

RESEARCH ARTICLE

Hsa-miR-181a-5p Expression and Effects on Cell Proliferation in Gastric Cancer

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Abstract

Purpose: MicroRNAs (miRNAs) are small endogenous, non-coding, single-stranded RNAs (approximately 22 nt). Accumulating evidence has shown that aberrant miRNA expression is pronounced and correlated with gastric cancer genesis and progression. **Materials and Methods:** Expression levels of miR-181a-5p in GC tissues and cell lines were assessed by qRT-PCR and tested for correlation with clinical features. In addition, effects of miR-181a-5p on GC cell growth were investigated. **Results:** Our findings indicate that miR-181a-5p is upregulated in GC, in correlation with lymph node invasion, nerve invasion and vascular invasion ($P < 0.05$). Enforced expression of miR-181a-5p promoted cell proliferation ability. **Conclusions:** This study suggested that increased miR-181a-5p is related to GC progression. MiR-181a-5p may represent a potential therapeutic target for GC.

Keywords: miR-181a-5p - GC - qPCR - cell proliferation

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Introduction

Gastric cancer (GC) is one of the most common cancers and ranks as the second leading cause of cancer death worldwide, probably accounting for about 10% of newly diagnosed cancers (Murray et al., 1997).

MicroRNAs (miRNAs) are small endogenous, non-coding, single-stranded RNAs. Mature miRNAs target the 3' untranslated region (3'UTR) of a specific mRNA by base pairing, leading to translational repression or mRNA degradation (Bartel, 2004; He et al., 2004). Bioinformatics prediction indicates that 30% of all the genes are regulated by miRNAs (Yu et al., 2007). miRNAs have been shown to play crucial roles in diverse biological processes, such as development, differentiation, apoptosis and proliferation (Chen et al., 2004; Harfe et al., 2005; Hwang et al., 2006). Accumulating evidence has strongly suggested that altered miRNA expression is a common and important feature of human malignancies (A. Esquela-Kerscher et al., 2006; S. Sassen et al., 2008). Emerging researches found that aberrant miRNAs could be important in tumorigenesis as oncogenes and tumor suppressor genes in GC (Du et al., 2009; Ding L et al., 2010; Guo et al., 2010; Tie et al., 2010; Wang et al., 2010; Guo et al., 2011). In this study, we choose miR-181a-5p as a candidate miRNA according to the miRNA microarray and then validate its expression in GC tissues and cell lines. Further research to assess

the effects of miR-181a-5p on GC cell growth. Finally, bioinformatics was used to predict the target genes of miR-181a-5p accounting for its function in GC.

Materials and Methods

Cell culture and tissue samples preparation

Human gastric adenoma cell lines (GES-1, SGC-7901, MGC-803, BGC-823 and AGS) were purchased from the Cell Bank of Shang Hai. Cells were routinely cultured with RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO₂.

Forty pairs of histopathologically confirmed GC and adjacent non-cancer tissue samples were obtained from patients in Nanjing Jiangning Hospital, China. Informed consent was taken from all patients and the procedure was approved by the Medical Ethics Committee of Nanjing Jiangning Hospital.

miRNA microarray analysis.

Prior to experimentation, five pairs of GC and adjacent non-cancer tissue samples were analyzed by miRNA microarray. Total RNA was harvested using TRIzol (Invitrogen) and an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. After RNA quantification using a Nanodrop spectrophotometer, the samples were labeled using the miRCURYHY3/Hy5 Power Labeling Kit

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Table 1. miRNAs are Expressed Differentially in GC Tissues

miRNA	upregulation
hsa-miR-181a-5p	14.20864382
hsa-miR-20b-5p	13.21065574
hsa-miR-708-5p	11.3383709
hsa-miR-196b-5p	7.880424301
hsa-miR-135a-5p	7.514186633
hsa-miR-210	4.18647541
hsa-miR-194-5p	3.62243605
hsa-miR-374a-5p	3.575597472
hsa-miR-199a-3p/hsa-miR-199b-3p	3.465471311

and hybridized to the miRCURYLNAArray (v. 11.0). The samples were hybridized using a hybridization station and the arrays were scanned with the Axon GenePix 4000B Microarray Scanner. The raw intensity of the image was read using GenePix Pro V6.0. The intensity of the green signal was calculated after background subtraction, and four replicated spots for each probe on the same slide were averaged. The Median Normalization Method was used to obtain 'Normalized Data' [Normalized Data = (foreground-background)/median]. The median was defined as the 50% quantile of microRNA intensity that was > 50 in all samples after background correction. The statistical significance of the differentially expressed miRNA was analyzed using the Student's t-test.

Real-time reverse transcriptase quantitative PCR

Total RNA was extracted from cells and tissues samples with Trizol reagent (Invitrogen). The quality and quantity of the RNA samples were assessed by standard electrophoresis and spectrophotometric methods. Real-time reverse transcriptase quantitative PCR (qRT-PCR) analysis were performed with locked nucleic acids (LNAs) linear primers (EXIQON) and SYBR Green I, and U6 small nuclear RNA was used as normalized PCR primers. All reagents for stem-loop RT and Q-PCR were obtained from TAKARA. The primers used for stem-loop RT-PCR and Q-PCR were synthesized and purified by RiboBio. The PCR conditions were 95°C for 30 s, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s. The reactions were monitored using a preheated real-time instrument (ABI step one).

The relative expression ratio of miR-181a-5p in gastric cancer tissues and cells was quantified by the $2^{-\Delta\Delta CT}$ method.

Cell Proliferation Assay---cck-8

The miR-181a-5p mimics (sense: 5'-AACAUUCAACGCUGUCGGUGAGU-3', antisense: 5'-UCACCGACAGCGUU GAAUGUUGU-3'), mimics control (sense: 5'-UUCUCCGAACGUGUCACGUTT-3', antisense: ACGUGACACGUUCGGAGAATT-3'), and hsa-miR-181a-5p inhibitor (5'-ACUCACCGACAGCGU UGAAUGUU-3'), inhibitor control (5'-CAGUACUUU UGUGUAGUACAA-3') were synthesized and purified by GenePharma Co. (Shanghai, China).

One day before transfection, 5.0 x 10³ BGC cells in 100ul growth medium were plated in each well of a 96-well plate. The cells were then transfected with 50

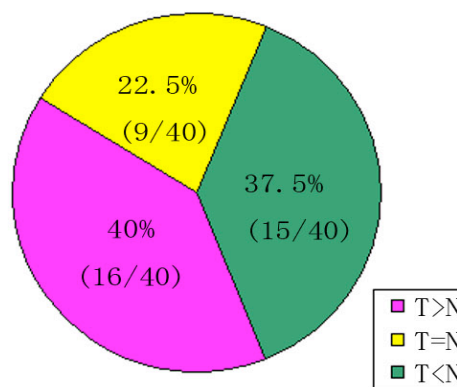


Figure 1. Hsa-miR-181a-5p Expression in Forty Matched GC Tissues, Q-PCR. N refers to normal tissues, T refers to tumor tissues

nM of various synthetic miRNAs mimics and 100 nM inhibitor using Lipofectamine2000 (Invitrogen) according to the manufacturer's instruction. Cell proliferation was assessed at different time points (0 h, 24 h, 48 h, and 72 h), using Cell Counting Kit 8 (Dojindo, Tokyo, Japan) according to manufacturer's protocol. The absorbance at a wave length of 450 nm, which shows positive relation to the capacity of cellular proliferation, was measured by a spectrophotometer.

Bioinformatic prediction for miR-181a-5p targeted genes

TargetScan (<http://www.targetscan.org>), miRanda (<http://www.microrna.org>) and PicTar (<http://pictar.bio.nyu.edu/>) online searching programs was used for the prediction of miR-181a-5p target genes.

Statistical analysis

Data were presented as mean \pm SD, the significance was analyzed with the Student's t-Test, the statistical significance of correlation was calculated by chi-square test and Spearman's rank correlation. Statistical analysis was performed using SPSS 16.0 software and differences were considered statistically significant when $p < 0.05$.

Research experience

We have enough experience in conducting medical researches, including clinical researches, and have published some results elsewhere (Jiang et al., 2010; Gao et al., 2011; Huang et al., 2011; Li et al., 2011; Li et al., 2011; Li et al., 2011; Xu et al., 2011; Xu et al., 2011; Xu et al., 2011; Yan et al., 2011; Zhang et al., 2011; Gong et al., 2012; Gong et al., 2012; Gu et al., 2012; Li et al., 2012; Yu et al., 2012; Zhan et al., 2012; Zhan et al., 2012; Deng et al., 2013; Huang et al., 2013; Liu et al., 2013; Liu et al., 2013; Lu et al., 2013; Wu et al., 2013; Yin et al., 2013; Yin et al., 2013).

Results

Differential expression miRNAs analysis in GC tissues by high through-out chip

Based on data of miRNA microarray assay, more than 90 miRNAs were increased expression in GC tissues with more than 3-folds change (Table 1) and were differentially

expressed between the normal gastric tissue and GC, among which miR-181a-5p was significantly upregulated. Therefore, miR-181a-5p was chosen as a candidate miRNA to evaluate the role in gastric carcinogenesis.

miR-181a-5p expression pattern and expression level was analyzed in forty matched GC tissues by qPCR

Q-PCR was used to validate miR-181a-5p expression in 40 pairs GC tissues. Compared to the normal tissues, hsa-miR-181a-5p was upregulated in GC tissues, accounting for 40% (Figure 1B).

miR-181a-5p expression in GC cell lines

Compared with normal Gastric cell line—GES-1, miR-181a-5p was highly expressed in SGC-7901, MGC-803, BGC-823 cell lines. The expression level was respectively 2.1-folds, 5.6-folds and 8.3-folds (Figure 2).

Correlation between miR-181a-5p expression and clinical features

To investigate miR-181a-5p role which is involved GC tumorigenesis and progression, we analysed the correlation between miR-181a-5p expression and clinical features. The results reveals that -miR-181a-5p expression is correlation with Lymph node invasion, nerve invasion,

vascular invasion (Table 2).

miR-181a-5p promotes the cell proliferation

To assess the effects of hsa-miR-181a-5p on GC cell growth, we transfected miR-181a-5p mimics/inhibitor respectively into BGC-823 cells, Cell growth curve assay reveals that miR-181a-5p mimics promoted cell proliferation, while hsa-miR-181a-5p inhibitor shows corresponding results (Figure 3).

Targeted genes of miR-181a-5p prediction

We observed many target genes (Table 3), including BCL2/K-RAS/GATA6/CDX2, Which suggested that hsa-miR-181a-5p may involved in the proliferation, apoptosis pathways.

Discussion

Recently, accumulating evidence indicated that miRNA is aberrant expressed in various human malignancies and play a role as oncogenes or tumor suppressor genes. Previous researches using microarray analysis suggested that a number of differentiated expression miRNAs were associated with GC, and could be potential markers for diagnosing and monitoring GC (Guo et al., 2012;

hsa-miR-181a-5p expression in GC cell lines

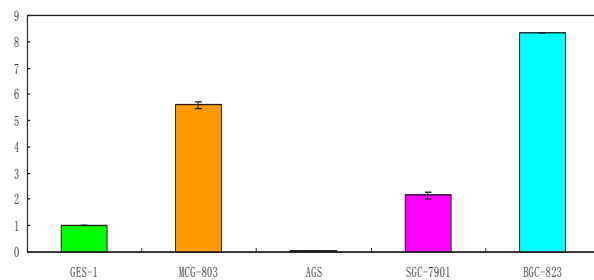


Figure 2. miR-181a -5p Expression in GC Cell Lines, Q-PCR. The relative expression ratio was quantified by the $2^{-\Delta\Delta CT}$ method

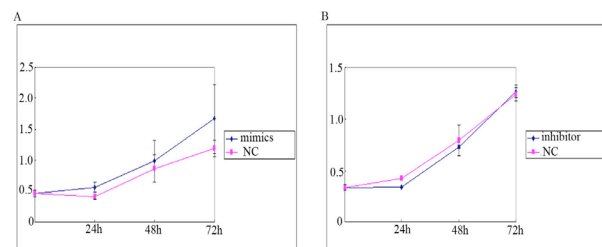


Figure 3. Effects of hsa-miR-181a -5p Over-expression or Knock-down on BGC-823 Cell Growth. NC refers to the negative control

Table 2. Correlation Between hsa-miR-181a-5p Expression and Clinical Features

features	upregulation	downregulation	P value	r value
Gender				
Male	11	16		
Female	5	8	0.547	0.096
Differentiation				
Low	4	11		
Moderate	12	13	0.182	0.211
Lymph node invasion				
No	5	16		
Yes	11	8	0.028	0.347
Vascular invasion				
No	6	18		
Yes	10	6	0.018	0.375
Nerve invasion				
No	12	9		
Yes	4	15	0.020	0.368
Lymphatic metastasis				
No	5	6		
Yes	11	18	0.668	0.069
Infiltration degree				
Tunica Mucosa	3	5		
Mucous Layer Outside	13	19	1.000	0.026
Stages				
III	4	10		
III IV	12	14	0.279	0.171

Table 3. hsa-miR-181a-5p Target Genes Predicted by Targetscan, miRanda, PicTar

BCL2	FBX011	ATP8A1	KLADCS	TIAL1	NUPL1	METAP1	PROX1
K-ras	SLC37A3	TMEM64	PDX3X	KIAA	CCNG1	BAPX1	DDIT4
GATA6	YOD1	NOCH2	SFRS7	LCLAT1	GPR137B	PLCL2	ANKRD13C
NRP1	AFTPH	NFYB	LBR	TGFBR3	METAP1	ARF6	RLF
CDX2	NR6A1	KLADCS	GIGYF1	PITPNB	BAPX1	SLC7A11	PLXDC2
NLK	ZNF445	PDX3X	FBX028	SCD	NUPL1	HMGB2	NOL4
PLAG1	TM9SF3	SFRS7	PUM1	FBXO33	CCNG1	BTBD3	TMED4
STCH	ZNF148	NFYB	RNF34	FAM160A2	GPR137B	MTMR12	CCDC6

Poliseno et al., 2012). It is noted that miR-181a is down-regulated expression in breast cancer, oral, hepatocellular, ovarian and involve many biological process , e.g. cell proliferation, cell apoptosis, etc (Guo et al., 2012; Poliseno et al., 2012; Shin et al., 2012; Zhou et al., 2012). However, few studies focus on the expression and function of miR-181a in GC, and no consistent result regarding miR-181a expression and its function in GC.

In this study, depending on miRNA microarray, we showed miRNAs were differentially expressed between normal gastric and GC tissue, and miR-181a-5p was significantly upregulated. Therefore, we choose miR-181a-5p as a candidate miRNA. We validated hsa-miR-181a-5p expression level in forty matched GC tissues and GC cell lines. Our results showed that expression of miR-181a-5p was upregulated in GC tissues (40%), but the difference was not statistically significant. While, the expression of miR-181a-5p in SGC-7901, MGC-803, BGC-823 cell lines is upregulated compared to immortalize GES-1, and to some extent, was consistent with the results of microarray analysis. Meanwhile, we analyzed the correlation between hsa-miR-181a-5p expression and clinical features. These results demonstrate that hsa-miR-181a-5p expression is correlated with lymph node, nerve, and vascular invasion ($p < 0.05$), suggesting that hsa-miR-181a-5p could be associated with GC tumorigenesis and progression. We found that hsa-miR-181a-5p overexpression promotes cell proliferation, by assessing the effects of miR-181a-5p on GC cell growth. Yang reported that miR-181a-5p was overexpressed and enhanced lymph-node metastasis through regulating migration in OSCC (Yang et al., 2011); and Bhattacharya proved that the expression of miR-181a-5p was upregulated in HCC (Bhattacharya et al., 2010).

In summary, our findings suggest that hsa-miR-181a-5p is correlated with GC tumorigenesis and progression. According to the relationship between the expression of miR-181a and the invasion of gastric cells, we suggest that hsa-miR-181a-5p could be a potential biomarker of GC and play a role in determining the prognosis of GC patients.

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