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Original Article

IER5 promotes irradiation- and cisplatin-induced apoptosis in human hepatocellular carcinoma cells

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Abstract: Purpose: To elucidate the mechanisms of the immediate-early response gene 5 (IER5) effect on the apoptosis induced by irradiation and cisplatin (CDDP) in human hepatocellular carcinoma ($HepG_2$) cells. Methods: We generated IER5 overexpression stable cells ($HepG_2$/IER5) using Lipofectamine 2000 transfection $HepG_2$ cells. Cell apoptosis was induced by irradiation and cisplatin treatments, and cell proliferation (viability) and apoptosis were evaluated by MTT and flow cytometry assays. Protein expression was determined by Western blot. Results: The growth of the IER5 overexpression cells was significantly inhibited after six days of ^{60}Co γ-irradiation exposure ($p<0.01$) compared with the cell growth of vector control cells. Furthermore, the $HepG_2$/IER5 cells were arrested at the G2/M phases. We also found that the expression of phospho-Akt was reduced, and the levels of cleaved caspase-3 and PARP were increased after the treatment of $HepG_2$/IER5 cells with γ-irradiation and cisplatin. Conclusion: Our results suggest that the overexpression of IER5 can inhibit cell growth and enhance the cell apoptosis induced by exposure to radiation or cisplatin. The overexpression of *IER5* can be utilized as a targeting strategy to improve the outcomes of radiotherapy used for the treatment of patients with liver cancer.

Keywords: IER5, apoptosis, γ-irradiation, cisplatin, human hepatocellular carcinoma

Introduction

Primary hepatocellular carcinoma (HCC) is one of the most widespread malignancies in the world. As indicated in human cancer statistics, the disease is the third most common cause of cancer-related deaths worldwide [1]. HCC often develops in patients suffering from chronic liver diseases associated with hepatitis B (HBV) or hepatitis C (HCV) virus infections. The natural development of this disease (without treatment) is exceedingly severe and leads to death in a short period of time [2]. Currently, liver resection and transplantation are both well-established options for the curative treatment of HCC. However, surgical resection is often accompanied by a high recurrence rate, and transplantation is not universally applicable. Radiofrequency ablation therapy and the percutaneous ethanol injection therapy are also used as curative treatments for HCC [1]. So far, there have been only limited effective therapeutic modalities for HCC patients [3]. Moreover, some of these treatment options have not been successfully applied in the therapy of HCC patients.

Radiotherapy is now becoming the most important treatment strategy in the treatment of unresectable HCC patients [4]. However, irradiation can indirectly activate H_2O and yield a large quantity of excessive free radicals, breaking the balance between the oxidant and antioxidant systems and leading to oxidative stress in the cells [5]. Recently, the more precise treatment by accurate delivery of radiation to the target tumor tissue using 3-dimensional conformal radiation therapy has improved tumor con-

trol and decreased treatment-related toxicity [5]. Nevertheless, radiotherapy still has some limitation in the treatment of HCC patients and early reports evidenced that liver tolerance to radiation was far lower than the required therapeutic radiation dose [1, 6]. Therefore, our strategy is to increase the liver tumor sensitivity to radiation therapy.

By using quantitative real-time PCR analysis, some studies have confirmed that the radiation treatment can increase *IER5* gene transcription. The authors found that the level of *IER5* mRNA was dependent on the radiation dose and the duration of the treatment. The expression level of IER5 in AHH-1 and HeLa cells was increased as early as 2 h after exposure to a radiation dose of 2 Gy and reached a peak shortly afterwards. The suppression of IER5 by RNA interference technology dramatically increased the radioresistance of HeLa cells up to radiation doses of 6 Gy and the radiation induced G2/M phase cell cycle arrest G2/M. These data suggested that IER5 expression could play important roles in the cell death induced by radiation [7].

IER5, which belongs to the group of immediate-early genes, was upregulated in waking and sleep deprivation [8]. IER5 is a nuclear protein which contains a PEST-like sequence and multiple phosphorylation sites and can cause rapid protein degradation. The promoter sequence of *IER5* gene contains some transcription factor-binding sites [9]. The *IER5* gene is well known as a key molecule in cell cycle control because of its specific and periodic expression during cell cycle progression [9].

Currently, improving the overall strategy for the treatment of liver cancer depends mainly on the combination of multiple therapies. The purpose of the combined multiple therapies for HCC is to increase the overall therapeutic efficiency and to reduce the side effects and medical complications. Inducing apoptosis has become an attractive strategy for cancer therapy.

In our study, we aimed to investigate the function of IER5 in ^{60}Co γ-irradiation-induced $HepG_2$ cell cycle progression and apoptosis and to examine the molecular mechanisms of tumor sensitivity to radiation therapy related to IER5 expression in human hepatocellular carcinoma cells. Herein, we highlighted that the overexpression of IER5 protein enhanced irradiation-induced cell apoptosis. The findings of this study can contribute to understanding the influence of IER5 on tumor sensitivity to radiation and facilitate the development of a new cancer treatment strategy.

Materials and methods

Reagents, antibodies, and cell lines

The anti-Flag and anti-β-actin antibodies were purchased from Sigma Aldrich; antibodies anti PARP, caspase-3, Akt, p-Akt, and p73 were obtained from Cell Signaling Technology; antibodies anti Bcl-2, Bcl-x, and Bax were acquired from Santa Cruz Biotechnology. The antibodies anti-p21 and p53 were purchased from Calbiochem, whereas the antibody anti-IER5 was purchased from Abcam. All reagents, including fetal bovine serum (FBS), penicillin G, streptomycin, G418, dimethyl sulfoxide (DMSO), ribonuclease (RNase), and propidium iodide (PI) were purchased from Invitrogen.

Cell lines

The human hepatocellular carcinoma cell line, $HepG_2$, was a generous gift from the Fourth Laboratory, Institute of Medical Radiology, the Academy of Military Science of China. The cells were cultured in DMEM (GIBCO) with 10% FBS (GIBCO), 2 mM L-glutamine, and 1% penicillin-streptomycin at in an incubator maintaining 37°C and a humidified atmosphere containing 5% CO_2.

Cell transfections

$HepG_2$ cells were transfected with Pcmv-3 × Flag or 3 × Flag-IER5 plasmids using Lipofectamine 2000TM (Invitrogen) according to manufacturer's instructions. Stable positive cell clones ($HepG_2$/IER5, $HepG_2$/Vector) were selected in medium supplemented with G418.

Flow cytometry analysis

The $HepG_2$/IER5 and $HepG_2$/Vector cells were plated in 6-well plates (5×10^4 cells/well) in DMEM growth medium and were cultured overnight. Then, the cells were exposed to 4 Gy of γ-ray irradiation and collected after treatment durations of 12 h and 24 h. Next, they were fixed by 70% ethanol and washed with PBS.

Further, the cell pellets were suspended in 200 uL of 1x propidium iodide (PI)+ RNase Staining Solution and incubated at 37°C for 30 min in the dark. The DNA histograms and cell cycle phase distributions of the 20,000 cells in the suspension were analyzed by flow cytometry (FACS Calibur instrument; Becton Dickinson), and the data were analyzed using the CELL-Quest software.

Cell viability assay (MTT)

The cells were seeded in 96-well plates at an initial density of 2000 cells per well and were cultured overnight. Then, the cells were exposed to 0 and 4.0 Gy of γ-ray irradiation at a dose rate of 5.0 cGy/min. After 24-h and 48-h exposure to radiation, the medium was removed. The MTT reagent (Sigma) was added, and the cells were incubated for an additional 4 h at 37°C. Afterwards, 10% sodium dodecyl sulfate was added to dissolve the blue formazan precipitate, and the absorbance at 492 nm was measured in an enzyme-linked immunosorbent assay (ELISA) reader. All experiments were run with at least five replicate cultures and repeated three times.

Cells and gamma-irradiation (γ-irradiation) treatment

The $HepG_2$/IER5 and $HepG_2$/Vector cells were plated in 6-well plates (5×10^4 cells/well) and were exposed to 0 and 4.0 Gy of γ-irradiation from a radioactive telecobalt therapy source at a dose rate of 5.0 cGy/min. Then, the cells were kept at 37°C in an atmosphere with 5% of CO_2 for the later experiments.

Western blot (protein immunoblot)

Cells (0.3×10^6) were collected at 0 h, 24 h, and 48 h after the irradiation treatment (irradiation dose of 4 Gy) and washed twice with ice-cold phosphate buffered saline (PBS). Further, they were resuspended and sonicated in lysis buffer (20 mM Hepes, pH 8.0, 150 mM KCl, 5% glycerol, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.02% NP-40, supplemented with NaF, $NaVO_4$, PMSF, and protease inhibitors). The cell supernatants were collected after centrifugation for 30 min at 14,000 rpm. The protein concentrations were determined using the standard Bradford assay (Bradford reagent was supplied by Bio-Rad). Equal amounts of protein were separated on 10% and 12% polyacrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting was performed using the indicated antibodies. The protein was transferred to PVDF membranes in transfer buffer (50 mM Tris, 190 mM glycin, and 10% methanol) at 100 V for 2 h. The membranes were incubated with blocking buffer (5% non-fat milk, 0.1% Tween in PBS, pH 7.4) for 1 h at 4°C. The membranes were then washed three times (10 min each time) with the washing buffer, (50 mM Tris, 200 mM NaCl, and 0.2% Tween 20). The blots were incubated in an antibody solution containing the indicated primary antibodies at 4°C overnight. After that, the blots were incubated in a secondary antibody solution for 1 h at room temperature. The membranes were washed three times (10 min each time). The indicated proteins were detected by the enhanced chemiluminescence Western blotting detection system and autoradiography according to the manufacturer's protocol. Each experiment was carried out in triplicate and performed at least three times.

Results

The overexpression of IER5 significantly inhibited the growth of $HepG_2$ cells

We generated a stable IER5 overexpression cell line ($HepG_2$/IER5) using IER5-3 × Flag vectors transfected in $HepG_2$ cells according to the Lipofectamin 2000 transfection procedure. G418 was used for cell selection. Stable cells without IER5 expression, $HepG_2$/vector (lacking an IRE5 cDNA insert as a negative control), were also produced through the Pcmv-3 × Flag vector. To reduce the influence of endogenous IER5 gene expression, we knocked down the endogenous *IER5* gene using specific IER5 siRNA. IER5 overexpression was verified by Western blot analysis using anti-IER5 or anti-Flag antibodies. High IER5 protein expression was manifested in $HepG_2$/IER5, but not in $HepG_2$/vector cells (**Figure 1A**, **1B**).

We first explored the effect of IER5 overexpression on the apoptosis induced by γ-ray irradiation. The stable cells were treated with γ-ray irradiation at a dose of 4 Gy for six days. We found that the number of $HepG_2$/IER5 cells was significantly reduced compared with that of the vector control cells ($P<0.05$). Interestingly, the growth of the untreated (IER5-0Gy) cells was

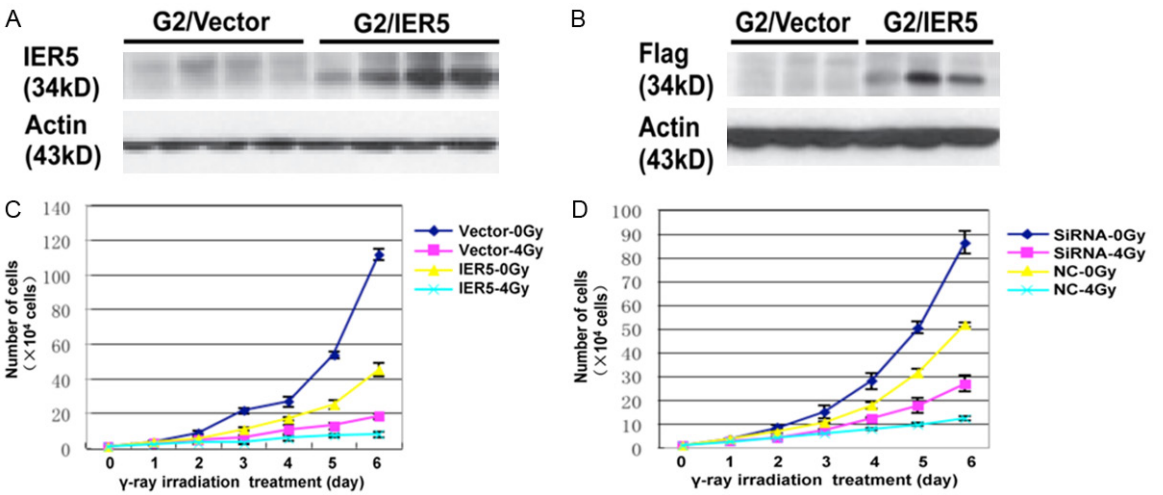

Figure 1. The overexpression of IER5 in $HepG_2$ suppressed cell growth. A, B. Detection of IER5 expression in $HepG_2$ cells by Western blotting using anti-IER5 (IER5) or Flag antibodies. Actin was used as a loading control. G2/Vector: $HepG_2$ cells transfected with the vector control plasmid; G2/IER5: $HepG_2$ cells transfected with the IER5 expression plasmid. The G2/IER5 cells showed higher IER5 expression compared with that of the G2/Vector cells. C. The overexpression of IER5 inhibited cell growth. $HepG_2$ cells transfected with IER5 expression (IER5-4Gy) or vector control (Vector-4Gy) plasmids were treated with γ-ray irradiation at a dose of 4 Gy for six days. The number of IER5-4Gy cells was significantly reduced compared with that of Vector-4Gy cells (P<0.05). IER5-0Gy: The $HepG_2$ cells transfected with the IER5 expression plasmid were not treated with γ-ray irradiation; Vector-0Gy: The $HepG_2$ cells transfected with the vector control plasmid were not treated with γ-ray irradiation. D. Knockdown of IER5 expression promoted cell growth. siRNA-4Gy: IER5 knocked down the cells transfected with IER5-siRNA that were treated with γ-ray irradiation at a dose of 4 Gy; siRNA-0Gy: the IER5 knocked down the untreated cells transfected with IER5-siRNA; NC-4Gy: $HepG_2$ cells were treated γ-ray irradiation at a dose of 4 Gy; NC-0Gy: $HepG_2$ cells were not treated with γ-ray irradiation.

also inhibited in comparison with that of the control cells (Vector-0Gy), which indicated that IER5 overexpression could suppress cell growth (**Figure 1C**). In confirmation of this finding, we also discovered that the knockdown of IER5 considerably promoted the cell growth in both the cells treated with γ-ray irradiation (SiRNA-4Gy) and those with no treatment (SiRNA-0Gy) (**Figure 1D**). Taken together, these results suggested that the γ-ray irradiation-induced IER5 overexpression inhibited cell growth and enhanced cell apoptosis.

IER5 enhanced the irradiation-induced $HepG_2$ cell apoptosis by increasing the cell cycle arrest at the G2/M phase

G2/M: To determine the influence of IER5 on the cell cycle distribution, we treated the $HepG_2$/IER5 cells with γ-ray irradiation and measured the cell cycle distribution using flow cytometric analysis. The results showed a significant increase of the cell population in G2/M phase compared with the control after both durations of the irradiation treatment, 12 h (p<0.01) and 24 h (p<0.05) (**Figure 2A**, **2B**). We then investigated if the knockdown of IER5 still exerted an effect on cell circle distribution, while the cell population showed no difference between the IER5 knockdown cells and the control cells (**Figure 2C**, **2D**). These results indicated that the overexpression of IER5 increased the cell cycle arrest at the G2/M phase and enhanced the cell apoptosis induced by irradiation.

IER5 enhanced the irradiation- and cisplatin-induced apoptosis by increasing the cleavage of caspase-3 and PARP

To further explore the mechanism of the influence of the overexpression of IER5 on tumor apoptosis, we examined the expression of some factors involved in the apoptosis signaling pathway by Western blot analysis. The protein lysates from $HepG_2$/IER5 and control cells were subjected to the Western blotting after these cells had been exposed to 4 Gy of γ-ray irradiation for 24 h and 48 h. The results indicated that cleavage caspase-3 and cleavage PARP were dramatically increased in the $HepG_2$/IER5 cells after their treatment with

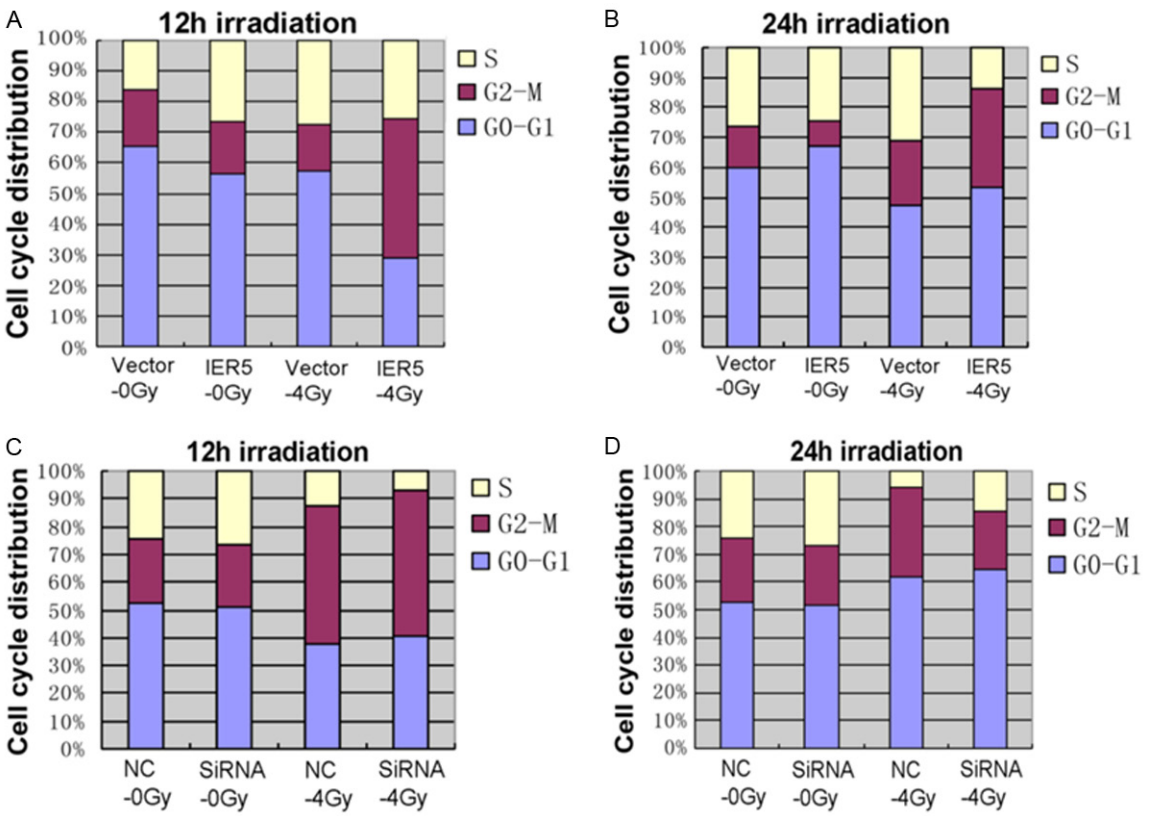

Figure 2. The overexpression of IER5 in $HepG_2$ affected the cell cycle distribution. A, B. The $HepG_2$/IER5 and $HepG_2$/Vector cells were treated with 4 Gy of γ-ray irradiation for 12 h and 24 h. The $HepG_2$/IER5 cells showed a significant increase in the cell population in G2/M phase compared with the $HepG_2$/Vector control cells treated with radiation for 12 h ($p<0.01$) or 24 h ($p<0.05$). C, D. The cell cycle distribution showed no difference between IER5 knockdown cells (SiRNA-4Gy) and the control cells (NC-4Gy) after the irradiation treatment. The cell cycle distribution was determined using flow cytometric analysis. Each value is expressed as the mean ± SD of three independent experiments.

γ-ray irradiation compared with $HepG_2$/Vector cells (**Figure 3A**). This suggested that the overexpression of IER5 could promote irradiation-induced tumor cell apoptosis by enhancing the cleavage of caspase-3 and PARP. Interestingly, the phospho-Akt level was reduced in the $HepG_2$/IER5 cells, but not in the $HepG_2$/Vector control cells (**Figure 3B**), which implied that the cell survival signaling pathway was blocked by IER5. Next, to evaluate the action of the overexpression of IRE5 on the cell apoptosis induced by the anti-malignancies chemotherapy drug cisplatin (CDDP), we treated the $HepG_2$/IER5 and $HepG_2$/Vector cells for 3 h, 6 h, and 9 h with 100 nM of cisplatin (CDDP). We found that the cleavage PARP and cleavage Caspase-3 were also dramatically increased in the $HepG_2$/IER5 cells in comparison with the $HepG_2$/Vector control cells (**Figure 3E**). Taken together, we confirmed that the overexpression of IER5 could enhance both the irradiation-induced and the cisplatin-induced apoptosis in human hepatocellular carcinoma cells. However, the results showed no changes in IER5 overexpression cells versus control cells treated with γ-ray irradiation for other tumor suppressor genes or oncogenes involved in the apoptosis signaling pathway, such as P53, P21, and P73, or the Bcl-2 family members, including the genes Bcl-2, Bcl-x, and Bax (**Figure 3C**, **3D**).

IER5 inhibited the survival of tumor cells by enhancing the irradiation-induced apoptosis

Then, to examine the impact of IER5 on tumor cell survival, we measured the $HepG_2$/IER5 cell viability using MTT assay, as described in the Materials and Methods. We found that the cell survival was significantly inhibited in the $HepG_2$/IER5 cells treated with γ-ray irradiation versus the no-treatment control at either the 24-h ($p<0.01$) or the 48-h ($p<0.05$) time point, but the cell survival was not suppressed in the $HepG_2$/vector control cells (**Figure 4A**, **4B**).

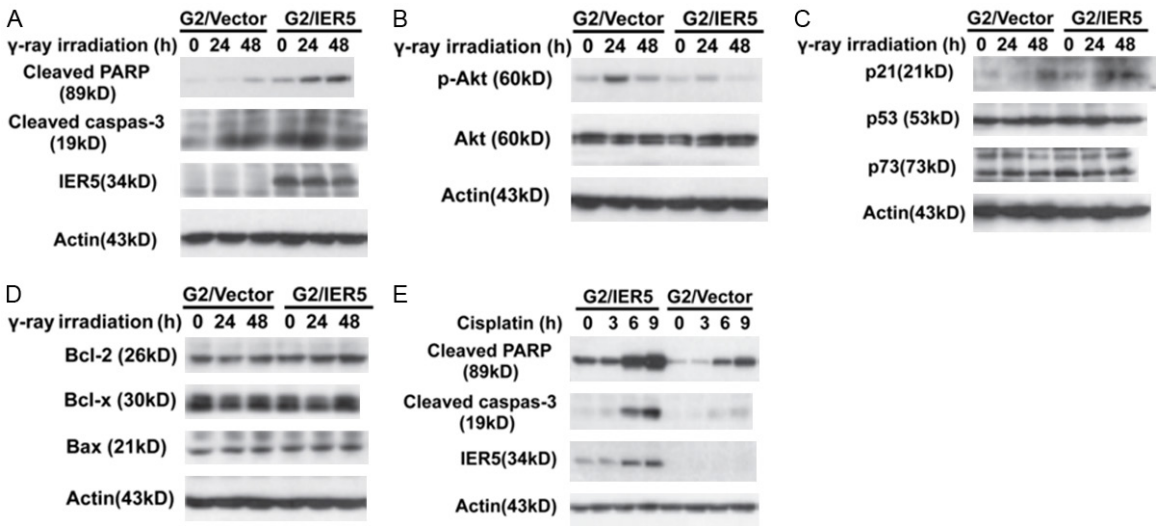

Figure 3. The overexpression of IER5 enhanced the cleavage of caspase-3 and PARP. (A-D) The $HepG_2$/IER5 and $HepG_2$/Vector cells were grown to 60% confluence and then were incubated overnight in medium with 10% serum. The cells were treated with 4 Gy of γ-ray irradiation and were harvested at 0, 24 h, and 48 h after the treatment. Protein lysates were subjected to Western blot using the indicated antibodies. (A) The levels of cleavage caspase-3 and cleavage PARP were increased in the $HepG_2$/IER5 cells treated with γ-ray irradiation compared with the $HepG_2$/Vector cells; (B) The phospho-Akt level was reduced in the $HepG_2$/IER5 cells treated with γ-ray irradiation; (C, D) No differences were detected between $HepG_2$/IER5 cells and $HepG_2$/Vector cells concerning the expression of other genes, such as P53, P21, P73, or the Bcl-2 family members, including Bcl-2, Bcl-x, Bax genes. Actin was used as loading control; (E) $HepG_2$/IER5 and $HepG_2$/Vector cells were treated with 100 nM of cisplatin (CDDP) for 3 h, 6 h, and 9 h. The protein lysates were subjected to Western blot analysis, and the levels of cleavage PARP and cleavage Caspase-3 were increased in the $HepG_2$/IER5 cells in comparison with the $HepG_2$/Vector control cells.

However, the inhibition of cell survival was not detected when we knocked down the *IER5* gene using *IER5*-specific siRNA (**Figure 4C**, **4D**). These results indicated that IER5 was the major factor that inhibited the tumor cell survival and enhanced irradiation-induced apoptosis.

Discussion

Human hepatocellular carcinoma (HCC) is the most common primary malignant tumor and the second leading cause of cancer mortality in China. Surgical resection has been accepted as the major therapy for primary liver cancer. Unfortunately, most patients are surgically unresectable. Radiation therapy has also been commonly used in the treatment of unamenable human hepatoma. However, the main disadvantage of this treatment is the tolerance to radiation exposure. New approaches that reduce side effects and provide good quality of life are required. Thus, it is imperative to improve tumor sensitivity to radiation therapy.

Radiotherapy is an efficient and widely used method for the treatment of cancer if the optimal and effective for tumor control radiation dose is correctly determined, and minimal exposure to the surrounding normal tissue is ensured. Tumor sensitivity to radiation exposure that is higher than that of the surrounding normal tissues would be exceedingly beneficial to HCC patients [10]. Up to now, some effective methods for prediction of optimal radiosensitivity and the most relevant parameters for its achieving have been discovered [11, 12]. Findings of experimental studies and clinical trials have been reported that molecular diagnosis or gene therapy strategies can be used in advanced symptomatic HCC patients to obtain an effective treatment outcome [13]. In our study, we discovered that the overexpression of IER5 can enhance the tumor cells sensitivity to radiation exposure. Further, we found that the proliferation of $HepG_2$/IER5 cells was significantly decreased compared with that of the $HepG_2$/Vector control cells after the γ-ray irradiation treatment (**Figure 4A**, **4B**). The results indicated that IER5 could successfully inhibit cell proliferation.

IER5 is an intronless gene which encodes a serum- and growth factor-inducible message of

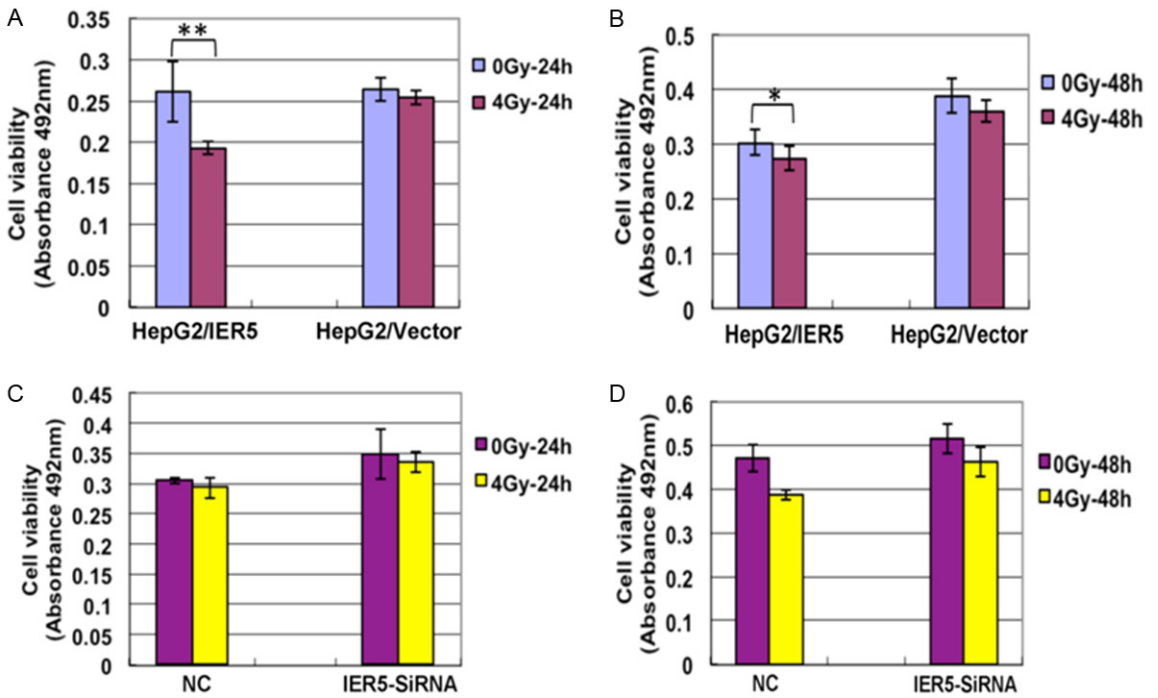

Figure 4. Detection of cell viability by MTT assay. (A, B) Cell viability was measured in the $HepG_2$/IER5 or $HepG_2$/vector control cells treated with γ-ray irradiation for 24 h (A) and 48 h (B). There was a significant difference between the viability of the $HepG_2$/IER5 cells treated with γ-ray irradiation and the untreated cells at 24 h ($p<0.01$) or 48 h ($p<0.05$) treatment time points. However, no difference was found in the $HepG_2$/vector control cells; (C, D) Cell viability was measured in IER5 knockdown cells (IER5-SiRNA) or control (NC) cells treated with γ-ray irradiation for 24 h (C) and 48 h (D). In the IER5 knockdown cells, no difference was established between the cell viability in the γ-ray irradiation treatment versus the untreated control at either 24 h or 48 h treatment duration. The values are expressed as the mean ± SD of three independent experiments (n=3). *, Represents a significant difference ($p<0.05$); **, Denotes a highly significant difference ($p<0.01$).

2123 nucleotides that is present in a wide variety of tissues [9]. *IER5* gene belongs to the slow-kinetics immediate-early gene family, which exhibits growth factor induction kinetics similar to that of *pip92/IER2/ETR101* [9]. In our investigation, we evidenced that IER5 was an important factor that regulates cell cycle progression and apoptosis. The pretreatment of hepatocellular carcinoma cells with overexpressed IER5 protein enhanced cell killing induced by irradiation. The overexpression of IER5 caused a significant increase in tumor cell death when it was combined with a treatment with moderate doses of radiation (**Figure 4A**, **4B**).

DNA is the major target for cell killing by radiation [10, 14]. Some rare hereditary conditions demonstrate a high cancer risk and hypersensitivity in response to exposures to agents, such as ultraviolet or ionizing radiation, and which are characterized by a defective processing of DNA damage [11]. Ionizing radiation exposure gives rise to a variety of lesions in DNA that result in genetic instability and potentially tumorigenesis or cell death. It has been confirmed that the individual risk of cancer may be related to the ability of a cell to identify and repair DNA damage. Moreover, individuals who were genetically susceptible to cancer manifested increased DNA radiosensitivity [12, 15]. This hypersensitivity is a response specific to G2-phase cells, and it is directly linked to the failure in the activation of the ataxia-telangiectasia mutated (ATM)-dependent early G2/M checkpoint after the application of low treatment doses [10, 16, 17]. The mechanisms of DNA damage-induced apoptosis are realized through the inactivation of DNA repair and eventually result in cell death [18].

We found that the overexpression of IER5 caused an apparent accumulation of cells in the G2/M phase at both 12 h ($p<0.01$) and 24

h ($p<0.05$) irradiation treatment durations (**Figure 2A**, **2B**). However, the cell population showed no increase in G2/M phase when we knocked down *IER5* gene using *IER5*-specific SiRNA (**Figure 2C**, **2D**). These results suggested that the overexpression of IER5 increased cell cycle arrest at the G2/M phase, promoted apoptosis, and enhanced the tumor sensitivity to radiation treatment. Our results were consistent with the findings of other studies revealing that the suppression of IER5 by RNA interference technology dramatically increased the radioresistance of HeLa cells to radiation treatment [7]. HCC has been reported to be exceedingly resistant to radio- or chemotherapy [13]. Our results indicated that the upregulation of IER5 protein could be used to promote tumor cell responses to the treatment with radiation and might become a new strategy for hepatocellular carcinomas therapy. The findings of the present examination might also contribute to understanding better tumor radiosensitivity, which will exert favorable effects on the therapeutic outcomes in liver cancer patients.

Previous studies have shown that cell proliferation and apoptosis are critical steps in tumor metastasis. In an investigation, the imbalance between cell proliferation and apoptosis enhanced the growth of tumor cells [19]. Apoptosis is essential for the maintenance of inherited genomic integrity and is a fundamental cellular process which orchestrates a series of controlled events and ultimately leads to cell death [18]. Caspases are major proteases involved in apoptosis. The caspase family members contribute to cellular disintegration via the action of cleavage proteins involved in many processes in the cell, such as DNA repair and checkpoint activation [20]. Caspase-3 is a key protease that controls other caspase members in the programmed cell death (PCD) [21]. Impaired apoptosis is a crucial step in the process of cancer development [22]. Of the members of the caspase family, caspase-3, caspase-6, and caspase-7 have been shown to be the major effectors in apoptosis [18, 23]. Many anticancer agents induce apoptosis, which trigger the apoptosis via caspase activation [3, 24-26]. Our results indicated that the overexpression of IER5 substantially increased the cleavage of caspase-3 and PARP after either γ-ray irradiation treatment or cisplatin treatment compared with the control cells with no IER5 expression (**Figure 3A**, **3E**). Interestingly, we also found that phospho-Akt (p-Akt) was inhibited in the $HepG_2$/IER5 cells investigated (**Figure 3B**). Therefore, it is possible that the overexpression of IER5 not only activates the DNA damage signaling pathway, but also inactivates the MAP kinase signaling pathway, as speculated in previous reports [27]. Our results are in agreement with those of Dr. Luo, who found that Akt deactivation was associated with both caspase-independent and caspase-dependent cell death in multiple cellular systems [28]. Herein, our research data indicated that IER5 could induce apoptosis by inhibiting p-Akt.

As reported earlier, similarly to p53, p73 could also be involved in cell apoptosis induced by irradiation [29]. There was also evidence that the radiation-induced bystander effect caused the activation of p21, nuclear factor-κB (NF-κB), Bax, Bcl-2, and the cleavage of poly(ADP-ribose) polymerase, which indicated that there were signals transmitted from the target cells to bystander cells in a paracrine manner [30]. However, in our IER5 overexpression models, we did not discover any evidence that those tumors suppressed the expression of genes or oncogenes, such as P53, P21, P73, or the Bcl-2 family members, including Bcl-2, Bcl-x, and Bax genes which are involved in the apoptosis signaling pathway (**Figure 3C**, **3D**).

Our results provide experimental evidence that the overexpression of IER5 activated and promoted both irradiation-induced and cisplatin-induced apoptosis and inhibited the growth of hepatocellular carcinoma cells. Our findings might facilitate the better understanding of tumor radiosensitivity and promote the development of new strategies for liver cancer treatment targeting *IER5* gene.

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Disclosure of conflict of interest

None.

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References

[1] Seong J. Challenge and hope in radiotherapy of hepatocellular carcinoma. Yonsei Med J 2009; 50: 601-612.

[2] Dienstag JL, Isselbacher KJ, Braunwald E, Fauci AS, Kasper DL, Hauser SL, Longo DL and Jameson JL. Tumors of the Liver and Biliary Tract. In: editors. Harrison's Principles of Internal Medicine. New York: McGraw-Hill, NewYork; 2001. p. 588-591.

[3] Chang J, Hsu Y, Kuo P, Kuo Y, Chiang L and Lin C. Increase of Bax/ Bcl-XL ratio and arrest of cell cycle by luteolin in immortalized human hepatoma cell line. Life Sci 2005; 76: 1883-1893.

[4] Song LH, Yan HL and Cai DL. Gene expression profiles in the liver of mice irradiated with (60) Co gamma rays and treated with soybean isoflavone. Eur J Nutr 2006; 45: 406-417.

[5] Purdy JA. Dose to normal tissues outside the radiation therapy patient's treated volume: a review of different radiation therapy techniques. Health Phys 2008; 95: 666-676.

[6] Lawrence TS, Robertson JM, Anscher MS, Jirtle RL, Ensminger WD and Fajardo LF. Hepatic toxicity resulting from cancer treatment. Int J Radiat Oncol Biol Phys 1995; 31: 1237-1248.

[7] Ding KK, Shang ZF, Hao C, Xu QZ, Shen JJ, Yang CJ, Xie YH, Qiao C, Wang Y, Xu LL and Zhou PK. Induced expression of the IER5 gene by gamma-ray irradiation and its involvement in cell cycle checkpoint control and survival. Radiat Environ Biophys 2009; 48: 205-213.

[8] Cirelli C and Tononi G. Gene expression in the brain across the sleep-waking cycle. Brain Res 2000; 885: 303-321.

[9] Williams M, Lyu MS, Yang YL, Lin EP, Dunbrack R, Birren B, Cunningham J and Hunter K. IER5, a novel member of the slow-kinetics immediate-early genes. Genomics 1999; 55: 327-334.

[10] Jeggo P and Lavin MF. Cellular radiosensitivity: how much better do we understand it? Int J Radiat Biol 2009; 85: 1061-1081.

[11] Pichierri P, Franchitto A and Palitti F. Predisposition to cancer and radiosensitivity. Genet Mol Biol 2000; 23: 1101-1105.

[12] Shahidi M, Mozdarani H and Bryant PE. Radiation sensitivity of leukocytes from healthy individuals and breast cancer patients as measured by the alkaline and neutral comet assay. Cancer Lett 2007; 257: 263-273.

[13] Blum HE. Molecular therapy and prevention of hepatocellular carcinoma. Hepatobiliary Pancreat Dis Int 2003; 2: 11-22.

[14] Ward JF. Some biochemical consequences of the spatial distribution of ionizing radiation-produced free radicals. Radiat Res 1981; 86: 185-195.

[15] Rothfuss A, Schutz P, Bochum S, Volm T, Eberhardt E, Kreienberg R, Vogel W and Speit G. Induced micronucleus frequencies in peripheral lymphocytes as a screening test for carriers of a BRCA1 mutation in breast cancer families. Cancer Res 2000; 60: 390-394.

[16] Krueger SA, Collis SJ, Joiner MC, Wilson GD and Marples B. Transition in survival from low-dose hyper-radiosensitivity to increased radioresistance is independent of activation of ATM Ser1981 activity. Int J Radiat Oncol Biol Phys 2007; 69: 1262-1271.

[17] Marples B and Collis SJ. Low-dose hyper-radiosensitivity: past, present, and future. Int J Radiat Oncol Biol Phys 2008; 70: 1310-1318.

[18] Bolderson E, Richard DJ, Edelmann W and Khanna KK. Involvement of Exo1b in DNA damage-induced apoptosis. Nucleic Acids Res 2009; 37: 3452-3463.

[19] Sheng H, Shao J, Morrow JD, Beauchamp RD and DuBois RN. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. Cancer Res 1998; 58: 362-366.

[20] Fischer U, Janicke RU and Schulze-Osthoff K. Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ 2003; 10: 76-100.

[21] Hao X, Qian J, Xu S, Song X and Zhu J. Location of caspase 3-like protease in the development of sieve element and tracheary element of stem in Cucurbita moschata. J Integr Plant Biol 2008; 50: 1499-1507.

[22] Johnstone RW, Ruefli AA and Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. Cell 2002; 108: 153-164.

[23] Kumar S. Caspase function in programmed cell death. Cell Death Differ 2007; 14: 32-43.

[24] Allen RT, Cluck MW and Agrawal DK. Mechanisms controlling cellular suicide: role of Bcl-2 and caspases. Cell Mol Life Sci 1998; 54: 427-445.

[25] Herr I and Debatin KM. Cellular stress response and apoptosis in cancer therapy. Blood 2001; 98: 2603-2614.
[26] Petak I and Houghton JA. Shared pathways: death receptors and cytotoxic drugs in cancer therapy. Pathol Oncol Res 2001; 7: 95-106.
[27] Ogura K, Magae J, Kawakami Y and Koana T. Reduction in mutation frequency by very low-dose gamma irradiation of Drosophila melanogaster germ cells. Radiat Res 2009; 171: 1-8.
[28] Luo HR, Hattori H, Hossain MA, Hester L, Huang Y, Lee-Kwon W, Donowitz M, Nagata E and Snyder SH. Akt as a mediator of cell death. Proc Natl Acad Sci U S A 2003; 100: 11712-11717.
[29] Hamer G, Gademan IS, Kal HB and de Rooij DG. Role for c-Abl and p73 in the radiation response of male germ cells. Oncogene 2001; 20: 4298-4304.
[30] Mitra AK and Krishna M. Radiation-induced bystander effect: activation of signaling molecules in K562 erythroleukemia cells. J Cell Biochem 2007; 100: 991-997.