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MiR-378 promotes the cell proliferation of non-small cell lung cancer by inhibiting FOXG1

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Abstract. – OBJECTIVE: To identify the functioning mode of miR-378 on non-small cell lung cancer (NSCLC) and provide therapeutic targets for NSCLC.

PATIENTS AND METHODS: Expression levels of miR-378 in human NSCLC tissue samples and NSCLC-derived cell lines were measured by using quantitative Real-time polymerase chain reaction (PCR). Cell proliferation capacity was assessed by methyl thiazolyl tetrazolium (MTT) assay and colony formation assay. Cell apoptosis and cell cycle distribution were identified by flow cytometry. Downstream target gene was confirmed by using luciferase and Western blotting assays.

RESULTS: MiR-378 was significantly elevated in NSCLC tissues when compared with para-carcinoma tissues (n=42). Decreased-miR-378 could attenuate cell proliferation capacity, as well as promoted cell apoptosis and induced cell cycle arrest at G0/G1 phase. FOXG1 was chosen as the target gene of miR-378 by bioinformatics analysis and luciferase reporter assay. Moreover, restoration of miR-378 could impair the tumor suppression role of downregulated-miR-378 on NSCLC growth.

CONCLUSIONS: Decreased-miR-378 exerted tumor-suppressive effects on NSCLC growth via targeting FOXG1 in vitro, which provided an innovative and candidate target for diagnosis and treatment of NSCLC.

Key Words:
microRNAs, miR-378, Proliferation, FOXG1, NSCLC.

Introduction

Lung cancer is a malignant tumor with the highest incidence and mortality rates in China. Non-small cell lung cancer (NSCLC) accounts for about 80-85% of lung cancer[1]. At present, the main treatment methods include operative treatment, chemotherapy, radiation therapy and targeted therapy. Although there are so many treatment methods, the 5-year survival rate of lung cancer is still less than 17%, and most patients eventually die from distant metastases[2]. Studying the molecular mechanisms of the occurrence and development of NSCLC will help find better therapeutic targets, so as to design better targeted therapeutic drugs.

Forkhead-box gene 1 (FOXG1), also known as brain factor 1, is a member of the forkhead-box family of transcription factors[3]. Recent studies have shown that the up-regulation and inverse proportion of FOXG1 are associated with the expression level of p21WAF1/cyclin-dependent kinase interacting protein 1 (CIP1) protein in ovarian cancer. Overexpression of FOXG1 is significantly related to high-grade ovarian cancer. Immunohistochemical analyses of ovarian cancer tissue microarray have further proved that high FOXG1 expression is significantly correlated with high-grade ovarian cancer[4]. FOXG1 contains a highly conserved deoxyribonucleic acid (DNA)-binding domain, which binds to specific DNA sequences and regulates gene expression. It has also been reported that overexpressed FOXG1 may help to maintain hepatocellular carcinoma and the undifferentiated state, which may be a potential target molecular therapy. In addition, excessive FOXG1 expression *in vivo* is correlated with the overgrowth of neural progenitor cells, which exerts an influence by binding to DNA and inhibiting activity. The role of FOXG1 is to maintain differentiation of normal neural stem/progenitor cell (NSPC), and its inactivation leads to premature NSPC differentiation in the cerebral cortex. FOXG1 protein plays a role by formation of transcription inhibition complexes and other regulatory proteins at least to some extent.

Micro ribonucleic acids (miRNAs) are a class of non-coding small RNAs, with approximate-

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ly 22 nucleotides in length. MiRNAs, which are commonly found in animal and plant cells, are post-transcriptional modification pathways for gene expression and regulation, and play regulatory roles primarily by acting on the 3' untranslated region (3'UTR) of the target gene mRNA[5]. It is estimated that more than 50% of mammalian genes are regulated by miRNAs. MiRNAs are involved in almost all-important processes of cell vital processes, including cell proliferation and differentiation, cell cycle regulation, death, apoptosis, migration, and invasion[6]. The expression dysregulation of miRNAs is involved in the occurrence and development of various diseases including tumors, and abnormal expressions of miRNAs can be detected in almost all tumors. These abnormally expressed miRNAs act as oncogenes or anti-oncogenes in tumors[7].

In this study, miRNAs that might directly target FOXG1 were firstly found via target gene prediction software and then, further verified in NSCLC cell lines. MiRNA-378, a new miRNA that could directly target FOXG1, was found. Next, the carcinogenic effect of miR-378 in NSCLC and its mechanism were further investigated, confirming that miR-378 may serve as a new research target in the treatment of NSCLC.

Patients and Methods

Patients

A total of 42 cases of lung cancer tissue specimens and adjacent tissue specimens were collected from NSCLC patients who underwent surgery in our hospital from May 2016 to May 2017. Surgically removed specimens were immediately put in liquid nitrogen for storage, and transferred to a refrigerator at -80°C. The whole collection and storage processes were performed in accordance with the principle of no enzyme. All patients did not receive any radiotherapy and chemotherapy before surgery, and received postoperative adjuvant chemotherapy. This investigation was approved by the Ethics Committee of the 2nd Affiliated Hospital of Harbin Medical University (Harbin, China). Signed written informed consents were obtained from all participants before the study.

Cell Culture

Human lung cancer cell lines (A549, H1299, H460, H520, H1975 and PC9 cells) and human normal lung epithelial cell line 16HBE were subcultured with Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin in a cell incubator with 5% CO_2 and saturated humidity at 37°C. The medium was replaced by fresh medium every 2-3 days, and cells in logarithmic growth phase were selected for experiments. Cells needing to be transfected were firstly cultured with RPMI-1640 medium containing 10% fetal bovine serum (FBS), then transfected and cultured with RPMI-1640 medium containing 5% FBS.

Plasmid and Transfection

Cells were digested with 0.25% trypsin, counted and seeded into a 96-well plate at 2×10^3 cells per well overnight. On the day of transfection, cell culture medium in 96 wells was replaced by fresh medium, and 150 μL RPMI-1640 medium containing 5% FBS was added into each well. Then, 50 μL serum-free RPMI-1640 medium was used to dilute 0.5 μL corresponding miRNA-inhibitor or NC-inhibitor, and0.75 μL Hiperfect transfection reagent was added. After that, the 96-well plate was taken out, and the above mixture was added dropwise with cell suspension and gently mixed to evenly distribute the transfection complex in the cell suspension.

RNA Extraction and qRT-PCR

A total of 40 mg lung cancer tissues and adjacent tissues were collected and added with TRIzol to extract RNA. Total RNA was extracted under aseptic RNase-free conditions in strict accordance with the instructions of RNA extraction kits (Invitrogen, Carlsbad, CA, USA). Then, extracted RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) according to the instructions of Prime-Script™ reverse transcription kits (TaKaRa, Otsu, Shiga, Japan). Real-time fluorescent quantitative polymerase chain reaction (PCR) reaction system was prepared according to the instructions of SYBR Green. After the reaction was completed, the cycle threshold (Ct) of each reaction tube was calculated, and the relative expression quantity of miR-378 gene was calculated by $2^{-\Delta\Delta Ct}$ method.

MTT Assay

NSCLC cells in logarithmic growth phase were collected and subjected to conventional trypsinization to prepare into single cell suspension. The cell count was adjusted to $2*10^4$/mL. 100 μL suspension were put into each well of the

96-well plate and placed in an incubator overnight. On the next day, the medium was changed to RPMI-1640 medium containing 5% FBS, and transfection with NC-inhibitor and miR-378 inhibitor was carried out. After 0-4 days of transfection, 20 μL 5 mg/mL methyl thiazolyl tetrazolium (MTT) were added to each well, and placed in the incubator for 4 h. Then, the medium was removed, and 200 μL dimethylsulfoxide (DMSO) were added into each well, followed by mixing on a shaker at room temperature in a dark place for 10 min to dissolve formazan crystals. Lastly, a microplate reader was used to read absorbance values at 570 nm, followed by drawing of the growth curve.

Colony Formation Assay

To further investigate cell growth of NSCLC cells, cells were plated in 6-well plates at a density of 600 per well, and maintained in normal medium for 10 d. The colonies were fixed in 70% methanol for 20 min and stained with 0.5% crystal violet for 10 min on ice, washing each well 3 times with phosphate-buffered saline (PBS).

Cell Apoptosis Analysis

Cells were seeded into a 6-well plate, transfected with miR-378 inhibitor or NC-inhibitor for 72 h, and subjected to apoptosis assay. Cells were digested with ethylene diamine tetraacetic acid (EDTA)-free trypsin. The digestion was stopped using complete medium. Then, cells were collected, 250 μL 1xBinding Buffer were added into each tube to re-suspend cells, and cell concentration was adjusted to 1×10^6/mL. After that, 100 μL cell suspension was taken, 5 μL Annexin V/ fluorescein isothiocyanate (FITC) and 10 μL 20 μg/mL propidium iodide (PI) solution were added and incubated at room temperature in darkness for 15 min. Lastly, 300 μL binding buffer were added into the reaction tube, and the suspension was loaded onto a flow cytometer for detection.

Cell Cycle Analysis

Cells were inoculated into the 6-well plate and transfected with miR-378 inhibitor or NC-inhibitor for 72 h, followed by cell cycle detection. Cells were collected after conventional trypsinization and fixed in 70% ethanol at 4°C for 48 h. Cells were harvested after centrifugation and stained with 500 μL propidium iodide (PI) staining solution containing RNase for 1 h. Lastly, a flow cytometer was used for detection.

Luciferase Reporter Assay

Dual luciferase reporter plasmids inserted with wild-type and mutant FOXG1-3'UTRs were established. Human embryonic kidney 293T (HEK 293T) cells were seeded into the 96-well plate and co-transfected with reporter plasmids and miR-378 inhibitor or NC-inhibitor according to experimental methods described above. After 48 h of transfection, luciferase activity was measured by dual luciferase reporter system (Promega, Madison, WI, USA).

Western Blot Analysis

Cells were collected after 72 h of transfection, added with cell lysis solution, and centrifuged at 15000 r/min for 30 min to extract total protein. Then, the concentration of extracted protein was determined by bicinchoninic acid (BCA) method and adjusted, followed by conventional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After that, the target protein was transferred onto a polyvinylidene difluoride (PVDF) membrane by semi-dry method and blocked with 5% non-fat milk. Lastly, protein was incubated with primary antibodies [anti-FOXG1 (1:500) and β-actin (1:2000)] overnight, then secondary antibodies [goat anti-rabbit-horseradish peroxidase (HRP) and goat anti-mouse-HRP (1:5000)] at room temperature for 1 h, followed by X-ray film color development.

Statistical Analysis

The target gene prediction software (microma.org, TargetScan) was used to screen the miRNAs that could be directly targeted to FOXG1. The experimental data were expressed with mean standard deviation; statistical product and service solutions (SPSS 20.0, IBM, Armonk, NY, USA) was used to analyze the data, *t*-test was used between the two groups. Comparison between groups was done using one-way ANOVA test followed by least significant difference (LSD). $p<0.05$ was considered as statistically significant.

Results

MiR-378 Expression was Elevated in NSCLC Tissues and Cell Lines

To examine the expression level of miR-378 in human NSCLC, we examined miR-378 expression level in 42 NSCLC tissue samples and adjacent samples. Results show that the expression levels of miR-378 were significantly higher in the tumor tissue than that in adjacent tissues ($p<0.05$) (Figure 1A).

Besides, we investigated the expression of miR-378 in several NSCLC cell lines and normal pulmonary epithelial cell line with qRT-RCR. Compared with 16HBE cell line, all these NSCLC cell lines expressed a relatively higher level of miR-378, in which H1299 expressed the relatively highest (Figure 1B). To identify the mode of action of miR-378 in NSCLC tumorigenesis *in vitro*, H1299 cell line was transfected with miR-378 inhibitor and C-inhibitor for downregulation of miR-378 (Figure 1C).

Downregulation of miR-378 Inhibited NSCLC Cell Growth In Vitro

Compared with NC-inhibitor, the viability of NSCLC cells was significantly suppressed after it was transfected with miR-378 inhibitor ($p<0.05$) (Figure 2A).

In addition, we used colony formation assay to further identify the effect of miR-378 on cell proliferation. Compared with NC-inhibitor group, a significant decrease regarding the number of colonies was observed in NSCLC cells transfected with miR-378 inhibitor (Figure 2B). Collectively, all these results showed that miR-378 could suppress NSCLC proliferation.

Downregulation of miR-378 Suppressed Cell Apoptosis and Attenuated Cell Cycle Arrest at G0/G1 Phase

Then, we detected the mechanism of how miR-378 inhibits the proliferation of NSCLC cells with cell apoptosis and cell cycle analysis. As shown in Figure 2C, compared with NC-inhibitor, miR-378 inhibitor significantly increased the apoptosis rate of H1299 cells ($p<0.01$). As shown in Figure 2D, compared with NC-inhibitor, miR-378 inhibitor reduced the proportion of G0/G1 phase in H1299 cells, but the proportion of S phase increased. The difference was statistically significant ($p<0.01$), that means miR-378 weakened the G0/G1 phase arrest of H1299 cells.

These results indicated that miR-378 inhibitor attenuated the growth of NSCLC cells by inducing cell apoptosis and weakening the G0/G1 phase block of cell cycle.

FOXG1 is Directly Targeted by miR-378

Conserved miRNAs that might act on 3'UTR of FOXG1 were predicted using network computer prediction software, microrna.org and TargetScan (Figure 3A). To verify whether miR-378 reduces the expression of FOXG1 by directly targeting it, dual luciferase reporter plasmids were established to express wild-type FOXG1 mRNA 3'UTR and mutant FOXG1 3'UTRm at miR-378 binding site, respectively (Figure 3A). In HEK293T cells, co-transfection of FOXG1-3'UTR/miR-378 inhibitor significantly lowered luciferase activity in comparison with co-transfection of FOXG1-3'UTR/NC-inhibitor, while there was no significant difference in luciferase activity between co-transfection of FOXG1-3'UTRm/NC-inhibitor and co-transfection of FOXG1-3'UTRm/miR-

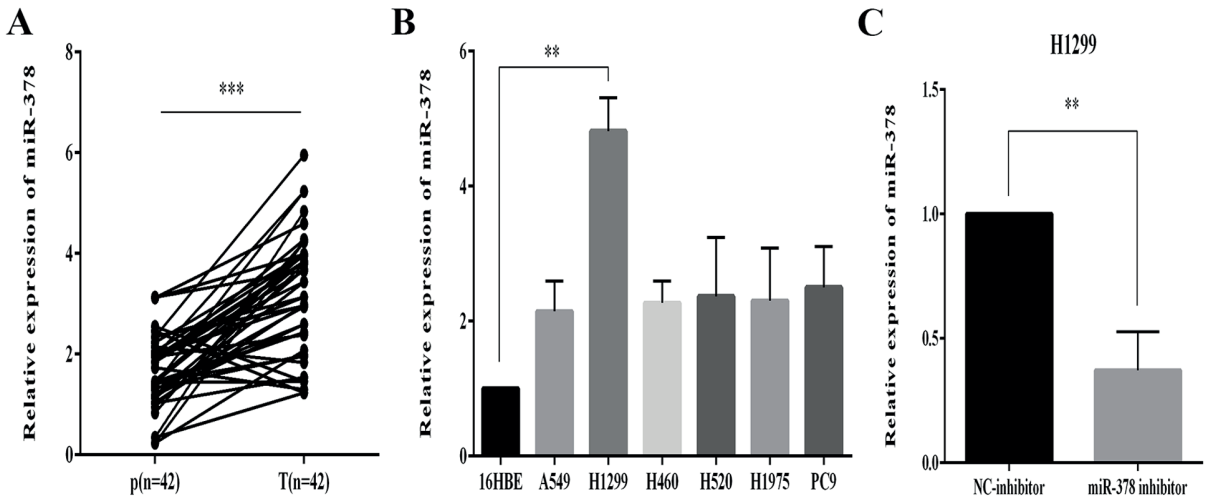

Figure 1. MiR-378 expression was elevated in NSCLC tissues and cell lines. ***A,*** Analysis of miR-378 expression in paracarcinoma tissues (P) and tumor tissues (T); ***B,*** Analysis of miR-378 expression in several NSCLC cell lines and normal cell line; ***C,*** Analysis of transfection efficiency in H1299 cells transfected with miR-378 inhibitor and NC-inhibitor. Total RNA was detected by qRT-PCR and GAPDH was used as an internal control. Data are presented as the mean ± SD of three independent experiments. $^{**}p<0.01$; $^{***}p<0.001$.

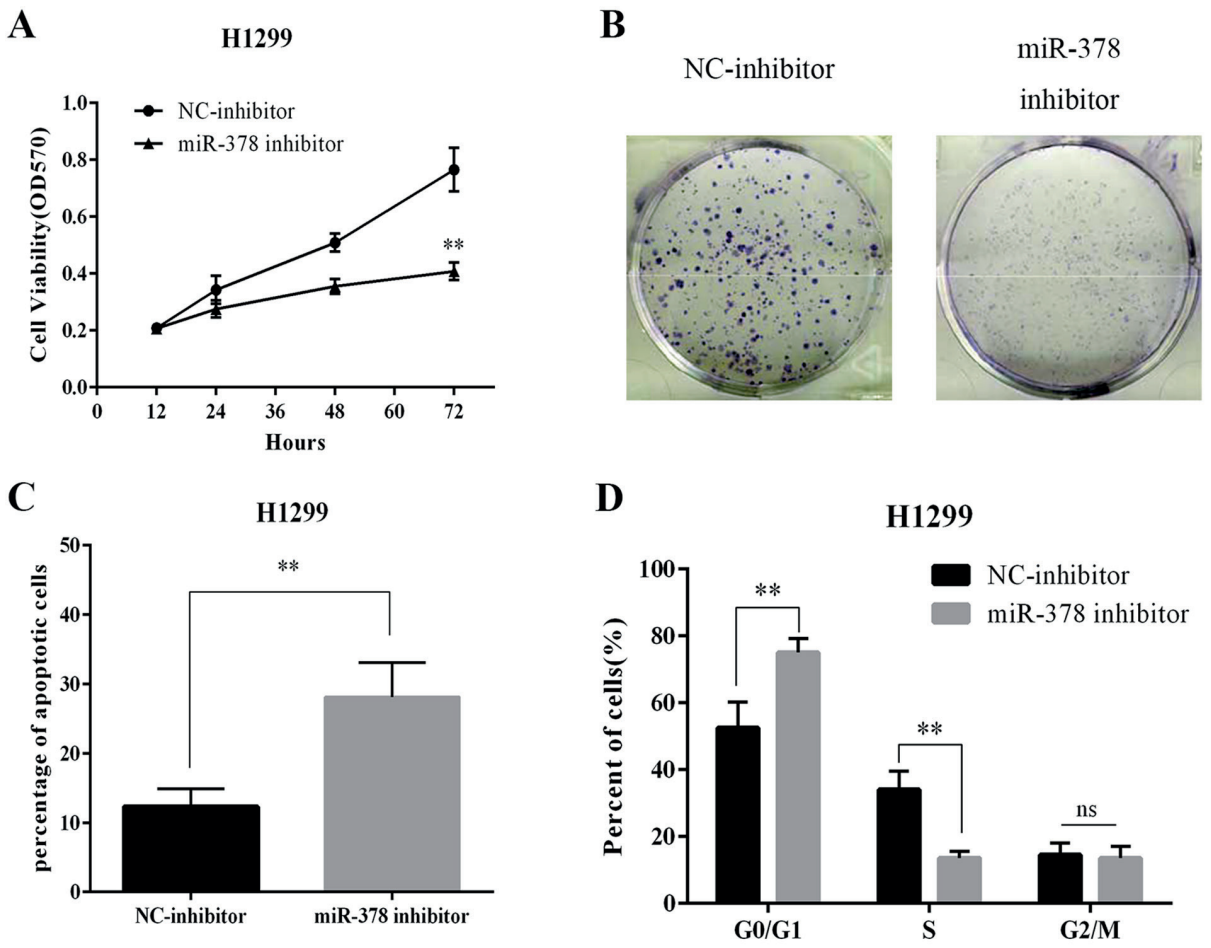

Figure 2. Downregulation of miR-378 inhibited NSCLC cell growth *in vitro*. ***A,*** MTT assay was performed to determine the viability of transfected H1299 cells; ***B,*** Colony formation assay was performed to determine the proliferation of transfected H1299 cells; ***C,*** Flow cytometric analysis was performed to detect the apoptotic rates of transfected H1299 cells; ***D,*** Flow cytometric analysis was performed to detect cell cycle progression of transfected MGC803 cells. $^{**}p<0.01$.

378 inhibitor (Figure 3B), indicating that FOXG1 mRNA 3'UTR is a direct target of miR-378, and miR-378 down-regulates the expression of FOXG1 by directly targeting FOXG1 mRNA 3'UTR.

Meanwhile, we further detected the expression level of FOXG1 in transfected NSCLC cells. The results indicated that FOXG1 was upregulated in H1299 cells transfected with miR-378 inhibitor on mRNA level and protein level when compared with NC-inhibitor (Figure 3C-D). All these results indicated that FOXG1 was directly targeted by miR-378.

Silencing of FOXG1 Recovered the Carcinogenesis Role of miR-378

To future identify the relationship of miR-378 and FOXG1, we firstly measured the expression of FOXG1 in NSCLC tissues. The results indicated that FOXG1 was significantly downregulated in NSCLC tissues compared with the para-carcinoma tissues on the mRNA level (Figure 4A), and the expression of FOXG1 was negatively correlated with the expression of miR-378 in NSCLC tissues (Figure 4B).

Secondly, we explored whether FOXG1 is responsible for the functional effects of miR-378 in RCCC tumorigenesis. We silenced FOXG1 expression by transfected with siRNA-FOXG1 in miR-378-decreased H1299 cells (Figure 4C). FOXG1 silencing not only increased the proliferation capacity (Figure 4D), but also attenuated cell apoptosis and promoted cell cycle distribution at G0/G1 phase (Figure 4E-F). These results implied that miR-378 promoted NSCLC tumorigenesis by inhibitingFOXG1expression partially.

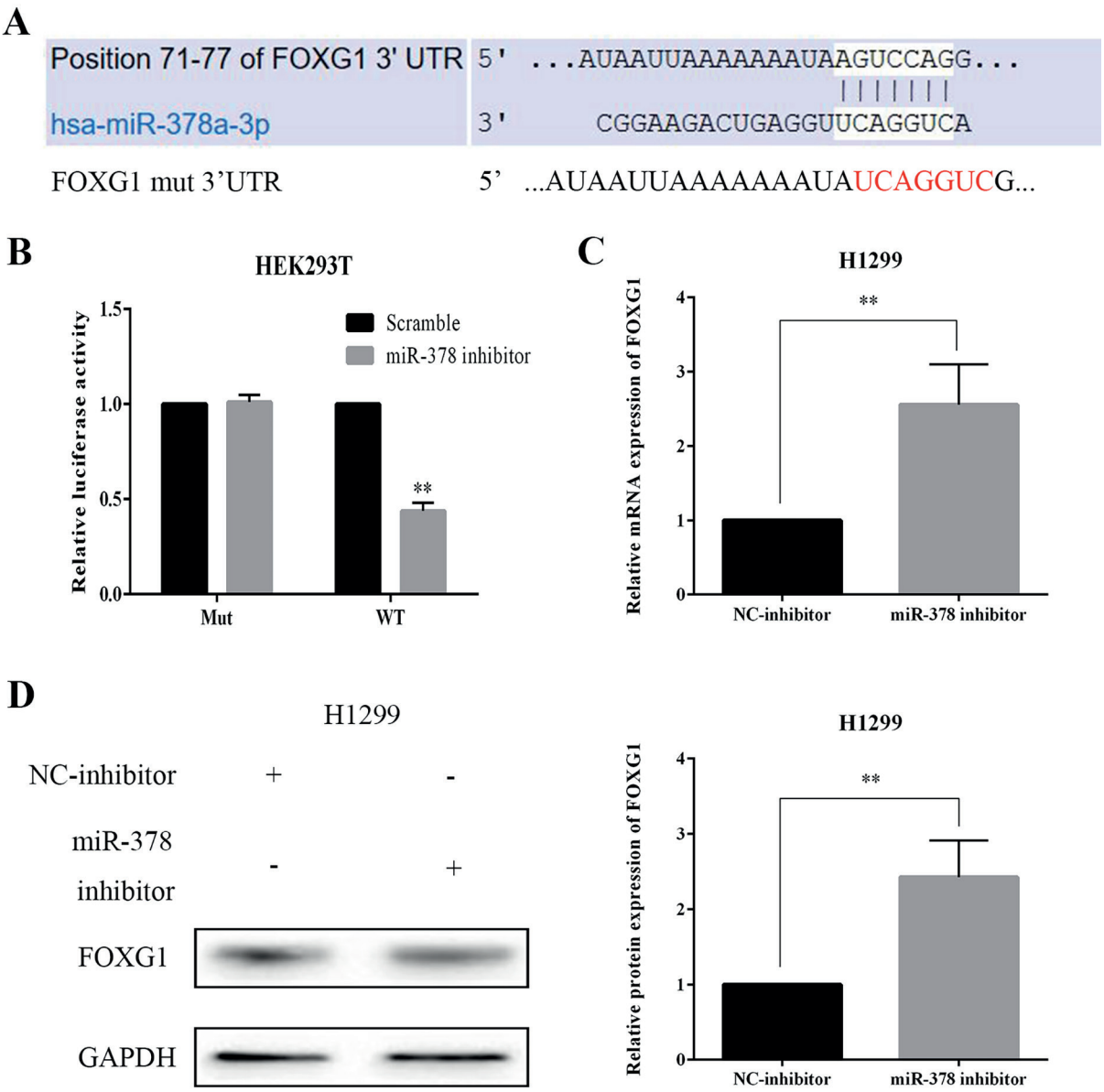

Figure 3. FOXG1 is directly targeted by miR-378. ***A,*** FOXG1 was selected as the potential downstream of miR-378 via using bioinformatics analysis; ***B,*** Luciferase activities of H1299 cells transfected with the wild-type or the mutated FOXG1 3′UTR together with miR-378 inhibitor or NC-inhibitor; ***C,*** Analysis of FOXG1 mRNA expression level of H1299 cells transfected with miR-378 inhibitor or NC-inhibitor; ***D,*** Analysis of FOXG1 protein expression level of H1299 cells transfected with miR-378inhibitor or NC-inhibitor. Data are presented as the mean ± SD of three independent experiments. **p<0.01.

Discussion

In recent years, with the in-depth research of the specific roles of miRNAs in tumors and their mechanisms, more and more miRNAs associated with tumorigenesis have been found. For example, miR-107 and miR-185 can inhibit NSCLC by inducing cell cycle arrest[8], miR-181a and miR-181b act as tumor suppressor genes in the pathological process of glioma[9]. In colon cancer, miR-143/miR-145 family has a down-regulated expression, while miR-21 has an increased expression, and colon cancer cell invasion is affected by inhibiting the expression of programmed cell death factor 4 (Pdcd4) gene[10]. Initially, miR-378 (formerly miR-422b) has been found to be expressed in some tumor cell lines and, therefore, considered to probably act as an oncogene. However, more and more studies[11-15] have shown that the expressions of miR-378 in adjacent tis-

sues are significantly higher than those in tissues of various tumors including liver cancer, gastric cancer, colon cancer, oral cancer and throat cancer. A report of ovarian cancer by Chan et al[16] showed that in high-grade serous ovarian cancer, patients with low miR-378 expression have short survival time. The above studies have shown that miR-378 may negatively regulate the occurrence and development of these tumors and may serve as a tumor suppressor gene in these tumors. A primary study of 24 cases of liver cancer tissues by Ma et al[17], found that miR-378 had the highest expression in normal liver tissue and decreased expression in adjacent tissue of liver cancer [hepatitis B virus (HBV)-positive], and its expression in liver cancer tissue is clearly lower than the previous two. Li et al[18] found that the expression of miR-378 is significantly lowered in hepatoma cell line HepG2.2.15 that is stably transfected and has HBV expression compared with that in hepatoma cell line HepG2. Pogribny et al[19] fed rats with hepatocarcinogenic agent tamoxifen to induce liver cancer. They detected miRNA expression profiles in rat liver after 12 weeks and 24 weeks, and found that tamoxifen feeding group has an evidently lowered miR-378 expression compared with that in control group, and this change occurred in the early stage of tumor formation. These results suggest that HBV or other hepatocarcinogenic agents (such as tamoxifen) may down-regulate the expression of miR-378, leading to the occurrence of liver cancer. However, how miR-378

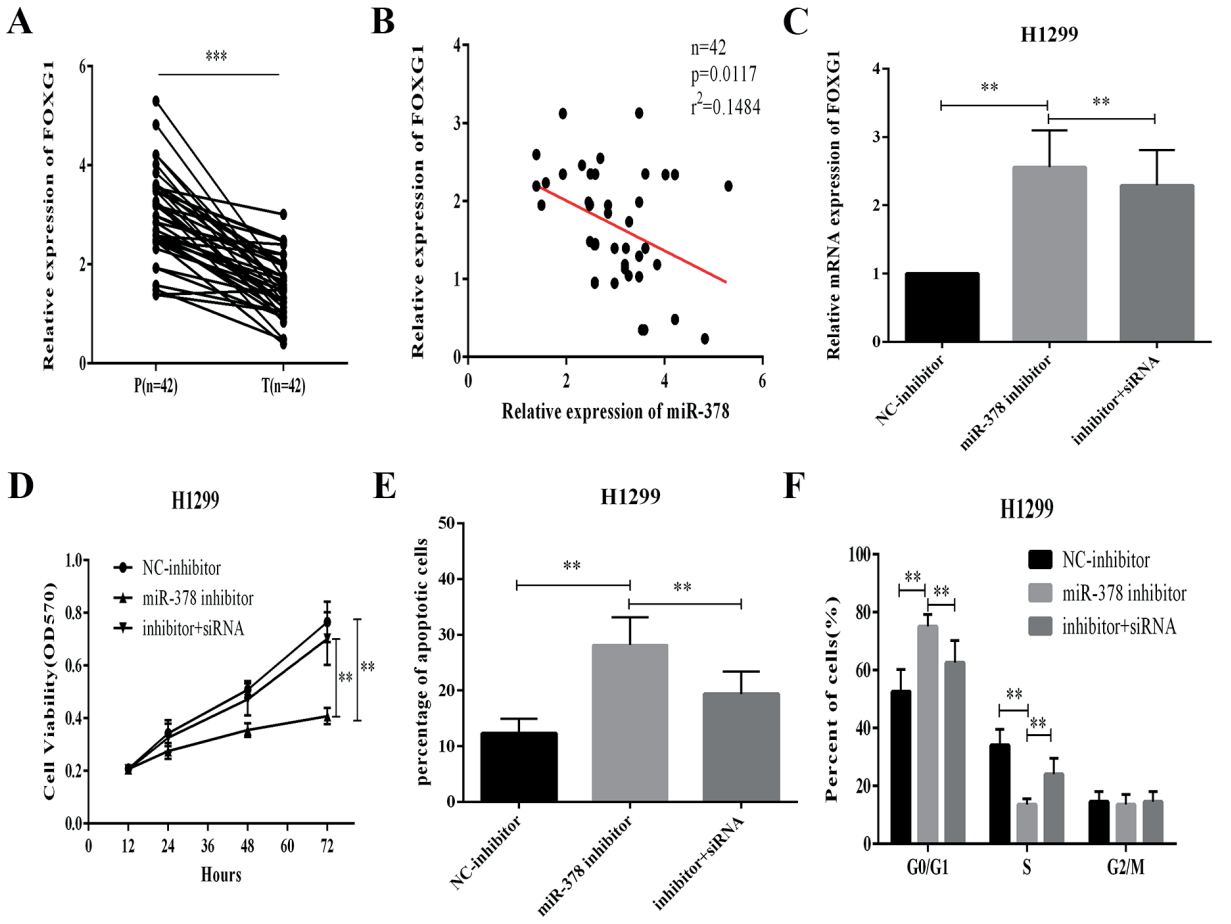

Figure 4. Silencing of FOXG1 recovered the carcinogenesis role of miR-378. ***A,*** Analysis of FOXG1 expression level in NSCLC tissues (T) and matched paracarcinoma tissues (N), n=42; ***B,*** Correlation between miR-378 and FOXG1 expression in NSCLC tissues (n=42); ***C,*** Analysis of transfection efficiency in H1299 cells transfected with miR-378 negative control (NC), inhibitor and/or si-FOXG1; ***D,*** Reduced FOXG1 rescued suppressed cell proliferation by miR-582-5p; ***E,*** Reduced FOXG1 attenuated cell apoptosis; ***F,*** ReducedFOXG1 attenuated cell cycle distribution at G0/G1 phase. Data are presented as the mean ± SD of three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$.

plays a role in NSCLC has not been explored yet. This work aimed to study the relationship between miRNAs and the occurrence and development of NSCLC. We demonstrated that miR-378 was significantly upregulated in NSCLC tissues compared with adjacent tissues, implying that miR-378 might play a potential role in the development of NSCLC. Besides, downregulated miR-378 inhibited NSCLC cell proliferation, promoted cell apoptosis, and induced cell cycle arrest at G0/G1 phase. All these findings suggested that downregulated miR-378 exerted its suppressive effect on cell proliferation of NSCLC. To further identify the underlying mechanism of how downregulated miR-378 suppressed NSCLC cell tumorigenesis, FOXG1 was predicted and selected as the novel target gene of miR-378 by bioinformatics analysis. Human FOX gene family is a gene family with rich functions in the body. Studies[20,21] have shown that Foxg1 expression is known to counteract the inducing of signal pathways, such as transforming growth factor beta (TGF-β), which represses β and bone morphogenetic protein 4 (BMP4) through the transcription of cyclin-dependent kinase inhibitors P15INK4B and p21CIP1. Also, it leads to overgrowth and apoptosis by reducing normal cellular programmed death or frequency. However, the underlying upstream mechanism of FOXG1 in NSCLC has not been well identified yet. We initially revealed that FOXG1 was directly targeted by miR-378, and FOXG1 expression was negatively correlated with miR-378 in NSCLC tissues and cell lines. Moreover, silencing of FOXG1 could rescue tumor suppression role by downregulated miR-378 on NSCLC cell proliferation. The evidence indicated that miR-378 might be the upstream of FOXG1 involved in NSCLC tumorigenesis.

Conclusions

We demonstrated that downregulated miR-378 had a tumor-suppressive effect on NSCLC proliferation via targeting FOXG1 *in vitro*. Our findings may help elucidating the molecular mechanisms underlying NSCLC progression and provide miR-378 as an innovative and candidate target for diagnose and treatment of NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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