Differences in miRNA expression profiles between wild-type and mutated NIFTPs

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Abstract

Noninvasive encapsulated follicular variants of papillary thyroid carcinomas have been recently reclassified as noninvasive follicular thyroid neoplasms with papillary-like nuclear features (NIFTPs). NIFTPs exhibit a behavior that is very close to that of follicular adenomas but different from the infiltrative and invasive follicular variants of papillary thyroid carcinomas (FVPTCs). The importance of miRNAs to carcinogenesis has been reported in recent years. miRNAs seem to be promising diagnostic and prognostic molecular markers for thyroid cancer, and the combination of miRNA expression and mutational status might improve cytological diagnosis. The aim of the present study was to evaluate the miRNA expression profile in wild-type, RAS- or BRAF-mutated NIFTPs, infiltrative and invasive FVPTCs, and follicular adenomas using the nCounter miRNA Expression assay (NanoString Technologies). To identify the significant Kyoto Encyclopedia of Genes and Genomes (KEGG) molecular pathways associated with deregulated miRNAs, we used the union of pathways option in DNA Intelligent Analysis (DIANA) miRPath software. We have shown that the miRNA expression profiles of wild-type and mutated NIFTPs could be different. The expression profile of wild-type NIFTPs seems comparable to that of follicular adenomas, whereas mutated NIFTPs have an expression profile similar to that of infiltrative and invasive FVPTCs. The upregulation of 4 miRNAs (miR-221-5p, miR-221-3p, miR-222-3p, miR-146b-5p) and the downregulation of 8 miRNAs (miR-181a-3p, miR-28-5p, miR-363-3p, miR-1285-5p, miR-152-3p, miR-25-3p, miR-30e-3) in mutated NIFTPs compared to wild-type ones suggest a potential invasive-like phenotype by deregulating the specific pathways involved in cell adhesion and cell migration (Hippo signaling pathway, ECM-receptor interaction, adherens junction, regulation of actin cytoskeleton, fatty acid biosynthesis and metabolism).

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Key Words

- NIFTPs
- miRNAs
- thyroid
- follicular-patterned thyroid lesions
**Introduction**

miRNA expression in thyroid cancer is an important and promising diagnostic marker since alterations have been identified not only between cancerous and either normal thyroid tissue or benign proliferative multinodular goiters (He et al. 2005, Tetzlaff et al. 2007), but also between different histological types of thyroid cancer (Dettmer et al. 2013, Borrelli et al. 2017). Moreover, from a prognostic point of view, miRNA expression correlates with clinicopathological features such as age, sex, tumor size and aggressive behavior (Yip et al. 2011, Wang et al. 2013).

Some studies have evaluated the association between genetic alterations and miRNA expression in papillary thyroid carcinomas (PTCs), but the results are still contradictory. Some groups have shown a correlation between mutational status and miRNA expression (Cahill et al. 2006, 2007, Nikiforova et al. 2008), while Sheu and coworkers (Sheu et al. 2009) have reported no significant differences in miRNA expression in BRAF V600E-positive PTCs compared with wild-type PTCs. Such results indicate that this mutation does not influence miRNA expression patterns (Sheu et al. 2009).

In recent years, the increasing incidence of thyroid cancer has been attributed to papillary thyroid carcinoma, and in particular to improvements in the diagnosis of the follicular variant of papillary thyroid carcinomas (FVPTCs). This variant includes both the encapsulated and non-encapsulated (or infiltrative) forms. Over the last ten years, many studies have reported that the noninvasive encapsulated follicular variant of papillary thyroid carcinoma has an indolent behavior that is similar to that of benign lesions (Liu et al. 2006, Piana et al. 2010, Vivero et al. 2013). Therefore, an international group of expert pathologists has recently proposed the reclassification of completely noninvasive encapsulated forms such as noninvasive follicular thyroid neoplasms with papillary-like nuclear features (NIFTPs) (Nikiforov et al. 2016). According to this study, NIFTPs are likely to represent the ‘benign’ counterpart or to be precursors of invasive FVPTCs. Their cytological diagnosis is controversial and demanding. Although the nuclear features of NIFTPs are different from those of benign follicular tumors and hyperplastic nodules, the differential diagnosis between NIFTPs and FVPTCs is almost impossible (Maletta et al. 2016). Furthermore, NIFTPs may harbor molecular alterations similar to those of thyroid tumors with a follicular growth pattern (follicular adenomas, follicular thyroid carcinomas and FVPTC), with a prevalence of RAS mutations.

Accurate molecular characterization may be important for cytological evaluation in order to prevent overdiagnosis or overtreatment.

Recently, it has been reported that the combination of mutational status and miRNA expression may improve the classification (into benign or malignant) of thyroid nodules with indeterminate cytology (Labourier et al. 2015). To date, the correlation between miRNA expression profile and mutational status in NIFTP lesions has not yet been studied.

To better characterize the molecular features of NIFTPs, the aim of this study was to evaluate whether miRNA expression correlates with mutational status in NIFTPs and to compare the expression profile with other follicular pattern thyroid tumors.

**Materials and methods**

**Patient samples**

This study included 53 patients who underwent surgery from 2013 to 2015 at the Department of Surgical, Medical, Molecular Pathology and Critical Area of the University of Pisa, Italy. Eighteen out of 53 patients were diagnosed with follicular adenomas, 19 with NIFTPs, 11 with infiltrative FVPTCs and 5 with invasive FVPTCs. All patients had a single nodule.

Tissue samples were fixed in 10% buffered formalin and embedded in paraffin for routine histopathological examination. The histological sections (2–4 µm thick) were stained with hematoxylin and eosin (Automatic Stainer Varistain Gemini Sheldon) and were assessed by two pathologists (C U and F B), with a diagnostic concordance rate of 98% between the two investigators. For all cases, the capsular and/or vascular invasiveness and the infiltration of thyroid and extrathyroid tissues were evaluated. To avoid contamination with other cellular types, cases with any grade of thyroiditis were excluded from the study.

Follicular adenomas and FVPTCs were classified according to the WHO 2004 histopathological criteria (DeLellis & Williams 2004). PTCs with follicular growth pattern and wide infiltration of thyroid parenchyma and/or of extrathyroid tissues were considered infiltrative FVPTCs. PTCs with follicular growth pattern and tumor capsular infiltration and/or intracapsular vascular infiltration were classified as invasive FVPTCs. NIFTP lesions were classified according to Nikiforov’s criteria.
miRNA expression in NIFTPs

et al. (Nikiforov et al. 2016). In brief, in this group, we included tumors with encapsulation and/or clear demarcation, follicular growth pattern and nuclear features of papillary thyroid carcinoma. Cases with only one papilla or with psammoma bodies, tumor necrosis, mitotic activity higher than 3 per 10 high power fields and with more than 30% of solid areas were excluded from the study.

In our comparative analyses, infiltrative and invasive FVPTCs were named IFVPTCs and evaluated as one group because infiltrative and invasive FVPTCs are malignant neoplasms requiring similar surgical treatment. On the contrary, NIFTPs are no longer considered carcinomas and a conservative therapeutic strategy has been proposed.

This retrospective study was conducted anonymously and complied with the Helsinki Declaration principles of 1975. Informed consent was obtained together with surgical consent.

miRNA extraction and Nanostring nCounter miRNA expression assay

miRNAs were isolated from formalin-fixed and paraffin-embedded tissues by using the miRNeasy Mini Kit (Qiagen), according to the manufacturer’s instructions. The concentration was assessed by means of a NanoDrop spectrophotometer (Thermo Scientific).

nCounter human v3 miRNA expression assays

nCounter human v3 miRNA expression assays designed and synthesized by NanoString Technologies (NanoString, Seattle, WA, USA) were used in this study. The miRNA panel included oligonucleotide tags onto 798 human miRNAs (from miRBase v21) and 5 housekeeping mRNAs for reference (ACTB, B2M, GAPDH, RPL19 and RPLP0). Twenty-five control probes recognizing either synthetic mRNA or miRNA targets were used to monitor the efficiency and specificity of each reaction step.

One hundred and fifty nanograms of total RNA were used for hybridization (16 h at 65°C) with probe pairs consisting of reporter probes carrying the signal on their 5’ end, and capture probes carrying biotin on their 3’ end. After hybridization, sample cleanup and digital report counts were performed according to the manufacturer’s instructions.

Raw data were analyzed using NanoString nSolver, version 2.5 software. Raw data were normalized by internal positive controls in order to remove variability in the hybridization and negative controls to subtract the background. In addition, miRNA input levels were normalized using the geometric mean of the top 100 miRNAs with the lowest variability coefficients in compliance with the manufacturer’s protocol.

The minimum threshold to consider an miRNA for further analysis was 20 counts (mean ± 2 s.d. of negative controls) detected in at least 10% of the samples. This threshold allowed us to obtain 142 miRNAs suitable for analyses.

DNA extraction and genotyping analysis

DNA was purified from formalin-fixed and paraffin-embedded tissues using the QIAamp DNA Mini Kit (Qiagen), according to the manufacturer’s protocol. DNA concentration and DNA quality were tested with a NanoDrop spectrophotometer (Thermo Fisher Scientific).

Direct DNA sequencing (3130 Genetic Analyzer Applied Biosystems) was performed to assess the mutational status of the BRAF (exon 15), NRAS (exons 2 and 3), HRAS (exons 2 and 3) and KRAS (exons 2 and 3) genes, according to standard procedures (Salvatore et al. 2004, Nikiforov et al. 2009).

Henceforward, we have indicated the lesions without mutations in the analyzed genes (BRAF, NRAS, HRAS, KRAS) as wild type.

DIANA-miRPath for miRNA pathway analysis

Pathway analysis using significantly upregulated and downregulated miRNAs was performed using DNA Intelligent Analysis (DIANA)-miRPath v3.0 software (Vlachos et al. 2015).

This software allowed us to link miRNAs to experimentally validated target genes from Tarbase, v7.0 and identifies the putative targeted molecular ‘KEGG’ pathways. As our analysis is hypothesis-free, we used the ‘pathways union’ option of the miRPath software. P values were obtained by the Fisher’s exact test as enrichment analysis method and the false discovery rate (FDR) was estimated using the Benjamini and Hochberg method (Benjamini & Hochberg 1995).

Statistical analysis

The data were analyzed using the Mann–Whitney U test followed by the Benjamini–Hochberg correction.

The Mann–Whitney U test was performed by the IBM SPSS software package, version 17.0.1; the R ‘stats’
package, version 3.4.0 was used for the Benjamini-Hochberg correction.

Given the exploratory nature of the present study to detect differentially expressed miRNAs to be validated in future experiments, a liberal cutoff of 0.15 for the FDR was used to identify miRNAs with different expression profiles.

Hierarchical clustering was performed with nSolver Analysis software 2.5 using Pearson’s correlation.

The pathway analysis was carried out by (DIANA)-miRPath v3.0 software using FDR <0.05 as significant threshold (Vlachos et al. 2015).

Results

We evaluated the RAS (NRAS, HRAS and KRAS) and BRAF genotypes and the expression profiles of 798 miRNAs by using the nCounter miRNA expression assay (NanoString Technologies) in 53 thyroid lesions, including 18 follicular adenomas (FAs), 19 NIFTPs, 5 invasive FVPTCs and 11 infiltrative FVPTCs.

Mutational analysis of follicular adenomas, NIFTPs and invasive and infiltrative FVPTCs

The results of our mutational analysis of the RAS (NRAS, HRAS and KRAS) and BRAF genes are reported in Table 1.

In the genes analyzed, we found that 94.0% of the follicular adenomas, 42.1, 40.0 and 45.4% of the NIFTPs, invasive FVPTCs and infiltrative FVPTCs respectively were wild type.

In detail, only one out of 18 of the follicular adenomas had a BRAF K601E mutation. The same mutation was also found in 2 of 19 of the NIFTPs (10.5%). In addition, 4 out of 11 of the invasive FVPTCs (36.4%) and 1 out of 19 of the NIFTPs (5.3%) had a mutation at codon 600 of the BRAF gene. Five of the NIFTPs (26.3%), 2 of the invasive FVPTCs (40.0%) and 2 of the infiltrative FVPTCs (18.2%) had an NRAS mutation. Three of the NIFTPs (15.8%) and only 1 of the invasive FVPTCs (20.0%) had mutations in the HRAS gene (Table 1). All cases were wild type for codons 12 and 13 of the RAS genes (data not shown).

NIFTPs: evaluation of miRNA expression profiles between wild-type and RAS- or BRAF-mutated lesions

As reported in Table 2, the miRNA expression profiles of 11 NIFTPs with BRAF or RAS mutations were compared to those of 8 wild-type NIFTPs.

We observed 12 differentially expressed miRNAs between the wild-type and mutated NIFTPs. Four of these (miR-221-5p, miR-221-3p, miR-222-3p and miR-146b-5p) were upregulated, while 8 were downregulated in the mutated lesions. The details are reported in Table 2.

To compare the expression profiles between wild-type and mutated NIFTPs, unsupervised hierarchical clustering was performed by using nSolver Analysis software.

This analysis allowed the system to clearly separate all the lesions into two main clusters.

Figure 1 shows the unsupervised hierarchical clustering of the wild-type NIFTPs and the RAS- or BRAF-mutated NIFTPs. Interestingly, as shown in Fig. 1, cluster 1 included 11 out of 12 (91.7%) NIFTPs with BRAF or RAS mutations. In contrast, cluster 2 consisted of the wild-type NIFTPs (7 out of 7, 100%).

Comparison of miRNA expression in wild-type and BRAF- or RAS-mutated follicular-patterned thyroid neoplasms

We compared the miRNA expression profiles of wild-type follicular adenomas, NIFTPs and IFVPTCs to those harboring mutations in the RAS or BRAF genes.

The results are reported in Table 3 and clearly indicate that the wild-type lesions had different miRNA expression profiles. In detail, 9 miRNAs were significantly upregulated and 5 miRNAs were downregulated in the mutation-positive lesions (FDRs are reported in Table 3). These results suggest that the presence of mutations in these genes might influence the miRNA expression.

To determine the miRNA profiles of wild-type and mutated lesions, an unsupervised hierarchical clustering analysis was performed by the differentially expressed miRNAs.

Four major clusters were obtained with this approach (Fig. 2).

Overall, clusters 1 and 2 included all the mutated cases: 11 NIFTPs (8 RAS and 3 BRAF-mutated), 9 IFVPTCs (5 RAS and 4 BRAF-mutated) and 1 follicular adenoma carrying the BRAF K601E mutation. However, 3 wild-type follicular adenomas and 6 out of 7 (85.7%) of the wild-type IFVPTCs were also present in these clusters.

None of the 22 lesions encompassed in clusters 3 and 4 harbored mutations in the RAS or BRAF genes, namely 7 NIFTPs, 14 follicular adenomas and 1 IFVPTC.

These data suggest that the NIFTPs carrying BRAF or RAS mutations had an miRNA profile similar to that of most IFVPTCs, irrespective of mutational status.
miRNA expression and functional pathways in NIFTPs harboring mutations in the RAS or BRAF genes

To understand the biological significance of the 12 miRNAs that were differentially expressed between the RAS- or BRAF-mutated and wild-type NIFTPs, we used DIANA-miRPath v.3 software (Vlachos et al. 2015).

This analysis revealed 29 enriched pathways with FDR <0.05. Among these, we selected the 15 pathways involved in cancer initiation and progression. The results are reported in Table 4.

According to the KEGG pathway maps, 3 pathways were cancer related, and 5 were environmental information processing related (4 involved in signal transduction, and 1 in signaling molecules and interactions).

The pathways related to cellular processes are involved in cell migration (adherens junction and regulation of the actin cytoskeleton), and in cellular growth and death (cell cycle and p53 signaling pathway).

In addition, metabolic (fatty acid biosynthesis and metabolism) and endocrine system pathways (thyroid hormone signaling pathway) were found to be significantly enriched.

The results indicate that the deregulation of these miRNAs influences not only the pathways related to cancer development, but also the pathways involved in cell motility, and probably induces the potentially invasive behavior of the cells harboring the RAS or BRAF gene mutations in NIFTPs.

**Discussion**

Several studies have evaluated the miRNA expression in thyroid lesions, and some authors have observed differences in expression among various tumor histotypes (Tetzlaff et al. 2007, Nikiforova et al. 2008). However, only a few reports have analyzed the correlation between miRNA expression profiles and mutational status. In particular, Cahill and coworkers showed that there was a significant overexpression of 21 miRNAs and a downregulation of 14 miRNAs in PTC cells harboring a RET/PTC1 rearrangement, compared with the Nthy-ori 3-1 normal thyroid follicular epithelial cell line (Cahill et al. 2006).

The same authors observed similar results when they compared the same control cells with a cell line derived from a papillary thyroid carcinoma harboring the BRAF V600E mutation (Cahill et al. 2007). Cahill and coworkers concluded that several of these deregulated miRNAs may be involved in the pathogenesis of PTC.

Similar results were also obtained in in vivo studies; Nikiforova and coworkers identified a correlation between miRNA expression and somatic mutations (RAS and BRAF point mutations, RET/PTC and PAX8/PPARγ rearrangements). These data were also validated in fine-needle aspiration (FNA) samples, and the authors

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**Table 1** Genotyping analysis results for follicular adenomas, NIFTPs and invasive and infiltrative FVPTCs.

<table>
<thead>
<tr>
<th>Hystological type</th>
<th>n (%)</th>
<th>Wild type</th>
<th>BRAF V600E</th>
<th>BRAF K601E</th>
<th>NRAS Q61R</th>
<th>HRAS Q61R</th>
<th>KRAS Q61R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular adenomas</td>
<td>19 (35.8)</td>
<td>17 (94.0%)</td>
<td>–</td>
<td>1 (6.0%)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NIFTPs 19 (35.8%)</td>
<td>8 (42.1%)</td>
<td>1 (5.3%)</td>
<td>2 (10.5%)</td>
<td>5 (26.3%)</td>
<td>3 (15.8%)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Invasive FVPTCs 5 (9.4%)</td>
<td>2 (40.0%)</td>
<td>–</td>
<td>–</td>
<td>2 (40.0%)</td>
<td>1 (20.0%)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Infiltrative FVPTCs 11 (20.8%)</td>
<td>5 (45.4%)</td>
<td>4 (36.4%)</td>
<td>–</td>
<td>2 (18.2%)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total 53</td>
<td>32 (60.4%)</td>
<td>5 (9.4%)</td>
<td>3 (5.7%)</td>
<td>9 (17.0%)</td>
<td>4 (7.5%)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

On the other hand, almost all the wild-type NIFTPs (7 out of 8, 87.5%) had miRNA expression profiles similar to those of the follicular adenomas.

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**Table 2** miRNAs with significantly different expression profiles between wild-type and RAS- or BRAF-mutated NIFTPs.

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Mutated vs WT</th>
<th>P value*</th>
<th>Adjusted P value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-221-5p</td>
<td>0.00033</td>
<td>0.04373</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-221-3p</td>
<td>0.00061</td>
<td>0.04373</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-222-3p</td>
<td>0.00148</td>
<td>0.05246</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-146b-5p</td>
<td>0.00439</td>
<td>0.10388</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-181a-3p</td>
<td>0.01111</td>
<td>0.05191</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-28-5p</td>
<td>0.00258</td>
<td>0.07325</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-363-3p</td>
<td>0.00567</td>
<td>0.10999</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-342-3p</td>
<td>0.00728</td>
<td>0.10999</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-1285-5p</td>
<td>0.00929</td>
<td>0.10999</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-152-3p</td>
<td>0.00929</td>
<td>0.10999</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-25-3p</td>
<td>0.00929</td>
<td>0.10999</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-30e-3p</td>
<td>0.00929</td>
<td>0.10999</td>
<td></td>
</tr>
</tbody>
</table>

*P values were obtained by using Mann–Whitney U test.
**Adjusted P values using the Benjamini–Hochberg method.
suggested that a limited number of miRNAs (miR-187, miR-221, miR-222, miR-146b, miR-155, miR-224, and miR-197) could be used for diagnostic purposes (Nikiforova et al. 2008).

In our study, we evaluated the expression of a wide panel of miRNAs (798 miRNAs) in follicular-patterned thyroid tumors (NIFTPs, follicular adenomas and IFVPTCs) using nCounter NanoString technology. We observed correlations between miRNA expression profiles and mutational status of the lesions recently reclassified as NIFTPs.

As a next step, we sought to understand whether the correlation between mutational status and miRNA expression profile could be confirmed for all lesions or whether the histotype would play a predominant role. Twelve miRNAs were found to be differentially expressed between the wild-type and RAS- or BRAF-mutated NIFTPs; of these, 4 were upregulated (miR-221-5p, miR-221-3p, miR-222-3p and miR-146b-5p) and 8 were downregulated.

The overexpression of miR-221 and miR-222 has already been reported not only in PTC but also in hepatocellular carcinoma, prostate carcinoma and colorectal carcinoma, suggesting that these miRNAs could be involved in cellular proliferation by regulating tumor suppressors such as p27kip1, p57 and PTEN (Bruni et al. 2000, Galardi et al. 2007, Visone et al. 2007, Fornari et al. 2008, Sun et al. 2011).

Additionally, the expression of miR-221, miR-222 and miR-146b-5p was also correlated with tumor aggressiveness and extrathyroidal invasion (Yip et al. 2011, Wang et al. 2013) and one possible mechanism might...

Table 3 miRNA expression profiles in wild-type and RAS- or BRAF-mutated follicular adenomas, NIFTPs and IFVPTCs.

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>P Value*</th>
<th>Adjusted P value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-221-5p</td>
<td>9.44 × 10⁻⁸</td>
<td>0.00001</td>
</tr>
<tr>
<td>hsa-miR-221-3p</td>
<td>3.10 × 10⁻⁶</td>
<td>0.00015</td>
</tr>
<tr>
<td>hsa-miR-222-3p</td>
<td>0.00003</td>
<td>0.00098</td>
</tr>
<tr>
<td>hsa-miR-135a-5p</td>
<td>0.0090</td>
<td>0.01835</td>
</tr>
<tr>
<td>hsa-miR-3151-5p</td>
<td>0.0125</td>
<td>0.02214</td>
</tr>
<tr>
<td>hsa-miR-181c-5p</td>
<td>0.00205</td>
<td>0.03181</td>
</tr>
<tr>
<td>hsa-miR-125b-5p</td>
<td>0.00216</td>
<td>0.03747</td>
</tr>
<tr>
<td>hsa-miR-551b-3p</td>
<td>0.00317</td>
<td>0.01001</td>
</tr>
<tr>
<td>hsa-miR-28-5p</td>
<td>0.00035</td>
<td>0.01175</td>
</tr>
<tr>
<td>hsa-miR-25-5p</td>
<td>0.00232</td>
<td>0.01175</td>
</tr>
<tr>
<td>hsa-miR-152-5p</td>
<td>0.00050</td>
<td>0.00604</td>
</tr>
<tr>
<td>hsa-miR-144-3p</td>
<td>0.00956</td>
<td>0.09697</td>
</tr>
</tbody>
</table>

*P values were obtained by using Mann–Whitney test.

**Adjusted P values using the Benjamini–Hochberg method.
be the deregulation of SMAD4 and/or TGFβ signaling, as recently reported by Lima and coworkers (Lima et al. 2016).

Unsupervised hierarchical clustering analysis allowed us to discover that the wild-type NIFTPs had a clearly distinct miRNA expression profile compared with the RAS- or BRAF-mutated NIFTPs. Interestingly, our data showed that the wild-type NIFTPs had miRNA expression profiles that were not different from the follicular adenomas. In contrast, the RAS- or BRAF-mutated NIFTPs were comparable to the infiltrative and invasive FVPTCs.

These data confirm our previous observation (data submitted for publication) that NIFTPs appear as heterogeneous lesions. Some of them have genotypes and expression profiles that are comparable to benign tumors, and others that more closely resemble PTCs. A follow-up of the patients with different NIFTP profiles will be crucial to our understanding of the clinical outcomes.

To clarify the biological role of deregulated miRNAs in wild-type and mutated NIFTPs, we investigated molecular pathways affected by altered miRNA expression using DIANA-miRPath software.

In our study, the enriched pathways were involved in several biological processes including signal transduction, cell proliferation, migration, differentiation and metabolism.

Among the signal transduction pathways, the most significant is the Hippo signaling pathway, which modulates not only cell proliferation and differentiation, but also cell contact inhibition and cancer development in mammals (Camargo et al. 2007, Zhao et al. 2007). In particular in thyroid tumors, Ugolini and coworkers reported the correlation of YAP-1 expression (a player of the Hippo pathway) with clinicopathologic characteristics such as extrathyroid invasion and absence of a tumoral capsule (Ugolini et al. 2016).

Among the other altered signal transduction pathways, the FoxO and PI3K-Akt signaling pathway is known to be involved in thyroid cancer (Zaballos & Santisteban 2013, Gunda et al. 2014, Sun et al. 2015, Nozhat & Hedayati 2016). However, previous high frequency genetic studies have reported the activation of somatic alterations of genes encoding effectors in the MAPK signaling pathway, including point mutations in
Table 4  Results from the DIANA-mirPath v3.0 predictions of KEGG pathways.

<table>
<thead>
<tr>
<th>KEGG pathway maps</th>
<th>Enriched pathway</th>
<th>Adjusted P value**</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human diseases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cancers: overview</td>
<td>Proteoglycans in cancer (hsa05205)</td>
<td>1.6563 × 10⁻⁸</td>
</tr>
<tr>
<td></td>
<td>Pathways in cancer (hsa05200)</td>
<td>0.00355</td>
</tr>
<tr>
<td></td>
<td>Thyroid cancer (hsa05216)</td>
<td>0.01557</td>
</tr>
<tr>
<td>Cancers: specific types</td>
<td>Hippo signaling pathway (hsa04390)</td>
<td>3.48765 × 10⁻¹²</td>
</tr>
<tr>
<td><strong>Environmental information processing</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Signal transduction</td>
<td>FoxO signaling pathway (hsa04068)</td>
<td>0.01557</td>
</tr>
<tr>
<td></td>
<td>TGF-beta signaling pathway (hsa04350)</td>
<td>0.01704</td>
</tr>
<tr>
<td></td>
<td>PI3K-Akt signaling pathway (hsa04151)</td>
<td>0.01748</td>
</tr>
<tr>
<td></td>
<td>ECM-receptor interaction (hsa04512)</td>
<td>&lt;1 × 10⁻²⁵</td>
</tr>
<tr>
<td><strong>Cellular processes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellular community</td>
<td>Adherens junction (hsa04520)</td>
<td>9.75901 × 10⁻⁶</td>
</tr>
<tr>
<td>Cell motility</td>
<td>Regulation of actin cytoskeleton (hsa04810)</td>
<td>0.03969</td>
</tr>
<tr>
<td>Cell growth and death</td>
<td>Cell cycle (hsa04110)</td>
<td>3.80975 × 10⁻⁸</td>
</tr>
<tr>
<td></td>
<td>p53 signaling pathway (hsa04115)</td>
<td>2.82533 × 10⁻⁵</td>
</tr>
<tr>
<td><strong>Metabolism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>Fatty acid biosynthesis (hsa00061)</td>
<td>9.93259 × 10⁻¹⁴</td>
</tr>
<tr>
<td>Global and overview maps</td>
<td>Fatty acid metabolism (hsa01212)</td>
<td>0.04484</td>
</tr>
<tr>
<td><strong>Organismal systems</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endocrine system</td>
<td>Thyroid hormone signaling pathway (hsa04919)</td>
<td>0.00942</td>
</tr>
</tbody>
</table>

**Adjusted P values using the Benjamini-Hochberg method.


Nikiforov and coworkers analyzed a group of NIFTPs and found that more than 60% of these lesions harbored mutations (the majority of which were RAS family mutations). The authors suggested that these lesions might be precursors of invasive FVPTCs (Nikiforov et al. 2016).

In our study, we observed that a group of NIFTPs with RAS and BRAF mutations (42.1% and 15.8%, respectively) have miRNA expression profiles influencing pathways involved in cell–cell/cell–extracellular matrix interactions, cell migration and cancer invasion processes. These observations allowed us to speculate that NIFTPs with the previously mentioned genetic alterations might progress to invasive and infiltrative forms of FVPTCs.

Specific interactions between cells and the extracellular matrix (ECM) are mediated by transmembrane molecules, mainly integrins. Many studies have reported significant differences in the expression and distribution of these molecules between pre-neoplastic tumors or normal tissues and malignant tumors (Mizejewski 1999, Hood & Cheresh 2002). A correlation between transmembrane molecules and invasive growth has been observed in several malignant tumors (melanoma, breast cancer, prostate cancer and ovarian cancer) (Desgrozslie & Cheresh 2010).

In particular, differences in the expression of certain integrin subunits between thyroid cancer and normal thyroid tissue have been reported (Dahlman et al. 1998, Illario et al. 2003). Serini and coworkers reported that α6β4 integrin was not expressed in normal thyroid cells, while its expression was correlated with progression and with a more aggressive phenotype in thyroid carcinoma (Serini et al. 1996).

Therefore, the altered expression of key mediators involved in cell–ECM interactions, in accordance with Serini’s report, could promote the progression of NIFTPs harboring RAS or BRAF mutations to lesions with an invasive-like phenotype.

Moreover, many studies have established that the altered expression of adherens junction (AJ) proteins and the disorganization of the actin cytoskeleton are involved in human cancer development (Yap et al. 2007, Collinet & Lecuit 2013).

The invasive behavior of cancer cells has been attributed to the dysfunction of both cell–cell adhesion molecules (E-cadherin) and cytoplasmic proteins (α-, β- and γ-catenin molecules). Huang and coworkers compared the distribution of the cadherin-catenin complex between a thyroid cancer cell line and normal human thyroid epithelial cells, demonstrating that the loss of cell adhesiveness in thyroid cancer cells might be due to the incomplete assembly of the cadherin-catenin complex at cell junctions (Huang et al. 1998).

In our analysis, we also observed alterations in the pathways involved in fatty acid biosynthesis and metabolism. Both alterations have been reported in other...
types of cancers (Menendez & Lupu 2007, Carracedo et al. 2013), suggesting that they are important for cancer progression and might be useful targets for therapies (Abramson 2011).

Recently, we have reported that miRNA expression profiles may have diagnostic potential and should therefore be assessed by presurgical FNA, in particular, for cytological lesions classified as indeterminate thyroid nodules (Borrelli et al. 2017).

Preoperative differential diagnosis between NIFTP and infiltrative FVPTCs is crucial because patients with NIFTPs could be treated in a conservative manner. According to our previous study, miRNA expression profiles seem to be useful for distinguishing between benign and malignant lesions, as well as between NIFTPs and both follicular adenomas and infiltrative FVPTCs (Borrelli et al. 2017).

Moreover, we reported that a wide group of deregulated miRNAs were involved in the infiltrative growth of FVPTCs, independently of mutational status (Borrelli et al. 2017). Among these, 5 deregulated miRNAs (miR-146b-5p, miR-221-3p, miR-181a-3p, miR-1285-5p and miR-25-3p) were also found to be differentially expressed in wild-type and mutated NIFTPs.

However, the present study has some potential limitations. In spite of the relatively large cohort of patients recruited, many miRNAs were considered for analyses even after pre-analytic selection. To overcome this potential issue, a cutoff of FDR <0.15 was then considered significant as a good compromise between true-positive and false-positive miRNAs.

Although RAS and BRAF are the most affected genes in the analyzed lesions, another limitation could be related to the presence of mutations other than those considered in this study and that could influence the miRNA expression profile.

In conclusion, our results suggest that the miRNA expression profiles of NIFTPs might differ according to their mutational status. The miRNA expression profile of wild-type NIFTPs seems to be similar to that of follicular adenomas, while NIFTPs harboring mutations in the RAS or BRAF genes probably have an miRNA expression profile similar to that of infiltrative and invasive FVPTCs. The upregulation of miR-221-5p, miR-221-3p, miR-222-3p and miR-146b-5p and the downregulation of a set of 8 miRNAs (miR-181a-3p, miR-28-5p, miR-363-3p, miR-342-3p, miR-1285-5p, miR-152-3p, miR-25-3p, miR-30e-3p) may promote an invasive phenotype of NIFTPs with mutations in the RAS or BRAF genes modulating specific pathways such as Hippo signaling pathway, ECM–receptor interaction, AJ, regulation of actin cytoskeleton, fatty acid biosynthesis and metabolism.

Future studies are warranted to validate these miRNAs and to elucidate their effects on the above-mentioned pathways.

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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miRNA expression in NIFTPs


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