

MiR-126 inhibits the invasion of gastric cancer cell in part by targeting Crk

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Abstract. – OBJECTIVE: Accumulating evidence has shown that microRNAs (miRNAs) are aberrantly expressed in human gastric cancer and crucial to tumorigenesis. Herein, we identified the role of miR-126 in human gastric cancer (GC) growth and development *in vitro*.

MATERIALS AND METHODS: MiR-126 expression was investigated in GC tissue samples and cell lines by real-time PCR. Crystal violet test and Transwell assay were conducted to explore the effects of miR-126 on the proliferation and invasion of human GC cell lines, respectively. The impact of miR-126 over expression on putative target Crk (v-crk sarcoma virus CT10 oncogene homolog) was subsequently confirmed via Western blot. Crk specific siRNA was used to suppress Crk expression.

RESULTS: MiR-126 expression was frequently and markedly downregulated ($p < 0.05$) in human gastric cancer tissues. Overexpression of miR-126 inhibited GC cells invasion but did not affect its proliferation *in vitro*. Moreover, overexpression of miR-126 significantly decreased ($p < 0.05$) the protein levels of Crk, which has previously been identified as a direct target of miR-126. Knockdown of Crk also markedly suppressed GC cells invasion.

CONCLUSIONS: Our results demonstrated that overexpression of miR-126 inhibited GC cells invasion in part by targeting Crk. These findings suggested that miR-126 played major roles in the malignant behavior of GC and it might be a promising therapeutic target of GC.

Key Words:

miR-126, Gastric cancer, Crk, Invasion.

of GC is a consequence of late-stage of diagnosis, the 5-year survival rate for advanced stages is extremely poor and around 5% to 15%³⁻⁴. Although diagnosis and treatment of GC have improved, the survival rate has not increased substantially in couple of years. Therefore, an improved understanding of the molecular pathways involved in the progression of gastric cancer will be helpful in improving prevention, diagnosis and therapy of this disease.

Recently many epigenetic events are widely investigated in the cancer development. Aberrant expression of miRNAs is reported in various types of cancers^{5,6}. MicroRNAs (miRNAs) are small non-coding RNAs of approximately 21-25 nucleotides and act as post-transcriptional regulators of gene expression. Mature miRNAs play important regulatory roles in cell growth, proliferation, differentiation and cell death^{7,8}. It reported that miRNAs can function either as oncogenes or tumor suppressors and are aberrantly expressed in several types of human cancer. Upregulated miRNAs in cancer may function as oncogenes by negatively regulating tumor suppressor genes. In contrast, downregulated miRNAs may normally function as tumor suppressor genes and inhibit cancer by regulating oncogenes⁹. Growing evidence has indicated that unique miRNA expression profiles for each cancer type would be a useful biomarker for cancer diagnosis and prognosis¹⁰. More recently, miR-126 has been widely studied in different type of cancers. For example, miR-126 may function as metastasis suppressor miRNA in human breast cancer¹¹, and miR-126 has been proved to have effects on the invasion of prostate cancer¹². However, the expression and function of miR-126 in gastric cancer was poorly understood.

In the present study, we investigated expression levels of miR-126 in primary GC clinical

Introduction

Gastric cancer (GC), one of the most common malignancies worldwide, is the second most frequent cause of cancer death¹⁻². The high mortality

specimens. Overexpression of miR-126 inhibited GC cells invasion but did not affect its proliferation *in vitro*. Moreover, miR-126 overexpression robustly reduced Crk (v-crk sarcoma virus CT10 oncogene homology) expression, thus, suppressed GC cells invasion. Therefore, we postulate that miR-126 inhibited GC cells invasion in part by regulation of Crk.

Materials and Methods

Clinical GC Specimens

Fifteen pairs of primary gastric cancer and corresponding adjacent normal tissues, as well as other 110 of human gastric cancer tissues were obtained from patients who underwent curative surgical resection for GC at the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China) from 2010 to 2013. Written consent of tissue donation for research purposes was obtained from each patient. The study protocol was approved by the Scientific and Ethical Committee of the First Affiliated Hospital of Zhengzhou University. Fresh samples were snap frozen in liquid nitrogen immediately after resection and stored at -80 °C. All samples were histologically confirmed by staining with hematoxylin-eosin. The histological grade of cancers was assessed according to criteria set by the World Health Organization.

Cell Lines and Cell Culture

Human gastric cancer cell lines BGC-823 and SGC-7901 were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI 1640 (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) in a humidified atmosphere containing 5% CO₂ at 37°C.

Cell Transfection

The miR-126 mimics and non-specific miR control (NC) were synthesized and purified by Genephama Biotech (Shanghai, China). The sequences of miR-126 were: Sense: 5'-UCGUACCGUGAGUAAUAAUGCG-3', Anti-sense: 5'-CAUUAUUACUCACGGUACGAUU-3'. The Cells cultured in 6-well plate were transfected with miR-126 mimics or NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA)

according to the manufacturer's protocol, respectively. After 48 hours transfection, cells were harvested for further experiments. For targeted knockdown of Crk, cells were transfected with Crk specific siRNA and scrambled siRNA (OriGene Technologies Inc., Rockville, MD, USA), then cells were harvested for Western blot analysis after 48 hours transfection. All experiments were performed in triplicate.

RNA Isolation and Real Time-PCR

Total RNA from tissues or cultured cells was extracted using TRIZOL reagent (Invitrogen, Carlsband, CA, USA). For analysis of miRNA expression, quantitative real-time PCR (qPCR) was performed using Platinum SYBR Green qPCR SuperMix-UDG system (Invitrogen) on an ABI7900HT System. The primer sequence of miR-126 is 5'-GGCTCGTACCGTGAG-TAAT-3' (sense) and GTGCAGGGTCCGAGGT (antisense). The relative levels of miR-126 transcripts were normalized to the control U6 mRNA; the primer sequence was 5'-TGCGGGTGCTCGCTCGGCAGC -3' (sense) and 5'- CCAGTGCAGGGTCCGAGG T -3' (antisense).

Cell Proliferation

BGC-823 and SGC-7901 cells after transfected with miR-126 mimics or NC were cultured in 96-well plates (3,000 cells/well). After 48 hrs of culture, the number of cells was determined. Briefly, cells were rinsed with phosphate buffered saline (PBS), fixed in methanol, and stained with 0.5% (w/v) crystal violet. Cells were rinsed with distilled water and air dried again. Once dry, cells were lysed with 2% (w/v) sodium deoxycholate solution with gentle agitation. Absorbance was measured at 570 nm on a microplate reader (BioTek Instrument, Winooski, VT, USA). Wells containing known cell numbers (0, 1,000, 2,000, 5,000, 10,000, 20,000, or 40,000 cells/well; six wells/cell density) were treated in the similar fashion to establish standard curves. Cell proliferation studies were run in four independent experiments.

Cell Invasion Assay

Cell invasion was performed in matrigel-coated Boyden chamber (BD Biosciences, San Jose, CA, USA). BGC-823 and SGC-7901 cells were plated in the 6-well plates and transfected with miR-126 mimics or NC. After 48 hrs transfection, cells were seeded onto the upper

compartment of an invasion chamber coated with matrigel. After 16 h incubation, cells that had invaded onto the bottom of the inserts were stained and counted under a microscope. The average number of invading cells per microscopic field over the random four fields was counted in each assay from four independent experiments.

Western Analysis

After 48hrs transfection, cells were washed in ice-cold PBS and added to radioimmunoprecipitation assay (RIPA) lysis and extraction buffer (Thermo Fisher Scientific, Franklin, MA, USA) containing Protease Inhibitor Cocktail I (Millipore, Billerica, MA, USA). Proteins (20 µg) were electrophoresed on SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. Proteins on the membranes were immunoblotted with an antibody against Crk (1:2000; Cell Signaling Technology, Beverly, MA, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:5000; Kangchen, Shanghai, China). Changes in Crk and GAPDH protein levels were quantified by scanning densitometry (model GS 670; Bio-Rad, Hercules, CA, USA). This study was run in three independent experiments.

Statistical Analysis

Correlations between miRNA expression and clinicopathological features were analyzed by Student's *t*-test between two groups. All values were expressed as means $\pm$ S.E.M. Cell proliferation, invasion and Western blot assays were tested using Student's *t*-test. $p < 0.05$ were considered to be statistically significant.

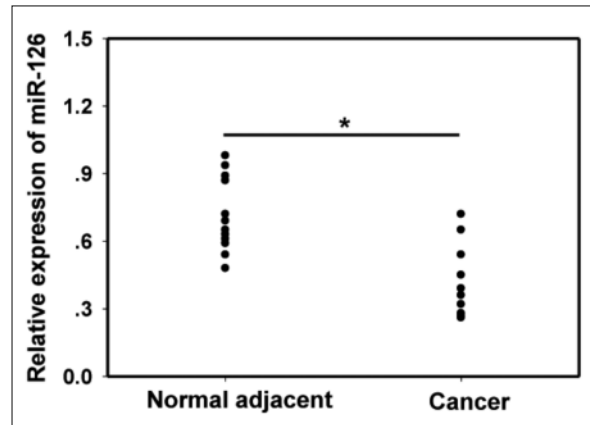


Figure 1. Decreased expression of miR-126 in primary GC tissues. Decreased miR-126 expression in GC tissues in comparison with the adjacent normal tissues. *Different ($p < 0.05$, $n=15$) from adjacent normal control.

Results

Relative expression of miR-126 in gastric cancer tissues and its correlation with clinicopathological features.

To determine whether miR-126 expression is associated with gastric cancer, we examined miR-126 expression levels in 30 frozen samples from GC patients (15 cancers and 15 adjacent normal controls) by TaqMan real time RT-PCR. Our results showed that miR-126 expression is significantly decreased ($p < 0.05$) in GC tissues in comparison with their pair-matched adjacent normal tissues (Figure 1). Student's *t*-test revealed that low expression of miR-126 was significantly associated with lymph-node metastasis ($p < 0.001$) and poor histological grade ($p < 0.001$) (Table I).

Table I. Correlation of miR-126 expression with clinical characteristics of gastric cancers.

		Total, (n=110)	miR-126	<i>p</i> value
Age	≤ 50	57	0.34 ± 0.021	0.121
	> 50	53	0.38 ± 0.014	
Sex	Male	71	0.39 ± 0.016	0.278
	Female	39	0.36 ± 0.023	
Tumor size (cm)	≤ 3	41	0.42 ± 0.007	0.072
	> 3	69	0.39 ± 0.012	
Histological grade	Well	33	0.46 ± 0.008	< 0.001
	Poor	77	0.26 ± 0.023	
pT stage	T1/T2	46	0.40 ± 0.031	0.073
	T3/T4	54	0.33 ± 0.024	
Metastasis lymph node	Negative	26	0.49 ± 0.007	< 0.001
	Positive	84	0.21 ± 0.016	

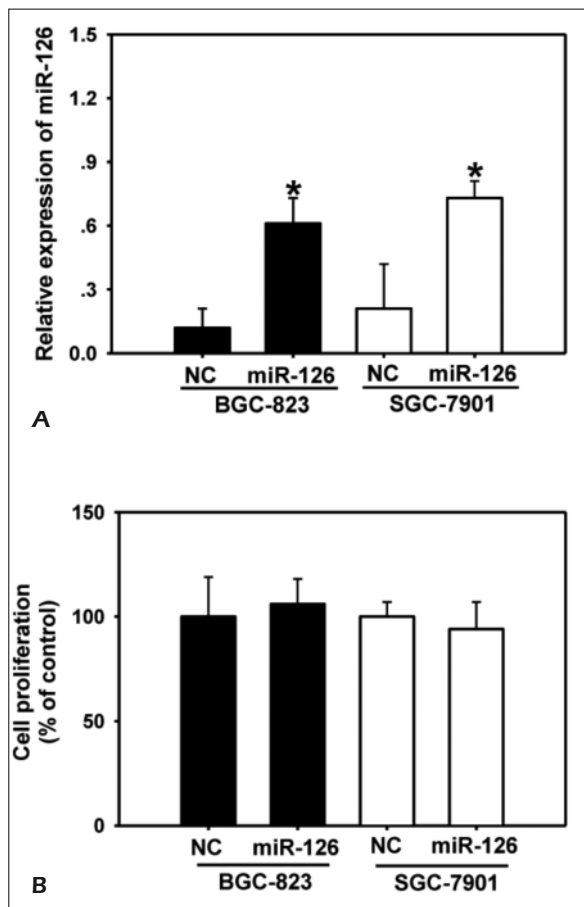


Figure 2. Effects of miR-126 over expression on cell proliferation in BGC-823 and SGC-7901 cells. **A**, Over expression of miR-126 by transfecting miR-126 mimics in BGC-823 and SGC-7901 cells, the expression levels of miR-126 were detected by real-time PCR. **B**, Cell proliferation was determined with crystal violet method. The results are represented as a percentage of cell number relative to the control cells ($n = 4$). *Different ($p < 0.05$) from their controls.

Effects of miR-126 on GC Cell Proliferation and Invasion

To validate if miR-126 regulated GC cell growth, we performed proliferation and invasion assays by transfection of miR-126 mimics or NC into GC cells. After transfection, the miR-126 is highly increased ($p < 0.05$) when compared to NC in both BGC-823 and SGC-7901 cells (Figure 2A). As expected, the increased expression of miR-126 induced significant inhibition ($p < 0.05$) on cell invasion in both these two cell lines (Figure 3). However, we did not observe any effects of miR-126 on cell proliferation in these GC cells (Figure 2B). Our results indicated that over expression of miR-126 significantly suppressed cell invasion in GC cell lines.

Over Expression of miR-126 Silenced Crk

To explore the mechanism of invasion inhibition induced by miR-126, we investigated whether miR-126 could regulate Crk expression in BGC-823 and SGC-7901 cells, since Crk was reported to be a target of miR-126 in different kind of cancer cells¹³⁻¹⁴. We transduced GC cells with miR-126 mimics at several different concentrations of 0, 20 and 40 nM and then examined Crk expression levels. As shown in Figure 4, over expression of miR-126 induced a dose-dependent decrease ($p < 0.05$) in Crk protein expression. Taken together, these data suggested that Crk is a direct target of endogenous miR-126.

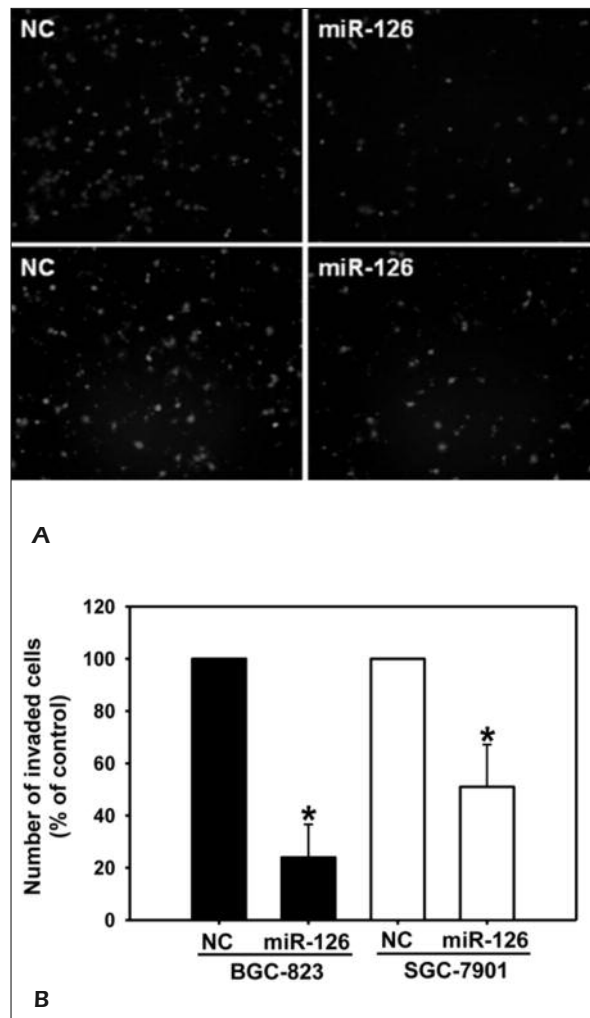


Figure 3. Effects of miR-126 over expression on cell invasion in BGC-823 and SGC-7901 cells. The invasion activity of GC cells was performed using transwell assay. **A**, Photographs depict the invasion of GC-823 and SGC-7901 cells. **B**, Quantified data are expressed as means $\pm$ S.E.M. *Different ($p < 0.05$) from the control.

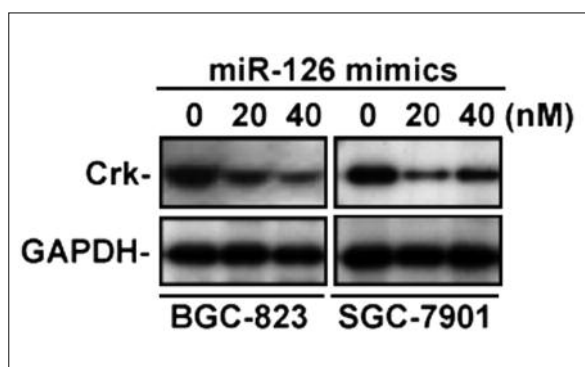


Figure 4. Over expression of miR-126 decreased the protein levels of Crk. The protein levels of Crk were detected by Western blot after transfected with different concentrations of miR-126 mimics. The relative expression of Crk was normalized to GAPDH. *Different ($p < 0.05$) from the control group.

Effects of Crk Knockdown on GC Cells Invasion

In order to address the functional role of Crk in GC cell invasion, BGC-823 and SGC-7901 cells were transfected with Crk specific siRNA. Compared with scrambled control, Crk specific siRNA significantly inhibited ($p < 0.05$) Crk protein expression, and this inhibitory effect lasted at least 72 hrs (Figure 5A). The results of Transwell assay indicated that knockdown of Crk led to inhibition of cell invasions ($p < 0.05$) in these two cell lines (Figure 5B).

Discussion

Within couple of years interest in miRNAs has grown greatly, especially in the regulation of gene expression during cancer development and progression. Previous studies have used an miRNA profiling approach to investigate the function of the miRNA in gastric cancer, showing many miRNAs aberrantly overexpressed or downregulated in gastric cancer progression¹⁵⁻¹⁷. In this study, we demonstrated that miR-126 was frequently down-regulated in GC clinical specimens. Overexpression of miR-126 inhibited GC cells invasion but did not affect its proliferation *in vitro*. Moreover, miR-126 overexpression robustly reduced Crk expression, thus, suppressed GC cells invasion. These findings suggested that miR-126 played major roles in the malignant behavior of GC by targeting Crk and it might be a promising therapeutic target of gastric cancer.

It is well known that miRNAs have been im-

plicated in the regulation of a wide range of biological processes, including cell proliferation, migration, differentiation, metabolism et al⁷⁻⁸. In the human genome, miR-126 is found on chromosome 9 within intron 7 of the EGFL7 gene¹⁸. MiR-126 is highly expressed in greatly vascularized tissues like the kidney, lung and heart in the adult¹⁹⁻²¹. Recently, decreased miR-126 expression were examined in different types of cancer, including lung cancer²², breast cancer²³ and cervical cancer²⁴. A recent study identified a functional role for miR-126 as a suppressor of tumor proliferation and metastases in gastric cancer²⁵. In agreement with these reports, we showed that miR-126 was significantly down-regulated in gastric cancer tissues compared with matched normal tissues, and was deeply associated with clinicopathological features, including lymph node metastasis, and histological grade. To reveal the functional roles of miR-126 in GC metastasis, we ectopically raised the miR-126 level in GC cells to investigate its effect on cell prolifera-

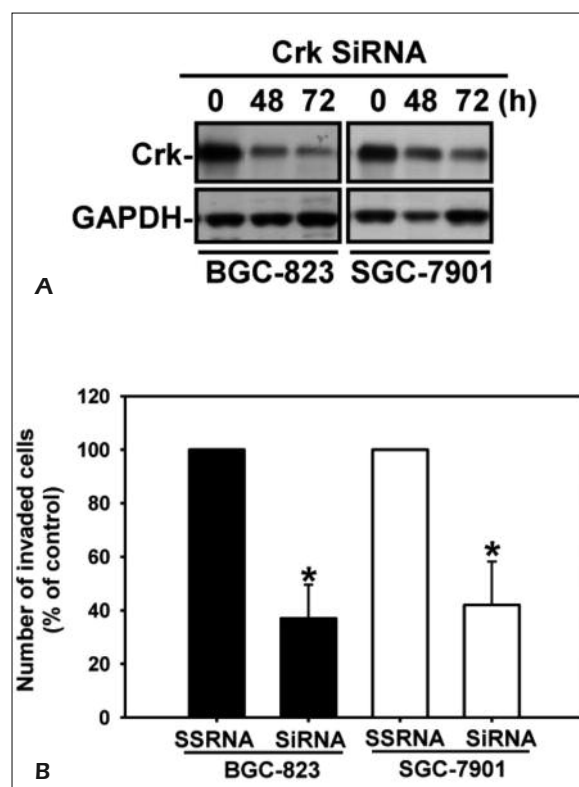


Figure 5. Effects of Crk knockdown on cell invasion in BGC-823 and SGC-7901 cells. **A**, The protein levels of Crk were detected by Western blot after transfected with Crk specific siRNA in GC-823 and SGC-7901 cells. **B**, Quantified data of cell invasion are expressed as means $\pm$ S.E.M. *Different ($p < 0.05$) from the control.

tion and invasion. Over expression of miR-126 could inhibit cell invasion in both BGC-823 and SGC-7901 cells, revealing its potential tumor suppressor role in GC metastasis.

Within couple of years, a lot of studies focused on the relationship between cancers and dysregulated miRNAs. Indeed, miRNAs can result in silencing of their target genes and control a wide range of biological processes²⁶. In the current study, Crk was found and validated to be a target of miR-126 in GCs¹³⁻¹⁴. It is well known that Crk was a key signaling integrators of migration and invasion of cancer cells²⁷. Crk was often found to be significantly increased in many human cancers, including of oral squamous cell carcinoma²⁸, glioblastomas²⁹, and breast cancer³⁰. Importantly, its expression levels was highly involved in the aggressive and malignant behavior of cancer cells. Knockdown of Crk induced in a significant decreased in migration and invasion of multiple malignant breast and other human cancer cell lines³⁰⁻³¹. Agreement with above findings, our results demonstrated that increase expression of miR-126 significantly inhibited Crk expression. Both over expression of miR-126 and knockdown of Crk all significantly reduced GC cell invasion, which strongly suggested that Crk may be a functional downstream target of miR-126.

Conclusions

We proved that miR-126 was markedly down-regulated in human gastric cancers. Increasing the expression of miR-126 may lead to GC cell invasion suppression by targeting Crk. However, the detailed mechanisms of GC invasion suppression by Crk were not clear yet and need further explored. Taken together, our findings may lead to new diagnostic and therapeutic approaches for gastric cancer.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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