

Radiosensitization by Inhibiting Survivin in Human Hepatoma HepG2 Cells to High-LET Radiation

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In this study, whether survivin plays a direct role in mediating high-LET radiation resistance in human hepatoma cells was investigated. Small interfering RNA (siRNA) targeting survivin mRNA was designed and transfected into human hepatoma HepG2 cells. Real-time PCR and western blotting analyses revealed that survivin expression in HepG2 cells decreased at both transcriptional and post-transcriptional levels after treatment with survivin-specific siRNA. Caspase-3 activity was determined with a microplate reader assay as well. Following exposure to high-LET carbon ions, a reduced clonogenic survival effect, increased apoptotic rates and caspase-3 activity were observed in the cells treated with the siRNA compared to those untreated with the siRNA. The cells with transfection of the survivin-specific siRNA also increased the level of G₂/M arrest. These results suggest that survivin definitely plays a role in mediating the resistance of HepG2 cells to high-LET radiation and depressing survivin expression might be useful to improve the therapeutic efficacy of heavy ions for radioresistant solid tumors.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors. Surgery and radiotherapy are the predominant treatment modalities for it. But surgery is only suitable for a minority of patients and owing to the influence of radiosensitivity and other factors, the efficacy of radiotherapy is not obvious.^{1,2} Therefore, the treatment for HCC is attracting growing interest all over the world.

Since high linear energy transfer (LET) charged particle radiation has several potential advantages over the treatment with low-LET radiation, such as an inverted depth-dose distribution, a higher relative biological effectiveness (RBE), a reduction in oxygen enhancement ratio (OER) and a lower cellular capability for repair of radiation injury,³ charged particle radiation may exert highly lethal effects even on radioresistant tumors.⁴ HCC also appears to respond favor-

ably to heavy-ion therapy, attaining a high local control rate of 95% at 5 years.⁵ Therefore, studies on biological effects on hepatoma cell lines and corresponding mechanisms are of great importance for the treatment of HCC using high-LET particle beams.

Cell apoptosis is a key mechanism by which chemotherapy and ionizing radiation kill tumor cells.⁶ It is regulated by a complex balance in signal transduction pathways between apoptosis-activating factors, such as p53 and bax, and anti-apoptotic factors, such as the bcl-2 family and the inhibitor of apoptosis protein (IAP) family. Survivin, as a member of the IAP family, originally identified in the late 1990s, shows regulatory functions for both regulation of cell division and inhibition of apoptosis.^{7,8} Interestingly, survivin is specifically up-regulated in cancer cells and completely down-regulated and undetectable in normal adult tissues. It has been suggested that survivin gene could represent a new and tumor-specific molecular target for cancer therapy.^{9,10} Since survivin overexpression desensitizes cancer cells to several anticancer agents¹¹ and low-LET radiation (such as X-rays),^{12,13} there is a possibility that survivin attenuates high-LET radiation induced apoptosis as well.

Recently, we reported that a differential survivin expression in human hepatoma HepG2 cells in response to low- and high-LET radiations was observed and more pronounced survivin expression occurred in HepG2 cells after exposure to high-LET heavy ions at moderate doses.¹⁴ These observations stimulate us to explore if inhibiting survivin expres-

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sion would enhance the radiosensitivity of cancer cells to heavy ion radiation. In this study, we utilized RNA interference (RNAi) strategy for tumor cell radiosensitization. We demonstrated that the treatment of survivin specific small-interfering RNA (siRNA) in human hepatoma HepG2 cells significantly reduces survivin messenger RNA (mRNA) and protein expressions, leading to cell radiosensitization *in vitro* to high-LET carbon ions mainly through the increase of spontaneous and radiation-induced apoptosis and G₂/M arrest. On the one hand, our study herein is driven by the need to explore terra incognita; on the other hand, the results of the present study provide some basic data and new ideas for the ongoing application of heavy ions in cancer therapy.

MATERIALS AND METHODS

Cell culture and irradiation

Human hepatoma HepG2 cells were obtained from the First Hospital of Lanzhou University and grown in DMEM (GIBCO, USA) supplemented with 10% fetal calf serum (Minhai, China) and antibiotics (penicillin 100 unit/ml, streptomycin 100 µg/ml GIBCO, USA). Irradiation was performed in the therapy terminal using a carbon ion beam supplied by the Heavy Ion Research Facility in Lanzhou (HIRFL) at the Institute of Modern Physics (IMP), Chinese Academy of Sciences (CAS). Uniform irradiation field was yielded using the irradiation system equipped in the therapy terminal. The energy of the carbon ions bombarding cell samples was calculated to be 68 MeV/u (dose averaged LET = 35 keV/µm), and the dose rate was adjusted to be about 4 Gy/min.

siRNA treatment

Survivin siRNA (sense, 5'-GGA-ACA-UAA-AAA-GCA-UUC-GTT-3'; antisense, 5'-CGA-AUG-CUU-UUU-AUG-UUC-CTC-3') and mis-matched were synthesized from SBS Genetech Co. Ltd, China. 5×10^5 HepG2 cells were seeded with 20nM siRNA using so-fast transfection reagent (Sunma Biotech, China) according to the instructions of the manufacturer. After the treatment with siRNA, the cells were then cultured in normal growth media for 12 h before irradiation.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells and the cells were directly lysed in Trizol reagent (Invitrogen, USA). mRNA silencing was quantified by realtime PCR using a Bio-rad icycler system. The mRNA value for each gene was normalized to the β-actin mRNA (internal marker) level in each RNA sample. All reagents necessary for running a TaqMan RT-PCR assay, including predesigned and optimized assays were purchased from TaKaRa biotechnology, China and used according to the manufacturer's instructions. All the measurements and results were analyzed with the icycler

detection software.

Western blotting

Western blotting analysis was performed following standard methods. Briefly, cell lysates were resolved by SDS-PAGE under reducing conditions at a concentration of 30 µg protein of each sample per lane. PVDF membranes were incubated overnight with primary antibody (anti-survivin antibody, 1:1500 dilution, Abcam, UK). Immunodetection with a secondary peroxidase-conjugated antibody (1:4000 dilution, Abcam, UK) and chemiluminescence was performed according to the manufacturer's protocol (Beyotime Biotech, China). To confirm equal protein loading per lane, the membranes were subsequently reprobed with a 1:5000 dilution of an anti-β-actin antibody (Abcam, UK) and developed as described above.

Cell survival assay

Immediately after irradiation, cells were washed with PBS buffer, trypsinized and counted using a hemacytometer. After diluting with fresh medium the cells were replated into 60 mm Petri dishes at various cell densities. After 10 days of growth at 37°C, the cells were fixed with methanol and acetic acid (v/v = 3:1), stained with Giemsa, and then the number of colonies with cells more than 50 was counted.

Detection of apoptosis

Apoptosis was quantified by a combined staining of Annexin V and propidium iodide (PI) using Annexin V-FITC Apoptosis Detection Kit (Keygentec, China). Briefly, cells were harvested and resuspended in 500 µl of Binding Buffer. After adding 2 µl of Annexin V-FITC solution and 5 µl of PI solution, the cells were incubated for 15 min at room temperature in darkness. After the incubation, 10,000 cells were analyzed by a flow cytometer (FACS Calibur, Becton Dickinson, USA). The apoptotic cells were quantified using the FlowJo 5.0 software.

Analysis of cell cycle

Cell cycle status was assessed by DNA content analysis. Cells were resuspended in 1ml of fluorochrome solution (PI at 0.5 mg/ml in 0.2 mg/ml RNAase and 0.1% Triton X-100) and incubated at room temperature for 30 min in darkness. Ten thousands cells were measured per sample using flow cytometer. The cells arrested at G₂/M phase (4N) were quantified using the cell-cycle data analysis software (FlowJo 5.0).

Measurement of caspase-3 activity

After collection and centrifugation at 600 g for 5 min, cells were washed two times with PBS (pH 7.4) and then resuspended with 50 µl lysis buffer and incubated on ice for 15 min. After centrifugation (12,000 g for 15 min), cell extracts were transferred to fresh tubes, and protein concen-

trations were measured. Each 50 μ l cell extract containing 100 μ g of protein were combined with equal volumes of 2 \times reaction buffer in a microplate followed by the addition of 5 μ l of peptide substrates of caspase-3 (Ac-DEVD-pNA). After overnight incubation at 37°C in darkness, samples were read in a microplate reader (Multiskan Spectrum, Thermo, USA) at 405 nm. Caspase-3 activity was valuated by the absorbance ratio of treated to control samples.

Statistical analysis

Because of the limited beam time for the irradiation with carbon ions at HIRFL, the data of apoptosis in this study came from one experiment and the survival and caspase-3 activity data were derived from the three replicates in one experiment. The other data were obtained from three independent experiments and are presented as mean \pm standard deviation (SD). Statistical comparison of the mean values was performed using the student's t-test. Differences with a *p*-value of less than 0.05 are considered to be statistically significant.

RESULTS

The effect of siRNA on survivin

We examined the effect of survivin-specific siRNA on survivin mRNA and protein expressions. HepG2 cells were transfected by the siRNA at various time points and mRNA expression was detected by means of realtime PCR as shown in Fig. 1A. Twelve hours after transfection, the level of survivin mRNA was decreased by 45% in contrast to that in untreated cells ($p < 0.01$). The expression level was reduced further at 24 h, 36 h and 48 h by 55%, 60% and 56% ($p < 0.01$), respectively, compared to that in untreated cells. Similar to the mRNA expression, the survivin protein level was decreased as well, as shown in Fig. 1B. The mismatched siRNA in the present experiment was used as the negative control to confirm the specificity of our survivin siRNA on survivin mRNA and protein expressions in HepG2 cells. The results indicate that the expression of survivin gene could be down-regulated specifically and effectively by our survivin-targeted siRNA.

Survivin siRNA enhances the radiosensitivity of HepG2 cells to high-LET radiation

To show the ability of the survivin siRNA transfection to sensitize HepG2 cells to the high-LET carbon ions, clonogenic survival assays were done for HepG2 cells under the conditions of irradiation alone (untreated group), irradiation plus the treatment with the mismatched siRNA (mismatched group) and irradiation plus the treatment with the survivin specific siRNA (siRNA group). Shown in Fig. 2 are the survival curves for HepG2 cells under the three conditions above. The survival fractions (SF) for the siRNA groups were significantly lower than those for the mismatched or untreated groups ($p < 0.05$ at the various doses); for exam-

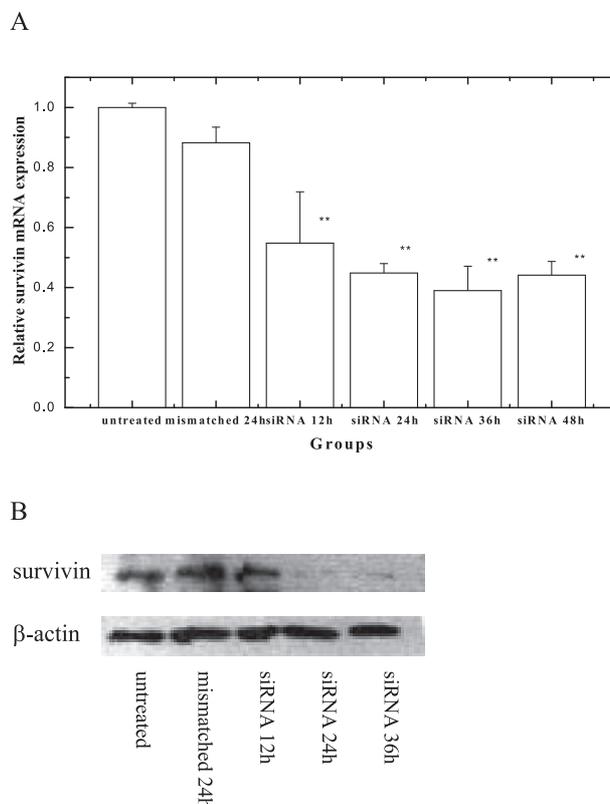


Fig. 1. Down-regulation of survivin mRNA and protein in following transient transfection with survivin-specific siRNA. A: total RNA was extracted at the indicated times after transfection and survivin mRNA was quantitated by a real-time PCR. Shown are the relative mRNA levels of survivin in reference to β -actin expression. **: $p < 0.01$ versus the untreated control. B: Western immunoblots from total cellular proteins extracted 12, 24, and 36 hours after transfection using antibodies against survivin (upper) and β -actin (lower) as a loading control.

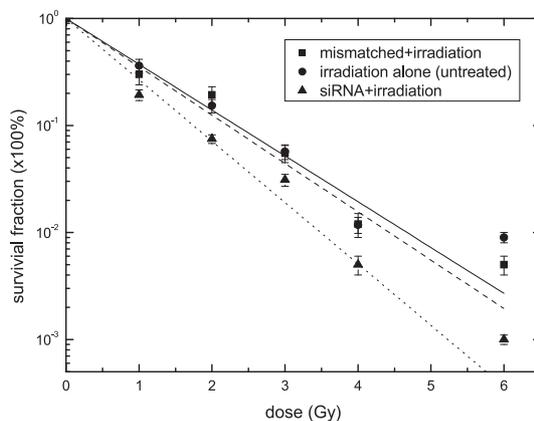


Fig. 2. The effect of survivin on the clonogenic survival of HepG2 cells transfected with either survivin-specific or mismatched siRNA. Untreated cells served as a control. Twelve hours after transfection, the cells were irradiated with the indicated doses. After incubation for 10 days, colonies with cells greater than 50 were counted.

ple, the survival fraction at 2 Gy (SF2) for the siRNA group was 7%, which was much lower than the SF2 of the mismatched (15%) and untreated (19%) groups by factors of 2.14 and 2.71, respectively. There was no significant difference in cell survival for the untreated and mismatched groups ($p > 0.05$). Clearly, the radiosensitivity of HepG2 cells to the carbon ions was enhanced remarkably by the treatment of the survivin siRNA.

To check the toxicity of siRNA, the plating efficiency (PE) were also done in three groups. The results show that PE of siRNA treated group (62.14%) is lower than other groups (untreated, 81.25%; mismatched, 79.82%), which cells treated with siRNA alone had reduced plating efficiency.

The effect of the combined treatment of survivin-specific siRNA and high-LET radiation on apoptosis and caspase-3 activity

To analyze whether down-regulation of survivin by siRNA

affects spontaneous and radiation-induced cell apoptosis, the proportion of apoptotic cells was detected with annexin V-PI staining. The results of spontaneous and radiation-induced cell apoptosis in the untreated, mismatched and siRNA groups are illustrated in Fig. 3, respectively. The percentage of spontaneous apoptotic cells was significantly higher in survivin siRNA-treated group (25%) as indicated by a 5- or 4.2-fold increase compared with the untreated (5%) or mismatched (6%) group. Furthermore, the apoptotic cells induced by the combination of the high-LET carbon ion irradiation and the survivin-siRNA treatment were greater than the corresponding control groups (35% in the siRNA group versus 26% in the mismatched or untreated group at 2 Gy, 49% in the siRNA versus 33% in the untreated or 35% in the mismatched group at 5 Gy, see Fig. 3). Shown in Fig. 4 are the results of caspase-3 activity in HepG2 cells irradiated with the carbon ions at 5 Gy under different conditions. Clearly, the survivin siRNA treatment also resulted in a sig-

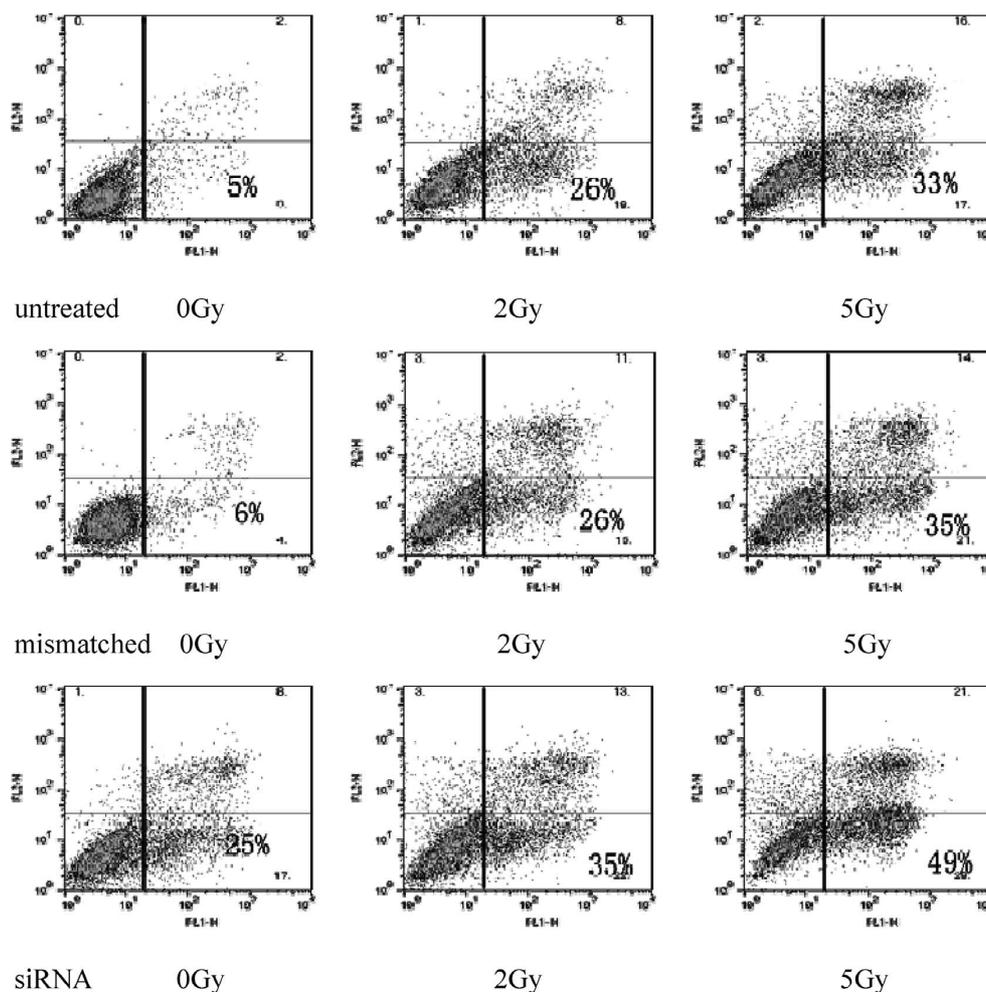


Fig. 3. Survivin attenuation by siRNA treatment (60 h after transfection) affects spontaneous and radiation-induced apoptosis. Apoptotic cells were determined with Annexin V- and PI-staining in non-irradiated cells and irradiated cells 48 hours after exposure to the carbon ions at 2 Gy and 5 Gy, respectively.

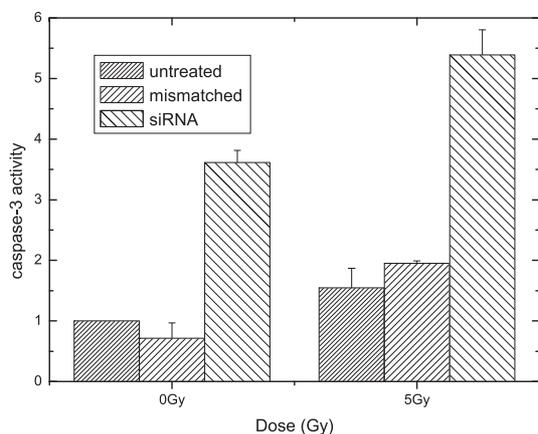


Fig. 4. Survivin attenuation by siRNA treatment (60 h after transfection) affects caspase-3 activity. Caspase-3 activities were determined with a microplate reader assay in non-irradiated (0 Gy) and irradiated (5 Gy) cells 48 hours after exposure to the carbon ions (three replicates in one experiment).

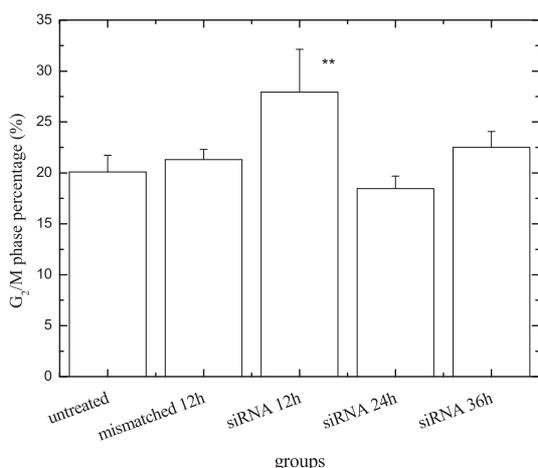


Fig. 5. Survivin attenuation by siRNA treatment influences cell cycle distribution. Various times (12 h, 24 h and 36 h) after transfection, fixed HepG2 cells were labeled with propidium iodide and flow cytometer was used to measure DNA content (**: $p < 0.01$ versus the untreated control).

nificant increase of caspase-3 activity.

Cell cycle analysis after siRNA treatment

To determine whether inhibition of survivin influences cell cycle distribution, cycle analyses were done in this study. Shown in Fig. 5 are the G₂/M phase percentages for cells in the untreated (20.1%), mismatched (21.3% at 12 h) and siRNA (27.9%, 18.5% and 22.5% at 12 h, 24 h and 36 h, respectively) groups. A light increase of G₂/M phase in the survivin siRNA-treated cells 12 hours after transfection (from 20.1% to 27.9%) was revealed, indicating that a larger fraction of cells might be blocked in a more radiosensitive phase compared with the untreated or mismatched cells

when the cells had been irradiated.

DISCUSSION

Recently, several groups showed that suppression of survivin effectively radiosensitizes human tumor cells to low-LET radiations.^{13,15–17} However, little information about survivin is available in tumor cells after exposure to high-LET radiations. Energy deposition by high-LET particles consists of a localized contribution along the trajectory of each particle and lateral extension of energetic electrons many microns from the particle's path. The difference in energy deposition between high-LET particles and low-LET X-rays leads to distinct spatial patterns of DNA lesions. For radiation effects on small DNA segments, heavy ions are several times more effective than X-rays.¹⁸ Also in the previous study, we found that survivin mRNA and protein expressions in HepG2 cells exposed to high-LET carbon ions increased in a dose-dependent manner at low dose (0–4 Gy).¹⁴ Therefore, we hypothesized that survivin also plays an important role in mediating the resistance of HepG2 cells to high-LET radiation. As expected, the cell survival experiments herein showed a clear radiosensitization by the survivin-specific siRNA transfection to the high-LET carbon ions in HepG2 cells as displayed in Fig. 2. This observation indicates that survivin functions as a factor conferring resistance to high-LET radiation.

In cells, survivin is an essential regulator of cell division and apoptosis.^{8,19} Survivin participates in the regulation of chromosome segregation and is involved in the spindle checkpoint pathway during mitosis.²⁰ Kapper *et al.* found a population of polyploid cells formed after treatment with survivin-specific siRNA.²⁰ Another pivotal function of survivin is its inhibition to apoptosis. Our experimental results show that inhibiting survivin expression enhances apoptosis-related cell death. Compared with the untreated cells, the survival fraction of the siRNA-treated cells was decreased by 12% (from 19% to 7%, see Fig. 2) and the cell apoptotic rate was increased by 9% (from 26% to 35%, see Fig. 3) at 2 Gy. The increment of the apoptotic cell death due to the siRNA treatment, therefore, contributed 75% to the enhancement of the radiosensitivity of HepG2 cells to the carbon ions. So the increase of apoptotic rate induced by siRNA transfection was the main reason for enhancing the sensitivity of HepG2 cells to high-LET radiations. The present caspase-3 results confirm the above assertion further. Caspase-3 is an effector caspase, which activity leads to the proteolytic degradation of substrates, resulting in the apoptotic morphology.²¹ We found the treatment with the siRNA obviously up-regulated the caspase-3 activity by 3.6 or 5.4 fold compared with the untreated HepG2 cells after irradiation with 0 Gy or 5 Gy as shown in Fig. 4.

Additionally, we observed that the survivin-specific siRNA treatment altered the cell cycle distribution slightly, resulting

in a small increased G₂/M phase fraction (6.8%) 12 hours after transfection as shown in Fig. 5. This is in good agreement with the observation of a marked G₂/M arrest after survivin-specific siRNA treatment in rectal tumor cell lines.^{13,22} Thus, at the time point of irradiation, the cells might be arrested in a more radiosensitive phase of the cell cycle. Therefore, we think this was an additional reason why the radiosensitivity of HepG2 cells to the carbon ions was increased. The underlying mechanisms by which anti-survivin strategies may improve cellular radiosensitivity appear to be multifaceted. In addition to apoptosis increase and cycle arrest as mentioned above, survivin knock-down by siRNA resulting in an impaired DNA double strand break repair was also reported by Capalbo *et al.*²³ They found that phosphohistone H2AX was increased after survivin attenuation with a maximum level at 60 min after irradiation and persisting elevated levels at 24 h compared with control.²³ This machinery may also contribute to decreased clonogenic survival after irradiation to cells in the presence of survivin inhibitors.

Because of the limited beam time for this study, the cells were irradiated at 12 h after siRNA transfection, when survivin expression was inhibited at mRNA level but not at protein level (see Fig. 1). However, we think this has not a significant impact on our present survival and apoptosis data. As we know, cell apoptosis is a very complex process. Although the exact mechanism that survivin inhibits cell apoptosis has not been understood completely, this inhibition usually occurs at downstream of apoptosis process,²⁴ that is, there is no effect on apoptotic signal pathways irrespective of survivin expression at the beginning of irradiation. Our apoptotic data at another time point (24 h) post-irradiation confirm this point indirectly, where cell apoptotic rates had no changes at 24 h after irradiation (data not shown) in three groups (untreated, mismatched and siRNA). Another consideration that we chose 12 h after siRNA transfection for irradiation is due to the results of cell cycle distribution. As shown in Fig. 5, we found that increased G₂/M phase fraction only appeared at 12 h after siRNA transfection, which made cell population more sensitive to radiation as mentioned above.

In summary, we demonstrate that survivin-specific siRNA significantly enhanced the radiosensitivity of human hepatoma HepG2 cells to high-LET radiation in this study. This radiosensitization is likely to come from the increase of spontaneous and radiation-induced apoptosis and G₂/M phase arrest caused by siRNA transfection. The present study suggests that RNAi technique targeting survivin has potential to be applied to radiation therapy with high-LET heavy ions for further cancerous cell killing.

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