Can the microRNA signature distinguish between thyroid tumors of uncertain malignant potential and other well-differentiated tumors of the thyroid gland?

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8Department of Biopathology, Laboratoire de Recherche Translationnelle, Gustave Roussy Institut, Villejuif, Paris, France

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*(P Barbry and P Hofman contributed equally to this work)

Abstract

The term ‘thyroid tumors of uncertain malignant potential’ (TT-UMP) was coined by surgical pathologists to define well-differentiated tumors (WDT) showing inconclusive morphological evidence of malignancy or benignity. We have analyzed the expression of microRNA (miRNA) in a training set of 42 WDT of different histological subtypes: seven follicular tumors of UMP (FT-UMP), six WDT-UMP, seven follicular thyroid adenomas (FTA), 11 conventional papillary thyroid carcinomas (C-PTC), five follicular variants of PTC (FV-PTC), and six follicular thyroid carcinomas (FTC), which led to the identification of about 40 deregulated miRNAs. A subset of these altered miRNAs was independently validated by qRT-PCR, which included 18 supplementary TT-UMP (eight WDT-UMP and ten FT-UMP). Supervised clustering techniques were used to predict the first 42 samples. Based on the four possible outcomes (FTA, C-PTC, FV-PTC, and FTC), about 80% of FTA and C-PTC and 50% of FV-PTC and FTC samples were correctly assigned. Analysis of the independent set of 18 WDT-UMP by quantitative RT-PCR for the selection of the six most discriminating miRNAs was unable to separate FT-UMP from WDT-UMP, suggesting that the miRNA signature is insufficient in characterizing these two clinical entities. We conclude that considering FT-UMP and WDT-UMP as distinct and specific clinical entities may improve the diagnosis of WDT of the thyroid gland. In this context, a small set of miRNAs (i.e. miR-7, miR-146a, miR-146b, miR-200b, miR-221, and miR-222) appears to be useful, though not sufficient per se, in distinguishing TT-UMP from other WDT of the thyroid gland.

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Introduction

The classification of well-differentiated tumors (WDT) of the thyroid gland according to histological criteria defines two distinct groups of neoplasia: benign (follicular thyroid adenoma, FTA) and malignant (papillary thyroid carcinoma, PTC; follicular thyroid carcinoma, FTC) neoplasia (De Lellis et al. 2004). However, there are borderline lesions that do not completely fulfill the different morphological criteria established to classify WDT of the thyroid gland, making the diagnosis and prognosis difficult. Besides, malignancy of some WDT of the thyroid gland can be difficult to diagnose based on the currently available diagnostic tools, which therefore lead to uncertain diagnosis and unpredictable prognosis (Suster 2006, Baloch & LiVolsi 2007). In this regard, some authors have defined other categories of thyroid tumors such as ‘thyroid tumors of uncertain malignancy potential’ (TT-UMP; Williams 2000, Rosai 2005, Fonseca et al. 2006). This definition includes two histological subtypes named WDT-UMP and ‘follicular tumor of UMP’ (FT-UMP; Williams 2000, Rosai 2005, Fonseca et al. 2006). Despite recent progress in immunohistochemistry as well as in molecular biology in identifying relevant biomarkers for WDT (Papotti et al. 2005, Sobrinho-Simões et al. 2005, Fontaine et al. 2008), robust diagnostic tools predicting the degree of malignancy of borderline WDT such as FT-UMP or WDT-UMP are not available.

High-throughput approaches (genomics, transcriptomics, and/or proteomics) can be useful in identifying diagnostic and prognostic tools in cancer (Ludwig & Weinstein 2005). Among these approaches, profiling of microRNAs (miRNAs), a class of small non-coding RNAs, has shown great promise for the diagnosis and prognosis of tumors (Calin & Croce 2006a,b). Altered expression of several miRNAs has already been reported in PTC (miR-21, miR-31, miR-146, miR-221, and miR-222), FTC (miR-197 and miR-346), and anaplastic thyroid carcinomas (miR-26a, miR-30a-5p, miR-30d, and miR-125b) (He et al. 2005a, Pallante et al. 2006, 2010, Weber et al. 2006, Tetzlaff et al. 2007, Visone et al. 2007, Chen et al. 2008). We therefore reasoned that some specific miRNA signatures might also be associated with ‘borderline’ WDT, i.e. TT-UMP, thus providing useful information for better diagnosis and prognosis of such lesions. We have thus conducted an extensive expression profiling study at the miRNA level on a training cohort of 42 WDT of the thyroid gland including 13 TT-UMP (seven FT-UMP and six WDT-UMP; Table 1). These 13 TT-UMP profiles were compared with those obtained from 16 PTC (11 conventional PTC (C-PTC) and five follicular variants of PTC (FV-PTC)), six FTC, and seven FTA in order to clarify the relationship existing between TT-UMP and the thyroid tumors already well characterized by morphological approaches (i.e. C-PTC, FV-PTC, FTC, and FTA). The obtained miRNA signature was then validated by qRT-PCR on an independent cohort including 18 additional tumors: eight WDT-UMP and ten FT-UMP.

Materials and methods

Patients and thyroid specimens

A total of 60 thyroid tumors consisting of 31 TT-UMP (17 FT-UMP and 14 WDT-UMP), 16 PTC (11 C-PTC and five FV-PTC), six FTC, and seven FTA, classified histologically according to the WHO classification (De Lellis et al. 2004), were included in this study. The samples were obtained at the Department of Pathology of Pasteur Hospital (Nice, France) and from the Gustave Roussy Institute (Villejuif, France). Of these 60 tumors, 13 TT-UMP, 16 PTC, six FTC, and seven FTA were assigned to the training/microarray set, whereas tumors in the training set and 18 supplementary TT-UMP (ten FT-UMP and eight WDT-UMP) were assigned to the validation/qRT-PCR set. Specimens of primary thyroid tumors and corresponding non-tumor thyroid tissues located at least 2 cm from the tumor were frozen in nitrogen and stored at $-80^\circ$C. All patients provided a signed agreement for participating in the study, and the protocol was approved by the local ethics committee of the University of Nice. The epidemiological, clinical, and pathological features of patients included in this study are summarized in Tables 1 and 2.

Genetics of the samples

Genomic DNA from tissue samples was extracted using the MagNA Pure Compact Nucleic Acid Isolation Large Volume kit (Roche) according to the manufacturer’s instructions. Total RNA was extracted from samples with TRIzol solution (Invitrogen), and the integrity of the RNA was assessed using an Agilent Bioanalyser 2100 (Agilent, Palo Alto, CA, USA). Synthesis of cDNA was performed with 1 μg total RNA at 37°C for 90 min, using random hexamer primers and reverse transcriptase in accordance with the manufacturer’s recommendations (Superscript III, Invitrogen). After initial denaturing at 95°C for 5 min, gDNA (25 ng) (for BRAF, KRAS, and NRAS analysis) or cDNA (for RET/PTC and PAX8/PPARγ analysis) was subjected to 40 cycles of PCR, 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, for BRAF, KRAS, and NRAS, and 94°C for 30 s, 55°C for 2 min, and
### Table 1 Epidemiological and pathological features of the training/microarray cohort

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FTA, follicular thyroid adenoma; C-PTC, classical papillary thyroid carcinoma; FV-PTC, follicular variant of papillary carcinoma; WDT-UMP, well-differentiated tumor of uncertain malignant potential; FT-UMP, follicular tumor of uncertain malignant potential; FTC, follicular thyroid carcinoma. Column 2 ‘ID’ gives the reference number of each patient. pTNM according to the UICC (6th edition).
72°C for 2 min, for RET/PTC1 and 3 using Taq platinum (Invitrogen), followed by final extension at 72°C for 7 min. DNA fragments from all patients were bidirectionally sequenced on an ABI 310 sequencer using the Big Dye Terminator kit (Applied Biosystems, Inc., Foster City, CA, USA).

miRNA isolation, microarray hybridization, and analysis

Following total RNA extraction, the miRvana miRNA isolation kit was used for the isolation and enrichment of small RNA fractions (Ambion, Austin, TX, USA), according to the manufacturer’s protocol. Each small RNA fraction (700 ng) was chemically labeled with Alexa Fluors as described previously (Babak et al. 2004). The RNA from each tumor was hybridized with the RNA from the corresponding non-tumor thyroid tissue. Experiments were performed with a miRNA microarray, referenced in GEO under the accession number GPL4717 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL4717). The array contained 1902 different sequences against 1449 distinct miRNAs (Sanger miRNA database, release 8.2, ftp://mirbase.org/pub/mirbase/8.2/) and 35 control RNAs. The microarray was derived from the one described elsewhere (Triboulet et al. 2007). It also included 167 sequences (Legendre et al. 2006). The specificity of the probes was defined by Le Brigand et al. (2006). All hybridization data have been deposited in GEO under the reference GSE15740 via the Mediane information system (Le Brigand & Barbry 2007). Normalization was obtained using the software limma from Bioconductor (www.bioconductor.org; Smyth 2005), according to the variance stabilization and calibration for microarray data (VSN) approach described by Huber et al. (2002).

Each pathological sample was compared with its matched control. This control was taken from the same patient at a large distance from the tumor. Results are therefore shown as log2(signaltumor/signalcontrol). The Bayesian approach developed by Smyth and colleagues was then used to identify probes modified in at least one of the different clinical conditions (Smyth 2005). To that end, intensities were background corrected and normalized according to Huber et al. (2002). A linear model of the data was calculated, and the 21 pairs of contrast between FTA, WDT-UMP, FT-UMP, FV-PTC, FTC, and C-PTC (mutated or not for BRAF) were evaluated. We used cutoff values equal to 7.5 for the log2(signal), 0.7 for the log2(ratio), and 0.01 for the adjusted P value.

Quantitative RT-PCR analysis

Values for miRNA expression, measured using microarrays, were confirmed by quantitative real-time RT-PCR for the selection of ten miRNAs (miR-31,

### Table 2 Epidemiological and pathological features of the validation/qRT-PCR cohort

<table>
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<td></td>
<td>08H01741</td>
<td>1.3</td>
<td>Microfollicular and trabecular</td>
<td>Female</td>
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WDT-UMP, well-differentiated tumor of uncertain malignant potential; FT-UMP, follicular tumor of uncertain malignant potential; NS, not specified. Column 2 ‘ID’ gives the reference number of each patient.
miR-34a, miR-100, miR-146, miR-152, miR-199a, miR-199b, miR-221, miR-222, and miR-451) according to the manufacturer’s protocol (Applied Biosystems, Carlsbad, CA, USA). Ten miRNAs compiled from our study and from other studies (He et al 2005a, Pallante et al. 2006, Chen et al. 2008, Nikiforova et al. 2008, Sheu et al. 2010) were also quantified independently, in the validation set (miR-7, miR-21, miR-31, miR-100, miR-146b, miR-181b, miR-199a, miR-200b, miR-221, and miR-222). Briefly, total RNA was reverse transcribed to cDNA with a miRNA-specific primer (TaqMan MicroRNA reverse transcription kit, Applied Biosystems). RT-PCR was performed using a 7500 Fast Real-Time PCR (Applied Biosystems), with TaqMan probes, as follows: 95°C for 10 min and 95°C for 15 s and 60°C for 1 min (for 40 cycles). Statistical significance was defined as a P value <0.05.

**In situ hybridization**

In situ hybridization (ISH) to detect miR-7, miR-200b, miR-221, and miR-222 was performed using frozen tissues including four normal thyroids, four C-PTC, four WDT-UMP, four FT-UMP, and two FTC. Probes for ISH were purchased from Exiqon (Copenhagen, Denmark) and consisted of locked nucleic acid (LNA) oligonucleotide probes labeled at both the 500 and 30 ends with digoxigenin. Microwave pre-treatment was performed in a 700 W oven in 10 mM citric acid, pH 6.0, for 12 min followed by cooling at room temperature for 20 min. Tissues were then digested in 25 mg/ml proteinase K solution at 37°C for 10 min. Hybridization was performed at 55°C overnight in a humidified chamber using 200 nM probe. Slides were washed in 2 M sodium chloride–sodium citrate (SSC) at 50°C for 20 min, followed by washing in 0.5 M SSC at 50°C for 20 min. The slides were then rinsed in Tris-buffered saline (TBS, pH 7.5; buffer A) and incubated with an anti-digoxigenin–alkaline phosphatase-labeled antibody (Roche) at 1:200 dilution in buffer A with 1% normal swine serum and 0.3% Triton X-100 for 3 h. After washing in buffer A and then in buffer C (TBS, pH 9.5), the slides were incubated with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate, at pH 9.5 in buffer C and developed between 30 min to 3 h. The negative control consisted of substituting a scrambled probe (Exiqon) at the same concentration as the hybridization probe. As a second control, the slides were treated with RNase A (250 μg/ml from Sigma) before hybridization.

**Statistical analysis**

The Student’s t-test was used to analyze the significant differences in the real-time RT-PCR experiments. A P value <0.05 with the two-tailed test was considered significant.

**Results**

The genetic mutations and rearrangements in thyroid tumor samples

A BRAF mutation was identified in six of the 11 C-PTC. No BRAF mutation was found in the group of TT-UMP (Table 3). Two WDT-UMP and one FT-UMP had NRAS mutations and one FT-UMP had a PAX8/PPARγ rearrangement. No RET/PTC rearrangement was found in TT-UMP.

The miRNA expression profile of well-differentiated thyroid tumors

Figure 1 shows hierarchical clustering of 42 pairs of miRNA samples (tumor versus control; training set) for the selection of 83 miRNAs. The distance was measured using the Spearman’s distance on the matrix of log2(ratio). The classification was performed using a complete agglomeration method. Two main arms were detected. The first arm contained all C-PTC samples (mutated or not in BRAF), four of the five FV-PTC samples, one WDT-UMP, one of the seven FTA, and one of the six FTC. The second arm is more heterogenous and contained most of the FTA (six of the seven), all FT-UMP (seven samples), and also five WDT-UMP and five FTC. Overall, Fig. 1 shows a clear consistency between miRNA profiling and papillary carcinoma diagnosis, corresponding either to C-PTC or to FV-PTC. FT-UMP showed some proximity with adenomas. The FTC and WDT-UMP samples did not appear in the same group as the others. Figure 1 also highlights some extreme log2(ratios) observed for miRNAs, such as miR-146a, miR-146b, miR-21, miR-221, and miR-222. The results are summarized in Table 4, which represent the average log2(ratios) for the 43 most discriminative miRNAs. The relationship between the different clinical groups was also visualized using paired comparison of the log2(ratio) (Fig. 2). A linear relationship between the average of two clinical conditions indicates proximity between them. This was the case when mutBRAF-C-PTC and wtBRAF C-PTC were compared, for which a coefficient of correlation close to 1 (i.e. 0.94) was observed. This representation illustrates the relationships existing between mutBRAF C-PTC,
wt*BRAF* C-PTC, and FV-PTC on the one hand and FTA, FTC, FT-UMP, and WDT-UMP on the other hand.

### Validation of differentially expressed miRNAs by real-time qRT-PCR

Both the training set of thyroid tumors analyzed using microarrays and an independent set of TT-UMP were analyzed using qRT-PCR (Fig. 3). The values for the average fold difference obtained by qRT-PCR TaqMan for each miRNA were plotted against the values for the fold change obtained from microarray data. A good correlation was obtained between the results of microarray and qRT-PCR (Fig. 3). In the samples obtained from the validation cohort, seven of the ten miRNAs quantified by qPCR showed significant modulation (Table 5). In particular, *miR-221* and *miR-222* were upregulated...
in WDT-UMP and FT-UMP, miR-146b and miR-34a were upregulated and miR-199a was downregulated in WDT-UMP. However, we were not able to identify in the supervision group any miRNA(s) showing differential expression between WDT-UMP and FT-UMP. This result clearly indicates that miRNAs cannot be used to distinguish between these two subgroups.

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**Figure 1** Heat map of 84 differentially expressed miRNAs analyzed for 42 patients. The cutoff values equal to 7.5 for the log2 (signal), ± 0.7 for the log2(ratio), and 0.01 for the adjusted P value were used. Each column corresponds to one patient: five C-PTC with wild-type BRAF; six C-PTC with mutated BRAF, five FV-PTC, seven adenomas, six FTC, six WDT-UMP, and seven FT-UMP were analyzed. Each line corresponds to one of the 84 miRNAs found differentially expressed between normal and pathological tissues, or differing between at least two experimental groups. The false color image represents the log2(ratio) between lesion and control biopsies. The log2(ratio) was measured between normal and pathological RNA extracted from the same patient. Control tissue was taken at a large distance from the lesion in order to minimize the risk of contamination with tumor tissue. Distances were measured according to Spearman. Clustering was performed using a complete method. wtC-PTC, wild-type classical papillary thyroid carcinoma (C-PTC non-mutated for BRAF V600); mutC-PTC, mutated classical papillary papillary thyroid carcinoma (C-PTC mutated for BRAF V600); FV-PTC, follicular variant of papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; FTA, follicular thyroid adenoma; WDT-UMP, well-differentiated tumors of uncertain malignant potential; FT-UMP, follicular tumor of uncertain malignant potential.
Expression of miRNA in thyrocytes is altered

ISH was performed on frozen tissue sections. An additional section was stained with HE for overall morphology (not shown). Figure 4A and B show that mir-7, mir-200b, mir-221, and mir-222 expression was restricted to thyrocytes. Normal thyroid tissues showed diffuse weak expression of these miRNAs (not shown). ISH with miR-221 and miR-222 showed moderate-to-strong expression in the C-PTC compared with other tumors. In particular, expression was strongly increased in C-PTC in comparison with WDT-UMP specimens. Low expression of mir-7 and mir-200b was observed in FTC (Fig. 4B, a, b), compared with that observed in
FT-UMP (Fig. 4B, d, e), in agreement with the microarray data. The specificity of the reaction was shown by RNAase digestion before ISH (not shown) and by using a scrambled LNA probe instead of specific miRNAs (Fig. 4A, c, f and B, c, f).

**Supervised classification of thyroid samples**

To evaluate more precisely the relationships between WDT-UMP and FT-UMP on the one hand, and FTA, C-PTC, FV-PTC, and FTC on the other hand, we used the supervised classification method described by Culhane et al. (2005). A between-group analysis (BGA) of microarray data was performed to ordinate the groups rather than the individual samples. It has been shown that the BGA of microarray data was performed by comparing other supervised classification methods, including support vector machines and neural networks (Culhane et al. 2005). Each three-dimensional plot in Fig. 5 illustrates the distribution of the training set of clinical samples in a three-dimensional space that integrates all the variations within the FTA, C-PTC, FTC, and FV-PTC samples. The coordinates of the supplementary samples (FT-UMP and WDT-UMP) were represented in the same three-dimensional space. A color code was used to position the data points according to the third axis of projection (CS3), and a vertical bar indicates the distance to the second axis of the projection (CS2=0). Each three-dimensional plot corresponds to one clinical condition (C-PTC, FV-PTC, WDT-UMP, FTA, FT-UMP, or FTC), and each point within each three-dimensional plot represents one patient from that group. These plots well illustrate the compactness of the FTA and C-PTC groups, as well as the more widespread distribution of the WDT-UMP, FT-UMP, FTC, and FV-PTC samples. This representation points to the fact that miRNA can be used to distinguish between C-PTC and FTA but are probably not sufficient *per se* to characterize the other groups.

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**Figure 2** Correlation between average log2(ratios) for C-PTC (wild-type BRAF), C-PTC (mutated BRAF), FV-PTC, adenoma, FTC, WDT-UMP, and FT-UMP. Each lower panel of the matrix scatter plot shows the relationship between the log2(ratios) of two groups with names given on the diagonal. Each point corresponds to one miRNA probe found differentially expressed by linear model analysis (Smyth 2005). The interpolation plot is shown in red. Each symmetric upper panel indicates the corresponding Pearson’s coefficient of correlation between the two groups.
miRNA expression profiles differ among categories of WDT tumors

Table 4 and Fig. 1 summarize the results obtained for miRNAs that displayed the largest range of variation. We observed that several groups of miRNAs were useful to define the sample class. For instance, group 1, composed of miR-513 and miR-494, appeared to be more strongly decreased in FTA than in other samples. Group 2, composed of miR-26a, miR-27a, miR-125a, miR-130a, and miR-143, appeared to be more strongly inhibited in FTC than in other samples. Group 4, composed of miR-7 and miR-345, appeared to be increased in TT-UMP and decreased in PTCs. Group 6, composed of miR-31, miR-375, miR-21 and miR-146a, appeared to be increased in PTCs. Group 7, composed of miR-15a, miR-181b, miR-22a, miR-221, miR-34a, and miR-146b, appeared to be increased in PTCs, but also in FTA and in WDT-UMP. Group 8, composed of miR-203, miR-136, miR-126-3p, miR-148a, and miR-152, was helpful in distinguishing mutBRAF and wtBRAF samples of C-PTC from other samples.

A direct comparison of the signature of FT-UMP and WDT-UMP was able to reveal differences in miRNA expression between the two conditions. For instance, differential expression of miR-221, miR-146b, miR-222a, miR-451, and miR-7 was observed between the two groups.

To better define the miRNA profile of the different PTCs studied, we also compared samples according to histological or genetic features. C-PTC and FV-PTC demonstrated a very similar miRNA signature. Interestingly, miRNA expression of C-PTC with a BRAF mutation versus C-PTC without a BRAF mutation showed that miR-203 was more highly expressed in BRAF-mutated C-PTC in comparison with non-BRAF-mutated cases.

Discussion

It is difficult to morphologically evaluate the risk of malignancy associated with some WDT of the thyroid gland, as well as the prognosis of WDT in general (Franc et al. 2003). In this regard, interest has been raised in defining additional categories of thyroid tumors, namely WDT-UMP and FT-UMP.
These tumors represent borderline lesions that are difficult to diagnose morphologically, since they lie between FTA, FV-PTC, and minimally invasive FTC. However, information about WDT-UMP and FT-UMP is still limited and largely debated (Baloch & LiVolsi 2007). Moreover, despite a number of recent studies, ancillary methods have had a low impact on aiding the diagnosis of such lesions (Rosai 2005, Fonseca et al. 2006, Fontaine et al. 2008).

In the last decade, there has been growing interest in the study of miRNA in various physiological and pathophysiological conditions (Bartel 2004, Baulcombe 2005, Croce & Calin 2005, Mattick & Makunin 2005, Pasquinelli et al. 2005, Waldman & Terzic 2009). Different analyses have shown that altered expression of specific miRNA genes contributes to the initiation and progression of several types of cancer including thyroid carcinoma (Calin et al. 2004, Takamizawa et al. 2004, Chan et al. 2005).

**Figure 4** Comparison of the expression of selected miRNAs using in situ hybridization with locked nucleic acid probes for miR-7, miR-200b, miR-221, and miR-222 for different thyroid tumors. (A) Overexpression of miR-221, and miR-222 is observed in C-PTC (a, b), compared to that observed in WDT-UMP (d, e). The scrambled control LNA probe showed no hybridization signal (c, f) (a–f, original magnification × 630). (B) Low expression of miR-7 and miR-200b is observed in FTC (a, b), compared to that observed in FT-UMP (d, e). The scrambled control LNA probe showed no hybridization signal (c, f) (a–c, f, original magnification × 400; d, e, original magnification × 630). miRNA expression was mainly observed in thyrocytes (indicated by arrowheads).

**Figure 5** Three-dimensional scatter plot of the 42 patients. The data set of the 42 patients was analyzed using the BGA function (between-group analysis) available from the MADE4 package from Bioconductor (Culhane et al. 2005). A principal component analysis was performed on groups rather than on individual samples to define a three-dimensional space maximizing variations between the four reference groups (C-PTC, FV-PTC, FTA, and FTC). All 42 samples were then projected into this three-dimensional space. WDT-UMP and FT-UMP were treated as supplementary data and were projected into the same three-dimensional space.
He et al. 2005a,b, Iorio et al. 2005, 2007, Lu et al. 2005, Ortholan et al. 2009). However, the determination and the use of a miRNA signature in TT-UMP have not been established previously.

The paucity of tools to evaluate the diagnosis and prognosis of some WDT of the thyroid gland was the initial justification for this study. We show that the different categories of WDT of the thyroid gland, including the so-called TT-UMPs, can be separated according to the level of expression of a small panel of miRNAs. Our data showed the existence of distinct profiles for WDT-UMPs, FT-UMPs, FTAs, PTCs (C-PTCs and FT-PTCs), and FTCs. The FT-UMPs were located in a region that is intermediate between FTAs and FTCs (Fig. 5), while WDT-UMPs samples were located in a region that is intermediate between FTAs, FV-PTCs, and C-PTCs samples. Supervised classification approaches were helpful in positioning these different groups of WDT. More specifically, we hypothesized that expression of some miRNAs might be altered in TT-UMP. However, rather than a specific alteration of expression for one or both groups of TT-UMP, we found that several miRNAs previously associated with FTC, FTA, or FTA were also deregulated in TT-UMPs, possibly at a lower or even sometimes at an opposite level. Moreover, as described previously (He et al. 2005a, Pallante et al. 2006, Tetzlaff et al. 2007, Nikiforova et al. 2008, 2009, Sheu et al. 2010), we confirmed in our cohort of 16 PTC that miR-221, miR-222a, miR-146a, miR-146b, miR-21, and miR-222 were robustly overexpressed in this category of WDT (both in C-PTC and in FV-PTC). Interestingly, our results also showed increased expression of miR-221 and miR-222a in some TT-UMP samples. The expression of miR-146a and miR-146b was strongly increased in PTC (both in C-PTC and in FV-PTC). miR-146b was slightly increased in WDT-UMP and FTA. Thus, a small set of miRNAs (i.e. miR-146a, miR-146b, miR-221, and miR-222a) can be useful to distinguish between WDT-UMP and PTCs (either FV-PTC or C-PTC) and FTA. In this regard, a previous study demonstrated that mir-146b was not overexpressed in a group of borderline lesions intermediate between FTA and FV-PTC (Chen et al. 2008). Sheu et al. (2010) have recently shown that only mir-146b was significantly upregulated when comparing mean values of FV-PTC with WDT-UMP. Minimally invasive FTC can be difficult to distinguish morphologically from some FTA and might sometimes be diagnosed as FT-UMP (Williams 2000, De Lellis et al. 2004, Rosai 2005). Our study shows that expression of mir-7 and mir-200b was altered in FTC relative to FT-UMP. Finally, the miRNA signature of FV-PTC was quite similar to that of C-PTC, sharing many traits, such as the overexpression of mir-221, mir-222, mir-146a, and mir-146b.

To localize the cellular origin of some of the miRNAs identified in our study, we performed ISH on a panel of WDT of the thyroid gland. Data presented in Fig. 4 confirmed the higher expression of miR-221 and miR-222 in C-PTC than in WDT-UMP and the higher expression of mir-7 and mir-200b in FT-UMP than in FTC. Moreover, ISH allowed us to demonstrate that this overexpression was specific to epithelial cells.

A previous study has demonstrated that mir-221 was also overexpressed in the non-tumor tissue of patients with C-PTC (He et al. 2005a). We could not confirm this data. Several elements can explain this discrepancy. First, it may be due to the fact that in our study, the different frozen specimens, used as paired non-tumor tissue, were taken from further away (i.e. at least 2 cm) from the C-PTC, in the same lobe or in the contralateral lobe, than in the study by He et al. (2005a). Secondly, we always checked the morphology of the corresponding non-tumor specimens on frozen sections before RNA extraction. This procedure allowed us to eliminate potential papillary microcarcinoma of the thyroid around the main tumor. Thirdly, in our study, the global analysis of miRNAs in the corresponding non-tumor tissue, in comparison with the miRNA profile in a unique normal thyroid tissue, did not reveal significant differences.

If major advances in the characterization of mutations and chromosomal rearrangements in WDT of the thyroid gland have been made, the impact of these different modifications on the miRNA signature in these tumors has been poorly investigated to date. BRAF mutations are detected in around half of the C-PTC (Xing 2005, Lassalle et al. 2010). In particular, the most frequent mutation, a V600E amino acid substitution, has been described in more than 800 C-PTC, with a prevalence of 20–69% in the different studies (Puxeddu et al. 2004, Trovisco et al. 2004, 2005, Xing 2005, Lassalle et al. 2010). Intragenic BRAF mutations seem to be almost completely restricted to non-radiation-induced cancers from the adult population (Lima et al. 2004). Several studies have reported that this mutation is related to poor prognosis in patients with C-PTC (Namba et al. 2003, Nikiforova et al. 2003, Giordano et al. 2005, Vasko et al. 2005, Xing et al. 2005, Adeniran et al. 2006, Lee et al. 2007). Conversely, the type and prevalence of BRAF mutations differ in FV-PTC from those detected in C-PTC (Trovisco et al. 2004). Thus, FV-PTC cases are characterized by a low prevalence (10%) of K601E (Trovisco et al. 2004). The most distinctive molecular
features of follicular carcinoma are a high prevalence of PAX8–PPARγ rearrangements and RAS mutations. The PAX8–PPARγ fusion gene has been detected in a high percentage of FTC (22–53%; Kroll et al. 2000; Nikiforova et al. 2002). This fusion gene has also been detected in FV-PTC (25%; Castro et al. 2006). RAS mutations are frequently detected in follicular adenomas and follicular carcinomas but do not provide any useful information from a diagnostic standpoint (Namba et al. 1990). Finally, distinct forms of RET mutations have been found in different WDTs of the thyroid gland. Rearrangements with other genes such as that of the RET/PTC oncogene have been demonstrated to play a causative role in a significant number of thyroid tumors (De Lellis et al. 2004). In our study, we were not able to find any miRNA signature significantly associated with the different mutations and rearrangements observed for WDT of the thyroid gland. This finding may imply that alterations in miRNA expression correspond to a late event in the pathogenesis of thyroid carcinogenesis. Further studies with additional cases, in particular for the PAX8–PPARγ rearrangement, will now be needed to associate specific miRNA profiles with different molecular characterization of WDTs. In our C-PTC samples, the impact of the mutations on BRAF appeared to be more quantitative than qualitative: both were associated with a similar miRNA signature, but levels of variation were often magnified when BRAF was mutated. Notably, miR-203 was differentially expressed in C-PTC with a BRAF mutation compared to all other conditions. This result has to be confirmed by qPCR on a larger group of BRAF-mutated and non-mutated C-PTC samples. Interestingly, Nikiforova et al. (2008) showed that miR-221, miR-222, and miR-146b were strongly upregulated in tumors carrying a BRAF mutation, whereas Chou et al. (2010) found that miR-146b had a more significant upregulation in BRAF-mutated C-PTC.

The miRNAs regulated in thyroid tumors are present on the chip designed for this study. However, it could be of interest to check the results with other commercially available miRNA chips containing a more exhaustive list of currently known sequences of human miRNAs. This should not only confirm the results of this study but also allow the discovery of new miRNA involved in thyroid pathology.

A potential criticism of the results lies in the fact that the miRNA signatures were obtained from whole tumor specimens since molecular heterogeneity can be present in the same tumor. To increase the variation in miRNA expression observed between the different sub-cohorts of tumors, an interesting approach may be to select different areas and foci of interest in these tumors using laser capture microdissection (LCM) before miRNA extraction. Inclusion of LCM to harvest a more homogenous cell population may avoid the heterogenous expression repertoire of miRNA. This approach and the interest in performing geographical mapping of thyroid tumors have been demonstrated in a previous study (Aherne et al. 2008). The inclusion of 35 TT-UMP cases, in this study, represents the largest panel of such samples ever studied by miRNA analysis. Our results demonstrate for the first time that FT-UMP and WDT-UMP samples differ slightly from PTCs (both C-PTCs and FV-PTCs), FTCs, and FTAs in their miRNA signatures. While we strongly believe that these results strengthen the existence of TT-UMPs as distinct clinical entities, this has to be confirmed on a larger panel of TT-UMP patients. Supervised classification suggests a distinct status for FT-UMP and WDT-UMP, the former being nearer to FTA than the latter.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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