RESEARCH

Association of serum miR-375, miR-155 and miR-146b levels with distinguish of papillary thyroid cancer from benign thyroid masses among Iranian patients

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Abstract

Background and aim Certain serum levels of microRNAs (miRNAs) throughout the body can be helpful for cancer diagnosis and prognosis. The miRNAs can be secreted from the papillary thyroid cancer (PTC) into the circulatory system. Accordingly, this study aimed to measure the serum levels of miR-146b, miR-155 and miR-375 to evaluate their diagnostic potentials in distinguish of benign from malignant lesions.

Materials and methods The serum levels of miRNAs were measured by real-time quantitative RT-PCR among100 patients with benign thyroid nodules and 30 patients with PTC.

Results The mean miR-375 and miR-155 expression levels in the PTC group were greater when compared with the benign group. The area under the ROC curve (AUC) was estimated at 0.81 for the miR-375 with 0.76% sensitivity and 0.80% specificity to distinguish between benign and PTC lesions. The AUC was calculated to be 0.75 for the miR-155 with 0.69% sensitivity and 0.90% specificity.

Conclusion According to the results of this study, the serum levels of miR-155 and miR-375 were increased in the patients with PTC, which may be useful as alternative seromarkers for the PTC.

Keywords Circulating miRNAs, Papillary thyroid cancer, Seromarker

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Introduction

Papillary thyroid cancer (PTC) is usually treated with thyroidectomy, but a subset of patients who are not timely diagnosed have an invasive disease that is characterized by recurrence or distant metastasis. Therefore, the diagnosis of patients with invasive phenotypes is important to determine the need for adjuvant therapy and prolonged follow-up. One of the promising solutions is to measure the level of miRNAs from the tumor released in the bloodstream. The specific expression patterns of these RNAs in tissue and their high durability in biological fluids are well documented [1]. Thyroid carcinoma as the most prevalent malignancy in the endocrine system has an incidence of 2% [2, 3]. The PTC affects

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approximately 90% of such cases, which still is rising [3, 4]. Despite recent advances in medical technology, it is difficult to detect the PTC in the early stages due to its asymptomatic nature, and thus, it has a poor prognosis when diagnosing. As a result, recent researches focused on novel prognostic parameters to detect the PTC, and the feasibility of using miRNA in this area is an attractive topic today [5–11]. One of the small non-coding RNAs is miRNAs responsible for numerous cellular pathways such as metastasis and cancer. According to documents, the miRNAs are highly associated with the cancer onset and progression [12]. Ultrasound-guided fine needle aspiration (FNA) is a commonly employed technique for the diagnosis of thyroid nodules, but the resulting histological findings are often ambiguous and different such as atypia of undetermined significance and suspicious malignancy. The majority of PTCs have unique genetic variations capable of triggering different pathways such as PAX8/PPAR, RET/PTC and BRAF, RAS signaling pathways [13]. Thus, special FNA-derived markers may be useful for physicians to distinguish malignant and benign lesions [13]. The circulating miRNAs are non-invasive biomarkers. They are easily obtained without severe damage to the patient. Observations have shown that it is possible to use the circulating miRNAs to evaluate the stage and progression of tumors. The expression pattern of total active miRNAs can show the prospects for cancer development during progression. The circulating miRNAs help monitor tumor metastasis. The circulating miRNAs can predict tumor sensitivity to clinical treatment [14]. In studies of the animal model and the cell line, the increase in miRNA 375 reduced the migration and invasion rates of the cancer cells. Increasing the expression of the ERBB2 molecule, whose activity is associated with the activation of PI3K-AKT and Ras-MAPK signaling pathways, increases cell proliferation and reduces apoptosis. The ERBB2 as the target of miRNA-375, after breakdown by this miRNA, inhibits the cell proliferation. Therefore, this miRNA is likely to play the role of a tumor suppressor in the thyroid cancer and indicates a reduction in expression in this cancer Table 1 [15, 16]. Increasing the expression of mRNA-155 in the cell line of thyroid cancer and laboratory animal models increases the growth of the tumor. According

to the findings, this miRNA has the oncogenic activity. The APC is a target for this miRNA and reduced APC enhances the cell growth. The Wnt/β-catenin signaling pathway is activated by this miRNA, which itself causes the activation of downstream pathways like TCF and C-MYC. This miRNA appears to have a central role in the diagnosis of thyroid cancer, which needs further investigation [17]. Investigating the miRNA-146b activity and function in the BCPAP and TPC1 cell lines, which are relevant to the PTC, suggest that inhibiting this miRNA significantly increases migration and invasion in cell lines and increasing this miRNA expression increases migration and invasion [18]. This miRNA targets and decomposes SMAD4 as a key protein in the TGF-β signaling pathway, cell migration inhibitor [19]. The cell cycle-regulatory protein of P27^{kip1}, through interaction with cyclins, inhibits the progression of the cell cycle at the G1 stage. A down-regulatory activity exists between this protein and the miRNA-146-b expression level [20]. Increasing the expression of this miRNA in the tumors following the BRAFT1799A mutation causes the invasion of surrounding tissues in thyroid cancer to occur more often. A positive relationship can be found between invasion in thyroid cancer and increasing the expression of this miRNA [21, 22]. The most prevalent prognostic markers detected for invasive tumors are lymph node metastasis, tumor size, extra-thyroidal invasion and BRAFT1799A mutation. The measurement of the miRNA-146-b expression is a suitable substitute for these invasive measures in the thyroid cancer. We hypothesized that the expression of miRNAs in the PTC is probably released into the circulatory system. Accordingly, the current study aimed to measure the serum miR-375, miR-155 and miR-146b levels to evaluate their diagnostic value to distinguish malignant from benign lesions in the PTC.

Materials and methods

The sampling process, the demographic profile of research units (Table 2) and histopathological findings were performed at Ayatollah Kashani Hospital of Shahrekord in Chaharmahal and Bakhtiari Province, Iran, under an institutional review board-approved protocol after obtaining written informed consent. The centrifugation of collected blood sample was performed at 4 °C for

Table 1 Candidate miRNAs for investigation in the serum of PTC patients

miRNAs of interest	Target genes functional activity, previous association with PTC	References
miR-375	ERBB2, overexpression of this miRNA increases cell proliferation and reduces apoptosis	[15, 16]
miR-155	APC, it activates the β-catenin pathway, which in turn leads to activation of downstream pathways such as C-MYC and TCF	[17]
miR-146b	SMAD4, P27, when this miRNA is inhibited, migration and invasion are significantly reduced in cell lines, and migration and invasion are increased by increasing its expression	[18–21]

Table 2 Characteristics of PTC patients and benign

Variables	Groups	
	PTC	Control group
Age in years at diagnosis (median, range)	45 (± 18.4)	52 (± 18.6)
Female	26	85
Male	4	15
Tumor size in millimeters (median, range)	19.9 (15–22) mm	–

Table 3 Sequences of primers used for measuring miRNA expression by qRT-PCR

miRNA name	miRNA Sequence (5′ → 3′)
Universal reverse primer	5′-GACGAGGACTCGAGCTCAAGCT-3′
miR-155	5′-TTAATGCTAATCGTGATAGGGGTA-3′
miR-146b	5′-ACACTCCAGCTGGGTGAGAACTGAATTCCAT-3′
miR-375	5′-GTCGAGGTCACCACTGGATT-3′

10 min with 3000 g, and then, the supernatant (or serum) was gathered and stored at −80 °C until testing. Overally, the inclusion criteria reported and included nodule diameter and histopathological confirmation of tumor type. The most common exclusion criteria are lymph node metastasis and extra-thyroidal extension. Because of the heterogeneous nature of tumoral and non-tumoral thyroid nodules as well as the lack of suitable clinico-pathological parameters, diagnostic criteria for thyroid cancer are highly variable. It is worth noting that this research was approved by the Ethics Committee of Shahrekord University of Medical Sciences with the Ethics Code: IR.SKUMS.REC.1396.1.

RNA extraction and cDNA synthesis
The total RNA of serum (200 µl) was extracted by miRNeasy extraction kit (Exiqon, Denmark). The purity and quantity of extracted RNA were, respectively, determined using the RNA concentration and NanoDrop Spectrophotometer (NanoDrop®, Thermo Fisher Scientific) and then, was analyzed by the agarose gel electrophoresis. The cDNA was constructed by reverse transcription using miRNeasy serum Reverse Transcription Kit (Exiqon, Denmark) in accordance with the manufacturer's protocol. As shown in Table 3, the design of primers specific for miRNA was on the basis of the miRNA sequences on database of miRBase (http://microrna.sanger.ac.uk/). The expected PCR product specificity was confirmed by melting curve analysis at the end of PCR cycles. The run was in duplicate for each sample, with cDNA-free blank controls. The required number of cycles for fluorescent

signal to cross the threshold in qPCR was considered as the cycle of threshold (Ct). The serum level of miRNAs was computed by $2^{-\Delta\Delta Ct}$ equation.

Amplification and quantification
The qRT-PCR analysis was performed to measure the expression levels of miR-146b, miR-155 and miR-375 based on the manufacturer's instruction. The endogenous control was considered to be Spike-in miRNA. The cDNA template in the real-time PCR was combined by SYBR Green (SG) Master Mix (Exiqon, Denmark). Forward and Reverse primers specific for miRNA were used to enrich the PCR array plate. A Real-Time PCR System (Qiagen, USA) was applied to carry out the real-time PCR, with the following thermal cycling profile: 95 °C for 2 min and then, 40 amplification cycles at 95 °C for 5 s and at 60 °C for 30 s. The required number of cycles for fluorescent signal to cross the threshold in real-time PCR was considered as the cycle of threshold (Ct).

Statistical analysis
The difference in serum miRNA levels between the subjects with PTC and benign thyroid nodules was compared by Mann–Whitney U test. Each test was two sided test at the significance level of $P < 0.05$. Receiver-operator characteristic (ROC) curves were drawn for the measurement of serum levels of miRNA to distinguish between the patients with PTC and those with benign thyroid nodules. Statistical analyses were performed by graphpad prism 5.

Results
Demographic properties
In the present study, the number of patients with PTC was 30 and 100 people were considered as healthy control. In this study, the number of women with PTC was 6 times greater than that of men. Also, the age range of the people was between 42 and 52 years.

Diagnostic performance of miR-375, miR-155 and miRNA-146b for PTC
Supporting Table 1, diagnostic performance of AUC and cut-off value, sensitivity, specificity of miR-375, miR-155, miR-146b for discriminating patients (PTC) from Control group. Fold differences (log2-DDCt) were carried out in the expression of serum miR-146b, miR-155b and miR-375 between PTC and benign group (Mann–Whitney U test). In the first step, the mean miR-375, miR-155 and miR-146b expression levels were measured in each group. The mean fold changes in miR-375 (−10.26 ± 0.040) and miR-155 (−6.08 ± 0.061) were higher in the PTC group compared to the benign group. The mean fold changes in miR-146b (−6.04 ± 0.052) were higher in the PTC group

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compared to the benign group, but not significant (supporting Table 1).

miRNA	Cut-off	Specificity	Sensitivity	AUC	Fold change	P value
miR-375	1.52	0.8	0.76	0.81	−10.26	0.007
miR-155	2.01	0.9	0.69	0.75	−6.08	0.04
miR-146b	2.9	1	0.61	0.72	−6.04	0.07

Diagnostic value of miR-375, miR-155 and miRNA-146b for PTC detection

The ROC curve analysis was used to know whether serum miR-375, miR-155, and miRNA-146b levels can have diagnostic value for PTC detection. It was documented that the serum levels of miR-375 and miR-155 were strong parameters to distinguish between PTC patients and the benign group. The serum miR-375 AUC was 0.81 with sensitivity and specificity of 0.76% and 0.80% at the cut-off point of 1.52, respectively (Fig. 1). The AUC of miR-155 was 0.75 with a sensitivity of 0.69% and a specificity of 0.90% at the cut-off point of 2.01 (Fig. 2). The miR-146b AUC was 0.72 with a sensitivity of 0.61% and a specificity of 100% at the cut-off point of 2.9 (Fig. 3).

Discussion

Few studies has been done to evaluate the circulating miRNAs of the patients with PTC, but none of these studies have emphasized the use serum miRNA375, 155, 146b to distinguish benign from malignant nodules.
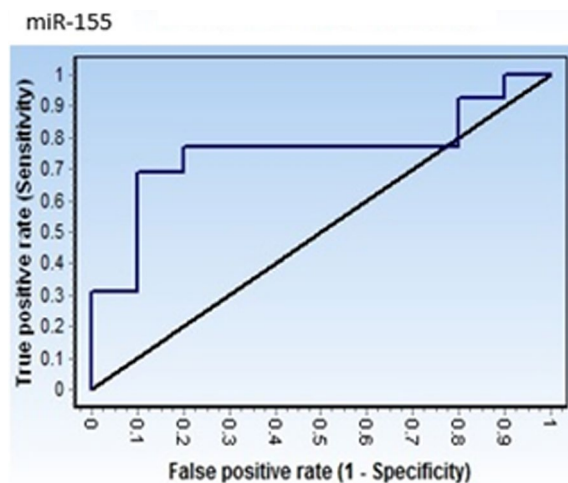

Fig. 2 Analysis of receiver operating characteristics (ROC) curve by serum miR-155 to distinguish benign tumors from papillary thyroid cancer

Our study showed that the serum miR-375 AUC was 0.81 with the sensitivity of 0.76% and the specificity of 0.80% at the cut-off point of 1.52. The AUC of miR-155 was 0.75 with the sensitivity of 0.69% and the specificity of 0.90% at the cut-off point of 2.01. The miR-146b AUC was 0.72 with the sensitivity of 0.61% and the specificity of 1 at the cut-off point of 2.9.

Excellent treatment outcomes have been reported for PTC, but a significant number of such patients experience recurrent disease or distant metastasis, possibly due to prognosis. The ultrasound-guided fine needle aspiration (US-FNA) is one of the widely used
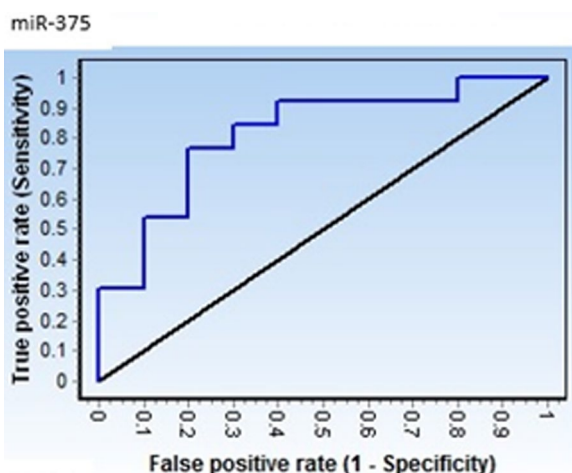

Fig. 1 Analysis of receiver operating characteristics (ROC) curve by serum miR-375 to distinguish benign tumors from papillary thyroid cancer
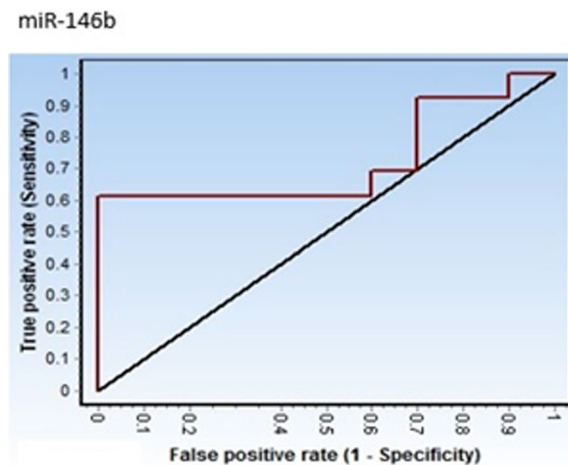

Fig. 3 Analysis of receiver operating characteristics (ROC) curve by serum miR-146b to distinguish benign tumors from papillary thyroid cancer

techniques for the detection of primary, metastatic and recurrent lesions [23, 24].

Nevertheless, the resulting vague findings of this approach can delay the proper therapeutic measures and increase the patient's concern. To solve these problems, various molecular tests have been proposed for the detection of somatic mutations in the primary lesion and the measurement of lymph node thyroglobulin content [13].

Early diagnosis of disease recurrence is the basis of PTC control after surgery. The fluorodeoxyglucose (FDG)-positron emission tomography (PET) / CT, CT and US-guided FNA are the current methods for the detection of metastatic and recurrent lesions, but with high cost and time. As with certain markers in a variety of tumors, including carcinoembryonic antigen (CEA) in colon cancer and alpha-fetoprotein (AFP) in hepatoma, identifying a seromarker for the PTC can help resolve this issue potentially. The serum and plasma miRNA can be used for the detection of cancer. The patterns of miRNA expression in blood samples have been clarified previously, and the resulting miRNAs are successful seromarkers for many cancers [25].

Current methods for the differential detection of benign malignant nodules, including pathologic tests, FNA, thyroid scan, ultrasound, physical examination and history taking, are based on surgical specimens. Gold standard is biopsy, and the FNA as the most valuable approach is used preoperatively to detect the thyroid nodules. There is limitation for the use of FNA prognostic potential among the patients with cytological profiles of suspected malignancy or follicular neoplasm and an atypia of undetermined significance, resulting in unwanted thyroidectomy or thyroid lobectomy [26].

According to findings, there are some circulating miRNAs with prognostic or diagnostic potentials for multiple cancers [27]. With regard to their proliferation and durability in the serum, the miRNA features are applied for the categorization of various types of human cancers [28, 29].

The majority of researches focused on the miRNA expression in tumor cells and tissues of the patients with PTC. As stated earlier, little research has been done to evaluate the circulating miRNAs of the patients with PTC, none of which tried to use serum miRNA375, 155 or 146b to distinguish benign from malignant nodules.

The current study decided to use qRT-PCR for the measurement of the serum miRNAs expression level in the patients with PTC and patients with benign nodules. Our findings revealed the upregulation of serum miRNAs (miR375 and miR-155) in the patients with PTC when comparing with those with benign nodules.

The two miRNAs resulted in the miR-375 AUC of 0.81 with the sensitivity of 0.76% and the specificity of 0.80%. The miR-155 AUC was 0.75 with the sensitivity of 0.69% and the specificity of 0.90%.

In distinguishing the PTC group from the benign nodule group, our results indicated that the combined use of miR375 and miR-155 can be considered a biomarker with the lowest invasive nature for preoperatively detection of PTC having a relatively high specificity and sensitivity.

In a study by Panebianco et al., the FNA samples were examined to detect the BRAF V600E mutation, followed by evaluating the expression of mRNA and miRNAs (KIT, TC1, miR-222, miR-146b) using qPCR for distinguishing the benign nodules and malignant nodules. The maximum diagnostic accuracy of the ROC curve was related to KIT and miR-146b. The miR-146b showed the AUC, sensitivity and specificity of 0.93, 87.8 and 100.0, respectively [30].

The miR-30d, miR-146b, miR-187 and miR-221 can reportedly distinguish the malignant lesions from benign ones. The highest differential expression between malignant and benign lesions was significantly related to miR-146b and miR-30d with sensitivity of 88.9% and specificity of 78.3% [31].

Mazeh et al. evaluated the FNA-extracted miR-21, miR-31, miR-146b, miR-187, miR-221, and miR-222 levels in distinguishing the thyroid benign from the malignant nodules, and their results showed the diagnostic accuracy of 90%, the sensitivity of 88% and the specificity of 100% [32]. Lee et al. measured the serum miR-146b, miR-221, miR-222, and miR-155 levels in the patients with TC and benign lesions. According to the results, the benign and malignant tumors were distinguished by the miR-146b with the sensitivity of 61.4% and the specificity of 74.3% as well as the miR-155 with the sensitivity of 57.9% and the specificity of 63.2% [33].

Yu et al. conducted a cohort study aiming to measure the serum miRNA levels in patients suffering from thyroid malignant or benign nodules, compared to healthy controls. They utilized Genome-wide serum miRNA expression profiles determined using Solexa sequencing and subsequently employed the RT-PCR technique.

A significant overexpression was observed for the serum levels of let-7e, miR151 and miR-222 in the thyroid cancer patients [34]. There is an in vitro report on the thyroid cancer, which determined the relative prevalence of miRNA-222 and miRNA-146b in exosomes from TPC1 cells [35]. Mazeh et al. applied the NGS method to detect 19 miRNAs in distinguishing the thyroid benign from malignant nodules using indeterminate cytology and reported the sensitivity of 91%, the specificity of 100%, negative predictive value of 87% and positive predictive value of 100% and overall diagnostic accuracy of 94%. The maximum

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diagnostic accuracy was related to the miR-146b with the AUC of 0.898 and more ability of discrimination [36].

Conclusion

The PTC is a heterogeneous condition in which diverse miRNAs act as agents for progression and invasion of the tumor. According to the findings from the current, the measurement of serum levels of miRNAs may be useful to distinguish between malignant tumors and benign lesions in the PTC. The measured expression levels of miR-146b and miR-155 were valuable for the differentiation of benign from malignant lesions. It is suggested that there is a need for further studies to scrutinize the importance of the serum levels of miRNAs as diagnostic tool. This study aimed to analyze the roles of some miRNAs possibly associated with the PTC. It should be noted that the sample size was not enough to draw a definite conclusion on the validity of serum levels of miRNAs.

Abbreviations
ROC   Receiver-operator characteristic
PTC   Papillary thyroid cancer
AUC   Area under the ROC curve

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This research was approved by the Ethics Committee of Shahrekord University of Medical Sciences with the Ethics Code: IR.SKUMS.REC.1396.1.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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