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miR-425 regulates cell proliferation, migration and apoptosis by targeting AMPH-1 in non-small-cell lung cancer

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ABSTRACT

Background: MicroRNAs (miRNAs) have been proved to act as vital roles on non-small-cell lung cancer (NSCLC), and miR-425 has been proven to serve an important function in several tumors. However, the functional role of miR-425 on NSCLC is still unclear.

Methods: The mRNA and protein expression of miR-425 and AMPH-1 were determined by qRT-PCR and western blot analysis, respectively. NSCLC cells (SK-MES-1 and A549) proliferation and migration were measured by CCK-8 and transwell assay, respectively. Cell apoptosis was assessed by flow cytometry and western blotting, In addition, luciferase reporter assay was carried out to confirm the direct targeting of AMPH-1 by miR-425. Xenograft experiments were performed to observe the tumorigenesis of miR-425 in vivo.

Results: The results showed that miR-425 was overexpressed and AMPH-1 expression was downregulated in SK-MES-1 and A549 cells. Silencing miR-425 inhibited proliferation, migration and promoted apoptosis of NSCLC cells. Moreover, we proved that miR-425 could target AMPH-1. The expression of AMPH-1was upregulated in A549 with miR-425 inhibitor. Moreover, miR-425 knockdown were less tumorigenic than the control in vivo.

Conclusions: Taken together, miR-425 could promote the proliferation, invasion and suppress apoptosis by targeting AMPH-1 in NSCLC cells. miR-425/AMPH-1 axis may represent a potential therapeutic strategy or novel prognostic biomarkers to NSCLC.

1. Introduction

Lung cancer, one type of common malignant tumors, has accounted for one third of all deaths caused by cancers [1,2]. Accounting for approximately 80% patients in all categories of lung cancers, non-small-cell lung cancer (NSCLC) comprises large cell lung cancer, lung adenocarcinoma and lung squamous carcinoma [3]. Various therapies such as platinum-based schemes have been applied in NSCLC, but the clinical and biological heterogeneity lead to variable responses and mixed outcomes, and patients still suffer from poor five-year survival rates for resistance to therapies, which brings a huge health challenge on society [4]. Moreover, current prognostic factors used in NSCLC are suboptimal for predicting fitness to therapy [5]. Therefore, the discovery of novel prognostic biomarkers is one unmet clinical need in NSCLC.

miRNAs are one type of very short (~20-24 nucleotides) non-coding RNA, which have been proved to take part in several biological processes, such as carcinogenesis, migration, cell proliferation, invasion, differentiation and apoptosis, angiogenesis, and play crucial roles in development and process of various tumors [6–8]. Previous studies revealed that many miRNAs are upregulated or downregulated in lung cancer tissues and cells compared with adjacent normal tissues [9–11]. Anti-oncogenes miRNAs regulate downstream signal pathways to reverse EGFR-TKIs resistance in the NSCLC, such as the pathway EGFR/PI3K/Akt/mTOR and RAS/ERK/MYC [12]. Oncogenic miRNAs, including miRNA-221/222 or miRNA-30b-c, have exert direct effects on epithelial-mesenchymal transition (EMT) associated with EGFR-TKIs resistance by the activation of PI3K/Akt pathway [13]. miR-425 has been proven to serve an important function in several types of cancer, including gastric cancer, cervical cancer, and hepatocellular carcinoma [14–16]. miR-425 was also found to play an important role in NSCLC development [17]. However, the underlying mechanism by which miR-425 regulates NSCLC and target genes were still unknown.

Amphiphysin (AMPH), a nerve terminal-enriched 128-kD protein [18], contains three identified functional domains [19]. Amphiphysin 1 (AMPH-1) was expressed highly to phosphoprotein in neurons, joining in synaptic vesicle endocytosis, as well as neurite outgrowth [20,21]. Several other studies also revealed that the yeast homologs of amphiphysin was related with the transition of cell growth stage, which

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implicates a possible biological role in progression of cancer [22,23]. Previous studies have showed the suppressive function of AMPH-1 in cancers. Activation of AMPH-1 may be a promising approach to treat breast cancer patients [24]. And AMPH-1 was reported to be a tumor suppressor in lung cancer by inactivating the Ras/Raf/MEK/ERK signal pathway [25]. However, the association between AMPH-1 and microRNA in NSCLC has rarely been reported.

In the present study, we have a strong interest in exploring the mechanism between AMPH-1 and miR-425. Our results indicated that miR-425 promotes NSCLC proliferation, migration and apoptosis by targeting AMPH-1. miR-425 may serves as a potential therapy biomarkers for patients with NSCLC.

2. Materials and methods

2.1. Cell culture

Human non-small cell lung cancer cell lines A549 and SK-MES-1 and human bronchial epithelial cell line BEAS-2B were gained from American Type Culture Collection (ATCC, Rockville, MD, USA). The cell lines were grown in Roswell Park Memorial Institute (RPMI) 1640 media (Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Gibco, Waltham, USA), 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA) at 37 °C with 5% CO_2.

2.2. Cell transfection

miR-425 inhibitor and corresponding negative control (miR-NC) were constructed by Invitrogen. A549 cells were seeded in six-well plates (1×10^6 cells/well) and transfected with miR-425 inhibitor or negative control by the Lipofectamine 2000 (Invitrogen-Life Technologies, USA) following the manufacturer's instructions. Cells were harvested at 48 h post-transfection.

2.3. RNA isolation and quantitative RT-PCR

Total RNA from the treated cells and tissues was isolated using TRIzol Reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. MiRNA detection and quantitative real-time PCR were performed using a 7500 real-time PCR system (Applied Biosystems) and SYBR green PCR master mix (Toyobo). The primer sequences are as follows: AMPH-1 forward: 5′- CGAGAACTCCGAGGA TATTTAGC-3′ and reverse: 5′−CCCATACCAGTCAGGCTCAT-3′; miR-425 forward: 5′-ACACTCCAGCTGGGAATGACACGATCACT-3′ and reverse: TGGTGCGTGGAGTCG-3′; GAPDH forward: 5′- GGAGCGAGATC CCTCCAAAAT-3′ and reverse: 5′- GGCTGTTGTCATACTTCTCATGG-3′; U6 forward: 5′- CTCGCTTCGGCAGCACA-3′ and reverse: 5′-AACGCTT CACGAATTTGCGT-3′. U6 was used as an internal control for miR-425 and GAPDH was used as an internal control for AMPH-1 within each sample using $2^{-\Delta\Delta Ct}$ method as relative quantification.

2.4. Western blot analysis

Total protein was extracted from whole cell lysates, which was detected using BCA Protein Assay kit (Pierce, USA). Equal protein quantities were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore). The membranes were incubated overnight at 4 °C with the following primary antibodies: anti-AMPH-1 (1:1000; Abcam, Cambridge, UK), anti-Bcl-2 (1:1000; Abcam, Cambridge, UK), anti-cleaved caspase 3 (1:1000; Abcam, Cambridge, UK) and anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (1:2000, Santa Cruz Biotechnology). After washing three times, the membranes were incubated with the anti-rabbit or mouse secondary antibodies (1:5000; Santa cruz biotechnology, USA) for 1h at room temperature. Proteins were visualized using an enhanced chemiluminescence (ECL) system (Amersham Biosciences, Buckinghamshire, UK) and quantified using Image *J* software.

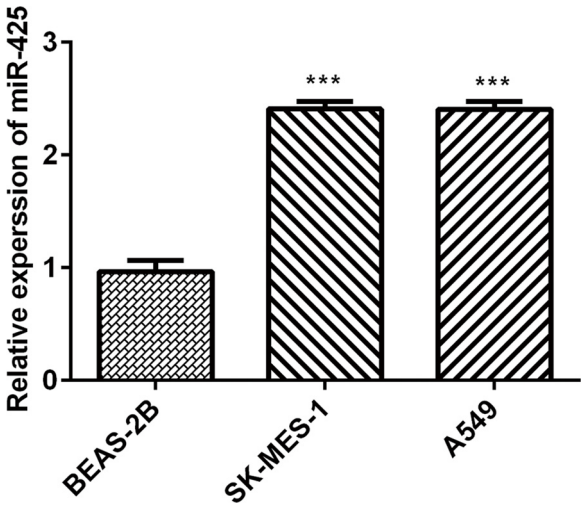

Fig. 1. The expression of miR-425 in NSCLC cell SK-MES-1 and A549 and lung epithelial cell BEAS-2B was detected by qRT-PCR. Data are expressed as mean ± SD. ***P < 0.001 versus the BEAS-2B cells.

2.5. Luciferase reporter assay

starBase (http://starbase.sysu.edu.cn/index.php) was used to predict the target gene of miR-425. Site-directed mutagenesis using a fast mutation kit (NEB, US) was used to gain mutations of miR-425 binding site [26,27]. The wild-type 3′-UTR of AMPH-1 (AMPH-1-WT) and the mutant 3′-UTR of AMPH-1 (AMPH-1-MUT) region containing the miR-425 binding site were synthesized and cloned into the p-MIR-reporter plasmid (Thermo Fisher Scientific, Inc.). Plasmids containing WT or mutant 3′-UTR DNA sequences were *co*-transfected with miR-425 mimic or negative control, and *co*-transfected cells were cultured at 37 °C for 24 h. Cells were detected using a dual luciferase reporter assay kit (Promega Corporation) and GloMax 20/20 illuminometer (Promega Corporation) was used to measure the fluorescence intensity.

2.6. Cell proliferation assay

For the CCK-8 assay (Beyotime), A549 cells transfected with miR-425 inhibitor or miR—NC were seeded in 96-well plates (5×10^3 cells/well, 100 µl each well) and incubated for 24 h, 48 h and 72 h. CCK-8 reagent (10 µl/well) was added in each group and used to detect cell proliferation index according to manufacturer's instructions, After that, the absorbance (optical density, OD) of 450 nm was measured on an enzyme immunoassay analyzer (Bio-Rad, USA).

2.7. Cell invasion assay

A549 cells invasion was measured using transwell assay as the reference. Cells transfected with miR-425 inhibitor or miR-NC were seeded in 24-well transwell plates (5×10^4 cells/well) in the upper chambers with Matrigel-coated membrane (BD Bioscience, San Jose, USA) without serum. And the cell culture medium containing 20% FBS was added the bottom of the chambers as a chemoattractant. Cells were incubated through the polyethylene terephthalate membrane for 72 h at 37 °C with 5% CO_2. Finally, the invasive cells which passed through the membrane were fixed and then stained with hematine. The number of invaded cells was counted using a light microscope, and the average value was determined through five different random fields.

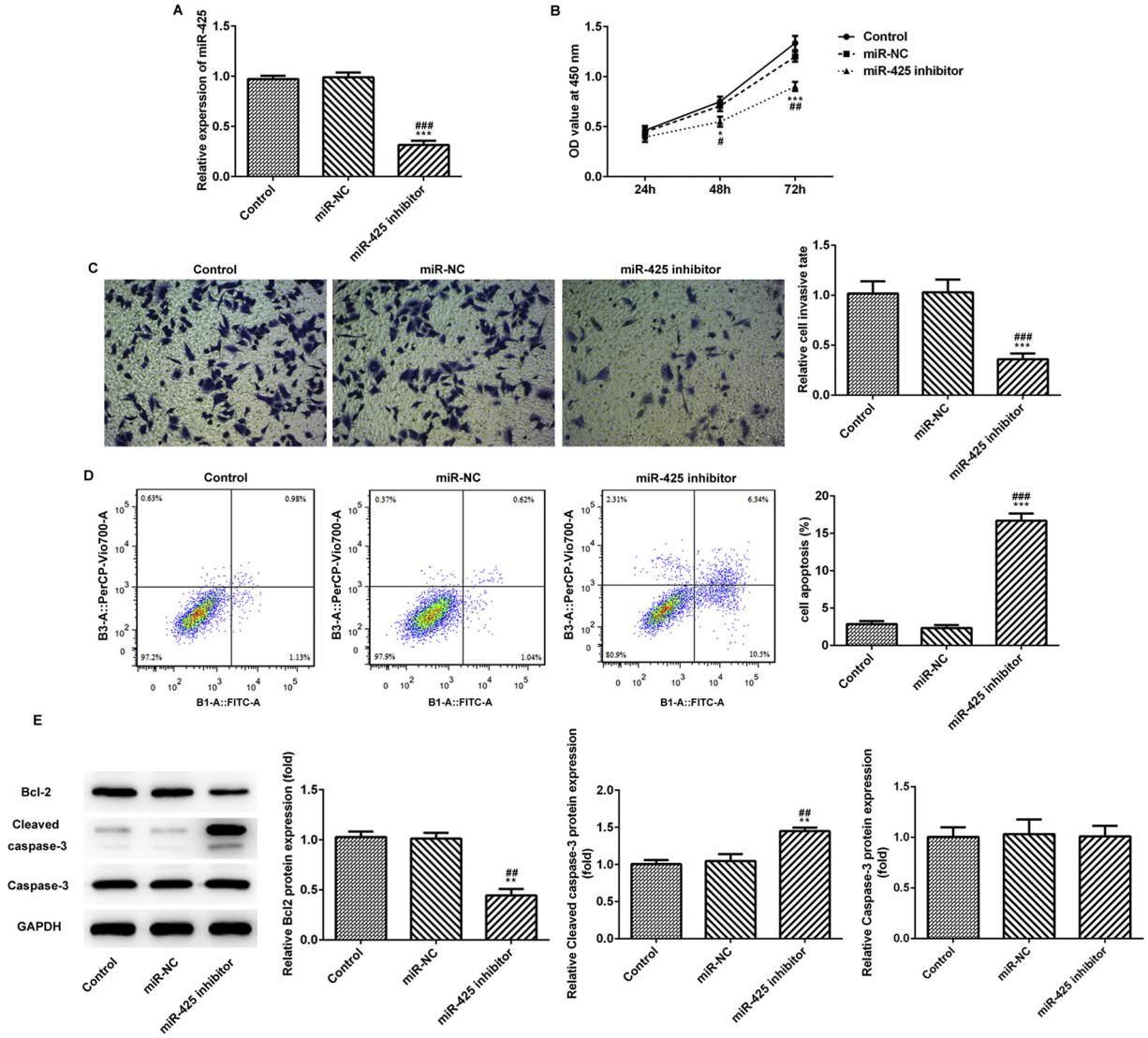

Fig. 2. Effects of knockdown of miR-425 on A549 cell proliferation, migration and apoptosis. A, The relative expression of miR-425 in A549 cells was measured after transfection with miR-425 inhibitor. B, CCK-8 assay was used to determine cell viability of A549 cells transfected with miR-425 inhibitor. C, The migratory capacity of A549 cells was confirmed by transwell assay. D, The cell apoptosis rate was increased after transfected with miR-425 inhibitor by flow cytometry. E, The levels of Bcl-2, cleaved caspase 3 and caspase 3 were measured by western blot analysis. Data are expressed as mean ± SD. **P < 0.01, ***P < 0.001 versus the control; ##P < 0.01, ###P < 0.001 versus miR-NC.

2.8. Cell apoptosis assay

Cell apoptosis was detected by annexin V-FITC/PI Apoptosis Detection Kit (Abcam, USA). Briefly, cells treated with miR-425 inhibitor or miR-NC were cultured for 72 h and resuspended by cold PBS at a density of 1×10^6 cells ml^{-1}. Then the cells were incubated with 5 μl annexin V-FITC solution and 5 μl of propidium iodide (PI) for 15 min at 4 °C in the dark. Apoptosis rates were analyzed by flow cytometry (Beckman Coulter).

2.9. Immunohistochemistry

Paraffin-embedded blocks was made and cut into continuous sections. Antigen retrieval was implemented by microwaving in citrate-buffered solution (pH 6.0) for 3 min. The sections were blocked with 10% goat serum for 30 min at room temperature and were incubated with AMPH-1 (1:500, ab90,434) antibodies at 4 °C overnight. Then, the sample was cultured with secondary antibody was executed for 1 h at room temperature, stained with 3, 3-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. Images were captured with Leica DMLA microscope (Leica Inc., Germany).

2.10. Xenograft experiments

A total 10 five-week female BALB/c nude mice (n = 5 each group) were chosen and anesthetized. A549 cells transfected with miR-425 inhibitor or miR—NC and control cells (1×10^6 cells/100 μl) were subcutaneously injected into the mice on flanks. Then the animals were housed under controlled conditions including a room temperature of 22 ± 1 °C with 12: 12 -h light-dark cycle and were bred in the Animal *Core* Facility by following procedures approved by Animal Ethics Committee. Tumor volume was measured with a caliper and calculated using the formula: volume = (length × width^2)/2. The tumors were stripped and the weights were determined after the mice were sacrificed with the way of cervical dislocation, and the tumors were collected for Immunohistochemistry. All experiments were conducted according to the guidelines established by the Animal Care and Use Committee of Harbin Medical University Cancer Hospital and were approved by this committee.

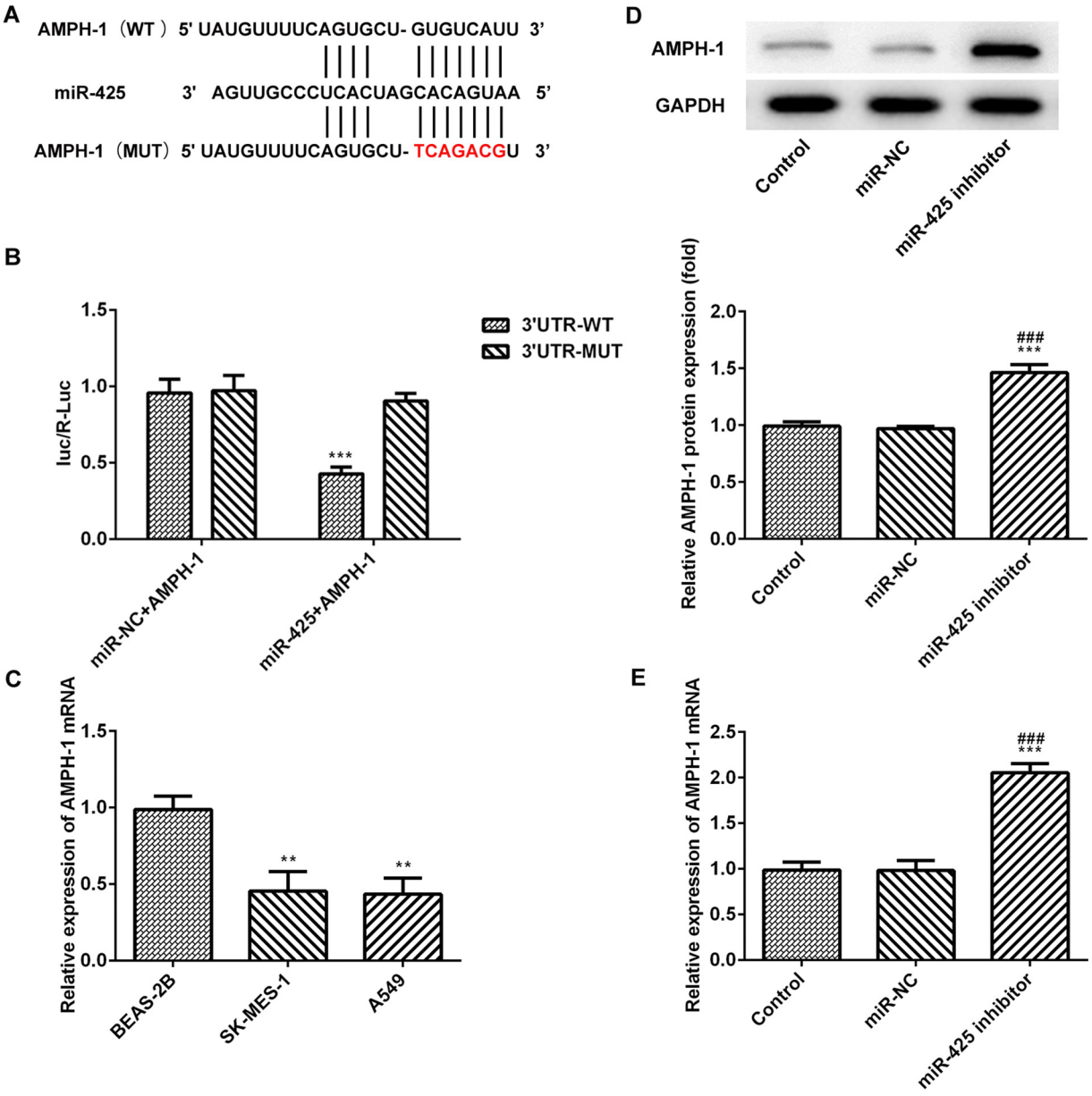

Fig. 3. AMPH-1 binds to miR-425 in NSCLC cells. A, starBase was carried out to predict the potential sequence between miR-425 and AMPH-1. B, The dual luciferase assay was used to examine the luciferase activity of wild type or mutational AMPH-1. C, The mRNA level of AMPH-1 in A549 and BEAS-2B cells was downregulated compared to the epithelial cell BEAS-2B. D and E, miR-425 inhibitor significantly promoted the protein level and mRNA expression of AMPH-1 in transfected A549 cells. Data are expressed as mean ± SD. **P < 0.01, ***P < 0.001 versus the control; $^{###}$P < 0.001 versus miR-NC.

2.11. Statistical analysis

Data was analyzed by using SPSS 17.0 (IBM, Armonk, NY, USA). A paired or unpaired *t*-test was applied to compare two groups with a parametric distribution, and one-way ANOVA was applied to analyze the significant differences among more than two groups. Data are presented as the means ± S.D. Each experiment was repeated at least three times. P < 0.05 was considered significant.

3. Results

3.1. miR-425 Is upregulated in NSCLC cell lines

To investigate the expression of miR-425 in NSCLC cell lines SK-MES-1 and A549 and the normal lung epithelial cell line BEAS-2B, qRT-PCR was performed. The results indicated that compared with BEAS-2B cells, miR-425 was significantly upregulated in the SK-MES-1 and A549 cells (Fig. 1). Based on the above-mentioned result, we chose one of the two NSCLC cell lines for the subsequent experiments.

3.2. miR-425 silence inhibits NSCLC cell proliferation, migration and induces cell apoptosis

To explore the role of miR-425 in NSCLC cell lines, transfection efficiency was determined using qRT—PCR at 48 h after transfection with miR-425 inhibitor. As depicted in Fig. 2A, miR-425 expression was remarkably decreased in A549 cells. Subsequently, cell viability was analyzed by using CCK-8 assay, and cell migration and apoptosis were analyzed with transwell assay and flow cytometry, respectively. In CCK-8 assay, the results showed that the miR-425 silence significantly inhibited A549 cells viability at 48 and 72 h (Fig. 2B). The results from transwell assay revealed that downregulation of miR-425 tremendously inhibited the cell migration compared with the control and miR—NC group (Fig. 2C). The results of flow cytometry and western blotting

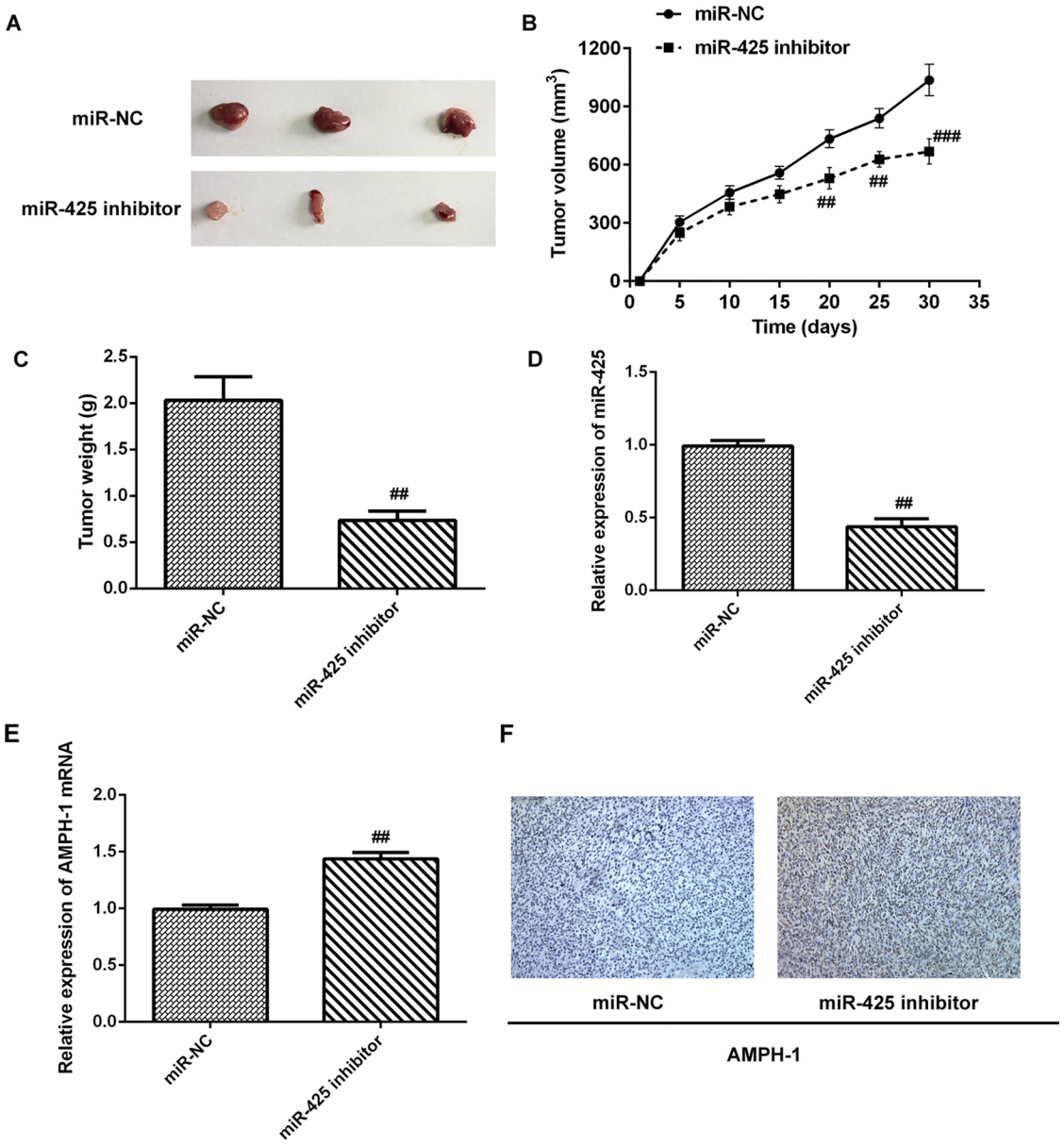

Fig. 4. NSCLC tumor growth in mice was restrained after knockdown of miR-425. A–C, The tumor volume and weight of mice injected with miR-425 inhibitor were repressed by the observation and measurement. D and E, The expressions of miR-425 and AMPH-1 in tissues were detected by qRT-PCR. F, Immunohistochemistry of AMPH-1 in tumor tissue from the miR-425 inhibitor mice. Data are expressed as mean ± SD. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus miR-NC.

showed that compared with the control and miR—NC group, the miR-425 inhibitor obviously induced cell apoptosis in NSCLC cells (Fig. 2D and E).

3.3. miR-425 directly targets AMPH-1

To explore the potential mechanisms that miR-425 inhibits cell proliferation, migration and induces cell apoptosis, bioinformatics analysis was used to predict the genes that potentially miR-425 targets and we found the sequence of AMPH-1could bind to 3′UTR of miR-425. Subsequently, we performed a luciferase reporter assay to validate the potential target, and mutations (3UTR-MUT) were introduced into the complementary sites of AMPH-1 3′-UTR (Fig. 3A). As expected, the luciferase activity was inhibited in cells *co*-transfected with miR-425 mimic and wild type AMPH-1 (3UTR-WT), whereas 3UTR-MUT had no effects on the luciferase activity in cells transfected with miR-425 overexpression (Fig. 3B). Furthermore, the result of qRT-PCR showed that the expression level AMPH-1 was notably decreased in NSCLC cell lines SK-MES-1 and A549 compared to the normal cell line BEAS-2B (Fig. 3C). In addition, the protein expression and mRNA level of AMPH-1 were upregulated when A549 cells were transfected with miR-425 inhibitor (Fig. 3D and 3E).

3.4. miR-425 silence suppresses NSCLC tumor growth in xenograft mouse model

To further investigate the effects of miR-425 on lung carcinogenesis in vivo, A549 stable cells transfected with miR-425 inhibitor were injected into the flanks of nude mice subcutaneously. As expected, the miR-425 inhibitor group showed significantly decreased tumor weights and volume compared with the miR—NC group (Fig. 4A-4C). Moreover, we found that miR-425 expression in cancer tissues was significantly decreased while AMPH-1 expression was increased after administration of miR-425 inhibitor compared to the miR—NC group (Fig. 4D and 4E). Immunohistochemical staining was used to measure the protein expression of AMPH-1. The results showed that the level of AMPH-1 was

extremely decreased in tumors from mice treated with miR-425 inhibitor (Fig. 4F). Thus, these results in vivo indicated that silencing miR-425 suppressed lung cancer growth at least partly dependent on the regulation of AMPH-1.

4. Discussion

Through above experiments, we identified the important role of miR-425 for lung cancer. Silencing miR-425 dramatically inhibited proliferation and promoted cell apoptosis of lung cancer cells. To investigate the further mechanism, we demonstrated that miR-425 directly targeted AMPH-1 and could markedly regulate AMPH-1 expression. In short, miR-425 might serves as one tumor promoter or carcinogenic biomarker by regulating the expression of AMPH-1.

miR-425 is located on human chromosome 3 and is aberrantly expressed in various types of cancer [28]. For example, miR-425 expression was upregulated in gastric cancer (GC) cells and could promote GC cell invasion and metastasis [29]. miR-425 may promote the expression of epithelial markers by targeting SATB homeobox 1, cyclin 2 (CCND2) and Fascin actin-bundling protein 1 in aggressive breast cancer cells [30]. Furthermore, miR-425 promoted hepatocellular carcinoma (HCC) cell invasion and metastasis via SCAI-mediated dysregulation of integrin β1-Fak/Src-RhoA/CDC42, PTEN-AKT and TIMP2—MMP2/MMP9 signaling pathways [16]. Through next-generation deep sequencing technology, it was suggested that some microRNAs, such as miR-425, might be a driving factor for tumor formation, growth, and development to higher staging in lung cancer [8]. miR-425 showed high expression in arterial plasma with borderline significance and were significantly up-regulated in exosomes extracted from LA peripheral plasma samples [10]. In the present study, we found that miR-425 was overexpressed in SK—MES-1 and A549 cells. miR-425 silence inhibited NSCLC cell proliferation, migration and induced cell apoptosis. Moreover, the tumor weights and volume of NSCLC in miR-425 inhibitor group were significantly decreased compared with control group in vivo. These results above showed that miR-425 could regulate cell proliferation, migration and apoptosis in NSCLC, which was also consistent with previous studies.

Human amphiphysin 1 has a putative role in endocytosis and concentrates in nerve terminals [31]. Previous studies identified anti-amphiphysin I antibodies in the patient serum with small-cell lung cancer (SCLC) [32]. In recent studies, AMPH-1 has proven to be one tumor suppressor in several cancers. AMPH-1 knockdown dramatically increased cell proliferation, attenuated cell apoptosis in human lung cancer cells and promoted development of tumor in vivo [33]. Another study demonstrated that AMPH-1 was upregulated in breast and AMPH-1 silence promoted cell proliferation, cell cycle progression and cell migration, and attenuated cell apoptosis [34]. In our study, miR-425 directly targets AMPH-1 by luciferase reporter assay and AMPH-1 was remarkably overexpressed after silencing miR-425. It was shown that miR-425 could regulate NSCLC cell proliferation, migration and apoptosis via targeting AMPH-1. To further verify the results from vitro experiment, we established the xenograft mouse model and explored whether miR-425 had the same effects and whether miR-425 can exert its functions via affecting potential target gene AMPH-1 in vivo. The results from mice revealed that both tumor weight and volume significantly were decreased and the protein expression of AMPH-1 in tumor tissues was obviously changed in miR-425 inhibitor treated mice, which is consistent with the cell experiments in this study.

Taken together, the results from our study indicated that miR-425 silence could inhibit NSCLC cell proliferation, invasion and promote cell apoptosis in vitro and inhibited tumor development in vivo. In addition, miR-425 regulated NSCLC cell at least partly by targeting AMPH-1. The findings suggest that miR-425/AMPH-1 axis may represent a potential therapeutic strategy or novel prognostic biomarkers for development of NSCLC.

Authors' contributions

Lei Jiang and Jingshu Geng designed the study, drafted and revised the manuscript. Wenyu Ge analyzed the data. Lei Jiang searched the literature. Lei Jiang and Wenyu Ge performed the experiments. All authors read and approved the final manuscript.

Conflict of interest

None.

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