

Downregulation of AATK mediates microRNA-558-induced resistance of A549 cells to radiotherapy

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Abstract. The deregulation of microRNAs (miRNAs) is often implicated in the control of sensitivity to radiotherapy. The objective of the present study was to identify the association between miR-558 and apoptosis-associated tyrosine kinase (AATK), and their importance in regulating the development of resistance to radiotherapy. The current study demonstrated that AATK, a radiosensitization-associated gene, is a target of miR-558 in lung cancer cells, using *in silico* analysis and a luciferase reporter system. Furthermore, it was determined that transfection of 30 or 50 nM miR-558 mimics and AATK specific siRNA markedly suppressed the mRNA and protein expression of AATK. To determine whether miR-558 was required for lung cancer cell radioresistance, A549 cells were treated with different doses of ionizing radiation, from 0 to 10 Gy, following transfection with miR-558 mimics or AATK specific siRNA. It was determined that the administration of miR-558 mimics or AATK specific siRNA alone did not significantly alter the survival rate of the cells. By contrast, in the cells exposed to 4, 6 or 8 Gy, the administration of miR-558 mimics or AATK specific siRNA significantly promoted cell survival rate and overexpression of AATK reversed this effect. In conclusion, these data demonstrate that the miR-558/AATK cascade is important for the radiosensitization of lung cancer cells and may be a potential radiotherapy target.

Introduction

Greater than 1,600,000 new cases of lung cancer occur each year worldwide. Lung cancer accounts for greater than 1,370,000 deaths annually and has become the leading cause of cancer-associated mortality. In China, lung cancer is the most common malignancy, and the incidence and mortality rates, based on ages, were higher compared with the worldwide

average (1). Among the various subtypes of lung cancer, non-small cell lung cancer (NSCLC) is the most common and accounts for 80% of lung cancer mortality (2). The 5 year overall survival rate for patients with NSCLC is less than 15% (3). Nearly 40% of patients with confirmed NSCLC have stage-III diseases when admitted to the hospital (4).

With the development of new technology and improved understanding of the underlying molecular mechanisms of the disease, the management of NSCLC has been greatly improved over the recent decades (5,6). However, it is currently difficult to manage patients with NSCLC. Radiotherapy has been considered to be the primary treatment for NSCLC. Unfortunately, the effectiveness of radiotherapy is often limited by resistance (2,7). Increasing evidence from clinical studies indicates that the response to radiotherapy varies between patients, ranging from complete recovery to relapse and distant metastasis due to resistance to radiotherapy. Additionally, normal tissues are exposed to inevitable toxic effects as a result from increasing radiation doses for treatment of radioresistant malignant cells (8-10). Resistance to radiotherapy is common in NSCLC cells (11), leading to induction of the local relapse of NSCLC (12,13). Radioresistance is most likely the result of tumor heterogeneity regarding cell of origin, etiology, pathology, and genetic/molecular pathogenesis (14). Therefore, it is necessary to develop new therapeutic measures for the treatment of NSCLC, including targeted gene therapy as a radiosensitizer in order to treat the disease and increase the survival rate of patients.

MicroRNAs (miRNAs) are small endogenous single-stranded non-coding RNA molecules. They act as a negative regulator of gene expression at the post-transcriptional level by either inhibiting translation of mRNA into functional protein or accelerating the degradation of the target mRNA (15). According to Sanger miRBase, greater than 2,000 miRNAs have been identified in the human genome. They have been reported to be essential in the control of various features in cancer, including the response to chemotherapy and radiotherapy by regulating apoptosis or cell cycle (16). Apoptosis-associated tyrosine kinase 1 (AATK1), also known as lemur kinase 1, was first identified as a protein that was significantly increased during final differentiation and apoptosis of 32Dcl3, which is a non-tumorigenic mouse myeloid cell line (17). AATK is a key regulator in the control of apoptosis, which mediates the toxicity of radiotherapy towards cells (18). Previous reports determined that AATK was negatively associated with the activity of SRC proto-oncogene,

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non-receptor tyrosine kinase (Src) in lung cancer (19), and Src is key in the occurrence of radiotherapy resistance in NSCLC (20). AATK has been determined to be subject to the regulation by miRNAs (21). The present study explored potential miRNAs that regulate the expression of AATK and their importance in the control of apoptosis induced by radiation exposure.

Materials and methods

Cell culture. The A549 lung cancer cell line was obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China), and incubated in Dulbecco's modified Eagle's medium supplemented with 100 μ g/ml streptomycin, 100 U/ml penicillin and 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in an atmosphere of 5% CO₂ in a humidified incubator. Cells the logarithmic phase of proliferation were used for the transfection.

Cell transfection. For the transfection, 1x10⁶ cells were treated with miR-558 mimics, anti-AATK siRNA or the scramble sequence (Shanghai GenePharma Co., Ltd., Shanghai, China). The transfection was performed using 1 μ l Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as specified by the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA was extracted using a RNeasy Mini kit (Qiagen, Hilden, Germany). Equal amounts of total RNA (30 ng) from each treatment group (treated with either miR-558 mimics, anti-AATK siRNA or scramble) were used for cDNA synthesis using miRNA specific primers purchased from Applied Biosystems (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The PCR cycles were as follows: 38°C for 15 min, 85°C for 5 sec for reverse transcription, followed by 40 cycles of 95°C for 10 sec, 95°C for 5 sec and 61°C for 20 sec. A TaqMan gene expression assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to determine the relative expression levels of miR-558, AATK and U6 (used as an internal control). RT-qPCR was conducted for 40 cycles in standard mode on the ABI 7900 HT Thermal cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction conditions were as follows: 40 cycles for 10 min at 50°C, 10 min at 95°C, 15 sec at 95°C and 45 sec at 60°C. The 2^{- $\Delta\Delta$ C_q} method was used to calculate the relative miRNA expression values (22).

Western blot analysis. Following lysis in lysis buffer [150 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM EGTA, 0.1 mM EDTA, 1% Triton X-100, 2 mM NaF, Complete Protease Inhibitor Mix and 2 mM sodium orthovanadate (Roche Diagnostics GmbH, Mannheim, Germany)] for 20 min on ice, cells were centrifuged at 16,000 x g at 4°C. A 10% SDS-PAGE gel was used to resolve proteins (30 μ g). Next, proteins were transferred onto nitrocellulose membranes. Subsequently, 5% nonfat dry milk in Tris-buffered saline Tween-20 (100 mM NaCl, 0.05% Tween-20 and 10 mM Tris-HCl pH 7.5) was used to block proteins to avoid non-specific binding. Following incubation with a primary anti-AATK antibody (1:5,000; cat. no. 87141; Cell Signaling Technology, Inc., Beverly, MA, USA) and anti- β -actin (1:10,000; cat. no. 3700; Cell Signaling Technology,

Inc.) for 12 h at 4°C, the blots were washed and cultured with horseradish peroxidase-conjugated secondary antibodies (1:12,000; cat. no. sc-53322; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h at 37°C. The proteins were visualized using an ECL chemiluminescence kit (GE Healthcare Life Sciences, Chalfont, UK) according to the manufacturer's protocol and exposed to X-ray film. The signal was determined using an Odyssey scanner (LI-COR Biosciences, Lincoln, NE, USA) and signals were normalized against that of β -actin.

Ionizing radiation. At 48 h after the transfection, γ -ray ionizing radiation (IR) from a ⁶⁰Co source was used to radiate sub-confluent cell monolayers at a rate of 2, 4, 6 and 8 Gy/min.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (200 μ l) were plated on a 96-well microtiter plate at a density of 5x10⁴ cells/ml 24 h prior to IR. Three days following IR, each well was supplemented with 10 μ l MTT reagent (Sigma-Aldrich St. Louis, MO, USA) and incubated at 37°C for 4 h. The supernatants were removed and 100 μ l dimethyl sulphoxide was added to terminate the reaction and the plates were mixed for 10 min. The absorbance was determined at 490 nm. Each experiment was repeated three times and the mean was calculated.

Plasmid construction. Human genomic DNA was used to amplify the 3'-UTR region of AATK using a TaqMan gene expression kit (Applied Biosystems), according to the manufacturer's protocol. Following amplification, it was inserted into the pmirGLO vector (Promega Corporation, Madison, WI, USA) next to the luciferase gene to establish a luciferase reporter plasmid (pmirGLO-WT-AATK-3'UTR). A QuikChange Site-Directed Mutagenesis kit (Promega Corporation) was used to perform site-directed mutagenesis of the miR-558 target site in the AATK 3'-UTR with pmirGLO-WT-AATK-3'UTR as the template, resulting to a mutant-type reporter plasmid (pmirGLO-MUT-AATK-3'UTR). In addition, the coding sequence of AATK was amplified and subcloned into pcDNA3.0 (Invitrogen; Thermo Fisher Scientific, Inc.) to make the construct (pcDNA3-AATK) for the purpose of ectopic overexpression.

Dual-luciferase reporter assay. A549 cells were seeded in 96-well plates at a density of 1x10⁵ cells/well and transfected with 200 ng luciferase reporter plasmid (pmirGLO-WT-AATK-3'UTR or pmirGLO-MUT-AATK-3'UTR; Promega Corporation), 50 nM mimics, scrambled microRNA or miR-558 using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were collected 48 h following transfection, and a dual-luciferase reporter assay (Promega Corporation) was used to determine luciferase activity.

Apoptosis. Flow cytometry was used to measure apoptosis using the Apoptest-FITC Kit with annexin V (Dako, Glostrup, Denmark) as specified in the manufacturer's protocol. Flow cytometry (LSR II, BD Biosciences, San Jose, CA, USA) was used to assess the proportion of annexin V-positive cells.

Online miRNA database search. The present study searched the online miRNA databases, including TargetScan (www.targetscan.org) and miRNA database (www.mirdb.org) to

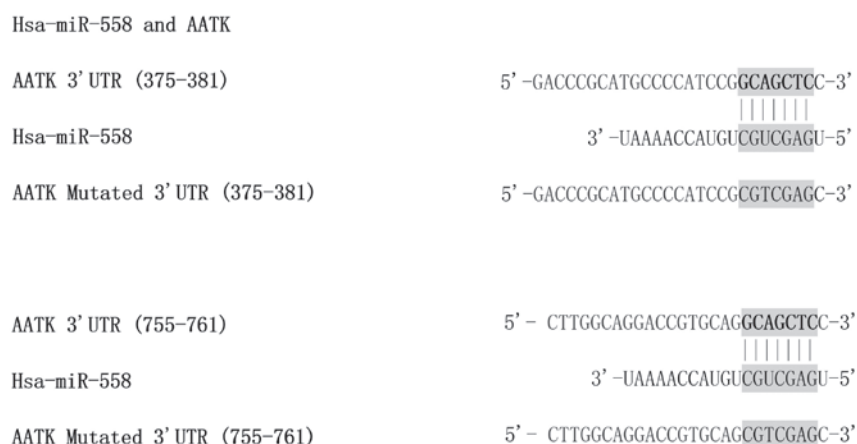


Figure 1. The 3'-UTR of AATK is targeted by miR-558 with potential two 'hits' in the 3'UTR of AATK. 3'UTR, 3' untranslated region; AATK, apoptosis-associated tyrosine kinase; miR, microRNA.

predict the virtual target gene of the miRNA with the potential binding site (at least 7 consecutive complementary bases) in the 3'UTR of target mRNA.

Statistical analysis. SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) was used to conduct statistical analysis. The results were presented as the mean \pm standard deviation based on three independent experiments. One way analysis of variance was used for statistical analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

AATK is a direct target of miR-558. The relationship between the AATK-3'-UTR and the miRNAs targeting it was predicted through bioinformatics analysis by miRanda, which indicated that the 3'-UTR of AATK may be targeted by miR-558 with two potential 'hits' in the 3'UTR of AATK (Fig. 1). To study the interaction between AATK and miR-558, the full length of the AATK 3'UTR containing potential miR-558 binding sites or the mutants was selected for further validation using luciferase reporter assays. miR-558 mimics significantly reduced the luciferase expression in the A549 cells co-transfected with the wild type AATK 3'UTR (Fig. 2). When the binding site (375-381 bp) was mutated, the suppression of the luciferase activity was reversed, while introduction of mutations into another binding site (755-761 bp) had minimal effects (Fig. 2). These assays demonstrated that AATK is one of the target genes of miR-558, and that the 375-381 bp region, instead of the 755-761 bp region of the AATK 3'UTR is the binding site of the miRNA.

miR-558 is a regulator of AATK. To test the hypothesis that miR-558 is a direct regulator of AATK, western blotting and RT-qPCR were used to analyze differences in AATK expression in the A549 cells transfected with scrambled control and miR-558 mimics. As presented in (Fig. 3A), transfection with 30 or 50 nM miR-558 mimics and AATK specific siRNA substantially suppressed the mRNA and protein expression levels of AATK. Transfection of the construct containing the coding sequence of AATK significantly increased the expression of AATK in A549 cells (Fig. 3B). The results further confirmed

the regulatory relationship between miR-558 and AATK in lung cancer cells, and the effect of miR-558 was specific as the scrambled miRNA did not exhibit such an effect.

miR-558 is required for radioresistance of lung cancer cells. To determine whether miR-558 was required for lung cancer cell radioresistance, A549 cells were treated with different doses of ionizing radiation, from 0 to 10 Gy, following transfection with miR-558 mimics or AATK-specific siRNA. IR treatment exhibited a dose-dependent inhibitory effect on the growth of the A549 cells (Fig. 4). In the A549 cells exposed to 2 Gy, the administration of miR-558 mimics or AATK specific siRNA did not significantly alter the survival rate of the cells (Fig. 4A). In contrast to the cells exposed to 4, 6 or 8 Gy, administration of miR-558 mimics or AATK specific siRNA significantly promoted the survival rate of the cells, with the overexpression of AATK reversing this effect (Fig. 4B-D). This suggests that the enhanced radioresistance of the A549 cells transfected with miR-558 may be due to the downregulation of AATK. Additionally, the effect of miR-558 promotion on IR-induced apoptosis in lung cancer cells was investigated. A549 cells transfected with miR-558 mimics and AATK siRNA were exposed to IR (4 Gy), and apoptosis measured in each group. This indicated that the introduction of miR-558 or anti-AATK siRNA significantly suppressed apoptosis in lung cancer cells, while ectopic overexpression of AATK partially but significantly reversed the effect on apoptosis (Fig. 5). This suggests that the increased radioresistance may have been due to a decreased rate of apoptosis resulting from downregulation of AATK expression. In addition, the effect of exposure to radiation on the expression of AATK and miR-558 was also evaluated, with the results not indicating alterations of the miRNA and AATK expression levels (data not shown).

Discussion

It has been established that resistance to radiotherapy leads to significantly elevated mortality and morbidity of patients with NSCLC, and it is a key issue in the treatment of NSCLC. Researchers have been seeking potent approaches to sensitize cases NSCLC that are often radioresistant.

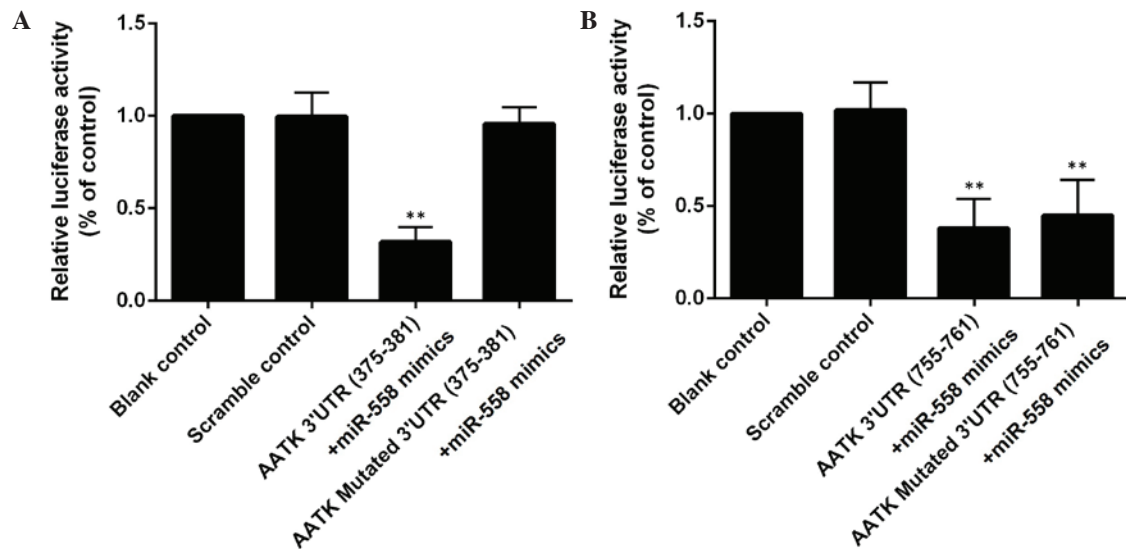


Figure 2. (A) miR-558 mimics significantly reduced luciferase expression in the cells co-transfected with the wild type AATK 3'UTR. When the 375-38 bp binding site was mutated, the effect of miR-558 mimics was prevented. (B) Introduction of the mutations into another binding site (755-761 bp) had minimal effects. Data are presented as the mean \pm standard deviation (** $P < 0.01$). miR, microRNA; AATK, apoptosis-associated tyrosine kinase; 3'UTR, 3' untranslated region.

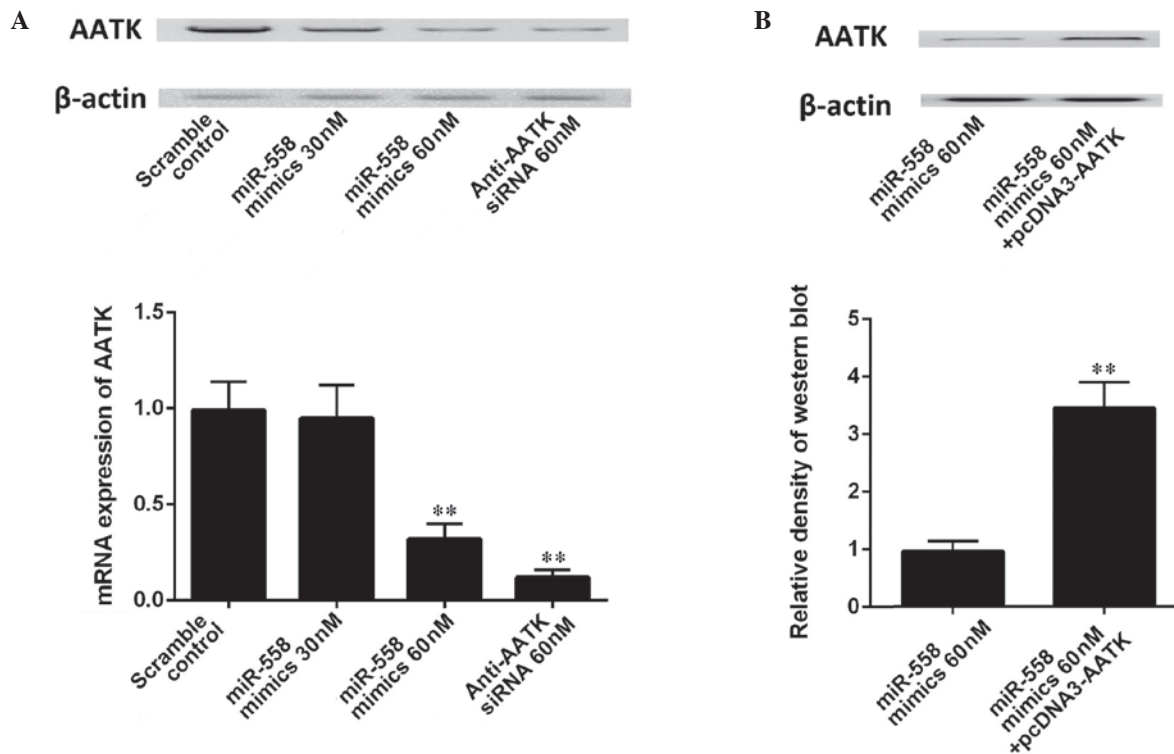


Figure 3. (A) Transfection with 30 or 50 nM miR-558 mimics and AATK specific siRNA significantly suppressed the mRNA and protein expression of AATK. (B) Transfection with the construct containing the coding sequence of AATK significantly increased the expression of AATK in A549 cells. Data are expressed as the mean \pm standard deviation (** $P < 0.01$). miR, microRNA; AATK, apoptosis-associated tyrosine kinase; siRNA, small interfering RNA.

AATK was identified to be significantly increased during final differentiation and apoptosis of 32Dcl3 cells, a non-tumorigenic mouse myeloid cell line (23). High expression of AATK predominantly occurs in different regions of the brain and it is detected in other tissues, including the kidney, lung, heart and skeletal muscle (23,24). The focus of functional studies of AATK has been placed on the nervous system. A previous study determined that during retinoic acid-induced neuronal differentiation of P19

embryonal carcinoma cells, the expression of AATK was significantly elevated (25). Previous reports have indicated that AATK is negatively associated with the activity of Src in lung cancer (19). Src is a member of the non-receptor tyrosine kinase family, which consists of nine members, including Fyn, Lyn, Src, Fgr, Yes, Yrk, Hck, Blk and Lck. Src is functionally associated with numerous cytokine and growth factor receptors and is important in the regulation of multiple cellular activities, including cell migration,

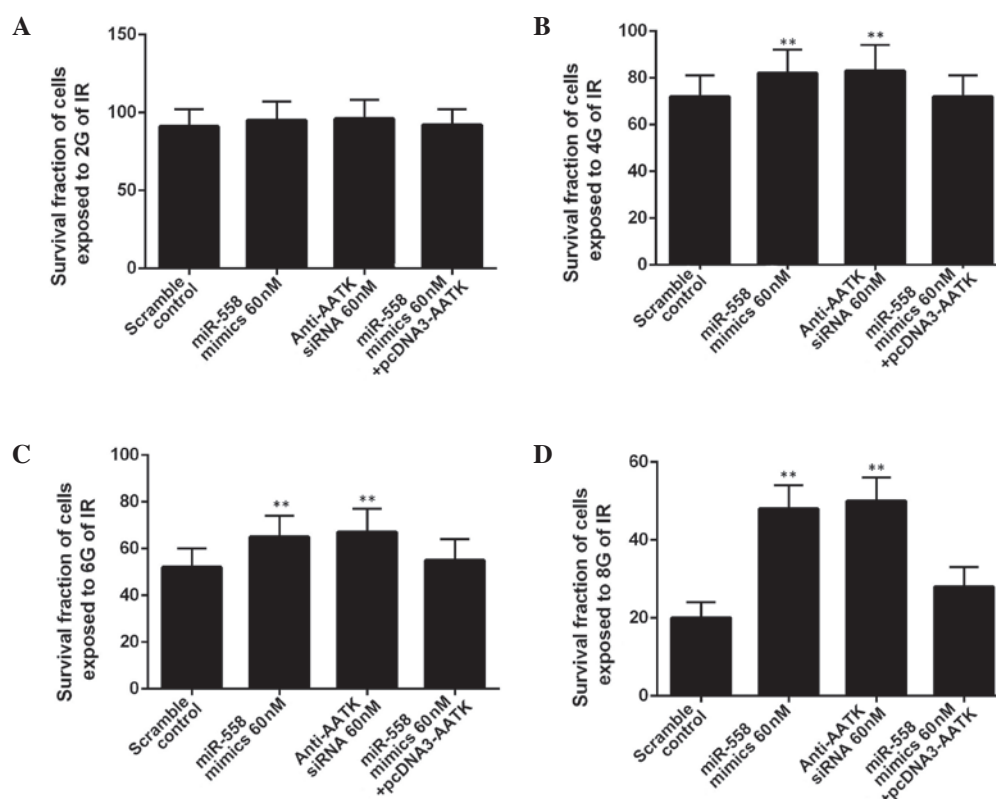


Figure 4. Ionizing radiation treatment exhibited a dose-dependent inhibitory effect on the growth of the A549 cells as determined using an MTT assay. (A) In A549 cells exposed to 2 Gy, the administration of miR-558 mimics or AATK specific siRNA did not significantly alter the survival rate of the cells. In the cells exposed to (B) 4 Gy, (C) 6 Gy or (D) 8 Gy, administration of miR-558 mimics or AATK specific siRNA significantly promoted the survival rate of the cells, and overexpression of AATK reduced this effect. Data are presented as the mean \pm standard deviation (** $P < 0.01$ compared with the control). miR, microRNA; AATK, apoptosis-associated tyrosine kinase; siRNA, small interfering RNA.

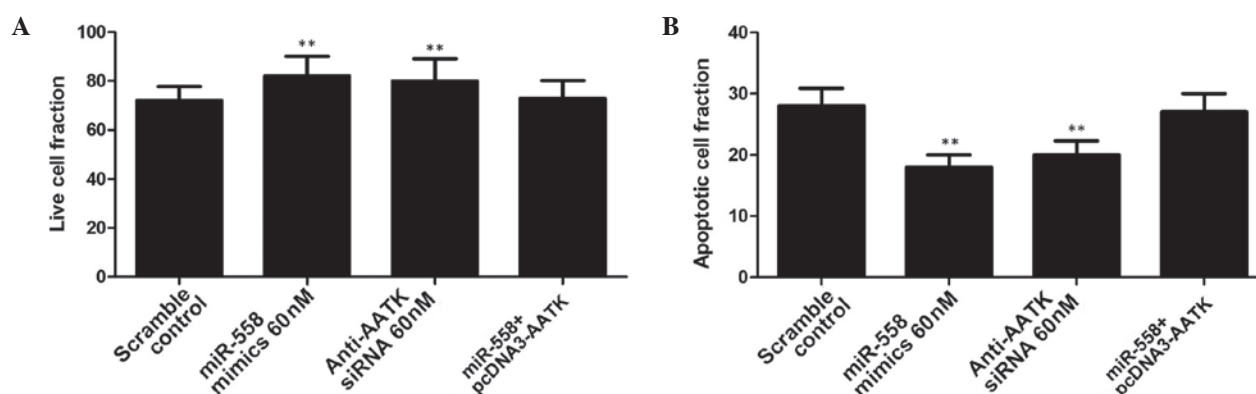


Figure 5. Introduction of miR-558 mimics or anti-AATK siRNA significantly suppressed apoptosis in lung cancer cells, while ectopic overexpression of AATK significantly reversed this effect on apoptosis. (A) Live cell fraction. (B) Apoptotic cell fraction. Data are presented as the mean \pm standard deviation (** $P < 0.01$ compared with the control). miR, microRNA; AATK, apoptosis-associated tyrosine kinase; siRNA, small interfering RNA.

invasion, adhesion, proliferation, survival, inflammation and angiogenesis (26). Notably, Src is commonly upregulated in lung cancer. Immunohistochemical analysis performed in a previous study indicated that the levels of Src were increased in 50-80% of NSCLC samples tested, and Src overexpression was significantly associated with poor differentiation (27). Src is key for the occurrence of radiotherapy resistance in NSCLC (20). Activating mutations of Src have been reported in colon cancer; however, no activating mutations and gene amplifications have been identified in lung cancer (28,29). Therefore, other regulators are potentially involved in the

control of AATK expression in NSCLC. The present study determined that AATK, a radiosensitization-related gene, is a target of miR-558 in lung cancer cells by using *in silico* analysis and luciferase reporter system. Additionally, it was identified that transfection of 30 or 50 nM miR-558 mimics and AATK specific siRNA significantly suppressed both the mRNA and protein expression levels of AATK.

Various miRNAs have been associated with the response to radiotherapy in different tumor types, such as miR-17-92 cluster, which interferes with the response of human mantle cell lymphoma to radiotherapy (30). miR-181 sensitizes human

gliomas to radiotherapy by acting on Bcl-2 (31). miR-221/miR-222 has been demonstrated to modulate the response of gastric carcinoma cells to radiotherapy through phosphatase and tensin homologue (32). It has been demonstrated that excessive expression of let-7 g and miRNA-9 in H1299 lung cancers cells leads to sensitization to radiotherapy by impeding nuclear factor- κ B pro-survival cascades (33). miR-558 is a miRNA that has been recently found in human cells. The upregulation of miR-558 has been observed in irradiated fibroblasts (34). It also has a regulatory effect on target genes associated with cell cycle checkpoints and apoptosis (35). In a previous study, significant underexpression of miR-558 was observed in breast cancer tissues (36). It has been determined that miR-558 in neuroblastoma tissues and cell lines was upregulated compared with in normal dorsal ganglia, with high expression of baculoviral IAP repeat containing 6, the host gene of miR-558, reported in a publicly available neuroblastoma microarray database (36). It has been previously determined that miR-558 acts as oncogene to increase colony formation, *in vivo* growth and *in vitro* proliferation of neuroblastoma cells (37). The current study exposed A549 cells to different doses of ionizing radiation, from 0 to 10 Gy, following transfection with miR-558 mimics or AATK specific siRNA, and determined that the administration of miR-558 mimics or AATK specific siRNA did not significantly alter the survival rate of the cells. In contrast to the cells exposed to 4, 6 or 8 Gy, administration of miR-558 mimics or AATK-specific siRNA significantly promoted the survival rate of the cells, and overexpression of AATK reduced this effect.

In conclusion, AATK was validated as a target gene of miR-558, and upregulation of miR-558 was observed in radioresistant lung cancer cells. Additionally, ectopic overexpression of AATK partially but significantly reduced the miR-558-induced radioresistance. The current study provides insight into miRNA-regulated sensitivity to radiotherapy in NSCLC and indicates that miR-558 may be a feasible novel target for the treatment of human NSCLC. Further *in vivo* experiments in animal models are required in the future to determine the efficacy of miR-558.

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