

# Role of autophagy in high linear energy transfer radiation-induced cytotoxicity to tumor cells

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## Key words

Apoptosis, autophagy, high-LET radiation, PI3K/Akt pathway, radiosensitivity

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The primary principle of radiotherapy lies on precise localization of sufficient dose in the target lesion while minimizing damage to the surrounding normal tissues. Charged particles such as carbon, neon, and other heavy ions, show an increase in energy deposition with penetration depth up to a sharp maximum at the end of their range, known as the Bragg peak. The dose deposition before this peak is low, and almost insignificant after it. Thus, the dose delivered to healthy tissues surrounding the tumor can be minimized.<sup>(1,2)</sup> Another advantage of heavy ions over X-rays is their higher linear energy transfer (LET). Compared with conventional radiations such as X-rays and  $\gamma$ -rays, high-LET heavy ions have excellent properties, such as higher relative biological effectiveness, reduction in oxygen enhancement ratio, and nearly unchanged radiosensitivity with the cell cycle.<sup>(3,4)</sup>

As a result, high-LET heavy ions have highly lethal effects, even on radioresistant tumors. It is conceivable that effective therapeutic strategies may be designed based on the genetic and biochemical events involved in cell death. Therefore, accurate characterization and quantification of the process by which radiation leads to cell response have become increasingly important in further understanding the biological effectiveness of high-LET radiation.

Heavy-ion radiotherapy has a potential advantage over conventional radiotherapy due to improved dose distribution and a higher biological effectiveness in cancer therapy. However, there is a little information currently available on the cellular and molecular basis for heavy-ion irradiation-induced cell death. Autophagy, as a novel important target to improve anticancer therapy, has recently attracted considerable attention. In this study, the effect of autophagy induced by high linear energy transfer (LET) carbon ions was examined in various tumor cell lines. To our knowledge, our study is the first to reveal that high-LET carbon ions could induce autophagy in various tumor cells effectively, and the autophagic level in the irradiated cells increased in a dose- and LET-dependent manner. The ability of carbon ions to inhibit the activation of the PI3K/Akt pathway rose with increasing their LET. Moreover, modulation of autophagy in tumor cells could modify their sensitivity to high-LET radiation, and inhibiting autophagy accelerated apoptotic cell death, resulting in an increase in radiosensitivity. Our data imply that targeting autophagy might enhance the effectiveness of heavy-ion radiotherapy.

Recently, autophagy has been revealed as a novel response of cancer cells to chemo-/radiation therapy. Autophagy is an evolutionarily conserved process by which cells recycle their components, such as long-lived proteins and damaged organelles, involving the sequestration of cytoplasmic components within a double membrane structure, termed autophagosome, and subsequent delivery to lysosomes for degradation.<sup>(5,6)</sup> Moreover, it is a dynamic process with an important role not only in the recycling of cytoplasmic constituents to support metabolism but also in overcoming adverse conditions to prevent the accumulation of damaged, toxic proteins and organelles.<sup>(7,8)</sup> Dysregulation of autophagy has severe consequences and is associated with several pathophysiological conditions, such as cancer, infection, autoimmunity, inflammatory diseases, neurodegeneration, and aging.<sup>(9,10)</sup> In cancer therapy, the role of autophagy is paradoxical. In some reports, autophagy appeared to function as a protective mechanism against cellular stress.<sup>(11–13)</sup> However, the induction of autophagy still played a pivotal role in cell death induced by radiations or reagents in other reports.<sup>(14–16)</sup> Therefore, whether autophagy helps to kill cancer cells or to sustain their survival under stressful conditions remains controversial.<sup>(8,17)</sup> Several groups have already reported high-LET carbon ion-induced autophagy

in tumor cells.<sup>(18–20)</sup> Systematic study of the relationship between high-LET radiation-induced cytotoxicity and autophagy, however, is still scarce.

In this study, using six different tumor cell lines originated from various organs, we examined whether cells after exposure to high-LET carbon ions undergo autophagy and explored the role of autophagy in high-LET radiation-induced cytotoxicity. In addition, the relationship between autophagy and apoptosis and relevant underlying mechanisms were investigated. Undoubtedly, elucidating the role of autophagy in high-LET radiation-induced cytotoxicity to tumor cells is useful to improving treatment against tumors by heavy-ion radiotherapy.

## Materials and Methods

**Reagents.** 3-Methyladenine (3-MA), chloroquine (CQ), and rapamycin purchased from Sigma-Aldrich (St Louis, MO, USA) were used at concentrations of 5 mM, 10  $\mu$ M, and 1  $\mu$ M, respectively, in all the experiments. These autophagy inhibitors and promoter at the indicated concentrations were evaluated to be non-cytotoxic or less cytotoxic to the tumor cells used in this study (Fig. S1). Cells were pretreated with 3-MA, CQ, and rapamycin for 4 h before irradiation.

**Cell culture.** Human cervical cancer cells (HeLa) and human glioblastoma cells (SHG44) were preserved in our laboratory. Established human breast cancer cell lines (MCF-7 and MDA-MB-231) and human hepatoma cell line (hepG2) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Human salivary gland tumor cell line (HSG) was a gift from Dr. Furusawa, National Institute of Radiological Sciences, Chiba, Japan. HeLa, MCF-7, MDA-MB-231, and HSG cells were maintained in DMEM. SHG44 and HepG2 cells were maintained in RPMI-1640 and MEM, respectively. All the cell cultures were supplemented with 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, and 10% (v/v) FBS (Gibco, Carlsbad, CA, USA) and kept at 37°C, 5% CO<sub>2</sub> in incubators.

**Irradiation.** Most of the irradiations were carried out with a carbon ion beam of 165 MeV/u in the heavy-ion therapy terminal of the Heavy-Ion Research Facility in Lanzhou at the Institute of Modern Physics, Chinese Academy of Sciences (Lanzhou, China). Some experiments were carried out with a carbon ion beam of 290 MeV/u in the Heavy-Ion Medical Accelerator in Chiba at the National Institute of Radiological Sciences, Japan. Dose-averaged LET of the carbon ion beams on cell samples was adjusted to 13 or 75 keV/ $\mu$ m according to our experimental requirements. All the irradiations were carried out at room temperature and control groups were sham-irradiated.

**Visualization of monodansylcadaverine-labeled vacuoles.** Monodansylcadaverine (MDC) is an autofluorescent weak base that accumulates in acidic lysosomal vacuoles, showing high selectivity for autophagosomes, due to the high level of unhydrolyzed membrane lipids from engulfed organelles.<sup>(12)</sup> Twenty-four hours after irradiation, cells were incubated with 0.1 mM MDC (Sigma-Aldrich) in PBS at 37°C for 10 min, washed, and immediately analyzed with fluorescence microscopy (BX51; Olympus, Tokyo, Japan).

**Vacuolar redistribution of GFP-LC3.** To visualize the formation of autophagic vesicles, the green fluorescent protein GFP-LC3 expression vector, purchased from (Addgene, Cambridge, MA, USA) (plasmid 11546) was used. Cells were transiently transfected with the Lipofectamine LTX and PLUS Reagents (Invitrogen, Carlsbad, CA, USA) according to the supplier's protocol. After incubation for 24 h, the cells were irradiated with

the high-LET carbon ions. Cells were fixed with 4% paraformaldehyde in PBS 4 or 24 h after irradiation and then analyzed with fluorescence microscopy.

**Quantitative analysis of autophagy with flow cytometry.** Autophagy is characterized by the formation of acidic vesicular organelles (AVOs).<sup>(21,22)</sup> To detect and quantify AVOs, cells were stained with 1.0  $\mu$ g/mL acridine orange (AO) for 15 min. In the AO stained cells, acidic compartments such as autophagosomes show bright red. Green (510–530 nm) and red (>650 nm) fluorescence, illuminated with blue (488 nm) light excitation, was measured with a flow cytometer (FACSCalibur; Becton-Dickinson, Franklin Lakes, NJ, USA) and analyzed using the FlowJo software (Treestar Inc., Ashland, OR, USA).

**Transfection with siRNA.** Short interfering RNA knockdown (KD) was used to inhibit mammalian Beclin 1 (ortholog of Atg6) and Atg5. Cells were transfected with siRNA of Beclin 1 and Atg5 (Cell Signaling Technology, Danvers, MA, USA) using the transfection reagent Lipofectamine 2000 (Invitrogen). The extent of Beclin 1 and Atg5 KD was determined by Western blot analysis of protein level.

**Apoptosis assay.** Flow cytometric measurements of Annexin V-FITC staining and DNA fragmentation were used to quantify apoptotic cells as reported previously.<sup>(23)</sup>

**Western blot analysis.** Total cellular extracts were prepared as described previously,<sup>(23)</sup> and transferred to PVDF membranes. Blots were incubated with the indicated antibodies and visualized by the enhanced chemiluminescence procedure. Primary antibodies such as LC3, SQSTM1/p62, Atg5, Beclin 1, caspase-3, p-Akt (ser473), p-mTOR (ser2448), p-p70S6K (ser235/236), and  $\beta$ -actin, purchased from Cell Signaling Technology, were used in this study.

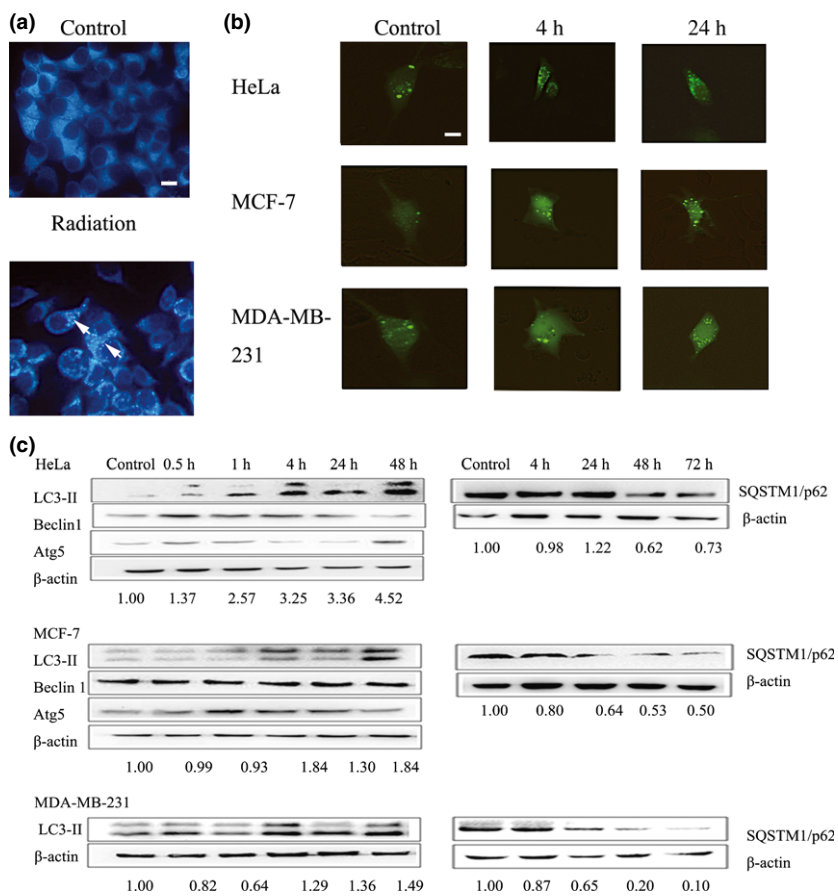
**Clonogenic assay.** After irradiation, cell survival was determined by the colony formation assay as reported previously.<sup>(23)</sup>

**Statistical analysis.** The statistical significance of the experimental results was evaluated using unpaired Student's *t*-tests. The data are expressed as means  $\pm$  standard deviations and are considered significant when  $P < 0.05$ .

## Results

**High-LET radiation induced autophagy effectively in tumor cells.** First, the autophagic effect induced by the carbon ions with LET of 75 keV/ $\mu$ m in tumor cells was observed at the morphologic level. The MDC dye was used to assess levels of mature autophagic vesicle formation in HeLa cells following irradiation. Unirradiated cells (control) showed diffusive MDC staining instead of a punctate staining pattern. In contrast, the irradiated cells showed extensive punctate staining of bright blue, as shown in the Figure 1(a). Figure 1(b) shows that the redistribution of GFP-LC3 from a diffusive cytosolic to a punctate autophagosome associated pattern was observed in HeLa, MCF-7, and MDA-MB-231 cells at 4 and 24 h post-irradiation. Taken together, the morphological results support that the carbon ions induced autophagy in the tumor cells effectively.

The LC3 protein is a marker for autophagy.<sup>(24)</sup> To assess the autophagic pathway on the carbon ions in HeLa cells, LC3 conversions from LC3-I to LC3-II were also analyzed at different time points post-irradiation (0.5, 1, 4, 24, and 48 h). As shown in Figure 1(c), unirradiated cells presented a low amount of LC3-II at any time. However, the level of LC3-II expression in the irradiated cells increased markedly with the time post-irradiation, implying the process of autophagy in the cells. The expression of LC3-II in MCF-7 and MDA-MB-231 cells differed slightly from that in HeLa cells. The LC3-II



**Fig. 1.** Autophagy induced by carbon ions with linear energy transfer of 75 keV/ $\mu$ m and 2 Gy in HeLa, MCF-7, and MDA-MB-231 cells at morphological and molecular levels. (a) HeLa cells were stained with monodansylcadaverine at 24 h post-irradiation, then the cells were observed using fluorescence microscopy (arrowheads). (b) GFP-LC3 staining patterns were analyzed by fluorescence microscopy. (a,b) Scale bars = 10  $\mu$ m. (c) LC3-II conversion and expression of sequestosome 1 (SQSTM1)/p62, Beclin 1, and Atg5 proteins in tumor cells following exposure to high-LET radiation; the relative level of LC3-II in comparison to  $\beta$ -actin is indicated below each immunoblot image.

content decreased for the first few hours (1 and 4 h after irradiation), then increased with time lapse in MCF-7 and MDA-MB-231 cells. In any case, we observed that the LC3-II expressions were enhanced in these three different cell lines after carbon ion irradiation. The amount of LC3-II at a certain time point does not indicate the autophagic flux.<sup>(25)</sup> Therefore, monitoring of the natural autophagic substrate p62 (also called sequestosome 1 or SQSTM1) has been widely used to assess autophagic flux. Figure 1(c) also shows the results of SQSTM1/p62 expression in these three cell lines. Initially, the SQSTM1/p62 expression levels showed slight attenuation at 4 h after irradiation. Subsequently, the levels of the protein expression decreased significantly at 24 h in MCF-7 and MDA-MB-231 or at 48 h in HeLa cells. Our results indicate that SQSTM1/p62 was degraded in autolysosomes and the autophagic flux was activated definitely after irradiation. The expression of other key proteins related to autophagy under the high-LET radiation stimulus was detected as well. Figure 1(c) shows Atg5 expression gradually increased and reached a maximum, then declined with time in HeLa and MCF-7 cells. Beclin 1 expression was similar to that of Atg5 in HeLa cells, but unchanged with time post-irradiation in MCF-7 cells.

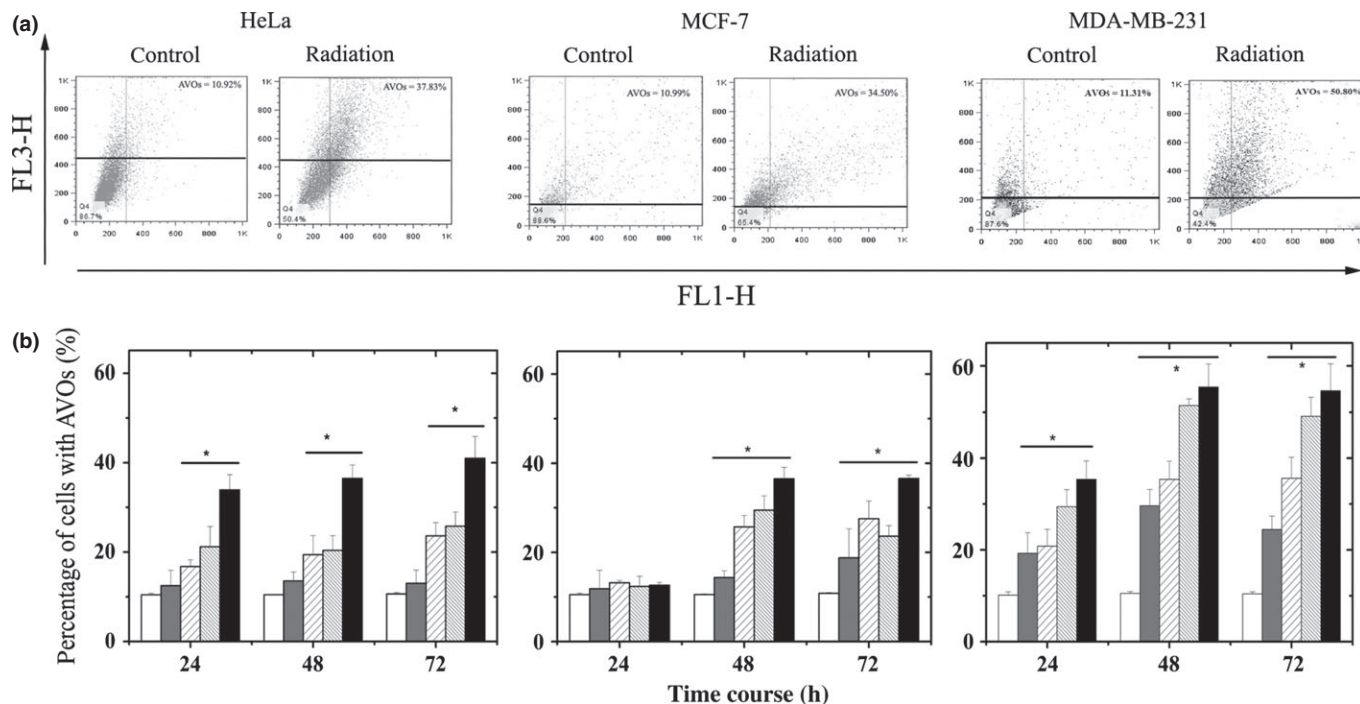
#### Autophagy level increased with LET and dose of carbon ions.

To quantify the possible induction of autophagy, we assayed the presence of acidic vesicular organelles, which are characteristic of this process and can be detected by flow cytometry in combination with AO staining. Shown in Figure 2 are the autophagy levels in HeLa, MCF-7, and MDA-MB-231 cells exposed to the carbon ions with LETs of 13 and 75 keV/ $\mu$ m at doses of 2 Gy or 5 Gy at 24, 48, and 72 h post-irradiation. Clearly, the autophagic rate of HeLa cells increased with LET

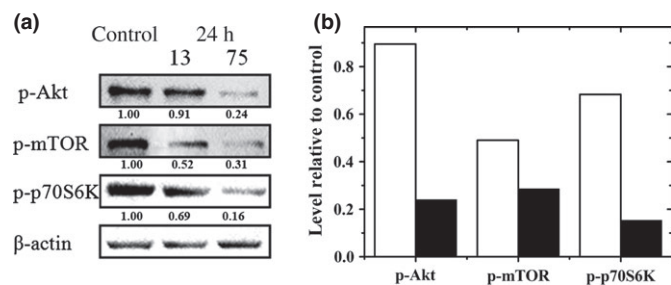
and dose at the time points under investigation after the carbon ion irradiations. Similar results were also observed in MCF-7 and MDA-MB-231 cells.

**Carbon ions induced autophagy in tumor cells by depressing the PI3K/Akt pathway.** To explore the molecular mechanism underlying the autophagy induction by the carbon ions with different LETs (LET = 13 and 75 keV/ $\mu$ m) in tumor cells, the alteration in expression level of serine/threonine kinase, mammalian target of rapamycin (mTOR), which is known to be associated with autophagic regulation,<sup>(26)</sup> was detected in HeLa cells. Because regulation of mTOR by the PI3K/Akt pathway has been revealed,<sup>(26)</sup> phosphorylation of PI3K/Akt-related proteins such as phospho-Akt and phospho-p70S6K were measured as well. Shown in Figure 3 are the phosphorylation levels of these proteins related to autophagy induction in HeLa cells at 24 h post-irradiation. Obviously, the protein expression level of phospho-mTOR decreased in HeLa cells irradiated with the carbon ions of 13 and 75 keV/ $\mu$ m. However, more reduction in the expression level was observed in HeLa cells after exposure to the carbon ions of 75 keV/ $\mu$ m than to those of 13 keV/ $\mu$ m. Compared with the unirradiated control cells, the phospho-mTOR level reduced to approximately 50% and 29% in the cells exposed to the carbon ions of 13 and 75 keV/ $\mu$ m at 24 h post-irradiation, respectively (Fig. 3b). As expected, phosphorylation of the PI3K/Akt-related proteins were effectively depressed by the carbon ion irradiations. The cellular contents of phospho-Akt and phospho-p70S6K proteins in HeLa cells irradiated with the relatively low LET carbon ions reduced to 90% and 68% of the corresponding amounts in the unirradiated cells, respectively, and to 24% and 15% in the cells irradiated with the high-LET carbon ions (Fig. 3b). These results





**Fig. 2.** Quantified assay of autophagy induced by high linear energy transfer carbon ions with flow cytometry in HeLa, MCF-7, and MDA-MB-231 cells. (a) Representative image of flow cytometry at 72 h after irradiation, where FL1-H (normal cells) and FL3-H (cells with autophagosomes) indicate green and red color intensities, respectively. Cells were irradiated with 75 keV/μm carbon ions at 5 Gy. (b) Statistical results of three independent experiments, where the white columns, gray columns, columns filled with sparse lines, columns filled with dense lines, and black columns represent the control groups, cells irradiated with 13 keV/μm carbon ions at 2 Gy, cells irradiated with 13 keV/μm carbon ions at 5 Gy, cells exposed to 75 keV/μm carbon ions at 2 Gy, and cells irradiated with 75 keV/μm carbon ions at 5 Gy, respectively. \* $P < 0.05$ , compared with control.



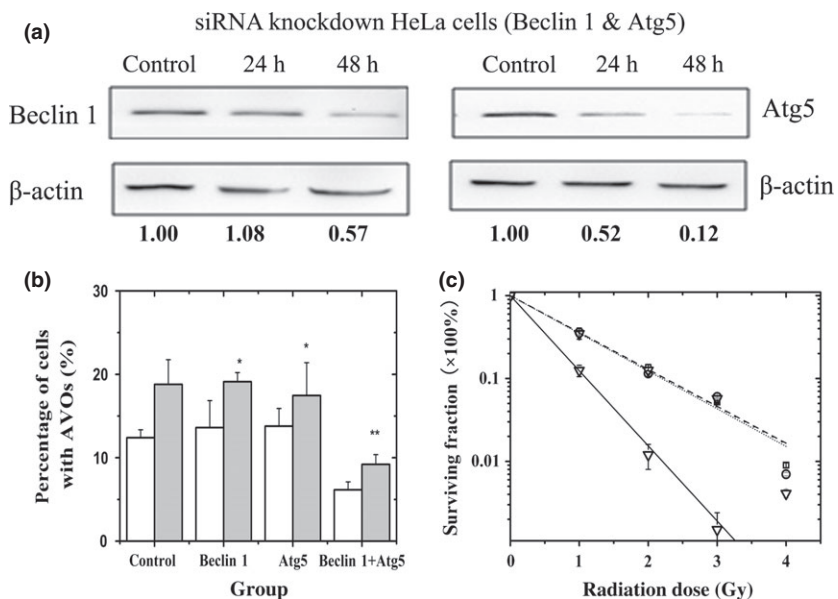
**Fig. 3.** The PI3K/Akt signaling pathway was effectively depressed by carbon ions with different linear energy transfer (LET). Protein expression levels at 24 h after irradiation at 2 Gy were detected. (a) Expression levels of phosphorylated (p-)Akt (ser473), p-mTOR (ser-2448), and p-p70S6K (ser235/236). The relative level of proteins in comparison to β-actin is indicated below each immunoblot image. (b) Each band is compared with the individual band density for β-actin. The relative amounts of the proteins were calculated by comparing them with the densities of the corresponding control samples. Black columns indicate the carbon ions with LET of 75 keV/μm; white columns indicate the carbon ions with LET of 13 keV/μm.

indicate that the carbon ion irradiations induced autophagy in HeLa cells by degrading the activation of the PI3K/Akt pathway, and this pathway was more effectively depressed by the carbon ions with high LET than relatively low LET.

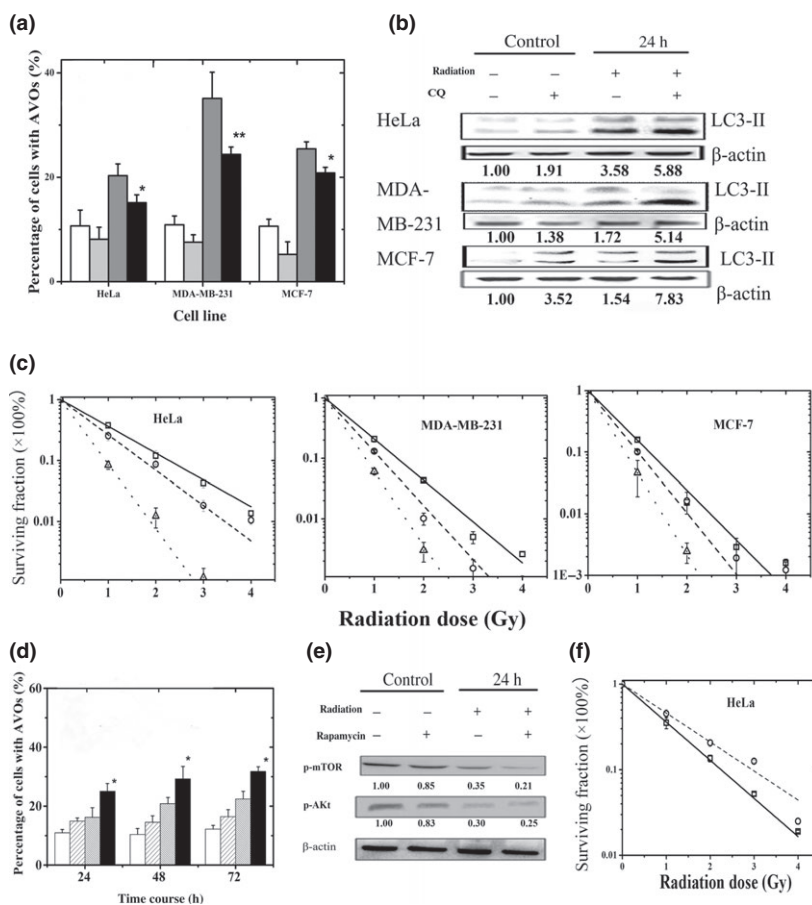
**Role of autophagy in radiosensitivity to high-LET radiation.** To assess whether autophagy contributes to the resistance or sensitivity of tumor cells to high-LET radiation, HeLa cells were used in our experiment. Two key autophagy regulators, Beclin 1 and Atg5, were depleted with siRNA. Beclin 1 and Atg5 are required at vesicle nucleation and elongation steps,

respectively.<sup>(27)</sup> These two genes were knocked down separately and together. The double KD result is shown in Figure 3(a). The degree of KD achieved for both genes was >50% at 24 and 48 h. Twenty-four hours after transfection, HeLa cells were irradiated with the carbon ions of 75 keV/μm. The inhibitory effect of siRNA on the proportion of AVO-positive cells was analyzed with flow cytometry. As shown in Figure 4(b), the KD of Beclin 1 together with Atg5 depressed the high-LET radiation-induced AVO formation significantly, whereas Beclin 1 or Atg5 KD alone had only a marginal effect. Shown in Figure 4(c) are the survival curves for HeLa cells under the conditions of irradiation, irradiation + siRNA Beclin 1, irradiation + siRNA Atg5, and irradiation + siRNA Beclin 1 and Atg5. The survival fractions of the cells treated with siRNA of both genes were significantly lower than those of the other three groups ( $P < 0.05$  at the various doses).

**Effect of pharmacological autophagy inhibition or promotion on cellular radiosensitivity to high-LET radiation.** HeLa, MDA-MB-231, and MCF-7 cells were used to investigate whether reported pharmacological inhibitor or promoter of the components of autophagy pathways could modulate the cellular radiosensitivity or reduce the resistance to high-LET radiation. Figure 5(a) shows the effects of autophagy inhibitor 3-MA, which inhibits autophagic sequestration during early stage autophagosome formation,<sup>(17)</sup> on autophagy induction by the carbon ions of 75 keV/μm in the three cancer cell lines. The percentages of AVO-harboring cells reduced to 15.1%, 24.4%, and 20.8%, compared with those (20.3%, 35.1%, and 25.5%) of the groups irradiated at 2 Gy alone, in the presence of 3-MA at 48 h post-irradiation in the three cell lines, respectively.



**Fig. 4.** Effect of Beclin 1 and Atg5 siRNA knockdown on the sensitivity of HeLa cells to high linear energy transfer radiation. (a) Western blot analysis of Beclin 1 and Atg5 levels in double knockdown (KD) HeLa cells. The relative level of proteins in comparison to  $\beta$ -actin is indicated below each immunoblot image. (b) Inhibition of Beclin 1 or Atg5 on autophagy induced by the carbon ions after 24 h, KD of Beclin 1 together with Atg5 significantly attenuated the high linear energy transfer radiation-induced formation of acidic vesicular organelles (AVOs). Beclin 1 or Atg5 KD alone had only marginal effects. The white and gray columns indicate the control groups and the irradiation groups with 75 keV/ $\mu$ m carbon ions at 2 Gy, respectively. \* $P > 0.05$  and \*\* $P < 0.05$ , compared with 2 Gy radiation alone. (c) Effect of inhibiting autophagy on the clonogenic survival of HeLa cells. Square, round, triangular, and diamond symbols represent the radiation groups, radiation + siRNA Beclin 1 groups, radiation + siRNA Atg5 groups, and radiation + siRNA Beclin 1 and Atg5 groups, respectively.



**Fig. 5.** Effect of pharmacological inhibition or promotion of autophagy on the sensitivity of HeLa, MCF-7, and MDA-MB-231 cells to high linear energy transfer (LET) radiation (75 keV/ $\mu$ m). (a) Autophagy analysis by flow cytometry (48 h after irradiation of 2 Gy) in tumor cells following combined treatment with 3-methyladenine (3-MA) and irradiation. White, gray, dark gray, and black columns represent the control groups, groups treated with 3-MA alone, groups irradiated alone, and the irradiation and 3-MA cotreatment groups, respectively. \* $P < 0.05$  and \*\* $P < 0.01$ , compared with irradiation alone. (b) Levels of LC3-II protein expression after treatment with chloroquine (CQ) and irradiation of 2 Gy. The relative level of LC3-II in comparison to  $\beta$ -actin is indicated below each immunoblot image. (c) Survival curves of the tumor cells irradiated with high-LET carbon ions. Square, round, and triangular symbols represent the radiation groups, radiation + 3-MA groups, and radiation + CQ groups, respectively. (d) Rapamycin enhanced the autophagy level induced by radiation of 2 Gy. White columns, columns filled with sparse lines, columns filled with dense lines, and black columns represent the control groups, rapamycin treated groups, irradiation groups, and irradiation and rapamycin cotreated groups, respectively. \* $P < 0.01$ , compared with irradiation alone. (e) Phosphorylation levels of mTOR and Akt proteins in HeLa cells irradiated with 75 keV/ $\mu$ m carbon ions at 2 Gy. The relative level of proteins in comparison to  $\beta$ -actin is indicated below each immunoblot image. (f) Survival curves of HeLa cells after exposure to the high-LET radiation in the presence and absence of rapamycin. Square and round symbols represent the radiation and radiation + rapamycin groups, respectively.

Chloroquine is a lysosomotropic agent that prevents lysosomal acidification to block autophagic catabolism.<sup>(28)</sup> When the late stage of autophagy is blocked, the cells may accumulate large numbers of autophagosomes. Therefore, the appearance of plenty autophagosomes in cells treated with CQ could indicate an inhibition of autophagy as well. As shown in Figure 5(b), the levels of LC3-II expression were accumulated in CQ pretreated HeLa, MDA-MB-231, and MCF-7 cells pretreated

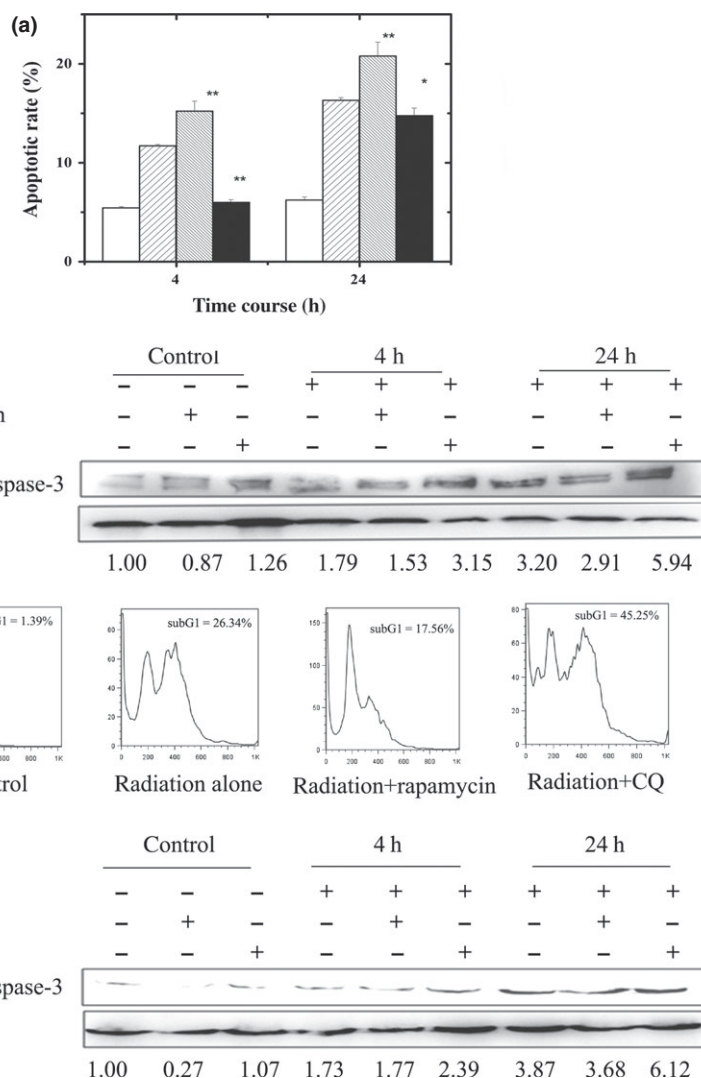
and irradiated with the carbon ions. Shown in Figure 5(c) are effects of the autophagy inhibitor 3-MA or CQ on the clonogenic survivals of HeLa, MDA-MB-231, and MCF-7 cells exposed to high-LET radiation. Clearly, the survival of the three cell lines reduced significantly in the presence of 3-MA or CQ. Both 3-MA and CQ definitely resulted in an increase of the radiosensitivity of the tumor cells to the carbon ions (Fig. 5c, Table S1).

To investigate whether promotion of the autophagic pathway has an influence on cell survival after carbon ion irradiation, rapamycin, a potent inhibitor of mTOR that has been shown to enhance autophagy,<sup>(29)</sup> was used in our study. The AVO-harboring percentages of HeLa cells, pretreated with rapamycin and followed by carbon ion irradiation, at 24, 48, and 72 h post-irradiation are displayed in Figure 5(d). We also examined the effect of rapamycin and radiation on the phosphorylation of mTOR and Akt. Consistent with the results shown in Figure 3, carbon ions depressed the phosphorylation of mTOR and Akt. Moreover, more reduction in the phosphorylation levels of mTOR and Akt was observed after cotreatment with radiation and rapamycin (Fig. 5e). The effect of rapamycin on the clonogenic survival of HeLa cells irradiated with carbon ions is shown in Figure 5(f). Obviously, the cotreatment led to higher autophagic rates and increased survival fractions in HeLa cells compared with those in the groups treated with irradiation alone, suggesting that rapamycin caused an increased radioresistance to the high-LET radiation in HeLa cells. All the data shown above indicated that autophagy promoted cell survival and then contributed to cellular radioresistance to high-LET radiation.

To verify our conclusion further, three cancer cell lines derived from different organs (HSG, hepG2, and SHG44

cells) were used for our experiments. Results demonstrated the autophagy inhibitor 3-MA degraded the clonogenic survival of all the cancer cell lines significantly and the autophagy promoter rapamycin clearly improved the survival fractions of irradiated HSG, hepG2, and SHG44 cells obviously (Fig. S2).

**Inhibition of autophagy enhanced high-LET radiation-induced apoptosis.** To elucidate the molecular mechanisms of radiosensitization by inhibiting autophagy, HeLa and MDA-MB-231 cells pretreated with autophagy inhibitor or promoter were irradiated with the carbon ions of 75 keV/μm at 2 Gy and then the cell apoptosis was measured at 4 or 24 h after irradiation. The apoptotic rates of HeLa cells induced by the carbon ions were significantly higher in the presence of 3-MA than those induced by irradiation alone at 4 and 24 h post-irradiation. On the contrary, cell apoptosis was suppressed in the presence of rapamycin at 4 h significantly and 24 h post-irradiation (Figs 6a,S3). Moreover, the protein expression of cleaved caspase-3 increased in HeLa cells cotreated with 3-MA but decreased in the case of cotreatment with rapamycin, as shown in Figure 6(b). Figure 6(c) details the flow cytometric measurements of DNA fragmentation in MDA-MB-231 cells at 24 h after irradiation. The profiles indicate that the high-LET radiation increased the apoptotic rate of MDA-MB-231 cells at 24 h post-irradiation,



**Fig. 6.** Influence of pharmacologic inhibition or promotion of autophagy on high linear energy transfer radiation-induced apoptosis in HeLa and MDA-MB-231 cells. (a) 3-Methyladenine (3-MA) or rapamycin was added to the culture medium 4 h before carbon ion irradiation (linear energy transfer = 75 keV/μm; dose = 2 Gy). Four and 24 h post-irradiation, the HeLa cells were collected and stained with annexin V-FITC and propidium iodide followed by flow cytometry analysis. White columns, columns filled with sparse lines, columns filled with dense lines, and black columns indicate the control groups, groups irradiated alone, irradiation and 3-MA cotreated groups, and irradiation and rapamycin cotreated groups, respectively. \**P* < 0.05 and \*\**P* < 0.01, compared with irradiation alone. (b,d) Protein expression of cleaved caspase-3 and β-actin in HeLa and MDA-MB-231 cells cotreated with irradiation and reagents. The relative level of cleaved caspase-3 in comparison to β-actin is indicated below each immunoblot image. (c) DNA fragmentation of MDA-MB-231 was induced at 24 h after irradiation.



whereas this effect was reduced or promoted by cotreatment with rapamycin or CQ. Western blot analysis of cleaved caspase-3 in MDA-MB-231 cells is shown in Figure 6(d). Once again, the results obtained in the apoptosis measurements above were confirmed at the level of protein expression. Clearly, these results indicated that inhibition or promotion of autophagy enhanced or mitigated the high-LET radiation-induced apoptosis in HeLa and MDA-MB-231 cell lines.

## Discussion

The rapid advance in heavy-ion radiotherapy has placed deep understanding of heavy-ion biological effectiveness and subsequent improvement of its curative effect at the center of current research. Modulation of autophagy on high-LET radiations is a novel area of research and still in its infancy. High-LET radiations have been shown to induce autophagy in diverse human cells. Hino *et al.*<sup>(18)</sup> observed ultrastructural changes and enhancement of autophagy in heavy-ion microbeam irradiated muscle fibers, isolated from skeletal muscle of SJL/J mice, with electron microscopy. Subsequently, they irradiated heavy-ion irradiation induced autophagy not only in irradiated cells but also in their bystander cells.<sup>(19)</sup> Jinno-Oue *et al.*<sup>(20)</sup> also showed that the major population of NP-2 cells died of apoptosis and autophagy when they were irradiated with carbon ions. Based on the observations at morphological and molecular levels in this study, we provided conclusive evidence that high-LET carbon ions could induce autophagy effectively in tumor cells. First, we found punctate MDC staining localized to the perinuclear regions in HeLa cells after exposure to carbon ions. The formation of large GFP-LC3 aggregates was observed 4 and 24 h post-irradiation in HeLa, MDA-MB-231, and MCF-7 cell lines (Fig. 1b). Moreover, the expression of LC3-II increased in tumor cells post-irradiation (Fig. 1c). In addition to LC3, SQSTM1/p62 protein serves as a link between LC3 and ubiquitinated substrates.<sup>(30)</sup> It joins into the completed autophagosome and is degraded in autolysosomes, thus serving as a marker of autophagic degradation.<sup>(31)</sup> In this study, we observed clear decreases in SQSTM1/p62 protein expression in the different cell lines, as shown in Figure 1(c), suggesting the activation of autophagic flux. Therefore, the data obtained in our study definitely confirmed that high-LET radiation elicited autophagy effectively and autophagy flux indeed occurred in tumor cells.

In the quantitative assay of autophagy induced by high-LET radiation, our results showed the autophagy level increased with increasing the LET and dose of carbon ions (Fig. 2). The alteration of autophagic levels may result from the inhibition of the PI3K/Akt molecular pathways. The PI3K/Akt pathway is a cell survival pathway that is important for normal cell growth and proliferation.<sup>(32)</sup> This pathway has also been implicated in tumorigenesis<sup>(33)</sup> and is becoming an important target for cancer treatment.<sup>(34)</sup> A 289-kDa serine/threonine kinase, mTOR is a downstream target of Akt. It exists in a phosphorylated form in normal conditions and suppresses autophagy. However, when its phosphorylation level is downregulated, as seen during rapamycin treatment or nutrient starvation, cell autophagy is induced.<sup>(26)</sup> Consistent with a previous study,<sup>(35)</sup> our results showed carbon ions apparently inhibited the PI3K/Akt pathway more efficiently and reduced the expression of phospho-mTOR depending on their LET (Fig. 3). Therefore, we think that more intense inhibition of the PI3K/Akt pathway by carbon ions along with increasing LET led to more severe inhibition of phospho-mTOR expression and thus elicited more

obvious autophagy in tumor cells. In addition, the expression level of phospho-mTOR is also negatively regulated by phospho-ATM in the cytoplasm,<sup>(36)</sup> which can be activated by high-LET radiation.<sup>(37,38)</sup> The exact mechanisms underlying the enhanced levels of autophagy induced by high-LET radiation in tumor cells remain the topic of future studies.

We revealed that modulating autophagy could modify the radiosensitivity of tumor cells to the high-LET radiation in this study. For the first time, we showed that inhibition of autophagy made tumor cells more sensitive to high-LET radiation. As shown in Figure 4, our study indicated that inhibiting autophagy enhanced the radiosensitivity of HeLa cells through co-eliminating the expressions of Beclin 1 and Atg5. Atg5 is a component of the Atg12-Atg5-Atg16 complex, which is essential for the formation of the pre-autophagosomes; Beclin 1 is a scaffold for the formation of the complex with the interaction of other proteins, to initiate autophagy.<sup>(27)</sup> In our study, we found that single KD of Atg5 or Beclin 1 had no effect on the radiosensitivity of HeLa cells to the carbon ions, but a double KD of both genes reduced the clonogenic survival of the irradiated cells. These results are similar to those acquired by O'Donovan *et al.*<sup>(12)</sup> using chemotherapeutic drugs on esophageal cancer cells. We suppose that there are at least two autophagy pathways that selectively involve these proteins. It has been reported that cells possess the Atg5-independent autophagy system, designated as "alternative autophagy."<sup>(39)</sup> Beclin 1-independent autophagy has been reported as well.<sup>(40,41)</sup> Moreover, our data definitely indicate that inhibiting or promoting autophagy in the six tumor cell lines under investigation altered the cellular radiosensitivity to the high-LET radiation, as shown in Figures 5 and S2. We provided further evidence that abrogation of autophagy by inhibitors such as 3-MA and CQ exacerbated cell death as well as radiosensitivity. Previous studies have indicated that rapamycin-induced autophagy prevents cell death.<sup>(42,43)</sup> Consistent with these studies, we found that cells cotreated with irradiation and rapamycin manifested resistance to the carbon ions. Thereafter, this was verified further in hepG2, HSG, and SHG44 cells, as shown in Figure S2. Our study coincides with previous ones where inhibition of autophagy was shown to increase cellular sensitivity to various therapies, including radiations and treatment with cisplatin, sulforaphane, and alkylating drugs.<sup>(11,12,44,45)</sup>

Inhibiting autophagy could sensitize tumor cells to high-LET radiation. However, what are the mechanisms underlying this process? In this study, we demonstrated that high-LET radiation simultaneously evoked two different responses in tumor cells: apoptotic cell death and autophagy, as shown in Figures 1 and 6. Our data reveal that abrogation of autophagy by the inhibitor 3-MA remarkably exacerbated annexin V-propidium iodide staining, cleaved caspase-3 expression, as well as apoptotic cell death induced by carbon ions in HeLa cells, whereas the autophagy promoter rapamycin led to the opposite results. The same findings were observed in MDA-MB-231 cells in our study. These results are consistent with the observations by Park *et al.*,<sup>(43)</sup> where inhibiting or enhancing autophagy with 3-MA or rapamycin greatly disturbed apoptosis induction by chlorpyrifos in the human dopaminergic SH-SY5Y cell line. We consider that there would be two pathways of molecular mechanism underlying this phenomenon. One, the elimination of damaged mitochondria by autophagy would prevent the release of proapoptotic substances from mitochondria, thus preventing apoptosis.<sup>(21,44)</sup> The other, some regulators may be involved in autophagy and apoptosis. Bcl-2, an anti-apoptotic protein, inhibits autophagy by disrupting the interaction of

Beclin 1/Atg6 with the class III PI3K complex, thus depressing the early stages of autophagosome formation.<sup>(22)</sup>

In conclusion, the present systematic study reveals that high-LET carbon ions can induce autophagy in various tumor cells effectively and autophagy flux occurs. The autophagic level in the irradiated cells increases in a dose- and LET-dependent manner and the ability of carbon ions to inhibit the activation of the PI3K/Akt pathway rises with increasing their LET. Modulating autophagy can modify the radiosensitivity of tumor cells to high-LET radiation. Inhibiting autophagy definitely accelerates apoptotic cell death, leading to an increased cellular sensitivity to high-LET radiation. Thus, our data might shed new light on improving the efficacy of new heavy-ion radiotherapy.

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## Disclosure Statement

The authors have no conflict of interest.



## Supporting Information

Additional supporting information may be found in the online version of this article:

**Fig. S1.** Clonogenic survival of various tumor cells following treatment with different reagents alone.

**Fig. S2.** Clonogenic survival of hepG2, HSG, and SHG44 cells following combined treatment with 3-methyladenine (3-MA) or rapamycin and carbon ion irradiation.

**Fig. S3.** Apoptotic rates of HeLa cells treated with different reagents alone.

**Table S1.** Survival fraction at 2 Gy for cells cotreated with high- linear energy transfer radiation and reagents.