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CIRCULATING MICRORNA-146B OVEREXPRESSION AS POTENTIAL BIOMARKER FOR DISTINGUISH BENIGN NODULES FROM PAPILLARY THYROID CARCINOMA

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ABSTRACT

Background: MicroRNA-146b is one of the widely studied microRNAs in thyroid cancers and has been shown to be frequently upregulated in papillary thyroid carcinoma and plays a crucial role in tumorigenesis, progression and may become a potential therapeutic target and biomarker of tumor diagnosis and prognosis. **Aim of the study:** Estimation of microRNA-146b gene expression levels in both serum and fresh tissues of the same patients of solitary thyroid nodule by using stem-loop follow by Taq-Man Real Time PCR technique to distinguish the benign nodule from papillary carcinoma and correlate with age, sex of patients, tumor size, lymph node involvement and stage of tumors. **Material and methods:** Stem-loop Real Time PCR was performed to identify the level of microRNA-146b gene expression in both serum and fresh tissues of the same patients of solitary thyroid nodule. The expression levels of microRNA-146b were normalized to housekeeping gene by using the livak method. **Results:** The expression level of serum miR-146b in papillary thyroid carcinoma cases was significantly higher than that benign cases and healthy controls. Receiver operating characteristic curve analyses indicated that use of serum microR-146b have a high diagnostic sensitivity and specificity for papillary thyroid carcinoma in correlated with nodal status and higher stage. **Conclusion:** The serum miR-146b may be used as novel and minimally invasive diagnostic and prognostic biomarker for papillary thyroid carcinoma.

KEYWORD: solitary thyroid nodule, serum and fresh tissues, miR-146b, RT-PCR.

INTRODUCTION

Thyroid nodules are very common and have a prevalence of 4–10% in randomly selected individuals[1]. Most are benign, and only 5–30% are malignant and require surgical intervention[2]. Papillary thyroid carcinoma (PTC) accounts for up to 90 % of thyroid carcinomas[3]. Patients with suspicious thyroid nodules should undergo ultrasound-guided fine-needle aspiration (FNA). FNA is currently the best diagnostic approach to rule out malignant thyroid neoplasm[4]. But thirty percent of FNA specimens from thyroid nodules are indeterminate, and up to 75 % of patients undergo a hemithyroidectomy for benign disease unnecessarily[5,6]. In addition the FNA is an invasive diagnostic method and heavily dependent on the technical performance and experience of the operators. Taken together, all of the above creates a gap in the clinical decision pathway and an additional burden in patients with thyroid nodules[7]. As the incidence of PTC increases, along with the detection of incidental thyroid nodules on computed tomography (CT) and ultrasound (U/S), considerable effort has been made to identify other reliable markers for primary PTC diagnosis is needed. An alternative to tumor tissue sampling is serum-based diagnostic testing. Serum testing would potentially allow for minimally invasive, safe and repeatable diagnosis, requiring less technical skill for collection. Although the feasibility of potential blood-based diagnostic testing has been shown[8], no biomarkers have been identified that reliably reflect elevated tumor tissue levels.

Recent studies have measured circulating microRNA(miR), and the results suggest that serum miR profiles may be useful as cancer biomarkers[9,10]. MiRs are non-coding RNA molecules involved in the regulation of gene expression[11]. MiRs bind to messenger RNA (mRNA) and promote their degradation or prevent their translation into proteins. Traditionally, miRs have been detected in tissue specimens; however, recent studies have also shown that miRs remains relatively stable and detectable in blood samples[12,13]. Numerous independent studies have identified miR expression profiles in differentiated thyroid carcinoma tissue, suggesting they may play a role in the development and progression of this carcinoma[14,15].

MiR-146 is one of the widely studied miRs in thyroid cancers and has been shown to be frequently upregulated in PTC[16,17], anaplastic thyroid cancer[18] and follicular thyroid cancer[19]. The miR-146 family includes two members: miR-146b and miR-146a. The miR-146b located on chromosome 10q24.32[16].

MATERIALS AND METHODS

Selected cases:-The study was conducted during the period from March 2014 to January 2016. This is a prospective study, where by 70 patients with newly diagnosed solitary thyroid nodules were recruited at the Surgical Department/AL-Diawania Teaching Hospital in Diawania City. Ultrasound-guided FNA cytology was performed as a part of the standard diagnostic protocol for patients with solitary thyroid nodule in Pathological Department/AL-Diawania Teaching Hospital in Diawania City.

Serum sample were collected from the patients (n=70) and apparently healthy controls(n=70) were diagnosed without any tumor or physical illness. After excision of the thyroid samples, further tested with the established criterion able to classify a nodule as benign or malignant on the basis of miR-146b expression values, take sevently-pairs of fresh tissues from same cases of thyroid nodule and normal adjacent tissues (NATs) which consider as internal control and preserved in Diethylpyrocarbonate (DEPC) water for total RNA extraction and for RT-PCR. Another 70 pairs specimens of both thyroid nodule and normal adjacent tissues for histopathological examination. The histopathological classification was performed according to the World Health Organization(WHO) classification, tumor staging was carried out according to American Joint Committee Cancer (AJCC)[20].

MiRNA isolation from serum and tissue: Serum samples were collected between 8:00 and 9:00 a.m. following centrifugation for 30 min at 2,650 g, then serum samples were stored at 80o c. Tissue samples were homogenized in adenaturing lysis solution and dissolved RNA was stored at -20°C before use. RNA was extracted from serum and fresh tissues using the Trizol reagent (Bioneer, Korea) according to the manufactures instructions. RNA quality was assessed with a NanoDrop 1000 spectrophotometer.

Real-time RT-PCR for miR-146b quantification: The miR-146b was analyzed using the TaqMan miR RT kit protocol (Applied Biosystems, Foster City, CA, USA) consisting in a first step of RT with an miR-specific primer (used miR-base ,as a database to design the primers)[21] and in a second step the real-time PCR with TaqMan probes. Reverse transcriptase reactions were carried out to produce cDNAs in a volume of 15 ml using 10 ng total RNA for each sample, 50 nM stem–loop RT primer, 1! RT buffer, 1 mM each of dNTPs, 3.33 U/ml Multi- Scribe reverse transcriptase, and 0.25 U/ml RNase inhibitor. After incubation on ice for 5 min, reactions were subjected to the following program of heating: 30 min at 16 8C, 30 min at 42 8C, 5 min at 85 8C, and hold at 4 8C. Real-time PCR was

performed in triplicate in a 96-well optical plate on the Applied Biosystems 7700 Sequence Detection System. The volume of 20 ml of each sample included 1! TaqMan Universal PCR Master Mix, 1 ml specific miR Assay Mix (Applied Biosystems), and 1.34 ml RT product. The reactions were incubated at 50 8C for 2 min and 95 8C for 10 min, followed by 40 cycles of 95 8C for 15 s and 60 8C for 1 min. All miR-146b quantification data were normalized to housekeeping gene like Glycer aldehyde 3-phosphate dehydrogenase (GAPDH). The mRNA of GAPDH gene primers and probe were designed by using NCBI- Gene Bank data base and Primer 3 plus design online. The cDNAs primer of GAPDH as design as Random Hexamer primer and the primer used in qPCR was: forward, CAGCCGCATCT-TCTTTTGC and reverse, TTAAAAGCAGCCCTGGTGAC. Taq-Man probe for mGAPDH was: FAM-CCAGCCGAGCCACATCGCTC-TAMRA. The data results of RT-qPCR for miR-146b and GAPDH were analyzed by the relative quantification gene expression levels (fold change) were based on the Ct values by using the Livak method (Fold change = $2^{-\Delta\Delta CT}$) that described by (Livak and Schmittgen)[22].

Statistical analysis: SPSS version 16 and Microsoft Office Excel 2007 were using in analysis of these data, Chi-square test and Fisher exact test were used to study association between any two nominal variables. P-value of less than or equal to 0.05 was considered significant.

RESULTS

1- Clinicopathological characteristics of patients

Table (1), shown a total of (n=70) solitary thyroid nodules patients, thirty one cases (44.29%) with PTC, other 39(55.71%) cases with BN, number and percentage of patients according to age, gender, lymph node (L.N) involvement and stage of tumor.

Table (1):- Clinicopathological characteristics of patients

Characteristic		PC (n = 31)		BN (n=39)	
		N	%	N	%
Age	< 45 years	15	48.39	10	25.64
	≥45 years	16	51.61	29	74.36
Gender	Male	10	32.26	23	58.97
	Female	21	67.74	16	41.03
LN	Positive	10	32.26	---	---
	Negative	21	67.74	---	---
Stage	I	18	58.06	---	---
	II	8	25.81	---	---
	III	5	16.13	---	---

2-Comparison the serum level of microRNA-146b gene expression between papillary carcinoma, benign thyroid nodule patients and apparently healthy control.

The mean serum level of miR-146b in PTC patients was statistical significantly higher than that apparently healthy control, (21.5±13.15) versus (1), respectively (P<0.001), as shown in table (2).

The mean serum level of miR-146b in BN patients was no statistical significantly difference from that apparently healthy control, (2.87±0.39) versus (1), respectively (P= 0.447), as shown in table (2).

The mean serum level of miR-146b in PC patients was statistical significantly higher than that BNs, (21.5±13.15) versus (2.87±0.39), respectively (P<0.001), as shown in table (2).

Table (2): comparison the serum level of microRNA-146b gene expression between papillary carcinoma, benign thyroid nodule patients and apparently healthy control.

Groups	N	Mean	SD	P1	P2	P3
Control	70	1	0.0	<0.001	0.477	<0.001
PC	31	21.5	13.15			
BN	39	2.87	0.39			

P1: Control vs PC; P2: Control Vs BN; P3: PC vs BN

3-Comparison the tissue microRNA-146b gene expression of papillary carcinoma, benign nodular thyroid and normal adjacent tissues.

Mean cancer tissue of miR-146b was statistical significantly higher than that NATs, 25.18±14.67 versus 1, respectively (P<0.001). The amplification plots of tissue miR-146b in PTC showed in figure (1).

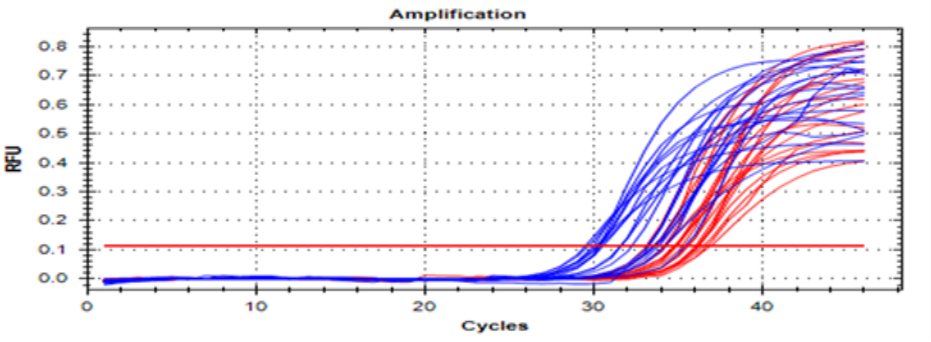

Figure (1): Stem- Loop RT-PCR amplification plots for microRNA-146b cDNA in papillary cancer tissue patients by using Taq-Man probe. (FAM), where (blue amplification plot as papillary cancer tissue samples) and (red amplification plot as normal adjacent tissue samples).

Mean BN thyroid tissues of miR-146b was statistical significantly higher than that NATs, 4.05± 0.23versus 1, respectively (P<0.001). The amplification plots of tissue miR-146b in BN showed in figure (2).

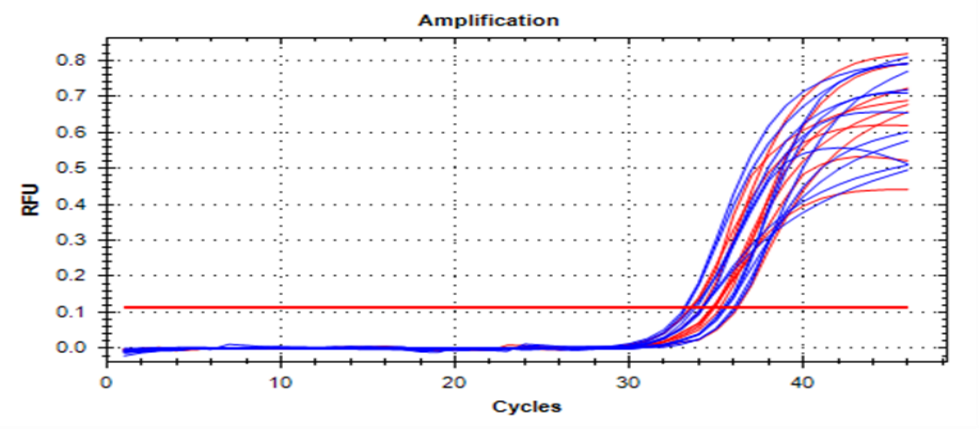

Figure (2): Stem- Loop RT-PCR amplification plots for microRNA-146b cDNA in patients with benign nodular thyroid by using Taq-Man probe. where (blue amplification plot as benign nodular thyroid tissue samples) and (Red amplification plot as normal adjacent tissue samples).

Mean cancer tissue of miR-146b was statistical significantly higher than that BN thyroid tissues, 25.18±14.67 versus 4.05±0.23, respectively (P<0.001).

4-The correlation of microRNA-146bgene expression pattern between papillary cancer tissues and paired serums

The results showed a statistical significant correlation between miR-146b gene expression in the PTC tissues with those in the paired serums, with r = 0.41 (p<0.001), as shown in figure (3).

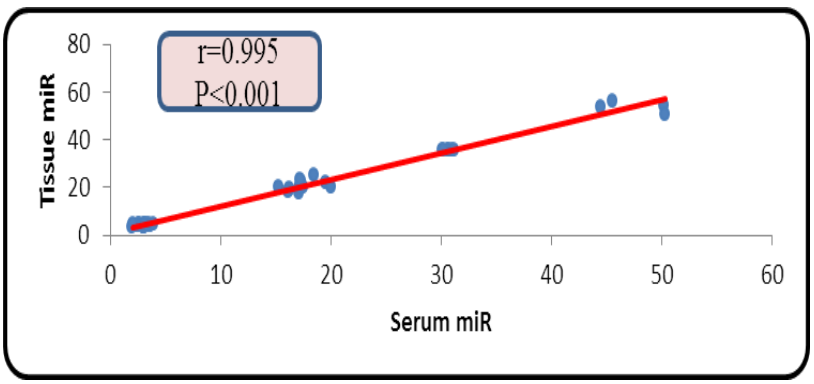

Figure (3): The correlation of microRNA-146b gene expression pattern between papillary cancer tissues and paired serums.

Also the results showed a statistical significant correlation between miR-146b gene expression in the BN tissues with those in the paired serums, with r = 0.31 (p<0.001).

5-Correlation of microRNA and clinicopathological features of papillary thyroid carcinoma and benign thyroid nodules.

A- Association between serum level of microRNA-146b and sex of patients.

The result of present study showed that there was no statistical significance correlation between serum level of miR-146b of PTC and BN with sex of patients (P>0.005), as shown in table (3).

B-Correlation between serum microRNA-146b gene expressions with age.

The result of present study showed that there was no statistical significance correlation between serum level of miR-146b of PTC and BN with age of patients (P>0.005), as shown in table(3), figure (4).

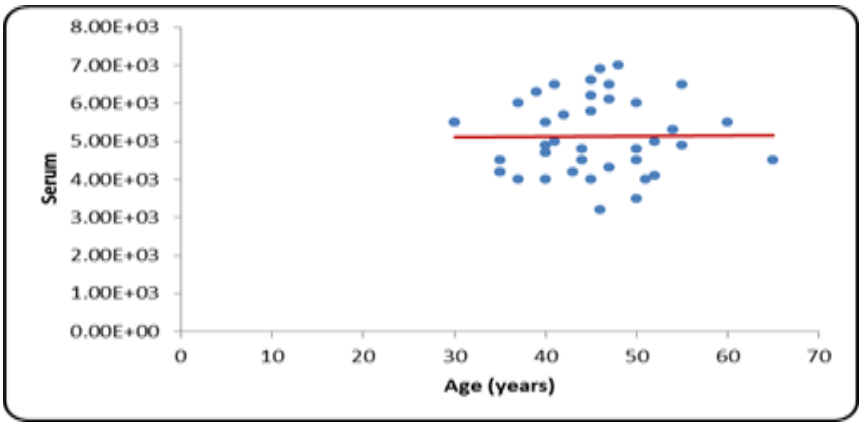

Figure (4): Spearman correlation between age of patients and serum of microRNA-146b.

Even when divided in two group (<45 and ≥45), no statistical significant between mean fold change of miR-146b serum levels of PTC and BN patients (p>0.05).As shown in table (3).

C- Association between serum level of microRNA-146b and size of thyroid nodules.

When divided in two group (≤ 2 and >2), no statistical significant between mean fold change of miR-146b serum levels of PTC and BN with size of tumors (p>0.05), as shown in table (3).

D- Association between serum level of microRNA-146b and L.N involvement

The mean miR-146b fold change of PTC patients with positive L.N was statistical significantly higher than that of patients with negative L.N involvement, (37.43± 8.96) versus (14.01± 6.14), respectively (P < 0.001), as shown in table (3).

E- Association between serum level of microRNA-146b and stage of tumor.

The mean fold change of miR-146b serum levels in PTC patients with higher stage (III) was statistical significantly higher than that early stage (I,II), (38.4±10.7) versus (18.32±11.00), (P <0.05), as shown in table (3).

Table (3): Clinicopathological features and their correlation with serum levels of miR-146b

Papillary carcinoma	N	%	Mean serum miR	SD	P-value
Gender					
Male	10	32.26	23.81	16.67	0.522
Female	21	67.74	20.50	11.43	
Age					
<45 years	15	48.39	20.99	13.25	0.818
≥ 45 years	16	51.61	22.11	13.47	
Size					
≤ 2 cm	21	67.74	22.70	13.20	0.497
> 2 cm	10	32.26	19.19	13.42	
LN					
Positive	10	32.26	37.43	8.96	<0.001*
Negative	21	67.74	14.01	6.14	
Stage					
I,II	26	83.87	18.32	11.00	0.001*
III	5	16.13	38.46	10.77	
Benign nodule					
Gender	23	58.97	2.88	0.40	0.803
Male	16	41.03	2.85	0.39	
Female					
Age					
<45 years	10	25.64	2.97	0.39	0.355
≥ 45 years	29	74.36	2.83	0.40	
Size					
≤ 2 cm	7	17.95	2.97	0.20	0.456
> 2 cm	32	82.05	2.85	0.42	

6-predictive value of microRNA-146b in serum of patients

To evaluate the diagnostic value of serum miR- 146b for PTC and BN, by using the RT-qPCR technique, an Receiver Operator Characteristic (ROC) curve analysis was done:

The best cutoff value for serum miR-146b in PTC was(10.55) with a specificity of 100%, sensitivity of 90.30% and excellent accuracy(AUC), as shown in table (4) and figure (5).

Table (4): The cut of value of serum microRNA-146b in the discrimination of patients with papillary thyroid carcinoma from benign nodular and healthy control groups

Parameter	Cutoff value	Sensitivity	Specificity	Accuracy (AUC)	P-value
PC serum mir	10.55	90.30%	100%	98.2 % (0.982)	<0.001

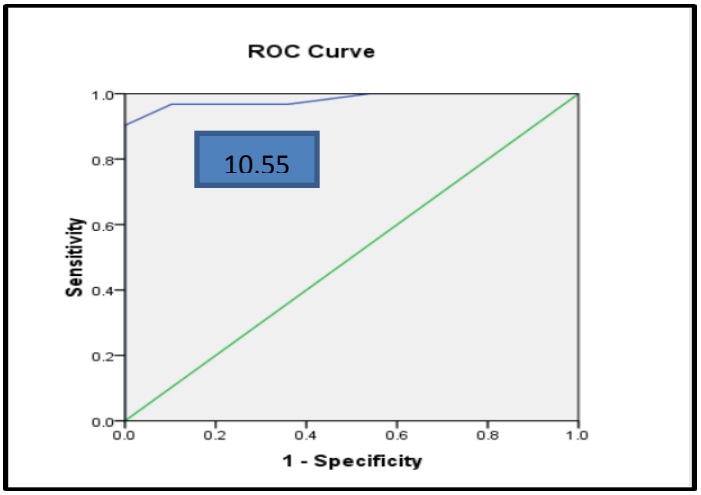

Figure (5): ROCcure analyses of serum microRNA-146b in the discrimination of patients with papillary thyroid carcinoma from benign nodular and healthy control groups.

And the best cutoff value for serum miR-146b in BN groups was (2.15) with a specificity of 100%, sensitivity of 96.80% and excellent AUC, as shown in table (5) and figure (6).

Table (5): The cut of value of of serum microRNA-146b in benign thyroid nodules.

Parameter	Cutoff value	Sensitivity	Specificity	Accuracy (AUC)	P-value
BN serum mir	2.15	96.80%	100%	99.7 % (0.997)	<0.001

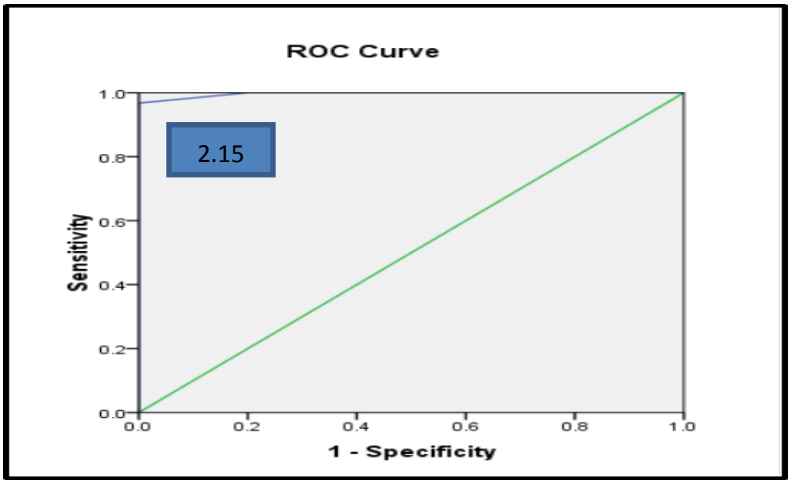

Figure (6): ROCcure analyses of serum microRNA-146b in the discrimination of patients with benign thyroid nodules from patients with papillary thyroid carcinoma.

7-Validity of microRNA-146b gene expression folds change as prognostic marker.

To find the cutoff value of fold change for miR-146b gene expression, that predict PTC patient with positive lymph node metastasis and higher stage (III), an ROC curve analysis was performed.

It was found that miR-146b gene expression fold change can predict positive lymph node and higher stage (III). The ROC results demonstrated that the excellent AUC was (100%), (98.5%), when the cutoff value was set to the optimal point, ≥ 15.2, ≥30.5; specificity was 100%, 97.1%; sensitivity was 100%, 100% respectively**.** As shown in figure (7).

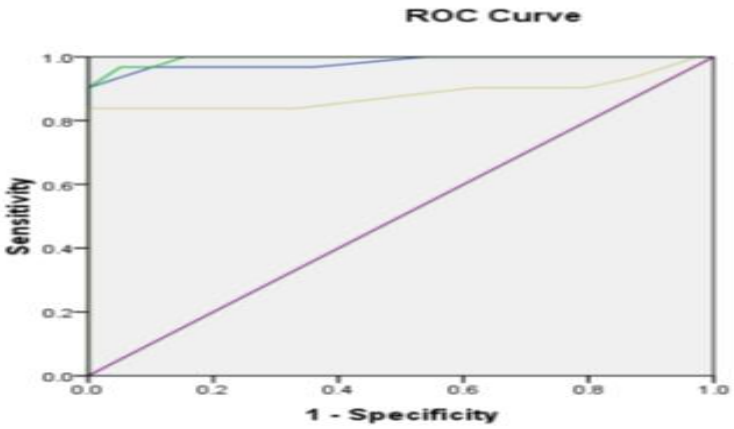

Figure (7): ROCcure analyses of serum microRNA-146b in the discrimination of patients with papillary thyroid carcinoma and lymph node involvement and higher stage. Where blue line for patients with papillary thyroid carcinoma. Yellow line for patients that predicts higher stage. Green line for patients that predicts positive L.N.

DISCUSSION

The current approaches for differential diagnosis of benign nodules from malignancy include history, physical examination, ultrasound, thyroid nuclear scanning, FNA, and pathological examination. The gold standard is surgical pathological examination. The FNA is considered the most reliable method for preoperative diagnosis of thyroid nodules. However, predictive value of FNA is still limited in subjects with cytological features of suspicious malignancy or follicular neoplasm and atypia of undetermined significance, leading to annecessary thyroid lobectomies or thyroidectomy[23]. Although the application of tissue tumor markers such as miR and gene profiling may potentially improve diagnostic accuracy, these must be obtained by FNA biopsy and therefore remain invasive, unpleasant and inconvenient for patients[24].

In the present study, a tissue-serum-paired samples for miR expression level by RT-PCR validation in patients with thyroid nodule and in healthy subjects. We identified the miR-146b in PTC was significant higher than that BNs and healthy controls. We speculated that circulating miR-146b may originate from tumor cells or a product of tumor cell death and lysis. The finding that tumor cells are the source of serum miR-146b serves as a tumor fingerprint. The results of present study were in concordance with other studies[16,25- 31].

Both neck U/S and CT have limited value for the prediction of L.N involvement; hence it is crucial to find novel biomarkers that can be used for this purpose and also as a standard for optimizing therapy and long-term follow-up care.

The results of present study demonstrated that the expression level of miR-146b in PTC with positive L.N and higher stage III was significant higher than that patients with negative L.N and early stage of cancer, suggesting its role in metastasis and functional analyses of miR-146b revealed its involvement in migration, invasion, proliferation and cell cycle[16,25-30].

The results of present study were in concordance with other studies[16,25-30] On the other hand, another study performed in (91)cases of PTC patients failed to find any significant association of miR-146b with LNM[31]. The reason for this remains unclear and it might be contributed by the use of a different platform and/or chemistry for detection and also possibly the intrinsic variability in the cohort of patients. In addition, even though the tissues used in the studies were verified by a pathologist, the authors did not mention about the percentage of cancer cells. Perhaps a more refined quality control and assessment of the tumor specimens

before being subjected to expression profiling will be able to explain such contradictory findings.

In the results of the present study, the expression level of serum miR-146b in PTC was no statistical significant difference in comparison to age, sex of patients and nodular tumor size. These results were in concordance with other study.[16,25-31]

The best for your knowledge, this study could be the first study of its type to be conducted in Iraq, evaluating tissue and serum level of miR-146b gene expression in same patients by RT-qPCR, in a sample of Iraqi patients. There was no baseline study regarding tissue and serum level of miR-146b gene expression stratification in apparently healthy control in Iraqi individuals. Although, similar studies were conducted abroad to stratify tissue and serum level of miR-146b in PTC and BN patients in other countries[16,25-31].

IN CONCLUSION

Serum miR-146b, an easy, minimally invasive, and effective diagnostic tool for preoperative assessment of thyroid nodules. The RT-PCR is a noval and applied means for investigation of serum and tissue miR samples and the extraction of RNA and identification of miR-146b from the serum and tissue of individuals diagnosed thyroid nodule is possible. Circulating miR-146b before operation can serve as a good biomarker for breast cancer screening, detection, discrimination the higher stage (III) with L.N positive from that of early stage (I,II) and negative L.N involvement. Long-term follow- up of the patients in the current study and a prospective study with a larger sample size are needed to further validate the usefulness of circulating miR in the diagnosis of thyroid carcinoma and the possibility of the miR as markers for monitoring tumor recurrence and predicting prognosis.

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