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MiR-20b Tissue Expression Level Displays the Diagnostic Value in Papillary Thyroid Carcinoma

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Abstract

Background: Detection of cancer in patients with thyroid nodules requires sensitive and specific diagnostic modalities that are accurate and inexpensive. This study aimed to identify a potential microRNA(miRNA) panel to detect papillary thyroid carcinoma (PTC).

Methods: Following a comprehensive literature review as well as miRNA target predictor databases, Real-time PCR was used to quantify the expression of candidate miRNAs in 59 tissue specimens from 30 patients with PTC and 29 patients with benign nodules. A receiver operating characteristic (ROC) curve analysis was used to assess the accuracy of miRNA expression levels compared to the pathology report as the gold standard. Based on prediction results, four miRNAs, including miR-9, miR-20b, miR-221, and miR-222, were selected to evaluate their expression level in Iranian thyroid samples.

Results: A significant difference between the tissue expression level of miR-20b, miR-9, miR-222, and miR-221 was detected in the PTC group compared with non-PTC ($P < 0.05$). The area under the curves for the included miRs were 1, 0.98, 0.99, 0.98, and 1, respectively.

Conclusion: Our results confirmed deregulations of miR-20b as well as miR-222, miR-221, and miR-9 in PTC and, therefore, could be used as a helpful miRNA panel to differentiate PTC from benign nodules, which results in the more efficient clinical management of PTC patients.

Keywords: Papillary thyroid cancer, miRNA, Pathology, Cytology

Conflicts of Interest: None declared
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Introduction

Thyroid nodules are relatively common and can be found in approximately 50% of adults through high-resolution ultrasound (1). Fortunately, most thyroid nodules are benign, and around 7% are cancerous (2). PTC has been known as the most frequent type of thyroid cancer (3).

Although the thyroid nodule's fine-needle aspiration (FNA) is a standard diagnostic procedure, equivocal cytology reports are challenging (4). Therefore, introducing molecular biomarkers that could predict malignancy in indeterminate samples is critical.

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↑What is "already known" in this topic:
Although the thyroid nodule's fine-needle aspiration (FNA) is a standard diagnostic procedure, equivocal cytology reports are challenging, and other methods may predict the nature of subspecies nodules. Introducing molecular biomarkers may predict malignancy in indeterminate samples of a thyroid nodule.

→What this article adds:
This study aimed to identify a potential microRNA (miRNA) panel to differentiate papillary thyroid carcinoma (PTC) from benign nodules. We have evaluated the miRNAs involved in different subtypes of thyroid cancer from the bioinformatics analysis so that we can distinguish the subtype-specific miRNA signatures in FNA samples.

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MicroRNAs (miRNAs) are a group of small and short-lived non-coding RNAs that regulate tissue-specific genes in eukaryotes (5, 6). As antisense regulators of mRNAs, miRNA binds to the 3'-untranslated region of target mRNAs, suppressing mRNA translation or degradation (7, 8). The contribution of several miRNAs in modulating cell proliferation, differentiation, and death processes and their tumor suppressor or oncogenic roles has been well indicated (9, 10). Previous reports have proposed miRNAs as possible biomarkers for cancer diagnosis, invasion, or progression (11, 12). Similarly, expressional deregulation of miRNAs' has been demonstrated in benign nodules compared with normal thyroid tissue (13-15).

Variability of tissue expression of miRNAs in PTC, follicular thyroid carcinoma, and anaplastic thyroid cancers has been shown. Previous studies have consistently demonstrated that some miRNAs, such as miR-220, miR-10b, miR-31, miR-222, miR-21, miR-146b, and miR-221, are differentially expressed in thyroid cancers without considering specific subtypes (16-19). However, the association of specific miRNAs' expression with certain types of thyroid cancer has remained to be discovered. In this study, we aimed to introduce a panel of miRNAs to differentiate PTC from benign thyroid nodules.

Methods

Study Setting

This study is a case-control study on 59 patients with thyroid lesions in two main groups: 1) Patients with PTC and 2) Patients with benign lesions. This study was conducted at Firoozgar General Hospital (A medical center affiliated with Iran University of Medical Sciences) from September 2017 until April 2021. The protocol of this study was approved by the ethics committee of the Iran University of Medical Sciences. The ethical approval of this study can be checked by the following code: "I.R.IUMS.REC 1396.31430".

Identification of miRNA and their target genes

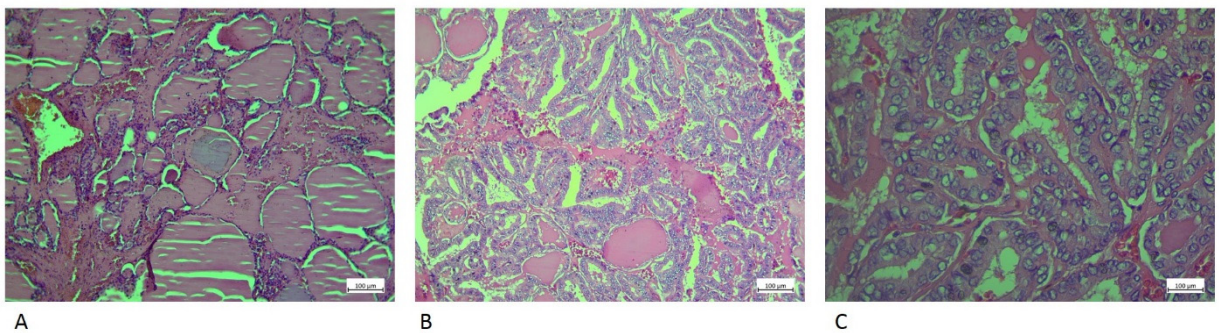
To select the candidate miRNAs, we performed a comprehensive literature review. Based on a comprehensive literature review as well as Target Scan 6.2, miRWalk, and RNAhybrid and miRDB.V6 databases, we selected candidate miRNAs with the most shared target genes involved in PTC development. In parallel, differential miRNA expression analysis on 282 stage 1 and 57 normal thyroid tissue were conducted on "htseq-count" files retrieved from TCGA (The Cancer Genome Atlas, HTTP: //cancergenome.nih. gov/). Each count file included 1046 miRNAs generated by TCGA through mapping of miRNAs sequencing data onto the human reference genome and subsequently counting the number of mapped reads(sequences) on each miRNA sequence on the reference genome. miRNAs with raw counts less than ten were removed from all samples before normalization by the DeSeq2 algorithm in the R 3.6.0 programming language. The list of expressed miRNAs was then filtered for adjusted p-value ≤ 0.05 and $|\log2 \text{ F.C.}| \geq 1$. Corresponding cellular pathways of the target genes were subsequently determined using EnrichR (20). Based on prediction results, we selected a set of four miRNAs, including miR-9, miR-20b, miR-221, and miR-222, to evaluate their expression level in Iranian thyroid samples. The results of miRNA expression level measurement of PTC obtained from TCGA confirmed our selected miRNA as well.

Experimental study

An experimental study was conducted to validate the deregulation of the identified differentially expressed miRNAs.Patients from whom total thyroidectomy was done between 2016 and 2017 were included. Fifty-nine Formalin-Fixed Paraffin-Embedded (FFPE) tissue specimens consisting of thirty PTC reports, eight follicular adenoma reports, eleven Hurtle cell adenoma reports, and ten multinodular goiter reports were examined in this study. Pathology slides were reviewed by a qualified pathologist blinded to the study protocol to reconfirm the previous pathology results and to ensure that no normal tissue was presented in the PTC samples (Figure 1). The demographic characteristics of the participants are shown in Table 1. This study was approved by the ethics committee of the Iran University of Medical Sciences (IR.IUMS.REC 1396.31430), Tehran, Iran. Written informed consent was signed by all participants.

RNA extraction and Real-time PCR

Total RNA was extracted from FFPE tissue samples according to the manufacturer's instructions. The quality of



A B C

Figure 1. Pathology results of thyroid tissue samples: (A)Non-PTCL: showing normal-follicular thyroid tissue including variable-sized thyroid follicle lined by benign-looking flattened follicular cells containing colloid material (H&E, medium power), (B) PTC: showing tumoral tissue including true papillary and follicular structured lined by atypical follicular cells (H&E, medium power), (C): PTC: showing nuclear details such as optically clearing and nuclear groove(H&E, high power)

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Table 1. Demographic characteristics of the study participants stratified by pathology reports

Pathological Vs. cytological reports			
		PTC (N: 30)	Benign (N: 29)
Gender, female, N (%)		22 (73.3)	24 (82.8)
Age (mean ± SD)†		42.2 ± 16.4	44.7 ± 10.5
Bethesda stage of cytology specimens	Nondiagnostic (I)	2	0
	Benign (II)	4	20
	Follicular neoplasm (IV)	1	3
	PTC and Suspicious for PTC (V &VI)	17	1
	NA	6	5

*Abbreviations: PTC: Papillary thyroid carcinoma, NA: not available
† *P*-value < 0.05

RNA samples was evaluated by Thermo Scientific™ NanoDrop™ One. The expression pattern of the four candidate miRNAs (miR-20b, miR-9, miR-221, and miR-222) was analyzed using the TaqMan MicroRNA RT kit protocol. The results were then normalized to the endogenous reference. Real-time PCR was performed in triplicate on the Applied Biosystems 7700 Sequence Detection System.

Statistical analysis

All the statistical analysis was performed using SPSS version 20 and GraphPad prism 8. We used the Kolmogorov-Smirnov test to check the normality of the numerical variables. For demonstrating the numerical variables, we used mean and standard deviation. In the case of non-parametric instances, median and range were considered. To compare the expression of miRs between two study groups, we used a t-test, and if the data failed to meet the parametric criteria, the Mann-Whitney test was implemented. The frequency and regarded percentage were employed to show the nominal and categorical variables. The chi-square test was considered as the method of choice for comparing non-numerical variables. Finally, to show the diagnostic function of the miR expression in patients with thyroid lesions, the receiver operator characteristic (ROC) curve was used. The $P < 0.05$ is considered statistically significant in this study.

Results

Differential Expression Analysis

The Appendix Table 1 represents deregulated miRNAs in patients with PTC. We found enormous variation among different studies on miRNA expression in thyroid cancer (20). Among previously reported deregulated miRNAs, the up-regulation of miR-221 and miR-222 has been commonly demonstrated (16, 17). Furthermore, some reports claim that miR-20b, miR-9-1, and miR-9-2 were down-regulated in early PTC (21). Figures 2A and 2B show the shared target genes and pathways between miR-9 and miR-20b, as well as miR-221 and miR-222 (2C), respectively. MiR-9 and miR-20b target several signaling pathways with a large number of shared genes, which mostly belong to MAPK and PI3K pathways. However, shared targets of miR-221 and 222 just belonged to the cellular senescence pathway (Figure 2).

Experimental validation

The demographic characteristics of the patients are shown in Table 1. A total number of 59 Formalin-Fixed Paraffin-Embedded (FFPE) tissue specimens consisting of 30 patients with stage I PTC, eight patients with follicular adenoma, 11 patients with Hurtle cell adenoma, and 10 patients with multinodular goiter were examined in this study. In eighteen patients with confirmed PTC, surgery had been done according to the Bethesda reporting of cytology specimens: 1 follicular neoplasm and 17 Bethesda categories V and VI, while in one patient, cosmetic problems and the effects of compression resulted in total thyroidectomy. In four patients with PTC pathology, cytology specimen reports were benign. Only one patient with benign pathology had a category V report in the cytology specimen.

We used qRT-PCR to measure the expression level of miRNA in tissue specimens. There was a significant difference between all miRNA levels in PTC and non-PTC groups. As shown in Figure 3, miR-221 and miR-222 were significantly overexpressed, while miR-9 and miR-20b were under-expressed in the PTC group.

According to the Bethesda reporting of cytology specimens, there were 26 patients with Bethesda categories I and II. Other 22 patients were reported as Bethesda categories IV, V, and VI (Table 2). There was a significant difference between miRNA expression levels in pathology samples and cytology reports. The association between miRNA tissue expression levels in tissue specimens and the category of Bethesda reporting of cytology specimens are shown in (Table 2). Interestingly, considering the Bethesda reporting of cytology specimens, we observed low expression levels of miR-9 and miR-20b in patients with Bethesda categories I and II, while miR-221 and miR-222 expression were notable in Bethesda categories IV to VI (Table 2).

Moreover, our ROC analysis showed that there was no overlap in the distribution of $\log_2$ miR-9 and $\log_2$ miR-222 expression between the PTC and benign groups. The expression of miR-20b and miR-221 also significantly differed between the two groups. The corresponding approximate area under the curves was 0.98 and 0.99, respectively (Figure 4).

Discussion

Although most thyroid nodules are benign, 7% are diagnosed with thyroid cancer (T.C.) (15). It is essential to distinguish between malignant and benign thyroid nodules. Overcoming the challenges of accurately assessing the risk for individual patients is critical to establishing appropriate therapeutic strategies and optimizing clinical outcomes.

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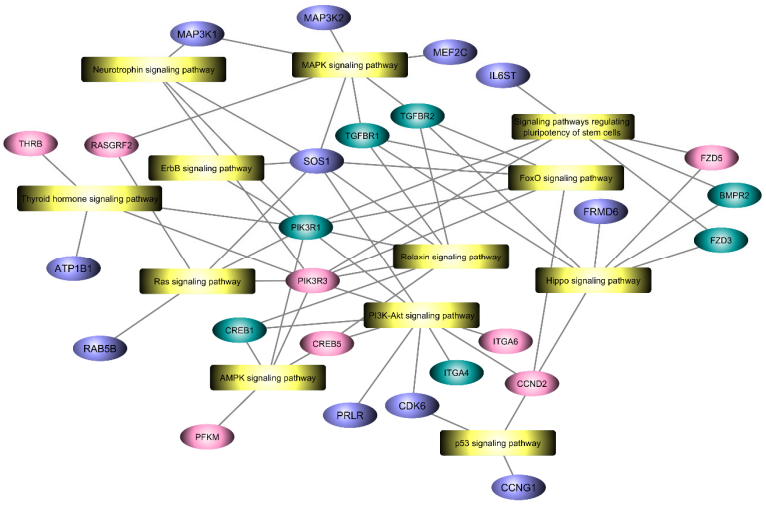

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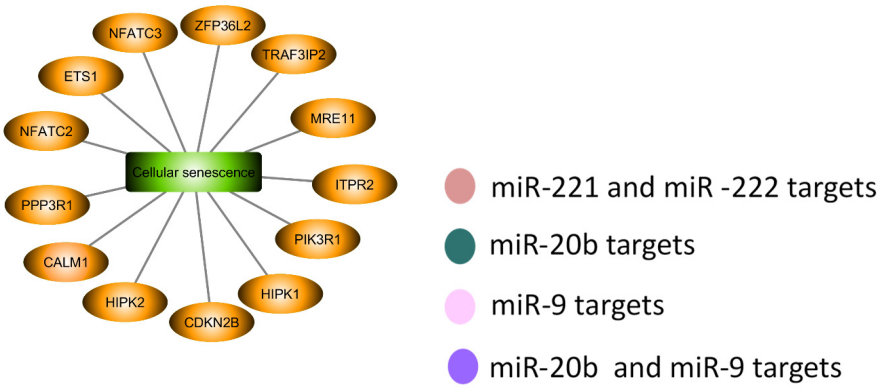

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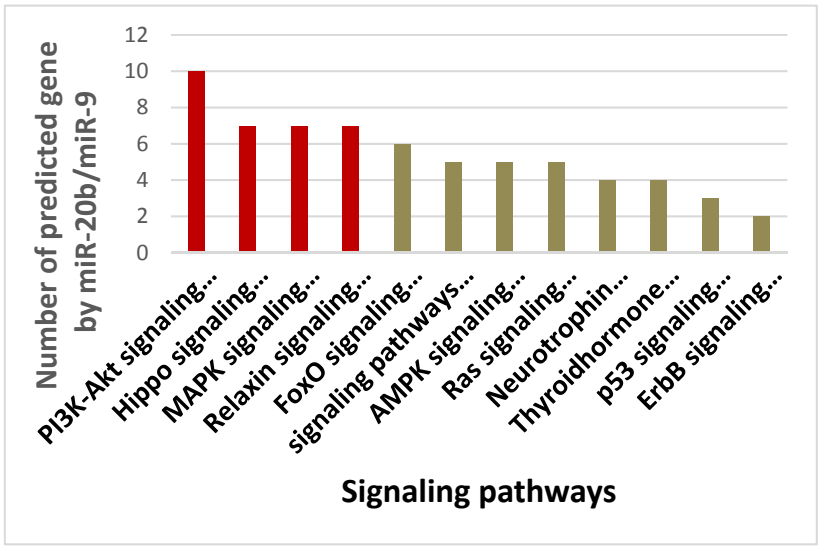

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Figure 2. Predicted genes and their related signaling pathway; (A-B) miR-9 and miR-20b, (C) miR-221 and miR-222

Currently, tissue diagnosis is the gold standard method. However, other methods could predict the nature of the nodule in an individual patient (4). Since the diagnostic accuracy of a combination of miRNA alteration is of greater value compared with a single miRNA (22), we decided to choose a panel of miRNAs. In this study, we introduce a novel panel of miRs for discriminating PTC in patients with thyroid lesions. According to our results, the panel of these four miRs, including miR-20b, miR-9, miR-221, and miR-222, can be a prominent diagnostic tool for detecting PTC in patients with thyroid lesions.

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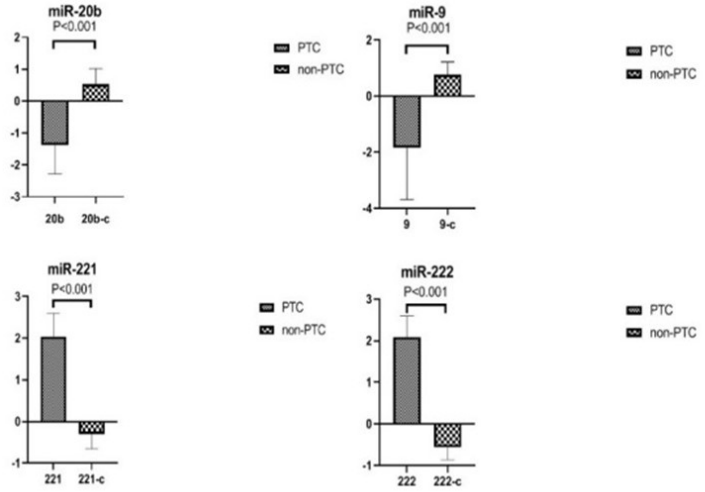

Figure 3. Real-time PCR analysis of miRNAs in pathology specimens. Box plots illustrate miRNAs expression (Log-FC) in benign (non-PTC) and PTC groups. It is indicated that our miR panel has a significantly expressed level in the PTC sample compared to non-PTCs. While miR-20b and miR-9 are overexpressed in non-PTC, PTC samples show mir-221 and 222 increased expression.

Table 2. Association between miRNAs tissue expression levels (Log_2) in tissue samples and the Bethesda category of the corresponding cytology specimen (median (IQR))

miRNA	Bethesda (I, II)	Bethesda (IV, V, VI)	*P*. Value
miR-9	0.48 (-0.03,1.00)	-0.90 (-1.45, -0.26)	<0.001
miR-20b	0.58 (-0.25,0.84)	-1.07 (-1.88, -0.51)	<0.001
miR-221	-0.15 (-0.48,0.83)	2.01 (0.89,2.14)	<0.001
miR-222	-0.47 (-0.73,0.26)	2.13 (1.48,2.47)	<0.001

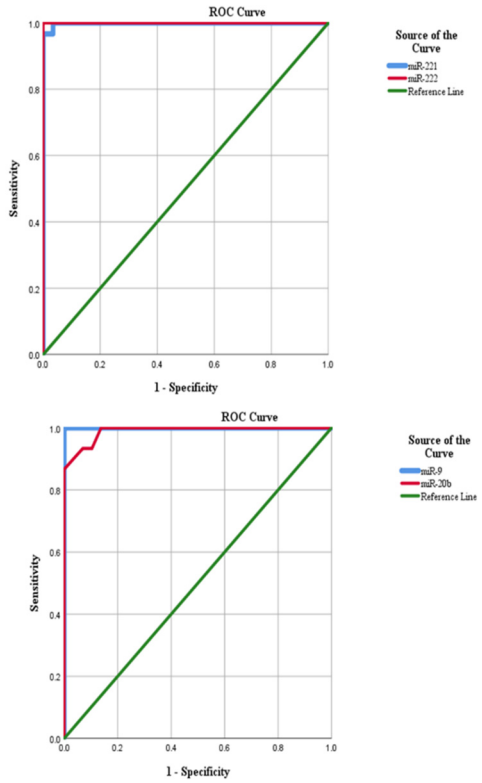

Figure 4. ROC curve analysis of included miRs among the patients with PTC or benign lesions.

Distinct molecular changes are associated with the tumorigenic process. The potential application of miRNA in thyroid cancer diagnosis has already been demonstrated (15, 23-26). In contrast to mRNAs, mature miRNAs are stable and remain largely intact in routinely collected FFPE tissue specimens (23). Previous research revealed that dysregulated miRNAs could have a diagnostic role in PTC cell lines and even in FNA samples (27).

According to our bioinformatic results, shared genes influenced by both miR-20b and miR- 9 were MAP3K, SOS1, CCNG1, CDK6, ATP1B1, FRMD6, and IL6 ST, which seems that their overexpression could have been involved in cancer signaling. Moreover, in vitro and in vivo studies revealed that miR-20b inhibits the MAPK/ERK signaling pathway through targeting (seven less homolog 1) SOS1 and (extracellular signal-regulated kinase 2) ERK2,c-Myc, and CCND1; it may have a role in PTC pathogenesis (28). It is also reported that SOS1 overexpression rescued the DUXAP8 downregulation-mediated inhibition of cell proliferation and promoted apoptosis in PTC cells through an oncogenic function (29).

As an important cell cycle regulator, CCNG1 gene abnormalities exist in a variety of tumors. However, The upstream mechanism of CCNG1 in PTC is still unclear (30). Interestingly, highly expressed CDK6 and ATP1B1 are detected in thyroid cancer tissues and cells, suggesting that they may play as oncogenic parts in thyroid cancer (31, 32). It has been shown that overexpression of inflammatory cytokines such as IL-6 facilitates tumor growth, and its gene polymorphisms were related to the decreased risk of tumorigenesis in thyroid cancer. Therefore, IL6 ST, as a part of

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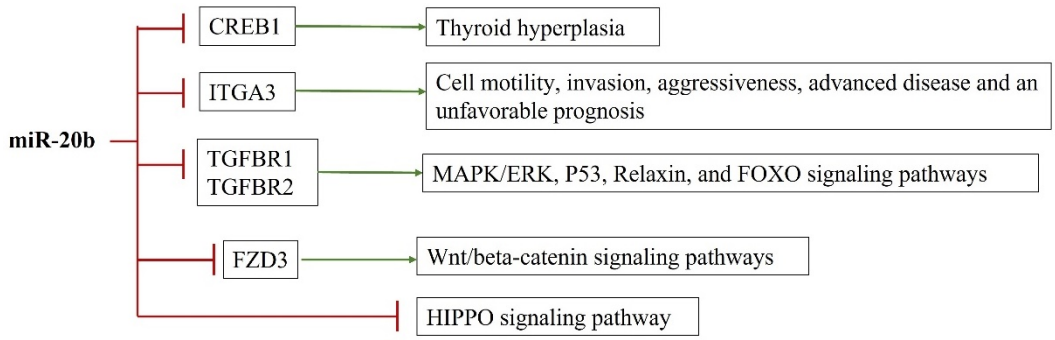

Figure 5. Important oncogenic genes or signaling pathways involved in PTC pathogenesis targeted by miR-20 overexpression

the IL-6 receptor complex, may have an effect on PTC (33).

The role of MEFC2 and FRMD6 in PTC pathogenesis is not completely clear. MEFC2 is highly expressed in some cancers and has synergistic activities with thyroid hormone receptors (34). FRMD6 seems to have a tumor-inhibiting role in thyroid cancer by antagonizing oncogenic Yes-associated protein (YAP1) (35). Based on computational analysis, it has been shown that PI3K-Akt, MAPK, Hippo, FOXO, and Relaxin signaling pathways are highly influenced by miR-20b/miR-9, respectively.

As an anti-cancer pathway, the Hippo pathway restricts cell proliferation and integrates PI3K–Akt signals to control tissue growth (36). In contrast, the Relaxin pathway increases the growth and invasiveness of cancers (37). On the other hand, the FOXO pathway has been known as a signaling pathway associated with the development of different types of cancers. The most important pathway interacting with FOXO is the PI3K/AKT, Ras/MEK/ERK, and AMPK pathways (38). Alterations of all these canonical pathways through miR-20b/miR-9 may have an impact on PTC pathogenesis.

Targetscan prediction result shows that FZD3, CREB1, TGFBR1, TGFBR2, ITGA4, BMPR2, and PIK3R1genes are only influenced by miR-20b; some of them are involved in thyroid cancer pathogenesis (Figure 5). For instance, an enhanced CREB1 activity and CREB-1 regulated genes are associated with thyroid hyperplasia (39) or PTC-specific ITGA3 expression displays the highest correlation with cell motility, invasion, aggressiveness, and advanced disease and is associated with an unfavorable prognosis (40).

EnrichR analysis has shown that Hippo, MAPK, and PI3K-Akt signaling pathways are highly influenced by miR-20b, respectively (Figure 1A). In the HIPPO signaling pathway, four membrane receptors, including TGFBR1, TGFBR2, BMPR2, and FZD3, are simultaneously targeted, indicating the down-regulation of miR-20b may play a significant role in the deregulation of this pathway. Also, FZD3 activates the Wnt/beta-catenin signaling pathway (41). Interestingly, TGFBR1 and TGFBR2 are also involved in MAPK, P53, Relaxin, and FOXO signaling pathways, so they may have an impact on cancer signaling.

Accordingly, the deregulation of miR-20b as a tumor suppressor biomarker can be used for the discrimination between benign and malignant thyroid lesions and may be noted as a therapeutic target to suppress tumor growth of PTC cells. In line with our finding, a Previous in vitro study confirmed that miR-9 might have potential prognostic value for predicting PTC recurrence (42, 21). Consequently, we found that FZD5, CREB5, CCND2, ITAG6, THRB, RASGRF2, PFKM, and PI3KR3 are bioinformatically targeted by miR-9.

Consistent with our findings, several studies showed that levels of miR-221 and miR-222 expressions are elevated in PTC. Some researchers claim that the overexpression of miR-222 is associated with high-risk features such as lymph node involvement, extrathyroidal extension, invasiveness, and recurrence in PTC (43-45). These studies indicate that miR-222 had a great sensitivity in identifying thyroid malignancies (46). It is also a potential biomarker for PTC stratification (47). Furthermore, miR-221 and miR-222 are associated with poor prognostic features (48, 49). It has been demonstrated that miR-222, as one of the most typically overexpressed miRNAs in PTC, is associated with important prognostic features such as-vascular invasion, capsular invasion, lymph node metastasis, and larger tumor size in PTCs (47).

Additionally, miR-137, miR-222, and miR-181b expression levels seem to be associated with malignancy in PTC (27). Yip et al. showed that a panel of miR-146b, miR-222, miR34b, and miR-130b were differentially expressed in aggressive BRAF-positive PTC (50).

Experimental studies showed that miR-221 and miR-222 are involved in thyroid cell proliferation and cell transformation via the suppression of cell cycle regulator (p27kip1) and long non-coding RNA; Growth Arrest-Specific 5 (Gas5), also the target of miR-222-3p (51-53). Analysis of TCGA data revealed that up-regulation of miR-222 and miR-221 may result in the down-regulation of cellular senesces through targeting more than ten components in this signaling pathway and, therefore, may potentially help cancer cells to be immortal.

Notably, all these deregulated miRNAs are related to several molecular pathways responsible for important biological processes. Conversely, correlations between the miRNA expression levels and the Bethesda reporting of cytology specimens showed that selected miRNAs could be measured on cytology specimens as well as pathology samples (Table 3). Hence, they might have a good potential to predict the nature of thyroid nodules. Given that the up-regulation of miR-20b and miR-9 targets may result in the

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Table 3. Micro RNA expression level difference between benign and PTC based on pathology results

miRNA	PTC	Benign	*P*. Value
miR-9	-1.25 (-2.21_-0.73)	0.77 (0.38_1.17)	<0.001
miR -20b	-1.15 (-1.74_-0.83)	0.58 (0.26_0.85)	<0.001
mirR -221	2.09 (1.77_2.38)	-0.32 (-0.59_0.32)	<0.001
miR -222	2.14 (1.71_2.46)	-0.54 (-0.74_-0.32)	<0.001

down-regulation of cancer-related pathways or genes, they may have a tumor-suppressive role in thyroid cells. They also could be utilized as a new therapeutic target in patients with PTC.

Conclusion

In this study, we yielded that the panel of miR-221, miR-222, miR-20b, and miR-9 would be a proper diagnostic tool for the diagnosis of PTC. Besides, understanding the molecular basis of thyroid nodule development will be helpful to identify novel diagnostic, prognostic, and therapeutic targets. Although the molecular mechanisms of the miRNAs in oncogenesis remain to be fully elucidated, evaluating the miRNA expression panel in cytology and tissue specimens provides a diagnostic tool to differentiate PTC from non-PTC. It may also serve as a novel therapeutic target for PTC in future drug design studies.

Ethical Approval

The research was supported by the Iran University of Medical Sciences (IR.IUMS.REC 1396.31430), Tehran, Iran.

Authors' Contributions

M.H. had the original idea for this work. M.H. and Z.M. designed the study. Data collection was done by A.K. M.P., who evaluated and reported tissue specimens. F.A. and M.H. performed an experimental process and analysis. Bioinformatics analysis was conducted by N.H. Data analysis was done by Z.M. and A.K. M.H., N.H. and Z.M. wrote the manuscript. M.K., M.H., and Z.M. reviewed and rewrote the paper. All authors critically revised the draft of the manuscript and approved its final version.

Conflict of Interests

The authors declare that they have no competing interests.

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Appendix Table 1

Up-regulated miRNAs (log_2 F.C.)		Down-regulated miRNAs (log_2 F.C.)
miR-146b (5.17)	miR-891a (1.65)	miR-7-3 (-2.23)
miR-551b (4.46)	miR-509-2 (1.59)	miR-1179 (-1.95)
miR-221 (3.18)	miR-508 (1.56)	miR-144 (-1.84)
miR-187 (2.97)	miR-181b-1 (1.52)	miR-873 (-1.82)
miR-222 (2.84)	miR-3065 (1.42)	miR-451 (-1.78)
miR-375 (2.73)	miR-514-2 (1.4)	miR-486 (-1.65)
miR-31 (2.26)	miR-181a-1 (1.4)	miR-675 (-1.61)
miR-34a (2.2)	miR-514-1 (1.4)	miR-9-1 (-1.51)
miR-181b-2 (2.04)	miR-503 (1.39)	miR-9-2 (-1.49)
miR-205 (1.79)	miR-514-3 (1.29)	miR-7-2 (-1.25)
miR-21 (1.78)	miR-935 (1.21)	miR-363 (-1.14)
miR-509-1 (1.74)	miR-183 (1.19)	
miR-509-3 (1.68)	miR-96 (1.177)	
miR-181a-2 (1.65)	miR-181d (1.048)	

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