

Serum Proteins as New Biomarkers for Whole-Body Exposure to High- and Low-LET Ionizing Radiation

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Abstract

Exposure to ionizing radiation is a major threat to human health and public security. Since the inherent limitations of current methods for indicating radiation exposure, new minimally invasive biomarkers that can be easily and quickly detected at an early stage are needed for optimal medical treatment. Serum proteins are attractive biomarkers and some radiosensitive proteins have been found, but the proteins in response to low-dose and high-linear energy transfer (LET) radiation have not been reported. In this study, mice were whole body exposed to a variety doses of carbon ions and X-rays. We performed Mouse Antibody Array to detect serum proteins expression profiles at 24 hours postirradiation. After conditional screening, insulin-like growth factor-I (IGF-I), insulin-like growth factor binding protein-I (IGFBP-I), and IGFBP-3 were further validated using enzyme-linked immunosorbent assay. After exposure to 0.05 to 1 Gy of carbon ions and 0.5 to 4 Gy of X-rays, only IGFBP-3 showed obvious increase with increased doses, both carbon ions and X-rays. Further, IGFBP-3 was detected for observation of its time-dependent changes. The results showed the expression difference of IGFBP-3 presented from 6 to 24 hours post-irradiation by carbon ions and X-rays. Moreover, the receiver–operating characteristic analysis showed that serum IGFBP-3 is efficient to triage exposed individuals with high sensitivity and specificity. These results suggest that serum IGFBP-3 is extremely sensitive to high- and low-LET ionizing radiation and is able to respond at an early stage, which could serve as a novel minimally invasive indicator for radiation exposure.

Keywords

serum proteins, biomarkers, carbon ions, X-rays, IGFBP-3

Introduction

In the events of manned space missions or radiation therapies, as well as industrial or terrorist nuclear accidents, people may be exposed to ionizing radiation with different doses and linear energy transfer (LET) features, including X-rays, gamma rays, protons, or heavy ions.¹⁻³ In general, exposure to high or lethal doses of radiation leads to acute radiation syndrome (ARS), such as gastrointestinal disorders, retinal or skin lesion, internal bleeding, and even death, which can be regarded as clinical evidences to diagnose radiation damage.⁴ However, in most cases, individuals are exposed to low or nonlethal doses of radiation which cause progressive damage or carcinogenesis instead of distinct ARS.⁵ The rapid and simple methods to triage low-dose exposed individuals within large populations are important and a big challenge for radiation safety management.

The current physical dosimeters have limitations in predicting individual absorbed dose because the same physical dose has different effects on different individuals.⁶ Radiosensitive biomarkers can indicate individual difference and provide more information about radiation-induced changes in

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biological processes. Traditional biomarkers based on DNA damage, such as chromosomal aberration, micronuclei formation, and γ -H2AX foci formation, have been developed for many years and applied to occupational workers in nuclear facilities and astronauts.^{7,8} However, traditional biomarkers have considerable limitations. For example, these methods are time consuming and require complex analysis by skilled workers.⁹ Chromosomal aberration and γ -H2AX foci are not sensitive to low dose of radiation and are easily influenced by the repair of DNA damage.^{10,11} Therefore, new rapid, simple, and efficient biomarkers are urgently needed to make up limitations or replace traditional biomarkers. In the recent years, many researches have been dedicated to the discovery of inherent radiation biomarkers in serum or plasma, and many molecules such as microRNA (miRNA), long noncoding RNA, and proteins have been found that respond to ionizing radiation.¹²⁻¹⁴ Proteins are relatively abundant in blood and easy for detection by enzyme-linked immunosorbent assay (ELISA) or antibody microarray, and these properties make them attractive candidates for use as minimally invasive biomarkers. Several researches reported some proteins in blood, such as serum amyloid A, C-reactive protein, and apolipoprotein E, are differentially expressed after whole-body exposure to X-rays or gamma rays.^{15,16} However, serum proteins that respond to low dose of radiation and high-LET radiation (heavy ions) which are common in solar winds and galactic cosmic rays, or widely used in heavy-ion cancer therapy,^{3,17} have not been reported.

Here, mice were whole body exposed to carbon ions generated by accelerator or X-rays. We measured the expression profiles of proteome by antibody microarrays. Specific radio-sensitive proteins were further detected by ELISA for their dose- and time-dependent changes. We sought to observe a set of serum proteins which are sensitive to different doses of high- and low-LET ionizing radiation to serve as new potential biomarkers.

Materials and Methods

Mice and Irradiation

Kunming male mice (8 week old) were bought from Gansu University of Chinese Medicine (Lanzhou, China) and kept in standard cages at common condition before exposure. Mice were exposed to carbon ions ($^{12}\text{C}^{6+}$) by total body irradiation (TBI). Carbon ions with energy of 80 MeV/u were generated by the Heavy Ion Research Facility (HIRFL) in Lanzhou, Institute of Modern Physics, Chinese Academy of Sciences (China) at the dose rates of 0.2 to 0.3 Gy/min, LET value is 30 keV/ μm . X-rays (225 kV, 13.3 mA) was generated by X-RAD 225 (Precision X-ray, North Branford, CT, USA) at the dose rate of 0.5 Gy/min. Control animals were sham-exposed in the same conditions. All experiments with mice were approved by the Animals Studies Committee of Gansu University of Chinese Medicine, and the approval number is 2019-184.

Serum Extraction

After anesthesia by chloral hydrate, peripheral blood of mice was collected by heart puncture at the designed time points in RNase-free centrifuge tubes (Kirgen, Shanghai, China). Whole blood samples in the tubes were stayed for 2 hours at room temperature. To harvest the cell-free serum, blood samples were first centrifuged at 4000 rpm for 10 minutes at room temperature, and then the supernatant was separated and centrifuged again at 4000 rpm for 10 minutes at 4°C to remove residual blood cells. The supernatant is pure serum sample and stored at -80°C.

Serum Proteome Expression Profiling

Serum protein was quantified by BCA Protein Assay Kit (Kangchen, Shanghai, China). L-Series Mouse Antibody Array 308 Membrane Kit (RayBiotech, Peachtree Corners, GA, USA) containing 308 assays was used to profile proteome expression in mouse serum after TBI at different doses with carbon ions and X-rays, according to the manufacturer's protocol. Antibody array images were detected by X-ray film scanner. By comparing the signal intensities, relative expression levels of proteins were made. The intensities of signals were quantified by densitometry. Positive controls were used to normalize the results from different membranes being compared and fold changes of protein expression in different groups were calculated.

Validation With ELISA Kit

The selected proteins in serum samples were measured using ELISA kits (Elabscience, Wuhan, China) according to the manufacturer's instructions. The assay was a solid-phase enzymatically amplified double antibody sandwich-type immunoassay. The standards and the diluted samples were incubated in ELISA plate wells for 2 hours at 37°C. Then, another biotin-conjugated antibody was added to the wells. After incubation at 37°C for 1.5 hours and washing, avidin-conjugated horseradish peroxidase was added to the wells. After incubation at 37°C for 1 hour and washing, the tetramethylbenzidine substrate solution was added to the wells for 20 minutes at 37°C. The reactions were stopped with strong acid solution. The optical density of each well was determined by Infinite 200Pro micro-plate reader (Tecan, Männedorf, Switzerland) at wavelength of 450 nm.

Statistical Analyses

One-way analysis of variance was used to assess differences in multiple groups, and Student *t* test was used to determine difference between treatment group and control group. All data were presented as the mean \pm standard error. Statistical analysis was performed using SPSS version 18.0 software (IBM Corp, Armonk, New York). The correlation analysis of dose and proteins' concentration was processed by MedCalc version 15.0 software (Ostend, Belgium). Receiver-operating

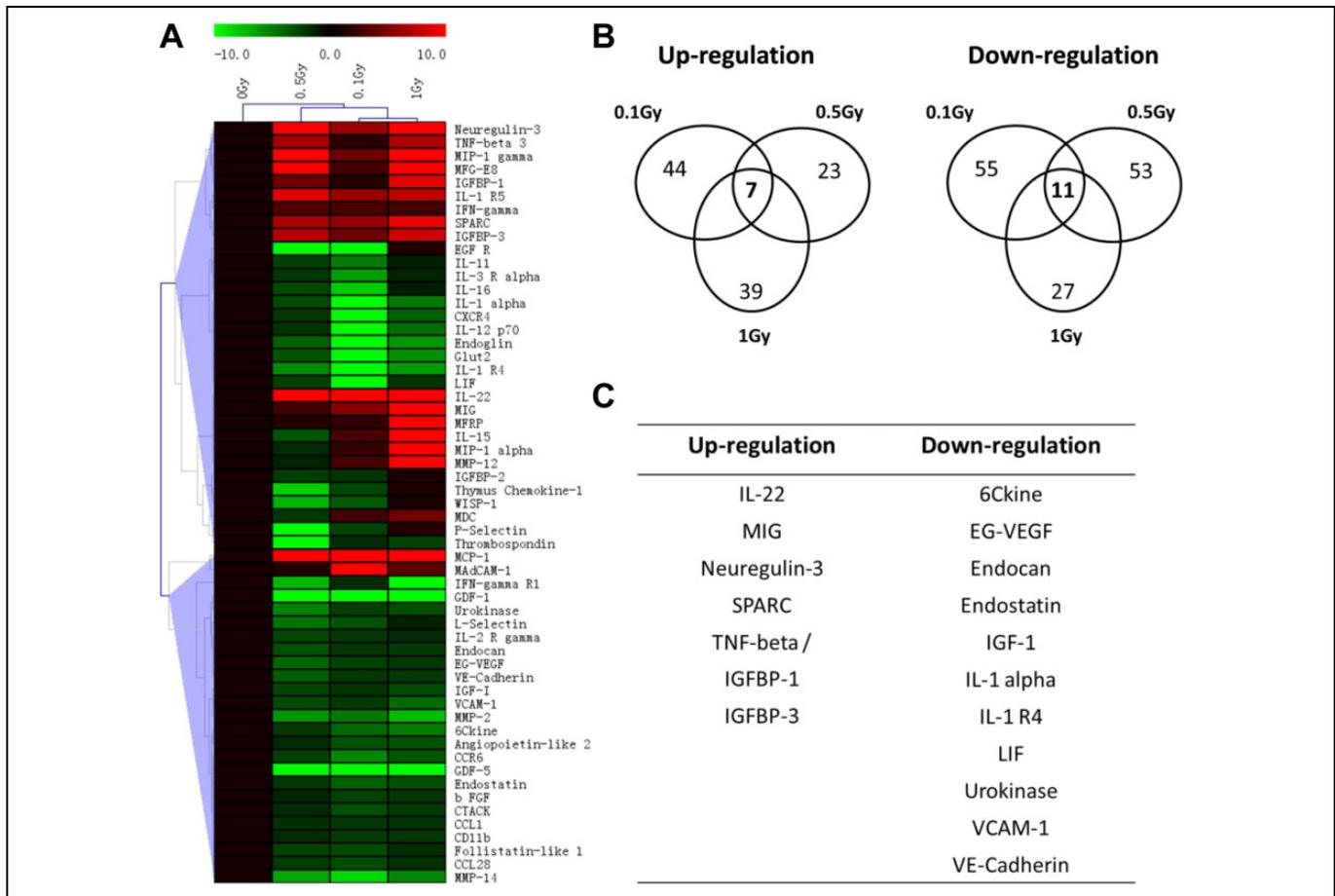


Figure 1. Identification of differentially expressed serum proteins after exposure to 0, 0.1, 0.5, and 1 Gy of carbon ions at 24 hours. A, Hierarchical clustering heatmap of 57 proteins with fold changes more than 1.5 times is shown using a red-black-green color scale (red indicates high expression and green indicates low expression). B, Venn diagram showing up- and downregulated proteins in different dose groups simultaneously. C, A list showing up- and downregulated proteins in different dose groups simultaneously.

characteristic (ROC) analysis curves was also processed by MedCalc 15.0.

Results

The Differential Expression Profile of Serum Proteins After Carbon Ion Irradiation

To identify serum proteins in response to high-LET ionizing radiation, 12 mice were whole body exposed to 0, 0.1, 0.5, and 1 Gy of carbon ions, 3 mice in each dose group. Serum samples were collected at 24 hours after irradiation, and samples from same dose were mixed together to ensure sufficient volume for investigation. Serum samples were analyzed using mouse antibody array containing 308 major mouse proteins in blood, and relative fold changes compared to control group (0 Gy) were calculated. A set of 57 proteins with fold changes more than 1.5 times was analyzed using hierarchical clustering heatmap to reveal expression patterns (Figure 1A). Among them, 7 proteins were upregulated and 11 proteins were downregulated in 3 dose groups at the same time (Figure 1B and C).

The Differential Expression Profile of Serum Proteins After X-ray Irradiation

To observe the proteins signature in response to low-LET radiation, mice were whole body exposed to X-rays. Because the relative biological effect (RBE) of X-rays is less than carbon ions, the doses of X-rays were set more than that of carbon ions in experiment. Here, 12 mice were whole body exposed to 0, 0.5, 2, and 4 Gy of X-rays, 3 mice in each dose group. At 24 hours postirradiation, serum samples were collected, and samples from the same dose were mixed together. Similarly, serum samples were analyzed using mouse antibody array and relative fold changes compared to the control group (0 Gy) were calculated. As shown in Figure 2, 47 proteins differentially expressed with fold changes more than 1.5 times, and hierarchical clustering heatmap of these proteins was performed to show expression patterns (Figure 2A). Among them, 11 proteins were upregulated and 9 proteins were downregulated in all 3 dose groups at the same time (Figure 2B and C). After screening of expression profiles of carbon ion and X-ray irradiation, we found the expression of interleukin 22, insulin-like

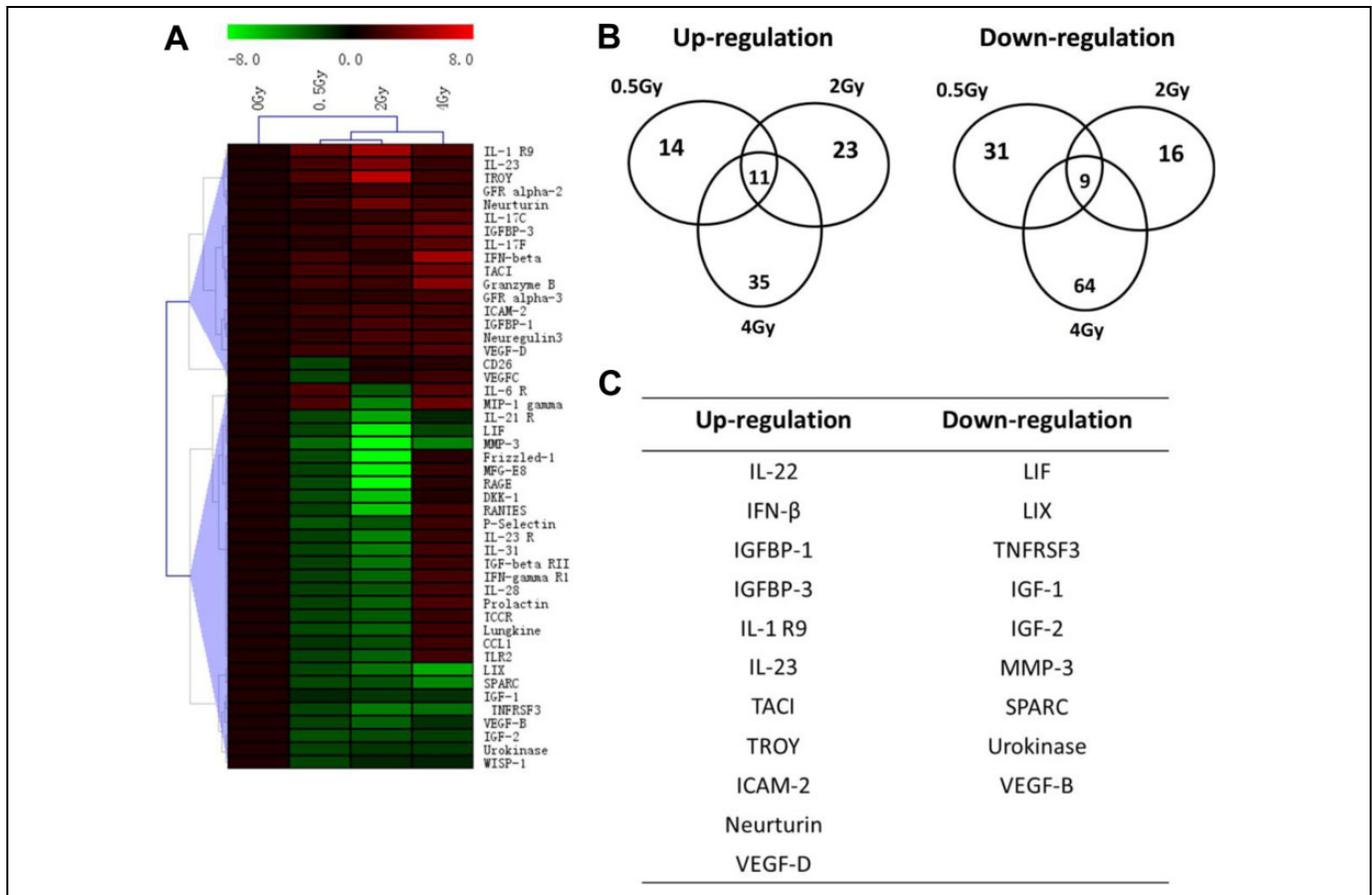


Figure 2. Identification of serum proteins in response to 0, 0.5, 2, and 4 Gy of X-rays at 24 hours postirradiation. A, Hierarchical clustering heatmap of 47 proteins with fold changes more than 1.5 times is shown using a red-black-green color scale (red indicates high expression and green indicates low expression). B, Venn diagram showing up- and downregulated proteins in different dose groups simultaneously. C, A list showing up- and downregulated proteins in different dose groups simultaneously.

growth factor binding protein-1 (IGFBP-1), and IGFBP-3 increased, while the expression of leukemia inhibitory factor (LIF), insulin-like growth factor-1 (IGF-1), and urokinase decreased after exposure to both carbon ions and X-rays. As a result, these 6 proteins were selected for next validation.

Expression Changes of the Selected Proteins After Irradiation to Different Doses of Carbon Ions or X-Rays

To ensure high abundance of proteins in serum for easy detection, we chose the proteins with higher signal readings in antibody chips. Insulin-like growth factor-1, IGFBP-1, and IGFBP-3 have higher signal readings than urokinase and LIF, and they are components of the GH-IGF-IGFBPs pathway. Therefore, IGF-1, IGFBP-1, and IGFBP-3 were chosen as potential biomarkers for ELISA detection. Then, mice were exposed to 0, 0.05, 0.1, 0.5, and 1 Gy of carbon ions ($n = 9/\text{dose}$), and 0, 0.5, 2, and 4 Gy of X-rays ($n = 9/\text{dose}$) separately. At 24 hours postirradiation, serum samples were collected, and the expression levels of IGF-1, IGFBP-1, and IGFBP-3 were quantified by ELISA. As shown in Figure 3, the concentration of serum IGF-1 had no changes (Figure 3A and D). The level of

serum IGFBP-1 slightly increased after exposure to carbon ions or X-rays (Figure 3B and E). Serum IGFBP-3 showed evidence of increase with increased doses of both X-ray and carbon ion irradiation (Figure 3C and F). Specifically, IGFBP-3 had an obvious dose-dependent effect after exposure to X-rays but not to carbon ions. From abovementioned results, IGFBP-3 was determined as the only proteins for further studies because it is the sole biomarker that strong responds to carbon ion and X-ray irradiation.

Temporal Expression of Serum IGFBP-3 After Exposure to Carbon Ions or X-Rays

An early response to radiation can make the biomarker detectable as soon as possible, so we investigated the kinetics of IGFBP-3 during 6 to 72 hours after carbon ion and X-ray irradiation. From the abovementioned results, we found IGFBP-3 obviously respond to 1 Gy of carbon ions or 4 Gy of X-rays, and the LET of carbon ions is more than X-rays. So, mice were exposed to 1 Gy of carbon ions and 4 Gy of X-rays separately to observe response at different time points. Here, serum was separately collected at 6, 24, and 72 hours

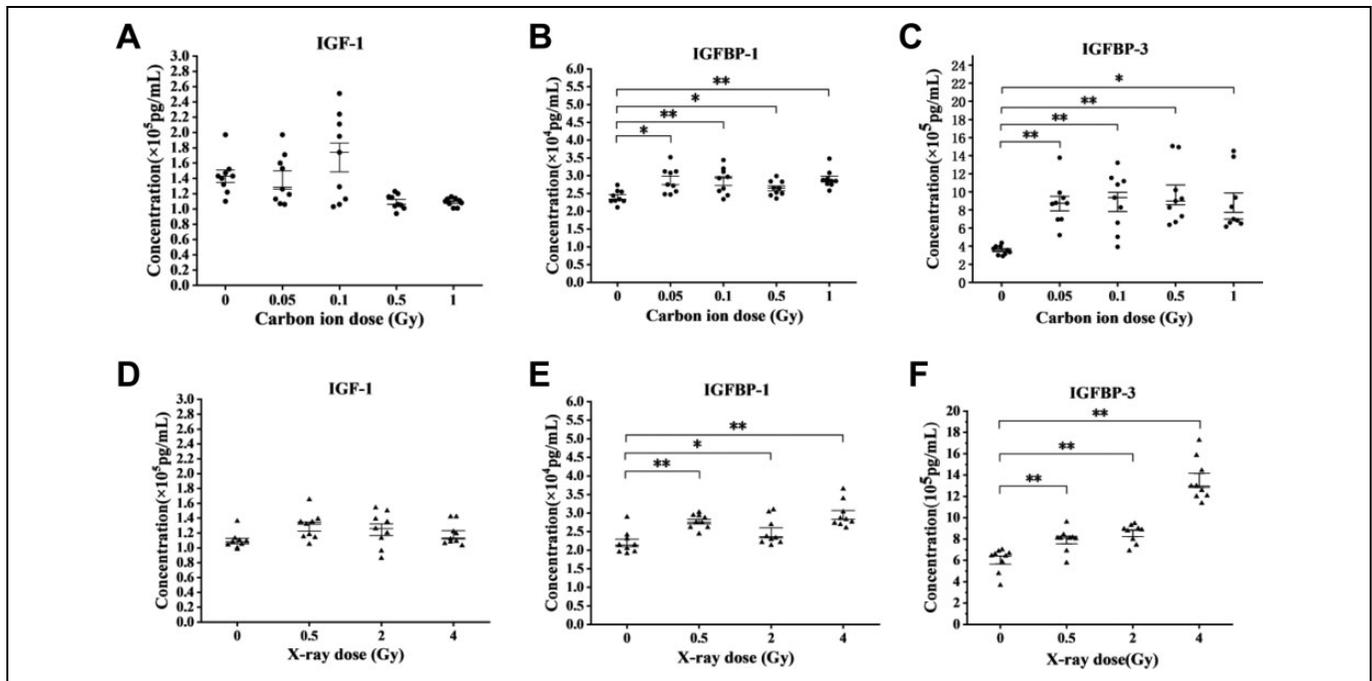


Figure 3. The expression changes of serum insulin-like growth factor-I (IGF-I), insulin-like growth factor binding protein-I (IGFBP-I), and insulin-like growth factor binding protein 3 (IGFBP-3) after exposure to different doses of carbon ions or X-rays. A-C, The concentration of serum IGF-I, IGFBP-I, and IGFBP-3 after exposure to carbon ions. D-F, The concentration of serum IGF-I, IGFBP-I, and IGFBP-3 after exposure to X-rays. (Data are showed by mean \pm standard error of mean [SEM]. * $P < .05$; ** $P < .01$, compared to 0 Gy group.)

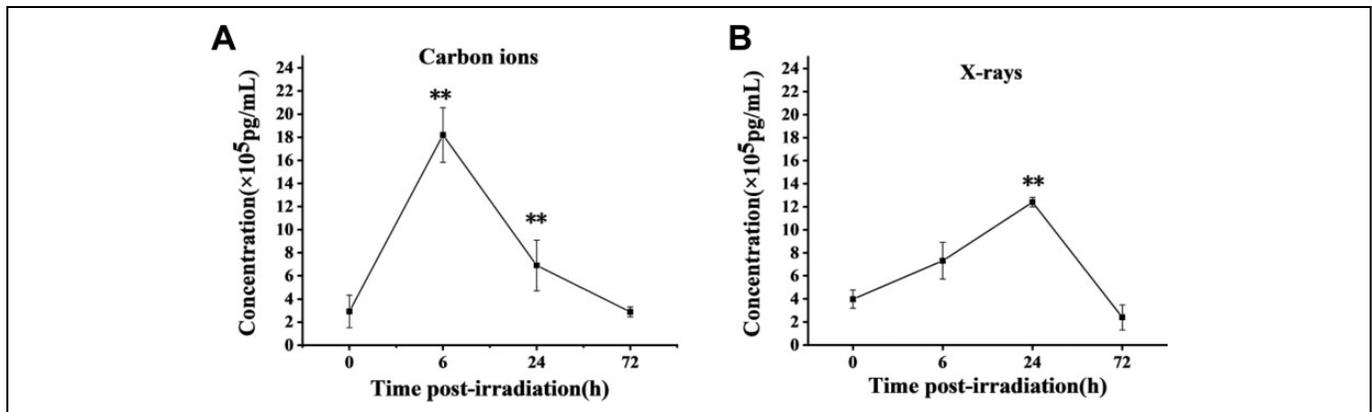


Figure 4. Temporal expression of serum insulin-like growth factor binding protein 3 (IGFBP-3) from 0 to 72 hours postirradiation in response to carbon ions or X-rays. A, Expression changes of IGFBP-3 in response to carbon ions. B, Expression changes of IGFBP-3 in response to X-rays. (Data are showed by mean \pm standard error of mean [SEM]. * $P < .05$; ** $P < .01$, compared to 0 hour group.)

postirradiation ($n = 3-4$ /time point), and the concentration of IGFBP-3 in each sample was detected by ELISA. After exposure to 1 Gy of carbon ions, the expression level of IGFBP-3 kept an increasing trend from 6 to 24 hours, reached a peak at 6 hours, and fell down to normal level (compared to 0 Gy) at 72 hours (Figure 4A). Similarly, the expression level of IGFBP-3 increased from 6 to 24 hours after exposure to X-rays, but reached a peak at 24 hours (Figure 4B). These results demonstrate that serum IGFBP-3 are able to respond to ionizing radiation at an early stage but fall down after 24 hours, so the best detection window is 6 to 24 hours after irradiation.

Estimation of serum IGFBP-3 in Predicting Radiation Exposure

To estimate the correlation between the expression levels of IGFBP-3 and exposure doses, we analyzed the dose–response data at 24 hours postirradiation by regression analysis to observe the best-fitting line. As shown in Figure 5, the concentration of serum IGFBP-3 had a positive linear correlation to doses after exposure to X-rays, with an R value of 0.8783 (Figure 5B). However, unlike the result of X-rays, the concentration of serum IGFBP-3 reached a relative plateau from 0.05

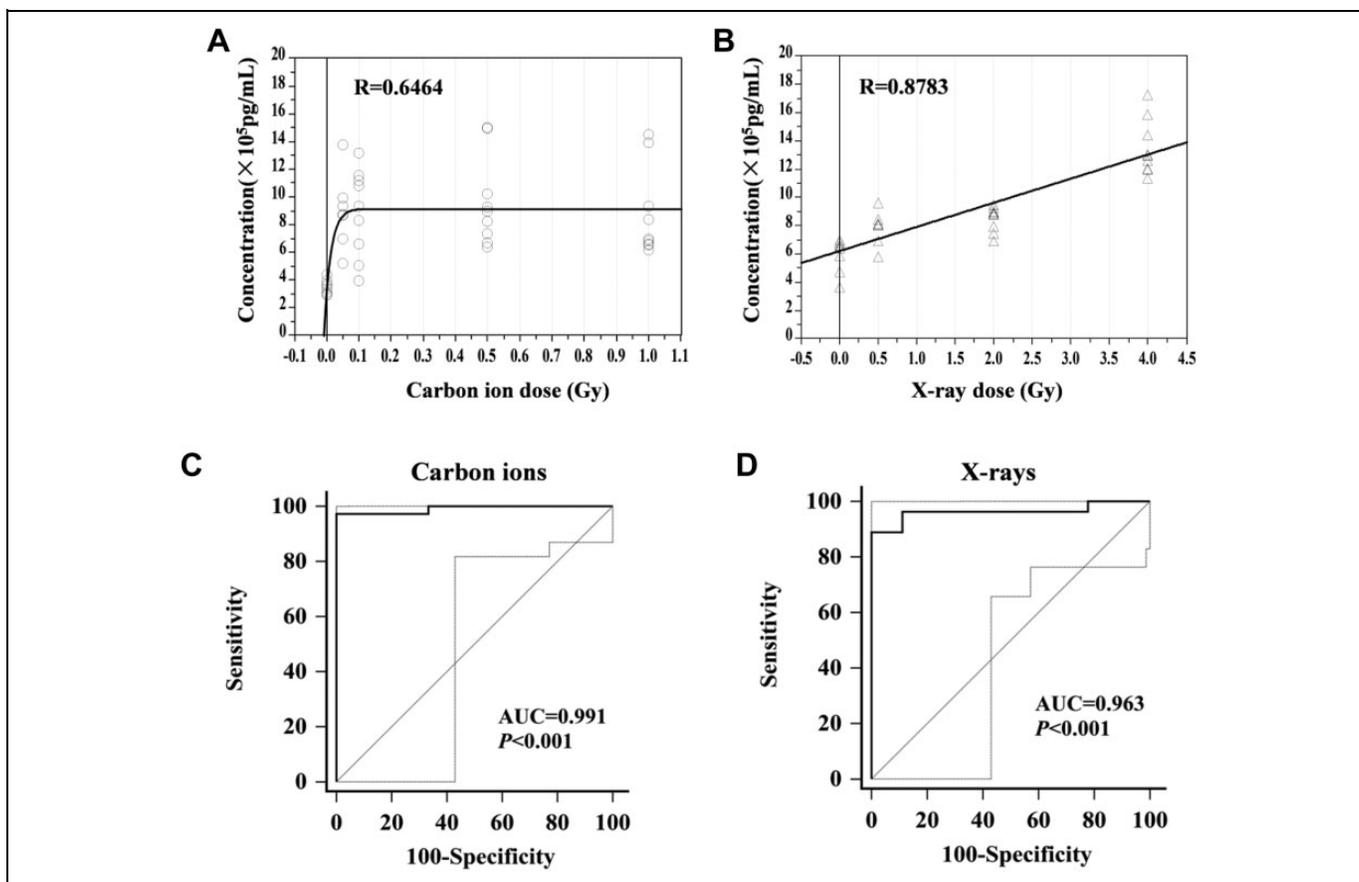


Figure 5. Estimation of serum insulin-like growth factor binding protein 3 (IGFBP-3) in predicting radiation exposure to carbon ions or X-rays. A and B, Correlation analysis between the concentration of serum IGFBP-3 and exposure doses to carbon ions and X-rays. C and D, Receiver-operating characteristic (ROC) analysis of IGFBP-3 in predicting radiation exposure to carbon ions and X-rays.

to 1 Gy after exposure to carbon ions (Figure 5A). The ROC analysis is often used to assess the sensitivity and specificity of a biomarker. Here, ROC curves of exposure to carbon ions and X-rays after 24 hours were depicted, and the area under the ROC curve (AUC) was calculated. The larger AUC value means the higher sensitivity and specificity. The ROC analysis showed that the AUC for carbon ions was 0.991 (Figure 5C), and the AUC for X-rays was 0.963 (Figure 5D), suggesting serum IGFBP-3 is efficient to triage exposed individuals with high sensitivity and specificity.

Discussion

Stride forward of manned spaceflight, nuclear industry, and radiotherapy follows growth of unexpected radiation exposure and more complex radiation environment. For many years, studies on radiation biomarkers have mainly concentrated on cell-target assays, such as in vitro colony or micronuclei formation, chromosome aberration, DNA damage-related proteins' foci, and so on.¹⁸⁻²⁰ But inherent limitations make them unsuitable for detection on orbit in space station or early detection for large population. Serum or plasma molecules originating from a variety of tissues and blood cells are ideal

minimally invasive biomarker because they are easy for collection and detection, their levels reflect human physiological or pathological states, and have been used for disease diagnosis and prognosis.^{21,22} Searching new radiation biomarkers from serum or plasma molecules is an anticipated direction. Our previous studies reported some specific serum miRNAs are potential biomarkers for carbon ion, iron ion, and X-ray irradiation,^{12,13} but the detection process of miRNA is composed of RNA extraction, reverse transcription, and real-time quantitative polymerase chain reaction. By comparison, serum proteins can be directly detected by ELISA or Meso Scale Discovery, simpler and faster. Several serum proteins such as C-reactive protein, transforming growth factor beta, serum amyloid A, and apolipoprotein E have been investigated in mice for their response to X-rays and γ -rays. However, most protein levels increased significantly when mice were exposed to a dose of radiation exceeding 2 Gy^{15,16,23}; the serum proteins in response to low dose (<1 Gy) of radiation are rarely reported. In addition, relevant published researches on high-LET radiation such as heavy ions are lacking. Heavy ion radiation leads to more DNA double-strand breaks and has higher RBE,²⁴ resulting in more serious consequence in carcinogenesis,²⁵ so it should be highlighted in future researches. The goal of this

work was to identify a set of serum proteins in response to high- and low-LET ionizing radiation, especially low dose of exposure.

The advance of proteomics technology enable the study of global expression changes for serum proteins.²⁶ Here, we chose mouse antibody array containing 308 major mouse proteins to screen the potential proteins. By using the carbon ions generated from the HIRFL in Lanzhou, China, and X-ray irradiator, we successfully identified 18 proteins in response to carbon ions and 20 proteins in response to X-rays (Figures 1 and 2). Insulin-like growth factor-1, IGFBP-1, and IGFBP-3 responded to both carbon ion and X-ray irradiation and are all part of the GH-IGFs-IGFBPs pathway. So, these 3 proteins were further validated using ELISA. The results showed serum IGF-1, IGFBP-1, and IGFBP-3 have a high abundance reaching 10^4 to 10^5 pg/mL that make it easy for microliquid detection. By determining the dose kinetics of 3 proteins, we found that only IGFBP-3 showed obvious increase with increased doses. In terms of sensitivity to low dose of radiation, the expression levels of IGFBP-3 were evidently increased in mice that received as low as 0.05 Gy of carbon ions or 0.5 Gy of X-rays (Figure 3). Moreover, the increased trend of IGFBP-3 presented from 6 to 24 hours after exposure to carbon ions and X-rays. Specifically, the expression level of IGFBP-3 reached a peak at 6 hours after carbon ion irradiation but peaked at 24 hours after X-ray irradiation (Figure 4). This difference of time reaching to an expression peak between carbon ions and X-rays may be caused by the different exposure doses or LET features. In conclusion, these results suggest serum IGFBP-3 is extremely sensitive to high- and low-LET ionizing radiation and is able to respond at an early stage. Many studies have shown that the serum IGFBP-3 level is stable in healthy subjects and some patients with different age or gender and is not influenced by circadian rhythm.²⁷⁻³⁰ These characteristics make serum IGFBP-3 an competitive biomarker for indicating radiation exposure. However, the observation in this study and the response of IGFBP-3 to other kinds of high- and low-LET radiations require further validation in other animal models or humans to ensure that they can be clinically used in unexpected radiation accidents.

The GH-IGFs-IGFBPs axis is an important hormone-related pathway. Generally, most of serum IGF-1 is combined with IGFBP-3, implying that high expression of IGFBP-3 will result in downregulation of IGF-1. In this study, ionizing radiation induced increase in serum IGFBP-3, and unexpectedly, serum IGF-1 had no expression changes and kept a stable levels. The reason is still not clear. Insulin-like growth factor binding protein-3 in blood is mainly secreted by liver, and it is possible that IGFBP-3 is a liver-specific marker for indicating radiation-induced liver damage. Although serum IGFBP-3 was founded extremely sensitive to ionizing radiation, its functions after exposure are not completely clear. The most important function of IGFBP-3 is to regulate the level of IGFs by high combination with IGFs, which impacts capabilities of IGFs such as promotion of metabolism, bone formation, hemopoiesis and immunity, and radiation response.³¹⁻³³ Besides, IGFBP-3 can work

independent through specific IGFBP receptors. For example, some studies reported that IGFBP-3 are able to promote the repair of DNA double-strand breaks as well as promote cell proliferation and induce cell apoptosis.^{34,35} Moreover, expression of P53, a critical gene-related with cell radiosensitivity, enable induce upregulation of IGFBP-3.³⁶ These studies suggest that high expression of serum IGFBP-3 after irradiation may involve in the process of radiation response or radiation damage repair and affect the functions of immune system, hematopoiesis, glycometabolism and lipid metabolism, and so on by targeting IGFs or IGFBP receptors. More studies need to be conducted to confirm these hypotheses in future.

Authors' Note

Wenjun Wei and Hao Bai contributed equally to this article.

Declaration of Conflicting Interests

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