Research article

MiR-34a REGULATES APOPTOSIS IN LIVER CELLS BY TARGETING THE KLF4 GENE

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Abstract: MicroRNAs (miRNAs) regulate gene expression by inhibiting translation or targeting messenger RNA (mRNA) for degradation in a post-transcriptional fashion. In this study, we show that ectopic expression of miR-34a-5p reduces the mRNA and protein levels of Krüppel-like factor 4 (KLF4). We also demonstrate that miR-34a targets the 3'-untranslated mRNA region of KLF4 and show that overexpression of miR-34a induces a significant level of apoptosis in BNL CL.2 cells exposed to doxorubicin or 10 Gy X-ray. Our data suggest that the effects of miR-34a on apoptosis occur due to the downregulation of KLF4.

Key words: MiR-34a, KLF4, Apoptosis, Liver, Irradiation, Doxorubicin

INTRODUCTION

MicroRNA (miRNA) is defined as a small regulatory RNA molecule consisting of non-coding RNA of about 22 nucleotides in length. In animals, miRNAs participate in the regulation of signaling pathways, proliferation, apoptosis, metabolism, hematopoietic organogenesis, and developmental timing [1-3].

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Many recent reports indicate that miRNA expression levels change in response to cellular stresses, such as hypoxia, drought, cold, oxidative stress and radiation [4-7]. The current data suggest that exposure to radiation provokes cellular responses controlled in part by gene expression networks [8, 9]. MicroRNAs regulate gene expression and have been shown to control multiple intracellular processes involved in the response to cellular stress [10, 11].

MiR-34a resides on chromosome 1 p36.23. Ectopic miR-34a expression induces apoptosis, cell cycle arrest and differentiation, or reduces protein migration [12-15]. The process of programmed cell death (apoptosis) has distinct morphological characteristics and generally involves energy-dependent biochemical mechanisms that eliminate aged, damaged or otherwise abnormal cells. MiR-34a is known to regulate a plethora of target proteins that induce cell apoptosis in a p53-dependent or independent manner [16, 17]. However, the function of miR-34a in radiation-induced apoptosis, which involves the inhibition of its target genes, is still unclear. Computational analyses predict that miR-34a can have hundreds of potential target genes [18]. We focused on Krüppel-like factor 4 (KLF4), which is an important factor in mediating the apoptosis induced by DNA damage [19].

In this study, we found that miR-34a-5p could be induced in BNL CL.2 cells by radiation. We used a transfection assay to discover that over-expression of miR-34a-5p markedly enhanced apoptosis after exposure to doxorubicin or radiation. We also found that the possible anti-apoptotic downstream target of miR-34a-5p is KLF4. Over-expression of miR-34a-5p and knockdown of KLF4 could significantly enhance the apoptosis of BNL CL.2 cells.

MATERIALS AND METHODS

Cell culture
BNL CL.2 is a liver cell line derived from BALB/c mice. The cells are not tumorigenic in immunosuppressed mice, but do form colonies in semi-solid medium. BNL CL.2 cells and human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco). The cells were grown in a 37ºC incubator with 5% CO_2.

Transfections with miRNA
The miR-34a-5p mimics and miR-34a-5p inhibitor were purchased from Shanghai GenePharma Company. The description of the mimics and inhibitor can be found on the GenePharma website [20]. The miR-34a-5p mimics and miR-34a-5p inhibitor were transfected into BNL CL.2 cells at a final concentration of 50 nM using lipofectamine 2000 (Invitrogen) in six-well plates according to the manufacturer's instructions.

Flow cytometry (FCM) analysis of apoptosis
BNL CL.2 cells were cultured in six-well plates and exposed to a single 10 Gy dose of X-ray (6 MV, Philips SL18, Berkshire, England, 200 cGy/min) for 0, 48 and

72 h, or treated with doxorubicin (final concentration 10 μg/ml) for 24 h. The cells were harvested and stained with the Annexin V(AV)-Propidium iodide(PI) Apoptosis Detection Kit (Biouniquer). The number of cells undergoing apoptosis was determined by FCM.

RNA isolation and quantitative RT-PCR
We isolated total RNA using TRIzol reagent (Invitrogen). Quantitative RT-PCR was performed using Assay-on-Demand Taqman probes for mouse KLF4 and β-actin (Applied Biosystems). The mRNA expression of KLF4 was compared using the values of the $\Delta\Delta$ crossing threshold (C_T). We used an miRNA isolation kit to isolate miR, a Taqman miRNA reverse transcription kit for amplification, and miR-34a-5p and U6 Taqman probes (all from Applied Biosystems) for the real-time PCR. U6 was used as an endogenous control to normalize the C_T values obtained for miR-34a-5p.

Western blot analysis
BNL CL.2 cells were lysed and the protein extracts were denatured. The solubilized proteins (50 μg) were subjected to electrophoresis, blotted to polyvinylidene difluoride membrane, and reacted with specific antibodies. The membranes were blotted with the appropriate KLF4 primary antibody (Santa Cruz Biotechnology) at dilutions of 1:100 to 1:200. The membranes were then incubated with the appropriate secondary antibody linked to horseradish peroxidase at a 1:2000 dilution for 2 h at room temperature. After Tris-buffered saline with Tween (TBST) washes, the blot was incubated in enhanced chemiluminescence detection reagent (ECL Advance Western Blotting Detection Kit, Amersham Bioscience) and exposed to a Hyper film ECL film (Pierce). Glyceraldehyde-3-phosphate dehydrogease (GAPDH) served as the loading control (Kang Cheng Biotechnology).

Lentiviral particle transduction of BNL CL.2 cells
The cells were plated into six-well plates for 24 h before transduction. The cells were then transduced with various lentiviral particles (Genepharma), specifically shNC (short hairpin RNA negative control) and shKLF4 (carrying interfered KLF4 short hairpin RNA that targets GGACGGCTGTGGATGGAAA) using 5 μg/ml polybrene (Santa Cruz Biotechnology) at a multiplicity of infection of 6. For transient transfection, the cells were collected after 48 h.

Luciferase assay
Firefly luciferase reporter vectors were constructed using the following methods. Wild-type 3'-UTRs containing predicted miRNA target sites were amplified via PCR from BNL CL.2 cell genomic DNA. Mutant 3'-untranslated regions (UTR) were generated using the overlap-extension PCR method. Both wild-type and mutant 3'-UTR were cloned in downstream of the firefly luciferase coding region (the Xba I site) of a modified pGL3-promoter plasmid (Promega).

Specific fragments including miR-34a -5p targeting site of KLF4 3'-UTR were generated using the following primers:
KLF4-3'UTR (forward) 5'-GGTATCTCTAGAATCCCACGTAGTGGATGT GACCCA-3', KLF4-3'UTR (reverse) 5'-GGTATCTCTAGACGTTCAGATAA AATATTATAGGT-3'.
KLF4 mutant 3'-UTR were generated using the following primers:
MutKLF4-3'UTR (forward) 5'-ATCCCACGTAGTGGATGTGACCCGCGCC GCAGGGAGAGAG-3', MutKLF4-3'UTR (reverse) 5'-CTCTCTCCCTGCG GCGCGGGTCACATCCACTACGTGGGAT-3'.
The seed region was mutated and is underlined in Fig. 1A. For the luciferase assay, HEK 293 cells were co-transfected with 100 ng of pGL3-KLF4-3'UTR or pGL3-KLF4-3'UTR mutant plasmid, and 50 nM of miRNA inhibitor control or miR-34a-5p inhibitor combined with lipofectamine 2000. In each transfection, 50 ng of pRL-TK (Promega) was used to correct for the transfection efficiency. After 48 h incubation, the cells were washed and lysed. The relative luciferase activity was assayed using the dual-luciferase reporter assay system (Promega).

Statistics
Data are presented as the means ± standard deviation (S.D.) and evaluated by analysis of variance (AVOVA) or Student's t-test when appropriate. Significance was established at a level of $P < 0.05$.

RESULTS

KLF4 is one of the target genes for miR-34a-5p in BNL CL.2 cells
The miR-34a-KLF4 target prediction was derived from miRanda [21], miRBase [22] and PicTar [23]. To examine whether miR-34a-5p directly binds the 3'UTR of KLF4 mRNA, we constructed the luciferase reporter plasmid pGL3-KLF4-3'UTR containing the putative miR-34a-5p binding site of KLF4 3'UTR downstream of the luciferase open reading frame (Fig. 1A). The pGL3-KLF4-3'UTR construct was transfected into HEK 293 cells together with miR-34a-5p mimics or miR-34a-5p inhibitor. As shown in Fig. 1B, miR-34a remarkably repressed the expression of luciferase containing an original miR-34a binding site (KLF4-3'UTR) but not that of luciferase containing a mutant binding site (KLF4-3'UTR-MUT). When miR-34a-5p inhibitor was co-transfected with pGL3-KLF4-3'UTR, the expression of firefly luciferase significantly increased compared to the values for the negative control co-transfected group. Mutations in seed complementary sites of the 3'UTR region of KLF4 could restore the luciferase expression, which strongly suggests a direct inhibitory effect of miR-34a-5p on KLF4 expression.
Recently, miR-34a was demonstrated to potentiate apoptosis in pancreatic cancer by inhibiting Notch-1 [24]. The γ-irradiated cells underwent apoptosis if KLF4 was absent [19]. In order to elucidate the relationship between miR-34a-5p

A

seed region

KLF4-3'UTR-WT   5' AGUGGAUGUGACCCACACUGCCA 3'

                         | | | | | | | | | |

miR-34a-5p   3' UUGUUGGUCGAUUCUGUGACGGU 5'

                         | | | | ||

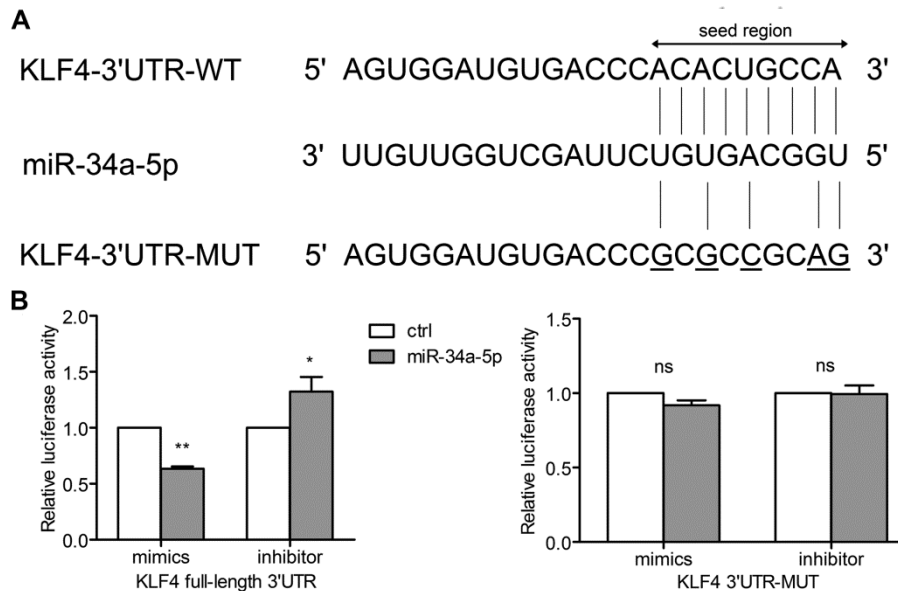KLF4-3'UTR-MUT   5' AGUGGAUGUGACCCGCGCCGCAG 3'

B

Fig. 1. Sequence-specific suppression of KLF4 gene expression by miR-34a-5p. A – The putative target site of KLF4 mRNA 3'UTR, as determined by computational predictions. The target sequence was cloned into pGL3-REPORT vector (pGL3-KLF4-3'UTR). The pGL3-KLF4-3'UTR mutant was also generated with QuikChange Site-directed Mutagenesis kit (Stratagene Products Division, Agilent Technologies) in the target site. B – HEK 293 cells were transfected with pGL3-KLF4-3'UTR or pGL3-KLF4-3'UTR mutant together with miR-34a-5p mimics or the miR mimics control, or together with miR-34a-5p inhibitor or the miR inhibitor control. Luciferase activity was measured using the Promega Dual-Luciferase reporter assay system. Values are means ± S.D. (n = 3). *P < 0.05, **P < 0.01 compared with control group.

and KLF4 in BNL CL.2 cells, we manipulated miR-34a-5p level in BNL CL.2 cells by transfecting with miR-34a-5p mimics or miR-34a-5p inhibitor. When 50 nM miR-34a-5p mimics was transfected into BNL CL.2 cells, the level of miR-34a-5p increased up to 4.2-fold (Fig. 2A, left panel). By contrast, transfection of the miR-34a-5p inhibitor decreased the miR-34a-5p level by 27% (Fig. 2A, right panel). These data suggest that transfection of miR-34a-5p mimics or miR-34a-5p inhibitor can significantly alter the miR-34a-5p level in BNL CL.2 cells. The over-expression of miR-34a-5p in BNL CL.2 cells reduced KLF4 mRNA expression by 38% (Fig. 2B, left panel). By contrast, KLF4 mRNA expression was increased in BNL CL.2 cells after miR-34a-5p inhibitor transfection (Fig. 2B, right panel).

Furthermore, over-expression of miR-34a-5p in BNL CL.2 cells reduced KLF4 protein expression by 38% (Fig. 2C, left panel). By contrast, KLF4 protein expression was increased in BNL CL.2 cells after miR-34a-5p inhibitor transfection (Fig. 2C, right panel). These results show that miR-34a suppressed KLF4 expression in liver cells through direct interaction with KLF4 3'UTR.
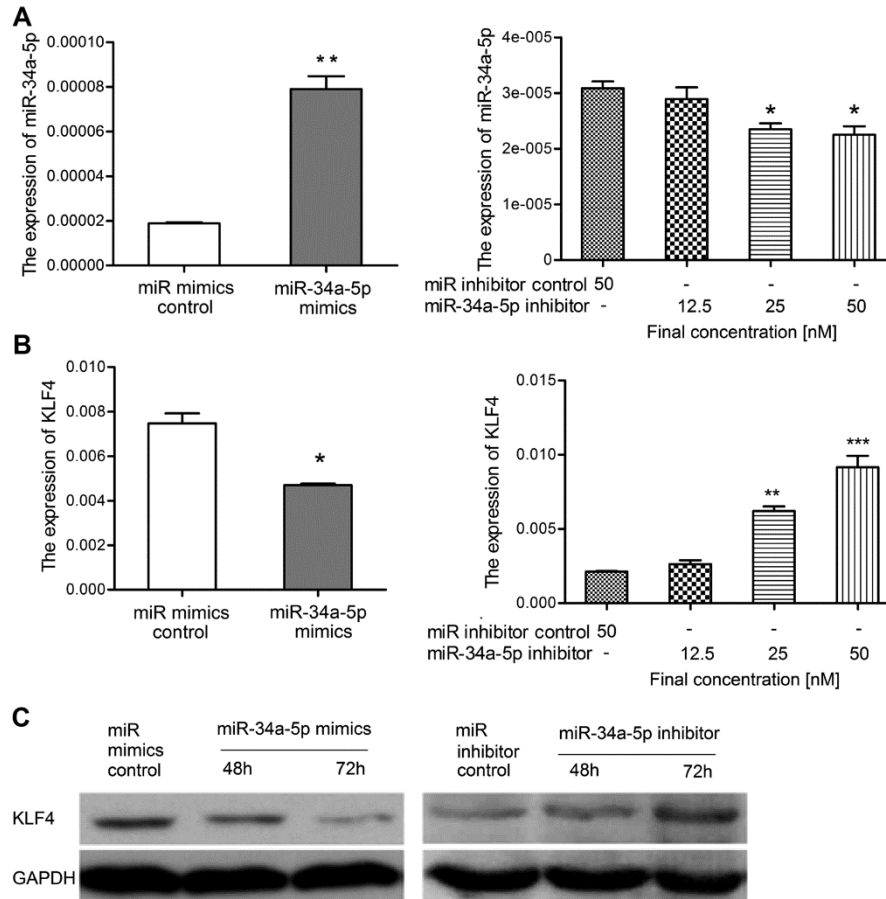
Fig. 2. KLF4 expression in BNL CL.2 cells after transfection of miR-34a-5p mimics or miR-34a-5p inhibitor. A – Manipulation of miR-34a-5p in BNL CL.2 cells. BNL CL.2 cells were transfected with miR-34a-5p mimics or inhibitor and the miR-34a-5p levels were measured via quantitative RT-PCR. B – BNL CL.2 cells were transfected with miR-34a-5p mimics or miR mimics control or transfected with miR-34a-5p inhibitor or miR inhibitor control. KLF4 mRNA expression was measured via quantitative RT-PCR. C – BNL CL.2 cells were transfected with miR-34a-5p mimics or miR mimics control, or transfected with miR-34a-5p inhibitor or miR inhibitor control. The KLF4 protein levels were measured by Western blot, and GAPDH expression was used for protein level normalization. Values are means ± S.D. (n = 3). *P < 0.05, **P < 0.01, ***P < 0.0001 compared with control group.

MiR-34a-5p is upregulated in BNL CL.2 cells undergoing apoptosis induced by X-ray radiation

To analyze the kinetics of miR-34a-5p expression in BNL CL.2 cells undergoing apoptosis induced by X-ray radiation, BNL CL.2 cells were exposed to X-rays for different lengths of time. BNL CL.2 cell apoptosis was detected with Annexin V/PI double staining and miR-34a-5p expression was quantified by qRT-PCR. As shown in Fig. 3A and B, treatment of BNL CL.2 cells with X-ray

radiation for 0 to 72 h resulted in a time-dependent induction of apoptotic cell death. With increasing apoptotic rate, miR-34a-5p was gradually upregulated, with a more than 3-fold increase in the miR-34a-5p amount observed at 48 h (Fig. 3C).
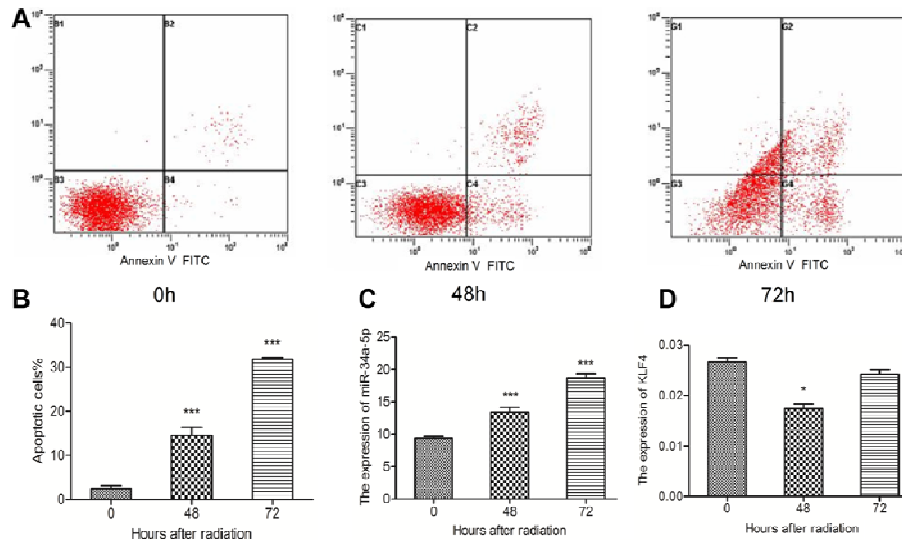

Fig. 3. Apoptotic effects and expression of miR-34a-5p in BNL CL.2 cells after X-ray exposure for 0, 48, and 72 h. A – Flow cytometric analysis of apoptosis. The lower left quadrants of each panel show the viable cells, which exclude PI and are negative for AV binding (AV−/PI−). The lower right quadrants and the upper right quadrants represent the apoptotic cells, which are AV positive and PI negative (AV+/PI−). B – Comparison of the apoptotic rate of BNL CL.2 cells. C – The relative expression level of miR-34a-5p in BNL CL.2 cells was analyzed using quantitative RT-PCR. D – The relative expression level of KLF4 in BNL CL.2 cells was analyzed using quantitative RT-PCR. Each bar represents the means ± S.D. (n = 3). *P <0.05, ***P < 0.0001 versus 0 h group.

The miR-34a-5p/KLF4 pathway modulates apoptosis in BNL CL.2 cells
To investigate the role of miR-34a-5p in BNL CL.2 cell apoptosis, we transfected BNL CL.2 cells with miR-34a-5p miRNAs and then subjected them to X-ray radiation. FCM were used to analyze changes in apoptosis. The apoptotic rate was upregulated in the presence of X-ray radiation. However, this effect was attenuated when the miR-34a-5p inhibitor was introduced into X-ray-stimulated BNL CL.2 cells (Fig. 4B). On the other hand, miR-34a-5p mimics increased the apoptosis of BNL CL.2 cells nearly two-fold compared to the apoptosis rate seen for the miRNA mimics control (Fig. 4A). The apoptotic rate increased up to 1.3-fold when miR-34a-5p mimics were introduced into X-ray-stimulated BNL CL.2 cells (Fig. 4C). In this study, lentiviruses were used to silence KLF4 expression. The disrupted KLF4 expression resulted in a significant increase in the percentage of apoptosis in X-ray-stimulated
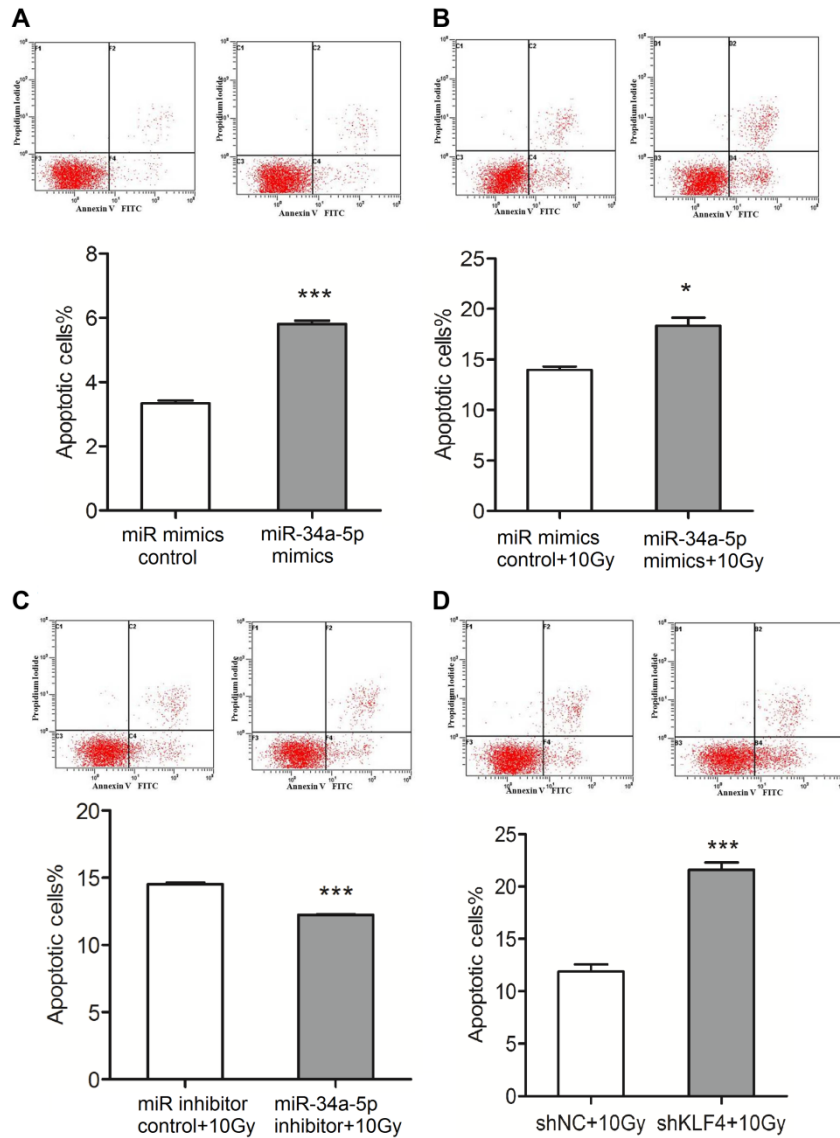
Fig. 4. Comparison of the apoptotic death rate of BNL CL.2 cells. A – Apoptosis in BNL CL.2 cells transfected with miR-34a-5p mimics. B – BNL CL.2 cells were transfected with miR-34a-5p mimics or miR mimics control, and further exposed to X-rays (10 Gy) for an additional 48 h. C – BNL CL.2 cells were transfected with miR-34a-5p inhibitor or miR inhibitor control, and further exposed to X-rays (10 Gy) for an additional 48 h. D – BNL CL.2 cells were transfected with sh KLF4 or sh NC, and further exposed to X-rays (10 Gy) for an additional 48 h. Quantitative measurement of apoptosis was performed by FCM after AV/PI double staining. The lower left quadrants of each panel show the viable cells, which exclude PI and are negative for AV binding (AV−/PI−). The lower right quadrants represent the apoptotic cells, which are AV positive and PI negative (AV+/PI−). Values are means ± S.D. (n = 3). *P < 0.05, ***P < 0.0001 compared with control group.

BNL CL.2 cells compared with the control group (Fig. 4D). The miR-34a-5p over-expression and inhibition data suggest that miR-34a-5p contributes to the apoptotic effect of X-ray radiation in BNL CL.2 cells by targeting KLF4.

We selected doxorubicin to repeat the experiment with a different pro-apoptotic agent. The results showed that the apoptotic rate was attenuated when miR-34a-5p inhibitor was introduced into doxorubicin-stimulated BNL CL.2 cells (Fig. 5). Furthermore, the apoptotic rate increased when miR-34a-5p mimics were introduced into doxorubicin-stimulated BNL CL.2 cells. The disrupted KLF4 expression resulted in a significant increase in the percentage of apoptosis in doxorubicin-stimulated BNL CL.2 cells compared with the control group, and the increase could be reduced when miR-34a-5p inhibitor was introduced. The miR-34a-5p over-expression and inhibition data suggest that miR-34a-5p contributes to the apoptotic effects of X-ray radiation and doxorubicin in BNL CL.2 cells by targeting KLF4.
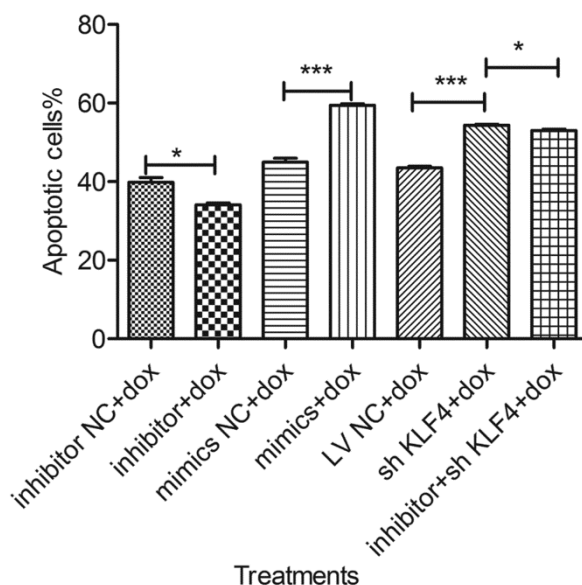

Fig. 5. Comparison of the apoptotic rate induced in BNL CL.2 cells exposed to doxorubicin. Values are means ± S.D. (n = 3). *P < 0.05, ***P < 0.0001 compared with control group.

DISCUSSION

Radiation can induce significant cellular damage, so the development of radioprotectants for medical and biodefense applications is very important [25-27]. Cells respond to DNA damage from X-ray irradiation either by activating cell cycle checkpoint and repair machinery or by triggering apoptosis. The damage to the DNA is believed to result from the production of free radicals [28]. Apoptosis is a self-destruct process that prevents cells from perpetuating mutations that might be harmful to the whole organism.

MicroRNAs are a family of 22-nt endogenous non-coding RNAs. They have been identified in organisms ranging from nematodes to humans [29]. MicroRNA attracted attention because it plays a crucial role in human disease and injury and could be a potential new therapeutic target [30, 31]. Studies show that miRNAs play pivotal roles in diverse processes such as cell development, differentiation and proliferation, the stress response, apoptosis, and cancer [1, 2]. Given the critical role miRNAs play in tumorigenic processes and their disease-specific expression, they hold potential as therapeutic targets and novel biomarkers [32]. It is apparent that miRNAs are involved in controlling the biological pathways associated with ionizing radiation-induced stress responses. MicroRNAs involved in regulating the expression of genes inducing apoptosis and other specific genes have been proposed for use in inducing the apoptosis of radioresistant cancer cells [33]. However, very limited study has been done demonstrating the role of miRNAs in radioprotection *in vivo* and *in vitro*.

MiR-34a has been reported to have anti-proliferative potential and to play a role in the regulation of cell cycle transition from G1 to S. It is also induced by p53, which leads to apoptosis or cell cycle arrest [34, 35]. It was reported that silencing miR-34a with miR-34a-specific locked nucleotide analogues (LNAs) could reduce the apoptosis induced by DNA damage [35]. Computational analyses show that miR-34a has hundreds of target genes. KLF4 is one of the target genes of miR-34a and this is an important factor in mediating the apoptosis induced by DNA damage. KLF4 is transcriptionally activated by p53 following DNA damage [36] and causes cell cycle arrest at both the G1/S and G2/M boundaries [37, 38]. Specific miRNAs regulate both cell-cycle progression and apoptosis, which reveals a new layer of complexity in cell cycle regulation [39, 40]. It has been sufficiently demonstrated that miR-34a mediates the negative regulation of cellular apoptosis through the synergistic effects of multiple targets. The anti-apoptotic protein B-cell lymphoma-2 (BCL2) is downregulated by miR-34a in several cell types, which is consistent with a role for miR-34a in p53-mediated apoptosis [41]. MiR-34a promotes apoptosis by down-regulating many anti-apoptotic proteins. For example, it could promote cardiomyocyte apoptosis by negatively regulating aldehyde dehydrogenase-2 (ALDH2) [42]. The miR-34a expression levels in glioblastoma cells after irradiation at 30 and 60 Gy were 0.17- and 18.7-times the BCL2 and caspase-9 expression levels, respectively [43]. This suggests that apoptosis might be promoted by regulating the action of miRNAs, even in cells that have acquired radioresistance.

Here, we report that miR-34a-5p directly regulates the expression of KLF4, affecting apoptosis in BNL CL.2 cells exposed to radiation. Our results reveal that miR-34a-5p negatively regulates KLF4 expression and promotes apoptosis. This research provides insight into the involvement of miR-34a in the mechanism of apoptosis induced by radiation and doxorubicin.

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