Macrophage migration inhibitory factor serves a pivotal role in the regulation of radiation-induced cardiac senescence through rebalancing the microRNA-34a/sirtuin 1 signaling pathway

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Abstract. Radiotherapy significantly increases survival innumerable cancer patients, although it may have delayed adverse effects, including significant short- and long-term effects on cardiovascular function, leading to significant morbidity and mortality. However, the mechanisms underlying these effects remain unclear. Cardiomyocyte senescence contributes to cardiovascular disease via impaired cardiac function. MicroRNA-34a (miR-34a) is a senescence-associated miR involved in the pathology of cardiovascular diseases, while macrophage migration inhibitory factor (MIF) is a cardioprotective cytokine with an important role in cardiovascular diseases. The present study aimed to determine whether MIF has a cytoprotective effect in cardiomyocytes exposed to radiation through modulating miR-34a. Human cardiomyocytes (HCMs) were incubated with MIF and then exposed to radiation. Cellular proliferation was measured using a Cell Counting Kit-8, while cellular senescence was evaluated based on the senescence-associated β-galactosidase activity and the gene expression levels of cyclin-dependent kinase inhibitor 1a (Cdkn1a) and Cdkn2c. Oxidative stress was evaluated by measuring the generation of reactive oxygen species and malondialdehyde, as well as the expression of antioxidant genes. In addition, HCMs were treated with small interfering RNA against sirtuin 1 (SIRT1) to examine the role of this gene in MIF-associated rejuvenation following radiation-associated senescence. miR-34a was significantly increased in HCMs exposed to radiation, while MIF inhibited senescence by suppressing miR-34a. SIRT1 was identified as a target gene of miR-34a, mediating the anti-senescence effect induced by MIF. Furthermore, MIF rejuvenation involved rebalancing the oxidation process disturbed by radiation. These results provided direct evidence that inhibition of miR-34a by MIF protected against radiation-induced cardiomyocyte senescence via targeting SIRT1. Inhibition of miR-34a by MIF may thus be a novel strategy for combating cardiac radiation-associated damage.

Introduction

Randomized trials have reported that radiotherapy can substantially reduce cancer recurrence and moderately reduce cancer mortality; however, cardiac radiation exposure increases the risk of heart failure, thus limiting the use of radiotherapy (1,2). Cardiac radiation exposure in a rodent model resulted in cardiomyocyte hypertrophy, left ventricular diastolic dysfunction, oxidative stress and myocardial fibrosis (3). However, the mechanisms responsible for these effects of radiotherapy remain unclear. A previous study suggested that exposure to radiation affected cardiac physiology-associated gene networks and molecular signaling in cardiomyocytes, with changes in cellular proliferation and cell cycle-associated genes and signaling during the early phase of radiation exposure (4). Furthermore, cardiomyocyte senescence has been demonstrated to be involved in cardiac pathological processes (5). Therefore, the present study aimed to determine whether radiation induced senescence in cardiomyocytes.

MicroRNAs (miRs) are a class of noncoding RNAs that have been reported to serve a significant role in gene regulation by targeting a variety of transcripts. miRs have also recently been identified as important regulators of cellular senescence and aging (6). miR-34a was demonstrated to be involved in the senescence process in cardiomyocytes (7), and exerted a pro-senescence effect by regulating the sirtuin 1 (SIRT1) signaling pathway (8), which serves a crucial role in the maintenance of cardiac homeostasis and energy balance, and is disturbed by radiotherapy (9). Therefore, the current study examined the role of the miR-34a/SIRT1 signaling pathway in radiation-induced cellular senescence.
Macrophase migration inhibitory factor (MIF) is a multifunctional cytokine secreted by various tissues, including the myocardium (10). Recent evidence has suggested that MIF may have a cardioprotective role in pathological conditions, including ischemia injury, hypertension and cellular senescence (11-13). Several mechanisms have been suggested to account for the MIF-induced biological responses, including modulation of the energy balance, which is known to be disrupted in radiation-induced ischemic heart disease (14). Thus, the role of MIF in the regulation of radiation-induced cellular senescence was also investigated in the present study.

SIRT1, a member of the SIRT family of class III histone deacetylases, is one of the targets of miR-34a, known to be widely involved in the regulation of cellular senescence (15,16). Furthermore, SIRT1 has been reported to be involved in the development and progression of heart failure through the regulation of cell senescence-associated signaling in chemotherapy-induced cardiac damage (17). Consequently, activation of SIRT1 may have a great potential in the treatment of radiation-associated cardiotoxicity.

Oxidative stress is an important inducer of cardiomyocyte senescence (18), with radiation being a major cause of reactive oxygen species (ROS) production (19). Upregulation of antioxidant proteins, such as endothelial nitric oxide synthase (eNOS) and catalase, has been demonstrated to protect against oxidative stress-mediated senescence (20). Growing evidence has also indicated that miR-34a functions as a senescence promoter by inhibiting SIRT1 and inducing oxidative stress (21). Therefore, the present study explored whether radiation-induced oxidative stress exerted a pro-senescence effect in cardiomyocytes.

In the current study, the aim was to examine whether radiation induces cardiomyocyte senescence, and whether MIF reduces cellular susceptibility to radiation-induced senescence by inhibiting miR-34a expression and oxidative stress. The results suggested that MIF may represent a promising therapeutic strategy for radiation-associated cardiac damage.

Materials and methods

Cell culture and treatment. Human cardiomyocytes (HCMs; PromoCell GmbH, Heidelberg, Germany) were cultured in Dulbecco’s modified Eagle’s medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal calf serum (HyClone; GE Healthcare Life Sciences) and 1% penicillin-streptomycin (Beyotime Institute of Biotechnology) at 37˚C with 5% CO2 and 1% penicillin-streptomycin (Beyotime Institute of Biotechnology). The plates were incubated for 1 h at 37˚C. The absorbance of cells at 450 nm was then measured with a microplate reader.

Cell proliferation assay. Cell proliferation was assessed using a Cell Counting Kit-8 assay (CCK-8) according to the manufacturer’s protocol (Beyotime Institute of Biotechnology). Briefly, cells were seeded at a density of 2x10^3 cells/well in 96-well plates and incubated at 37˚C for 24, 48 and 72 h, respectively. CCK-8 solution (10 µl) was added to each well, and the plates were incubated for 1 h at 37˚C. The absorbance of cells was measured by RT-qPCR analysis.

Materials and methods

Small interfering RNA (siRNA) gene knockdown. HMCs (1x10^5) were seeded in 6-well plates at 30-40% confluence 1 day prior to transfection and reached 70-80% confluence the following day. Cells were then transfected with 100 nM SIRT1 siRNA and siRNA-non targeting (siRNA-NT; both Invitrogen; Thermo Fisher Scientific, Inc.) using X-tremeGENE HP DNA transfection reagent according to the manufacturer’s protocol. The siRNA sequences are listed in Table I. Cells were irradiated with 5 Gy, treated with 100 ng/ml MIF and harvested 48 h after transfection for further analysis. The transfection efficiency was analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Western blotting. Cells were lysed in ice-cold lysis buffer to obtain the total protein (Beyotime Institute of Biotechnology), and protein concentrations were measured using the BCA Protein Assay kit (Beyotime Institute of Biotechnology). Samples were then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride membranes. The membranes were incubated with SIRT1 (cat. no. 9475) and β-actin (cat. no. 4970) primary antibodies (both 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (cat. no. 7074; 1:2,000; Cell Signaling Technology, Inc.). Subsequently, the membranes were visualized using the BeyoECL Plus (Beyotime Institute
of Biotechnology). The stained protein bands were visualized using a Bio‑Rad ChemiDoc XRS system and analyzed using Quantity One software (Bio‑Rad Laboratories, Inc., Hercules, CA, USA).

Relative telomere length. The relative telomere length in HCMs was measured using a qPCR approach, as described previously (24), with GAPDH serving as the normalizing gene. Table II lists the primers used for the detection of the telomere length.

Relative telomerase activity. A TeloTAGGG Telomerase PCR ELISA PLUS Assay kit (Sigma‑Aldrich; Merck KGaA, Darmstadt, Germany) was used to analyze the relative telomerase activity, according to the protocol provided by the manufacturer.

Senescence‑associated β‑galactosidase (SA‑β‑gal) assay. Cellular senescence was measured by SA‑β‑gal assay (Cell Signaling Technology, Inc., Danvers, MA, USA). Briefly, cells were seeded in a 6‑well plate at a density of 2x10⁴ cells/well were washed with phosphate-buffered saline, fixed with 2% paraformaldehyde for 30 min at room temperature and then incubated with fresh SA‑β‑gal staining solution, as described previously (25). SA‑β‑gal activity was measured using a microplate reader at a wavelength of 50 µm.

ROS measurement. Intracellular ROS levels were measured using 2,7‑dichlorodihydrofluorescein diacetate (Beyotime Institute of Biotechnology), according to the protocol described by the manufacturer. A fluorescence spectrophotometer was used to determine the fluorescence intensity of the cells, at an excitation and emission wavelength of 488 and 525 nm, respectively.

Lipid peroxidation assays. Malondialdehyde (MDA) levels in HMCs were measured using the Lipid Peroxidation (MDA) Assay kit (Abcam, Cambridge, UK). Briefly, 2 ml HMCs at a density of 1x10⁶ cells/ml were homogenized on ice in 300 µl MDA lysis buffer, followed by centrifugation at 12,000 x g and

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tr>
<td>miR‑34a mimic</td>
<td>5'-UGGCAGUGCUUAGCUGGUUGUAACCAGCUAAGACACUGCAUU-3'</td>
</tr>
<tr>
<td>miR‑negative control</td>
<td>5'-UGUCAGCUUUGGAGCUUUGUAACCUAAGAUGCCACCAGCAUU-3'</td>
</tr>
<tr>
<td>siRNA‑Sirtuin 1</td>
<td>F: 5'-AAGTACAATCCACCAGGAA TGA-3'</td>
</tr>
<tr>
<td>siRNA‑non targeting</td>
<td>F: 5'-CTCTATGAACACCTAGCT-3'</td>
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miR, microRNA; NC, si, small interfering; F, forward

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
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<tr>
<td>microRNA‑34a</td>
<td>F: 5'-CAGAGCATCACACGCAAGC-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CAGGAAACAGAAACCCCCAGC-3'</td>
</tr>
<tr>
<td>Cdkn1a</td>
<td>F: 5'-TCACTGTCTTTGACCTTGAC-3'</td>
</tr>
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<td></td>
<td>R: 5'-GGCGTGTGTGAGTGTAGAAA-3'</td>
</tr>
<tr>
<td>Cdkn2c</td>
<td>F: 5'-CGGGAGGTCTTGTCTG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-TTTGTGTGCTTGAC-3'</td>
</tr>
<tr>
<td>Sirtuin 1</td>
<td>F: 5'-CAGAGCATCACACGCAAGC-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CAGGAAACAGAAACCCCCAGC-3'</td>
</tr>
<tr>
<td>Telomere length</td>
<td>F: 5'-CGGTTTGTTTGGGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTT-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GGCTTGCTTACCTTACCTTACCTTACCTTACCTTACCTTACCTTACCTTACCT-3'</td>
</tr>
<tr>
<td>Endothelial nitric oxide synthase</td>
<td>F: 5'-GCAACCCACATCAAGTATGACCAAA-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GCTGTGTCAGTGAGAAGTGCCTCT-3'</td>
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<tr>
<td>Catalase</td>
<td>F: 5'-CAAGCTGTGTAAATCGAATGG-3'</td>
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<tr>
<td></td>
<td>R: 5'-TTGAAAAAGATCTCGAGCGGC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5'-CTGACCACACAAGCTGATCAG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AGGTCCACCCACTGACAGCT-3'</td>
</tr>
</tbody>
</table>

miR, microRNA; Cdkn, cyclin‑dependent kinase inhibitor; F, forward; R, reverse.
4°C for 1 min to remove insoluble material. Thiobarbituric acid was then added to the supernatant and incubated at 95°C for 60 min. The samples were allowed to cool at room temperature for 10 min, and the absorbance at 532 nm was measured with a spectrophotometer.

**Statistical analysis.** Data are expressed as the mean ± standard deviation. Differences among groups were tested by one-way analysis of variance, and comparisons between two groups were evaluated by Student’s t-tests using SPSS version 19.0 software (IBM Corporation, Armonk, NY, USA). A value of P<0.05 was considered to denote a difference that was statistically significant.

**Results**

Radiation induces miR-34a expression and senescence in HCMs. miR-34a expression levels in HCMs were significantly increased following exposure to radiation (Fig. 1A). Radiation markedly inhibited cellular proliferation
(Fig. 1B) and increased the expression levels of the cellular senescence-associated genes Cdkn1a and Cdkn2c (Fig. 1C and D). Radiation exposure also significantly shortened the relative telomere length (Fig. 1E), impaired the relative telomerase activity (Fig. 1F) and increased the percentage of SA-β-gal-positive cells (Fig. 1G and H).

Exogenous MIF alleviates radiation-induced senescence via inhibition of miR-34a. The anti-senescence effect of MIF was explored by adding 100 ng/ml MIF to HCMs prior to exposure to radiation. Exogenous MIF significantly decreased the expression of miR-34a induced by radiation (Fig. 2A). Subsequently, the study further examined whether MIF
exerted its anti-senescence effect via inhibition of miR-34a by transfecting HCMs with a miR-34a mimic to induce the overexpression of miR-34a (Fig. 2B). MIF treatment in cells exposed to radiation increased cellular proliferation (Fig. 2C), decreased the expression levels of the senescence-associated genes Cdkn1a and Cdkn2c (Fig. 2D and E), recovered the impaired relative telomere length and activity (Fig. 2F and G), and reduced the percentage of SA-β-gal-positive cells (Fig. 2H and I). However, the effects of MIF treatment were reversed by miR-34a overexpression in cells exposed to radiation.  

**Rebalancing of the miR-34a/SIRT1 signaling pathway by MIF is essential for preventing radiation-associated senescence.** SIRT1 is a well-known target of miR-34a and is closely associated with cellular senescence (8). The present study analyzed SIRT1 expression to investigate the miR-34a target and clarify the mechanism of MIF-mediated suppression of radiation-associated cellular senescence in HCMs. SIRT1 protein expression was significantly inhibited by radiation and then recovered by MIF; however, the MIF-mediated recovery was reversed by miR-34a overexpression (Fig. 3A and B). Furthermore, the mechanism responsible for the anti-senescence effect of MIF in relation to the miR-34a/SIRT1 signaling pathway was further explored by silencing SIRT1 using siRNA (Fig. 3C). Downregulation of SIRT1 in MIF-treated radiation-exposed cells decreased the recovery of cell proliferation induced by MIF (Fig. 4A), induced the expression of the senescence-associated genes Cdkn1a and Cdkn2c (Fig. 4B and C), reduced the telomere length and activity (Fig. 4D and E), and increased the percentage of SA-β-gal-positive cells (Fig. 4F and G), as compared with the cells treated with only MIF and radiation. By contrast, none of these effects were observed in HCMs treated with the control, siRNA-NT.

**Modulation of oxidative stress by MIF inhibits the radiation-induced senescence.** The effects of MIF on oxidants and antioxidant gene expression levels were assessed to determine whether MIF exerted its anti-senescence effect via modulation of oxidative stress in cardiomyocytes. Quantitative analysis demonstrated that MIF inhibited the radiation-induced accumulation of cellular ROS and generation of MDA in HCMs (Fig. 5A and B), and reversed the radiation-mediated inhibition of eNOS and catalase gene expression levels (Fig 5C and D). These antioxidant effects of MIF were abolished by overexpression of miR-34a or silencing of SIRT1 (Fig. 5).

**Discussion**

Recent progress in radiotherapy over the past two decades has led to substantial improvements in life expectancy for cancer patients, however, at the cost of increased risks of unintended side effects (26). Radiotherapy has been closely associated with cardiotoxicity and an increased risk of symptomatic cardiac dysfunction (1,27). Given the increasing number of long-term survivors following treatment with radiotherapy, cardiotoxicity has become recognized as a major concern in oncology (28). However, the mechanisms responsible for radiotherapy-associated cardiac damage remain unclear. Heart aging is associated with functional decline and increased vulnerability to cardiovascular damage, and is the leading cause of mortality worldwide (29). Age-associated changes include decreased cardiac output, elevated susceptibility to arrhythmia and impaired relaxation with increased myocardial stiffness, all of which are included in the phenotype of radiotherapy-associated cardiac damage (1,30). The results of the current study revealed that radiation induced senescence in HCMs.

miRs have emerged as a new class of modulators of gene expression. Among these, miR-34a has been implicated in cardiac-associated damage, particularly cardiac senescence (31). Researchers recently demonstrated that miR-34a was involved in modulating radiation-induced senescence, and that radiation-induced senescence was correlated with the upregulation of miR-34a expression (32). Accordingly,
the present study found that radiation exposure induced miR-34a expression in HCMs, accompanied by cellular senescence. MIF is known as a prototype cytokine that regulates macrophage function in inflammation and serves an important role in cardiac metabolism (33). Previously, it was confirmed that MIF was highly expressed in cardiomyocytes and regulated cellular senescence (10). As a promoter of miR-34a, p53 is associated with accelerated cellular senescence and is inhibited by MIF (34,35). The results of the present study revealed that MIF alleviated radiation-associated cellular senescence through inhibiting miR-34a expression, while overexpression of miR-34a abolished the anti-senescence effect of MIF. miR-34a has been reported to trigger senescence.
parly through genetic inhibition of SIRT1 (36); therefore, this observation prompted us to explore the potential role of MIF in modulating miR-34a-dependent SIRT1 expression in cardiomyocytes. It was observed that MIF significantly suppressed the radiation-induced expression of miR-34a, while silencing SIRT1 blocked the anti-senescence effects of MIF. These results revealed that MIF reduced cardiomyocyte senescence by interacting with miR-34a and upregulating SIRT1.

Cellular senescence is defined as the stable cell cycle arrest elicited in response to a variety of stressors (37). The accumulation of oxidative stress in cardiomyocytes has been reported to cause senescence and reduce cardiac function (18). Radiation-associated oxidative stress and telomere loss have all been linked to the induction of the senescent phenotype through replication stress-induced DNA damage (38, 39). The current study demonstrated that cardiomyocyte senescence was accompanied by radiation-induced oxidative stress and telomere loss. MIF has been reported to function as an antioxidant factor (40). Accordingly, the present study found that MIF significantly inhibited ROS and MDA generation, and stimulated eNOS and catalase gene expression in cardiomyocytes. Furthermore, overexpression of miR-34a by transfection of anti miRNA mimic or silencing SIRT1 abolished the MIF-induced antioxidant gene expression.

In conclusion, the results of the present study demonstrated that MIF had a protective role in radiation-induced cardiac senescence. This cardioprotective effect of MIF may mainly be attributed to the suppression of oxidative stress by preventing miR-34a-mediated inhibition of antioxidant gene expression.

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Availability of data and materials

All data generated or analyzed during the present study are included in the published article.

Authors' contributions

YH and WX made substantial contributions to the acquisition, analysis and interpretation of data. MH was involved in conception and design of the study, and in drafting the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University. All animal procedures were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


