ISSN 2472-1972

Identification of Thyroid-Associated Serum microRNA Profiles and Their Potential Use in Thyroid Cancer Follow-Up

Francesca Rosignolo,[1]* Marialuisa Sponziello,[1]* Laura Giacomelli,[2] Diego Russo,[3] Valeria Pecce,[1] Marco Biffoni,[2] Rocco Bellantone,[4] Celestino Pio Lombardi,[4] Livia Lamartina,[1] Giorgio Grani,[1] Cosimo Durante,[1] Sebastiano Filetti,[1] and Antonella Verrienti[1]

[1]Dipartimento di Medicina Interna e Specialità Mediche and [2]Dipartimento di Scienze Chirurgiche, Università di Roma "Sapienza", Viale del Policlinico 155, 00161 Rome, Italy; [3]Dipartimento di Scienze della Salute, Università di Catanzaro, 88100 Catanzaro, Italy; and [4]U.O. Chirurgia Endocrina e Metabolica, Policlinico "A. Gemelli", Università Cattolica del Sacro Cuore, L.go A. Gemelli 8, 00168 Rome, Italy

Context: Trends toward more conservative management of papillary thyroid cancer (PTC) diminish the primacy of serum thyroglobulin (Tg) assays as a posttreatment surveillance tool.

Objective: To identify thyroid tumor-associated microRNAs (miRNAs) in the serum with potential for development as unique biomarkers of PTC recurrence.

Methods: We measured expression of 754 miRNAs in serum samples collected from 11 patients with PTC before and 30 days after thyroidectomy. Major candidates were then re-evaluated by absolute quantitative polymerase chain reaction analysis in an independent cohort of patients with PTC (n = 44) or benign nodules and 20 healthy controls (HCs). The 2 miRNAs most significantly associated with thyroid tumors were then assessed in matched serum samples (before and 30 days and 1 to 2 years after surgery) from the 20 PTC patients with complete follow-up datasets and results correlated with American Thyroid Association (ATA) responses to therapy.

Results: Eight miRNAs (miR-221-3p, miR-222-3p, miR-146a-5p, miR-24-3p, miR-146b-5p, miR-191-5p, miR-103a-3p, and miR-28-3p) displayed levels in prethyroidectomy serum samples from patients with PTC that significantly exceeded those measured after thyroidectomy and those found in samples from HCs. The 2 most promising candidates—miR-146a-5p and miR-221-3p —were further analyzed in the 20 PTC patients mentioned earlier. Serum levels of both miRNAs after 1 to 2 years of follow-up were consistent with ATA responses to therapy in all patients, including 2 with structural evidence of disease whose Tg assays remained negative (<1 ng/mL).

Conclusion: miR-146a-5p and miR-221-3p hold remarkable promise as serum biomarkers for post-treatment monitoring of PTC patients, especially when Tg assay results are uninformative.

Copyright © 2017 by the Endocrine Society

This article has been published under the terms of the Creative Commons Attribution License (CC BY-NC-ND; https://creativecommons.org/licenses/by-nc-nd/4.0/).

Freeform/Key Words: papillary thyroid carcinoma, microRNA, circulating, biomarker, follow-up

Serum thyroglobulin (Tg) is the main biomarker for detecting persistent/recurrent thyroid cancer [1–3], although its specificity is limited in the presence of anti-Tg autoantibodies and

*These authors contributed equally to this study.

Abbreviations: ATA, American Thyroid Association; BN, benign nodule; cDNA, complementary DNA; Ct, cycle threshold; HC, healthy control; miRNA, microRNA; NF, normalization factor; PTC, papillary thyroid cancer; ROC, receiver-operating characteristic; RRA, radioiodine remnant ablation; Tg, thyroglobulin

Received 25 October 2016
Accepted 8 December 2016

residual normal thyroid tissue. The latter is being encountered with increasing frequency owing to the declining use of total thyroidectomy followed by radioiodine remnant ablation [4–13]. Consequently, the search is on for new, more specific tumor biomarkers to ensure optimal postoperative follow-up of today's thyroid cancer patients.

One of the most promising solutions involves measuring the levels of tumor-derived microRNAs (miRNAs) in the bloodstream. The tissue-specific expression patterns of these RNA species and their high stability in biological fluids are well established [14]. Dysregulated miRNA expression has been documented and characterized at the tissue level in many malignancies, including papillary thyroid cancer (PTC), the most common form of differentiated thyroid cancer [15–19]. Less is known about the circulating miRNAs derived from normal and neoplastic thyroid tissues. Attempts have been made to identify miRNAs associated with thyroid tissue in general [20–22], thyroid tumor tissue [20–25], and specific clinicopathological variables of thyroid tumors [20, 22, 23, 25]. However, the few studies that have explored these issues have generated discordant results. The discrepancies can probably be attributed in large part to the variety of approaches used to quantify circulating miRNAs (*e.g.*, RNA sequencing *versus* real-time polymerase chain reaction *versus* microarrays, as well as miRNA profiling *versus* selected miRNA panels) and other methodological differences (*e.g.*, characteristics and size of patient and control groups, sampling time, or use of plasma or serum).

The aim of the current study was to identify circulating miRNAs that might be developed into thyroid cancer biomarkers for use during postoperative follow-up. To this end, we set out to identify (1) circulating thyroid-derived miRNAs by analyzing the expression of 754 miRNAs in preoperative and postoperative serum samples from patients with PTC; (2) thyroid tumor–derived miRNAs by analyzing serum levels of a selected panel of miRNAs in healthy controls (HCs), patients with benign thyroid nodules, and patients with PTC; and (3) the potential value of selected miRNAs as clinical biomarkers of PTC by analyzing their postoperative serum levels in a subset of patients with PTC classified according to the response-to-therapy criteria recommended by the American Thyroid Association (ATA) [1].

1. Materials and Methods

A. Study Design and Patient Samples

We conducted a prospective observational study from 2012 to 2016 at the Sapienza University of Rome Hospital. Two cohorts were enrolled (Table 1). The screening cohort consisted of 11 patients who consecutively underwent total thyroidectomy for sporadic PTCs. The validation cohort included 44 patients with sporadic PTCs consecutively treated with total thyroidectomy and 39 age- and sex-matched controls. Nineteen of the 39 controls in the validation cohort were patients who underwent total thyroidectomy for nodules with indeterminate cytology (Bethesda Class IV) that were subsequently diagnosed as benign on the basis of surgical histology (benign nodule [BN] group). The other 20 were HCs seen in the hospital's outpatient clinics for periodic health assessments or complaints unrelated to the thyroid. All the HCs had negative workups and no evidence of thyroid-disease based on the results of a screening examination performed by the study team (complete patient and family histories, neck ultrasound findings, and results of thyroid hormone and thyroid antibody assays).

The protocol called for collection of serum samples from enrolled participants according to the following schedule. For PTC patients of both cohorts and BN patients in the validation cohort, we collected a preoperative sample on the day of thyroidectomy, right before induction of anesthesia. A second sample was collected from PTC patients 30 days after surgery. For HCs in the validation cohort, a single sample was taken at study enrollment. Blood samples (8 to 10 mL) were collected in a BD Vacutainer® Tube (Becton, Dickinson and Company, Melbourne, Australia) and centrifuged at 3000 rpm for 10 minutes, and the serum obtained was stored at –80°C prior to analysis.

Clinical data on PTC patients were collected from hospital and outpatient clinic charts. Tumors were staged according to the American Joint Committee on Cancer criteria [26].

Table 1. Characteristics of PTC Patients in the 2 Cohorts

Clinicopathological Features	Screening Cohort (n = 11)	Validation Cohort (n = 44)
Age in years at diagnosis (median, range)	47 (23–67)	46 (22–75)
Sex (n, rate)		
Female	7 (64%)	34 (77%)
Male	4 (36%)	10 (23%)
Tumor size in millimeters (median, range)	11 (5–33)	10 (1–60)
Tumor foci (n, rate)		
Unifocal	9 (82%)	31 (70%)
Multifocal	2 (18%)	10 (23%)
NA	0	3 (7%)
Extrathyroidal extension (n, rate)		
No	5 (45%)	23 (52%)
Yes, microscopic	6 (55%)	17 (39%)
Yes, macroscopic	0	1 (2%)
NA	0	3 (7%)
AJCC/UICC T class (n, rate)		
T1a	4 (36%)	13 (30%)
T1b	1 (9%)	5 (11%)
T2	0	4 (9%)
T3	6 (55%)	18 (41%)
T4a	0	1 (2%)
NA	0	3 (7%)
AJCC/UICC N class (n, rate)		
Nx	7 (64%)	14 (32%)
N0	1 (9%)	15 (34%)
N1	3 (27%)	15 (34%)
N1a	2 (18%)	5 (11%)
N1b	0	7 (16%)
NA	1 (9%)	3 (7%)
AJCC/UICC M class (n, rate)		
Mx	10 (91%)	25 (57%)
M0	1(9%)	16 (36%)
M1	0	3 (7%)
2009 ATA risk (n, rate)		
Low	4 (36%)	19 (43%)
Intermediate	7 (64%)	21 (48%)
High	0	4 (9%)
Radioiodine remnant ablation (n, rate)		
Yes	5 (45%)	10 (23%)
No	0 (0%)	24 (54%)
NA	6 (55%)	10 (23%)

Continuous variables are reported as median values and ranges; categorical variables are reported as absolute numbers and percentages.
Abbreviation: AJCC/UICC, American Joint Committee on Cancer/ Union Internationale Contre le Cancer; M, metastasis; N, node; NA, information not available; T, tumor.

Risks of recurrence were classified as high, intermediate, or low using ATA-recommended criteria [27]. Posttreatment surveillance involved visits every 1 to 2 years, each including a serum Tg assay (DYNO test Tg-plus, Brahms Diagnostics GmbH, Berlin, Germany; functional sensitivity, 0.2 ng/mL), radioimmunometric assay of circulating Tg antibodies (Architect System Anti-Tg, Abbott Laboratories, Abbott Park, IL; functional sensitivity, 0.31 IU/mL), and high-resolution grayscale and color Doppler ultrasound of the thyroid bed and cervical lymph node compartments. Outcomes at the 1- to 2-year visit were classified according to the response-to-therapy criteria recommended by the ATA in its 2015 guidelines [1]. For the purposes of the study, 3 response categories were considered: (1) structural incomplete responses, (2) biochemical incomplete responses, and (3) excellent or indeterminate responses.

The study protocol was approved by the local ethics committee, and written informed consent was obtained from all patients whose serum samples were analyzed.

B. RNA Isolation From Serum Samples

Total RNA (containing small RNAs) was extracted from 200 μL of serum using the miRNeasy Serum/Plasma kit and *cel-miR-39* as the spiked-in control (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. To perform miRNA profiling without the preamplification step, we used a multiple extraction approach, which involved isolation of total RNA from up to 5 200-μL aliquots of each serum sample. This solution allowed us to obtain over 1000 ng of total RNA from each sample. The quality and quantity of RNA samples were verified with a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA).

C. Circulating miRNA Analysis

C-1. Screening cohort

miRNA profiling was performed with TaqMan Array Human MicroRNA A+B Cards, version 3.0 (Thermo Fisher Scientific, Inc.), a set of 2 384-well microfluidic cards that allow quantitative expression analysis of 754 miRNAs. As noted earlier, reverse transcription and real-time polymerase chain reaction reactions were performed without a preamplification step, in accordance with the manufacturer's instructions, which call for preamplification only if the total amount of RNA is <350 ng. Expression Suite software, version 1.0.3 (Thermo Fisher Scientific, Inc.), was used to calculate cycle threshold (Ct) values (cutoff, 35) and for data analysis. U6 was chosen as the endogenous control because it displayed the lowest between-sample variance, and relative miRNA expression levels were calculated using the comparative $2^{-\Delta\Delta Ct}$ method. Differences in preoperative and postoperative miRNA expression levels were considered significant if the fold change was >2 or <0.5 and $P \leq 0.05$.

C-2. Validation cohort

The miRNAs analyzed in the validation cohort were selected on the basis of the results of circulating miRNA profiling in the screening cohort and data in the literature (see "Results" for details). Expression levels were quantified in duplicate using specific TaqMan MicroRNA Assays (Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. Circulating miRNA abundance was quantified with the aid of standard curves constructed with miScript miRNA mimics (Qiagen), synthetic RNA oligonucleotides corresponding to mature miRNA sequences (miRBase Release, version 21). The mimics were reverse transcribed with specific reverse transcription primers (5x) over an empirically derived range of copy numbers, and 10-fold serial dilutions of the corresponding complementary DNA (cDNA) were used to generate standard curves for each miRNA TaqMan assay. The absolute number of miRNA copies was determined from each sample Ct using the following equation: $y = kx + m$, where y is the Ct value, k is the slope, x is the logarithm of the number of miRNA copies, and m is the y intercept. A normalization factor (NF) was calculated for each sample using the following formula: NF = 1/[2(median spike in Ct value) – (spike in average Ct value of the given sample)]. A normalized copy number value was then obtained for each sample by multiplying the number of copies of the given miRNA by the NF. Normalized copies of each miRNA in the quantitative polymerase chain reaction were converted to normalized copies of miRNA per nanogram of cDNA loaded into the reaction mix (total: 0.4 ng per sample) and then to normalized copies of miRNA per milliliter of serum (considering the total amount of RNA isolated from 200 μL of serum).

D. Statistical Analysis

The Mann-Whitney test was used to assess differences in miRNA expression levels between preoperative and postoperative serum samples. The Kruskal Wallis test followed by Dunn's multiple comparisons test was used when more than 2 groups were compared. P values lower than 0.05 were considered statistically significant. All these analyses were performed with GraphPad Prism software, version 5.0 (GraphPad Software Inc., San Diego, CA). Receiver-operating characteristic (ROC) curves and areas under the ROC curve were analyzed with the *p*-ROC package in R software, version 3.1.1 [28, 29], using the Youden Index and the DeLong method.

3. Results

A. Screening Analysis

miRNA profiling was performed on preoperative and 30-day postoperative serum samples from the PTC patients of the screening cohort. Of the 754 miRNAs that were quantified, 11 displayed significantly lower levels in the 30-day postoperative samples than in the samples collected right before thyroidectomy (Supplemental Table 1). None of the 754 miRNAs exhibited significantly increased expression after surgery.

B. Validation Analysis

The 6 miRNAs in Supplemental Table 1 whose expression declined most significantly after thyroidectomy (defined arbitrarily as $P < 0.02$ *versus* mean preoperative level) were investigated further in the validation cohort. Four additional miRNAs that did not meet this criterion were also included in the validation analysis because their association with PTC is supported by substantial evidence in the literature. They included miR-146b-5p and miR-221-3p, whose circulating levels have been reported by others to decrease significantly after surgery for PTC and whose expression in PTC tissues has proved to be substantially upregulated with respect to normal thyroid tissue in multiple studies [19, 21, 24, 30], and miR-95-3p and miR-190a-5p, which were identified as circulating thyroid tumor miRNAs in a large, methodologically robust study that included both screening and validation steps [23]. Levels of these 10 miRNAs (miR-146a-5p, miR-28-3p, miR-103a-3p, miR-222-3p, miR-191-5p, miR-24-3p, miR-146b-5p, miR-221-3p, miR-95-3p, and miR-190a-5p) were then analyzed in serum samples from the 44 patients with sporadic PTCs (preoperative and 30-day postoperative), 19 patients with benign nodular disease (preoperative only), and 20 HCs (study-entry sample).

Levels of miR-95-3p and miR-190a-5p were very low in all the serum samples we tested—in many cases, below the detection limits of our assay (Ct values higher than 35). This precluded comparative assessment of their expression in the various sample subgroups. Analysis of data on the remaining 8 miRNAs revealed that all were less abundant in the 30-day postoperative serum samples than in those collected before thyroidectomy (Fig. 1). The most significant decreases were observed for miR-146a-5p, miR-221-3p, and miR-222-3p ($P = 0.0007$, $P < 0.0001$, and $P < 0.0001$, respectively).

All 8 miRNAs—including 3 (miR-146b-5p, miR-221-3p, and miR-222-3p) that are reported overexpressed in PTC tissues [18, 19, 30]—were significantly more abundant in the preoperative sera from the PTC group than in sera from HCs (Fig. 1). None of the 8 miRNAs displayed expression levels in preoperative PTC serum samples that differed significantly from those in the BN group, although the mean preoperative serum level of miR-221-3p in the PTC group appreciably exceeded that found in samples from patients with BN (653,242,460 ± 490,942,066 copies/mL *versus* 316,148,953 ± 153,115,869 [$P = 0.076$]).

The 3 miRNAs that decreased most markedly after surgery in the PTC group (*i.e.*, miR-146a-5p, miR-221-3p, and miR-222-3p) were subjected to ROC curve analysis. All 3 displayed

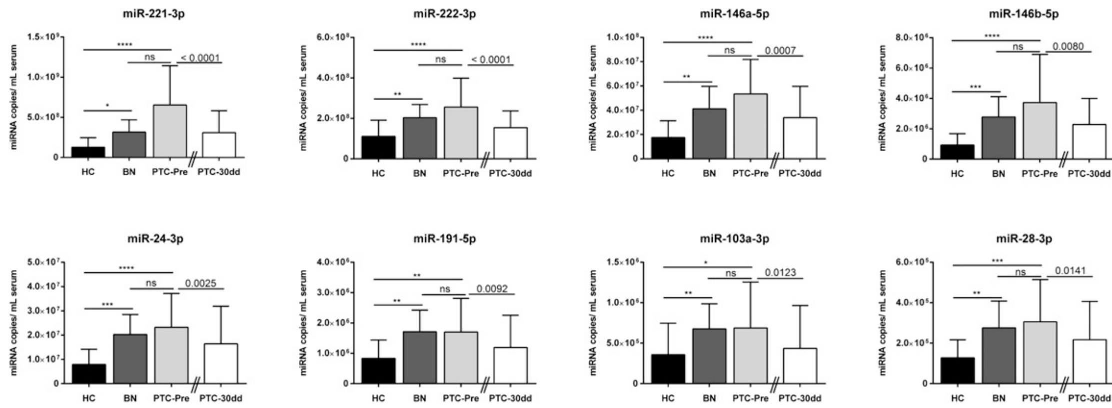
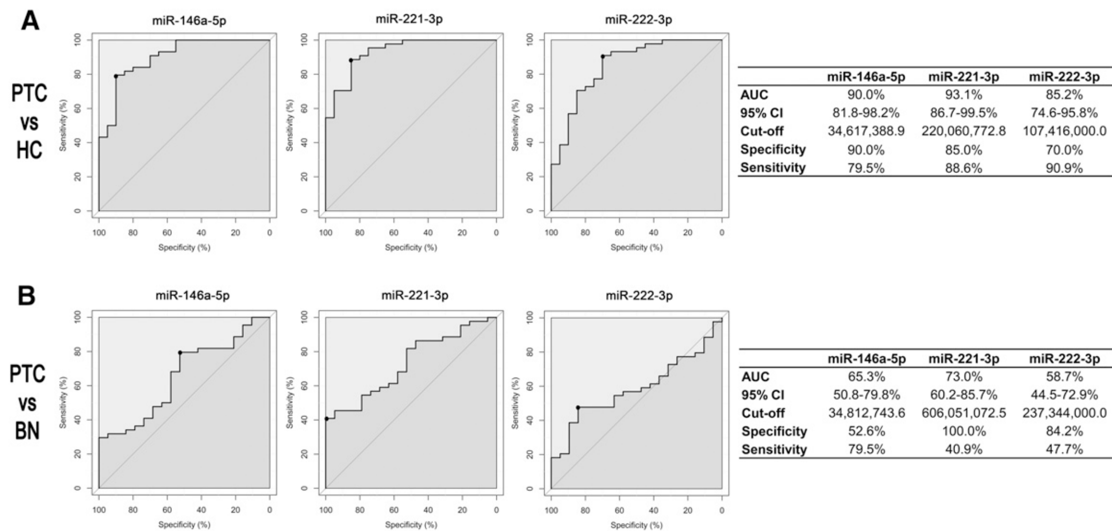


Figure 1. Validation of 8 miRNA serum levels in 44 patients with PTC (preoperative and 30-day postoperative serum samples), 19 BN patients (preoperative serum sample), and 20 HCs (study-entry serum sample). Data are reported as mean copies/mL ± standard deviation; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (Kruskal-Wallis test followed by Dunn's multiple comparison test for intergroup differences; Mann-Whitney test for intraindividual differences.) ns, not significant.

good accuracy in discriminating between PTC patients and HCs [Fig. 2(A)], but their performances declined substantially in the differentiation of PTCs and BNs [Fig. 2(B)]. In both comparisons, miR-146a-5p and miR-221-3p proved to be more accurate discriminators than miR-222-3p (Fig. 2).

These 2 miRs were therefore selected for further characterization. To this end, we analyzed their expression levels in additional serum samples collected 1 to 2 years after surgery from 20 of the 44 PTC patients (selected on the basis of availability alone). As shown in Fig. 3, at this point in time, 15 of the 20 patients had excellent or indeterminate responses to therapy as defined by 2015 ATA guidelines [1]. In these 15 cases, and in the single patient with a biochemical incomplete response, expression levels of miR-146a-5p and miR-221-3p decreased after surgery and remained low at the 1- to 2-year visit. Trends were very different in the 4 patients with structural incomplete responses at 1 to 2 years. In this subgroup, initial postoperative



	miR-146a-5p	miR-221-3p	miR-222-3p
AUC	90.0%	93.1%	85.2%
95% CI	81.8-98.2%	86.7-99.5%	74.6-95.8%
Cut-off	34,617,388.9	220,060,772.8	107,416,000.0
Specificity	90.0%	85.0%	70.0%
Sensitivity	79.5%	88.6%	90.9%

	miR-146a-5p	miR-221-3p	miR-222-3p
AUC	65.3%	73.0%	58.7%
95% CI	50.8-79.8%	60.2-85.7%	44.5-72.9%
Cut-off	34,812,743.6	606,051,072.5	237,344,000.0
Specificity	52.6%	100.0%	84.2%
Sensitivity	79.5%	40.9%	47.7%

Figure 2. ROC curve analyses performed to assess the diagnostic value of serum miR-146a-5p, miR-221-3p, and miR-222-3p levels for discriminating (A) between PTC patients and HCs and (B) between PTC patients and those with BNs. Tables on the right show areas under the curve (AUC) with 95% confidence intervals (95% CI) and cutoffs (indicated on curves as solid black circles and reported as copies per milliliter) with associated sensitivity and specificity.

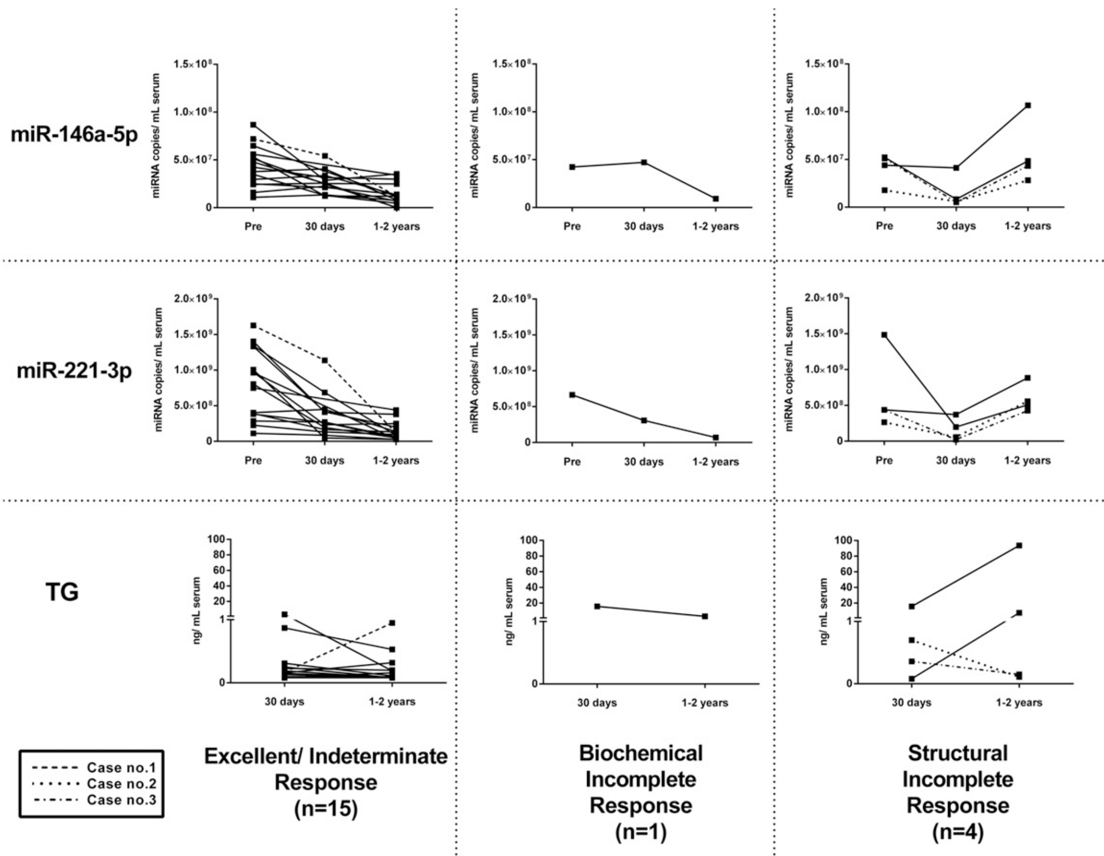


Figure 3. Levels of miR-146a, miR-221-3p, and Tg in preoperative and postoperative (30 days and 1 to 2 years after thyroidectomy) serum samples of PTC patients classified according to response-to-therapy classes defined by the ATA. Case 1: Patient with an indeterminate response and increasing serum Tg levels. Cases 2 and 3: Patients with structural incomplete responses and decreasing serum Tg levels that were consistently less than 1 ng/mL.

declines in serum miR-146a-5p and miR-221-3p levels were followed by increases to levels at the 1- to 2-year visit that were similar to or higher than those found prior to surgery.

Serum Tg levels measured in these 20 patients at the 30-day and 1- to 2-year visits were consistent with clinical findings and with circulating levels of the 2 miRNAs in all but 3 cases. The first exception (Fig. 3, case 1) was a patient with an indeterminate response consisting of negative cervical ultrasound findings and increasing serum Tg levels, which rose from 0.17 ng/mL (serum thyroid-stimulating hormone, 0.97 mIU/L) at 30 days to 0.95 ng/mL (serum thyroid-stimulating hormone, 0.2 mIU/L) at 1 year. Radioiodine remnant ablation (RRA) had been omitted in this case, and the patient's Tg antibody assays were also positive but declining overtime (from 61.4 IU/mL to 14.3 IU/mL). More consistent with the negative imaging data was the steady postoperative decline in circulating levels of miR-146a-5p and miR-221-3p. The other 2 exceptions (Fig. 3, cases 2 and 3) were also patients whose thyroidectomies had not been followed by RRA. Both were classified as having structural incomplete responses owing to sonographic findings of lymph node lesions with suspicious features [31]. In case 3, the metastatic nature of the nodal lesions had also been confirmed cytologically. Serum Tg levels in both cases remained consistently below the 1-ng/mL cutoff and were even lower at the 1- to 2-year assessment (0.11 and 0.15 ng/mL) than they had been 30 days after surgery (0.7 and 0.36 ng/mL). Tg antibody titers were low and declining in case 2 and always negative in case 3. In both cases, the rising serum levels of miR-146a-5p and miR-221-3p were more consistent with the presence of structural disease.

4. Discussion

For the growing population of patients with PTC, the 2015 ATA guidelines [1] recommend less extensive surgery, more selective use of RRA, and, in some cases, even active surveillance alone. One "side effect" of this change is a diagnostic gap in our posttreatment surveillance protocols. The reliability of serum Tg assay results declines substantially in patients with residual normal thyroid tissue. Consequently, clinicians are increasingly faced with a need for more accurate tumor biomarkers to monitor these patients for persistent/recurrent disease.

The results of our study suggest that serum miRNA assays are a promising tool for meeting this need. Our screening analysis of expression levels of 754 miRNAs in pre- and postthyroidectomy serum samples from PTC patients pinpointed 11 miRNAs (Supplemental Table 1) whose serum levels dropped significantly after thyroidectomy—a pattern suggestive of miRNAs derived largely from thyroid tissue. In the validation cohort, this list was refined to include 8 miRNAs: In addition to displaying down-regulated expression after thyroidectomy, these miRNAs appeared more likely to be closely associated with thyroid tumorigenesis, because their preoperative serum levels in PTC as well as in BN were significantly higher than those found in HCs (Fig. 1). Moreover, 3 (miR-146b-5p, miR-221-3p, and miR-222-3p) out of 8 miRNAs were previously reported overexpressed in PTC tissues [18, 19, 30]. In our series, expression of none of the 8 miRNAs was significantly different between BN and PTC patients, although the mean preoperative serum level of miR-221-3p in the PTC group was appreciably higher than that found in samples from patients with BN (653,242,460 ± 490,942,066 copies/mL *versus* 316,148,953 ± 153,115,869 copies/mL). ROC curve analyses revealed that miR-221-3p and miR-146a-5p were the most accurate in discriminating between PTC patients and HCs [Fig. 2(A)], but their performances declined substantially in the differentiation of PTCs and BNs [Fig. 2(B)]. To definitely ascertain the diagnostic role of miRNA, larger cohorts of patients will be needed. The 2 most promising miRNAs—miR-146a-5p and miR-221-3p—were subjected to additional analyses to further evaluate their potential roles in predicting tumor persistence or relapse. We measured their serum levels 1 to 2 years after surgery in a subset of 20 patients classified according to the response to treatment [1]. In the patients in this subset with excellent/indeterminate responses or biochemical incomplete responses, circulating levels of both miRNAs continued to decline between the 30-day and 1- to 2-year observation points. In contrast, in the patients with structural incomplete responses, serum miRNA levels at the 1- to 2-year visit were both higher than those at 30 days (Fig. 3). Serum levels of miR-146a-5p and miR-221-3p were also consistent with serum Tg assay results in all but 3 (15%) of the 20 patients analyzed (Fig. 3). Thus, both miRNA assays accurately predicted the disease status of all patients, and both were better for detecting lymph node metastases than serum Tg assays, which were consistently negative and even revealed decreasing levels between the 30-day and 1- to 2-year observation points. These findings are consistent with reports of late increases in the serum Tg level in certain settings characterized by documented recurrence/persistence [32]. Of note, alterations of miR-146a-5p and miR-221-3p circulating levels were previously reported in patients with cardiovascular diseases, acute cerebral infarction, systemic inflammation, and osteoarthritis [33, 34]. In our series, only 1 patient had a history of ischemic cardiovascular disease, and none had a history of stroke, systemic inflammation, or osteoarthritis; thus we can exclude a role of systemic disorders in affecting miRNA expression levels.

Our study attempts to document the kinetics of circulating miRNA levels in PTC patients during the first 1 to 2 years of follow-up and to analyze these results in light of the patients' clinical status. The cohorts we studied were admittedly small, and much larger studies are needed to confirm our findings. An important strength of our study, however, is the quality of the protocol we used to assay circulating miRNA levels. To minimize intrinsic interindividual variability related to thyroid-independent factors (*e.g.*, sex, age, individual genetic background, diet, lifestyle, and comorbidities), our initial screening analysis involved comparison of post-thyroidectomy miRNA levels with those found in preoperative samples from the same PTC patient population. Intersample variability can also be introduced by technical factors,

such as sample preparation, the addition of a preamplification step, and normalization strategies [35–38]. We selected the miRNeasy Serum/Plasma kit (Qiagen), which facilitates the detection of low-abundance miRNAs and allows the isolation of more concentrated RNA samples than other kits of this type [37, 39]. In addition, the multiple-extraction approach we adopted for miRNA profiling allowed us to obtain over 1,000 ng total RNA from each serum sample, thereby eliminating the need for cDNA preamplification. Because consensus is lacking on reliable endogenous controls for circulating miRNA analysis [37], we used absolute quantitative polymerase chain reaction for all the analyses carried out in the validation cohort.

Preamplification is an important potential source of technical bias [35, 36], and it might at least partially explain the discrepancy that emerged between our findings regarding miR-95-3p and miR-190a-5p and those of other researchers [23]. Using a protocol that involved cDNA preamplification, Cantara *et al.* [23] found serum miR-95-3p and miR-190a-5p levels in PTC patients to be significantly different (lower and higher, respectively) from those in HCs. In our study, however, the levels of both miRNAs in all the serum samples were so low (in many cases, below the detection limits of our assay) that they had to be excluded from subsequent analyses. More sensitive techniques are needed to allow reliable quantification of even low-abundance circulating miRNAs without the use of the preamplification step and endogenous controls.

In summary, postoperative changes in circulating levels of miR-146a-5p and miR-221-3p in PTC patients display good correlation with ATA-defined response-to-therapy classes, even in cases in which serum Tg assay results are unreliable or difficult to interpret. Studies are already underway in our laboratory to obtain additional validation for our findings in a larger PTC patient population that includes cases with locoregional spread at diagnosis as well as groups with and without evidence of recurrence/persistence during follow-up. Our current results suggest, however, that serum levels of miR-146a-5p and miR-221-3p might one day be used as complementary biomarkers for the early noninvasive detection of persistent/recurrent PTC, particularly in the expanding population of patients undergoing more conservative treatment of these tumors.

Acknowledgments

Address all correspondence to: Sebastiano Filetti, MD, Dipartimento di Medicina Interna e Specialità Mediche, Università di Roma “Sapienza”, Viale del Policlinico 155, 00161 Roma, Italy. E-mail: sebastiano.filetti@uniroma1.it.

This work was supported by the Umberto Di Mario Foundation, Banca d’Italia, University of Rome Sapienza (grants C26A13C8C7 and C26A15Z4JM to C.D.), the program of Biotechnologies and Clinical Medicine of the University of Rome Sapienza (to G.G., L.L., and V.P.), Marian Everett Kent (European Medical Writers Association), and the Umberto Di Mario Foundation.

Disclosure Summary: The authors have nothing to disclose.

References and Notes

1. Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, Nikiforov YE, Pacini F, Randolph GW, Sawka AM, Schlumberger M, Schuff KG, Sherman SI, Sosa JA, Steward DL, Tuttle RM, Wartofsky L. 2015 American Thyroid Association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer. *Thyroid*. 2016;**26**(1):1–133.
2. Pacini F, Molinaro E, Lippi F, Castagna MG, Agate L, Ceccarelli C, Taddei D, Elisei R, Capezzone M, Pinchera A. Prediction of disease status by recombinant human TSH-stimulated serum Tg in the postsurgical follow-up of differentiated thyroid carcinoma. *J Clin Endocrinol Metab*. 2001;**86**(12): 5686–5690.
3. Torlontano M, Crocetti U, D’Aloiso L, Bonfitto N, Di Giorgio A, Modoni S, Valle G, Frusciante V, Bisceglia M, Filetti S, Schlumberger M, Trischitta V. Serum thyroglobulin and 131I whole body scan after recombinant human TSH stimulation in the follow-up of low-risk patients with differentiated thyroid cancer. *Eur J Endocrinol*. 2003;**148**(1):19–24.

4. Durante C, Tognini S, Montesano T, Orlandi F, Torlontano M, Puxeddu E, Attard M, Costante G, Tumino S, Meringolo D, Bruno R, Trulli F, Toteda M, Redler A, Ronga G, Filetti S, Monzani F; PTC Study Group. Clinical aggressiveness and long-term outcome in patients with papillary thyroid cancer and circulating anti-thyroglobulin autoantibodies. *Thyroid*. 2014;**24**(7):1139–1145.
5. Spencer C, Petrovic I, Fatemi S, LoPresti J. Serum thyroglobulin (Tg) monitoring of patients with differentiated thyroid cancer using sensitive (second-generation) immunometric assays can be disrupted by false-negative and false-positive serum thyroglobulin autoantibody misclassifications. *J Clin Endocrinol Metab*. 2014;**99**(12):4589–4599.
6. Latrofa F, Ricci D, Sisti E, Piaggi P, Nencetti C, Marinò M, Vitti P. Significance of low levels of thyroglobulin autoantibodies associated with undetectable thyroglobulin after thyroidectomy for differentiated thyroid carcinoma. *Thyroid*. 2016;**26**(6):798–806.
7. Vaisman F, Momesso D, Bulzico DA, Pessoa CHCN, Dias F, Corbo R, Vaisman M, Tuttle RM. Spontaneous remission in thyroid cancer patients after biochemical incomplete response to initial therapy. *Clin Endocrinol (Oxf)*. 2012;**77**(1):132–138.
8. Lamartina L, Montesano T, Trulli F, Attard M, Torlontano M, Bruno R, Meringolo D, Monzani F, Tumino S, Ronga G, Maranghi M, Biffoni M, Filetti S, Durante C. Papillary thyroid carcinomas with biochemical incomplete or indeterminate responses to initial treatment: repeat stimulated thyroglobulin assay to identify disease-free patients. *Endocrine*. 2015;**54(2)**:467–475.
9. Durante C, Attard M, Torlontano M, Ronga G, Monzani F, Costante G, Ferdeghini M, Tumino S, Meringolo D, Bruno R, De Toma G, Crocetti U, Montesano T, Dardano A, Lamartina L, Maniglia A, Giacomelli L, Filetti S; Papillary Thyroid Cancer Study Group. Identification and optimal postsurgical follow-up of patients with very low-risk papillary thyroid microcarcinomas. *J Clin Endocrinol Metab*. 2010;**95**(11):4882–4888.
10. Vaisman F, Momesso D, Bulzico DA, Pessoa CHCN, Cruz MDG da, Dias F, Corbo R, Vaisman M, Tuttle RM. Thyroid lobectomy is associated with excellent clinical outcomes in properly selected differentiated thyroid cancer patients with primary tumors greater than 1 cm. J Thyroid Res. 2013;2013:1–5.
11. Miyauchi A. Clinical trials of active surveillance of papillary microcarcinoma of the thyroid. *World J Surg*. 2016;**40**(3):516–522.
12. Durante C, Montesano T, Attard M, Torlontano M, Monzani F, Costante G, Meringolo D, Ferdeghini M, Tumino S, Lamartina L, Paciaroni A, Massa M, Giacomelli L, Ronga G, Filetti S; PTC Study Group. Long-term surveillance of papillary thyroid cancer patients who do not undergo postoperative radioiodine remnant ablation: is there a role for serum thyroglobulin measurement? *J Clin Endocrinol Metab*. 2012;**97**(8):2748–2753.
13. Momesso DP, Vaisman F, Yang SP, Bulzico DA, Corbo R, Vaisman M, Tuttle RM. Dynamic risk stratification in differentiated thyroid cancer patients treated without radioactive iodine. *J Clin Endocrinol Metab*. 2016;**101(7)**:2692–2700. jc20154290.
14. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA*. 2008;**105**(30):10513–10518.
15. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer*. 2006;**6**(11):857–866.
16. Calin GA, Croce CM. MicroRNA-cancer connection: the beginning of a new tale. *Cancer Res*. 2006; **66**(15):7390–7394.
17. Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor suppressors. *Dev Biol*. 2007;**302**(1):1–12.
18. Dettmer M, Perren A, Moch H, Komminoth P, Nikiforov YE, Nikiforova MN. Comprehensive MicroRNA expression profiling identifies novel markers in follicular variant of papillary thyroid carcinoma. *Thyroid*. 2013;**23**(11):1383–1389.
19. Cancer Genome Atlas Research Network. Integrated genomic characterization of papillary thyroid carcinoma. *Cell*. 2014;**159**(3):676–690.
20. Yu S, Liu Y, Wang J, Guo Z, Zhang Q, Yu F, Zhang Y, Huang K, Li Y, Song E, Zheng XL, Xiao H. Circulating microRNA profiles as potential biomarkers for diagnosis of papillary thyroid carcinoma. *J Clin Endocrinol Metab*. 2012;**97**(6):2084–2092.
21. Lee JC, Zhao JT, Clifton-Bligh RJ, Gill A, Gundara JS, Ip JC, Glover A, Sywak MS, Delbridge LW, Robinson BG, Sidhu SB. microRNA-222 and microRNA-146b are tissue and circulating biomarkers of recurrent papillary thyroid cancer. *Cancer*. 2013;**119**(24):4358–4365.
22. Yoruker EE, Terzioglu D, Teksoz S, Uslu FE, Gezer U, Dalay N. microRNA expression profiles in papillary thyroid carcinoma, benign thyroid nodules and healthy controls. *J Cancer*. 2016;**7**(7):803–809.

23. Cantara S, Pilli T, Sebastiani G, Cevenini G, Busonero G, Cardinale S, Dotta F, Pacini F. Circulating miRNA95 and miRNA190 are sensitive markers for the differential diagnosis of thyroid nodules in a Caucasian population. *J Clin Endocrinol Metab*. 2014;**99**(11):4190–4198.
24. Graham ME, Hart RD, Douglas S, Makki FM, Pinto D, Butler AL, Bullock M, Rigby MH, Trites JR, Taylor SM, Singh R. Serum microRNA profiling to distinguish papillary thyroid cancer from benign thyroid masses. *J Otolaryngol Head Neck Surg*. 2015;**44**(1):33.
25. Lee YS, Lim YS, Lee JC, Wang SG, Park HY, Kim SY, Lee BJ. Differential expression levels of plasma-derived miR-146b and miR-155 in papillary thyroid cancer. *Oral Oncol*. 2015;**51**(1):77–83.
26. Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. *Ann Surg Oncol*. 2010;**17**(6):1471–1474.
27. Cooper DS, Doherty GM, Haugen BR, Kloos RT, Lee SL, Mandel SJ, Mazzaferri EL, McIver B, Pacini F, Schlumberger M, Sherman SI, Steward DL, Tuttle RM; American Thyroid Association (ATA) Guidelines Taskforce on Thyroid Nodules and Differentiated Thyroid Cancer. Revised American Thyroid Association management guidelines for patients with thyroid nodules and differentiated thyroid cancer. *Thyroid*. 2009;**19**(11):1167–1214.
28. R Development Core Team. R: a language and environment for statistical computing. Available at: www.R-project.org.
29. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez J-C, Müller M. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics*. 2011;**12**:77.
30. Hu Y, Wang H, Chen E, Xu Z, Chen B, Lu G. Candidate microRNAs as biomarkers of thyroid carcinoma: a systematic review, meta-analysis, and experimental validation. *Cancer Med*. 2016;**5**(9):2602–2614 .
31. Lamartina L, Grani G, Biffoni M, Giacomelli L, Costante G, Lupo S, Maranghi M, Plasmati K, Sponziello M, Trulli F, Verrienti A, Filetti S, Durante C. Risk stratification of neck lesions detected sonographically during the follow-up of differentiated thyroid cancer. *J Clin Endocrinol Metab*. 2016;**101**(8):3036–3044.
32. Torlontano M, Crocetti U, Augello G, D'Aloiso L, Bonfitto N, Varraso A, Dicembrino F, Modoni S, Frusciante V, Di Giorgio A, Bruno R, Filetti S, Trischitta V. Comparative evaluation of recombinant human thyrotropin-stimulated thyroglobulin levels, 131I whole-body scintigraphy, and neck ultrasonography in the follow-up of patients with papillary thyroid microcarcinoma who have not undergone radioiodine therapy. *J Clin Endocrinol Metab*. 2006;**91**(1):60–63.
33. Marques-Rocha JL, Samblas M, Milagro FI, Bressan J, Martínez JA, Marti A. Noncoding RNAs, cytokines, and inflammation-related diseases. *FASEB J*. 2015;**29**(9):3595–3611.
34. Coskunpinar E, Cakmak HA, Kalkan AK, Tiryakioglu NO, Erturk M, Ongen Z. Circulating miR-221-3p as a novel marker for early prediction of acute myocardial infarction. *Gene*. 2016;**591**(1):90–96.
35. Reiter M, Kirchner B, Müller H, Holzhauer C, Mann W, Pfaffl MW. Quantification noise in single cell experiments. *Nucleic Acids Res*. 2011;**39**(18):e124.
36. Jarry J, Schadendorf D, Greenwood C, Spatz A, van Kempen LC. The validity of circulating microRNAs in oncology: five years of challenges and contradictions. *Mol Oncol*. 2014;**8**(4):819–829.
37. Farina NH, Wood ME, Perrapato SD, Francklyn CS, Stein GS, Stein JL, Lian JB. Standardizing analysis of circulating microRNA: clinical and biological relevance. *J Cell Biochem*. 2014;**115**(5):805–811.
38. Moldovan L, Batte KE, Trgovcich J, Wisler J, Marsh CB, Piper M. Methodological challenges in utilizing miRNAs as circulating biomarkers. *J Cell Mol Med*. 2014;**18**(3):371–390.
39. Page K, Guttery DS, Zahra N, Primrose L, Elshaw SR, Pringle JH, Blighe K, Marchese SD, Hills A, Woodley L, Stebbing J, Coombes RC, Shaw JA. Influence of plasma processing on recovery and analysis of circulating nucleic acids. *PLoS One*. 2013;**8**(10):e77963.