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ORIGINAL ARTICLE

# Influence of MiR-126-3p and MiR-146a-5p Dysregulation on The Risk and Clinicopathological Features of Papillary Thyroid Cancer in Patients with Thyroid Nodules

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## ABSTRACT

**Background:** Papillary thyroid cancer (PTC) is the most frequent endocrine cancer. Aberrant expression of miRNAs can increase the risk of thyroid cancer. We aimed to investigate the miR-126-3p and miR-146a-5p plasma levels in patients with thyroid nodules and to identify their associations with clinicopathological characteristics, and progression of PTC.

**Methods:** We enrolled forty healthy control and forty patients with thyroid nodules; benign thyroid nodules (BTN), (n=30), and PTC(n=10). We analyzed miR-126-3p and miR-146a-5p levels by using real-time PCR.

**Results:** miR-126-3p expression level was downregulated in PTC patients (0.40±0.11) compared with BTN (0.58±0.12) and healthy subjects (0.87±0.31), P<0.001*. Regarding miR-146a-5p, its level was upregulated in PTC patients (4.59±1.61) compared with BTN (3.77±0.96) and healthy subjects (0.99±0.41), Additionally, miR-126-3p and miR-146b-5p levels were significantly associated with tumor progression, lymph node metastasis, TNM staging, and thyroid function tests. The diagnostic power of miR-126-3p in the prediction of PTC, the AUC was 0.860 with a sensitivity of 60 % and specificity of 87.3% at cutoff values =0.49. miR-146a-5p level in the prediction of PTC, the AUC was 0.742 with a sensitivity of 80 % and specificity of 74.3% at cutoff values =3.4.

**Conclusions**: miR-146a-5p and miR-126-3p levels were dysregulated in patients with PTC compared to patients with BTN and the control group. The dysregulated miRNAs were associated with clinicopathological features of PTC.

**Keywords**: miR-146b-5p; MiR-126-3p; papillary thyroid cancer; benign thyroid nodules.

## INTRODUCTION

The prevalence of thyroid nodules (TN) is growing worldwide, and the risk attributed to TN has substantially increased. Unfortunately, it is estimated that approximately 5–15% of TN bears a malignancy tendency [1]. Papillary thyroid cancer (PTC) is the most frequent endocrine cancer [2]. There is a body of knowledge related to the histopathological classification of thyroid cancer. Emerging evidence describes PTC as it is a highly prevalent and differentiated thyroid cancer, approximately estimating 80–85% of thyroid cancer [3]. For PTC, metastasis, recurrence, and prognosis were suggested to be affected by different factors, such as genetic and epigenetic dysregulation [4].

It is well established that the burden of cancer not only gravely affects physical health but also the

mental and psychological health of patients. Screening for thyroid cancer can be performed for early detection which could make treatment more effective [5]. Regrettably, conservative management of PTC is currently useless in patients with metastatic PTC [6].

It is reported that genetic and epigenetic dysregulations display various functions in cancer initiation and progression. Nonetheless, it is poorly understood till now. Meanwhile, further examination of the effects of those dysregulations on the risk and clinicopathological features of PCT is mandatory [7]. MicroRNAs (miRNAs) have an essential role in the pathogenesis of cancer [8,9]. In addition, an intriguing discovery detected that the dysregulated miRNAs are linked to cancer progression, and metastasis [10]. Among miRNAs, the dysregulated miR-126 and miRNA-146b-5p are associated with several types of cancers [11] such as hepatocellular carcinoma [12], and thyroid cancer [13]. To establish highly accurate non-invasive precision medicine for patients with PTC we investigated plasma levels of miR-126-3p and miR-146a-5p in patients with TN and PTH. To the best of our knowledge, this study is the first Egyptian study to explore the miR-126-3p and miR-146a-5p values in TN to recognize their associations with the prediction of PTC about its clinicopathological features.

## METHODS

This case-control study included forty healthy controls and forty patients with TN. Among the forty patients with TN, we had 30 cases of benign thyroid nodules (BTN) and 10 cases with PTC and the diagnosis of TN, PTC as well as study design are described in **Figure 1** (the flowchart of the study). Authored detailed agreement including thyroid biopsy and histopathological examination was gained from all members according to recommendation of the research ethical committee of the Faculty of Medicine, Zagazig University and the research protocol was approved (Ethics number. 11235), The research has been performed by the Code of Ethics of the World Medical Association (Declaration of Helsinki) for students involving humans.

Routine diagnostic analyses were carried out according to Zagazig University Hospital. Real-time quantitative PCR for miR-126-3p and miR-146a-5p was applied according to manufacture structures. The primer sequence of miR-126-3p: forward: 5'-TTGGCGGTCGTACCGTGAGTAAT-3′, reverse: 5′-ATCCAGTGCAGGGTCCGAGG-3′. The primer sequence of miR-146a-5p: forward: 5'-UGAGAACUGAAUUCCAUGGGUU3-3', reverse: 5'- AACCCAUGGAAUUCAGUUCUCA-3' U6 primers: forward: 55′-ATGACGTCTGCCTTGGAGAAC-3″, reverse: 5′-TCAGTGTGCTACGGAGTTCAG-3′, We extracted total RNA using TRIzol reagent (Invitrogen, USA). The relative amounts of hsa-miR-126-3p and U6 mRNA were expressed as $2^{-\triangle CT}$ ($\triangle CT = CT$ value of the target gene (U6) - CT value of internal control).

***Statistical Analysis***

All statistics were performed using SPSS software (version 26.0; IBM Corp.). The normality of variables was confirmed with the Kolmogorov-Smirnov test. Quantitative data were described by mean and standard deviation. An independent sample t-test and Mann-Whitney-U-tests were performed. For descriptive characterization, frequencies were calculated using crosstabs followed by $\chi 2$-tests. The associations of miR-126-3p and miR-146a-5p mRNA levels with other parameters were assessed with the Spearman and Pearson correlation, and further evaluation of independent factors correlated with miR-126-3p and miR-146a-5p levels in the case group was investigated with a linear regression test. The diagnostic power of miR-126-3p and miR-146a-5p was explored by the ROC test. $p < 0.05$ were regarded as statistically significant.

## RESULTS

We conducted the current research on eighty participants; 40 healthy control and forty patients with TN, age, sex, and smoking status were matched in both groups to avoid their influence on miR-126-3p and miR-146a-5p expression levels. As expected, family history of PTC and radiological as well as laboratory features of thyroid diseases were higher in TN in comparison to controls, **Table 1**.

We enrolled 30 patients with BTN. The ultrasonographic features (TI-RADS) of the included patients with TN and FNAC of the included patients with TN are shown in supplementary **Figures s1** and **s2**, respectively. Among patients with TN (n=40), 10 patients had PTC which was confirmed by histopathological examination of thyroid tissue obtained after total thyroidectomy. In T**able 2**, the clinical, demographic, and laboratory features are shown, that there was a significantly higher prevalence of positive family history of PTC in patients with PTC compared to patients with BTN, P<0.001*. Interestingly, regarding radiological features, we observed that there was a significantly

higher prevalence of solitary thyroid nodule (STN) in PTC compared to patients with BTN, P<0.05*. In addition, TSH, thyroglobulin (TG), and anti-TG levels were significantly higher in PTC compared to patients with BTN, P<0.001*. On the other hand, free T4 was significantly lower in PTC compared to those with BTN, P<0.001*.

**Clinicopathological characteristics of PTC patients**

In the current research, ten patients had histopathological confirmed PTC and their features were described in supplementary **Table s1**. The TNM staging of PTC [14] shows that there were 30%, 20%, 40%, and 10% of cases had I, II, III, and IV stages, respectively. There were 60% with tumor size >1cm, 80% with multifocal tumor, 60% with capsular invasion, 60% with calcification, 40% with hemorrhagic area, 40% with necrosis, 50% with LN metastasis, 60% with vascular permeation, 70% with lymphatic permeation, and 70% of the lesions were bilateral, **Table 3**.

Interestingly, we detected that miR-126-3p expression level was downregulated in PTC patients (0.40±0.11) compared with BTN (0.58±0.12) and healthy subjects (0.87±0.31), supplementary **Figure s3**, P<0.001*. The lower level of miR-126-3p was significantly negatively associated with tumor size, advanced PTC stage, multifocality LN metastasis, lymphatic permeation TSH, thyroglobulin, and anti-TG (<0.001), while was positively correlated with free T4 (p < 0.001), **Table 3**.

Regarding miR-146a-5p, its level was upregulated in PTC patients (4.59±1.61) compared with BTN (3.77±0.96) and healthy subjects (0.99±0.41), supplementary **Figure s4**, P<0.001*. The higher level of miR-126-3p was significantly positively associated with tumor size, advanced PTC stage, multifocality LN metastasis, lymphatic permeation TSH, thyroglobulin, and anti-TG (<0.001), while was negatively correlated with free T4 (p < 0.001), **Table 3**.

Linear regression analyses examined the main independent variables against the relative expression level of miR-126-3p and miR-146a-5p levels in the PTC group noticed that tumor size was the solitary independent factor linked to miR-126-3p, while tumor size and tumor stage were the only independent variables associated with miR-146a-5p levels among other studied parameters, P <0.001*, **Table 4**.

To further assess the current analytical test, we applied ROC analysis to differentiate patients with BTN from healthy subjects. Regarding miR-126-3p, the sensitivity =92.3 %, specificity = 95.1%, (**Figure 2a**). Concerning miR-146a-5p, the sensitivity =94.9 %, specificity = 99%, (**Figure 2b**).

To recognize patients with PTC from others BTN, the AUC of miR-126-3p was 0.860 (95% CI = 0.741–0.979) with sensitivity =60 %, specificity = 87.3%, (**Figure 3a)**, while the AUC of miR-146a-5p was 0.742 (95% CI = 0.565–0.918) with sensitivity =80 %, specificity = 74.3%, (**Figure 3b**), P <0.001*.

**Table 1:** Clinical, demographic and laboratory characteristics of studied groups

| Characteristics | Control (N=40) | Thyroid nodule (N=40) | P value |
|---|---|---|---|
| **Age**<br><45<br>>45 | <br>25(62.5%)<br>15(37.5%) | <br>27(67.5%)<br>13(32.5%) | 0.639 |
| **Sex**<br>Male<br>Female | <br>7(17.5%)<br>33(82.5%) | <br>8(20%)<br>32(80%) | 0.774 |
| **Family history of PTC** | 1(2.5%) | 18(45%) | <0.001* |
| **Smoking status**<br>Never<br>Current | <br>37 (82.5%)<br>3 (7.5%) | <br>37(80%<br>3(10%) | 0.707 |
| **Thyroid function**<br>Hypothyroid<br>Euthyroid<br>Hyperthyroid | <br>0(0%)<br>40(100%)<br>0(0%) | <br>8 (20%)<br>29 (72.5%)<br>3 (7.5%) | <0.001* |
| **Radiologic**<br>STN<br>MNG | <br>-<br>- | <br>7(17.5%)<br>33(82.5%) | <br><0.001*<br><0.001* |

| | | | |
|---|---|---|---|
| Free T4, ng/dL | 1.23±0.42 | 0.9±0.33 | <0.001* |
| TSH, µIU/mL | 3.78±1.35 | 6.5±2.5 | <0.001* |
| Thyroglobulin, ng/mL | 26.46±2.35 | 91.25± 17.6 | <0.001* |
| Anti-TG (IU/mL) | 33.08±89.05 | 156.5±32.5 | <0.001* |

STN: solitary thyroid nodule; MNG: multinodular goiter; PTC: papillary thyroid cancer; Anti-TG: Anti thyroglobulin. P <0.05 is

**Table 2:** Clinical, demographic and laboratory characteristics of studied patients with thyroid nodules.

| Characteristics | BTN (N=30) | PTC (N=10) | P value |
|---|---|---|---|
| **Age**<br><45<br>>45 | <br>23(76.7%)<br>7 (23.3%) | <br>4 (40%)<br>6(60%) | 0.874 |
| **Sex**<br>Male<br>Female | <br>6(20%)<br>24(80%) | <br>2(20%)<br>8(80%) | 0.730k |
| **Family history of PTC** | 2(5.6%) | 34(42.5%) | <0.001* |
| **Smoking**<br>No<br>Yes | <br>28(93.7%)<br>2 (6.7%) | <br>9 (90%)<br>1(10%) | 0.082 |
| **Thyroid function**<br>Hypothyroid<br>Euthyroid<br>Hyperthyroid | <br>6(20%)<br>22(73.3%)<br>2(5.6%) | <br>2(20%)<br>7(70%)<br>1(10%) | 0.941 |
| **Radiologic**<br>STN<br>MNG | <br>3(10%)<br>27(90%) | <br>4(40%)<br>6(60%) | <0.05* |
| Free T4, ng/dL | 1.01±0.41 | 0.65±0.66 | <0.001* |
| TSH, µIU/mL | 5.38±0.93 | 7.5±1.55 | <0.001* |
| Thyroglobulin, ng/mL | 87.76±7.35 | 105.25±7.92 | <0.001* |
| Anti-TG (IU/mL) | 42.08±9.05 | 140.5±26.8 | <0.001* |

BTN: benign thyroid nodule; PTC: papillary thyroid cancer; STN: solitary thyroid nodule; MNG: multinodular goiter; Anti-TG: Anti thyroglobulin

**Table 3:** Correlation of miR-126-3p and miR-146a-5p levels with clinicopathological characteristics of PTC patients

| Parameters | miR-126-3p | | miR-146a-5p | |
|---|---|---|---|---|
| | *r* | P | | |
| Tumor size | -0.611 | <0.001* | 0.546 | <0.001* |
| Stage | -0.519 | <0.001* | 0.583 | <0.001* |
| Multifocality | -0.631 | <0.001* | 0.496 | <0.001* |
| Capsular invasion | -0.188 | 0.119 | 0.188 | 0.119 |
| Calcification | -0.068 | 0.578 | 0.068 | 0.578 |
| Hemorrhagic area | -0.182 | 0.211 | 0.182 | 0.211 |
| Necrosis | -0.130 | 0.236 | 0.130 | 0.236 |
| LN metastasis | -0.264 | <0.05* | 0.368 | <0.05* |
| Vascular permeation | -0.221 | 0.050 | 0.221 | 0.050 |
| Lymphatic permeation | 0.457 | <0.001* | 0.457 | <0.001* |
| Bilaterality | -0.154 | 0.203 | 0.010 | 0.951 |

| | | | | |
|---|---|---|---|---|
| Free T4 | 0.543 | <0.001* | -0.611 | <0.001* |
| TSH | -0.657 | <0.001* | 0.545 | <0.001* |
| Thyroglobulin | -0.574 | <0.001* | 0.474 | 0.203 |
| Anti-TG | -0.551 | <0.001* | 0.509 | <0.001* |

P <0.05 is significant

**Table 4**: Linear regression analyses examine the main independent variables against the relative expression level of miR-126-3p and miR-146a-5p levels in PTC group.

| Model | | Unstandardized Coefficients | | Standardized Coefficients | t | P value | 95% C.I. | |
|---|---|---|---|---|---|---|---|---|
| | | B | S.E. | Beta | | | Lower Bound | Upper Bound |
| **miR-126-3p** | Constant | -1.636 | 1.001 | | -1.633 | 0.178 | -4.416 | 1.145 |
| | Tumor size | -0.220 | 0.072 | -1.007 | -3.035 | <0.05* | -0.421 | -0.019 |
| | Tumor stage | 0.004 | 0.006 | 0.221 | 0.673 | 0.538 | -0.014 | 0.022 |
| | Multifocality | 0.035 | 0.016 | 0.676 | 2.182 | 0.094 | -0.009 | 0.079 |
| | LN metastasis | -0.102 | 0.064 | -0.467 | -1.594 | 0.186 | -0.280 | 0.076 |
| **miR-** | TSH | -0.080 | 0.161 | -0.139 | -0.498 | 0.645 | -0.527 | 0.367 |
| **146a-5p** | Constant | -15.693 | 15.361 | | -1.022 | 0.365 | -58.34 | 26.956 |
| | Tumor size | -3.148 | 1.111 | -0.941 | -2.834 | <0.05* | -6.233 | -0.063 |
| | Tumor stage | 0.031 | 0.005 | 0.725 | 5.663 | <0.001* | 0.020 | 0.042 |
| | Multifocality | 0.300 | 0.243 | 0.381 | 1.232 | 0.285 | -0.376 | 0.975 |
| | LN metastasis | -0.802 | 0.981 | -0.240 | -0.817 | 0.460 | -3.526 | 1.922 |
| | TSH | -01.714 | 2.467 | -0.194 | -0.695 | 0.525 | -8.564 | 5.136 |

P <0.05 is significant

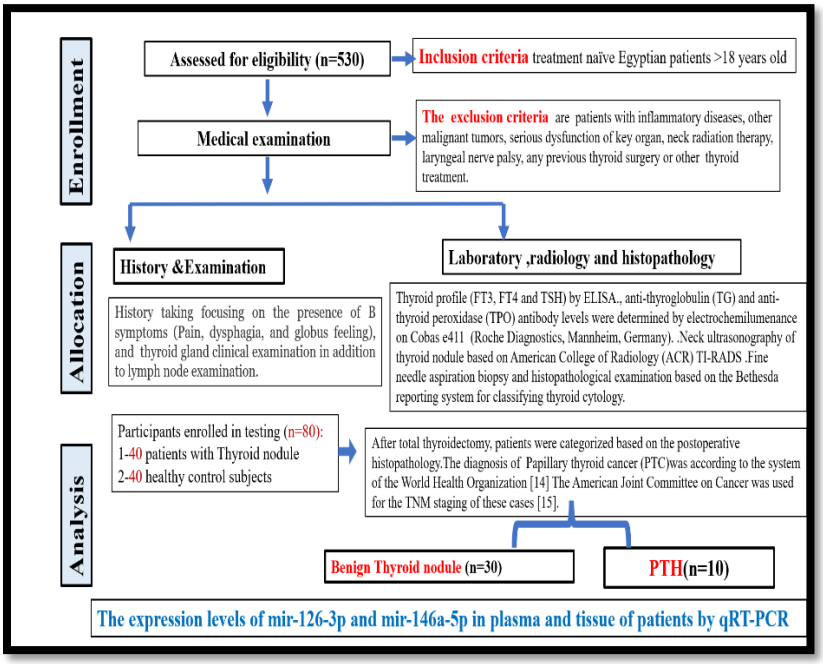


**Figure 1:** Flowchart of the present study

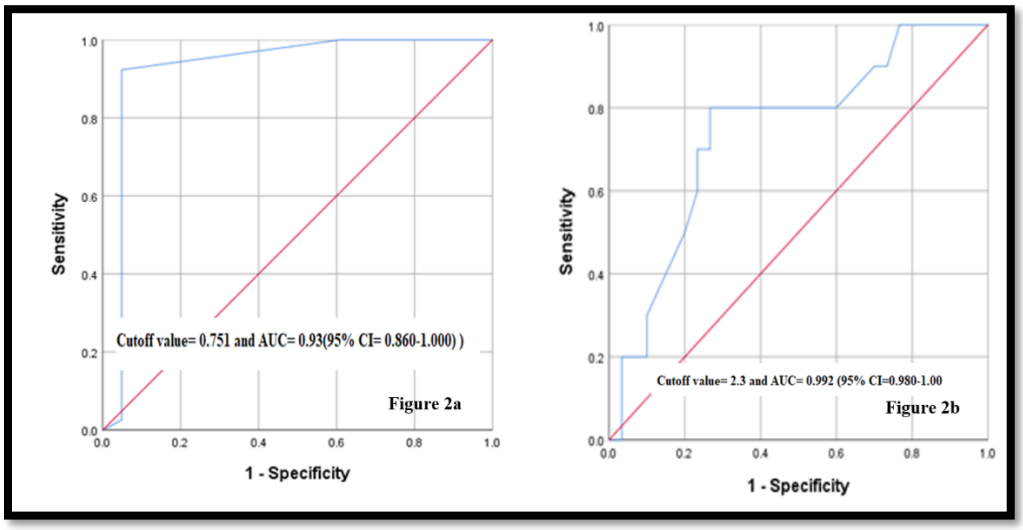


**Figure 2a:** ROC curve of miR-126-3p levels for prediction of patients with BTN among studied groups.
**Figure 2b:** ROC curve of miR-146a-5p levels for prediction of patients with BTN among studied groups.

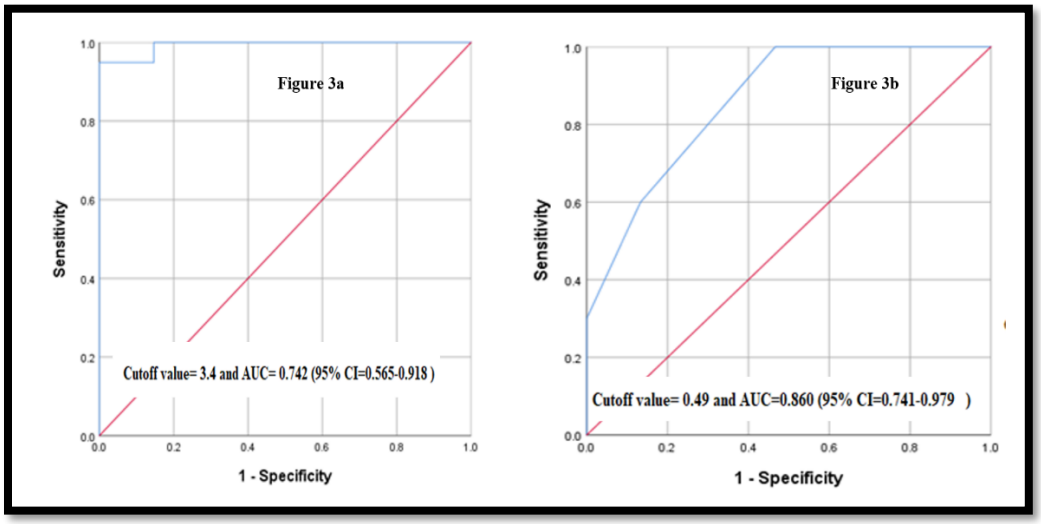


**Figure 3a:** ROC curve of miR-126-3p levels for prediction of patients with BTN among studied groups.
**Figure 3b:** ROC curve of miR-146a-5p levels for prediction of patients with BTN among studied groups.

## DISCUSSION

Recent accumulating studies detected the essential role of miRNAs in the control of many diseases [15]. Additionally, interesting studies confirmed that miRNA has been demonstrated to be of significance in cancer prediction [16]. Several reports have revealed that genetic dysregulation recognition in the progression of thyroid cancer is important for treatment of different thyroid cancer classes [17]. Remarkably, miRNA dysregulations appear in various body fluids and tissues [18].

MiRNAs are not only noteworthy markers for the diagnosis but also for the treatment of different diseases [15]. To the best of our knowledge, this is the first Egyptian study to investigate the miR-126-3p and miR-146a-5p plasma levels in patients with thyroid nodules (TN) to evaluate their associations with the prediction of PTC in relation to its clinicopathological features.

We proved that the miR-126-3p expression level decreased in PTC compared with BTN and control. Additionally, we further confirmed that the statistically significant reduction level of miR-126-3p was significantly negatively associated with tumor size, advanced PTC stage, multifocality LN metastasis, lymphatic permeation TSH, thyroglobulin (TG), and anti-TG. Whereas it was positively correlated with free T4. The only variable independently associated with miR-126-3p in the PTC group was tumor size. To further explore the diagnostic power of miR-126-3p in discriminating patients with BTN from healthy subjects, we applied

ROC analysis and found that the AUC was 0.93 with a sensitivity of 92.3 % and specificity of 95.1% at cutoff values =0.75. However, in the case of the prediction of PTC, the AUC was 0.860 with a sensitivity of 60 % and specificity of 87.3% at cutoff values =0.49. Overall, our findings suggest that miR-126-3p expression could be used as a non-invasive predictive marker of PTC and inversely correlated with the severity and progression of the tumor.

Previous studies conducted on different malignancies provided evidence that miR-126 levels can differentiate between cancerous and para-cancerous tissues such as gastric cancer and normal tissue by regulating the oncogene target gene [19]. Also, Akbari Moqadam et al detected lower expression of miR-126 in the acute lymphoblastic leukemia group compared to the healthy control group [20]. Concerning thyroid cancer, similar to our results Kitano et al found lower miR-126-3p levels compared to non-cancerous tissues [21]. Comparable results were confirmed in prior studies, they observed lower levels of miR-126-3p in breast, gastric, and colonic cancer [22-24]. Additionally, Liu et al detected that miR-126 level was downregulated in lung cancer cells via targeting VEGF [25].

Inconsistent with our results, Xiong et al detected that the miR-126-3p was down-expressed in thyroid cancer with capsular as well as vascular invasion, and they confirmed that the downregulated miR-126-3p regulates tumor progression and spread through targeting VEGF [26].

In a study conducted by Du et al, they examined miR-126-3p in hepatocellular carcinoma tissues and cells and they revealed that miR-126-3p level declined in hepatocellular carcinoma and inversely associated with hepatocellular carcinoma progression through different pathways [27].

Regarding miR-146b-5p expression, the current research results detected overexpression of miR-146b-5p in PTC patients compared to BTN and control. Moreover, the higher level of miR-126-3p was significantly positively associated with tumor size, advanced PTC stage, multifocality, LN metastasis, lymphatic permeation, TSH, TG, and anti-TG, although its value was negatively correlated with free T4. Based on these results, we further confirmed that the tumor size and stage were the only independent variables associated with miR-146a-5p levels among other studied parameters in the PTC group. Interestingly, the diagnostic power of the over-expressed circulatory miR-146a-5p level in discriminating BTN from healthy control had an AUC of 0.992 with a sensitivity of 94.9 % and specificity of 99 % at cutoff values =2.3. However, in the case of PTC prediction, the AUC was 0.742 with a sensitivity of 80 % and specificity of 74.3% at cutoff values =3.4.

Similarly, Lee et al detected overexpressed miR-146b-5p in PTC [28]. Additionally, the tissue expression of miR-146b-5p in PTC was higher than para cancerous normal tissues. Similar findings were observed by Li et al, they found that miR-146b-5p expression level was associated with tumor size, lymph node metastasis, and TNM stage. They also observed overexpression of miRNA-146b-5p in both Hashimoto thyroiditis and thyroid cancer and confirmed the link between this genetic marker with PTC progression features [29].

The result findings of the research conducted by Taganov and his colleague's detected overexpression of miR-146b-5p in mixed thyroid cancer and Hashimoto thyroiditis than in thyroid cancer. They explained their findings by the hypothesis of altered miR-146b-5p expression in various disorders such as tumor and autoimmune diseases [30]. A similarly interesting study detected higher values of miR-146-5p in PTC compared to healthy tissues [31]. As confirmed by Rogucki et al the overexpressed miR-146b increased the risk of PTC by targeting different pathways, consequently it increased the risk of vascular invasion and distant metastasis in patients with PTC [32].

**Conclusions**

miR-146a-5p and miR-126-3p levels were dysregulated in patients with PTC compared to patients with BTN and the control group. The dysregulated miRNAs were associated with clinicopathological features of PTC and might be utilized as non-invasive predictive and prognostic markers of PTC.

**Study Strengths and Limitations**

This study has several unique strengths. To date, according to our information, no study has examined the role of miR-126-3p and miR-146a-5p in diagnosing thyroid cancer in association with its clinicopathological features and these miRNAs could be used as predictive and prognostic biomarkers of PTC as well as in the treatment of PTC.

**Limitations**

Our study also has a few potential limitations. We investigated the miR-126-3p and miR-146a-5p in plasma only not in tissue and plasma, in addition to the small sample size and the participants were Egyptians only. Consequently, we recommend

further research on larger sample size of participants from different ethnicities and investigate the genetic markers in both tissue and plasma to further enforce the current research results.

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**Conflict of interest**

The authors declared that they have no conflicts of interest with respect to the authorship and/ or publication of this article.

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**Table s1:** Clinicopathological characteristics of PTC patients.

| Characteristics | N | (%) |
|---|---|---|
| **Tumor size** | | |
| <1cm | 4 | (40%) |
| >1cm | 6 | (60%) |
| **Stage** | | |
| I | 3 | (30%) |
| II | 2 | (20%) |
| III | 4 | (40 %) |
| IV | 1 | (10 %) |
| **Multifocality** | 2 | |
| Unifocal | 8 | (20%) |
| Multifocal | | (80%) |
| **Capsular invasion** | | |
| No | 4 | (40%) |
| Yes | 6 | (60%) |
| **Calcification** | | |
| No | 4 | (40%) |
| Yes | 6 | (60%) |
| **Hemorrhagic area** | | |
| No | 6 | (60%) |
| Yes | 4 | (40%) |
| **Necrosis** | | |
| No | 6 | (60%) |
| Yes | 4 | (40%) |
| **LN metastasis** | | |
| N0 | 5 | (50%) |
| N1 | 5 | (50%) |
| **Vascular permeation** | | |
| No | 4 | (40%) |
| Yes | 6 | (60%) |
| **Lymphatic permeation** | | |
| No | 3 | (30%) |
| Yes | 7 | (70%) |
| **Bilaterality** | | |
| Unilateral | 3 | (30%) |
| Bilateral | 7 | (70%) |

P <0.05 is significant

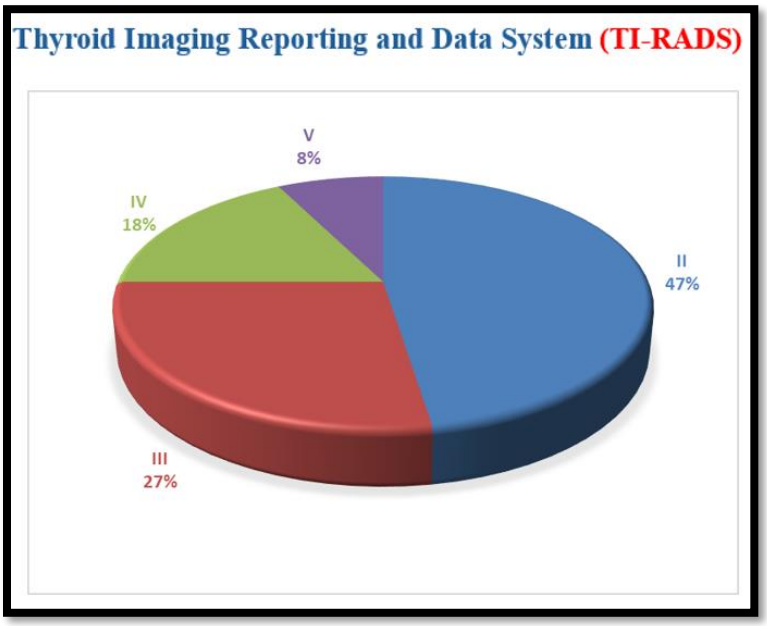


**Figure s1:** Thyroid Imaging Reporting and Data System (TI-RADS) of the included patients with thyroid nodules

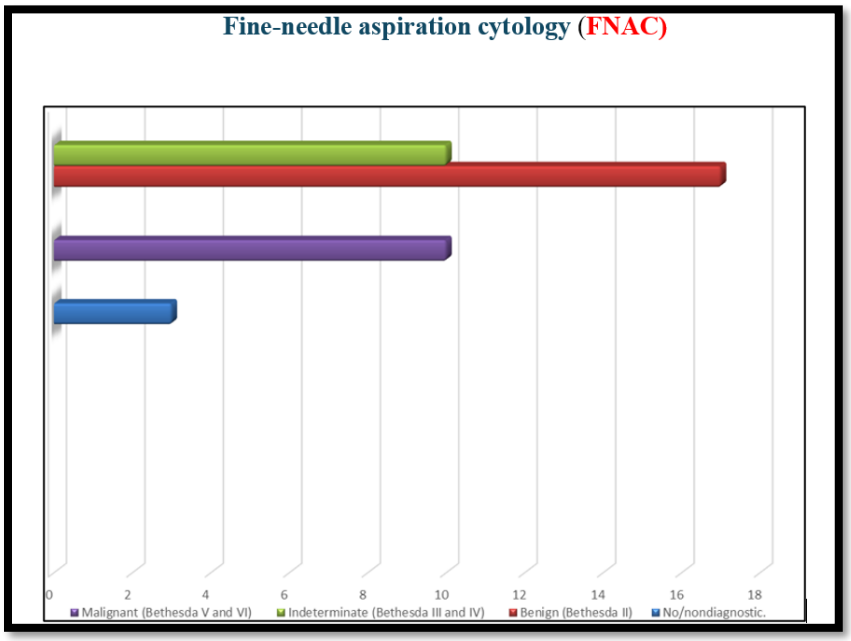


**Figure s2:** Fine-needle aspiration cytology (FNAC) of the included patients with thyroid nodules.

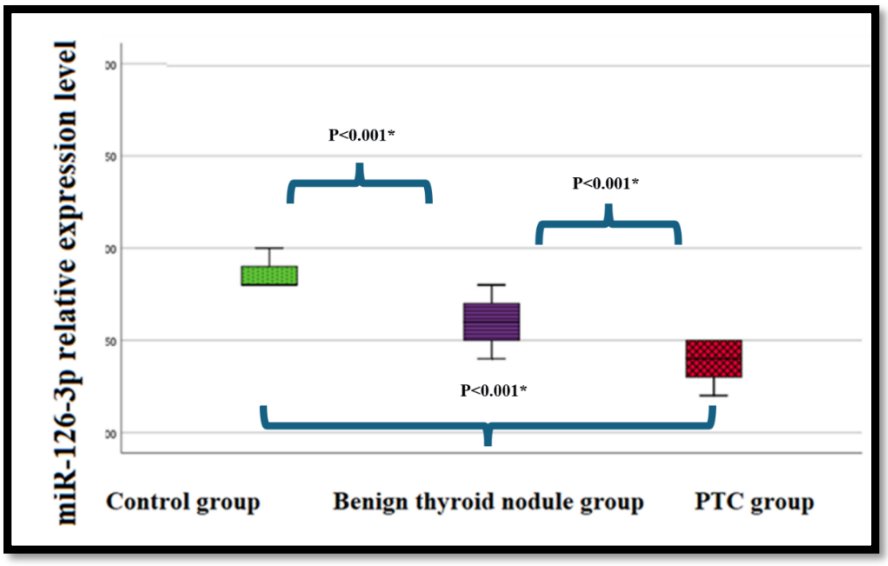


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**Figure s3:** Comparison between the relative expression of miR-126-3p levels between studied groups.

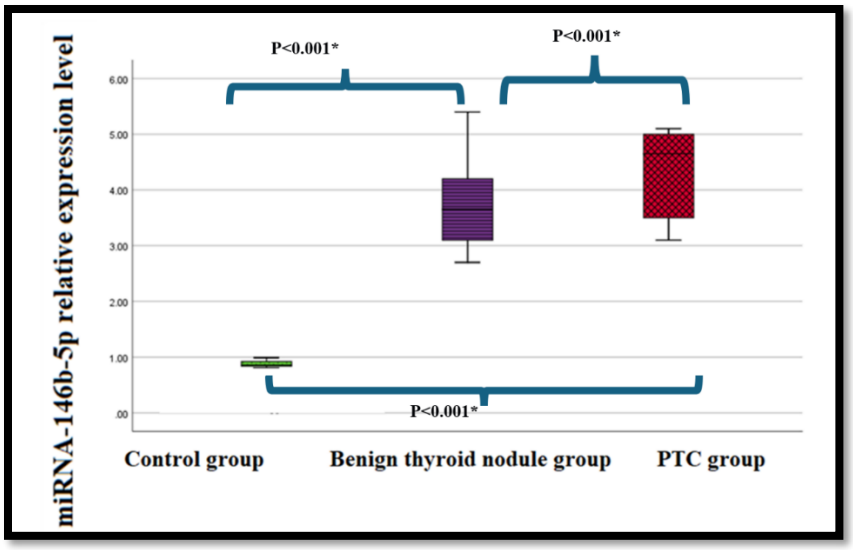


**Figure s4:** Comparison between the relative expression of miR-146a-5p levels between studied groups.