

# Regulation Effect of miR-34a Expression on Radiosensitivity of Lung Adenocarcinoma Cells by Targeting Bcl-2 and CDK4/6 Signaling Pathways

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## Abstract

**Objective:** Radiotherapy has been widely used to treat lung cancer. However, non-small lung cancer cells are insensitive to radiation, diminishing their radiotherapy effects. Although the radiosensitivity of the non-small lung cancer cells was reported to be enhanced through regulating miR-34a, the regulation effects of miR-34a expression on radiosensitivity of lung adenocarcinoma cells through target genes CDK4, CDK6, CyclinD1, and Bcl-2/Bax have not been systematically investigated. **Methods:** In this study, we investigated the effect of miR-34a expression on the Bcl-2, CDK4, and CDK6 pathways in lung adenocarcinoma cells, to provide new insights into the sensitization treatment of lung cancer. We first studied the effect of miR-34a expression on H1299 and A549 cell activity. Then to investigate the mechanisms of radiosensitivity, we focused on apoptosis, cell cycle, and target genes. **Results:** We find that overexpression of miR-34a in lung adenocarcinoma cells inhibits cell activity, and improves radiosensitivity. Specifically, overexpression of miR-34a suppresses the expression of target genes CDK4, CDK6, CyclinD1, and Bcl-2/Bax, which leads to cell cycle arrest and promotes apoptosis of lung adenocarcinoma cells. **Conclusions:** Overall, our results demonstrate that the overexpression of miR-34a enhances the radiosensitivity of lung adenocarcinoma cells, indicating that miR-34a is a sensitizer for lung adenocarcinoma radiotherapy.

## Keywords

miR-34a, p53, Lung Adenocarcinoma Cells, Radiosensitivity, Cell Apoptosis, Cell Cycle

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## 1. Introduction

Lung cancer has been regarded as one of the main diseases of human beings. Radiotherapy has been widely used in lung cancer treatment. Cells exposed to ionizing radiation will cause DNA damage directly or indirectly, which activates p53 and induces a series of other reactions, like cell cycle arrest, senescence, cell apoptosis, and cell proliferation inhibition [1] [2] [3] [4]. During this chain process, p53 plays a controlling role and p53 mutant cancer cells respond poorly to radiation [5] [6] [7] [8]. Numerous studies have reported that microRNAs (miRNAs) acted as p53 target genes and were directly regulated by p53 [9] [10] [11] [12]. Among the p53-regulated miRNAs, the miR-34 family (miR-34a, miR-34b, and miR-34c) is considered as the most obvious one regulated by p53 [13] [14] [15] [16] [17]. However, the inactivation of miR-34a has been identified in many types of cancers (lung, pancreatic, colon, and breast cancers) [18]-[28]. In addition, over-expression of miR-34a induces cell cycle arrest, cell proliferation inhibition, senescence, and apoptosis [29]-[33].

Due to the radiation tolerance of non-small cell lung cancer (NSCLC), the efficacy of radiotherapy is usually unsatisfactory. Several studies have investigated the effect of miR-34a expression on the radiosensitivity of a variety of tumors [29] [34]. miR-34a also regulates a variety of target mRNAs, such as cyclin-dependent kinase 4/6 (CDK4/6), E2F transcription factor 3 (E2F3), Cyclin E2, B-cell lymphoma 2 (Bcl-2), NAD-dependent deacetylase sirtuin-1 (SIRT1), Notch, and CD44 [35] [36]. Specifically, experimental results have shown that miR-34a can improve the radiation sensitivity of NSCLC cells by regulating target genes such as LyGDI [29], Notch-1 [37], and RAD51 [38].

Among all types of NSCLC, lung adenocarcinoma is found to be one of the most insensitive kinds to radiotherapy, which renders it an acritical research topic for future application of radiotherapy on lung cancer. Although a previous study has demonstrated that ectopic expression of miR-34a enhances radiosensitivity and promotes apoptosis by suppressing the LyGDI pathway in NSCLC cells [29], little is reported regarding the radiosensitivity of lung adenocarcinoma in Bcl-2 and CDK4, CDK6 pathway signaling. Therefore, we aimed to investigate the effect of the expression of miR-34a on the Bcl-2, CDK4, and CDK6 pathways in lung adenocarcinoma cells, to provide new insights into the sensitization treatment of lung cancer.

## 2. Materials and Methods

### 2.1. Cell Lines, Cell Culture, and Irradiation

Normal lung tissue RNA, human lung adenocarcinoma cell lines A549 (p53+/+) and H1299 (p53-/-) were prepared by the cell center of the Cancer Hospital Chinese Academy of Medical Sciences. After being cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA), in a humidified chamber stabilized at 37°C and under 5% CO<sub>2</sub>, cells were treated with different 60Co  $\gamma$ -ray irradiation doses (2, 4, 6, and 8 Gy) at the dose rate of 0.5

Gy/min.

## 2.2. Transfection

Pre-miR-34a was transiently transfected into H1299 cells, and anti-miR-34a was transiently transfected into A549 cells. Briefly, 15  $\mu$ l of pre-miR-34a, anti-miR-34a, or negative control (ABI, USA) in 100  $\mu$ l of serum-free RPMI1640 medium were mixed with 5  $\mu$ l of siPORT transfection reagent (Invitrogen, USA) in 100  $\mu$ l of the same medium, and allowed to stand at room temperature for 10 min. 2.3 ml of cell suspension was added to the mixture, and transfection efficiency could be detected after incubation for 8 - 72 h.

## 2.3. Real-Time PCR

Total RNA was extracted using Trizol agent (Invitrogen, USA). The TaqMan miRNA reverse transcription kit (ABI, USA) was used to reverse-transcribe RNA into cDNA. 7500 fast real-time PCR analyzer (ABI, USA) was used to detect expression levels of RNA. Real-time PCR reactions: 95°C, denaturation for 10 min; 95°C, denaturation for 15 s, Reaction at 60°C for 1 min, 40 cycles for amplification.

## 2.4. Western Blot Analysis

Cells were harvested, lysed by cell lysate, and then transferred to NC membranes. The membranes were probed with a primary antibody (Nanjing Ba aode Biotechnology Company, China), followed by a peroxidase-conjugated secondary antibody (Beijing Zhongshan Golden Bridge Biotechnology Company, China). Immunoreactive proteins were visualized using the ECL Plus reagent (Beijing Pu lilai Gene Technology Company, China).

## 2.5. CCK-8 Cell Activity Assay

Cells were seeded in 96-well plates, every group with five wells, each well was added 100  $\mu$ l of cell suspension, and the culture plate was placed in an incubator (37°C, 5% CO<sub>2</sub>) for 1 - 4 h. Each well was added 10  $\mu$ l of CCK-8 solution (Beijing Tongren Chemical Company, China), incubated for 1 - 4 h at 37°C, and measured with a microplate reader at absorbance values of 450 nm.

## 2.6. Apoptosis Analysis

Cells ( $1 \times 10^5$ ) were harvested at 24 h after transfection and 48 h after irradiation, and suspended in 500  $\mu$ l of Binding Buffer, then stained with 5  $\mu$ l of Annexin V-FITC and 5  $\mu$ l of the PI (Nanjing KGI biotech companies, China), and kept in a dark place 15 min before being analyzed by flow cytometry.

## 2.7. Cell Cycle Analysis

Cells ( $1 \times 10^5$ ) were harvested at 24 h after transfection and 48 h after irradiation, and fixed with paraformaldehyde for 2 h. Cells were stained in 500  $\mu$ l of the

PI solution containing RNA enzyme (0.1  $\mu\text{g}/\text{ml}$ ) (Promega, USA), then allowed to stand in a dark place at room temperature for 30 min, and analyzed using flow cytometry.

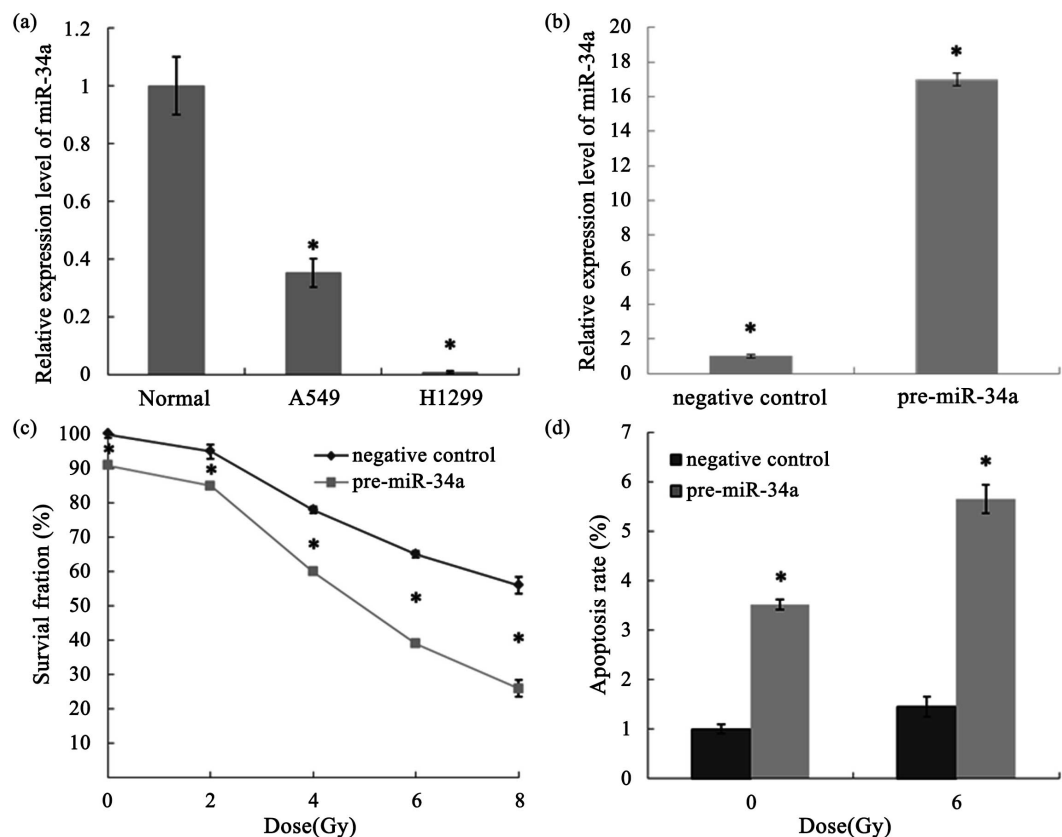
## 2.8. Statistical Analysis

Data of the two comparing groups were analyzed by the Student's t-test using the SPSS, version 19.0 and values less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. Restoration of miR-34a Expression on H1299 Cell Activity

As shown in **Figure 1(a)**, when being compared to normal lung tissue RNA, miR-34a expression levels of A549 and H1299 cells both decreased. The expression of miR-34a between A549 (p53+/+) and H1299 (p53-/-) cells also had significant differences. Several studies have reported the low expression of miR-34a in H1299 and A549 cells [13] [14] [15] [16] [17], which was confirmed by our results in **Figure 1(a)**.



**Figure 1.** Effects of miR-34a on cell activity and apoptosis. (a) miR-34a expression in A549 and H1299 lung adenocarcinoma cells (\* $P < 0.05$ ); (b) Transfection efficiency of pre-miR-34a in H1299 cells (\* $P < 0.05$ ); (c) Restoration of miR-34a expression on the activity of H1299 cells, with cell activity determined at 48 h after irradiation (0, 2, 4, 6, and 8 Gy) by CCK-8 kit (\* $P < 0.05$ ); and (d) Cell apoptosis in H1299 cells at 48 h after 6 Gy irradiation (\* $P < 0.05$ ).

Since miR-34a showed obviously reduced expression in H1299 cells, we restored miR-34a expression in H1299 to observe cell proliferation. Pre-miR-34a or negative control miRNA (NC) was transfected into H1299 cells, and transfection efficiency was detected by the flow cytometry, which was 93%. Real-time PCR detection results showed that the expression of miR-34a in the pre-miR-34a transfection group was significantly increased ( $P < 0.01$ ) up to 17 times (shown in **Figure 1(b)**), when compared with the negative control transfection group. Therefore, these results demonstrate a significant increase in miR-34a expression in miR-34a transfection.

Cell activity of H1299 cells was detected by the CCK-8 kit, which was shown in **Figure 1(c)**. As expected, H1299 cells transfected with pre-miR-34a showed dose-dependent growth inhibition after different doses of  $^{60}\text{Co}$   $\gamma$ -ray irradiation. These results suggest that restoration of miR-34a expression enhanced the radiosensitivity of H1299 cells.

### 3.2. Effect of miR-34a Up-Regulation on Apoptosis and Cell Cycle of H1299

To further investigate the mechanism of miR-34a on radiosensitivity of H1299 cells, we performed cell apoptosis and cell cycle analyses, and the results were shown in **Figure 1(d)** and **Table 1**.

When compared to NC transfected group, the cell apoptosis rate in pre-miR-34a transfected group increased significantly. **Table 1** showed that, compared to NC transfected group, G0/G1 arrested and S phase decreased in pre-miR-34a transfected group. Since CDK4, CDK6, and CyclinD1 are proved to be cell cycle-related target genes of miR-34a, and Bcl-2 and Bax are apoptosis-related target genes, we focused on CDK4, CDK6, CyclinD1, Bcl-2, and Bax, as targets of miR-34a. As shown in the results of real-time PCR, transcript levels of CDK4, CDK6, and Bcl-2/Bax in the pre-miR-34a transfected group were significantly lower than those for the NC transfected group. The results were further confirmed by western blot analysis. Protein levels of Bcl-2, CDK4, and CDK6 in the pre-miR-34a transfected group were significantly lower than those for the NC transfected group. These results indicated that miR-34a suppressed its target genes expression, enhanced radiation-induced apoptosis and cell cycle arrest (**Figure 2(a)** and **Figure 2(b)**).

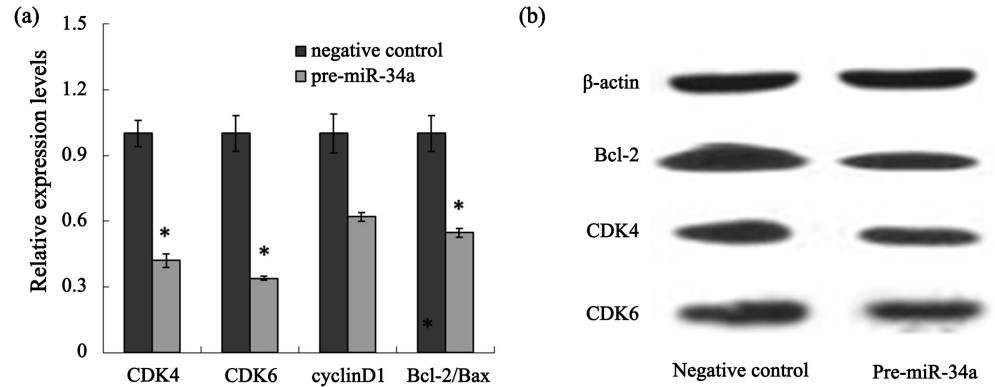
### 3.3. Effect of Inhibition of miR-34a Expression on Radiosensitivity of A549 Cells

In order to verify the impacts of miR-34a expression on H1299 cells, we inhibited the expression of miR-34a in A549 cells to observe the cell proliferation of A549. Anti-miR-34a was transfected into A549 cells to observe cell activity, cell cycle, and apoptosis. When compared to the NC transfected group, cell activity in anti-miR-34a transfected group was significantly increased after irradiation (**Figure 3(a)**), and the difference was a dose-dependent increase.

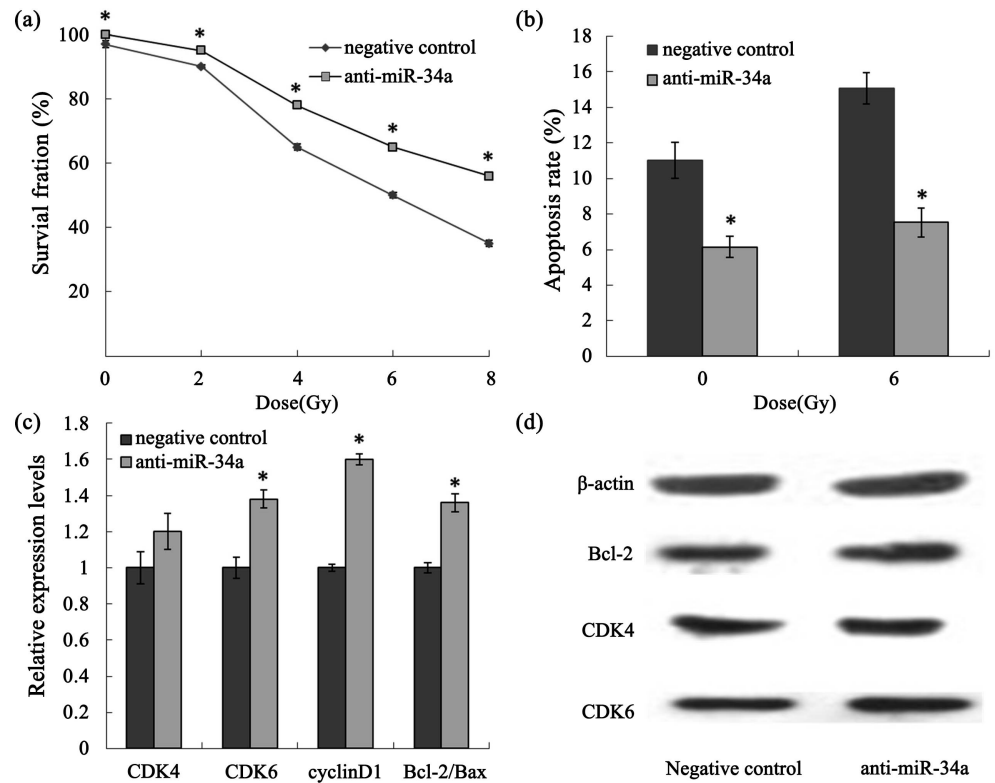
**Table 1.** Effects of the miR-34a regulation on cell cycle of H1299 (x ± s).

Groups	n	G0/G1 (%)	G2/M (%)	S (%)
Negative control	3	1.82 ± 0.29	13.13 ± 1.26	85.05 ± 2.89
Pre-miR-34a	3	17.21 ± 2.17 *	10.50 ± 2.36	76.79 ± 2.05*

Note: compared to the NC transfected group, \*P < 0.05.



**Figure 2.** Influence of miR-34a up-regulation on expression of target genes. Pre-miR-34a and negative control transfected cells were irradiated at 6 Gy, and the cells were collected at 48 h after irradiation. (a) Realtime PCR to detect the target genes (\*P < 0.05); (b) Western blot to detect the targets.



**Figure 3.** Effect of transfection with anti-miR-34a on A549 cells. (a) inhibition of miR-34a expression on the activity of A549 cells (\*P < 0.05). (b) Anti-miR-34a transfection on apoptosis of A549 cells (\*P < 0.05). (c) Realtime PCR to detect the target genes (\*P < 0.05); (d) Western blot to detect the targets.

As shown in **Figure 3(b)**, the rate of cell apoptosis in the anti-miR-34a transfected group was significantly lower. **Table 2** showed that the percentage of the G0/G1 phase in anti-miR-34a transfected cells significantly reduced, and the S phase increased significantly. The target gene (CDK6, CyclinD1, and Bcl-2/Bax) expressions of A549 cells increased significantly in anti-miR-34a transfected cells, as shown in Figures 3C and 3D. These results suggested that down-regulation of the miR-34a expression can reduce the radiosensitivity of A549 cells, which was mutual verification with the results of H1299 cells.

#### 4. Discussion

The key to tumor radiotherapy is reducing the radioresistance and improving radiosensitivity of tumors which are associated with miRNAs levels [39]. Among various miRNAs, miR-34a played an important role as predictive biomarker of tumor radiation [31]. In addition, the miR-34 family has been identified as a target of p53 and a potential tumor suppressor that plays a role downstream of the p53 pathway [13] [14] [15] [16] [17]. More than 50% of cancers, including NSCLC, have a p53-mutant and a low level of miR-34 expression [40]. In the present study, we also observed low levels of expression of miR-34a in lung adenocarcinoma cells, especially in p53-mutant cells H1299, which was consistent with previous studies. Our experimental results proved that miR-34a is an important molecule of lung adenocarcinoma cells.

Although ectopic expression of miRNAs has been frequently investigated for various types of cancer, the current cognition about the role of miRNAs on lung adenocarcinoma cells and corresponding mechanisms is still limited. Therefore, it is essential for promising the therapeutic effect of lung adenocarcinoma to identify the valuable miRNAs and targets. Previous studies have emphasized the key role of miR-34a as a radiosensitizer. Balca-Silva's study showed that overexpression of miR-34b in NSCLC cells increased radiosensitivity at low doses of radiation [41]. Liu *et al.* [2011] found that miR-34a played a key role in increasing radiosensitivity in different tissues by promoting cell apoptosis and decreasing cell viability [34]. Cortez *et al.* [2015] identified RAD51 as a new direct target of miR-34a to increase radiosensitivity in NSCLC cells [38]. Here, we found that overexpression of miR-34a inhibited cell activity of H1299. We further revealed that restoration of miR-34a enhanced the radiosensitivity of H1299 cells, suggesting that target genes of the miR-34a were sufficient to inhibit the cell activity of H1299. To investigate the mechanisms of radiosensitivity, we focused on apoptosis, cell cycle, and target genes. We profiled 5 genes from the cell cycle and apoptosis pathways after enforced overexpression of miR-34a in lung adenocarcinoma cell lines.

It is well known that exposure to ionizing radiation causes changes in the cell cycle. The changes at each stage of the cell cycle are a direct response to external injury factors. Among the various phases of the cell cycle, G1 is critical for regulating the cell cycle, providing enough time to promote damaged DNA repair. It has been previously shown that CyclinD1, CyclinE2, CDK4, and CDK6 are

**Table 2.** Effects of anti-miR-34a transfection on cell apoptosis in A549 ( $x \pm s$ ).

groups	n	G0/G1 (%)	G2/M (%)	S (%)
Negative control	3	37.59 $\pm$ 2.01	10.16 $\pm$ 1.22	52.25 $\pm$ 2.99
Anti-miR-34a	3	21.8 $\pm$ 0.98 *	12.83 $\pm$ 1.19	65.37 $\pm$ 5.09 *

Note: compared to the NC transfected group, \*P < 0.05.

important molecules in the G1 checkpoint [42]. Moreover, CyclinD1, CyclinE2, CDK4, and CDK6 have been shown to be miR-34 target genes [42]. However, whether miR-34a could directly target CDK4 and CDK6 in lung adenocarcinoma is unclear. Our study showed that overexpression of miR-34a blocked in G0/G1 and G2 phases, and CDK4, CDK6, and CyclinD1 were all down-regulated in lung adenocarcinoma cells. These results indicated that miR-34 plays an important role in regulating the cell cycle, thereby enhancing the radiosensitivity of lung adenocarcinoma cells.

Apoptosis is a strictly controlled process of multiple genes, including the Bcl-2 family, caspase family, Bax, oncogenes such as C-myc, and tumor suppressor gene p53. Studies have shown that regulating the expression of the Bcl-2 family can inhibit cell proliferation and promote apoptosis [43] [44]. Bcl-2 expression is decreased by knockdown in brain glioblastoma and colon cancer cell lines, the apoptosis is enhanced after irradiation and the radio-protective role of miR-34a inhibitor is attenuated [34]. In different types of cancer, it has been reported that the expression of anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax is negatively regulated by miR-34a. Accordingly, our results showed that the overexpression of miR-34a increased apoptosis, inversely correlated with Bcl-2/Bax ratio. This is different from the effect of miR-34b on H1299 cell apoptosis and Bcl-2 level [41], suggesting the complex mechanism of the miR-34 family's influence on the radiosensitivity of tumor cells. Our results verified that miR-34a can promote cell apoptosis by regulating the expression of target genes, which is consistent with the previous results.

It is worth noting that the apoptosis rate of lung adenocarcinoma cells after irradiation increased, but the increase in apoptosis rate caused by overexpression of miR-34a was much smaller. The reason is that lung adenocarcinoma cells are not sensitive to  $\gamma$ -rays, and even larger doses of  $\gamma$ -rays cannot have a greater impact, indicating that lung adenocarcinoma cells have lower radiation sensitivity. Restoring the expression of miR-34a, the apoptosis rate of lung adenocarcinoma cells was significantly increased, which indicated that miR-34a can enhance the radiosensitivity of lung adenocarcinoma cells, promote cell apoptosis, and have a synergistic effect with the pro-apoptotic effect of  $\gamma$ -rays. Therefore, the combined effects of overexpression of miR-34a and  $\gamma$ -rays provide a new understanding of the treatment of lung adenocarcinoma.

## 5. Conclusion

In conclusion, our experimental study verified both positive and negative sides,



demonstrating that miR-34a could increase the radiosensitivity of lung adenocarcinoma cells by targeting the expression of CDK4, CDK6, CyclinD1, and Bcl-2/Bax. Altogether, this study suggested that miR-34a is a sensitizer for lung adenocarcinoma radiotherapy. To properly apply the treatment of lung adenocarcinoma, further experiments are needed to study samples in clinical cancer radiotherapy.

### Authors' Contributions

All authors were involved in the design and implementation of the experiment. Manuscripts have been reviewed and approved by all authors.

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### Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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