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ORIGINAL ARTICLE

The relationship between miR-17-5p, miR-92a, and let-7b expression with non-small cell lung cancer targeted drug resistance

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Summary

Purpose: *To investigate the relationship between microRNA (miR)-17-5p, miR-92a, and let-7b expression and resistance to the non-small cell lung cancer (NSCLC) targeted drug Gefitinib.*

Methods: *The human NSCLC cell line A549 and its drug resistant strain A549/GR (Gefitinib Resistant) was used in this study. The expression of miR-17-5p, miR-92a, and let-7b in different NSCLC cell lines was detected before and after transfection using real-time fluorescent PCR. Cell viability was detected using the CCK8 method. Cell cloning was performed to examine cell proliferation; cell apoptosis before and after transfection was evaluated using flow cytometry.*

Results: *miR-17-5p and miR-92a expression in A549/GR cells was 3.23 ± 0.92 and 9.29 ± 3.13 fold higher than in A549 cells respectively ($p<0.05$). In addition, let-7b expression in A549/GR cells was 29.37 ± 9.32% fold higher than in A549 cells ($p<0.05$). A549 cell sensitivity to Gefitinib was significantly decreased after transfection with the miR-17-5p mimic, miR-92a mimic, or the let-7b inhibitor ($p<0.05$), whereas the sensitivity of A549/GR cells to Gefitinib was significantly increased after transfection with miR-17-5p inhibitor, miR-92a inhibitor, or the let-7b mimic ($p<0.05$). A549 transfected with miR-17-5p mimic, miR-92a mimic, and/or let-7b inhibitor formed more colonies than non-transfected controls ($p<0.05$); A549/GR transfected with miR-17-5p inhibitor, miR-92a inhibitor and let-7b mimic formed fewer colonies than the control group ($p<0.05$). The apoptosis rate of A549 cells transfected with miR-17-5p mimic, miR-92a mimic, or let-7b inhibitor was significantly lower than that of the control group ($p<0.05$); the apoptosis rate of A549/GR cells transfected with miR-17-5p inhibitor, miR-92a inhibitor, or let-7b mimic was significantly higher than that of the control group ($p<0.05$).*

Conclusions: *Increased miR-17-5p and miR-92a expression and decreased let-7b expression can significantly induce proliferation and inhibit apoptosis of lung cancer cells, while reducing lung cancer cell sensitivity to Gefitinib.*

Key words: *Gefitinib, miR-17-5p, miR-92a, let-7b, non-small cell lung cancer, sensitivity*

Introduction

With serious and continuously increasing environmental pollution, the incidence of lung cancer in China has increased year by year. Lung cancer has become one of the most common malignant tumors in China, with morbidity and mortality levels among the highest in the world [1]. Among lung cancers, NSCLC is the most important disease subtype, with 70-80% of patients diagnosed with advanced disease stage, losing the operative window. Therefore, chemotherapy has become the most prominent therapeutic approach [2]. However, resistance to chemotherapeutic drugs has become a major issue in NSCLC treatment [2-5]. An increasing number of studies have

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shown that miRs play an important role in the occurrence and development of various cancers, and impact tumor cell proliferation, apoptosis, and resistance to chemotherapeutic drugs [6,7]. Studies have shown that a large number of miR expression abnormalities exist between the A549 expression profile and A549/GR expression profile within the miR-17-92 family and let-7b. At present, a significant amount of prior research has confirmed that the miR-17-92 family is a proto-oncogene which can promote the occurrence and development of cancer, and that let-7b is a tumor suppressor gene which can inhibit the occurrence of cancer [9-11].

Gefitinib (GR) is a selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor and is indicated for the treatment of locally advanced or metastatic NSCLC patients that have been previously treated with chemotherapy or are unsuitable for chemotherapy [12]. Tyrosine kinases are usually expressed in epithelial-derived solid tumors, and inhibition of EGFR tyrosine kinase activity can inhibit tumor growth, metastasis, and angiogenesis, and can increase tumor cell apoptosis [13]. *In vivo*, Gefitinib broadly inhibits tumor growth in nude mice bearing xenograft human tumor derived cell lines and enhances the antitumor activity of chemotherapy, radiotherapy and hormone therapy. In clinical trials, Gefitinib has been confirmed to exert an objective antineoplastic response to locally advanced or metastatic NSCLC and to be able to ameliorate disease-related symptoms [14]. However, along with the popularity of targeted drugs and their long-term use, clinical resistance to targeted drugs has also been confirmed [15]. We therefore examined the relationship between the expression of miR-17-5p, miR-92a, and let-7b and Gefitinib resistance in NSCLC through cell and molecular biology experiments. The aim of this study was to investigate the mechanism of resistance to chemotherapy in NSCLC and provide an important theoretical basis and method for the development of individualized treatment plans.

Methods

Materials

The lung cancer cell line A549 and its induced Gefitinib-resistant cell line A549/GR used in this study were purchased from the Jingzhou Central Hospital. Other reagents used included RMPI 1640 medium (Invitrogen, CA, USA), Trizol (Invitrogen, CA, USA), PCR reagents, primers (Shanghai Shenggong Co., Ltd), and kits (Dalian Baoshengwu), lipofectamine RNAimax (Invitrogen, CA, USA); miR-17-5p, miR-92a, and let-7b mimic, as well as miR-17-5p, miR-92a, and let-7b inhibitors were purchased from Guangzhou Ribobio Co., Ltd.(China).

Cell culture

A549 and A549/GR cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and double antibody at 37°C, 5% CO_2, and 85% saturated humidity. The cells were passaged by 0.25% trypsin digestion or cryopreserved after centrifugation, removal of media, and resuspension in frozen stock solution (DMSO:serum 1:9 ratio). The A549/GR medium contained 2 μg/mL of Gefitinib to maintain cellular drug resistance. Cells in the logarithmic growth phase were used for experimentation.

Total RNA extraction and integrity test

A549 and A549/GR cells were inoculated into culture flasks and cultured in 5 mL RPMI 1640 complete medium. Total RNA was extracted by addition of Trizol reagent when cells reached about 85% confluence.

Real-time quantitative PCR

Examination of the expression levels of miR-17p, miR-92a, and let-7b in A549 and A549/GR cells before and after transfection was conducted using real-time PCR. Primer sequences were as follows: miR-17-5p forward: 5'-TCAAAGTGCTTACAGTGCAGGT-3'; reverse: 5'- CCAGGCAGATTCTAC ATCGAC- 3'; PCR product: 426 bp. miR-92a forward : 5'-CTGATGGTGGCCTGCTATTT-3'; reverse: 5'- ACAGTGGAAGTCGAAATCTTCAG- 3'; PCR product: 496 bp. let-7b forward primer: 5'-CCGGATCCCCTGGATGTTCTCTTCACTG-3', reverse: 5'-CCAAGCTTGCCTGGATGCAGACTTTTCT-3'. The PCR amplification reaction system totaled 50 μl and comprised the following: DNA template 1 μl, 10 × PCR buffer 5 μl, 25 mmol/l MgCl 24 μl, 10 mmol/l dNTP 1 μl, primers (10 μmol/L) 1 μl, Taq DNA polymerase 1 U. The amplification conditions were: 94°C for 2 min; 32 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min; 72°C for 10 min, and stored at 4°C.

Transfection efficiency test

miR-17-5p, miR-92a, let-7b mimic, miR-17-5p, miR-92a, let-7 inhibitors, as well as negative control (NC) were transfected respectively using lipofectamine RNAimax.

CCK8 detection

A549, A549/GR cells were inoculated into 96-well plates at a density of about 2,000-3,000 cells/well. 24 hrs after cell adherence, 100 μL complete medium containing GR was added to each well. The investigated GR concentration gradient in the A549 cell line was 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0 μg/mL. The GR concen-

tration gradient in the A549/GR cell line was 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 16.0 μg/mL. Blank wells containing only medium and control wells containing only cell suspensions were set up at the same time, with each group having 3 auxiliary wells. After 48-h incubation, the culture medium was changed to complete medium containing 10% CCK-8, and cells were further cultured for 3-6 hrs. The absorbance at 450 nm wavelength was measured by an enzyme-labeling measuring instrument, and cell viability % was calculated as $(A_{dosing}-A_{blank}/A_{0dosing}-A_{blank})*100\%$. IC_{50} values were calculated using the SPSS 19.0 statistical analysis software (SPSS, IL, USA) probit regression model.

Cell cloning experiment

Cell monolayers in the logarithmic growth phase were digested using 0.25% trypsin and pipetted to create single cell suspensions by adding 1640 medium with serum. Cell suspensions were diluted to 1,000 cells per well and inoculated into 6-well plates, where the cells were evenly distributed through gentle agitation. Plates were incubated at 37°C and 5% CO_2 in a saturated humidity environment for 1-2 weeks of static culturing. Cells were often observed, and when a visible clone was detected, the culture was terminated. The supernatant was then discarded and the plate was carefully washed twice with PBS. Each well of the 6-well plate was treated with pure methanol and fixed for 15 min. Following this, the fixative was discarded and 1 ml Giemsa staining solution was added to each well and allowed to stand for 30 min, after which the staining solution was washed with flowing water and dried by air. The 6-well plate was then inverted and overlaid with a transparent film containing a mesh, and clones were counted with the naked eye or under low power microscopy for clones greater than 10 cells.

Apoptosis analysis

Sample data were analyzed using flow cytometry software Flowjo 7.6.1 (Stanford University, CA, USA). Apoptotic rate = early apoptotic rate + late apoptotic rate. All experiments were repeated 3 times.

Statistics

SPSS 13.0 software (SPSS, IL, USA) software was used for statistical analyses. Experimental data were expressed as mean ± standard deviation, and quantitative data between groups were analyzed by t test. P value <0.05 was considered as statistically significant.

Results

The expression of mir-17-5p, mir-92a, and let-7b before and after transfection

Figures 1 and 2 show the expression of miR-17-5p, miR-92a, and let-7b in NSCLC cells before and after transfection as detected by RT-PCR. The expression of miR-17-5p and miR-92a in A549/GR cells was significantly higher than that in A549 cells prior to transfection (3.23 ± 0.92 and 9.29 ± 3.13 fold greater, respectively ($p<0.05$). The expression of let-7b in A549/GR cells was 29.37±9.32% lower than in A549 cells ($p<0.05$). Therefore, we decided to use the A549 and A549/GR cell lines for further study. 48 hrs post-transfection with the corresponding mimic, miR-17-5p and miR-92a expression in A549 cells was 10.64±1.17 and 10.17±1.55 fold greater respectively than in miR-NC cells ($p<0.05$). Likewise, miR-17-5p and miR-92a expression in A549/GR cells was 23.13±1.50 and 17.12±2.26 fold greater respectively than in miR-NC cells ($p<0.05$).

The expression of let-7b in the A549 cell let-7b inhibitor group was 19.90±1.67% of miR-NC cells ($p<0.05$), whereas let-7b levels in the A549/GR cell let-7b mimic group was 12.78±1.65 fold higher than in the miR-NC cells ($p<0.05$). Taken together, these findings suggest high transfection efficiency.

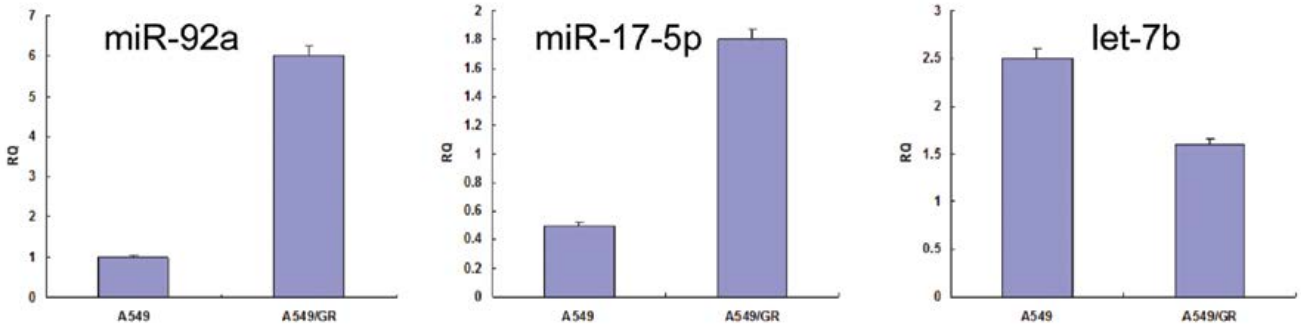

Figure 1. Basal expression of miR-17-5p, miR-92a, and let-7b in A549 and A549/GR cell lines. The expression of miR-17-5p and miR-92a in A549/GR cells was significantly higher (3.23 ± 0.92 and 9.29 ± 3.13 fold respectively) than in A549 cells before transfection ($p<0.05$). The expression of let-7b in A549/GR cells was significantly lower (29.37±9.32% decrease) than in A549 cells ($p<0.05$).

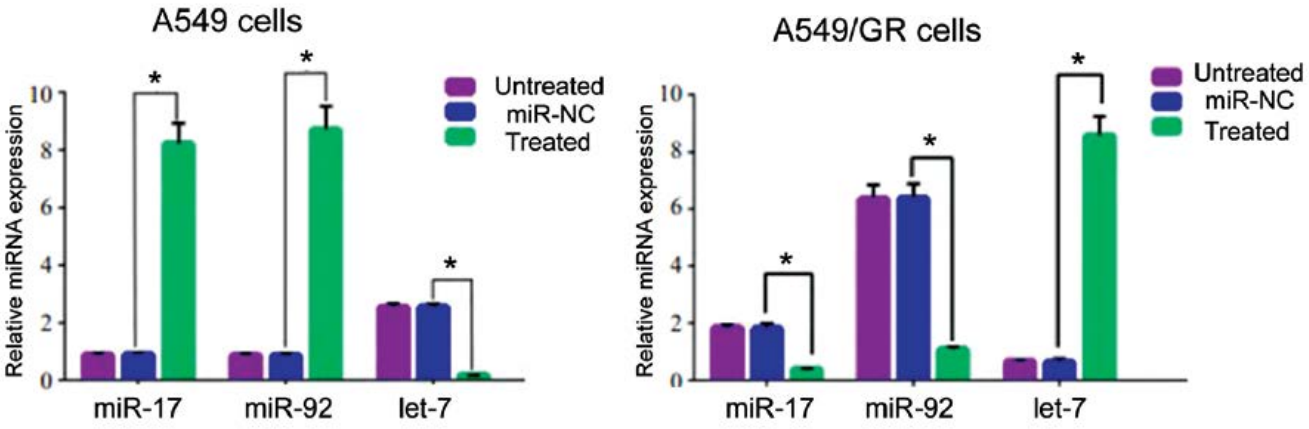

Figure 2. The expression of miR-17-5p, miR-92a, and let-7b in A549 cells after transfection with miR-17-5p mimic, miR-92a mimic, and let-7b inhibitor. miR-17-5p and miR-92a levels increased by 10.64±1.17 and 10.17±1.55 fold respectively in A549 cells post-transfection vs negative controls (*p<0.05). let-7b expression post-transfection in A549 cells decreased to 19.90 ± 1.67% of the NC group (*p<0.05). miR-17-5p and miR-92a expression decreased to 23.13±1.50 and 17.12±2.26% respectively of the negative control group after transfection with the corresponding inhibitors (*p<0.05). let-7b expression post-transfection in A549/GR cells was 12.78 ± 1.65 fold higher than the NC group (*p<0.05).

The effects of miR-17-5p, miR-92a, and let-7b on cellular sensitivity to Gefitinib

After transfection with miR-17-5p mimic, the CCK-8 detection kit showed that IC_{50} for Gefitinib in A549 cells was 0.606 μg/mL and the 95% confidence interval (95% CI) was 0.403-0.784 μg/mL. After transfection with miR-92a mimic, Gefitinib IC_{50} in A549 cells was 0.634 μg/mL and the 95% CI was 0.424-0.817 μg / mL. After transfection with let-7b inhibitor, Gefitinib IC_{50} in A549 cells was 0.607 μg/mL and the 95% CI was 0.408-0.782 μg/mL. Gefitinib IC_{50} in control A549 cells was 0.402 μg/mL and the 95% CI was 0.358-0.444 μg/mL (Table 1). The Gefitinib IC_{50} values for A549 cells transfected with miR-17-5p mimic, miR-92a mimic and let-7b inhibitor were significantly higher than that of control cells (p<0.05), which indicated that the sensitivity of A549 cells to Gefitinib was decreased (p<0.05).

After transfection with miR-17-5p inhibitor, A549/GR cell Gefitinib IC_{50} was 4.400 μg/mL and the 95% CI was 3.990-4.793 μg/mL. After transfection with miR-92a inhibitor, A549/GR cell Gefitinib IC_{50} was 4.231 μg/mL and the 95% CI was 3.824-4.621 μg/mL. After transfection with let-7b mimic, A549/GR cell Gefitinib IC_{50} was 4.430 μg/mL and the 95% CI was 4.049-4.797 μg/mL. In the control group A549/GR cell line Gefitinib IC_{50} was 5.770 and the 95% CI was 5.392-6.136 μg/mL (Table 1). These data indicate that transfection with miR-17-5p inhibitor, miR-92a inhibitor, and let-7b mimic decreased the IC_{50} for Gefitinib of A549/GR cells vs the control group, suggesting increased

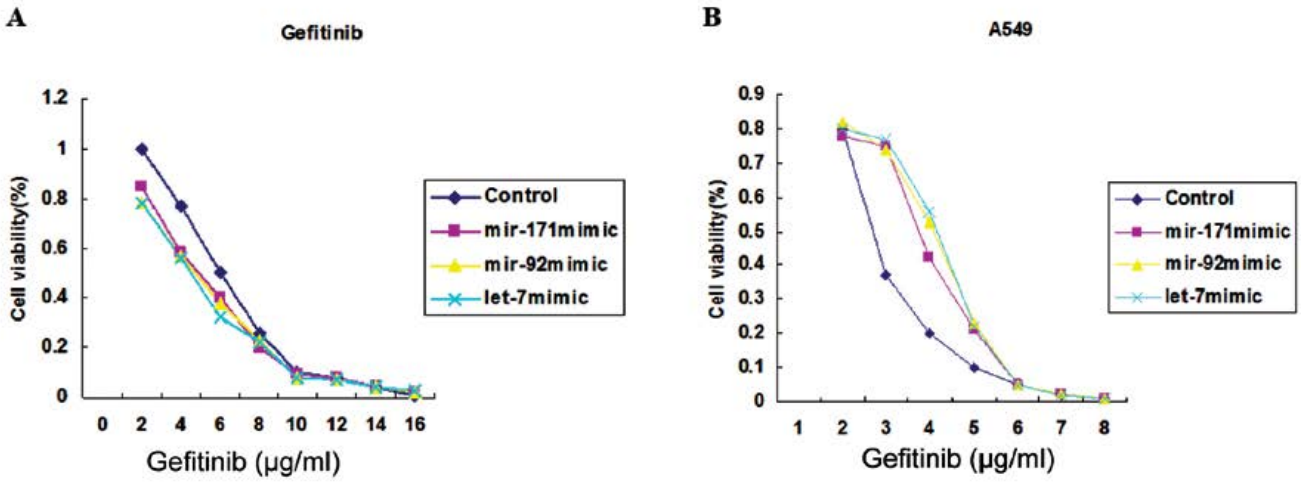

Figure 3. CCK-8 detection results. **A:** A549 cell line; **B:** A549/GR cell line. After transfection with miR-17b inhibitor, miR-92a inhibitor or let-7b mimic, A549/GR cell line Gefitinib IC_{50} values were significantly decreased vs the control group, suggesting increased A549/GR cell line sensitivity to Gefitinib (p<0.05).

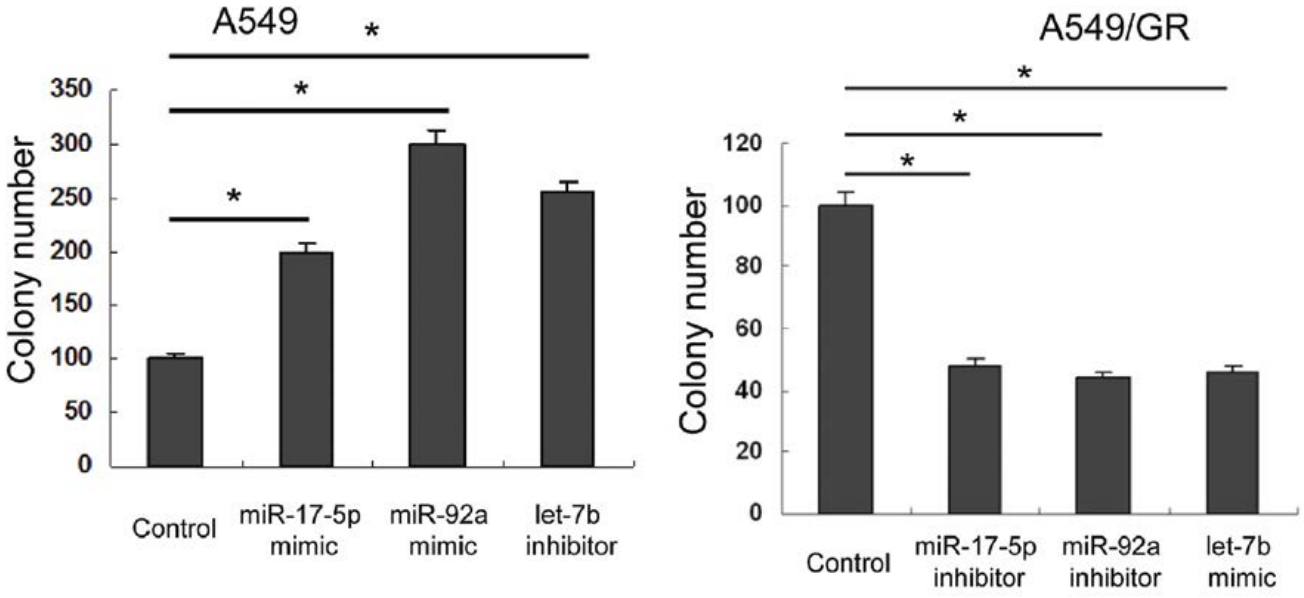

Figure 4. Plate clone test colony counts. **A:** A549 cell line; **B:** A549/GR cell line (*p<0.05). After transfection with miR-17-5p, miR-92a mimics or let-7b inhibitor, the number of cell colonies formed in the A549 cell line was greater vs the control (*p<0.05), suggesting enhancement of A549 cell line proliferation. However, after transfection with miR-17-5p, miR-92a inhibitor or let-7b mimic, the number of cell colonies formed in the A549/GR cell line was decreased vs the control group (*p<0.05).

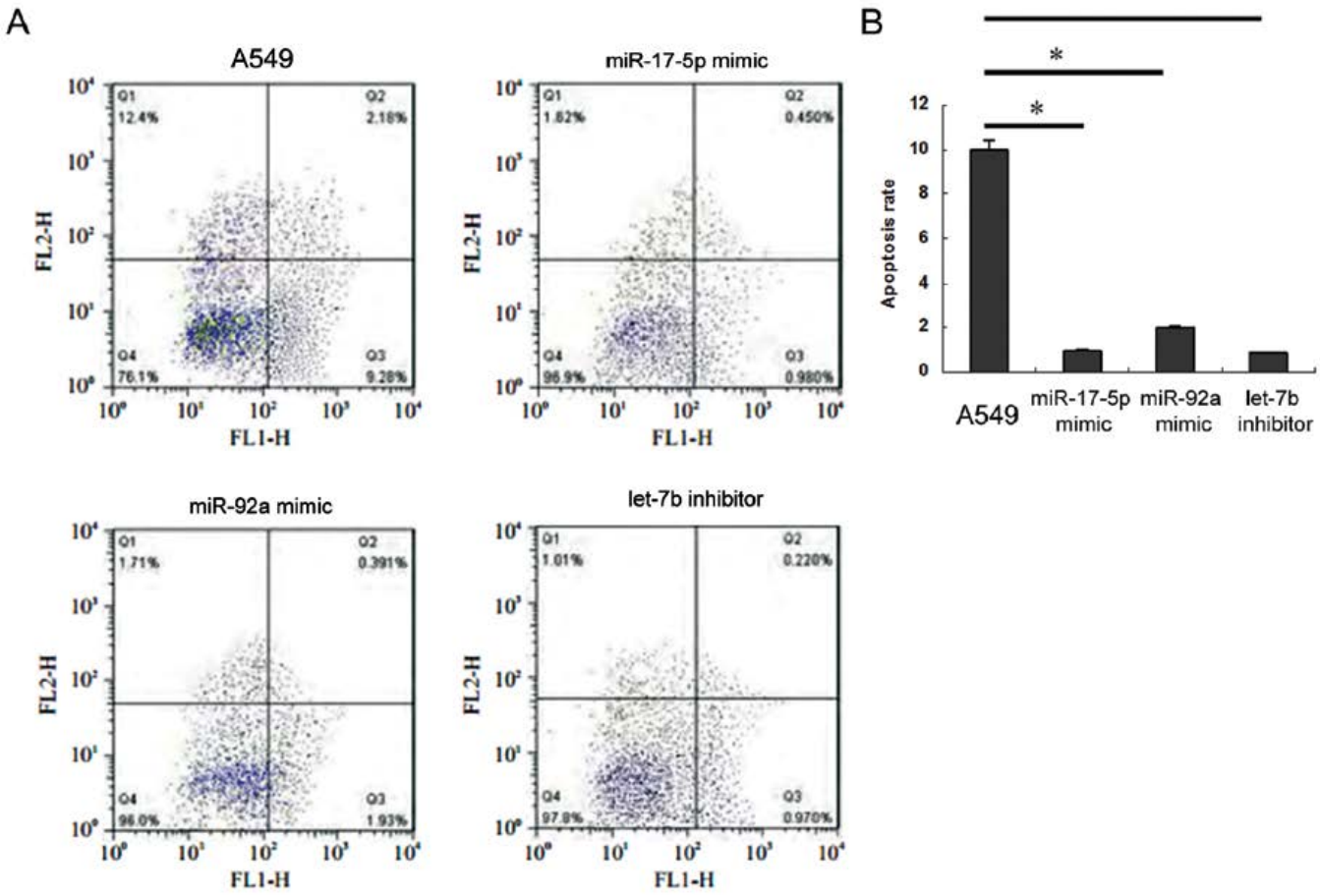

Figure 5. Apoptosis in A549 cells transfected with miR-17-5p, miR-92a mimics or let-7b inhibitor. **A:** apoptosis; **B:** apoptosis rate. After transfection with miR-17-5p, miR-92a mimic or let-7b inhibitor, the apoptosis rate of A549 cells was significantly lower vs the control group (*p<0.05).

A549/GR cell line sensitivity to Gefitinib ($p < 0.05$, Figure 3).

Plate clone testing of miR-17-5p, miR-92a, and let-7b effects on cell proliferation

After transfection with miR-17-5p, miR-92a mimics or let-7b inhibitor, the number of formed cell colonies in A549 cells was increased vs the control group ($p<0.05$), suggesting enhancement of proliferation. However, after transfection with miR-17-5p, miR-92a inhibitor or let-7b mimic, the number of formed cell colonies in A549/GR cells was decreased vs the control group ($p<0.05$), suggesting decreased proliferation (Figure 4).

Effect of miR-17-5p, miR-92a and let-7b on apoptosis

As shown in Figures 5 and 6, after transfection with miR-17-5p, miR-92a mimics or let-7 inhibitor, the apoptosis rate of A549 cells was significantly lower vs the control group ($p<0.05$), suggesting that Gefitinib induced inhibition of cell apoptosis. However, after transfection with miR-17-5p, miR-92a inhibitors or let-7b mimic, the apoptosis rate of A549/GR cells was significantly higher vs the control group ($p<0.05$).

Discussion

Gefitinib has an important effect on inhibiting lung cancer cell proliferation in patients treated with chemotherapy for advanced NSCLC. However, the long-term use of Gefitinib can lead to primary or secondary chemotherapeutic drug resistance, thereby decreasing treatment efficacy [12-14]. This situation presents an urgent problem that needs to be resolved in order to accurately improve targeted drug therapy in patients with advanced NSCLC. Studies have found that a large number of miR expression abnormalities exists in tumor tissues or cells. miR is involved in post-transcriptional regulation, and can therefore affect various molecules within cell signaling pathways by inhibiting the expression of target genes, leading to abnormalities in tumor cell survival and apoptosis pathways [9,11,15-17].

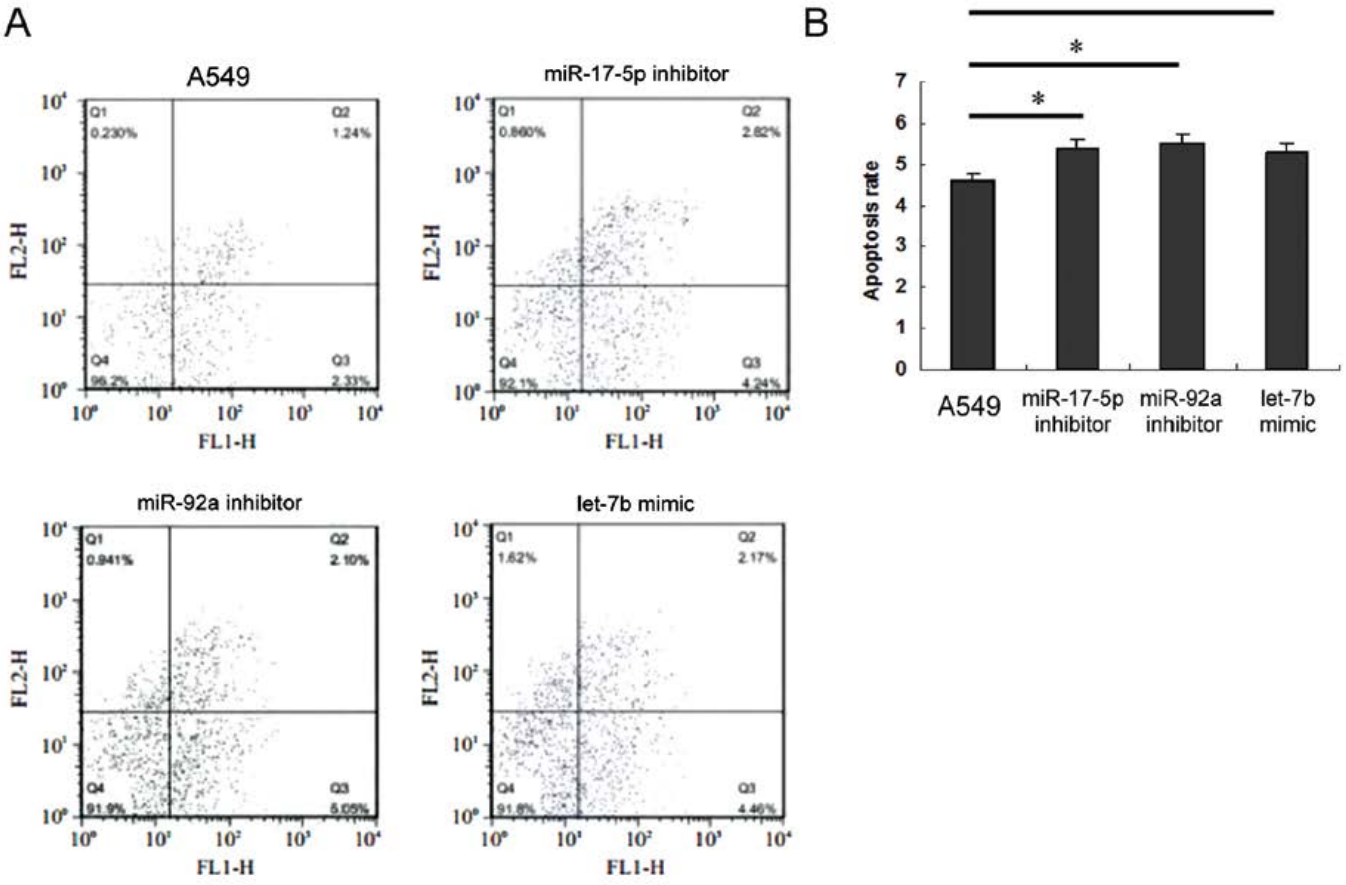

Figure 6. Apoptosis in A549/GR cells transfected with mir-17-5p, mir-92a inhibitors or let-7b mimic. **A:** apoptosis; **B:** apoptosis rate. After transfection with miR-17b, miR-92a inhibitors or let-7b mimic, the apoptosis rate of A549/GR cells was significantly higher vs the control group (*$p<0.05$).

In this study, three miRs (miR-17, miR-92a, and let-7b) with expression differences were identified through previous bioinformatics analysis [9,10]. Compared with A549 cells, high expression of miR-17-5p and miR-92a, and low expression of let-7b were found in A549/GR cells by RT-PCR. This result is consistent with other studies which found that the miR-17-92 family played a proto-oncogenic role in a variety of malignant tumors, and that let-7b acted as a tumor suppressor gene [18]. Further cytotoxicity assays showed that high expression of miR-17-5p or miR-92a or low expression of let-7b in the A549 cell line resulted in decreased sensitivity and increased resistance to Gefitinib. In contrast, lower expression of miR-17-5p or miR-92a or high expression of let-7b in A549/GR cells resulted in an increased sensitivity to Gefitinib. Cell cloning assays showed that high expression of miR-17-5p or miR-92a or low expression of let-7b in A549 cells resulted in enhancement of cell proliferation and increase of cell clone numbers. In contrast, low expression of miR-17-5p or miR-92a or high expression of let-7b in A549/GR resulted in a decrease in cell proliferation and in the number of cell clones.

The main mechanism of Gefitinib is via DNA binding in the nuclei of tumor cells, inducing DNA damage and apoptosis. This study showed that high expression of miR-17-5p and miR-92a and low expression of let-7b in A549/GR cells resulted in enhanced proliferation and antiapoptotic activity. Furthermore, this study showed that miR-17-5p and miR-92a can act as proto-oncogenes and promote cell proliferation, and that let-7b can act as a tumor suppressor gene and inhibits cell proliferation. Changes in the expression of miR-17-5p, miR-92a and let-7b can affect cell proliferation, apoptosis, and cell sensitivity to Gefitinib. Enhancement in antiapoptotic ability may directly reduce the killing effect of Gefitinib on tumor cells, therefore leading to cell resistance to Gefitinib.

We have explored the molecular mechanism of drug resistance in NSCLC chemotherapy. This will provide a new theoretical basis and technical approach for improving the overall efficacy of treatment against NSCLC, and for establishing a rational individualized treatment plan.

Conflict of interests

The authors declare no confict of interests.

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