RESEARCH ARTICLE

Effects of MicroRNA-106 on Proliferation of Gastric Cancer Cell through Regulating p21 and E2F5

Yong-Liang Yao, Xiao-Yang Wu, Jian-Hong Wu, Tao Gu, Ling Chen, Jin-Hua Gu, Yun Liu, Qing-Hui Zhang*

Abstract

Objective: To investigate the effects of miR-106b on malignant characteristics of gastric cancer cells, and explore possible mechanisms. Methods: Expression of miR-106b, p21 and E2F was determined by real-time PCR. Transfection with miR-106b mimics was conducted, and gastric cancer cells with miR-106b overexpression were obtained. Cells transfected with mimic mutants and those without transfection served as negative and blank controls, respectively. Flow cytometry and transwell assays were adopted to detect the effects of miR-106b overexpression on cell cycle, migration and invasion of gastric cancer cells. Results:. The expression of miR-106b in gastric cancer cells was significantly higher than that in normal gastric mucosa cells. Furthermore, the expression level of miR-106b rose according to the degree of malignacy among the three GC cell strains (MKN-45 > SGC-7901 > MKN-28). Overexpression of miR-106b shortened the G0/G1 phase and accelerated cell cycle progression, while reducing p21 and E2F5, without any significant effects on the capacity for migration and invasion of gastric cancer cells. Conclusions: miR-106b may promote cell cycling of gastric cancer cells through regulation of p21 and E2F5 target gene expression.

Keywords: Gastric cancer - microRNA - proliferation - cell cycle - migration - invasion

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Introduction

In recent years, with the in-depth research of Nucleic Acids, the study of genome's "dark matter" - non-coding RNA has become hot spots to explore life sciences, such as miRNAs are widely used in the research of development and diseasea (Martin et al., 2012). In the tumor related miRNAs positioning study, we have found that 98 (52.5%) of the 186 miRNA-encoding genes located in the tumor-associated region of chromosome fragile sites, prompted miRNA may play an important role in the process of tumor formation (Calin et al., 2001). Currently, only a small part of the biological function of miRNAs are articulated, these miRNAs regulate cell growth and tissue differentiation, involved in several physiological processes (Moss., 2002; Anglicheau et al., 2010; Herranz et al., 2010).

MiR-106b is located on chromosome 7q21 amplified region of gastric cancer. In a previous study, found its target genes are p21 and E2F5 (Li et al., 2011; Trompeter et al., 2011). It is significantly overexpressed in gastric cancer, but its main role is still unclear. This article is to explore the effect of miR-106b on malignant features of gastric cancer cells, by analyzing itself and its target genes expression in different gastric cancer cell lines. With the elucidation of miRNAs mechanism, we found miRNAs also closely related to the development of a variety of human tumors, including gastric cancer (Gong et al., 2005). Numerous studies have shown that the use of miRNAs microarray\, Northern Blotting, quantitative stem-loop PCR technology, each stage of the tumor tissue-specific expression of miRNAs can be efficiently identified, thus further to diagnoses and foresee its prognosis (Manikandan et al., 2008). MiRNAs has become a new biological marker in the diagnosis of diseases, may also be to explore molecular drug targets or to simulate drug research, to provide new ideas and means to the treatment of a variety of human diseases in the future.

Materials and Methods

Cell culture

Human gastric cancer cell lines MKN-28, SGC-7901 and MKN-45, and normal gastric mucosa cell line GES were obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in 25-cm^2 cell culture flask at 37°C in a humidified atmosphere of 5% CO2 with RPMI-1640 Medium (Life Technologies, Grand Island, NY, USA) containing 10% fetal calf serum with 50 U/ml penicillin and 50 μg/ml streptomycin.

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Table 1. Primers for Real-time RT- PCR

Gene	Primers	Primer Sequence
miR-106b	RT primer (5'-3')	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATCTGC
	Forward (5'-3')	CGCTAAAGTGCTGACAGTG
	Reverse (5'-3')	GTGCAGGGTCCGAGGT
U6 RNA	RT primer (5'-3')	CGTTCACGAATTTGCGTGTCAT
	Forward (5'-3')	GCTTCGGCAGCACATATACTAAAAT
	Reverse (5'-3')	CGCTTCACGAATTTGCGTGTCAT
E2F5	Forward (5'-3')	CCTGTTCCCCCACCTGATG
	Reverse (5'-3')	TTTCTGTGGAGTCACTGGAGTCA
P21	Forward (5'-3')	CCTCATCCCGTGTTCTCCTTT
	Reverse (5'-3')	GTACCACCCAGCGGACAAGT
β-actin	Forward (5'-3')	CTGGAACGGTGAAGGTGACA
	Reverse (5'-3')	AAGGGACTTCCTGTAACAATGCA

Cell transfection

MiR-106b mimics were transfected into MKN-45 cell line. Cells with mutant mimics and without anything were used as the randomized and blank control groups, respectively. 3 mmol mimics were diluted in 200 μl serum-free RPMI-1640 medium, and then 10 μl the liposome Lipofectamine TM2000 were diluted in 200 μl RPMI-1640 medium by mixing gently. After incubation at room temperature for 5 min, the two agents were mixed gently and incubated at room temperature for 20 min. The resulting complexes were added into cells and shaken gently, and were incubated overnight at a temperature of 37°C, and humidity of 5% CO_2. In the follow-up experiments, the medium was replaced with containing 10% fetal calf serum and cultured for 48h.

Real-time polymerase chain reaction (Real-time PCR)

Total RNAs were extracted by using Trizol in accordance with the manufacturer's instructions. Reverse transcriptase reactions contained RNA samples, 50 nM stem-loop RT primer (Table 1), 1×RT buffer (Biosystems), 0.25 mM each of dNTPs, 3.33 U/ml MultiScibe reverse transcriptase (Biosystems), and 0.25 U/ml RNase inhibitor (Applied Biosystems). The 7.5 μl reactions were incubated in a ABI 7900 cycler for 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C and then held at a constant temperature of 4 °C. Quantitative PCR reaction conditions: 95 ° C for 10 min, 95 ° C for 15 s, 60 ° C for 1 min, 35 cycles (Chen et al., 2005; Xu et al., 2013), the specific primer sequences are shown in Table 1. Each sample was measured three times.

Western blot

For detecting the changes of miR-106b targets in protein levels, Western blot analysis were performed in MKN-45 cell lines transfected with miR-106b mimics and several control groups. For the detection of E2F5 and P21 proteins, antibodies for proteins (Santa Cruz Biotechnology, at 1:500 dilution) were diluted. The secondary HRP conjugate antibodies were diluted 1:1500. The immunocomplexes were detected using the ECL system (Beyotime Biotechnology, China).

Analysis of cell viability

Cell viability was measured by using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan), which assessed the number of cells based on the reduction of a water- soluble formazan by the dehydrogenases present in viable cells. Briefly, cells (1~2×10^3 cells/well) were seeded in 96- well plates (200 μl medium/well). Three wells of each group were used in repeated experiments. After 24 h post-attachment, cells were transfected. At the appropriate times , 10 μl of WST-8 solution was added to each well, and cells were incubated at 37°C for 3 hours. Absorbance was read at 450 nm on a microplate reader (SPECTRAFLUOR, TECAN, Sunrise, Austria).

Flow cytometry cell cycle

collection suppress most efficient at 48 h after transfection of the interference group of MKN-45 cells were washed twice with PBS, fixed with 70% ethanol, 4 °C overnight adding 50 μg/mL propidium iodide piperidine (PI) 250 μl of 1mg/mL of RNase 10 μl of dark 30min, flow cytometry measurement of cell cycle.

Cell migration and invasion assay

Cell migration assays were performed using transwell chambers with 8 μm pores (Chemicon). The lower chambers of the transwell plates were filled with 500 μl medium containing 10% fetal bovine serum as a chemoattractant. The cell suspension (300 μl) was then added to the upper chamber, and plates were incubated at 37°C for 24 h. Cells that migrated to the lower surface of the polycarbonate membrane were stained with Giemsa solution. The cells that migrated to the lower surface were quantified by counting 5 randomly selected microscope fields at ×400 magnification. Cell invasion assays were performed using same way, transwell chambers covered wiht Matrigel and calculated to cultivate through the number of cells after 36 hours.

Statistical analysis

All results are expressed as mean ± standard deviation (X ± s). Real time-PCR data were analyzed using paired t-test comparison analysis (Livak et al., 2001). Western blot, cell cycle, transwell migration and invasion assay were used ANOVA test for analysis. P <0.05 was statistically significant.

Results

The expression of miR-106b and its target gene is increased in gastric cancer cell lines

The results showed that have different expression levels of miR-106b in gastric cancer cell line MKN-45,

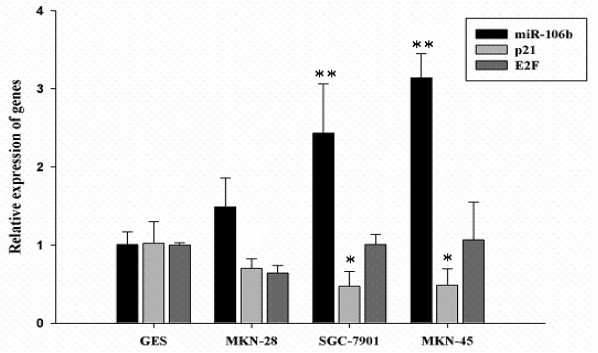

Figure 1. The Expression of miR-106b and its Target genes in Gastric Cancer Cell Lines and Normal Gastric Mucosa Cell. $p < 0.05$ (*), $p < 0.01$ (**) was considered a statistically significant difference

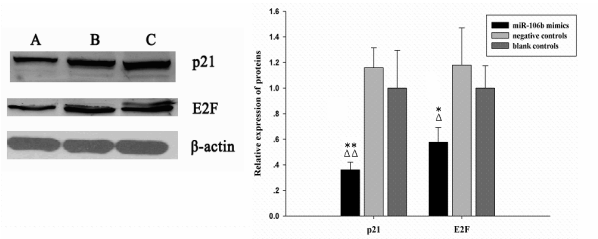

Figure 2. Comparison of Relative Expression Levels of p21, E2F5 Protein after miR-106b Mimics Transfection, as Determined by Western Blotting. A: mib-106b mimics transfected groups, B: negative control groups, C: blank control groups. *P<0.05 vs negative control ; **P<0.01 vs negative control; $^{\Delta}P$<0.05 vs blank control, $^{\Delta\Delta}P$<0.01 vs blank control

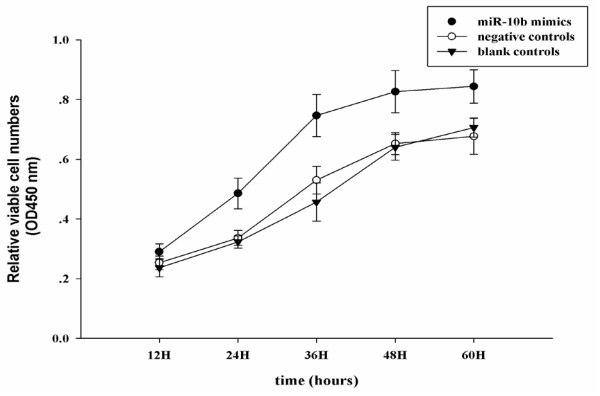

Figure 3. Cell Growth Curve of Gastric Cancer Cell Transfected with miR-106b Mimics. miR-106b effects on the cell cycle of gastric cancer cell

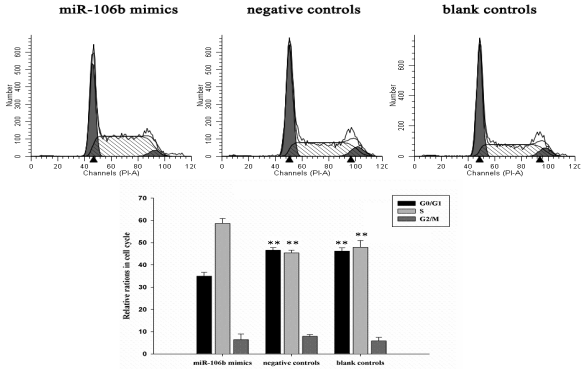

Figure 4. Effects on Tumor Cell Cycle of Gastric Cancer Cell after miR-106b Mimics Transfection. **P<0.01 vs negative control

SGC-7901 and MKN-28 and normal gastric mucosa cell line GES, but expressed in gastric cancer were significantly higher than normal strains. MiR-106b expression were significantly increased in MKN-45 (3.14 ± 0.31, $P < 0.001$) and SGC-7901 (2.43 ± 0.63, $P = 0.003$), MKN-28 (1.49 ± 0.37, $P = 0.182$), compared with normal gastric mucosa cell line GES (1.01 ± 0.16). Meanwhile, we found that the expression of miR-106b presents increasing trend in accord with malignancy (MKN-45 > SGC-7901 > MKN-28). Similarly, the expression of its target gene p21 was significantly increased in MKN-45 (0.49 ± 0.21, $P = 0.013$) and SGC-7901 (0.47 ± 0.19, $P = 0.011$) compared to the mRNA the GES (1.02 ± 0.28), but there was no significant difference in MKN-28 (0.70 ± 0.12, $P = 0.093$). The expression of another target gene E2F was not significant change in various cell lines (Figure 1).

Overexpression for miR-106b effects on target proteins

miR-106b mimics were transfected in MKN-45, the highest gastric cancer cell lines for miR-106b expression, for 48 hours to explore its target proteins p21 and E2F5. The results demonstrated that, along with the increase of miR-106, p21 expression was significantly decreased in the experimental group (0.58 ± 0.11) ($P = 0.007$, $P = 0.002$), compared to blank control group (1.01 ± 0.18, $P = 0.046$) and random control group (1.18 ± 0.29, $P = 0.046$). Meanwhile, E2F5 protein expression was also uncommonly reduced (0.58 ± 0.11) (Figure 2).

To characterize the function of miR-106b on GC cell proliferation, miR-106b mimics transfected MKN-45 cells were cultured and detected the cell proliferation rate in different time using cell counting kit 8 (CCK8). The assay manifested that miR-106b can play a catalytic role of gastric cancer cell proliferation ($F = 18.204$, $P < 0.001$), as Figure 4 shows in vitro miR-06b can significantly enhanced cell proliferation effect, and presented in a certain range of time-dependent, and then the slope of the proliferation rate was decline after 48 hours, suggesting the efficiency begins to reduce in unit concentration of miR-106b, its effect gradually reached a plateau.

In this study, we found that miR-106b could increase proliferation of the GC cells. Therefore, we investigated the mechanism of this phenomenon. The effect of miR-106b on cell cycle was tested using flow cytometry analyses. miR-106b overexpressed cells accelerated cells in the G0/G1 phase ($34.93 \pm 1.72\%$), S phase cells$58.65 \pm 2.20\%$, compared with the negative control (G0/G1, $46.64 \pm 1.06\%$; S, $45.44 \pm 1.14\%$) and the blank control (G0/G1, $46.20 \pm 1.49\%$; S, $47.92 \pm 3.05\%$) were significantly different ($P = 0.004$, $P = 0.005$), our results reveal that miR-106b can promote gastric cell cycle transition, accelerated proliferation through shortening G0/G1 (Figure 4).

miR-106b has no effect on gastric cancer cell migration and invasion

Metastasis is an important character of gastric cancer that influences both clinical treatment and prognosis. To test the impact of miR-106b is necessary in GC cell metastasis, the function of miR-106b on GC cell migration

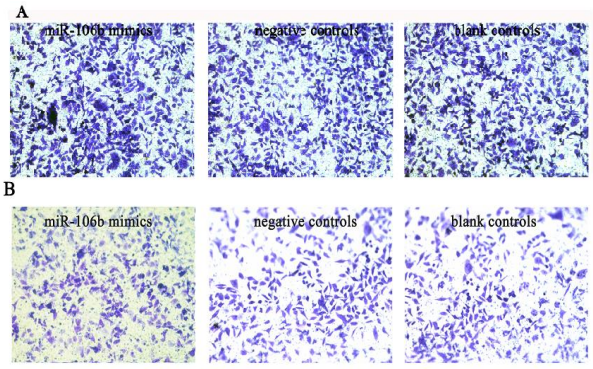

Figure 5. Effects on Cell Migration and Invasion of Gastric Cancer Cell after miR-106b Mimics Transfection. A: effects on cell migration, B: effects on cell invasion

and invasion is studied. The results of Transwell chamber assay showed that the relative percentage of cell migration and invasion were similar between the transfected cells group and negative or blank control groups. The migration or invasion ability of the cells of the experimental group is no significant changes, indicating that miR-106b can not affect the invasive ability of cancer cells (Figure 5A and B).

Discussion

MicroRNAs (miRNAs) are small, non-coding RNA controlling the activity of protein-coding genes by combining 3'-UTR of target mRNA and impacting its transcription or post-transcription level (Zeng et al., 2002). MiRNAs involved in various processes from early development of life processes to cell differentiation and apoptosis. With the depth of miRNAs research, in a variety of human tumors detected changes in miRNA expression levels, and the expression of different combinations of abnormal miRNA expression profiling in different types of tumors, suggesting that tumors have a tissue-specific miRNA expression (Ferdin et al., 2010; Kahlert et al., 2013). Multiple miRNA is associated with the formation of gastric cancer, which functions as a tumor suppressor, can also play the role of oncogenes. A lot of specific miRNAs differentially expressed in gastric cancer and normal gastric mucosa, such as miR-21, miR-34b/c, miR-221/222 and miR-106a is highly expressed in gastric cancer, compared with low expression of miR-124a, miR-128b, miR-148 and miR-129 (Konishi et al., 2012; Li et al., 2012). Murakami found that miR-222, miR-106a, miR-92, miR-17-5p, miR-20 and miR-18 are related to the degree of differentiation, indicating that specific miRNAs and disease processes (Chu et al., 2008).

In this study, we found significant differences in the differentiation of gastric cancer cells, and to investigate the effects on gastric cancer cell growth, proliferation and migration and invasion. The results showed, with expression of mkiR-106b, its target genes p21 and E2F5 transcriptional and translational levels are suppressed, further study found that miR-106b can effectively promote the growth and proliferation of tumor cells, gastric cancer cells were shorten in the G0/G1 phase and facilitate their entry into the subsequent mitosis proliferation, and speed up cell cycle progression, but had no effect on gastric cancer cell migration and invasion abilities. So we're guessing that the abnormal expression of miR-106b in gastric cancer upset the balance of cell cycle network, and promote gastric cancer progress which may is possible mechanisms of miR-106b for cancers.

Gastric cancer is one of the most common malignant tumor, its occurrence and development involved in multiple genes, multi-step and multi-stage molecular patterns of events, including activation of oncogenes, inactivation of tumor suppressors, abnormalities of mismatch repair genes, and mutations of cell cycle regulatory factors. MiRNAs were considered the switch of the regulation of tumor, because it can control the activity of oncogenes and tumor suppressors in the process of neoplastic transformation network, which impact on important characteristics of the tumor cells. Currently miRNAs are considered that can be formed microvesicles, similar to the endocrine cells of the vesicles, secreted out of the cell, to transfer information between cells through the fluid circulation, regulation of gene transcription and expression of a variety of cell (Carlsbecker et al., 2010; Zhang et al., 2010). Visible, miRNAs have hormone-like role, regulation and participate in a wide range of physiological and pathological reactions, their researches will contribute to the deepening of a variety of malignancies, including gastric cancer, future clinical diagnosis, treatment and prevention.

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