Original Research

Apoptosis and injuries of heavy ion beam and x-ray radiation on malignant melanoma cell

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Impact statement

Malignant melanoma is a malignant skin tumor derived from melanin cells, which has a high malignant degree and high fatality rate.

In this study, proliferating cell nuclear antigen (PCNA) can induce the apoptosis of malignant melanoma cells and inhibit its proliferation, and its induction effect on apoptosis is significantly higher than low LET X-ray; hence, it is expected to overcome its lower sensitivity to radiation.

This study can provide theoretical basis for clinical trials, in which malignant melanoma is treated by heavy ion ($^{12}C^{6+}$), in order to accurately determine the clinical efficacy of heavy ion therapy.

Clinical applications has revealed that local tumor control rate is high when heavy ion is used to treat malignant melanoma, indicating that heavy ion is an important direction in treating melanoma in the future.

Abstract

This study aims to investigate the influence of high linear energy transfer (LET) heavy ion ($^{12}C^{6+}$) and low LET X-ray radiation on apoptosis and related proteins of malignant melanoma on tumor-bearing mice under the same physical dosage. C57BL/6 J mice were burdened by tumors and randomized into three groups. These mice received heavy ion ($^{12}C^{6+}$) and X-ray radiation under the same physical dosage, respectively; their weight and tumor volumes were measured every three days post-radiation. After 30 days, these mice were sacrificed. Then, median survival time was calculated and tumors on mice were proliferated. In addition, immunohistochemistry was carried out for apoptosis-related proteins to reflect the expression level. After tumor-bearing mice were radiated to heavy ion, median survival time improved and tumor volume significantly decreased in conjunction with the upregulated expression of pro-apoptosis factors, Bax and cytochrome C, and the downregulated expression of apoptosis-profilin (Bcl-2, Survivin) and proliferation-related proteins (proliferating cell nuclear antigen). The results indicated that radiation can promote the apoptosis of malignant melanoma cells and inhibit their proliferation. This case was more suitable for heavy ion ($^{12}C^{6+}$). High LET heavy ion ($^{12}C^{6+}$) radiation could significantly improve the killing ability for malignant melanoma cells by inducing apoptosis in tumor cells and inhibiting their proliferation. These results demonstrated that heavy ion ($^{12}C^{6+}$) presented special advantages in terms of treating malignant melanoma.

Keywords: Malignant melanoma, heavy ion, X-ray, apoptosis, proliferation

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Introduction

Malignant melanoma is a malignant skin tumor derived from melanin cells, which has a high malignant degree and high fatality rate. Its incidence has continuously increased throughout the world for the past 40 years, and the ratio of tumors at the middle and advanced stages, as well as operation infeasibility, has also increased.[1–6] Radiotherapy is an important local therapy that plays an important role in the treatment of malignant tumors. However, clinical studies indicate that malignant melanoma resists to conventional rays, and conventional radiotherapy is restricted in the clinical applications of malignant melanoma. Studies have shown that the shoulder section of the survival curve widens after malignant melanoma cells are radiated by conventional rays and the strong repair ability to sublethal cell injury results in the relative insensitivity of melanoma to conventional rays.

The mechanism for rays to destroy tumor cells is that the single strand and/or double strand DNA are broken by direct ionization injuries and indirect actions of active oxygen on DNA; thus, inducing cell death. Hence, apoptosis is the main mechanism of sublethal cell injury repair and tumor cell death (radiotherapy is achieved through the induction of cell apoptosis).[7] Malignant melanoma cannot induce massive cell apoptosis under the action of

conventional rays due to radiation resistance, and its treatment effects are not significant. Heavy ion can provide better cell killing effects due to the unique physical and biological effects of its radiation, such that it is valued in terms of treating tumors with radiation resistance. Clinical applications has revealed that local tumor control rate is high when heavy ion is used to treat malignant melanoma, indicating that heavy ion is an important direction in treating melanoma in the future.

Materials

Reagents and instruments. Fetal bovine serum (FBS; purchased from BI); trypsin, 100 U/mL of penicillin and 100 μg/mL of streptomycin (purchased from Hyclone, USA); dimethyl sulfoxide (DMSO; Sigma, USA); Paraformaldehyde (purchased from Linyi Taier Chemtech Corporation, Linyi City, Shandong Province, China); phosphate buffer saline (PBS), anti-rat monoclonal antibodies and S-P kit were purchased from Fuzhou MX Biotechnologies Co., Ltd.; all models of culture bottles and culture dishes (purchased from Corning, USA); other reagents were made in China and pure upon analysis.

Cell culture. Mice with B16F10 melanoma cells were purchased from Shanghai OBIO Co., Ltd. B16F10 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Then, cells were cultured and generated in an incubator with 5% CO_2 at 37°C. Cells at the logarithmic phase were chosen for the experiments.

Animals. Pure C57BL/6J mice, weighting 17 ± 2 g, were selected (female: 50%, male: 50%) and purchased from the Experiment Animal Center of The Fourth Military Medical University, License no. SCXK-(J)-2012-0007.

Methods

Tumor-bearing methods. B16F10 melanoma cells at the logarithmic phase were selected and digested by pancreatin, and cell concentration was adjusted to 2×10^7/mL. A 0.1-mL solution was cultivated into the C57BL/6 mice foot pad, and was closely observed for leakage, inflammation and swelling and diabrosis in the injection site of the mice foot pad, together with the mental status and defecation.

A solid tumor was subcutaneously formed in the mice foot pad (approximately on the 11th day after tumor cells were inoculated), and a lump could be observed in the plantapedis of all mice (with a diameter of approximately 2.5×2.5 mm). The tumor-bearing ratio was 100%. During this time, mice were randomized to three groups: control group ($n = 20$; female: 50%, male: 50%), heavy ion ($^{12}C^{6+}$) experimental group ($n = 20$; female: 50%, male: 50%), and X-ray experimental group ($n = 20$; female: 50%, male: 50%).

Radiation on tumor-bearing mice. Self-customized units were used to fix the mouse pads for the purpose of radiation. Heavy ion rays were supplied by Lanzhou Heavy Ion Study Unit of the Institute of Modern Physics, Chinese Academy of Sciences, with an energy of 300 MeV/nucleon, linear energy transfer (LET) of 75 keV/μm, and a dose rate of 0.15 Gy/min. The $^{12}C^{6+}$ low LET X-rays were provided by a 21EX type medical electron linear accelerator by Lanzhou General Hospital of Lanzhou Military Region, with the dose ratio of 4 Gy/min. The heavy ion group and X-ray group gave a total radiation dosage of 10 Gy to the tumor site of the mice pad, while the control group was not radiated and other conditions remained the same.

Treatment methods of the tumor. Tumor-bearing mice were dislocated and killed at 30 days following incubation. Then, the weight and tumor volume ($L \times W \times T$) were measured. Ophthalmic scissors were used to carefully separate the tumor from the pad, which was placed on a scaled paper, photographed, and encoded. Afterwards, the tumor was stored in 4% paraformaldehyde container.

Immunohistochemical examination. Slices were obtained for routine dewaxing, and cultivated with 3% hydrogen peroxide at room temperature for 10 min to eliminate the enzymatic activity of the endogenous peroxidase. Then, these were rinsed with PBS for 10 min three times. Upon antigen microwave repairing, these were diluted by target antibodies over night at 4°C. Afterwards, these were incubated by secondary antibodies at 37°C for 30 min, and a 3,3′-diaminobenzidine (DAB) developer and hematoxylin were used for counterstaining and sealing slices. The above slices were observed under a microscope. PBS was used to substitute the first antibodies, and other steps were kept the same. The result was used as the negative control.

Statistical methods. Measurement data were expressed as $z \pm s$, and all data used the SPSS 20.0 software for statistical processing. In case of single factor analysis, numerical variables were analyzed by *t*-test, and categorical variables were analyzed using X^2-test or Fisher's exact test. In addition, indicators that have statistical significance were used for logistic multi-factor regression analysis. The survival analysis was conducted using the Kaplan-Meier method and Log-rank test. $P < 0.05$ was considered statistically significant.

SPSS16.0 software was used to conduct the statistical analysis of the expression of proteins via the Kruskal-Wallis method.

Results

The influence of low LET ray and high LET particle radiation on the growth of tumor-bearing mice

After the tumor underwent radiotherapy, the superficial skin did not present with the bleeding phenomenon, and the tumor was hardened. Upon X-ray radiotherapy, the tumor volume decreased and did not grow, and the number of diabrosis was reduced. However, the tumor did not disappear, and the movement of mice was restricted. In addition, one mouse presented with a new tumor around the primary site. Upon heavy ion radiation for 4–5 days, the tumor volume significantly decreased, the mental status of mice was relatively satisfactory, and the

hair color was smooth with active actions. Among these mice, the tumor of two mice basically disappeared at approximately 13 days of radiation. In the control group, at 30 days after mice were burdened by the tumor, three male mice and four female mice survived; and the status of mice was unsatisfactory with delayed actions, whitened hair color, and exhibited a sharp decrease in weight. After the tumor was separated, it can be observed that the tumor volume significantly decreased upon heavy ion ($^{12}C^{6+}$) radiation (Figure 1).

At one week after X-ray radiation, the growth inhibition ratio of the tumor reached 37.5%, compared with the 95% ratio at one week after heavy ion radiation. At two weeks after treatment, the growth inhibition ratio of the tumor reached 42% in the X-ray treatment group, while this ratio reached 96% in the heavy ion treatment group. In addition, the tumor volume significantly shrunk compared with the control group (Figure 1).

The influence of low LET ray and high LET particle radiation on the morphology of tumor-bearing mice tumor cells

In the control group, melanoma cells grew actively with intensive cells, and the nuclear atypia was significant with large nucleus and deep dyeing. Multi-direction differentiation was significant, and the multi-nuclear and fission phase were observed. In the X-ray treatment group, no significant necrosis was observed in cancer cells, and the partial nucleus was small with irregular shapes. Spindle-shaped, oval and triangular tumor cells were arranged around the vessels like a sleeve. These had a thick vascular wall, and extensive bleeding and necrosis occurred. However, necrosis sites increased in the heavy ion treatment group compared with the X-ray group. Furthermore, cellular morphology trends normal and the arrangements were regularly relative to the control group (Figure 2).

Influence of low LET ray and high LET particle radiation on the median survival time of mice

Upon analysis on the survival rate by Kaplan-Meier method, $P < 0.01$ was obtained after each survival curve and was adjusted by Log-rank test, indicating the statistical significance. Figure 3 shows that the survival rate of mice was the highest in the heavy ion treatment group. Furthermore, median survival time of mice was 30 days after heavy ion radiation, 27 days after X-ray radiation, and 23 days in the control group. This indicates that heavy ion radiation can effectively improve the median survival time of mice with malignant melanoma.

Influence of low LET ray and high LET particle radiation on the expression of Bax and Bcl-2 proteins in tumor cells

Bcl-2 and Bax were positioned in the cell cytoplasm and membrane, and immunohistochemical assay revealed that the cytoplasm presented with yellow particles during their expression. After tumor-bearing tumors were radiated, the positive ratio of Bax protein significantly increased compared with the control group. A indicated the expression of the control group, and this low expression level was basically negative. Compared with X-ray group, the positive ratio in the heavy ion radiation group significantly increased. However, the positive expression of Bcl-2 protein decreased (Figure 4) and controls revealed a highly positive expression, while the positive ratio after heavy ion radiation significantly reduced. Statistical analysis described that the positive expression of Bax was not statistically significant in the X-ray and control groups ($P = 0.218$). Statistically significant differences were observed between the heavy ion group, control group, and X-ray group ($P = 0.000$, extremely significant). The difference in the positive expression of Bcl-2 was statistically significant in the X-ray and control groups ($P = 0.037$). Statistically significant differences were observed between the heavy ion group, control group, and X-ray group ($P = 0.000$, extremely significant).

Influence of low LET ray and high LET particle radiation on the expression of cytochrome C protein in tumor cells

Dyeing results demonstrated that the positive expression of cytochrome C increased after the tumor was radiated, when compared with the control group; and these presented with

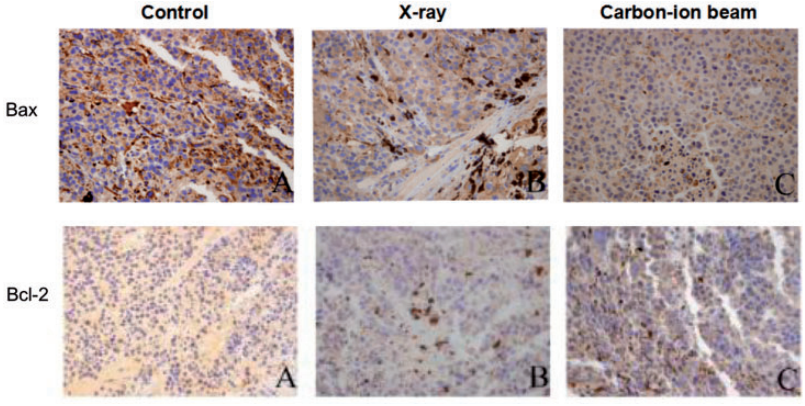

Figure 1 Effect of high linear energy transfer (LET) and low LET irradiation on the development of the mice. (a) Control tumor; (b) after X-ray radiation; (c) after heavy ion radiation. (A color version of this figure is available in the online journal.)

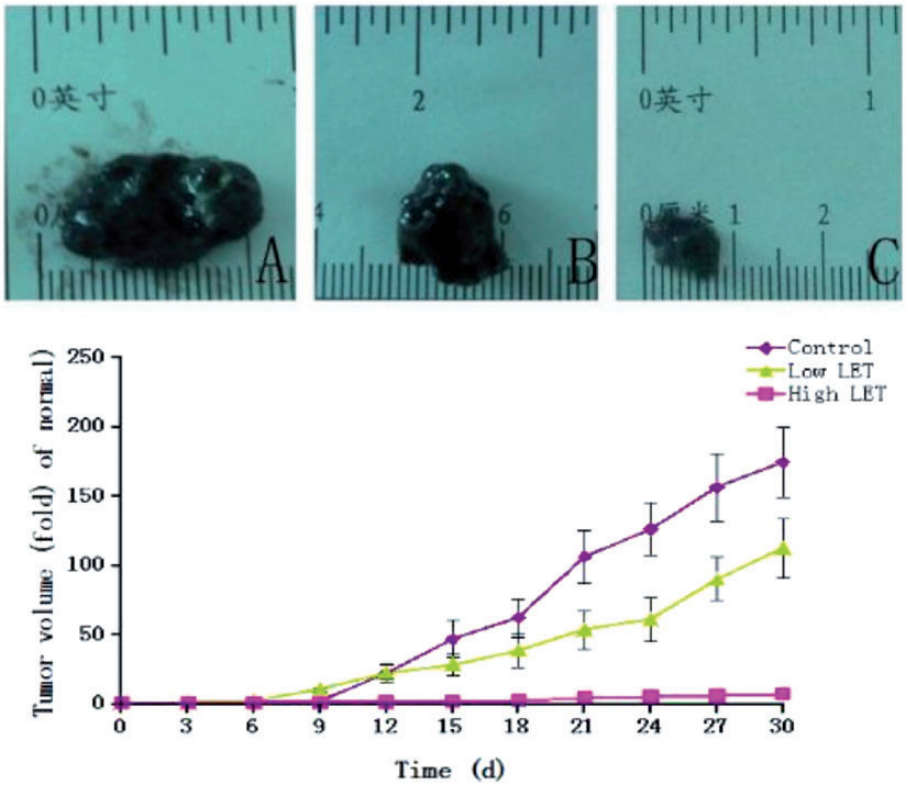

Figure 2 Effect of high linear energy transfer (LET) and low LET irradiation on cellular morphology in mice. (A color version of this figure is available in the online journal.)

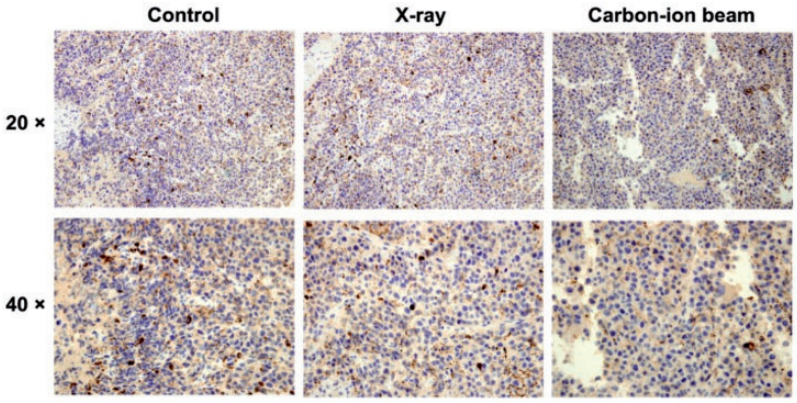

Figure 3 Survival curves of mice model after high linear energy transfer (LET) and low LET irradiation. (A color version of this figure is available in the online journal.)

yellow particles, indicating tumor cells commenced apoptosis after radiation. However, heavy ion radiation indicated that this high positive expression was observed in cytochrome C in cells, indicating that heavy ion radiation can significantly induce apoptosis in tumor cells (Figure 5). Statistical analysis indicated that the positive expression of cytochrome C in the X-ray and control groups had no statistical significance ($P = 0.435$), and statistically significant differences were observed between the heavy ion group, control group, and X-ray group ($P = 0.001$ and $P = 0.012$, respectively).

Influence of low LET ray and high LET particle radiation on the expression of survivin protein in tumor cells

The positive expression of survivin protein indicated brown particle dyeing in the cytoplasm and/or nucleus under a light microscope. In the control group, melanoma cells were densely stained in brown, and survivin protein was highly expressed. Furthermore, X-ray group revealed a brown cytoplasm with a lightened color. Statistical analysis described that the positive expression of survivin had no statistical significance in the X-ray and control groups

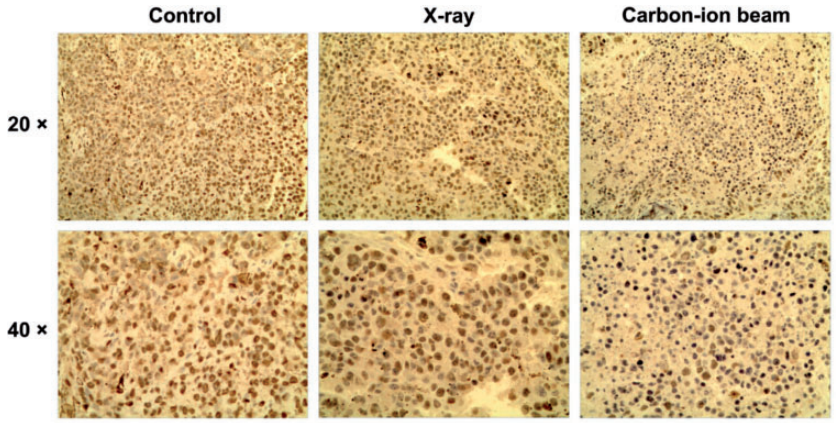

Figure 4 Expression of Bax and Bcl-2 in tissues after high linear energy transfer (LET) and low LET irradiation. (A color version of this figure is available in the online journal.)

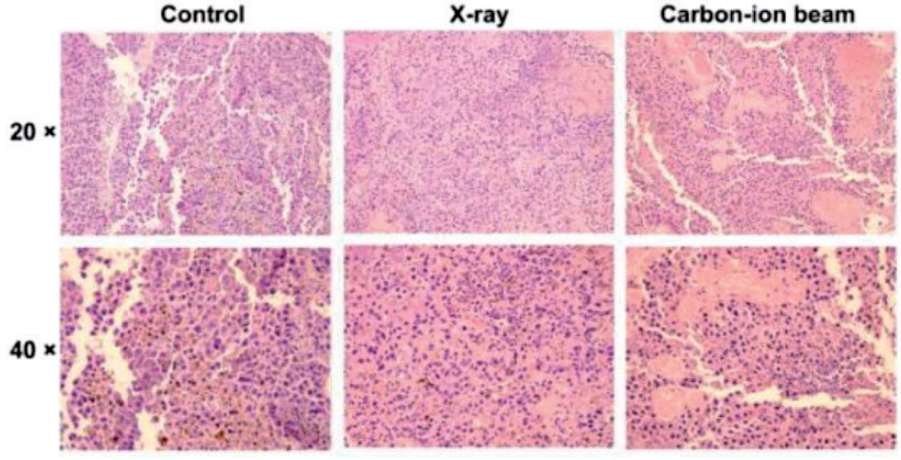

Figure 5 Expression of cytochrome C in tissues after high linear energy transfer (LET) and low LET irradiation. (A color version of this figure is available in the online journal.)

($P = 0.06$). Statistically significant differences were observed among the heavy ion group, control group, and X-ray group ($P = 0.001$, $P = 0.01$). The positive expression ratio of survivin protein was significantly decreased in the heavy ion radiation group (Figure 6).

Influence of low LET ray and high LET particle radiation on the expression of PCNA proteins in tumor cells

Proliferating cell nuclear antigen (PCNA) in the control group indicated highly positive expression levels, and was mainly positioned in the nucleus of tumor cells, which was diffusedly distributed in brown-yellow or dark-brown (Figure 7). PCNA positive cells in the X-ray group were focally distributed, while PCNA positive cells in the heavy ion group were individually distributed. Statistical analysis revealed that the positive expression had no statistical significance in the X-ray and control groups ($P = 0.157$). Statistically significant differences were observed between the heavy ion group, control group, and X-ray group ($P = 0.000$, $P = 0.004$).

Discussion

Duan Xin *et al.* expressed that heavy ions can promote exogenous P53 genes to induce the apoptosis of cells and increase their sensitivity to radiation. The increase in cell apoptosis ratio induced by radiation depends on LET, regardless of whether it is high LET or low LET; exogenous P53 proteins can effectively induce cell apoptosis. However, the P53 in this study is an exogenous inductive gene with a lower wild mutation rate.[8] Meanwhile, some literatures reported that P53 in human malignant melanoma cells cannot regulate target genes related to apoptosis. It is very important to select one apoptosis pathway expressed in melanoma, which can better investigate the biological mechanism of moderate and heavy ions and X-ray. Studies have indicated that bcl-2, Bax, and survivin are expressed in human and mice, based on genes that are

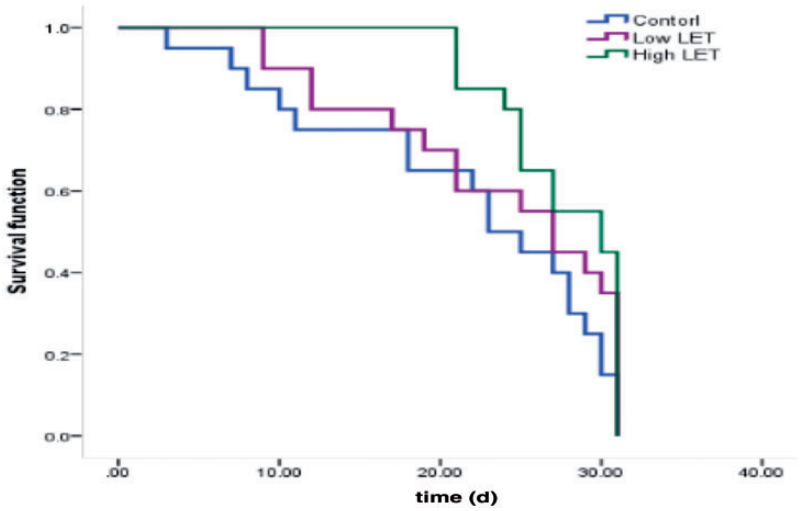

Figure 6 Expression of Survivin in tissues after high linear energy transfer (LET) and low LET irradiation. (A color version of this figure is available in the online journal.)

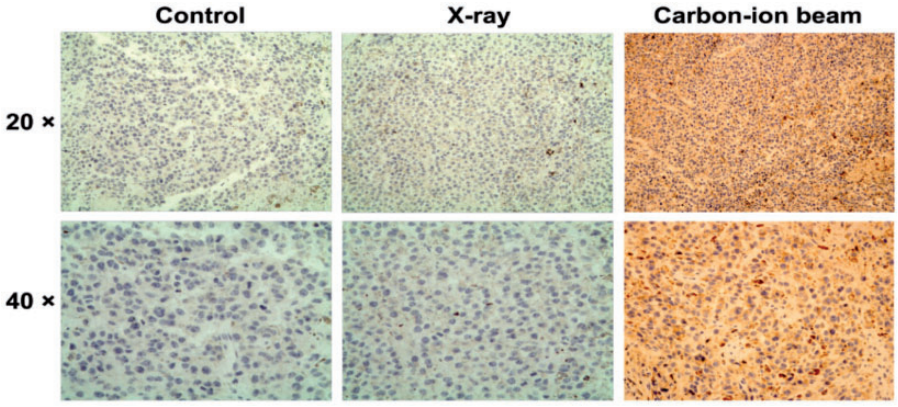

Figure 7 Expression of proliferating cell nuclear antigen (PCNA) in tissues after high linear energy transfer (LET) and low LET irradiation. (A color version of this figure is available in the online journal.)

directly expressed in melanoma, which can better indicate the expression mechanism of their heavy ions and X-ray, and compare the influence of two kinds of rays on the apoptosis of melanoma. Most of the previous studies have adopted the same biological dosage to observe their apoptosis, but the expression of their apoptotic genes have not been reported under the same physical dosage. However, 10 Gy is a critical value in clinical and basic studies, which is extensively applied in clinical treatment.

Apoptosis, also called programmed death, plays an important role in maintaining normal body physiological equilibrium and eliminating aging body cells. Excessive proliferation or the apoptosis inhibition of tumor cells is one of the important reasons for the generation and development of tumors. Furthermore, whether tumor cells can enter into the apoptosis pathway remains as one of the important signs whether one treatment method is effective or not. Bcl-2 family is closely correlated with cell apoptosis, and its family can be categorized into anti-apoptosis proteins including Bcl-2 and Bcl-xl, as well as pro-apoptotic proteins such as Bax and bak.[9] Among these, Bax and Bcl-2 are typical positive regulator proteins and negative regulator proteins of apoptosis, respectively. Bcl-2/Bax jointly acts on the outer membrane of the mitochondria, and the ratio between these affects the apoptosis or survival of cells.[10] The overexpression of Bax can antagonize the protective effect of Bcl-2, and induce cell death. The overexpression of Bcl-xL can be found in many tumors; hence, the inhibition of the overexpression of Bcl-2 and Bcl-xL and other antiapoptotic proteins, as well as the recovery of their apoptosis pathway signals and protein expression, are strategies and directions of tumor treatment that are currently to some extent.[11] At present, it is thought that Bcl-2 transduces pathways mainly by interrupting the public signals of tumor cell apoptosis, in order to inhibit or interrupt the apoptosis process of many kinds of cells and cell lines.[12] The mechanism for heavy ions to induce the apoptosis of tumor cells remains unknown. At present, it is considered that the injury target to tumor cells in radiotherapy is DNA, since injuries to the DNA would destroy the mechanism of cell

division, and consequently induce apoptosis.[13] Studies indicate that Bcl-2 can interrupt the apoptosis actions of products of genetic transcription caused by ionizing radiation and other factors on cells. On the contrary, the expression of Bax is positively correlated with its apoptosis.[14] This test indicates that radiotherapy can inhibit the expression of Bcl-2 genes by inducing the increase in the expression level of Bax genes, based on combined actions, in order to jointly induce apoptosis using high LET (heavy ion) and low LET (X-ray) radiation on tumor-bearing mice, as shown in Figure 4. Compared with X-ray, heavy ion presents with more significant pro-apoptosis phenomenon to promote cells apoptosis. It is speculated that heavy ion radiation may break the DNA double strands of tumor cells.

To date, studies on cell apoptosis have been transformed into the apoptosis system, which is induced by the mitochondria from a previous nucleus. A number of studies have demonstrated[15] that proteins of the Bcl-2 family can regulate the release of cytochrome C. After the activated Bax is transported into the mitochondria, combined with mitochondria apoptosis pathways mediated by Bax that started on the outer membrane of the mitochondria, cytochrome C can also result in the release of cytochrome C through the release of this pathway even during the presence of excessive Bax in the absence of other death signals. Cytochrome C is an apoptosis signal derived from the mitochondria, which can magnify key apoptosis signals in several cell apoptosis pathways.[16] To date, it is considered that cytochrome C is one of three apoptotic protease activating factors (APAFs),[17] which also participates in the process of cell apoptosis. Generally, cytochrome C is located in the inner membrane of the mitochondria, and cannot pass through the outer membrane. However, cytochrome C can be released into the cytoplasm from the inner membrane of the mitochondria under the stimulation of some factors, and such release of cytochrome C magnifies the death receptor pathway and induces cell apoptosis or necrosis. Apoptosis or necrosis depends on the stability degree of ATP. If ATP can be stabilized in a certain level, then caspase would be activated to induce cell apoptosis.[18,19] Cytochrome C is first separated from the respiratory chains, and released into the cytoplasm based on which the apoptosis body compound is formed by it with Apaf-1 and caspase9. Then, the apoptosis body continuously activates caspase3 in the downstream, resulting in a cascade reaction, and finally inducing cell apoptosis.[20] This study shows that the expression level of cytochrome C increases after tumors are radiated, and this positive expression is higher in the heavy ion group. As a result, after mice with melanoma receive cell radiation, cells are induced in the mitochondria apoptosis pathway; and this is more significant for heavy ion radiation.

The study of Shin *et al*.[21] and Suzuki *et al*.[22] indicated that the survivin gene, the strongest apoptosis-profilin so far, regulates cells by directly inhibiting the end effector molecule caspase-3 and caspase-7, as well as the interaction with CDK4 and CDK2. Furthermore, survivin is not expressed in normally differentiated human tissues, and is only expressed in malignant tumor tissues. However, it was found that 85% of survivin exists in invasive melanoma. The antisense transfection or dominant negative phase of survivin in melanoma, without other irritations, can also effectively result in cell apoptosis; hence, the malignancy of malignant melanoma can be diagnosed by the expression level of survivin[23,24] and be positively correlated with survivin. As shown in Figure 6, this highly positive expression in the control group may lead to abnormal apoptosis regulation, and result in apoptosis blocking, which play an important promotion in the generation and development of tumors, and indicate higher malignancy. The positive expression in the X-ray group had no significant changes compared with the control group, which may be related to the insensitivity of malignant melanoma to low LET rays. In the heavy ion radiation group, it can be obviously observed that the expression level of survivin is reduced, indicating the reduction in malignancy of malignant melanin tumor cells, and showing that the inhibition of apoptosis is significantly weakened upon heavy ion radiation. Its sensitivity to radiation is stronger than that of the X-ray.

PCNA exists in actively proliferated cells without species and tissue specificity.[25–27] Bravo *et al.* demonstrated that the content change of PCNA in proliferated cell cycles has significant periodicity and consistency with the phase change of DNA duplicates, indicating that the ''trace amount'' expression at the G0 stage increased from the early stage of G1, and quickly elevated at the advanced stage, reaching a peak to the S stage, while continuously decreasing at the G2 and M stages.[28] PCNA is mainly expressed in the S stage of proliferated cell cycles, which is a key factor for controlling DNA duplicates and cell division. Hence, its expression is positively correlated with cell proliferation activity. As a regulatory protein of cell cycle, its synthesis level reflects the ratio of cell proliferation and DNA synthesis, and such protein is closely correlated with multiple regulatory factors of the cell cycle; which is a hot marker at the proliferation stage in tumor proliferation activities to date.[29,30] Some reports indicate that PCNA is positively expressed in different human tumor tissues, and it has been considered that the positive degree of PCNA is used as a parameter of tumor grading, staging, and prognosis, which has clinical significance. As shown in Figure 7, PCNA is highly and positively expressed in the control group, indicating that melanoma cells are proliferated actively, and most cells are at the proliferation stage with high malignancy and fast tumor growth rate. Upon X-ray radiation, the expression level of PCNA decreased. The inhibition degree of heavy ion radiation is higher, indicating that its inhibition of tumor cell proliferation is more significant, compared with that of the X-ray. PCNA is expressed in few cells, which indicates that only partial cells are at the proliferation stage and is continuously divided, and DNA duplicates are restricted. The expression level of PCNA can reflect cell proliferation activity, which indicates that the growth speed of malignant melanoma cells is reduced and its malignancy is lowered after radiation, especially heavy ion radiation.

In this study, PCNA can induce the apoptosis of malignant melanoma cells and inhibit its proliferation, and its induction effect on apoptosis is significantly higher than

low LET X-ray; hence, it is expected to overcome its lower sensitivity to radiation. This study can provide theoretical basis for clinical trials, in which malignant melanoma is treated by heavy ion ($^{12}C^{6+}$), in order to accurately determine the clinical efficacy of heavy ion therapy.

Authors' contributions: SL and JQ designed the study and the experiments. CZ, D-WG, QL, HZ, and X-DJ performed the experiments. SL and CZ helped designing and optimizing experimental protocols. YL helped in statistical analysis of results. All authors contributed in writing the paper, and SL supervised the research.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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