

Knockdown of RAD54B expression reduces cell proliferation and induces apoptosis in lung cancer cells

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Abstract

Objective: RAD54 homolog B (RAD54B), a member of the SNF2/SWI2 superfamily, is implicated in homologous recombination, and high RAD54B expression predicts the prognostic outcomes of lung adenocarcinoma. However, its role in lung carcinogenesis was unclear so this was determined in the present study.

Methods: We evaluated the gene and protein expression of RAD54B in 15 lung adenocarcinoma tissues and matched adjacent healthy lung tissues by real-time PCR, immunohistochemical staining, and western blotting. A549 lung cancer cells were transduced with lentivirus carrying small hairpin RNA (shRNA) against RAD54B (shRAD54B) or control shRNA (shCtrl), and cell proliferation, viability, apoptosis, and caspase 3/7 activity were evaluated.

Results: RAD54B protein expression was significantly higher in lung adenocarcinoma tissues than in healthy lung tissues. *RAD54B* gene expression was high in A549 cells but was efficiently knocked down using shRAD54B with an infection efficiency of 80% and a knockdown ratio of 72.2% compared with shCtrl. Suppressing RAD54B expression in A549 cells significantly reduced cell proliferation and caspase 3/7 activity, and significantly increased the apoptotic rate.

Conclusions: RAD54B exerts an oncogenic role in lung cancer cell proliferation.

Keywords

Knockdown, lung cancer, RAD54B, proliferation, apoptosis, oncogenesis, DNA damage response

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Introduction

Lung cancer is one of the most frequent forms of neoplasia. It is the leading cause of cancer-related mortality worldwide, resulting in over 1 million deaths every year.¹ Lung adenocarcinoma (ADC), as the most common histological subtype of lung cancer, is a global public health problem with a low 5-year survival rate of only 15%.¹ Therefore, there is an urgent need to investigate the molecular mechanisms of the pathogenesis and progression of lung cancer.²⁻⁴

RAD54 homolog B (RAD54B) was first identified as a homolog of RAD54 in 1999 by Hiramoto et al.⁵ Human *RAD54B*, located on human chromosome 8q21.3-q22 in a region associated with cancer-related chromosomal abnormalities, encodes a protein containing ATPase domains that is a member of the SNF2/SWI2 family. RAD54B, like its homolog, is actively involved in homologous recombination (HR) and DNA repair processes.⁶⁻¹⁰ Aside from these, RAD54B functions as a scaffold for the heterodimerization of MDM2-MDMX, promoting p53 degradation and cell cycle progression under conditions of DNA damage.¹¹ Emerging evidence has also suggested the association of HR genes, including *RAD54B*, with various human cancers, while homozygous mutations of *RAD54B* were identified in human primary lymphoma and colon cancer.⁵ Moreover, high RAD54B expression was found to predict the prognostic outcomes of patients with glioblastoma,¹² colorectal cancer,¹³ and lung ADC.¹⁴ However, the roles of RAD54B in the development of these tumors have not been clarified.

In the current study, we examined the expression of RAD54B in human lung ADC tissues and lung cancer cell lines. We also investigated the biological functions of RAD54B in the proliferation and apoptosis of lung cancer cell lines after

knocking down its expression by lentivirus-mediated RNA interference.

Materials and methods

Immunohistochemistry staining (IHC) of RAD54B expression

A lung adenocarcinoma tissue microarray containing 15 lung adenocarcinoma tissues and matched healthy lung tissues (Outdo Biotech, Shanghai, China) was used for IHC staining. Slides were deparaffinized with xylene and then rehydrated in an ethanol series. Endogenous peroxidases were inactivated by incubation with 3% H₂O₂ for 10 minutes at room temperature. Antigen retrieval was then conducted by microwaving in 0.01 M citrate buffer (pH 6.0) for 20 minutes. Non-specific antigens were blocked by incubating with 10% normal goat serum for 30 minutes. Subsequently, the slides were incubated with a rabbit anti-RAD54B primary antibody at 1:500 dilution (HPA007087; Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C, then with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG at 1:100 dilution (RABHRP1; Sigma-Aldrich) for 1 hour at room temperature. Immunoreactivity was developed with the 3, 3'-diaminobenzidine substrate and counterstaining was performed with hematoxylin. The intensity of the immunoreactivity score (IRS) was assessed by two investigators independently as IRS = staining intensity (SI) × percentage of positive cells (PP). SI was graded as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong); PP was scored as 0 (negative), 1 (<25%), 2 (26%–50%), 3 (51%–75%), and 4 (>75%). IRS ≥ 6 was considered to represent high RAD54B expression (Figure 1 and Table 1). Written informed consent was obtained from all patients, and the study protocol was approved by the ethics committee of

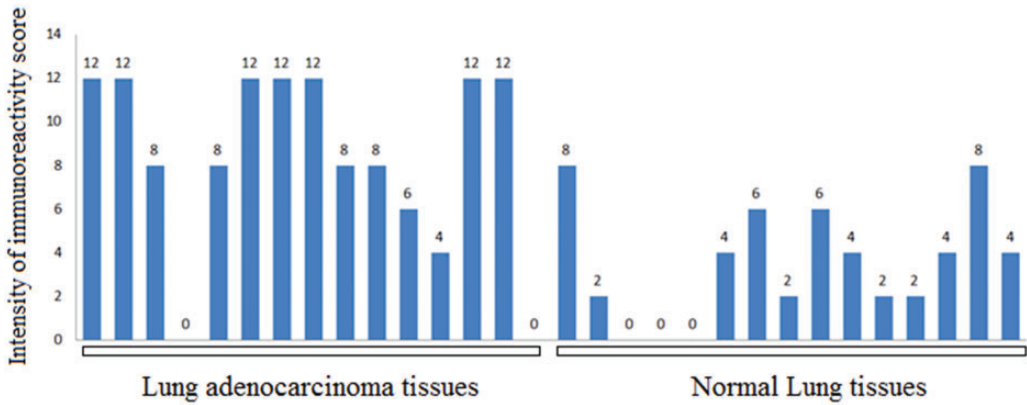


Figure 1. Sign test of the immunoreactivity score between lung adenocarcinoma and healthy lung tissues.

Table 1. RAD54B expression in lung adenocarcinoma tissues and matched healthy lung tissues.

RAD54B expression	Normal lung tissues	Lung adenocarcinoma tissues	P
Low	11	3	<0.005
High	4	12	<0.001
n	15	15	

Guizhou Provincial People’s Hospital (dated October 20, 2015; Guizhou, China).

Tumor cell lines

A549 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained at 37°C under 5% CO₂. Cells were cultured in F12K medium (Corning, New York, NY, USA) supplemented with 10% fetal bovine serum (Ausbian, Sydney, Australia).

Real-time PCR

Total RNA was extracted from cells using TRIzol (Shanghai Pufei Biotechnology, Shanghai, China) according to the manufacturer’s protocol. A total of 2 µg RNA was reverse-transcribed into cDNA with the

oligo (dT) primer using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). cDNA was then used for quantitative PCR with the SYBR Master Mixture (Takara, Shanghai, China) according to the manufacturer’s instructions. *GAPDH* was used for normalization. Primers were: RAD54B forward, 5'-GCCAAACACTGATGATTTGTGG-3' and RAD54B reverse, 5'-CCTGAGAAGAATGCGAGATAGC-3'; GAPDH forward, 5'-TGA CTTCAACAGCGACACCCA-3' and GAPDH reverse, 5'-CACCTGTGCTGTAGCCAAA-3'. Real-time PCR was carried out in an Agilent MX3000p qPCR system (Agilent, Palo Alto, CA, USA) using an initial denaturation step at 95°C for 30 seconds, followed by 45 cycles of amplification with denaturation at 95°C for 5 seconds, and annealing and extension at 60°C for 30 seconds. *RAD54B* expression was calculated in triplicate using the 2^{-ΔΔCt} method.

Western blotting

Cell extracts were separated on a 10% sodium dodecyl sulfate polyacrylamide gel and proteins were transferred to nitrocellulose membranes. These were incubated overnight at 4°C with a rabbit anti-RAD54B primary antibody at 1:100 dilution

(HPA007087, Sigma-Aldrich) and an anti β -actin antibody at 1:200 dilution (SAB5500001, Sigma-Aldrich), then with HRP-conjugated anti-rabbit IgG at 1:1000 dilution (RABHRP1, Sigma-Aldrich) for 2 hours. Western blotting signals were developed by the BCA Protein Assay Kit (Tiangen, Beijing, China). After color development, the image was analyzed using a ChemiScope Mini chemiluminescence meter to calculate the optical density of the target and the internal control band for protein expression as follows:

Protein expression
= integral optical density value of
target protein/integral optical
density value of β -actin.

Small hairpin RNAs (shRNAs) and lentiviruses

The lentiviral vector GV115 (Genechem, Shanghai, China), containing hU6-MCS-CMV-EGFP, was used for shRNA-mediated RNA interference. EGFP was used as an indicator of infection efficiency. shRNAs targeting human RAD54B (shRAD54B, AGATTGTTGATGGCTTAA) and control shRNA (shCtrl, TTCTCCGAACGTGTCACGT) were separately synthesized and inserted between the *AgeI* and *EcoRI* sites of the multiple cloning site of GV115, and confirmed by sequencing. Lentiviruses were produced by transfecting the lentiviral constructs pHelper 1.0 and pHelper 2.0 (Genechem) into HEK293T cells. At 48 hours after transfection, viruses were collected and filtered through a 0.45- μ m filter. A549 cells were then transduced with the lentiviruses at a multiplicity of infection of 10 in the presence of 8 μ g/ml polybrene. After 72 hours, the infection efficiency was evaluated under fluorescence microscopy (IX71; Olympus, Tokyo, Japan). To confirm RAD54B knockdown, its expression was

evaluated by real-time PCR and western blotting in shRAD54B-A549 cells and shCtrl-A549 cells.

Analysis of cell proliferation using Celigo Imaging cytometer

A549 cells (2×10^3 /well) were plated into 96-well plates and cultured overnight in F12K medium supplemented with 10% fetal bovine serum. Lentiviruses were added to cultures the next day (Day 1), and cells were then imaged and counted daily using the Celigo Cell Counting application (Nexcelom Bioscience, Lawrence, MA, USA) for 5 days. The cell proliferation rate was calculated as the fold change in cell number per unit time (days) based on the Day 1 value.

3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay cell viability

A549 cells (2×10^3 /well) were plated and treated as described above. The MTT assay was performed daily for 5 days. Briefly, 20 μ L of MTT solution (5 mg/mL; Genview, Houston, TX, USA) was added to each well and mixed well. A549 cells were then incubated at 37°C for 4 hours, after which medium containing MTT was removed and 100 μ L dimethyl sulfoxide was added. The optical density at 490 nm was measured using the Infinite M2009PR plate reader (Tecan, Crailsheim, Germany).

Cell apoptosis

At 72 hours after lentivirus infection, A549 cells were plated into 6-well plates for 48 hours, then labeled by the Annexin V Apoptosis Detection kit (eBioscience, San Diego, CA, USA) and analyzed by flow cytometry (Millipore, Bedford, MA, USA). In brief, A549 cells were collected by trypsinization, washed with binding

buffer, resuspended in binding buffer, and stained with Annexin V-APC in the dark at room temperature for 15 minutes. All assays were performed in triplicate.

Activity of caspases 3/7

The activity of caspases 3/7 was evaluated in A549 cells 72 hours post-lentivirus infection using the Caspase-Glo® 3/7 Assay kit (Promega) according to the manufacturer's instructions. Briefly, A549 cells were plated into 96-well plates (1×10^4 /well in 100 μ L medium), then 100 μ L of Caspase-Glo® 3/7 Reagent was added to all wells and mixed on a plate shaker at 300 rpm for 30 seconds. Cells were incubated at room temperature for 1 to 2 hours and the optical density was measured using a plate reader (Tecan).

Statistical analyses

Nonparametric IHC data were analyzed by the Sign test.¹⁵ All *in vitro* assays were

conducted at least three times and data are shown as the means \pm standard deviation (SD). The Student's t test was used to evaluate differences between two groups. A *P* value < 0.05 was defined as statistically significant.

Results

IHC analysis of RAD54B expression in lung adenocarcinoma

RAD54B expression was evaluated by IHC staining in 15 pairs of lung adenocarcinoma tissues and healthy lung samples. RAD54B was clearly localized in the nucleus of tumor cells (Figure 1 and 2). Its expression was low (staining index < 6) in most healthy lung samples (Table 1), and high (staining index ≥ 6) in 80.0% of lung adenocarcinoma samples (12/15, Table 1). The Sign test revealed that this difference was significant ($P < 0.001$; Table 1 and Figure 1).

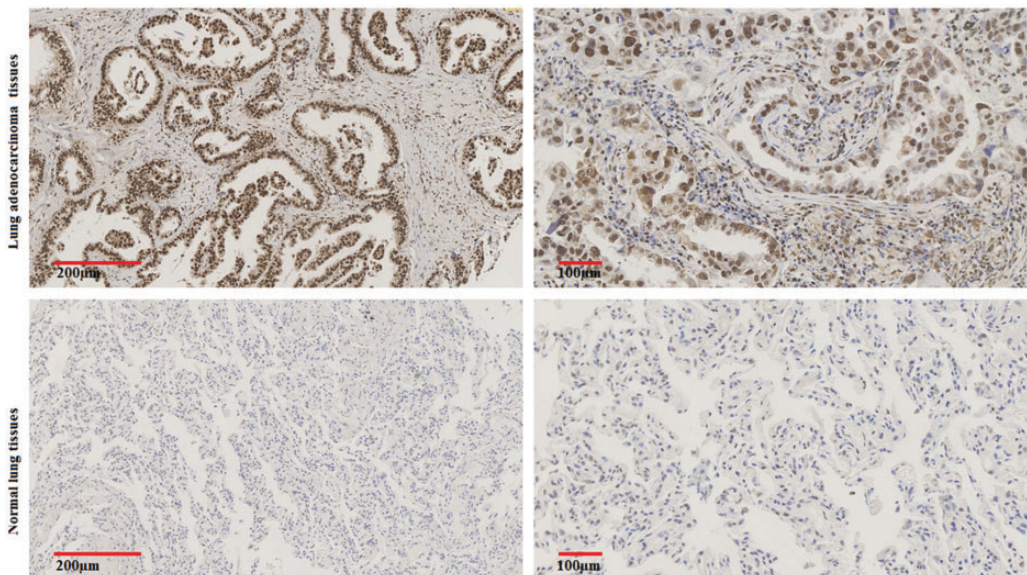


Figure 2. Immunohistochemical staining of RAD54B protein in lung adenocarcinoma and adjacent healthy tissues. RAD54B protein expression is seen as brown staining in the nucleus of tumor cells. Scale bars: 100 μ m and 200 μ m.

Expression of *RAD54B* and knockdown in A549 cells

RAD54B expression in A549 cell lines was first confirmed by real-time PCR (Figure 3a). To gain further insights into its potential role in lung carcinogenesis, we knocked down its expression by lentivirus-mediated shRNA interference. At 72 hours after viral infection, most cells showed a normal morphology. After knockdown, *RAD54B* expression was significantly reduced by 72.2% compared with shCtrl ($P < 0.001$, Figure 3a), while *RAD54B* protein expression was significantly reduced by 46.37% compared with shCtrl ($P < 0.05$, Figure 3b, c).

Suppression of cell proliferation by *RAD54B* knockdown

Data generated from the Celigo assay showed that the number of A549 cells was significantly decreased after the knockdown of endogenous *RAD54B* expression on Days 2, 3, 4, and 5 ($P < 0.01$, Figure 4a). MTT assays revealed similar findings ($P < 0.01$, Figure 4b). These data indicate that *RAD54B* may exert proliferation-promoting properties in lung cancer cells.

Induction of cell apoptosis by *RAD54B* knockdown

The apoptotic function of *RAD54B* in lung cancer cells was then assessed by Annexin V staining and flow cytometry analysis (Figure 5a and 5b). Flow cytometry showed that shRNA transduction significantly induced cell apoptosis compared with shRNA-treated cells ($P < 0.001$, Figure 5c). Additionally, the activity of caspases 3/7 was significantly induced after *RAD54B* shRNA ($P < 0.001$, Figure 5d), which may have caused the increased apoptotic rate.

Discussion

RAD54B was first identified by Hiramoto et al.⁵ who reported homozygous *RAD54B* mutations in human lymphoma and colon cancer. Since then, several studies have detected abnormal *RAD54B* expression in several types of tumors. A gene expression profile study indicated that *RAD54B* and seven other genes could predict overall survival in patients with glioblastoma receiving concurrent capecitabine and radiation.¹² Nagai et al.¹³ reported that high *RAD54B* expression was an independent predictor for the prognosis of colorectal cancer, while Hwang et al.¹⁴ suggested that lung adenocarcinoma patients with high expression of *RAD54B* and *FEN1* had a worse prognosis than those with lower expression. The present study confirmed the overexpression of *RAD54B* protein in lung adenocarcinoma tissues by IHC (Figure 1 and Table 1) and revealed high *RAD54B* expression in lung cancer cell lines by real-time PCR.

Cells respond to DNA damage by activating a complex DNA damage response (DDR) pathway, including cell cycle arrest, the activation of DNA repair-associated genes, and in some cases the triggering of apoptosis.¹⁶ The DDR plays crucial roles in the maintenance of homeostasis, and deficiencies in its components lead to genomic instability and tumor development.¹⁷ That *RAD54B* participates in HR and DNA repair processes^{6–10} prompted us to investigate its possible functions in lung carcinogenesis through *in vitro* *RAD54B* loss-of-function studies. In combination with previous findings,¹⁴ our current results suggest that *RAD54B* has the potential to be used as a novel therapeutic target for lung cancer.

Deregulated proliferation is one of the most distinguishing hallmarks of cancer cells,¹⁸ and *RAD54B* promotes cell cycle progression under conditions of DNA

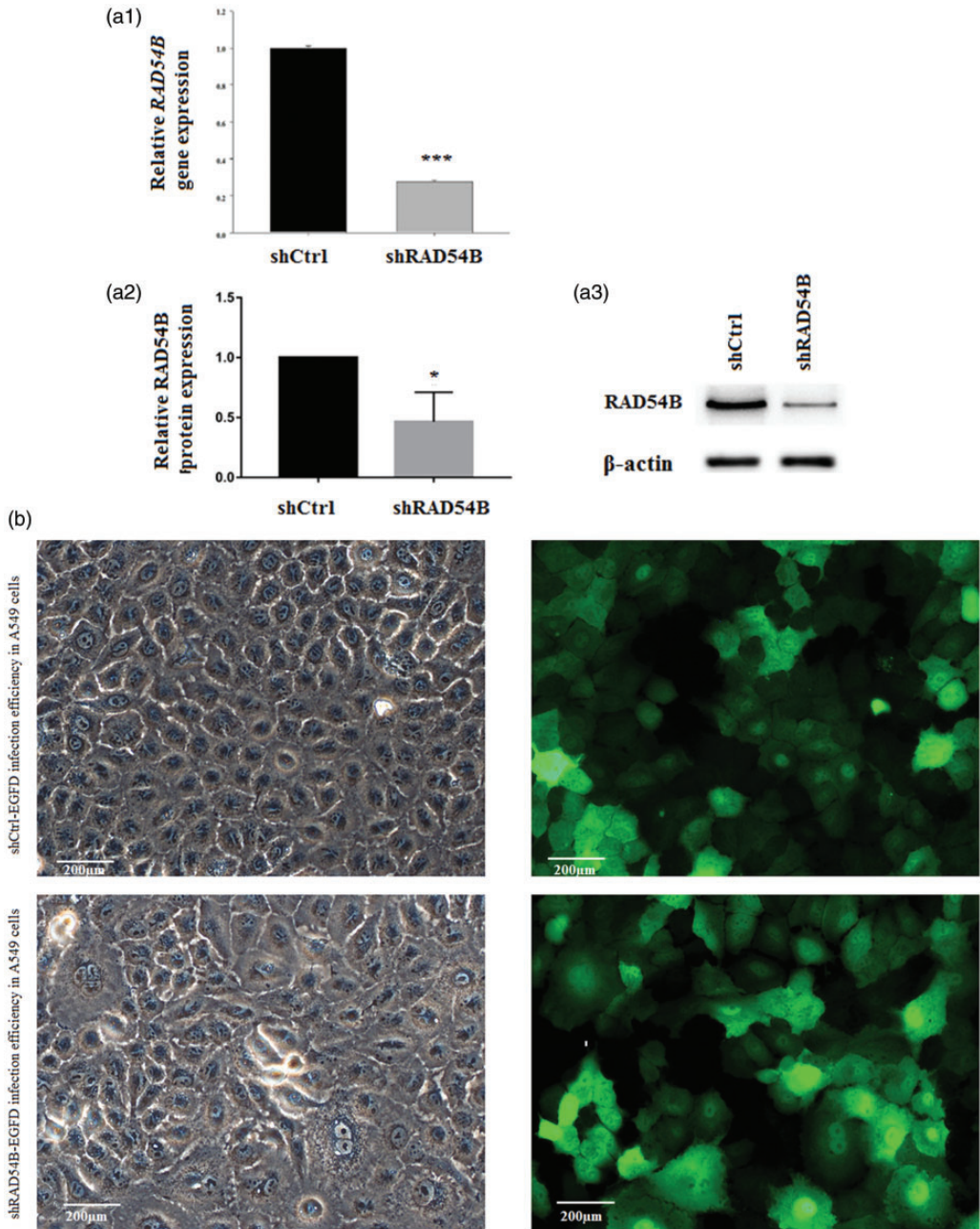


Figure 3. (a) RAD54B expression in A549 cells as shown by real-time PCR, (***) $P < 0.001$ shRAD54B vs. shCtrl) and (b, c) western blotting (* $P < 0.05$ shRAD54B vs. shCtrl). (d) Infection efficiency shown by green fluorescence 72 hours after infection. Scale bar: 200 μ m.

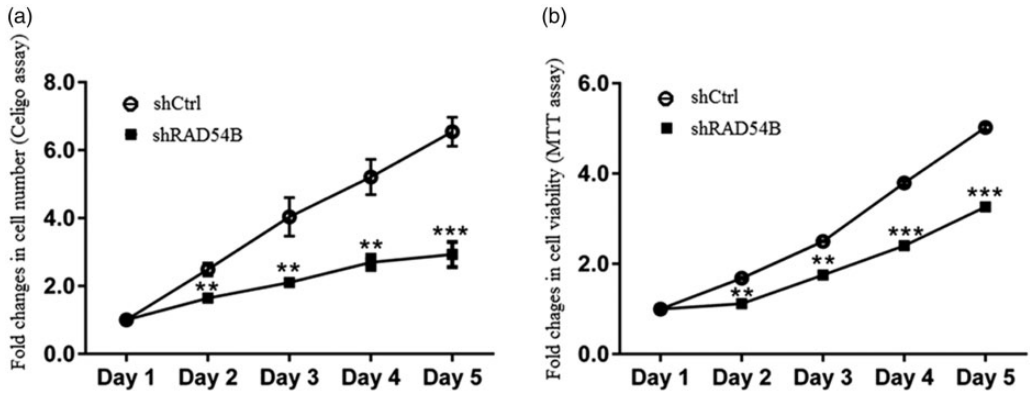


Figure 4. (a) Celigo counting of the A549 cell number after RAD54B shRNA (shRAD54B) or control shRNA (shCtrl) treatment. (b) A549 cell viability after RAD54B shRNA (shRAD54B) or control shRNA (shCtrl) treatment by MTT assays. $**P < 0.01$, $***P < 0.001$ shRAD54B vs. shCtrl.

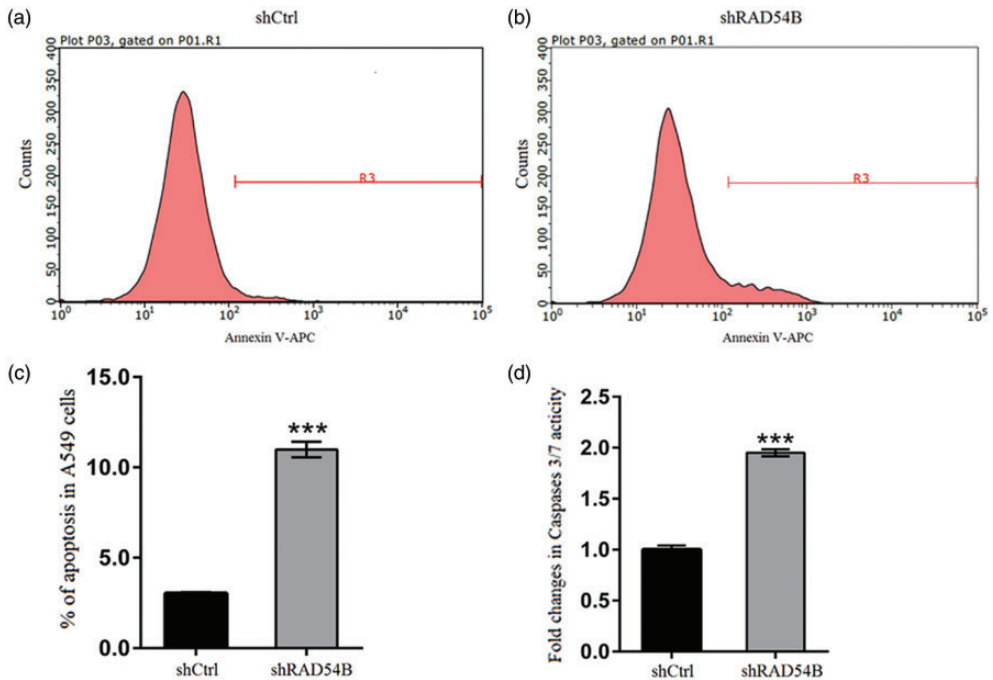


Figure 5. Flow cytometry data showing higher apoptosis in shRAD54B-treated A549 cells than shCtrl-treated cells (a–c). (d) Caspases 3/7 activity in shRAD54B-treated A549 cells was significantly higher than that in shCtrl-treated A549 cells. $***P < 0.001$.

damage.¹¹ These findings suggest a possible involvement of RAD54B in the growth of cancer cells. In the present study, after successfully knocking down the expression of

RAD54B in A549 cells (Figure 3), cell counting with the Celigo Image cytometer and MTT assays revealed significantly suppressed A549 cell proliferation and viability

(Figure 4a and 4b). This indicated that under normal conditions, RAD54B activity is essential for cell proliferation in lung cancer.

Suppressed apoptosis is a possible reason for the deregulation of cell proliferation during cancer progression.¹⁹ RAD54B knockdown was shown by flow cytometry to significantly induce the apoptosis of A549 cells (Figure 5a–c), suggesting that RAD54B shRNA exerted anti-proliferation effects on A549 cells.

The mitochondrial pathway, also known as the intrinsic pathway, is one of the best-understood apoptosis mechanisms.²⁰ Caspases, including initiator and effector caspases, are involved in this process,²¹ and caspases 3 and 7 are critical mediators of the intrinsic pathway.²² We found that suppressing *RAD54B* expression significantly enhanced the activity of caspases 3/7 in A549 cells (Figure 5d), indicating that RAD54B affects lung cancer cell apoptosis via the mitochondrial pathway. However, further investigation is required to understand the mechanisms by which RAD54B regulates the activation of effector caspases.

Conclusion

RAD54B is able to modulate the proliferation and apoptosis of lung cancer cells, so it could be used as a biomarker and therapeutic target for lung cancer.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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