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Original Article

Higher miR-10b expression contributes to malignant behaviors in gastric cancer in ex vivo and in vitro

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Abstract: Overexpression of miR-10b was associated with human carcinogenesis and cancer progression, whereas miR-10b expression and functions in gastric cancer remain inclusive and controversial. This study assessed miR-10b expression in gastric cancer tissues and cell lines for association with clinicopathological data from patients and then investigated miR-10b overexpression in regulation of tumor cell malignant behaviors in vitro. Levels of miR-10b expression was detected using quantitative PCR in eight human gastric cell lines and 70 tissue samples from gastric cancer patients. Expression of miR-10b was induced in gastric cancer and then assessed for changed cell migration and invasion ability using wound healing and Transwell invasion assays. Levels of miR-10b were higher in gastric cancer cell lines than that of normal gastric epithelial cell GES-1 and associated with tumor cell differentiation and lymph node metastasis potential. Moreover, miR-10b expression was also up-regulated in gastric cancer tissues compared to non-tumor tissues, upregulated expression of which was associated with larger tumor size ($P = 0.007$), tumor location ($P = 0.038$), tumor cell de-differentiation ($P = 0.042$), and tumor local invasion depth ($P = 0.001$). In addition, overexpression of miR-10b using gene transfection technique showed induction of AGS cell migration and invasion capacity. The data from our current study indicated that miR-10b could involve gastric cancer development and progression. Further study will assess whether target of miR-10b can be useful as a novel strategy in control of gastric cancer progression.

Keywords: miR-10b, gastric cancer, differentiation, invasion

Introduction

Gastric cancer is one of the most commonly occurring malignancies in the world and remains to be the second leading cause of cancer morbidity and mortality in the developing countries, despite there is a rapid decline in the global gastric cancer incidence for the past two decades [1]. It was estimated that there were a total of 989,600 new gastric cancer cases and 738,000 cancer-related deaths in 2008 globally, but more than 70% of new cases and deaths occur in developing countries [1] where most gastric cancer cases are diagnosed at the advanced stages of disease. Early staged disease is usually cured by surgical resection, whereas the advanced staged diseases are treated by chemotherapy, which is not sensitive at all to gastric cancer patients. To date, a number of risk factors have been identified to associate with gastric cancer development but the precisely underling molecular mechanism of gastric cancer development is to be defined. Thus, further study of gastric cancer could lead to biomarker discovery and novel strategy to control gastric cancer development and disease progression.

Towards this end, microRNA (miRNA) is a class of endogenous noncoding RNA molecules with approximately up to 24 nucleotides in length and can regulate expression of other genes that are involved in various biological and metabolic processes [2]. Increasing evidence showed that dysregulation of miRNA expression and functions were able to influence cell growth, differentiation, and apoptosis, and thus play a key role in tumorigenesis and cancer development [3, 4]. Among them, miR-10b has been frequently studied in the literature [5-14] and miR-

Table 1. Sources and biological characteristics of the gastric epithelial cell lines

Cell line	Biological characteristics	Source
GES-1	A human normal gastric epithelial cell line	CBCAMC*
BGC-803	A human gastric cancer cell line, moderately differentiated	FAHZUMC**
MGC-803	A human gastric mucous adenocarcinoma cell line, poorly differentiated	CBCAMC*
AGS	A human gastric cancer cell line, poorly differentiated	FAHZUMC**
BGC-823	A human gastric cancer cell line, undifferentiated	CBCAMC*
MKN-45	A human gastric cancer cell line of signet-ring carcinoma, poorly differentiated	CBCAMC*
SGC-7901	A human gastric cancer cell line deriving from metastatic lymph node, moderately differentiated	CBCAMC*
HGC-27	A human gastric cancer cell line deriving from metastatic lymph node, undifferentiated	FAHZUMC**

*CBCAMC: Cell Bank of Chinese Academy of Medical Sciences, Beijing, China; **FAHZUMC: the First Affiliated Hospital of Zhejiang University Medical College, Zhejiang, China.

10b is located at chromosome 2q31.1 within the HOXD gene cluster, which was first identified as one of the most significantly downregulated miRNAs in breast cancer compared with normal breast samples [5]. A later research in 2007 reported by Ma et al. [6] demonstrated that miR-10b acted as a metastasis-associated miRNA in breast cancer. Upregulation of miR-10b expression was found in metastatic breast cancer cells and consequently promoted tumor cell migration and invasion capacity both in vitro and in vivo. Subsequently, upregulated expression of miR-10b was extensively reported in various human cancer tissues and cells and therefore, miR-10b was proposed as a key player in malignant tumors [7-10]. However, conflicting evidence did raise; for example, expression and role of miR-10b in gastric cancer was inclusive and controversial. Wang et al. [11] showed that overexpression of miR-10b in gastric cancer was positively correlated with tumor size, Lauren classification, invasion depth, TNM stage, lymph node and distant metastasis, and prognosis. The data from Liu et al. [12] suggested that miR-10b levels were dramatically elevated in lymphoma node metastasized tumor tissues and in strong metastatic potential gastric cancer cell lines and miR-10b expression associated with tumor cell invasiveness. In contrast, Kim et al. [13] demonstrated that miR-10b expression was frequently silenced in gastric cancer through methylation of miR-10b gene promoter and that miR-10b may act as a tumor suppressor in gastric cancer. Li et al. [14] showed that miR-10b expression was regulated by DNA methylation and significantly down-regulated in gastric cancer cell lines and tissues; thus, miR-10b could act as a tumor suppressor in gastric cancer. To further clarify and elucidate this discrepancy, we in this study, assessed miR-10b expression in various differentiation-grade gastric cell lines and gastric cancer tissues and then investigated the effect of overexpression miR-10b on regulation of malignant behaviors in gastric cancer cells in vitro.

Materials and methods

Cell lines and culture

Human gastric cancer cell lines BGC-803, MGC-803, AGS, BGC-823, HGC-27, MKN-45, and SGC-7901 and a human normal gastric epithelial cell line GES-1 were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) in a humidified atmosphere of 5% CO_2 (Thermo, Waltham, MA, USA) at 37°C. Characteristics of these cell lines are summarized in **Table 1**.

Tissue specimens

This prospective study was approved by the Ethics Committee of Huzhou Centre Hospital, and all participants provided an informed consent. A total of 70 patients with newly diagnosed gastric cancer according to the 7th UICC/TMN staging system [15] were enrolled into this study between September 2010 and December 2013 from Huzhou Centre Hospital (Huzhou, China). Their clinicopathological characteristics were listed in **Table 2**. All paired tissue specimens (gastric cancer and pair-matched normal adjacent tissues) were surgically removed from these patients and instantly frozen in liquid nitrogen and then stored at -80°C until use. None of these patients had received radiotherapy or chemotherapy before surgery.

Table 2. Relative miR-10b expression in GC patients and clinicopathological features

Parameter	N	Normalized miR-10b expression in GC tissues ($2^{-\Delta\Delta CT}$)	Z	P
Age (years)				
< 60	29	1.245±0.823	0.168	0.872
≥ 60	41	1.281±0.896		
Gender				
Male	56	1.305±0.915	1.133	0.268
Female	14	1.080±0.518		
Tumor size				
< 7 cm	50	1.098±0.740	2.787	0.007
≥ 7 cm	20	1.730±1.202		
Location				
Upper	11	0.777±0.747	3.458	0.038
Middle	12	0.974±0.834		
Lower	47	1.445±0.849		
Differentiation				
Well-moderate	20	1.080±0.914	2.887	0.042
Poor	26	1.097±0.748		
SRC	15	1.616±0.664		
Mucinus	9	2.005±1.223		
Local invasion				
T1	10	0.990±0.811	6.514	0.001
T2	7	0.714±0.358		
T3	18	1.929±0.724		
T4	35	1.100±0.841		
Lymph node metastasis				
N0	17	1.264±0.979	1.232	0.306
N1	8	1.061±0.920		
N2	15	1.660±0.891		
N3	20	1.151±0.749		
TNM stage				
I	12	0.866±0.738	1.63	0.204
II	13	1.397±0.933		
III-IV	45	1.344±0.863		
Roukos type				
I	10	1.000±0.765	0.623	0.539
II	15	1.268±0.944		
III	45	1.324±0.863		
Lymphatic invasion				
Positive	54	1.311±0.965	0.388	0.699
Negative	16	1.228±0.771		

RNA isolation and reverse transcription

Total mRNA was isolated from cells and tissues using a mirVana PARIS Kit (Ambion, Austin, TX, USA) and eluted in 80 μl pre-heated (95°C) elution solution according to the manufacturer's protocol. For reverse transcription (RT) reactions, a stem-loop primer [16] (**Table 3**) was applied for cDNA synthesis using a PrimeScript™ First Strand cDNA Synthesis Kit (#D6110A, TaKaRa, Dalian, China). In brief, 2 μl each of miRNA samples were mixed with 1 μl dNTP mixture (10 mM each), 0.1 μl reverse transcription primers (10 μM), and then adjusted to a total volume of 10 μl with ddH_2O, and the mixture was then incubated at 65°C for 5 min and cooled down on ice immediately. After that, 10 μl of denatured miRNA mixtures were added with 4 μl 5 × PrimeScript™ Buffer, 0.5 μl RNase inhibitor (40 U/μl), 1 μl PrimeScript™ RTase (200 U/μl), and 4.5 μl ddH_2O and then incubated at 50°C for 60 min, 70°C for 15 min, and then stored at 4°C for PCR amplification.

Quantitative PCR

Quantitative PCR was performed by using a standard SYBR Green I PCR kit (TaKaRa) on a 7500 real-time PCR System (Applied Biosystems, Foster city, CA, USA). PCR amplification was set to include 2 μl cDNA from reverse transcription, 12.5 μl of 2 × SYBR Green I PCR master mix, 0.5 μl of each 10 μM forward and reverse primer, 0.5 μl ROX Reference Dye, and 9 μl ddH_2O. The qPCR conditions were set to be at 95°C for 5 min and 40 cycles of 95°C for 5 s and 60°C for 34 s. Increment 0.5°C for 10 s was used to yield the melt curve. The DNA sequences used for amplification are listed in **Table 3**. For normalization, miR-16 was used as an internal reference gene according to a previous study [17]. The level of miR-10b expression was analyzed by $2^{-\Delta\Delta CT}$ [18] method, where

Table 3. Reverse transcription and stem-loop primers for Real-Time PCR

Gene name	Reverse transcription primers (5'-3')	PCR primers (5'-3')
miR-10b	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCACAAA	F: CGTCGTACCCTGTAGAACCGA
		R: GTGCAGGGTCCGAGGT
miR-16	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGCCAA	F: CGCGCTAGCAGCACGTAAAT
		R: GTGCAGGGTCCGAGGT

F: forward primer, R: reverse primer.

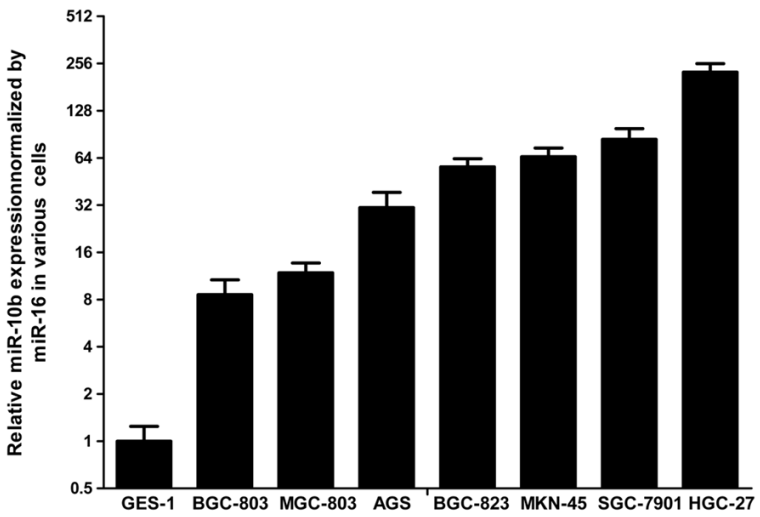

Figure 1. Detection of relative miR-10b levels in eight gastric cell lines using qRT-PCR with an SYBR Green I kit. Data are presented as fold change in gastric cancer cell lines relative to human normal gastric epithelial cell line GES-1. MiR-10b expression is upregulated in gastric cancer cell lines (BGC-803, MGC-803, AGS, BGC-823, MKN-45, SGC-7901, and HGC-27 vs. GES-1). Levels of miR-10b expression are associated with tumor cell differentiation (BGC-823, MKN-45, SGC-7901, and HGC-27 vs. BGC-803, MGC-803, and AGS) and with lymph node metastatic potential (SGC-7901 and HGC-27 vs. BGC-803, MGC-803, AGS, BGC-823, and MKN-45).

$\Delta\Delta CT = \Delta CT_{\text{Gastric cancer}} - \Delta CT_{\text{normal tissue}}$. The miR-10b expression level was defined as being upregulated in tumor tissue with a relative expression ratio > 1, and was defined as downregulated in tumor tissue with a relative expression ratio < 1. An empty control with ddH_2O was used as negative and quality controls and each sample was performed in triplicate.

Generation of stably miRNA-expressed gastric cancer cell sub-lines

Lentiviruses containing GFP-miR-10b mimics (miR-10b mimic) or GFP negative control (Scramble) miRNA vector were purchased from Genepharma (Shanghai, China). Specifically, gastric cancer AGS cell line was seeded into a 6-well plate and grown overnight. On the next day, the cells were infected with 200 μl of the viral particles for 48 h and the cell growth medium was added with 1 μg/ml puromycin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and cells were further cultured in a humidified atmosphere of 5% CO_2 at 37°C. After 4-5 cell passages, the stable cells were verified by qRT-PCR and fluorescence microscopic analysis of miR-10b expression. AGS cells without any transfection were used as a blank control.

Tumor cell wound healing assay

Parental AGS cells and miR-10b mimic and Scramble-infected AGS cells were plated into six-well plates at a density of 5×10^5 cells/well. After overnight incubation, cells formed more than 95% confluency monolayer and wounds were then created using a sterile 200 μl pipette tip. After that, cells were then washed three times with phosphate buffered saline (PBS), and further incubated in RPMI-1640 without serum or other growth factors for 24 h. The wound distance (wound healing capacity) was photographed at 0 and 24 hours and analyzed by using the Image Pro Plus 6.0 software (Media Cybernetics, Inc., Bethesda, MD, USA).

Tumor cell invasion assay

For tumor cell invasion assay, we first diluted Matrigel (BD Biosciences, San Jose, CA) in cell growth medium without serum or growth factors into 0.3 mg/ml and then added 50 μl of the dilute onto each of Transwell membrane (24-well insert with a pore size of 8 μm; BD Biosciences, San Jose, CA). Parental AGS cells

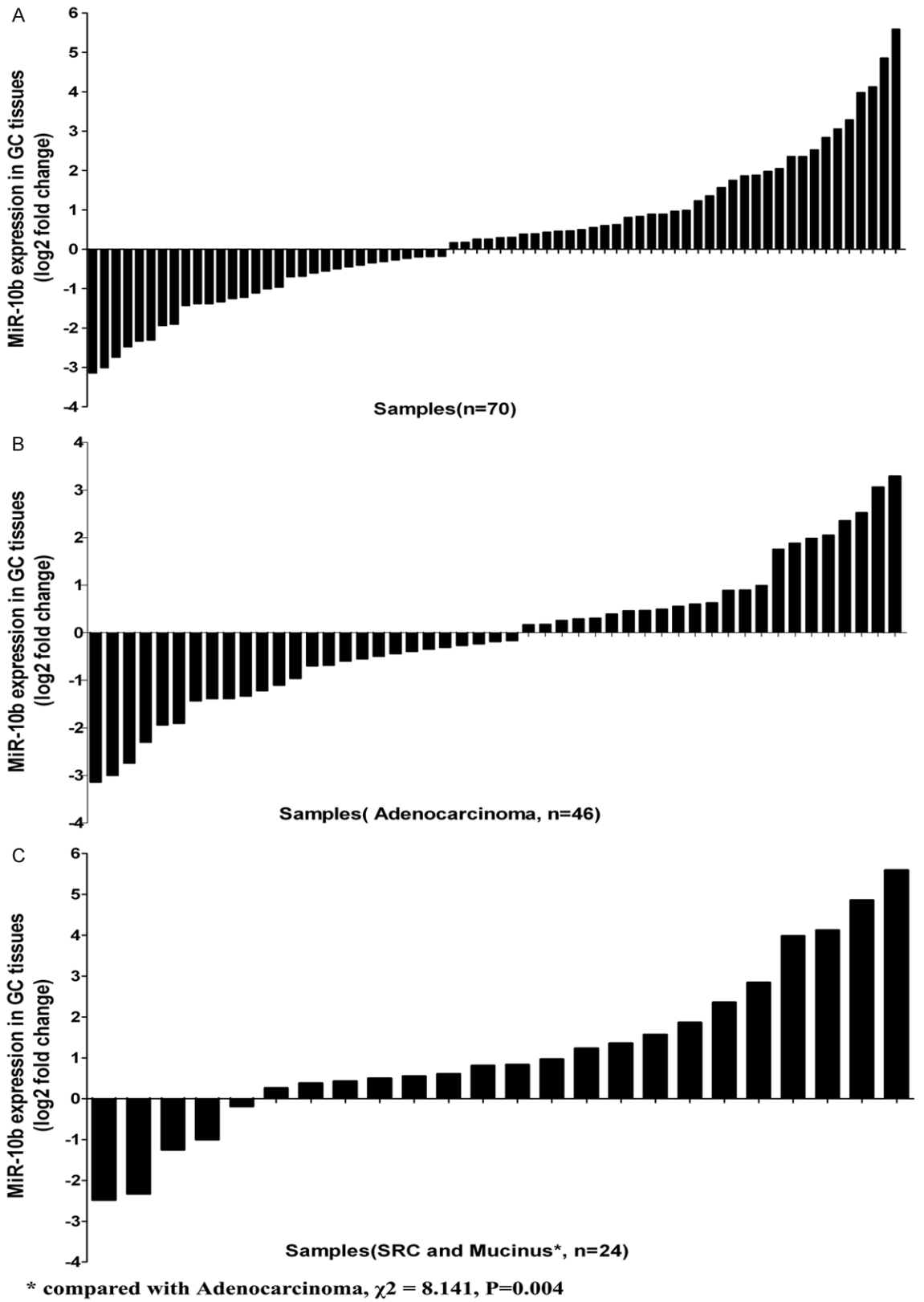

Figure 2. Detection of relative miR-10b levels in 70 cases of gastric cancer tissues. Tissue samples were subjected to qRT-PCR analysis of miR-10b level with an SYBR Green I kit. All data on the fold change were assessed as log2 values. A. Relative expression of miR-10b in 70 primary gastric cancer tissues compared with their normal muco-

sae. B. Relative expression of miR-10b in 46 tissues with adenocarcinoma relative to their normal mucosae and 20 of 46 (43.48%) cases overexpressed miR-10b (relative expression ratio > 1.0). C. Relative expression of miR-10b in 21 SRC and mucinus tissues compared with their normal mucosae and 19 out of 24 (79.17%) cases overexpressed miR-10b (relative expression ratio > 1.0).

and miR-10b mimic and Scramble-infected AGS cells were seeded into the upper chambers at a density of 5×10^4 cells and normal growth medium were placed in underneath chambers and growth for 24 h. After that, cells on the upper surface of the membrane were removed by a cotton swab, while cells on the lower chamber were stained with 0.1% crystal violet and counted at × 200 magnification in five different fields of each membrane. The experiments were in triplicate and repeated at least once.

Statistical analysis

All statistical analyses were performed using SPSS 18.0. (SPSS, Chicago, IL, USA) and the continuous variable values were expressed as Mean ± SD. Comparison of the means between two groups was analyzed using Student *t* test, while comparison of the means among multiple groups was analyzed using the one-way ANOVA test. A two-tailed *P*-value ≤ 0.05 was considered statistically significant.

Results

Up-regulated miR-10b expression in gastric cancer cells and tissues

We first assessed miR-10b levels in various gastric cell lines, i.e., a human normal gastric epithelial cell line GES-1 and seven cell lines derived from gastric cancers with various differentiation degrees and lymph node metastasis potential (**Table 1**). Our data showed that miR-10b expression was substantially higher in BGC-803 (8.6-fold compared to normal cells), MGC-803 (11.8-fold), AGS (30.9-fold), BGC-823 (56.1-fold), MKN-45 (65.2-fold), SGC-7901 (84.1-fold), and HGC-27 (225.7-fold) cell lines compared with GES-1 cells (**Figure 1**). Expression of miR-10b was also associated with tumor cell differentiation and lymph node metastasis.

Moreover, we detected miR-10b expression in 70 cases of gastric cancer tissue samples and found that level of miR-10b expression was also higher in gastric cancer tissues than in normal mucosae. After histologically divided the patients into SRC-mucinus (including Signet-ring carcinoma and mucinous adenocarcinoma) and adenocarcinoma (including moderated or poorly differentiated adenocarcinoma and neuroendocrine carcinoma), suggesting different tumor cell differentiation, miR-10b expression was upregulated in 20 out of 46 (43.48%) adenocarcinoma patients and in 19 out of 24 (79.17%) SRC-mucinus patients (**Figure 2B**, **2C**; $\chi^2 = 8.141$, P = 0.004). Altered miR-10b expression was significantly associated with tumor size (P = 0.007), location (P = 0.038), differentiation (P = 0.042), and local invasion depth (P = 0.001). However, there was no association found between miR-10b expression and clinical characteristics of these patients, such as gender, age, TNM stage, Roukos type, lymphatic invasion, and lymph node metastasis (**Table 2**).

Expression of miR-10b promoted tumor cell migration and invasion in vitro

To explore the role of miR-10b in gastric cancer, we restored miR-10b expression a gastric cancer cell line using miR-10b mimics and negative control lentiviruses. The stable cell line expressed miR-10b detected by qRT-PCR in miR-10b mimic-infected AGS cells compared to Scramble negative control cells and parental AGS cells (**Figure 3A**). The level of miR-10b expression was approximately 120-fold induced in miR-10b mimic-infected gastric cancer cells compared to that of Scramble or parental cells (both P < 0.001). The wound healing assay showed that miR-10b overexpression enhanced the migration speed of AGS cells compared with that of Scramble or parental cells after 24 h (**Figure 3B**, **3C**). Furthermore, and tumor cell invasion assay also showed that miR-10b expression induced gastric cancer cell invasion capacity (**Figure 3D**, **3E**). Collectively, our current data strongly suggest that overexpression of miR-10b promoted gastric cancer cell migration and invasion capacity in vitro.

Discussion

In this study, we first performed qRT-PCR with a stem-loop SYBR Green I miRNA kit to detect

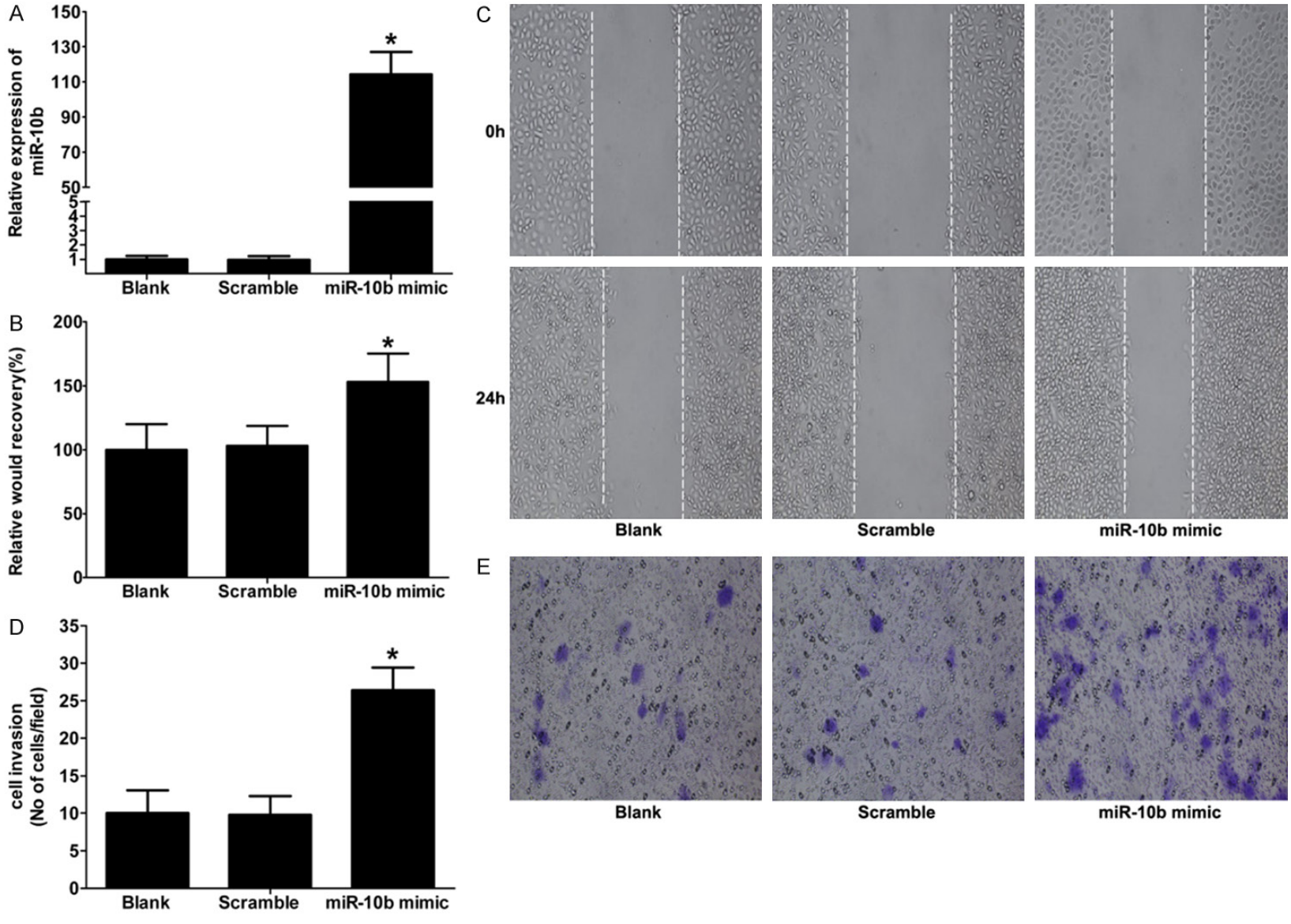

Figure 3. Effects of miR-10b on regulation of gastric cancer cell migration and invasion. A. qRT-PCR analysis of miR-10b level in parental AGS cells (Blank) and miRNA mimic or Scramble-infected AGS cells. B and C. Wound healing assay. Parental AGS cells (Blank) and miRNA mimic or Scramble-infected AGS cells were

grown and subjected to wound healing assay and photographed. The relative ratio of the wound closure per field was measured B. D and E. Transwell tumor cell invasion assay. Parental AGS cells (Blank) and miRNA mimic or Scramble-infected AGS cells were subjected to Transwell tumor cell invasion assay. The relative ratio of invasive cells per field was shown in D.

levels of miR-10b expression in gastric cells and tissues. Our data revealed higher levels of miR-10b expression in gastric cancer cells, expression of which was associated with tumor cell differentiation and lymph node metastasis potential. We also found that miR-10b expression was upregulated in gastric cancer tissue samples compared to normal mucosae. The upregulated miR-10b expression was associated with tumor size, location, differentiation, and local invasion depth. Furthermore, our in vitro experimental data showed that overexpression of miR-10b could significantly promote cell migration and invasion in vitro. Thus, our current study suggests that altered expression of miR-10b could contribute to gastric cancer development and progression. Further study will explore whether detection of miR-10b expression could be used as biomarker for gastric cancer diagnosis and whether target of miR-10b expression is useful as a novel therapeutic strategy in future clinical control of gastric cancer.

Indeed, miR-10b was originally discovered to have a tumor metastasis activity in breast cancer cells [6]. In the past few years, miR-10b has been reported to be overexpressed in various human malignant tumors, such as malignant glioma [7, 8], esophageal cancer [9], neurofibromatosis type 1 (NF1) tumorigenesis [10], nasopharyngeal carcinoma [19], human pancreatic cancer [20], hepatocellular carcinoma [21], breast cancer [22, 23], non-small cell lung cancer [24], ovarian cancer cells [25], and gastric cancer [11-14]. These studies have suggested that miR-10b overexpression participated in initiation and progression of different human cancers. However, to date, there is a debate and argument on miR-10b expression in gastric cancer continuously existed. Thus, in this study, we notably supported the view that up-regulated expression of miR-10b contributes to malignancy potential in gastric cancer.

Furthermore, our current data showed that upregulated miR-10b expression was significantly associated with tumor size, location, differentiation, and local invasion depth. As we know, characteristics of malignant neoplasms include more rapid growth and increase in tumor size, tumor cell de-differentiation (or lack of differentiation, called anaplasia), tendency to invade the surrounding tissues, and ability to metastasize to distant tissues [26, 27]. Our current study further confirmed that overexpression of miR-10b is associated with malignant phenotypes, such as tumor size and tumor differentiation, suggesting that miR-10b expression contributed to tumor growth and differentiation of gastric cancer. Furthermore, our current finding showing that miR-10b overexpression in AGS cells led to a dramatic increase in tumor cell invasion ability and that miR-10b expression was associated with tumor local invasion suggests that up-regulated miR-10b expression could promote gastric cancer progression and invasion ability. In addition, miR-10b expression was also associated with lymph node metastasis potential in gastric cancer SGC-7901 and HGC-27 cells. Our in vitro data also confirmed that up-regulated miR-10b in AGS cell could dramatically increase AGS cell migration and invasion capacity, although miR-10b expression was not associated with lymph node metastasis in ex vivo. This discrepancy may be because primary cancer lesions contain a heterogeneous mass of cells, such as mesenchymal cells, blood cells, and cancer cells. Metastatic or invasive cells might be only a small fraction of the whole cancer lesions. Alternatively, this discrepancy may be solely due to the variance of in vivo and in vitro environments. Again, histological grade and interstitial invasive depth are considered as important indicators for cancer differentiation and progression; thus, miR-10b overexpression is suggested closely associating with gastric cancer malignancy potential.

In summary, miR-10b expression was upregulated in gastric cancer tissues and cells and associated with tumor size, location, differentiation, and local invasion depth in ex vivo. MiR-10b overexpression promoted gastric cancer cell migration and invasion capacity in vitro. Future study with a larger sample size will confirm use of miR-10b levels as a biomarker for gastric cancer early detection and prediction of tumor progression or inhibition of miR-10b

expression as a novel strategy in clinical control of gastric cancer.

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Disclosure of conflict of interest

None.

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