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MiR-377 suppresses cell proliferation and metastasis in gastric cancer via repressing the expression of VEGFA

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Abstract. – OBJECTIVE: In recent years, microRNAs have been identified to participate in tumor genesis and progression of different tumors including gastric cancer. However, the role of miR-377 played in gastric cancer (GC), and its mechanisms have not been demonstrated.

PATIENTS AND METHODS: We detected miR-377 expression level in 86 GC and adjacent normal tissue samples by quantitative reverse transcription PCR (qRT-PCR) as well as in GC cell lines. The relationship between miR-377 and clinical pathological features was analyzed. Using miR-377 mimics and inhibitors, we interfered with miR-377 level and employed several functional experiments to study the miR-377 effects on cell proliferation, migration, and invasion. Western blot assay and dual-luciferase assay were used to verify the target of miR-377.

RESULTS: miR-377 expressed significantly lower in GC tissues and cell lines compared to normal tissues and GES-1 cells. Overexpression of miR-377 inhibited cell growth, migration and invasion, while downregulation miR-377 obviously promoted cell growth and metastasis. Furthermore, vascular endothelial growth factor A (VEGFA) was confirmed as a direct target of miR-377 and reversed the influence of mir-377 over-expression.

CONCLUSIONS: miR-377 expressed lower in GC and suppressed cell proliferation, migration and invasion partly via repressing the VEGFA expression, which could provide a potential target for GC diagnosis and therapy.

Key Words:
miR-377, Gastric cancer, Proliferation, Metastasis, VEGFA.

Abbreviations

qRT-PCR = quantitative reverse transcription PCR; VEGFA = vascular endothelial growth factor A; GC = Gastric cancer; miRNA = MicroRNA; LncRNAs = Long noncoding RNAs; circRNAs = Circular RNAs; RPMI = Roswell Park Memorial Institute; FBS = fetal bovine serum.

Introduction

Gastric cancer (GC) is the fourth highest incidence cancer, and one of the top three tumors mortality, which greatly threaten the survival and life of mankind[1]. Although the current diagnostic techniques and treatment programs advanced rapidly, early diagnosis and prognosis prediction of GC still face a huge challenge[2,3]. As many mechanisms are involved in GC development and progression, it is very important to understand underlying biomarkers or factors to improve the understanding of GC.

In recent years, several non-coding RNAs including microRNAs, long noncoding RNAs (lncRNAs) and circular RNAs (circRNAs) have been identified as useful methods for biological diagnosis and therapy[4]. MicroRNA (miRNA) as long as 21-24 nucleotides, is a class of non-coding RNA molecules, which is widely present in eukaryotic cells that are highly conserved in evolution[5,6]. They have become one of the most popular bio-molecules being studied in recent years. They play as negative regulators of gene expression at post-transcriptional levels via binding to the 3'-UTR of target genes[7]. Several investigations[8-12] have revealed that miRNAs were related to the tumorigenesis and progression of several different kinds of cancers including GC. For example, miR-29c was overexpressed in GC and inhibited cell proliferation by regulator of chromosome/condensation 2 (RCC2); miR-423-5p regulated cell growth and invasion by suppressing TF1 expression in GC cells. In addition, miR-93 could function as an oncogene via downregulating programmed cell dea-

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th 4 (PDCD4) in gastric carcinoma. What's more, microRNA-524-5p enhanced the sensitivity of cisplatin of gastric cancer via targeting SOX9, which provided a target for GC chemotherapy.

MiR-377 was reported to participate in the development and progression of many tumors via different genes. For example, it directly targeted SP1 transcription factor and inhibited human glioblastoma proliferation and invasion. In hepatocellular carcinoma, miRNA-377 suppressed tumor progression by inhibiting T-Cell Lymphoma Invasion and metastasis (TIAM1) or Iroquois homeobox protein 3 (IRX3)[13,14]. In human clear cell renal cell carcinoma, miR-377 functioned as a tumor suppressor via targeting ETS1 (ETS Proto-Oncogene 1, Transcription Factor)[15]. In malignant melanoma, esophageal cancer, non-small-cell lung cancer, and oral squamous cell carcinoma, miR-377 could suppress tumor initiation and progression by targeting specific gene[16-20]. Also, miR-377 has been reported to predict poor prognosis of GC; however, the concrete mechanism of miR-377 in GC still remains to be elucidated[21]. In this study, we first surveyed the expression of miR-377 in 86 pairs of GC and adjacent normal tissue samples and analyzed the relationship between miR-377 and clinical pathological variables. Furthermore, we established the model of upregulating and downregulating miR-377 cell lines for several functional experiments to confirm that overexpressing miR-377 inhibited cell growth, invasion, and migration abilities of GC, though repressing vascular endothelial growth factor A (VEGFA). These findings might provide a novel target for biological treatment for GC.

Patients and Methods

GC Tissue Samples

A total of 86 GC tissue samples and adjacent normal samples were collected from Yancheng City NO.1 People's Hospital and The Second Affiliated Hospital of Nanjing Medical University. No patients have received any chemotherapy or radiotherapy before the surgical ectomy. The tissues were stored in liquid nitrogen at once after surgical resection before the next experiments. The clinical pathological features were collected according to the American Joint Committee on Cancer (AJCC) standards. All 86 patients have signed the written consent and the study was under the approval of the Ethics Committee of Yancheng City NO.1 People's Hospital collage.

GC Cell Lines and Culture

Five GC-derived cell lines MKN-45, BGC-823, MKN-7, MGC-803, SGC-7901 and human gastric mucosal epithelial cell line GES-1 were bought from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All the six cell lines were incubated with Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and cultured in humid atmosphere containing 5% CO_2 at 37°C.

MiR-377 Interference and VEGFA Up-regulation

MiR-377 mimics or inhibitors synthesized by GenePharma (Shanghai, China) were used to upregulate or downregulate miR-377 level in SGC-7901 or MKN-45 cells. Cells were seeded in six-well plates and cultured to a density of 60%; then, the miR-377 mimics, inhibitors, control, or negative controls were added into the plates together with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufactures instruction, respectively. After miR-377 was incubated for 24 h, the transfection efficiency of miR-377 was confirmed using qRT-PCR.

For VEGFA over-expression, cells were cultured for 24 h in six-well plate, and then incubated with pcDNA3.1-VEGFA (GenePharma, Shanghai, China) using Lipofectamine 2000 in FBS-free RPMI 1640. After incubation for 48 h in normal culture atmosphere, the level of VEGFA was measured.

RNA Isolation and Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA of GC tissue or cell samples were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reversed using a miRNA Reverse Kit (TaKaRa, Dalian, China). The miR-377 expression levels were detected using SYBR Premix kits (TaKaRa, Dalian, China) with ABI 7900 (ABI, Waltham, MA, USA) and U6 was used as internal control. The relative expression levels were measured using the $2^{-\Delta\Delta CT}$ method and each experiment was confirmed for three times.

Colony Formation Assay

For colony formation assay, a total of 300 cells were planted in each six-well plate after transfection, and maintained in normal medium for 10 days. Then, colony containing more than 50 cells was identified as a colony, and the numbers of colonies were calculated.

CCK8 Assay

CCK8 assay (Keygen Biotech, Nanjing, China) was used to study the cell proliferation ability. A total of 2000 cells after transfection were put into 96-well plate suspended in 100 μL RPMI-1640 medium. Then, they were maintained for 24, 48, 72, 96 h. For detection, 10 μL per well CCK8 reagent was mixed into each well and absorbance at 450 nm was recorded after 2 h incubation in dark. Each group was replicated for five times.

Wound-Healing Assay

Wound-healing assay was applied to study the cell migration ability. Cells after treatments were cultured in six-well plates to a density of 100%; then, three scratches were made using 200 μL tips on the surface of cells. Cells were cultured in FBS-free medium for 48 h after being washed three times using phosphat-buffered saline (PBS). The wound healing condition was measured using microscope at 0 h and 48 h, each scratch was measured for three times.

Transwell Assay

To detect the cell migration and invasion ability, transwell assay was recruited with using 8-μm inserts (Millipore, Billerica, MA, USA). For migration, a total of $1x10^5$ treated cells in 10% FBS medium were seeded into the top chamber of the insert, and the lower chamber was immersed in 500 μL FBS-free RPMI 1640 medium. After 48 h incubation, cells that failed to pass through the membrane were removed. The membrane containing cells on its lower surface was fixed with pre-cooling methanol and stained with 0.5% crystal violet. Then, the cells stained were calculated after pictures taken using a microscope in five random visions.

For invasion, the upper surface of the membrane was covered with matrigel (BD Biosciences, San Jose, CA, USA), and other steps were the same as the migration one.

Dual-luciferase Assay

Dual-luciferase assay (Promega, Madison, WI, USA) was enlisted to measure the luciferase activity. The wild type or mutant miR-377 binding site VEGFA 3'-UTR cDNA fragment, after being amplified, was cloned into pGL3 luciferase vector (Promega, Madison, WI, USA), relatively. Then, SGC-7901 cells were transfected with miR-377 mimics and the conducted PGL3 vector. The cells were maintained for 24 h, and then, the activity of luciferase was determined using luminometer (Promega, Madison, WI, USA) and measured. The experiment was repeated for three times.

Protein Isolation and Western Blot

The proteins of established cell lines were extracted using Radioimmunoprecipitation assay (RIPA) reagent (Beyotime, Shanghai, China). The protein concentration was analyzed using bicinchoninic acid (BCA) quantitative detection reagent kit (Beyotime, Shanghai, China). A total of 20 μg extracted protein was degenerated and chilled on ice. Next, the protein was separated using 8% SDS-PAGE and shifted to PVDF membranes (Millipore, Billerica, MA, USA). After blocking non-specific protein interactions using 5% fat-free milk, the membranes loaded with proteins were immersed within the fat-free milk with the primary antibody against VEGFA or GAPDH at 4°C overnight. After the membranes were washed with Tris-buffered saline-Tween (TBST) buffer for three times, they were incubated with secondary antibody carrying horseradish peroxide (HRP) for 1 h at room temperature. We next detected the membranes using ECL (Millipore, Billerica, MA, USA) following the instructions. All these antibodies were purchased from Abcam (Cambridge, MA, USA).

Statistical Analysis

Statistical analysis was done using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA), all data were showed as mean ± SD. Comparison between groups was done using One-way ANOVA test followed by Least Significant Difference (LSD). p values < 0.05 were considered having significant difference.

Results

MiR-377 was Downregulated in GC Tissues and Cell Lines

To study the relationship between miR-377 and gastric cancer (GC), we detected the miR-377 expression level in 86 GC and adjacent normal tissue samples using qRT-PCR. Figure 1A showed that the miR-377 level of GC tissues expressed significantly lower than that of normal group. Also, the level of miR-377 in GC cell lines was lower than that in normal GES-1 cell line (Figure 1B). Furthermore, we analyzed the clinical pathological characteristics of 86 patients, which suggested that lower miR-377 level indicated more distant metastasis, more lymph node metastasis and advanced TNM stage (Table I). These data suggested that miR-377 might inhibit GC progression. To further explore the miR-377 function, we cho-

Table I. Correlation between miR-377 level and clinicopathological features in gastric cancer.

		miR-377 expression		
Characteristics	**Total**	**Low**	**High**	**p-value**
Age				
<60 years	38	17	21	0.3851
>60 years	48	26	22	
Gender				
Male	56	31	25	0.1746
Female	30	12	18	
Tumor size				
<4.5 cm	57	31	26	0.2541
≥4.5 cm	29	12	17	
Lymph node metastasis				
Yes	41	29	12	0.0002*
No	45	14	31	
Distant metastasis				
Yes	44	32	12	0.0000*
No	42	11	31	
TNM stage				
I-II	33	9	24	0.0009*
III-IV	53	34	19	

The expression level of miR-377 was cut-off by median expression level and *indicated $p < 0.05$.

se SGC-7901 cell line to over-express miR-377 while MKN-45 to interfere miR-377 level, which provide established cell lines for next functional experiments (Figure 1C-D).

MiR-377 Over-expression Suppressed GC Cell Proliferation

To determine whether miR-377 could affect cell proliferation of GC cells, we used the colony formation assay and CCK8 assay. Overexpression of miR-377 suppressed SGC-7901 cells colony formation ability (Figure 2A-B) while miR-377 knocking-down increased MKN-45 cells colonies numbers (Figure 2C-D). Also, CCK8 assay showed that the proliferation ability of SGC-7901 cells was markedly decreased after miR-377 mimics treatment, but MKN-45 cells raised after miR-377 inhibitors transfection compared to negative control group relatively. These results indicated that miR-377 inhibited cell proliferation of GC.

Ectopic miR-377 Effected Cell Migration and Invasion of GC Cells

Next, we explored the influence of miR-377 on cell metastasis. Wound-healing assay displayed that SGC-7901 cells migrated shorter distance after miR-377 over-expression than the control group (Figure 3A-B). However, the migration ability of MKN-45 cells increased after miR-377 inhibitors interference when compared to the negative control group (Figure 3C-D). Furthermore, transwell assay was recruited to detect how the miR-377 effected cell migration and invasion. Figure 4A-B showed SGC-7901 invaded less cells than control group because of miR-377 mimics transfection and the migrated SGC-7901 cell numbers decreased after miR-377 over-expression (Figure 4C-D). On the contrast, down-regulation of miR-377 in MKN-45 cells remarkably increased cell migration and invasion abilities (Figure 4E-H). These results demonstrated that miR-377 could inhibit cell migration and invasion.

VEGFA was a Direct Target Gene of miR-377

To explain the underlying mechanism of miR-377 in GC, we explored several databases including TargetScan, miRanda, and miRWalk. VEGFA was found having a direct bonging site in its 3'-untranslated regions (3'-UTR) for miR-377 (Figure 5A). Next, we established dual-luciferase assay by constructing wild type or mutant 3'-UTR miR-377-binding region VEGFA plasmids to verify our assumption. As shown in Figure 5B, we found that the luciferase activity significantly decreased in wild type

group but there was no difference in mutant group. Furthermore, Western blot experiments showed that VEGFA protein level decreased in miR-377 mimics treated SGC-7901 cells (Figure 5C-E) but increased in miR-377 inhibitors transfected MKN-45 cells (Figure 5C-F). All these data suggested that VEGFA was a direct target of miR-377.

VEGFA Restored Influences of miR-377 Up-regulation in GC Cells

As mentioned before, upregulation miR-377 could decrease the cell proliferation and metastasis abilities, via repressing the VEGFA expression. We further constructed pcDNA 3.1-VEGFA to reverse the effect of miR-377 mimics to verify these results. CCK8 assay showed that pcDNA 3.1-VEGFA transfection in miR-377 mimics treated SGC-7901 cells significantly rescued the cell proliferation ability (Figure 6A). Furthermore, the inhibition of invasion activity caused by miR-377 over-expression was reversed by VEGFA (Figure 6B). In addition, the VEGFA protein expression was confirmed using Western blot that showed VEGFA restored by pcDNA 3.1-VEGFA (Figure 6C-D). These data demonstrated that miR-377 could inhibit GC progression via down-regulating VEGFA.

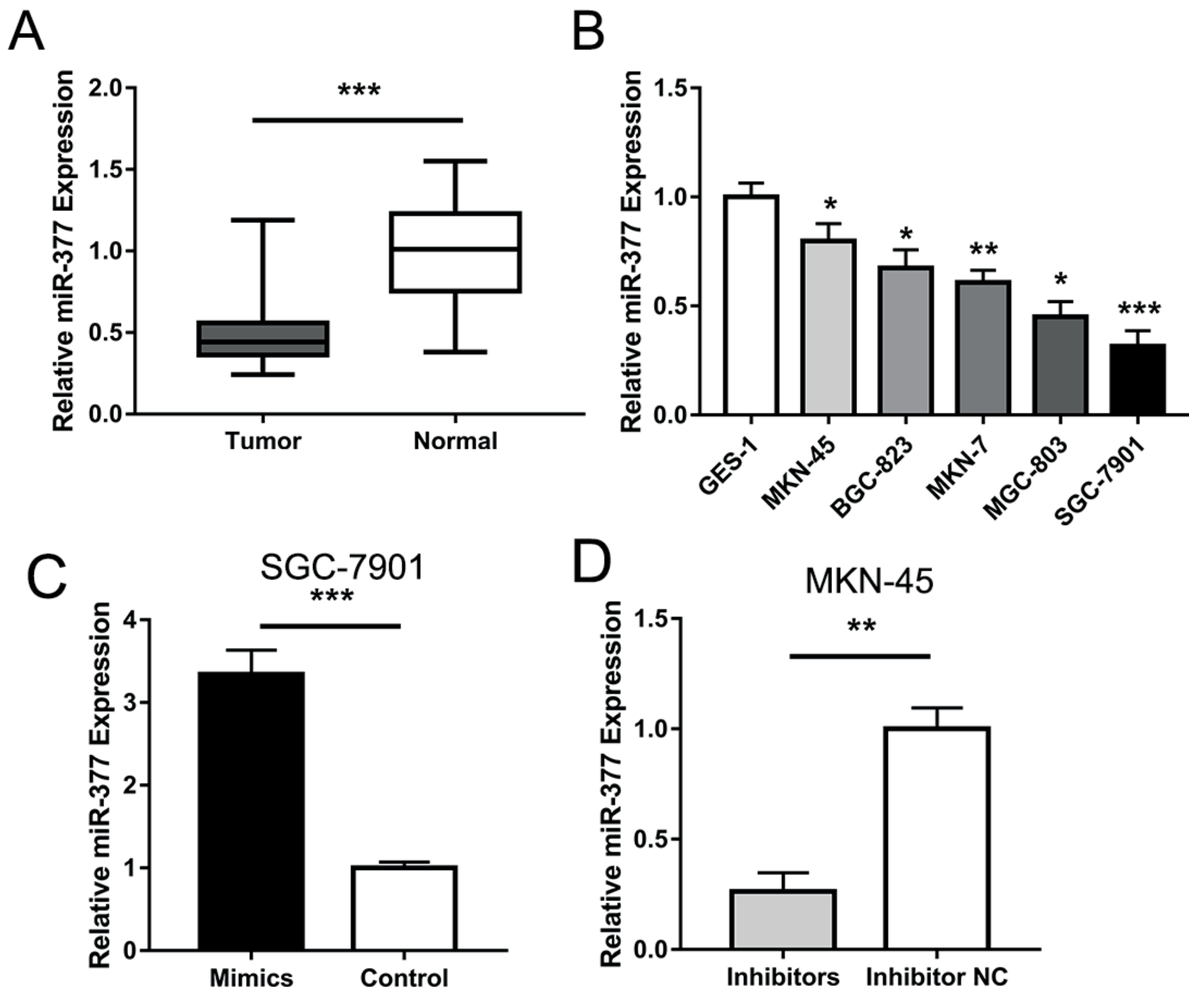

Figure 1. MiR-377 was downregulated in GC tissues and cell lines. ***A,*** Analysis of the expression level of miR-377 in 86 pairs GC tumor and adjacent tissues. ***B,*** Analysis of miR-377 expression level in GC cell lines (MKN-45, BGC-823, MKN-7, MGC-803, SGC-7901) and breast epithelial cell line (MCF10A). ***C,*** Expression of miR-377 in miR-377 mimics treated SGC-7901 cells. ***D,*** Expression of miR-377 in miR-377 inhibitors treated MKN-45 cells. MiR-377 was detected by qRT-PCR and U6 was used as an internal control. Data are presented as the mean ± SD of three independent experiments. $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$.

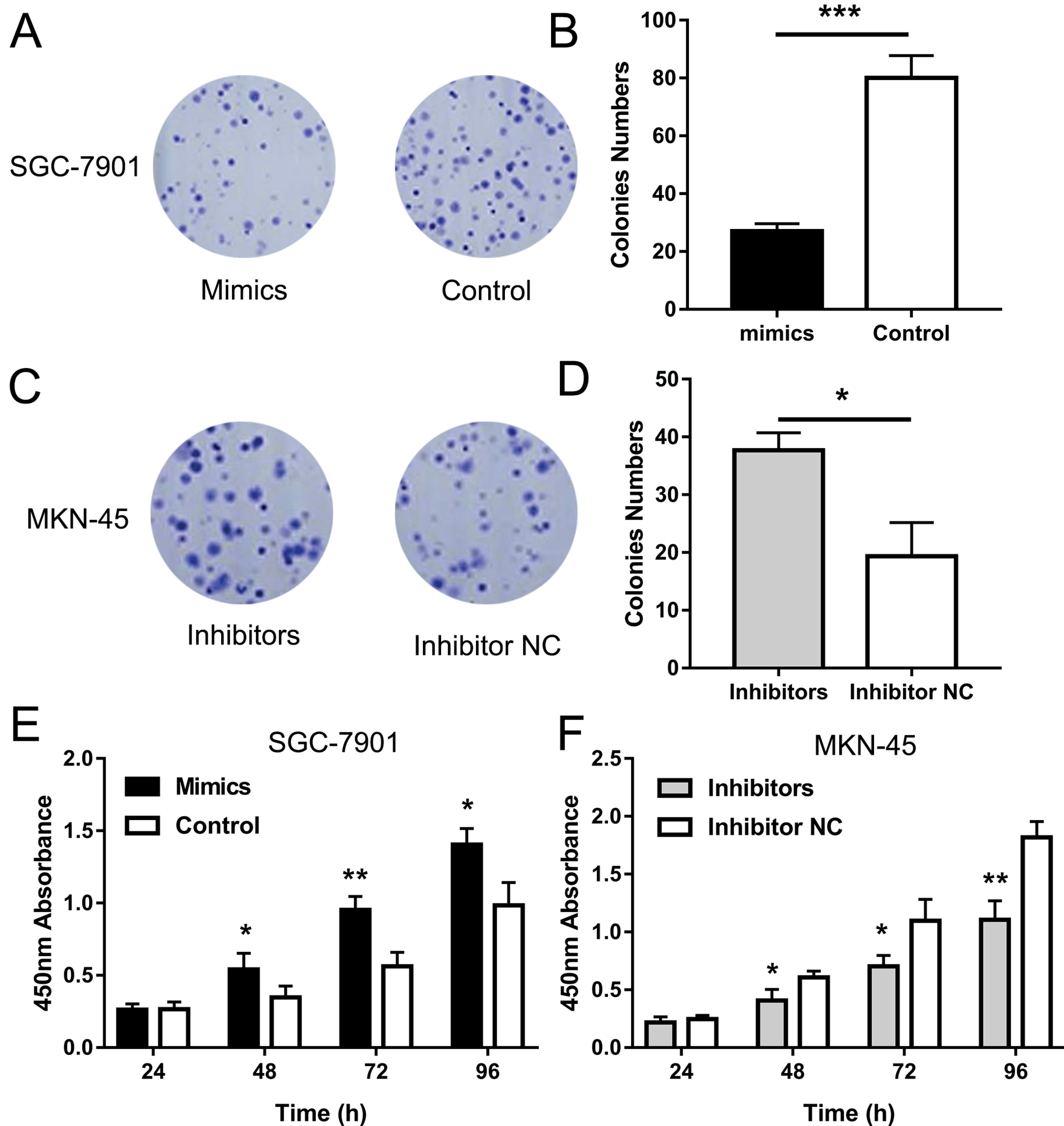

Figure 2. MiR-377 effected the proliferation of GC cells. ***A-D,*** Colony formation assay was performed to determine the growth of SGC-7901 ***(A, B)*** or MKN-45 ***(C, D)*** cells transfected with mimics or inhibitors, respectively. ***E, F,*** CCK8 assay was performed to determine proliferation of SGC-7901 ***(E)*** or MKN-45 ***(F)*** cells treating with miR-377 mimics or inhibitors compared to each negative control Data are presented as the mean ± SD of three independent experiments. $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$.

Discussion

In this study, we first demonstrated miR-377 was suppressed in GC tissues as well as in GC-derived cell lines. Lower miR-377 expression level indicated poor clinical pathological features. Through loss- and gain- of function experiments, we found that miR-377 could inhibit cell proliferation, migration, and invasion abilities of GC cells. Furthermore, we verified VEGFA as a potential target for miR-377 and over-expression of VEGFA could rescue the effects of miR-377. To our known, this is the first time that we explained the relationship between miR-377 and VEGFA in GC. Despite the current treatment methods of gastric cancer, including

surgical treatment, chemotherapy and radiotherapy, developed continuously, the five-year survival rate of gastric cancer is still maintained at a low level[22,23]. In recent years, some miRNAs have been found to serve as new targets for the diagnosis and treatment of gastric cancer[5,6]. Here, we found miR-377 could inhibit GC cell growth and metastasis via several experiments, which verify miR-377 as a target for GC diagnosis and therapy. Furthermore, VEGFA was justified as a target for miR-377. The VEGF gene is located at 6p21.3 of the chromosome and consists of 8 exons and 7 introns. VEGF expression was reported to be related with increased vascular distribution, lymph node metastasis, liver metastasis and poor prognosis of gastric cancer[24-26]. Growing evidence showed that VEGF promoted the cell division, proliferation, and migration of cancers including GC[27]. It was an important molecular involved in different tumor regulating mechanism and targeting VEGFA has become a therapy for cancer[28-30]. In gastric cancer, VEGFA could be regulated by some miRNAs according to existing research[31,32]. For instance, miR-126 facilitated angiogenesis of GC through VEGFA, miR-874 functioned as a tumor suppressor via

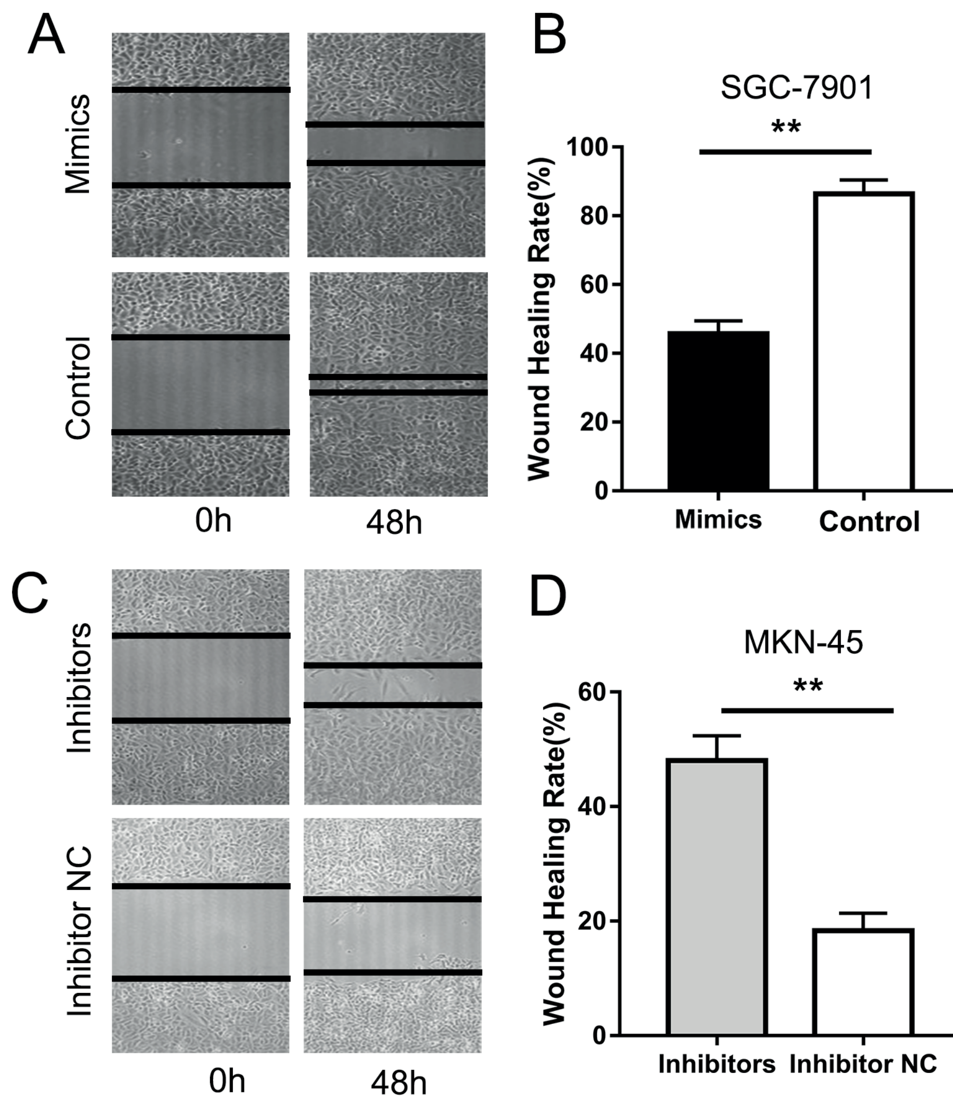

Figure 3. MiR-377 effected the migration of GC cells. Wound-healing assay was performed to determine proliferation of SGC-7901 ***(A, B)*** or MKN-45 ***(C, D)*** cells treating with miR-377 mimics or inhibitors compared to each negative control. Data are presented as the mean ± SD of three independent experiments. $^{**}p < 0.01$.

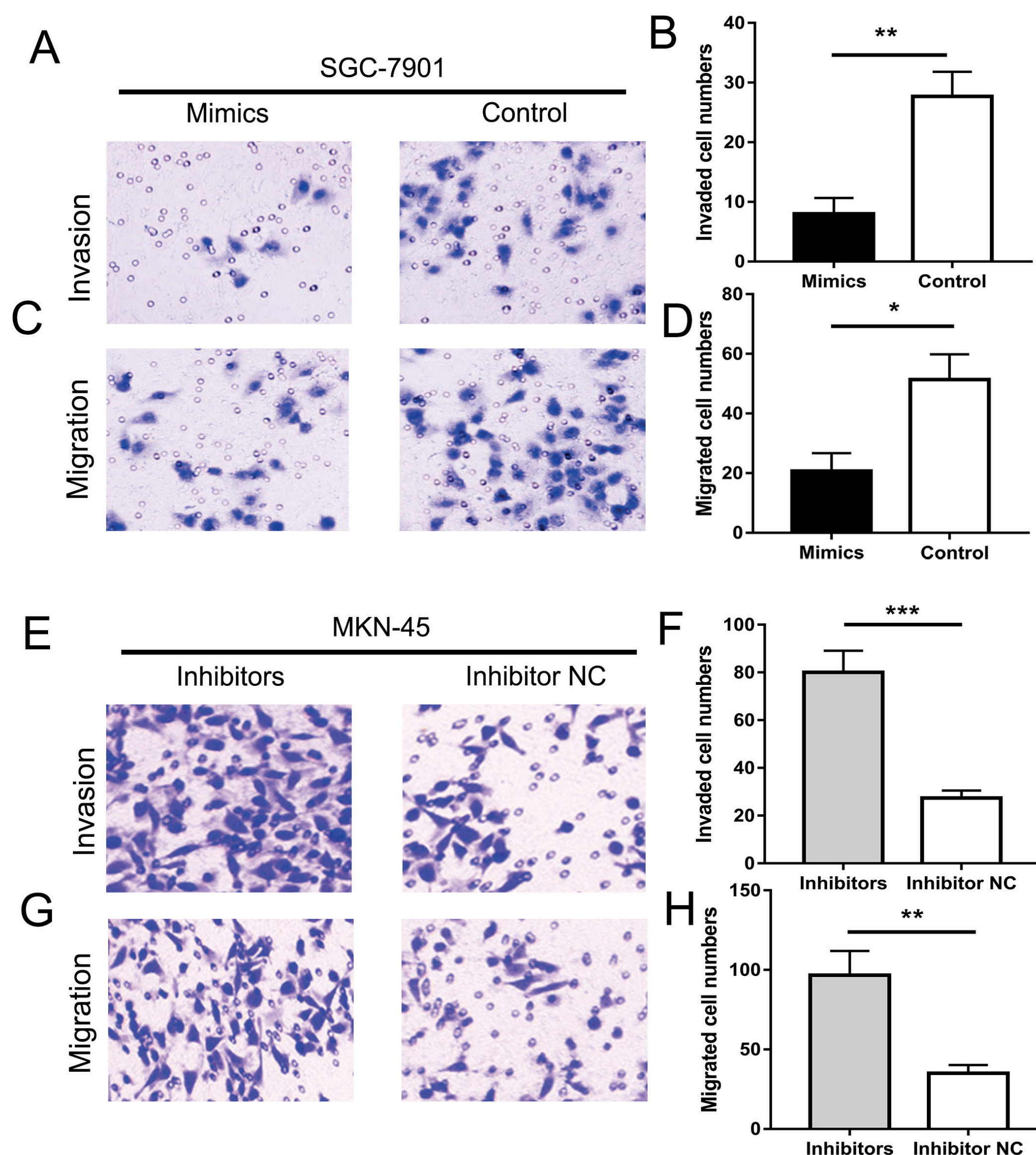

Figure 4. MiR-377 effected the migration and invasion of GC cells. ***A-F,*** Wound-healing assay was performed to determine migration of SGC-7901 ***(A, B)*** or MKN-45 ***(E, F)*** cells treating with miR-377 mimics or inhibitors compared to each control. ***C-H,*** Transwell invasion assay was used to detect the invasion ability of miR-377 mimics treated SGC-7901 cells ***(C, D)*** or miR-377 inhibitors treated MKN-45 cells ***(G, H)***. Data are presented as the mean ± SD of three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

STAT3/VEGF-A axis, and miR-1 inhibited tumor development by inhibiting VEGFA[33-35]. We elucidated VEGFA could be repressed by miR-377 using Western blot. We next overexpressed VEGFA in miR-377 upregulated SGC-7901 cells and found the influence of miR-377 was restored, which confirmed VEGFA as a direct binding gene for miR-377. All these findings provided miR-377 as a new target for VEGFA-related biotherapy.

Conclusions

We showed that miR-377 was downregulated in GC tissues and cells and inhibited cell proliferation, invasion, and migration via VEGFA. However, more experiments, especially *in vivo* evidence, are needed to explain more details about miR-377 function in GC. Taken all together, this study could provide miR-377 a new target for GC diagnosis and for biotherapy, especially related with VEGFA.

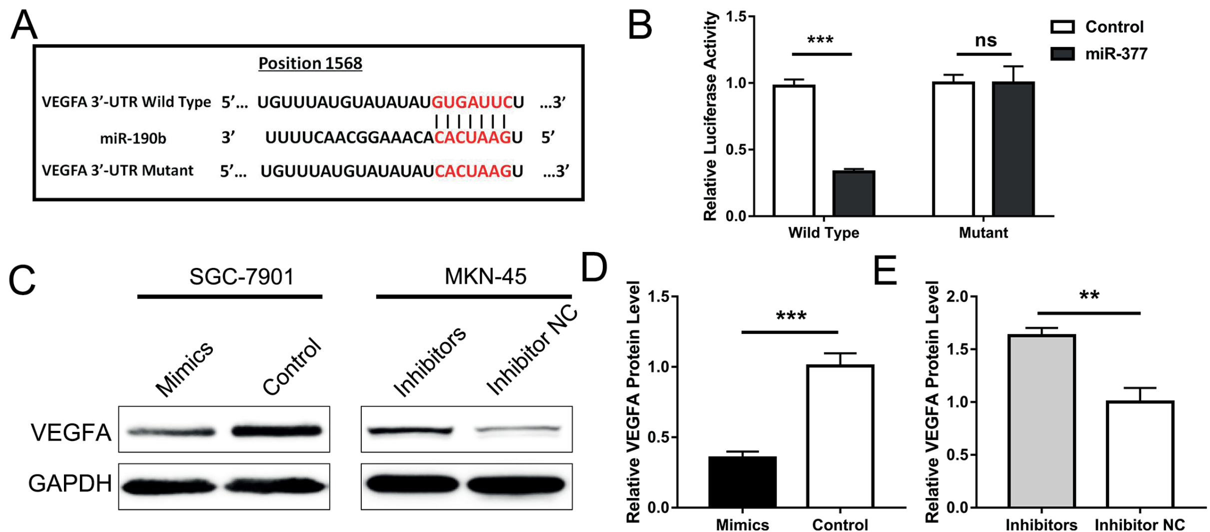

Figure 5. VEGFA was a direct target of miR-377. ***A,*** The predicted binding sites of miR-377 in the 3'-UTR of VEGFA. ***B,*** Dual-luciferase reporter assay was used to determine the binding site. SGC-7901 cells treated by mimics or control were transfected with pGL3 construct containing the WT or mutant VEGFA 3'-UTR site. ***C,*** Levels of VEGFA and GAPDH protein measured by Western-blot in miR-377 overexpression SGC-7901 cells and miR-377 knockdown MKN-45 cells. ***D, E,*** the relative protein level of VEGFA and GAPDH. Data are presented as the mean ± SD of three independent experiments. $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$, ns: no significant.

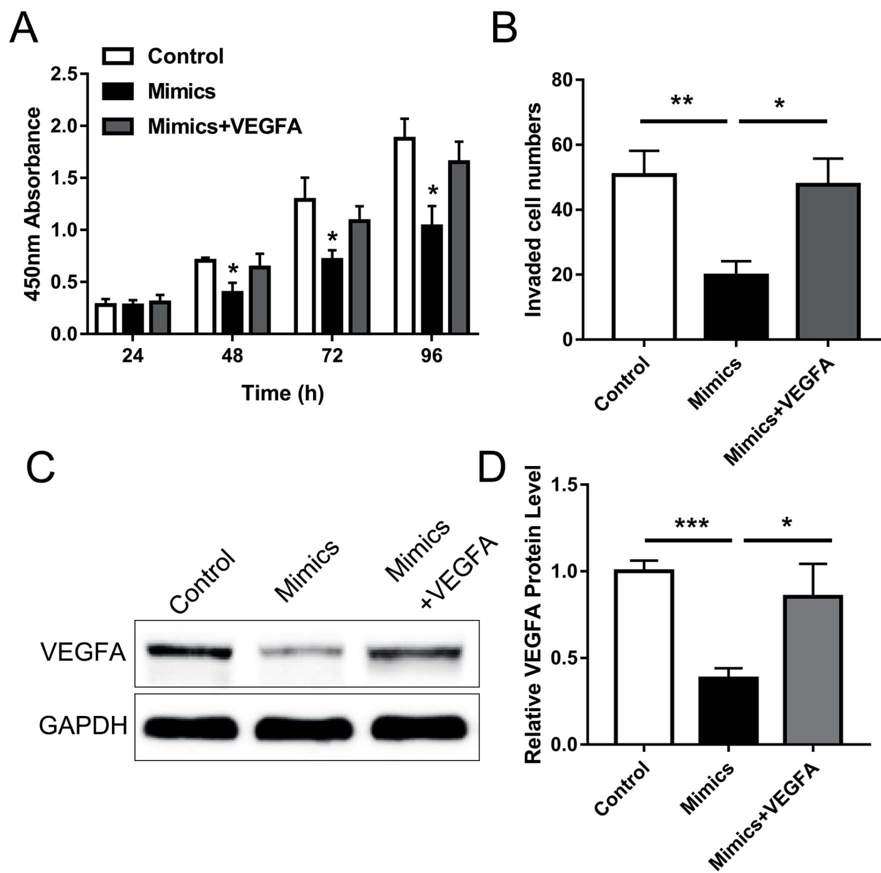

Figure 6. VEGFA restored the effects of miR-377 mimics in SGC-7901 cells. ***A,*** Analysis of the cell proliferation ability by CCK8 assay in miR-377 control, mimics, or mimics+VEGFA treated SGC-7901 cells; ***B,*** Cell invasion ability was measured by Transwell assay; ***C,*** Western-blot analyses of VEGFA and GAPDH expression level. ***D,*** Relative protein band densities of VEGFA and GAPDH. Data are represented as the mean ± SD of three replicates. $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$.

Conflict of interest

The authors declare no conflicts of interest.

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