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

Gang Chen¹&, Zhi-Li Shen¹&, Ling Wang²&, Chun-Ye Lv¹, Xin-En Huang³*, Rong-Ping Zhou¹*

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

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; DingL 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 Shanghai. 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 RNaseasy 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...
and hybridized to the miRCURY LNAArray (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’-AACAUUCAACGCGUCCGUGAGU-3’, antisense: 5’-UCACCGGACGUGUAAUGUUGU-3’), mimics control (sense: 5’-UUCUCCGAACGUGACUGUTT-3’, antisense:ACUGAGACCGUUGUGAGATT-3’), and hsa-miR-181a-5p inhibitor (5’-ACUCAGCAGCGUUGAUGUUGU-3’), inhibitor control (5’-CAGUACUUGUGUAGUACAA-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 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 ± 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 $p< 0.05$.

**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.
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;
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.

Acknowledgements
Dr. Xin-En Huang is supported in part by a grant from Jiangsu Provincial Administration of Chinese Medicine (LZ11091), and in part from a special research fund of Organization Department of Jiangsu Provincial Party Committee, Talent Work Leading Group of Jiangsu Province (333 High-level Talents Training Project).

References
Bartel DP, MicroRNAs (2004). Genomics, biogenesis, mechanism, and function. Cell, 116, 281-97. Bhattacharya SD, Garrison J, Guo H, et al (2010). Micro-RNA-181a regulates osteopontin-dependent metastatic function in hepatocellular cancer cell lines[J]. Surgery, 148, 2, 291-7. Chen CQ, Li L, Lodish HF, Bartel DP (2004). MicroRNAs modulate hematopoietic lineage differentiation. Science, 303, 83-6. Deng QQ, Huang XE, Ye LH, et al (2013). Phase II trial of Loubo (Lobaplatin) and pemetrexed for patients with metastatic breast cancer not responding to anthracycline or taxanes. Asian Pac J Cancer Prev, 14, 413-7. Ding L, Xu Y, Zhang W, et al (2010). MiR-375 frequently downregulated in gastric cancer inhibits cell proliferation by targeting JAK2. Cell Res, 20, 784-93. Du Y, Xu Y, Ding L, et al (2009). Down-regulation of miR-141 in gastric cancer and its involvement in cell growth. J Gastroenterol, 44, 556-61. Esquela-Kerscher A, Slack FJ (2006). Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer, 6, 259-69. Gao LL, Huang XE, Zhang Q, et al (2011). A Cisplatin and vinorelbine (NP) regimen as a postoperative adjuvant chemotherapy for completely resected breast cancers in China: final results of a phase II clinical trial. Asian Pac J Cancer Prev, 12, 77-80. Gong P, Huang XE, Chen CY, et al (2012). Comparison on complications of peripherally inserted central catheters by ultrasound guide or conventional method in cancer patients. Asian Pac J Cancer Prev, 13, 1873-5. Gu M, Li SY, Huang XE, et al (2013). A phase II study on continuous infusional paclitaxel and 5-Fu as first-line chemotherapy for patients with advanced esophageal cancer. Asian Pac J Cancer Prev, 13, 5587-91. Guo LJ, Zhang QY (2012). Decreased serum miR-181a is a potential new tool for breast cancer screening. Int J Mol Med, 30, 680-6. Guo X, Guo L, Ji J, et al (2010). miRNA-331-3p directly targets E2F1 and induces growth arrest in human gastric cancer. Biochem Biophys Res Commun, 398, 1-6. Guo XB, Jing CQ, Li LP, et al (2011). Down-regulation of miR-622 in gastric cancer promotes cellular invasion and tumor metastasis by targeting ING1 gene. World J Gastroenterol, 17, 1895-902. Harfe BD (2005). MicroRNAs in vertebrate development. Curr Opin Genet, 15, 410-5. He L, Hannon GJ (2004). MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet, 5, 522-31. Huang XE, Li CG, Li Y, et al (2011). Weekly TP regimen as a postoperative adjuvant chemotherapy for completely resected breast cancer in China: final result of a phase II trial. Asian Pac J Cancer Prev, 12, 2797-800. Huang XE, Wei GL, Huo JG, et al (2013). Intraperitoneal or intraperitoneal lobaplatin for treatment of patients with malignant pleural effusion or ascites. Asian Pac J Cancer Prev, 14, 2611-4. Hwang HW, Mendell JT (2006). MicroRNAs in cell proliferation, cell death, and tumorigenesis. Br J Cancer, 94, 776-80. Jiang Y, Huang XE, Yan PW, et al (2010). Validation of treatment efficacy of a computer-assisted program for breast cancer patients receiving postoperative adjuvant chemotherapy. Asian Pac J Cancer Prev, 11, 1059-62. Li CG, Huang XE, Li Y, et al (2011). Clinical observations on safety and efficacy of OxyContin administered by rectal route in treating cancer related pain. Asian Pac J Cancer Prev, 12, 2477-8. Li CG, Huang XE, Li Y, et al (2011). Phase II trial of irinotecan plus nedaplatin (INP) in treating patients with extensive stage small cell lung cancer. Asian Pac J Cancer Prev, 12, 487-90. Li CG, Huang XE, Xu L, et al (2012). Clinical application of serum tumor associated material (TAM) from non-small cell lung cancer patients. Asian Pac J Cancer Prev, 13, 301-4. Li Y, Yan PW, Huang XE, et al (2011). MDR1 gene C3435T polymorphism is associated with clinical outcomes in gastric cancer patients treated with postoperative adjuvant chemotherapy. Asian Pac J Cancer Prev, 12, 2405-9. Liu J, Huang XE, Tian GY, et al (2013). Phase II Study on Safety
and Efficacy of Yadranzi (Javanica oil emulsion injection) Combined with Chemotherapy for Patients with Gastric Cancer. *Asian Pac J Cancer Prev*, 14, 2009-12.

Liu W, Li SY, Huang XE, et al (2012). Inhibition of tumor growth in vitro by a combination of extracts from rosa roxburghii tratt and fagopyrum cymosum. *Asian Pac J Cancer Prev*, 13, 2409-14.

Liu YC, Zhou SB, Gao F, et al (2013). Chemotherapy and Late Course Three Dimensional Conformal Radiotherapy for Treatment of Patients with Stage III Non-small Cell Lung Cancer. *Asian Pac J Cancer Prev*, 14, 2663-5.

Lu YY, Huang XE, Xu L, et al (2013). Potential Predictors of Sensitivity to Pemetrexed as First-line Chemotherapy for Patients with Advanced Non-Squamous NSCLCs. *Asian Pac J Cancer Prev*, 14, 2005-8.

Murray CJ, Lopez AD (1997). Alternative projections of mortality and disability by cause 1990-2020: global burden of disease study. *Lancet*, 349, 1498-504.

Poliseno L, Haimovic A, Segura MF, et al (2012). Histology-specific microRNA alterations in melanoma. *J Invest Dermatol*, 132, 1860-8.

Sassen S, Miska EA, Caldas C (2008). MicroRNA: implications for cancer. *Virchows Arch*, 452, 1-10.

Shin KH, Bae SD, Hong HS, et al (2011). miR-181a shows tumor suppressive effect against oral squamous cell carcinoma cells by downregulating K-ras. *Biochem Biophys Res Commun*, 404, 896-902.

Shu J, Li CG, Liu YC, et al (2012). Comparison of Serum Tumor Associated Material (TAM) with Conventional Biomarkers in Cancer Patients. *Asian Pac J Cancer Prev*, 13, 2399-403.

Sun MQ, Meng AF, Huang XE, et al (2013). Comparison of psychological influence on breast cancer patients between breast-conserving surgery and modified radical mastectomy. *Asian Pac J Cancer Prev*, 14, 149-52.

Tie J, Pan Y, Zhao L, et al (2010). MiR-218 inhibits invasion and metastasis of gastric cancer by targeting the Robo1 receptor. *PLoS Genet*, 6, e1000879.

Wang HJ, Ruan HJ, He XJ, et al (2010). MicroRNA-101 is down-regulated in gastric cancer and involved in cell migration and invasion. *Eur J Cancer*, 46, 2295-303.

Wu XY, Huang XE, You SX, et al (2013). Phase II study of pemetrexed as second or third line combined chemotherapy in patients with colorectal cancer. *Asian Pac J Cancer Prev*, 14, 2019-22.

Xu YC, Huang XE, Wang L, et al (2011). Clinical study on safety and efficacy of Yadanzi (Javanica oil emulsion injection) Combined with Chemotherapy for Patients with Metastatic Gastric Cancer. *Asian Pac J Cancer Prev*, 12, 2295-9.

Xu JW, Li CG, Huang XE, et al (2011). Ubenimex capsule improves general performance and chemotherapy related toxicity in advanced gastric cancer cases. *Asian Pac J Cancer Prev*, 12, 985-7.

Xu T, Xu ZC, Zou Q, Yu B, Huang XE (2012). P53 Arg72Pro polymorphism and bladder cancer risk--meta-analysis evidence for a link in Asians but not Caucasians. *Asian Pac J Cancer Prev*, 13, 2349-54.

Yan PW, Huang XE, Yan F, et al (2011). Influence of MDR1 gene codon 3435 polymorphisms on outcome of platinum-based chemotherapy for advanced non small cell lung cancer. *Asian Pac J Cancer Prev*, 12, 2291-4.

Yang CC, Hung PS,Wang PW, et al (2011). miR-181 as a putative biomarker for lymph-node metastasis of oral-squamous cell carcinoma[J]. *J Oral Pathol Med*, 40, 397-404..