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Original Article

Establishments of IER5 silence and overexpression cervical cancer SiHa cell lines and analysis of radiosensitivity

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Abstract: Objective: This study is to investigate the role of IER5 in radiosensitivity of cervical cancer squamous cells. Methods: Real-time PCR and Western Blotting assay were used to examine the mRNA and protein expression of IER5 in different time and dose irradiated cervical cancer cell lines, including SiHa and HeLa cells. We established IER5 silence and overexpression eukaryotic expression vectors and transfected SiHa cells. Then the role of IER5 was studied including cell morphorlogy, proliferation, counting, survival, and apoptosis as well as cell cycle with IER5 gene silencing or overexpression via CCK-8 assay and flow cytometry after radiation. Results: IER5 gene silencing SiHa cells increased slightly, and the speed of cell proliferation after radiation increased. And IER5 gene overexpression made SiHa cells shrink assumes as circle, and cell proliferation slowed down. IER5 gene silencing reduced apoptosis rate by radiation, while IER5 gene overexpression promoted the apoptosis rate by radiation. The G_2-M phase retardation effect on cell cycle after radiation was promoted along with the decreasing of IER5 expression, which indicated that IER5 gene silencing promoted cell proliferation. Conclusion: The expression of IER5 was increased in irradiated cervical cancer cell lines. IER5 is a radiation-inducible gene in cervical cancer SiHa cell line because of enhanced radiosensitivity of cervical cancer cells in vivo. IER5 may be a potential target to improve the results if radiation therapy for patients with cervical cancer.

Keywords: IER5, cervical cancer, radiosensitivity, SiHa cell line

Introduction

Cervical cancer is the 4th causes of death in women globally which is 7.9% according to GLOBOCAN 2012. Radiation therapy, as equally significant as radical hysterectomy, has a wider range of indication in clinic. However, it still remains difficult to further improve on the cervical cancer cells' sensitivity to radiotherapy and pelvic normal tissues' tolerance for radiation [1]. Cervical cancer radiotherapy target genes have become a research hotspot in recent years.

IER5, a 43-kDa protein, isdescribed as a slow-kinetics immediate-early gene, encoding a serum- and growth factor-inducible message of 2350 nucleotides, which is mainly responsible for the rapid response to external stimuli and the regulation of cell cycle [2]. Scholars found that IER5 gene's transcription or translation in a variety of tissue cells were multiplied after radiation by gene microarray, [3-5] which hintedthat IER5 gene was closely related to the biological changes after radiation. IER5 has been reported as a radiation-inducible genes by using microarray analysis recently in human lymphoblastoid AHH-1 cells after γ-ray irradiation [6]. In early studies, inhibition of IER5 gene expression in cervical adenocarcinoma cell line HeLa promoted cell mitosis, improved radiation resistance. However, whether IER5 gene has the same effect in different pathological type of cervical cancer cells, and the roleof IER5 gene in cell apoptosis and cell cycle, were studied in this article.

Materials and methods

Cell culture and ionizing radiation treatment

The cervical cancer cell lines SiHa and HeLa from National Platform of Experimental Cell Resources for Sci-Tech were cultured in MEM or DMEM (Hyclone) containing 10% fetal bovine serum (Hyclone), 100 IU/ml penicillin, 100 ug/ml streptomycin at 37°C in a humidified, 5% CO_2 atmosphere.

For each experiment, the exponentially growing cells were irradiated at room temperature using a cobalt-60 γ-ray source at a single dose of 0, 2, 4, 6, 8, 10 Gy at a dose rate of 1.7 Gy/min. Then cells were returned to the incubator and incubated at 37°C, 5% CO_2.

Construction of IER5-siRNA vectors

Oligonucleotide sequences of the siRNAs targeting the IER5 gene (NCBI Reference Sequence: NM_016545.4) were analyzed by online software WhiteHead and using BLAST to ensure they did not target any other gene transcripts. Two sequences encoding IER5 siRNAs were as follows (targeting sequences are underlined):

IER5-2^{siRNA}: Sense-5'GATCCTAACCTCATCAGCATCTTCTTCAAGAGAGAAGATGCTGATGAGGTTAGCA 3'; Antisense-5'AGCTTGCTAACCTCATCAGCATCTTCTCTCTTGAAGAAGATGCTGATGAGGTTAG 3'.

IER5-3^{siRNA}: Sense-5'GATCCCACAGGATTTCCTAAGACGTTCAAGAGACGTCTTAGGAAATCCTGTGCGA 3'; Antisense-5'AGCTTCGCACAGGATTTCCTAAGACGTCTCTTGAACGTCTTAGGAAATCCTGTGG 3'.

BamHI and HindIII restriction sites were added upstream and downstream of each oligonucleotide, respectively. After annealing, these duplex oligonucleotides were inserted between the BamHI and HindIII sites of the pSilencer^{TM}4.1 vector to generate the IER5-siRNA vectors. All constructs were sequence-verified before use.

A vector containing the following non-specific siRNA (NS^{siRNA}) was used as an experimental control:

Sense-5'GATCCCACCATTCCAAGCACTTCTTTCAAGAGAAGAAGTGCTTGGAATGGTGACA 3'; Antisense-5'AGCTTGTCACCATTCCAAGCACTTCTTCTCTTGAAAGAAGTGCTTGGAATGGTGG3'.

Construction of IER5-overexpressed vectors

According to IER5 mRNA sequence in NCBI genebank, IER5 gene was synthesized to the vector pCMV-Tag, whose size is 4.3 kb. The building process was done according to manufacture's instruction. The sequences of primer encoding IER5 open reading frame were as follows:

Up-primer 5' TGCAAGCTTATGGAGTTCAAGCTGGAG 3'; Down-primer 5' TAACTCGAGTCAGAAGGCCACGATG 3'.

HindIII-HF and XhoI restriction endonuclease were chose to insert IER5 sequence into pCMV-Tag2B vector. Then the linked products were transducted into competent cells DH5α, and IER5-overexpressed vectors were extracted from positive clones and identified by electrophoresis and sequencing.

Transfection and establishment of IER5-siRNA-SiHa and IER5-overexpressed-SiHa stable cell lines

For vector DNA transfection, SiHa cells were plated in 60 mm Petri dishes at a density of 5×10^5 cells per dish. The cells were transfected with IER5-siRNA and IER5-overexpressed vectors using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) reagent 24 hrs after plating according to the manufacturer's instructions [7]. Transfected cultures were maintained in conditioned MEM supplemented with 50 ug/ml hygromycin B (Roche, Basel, Switherland) and G418 to select for transfected clones. A non-specific siRNA vector and pCMV-Tag2B vector were used as control.

Western blotting assay

The cells were washed twice with cold PBS before being lysed in mammal cell lysis buffer (Thermo, USA). The proteins (40 μg/lane) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose membranes (Sigma). The nitrocellulose membranes were probed with the primary antibody against IER5 (1:500, Abcam, UK), and then with peroxidase-conjugated secondary antibodies at optimized concentrations. The blots were devel-

Establishments of IER5 silence and SiHa cell lines

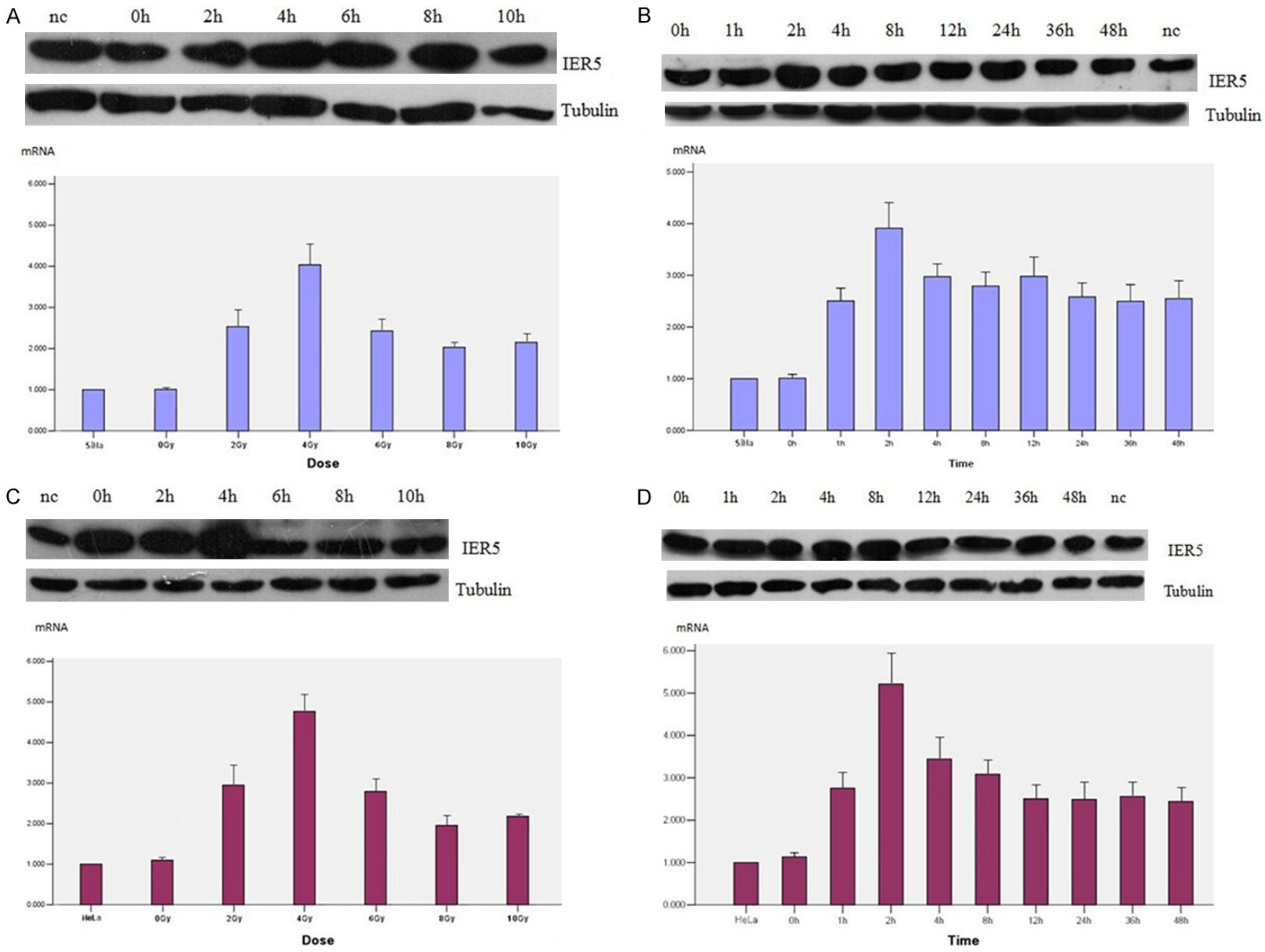

Figure 1. The transcription peak of IER5 gene of cervical cancer SiHa cells appeared at 2-hour after radiation, and offered upgrade firstly than descending later tendency. A. SiHa cells radiation dose-effect. B. SiHa cells radiation time-effect. C. HeLa cells radiation dose-effect. D. HeLa cells radiation time-effect.The translation peak of protein appears at 2-hour too. The mRNA of IER5 gene's time-effect and dose-effect relationship of HeLa cells after radiation reaches concensus with SiHa cells. However, the peak of protein expression is later than SiHa, which is 4-hour after radiation, while there is also a small peak at 36-hour.

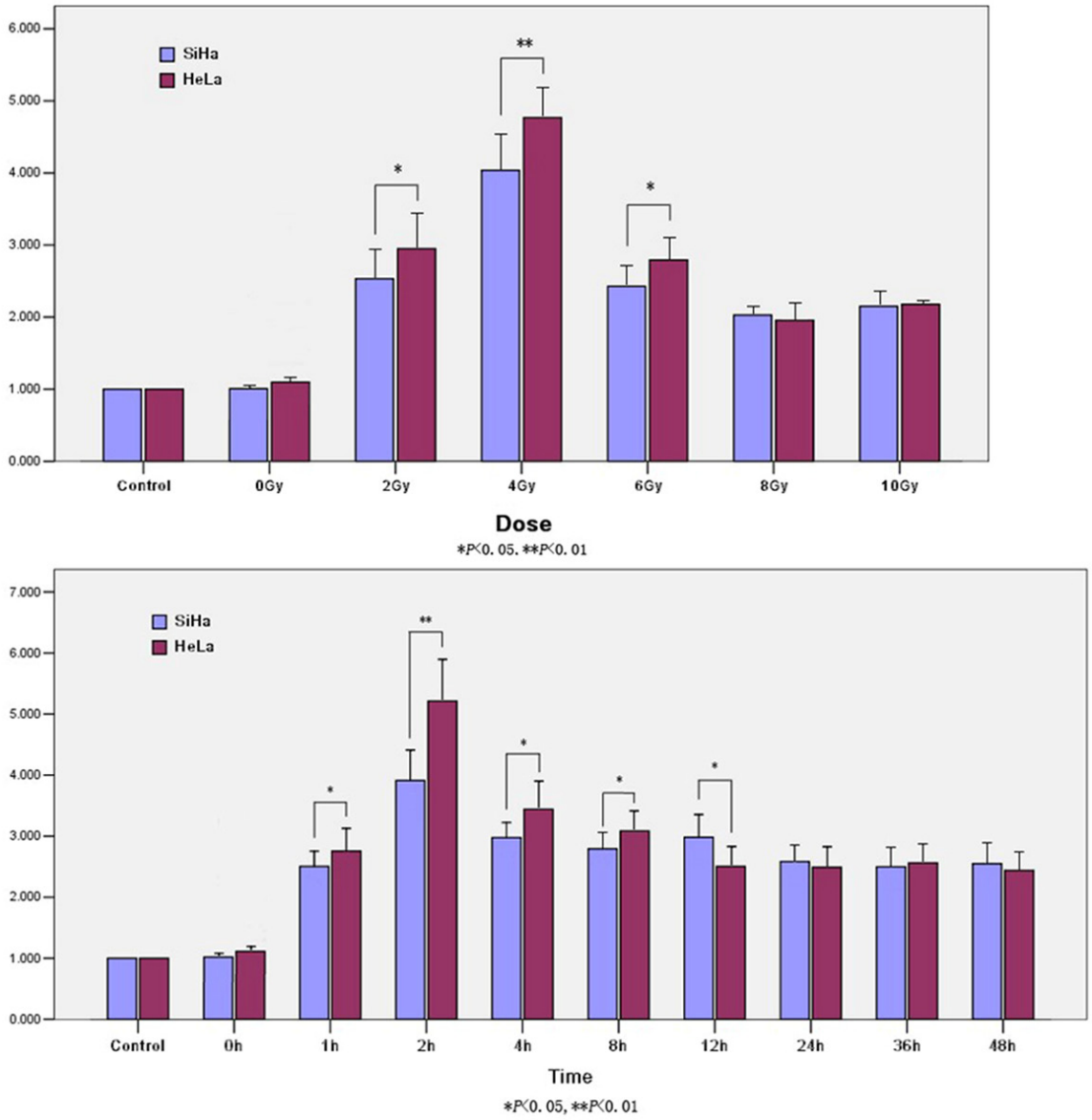

Figure 2. Data in bar graphs represent means±standard deviations from three independent experiments. Statistical analysis of the mRNA data of SiHa cells versus HeLa cells using two-tailed t test: P=0.023, 0.006, 0.035 for 2, 4, and 6Gy irradiation, respectively on the left; P=0.042, 0.000, 0.022, 0.036, 0.018 for 1, 2, 4, 8, and 12 hours after irradiation, respectively on the right.

oped by using an enhanced chemiluminescence (ECL) kit (Sino-American Biotechnology) [8]. β-Tubulin monoclonal antibody was a control (1:2000, Sigma-Aldrich).

Quantitative real-time PCR

Total RNA was isolated by the use of a Trizol reagent (Invitrogen) as described [9]. Then 1 ug

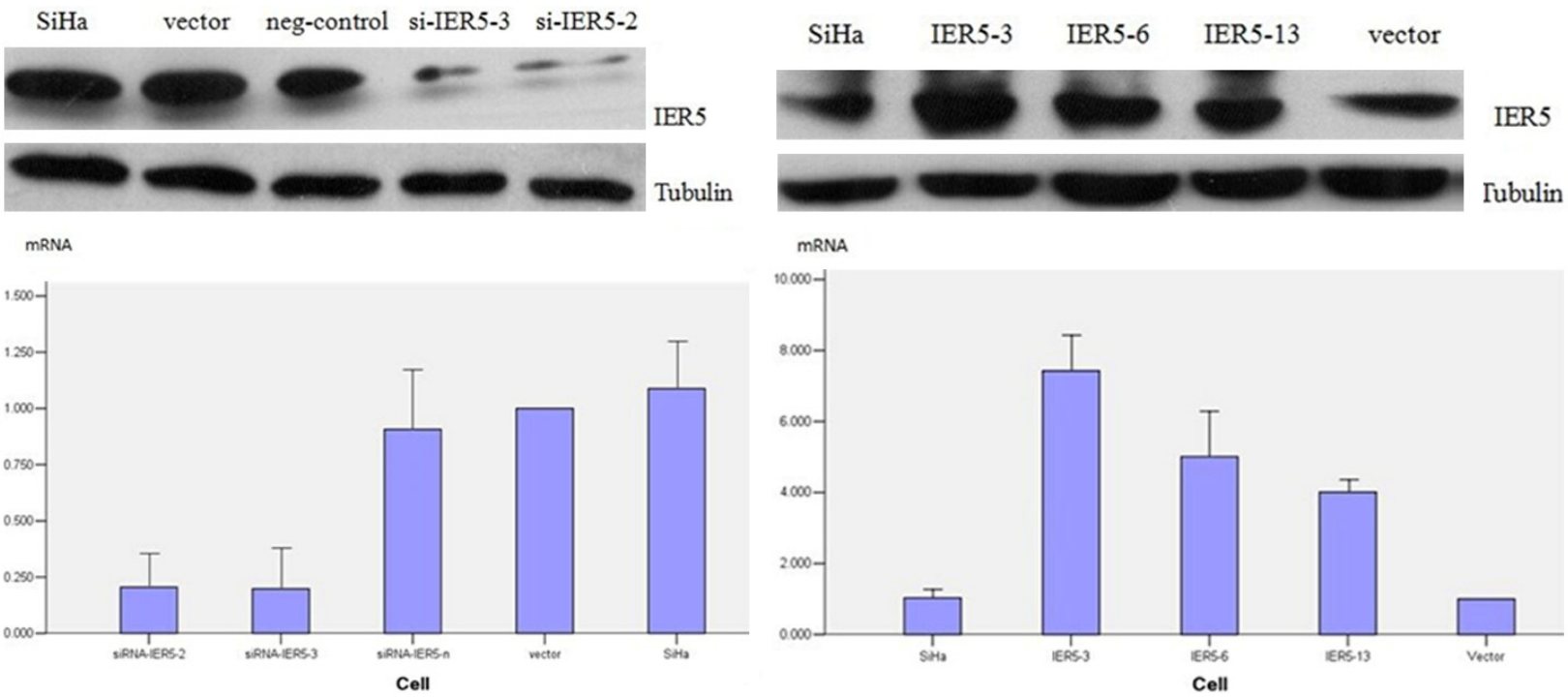

Figure 3. Data in bar graphs represent means±standard deviations from three independent experiments. Statistical analysis of the mRNA data of new cell lines versus vector cells using two-tailed t test: P=0.000, 0.000, 0.751 for siRNA-SiHa-2, siRNA-SiHa-3, and siRNA-SiHa-n cells versus vector cells, respectively on the left; P=0.000, 0.000, 0.000 for IER5-3, IER5-6, IER5-13 cells versus vector cells, respectively on the right.

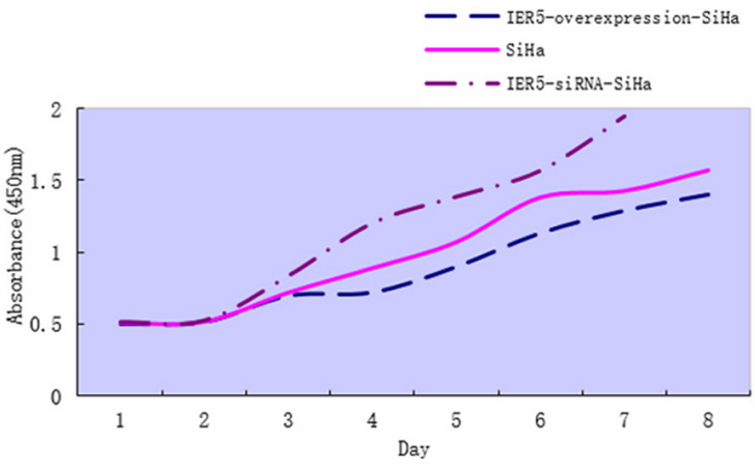

Figure 4. We used CCK-8 assay to observe cell propagation after irradiation of different IER5-expressing SiHa cells. Each cell line was plated in triplicate in three independent well. We constructed the growth curve of each cell line based on the OD value.

total RNA was reverse transcribed to cDNA by the use of the real-time PCR Master Mix (TOYOBO, Osaka). To quantify mRNA levels, quantitative real-time PCR was performed using an Chromo 4 real-time PCR System (Bio Rad, Hercules, California, USA). The reaction mixture was composed of SYBR Green PCR Master Mix, containing 9 ul 2.5× RealMasterMix (add SYBR before use), 1 μM of each primer, and 1 ul cDNA template (obtained after RT as described above) in a final volume of 20 ul. The cycle number at which other fluorescent signal crossed the detection threshold was denoted as the threshold cycle (CT). CT values obtained for the detected genes were normalized using β-actin as an internal standard. Each PCR reaction was run in triplicate in three independent experiments. The primer pairs for human IER5 amplification were as follows: forward primer 5'-CCGGGAACGTGGCTAACC-3', reverse primer 5'-CCGGGAACGTGGCTAACC-3', and those for human β-actin amplification were as follows: forward primer 5'-GCGCGGCTACAGCTTCA-3', reverse primer 5'-CTTAATGTCACGCACGATTTCC-3'.

Cell proliferation and survival (CCK-8 assay)

For cell proliferation assays, subjected cells to γ-ray irradiation of 4 Gy, and then seeded 5000 cells per well in 96-well culture plates with 200 μl MEM. Add 100 μl 10% CCK-8 solution into each well of the first row, including a blank well without cells, and measure the absorbance at 450 nm after 2 hrs incubation. Repeat the absorbance measure on 2nd to 8th day at the same time. Each cell line was plated in triplicate in three independent well. Construct the growth curve of each cell line based on the OD value.

Flow cytometry to detect apoptosis and cell cycle

Cells transfected stably with IER5-overexpressed plasmid or siRNA were plated in 60-mm dishes, then exposed to 10 Gy irradiation. After 0, 24, 48, 72 h of incubation, cells were washed twice with PBS and collected in tubes. For apoptosis analysis, cells were collected and stained by the use of a cell apoptosis kit (Annexin V-FITC) [10]. Cells were then assayed by flow cytometry (FACSCalibu, USA). For cell cycle analysis, cells were exposed to 4 Gy irradiation and after 0, 1, 2, 4, 8, 12, 24, 36, 48 h of incubation, cells were fixed and stained as described [11]. Then cells were assayed by flow cytometry.

Statistical analysis

All experiments were performed at least in triplicate and results were recorded. The SPSS 16.0 software package was used for statistical analysis. Data were expressed as means ± SD and compared by two-tailed t test. $P<0.05$ was considered statistically significant.

Results

IER5 is an irradiation-inducible gene in cervical cancer SiHa and HeLa cell lines

Radiation treatment significantly increased both the mRNA and protein levels of IER5 in SiHa and HeLa cells (**Figure 1**). Thus, IER5 might be an irradiation-inducible protein in cervical cancer cells, for cervical cancer HeLa cell line had the same effects after irradiation [7]. The content of IER5 gene in cervical adenocarcinoma HeLa cells was more than cervical squamous carcinoma SiHa cells (**Figure 2**). 4 Gy was confirmed to be the optimal dose of

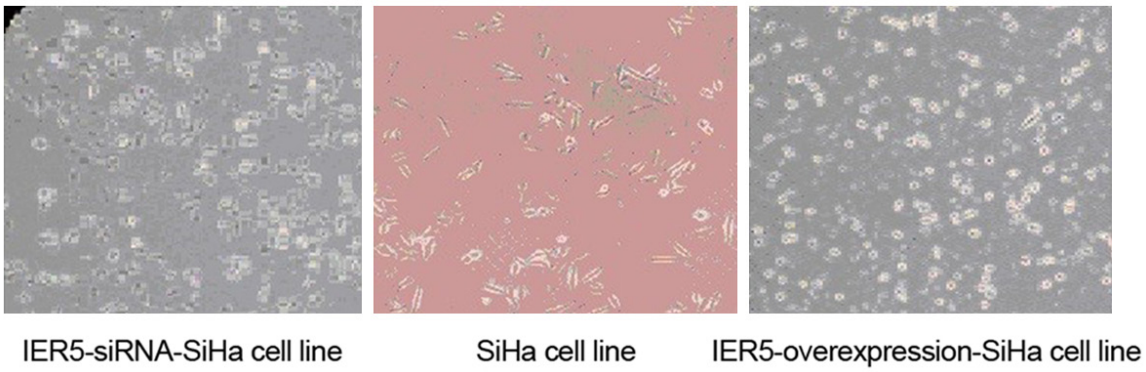

Figure 5. We observed different IER5-expressing cell lines in the same inverted microscope of high power lens and took pictures. IER5 gene knock-down SiHa cells are slightly larger than normal SiHa cells, and its forms included rhombus, fusiform, triangular, long spindle, etc. IER5 gene overexpression of cell is smaller than normal SiHa cells, and most of its shape was round, where there was also long spindle and fusiform.

radiation to observe IER5 gene mRNA transcription and protein translation in both kinds of cervical cancer cell lines (**Figure 2** left).

The effects of the established IER5 gene silencing cervical cancer SiHa cell line and IER5 overexpression SiHa cell line

The results of Western Blotting and Real-Time PCR showed that protein expression and mRNA in IER5-siRNA-SiHa cell line are significantly decreased, IER5-overexpression-SiHa cell line's protein expression and mRNA increased significantly. Based on those results, the IER5 knock-down and overexpression cell lines were established successfully (**Figure 3**).

Gene silencing of IER5 expression attenuates cellular response to radiation in cervical cancer SiHa cell line, and IER5 overexpression has the opposite effect

To detect whether IER5 gene was related to the radiation sensitivity in cervical cancer, we suppressed IER5 gene expression in SiHa cells by siRNA targeting IER5. siRNA knockdown of IER-2/3 inhibited IER5 expression. siRNA knowkdown of IER5 enhanced radiation-induced cell proliferation by both increased cell proliferation and cell number (**Figure 4**).

IER5 gene silencing SiHa cells increased in volume slightly, and the speed of cell proliferation after radiation increased. And IER5 gene overexpression made SiHa cells shrink assumes as circle, and cell proliferation slowed down (**Figure 5**).

IER5 promotes radiosensitivity by enhancing G_2/M phase cell-cycle arrest and inducing cell apoptosis in cervical cancer SiHa cell line

To further clarify and evaluate the potential role of IER5 as a therapeutic target in radiation therapy of cervical cancer, we built stable cell lines overexpressing IER5 gene in SiHa cell. IER5 overexpression combined with radiation had a greater effect on inhibiting cell proliferation and viability of cervical cancer SiHa cells than radiation alone, as seen by reduced cell proliferation and cell number and survival, which proved to us that IER5-overexpressed markedly enhanced the expression of IER5 in cells and promoted their radiosensitivity (**Figure 6**). Thus, alteration of IER5 expression affected the survival and viability of cervical cancer SiHa cells with radiation treatment.

We next analyzed the role of IER5 in the cell cycle in response to radiation. The G_2/M phase arrest was more marked in IER5-suppressed SiHa cells treated with radiation than with the control treatment (**Figure 7**). As well, IER5-overexpression in SiHa cells significantly enhanced cell apoptosis induced by radiation treatment. Consistently, siRNA knockdown of IER5 with radiation inhibited the apoptotic rate as compared with control treatment.

Discussion

Cervical cancer as a main opponent of gynecologic oncologists, its incidence and mortality of malignant tumors in women still remains the 4th. Radiotherapy as a significant treatment for

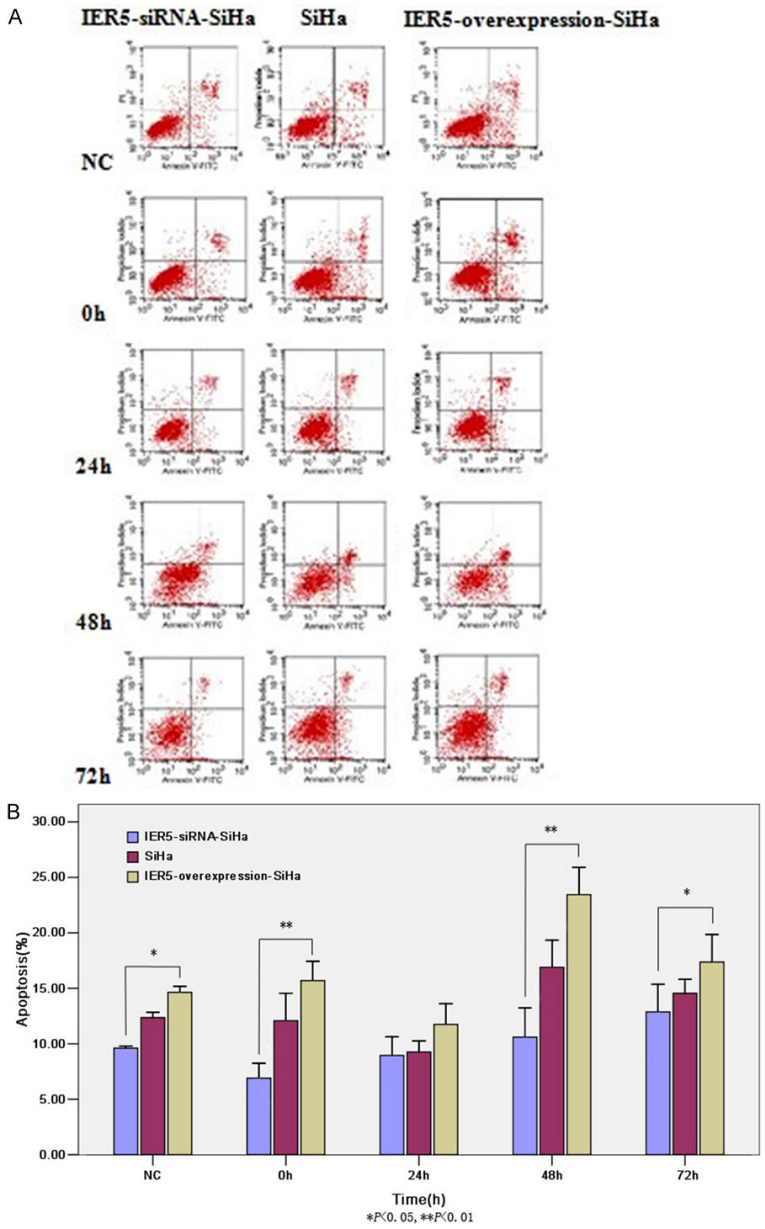

Figure 6. We used flow cytometry to detect cell apoptosis via cell apoptosis kit (A). Data in bar graphs (B) represent means±standard deviations from three independent experiments. Statistical analysis of the apoptosis% data of IER5-siRNA-SiHa versus IER5-overexpression-SiHa cells using two-tailed t test: P=0.016, 0.000, 0.056, 0.000, 0.021 for without irradiation, 0, 24, 48, and 72 hours after irradiation, respectively.

cervical cancer, is appropriate for any stage of patients in clinical, especially for stage IIa_2 or even more advanced patients [12]. At present, the direction of cancer gene therapy is focused on the gene level, where the strategy is to understand suppress the growth of cancer cells via improvement of molecular level therapeutic methods administered to human body in vivo [13]. IER5 encodes a 327-amino-acid protein with multiple nuclear localization signals and a PEST-like sequence implying rapid protein degradation. The explicit biological function of IER5 gene still remains unclear, although it is known to play an important role in cellular responses to a number of exogenous stimuli including ionizing radiation. Based on the previous observations from microarrays and different IER5-expressing of cervical cancer adenoma HeLa cell lines, liver cancer HepG2 cell lines, we have further confirmed the function of IER5 in radiation by establishing different IER5-expressing SiHa cell lines, which is cervical cancer squamous cells.

In this study, we observed dose-dependent effects and time-dependent effects on radiation-induced expression of IER5 both in cervical cancer squamous SiHa cell line and adenoma HeLa cell line. Based on our observation, we found out 4 Gy is the best dose for cervical cancer cells ionizing radiation experiments, for both transcription and translation are markedly increased. A characteristic of immediate-early genes, including transcriptional regulators, is a rapid induction by growth factors or other stimuli that is not prevented by the presence of protein synthesis inhibitors. Activation of the immediate-early genes is thought to be an important initial step in response to exogenous stimuli or stress. We observed the same phenomenon for IER5 mRNA was promoted rapidly after irradiation in 2 hours. There was another significant result that the content of IER5 was more in the adenoma HeLa cells than squamous SiHa cells, which implied to be a key to

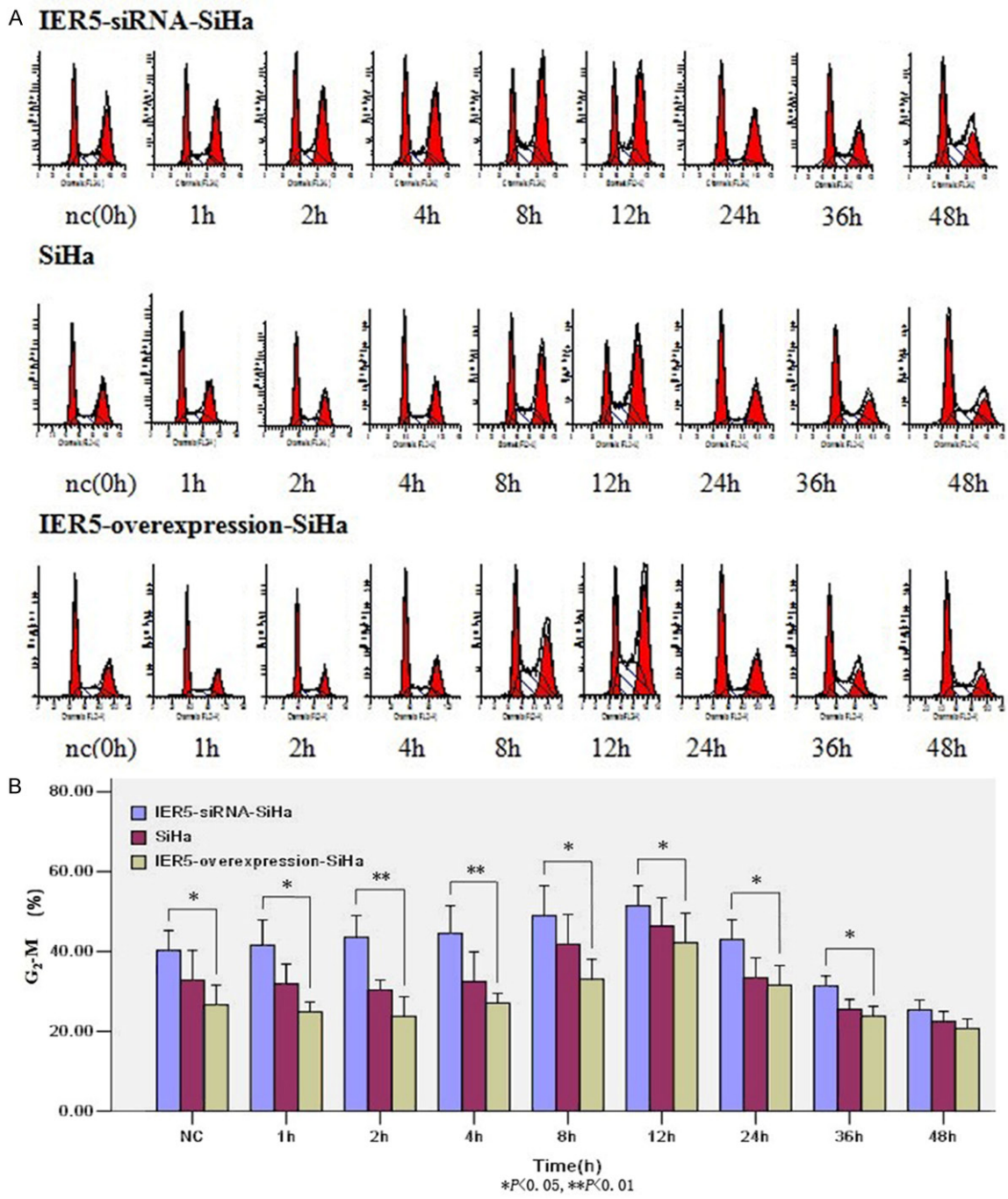

Figure 7. We used flow cytometry to detect cell cycles for different incubation hours after irradiation (A). Data in bar graphs (B) represent means±standard deviations from three independent experiments. Statistical analysis of the G2/M% data of IER5-siRNA-SiHa versus IER5-overexpression-SiHa cells using two-tailed t test: P=0.021, 0.017, 0.000, 0.000, 0.026, 0.030, 0.042, 0.217 for without irradiation, 1, 2, 4, 8, 12, 24, 36, and 48 hours after irradiation, respectively.

promote cervical adenocaicinoma's sensitivity to radiotherapy.

In order to investigate the role of IER5 in the cellular response to ionizing radiation, we established a SiHa cell line with reduced IER5 expression by using siRNA technology and a SiHa cell line with IER5 overexpressed. Knocking down IER5 resulted in an increased propagation of SiHa cells, which was opposite

in IER5-overexpressing. In additional, the overexpressed IER5 caused more cell apoptosis. These results suggest that IER5 gene expression could play an important role in the induction of cell death caused by irradiation and increased radiosensitivity.

It is known that cells in different phase of cell cycle has different sensitivity to irradiation. G_2 and M phases are more sensitive than G_0, G_1 and S phases [14]. According to our results, there was an accumulation of G_2/M cells in IER5-reduced SiHa cells after irradiation, which suggested a potential activation of G_2 checkpoint induced by ionizing radiation related to IER5. In further study, we planned to investigate the relationship between IER5 gene and DNA repair, and its role in apoptosis.

In conclusion, results in this study showed initial evidence that IER5 gene could improve the radiosensitivity in cervical cancer cells in vitro. Our research preliminarily found out IER5 gene's function, which is participating in the regulation of cell cycle after irradiation, and its overexpressing could reduce cell division, promote cell apoptosis after irradiation. However, research of the mechanism of how it involved in regulating cell apoptosis and cell cycle will continue, as well as in vivo experiments. Combined with the clinical tissues experiments, our study will lay a foundation for its application in the clinical treatment.

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Disclosure of conflict of interest

None.

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