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Research Article

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Investigating the expression levels of miR-146b as a tumor marker for early diagnosis of thyroid cancer

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ABSTRACT

Introduction: *Micro-RNAs are a new class of regulatory RNAs with length of 18-22 nucleotides involved in the pathogenesis of many human cancers including papillary thyroid cancer (PTC). In general, it acts as an oncogenes or tumor suppressor. This study examined the expression levels of miR-146b in patient and healthy people plasma to find out that if it can be used as an early diagnostic indicator in papillary thyroid cancer.* ***Materials and Methods:*** *in this study, we examined the expression level of miR-146b using q rt-PCR method in plasma of 40 patients, divided into four groups (10 PTC patients on the day before the operation, 10 patients with benign nodules and 10 patients after the Thyroidectomy and 10 healthy subjects as control). All data were normalized with expression level of GAPDH, as an internal control.* ***Findings:*** *expression level of miR-146b in plasma samples of patients was about 5.5 times greater than that in control patients (P <0.05), and generally statistical difference was found in plasma levels for the expression of miR-146b between controls and patients with PTC. However, significant difference was not found in the expression level of miR-146b between control subjects, thyroidectomy and benign patients.* ***Conclusion:*** *This study showed that increased expression of miR-146b and its presence in blood circulation are directly correlated with PTC. The results also indicated the capacity of this technique in distinguishing benign, thyroidectomy, PTC, and normal groups. Therefore, this method can be introduced as a non-invasive method, versus previous methods like FNA, aid in diagnosis to pathologist.*

Keywords: *papillary thyroid carcinoma (PTC), miR-146b, biomarker, q RT-PCR*

INTRODUCTION

Cancer is the result of interruption in the correct regulation, proliferation, and differentiation paths. Lack of sensitivity to the growth inhibitory signals, escaping from programmed cell death, unlimited proliferation potential, maintaining angiogenesis,and tissue invasion and metastasis lead to malignant cancer. Thyroid cancer is one of the most common endocrine malignancies that its prevalence has increased in recent decades in the world (2). Thus, developing methods that can detect disease at its early stage is important. MiRNAs are small(approximately 18-22 nucleotides), single-stranded, non-coding RNAs regulating the expression of many genes at the post-transcriptional level, and they are highly protected (3). According to recent studies, miRNAs can be transferred into blood circulation via exosomes or extracellular vesicles and they are exchanged among cells through specific RNA binding proteins and by direct binding of cells to each other (4 and 5). MiRNA is bound to untranslated region (3'-UTR) of mRNAs and disrupts their translation and stability. It also prevents protein or target product expression (6). Depending on the type of mRNA that miRNAs inhibit, they can be oncogene or tumor suppressor and thereby they can alter the expression of target genes related to cancer (7, 8). MiR-146b causes cell stimulation, proliferation, and invasion, and it inhibits apoptosis (9). MiRNAs interaction with target genes specifies their role in cell cycle and confirms direct influence of micro-RNAs in cancer. MiRNAs structure and their performance suggest that miRNAs are expressed in various cancers abnormally (10). Investigation of miRNAs as diagnostic markers is feasible by studying serum or plasma of humans. Therefore, miRNAs related to cancer cells present in serum or plasma can be

diagnosed without any invasive procedure, with the exception of leukemia in which malignant cells are readily available (11). The use of micro RNAs that are closely related to the malignant phenotype can be very helpful as a tumor marker for diagnosis of disease in early stages (12). Considering the fact that most of methods have been used so far to screen the cancer are not able to diagnose and prevent the disease at early stages. Identification of tumor microRNAs released during the gradual progress of cancer in blood circulation can be noteworthy in timely diagnosis of cancer (12, 13). Therefore, the aim of this study was to examine the expression levels of miR-146b in the peripheral blood of patients either with PTC or have nodular goiter as well as patients who have been undergoing thyroidectomy surgery and its comparison with the control group to determine if it can be used as markers.

Materials and methods

The population of this study included patients referred to Boali Laboratory in Zanjan, who have been introduced to center by an endocrinologist based on papillary cancer diagnosis. Patient people were classified into three groups, each containing ten patients with this sub-category; Ten patients with PTC that their plasma samples were prepared in one day, ten patients with nodular goiter, and ten patients who previously underwent thyroidectomy surgery, and finally ten healthy individuals as control group were collected. The criteria to select the samples was in a way that sample was obtained in the case of patients affected with papillary cancer after studying the pathology answer and one day before surgery. In the case of thyroidectomy patients, sampling was performed regardless of surgery time in past and regardless of gender. In the case of benign nodules, after FNA and after determining that sample response was benign, blood sample was collected from the patient. Normal individuals, who had no abnormality in thyroid gland, used no drug in this regard. All people were participated in this study with their written consent and this study imposed no treatment limitation or physical damage for people studied in this research. The Ethics Committee of Medical Sciences of Zanjan University approved this study. Preparation of plasma from patients: 3 ml of blood were collected from studies people, while they were fasting, in tubes containing EDTA. Then, it was centrifuged at room temperature at 3000 rpm for 5 minutes, and then plasma samples were collected in RNAase Free micro-tubes. Total RNA of plasma was extracted using GeneMATRIX Universal RNA / miRNA EURX kit. In order to reduce miRNA destruction, cDNA production stages are performed, immediately. Then, it was kept at -20 c˚.

Construction of cDNA: to build complementary strand of cDNA from RNA extracted in the previous step, Cinnagen Company kit was used (CinnaGen First Strand cDNA synthesis Kit). First, nearly 200 ng of the RNA with 1 mM of specialized primer of Stem-Loop was treated for 5 min at 65 ° C. The obtained mixture was placed on ice for 2 minutes. Then, Special 1X buffer solution, 200U of reverse transcriptase enzyme, 10 mM of dNTPs and 0.2 mM of GAPDH reverse primer were added . Finally, by adding RNase free water, the total volume became 20 µl. The RT reaction was carried out at 42 ° C for 60 minutes in a thermoblock.

Real-Time PCR reaction: Real-Time PCR was performed using specific primers (Table 1) and SG qPCR Master Mix (2x) EURx / E0401-01 / 100reactions kit.

Table 1: The sequence of primers used in q RT-PCR

Name	3'sequence 5'
RT primers STmiR-146b	**GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGA TACGACCCAGAAC**
Forward miR-146b	**5'-TGCCCTGTGGACTCAGT-3'**
Reverse miR-146b	**5'- GTGCAGGGTCCGAGGT-3'**
F- GAPDH	**5'- GGTCATCATCTCTGCCCCCT-3'**
R- GAPDH	**5'- ATCATCTCTGCCCCCT-3'**

Before Real-time PCR reaction, to control the product and accuracy of designed primers performance, a gradient PCR followed by agarose gel electrophoresis was accomplished. Then, for each of Real-Time PCR reaction, 1X SG

qPCR Master Mix, 0.2 mM of reverse primer, and 0.2 of forward primer, and 30ng of synthesized CDNA related to each sample were added. The control sample was composed of 0.2 mM of GAPDH forward primer instead of specific primers. The RT-PCR program was as follows: 95 ° C for 10 min (1 cycle, initial denaturation), 40 cycles consisted of three stages with 94 ° C for 15 s, 50 ° C for 30 s, 72 ° C for 30 seconds respectively and five minutes for 72 ° C as the final extension and eventually 90-50 ° C were set for melting curve analysis and investigating specific proliferation of considered product. All samples as well as control sample were measured in pairs. GAPDH gene as an internal control was measured for normalizing the measurement of target miRNA in all stages simultaneously (14 ,15). Also, ten healthy samples were mixed and one pooled sample was prepared after vortex. The recent pooled sample was used as a calibrator.

Calculation of miRNA-146b gene expression level of studied samples:

Data were analyzed by several statistical methods. The first method was comparative method of ΔΔ Ct. Firstly, Ct of each sample was determined. ΔCt value is calculated according to the following formula: [ΔCt= Ct (each sample) - Ct (GAPDH)]. In the next step, ΔΔCt value and normalized Ct value were calculated for each sample: ΔΔCt= ΔCt (plasma sample of cancer patients or control) ΔCt - (Plasma sample of calibrator). The normalized miRNA value of each sample will be equal to the 2-ΔΔct. The Data of study were analyzed statistically by using SPSS VOL.16 software. Statistical difference of miR-146b values between patients and control people was evaluated in terms of test of equality of means of four groups using One-way ANOVA method with value of $p<0.05$. Then, the complementary test of Bonferroni test was used for the final analysis. Finally, numbers obtained by efficiency of all conducted cycles were placed in Rest 2009 software. Then, calculations related to investigation of equality of groups were conducted. Finally, the difference of miR-146b was evaluated among various groups in the form of a graph.

Findings

Specific amplification of miRNA-146b:

The specific amplification of miRNA-146b and melting curve analysis was indicated in figure1. The single peak was observed for miRNA-146b amplification and was ensured the specificity of Real-time PCR reaction (Figure1).

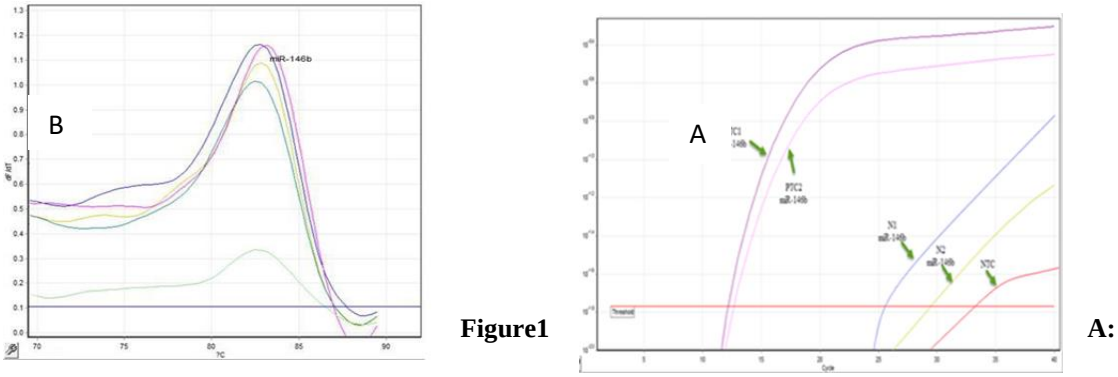

Figure1 A: Amplification plot; the red horizontal line indicates the manual quantification cycle threshold set at 0.01. The red and purple lines related to PTC sample and the blue and yellow lines related to normal samples. B: specific and single peak of miR-146b was ensured of specialty of Real-Time PCR amplification.

Analysis of miRNA-146b expression level among different groups:

As indicated in figure2, a significant difference in mean Ct values was observed among four groups (One-way ANOVA, $P<0.05$).

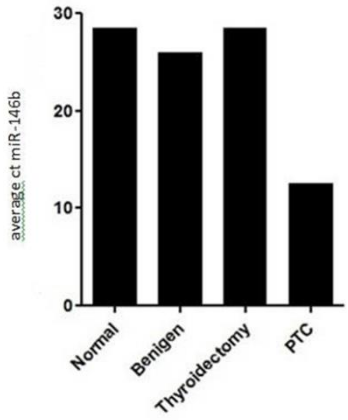

Figure2. The graph illustrated a mean difference of Cts obtained for four groups using one-way ANOVA method with value of P<0.05.

Also, supplementary Bonferroni test was performed and the following results were obtained. Since, a significant difference among patients with papillary cancer group from other three groups was observed by complementary Bonferroni test (P <0.05). Therefore, it suggests that investigation of this miRNA can distinguish this group from other groups. No significant difference was observed among normal group with benign and thyroidectomy groups (P = 0.056 and P = 0.770). In comparing normal group with benign group (P = 0.056) as comparing normal group with benign group, null hypothesis was rejected. However, the observed difference was very lower than PTC group. This very low difference might be used in making distinction between these two groups. Comparing thyroidectomy group with normal group also showed no significant difference (P = 0.770), and null hypothesis was not rejected, but the difference was very small.

Then, the expression level of miR-146b in plasma was examined among normal, PTC, benign and thyroidectomy groups using Rest 2009 software. A significant difference was obtained in index of miR- 146b expression level in people with papillary thyroid cancer and other three groups. The miRNA expression level in patients with benign nodules had slight difference with normal and thyroidectomy groups, but this difference was not as much as that in papillary samples. The result of Rest software calculations showed that the presence of miR-146b in blood samples of patients with papillary thyroid cancer is significantly higher in comparison to that in normal, benign, thyroidectomy groups. The diagram depicted by

software showed the maximum expression level of approximately 5.5 times increasing in patients compared to normal individuals (Figure 3).

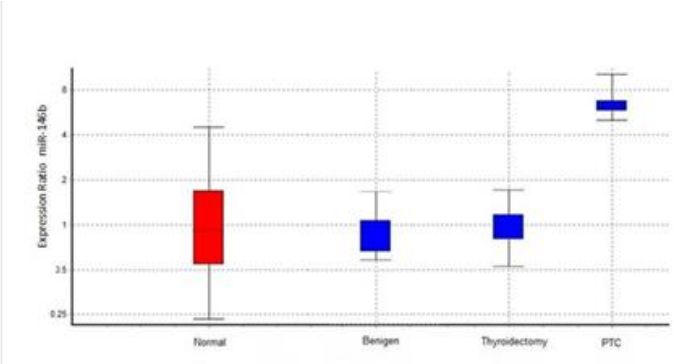

Figure3. The comparing of miR-146b expression level in plasma of four groups, The miR- 146b expression level in plasma of patients was 5.5 times higher than that in three other groups.

Discussion and conclusion

Thyroid cancers are the most common endocrine system cancers. Among them, the most common type is papillary thyroid cancer including 70 to 80% of thyroid cancers. This cancer can occur at any age (16,17). The mean incidence of this cancer in Iran suggests that it is increasing since 2002 to 2010 (18,19). According to case mortality rate of this cancer worldwide, and its rising incidence every day, considering early detection of this cancer and disease control programs can help reduce mortality from cancer. Therefore, tumor markers can be an appropriate alternative for tissue sampling by a needle using like FNA procedure (22). Unlike molecular markers, such as BRAF, P_{27}, P_{21}, CEACAM-1 (Casrcinoembryonic antigen-related cell adhesion molecule 1), osteopontin and E-cadherin, that are lacking predict the risk of PTC recurrence (20), miRNA are considered by researchers for diagnosis and prediction of PTC (10,16).

Due to over expression of miR-146b in cancerous tissues, it can be used for timely diagnosis and prevention of this cancer and as this miRNA affects tumor cells and it has oncogene and anti-apoptosis property, it causes disruption in apoptotic path that is beneficial to the survival of cancer cells (21). Through to investigations, it was revealed that miRNAs found in the exosomes are associated with a variety of specific diseases such as cancer, for example, research has shown that exosomes in blood circulation are similar to exosomes in the origin of their cancer cells. This suggests that miRNAs in exosomes have the potential to diagnose cancer. In patients with papillary thyroid cancer, over expression of miR-146b in the blood might act as a diagnostic indicator (21). Additionally, a large number of studies have reported advantage of using miRNAs isolated from exosomes as non-invasive and easy access to them from body fluids such as saliva and the plasma and serum (23).

In this study, by using q RT-PCR, we confirmed that miR-146b levels was significantly over expressed in PTC groups compared to benign, thyroidectomy and normal groups.

In 2005, use of miRNAs as tumor markers was reported firstly by Chen YT et al. They used a number of tumor tissues embedded in paraffin and some samples collected by FNA. They examined the presence of miRNAs including miR- 146b, miR- 221, and miR-222 to compare expression between malignant papillary and benign follicular tissues. They concluded that the amount of the miRNAs is several times more than the normal tissues and they have a potential for tumor diagnosis in the early stages and they can be an alternative to invasive sampling method. According to our results and the study was performed by Chen Y-T et al, it was revealed that expression level of miR-146b in papillary cancer people is greater than that in normal people (24). Similarly, in studies conducted by Chruścik A et al in 2015 on miRNAs of 221 and 222 and 146b levels in various stages of papillary thyroid cancer, it was also revealed that these miRNAs have been changed. They also emphasized that there is a significant relationship between expression level and presence of these micro RNAs in the lymph nodes and metastases in patients with papillary thyroid cancer. In 2015, Lee YS et al conducted studies on miRNAs of 146b and 155 extracted from serum samples of patients with papillary cancer and they performed test measurement on the level of miRNAs compared to normal individuals. They concurred to effectiveness of these non-invasive biomarkers in early diagnosis of this cancer. In this study, we also examined the expression levels of miR-146b and its difference in four benign, thyroidectomy, normal and papillary groups, and obtained results were in accordance with results by Yoon Se Lee et al, (25). In general, all studies that have been conducted so far assent on feasibility of using miRNAs as diagnostic markers. MiR-146b expression level in serum samples of patients with benign nodules had no significant difference compared to that in normal people. However, it may be suggested that by using it we can find out that if tumor is benign or malignant. The expression level of miR-146b in serum sample of thyroidectomy patients showed no significant difference with normal people samples and this issue might be used in individuals who are concerned about recurrence of the disease. Finally, measuring the expression level of miR-146b can be used as a tumor marker for diagnosis of papillary thyroid cancer in the early stages.

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