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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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°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/PPAR γ* 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

Diagnosis	ID	Tumor size (cm)	Histopathology	pTNM	Gender	Age (years)	History of thyroid disease
FTA	2	3.5	Microfollicular	–	Female	46	None
	10	2.8	Microfollicular	–	Male	31	None
	11	2.0	Microfollicular	–	Male	68	None
	20	1.4	Heterofollicular	–	Male	77	None
	32	2.0	Heterofollicular	–	Female	51	None
	37	4.0	Microfollicular	–	Male	42	None
	40	4.0	Heterofollicular	–	Male	74	None
C-PTC	15	3.5	Papillary	pT3 N1M0	Male	23	None
	23	1.5	Papillary	pT1 N0M0	Female	46	Hyperplastic nodules
	26	1.0	Papillary	pT1 N0M0	Female	80	None
	31	1.5	Papillary	pT3 N0M0	Female	53	Hyperplastic nodules
	39	2.8	Papillary	pT2m N1M0	Female	41	None
	12	1.3	Papillary	pT3m N1M0	Female	43	None
	24	2.3	Papillary	pT3 N1M0	Male	34	None
FV-PTC	28	1.5	Papillary	pT1 N0M0	Female	69	None
	33	1.1	Papillary	pT1 N1M0	Female	18	None
	34	1.8	Papillary	pT1 N1M0	Female	39	None
	35	1.2	Papillary	pT1 N1M0	Female	43	None
	4	3.0	Heterofollicular, invasive	pT2 N0M0	Female	28	None
	6	3.0	Heterofollicular, invasive	pT2 N0M0	Male	36	None
	13	1.4	Heterofollicular, invasive	pT3 N0M0	Female	50	None
WDT-UMP	17	2.5	Heterofollicular, encapsulated	pT2 N0M0	Female	39	None
	29	1.3	Vesicular predominant, invasive	pT3 N0M0	Female	42	None
	3	3.0	Heterofollicular	–	Female	29	Hyperplastic nodules
	9	2.8	Heterofollicular	–	Female	66	None
	16	8.0	Microfollicular	–	Female	62	Goiter
	18	2.5	Microfollicular	–	Female	34	None
	22	2.5	Microfollicular	–	Male	66	None
FT-UMP	30	6.0	Microfollicular and trabecular	–	Female	57	None
	1	6.0	Heterofollicular	–	Female	78	None
	5	2.5	Microfollicular	–	Female	66	Hyperplastic nodules
	7	3.0	Heterofollicular	–	Male	45	None
	14	6.5	Microfollicular	–	Male	60	None
	25	3.0	Microfollicular	–	Male	53	None
	27	2.5	Heterofollicular	–	Female	36	None
FTC	36	3.5	Microfollicular	–	Female	55	None
	8	3.2	Well-differentiated minimally invasive	pT2N0M0	Female	54	None
	19	3.5	Well-differentiated minimally invasive	pT2N0M0	Male	61	None
	21	2.2	Oncocytic, minimally invasive	pT2N0M0	Female	30	None
	38	2.7	Well-differentiated minimally invasive	pT2N0M0	Female	78	None
	41	1.3	Oncocytic, minimally invasive	pT3N0M0	Female	27	None
	42	2.0	Well-differentiated minimally invasive	pT1N0M0	Female	28	None

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).

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

Diagnosis	ID	Tumor size (cm)	Histopathology	Gender	Age (years)	History of thyroid disease
WDT-UMP	43	3.0	Heterofollicular	Female	26	None
	44	4	Heterofollicular	Female	31	None
	45	2.4	Heterofollicular	Female	44	None
	46	1.5	Microfollicular	Male	40	None
	48	3	Microfollicular	Male	26	None
	49	3.5	Heterofollicular	Female	20	None
	50	5	Microfollicular	Female	77	None
	51	1.5	Microfollicular	Female	59	None
FT-UMP	53	2	Oncocytic microfollicular and trabecular	Male	74	None
	54	1.8	Microfollicular	Male	46	None
	55	3	Oncocytic microfollicular and trabecular	Female	43	None
	56	3.0	Oncocytic microfollicular and trabecular	Female	60	None
	57	0.8	Heterofollicular	Female	35	None
	58	1.2	Heterofollicular	Female	16	None
	380326	2	Heterofollicular	Female	51	NS
	07H01751	2.5	Heterofollicular	Female	51	NS
	08H01741	1.3	Microfollicular and trabecular	Female	25	NS
	08H05210	3.3	Heterofollicular	Female	51	NS

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.

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 Mediant 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 $\log_2(\text{signal}_{\text{tumor}}/\text{signal}_{\text{control}})$. 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 $\log_2(\text{signal})$, 0.7 for the $\log_2(\text{ratio})$, 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*,

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 nitrobluetetrazolium 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 $\log_2(\text{ratio})$. 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 heterogeneous 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 $\log_2(\text{ratio})$ s 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 $\log_2(\text{ratios})$ for the 43 most discriminative miRNAs. The relationship between the different clinical groups was also visualized using paired comparison of the $\log_2(\text{ratio})$ (Fig. 2). A linear relationship between the average of two clinical conditions indicates proximity between them. This was the case when mut*BRAF*-C-PTC and wt*BRAF* 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 mut*BRAF* C-PTC,

Table 3 Mutations and rearrangements detected in the different tumor specimens of the training set used for miRNA analysis

Diagnosis	References	B-RAF	K-RAS	N-RAS	H-RAS	RET/PTC1–3	PAX8/PPAR γ
FTA	2	NS	NS	NS	NS	NS	Negative
	10	NS	NS	NS	NS	NS	Negative
	11	NS	NS	NS	NS	NS	Negative
	20	NS	NS	NS	NS	NS	Negative
	32	NS	NS	NS	NS	NS	Negative
	37	NS	NS	NS	NS	NS	Negative
	40	NS	NS	NS	NS	NS	Negative
C-PTC	15	Negative	Negative	Negative	Negative	Negative	Negative
	23	Negative	Negative	Negative	Negative	Negative	Negative
	26	Negative	Negative	Negative	Negative	Negative	Negative
	31	Negative	Negative	Negative	Negative	Negative	Negative
	39	Negative	Negative	Negative	Negative	Negative	Negative
	12	Positive	Negative	Negative	Negative	Negative	Negative
	24	Positive	Negative	Negative	Negative	Negative	Negative
	28	Positive	Negative	Negative	Negative	Negative	Negative
	33	Positive	Negative	Negative	Negative	Negative	Negative
	34	Positive	Negative	Negative	Negative	Negative	Negative
	35	Positive	Negative	Negative	Negative	Negative	Negative
FV-PTC	4	Negative	Negative	Negative	Negative	Positive	Negative
	6	Negative	Negative	Negative	Negative	Negative	Negative
	13	Negative	Negative	Negative	Negative	Negative	Negative
	17	Negative	Negative	Negative	Negative	Negative	Negative
	29	Negative	Negative	Negative	Negative	Negative	Negative
WDT-UMP	3	Negative	Negative	Negative	Negative	Negative	Negative
	9	Negative	Negative	Negative	Negative	Negative	Negative
	16	Negative	Negative	Positive	Negative	Negative	Negative
	18	Negative	Negative	Negative	Negative	Negative	Negative
	22	Negative	Negative	Positive	Negative	Negative	Negative
	30	Negative	Negative	Negative	Negative	Negative	Negative
FT-UMP	1	Negative	Negative	Negative	Negative	Negative	Negative
	5	Negative	Negative	Negative	Negative	Negative	Negative
	7	Negative	Negative	Negative	Negative	Negative	Positive
	14	Negative	Negative	Negative	Negative	Negative	Negative
	25	Negative	Negative	Negative	Negative	Negative	Negative
	27	Negative	Negative	Positive	Negative	Negative	Negative
	36	Negative	Negative	Negative	Negative	Negative	Negative
FTC	8	NS	Negative	Negative	Negative	Negative	Negative
	19	NS	Negative	Negative	Negative	Negative	Negative
	21	NS	Negative	Negative	Negative	Negative	Positive
	38	NS	Negative	Negative	Negative	Negative	Negative
	41	NS	Negative	Negative	Negative	Negative	Positive
	42	NS	Negative	Negative	Negative	Negative	Negative

FTA, follicular thyroid adenoma; C-PTC, classical papillary thyroid carcinoma; FV-PTC, follicular variant papillary carcinoma; WDT-UMP, well-differentiated tumor of uncertain malignant potential; FT-UMP, follicular tumor of uncertain malignant potential; FTC, follicular thyroid carcinoma; NS, not searched. Column 2 of the table gives the reference of each patient.

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

Figure 1 Heat map of 84 differentially expressed miRNAs analyzed for 42 patients. The cutoff values equal to 7.5 for the $\log_2$ (signal), ± 0.7 for the $\log_2$ (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 $\log_2$ (ratio) between lesion and control biopsies. The $\log_2$ (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.

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.

Table 4 A summary of the differentially expressed miRNAs between tumor and unaffected thyroid tissue (training set). Mean of log₂ (fold changes) are represented

miRNA	FTA	FTC	FT-UMP	WDT-UMP	FV-PTC	wtC-PTC	mutC-PTC
<i>mir-513</i>	-1.86±0.67	-0.54±0.5	-0.03±0.93	0.37±0.86	0.2±1.2	0.17±0.47	-0.78±0.62
<i>mir-494</i>	-0.74±0.21	-0.1±0.36	-0.25±0.73	0.74±0.3	0.03±0.83	0.61±0.54	-0.7±0.43
<i>mir-29ac</i>	-0.23±0.24	-0.47±0.2	0.03±0.15	0.26±0.13	0±0.23	-0.45±0.15	0.22±0.05
<i>mir-15b</i>	0.1±0.19	-0.6±0.17	-0.53±0.2	-0.08±0.19	-0.58±0.23	-0.22±0.15	-0.03±0.17
<i>mir-125a</i>	0.03±0.1	-0.91±0.33	-0.24±0.1	-0.46±0.11	-0.48±0.15	-0.07±0.07	0.03±0.09
<i>mir-27a</i>	-0.04±0.13	-0.84±0.1	-0.54±0.39	-0.24±0.25	0.19±0.67	0.23±0.28	0.55±0.14
<i>mir-26a</i>	-0.23±0.3	-1.03±0.3	-0.55±0.47	-0.58±0.45	-0.08±0.42	-0.14±0.18	-0.28±0.13
<i>mir-130a</i>	-0.24±0.37	-0.93±0.51	-0.62±0.32	-0.24±0.6	-0.58±0.35	-0.91±0.4	-0.23±0.15
<i>mir-143</i>	0.3±0.08	-0.82±0.31	-0.8±0.21	-0.19±0.21	-0.26±0.21	-0.11±0.25	-0.48±0.15
<i>mir-497</i>	0.42±0.2	-0.4±0.14	0.06±0.23	-0.53±0.5	-0.02±0.15	-0.49±0.28	-0.57±0.16
<i>mir-98</i>	0.17±0.23	-0.38±0.08	-0.36±0.46	-0.63±0.39	-0.19±0.26	0.01±0.15	-0.45±0.3
<i>mir-200b</i>	-0.46±0.36	-0.34±0.31	0.47±0.26	-0.14±0.41	0.11±0.47	0.52±0.13	0.41±0.09
<i>mir-429</i>	-0.11±0.15	-0.5±0.22	0.58±0.15	0.39±0.16	0.63±0.31	0.7±0.2	0.47±0.21
<i>mir-181d</i>	0.6±0.25	-0.26±0.25	0.17±0.23	0±0.23	0.47±0.26	0.75±0.27	0.99±0.24
<i>mir-99a</i>	-0.59±0.29	-0.8±0.46	-1.36±0.25	-0.71±0.31	-0.33±0.15	-0.46±0.26	-0.62±0.16
<i>mir-100</i>	-0.99±0.27	-1.33±0.25	-1.5±0.24	-1.16±0.45	-1.33±0.3	-0.8±0.32	-0.81±0.13
<i>mir-199b</i>	-1.63±0.17	-2.03±0.18	-1.78±0.4	-2.07±0.14	-0.86±0.23	-1.26±0.55	-1.05±0.22
<i>mir-199a</i>	-0.86±0.12	-2.16±0.21	-2.35±0.2	-1.98±0.17	-0.85±0.26	-0.67±0.3	-0.27±0.05
<i>mir-15a</i>	0.41±0.18	0.01±0.32	0.38±0.26	1±0.33	0.44±0.53	1.16±0.18	1.01±0.16
<i>mir-181b</i>	0.58±0.33	-0.07±0.17	0.31±0.21	0.74±0.19	1.03±0.33	0.79±0.2	1.14±0.19
<i>mir-222a</i>	0.89±0.47	-0.34±0.54	-0.3±0.5	1.45±0.23	2.41±0.55	3.27±0.18	3.81±0.22
<i>mir-221</i>	1.19±0.64	0.2±0.56	-0.43±0.51	1.56±0.47	2.47±0.54	3.36±0.11	3.87±0.11
<i>mir-34a</i>	1.36±0.27	0.25±0.34	0.78±0.47	1.63±0.21	1±0.26	1.56±0.24	2.2±0.24
<i>mir-146b</i>	1.15±0.58	-0.3±0.3	-0.22±0.53	1.75±0.87	2.87±1.21	4.17±0.48	4.82±0.15
<i>mir-345</i>	0.24±0.24	0.06±0.33	0.93±0.16	0.78±0.5	-0.06±0.14	-0.65±0.22	-0.79±0.15
<i>mir-7</i>	0.56±0.43	-0.49±0.96	1.87±0.65	0.09±1	-1.09±0.19	-1.67±0.31	-3.11±0.28
<i>mir-375</i>	0.22±0.22	-0.04±0.23	-0.04±0.14	0.33±0.32	1.16±0.41	0.92±0.23	1.39±0.2
<i>mir-21</i>	0.62±0.23	-0.15±0.39	0.11±0.36	0.17±0.27	1.07±0.74	1.44±0.65	2.13±0.14
<i>mir-146a</i>	0.19±0.18	-0.61±0.48	-0.62±0.32	0.3±0.08	1.63±0.99	2.05±0.64	2.7±0.23
<i>mir-31</i>	-0.42±0.31	-1.39±0.15	-1.14±0.11	-0.63±0.08	1.1±0.71	1.67±0.13	1.82±0.26
<i>mir-150</i>	-0.52±0.06	-1.38±0.47	-1.46±0.23	-0.88±0.08	-0.87±0.21	-0.92±0.33	-0.29±0.1
<i>mir-197</i>	-0.65±0.17	-1.01±0.42	-1.09±0.12	-0.78±0.27	-0.66±0.21	-0.23±0.25	-0.13±0.21
<i>mir-328</i>	-0.61±0.11	-1.43±0.18	-0.88±0.06	-1.11±0.14	-0.96±0.15	-0.44±0.12	-0.22±0.39
<i>mir-203</i>	0.06±0.17	0.42±0.13	0.11±0.2	-0.01±0.13	0.29±0.27	0.01±0.28	1±0.2
<i>mir-136</i>	0.18±0.06	0.13±0.14	0.08±0.09	0.32±0.11	0.59±0.12	0.59±0.19	1.02±0.11
<i>mir-126-3p</i>	0.32±0.14	0±0.17	-0.36±0.59	0.09±0.35	0.2±0.33	-0.46±0.24	-0.8±0.16
<i>mir-148a</i>	0.4±0.32	-0.22±0.55	0.49±0.17	0.11±0.52	-0.29±0.23	-0.82±0.2	-0.58±0.18
<i>mir-152</i>	0.26±0.29	0.45±0.28	0.07±0.19	-0.06±0.52	-0.14±0.14	-0.84±0.2	-1.05±0.2
<i>mir-29b</i>	0.89±0.19	-0.22±0.34	-0.15±0.21	0.77±0.14	0.6±0.45	-0.01±0.11	1±0.13
<i>mir-182</i>	0.58±0.11	0.45±0.09	0.72±0.07	0.46±0.06	0.16±0.08	0.44±0.11	0.29±0.1
<i>mir-141</i>	0.33±0.54	0.22±0.52	0.86±0.28	0.74±0.47	0.8±0.52	0.03±0.29	0.6±0.35
<i>mir-451</i>	-1.3±0.2	-1.79±0.3	-1.73±0.78	-0.58±0.73	-2.21±0.44	-1.95±0.08	-2.04±0.24
<i>mir-638</i>	-1.45±0.25	-0.77±0.5	-1.29±0.54	-1.19±0.5	-1.07±0.23	-0.83±0.36	-1.47±0.11

The different probes spotted on chips showed no significant fold variation. wtC-PTC, classical papillary thyroid carcinoma *BRAF* wild type; mutC-PTC, classical papillary thyroid carcinoma *BRAF* V600E; FV-PTC, follicular variant of papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; FTA, follicular thyroid adenoma; WDT-UMP, well-differentiated tumour of uncertain malignant potential; FT-UMP, follicular tumour 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

Figure 2 Correlation between average $\log_2$ (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 $\log_2$ (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.

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.

Figure 3 Quantitative RT-PCR analysis of a set of selected miRNA strongly regulated in different families of thyroid tumors. Comparison of qRT-PCR results (red) and microarray data (blue) showed similar expression levels for *miR-31*, *miR-146b*, *miR-221*, *miR-222*, and *miR-451* on C-PTC; *miR-146b*, *miR-221*, *miR-222*, and *miR-451* on FV-PTC; *miR-199a*, *miR-34a*, *miR-146b*, *miR-221*, and *miR-222* on WDT-UMP; *miR-199a* and *miR-199a* on FTA; *miR-199a*, *miR-199b*, and *miR-152* on FTC; and *miR-100*, *miR-199a*, *miR-221*, and *miR-222* on FT-UMP.

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-222a*, *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 mut*BRAF* and wt*BRAF* 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

Table 5 qPCR mean of fold changes of the validation cohort between non-tumoral and tumoral tissue

MicroRNAs	FT-UMP		WDT-UMP	
	qPCR	P values	qPCR	P values
<i>mir-221</i>	2.62	≤0.001	3.56	≤0.001
<i>mir-222</i>	1.83	≤0.001	3.01	≤0.001
<i>mir-146b</i>	0.50	NS	1.96	≤0.01
<i>mir-200b</i>	0.73	NS	−0.34	NS
<i>mir-7</i>	1.25	≤0.001	−1.30	≤0.01
<i>mir-181b</i>	0.31	NS	0.78	NS
<i>mir-21</i>	−0.43	NS	−0.80	NS
<i>mir-31</i>	−1.88	≤0.05	−1.35	NS
<i>mir-100</i>	−1.22	≤0.01	−0.90	NS
<i>mir-199a</i>	−1.92	NS	−3.50	≤0.001

NS, not significant.

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 $\times 630$). (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 $\times 400$; d, e, original magnification $\times 630$). miRNA expression was mainly observed in thyrocytes (indicated by arrowheads).

(Williams 2000, Rosai 2005, Fonseca *et al.* 2006). 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 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 PTC, FTC, 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-31* 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-222a* 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–PPAR γ* rearrangements and *RAS* mutations. The *PAX8–PPAR γ* 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–PPAR γ* 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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