Tumor Suppressor Role of miR-363-3p in Gastric Cancer

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Data Collection **B**
Statistical Analysis **C**
Data Interpretation **D**
Manuscript Preparation **E**
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Background: Gastric cancer (GC) is the most common cancer in the world. Despite the advancement of the treatment of GC, the 5-year overall survival rate is still very low. MicroRNAs (miRNAs) play important roles in the pathogenesis of GC. A recent study suggested that miR-363-3p plays a role in the development of GC. However, the function of miR-363-3p in GC is not fully understood.

Material/Methods: The network of NOTCH1 and the involved molecules was constructed by use of Cytoscape software. MiR-363-3p levels in GC tissues and cells were tested by qRT-PCR. Cells were miR-363-3p mimics or anti-miR-363-3p transfected by Lipofectamine. Bioinformatics algorithms from TargetScanHuman were used to predict the target genes of miR-363-3p. The NOTCH1 protein level was tested by Western blot. The interaction between miR-363-3p and NOTCH1 was confirmed by dual luciferase assays.

Results: MiR-363-3p showed low levels in GC tissues and cells. Enforced expression of miR-363-3p inhibited cell growth and migration of GC cells and vice versa. NOTCH1 is the targeted gene of miR-363-3p.

Conclusions: MiR-363-3p plays a tumor suppressor role in GC.

MeSH Keywords: Cell Proliferation • MicroRNAs • Receptor, Notch1 • Stomach Neoplasms

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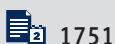
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Background

GC is the most common cancer in the world. Almost two-thirds of the cases occur in developing countries and 42% in China alone. In 2014 there were 221,478 GC-related deaths in China, accounting for nearly half of all the GC deaths worldwide [1,2]. Importantly, Chinese GC patients have a worse outcome than GC patients in the USA. Despite advancements in the treatment of GC, the 5-year overall survival rate is still very low [3,4]. Thus, development of novel therapeutic methods is urgently required. However, the molecular mechanism of the pathogenesis of GC remains unclear.

MiRNAs are evolutionarily conserved small non-coding RNAs involved in the regulation of gene expression and protein translation [5]. It has been shown that many miRNAs are involved in tumor progression and development in GC. For example, miR-421 is up-regulated in GC tissues and the overexpression of miR-421 is an early diagnostic marker [6]. A recent study showed a prognostic signature for GC that consists of 4-risk miRNAs profile: miR-10b, miR-21, miR-223, and miR-338. The 4-miRNAs profile was associated with clinical outcomes [7].

The roles of miR-363-3p have not been fully investigated in GC. A recent study suggested that miR-363-3p plays a role in the development of GC. MiR-363-3p is down-regulated in GC tissue and the level of miR-363-3p is significantly associated with the degree of tumor differentiation [8]. However, the function of miR-363-3p in GC is not fully understood, and the underlying mechanism remains unclear.

In this study, we initially tested the level of miR-363-3p in GC, and studied the function of miR-363-3p and the involved pathogenesis. We hope our data will prove useful in future research.

Material and Methods

Integrative network analysis

The network of NOTCH1 and the involved molecular factors was constructed by use of Cytoscape software. The network deciphered the complex interplay of miR-363-3p and NOTCH1 and suggested possible roles in the pathogenesis of GC [9].

Cell culture

The human gastric epithelial mucosa cell line GES-1 and the human gastric cancer cell lines SGC-7901 and AGS were purchased from Sichuan University (Chengdu, China). The GES-1, SGC-7901, and AGS cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) (Invitrogen Corp, Grand Island, NY, USA).

Detection of miR-363-3p level in GC tissues or cells

Trizol reagent (Invitrogen Corp, Grand Island, NY, USA) was used to extract the total RNAs of GC tissues or cell lines according the manufacturer's instructions. The mir-VanaTM isolation kit (Ambion, Shanghai, China) was used to isolate total RNA, including microRNA, from GC tissues and SGC-7901 and AGS. The total RNAs were reverse-transcribed to cDNA by using the All-in-oneTM miRNA First-Strand cDNA Synthesis Kit (Invitrogen Corp, Grand Island, NY, USA). The primers were designed and synthesized by Shengong Company (Shengong, Shanghai, China). Real-time PCR assay was performed as described previously [10,11].

miRNA transfection

miR-363-3p mimics and anti-miR-363-3p (antisense oligonucleotides) and the miRNA mimics negative control were purchased from Ambion (Ambion Life Technologies, Austin, TX). SGC-7901 and AGS cells were seeded into 24-well plates and then transfected with miR-363-3p mimics or anti-miR-363-3p using Lipofectamine (Invitrogen, Shanghai, China), according to the manufacturer's instructions [11].

Cell proliferation and migration assay

After 24 h of transfection of miR-363-3p mimics and anti-miR-363-3p, SGC-7901 and AGS cells (5×10^3/well) were seeded into 96-well plates. Then MTT experiments were performed as previously described [12,13]. Absorbance in each well was measured by using a microplate reader set at 570 nm. To measure cell migration, 8-mm pore size culture inserts (Transwell; Falcon, BD Biosciences) were placed into wells of 24-well culture plates, separating the upper and lower chambers. In the lower chamber, 600 µL DMEM containing 10% FBS was added, then 1×10^5/well cells were added to the upper chamber. After 24-h incubation, migrated cells were counted by use of a counting chamber [14].

Target prediction

Bioinformatics methods were used for the prediction of the targeted genes of miR-363-3p using the bioinformatics algorithms from TargetScanHuman [15–20].

Patients

Ten GC specimens were collected from the Division of Intestinal Surgery, Department of Abdominal Surgery, Sichuan Cancer Hospital, Chengdu. Tissue samples were immediately frozen in liquid nitrogen after isolation. Informed consent was obtained from each patient. The Ethics Committee of Sichuan Provincial Cancer Hospital and the Ethics Committee of Sichuan

Supplementary Table 1. The clinical data of 10 GC patients.

No.	Age (years)	Gender	Stage	Differentiation
1	63	Male	II	Low
2	59	Female	I	Low
3	57	Female	III	Low
4	36	Male	IV	Moderate
5	68	Male	III	Moderate
6	49	Female	III	Low
7	57	Female	IV	Moderate
8	73	Male	IV	Moderate
9	65	Male	II	Moderate
10	75	Male	II	Low

University approved this study. The senior pathologists of Sichuan Provincial Cancer Hospital evaluated the histological features of the specimens [21]. The clinical data from these 10 GC patients are provided in Supplementary Table 1.

Dual luciferase assays

To confirm whether NOTCH1 have the direct binding of miR-363-3p, a dual luciferase assay was performed [22,23]. The 3'UTR of NOTCH1 was amplified by using PCR from genomic DNA. The production was inserted into the downstream of NOTCH1 3'UTR reporter plasmids (pRL-NOTCH1) (Biotech, Chengdu, China), Mutants of NOTCH1 3'UTR were generated by use of the Site-Directed Mutagenesis kit (Shanghai, China), then the whole plasmid was confirmed by sequencing. Mutations in the miR-363-3p binding site of the NOTCH1 3'UTR were constructed by Shengong Company (Shengong, Chengdu, China). The luciferase reporter-containing mutant was constructed. For luciferase assays, SGC-7901 was transfected with luciferase reporter plasmid along with miR-363-3p mimics or negative control by using Lipofectamine 2000 (Invitrogen, Shanghai, China). At 24 h after transfection, these cells were analyzed by using a luciferase assay kit (Promega, Madison, WI, USA) [24].

Western blot

At 48 h after treatment, cells were washed with cold PBS and subjected to a lysis buffer. Protein lysates were separated using 8% SDS-polyacrylamide gel electrophoresis, then electro-transferred onto nitrocellulose filter membranes. The membranes were blocked with a buffer containing 5% non-fat milk in PBS with 0.05% Tween-20 for 2 h and incubated with primary antibody (anti-NOTCH1) overnight. Then the membranes were incubated with peroxidase-conjugated secondary antibodies

(Milli-pore, Darmstadt, Germany) and developed using an enhanced chemiluminescence detection kit (Pierce, Rockford, IL). β-actin was used as blank control.

Statistical analysis

The 2-tailed Student's *t* test was used to analyze the difference between the 2 groups. ANOVA was used to analyze the difference among 3 groups. SPSS Version 19 was used to perform all statistical analysis. Values are expressed as the mean ±SD from the 3 tests. All values of $P<0.05$ are marked with an asterisk and were considered to be statistically significantly different.

Results

The miR-363-3p and its predicated target genes were shown in the protein network in GC

We initially predicted the target of miR-363-3p and the integrative network by bioinformatics methods. The target of miR-363-3p was predicted by TargetScanHuman and the integrative network analysis was performed by Cytoscape software. The target prediction indicated that NOTCH1 could be targeted by miR-363-3p (Figure 1A). A previous study showed that miR-139-5p exerts a tumor suppressor function by targeting NOTCH1 in colorectal cancer [14]; therefore, NOTCH1 was chosen for further study. The GC protein-protein network was shown (Figure 1B)

The role of miR-363-3p in GC cells

Next, we tested the function of miR-363-3p *in vitro*. The miR-363-3p levels in 2 GC cell lines, SGC-7901 and AGS, were tested by qRT-PCR, and the miR-363-3p level in the immortalized
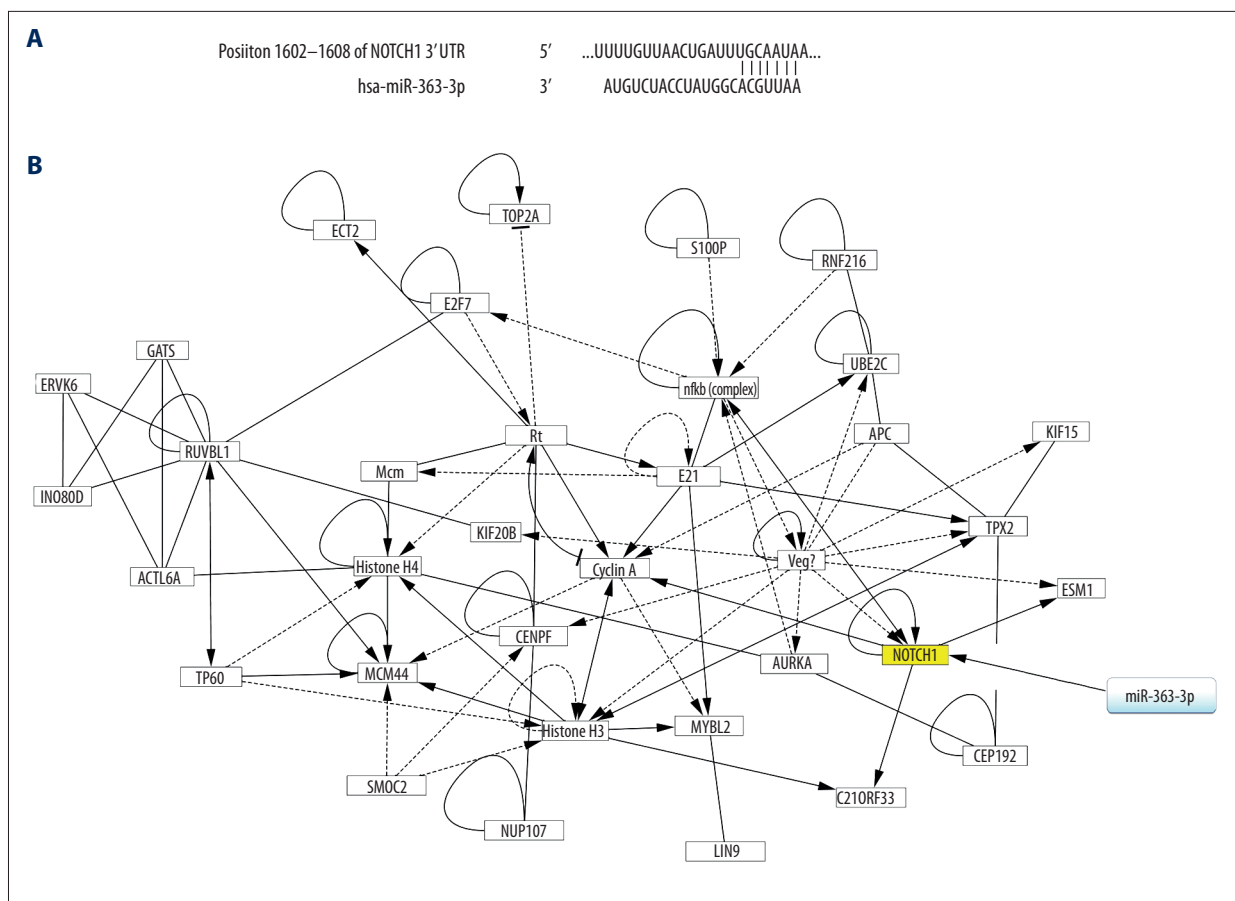
Figure 1. Regulatory networks involved in GC. TargetScanHuman (www.targetscan.org) showed that NOTCH1 is a direct target of miR-363-3p (**A**). The network of NOTCH1 and the involved molecular was performed by use of Cytoscape software (**B**).

human gastric epithelial mucosa cell line GES-1 was used as the negative control. We found that SGC-7901 and AGS showed lower miR-363-3p levels than GES-1 (Figure 2A). Next, SGC-7901 and AGS cells were transfected with miR-363-3p mimics or anti-miR-363-3p. The miR-363-3p levels in these transfected cells were examined by qRT-PCR 48 h later. We found that miR-363-3p mimics up-regulated the miR-363-3p levels in SGC-7901 and AGS cells, and anti-miR-363-3p inhibited the expression of miR-363-3p (Figure 2B, 2C). After the transfection, we assayed the growth of SGC-7901 and AGS cells by MTT analysis and found that miR-363-3p mimics inhibited cell growth and anti-miR-363-3p promoted cell growth (Figure 2D). Migration assay showed that miR-363-3p mimics inhibited the migration of SGC-7901 and AGS cells. In contrast, anti-miR-363-3p promoted cell migration (Figure 2E). We then assayed the miR-363-3p levels in 10 GC specimens and the found that the GC tissues had lower levels than the normal gastric tissues (Figure 2F).

NOTCH1 is the targeted gene of miR-363-3p

We next mutated the binding site for miR-363-3p in NOTCH1, and the mutated version of NOTCH1 is listed in Figure 3A. The

effect of miR-363-3p on the NOTCH1 translation was tested by luciferase reporter assay and the luciferase reporter plasmid with wild-type 3'UTR of NOTCH1 or mutant. We found that overexpression of miR-363-3p significantly reduced the luciferase activity of the reporter gene with wild-type 3'UTR of NOTCH1, but not with the mutant (Figure 3B). Furthermore, enforced expression of miR-363-3p decreased the protein levels of NOTCH1 in SGC-7901 cells (Figure 3C).

Disscusion

The worldwide incidence of GC is declining, but Chinese incidence remains very high. Thus, development of novel therapeutic methods is urgently required, especially in China. In this study, we explored the role of miR-363-3p in GC. We found that miR-363-3p showed low levels in GC tissues and cells. Enforced expression of miR-363-3p inhibited cell growth and migration of GC cells. NOTCH1 is the targeted gene of miR-363-3p.

The roles of NOTCH1 in GC have been investigated in several studies [25–33]. A previous study showed that NOTCH1
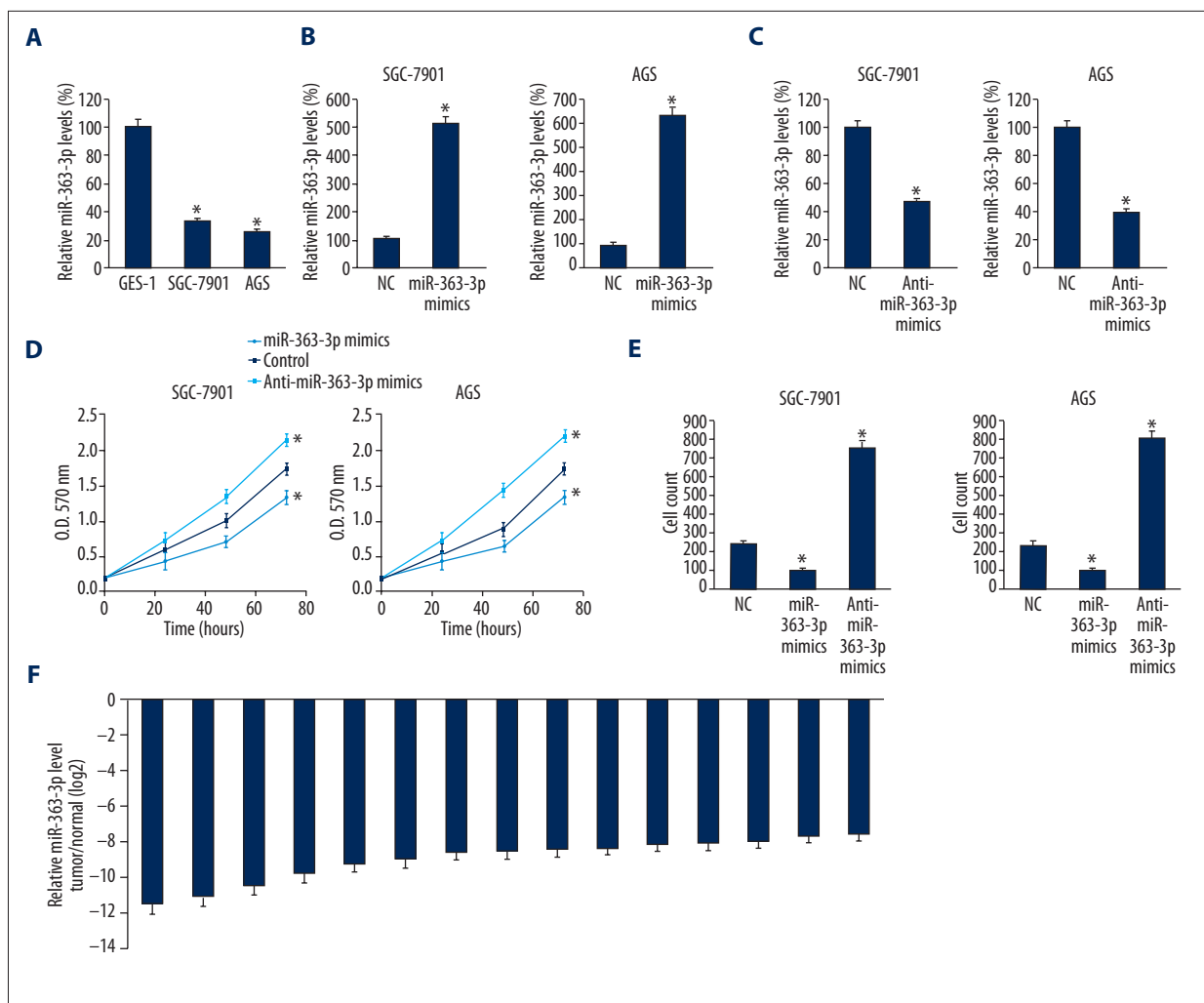
Figure 2. Overexpression of miR-363-3p inhibited cells growth and invasion and vice versa. The miR-363-3p levels in GES-1, SGC-7901, and AGS cells were tested by qRT-PCR. The miR-363-3p level in GES-1 cells was deliberately treated as 100% (**A**). After transfection of miR-363-3p mimics, the miR-363-3p levels in SGC-7901 and AGS cells were examined. The miR-363-3p level in negative controls was deliberately treated as 100% (**B**). After anti-miR-363-3p transfection, the miR-363-3p levels in SGC-7901 and AGS cells were examined. The miR-363-3p level in negative control transfected cells was deliberately treated as 100% (**C**). After miR-363-3p mimics or anti-miR-363-3p transfection, the cellular proliferation of SGC-7901 and AGS were tested by MTT analysis (**D**). After miR-363-3p mimics or anti-miR-363-3p transfection, cells were collected for migration testing (**E**). Ten GC specimens were collected. The miR-363-3p levels in tumor tissues and adjacent normal gastric tissues were examined. Data are shown as log2 of fold change of GC tissues relative to adjacent normal tissues (**F**). The experiments were performed 3 times. Data are mean ±SD *P<0.05

activation is an independent poor prognostic factor in GC patients [25]. Furthermore, NOTCH1 silencing inhibits the proliferation of gastric SGC-7901 cells by decreasing the expression of cyclins D1 and A1, and reduces the invasive ability of SGC-7901 cells through the down-regulation of MMP-2 and COX-2 genes [27]. In the present study, NOTCH1 is the targeted gene of miR-363-3p. We hypothesized that miR-363-3p might also be a prognostic factor in GC patients. We will investigate this in future studies by collecting more GC tissues. Our data also showed that miR-363-3p inhibited the cells growth and

migration of SGC-7901 and AGS cells. This data is consistent with previously reported results [27].

A recent study showed that, in adenocarcinoma of the uterine cervix, overexpression of miR-363-3p by more than 2.5-fold relative to the normal control was a strong predictor of favorable prognosis [34]. This is consistent with our data, and we will investigate the role of miR-363-3p in GC patients in further investigations. Another study indicated that bezo(a)pyrene initiates gastric cancer in a dose-dependent manner [35], and we will use this model for further study.
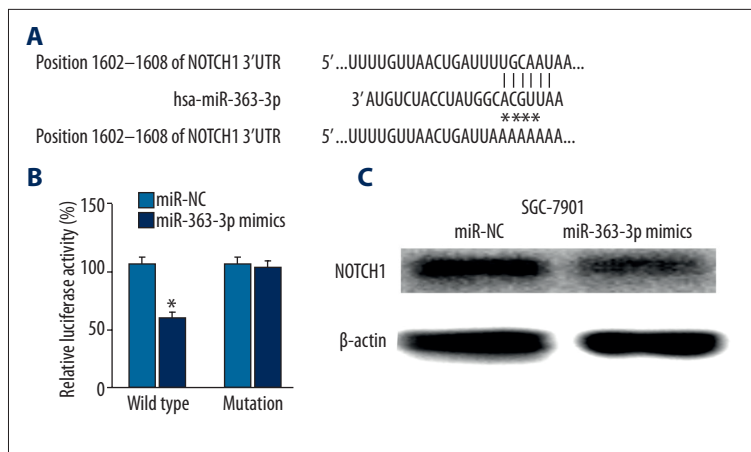
Figure 3. MiR-363-3p targeted NOTCH1. The binding site for miR-363-3p in NOTCH1 was mutated (**A**). SGC-7901 cells were co-transfected with miR-363-3p mimics or control and reporter plasmid or the mutant 3'UTR of NOTCH1, together with the controls. At 48 h after transfection, the luciferase activity was measured (**B**). MiR-363-3p mimics was transfected into SGC-7901 cells, and the NOTCH1 protein level was tested by Western blot 48 h later (**C**). The experiments were performed 3 times. Data are mean ±SD *P<0.05

Conclusions

Our study identified miR-363-3p as a novel tumor suppressor that inhibits human GC cell proliferation and invasion. Our data indicate that miR-363-3p might be a potential molecular target for the development of novel treatments of GC.

Conflicts of interest

The authors declare no conflicts of interest.

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