Original Article
Radiation modulated the interaction of IER5 protein and CDC25B promoter DNA in primary hepatocellular carcinoma

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Abstract: Objective: Primary hepatocellular carcinoma (PHC) is associated with increasing incidence and poor survival. The specific carcinogenesis is still not completely understood, which disables the identification of new effective anti-tumor therapy in treating this deadly disease. Our present study aimed to further investigate the functional role of IER5 and CDC25B in the development of PHC. Methods: HepG2 cells were transiently transfected to modulate the endogenous IER5 expression and treated under ^{60}Co-γ ray radiation. Real-time RT-PCR and Western blot were used to detect the mRNA and protein level of the IER5 and CDC25B, respectively. CHIP was conducted to analyze the interaction of IER5 protein with CDC25B promoter DNA. Results: The radiation could regulate the expressions of IER5 and CDC25B mRNA, which was time-dependent. IER5 mRNA level was increased, and then decreased after reaching the peak at 6 h. An opposite trend was observed on CDC25B mRNA. This effect of radiation on IER5 and CDC25B expression was abolished in IER5 silencing HepG2 cells. CHIP proved that IER5 protein could competitively bind to the CDC25B promoter, thus regulating the gene transcription. Conclusion: The radiation could control the IER5 expression, which may modulate the transcription of CDC25B by competitively binding to the CDC25B promoter in PHC.

Keywords: IER5, CDC25B, radiation, HepG2 cells, promoter

Introduction

Primary hepatocellular carcinoma (PHC) is one of the common malignant digestive tract tumors with very high incidence and mortality [1]. Patients with chronic liver diseases like hepatitis B or C virus infection and fatty liver could develop into PHC. There is no specific symptom in early stage, and the overall survival is very poor once the patients with PHC were diagnosed. Currently, a variety of treatments for PHC are available, including surgery, chemotherapy, radiation, transarterial chemoembolism and radiofrequency ablation, which still could not cure this devastating disease [2]. Thus, the development of new effective therapeutic agents is of vital importance to improving life quality and survival of such patients.

CDC25 family (cell division control Cdc25 phosphatase) is an important regulator in the control of cell cycle by modulating CDKs activity. CDC25 family has three homologous isomers (CDC25A, CDC25B, CDC25C), which play a tremendous role in the function of Thr/Tyr kinase [3]. Previous studies have proved that CDC25B overexpression could promote the tumor cell proliferation, while CDC25B silencing could inhibit the tumor growth [4]. Although it was reported that CDC25B could induce cell cycle arrest in S phase or even earlier, the specific molecular mechanism is still unclear, which needs further exploration.

Immediate early response 5 (IER5) belongs to the early slow reaction gene family. IER5 gene was located in the chromosome 1q25.3.
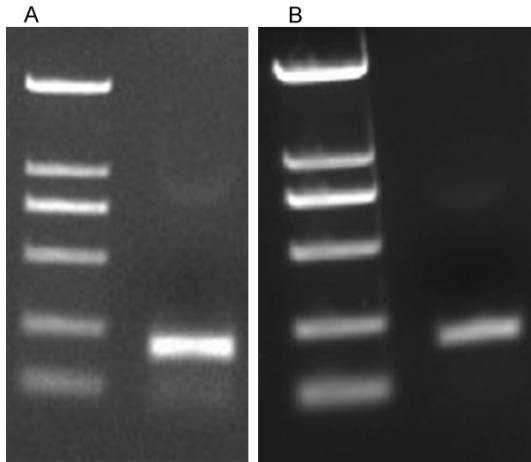
Figure 1. Agarose gel electrophoresis showed that CDC25B (A) and IER5 (B) could be amplified by PCR using specific primers designed. The marker and product were loaded in the left and right well, respectively.
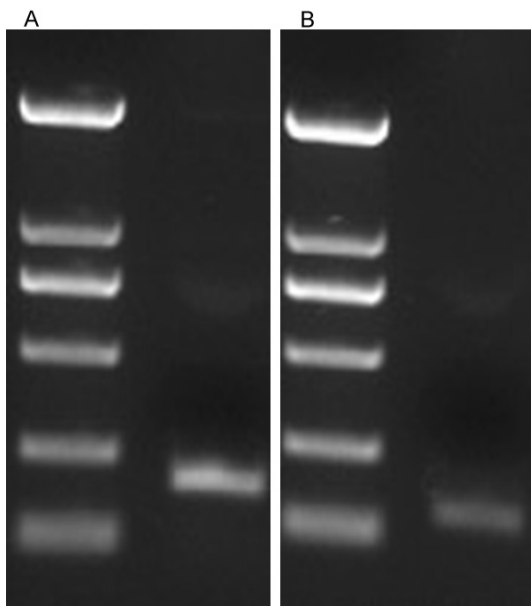

Figure 2. Real-time RT-PCR products using specific primers for IER5 (A) and CDC25B (B) were further validated by agarose gel electrophoresis and clear band could be seen. The marker and product were loaded in the left and right well, respectively.

Experimental investigations conducted by Williams et al, demonstrated that IER5 is kind of protein rich in proline with multiple nucleic acid sites [5]. Radiation can induce IER5 transcription levels, which enhance the radiation induced apoptosis. It is supposed that IER5 might exhibit regulatory effects on the cell mitosis by activating the phosphorylation and/or

phosphorylation under certain stimulus. In addition, increased IER5 protein level but reduced CDC25B mRNA expression were observed in chronic myeloid leukemia [6]. However, how IER5 interacts with CDC25B in hepatoma under radiation has not been ever evaluated. Therefore, we conducted this study, aiming to clarify the association of IER5 and CDC25B mRNA expression in HepG2 cells when treated by radiation, and further detect the changes and interactions of IER5 protein and CDC25B promoter.

Materials and methods

Cell culture

Human hepatocellular carcinoma cell line HepG2 was purchased from American Type Culture Collection (ATCC) and cultured in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% sodium pyruvate. Culture medium was exchanged every 3 days. HepG2 cells were incubated at 37°C in humidified atmosphere with 5% CO_2. PCMV-IER5-3xFlag and PCMV-3xFlag Vector stably transfected HepG2 cells were previously established in our laboratory, which were validated and recovered. A dosage of 2Gy and 4Gy ⁶⁰Co-γ ray radiation at 148.2cGy/min was applied to treat HepG2 cells, respectively. Cells were collected at 2 h, 4 h, 6 h, 8 h, and 12 h after radiation, and untreated cells serve as controls.

Construction of plasmid and transfection

Plasmid pSilencer™3.1-H1 hygro vector was used and pSilencer™3.1-H1 hygro siIER5 was constructed and extracted by using Plasmid Maxi Kit (TIANGEN, China). The plasmids were further validated by sequencing before transfecting cells. A count of 1×10⁶ HepG2 cells was seeded in a 60 mm dish. After culturing for 24 h, the cells were transiently transfected by pSilencer™3.1-siIER5 and pSilencer™3.1-Vector using Lipofectamine 2000™ (Invitrogen, USA). After 36 h, the transfected cells were collected for total RNA extraction.

Total RNA extraction and real-time RT-PCR

The total RNA was extracted by Trizol (Invitrogen, USA), and quantified by Nanodrop2000 (Thermo, USA). 1 µg total RNA was reversely
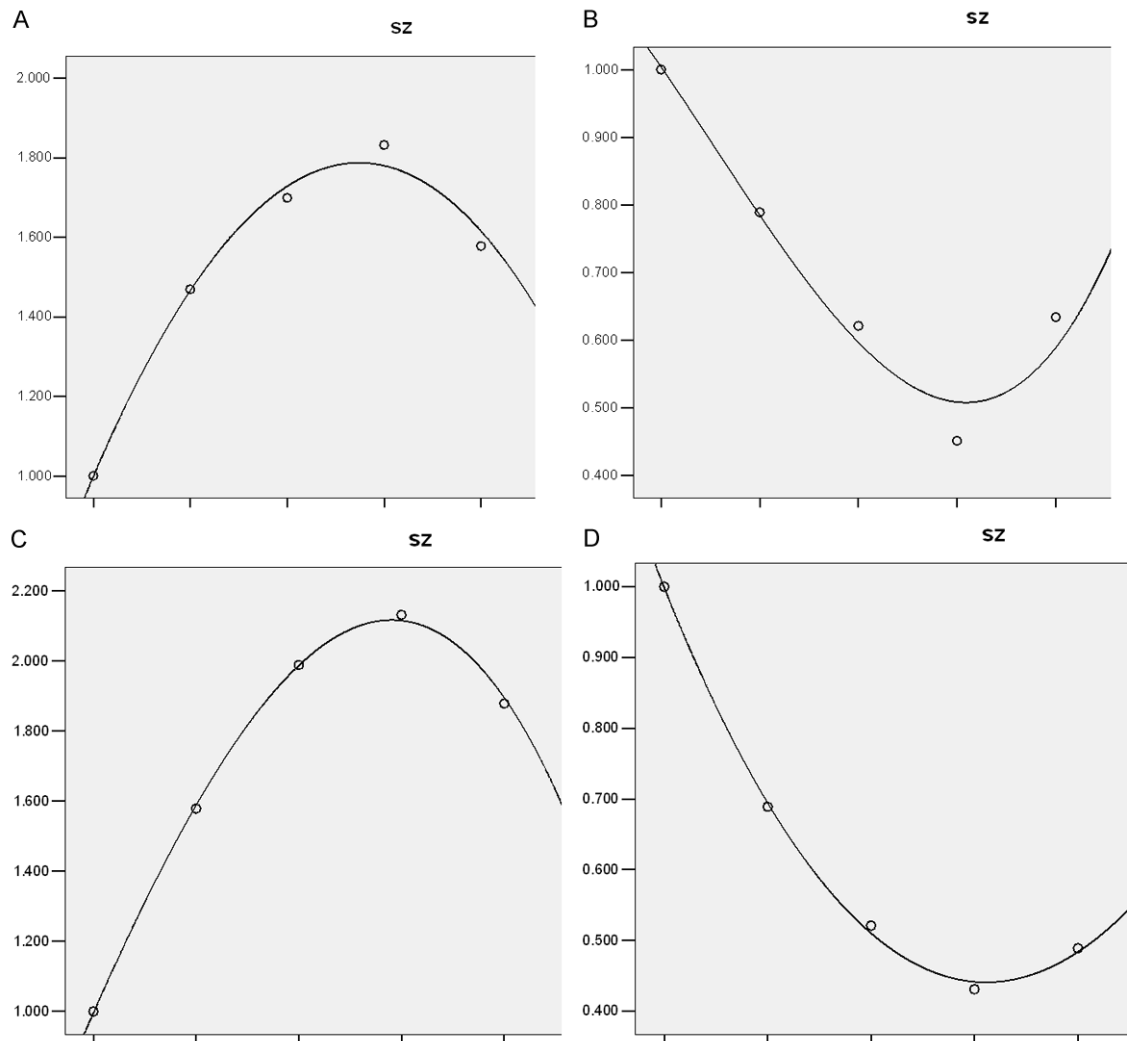
Figure 3. Radiation could regulate the IER5 and CDC25B transcription in HepG2 cells. Real-time RT-PCR showed that 2Gy radiation induced changes of the IER5 (A) ($Y=0.331+0.765X-0.097X^2-0.001X^3$, $R^2=0.989$, $P=0.016$) and CDC25B mRNA expression (B) ($Y=1.186-0.140X-0.053X^2+0.011X^3$, $R^2=0.970$, $P=0.045$). Similar trends were observed in 4Gy treated Hep2G cells on IER5 (C) ($Y=0.288+0.741X-0.019X^2-0.013X^3$, $R^2=0.999$, $P=0.001$) and CDC25B mRNA expression (D) ($Y=1.428-0.494X+0.065X^2-0.001X^3$, $R^2=0.999$, $P=0.002$).

transcribed into cDNA using Reverse Transcription Kit (Promega, USA). Specific primers for real-time RT-PCR were listed as follows: IER5 forward 5'-AGGCTCATCGCATCGTCAG-3', IER5 reverse 5'-CGCTCAGGTAGACTTGGCG-3'; CDC25B forward 5'-ACGCACCTATCCCTGTCT-C-3', CDC25B reverse 5'-CTGGAAGCGTCTG-ATGGCAA-3'; β-actin forward 5'-ATCACCATT-GGCAATGAGCG-3', β-actin reverse 5'-TTGAA-GGTAGTTTCGTGGGAT-3'. The specificity of the primers was tested by 1% agarose gel eletro-phoresis (**Figure 1**). The Real-time RT-PCR Kit (TIANGEN, China) and 7300 real-time PCR system were introduced to detect the relative expression of IER5 and CDC25B mRNA and

$\Delta\Delta$Ct was calculated. The program includes 95°C for 10 min and 45 cycles of 95°C for 30 s, 63°C for 20 s and 68°C for 20 s. The products were also validated by 1% agarose gel electro-phoresis (**Figure 2**).

Western blot

A 60 mm dish of HepG2 cells at 90% confluency were washed by phosphate buffered saline (PBS) and collected. Cell lysis buffer (5 mM PIPES at pH 8.0; 85 mM KCL; 0.5% NP-40; protease inhibitors) was added and the supernatant was kept after 12,000 rpm centrifugation for 5 min. The protein samples were quantified
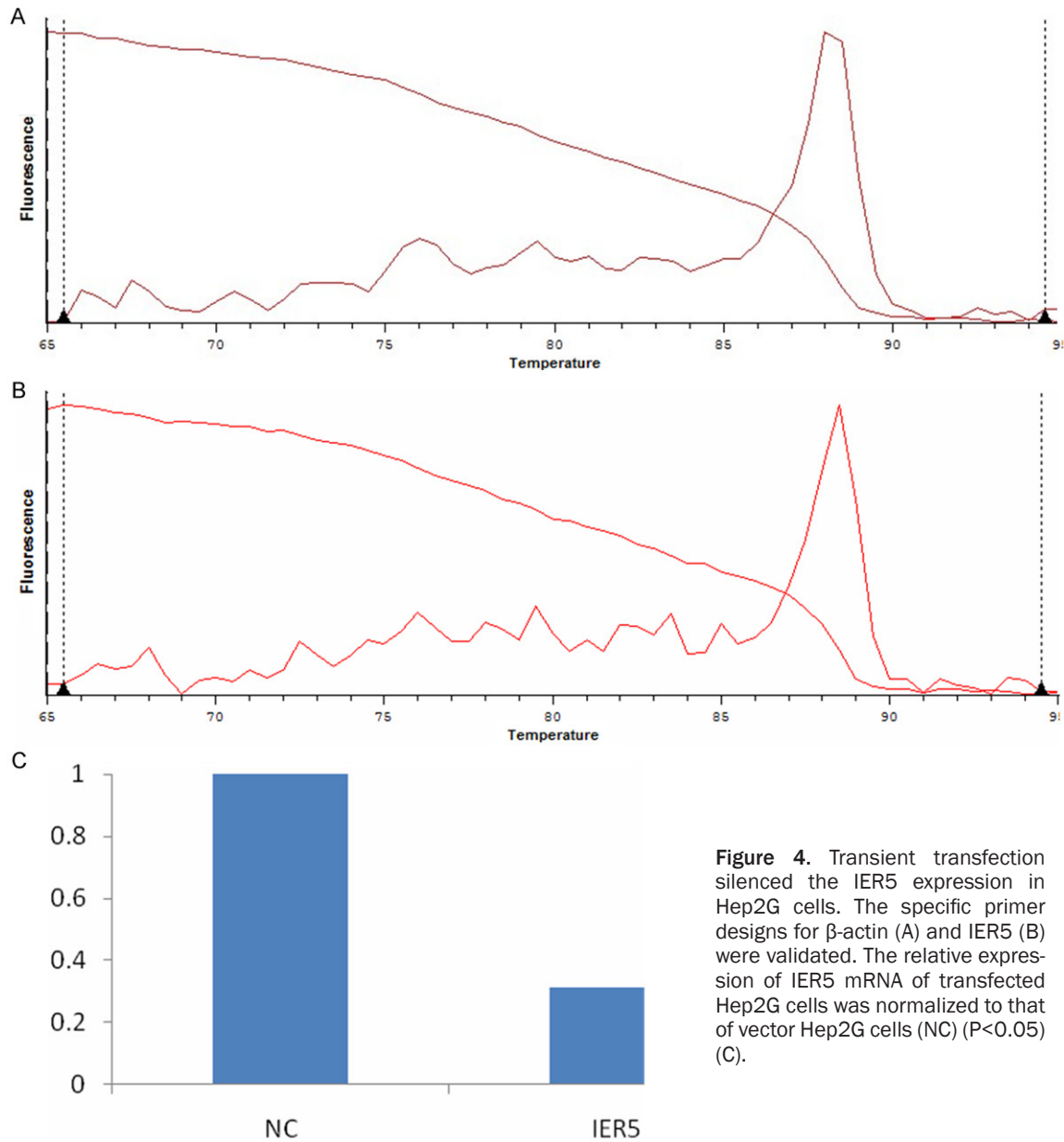
Figure 4. Transient transfection silenced the IER5 expression in Hep2G cells. The specific primer designs for β-actin (A) and IER5 (B) were validated. The relative expression of IER5 mRNA of transfected Hep2G cells was normalized to that of vector Hep2G cells (NC) ($P<0.05$) (C).

Figure 5. IER5 protein level was detected by Western blot. The result confirmed that IER5 were overexpressed in HepG2 cells after transfection.

The membrane was incubated in 8% nonfat milk TBST blocking buffer for 1 h, 1:500 diluted goat Anti-IER5 antibody (Abcam, USA) for 2 h and rabbit anti-goat IgG antibody (Abcam, USA) for 1 h at room temperature. β-actin was the internal control and the intensity of the band was detected by ECL Western Blotting Substrate (Pierce, USA).

Chromatin immune coprecipitation (CHIP)

by BCA Protein Assay Kit (Pierce, USA). SDS-PAGE gel was freshly prepared and 40 μg total protein was loaded in each well. Then, the gel was transferred to nitrocellulose membrane.

A count of $1×10^6$ HepG2 cells in a 100 mm dish were treating by 1% formaldehyde at 37°C for 10 min and then 0.125 M Glycine at 25°C for 5 min. After two washes by cold PBS, cells were

scraped and collected. Cell lysis buffer and nucleus lysis buffer (50 mM Tris-HCl at pH 8.1; 10 mM EDTA; 1% SDS; protease inhibitors) were added. The samples were then diluted by ChIP Dilution buffer (0.01% SDS; 1.1% Triton X-100; 1.2 mM EDTA; 16.7 mM Tris-HCl at pH 8.1) and incubated with IER5 antibody at 4°C overnight. After removing the supernatant, the precipitation was washed by CHIP high salt wash buffer (25 ml 1 M HEPES at pH 7.5; 62.5 ml 4 M NaCl; 50 ml 10% Triton X-100; 5 ml 10% Sodium Deoxycholate; protease inhibitors for 500 ml), CHIP low salt wash buffer (25 ml 1 M HEPES at pH 7.5; 18 ml 4 M NaCl; 50 ml 10% Triton X-100; 5 ml 10% Sodium Deoxycholate; protease inhibitors for 500 ml) and CHIP LiCl wash buffer (5 ml 1 M Tris at pH 8.0; 5.3 g LiCl; 25 ml 10% NP-40; 25 ml 10% Sodium Deoxycholate; 1 ml 0.5 M EDTA for 500 ml), and TE buffer (10 mM Tris-HCl at pH 8.0; 1 M EDTA) in turn, and finally by Elution buffer (0.5 ml 1 M Tris at pH 8.0; 1 ml 10% SDS; 0.2 ml 0.5 M EDTA for 10 ml). DNA was extracted and precipitated. The target gene CDC25B was amplified by PCR (forward primer 5'-AGCCGGGTTG-ACAGAGGGAGAC-3'; primer 5'-AACGGTGGAAC-TAGGAATGGA-3'), and validated by 2% agarose gel electrophoresis.

Statistical analyses

The statistical analyses were conducted using SDS software. Continuous data are shown as mean ± standard deviation (SD), and categorical data are described as percentage (%). The differences between treat and non-treat groups were tested by the Student's t test. A *P* value <0.05 was considered to be statistically significant.

Results

Radiation regulated IER5 and CDC25B mRNA levels in Hep2G cells

The effect of radiation on the expression of IER5 and CDC25B was investigated in PHC in vitro. Two dosages of 2Gy and 4Gy ^{60}Co-γ ray were selected to treat Hep2G cells. The IER5 and CDC25B mRNA levels at different time points were compared, using untreated Hep2G cells as controls. Real-time RT-PCR was used to detect the changes of IER5 and CDC25B mRNA under radiation. Results showed that the IER5 mRNA was increased and then decreased,

reaching the peak at 6 h, while CC25B mRNA tended to decrease first and then increase with the lowest point at 6 h. These data indicated that radiation could regulate the IER5 and CDC25B mRNA level. The dosage-dependent correlation of radiation with IER5 and CDC25B mRNA levels were shown in **Figure 3**.

IER5 silencing abolished the effect of radiation on CDC25B expression

The IER5 level in Hep2G cells was knocked down by transient transfection (**Figure 4**). In IER5 silencing Hep2G cells, radiation could not induce similar changes on the IER5 and CDC25B mRNA levels, indicating that IER5 may play a key role in modulating the function of CDC25B.

IER5 overexpression decreased the expression of CDC25B in Hep2G cells

The IER5 was overexpressed in Hep2G cells, validated by Western blot (**Figure 5**). In IER5 overexpressing Hep2G cells, the radiation of 2Gy and 4Gy could also induce the increase on IER5 mRNA, but the decrease on CDC25B mRNA, which also supported that the IER5 and CDC25B mRNA expressions were negatively correlated (**Figure 6**).

IER5 upregulation by radiation could competitively bind to CDC25B promoter DNA

After being treated by 4Gy radiation for 6 h, Hep2G cells were collected and CHIP was conducted. The results demonstrated that IER5 could competitively bind with the promoter region of CDC25B gene, which is a key gene in regulating cell cycle (**Figure 7**). Taken together, the protein level of IER5 is supposed to modulate the transcription of CDC25B, thus controlling the cell cycle in Hep2G cells.

Discussion

IER5 is one member of the early slow reaction gene family, which could be dynamically induced by c-fos. There were 2123 nucleotides in IER5 gene and no introns. The protein encoded by IER5 gene is a proline-rich nuclear protein with three nuclear targeting sites and multiple potential phosphorylation sites. External and/or internal stimulus could modulate the phosphorylation/dephosphorylation of IER5,
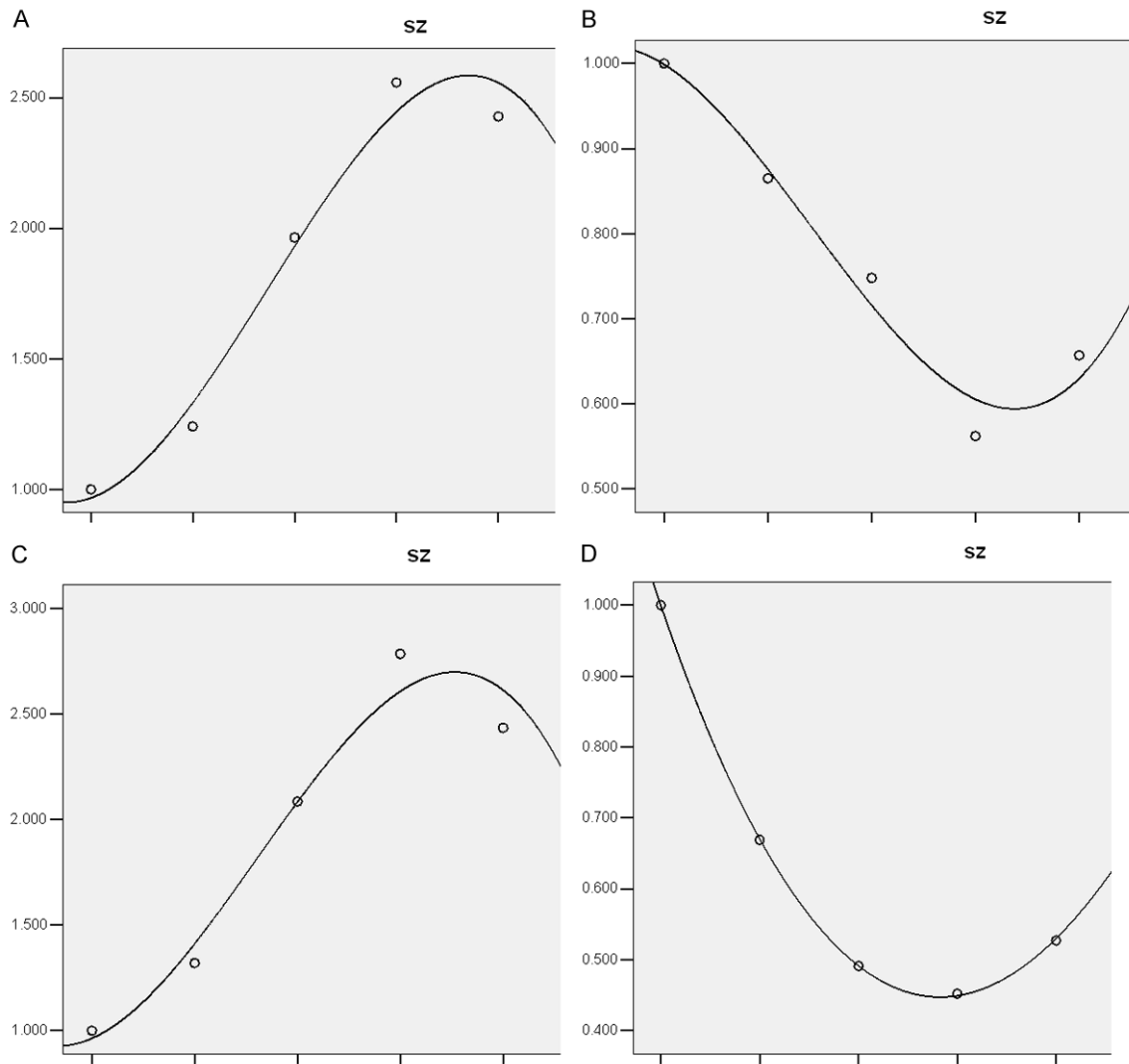
Figure 6. Real-time RT-PCR detected increased IER5 mRNA expression (A) (Y=1.157-0.574X-0.437X2-0.053X3, R2=0.979, P=0.032) but decreased CDC25B mRNA expression (B) (Y=1.000+0.089X-0.104X2+0.014X3, R2=0.970, P=0.044) in IER5 overexpressing HepG2 cells under 2 Gy radiation. Similar trends on IER5 (C) (Y=1.109-0.566X+0.482X2-0.062X3, R2=0.966, P=0.050) and CDC25B mRNA expression (D) (Y=1.493-0.580X+0.089X2-0.002X3, R2=1.000, P=0.000) under 4 Gy radiation were also observed.
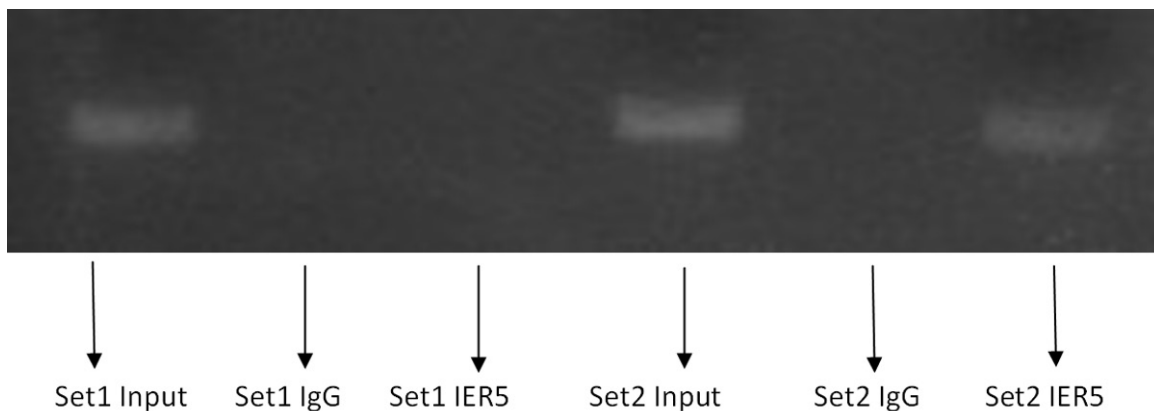

Figure 7. CHIP supported that IER5 could bind to CDC25B promoter.

thus functioning as a regulator of the cell mitosis [7]. The presence of PEST sequence could rapidly degrade, which widely exists in all kinds of cells and tissues. Studies have ever demonstrated that IER5 plays important roles in regulating cell cycle and cell apoptosis. But the specific molecular mechanism remains unclear. It is supposed that the identification of IER5 as one member of early response family may be due to its ability to directly upregulate the expression by promoting the protein synthesis when stimulated. The expression of IER5 is dependent on the phase of cell cycle, indicating IER5 may be a key player in the control of the cell cycle. The function of IER5 gene is also sensitive to radiation. It has been reported that the radiation of 2Gy and 4Gy could increase the transcription within a short period in AHH-1 and Hela cells, which is time-dependent [8].

CDC25B gene is located in chromosome 20p13 [3]. For one thing, CDC25B could activate RAS gene, which may induce the malignant transformation of specific cells. CDC25B gene also regulates the gene transcription in the early stage of the cell cycle. CDC25B knockdown might lead to cell cycle arrest at S phase or even earlier. For another thing, CDC25B serves as a checkpoint of the cell cycle, and the activation of CDK1/Cyclin B by CDC25B will further trigger the process of cell mitosis. In addition, CDC25B is differentially overexpressed in multiple human malignant diseases like hepatocellular carcinoma, esophageal squamous cell carcinoma, pancreatic cancer, gastric carcinoma, colon cancer, breast cancer, ovary cancer, uterine endometrial carcinoma, head and neck cancer, lung non-small cell carcinoma, non-Hodgkin lymphoma, neuroblastoma and thyroid cancer [9-14], which is associated with malignancy, metastasis and prognosis [15]. The current findings suggest that CDC25B might be a potential biomarker for diagnosis and therapy, considering that CDC25B plays key roles in the development and progression of human cancers [16].

Our present study indicated that radiation could regulate the expressions of IER5 and CDC25B in Hep2G cells. Furthermore, IER5 level was negatively correlated with CDC25B level. To better clarify the association of IER5 with CDC25B, we then conducted CHIP assay, which proved that IER5 protein could bind to the promoter of CDC25B gene, and the com-

plexes may influence the cell cycle and cell mitosis. Nowadays, more and more evidence has reached the conclusion that CDC25B is a very important regulatory factor in the carcinogenesis and our findings in this study might enrich the current understanding on the function and molecular mechanism of IER5 in cell cycle and cell apoptosis. However, further investigations on the IER5 related signaling pathways still need to be conducted, which will provide a new therapeutic target for treating human cancers.

Disclosure of conflict of interest

None.

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