INVESTIGATION OF THE PATHOMECHANISM AND POTENTIAL THERAPEUTIC TARGETS IN RADIATION-INDUCED HEART DISEASE IN A RAT MODEL

Summary of the Ph.D. Thesis

Mónika Gabriella Kovács MD

Supervisor: Márta Sárközy MD Ph.D.

Department of Biochemistry

Doctoral School of Multidisciplinary Medical Sciences

Albert Szent-Györgyi Medical School

University of Szeged

Szeged

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List of publications

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I. **Mónika Gabriella Kovács**, Zsuzsanna Z. A. Kovács, Zoltán Varga, Gergő Szűcs, Marah Freiwan, Katalin Farkas, Bence Kővári, Gábor Cserni, András Kriston, Ferenc Kovács, Péter Horváth, Imre Földesi, Tamás Csont, Zsuzsanna Kahán, Márta Sárközy. Investigation of the Antihypertrophic and Antifibrotic Effects of Losartan in a Rat Model of Radiation-Induced Heart Disease. *Int. J. Mol. Sci.* 2021, *22*(23), 12963; https://doi.org/10.3390/ijms222312963 (**IF**: 5.923, **D1**)

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1. Introduction

Cardiovascular diseases and cancer are the leading causes of morbidity and mortality worldwide. The most common cancerous diseases are breast and lung cancers in women and men, respectively. Cancer therapy has undergone significant improvement, which led to increased long-term survival rates among cancer patients. About 50% of cancer patients receive radiotherapy (RT), which also has an important role in the treatment of malignancies superposed on the chest wall, such as breast cancer and thoracic malignancies including lung, and esophageal cancers, Hodgkin's lymphoma, and thymoma. While high-energy ionizing radiation (i.e., RT) successfully kills tumor cells, it could have harmful effects on the surrounding healthy tissues. Depending on the RT technique and dose used in thoracic and breast malignancies, the heart can be at risk of being exposed to ionizing radiation resulting in radiogenic sequelae in a dose-dependent manner.

The syndrome of unwanted cardiovascular side effects of thoracic RT is termed radiation-induced heart disease (RIHD), which is a critical concern in current oncology and cardiology practice. RIHD is a progressive multifactorial disease that covers a broad spectrum of cardiac pathologies. Its clinical manifestation includes acute and chronic pericarditis, conduction system abnormalities, ischemic heart disease, cardiomyopathy, heart failure with preserved ejection fraction (HFpEF) or reduced ejection fraction (HFrEF), and valvular heart disease. RT simultaneously causes damage to the cardiac macrovasculature (i.e., coronary arteries), microvasculature, and the myocardium (i.e., diffuse injury), leading to the complex pathomechanism of RIHD. However, the precise molecular mechanisms in the progression of RIHD from acute to chronic heart diseases are not clearly understood yet. Evidence suggests that RT-induced direct nitro-oxidative damage of macromolecules, including DNA, proteins,

and lipids, initiates the development of RIHD. At this acute phase of RIHD, the elevated nitro-oxidative stress causes injury to the endothelial and other cells, eventually leading to various forms of cell death and acute inflammation. In the early chronic phase of RIHD, the sublethally damaged surviving cardiomyocytes develop hypertrophy accompanied by endothelial cell proliferation as a compensatory mechanism. If these compensatory mechanisms are exhausted, chronic inflammatory processes, fibrosis, and endothelial senescence play the primary role in the progression of RIHD. Several pathomechanisms, including nitro-oxidative stress, cell death, and inflammatory processes, overlap in the acute and chronic phases of RIHD. The injury of the capillaries or coronary arteries disturbs circulation and leads to hypoxia which aggravates tissue damage. These mechanisms seem to activate and potentiate each other leading to a vicious cycle in the progression of RIHD. Unfortunately, therapeutic options for RIHD are currently insufficient. Therefore, understanding the exact molecular mechanisms in the progression of RIHD is essential for developing preventive and therapeutic strategies together with testing drugs that do not interfere with the anti-cancer effects of RT.

Endogenous microRNAs (miRs, 22 bp) are non-coding RNA species that are post-transcriptional regulators targeting specific mRNAs, resulting in an increase of mRNA degradation via complementary binding and the suppression of protein synthesis, thus influencing cellular function, including cell death and antioxidative mechanisms, inflammatory processes, cardiac hypertrophy, and fibrosis. MiRs have been described as “master switches” in cardiovascular biology, and the dysregulation of specific miRs are key pathological factors in many cardiovascular diseases. The miR-212/132 cluster is considered to be a central regulator in the development of pressure-overload-induced left ventricular hypertrophy (LVH) and heart failure via the repression of the antihypertrophic transcription factor forkhead box O3 (FOXO3) in mice with transverse aortic constriction (TAC). Moreover, the overexpression of miR-212, separate from miR-132, was reported to play a role in the development of LVH and heart failure via fetal gene reprogramming in human hearts. Furthermore, the pro-hypertrophic potential of miR-212 was also confirmed in primary neonatal rat cardiomyocytes. So far, there is no literature data available on cardiac miR-212 and FOXO3 in the development of RIHD.

Chronic activation of the renin-angiotensin-aldosterone system (RAAS) plays a pivotal role in cardiovascular pathophysiology, including hypertension, cardiac hypertrophy, and heart failure via different systemic and tissue-specific mechanisms such as elevated nitro-oxidative and endoplasmic reticulum stress, inflammation, apoptosis, and fibrosis via transforming growth factor-β (TGF-β) signaling, and the transactivation of various intracellular protein kinases such as ERKs and AKT. There is some preclinical evidence that irradiation could upregulate

angiotensin-II (AngII) expression in the rat heart and lungs in a dose-dependent manner. Interestingly, preclinical studies evaluating the cardiac effects of the widely used selective AngII type 1 receptor (AT1R) blockers in RIHD are lacking in the literature. Only two clinical studies investigated the effects of angiotensin II receptor blockers (ARBs) in cancer patients treated with thoracic RT, and their results were controversial.

2. Aims of the thesis

The aims of the present thesis were the following: i) set up a rat model with diastolic dysfunction in the acute, mild LVH in the early chronic, and severe LVH and HFpEF in the late chronic phases of RIHD; ii) investigation of the potential role of miR-212 and iii) its selected target FOXO3 in the development of radiation-induced cardiac pathologies, particularly hypertrophy and fibrosis, and; iv) test the effects of the ARB losartan (widely used in standard heart failure therapy) on the development of radiation-induced cardiac remodeling in the acute, early chronic, and late chronic phases of RIHD in our rat model.

3. Materials and methods

The studies were conducted according to the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996) and guidelines of the Declaration of Helsinki. All institutional and national guidelines for the care and use of laboratory animals were followed. The studies were approved by the University of Szeged and the regional Animal Research Ethics Committee of Csongrád County (Csongrád county, Hungary; project licenses: XV.1181/2013 for the model set up study and XV./800/2019 for the treatment study).

3.1. Experimental setup

3.1.1. Setup of the rat model in RIHD

A total of 48 male Sprague-Dawley rats (200-220 g, 6-7 weeks old) were divided into three control and three irradiated groups in separate experiments (n=8 in each group). A total of 24 animals received selective heart irradiation (50 Gy) to induce RIHD, and a total of 24 animals served as controls. Groups were followed up for 1, 3, and 19 weeks, respectively. Cardiac morphology and function were assessed by transthoracic echocardiography in all time points. The development of LVH and fibrosis in chronic RIHD was verified by the measurement of myocardial fiber diameters as well as picrosirius red and fast green staining for collagen at week 19. The myocardial expression of miR-212 and its direct target *Foxo3* were measured by qRT-

PCR at every time point. Moreover, cardiac expression of total FOXO3 and phospho-FOXO3 (pFOXO3) was measured by Western blot technique at week 19.

3.1.2. Investigation of the cardiac effects of losartan in our rat model of RIHD

A total of 63 male Sprague-Dawley rats (220–300 g, 6–8 weeks old) were used in three separate experiments. A total of 21 animals served as controls, and a total of 42 animals received a single dose of 50 Gy delivered to the whole heart to induce RIHD as described previously. Rats were divided into three groups (n = 6–9 in each group) and treated via oral gavage daily for 1, 3, and 15 weeks, respectively, as follows: (i) control group treated with tap water (per os 2 mL/kg/day, n = 7), (ii) RT only group treated with tap water (per os 2 mL/kg/day, n = 6–7), and (iii) RT plus losartan group treated with losartan (per os 10 mg/kg/day dissolved in tap water in 2 mL/kg end volume, n=7–9). Cardiac morphology and function were assessed by transthoracic echocardiography at the endpoint of each experiment. At the end of the different follow-up times, rats were anesthetized with sodium pentobarbital; then blood was collected to measure routine laboratory parameters. Hearts, lungs, and tibias were also isolated. The development of LVH and fibrosis in the irradiated groups was verified by measuring cardiomyocyte cross-sectional areas on hematoxylin-eosin (HE)-stained slides and picrosirius red and fast green-stained (PSFG) slides. Total RNA was isolated from the left ventricles, and the expression of hypertrophy and fibrosis (i.e., *Myh6, Myh7, Ctgf, Tgfb, Col1a1*, and *Mmp2*), RAAS-associated (i.e., *Cma* and *Agt*), and inflammatory (i.e., *Il1*, *Il6*, and *Tnf*) markers were measured at the transcript level by qRT-PCR in every time point. Moreover, left ventricular protein levels of angiotensin II receptor type 1 (AT1R), angiotensin II receptor type 2 (AT2R), transforming growth factor-beta receptor type 2 (TGF-βRII), mothers against decapentaplegic homolog 2/3 (SMAD2/3), total and phosphorylated signal transducer and activator of transcription 3 (STAT3 and pSTAT3), total and phosphorylated protein kinase B (AKT and pAKT), total and phosphorylated extracellular signal-regulated kinase 1 and 2 (ERK1, ERK2, pERK1, and pERK2), were measured by using Western blot technique at weeks 1, 3, and 15.

3.2. Heart irradiation

Heart irradiation with a single dose of 50 Gy in the RT groups of both studies was carried out. Before the irradiation, rats were anesthetized with sodium pentobarbital, then fixed in the supine position to a flat surface couch. Briefly, the planning of the irradiation was based on a 3D model. For better coverage of the heart and lung protection, a 6 MeV electron radiation was given with a circle-shaped aperture with a 2 cm diameter. The radiation dose was delivered with a Primus

linear accelerator at a dose intensity of 5 Gy/min if the appropriate position of the animal was proven using a built-in electronic portal imaging device.

3.3. Transthoracic echocardiography

Cardiac morphology and function were assessed by transthoracic echocardiography in both studies as described previously, at weeks 1, 3, and 15 or 19. Rats were anesthetized with 2% isoflurane, then two-dimensional, M-mode, Doppler, tissue Doppler, and four chamber-view images were performed by the criteria of the American Society of Echocardiography. In the model setup study, a Vivid 7 Dimension ultrasound system was used with a phased array 5.5–12 MHz transducer. In the treatment study, a Vivid IQ ultrasound system with a phased array 5.0–11 MHz transducer was used. Data of three consecutive heart cycles were analyzed by an experienced investigator in a blinded manner.

3.4. Blood parameters

In the treatment study, blood was collected from the abdominal aorta at weeks 1, 3, and 15. Total blood count and hematocrit were measured from whole blood by a hematology analyzer to characterize the severity of systemic inflammation and the compensatory increase in red blood cell synthesis associated with lung and heart damage due to RT.

3.5. Tissue harvesting

At weeks 1, 3, and 19 (in the model setup study) or 1, 3, and 15 (in the treatment study), hearts of the respective subgroups were isolated under pentobarbital anesthesia. Then left ventricular (LV) samples were fixed in 4% buffered formalin for histology or freshly frozen in liquid nitrogen until further biochemical measurements. In the case of the model setup study, LV samples were prepared for histological analysis only at week 19. In the case of the treatment study, LV samples were prepared for histology at weeks 1, 3, and 15.

3.6. Hematoxylin-eosin and picrosirius red and fast green stainings

In the case of the model setup study, the development of LVH was verified by measuring cardiomyocyte diameters. In the treatment study, cardiomyocyte cross-sectional areas were measured to verify the development of LVH at the cellular level. The Biology Image Analysis Software (BIAS) was used for the evaluation. In both studies, cardiac fibrosis was assessed on PSFG slides with an in-house developed program.

3.7. MicroRNA expression profiling by qRT-PCR

In our model setup study, quantitative RT-PCR was performed with miR-specific primers to monitor miR expression as described earlier. In the case of miR-212, RNA was isolated using Trizol reagent. For quantitative detection of miR-212, TaqMan MicroRNA Reverse Transcription Kit, TaqMan miR-212, snoRNA (U64702) Assays, and Absolute Blue qPCR Mix were used according to the manufacturer's instructions. SnoRNA was used as a control for normalization.

3.8. mRNA expression profiling by qRT-PCR

Quantitative RT-PCR was performed with gene-specific primers to monitor mRNA expression. RNA was isolated from LV tissue using Qiagen RNeasy Fibrous Tissue Mini Kit. In the model setup study, 3 μg of total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit, specific primers for *Foxo3*, and FastStart Essential DNA Green Master Mix according to the manufacturer's instructions. Hypoxanthine phosphoribosyl transferase 1 (*Hgprt1*), peptidylprolyl isomerase A (*Ppia*), ribosomal protein lateral stalk subunit P2 (*Rplp2*), and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) were used as controls for normalization. At week 19, *Foxo3* expression was also measured by another method using iScript™cDNA Synthesis Kit and Specific primers for *Foxo3* and *Gapdh*, SsoAdvanced™ Universal SYBR® Green Supermix according to the manufacturer's instructions.

In the treatment study, 100 μg of total RNA was reverse transcribed using iScript™ cDNA Synthesis Kit. Specific primers [angiotensinogen (*Agt*), chymase *(Cma1)*, collagen type 1 alpha 1 chain (*Col1a1)*, connective tissue growth factor (*Ctgf)*, interleukin-1 (*Il1)*, interleukin-6 (*Il6)*, matrix metalloproteinase 2 (*Mmp2)*, α-myosin heavy chain (*Myh6)*, β-myosin heavy chain (*Myh7)*, transforming growth factor-β (*Tgfb)*, tumor necrosis factor-α (*Tnf-a*)] and SsoAdvanced™ Universal SYBR® Green Supermix were used according to the manufacturer's instructions. Ribosomal protein lateral stalk subunit P2 (*RpIp2*) was used as a housekeeping control gene for normalization.

3.9. Western blot

To investigate gene expression changes at the protein level, standard Western blot techniques were used in both studies. In the model setup study, we measured the LV protein expression of pFOXO3, and FOXO3 with GAPDH loading control. In our treatment study, we measured the LV protein expression of AT1R, AT2R, TGF-βRII, SMAD2/3, STAT3, pSTAT3, AKT, pAKT, ERK1, ERK2, pERK1, and pERK2 with GAPDH loading background. LV samples were

homogenized, and after quantifying the supernatants' protein concentrations, sodium dodecyl-sulfate polyacrylamide gel electrophoresis was performed, followed by the transfer of proteins onto a nitrocellulose membrane. Membranes were blocked and then incubated with primary antibodies overnight. Then the membranes were incubated with secondary antibodies. In the model setup study, a chemiluminescence kit was used to develop signals on the membranes. In the treatment study, the fluorescent signals on the membranes were detected by the Odyssey CLx machine.

3.10. Statistical analysis

$P < 0.05$ was accepted as a statistically significant difference. In the model setup study, the data measured at different follow-up time points in separate experiments, including body weight, heart weight, heart weight to body weight ratio, lung weight, and echocardiographic parameters, were compared using One-Way ANOVA among the groups. Bonferroni test was used as a *post hoc* test. Two sample t-tests (in case of the normal distribution of the data) or Mann Whitney U tests (in case of the non-normal distribution of the data) were used to determine the effect of RT on all measured parameters within each time point. In the case of target genes, the analysis of relative gene expression data was performed using the $2^{-\Delta\Delta Ct}$ method. Gene expression ratios with a p-value of <0.05 and fold change of <-2.00 or fold change of >2.00 were considered as repression or overexpression respectively in gene activity. One-Way ANOVA was used in the treatment study to determine the statistical significance between all measured parameters within each time point. A Holm-Sidak test was used as a post hoc test.

4. Results

The presented echocardiography and histology findings in both studies are consistent with the literature data and our previous results on developing a rat model of chronic RIHD. We detected diastolic dysfunction (DD) in the irradiated animals at every pre-specified follow-up time point in our rat model of RIHD. In fact, the molecular markers (the expression of *Myh7* and *Myh6* in the left ventricle and their ratios), echocardiographic findings, and histological signs of LVH became more severe over time. Interestingly, the weights of the left ventricles were significantly lower in the RT groups compared to that in the control group throughout the experiment, probably due to the cell death and developmental retardation caused by the RT. Despite the smaller LV weights, the surviving cardiomyocytes might develop compensatory LVH to maintain the ejection fraction and sufficient oxygen supply to the whole body.

In the treatment study, a compensatory increase in the red blood cell counts at week 1 might be related to the hypoxia caused by radiation-induced heart and lung damage. Indeed, at week 1, *Il1* was significantly overexpressed in the left ventricles, pointing to the development of tissue inflammation in RIHD. The systemic inflammatory marker white blood cell count was significantly increased at week 3 in the irradiated animals. Accordingly, at week 3, the left ventricular expression of all investigated inflammatory markers, including *Il1*, *Il6*, and *Tnfα*, were significantly higher post-RT, indicating a more severe tissue inflammation at this phase. At week 15, *Il6* expression (which is a well-known mediator of myocardial fibrosis leading to concentric hypertrophy and secondary DD) remained high. In the fibrotic phase with severe concentric LVH at week 15, hemoglobin and hematocrit levels significantly increased, probably, in response to the chronic hypoxia caused by radiogenic heart failure and lung fibrosis.

In both studies, picrosirius red staining indicated interstitial fibrosis in response to RT at the endpoints (19 or 15 weeks). Generally, the increased collagen deposition could be explained by either the stimulation of production or the reduced turnover of the mRNAs and/or proteins of different collagen types. The key molecular mechanism of radiation-induced cardiac fibrosis is thought to be the TGF-β/SMAD2/3-mediated fibrotic pathway. According to our experimental data, the heart 19 weeks postirradiation is best characterized by hypertrophy, mild interstitial fibrosis, and diastolic dysfunction, demonstrating characteristic HFpEF. In our treatment study, molecular signs (i.e., overexpression of *Ctgf*, TGF-βRII, and SMAD2/3) preceded the histologic signs of fibrosis 3 weeks after the irradiation, showing the initiation of fibrotic remodeling in the heart in our RIHD model. At week 15, left ventricular *Ctgf* and SMAD2/3 expressions remained high with the overexpression of *Tgfb* and *Col1a1*. At week 15, the significantly increased interstitial collagen content of the left ventricle revealed the presence of fibrosis in RIHD. These molecular, morphological, and functional changes were similar to those reported in other studies.

In our model setup study, LVH was accompanied by the overexpression of miR-212 at weeks 3 and 19 after the selective heart irradiation. Interestingly, the repression of *Foxo3* at the mRNA level was present in cardiac tissue samples in RIHD only at week 19. In contrast, the FOXO3 protein level failed to decrease, and the pFOXO3/FOXO3 ratio showed a non-significant trend toward an increase in RIHD at week 19. Although the increased pFOXO3/FOXO3 ratio is a characteristic shift in cardiac hypertrophy forms, it seems to be independent of the effect of miR-212 in chronic RIHD. It has also been demonstrated in other LVH models that the repression of the FOXO3 level could indirectly lead to the overactivity of the hypertrophic

calcineurin/NFAT pathway. The development of the LVH and HFpEF in RIHD seems to be unique and very different from other forms of cardiac hypertrophies, such as the commonly studied pressure-overload-induced compensatory hypertrophy and subsequent heart failure.

In our treatment study, we also investigated selected molecules, including AKT, ERK1,2, and STAT3 and their phosphorylated forms, in the non-canonical non-SMAD-dependent fibrotic pathway. Significantly increased pERK1,2/tERK1,2 and pAKT/tAKT ratios were detected after RT at week 15. Since the ERK-mediated pathways are also considered survival pathways regulating cell death and hypertrophy, the increased pERK1,2/tERK1,2 ratios in the RT groups could be explained as compensatory defense mechanisms against cell death. AKT was also described as a molecule mediating cardiomyocyte survival and hypertrophy, and this, in fact, might explain the higher pAKT/tAKT ratios in the RT groups at week 15. In summary, the canonical SMAD-dependent TGF-β pathway seems to be responsible for the cardiac fibrosis in our RIHD model. The non-canonical SMAD-independent pathways, including the AKT and ERK1,2-mediated mechanisms, seem to be involved in cell survival and compensatory hypertrophy in RIHD.

It has been reported that irradiation could induce AngII overexpression in the rat heart and lungs in a dose-dependent manner. The AT1R is mainly involved in pro-inflammatory, prohypertrophic, and profibrotic mechanisms, whereas the AT2R is associated with counter-regulatory anti-inflammatory, antihypertrophic and antifibrotic pathways in the cardiovascular system. Therefore, selective AT1R blockade by losartan seems to be a rational therapeutic option to ameliorate cardiac remodeling by reducing nitro-oxidative stress and inflammatory mechanisms in RIHD.

In the treatment study, at week 1, losartan-treatment did not influence the early molecular (i.e., LV expressional changes of *Myh7* and *Myh6* and their ratios) and echocardiographic signs of LVH as assessed by M-mode echocardiography, probably due to the acute compensatory and surviving mechanisms in response to tissue damage caused by RT. At week 3, in the losartan-treated animals, various experimental parameters improved as compared to that in the RT only group, such as several echocardiographic signs of LVH and consequential DD together with the overexpression of *Myh7* and SMAD2/3 in the left ventricle indicating a possible antihypertrophic and antifibrotic effect of losartan in the early chronic phase of RIHD. In contrast, white blood cell counts remained elevated irrespective of losartan treatment in RIHD at week 3, supposing the presence of systemic inflammation. At week 15, losartan treatment did not reduce the molecular signs of LVH (the changes in the expression of *Myh7* and *Myh6* and their ratios), suggesting that the hypertrophic process is also active in this late chronic phase

of RIHD as a compensatory mechanism to cardiomyocyte loss after RT and secondary hypoxia. However, according to the echocardiography and histology findings at week 15, losartan reduced the severity of LVH. Losartan reduced the interstitial collagen content by reducing the *Ctgf*, *Tgfb*, and *Col1a1* overexpression and SMAD2/3 levels in RIHD. Similar to our results, losartan reduced cardiac fibrosis in heart failure by reducing the CTGF and SMAD2/3 expression in other studies. We also investigated molecules in the non-canonical SMAD-independent fibrotic pathway and found that pERK1,2/tERK1,2 ratios were significantly increased in the RT groups and were further increased by losartan at week 15. In contrast, several studies found that losartan reduced the pERK1,2/tERK1,2 ratio in heart failure. Our result could be explained by the fact that ERK1,2 phosphorylation can be increased by MMP2. Indeed, losartan failed to significantly reduce the overexpression of *Mmp2* in our RIHD model. Interestingly, at weeks 3 and 15, angiotensinogen expression was not significantly different between the groups, but the AT1R levels were decreased, probably, due to increased AngII levels after RT. At week 15, the repression of the AT1R was weakened by losartan, supposedly via blocking the cleavage of AngII from AngI. Indeed, the left ventricular expression of the mast cell *Cma* was higher both at week 3 and 15 in RIHD, likely mediated by cardiac inflammation and *Mmp2* overexpression. *Cma* overexpression was reduced by losartan at weeks 3 and 15, possibly explained by its mild anti-inflammatory effects in RIHD (i.e., repression of *Il6* at week 3, and decreased expression of *Tnfα* at week 15). Indeed, it has been described that losartan was more effective than the angiotensin-converting enzyme (ACE)-inhibitor captopril in controlling ongoing vascular inflammation if AngII-dependent components of atherogenesis were present in mice. However, DD was not improved by losartan in our RIHD model at week 15, which may have been due to the presence of local inflammation (i.e., *Il6* overexpression) and more severe LVH and fibrosis at this late chronic stage of RIHD. We found here antiremodeling effects of losartan and hypothesized that losartan could have antifibrotic effects by ameliorating the TGF-β/SMAD2/3-mediated pathway and reducing the overexpression of *Cma*. Moreover, the non-canonical SMAD-independent pathways, including the AKT and ERK1,2-mediated mechanisms, seem to be involved in maintaining compensatory hypertrophy in our chronic RIHD model. Inhibition of the TGF-β /SMAD2/3 pathway or the non-SMAD-dependent pathways in further experiments is needed to determine their exact role in losartan-mediated antiremodeling effects in RIHD.

5. Conclusions

The purpose of this study was to i) set up a rat model with similar pathologic changes in the acute and chronic phases of RIHD seen in humans, ii) investigate the potential role of miR-212, and iii) its selected target FOXO3 in the development of RIHD-associated LVH and fibrosis, iv) and to test the effects of the ARB losartan on the development of radiation-induced cardiac remodeling in the acute, early chronic, and late chronic phases of RIHD in our rat model. Based on our results, we can conclude that:

1. Our rat models in the acute and chronic phases of RIHD seem to be appropriate for modeling several characteristics of radiation-induced heart disease in humans, particularly LVH, inflammation, and fibrosis.
2. LVH was associated with cardiac overexpression of miR-212 in our RIHD model. However, miR-212 seems to play a role in the development of LVH via FOXO3-independent mechanisms in our RIHD model.
3. In our rat model, RIHD-associated LVH and fibrosis development were prevented or markedly slowed down by losartan if its administration started early after RT.
4. The antiremodeling effects of losartan seem to be associated with the repression of chymase and several elements of the canonical SMAD-dependent TGF-β signaling pathway.
5. The non-canonical SMAD-independent pathways, including the AKT and ERK-1,2-mediated mechanisms, seem to be involved in the maintenance of compensatory hypertrophy in our late phase chronic RIHD model.

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List of abbreviations

ACE: angiotensin converting enzyme
Agt: angiotensinogen
AKT: protein kinase B
AngII: angiotensin II
ARB: angiotensin II receptor blocker
AT1R: angiotensin II type 1 receptor
AT2R: angiotensin II type 2 receptor
Cma: chymase
Col1a1: collagen type I alpha 1 chain
Ctgf: connective tissue growth factor
DD: diastolic dysfunction
ERK1: extracellular signal-regulated kinase 1
ERK2: extracellular signal-regulated kinase 2
FOXO3: forkhead box O3
Gapdh: glyceraldehyde-3-phosphate dehydrogenase
HE: hematoxylin-eosin
HFpEF: heart failure with preserved ejection fraction
HFrEF: heart failure with reduced ejection fraction
Hgprt: hypoxanthine-guanine phosphoribosyl transferase 1
IL1: interleukin-1
IL6: interleukin-6
LVH: left ventricular hypertrophy
Mmp2: matrix metalloproteinase 2
Myh6: α-myosin heavy chain
Myh7: β-myosin heavy chain
NFAT: nuclear factor of activated T cells
PSFG: picrosirius red/fast green
Ppia: peptidyl prolyl isomerase A
RAAS: renin-angiotensin-aldosterone system
RIHD: radiation-induced heart disease
RpIp2: ribosomal protein lateral stalk subunit P2
RT: radiotherapy
SMAD2/3: mothers against decapentaplegic homolog 2/3
STAT3: signal transducer and activator of transcription 3
TAC: transverse aortic constriction
TGFβ: transforming growth factor-β
TGFβRII: transforming growth factor-β receptor II
Tnfa: tumor necrosis factor-α