miR-296-5p suppresses cell viability by directly targeting PLK1 in non-small cell lung cancer

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Abstract. Polo-like kinase 1 (PLK1), a critical kinase for mitotic progression, is overexpressed in a wide range of cancers. MicroRNAs (miRNAs) are a class of small non-coding RNA molecules and proposed to play important roles in the regulation of tumor progression and invasion. However, the relationship between PLK1 and miRNAs have remained unclear. In the present study, the association between PLK1 and miR-296-5p was investigated. The upregulation of PLK1 mRNA expression levels combined with the downregulation of miR-296-5p levels were detected in both non-small cell lung cancer (NSCLC) tissues and cell lines. Functional studies showed that knockdown of PLK1 by siRNA inhibited NSCLC cells proliferation. Impressively, overexpression of miR-296-5p showed the same phenocopy as the effect of PLK1 knockdown in NSCLC cells, indicating that PLK1 was a major target of miR-296-5p. Furthermore, using western blot analysis and luciferase reporter assay, PLK1 protein expression was proved to be regulated by miR-296-5p through binding to the putative binding sites in its 3'-untranslated region (3'-UTR). Taken together, the present study indicated that miR-296-5p regulated PLK1 expression and could function as a tumor suppressor in NSCLC progression, which provides a potential target for gene therapy of NSCLC.

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Introduction

Lung cancer is the most common malignancy and is still increasing both in incidence and mortality worldwide (1,2). Non-small cell lung cancer (NSCLC) comprises 85% of all lung cancer cases (3). Therefore, development of early diagnosis and effective therapies for NSCLC are urgently needed.

Polo-like kinase 1 (PLK1), belongs to the polo-like kinase protein family, is a serine/threonine kinase that regulates a multitude of mitotic processes (4,5). Importantly, PLK1 is highly expressed in a wide range of human malignancies, such as lung, prostate, esophageal and colon cancer (6-9). In addition, it is often correlated with poor prognosis in patients suffering from NSCLC (5,10,11). Previous studies *in vitro* have shown that downregulation of PLK1 could inhibit the growth of lung cancer cells (11). Given the increasing appreciation of PLK1 as an important oncogene, the mechanisms underlying the upregulation of PLK1 expression in cancer cells have been investigated.

MicroRNAs (miRNAs) are a conserved class of non-coding RNAs that regulate protein expression by binding to the mRNA 3'-untranslated region (3'-UTR) (12,13). miRNAs likely have been implicated in diverse biological processes, and have contributing roles in cancer initiation and progression (14,15). Changes in miRNA expression have profound effects on human carcinogenesis and cancer progression (16). In this regard, the role of miRNAs in regulating PLK1 expression in cancer cells has also been investigated. Ito *et al* (17) demonstrated that miR-593* has an important role in downregulating PLK1 expression, and is responsible for the increased expression of PLK1 in human esophageal cancer. Another miRNA, miR-100 acts as a tumor suppressor by targeting PLK1 and downregulates its expression in NSCLC (18). However, whether there are additional miRNAs which are responsible for the increase of PLK1 expression in NSCLC have not been reported.

To address this problem, we identified miR-296-5p as a potential miRNA which could target PLK1 mRNA. Although the tumor suppressor function of miR-296-5p has been

proved in previous studies (19,20), the oncogenic function of miR-296-5p has not been fully investigated. miR-296-5p expression is downregulated in a diverse array of tumors including lung cancer (21-23).

In the present study, the miR-296-5p/PLK1 pathway was investigated, in addition to the mechanistic roles of miR-296-5p and PLK1 in NSCLC cells.

Materials and methods

Human samples. Twenty-four paires of NSCLC and corresponding normal lung tissue specimens were obtained from the First Affiliated Hospital of Soochow University. The adjacent macroscopically non-tumor tissues were taken at least 6 cm distant from the tumor. The patients with NSCLC had received neither radiotherapy nor chemotherapy prior to tissue sampling. All tissues were snap-frozen in liquid nitrogen and stored at -80˚C until used. The study protocol was approved by the ethics committee of Soochow University.

Cell culture. Human non-small cell lung cancer cell lines A549, H1299, LTEP-α-2, 95C and 95D were obtained from the Chinese Academy of Sciences (Shanghai, China). Human bronchial epithelial (HBE) cells were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). All cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco) and maintained in a 5% CO_2 humidified sterile atmosphere at 37˚C.

RNA extraction, cDNA synthesis and real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Total RNA from NSCLC tissues and cell lines were extracted using TRIzol reagent (Invitrogen; Life Technologies) and RNA concentration was measured on a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Synthesis of cDNA with reverse transcriptase (RT) was performed with an M-MLV first strand kit (Invitrogen; Life Technologies). For miR-296-5p quantification, RNA was reverse-transcribed with stem-loop RT primer: GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACACAGGATTG, and the amplification primers were as follows: forward, 5'-GTATCCAGTGCAGG GTCCGA-3' and reverse, 5'-CGACGAGGGCCCCCCCT-3'. For PLK1 mRNA quantification, RNA was reverse-transcribed with a random primer. The amplification primers were as follows: forward, 5'-GCCTAAGTCTCTGCTGCTCAA-3' and reverse, 5'-CAACACCACGAACACGAAGT-3'.

Quantitative real-time PCR (qRT-PCR) of miR-296-5p and PLK1 mRNA were performed using Takara SYBR-Green PCR kit (Takara, Dalian, China) and an ABI 7500 Real-Time system (Applied Biosystems, Carlsbad, CA, USA) was used to analyze the expression of RNA.

We used U6 small nuclear RNA (U6 snRNA) and GAPDH mRNA as an endogenous control to normalize miR-296-5p and PLK1 mRNA expression level separately. Primer sequences for U6 detection were: forward, 5'-CGAGCA CAGAATCGCTTCA-3' and reverse, 5'-CTCGCTTCGGC AGCACATAT-3'. Primer sequences for GAPDH detection were: forward, 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse, 5'-GAAGATGGTGATGGGATTTC-3'. Relative expression was calculated using the comparative cycle threshold (Ct) method.

Plasmid construction and transient transfection of A549 cells. The genome cDNA was generated by reverse transcription-polymerase chain reaction (RT-PCR). The cDNA encoding PLK1 (without 3'-UTR) was further amplified by PCR with sense primer (5'-CCGGAATTCGCAGCTTCGGGAGCAT GAGTGC-3') containing a *Bam*HI restriction site and antisense primer (5'-CCGCTCGAGCGGGAGCCAACCAGTATG GG-3') containing an *Xho*I restriction site. The PCR products were purified and then cloned into the *Bam*HI/*Xho*I sites of pcDNA3.1(+) (Invitrogen) to create pcDNA3.1(+)-PLK1. The identification of plasmid was verified by DNA sequencing. A549 cells were seeded at $2x10^5$/well in a 6-well plate 24 h prior to transfection. The cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocols. Transfected cells were selected in the presence of G418 (600 μg/ml).

miRNA and siRNA transfection. A549 cells were transfected with miRNA or siRNA 24 h after being seeded in 6-well plates. miR-296-5p mimics or PLK1 siRNA (100 pmol) and Lipofectamine 2000 (5 μl) were added into 250 μl of serum-free medium separately and then mixed at room temperature for 20 min. The complexes were then added dropwise to the cells and mixed gently. The cells were incubated for 48 h for further study.

Western blot assay. Cells were lysed in a RIPA buffer (Cell Signaling Technology, Boston, MA, USA) with protease inhibitor (Sigma-Aldrich, Dorset, UK) at 48 h post-transfection. The total proteins were then separated on 12% SDS-PAGE and blotted onto PVDF membranes. The membranes were blocked by 1% BSA for 30 min and incubated with the primary antibody (anti-PLK1 or anti-GAPDH; Santa Cruz Biotechnology, Santa Cruz, CA and Bioworld Technology, Inc., St. Louis Park, MN, USA, respectively) overnight at 4˚C. After 3 washes with TBST, the membranes were incubated with anti-mouse IgG (Santa Cruz Biotechnology, Dallas, TX, USA) or anti-rabbit IgG (Santa Cruz Biotechnology) at room temperature for 2 h. Proteins were visualized using ECL detection system (Pierce, Rockford, IL, USA). PLK1 protein level was normalized to GAPDH protein and the density was quantified using Quantity One 4.6 software (Bio-Rad Laboratories, Hercules, CA, USA).

Luciferase activity assay. The potential binding site of miR-296-5p in the PLK1-3'UTR was predicted at the position 51-58 of the 3'-UTR of PLK1 (NM_005030) by TargetScan software (www.targetscan.org). PsiCHECK-2 vector (Promega, Madison, WI, USA) was used to construct the plasmid containing 3'-untranslated region (3'-UTR) of PLK1. The fragments containing the predicted wild and mutant sites were directly synthesized by Genewiz (Suzhou, China) and then subcloned into psiCHECK-2 vector. After 16 h of culture in a 24-well plate, A549 cells were co-transfected with psiCHECK-2-PLK1-3'-UTR-wild/mutant vector (50 ng) and miR-296-5p mimics or scrambled microRNA negative control (miR-NC). After 48 h of culture, the luciferase activity was measured using a Dual-luciferase assay kit (Promega) on

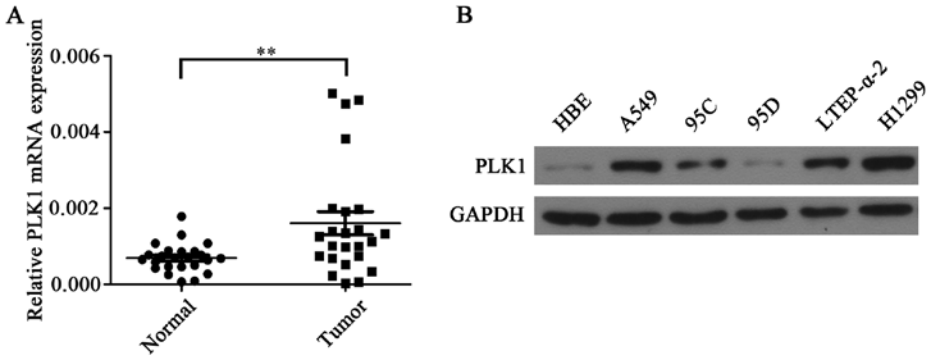

Figure 1. PLK1 expression was shown increased in human NSCLC tissues and cell lines. (A) PLK1 mRNA levels expressed in 24 NSCLC tissues and paired non-cancerous lung tissues. (B) PLK1 protein levels were examined by western blot analysis in HBE cells and A549, 95C, 95D, LTEP-α-2 and H1299 cells. GAPDH was used for loading control. **P<0.01.

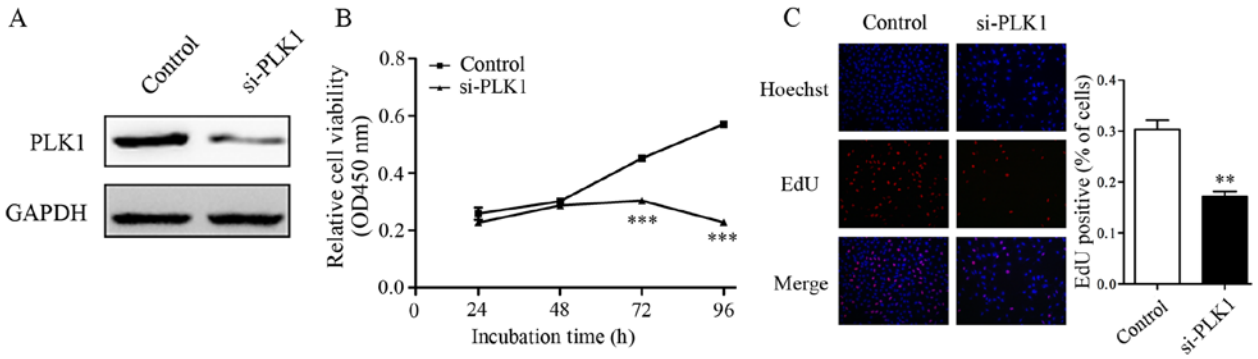

Figure 2. The proliferation of NSCLC cells were suppressed by transfection of PLK1 siRNA. (A) Detection of PLK1 protein levels in A549 cells by transfection of PLK1 siRNA. (B) CCK-8 analysis shows cell proliferation in A549 cells was suppressed by transfecting with PLK1 siRNA. (C) EdU was detected in A549 cells by transfecting with PLK1 siRNA. The percentage of EdU-positive cells in groups was calculated. At least 200 cells were counted per well. **P<0.01, ***P<0.001.

a TD20/20 Luminometer (Turner Designs, Westport, MA, USA). Expression values were normalized to *Renilla* luciferase. All assays were performed in triplicate and repeated at least three times.

Cell proliferation assay. A549 cells were seeded into 96-well plates with the density of $5x10^3$ cells/well after transfected with miR-296-5p or PLK1-siRNA for 24 h. Cell proliferation was detected by CCK-8 and EdU assay.

For the CCK-8 assay, cells were harvested over four consecutive days and incubated with 20 μl of the Cell Counting kit-8 (CCK-8; Beyotime Institute of Biotechnology, Shanghai, China) reagent at 37˚C for 2 h. The OD value was measured at 450 nm.

5-Ethynyl-2'-deoxyuridine (EdU) assay (Guangzhou RiboBio, Co., Ltd., Guangzhou, China) was carried out to label cells undergoing DNA replication. Cells were planted in 96-well plates and treated, and then were incubated with EdU for 2 h before fixation. The cells were fixed in 4% paraformaldehyde for 30 min and permeabilized in 0.5% Triton X-100 for 25 min at room temperature. Apollo dyeing reaction buffer was added and shaken in the dark for 30 min. Then, the DNA was stained with Hoechst 33342 for 30 min. The proportion of nucleated cells incorporating EdU was observed by fluorescence microscopy. Cell proliferation rate was calculated as the percentage of EdU-positive nuclei to total nuclei in five high-power fields/well. The assay was performed in triplicate and repeated three times.

Statistical analysis. All data are presented as means ± SEM from at least three separate experiments. Statistical analysis was performed by non-parametric tests (Mann-Whitney U test for 2 groups). For cell lines, unpaired t-test (2-tailed) was used to assess the differences between groups. Data analysis was performed using the SPSS 17.0 software package. Differences were considered statistically significant at P<0.05.

Results

PLK1 expression is increased in NSCLC samples and cell lines. To determine the role of PLK1 in NSCLC, PLK1 mRNA expression levels were determined in 24 paired NSCLC tissues and adjacent non-tumor tissues. It is shown that the average expression of PLK1 mRNA was significantly higher in NSCLC tissues when compared with adjacent non-tumor tissues (Fig. 1A). In addition, the higher levels of PLK1 protein were also detected in NSCLC cells lines including A549, 95C, 95D, LTEP-α-2 and H1299 when compared with HBE cells (Fig. 1B).

Functional role of PLK1 in NSCLC cells. It is shown that PLK1 acted as an oncogene, since the increased expression of

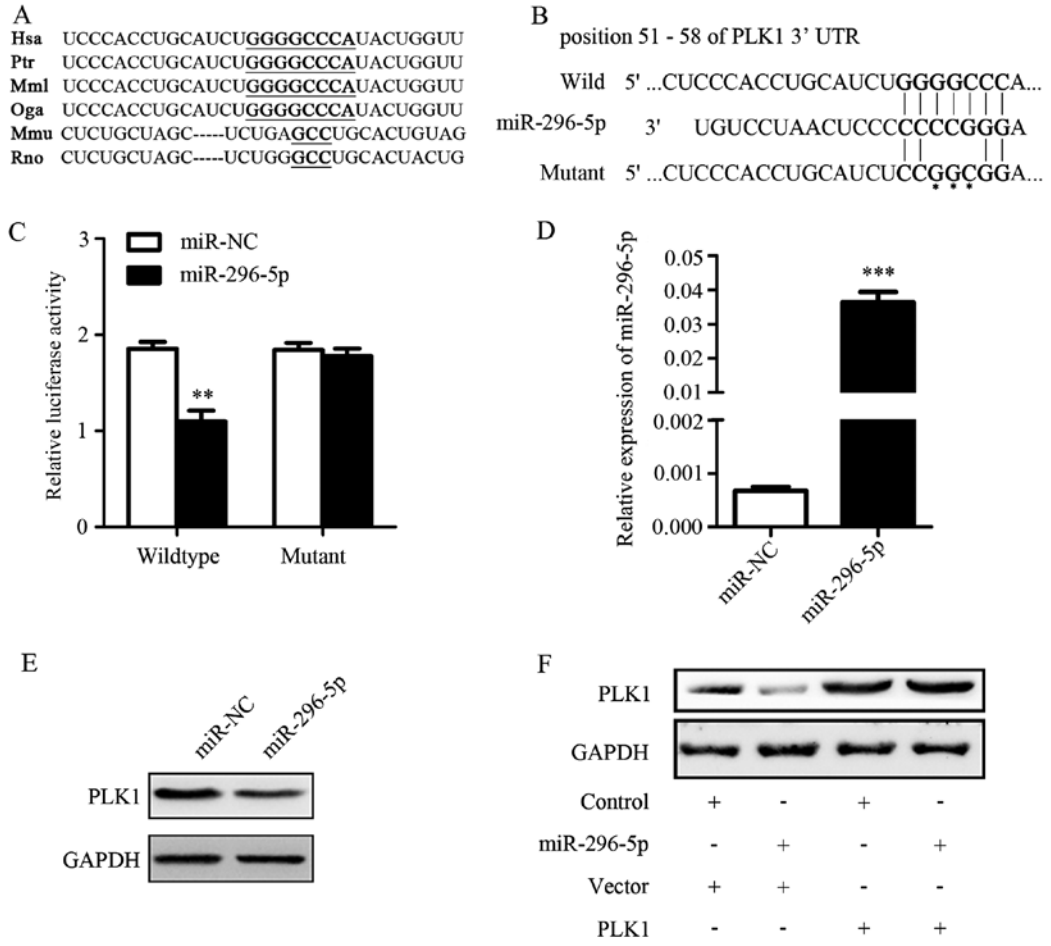

Figure 3. PLK1 is a direct target of miR-296-5p. (A and B) The sequence alignment of predicted miR-296-5p target sites on PLK1 3'-UTR is shown between human and other species. Potential association between the predicted miR-296-5p target sites and miR-296-5p is shown. (C) Luciferase activity of the wild-type or mutant-type PLK1-3'-UTR reporter gene was detected in A549 cells transfected with miR-NC (control) or miR-296-5p. Relative *Renilla* luciferase activity was obtained after normalizing to the firefly luciferase activity. (D) The efficiency of transfection with miR-296-5p was detected by qRT-PCR analysis. (E) PLK1 protein levels in A549 cell transfection with miR-296-5p mimics or miR-NC were detected by western blot analysis. (F) Western blot analysis of PLK1 protein levels in A549 cells which were co-transfected with miR-296-5p mimics or miR-control and the PLK1 plasmid (without 3'-UTR) or vector control. **P<0.01, ***P<0.001.

PLK1 was examined in NSCLC tissues and cell lines. We then investigated the promotion effect of PLK1 on cell proliferation in NSCLC. siRNA which could downregulate PLK1 expression was introduced into A549 cells (Fig. 2A). CCK-8 results showed that the proliferation rate of A549 cells was significantly lower in the PLK1 siRNA treated group than control (Fig. 2B). EdU assay also showed that A549 cells treated with miR-296-5p mimics had significantly lower rates of proliferation (Fig. 2C). These results suggested that downregulation of PLK1 significantly inhibited the proliferation of A549 cells.

PLK1 protein expression is downregulated by miR-296-5p in NSCLC cells. To explore the underlying mechanism of PLK1 regulation, we used TargetScan software to predict the possible microRNAs that targeted PLK1 mRNA. The conserved predicted target site among species on the 3'-UTR of PLK1 mRNA was identified (Fig. 3A). It showed that miR-296-5p was one of the predicted microRNAs which targeted PLK1 mRNA 3'-UTR (Fig. 3B). The results of luciferase reporter assays showed that the luciferase activity decreased significantly in cells co-transfected with psiCHECK-2-PLK1-3'-UTR-wild vector and miR-296-5p mimics when compared with control. However, the luciferase activities did not change much in A549 cells with the mutant construct (Fig. 3C). The above results suggested that miR-296-5p could directly target 3'-UTR of PLK1.

Next, we detected whether PLK1 protein expression could be suppressed by miR-296-5p. The transfection efficiency of miR-296-5p was determined by qRT-PCR (Fig. 3D). The effect of miR-296-5p on levels of endogenous PLK1 protein was determined by western blot assay in A549 cells. As expected, PLK1 protein expression was significantly downregulated in miR-296-5p transfected group when compared with miR-NC transfected group (Fig. 3E). Furthermore, A549 cells were transfected with pcDNA3.1(+)-PLK1 in combination with miR-296-5p transduction. The results of western blotting assay revealed that miR-296-5p downregulated PLK1 expression, but the effect could be rescued by transfection with PLK1 plasmid (Fig. 3F). These results further proved that PLK1 expression could be mediated by miR-296-5p through targeting its mRNA 3'-UTR.

miR-296-5p expression is decreased in NSCLC samples and cell lines. The expression levels of miR-296-5p were further

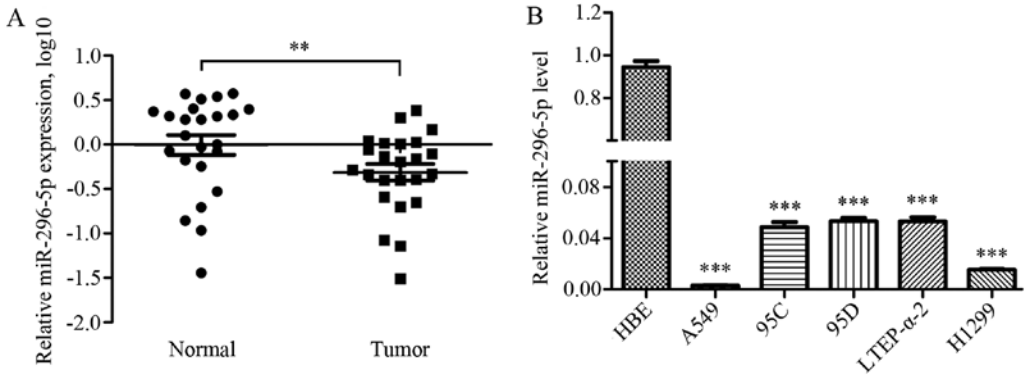

Figure 4. miR-296-5p expression was reduced in human NSCLC tissues and NSCLC cells. (A) miR-296-5p levels expressed in 24 NSCLC tissues and paired non-cancerous lung tissues. (B) miR-296-5p levels were examined by qRT-PCR in HBE cells and A549, 95C, 95D, LTEP-α-2 and H1299 cells. U6 was used for loading control. **P<0.01, ***P<0.001.

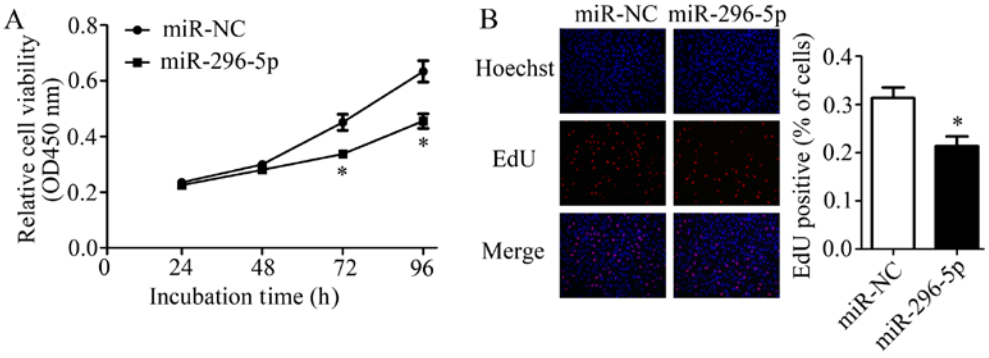

Figure 5. The proliferation of NSCLC cells were suppressed by restoration of miR-296-5p. (A) CCK-8 analysis shows cell proliferation in A549 cells was inhibited by transfection with miR-296-5p mimics. (B) EdU was detected in A549 cells by transfection with miR-296-5p mimics. The percentage of EdU positive cells in groups was calculated. At least 200 cells were counted per well. *P<0.05.

investigated in 24 pairs of NSCLC samples by qRT-PCR. The results showed that the expression levels of miR-296-5p were significantly reduced in NSCLC specimens when compared with paired normal tissues (Fig. 4A). Furthermore, the decreased expression levels of miR-296-5p were also detected in NSCLC cell lines including A549, 95C, 95D, LTEP-α-2 and H1299 when compared with HBE cells (Fig. 4B).

miR-296-5p inhibits NSCLC cell growth in vitro. Since reduced expression of miR-296-5p was observed in NSCLC tissues and cells, we explored whether restoration of miR-296-5p had similar effect as PLK1 knockdown in A549 cells. After transfected with miR-296-5p mimics or miR-NC, cell viability was measured by CCK-8 and EdU assays. The results of CCK-8 assay indicated that cells treated with miR-296-5p mimics showed a significantly decreased proliferation rate when compared with control (Fig. 5A). Similar results were also observed in EdU assay (Fig. 5B). These results implied that miR-296-5p has a strong ability to suppress NSCLC cell proliferation.

Discussion

PLK1 is an important member of the Polo-like kinases (PLKs), which are a family of highly conserved serine/threonine kinases (24). It is involved in several crucial physiological process including cell cycle progression, spindle formation and chromosome segregation during mitosis (25,26). Additionally, PLK1 is upregulated in various human malignancies, such as non-small cell lung cancer, breast cancer, colorectal cancer, and melanomas (27-30). In view of the important role of PLK1 in tumors, it is necessary to study the mechanism of aberrantly expressed PLK1 in NSCLC.

In general, miRNAs have been suggested to be classified into two main classes: oncosuppressor genes and oncogenes. Some low abundance miRNAs act as oncosuppressor genes by negatively regulating oncogenes, whereas some highly expressed miRNAs act as oncogenes by repressing tumor suppressor genes (31,32). Sometimes the same microRNA performs distinct functions in different tumor circumstances. For example, miR-296-5p is upregulated in esophageal squamous cell cancer tissues and downregulation of miR-296-5p is shown to suppress esophageal cancer cell progression (33). However, more studies have found that miR-296-5p acted as a tumor suppressor and was downregulated in breast, prostate and lung cancer (19-21). These controversial results indicate that the roles of miR-296-5p were highly dependent on its targets in different cancer cells.

PLK1 has been shown to be a target of several miRNAs including miR-100 and miR-593*, and these miRNAs are known to be responsible for the regulation of cancer cell proliferation, differentiation and invasion (17,18). In the

present study, we investigated whether miR-296-5p suppresses NSCLC cell viability by targeting PLK1. Using a luciferase reporter assay, we demonstrated conclusively that miR-296-5p directly targeted PLK1 mRNA by binding to the potential 3'-UTR binding site.

It is known that one transcript can be regulated by one or more miRNAs while each miRNA also can have multiple target transcripts (34). For instance, miR-128 acts as a tumor suppressor by directly targeting Bmi1 and CYP2C9 in prostate cancer and hepatocellular carcinoma, respectively (35,36). Bmi1 can be targeted by miR-203, miR-218 and miR-135a in different malignancies (37-39). Although abundant transcriptional targets are predicted, most of these have not been fully elucidated. In the present study, we found that PLK1 mRNA could be targeted by miR-296-5p, and PLK1 protein could be downregulated by miR-296-5p transfection.

Our analysis confirmed the downregulation of miR-296-5p expression in all the NSCLC cell lines tested compared with HBE. To confirm this result, we examined primary tissues from 24 cases of NSCLC and compared them with paired non-tumor tissues, and found that miR-296-5p expression in NSCLC was significantly decreased.

Next, we investigated whether the downregulation of miR-296-5p was responsible for the uncontrolled growth of tumor cells. Our results showed that transfection of miR-296-5p mimics in A549 cells resulted in a significant suppression in tumor growth, and this result is in agreement with a previous study in breast cancer (40). Vaira *et al* (21) also revealed that miR-296-5p could inhibit lung cancer cell migration and invasion.

Our research indicates that miR-296-5p is involved in the PLK1 oncogene network, and the introduction of miR-296-5p is able to rebuild the tumor-suppressing signaling pathway in A549 cells. Importantly, miR-296-5p potently inhibites cell viability in PLK1-overexpressing NSCLC cells, providing the first evidence that there is a potential link between the tumor suppressor miR-296-5p and NSCLC cell self-renewal. The present study also suggests that miR-296-5p could contribute to human NSCLC therapy.

Acknowledgements

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