Radiation-induced heart energy metabolism dysfunction leads to heart fibrosis

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Abstract

Thoracic radiotherapy increases the risk of radiation-induced heart disease (RIHD), but its molecular mechanisms are not fully understood. We aimed to explore the effects of radiation on the mouse heart using high-throughput proteomics. An RIHD mouse model was established by exposing the whole heart to 16 Gy high-energy X-rays, and cardiac injuries were verified by cardiac echocardiogram, serum BNP, HE and Masson staining 5 months after irradiation. Proteomics experiments were performed using the whole heart tissue of the irradiated mice and the control mice not exposed to irradiation. The proteomics data were subjected to bioinformatics analysis, and they indicated that irradiated mouse hearts showed alterations in cardiac fibrosis and energy metabolism proteins. Then, we confirmed the cardiac fibrosis and energy metabolism changes by IHC staining and WB analysis. Extracellular matrix proteins such as Col1a1, Col3a1, Vimentin and CTGF, along with metabolism-related proteins such as Pfkfb3 and Slc25a1, were overexpressed after exposure to ionizing radiation. Additionally, myocardial mitochondria inner membranes presented with injury, ATP declined and lactic acid accumulated in the irradiated heart tissues. This study suggests that high doses of ionizing radiation lead to structural remodeling, functional injury and fibrosis alterations in the mouse heart. Radiation-induced mitochondrial damage and metabolic alterations of the cardiac tissue may be one of the pathogenic mechanisms of RIHD.

1. Introduction

Thoracic radiotherapy is an important cancer treatment for patients diagnosed with malignant tumors such as breast cancer, lung cancer, Hodgkin's lymphoma and esophageal cancer[1–4]. Radiotherapy improves overall survival in thoracic cancer patients, but it involves some inevitable complications, especially RIHD, which has gradually become a concern to oncologists and cardiologists [5, 6].

The incidence of RIHD has increased due to the overall survival of cancer patients being prolonged, and RIHD usually only occurs 5 to 10 years after radiation therapy[7, 8]. It has been reported that RIHD comprises a number of heart diseases, including cardiomyopathy, coronary artery disease, pericarditis, valvular disease and conduction system abnormalities[9–11]. The rates of major coronary events increase as the heart mean dose is increased by 7.4% per gray, and these coronary events usually occurred 5–20 years after radiotherapy among breast cancer survivors[12]. The risk of coronary heart disease is increased 2.5-fold in the 20 Gy mean heart dose (MHD) group compared with Hodgkin lymphoma patients treated without radiation[2]. In our previous study, we identified a cardiac biomarker, serum ST-2, that was increased among cancer patients after thoracic radiotherapy, and it was positively associated with the radiation parameters V_5 , V_{10} , V_{20} and MHD[13].

High-energy X-rays could cause cardiac injury, but the underlying mechanisms of RIHD have not been fully investigated. Our understanding of the mechanisms involved in RIHD progression begins with energy deposition and reactive oxygen species (ROS) generation followed by molecular changes, damage to DNA, lipids and proteins, as well as the activation of early response transcription factors, cytokines and signal transduction pathways[14–17]. In addition, it is also believed that RIHD is associated with

endothelial cell injury[18, 19]. Irradiated endothelial cells are surrounded by large amounts of pro-inflammatory cytokines and then cellular degeneration occurs; cardiac dysfunction could be a result of many years of persistent inflammatory stimulation[19]. Our previous study also indicated that microvascular endothelial cell dysfunction may be a predominant mechanism of RIHD[20].

The latest advances in the high-throughput technologies of multiple "combinatorial data", such as genomics and proteomics, may explain the molecular mechanism of diseases more directly and more precisely[21, 22]. Notably, proteomics are used to provide a functional context to interpret genomic abnormalities and can present a novel paradigm for understanding cancer biology[23, 24]. Proteomics has gradually become an important technique in the fields of disease diagnosis, drug research and development, while also playing a predominant role in the much larger field of the molecular basis of diseases and biological processes at the protein level[25, 26]. The proteomics of radiation injury may provide novel evidence for the study of the mechanism of radiation heart injury, so we tend to use proteomics analysis to reveal the changes of cells or tissues by detecting the alterations of proteins.

This study aimed to use a novel high-throughput proteomics technology to explore the effects of radiation on heart proteins through establishing an RIHD mouse model to better understand the molecular mechanisms of RIHD.

2. Methods And Materials

Animal model and local cardiac irradiation

C57BL/6 male mice aged 8 weeks were purchased from the Shanghai Institute of Biochemistry and Cell Biology. The mice were irradiated at the age of 8–9 weeks. All mice lived in a 12:12 light:dark cycle environment with free access to food and water. The mice were sacrificed at 1 month, 3 months, or 5 months after irradiation or sham-irradiation. Each cohort included 3–6 mice. All animal procedures in this study were approved by the Animal Care and Use Committee of China. Ethical approval was obtained from the Institutional Review Board of the Second Affiliated Hospital of Nanchang University.

The whole heart was locally irradiated with a dose of 16 Gy by a precise small-animal radiation research platform (SARRP, XStrahl Medical and Life Sciences, USA) in the Zhejiang Key Radiation Laboratory. Mice were anesthetized by intraperitoneal injection of 75 gm/kg pentobarbital sodium and then were placed in the supine position in the irradiation area of the small animal X-ray radiometer; the laser system was used to establish a three-dimensional coordinate system.

Cone-beam computed tomography (CBCT) using 50 kV and 0.8 mA photons filtered with aluminum (1 mm) was performed for each mouse to visualize the tomographic scanning of the thorax. Heart, lung and spinal cord were drawn by the same physicist on the tomographic scanning of the thorax, then the physicist designed and evaluated the radiotherapy plan, and limited the irradiated volume of the lung tissue and spinal cord in the mice as much as possible. A dose-volume histogram (DVH) of the heart,

lung and spine could be obtained. The whole heart was irradiated using 220 kV and 13 mA X-ray beams filtered with a copper filter (0.15 mm). Control mice received sham irradiation (0 Gy).

Cardiac Echocardiogram

Transthoracic echocardiography was performed using the Vevo 2100 ultrasound system (Visualsonics, Toronto, Canada) according to our previous study[20]. Two-dimensional guided M-mode echoes were obtained at the level of the largest left ventricle (LV). The left ventricular posterior wall at the end of diastole was measured from the M-mode image. The LV ejection fraction (EF) and fractional shortening (FS) were calculated from the measured ventricle dimensions.

Measurement of Serum BNP

Mouse blood samples were collected in tubes with EDTA and the serum was separated by centrifugation for 10 min at $600 \times g$. BNP was determined using a high sensitivity Enzyme-linked Immunosorbent assay (ELISA) kit (Presage BNP assay, USCNK, China) according to the manufacturer's instructions. BNP levels were evaluated after determining the optical density of the samples at 450 nm (Thermo Scientific Microplate Reader, Varioskan LUX, Finland).

HE staining and immunohistochemistry

Whole mouse hearts were quickly excised, immersed in 10% paraformaldehyde and embedded in paraffin. The whole heart was cut into 5 μm thick sections. The slides were stained with hematoxylin and eosin (HE). Immunohistochemical (IHC) staining was performed as described previously [27]. Sections were incubated with primary antibodies mouse anti-Col1a1 or mouse anti-Col3a1 (Proteintech, Wuhan, China) at 4 °C overnight and were then incubated with a secondary antibody (ZSJQ-Bio, Beijing China), which was followed by DAB staining.

LC-MS/MS

Heart tissue protein extraction, trypsin digestion and TMT labeling

The heart tissue samples were ground in liquid nitrogen and then mixed with lysis buffer, followed by sonication three times on ice (Scientz). The supernatant was collected after centrifugation and we measured the protein concentration. The protein solution was reduced with dithiothreitol and alkylated with iodoacetamide in the dark. The protein sample was diluted by adding tetraethylammonium bromide (TEAB). Finally, trypsin was added at a trypsin-to-protein mass ratio for the first digestion overnight, repeated for a second 4 h-digestion. After the trypsin digestion, the peptides were desalted with a Strata X C18 SPE column (Phenomenex) and vacuum-dried. The peptides were reconstituted in 0.5 M TEAB and processed following the manufacturer's instructions for the TMT kit.

HPLC Fractionation

A high pH reverse-phase HPLC using Agilent 300Extend C18 column (5 μ m particles, 4.6 mm ID, 250 mm length) was used to fractionate the tryptic peptides into fractions. First, the peptides were isolated by using a gradient of 8–32% acetonitrile (ACN, pH 9.0) to separate them into 60 fractions over 1 h. Then, the peptides were merged into 18 fractions and dried by vacuum freeze-drying.

LC-MS/MS Analysis

The tryptic peptides were dissolved in solvent A (0.1% formic acid in 2% ACN) and separated by an EASY-nLC 1000 UPLC system. The liquid gradient setting consisted of an increase from 9–25% solvent B (0.1% formic acid in 90% ACN) over 24 min, 25–36% over 30 min, and increasing to 36 ~ 80% over 32 min, then holding at 80% for the last 36 min, and all of the above settings were maintained at a continuous flow rate of 350 nL/min.

The peptides were subjected to an NSI source, which was followed by tandem mass spectrometry (MS/MS) in Q Exactive TM Plus (Thermo) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. Secondary fragments of the peptides were detected and analyzed by a high-resolution Orbitrap. The scanning range of the primary mass spectrometry was set to 350–1800 m/z, and the scanning resolution was set to 700000; the scanning range of the secondary mass spectrometry was set to a fixed starting point of 100 m/z, while the secondary scanning resolution was set to 17500. The data acquisition mode used a data-dependent scanning (DDA) program; that is, after the first-level scanning, the first 20 peptide parent ions with the highest signal strength were selected to enter the high-energy C-trap dissociation (HCD) collision pool in turn to use 31% fragmentation energy for fragmentation, and the second-stage mass spectrometry analysis was also carried out in turn. To improve the effective utilization of the mass spectrometry, the automatic gain control (AGC) was set to 5E4, the signal threshold was set to 10000 ions/s, the maximum injection time was set to 200 ms, and the dynamic exclusion time for the tandem mass spectrometry scanning was set to 30 seconds to avoid repeated scanning of the parent ions. LC-MS/MS was conducted and analyzed by Jingjie PTM Biolab Co. Ltd. (Hangzhou, China).

Database Search

Maxquant search engine (v.1.5.2.8) was used to process the MS/MS data results, and the tandem mass spectra were analyzed through the SwissProt Mouse database concatenated with the reverse decoy database to calculate the false positive rate (FDR) caused by random matching. In addition, common pollution databases were added to the database to eliminate the influence of contaminating proteins in the identification results. Trypsin/P was regarded as the cleavage enzyme, allowing up to 2 missing cleavages, and the minimum length of the peptides was 7 amino acid residues. The mass error tolerance of the primary parent ion of the first search and main search were 20 ppm and 5 ppm, respectively. The mass error tolerance of the secondary fragment ion was 0.02 dalton (Da). The FDR was adjusted to < 1% and the minimum score for the peptides was set to > 40.

Annotation Methods

GO Annotation

The Gene Ontology (GO) annotation proteome was derived from the UniProt-GOA database ([www.http://www.ebi.ac.uk/GOA/](http://www.ebi.ac.uk/GOA/)). First, we used UniProt ID to match the GO ID, and then we retrieved the corresponding information from the UniProt-GOA database according to the GO ID. InterProScan software would be used to annotate the protein's GO function if the proteins were not annotated by the UniProt-GOA database. Then, the proteins were classified by the GO annotation based on the biological process, cellular component and molecular function.

Subcellular Localization

We used 'Wolfpsort' (http://www.genscript.com/psort/wolf_psort.html), a subcellular localization predication program, to predict the subcellular localization of the proteins.

Functional Enrichment

Enrichment of Gene Ontology analysis

GO annotations can be divided into three categories: Biological Process, Cellular Component and Molecular Function, which categorize the biological functions of proteins based on different features. A two-tailed Fisher's exact test was employed to test the enrichment of the differentially expressed proteins against all identified proteins. A GO analysis with $p < 0.05$ is considered significant.

Enrichment of the pathway analysis

The Encyclopedia of Genes and Genomes (KEGG) database (<http://www.kegg.jp/kegg/>) was used to study the enriched pathways by using a two-tailed Fisher's exact test. The enrichment of pathway analysis with a $p < 0.05$ was considered significant. These pathways were classified according to the hierarchical classification method of the KEGG website.

Enrichment of the protein domain analysis

InterPro (<http://www.ebi.ac.uk/interpro/>), a database providing functional analysis of protein sequences and predicting the presence of domains and important sites, was applied and a two-tailed Fisher's exact test was used to test the enrichment of the differentially expressed proteins. Protein domains analysis with a $p < 0.05$ was considered significant.

Transmission electron microscope (TEM)examination

Fresh heart apical portions of the mice at 5 months was quickly cut into 1 mm cubes and fixed in paraformaldehyde (Solarbio, China) for 2 h at 4 °C, and then fixed in 1% osmium tetroxide for 2 h at room temperature, followed by stepwise dehydration in graded acetone, and then it was infiltrated, embedded and polymerized. The pieces were sectioned with an ultramicrotome into 1–2 µm pieces and then stained

with a toluidine blue dye solution. The myocardial ultrastructure of the hearts were observed on a HITACHI-7700 electron microscope (Hitachi, Japan).

ATP and Lactic Acid Assays

Heart tissue samples were mixed with lysis fluid, homogenized with a homogenizer, and then centrifuged at 12000 g/min for 5 min. The ATP production was determined according to the manufacturer's instructions (Beyotime, China). Relative light unit (RLU) values were collected using a multimode microplate reader with the Luminometer mode (Thermo Scientific Microplate Reader, Varioskan LUX, Finland). The lactic acid was detected using a Lactic Acid Kit (Nanjing Jiancheng Bioengineering, China) following the manufacturer's instructions. The OD value was collected using a multimode microplate reader at 530 nm. The protein concentration was detected using the BCA method. Absolute ATP and lactate levels were calculated from the corresponding standard curve and normalized by the total protein concentration. Each group contained 3 mice.

Western Blot Analysis

Western blotting was carried out according to standard methods, as described previously[28], using Col1a1, Col1a3, GTGF, Vimentin, Fasn, Slc25a1 and anti- α -tubulin antibodies. All primary antibodies (Boster, Wuhan, China) were diluted 1:3000. Goat anti-rabbit (1:20000, Abcam, USA) or goat anti-mouse secondary antibodies conjugated with horseradish peroxidase were used. ECL reagent (Thermo Scientific, USA) was used for chemiluminescence detection.

Statistical analysis

All data are presented as the mean $\pm$ standard deviation (SD). Statistical differences were determined by one-way ANOVA or Student's t test using SPSS 20 (IBM Corporation, Armonk, NY, USA). $P < 0.05$ was considered significant. All experiments were performed with at least three biological replicates.

3. Results

3.1 High-dose ionizing radiation causes cardiac structural remodeling and functional injury to the hearts of mice

To investigate the effects of high-energy X-rays on mouse heart tissue, we first established an RIHD animal model with local 16 Gy heart irradiation. Cardiac echocardiography, serum myocardial biomarkers, and HE and Masson staining were used to verify the cardiac injury at different time points. The results showed that LVEF, systolic thickness of the left ventricular posterior wall and diastolic thickness of the left ventricular posterior wall at 5 months after radiation ionizing were significantly changed ($p < 0.05$) compared with those of the control mice (Fig. 1A). Pericardial effusion was not present in all groups (Fig. 1A). In addition, the serum cardiac biomarker BNP (Fig. 1A) was obviously increased 5 months after exposure to ionizing radiation ($p < 0.05$).

Moreover, the HE staining sections (Fig. 1B) indicated that the cardiomyocytes had degenerated and the myofilament boundary was blurred after radiation, and the myocardium changes were more obvious as the follow-up time increased. In addition, compared with the control mice, collagen fibers in the 5-month irradiated heart tissues had obviously increased (Fig. 1C). Cardiomyocyte degeneration, fibrosis deposition, ventricular wall remodeling, BNP elevation, and left ventricular systolic dysfunction were evident in the 5-month irradiated mice. This indicated that myocardial damage and cardiac remodeling manifested in the 5-month mice after local heart radiation exposure.

3.2 Proteomic analysis

3.2.1 Proteins quantification

In the above results, we proved that mice manifested the RIHD phenotype 5 months after 16 Gy ionizing radiation. Therefore, proteomics was performed in control and 5-month irradiated mice heart tissue (Fig. 2A). Principal component analysis (PCA) and relative standard deviation (RSD) were used to evaluate the quantitative repeatability of the proteins (Fig. 2B-C). A total of 269637 secondary spectra were obtained by MS. After the secondary spectrum of the MS went through the protein theory database, the available number of available spectra was 64186 and the utilization rate of the spectrum was 23.8%. A total of 29161 peptides were identified by the spectral diagram, among which there were 28194 specific peptides. From these peptides, 3777 proteins were identified, and 3274 were quantifiable (Fig. 2D). High-throughput proteomics analysis identified 234 proteins in total, and 219 proteins were found to be significantly increased ($\log_2 > 1.2$), while only 15 proteins were certified as downregulated ($\log_2 < -1/1.2$) in the irradiated mouse heart (Fig. 2E).

3.2.2 Subcellular localization and functional classification analysis

The 234 differentially expressed proteins were searched in the Wolf Psort database for the prediction of their subcellular localization. The subcellular localization analysis found that the distribution of the differentially expressed proteins was extracellular (46.58%), cytoplasm (18.8%), nucleus (14.1%), plasma membrane (7.26%), mitochondria (6.41%), endoplasmic reticulum (2.99%), nucleus (2.90%), and other (0.85%) (Fig. 3A).

According to the GO (Gene Ontology) category results, the proteins related to Biological Process were mainly cellular processes, biological regulation, single organelle treatment, response to stimulation and metabolic related processes; Cell Component was mainly composed of extracellular components and organelle components. Binding proteins and catalases were the main Molecular Functions (Fig. 3B).

The differentially expressed proteins in the heart tissue induced by radiation were classified by COG/KOG (Fig. 3C). The results of the COG/KOG functional classification showed that the differentially expressed proteins were mainly distributed in the following functions: (W) Extracellular structure, (O) Posttranslational modification and protein turnover, (T) Signal transduction mechanism, and (V) Defense

mechanism. Moreover, there are also dramatically differently expressed proteins in substance metabolism after radiation, such as (C) energy metabolites and conversion, (E) amino acid metabolism and transport, (F) nucleotide metabolism and transport, (G) glucose transport and metabolism and (I) lipid transport and metabolism. Metabolism-related proteins were significantly changed according to their subcellular localization and functional classification analysis.

3.2.3 Functional enrichment analysis

To find the significant enrichment trend of the differentially expressed proteins among the functional types, we analyzed the enrichment of the differentially expressed proteins in the cardiac irradiation and control group through three aspects: GO category, KEGG pathway and protein domain.

The biological process enrichment analysis results of the GO secondary classification showed that the expression of proteins regulated by protein metabolism, negative regulation of protein metabolism and proteolysis increased after irradiation. Collagen fiber tissue, protein activation cascade and invagination of the cytoplasmic membrane were among the most apparent differences (Fig. 4A). Cell component enrichment analysis demonstrated that the proteins related to vesicles, extracellular cellular components, extracellular vesicles, exocrine and extracellular region or the parts of extracellular structures increased significantly 5 months after irradiation (Fig. 4B). Molecular functions such as protein complex binding, receptor binding, molecular function regulation and enzyme activity regulated calcium binding proteins were significantly increased after irradiation (Fig. 4C).

The domains of these differential proteins were mainly enriched in immunoglobulin subtypes, immunoglobulin folding, EGF-like domains, immunoglobulin-like domains and so on (Fig. 4D). KEGG enrichment analysis found that the radiation-induced altered proteins were mainly enriched in *Staphylococcus aureus* infection, complement and collagen cascade, protein digestion and absorption, extracellular matrix interaction, folding and adhesion related signal pathways (Fig. 4E). From the three functional enrichment analyses, fibrosis was considered to be increased after irradiation.

3.3 Irradiation induces alterations in the ECM and metabolism proteins

According to the proteomic analysis results, it was found that irradiation led to the overexpression of extracellular matrix proteins such as Col14a1, Postn, Lgals3, Hpx, Tgfb1, Col2a1, Col5a2, Col3a1, Col1a2, Col1a1, Sparcl1, Col5a1, Vtn, Col6a1, and Lama5 (Table 1). COL1a1 and COL3a1 in the irradiated heart have been shown to be overexpressed by IHC and WB experiments (Fig. 5C, D). Additionally, we confirmed that other typical extracellular matrix proteins (Vimentin and CTGF) were also overexpressed (Fig. 5D). Moreover, the proteomic analysis results indicated that metabolism-related proteins associated with carbohydrate transport and metabolism, energy production and conversion, amino acid transport and metabolism, nucleotides transport metabolism and lipid transport metabolism were increased (Table 2). Metabolism-related proteins such as Fasn and Slc25a1 were verified to be overexpressed by WB (Fig. 5D).

Table 1
Upregulation of extracellular matrix proteins in mouse hearts 5 months after exposure to ionizing radiation

<i>Category</i>	<i>Gene name</i>	<i>Ratio</i>	<i>Regulated Type</i>	<i>P value</i>
Extracellular structures	Col14a1	1.832	Up	0.0032566
	Postn	1.643	Up	0.0184358
	Lgals3	1.637	Up	0.0100236
	Hpx	1.531	Up	0.0092418
	Tgfbi	1.468	Up	0.0008027
	Col2a1	1.419	Up	0.0005821
	Col5a2	1.406	Up	0.0023836
	Col3a1	1.342	Up	0.0002423
	Col1a2	1.334	Up	0.0001224
	Col1a1	1.331	Up	0.0004185
	Sparcl1	1.314	Up	0.043479
	Col5a1	1.299	Up	0.0041625
	Vtn	1.287	Up	0.0076994
	Col6a1	1.24	Up	0.0013383
	Lama5	1.219	Up	0.0004591

Table 2
Upregulation of metabolism-related proteins in the mouse heart 5 months after exposure to ionizing radiation

Category	Gene name	Ratio	Regulated Type	P value
[C] Energy production and conversion	Slc25a1	1.228	Up	0.0136395
	Aldh1a1	1.268	Up	0.0105642
	Aldh1b1	1.257	Up	0.013897
[G] Carbohydrate transport and metabolism	Hexb	1.245	Up	0.0017016
	Manba	1.311	Up	0.038884
	Slc2a1	1.309	Up	0.0061217
	Tkt	1.217	Up	0.0160025
[E] Amino acid transport and metabolism	Cfi	1.318	Up	0.0001813
	Abat	1.335	Up	0.0140643
	Cfb	1.429	Up	0.0005422
	Klkb1	1.605	Up	0.0008644
	Lta4h	1.204	Up	0.0122628
[F] Nucleotide transport and metabolism	Dpysl3	1.247	Up	0.0009192
	Entpd1	1.232	Up	0.022776
[I] Lipid transport and metabolism	Fasn	1.617	Up	0.0052028
	Lta4h	1.204	Up	0.0122628
	Ttr	1.444	Up	0.0011445

Subsequently, the electron micrograph results of the irradiated heart mitochondria were found to have myofilaments with fuzzy boundaries, swollen and cavitation of the mitochondria, and fewer mitochondria (Fig. 5A). ATP production (Fig. 5B) was decreased after irradiation, from 133.2 $\mu\text{mol/gprot}$ in the control to 103.0 $\mu\text{mol/gprot}$ in the irradiated mice ($p < 0.05$). However, lactate (Fig. 5B) in the irradiated heart tissue was obviously increased ($p < 0.05$), from 110.7 $\mu\text{mol/L}$ in the control to 269.4 $\mu\text{mol/L}$ in the irradiated mice ($p < 0.05$).

4. Discussion

An RIHD animal model was established 5 months after subjecting mice to 16 Gy local heart irradiation. The mice exhibited cardiac structural remodeling and functional injury. High-throughput proteomics was used to investigate the protein alterations of the irradiated mice hearts.

We obtained 29161 peptides, including 28194 specific peptides and 3272 quantitative proteins from among 3777 identified proteins. We found 219 proteins were significantly upregulated ($\log_2 > 1.2$) and 15 proteins were downregulated ($\log_2 < -1.2$). In contrast, Azimzadeh O et al. investigated the proteomics of 16-weeks irradiated mouse heart (16 Gy) and found that there were 662 myocardial proteins identified and 371 quantified proteins [29]. Subramanian V et al. studied the proteomics of the mouse heart at 40 weeks after 16 Gy, and 1038 proteins were identified and 940 proteins were quantified [30]. We screened 3777 proteins and quantified 3274 proteins by TMT labeling, which might be the highest flux and labeling rate in the histological data of RIHD. Abundant quantified proteins could present more comprehensive and efficient bioinformatics data to investigate RIHD.

Myocardial cell energy metabolism is very important to maintain the autonomic and contractile function of cardiac myocytes [31]. ATP is the direct energy supply and fatty acid β oxidation is a vital method to produce myocardial ATP [32]. Moreover, the mitochondria is the main organelle involved in energy metabolism [33, 34]. Therefore, derangement of energy and fatty acid metabolism could be a factor involved in cardiac diseases [35, 36]. High-throughput proteomics results of the annotation and functional enrichment indicated that the metabolism and ECM-related proteins were altered.

A large number studies have reported that the mechanism of RIHD may be associated with endothelial cell injury, inflammatory reactions, and reactive oxygen species (ROS) [9, 14, 19]. Irradiation leads to endothelial cell injury by pro-inflammatory cytokines and DNA or proteins damaged by ROS. Endothelial dysfunction influences the vascular intimal collagen deposition, which could cause pipe wall thickening and luminal stenosis [18]. A series of pathological effects such as ischemia and hypoxia of the irradiated heart may aggravate the inflammatory reaction [19]. With the continuous release of inflammatory cytokines such as TNF, IL-1, IL-6, and IL-8, cardiac parenchyma cell necrosis and excessive deposition of extracellular matrix could be found in irradiated heart tissue [9]. Radiation-induced myocardial fibrosis may be a late pathological process of RIHD [14, 37]. Fibrosis is an abnormal deposition of extracellular matrix, which can lead to organ dysfunction, morbidity and necrosis [38–40]. Radiation-induced myocardial fibrosis is often regarded as the final pathological process of RIHD and is the main risk factor for adverse myocardial remodeling and vascular changes [41, 42]. Our proteomics data and verified experiments also indicated that the mouse heart showed significant fibrosis deposition after receiving 16 Gy radiation, and the common extracellular matrix proteins such as Col14a1, Postn, Lgals3, Hpx, Tgfb1, Col2a1, Col5a2, Col3a1, Col1a2, Col1a1, Sparcl1, Col5a1, Vtn, Col6a1, and Lama5 were significantly upregulated.

In brief, the results of electron micrographs, ATP and lactate indicated that high-dose ionizing radiation actually led to cardiac mitochondria structural alterations. Consistent with the histological results, changes in the inner membrane of the mitochondria led to alterations in metabolism-related enzymes. In addition, the mitochondria are usually regarded as a vital energy-producing organelle of cells, which produce ATP via the TCA cycle in the mitochondrial inner membrane. Furthermore, one of the most vital functions of the mitochondria is the generation of ATP by aerobic respiration while lactate is a metabolite produced in hypoxic conditions.

Moreover, several ECM and metabolism-related proteins have been proven to be overexpressed after RT by WB verification. There are emerging studies about metabolic processes promoting factors involved in fibrosis pathogenesis [43–45]. Glucose metabolism can provide energy for anabolic processes and collagen production. It is well known that not only glucose metabolism but also protein and lipid metabolism are closely correlation with mitochondria [46–48]. Indeed, mitochondria are considered to be the major suppliers of cellular energy in the form of adenosine triphosphate (ATP) produced by oxidative phosphorylation and it is the main energetic organelle [49, 50].

Vincent AS et al. investigated keloid scars with excessive amounts of collagen production concluded that keloid-associated fibroblasts consume unusually large amounts of glucose and produce more lactate to fulfill their ATP needs, which means the secretion of collagen relies on ATP produced by glucose, protein and lipids metabolism[51, 52].

Meanwhile, metabolic changes are regarded as an important pathogenic process of fibrosis in various organs, and metabolic targeted therapy may become an important strategy to reduce fibrosis [33, 37, 39]. The disturbance of myocardial energy metabolism is an important cause of myocardial heart disease, and mitochondria are the main organelles of energy metabolism [53–55].

Similarly, mitochondrial dysfunction may also be an important cause of radiation-triggered heart disease. The myocardial mitochondria of C57BL/6 mice were significantly decreased 40 weeks after local 2 Gy radiation, but not in the 0.2 Gy group[56]. Marjan Boerma[57], using a model of radiation injury to the heart of SD rats, found that the mitochondrial permeability transition pore (MPTP) was opened for 6 h and 9 months after SD rats were exposed to different doses of radiation, and further studies confirmed that late application of a palladium lipoate complex (POLY-MVA) could improve myocardial mitochondrial function by reducing the cellular inflammatory microenvironment. However, it had no effect on repairing the damaged mitochondrial structure[58]. In 2019, Chen Tianfeng's team also confirmed that radiation can cause damage to the heart function caused by ROS produced by the mitochondria. The simple Ganoderma lucidum spore oil system they developed can target the myocardial mitochondria and slow down the production of ROS, thus having a preventive and therapeutic effect on RIHD. The above studies showed that mitochondrial damage is an important target of radiation heart disease, and drugs/drug-loaded materials for the repair of mitochondrial damage can reduce radiation-induced heart injury. Therefore, the mitochondria damage caused by ionizing radiation may be a significant cause of the pathogenic mechanisms of RIHD.

In this study, we analyzed the differentially expressed proteins in irradiated mice heart tissue and found fibrosis and metabolic alterations. Next, we may concentrate on the relationship between mitochondrial damage and RIHD to search for further pathogenic mechanisms.

Conclusion

The results of this study suggest that ionizing radiation causes structural remodeling, functional injury and fibrosis alterations in the heart. Radiation-induced mitochondrial damage and metabolic alterations

in cardiac tissue may be some of the pathogenic mechanisms of RIHD.

Abbreviations

RIHD

Radiation-Induced Heart Disease

BNP

Brain natriuretic peptide

IHC

Immunohistochemistry

HE

Hematoxylin and Eosin.

WB

Western Blot

ATP

Adenosine Triphosphate

MHD

Mean Heart Dose

ROS

Reactive Oxygen Species

SARRP

Small-Animal Radiation Research Platform

CBCT

Cone-Beam Computed Tomography

DVH

Dose-Volume Histogram

FS

Fractional Shortening

TEAB

Tetraethylammonium Bromide

TEM

Transmission Electron Microscope

PCA

Principal Component Analysis

RSD

Relative Standard Deviation

GO

Gene Ontology

LVEF

Left Ventricle Ejection Fraction

Declarations

Ethics approval and consent to participate

Animal ethical approval was obtained from the Institutional Review Board of the Second Affiliated Hospital of Nanchang University.

Consent for publication:

Not applicable.

Availability of data and materials:

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests:

The authors declare that they have no competing interests.

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Authors' Contributions

Anwen Liu and Zhimin Zeng designed the study, Zhimin Zeng revised the final manuscript. Peng Xu performed experiments and wrote the manuscript. Yali Yi and Yilin Xu performed part of experiments. Yijing Luo and Zhicheng Liu assisted histopathological diagnosis and immunohistochemistry analysis. Zhimin Zeng and Peng Xu performed bioinformatics analysis. Long Huang and Jing Cai gave final approval of the version to be published and participated in data analysis. All authors approved the final version of the manuscript.

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Footnotes

Not applicable.

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Figures

Fig1:

Figure 1

Sixteen Gy local heart irradiation led to RIHD after 5 months in mice (A). Mouse representative images of cardiac echocardiography and BNP alterations of the control, 3 months and 5 months after local heart 16 Gy irradiation. Red arrow: systolic thickness of the left ventricular posterior wall at 3 months post ionizing irradiation; green arrow: systolic thickness of the left ventricular posterior wall at 5 months post ionizing irradiation. (B). HE staining of control, 1-month, 3-month and 5-month groups. (C). Masson's staining of control, 3-month and 5-month groups. * and # indicate significant differences ($P < 0.05$) compared to the sham-irradiated group.

Fig2

Figure 2

Proteomics process and protein quantification. (A). LC-MS/MS analysis schedule. (B). Two-dimensional scatter plot of PCA (principal component analysis) distribution of all samples using the quantified proteins. (C). Box plot of RSD (relative standard deviation) distribution of the repeated samples using the quantified proteins. (D). Basic statistical figure of the MS results. (E). Volcano plot of the differentially expressed proteins.

Fig3:

Figure 3

Subcellular localization and functional classification. (A). Subcellular localization chart of the differentially expressed proteins. (B). Statistical distribution chart of the differentially expressed proteins under each GO category. (C). COG/KOG functional classification chart of the differentially expressed proteins.

Fig4:

Figure 4

Functional enrichment analysis. (A-C). GO (Gene Ontology) enrichment bubble plot of the differentially expressed proteins in three categories (A. Biological Process B. Cellular Component C. Molecular Function). (D). Protein domain enrichment bubble plot of the differentially expressed proteins. (E). KEGG pathway enrichment bubble plot of the differentially expressed proteins.

Fig5:

Figure 5

Irradiation induces alterations in the ECM and metabolism proteins. (A). Electron micrographs of the cardiac mitochondria from the sham-irradiated and 5-month group after 16 Gy radiation. Green arrow: myofilaments fuzzy boundaries; red arrow: swollen and cavitation mitochondria. (B). Measurement of ATP and lactate in sham-irradiated and 5 month mouse heart tissues. (C). IHC staining analysis of COL1a1 and COL3a1 in the heart tissues ($\times 400$ magnification). (D). WB analysis of Col1a1, Col3a1, Vimentin and CTGF and metabolism-related Fasn and Slc25a1. Relative protein levels of Col1a1, Col3a1, Vimentin, CTGF, Fasn and Slc25a1 were normalized to Tubulin. *and # indicates a significant difference ($P < 0.05$) when compared to the sham-irradiated group.