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Serum MicroRNA-146b Expression for Malignancy Prediction in Euthyroid Patients with Indeterminate Thyroid Nodules

Amal O. Abdelfadeil[1], Mohamed R. Halawa[1], Iman Zaky[1], Merhan Samy[1], Lamyaa Salem[2] and Ahmed M. Bahaaeldin[1]

[1]Department of Internal Medicine & Endocrinology, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

[2]Department of Clinical Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

Corresponding author: Amal O. Abdelfadeil, Department of Internal Medicine & Endocrinology, Faculty of Medicine, Ain Shams University, Cairo, Egypt.
Email: am_om@ymail,com.

Abstract

Thyroid nodules are frequently found, but the vast majority of them are benign. The difficulty in managing thyroid nodules is correctly diagnosing the minority of those who have malignancy. Thyroid fine-needle aspiration cytology (FNAC) with indeterminate cytology continues to raise doubts about the presence of thyroid cancer, leading to an unnecessary thyroidectomy. Circulating miRNAs may be useful as diagnostic and prognostic markers for a variety of cancers, including thyroid cancer. The goal of the present study was to determine the predictive value of serum miRNA-146b expression level for thyroid cancer by estimating its level in a group of euthyroid patients with thyroid nodules with indeterminate FNAC results. This cross-sectional study included 45 euthyroid patients with indeterminate thyroid nodules who visited the Endocrine Outpatient Clinic and Endocrine Surgical Ward at Ain Shams University Hospitals. For all patient thyroid profiles, ultrasound of the thyroid gland and FNAC of the thyroid nodule were performed. In addition, preoperative assessment of serum microRNA-146b expression by real-time PCR was achieved and the results correlated with post-operative thyroid histopathology. There was no difference in serum miRNA-146b expression between patients with benign thyroid nodules versus patients with malignant nodules (p= 0.789). The risk of malignancy increased with the increase in size of the dominant thyroid nodules, as larger nodules had a higher risk of malignancy (p= 0.027). In conclusion, in euthyroid patients with indeterminate thyroid nodules, serum miRNA-146b is a poor predictor of thyroid malignancy, however, the larger the nodule size, the higher the risk of cancer.

Keywords: Thyroid nodules, microRNA-146b, Thyroid fine-needle aspiration cytology (FNAC), thyroid profile.

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Introduction

Thyroid nodules are becoming more common, and it is critical to differentiate between benign and malignant thyroid nodules to avoid unnecessary thyroidectomy and surgical complications.[1] The clinical management of patients with indeterminate thyroid nodules is a

challenging issue. Ultrasound (US) examination is an essential tool for the initial assessment of thyroid nodules as well as patient follow-up. Several US-based systems of stratification have been used to better characterize thyroid nodules, but their overall efficacy in the context of indeterminate cytology is poor.[2]

Indeterminate thyroid nodules, including classes III and IV according to the Bethesda system, have a high rate of expressing a benign lesion in postoperative histopathology, and most patients are prone to unneeded thyroidectomy3. Therefore, indeterminate thyroid nodules should be characterized preoperatively in order to select the best possible management and avoid unnecessary thyroidectomy.[3]

Thyroid cancer is frequently missed by patients, because of the lack of obvious clinical findings at an early stage, causing delayed management. Misdiagnosis in clinics is also possible due to the occult features of thyroid cancer, like the slow growth of the disease and its resemblance to a nodular goiter. As a result, a valid biomarker is required for better thyroid cancer diagnosis.[4]

The involvement of microRNAs (miRNAs) in various human diseases has recently become a topic of interest. MiRNAs are a group of short non-coding molecules of RNA consisting of 19–22 nucleotides. They are extremely conserved molecules that regulate specific genes by binding to 3' untranslated regions (3'-UTRs) of the selected gene in a totally or partially complementary manner.[5] According to the literature, a number of miRNAs are linked to malignancy onset and progression through upregulation of some miRNAs, resulting in the silencing of tumor suppressor genes. In addition, some miRNAs may result in increased oncogene expression.[6]

Our goal was to determine the predictive value of serum miRNA-146b for malignant thyroid nodules in euthyroid patients with thyroid nodules with indeterminate thyroid fine-needle aspiration cytology (FNAC) results.

Subjects and Methods

Study population

This cross-sectional study included 45 euthyroid subjects aged ≥ 18 years during the period from December 2021 to December 2022. Cases were enrolled at Ain Shams University Hospitals (Endocrine Outpatient Clinic as well as the Endocrine Surgery Ward). They included patients with suspicion of thyroid nodules [Thyroid Imaging Reporting and Data System, TI-RADS score ≥ 3 by ultrasound (US)] and Bethesda classifications III and IV by FNAC (indeterminate FNAC). Patients underwent total thyroidectomy and were divided based on the postoperative histopathology outcome into two groups. Group (I): cases with confirmed benign thyroid nodules, and Group (II): cases with confirmed malignant thyroid nodules.

In addition, 25 apparently healthy individuals matched in age and sex were included as controls in the miRNA-146b expression assay. Patients with diseases affecting the level of serum miRNA-146b, such as chronic kidney disease and tumors of the liver, lung, breast, and prostate, were excluded from the study.

Clinical and Biochemical analyses

All cases underwent detailed history-taking, including the onset of thyroid nodules, duration of illness, toxic symptoms, and presence of thyroid complications. A thorough clinical examination included vital data and a neck examination. Peripheral venous blood samples (5 ml) were withdrawn under aseptic conditions from each subject into 2 sterile tubes (2.5 ml in each tube). The serum was separated after centrifugation at 2000xg for 10 minutes at room temperature. One tube was used for measurement of serum thyroid-stimulating hormone (TSH) (cat.No.11731459122), free thyroxine (FT4) (cat.No.06437281190), and free triiodothyronine (FT3) (cat.No.06437206190) by chemiluminescent microparticle immunoassay kits (Cobas® kits) on an immunoassay analyzer (Cobas e411 autoanalyzer, Roche Diagnostics, GmbH, Mannheim, Germany), according to the manufacturer's instructions.

The serum in the second tube was used for assessment of serum miRNA-146b expression by real-time polymerase chain reaction (PCR) assay. This involved multiple steps. Briefly, extraction of miRNA was performed by commercial kits (cat. No. 217184) miRNeasy Mini Kit provided by Qiagen®, QIAGEN Strasse 1 40724 Hilden, Germany), according to the manufacturer's instructions. Then the extracted RNA was used in reverse transcription to prepare complementary DNA (cDNA) using specific miRNA primers (TaqMan® MicroRNA Assays, ThermoFisher Scientific, Germany) using Commercial kits (cat. No. 4366596, TaqMan® MicroRNA Reverse Transcription Kit, ThermoFisher Scientific, Frankfurter Strasse 129 b, 64293 Darmstadt, Germany), according to the manufacturer's instructions.

Candidates of miRNAs, namely miRNA-146b (hsa-miR-146b, cat. No. 4427975, ID: 001097) and miRNA-16, which was used as an endogenous reference gene (hsa-miR-16 cat. No. 4427975, ID: 000391), were reversibly transcribed. The PCR reaction mixture (20 µl) was prepared according to the manufacturer's instructions, containing 1.33 µl cDNA from the reverse transcription reaction as template, 10 µl of TaqMan Universal PCR Master Mix, 1 µl of TaqMan microRNA assay (20×), and 7.67µl nuclease-free water. The thermal cycling profile included holding at 95 °C for 10 minutes, and then, 40 cycles each of 15 seconds at 95 °C and 60 seconds at 60 °C. The reaction was performed using an automated RT-PCR detection system (5 Plex Rotor Gene Real-Time PCR Analyzer, Qiagen, Germany). Sera from the 25 controls were used in the assay of serum miRNA-146b and miRNA-16. Finally, the relative expression (fold change) of miR-146b and miR-16 in each sample was calculated using the delta-delta Ct (2-ΔΔCT) algorithm[7].

Ultrasonography (US) of the thyroid gland was performed and evaluated by a radiology specialist for all patients. All data were analyzed and interpreted using the **American College of Radiology** (ACR)-TI-RADS scoring system for malignant risk [8, 9]. Patients with suspicious lesions with TI-RADS scores of III, IV, and V were selected in this study and referred for FNAC.

FNAC was performed by an interventional radiologist for all patients in accordance with the principles described by Werga et al., 2000.[10] A qualified pathologist interpreted all slides, and findings were reported and calculated in accordance with the Bethesda system classification.[2] Only patients with indeterminate lesions of Bethesda III and IV were selected and referred for preoperative measurement of miRNA-146b and surgery.

According to the case, a qualified surgeon performed a total thyroidectomy, and the specimens were sent for histopathology examination. The results were correlated to the preoperative expression level of serum miRNA-146b, US criteria, and FNAC results.

Statistical Analysis

The data were processed using the Statistical Package for Social Sciences (SPSS, IBM) version 23. Quantitative data were presented as mean, standard deviations and ranges when found to be parametric and median and inter-quartile range (IQR) when found to have a non-parametric distribution. Also, qualitative variables were presented numerically and as percentages. The comparison between groups for qualitative data was done by using the Chi-square test. The comparison between two groups with quantitative parametric data were analyzed by using the independent t-test test while comparison between two groups for quantitative non-parametric data were done by using the Mann-Whitney test. p-value of < 0.05 was considered significant.

Results

In the present study, 44/45 (97.8%) of patients were females, and 1/45 (2.2%) male patients. According to the postoperative histopathology, 37/45 patients (82%) had benign thyroid nodules, representing group I, while 8/45 patients (18%) had malignant thyroid nodules, representing group II.

Among the eight patients with malignant thyroid nodules, four (50%) had classic papillary thyroid carcinoma (PTC), two (25%) had invasive follicular variant of papillary carcinoma, one (12.5%) had medullary thyroid carcinoma, and

one (12.5%) had hürthle cell neoplasm with encapsulated medullary micro-carcinoma.

There was no difference in the mean age of patients with benign thyroid nodules 41.405±11.687 years, and the mean age of patients with malignant thyroid nodules 40.375±12.397 years (p = 0.824) (Table 1).

On comparing ultrasound results, the mean size of dominant thyroid nodules in patients with benign thyroid nodules was 3.180±1.431 cm, significantly lower than the mean size in patients with malignant thyroid nodules 4.6±2.258 cm as larger nodules size was associated with higher risk of malignancy (*p*=0.027) (Table 1).

The median miRNA-146b relative expression level in patients with benign thyroid nodules was 4.89 (1.31-7.94) fold change, not different than in patients with malignant thyroid nodules 3.53 (1.38-7.99) fold change, (*p*=0.789) (Table 1) (Figure 1).

The prevalence of different TI-RADS scores and Bethesda categories showed no difference between patients with benign thyroid nodules (group I) and patients with malignant thyroid nodules (group II) (*p* = 0.727 and *p* = 0.059, respectively). (Table 2)

Table 1. Comparison of age, size of dominant nodule, and miRNA-146b expression level between patients with benign thyroid nodules (group I) and patients with malignant thyroid nodules (group II).

Studied parameter		Group I n=37	Group II n=8	*p* value
Age (years)	Range	19 – 70	25 – 59	NS[t]
	Mean ±SD	41.405±11.687	40.375±12.397	
Size of the dominant Nodule (cm)	Range	1.1 - 8.6	1.5 – 9	0.027[t]
	Mean ±SD	3.180±1.431	4.600±2.258	
miRNA-146b expression level (Fold change)	Range	0.16 – 74.54	0.97 – 9.58	NS[z]
	Median (IQR)	4.89 (1.31-7.94)	3.53 (1.38-7.99)	

P > 0.05 is not significant (NS). t: Independent t-test, z: Mann-Whitney test

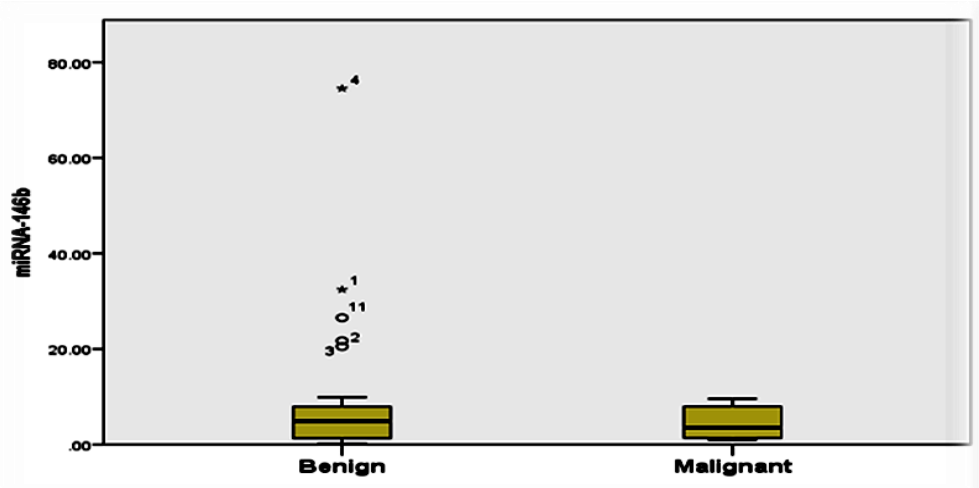

Figure 1. Comparison of the expression of serum miRNA-146b between patients with benign thyroid nodules and patients with malignant thyroid nodules.

Table 2. Comparison of the prevalence of different TIRADS scores and Bethesda scores between patients with benign thyroid nodules (group I) and patients with malignant thyroid nodules (group II).

	Group I		Group II		Total		Chi-square
	N	%	N	%	N	%	*p*-value
TI-RADS 3	21	56.76	4	50.00	25	55.56	NS
TI-RADS 4	13	35.14	3	37.50	16	35.56	
TI-RADS 5	3	8.10	1	12.5	4	8.88	
Total	37	100.00	8	100.00	45	100.00	
BETHSEDA III	33	89.19	5	62.50	38	84.44	NS
BETHSEDA IV	4	10.81	3	37.50	7	15.56	
Total	37	100.00	8	100.00	45	100.00	

P > 0.05 is not significant (NS). TI-RADS: thyroid Imaging Reporting and Data System.

Discussion

The goal of the present study was to determine the predictive value of serum miRNA-146b for malignant thyroid nodules by assessment of serum miRNA-146b expression level in euthyroid patients with thyroid nodules with indeterminate FNAC results. Our findings revealed no difference between the two malignant and benign thyroid nodule groups, indicating that serum miRNA-146b could not distinguish between malignant and benign thyroid nodules ($p = 0.789$). However, Soudeh et al., 2020,[11] found that miRNA-146b expression levels were higher in patients with malignant thyroid nodules. Rosingolo and his colleagues (2017) studied 11 patients with PTC and found that serum miRNA 146b was higher in patients compared to healthy control subjects. In addition, they found that pre-thyroidectomy serum miRNA-146b levels significantly exceeded the measurement after thyroidectomy, denoting potential for its use as a biomarker of PTC recurrence.[12]

In addition, a study by Lee and his colleagues (2013) found that circulating miRNA-146b was elevated in PTC and had a diagnostic value, suggesting that it may be used as a new noninvasive biomarker for the preoperative diagnosis of malignant nodules of the thyroid.[13] In our study, only 4 out of the 8 patients who were confirmed to be malignant had PTC, and the other 4 had different types of thyroid cancer, namely anaplastic, hürthle cell neoplasm, and follicular variants of PTC.

Our findings were consistent with Yoruker et al., 2016, who found no significant difference in serum miRNA-146b expression levels between patients with papillary thyroid cancer and multinodular goiter ($p = 0.3$).[14] Furthermore, a study by Kondrotienė et al., 2020, assessed five miRNAs (miR-146b, miR-221, miR-222, miR-21, and miR-181b) as non-invasive biomarkers for PTC and compared their expression profiles in serum samples of PTC, nodular goiter, and healthy control groups and analyzed their relation to the clinicopathologic characteristics of PTC. They found a significant increase in expression of miR-146b, miR-221, miR-222, miR-21, and miR-181b in serum samples of patients with PTC in comparison to healthy controls. In addition, the expression of the studied miRNAs (miR-221, miR-21, miR-146b, and miR-181b) did not differ between patients with PTC and nodular goiter groups.[15] The variability of results reported by the different studies could be attributed to differences in study populations. To the best of our knowledge, we are the first in the Middle East to study the predictive value of serum miRNA-146b for thyroid cancer in a sample of euthyroid patients with indeterminate cytology.

We also found no difference in age between patients with benign thyroid nodules (group I) and patients with malignant thyroid nodules

(group II). This was aligned with the findings of a study by Witczak et al., 2016.[16] However, Boonrod et al., 2021, stated that the age of patients was a predictor of thyroid malignancy; however, their study focused on follicular and hürthle cell neoplasms, and they suggested a cutoff of age for the prediction of malignancy as > 55 years.[17] Another study by Hughes et al., 2011, found that the prevalence of thyroid cancer was considerably higher in patients over the age of 45 than in those under the age of 45 [18]. Similarly, according to Reynolds et al., 2005, thyroid cancer was more prevalent in older age groups than in younger age groups.[19]

In the present study, we found that the prevalence of malignancy was 16% of nodules with TI-RADS 3, 18.75% of nodules with TI-RADS 4, and 25% of nodules with TI-RADS 5. While a study by Middleton et al., 2017, found that the prevalence of malignancy for TI-RADS 3 was 4.8%, for TI-RADS 4, 9.1%, and for TI-RADS 5, 35% in their study.[20] Also, in 2019, Barbosa and his colleagues postulated that the risk of malignancy with TI-RADS 3 was 23.3%, TI-RADS 4 49.6%, and TI-RADS 5 92.9%.[21] However, Chaigneau and his colleagues (2018) examined 602 indeterminate thyroid nodules with TI-RADS scores of 3, 4a, 4b, and 5 and found that the malignancy risks were 20.5%, 29%, 63.4%, and 100%, respectively.[22]

In the current study, we found that patients with malignant thyroid nodules had significantly larger nodules than patients with benign nodules based on ultrasound dimensions. These findings agreed with those of Shin et al., 2015, who stated that nodule size affects cancer risk.[23] Furthermore, Kuru et al., 2010, found that nodule size larger than or equal to four cm in diameter was an independent factor associated with malignancy.[24] Also, Kamran and his colleagues (2013) found that nodules larger than or equal to four cm in diameter had a higher prevalence of malignancy than nodules with smaller diameter.[25]

Albuja-Cruz et al., 2013, on the other hand, stated that a nodule size larger than or equal to four cm is not associated with a higher overall prevalence of malignancy.[26] In addition, Cavallo and his colleagues (2017) stated that the rate of cancer is inversely related to the size of the nodules. In the last-mentioned study, the authors declared that nodule size should not be considered an independent risk factor for cancer. Their results were based on 1003 nodules in 659 patients and revealed that 26% were malignant. Nodules less than 2 cm in size had a 30% malignancy rate, while nodules larger than 2 cm had a 20% risk of malignancy.[27] According to a study by Shrestha et al., 2012, there was no evidence of a difference in malignancy prevalence based on size.[28]

In the present study, the risk of malignancy was 13.15% in thyroid nodules with Bethesda III and 42.85% in thyroid nodules with Bethesda IV. These findings agreed with those of Iskandar et al., 2015, and Faquin et al., 2016, as they found that the malignancy prevalence in resected thyroid nodules was higher in Bethesda IV than in Bethesda III.[29, 30] Finally, in a study by Yaprak and Eruyar, 2020, malignancy rates ranged from 10 to 30% for Bethesda category III and 25–40% for Bethesda category IV.[31]

In conclusion, based on our study findings, serum miRNA-146b is a poor predictor of thyroid malignancy in euthyroid patients with indeterminate thyroid nodules, however, the larger the nodule size, the higher the risk of malignancy.

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Author Contributions

All authors shared the design of this study. AO, MH, and IZ proposed the idea. Data collection and sampling were done by AO. Laboratory work was conducted by LS. Data analysis and interpretation were done by IZ, MS, AM, LS, and AO. Writing and revision of the manuscript were done by MH, IZ, MS, AM, LS, and AO. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The protocol of the study was reviewed and approved by the Research Ethical Committee of the Faculty of Medicine, Ain Shams University. (MD 244/2019).

Informed consent

All the study subjects provided informed written consents before participating in this study.

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