

Expression of miRNA-146b-5p in patients with thyroid cancer in combination with Hashimoto's disease and its clinical significance

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Abstract. The expression level of microRNA (miRNA)-146b in patients with thyroid carcinoma (TC) in combination with Hashimoto's thyroiditis (HT) was evaluated to analyze the clinical significance. Eighty-seven patients who underwent thyroid surgery in The Third Affiliated Hospital of Southern Medical University from March 2010 to February 2013 were enrolled. Of the patients, 37 were diagnosed with TC (group A), and 50 were diagnosed with TC in combination with HT (group B). Forty patients were diagnosed with HT (group C). The expression levels of miRNA-146b-5p in cancer tissue and paracancerous tissue of patients in the two groups were measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Correlation between the miRNA-146b-5p levels and the clinicopathological characteristics of patients with combined TC and HT were evaluated. According to the expression of miRNA-146b-5p, patients in group B were separated into the high and low expression groups to observe the five-year cancer recurrence rate. There was no significant difference in miRNA-146b-5p expression between the papillary carcinoma group, the follicular carcinoma group and the medullary carcinoma group ($P>0.05$). The miRNA-146b-5p expression in the lesion tissue of group B was significantly higher than that of groups A and C, while the miRNA-146b-5p expression in the lesion tissue of group A was significantly higher than that of group C ($P<0.05$), and the lesion tissue of the three groups was higher than that of the corresponding parastatal tissue ($P<0.05$). The miRNA-146b-5p expression level was associated with tumor size, lymph node metastasis and TNM stage ($P<0.05$), while not associated with sex, age,

lesion multiplicity, smoking history, diabetes history and pathological type in group B ($P>0.05$). The non-recurrence rate of group B high expression subgroup was lower than that of group B low expression subgroup ($P=0.045$). High expression of miRNA-146b-5p was found in the cancer tissues of patients with combined TC and HT. The expression level of miRNA-146b-5p was associated with tumor size, lymph node metastasis and TNM stage. It is a potential prognostic indicator for patients with combined TC and HT.

Introduction

Hashimoto's thyroiditis (HT), also known as chronic lymphocytic thyroiditis, is a specific autoimmune disease. Currently its pathogenesis remains unclear (1). According to literature (2), HT may be associated with genetic deletions. Congenital immunodeficiency, as a result of genetic deletions, causes immune dysfunction in patients. Thyroiditis occurs when thyroid follicular epithelium is damaged. In clinic, HT is a common form of thyroiditis. Patients with HT do not have prominent clinical manifestations at the early stage. When patients are admitted to the hospital, the disease is already serious (3). HT can be complicated by a variety of thyroid diseases, of which, thyroid carcinoma (TC) is the most serious one (4).

TC is a common cancer treated in Department of General Surgery, accounting for only 1% of all malignant tumors. However, its incidence ranks first among malignant tumors in the human endocrine system (5). Statistics show that the incidence of TC is increasing year by year, and the patients tend to be diagnosed with TC at a younger age (6). It was reported that TC patients are mostly females, and the ratio of male to female is approximately 1:2.5-3 (7). TC has a wide age range of onset, ranging from adolescents to elderly, and the average age of onset is approximately 40 years. Surgical resection is the major treatment of TC. Surgery combined with chemotherapy can significantly improve patient survival (8). Due to the absence of prominent clinical manifestations at the early stage, TC is easily neglected by patients, resulting in missing the optimal time for treatment. Misdiagnosis can also occur in clinic due to the occult nature of TC, such as slow disease progression and similarity to nodular goiter (9). Therefore, a reliable biomarker is needed in the diagnosis and treatment of combined HT and TC.

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The role of microRNAs (miRNAs) in various human diseases has become a hot research topic in recent years. miRNAs are a class of short non-coding RNA molecules containing 19-22 nucleotides. They are highly conserved molecules and are involved in the regulation of target genes by binding to 3' untranslated regions (3'-UTRs) of the target gene in a completely or incompletely complementary manner (10). According to literature, a variety of miRNAs are closely associated with cancer onset and progression, often playing the role of cancer suppressor genes or cancer promoting genes (11). It was reported that miRNA-146b-5p is associated with TC invasion and migration, but not associated with HT (12). To the best of our knowledge, association of miRNA-146b-5p with combined TC and HT has not been reported. In this study, the tissue expression levels of miRNA-146b-5p in patients with TC in combination with HT were evaluated, and its clinical significance was analyzed, in order to provide a reference for clinical practice.

Patients and methods

Eighty-seven patients who underwent thyroid surgery in The Third Affiliated Hospital of Southern Medical University (Guangzhou, China) from March 2010 to February 2013 were enrolled in this study. Fresh specimens of cancer tissues and paracancerous tissues (5 mm away from the cancer border) were collected and transferred to liquid nitrogen for storage 5-10 min after surgery. After pathological tests, 37 of 87 patients were diagnosed with TC (group A), and 50 were diagnosed with TC in combination with HT (group B). In group A, there were 10 males and 27 females, and the patients were aged 33-58 years, with an average age of 44.2 ± 9.9 years. The pathological types in group A included 30 cases of papillary carcinoma, 5 cases of follicular carcinoma, and 2 cases of medullary carcinoma. In group B, there were 15 males and 35 females, and the patients were aged 24-62 years, with an average age of 45.8 ± 8.9 years. The pathological types in group B included 40 cases of papillary carcinoma, 7 cases of follicular carcinoma, and 3 cases of medullary carcinoma. During the same time period, further 40 patients with HT only were collected (group C). In group C, there were 15 males and 25 females, and the patients were aged 30-55 years, with an average age of 44.8 ± 9.1 years. Variables such as sex and age were comparable in the three groups, and the differences were not statistically significant. The study was approved by the Medical Ethics Committee of The Third Affiliated Hospital of Southern Medical University. The patients and their families were informed and signed an informed consent.

Inclusion and exclusion criteria. Patients who met the following criteria were eligible for the study: i) Patients older than 18 years; ii) patients without preoperative treatment of radiotherapy and chemotherapy; iii) patients with all limbs and normal cognitive function; iv) patients with complete medical records; and v) patients and their families who were cooperative in treatment. Patients who met the following criteria were excluded from the study: i) Patients with immunodeficiency; ii) patients with congenital heart disease; iii) patients with other malignant tumors; iv) patients with serious dysfunction of key organs; and v) patients not willing to be followed up.

Reagents and instruments. The following reagents and instruments were purchased: i) TRIzol RNA extraction reagent from Thermo Fisher Scientific, Inc. (Shanghai, China, A33251); ii) RT-PCR kit for PrimeScript™ and PrimeScript™ II 1st Strand cDNA Synthesis kit from Takara Biomedical Technology (Beijing) Co., Ltd. [Beijing, China (6210A, RR055B)]; and iii) PCR Amplifier ABI7900 from Applied Biosystems; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The miRNA-146b-5p primers were designed and synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). The primer sequences are shown in Table I.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA reverse transcription was performed in strict accordance with the PrimeScript™ II 1st Strand cDNA Synthesis kit instructions. The system was: 2 μ l 5X buffer, 0.5 μ l RT Enzyme Mix, 0.5 μ l Oligo (dT) primer, 2 μ l Random 6 mers, 1 μ g total RNA, and RNase free H₂O to 10 μ l. The reaction conditions were: 37°C for 15 min and then 85°C for 5 min. After the reverse transcription, cDNA was collected. The SYBR Green PCR reaction system was: (Thermo Fisher Scientific, Inc.): PrimeScript 1 Step Enzyme Mix 1 μ l, upstream and downstream primers 0.5 μ l each, 2X 1 step buffer 12.5 μ l, and cDNA 1 μ l. Finally, cDNA RNase Free dH₂O was used to add up to 25 μ l. The PCR reaction conditions were: 50°C for 30 min, 94°C degeneration for 2 min, 98°C degeneration for 10 sec, and 68°C for 1 min. Thirty circles were performed, and U6 was used as the internal reference. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method (13). The experiment was carried out three times. The median relative expression of miRNA-146b-5p in patient tissues was selected and divided into the high expression and low expression groups.

Follow-up. The patients were followed up for 60 months after discharge. The recurrence of cancer was tracked by follow-up phone calls at 1, 3, 6, 12, 24, 36, 48 and 60 months.

Statistical analysis. The SPSS 20.0 statistics software package (IBM Corp., Armonk, NY, USA) was used for statistical analysis on the collected data. The GraphPad Prism 7 software was used for figure drawing. Enumeration data were expressed in rate (%) and compared using the Chi-square test. Measurement data were expressed in a mean \pm standard deviation (mean \pm SD). Independent t-test was used for inter-group comparison, represented by t. Single-factor ANOVA was used for multi-group comparison, and LSD t-test was used for pairwise comparison. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Relative expression levels of miRNA-146b-5p in patient cancer tissues. After the detection of miRNA-146b-5p, no significant difference was found in the expression of miRNA-146b-5p in patient cancer tissues among the three groups ($P < 0.05$). The miRNA-146b-5p expression in patient cancer tissues in group B was significantly higher than those in groups A and C. The miRNA-146b-5p expression in patient cancer tissues in group A was significantly higher than that in group C ($P < 0.05$). The miRNA-146b-5p expression levels in patient cancer tissues

Table I. Primer sequences.

Genes	Upstream primers	Downstream primers
miRNA-146b-5p	5'-GCGTGAGAACTGAATTCCA-3'	5'-GTGCAGGGTCCGAGGTAT-3'
U6	5'-GCGCGTCGTGAAGCGTTC-3'	5'-GTGCAGGGTCCGAGGT-3'

Table II. Relative expression levels of miRNA-146b-5p in patient cancer tissues.

Groups	Pattern of organization		t value	P-value
	Lesion tissue	Perifocal tissue		
Group A (n=37)	1.864±0.235	1.005±0.022	22.138	<0.001
Group B (n=50)	2.532±0.422 ^a	1.009±0.038	25.417	<0.001
Group C (n=40)	1.384±0.102 ^{a,b}	1.002±0.020	23.243	<0.001
F value	166.929	0.663		
P-value	<0.001	0.517		

^aP<0.05, differences compared with group A, and ^bP<0.05, differences compared with group B.

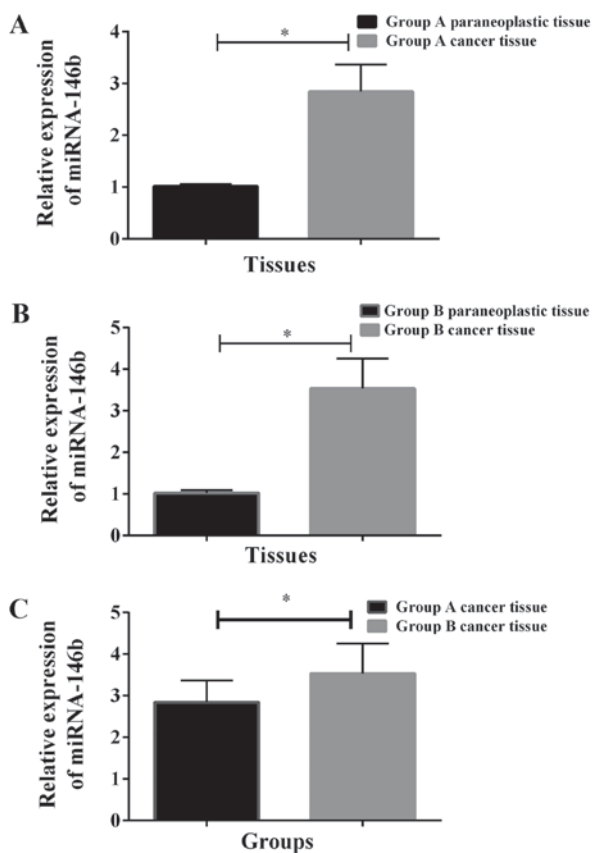


Figure 1. Tissue expression of miRNA-146b-5p in patients of the two groups. (A) Measurement of miRNA-146b-5p expression in group A patients by RT-qPCR indicated that the expression level in cancer tissue was higher than that in paracancer tissue (P<0.05). (B) Measurement of miRNA-146b-5p expression in group B patients by RT-qPCR indicated that the expression level in cancer tissue was higher than that in paracancer tissue (P<0.05). (C) Measurement of tissue miRNA-146b-5p expression in both groups A and B patients by RT-qPCR indicated that the expression level in cancer tissue was higher in group B than that in group A (P<0.05). The difference between the two groups was statistically significant (*P<0.05). RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

in the three groups were higher than those in para-carcinoma tissues (P<0.05) (Table II and Fig. 1).

Expression of miRNA-146b-5p in patients with different pathological tissue types. According to the pathological tissue types, 87 patients were divided into the papillary carcinoma group, the follicular carcinoma group and the medullary carcinoma group. We found that there was no significant difference in the miRNA-146b-5p expression in all three groups (P>0.05). (Table III).

Association of relative expression levels of miRNA-146b-5p with clinical records in group B. The patients in group B were further separated into two subgroups based on the median miRNA-146b-5p relative expression level: group B high expression subgroup and group B low expression subgroup. There were 22 patients in group B high expression subgroup, and 28 patients in group B low expression subgroup. The miRNA-146b-5p expression level was found to be associated with tumor size, lymph node metastasis and TNM stage (P<0.05), while not associated with sex, age, lesion multiplicity, smoking history, diabetes history and pathological type in group B (P>0.05) (Table IV).

Recurrence rate of group B. Patients in group B were followed up for 5 years. The 5-year non-recurrence rate of group B high expression subgroup was lower than that of group B low expression subgroup (P<0.05). The results are shown in (Table V and Fig. 2).

Discussion

HT, also known as chronic lymphocytic thyroiditis, is a human autoimmune disease. Under the microscope, diffuse lymphocytic infiltration and fibrosis, as well as lymphoid follicles, are identifiable (14). In clinic, symmetrical and diffuse

Table III. Relative expression of miRNA-146b-5p in patients with different histological types.

Group	Papillary carcinoma group (n=70)	Follicular carcinoma group (n=12)	Medullary carcinoma group (n=5)	F value	P-value
Relative expression of miRNA-146b-5p	2.254±0.434	2.362±0.634	2.358±0.367	0.365	0.695

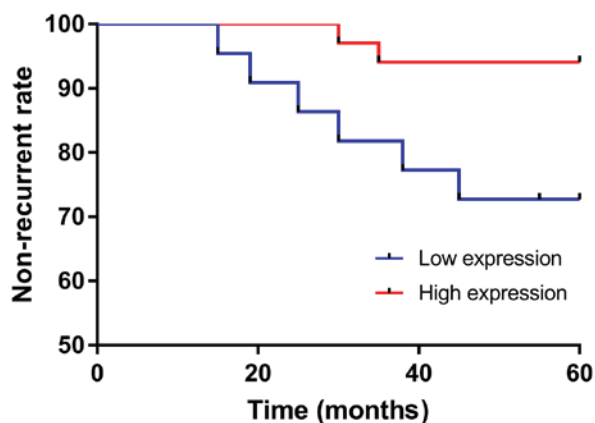


Figure 2. Non-recurrence rates of group B high expression subgroup and group B low expression subgroup.

enlargement of the thyroid gland at both sides is typical of HT. Most patients slowly develop hypothyroidism as the patients' disease course progresses. When this destruction goes on long enough, permanent hypothyroidism can be caused, posing a serious threat to patients' health and quality of life (1). In addition, a variety of other conditions may develop on the basis of primary HT, of which TC is the most serious one. Among the pathological types of combined TC, thyroid papillary carcinoma is the most common (15).

In clinic, TC is the most common malignancy of the endocrine system, impacting individuals' life, though its incidence is lower than many other tumors (16). According to the pathological classification, TC can be divided into four types: papillary carcinoma, follicular carcinoma, undifferentiated carcinoma and medullary carcinoma. Among them, papillary carcinoma accounts for 80-85% of the total incidence of TC (17). The distribution of pathological types in current study is basically similar to this general distribution. Although the 35- or 40-year survival rate of TC can reach 80% or even higher with timely treatment (18), there are currently no effective means of preventing TC.

Recent technological advances in molecular biology have allowed a deeper understanding of the mechanisms underlying TC onset and progression. miRNAs as a new type of molecules are gradually gaining widespread interest. miRNAs are a type of endogenous short non-coding single-stranded RNA molecules, which are highly chronological, conserved and specific (19). It was reported that miRNAs are involved in disease onset and progression by specific binding to the target gene of interest, for example, miRNAs play an important role in cell proliferation, apoptosis and growth in tumor onset and progression processes (20). miRNA-146b-5p belongs to the miRNA-146 family and is a newly discovered

immune regulatory factor. It is associated with the onset and progression of autoimmune diseases. For example, miRNA-146 may play a role in persistent inflammation in rheumatoid arthritis through a T cell network (21). According to literature, miRNA-146b-5p can suppress TC cell invasion by regulating its target gene IRAK1 (22). However, it is unclear if miRNA-146b-5p is associated with TC complicated by HT. In this study, the tissue expression levels of miRNA-146b-5p in patients with TC in combination with HT were evaluated, and possible association between miRNA-146b-5p and the disease was analyzed, in order to provide a reference for clinical practice.

In this study, the expression levels of miRNA-146b-5p in cancer tissues, as well as paracancerous tissues, of patients with TC (group A), patients with combined TC and HT (group B) and patients with HT (group C) were evaluated. It was found that in the three groups, the expression levels of miRNA-146b-5p in cancer tissues were higher than those in corresponding paracancerous tissues. The expression level of miRNA-146b-5p in cancer tissues was higher in group B than those in groups A and C, and the differences were statistically significant. The expression level of miRNA-146b-5p in cancer tissues was higher in group A than that in group C, and the differences were statistically significant. The above findings were consistent with previous studies. For example, the tissue expression of miRNA-146b-5p in patients with papillary thyroid carcinoma was higher than that in healthy subjects (23), and the miRNA-146b-5p expression in cancer tissues of patients with papillary thyroid carcinoma was also higher than that in corresponding paracancerous tissues (24). Moreover, higher tissue expression of miRNA-146b-5p in combined TC and HT than in TC only suggested that miRNA-146b-5p expression was associated with HT. In the study of Taganov *et al* (25), miRNA-146b-5p is a natural immune protein inhibitor, while TC, an autoimmune disease, is significantly higher than that in the lesion tissue of HT patients, suggesting that the expression of miRNA-146b-5p is associated with the occurrence and development of HT. The possible reason of higher expression of miRNA-146b-5p in patients with combined TC and HT was that the miRNA-146b-5p expression was increased in TC with HT patients under the influence of the dual factors of cancer lesions and immune inflammatory response. We also analyzed the expression of miRNA-146b-5p in tissues of patients with different pathological types and found no significant difference in miRNA-146b-5p expression in tissues of patients with papillary carcinoma group, follicular carcinoma group and medullary carcinoma group, but the small number of sample size may affect the results. The statistical analysis of the clinical records of patients in group B high expression subgroup and group B low expression subgroup found that miRNA-146b-5p

Table IV. Association of relative expression levels of miRNA-146b-5p with clinical records in group B (n=50) [n (%)].

Variables	Case (%)	Group B subgroup		χ^2 value	P-value
		Low expression (n=28)	High expression (n=22)		
Sex				0.990	0.319
Male	15 (30.00)	10 (35.71)	5 (22.73)		
Female	35 (70.00)	18 (64.29)	17 (77.27)		
Age, years				0.325	0.569
>45	25 (50.00)	15 (53.57)	10 (45.45)		
≤45	25 (50.00)	13 (46.43)	12 (54.55)		
Tumor size				5.864	0.015
≥1 cm	40 (80.00)	19 (67.86)	21 (95.45)		
<1 cm	10 (20.00)	9 (32.14)	1 (4.55)		
Lesion multiplicity				0.487	0.485
Single	20 (40.00)	10 (35.71)	10 (45.45)		
Multiple	30 (60.00)	18 (64.29)	12 (54.55)		
Lymph node metastasis				4.818	0.028
Yes	41 (82.00)	20 (71.43)	21 (95.45)		
No	9 (18.00)	8 (28.57)	1 (4.55)		
TNM stage				5.009	0.025
Stage I-II	35 (70.00)	16 (57.14)	19 (86.36)		
Stage III-VI	15 (30.00)	12 (42.86)	3 (13.64)		
Smoking history				0.139	0.709
Yes	15 (30.00)	9 (32.14)	6 (27.27)		
No	35 (70.00)	19 (67.86)	16 (72.73)		
Diabetes history				0.033	0.856
Yes	13 (26.00)	7 (25.00)	6 (27.27)		
No	37 (74.00)	21 (75.00)	16 (72.73)		
Pathological type				3.449	0.178
Papillary carcinoma	40 (80.00)	25 (89.29)	15 (68.18)		
Follicular carcinoma	7 (14.00)	2 (7.14)	5 (22.73)		
Medullary carcinoma	3 (6.00)	1 (3.57)	2 (9.09)		

Table V. Group B patients' 1-, 3- and 5-year non-recurrence rates (%).

Group B subgroup	1-year	3-year	5-year
High expression (n=22)	100.00	81.81	68.18
Low expression (n=28)	100.00	92.86	92.86
χ^2 value	0	1.422	5.082
P-value	>0.999	0.233	0.024

expression was associated with tumor size, lymph node metastasis and TNM stage. At the end of this study, we conducted a 5-year statistical analysis on the recurrence of patients with high and low expression in group B and found that the recurrence rate of patients with high expression was higher than that of patients with low expression. In a recent study, the miRNA-146b-5p expression was found to be associated with TC tumor size and lymph node metastasis, suggesting

that miRNA-146b-5p could be used as a potential prognostic indicator for combined TC and HT (26). However, in the study of Lee *et al* (26), compared to PTC patients without HT, patients with HT had favorable clinicopathologic features by a meta-analysis, which is inconsistent with our study. It may be due to the small sample size in this study. Besides, only 2,471 PTC patients of the 10,648 cases were combined with HT, accounting for 23.2%, while in this study, 57.47% PTC patients had HT. The significant difference between the two studies may be caused by the exclusion criteria in this study.

It is undeniable that there are certain limitations in this study. Both the sample size and the group numbers were small. In addition, the expression level of miRNA-146b-5p in patients with HT only was unclear, and it was unclear as to which target gene played a role in combined TC and HT.

Collectively, miRNA-146b-5p was overexpressed in tissues of patients with combined TC and HT. It was associated with tumor size, lymph node metastasis, and TNM staging. miRNA-146b shows potential as a prognostic indicator for combined TC and HT.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

NL and XL conceived the study and drafted the manuscript. LH, RZ and JY acquired the data. GZ and LL analyzed the data and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of The Third Affiliated Hospital of Southern Medical University, (Guangzhou, China). The patients and their families were informed and signed an informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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