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Original Article

Decreased miR-452 expression and its tumor suppressive function in human gastric cancer

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Abstract: Objective: microRNAs (miRs) have been proved to play important roles in the tumorigenesis and development of human gastric cancer (GC). Our study aims to investigate the expression and function of miR-452 in GC. Methods: miR-452 expression was detected in GC tissues and cell lines by using qRT-PCR. Association between miR-452 levels and clinicopathological factors and patient prognosis was also analyzed. MTT, flow cytometry, and transwell invasion and migration assays were used to investigate the role of miR-452 in the regulation of biological behaviors of GC cells. Results: MiR-452 was significantly down-regulated in GC cell lines and clinical specimens. Decreased miR-452 expression was significantly associated with larger tumor size, deeper invasion depth, positive lymph node metastasis, and advanced clinical stage. Multivariate regression analysis identified low miR-452 expression as an independent predictor of poor survival. Overexpression of miR-452 inhibited GC cell proliferation, invasion and migration, and promoted cell apoptosis *in vitro*, while down-regulation of miR-452 showed the opposite effect. Conclusions: These findings indicate that miR-452 may act as a tumor suppressor in GC and would serve as a novel therapeutic agent for miR-based therapy.

Keywords: MiR-452, gastric cancer, prognosis, invasion

Introduction

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer-related death worldwide [1]. Despite recent advances in experimental and clinical oncology, the detection of early GC is <15%, and the overall 5-year survival rate of GC patients is less than 30% [2]. Even after a curative resection, the majority of patients with advanced-stage GC will eventually have and die of tumor recurrence or metastasis [3]. Like other cancers, the development of GC is a multistep process with accumulation of genetic and epigenetic changes [4]. However, the precise mechanisms underlying its carcinogenesis and prognosis remain unclear. Therefore, it is urgent to elucidate the regulatory network underlying GC and develop novel biomarkers for its early diagnosis, accurate assessment, targeted therapy, and prognosis evaluation.

MicroRNAs (miRs) are a class of naturally occurring, small (19-24 nucleotides nucleotides in length), single-stranded, non-protein-coding RNAs, which suppress translation or promote the degradation of target messenger RNAs (mRNAs) through base pairing with the 3'-untranslated regions (3'-UTRs) [5, 6]. It has been shown that miRs regulate many biological events including cancer development [7, 8]. Some highly expressed miRs could function as oncogenes by repressing tumor suppressor genes, whereas low-expressed miRs could function as tumor suppressors by negatively regulating oncogenes. In terms of GC, abnormal expression of several miRs and their function has been reported. For example, miR-451 showed decreased expression in GC tissues, and its down-regulation was correlated with positive lymph node metastasis, advanced clinical stage, and shorter overall survival [9]. Ectopic expression of miR-133a inhibited GC cell proliferation, migration, and invasion, and induced cell apoptosis [10]. Decreased plasma miR-940 may serve as a novel diagnostic biomarker for GC with a satisfactory sensitivity [11]. Overexpression of miR-23b-3p reversed

Table 1. Correlation between miR-452 expression and different clinicopathological features in gastric cancer patients

	Low miR-452 expression	High miR-452 expression	*P*-value
Age			
≥60	37 (48.1%)	40 (51.9%)	
<60	18 (56.2%)	14 (43.8%)	0.285
Gender			
Male	29 (46.8%)	33 (53.2%)	
Female	26 (55.3%)	21 (44.7%)	0.245
Differentiation			
Well-moderate	15 (42.9%)	20 (57.1%)	
Poor	40 (54.1%)	34 (45.9%)	0.188
Lauren type			
Intestinal	30 (44.8%)	37 (55.2%)	
Diffuse and mixed	25 (59.5%)	17 (40.5%)	0.096
Tumor size			
≥5 cm	39 (60.9%)	25 (39.1%)	
<5 cm	16 (35.6%)	29 (64.4%)	0.008
Invasion depth			
T1, T2	17 (35.4%)	31 (64.6%)	
T3, T4	38 (62.3%)	23 (37.7%)	0.009
TNM stage			
I/II	14 (36.8%)	24 (63.2%)	0.030
III	41 (57.7%)	30 (42.3%)	
Lymphatic metastasis			
Negative	13 (33.3%)	26 (66.7%)	0.007
Positive	42 (60.0%)	28 (40.0%)	

GC cell resistance to multiple chemotherapeutics in vitro [12]. Thus, functional miRs may be applied for GC diagnosis and prognosis, and also act as potential novel therapeutic targets.

MiR-452 is a recently identified cancer-related miR. It is observed to be up-regulated in bladder cancer [13], urothelial carcinoma [14], and hepatocellular carcinoma [15], and acts as a potential oncogene in these tumors. On the contrary, miR-452 is found to be significantly decreased in non-small cell lung cancer (NSCLC) [16], glioma [17], and prostate cancer [18], and acts as a candidate tumor suppressor. Notablely, Hashimoto et al. revealed that miR-452 was down-regulated in GC cells, and that ectopic miR-452 expression decreased GC cell proliferation [19]. However, the potential effect of miR-452 on GC cell apoptosis, invasion and migration was not involved in his study, and very little is known about the links of miR-452 dysregulation to clinicopathological characteristics of GC patients till now. In the present study, we examined miR-452 expression in GC tissues and cell lines. We also investigated the correlation between miR-452 levels and clinicopathological characteristics and patient's survival. Moreover, we explored the role of miR-452 in the regulation of biological behaviors of GC cells.

Materials and methods

Patients and clinical specimens

This study was approved by the Research Ethics Committee of Logistics University of Chinese People's Armed Police Force. Written informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

Fresh primary GC tissues and matched normal adjacent tissues (≥3 cm away from tumor margin) were obtained from 109 consecutive patients who received radical excision at The Affiliated Hospital of Logistics University of Chinese People's Armed Police Force between January 2008 and July 2010. The diagnosis of all samples was histopathologically confirmed by two pathologists. All specimens were frozen immediately in liquid nitrogen and stored at -80°C until analysis. Patients with two or more different malignancies were excluded. None of the patients had received preoperative radiotherapy or chemotherapy. Patient characteristics are shown in **Table 1**. Follow-up data were available for all patients. Overall survival was defined as the amount of time from the day of primary surgery to the date of death or the end of follow-up (for living patients).

Cell culture and miR transfection

Human GC cell lines (AGS, SGC-7901, BGC-823, and MKN-28) and human normal gastric epithelial cell line GES-1 were purchased from the Cell Resource Center, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100

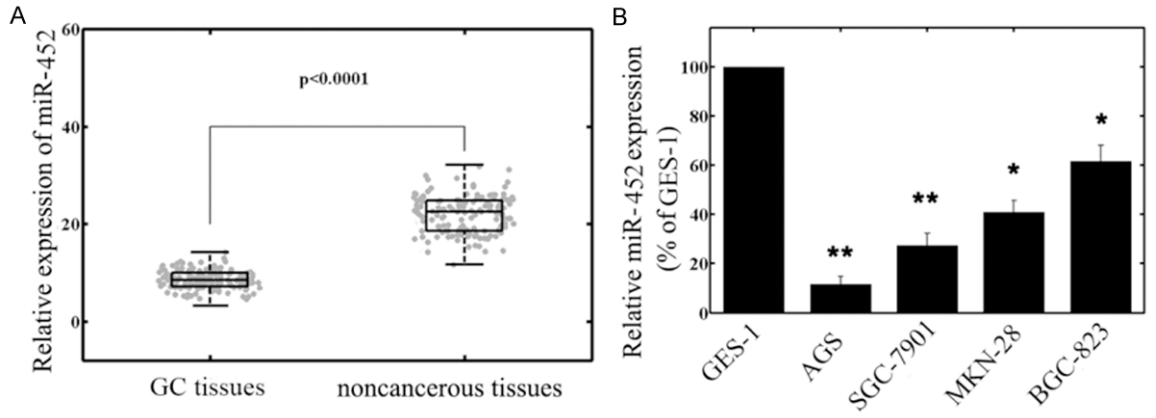


Figure 1. Expression of miR-452 in gastric cancer (GC) tissues and cell lines. A. MiR-452 expression was significantly lower in GC tissues than in the corresponding non-cancerous tissues. MiR-452 expression levels were calculated by the $2^{-\Delta Ct}$ method and normalized to U6 small nuclear RNA. B. miR-452 expression was down-regulated in GC cell lines AGS, SGC-7901, BGC-823, and MKN-28, compared to normal gastric epithelial cell line GES-1. *P<0.05; **P<0.01.

U/ml of penicillin and 100 μg/ml streptomycin sulfate. Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C.

For RNA transfection, GC cells were seeded into each well of 24-well plate and incubated overnight, then transfected with mature miR-452 mimics, miR-452 inhibitors (Anti-miR-452), or negative control (miR-NC or anti-miR-NC) (GenePharma, Shanghai, China) at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen, California, USA). The cells were collected for further experiments 24 hours post-transfection.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from GC cells and clinical specimens by using Trizol reagent (Invitrogen Corp, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized from isolated RNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed with a Taqman MicroRNA Assay Kit (Applied Biosystems) on ABI7500 real-time PCR detection system. Quantitative PCR was conducted at 95°C for 10 min followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. U6 small nuclear RNA was used as an internal control. All reactions were run in triplicates. The cycle threshold (Ct) values were recorded, and the relative amount of miR-452 to U6 was calculated using the equation $2^{-\Delta Ct}$, where $\Delta CT = (CT_{miR\text{-}452} - CT_{U6})$.

MTT assay

The *in vitro* cell proliferation was measured using the MTT method. Briefly, GC cells were seeded into 96-well plates (about 4×10^3 cells/well) and incubated at 37°C after transfection. At different time points (24, 48, 72 or 96 h), 20 *u*L of MTT solution (5 mg/mL; Sigma, USA) was added into each well and incubated for another 4 hours. Then, 150 *u*L of dimethyl sulfoxide (DMSO) was added to stop the reaction. The plates were gently shaken on a swing bed for 10 min, and the absorbance value (OD) was measured at 490 nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Detection of apoptosis by flow cytometry

The GC cells were harvested, washed and resuspended in ice-cold PBS after miR transfection. Then the cells were treated with propidium iodide (10 μg/ml; Sigma) and Annexin V-FITC (50 *u*g/ml, BD) in the dark for 15 min at room temperature, and examined by a FACScan flowcytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Transwell invasion and migration assays

Cell migration and invasion assays were performed using a 24-well transwell plate (8-mm pore size, Corning, New York, USA). For migration assay, about 1×10^5 GC cells suspended in serum-free media were added to the upper chamber. The lower chamber contained medi-

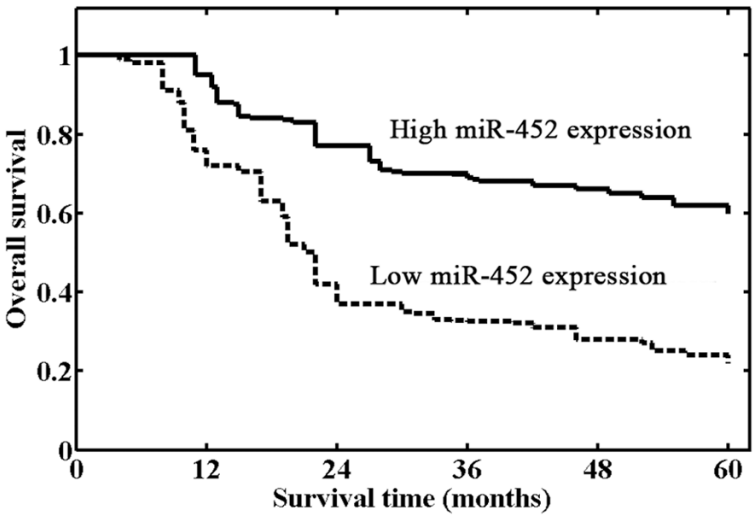


Figure 2. Kaplan-Meier survival curves of 109 gastric cancer patients based on miR-452 expression status. Patients in the low expression group had significantly poorer prognosis than those in high expression group (P<0.001, log-rank test).

um with 10% FBS as a chemoattractant. Following a 48 h-incubation, the cells located on the lower surface of the chamber were stained and counted using a microscope (Olympus Corp., Tokyo, Japan). The invasion assay protocol was similar to the migration assay except that the upper chambers were first covered with Matrigel.

Statistics

All statistical analyses were performed using the SPSS 16.0 software package (SPSS, Chicago, IL, USA). The significance of differences between groups was estimated by Student's t-test and Chi-square test. Survival curves were constructed with the Kaplan-Meier method and compared by log-rank test. The significance of survival variables was evaluated using a multivariate Cox proportional hazards regression analysis. P<0.05 was considered statistically significant.

Results

Downregulation of miR-452 in GC tissues and cell lines

We performed quantitative RT-PCR analysis to detect miR-452 expression in GC tissues and cell lines. As in **Figure 1A**, the results showed that the expression levels of miR-452 were significantly lower in GC specimens (mean ± SD: 8.3 ± 1.9) than those in the corresponding adjacent non-cancerous tissues (mean ± SD: 20.4 ± 3.8; P<0.001). The miR-452 expression in four GC cell lines was also clearly downregulated (**Figure 1B**). Because AGC cells exhibited the lowest miR-452 expression while BGC-823 cells expressed relatively high levels of miR-452 among the four GC cell lines, these two cell lines were selected for miR-452 mimics or miR-452 inhibitors transfection and further studies.

Correlation between miR-452 expression and clinical features of GC

We further analyzed the association between miR-452 expression levels and clinicopathological characteristics of GC. GC samples were classified into low miR-452 expression group (n = 55) and high miR-452 expression group (n = 54) according to the median miR-452 expression level of all GC samples. The association between clinicopathological characteristics and miR-452 expression was summarized in **Table 1**. We found that miR-452 level was associated with tumor size, tumor depth, lymph node metastasis, and clinical stage. However, we did not find any significant correlation between miR-452 levels and other clinicopathological features, such as patient's gender, age, Lauren type, and cancer differentiation.

Prognostic values of miR-452 expression in GC patients

We further evaluated the association of miR-452 expression level with survival of GC patients. Survival analysis indicated that patients in high miR-452 expression group had better 5-year overall survival than those in low miR-452 expression group (P<0.001, **Figure 2**). Univariate analysis revealed that miR-452 expression, tumor size, tumor depth, lymph node metastasis, and TNM stage were prognostic factors for patient's overall survival (**Table 2**). Multivariate analysis confirmed low miR-452 expression (P = 0.015, RR = 2.41) as

Table 2. Univariate and multivariate analysis of overall survival in 109 gastric cancer patients

Variables	Univariate analysis		Multivariate analysis	
	RR	*P*-value	RR	*P*-value
Age	0.85	0.228	———	———
Gender	1.32	0.135	———	———
Differentiation	1.46	0.096	———	———
Lauren type	1.24	0.144	———	———
Tumor size	3.02	0.006	1.45	0.084
Invasion depth	2.76	0.015	3.07	0.003
Lymphatic metastasis	2.48	0.028	2.68	0.012
TNM stage	3.86	<0.001	2.85	0.008
MiR-452 expression	3.71	<0.001	2.41	0.015

an unfavourable prognostic indicator independent of other clinicopathological factors, including depth of infiltration (P = 0.003, HR = 3.07), lymph node status (P = 0.012, HR = 2.68), and TNM stage (P = 0.008, HR = 2.85; **Table 2**).

Effects of miR-452 on the biological behaviours of GC cells

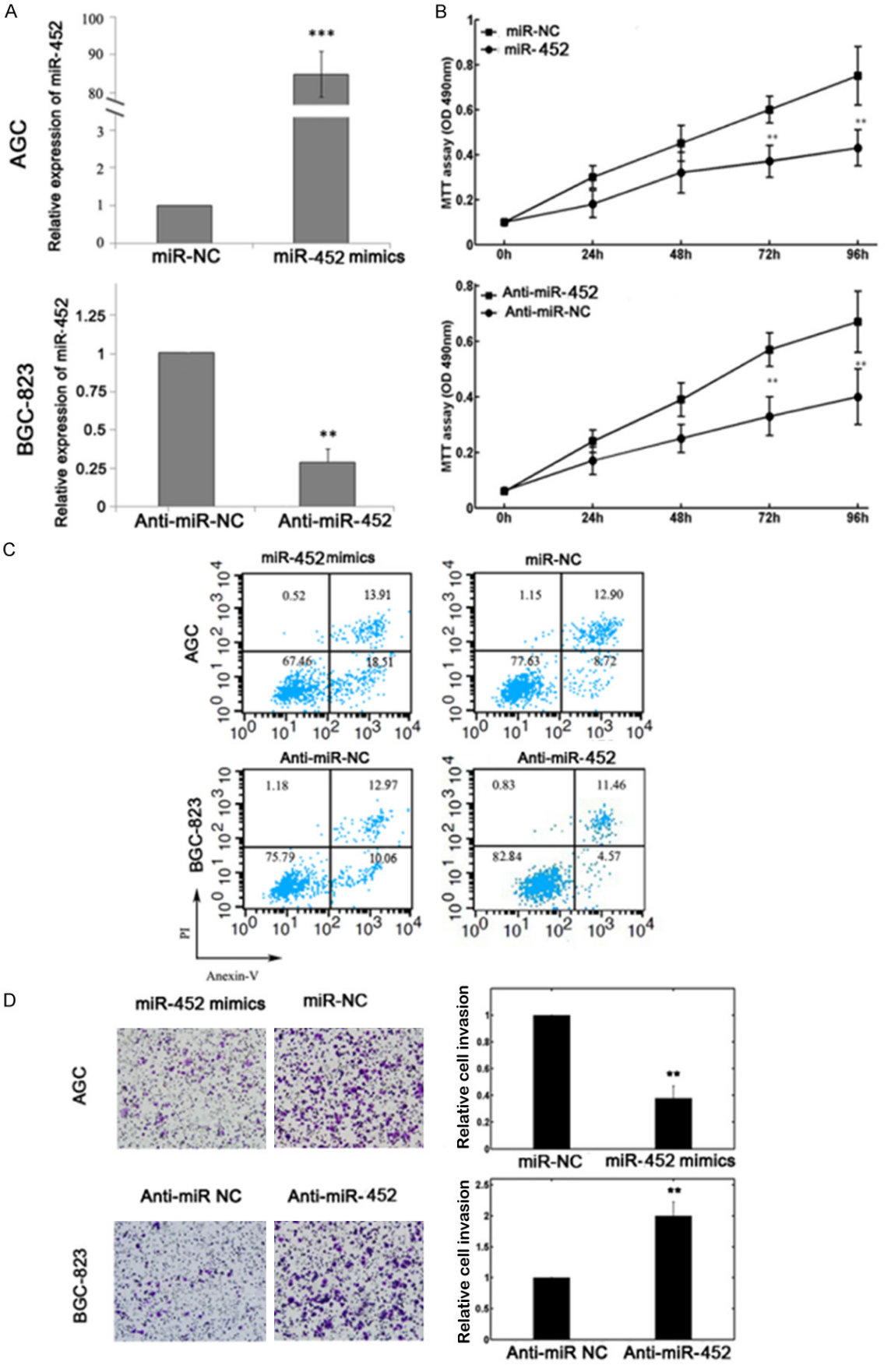
To selectively over-express or down-regulate miR-452, mature miR-452 mimics or miR-452 inhibitors were transfected into AGC cells or BGC-823 cells. qRT-PCR analysis confirmed increased miR-452 expression after miR-452 mimics transfection and decreased miR-452 expression following miR-452 inhibitors transfection (**Figure 3A**). MTT assay showed that cell proliferation was significantly impaired in AGC cells transfected with miR-452 mimics, while proliferation of BGC-823 cells was increased in miR-452 inhibitors transfected cells compared with controls (**Figure 3B**). Flow cytometry demonstrated promoted cell apoptosis after miR-452 mimics transfection and reduced cell apoptosis after miR-452 inhibitors transfection (**Figure 3C**). As shown in **Figure 3D** and **3E**, up-regulation of miR-452 impeded the invasion/migration of AGC cells. Conversely, transfection of BGC-823 cells with miR-452 inhibitors promoted cell invasion/migration ability.

Discussion

Identifying novel molecules that take part in GC formation and progression may be helpful for improving the diagnosis, prevention and treatment of this disease. The relationship between miRs and tumors has currently become one of the focuses of cancer studies. In the present study, we revealed decreased miR-452 expression in GC and its correlation with aggressive clinicopathological features. Low miR-452 expression was confirmed as an independent predictor of poor survival. Over-expression of miR-452 could significantly reduce cell proliferation, enhance cell apoptosis, and impair cell invasion and migration *in vitro*. These findings revealed that miR-452 might be involved in GC initiation and progression, and contribute to molecular-targeted therapy.

Previous research has demonstrated the tumor-suppressive functions of miR-452 in several human cancers. *In vitro*, ectopic miR-452 expression inhibited proliferation, migration, and invasion of prostate cancer cells [18]. Down-regulated miR-452 in NSCLC cells could enhance cell invasion capability by targeting oncogene BMI1 [16]. Overexpression of miR-452 could sensitize MCF-7 breast cancer cells to chemotherapy and induce cell apoptotic via targeting insulin-like growth factor-1 receptor (IGF-1R) [20]. *In vivo*, He et al. confirmed decreased miR-452 expression in NSCLC tissues and its association with advanced tumor stage and lymph node metastasis [16]. Liu et al. showed that miR-452 was markedly down-regulated in clinical glioma tissues [17]. miR-452 overexpression would reduce tumorigenesis of glioma cells in nude mice. However, miR-452 was reported to be up-regulated in some other cancers such as bladder cancer and hepatocellular carcinoma [13-15], indicating that miR-452 may not behave as a tumor suppressor in all cases. Overexpression of miR-452 in hepatocellular carcinoma cells dramatically accelerated proliferation, induced cell cycle from G1 to S transition, blocked apoptosis, and promoted cell migration and invasion through targeting cyclin-dependent kinase inhibitor 1B (CDKN1B) [15]. miR-452 upregulation in bladder urothelial carcinomas was correlated with lymph nodes metastasis and shorter overall survival [14]. So, miR-452 plays dual functions in cancer pathogenesis and progression, and the role of miR-452 should be tumor specific and possibly dependent on its targets in different cancer types.

We are aware of some limitations in our work. First, the clinical part was a retrospective study,


A
AGC
Relative expression of miR-452
miR-NC
miR-452 mimics
BGC-823
Anti-miR-NC
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B
miR-NC
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Anti-miR-NC
MTT assay (OD 490nm)
0h
24h
48h
72h
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C
miR-452 mimics
miR-NC
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Relative cell invasion

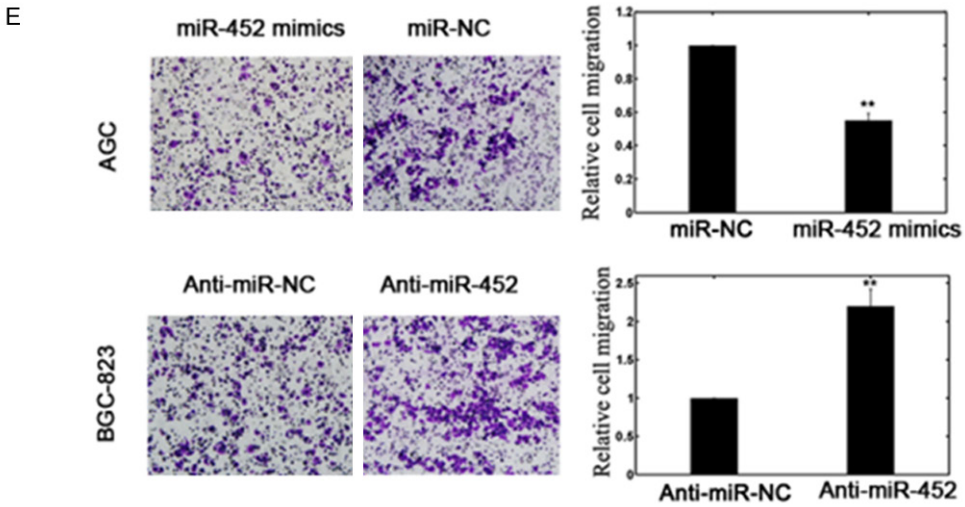

Figure 3. Effects of miR-452 mimics or inhibitors transfection on biological behaviours of AGS and BGC-823 cells. A. qRT-PCR analysis confirmed increased miR-452 expression in AGS cells transfected with miR-452 mimics, and decreased miR-452 expression in BGC-823 cells transfected with miR-452 inhibitors. U6 RNA was used as an internal control. **P<0.01, ***P<0.001. B. MTT assay showed that miR-452 reduced cell proliferation *in vitro*. **P<0.01. C. Cell apoptosis was detected by flow cytometric analysis after transfection with miR-452 mimics, miR-452 inhibitors, or negative control. D, E. Transwell invasion and migration assays showed that up-regulation of miR-452 impeded the invasion/migration of AGS cells, while transfection of BGC-823 cells with miR-452 inhibitors promoted cell invasion/migration. **P<0.01.

and the tumor sample size was relatively small. Second, although we revealed the role of miR-452 as a candidate tumor suppressor in GC, the complex molecular mechanisms underlying low miR-452 expression in GC and its function are still incompletely known. It is now clear that miRs exert their functions by regulating the expression of target genes [21]. Therefore, identification of probable downstream mediators of miR-452 in GC would be an important facet of future investigations.

In conclusion, our research confirmed decreased miR-452 expression in GC tissues and cell lines. Our study also showed that low miR-452 levels correlated tumor progression and poor prognosis in GC patients. Regulation of miR-452 expression influenced biological behaviors of GC cells. These findings suggested that miR-452 may act as a tumor suppressor in GC initiation and development, and would be not only a novel prognostic marker but also a potential therapeutic target for this disease.

Disclosure of conflict of interest

None.

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