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miR-126 Functions as a Tumor Suppressor by Targeting SRPK1 in Human Gastric Cancer

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The expression of miR-126 and serine–arginine protein kinase 1 (SRPK1) are linked to tumor development; nevertheless, its role in the tumor growth and invasion of gastric cancer (GC) and the underlying mechanism have not been clarified. Here the expression and role of miR-126 and SRPK1 were investigated in GC tissues and cells by in vitro assay, and then targets of miR-126 were identified by dual-luciferase reporter assay. In this study, miR-126 expression was downregulated and associated with lymph node metastasis and poor prognosis as well as SRPK1 expression. In vitro assay revealed that miR-126 obviously inhibited the proliferative and invasive capabilities of GC cells. The dual-luciferase reporter assay showed that miR-126 targets the 3′-UTR of SRPK1 and downregulates its expression. SRPK1 overexpression promoted cell migration and invasion. In conclusion, the reduced expression of miR-126 is suggestive of the risk of GC recurrence and metastasis, and miR-126 functions as a tumor suppressor by targeting SRPK1 expression in the development of GC.

Key words: miR-126; SRPK1; Gastric cancer (GC)

INTRODUCTION

Gastric cancer (GC) is one of the most common causes of cancer deaths and is reported to be the fourth highest cancer incidence[1]. In recent decades, GC has strongly threatened the survival and life span of humans[2]. Despite the rapid improvement in recent diagnosis methods and therapy, early diagnosis and treatment of GC patients remain unsatisfactory[3,4]. Because some molecular mechanisms are implicated in GC development and progression, it is essential to hunt for potential biomarkers to promote the progression of GC treatment.

MicroRNAs (miRNAs) are composed of endogenous, single-stranded, noncoding RNAs of approximately 22 nucleotides[5]. miRNAs negatively modulate gene expression by traditional base pairing with the 3′-untranslated regions (3′-UTRs) of their target mRNA, leading to destabilization and degradation of the target mRNA[6]. Until now, miRNAs have been linked to diverse biological processes, including cell proliferation, cell cycle, cell differentiation, and metastasis[7,8]. Dysregulation of miRNAs has been identified in different kinds of human cancers, suggesting that miRNAs play an important role in the initiation and development of tumors[9]. miRNAs have different functions in different human cancers, acting as tumor suppressors or oncogenes depending on the characteristics of their target mRNAs[10]. Notably, few studies on miR-126 expression and GC development can be found in recent decades.

In this work, we first investigated the expression of miR-126 in 30 cases of GC tissues and adjacent normal tissues and then analyzed the association between miR-126 and clinical pathological indicators. In vitro, the miR-126 overexpression cell model was established for further functional assay. These findings might offer a novel target for biological treatment for GC patients.

MATERIALS AND METHODS

Specimen Collection

GC specimens, including adjacent nontumor tissues, were obtained from the Shandong Provincial Third Hospital (Jinan, Shandong, P.R. China). The Institutional Ethics Committee approved the study protocol and the use of clinical specimens. Written, voluntary, informed consent was obtained from all the patients. The specimens were

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obtained after surgical resection, immediately frozen, and stored in liquid nitrogen. Histological grade was determined blindly by two pathologists.

Cell Lines

GES-1, an immortalized human gastric epithelial cell line, was purchased from the ATCC, and cells (passages 5–10) were maintained in Gibco RPMI-1640 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, and 100 mg/ml streptomycin. Human GC cell lines BGC-823 and MKN-28 were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) and supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco, Grand Island, NY, USA). Cell culture was conducted at 37°C in a humidified 5% CO_2 incubator.

Transfection

miR-126 mimic and corresponding miRNA negative control (miR-NC) were chemically synthesized and purified by Guangzhou RiboBio Co., Ltd. (Guangzhou, P.R. China). Overexpression of the serine–arginine protein kinase 1 (SRPK1) plasmid (pcDNA3.1-SRPK1) and blank vector (pcDNA3.1) was obtained from GeneCopoeia (Guangzhou, P.R. China). Cells were seeded into six-well plates at a density of 8×10^5 cells per well and maintained in DMEM without antibiotics. When the cell density reached 60%–70%, transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

According to the manufacturer's instructions, total RNA was isolated from the tissue specimens or cells using TRIzol (Invitrogen) and stored at −80°C. To determine miR-126 expression levels, cDNA was generated by reverse transcription using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Quantitative PCR (qPCR) was performed with TaqMan MicroRNA PCR Kit (Applied Biosystems) on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). To quantify SRPK1 mRNA expression, cDNA was synthesized with PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, P.R. China), and qPCR was conducted with SYBR Premix Ex Taq™ (Takara Biotechnology Co., Ltd.). U6 and GAPDH were used to normalize the level of miR-126 and SRPK1 mRNA expression, respectively. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method.

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded into 96-well plates at 3×10^3 cells per well. After overnight incubation, cell transfection was performed, and the cells were incubated at 37°C in humidified air with 5% CO_2. Cell proliferation was examined at 0, 24, 48, and 72 h after transfection. Briefly, 10 μl of CCK-8 reagent (Dojindo, Kumamoto, Japan) was added into each well and incubated at 37°C for another 2 h. Finally, the optical density (OD) was detected at a wavelength of 450 nm using the ELISA plate reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA). At least three independent experiments were performed.

Transwell Assay

Transwell assay was performed to assess cell migration and invasion capacities using Matrigel-coated Transwell chambers (Millipore, Billerica, MA, USA). A total of 1×10^5 transfected cells in 100 μl of FBS-free DMEM were placed in the upper chambers. DMEM with 10% FBS was added into the lower chamber as chemoattractant. After 24 h of incubation, the upper surface of the membrane was wiped with a cotton tip. Subsequently, cells were fixed with methanol, stained with 0.5% crystal violet (Sinopharm Chemical Reagent Co., Shanghai, P.R. China), washed with PBS, and photographed under an inverted microscope at 200× magnification (X71; Olympus, Tokyo, Japan). The number of cells was counted at five randomly selected fields.

Bioinformatics Analysis

To predict the potential targets of miR-126, bioinformatics analysis was performed with TargetScan (http://www.targetscan.org) and miRanda (http://www.microrna.org/microrna/getExprForm.do).

Dual Luciferase Reporter Assay

The pMIR-wild type and mutation vector of SRPK1 3′-UTR containing the putative binding site of miR-126 were synthesized and sequenced by GenePharma, Co., Ltd. (Shanghai, P.R. China). Cells were seeded in 24-well plates and transfected with reporter vectors together with miR-126 mimics, or the corresponding miR-NC. After 48 h of incubation, the activities of firefly and *Renilla* luciferases were determined in transfected cells using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA) following the manufacturer's instructions. *Renilla* luciferase activity was used for normalization.

Western Blot Analysis

Total protein was isolated from tissue samples or cells with RIPA lysis buffer (Beyotime Biotechnology, Jiangsu,

P.R. China) containing 1% protease inhibitors (Pierce, Rockford, IL, USA). The concentration of total protein was examined by Bradford assay (Bio-Rad Laboratories). Equal amounts of protein samples (about 30 μg) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore). After blocking with 5% nonfat milk in Tris-buffered saline with Tween (TBST; Sigma-Aldrich, St. Louis, MO, USA), the membranes were incubated with primary antibodies overnight at 4°C. The primary antibodies used in this study include rabbit anti-human polyclonal SRPK1 (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-human monoclonal GAPDH antibody (1:1,000 dilution; Santa Cruz Biotechnology). The membranes were then washed with TBST and incubated with corresponding HRP-conjugated secondary antibodies (1:1,000 dilution; Santa Cruz Biotechnology) at room temperature for 2 h. Band signals

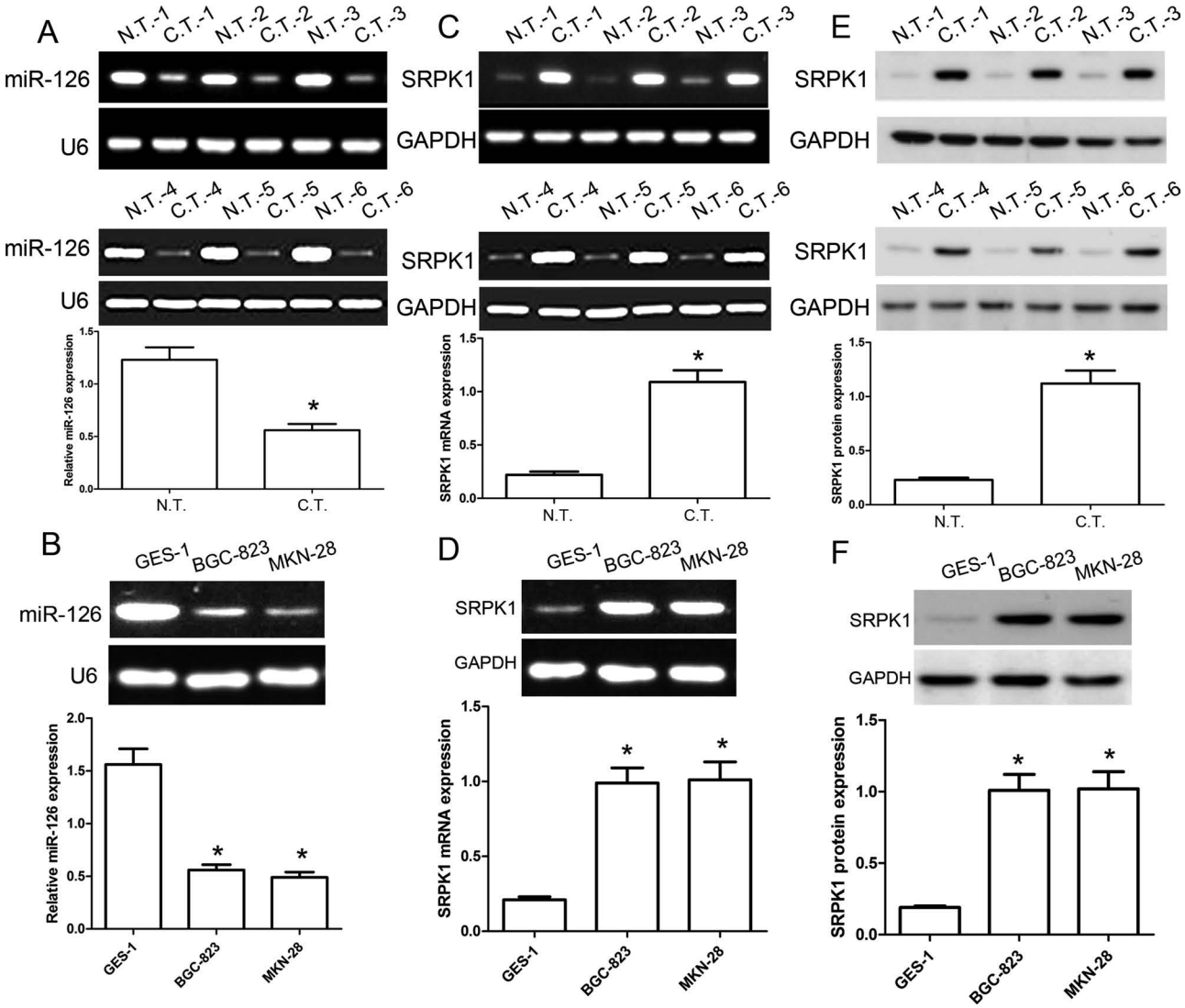

Figure 1. MicroRNA (miR)-126 and serine–arginine protein kinase 1 (SRPK1) expression in gastric cancer (GC) tissues and cells. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed to measure the expression of miR-126 (A) in representative GC tissue (C.T.) ($n=6$) and normal tissues (N.T.) ($n=6$), and (B) normal GES-1 cells, and GC BGC-823 and MKN-28 cells. RT-qPCR (C, D) and Western blot (E, F) were performed to measure the expression of SRPK1 mRNA and protein in representative GC tissue ($n=6$) and normal tissues ($n=6$), normal GES-1 cell, and BGC-823 and MKN-28 cells. The expression of miR-126 was normalized to U6, and levels of mRNA and proteins were normalized to GAPDH in each sample. Data are presented as the mean±standard deviation (SD) of at least three independent experiments or six cases of representative samples. *$p<0.001$ versus N.T. or GES-1 cells.

Table 1. miR-126 and SRPK1 With Clinicopathological Indicators

Indicators	*N*	miR-126*	*p* Value	SRPK1 mRNA*	*p* Value	SRPK1 Protein	*p* Value
Age			0.184		0.563		0.496
<50	15	2.39±0.18		8.91±0.96		0.98±0.09	
≥50	15	2.21±0.22		9.12±1.01		1.02±0.12	
Gender			0.408		0.136		0.134
Male	20	2.28±0.23		9.19±0.91		1.07±0.23	
Female	10	2.35±0.18		8.66±0.85		0.92±0.29	
Tumor size			0.485		0.556		0.085
<5 cm	19	2.33±0.22		8.93±0.97		0.94±0.25	
≥5 cm	11	2.26±0.31		9.14±0.85		1.13±0.33	
Tumor differentiation			0.023		0.025		0.005
Well/moderate	21	2.37±0.22		8.75±0.65		0.89±0.11	
Poor	9	2.15±0.25		9.34±0.55		1.17±0.39	
LN metastasis			<0.001		0.044		0.006
Present	22	2.11±0.19		9.31±0.91		1.20±0.33	
Absent	8	2.67±0.27		8.54±0.81		0.78±0.36	
TNM stage			<0.001		0.017		<0.001
I–II	10	2.65±0.29		8.33±0.99		0.65±0.19	
III–IV	20	2.15±0.22		9.38±1.11		1.21±0.21	

LN, lymph node; SRPK1, serine–arginine protein kinase 1.
*The expression of miR-126 and SRPK1 mRNA was detected by reverse transcription quantitative polymerase chain reaction (RT-qPCR).

were visualized using an enhanced chemiluminescence kit (Pierce, Minneapolis, MN, USA) and analyzed with Quantity One software version 4.6.2 (Bio-Rad Laboratories). GAPDH was used as an internal control.

Statistical Analysis

The statistical analyses were performed using the SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). All data were presented as mean±standard deviation (SD), and differences between groups were analyzed using two-tailed Student's *t*-test or a one-way ANOVA. A value of $p<0.05$ was considered to indicate a statistically significant difference.

RESULTS

miR-126 Expression Is Decreased in GC Tissues and Cells

In order to address the miR-126 expression in GC, we acquired 30 pairs of clinical GC samples to examine miR-126 levels by RT-PCR. The expression levels of miR-126 were downregulated in GC tissues compared to the adjacent nontumor tissues, which is consistent with the TCGA clinical data ($p<0.001$) (Fig. 1A). Additionally, to determine whether miR-126 was downregulated in GC cell lines, RT-PCR was performed on GC cells as well as normal GES-1 cell line. The results showed that miR-126 was significantly lower in BGC-823 and MKN-28 cell lines compared with GES-1 cells ($p<0.001$) (Fig. 1B). The results indicated miR-126 was decreased in GC cells.

SRPK1 Expression Is Increased in GC Tissues and Cells

Subsequently, we also measured the SRPK1 mRNA and protein expression in GC tissues and cell lines using RT-PCR and Western blot. The results showed that a significant increase of SRPK1 mRNA and protein in GC tissues compared to normal tissues ($p<0.001$) (Fig. 1C and E). Moreover, the expression levels of SRPK1 mRNA and protein in the BGC-823 and MKN-28 cells were significantly increased compared with normal GES-1 cells ($p<0.001$) (Fig. 1D and F). All these results indicated that SRPK1 was increased in GC.

Association of miR-126 and SRPK1 Expression With Lymph Node Metastasis and Poor Prognosis

To evaluate the quantitative expression and significance of miR-126 and SRPK1 in GC tissues, miR-126 and SRPK1 expression was divided into low and high expression in the cohort of patients according to the median value from the RT-qPCR and Western blot (median miR-126 value: 2.31; median SRPK1 mRNA value: 8.99; median SRPK1 protein value: 1.01). As shown in Table 1, miR-126 expression in GC tissues was negatively correlated with lymph node metastasis and TNM stage (all $p<0.05$), whereas SRPK1 expression was positively correlated with lymph node metastasis and TNM stage (all $p<0.05$). miR-126 and SRPK1 had no correlations with age and gender. As shown in Table 2, multivariate analyses were performed to analyze the prognostic role of miR-126 and SRPK1 for GC. The results revealed that miR-126 and SRPK1 (mRNA

Table 2. Multivariate Cox Regression Analysis of Gastric Cancer Patients ($N=30$)

	5-Year Overall Survival		
Variables	HR	95% CI	p Value
Lymph node metastasis (present vs. absent)	2.14	1.05–4.34	0.035*
Tumor size (>5 vs. ≤5) (cm)	1.46	0.94–2.26	0.091
Tumor differentiation (poor/signet vs. well/moderate)	1.37	0.69–2.70	0.364
TNM stage (III+IV vs. I+II)	3.23	1.38–7.57	0.007*
miR-126 expression (low vs. high)	2.12	1.16–3.86	0.014*
SRPK1 mRNA expression (high vs. low)	2.23	1.17–4.26	0.015*
SRPK1 protein expression (high vs. low)	2.19	1.23–3.91	0.008*

HR, hazard ratio; CI, confidence interval.
*Statistically significant.

and protein) expression was an independent risk factor for poor prognosis of GC patients ($p=0.014$, $p=0.015$, $p=0.008$, respectively).

Based on the quantitative expression levels of miR-126 and SRPK1 mRNA by RT-qPCR, we identified an inverse correlation between the expressions of miR-126 and SRPK1 mRNA in GC tissues ($R^2=0.8648$, $p<0.001$) (Fig. 2). An inverse correlation between the expressions of miR-126 and SRPK1 proteins ($R^2=0.8318$, $p<0.001$) (Fig. 2) in GC tissues was also identified. Thus, we assumed that increased SRPK1 expression levels in GC may be attributed to downregulation of miR-126.

miR-126 Affects Proliferation, Migration, and Invasion of GC Cells

To elucidate the role of miR-126 in the development and progression of GC, we transfected BGC-823 and MKN-28 cells with miR-126 mimic or miR-NC. Then we evaluated the proliferation, migration, and invasion of GC cells. Compared with control, transfection of miR-126 mimics markedly increased the expression level of miR-126 in BGC-823 and MKN-28 cells ($p<0.001$) (Fig. 3A). Functional analysis showed that miR-126 overexpression resulted in a significantly decreased proliferation ability of BGC-823 and MKN-28 cells ($p<0.01$) (Fig. 3B). Transwell assay further showed that upregulation of miR-126 significantly reduced the migration and invasion abilities of BGC-823 and MKN-28 cells ($p<0.01$) (Fig. 4A and B).

SRPK1 Is a Target Gene of miR-126

First, we used bioinformatical analysis to explore the putative targets of miR-126. TargetScan software validated that SRPK1 was a potential target of miR-126, and the relationship between miR-126 and SRPK1 was evolutionally conserved (Fig. 5A). To elucidate this relationship, we generated luciferase reporter vectors containing WT and MUT 3′-UTR of SRPK1 mRNA, and subsequently conducted the dual-luciferase reporter assay. We found that luciferase activity was significantly decreased in BGC-823 cells cotransfected with the WT SRPK1 3′-UTR vector and miR-126 mimic ($p<0.001$) (Fig. 5B). However, luciferase activity was not changed in BGC-823 cells cotransfected with the MUT SRPK1 3′-UTR vector and miR-126 mimic ($p>0.05$) (Fig. 5C). These results suggest that miR-126 directly binds to the

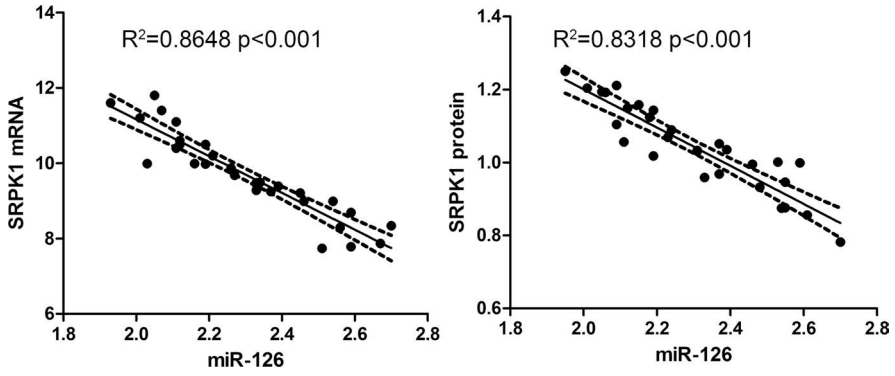

Figure 2. The correlation of miR-126 and SRPK1 expression in GC tissues. The expression of miR-126 and SRPK1 mRNA was detected by RT-qPCR. An inverse correlation between the expressions of miR-126 and SRPK1 mRNA ($R^2=0.8648$, $p<0.001$) and proteins ($R^2=0.8318$, $p<0.001$) in GC tissues was identified.

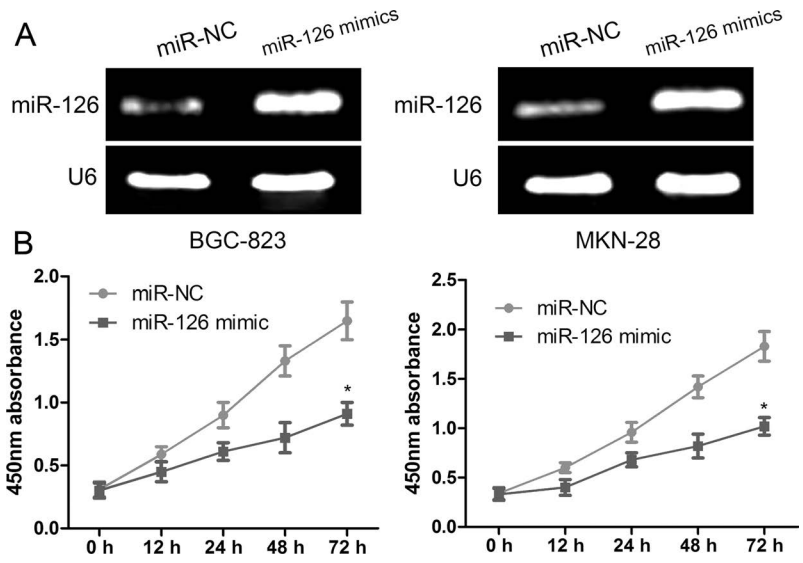

Figure 3. miR-126 inhibits cell proliferation, migration, and invasion in GC cells. (A) miR-126 mimic was transfected into the BGC-823 and MKN-28 cell lines to overexpress miR-126. (B) Cell proliferation was determined in BGC-823 and MKN-28 cells by cell counting kit-8 (CCK-8) assay. Cells were transfected with miR-126 mimics or miRNA negative control (miR-NC). The data were represented as mean ± SD. *$p < 0.001$, versus miR-NC.

3′-UTR of SRPK1 mRNA. In an attempt to determine the role of miR-126 in regulating SRPK1 expression in GC, we transfected the BGC-823 and MKN-28 cells with miR-126 mimics and miR-NC. Following transfection, we performed Western blot analysis to detect SRPK1 protein expression. The results revealed that SRPK1 protein was significantly inhibited by miR-126 ($p < 0.01$) (Fig. 6A). To examine whether miR-126 can degrade SRPK1 mRNA, we performed RT-PCR and real-time PCR and we found that miR-126 did affect the SRPK1 mRNA level ($p > 0.05$) (data not shown).

SRPK1 Overexpression Reverses miR-126-Repressed Cell Migration and Invasion

Finally, we investigated whether SRPK1 was involved in the inhibitory effects of miR-126 on the proliferation

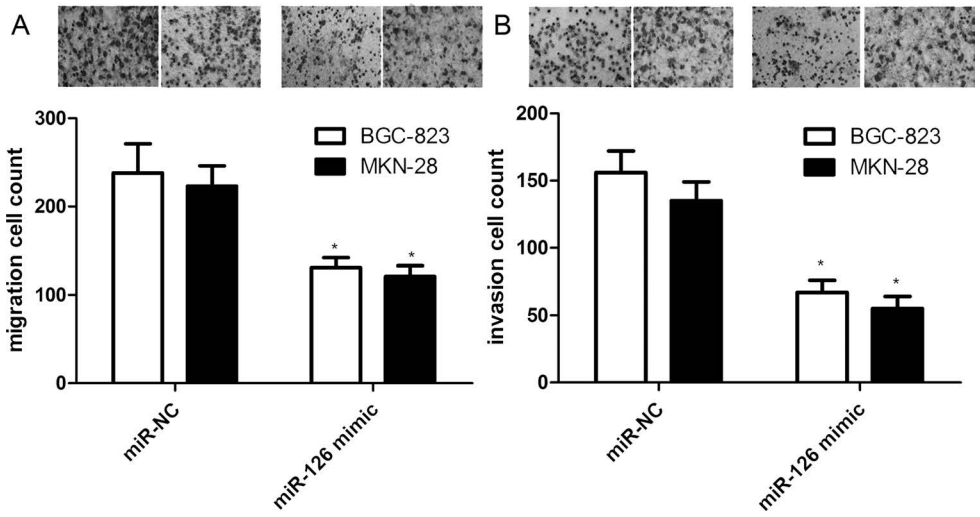

Figure 4. miR-126 inhibits cell migration and invasion in GC cells. (A) Cell migration assay was determined in BGC-823 and MKN-28 cells by Transwell assay. (B) Cell invasion assay was determined in BGC-823 and MKN-28 cells by Transwell assay. Cells were transfected with miR-126 mimics or miR-NC. The data were represented as mean ± SD. *$p < 0.001$, versus miR-NC.

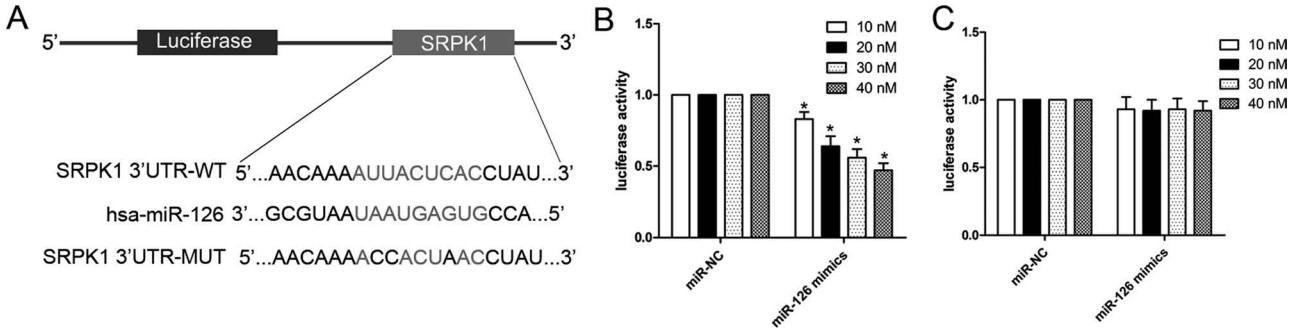

Figure 5. SRPK1 is a direct target of miR-126. (A) The sequence relation between miR-126 and the 3′-untranslated region (3′-UTR) of SRPK1. Luciferase reporter vectors containing WT and MUT SRPK1 3′-UTR were constructed. (B) Luciferase activity was significantly decreased in BGC-823 cells cotransfected with the WT SRPK1 3′-UTR vector and miR-126 mimic (C) but was unaffected in cells cotransfected with the MUT SRPK1 3′-UTR vector and miR-126 mimic, relative to the control group. Data are presented as mean ± SD of at least three independent experiments *$p < 0.001$ versus miR-NC. Wt, wild type; MUT, mutant.

and invasion of GC cells. BGC-823 and MKN-28 cells were cotransfected with miR-126 mimics and SRPK1 plasmids. Then we conducted Transwell assay to assess their effects on cell migration and invasion. The Transwell assay revealed that SRPK1 overexpression promotes miR-126-inhibited cell migration and invasion compared with BGC-823 and MKN-28 cells cotransfected with miR-126 mimics and vector ($p < 0.001$) (Fig. 6B and C). Together with the data above, miR-126 indeed inhibited the migration and invasion of BGC-823 and MKN-28 cells through direct targeting of SRPK1 expression.

DISCUSSION

miRNAs are associated with numerous cellular processes, which are also identified as critical regulators in the initiation and metastatic progression of cancers[11]. Some miRNAs including miR-206, miR-34a, and miR-335 are decreased in GC tissue and are associated with lymph node metastasis[12–15]. However, miRNAs such as miR-199, miR-223, and miR-107 are increased and promote metastasis[16–19]. Tumor metastasis is a crucial hallmark of cancer progression and the major cause of cancer morbidity and

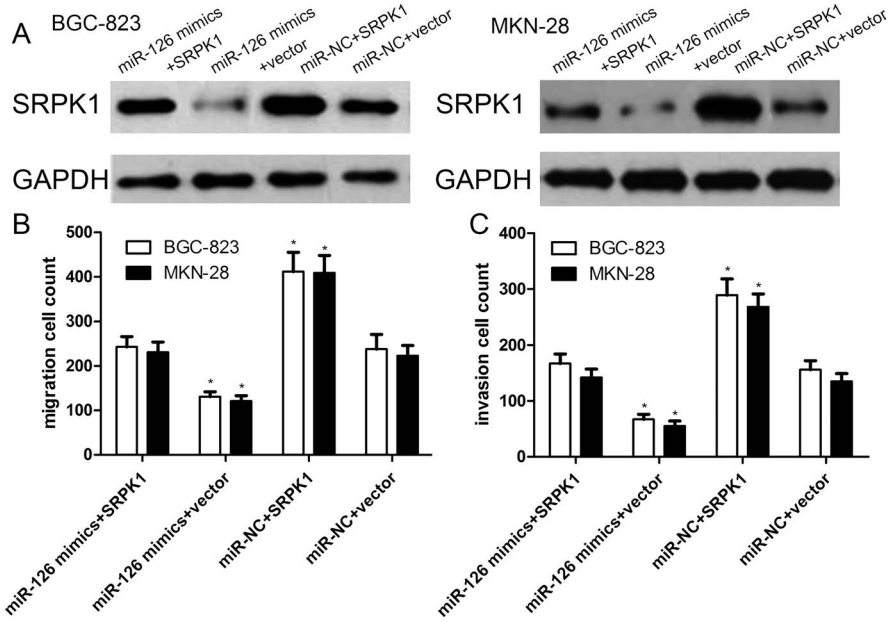

Figure 6. SRPK1 is a downstream effector of miR-126 in GC. BGC-823 and MKN-28 cells were transfected with miR-126 mimic or miR-NC, and then cotransfected with SRPK1 plasmid or vector. (A) Western blot analysis was conducted to measure the protein expression of SRPK1. Transwell assays were performed to measure the (B) migration and (C) invasion using crystal violet staining (magnification: 40×). *$p < 0.001$ versus miR-NC + vector.

mortality[20]. Although remarkable progress has been made in understanding the molecular mechanism of metastasis during the last decades, there are still a lot of questions that remain unanswered.

Some reports investigated the association of a GC-specific miRNA with the prognosis of GC patients and identified that miRNAs were an independent predictor of overall survival and relapse-free survival.[12–16] However, the exact role of miR-126 in GC remains unknown. In this study, we observed that miR-126 was significantly decreased in GC tissues compared with nontumor gastric tissues. In addition, miR-126 expression was also downregulated in GC cell lines, suggesting that miR-126 may serve as a tumor suppressor in the development and progression of GC. To our knowledge, this is the first study to demonstrate that overexpression of miR-126 significantly inhibited the proliferation and invasion of GC cells, indicating that miR-126 may also exert an inhibitory effect on the growth and metastasis of GC.

According to recent reports, miRNAs modulate gene expression by base-pairing with the 3′-UTR of their target mRNAs. Depending on the degree of complementarity between the miRNA and its mRNA target, the interaction leads to either inhibition of translation or degradation of the mRNA. In the present study, bioinformatical analysis and dual-luciferase reporter assay indicated that miR-126 directly bound to the 3′-UTR of SRPK1 mRNA to repress SRPK1 expression. SRPK1 was reported to regulate posttranscriptional processes of some mRNAs[21–23]. In addition, SRPK1 expression was often increased and affected cancer cell progression in prostate cancer, breast cancer, lung cancer, and glioma[24]. In other studies, SRPK1 was reported as a novel therapeutic target for cancer patients[24,25]. Together with evidence above, we assumed that miR-126-SRPK1 signaling pathway may be a potential way to treat GC patients.

In conclusion, this study demonstrated that miR-126 functions as a tumor suppressor by targeting SRPK1 expression in the development of gastric cancer. Thus, miR-126-SRPK1 may be a potential therapeutic target for GC patients.

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