100 μg/ml, 5 Fluorouracil (5-Fu) : 0, 1, 2, 4, 8, 16 μg/ml. All kinds of chemotherapeutic drugs were diluted to the required concentration in Eagle's Minimum Essential Medium (EMEM) complete medium without antibiotics. Old culture medium was gently absorbed, the fresh medium containing chemotherapeutic drugs was added, which was put into the cell culture box with 5% CO_2 at 37°C to be incubated for 48 h. Color: after incubated for 48 h with drugs, 96-well plate was taken out, 20 μl MTT solution (5 mg/ml) was added to each well, and incubated in a cell culture box for 4 h. Under microscope, blue and purple crystals were formed in the pores, then the inoculum was carefully absorbed and discarded. Dimethyl sulfoxide (DMSO) 200 l were added to each hole, and then oscillated at low speed in the shaking bed for 5 min. The crystallization was fully dissolved for 10 min (the whole process was operated avoiding light). The detection wavelength was the absorbance value of each hole at 550 nm. The relative survival rate of the cells was drawn with the drug concentration as the transverse coordinate and the relative survival rate of the cells as the longitudinal coordinate. The relative survival rate of cells was calculated according to the following formula: the relative survival rate of cells = (the average absorbance value of adding medicine group – the average absorbance value of zero adjusting hole) / (the average absorbance value of control group – the average absorbance value of zero adjusting hole). The experiment was repeated 3 times.

QRT-PCR to Test the Correlation between GREM1 and Radiotherapy of SiHa Cells

Cell irradiation: the cervical carcinoma SiHa cells in the logarithmic growth phase were digested with conventional pancreatin, 10% fetal bovine serum (FBS)/Dulbecco's Modified Eagle's Medium (DMEM)/MEM was used to stop the digestion, and the 6-well plate was inoculated with 3×10^3 cells per well, and 0,4 and 8 Gy of radiotherapy were given after 16 h. Irradiation condition: the dose rate was 200 cGY/min, the single absorption dose was set according to the experimental setting (0-8 Gy), the distance of source skin was 100 cm, the irradiation field was 10 cm × 10 cm, and the plexiglass group was compensated. Real-time Fluorescence Quantitative PCR was used to detect the mRNA expression of GREM1 in SiHa cells irradiated with different doses. The protein expression of GREM1 was tested by Western blot.

Statistical Analysis

The statistical analysis of all the experimental data was carried out by using GraphPad Prism software (San Diego, CA, USA), and the rate or percentage of counting data was expressed, and the analysis was carried out by χ^2-test. The measurement data were expressed by ($\bar{x} \pm s$), the matched sample *t*-test was used for the comparison of cancer tissues and paracancerous tissues, the independent sample *t*-test was used for the comparison between the other two groups, and the single factor variance analysis (One-Way ANOVA) was used for the comparison of multiple groups of data. When $p<0.05$, it had a statistically significant difference.

Results

Expression of GREM1 in Cervical Carcinoma Tissue

The results of qRT-PCR showed that the expression levels of GREM1mRNA in cervical carcinoma tissues and paracancerous cervical tissues were 2.005 ±0.153 and 0.980±0.038, respectively. The expression levels of GREM1mRNA in cervical carcinoma tissues were significantly hi-

Table II. Expression of GREM1 in cervical carcinoma tissue.

Group	Cases	Cancer tissue	Paracancerous tissue	*p*-value	*t*-value
GREM1mRNA	72	2.005±0.153	0.980±0.038	0.000	54.343
GREM1 Protein	72	0.960±0.115	0.333±0.088	0.000	36.049

gher than those in paracancerous cervical tissues (t = 54.343, p<0.001). The results of Western blot showed that the expression levels of protein in cervical carcinoma tissues and paracancerous cervical tissues were 0.960±0.115 and 0.333±0.088, respectively, which were significantly higher than those in paracancerous cervical tissues (t = 36.049, p<0.001; Table II).

Further analysis of the relationship between GREM1 mRNA expression and clinicopathological features in cervical carcinoma is shown in Table III: there was no significant relationship between the expression level of GREM1 mRNA and age in cervical carcinoma (p>0.05). In cervical carcinoma, the expression level of GREM1 mRNA in patients with FIGO phasing lower than Phase II was significantly lower than that in patients with Phase II or more (p<0.05). The expression level of GREM1 in patients without lymph node metastasis was significantly lower than that in patients with lymph node metastasis (p<0.05). The expression level of GREM1 in patients without vascular infiltration was significantly lower than that in patients with vascular infiltration (p<0.05). The expression level of GREM1 in patients with high differentiation was significantly lower than that in patients with low differentiation (p<0.05). The expression level of GREM1 in patients with tumor size ≤4 cm was significantly lower than that in patients with tumor size > 4 cm (p<0.05). The results showed that GREM1 may be involved in the occurrence, development, and metastasis of cervical carcinoma, which is related to the worse prognosis (Figure 1).

Expression of GREM1 in Siha Cells of Cervical Squamous Carcinoma

The expression of GREM1 mRNA in cervical carcinoma cells and normal cervical epithelial cells was tested by qRT-PCR. The expression of GREM1 protein in cervical carcinoma cells and normal cervical epithelial cells was tested by Western blot (Figure 2C). QRT-PCR test results (Figure 2A) and the expression level of GREM1 tested (Figure 2) showed that: the expressions of GREM1 mRNA and protein in SiHa cell line of

Table III. Relationship between GREM1 mRNA expression and clinicopathological features.

Clinicopathological factors	Cases	GREM1mRNA level	p-value
Age			
≤40	32	2.007±0.150	0.928
>40	40	2.003±0.158	
Phase			
<Phase II	39	1.963±0.161	0.010
≥Phase II	33	2.054±0.130	
Lymphatic metastasis			
No	44	1.976±0.169	0.030
Yes	28	2.050±0.113	
Vascular infiltration			
No	37	1.954±0.162	0.003
Yes	35	2.059±0.124	
Differentiation degree			
Poorly differentiated	24	2.064±0.161	0.000
Moderately differentiated	26	2.051±0.132	
Well-differentiated	22	1.885±0.094	
Size of tumor (cm)			
≤4	39	1.953±0.135	0.001
>4	33	2.066±0.153	

Note: The data were expressed as ($\bar{x}$±s), and the difference between groups was analyzed by t-test. One-way ANOVA was used for multi-group data analysis.

cervical carcinoma were significantly higher than those in normal cervical epithelial cell line HCerEPic. The difference was of statistical significance ($p<0.05$).

Effect of GREM1 on Chemotherapy Sensitivity of SiHa Cells

The effect of GREM1 on chemotherapy sensitivity of SiHa cells was tested by MTT. After treated with DDP, paclitaxel and 5-Fu, respectively, the relative survival rate of cells decreased with the increase of drug dose. The relative cell survival rate of interfering GREM1 group (siRNA GREM1) was significantly lower than that of blank control group (NC group) and empty vector group (Vector) ($p<0.05$). The relative survival rate of GREM1 overexpression group (GREM1 group) was significantly higher than that of blank control group (NC group) and empty vector group (Vector). When GREM1 was down-regulated, the relative survival rate of cells in the GREM1 group was significantly decreased. When GREM1 was overexpressed, the relative survival rate of cells in GREM1 group was significantly increased (Figure 3).

Correlation between GREM1 and Radiotherapy Sensitivity of SiHa Cells

After SiHa cells were irradiated with different doses of high energy X-ray, the protein expression of GREM1 and the level of mRNA in SiHa cells decreased in a dose-dependent manner with the increase of irradiation dose (Figure 4).

Discussion

GREM1 is an antagonist of bone morphogenetic protein and a glycoprotein containing highly conserved 184-AA cysteine domain. It belongs to the secretory glycoprotein family[10,11].

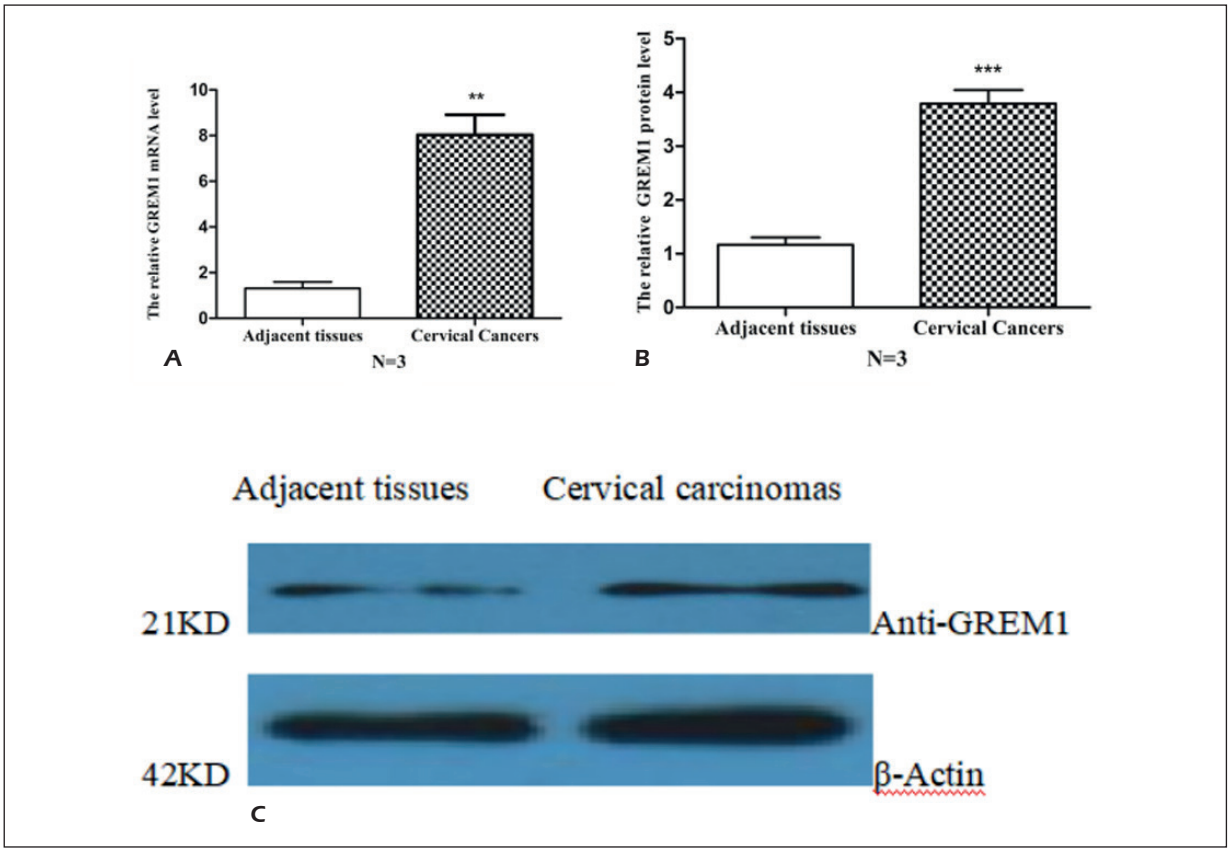

Figure 1. Expression of GREM1 in Paracancerous Cervical Tissues and Cervical Squamous Carcinoma (N = 72 Cases). Note: **A**, Expression histogram of GREM1 mRNA tested by RT-PCR, normalized to adjacent tissues. **B**, Each related protein expression histogram tested by Western blot, normalized to β-actin. **C**, Electrophoresis band diagram of GREM1 protein expression tested by Western blot, **expressed that compared with paracancerous tissues, $p<0.01$, and ***expressed that compared with paracancerous tissues, $p<0.001$. The data were expressed as ($\bar{x}\pm s$), and *t*-test of matched samples was used in the analysis.

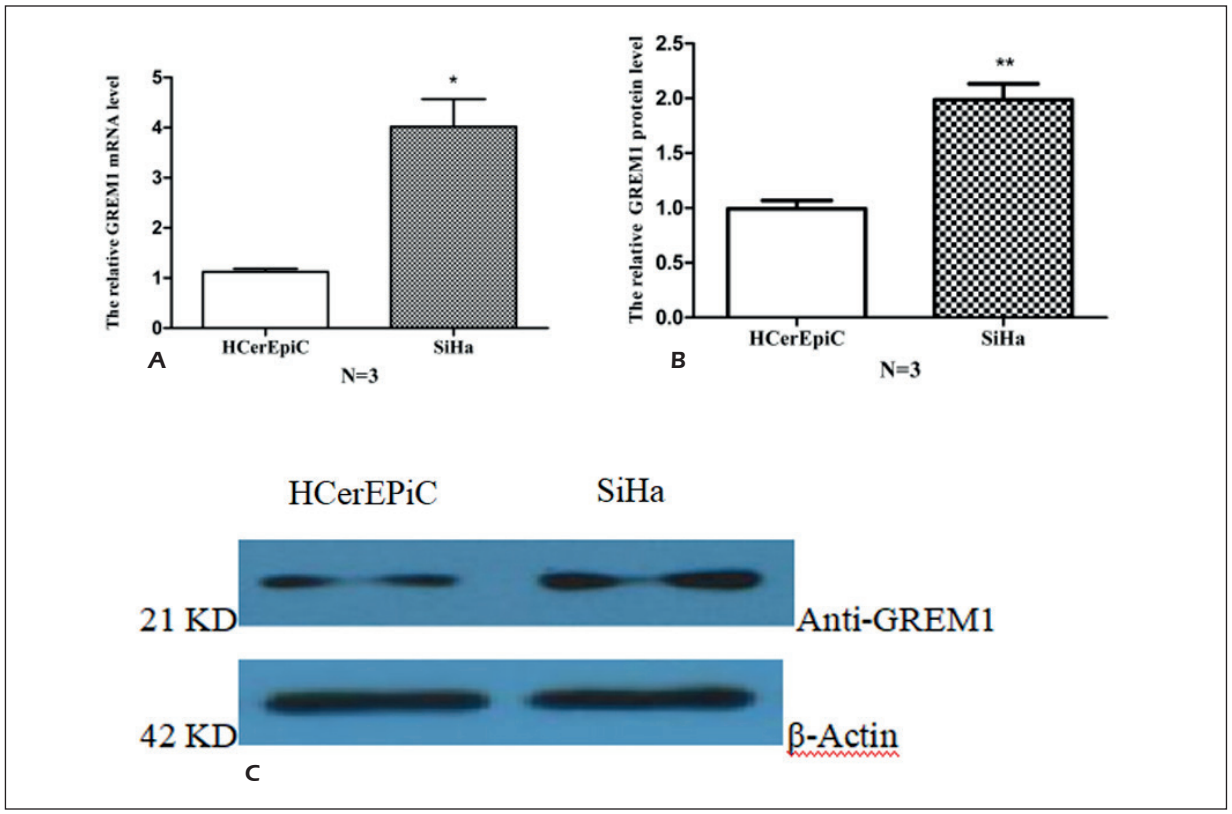

Figure 2. Expression of GREM1 in cervical SiHa cell line. Note: **A**, Expression histogram of GREM1 mRNA tested by RT-PCR, normalized to HCerEpiC. **B**, Each related protein expression histogram tested by Western blot, normalized to β-actin. **C**, Electrophoresis band diagram of GREM1 protein expression tested by Western blot, *expressed that compared with paracancerous tissues, $p<0.05$, and **expressed that compared with paracancerous tissues, $p<0.01$. The data were expressed as ($\bar{x} \pm s$), and t-test of matched samples was used in the analysis.

Namkoong et al[12] have shown that GREM1 may be a key molecule in the development of human tumorigenesis. The results showed that the high expression of GREM1 in cervical carcinoma was significantly correlated with pathological stage, lymph node metastasis, vascular invasion, tumor size and differentiation, suggesting that GREM1 was not only involved in the occurrence and development of cervical carcinoma, but also its high expression may predict a worse prognosis. With the application of HPV vaccine and the improvement of cervical carcinoma screening level, the incidence of cervical carcinoma in developed countries has a downward trend, but in developing countries, the incidence and mortality of cervical carcinoma are still on the rise[13], and the incidence and mortality of cervical carcinoma are more common in locally advanced patients[14]. NCCN guidelines recommend DDP combined with synchronous radiotherapy as the main treatment for locally advanced cervical carcinoma[4]. However, with the continuous progress of radiotherapy technology, there are still about 25-40% of the patients who recur after treatment[15] after simultaneous radiotherapy and chemotherapy. The main reason is that this part of patients is resistant to radiotherapy and chemotherapy. Scholars[16-19] have shown that cisplatin, paclitaxel, and 5-Fu have better chemotherapeutic effects on cervical carcinoma. Therefore, cisplatin, 5 Fu, and paclitaxel are commonly used therapeutic agents recommended in NCCN guidelines for cervical carcinoma[4]. Studies showed that P-glycoprotein (P-gp), Glutathione S-transferase-pi (GST-1), and DNA topoisomerase II (TOPO II) were found to be involved in the chemotherapy resistance of cervical carcinoma[20]. It has been found that the overexpression of OTUBl gene can inhibit the proliferation of HeLa cells and promote the apoptosis of HeLa cells, thus increasing the sensitivity of cervical carcinoma Hela cells to DDP chemotherapy[21]. In our study, the effect of GREM1 on chemotherapy sensitivity of SiHa cells was tested by MTT. After treated with different concentrations of DDP, paclitaxel and 5-Fu, it was found that the survival rate of the three groups decreased with the increase of drug dose.

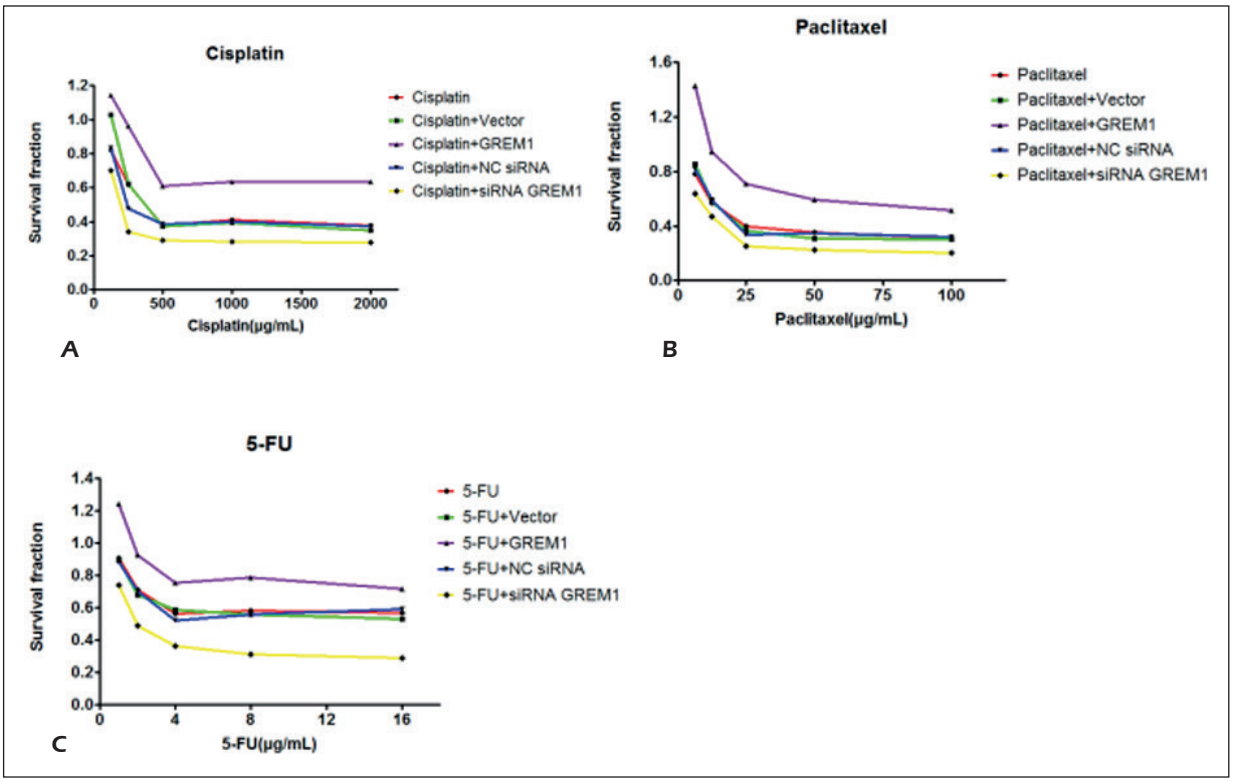

Figure 3. Effect of GREM1 on chemotherapy sensitivity of SiHa cell line. Note: **A**, Down-regulating GREM1 can increase the sensitivity of SiHa cells to DDP, while over-expressing GREM1 will decrease the sensitivity of SiHa cells to DDP. **B**, Down-regulating GREM1 can increase the sensitivity of SiHa cells to paclitaxel, while over-expressing GREM1 will decrease the sensitivity of SiHa cells to paclitaxel. **C**, Down-regulating GREM1 can increase the sensitivity of SiHa cells to 5-Fu, while over-expressing GREM1 will decrease the sensitivity of SiHa cells to 5-Fu.

The relative cell survival rate of interference group (siRNA GREM1 group) was significantly lower than that of empty vector group and control group (Vector group and NC group) ($p<0.05$; Figure 3). The relative survival rate of overexpressed cells was significantly higher than that of transfected empty vector group and control group (Vector group and NC group) ($p<0.05$; Figure 3). The results showed that down-regulation of the GREM1 expression significantly increased the chemosensitivity of SiHa cells to DDP, paclitaxel, and 5-Fu. On the contrary, the overexpression of GREM1 decreased the chemosensitivity of SiHa cells to DDP, paclitaxel, and 5-Fu. Therefore, GREM1 may be an important factor in the control of cervical carcinoma, the effect of the tumor cells on the radiotherapy and chemotherapy has cross-tolerance[22]. Therefore, to observe the effect of GREM1 on the radiotherapy response of SiHa cells, SiHa cells were irradiated with different doses of high energy X-ray, and the expression of GREM1 in SiHa cells was detected by Western blot and qRT-PCR, respectively. The results showed that the expression of GREM1 protein and the level of mRNA in the SiHa cells decreased in a dose-dependent manner with the increase of the irradiation dose (Figure 4) .Our experimental results showed that, GREM1 was correlated with SiHa cell radiotherapy response, and it was likely to be one of the main regulators of radiotherapy sensitivity of cervical carcinoma. The specific mechanism will be the focus of our next research work.

Conclusions

GREM1 may be involved in the occurrence and development of cervical carcinoma and may be an important factor in the regulation of radiotherapy and chemotherapy sensitivity of cervical carcinoma. It is likely to be the key molecule to judge the prognosis of cervical carcinoma and improve the therapeutic effect of cervical carcinoma, and to provide a new therapeutic target and predictive index for the diagnosis and treatment and prognosis of cervical carcinoma.

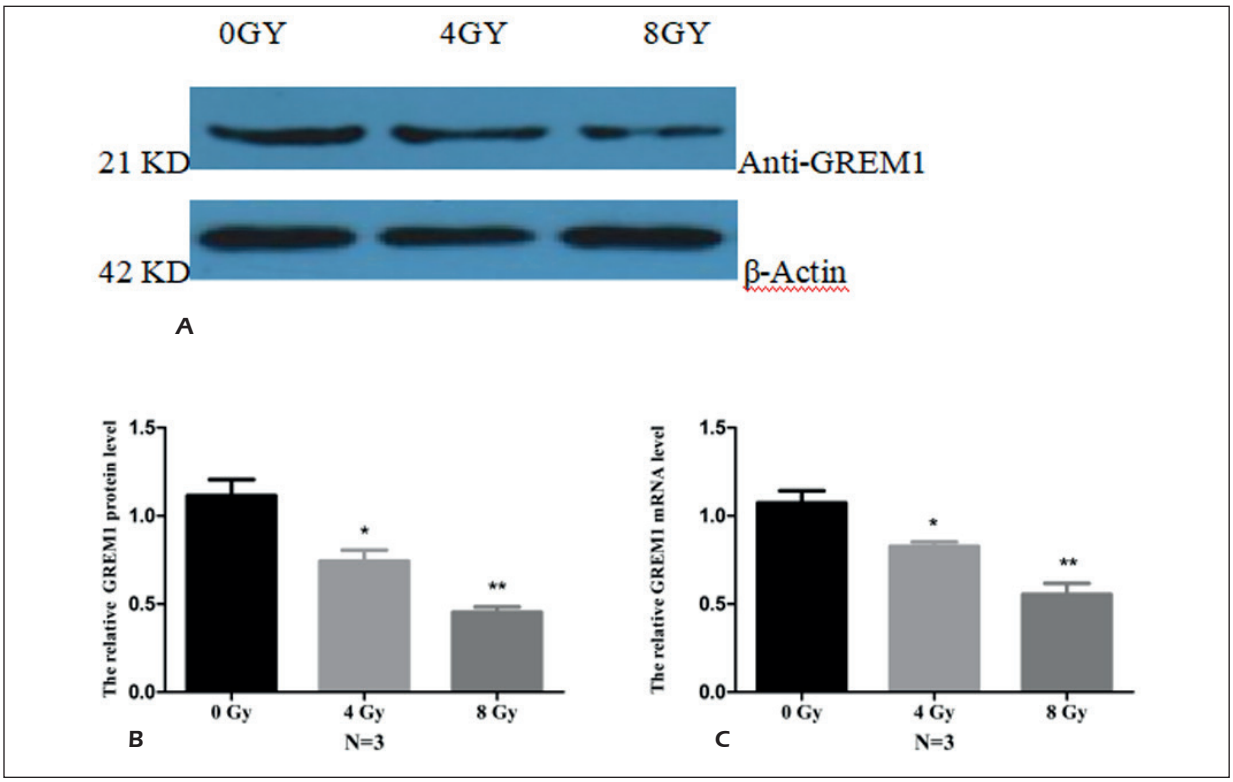

Figure 4. Effect of Radiotherapy on GREM 1 Expression in SiHa Cell Line. Note: **A**, Electrophoresis band diagram of GREM1 protein expression in SiHa cells after different radiotherapy doses tested by Western blot. **B**, Expression histogram of GREM1 protein in SiHa cells after different radiotherapy doses tested by Western blot. **C**, Expression histogram of GREM1 mRNA in SiHa cells after different radiotherapy doses tested by qRT-PCR. *expressed as a comparison with an unirradiated cell line, $p<0.05$, **expressed as a comparison with an unirradiated cell line, $p<0.01$. The data were expressed as ($\bar{x}\pm s$), and t-test of matched samples was used in the analysis.

Conflict of Interests

The authors declare that they have no conflict of interests.

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