

Original Article

Immediate early response gene 5 promotes irradiation combined with cisplatin-induced apoptosis in HeLa cells

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Received November 2, 2017; Accepted November 21, 2017; Epub January 1, 2018; Published January 15, 2018

Abstract: The objective of this study was to explore the action and mechanism of immediate early response gene 5 (IER5) on the apoptosis induced by irradiation combined with cisplatin in HeLa cells. The IER5 gene was knocked down using IER5-specific small interfering (si)RNA to generate HeLa cells stably expressing low levels of IER5 (HeLa-siIER5). Apoptosis was induced by ⁶⁰Co γ-radiation, cisplatin, and radiation combined with cisplatin. Apoptosis and cell viability were evaluated by flow cytometry and Cell Counting Kit-8, respectively. Protein expression was determined by western blotting. Apoptosis was significantly inhibited in HeLa-siIER5 cells after γ-radiation combined with cisplatin exposure compared with siRNA control cells ($P < 0.01$). We also found that the expression of Bcl-2 was increased, and the levels of cleaved caspase-9 and cleaved PARP were reduced after γ-radiation combined with cisplatin treatment of HeLa-siIER5 cells. These results indicated that decreased expression of IER5 can reduce apoptosis induced by exposure to γ-radiation combined with cisplatin. Enhancing the expression of IER5 in tumor cells, and reducing its expression in normal cells, may be utilized as targeting strategies to improve the outcomes of chemo/radiotherapy for the treatment of patients with cervical cancer.

Keywords: IER5, cervical cancer, apoptosis, γ-radiation, cisplatin

Introduction

Cervical cancer is the fourth most common cancer among women worldwide [1] and remains a significant public health concern despite the existence of highly effective prevention and screening methods. As a result, cervical cancer causes more than 250,000 deaths per year as a result of grossly deficient treatments in many developing countries.

Primary treatment options for patients with cervical cancer may include surgery, or external beam radiotherapy and brachytherapy. Recent research has focused on systemic regimens that are able to improve survival for patients with persistent, recurrent, or metastatic cervical cancer. Historically, cisplatin has been considered the most active and effective agent for metastatic cervical cancer [2, 3]. A concurrent chemoradiotherapy regimen including cisplatin-based chemotherapy is recommended for patients with medium-term and advanced cer-

vical cancer [4]. How to enhance the sensitivity of cervical cancer to radiotherapy and chemotherapy, and improve the survival rate, is currently the subject of intense research.

Using cDNA microarray technology, immediate early response gene 5 (IER5) was found to be induced by ionizing radiation [5]. IER5, which belongs to the group of immediate early genes, is upregulated in waking and sleep deprivation [6]. It can be induced by growth-promoting activities, oncogenic signals, irradiation, and heat shock [7]. Several studies have confirmed that the IER5 can arrest the cell cycle, suppress proliferation, and accelerate apoptosis to enhance sensitivity to radiation and chemotherapeutics. However, there are no reports about the relationship between IER5 and cisplatin [8].

In this study, apoptosis was induced by irradiation and cisplatin, alone and in combination, in HeLa cells stably expressing decreased levels of IER5. We examined cell viability and the

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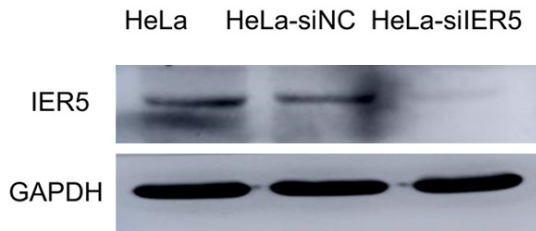


Figure 1. Western blot for IER5 protein expression in different cells.

expression of signal pathway-related proteins to elucidate the mechanism by which IER5 affected chemotherapy of cervical cancer. The results suggest that IER5 may become a new target for cervical cancer radiotherapy and chemotherapy. We hope these findings can provide new insights for the sensitization of cells to pharmacotherapy.

Materials and methods

Cell lines

HeLa cells were obtained from the Fourth Laboratory, Institute of Medical Radiology, the Academy of Military Science of China. Cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) containing 10% fetal calf serum (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Hyclone) at 37°C under 5% CO₂. The IER5 gene was knocked down using IER5-specific small interfering (si)RNA to generate HeLa cells stably expressing low levels of IER5 (HeLa-siIER5). HeLa cells lacking an siIER5 cDNA insert (HeLa-siNC) were used as the control.

Antibodies and key reagents

The antibodies used were: goat polyclonal anti-IER5 (Abcam, Cambridge, UK), rabbit polyclonal anti-IER5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Zhongshan Jinqiao Biotechnology, Beijing, China), anti-Bcl-2, anti-cleaved-poly (ADP-ribose) polymerase (PARP) (Shandong Ruiying Pioneer Pharmaceutical Co., Ltd. China), anti-P53 (Santa Cruz Biotechnology), and anti-cleaved caspase-3 (Zhongshan Jinqiao Biotechnology). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies (Japan), cisplatin was obtained from Qilu Pharmaceutical

(Hainan, China), and the Annexin V-FITC/propidium iodide Apoptosis Detection Kit was from Jiamay Biotechnology (Beijing, China).

Experimental treatments

Exponentially growing HeLa-siIER5 and -siNC cells were plated in 96-well plates at 5×10^3 cells/well (five wells per group) and were irradiated with 4 and 10 Gy ⁶⁰Co γ-rays at a dose rate of 1.98 Gy/min. After a 4 h exposure to radiation, cisplatin was added at 4, 6, or 8 µg/mL.

CCK-8 assay

CCK-8 assays were used to assess the number of viable cells. After 24 h with different treatments, the medium was removed and 10 µL CCK-8 reagent was added to the cells. The cells were incubated for 4 h at 37°C and absorbance values were measured at 450 nm using an enzyme-linked immunosorbent assay reader. All experiments were repeated three times. The percentage change in viability was calculated according to the following formula: inhibition ratio = [(experimental group absorbance - blank group absorbance)/blank group absorbance] × 100.

Flow cytometry analysis

A total of 1.5×10^5 cells/well were seeded in six-well plates and cultured overnight. Cells were treated with γ-radiation, cisplatin, or both. After 24 h of incubation, the cells were collected and washed with cold phosphate-buffered saline. The cells were resuspended in 300 µL 1 × binding buffer. Then, 5 µL Annexin V-FITC reagent were added and the cells were cultured for 15 min at room temperature in the dark. The cells were then dyed with 5 µL propidium iodide 5 min before analysis. Finally, 200 µL of 1 × binding buffer were added to each tube. In total, 8000 events were analyzed using a FACScan™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot

After 24 h of the different treatments, cells were collected in lysis buffer (Thermo Scientific, Rockford, IL, USA). The Bradford assay was used to determine protein concentrations. Equal quantities of protein were separated by sodium dodecyl sulfate-polyacrylamide gel el-

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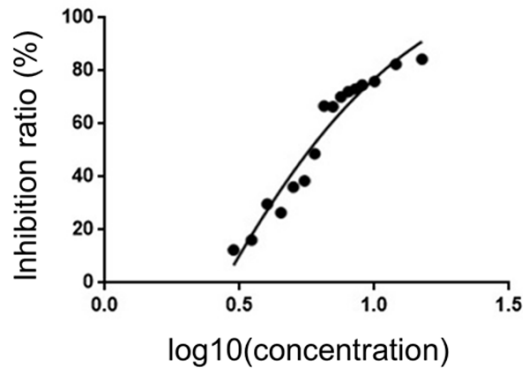


Figure 2. Percentage viability of HeLa-siNC cells treated with cisplatin for 24 h. The IC_{50} value was 6 $\mu\text{g}/\text{mL}$.

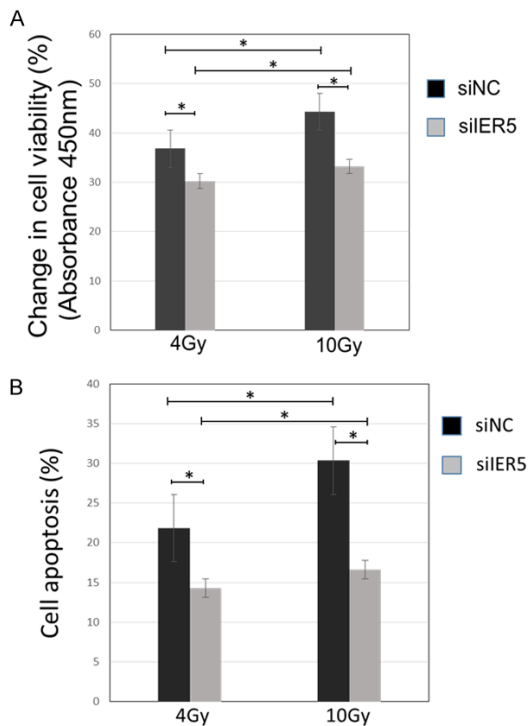


Figure 3. The relationship between IER5 expression and irradiation. A. Nonviable percentages of HeLa-siIER5 and -siNC cells exposed to 4 or 10 Gy of ^{60}Co γ -radiation. Viability was assessed by the CCK-8 assay. B. Apoptosis percentages of HeLa-siIER5 and -siNC cells exposed to 4 or 10 Gy of ^{60}Co γ -radiation. Apoptosis was assessed by flow cytometry using Annexin V. Data are expressed as means \pm SD of three independent experiments. * $P < 0.01$.

ectrophoresis and transferred to polyvinylidene membranes. After blocking the membranes with 5% milk in Tris-buffered saline-Tween 20, primary antibodies were added and the membranes were incubated overnight at 4°C.

Then, the membranes were labeled by appropriate secondary antibodies for 1 h at room temperature. The membranes were washed with Tris-buffered saline-Tween 20 and immunoreactive bands were visualized using ImageQuant LAS500 (GE Healthcare, Pittsburgh, PA, USA). GAPDH was used as the loading control.

Statistical analysis

Data are expressed as means \pm standard deviation. SPSS 21.0 software (IBM, Chicago, IL, USA) was used for statistical analyses. One-way analysis of variance was used to assess differences in the different experiments. Student's t-test was used to assess differences in HeLa-siNC cells and HeLa-siIER5 cells after the same treatments. $P < 0.05$ was considered statistically significant. Half-maximal-inhibitory concentrations (IC_{50} s) were calculated using GraphPad Prism 6 software (GraphPad, San Diego, CA, USA).

Results

Expression of IER5 protein

The expression of IER5 protein in HeLa cells and HeLa-siNC cells was similar. However, IER5 was barely detectable in HeLa-siIER5 cells (Figure 1). Based on these findings, HeLa-siIER5 and HeLa-siNC cells were used for experimental and control cells, respectively.

IC_{50} value

After HeLa-siNC cells were cultured overnight in 96-well plates (5×10^4 cells/well), the culture medium was removed and replaced by 100 μL complete culture medium containing cisplatin. Final concentrations of cisplatin were 3, 4, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 10, 12, 14 and 15 $\mu\text{g}/\text{mL}$. The percent changes in cell viability were calculated after the CCK-8 assays (Figure 2). The IC_{50} value was 6 $\mu\text{g}/\text{mL}$. Thus, 4, 6, and 8 $\mu\text{g}/\text{mL}$ cisplatin were used to treat cells in further studies.

Decreased expression of IER5 significantly reduced the toxicity of ^{60}Co γ -irradiation for HeLa cells

HeLa-siIER5 and -siNC cells were exposed to ^{60}Co γ -radiation at doses of 4 or 10 Gy, and then cultured for 24 h. For both cells, the percentages of nonviable and apoptotic cells in-

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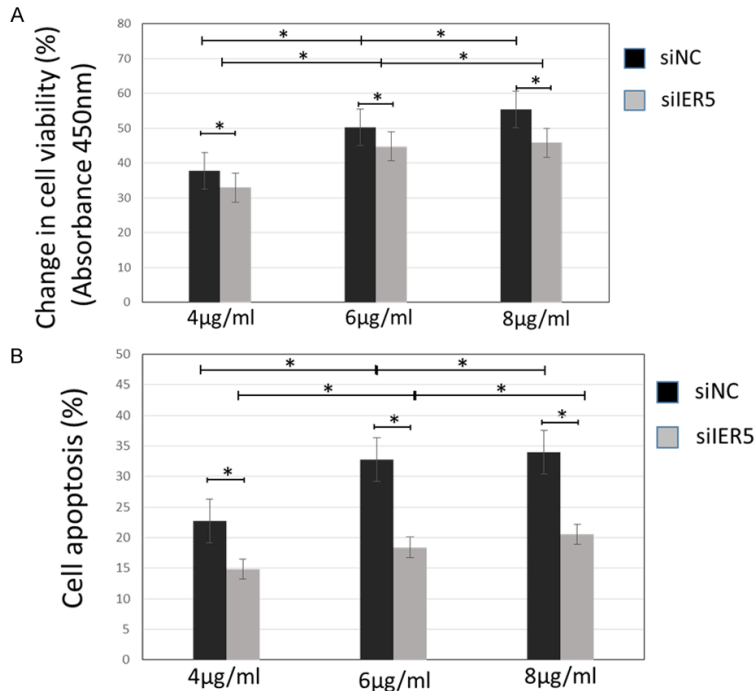


Figure 4. The relationship between IER5 levels and cisplatin toxicity. A. Non-viable percentages of HeLa-siIER5 and -siNC cells treated with 4, 6, or 8 µg/ml cisplatin for 24 h. B. Percentages of apoptotic HeLa-siIER5 and -siNC cells following treatment with 4, 6, or 8 µg/ml cisplatin for 24 h. * $P < 0.01$, ** $P < 0.05$.

creased with the dose of radiation (Figure 3; $P < 0.01$). The percentages of nonviable and apoptotic HeLa-siIER5 cells were significantly lower than HeLa-siNC cells ($P < 0.01$). This indicates that the sensitivity of cells to γ -irradiation was reduced when the cells expressed decreased amounts of IER5.

Decreased expression of IER5 reduced the toxicity of cisplatin for HeLa cells

Different final concentrations of cisplatin (4, 6, and 8 µg/mL) were added to HeLa-siIER5 and -siNC cells. Viability was decreased and apoptosis was increased in both cell types in a concentration-dependent manner ($P < 0.01$). The numbers of nonviable and apoptotic HeLa-siIER5 cells were reduced significantly compared with HeLa-siNC cells ($P < 0.01$). This indicates that sensitivity to cisplatin was decreased when the cells lacked IER5 (Figure 4).

Decreased expression of IER5 reduced the toxicity of cisplatin combined with irradiation

HeLa-siIER5 and -siNC cells were irradiated with 4 or 10 GY of ^{60}Co γ -rays. After a 4 h expo-

sure to radiation, cisplatin was added at different concentrations. The results showed that the decreases in cell viability and apoptosis induced by cisplatin and radiation, each alone and in combination, were highest in siNC cells (Table 1, $P < 0.01$). This indicates that IER5 protects against the toxicity of cisplatin and irradiation. Cell inhibition and apoptosis of both cell lines were higher in radiation combined with cisplatin than radiation or cisplatin treatment alone ($P < 0.01$).

IER5 augmented radiation- and cisplatin-induced apoptosis by decreasing Bcl-2 and increasing p53 and the cleavage of PARP and caspase-3

The expression of several common markers in the apoptosis signaling pathway was determined by western blot to illuminate the mechanism by which

IER5 affected apoptosis. The blots shows that the expression of P53, cleaved caspase-3, and cleaved PARP was significantly increased in HeLa-siIER5 and -siNC cells after irradiation, cisplatin, or both treatments. In contrast, the expression of Bcl-2 was decreased. These changes were more obvious at higher doses of irradiation and cisplatin. The degree of changes in HeLa-siIER5 cells was lower than HeLa-siNC cells (Figure 5).

Discussion

Concomitant chemotherapy and radiotherapy improves overall and progression-free survival, and reduces local and distant recurrences in patients with cervical cancer [9]. Radiosensitivity and chemosensitivity are important prognostic factors for this disease. Therefore, accurately predicting sensitivity is of essential importance for treating cervical cancer patients. Cisplatin resistance and radioresistance are relevant to the mechanism of DNA lesions and repair, and apoptosis [10]. Although various techniques are used for studying radiosensitivity and chemosensitivity, the molecular

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Table 1. Viability and apoptosis in HeLa-siER5 and -siNC cells after different treatments

Treatment	Nonviable cells (%)		Apoptotic cells (%)	
	siNC	siER5	siNC	siER5
4 Gy	36.80 ± 1.13	30.20 ± 1.70	21.84 ± 0.52	14.27 ± 1.29
10 Gy	44.30 ± 0.49	33.20 ± 1.71	30.34 ± 2.24	16.59 ± 0.78
4 µg/mL cisplatin	37.80 ± 1.20	32.90 ± 1.27	22.70 ± 0.19	14.87 ± 0.09
6 µg/mL cisplatin	50.20 ± 3.39	44.70 ± 1.48	32.74 ± 0.52	18.42 ± 1.97
8 µg/mL cisplatin	55.40 ± 4.67	45.80 ± 0.92	34.02 ± 0.72	20.54 ± 0.33
4 Gy + 4 µg/mL cisplatin	48.10 ± 3.89	34.50 ± 1.70*	31.81 ± 4.11	18.96 ± 1.52*
4 Gy + 6 µg/mL cisplatin	62.40 ± 0.92	46.70 ± 1.27*	39.93 ± 3.33	28.81 ± 0.79*
4 Gy + 8 µg/mL cisplatin	64.80 ± 0.85	50.20 ± 0.92*	44.70 ± 2.04	30.64 ± 1.16*
10 Gy + 4 µg/mL cisplatin	65.30 ± 1.31	47.60 ± 1.34*	47.18 ± 2.76	32.43 ± 0.90*
10 Gy + 6 µg/mL cisplatin	78.30 ± 0.78	60.50 ± 1.31*	57.22 ± 1.22	36.54 ± 0.88*
10 Gy + 8 µg/mL cisplatin	79.60 ± 0.49	62.40 ± 2.33*	58.89 ± 1.97	38.88 ± 0.98*

Data are presented as means ± standard deviations from three independent experiments. **P* < 0.01 vs. siNC.

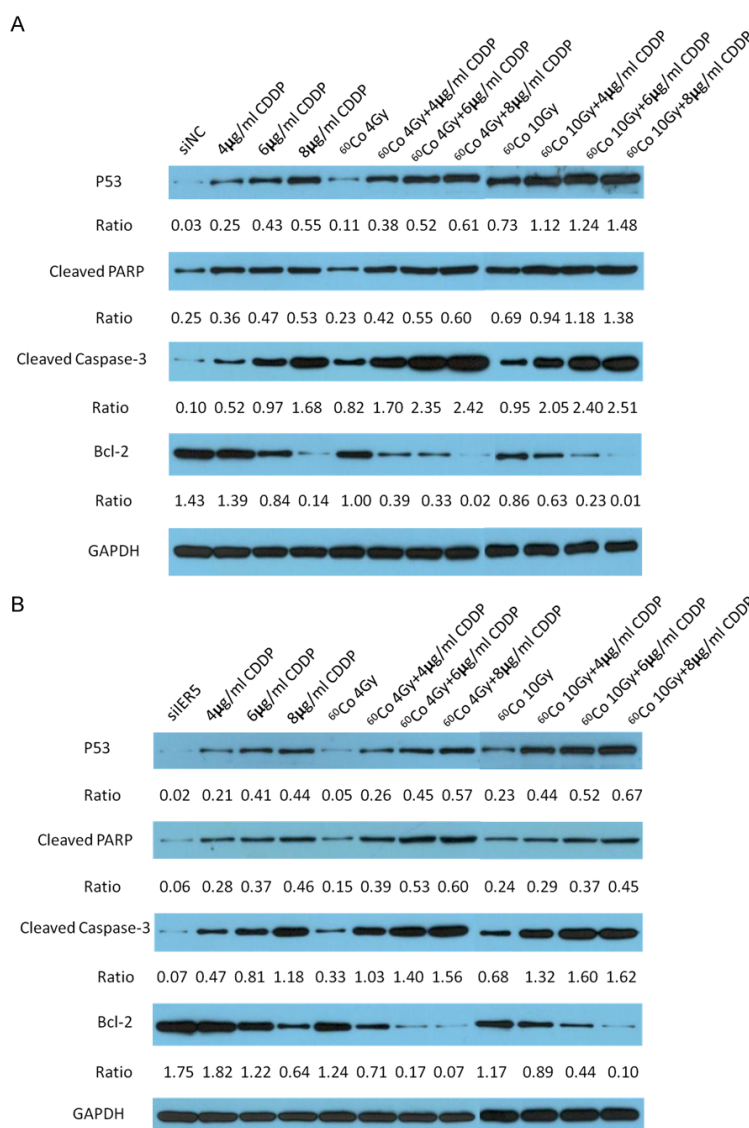


Figure 5. The expression of apoptosis markers in HeLa-siNC and -siER5 cells. P53, cleaved caspase-3, cleaved PARP, and Bcl-2 protein levels were measured by western blots in (A) HeLa-siNC and (B) HeLa-siER5 cells treated with 4, 6, or 8 µg/mL cisplatin with or without 4 or 10 Gy ⁶⁰Co irradiation. HeLa-siNC cells without any treatment were used as the control. GAPDH protein expression is shown as the internal control.

mechanisms responsible for sensitivity are still not clearly understood.

The IER5 gene belongs to the slow-kinetics immediate-early gene family [11]. Several studies confirmed that it is one of the genes upregulated in response to radiation. It is a time- and dose-dependent gene that could modulate cell cycle checkpoints, leading to apoptosis and decreased cancer cell survival in response to radiation [8, 12-15]. Long et al., using microarray analysis, identified a series of radiation-inducible genes, including IER5, in human lymphoblastoid AHH-1 cells at 4 h after γ -irradiation at doses from 0.02 to 10 Gy [16, 17]. Li et al.

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observed that 4 Gy was the best ionizing radiation dose for experiments on cervical cancer cells because both transcription and translation were increased markedly [18].

We previously examined cervical cancer patients at stage IIb-IIIb who received a cisplatin-concurrent chemoradiotherapy regime. There was a significantly positive association between IER5 expression and the radiation dose. That study also showed a strong correlation between the expression of IER5 and the reduction in tumor size induced by irradiation; i.e., the smaller the cervical cancer tumor, the greater variation of IER5 expression. This suggested that IER5 could be a promising predictive biomarker for the responses of cervical cancer patients receiving concurrent chemoradiotherapy [19].

Yang et al. found that overexpression of IER5 activated and prompted both radiation- and cisplatin-induced apoptosis, and inhibited the growth of hepatocellular carcinoma cells [20]. Nevertheless, the relationship between IER5 and cisplatin in HeLa cells has not been verified. In agreement with previous studies, we found that suppression of IER5 reduced cell apoptosis and increased both radioresistance and cisplatin resistance. This tendency was significant in experimental groups (radiation with cisplatin) and dose-dependent. The results demonstrated that radiation combined with cisplatin was a more effective way to inhibit HeLa cell growth. Thus, downregulation of IER5 in normal cells could be used to prevent adverse responses to treatment with radiation and cisplatin.

Caspases are major proteases involved in apoptosis. Caspase family members contribute to cellular disintegration via cleavage of proteins involved in many cell processes, such as DNA repair and checkpoint activation. Caspase-3 is a main protease that controls other caspase members during programmed cell death. Impaired apoptosis is a crucial step in the process of cancer development [21-23]. In contrast, overexpression of BCL-2 protein family members that block apoptosis contributes to malignant transformation [24]. This was first recognized as the mechanism of action of the recurrent t(14;18) translocation in follicular lymphoma [25].

In the present study, the expression of Bcl-2 was significantly lower in the treated groups

compared to the control. By contrast, the expression of p53, cleaved caspase-3, and cleaved PARP were significantly higher. Importantly, the changes were less in cells with downregulated IER5 expression. Therefore, it is possible that IER5 regulates oncogenes and several factors in the apoptosis pathway such as caspase-3, PARP, P53, and Bcl-2.

In conclusion, this pilot study found that decreased expression of IER5 increased radioresistance and cisplatin resistance, especially in groups treated with radiation combined with cisplatin. Based on these findings, we speculate that IER5 might become a new target for cervical cancer therapy. It might also give us the opportunity to modify irradiation and cisplatin responses with pharmaceutical agents to increase the radiation and chemotherapeutic drug resistance of normal tissues and decrease the resistance of tumor cells. Meanwhile, a better understanding of radiation- or chemotherapy-induced processes is still urgently required.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (Grant No. 81272888) and the Beijing Obstetrics and Gynecology Hospital Specialized Youth Foundation Project (Grant No. FCY-QN-201604) and Beijing Municipal Administration of Hospitals Clinical Medicine Development of Special Funding Support (Grant No. ZYLX201705).

Disclosure of conflict of interest

None.

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