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Original Research

The expression levels of Mir-146b and Mir-221 in thyroid carcinoma tissues and their correlation with malignancy degree

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Abstract: This experiment aimed to investigate the correlation between the expressions of mir-146b and mir-221 and the proliferation, invasion, and malignancy of tumor cells. For this purpose, 135 patients with thyroid cancer treated in our hospital and 120 patients with non-malignant thyroid cancer in the outpatient clinic were selected as subjects. Fine needle biopsy tissues of the two groups were taken as samples to detect the expression levels of mir-146b and mir-221 in the tissues. The effect of the two microRNA on the proliferation and migration of cancer cells was observed by the transfection of cell lines. The contents of gal-3 and MMP9 in serum were further detected, and their relationship with the expressions of mir-146b and mir-221 was analyzed. The results showed that the expressions of mir-146b and mir-221 were significantly decreased and increased in thyroid carcinoma fine-needle aspiration tissues. Mir-146b inhibited the proliferation and migration of cancer cells, while mir-221 promoted this process. In the more cancer cells, the expression levels of these two genes changed more, and the serum levels of gal-3 and MMP9 also increased. It was concluded that the expression levels of mir-146b and mir-221 were correlated with the degree of tumor malignancy, and the low expression of mir-146b and the high expression of mir-221 were correlated with the proliferation, invasion, and malignancy of thyroid cancer tumor cells.

Key words: Mir-146b; Mir-221; Thyroid carcinoma tissues; Malignancy degree.

Introduction

Thyroid cancer (TC) is common in young and middle-aged people and is more common in women. In most cases, the cancer is completely disappeared. An important property of thyroid cancer cells, which sets them apart from other cancers, is that they strongly absorb iodine. The most obvious feature is the feeling of a bulge in the throat. Of course, thyroid enlargement can have many other causes besides cancer. The incidence rate of TC is not high in the clinic. However, when the diagnosis was found, the surrounding tissues were invaded by cancer cells. According to the literature, 50% of TC patients were found that liver and lung have been invaded by cancer cells when diagnosed, while the latter treatment only maintained 5-9 months of life (1-4). Therefore, it is of great clinical significance to explore the diagnosis scheme of TC earlier, which is helpful to alleviate the sufferings of patients and improve the survival rate. miRNAs, the gene product of non-coding small RNA, are about 20-25 nucleotides in length. They are found in animals and plants. They negatively regulate the expression of target genes at the post-transcription level by recognizing specific mRNA (4-6). It plays an important role in the early development of the embryo, tissue differentiation, virus infection, cell proliferation, cell apoptosis and other aspects (7-11). MicroRNA is closely related to tumorigenesis and may become a biological marker for diagnosis. In the process of occurrence and development of thyroid cancer, with the proliferation and invasion of thyroid cancer cells, the content of Gal-3 and MMP9 in serum will increase, which can be seen that these factors are related to the malignancy of the tumor (12-14). Therefore, to explore the relationship between microRNA and the proliferation, migration and invasion of thyroid cancer cells is helpful to analyze whether microRNA can be used as a biological marker for diagnosis; to further determine the content of tumor-related molecules such as Gal-3 and MMP9 in the serum of patients with different expression levels of microRNA can reflect the correlation between the malignant degree of the tumor with different expression levels of microRNA. Previous studies have shown that the expression of mir-146b and miR-221 in thyroid cancer patients' fine needle aspiration tissue has significant changes, but there are many studies to explore the correlation between the expression of mir-146b and miR-221 and tumor cell proliferation, invasion and tumor malignancy. This research has carried on the discussion to this question.

Materials and Methods

Experimental method

Subjects

Subjects from June 2016 to January 2018, 135 pa-

tients with thyroid cancer were treated in our hospital. The median age was 55. 120 cases in the control group were non-malignant thyroid cancer patients. Fine needle aspiration tissues of both groups were taken as samples.

Research methods

Group design

The subjects were divided into cancer groups, cancer tissue obtained by reorganizing the patients, normal tissue obtained by non-malignant group (normal control group), and classified according to different characteristics of thyroid cancer tissue. The expression levels of mir-146b and miR-221 were observed and compared.

Cell Extraction

The total RNA of cells extracted by Trizol refers to the steps of the Trizol method.

cDNA synthesis process of miRNA and Poly (A) tail

The total reaction system is 25 μ L, including total RNA 2 μ L, 10 × Poly (A) Polymerase Buffer 2.5 UL, *E. coli* Poly (A) polymer (50u/ul) 0.5 μ L, diluted ATP 1 μl. The remaining 25 ul was supplemented with RNase-free Water. The reaction liquid was gently mixed and briefly centrifuged to collect the liquid at the bottom of the tube, and then incubate at 37°C for 15 minutes.

Primer design

miRNA quantitative primers are designed according to http:/ www.sanger.ac.uk/Software/Rfam/mima. The miRNA sequence provided by the website. Because of the short sequence of miRNAs, a common primer with a neck ring structure is used to reverse transcribe microRNA into cDNA. The sequence is

GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC. The downstream primers of PCR are aimed at the loop, and their sequences can be used universally. The internal reference of miRNA expression was U6RNA.

Quantitative analysis of real-time fluorescence quantitative RT-PCR products

In the PCR reaction system, fluorescent groups were added, and the whole PCR process was monitored in real-time by the accumulation of fluorescent signals. After the reaction, the reaction quality was judged according to the amplification curve and dissolution curve. The expression of miR-125b and miR-616 was identified by the 2-CT method, and the statistical analysis was carried out compared with the normal control group. The Ct value is the number of cycles corresponding to the critical value of the amount of amplified product.

Calculation formula: $F=2^{-\Delta\Delta CT}$ ΔΔCT= (CT value of target gene in the test group - CT value of reference gene in the test group) - (CT value of target gene in the control group - CT value of reference gene in the control group).

Cell transfection

Mir-146b mimics, miRNA-146b inhibitor, Mir-221 mimics, miRNA-221 inhibitor, Mir-NC, NC-inhibitor were synthesized by Gma Shanghai (Shanghai, China). The above samples were diluted to 50nm with 250 μl OPTI-MEM in a six-hole plate, and the inoculation density was controlled at about 40%. The Lipofectamine 2000 was added and incubated at room temperature for 20 minutes.1500 μl non-resistant medium was added. After incubation for 6 hours, the mixture was sucked out and the fresh medium was replaced, then was incubated in a constant temperature incubator at 37 °C and 25% CO. The transfected cells were placed under laser confocal microscopy, and the transfection status was observed and counted.

Serum Molecular Detection Method

On the day of fine needle biopsy, 5 mL of peripheral blood was collected and centrifuged at room temperature for 10 min. The contents of galectin-3 (Gal-3) and matrix metalloproteinase 9 (MMP9) in the isolated serum were determined by ELISA kit with a microplate reader.

Statistical analysis

SPSS 16.0 statistical software was used for analysis, and measurement data were expressed as mean ± standard deviation. For data with normal distribution and same variance, one-way ANOVA was used for multi-group comparison. If the overall mean was different, a two-way comparison was made by an independent sample t-test. For data not in accordance with normal distribution or with the same variance, a non-parametric test was applied. Pearson correlation analysis was used for correlation comparison. Taking $\alpha=0.05$ as a significance test level, $P<0.05$ as difference has statistical significance.

Results

Effects of miR-146b and miR-221 on cell proliferation

We classified the fine needle aspiration tissues. As shown in Table 1, the mRNA contents of miR-146b and miR-221 in thyroid cancer tissues with different pathological types, TNM stages and tumor diameters were significantly different, while the change trends of the two microRNAs were opposite. The mRNA content of miR-146b in thyroid cancer tissues with lymph node metastasis was significantly lower than that in thyroid cancer tissues without lymph node metastasis, while miR-221 was significantly higher. There was no difference in the mRNA content of miR-146b and miR-221 in thyroid cancer tissues with different pathological types and tumor diameters, while the mRNA expression of miR-

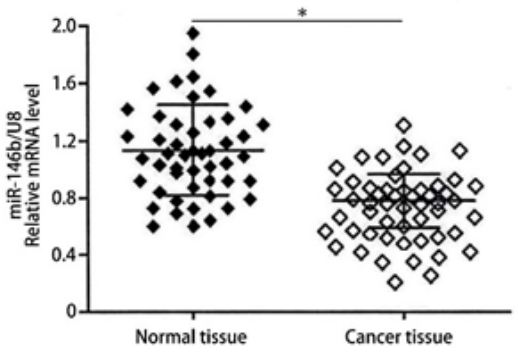

Figure 1. Changes in the expression of miR-146b in different tissues.

Table 1. Expression of miR-146 B and miR-221 in thyroid cancer tissues with different characteristics（ $\bar{x}\pm s$）

Category		miR-146b/U8	miR-221/U8
Pathological classification	Papillary carcinoma	0.53±0.04	1.67±0.08*
	Follicular carcinoma	0.47±0.06	1.62±0.09*
	Medullary carcinoma	0.48±0.07	1.55±0.08*
	Anaplastic carcinoma	1.01±0.05	1.08±0.08
TNM staging	TNMI-II stage	0.57±0.08	1.72±0.10*
TNM	TNMIII-IV stage	0.34±0.03	2.05±0.02*
Tumor diameter	<1cm	0.37±0.04	0.46±0.09
	≥1cm	0.52±0.06	0.63±0.12
Lymphatic metastasis	No	0.78±0.10	0.71±0.12
	Yes	0.24±0.04*	1.33±0.07*

146b in thyroid cancer tissues with TNM stage III-IV and lymph node metastasis was significantly lower than that in thyroid cancer tissues with stage II and no lymph node metastasis, while the expression of miR-221 was significantly higher.

We speculated that the functions of Mir-146b and Mir-221 in the proliferation of cancer cells were different, so we transfected human thyroid cancer cell lines FTC-133 with the analogues and controls of microRNA. As shown in Figure 3, after transfection with mir-146b analogues and inhibitors, the number of cells decreased and increased significantly, indicating that Mir-146b inhibited the proliferation of cancer cells. However, after transfection with mir-221 analogues and inhibitors, the number of cells increased and decreased significantly, indicating that Mir-221 promoted the proliferation of cancer cells.

Effects of Mir-146b and Mir-221 on cell migration

Mir-146b and Mir-221 may also function in cancer cell migration, so we transfected human thyroid cancer cell lines FTC-133 with microRNA analogues and controls. As shown in Figure 4, after transfection with mir-146b analogues and inhibitors, the number of cells decreased and increased significantly, indicating that Mir-146b inhibited the migration of cancer cells. However, Mir-221 had an opposite effect on the amount of cell data, suggesting that Mir-221 promoted the migration of cancer cells.

Effects of Mir-146b and Mir-221 expression on serum molecular content

The levels of Gal-3 and MMP9 in serum were correlated with the malignant degree of tumors. As shown in Table 2, the serum Gal-3 and MMP9 levels of patients with positive expression of miR-146b in thyroid cancer tissues were significantly lower than those of patients with negative expression in thyroid cancer tissues; the serum Gal-3 and MMP9 levels of patients with positive expression of miR-221 in thyroid cancer tissues were significantly higher than those of patients with negative expression in thyroid cancer tissues. It can be seen that changes in the expression of miR-146b and miR-221 are associated with the malignant degree of human tumors.

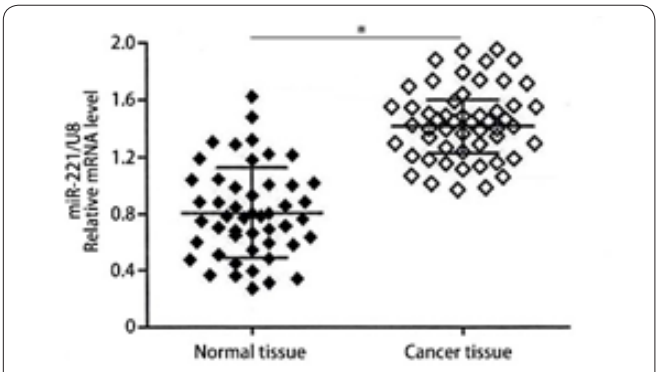

Figure 2. Changes of expression of miR-221 in different tissues.

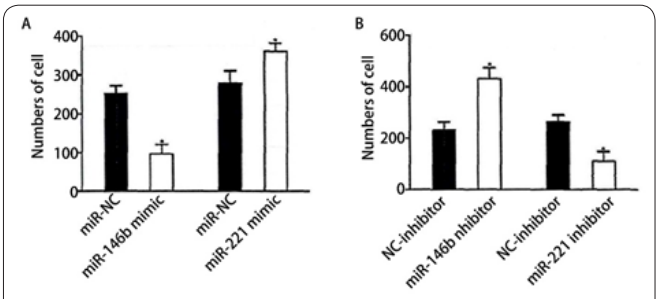

Figure 3. Effects of Mir-146b and Mir-221 on cell proliferation.

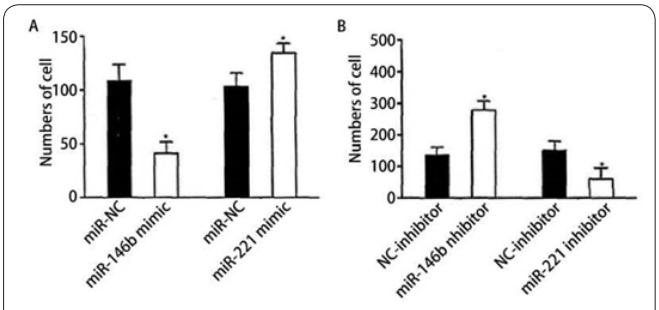

Figure 4. Effects of miR-146b and miR-221 on cell migration.

Discussion

In this study, we found that the expression of mir-146b and miR-221 decreased and increased significantly in the tissue of thyroid cancer by fine-needle aspiration. By using the analogues and inhibitors of mir-146b and miR-221 to transfect the cells, we found that they had a certain impact on the proliferation and migration of the cancer cells. It can be seen that these two microRNAs participated in the growth and development of thyroid cancer. In addition, through the classification of cancer cells, it was found that the expression of these two genes changed more in cancer cells with higher malignancy; these two genes changed more in cancer cells with higher malignancy. The higher the expression of these two genes, the higher the content of Gal-3 and MMP9 in the serum, and the content of Gal-3 and MMP9 is

Table 2. Effects of Mir-146b and Mir-221 expression on serum molecular content.

Category		Gal-3(ng/mL)	MMP9(ng/mL)
miR-146b expression	Positive	3.05±0.41*	64.98±7.93*
	Negative	6.12±0.88	147.31±15.46
miR-221 expression	Positive	5.62±0.76	163.42±27.25
	Negative	3.72±0.38*	60.21±6.81*

related to the degree of malignancy of the tumor.

It can be seen that the expression of the length of single-stranded non-coding microRNA is about 22 NT, and miRNA is involved in promoting and inhibiting apoptosis of tumor (13-15). Recently, miR-146 was found to be an immune-regulatory factor. At present, the research focuses on its role in autoimmune diseases. It has been reported that miR-146 decreased expression in pancreatic cancer cells, and regulated the proliferation of pancreatic cancer cells. In this study, the expression of mir-146b in thyroid cancer tissue by fine-needle aspiration was significantly lower than that in normal thyroid tissue, indicating that the low expression of mir-146b is expected to become a biological marker for diagnosis (16-18). In breast cancer, prostate cancer, pancreatic cancer, liver cancer, colorectal cancer, thyroid cancer and other cancer cells, the expression level of miR-221 was increased; in 30 cases of thyroid cancer, the expression level of miR-221 was significantly increased; in biopsy samples of thyroid cancer, the expression of miR-221 was increased. The results showed that the expression of miR-221 in thyroid cancer tissue was significantly higher than that in normal thyroid tissue, indicating that the high expression of miR-221 can be used as a potential characteristic molecular event in thyroid cancer tissue (19-21). The results of this study showed that mir-146b and miR-221 were significantly lower in thyroid cancer tissues with higher TNM stage (III and IV), higher than other thyroid cancer tissues with lower TNM stage, which indicated that mir-146b and miR-221 may play an important role in the development and deterioration of thyroid cancer. Further study showed that the low expression of mir-146b and the high expression of miR-221 promoted the proliferation and migration of thyroid cancer cells, and the possible mechanism was that mir-146b / miR-221 regulated the expression of the genes involved in the proliferation and migration of cancer cells. The proliferation and migration of thyroid cancer cells will increase the content of many molecules in serum. The content of these molecules can reflect the malignant degree of the tumor (22-24). Galactose lectin-3 (Gal-3) can promote cell proliferation, cell-extracellular matrix interaction and angiogenesis; matrix metalloproteinase (MMPs) - MMP9 synthesized and secreted by cancer cells can destroy the basement membrane and cause infiltrative growth of cells. MMP9 is the main member of collagen IV in the degradation of the extracellular matrix and basement membrane (25-27). It has been reported that the levels of Gal-3 and MMP9 in serum are significantly increased and correlated with the malignant degree of thyroid cancer (28-30). In order to further clarify the correlation between the expression of mir-146b and miR-221 in thyroid cancer tissue and the degree of tumor malignancy, we analyzed the levels of Gal-3 and MMP9 in the serum of thyroid cancer patients with different expression of mir-146b and miR-221. The results showed that the levels of serum Gal-3 and MMP9 in patients with negative expression of mir-146b were significantly higher than those in patients with positive expression. However, the levels of Gal-3 and MMP9 in the serum of patients with miR-221 positive expression increased significantly. This shows that the positive expression of mir-146b and the negative expression of miR-221 can inhibit the malignant of thyroid cancer. There are several ways to treat thyroid cancer, such as radiation (31-40) and regulating the expression of genes and biotechnological methods (41-44). In this regard, the importance of studying and controlling gene expression is very high and it is necessary to conduct extensive research on the expression of genes associated with different traits. (45-58). In this research, the expression of mir-146b and miR-221 have been investigated.

To sum up, the low expression of mir-146b and the high expression of miR-221 in thyroid cancer fine needle aspiration are related to the proliferation, invasion and malignant degree of tumor cells.

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