Radiation-induced expression of *IER5* is dose-dependent and not associated with the clinical outcomes of radiotherapy in cervical cancer

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Abstract. This study aimed to investigate the expression of the immediate-early response 5 (*IER5*) gene in cervical cancer tissues and explore the association between the expression of *IER5* and the clinical outcomes of radiotherapy. We collected specimens by surgery or biopsy and obtained 53 specimens from tissues after radiotherapy and 16 specimens from tissues before radiotherapy. Immunohistochemistry and western blotting were used to assess the protein expression levels of *IER5*. Quantitative polymerase chain reaction (qPCR) was performed to assess the mRNA expression levels of *IER5*. The protein and mRNA expression levels of *IER5* in cervical cancer patients treated with radiation doses ≥20 Gy were significantly higher than in those treated with radiation doses <20 Gy (P<0.05) and before treatment with radiotherapy. Moreover, the expression of *IER5* was significantly positively correlated with the radiation dose (immunohistochemistry: r=0.548, P=0.019; qPCR: r=0.671, P=0.002; western blotting: r=0.573, P<0.0001). Radiotherapy induced the upregulated expression of *IER5* and this was dependent on the radiation dose. However, the radiation-induced expression of *IER5* was not associated with the clinical outcomes of radiotherapy in cervical cancer.

Introduction

Cervical cancer, which is associated with sexual, bowel and bladder function, is caused by the infection of the human papillomavirus in 99.8% of cases (1). It is the leading malignancy among females and a common cause of mortality among middle-aged females (2,3). Statistics have revealed that in 2008, the incidence and mortality rates were 9.0% and 3.2% in more developed areas, and 17.8% and 9.8% in less developed areas, respectively (4). The estimated numbers of new cases and mortalities, which have been increasing in recent years, reached 12,360 and 4,020, respectively, in 2014 in the United States (5-7). In view of these figures, it is essential to the health of females to improve the efficacy of treatment for cervical cancer.

At present, radiotherapy continues to be the cornerstone in the treatment of cervical cancer (8,9). However, the radiosensitivity of cervical cancer cells, which is associated with genetic factors, restricts the efficacy of radiotherapy (10). Thus, it is essential to investigate the mechanism of radiosensitivity of cervical cancer cells in order to improve the efficacy of radiotherapy. It was reported that radiotherapy-induced expression of encoding immediate-early response 5 gene (*IER5*) affected the radiosensitivity of HeLa cells by disturbing radiation-induced cell cycle checkpoints (11,12). In addition, it was reported that the radiation-induced expression of *IER5* in human lymphoblastoid cells was dose-dependent (13,14). Thus, we speculated that the expression of *IER5* might be also induced by radiotherapy, and then correlated the efficacy of radiotherapy through its influence on radiosensitivity in cervical cancer cells.

However, no studies exist concerning the correlation between radiotherapy-induced expression of *IER5* and the efficacy of radiotherapy in cervical cancer. Thus, we investigated the expression of *IER5* in cervical cancer patients treated with various radiation doses to explore the association between the expression of *IER5* and radiotherapy. In addition, the correlation between the expression of *IER5* and clinical outcomes of radiotherapy was also analyzed. These investigations are likely to provide a new direction for assessing the improvement and predicting the clinical outcomes of radiotherapy in treating cervical cancer.

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Materials and methods

Patients and treatment. A total of 53 cervical cancer patients aged between 47 and 68 years old (average, 58.3±3.2) and

treated in the Department of Gynecology, Liaoning Cancer Hospital and Institute, China, between October 2011 and July 2013 were included in this study. The inclusion criteria were: i) patients were first diagnosed and treated; ii) patients were diagnosed with cervical squamous cell carcinoma by biopsy; iii) patients were in clinical stage II-III of cervical cancer based on the International Federation of Gynecology and Obstetrics (FIGO) cancer staging system (15); iv) the results of complete blood count (CBC), urinalysis, electrocardiogram, and liver and kidney function tests were normal; v) there were no contraindications to radiotherapy in the patients. The exclusion criteria were: i) patients had bone marrow suppression with white blood cells less than $3x10^9/l^{-1}$ and platelets less than $7x10^9/l^{-1}$ in the CBC examination; ii) patients had complications due to other critical diseases, including serious cardiovascular and cerebrovascular diseases, acute hepatitis and uremia; iii) acute or subacute pelvic inflammatory disease was not under control.

All the included patients were treated with pelvic external irradiation by a 10-MV X-ray at a dose of 180-200 centigrays (cGy) once a day and five times a week. The treatment was continued until all tumors had fully regressed. For all patients, the maximum cumulative dose of radiation was 50 Gy.

The study was approved by the Liaoning Provincial Tumor Hospital Ethics Committee and all included patients provided their informed consent.

Specimen collection. The 3-5 mm^3 cervical cancer tissues of patients were obtained by surgery or biopsy before and after radiotherapy. Each fresh tissue was immediately placed into an Eppendorf tube (RNase-free; Eppendorf, Hamburg, Germany) and preserved in liquid nitrogen. As a result, a total of 53 specimens from tissues after radiotherapy and 16 specimens from tissues before radiotherapy were obtained. According to the cumulative dose of radiation, the specimens were randomly divided into three groups: the 0 Gy group (16 specimens which were obtained from tissues before radiotherapy), the <20 Gy group (20 specimens) and the ≥20 Gy group (33 specimens).

Immunohistochemistry. The immunohistochemical staining was performed using a SuperPolymer rabbit and mouse horseradish peroxidase (HRP) kit (CoWin Bioscience Co., Ltd., Beijing, China). Firstly, 4-μm paraffin-embedded sections were deparaffinized with xylene and dehydrated in alcohol. The sections were then subjected to microwave antigen retrieval in 10 mM sodium citrate buffer at pH 6 (CoWin Bioscience Co., Ltd.) for 10 min. After cooling for 20 min and washing in phosphate-buffered saline (PBS), these sections were immersed in methanol with 0.3% hydrogen peroxide for 10 min to inactivate endogenous peroxidase activity, followed by normal horse and goat serum for 30 min to block non-specific reactions. Secondly, these sections were incubated in primary antibody solution (1:20) for 60 min. After washing with PBS, they were incubated with secondary antibody solution for 10 min at room temperature. Then sections were rewashed with PBS and incubated with streptavidin-HRP solution for 10 min at room temperature. Finally, sections were stained with 3,3-diaminobenzidine solution and counterstained with hematoxylin (CoWin Bioscience Co., Ltd.). In addition, the PBS instead of the primary antibody solution was considered as the negative control. Color images of immunohistochemically stained sections were captured with a microscopic imaging system (Leica Q500MC; Leica, Cambridge, UK). The shade of positive tissue staining was observed, with brown color representing positive expression of *IER5* protein. The optical density (OD) value of positive tissue staining was measured and analyzed using an Alpha Imager 2000 (Alpha Innotech Corp., CA, USA).

RNA extraction and reverse transcription. Frozen samples were thawed, and then total RNA was extracted using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). The quantity and quality of RNA were analyzed by NanoDrop (NanoDrop Technologies, Wilmington, DE, USA) using 260/280 nm and gel analysis. Moreover, pretreatment of the RNA samples with RNAse-free DNAse was conducted to avoid genetic DNA causing false positive amplifications (16). Complementary DNA (cDNA) was synthesized from RNA using a reverse transcription kit (Toyobo Biotech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The reaction conditions were 37˚C for 15 min, followed by 50˚C for 5 min and 98˚C for 5 min.

Quantitative polymerase chain reaction (qPCR). qPCR was performed using a real-time PCR instrument (Bio-Rad Laboratories, Hercules, CA, USA) and data were analyzed by MJ Opticon Monitor software (Bio-Rad Laboratories). *IER5* and *β-actin* were used as shown in Table I. The reactions were carried out in a volume of 20 μl containing 9 μl 2.5X Real Master mix/20X SYBR solution, 4 μl each primer, 0.33 μl cDNA template and 6.77 μl nuclease-free water. The amplification of cDNA was started with an initial denaturation step at 95˚C for 1 min, then 40 consecutive cycles of the following series of steps were performed: denaturation at 95˚C for 30 sec, annealing at 63˚C for 45 sec and extension at 68˚C for 45 sec. Results were collected and analyzed with MJ Opticon Monitor analysis software. The comparative Ct method (ΔΔCt) was used for quantification of gene expression. The relative expression was calculated as $2^{-\Delta\Delta Ct}$ according to the Perkin Elmer Instruction Manual (17), where ΔΔCt = [Ct (*IER5*) - Ct (*β-actin*)] - [Ct (*IER5*, calibrator) - Ct (*β-actin*, calibrator)].

Western blotting. Frozen tissue samples were pulverized under liquid nitrogen using a mortar and pestle immersed in liquid nitrogen. Then tissue cells were lysed on ice by using protein lysates (CoWin Bioscience Co., Ltd.) and phenylmethylsulfonyl fluoride (CoWin Bioscience Co., Ltd.). Cell disruption was performed in an ice bath using an ultrasonic processor (Q700 Sonicator; Qsonica, LLC, Newtown, CT, USA) for 5 min. The proteins were released after cell disruption. Protein content was quantitated using a bicinchoninic acid protein assay kit (Tiangen Biotech Co., Ltd., Beijing, China). Western blotting was performed as follows. Firstly, proteins were heated for 2 min in a boiling water bath prior to loading on a sodium dodecyl sulfate (SDS) polyacrylamide gel (12%), and then electrophoretically transferred to a nitrocellulose membrane. After blocking, membranes were incubated overnight at 4˚C with goat polyclonal anti-IER5 (1:500; Abcam Inc., Cambridge, MA, USA) or mouse anti-β-actin (1:1000; Santa

Table I. Primers of *IER5* and *β-actin*.

Gene name	Forward primer (5'→ 3')	Reverse primer (5'→3')
IER5	GGACGACACCGACGAGGAG	GCTTTTCCGTAGGAGTCCCG
β-actin	GCGCGGCTACAGCTTCA	CTTAATGTCACGCACTTTCC

IER5, immediate-early response 5.

Table II. mRNA and protein expression of *IER5* in each group.

Groups	Immunohistochemistry expression levels of *IER5* protein	qPCR mRNA expression of *IER5*	Western gray scale ratio of *IER5*/*β-actin*
0 Gy group	0.241±0.030	0.813±0.145	0.653±0.154
<20 Gy group	0.239±0.014	0.785±0.238	0.847±0.359
≥20 Gy group	0.272±0.019[a,b]	1.227±0.216[a,b]	1.300±0.376[a,b]

IER5, immediate-early response 5. qPCR, quantitative polymerase chain reaction. [a]Compared with 0 Gy group, P<0.05; [b]Compared with <20 Gy group, P<0.05.

Cruz Biotechnology, Inc., Santa Cruz, CA, USA) monoclonal antibody. Afterwards, membranes were washed three times with Tris-buffered saline containing 0.05% Tween-20 (8 min each time) before and after incubating with anti-goat IgG and anti-mouse IgG for 1 h at room temperature (1:1000, Santa Cruz Biotechnology, Inc.). Finally, the membrane was assayed using an enhanced chemiluminescent kit (ECL; Thermo Scientific, Rockford, IL, USA) and scanned with a ChemiDoc™ Doc XRS+ system (Bio-Rad Laboratories). The relative protein content was represented through the gray value ratio of IER5 protein bands/β-actin protein bands, and the results were analyzed with Quantity One software (Version 4.3.0, Bio-Rad Laboratories).

Follow-up. The outcomes of treatment were obtained by telephone follow-up or medical record review with the deadline of March 2014. The survival and recurrence rate during the follow-up were calculated.

Data analysis. Data are presented as the means ± standard deviation. The data were analyzed by using the SPSS statistical package 19.0 (IBM SPSS, Armonk, NY, USA). One-way analysis of variance was used to compare differences among groups. The least significant difference method was used to test the difference between groups. Spearman's rank correlation method was used to assess the association. A likelihood ratio Chi-square test was used to compare the difference between two values. For all above statistical analyses, P<0.05 was considered to indicate a statistically significant difference.

Results

Immunohistochemistry analysis. A total of 18 tissue specimens (0 Gy group, 6 specimens; <20 Gy group, 4 specimens; ≥20 Gy group, 8 specimens) were randomly selected for the immunohistochemistry analysis. We observed that the brown staining was increasingly deep with the increase of radiation dose in immunohistochemically stained sections (Fig. 1), indicating that the protein expression level of *IER5* was raised with the increase in radiation dose. For the OD values, there was no significant difference between the 0 Gy and <20 Gy groups (0 Gy group, 0.241±0.030; <20 Gy group, 0.239±0.014; P=0.915; Table II). However, the protein expression level of *IER5* in the ≥20 Gy group was significantly higher than that in the other two groups (≥20 Gy group, 0.272±0.019; compared with <20 Gy group, P=0.030; compared with 0 Gy group, P=0.021; Table II). Moreover, the results of the correlation analysis indicated that there was a significant positive correlation between the protein expression of *IER5* and the dose of radiation (r=0.548, P=0.019).

mRNA level of IER5. A total of 18 tissue specimens (0 Gy group, 6 specimens; <20 Gy group, 4 specimens; ≥20 Gy group, 8 specimens) were randomly selected for qPCR. The results revealed that there was no significant difference between the 0 Gy and <20 Gy groups (0 Gy group, 0.813±0.145; <20 Gy group, 0.785±0.238; P=0.830; Table II). However, the mRNA level of *IER5* in the ≥20 Gy group was significantly higher than that in the other two groups (≥20 Gy group, 1.227±0.216; compared with <20 Gy group, P=0.003; compared with 0 Gy group, P=0.002; Table II). In addition, the results of the correlation analysis indicated that there was a significant positive correlation between the mRNA expression of *IER5* and the dose of radiation (r=0.671, P=0.002).

Western blotting. A total of 33 tissue specimens (0 Gy group, 4 specimens; <20 Gy group, 12 specimens; ≥20 Gy group, 17 specimens) were randomly selected for western blot analysis. The results of SDS-polyacrylamide gel electrophoresis are shown in Fig. 2. Based on the gray scale ratio of *IER5*/*β-actin*,

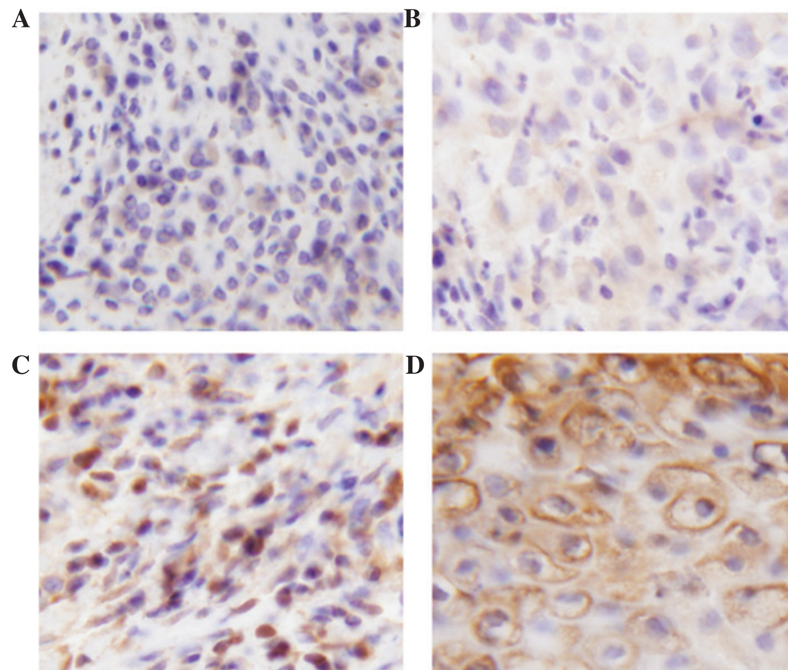

Figure 1. Immunohistochemically stained tumor sections from patients treated with (A) 0, (B) 7, (C) 30 and (D) 50 Gy radiation (3,3'-diaminobenzidine and hematoxylin staining; magnification, x40).

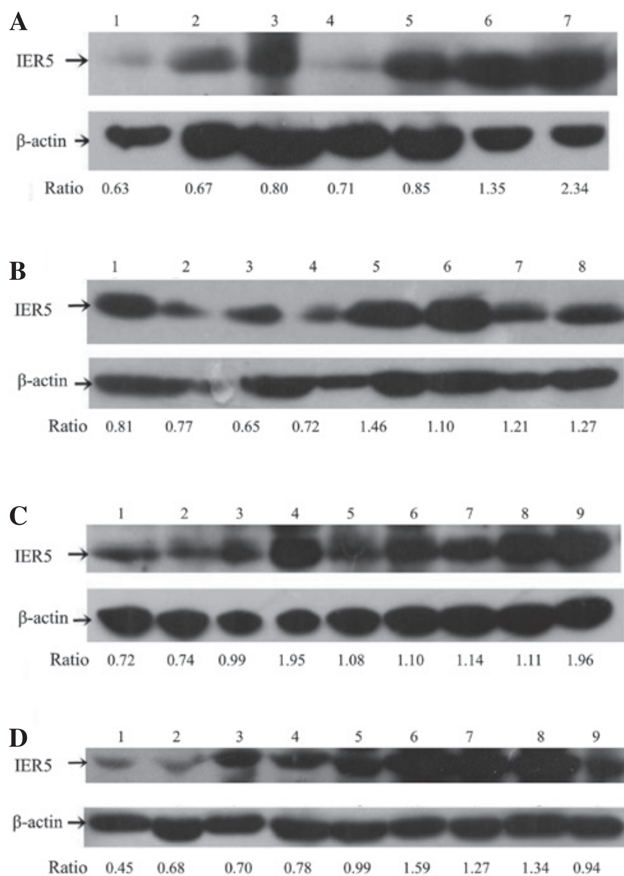

Figure 2. Results of sodium dodecyl sulfate polyacrylamide gel electrophoresis of immediate-early response 5 (*IER5*) and *β-actin*. (A) 1, 0 Gy; 2, 3 Gy; 3, 10 Gy+1 TC; 4, 10 Gy; 5, 20 Gy; 6, 30 Gy; 7, 30 Gy. (B) 1, 0 Gy+1 TC; 2, 2 Gy; 3, 3 Gy; 4, 10 Gy; 5, 20 Gy; 6, 30 Gy; 7, 40 Gy; 8, 40 Gy. (C) 1, 0 Gy; 2, 3 Gy; 3, 6 Gy; 4, 10 Gy; 5, 20 Gy; 6, 30 Gy; 7, 40 Gy; 8, 50 Gy; 9, 50 Gy. (D) 1, 0 Gy; 2, 3 Gy; 3, 6 Gy; 4, 10 Gy; 5, 20 Gy; 6, 20 Gy+1 TC; 7, 30 Gy; 8, 40 Gy; 9, 50 Gy. 1 TC, one course of paclitaxel-cisplatin chemotherapy. Ratio, gray scale ratio of *IER5*/*β-actin*.

the protein expression level of *IER5* in ≥20 Gy group was significantly higher than that in the other two groups (≥20 Gy group, 1.300±0.376; compared with <20 Gy group, P=0.002; compared with 0 Gy group, P=0.003; Table II). However, no significant difference was observed between the 0 Gy and <20 Gy groups (0 Gy group, 0.653±0.154; <20 Gy group, 0.847±0.359; P=0.349; Table II). In addition, the results of the correlation analysis indicated that there was a significant positive correlation between the protein expression of *IER5* and the dose of radiation (r=0.573, P<0.0001).

Correlation between expression of IER5 and clinical outcomes of radiotherapy. Based on the mRNA and protein expression of *IER5*, the patients were divided into two groups: a low *IER5* expression group (including the 0 Gy group and <20 Gy group) and a high *IER5* expression group (including the ≥20 Gy group). There were 36 patients in the low expression group and 33 patients in the high expression group. The results of the Chi-square test indicated that there was no significant difference between the two groups with regard to the survival rate (low expression group, 93.3%; high expression group, 96.8%; Chi-square test; P=0.490) and recurrence rate (low expression group, 6.67%; high expression group, 6.25%; Chi-square test; P=0.914).

Discussion

IER5 is possibly an intronless gene, which encodes a transcript of 2110 nucleotides in length and shares a number of nucleic acid and protein homologies with other members of the growth factor-inducible genes (18,19). Previous studies have reported that the expression of *IER5* may be induced by radiation in cells including HeLa cells, human lymphoblastoid cells, HepG2 and A549 (20-23). In this study, we investigated

the radiation-induced mRNA and protein expression levels of *IER5* in cervical cancer. The results indicated that the protein and mRNA expression of *IER5* in the ≥20 Gy group was significantly higher than that in the other two groups, which suggested that the expression of *IER5* in cervical cancer tissue was significantly increased after receiving radiotherapy at a dose of ≥20 Gy. Moreover, the mRNA and protein expression of *IER5* was significantly positively correlated with the radiation dose. The results suggested that radiotherapy could induce the upregulation of mRNA and protein expression of *IER5* in cervical cancer and that this induction was dose-dependent. In addition, there was no significant difference between the low *IER5* expression group and high *IER5* expression group in terms of the survival and recurrence rate. This suggested that the radiation-induced upregulation of *IER5* expression was not associated with the clinical outcomes of radiotherapy in cervical cancer.

The results of this study were consistent with those of Kis *et al*, who observed that the mRNA expression of *IER5* was dependent on radiation dose and time (24). Moreover, the dose- and time-dependent patterns of radiation-induced expression of *IER5* varied with cell types (18). This indicated that there was a complex transcriptional responsiveness of *IER5* to ionizing radiation. In addition, in an study of estrogen-dependent gene expression in the rat uterus, investigators identified that the expression of *IER5* would decrease following ovariectomy but increase following an injection of estrogen (25). In addition, Tavakoli *et al* observed that the radiation-induced transcription alterations of *IER5* were associated with gender (13). Given that cervical cancer is a female malignancy, we inferred that the radiation-induced expression of *IER5* might be associated with the secretion of estrogen in the cervical cancer tissues.

Furthermore, the radiation-induced expression of *IER5* may be associated with the radiosensitivity of cervical cancer. Ding *et al* observed that suppression of *IER5* potentiated radiation-induced arrest at the G2/M transition and led to an increase in the fraction of S-phase cells (11). It was also reported that low-dose hyper-radiosensitivity is linked to the early G2/M checkpoint through the damage response of G2-phase cells (26-28). Thus, we inferred that the radiation-induced expression of *IER5* was associated with the radiosensitivity of cervical cancer via the damage response of G2-phase cells. Further studies are required to prove this speculation.

Certain limitations must be noted in this study. Firstly, due to the lack of detailed information on follow-up, survival curves could not be obtained. More studies should be carried out to investigate the correlation between the expression of *IER5* and the efficacy of radiotherapy. Secondly, three of the patients were treated with a combination of radiotherapy and chemotherapy, which may affect the results of this study. Thus, further studies are required to verify the results of this study.

In conclusion, we observed that radiotherapy could induce the upregulated expression of *IER5* in cervical cancer and that the induction was dependent on the dose of radiation. *IER5* may play crucial roles in the radiosensitivity of cervical cancer cells. This study provides data for the investigation of the radiosensitivity mechanism of cervical cancer, which is the main constraint in the efficacy of radiotherapy. However, no association between the radiation-induced expression of *IER5* and the clinical outcomes of radiotherapy in cervical cancer was identified in this study. The results suggested that although *IER5* may be a key gene in the radiosensitivity mechanism of cervical cancer, there may be no direct association between the expression of *IER5* and clinical efficacy of radiotherapy. The possible roles of *IER5* in the radiosensitivity mechanism of cervical cancer still require further investigation.

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