Original Article
MicroRNA-17-5p promotes gastric cancer proliferation, migration and invasion by directly targeting early growth response 2

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Abstract: MicroRNA-17-5p (miR-17-5p) has previously been reported to play an important role in tumor development and progression. However, it functions differently regarding different kinds of malignant tumor, and its role and mechanism in gastric cancer (GC) still lacks investigation. In this study, we detected the relationship between miR-17-5p and the development of GC by qRT-PCR, and it turned out that the level of miR-17-5p was significantly higher in GC patients than that in normal controls, and the aberrant expression of miR-17-5p was correlated with lymph node metastasis. After that, we examined the effect of miR-17-5p taking on the proliferation, apoptosis, migration and invasion of GC cells and the underlying mechanism. Experiments indicated that knockdown of miR-17-5p inhibited the proliferation, invasion and migration, while promoting apoptosis of SGC7901 cells. Early Growth Response 2 (EGR2) protein or mRNA levels were downregulated or upregulated after overexpression or knockdown of miR-17-5p, respectively. By using dual luciferase assay and Western blot, we identified EGR2 as a functional target of miR-17-5p. As far as we know, this could be the first study to demonstrate that miR-17-5p is associated with tumor stage of GC and that it could possibly become a new therapeutic method for the treatment of GC.

Keywords: Gastric cancer, miR-17-5p, SGC7901, early growth response 2, tumor

Introduction

Gastric cancer (GC) ranks the fifth most common and the third leading deadly cancer in the world [1]. Although general trends of decreasing incidence and mortality has occurred in most industrial countries, it is more common in developing countries, predominantly in China [2]. Despite great improvements in the diagnosis and treatment of GC, the survival rate of GC is disappointing with overall 5-year survival rates leveling up to 25% [3]. A majority of patients have developed an aggressive form of GC by the time of diagnosis, and they virtually are incurable with the metastatic disease. Thus, it is essential to identify novel therapeutic strategies to improve treatment outcome.

MiRNAs are a family of endogenous small noncoding RNA molecules that directly bind to the target messenger RNAs (mRNAs) in the 3'-untranslated regions (3'-UTRs) to regulate gene expression by causing mRNA degradation or translational suppression [4-6]. It is reported that miRNAs are implicated in a wide range of important physiological events [7, 8]. Emerging evidence indicate that each miRNA regulates hundreds of target genes which take part in a variety of physiological and pathological processes, such as proliferation, differentiation, cell cycle regulation, apoptosis, tumorigenesis, migration, and invasion [9]. A number of studies have illustrated that some miRNAs can influence the tumorigenesis property of GC by changing its physiological process [10, 11]. A growing number of evidence demonstrates that miRNAs can function either as oncogenes or tumor suppressors given the roles of their target genes, and that aberrant miRNA expression is common in various types of human cancers including GC [11, 12].

MiR-17-5p belongs to the miR-17-92 cluster located in the third intron of chromosome 13

[13]. Previous reports have found that miR-17-5p is upregulated in several types of cancer, suggesting it functions pleiotropically during tumorigenesis and progression by promoting proliferation, inhibiting differentiation as well as sustaining cell survival [14]. On the contrary, in some cancer cell lines, miR-17-5p acts as a tumor suppressor [15, 16]. These conflicting results suggest that miR-17-5p might have different functions in various cells. However, neither the role nor its underlying molecular mechanism of miR-17-5p in the progression of GC remains to be explored.

In current research, we demonstrated that miR-17-5p was significantly up-regulated in GC tissues and cell lines. We found that downregulation of miR-17-5p inhibited GC cell growth, migration and invasion, while promoting apoptosis *in vitro*. Moreover, lentivirus mediated anti-miR-17-5p can significantly suppress the growth of xenografts *in vivo*. We also identified EGR2 as a direct target of miR-17-5p in GC. Our research for the first time suggests that miR-17-5p should be a promising target for developing miRNA-based GC therapy in the future.

Materials and methods

Ethics statement

This study was approved by the Medical ethics committee of Hospital and each participant signed a written informed consent before participation of this study.

Clinical specimens

A total of 40 human GC tissues and 40 matched normal tissue samples (located > 5 cm away from the tumor) were collected from patients who underwent primary surgical treatment in our hospital. All the clinical specimens were snapped-frozen in liquid nitrogen and stored at -80°C.

Cell lines and culture

Human GC cell lines AGS, HGC27, MKN45, and SGC7901 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human GC cell line GES-1 was purchased from the Chinese Academy of Sciences (Shanghai, China). All of these cell lines were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 10 units/mL penicillin and 10 mg/mL streptomycin at 37°C in a 5% CO_2 incubator.

Establishment of stable anti-miR-17-5p SGC7901 cell line by lentivirus

The lentiviral vectors with miRNAs based on the miR-17-5p framework were constructed by the GeneChem Company (Shanghai, China). In brief, the double-stranded oligonucleotide encoding anti-miRNA and negative control were annealed and inserted into the linearized eukaryotic pFU-GW-009 vector (GeneChem) and further confirmed by sequencing. The recombinational vectors and the packaging vectors (pHelper 1.0 and pHelper 2.0) (GeneChem) were co-transfected into 293T cells (Invitrogen, Carlsbad, CA, USA) with Lipofectamine 2000 (Invitrogen). The culture supernatants were collected after transfection for 48 h. After transfection, cells were collected and subjected to qRT-PCR analysis of miR-17-5p expression.

RNA extraction and quantitative RT-PCR

Total cellular RNA was isolated from tissues and cell lines using TRIzol reagent (Life Technologies, USA) following the manufacturer's instructions. For miR-17-5p expression level detection, total RNA was reverse-transcribed with a miR-17-5p-specific RT primer (RiboBio, China) and amplified with PCR primers (RiboBio, China) by using the ABI 7300 Real-Time PCR System (Life Technologies, USA). The relative expression of miR-17-5p was normalized to U6. SYBR Green PCR kit (Takara) was used to quantify the mRNA levels of EGR2 by normalizing to GAPDH according to the manufacturer's protocols. Difference between miR-17-5p and EGR2 expressions in different treated-method cells was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Proteins were extracted using RIPA lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM DTT, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 mg/ml leupeptin, 2 mg/ml aprotinin, 2 mg/ml pepstatin, 1% NP-40, and 0.1% SDS). BCA protein assay kit (Beyotime, Beijing, China) was used to quantify protein concentrations. 40 μg protein each sample were separated by

10% gradient SDS polacrylamide gel and transferred onto PVDF membranes. After that, the membranes were blocked in 5% skim milk in Tris-based saline-Tween 20 (TBST) for 1 h at room temperature, and further incubated with rabbit anti-human antibodies at the recommended dilution (1:1000) overnight at 4°C. After being washed by TBST, the membranes were further incubated with a secondary antibody (1:2000) for 1 h. Enhanced chemiluminescence (ECL) solution was added onto the membranes and protein expression was measured using ImageJ software. β-actin as a loading control.

Cell proliferation assay

SGC7901 cells were seeded into 96-well plates at a density of 3×10^3 and cultured overnight. The transfected cells proliferation was measured using Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) assay. Briefly, 10 μL CCK-8 solutions were added into each well and further incubated for another 2 h. The optical density was then measured by FACS analysis at the absorbance of 450 nm.

Soft-agar colony formation assay

SGC7901 cells were seeded into a 12-well plate at a density of 500 cells per well and were suspended in RPMI-1640 with 0.3% agar and 10% FBS. The cells were layered in RPMI-1640 with 0.6% agar, 10% FBS and 400 ng/mL puromycin. After 2 weeks, the colonies were counted and imaged under a light microscope.

Cell migration and invasion assay

In the migration examination, the transfected cells were harvested and resuspended in 100 μL serum-free medium and then transferred to the upper chambers (2×10^4 cells per well). 600 μL medium supplemented with 10% FBS was added to the lower chamber. After incubation for 24 h, the membranes were fixed in 4% paraformaldehyde/PBS for 10 min and stained with 2% crystal violet, and then be counted under a light microscope. For the invasion assay, we pre-coated the Transwell membrane with 30 μL of Matrigel (1:3 mixed with PBS; BD Biosciences, Heidelberg, Germany) for 30 min; the remaining experimental procedures were similar to the migration assay.

Flow cytometry apoptosis assay

After transfection, SGC7901 cells were harvested and seeded at a density of 5-10 × 10^6 cells/mL. Cells were then incubate with 5 μL of Annexin V-FITC and 5 μL PI (BD Biosciences, USA) for 15 min before they were subjected to FACS analysis.

Dual luciferase report assay

To construct a luciferase reporter vector, the EGR2 3'-UTR fragment containing putative binding sites for miR-17-5p was amplified using PCR and cloned in the psiCHECK-2 vector (Promega, Madison, WI, USA). 1 μg constructs were co-transfected with 1 μg miR-17-5p precursor or control into SGC7901 cells. After post-transfection for 48 h, dual-luciferase activity assays (Promega, Madison, WI, USA) were assayed following the manufacturer's instructions.

Gastric cancer xenografts

4-5 weeks old female nude (BALB/c-nu) mice were purchased from Vital River (Beijing, China). SGC7901 cells transfected with miR-17-5p inhibitor or inhibitor negative control were injected subcutaneous into the left axillae of mice. Tumor volume and the whole body weight were measured every 2 days after tumor inoculation. At the end of the experiment, all mice were sacrificed and tumors were harvested to evaluate their weight and further study.

Statistical analysis

The data are expressed as mean ± SD. GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA) was used for statistical analysis. Difference was analyzed using one-way ANOVA. Significant difference was considered if $P < 0.05$.

Results

miR-17-5p is up-regulated in patients with GC tissues and GC cell lines

To investigate the role of miR-17-5p in human GC occurrence, we detected the expression levels of miR-17-5p by RT-qPCR in tumorous tissue and adjacent normal tissue of 40 GC patients. The results revealed that expression of miR-17-5p in tumor tissues was significantly higher
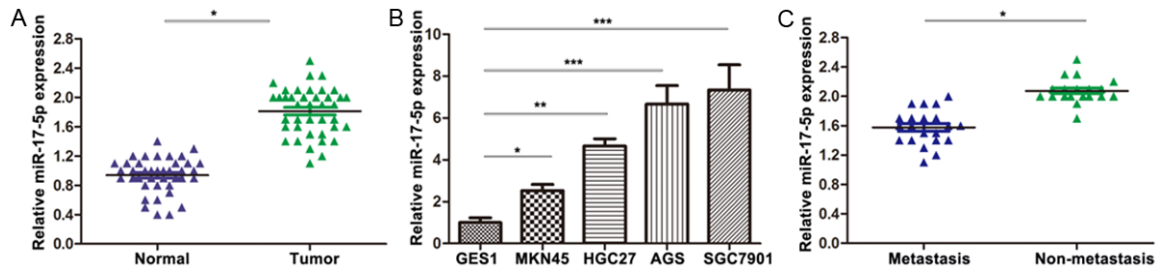
Figure 1. qRT-PCR analysis of miR-17-5p in GC tissues and cell lines. A. The relative mRNA expression of miR-17-5p was performed by qRT-PCR and normalized to an endogenous control (U6 RNA) using 40 GC tissue samples and 40 adjacent non-tumor normal tissues. B. Average relative expression of miR-17-5p in four GC cell lines MKN45, HGC27, AGS, and SGC7901 and one normal cell line GES1. C. Average relative expression of miR-17-5p levels in metastatic (n=19) and non-metastatic (n=21) gastric cancers. Data are means ± SD of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

than that in normal tissues (**Figure 1A**). To further confirm such relationship, we also monitored miR-17-5p levels in four GC cell lines MKN45, HGC27, AGS and SGC7901 as well as one normal cell line GES1. We found that all four tested GC cells had significantly upregulated miR-17-5p levels compared with GES1 cells (**Figure 1B**). As SGC7901 cell linehas the highest level of miR-17-5p, it is used for further molecular mechanisms investigation. By dividing the 40 GC patients into two groups, 21 patients with lymph node metastasis, 19 patients without metastasis, we found that the there is a higher miR-17-5p expression level in the patients with metastasis than in those without metastasis (**Figure 1C**). These data suggested that miR-17-5p may serve as a prognostic marker for GC patients.

MiR-17-5p promotes growth of GC cells and inhibits apoptosis of GC cells

SGC7901 cells without infection of virus or transfected with control or anti-miR-17-5p lentiviral vectors were termed as Blank or NC-LV or anti- miR-17-5p-LV, respectively. The expression levels of miR-17-5p were further confirmed by qPCR. The expression of miR-17-5p in the anti- miR-17-5p-LV cells was decreased when compared to the Blank or NC-LV cells ($P < 0.05$) (**Figure 2A**). To illustrate functional significance of miR-17-5p in SGC7901 cell line, a series of experiments were performed. We first determined the effect of miR-17-5p on miR-17-5p high expressed SGC7901 cell growth. After transfection of control or anti-miR-17-5p lentiviral vectors, CCK8 assay was performed. It was found that SGC7901 proliferation decreased significantly in anti- miR-17-5p-LV group (**Figure**

2B). To determine the long term growth effect of miR-17-5p in SGC7901 cells, colony formation assay was performed. Cells transfected with miR-17-5p inhibitor showed lower colony formation than cells transfected with control (**Figure 2C**). To further elucidate the mechanism of miR-17-5p-mediated cell growth in SGC7901 cells, apoptosis analysis was performed. Results showed that apoptosis rate increased in the cells transfected with anti-miR-17-5p lentiviral vectors (23.63±5.78%) compared with that in the blank cells (2.12±0.25%) or NC-LV cells (2.95±0.73%), indicating that miR-17-5p suppressed the apoptosis of SGC-7901 (**Figure 2D**).

Inhibition of miR-17-5p expression suppresses the invasion and metastatic capacity of GC cells by regulating cadherins

To further assess the effects of miR-17-5p on malignant tumor progression and metastasis, we used migration and invasion method to determine the role of miR-17-5p. As shown in **Figure 3A-D**, knockdown of miR-17-5p significantly suppressed the cell migration and invasion of SGC7901. In addition, we found that transfection of miR-17-5p inhibitor significantly decreased the expression of vimentin and N-cadherin, while increasing the expression of E-cadherin in SGC7901 cells (**Figure 3E** and **3F**). The above results suggested that miR-17-5p can boost migration and invasion abilities of SGC7901 cells.

EGR2 was a direct target of miR-17-5p

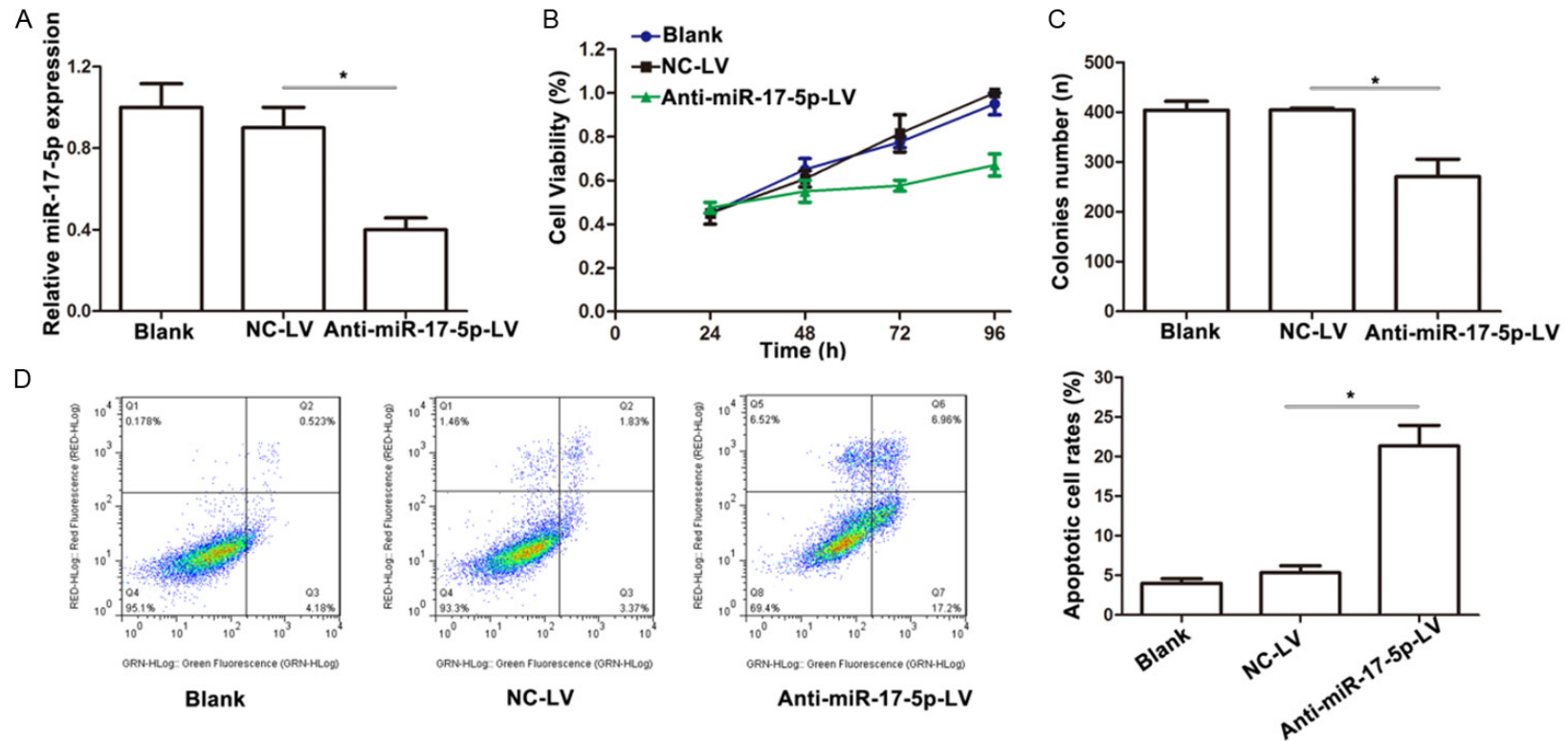EGR2 was selected for further validation by bioinformatics predictions as a potential target of

Figure 2. Effect of miR-17-5p on cell proliferation and apoptosis in SGC7901. A. miR-17-5p was lower expressed in stable anti-miR-17-5p SGC7901 cell line compared with cells infected with negative control. B. Cell proliferation CCK-8 assay. SGC7901 cells were grown and transfected with anti-miR-17-5p lentivirus and control, then subjected to CCK-8 assay. C. Representative results of colony formation assay in SGC7901 cells transfected with anti-miR-17-5p lentivirus or the corresponding control, respectively. D. Annexin-V FITC/PI assay was used to detect apoptotic cells in SGC7901 cells transfected with anti-miR-17-5p lentivirus or the corresponding control. The rates of apoptosis in SGC7901 cells were quantified. Error bars represent the mean ± SD from three independent experiments. *$P < 0.05$.
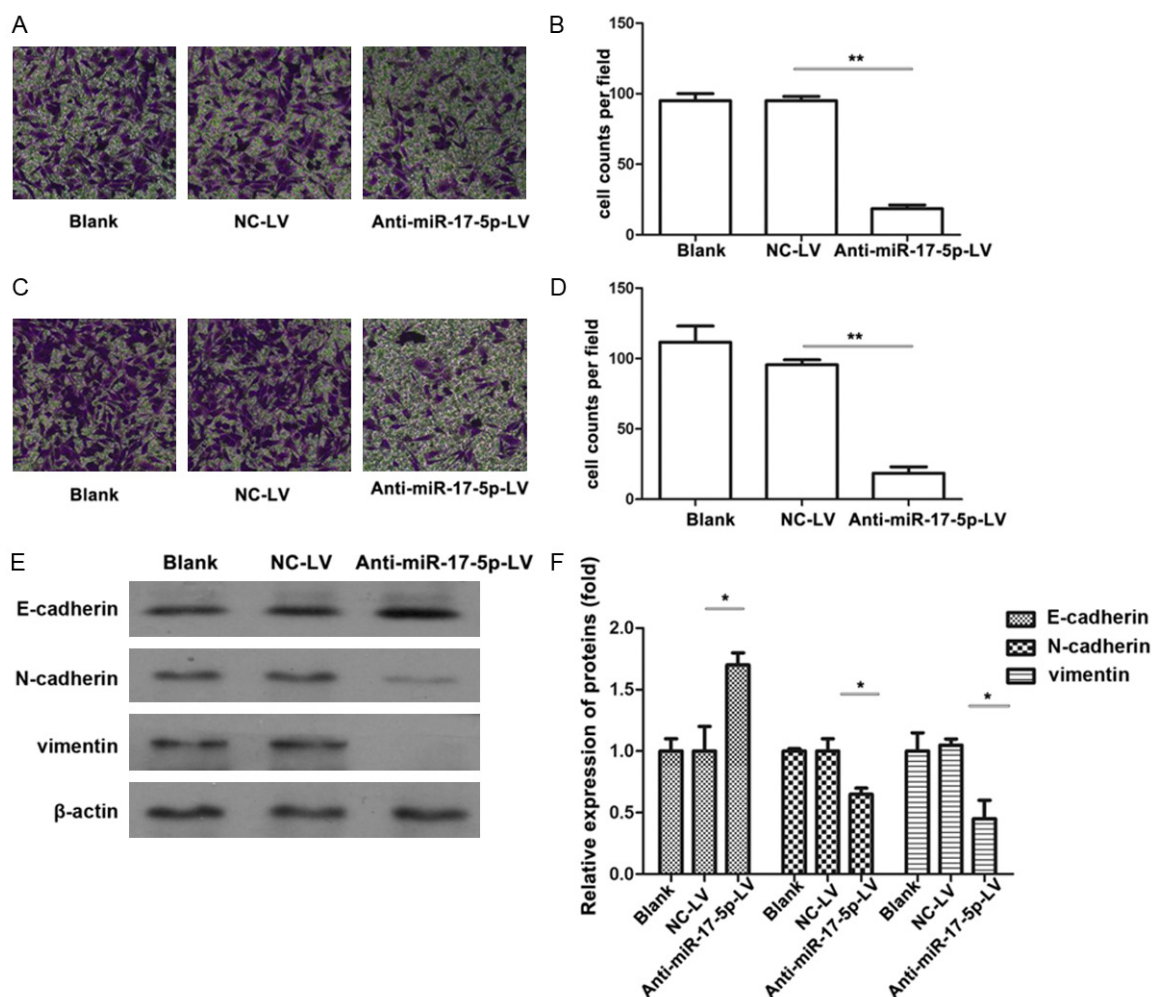
Figure 3. Effects of miR-17-5p on migration and invasion in GC cell SGC7901. Cells were grown and transfected with anti-miR-17-5p lentivirus or the corresponding control, then subjected to migration or invasion assay. A, B. Representative microscopy images and quantitative data of migration cells (100 ×). C, D. representative microscopy images and quantitative data of invasion cells (100 ×). E, F. proteins expression of E-cadherin, N-cadherin, and vimentin was detected by Western blot in SGC7901 cells. Error bars represent SD, n=3, *$P < 0.05$, **$P < 0.01$.

miR-17-5p (**Figure 4A**). EGR2 is a member of a multi-gene family encoding zinc finger proteins and is a central player throughout cellular proliferation and cell cycle. Thus, we selected EGR2 as a target for further analysis. Dual-luciferase reporter assay was performed to verify whether EGR2 was a potential target of miR-17-5p (**Figure 4B**). We amplified the EGR2 3'UTR that contains the target sequences, or the mutants, into a luciferase reporter vector. The results showed that miR-17-5p suppressed luciferase activity, while knockdown of miR-17-5p increased luciferase activity of the wild type EGR2 3' UTR (WT), which were not observed in mutation of the miR-17-5p binding sites (Mut) in SGC7901 cells. These data suggest that miR-

17-5p may repress the expression of EGR2 by binding to the specific sequences in the 3'-UTR of EGR2. We found that miR-17-5p overexpression decreased EGR2 mRNA level, while miR-17-5p inhibitor increased EGR2 mRNA level (**Figure 4C**). Western blot also demonstrated that overexpression of miR-17-5p reduced EGR2 expression in SGC7901 cells, while miR-17-5p inhibitor elevated EGR2 protein level (**Figure 4D**).

To determine whether EGR2 is a functional target of miR-17-5p, we performed 'rescue' experiments by co-transfecting SGC7901 cells with miR-17-5p mimic and EGR2 overexpressing construct containing the EGR2 cDNA lacking its 3' UTR, which could not be inhibited by miR-17-
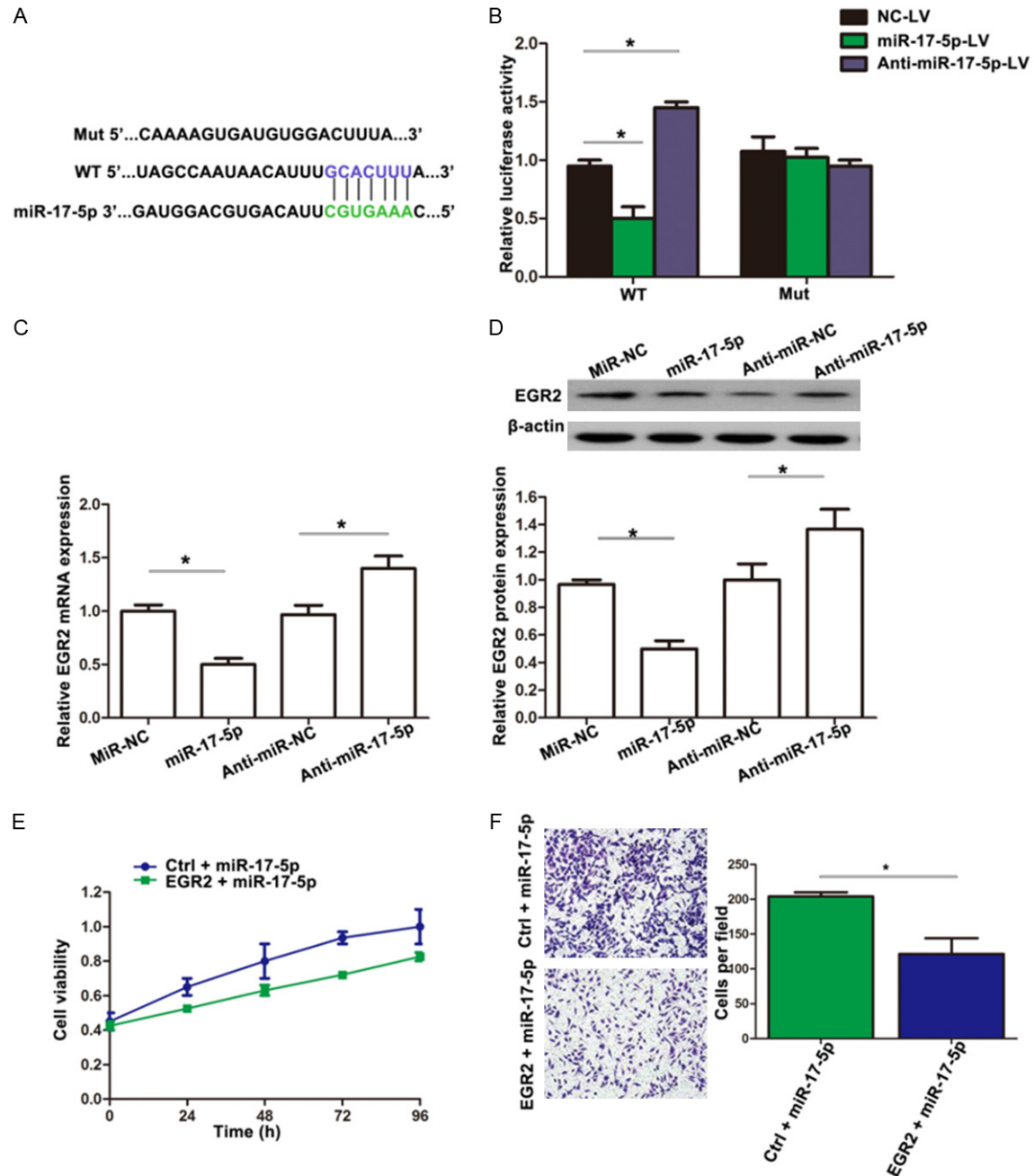
Figure 4. Identification of EGR2 as target of miR-17-5p. A. Wild type of the mutated sequences of EGR2 3' UTR (nucleotides 458-465). B. Luciferase activity was detected after SGC7901 cells were co-transfected with miR-17-5p precursor or miR-17-5p inhibitor with EGR2 3' UTR fragment with either the miR-17-5p target sequence (WT), or a mutant (Mut). C. qRT-PCR was used to detect expression of EGR2 mRNA in cells transfected with miR-17-5p precursor/inhibitor or the corresponding control. D. Western blot was used to detect EGR2 protein level in cells transfected with miR-17-5p precursor/inhibitor or the corresponding control. β-actin was used as an internal control. E. SGC7901 cells transfected with miR-17-5p were treated with EGR2 or control, then subjected to CCK-8 assay. F. Representative microscopy images and quantitative data of invasion cells transfected with miR-17-5p were treated with EGR2 or control (100 ×). Error bars represent SD, n=3, *$P < 0.05$.

5p. EGR2 overexpression was able to inhibit both proliferation (**Figure 4E**) and metastasis (**Figure 4F**). Altogether, these data support the statement that EGR2 is a direct and functional target mediating the proliferative and tumorigenic effects of miR-17-5p in SGC7901 cells.
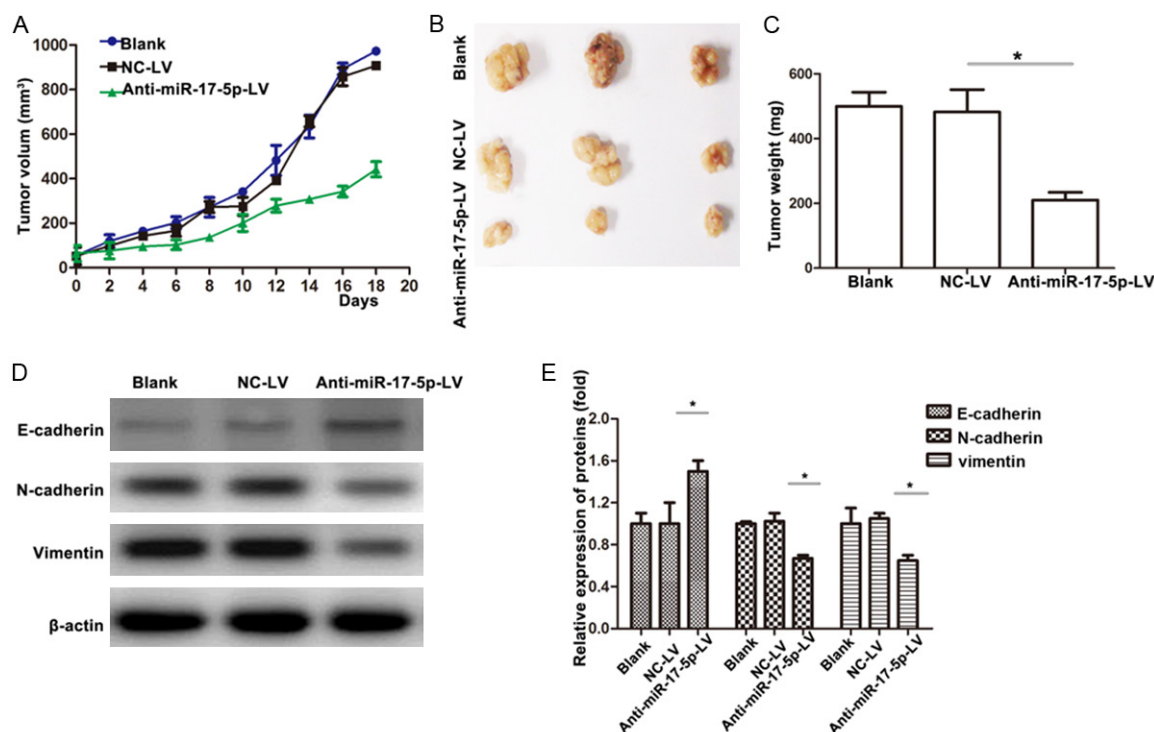
Figure 5. Inhibition of growth of SGC7901 xenografts in nude mice by anti-miR-17-5p-LV. 9 female Balbc/nu mice were randomly separated into 3 groups of 3 each. SGC7901 cells were transfected with anti-miR-17-5p lentivirus or the corresponding control, then transplanted subcutaneously into mice. A. Tumor growth was measured every 2 days of injection. B. Images of xenograft tumors. Tumors were harvested on day 18. C. Mean tumor weights. D, E. Proteins expression of E-cadherin, N-cadherin, and vimentin was detected by Western blot in tumors. Error bars represent SD, n=3, *$P < 0.05$.

MiR-20a promotes tumorigenicity and metastasis in vivo

To further demonstrate the role of miR-17-5p, we injected SGC7901 cells infected with control or anti-miR-17-5p lentiviral vectors into the female nude mice. As shown in **Figure 5A**, tumors infected with anti-miR-17-5p lentiviral vectors grew much more slowly than the Blank or NC-LV group, and the tumor weight was also significantly less than the Blank or NC-LV group (**Figure 5B** and **5C**). To further confirm the effects of miR-17-5p on tumor metastasis, we evaluated its effect on metastasis marker proteins. As exhibited in **Figure 5D** and **5E**, knockdown of miR-17-5p led to the upregulation of E-cadherin expression and the downregulation of N-cadherin and Vimentin expression *in vivo*.

Discussion

Although it is clear that several miRNAs changed their expression pattern in tumor development, less has been known concerning the relationship between particular miRNA and GC. Hence, it is of great value to explore the function of miRNAs specifically involved in GC carcinogenesis and dig out the underlying mechanisms.

miR-17-5p belongs to the miR-17-92 cluster, and it reside in intron 3 of the C13orf25 gene at 13q31.3 [15]. Aberrant expression of miR-17-5p had been found in several cancers such as gastric cancer, lung cancer, and prostate cancer [17-19]. It is reported that miR-17-5p overexpression can inhibit cellular proliferation, invasion, and tumor metastasis in some cancer cell lines [20], suggesting a tumor suppressor role for miR-17-5p. On the contrary, some studies have shown that miR-17-5p may promote proliferation in ovarian carcinoma, and colorectal cancer [21, 22], indicating the oncogenic role of miR-17-5p in tumor development. The opposite difference may be down to the fact that miR-17-5p functions differently in different cancers depending on the specific cell type and extracellular factors. However, conflicting resu-

Its still exist concerning the exact functions of miR-17-5p in human GC progression. Thus, we aim to illustrate the precise role of miR-17-5p in the malignant progression of GC *in vitro* and *in vivo*.

Previous studies have indicated that miR-17-5p is associated with cellular proliferation and apoptosis [23]. In this study, we showed that the expression of miR-17-5p was significantly increased in human GC tissues and in highly metastatic GC cells than that in corresponding normal tissues and normal cells respectively, which was consistent with a previous study [24]. Thus, we speculate that miR-17-5p may be a novel oncogenic miRNA and its deregulation may involve the advanced progress of human GC. Therefore, the main focus of this research was to clarify the functions and molecular mechanisms of miR-17-5p in human GC.

In this study, we found that miR-17-5p is related to cell growth, knockdown of miR-17-5p potently decreased the proliferation of SGC7901. Soft-agar colony formation assay showed that miR-17-5p can promote cell growth in SGC7901. In addition, we found that knockdown of miR-17-5p can promote apoptosis. Invasion assay showed that miR-17-5p was associated with invasion activity in GC cells. Similar results were also observed in Wang M et al.'s studies, they reported that the levels of circulating miR-17-5p/20a might be a molecular marker for GC [25]. They also found that knockdown of miR-17-5p/20a resulted in cell cycle arrest and increased apoptosis in AGS cells and *in vivo* [26]. To investigate the *in vivo* potential of miR-17-5p in GC, we established the model of down-expressed miR-17-5p GC cancer xenografts in nude mice. Growth curves results showed that lentivirus mediated anti-miR-17-5p can significantly suppress growth of GC cancer xenografts in nude mice. *In vivo* Western blot also demonstrated that the downstream proteins E-cadherin and N-cadherin were involved in the miR-17-5p-mediated oncogenic roles.

To identify the targets of miR-17-5p, we used miRNA target prediction programs (Targetscan 6.2 and miRDB) to search candidate targets. EGR2, the candidate target of miR-17-5p, was further confirmed by using Luciferase activity assay. We also found that both mRNA and protein levels of EGR2 decreased in SGC7901 cells transfected with miR-17-5p but increased in SGC7901 cells transfected with anti-miR-17-5p. These results suggested that EGR2 was a target of miR-17-5p in GC cells. EGR2 belongs to the Egr family genes encoding C2H2-type zinc-finger proteins [27]. EGR2 plays an important role in the regulation of cell proliferation and cell cycle [28]. EGR2 was a target of the p53 family, and overexpression of EGR2 led to apoptosis, while down-regulation of EGR2 attenuated p53-mediated apoptosis [29]. It is reported that EGR2 could be also regulated by other miRNAs [30]. We also found that EGR2 suppressed both SGC7901 cell growth and invasion, suggesting that it might function as a tumor suppressor.

In conclusion, for the first time our study demonstrates that miR-17-5p expression is upregulated in GC tissues, lymph node metastases and GC cells. miR-17-5p promotes cell growth, migration and invasion by targeting EGR2 in human GC cells. Moreover, we confirmed EGR2 as a target of miR-17-5p. To the best of our knowledge, we are the forerunner to illustrate that miR-17-5p may be a biomarker and therapeutic target in GC therapy one day.

Acknowledgements

Disclosure of conflict of interest

None.

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