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Downregulation of miR-497 promotes tumor growth and angiogenesis by targeting HDGF in non-small cell lung cancer

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ABSTRACT

MicroRNAs (miRNAs) play important roles in the development of various cancers. MiRNA-497 functions as a tumor-suppressor that is downregulated in several malignancies; however, its role in non-small cell lung cancer (NSCLC) has not been examined in detail. Here, we showed that miR-497 is downregulated in NSCLC tumors and cell lines and its ectopic expression significantly inhibits cell proliferation and colony formation. Integrated analysis identified HDGF as a downstream target of miR-497, and the downregulation of HDGF by miR-497 overexpression confirmed their association. Rescue experiments showed that the inhibitory effect of miR-497 on cell proliferation and colony formation is predominantly mediated by the modulation of HDGF levels. Furthermore, tumor samples from NSCLC patients showed an inverse relationship between miR-497 and HDGF levels, and ectopic expression of miR-497 significantly inhibited tumor growth and angiogenesis in a SCID mouse xenograft model. Our results suggest that miR-497 may serve as a biomarker in NSCLC, and the modulation of its activity may represent a novel therapeutic strategy for the treatment of NSCLC patients.

1. Introduction

Lung cancer is the leading cause of cancer-related death worldwide, and non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases [1]. The overall 5-year survival for all lung cancer patients is 15%; however, the 5-year survival for patients with pathologic stage I NSCLC is 58–73%, underscoring the importance of the early detection of this disease. Angiogenesis, which is an early event of cancer development, is known to play a critical role in tumor growth, invasion and metastasis, and several angiogenic factors have been identified as poor prognostic indicators of tumor aggressiveness and survival in NSCLC [2,3].

Hepatoma-derived growth factor (HDGF) is a heparin-binding growth factor that has been implicated in angiogenesis and its overexpression is correlated with poor clinical outcomes in several cancers. HDGF expression is associated with poor prognosis in patients with HCC [4], and has been correlated with poor disease free survival and overall survival in gastric carcinoma [5]. In NSCLC, a high level of HDGF in tumors was found to be correlated with a high incidence of tumor relapse and distant metastasis [6].

MicroRNAs (miRNAs) are small non-coding RNAs that function in the regulation of gene expression leading to the degradation of target mRNAs [7] or by inhibiting the mRNA from being translated into proteins [8]. MiRNAs regulate the expression of a wide variety of target genes, and are therefore involved in a wide range of biological processes including cell proliferation, differentiation and apoptosis [9]. Alterations in miRNA expression have been suggested to play important roles in tumorigenesis and cancer progression [10]. Recently, Tan and colleagues found that miR-497 is significantly deregulated in squamous cell carcinoma (SCC) compared with normal specimens [11]. The involvement of miR-497 in the tumorigenesis and development of breast, colorectal and cervical cancers has also been reported [12–14]. However, to our knowledge, its role in NSCLC remains undefined.

In the present study, we show that miR-497 is downregulated in NSCLC tissues and cell lines. Ectopic expression of miR-497 inhibited NSCLC cell proliferation and colony formation, and suppressed tumor growth in a xenograft mouse model. Furthermore, we identified HDGF as a target gene of miR-497 and showed that miR-497 exerts its effect on the inhibition of tumor development and growth by downregulating HDGF and suppressing its pro-angiogenic activity. Our findings demonstrate a novel role of miR-497 as a tumor suppressor in NSCLC.

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2. Materials and methods

2.1. Cell lines and patient samples

Four NSCLC cell lines, A549, SPC-A1, H1299 and H460, and a normal bronchial epithelial cell line (16HBE) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) in humidified air at 37 °C with 5% CO_2. Paired NSCLC and adjacent non-tumor lung tissues were obtained from 20 patients at Tianjin Tumor Hospital (Tianjin, China). Informed consent was obtained from each patient and this study was approved by the Ethics Committee of Tianjin Tumor Hospital.

2.2. Quantitative realtime PCR (qRT-PCR)

Total RNA was extracted from cells or tissues using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. For the detection of miR-497, reverse transcription and qRT-PCR reactions were performed using the standard SYBR Green Assay protocol and the ABI PRISM 7500 Sequence Detection System (ABI). The primers for miR-497 were 5′-GTGCAGGG TCC GAGGT-3′ (forward) and 5′-TAGCCTGCAGCACACTGTGGT-3′ (reverse). U6 snRNA was used as a normalization control. qRT-PCR analyses for HDGF and the normalization control gene GAPDH were performed using SYBR Premix Ex Taq (TaKaRa, Dalian, China). The relative expression of each gene was calculated and normalized using the $2^{-\Delta\Delta Ct}$ method relative to U6 snRNA or GAPDH.

2.3. Lentivirus production and infection

The human miR-497 precursor sequences were cloned into the lentivirus-based expression plasmid pLenti-6.3 (Invitrogen). The primers for pre-miR-497 were 5′-AGAATT CCCCCTCCAGTCATTCCC-TATTTCTT-3′ (forward) and 5′-ACTCGAGCTCAGCCCCTC CTCGGT AGTTTTCA-3′ (reverse). Viral packaging and infection were performed according to standard protocols as recommended by the manufacturer. Cells (1×10^6) were infected with 1×10^7 lentivirus transducing units in the presence of 10 μg/mL polybrene (Sigma–Aldrich, St. Louis, Missouri, USA). An empty lentiviral vector was used as negative control.

2.4. Plasmid construction and luciferase reporter assays

The full-length open reading frame of HDGF was cloned into pcDNA3.1 (+) to generate HDGF expression vectors. The primers for HDGF were 5′-GCCTAGGAACACAAACAACTG CACGAGCG-3′ (forward) and 5′-TGAATTCATTGGTGGCTACAGGCTCTCAT-3′ (reverse). The wild-type HDGF 3′UTR (WT) was cloned into the pGL3 Basic Vector (Promega, Madison, WI, USA). Site-directed mutagenesis of the miR-497 seed sequence in the HDGF 3′-UTR (Mut) was performed using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The primers for HDGF 3′-UTR were 5′-ACCGCGGATGAGAGCCTGTAGCCACCAA T-3′ (forward) and 5′-T CTCGAGTGAGTAGAAGAGGAGAGCAGGT-3′ (reverse). For the reporter assays, stable miR-497 overexpressing cells were transiently transfected with WT or Mut reporter plasmids using Lipofectamine 2000 reagent (Invitrogen). After 48 h, the cells were harvested and lysed, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Renilla-luciferase was used for normalization.

2.5. Cell viability assays

Cells were plated in 96-well microtitre plates at a density of 5000 cells/well. At the completion of incubation, cell viability was assessed by MTT assay as described previously [15].

2.6. Colony formation assay

Twenty-four hours after infection, 500 infected cells were placed in a fresh six-well plate and maintained in DMEM medium containing 10% FBS for 2 weeks. Colonies were fixed with methanol and stained with 0.1% crystal violet in 20% methanol for 15 min. Colony numbers in each assay were quantified by Alpha Innotech (San Leandro, CA, USA) imaging software and percentage colony formation was calculated by adjusting control cells to 100.

2.7. Western blotting

For each sample, 25 mg of total protein extracts were separated on SDS–PAGE gels and transferred to PVDF membranes, which were blocked with 5% non-fat dry milk for 2 h and incubated with primary antibodies against HDGF (Cell Signaling Technology Inc., Danvers, MA, USA) and β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). All the primary antibodies were incubated overnight at 4 °C, followed by incubation with AP-conjugated goat anti-rabbit secondary antibody and detection.

2.8. Tumor xenograft model

1×10^6 SPC-A1-miR-497 or control cells were mixed with 100 μl of Matrigel and injected subcutaneously in the flanks of SCID mice ($n = 5$) as described previously [16]. The length and width of the tumors were measured every 5 days using a digital caliper and tumor volumes were calculated using the formula Volume (mm^3) = $L \times W^2/2$ (length L, mm; width W, mm). After 25 days, tumor samples were carefully removed and weighed. All animal procedures were performed in accordance with institutional guidelines.

2.9. Statistical analysis

Data were expressed as the mean ± SEM from at least three independent experiments. The Student's *t*-test was used to compare the differences between two groups. Correlation between the miR-497 and HDGF level was evaluated using Spearman's correlation coefficient. $P < 0.05$ was considered statistically significant.

3. Results

3.1. MiR-497 is down-regulated in NSCLC cells and tissues

The expression of miR-497 was examined in four NSCLC cell lines (A549, SPC-A1, H1299 and H460) and normal human bronchial epithelial cells (16-HBE) by qRT-PCR. The results showed that NSCLC cells expressed significantly lower levels of miR-497 than 16-HBE cells, with the lowest expression levels detected in SPC-A1 and H1299 cells (Fig. 1A). These two cell lines were therefore used for subsequent experiments. Assessment of miR-497 expression in tumor tissues and paired adjacent normal tissues of 20 patients with NSCLC by qRT-PCR showed that miR-497 was significantly downregulated in NSCLC tissues compared with adjacent normal tissues (Fig. 1B).

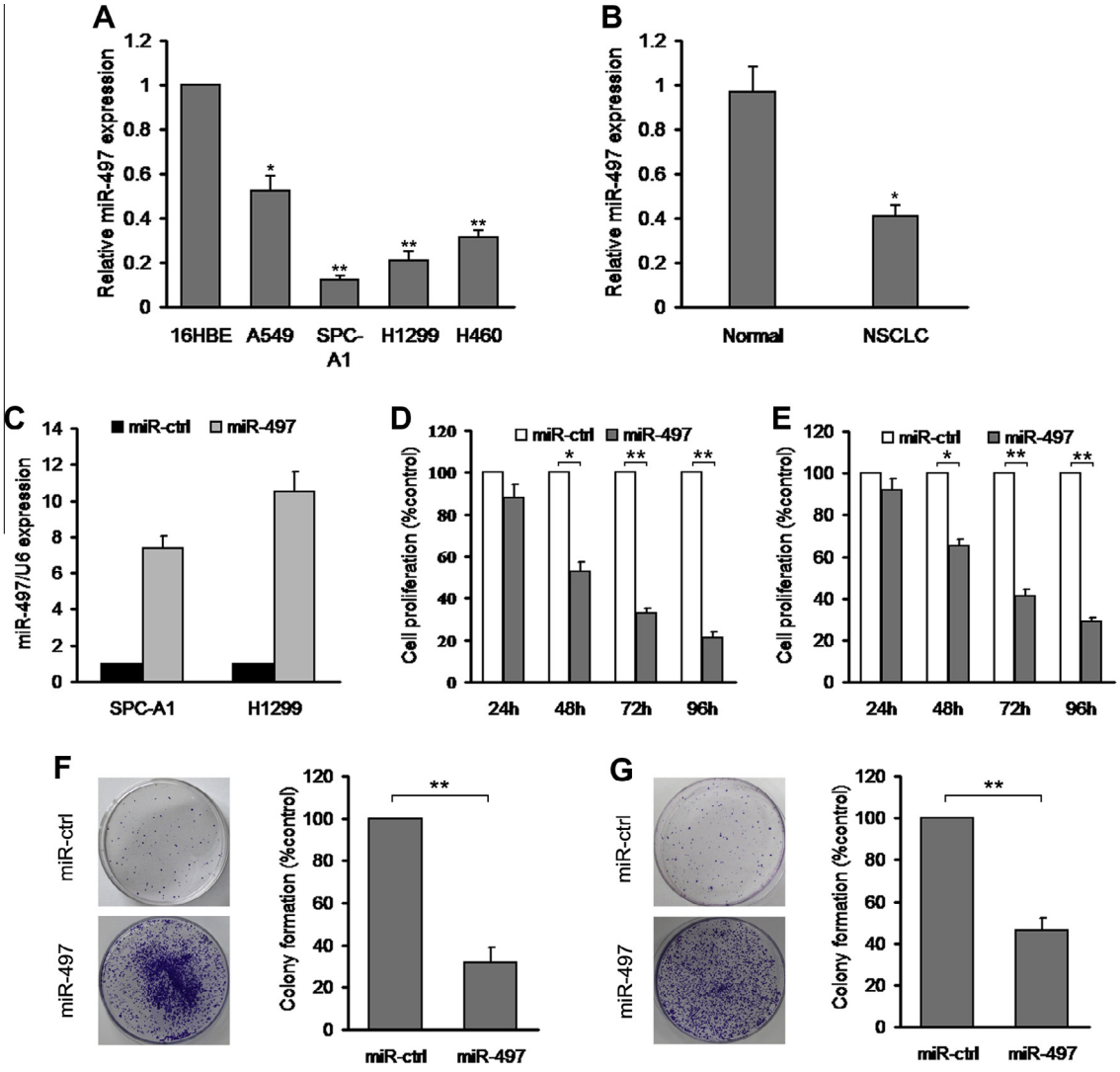

Fig. 1. MiR-497 is downregulated in human NSCLC tissues and cell lines, and overexpression of miR-497 inhibits NSCLC cell proliferation and colony formation in vitro. (A) MiR-497 expression levels in four NSCLC cell lines were measured by qRT-PCR with snRNA U6 levels as an internal control. (B) The expression levels of miR-497 were analyzed by qRT-PCR in 20 pairs of NSCLC tissues and adjacent normal tissues. (C) SPC-A1 and H1299 cells were infected with miR-497 or miR-control lentivirus, and miR-497 expression levels were analyzed by qRT-PCR. (D, E) Cell proliferation assay (MTT). The percentage cell proliferation of tumor cells infected with miR-497 was calculated by adjusting the proliferation index of tumor cells infected with miR-control to 100. (F, G) Colonogenic assays. The percentage colony formation for tumor cells infected with miR-497 was calculated by adjusting tumor cells infected with miR-control to 100.

3.2. MiR-497 inhibits NSCLC cell growth in vitro

To examine the role of miR-497 in NSCLC cell growth, SPC-A1 and H1299 cells were infected with lentivirus particles carrying miR-497 gene. Increased expression of miR-497 upon infection in these cells was confirmed by qRT-PCR (Fig. 1C). Overexpression of miR-497 significantly decreased the growth of SPC-A1 and H1299 cells when compared to their corresponding controls (Fig. 1D and E). Accordingly, miR-497 overexpression significantly inhibited colony formation in both NSCLC cell lines (Fig. 1F and G). These results indicate a growth-inhibitory role of miR-497 in NSCLC cells.

3.3. HDGF is a target gene of miR-497

To explore the function of miR-497 in NSCLC, we used TargetScan and miRanda algorithms to search for target genes of miR-497. Among mRNAs containing miR-497 recognition sites in their 3′-UTRs, we focused on HDGF (Fig. 2A), a protein involved in tumorigenesis and progression of NSCLC. To verify that HDGF is a direct target of miR-497, HDGF wild-type (WT) or mutant 3′-UTR was subcloned into a luciferase reporter vector and co-transfected with miR-497 or scrambled control into SPC-A1 and H1299 cells. The results showed that miR-497 significantly inhibited the luciferase activity of the HDGF WT 3′-UTR but not that of the mutant in both cell lines (Fig. 2B). Furthermore, overexpression of miR-497 significantly downregulated HDGF mRNA and protein levels in both NSCLC cell lines (Fig. 2C and D). These results indicated that HDGF is a target gene of miR-497.

3.4. MiR-497 inhibits NSCLC cell growth by downregulating HDGF

To further examine whether miR-497 exerts its tumor suppressor function through inhibition of HDGF, we performed rescue studies. SPC-A1 cells were infected with miR-497 or scrambled control for 72 h followed by transfection with HDGF vector (without 3'UTR). As shown in Fig. 3B and C, miR-497 overexpression significantly inhibited SPC-A1 cell proliferation and colony formation, whereas co-transfection with an HDGF-overexpressing vector partially blocked the miR-497 induced inhibition of cell proliferation

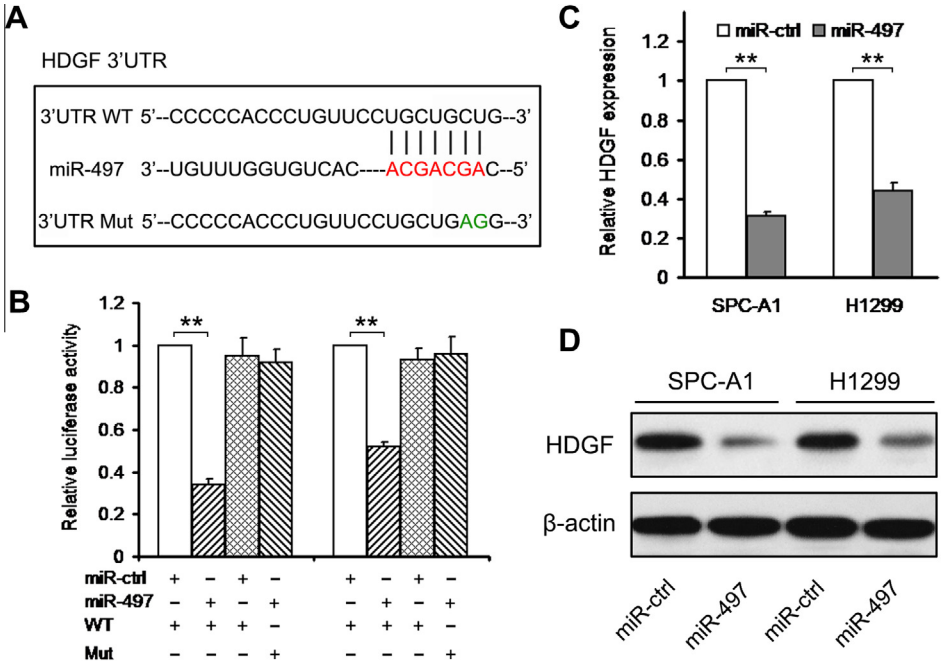

Fig. 2. HDGF is a downstream gene target of miR-497. (A) Sequence alignment of miR-497 and the HDGF 3′-UTR, which contains one predicted miR-497-binding site. The miR-497 seed regions and the seed-recognizing sites in the HDGF 3′-UTR are indicated in red. (B) Luciferase assay in SPC-A1 and H1299 cells co-transfected with miR-497 and a luciferase reporter containing the HDGF 3′-UTR (WT) or a mutant (Mut). Luciferase activities were measured 48 h post-transfection. (C) HDGF mRNA levels were analyzed after miR-497 transfection by qRT-PCR. (D) miR-497 transfection affects HDGF protein levels.

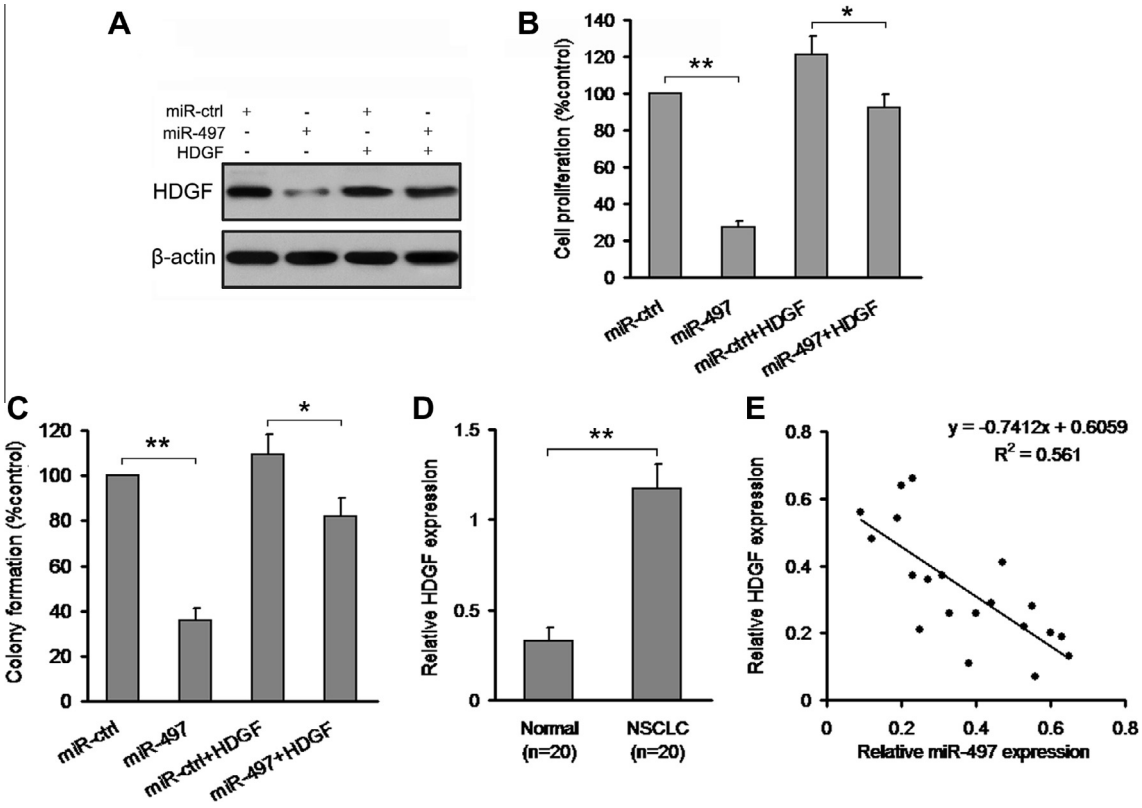

Fig. 3. The biological function of miR-497 is mediated by the downregulation of HDGF. (A) SPC-A1 cells were infected with miR-497 or miR-control. Seventy-two hours after transfection, whole cell lysates were prepared and analyzed by Western blotting using a specific antibody against HDGF. (B) SPC-A1 cells overexpressing miR-497 were transfected with HDGF or a scrambled control. After 72 h, cell proliferation was measured. (C) Clonogenic assays. (D) The relative expression levels of HDGF in 20 NSCLC tissues and adjacent normal tissues were determined. (E) The inverse correlation between HDGF and miR-497 expression in 20 NSCLC samples was determined using Spearman's correlation analysis.

and colony formation. These results indicated that miR-497 reduces the viability of SPC-A1 cells and inhibits colony formation by downregulating HDGF. The association between HDGF and NSCLC was further examined by analyzing the levels of expression of HDGF in tissues from 20 NSCLC patients, which showed that HDGF levels were significantly higher in tumor tissues than in the matched adjacent non-tumor tissues (Fig. 3D). An inverse correlation between the expression of HDGF and that of miR-497 in these tissues (Fig. 3E) confirmed that HDGF is a target of miR-497.

3.5. MiR-497 inhibits tumor growth and angiogenesis in vivo

To examine the role of miR-497 in NSCLC tumor development, we used a SCID xenograft mouse model in which mice were transplanted with miR-497 overexpressing and scramble control SPC-A1 cells. After 25 days, miR-497 overexpressing tumors were significantly smaller than those of mice transfected with the scrambled control (Fig. 4A). Furthermore, overexpression of miR-497 significantly reduced xenograft tumor volume (Fig. 4B) and tumor weight (Fig. 4C). Given that HDGF has been implicated in the regulation of angiogenesis, we investigated the potential mechanisms of antitumor activity of miR-497 involving HDGF by assessing the vascular density of tumor samples by immunohistochemical staining with an antibody against CD31. The results showed that microvessel density was significantly lower in miR-497 overexpressing tumors than in control tumors (Fig. 4D), suggesting that the mechanism underlying the effect of miR-497 on the inhibition of tumor development and growth involves the downregulation of HDGF and thus the suppression of its pro-angiogenic activity.

4. Discussion

In the present study, we showed that miR-497 is downregulated in NSCLC tissues and cell lines and identified HDGF as a target gene of miR-497. The correlation between miR-497 downregulation and NSCLC cell proliferation and tumor growth suggests that it plays a tumor suppressor role.

MiRNAs play important roles in a variety of biological processes including metabolism, proliferation, development, differentiation, apoptosis and immune responses [17]. Deregulation of miRNAs is associated with several diseases including cancer [18], and tumor-associated miRNAs can function as tumor suppressors or oncogenes depending on whether they target oncogenes or tumor suppressor genes. In addition, the deregulation of miRNA activity has been shown to be associated with the development and progression of several cancers through their effect on different regulatory pathways [11]. In NSCLC, a distinct pattern of up- and down-regulation of different miRNAs suggests their potential as diagnostic, prognostic and predictive markers [19]. Yanahaira et al. used microarray analysis to identify a unique microRNA profile capable of discriminating lung cancers from noncancerous tissues and molecular signatures distinguishing specific NSCLC histological sub-types [7]. These authors showed that overexpression of the precursor of miR-155 and downregulation of let-7a-2 were correlated with poor survival of NSCLC patients. In addition, the miR-29 family, composed of miR-29a, 29b and 29c, was shown to be downregulated in NSCLC [20].

MiR-497 belongs to the miR-15/16/195/424/497 families, whose members share the same 3′-UTR seed sequence and regulate several genes involved in cell cycle progression [13]. MiR-497 is downregulated in cervical cancer and has been shown to target IGF-1-R in colorectal cancer and cervical cancer [13,14]. However, the specific mechanism by which miR-497 downregulation affects tumor development and progression has not been elucidated and its involvement in NSCLC has not been addressed in detail.

In the present study, we showed that miR-497 specifically targets HDGF in NSCLC cell lines, suggesting that the mechanism of action of miR-497 may involve the modulation of HDGF

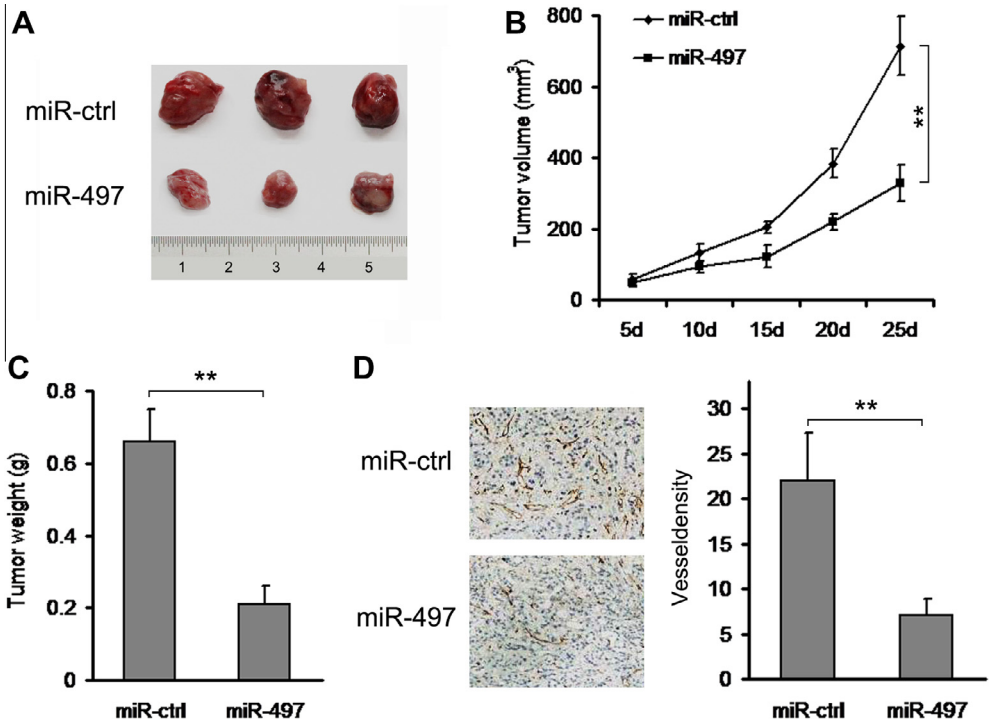

Fig. 4. MiR-497 inhibits tumor growth and angiogenesis in vivo. (A) Representative image of tumors formed. (B) Growth curve drawn by measuring tumor volumes at the indicated times. (C) Weight of xenograft tumors. (D) Tumors were stained with CD31 antibody and positively-stained blood vessels were counted in 5 areas with maximum number of microvessels. Bar, 50 μm.

expression. HDGF is highly expressed in lung vascular endothelial cells and it acts as an angiogenic factor by directly inducing vascular development and by promoting the expression of vascular endothelial growth factor (VEGF) [21]. Knockdown of HDGF inhibited growth and colony formation in NSCLC cells and suppressed vessel formation in NSCLC tumors, suggesting that HDGF is involved in anchorage-independent growth, cell invasion and angiogenesis in NSCLC [22]. Our results showed that miR-497 inhibited tumor angiogenesis in a mouse xenograft model and miR-497 downregulation was correlated with HDGF upregulation in NSCLC tumors. These results indicate that the downregulation of miR-497 in NSCLC tumors may promote angiogenesis through the upregulation of HDGF expression. A similar result was obtained in hepatocellular carcinoma, where the downregulation of miR-214, which was associated with recurrence and poor clinical outcome, induced expression and secretion of HDGF, leading to angiogenesis and increased tumor growth [23].

In conclusion, the present study showed that miR-497 is downregulated in NSCLC cells and tissues, and its ectopic expression inhibits cell proliferation and colony formation in vitro and tumor growth and angiogenesis in vivo. The inverse correlation between miR-497 and HDGF expression, and the downregulation of HDGF in response to ectopic expression of miR-497 identified HDGF as a target of miR-497 in NSCLC. The present results indicate that miR-497 could be a useful marker in NSCLC and a potential therapeutic target for the treatment of NSCLC patients.

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