Epigenetic silencing of microRNA-199a-5p promotes the proliferation of non-small cell lung cancer cells by increasing AKAP1 expression

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Abstract. MicroRNA (miR)-199a-5p expression is downregulated in a variety of malignancies, including non-small cell lung cancer (NSCLC), and its low expression is associated with a poor prognosis. However, to the best of our knowledge, the mechanism underlying miR-199a-5p downregulation in NSCLC and its target effectors remain to be elucidated. The present study revealed the downregulation of miR-199a-5p expression in NSCLC tissues and cell lines compared with in para-carcinoma tissues and a lung epithelial cell line. Further experiments indicated that the methylation levels of the miR-199a promoter were markedly higher in NSCLC tissues compared with in para-carcinoma tissues. The DNA methyltransferase inhibitor 5-Aza-2'-deoxycytidine markedly increased the expression levels of miR-199a-5p in NSCLC cells. Furthermore, it was identified that miR-199a-5p mimics transfection decreased the expression levels of A-kinase anchoring protein 1 (AKAP1) at both the mRNA and protein levels by targeting the 3' untranslated region of AKAP1 mRNA. The *in vitro* experiments demonstrated that miR-199a-5p overexpression inhibited the proliferation and tumorigenicity of NSCLC cells, whereas overexpression of AKAP1 partially recovered the malignant phenotypes, suggesting that AKAP1 may be a downstream effector targeted by miR-199a-5p. Collectively, the present findings indicated that miR-199a-5p may be a novel regulator of AKAP1, and that miR-199a-5p may be a potential tumor suppressor in NSCLC.

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Introduction

Lung cancer is the leading cause of cancer-associated mortality worldwide (1). In most countries, for lung cancer patients diagnosed during 2005-2009, the 5-year survival rate ranges between 10 and 20% (1), which highlights the inadequacy of the current treatment strategies for lung cancer (1). Lung cancer is classified into two main types, small cell lung cancer and non-small cell lung cancer (NSCLC), and NSCLC accounts for ~90% of all cases (2). In the majority of patients with NSCLC, local or advanced metastasis occurs after diagnosis (3). Therefore, the mechanisms underlying NSCLC development have become a focus of research in recent years.

MicroRNA (miRNA/miR)-199a-5p expression is generally downregulated in tumors and can inhibit the progression of various tumor types, including prostate cancer, breast cancer, hepatocellular carcinoma, ovarian cancer, colorectal cancer and lung cancer, by downregulating different downstream genes (4-9). For example, Hu *et al* (10) reported that miR-199a-5p could inhibit the migration and invasion of colorectal cancer by targeting discoidin domain receptor-1. In liver cancer, miR-199a-5p inhibits glucose consumption and lactic acid generation by targeting hexokinase 2, thus attenuating the proliferation and tumorigenesis of cancer cells (11). In addition, Li *et al* (12) revealed that miR-199a-5p-mediated silencing of ETS proto-oncogene 1, transcription factor represses the invasion of breast cancer cells by decreasing the levels of integrin 1 (12). Additionally, miR-199a-5p has been reported to be a tumor suppressor in NSCLC (9,13). However, the mechanisms of the downregulation of miR-199a-5p expression and the downstream effectors targeted by miR-199a-5p in NSCLC are not fully understood.

A-kinase anchoring protein 1 (AKAP1) is a scaffold protein, which recruits protein kinase A, other signaling proteins and RNA to the outer mitochondrial membrane (14). In hepatocellular carcinoma, high expression levels of AKAP1 are associated with a poor prognosis, suggesting that AKAP1 may serve as a biomarker (15). Another previous study reported that knockdown of AKAP1 sensitizes gastric cancer cells to cisplatin treatment (16). Furthermore, AKAP1 is expressed at

high levels in lung cancer cells compared with non-tumoral cells, and is associated with the decreased survival rate of patients with lung cancer (17). However, to the best of our knowledge, the mechanisms underlying the aberrant expression of AKAP1 in NSCLC and its cellular function remain unknown.

The present study demonstrated that the hypermethylation of the promoter of miR-199a led to downregulation of miR-199a-5p in NSCLC cells. Exogenous miR-199a-5p suppressed the proliferation and tumorigenicity of NSCLC cells by decreasing AKAP1 expression, which was achieved via direct targeting of the 3' untranslated region (UTR) of AKAP1 mRNA. These findings indicated a novel regulatory mechanism of AKAP1, as well as its role in NSCLC proliferation.

Materials and methods

Collection of NSCLC tissues. All patients with NSCLC (n=41; median age, 61 years; age range, 35-84 years; 14 female and 27 male patients) were diagnosed according to the International Association for Lung Cancer Research 2015 version guidelines (18). NSCLC and paired para-carcinoma tissues (2 cm away from the tumor edge) were collected between January 2019 and June 2020 at The First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China). Patients with NSCLC were included that had been diagnosed on the basis of pathological examinations. Histological classification was performed according to WHO Classification of Tumors of the Lung (2015) (19) and tumor staging was performed according to the staging criteria of International Union Against Cancer (UICC, 2017) (20). Patients with NSCLC without radiotherapy or chemotherapy prior to the surgical lung resection were included. Pregnant patients, patients with mental or cognitive impairment and patients with another lung disease were excluded from the present study. Written informed consent was obtained from all patients, and the present study was approved by the Ethics Committee in Clinical Research of The First Affiliated Hospital of Wenzhou Medical University (issuing no. 2019-128; Wenzhou, China).

Online analysis of the cancer genome atlas (TCGA) data. Kaplan-Meier analysis of TCGA database (https://cancergenome.nih.gov/abouttcga/overview) was performed by OncoLnc (version 2016: http://www.oncolnc.org/) using the lung adenocarcinoma (LUAD) dataset (21). Patients were divided into high miR-199a-5p expression (40% upper percentile) and low miR-199a-5p expression (40% lower percentile) groups.

Cell culture. The BEAS-2B normal human lung epithelial cell line and the H1299 and H460 NSCLC cell lines were purchased from American Type Culture Collection. Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 mg/ml streptomycin and 100 IU/ml penicillin in a 5% CO_2 atmosphere at 37°C. Cell passage was performed every 2 days. 5-Aza-dC (2 μM) purchased from Sigma-Aldrich; Merck KGaA was used to treat H1299 cells for 48 h at 37°C.

Plasmids and mimic RNAs. miR-199a-5p or negative control (NC) mimic RNAs (miR-199a-5p mimics, 5'-CCCAGUGUU CAGACUACCUGUUC-3'; NC mimics, 5'-GGUUCGUAC GUACACUGUUCA-3', both 20 μM) were purchased from Shanghai GenePharma Co., Ltd. miR-199a-5p and AKAP1 overexpression plasmids (ov-miR-199a-5p and ov-AKAP1) were constructed using the pcDNA3.1 vector (both 500 ng/μl, Invitrogen; Thermo Fisher Scientific, Inc.). The 3'UTR of the AKAP1 gene was amplified based on cDNA and then cloned into the pGL3 luciferase reporter vector (500 ng/μl; Promega Corporation) to obtain the AKAP1-3'UTR reporter plasmid (pGL3-AKAP1-3'UTR). A mutation reporter vector (pGL3-AKAP1-3'UTR-mut) was constructed via subcloning (the sequence ACU at the miR-199a-5p binding site was mutated to UGA). Transfection was performed using Lipofectamine® 3000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols at 37°C for 24 h. At 48 h after transfection, subsequent experiments were performed or G418 (Sigma-Aldrich; Merck KGaA) was used to screen H1299 cells with stable transfection.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells and tissues using an RNAiso Plus kit (Takara Bio, Inc.). The RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.).

For mRNA, cDNA was reverse transcribed from 0.5 μg mRNA using a PrimeScript RT reagent kit (Takara Bio, Inc.), and the following conditions were used for RT: 37°C for 30 min, 85°C for 15 sec. qPCR for AKAP1 (forward, 5'-TCC GTGGATAGCTGTTGCAG-3'; reverse, 5'-CTGCTTGCC AATTAGCCGAC-3') was performed using SYBR Green PCR Master Mix (Takara Bio, Inc.). The following thermocycling conditions were used: Initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 1 min. β-actin (forward, 5'-CTCCATCCTGGCCTCGCTGT-3'; reverse, 5'-GCTGTC ACCTTCACCGTTCC-3') was used as an internal control to normalize mRNA levels. The relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (22).

For miRNA, a TaqMan microRNA RT kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for reverse transcription, and the following conditions for RT were used: 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. A TaqMan miRNA assay (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to measure the expression levels of mir-199a-5p (forward, 5'-AACCATGCCCAGTGTTCAGAC TA-3'; reverse, 5'-CAGTGCAGGGTCCGAGGT-3'). The following thermocycling conditions were used: Initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, annealing and extension at 60°C for 1 min. U6 (forward, 5'-CTCGCTTCGGCAGCACA-3'; reverse, 5'-AACGCT TCACGAATTTGCGT-3') was used as an internal control to normalize miRNA levels. The relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (22).

DNA sodium bisulfite conversion. Genomic DNA was extracted from NSCLC and para-carcinoma tissues using the phenol-chloroform technique. Bisulfite conversion was performed using an EpiJET Bisulfite Conversion Kit
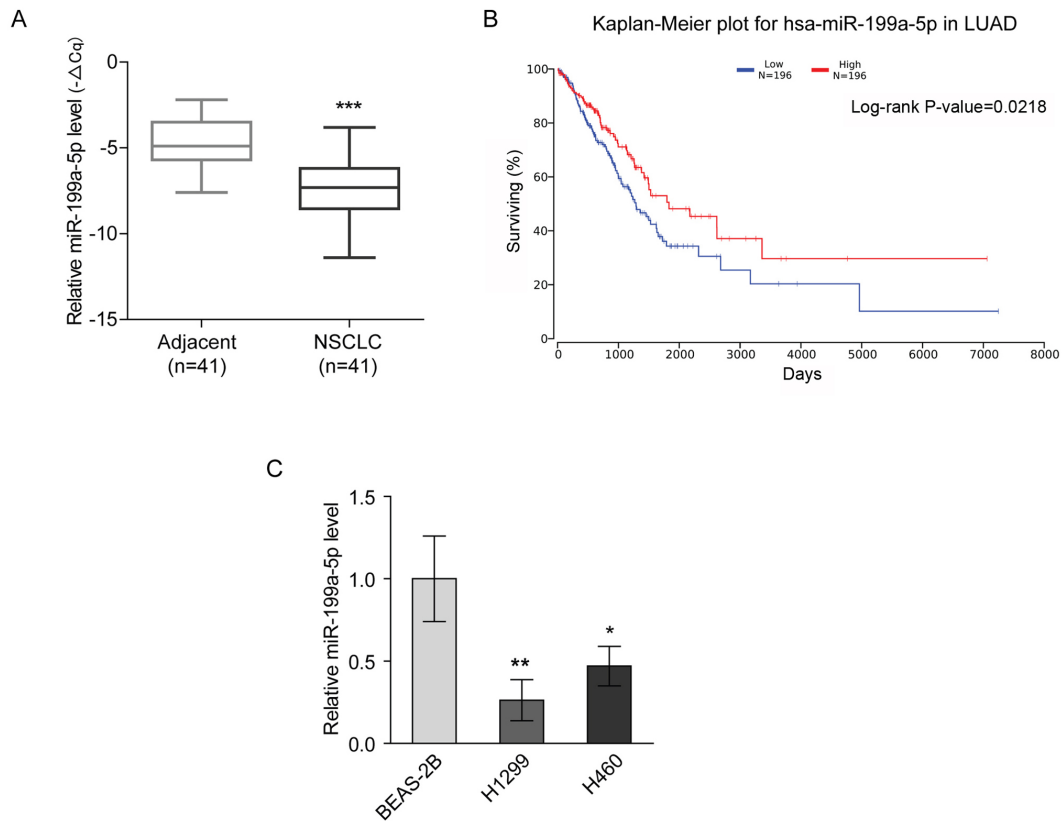
Figure 1. Expression levels of miR-199a-5p are decreased in NSCLC. (A) Expression levels of miR-199a-5p in 41 paired NSCLC and adjacent normal tissues were quantified using RT-qPCR using U6 as an internal control. -ΔCq [ΔCq: Cq (miR-199a-5p)-Cq (U6)] represented the relative expression level. A paired t-test was used for analysis. ***$P<0.001$ vs. adjacent tissue. (B) Overall survival rates of patients with LUAD with high or low miR-199a-5p expression (high, n=196; low, n=196) were analyzed based on The Cancer Genome Atlas database using OncoLnc. (C) Expression levels of miR-199a-5p in BEAS-2B, H1299 and H460 cells were quantified via RT-qPCR using U6 as an internal control, and were normalized to BEAS-2B. One-way ANOVA with Tukey's post hoc test was used for analysis. *$P<0.05$, **$P<0.01$ vs. BEAS-2B cells. LUAD, lung adenocarcinoma; miR, microRNA; NSCLC, non-small cell lung cancer; RT-qPCR, reverse transcription-quantitative PCR.

(Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols (incubation conditions: 98˚C for 10 min, 60˚C for 150 min). Specific primers [Forward, 5'-TATATTTGGAAT TGTTTATAGT-3'; reverse, 5'-AAAAAAATATCTAACTCT TTAA-3' (23)] for the converted promoter region were used to generate the PCR product using Platinum™ Taq Green Hot Start DNA Polymerase (Invitrogen; Thermo Fisher Scientific, Inc.). Amplification was conducted by performing initial denaturation at 94˚C for 5 min, followed by 35 cycles of 94˚C for 2 min, 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 1 min. PCR products were separated by 2% agarose gel and cloned into pGEM-Teasy (Promega Corporation) followed by sequencing with Sp6 primer. The dideoxy chain-termination method (Sanger method) was used to sequence the inserted fragments. The lollipop diagram from the sequencing data was generated by BiQ Analyzer v.2.0 (Max-Planck-Institut Informatik). Filled (black) circles corresponded to methylated Cs, and unfilled (white) circles corresponded to unmethylated Cs.

Western blotting. Whole cell protein was obtained using cold cell lysis RIPA buffer (cat. no. 20-188; EMD Millipore), and the total protein concentration was measured using a Bradford protein assay (cat. no. 23236; Bio-Rad Laboratories, Inc.). Equivalent amounts of protein (30 µg/lane) were separated via 10% SDS-PAGE and transferred to a nitrocellulose membrane

(Whatman plc; Cytiva). The membrane was blocked with 5% milk for 2 h at room temperature, and incubated at 4˚C overnight with the following primary antibodies: AKAP1 (dilution, 1:1,000; cat. no. 5203; Cell Signaling Technology, Inc.) and GAPDH (dilution, 1:2,000; cat. no. sc-47724; Santa Cruz Biotechnology, Inc.). Subsequently, the membrane was incubated with an appropriate fluorescent secondary antibody (IRDye® 800CW- or IRDye® 680RD-conjugated antibodies (dilution, 1:10,000; cat. nos. 926-32211 and 926-68070; LI-COR Biosciences) at room temperature for 1 h. Subsequently, protein bands were detected using an Odyssey® infrared imaging system (LI-COR Biosciences). The obtained signals were converted into grayscale images using Application Software v.2.1.12 (LI-COR Biosciences).

Prediction of miR-199a-5p targets. TargetScan (TargetScan Release 3.1; http://www.targetscan.org) online prediction tools were used to predict miR-199a-5p target genes. Briefly, the default settings were adopted when setting up the run parameters. The gene was considered as a potential target gene of miR-199a-5p when its total context++ score was <-0.6.

Luciferase reporter assay. H1299 or H460 cells (1x10^5 cells/well) were plated onto a 24-well plate. The following day, the cells in each well were co-transfected
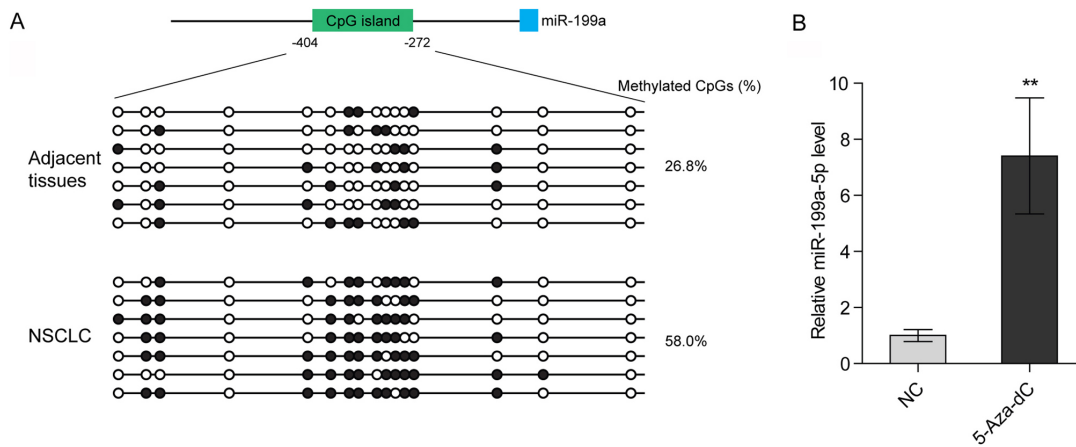
Figure 2. Hypermethylation of the promoter of miR-199a contributes to the low expression levels of miR-199a-5p in NSCLC. (A) Methylation levels of the miR-199a gene promoter in NSCLC and para-carcinoma tissues were determined using bisulphite DNA sequencing, showing the methylation status of CpGs in NSCLC and adjacent tissues. (B) Expression levels of miR-199a-5p in H1299 cells after 5-Aza-dC treatment were measured using reverse transcription-quantitative PCR using U6 as an internal control, and normalized to the NC group. An unpaired t-test was used for analysis. **P<0.01 vs. NC group. 5-Aza-dC, 5-Aza-2'-deoxycytidine; miR, microRNA; NC, negative control; NSCLC, non-small cell lung cancer.

with 0.5 μg firefly luciferase report plasmid (pGL3 with AKAP1 3'UTR) and 0.01 μg *Renilla* luciferase report plasmid (pRL-SV40; Promega Corporation). The pRL-SV40 plasmid was used to standardize the transfection efficiency. Meanwhile, the cells in each well were also transfected with 40 pmol miR-199a-5p or negative control (NC) mimic RNAs (miR-199a-5p mimics, 5'-CCCAGUGUUCAGACU ACCUGUUC-3'; NC mimics, 5'-GGUUCGUACGUACAC UGUUCA-3', both 20 μM, Shanghai GenePharma Co., Ltd.). Transfection was performed using Lipofectamine® 3000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols at 37˚C. The cells were lysed using 100 μl passive lysis buffer (Promega Corporation) at 2 days after transfection. The reporter activity was measured using the dual luciferase reporter assay system (cat. no. E1910; Promega Corporation) according to the manufacturer's protocols, and a luminometer (model no. LB9507; Titertek-Berthold). Briefly, 20 μl cell lysate was mixed with 100 μl LAR II in a luminometer tube and then the tube was placed in the luminometer for the first measurement (firefly luciferase activity). Subsequently, 100 μl Stop & Glo® reagent was added and followed by gentle mixing. The tube was then placed in the luminometer again for the second measurement (*Renilla* luciferase activity). Relative luciferase activity was calculated by dividing the first measurement by the second.

Cell proliferation assay. Cell proliferation was analyzed using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) assay. Cells ($3x10^3$) were added into each well of a 96-well plate. After the indicated time (12, 24, 36, 48 and 60 h), 10% CCK-8 reagent was added to each well and the cells were incubated for 2 h. Measurements were obtained at a wavelength of 450 nm using a spectrophotometer (Bio-Rad Laboratories, Inc.).

EdU incorporation assay. The EdU (5-ethynyl-2-deoxyuridine) incorporation assay was used to represent DNA synthesis in cells. H1299 and H460 cells ($5x10^4$/well) were transfected with miR-199a-5p mimics or AKAP1 expression plasmids for

48 h at 37˚C. Next, cells were washed 3 times with PBS, and then incubated in serum-free RPMI 1640 with 10 mM EdU (Sigma-Aldrich; Merck KGaA) for 2 h at 37˚C. After extensive washing with PBS, cells were blocked with 10% FBS in PBS for 30 min at 37˚C. Incorporated EdU was detected by the fluorescent azide coupling reaction (Invitrogen; Thermo Fisher Scientific, Inc.). Images of the cells were captured with a fluorescence microscope (Nikon Ti-E) (magnification, x40) and analyzed by Image J v.1.4.3.67 (National Institutes of Health).

Colony formation assay. H1299 cells ($5x10^3$) were suspended in 2.5 ml of 0.3% agar and then placed in each well of a 6-well plate, which was pre-coated with 1.0 ml 0.6% agar/well. Cells were cultured in an incubator at 37˚C with 5% CO_2 and 95% air. The RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) was replaced with fresh medium every 3 days. After 21 days, the colonies were captured by a light microscope (Eclipse Ti), and at least 5 individual fields at magnification, x100 were chosen. Then colonies were counted using ImageJ software (version 1.4.3.67; National Institutes of Health).

Statistical analysis. SPSS v13.0 (SPSS, Inc.) and GraphPad Prism 5 (GraphPad Software, Inc.) were used for statistical analysis. Experimental data were presented as mean ± SD from at least 3 independent experiments. An unpaired Student's t-test was used for comparisons between two independent groups. A paired t-test was used for comparisons between two paired groups. ANOVA (data with univariate change were analyzed using one-way with Tukey's post hoc test; data with multivariate change were analyzed using two-way with Sidak's post hoc test) was used for multiple group comparisons. A Pearson test was used for correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-199a-5p expression is decreased in NSCLC. miR-199a-5p has been reported to be a tumor suppressor in lung cancer (9,13).
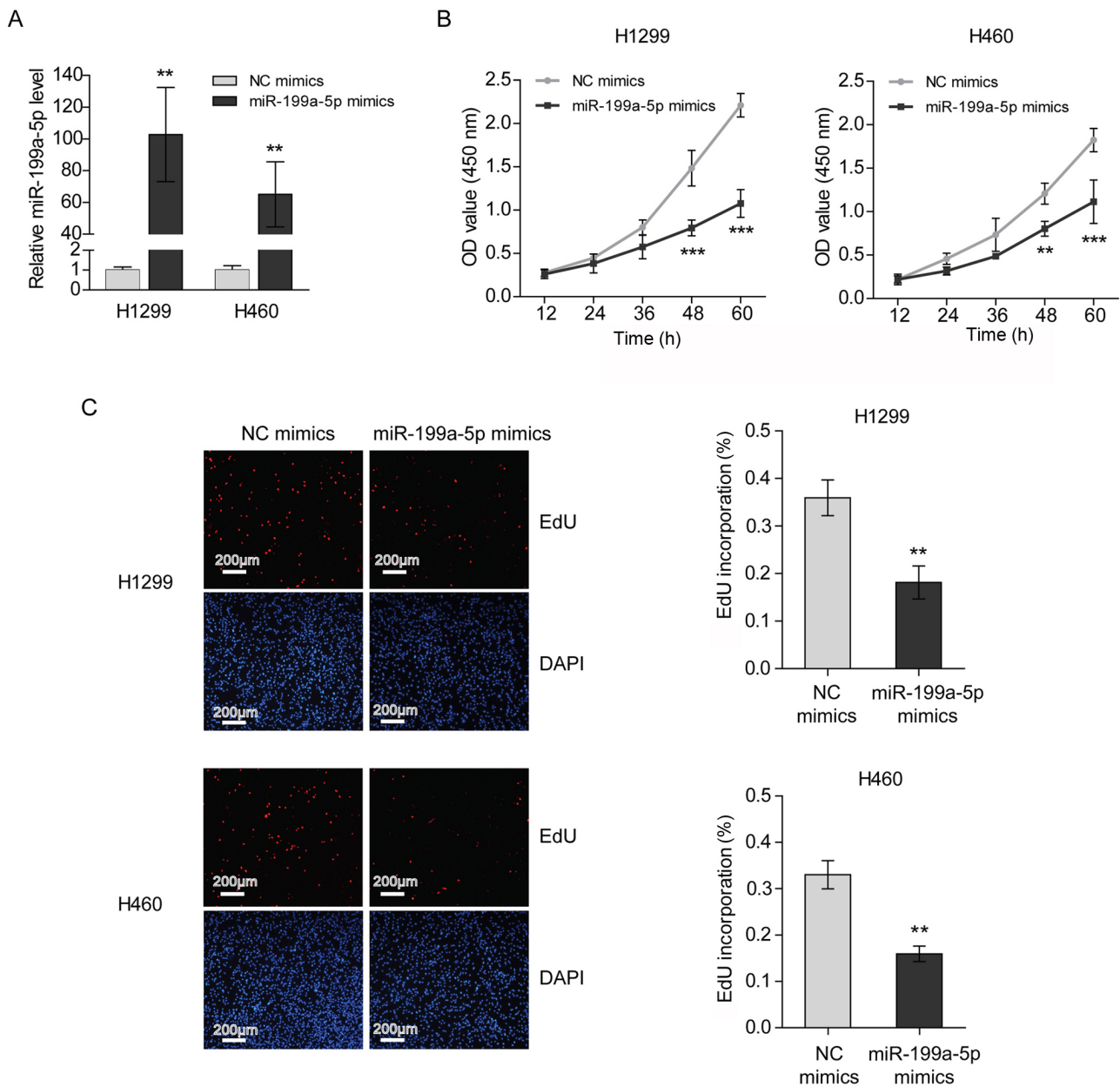
Figure 3. Transfection with miR-199a-5p mimics suppresses the proliferation of non-small cell lung cancer cells. (A) Transfection efficiency of miR-199a-5p mimics in H1299 and H460 cells was verified using reverse transcription-quantitative PCR using U6 as an internal control. miR-199a-5p expression was normalized to the NC mimics group. An unpaired t-test was used for analysis. (B) Cell proliferation was measured using a Cell Counting Kit-8 assay in H1299 and H460 cells transfected with miR-199a-5p mimics. Two-way ANOVA with Sidak's post hoc test was used for analysis. (C) DNA synthesis was measured using an EdU incorporation assay in H1299 and H460 cells transfected with miR-199a-5p mimics. The percentage of cells with EdU incorporation was measured using ImageJ software. An unpaired t-test was used for analysis. **P<0.01, ***P<0.001 vs. NC mimics group. EdU, 5-ethynyl-2'-deoxyuridine; miR, microRNA; NC, negative control; OD, optical density.

The present study revealed that miR-199a-5p expression was downregulated in NSCLC tissues compared with para-carcinoma tissues (Fig. 1A). Furthermore, Kaplan-Meier analysis of a TCGA dataset using OncoLnc demonstrated that the overall survival rate of the patients with LUAD with high expression levels of miR-199a-5p was significantly increased compared with that of the patients with low miR-199a-5p expression (Fig. 1B). Consistently, the expression levels of miR-199a-5p were significantly lower in NSCLC cell lines (H1299 and H460) compared with in the BEAS-2B normal lung epithelial cell line (Fig. 1C). These findings suggested that the downregulation of miR-199a-5p expression was associated with NSCLC.

Hypermethylation of the promoter of the miR-199a gene contributes to the low expression levels of miR-199a-5p in NSCLC. Deng *et al* (23) reported that promoter methylation of the miR-199a gene suppresses miR-199a-3p in ovarian cancer. To determine whether the loss of miR-199a-5p in NSCLC also arises from promoter hypermethylation, bisulfite genomic sequencing was performed to analyze the methylation levels of the CpG island (-404/-272) located at the miR-199a promoter. It was revealed that the methylation levels of the CpG island were markedly higher in NSCLC tissues compared with in para-carcinoma tissues (Fig. 2A; Table SI). Additionally, treatment with a selective inhibitor of DNA methyltransferases
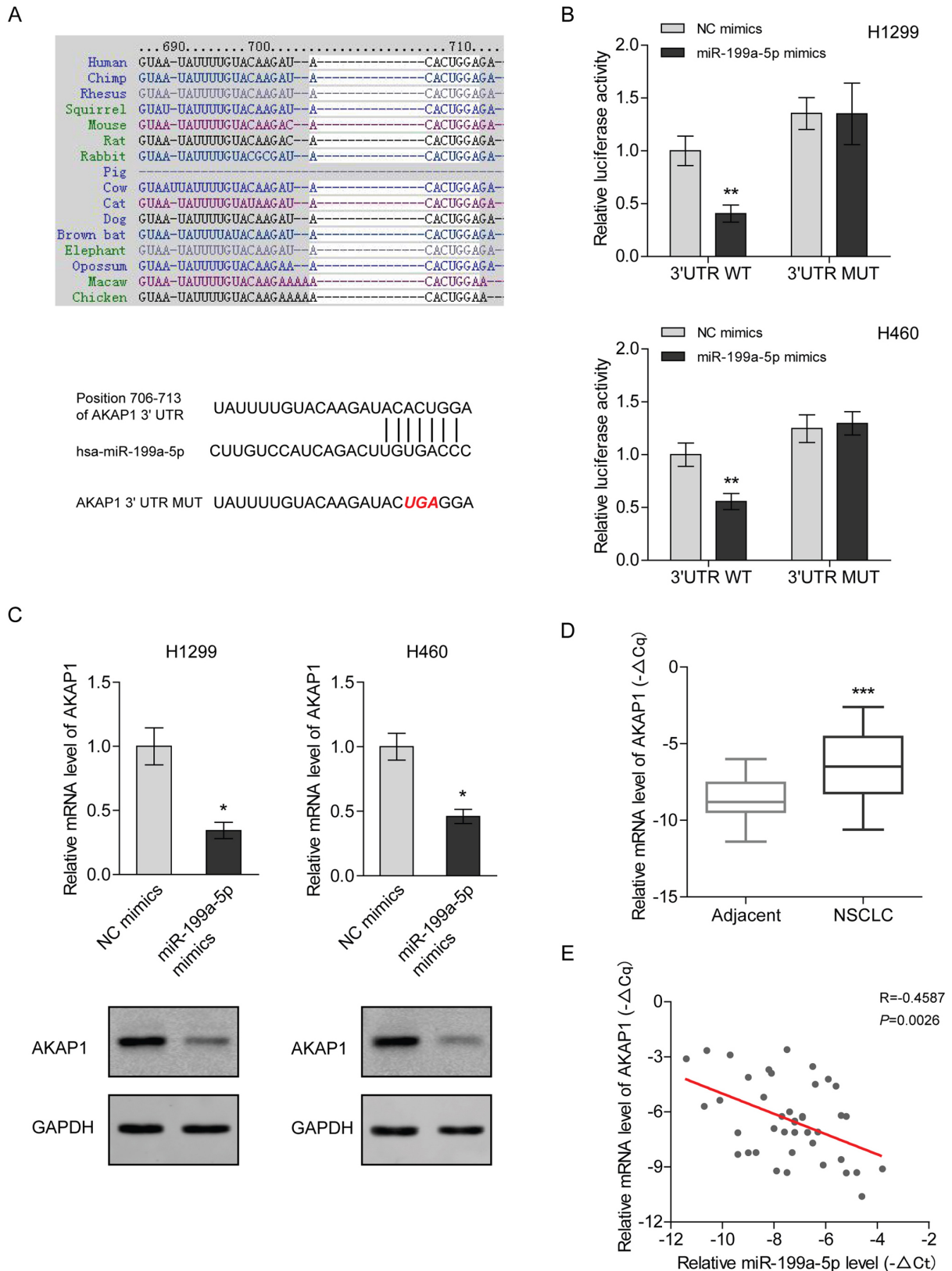
Figure 4. miR-199a-5p directly targets the mRNA of AKAP1 in NSCLC. (A) miR-199a-5p seed sequence complementary to the 3'UTR of AKAP1 mRNA was analyzed using TargetScan Human 7.2. (B) Luciferase activity was measured in H1299 and H460 cells co-transfected with the luciferase constructs containing the WT or MUT AKAP1 3'UTR, as well as miR-199a-5p mimics or NC mimics, and was normalized to the WT/NC mimics group. Two-way ANOVA with Sidak's post hoc test was used for analysis. **P<0.01 vs. WT/NC mimics group. (C) mRNA and protein expression levels of AKAP1 were analyzed using RT-qPCR and western blotting in H1299 and H460 cells transfected with miR-199a-5p mimics. mRNA expression levels were normalized to those of the NC mimics group. An unpaired t-test was used for analysis. *P<0.05 vs. NC mimics group. (D) mRNA expression levels of AKAP1 in 41 paired NSCLC and adjacent tissues were quantified via RT-qPCR, using β-actin as an internal control. -ΔCq [ΔCq: Cq (AKAP1)-Cq (β-actin)] represented the relative expression level. A paired t-test was used for analysis. ***P<0.001 vs. adjacent tissue. (E) Correlation between the mRNA expression levels of AKAP1 and miR-199a-5p in 41 NSCLC tissues. A Pearson test was used for analysis. AKAP1, A-kinase anchoring protein 1; miR, microRNA; MUT, mutant; NC, negative control; NSCLC, non-small cell lung cancer; RT-qPCR, reverse transcription-quantitative PCR; UTR, untranslated region; WT, wild-type.
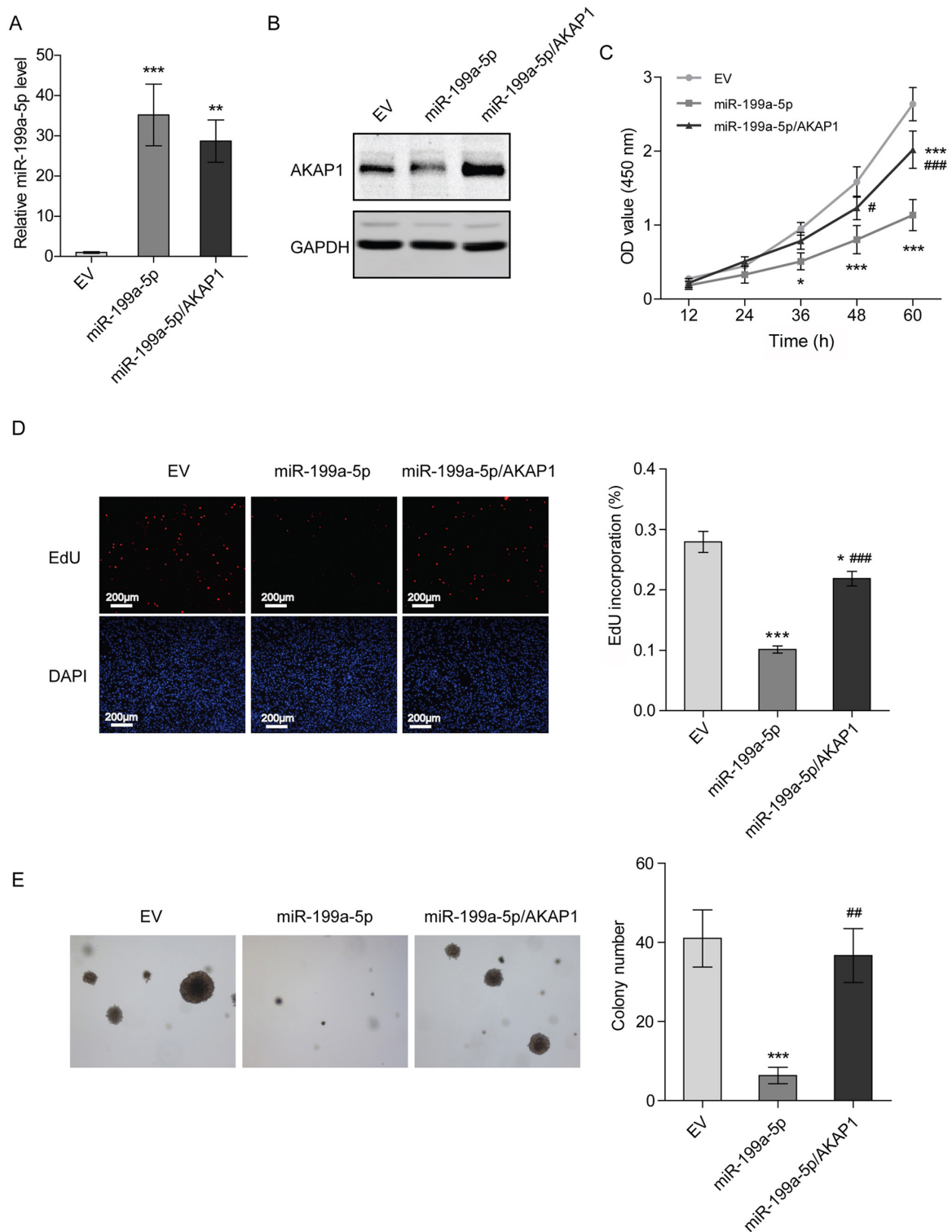
Figure 5. Overexpression of AKAP1 restores the proliferation of non-small cell lung cancer cells transfected with miR-199a-5p overexpression plasmid. Stable overexpression of (A) miR-199a-5p and (B) AKAP1 in H1299 cells was verified using reverse transcription-quantitative PCR and western blotting. miR-199a-5p expression was normalized to the EV group. One-way ANOVA with Tukey's post hoc test was used for analysis. (C) Cell proliferation was measured using a Cell Counting Kit-8 assay in H1299 cells stably overexpressing miR-199a-5p or AKAP1. Two-way ANOVA with Sidak's post hoc test was used for analysis. (D) DNA synthesis was measured using an EdU incorporation assay in H1299 cells stably overexpressing miR-199a-5p or AKAP1. The percentage of cells with EdU incorporation was measured using ImageJ software. One-way ANOVA with Tukey's post hoc test was used for analysis. (E) Colony formation of H1299 cells stably overexpressing miR-199a-5p or AKAP1. Colony numbers were counted using a microscope (magnification, x100). One-way ANOVA with Tukey's post hoc test was used for analysis. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. EV group; $^{#}P<0.05$, $^{##}P<0.01$ and $^{###}P<0.001$ vs. miR-199a-5p group. AKAP1, A-kinase anchoring protein 1; EdU, 5-ethynyl-2'-deoxyuridine; EV, empty vector; miR, microRNA; OD, optical density.

[5-Aza-2'-deoxycytidine (5-Aza-dC)] significantly increased the expression levels of miR-199a-5p in H1299 cells (Fig. 2B).

These data indicated that promoter hypermethylation may lead to the loss of miR-199a-5p in NSCLC.

Transfection with miR-199a-5p mimics suppresses the proliferation of NSCLC cells. To investigate the role of miR-199a-5p in NSCLC development, the proliferation of H1299 and H460 cells transfected with miR-199a-5p mimics was measured. The transfection efficiency was verified by RT-qPCR (Fig. 3A). The results of the CCK-8 assays demonstrated that the proliferation rates of H1299 and H460 cells were decreased following miR-199a-5p mimics transfection (Fig. 3B). Furthermore, it was revealed that the transfection of miR-199a-5p mimics interrupted 5-ethynyl-2'-deoxyuridine (EdU) incorporation, indicating that miR-199a-5p inhibited the DNA synthesis of NSCLC cells (Fig. 3C).

miR-199a-5p directly targets AKAP1 mRNA in NSCLC. To understand the mechanism underlying the antitumor function of miR-199a-5p in NSCLC, TargetScan was used to predict the potential transcripts targeted by miR-199a-5p. The seed sequence of miR-199a-5p was complementary to the 3'UTR of AKAP1 mRNA and is highly conserved among different species (Fig. 4A). Recently, the unique involvement of AKAP1 in tumor growth has been reviewed (14). In the present study, the luciferase assay results demonstrated that miR-199a-5p inhibited the activity of the reporter with wild-type 3'UTR of AKAP1 mRNA, but was not able to change the activity of the reporter when the miR-199a-5p binding site was mutated (Fig. 4B). Consistently, the mRNA and protein expression levels of AKAP1 were decreased following miR-199a-5p mimic transfection in H1299 and H460 cells (Fig. 4C). Since miR-199a-5p expression was induced by 5-Aza-dC treatment, the expression levels of AKAP1 were measured after 5-Aza-dC treatment. The results indicated that 5-Aza-dC decreased the mRNA and protein expression levels of AKAP1 (Fig. S1). In contrast to miR-199a-5p, clinical analysis demonstrated that the mRNA expression levels of AKAP1 were significantly higher in NSCLC tissues compared with in para-carcinoma tissues (Fig. 4D). Furthermore, the mRNA expression levels of AKAP1 were negatively correlated with miR-199a-5p expression in NSCLC tissues (Fig. 4E). Collectively, these findings suggested that miR-199a-5p may downregulate AKAP1 in NSCLC.

Overexpression of AKAP1 restores the proliferation of NSCLC cells transfected with miR-199a-5p mimics. To verify whether miR-199a-5p represses the proliferation of H1299 cells by downregulating AKAP1, H1299 cells stably overexpressing miR-199a-5p and AKAP1 were constructed (Figs. 5A, B and S2). Compared with the control group (EV), a suppression of cell proliferation and DNA synthesis was observed in H1299 cells stably overexpressing miR-199a-5p after 36 h of culturing, while overexpression of AKAP1 partially rescued cell proliferation and DNA synthesis (Fig. 5C and D). Additionally, overexpression of miR-199a-5p decreased the colony formation ability of H1299 cells, and overexpression of AKAP1, to a certain extent, recovered this attenuated colony formation (Fig. 5E). These results suggested that miR-199a-5p inhibited the proliferation of NSCLC cells by targeting AKAP1.

Discussion

The present study demonstrated that promoter hypermethylation inhibited the transcription of miR-199a-5p, which decreased the expression levels of AKAP1 by targeting AKAP1 mRNA. Furthermore, increased AKAP1 expression promoted the proliferation and colony formation of NSCLC cells.

Abnormal regulation of miRNAs serves an important role in the occurrence and development of lung cancer (24). The inhibition of miRNA biosynthesis has been observed in lung cancer (24). For example, Karube *et al* (25) reported that the expression levels of Dicer, a key endoribonuclease in miRNA-mediated gene silencing was decreased in NSCLC and low Dicer expression was associated with poor prognosis. Additionally, the results of animal experiments have demonstrated that conditional knockout of Dicer promotes tumor development in K-RAS-induced lung cancer (26). In rats, the tobacco carcinogen nicotine-derived nitrosamine ketone induces the dysregulation of various miRNAs (27). In addition, changes in the miRNA expression profile have been detected in precancerous lesions of the bronchial epithelium (28). However, it is difficult to identify specific miRNA markers for NSCLC due to tumor heterogeneity and different analysis methods. Wang *et al* (29) retrospectively analyzed four studies comparing the miRNA expression profiles between NSCLC and corresponding para-cancerous tissues; however, the differentially expressed miRNAs were different in each study.

The present study demonstrated that low expression levels of miR-199a-5p in NSCLC were associated with a poor survival, suggesting that miR-199a-5p may be a potential miRNA marker for the prediction of the prognosis of patients with NSCLC. Previously, Sanfiorenzo *et al* (30) reported that low expression levels of miR-199a-5p in the plasma are associated with poor survival of patients with NSCLC. Recently, it was revealed that miR-199a-5p could increase the doxorubicin sensitivity of NSCLC (31). Therefore, miR-199a-5p may function as a crucial tumor suppressor miRNA for NSCLC.

Several studies have revealed that long non-coding RNAs promote the proliferation of NSCLC via sponging of miR-199a-5p (32,33). In addition, hypermethylation of the CpG island has been identified to be closely associated with the abnormal miRNA expression profiles in tumors (34). In total ~1/2 of all miRNAs are regulated by the CpG island (35). Previous studies have reported that the altered status of DNA methylation leads to the abnormal regulation of miRNA expression in NSCLC (36-38). The miR-199a gene is located at two different chromosomes (miR-199a1 is located at chromosome 19 and miR-199a2 is located at chromosome 1) (23). Therefore, the present study first analyzed the distribution of CpG islands at the promoter regions of the two gene loci using bioinformatics software, and identified that there was a CG-rich region (-404/-272) upstream of the transcription start site of miR-199a1, while no potential CpG island was found at the miR-199a2 promoter, which was consistent with a previous study (23). Therefore, the present study only analyzed the transcriptional regulation of the miR-199a1 gene. Bisulfite sequencing demonstrated that the methylation levels of the CpG island at the miR-199a promoter were increased in NSCLC compared with in para-cancerous tissues. Furthermore, treatment with a DNA methyltransferase inhibitor increased the expression levels of miR-199a-5p in NSCLC cells. These findings suggested that promoter hypermethylation suppressed miR-199a-5p expression in NSCLC.

Deng *et al* (23) revealed that knockdown of DNA methyltransferase 3α (DNMT3A) reverses the hypermethylation

of the miR-199a gene in ovarian cancer. Furthermore, DNMT3A is highly expressed in various cancer types, including NSCLC, and can mediate the epigenetic silencing of tumor suppressor genes (39). Therefore, it was hypothesized that the promoter hypermethylation of the miR-199a gene may also be induced by aberrantly expressed DNMT3A in NSCLC.

The present study demonstrated that miR-199a-5p inhibited the proliferation of NSCLC cells by targeting AKAP1. Rinaldi *et al* (17) reported that AKAP1 promotes glioblastoma (GBM) growth by enhancing the mTOR signaling pathway. In addition, these authors observed that knockdown of AKAP1 severely impaired the oxidative metabolism of GBM cells. In general, high oxidative phosphorylation (OXPHOS) is adopted to promote tumor growth (40,41), and AKAP1 stimulates mitochondrial respiration (42). Therefore, deregulated AKAP1 may enhance OXPHOS to induce the proliferation of NSCLC cells.

In conclusion, the present study indicated that promoter hypermethylation caused the downregulation of miR-199a-5p expression in NSCLC, and miR-199a-5p was identified as a novel regulator of AKAP1 expression influencing NSCLC proliferation. Therefore, the present findings may provide a prognostic miRNA marker for patients with NSCLC.

Acknowledgements

Not applicable.

Funding

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

NY and LJ conceived and designed the study. NY, YFL, TZ and YXL performed the experiments and wrote the manuscript. NY, ZC and XZ analyzed and interpreted the data. NY, XZ and LJ confirmed the authenticity of all the raw data. NY, XZ and LJ drafted the manuscript. All other authors helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Experiments using human tissues were approved by the Ethics Committee in Clinical Research of the First Affiliated Hospital of Wenzhou Medical University (issuing no. 2019-128; Wenzhou, China) and written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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