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Identification of a Novel, Diagnostic MicroRNA Signature in Papillary Thyroid Cancer

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Abstract

Diagnosis is based on ultrasound and fine needle aspiration pathological findings. Screening asymptomatic people as well as those being at normal risk for thyroid cancer is not recommended. "Molecular signatures" consisting of miRNAs could be accounted for as promising diagnostic and/or prognostic biomarkers for different human malignancies, including thyroid cancer. MicroRNA molecules, being stable in blood and urine, are ideal components of molecular signatures. In this study, we sought to identify a molecular signature that could be used to diagnose papillary thyroid cancer with high sensitivity and specificity. For this purpose, we performed miRNA-seq to identify and quantify miRNAs showing high difference in their expression levels in serum of patients with papillary thyroid cancer and other diseases of thyroid and/or normal population. Validation was performed using real-time PCR. Using this approach, we found eight miRNAs being significantly over- or underexpressed in papillary thyroid cancer tissues compared to their normal counterparts (normal thyroid tissues): miR-144-3p, miR-622, miR-361-5p, miR-146a-3p, miR-340-5p, miR-199a-5p, miR-335-5p, and miR-129-5p. Five out of these eight miRNAs are also significantly over- or under-represented in blood serum of patients with papillary thyroid cancer. In conclusion, our study shows, for the first time, that a molecular signature consisting of miR-144-3p, miR-146a-3p, miR-340-5p, miR-199a-5p and miR-335-5p, is able to diagnose with high sensitivity and specificity papillary thyroid cancer in patients' blood serum.

Keywords: Thyroid gland; Thyroid malignancy; Biomarkers; miRNA; Diagnostic utility

Introduction

Thyroid cancer is a malignancy that originates from the tissues of the thyroid gland. It is a disease in which cells proliferate without being under control and have the potential to spread to other parts of the body. Thyroid cancer symptoms include swelling or a lump in the neck. Cancer can also occur in the thyroid secondarily, after spread from other organs of the body; however, these cases are not considered as thyroid cancer. Risk factors include radiation exposure at a young age, an enlarged thyroid, and family history. There are four main thyroid cancer types, namely papillary, follicular, medullary, and anaplastic thyroid cancer [1]. Diagnosis is based on ultrasound and fine needle aspiration pathological findings. Screening asymptomatic people as well as those being at normal risk for thyroid cancer is not recommended [2]. Treatment options usually include surgery, radiation therapy including radioactive iodine, chemotherapy, thyroid hormone, targeted therapy, and watchful waiting. Five-year overall survival rates reach 98% in the Western world countries [3].

MicroRNAs (miRNAs) are single-stranded, small non-coding RNA molecules that epigenetically control gene expression, mainly *via* binding to the 3'-untranslated region of targeted mRNA molecules. miRNAs mediate translational repression, most often with concomitant mRNA degradation. It is estimated that the human genome is able to produce two thousands of different miRNAs, which regulate the expression of approximately 95% of protein-coding genes, thus controlling the activity and function of key signaling pathways and cellular processes, including cell proliferation, apoptosis, cell differentiation, and response to hypoxia [4]. miRNAs can function either as oncogenes or tumor suppressors and some of them play a particular role in papillary thyroid cancer [5]. From a clinical perspective, particular miRNAs can be exploited as diagnostic and/or prognostic biomarkers in papillary thyroid cancer [6]. During the last ten years, scientists have put intensive efforts on deciphering the molecular impact of miRNA expression in thyroid cancer. A great number of miRNAs regulate the expression of important genes, assisting in the elucidation of molecular aspects of thyroid cancer onset and/or progression [5].

In this study, we identified a miRNA signature that could be used for diagnostic purposes in papillary thyroid cancer, at it can discriminate those with papillary thyroid cancer from the entire population. Moreover, we showed that alterations in the expression levels of five miRNAs are associated with clinicopathological characteristics of patients with papillary thyroid cancer.

Materials and Methods

Patients

In the present study, we used 34 Formalin Fixed Paraffin Embedded (FFPE) thyroid cancer tissue samples and the respective normal adjacent thyroid tissue samples. For 16 out of 35 patients, we collected blood serum prior to surgery. Moreover, blood sera from 48 patients with papillary thyroid cancer as well as from 72 patients with other thyroid diseases were collected (e.g. benign thyroid tumors, hyperparathyroidism, etc.).

Patients' age fluctuated from 19 to 68 years, with a median standard error of 45 ± 2.2 years. Clinicopathological data were recorded in a database and included tumor size, lymph node infiltration, extra thyroidal infiltration, and the existence of multifocal tumors.

This original research study was conducted with respect to the ethical standards of the Declaration of Helsinki. Each patient was informed for the content of the study and consented to provide sample for research purposes.

Total RNA extraction from thyroid FFPE tissues and blood sera

The tissue mass was very small with regard to most samples; thus, we used 1 mL TRIzol to extract total RNA from homogenized tissues. Regarding blood serum samples, we used 1 mL TRIzol BD, according to the manufacturer's guidelines.

Concentration assessment and integrity control of the extracted total RNA

The concentration of total RNA was assessed spectrophotometrically, using 1 L of total RNA in a nano-spectrophotometer. In order to determine the purity of the total RNA extracts, the ratio of absorbance at 260 nm and 280 nm was calculated. In order to assess the integrity of total RNA, 1 g of total RNA from each extract was electrophoresed on an agarose gel (1.5%, w/w).

miRNA-seq using next-generation sequencing (NGS)

Eight small-RNA libraries were constructed according to the manufacturer's instructions, starting from total RNA samples. 4 out of the 8 samples were papillary thyroid cancer tissues and the rest 4 samples were adjacent non-cancerous thyroid tissues. After template preparation and miRNA-seq using next-generation sequencing (NGS) following the manufacturer's guidelines, advanced bioinformatics analysis was carried out. Read alignment was performed using Partek Genomics Suite software and miRbase v.21. Thus, we carried out quantification of all miRNA sequences that were detected. In order to identify miRNAs the levels of which differed significantly between paired thyroid tissue samples, the Wilcoxon signed-rank test was used.

Polyadenylation of total RNA and its reverse transcription into first-strand cDNA

We carried out *in vitro* polyadenylation of total RNA and first-strand cDNA synthesis using an oligo-dT-adaptor primer. In more detail 1 μg of total RNA was polyadenylated and then reversely transcribed using MMLV, following the manufacturer's instructions [7].

Quantitative real-time PCR

We performed real-time PCR to validate miRNA expression levels of the five most variable mRNAs, based on our analyzed miRNA-seq data. miRNA-specific forward primers and a universal reverse primer were used to quantify miRNA expression in each sample, as presented in Supplementary Table 1 [8].

Mature miRNA	Accession number (miRbase v.21)	Expression in cancerous thyroid tissue *vs.* adjacent normal thyroid tissue	Log_2 fold change of expression (cancerous thyroid tissue *vs.* adjacent normal thyroid tissue)
miR-125a-5p	MIMAT0000443	Lower	-2.75
miR-144-3p	MIMAT0000436	Higher	2.39
miR-622	MIMAT0003291	Lower	-3.27
miR-361-5p	MIMAT0000703	Lower	-2.08
miR-146a-3p	MIMAT0004608	Higher	2.5
miR-340-5p	MIMAT0004692	Higher	3.1
miR-199a-5p	MIMAT0000231	Lower	-3.47
miR-205-5p	MIMAT0000266	Lower	-1.75
miR-335-5p	MIMAT0000765	Lower	-1.84
miR-129-5p	MIMAT0000242	Lower	-3.45

Table 1: miRNAs with the most important alterations in their expression levels, as quantified using NGS in 4 pairs of cancerous thyroid and adjacent normal thyroid tissues.

Biostatistical analysis

Following the expression analysis of the 5 selected miRNAs in all samples, biostatistical analysis was performed. We carried out descriptive biostatistical analysis and used non-parametric tests. We checked for the association of the expression levels of each miRNA with clinicopathological characteristics of the thyroid tumor. Moreover, we used ROC analysis to check the diagnostic and utility of each of these 5 miRNAs, composing a "miRNA signature".

Results

Parallel identification and quantification of miRNAs in cancerous and normal thyroid tissues and validation of the results regarding the 10 most variable miRNAs

Based on miRNA-seq data analysis regarding 4 selected papillary thyroid cancer tissues and their normal counterparts, we proceeded with 10 miRNAs showing the most variable expression levels: miR-125a-5p, miR-144-3p, miR-622, miR-361-5p, miR-146a-3p, miR-340-5p, miR-199a-5p, miR-205-5p, miR-335-5p, and miR-129-5p. The levels of these miRNAs were significantly different ($P<0.050$) between the cancer tissues and their normal counterparts, as shown using the Wilcoxon signed-rank (Table 1). Other significant changes in miRNA levels were also observed; yet, the statistical significance was marginal in these cases.

Before applying real-time PCR for the quantification of the expression levels of each of the 10 selected miRNAs, the respective molecular qPCR assays were developed including primer optimization for the amplification of each miRNA (cDNA) and quality control of each assay and a representative amplification plot and a melting curve of the real-time PCR product corresponding to miR-144-3p was obtained.

Using real-time PCR, these 10 miRNAs were quantified in each one of the 8 selected tissue samples (4 tissue pairs) and next in each of the other 31 tissue pairs. 8 out of these 10 miRNAs (all except for miR-125a-5p and miR-205-5p) were significantly different ($P<0.050$) between the cancer and the normal samples (Table 2).

miRNA expression (RQU)	**Mean value ± S.E**	**Range**	**Percentiles**		
			25th (Median)	50th (Median)	75th (Median)
miR-125a-5p expression					
In malignant tumors	0.2 ± 0.01	0.03-0.5	0.09	0.1	0.2
In benign tumors	0.4 ± 0.02	0.09-1.1	0.2	0.3	0.5
miR-144-3p expression					
In malignant tumors	27.2 ± 2.5	0.5-99.7	7.9	16.8	40.2
In benign tumors	5.8 ± 0.7	0.7-25.3	2	4.2	7.5
miR-622 expression					
In malignant tumors	18.7 ± 1.7	2.3-70.7	9.6	14.8	25.9
In benign tumors	50.0 ± 4.5	0.03-217.2	16.7	32	67.9
miR-361-5p expression					
In malignant tumors	15.8 ± 1.2	2.1-63.2	6.1	11.3	21.2
In benign tumors	47.3 ± 4.0	1.8-187.7	16.1	34.2	64.3
miR-146a-3p expression					
In malignant tumors	25.4 ± 12.5	0.1-91.7	0.8	2.4	60
In benign tumors	2.1 ± 0.8	0.01-43.8	0.09	0.04	0.3
miR-340-5p expression					
In malignant tumors	6.7 ± 0.5	0.2-19.4	2.4	6.5	10.6
In benign tumors	0.5 ± 0.1	0.06-2.1	0.2	0.3	0.8
miR-199a-5p expression					
In malignant tumors	0.8 ± 0.1	0.03-5.0	0.3	0.5	0.8
In benign tumors	19.3 ± 2.3	0.4-96.1	4.1	14.9	25.9
miR-205-5p expression					
In malignant tumors	25.8 ± 2.3	1.5-85.2	11	19.6	36.8
In benign tumors	29.2 ± 2.1	1.8-105.2	12.8	23.5	38.3
miR-335-5p expression					
In malignant tumors	10.0 ± 1.4	0.2-82.6	0.6	6.35	13.5
In benign tumors	108.9 ± 39.6	5.9-256.0	15.6	90.1	126.4
miR-129-5p expression					
In malignant tumors	1.6 ± 0.2	0.08-4.3	0.7	1.5	2.2

In benign tumors	4.2 ± 0.5	0.08-16.4	0.5	1.5	5.9

Table 2: Results of expression analysis of ten selected miRNAs quantified using real-time PCR in 35 pairs of cancerous thyroid and adjacent normal thyroid tissues.

Next, the continuous variables representing expression of each of the 8 selected miRNAs were transformed into the respective dichotomous variables using the median values as cut-off points. Therefore, expression values of all miRNAs in each sample were categorized into negative and/or positive samples. High expression levels of miR-144-3p, miR-146a-3p and miR-340-5p, as well as low expression levels of miR-622, miR-361-5p, miR-199a-5p, miR-335-5p, and miR-129-5p are significantly associated (P<0.050) with particular clinicopathological traits of patients, such as lymph node infiltration, extrathyroidal infiltration, and the existence of multifocal tumors (Table 3).

miRNA	Lymph node infiltration	Extrathyroidal infiltration	Existence of multifocal tumors	Tumor size
miR-144-3p (overexpression)	0.02	0.054	0.1	0.32
miR-622 (underexpression)	0.07	0.013	0.096	0.26
miR-361-5p (underexpression)	0.05	0.018	0.13	0.4
miR-146a-3p (overexpression)	0.04	0.094	0.089	0.3
miR-340-5p (overexpression)	0.2	0.08	0.044	0.57
miR-199a-5p (underexpression)	0.05	0.004	0.077	0.22
miR-335-5p (underexpression)	0.1	0.16	0.04	0.62
miR-129-5p (underexpression)	0	0.04	0.21	0.66

Table 3: Significance of the associations (P values) between the expressions of each selected miRNA and clinicopathological characteristics of tumors.

Quantitative expression analysis of 8 selected miRNAs in blood serum of patients with papillary thyroid cancer and patients with other non-malignant thyroid diseases

Using real-time PCR, we identified and quantified the 8 selected miRNAs (miR-144-3p, miR-622, miR-361-5p, miR-146a-3p, miR-340-5p, miR-199a-5p, miR-335-5p, and miR-129-5p) in blood sera of both patient cohorts. The expression levels of the 8 miRNAs were significantly different (P<0.050) between the blood sera of patients with papillary thyroid cancer and blood sera of other (non-cancer) patients (Table 4). These miRNAs include: miR-144-3p, miR-146a-3p, miR-340-5p, miR-199a-5p, and miR-335-5p.

miRNA expression (RQU)	Mean value ± S.E.	Range	Percentiles		
			25th	50th (Median)	75th
miR-144-3p expression					
In serum of cancer patients	57.2 ± 4.5	2.5-149.1	17.9	36.8	120.2
In benign tumors	15.8 ± 1.9	1.7-75.3	6	14.2	47.5
miR-622 expression					
In serum of cancer patients	7.7 ± 1.0	0.3-100.7	4.6	24.8	78.1
In benign tumors	10.0 ± 2.5	0.1-155.0	6.2	23	100.9
miR-361-5p expression					
In serum of cancer patients	132.8 ± 5.0	11.1-343.0	46.1	98.3	287.2
In controls	147.3 ± 4.1	10.8-387.7	56	104	244.3
miR-146a-3p expression					
In serum of cancer patients	50.1 ± 16.5	5.1-101.1	8.8	40.4	90
In controls	12.1 ± 1.8	0.1-53.5	3.3	10.4	48.3
miR-340-5p expression					
In serum of cancer patients	12.7 ± 1.5	1.2-29.2	2.1	7.5	20.6
In controls	4.5 ± 0.9	0.6-12.1	1.2	3.3	8.9
miR-199a-5p expression					
In serum of cancer patients	1.2 ± 0.1	0.08-6.1	0.4	1.2	3.8
In controls	12.3 ± 1.3	2.4-56.0	8.2	18	45.1
miR-335-5p expression					
In serum of cancer patients	23.1 ± 3.9	2.2-62.6	0.4	16.3	33.5
In controls	128.9 ± 21.6	13.9-211.0	25.6	101	166.8
miR-129-5p expression					
In serum of cancer patients	0.6 ± 0. 1	0.01-2.3	0.03	0.15	1.2
In controls	1.0 ± 0.06	0.09-10.4	0.5	1.5	5.9

Table 4: Results of expression analysis of eight selected miRNAs in serum of patients with papillary thyroid cancer and controls, using real-time PCR.

Covariate	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value

miR-144-3p expression						
Negative	1			1		
Positive	2.56	1.22-5.39	0.013	1.79	1.21-2.94	0.025
miR-622 expression						
Negative	1			1		
Positive	0.85	0.45-1.64	0.24	0.52	0.23-1.15	0.21
miR-361-5p expression						
Negative	1			1		
Positive	0.93	0.43-2.00	0.35	0.87	0.37-2.04	0.37
miR-146a-3p expression						
Negative	1			1		
Positive	2	1.31-3.06	0.001	1.76	1.11-2.80	0.016
miR-340-5p expression						
Negative	1			1		
Positive	8.4	4.17-16.94	<0.001	6.63	2.41-18.24	<0.001
miR-199a-5p expression						
Negative	1			1		
Positive	0.54	0.36-0.79	0.002	0.69	0.42-0.84	0.04
miR-335-5p expression						
Negative	1			1		
Positive	0.48	0.28-0.81	0.006	0.62	0.54-0.84	0.032
miR-129-5p expression						
Negative	1			1		
Positive	0.59	0.32-1.11	0.1	0.83	0.42-1.61	0.57

Table 5: Univariate and multivariate logistic regression results regarding the selected miRNAs that were quantified in serum.

Moreover, ROC curve analysis and univariate logistic regression uncovered the diagnostic potential of these miRNAs. Multivariate logistic regression showed that these miRNAs can be combined in order to effectively diagnose papillary thyroid cancer (Table 5). The serum levels of the 5 selected miRNAs, namely miR-144-3p, miR-146a-3p, miR-340-5p, miR-199a-5p, and miR-335-5p are significantly associated (P<0.050) with particular clinicopathological traits of patients (Table 6).

miRNA	Lymph node infiltration	Extrathyroidal infiltration	Existence of multifocal tumors	Tumor size
miR-144-3p (high levels)	0.02	0.15	0.27	0.62
miR-146a-3p (high levels)	0.05	0.19	0.099	0.44
miR-340-5p (high levels)	0.31	0.07	0.034	0.3
miR-199a-5p (low levels)	0.09	0.002	0.1	0.29
miR-335-5p (low levels)	0.13	0.2	0.029	0.52

Table 6: Significance of the associations (P values) between the serum levels of each selected miRNA and clinicopathological characteristics of tumors.

Discussion

miRNAs are the most extensively studied small non-coding ncRNA molecules, since they are involved in transcriptional and post transcriptional regulation of protein-coding genes. The 5' seed region (between nucleotides 2-7) of miRNAs binds within the 3′untranslated region of mRNA molecules, leading to the degradation or repression of the targeted mRNAs, depending whether or not a perfect miRNA/mRNA complementarity is mediated [9]. Furthermore, *in silico* predictions suggest that over 90% of protein-coding genes are potential targets of miRNAs [10]. Therefore, essential cellular processes including cell proliferation, differentiation, migration, angiogenesis, and/or apoptosis are regulated by a wide, complicated miRNA network [4]. Consequently, any dysregulation of the pathway of miRNA biogenesis is strongly associated with malignant transformation and hence renders them as key players during thyroid cancer initiation, metastasis promotion, and progression of the disease [11,12].

miRNAs play an important role in various processes, including carcinogenesis. Moreover, due to their small size and their resistance to nucleolytic cleavage by RNases, these tiny RNA molecules are stable in blood and urine, as they are not subjected to degradation. Intracellular differences in miRNA levels are usually reflected in blood, too. Therefore, it has been suggested that these "molecular signatures" consisting of miRNAs could be accounted for as promising diagnostic and/or prognostic biomarkers for different human malignancies, including thyroid cancer [13,14].

Our study shows, for the first time, that a molecular signature consisting of miR-144-3p, miR-146a-3p, miR-340-5p, miR-199a-5p and miR-335-5p, is able to diagnose with high sensitivity and specificity papillary thyroid cancer in patients' blood serum. [14]. These five miRNAs are known to regulate the expression of protein with pivotal roles in this type of cancer. For instance, miR-144-3p promotes tumor growth and metastasis of papillary thyroid cancer cells by targeting the expression of PAX8 and WWTR1 [15,16]. A genetic polymorphism in the sequence of miR-146a-3p is associated with worse prognosis of patients with papillary thyroid cancer [17]. Moreover, upregulation of miR-340-5p promotes thyroid cancer progression by targeting and downregulating BMP4 [18]. On the other hand, miR-199a-5p inhibits thyroid cancer progression by targeting the *SNAI1* oncogene [19]. miR-335-5p is underexpressed in papillary thyroid cancer and suppresses tumor growth and cancer cell proliferation, invasion and metastasis *via* direct inhibition of ZEB2 expression [20].

In this study, we determined the cut-off value for the expression levels of each one of these five miRNAs in blood serum, in order to categorize expression of each miRNA into positive or negative. This miRNA signature could be used for diagnostic purposes, at it can discriminate those with papillary thyroid cancer from the entire population. Moreover, alterations in the expression levels of these 5 miRNAs are associated with clinicopathological characteristics of patients with papillary thyroid cancer.

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