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### RESEARCH ARTICLE

## THE MICRORNA-146B OVEREXPRESSION HELPS TO DIFFERENTIATE BENIGN NODULES FROM PAPILLARY THYROID CARCINOMA.

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##### Manuscript History

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##### Key words:-

Solitary thyroid nodule, fresh tissues, miR-146b, RT-PCR

#### 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 fresh tissues of solitary thyroid nodule in comparison to normal adjacent tissue of same patients by using stem-loop followed by Taq-Man Real Time PCR (RT-PCR) technique to distinguish the benign nodule from papillary carcinoma and correlate with age, gender of patients, size of tumor, 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 fresh tissues of solitary thyroid nodule in comparison to normal adjacent tissues of same patients. The expression levels of microRNA-146b were normalized to housekeeping gene by using the Livak method.

**Results:-** The expression level of tissue microRNA-146b in papillary thyroid carcinoma cases was significantly higher than that in benign cases and normal adjacent tissues. Receiver operating characteristic curve analyses indicated that use of tissue microRNA-146b has a high diagnostic sensitivity and specificity for papillary thyroid carcinoma and is correlated with nodal status and advanced tumor stage.

**Conclusion:-** The microRNA-146b as a new novel diagnostic and prognostic biomarker for papillary thyroid carcinoma.

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#### Introduction:-

Thyroid cancer, which usually presents as a nodule, accounts for approximately 1% of all newly diagnosed cancer cases and its incidence is increasing faster than any other cancer types, thus representing one of the most common and clinically worrying malignant tumors of the endocrine system. Papillary thyroid carcinoma (PTC) represents the most frequent typology of thyroid malignancy, with a prevalence of about 90% of all diagnosed cases<sup>(1)</sup>. Fine-needle aspiration (FNA) biopsy and subsequent cytological analysis represents the most reliable procedure to date to diagnose thyroid nodules<sup>(2)</sup>. FNA is highly specific for thyroid cancer; however, it has low sensitivity. In fact, 10%–

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40 % of the analyzed nodules are detected as indeterminate lesions, thus creating difficulties for the optimal management of these patients<sup>(3)</sup>. Moreover, only 10 %–30 % of indeterminate thyroid nodules that are surgically resected are confirmed to be malignant<sup>(4)</sup>. As a result, most diagnostic surgeries are performed for benign thyroid nodules (BN). Conversely, patients who have undergone a surgical lobectomy and are found to have a tumor larger than 1 cm, may require a second surgery to remove the remaining thyroid lobe, thereby creating an important gap in the clinical decision pathway for thyroid nodules. Clearly, additional diagnostic markers are needed to guide the management of patients with indeterminate thyroid nodules. In the past few years, significant progress has been made in developing molecular markers for clinical use in FNA specimens, such as gene mutation panels and gene expression classifiers<sup>(5)</sup>. BRAF and KIT mutation are the best known and studied prognostic markers for the diagnosis of PTC. The genetic characterization of BRAF and KIT status leads to an increase of preoperative diagnostic accuracy up to 20 %–30 %<sup>(6,7)</sup>, but still left a percentage of indeterminate samples. This is due to the small number of screened patients and some malignant tumors do not have the BRAF and KIT mutation, confirming the necessity of finding other molecular markers able to provide a more accurate diagnosis<sup>(7)</sup>.

Recently, new prognostic markers are currently being explored for the effective diagnosis and treatment of PTC, and research into the potential for microRNAs (miRs) use in this area is currently a popular research topic<sup>(8,9)</sup>.

miRs are non-coding RNA molecules involved in the regulation of gene expression<sup>(10)</sup>. miRs bind to messenger RNA (mRNA) and promote their degradation or prevent their translation into proteins. As miRs have been reported to be deregulated in thyroid cancer<sup>(11)</sup>, and they have been shown to function both as tumor suppressors and oncogenes<sup>(12)</sup>, we decided to assess the prediction value of two miRs targeting the KIT gene; namely, miR-146b and miR-222.

MiR-146 is one of the widely studied miRs in thyroid cancers and has been shown to be frequently upregulated in PC<sup>(8,13)</sup>, anaplastic thyroid cancer<sup>(14)</sup> and follicular thyroid cancer<sup>(9)</sup>. The miR-146 family includes two members: miR-146b and miR-146a. The miR-146b located on chromosome 10q24.32<sup>(8)</sup>.

## Materials and methods:-

### Selected cases:-

The study was conducted during the period from March 2013 to January 2016. This is a prospective study, where by 70 patients with newly diagnosed solitary thyroid nodule, 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.

After excision of the thyroid nodule samples, taken seven pairs of fresh tissues from thyroid nodule and normal adjacent tissues (NATs) of same cases 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 NATs 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)<sup>(15)</sup>.

### MiRNA isolation from tissue:-

RNA was extracted from tissues using the Trizol reagent (Bioneer, Korea) according to the manufacturer's instructions. RNA quality was assessed with a NanoDrop 1000 spectrophotometer. Total RNA samples of tissues were transferred to a -20 °C freezer for preservation. The quality of RNA samples was analyzed by Nano-Drop 1000.

### 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)<sup>(16)</sup> 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 µl 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 °C, 30 min at 42 °C, 5 min at 85 °C, and hold at 4 °C. 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 mlspecific miRNA 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)<sup>(17)</sup>.

#### 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:-

##### Clinicopathological characteristics of patients:-

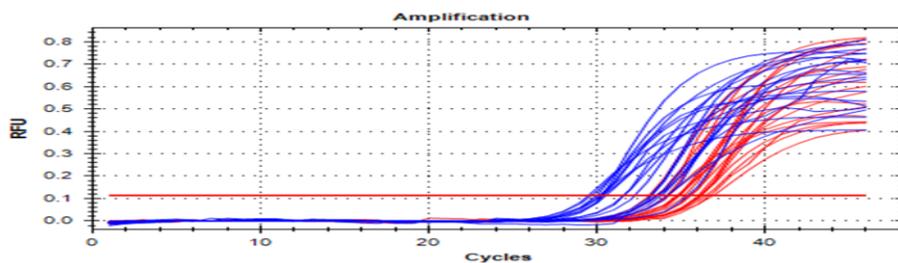
Table (1) , shown a total of (n=70) solitary thyroid nodules patients , thirty cases (42.86%) with PTC, other 40(57.14%) cases with BN , number and percentage of patients according to age ,gender ,lymph node (L.N) involvement and stage of tumor .

**Table 1:-** Clinicopathological characteristics of patients.

PC (n = 30)		BN (n=40)			
Characteristic		N	%	N	%
Age	< 45 years	14	46.66	10	25.64
	≥45 years	16	51.61	30	75
Gender	Male	10	32.26	23	58.97
	Female	20	66.66	17	42.5
LN	Positive	10	32.26	---	---
	Negative	20	66.66	---	---
Stage	I	17	56.66	---	---
	II	8	25.81	---	---
	III	5	16.13	---	---

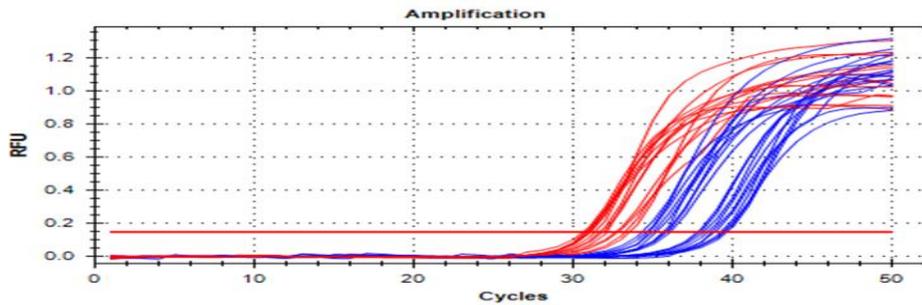
##### Comparison the tissue microRNA-146b gene expression of papillary carcinoma , benign nodular thyroid and normal adjacent tissues .

Mean cancer tissue of miR-146b was statistical significantly higher than that NATs ,  $26.18 \pm 14.67$  versus , 1 respectively ( $P < 0.001$ ) . The amplification plots of tissue miR-146b in PTC showed in figure (1) .



**Figure 1:-** Stem- Loop qRT-PCR amplification plots for miR-156b cDNA 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 ,  $4.05 \pm 0.23$  versus 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 from BN thyroid tissues,  $26.18 \pm 14.67$  versus  $4.05 \pm 0.23$ , respectively ( $P < 0.001$ ) .

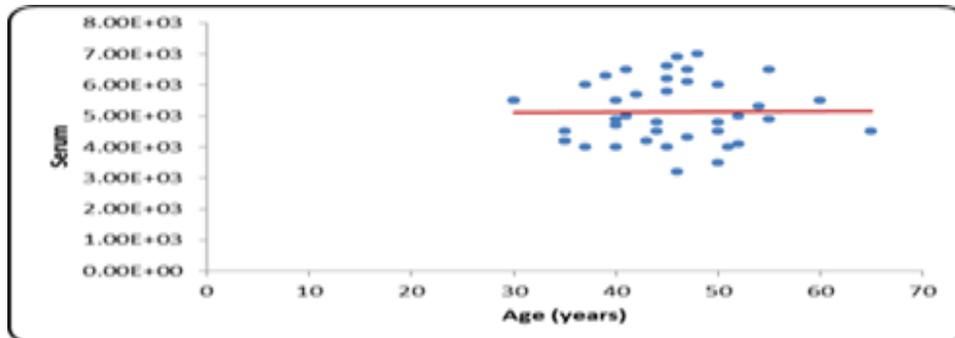
#### **Correlation of microRNA and clinicopathological features of PTC and BN:-**

##### **Association between tissue level of microRNA-146b and sex of patients:-**

The result of present study showed that there was no statistical significance correlation between tissue level of miR-146b of PTC and BN with sex of patients ( $P > 0.005$ ) , as shown in table (2) .

##### **Correlation between tissue microRNA-146b gene expressions with age:-**

The result of present study showed that there was no statistical significance correlation between tissue level of miR-146b of PTC and BN with age of patients ( $P > 0.005$ ),as shown in table(2),figure (3) .



**Figure 3:-** Spearman correlation between age of patients and tissue of microRNA-146b.

Even when divided in two group ( $< 45$  and  $\geq 45$ ) , no statistical significant between mean fold change of miR-146b tissue levels of PTC and BN with age of patients ( $p > 0.05$ ).As shown in table (2) .

##### **Association between tissue level of microRNA-146b and size of thyroid nodules:-**

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

##### **Association between tissue level of microRNA-146b and L.N involvement:-**

The mean tissue miR-146b fold change of PTC patients with positive L.N was statistical significantly higher from that of patients with negative L.N involvement, ( $40.43 \pm 6.92$ ) versus ( $20.02 \pm 5.12$ ) , respectively ( $P < 0.001$ ),as shown in table (2) .

**Association between tissue level of microRNA-146b and stage of tumor:-**

The mean fold change of tissue miR-146b in PTC patients with higher stage (III) was statistical significantly higher than that early stage (I,II), (40.4±19.66 ) versus , (20.32±10.00 ) , ((P <0.05 ) , as shown in table (2) .

**Table 2:-** Clinicopathological features and their correlation with tissue levels of miR-146b

Papillary carcinoma	N	%	Mean tissue miR	SD	P-value
Gender					
Male	10	32.26	27.81	13.67	0.422
Female	20	66.66	25.50	10.43	
Age					
<45 years	14	46.66	25.99	10.25	0.718
≥ 45 years	16	51.61	27.11	11.47	
Size					
≤ 2 cm	20	66.66	25.70	11.20	0.397
> 2 cm	10	32.26	27.19	12.42	
LN					
Positive	10	32.26	40.43	6.92	<0.001*
Negative	20	66.66	20.02	5.12	
Stage					
I,II	25	83.33	20.32	10.00	0.001*
III	5	16.13	40.46	19.66	
Benign nodule					
Gender	23	58.97	4.5	0.20	0.702
Male	17	42.5	4.2	0.46	
Female					
Age					
<45 years	10	25.64	4.4	0.19	0.265
≥ 45 years	30	75	4.5	0.31	
Size					
≤ 2 cm	7	17.95	4	0.41	0.374
> 2 cm	33	82.5	4.5	0.33	

**predictive value of tissue microRNA-146b in the patients:-**

To evaluate the diagnostic value of tissue 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 tissue miR-146b in PTC was(14.55) with a specificity of 100%, sensitivity of 90.30% and ) excellent accuracy(AUC, as shown in table (3) and figure (4) .

**Table 3:-** The cut of value of tissue 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	14.55	90.30%	100%	98.2 % (0.982)	<0.001

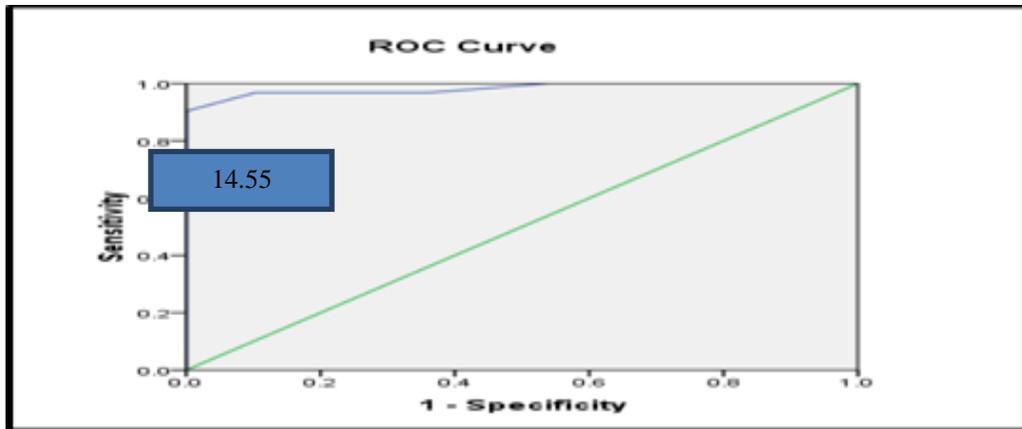


Figure 4:- ROCcure analyses of tissue microRNA-146b in the discrimination of patients with papillary thyroid carcinoma from benign nodular and normal adjacent tissues .

And the best cutoff value for tissue miR-146b in BN groups was (3.12) with a specificity of 100%, sensitivity of 96.80% and excellent AUC, as shown in table (4) and figure (5) .

Table 4:- The cut of value of oftissue microRNA-146b in the discrimination of patients with benign thyroid nodules from papillary thyroid carcinoma.

Parameter	Cutoff value	Sensitivity	Specificity	Accuracy (AUC)	P-value
BN serum mir	3.12	96.80%	100%	98.2 % (0.982)	<0.001

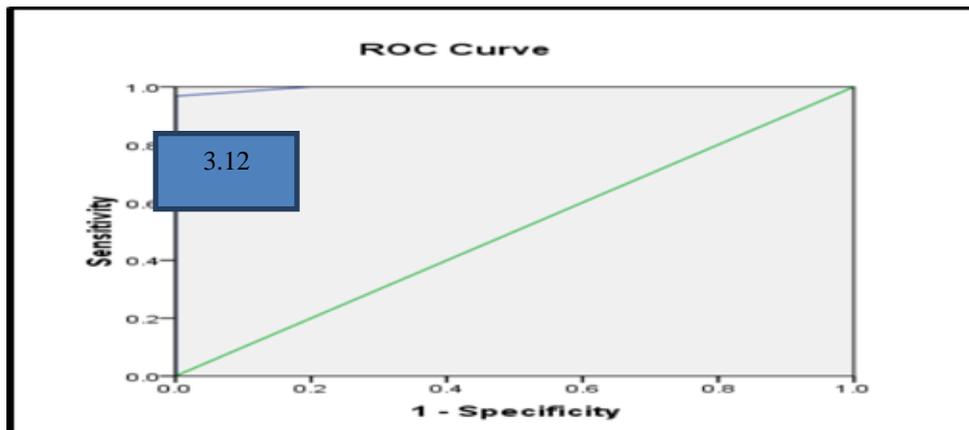
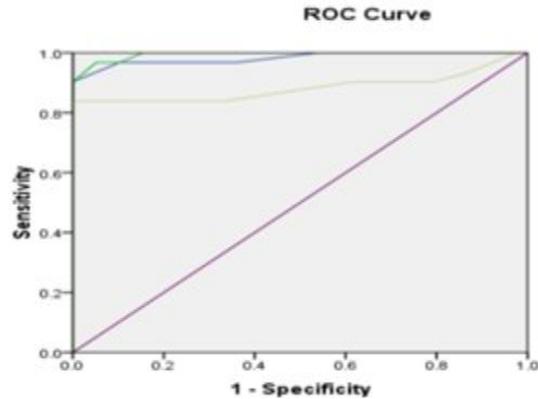


Figure 5:- ROCcure analyses of tissue microRNA-146b in the discrimination of patients with benign thyroid nodules from papillary thyroid carcinoma .

**Validity of tissue 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,  $\geq 20.1$  ,  $\geq 35.5$ ; specificity was 100% , 97.1%; sensitivity was 100% ,100% respectively. As shown in figure (6) .



**Figure 6:-** ROC curve 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 primary goal of the evaluation of patients with nodular thyroid disease is the exclusion of thyroid malignancy, alteration of gene expression for target gene therapy and for follow up patients and management. At present, miR have become the rising stars in cancer genetics. MiRs are excellent candidates for novel molecular targeting treatments because of their ability to regulate multiple genes in molecular pathways. Chemotherapy is an important component in the treatment paradigm for cancers. However, the resistance of cancer cells to chemotherapeutic agents frequently results in the subsequent recurrence and metastasis. Recently, a new data suggest that the expression level of miR-146b in tumor tissue and plasma might be used as a biomarker to predict adjuvant platinum-based chemotherapy response and disease free survival in patients with non-small-cell lung cancer. Thus, it may serve as a novel therapeutic target to modulate platinum-based chemotherapy<sup>(9)</sup>.

In the present study, a tissue samples for miR expression level by RT-PCR validation in patients with PTC, BN and in NATs. We identified the miR-146b in PTC was significant higher than that BNs and NATs. We speculated that tissue miR-146b may originate from tumor cells or a product of tumor cell death and lysis. The results of present study were in concordance with other studies<sup>(9,18-24)</sup>.

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 tissue miR-146b in PTC with positive L.N and higher stage III was statistical 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<sup>(9)</sup>. The results of present study were in concordance with other studies<sup>(9,18-23)</sup>. On the other hand, another study performed in (91) cases of PTC patients failed to find any significant association of miR-146b with L.N involvement<sup>(24)</sup>. 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, no statistical significant difference in PTC and BN in comparison to age, sex of patients and nodular size. These results were in concordance with other study<sup>(9,18-24)</sup>.

Best for the present knowledge, this study could be the first study of its type to be conducted in Iraq, evaluating tissue miR-146b gene expression by RT-qPCR, in the same series fresh tissue of thyroid nodule /apparently NAT samples in a sample of Iraqi patients. There was no baseline study regarding miR-146b gene expression stratification in apparently NATs in Iraqi individuals. Although, similar studies were conducted abroad to stratify miR-146b in other countries such as<sup>(9,18-24)</sup>.

**Conclusion:-**

The extraction of RNA and identification of miR-146b from the fresh tissue of individuals diagnosed with thyroid nodule is feasible and RT-PCR is a novel and practical means for investigation of tissue miR samples. MiR-146b could be a useful biomarker for detection of PTC, detect of higher stage and positive L.N involvement. However, studies with larger number of patients are needed to validate the present study and to investigate whether other miRs are also capable of indicating PTC detection and progression and more importantly whether miR-146b is the best choice among potential PTC biomarkers.

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