Am J Transl Res 2017;9(8):3654-3664
www.ajtr.org /ISSN:1943-8141/AJTR0051539

Original Article

MIR-519d suppresses the gastric cancer epithelial-mesenchymal transition via Twist1 and inhibits Wnt/β-catenin signaling pathway

Hailing Yue, Bo Tang, Yongliang Zhao, Yanyang Niu, Peng Yin, Weijun Yang, Zhigang Zhang, Peiwu Yu

Department of General Surgery and Center of Minimal Invasive Gastrointestinal Surgery, Southwest Hospital, Third Military Medical University, Chongqing 400038, China

Received November 22, 2016; Accepted March 6, 2017; Epub August 15, 2017; Published August 30, 2017

Abstract: MicroRNAs (miRNAs) deregulation is frequent in human gastric cancer (GC). MiR-519d has been reported to function as tumor suppressor microRNA in some tumors. However, the role of miR-519d in GC progression remains unclear. In the study, we demonstrated that the expression of miR-519d was down-regulated in gastric cancer tissues and cell lines, and lower miR-519d expression was associated with distant metastasis, lymph node metastasis and clinical stage for patients with GC. Univariate and multivariate Cox analysis showed that lower miR-519d expression was positively associated with shorter disease-free survival (DFS) and the over survival (OS) time for GC patients and was an independent predictor. Kaplan-Meier curve and log-rank test also demonstrated that lower miR-519d had a poor shorter DFS and OS for GC patients. Function analysis showed that the inhibition of miR-519d expression was able to promote the cell proliferation, migration and invasion and over-expression of miR-519d in GC cells had inhibited effects. Moreover, we demonstrated that over-expression of miR-519d significantly inhibited the process of epithelial mesenchymal transition (EMT) in GC cells and miR-519d can directly target at 3'-untranslation region of Twist1 and regulate its expression. We also demonstrated that miR-519d could suppress the Wnt/β-catenin signaling pathway in GC cells. In vivo, we showed that miR-519d inhibited the tumor growth. Thus, our results suggested that miR-519d functioned as a tumor suppressor in GC and could be a promising therapeutic target for GC.

Keywords: Gastric cancer, miR-519d, Twist1, epithelial mesenchymal transition, cell invasion

Introduction

Gastric cancer (GC) is the fourth most prevalent cancer and the second most frequent cause of cancer related death in the world [1]. GC is an asymptomatic disease at early stages and is therefore often detected at late time. The majority of GC deaths are caused by cancer cell invasion and metastasis. the 5-year survival rates was only 20-30% [2]. The underlying mechanisms of tumor invasion and migration in GC progression remain unclear, thus, it is urgent to identify novel biomarkers to predict GC prognosis and investigate novel therapeutic strategies for patients.

MicroRNAs (miRs) are ~22-nt, single-stranded, non-coding RNAs that serve as key roles in regulating carcinogenic pathways [3]. MicroRNAs could bind primarily to 3'-untranslated regions (3'-UTRs) of target gene mRNAs, which leads to translational repression or mRNA cleavage [4]. Studies had identified that miRNAs functioned as tumor suppressors or oncogenes to participate in cell activities including development, differentiation, proliferation, apoptosis, metabolism and immunity [5, 6].

MiR-519d, a miRNA within the chromosome 19 miRNA cluster, was down-regulated in some human tumors and functioned as a tumor suppressors. MiR-519d was down-regulated in human HCC and could suppress HCC cell line QGY-7703 growth [7]. MiR-519d suppressed cell proliferation and sensitized ovarian cancer cells to cisplatin-induced cell death by targeting the XIAP transcript [8]. MiR-519d also functioned as a tumor suppressor in breast cancer by suppressing STAT3 expression [9], however, its biological function in GC is unclear so for.

In the study, we demonstrated that the expression of miR-519d was down-regulated in gastric cancer and cell lines, and lower miR-519d expression was associated with short DFS and OS time in patients with GC. The inhibition of miR-519d expression was able to promote the cell proliferation, migration, invasion and cell epithelial mesenchymal transition process. In vivo, we also showed that miR-519d inhibited tumor growth. Furthermore, we showed that miR-519d could directly target at 3'-untranslation region of Twist1 and regulate its expression. Thus, we demonstrated that miR-519d functioned as a tumor suppressor in GC at least partly through inhibiting epithelial mesenchymal transition process by targeting Twist1. Thus, our findings indicated that miR-519d could act as a promising therapeutic target for GC treatment.

Material and methods

Human tissues samples

The 112 cases human GC tissues and their corresponding non-tumorous gastric samples were obtained from surgical resection at General Surgery Center, the Southwest Hospital, the Third Military Medical University. The tissues were verified as gastric cancer by two trained pathologist. Collected tissue samples were immediately frozen in liquid nitrogen and subsequently stored at -80°C freezer for further analysis. None of the patients had received radiotherapy or chemotherapy before operation. Written informed consent had been obtained from all the patients and the study was approved by the Ethics Committee of the Southwest Hospital, the Third Military Medical University.

Cell lines culture

The four human GC cell lines (SGC-7901, BGC-823, SGC-7901 and HGC-27) and a human gastric epithelial immortalized GES-1 cell line were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All of cells were cultured in RPMI 1640 medium or Dulbecco's modified Eagle medium and were supplemented with 10% foetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified incubator at 37°C with 5% CO_2.

Cell transfection

The hsa-miR-519d mimic, miR-519d plasmid, antago-miR-519d and negative control (NC) oligos were purchased from Ribobio (Guangzhou, China). Cells were transfected by has-miR-519d mimic, miR-519d plasmid, antago-miR-519d and negative control (NC) oligos using Lipofectamine 3000 reagent (Invitrogen) at a final concentration of 100 nM according to the manufacturer's instructions.

RNA extraction, reverse transcription and RT-PCR

The Total RNA derived from cells or tissues were extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription of RNAs was performed with M-MLV reverse transcriptase (Fermentas, Vilnius, Lithuania). Expression of Twist1 and miR-519d were assessed by qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China). QRT-PCR was performed in an ABI7500 sequence detector (Applied Biosystems, Foster City, CA, USA). GAPDH or U6 small nuclear RNA were used as the control for detection of Twist1 mRNA or miR-519d, respectively. The primers are miR-519d-F, 5'-ACA CTC CAG CTG GGC AAA GTG CCT CCC T-3'. GAPDH-F, 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3' and GAPDH-R, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'. Twist1-F, 5'-GTCCGCAGTCTTACGAGGAG-3', and Twist1-R, 5'-GCTTGAGGGTCTGAATCTTGCT-3'. U6-F, GCTTCGGCAGCACATATACTAAAAT.

Cell proliferation assays

Cell proliferation ability was detected using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Co. Ltd., Japan). Briefly, 5000 cells/well were seeded in 96-well plates. Cells after transfection were evaluated at 1, 2, 3, 4 and 5 days, the 10 μl of the CCK-8 solution was added to each well, respectively, then incubated for another 2 hours at 37°C. After incubation, the absorbance was measured at 450 nm using a microplate reader.

Cell migration and invasion assays

Cell migration and invasion assays were performed using transwell chambers (8-μm pore size membranes; Corning, Cambridge, MA, USA) as previously described [10]. For the

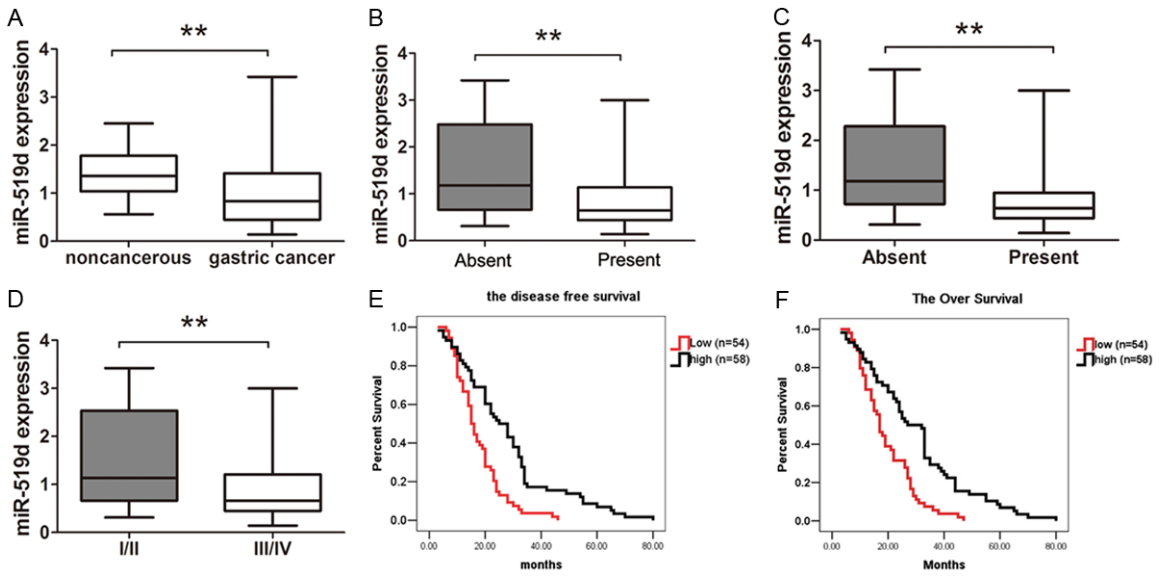

Figure 1. MiR-519d was down-regulated in gastric cancer tissue samples and cell lines. A. QRT-PCR analysis of miR-519d expression in human GC tissue samples and their matched normal GC tissues from 112 GC patients. B. Correlation between miR-519d expression and distant metastasis of GC. C. Correlation between miR-519d expression and lymph node metastasis of GC. D. Correlation between miR-519d expression and TNM stage of GC. E, F. Kaplan-Meier survival curve and log-rank test were performed to analyze the correlation between miR-519d expression and DFS or OS. **: P<0.05.

migration or invasion assays, the upper surface of the insert membrane was first coated without or with matrigel (BD Biosciences, San Jose, CA, USA), 1×10^5 MKN45 or SGC-7901 cells were seeded in the upper chamber supplemented serum-free medium and fetal bovine serum (FBS) (10%) was added to the lower chamber. After 24 h incubation, the cells remaining on the upper surface of the chamber were gently removed, whereas the cells adhering to the lower surface were fixed with methanol, stained with crystal violet and counted under a microscope. The cells were counted under a microscope in five randomly selected fields. All experiments were performed in triplicates.

Western blotting analysis

Cells were washed with ice-cold PBS and lysed on ice in Radio-Immunoprecipitation Assay (RIPA) buffer (Sigma-Aldrich, St. Louis, MO, USA) with Protease Inhibitor Cocktail (Sigma). Equal amounts of proteins (40 μg) were loaded into the wells of the SDS-PAGE gel and then transferred onto polyvinylidene fluoride membranes (Millipore, USA). The membranes were blocked with 5% non-fat milk in TBST for 1.5 hours. After blocking, the membranes were incubated with the respective primary antibodies overnight at 4°C. The primary antibodies used were as follows: anti-β-Actin antibody (1:1000; Santa Cruz Biotechnology, USA), or rabbit anti-Twist1 (Abcam, USA), anti-E-cadherin, vimentin and N-cadherin (1:1000, Santa Cruz Biotechnology, USA) anti-β-catenin (1:1000, CST, USA), anti-p-GSK-3β (Ser 9), anti-GSK-3β, anti-NKD1 also were purchased from Santa Cruz Biotechnology (1:1000, USA). After washing, membranes were incubated in PBS with 0.1% Tween with antimouse or anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) for 2 hours. Blots were detected using enhanced chemiluminescence substrate kit (GE Healthcare, Piscataway, NJ, USA).

Construction of reporter plasmid and luciferase activity assay

The wild-type 3'-UTR of human Twist1 mRNA containing miR-519d binding site was amplified by PCR and inserted into the SpeI/HindIII sites of pMIR-REPORT™ luciferase reporter plasmid to generate pMIR-Twist1-wt plasmid. The complementary sequence for miR-519d seed sequence in Twist1 3'-UTR was mutated and named as pMIR-Twist1-mut plasmid. The cells

Table 1. Correlation between miR-519d expression and clinicopathological features in 112 cases of GC patients

Variable	Patients number	MiR-519d expression levels		*p*-value
		Lower	Higher	
Age				0.073
≤55	81	43	38	
>55	31	11	20	
Gender				0.872
Male	78	38	40	
Female	34	16	18	
Location				0.507
Distal	34	16	18	
Middle	46	24	22	
Proximal	32	14	18	
Distant metastasis				0.001**
Absent	82	32	50	
Present	30	22	8	
Lymph node metastasis				0.001**
Absent	42	10	32	
Present	72	46	26	
Depth of invasion				0.168
T1, T2	58	28	30	
T3, T4	54	26	28	
Histological grade				0.543
High differentiation	29	10	19	
Middle differentiation	42	20	22	
Low differentiation	41	24	17	
TNM stage				0.001**
I/II	42	10	32	
III/IV	70	44	26	

**indicates that statistical significance of *P* value is less than 0.05.

were co-transfected with miR-519d mimic and pMIR-Twist1-MUT or pMIR-Twist1-WT by Lipofectamine 3000 reagent (Invitrogen). 48 h after transfection, cells were lysed and assayed for luciferase activity using a dual luciferase reporter assay (Promega).

Xenograft tumor formation assay

2×10^6 MKN45 cells transfected with pre-miR-519d plasmid or negative control were injected subcutaneous into the left axillae of 3-4 weeks old female nude mice. Tumor volume and the whole body weight were evaluated every 7 days after tumor inoculation. At the end of the experiment, mice were sacrificed and tumor tissues were performed for further study. All animal experiments were performed in animal laboratory center of Southwest Hospital, Third Military Medical University and in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication number 85-23, revised 1996).

Statistical analysis

Statistical analysis was used by SPSS 18.0 (IBM, Armonk, NY, USA). All experiments were performed in triplicate, and data are expressed as mean ± S.D. Univariate and multivariate analysis was used to analyze the risk factors of prognosis in patients. Kaplan-Meier curve and log-rank test was used to determined the association between miR-519d expression and DFS and OS for GC patients. Statistical analysis was conducted using the independent samples t-test or one-way anova. *P* values of less than 0.05 were considered statistically significant.

Result

MiR-519d is down-regulated in GC tissues and cells

To investigate the roles of miR-519d in gastric cancer, firstly, we detected the expression of miR-519d in gastric cancer tissues and adjacent normal tissues in 112 cases GC patients using quantitative polymerase chain reaction (qRT-PCR). Our results from qRT-PCR analysis demonstrated that the miR-519d expression levels in gastric cancer tissues were down-regulated compared with their compared normal tissues, As shown in **Figure 1A**, the average level of miR-519d expression was significantly reduced in tumor tissues compared with their adjacent normal tissues. Lower miR-519d expression was negative correlation with distant metastasis, lymph node metastasis and TNM stage (**Figure 1B-D**; **Table 1**). These results confirmed that that miR-519d was significantly down-regulated in gastric cancer. Furthermore, univariate and multivariate Cox analysis showed that miR-519d expression was an independent prognostic indicator for DFS and OS in patients with GC (**Tables 2** and **3**). Moreover, Kaplan-Meier survival curve and log-rank test also demonstrated that patients with lower

Table 2. Univariate and multivariate Cox proportional hazards analysis of miR-519d expression and disease free survival (DFS) for patients in gastric cancer

Factor	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age	0.837 (0.572-1.224)	0.359		
Gender	1.159 (0.785-1.710)	0.458		
Location	0.955 (0.631-1.455)	0.512		
Distant metastasis	2.241 (1.515-3.141)	P<0.001**	1.882 (1.223-2.589)	0.008**
Lymph node metastasis	2.012 (1.386-2.972)	P<0.001**	1.689 (1.056-2.493)	0.019**
Depth of invasion	1.001 (0.783-1.551)	0.498		
Histological grade	1.101 (0.753-1.624)	0.608		
TNM stage	2.073 (1.416-3.035)	P<0.001**	1.713 (1.129-2.487)	0.012**
miR-519d	2.578 (1.730-3.840)	P<0.001**	2.041 (1.415-3.041)	P<0.001**

**indicates that statistical significance of *P* value is less than 0.05.

Table 3. Univariate and multivariate Cox proportional hazards analysis of miR-519d expression and overall survival (OS) for patients in gastric cancer

Factor	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age	0.766 (0.445-1.098)	0.423		
Gender	1.044 (0.665-1.554)	0.489		
Location	0.889 (0.566-1.245)	0.612		
Distant metastasis	2.121 (1.386-3.039)	P<0.001**	1.782 (1.144-2.443)	0.009**
Lymph node metastasis	1.913 (1.254-2.899)	P<0.001**	1.627 (0.912-2.344)	0.027**
Depth of invasion	0.992 (0.644-1.338)	0.501		
Histological grade	1.001 (0.695-1.506)	0.499		
TNM stage	1.889 (1.166-2.725)	0.007**	1.678 (1.036-2.324)	0.023**
miR-519d	2.374 (1.655-3.216)	P<0.001**	1.988 (1.229-2.901)	0.003**

**indicates that statistical significance of *P* value is less than 0.05.

miR-519d expression had poorer DFS and OS than those with higher miR-519d expression in GC patients (**Figure 1E**, **1F**, log-rank =19.39, P<0.05, and log-rank =18.33, P<0.05, respectively). Thus, these results indicated that miR-519 might be an independent tumor biomarker for evaluating prognosis for GC patient.

MiR-519d inhibited cell proliferation, migration and invasion in gastric cancer

To further investigate the biological role of miR-519d expression in gastric cancer, we detected the expression of miR-519d in four gastric cancer cell lines by qRT-PCR. The data showed that miR-519 expression was much lower in human gastric cancer cells including MKN45, BGC-823, HGC-27 and SGC7901, compared with GES-1 normal cells, the results showed that miR-519d expression significantly decreased in four gastric cancer cell lines (**Figure 2A**). MKN45 and SGC-7901 cells were transfected using miR-519d plasmid or antagomiR-519d, the results of transfection effect indicated that miR-519d was increased significantly in MKN45 cells and was significantly inhibited in SGC-7901 cells by qRT-PCR analysis (**Figure 2B**, **2C**). CCK8 cell proliferation assay displayed that miR-519d inhibited cell proliferation in MKN45 cells, but cell proliferation ability was enhanced by transfecting antagomiR-519d into SGC-7901 cells (**Figure 2D**, **2E**). It was also shown that cell migration abilities were inhibited by transfecting miR-519d into MKN45 cell lines compared with their controls (**Figure 3A**, **3B**), however, cell migration abilities were enhanced by transfecting antagomiR-519d into SGC-7901 cell lines (**Figure 3C**, **3D**). Moreover, the cell invasion abilities were inhibited by transfecting miR-519d into MKN45 cell lines compared with their controls (**Figure 3E**, **3F**), but were enhanced by transfecting antagomiR-

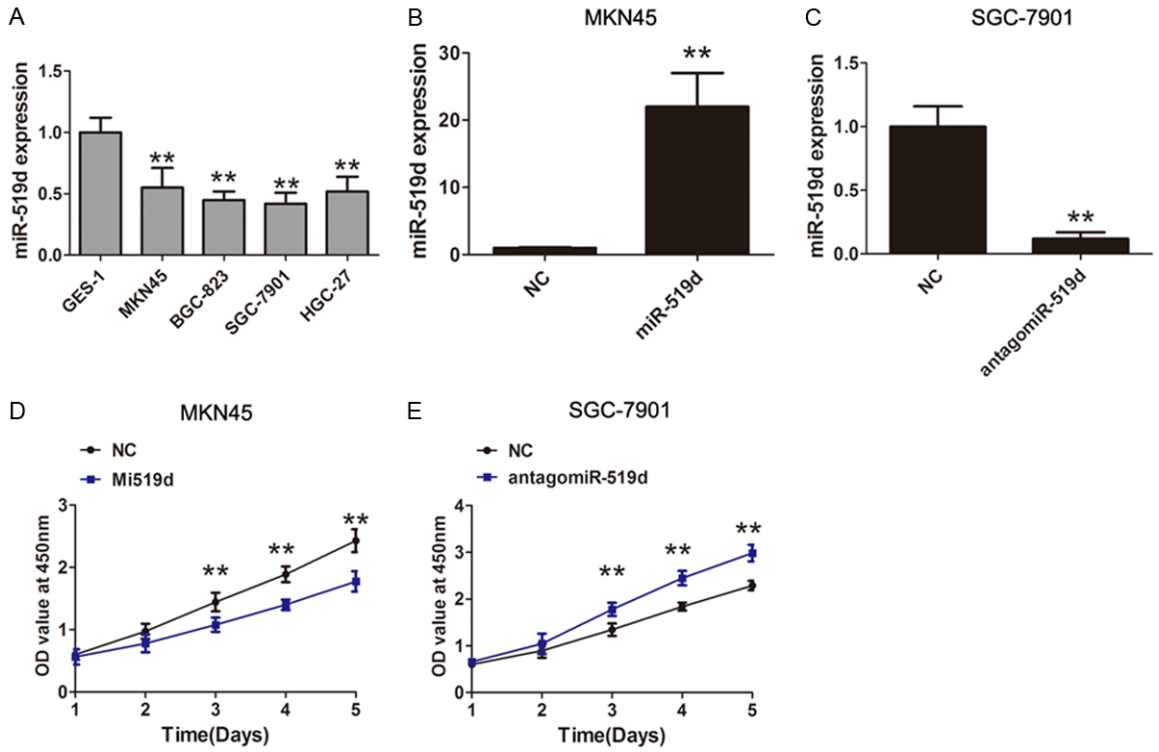

Figure 2. MiR-519d inhibited the cell proliferation in GC cells. A. QRT-PCR analysis of miR-519d expression in four human GC cell lines MKN45, HGC27, BGC-823, and SGC-7901 and one normal cell line GES-1. B. QRT-PCR analysis of miR-519d expression by transfecting miR-519d plasmid into MKN45 cells. C. QRT-PCR analysis of miR-519d expression by transfecting antagomiR-519d into SGC-7901 cells. D. CCK8 cell proliferation assays were used to analyze the cell proliferation ability by transfecting miR-519d plasmid or negative control into MKN45 cells. E. CCK8 cell proliferation assays were used to analyze the cell proliferation ability by transfecting antagomiR-519d or negative control into SGC-7901 cells. Data are means ± SD of three independent experiments. **: P<0.05.

519d into SGC-7901 cell lines (**Figure 3G**, **3H**). Thus, the above data indicated that miR-519d inhibited cell proliferation, migration and invasion in GC.

MiR-519d suppressed gastric cancer cell epithelial-mesenchymal transition

The above findings suggested miR-519d may participate in GC invasion progression. Hence, we speculated that miR-519d might be associated with the cells epithelial-mesenchymal transition in GC. To investigate the potential effect of miR-519d on GC cells EMT, the miR-519d plasmid or antagomiR-519d was transfected into MKN45 and SGC-7901 cells, respectively. The western-blot assays results showed that over-expression of miR-519d inhibited the expression levels of transcription factor Twist1, EMT related marker N-cadherin, Vimentin, but upregulating the E-cadherin expression level (**Figure 4A**). However, down-regulation of miR-519d enhanced the expression levels of transcription factor Twist1 and EMT related marker N-cadherin and Vimentin, but down-regulating the E-cadherin expression level (**Figure 4B**). Therefore, these results showed that miR-519d suppressed gastric cancer cell epithelial-mesenchymal transition.

Twist1 was a target gene of miR-519d in gastric cancer cells

We then investigated the mechanisms by which miR-519d inhibited gastric cancer invasion. Bioinformatics analysis by online predicted software miRanda (www.mircrorna.org) showed that Twist1 was potential target of miR-519d (**Figure 4C**). It is generally believed that Twist1 was critical regulators of EMT modulation [11]. To confirm whether miR-519d suppressed EMT by targeting Twist1, we initially constructed two types of plasmids containing the luciferase reporting gene wild-type (pmiR-Twist1-WT) or mutant Twist1 (pmiR-Twist1-MUT) 3'UTR and co-transfected a miR-519d mimic into MKN45

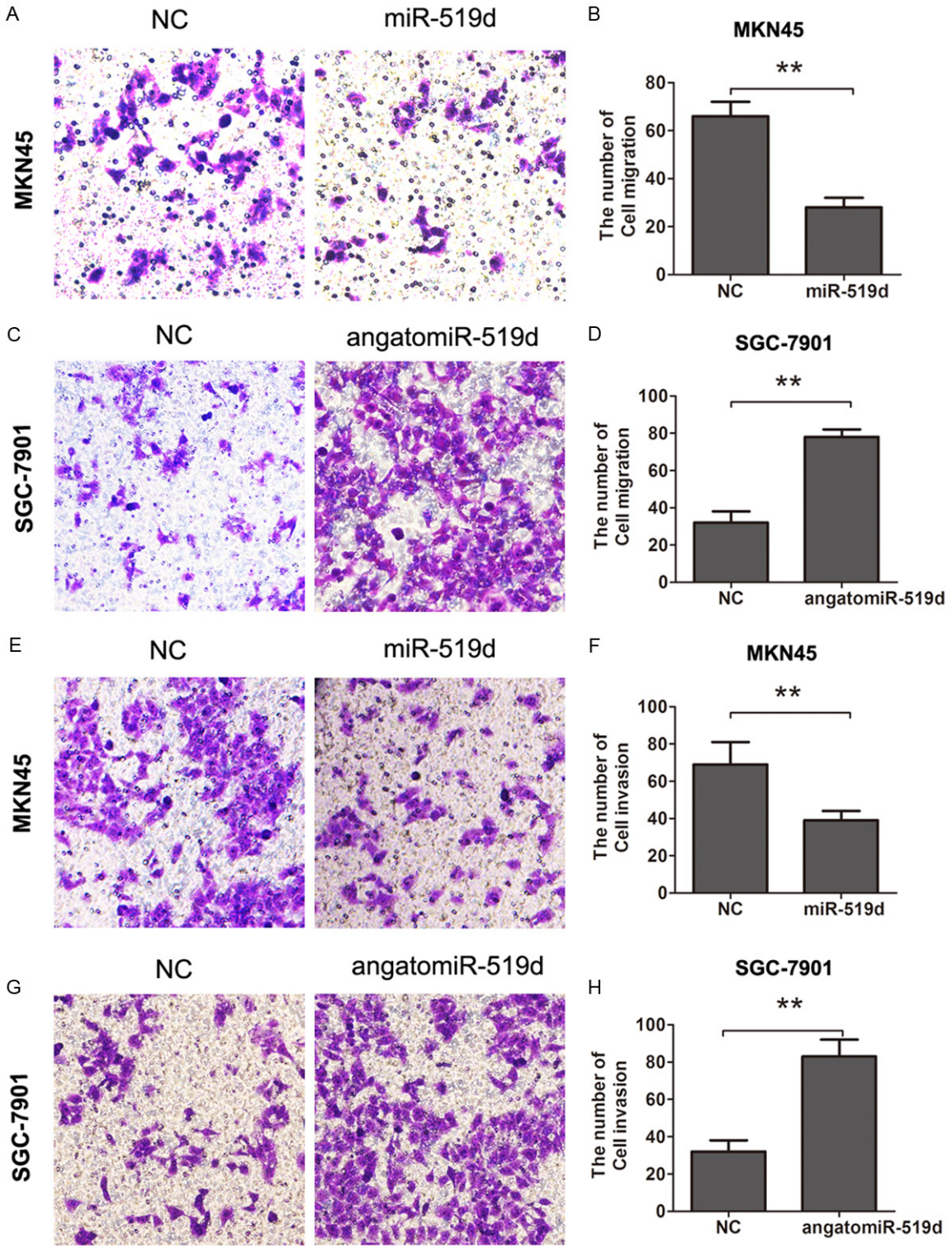

Figure 3. MiR-519d inhibited the cell migration and invasion in GC cells. A, B. Transwell cell migration assays and cell number analysis were used to analyze the cell migration ability by transfecting miR-519d plasmid or negative control into MKN45 cells. C, D. Transwell cell migration assays and cell number analysis were used to analyze the cell migration ability by transfecting antagomiR-519d or negative control into SGC-7901 cells. E, F. Transwell cell invasion assays and cell number analysis were used to analyze the cell invasion ability by transfecting miR-519d plasmid or negative control into MKN45 cells. G, H. Transwell cell invasion assays and cell number analysis were used to analyze the cell invasion ability by transfecting antagomiR-519d or negative control into SGC-7901 cells. Data are means ± SD of three independent experiments. **: $P<0.05$.

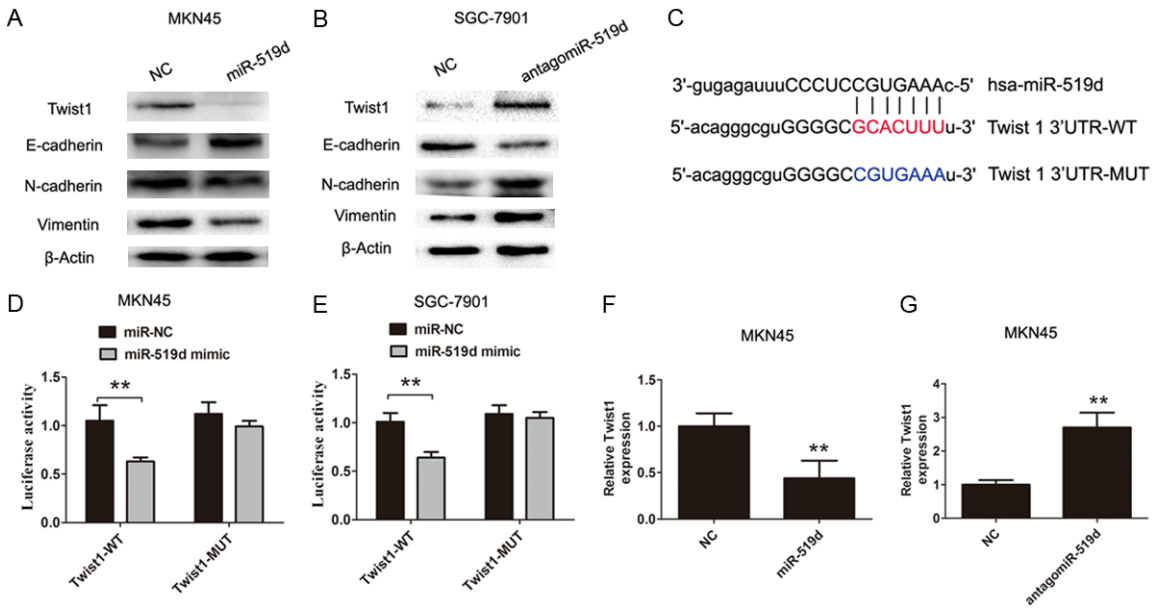

Figure 4. MiR-519d inhibited the cell EMT by targeting Twist1 in GC cells. A. Western-blot analysis was performed to analyze the protein expression of Twist1, E-cadherin, N-cadherin and Vimentin by transfecting miR-519d plasmid or negative control into MKN45 cells, the β-actin was used the internal control. B. western-blotting analysis was performed to analyze the protein expression of Twist1, E-cadherin, N-cadherin and Vimentin by transfecting antagomiR-519d or negative control into SGC-7901 cells, the β-actin was used the internal control. C. Bioinformatics analysis of between miR-519d and Twist1 recognition sequences by miRanda (http://mircorna.org) revealed the miR-519d had a binding site with Twist1. D, E. miR-519d mimic decreased the luciferase activities in Twist1-WT + miR-519d mimic group, but did not affect luciferase activity by transfecting miR-519d mimic + Twist1-MUT group into MKN45 or SGC-7901 cells. F, G. The Twist1 expression was determined by qRT-PCR analysis by transfecing miR-519d plasmid or antagomiR-519d into MKN45 cells. Data are means ± SD of three independent experiments. **: P<0.05.

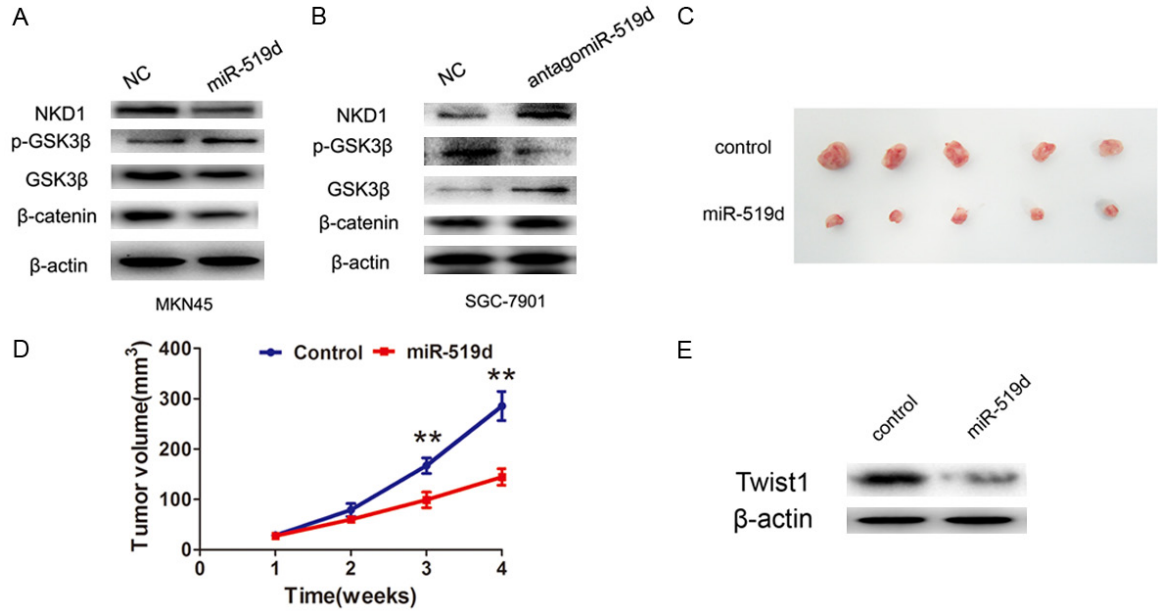

Figure 5. MiR-519d suppresses the Wnt/β-catenin signaling pathway in GC cells. A. Western-blot analysis was performed to analyze the protein expression of β-catenin, p-GSK-3β, GSK-3β, NKD1 by transfecting miR-519d plasmid or negative control into MKN45 cells, the β-actin was used the internal control. B. Western-blot analysis was performed to analyze the protein expression of β-catenin, p-GSK-3β, GSK-3β, NKD1 by transfecting antagomiR-519d or negative control into SGC-7901 cells, the β-actin was used the internal control. C. The tumor was showed in pre-miR-519d plasmid or negative control group. D. The tumor size was showed in pre-miR-519d plasmid or negative control group. E. Western-blot analysis was performed to analyze the protein expression of Twist1 in pre-miR-519d plasmid or negative control group. Data are means ± SD of three independent experiments. **: P<0.05.

and SGC-7901 cells. The results showed that relative luciferase activity was reduced by 39% and 41% in pmiR-Twist1-WT-3'UTR in MKN45 or SGC-7901, respectively, but not in those with respective mutant 3'UTR (**Figure 4D**, **4E**). Furthermore, we found that Twist1 mRNA levels was decreased by transfecting miR-519d plasmid into MKN45 cells, but was significantly increased by transfecting antagomiR-519d into SGC-7901 cells (**Figure 4F**, **4G**). In summary, these data showed that miR-519d inhibited the GC cell EMT process by targeting Twist1.

MiR-519d suppresses the Wnt/β-catenin signaling pathway in GC cells

Wnt/β-catenin signaling pathway is considered as a key player in the regulation of tissue homeostasis, organ size, tumorigenesis and E-cadherin/β-catenin complex and plays an important role in maintaining epithelial integrity and disrupting this complex affect not only the adhesive repertoire of a cell, but also the Wnt-signaling pathway. Aberrant expression of the complex was associated with a wide variety of human malignancies and disorders of fibrosis resulting from epithelial-mesenchymal transition [12]. In the study, the western blotting analysis revealed that over-expression of miR-519d markedly reduced the expression levels of NKD1, GSK3β and β-catenin and up-regulated the p-GSK3β (**Figure 5A**). In contrast, miR-519d inhibition increased the expression levels of NKD1, GSK3β and β-catenin and down-regulated the p-GSK3β (**Figure 5B**). Collectively, our results also revealed that miR-519d suppressed Wnt/β-catenin signaling and subsequent inhibited the cell epithelial-mesenchymal transition.

MiR-519d suppresses the tumor growth in vivo

Furthermore, we stably transfected MKN-45 cells with pre-miR-519d or negative control and established xenograft tumor formation for GC. The results showed that overexpressing miR-519d had a significantly reduced tumor volume, compared to the control group, and slower median rate of growth (**Figure 5C**, **5D**). Moreover, we detected the expression of Twist1 in miR-519d overexpression group or negative group and the results showed that Twist1 was significantly down-regulated in miR-519d group (**Figure 5E**). Thus, these results showed that MiR-519d suppresses the tumor growth in vivo.

Discussion

Recent studies indicate that miRNAs could suppress protein translation at the posttranscriptional level and function as tumor suppressors in human cancers. Such as, microRNA-126 inhibited cell proliferation in gastric cancer by targeting LAT-1 [13]. The microRNA-217 functioned as a potential tumor suppressor in gastric cancer by targeting GPC5 [14]. MicroRNA-449a inhibited proliferation and induced apoptosis by directly repressing E2F3 in gastric cancer [15]. MiR-124 inhibited GC cell growth, migration and invasion, and induced cell cycle arrest by negatively regulated Notch1 signaling targeting JAG1 [16], and so on. In this study, down-regulation of miR-519d was observed in GC tissues specimens and cell lines. Multivariate Cox analysis showed that miR-519d expression was an independent prognostic indicator for DFS and OS in patients with GC. Moreover, lower miR-519d expression had poorer DFS and OS than those with higher miR-519d expression in GC patients. Our gain-and loss-of-function experiments demonstrated that up-regulation of miR-519d significantly inhibited the cell proliferation and reduced the number of migrated and invaded MKN-45 cells, and down-regulation of miR-519d promoted cell proliferation and increased the number of migrated and invaded SGC-7901 cells. In vivo, we also showed that miR-519d inhibited the tumor growth. Collectively, these results suggested a potential tumor suppressor of miR-519d in GC progression.

Furthermore, we demonstrated that miR-519d inhibited EMT process in gastric cancer cells by up-regulating E-cadherin expression and inhibited the expression levels of Twist1, N-cadherin and Vimentin. Previous study demonstrated that miR-519d-3p suppressed invasion and migration of trophoblast cells via targeting MMP-2 [17]. Resistin promoted chondrosarcoma metastasis and MMP-2 expression through activation of the AMPK/p38 signaling pathway and down-regulated miR-519d expression [18]. Another study also showed that microRNA-519d targeted MKi67 and suppressed cell growth in the hepatocellular carcinoma cell line [7]. In the study, we demonstrated that miR-519d inhibited the GC cell EMT process, but down-regulated miR-519d had the opposite effects. We then investigated the mechanisms

by which miR-519d inhibited gastric cancer EMT. The results from luciferase reporter assays showed that Twist1 was a target of miR-519d. Thus, our results revealed that miR-519d inhibited the GC cell invasion by downregulating the expression of Twist1.

Several signaling pathways including the Wnt/β-catenin, Notch, and PI3K/Akt pathways have been reported to be aberrantly activated in the development and progression of gastric cancer [16, 19, 20]. Our data revealed that overexpression of miR-519d markedly reduced the expression levels of NKD1, GSK3β and β-catenin and up-regulated the p-GSK3β. In contrast, inhibition of miR-519d increased the expression levels of NKD1, GSK3β and β-catenin and down-regulated the p-GSK3β. Thus, these results suggested that miR-519d inhibited the cell invasion by partly suppressing the Wnt/β-catenin signaling.

In summary, our results showed that miR-519d functioned as a tumor suppressor to inhibit cell invasion in GC by targeting Twist1 and finally inhibiting EMT process. Our findings indicated that the miR-519d could be a promising therapeutic target for GC treatment.

Acknowledgements

We thank the staff of Department of General Surgery and Center of Minimal Invasive Gastrointestinal Surgery, Southwest Hospital, Third Military Medical University, for their help.

Disclosure of conflict of interest

None.

Address correspondence to: Peiwu Yu, General Surgery Center, The Southwest Hospital, The Third Military Medical University, Gaotanyan Street 30, Shapingba District, Chongqing 400038, China. Tel: 86-23-68754161; Fax: 86-23-68754161; E-mail: yupeiwu112@126.com

References

[1] Hohenberger P and Gretschel S. Gastric cancer. Lancet 2003; 362: 305-315.
[2] Thiel A and Ristimaki A. Gastric cancer: basic aspects. Helicobacter 2012; 17 Suppl 1: 26-29.
[3] Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136: 215-233.
[4] Gramantieri L, Fornari F, Callegari E, Sabbioni S, Lanza G, Croce CM, Bolondi L and Negrini M. MicroRNA involvement in hepatocellular carcinoma. J Cell Mol Med 2008; 12: 2189-2204.
[5] Wang Y and Lee CG. MicroRNA and cancer--focus on apoptosis. J Cell Mol Med 2009; 13: 12-23.
[6] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.
[7] Hou YY, Cao WW, Li L, Li SP, Liu T, Wan HY, Liu M, Li X and Tang H. MicroRNA-519d targets MKi67 and suppresses cell growth in the hepatocellular carcinoma cell line QGY-7703. Cancer Lett 2011; 307: 182-190.
[8] Pang Y, Mao H, Shen L, Zhao Z, Liu R and Liu P. MiR-519d represses ovarian cancer cell proliferation and enhances cisplatin-mediated cytotoxicity in vitro by targeting XIAP. Onco Targets Ther 2014; 7: 587-597.
[9] Deng X, Zhao Y and Wang B. miR-519d-mediated downregulation of STAT3 suppresses breast cancer progression. Oncol Rep 2015; 34: 2188-2194.
[10] Li J, Wang Q, Wen R, Liang J, Zhong X, Yang W, Su D and Tang J. MiR-138 inhibits cell proliferation and reverses epithelial-mesenchymal transition in non-small cell lung cancer cells by targeting GIT1 and SEMA4C. J Cell Mol Med 2015; 19: 2793-2805.
[11] Zhao XL, Sun T, Che N, Sun D, Zhao N, Dong XY, Gu Q, Yao Z and Sun BC. Promotion of hepatocellular carcinoma metastasis through matrix metalloproteinase activation by epithelial-mesenchymal transition regulator Twist1. J Cell Mol Med 2011; 15: 691-700.
[12] Tian X, Liu Z, Niu B, Zhang J, Tan TK, Lee SR, Zhao Y, Harris DC and Zheng G. E-cadherin/beta-catenin complex and the epithelial barrier. J Biomed Biotechnol 2011; 2011: 567305.
[13] Wang J, Chen X, Su L, Li P, Cai Q, Liu B, Wu W and Zhu Z. MicroRNA-126 inhibits cell proliferation in gastric cancer by targeting LAT-1. Biomed Pharmacother 2015; 72: 66-73.
[14] Wang H, Dong X, Gu X, Qin R, Jia H and Gao J. The MicroRNA-217 functions as a potential tumor suppressor in gastric cancer by targeting GPC5. PLoS One 2015; 10: e0125474.
[15] Li X, Li H, Zhang R, Liu J and Liu J. MicroRNA-449a inhibits proliferation and induces apoptosis by directly repressing E2F3 in gastric cancer. Cell Physiol Biochem 2015; 35: 2033-2042.
[16] Jiang L, Lin T, Xu C, Hu S, Pan Y and Jin R. miR-124 interacts with the Notch1 signalling pathway and has therapeutic potential against gastric cancer. J Cell Mol Med 2016; 20: 313-322.
[17] Ding J, Huang F, Wu G, Han T, Xu F, Weng D, Wu C, Zhang X, Yao Y and Zhu X. MiR-519d-3p sup-

presses invasion and migration of trophoblast cells via targeting MMP-2. PLoS One 2015; 10: e0120321.

[18] Tsai CH, Tsai HC, Huang HN, Hung CH, Hsu CJ, Fong YC, Hsu HC, Huang YL and Tang CH. Resistin promotes tumor metastasis by down-regulation of miR-519d through the AMPK/p38 signaling pathway in human chondrosarcoma cells. Oncotarget 2015; 6: 258-270.

[19] Yu Y, Yan W, Liu X, Jia Y, Cao B, Yu Y, Lv Y, Brock MV, Herman JG, Licchesi J, Yang Y and Guo M. DACT2 is frequently methylated in human gastric cancer and methylation of DACT2 activated Wnt signaling. Am J Cancer Res 2014; 4: 710-724.

[20] Wu XY, Liu WT, Wu ZF, Chen C, Liu JY, Wu GN, Yao XQ, Liu FK and Li G. Identification of HRAS as cancer-promoting gene in gastric carcinoma cell aggressiveness. Am J Cancer Res 2016; 6: 1935-1948.