

## Original Article

# microRNA-377 inhibits non-small-cell lung cancer through targeting AEG-1

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**Abstract:** Non-small-cell lung cancer (NSCLC) is the leading cause of cancer-related deaths. MicroRNAs (miRNAs) have been reported to be involved in tumorigenesis. However, the underlying mechanisms of microRNA-377 (miR-377) in NSCLC remain unknown. Hence, in the present study, we aimed to explore the role of miR-377 in the development of NSCLC, with identifying its target genes. The results showed that miR-377 expression was significantly decreased in NSCLC tissues as well as in NSCLC cell lines. Moreover, high expression of miR-377 could markedly inhibit the viability, proliferation, migration and invasion of NSCLC cells. The bioinformatics analysis results showed that astrocyte elevated gene-1 (AEG-1), an oncogene as previously reported, was a potential target gene of miR-377, which was further validated by dual-luciferase reporter assay. Besides, the expression of AEG-1 in protein level was decreased by miR-377 overexpression, but not in mRNA level. In addition, AEG-1 overexpression could reverse the inhibitory effects on NSCLC cells caused by miR-377 transfection. In conclusion, our results suggested that miR-377 played an important role in the development of NSCLC by regulating AEG-1 and may be a potential therapeutic target for NSCLC.

**Keywords:** Non-small-cell lung cancer, miR-377, AEG-1, miRNAs, proliferation

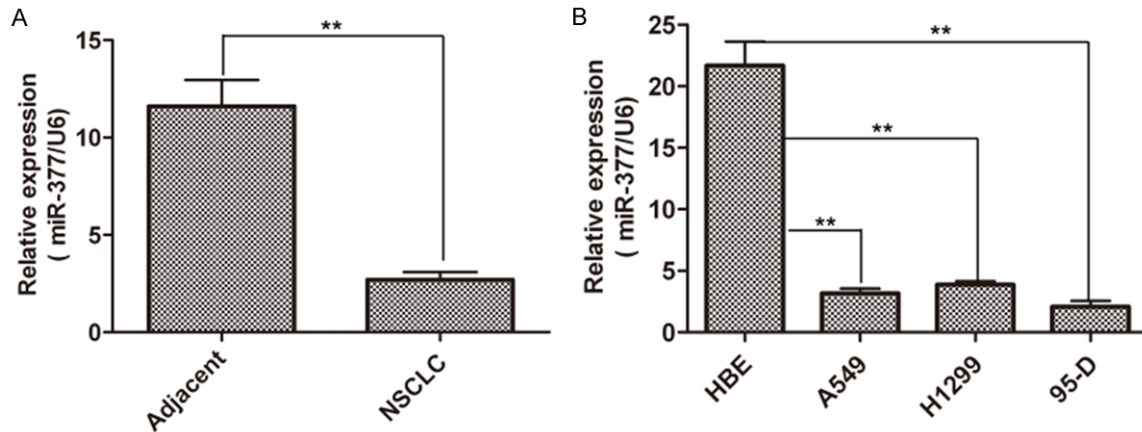
## Introduction

Lung cancer is the most common reason for cancer-related deaths, among which non-small-cell lung cancer (NSCLC) is the leading cause [1]. MicroRNAs (miRNAs) are a group of endogenous, single-stranded and non-coding RNA with about 22 nucleotides [2]. MiRNAs have been emerging as oncogenes or tumor suppressors depend on the functions of their target genes [3]. According to the previous reports, the target genes of miRNAs were negatively regulated by miRNAs through the inhibition of post-transcriptional translation without changing DNA sequences via pairing with the 3'-untranslated region (UTR) of the target genes [4, 5]. So far, miRNAs were shown to be involved in regulating more than half of human gene expression [6-8] and a large number of them have been found to be abnormally expressed (up-regulated or down-regulated) in cancer cells compared with corresponding normal cells [9, 10].

To date, several studies have focused on the relationship between miRNAs and NSCLC. For

example, miR-133a was shown to be a tumor-suppressive miRNA in human NSCLC, and its down-regulation suggested deterioration in NSCLC patients [11]. Similarly, miR-675-5p was found to be a novel tumor suppressor in NSCLC, whose target gene was GPR55 [12]. In addition, miR-489 and its target gene SUZ12 [13], miR-15a and its target gene BCL2L2 [14], miR-30d-5p and its target gene CCNE2 [15], were all shown to be involved in human NSCLC development.

miR-377 is another cancer-related miRNA. Ze-havi *et al.* reported that miR-377 expression was absent in malignant melanoma, and both E2F3 and MAP3K7 were found to be direct targets of miR-377 [16]. The results reported by Wang *et al.* showed that miR-377 was down-regulated in human clear cell renal cell carcinoma, proving that miR-377 could serve as a tumor suppressor through targeting ETS1 [17]. miR-377 was also shown to inhibit cell proliferation and invasion in hepatocellular carcinoma [18], osteosarcoma [19] and glioblastoma [20]. However, the relationship between miR-



**Figure 1.** The comparison of miR-377 expression in normal lung and NSCLC tissues and cells. A. miR-377 was highly expressed in normal lung tissues, but lowly expressed in NSCLC tissues. B. miR-377 was highly expressed in normal human bronchial epithelial cells HBE, but down-regulated in NSCLC cell lines, including A549, H1299 and 95-D. Each column represents mean  $\pm$  SD;  $n = 3$  per group. \* $P < 0.05$  and \*\* $P < 0.01$ .

377 and NSCLC remains unknown. Therefore, this study set out to investigate the role of miR-377 in NSCLC. The expressions of miR-377 in NSCLC and normal tissues and cells were compared. Next, the effects of miR-377 high expression on the viability, proliferation, migration and invasion of NSCLC cells were also measured.

Astrocyte elevated gene-1 (AEG-1), also known as Lysine-rich CEACAM1 (LYRIC) or Metadherin, is a novel oncogene in malignant tumors, which is also a protein that in humans is encoded by the Metadherin gene [21]. AEG-1 is first discovered in human fetal astrocytes by Su et al. in 2002 [22], which is ubiquitously expressed in all organs and distributed in the cell cytoplasm, membrane, nucleus and endoplasmic reticulum [23]. To date, AEG-1 has been found to be closely related to epithelial-mesenchymal transition (EMT) process [24, 25]. In addition, AEG-1 was also found to be associated with gastric cancer [26], breast cancer [27] and NSCLC [28]. In general, AEG-1 was overexpressed in tumors and correlated with poor prognosis [29]. We found that AEG-1 was a potential target gene of miR-377. The connections between AEG-1 and miR-377 were investigated via dual luciferase reporter assay. In conclusion, miR-377 could regulate the tumorigenesis of NSCLC, possibly through targeting AEG-1. Our results showed an inhibitory effect of miR-377 on NSCLC and provided an experimental basis for the potential diagnostics, prognostics and therapeutics.

## Materials and methods

### Sample collection

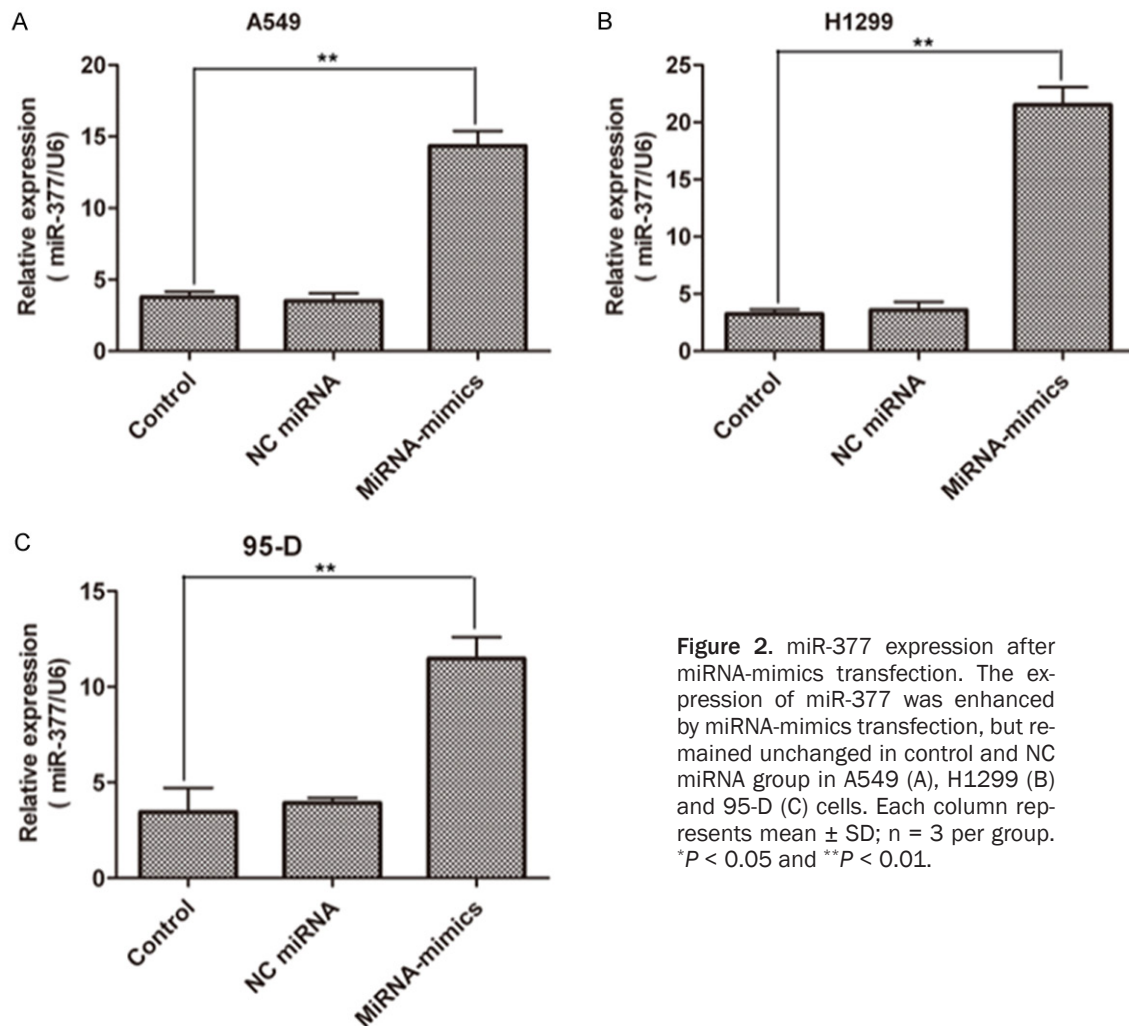
All the samples were collected from Tianjin Medical University General Hospital. The informed consents from all of the patients involved were obtained prior to the initiation of this study. No any other drugs were employed before sample collection. NSCLC samples and paired adjacent non-tumor samples were collected from 40 patients via lung resection. The samples were immediately stored in liquid nitrogen in preparation for use.

### Cell culture

Three human NSCLC cell lines (A549, H1299 and 95-D) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). A normal human bronchial epithelial cell line (HBE), were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>.

### Ectopic expression of miR-377 and AEG-1

For miR-377 up-regulation, the negative control miRNA (NC miRNA) and miRNA-mimics (GenePharma, Shanghai, China) were employed. For AEG-1 overexpression, AEG-1 cDNA were



**Figure 2.** miR-377 expression after miRNA-mimics transfection. The expression of miR-377 was enhanced by miRNA-mimics transfection, but remained unchanged in control and NC miRNA group in A549 (A), H1299 (B) and 95-D (C) cells. Each column represents mean  $\pm$  SD; n = 3 per group. \* $P < 0.05$  and \*\* $P < 0.01$ .

purified and cloned into a pGL3 vector (Promega, Madison, WI, USA) to generate pGL3-AEG-1 recombinant plasmids. An empty vector served as scramble group. All miRNAs and plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

#### Real-time quantitative polymerase chain reaction

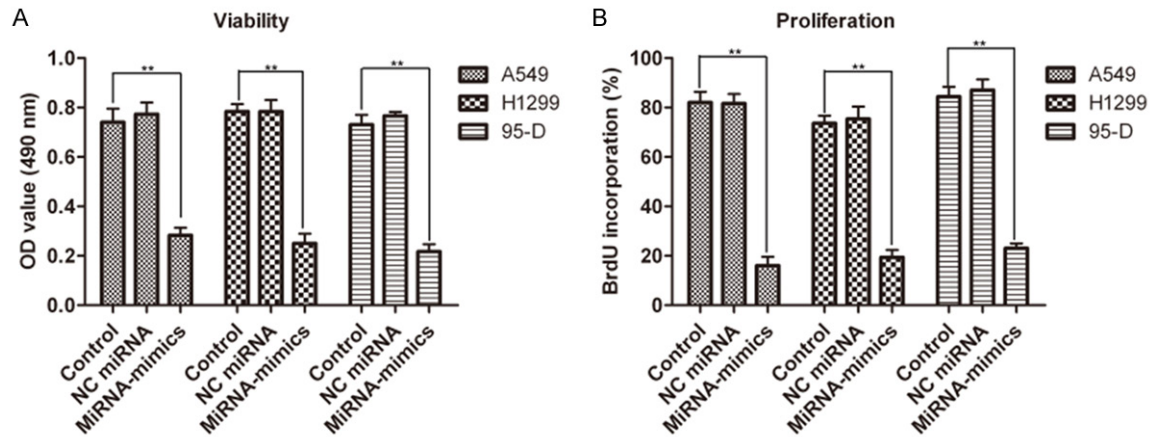
Total RNA was isolated from the tissues and cells using TRIzol reagent (Invitrogen). The reverse transcription reaction was performed using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocols. Real-time quantitative PCR was conducted using SYBR Green PCR kit (Takara, Dalian, China) on CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA).  $\beta$ -actin was used as an endogenous control for

AEG-1 and U6 small nuclear RNA (snRNA) for miR-377. The primer sequences were as follows: miR-377 forward: 5'-AUC ACA CAA AGG CAA CUU UUG U-3' and reverse: 5'-AAA AGU UGC CUU UGU GUG AUU U-3'. U6 forward: 5'-GTA GAT ACT GCA GTA CG-3' and reverse: 5'-ATC GCA TGA CGT ACC TGA GC-3'. AEG-1 forward: 5'-TGC CTC CTT CAC AGA CCA A-3' and reverse: 5'-TCG GCT GCA GAT GAG ATA G-3'.  $\beta$ -actin forward: 5'-CCT AGA AGC ATT TGC GGT GG-3' and reverse: 5'-GAG CTA CGA GCT GCC TGA CG-3'. The threshold cycle (Ct) value was recorded. Each sample was measured in triplicate, and the relative expression of miR-377 to U6 and AEG-1 to  $\beta$ -actin was calculated using the  $2^{-\Delta\Delta Ct}$  method.

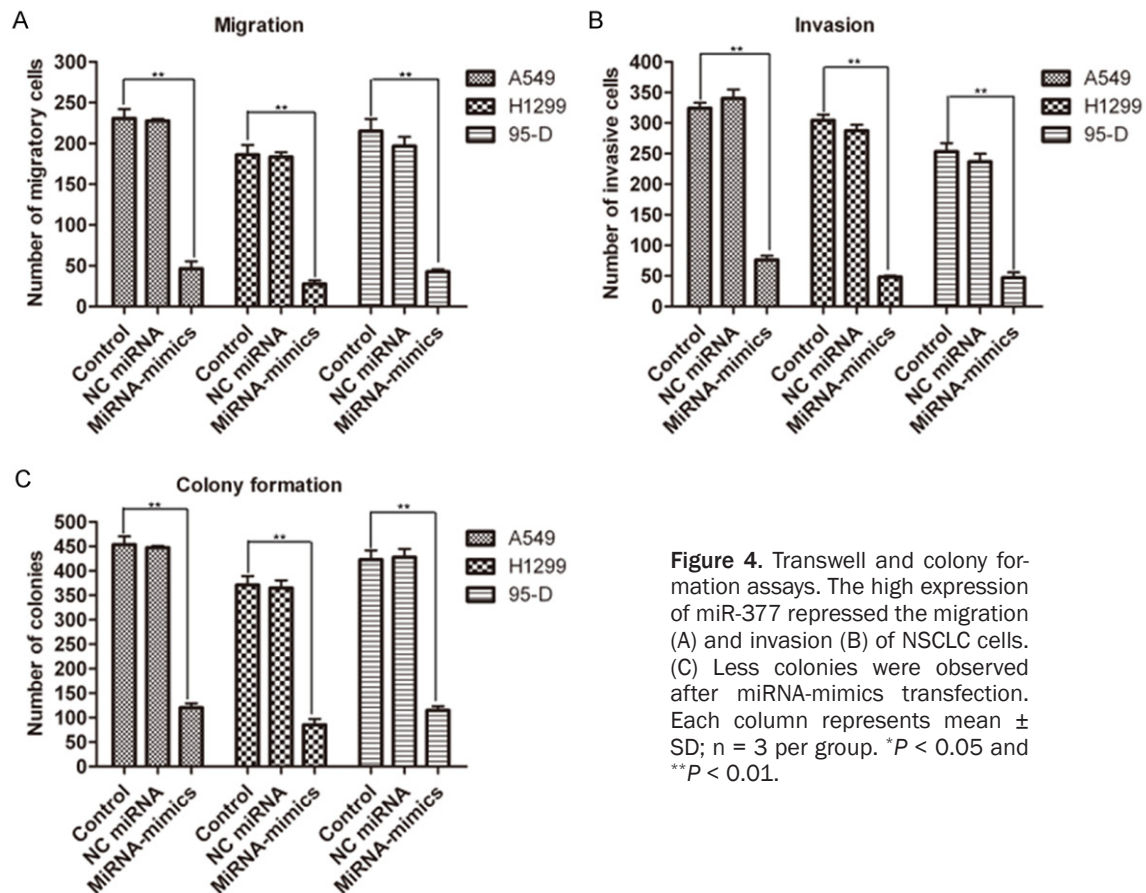
#### Western blotting

Proteins were extracted using RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) containing 1 mM phenylmethanesulfonyl fluoride (PMSF;

## miR-377 inhibits NSCLC through AEG-1



**Figure 3.** MTT and BrdU assays. The high expression of miR-377 inhibited the viability (A) and proliferation (B) of NSCLC cells. Each column represents mean  $\pm$  SD;  $n = 3$  per group. \* $P < 0.05$  and \*\* $P < 0.01$ .

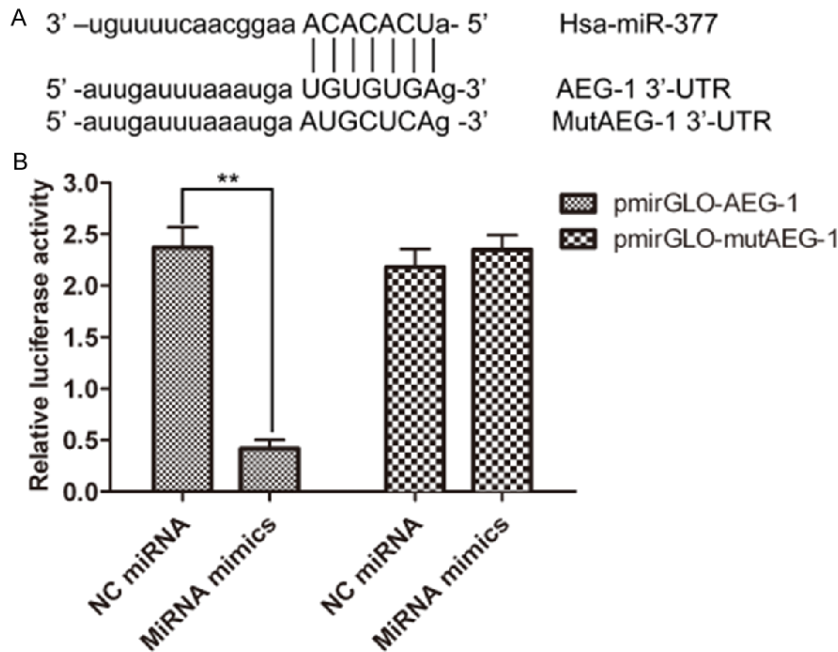


**Figure 4.** Transwell and colony formation assays. The high expression of miR-377 repressed the migration (A) and invasion (B) of NSCLC cells. (C) Less colonies were observed after miRNA-mimics transfection. Each column represents mean  $\pm$  SD;  $n = 3$  per group. \* $P < 0.05$  and \*\* $P < 0.01$ .

Sigma-Aldrich) and centrifuged at 13000 r/min for 15 min at 4°C. The protein concentration was measured using BCA Protein Assay Kit (BioTeke, Beijing, China). The proteins were isolated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF)

membrane (Thermo Fisher Scientific). Subsequently, the membrane was incubated with primary antibodies against AEG-1 (1:1000) and  $\beta$ -actin (1:2000; all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 4 h at 37°C. Next, the membrane was incubated with horseradish peroxidase (HRP) conjugated secondary





**Figure 5.** Dual luciferase reporter assay. A. The predicted binding sequences for miR-377 in the 3'-UTR of AEG-1. B. miR-377 significantly inhibited luciferase activity in the presence of pmirGLO-AEG-1 vectors, indicating that AEG-1 was a target gene of miR-377. Each column represents mean  $\pm$  SD; n = 3 per group. \* $P$  < 0.05 and \*\* $P$  < 0.01.

antibodies (1:3000; Santa Cruz Biotechnology) for 2 h at room temperature. Finally, the protein binding was detected by an enhanced chemiluminescence (ECL) detection system (Amersham, Little Chalfont, UK).

#### MTT and BrdU assays

For MTT assay, cells were seeded into 96-well plate. Next, 20  $\mu$ l of MTT was added to each well and incubated for 4 h at 37°C with 5% CO<sub>2</sub>. Dimethylsulfoxide (DMSO; Sigma-Aldrich) was added to dissolve the formazan crystals and the absorbance was measured at 490 nm. The data were presented as optical density (OD) value. For BrdU assay, cells were also seeded into 96-well plate. Next, the medium was replaced with fresh-BrdU-medium and incubated in 5% CO<sub>2</sub> at 37°C for 1 h. After fixation with 70% ethanol, cell slides were incubated with the primary human anti-BrdU antibody and secondary fluorescein isothiocyanate (FITC)-conjugated-anti-human antibody. The cells were observed and counted in randomly selected fields under a fluorescence microscope (Olympus). The data were presented as percentages of BrdU-positive cells.

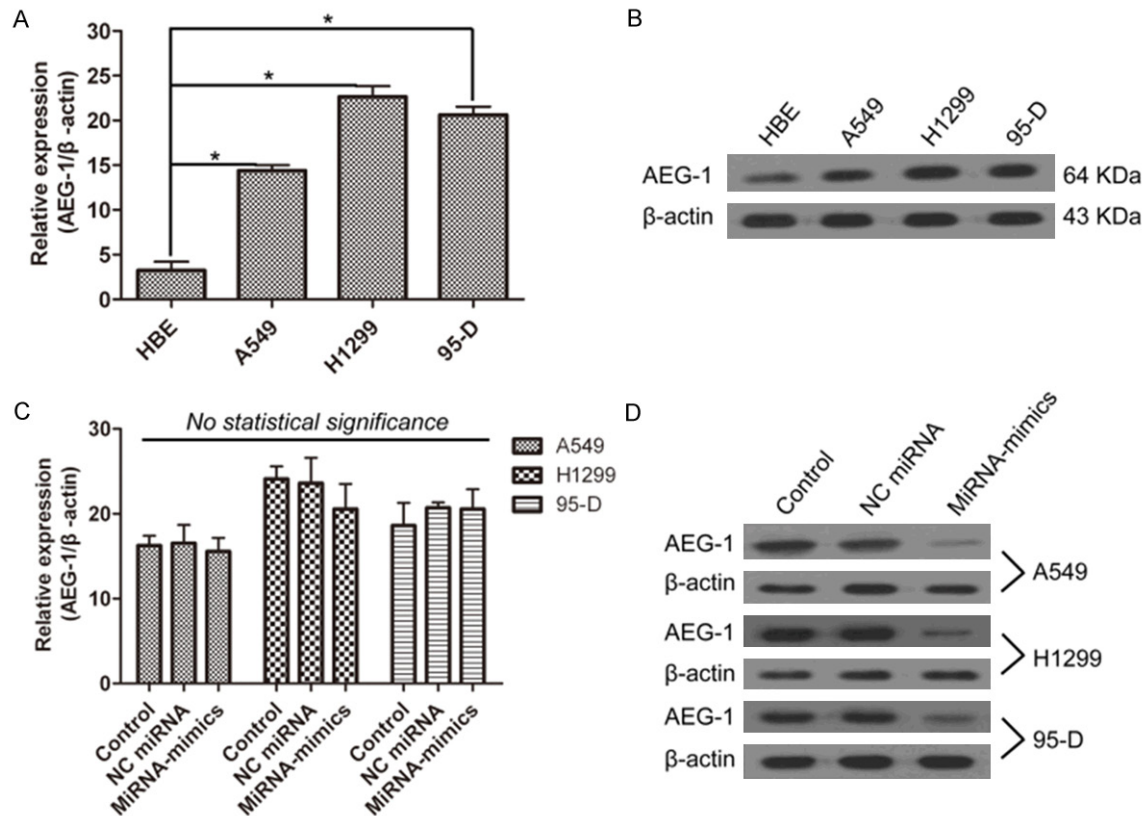
#### Migration, invasion and colony formation assays

Cell migration and invasion were performed in 24-well plate using transwell chambers (BD Biosciences, San Jose, CA, USA). Briefly, 2 $\times$ 10<sup>5</sup> cells resuspended in 100  $\mu$ l serum-free medium were added to the upper chamber. For invasion assay, the chamber was pre-coated with 40  $\mu$ l Matrigel at 37°C for 2 h to form a reconstituted basement membrane; whereas the migration assay was not. The lower chamber contained medium supplemented with 10% FBS. The non-invading cells were removed by scraping 12

h (for migration assay) or 24 h (for invasion assay) after incubation at 37°C in 5% CO<sub>2</sub>. Correspondingly, the invasive cells attached on the lower chamber were fixed with 100% methanol and stained with 0.1% crystal violet. Subsequently, the invasive cells were counted in randomly selected fields under a microscope (Olympus, Tokyo, Japan). For colony formation assay, 5 $\times$ 10<sup>2</sup> cells were seeded into 60 mm tissue culture plates. After 2 weeks, visible colonies were fixed with 100% methanol and stained with 0.1% crystal violet. Visible colonies were manually counted under a microscope (Olympus). All experiments were performed in triplicate.

#### Dual-luciferase reporter assay

The possible miR-377 binding sites in AEG-1 gene 3'-UTR were predicted using bioinformatics software (DIANA TOOL, Targetscan, miRanda). The predicted and mutated sequences targeting on AEG-1 3'-UTR were both amplified and cloned into pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega; named pmirGLO-AEG-1 and pmirGLO-mutAEG-1, respectively). Subsequently, HEK-293T cells were co-transfected with 0.5  $\mu$ g of pmirGLO-



**Figure 6.** AEG-1 expression after miRNA-mimics transfection. AEG-1 was highly expressed in NSCLC cells in mRNA (A) and protein (B) levels. AEG-1 expression was not altered in mRNA level (C), but inhibited by miRNA-mimics transfection in protein level (D). Each column represents mean  $\pm$  SD;  $n = 3$  per group. \* $P < 0.05$  and \*\* $P < 0.01$ .

AEG-1 or pmirGLO-mutAEG-1 vectors, 100 nM NC miRNA or miRNA-mimics using Lipofectamine 2000 (Invitrogen). The luciferase activities were measured using the Dual-Luciferase Reporter Assay Kit (Promega) 48 h after transfection.

#### Statistical analysis

The data were presented as mean  $\pm$  standard deviation (SD). The differences were analyzed by the Students't test or one-way ANOVA analysis. A \* $P < 0.05$  was considered to be statistically significant and \*\* $P < 0.01$  extremely significant.

#### Results

##### miR-377 expression is down-regulated in NSCLC tissues and cells

Firstly, miR-377 expression in NSCLC tissues and cells was evaluated. As shown in **Figure 1A**, miR-377 showed a significantly lower expression in NSCLC tissues than in adjacent lung tissues. Similarly, the expression of miR-

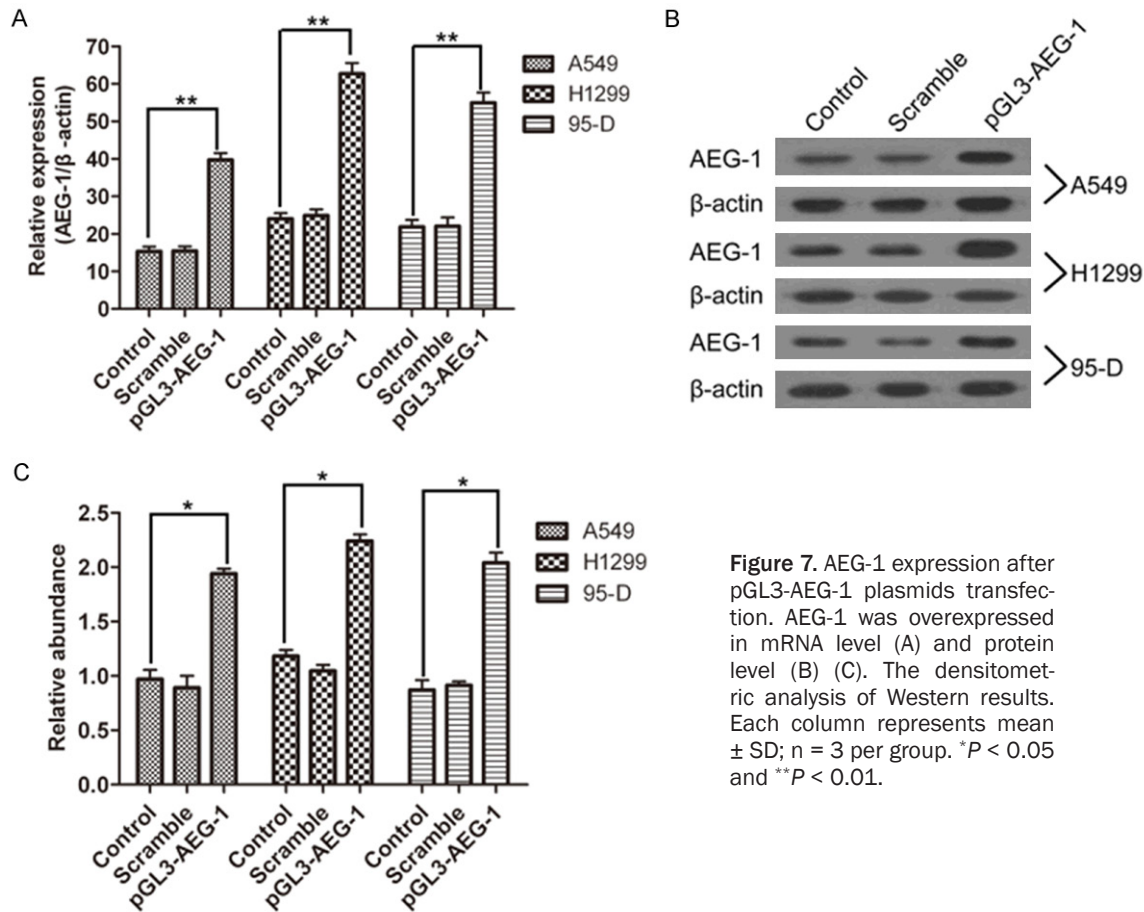
377 in NSCLC cell lines (A549, H1299 and 95-D cells) was also lower compared with HBE cells (**Figure 1B**). These results suggested that miR-377 was associated with NSCLC, characterized by its low expression.

##### miR-377 expression is up-regulated after miRNA-mimics transfection in NSCLC cells

Next, we aimed to up-regulate the expression of miR-377 artificially. As shown in **Figure 2A**, after miRNA-mimics transfection, miR-377 showed an average of  $14.35 \pm 1.04$  fold expression in A549 cells,  $21.52 \pm 1.56$  fold expression in H1299 cells (**Figure 2B**), and  $11.46 \pm 1.13$  fold expression in 95-D cells (**Figure 2C**), indicating that the expression of miR-377 was higher in miRNA-mimics group compared with NC miRNA and control group.

##### High expression of miR-377 reduces viability and proliferation of NSCLC cells

The effects of high miR-377 expression on the viability and proliferation were subsequently investigated via MTT and BrdU assay, respec-



**Figure 7.** AEG-1 expression after pGL3-AEG-1 plasmids transfection. AEG-1 was overexpressed in mRNA level (A) and protein level (B) (C). The densitometric analysis of Western results. Each column represents mean  $\pm$  SD; n = 3 per group. \* $P$  < 0.05 and \*\* $P$  < 0.01.

tively. As shown in **Figure 3A**, there was a sharp decrease in OD value in the presence of miR-377 high expression, indicating that the cell viability was inhibited by the high expression of miR-377. Also, the BrdU-positive cells were also reduced by miR-377 high-expression, implying that the cell proliferation was inhibited (**Figure 3B**).

#### High expression of miR-377 inhibits migration, invasion and colony formation of NSCLC cells

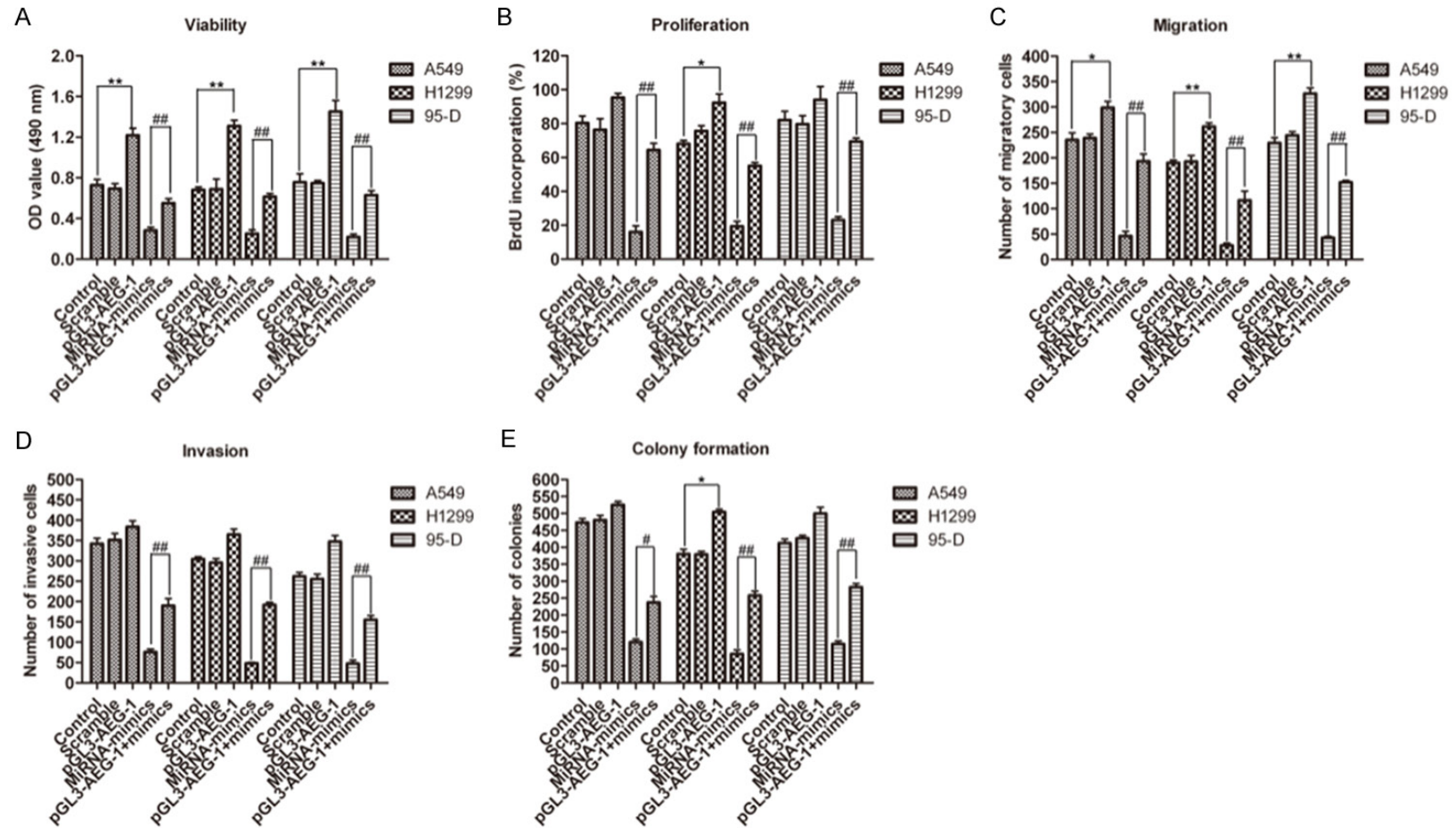
We further investigated the effects of miR-377 on the migration and proliferation of NSCLC cells. A549, H1299 and 95-D cells were subjected to transwell assay. All the three NSCLC cell lines with high expression of miR-377 showed significantly less migration (**Figure 4A**) and invasion (**Figure 4B**) compared with control and NC miRNA group, indicating that up-regulation of miR-377 inhibited the metastasis of NSCLC cells. The colony formation assay demonstrated that the enforced expression of miR-

377 significantly reduced the number of colonies in A549, H1299 and 95-D cells (**Figure 4C**).

#### AEG-1 is a target gene of miR-377

Through bioinformatics analysis, AEG-1 was regarded as a target gene of miR-377 in humans. AEG-1 was usually over-expressed in various tumors and high expression of AEG-1 was often correlated with poor prognosis [23]. Hence, AEG-1 was selected for further analysis. As shown in **Figure 5A**, AEG-1 was shown to bind with miR-377 within the 3'-UTR, but mut-AEG-1 had no binding sites with miR-377. Next, dual luciferase reporter assay was performed to verify the mutual binding of miR-377 and the 3'-UTR of AEG-1. As shown in **Figure 5B**, the luciferase activity was significantly repressed after co-transfection with miRNA-mimics and pmirGLO-AEG-1 plasmids, whereas no significant variation in luciferase activity was observed for either pmirGLO-mutAEG-1 or NC

# miR-377 inhibits NSCLC through AEG-1



**Figure 8.** The effects of AEG-1 overexpression on NSCLC cells. AEG-1 overexpression promoted the viability (A), proliferation (B), migration (C), invasion (D) and colony formation (E) of NSCLC cells. Moreover, AEG-1 overexpression could counteract the inhibitory effects caused by the high expression of miR-377. Each column represents mean  $\pm$  SD; n = 3 per group. \* $P$  < 0.05 and \*\* $P$  < 0.01.



miRNA, implying that miR-377 could directly bind to AEG-1 3'-UTR; in other words, AEG-1 is a target gene of miR-377.

*AEG-1 expression is regulated by miR-377 in the protein level but not in the mRNA level*

Since it had been proved that AEG-1 is a target gene of miR-377, the effects of miR-377 on AEG-1 expression were investigated subsequently. As shown, AEG-1 was highly expressed in NSCLC cells (**Figure 6A and 6B**); interestingly, the expression of AEG-1 was not altered in mRNA level (**Figure 6C**), but inhibited in protein level after miRNA-mimics transfection (**Figure 6D**), verifying the regulation of AEG-1 by miR-377 in the post-transcriptional translation level.

*AEG-1 overexpression diminishes the inhibitory effects of miR-377 on NSCLC cells*

From the above-mentioned results we understood that the enforced expression of miR-377 inhibited NSCLC cells and repressed AEG-1 expression. We wanted to further understand whether miR-377 inhibited NSCLC cells through targeting AEG-1. The pGL3-AEG-1 vectors harboring no specific binding sequences of miR-377 in the 3'-UTR and miRNA-mimics were mono- or co-transfected into A549, H1299 and 95-D cells. As a result, AEG-1 was overexpressed in both mRNA and protein levels (**Figure 7A-C**). The viability, proliferation, migration, invasion and colony formation were measured as described above. AEG-1 overexpression enhanced the viability (**Figure 8A**) and promoted the proliferation (**Figure 8B**) of NSCLC cells. In addition, the migration (**Figure 8C**) and invasion (**Figure 8D**) ability of NSCLC cells was also improved. More colonies were observed after AEG-1 overexpression (**Figure 8E**). Besides, the inhibitory effects of miR-377 could be counteracted by the ectopic expression of AEG-1. These results suggested that miR-377 played a role in NSCLC, possibly through targeting AEG-1.

## Discussion

In the present study, miR-377 was found to be a unique molecule in NSCLC, characterized by its low expression. We wondered if miR-377 participated in the regulation and development of NSCLC; if so, miR-377 would be a diagnostic and therapeutic target for NSCLC. NSCLC is worth noting because most of the lung cancer

related deaths are attributed to NSCLC [30]. miR-377 expression was up-regulated manually by miRNA-mimics transfection in three NSCLC cell lines, A549, H1299 and 95-D cells. The viability, proliferation, migration, invasion and colony formation abilities of NSCLC cells were all inhibited by miR-377 high expression. Next, AEG-1 was identified as a target gene of miR-377 in humans via dual luciferase reporter assay. Furthermore, AEG-1 overexpression could diminish the inhibitory effects on NSCLC cells induced by miR-377, indicating that miR-377 acted as a tumor repressor possibly through down-regulating AEG-1.

miRNAs are a group of single-stranded, non-coding molecules, participating in the regulation of their target gene at the level of translation [2, 31]. The effects of miRNAs on cancers were often produced through their target genes. For example, in NSCLC, miR-27b was shown to inhibit the growth and invasion of cancer cells through targeting Sp1 [32], and miR-30b/c through targeting Rab18 [33]. As we know, miR-377 participated in the regulation of human clear cell renal cell carcinoma [17], malignant melanoma [16], human glioblastoma [20], hepatocellular carcinoma [18] and osteosarcoma [19], acting as a tumor suppressor, which usually decreased the proliferative and migratory capacity of cancer cells. In our study, miR-377 was also shown to inhibit NSCLC cells through regulating AEG-1, which was consistent with the above results. However, some problems remain to be solved. The effects of miR-377 on NSCLC development *in vivo* are absent. Hence, in the future studies, animal models are supposed to be established for the evaluation of tumor formation ability. Besides, the relative signaling pathway is also supposed to be involved. As a result, more experiments will be performed to solve these problems.

AEG-1 was shown to be an oncogene in various tumors. As reported by Ke *et al.*, AEG-1 expression was closely correlated with the clinical staging, differentiation and lymph node metastasis of NSCLC [34]. Also, Song *et al.* reported that AEG-1 overexpression was a main indicator of poor prognosis for NSCLC [28]. Here in this study, overexpression of AEG-1 was also demonstrated to play a role in promoting tumorigenesis of NSCLC. Besides, the functions of AEG-1 were proved to be negatively regulated by miR-377. In conclusion, NSCLC is characterized by

the low expression of miR-377. Our data also provided clear evidence that miR-377 served as a tumor suppressor, possibly through down-regulating AEG-1. These results qualified miR-377 as a better target for the diagnosis, treatment and prognosis of NSCLC.

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### Disclosure of conflict of interest

None.

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