

Circulating miRNA95 and miRNA190 Are Sensitive Markers for the Differential Diagnosis of Thyroid Nodules in a Caucasian Population

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Context: MicroRNA (miRNAs) are nonprotein-encoding RNAs that regulate gene expression and enable the distinction of benign from malignant tissues in human cancers.

Objective: We investigated the role of miRNA circulating in the blood for the differential diagnosis of thyroid nodules.

Setting and Design: miRNA profiling was assessed by TaqMan Array Human MicroRNA A Cards v2.0 in pooled sera from 12 healthy subjects (HS), 12 nodular goiters (NG), and 12 patients with papillary thyroid cancer (PTC) (cohort 1). From this analysis, we selected eight miRNAs that were validated in individual samples (same of cohort 1) by qRT-PCR. Four miRNAs were confirmed differentially expressed in PTC and were analyzed in a larger second cohort.

Results: The profiling analysis revealed eight miRNAs (miRNA579, -95, -29b, 5-01-3p, -548d-5p down-regulated, and miR190, -362-3p, -518a-5p up-regulated) which differ in PTC compared with NG and HS. After the validation in individual samples, we confirmed as differentially expressed miRNA579, -95, -29b, and miRNA190. These miRNAs were further validated in a second cohort of sera from 79 PTC, 80 NG, and 41 HS. MiRNA95 had a sensitivity of 94.9%, which reached 100% in a multivariate risk model combined with miRNA190. We developed a mathematical formula that calculates the probability of malignancy with a cut-off value of 0.5 above which the patient was at high risk of malignancy.

Conclusions: We have identified for the first time two miRNAs differently expressed in serum of PTC patients who in combination allow the differential diagnosis of thyroid nodules with great accuracy in our study population. Additional studies are required; however, to define whether these results will also be generalized across other patient populations." (*J Clin Endocrinol Metab* 99: 4190–4198, 2014)

Thyroid nodules are frequent in the general population and their detection is increasingly frequent due to intensive screening by neck ultrasound (1–3). Most thyroid nodules are benign and only approximately 5–10% are malignant, usually represented by papillary thyroid cancer (PTC) (4–5). In this view, it is of paramount importance to limit surgical treatment only to malignant/suspicious nodules. Nowadays, fine-needle aspiration cy-

tology (FNAC) is the gold standard for the differential diagnosis of thyroid nodules (4, 5). In general, in expert hands, FNAC has good specificity and acceptable sensitivity (6). However, this procedure has some limitations related to inadequate sampling or to the difficulty to discriminate follicular lesions. Therefore, a significant proportion of patients who do not have malignant lesions undergo unnecessary thyroidectomy.

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Abbreviations: ATC, anaplastic thyroid cancer; AUC, area under the ROC curve; CI, confidence interval; FTC, follicular thyroid cancer; HS, healthy subjects; MTC, medullary thyroid cancer; NG, nodular goiter; OR, odds ratio; PTC, papillary thyroid cancer.

MicroRNA (miRNAs) are small (approximately 22 nucleotides), nonprotein-encoding RNAs that post-transcriptionally regulate gene expression via suppression of specific target mRNAs (7). Of note, miRNAs circulate in a highly stable, cell-free form in blood and they can be detected in plasma and serum (8). Furthermore, tumor cells have been shown to release miRNAs into the circulation (8) and miRNA profiles in plasma and serum have been found to be altered in some cancers and in other diseases (9–11). Large-scale miRNAs analysis of surgical specimens has proven that miRNA expression enables the distinction of benign tissues from their malignant counterpart in several human cancers (12, 13) including thyroid cancer (14–17). Tetzlaff et al (14) identified 13 miRNAs up-regulated and 26 miRNAs down-regulated in PTC vs multinodular goiter in formalin-fixed paraffin-embedded tissues. Chen et al (15) identified miRNA-146b, miRNA-221, and miRNA-222 as overexpressed in PTC and found an increased expression of miRNA-146b in follicular thyroid cancer (FTC) both in FNAC and surgical pathological specimens. Some miRNAs were found to be related to PTC formation in another study by He et al (16). Another group demonstrated that a specific limited set of miRNAs (miRNA-197 and miRNA-346) is overexpressed in FTC cell lines and in tumor samples (17). Although tissue miRNA profiles may be useful to distinguish benign from malignant lesions, they do not represent minimally invasive biomarkers for presurgical diagnosis. A study conducted on 106 patients with primary PTC, 95 patients with benign thyroid lesion and 44 healthy controls in China, revealed that serum let-7e, miRNA-151-5p, and miRNA-222 were significantly increased in PTC cases relative to benign cases and healthy controls offering, for the first time, the basis for the development of an easy, non-invasive, and effective diagnostic tool for the preoperative diagnosis of thyroid nodules (18).

In this study, we aimed to investigate the role of serum miRNA profiling in differential diagnosis of thyroid nodules in a cohort of Caucasian patients.

Study design

The experimental workflow comprises four sequential steps and was performed on a surgical cohort of patients.

A graphic representation of the study design is reported in Figure 1.

Step 1. MicroRNAs profiling analysis was performed employing TaqMan Array Human MicroRNA A Cards v2.0 (Life Technologies), which contain 384 TaqMan MicroRNA Assays, in pooled sera from 12 healthy subjects (HS), 12 nodular goiters (NG), and 12 patients with papillary thyroid cancer (PTC) (first cohort).

Step 2. Eight miRNAs, resulting as differentially expressed in Patients with PTC in step 1, were selected and validated in individual samples (the same used in step 1) by qRT-PCR; 4/8 were confirmed to be differentially expressed in cancers.

Step 3. The 4 miRNAs identified in step 2 were analyzed by qRT-PCR in a second cohort of subjects comprising 79 PTCs, 80 NGs, and 41 HSs.

step 4. By qRT-PCR we evaluated the expression of the four miRNAs of interest in thyroid tissue samples of each patient (12 PTCs and 12 NGs) of step 1.

Patients

For both cohorts, we selected HSs among patients referred to our outpatient clinic for a suspicion of thyroid disease who were not found to have any thyroid disorders after neck ultrasound and thyroid hormone measurement, and we defined as Patients with NG having a single or multiple nodules in the context of a normal thyroid or a goiter.

First cohort

We selected 12 HSs (M:F = 1:11; mean age \pm SD = 37.58 ± 19.43 y), 12 patients with benign nodular disease (M:F = 3:9; mean age \pm SD = 55.25 ± 10.40 y), and 12 Patients with PTC (M:F = 3:9; mean age \pm SD = 45.67 ± 17.15 y).

There was no difference in terms of age among the three groups, although TSH levels were higher in HS compared with Patients with NG (mean \pm SD = 2.16 ± 0.81 vs 0.58 ± 0.45 ; $P = .0003$) but not different with respect to Patients with PTC.

Among 12 patients with PTC, five showed intrathyroidal tumor whereas 7/12 patients had minimal extrathyroidal extension of the tumor; cervical lymph node metastases were present in 5/12 patients, and no patients had distant metastases; 4/12 patients showed multifocal disease and in 4/12 cases the tumor was localized in both lobes; 6/12 patients had classical variant of PTC, follicular variant of PTC was present in one case of 12, 2/12 patients showed sclerosing variant of PTC, tall/columnar cell PTC was present in 2/12 cases, and 1 patients had a less common variant of PTC.

Second cohort

We examined 41 HSs (M:F = 35:6; mean age \pm SD = 38.63 ± 17.79), 80 patients with benign nodular disease (M:F = 23:57; mean age \pm SD = 54.35 ± 11.72) and 79 patients with PTC (M:F = 13:63; mean age \pm SD = 42.18 ± 14.33).

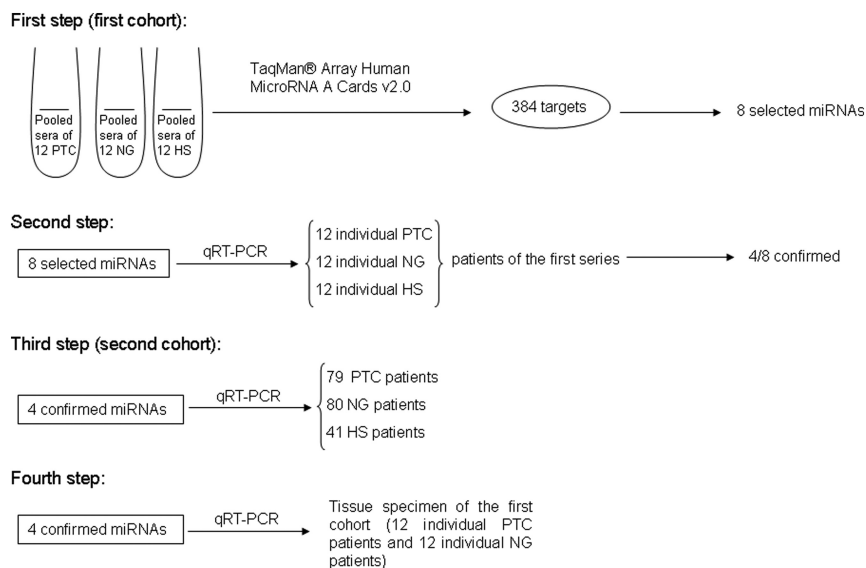


Figure 1. Graphical schematization of the study design. In step 1, microRNAs profiling analysis was performed in pooled sera from 12 HS, 12 NG, and 12 patients with PTC. In step 2, eight miRNAs were selected and validated in individual samples (the same used in step 1) and 4/8 were confirmed to be differentially expressed in cancers. In step 3, these four miRNAs were analyzed in a larger validation series. In step 4, expression of the four miRNAs of interest was evaluated in thyroid tissue samples of 12 PTCs and 12 NGs used in step 1.

Patients with NG were older than patients with PTC and HSs (mean \pm SD, 54.35 ± 11.72 vs 42.18 ± 14.33 and vs 38.63 ± 17.79 years, respectively; $P = < .0001$) whereas age was not different between HS and PTC. TSH levels were lower in patients with NG compared with PTC and HS (mean \pm SD, 0.70 ± 0.42 vs 1.74 ± 1.31 and vs 2.13 ± 0.82 , respectively; $P = < .0001$) and lower in patients with PTC with respect to HS ($P = .0024$).

Among 79 patients with PTC, 32 had intrathyroidal tumor whereas 47/79 had minimal extrathyroidal extension of the tumor; cervical lymph node metastases were present in 30/79 patients and only one patient had lung metastases; 33/79 patients showed multifocal disease, and in 26/79 cases the tumor was localized in both lobes; 49/79 patients had classical variant of PTC, follicular variant of PTC was present in 12/79 cases, 7/79 patients showed sclerosing variant of PTC, tall/columnar cell PTC was present in 6/79 cases, and five patients had less common variants of PTC.

miRNA extraction and qRT-PCR analysis

Blood samples used in this study were obtained after informed consent, in accordance with local ethical committee guidelines, and always collected before FNAC procedure.

MiRNAs were extracted from serum and tissues using the miRVana Paris kit (Life Technologies). As reference control for RNA extraction, we added the spike-in synthetic exogenous miRNA ath-miR-159a at a concentration of 25pM. MiRNAs were reverse transcribed using

Megaplex Human microRNA RT primers pools v2.1 and then preamplified. Briefly, $5 \mu\text{l}$ of RNA was used for each reaction adding $1.33 \mu\text{l}$ of 10X Megaplex human microRNAs RT primers pool A v2.1, $0.33 \mu\text{l}$ of 100mM deoxynucleotide triphosphate, $1.33 \mu\text{l}$ of 10X RT Buffer, $1.50 \mu\text{l}$ of 25mM MgCl_2 , $0.17 \mu\text{l}$ of 20 U/ μl RNase inhibitor, $2.50 \mu\text{l}$ of 50 U/ μl MultiScribe Reverse Transcriptase, and $0.33 \mu\text{l}$ H_2O (all from Applied Biosystem). The reaction was then incubated for 40 cycles at $16 \text{ C} \times 2$ minutes, $42 \text{ C} \times 1$ minute, $50 \text{ C} \times 1$ second, and then at $85 \text{ C} \times 5$ minutes. cDNA was then preamplified using $4.16 \mu\text{l}$ of final RT reaction volume by adding $20.8 \mu\text{l}$ of 2X Taqman Preamp Master mix, $4.16 \mu\text{l}$ of 10X Taqman Human Preamp primers pool v2.1, and $12.5 \mu\text{l}$ of H_2O (all from LifeTechnologies).

The reaction was then incubated $95 \text{ C} \times 10'$, $55 \text{ C} \times 2'$, $72 \text{ C} \times 2'$, 12 cycles at $95 \text{ C} \times 15$ seconds, $60 \text{ C} \times 4$ minutes, and finally, $99.9 \text{ C} \times 10$ minutes. Preamplification reaction was diluted 1:4 in Tris-EDTA buffer, pH 8.0. Samples were loaded onto the TaqMan Array Human MicroRNA A Cards v2.0. Relative expression quantification was performed by the comparative cycle threshold method ($2^{-\Delta\Delta\text{Ct}}$). Synthetic exogenous miRNA ath-miR-159a and endogenous miRNA16 and miRNA451 were selected as reference to normalize microRNA expression values. MiRNAs were considered down-regulated for fold-change values < 0.35 and up-regulated for values > 2.5 . Data from Taqman Human MicroRNA Array Cards were exported using ViiA7 RUO software and then analyzed using Expression Suite software v1.0.1 (Applied Biosystem). Only miRNAs with $\text{Ct} < 35$ and with a high-efficiency amplification plot were taken into consideration for subsequent analysis. Graphical analysis for Hierarchical clustering was performed using Spotfire software v5.0 (Tibco). For single assay miRNAs validation, $2.5 \mu\text{l}$ of preamplification product were used in real-time PCR reaction for each microRNAs analyzed. The same reference miRNAs as those used in the array profiling were adopted to normalize individual miRNAs expression. ViiA7 Real-time PCR instrument (Applied Biosystem) was used to perform Array cards and single assay reactions.

Statistics

Kruskal-Wallis test with Dunn's post test was used to determine the significance of different serum levels of

miRNA expression in the three groups. Mann-Whitney *U* test was used to compare miRNA expression levels between NG and PTC tissues. Spearman's rank correlation was used to correlate clinical parameters and miRNA expression values.

Multivariate stepwise logistic regression analysis was carried out to identify a prognostic and/or diagnostic model able to identify the risk of malignancy in thyroid nodules, taking the four confirmed miRNA expressions as a predictive variable. For this purpose, the miRNA variables have been suitably transformed logarithmically. In the stepwise process, one independent variable related to the different miRNA expressions was added to, or removed from, the discriminant model at each step, on the basis of the criterion of maximum likelihood ratio. The process stopped when no more statistical significant variables could be entered or removed. Model diagnostic accuracy was evaluated by the area under the ROC curve

(AUC) and its 95% confidence interval (CI). The Hosmer-Lemeshow goodness-of-fit test was used to evaluate model prognostic reliability. Odds ratio (OR) and its 95% CI were evaluated for model-selected miRNA variables. The patient OR was expressed by a formula obtained by approximating the coefficients of the logistic regression model to integer or simple fractional values. In this way, it was possible to express the individual OR and the corresponding probability of malignancy directly through a mathematical relationship of model-selected miRNA expressions. Such prognostic probability of malignancy, *pmiRNA*, allowed discrimination between benignity and malignancy, taking a cut-off value of *pmiRNA* = 0.5, above which the patient was considered at high risk of malignancy. A statistical significance level of 95% ($P < .05$) was considered for all statistical analyses that were performed with both the SPSS and Matlab computer packages.

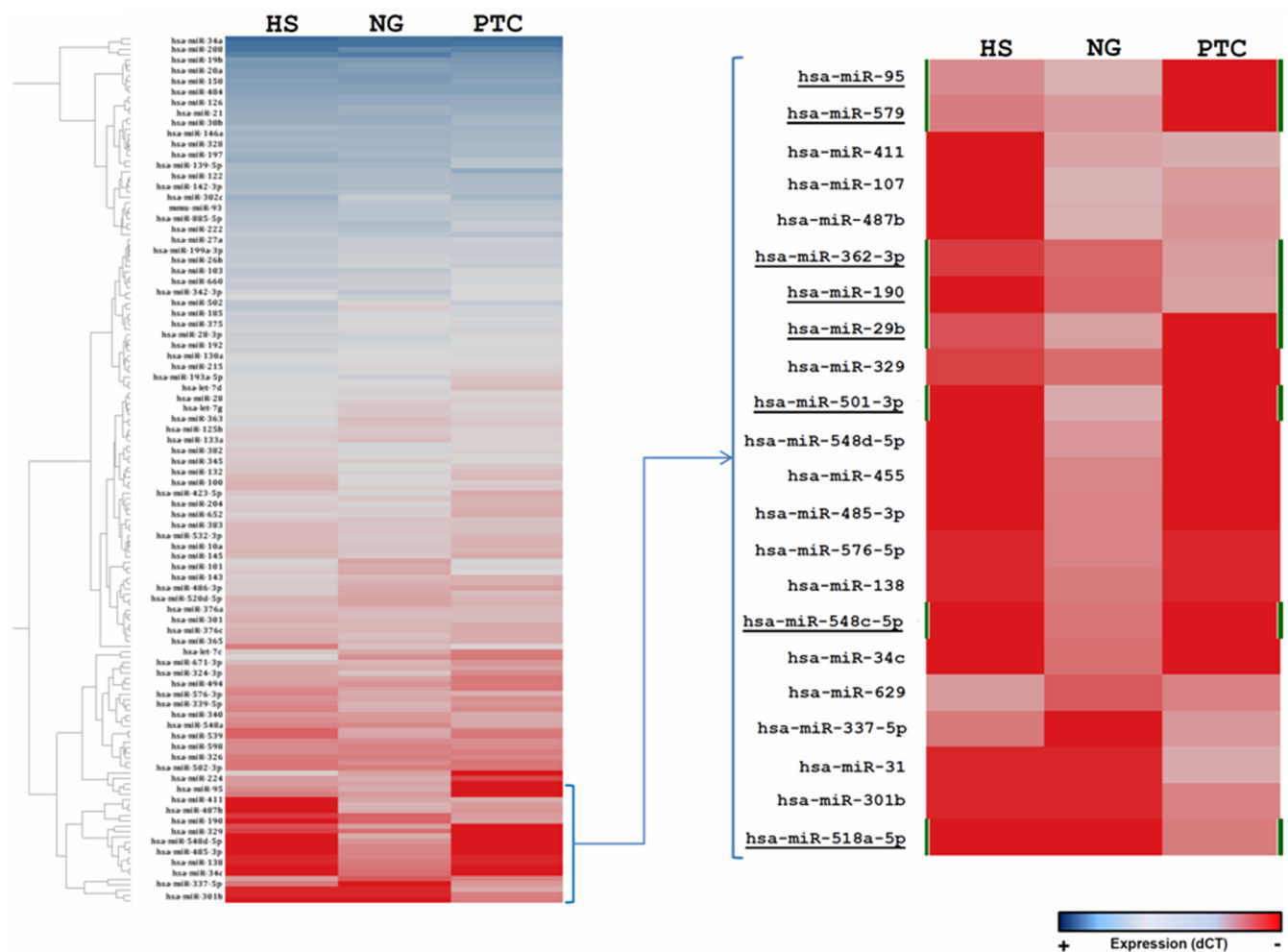


Figure 2. Hierarchical clustering and heat map analysis of miRNA array profiling. MicroRNAs expressed in sera of the three groups analyzed (HS, NG, and PTC) are reported in the hierarchical clustering heat map analysis in which each row represents a detected miRNA. The scale color from blue (high expression) to red (low expression) reports the expression levels of each miRNA (expressed as average of dCT value normalized by using exogenous ath-miR-159a and endogenous miR-16 and miR-451). On the right side, a high magnification of the heat map shows a graph zone reporting the eight selected miRNAs (underlined).

Results

Step 1: MicroRNA array profiling and identification of differentially expressed miRNAs

To search for suitable microRNAs whose differential expression in serum might define the presence of PTC, we analyzed microRNAs expression profiles in pooled sera (first cohort). Among 384 analyzed miRNAs, we detected the expression of 150, 165, and 151 microRNAs, in HS,

NG, and PTC sera, respectively. The stability of house-keeping small RNAs (ath-miR159a, miRNA16, and miRNA451) used to normalize the miRNA expression resulted optimal in the three groups and then used to normalize the expression of microRNAs detected in the analysis.

From this analysis, we specifically selected five miRNAs (miRNA579, -95, -29b, -501-3p, and miRNA-548d-5p)

that were strongly down-regulated and three miRNAs (miR190, -362-3p, and -518a-5p) that were up-regulated in PTC vs NG and HS sera (Figure 2).

Step 2: Expression profiles of serum miRNAs of interest selected in Step 1

The expression of the eight miRNAs selected in Step 1 was validated in the individual samples by qRT-PCR. As shown in Figure 3, 4/8 selected miRNAs were confirmed to be significantly different in PTC compared with NG and HS. Specifically, miRNA579, miRNA95, and miRNA29b levels were down-regulated whereas miRNA190 was up-regulated in PTC. These four miRNAs did not result differentially expressed between HS and NG serum samples.

Step 3: Validation series

We validated results for miRNA190, miRNA95, miRNA579 and miRNA29b in a second cohort of sera from 79 PTC, 80 NG and 41 HS patients. As shown in Figure 4, miRNA95, miRNA579, and miRNA-29b were confirmed to be down-regulated in PTC compared with the other groups whereas miRNA190 was up-regulated in PTC; no difference between HS and NG was found in all analyzed miRNAs. No correlation was found between the miRNAs and the histological parameters and clinical outcome of the patients with PTC (data not shown).

ROC curve analysis was performed for serum levels of miRNA29b, miRNA190, miRNA95, and miRNA-579 and the best diagnostic accuracy was found for miRNA95 with a sen-

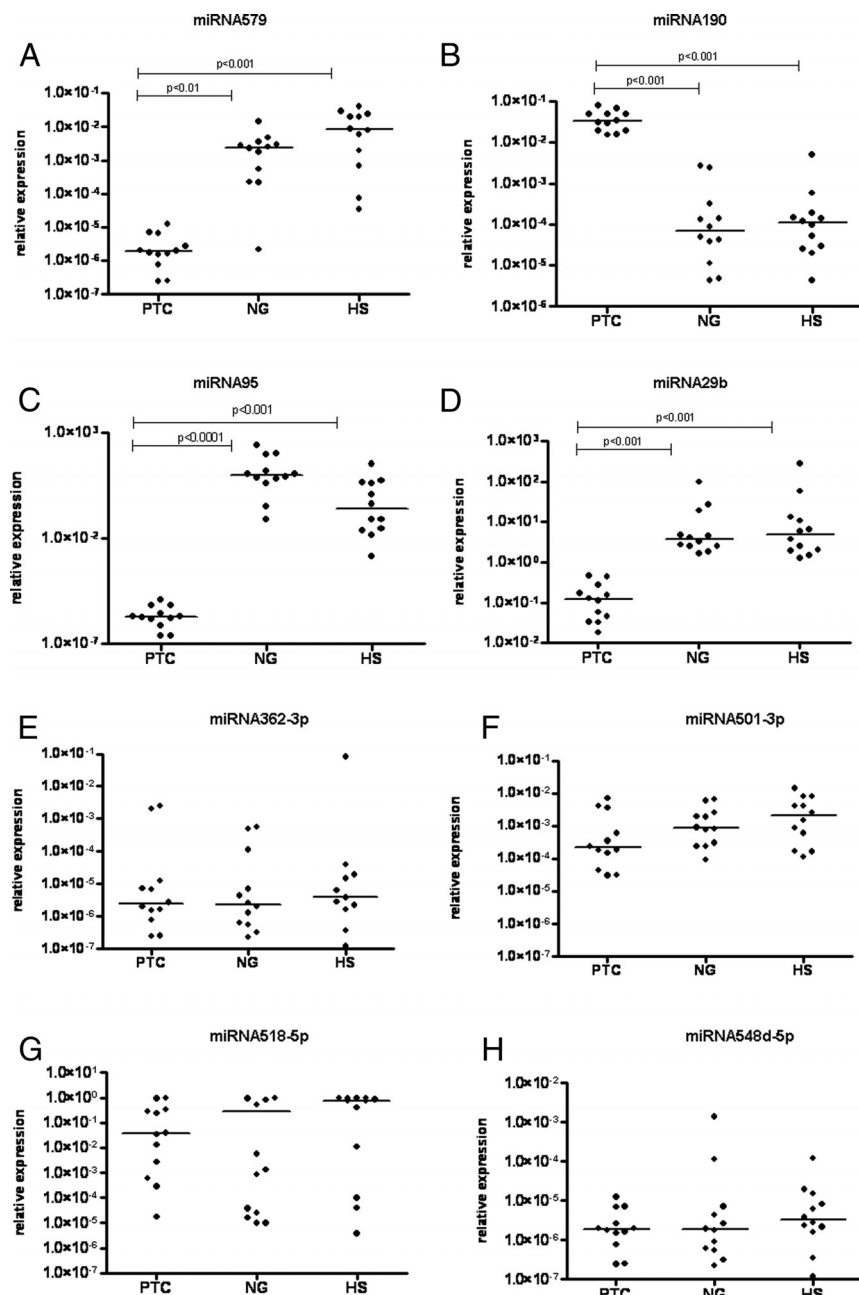


Figure 3. Validation of eight miRNA expression levels in sera of 12 PTCs, 12 NGs and 12 HSs (same patients used for initial pools). Medians are suggested. *P* values are calculated using Kruskal Wallis test with Dunn’s correction. Levels of serum (E), miRNA362-3p; miRNA501-3p (F); miRNA518a-5p (G); and miRNA548d-5p (H) were not significantly different in PTC compared with HS and NG. Levels of serum A, miRNA579; C, miRNA95; and miRNA29b (D) were significantly (*P* < .001) lower in PTC compared with other groups, whereas serum levels of B, miRNA190 were significantly (*P* < .001) higher in PTC compared with NG and HS.

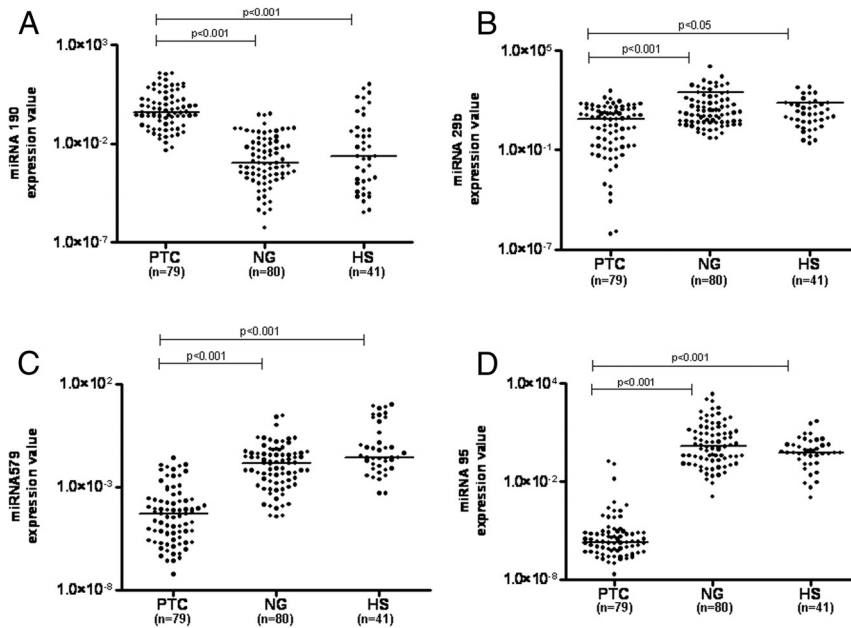


Figure 4. Results for serum miRNA190, miRNA95, miRNA579, and miRNA29b in the second validation series. Lines suggest the medians. *P* values are calculated using Kruskal Wallis test with Dunn's correction. Serum levels of miRNA190 (A) were significantly ($P < .001$) higher in PTC compared with NG and HS. Levels of serum miRNA95 (D, $P < .001$) and miRNA579 (C, $P < .001$) were significantly lower in PTC compared with other groups. Expression levels of miRNA29b were lower compared with NG ($P < .001$) and HS ($P < .05$). No differences between HS and NG were found in all analyzed miRNAs.

sitivity of 94.9% and a specificity of 98.7% for a cutoff of 0.0005 (Supplemental Table 1).

Multivariate risk model

A further improvement of sensitivity was achieved by applying a multivariate risk model. The logistic regression model identified the expression of two miRNAs (miRNA190 and miRNA95) as statistically significant to predict malignancy. The Hosmer-Lemeshov test proved the model goodness of fit ($P > .05$), that is the prognostic reliability of the risk probability in an estimated patient.

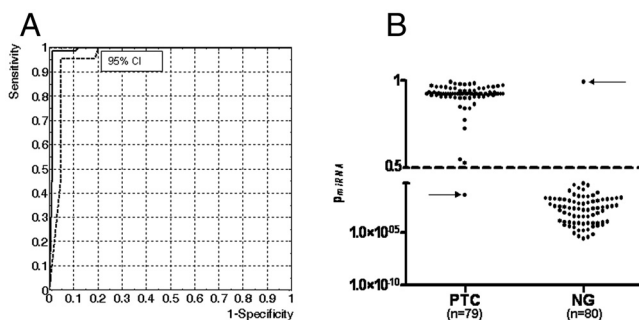


Figure 5. A, ROC curve (continuous line) and related 95% CIs (dashed lines) of the miRNA diagnostic model. B, P_{miRNA} (probability of malignancy) calculated for PTC and NG sample with the multivariate risk model. Arrows indicate the false-negative and false-positive samples.

From the analysis of model regression coefficients and their approximation, the patient OR and the corresponding prognostic probabilities could be calculated by using the following formulas:

$$OR_{miRNA} = \frac{1}{4} \sqrt[4]{\frac{miRNA_{190}^3}{miRNA_{95}^2}}$$

$$P_{miRNA} = \frac{OR_{miRNA}}{1 + OR_{miRNA}}$$

where OR_{miRNA} and p_{miRNA} are the model-estimated patient OR and risk probability, respectively.

The ROC curve of this model is shown in Figure 5A, together with its 95% CI. The area under ROC curve, AUC, demonstrates a great degree of diagnostic accuracy: AUC = 99.0%, 95% CI = 96.9–100%. In particular, in our sample only one patient was misclassified as false positive and only one patient as false negative (Figure 5B). The false negative case was a young female patient

with a diffuse-sclerosing variant of PTC (maximum diameter, 1 cm), with minimal extrathyroidal invasion and lymph node metastasis, whereas the false-positive case was a middle-age women with a large nodule (maximum diameter, 5 cm) in the left lobe of the thyroid. Both patients were euthyroid and no thyroiditis was found at the histological examination.

Step 4: Correlation of miRNA expression between thyroid tissue and serum samples

Because serum levels of miRNA579, miRNA95, miRNA29b, and miRNA190 were significantly different in patients with PTC compared with NG, we analyzed the expression of these miRNAs also in the thyroid tissues of patients used for the initial pools (12 PTC and 12 patients with NG). As shown in Figure 6, we found that tissue levels of miRNA579, miRNA95, and miRNA190 were similar to those observed in the serum; in details: miRNA579 and miRNA95 were significantly down-regulated in PTC compared with NG ($P < .0001$ and $P = .0006$, respectively), whereas miRNA190 was up-regulated ($P = .0014$). On the contrary, tissue levels of miRNA29b did not differ between PTC and patients with NG, and no correlation was found with its serum levels.

Clinical correlation

MiRNA levels were not correlated with age, sex, serum TSH, presence of thyroiditis, any histological parameters

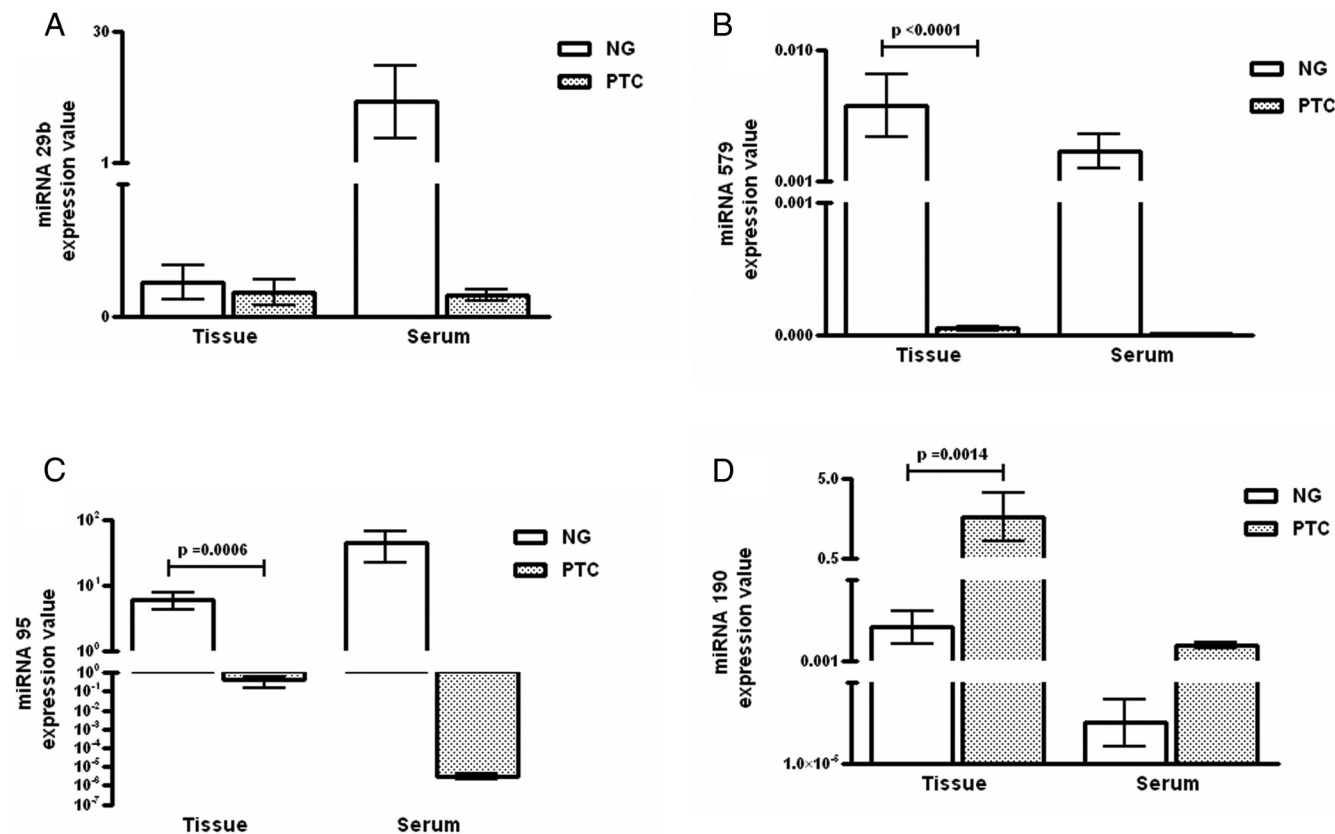


Figure 6. Correlation of miRNA expression in tissue and serum. *P* values are calculated using the Mann-Whitney *U* test. A, No correlation between serum and tissue miRNA29b in which expression levels were similar in tissues of NG and Patients with PTC. On the contrary we found a correlation between serum and tissue expression levels for miRNA579 (B); miRNA95 (C); and miRNA190 (D). In details, tissue miRNA579 and miRNA95 were higher ($P < .0001$ and $P = .0006$, respectively) in NG than in PTC and tissue miRNA190 was up-regulated ($P = .0014$) in PTC than NG tissues.

(tumor diameter, presence of lymph node or distant metastasis, tumor multifocality, and bilaterality, histological variants) and clinical outcome of the patients with PTC.

Discussion

Nowadays, FNAC is considered the gold standard for the differential diagnosis of thyroid cancer (4–5). However, predictive value of FNAC is still inadequate for follicular lesions or samples with inadequate material. A significant improvement has been obtained by searching genetic alterations specific to differentiated thyroid cancer in the material obtained by fine needle aspiration (19, 20). Nevertheless, some patients still undergo unnecessary thyroidectomy due to inconclusive results.

Previous studies have analyzed miRNA expression profiles in thyroid cancer specimens and found significant differences in miRNA signatures between malignant and benign lesions (11–14). Recently, several authors have reported (21–23) that circulating miRNAs are stable and easy to detect in the serum and may have potential utility as diagnostic or prognostic markers for several cancers. In

this view, a study performed in China (18) identified three miRNAs (let-7e, miRNA-151-5p, and miRNA-222), which were significantly overexpressed in the serum of patients with PTC compared with subjects with benign nodules and healthy, control individuals.

Our study is the first to report the search of miRNA in the blood in a Caucasian population, which led to the identification of a specific signature for PTC. To reach these results we performed initial screening of 384 miRNAs in pooled sera of PTC, patients with NG and HS, identifying eight miRNAs, which were differentially expressed in PTC vs NG and HS. We then applied qRT-PCR validation to confirm the results in the same patients used for pools. Four of eight miRNAs were confirmed differently expressed in PTC compared with HS and patients with NG. In particular, miRNA190 was up-regulated whereas miRNA95, -579, and miRNA29b were down-regulated. These data were further validated in a larger confirmatory series of patients, and the best diagnostic accuracy was found for miRNA95 and miRNA190. Moreover, combining these two miRNAs into a mathematical formula we were able to achieve a very high di-

agnostic power with an AUC% = 99.0%, 95% CI = 96.9–100%. The logistic model used to obtain the formula was designed with a stepwise method of variable selection, which generally leads to good generalization (statistically equivalent predictive power on new cases), thus limiting the need for studying a third additional confirmatory series. Moreover, the Hosmer-Lemeshov test was used to verify goodness of fit, which is the prognostic accuracy of model-estimated probabilities. With this multivariate risk model, it is possible to express the individual OR and the corresponding probability of malignancy directly through a mathematical relationship with miRNA expression levels. Such prognostic probability of malignancy (pmiRNA), allowed to discriminate between benignity and malignancy, taking a cut-off value of pmiRNA = 0.5, above which the patient is considered at high risk of malignancy. By applying this formula to our data we misclassified only two patients. Although the false negative case was found to have a microcarcinoma with PTC sclerosing variant, these features are likely not to be the reason for the misclassification, because we correctly identified as cancer 6/7 patients with PTC sclerosing variant, and 25/26 patients with microPTC. Overall, miRNAs do not seem to be affected by the histological variant of PTC because we did not find any correlation between histology and miRNA expression levels, including the follicular variant of PTC.

Our study was not intended to compare the diagnostic ability of circulating miRNA profile with any other methodology at the tissue level, such as the search for oncogene mutations in fine needle aspiration material. The reason is self evident because a blood test is less invasive than any other methodology. Nevertheless, we must point out that we did not find any correlation between serum miRNA levels and the presence or absence of *RET/PTC* rearrangements, *BRAF*, or *RAS* point mutations.

We have also compared miRNA expression levels in the tissues with those obtained in the sera, and we found a good correlation for three of four selected miRNAs (miRNA579, miRNA95, and miRNA190). On the contrary, no correlation was found for miRNA190, which was also the miRNA with the poorest diagnostic accuracy when its circulating levels were analyzed by ROC curve. However, this limited discrepancy might be expected somehow because we do not know the mechanisms underlying the regulation of miRNAs at the tissue level, in the tumor environment, compared with what happens in the serum.

A potential limitation of our study for the application in clinical practice might be that we analyzed only PTC without considering follicular, anaplastic (ATC), or medullary thyroid cancer (MTC). However, this represents a

minor issue considering that nearly 90% of thyroid cancers are represented by papillary histotype (4–5) and that ATC and MTC are easily diagnosed by other clinical and biochemical parameters such as measurement of serum calcitonin in MTC and clinical and ultrasound examination for ATC.

In the context of well-differentiated thyroid cancers there is an important area of diagnostic uncertainty represented by the lesions with indeterminate cytology. We are aware that some patients found to have a follicular lesion at cytology may harbor a follicular carcinoma (FTC), but this type of thyroid tumor is less and less frequent. Indeed, at our center, during the last decade we have witnessed only a few cases of FTC. Thus we do not think that our formula, designed on the miRNA profile in patients with PTC, might be limited by the chance of missing an exceptionally rare thyroid cancer.

Finally, the miRNAs identified in our study were different from those of the Chinese report (18). We do not know the intimate reason for this discrepancy, but we can speculate that the different genetic background may be the cause.

In conclusion, we have identified two miRNAs differently expressed in serum of PTC patients which, in combination can be used for the differential diagnosis of thyroid nodules with great accuracy.

Prospective studies in larger groups of patients, possibly in different countries with different ethnic origins, are needed to validate the clinical importance of our findings.

Acknowledgments

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Disclosure Summary: The authors have nothing to disclose.

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