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Original Article

miR-371 promotes cell growth and tumorigenesis in non-small cell lung cancer by targeting CSMD1

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Abstract: MicroRNAs (miRNAs) play an important role in cancer development and progression. The present study explored expression and the biological roles of miR-371 in non-small cell lung cancer (NSCLC). Present data shows that miR-371 was expressed at greater levels in NSCLC tissues and cell lines. Overexpression of miR-371 significantly promoted cell proliferation and colony formation in H460 NSCLC cells, whereas inhibition of miR-371 suppressed cell proliferation and induced apoptosis in H1299 NSCLC cells. Bioinformatic analysis and luciferase reporter assays revealed that CUB and Sushi Multiple Domains 1 (CSMD1) is a direct target of miR-371. miR-371 showed the ability to negatively regulate endogenous CSMD1 expression in NSCLC cells. CSMD1 was underexpressed in NSCLC specimens compared to adjacent normal tissues. Small interfering RNA-mediated silencing of CSMD1 led to a significant ($P < 0.05$) induction of NSCLC cell proliferation. Rescue experiments with a CSMD1 construct lacking the 3'-UTR further confirmed that miR-371-induced H460 cell proliferation was prevented by overexpression of CSMD1, accompanied by significant apoptotic response. *In vivo* studies demonstrated that overexpression of miR-371 accelerated growth and reduced CSMD1 expression in H460 xenograft tumors. Overall, present findings indicate that miR-371 promotes NSCLC growth and tumorigenesis by targeting CSMD1, serving as a potential therapeutic target for NSCLC.

Keywords: Apoptosis, growth, lung cancer, miR-371, therapeutic target

Introduction

Non-small-cell lung carcinoma (NSCLC), accounting for about 85% of all lung cancers, is the most common type of cancer and a leading cause of cancer-related deaths worldwide [1]. Although great advances have been made in cancer diagnosis and treatment, the prognosis of NSCLC remains poor, with a 5-year overall survival rate of less than 15% [2]. It has been established that downregulation of tumor suppressors represents a critical mechanism for tumor development and progression [3]. CUB and Sushi Multiple Domains 1 gene (CSMD1) has been identified as a candidate tumor suppressor gene located on human chromosome 8 [4]. Reduction or loss of CSMD1 gene expression frequently occurs in many cancers, including lung, head and neck, breast, and skin cancers [5]. It has been documented that reduced expression of CSMD1 is significantly associated with shorter overall survival of patients with invasive ductal breast carcinoma [6]. Preclinical studies have provided direct evidence for the tumor suppressive activity of CSMD1 in melanomas [7]. However, the roles of CSMD1 in NSCLC remain unclear.

MicroRNAs (miRNAs) are a family of endogenous small non-coding RNAs of 20-25 nucleotides in length. They can negatively regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of target mRNAs [8]. Compelling evidence has indicated that miRNAs play a fundamental role in the pathogenesis of cancers, acting as tumor suppressors or oncogenes [9, 10]. miR-371 has been shown to promote cell proliferation in pancreatic cancer [11] and hepatocellular carcinoma [12] cells. However, this miRNA exerts opposite effects in

several other types of cancers, such as colorectal cancer [13].

The present study determined the expression and biological relevance of miR-371 in NSCLC. To explore the molecular mechanisms for miR-371 action, this study searched for and functionally characterized the target genes of miR-371.

Materials and methods

Tissue specimens

Primary NSCLC tumors and adjacent non-tumorous lung tissues were obtained from 27 NSCLC patients that underwent surgical resection of tumors at Cixi People's Hospital Affiliated to Wenzhou Medical College (Cixi, China). Tumors were staged according to the seventh tumor-node-metastasis (TNM) staging system of the American Joint Committee on Cancer (AJCC) for lung cancer [14]. The tumors studied consisted of 13 cases of TNM I, 8 TNM II, and 6 TNM III.

Tissue samples were snap-frozen and stored at -80°C until gene expression analysis. All cases were confirmed by pathological examination. Patients receiving any anticancer treatment before surgery were excluded. Written informed consent was obtained from each patient. The study protocol was approved by the Ethics Committee of Wenzhou Medical College.

Cells

Five human NSCLC cell lines (A549, H460, H1299, H1373, and H1975) and one normal immortalized lung cell line (BEAS-2B) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). HEK293 cells were obtained from Shanghai Institute of Cell Life Sciences Resource Center (Shanghai, China). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO_2 incubator.

Measurement of miRNA expression

RNA was isolated from tissue and cell samples using TRIzol Reagent (Invitrogen), according to manufacturer protocol. cDNA synthesis was performed with a specific stem-loop primer for miR-371 (Applied Biosystems, Foster City, CA, USA). Mature miR-371 was quantified using the TaqMan microRNA Assay kit, following manufacturer instructions (Applied Biosystems). RNU6B was used as an endogenous control.

Oligonucleotides, plasmids, and cell transfection

miR-371 mimic, miR-371 inhibitor, and their negative controls were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). CSMD1 small interfering RNA (siRNA) and negative control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A miR-371-expressing plasmid was generated by inserting the DNA fragment containing human miR-371 precursor into pcDNA3.1(+) vector (Invitrogen). For the luciferase reporter assay, the 3'-UTR of human CSMD1 mRNA was amplified by PCR and cloned into pMIR-REPORT vector (Applied Biosystems). Using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA), a mutated CSMD1 3'-UTR was generated where a putative binding site for miR-371 was disrupted. For CSMD1 overexpression studies, a fragment containing full-length human CSMD1 open reading frame was amplified by PCR and inserted into pcDNA3.1(+) vector. All constructs were confirmed by DNA sequencing.

Cell transfection was performed using Lipofectamine 2000, according to manufacturer instructions (Invitrogen). miR-371 mimic, miR-371 inhibitor, CSMD1 siRNA, and their negative controls were used at a final concentration of 40-60 nM. At 24 hours post-transfection, cells were collected and subjected to gene expression, proliferation, and survival analysis. For generation of stable cell lines, H460 cells were transfected with the miR-371-expressing plasmid or empty vector (1 μg) and selected for 10 days in the presence of G418 (600 μg/mL; Sigma-Aldrich, St. Louis, MO, USA).

Cell proliferation and clonogenic assays

Transfected cells were seeded into 96-well plates (5×10^3 cells/well) and allowed to grow for 48 or 72 hours. The 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (0.5 mg/ml, Sigma-Aldrich) was added to each well and incubated for 4 hours at 37°C. Generated crystals were dissolved by

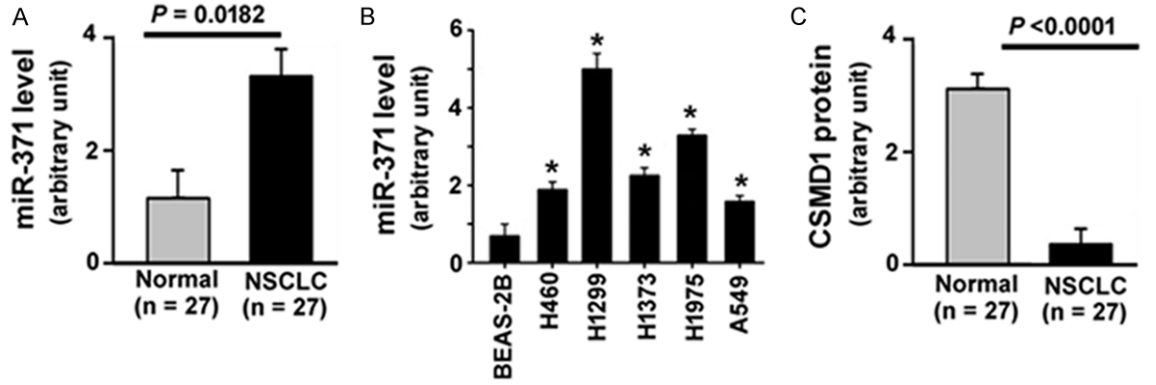

Figure 1. miR-371 was overexpressed in human NSCLC tissues and cells. A. Determination of miR-371 levels in 27 pairs of NSCLC and their adjacent non-tumorous lung tissues. B. Analysis of miR-371 in 5 NSCLC cell lines and BEAS-2B normal immortalized lung cells. C. Western blot analysis of CSMD1 protein levels in 27 pairs of NSCLC and their adjacent non-tumorous lung tissues. $^{*}P < 0.05$ vs. BEAS-2B cells.

dimethyl sulfoxide. Absorbance at 570 nm was measured using a microplate reader. Cell proliferation was measured using the BrdU Cell Proliferation Assay Kit (Cell Signaling Technology, Danvers, MA, USA). Briefly, cells labeled with 5-bromo-2'-deoxyuridine (BrdU) were incubated with mouse anti-BrdU antibody and horseradish peroxidase (HRP)-conjugated anti-mouse IgG. The substrate 3,3',5,5'-tetramethylbenzidine was used. Absorbance was measured at 450 nm. For assessment of colony formation, cells were plated onto 6-well plates at a density of 600 cells per well. After culturing for 14 days, cells were fixed, stained with 0.1% crystal violet (Sigma-Aldrich), and counted under a microscope.

Apoptosis detection

Cell apoptosis was detected by flow cytometry using an Annexin V-FITC apoptosis detection-kit (Nanjing KeyGen Biotechnology, Nanjing, China), according to manufacturer instructions.

Target prediction

The present study predicted target genes for miR-371 using miRDB algorithm (http://mirdb.org/miRDB/). CSMD1 was among the list of candidate target genes with a high score.

Luciferase reporter assay

To determine if miR-371 can target CSMD1 3'-UTR, HEK293 cells (5×10^4) were seeded onto 24-well plates and co-transfected with miR-371 mimic or control miRNA (40 nM), together with CSMD1 3'-UTR reporters (200 ng). *Renilla* luciferase-encoding plasmid pRL-SV40 (10 ng; Promega, Madison, WI, USA) was also transfected as an internal control. After 24 hours, transfected cells were lysed and assayed using the dual-luciferase reporter assay system kit (Promega), according to manufacturer instructions. Relative luciferase activity was calculated by normalization to the *Renilla* luciferase activity.

Western blot analysis

Tissue and cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was incubated with anti-CSMD1 (1:300, Santa Cruz Biotechnology) and anti-β-actin (1:2000, Santa Cruz Biotechnology) antibodies overnight at 4°C, followed by secondary antibodies conjugated to HRP (Santa Cruz Biotechnology). Protein bands were detected with the enhanced chemiluminescent reagent (Millipore, Billerica, MA, USA) and quantified with Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Animal experiments

H460 cells stably transfected with miR-371-expressing plasmid or vector were harvested and washed. They (2×10^6 cells/mouse) were subcutaneously injected into the flanks of male BALB/c nude mice (Shanghai Laboratory Animals Center of Chinese Academy of Sc-

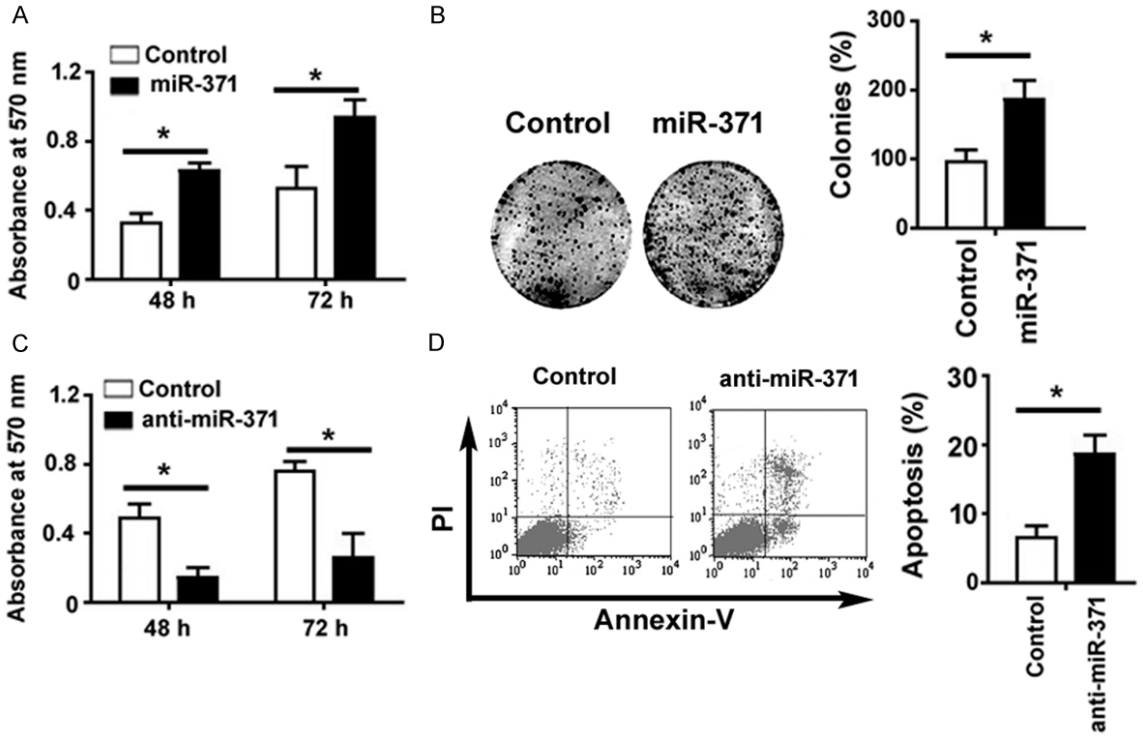

Figure 2. miR-371 promotes NSCLC cell proliferation and survival. A. MTT assay was performed to measure the viability of H460 cells transfected with control miRNA or miR-371 mimic after culturing for 48 and 72 hours. B. Colony formation assay was done to detect the abilities of H460 cells stably transfected with control miRNA or miR-371-expression plasmid to form colonies. *Left*, representative images of colonies. *Right*, quantitative results from three independent experiments. C. Analysis of the viability of H1299 cells transfected with control miRNA or anti-miR-371 after culturing for 48 and 72 hours. D. Measurement of apoptosis by flow cytometry after staining with annexin-V/PI. *Left*, representative dot plots of flow cytometric analysis. *Right*, quantitative results from three independent experiments. $^{*}P < 0.05$.

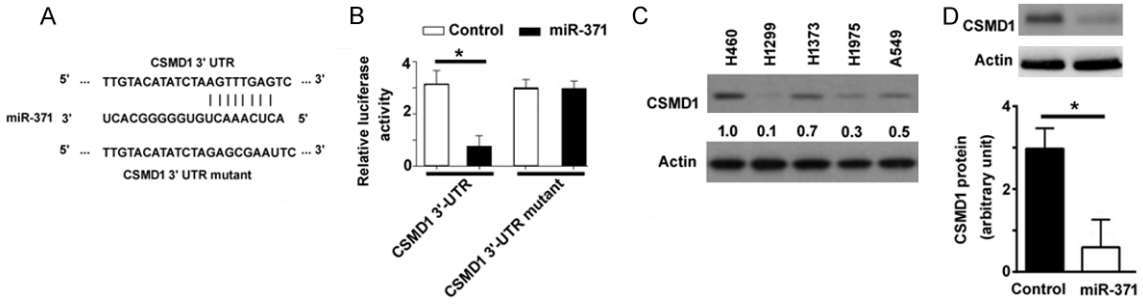

Figure 3. miR-371 targets CSMD1 via binding to its 3'-UTR. A. Bioinformatics analysis suggested a potential target site in the 3'-UTR of CSMD1 for miR-371. B. Luciferase reporter assay. HEK293 cells were transfected with wild-type or mutant CSMD1 3'-UTR reporters and control miRNA or miR-371 mimic and the activitis of the reporters were measured. C. Western blot analysis of CSMD1 protein levels in NSCLC cell lines. Numbers below the blots represent fold change relative to the level of CSMD1 in H460 cells. D. H460 cells were transfected with control miRNA or miR-371 mimic and CSMD1 protein levels were examined. *Top*, representative Western blots. *Bottom*, quantitative results from three independent experiments. $^{*}P < 0.05$.

iences, Shanghai, China). Tumor volumes were measured every week. At 4 weeks after cell injection, the mice were sacrificed. Tumors were removed and weighed. Tumor tissues were homogenized and processed for Western blot analysis. Animal studies were approved by the local Ethics Committee of Wenzhou Medical College.

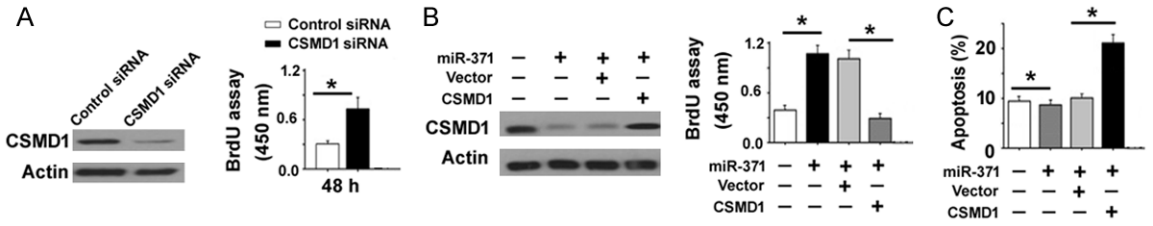

Figure 4. miR-371 accelerates NSCLC cell proliferation through downregulation of CSMD1. A. *Left*, Western blot analysis of CSMD1 in H460 cells transfected with control or CSMD1 siRNA. *Right*, H460 cells were transfected with control or CSMD1 siRNA and tested for cell proliferation by the BrdU incorporation assay at 48 hours after transfection. B. *Left*, Western blot analysis of CSMD1 in H460 cells transfected with indicated constructs. *Right*, analysis of cell proliferation in H460 cells transfected with indicated constructs by the BrdU incorporation assay. C. Measurement of apoptosis by flow cytometry after staining with annexin-V/PI. $^{*}P < 0.05$.

Statistical analysis

Data are expressed as mean ± SEM from 3 independent experiments. miR-371 and CSMD1 expression between NSCLC and adjacent lung tissues was analyzed using Mann-Whitney U-test. Differences in group means were determined using one-way analysis of variance or Student's *t*-test. *P*-values less than 0.05 are considered statistically significant.

Results

Expression of miR-371 was augmented in NSCLC tissues and cells

Expression of miR-371 was examined in 27 cases of NSCLC and their adjacent non-tumorous lung tissues. As shown in **Figure 1A**, there was a significant increase in the abundance of miR-371 in NSCLC tissues, compared to adjacent lung tissues ($P = 0.0182$). Next, this study measured relative miR-371 expression levels in a panel of human NSCLC cell lines. Consistent with the findings in NSCLC tissues, miR-371 was expressed at greater levels in the 5 NSCLC cell lines tested than in BEAS-2B normal immortalized lung cells ($P < 0.05$; **Figure 1B**). Of the NSCLC cell lines, H1299 cells had a relatively higher level of miR-371. Western blot analysis demonstrated that CSMD1 was underexpressed in cancer tissues, compared to adjacent lung tissues ($P < 0.0001$; **Figure 1C**).

miR-371 promotes NSCLC cell proliferation and survival

The present study explored the roles of miR-371 in NSCLC growth. MTT assay demonstrated that transfection with miR-371 mimic significantly ($P < 0.05$) enhanced cell proliferation in H460 cells, compared to control cells (**Figure 2A**). Colony formation assays showed that overexpression of miR-371 promoted colony formation by H460 cells *in vitro* (**Figure 2B**). Similarly, overexpression of miR-371 facilitated cell proliferation and colony formation of A549 cells (data not shown). To further check whether miR-371 was required for NSCLC growth, this miRNA was knocked down with an anti-miR-371 inhibitor and cell proliferation was examined. As shown in **Figure 2C**, inhibition of miR-371 led to a significant decline in the proliferation of H1299 cells. Moreover, a significant apoptotic response was detected in H1299 cells transfected with the anti-miR-371 inhibitor (**Figure 2D**).

miR-371 targets CSMD1 via binding to its 3'-UTR

Bioinformatic analysis based on miRDB algorithm was performed to search for potential target genes of miR-371, revealing CSMD1 as a candidate target gene (**Figure 3A**). To validate the targeting of the 3'-UTR of CSMD1 by miR-371, this study constructed the CSMD1 3'-UTR reporter and transfected it to HEK293 cells together with miR-371 mimic or control miRNA. The normalized luciferase activity of the reporter containing wild-type CSMD1 3'-UTR was significantly ($P < 0.05$) reduced by miR-371 (**Figure 3B**). However, mutation of the putative miR-371 binding site in the CSMD1 3'-UTR abrogated miR-371-mediated repression of reporter gene activity. Western blot analysis revealed that CSMD1 was highly expressed in H460 cells and to a lower extent in H1373, A549, and H1975 cells, but almost absent in H1299 cells (**Figure 3C**). Next, this study tested the ability of miR-

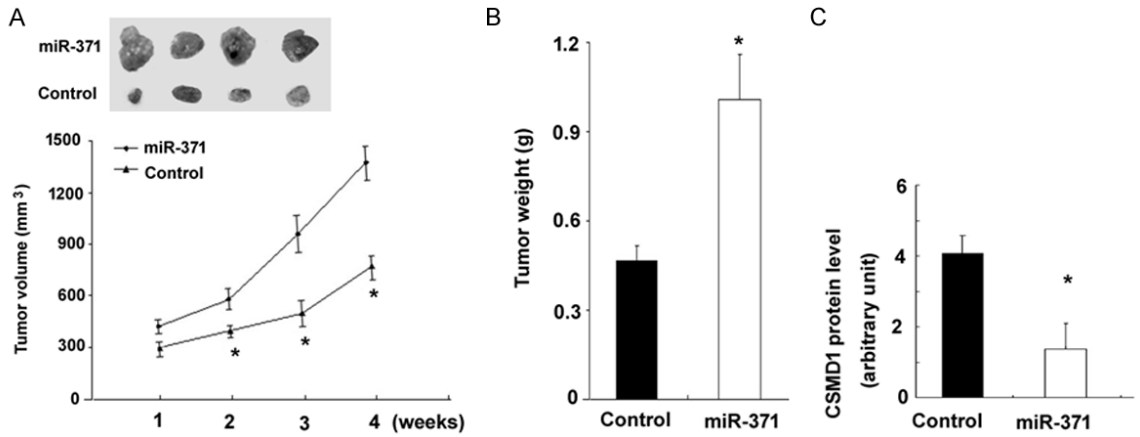

Figure 5. miR-371 enhances the growth of NSCLC xenograft tumors *in vivo*. H460 cells stably transfected with miR-371-expressing plasmid or empty vector were subcutaneously injected into nude mice (n = 4 for each group), while tumor growth and gene expression were analyzed. A. *Top*, representative images of tumors 4 weeks after cell injection. *Bottom*, growth curves of xenograft tumors are shown. B. Mean tumor weight was determined for each group. C. Western blot analysis of CSMD1 in the xenograft tumors. $^{*}P < 0.05$ vs. control.

371 to regulate endogenous CSMD1 expression. The delivery of miR-371 mimic significantly decreased protein levels of CSMD1 in H460 cells (**Figure 3D**). Similar results were observed in A549 cells after transfection with miR-371 mimic (data not shown). Collectively, these results pinpointed CSMD1 as a direct target of miR-371 in NSCLC cells.

miR-371 accelerates NSCLC cell proliferation through downregulation of CSMD1

The present study asked whether miR-371 exerted its positive effects on NSCLC cell proliferation by targeting CSMD1. To this end, CSMD1 expression was knocked down in H460 cells via siRNA technology, examining its silencing on cell proliferation. In accord with that induced by miR-371 mimic, silencing of CSMD1 led to a significant ($P < 0.05$) induction of NSCLC cell proliferation, as assessed by BrdU incorporation assay (**Figure 4A**). Rescue experiments with a CSMD1 construct lacking the 3'-UTR further demonstrated that miR-371-induced H460 cell proliferation was abolished by enforced expression of CSMD1 (**Figure 4B**). Moreover, overexpression of CSMD1 significantly induced apoptosis in miR-371-overexpressed H460 cells (**Figure 4C**).

miR-371 enhances the growth of NSCLC xenograft tumors in vivo

To confirm the tumor-promoting effects of miR-371 *in vivo*, this study implanted H460 cells stably transfected with miR-371-expressing plasmid or empty vector into the flanks of nude mice. Overexpression of miR-371 was found to accelerate the growth of H460 xenograft tumors, compared to control counterparts (**Figure 5A**). At the end of the experiment (4 weeks after cell implantation), tumors were excised and weighed. Mean tumor weight in the miR-371 group was 3-fold greater than that in the vector group (**Figure 5B**). Western blot analysis confirmed the downregulation of CSMD1 in miR-371-overexpressing tumors (**Figure 5C**). Results collectively indicate the role of miR-371 in the promotion of tumorigenesis of NSCLC cells.

Discussion

MicroRNAs are frequently dysregulated in cancers and participate in multiple aspects of tumor biology, including proliferation, adhesion, migration, invasion, and tumorigenesis [8-10]. It has been documented that downregulation of miR-145 and upregulation of miR-10b are correlated with lymph node metastasis in NSCLC [15]. miR-10b can promote the migration and invasion of A549 NSCLC cells, while miR-145 displays opposite effects on the invasiveness of A549 cells [15]. miR-613 has been reported to be downregulated in NSCLC and negatively regulate cancer cell growth by inducing cell cycle arrest [16]. The present study demonstrated that miR-371 was overexpressed in NSCLC tissues relative to adjacent normal lung tissues, suggesting its potential involvement in

the pathogenesis of NSCLC. Functional studies have revealed that delivery of miR-371 mimics significantly augmented NSCLC cell proliferation and colony formation, whereas inhibition of miR-371 impaired cell proliferation and induced apoptosis in NSCLC cells. *In vivo* studies further confirmed that miR-371 overexpression conferred a proliferative advantage to NSCLC xenograft tumors. These results highlight the importance of miR-371 in NSCLC growth. In agreement with present findings, miR-371 shows favorable effects on cell proliferation in pancreatic cancer [11] and hepatocellular carcinoma [12] cells. However, it is noteworthy that miR-371 can exert anti-proliferative effects against some types of cells [13, 17]. Therefore, the functional consequence of miR-371 dysregulation is cellular context dependent.

It has been established that a single miRNA can target many mRNAs, usually in a partially complementary manner, thus yielding distinct biological effects [18, 19]. Several target genes of miR-371 have been identified, such as PRPF4B [12] and SOX2 [13]. This study identified a novel target gene for miR-371. Bioinformatics analysis and the luciferase reporter assay demonstrated that miR-371 had the ability to repress expression of CSMD1 by binding to its 3'-UTR. Of the 5 NSCLC cell lines examined, H1299 cells had the highest level of miR-371, which was associated with weak expression of CSMD1. Overexpression of miR-371 led to a significant suppression of endogenous CSMD1 in NSCLC cells and xenograft tumors. Given that CSMD1 is known as a potential tumor suppressor gene [4], it was proposed that targeting CSMD1 may be an important mechanism for miR-371-mediated NSCLC growth. To test this hypothesis, loss- and gain-of-function studies were performed. Results showed that knockdown of CSMD1 phenocopied the effects of overexpression of miR-371 in NSCLC cells, facilitating cell proliferation, and that ectopic expression of a miRNA-resistant variant of CSMD1 reversed the induction of cell proliferation by miR-371. Consistent with present results, CSMD1 has shown antitumor activity in A375 melanoma cells [7]. The growth-suppressive activity of CSMD1 in NSCLC cells may explain its downregulation in this disease [5]. It has been documented that epigenetic inactivation contributes to reduced expression of CSMD1 in squamous cell carcinoma cells [21]. Present observations suggest that, in NSCLC cells, CSMD1 can be downregulated by another mechanism which involves the upregulation of miR-371.

Regarding the induction of miR-371 expression, several molecular mechanisms have been suggested. In colorectal cancer, demethylation of SOX17 has been reported to stimulate miR-371 expression [13]. Inhibitor of growth 1 (ING1) was found to be able to interact with the promoter of miR-371 [11]. Downregulation of ING1 accounts for overexpression of miR-371 in pancreatic cancer cells. However, the exact mechanisms for the upregulation of miR-371 in NSCLC need to be clarified in future work.

In conclusion, present data demonstrates that miR-371 is upregulated in NSCLC, providing a significant proliferative advantage to NSCLC cells. The oncogenic roles of miR-371 are largely mediated through repression of CSMD1, which shows anti-tumor activity in NSCLC cells. Therefore, miR-371 represents a potential therapeutic target for NSCLC.

Disclosure of conflict of interest

None.

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