Down-regulation of miR-326 is associated with poor prognosis and promotes growth and metastasis by targeting FSCN1 in gastric cancer

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Abstract

Background: MicroRNAs (miRNAs) have been documented as playing important roles in diverse biological processes including tumorigenesis. However, the function and mechanism of miR-326 in gastric cancer are still unknown. The aim of this study is to identify the role of miR-326 in gastric cancer and clarify the regulation of Fascin1 (FSCN1) by miR-326.

Methods: The expression levels of miR-326 were detected in gastric cancer samples and cell lines by real-time PCR. The clinical and prognostic significance of miR-326 in gastric cancer patients were analyzed. Furthermore, the function of miR-326 on tumor cell growth and mobility were explored through MTT, colony formation, Transwell migration and invasion assays in vitro. A miR-326 target was confirmed using luciferase reporter assays, real-time PCR and Western blot.

Results: Our study showed that miR-326 expression was decreased in gastric cancer tissues and cell lines, and low expression of miR-326 was associated to clinical stage, tumor depth, lymph node metastasis and distant metastasis. In survival analysis, low expression of miR-326 was a poor independent prognostic factor for gastric cancer patients. Gain-of-function and loss-of-function studies showed that miR-326 served as a tumor suppressor regulating gastric cancer cells growth, migration and invasion. Furthermore, we identified FSCN1 as the functional target of miR-326 by directly targeting the 3'-UTR of FSCN1.

Conclusions: Our study demonstrated that miR-326 overexpression was a poor prognostic marker for gastric cancer patients, and miR-326 served as a tumor suppressor in gastric cancer via directly regulating FSCN1.

Keywords

FSCN1, gastric cancer, miR-326, prognosis

History

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Introduction

Gastric cancer is the second most common cause of cancer-related death in the world (Torre et al., 2015). Based on 2015 Cancer Statistics of America, a total of estimated 24,590 new gastric cancer cases and 10,720 gastric cancer deaths occur in 2015 (Siegel et al., 2015). In China, the morbidity of gastric cancer ranked second with 3,621,000 new gastric cancer cases, and the mortality reached to third with the proportion of 14.33% each year (Chen et al., 2013). Despite recent advances in systematic treatment including surgery, radiotherapy and chemotherapy, the 5-year overall survival rate is still unsatisfactory. Major reasons of high mortality rate in gastric cancer are recurrence distant metastasis (Meyer & Wilke, 2011). Up to now, the molecular mechanism underlying the pathogenesis and progression of gastric cancer remains unknown fully. Thus, a better understanding of the molecular mechanism about gastric cancer progression is necessary for the development of novel therapeutic strategies for gastric cancer patients.

MicroRNAs (miRNAs), a small non-coding RNA molecules about 19–23 nt, exist in many organisms and modulate gene expression at the post-transcriptional level by combining with the 3'-untranslated region (3'-UTR) of their target genes (He & Hannon, 2004; Zamore & Haley, 2005). Recently, miRNAs serve as potential biomarkers and therapy targets have been widely explored in many kinds of cancers (Du et al., 2014; Janaki Ramaiah et al., 2014; Zhang et al., 2014; Zhou et al., 2014).

miR-326, a 20 nt miRNA on chromosome 11, was originally identified as a neural-specific miRNA in neurons (Ferretti et al., 2008). Recently, more and more evidences show that miR-326 serves as a tumor suppressor involving in human cancer development and progression, such as colorectal cancer (Wu et al., 2015), non-small cell lung cancer (Wang et al., 2015), glioma (Zhou et al., 2013), breast cancer (Liang et al., 2010) and pancreatic ductal adenocarcinoma (Zhang et al., 2015). However, the significance of miR-326 in gastric cancer is still unknown. Thus, we primarily detected the expression of miR-326 in 136 gastric cancer tissue...
samples, and analyzed the association of miR-326 expression to gastric cancer patient’s clinicopathological characteristics including prognosis. Our study showed that miR-326 expression was decreased in gastric cancer tissues and cell lines, and conversely correlated with malignant status and poor prognosis of gastric cancer patients.

In order to further elucidate the functions and mechanisms of miR-326 in gastric cancer development and progression, we analyzed miRNA target databases, and found that Fascin1 (FSCN1) was to be a potential target of miR-326. FSCN1 is an important factor in gastric cancer progression. FSCN1 overexpression correlated directly with advanced clinical stages and poor tumor differentiation, and inversely with survival rates in gastric cancer patients (Tsai et al., 2007). Moreover, knockdown of FSCN1 expression effectively suppressed gastric cancer cell proliferation and metastasis both in vitro and in vivo (Fu et al., 2009). Thus, we supposed that FSCN1 as a major functional target of miR-326 regulating cell proliferation and metastasis.

In gain-of-function and loss-of-function studies, we found that miR-326 acted as a tumor suppressor regulating gastric cancer cells growth, migration and invasion. Meanwhile, we proved that FSCN1 was directly negatively regulated by miR-326 at a posttranscriptional level, and as a major functional target of miR-326 regulating cell proliferation and metastasis.

**Materials and methods**

**Cell culture**

MKN-28 (well differentiated), NCI-N87 (well differentiated), MKN-45 (poorly differentiated), AGS (poorly differentiated) and GES-1 (a normal gastric epithelial cell line) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified CO₂ (5%) atmosphere.

**Ethics statement**

This study was approved by the Research Ethics Committee of Shandong Provincial Cancer Hospital. The informed written consents were collected from all eligible patients and the entire study was performed based on the Declaration of Helsinki.

**Sample collection**

One hundred and thirty-six freshly-frozen gastric cancer samples and 20 paired adjacent normal gastric tissue samples were collected from Shandong Provincial Cancer Hospital. The histopathological diagnosis of all samples was, respectively, diagnosed by two pathologists. All fresh samples were immediately preserved in liquid nitrogen. The system treatments were performed based on NCCN guideline after diagnosis. In the 136 cases, there were 86 males and 50 females with a median age of 58.9 years (range 18–81 years). The clinical follow-up time of patients ranged from 8 to 93 months. Overall survival (OS) was defined as the interval from the date of diagnosis to gastric cancer-related death.

**Real-time PCR**

To quantitate mRNA expression, total RNA was extracted from clinical samples with RNAiso Plus (Takara, Japan). The isolated total RNA was reverse transcribed using the PrimeScript RT Master Mix (Takara, Japan) for FSCN1 and One Step PrimeScript miRNA cDNA Synthesis Kit (Takara, Japan) for miR-326, according to manufacturer’s instructions. Relative expression was calculated via the comparative cycle threshold (Ct) method and was normalized to the expression of GAPDH or U6. The sequence-specific forward and reverse primers sequences for FSCN1 mRNA were 5'-ACCTGGTC TGCCAATCAGGAC-3' and 5'-CCCACTCTTGAAGGCTCA-3', respectively. Forward and reverse primers sequences for GAPDH mRNA were 5'-GCACCGTAAAGCTGAGAAC-3' and 5'-TGGTGAAGACGCGAGTGA-3', respectively. The sequence-specific forward primers for mature miR-326 and U6 internal control were 5'-CCTCTGGGCCCCCTCCTCCAG-3' and 5'-CTCGCTTCGGCAGCACA-3', respectively. The UnimqPCR Primer was included in the kit. The reactions were performed using SYBR Premix Ex TaqTM II (Takara, Japan) on a LightCycler (Roche Diagnostics). Relative quantification was calculated by using the 2^-ΔΔCt method. All qRT-PCRs were performed in triplicate.

**Western blot**

Frozen tissues were lysed in lysis buffer, and protein concentrations were measured using the BCA protein assay kit (Beyotime). Total protein was separated by SDS-PAGE using a 10% polyacrylamide gel and electroblotted onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membrane was immunoblotted overnight at 4°C with primary antibodies: rabbit monoclonal antibody against human FSCN1 (1:1000 dilution; Abcam, Cambridge, UK) and GAPDH (1:2000 dilution; Beyotime, Shanghai, China). A secondary antibody, HRP-conjugated anti-rabbit IgG antibody (1:2000 dilution; Cell Signaling Technology, Danvers, MA), was incubated with the membrane for 1 h after three washes with TBST. Signals were detected with ECL detection reagent (Pierce, Rockford, IL). The images were obtained by Quantity One (Bio-Rad).

**Cell transfection**

The gain-of-function study was performed using miR-326 mimics and its negative control on the AGS cell line. The loss-of-function study was performed with miR-326 inhibitor and its negative control on the MKN-28 cell line. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) in Opti-MEM (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. The relative level of miR-326 in transfected cells was examined by qRT-PCR.

FSCN1 siRNA (si-FSCN1) and non-targeting siRNA (si-NC) were purchased from GenePharma (Shanghai, China) and used at 20 nM Opti-MEM transfection media and Lipofectamine 2000 (both from Invitrogen) were used to transfect the cells once they reached 60% confluency. Knockdown efficiencies were assessed by qRT-PCR and western blotting after 48 h of transfection.
Luciferase reporter assay

The FSCN1 wild-type (wt) and mutant (mut) 3’-UTR were created and cloned to the firefly luciferase-expressing vector the pmiR-RB-REPORT luciferase reporter plasmid (RiboBio, China). For the luciferase assay, AGS and MKN-28 cells were seeded in 6-well plates the day before transfection, and cotransfected with the pmiR-FSCN1-wt or pmiR-FSCN1-mut, and miR-326 mimics and miR-326 inhibitor using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Luciferase activities were determined with the Dual-Luciferase Reporter System (Promega, Madison, WI).

Cell proliferation assays

Cell proliferation was analyzed using MTT assay. Briefly, 1 × 10^5 cells were seeded into a 96-well plate with quadruplicate repeat for each condition. The cells were incubated for 1, 2, 3 and 4 days. Twenty microliters of MTT (5 mg/ml) (Sigma, St. Louis, MO) was added to each well and incubated for 4 h. At the end of incubation, the supernatants were removed and 150 µl of DMSO (Sigma, St. Louis, MO) was added to each well. The absorbance value (OD) of each well was measured at 490 nm. Experiments were performed three times.

Colony formation assay

Briefly, cells (0.5 × 10^3) were plated into 6-well plates and cultured for 10 days. Colonies were then fixed for 5 min with 10% formaldehyde and stained with 1.0% crystal violet for 30 s. The number of colonies containing ≥50 cells was counted under a microscope. Experiments were performed three times.

Cell migration and invasion assays

Briefly, 1 × 10^5 cells were seeded on a fibronectin-coated polycarbonate membrane insert in a transwell apparatus (Corning Inc., Corning, NY). After the cells were incubated for 12 h, Giemsa-stained cells adhering to the lower surface were counted under a microscope in five predetermined fields (100×). For the cell invasion assay, the procedure was similar to the cell migration assay, except that the transwell membranes were pre-coated with 24 mg/ml Matrigel (Corning Inc., Corning, NY). Experiments were performed three times.

Statistical analysis

All data were analyzed for statistical significance using SPSS 13.0 software and GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA). The Chi-square test was applied to the examination of correlation between miR-326 expression and clinicopathological characteristics. Survival curves were plotted using the Kaplan–Meier method and the log-rank test. The significance of survival variables was analyzed using the Cox multivariate proportional hazards model. The relationship between FSCN1 and miR-326 expression was explored by Pearson test. Two-tailed Student’s t test was used for comparisons of two independent groups. One-way ANOVA was used to determine the differences between groups or all in vitro analyses. A p value of less than 0.05 was considered statistically significant.

Results

MiR-326 expression is decreased in gastric cancer tissues and cell lines

We measured the expression levels of miR-326 in gastric cancer tissues and cell lines. Real time-PCR analysis indicated that the miR-326 expression level was significantly decreased in gastric cancer tissues compared with paired adjacent normal gastric tissues (Figure 1A, p < 0.001). Meanwhile, the expression of miR-326 was also decreased in gastric cancer cell lines (NCI-N87, MKN-28, AGS, MKN-45) compared with normal gastric epithelial cell line (GES-1) (Figure 1B, p < .05 for MKN-45, other p < .001). According to this expression pattern, we therefore chose AGS and MKN-28 cells for the following gain-of-function and loss-of-function studies, respectively (Figure 1B).

Decreased expression of miR-326 is associated with malignant status in gastric cancer patients

We next analyzed the association between the expression of miR-326 and clinicopathological characteristics of gastric cancer patients. Gastric cancer tissue samples were classified into the low expression group (n = 68) and the high expression group (n = 68) according to the median expression level of all gastric cancer samples (median expression value 4.31). This classification was based on published study.

Figure 1. miR-326 expression is decreased in gastric cancer tissues and cell lines, and associates with overall survival in gastric cancer patients. (A) Expression of miR-326 is decreased in gastric cancer tissues compared with normal gastric tissues (**, p < 0.001). (B) miR-326 expression is elevated in gastric cancer cell lines (NCI-N87, MKN-28, AGS, MKN-45) compared with normal gastric epithelial cell line (GES-1) (*, p < 0.05; **, p < 0.001). (C) Low expression of miR-326 predicts a poor prognosis in gastric cancer patients. The association between gastric cancer patient survival and miR-326 expression was estimated using the Kaplan–Meier method and the log-rank test (p < 0.001). Each experiment was performed in triplicates.
miR-326 expression is associated with overall survival in gastric cancer patients

In gastric cancer patients with prognosis information, we found that the level of miR-326 expression was significantly associated with the overall survival of gastric cancer patients, as patients with lower levels of miR-326 expression had poorer survival than those with higher levels of miR-326 expression (p < 0.001, Figure 1C). Multivariate analysis showed that decreased miR-326 expression was a poor independent prognostic factor for gastric cancer patients (p < 0.022, Table 2).

miR-326 serves as a tumor suppressor regulating gastric cancer cells growth, migration and invasion

To study the biological functions of miR-326 in gastric cell lines, we induced up-regulation of miR-326 expression in AGS by miR-326 mimics and down-regulation of miR-326 expression induced by miR-326 inhibitor in MKN-28, and these efficiencies were confirmed by qRT-qPCR (Figure S1A). The growth curves and colony formation assay showed that miR-326 mimics significantly inhibited AGS cell proliferation in comparison to mimics-NC, and miR-326 inhibitor significantly promoted MKN-28 cell proliferation in comparison to inhibitor-NC (all p < 0.05, Figure 2A–B). In cell migration and invasion assays, up-regulation of miR-326 expression significantly reduced the migration and invasion of AGS cells, which was contrary to the phenotype of migration and invasion induced by down-regulation of miR-326 expression (all p < 0.001, Figure 2C–D).

miR-326 down-regulates FSCN1 expression by directly targeting its 3′-UTR

To address the molecular mechanism by which miR-326 suppressed gastric cancer cell growth and metastasis, we identified FSCN1 as a potential target of miR-326 using microRNA target databases (Figure 3A). Moreover, we measured miR-326 and FSCN1 expressions in the same gastric cancer specimens, and found that a significant inverse correlation between miR-326 and FSCN1 (Spearman’s correlation, r = −0.542; p = 0.002, Figure 3C). Furthermore, we constructed luciferase reporter vectors that contained wild-type or mutant miR-326 target sequences of the FSCN1 3′-UTR, and performed luciferase reporter assay to detect whether FSCN1 was a direct target of miR-326. Then, we observed that miR-326 mimics could down-regulate the luciferase activity of the pmiR-FSCN1-wt (Figure 3B, p < 0.001). Conversely, miR-326 inhibitor could up-regulate the luciferase activity of the pmiR-FSCN1-wt (Figure 3B, p < 0.001). These data indicated that miR-326 can bind to the 3′-UTR of FSCN1. Furthermore, we found that increased expression of miR-326 could suppress the mRNA and protein expression of FSCN1, and decreased expression of miR-326 could elevate the mRNA and protein expression of FSCN1 (Figure 3D–E). These data suggested that miR-326 negatively regulates FSCN1 expression by directly targeting its 3′-UTR.

FSCN1 is a functional target of miR-326 and involve in gastric cancer cell growth, migration and invasion

Because miR-326 served as a tumor suppressor regulating gastric cancer cells growth, migration and invasion and directly inhibits FSCN1 expression, we were interested in exploring whether miR-326 functions in cell growth, migration and invasion are via targeting to FSCN1. A previous study has proved that knockdown of FSCN1 effectively suppressed gastric cancer cell proliferation and metastasis both in vitro and in vivo (Fu et al., 2009). Based on qRT-PCR and Western blot analyses, FSCN1 mRNA and protein decreased significantly after 48 h in both AGS and MKN-28 cells transfected with si-FSCN1 (Figure S1B–C). To further verify whether miR-326 regulated cell proliferation, migration and invasion by targeting FSCN1, we co-transfected miR-326 mimics and si-FSCN1 into AGS, and miR-326 inhibitor and si-FSCN1 into MKN-28. We found that co-transfection of miR-326 mimics and si-FSCN1 did not profoundly inhibit AGS cell proliferation, migration and invasion in comparison to miR-326 mimics (Figure 2A–D). Meanwhile, co-transfection of miR-326 inhibitor and si-FSCN1 could rescue facilitation of miR-326 inhibitor in MKN-28 cell proliferation, migration and invasion (Figure 2A–D). Thus, FSCN1 was a functional target of miR-326 involved in gastric cancer cell growth, migration and invasion.

Discussion

miR-326, a 20 nt miRNA on chromosome 11, was originally identified as a neural specific miRNA in neurons
Figure 2. miR-326 serves as a tumor suppressor regulating gastric cancer cells growth, migration and invasion. Transfection with miR-326 mimics inhibited AGS cells proliferation (A–B), migration (C) and invasion (D), and transfection with miR-326 inhibitor promoted MKN-28 cells proliferation (A–B), migration (C) and invasion (D). Co-transfection of miR-326 mimics and si-FSCN1 did not profoundly inhibit AGS cell proliferation (A–B), migration (C) and invasion (D) in comparison to miR-326 mimics. Meanwhile, co-transfection of miR-326 inhibitor and si-FSCN1 could rescue facilitation of miR-326 inhibitor in MKN-28 cell proliferation (A–B), migration (C) and invasion (D). (*, p < 0.05; **, p < 0.001). All assays were independently repeated at least three times.
(Ferretti et al., 2008). Recently, more and more evidences show that miR-326 involves in human cancer development and progression. miR-326 overexpression was observed in a variety of human cancer tissues, such as colorectal cancer (Wu et al., 2015), glioma (Kefas et al., 2009; Wang et al., 2013; Zhou et al., 2013), breast cancer (Liang et al., 2010) and pancreatic ductal adenocarcinoma (Zhang et al., 2015). Wang et al. showed that the low expression of miR-326 in glioma was obviously correlated with advanced pathological grade and low Karnofsky performance score, and predicted poor prognosis for patients with glioma (Wang et al., 2013). In colorectal cancer patients, decreased expression of miR-326 was associated with tumor metastasis and recurrence, and was an unfavorable independent prognostic factor (Wu et al., 2015). In pancreatic ductal adenocarcinoma patients, Zhang et al. demonstrated that low levels of miR-326 was correlated with venous invasion, and high expression of miR-326 was associated with a better prognosis for patients with pancreatic ductal adenocarcinoma (Zhang et al., 2015). However, the clinical and prognostic significance of miR-326 in gastric cancer are still unknown.

In our study, we found that miR-326 expression was significantly decreased in gastric cancer tissues and cell lines compared with adjacent normal gastric tissues and normal gastric epithelial cell line. Moreover, low expression of miR-326 was significantly associated with clinical stage, tumor depth, lymph node metastasis and distant metastasis. In survival analysis, we found that the level of miR-326 expression was significantly associated with the overall survival of gastric cancer patients, as patients with lower levels of miR-326 expression had poorer survival than those with higher levels of miR-326 expression. These data suggested that miR-326 expression may serves as a tumor suppressor in gastric cancer, and decreased miR-326 expression may promote tumor proliferation, and accelerate tumor cell metastasis. However, the biological function of miR-326 in gastric cancer is not fully understood. In order to identify the function of miR-326 in gastric cancer, we performed gain-of-function and loss-of-function studies to test the effect of miR-326 on gastric cancer cell lines. Our results showed that miR-326 could suppress gastric cancer cell proliferation, migration and invasion, which verified its role as a tumor suppressor.
miR-326 has the potential to target hundreds of genes that harbor target sequence in their 3′-UTR complementary to the seed region of the miRNA (Bartel, 2004). Several targets of miR-451, such as NOB1 (Wu et al., 2015; Zhou et al., 2013), ABC1 (Liang et al., 2010; Ma et al., 2015), pyruvate kinase type M2 (PKM2) (Kefas et al., 2010), histone deacetylase-3 (HDAC3) (Kim et al., 2014), have been identified. To explore the molecular mechanism responsible for the proliferation and migration inhibition induced by miR-326 in gastric cancer cells, we identified FSCN1 as a potential target of miR-326 by using microRNA target databases. In gastric cancer patients, overexpression of FSCN1 was correlated with tumor differentiation, T classification, N classification, clinical stage and prognosis (Kim et al., 2012; Tsai et al., 2007). Interestingly, we found that low expression of miR-326 was correlated with clinical stage, tumor depth, lymph node metastasis, distant metastasis and prognosis, and observed a significant inverse correlation between miR-326 and FSCN1 in clinical gastric cancer specimens. Furthermore, we found that miR-326 could bind to the 3′-UTR of FSCN1 by luciferase reporter assay, and suppressed mRNA and protein expression of FSCN1. These data suggested that miR-326 negatively regulates FSCN1 expression by directly targeting its 3′-UTR.

FSCN1 acted as a key role in tumor cell growth and motility. Fu et al. showed that knockdown of FSCN1 expression inhibited the growth and metastasis of gastric cancer cells in vitro and in vivo (Fu et al., 2009). Feng et al. present evidence that FSCN1 was a functional target of miR-145 to regulate tumor growth and metastasis in colorectal cancer (Feng et al., 2014). Moreover, we have found that miR-326 served as a tumor suppressor regulating gastric cancer cells growth, migration and invasion, and directly inhibits FSCN1 expression. Thus, we supposed FSCN1 was a functional target of miR-326 in regulating gastric cancer cell growth and metastasis.

In conclusion, our study showed that miR-326 expression was decreased in gastric cancer tissues and cell lines, and low expression of miR-326 was associated to malignant status and poor prognosis in gastric cancer patients. miR-326 could regulate gastric cancer cell growth and mobility by directly targeting FSCN1. This newly identified miR-326/FSCN1 pathway provides new insight into the molecular mechanisms of gastric cancer progression, and may be a novel diagnostic marker and potential therapeutic target in gastric cancer.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Supplementary material available online
Supplementary Figure S1