Effects of downregulated expression of microRNA-187 in gastric cancer

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Abstract. microRNAs (miRNAs) are involved in cancer development and progression, and have regulatory roles as tumor suppressors or oncogenes. Although aberrant expression of miR-187 has been observed in several types of cancer, its pathophysiological role and relevance to tumorigenesis in gastric cancer (GC) remains unknown. In the present study, the expression and biological role of miR-187 was investigated in 32 specimens of GC tissues and their adjacent non-tumorigenic controls, and the association between miR-187 expression and clinical features of GC were analyzed further. Kaplan-Meier survival curves determined the clinical significance of miR-187 expression in GC. Following transfection with miR-187 mimics, the biological functions of miR-187 were determined by cell proliferation and cell cycle assays. Moreover, following transfection with miR-187 mimics, the targets regulated by miR-187 were investigated using western blotting. Luciferase reporter assays confirmed whether miR-187 regulated MAD2 mitotic arrest deficient-like 2 (MAD2L2) and stomatin (EPB72)-like 2 (STOML2) expression. The data of the present study revealed that miR-187 was significantly downregulated in GC compared with adjacent non-tumorigenic counterparts. Furthermore, decreased expression of miR-187 correlated with cell differentiation (P<0.05), TNM staging (P<0.05) and poor prognosis in GC patients. Functional studies indicated that miR-187 overexpression evidently inhibited MGC-803 cell proliferation *in vitro* and altered the cell cycle by arresting cells in the G_0/G_1 phase. In addition, the luciferase assay and western blotting revealed that MAD2L2 and STOML2 were targeted by miR-187. In conclusion, it is suggested that miR-187 functions as a tumor suppressor in GC, and is important in the development and progression of GC. Moreover, miR-187 may be a potential biomarker and therapeutic target in GC.

Introduction

Gastric cancer (GC) is one of the most common malignancies, and is considered as the second leading cause of cancer-related mortality worldwide (1). Although the five-year post-operative survival rate of GC has improved to some extent and concordantly with advances made in specific treatment, the prognosis for GC remains very poor. Further clarification of the mechanism underlying GC development from the molecular level and seeking of novel biological cancer markers and effective therapeutic targets may aid in improving the prognosis of GC.

microRNAs (miRNAs) are a class of endogenous, small non-coding regulatory RNAs that are 19-25 nucleotides in length, and are known to be involved in the regulation of gene expression by repressing translation or by decreasing the stability of mRNAs (2). In total, >2,578 types of miRNA have been discovered, and they regulate ~1/3 of human protein-encoding genes (3). Accumulating evidence has indicated that miRNAs are important in numerous crucial biological processes, including cell proliferation, apoptosis and energy metabolism (2,4,5). Moreover, it has been previously reported that ~50% of miRNAs are located in the chromosomal regions that are known to be frequently deleted or amplified in human cancer cells (6). Previous studies revealed that miRNAs may function as oncogenes or tumor suppressors that are involved in tumorigenesis (7,8). In addition, emerging evidence has confirmed that aberrant expression of miRNAs in GC, including miR-21, miR-27a and miR-218, are involved in tumor growth, invasion and metastasis (9-11). These data have indicated the importance of miRNAs in GC development and have provided insights into the mechanisms that underlie tumorigenesis.

miR-187 has been revealed to be associated with numerous neoplasms (12,13). However, mir-187 is differentially expressed between normal and different cancer tissues, which has revealed the various effects of prognostic evaluation (13). Mulrane *et al* (12) reported that miR-187 expression in breast cancer leads to a more aggressive, rapidly progressing and invasive phenotype, which serves as an independent predictor of the outcome. However, Zhao *et al* (13) revealed that miR-187

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was downregulated in renal cell carcinoma tissues, and lower miR-187 expression levels were associated with higher tumor grade and staging. To date, the expression of miR-187 and its clinical significance in GC remain poorly understood.

In the present study, the expression and clinical significance of miR-187 in the tissues of GC were explored. Cell proliferation *in vitro* and cell cycle phase analysis were further investigated by the ectopic expression of miR-187 in a GC cell-line. Furthermore, the potential target genes and their expression levels were determined. The observations of the present study may assist in the elucidation of the functions and pathophysiological roles of miRNA-187 and provide a formal basis for further research into the role of mir-187 in gastric carcinoma.

Materials and methods

Patients and tumor tissues. In total, 32 pairs of human gastric tissue samples were obtained from patients who underwent surgical resection in the Department of Gastrointestinal Surgery at the First Hospital of Wenzhou Medical College (Wenzhou, China) between 2008 and 2012. Patients were aged between 42 and 80 years, with a mean age of 65 years, and were diagnosed with GC based on histopathological evaluation. The matched normal adjacent tissue was obtained from a segment of the resected specimens that was the farthest from the tumor (i.e., >5 cm). The samples were snap-frozen in liquid nitrogen and stored at -80˚C. None of the patients received chemotherapy or radiotherapy prior to surgical excision. In addition, the tumor histological grade was staged using the TNM staging of the International Union against Cancer/American Joint Committee on Cancer (AJCC) system (2002) (14). The study was then approved by the Research Ethics Committee of Wenzhou Medical College, and written informed consent was obtained from all patients.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) quantification of miR-187. Total RNA was extracted from the specimens using TRIzol reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer's instructions. In order to remove potentially contaminated DNA, the extracted RNA samples were further purified using DNase I reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Inc.) detected RNA purity by measuring the OD_{260}/OD_{280} ratio and calculating the RNA concentration and purity. Only an OD_{260}/OD_{280} ratio >1.8 was considered suitable for subsequent experiments. At last, the integrity of the RNA was checked by 1.0% denaturing agarose gel electrophoresis (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Xue *et al* (15) reported a method of detecting miRNA by stem-loop qPCR, where GAPDH RNA was used as an endogenous control. Using this approach, the expression of miR-187 was analyzed in tissue samples. The first-strand cDNA synthesis was performed with the ReverTra Ace® qPCR RT kit (cat. no. FSQ-101; Toyobo Co., Ltd., Osaka, Japan) using 0.5 μg total RNA as the template and specific reverse primers at 16˚C for 30 min, 42˚C for 30 min and 95˚C for 5 min of reverse transcription. The resulting cDNA was amplified by PCR using miRNA specific primers with SYBR® Green Real-Time PCR Master Mix (Toyobo Co., Ltd.) and was performed using an Eppendorf Mastercycler Ep Realplex (SABiosciences; Qiagen Sciences, Inc., Gaithersburg, MD, USA). The primers used for qPCR are listed in Table I. PCR parameters were as follows: 95˚C for 5 min, followed by 40 cycles of 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 20 sec. At the end of the PCR cycles, a melting curve analysis was performed, and PCR products were analyzed using 2.5% PAGE electrophoresis.

The relative expression level of miR-187 in tissues was compared to the endogenous control or paired normal tissues. Thus, the $2^{-\Delta Cq}$ and $2^{-\Delta\Delta Cq}$ methods were used to evaluate the relative expression levels of the target (16). In these analyses; $\Delta Cq=Cq_{miR-187}-Cq_{GAPDH,}$ and $\Delta\Delta Cq=[(Cq_{miR-187}-Cq_{GAPDH})_{tumor}]-[(Cq_{miR-187}-Cq_{GAPDH})_{control}]$. When the data were calculated by the $\Delta\Delta Cq$ method, the value of the relative expression ratio <1.0 was considered to be low expression in cancer relative to the control, and a ratio >1.0 was considered to indicate a high level of expression. Moreover, all qPCR assays were run in triplicate.

Cell line and culture conditions. MGC-803 is a poorly differentiated GC cell-line and was purchased from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). MGC-803 cells were cultured in RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences, Chalfont, UK) and were all supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences), 50 U/ml penicillin-G (Invitrogen; Thermo Fisher Scientific, Inc.) and 50 μg/ml streptomycin sulfate (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C and 5% CO_2 in the air.

RNA oligoribonucleotides and cell transfection. The miRNA mimics were designed and synthesized by the GenePharma Company (Shanghai, China). miR-187 mimics (Table II) were an RNA duplex and negative control (NC) RNA duplex and were non-homologous to any human genomic sequences. All the oligonucleotide sequences used in this experiment are listed in Table II. The mimic transfection was performed with Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. The GC cells were seeded into 6-well plates and were grown to 60-70% confluence before transfection. Moreover, transfection efficiency was then monitored using qPCR.

Cell Counting Kit-8 (CCK-8) proliferation assay. The effect of miR-187 on the proliferation of GC cells was evaluated using the CCK-8 assay kit (Dojindo Molecular technologies, Inc., Kumamoto, Japan), according to the manufacturers instructions. MGC-803 cells were plated into 96-well plates at a density of $8x10^3$ cells/well in three parts. After 24 h of static culture, the cells were transfected with miR-187 mimics and the negative control (NC) using Lipofectamine 2000. The cellular proliferation capacity for the untransfected blank control (Blank), cells transfected with miR-187 mimic NCs and cells transfected with miRNA-187 mimics were measured using the CCK-8 assay. Moreover, the transfected miRNA-187 mimics group was set to final concentrations of 20, 40 and 80 nM. The cells were then incubated for 24, 48 and 72 h at 37˚C in an atmosphere of 5% CO_2 in the air. Next, a total 10 μl of the

Table I. RT-qPCR primer sequences for the amplification of miR-187 and GAPDH.

Gene	Primer	Sequence	Product (bp)
miR-187	RT stem-loop	5'-GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACCCGGCTGC-3'	61
	Forward	5'-TCGTGGGTCGTGTCTTGTGTTGC-3'	
	Reverse	5'-GCAGGGTCCGAGGTATTC-3'	
GAPDH	Forward	5'-CAGGGCTGCTTTTAACTCTGGTAA-3'	101
	Reverse	5'-GGGTGGAATCATATTGGAACATGT-3'	

RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR, microRNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; bp, base pair.

Table II. Sequence of miR-187 mimics and negative control.

RNA oligo	Sequence
miR-187 mimic	5'-UCGUGUCUUGUGUUGCAGCCGG-3' 3'-UUAGCACAGAACACAACGUCGG-5'
Negative control	5'-UUCUCCGAACGUGUCACGUTT-3' 3'-TTAAGAGGCUUGCACAGUGCA-5'

CCK-8 solution was added to each well, and the cells were incubated for a further 4 h. The absorbance (A) values were determined using a spectrophotometer (EPOCH2; BioTeK Instruments, Inc., Winooski, VT, USA) at a wavelength of 450 nm, and the experiment was repeated three times. The inhibition rate of cell proliferation=1-($A_{450 \text{ nm}}$ of the study group/$A_{450 \text{ nm}}$ of the control group) x 100%. In addition, the relative activity of the cells=$A_{450 \text{ nm}}$ of the study group/$A_{450 \text{ nm}}$ of the control group.

Cell cycle analysis. For the cell cycle analysis, cells were seeded into 6-well plates at a density of 2×10^5 cells/well, and transfected according to the protocol described above. The experimental groups and time points were set as described for the proliferation assay above. The cells were obtained after being cultured for an appropriate time by trypsinization, and were then pooled with the floating cells, centrifuged at 1,000 x g for 5 min at 4°C and stored at -20°C overnight. DNA staining solution that contained (PI) iodide and RNaseA reagent [MultiSciences (Lianke) Biotech Co., Ltd., Hangzhou, China] were added to the cells. Moreover, cell cycle analysis was performed by fluorescence-activated cell sorting flow cytometry using CellQuest software 1.0 (BD Biosciences, San Jose, CA, USA).

Western blot analysis. miR-187 mimics (5'-UCGUGUCUU GUGUUGCAGCCGG-3'; Applied Biological Materials, Inc., Richmond, BC, Canada) or negative control oligonucleotides (5'-UUCUCCGAACGUGUCACGUTT-3'; Applied Biological Materials, Inc.) at a concentration of 40 nM were transfected into GC MGC-803 cells using the Lipofectamine™ 2000 Transfection Reagent kit (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.) and cells were harvested 48 h after transfection. Proteins were extracted and separated on a 12% SDS-PAGE gel and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.). The membrane was then blocked with 5% non-fat milk proteins and incubated with anti-MAD2L2 (cat. no. ab180579; 1:2,000), anti-STOML2 (cat. no. ab37531; 1:2,000; each Abcam, Cambridge, UK) or anti-β-actin antibody (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). After being washed extensively, a goat anti-mouse secondary antibody (Pierce Biotechnology, Inc., Rockford, IL, USA) was added to the system. The proteins were detected using enhanced chemiluminescence reagents (cat. no. 32106; Pierce Biotechnology, Inc.). Protein expression levels were quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Luciferase reporter assays. In order to verify the direct interaction of miR-187 to the target genes MAD2L2 and STOML2, human mRNA sequence were cloned into the pMIR-reporter construct (Ambion; Thermo Fisher Scientific, Inc.) in order to synthesize the luciferase reporter construct. Wild-type and mutant MAD2L2 and STOML2 mRNA fragments were amplified and sub-cloned into *Hind*III sites of the luciferase reporter, and luciferase reporter assays were performed as previously described (17). MGC803 cells were co-transfected with 50 nM single-stranded miRNA mimics or internal control oligonucleotides, then plated into 24-well plates with 10 ng pRL-TK (Promega Corporation, Madison, WI, USA) and 50 ng firefly luciferase reporter using the JetPRIME reagent (Polyplus-transfection) according to the manufacturer's instructions. Cells were collected at 48 h after transfection (performed as aforementioned) and analyzed using the Dual-Luciferase Reporter Assay System (Promega Corporation).

Immunohistochemistry. Paired paraffin-embedded tissue sections (n=20) were cut into 5-μm sections, deparaffinized in xylene and rehydrated in graded series of ethanols followed by heat-induced epitope retrieval in citrate buffer (pH 6.0). The expression levels of MAD2L2 and STOML2 were detected using polyclonal antibodies targeted to MAD2L2 (cat. no. ab180579; 1:50) and STOML2 (cat. no. ab37531; 1:50; each Abcam). Next, samples were incubated with horse-radish peroxidase-conjugated secondary goat anti-mouse antibodies (cat. no. SPKIT-C7; Fuzhou Maixin Biotech Co.,

Ltd., Fuzhou, China) and visualized with 3,3'-diaminodbenzidine (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). Images obtained were processed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. The expression of miR-187 was calculated for paired groups (i.e., the tumor and paired non-tumor) were compared using non-parametric Wilcoxon tests. The statistical significance of correlation between the expression of miR-187 and the clinicopathological parameters was calculated using non-parametric tests (Mann-Whitney U test between two groups and Kruskall-Wallis test for three or more groups). The association between miR-187 expression and prognosis was analyzed using the Kaplan-Meier survival curve statistics. In the Kaplan-Meier survival curve, high expression was defined as the fold-change of >1 and low expression levels as <1 compared to the probability of survival by the Log-rank test. Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Differences were considered statistically significant at a value of $P<0.05$.

Results

Expression of miR-187 in GC tissues and matched non-tumor tissues. PCR products of miR-187 and GAPDH cDNA revealed a single band at the appropriate position (67 bp for miR-187 and 101 bp for GAPDH) on the electrophoretic gel (Fig. 1A). In addition, the melting curves of the products were sharply defined curves with a narrow peak (Fig. 1B). The combination of melting curves and gel electrophoresis confirmed PCR specificity. Furthermore, the expression of miR-187 in all 32 pairs of GC tissues and their matched non-cancerous tissues was detected using qPCR. As shown in Fig. 1C, the expression levels of miR-187 were lower in gastric tumors than in normal control tissues. The median relative expression levels of miR-187 was 0.031 (25-75th percentile, 0.013-0.102) in tumor samples, and was compared to that in non-tumor control samples, which was set at 0.086 (25-75th percentile, 0.028-0.192). Moreover, the difference in expression of miR-21 between the tumor and the control samples was statistically significant (P=0.037, Wilcoxon test).

Association between the expression of miR-187 and clinicopathological features of GC. The association between miR-187 expression and the clinical and pathological characteristics of GC were explored further (Table III). The downregulated expression levels of miR-187 were associated with cell differentiation (P=0.042) and TNM stage (P=0.034) in GC patients. However, no significant association was identified between the expression of miR-187 and the demographic or clinical variables, including gender, age, tumor location, tumor size, depth of tumor invasion and lymph node metastasis (P>0.05). According to the relative expression levels of miR-187 of paired tumor and normal tissues, specimens with a relative expression level <1 were set as Group One, and specimens with a relative value ≥1 were set as Group Two. A Kaplan-Meier survival analysis illustrated that the cohort with higher expression levels of miR-187 demonstrated greater rates of survival than those with lower levels of expression of miR-187 (Fig. 2).

Moreover, the 5-year survival rate for Group One was 66.7%, while that for Group Two was 13.5%.

miR-187 inhibited cell proliferation and induced cell cycle arrest at the G_0/G_1 phase in vitro. The significant reduction in miR-187 expression in GC samples prompted us to explore the possible biological roles of miR-187 in the mechanism of tumorigenesis. Thus, the effect of miR-187 expressing again was investigated on the proliferative capacity of the GC cell-line. Compared with that of the cells transfected with either the negative or blank control, MGC-803 cells that were transiently transfected with miR-187 mimics had a significant growth inhibition to different degrees (P<0.05; Fig. 3A). Moreover, the cell vitality of GC cells was markedly associated with different transfected concentrations and was time-dependent (P<0.05; Fig. 3A). In order to investigate further whether inhibition of MGC-803 proliferation reflected cell cycle arrest, the progression of cell cycle phases was analyzed by PI staining and flow cytometry. The results revealed that MGC-803 cells that were dose-dependently transfected with miR-187 mimics had an evident effect on cell cycle arrest at the G_0/G_1 phase (Fig. 3B and C) as compared with the blank and the negative control group (P<0.05). However, the proportion of cells that were transfected with different doses of miR-187 mimics had little effect on the cell cycle phases (P>0.05).

miR-187 targets MAD2L2 and STOML2. As miRNAs function mainly through the inhibition of target genes, the target of miR-187 that functions in the pathogenesis of GC was further analyzed. Based on previously published CLASH data, which provided direct experimental data of miRNA-targeted pairs (18), MAD2L2, STOML2 and tubulin, γ 1 (TUBG1) were identified as potential targets of miR-187. In order to confirm whether these genes that had been regulated by miR-187 were involved in the pathogenesis of GC, MGC-803 cells were transfected with miR-187 mimics or negative control oligonucleotides, and the protein expression levels of the genes were examined by western blotting. Data indicated that the expression of MAD2L2 and STOML2 were consistently and substantially downregulated by the ectopic expression of miR-187, whereas TUBG1 expression was not significantly affected by miR-187 (Fig. 4A).

Luciferase reporter assay. In order to verify the interaction between miR-187, MAD2L2 and STOML2, luciferase reporter assays were performed in MGC803 cells. The luciferase reporter plasmid with sequences of MAD2L2 and STOML2 mRNA or mutant sequences were co-transfected into MGC803 cells for 48 h with miR-187 or NC, respectively. Moreover, the luciferase activity was measured in transfected cells. The results demonstrated that the reporter plasmid of MAD2L2 and STOML2 mRNA caused a significant decrease in the luciferase activity in cells that were transfected with miR-187. By contrast, the luciferase activity of the reporter plasmid with mutant sequences of MAD2L2 and STOML2 did not change (Fig. 4B). Furthermore, GC tissues with a low miR-187 demonstrated much higher MAD2L2 and STOML2 expression when compared with paired normal gastric tissues (Fig. 5). Altogether, the results suggested that MAD2L2 and STOML2 was the target of miR-187 in GC cells.
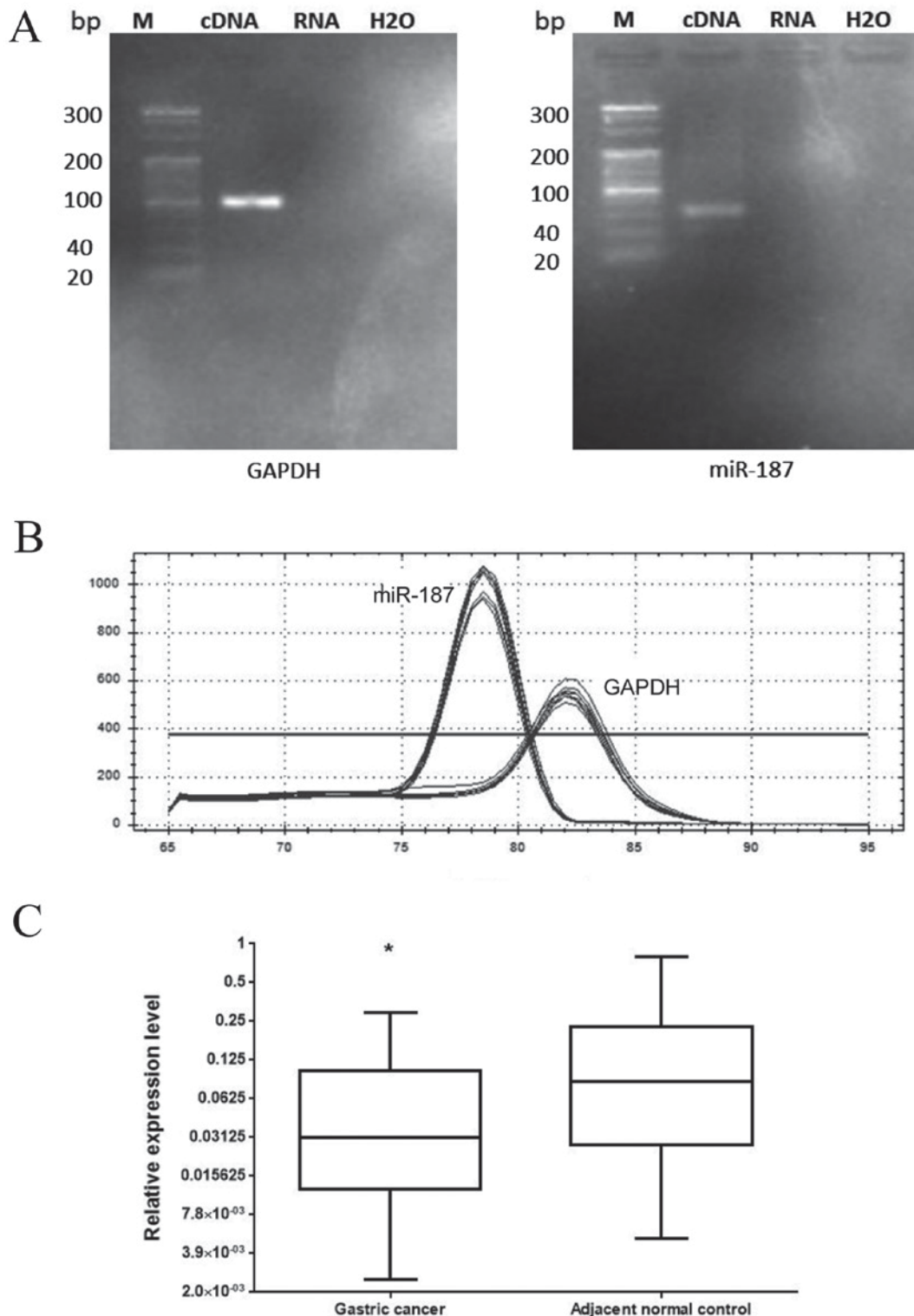
Figure 1. miR-187 was downregulated in 32 pairs of human gastric tissue samples. (A) PCR products of miR-187 and endogenous control GAPDH (Lane M, 20 bp DNA marker; lane RNA, RNA control; lane H$_2$O, Negative control). (B) Melting curve of miR-187 RT-qPCR amplified products. (C) Expression of miR-187 was measured by RT-qPCR, and the expression levels of miR-187 were normalized against GAPDH RNA expression for subsequent analyses. A P-value was obtained using a one-tailed Student's t-test. *P<0.05 vs. the control. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Discussion

Accumulating evidence has indicated that the abnormal expression of miR-187 is closely associated with cancer cell proliferation and apoptosis, and that they may serve as tumor suppressors or oncogenes (19,20). Previously, numerous studies have shown that miR-187 is anomalously expressed in various tumor types, including thyroid (21), nasopharyngeal carcinoma (22), esophageal (23) and pancreatic cancer (24) and neuroblastoma cell tumors (25). However, in different cancers,

Table III. Association between the expression of miR-187 with clinicopathological features of gastric cancer.

Clinical characteristics	Cases (n)	miR-187 expression[a]	P-value
Age (years)			0.258
<60	8	0.60±0.63	
≥60	24	0.97±0.89	
Sex			0.630
Male	23	0.86±0.87	
Female	9	0.91±0.82	
Tumor size (cm)			0.440
<5	26	0.88±0.91	
≥5	6	0.87±0.53	
Location			0.967
Upper area	4	0.59±0.17	
Middle area	7	0.77±0.87	
Lower area	21	0.96±0.92	
T stage			0.500
T1/T2	7	1.21±1.00	
T3	10	0.72±0.63	
T4	15	0.82±0.89	
Cell differentiation			0.042
Well or Moderately-differentiated	9	1.45±0.92	
Poorly-differentiated	23	0.65±0.71	
Lymph node metastasis			0.090
No	9	1.21±0.86	
Yes	23	0.74±0.82	
N stage			0.270
N1	3	1.65±1.18	
N2	12	0.65±0.85	
N3	8	0.52±0.34	
TNM stage			0.034
I/II	11	1.23±0.84	
III/IV	21	0.69±0.80	

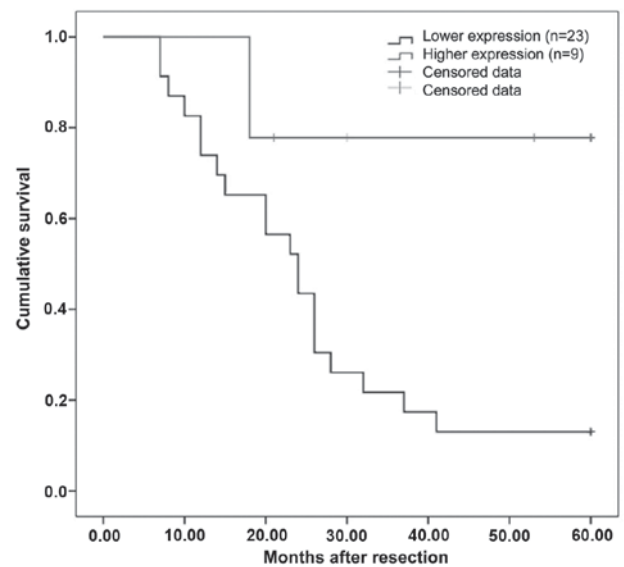[a]Mean of relative expression ± standard deviation. miR, microRNA; TNM, tumor node metastasis.

Figure 2. Analysis of the survival curves. Gastric cancer patients with high and low expression of miR-187 demonstrated differential survival statistics.

the changes in expression of miR-187 varied greatly, indicating that it may be involved in carcinogenesis and progression in a specific way.

A previous study demonstrated that miR-187 that was downregulated in tumor tissue was involved in ovarian cancer progression (26). Mulrane *et al* (12) revealed that the expression levels of miR-187 in breast cancer tissue was correlated with breast cancer invasion, and could be used as an independent prognostic factor. However, the expression level and the biological impact of miR-187 in GC remain unclear. In order to investigate the association of miR-187 and GC pathogenesis, qPCR was used to profile the expression of miR-187 in 32 matched gastric tumor tissues, and clarified the association between miR-187 and the clinicopathological characteristics of GC.

In the present study, the expression of miR-187 was found to be downregulated in gastric tumors compared to non-tumor tissues. Besides, miR-187 expression was associated with cell differentiation and TNM staging in GC patients. In addition, the Kaplan-Meier survival analysis illustrated that the cohort with a higher expression of miR-187 demonstrated higher survival rates than those cells with lower levels. These results indicated that miR-187 is important in the development and progression of GC. In view of the above, miR-187 is speculated to function as a tumor suppressor in GC, and the downregulation of miR-187 may promote its occurrence and development of GC.

To further clarify this point, the biological function of miR-187 was investigated. Deregulated cell proliferation is a key biological characteristic of neoplastic progression (27). In the present study, miR-187 mimics were transfected into MGC-803 cells, then the cell proliferation ability was evaluated. The CCK-8 assay data indicated that the overexpression of miR-187 could suppress the proliferation of GC cells. However, the reason for miR-187 induced inhibition of cell proliferation remains to be investigated. Overall, cell cycle arrest was likely to be an important factor (28,29). Indeed, the subsequent cell cycle analyses of the present study revealed that MGC-803 cells that were transfected with miR-187 mimics had an evident cell cycle arrest at the G_0/G_1 phase. Nevertheless, the proportion of cells of the different transfected groups demonstrated no difference. It is likely that the process of cell cycle arrest induced by miR-187 was influenced by another molecular mechanism since indirect regulation and miRNA may have complex roles that may formally involve multiple aspects and factors in cell cycle regulation.

The main function of miRNA is to regulate target gene expression by direct cleavage of the mRNA or by inhibition of protein synthesis according to the degree of complementarities with the 3'untranslated region of target genes (30,31). Perfect or nearly perfect base-pairing induced targeted mRNA cleavage whereas imperfect base pairing induced mainly translational
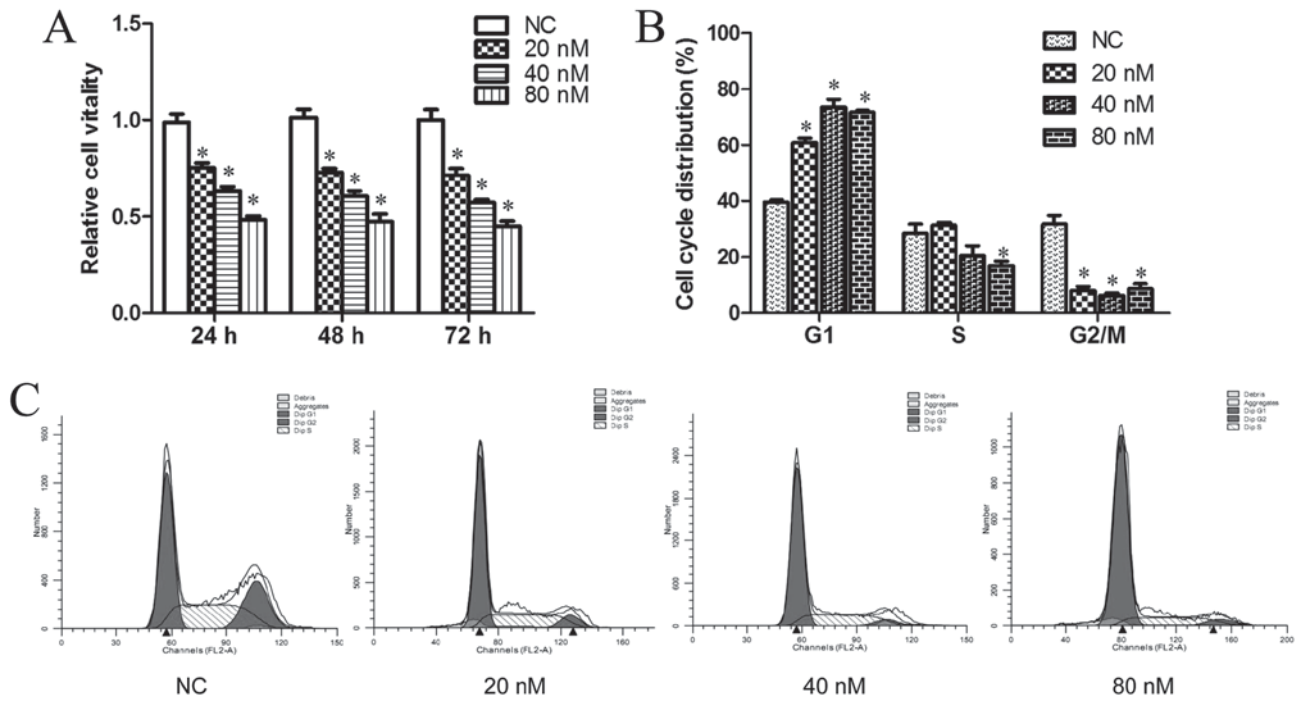
Figure 3. miR-187 restoration inhibited the viability of GC cells. (A) Relative cell vitality of the GC MGC-803 cell line was measured by the CCK-8 cell proliferation assay after being transfected with different concentrations of miR-187 mimics and at different time-points. (B and C) Cell-cycle distribution was determined by FACS analysis. MGC-803 cells were transfected with different concentrations of miR-187 mimics or negative control oligonucleotides for 24 h. Data were subjected to Student's t-test. *P<0.05 vs. NC group. NC, negative control; CCK-8, Cell Counting Kit-8; FACS, fluorescence-activated cell sorting.
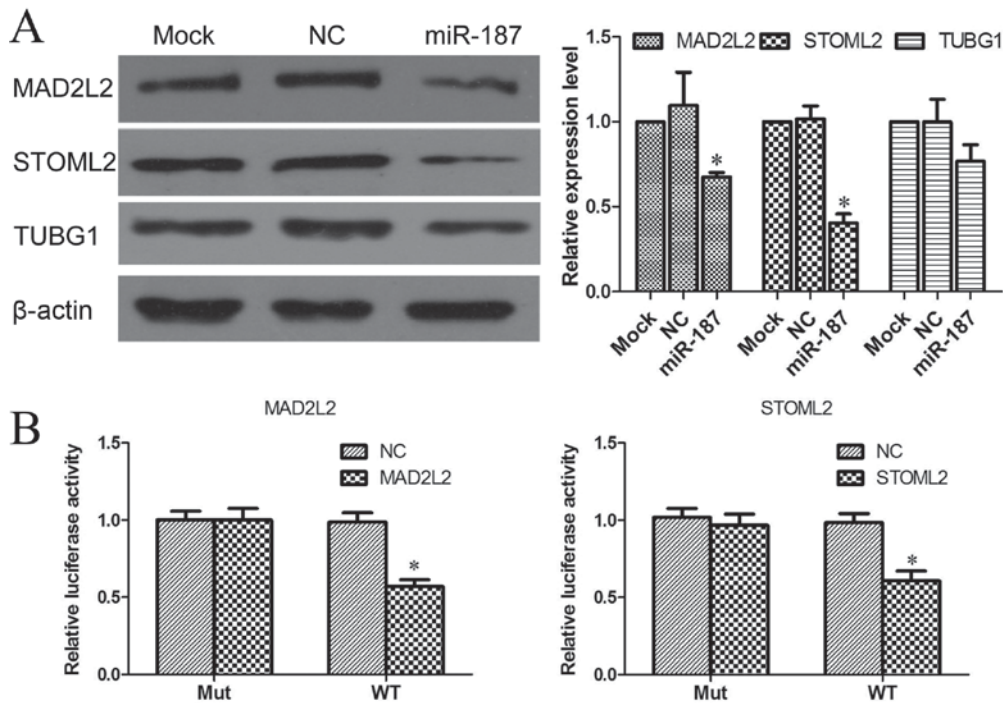

Figure 4. Effects of miR-187 on the protein expression levels of potential target genes. (A) In total, 48 h after transfection with the 40 nM miR-187 mimics or NC oligonucleotides, MGC-803 cells were analyzed by western blotting. (B) Analysis of the luciferase activity. MGC803 cells were co-transfected with miR-187 mimics and the firefly luciferase reporter plasmid containing putative miR-187 targeting sequences of MAD2L2 and STOML2. The plasmid construct pRL-TK served as an internal control. The normalized luciferase activity of the control oligonucleotides was set as the relative luciferase activity. *P<0.05 vs. blank (mock) or NC groups. MAD2L2, mitotic arrest deficient-like 2; STOML2, stomatin (EPB72)-like 2; TUBG1, tubulin, gamma 1; NC, negative control; WT, wild type.

silencing of the target (32). Chao *et al* (26) demonstrated that miR-187 inhibited the epithelial-mesenchymal transition

process and migration by targeting Dab2 in ovarian cancer cells. Furthermore, Mulrane *et al* (12) reported that miR-187
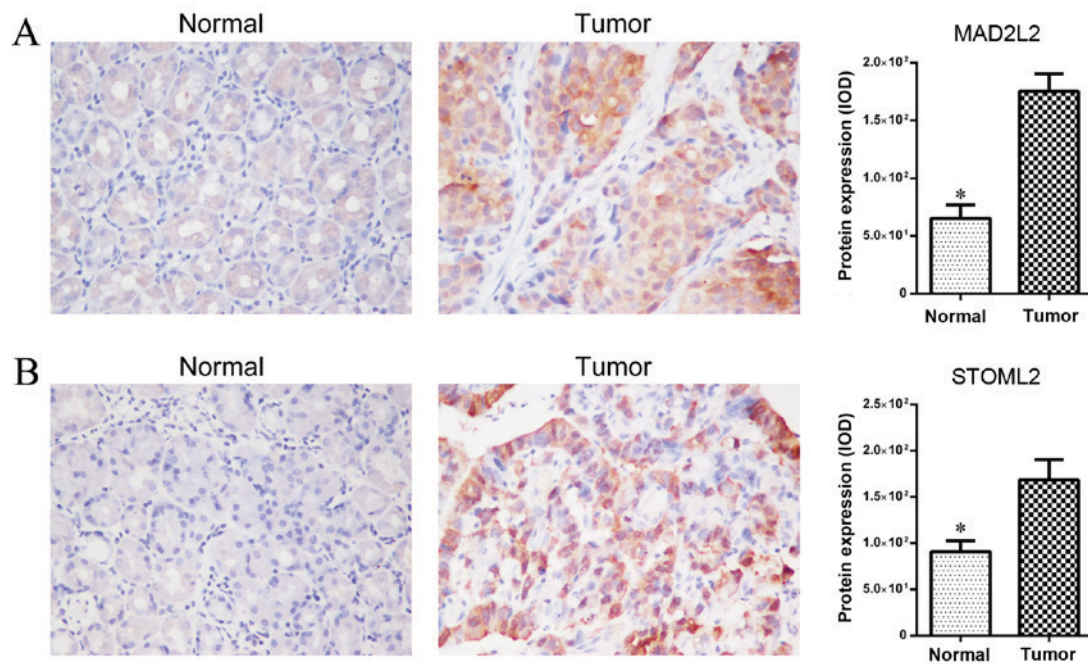
Figure 5. MAD2L2 and STOML2 were highly expressed in human gastric cancer tissues. Expression of (A) MAD2L2 and (B) STOML2 in gastric cancer tissues were determined by immunohistochemistry (magnification, x400). Normal gastric tissue served as the control (magnification, x400). The protein expression levels were measured in different tissues by semi-quantitative immunohistochemistry (n=20). *P<0.05 vs. tumor group. MAD2L2, mitotic arrest deficient-like 2; STOML2, stomatin (EPB72)-like 2; IOD, integrated optical density.

regulated collagenase (matrix metallopeptidase 13) expression by either direct or indirect pathways in breast cancer, and that this pathway may have also been involved in the migration of tumor cells. Moreover, Sirotkin *et al* (33) revealed that overexpression of miR-187 could reduce the expression of the apoptosis-related B-cell lymphoma 2-associated X protein and the proliferation marker proliferating cell nuclear antigen in human ovarian granulosa cells. However, whether or not other target genes are responsible for the effect in GC is currently unknown.

A recent advance in high-throughput direct mapping of miRNA-mRNA binding sites from CLASH experiments directly identified the miRNA-mRNA target pairs that were associated with the human AGO1 protein, and presents an improved determination of the miRNA target (18). Based on the CLASH experiment data and combined with the analysis of miR-187 interference, MAD2L2 and STOML2 were consistently and substantially downregulated by the ectopic expression of miR-187.

In the present study, luciferase reporter assays further confirmed the interaction between miR-187, MAD2L2 and STOML2. Thus, MAD2L2 and STOML2 could be considered to be potential targets that were involved in the development of GC. It has been demonstrated that MAD2L2, which is a member of a family of genes involved in mitotic checkpoint control mechanisms, were significantly upregulated in colon cancer as compared to matched normal tissue (34). In addition, tumors that show upregulated MAD2L2 expression had significantly higher numbers of aberrant mitotic figures (anaphase bridges), an indication of chromosomal instability and a poor prognosis in colorectal cancer (34). STOML2 is a member of the stomatin superfamily, and has been identified as an oncogenic-related protein, whose functional expression

is enhanced in numerous cancer types (35-38). Moreover, overexpression of STOML2 has been reported in the tissues of GC compared with the adjacent normal gastric epithelium. In addition, a high-level STOML2 expression was significantly correlated with the depth of invasion, lymph node and distant metastasis, and staging according to the AJCC and poor prognosis in GC (39).

The present data revealed that the expression of MAD2L2 and STOML2 were significantly inhibited in MGC-803 cells transfected with miR-187 mimics. Furthermore, high levels of expression of MAD2L2 and STOML2 were also confirmed in GC tissues compared with adjacent normal tissues. These results provided insights into the regulatory mechanism of miR-187 in GC. Downregulation of miR-187 in GC cells resulted in enhanced expression of its target genes, which may consequently favor tumor progression. However, miRNA displays multi-target characteristics, and has complex regulatory networks that are involved in numerous aspects of biological functions (2,40,41). Therefore, the consequent molecular mechanism of miR-187 remains to be further investigated.

In conclusion, the present study demonstrated that there was a significantly reduced expression of miR-187 in GC tissues compared with their non-tumor counterparts. Moreover, the expression of miR-187 was associated with the degree of cell differentiation, pathological staging and prognosis in GC patients. The data of the present study also indicated that miR-187 could inhibit cell proliferation *in vitro*, and could induce cell cycle arrest at the G_0/G_1 phase. To the best of our knowledge, the present study confirmed the tumor suppressive roles of miR-187 in GC for the first time, and provided evidence for the potential utility of miR-187 as a biomarker and therapeutic target against GC.

Acknowledgements

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors have approved this manuscript and agreed to be accountable for all aspects of this work. WC, JC and SS contributed in the conception and design and revision of the manuscript, the acquisition, analysis and interpretation of data. XS and LZ prepared the initial draft of the manuscript, contributed in the acquisition, analysis and interpretation of data, and helped perform the cell biology experiments. YC, JW and YY made substantial contributions to conception and design, acquisition of data, and analysis of data, and performed the western blot experiments.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Wenzhou Medical College and written informed consent was obtained from all patients.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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