

# MicroRNA-4284 promotes gastric cancer tumorigenicity by targeting ten-eleven translocation 1

YANSEN LI<sup>1-3</sup>, ZHANLONG SHEN<sup>1</sup>, HONGPENG JIANG<sup>2,3</sup>, ZHIYONG LAI<sup>1</sup>,  
ZHU WANG<sup>3</sup>, KEWEI JIANG<sup>1,2</sup>, YINGJIANG YE<sup>1</sup> and SHAN WANG<sup>1-3</sup>

<sup>1</sup>Department of Gastroenterological Surgery; <sup>2</sup>Laboratory of Surgical Oncology; <sup>3</sup>Beijing Key Laboratory of Colorectal Cancer Diagnosis and Treatment Research, Peking University People's Hospital, Beijing 100044, P.R. China

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**Abstract.** Increasing evidence has shown that abnormal expression of miR-4284 participates in the progression of several types of cancer. However, the expression and the role of miR-4284 in gastric cancer remain largely unknown. Therefore, in the present study the miR-4284 expression levels in gastric cancer tissues and cell lines, was examined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and found that miR-4284 was significantly upregulated in 40 pairs of gastric cancer tissues and five gastric cancer cell lines compared to the corresponding normal tissues and GES-1 cell line. In addition, increased miR-4284 expression was positively associated with TNM stage ( $P=0.035$ ), distal metastasis ( $P=0.022$ ) and poor prognosis in gastric cancer patients. Furthermore, the overexpression of miR-4284 expression was shown to promote cell proliferation, clone formation, invasion and migration, while the suppression of miR-4284 expression induced opposite effects. Additionally, luciferase reporter assay was conducted and showed that ten-eleven translocation 1 (TET1), a tumor suppressor gene that regulating cell survival and metastasis, was a direct target of miR-4284. Upregulated miR-4284 decreased the mRNA and protein levels of TET1 in SGC-7901 cells and downregulated miR-4284 increased the mRNA and protein levels of TET1 in AGS cells. In addition, miR-4284 expression was negatively correlated with the TET1 expression in gastric cancer tissues. Moreover, inhibition of TET1

suppressed the effect of miR-4284 inhibitors on cell proliferation in AGS cells. Therefore, data demonstrated that miR-4284 could promote tumor cell growth, migration and invasion by directly targeting TET1 in gastric cancer, which may provide a potential therapeutic target for gastric cancer treatment.

## Introduction

Gastric cancer, one of the most common malignant tumors of the digestive tract, represents a serious health threat. In China, gastric cancer is the second most common type of cancer, and the third highest cause of death of malignant tumors (1). Despite the improvements in cancer treatment in recent years, the prognosis remains unsatisfactory, especially since there is no biomarker suitable for early diagnosis (2). Therefore, the identification of an effective biomarker, elucidation of the underlying mechanisms of development, and the improvement of the treatment strategies are necessary.

Recently, microRNAs (miRNAs/miRs; ~22 nucleotides long), which negatively regulate target gene expression, attracted a lot of research attention. Aberrant expression of miRNAs has been identified in numerous cancers, and these molecules can act as tumor-promoting or suppressor genes. Increasing body of evidence demonstrates that the abnormal expression of miRNAs may be involved in the development and progression of cancers in humans (3-5). Moreover, various miRNAs were shown to be involved in the development of gastric cancer, including miR-584-5p (6), miR-27a (7), and miR-545 (8). Recently, a novel miRNA, miR-4284, was identified, and shown to promote the development of diffuse large B-cell lymphoma (9), in addition to its anti-tumor effects in glioblastoma (10). However, the expression of miR-4284 and its relationship with clinically observed digestive tract alterations remain unclear, especially in gastric cancer.

Ten-eleven translocation 1 (TET1), a member of TET family, was shown to be downregulated in different cancer types, and to decrease cell proliferation and metastasis in different cancer types, including breast (11), renal (12), and colon cancers (13). Furthermore, it was shown to represent a direct target of other miRNAs, such as miR-29a and miR-520b (14,15).

Therefore, in this study, we aimed to investigate the expression, functions, and the underlying mechanisms of miR-4284 in gastric cancer. We analyzed the expression of this molecule

*Correspondence to:* Dr Shan Wang, Beijing Key Laboratory of Colorectal Cancer Diagnosis and Treatment Research, Peking University People's Hospital, 11 Xizhimen South Street, Xicheng, Beijing 100044, P.R. China  
E-mail: shanwang60@sina.com

Dr Zhanlong Shen, Department of Gastroenterological Surgery, Peking University People's Hospital, 11 Xizhimen South Street, Xicheng, Beijing 100044, P.R. China  
E-mail: shenlong1977@163.com

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in 40 paired gastric cancer tissue samples, and the potential correlations with clinical features. Afterward, miR-4284 functions in gastric cancer cells *in vitro* were further explored. Finally, we found that TET1 was a direct target of miR-4284, which elucidated the potential mechanisms underlying the observed effects.

## Materials and methods

**Clinical specimens.** Forty pairs of frozen gastric cancer and the corresponding normal tissue samples, preserved at -80°C in our laboratory, were collected from January 2011 to August 2011 and underwent pathological examination. All patients were followed-up for at least 5 years following the tissue collection and received no anti-tumor treatment before operation. All patients provided informed consent and the study was approved by ethics committee of Peking University People's Hospital (Beijing, China).

**Cell lines and cultures.** Human gastric cancer cell lines, AGS and NCI-N87 and gastric mucosal normal cell line GES-1 were obtained from ATCC, SGC-7901, HGC-27, were obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and FU97 was purchased from JCRB. FU97 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10 mg/l insulin, while others were grown in RPMI-1640 medium (Thermo Fisher Scientific, Inc.). All media were supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), and the cells were incubated at 37°C in the atmosphere with 5% CO<sub>2</sub>.

**Cell transfection.** The miR-4284 mimics, inhibitors, and negative control (mimic NC or inhibitor NC) were purchased from Suzhou GenePharma Co., Ltd. (Suzhou, China). After incubating 8x10<sup>4</sup> gastric cells in 12-well plates for 16 h, the cells were transfected with miRNAs using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The final concentrations of mimics and inhibitors were 50 nM.

**RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and reversely transcribed using transcription kit (Takara Biotechnology Co., Ltd., Dalian, China; Tiangen, Biotech, Co., Ltd., Beijing, China) according to the manufacturer's instruction. mRNA was performed with the SYBR-Green PCR kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). Primers for miR-4284 and U6 were synthesized by Tiangen (Tiangen, Biotech, Co., Ltd.), while those for TET1 and GAPDH were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The primers used were: TET1 forward: 5'-CTGGCTCAAACGAGGTCCAT-3', reverse: 5'-TGCCATCACGTTAGCACACT-3'. Expression levels were normalized to those of U6 or GAPDH.

**Colony formation.** For colony formation assays, 1x10<sup>3</sup> gastric cancer single-cell suspensions were added to the 6-well plates

and cultured for 2 weeks. Colonies with at least 50 cells were counted. The experiments were performed three times.

**CCK-8 assays.** To assess the proliferation, following the treatment of cells with miRNA mimics or inhibitors for 24 h, 1,500 single cells in 100 µl of medium were seeded into 96-well plates, incubated for 1.5 h, after which CCK8 reagents (cat. no. C0038; Dojindo Molecular Technologies, Kumamoto, Japan) were added. Proliferation rates at 0, 24, 48, 72, and 96 h were determined by measuring the absorbance at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.). Each group was assayed five times.

**Migration and invasion assays.** For migration assays, 4x10<sup>4</sup> cells in 200 µl of medium with 1% FBS were plated into the upper chamber, separated by a membrane from the lower chamber (24-well insert; 8-µm pore size; Corning Costar, Corning, NY, USA), which contained 600 µl medium with 10% FBS. After 24 h, the membranes were stained with 0.1% crystal violet and photographed.

**For invasion assays, 8x10<sup>4</sup> cells were seeded into the upper chambers.** All other conditions remained as described, except the addition of 50 µl Matrigel on the membranes and incubation time of 48 h. **Western blot analysis.** After washing the samples three times with phosphate-buffered saline (PBS; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), cells were lysed with radioimmunoprecipitation assay (RIPA) buffer at 4°C and collected. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotting were performed as previously described (16). Anti-TET1 antibody was purchased from Santa Cruz Biotechnology, Inc. (1:1,000, cat. no. sc-293186; Dallas, TX, USA).

**miRNA target gene prediction and Luciferase reporter assays.** miR-4284 binding site at TET1 molecule was predicted by Microna (<http://www.microna.org/>). The mRNA 3'-UTR of TET1, which carrying the predicted binding site or mutant binding site of miR-4284, was amplified by PCR and was inserted between the XhoI and SacI restriction sites of the pMIR-GLO™ Luciferase vector (Promega Corporation, Madison, WI, USA). The cloning procedure was performed by GenePharma. For Dual-Luciferase assay, cells were seeded into 96-well plates and co-transfected with Pmir-GLO-TET1-3'UTR Luciferase vector or mutated sequences with 50 nM miR-4284 mimics or NC. After 24 h incubation, luciferase activity was detected using dual-luciferase reporter assay system (Promega, Ltd., Shanghai, China). Relative luciferase activities were normalized to Renilla Luciferase activity levels.

**Statistical analysis.** All data were expressed as means ± standard deviation (SD) and were analyzed using SPSS 18.0. Differences between clinicopathological variables were assessed using  $\chi^2$  test analysis; the biological variables between the groups were compared using Student's t-test; Multiple comparisons using One-way ANOVA (post hoc is LSD). The overall survival was analyzed by Kaplan-Meier method and the log-rank test and the median was used to define the thresholds for miR-4284 expression. P<0.05 was considered to indicate a statistically significant difference.

Table I. Patient characteristics and miR-4284 expression in gastric cancer tissues.

Factors	No. of patients	miR-4284 expression (mean $\pm$ SD)	P-value
Age (year)			0.459
$\leq 60$	13	0.94 $\pm$ 0.78	
$> 60$	27	1.18 $\pm$ 1.03	
Sex			0.837
Male	26	1.13 $\pm$ 1.01	
Female	14	1.06 $\pm$ 0.87	
Tumor size (cm)			0.338
$\leq 4$	20	1.25 $\pm$ 1.10	
$> 4$	20	0.96 $\pm$ 0.79	
Tumor differentiation			0.456
Well/Moderate	13	1.27 $\pm$ 0.95	
Poor	27	1.02 $\pm$ 0.96	
TNM stage			0.035 <sup>a</sup>
I+II	18	0.77 $\pm$ 0.60	
III+IV	22	1.38 $\pm$ 1.11	
Lymph node metastasis			0.648
Positive	17	1.02 $\pm$ 0.92	
Negative	23	1.16 $\pm$ 1.00	
Distant metastasis			0.022 <sup>a</sup>
Positive	5	2.01 $\pm$ 0.51	
Negative	35	0.98 $\pm$ 0.94	

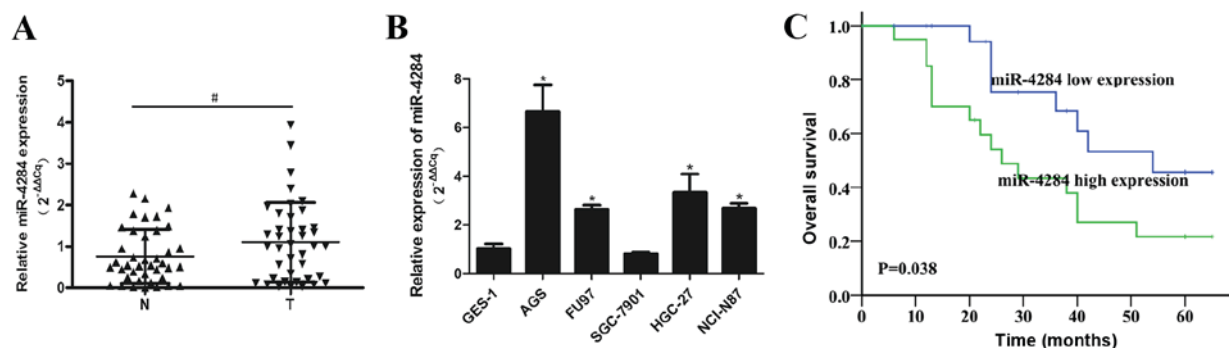
<sup>a</sup>P<0.05. SD, standard deviation; miR, microRNA.

Figure 1. miR-4284 expression levels in gastric cancer tissue samples and prognostic value. (A) miR-4284 in human gastric cancer tissues (n=40) and adjacent normal tissues (n=40). (B) Relative miR-4284 expression in gastric cancer cells *in vitro* compared with that in normal gastric cells, GES-1. (C) Kaplan-Meier survival analysis of 40 gastric cancer samples with high 20 miR-4284 expression. Log-rank test was used. #P<0.05 vs. adjacent normal tissues. \*P<0.05 vs. GES-1. T, tumor tissues; N, normal tissues.

## Results

**miR-4284 expression is upregulated in gastric cancer tissues.** miR-4284 expression was shown to be significantly upregulated in gastric cancer tissues, compared with that in the corresponding normal tissues (Fig. 1A). The levels of this miRNA were increased in gastric cancer cells (AGS, FU97, HGC-27 and NCI-N87) *in vitro*, than those in the normal GES-1 cells, with the exception of SGC-7901 cells (Fig. 1B). Furthermore, Kaplan-Meier analysis of the relationship between miR-4284 expression and gastric cancer patient

prognosis showed that high miR-4284 expression correlates with a significant decrease in patient survival rate (P=0.038; Fig. 1C). Increased miR-4284 expression was shown to be significantly associated with TNM stage (P=0.035) and distant metastasis rate (P=0.022), but was not associated with age, gender, tumor size, differentiation, or lymph node metastasis in gastric cancer patients (Table I).

**miR-4284 promotes gastric cancer cell proliferation, invasion, and migration.** To assess miR-4284 effects in gastric cancer cells, this molecule was overexpressed in SGC-7901 cells



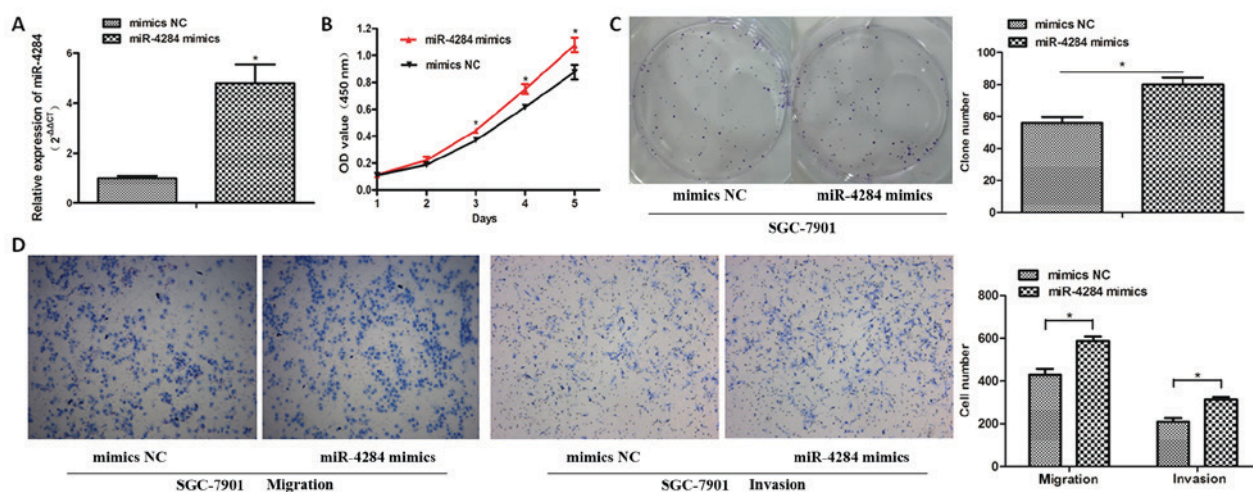


Figure 2. miR-4284 promotes proliferation, invasion, and migration of SGC-7901 cells. (A) Increased miR-4284 levels in cells treated with miR-4284 mimics. (B) Cell proliferation in the miR-4284 overexpressing and negative-control (NC) groups. (C) Colony formation assay results. (D) Migration (left) and invasion (right) were evaluated in the investigated groups (magnification, x100). \*P<0.05 vs. control.

using miR-4284 mimics, and the efficiency of overexpression was confirmed by RT-qPCR analysis (Fig. 2A; P<0.05). The proliferation of SGC-7901 cells was shown to be significantly increased following the treatment with miR-4284, compared with that in cells treated with the NC (Fig. 2B and C; P<0.05). Additionally, increased miR-4284 expression considerably enhanced the ability of migration and invasion of SGC-7901 cells compared with the NC (Fig. 2D; P<0.05).

**Decreased miR-4284 expression inhibits gastric cancer cell proliferation, invasion, and migration.** To explore the role of miR-4284 in gastric cancer further, we inhibited the expression of miR-4284 in AGS cells using inhibitors, which was confirmed by RT-qPCR analysis (Fig. 3A; P<0.05). CCK-8 and colony formation assays showed that the proliferation of AGS cells decreases following the suppression of miR-4284 expression, compared with that in the NC group (Fig. 3B and C; P<0.05). The migration and invasion of AGS cells treated with miR-4284 inhibitors were significantly inhibited (Fig. 3D; P<0.05).

**TET1 is a direct miR-4284 target.** To elucidate the molecular mechanisms underlying miR-4284 effects on the proliferation and migration of gastric cancer cells, we employed microRNA.org to identify miR-4284 target genes, which led to the identification of TET1 as a direct target gene. Therefore, we cloned the wild-type and mutant TET1 3'UTR sequences into a dual-luciferase reporter (Fig. 4A), which showed that miR-4284 induces a significant decrease in the relative luciferase activity of wild-type TET1 3'UTR (TET1-UTR-WT) (Fig. 4B; P<0.05), compared with the control, whereas this activity in the mutant group was not affected (Fig. 4B; P>0.05). Furthermore, TET1 mRNA and protein expression following the treatment with miR-4284 mimics or inhibitors was determined, showing that miR-4284 inhibits TET1 mRNA (Fig. 4C; P<0.05) and protein expression (Fig. 4D; P<0.05).

**The correlation between miR-4284 and TET1 expression.** We showed that the expression of TET1 in gastric cancer tissue samples is significantly decreased compared with that in the

adjacent normal tissue samples (Fig. 5A; P<0.05). Pearson correlation analysis showed a negative correlation between miR-4284 and TET1 expression levels in gastric cancer tissues (Fig. 5B;  $r=-0.319$ , P<0.05).

## Discussion

In most gastric cancer cases, the cancer is already in an advanced stage when diagnosed, with unsatisfactory prognosis (17). The underlying molecular mechanisms remain unclear, and no current prognostic biomarker is effective.

The dysregulation of miRNA expression was shown to play important roles in gastric cancer development, by affecting cell proliferation, invasion, and migration (18-20). Furthermore, miR-4284 was shown to be involved in physiological and pathological process, including diffuse large B-cell lymphoma and glioblastoma development (9,10). However, the role of miR-4284 in gastrointestinal tumors, especially in gastric cancer, has not been fully elucidated, which is why we focused on determining the levels and biological functions of this molecule in gastric cancer. We showed that miR-4284 expression is significantly upregulated in gastric cancer tissues in comparison with that in the matched normal tissues, suggesting that miR-4284 may be a tumor-driving factor in gastric cancer. Furthermore, our results show that high miR-4284 expression in gastric cancer correlates with TNM stage, distant metastases, and poor prognosis, indicating that miR-4284 may be a prognostic and potentially an early diagnostic biomarker in gastric cancer.

Furthermore, we analyzed miR-4284 expression levels in five gastric cancer cell lines and in one normal gastric mucosa epithelial cell line (GES-1), which showed that these levels are significantly higher in all gastric cancer cells, except SGC-7901 cells, compared with those in GES-1 cells. We further overexpressed miR-4284 in SGC-7901 cells, and inhibited its expression in AGS cells, which were shown to have highest levels of miR-4284 in all five gastric cancer cell lines. Increase in miR-4284 expression significantly induced gastric cancer cell proliferation, while the decrease

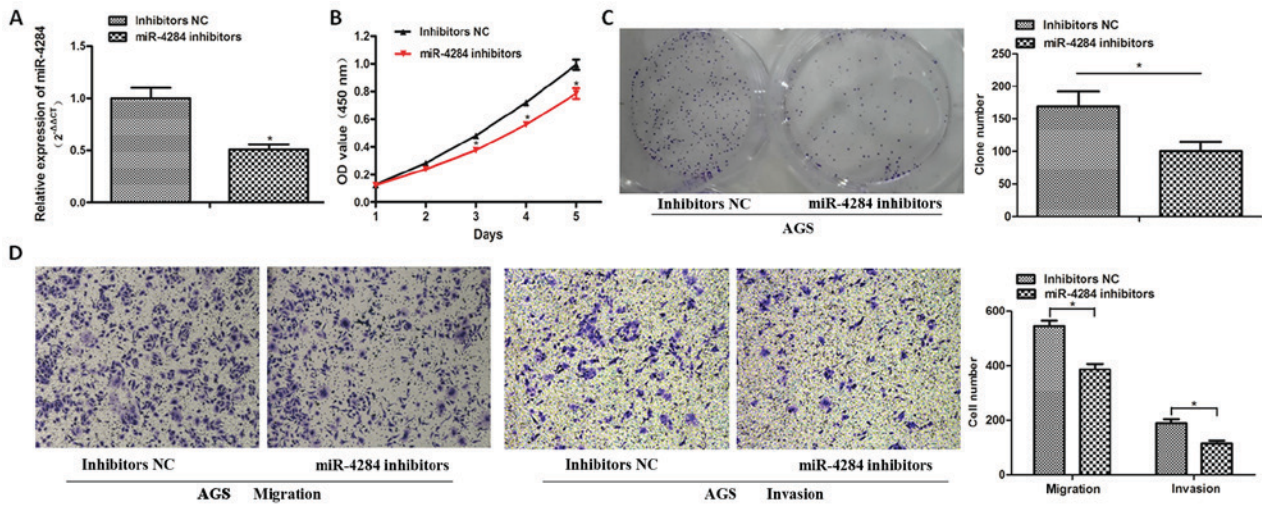


Figure 3. Inhibition of miR-4284 expression suppresses AGS cell proliferation, invasion, and migration. (A) Efficacy of miR-4284 suppression (approximately 70%). (B) Cell proliferation following the treatment with miR-4284 inhibitors and the negative control (NC). (C) Colony formation assay, evaluating cell proliferation in both groups. (D) Cell migration (left) and invasion (right) in the analyzed groups (magnification, x100). \*P<0.05 vs. control.

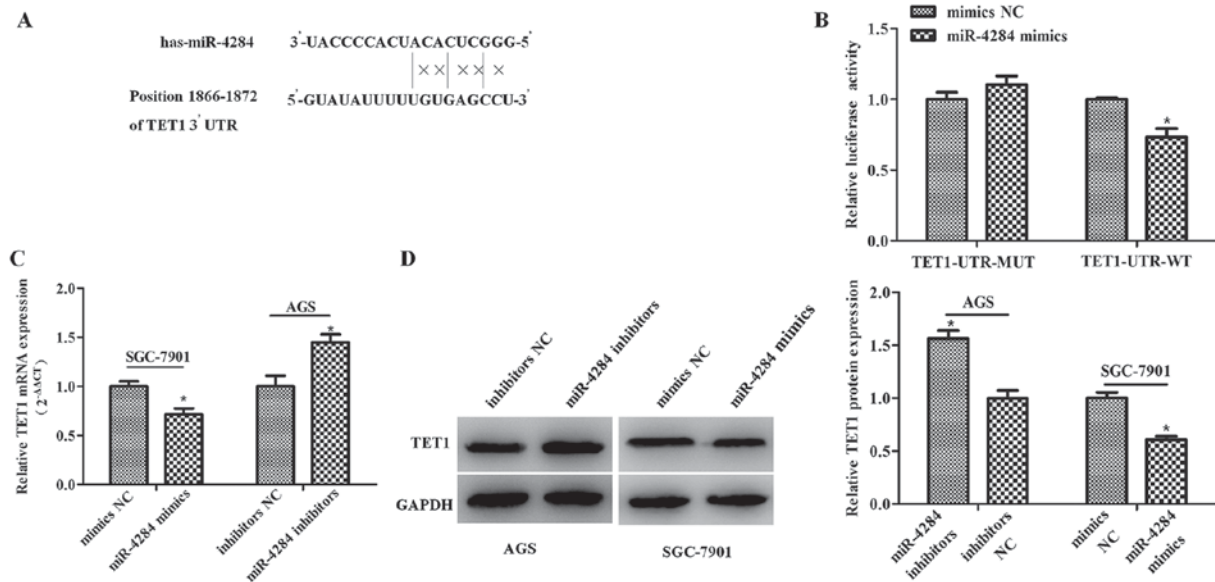


Figure 4. miR-4284 directly targets TET1 in 293T cells. (A) Predicted miR-4284 binding site in TET1 molecule. (B) Relative luciferase activity following the transfection of cells with the *wild-type* or mutant TET1 molecules and miR-4284 mimics or negative controls (NC). (C) *TET1* expression after miR-4284 mimic and inhibitor transfection. (D) TET1 levels after cell transfection with miR-4284 mimics or inhibitors. \*P<0.05 vs. control. TET1, ten-eleven translocation 1; MUT, mutant; WT, wide-type; UTR, untranslated region; X, mutation site; line, complementary base pairing site.

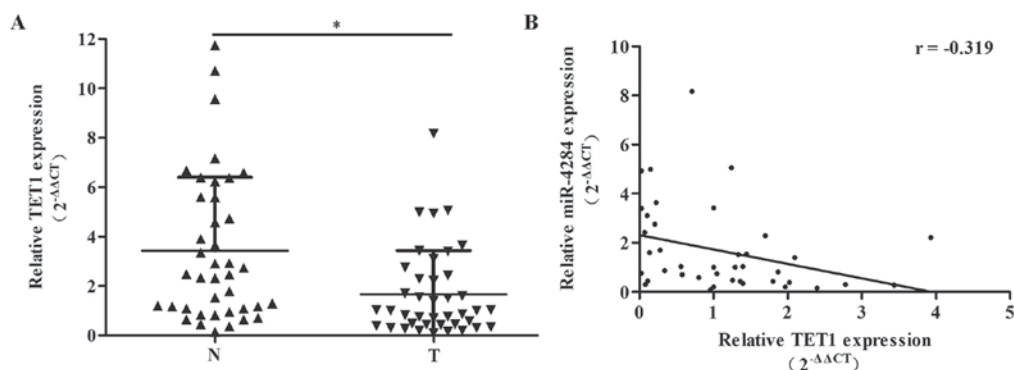


Figure 5. TET1 is downregulated in gastric cancer tissues and its expression is negatively correlated with miR-4284 expression in gastric cancer tissue samples. (A) TET1 expression in gastric cancer tissues and adjacent normal tissue samples. (B) Correlation between miR-4284 and TET1 expression in gastric cancer tissues. \*P<0.05 vs. cancer tissue samples. T, tumor tissues; N, normal tissues; TET1, ten-eleven translocation 1.

in miR-4284 expression significantly inhibited it. Since enhanced cell migration leads to tumor metastasis, this represents a major factor affecting cancer prognosis (21). Here, cell invasion and migration assays showed that the treatment of SGC-7901 cells with miR-4284 mimics and AGS cells with miR-4284 inhibitors led to an increase and decrease, respectively, in invasiveness and migratory rate of these cells. To the best of our knowledge, this is the first study showing the roles of miR-4284 in the development of gastric cancer.

The effects of miRNAs are exerted primarily through the binding to the tumor-related genes, inhibiting their expression (22). Therefore, we aimed to identify potential miR-4284 target genes, and among a number of potential targets, we focused on TET1, which was previously shown to be a tumor suppressor. Here, TET1 expression in gastric cancer tissues was shown to be significantly lower than that in the corresponding normal tissues, consistent with previous studies (23-25). To ascertain whether TET1 was a direct target of miR-4284, luciferase reporter assay was performed, and we showed that miR-4284 overexpression significantly decreased the luciferase activity in the *wild-type* TET1 group, which was not observed in the mutant group. Additionally, we overexpressed miR-4284 in SGC-7901 cells and inhibited miR-4284 expression in AGS cells, which led to a significant decrease and increase, respectively, in TET1 levels. TET1 expression was shown to correlate negatively with miR-4284 levels in gastric cancer tissues. These findings suggest that miR-4284 may contribute to gastric cancer progression by targeting TET1, which is the first time this potential mechanism has been described.

In conclusion, the results obtained here show miR-4284 expression is significantly upregulated in gastric cancer tissues and cells, and that this molecule may represent a novel predictive and prognostic biomarker for gastric cancer. Moreover, we elucidated miR-4284 roles in cell proliferation and migration. However, further research, confirming miR-4284 as a potential therapeutic target, is required.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

SW and ZLS conceived and designed the study. YSL performed the experiments. HPJ, ZYL and ZW collected and analyzed the clinical data. KWJ and YJY analyzed and interpreted the data. YSL wrote the manuscript.

## Ethics approval and consent to participate

All patients provided their informed consent and the study was approved by Ethics Committee of Peking University People's Hospital.

## Consent for publication

All patients provided their informed consent for publication of the data.

## Competing interest

The authors declare that they have no competing interests.

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