MiRNA-146b-5p inhibits the malignant progression of gastric cancer by targeting TRAF6

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) are 22 nucleotides long that are extensively expressed in eukaryotes. They are vital regulators in pathological processes. This study aims to illustrate the role of miRNA-146b-5p in the development of gastric cancer (GC).

PATIENTS AND METHODS: MiRNA-146b-5p levels in 62 GC species and matched paracancerous ones were detected. Influences of miRNA-146b-5p level on clinical parameters of GC patients were assessed. Phenotype changes of AGS and SGC-7901 cells overexpressing miRNA-146b-5p were evaluated by Cell Counting Kit-8 (CCK-8) and transwell assay, respectively. Luciferase assay and rescue experiments were conducted to uncover the mechanism of miRNA-146b-5p in regulating the development of GC.

RESULTS: MiRNA-146b-5p was downregulated in GC species than paracancerous ones. Lower level of miRNA-146b-5p was observed in GC patients combined lymphatic metastasis and distant metastasis than those without metastases. In vitro overexpression of miRNA-146b-5p inhibited proliferative and migratory potentials of GC cells. TRAF6 was the target of miRNA-146b-5p, which was responsible for the development of GC regulated by miRNA-146b-5p.

CONCLUSIONS: MiRNA-146b-5p negatively correlated to lymphatic metastasis and distant metastasis rates of GC. It suppresses the malignant development of GC by targeting TRAF6.

Keywords: MiRNA-146b-5p, TRAF6, Gastric cancer (GC), Malignant development.

Introduction

Gastric cancer (GC) is a popular digestive system cancer. Its incidence has been sharply reduced in developed countries. In the United States, the incidence of GC reduced about 20% in the past two decades. However, Asia is the area with high morbidity and mortality of GC. GC remains to be the second leading fatal disease in Asian countries. It is estimated that there are 1.08 million newly onsets of GC, more than 80% of people die of this tumor each year, about 1/4 of GC patients are initially diagnosed as advanced GC. Seriously, more than 70% GC patients develop postoperative recurrence, with a median survival of shorter than 10 months. Low detective rate of early-stage GC, unsensitive chemotherapy and radiotherapy, as well as deficiency of effective biomarkers, all result in the poor prognosis of GC. It is urgent to clarify the genesis and etiology of GC, thus develop preoperative biomarkers of GC.

MicroRNAs (miRNAs) are non-coding RNAs expressed in eukaryotes. They exert post-transcriptional regulation on target gene silencing through degrading or inhibiting translation of mRNAs after recognizing their 3'UTRs. MiRNAs are featured by high conservation and tissue specificity, which are involved in cell behavior regulations. By targeting oncogenes or tumor-suppressor genes, miRNAs are able to influence tumor development. Previous studies have shown the potentials of miRNAs as diagnostic and therapeutic targets of GC.

In this paper, a total of 62 GC species and paired paracancerous ones were collected. The role of miRNA-146b-5p/TRAF6 axis in mediating the malignant progression of GC was explored.

Patients and Methods

Patients and Samples

62 paired GC species and paracancerous ones (5 cm away from tumor edge) were collected and stored at -80°C. None of enrolled subjects were preoperatively treated with anti-tumor therapy. Their clinical data were recorded. Patients and their families in this study have been fully informed. This study was approved by Ethics Committee of the Second Hospital of Shandong University.
Cell Culture
GC cell lines (AGS, BGC-823, SGC-7901, MKN28, and MKN45) and epithelial cells of gastric mucosa (GES-1) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 UI/mL penicillin, and 100 μg/mL streptomycin in a 5% CO₂ incubator at 37°C. Cell passage was conducted in 1×trypsin+EDTA (ethylenediaminetetraacetic acid) at 80-90% confluence.

Transfection
Cells were grown at 50-70% confluence in 6-well plates and transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfected cells for 48 h were harvested for functional experiments.

Cell Proliferation Assay
Cells were inoculated in a 96-well plate with 2×10³ cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the Cell Counting Kit-8 (CCK-8) kit (Dojindo Molecular Technologies, Kumamoto, Japan) for plotting the viability curves.

Transwell Assay
200 μL of suspension (1.0×10⁵/mL) was applied in the upper side of the transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate with 700 μL of medium containing 10% FBS in the bottom. After 48 h of incubation, the cells in the bottom were fixed in methanol for 15 min, dyed with crystal violet for 20 min, and counted using a microscope. Migratory cell number was counted in 5 randomly selected fields per sample (magnification 40×).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)
Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and then transcribed into complementary deoxyribonucleic acids (cDNAs) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNAs underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Japan). β-actin and U6 were used as the internal references. Each sample was performed in triplicate, and the relative level was calculated by 2-ΔΔCt. miRNA-146b-5p: forward: 5’-TGACTCCATCTGGGCCTAA-3’, reverse: 5’-GGCAGAAGCTTCG-3’; U6: forward: 5’-CGTCTGGCAGCACATATA-3’, reverse: 5’-GGTGTCCTTTG-3’. TRAF6: forward: 5’-TGCTTGAATGGCCATATCAT-3’, reverse: 5’-ATTTGAGACGCT-3’; β-actin: forward: 5’-CGCTTCGGGAGCACCCAGC-3’, reverse: 5’-TTGGTGGTCTTGGT-3’.

Western Blot
Cells were lysed for isolating total proteins and electrophoresed onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

Luciferase Assay
Cells inoculated in a 24-well plate were co-transfected with pmirGLO-TRAF6-WT/pmirGLO-TRAF6-MUT/pmirGLO and NC/miRNA-146b-5p mimics, respectively. 48 hours later, the cells were lysed for determining relative Luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis
GraphPad Prism 5 V6.01 (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation (SD). The differences between the two groups were analyzed by the t-test. Chi-square analysis was used to assess the relationship between miRNA-146b-5p level and clinical parameters of GC patients. p<0.05 was considered as statistically significant.

Results
Downregulated MiRNA-146b-5p in GC
Compared with paracancerous species, miRNA-146b-5p was downregulated in GC tissues (Figure 1A). Similarly, miRNA-146b-5p was lowly expressed in GC cell lines than that of GES-1 cell line (Figure 1B).

MiRNA-146b-5p Level was Linked to Lymphatic Metastasis and Distant Metastasis of GC
Based on median level of miRNA-146b-5p in enrolled GC patients, they were assigned into
high or low-level groups. Chi-square analysis showed that miRNA-146b-5p level was linked to rates of lymphatic metastasis and distant metastasis, while it was not correlated to age, sex, and tumor grade of GC patients (Table I). Moreover, lower level of miRNA-146b-5p was observed in GC patients combined lymphatic metastasis and distant metastasis than those without metastasis (Figure 1C, 1D). MiRNA-146b-5p may be a novel biomarker of GC.

Table I. LncRNA FAM83H-AS1 expression and clinical features of patients with ovarian cancer.

<table>
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<th>Number of cases</th>
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MiRNA-146b-5p Targeted TRAF6

Potential binding sequences in 3'UTR of miRNA-146b-5p and TRAF6 were predicted (Figure 3D). Decreased Luciferase activity in GC cells co-transfected with miRNA-146b-5p mimics and pmirGLO-TRAF6-WT further showed the binding between miRNA-146b-5p and TRAF6. Protein and mRNA levels of TRAF6 were downregulated in AGS and SGC-7901 cells overexpressing miRNA-146b-5p (Figure 3A). In addition, TRAF6 was highly expressed in GC species and cell lines (Figure 3B, 3C).

Overexpression of TRAF6 Abolished Inhibitory Effect of overexpressed MiRNA-146b-5p on Malignant Phenotypes of GC

Rescue experiments were designed for clarifying the involvement of TRAF6 in GC development regulated by miRNA-146b-5p. First of all, the over-
MiRNA-146b-5p and gastric cancer

expression of TRAF6 was able to downregulate miRNA-146b-5p in GC cells overexpressing miRNA-146b-5p (Figure 4A) and upregulate TRAF6 as well (Figure 4B). Notably, the overexpression of TRAF6 reversed the reduced migratory potential in GC cells overexpressing miRNA-146b-5p (Figure 4C). Hence, TRAF6 was responsible for malignant development of GC regulated by miRNA-146b-5p.
Figure 4. Overexpression of TRAF6 abolished inhibitory effect of overexpressed miRNA-146b-5p on malignant phenotypes of GC. AGS and SGC-7901 cells were transfected with NC mimics+NC, miRNA-146b-5p mimics+NC or miRNA-146b-5p mimics+pcDNA-TRAF6, respectively. A, The mRNA level of miRNA-146b-5p. B, Protein and mRNA levels of TRAF6. C, Migration (magnification 40×). Data were expressed as mean±SD. **p<0.01.
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Discussion

Despite great advances have been made in the treatment and improved prognosis of GC, its mortality in China remains high\(^5\). Postoperative recurrence and metastasis are the main causes of GC-induced death. Therefore, it is of great significance to elucidate the mechanism underlying metastasis and recurrence of GC\(^6,8\). The occurrence and development of GC are complicated and regulated by epigenetics and genetics, involving multiple factors and genes\(^7,9\). In the past, abnormally expressed oncogenes and tumor-suppressor genes were believed to be the key links in the pathogenesis of GC\(^9,10\).

MiRNAs are highly conserved non-coding RNAs\(^12,13\). They extensively participate in GC development\(^17\). Differences in pathological subtypes and differentiation levels of GC may be attributed to different pathways in which miRNAs are involved\(^17,18\). It is reported that miRNA-146b-5p dysregulation is closely linked to malignant development of tumor cells\(^21,22\).

The strong invasive and metastatic potentials of GC result in the poor prognosis of affected patients. In this paper, our findings uncovered that miRNA-146b-5p was downregulated in GC species than paracancerous ones. GC patients combined lymphatic metastasis or distant metastasis expressed a lower level of miRNA-146b-5p than those without metastases. In vitro overexpression of miRNA-146b-5p attenuated proliferative and migratory potentials of GC cells.

MiRNAs negatively regulate gene expression through complementary base pairing, and miRNAs specifically recognize target mRNA, which results in inhibition of translation\(^14,15\). Differentially expressed miRNAs in different types of tumors may be potential biomarkers\(^16,17\). Our findings show that TRAF6 was the direct target of miRNA-146b-5p. TRAF6 is a member of the adaptor family that couples the TNF receptor family to the signaling pathway\(^23,24\). It is reported that the activation of the downstream TNF receptor family, including NF-κB pathway, is involved in NF-κB-induced tumorigenesis\(^25\). Here, miRNA-146b-5p is responsible for the development of GC regulated by miRNA-146b-5p, which is associated with the malignant development of GC by targeting TRAF6.

Conclusions

MiRNA-146b-5p level is negatively correlated to lymphatic metastasis and distant metastasis rates of GC. It suppresses the malignant development of GC by targeting TRAF6.

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Conflict of Interests

The authors declare that they have no conflict of interests.

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