

Original Article

MiRNA-1469 promotes lung cancer cells apoptosis through targeting STAT5a

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Received November 27, 2014; Accepted February 10, 2015; Epub February 15, 2015; Published March 1, 2015

Abstract: MicroRNAs play key roles in cell growth, differentiation, and apoptosis. In this study, we described the regulation and function of miR-1469 in apoptosis of lung cancer cells (A549 and NCI-H1650). Expression analysis verified that miR-1469 expression significantly increased in apoptotic cells. Overexpression of miR-1469 in lung cancer cells increased cell apoptosis induced by etoposide. Additionally, we identified that Stat5a is a downstream target of miR-1469, which can bind directly to the 3'-untranslated region of the Stat5a, subsequently reducing both the mRNA and protein levels of Stat5a. Finally, co-expression of miR-1469 and Stat5a in A549 and NCI-H1650 cells partially abrogated the effect of miR-1469 on cell apoptosis. Our results show that miR-1469 functions as an apoptosis enhancer to regulate lung cancer apoptosis through targeting Stat5a and may become a critical therapeutic target in lung cancer.

Keywords: MicroRNA, lung cancer, Stat5a, apoptosis

Introduction

Lung cancer is one of the leading causes of cancer-related deaths in men and women and accounts for over a million deaths worldwide each year [1-3]. Lung cancer can be categorized into two clinically relevant groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC includes adenocarcinoma, squamous cell carcinoma and large cell carcinoma [4]. Recently, accumulated evidences have shown that miRNAs are deregulated in lung cancer cells and may act as oncogenes or tumor suppressor genes. For example, miR-197 [5], miR-21 [6, 7], and miR-198 [8] are deregulated in lung cancer apoptosis. These studies provide new insights into lung cancer biology and deserve broad investigation.

MicroRNAs (miRNAs) are small, non-coding RNAs, which are 18-23 nucleotides (nt) in length and regulate gene expression post-transcriptionally by binding to the 3'-untranslated region (UTR) of their target mRNAs [9]. It was reported that miRNAs are involved in crucial biological processes, including development, differentiation, proliferation and apoptosis [10, 11]. Many studies have shown that miRNAs

such as miR-372, miR-202 and miR-509 [12-14] regulate cellular apoptosis.

Recently, miR-1469 was found to be located on chromosome 15 and was upregulated during metastasis in clear cell renal cell carcinoma [15]. In addition, miR-1469 was downregulated after treatment of Polyphenon-60, which has been used to treat acne clinically [16, 17] and significantly inhibited human breast cancer MCF-7 cell growth. In this study, we examined the role of miR-1469 in apoptosis of lung cancer cells. MiR-1469 was shown to enhance etoposide (VP16)-induced cell apoptosis. Further investigation revealed that Stat5a, which regulates proliferation and apoptosis in many cancers [18-23], is a direct and functional target of miR-1469. In addition, Stat5a can partly inhibit the A549 and H1650 cells apoptosis induced by miR-1469.

Materials and methods

Cell culture

Both A549 and NCI-H1650 (H1650) cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml strepto-

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mycin, and 100 units/ml penicillin. The medium was changed at alternate days and the cells were split before they reached 100% confluency.

Oligonucleotide, plasmids, and transfection

MiRNA-1469 mimics and miRNA-1469 inhibitors were synthesized by Genepharma group (Shanghai, China). The full-length 3'UTR of Stat5a was subcloned into the pISO luciferase plasmid [24] to generate pISO-Stat5a-3'UTR. Mutant construct of Stat5a-3'UTR, named pISO-Stat5a-3'UTR-mut, which carried a substitution of three nucleotides within the core binding sites of Stat5a-3'UTR, was conducted using mutant PCR primers. Primers used in this study are shown in [Supplementary Table 1](#). To construct FLAG-tagged Stat5a, PCR was performed using plasmid pMD-Stat5a as a template. The plasmid was a generous gift from Dr. Liu (State Key Laboratory of Molecular Oncology, Cancer Institute and Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China). Lipofectamine 2000 (Life Technologies Corporation, Grand Island, NY, USA) was used for DNA plasmid and oligonucleotide transfection according to the manufacturer's protocol.

RNA extraction and real-time PCR

Total RNA was extracted with TRIzol reagent (Life Technologies Corporation). Reverse transcription was performed using FastQuant RT Kit (with gDNase) (TianGen, Beijing, China) according to the manufacturer's protocol. MiRNA-1469 was reversely transcribed by the looped primer, which binds to six nucleotides at the three portions of miRNA-1469 molecules. Reverse transcription of Stat5a mRNA was performed according to the manufacturer's protocol. Real-time PCR was performed using SuperReal PreMix Plus (TianGen) according to the manufacturers' recommendations. The U6 small nuclear RNA and β -actin mRNA were used as internal controls for miRNA-1469 and Stat5a mRNA, respectively. Primers for PCR are shown in the [Supplementary Table 1](#). All of the reactions were run in triplicate.

Luciferase assay

Cells were cultured in 96-well plates and transiently co-transfected with firefly luciferase

reporter gene constructs and miRNA-1469 mimics using Lipofectamine 2000 (Life Technologies Corporation). After 48 h, luciferase activity was measured using a dual luciferase reporter assay system according to the manufacturer's protocol (Promega, Madison, WI). The pRL-TK Renilla was used as an internal control.

Analysis of cell apoptosis and cell cycle

Cells transfected with negative control or miRNA-1469 mimics for 24 h were starved overnight and then treated with etoposide for 48 h. The flow cytometry method (FCM) assay was performed to analyze cell apoptosis and cell cycle. The Annexin V FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) was used to detect cell apoptosis and the BD cycletest Plus DNA Reagent Kit (BD Biosciences) was used to detect cell cycle according to the manufacturer's instructions.

Western blot

Cellular proteins were extracted after treatment as described previously [25]. Clarified cell lysates were equalized for protein concentration using the BCA protein assay. The protein samples were resolved by SDS-PAGE and processed with Western blot. The antibodies against Stat5a (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-1081), phosphorylated H2AX (gH2AX) (Cell Signaling Technology, Beverly, MA, USA, # 9718), Bcl-2 and b-actin (Cell Signaling Technology, #4970) were respectively used to detect their targeting proteins:

Statistical analysis

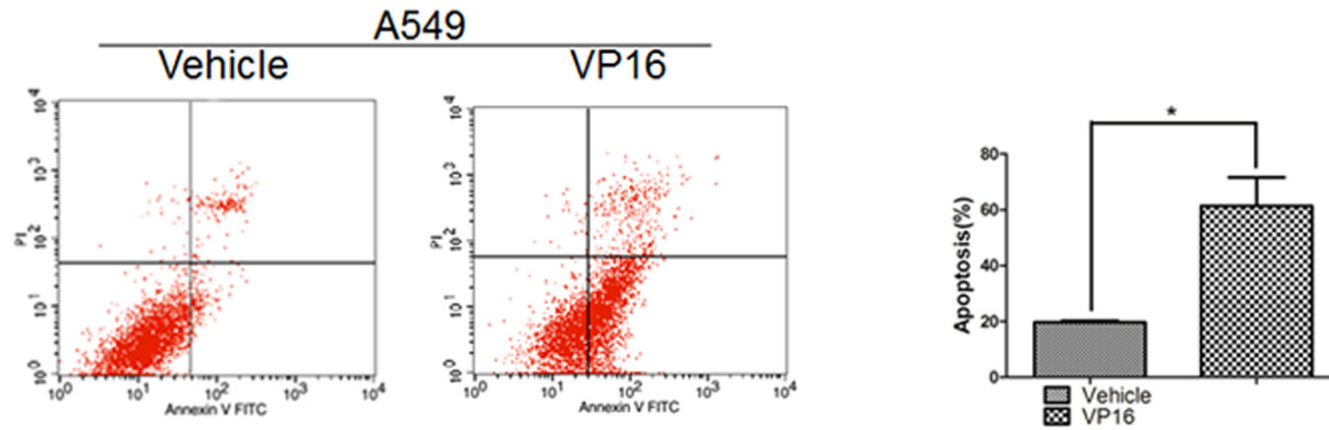
Data were presented as mean \pm SD from at least three separate experiments, and Student's t-test analysis was performed using SPSS 17.0 software. Statistical significance was set at $p < 0.05$.

Results

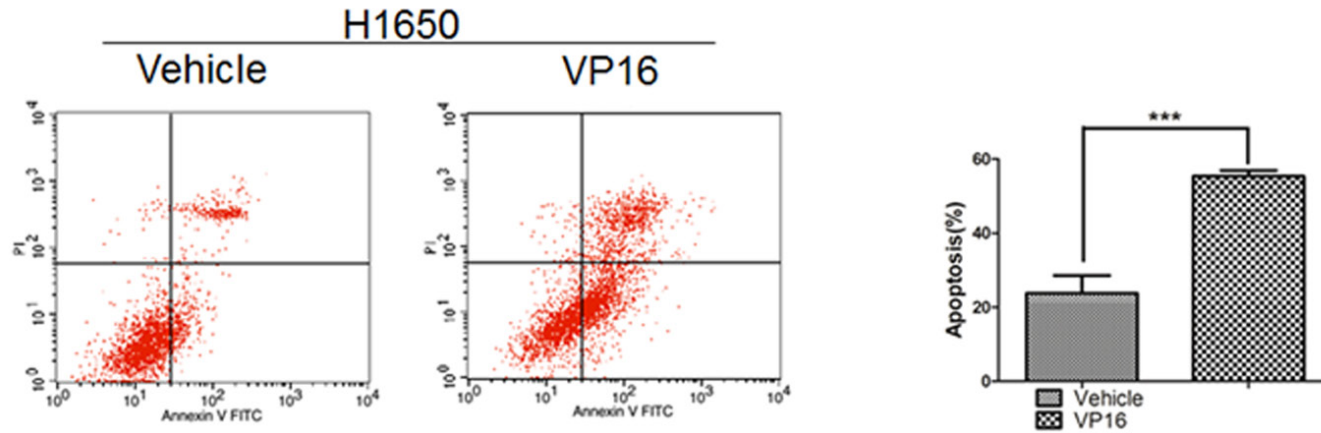
MiR-1469 is upregulated during apoptosis of lung cancer cells

Etoposide (VP16), a chemotherapy drug derived from a type of plant alkaloid known as a podophyllotoxin, is thought to work by blocking the action of an enzyme in cells called topoisomerase II [26]. Etoposide has been used for the

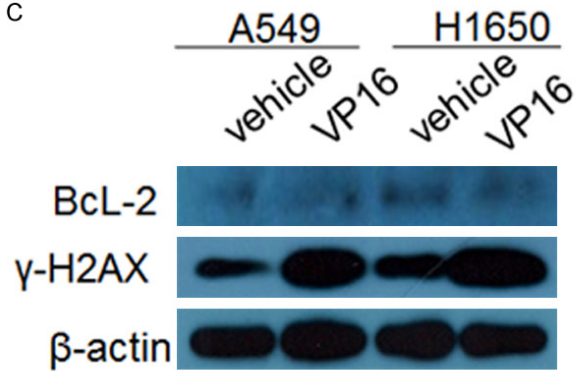
A



B



C



D

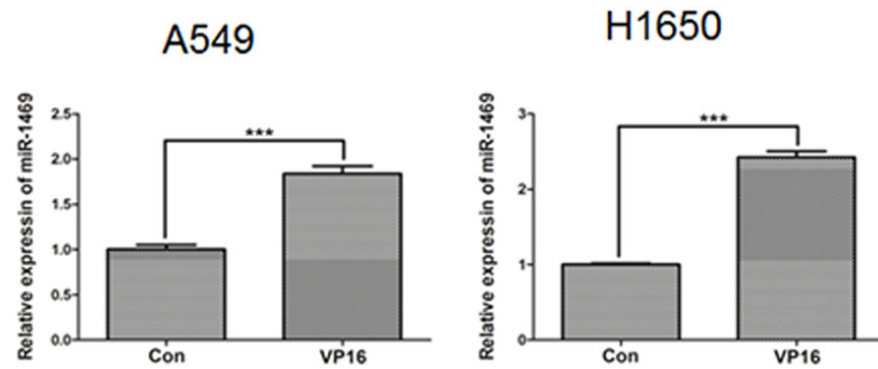


Figure 1. MiR-1469 is upregulated during apoptosis of lung cancer cells. A. The apoptosis of A549 cells was induced by treating with VP16 (100 μ M) for 48 h. The histogram shows the apoptotic cell percentage detected by FCM, and error bars denote mean \pm SD (right panel). B. Flow cytometry showed apoptosis of H1650 cells after VP16 (100 μ M) treatment. The histogram shows the apoptotic cell percentage detected by FCM, and error bars denote mean \pm SD (right panel). C. Western blot analysis was used to detect the expression of Bcl-2 and γ -H2AX in A549 and H1650 cells both treated by VP16 (100 μ M) for 48 h. β -actin was detected as a loading control. D. The histogram shows the expression of miRNA-1469 in A549 (left panel) and H1650 cells (right panel) 48 h after VP16 treatment (100 μ M). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

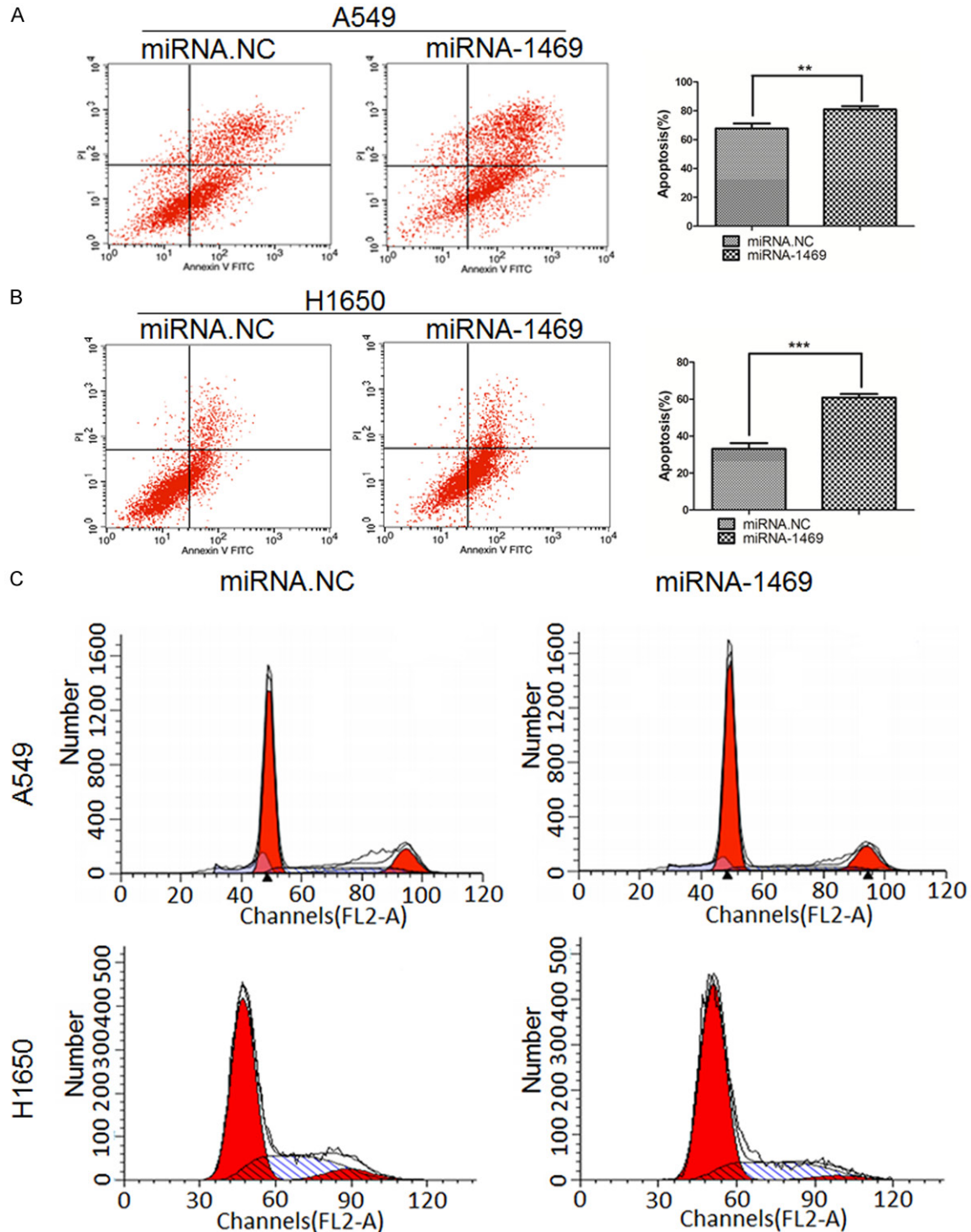


Figure 2. Overexpression of miRNA-1469 promotes VP16-induced cell apoptosis. A. The apoptosis of A549 cells was induced by treatment with VP16 (100 μ M) for 48 h after miRNA-1469 mimics (20 nM) transfection for 24 h. The histogram shows the apoptotic cell percentage detected by FCM, and error bars denote mean \pm SD (right panel). B. Flow cytometry was used to detect apoptosis of H1650 cells induced by treating with VP16 (100 μ M) after miRNA-1469 mimics (20 nM) transfection for 24 h. The histogram shows the apoptotic cell percentage and error bars denote mean \pm SD (right panels). **, $p < 0.01$; ***, $p < 0.001$. C. Flow cytometry was used to detect the cell cycle of A549 and H1650 cells 48 h after miRNA-1469 mimics (20 nM) transfection.

treatment of a wide variety of cancers [27] and can induce many types of cancer cells apoptosis [28-31]. Here we showed that the number of apoptotic cells markedly increased after treating with VP16 both in lung cancer cells A549 and H1650 compared with the control (**Figure 1A, 1B**). There are several proteins including Bcl2 and γ -H2AX (phosphorylated H2AX), which were reported to be related to apoptosis. In addition, we found that the level of Bcl2 did not change, but γ -H2AX was significantly upregulated in A549 and H1650 cells during the treatment (**Figure 1C**). In the meantime, Real-time PCR was performed to detect whether the level of miRNA-1469 was changed. The results showed that miRNA-1469 was significantly upregulated during VP16-induced cell apoptosis (**Figure 1D**), suggesting that miRNA-1469 may be involved in apoptosis of lung cancer cells.

Overexpression of miRNA-1469 promotes VP16-induced lung cancer cell apoptosis

To investigate whether miRNA-1469 regulates apoptosis, we transfected miRNA-1469 mimics and control miRNA into A549 and H1650 cells respectively, followed by the treatment of VP16 for 48 h. FCM was performed to detect the number of apoptotic cells including early and late apoptosis. The results indicated that overexpression of miRNA-1469 could significantly promote VP16-induced cell apoptosis compared with control miRNA (**Figure 2A, 2B**). These data demonstrated that miRNA-1469 acts as a tumor suppressor in apoptosis of lung cancer cells. However, overexpression of miRNA-1469 did not affect the cell cycle in both A549 and H1650 cells (**Figure 2C**).

MiRNA-1469 specifically targets stat5a and decreases its expression

To explore the molecular mechanism of miRNA-1469 in the regulation of lung cancer cells apoptosis, we used TargetScan, miRanda and miRbase to identify the potential downstream

targets of miRNA-1469. The result of the analysis showed that Stat5a is one of the predicted targeting genes and that there is a miRNA-1469 binding site at nucleotides 226-232 of Stat5a-3'UTR (**Figure 3A**, upper panel). Homology search showed that the miRNA-1469 targeting sequence at nucleotides 226-232 of the Stat5a-3'UTR was highly conserved in human, chimpanzee and rhesus (**Figure 3A**, bottom panel). To determine whether Stat5a is regulated by miRNA-1469 through direct binding to its 3'UTR, we constructed pISO-Stat5a-3'UTR and pISO-Stat5a-3'UTR-mut (3'UTR was mutated to block binding by miRNA-1469) (**Figure 3B**). Co-transfection of the luciferase reporter pISO-Stat5a-3'UTR and miRNA-1469 into A549 cells produced nearly 80% decrease in the luciferase activity compared to the negative control (**Figure 3C**, right panel). This suppressive effect was rescued by pISO-Stat5a-3'UTR-mut (three nucleotide substitutions in the core binding sites) as shown (**Figure 3C**, right panel). The similar effect was also found in H1650 cells (**Figure 3C**, left panel). Consistent with these results, we found a significance decrease of endogenous Stat5a protein and mRNA level in A549 (**Figure 3D, 3E** left panels) and H1650 cells (**Figure 3D, 3E** right panels) transfected with miRNA-1469 mimics (**Figure 3F**, left panel). On the contrary, transfection with miR-1469 inhibitor (**Figure 3F**, right panel) induced a significant increase in Stat5a expression at mRNA (**Figure 3E**) and protein levels (**Figure 3D**). These results suggest that miRNA-1469 down-regulates Stat5a expression by directly targeting its 3'UTR.

MiR-1469 regulates apoptosis through Stat5a

We also investigated whether miRNA-1469 functions in cell apoptosis via targeting to Stat5a. To examine the role of Stat5a in lung cancer cell apoptosis, a vector carrying Stat5a coding sequence (CDS), which lacked the 3'UTR but contained the miR-1469 binding sites, was introduced into A549 cells for the overexpression of Stat5a. The results showed that overex-

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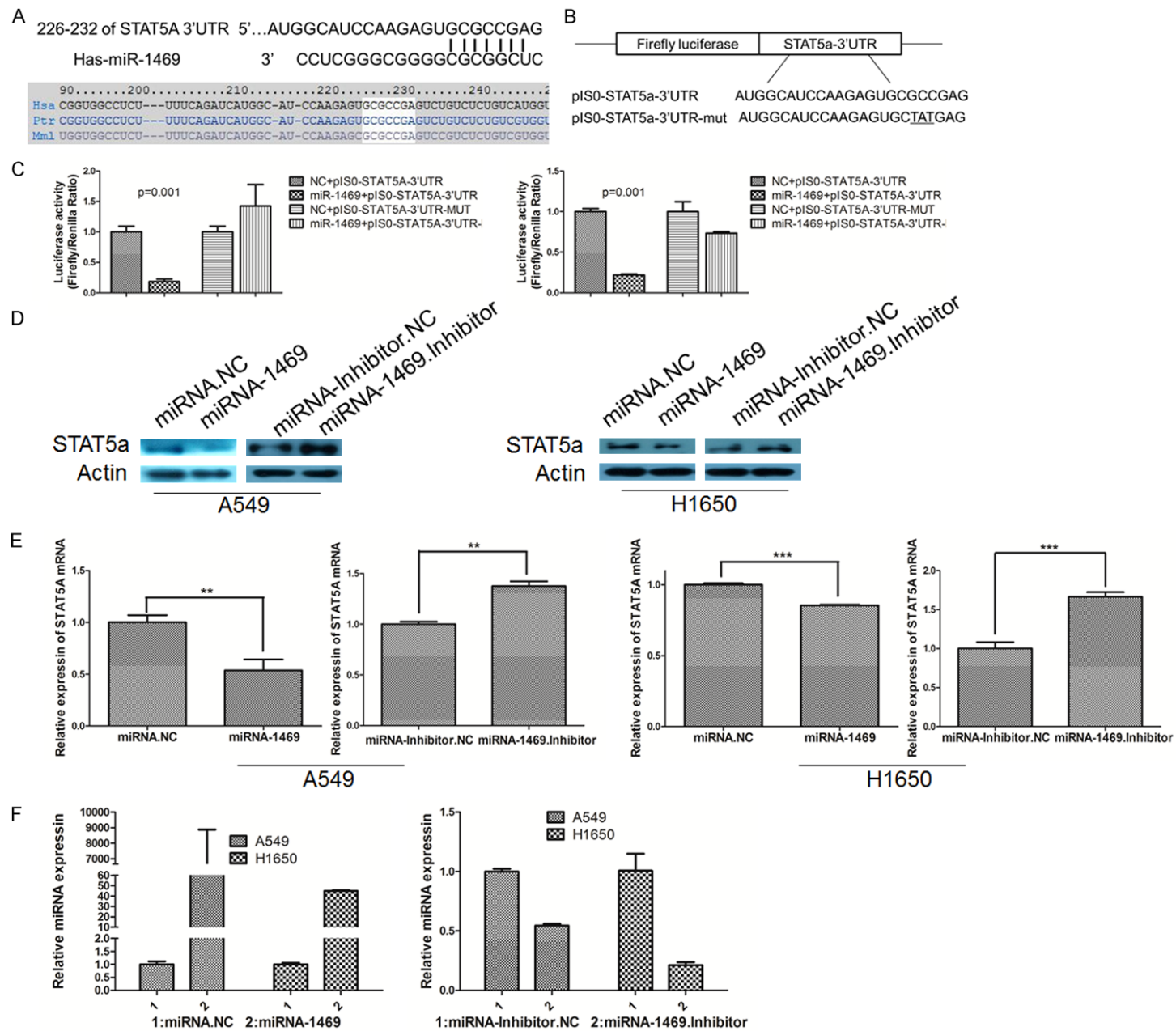


Figure 3. Stat5a is a direct target of miRNA-1469. A. MiR-1469 targeting site resides at nucleotides 226-232 of Stat5a-3'UTR and is highly conserved in different species. Upper panel: sequence alignment of miR-1469 with binding sites on the Stat5a-3'UTR. Bottom panel: sequence of the miR-1469 binding site within the Stat5a-3'UTR of three species (human, chimpanzee and rhesus). B. Diagram of the luciferase reporter plasmids including plasmid with the full-length Stat5a-3'UTR insert (pISO-Stat5a-3'UTR) and plasmid with a mutant Stat5a-3'UTR (pISO-Stat5a-3'UTR-mut) which carried a substitution of three nucleotides within the miR-1469 binding site. C. Luciferase activity assay demonstrates a direct targeting of the Stat5a-3'UTR by miR-1469. A549 cells (left panel) and H1650 cells (right panel) were transfected with miR-1469 mimics (20 nM) and pISO-Stat5a-3'UTR /pISO-Stat5a-3'UTR-mut. pRL-SV40 Renilla was used for the normalization of transfection efficiency. After 48 h, the luciferase activities were measured. D. Western blotting was used to detect the expression of the Stat5a protein after miRNA-1469 mimics (20 nM) or miRNA-1469; Inhibitor (40 nM) transfection of A549 (left panel) or H1650 (right panel) cells. E. Stat5a mRNA in the A549 (left panel) or H1650 (right panel) cell lines treated as above was measured with real-time RT-PCR. β -actin was used as internal control. F. Overexpression of miR-1469 mimics (20 nM) or miRNA-1469; Inhibitor (40 nM) in A549 or H1650 cell lines transfected miRNA-1469 mimics or miRNA-1469; Inhibitor was measured by real-time RT-PCR. U6 was used as internal control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

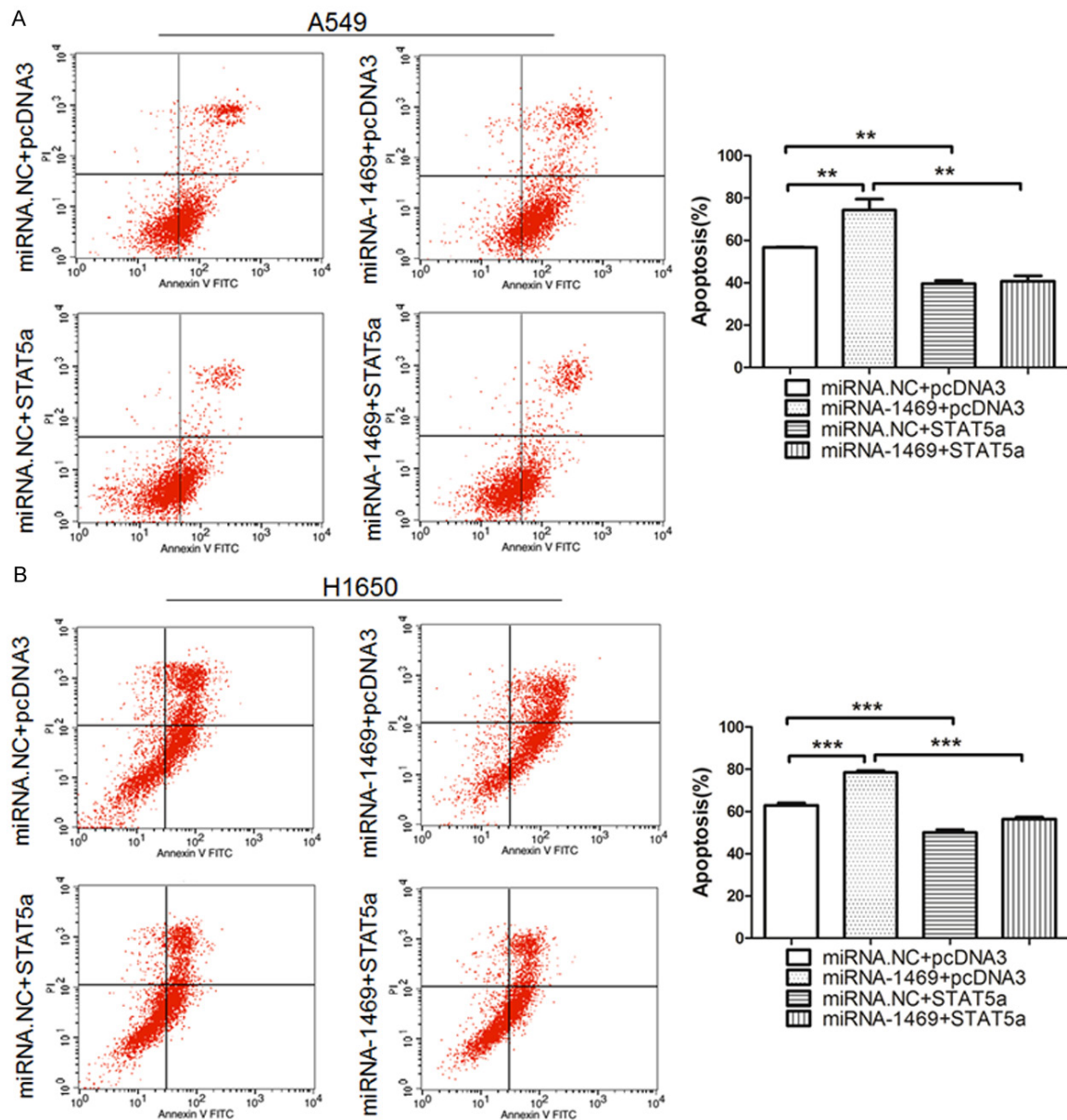


Figure 4. MiR-1469 regulates apoptosis through Stat5a. A. Apoptosis of A549 cell were detected by FCM 48 h after miRNA-1469 mimics and combined with Stat5a transfection (left panel). Error bars denote mean \pm SD (right panel).

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B. The apoptosis of H1650 cells 48 h after transfection of miRNA-1469 mimics and combined with Stat5a were detected by FCM (left panel). Error bars denote mean \pm SD (right panel). **, $p < 0.01$; ***, $p < 0.001$.

pression of miRNA-1469 increased the number of VP16-induced apoptotic cells while on the contrary, co-expression of miRNA-1469 with Stat5a reduced apoptosis induced by VP-16 (**Figure 4A**). The same phenomenon was also observed in H1650 cells by FCM (**Figure 4B**). These results suggest that miRNA-1469 regulation of lung cancer cells apoptosis, at least in part, depends on its regulation of Stat5a.

Discussion

Abnormal expression of miRNA-1469 was observed in some cancers [15, 17, 32], and was not previously reported to be implicated in lung cancer [33]. However, in this study, we found that miR-1469 is related to apoptosis of lung cancer cells.

Different methods have been developed for computation of miRNA target prediction. Currently, such available methods include TargetScan, PicTar, rna22, miRanda and miRbase. In this study, we used two strategies to identify the potential downstream targets of miR-1469. Firstly, we adopted three widely used bioinformatic algorithms (TargetScan, miRanda and miRbase) to help identify miR-1469 targets in humans. Secondly, we compared the human sequence with interspecies homology. These methods suggest that Stat5a might be a target of miRNA-1469 (**Figure 3A**).

It is a well-known fact that signal transducer and activator of transcription-5 (STAT5) is involved in a variety of cellular processes, including survival, proliferation, invasion, angiogenesis and immune evasion. In fact, STAT5 includes two closely related proteins, Stat5a and Stat5b [34-37]. Presently, STAT5a was found to mediate chemotherapeutic sensitivity in blood malignancies [38]. In this study, we used detailed experiments to prove that Stat5a was a target of miRNA-1469. By interacting directly with the 3'UTR of Stat5a mRNA, miRNA-1469 regulates Stat5a expression at the post-transcriptional level. Overexpression of miRNA-1469 could promote VP16-induced apoptosis and significantly decrease endogenous Stat5a protein in tumor cells. The finding expanded the list of miRNA members involved in regulating cellular apoptosis.

In conclusion, we have shown, for the first time that the miR-1469 level was significantly upregulated during the apoptosis of lung cancer cells. Also, the overexpression of miRNA-1469 strongly promotes VP16-induced apoptosis. Additionally, we identified Stat5a as a direct and functional target of miRNA-1469 and also revealed that miR-1469 regulates apoptosis through Stat5a. This finding might lead to unique therapeutic options for treating human cancers.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81070424 and No. 81272303).

Disclosure of conflict of interest

None.

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Supplementary Table 1. Primers used in this study

The primers for plasmid construction

| | |
|----------------|--|
| miRNA-1469-F | ACACTCCAGCTGGG CTCGGCGCGGGGCGCG |
| miRNA-1469-R | CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGAGCCCG |
| STAT5a-F | GGTTTGAGTGAGGGTTTCT |
| STAT5a-R | GGCAACAGCATCATAGACT |
| STAT5a-UTR-F | TTGAGCTCTATGCAATGTGAAGCGGTCG |
| STAT5a-UTR-R | GCTCTAGAGAGTCTGGAGTCCACGTTCA |
| STAT5a-UTR-M-F | CCAAGAGTGCTATGAGTCTGTCT |
| STAT5a-UTR-M-R | AGACAGACTCATAGCACTCTTGG |
| U6-F | CTCGCTTCGGCAGCACA |
| U6-R | AACGCTTCACGAATTTGCGT |
| miRNA URP | TGGTGTCGTGGAGTCG |