

## Original Article

# MircoRNA-383 suppress cell proliferation and invasion by targeting GPC5 in human non-small cell lung cancer

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**Abstract:** Objective: To investigate the role of miR-383 in human non-small cell lung cancer (NSCLC). Materials and methods: The expression level of miR-383 was detected in non-small cell lung cancer tissues and cell lines by using qRT-PCR. A549 cells were transfected with miR-383 mimics and mimic controls. The proliferation, invasion and migration abilities were measured by colony formation assay, transwell and wound healing analysis. The targeting gene of miR-383 was detected by western blot, qRT-PCR and luciferase activity assays. Results: We illustrated that the level of miR-383 was downregulated in non-small lung cancer tissues when compared with the adjacent normal tissues. The expression of miR-383 was also downregulated in lung cancer cell lines. Upregulation of miR-383 suppressed A549 cells proliferation, invasion and migration. What's more, we demonstrated that Glypican (GPC5) was a target gene of miR-383. Upregulation of GPC5 can ameliorate the inhibiting effect of miR-383 on tumor cells proliferation and migration. Conclusions: MiR-383 acted as an anticarcinogenic miRNA, partly through targeting GPC5 in non-small cell lung cancer, it would become a therapeutic target for NSCLC.

**Keywords:** MiR-383, non-small cell lung cancer, GPC5, proliferation, invasion, migration

## Introduction

Lung cancer is one of the most commonly malignancies among both women and men, and it is the leading cause of tumor-related death worldwide [1-4]. There are two kinds of lung cancers: small cell lung cancer and NSCLC. NSCLC is the most common sub-type of lung cancers, which accounts for about ~85% of all lung cancers [5, 6]. Although the incidence rate during the past decades continuously reducing, there has no progress in relative 5-year overall survival rate for patients with lung cancer, mainly due to the fact that no biomarker is available for early diagnosis of lung cancer [7]. Therefore, it is very important to recognize novel biomarkers to afford precise diagnosis and personalized treatment plans for patients with lung cancers.

MicroRNAs (miRNAs) are a new groups of endogenous, short-length single-stranded, conserved and small RNAs that suppress protein translation through binding to the 3'-untrans-

lated region (3'-UTR) of target messenger RNAs (mRNAs) [8-10]. MiRNAs are participated in many processes such as cell proliferation, differentiation, development, inflammation, apoptosis, migration and stress response [11-15]. During past decades, strong evidence has demonstrated that miRNAs are dysregulated, either upregulated or downregulated, and play a critical role in the carcinogenesis, cancer development and cancer death such as gastric cancer, hepatocellular carcinoma, gallbladder carcinoma, bladder cancer, cutaneous squamous cell carcinoma and colorectal cancer [15-18]. Therefore, researches of miRNAs in lung cancer are very important to find new diagnostic biomarker for lung cancer diagnosis.

Recently, increasing studies have certified that micro-RNA-383 (miR-383) was proved to be downregulated in many kinds of carcinoma tissues, including glioma, testicular cancer, hepatocellular carcinoma and medulloblastoma [19-22]. In lung disease, a recent study found that among patients with Chronic Obstructive Pul-

**Table 1.** Clinical characteristics of 78 patients and the level of miR-383 in NSCLC tissues

Clinicopathological features	Patients	MiR-383 Median (range)	P
Age (year)			0.469
<54	40	0.89 (0.17-1.25)	
≥54	38	1.04 (0.17-1.25)	
Sex			0.365
Male	48	0.79 (0.17-1.25)	
Female	30	0.85 (0.17-1.25)	
TNM Stage			0.026*
Early stage: I & II	32	0.57 (0.17-0.96)	
Advanced stage: III	46	1.09 (0.48-1.25)	
Tumor size			0.124
≤3 cm	38	0.78 (0.17-1.25)	
>3 cm	40	0.89 (0.17-1.25)	
Pathology			0.233
Adenocarcinoma	42	0.83 (0.17-1.25)	
Squamous cell carcinoma	36	0.92 (0.17-1.25)	
Lymph Node Metastasis			0.015*
Negative	34	0.48 (0.17-0.97)	
Positive	44	0.94 (0.22-1.25)	

\*P<0.05 was considered to be statistically significant differences.

monary Disease (COPD) miR-383 was dysregulated in the plasma of COPD patients than in healthy control patients [23]. Moreover, there is an in vitro study on NCLC cell lines, Bae and his colleagues found that resveratrol, an anti-tumor reagent, was able to regulate the expression level of miR-383 among A549 NSCLC cells [24]. However, the role of miR-383 in NSCLC was still unknown. In this study, we investigated that miR-383 expression was down-regulated in NSCLC cell lines, and clinical NSCLC tissues. We also found that GPC5 is a direct target gene of miR-383. The effects of miR-383 inhibit lung cancer development is relying on downregulation of GPC5. Our results showed a new role and the mechanism for miR-383 as a cancer suppressor in NSCLC.

## Materials and methods

### Clinical tissues and cell culture

Clinical tissues, including paired cancer tissues and their adjacent non-cancer lung epithelial tissues, were received from 78 patients with NSCLC in stage I/II/III who underwent surgery in our hospital from May 2015 to February 2016. All of these patients agreed to partici-

pate in this study and gave written informed consent. Both the study and consent were met the standards with the Declaration of Helsinki. None of the patients obtained any adjuvant therapies, or major surgeries within 6 months prior to surgery. Classification on Tumor, Lymph Node, Metastasis (TNM) stage was decided by three independent and experienced pathologists in conformity to the International Union Against Cancer and the American Joint Committee on Cancer [25]. Once the clinical samples surgically obtained from patients, they were immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction. All the patients' clinicopathological features were summarized in **Table 1**. Human normal bronchial epithelium cell line NuLi-1, and NSCLC cell lines, A549, SW900, H226 were all purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All the cells were cultured with RPMI 1640 medium

supplemented with heat-inactivated 10% FBS and penicillin/streptomycin (100 U/ml and 100 mg/ml, respectively) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

### RNA extraction and real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from cell lines and clinical samples by using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the operating instructions. RNA was quantified by using UV absorbancies at 260 and 280 nm (A<sub>260</sub>/A<sub>280</sub>). Subsequently the RNA was reverse-transcribed into cDNA using reverse transcription system (Thermo Scientific, CA, USA). The mRNA expression level of miR-383 was detected by the ABI PRISM 7500 Sequence Detection System (ABI) using the TaqMan MicroRNA assay kits (Applied Biosystems, California, USA). U6 small nuclear RNA (snRNA) was used as the control normalize. The gene expression of GPC5 also analyzed by SYBR Green and normalized to GAPDH. The judgment of primer sequences' specificity was based on dissociation curve, 2<sup>-ΔΔCt</sup> (cycle threshold) was used to calculate the relative gene expression levels.

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### *Western blot*

Protein was extracted by using RIPA buffer which contain a protease inhibitor cocktail and phosphatase inhibitors (Sigma, St. Louis, MO, USA), according to the operating instructions. 50 µg of protein samples were separated by SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF, Millipore, Bedford, MA, USA) membranes using the Bio-Rad transfer system. Western blotting was performed using anti-β-actin and anti-GPC5 (CST, Denver, CO, USA). The protein levels were detected with an ECL kit (Thermo Scientific, CA, USA) following the manufacturer's instructions.

### *Lentivirus production and infection*

The miR-383 mimics and miR-383 mimics control were synthesized chemically from Gene-Chem (Gene-Chem, Shanghai, China). Cells were transfected by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocols.

### *Cell migration assay*

Cell migration was measured by the wound healing analysis. Human lung cancer cell lines were seeded into 6 well plates. The wounds were created by a sterile pipette tip. The debris cells were removed by washing with PBS for three times. The cells were continuing cultured with medium for another 24 or 48 hours at 37°C. The wound width was measured by using microscope (Nikon, Tokyo, Japan).

### *Cell invasion assay*

Cells were transfected with the miR-383 mimics and miR-383 mimics control for 24 h. Then plating the cells onto the 24-well upper chamber with a membrane that was pre-treated with matrigel (100 µg per well, BD Biosciences, San Jose, CA, USA). In the lower portion of the chamber, adding fresh medium with 10% FBS. After the cells were incubated for 24 h at 37°C, we carefully removed the cells in the upper chamber. Invaded cells were fixed with 4% formaldehyde, stained with 0.5% crystal violet, and counted under a microscope (Nikon, Tokyo, Japan).

### *Cell proliferation assay*

Cell proliferation was measured by colony formation tests, cells were treated with miR-383

mimics and miR-383 mimics control then cultured in the 6-well plate. After cultured for 2 weeks, the colonies were stained with crystal violet and then counted.

### *Plasmid construction and dual luciferase activity assay*

The eukaryotic expression vector pcDNA3.1(+) was subcloned with full-length GPC5 cDNA which lacking the 3'-UTR (Invitrogen, Carlsbad, California, USA). The GPC5 3'UTR target site for miR-383 was amplified by PCR and cloned into the XhoI site of pGL3 control (Promega, Madison, USA). This vector was called WT GPC5 3'UTR. The Quick-change mutagenesis kit (Strata-gene, Heidelberg, Germany) was used to carry out the site-directed mutagenesis of the miR-383 target-site in the GPC5 3'UTR and known as Mut GPC5 3'UTR. For the luciferase activity assay, Wt or Mut GPC5 3'UTR vector and the control vector pRL-CMV ((cytomegalovirus) coding for Renilla luciferase, Promega) were cotransfected. Dual-Luciferase Reporter Assay System (Promega, Madison, USA) was used to detect the luciferase activity 36 h after transfection.

### *Statistical analysis*

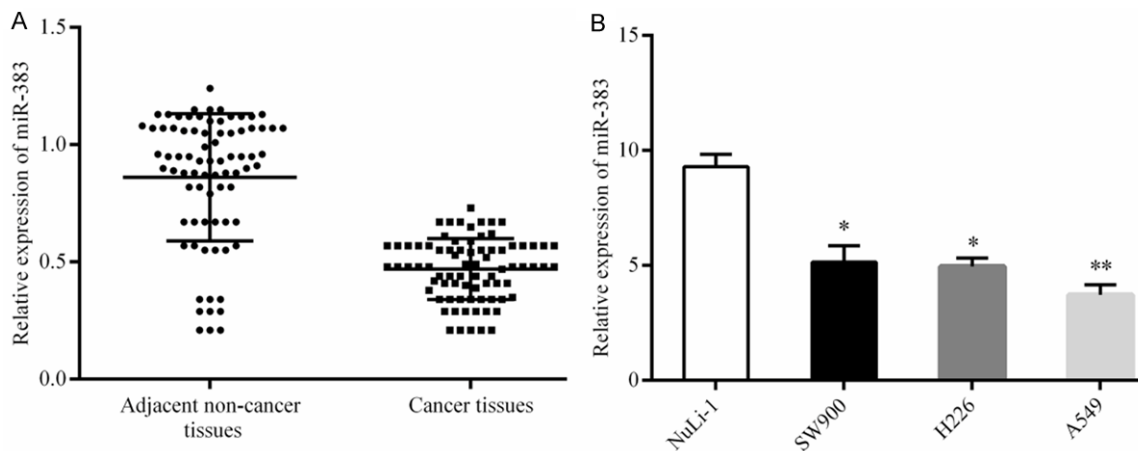
All the results analyses were performed by SPSS 19.0. Dates were presented as the means ± SD (standard deviation). One-way ANOVA test and Student's t-test were used to measure the differences between the groups.  $P < 0.05$  was considered to be statistically significant differences.

## **Results**

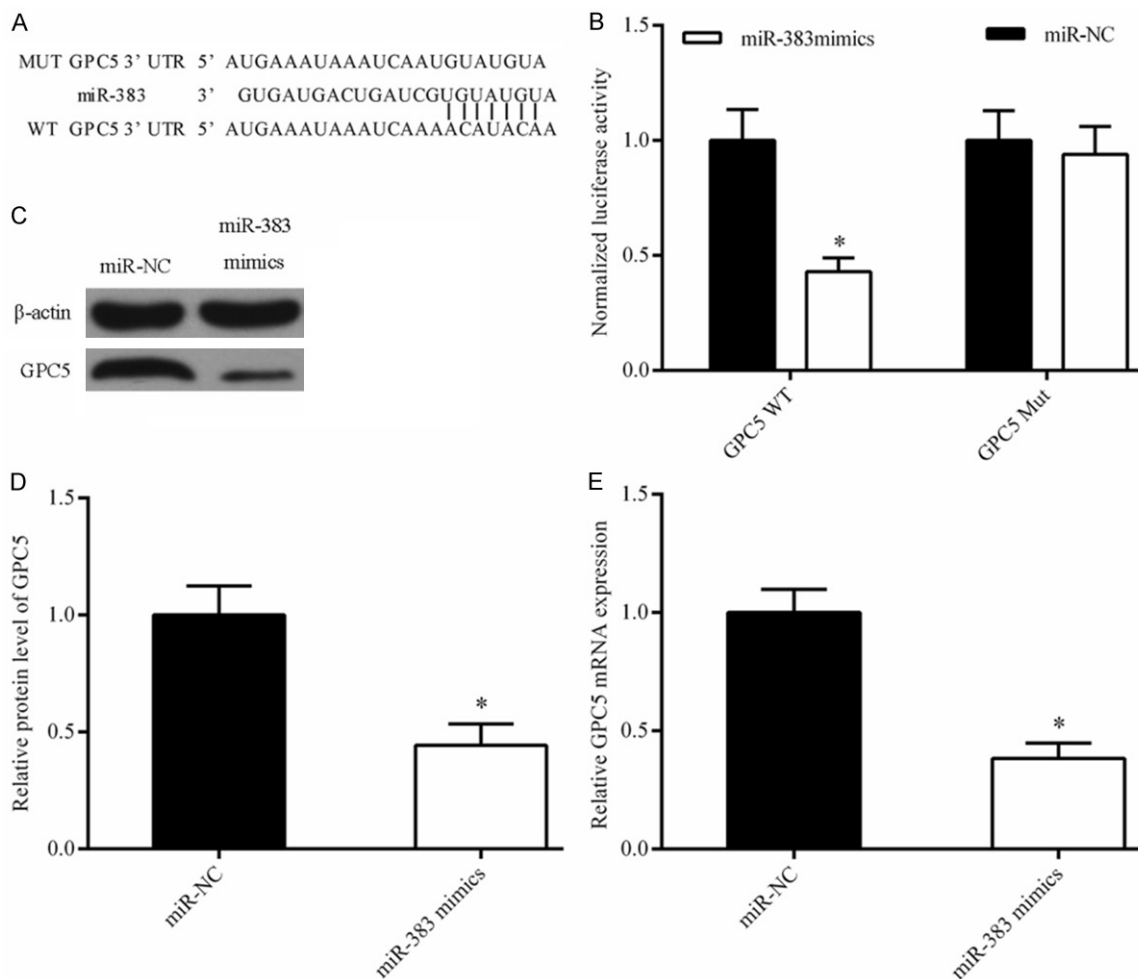
### *MiR-383 expression was down-regulated in lung cancer tissues and NSCLC cell lines*

In order to state the role of miR-383 in cancer metastasis and cancer growth, we subsequently researched the expression level of miR-383 in lung cancer tissues and whether miR-383 affected the growth, invasion and migration of NSCLC cell lines. We first investigated the level of miR-383 in human lung cancer tissues. As shown in **Figure 1A**, miR-383 expression level was down-regulated in lung cancer tissues when compared with the adjacent normal tissues. In addition, the expression level of miR-383 in NSCLC cell lines was lower than in human normal bronchial epithelium cell line

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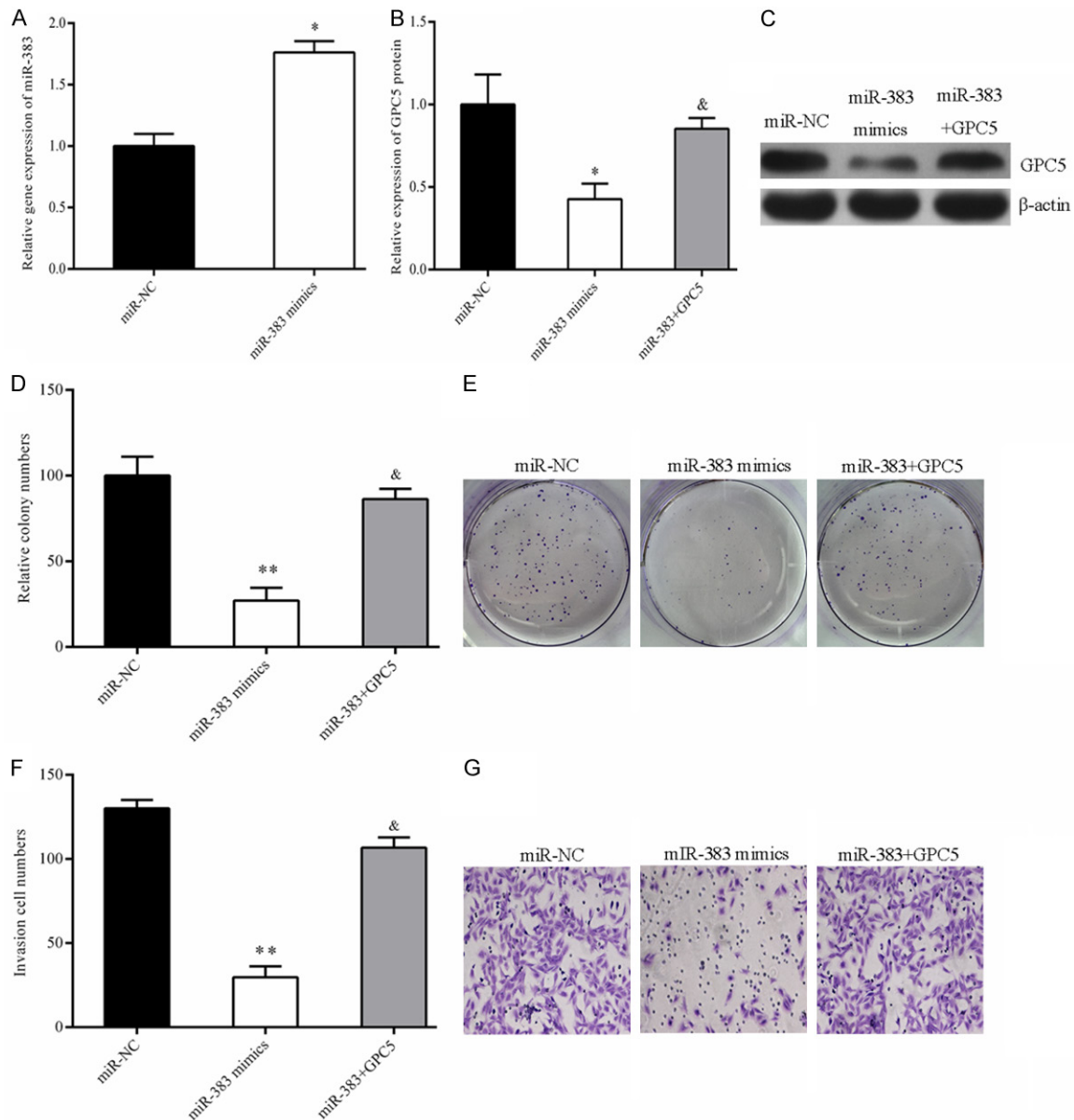


**Figure 1.** MiR-383 is down-regulated in NSCLC tissues and cell lines. A: The expression of miR-383 was detected by qRT-PCR, the level of miR-383 in NSCLC tissues is reduced when compared with the adjacent non-cancer tissues. B: The expression of miR-383 was decreased in NSCLC cell lines than in human normal bronchial epithelium cell line (\* $P < 0.05$  when compared with the Nuli-1, \*\* $P < 0.01$  when compared with the Nuli-1).



**Figure 2.** GPC5 is a direct target of miR-383. A: Sequence of miR-383 and the GPC5 3'-UTR, which contains a predicted miR-383 binding site. B: Luciferase assay in A549 cells co-transfected with miR-383 mimics and mimic control which containing the GPC 3'-UTR (WT) or a mutant (Mut) (\* $P < 0.05$  when compared with the miR-NC). C, D: MiR-383 transfection suppressed the GPC5 protein levels (\* $P < 0.05$  when compared with the miR-NC). E: MiR-383 transfection suppressed the GPC5 mRNA levels (\* $P < 0.05$  when compared with the miR-NC).

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**Figure 3.** MiR-383 inhibits the proliferation and invasion of NSCLC cells by targeting GPC5. A: The level of miR-383 can be regulated by transfected with miR-383 mimics. B, C: Western blot showed that transfection of GPC5 recovered the miR-383 induced down-regulation of GPC5 (\* $P < 0.05$  when compared with the miR-NC, & $P < 0.05$  when compared with the miR-383 mimics). D, E: MiR-383-overexpressing NSCLC cells had suppressed colony forming abilities when compared with mock cells (\*\* $P < 0.01$ ). Restoration of GPC5 increased the colony forming abilities of miR-383-overexpressing cells (& $P < 0.05$ ). F, G: Invasion assay demonstrated that miR-383 made the invasion cell numbers reduced while GPC5 could increase it (\* $P < 0.05$  when compared with the miR-NC, & $P < 0.05$  when compared with the miR-383 mimics).

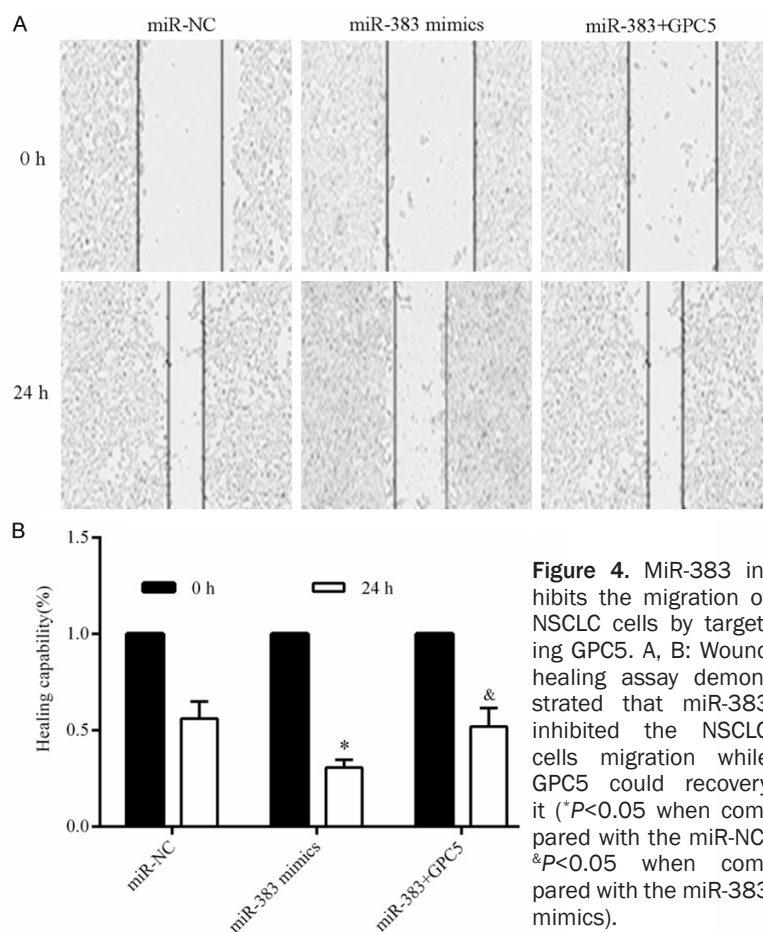
(Figure 1B). To summarize, the results verified that in human lung cancer tissues and cells the expression level of miR-383 decreased.

### MiR-383 directly targets GPC5 in A-549 cells

We used targets can to search for target genes of miR-383. Among mRNAs involving miR-383

recognition sites in their 3'-UTRs, we focused on GPC5. With the purpose of certifying GPC5 was a direct target of miR-383, GPC5 wild-type (WT) or mutant 3'-UTR (Figure 2A) was subcloned into a luciferase reporter vector and co-transfected with miR-383 mimics or mimics control into A549 cells. Results showed that in A549 cell lines the luciferase activity of GPC5

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**Figure 4.** MiR-383 inhibits the migration of NSCLC cells by targeting GPC5. A, B: Wound healing assay demonstrated that miR-383 inhibited the NSCLC cells migration while GPC5 could recovery it (\* $P < 0.05$  when compared with the miR-NC, & $P < 0.05$  when compared with the miR-383 mimics).

WT 3'-UTR was dramatically suppressed by miR-383 but had no influence on the mutant (Figure 2B). To further confirmed the effect of miR-383 on GPC5 expression, we transfected miR-383 into A549 cells and discovered that overexpression of miR-383 reduced the mRNA and protein levels of GPC5 (Figure 2C-E). These findings illustrated that GPC5 is a direct target gene for miR-383 in lung cancer cells.

### *MiR-383 inhibits A-549 cells proliferation, migration and invasion by targeting GPC5 in vitro*

As we found the low expression level of miR-383 in lung cancer tissue and cells, we subsequently analyzed the role of miR-383 in the proliferation, migration and invasion of lung cancer cells. MiR-383 mimics and miR-NC were transfected into A-549 cells and the expression level of miR-383 was examined by qRT-PCR. The gene expression level of miR-383 in the mimics group was dramatically upregulated when compared with the mimics control (Figure 3A). The results showed that the level of miR-383

can be regulated. Glypicans (GPCs) are a kind of heparan sulphate proteoglycans, and GPC5 is one of the most important to regulate the progression of many kinds of cancers. GPC5 has been found to be upregulated in NSCLC tissues and the level of GPC5 was concerned with poor differentiation, metastasis and invasion [26]. The low expression level of miR-383 in NSCLC cells, and its suppression function on GPC5 implies that miR-383 may play a critical role in NSCLC cells proliferation by reducing the expression of GPC5. We therefore studied the role of miR-383 overexpression and GPC5 restoration on NSCLC cells. Western blot assay demonstrated that reconstruction of GPC5 recovered the expression level of GPC5 which inhibited by miR-383 (Figure 3B, 3C). In our colony formation assays, we found that transfection of miR-383 mimics markedly suppressed

the proliferation of A549 cells when compared to the miR-383 controls (Figure 3D, 3E). What's more, the transwell invasion assay revealed that enhanced the level of miR-383 significantly reduced the cells invaded when compared with the cells transfected with mimics control (Figure 3F, 3G). In addition, a similar tendency was discovered in the wound healing assays, we discovered that the migration ability of cells transfected with miR-383 was dramatically limited than the miR-NC transfection cells (Figure 4A, 4B). This above results illustrated that upregulation of miR-383 significantly reduced the level of GPC5 when compared with mimics control, and it was dramatically improved by tranfection of GPC5. What's more, the abilities of colony formation, migration and invasion were restored by transfection GPC5 into NSCLC cell lines (Figures 3, 4).

### Discussions

Over the past decade, studies have shown that miRNAs can be functional as a tumor regulator,

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either as cancer suppressor or oncogene [27, 28]. In our study, we demonstrated that miR-383 was downregulated in lung cancer tissues when compared with the adjacent normal tissue in vivo. Furthermore, we also found that miR-383 was downregulated in NSCLC cell lines in vitro. It is interesting to note that in previous studies, miR-383 was discovered to be dysregulated in blood plasma of patients with COPD, but not in patients with NSCLC [23]. The difference expression of miR-383 between tumorous and plasma in NSCLC patients may suggest that the level of miR-383 in NSCLC may be more positively correlated with NSCLC patients' tumor progress and prognosis than in their circulating systems. It is exciting that the results of our study are in accordance with other scholars' studies demonstrating miR-383 was lower expression in many kinds of human cancers, such as hepatocellular carcinoma, glioma, testicular cancer and medulloblastoma [19-22, 29].

In addition, in this present study, we surveyed functional roles of miR-383 in NSCLC. The colony formation assay showed that upregulation of miR-383 severely inhibited A549 cell lines proliferation than the cells transfected with miR-NC, so as to the cell migration and invasion abilities. In order to investigate the molecular mechanism of the cancer inhibitor role of miR-383 in lung cancer, we made use of luciferase reporter assay and western blot to illustrate that GPC5 was a target gene of miR-383 in A549 cells. Furthermore, both the qRT-PCR and western blot results demonstrated that miR-383 had the ability to negatively regulate the expression of GPC, which was play the role by binding with a site in the VEGFA 3'-UTR. Transfection of GPC5 into miR-383-overexpressing A549 cells increased the GPC5 expression levels. GPCs are a kind of heparan sulphate proteoglycans (HSPGs) that combine with the plasma membrane by a glycosyl-phosphatidylinositol (GPI) anchor [30, 31]. More and more evidences have illustrated that GPCs, especially GPC5 and GPC3, play an important role in the progression of many kinds of cancers [32, 33]. Recent studies have shown that GPC5 plays critical roles in the development of lung tumor [34-36]. For instance, Li Y et al. found that the expression level of GPC5 was increased in NSCLC cell lines and upregulation of GPC5 accelerated the NSCLC cell migration and invasion [37]. What's more, the level of GPC5 was upregulated in NSCLC tissues and

the expression level of GPC5 was correlated with poor differentiation, metastasis and invasion. In our present study, we transfection of GPC5 into miR-383-overexpressing cells, results demonstrated that the colony forming abilities, the invasion ability and migration ability recovery. These results illustrated that miR-383 might act as a tumor suppressor in NSCLC by targeting GPC5.

In conclusion, our results confirmed that miR-383 expression level was downregulated in lung cancer tissues and cell lines. Overexpression of miR-383 suppressed the lung cancer cell proliferation, migration and invasion. We also revealed that GPC5 was a potential target gene of miR-383. This study demonstrated that miR-383 play as a tumor suppressor in the development and progression of NSCLC, it may serve as a predictor for prognosis and a therapeutic target for NSCLC patients.

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### Disclosure of conflict of interest

None.

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