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Clinical Research Article

Circulating MicroRNA Profiles as Potential Biomarkers for Differentiated Thyroid Cancer Recurrence

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Abstract

Context: Circulating microRNAs (miRNAs) are emerging biomarkers of thyroid cancer.

Objective: This study sought to identify the profile of circulating miRNAs and its response to human recombinant TSH (rhTSH) in thyroid cancer patients with recurrent/persistent disease.

Methods: We obtained serum samples from 30 patients with differentiated thyroid cancer, 14 with recurrent/persistent disease and 16 with complete remission. We used next-generation sequencing to define the miRnomes along with a comprehensive quantitative PCR (qPCR) validation using 2 different platforms. We made a transversal study by comparing serum miRNA profiles of patients with or without recurrent/persistent disease and a longitudinal study looking at differences before and after rhTSH stimulation. Selected miRNAs were then studied in human thyroid cancer cell lines TPC-1, FTC-133, and OCUT-2 in response to TSH stimulation.

Results: We could not demonstrate any consistent differences in serum profiles of known miRNAs between patients with and without recurrent/persistent disease or before and after rhTSH stimulation. However, our sequencing data revealed 2 putative novel miRNAs that rise with rhTSH stimulation in the serums of patients with recurrent/persistent disease. We further confirmed by qPCR the upregulation of these putative miRNAs both in serums and in TSH-stimulated cells. We also show miRNAs that are good candidates for housekeeping genes in the serum of patients independently of the levels of TSH.

Conclusions: The present study does not provide evidence that known miRNAs can be used as circulating markers for recurrence of thyroid cancer. However, we suggest that novel miRNA molecules may be related to thyroid cancer pathogenesis.

Key Words: microRNA, serum, thyroid cancer, recurrence, NGS, novel microRNA

Abbreviations: CV, coefficient of variation; FDR, false discovery rate; miRNA, microRNA; NGS, next-generation sequencing; NR, nonrecurrent group; PTC, papillary thyroid cancer; qPCR, quantitative PCR; R, persistent/recurrent group; RAI, radioactive iodine; rhTSH, recombinant human TSH; Tg, thyroglobulin

Papillary thyroid cancer (PTC) is the most common form of thyroid malignancy, with a rising incidence worldwide (1, 2). Generally, PTC has an excellent prognosis with > 90% 10-year survival (3, 4). However, long term follow-up of patients is necessary as 20% of cases will present with recurrence in the first 10 years, and 10% to 20% will recur after the 10-year mark (5, 6). The measurement of serum thyroglobulin (Tg), a thyrocyte-specific glycoprotein, presents the basis for detecting thyroid cancer recurrence in patients with total thyroidectomy followed by radioactive iodine (RAI) ablation. After surgical removal of thyroid gland and RAI treatment, patients are treated with levothyroxine to lower the levels of TSH, which decreases the chances of relapse. Because up to 20% of patients with recurrence will not have detectable Tg at low TSH levels, Tg levels are measured either after thyroid hormone withdrawal, or after administration of recombinant human TSH (rhTSH) (7, 8).

However, the use of Tg as a marker can be hampered for several reasons. First, ~25% of cancer patients will have antithyroglobulin antibodies, rendering Tg measurements unreliable (9-11). The variability of antithyroglobulin antibody assays can also make them undetectable, causing false-negative results in Tg detection (12). Moreover, not all patients are treated with total thyroidectomy followed by radioiodine ablation of remnant thyroid tissue. Given the rise in incidence of low-risk papillary microcarcinoma, more conservative

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treatments such as lobectomy or total thyroidectomy without radioiodine ablation are being chosen. In both cases, healthy thyroid tissue remains present, and because Tg is a marker of thyrocytes, its use for detecting recurrence would give uninformative results (13).

In search of new serum biomarkers for thyroid cancer recurrence, microRNAs (miRNAs) have recently emerged. They present small, noncoding RNA species, 18 to 24 nt in length, which negatively regulate gene expression by binding to 3′ untranslated regions of target mRNAs. Their role in the pathophysiology of cancer is well established (14-16), whereby in thyroid cancer, miRNAs have shown to be dysregulated (17) and implicated in differentiation of PTC (18). Also, thyroid cancer patients can be risk-stratified into several groups according to miRNA expression in the primary tumor (19). In the past decade, it was observed that tumor-derived miRNAs are present in serum (20, 21), where they circulate enclosed in extracellular vesicles or complexed with Ago2 proteins (22, 23). This makes them remarkably stable in the circulation and resistant to pH and temperature changes as well as RNases, which altogether puts them in the spotlight of cancer biomarker research. Considering thyroid cancer, several groups have made attempts at identifying the circulating miRNAs that would differentiate patients with PTC from patients with benign thyroid tumors (24-28), reaching conflicting conclusions. High-throughput screening studies failed to overlap, pinpointing different miRNAs as being the most differentially expressed miRNAs. Very few studies addressed the question whether miRNAs can mark recurrence of thyroid cancer (29, 30), with none of them determining the whole miRnomes of recurrence.

Our aim was to determine the circulating miRNA profiles of patients undergoing follow-up for thyroid cancer to identify miRNA markers of recurrence. For this purpose, we used the following approach: a discovery study using a high-throughput method (ie, next-generation sequencing (NGS) of small RNA to define the circulating miRNome); a validation study using 2 different quantitative PCR (qPCR) platforms, and a search for novel miRNAs. We compared miR expression (1) transversally by looking at serum miR profiles of patients in complete remission and patients with recurrent/persistent disease and (2) longitudinally by investigating serum miR content in patients with recurrence before and after rhTSH stimulation.

Material and Methods

This study was granted ethics approval by the local ethics committee before commencement.

Patients

Samples were collected between 2015 and 2018 from the University Hospital Mostoles, Madrid. All patients (n = 30) underwent total thyroidectomy and were treated with RAI. Two groups of patients were recruited: those with PTC who had biochemically and/or structurally confirmed recurrence after their initial treatment (persistent/recurrent disease group) (n = 14) and those with PTC who have not been diagnosed with recurrence at their latest follow-up (nonrecurrent or complete remission group) (n = 16). Persistent/recurrent disease was defined as the presence of basal serum Tg levels > 1 ng/mL or rhTSH, stimulated Tg levels higher than 10 ng/mL (biochemical recurrence), and/or ultrasound- or I^{125} whole-body scan-detected malignant lesions (structural recurrence). Contrary to that, complete remission was defined as the lack of detectable lesions and basal Tg levels below 0.2 ng/mL or below 1 ng/mL upon rhTSH stimulation. Tg was measured before administration of rhTSH (basal levels) and at 2 timepoints upon rhTSH stimulation (ie, 24 and 72 hours after stimulation). Samples used in the transversal study included serums of patients in complete remission and with recurrent/persistent disease taken at basal TSH. In the longitudinal part of our study, for comparison with the basal time point, the 72-hour post-stimulation samples were chosen because of the higher rise in Tg levels that reflected the influence of rhTSH on thyroid cancer cells.

A total of 14 patients in the persistent/recurrent disease group (R) were recruited between 2015 and 2018, of which there were 9 with paired samples of pre- and post-rhTSH treatment (R Thy0 and R Thy2, respectively) and 16 patients in the nonrecurrent group (NR), of which there were 6 with paired samples (NR Thy0 and NR Thy2). All patients signed written informed consent forms. We used 2 cohorts for the different phases of our study. The first, smaller cohort, was used in the discovery study (NGS), and the second, larger cohort was used for the validation phase with 2 different qPCR platforms (Table 1). Because of sample availability, some samples from the discovery study cohort and the validation study cohort did not overlap, as detailed in Table 1.

An independent cohort of patients recruited between 2018 and 2020 was used exclusively for the validation of new putative miRNAs found by NGS. A total of 11 patients, of which 5 had persistent/recurrent disease and 6 were in remission are shown in Supplementary Table 1 included in the Supplementary Material deposited in digital repository Figshare and accessible through DOI https://doi.org/10.6084/m9.figshare.17019077.v3 (31).

Serum Samples

Approximately 4 to 5 mL of nonfasting venous blood was drawn before rhTSH stimulation, at 24 and 72 hours upon stimulation. Samples were collected in BD Vacutainer SST II Advance Tubes (Becton, Dickinson and Company). After clotting, serums were separated centrifuging at 1000*g* for 30 minutes and immediately frozen at -80°C until NGS or RNA extraction.

Next-generation Sequencing

Small RNA sequencing and subsequent data analysis was performed by Qiagen Genomic Services on an Illumina platform NextSeq500 sequencing instrument. We included 11 patients in this analysis and used 2 samples from each patient, 1 before and 1 72 hours after rhTSH stimulation. In total, 22 samples were used for RNA sequencing: 8 paired samples (before and 72 hours after rhTSH) from 4 patients in complete remission and 14 paired samples (before and 72 hours after rhTSH) from 7 patients with persistent/recurrent disease. Before RNA isolation, all the samples were checked for hemolysis contamination and confirmed to be negative, as described later. A total of 200 µL of each sample were used for RNA isolation using miRNeasy Serum/Plasma kit (Qiagen) according to the manufacturer's instructions. During the process of isolation UniSp2, 4, and 6 spike-ins were added to the sample for subsequent quality control. Total RNA was eluted in 14 µL of RNAse-free water. Library preparation, sequencing, and raw data analysis is detailed in Supplementary Materials and

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Table 1. Clinicopathological characteristics of patients

Patients	Age (y)	Sex	TT	Lymphadenectomy	Histology	TNM	Cumulative RAI (mCi)	ATG	Basal Tg (ng/mL)	Basal TSH (mUI/L)	Tg-stim (ng/mL)	TSH-stim (mUI/L)	Time after surgery (mo)	Time after RAI (mo)	Persistent/ recurrent disease	NGS	qPCR miScript	qPCR LNA	put-miR
1	44	Male	Yes	Central	PTC classic	T3N1bM0	450	No	9	0.01	30.6	12.09	74	73	Mediastinal lymph nodes	Yes	Yes	Yes	Yes
2	30	Female	Yes	Lateral	PTC classic	T2N1aM0	250	No	2	0.01	13.5	5.48	38	35	Locoregional	Yes	Yes	Yes	Yes
3	50	Female	Yes	No	PTC classic	T3N0M1	708	No	9.3	0.03	143.2	18.47	139	133	Bone and axillary lymph nodes		Yes		
4	63	Female	Yes	No	PTC classic	T2NxM0	240	No	0.6	0.01	10.8	49.69	58	54	Biochemical		Yes	Yes	Yes
5	26	Female	Yes	Central	PTC classic	T1N1aM0	150	No	1.1	0.01	13.2		26	22	Biochemical		Yes		
6	23	Female	Yes	Lateral	PTC classic	T4N1bM1	963	No	2.8	0.01	NA	NA	249	247	Lungs		Yes	Yes	Yes
7	47	Female	Yes	Central	PTC classic	TxN1aM0	251	Yes	0.1	0.21	0.03	12.8	103	98	Locoregional		Yes	Yes	
8	32	Female	Yes	Central	PTC classic	TxN1bM0	NA	No	3.3	0.07	NA	NA	249	248	Locoregional		Yes	Yes	
9	25	Female	Yes	Lateral	PTC classic	T1N1bM0	650	Yes	0.1	0.01	NA	NA	199	197	Lungs		Yes	Yes	
10	39	Female	Yes	Central	PTC classic	T3N1aM0	450	No	3.1	0.01	NA	NA	136	125	Biochemical	Yes	Yes	Yes	Yes
11	74	Male	Yes	Central and lateral	PTC classic and follicular	T1N1bM0	220	No	4.3	0.18	NA	NA	19	14	Locoregional	Yes		Yes	Yes
12	51	Female	Yes	No	PTC follicular variant	T3NxM0	670	No	11.4	0.01	NA	NA	280	270	Lungs and locoregional	Yes		Yes	yes
13	38	Female	Yes	Central	PTC classic	T1bN1aM0	200	No	3.8	0.04	27.7	29.48	46	43	Biochemical	Yes		Yes	yes
14	43	Female	Yes	Central	PTC classic and follicular	T1bN1aM0	220	No	11.4	0.03	NA	NA	13	8	Locoregional	Yes		Yes	yes
15	40	Female	Yes	Lateral	PTC classic	T2N1bM0	120	No	0.1	0.42	0.1	13.10	80	78	No		Yes	Yes	
16	18	Female	Yes	Central	PTC classic	TxN1bM0	300	No	0.1	0.01	0.1	17.78	195	194	No		Yes	Yes	
17	59	Female	Yes	Central	PTC classic	T3N1aMx	150	No	0.1	0.01	0.1	45.38	12	8	No		Yes	Yes	
18	67	Female	Yes	No	PTC classic	T1NxM0	100	No	<0.1	0.89	<0.1	49.98	65	59	No		Yes	Yes	
19	27	Female	Yes	No	PTC classic	T1aN0M0	100	No	0.1	1.51	0.1	7.2	123	117	No		Yes		
20	27	Female	Yes	Central	PTC classic	T2N1aM0	150	No	0.6	0.06	0.1	7.94	145	141	No		Yes		
21	20	Female	Yes	Central	PTC classic	T1N1aM0	125	No	<0.1	0.03	<0.1	29.48	141	138	No	Yes	Yes	Yes	
22	51	Female	Yes	Central	PTC classic	pT1N1M0	150	No	0.1	0.01	0.1	21.21	34	29	No		Yes	Yes	
23	48	Male	Yes	No	PTC classic	T1N0M0	100	No	<0.1	1.33	<0.1	7.55	82	78	No		Yes	Yes	
24	36	Female	Yes	No	PTC classic	T3NxM1	113	No	<0.1	0.2	0.1	29.52	192	187	No		Yes	Yes	
25	67	Female	Yes	No	PTC classic	T1bNxM0	100	No	<0.1	0.92	0.1	24	25	20	No		Yes		
26	25	Female	Yes	No	PTC follicular variant	T1N0M0	100	No	0.1	1.07	0.1	180	173	NA	No	Yes		Yes	
27	49	Female	Yes	No	PTC classic	T1N1bM0	150	No	0.1	0.01	0.1	14	9	NA	No	Yes		Yes	

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Table 1. Continued

Patients	Age (y)	Sex	TT	Lymphadenectomy	Histology	TNM	Cumulative RAI (mCi)	ATG	Basal Tg (ng/mL)	Basal TSH (mUI/L)	Tg-stim (ng/mL)	TSH-stim (mUI/L)	Time after surgery (mo)	Time after RAI (mo)	Persistent/recurrent disease	NGS	qPCR miScript	qPCR LNA	put-miR
28	38	Female	Yes	Central	PTC follicular variant	T1aN1aM0	132	No	0.1	0.01	0.1	14	11	NA	No	Yes		Yes	
29	42	Female	Yes	Central and lateral	PTC classic	T1aN1bM1	120	No	0.1	0.01	0.1	NA	NA	NA	No			Yes	
30	55	Male	Yes	Central and lateral	PTC classic	T3N1bM0	150	No	0.1	0.21	0.1	NA	NA	NA	No			Yes	

Abbreviations: ATG, antithyroglobulin antibodies; NA, not available; PTC, papillary thyroid cancer; qPCR, quantitative PCR; RAI, radioactive iodine; Tg, thyroglobulin; Tg-stim, 72-h stimulated thyroglobulin; TSH-stim, 72-h stimulated TSH; TT, total thyroidectomy.

Methods (31). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (32) and are accessible through GEO Series accession number GSE173248 deposited on April 25, 2021 (33).

Serum RNA Extraction and miScript miRNA Quantification

Total RNA was isolated from a total of 1 mL of serum using TRI reagent BD (Sigma-Aldrich) and purified with a RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. Reverse transcription was performed using miScript II RT Kit (Qiagen), using 6 µL of input RNA as detailed in Supplementary Material and Methods (31). We selected the endogenous control based on the results from a study of serum miRNAs in a Spanish cohort (34). We analyzed the 3 microRNAs used by the authors as reference genes (hsa-miR-223, hsa-miR-19a, and hsa-miR-106) and determined their stability using BestKeeper software tool for reference gene comparison. Comparing the SD and coefficient of variation (CV), the indexes of stability, hsa-miR-223 was selected as the endogenous control because it had the best scores (SD and CV for hsa-miR-223 were 2.35 and 9.53%, for hsa-miR-19a 3.59 and 11.09%, and for hsa-miR-106 3.48 and 11.47%).

Serum RNA Extraction, Quality Control, and miRCURY LNA miRNA Quantification

Serums were thawed and centrifuged 5 minutes on 4°C at 16 000*g* to remove cryoprecipitates and cellular debris from damaged cells. Quality control of the samples included measuring hemolysis levels (a process known to influence the levels of circulating miRNAs) and controlling for the efficiency of RNA extraction and reverse transcription. To determine the levels of hemolysis, 1 µL of serum sample was used to measure the absorbance at 414 nm on Nanodrop ND-1000 spectrophotometer. Samples showing a characteristic absorbance peak were tested for 2 miRNAs know to be influenced by hemolysis: miR-23a-3p and miR-451a. If their cycle quantification (Cq) ratio was less than 7, hemolysis was considered not to have a significant influence on the results. We tested these miRNAs in 2 samples that had high absorbance peaks at 414 nm, confirming that their ratios were less than 7. A total of 200 µL of serum was used for RNA isolation on a Qiacube workstation (Qiagen) with miRNeasy serum/plasma Advanced kit (Qiagen) according to the manufacturer's instructions. MS2 carrier was added to increase recovery. To control for efficiency of RNA extraction, 3.5 µL of miRNeasy serum/plasma Spike-in controls (UniSp2, Sp4, and Sp5) were added. Reverse transcription was performed with miRCURY LNA RT Kit (Qiagen) with 1 µL of input RNA and UniSp6 spike-in as a control for RT efficiency. For the samples to pass the quality control the levels of spike-ins in a 1:30 dilution of samples needed to be in the following range: UniSp2 (18-20); UniSp4 (24-26); UniSp5 (30-32); and UniSp6 (18-20). Before commencing the validation study, we first assayed UniSp2, UniSp4, UniSp5, and UniSp6 spike-ins and confirmed that the levels in all samples were in the expected range. Details on RT conditions and miRCURY LNA microRNA quantification are given in Supplementary Materials and Methods (31). In this case, based on our NGS results, hsa-miR-16-5p was used as an endogenous control because it showed higher indexes of stability than hsa-miR-223 in BestKeeper software (SD 0.89 vs 1.16, CV 4.11% vs 4.92%, respectively).

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Cell Culture

The human PTC cell line TPC-1, the follicular thyroid cancer cell line FTC-133, and the anaplastic thyroid cancer cell line OCUT-2 were cultured as described (35). After 24 to 48 hours' starvation, cells were treated with bovine TSH for 24 hours in the following concentrations: 0.5 mIU/mL; 5 mIU/mL; and 50 mIU/mL. Total RNA was isolated using Trizol reagent (Invitrogen) according to manufacturer's instructions. RT and qPCR were performed using miRCURY LNA Assays as described in Supplementary Materials and Methods (31). Protein isolation and Western blot was performed as described previously (18). Anti-CREB, anti-vinculin, and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology Inc (RRID:AB_627300, RRID:AB_1131294, RRID:AB_2714189); anti-pCREB was from Millipore (RRID:AB_310153).

Statistical Analysis

Differential expression analysis of sequencing data was performed using the EdgeR statistical software package (Bioconductor, http://bioconductor.org/). For normalization, the trimmed mean of the M-values method based on log-fold and absolute gene-wise changes in expression levels between samples (TMM normalization) was used. Principal component analysis and hierarchical clustering are detailed in Supplementary Material and Methods (31). When comparing the expression of microRNA between groups, an exact test assuming negative binomial distribution was applied. After that, the Benjamini-Hochberg method was used for correcting P values after multiple testing, and the criteria for significantly differentially expressed miRNAs was false discovery rate (FDR) < 0.05. Statistical analysis of qPCR data was performed using GraphPad Prism (version 7). Depending on the distribution, 2-tailed Student t test or Mann-Whitney U test were used to determine statistical significance between distributed variables. Differences were considered significant at $P < 0.05$.

Results

Patient Characteristics

Clinical-pathological characteristics of patients are summarized in Table 1. All patients (n = 30) enrolled in this study were treated for PTC with total thyroidectomy followed by radioiodine ablation. Patients were followed for a median period of 81 months (interquartile ratio = 30-159 months) and their levels of Tg were used as a biochemical indicator of recurrence. Basal Tg level in patients with recurrent disease was 4.45 ng/mL (0.1-11.4), whereas the post-rhTSH stimulation Tg level was 26.4 ng/mL (0.01-143.1). For patients in complete remission, basal Tg levels ranged from < 0.1 to 0.1 ng/mL, with 1 sample having 0.6 ng/mL, whereas the post-rhTSH stimulation Tg levels ranged from < 0.1 to 0.1 ng/mL. Of 14 patients with recurrent disease, 10 suffered from structural recurrence: 3 had distant metastasis at the lungs, 1 had cancer spread to the bone and 1 in mediastinal lymph nodes, and 5 had locoregional structural recurrence. Anti-thyroglobulin antibodies were detected in 2 patients that belonged to the recurrent disease group.

MicroRNA Sequencing of Serum Samples Shows no Significant Differences Between Recurrent and Nonrecurrent Patients

We performed next-generation small-RNA sequencing to assess the differences between circulating miRNA profiles in patients with recurrent thyroid cancer and patients in complete remission (transversal study). We determined the circulating miRNomes in a discovery study cohort that consisted of 7 patients with recurrence and 4 patients in complete remission.

The number of known miRNAs detected in all samples was 210 and 132 at ≥ 1 TPM and ≥ 10 TPM thresholds, respectively. Patients were compared using methods of unsupervised analyses using 50 miRNAs that have the largest variation across all samples. Principal component analysis did not reveal the presence of any naturally arising sample clusters (Fig. 1A). Two-way hierarchical clustering of 50 miRNAs and samples revealed no distinct groups between the patients with and without recurrence (Fig. 1B). When comparing the expression of miRNA in these 2 groups of patients using an exact test, several miRNAs showed significantly different expression levels (Fig. 1C). However, no miRNAs passed the FDR after Benjamini-Hochberg correction for multiple testing, suggesting that the microRNAs secreted in the serum are not different between patients who have recurrent thyroid cancer and patients who are in complete remission.

Stimulation of Patients With rhTSH in Thyroid Cancer Follow-up Does Not Change Their Circulating miRNome

The longitudinal part of the study included the comparison of the miRNomes before and after stimulation with rhTSH between the recurrent/persistent disease group and the remission group. We sequenced the serum miRNAs in 8 paired samples (before and 72 hours after rhTSH) from 4 patients in complete remission and 14 paired samples (before and 72 hours after rhTSH) from 7 patients with recurrence. Principal component analysis and hierarchical clustering of the 50 most differentially expressed miRNAs did not reveal any clustering of these samples (Fig. 2A, 2B). Also, there were no differentially expressed miRNAs that passed the FDR (Fig. 2C), suggesting that treatment with recombinant human TSH does not influence the secretion of miRNAs from thyroid cancer cells into the circulation.

Validation With miScript qPCR Platform Shows microRNAs Differentially Expressed Between Patients With Persistent/Recurrent Thyroid Cancer and Patients in Complete Remission

To confirm the negative results obtained by small RNA sequencing, we performed the validation of these findings first on Qiagen's miScript, a commonly used qPCR platform for miRNA exploration. Because the discovery phase of the study did not reveal any differentially expressed miRNAs known to miRBase, we decided to test the expression of miRNAs reported in the literature. MiRNAs were preselected according to their differential expression in the primary tumors and in the circulation of patients with thyroid cancer recurrence. We preselected 17 miRNAs representative of miRNA clusters defined in The Cancer Genome Atlas study of PTC (19), and miRNAs that showed high expression in primary tumors compared with healthy thyroid tissue in our previous study (18) (ie, miR-146b-5p, miR-146b-3p, miR-21-5p, miR-221-3p, miR-222-3p, miR-34a-5p, miR-181-3p, miR-181-5p, miR-182-5p, miR-183-5p, miR-30a-5p, miR-30a-3p, miR-let-7f-5p, miR-let-7a-5p, miR-143-3p, miR-155-5p, and miR-375-5p). The validation study cohort used for miScript qPCR included 11 patients in complete remission and 10 patients with persistent/recurrent disease.

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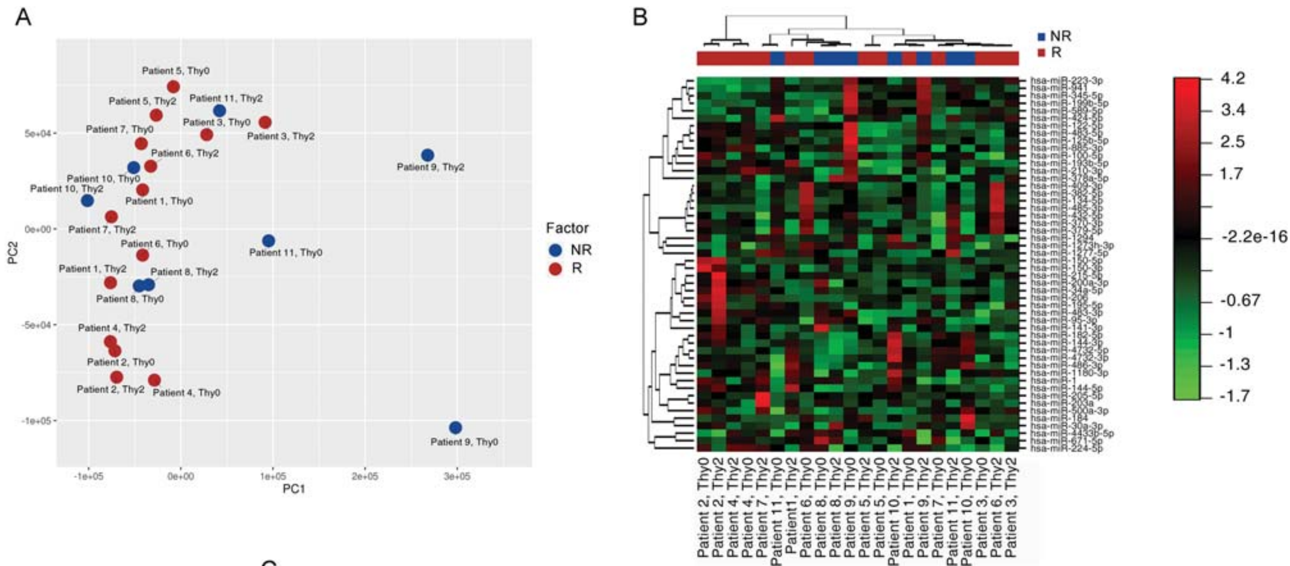

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Name	NR Thy0 TMM	R Thy0 TMM	Log Fold Change	P Value	FDR
hsa-miR-941	335	767	1,20	0,0020	0,4952
hsa-miR-144-5p	174	82	-1,05	0,0022	0,4952
hsa-miR-345-5p	188	394	1,09	0,0069	0,8904
hsa-miR-5193	1	13	2,53	0,0133	0,8904
hsa-miR-1296-5p	11	36	1,37	0,0139	0,8904
hsa-miR-4433b-3p	29	5	-1,82	0,0141	0,8904
hsa-miR-199b-5p	73	192	1,41	0,0172	0,8904
hsa-miR-27a-5p	10	0	-3,75	0,0257	0,8904
hsa-miR-103a-3p	5430	3897	-0,48	0,0299	0,8904
hsa-miR-192-5p	913	1602	0,82	0,0299	0,8904
hsa-miR-1247-5p	7	23	1,61	0,0306	0,8904
hsa-miR-1299	4	19	1,97	0,0318	0,8904
hsa-miR-505-3p	26	59	1,07	0,0325	0,8904
hsa-miR-107	315	207	-0,65	0,0330	0,8904
hsa-miR-1304-3p	23	51	1,12	0,0378	0,8904
hsa-miR-181d-5p	20	4	-2,07	0,0381	0,8904
hsa-miR-155-5p	592	374	-0,67	0,0414	0,8904
hsa-miR-27a-3p	704	1000	0,51	0,0415	0,8904
hsa-miR-197-3p	808	1187	0,55	0,0420	0,8904
hsa-miR-4645-3p	8	0	-3,47	0,0432	0,8904
hsa-miR-483-3p	150	297	1,00	0,0476	0,8904
hsa-miR-151a-3p	3786	2581	-0,55	0,0488	0,8904
hsa-miR-584-5p	1133	630	-0,86	0,0491	0,8904
hsa-miR-194-5p	593	994	0,75	0,0497	0,8904
hsa-miR-3688-3p	5	21	1,62	0,0553	0,8904

Figure 1. Small RNA sequencing of serum samples comparing patients in complete remission and patients with persistent/recurrent disease reveals no significant differences in the levels of known miRNAs. (A) Principal component analysis using the 50 miRNAs that have the largest coefficient of variation based on TMM normalized counts. Each circle represents a sample. (B) Heat map and unsupervised hierarchical clustering by sample and by genes performed using 50 miRNAs with the largest coefficient of variation based on TMM normalized counts. (C) Between-group comparison of the 25 miRNAs that have shown the highest changes in expression levels. FDR, false discovery rate calculated after Benjamini-Hochberg correction for multiple testing; NR, patients in complete remission; R, patients with persistent/recurrent disease; Thy0, serums taken at basal Tg levels; TMM, trimmed mean of the M-values method. Differences considered significant if FDR < 0.05.

When comparing patients in complete remission and patients with persistent/recurrent disease, 3 miRNAs showed significant upregulation: miR-221-3p (Mann-Whitney *U* test $P = 0.010$; log2 fold change = 3.5); miR-34a-5p (Mann-Whitney *U* test $P = 0.003$; log2 fold change = 3.4), and miR-143-3p (Mann-Whitney *U* test $P = 0.043$; log2 fold change = 4.6). The results of miScript miRNA quantification in serum samples of significantly dysregulated miRNAs are shown in Fig. 3; the complete data of this analysis are presented in Supplementary Figure 1 (31). These results were different to what we observed with small-RNA sequencing because NGS did not show these miRNAs to be differentially expressed and prompted us to do further qPCR investigation. It has been reported that miScript platform has limitations in terms of specificity (36); therefore, for the second validation we searched for a qPCR kit with higher testing accuracy.

qPCR Validation With LNA-based Assays Corroborates the Results Obtained by NGS miRNA Sequencing

The second qPCR validation was performed with LNA miRCURY assays. These assays were chosen because they

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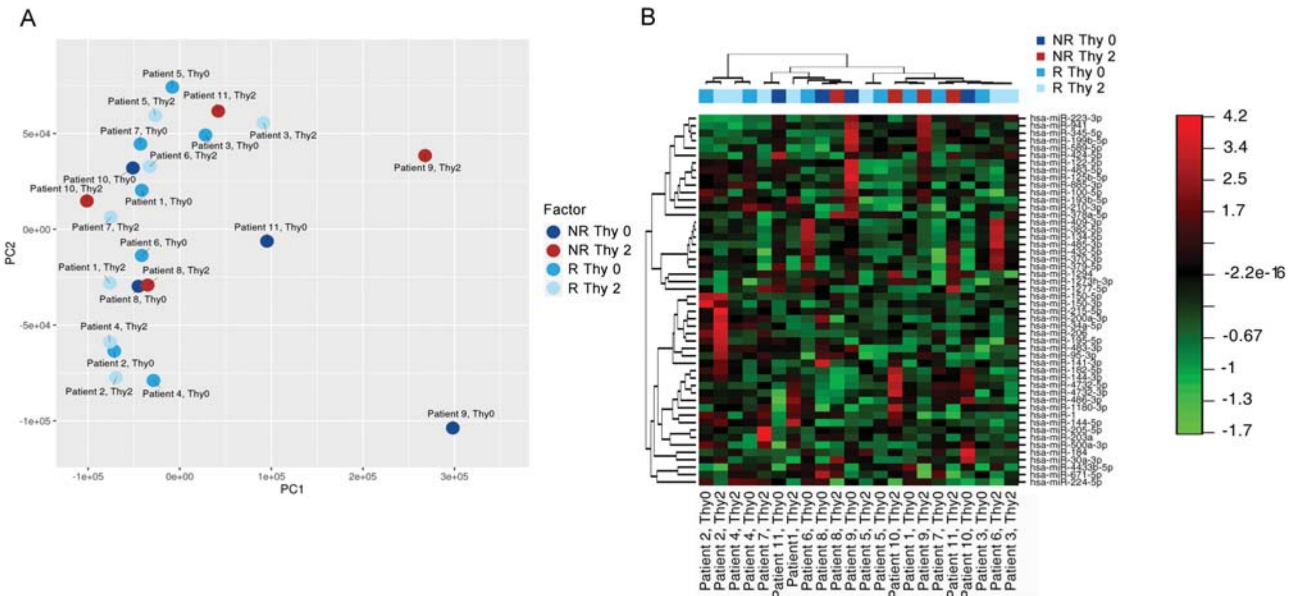

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Name	R Thy0 TMM	R Thy2 TMM	Log Fold Change	P Value	FDR
hsa-miR-3124-5p	11	1	-2,08	0,0196	1,0000
hsa-let-7i-3p	21	1	-1,23	0,0298	1,0000
hsa-miR-342-5p	3	1	1,71	0,0303	1,0000
hsa-miR-452-5p	4	14	1,65	0,0307	1,0000
hsa-miR-148b-5p	19	7	-1,40	0,0416	1,0000
hsa-miR-4662a-5p	11	2	-1,74	0,0457	1,0000
hsa-miR-29b-2-5p	7	0	-2,21	0,0467	1,0000
hsa-miR-7850-5p	9	2	-1,73	0,0498	1,0000
hsa-miR-205-5p	163	357	1,12	0,0528	1,0000
hsa-miR-130b-3p	50	81	0,62	0,0531	1,0000
hsa-miR-30c-1-3p	8	1	-1,92	0,0543	1,0000
hsa-miR-203a	81	154	0,93	0,0560	1,0000
hsa-miR-181c-3p	31	12	-1,21	0,0565	1,0000
hsa-miR-130a-3p	140	191	0,48	0,0622	1,0000
hsa-miR-1914-5p	0	5	2,87	0,0694	1,0000
hsa-miR-454-5p	22	9	-1,32	0,0749	1,0000
hsa-miR-4645-3p	8	1	-1,33	0,0805	1,0000
hsa-miR-4433b-3p	30	55	0,90	0,0855	1,0000
hsa-miR-214-3p	20	9	-1,00	0,0858	1,0000
hsa-miR-1246	30	16	-0,83	0,0937	1,0000
hsa-miR-664a-3p	21	9	-1,00	0,1082	1,0000
hsa-miR-942-5p	102	72	-0,54	0,1087	1,0000
hsa-miR-145-5p	181	124	-0,54	0,1101	1,0000
hsa-miR-504-5p	8	2	-1,41	0,1180	1,0000
hsa-miR-141-3p	89	137	0,60	0,1195	1,0000

Figure 2. Small-RNA sequencing of serum samples comparing patients before and after treatment with rhTSH reveals no significant differences in the levels of known miRNAs. (A) Principal component analysis using 50 miRNAs that have the largest coefficient of variation based on TMM normalized counts. Each circle represents a sample. (B) Heat map and unsupervised hierarchical clustering by sample and by genes performed using 50 miRNAs with the largest coefficient of variation based on TMM normalized counts. (C) Between-group comparison of the 25 miRNAs that have shown the highest changes in expression levels. FDR, false discovery rate calculated after Benjamini-Hochberg correction for multiple testing; NR, patients in complete remission; R, patients with persistent/recurrent disease; Thy0, serums taken at basal Tg levels; Thy2, serums taken at 72-hour stimulated Tg levels; TMM, trimmed mean of the M-values method. Differences considered significant if FDR < 0.05.

are considered superior to all other qPCR platforms for miRNA research (36). Of 17 miRNAs that were selected for the first qPCR study, with LNA-based assays we tested 9 miRNAs that were both differentially expressed in the miScript analysis and that had the highest expression in primary tumors and/or miRNA clusters (ie, miR-146b-3p, miR-146b-5p, miR-375, miR-21-5p, miR-221-3p, miR-143-3p, miR-34a-5p, miR-182-5p, let-7f-5p) (Fig. 4). Because of insufficient serum amounts for RNA extraction, the validation study cohort used for miRCURY LNA qPCR somewhat differed from the cohort used for MiScript. Eight patients in complete remission and 8 with persistent/recurrent disease overlapped, and the cohort was further extended to finally include 13 samples in complete remission and 12 samples of patients with persistent/recurrent disease. We were also able to include samples from patients before and after rhTSH administration: 6 patients in complete remission and 9 with recurrence.

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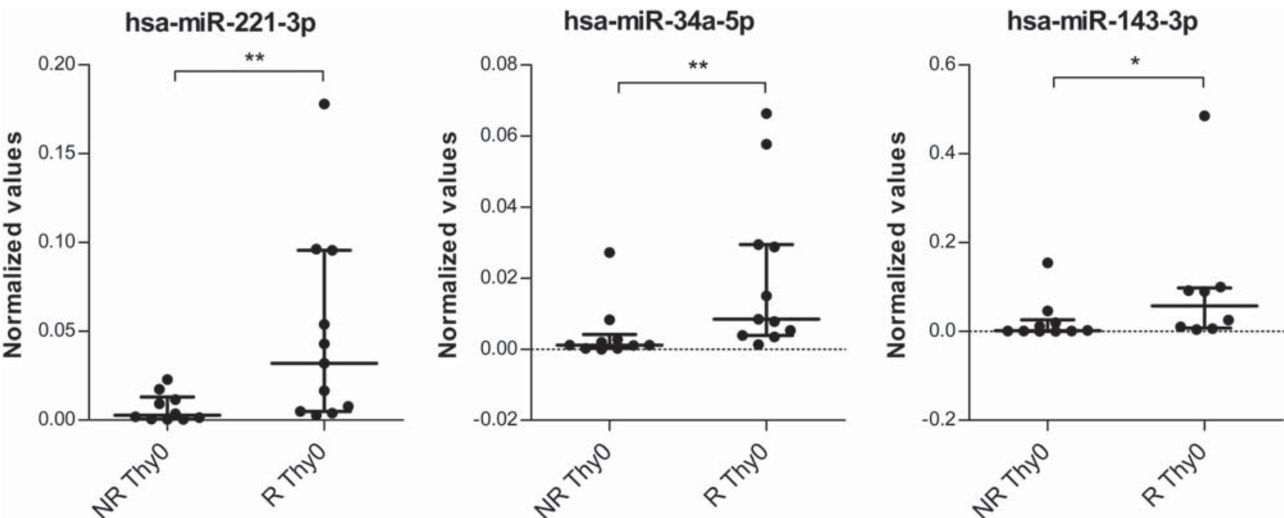

Figure 3. MiScript RT-quantitative PCR of miRNA expression levels in serum samples shows that 3 miRNAs are significantly upregulated in the serum of patients with recurrence compared with the patients in complete remission at basal Tg levels. NR, patients in complete remission; R, patients with persistent/recurrent disease; Thy0, serums taken at basal Tg levels. Lines represent median with interquartile range. $*P < 0.05$; $**P < 0.01$.

The results of LNA miRCURY qPCR showed that among the selected miRNAs, 3 have significant differences in the compared groups (Fig. 4). MiR-143-3p levels were lower in the group of patients with persistent/recurrent disease than in those with complete remission (unpaired *t* test, $P = 0.001$, log2 fold change = -1.15); miR-182-5p levels were higher in the patients with persistent/recurrent disease than in patients with complete remission under rhTSH stimulation (unpaired *t* test, $P = 0.042$, log2 fold change = 0.8) and miR-146b-5p levels were downregulated after rhTSH stimulation in patients with persistent/recurrent disease (unpaired *t* test, $P = 0.044$, log2 fold change = -0.7). Although these miRNAs showed differences between the 2 groups that were statistically significant, the magnitude (fold change) was small, and we do not consider them clinically relevant. Also, these results were not concordant with the mirScript analysis, showing different trends in the levels of miRNAs in the compared groups. Even when we selected only the samples that overlap between the 2 qPCR platforms, the results differed. Bearing this in mind, the results generated with LNA assays generally corroborate the findings from small-RNA sequencing in that no known miRNA can be an indicator of thyroid cancer recurrence in the circulation.

Putative Novel microRNAs Are Detected in the Serum of Patients After rhTSH Stimulation and Thyroid Cancer Cells

To explore our third goal, we searched for novel miRNAs in our sequencing data by using the miRPara software tool (37). With this tool, the detected sequences that do not match any known miR in miRBase but the structural properties of the genome in the indicated regions resemble that of known miRNAs are designated as putative, novel miRNAs. In this study, miRPara designated 92 sequences as being putative miRNA; they are listed in Supplementary Table 2 (31). The numbers assigned to these sequences are specific to this study only.

Next, we looked at the differences between the expression levels of these putative miRNAs in our study groups. We saw no differences between nonstimulated groups of patients in remission and patients with recurrence. However, 5 sequences showed significant differences in the expression in patients with recurrence before and after rhTSH treatment (put-miR-79, put-miR-29, put-miR-17, put-miR-35, put-miR-49; Table 2). We then aimed to validate these findings with LNA qPCR. Because put-miR-29 also showed significant differences in patients with complete remission before and after rhTSH treatment, it was excluded as a potential marker. Among the 4 left, we validated the expression of 2 sequences with the highest fold changes, put-miR-17 and put-miR-49, with LNA qPCR. We checked the expression using the discovery study cohort with additional 2 samples from validation study cohort ($N = 9$, Table 1). We were able to detect the same difference in expression levels of put-miR-49 (paired *t* test, $P = 0.03$, log2 fold change = 1.6) (Fig. 5A), which showed upregulation upon rhTSH administration. Interestingly, samples that had a pronounced rise in put-miR-49 expression levels after rhTSH stimulation had evidence of structural recurrence. The levels of put-miR-17 also showed a trend of rising after rhTSH stimulation, implying a possibility that its detection would reach significance should the number of patients be increased. Additionally, levels of put-miR-49 showed a strong positive correlation with the levels of thyroglobulin (Pearson coefficient = 0.623; $P = 0.01$). Moreover, we further analyzed an independent cohort of patients with PTC ($n = 11$, 5 recurrent disease and 6 in remission) and confirmed that put-miR-49 significantly increased upon rhTSH administration only in patients with recurrent disease and correlated with the levels of thyroglobulin (Pearson coefficient = 0.866; $P = 0.01$) (Fig. 5B).

Finally, we wanted to analyze if these sequences could be detected in immortalized human thyroid cancer cell lines and whether they change in response to TSH stimulation. TPC-1, FTC-133, and OCUT-2 cell lines were treated with increasing levels of TSH, aiming to resemble concentrations used for patients (0.5 mIU/mL, 5 mIU/mL, and 50 mIU/mL). We first checked that the 3 cell lines expressed TSH receptor and then confirmed the activation of TSH receptor by measuring the levels of CREB1 and pCREB (Supplementary Figure 2) (31). When we analyzed the levels of put-miR-17 and put-miR-49,

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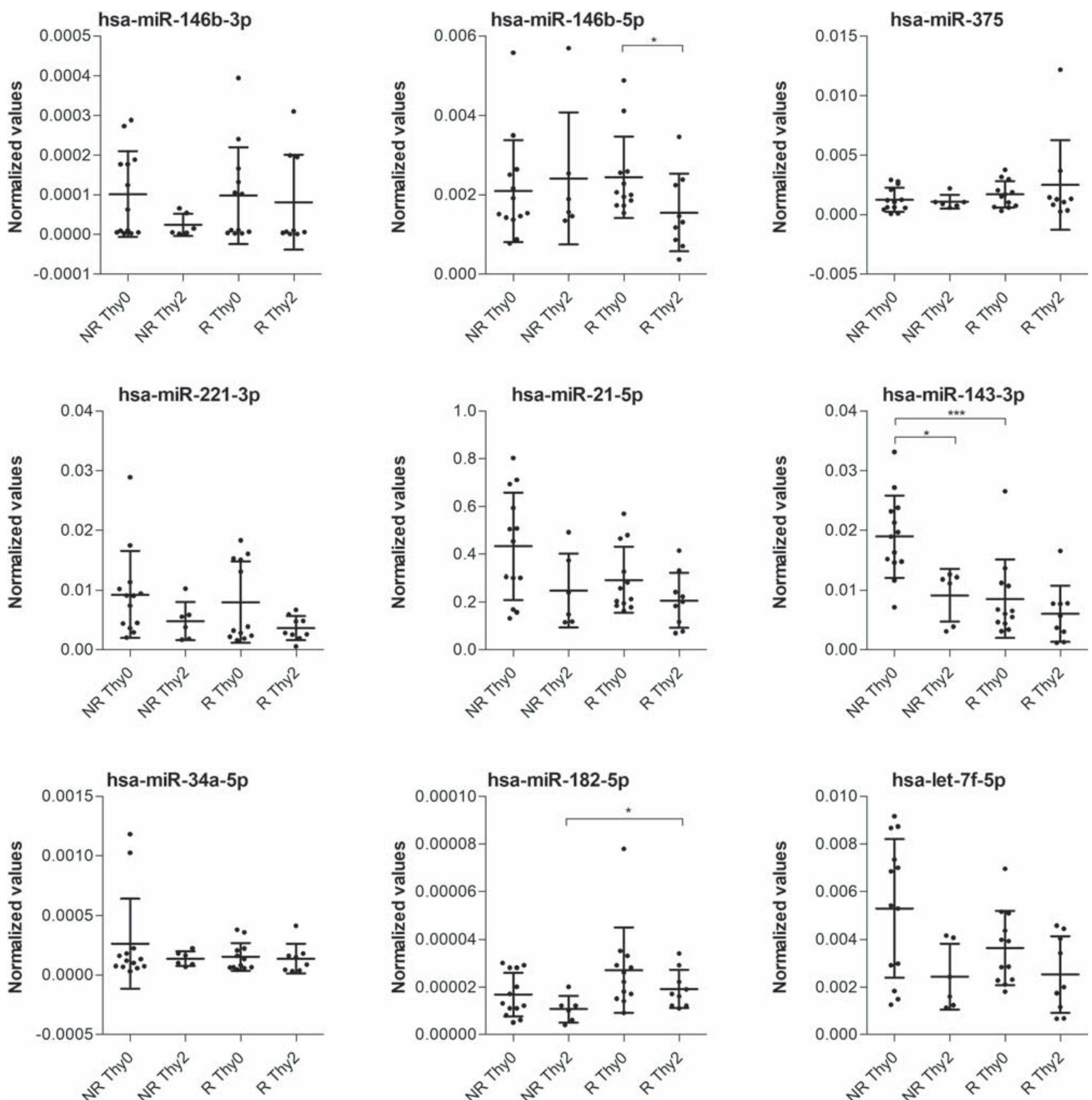

Figure 4. MiRCURY RT-quantitative PCR of miRNA expression levels in serum samples shows that the levels of miR-143-3p are lower in the group of patients with persistent/recurrent disease than in those with complete remission; miR-182-5p levels are higher in the patients with persistent/recurrent disease than in patients with complete remission at stimulated Tg levels and miR-146b-5p levels are downregulated after rhTSH stimulation in patients with persistent/recurrent disease. NR, patients in complete remission; R, patients with persistent/recurrent disease; Thy0, serums taken at basal Tg levels; Thy2, serums taken at 72-hour stimulated Tg levels. Lines represent mean ± SD. $*P < 0.05$; $***P < 0.001$.

Table 2. Differentially expressed putative microRNAs detected by small-RNA sequencing in the serum of patients with recurrent/persistent disease before and after rhTSH stimulation

Transcript	R Thy0 TMM	R Thy2 TMM	Log Fold Change	*P* value	FDR
Put-miR-79	408.29	2922.57	2707	0.0000	0.005
Put-miR-29	383.43	4578.14	3399	0.0001	0.005
Put-miR-17	351.86	4259.29	3446	0.0004	0.013
Put-miR-35	997.29	5108.86	2321	0.0007	0.014
Put-miR-49	188.71	8372.29	5290	0.0008	0.014

Differences are considered significant if FDR < 0.05.
Abbreviations: FDR, false discovery rate calculated after Benjamini-Hochberg correction for multiple testing; TMM, trimmed mean values.

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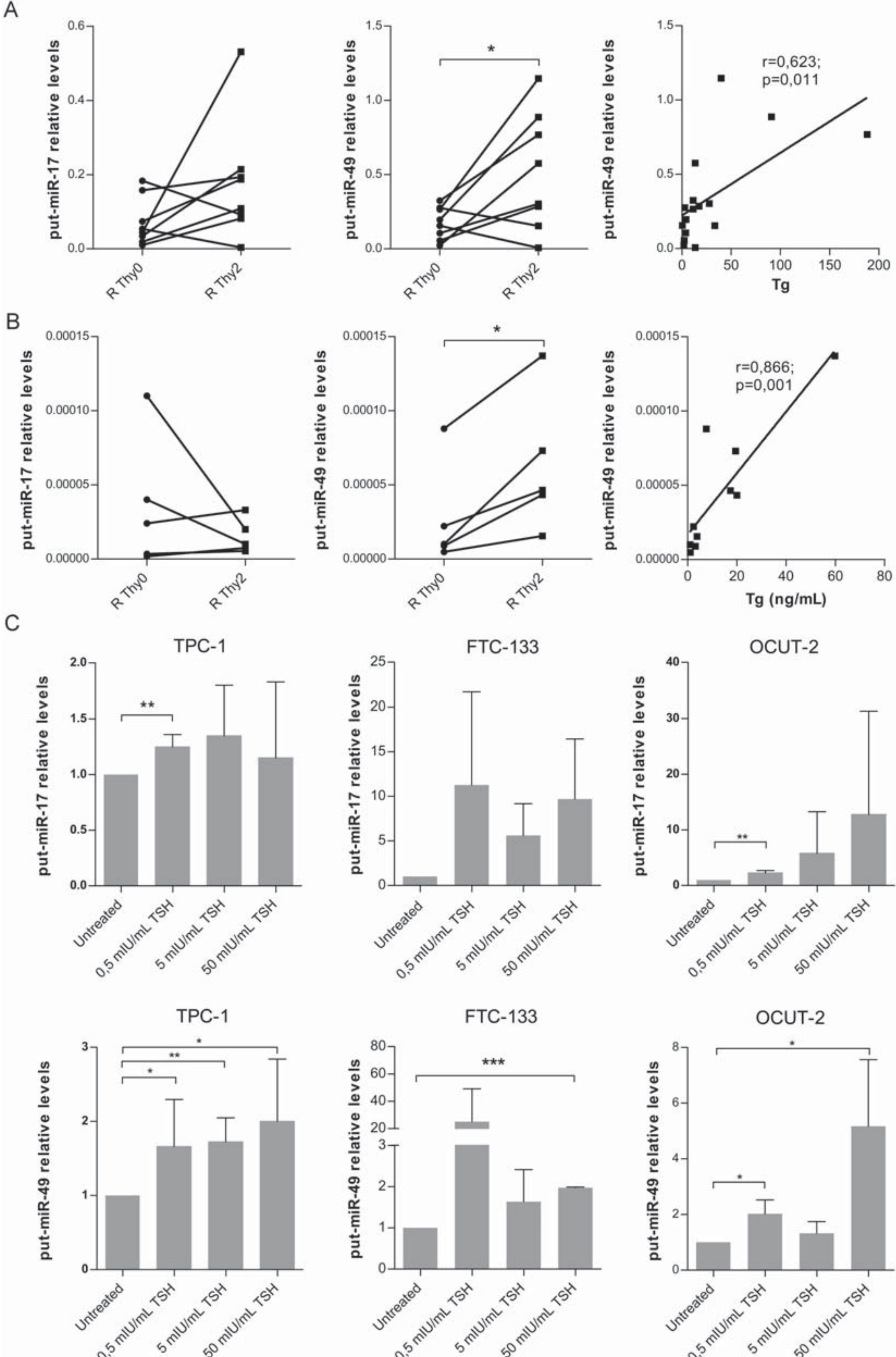

Figure 5. The expression of putative novel microRNAs in serums of patients with persistent/recurrent disease before and after rhTSH stimulation and TSH-stimulated TPC-1, FTC-133, and OCUT-2 cell lines measured by miRCURY RT-qPCR. (A) In the first validation cohort and (B) in the second, independent validation cohort the levels of put-miR-17 show no difference whereas put-miR-49 expression levels rise in the serum of patients after rhTSH stimulation and put-miR-49 shows a strong positive correlation with the levels of Tg. (C) Expression of put-miR-17 and put-miR-49 is rising in TPC-1, FTC-133, and OCUT-2 cell lines stimulated with different concentrations of TSH. R Thy0, serums taken at basal Tg levels from patients with recurrence; Thy2, serums taken at 72 hours stimulated Tg levels from patients with recurrence. Bars represent mean ± SD results from 3 measurements; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

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we detected consistently increasing levels of both sequences in the 3 cell lines, which was in accordance with what we observed in the human serum (Fig. 5C).

Sequencing Small RNAs Reveals Stably Expressed miRNAs Across all Samples

One of the advantages of using small-RNA sequencing is detecting miRNAs that are stably expressed across all samples. This may help to select proper endogenous control for circulating microRNA normalization in specific clinical settings (ie, thyroidectomized patients on levothyroxine treatment). Analyzing the data with Normfinder, a software tool for endogenous control discovery, 25 miRNAs with a stable and sufficient expression levels were unveiled (Supplementary Table 3) (31). Using Stability/TPMaverage as a measure of stability, mir-24-3p was shown to have the highest score. Additionally, this analysis showed that miR-16-5p, an miRNA often used as a normalizer in serum miRNA qPCR studies (24, 25), has high stability across samples. These miRNAs can be good candidates for normalizers/housekeeping genes in qPCR studies using serum samples of patients who have been treated for thyroid cancer with total thyroidectomy followed by RAI ablation, independently of the levels of TSH.

Discussion

The aim of this study was to assess the utility of circulating miRNAs in thyroid cancer follow-up. To the best of our knowledge this is the first study that performed small RNA sequencing (NGS) followed by extensive qPCR validation to establish the circulating miRnomes of patients with thyroid cancer recurrence. However, using this approach based on NGS as a discovery method, followed by 2 qPCR platforms to validate its findings, we could not demonstrate any consistent differences in serum profiles of known miRNAs between patients with or without recurrent/persistent disease, not even after TSH stimulation. On the other hand, sequencing results revealed a significant upregulation upon rhTSH administration in the levels of several sequences characterized as putative miRNAs. We have validated with qPCR that 1 of these putative miRNAs, named put-miR-49, is upregulated in the serum of patients with recurrent/persistent disease after TSH stimulation. We have also shown that the levels of this novel putative miRNA are upregulated in three thyroid cancer cell lines after TSH stimulation.

Few studies have addressed the question of whether circulating miRNAs can be used in the follow-up of thyroid cancer. A study from Italy (29) screened the levels of 754 different miRNAs using TaqMan Array Cards in the serum of patients with PTC before and 30 days after surgery and validated the results with qPCR (TaqMan Assays). This study pinpointed several miRNAs whose levels decreased after surgery and further observed through qPCR analysis that 2 of these miRNAs, miR-221-3p and miR-146a-5p, increased in 4 patients with structural recurrence after 1 to 2 years during the follow-up but remained low in the group of patients in complete remission. Of note, miR-221-3p was not detected in their initial screening method, it was included a posteriori in the qPCR analysis based on the evidence supported by the literature. In our study, we detected increased levels of miR-221-3p through 1 of our qPCR platforms (mirScript) but failed to detect any differences in another more specific qPCR platform (LNA) and in our NGS analysis. A second study by Zhang et al (30) tested through qPCR the levels of several preselected miRNAs based on the literature. They showed that miR-221, miR-222, and miR-146b were significantly lower in the serum of patients in complete remission (n = 9) compared with patients that had thyroid cancer recurrence (n = 12). This study had no high-throughput method such as screening or discovery analysis. The discrepancies between this study and our study may arise from the different patient cohorts (Caucasian vs Chinese cohort) but also from the different detection techniques (miRcute vs miRCURY LNA).

The importance of the technique used to detect circulating miRNAs has been explored thoroughly in the miRQC study (36). This seminal paper alerted about studies reaching different conclusions in similar clinical settings, possibly because of a variety of methods used for generating results, which creates difficulties in comparing these studies. For instance, in patients with benign and malignant thyroid nodules, studies that used different high-throughput methods for screening the levels of circulating miRNAs have suggested different miRNAs to be differentially expressed (25, 29, 38, 39), some of which had questionable thyroid origin never being detected in primary thyroid tumors (27, 40). The miRQC study made a great effort to compare the performance of 12 platforms from 3 different technologies (NGS, microarray cards, and qPCR) used both for screening and specific validation of miRNA levels in human serums under the same preanalytical conditions. The authors pointed out that studies analyzing differential miRNA expression should use at least 2 different technologies for validation of results, and we followed this recommendation. In our study, we used the Illumina NGS platform for the complete miRNA profiling and validated these results with 2 different qPCR-based platforms: miScript qPCR and miRCURY LNA qPCR. The results of NGS correlated with miRCURY LNA, whereas miScript showed distinct results. According to the miRQC performance metrics, Illumina NGS platform has superior performance as a screening method compared with microarray cards. The miRCURY LNA qPCR kit has the highest specificity among all methods, showing no cross-reactivity between similar miRNA sequences. Also, miRCURY LNA shows superiority in sensitivity in relation to the sample input given that sample size for this platform was over 10× lower compared with the other platforms. MiScript performs well in terms of accuracy and sensitivity but has a lower score for specificity. Moreover, miRCURY LNA is a qPCR method that has the highest correlation with sequencing platforms. Taking this into account, the results we obtained by both Illumina NGS and miRCURY LNA seem to be a more accurate representation of miRNA levels in the selected groups. To the best of our knowledge, our study is the only one that used NGS sequencing rather than microarray cards in the screening part of the research.

In our study, although the results of NGS mostly correlated with miRCURY LNA qPCR under the same preanalytical conditions, we did observe some discrepancies in that we detected significant differences in the levels of 3 miRNAs with miRCURY LNA qPCR that were not seen with NGS. However, it is known and once again proved in the miRQC study that qPCR platforms generally have much higher sensitivity than NGS, so we argue that this is the reason for the discrepancy. Still, the observed changes in miRNA levels on qPCR are very small and unlikely to be related with thyroid cancer recurrence, representing thyroid-independent events, and therefore corroborating the results of sequencing.

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In addition, the number of samples subjected to sequencing was small, and some differences might be detected in a larger sample size.

We are aware that in our study there are some preanalytical variabilities affecting mainly the 2 qPCR methods used. Different extraction kits for RNA isolation were employed, not all samples for miScript were checked for hemolysis, and we used 2 different miRNAs as endogenous controls in each qPCR. However, RNA was isolated using the same extraction principles (phenol/chloroform extraction followed by silica-membrane purification), most samples (70%) were negative for hemolysis contamination, and the 2 endogenous controls were proved to be stable in the different clinical conditions of the cohort. These differences may have contributed to a certain degree to the differences between qPCR results, stressing even more the need for standardization of microRNA research methods, and may help to make better experimental decisions.

Our sequencing analysis also detected sequences strongly suggested to be novel miRNAs. The script used for predicting and testing if a sequence might be a miRNA, miRPara, is based on 25 important biological characteristics of a miRNA considering, among others, size (length and number of paired bases in the hairpin structure), stability (mean free energy of secondary structure), and sequence (GC content and first base of miRNA). Experimental evidence has shown that these are good criteria for proving that a sequence is a bona fide miRNA (41, 42). More importantly, 2 of the novel miRNAs detected in our sequencing analysis were further validated through qPCR in the serum of the patients, and 1 showed a strong positive correlation with the levels of thyroglobulin. Moreover, we detected both miRNA sequences to be expressed in 3 human thyroid cancer cell lines (TPC1, FTC-133, and OCUT-2) being upregulated under TSH stimulation. We are currently obtaining more experimental evidence to fully characterize these promising putative miRNAs. Taken together, these results show that put-miR-49 has a high potential for being a novel miRNA and deserves further investigation on its role in thyroid cancer pathogenesis.

This study has some limitations and strengths. One of the limitations was that we used a relatively small sample size for both profiling and validation, all of them belonging to a single center. Because PTC recurrence is not a common event, not many samples with thyroid cancer recurrence could be collected. Therefore, we cannot rule out that a larger sample size could detect significant differences among groups, although it is unlikely that the magnitude of such difference would be clinically relevant, particularly when compared with Tg, the current biomarker for thyroid cancer recurrence. The strength of this study lies in several points. First, our study was performed using the highest available standards for the technical execution in the field of miRNA research. We performed extensive quality control of the samples used in NGS and LNA assays. We controlled for critical determinants of miRNA circulating levels such as hemolysis, the RNA isolation process, and the reverse transcription efficiency using specific markers and spike-ins. We also carefully selected endogenous controls for qPCR that were confirmed to be stably expressed in all samples. Next, we opted for using NGS as the most accurate high-throughput analysis method, and a different technology (ie, qPCR) for confirming these results. We took a step forward in strengthening the conclusions from NGS by using 2 different platforms for qPCR, 1 of which is superior to all other qPCR platforms in terms of specificity and reproducibility. A second strength lies in that most of the patients had the same histological type (PTC) and the same clinical condition (ie, all the patients were treated in the same way with total thyroidectomy followed by radioiodine ablation and TSH suppression), making the cohort highly homogenous. Moreover, the discovery phase revealed stably expressed miRNAs that can be potential endogenous controls for qPCR experiments performed in similar cohorts. Finally, by means of small-RNA sequencing, we discovered sequences that might represent novel miRNA molecules, with one of them showing a potential of being related to thyroid cancer pathogenesis.

In summary, our study is the first to perform small-RNA sequencing for profiling of miRNAs in serum of patients monitored for thyroid cancer recurrence, showing that no known miRNAs can be used as markers of recurrence. However, our results point to a putative novel miRNA, put-miR-49, that rises with rhTSH stimulation in the serum of patients with recurrence and in 3 thyroid cancer cell lines. Further functional studies are needed to confirm this sequence is indeed an miRNA and strengthen its link with carcinogenesis of thyroid cancer. Finally, our study showed a necessity for the field to tackle the challenge of standardizing the methodology for miRNA analysis in low sample amount such as plasma and serum.

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Disclosures

The authors have nothing to disclose.

Data Availability

Some or all data generated or analyzed during this study are included in this published article or in the data repositories

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listed in References (31) (Supplementary material, DOI: https://doi.org/10.6084/m9.figshare.17019077.v3) and (33) (small RNA sequencing data deposited in GEO expression omnibus under accession number GSE173248).

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