Objectives: The aim of the study was to study the role of dysregulated expression of a microRNA (miRNA), miR-503, in non-small-cell lung cancer (NSCLC) and investigate the underlying mechanism.

Methods: Quantitative real-time PCR (qRT-PCR) and in situ hybridization staining (ISH) were used to evaluate the expression level of miR-503 in NSCLC tissues and paired adjacent tissues. CCK-8, colony formation and flow cytometry were performed to explore the effects of miR-503 overexpression on cell proliferation, colony formation and apoptosis. Cells with miR-503 overexpression were used to initiate xenograft models. Dual luciferase reporter assay, qRT-PCR, immunohistochemistry and Western blotting were conducted to investigate the interaction of miR-503 and its potential target.

Results: Significantly downregulated miR-503 was found in NSCLC tumor tissues and cell lines. miR-503 overexpression significantly inhibited NSCLC cell proliferation, migration and invasion. PDK1 was predicted as the direct targets of miR-503. PDK1 overexpression reversed the inhibitory effects of miR-503 on biological functions, while PDK1 silencing significantly counteracted miR-503 inhibitor-induced pro-tumor effects in A549 cells. Mechanistically, upregulation of miR-503 inhibited PDK1 expression and subsequently caused the inactivation of PI3K/AKT pathway.

Conclusion: Our results suggest that miR-503 inhibits NSCLC progression by targeting PDK1/PI3K/AKT pathway, potentiating the use of miR-503 as a biomarker and therapeutic target for NSCLC.

Keywords: miR-503, non-small cell lung cancer, PDK1, proliferation, mortality

Introduction
Non-small cell lung cancer (NSCLC) is a malignant type of lung cancer and responsible for the high mortality and morbidity of lung cancer. It is increasingly recognized that molecular mechanisms that drive the progression and chemoresistance of cancers are valuable therapeutic targets of NSCLC. Therefore, strategies have been devised to counteract molecular abnormality associated with cancer malignancy, an approach that is precise and individualized, to improve the treatment efficiency.

MicroRNAs (miRNAs) are a class of non-coding RNAs with the length of 21–23 nucleotides. It has been shown that miRNAs are versatile regulators of cancers and the upregulation or downregulation of certain miRNAs may be indicative of lung cancer tumorigenesis. A number of putative miRNAs have been reported as...
A miRNA, miR-503, is recently discovered as a potent tumor suppressor in esophageal cancer, hepatocellular cancer, glioma, breast cancer, etc. The overexpression of miR-503 demonstrated marked anti-tumor effects, by suppressing the proliferation, migration, invasion and metastasis of cancer cells. The role of miR-503 in NSCLC has also been implicated. For example, downregulation of miR-503 was shown to be linked to poor prognosis in patients with NSCLC. miR-503 was also demonstrated to be a therapeutic target in NSCLC to impede cancer progression and to reverse chemoresistance.

PDK1/PI3K/AKT pathway is a well-known driver of lung cancer progression. The activation of PDK1/PI3K/AKT pathway has also been linked to cancer metastasis and resistance, making it accountable for the high aggressiveness of cancers. Emerging evidences have shown that several putative miRNAs regulate lung cancer progression through the PDK1/PI3K/AKT pathway. For example, miR-375 was found to target PDK1 in esophageal cancer and pancreatic cancer to suppress cancer progression.

Herein, the aim of the study was to elucidate the role of miR-503 in NSCLC and investigate the link between miR-503 and PDK1/PI3K/AKT pathway. To this end, we compared miR-503 levels in tumor and normal tissue, as well as NSCLC cells. To investigate the functional role of miR-503, miR-503 overexpression, achieved by miR-503 mimic transfection, and miR-503 inhibition, achieved by miR-503 silencing, were performed, followed by examination of cell proliferation, colony formation, migration and invasion. The interaction of miR-503 and PDK1/PI3K/AKT pathway was also investigated. Our results could potentially provide a novel diagnostic and therapeutic target in NSCLC.

Materials and Methods

Clinical Specimen and Cell Culture

Our study was approved by the Ethics Committee of Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, and all patients provided informed consent. NSCLC tumor and adjacent normal tissue specimens were acquired from a total of 42 patients, who had no preoperative chemotherapy or radiotherapy. The clinical characteristics of the patients, including TNM staging in adherence to AJCC staging system (the 7th edition), were recorded. Human NSCLC cell lines including SPCA1, A549, H1299, PC9, H358, 16HBE and human bronchial epithelial cells, HEK293 cells were acquired from the Shanghai Academy of Sciences. RPMI-1640 medium (Gibco, NY, USA) supplemented with 10% foetal bovine serum (Gibco, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, NY, USA) was used for cell culture at 37°C in a humidified cell incubator in a 5% CO2 atmosphere.

Quantitative Reverse-Transcription PCR

Total RNA extraction from tissues and cells was performed using the TRIzol reagent (Invitrogen, CA, USA). cDNA synthesis was performed using the PrimeScript RT reagent (Takara, Kusatsu, Japan), and real-time PCR was performed on an ABI 7900 fast real-time PCR system (ABI, CA, USA) with SYBR Green Master Mix II (Takara). The mRNA and miRNA levels were normalized using GAPDH and small RNA RNU6B (U6), respectively, as house-keeping genes. The relative levels of miR-503 and PDK1 mRNA was quantified using the 2-ΔΔCT method. The oligonucleotides used in this study are shown in Table 1.

Cell Transfection

Plasmid PDK1, empty vector, siRNA-PDK1, siRNA-NC and the lentiviral systems (LV-hsa-miR-503-mimics, LV-NC, LV-hsa-miR-503-inhibitor, LV-NC-inhibitor) were acquired from Gene Pharma (Shanghai, People’s Republic of China). Transfection was conducted using the lipofectamine 3000 reagent (Invitrogen) following the manufacturer’s instructions.

Cell Proliferation Assay

Cells were seeded into 96-well plates at 1000 cells/well. Each group was seeded into 3 wells. During the last 2 hrs of incubation, each well was added 10 μL/well of CCK8. The absorbance at 450 nm was used to determine cell viability.

To evaluate colony formation ability, cells seeded on 6-well plates (400 cells/well), were cultured for 14 days. After fixation with methanol for 20 mins, the cells were stained with crystal violet (Nanjing Jiancheng Bioengineering Institute, Nanjing, People’s Republic of China), following which the number of colonies were counted.

Migration and Invasion Assays

To assess cell migration and invasion, 5×10^4 or 1×10^5 cells were plated in the upper chamber of each insert (BD Biosciences) with a non-coated or Matrigel-coated membrane for migration or invasion assays. For both assays, the lower chambers were filled with 800 μL medium supplemented with 10% fetal bovine serum. Cells on the inserts, which
were fixed and stained with 0.1% crystal violet and 20% methanol, were counted, using an IX71 inverted microscope (Olympus, Tokyo, Japan). Quantification was made based on five randomly selected fields.

**Flow Cytometric Analysis**
Cells underwent fixation in 75% ethanol overnight at −20°C. After PBS washing, cells were stained with 25 μg/mL propidium iodide (PI; Kaji Biotech, Nanjing, People’s Republic of China), 10 μg/mL RNase A (Sigma-Aldrich), 0.05 mM ethylene diamine tetraacetic acid, and 0.2% Triton X-100 for 30 mins, followed by flow cytometry measurement with a FACSCalibur flow cytometer (BD Biosciences, New Jersey, USA), and analysis using ModFit software (BD Biosciences).

**Luciferase Reporter Assay**
The wild-type or mutated 3′-UTR sequence of PDK1 was cloned into the pmirGLO-promoter vector (Promega, Madison, USA), yielding pmirGLO-PDK1 WT and pmirGLO-PDK1-MUT. HEK293T cells, which were seeded in 24-well plates, underwent transfection for 24 hrs with 100 ng of pmirGLO-PDK1 or pmirGLO-PDK1-MUT, miR-503 mimics and NC mediated by Lipofectamine 3000 (Invitrogen). A Dual Luciferase Assay Kit (Promega) was used to quantify the relative luciferase activity.

**In Vivo Experiments**
All animal experiments were carried out in adherence to the regulations of the Institutional Animal Protection and Use Committee of Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology. BALB/c-nu/nu mice (4–5 weeks old, n=40) were randomly divided into 4 groups. One group of mice were injected subcutaneously on both sides of the armpit with A549 cells stably expressing miR-503 mimic or NC. Another group of mice were injected with PC9 cells stably expressing miR-503 inhibitor or NC inhibitor. Tumor size, which was calculated as length×width×0.5 (mm³), was measured every week.

### Table 1 Correlations Between miR-503, PDK1 Expression And Clinicopathological Parameters In 42 NSCLC Patients

| Parameters                  | n  | miR-503 | P     | PDK1 | P     |
|-----------------------------|----|---------|-------|------|-------|
|                            |    | Low (n) |       |      |       |
|                            |    | High (n)|       |      |       |
| Age (years)                 |    |         |       |      |       |
| ≤60                         | 22 | 12      | 10    | 0.801| 9     | 13    | 0.658 |
| >60                         | 20 | 9       | 11    |      | 12    | 8     |      |
| Gender                      |    |         |       |      |       |
| Female                      | 17 | 9       | 8     | 0.752| 10    | 7     | 0.134 |
| Male                        | 25 | 12      | 13    |      | 11    | 14    |      |
| Smoker                      |    |         |       |      |       |
| Yes                         | 23 | 10      | 13    | 0.210| 11    | 12    | 0.855 |
| No                          | 19 | 11      | 8     |      | 10    | 9     |      |
| Histology                   |    |         |       |      |       |
| LSC                         | 16 | 7       | 9     | 0.274| 9     | 7     | 0.274 |
| LAC                         | 26 | 14      | 12    |      | 12    | 14    |      |
| Tumor size (cm)             |    |         |       |      |       |
| >5                          | 15 | 12      | 3     | <0.001*| 4    | 11    | 0.025* |
| ≤5                          | 27 | 9       | 18    |      | 17    | 10    |      |
| Lymph node metastases       |    |         |       |      |       |
| Yes                         | 16 | 11      | 5     | 0.018*| 4    | 12    | 0.001* |
| No                          | 26 | 10      | 16    |      | 17    | 9     |      |
| TNM stage                   |    |         |       |      |       |
| A-IIB                       | 25 | 16      | 9     | 0.025*| 10   | 15    | 0.037* |
| IIIA                        | 17 | 5       | 12    |      | 11    | 6     |      |

Note: *indicates P<0.05 (Chi-square test).
After 4 weeks, mice were sacrificed, and tumors were weighed.

Western Blotting Analysis
Protein extraction was performed using the RIPA reagent (Beyotime, Shanghai, People’s Republic of China) supplemented with 100 μg/mL PMSF (Beyotime, Shanghai, People’s Republic of China) and 2 μg/mL aprotinin (Beyotime, Shanghai, People’s Republic of China). Proteins were separated by 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. Five percent skim milk was used to block the membrane for 2 hrs, and the following specific primary antibodies were added to the membrane and incubated overnight at 4°C: PDK1 (Abcam), AKT (Cell Signalling Technology), p-AKT (Cell Signalling Technology) and GAPDH (CST, 2118). After washing, HRP-conjugated anti-rabbit IgG (1:2000) was used to incubate the membrane for 2 hrs at room temperature and followed by washing 3 times with TBST buffer. An enhanced chemiluminescence (ECL) system (Pierce Biotechnology), p-AKT (Cell Signalling Technology), and GAPDH (CST, 2118) were used to detect the bound antibodies. GAPDH was used as a loading control.

Statistical Analysis
All data were analyzed using SPSS 19.0 and GraphPad software 7.0. The P-values were acquired using one-way ANOVA, Student’s t-test, and Spearman’s test. Difference with P<0.05 was considered statistically significant.

Results
MiR-503 DownRegulation In NSCLC Tissues And Cell Lines
Quantitative real-time PCR (qRT-PCR) was performed to evaluate miR-503 expression in NSCLC tissues. MiR-503 expression in NSCLC tissues was markedly lower than that in the adjacent normal tissues (P<0.05, N=42 per group, Figure 1A). Similarly, miR-503 expression in NSCLC cells, including A549, H1299, SPCA1, H358 and PC9, was prominently downregulated compared to that in human bronchial epithelial cells (16HBE) (Figure 1B, P<0.05 for SPCA1, H358, and PC9, P<0.01 for H1299, and P<0.001 for A549). A549 cells and PC9 cells, which showed the lowest or highest miR-503 expression, were chosen for subsequent experiments. Correlation analysis demonstrated that miR-503 expression was negatively correlated with lymph node metastasis, TNM staging, and tumor sizes (Table 1).

MiR-503 Overexpression Inhibited The Proliferation Of NSCLC Cells In Vitro
To explore the biological functions of miR-503 in NSCLC progression, A549 cells or PC9 cells were transfected with miR-503 mimic or miR-503 inhibitor to up-regulate or downregulate miRNA-503 expression, respectively. As shown in Figure 1C and D, the transfection of miR-503 inhibitor or mimic significantly reduced (P<0.001) or increased (P<0.01) miR-503 expression. CCK-8 and colony formation assays indicated that miR-503 overexpression significantly suppressed cell proliferation and colony formation ability of A549 cells. However, miR-503 inhibition significantly enhanced the proliferation (Figure 2A, P<0.05 at 48 and 72 hrs; P<0.01, for 96 hrs) and colony formation abilities of PC9 cells (Figure 2B). As shown in Figure 2C, flow cytometry analysis indicated that the transfection of miR-503 mimic into A549 cells resulted in a significant increase in the percentage of G0/G1 phase cells (P<0.01), whereas the opposite results were observed in miR-503 inhibitor-transfected PC9 cells (P<0.05). Altogether, these results suggested that miR-503 inhibited NSCLC cell proliferation and induced G0/G1 cell cycle arrest.

MiR-503 Overexpression Attenuated NSCLC Cell Migration And Invasion
Migration assay showed that miR-503 mimics dramatically inhibited the migration of A549 cells (P<0.01), while miR-503 inhibitor remarkably enhanced the migration of PC9 cells (Figure 3A, P<0.01). Similarly, it was shown that the invasive capacities of A549 cells were significantly inhibited by miR-503 overexpression. In contrast, the invasive capability of PC9 cells was augmented after miR-503 silencing (Figure 3B, P<0.01). These results showed that miR-503 overexpression suppressed the migration and invasion of NSCLC cells.

Identification Of PDK1 As A Direct Target Of miR-503 In NSCLC
To determine the underlying mechanisms of miR-503 in NSCLC, bioinformatics analysis was performed, which identified PDK1 as a potential target of miR-503 (Figure 4A). The miR-503 binding region in the PDK1 3’-UTR was mutated to verify the interaction between miR-503 and the PDK1. The wild-type PDK1 luciferase vector (PDK1-WT) and mutant type PDK1 luciferase vector (PDK1-MUT) were co-transfected with miR-503 mimic into HEK293T cells. As shown in Figure 4B, the relative luciferase activity was
pronouncedly decreased by miR-503 mimics in HEK293T cells transfected with the PDK1 (WT) vector (p<0.01), while no significant effects on the luciferase activity were observed due to the mutations in the 3′-UTR of PDK1.

Further, it was demonstrated that PDK1 is remarkably up-regulated in the cancerous tissues of NSCLC patients (Figure 4C). As shown in Table 1, the expression of PDK1 correlated with tumor size, lymph node metastases and TNM stage. Importantly, PDK1 mRNA levels were negatively correlated with miR-503 in 42 NSCLC human specimens (Figure 4D). Additionally, PDK1 expression was significantly suppressed by the miR-503 mimics transfection and was significantly enhanced by the transfection of miR-503 inhibitor in NSCLC cells (P<0.01, Figure 4E). In addition, PDK1 inhibition by miR-503 led to an inactivation of p-AKT compared to the levels in the control group, while PDK1 upregulation increased the activation of p-AKT (Figure 4E). Altogether, these evidences indicated that PDK1/PI3K/AKT may be a direct target of miR-503 in NSCLC.

**MiR-503 Inhibited NSCLC Cell Proliferation, Migration And Invasion By Regulating PDK1**

Further determine the roles of PDK1 in the miR-503-induced anti-tumor effects in NSCLC, A549 cells or
miR-503 induces NSCLC cell proliferation inhibition and cell cycle arrest. 

(A) The cell proliferation of A549 cells or PC9 cells was measured by using CCK-8 assay.

(B) The colony formation ability of A549 cells or PC9 cells was measured by using colony formation assay.

(C) The cell cycle distribution of A549 cells or PC9 cells was measured by using flow cytometry. *P<0.05; **P<0.01 compared with the indicated controls.
PC9 cells were transfected with PDK1 plasmid or PDK1 siRNA. As shown in Figure 5A, PDK1 plasmid significantly increased PDK1 expression in miR-503-overexpressing A549 cells, while PDK1 expression was significantly downregulated by the transfection of miR-503 inhibitor and PDK1 siRNA into PC9 cells.

Figure 3 miR-503 suppresses NSCLC cell migration and invasion. (A, B) The cell migration and invasion capacities of A549 cells or PC9 cells were measured by using Transwell assay. **P<0.01 compared with the indicated controls.
Figure 4 miR-503 directly targets PDK1 by binding its 3'-UTR and regulating PI3K/AKT pathway. (A) A putative miRNA-503 target site in the 3'-UTR of PDK1 mRNA was predicted by using bioinformatics analysis. (B) Wild-type (PDK1-WT) or mutant (PDK1-Mut) luciferase reporter and/or miRNA-503 mimic were co-transfected into HEK293T cells to measure the luciferase activity. (C) The expression level of PDK1 mRNA in 42 NSCLC tissues and adjacent tissues was measured by qRT-PCR. (D) Negative correlation between miR-503 and PDK1 mRNA in NSCLC tissues. (E) The expression level PDK1 protein and PI3K/AKT proteins in A549 cells or PC9 cells was measured by Western blotting. *P<0.05; **P<0.01 compared with the indicated controls.
Subsequently, it was demonstrated that PDK1 up-regulation effectively reversed the inhibitory effects of miR-503 on A549 cell proliferation. Moreover, PDK1 down-regulation significantly reversed the promoting effects of miR-503 inhibition on PC9 cell proliferation (Figure 5B and C, p<0.01). The effects of miR-503 on cell cycle arrest (Figure 5D), which was attenuated by miR-503 but enhanced by miR-503 inhibitor, can also be reversed by PDK1 overexpression and PDK1 inhibition, respectively (p<0.01). Next, upregulating PDK1 significantly reversed miR-503-induced inhibition on cell migration and invasion, whereas the downregulation of PDK1 showed an opposite effect (Figure 5E and F, p<0.01). Altogether, these evidences indicated that PDK1 played pivotal roles in the inhibitory effects of miR-503 on NSCLC cells.

**MiR-503 Overexpression Inhibited The Proliferation Of NSCLC Cells In Vivo**

To further evaluate the effects of miR-503 overexpression on NSCLC tumor growth in vivo, A549 cells or PC9 cells with miR-503-overexpression or knockdown were subcutaneously inoculated into the flanks of nude mice. We showed here that upregulation of miR-503 significantly inhibited the growth of tumors in vivo, while downregulation of miR-503 showed the opposite effects (Figure 6A and B). Furthermore, PDK1 and p-AKT protein expression were significantly decreased
Figure 6 miR-503 inhibited the tumorigenicity xenograft by regulating PDK1/P38K/AKT signaling pathway in vivo. (A) Representative photographs of tumors were obtained from four groups of mice transfected with miR-503, NC, miR-503 inhibitor and inhibitor NC at indicated times. (B) The volume of tumors was measured and analyzed. (C) The expression levels of PDK1 protein in the implanted tumors were explored by Western blotting. *P<0.05; **P<0.01 compared with the indicated controls.
in the miR-503-overexpression group and were significantly increased in the miR-503-knockdown group (Figure 6C).

Discussions

We here report that miR-503 downregulation is a characteristic of NSCLC. This is further supported by the fact that NSCLCs of higher clinical stages had a lower miR-503.10 Our data are consistent with a previous report, which compared the level of miR-503 in NSCLC tissue and normal tissue, demonstrating an approximately two-fold downregulation of miR-503 in tumor.10 In addition, patients with high miR-503 level were shown to be associated with the presence of metastases. These evidences suggest that miR-503 is a potential predictor of NSCLC, and may be used to guide the detection, staging, and prognosis of NSCLC patients. Here we focus on the tissue-level expression of miR-503 and it may be helpful to further determine the serum-level expression of miR-503, as studies have demonstrated the use of serum-level miRNAs as a diagnostic tool for a variety of cancers, including NSCLC.4,5,19 Serum level of miR-503 has also been employed as a marker for detection of nephrotic syndrome.20 The correlation of serum miR-503 to NSCLC aggressiveness, which is a more convenient and clinically feasible method, will enhance the translational potential of miR-503 as a clinical tool for NSCLC diagnosis.

To further characterize miR-503 as a therapeutic target, miR-503 overexpression or downregulation was achieved by transfection of miR-503 mimics or inhibitors, followed by monitoring changes in cell proliferation, migration and invasion. Our results echoed prior evidences that miR-503 overexpression is capable of exerting tumor-suppressive effects.6–9,21 Further, cells with miR-503 overexpression demonstrated slower in vivo tumor growth, while cells with miR-503 inhibition demonstrated fast in vivo tumor growth, indicating that the anti-tumor effects of miR-503 can be potent in vivo. This piece of evidence potentiates the further development of therapeutic strategy based on in vivo miR-503 delivery, which unlike our experiment that employed cells pre-treated to overexpress or downregulate miR-503. Indeed, the in vivo delivery of miR-503 has been explored by a study, using polyamine-coated carbon tubes, to achieve efficient transfection of this therapeutic gene to target cells,22 which is also worthwhile to pursue in future studies. As shown as an advantage of delivering tumor-suppressor miRNA by other studies,23,24 this strategy is presumably of less side effects to normal tissue as normal tissues have a high miR-503 level.

PDK1/PI3K/AKT signaling was shown as a target of miR-503 in our study. PDK1/PI3K/AKT signaling has been long sought as a therapeutic target in NSCLC14,25–27 and other cancers.28,29 The suppression of this signaling pathway, through chemotherapy drugs or small-interfering RNAs (siRNA), was shown to pronouncedly attenuate NSCLC progression. Here, using bioinformatics analysis, we identified that PDK1 is a target of miR-503. A negative correlation was found between miR-503 and PDK1. miRNAs are able to post-transcriptionally inhibit target gene expression and our results clearly show an antagonizing effect between miR-503 and PDK1. In tumors with miR-503 overexpression, PDK1 downregulation was also observed. Therefore, transfection of miR-503 mimic is also a potential approach to inhibit PDK1/PI3K/AKT signaling. It should however be noted that other oncogenic molecules have also reported as target of miR-503, including PRMT1,7 L1CAM,8 CCND1,9 insulin-like growth factor 1 receptor.21 It is likely that the alteration of these targets, in addition to the PDK1/PI3K/AKT signaling, synergistically contributed to the anti-tumor effects of miR-503. Further studies are warranted to fully elucidate the mechanism of miR-503 in NSCLC.

Conclusion

In summary, this study accessed the functional mechanism of miR-503 in the diagnosis and therapy of NSCLC. The results indicated that miR-503 overexpression could attenuate NSCLC cell proliferation, migration, invasion and in vivo tumor growth. Moreover, PDK1 was confirmed to be a direct target of miR-503 and a negative correlation exists between PDK1 and miR-503. These results provided new ideas for the treatment of NSCLC.

Ethical Statement

Our study was approved by the Ethics Committee of Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology. Patients provided written informed consent, and that this was conducted in accordance with the Declaration of Helsinki. Ethical and legal approval was obtained prior to the commencement of the study. All experiments were performed following Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology and national guidelines and regulations.
Disclosure

The authors report no conflicts of interest in this work.

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