

## Original Article

# Effects of low dose $^{12}\text{C}^{6+}$ ion irradiation on human cervical cancer cell line

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Received March 5, 2018; Accepted October 9, 2018; Epub May 15, 2019; Published May 30, 2019

**Abstract:** Cancer is one of the major causes of morbidity and mortality worldwide. Five- or 10-year survival rate for cancer patients is still low. There is still much room to improve the survival rate. As one of the most three common types of cancer treatment used in the clinical practice, radiation therapy includes x-rays, gamma rays, electrons, and proton. Currently, there is an increasing interest in heavy ion radiotherapy for both research and clinical purposes. Since 2006, the first facility for heavy ion radiotherapy has been put into use to treat cancer patients in China. Here, we investigate the effect of low dose  $^{12}\text{C}^{6+}$  ion irradiation on human cervical cancer cell line HeLa. Results showed that heavy carbon ion treatment has an inhibitive effect on HeLa cell proliferation in a dose-dependent manner. Further analysis indicated that molecules involved in both Akt-P53 and MAPK signal pathways contributed to the apoptosis induced by heavy ion treatment. In addition, carbon ion treatment induced the imbalance of oxidative stress, which could be the major cause to inhibit the cell proliferation. This is the first study to explore the basic mechanism of effects of low dose  $^{12}\text{C}^{6+}$  ion irradiation on human cervical cancer HeLa cell line. Our study suggests that carbon ion irradiation could inhibit proliferation of the cancer cells through primary apoptosis and secondary imbalance of oxidative stress.

**Keywords:** Carbon ion irradiation, low dose, treatment, cervical cancer cell line HeLa

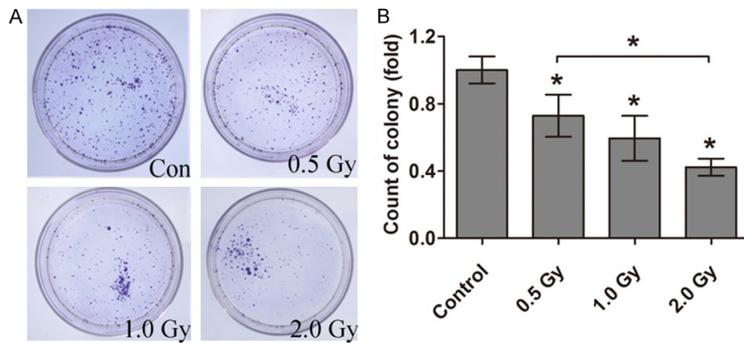
## Introduction

Radiotherapy using X-ray, gamma ray, and heavy ion beams is one of the major approaches for cancer treatment. In 1946, the therapeutic use of charged-particle beams was proposed in terms of their unique physical characteristics [1]. Heavy ions concentrated the dose at the end of the particle range, exhibiting a high linear energy transfer (LET) and an inverted depth dose profile (Bragg peak). Currently, the heavy ion is internationally recognized as the best radiotherapy radiation, especially in the application of surgery, chemotherapy, conventional radiotherapy, and refractory recurrent cases. Heavy ion radiotherapy has been applied to thousands of patients in the world [2-4].

In November 2006, the Institute of Modern Physics of the Chinese Academy of Sciences (IMP) used the  $^{12}\text{C}^{6+}$  ions produced by the high

energy heavy ion accelerator (HIRFL) to treat hundreds of patients in China [5]. In addition to the clinical applications, several studies have since been conducted to explore the mechanisms of heavy ion beam treatment in China. It was reported that a low dose of  $^{12}\text{C}^{6+}$  irradiation at 0.05 Gy could enhance the mouse immunity with a stimulatory effect [6]. Carbon irradiation treatment of peripheral blood lymphocytes (PBL) from healthy human donors at a dose of 0.05 Gy increased cytotoxic activity and its mRNA expression of IL-2, IFN- $\gamma$  and TNF- $\alpha$  [7], but there was no significant change in the percentage of T and NK cells subsets. By contrast, the same treatment of PBL from patients with alimentary tract cancer increased the percentage of T cells subset, and mRNA expression of IL-2 and IFN- $\gamma$ , but no significant change in the percentage of NK cell subset and TNF- $\alpha$  production [8]. These data suggest that low

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**Figure 1.** The proliferative activity of HeLa cells being hit or not by carbon ions. Representative images showing colonies formed with or without irradiation (A). (B) Quantitative analysis of the number of the colony formation assay by different groups of cells. Data shown are the means from three independent experiments (\* $P < 0.05$  VS. Control or other groups).

dose irradiation may mitigate immune suppression in cancer patients.

A comparative proteomic analysis of effects by X-ray and heavy ion in HeLa cells suggests that unlike the radioresistance of cancer cell to X-ray treatment due to glycolysis enhancement, the unchanged glycolysis and decreased amino acid metabolism could contribute to the greater killing effect of heavy ion carbon on cancer cells [9]. However, it remains unknown about the mechanism of low-dose  $^{12}\text{C}^{6+}$  ion irradiation on human cervical cancer cell line HeLa. In the present study, we evaluated the cellular changes in HeLa treated with heavy ion carbon.

### Materials and methods

#### Cell culture

The human cervical cancer cell line HeLa was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific) and cultured at  $37^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$  incubator.

#### Heavy-ion irradiation

When in the mid-log phase of growth after 24 h culture, the cells were irradiated at a dose rate 0.5 Gy/min at room temperature using 80.55 MeV/u  $^{12}\text{C}^{6+}$  beam derived from the Heavy-ion Research Facility in Lanzhou (HIRFL, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, China). The Heavy-ion dose is calculated based on a formulation previously described:  $D$  (Gy) =  $1.602 \times 10^{-9} \times P \times (dE/dx) \times$

$\rho^{-1}$ , where  $D$  is the irradiation dose (Gy),  $P$  the ion flux (ions/cm $^2$ ),  $\rho$  the medium absorption index ( $\rho_{\text{water}} = 1$ ),  $dE/dx$  the energy loss given by a charged particle in water (keV/ $\mu\text{m}$ ) (Pre-irradiation with low-dose, Liu bing). The radiation doses were 0.5, 1.0, and 2.0 Gy.

#### Colony formation assay

Cell survival was assessed using a colony formation assay. Twelve-hour post irradiation with different doses of Gy  $^{12}\text{C}^{6+}$  ion beams, the cells were collected by trypsinization and

resuspended in DMEM medium complemented with 10% FBS. Cells were diluted with medium and seeded in 60-mm Petri dishes (3002 Falcon) pretreated with 0.1% polylysine to provide 1000 cells per dish. The plate was placed at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  and humidity incubator for 12 days, followed by staining with 0.1% Giemsa stain. The number of cell clones containing more than 50 cells was counted. Colony formation rate of each group (%) = number of cell clones/inoculated cells  $\times 100\%$ .

#### Measurement of oxidative stress

To determine oxidative stress, lactate dehydrogenase (LDH) release, the activity of superoxide dismutase (SOD), and the content of malondialdehyde (MDA) were performed by colorimetric assay using commercial kits as previously described [10].

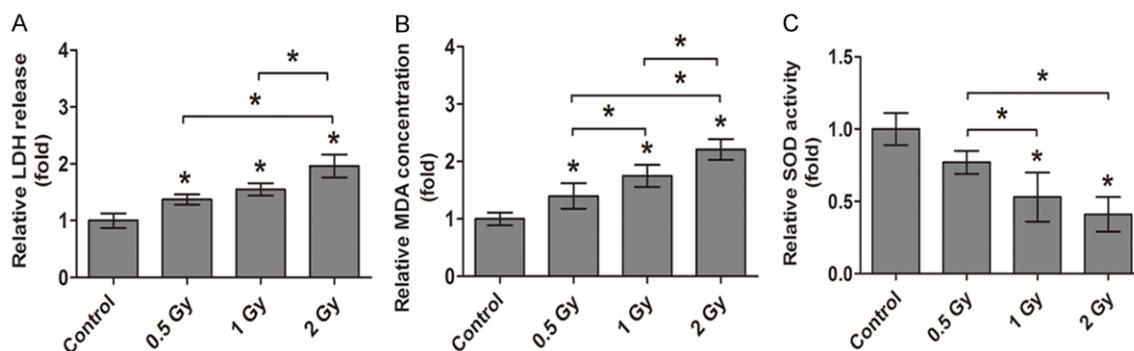
#### Apoptosis assay

Following irradiation and subsequent 72-h incubation at  $37^{\circ}\text{C}$ , cells were harvested and resuspended in 100  $\mu\text{L}$  of  $1 \times$  binding buffer after wash twice with cold PBS. Cells were then stained using Annexin V/PI double staining kit (BD, USA) and analyzed using a FACS can flow cytometer.

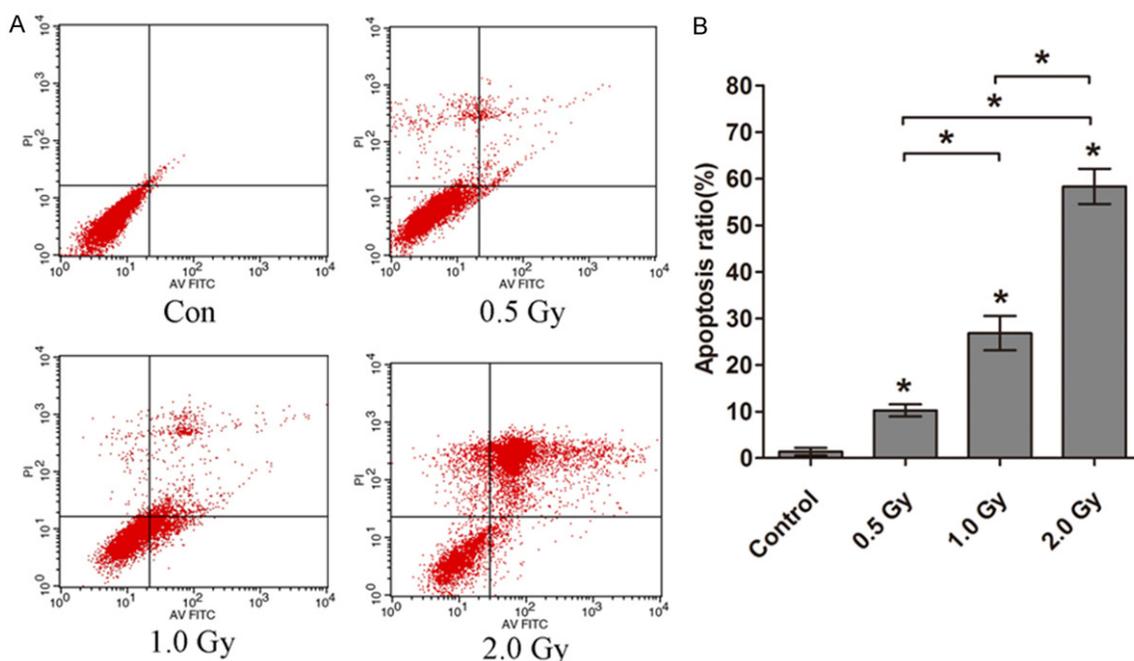
#### Measurement of expression of proteins involved in both Akt-P53 and MAPK signal pathways

Following irradiation and subsequent 72-h incubation at  $37^{\circ}\text{C}$ , HeLa cells were lysed in 250  $\mu\text{L}$  RIPA buffer and sonicated. After a 15-min centrifugation at  $4^{\circ}\text{C}$ , Western-blot analysis of

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**Figure 2.** Carbon ion radiation-induced imbalance of oxidative stress in HeLa cells. Antioxidant activity was determined by measuring (A) LDH, (B) malondialdehyde (MDA) and superoxide dismutase (SOD) activity (C). Data shown are the means of three independent experiments (\* $P < 0.05$  VS. Control or other groups).



**Figure 3.** Effect of carbon ion radiation on apoptosis in HeLa cells. Detection of apoptotic cells by Annexin V/PI double-staining. HeLa cells were treated with carbon ion radiation, stained with Annexin V and PI, and then analyzed by flow cytometry (A). (B) Quantitative analysis of apoptosis. Data shown are the means of three independent experiments (\* $P < 0.05$  VS. Control or other groups).

supernatants was used to detect the effect of irradiation on expression of Ras (CST, USA), Akt (CST, USA), pAkt (CST, USA), P53 (CST, USA), Bcl-2 (CST, USA), Bax (CST, USA), caspase 3 (CST, USA), P38 (CST, USA), pP38 (CST, USA), ERK (CST, USA), and pERK (CST, USA).

### Statistical treatment

The data is represented by mean SD. The data were analyzed by one-way ANOVA using SPSS 17.0 statistical software (SPSS, Chicago, IL,

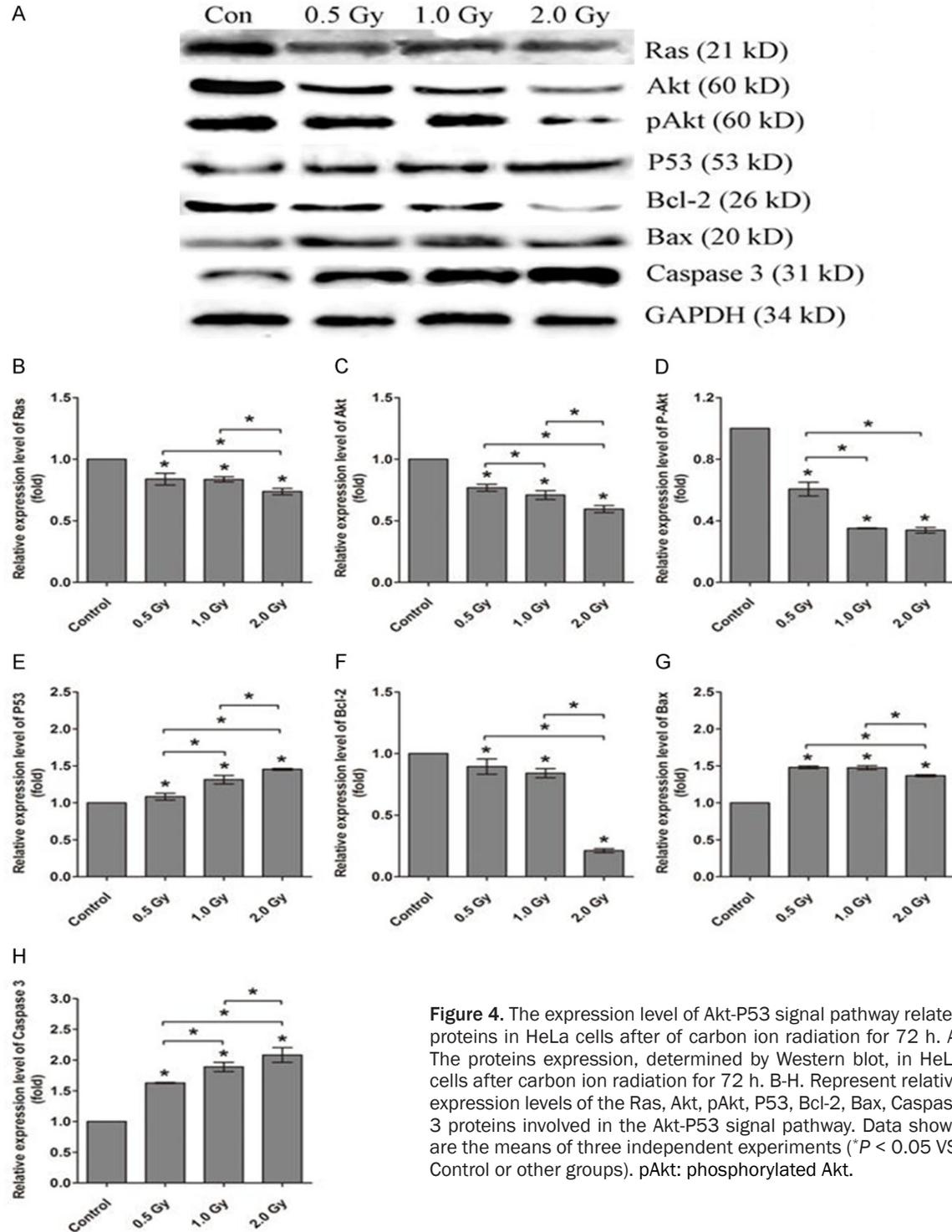
USA).  $P < 0.05$  for the difference was significant.

### Results

#### Cell viability of HeLa cells following treatment of $^{12}\text{C}^{6+}$ ion irradiation

Different doses of  $^{12}\text{C}^{6+}$  ion beam have an inhibition effect on cell proliferation of HeLa. After irradiation with 0.5, 1.0 and 2.0 Gy of the  $^{12}\text{C}^{6+}$  ion beam, compared to the untreated negative

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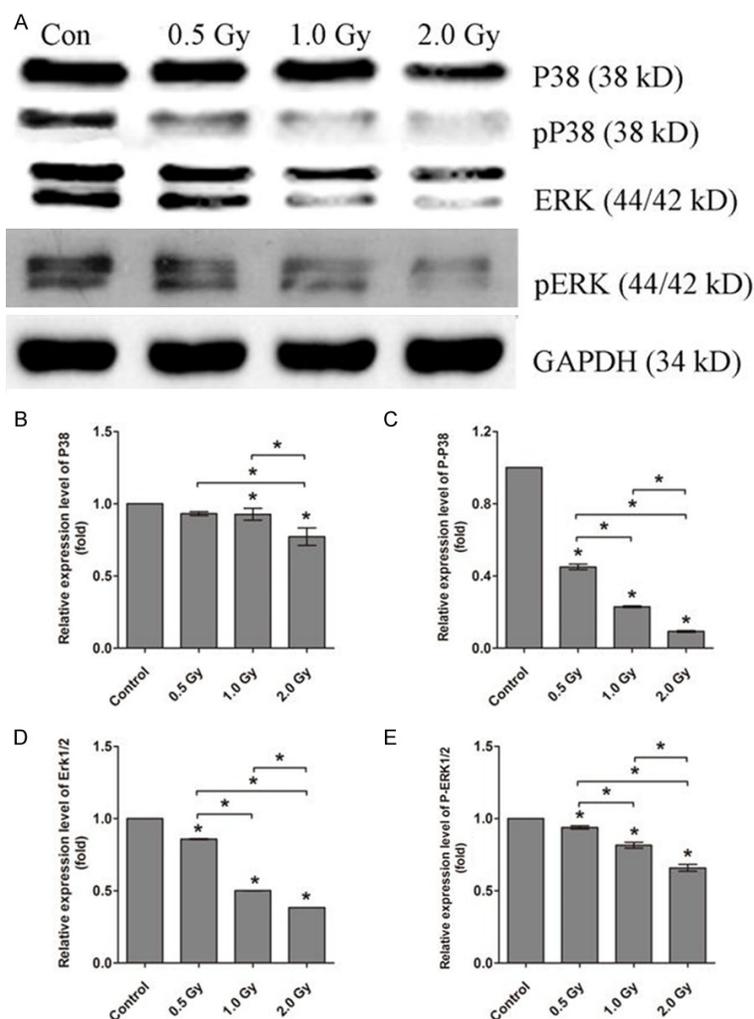


control group, the ratio of colony formation rate of HeLa cell was  $0.73 \pm 0.12$ ,  $0.59 \pm 0.13$  and  $0.42 \pm 0.11$ , respectively. Significant differences were observed between different doses of 0.5 Gy and 2.0 Gy irradiation groups and between irradiation and control groups ( $P < 0.05$ ) (Figure 1).

*Carbon ion irradiation induced imbalance of oxidative stress*

The Colorimetric assay showed that carbon ion irradiation significantly promoted the LDH release (Figure 2A). The same treatment also significantly upregulated the level of MDA, but

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**Figure 5.** The expression level of P38, pP38, ERK, and pERK in HeLa cells after carbon ion radiation for 72 h. A. The expression levels of proteins were determined by Western blot analysis. GAPDH was used as a loading control. B-E. Represent relative expression levels of the proteins. Data shown are the means of three independent experiments. (\* $P < 0.05$  VS. Control or other groups). pP38: phosphorylated P38, pERK: phosphorylated ERK.

downregulated the SOD level (Figure 2B and 2C). The effects of  $^{12}\text{C}^{6+}$  on LDH, MDA, and SOD were dose-dependent. These data suggest carbon ion irradiation induced imbalance of oxidative stress.

### Effect of ion beam on apoptosis of HeLa cells

Compared with non-treatment cell control group, carbon ion irradiation treatment significantly increased the apoptotic fraction of cells to 10.26, 26.82, and 58.34 at doses of 0.5, 1.0 and 2.0 Gy, respectively. This data indicates that  $^{12}\text{C}^{6+}$  ion beam could induce HeLa cervical cancer cell apoptosis.

### Effect of carbon ion irradiation on Akt-P53 and MAPK signal pathways

Compared with non-treatment control group, the expression levels of proteins (Ras, Akt, pAkt, Bcl-2) involved in Akt-P53 in HeLa cells was significantly decreased after irradiation with different dose of  $^{12}\text{C}^{6+}$  ion beam ( $P < 0.05$ ), whereas the levels of proteins (P53, Bax, and caspase 3) significantly increased ( $P < 0.05$ ) (Figure 4). The effect of irradiation on expression levels of all proteins except Bax is in a dose-dependent manner (Figure 4A-F, 4H), in contrast, there was a dose-dependent reverse effect on the level of Bax (Figure 4A and 4G). Furthermore, the carbon ion irradiation reduced the expression level of proteins (P38, pP38, ERK, and pERK) involved in the MAPK signal pathway in a dose-dependent manner (Figure 5A-E).

### Discussion

Cancer is one of the leading causes of death worldwide. It was reported that 14 million new cases were diagnosed and there were 8.2 million cancer-related deaths worldwide in 2012 (<https://www.cancer.gov/about-cancer/understanding/statistics>).

In general, cancer treatment includes surgery, chemotherapy, radiation therapy, or combination of surgery with chemo- or radiation-therapy. In case of radiation therapy, X-rays, gamma rays, and charged particles are used to shrink tumors and kill cancer cells. Application of irradiation therapy has a history of over one hundred of years. Among the two-thirds of cancer patients treated with radiotherapy, 0.8% receives charged-particle therapy [11]. In addition, carbon ion radiotherapy has been applied to treat different types of cancers including skull-base tumor, head and neck tumor, prostate, bone and soft tissue sarcomas, and lung and

liver tumors [12]. In recent years, there has been rapidly increasing interests in applying this treatment in the clinics. Therefore, the research to explore the basic mechanism about how the charged-particle therapy works is urgently needed.

In the present study, we evaluated the effect of low dose heavy ion radiation on human cervical cancer cell line HeLa. Although a previous study investigated proteomic analysis of effects by both x-rays and heavy ion in HeLa cells [9], our study is the first to explore the cellular mechanism of heavy carbon ion treatment on HeLa cells. Low doses of  $^{12}\text{C}^{6+}$  ion beam irradiation significantly decreased the proliferative activity of HeLa cell, which correlates with significantly increased apoptosis ratios (**Figures 1 and 3**). Upregulated levels of both p53 and Caspase 3 correlated with the increased percentage of apoptosis ratio in HeLa cells treated with carbon ion radiation (**Figures 3 and 4**). Further analysis by the colorimetric assays demonstrated that carbon ion irradiation treatment disturbed the balance of oxidative stress, which could be the main reason to reduce the cellular proliferative activity. The inhibitive effect on HeLa cell proliferation could be related to an enhanced accumulation of cells in G2/M, as previously reported [13].

There are many biological pathways involved in tumorigenesis. Some of the most important pathways involved in cancer biology include Akt-P53 and MAPK signal pathways. In our study, several proteins involved in both Akt-P53 and MAPK pathways were analyzed. All proteins of both pathways except P53, Bax, and caspase 3 were down-regulated in HeLa cell treated with  $^{12}\text{C}^{6+}$  ion beam irradiation. A previous study showed that there was a no significant decrease of the Akt level in HeLa cells treated with a dose of 2 Gy carbon beam, which is contrary to our findings [9]. However, it remains unknown about the details of factors associated with this discrepancy.

In conclusion, our study for the first time investigated the role of low dose  $^{12}\text{C}^{6+}$  ion irradiation on human cervical cancer HeLa cell line and demonstrated that low dose of  $^{12}\text{C}^{6+}$  ion beam irradiation inhibited the HeLa cell proliferation through an apoptosis mechanism and disturbing balance of oxidative stress. Moreover, carbon ion irradiation caused both up-regulation

and down-regulation of proteins involved in Akt-P53 and MAPK pathways.

### Acknowledgements

This work was funded by grant from the National Natural Science Foundation of China, No. 81360141. This study was also supported by a grant from the Traditional Chinese Medicine Scientific Research Funds of Health and Family Planning Committee of Shanghai, (Number: 2014.JP024A).

### Disclosure of conflict of interest

None.

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