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Association of Serum and Tumor Tissue microRNA Profile with Aggressiveness of Papillary Thyroid Carcinoma in an Iranian Population

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Abstract

Objectives: Papillary thyroid carcinoma (PTC) is the most common malignancy of thyroid. We aimed to investigate the association of *let-7f*, *miR-146b-5p*, *miR-34b*, *miR-16* and *miR-877-5p* expression in blood circulation and tumor with aggressiveness of PTC.

Methods: A total of 18 patients with aggressive PTC and 18 patients with non-aggressive PTC were studied. The microRNAs expressions were evaluated using real-time PCR. Fold changes (FC) of the miRs in aggressive PTC patients were calculated via calibration with mean of expression of the miRs in non-aggressive groups.

Results: *MiR-16* showed significant up regulation in blood (FC=2.85; P=0.024), *miR-34* showed significant down regulation in blood (FC=0.19; P<0.001) and tumor tissue (FC=0.19; P<0.001), *miR-146* showed significant up regulation in blood (FC=48.10; P<0.001) and tumor tissue (FC=60.61; P<0.001), *miR-877* showed significant down regulation in blood (FC=0.22; P<0.001), and *let-7* showed significant down regulation in blood (FC=0.09; P<0.001) and tumor tissue (FC=0.13; P<0.001).

Conclusion: In general, our study in an Iranian population supported the previous results. Up regulation of *miR-146* was associated with aggressiveness of PTC.

Keywords: Papillary thyroid carcinoma • microRNA • Aggressive tumor • *mir-146b*

Introduction

Papillary thyroid carcinoma (PTC) is the most common malignancy of thyroid gland originating from thyroid follicular cells. PTC has generally a good survival rate; however the cases having certain clinico-pathological parameters have poorer prognosis [1]. A population-based cohort study showed that incidence of total thyroid cancers increased from 3.6 per 100000 in 1973 to 8.7 per 100000 in 2002. No significant change was reported for incidence of the less common types including follicular, medullary, and anaplastic carcinomas. In other words the entire increase is attributable to an increase in incidence of PTC, which increased from 2.7 to 7.7 per 100000. Crude death rate of PTC was approximately 0.5 deaths per 100000 individuals from 1973 to 2002 [2]. Point mutations in proto-oncogenes including rearranged during transformation (*RET*), *BRAF*, *V-Raf* and rat sarcoma viral oncogene homolog (*RAS*) were frequently observed in PTC [3].

microRNAs (miRs) are endogenous single stranded non coding RNAs that bind to the 3' non coding region of the target mRNAs, resulting in their selective degradation or inhibition of translation. Therefore miRs are involved in regulation of biological functions [4]. *Let-7* family is known as the first group of discovered miRs in human. It has been observed that down regulation of these miRs is associated with malignancies [5]. *Let-7* miRs bind to RAS oncogenes and result in their down regulation. Therefore they have been investigated in PTC. In addition, this family has been observed in normal thyroid gland suggesting that it may play a role in function of thyroid gland [6]. Other than *let-7* family, other miRs were notable. MiR-146b regulates signal transduction of transforming growth factor-beta (TGF-β) and therefore may play a role in PTC. In addition, oncogene activation has resulted in increase in expression of miR-146b [7]. It has been observed that patients with *BRAF* mutation have a higher expression level of miR-146b in comparison to patients with wild type of *BRAF* [8]. MiR-34b is involved in oncogenesis. In has been observed that it was down regulated in cancer cells [9]. A study on different cancer cell lines showed that many genes such as *RAS* family are targeted by miR-16 [10]. MiR-877 is another cancer related miR which is down regulated in hepatocellular carcinoma via targeting cyclin dependent kinase (CDK) 14 [11].

Previously diagnostic role of miRs in PTC have been investigated [12]. However, other than diagnosis, it is important to have ability to differentiate aggressive cases from the non-aggressive ones. Therefore, the present study was designed to investigate the association of *let-7f*, *miR-146b-5p*, *miR-34b*, *miR-16* and *miR-877-5p* expression in blood serum and tumor tissue with aggressiveness of PTC in an Iranian population.

Materials and Methods

Study design and patients

The present work was a case control study to compare blood and tumor tissue expression of the miRs between aggressive and non-aggressive patients of PTC using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). A total of 18 patients with aggressive PTC were considered as the case group and 18 patients with non-aggressive PTC were considered as the

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control group. The samples were collected from 3 centers Toos, Shariyati and Firoozgar (Tehran, Iran) from February to September 2019 through convenient sampling. This study was approved by the ethics committee of Shariyati hospital (IR.TUMS.EMRI.REC.1398.008). Written informed consents were obtained from all the participants.

Blood and tumor tissue samples collection

Fresh tissue specimens of aggressive and non-aggressive PTC tissues were obtained from tumors and immediately kept at -80°C until analysis. All the samples were diagnosed by two independent pathologists. Confirmation of PTC was through light microscope using hematoxylin and eosin staining. Clinical staging was according to prognostic factor for recurrence in N1b [13]. From each participant, 2 ml of peripheral blood was collected in EDTA containing tubes and immediately kept at -80°C until analysis.

Total RNA extraction

Total RNA was obtained from the tumors and serum with columnar extraction method using total RNA purification kit (Norgen, Canada) according to the manufacturer's instructions. Total RNA quantification was performed using nanodrop spectrophotometry (Thermo Scientific Wilmington, DE, USA). Sample quality control using cel-mir-39 Spike-In Kit (Norgen, Canada) offers a quantified synthetic RNA (cel-miR-39) for spike-in during RNA extraction procedures and subsequent normalization in RT-qPCR assays. The amount of cel-miR-39 RNA recovered after RNA extraction is directly correlated with the amount of total RNA recovered. Therefore cel-miR-39 was used as the reference to report ΔCt.

miR reverse transcriptase PCR

cDNA was synthesized with poly A polymerase method using microScript microRNA cDNA Synthesis kit (Norgen, Canada). Then the cDNA was subjected to be used for real-time reverse transcriptase polymerase chain reaction (RT-PCR) using SYBR Green mastermix (Norgen, Canada) according to the manufacturer's instructions (after a first 30 min at 37°C, then 30 min at 50°C and finally 15 min at 70°C). All RT-PCR reactions were performed in triplicates. Levels of miR expression were calculated by relative quantification using Rotor Gene Q Real-Time PCR SDS 2.3.1 software (Applied Biosystems Inc., Foster city, CA). The results were presented as normalized Ct values. Previously published literatures were used for primary selection of the miRs. miR cancer database was used to find associations of miRs with cancers according to the literatures of PubMed. The candidate miRs were *let-7f, miR-146b-5p, miR-34b, miR-16* and *miR-877-5p*. Mirbase database was used to find the sequences of the miRs in order to convert them to primer sequence. The used primers are shown (Table 1). miRDB database was used to find the targeted genes. According to this, these miRs can target *RAS* and *Braf*. Mirandola database was used to understand whether the miRs could be detected in circulating blood and the tissue. Bioinformatics characteristics of the used miRs are shown (Table 2).

Statistical analysis

Rest 2009 was used to investigate relative expression. Fold changes of each miR were calculated via the formula $2^{-\Delta\Delta CT}$ in Excel 2013 calibrating with the mean of expression in non-aggressive PTC patients. Significance of

Table 1. Sequences of the primers used in this study.

miR	Primers
Mir16-5p	TAGCAGCGTAAATATTGGCG
Mir34b-5p	TAGGCAGTGTCATTAGCTGATTG
Mir146b-5p	TGAGAACTGAATTCCATAGGCTG
Mir877-5p	GTAGAGATGGCGCAGGG
Let7f-5P	TGAGGTAGTAGATTGATAGTT

Table 2. Bioinformatics of the used miRs.

MicroRNA	Location	Target	Score	Position	Sequence
Mir-16-5p	Chr 13: 50,048,973-50,049,061	PLSCR4	70	1568-1575 of PLSCR4 3' UT R	UAGCAGCACGUAAAUAUUGGCG
		ANO3	98	2517-2523 of ANO3 3' UTR	
		PHF19	100	1377-1383 of PHF19 3' UTR	
		AQP11	65	227-234 of AQP11 3' UTR	
Mir-146b-5p	chr10: 102,436,512-102,436,584	NOVA1	98	682-689 of NOVA1 3' UTR	UGAGAACUGAAUUCCAUAGGCUG
		TRAF6	100	1272-1279 of TRAF6 3' UTR	
		CD80	90	839-846 of CD80 3' UTR	
		SEC23IP	99	3634-3641 of SEC23IP 3' UTR	
Mir-877-5p	chr6: 30584332-30584417	ZNF174	89	329-336 of ZNF174 3' UTR	GUAGAGGAGAUGGCGCAGGG
		TP53INP2	80	3069-3075 of TP53INP2 3' UTR	
		RP11-204N11.1	80	2289-2296 of RP11-204N11.1 3' UTR	
		TMCC2	67	1200-1207 of TMCC2 3' UTR	
Mir-34b-5p	chr11: 111512938-111513021	TENM1	100	1314-1321 of TENM1 3' UTR	UAGGCAGUGUCAUUAGCUGAUUG
		ELMOD1	98	1100-1106 of ELMOD1 3' UTR	
		RFX3	98	3622-3628 of RFX3 3' UTR	
		DLL1	97	294-300 of DLL1 3' UTR	
Let-7f-5p	chr9: 94176347-94176433	STARD13	100	Position 2362-2368 of STARD13 3' UTR	UGAGGUAGUAGAUUGUAUAGUU
		C14orf28	100	437-444 of C14orf28 3' UTR	
		LIN28B	100	44-51 of LIN28B 3' UTR	
		BZW1	92	84-90 of BZW1 3' UTR	

individual fold changes was investigated with one sample t test (fold change=1 was the null hypothesis) and comparison of the fold changes between blood serum and tumor tissue was through independent t test. SPSS 24 software (IBM, US) was used for data analysis. Two-tailed P value less than 0.05 was considered as the significance level.

Results

A total of 36 Iranian patients of PTC with age range 20-72 were investigated. The range of the tumor size among the PTC patients was 0.5-8.0 cm with number of lymph node metastasis ranged 0-5 (Table 3). Real-time RT-PCR was performed and after approving the melting curves, fold changes were compared between blood serum and tumor tissue expression. Up regulation and down regulation of the miRs based on relative expression were calculated with rest program. This relative expression was calculated for expression of the miRs as aggressive versus non-aggressive groups (Table 4). Fold changes of the miRs in aggressive PTC patients were calculated via calibration with mean of expression of the miRs in non-aggressive groups using one sample t test. According to this, *MiR-16* showed significant up regulation in blood (fold change [FC]=2.85; P=0.024), *miR-34* showed significant down regulation in blood (FC=0.19; P<0.001) and tumor tissue (FC=0.19; P<0.001), *miR-146* showed significant up regulation in blood (FC=48.10; P<0.001) and tumor tissue (FC=60.61; P<0.001), *miR-877* showed significant down regulation in blood (FC=0.22; P<0.001), and *let-7* showed significant down regulation in blood (FC=0.09; P<0.001) and tumor tissue (FC=0.13; P<0.001). Fold change of each miR (blood expression versus tumor tissue expression) was compared using independent t test. *MiR-16* showed a significant more up regulation in aggressive PTC patients (2.85 *vs*. 0.92; P=0.020). No significant difference was observed between blood and tumor tissue fold changes for other miRs (Table 5 and Figure 1).

Discussion

The present study was designed in order to find the role of circulating and tumor tissue miRs in invasion of PTC. At this level we could find significant association of 3 miRs with invasion of PTC in both blood and tumor tissue, and association of 2 miRs with invasion of PTC in blood. *MiR-16* showed significant up regulation in blood, *miR-34* showed significant down regulation in blood and tumor tissue, *miR-146* showed significant up regulation in blood and tumor tissue, *miR-877* showed significant down regulation in blood, and *let-7* showed significant down regulation in blood and tumor tissue. Expression change of *miR-16* was significantly more dominant in blood in favor of up regulation. Since there was no significant difference between the fold changes of the miRs in blood and tissue (except *miR-16*), blood serum expression study of these miRs can be used in clinics as an available and representative source instead of tumor tissue.

PTC is a cancer with good prognosis. The necessity of total thyroidectomy may be affected by its aggressive behavior. Since aggressive behavior of PTC was hard to predict, many studies were trying to have research on biomarkers. Linwah et al. studied 17 aggressive and 15 non-aggressive PTC patients in USA in order to find the role of tissue miR signature. They found up regulation for *miR-146b, -221, -222, -155, -31*, and down regulation for *miR-1, -34b, -130b, -138* in aggressive PTC. Our study supported this finding for the common *miRs* [14]. Yang et al. studied 20 aggressive and 20 non-aggressive patients in China. They found up regulation of miR *146b 5p* and *miR 221/222* and down regulation of *miR 16* and *miR 613* in aggressive PTC. In contrast,

Table 3. Clinico-pathological features of the participants.

Variables	Group 1 (Aggressive PTC)	Group 2 (Non-aggressive PTC)
Gender		
Female	13	13
Male	5	5
Age		
45›	4	6
45‹	14	12
Pathological characteristics		
Metastatic lymph node	03-May	0-4
Tumor size	2.5-8 cm	0.5-4 cm
TNM staging	III, IV, V	I, II
Lobectomy	2	3
Thyroidectomy	16	15

Table 4. Comparison of relative expressions with their 95% confidence intervals are shown. *Significant at P<0.05.

miR	Source	Relative expression (95% CI)	P value (Rest)	Effect direction
Mir16-5p	Tissue	1.206 (0.206-6.571)	0.407	Non-significant
	Blood	1.162 (0.214-5.852)	0.485	Non-significant
Mir34b-5p	Tissue	0.289 (0.010-4.225)	0.001*	Down regulation
	Blood	0.294 (0.018-5.513)	0.002*	Down regulation
Mir146b-5p	Tissue	6.834 (1.007-62.775)	<0.001*	Up regulation
	Blood	4.289 (0.350-35.198)	<0.001*	Up regulation
Mir877-5p	Tissue	1.096 (0.082-13.296)	0.774	Non-significant
	Blood	0.858 (0.056-20.990)	0.661	Non-significant
Let7f-5P	Tissue	0.555 (0.065-4.324)	0.028*	Down regulation
	Blood	0.108 (0.012-1.253)	<0.001*	Down regulation

Table 5. Fold changes of the miRs in aggressive PTC and comparison of them in blood and tissue. *Significant at P<0.05.

Group		N	Mean of fold change	Std. Deviation	Std. Error Mean	One sample t test P value	Independent t test P value
mir_16	blood	18	2.854247	3.1674688	0.7465796	0.024*	0.020*
	tissue	18	0.920071	1.0990411	0.2590465	0.761	
mir_34	blood	18	0.198956	0.1896811	0.0447083	0.000*	0.377
	tissue	18	0.286675	0.3702283	0.0872637	0.000*	
mir_146	blood	18	48.101287	30.1388044	7.1037843	0.000*	0.239
	tissue	18	60.617704	32.4837004	7.6564816	0.000*	
mir_877	blood	18	0.220025	0.1853129	0.0436787	0.000*	0.088
	tissue	18	0.700225	1.144368	0.2697301	0.282	
let_7	blood	18	0.093891	0.0760213	0.0179184	0.000*	0.265
	tissue	18	0.136635	0.1406197	0.0331444	0.000*	

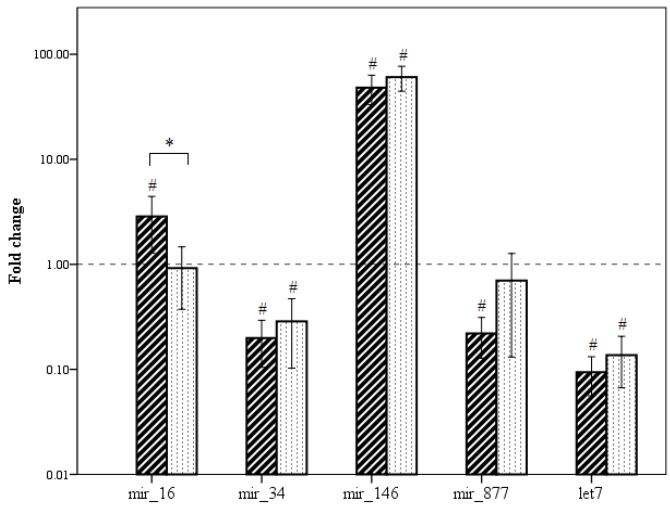

Figure 1. Fold changes of the expression of the miRs. Black (left) columns are for blood expression and white (right) columns are for tissue expression. The error bars indicate 95% confidence interval. The reference line fold change =1 shows calibration line (aggressive PTC vs non-aggressive PTC). *Significant at P<0.05; independent t-test. #Significant at P<0.05; one sample t test (the baseline fold change=1 is the null hypothesis).

our study did not show down regulation for *miR 16* [4]. Lee et al. in Australia found that *miR-222* and *miR-146b* had over expression in PTC via comparing tumor tissue and plasma of 9 recurrent and 17 non-recurrent PTC patients [15]. Rosignolo et al. in Italy found association of *miR 146b-5p* and *miR 222 3p* up regulation with increased risk of recurrence [16]. Chou et al. introduced *miR-146b* as novel biomarker in PTC. They believed that this miR was associated with aggressiveness and prognosis. Different mechanisms had been suggested for the initiating role of *miR 146b* in oncogenic pathways. One of them was that *miR 146b* inhibited TGF-β anti-signal via down regulation of *SMAD4* and therefore inhibition of cell cycle arrest [17].

The most important limitation of this study and the previous studies was the study design. Although such studies named their groups as cohorts of PTC, but from the critical appraisal point of view in evidence-based medicine, they were not eligible cohort studies. Since the practical aim of this topic is prediction of aggressiveness, occurrence of PTC and its aggression should be subsequent to these biomarker changes.

Conclusion

In general, our study in an Iranian population supported the previous results. miRs can help to differentiate invasive PTC from non-invasive PTC. Briefly, *miR-16* and *miR-877* showed up regulation in blood, *miR-34* and *let-7* showed down regulation in blood and tumor tissue, *miR-146* showed significant up regulation in blood and tumor tissue, and *miR-146* showed significant up regulation in blood and tumor tissue. Expression change of *miR-16* was significantly more dominant in blood in favor of up regulation. Predicting role of these biomarkers should be investigated in well-designed cohort studies. Then the results can be used as personalized medicine in management of PTC.

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