MicroRNA Signature in Papillary Thyroid Cancer; A Novel Diagnostic Panel

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Abstract

Background

Detection of cancer in patients with thyroid nodule requires sensitive and specific diagnostic modalities that are accurate and inexpensive. The aim of this study was to identify potential microRNA panel to detect papillary thyroid carcinoma (PTC).

Method:

Following conducting differential expression analysis on stage 1 PTC and normal tissues available in TCGA, 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 access accuracy of miRNA expression levels compared to the pathology report as the gold standard.

Results

There was a significant difference between tissue expression level of miR-9, miR-20b, miR-222 and miR-221 in the PTC group Compared with non-PTC (P value < 0.05). The area under the curves were 1, 0.98, 0.99, 0.98 and 1 irrespectively

Conclusion

A panel of miRNA including miR-20b, miR-222, miR-221 and miR-9 expression could be used as a useful to differentiate PTC from benign nodules.

Introduction

Thyroid nodules are relatively common and could found in approximately 50% of the adult population by high-resolution ultrasound [1]. Fortunately, the majority of thyroid nodules are benign and approximately 7% are cancerous [2]. PTC is the most frequent thyroid cancer [3].

Fine needle aspiration (FNA) of the thyroid nodule is a standard diagnostic procedure. However, equivocal cytology reports are challenging[4]. Therefore, introducing biomarkers that could predict malignancy in indeterminate samples are highly important.

MicroRNAs (miRNAs) are a group of small and short-lived non-coding RNAs which regulate tissue specific genes in eukaryotes [5, 6]. As antisense regulators of mRNAs, miRNA binds to 3'-untranslated region of target mRNAs, suppressing mRNAs translation or degradation [7, 8]. MiRNAs contribute as tumor

suppressors or oncogenes [9]. Large number of miRNAs participate in various cellular functions involved in cell proliferation, differentiation, and death [10]. Previous reports have proposed miRNAs as possible biomarkers to diagnose cancer, its invasiveness, or progression [11, 12]. Recent studies introduce transcriptionally deregulated miRNAs in benign nodules compared with normal thyroid tissue [13–15].

The tissue expression of miRNAs in PTC, follicular thyroid carcinoma, and anaplastic thyroid cancers is variable. Former researches 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 [16–19]. However, the association of certain miRNAs' expression and certain types of thyroid cancer, remained to be discovered.

In this study we aimed to introduce a panel of miRNAs to differentiate PTC from benign thyroid nodules.

Material And Methods

Identification of miRNA and their target genes

To select the miRNA targets, we performed a comprehensive literature review followed by a bioinformatics analysis on 282 stage 1 and 57 normal thyroid tissue mirSeq htseq-count files retrieved from TCGA (The Cancer Genome Atlas, <http://cancergenome.nih.gov/>) covering 1046 miRNAs. miRNAs with raw counts less than 10 were removed from all samples before normalization by DeSeq2. The list of differential expressed miRNAs was then filtered for adjusted p value ≤ 0.05 and $|\log_2 FC| \geq 1$. Based on a comprehensive literature review and Target Scan 6.2, miRWalk, and RNAhybrid. Then we selected miRNAs that targeted most shared important genes in PTC development pathway.

To identify interactions between deregulated miRNAs and their corresponding genes, the lists of four selected deregulated miRNAs were separately introduced into miRDB.V6 (<http://mirdb.org>) and their corresponding target genes were identified [20]. Corresponding cellular pathways of the target genes were subsequently determined using EnrichR [21].

Validation study

Patients

To validate deregulation of the identified differentially expressed miRNAs, an experimental study was done. Patients from whom total thyroidectomy was done between 2016 and 2017 were included. A total number of 59 Formalin-Fixed Paraffin-Embedded (FFPE) tissue specimens from 30 patients with stage I PTC, 8 patients with follicular adenoma, 11 patients with Hurtle cell adenoma and 10 patients with multinodular goiter were examined. Pathology slides were reviewed by a qualified pathologist who was blinded to the study protocol to reconfirm the previous pathology results and to ensure that no normal tissue was presented in the PTC samples. 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 FFPE tissue samples according to the manufacturer's instructions. The quality of RNA samples analyzed 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.

Results

Based on measurement of the miRNAs expression level of stage I PTC obtained from TCGA and literature review, we selected a set of four miRNAs including miR-9, miR-20b, miR-221 and 222 :

Differential Expression Analysis

Deregulated miRNAs and related pathways

Table 1 represents deregulated miRNAs in patients with PTC. We found, huge variation among different studies on miRNAs expression in thyroid cancer [20]. Previously reported deregulated miRNAs are shown in bold. 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[22].

Figures 1A and 1B show the shared target genes and pathways between miR-9 and miR-20b, as well as miR-221, and miR-222 respectively. MiR-9 and miR-22 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 (Fig 2).

Validation study

Demographic

Demographic characteristics of the patients are shown in table 2: 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 cytology specimen. (patient code 797495) (Table 2).

MiRNAs expression

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, both miR-221 and miR-222 expression levels were significantly greater in the PTC group, while the expression of miR-9 and miR-20b was greater in non-PTC patients.

There was no overlap in the distribution of log miR-9 and log miR-222 expression between the PTC and benign groups. The expression of miR-20b and miR-221 were also significantly differed between the two groups. The corresponding approximate area under the curves was 0.98 and 0.99 irrespectively. (Figure 3 and Apendix1)

According to the Bethesda reporting of cytology specimens, there were 26 patients with bethesta categories I and II. Other 22 patients were reported as Bethesta categories IV, V and VI (Table 2). There was a significant difference between microRNA expression levels in pathology samples and cytology reports. Correlations between miRNAs tissue expression levels in tissue specimens and the category of Bethesda reporting of cytology specimens is was shown in table 3.

Interestingly, considering the Bethesda reporting of cytology specimens, we observed low level expression of miRNA -9 and 20b levels in patients with Bethesda categories I and II, while, miR-221 and miR-222 expression were greater in Bethesda categories IV to VI (Table 3).

Discussion

Although the majority of thyroid nodules are benign, 7% are diagnosed as thyroid cancer (TC) [15]. It is highly important to distinguish between malignant and benign thyroid nodule. Overcoming the challenges of accurate assessments of the risk for individual patients is important to establish appropriate therapeutic strategies and to optimize clinical outcomes.

Currently, tissue diagnosis is the gold standard method. However, other methods could predict the nature of the nodule in an individual patient.

Distinct molecular changes are associated with tumorigenic process. Numerous studies have demonstrated that there is a potential application of miRNA in thyroid cancer diagnosis.[15, 23–26]. In contrast to mRNAs, mature miRNAs are comparatively stable and remain largely intact in routinely collected, formalin-fixed paraffin embedded (FFPE) Tissue specimens.[23]. Previous research revealed that dysregulated miRNAs could have diagnostic role in PTC cell line and even in FNA samples. [27].

In our study, using 282 patients with stage I PTC, and 57 normal thyroid tissue samples from TCGA, differentially expressed miRNAs were identified. Since diagnostic accuracy of a combination of miRNAs alteration is greater value compared with a single miRNA [28], we decided to choose a panel of miRNAs (miR-221, miR-222, miR-9 and miR-20b) based on the bioinformatics analysis of TCGA data sets and also a comprehensive literature review in which all selected miRNAs could play a biological role in tumor genesis pathways. Our miRNA- target prediction result showed that, although miR-9 and miR-20b target

several shared proteins involved in cancer signaling pathways, there is limited shared results for miR-221 and miR-222 (involved exclusively in cellular senescence pathway). Next, we sought to evaluate the diagnostic accuracy of selected miRNAs in PTC Compared with benign tissue samples. Interestingly miR-20b and miR-9 indicated an up-regulated pattern in benign samples, while miR-221 and miR-222 were highly expressed in PTC.

Since we observed down-regulation of miR-9 and miR-20b in thyroid cancer samples, we can expect the up-regulation of their target genes. Based on bioinformatics analysis, MAPK and PI3K-Akt are the two signaling pathways highly influenced by these two miRNAs, with 14 and 12 target genes, respectively. In HIPPO signaling pathway, four membrane receptors including TGFBR1, TGFBR2, BMPR2 and FZD3 are simultaneously targeted by these two miRNAs, indicating that the down-regulation of miR-9 and miR-222 plays a significant role in the activation of this pathway. TGFBR1 and TGFBR2 are also involved in MAPK, P53, Relaxin, and FOXO signaling pathways. Inspecting the activation or inhibition role of target genes in different pathways showed that the up-regulation of most genes results in cell survival, cell cycle progression and angiogenesis.

Given that the up-regulation of miR-9 and miR-20b targets may result in down-regulation of MAPK, PI3K, Hippo and other cancer related pathway, they may have tumor suppressive role in thyroid cells and could be utilized as a new therapeutic target, strategy for patient with PTC.

In line with our finding, several studies determined that levels of miR-221 and - 222 are up-regulated in PTC. Some researchers claim that the overexpression of miR-222 is associated with high-risk features such as lymph node involvement, extra thyroidal extension, invasiveness, and recurrence in PTC [29–31]. These studies indicate that miR-222 had a great sensitivity in identifying thyroid malignancies [32], as well as a potential biomarker for PTC stratification.[33] furthermore, miR-221 and miR-222 are associated with poor prognostic features [34, 35]. It has been determined 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[33].

Additionally, miR-137, -222 and 181b 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[36].

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) as a target of miR-222-3p [37] ,[38] ,[39]. Our bioinformatics analysis revealed that up-regulation of miR-222 and miR - 221 may result in the down-regulation of cellular senescence through targeting more than ten components in the signaling pathway and therefore potentially may help cancer cells to be immortal.

On the other hand, correlations between the miRNA expression levels and the Bethesda reporting of cytology specimens, showed that selected miRNAs could be measured on cytology specimens and might

have a good potential to predict the nature of thyroid nodules (Table 3).

It should be noted that a panel of miRNAs could be used to differentiate malignant nodule from a benign nodule.

Understanding the molecular basis of thyroid nodule development will be useful to identify novel diagnostic, prognostic, and therapeutic targets. Although the molecular mechanisms of the miRNAs in oncogenesis are remained 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 the future.

Declarations

Ethical Approval and Funding: The research was supported by Iran University of Medical Sciences, (IR.IUMS.REC 1396.31430), Tehran, Iran.

Conflict of Interest: The authors declare that they have no conflict of interest.

Consent to Participate and Consent to Publish: Not applicable.

Authors Contributions: M.H had the original idea of this work. M.H and Z.M designed study. M.K supervised the project. Data collection was done by AK. M.P evaluated and reported tissue specimens. F.A and M.H performed experimental process and analysis. Bioinformatics analysis was conducted by N.H. Data analysis was done by Z.M. 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.

Availability of data and materials: All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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Tables

Table 1
Deregulated miRNAs and corresponding (log₂ FCs) in PTC compared to normal thyroid tissue

Up-regulated miRNAs (log ₂ FC)		Down-regulated miRNAs (log ₂ FC)
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)	
Known deregulated miRNAs are shown in bolds		

Table 2
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 $\pm$ SD) [§]		42.2 $\pm$ 16.4	44.7 $\pm$ 10.5
Bethesda reporting 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			

Table 3
Correlations between miRNAs tissue expression levels (Log) 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

Figures

Figure 1

Shared miRNA targeted genes and their corresponding pathways between (A) miR-9 and miR-22 and (B) miR-221 and miR-222 retrieved from miRDB and EnrichR databases.

Figure 2

Number of shared target genes between miR-9 and miR-22 (yellow) and miR-221 and miR-222 (green) in different signaling pathways

Figure 3

Real Time PCR analysis of miRNAs in pathology specimens. Box plots illustrate miRNAs expression in benign (non-PTC) and PTC groups

Supplementary Files

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