

Heavy Ion Beams Induce Survivin Expression in Human Hepatoma SMMC-7721 Cells More Effectively than X-rays

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Abstract High linear energy transfer (LET) heavy ion radiation is more effective in inducing biological damage than low-LET X-rays or γ -rays. Heavy ion beam provides good dose localization (Bragg peak) in critical cancer tissue and gives higher relative biological effectiveness in cell killing across the dose peak, so high-LET heavy ion beam is superior to low-LET radiation in cancer treatment. Survivin, as a member of the inhibitor of apoptosis protein family, might help cancerous cells to overcome the G₂/M apoptotic checkpoint and favor the aberrant progression of transformed cells through mitosis. Survivin expression in the human hepatoma SMMC-7721 cell line after exposure to low-LET X-ray and high-LET carbon ion irradiation was investigated in this study. Compared with X-ray irradiation, the carbon ion beam clearly caused G₂/M arrest and promoted the expression of the survivin gene in a dose-dependent manner. Clonogenic survival assay showed that SMMC-7721 cells were more radiosensitive to the high-LET carbon ions than to the X-rays, and the radiosensitivity was promoted after treatment with specific survivin short interfering RNA. Differential survivin expression at both transcriptional and translational levels was found for SMMC-7721 cells following low- and high-LET irradiation. The overexpression of survivin in SMMC-7721 cells is probably an important reason why the cancerous cells have radioresistance to strong stimulus such as dense ionizing high-LET radiation. However, the direct killing effect on cancerous cells by high-LET radiation might be more significant than the apoptosis inhibition through the overexpression of survivin following heavy ion irradiation.

Keywords survivin; human hepatoma SMMC-7721 cell; high-LET carbon ion; low-LET X-ray; radiosensitivity

Malignant tumor is a disease that seriously threatens human health. Conventional radiations such as low linear energy transfer (LET) X-rays and γ -rays are extensively applied in cancer radiotherapy. The absorbed dose delivered by these radiations usually undergoes an exponential attenuation along the penetration depth in patient bodies.

Consequently, these radiations can not only destroy a tumor but can also lead to serious damage to normal tissues around the tumor. High-LET heavy ion radiation is more effective than low-LET X-rays or γ -rays to induce biological damage [1]. It is generally characterized by an energy deposition peak (Bragg peak) at the end of its range, which provides good dose localization in a critical cancer tissue [2] and gives an increased relative biological effectiveness in cell killing [3] within the peak [4]. Tolerable damage to normal tissues and destructive strike to cancerous cells, therefore, are expected with the use of heavy ion beam in cancer treatment [5]. Comparative studies on biological effects induced by low- and high-LET radiations are always interesting in the field of radiobiology.

Received: March 15, 2007 Accepted: April 26, 2007

This work was supported by the grants from the Century Program of the Chinese Academy of Sciences (0506120BR0), the Key Directional Project of the Chinese Academy of Sciences (0713030YF0) and the National High Technology Research and Development Program of China (2006AA02Z499).

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DOI: 10.1111/j.1745-7270.2007.00314.x

Survivin is a bifunctional protein that regulates tumor cell division and suppresses apoptosis [6,7]. It is present in only small amounts in terminally differentiated normal cells, but is found to be prominently overexpressed in a variety of human tumors, including cancers of the lung, stomach, breast, melanoma, pancreas, esophagus and colon [8–15]. Therefore, survivin is an important target and prognostic marker in tumor treatment. As the expression of survivin is high in many human cancers, the protein might be involved in cell resistance to chemotherapeutic agents and ionizing radiations. The selective expression of survivin as well as its important anti-apoptotic function have stimulated studies using survivin as a therapeutic target in cancer treatment [16,17].

In this work human hepatoma SMMC-7721 cells were irradiated with both high- and low-LET radiations, and the expression of survivin and relevant biological effects, such as radiation-induced apoptosis, in the cell line following both the irradiations are investigated. Moreover, the influence of specific survivin short interfering (si)RNA on the radiosensitivity of SMMC-7721 cells to heavy ion beam is also examined. The studies herein provide necessarily basic data for promising heavy ion cancer therapy.

Materials and Methods

Cell culture

Human hepatoma cell line SMMC-7721 conserved in our laboratory was used in this study. SMMC-7721 is a wild-type p53 cell line. The cells were routinely cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Carlsbad, USA) supplemented with 10% heat inactivated fetal calf serum (Minghai Biochem, Lanzhou, China), 100 U/ml penicillin and streptomycin, 1% sodium pyruvate and 2 mM glutamine at 37 °C, 5% CO₂, 95% humidity. All supplements were from Sigma-Aldrich (St. Louis, USA). One day before irradiation, for heavy ion irradiation SMMC-7721 cells were plated into 35 mm Petri dishes (Nunc, Wiesbaden, Germany) and 2 ml fresh medium was furnished; for X-ray irradiation cells were seeded into 75 cm² plastic flasks (Nunc) and 10 ml fresh medium was added.

Irradiation

A carbon ion beam of 80.55 MeV/u was supplied by the Heavy Ion Research Facility in Lanzhou (HIRFL) at the Institute of Modern Physics, Chinese Academy of Sciences (Lanzhou, China). Cell exposures were carried out at the therapy terminal of the HIRFL, which has a vertical beam

line. SMMC-7721 cells in Petri dishes were irradiated at room temperature with the carbon ion beam. Due to the energy degradation by the vacuum window, air gap, Petri dish cover and medium, the energy of the ion beam on cell samples was calculated to be 68 MeV/u, corresponding to an LET of 35 keV/μm, and the dose rate was adjusted to be approximately 4 Gy/min. The X-ray irradiation (8 MV using an electron linear accelerator, CL 2100; Varian, Darmstadt, Germany) to cells was carried out at room temperature in the therapy unit of the General Hospital of the Lanzhou Military Area at a dose rate of 4 Gy/min. For cell sample transportation, the culture flasks were covered with ice bags in a foam box. After irradiation, the cells were incubated under normal culture conditions for up to 72 h. The doses applied in this experiment varied from 0 to 6 Gy for both the irradiations.

Survivin expression at mRNA level

Total RNA was isolated from the cell line and the cells were directly lysed in Trizol reagent (Invitrogen, Carlsbad, USA). Human survivin-specific reverse transcription-polymerase chain reaction (RT-PCR) primers (GenBank accession No. NM-001168) [18] used for the analysis of survivin mRNA expression by RT-PCR were purchased from SBS Genetech (Beijing, China). Total RNA (1 μg) from each sample was used in the RT reaction to synthesize the first strand of cDNA. One microliter of cDNA was used in each PCR. All of the RT-PCR experiments were carried out three times. Primer sequences of the genes under consideration were as follows. For survivin, forward primer is 5'-ATGGGTGCCCCGACGTTG-3' and reverse primer is 5'-GGCCAGAGGCCTCAATCCAT-3', yielding a 441 bp segment. Cycling conditions included denaturing at 94 °C for 1 min (one cycle), then denaturing at 94 °C (30 s), annealing at 56 °C (30 s) and extending at 72 °C (30 s) for 35 cycles, with a final extension at 72 °C (7 min). For β-actin, forward primer is 5'-AACCGCGAGAAGATGACCCAGATCATGTTT-3' and reverse primer is 5'-AGCAGCCGTGGCC-ATCTCTTGCTCGAAGTC-3'. Thermocycling conditions included denaturing at 94 °C for 1 min (one cycle), then denaturing at 94 °C (1 min), annealing at 60 °C (1 min), and extending at 72 °C (1 min) for 30 cycles, with a final extension at 72 °C (7 min). The β-actin primers were used as positive controls [19]. Quantitative PCR mRNA subsequences (survivin, 127 bp; β-actin, 186 bp) were amplified from cDNA in duplicate experiments by ready-to-use quantitative PCR assay (TaKaRa, Dalian, China). The survivin primers were as follows: forward primer, 5'-TTCTCAAGGACCACCGCATC-3'; reverse primer, 5'-

GCCAAGTCTGGCTCGTTCTC-3'. β -actin acted as the internal control and the primers were shown as follows: forward primer, 5'-TGGCACCCAGCACAATGAA-3'; reverse primer 5'-CTAAGTCATAGTCCGCCTAG-AAGCA-3'. PCR amplification and detection were carried out with a TaqMan sequence detection system (BioRad Laboratories, Hercules, USA).

Survivin expression at protein level

Cells were treated with various doses and collected at different time points. The cells were counted then washed with ice-cold phosphate-buffered saline (PBS) twice before the addition of lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml leupeptin). Protein concentration was determined using the Bradford assay. Equal amounts of protein (30 μ g) were loaded into each well and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, followed by transfer onto polyvinylidene difluoride membranes (Millipore, Bedford, USA) using a Protean system (BioRad Laboratories). Membranes were blocked in 5% non-fat dry milk in PBS overnight at 4 °C. The blots were then probed with the rabbit anti-human survivin antibody (dilution 1:1500; BioLegend, San Diego, USA) for 1 h at 37 °C. Blots were then washed with PBST (PBS/0.05% Tween-20) then incubated in horseradish peroxidase-linked secondary antibody (dilution 1:4000; Southern Biotech, Birmingham, USA) for 1 h at 37 °C. Immunoblots were developed using 20 ml of PBS solution containing 1 mg/ml 3,3'-diaminobenzidine-tetrachloride, 1 mM CoCl₂ and 0.075% hydrogen peroxide. To confirm equal protein loading per lane, membranes were subsequently reprobed with a 1:5000 dilution of an anti- β -actin antibody (Lab Vision, Fremont, USA) and developed as described previously. For densitometric analysis, scanned photographs were quantified using the AlphaEasy FC software package (Alpha Innotech, San Leandro, USA).

Cell cycle analysis

Cells were trypsinized, rinsed once, resuspended in PBS, and fixed with ice-cold 70% ethanol for at least 20 min. Fixed cells were then rinsed again with PBS and resuspended in 50 μ g/ml propidium iodide (Sigma-Aldrich) with 40 kU/ml of DNase-free RNase (Stratagene, La Jolla, USA). The cells were then analyzed with a flow cytometer (FACScan; Becton Dickinson, San Jose, USA), and the percentage of the cells in each phase was calculated using ModFit software (Verity Software House, Topsham, USA).

Detection and quantification of apoptosis

Apoptotic cells were identified using the flow cytometer. Approximately 1×10^5 cells were collected at 24, 48 and 72 h after irradiation. The cells were washed twice with PBS then fixed in 75% ethanol pre-cooled at 4 °C overnight. The cells were then stained with solution containing RNase and propidium iodide for 30 min. The samples were analyzed immediately by flow cytometry in a FACScan (Becton Dickinson) using a logarithmic fluorescence scale. The apoptosis data are presented as the percentage of sub-G₁ staining in the cell samples where the relevant background staining was subtracted.

Antisense oligonucleotides and transfection

Antisense oligonucleotides were synthesized (SBS Genetech) using the following sequence at the position 1172–1189 of human survivin mRNA (GenBank accession No. NM-001168): 5'-TGTGCTATTCTGTGAATT-3'. The nucleotides were phosphorothioated modified. SMMC-7721 cells with 50%–70% confluence were transfected with the oligonucleotides, using a mixture of Lipofectin (Invitrogen) and oligonucleotides in serum-free Dulbecco's modified Eagle's medium (Gibco BRL) at a ratio of 3 μ l Lipofectin/ml medium per 100 nM oligonucleotide. After 4 h of incubation, the transfection medium was replaced by the regular complete medium for subsequent experiments.

Determination of cell survival

Cell survival was determined by a standard colony-forming assay. Briefly, cells were rinsed with PBS, trypsinized and resuspended in PBS immediately after irradiation. Cell concentration was measured by use of a hemocytometer. After diluted appropriately with fresh medium, irradiated cells for each dose were seeded into three 60 mm Petri dishes then cultivated in an incubator for 10–15 d. Colonies in the dishes were fixed and stained with methylene blue for 30 min. The colonies greater than 50 cells were counted as survivors, and survival data were fitted according to the linear quadratic equation using Origin 7.0 software (OriginLab, Northampton, USA). In all cases, each survival rate is presented as the mean \pm SD, which comes from the three replicates.

Results

Survivin mRNA and protein expression

The expression of survivin at mRNA and protein levels

was observed in human hepatoma SMMC-7721 cells during the logarithmic growth phase. For SMMC-7721 cells irradiated with the heavy ion beam, we observed that survivin mRNA expression escalated as the radiation dose increased [Fig. 1(A,B)]. Furthermore, this observation was confirmed for survivin protein by Western blot analysis [Fig. 1(C)]. Compared with the heavy ion beam, the X-ray irradiation induced a moderate differential survivin expression at the mRNA level in SMMC-7721 cells with an increasing radiation dose. However, there was no obvious difference in survivin expression at the protein level in SMMC-7721 cells exposed to the various doses of X-rays (Fig. 1).

Cell cycle effect

To determine whether survivin could affect cellular radiosensitivity to irradiation through cell cycle redistribution, SMMC-7721 cell cycles were analyzed by means of flow cytometry. The relative values of SMMC-7721 cells at the G₂/M phase in relation to the controls at different radiation doses are shown in Fig. 2. Compared with the X-ray

irradiation, cells exposed to the heavy ion beam showed obvious G₂/M blocks at the various doses, and the highest percentage of G₂/M phase was observed at 4 Gy within 24 h after irradiation. But the G₂/M percentage reduced with the dose of 6 Gy, as shown in Fig. 2(A). With the culture time up to 72 h after heavy ion beam irradiation, cells showed a reduced percentage of G₂/M phase. However, the dose of 6 Gy might be too high to be tolerable for the cells. Compared with the total number of the cells, few cells could survive the challenge of the particle beam at 6 Gy. Consequently, the percentage of G₂/M decreased with the dose of 6 Gy. Interestingly, when the cells were cultured over 24 h following the heavy ion irradiation, the percentage of G₂/M decreased as a whole, as shown in Fig. 2(B,C). After 24 h culture post-irradiation, surviving cells finished damage repair and surpassed the G₂/M phase arrest, then went on with their programmed processes.

Radiation-induced cell apoptosis

Shown in Fig. 3 are the relative apoptotic rates of

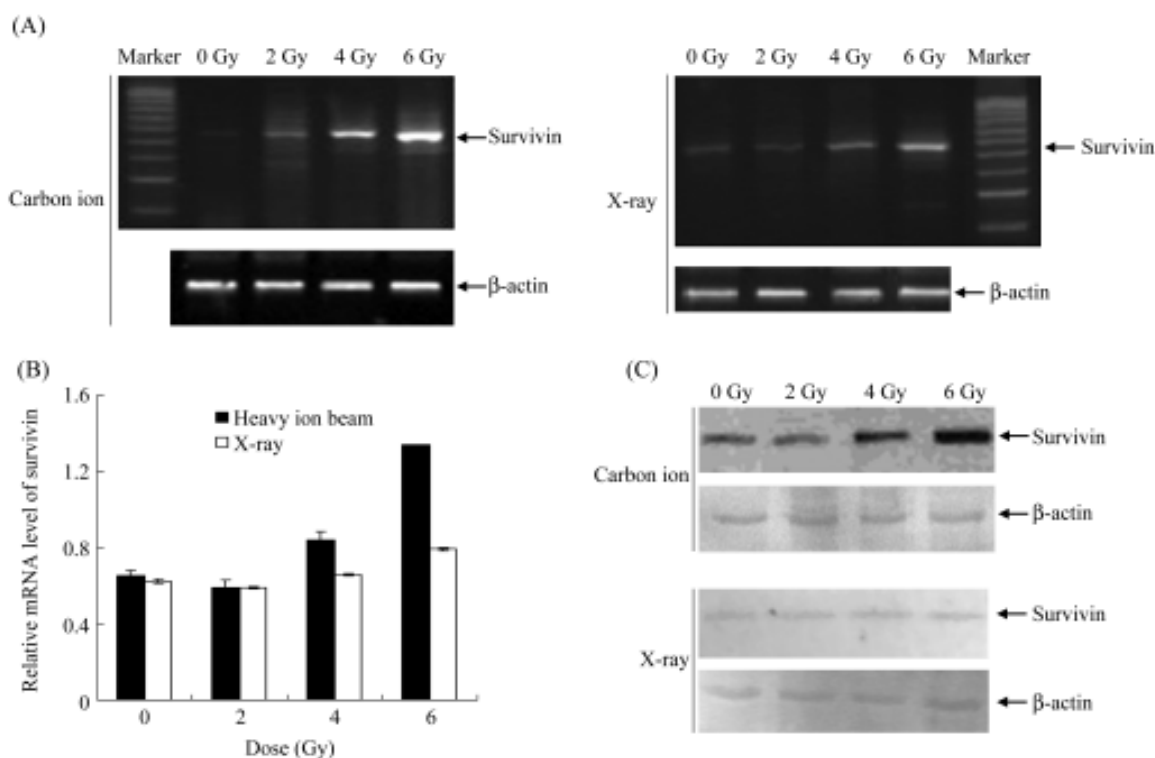


Fig. 1 Survivin mRNA and protein expressions in human hepatoma SMMC-7721 cells after exposure to carbon ion and X-ray irradiation

(A) Reverse transcription-polymerase chain reaction (RT-PCR) analyses. Total RNA was isolated from SMMC-7721 cells cultured for 24 h after X-ray and heavy ion irradiation and analyzed by RT-PCR using survivin and β -actin-specific primers. (B) Real-time PCR analysis showed different survivin expressions after exposure to high- and low-LET irradiation. (C) Western blot analysis. Soluble proteins (30 μ g) lysed from cultured SMMC-7721 cells for 24 h after exposure to different radiation doses were analyzed by Western blot as described above. β -actin antibody was used as a control for equal sample loading.

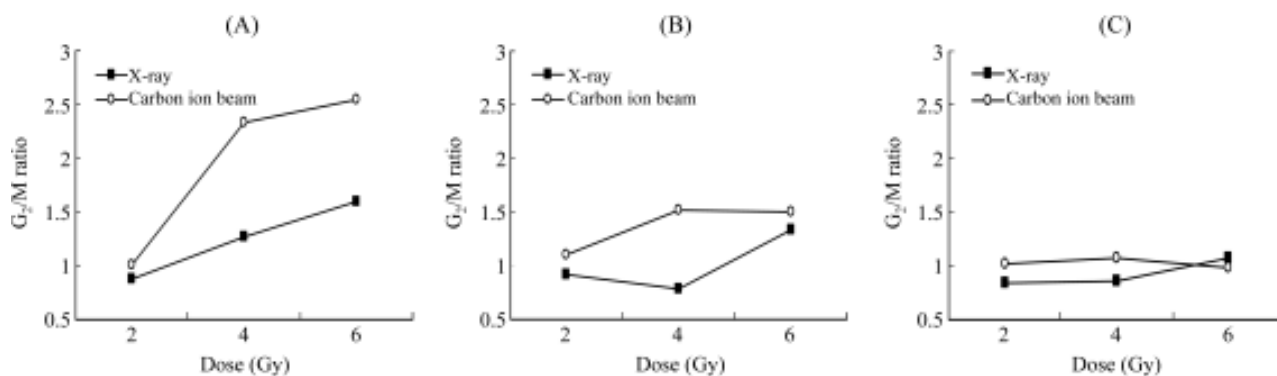


Fig. 2 Dependence of the G₂/M phase ratios (compared with controls) of human hepatoma SMMC-7721 cells at 24 h (A), 48 h (B) and 72 h (C) of culture after irradiation on radiation dose

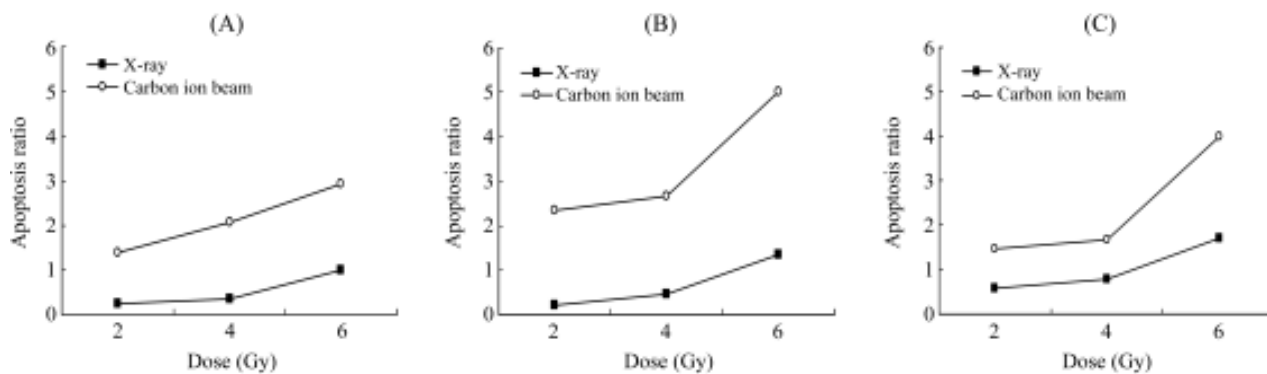


Fig. 3 Dependence of the relative apoptotic ratios (compared with controls) of human hepatoma SMMC-7721 cells at 24 h (A), 48 h (B) and 72 h (C) of culture after irradiation on radiation dose

SMMC-7721 cells exposed to both the low- and high-LET irradiations in comparison with the corresponding controls. The results show that the high-LET carbon ion beam induced higher apoptotic rates than the low-LET X-rays at the different doses applied in this experiment. This observation indicates that SMMC-7721 cells could be restrained at the G₂/M phase and clearly induced cell apoptosis more following the irradiation with carbon ions, in contrast to X-rays. Moreover, the apoptotic rate increased with increasing doses of each form of irradiation.

Clonogenic survival

After exposure to the low- and high-LET irradiations at doses varying from 0 to 6 Gy, SMMC-7721 cell survival fractions were examined using a clonogenic assay and the results are shown in Fig. 4. The survival curves of SMMC-7721 cells, as shown in Fig. 4, were obtained from data fitting according to the linear quadratic model. It is clear that SMMC-7721 cells are more radiosensitive to the high-

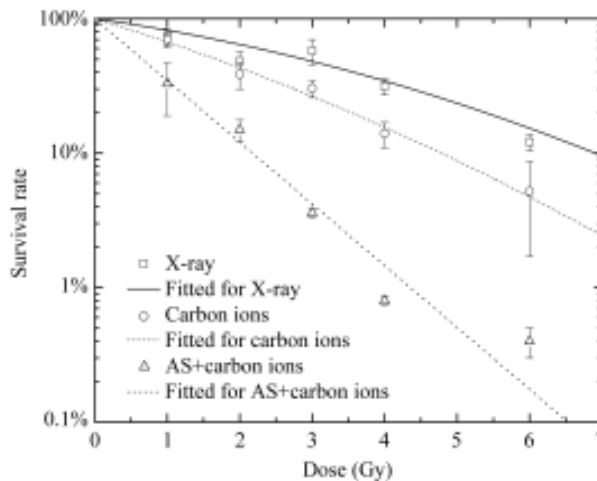


Fig. 4 Survival curves of human hepatoma SMMC-7721 cells exposed to the low linear energy transfer (LET) X-ray and high-LET carbon ion irradiations, determined by clonogenic assay

The cells transfected by the antisense oligonucleotides (AS) are more sensitive to the carbon ion beam. Lines represent data fit according to the linear quadratic model.

LET carbon ions than to the low-LET X-rays. When SMMC-7721 cells were treated with specific survivin siRNA, the cells showed markedly increased radiosensitivity to the carbon ions.

Discussion

Expression of survivin is up-regulated in a variety of human cancers, with high levels documented in breast and lung cancers [20]. Overexpression of survivin in tumors correlates with resistance to radiotherapy and poor prognosis in patients with colon [15], colorectal [21] and pancreatic cancers [22]. Radiation results in DNA damage and generation of free radicals in cells. Cells exposed to radiation would be arrested at the G₂/M phase and finally die through both apoptosis and necrosis [23]. Apoptosis might be regulated by a complex balance in signal transduction pathways between apoptosis-activating and anti-apoptotic factors [24,25]. Survivin is a strong apoptosis inhibitor, and the expression of survivin might confer a certain degree of radioresistance to some kinds of cancerous cells.

It is well known that ionizing radiation could lead to serious damage so as to induce cell death. However, cancerous cells usually show resistance to radiation. The expression of survivin in cancerous cells might be one of the reasons why the cells show signs of radioresistance. The present experimental results show that the high-LET carbon ion beam induced the accumulation of survivin expression with increasing radiation dose (**Fig. 1**) at both transcriptional and translational levels. In contrast, there was no obvious difference in survivin expression at transcriptional or translational levels after irradiation with different doses of low-LET X-rays [**Fig. 1(A,C)**]. The different expressions of survivin in SMMC-7721 cells were identical to the different resistant responses to each form of LET radiation. Previous studies have revealed that survivin gene expression following conventional radiation or chemotherapeutic agents was negatively regulated by wild-type p53, and the down-regulation of survivin was not apparent in cell lines with mutated or null p53 alleles, such as survivin expression in colorectal cancer cell lines [26,27] and lung cancer cell lines [28]. After the X-ray irradiation, survivin expression seemed to be depressed due to the wild-type p53 status of SMMC-7721 cells [**Fig. 1(C)**]. The same result was observed in human colorectal cancer cell lines and pancreatic cell lines for low-LET radiations [25,26]. However, this is not the case for the high-LET carbon ions. We infer that the expression of

survivin in cells after high-LET irradiation might increase regardless of the p53 status of the cell line. Because high-LET heavy ion beam deposits its energy in a dense manner around its track, this physical characteristic might explain why the wild-type p53 could not negatively regulate the expression of survivin in response to severe DNA damage caused by the heavy ion beam.

The presence of arrest at the G₂/M phase is thought to be correlated with the radiosensitivity of the cells [21] and high-LET irradiation is more effective to induce apoptosis [29]. Because SMMC-7721 cells are more sensitive to high-LET carbon ions, the population of G₂/M cells increased more significantly after the particle beam irradiation than the low-LET X-rays within 24 h of culture, as shown in **Fig. 2**. Survivin is maximally expressed at the G₂/M phase of the cell cycle and is associated with the mitotic spindle of dividing cells. The overexpression of survivin in cancers might help cancerous cells to overcome the G₂/M apoptotic checkpoint and favor the aberrant progression of transformed cells through mitosis [30]. We supposed that increased survivin expression in SMMC-7721 cells irradiated with the high-LET carbon ions was an indication of radioresistance and helped the cells proceed with their mal-division. The G₂/M block gave them enough time to overexpress survivin and the resulting survivin protein participated in the improperly constructed spindle and mitosis [30]. Although the cancerous cells had the potential to repair the damage induced by the high-LET radiation and underwent mal-division, compared with the damage-repaired cells, there were still many irradiated cells with lesions that could not be repaired. These cells trended towards apoptosis. Correspondingly, the high-LET carbon ion beam caused a higher relative apoptotic rate in comparison with the low-LET X-rays, which increased with the increasing radiation dose (**Fig. 3**).

The toxicity of radiation to living tissue was found after the discovery of radiosensitivity itself and this toxicity is the basis for cancer therapy with radiation. Although radiotherapy is often effective, normal tissues, as well as tumor cells, are also sensitive to radiation so that the dose applied is often limited by the tolerance of the normal tissues [31]. Due to the favorable characteristics of heavy ion beams, such as dose localization, as well as a high relative biological effectiveness, heavy ion cancer therapy is attracting growing interest all over the world. Cancer therapies with heavy ions have achieved unprecedented success against several kinds of tumors [32–34]. SMMC-7721 cells are more sensitive to the carbon ion beam than to the low-LET X-rays, as shown in **Fig. 4**. Our experimental results show that the cell apoptosis clearly increased

following irradiation with the carbon ion beam, whereas the cell survival fraction decreased significantly compared with the low-LET X-rays. Because high-LET particle beams lead to more dense energy deposition than conventional radiations, carbon ions could induce more serious DNA damage than low-LET radiation so as to lower the repairing ability of the cells. Based on the differential expression of survivin in human hepatoma SMMC-7721 cells after exposure to high- and low-LET radiations, it is indeed interesting to observe the change in the radiosensitivity to high-LET radiation after specific survivin siRNA was applied to SMMC-7721 cells. The radiosensitivity of SMMC-7721 cells was shown to be markedly enhanced compared with that of the X-ray irradiation or the carbon ion beam alone. This observation hints that the specific survivin siRNA would be useful in heavy ion cancer therapy, and the therapeutic efficacy of heavy ions would be promoted.

In summary, for the first time we have found a differential survivin expression in human hepatoma SMMC-7721 cells after exposure to low- and high-LET irradiations. The survivin gene is expressed in a dose-dependent fashion in SMMC-7721 cells following high-LET heavy ion irradiation, and the irradiated cells are significantly arrested at the G₂/M phase because of the susceptibility of SMMC-7721 cells to heavy ions and the overexpression of survivin at this phase. Survivin expression in SMMC-7721 cells is regarded as a kind of stress reaction triggered by resistance following particle beam treatment. Specific survivin siRNA greatly enhances the radiosensitivity of SMMC-7721 cells to heavy ion beam irradiation. However, despite the overexpression of the survivin gene, cell apoptosis is still enhanced after cells exposed to high-LET heavy ion irradiation because of the severe DNA damage caused by the heavy ions in the penetrate path of Bragg Peak tail.

Acknowledgements

The authors would like to thank Dr. Lijing ZHANG and Jianfeng CHANG at Lanzhou University (Lanzhou, China) for their skillful technical assistance and the operating crew of the HIRFL accelerator complex for supplying the carbon ion beam. We are also grateful to Dr. Sha LI at the General Hospital of the Lanzhou Military Area (Lanzhou, China) for her help in the X-ray irradiation experiments.

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Edited by
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