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ORIGINAL ARTICLE

Reduced expression of *THRβ* in papillary thyroid carcinomas: relationship with *BRAF* mutation, aggressiveness and miR expression

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Abstract
Purpose Down-regulation of thyroid hormone receptor beta (*THRβ*) gene has been described in several human malignancies, including thyroid cancer. In this study, we analyzed *THRβ* mRNA expression in surgical specimens from a series of human papillary thyroid carcinomas (PTCs), characterized by their genotypic and clinical–biological features.
Methods Thirty-six PTCs were divided into two groups according to the 2009 American Thyroid Association risk classification (17 low, 19 intermediate), and each group was divided into subgroups based on the presence or absence of the *BRAF*V600E mutation (21 *BRAF* mutated, 15 *BRAF* wild type). Gene expression was analyzed using fluidic cards containing probes and primers specific for the *THRβ* gene, as well as for genes of thyroperoxidase (*TPO*), sodium/iodide symporter (*NIS*), thyroglobulin (*Tg*) and thyroid stimulating hormone receptor (*TSH-R*) and for some miRNAs involved in thyroid neoplasia and targeting *THRβ*. The mRNA levels of each tumor tissue were compared with their correspondent normal counterpart.
Results *THRβ* transcript was down-regulated in all PTCs examined. No significant differences were found between intermediate- vs low-risk PTCs patients, and *BRAF*-mutated vs *BRAF* wild-type groups. *THRβ* expression was directly correlated with *NIS*, *TPO*, *Tg* and *TSH-R*, and inversely correlated to miR-21, -146a, -181a and -221 expression.
Conclusions Our results demonstrate that down-regulation of *THRβ* is a common feature of PTCs. While it is not associated with a more aggressive phenotype of PTC, it correlates with the reduction of all the markers of differentiation and is associated with overexpression of some miRNAs supposed to play a role in thyroid tumorigenesis.

Keywords Thyroid hormone receptor · Papillary thyroid carcinoma · miRNA · *BRAF*

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Introduction

Thyroid hormone receptors (THRs) mediate the biological activities of the thyroid hormones in development, growth, differentiation and metabolism [1, 2]. Detection of alterations in the expression levels and/or integrity of *THR* genes in many neoplasms has made the analysis of these genes in human cancers an area of considerable interest. Several alterations have been described, including hypermethylation in breast cancer [3, 4], altered splicing in hepatocellular cancer [5] and loss of heterozygosity/deletions in breast, lung and melanoma cancer [2, 6–8], all resulting in reduced/lost expression of *THR* in tumor cells. Moreover, Martinez-Iglesias et al. have

shown that THRβ1 re-expression in hepatocarcinoma and breast cancer cells was able to reduce tumor growth, enhancing expression of epithelial markers and diminishing that of the mesenchymal marker vimentin [9]. Gene mutations, small deletions and variations in the THR RNA and protein levels [10] have also been reported in thyroid cancers, in which a role of THRβ1 as potential suppressor of thyroid tumorigenesis has been proposed [11].

In the last years, miRNA expression profiling of human tumors has permitted to identify signatures associated with the diagnosis, staging, prognosis and response to treatment [12] and several studies have demonstrated that up-regulation of oncogenic miRNAs or down-regulation of miRNAs functioning as tumor suppressors is associated with carcinogenesis [13, 14]. Many groups have analyzed the expression of miRNAs in thyroid neoplasia and have shown an altered regulation of several miRNAs, proposing a role for some of them in papillary thyroid cancer (PTC) tumorigenesis [reviewed in 15, 16]. Noteworthy, a subset of miRNAs that are up-regulated in PTC tumors (miR-21, miR-146a, miR-181a and miR-221) has been described to target and to inhibit the expression of *THRβ* [17].

In this work, we have examined 36 PTCs, well characterized in their clinical–biological properties and genotypic alterations, to study *THRβ* expression and its correlation with the degree of aggressiveness, the presence of *BRAF* oncogene activating mutation and the expression of markers of thyrocyte differentiation thyroperoxidase (*TPO*), sodium/iodide symporter (*NIS*), thyroglobulin (*Tg*) and thyroid stimulating hormone receptor (*TSH-R*). Moreover, in the same tumors, we analyzed the expression levels of miRNAs targeting *THRβ*.

Materials and methods

Patients and tissues

Thirty-six patients with sporadic PTCs have been enrolled at the University Hospital of Rome, Sapienza. For each patient, a sample of thyroid tumor tissue and contralateral non-tumor (normal) tissue were collected immediately after thyroidectomy, snap-frozen and stored in liquid nitrogen. All samples were reviewed by a single pathologist, who confirmed the diagnosis of PTC, identified the histological variant of the tumor and evaluated the percentage of tumor cells (all tumor tissues selected had a percentage of tumor cells higher than 60 %). Clinical data were collected by retrospective review of hospital charts, and tumors were staged according to the criteria of the AJCC/UICC TNM classification, 7th edition [18]. The 36 cases were risk-stratified on the basis of clinical and histological data in accordance with the 2009 American Thyroid Association (ATA) risk of recurrence staging system [19]. The study was approved by the local medical ethics committee and written informed consent was obtained from all patients whose tissues were analyzed.

Mutational analysis of *BRAF*

The mutational status of *BRAF* was analyzed by direct sequencing in cDNA samples of tumor tissues. The exon 15 of *BRAF* was amplified by PCR (Table S1) and the products sequenced with the BigDye Terminator version 3.1 Cycle Sequencing kit in an automated 3130xl analyzer (Life Technologies, Foster City, CA, USA). All PCR and sequencing reactions were repeated at least twice to confirm the presence of the mutation V600E.

RNA isolation from thyroid tissues and reverse transcription

Total RNA was extracted from tissue samples using Trizol reagent (Life Technologies) [20] according to the manufacturer's instructions. RNA concentration was measured by a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). First-strand cDNA synthesis was performed following the protocol provided with the High Capacity cDNA Reverse Transcription kit (Life Technologies), as previously described [21].

Gene expression analysis

In the screening step, mRNA levels of *THRβ*, *NIS*, *TPO*, *Tg* and *TSH-R* genes were assessed in thyroid tissues by real-time PCR using custom Taqman Low Density Arrays (TLDA, Life Technologies). Each TLDA was configured with specific predesigned assays (TaqMan Gene Expression Assays, Life Technologies) and four housekeeping genes (Glyceraldehyde-3-Phosphate Dehydrogenase; Beta-actin; Hypoxanthine Phosphoribosyltransferase 1 and Beta-2 microglobulin) were included to normalize RNA expression levels. The TaqMan arrays were set up as previously described [22] and all the PCR reactions were performed on a 7900 HT Fast Real-time PCR System (Life Technologies). Ct values were calculated with SDS 2.4 software (Life Technologies) and data analysis was carried out using RQ Manager 1.2.1 software (Life Technologies). Beta-actin was chosen as endogenous control because of its least variance among samples. Final results were determined by the comparative $2^{-\Delta\Delta Ct}$ method and expressed as relative expression normalized to a calibrator sample.

miRNA expression analysis

TaqMan® Array Human MicroRNA Card Set v3.0 (Life Technologies) was used to evaluate the expression levels

of miR-21, miR-146a, miR-181a and miR-221. Total RNA (500 ng) was reverse transcribed using high-capacity cDNA reverse transcription kit (Life Technologies) and MegaplexTM RT primer pool (Life Technologies). Real-time PCR was performed on a 7900 HT Fast Real-Time PCR System (Life Technologies). Ct values were calculated with SDS 2.4 software (Life Technologies) and data analysis was carried out using Expression Suite v1.0.3 (Life Technologies). U6 was chosen as endogenous control due to its least variance among samples. The relative level of miRNA expression was calculated by the comparative $2^{-\Delta\Delta Ct}$ method using Expression Suite v1.0.3 (Life Technologies).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 5.0 statistical software (GraphPad Software Inc., San Diego, CA, USA). Real-time PCR results are expressed as mean ± standard deviation (SD). Mann–Whitney test and Student's *t* test were used to evaluate intergroup differences. Correlations between quantitative variables were analyzed by the Spearman rho rank correlation coefficient. *p* values lower than 0.05 were considered statistically significant.

Results

Clinical and pathological features

We examined 36 tissues from patients with apparently sporadic PTC. They included 17 cases with a low risk of recurrence, and 19 with an intermediate risk based on the 2009 ATA risk stratification system. Table 1 summarizes the characteristics of study population at the time of the primary treatment, which consisted in total or near total thyroidectomy, with or without radioactive iodine remnant ablation. Among the low-risk patients and those at intermediate risk, 7 (41.1 %) and 14 (73.7 %), respectively, carry the *BRAF* V600E mutation (Fig. 1).

Expression of THRβ and correlation with thyroid-specific genes in PTCs

Analysis of mRNA showed that expression levels of *THRβ* were lower in tumor tissues than in the normal counterpart tissues (Normal tissues = 1 ± 0.536, Tumor tissues = 0.618 ± 0.434, $p = 0.0021$) (Fig. 2). By comparison of the subgroups, no significant deregulation was found between low-risk PTCs and intermediate-risk PTCs (Fig. 2), as well as between *BRAF* V600E PTCs and *BRAF* wild-type PTCs (Fig. 2). The expression of *THRβ* was then compared to mRNA levels of the thyroid-specific genes *NIS*, *TPO*, *Tg* and *TSH-R*. A statistically significant and direct correlation with *THRβ* levels was detected for all of the thyrocyte-specific genes examined (Table 2). In addition, as reported in Fig. 3, *NIS* mRNA levels showed the major significant reduction and *TSH-R* the minor. Moreover, expression levels of *TPO* were significantly lower in PTC with intermediate risk and in *BRAF* V600E PTCs (Table S2). No significant deregulation was found for *NIS*, *Tg* and *TSH-R* (Table S2).

Expression of miRNAs in PTC tissues

Twenty-one PTC tissues of the present cohort were also analyzed for the expression level of a subset of miRNA previously predicted to target *THRβ* (miR-21, miR-146a, miR-181a and miR-221) [22]. Expression levels of miR-21, miR-181a and miR-221 resulted significantly higher in tumor tissues compared to normal tissues (Table S3). According to ATA risk and *BRAF* mutational status, no significant deregulation was found (Table S3).

As reported in Fig. 4, a higher expression of miR-21, miR-146a, miR-181a and miR-221 was observed in almost all those tumor tissues displaying lower levels of *THRβ*. However, Spearman correlation of miRNA and *THRβ* data did not show a statistically significant association (data not shown). miRNA expression levels were then compared with those of thyroid-specific genes *NIS*, *TPO*, *Tg* and *TSH-R*. As shown in Fig. S1, a statistically significant and inverse correlation was found between expression levels of *TG* and both miR-21 and miR-146a, and between *TPO* and miR-21.

Discussion

In the last two decades, the incidence of thyroid cancer, the most common malignancy in the endocrine system, has greatly increased [23] and about 80 % of all cases are represented by PTCs. Although most of these tumors present a good prognosis (survival rates of 90–95 % at 5 years), there is a minority of patients unresponsive to treatment based on association of surgery and radioactive iodine. The loss of differentiation of the transformed cells, and in particular the reduction/loss of expression of functioning NIS protein represents a major cause of lack of responsiveness to radioiodine treatment in case of recurrent/metastatic disease [24–27]. Several genetic and epigenetic alterations are involved in the loss of differentiation of PTCs and the acquisition of more aggressive phenotype [28–31]. Thus, elucidating the molecular mechanisms that contribute to dedifferentiation and aggressiveness of PTCs may help to tailor diagnostic and therapeutic strategies and to propose novel targeted therapy approaches.

Table 1 Characteristics of the study population at the time of primary treatment

No.	Sex	Age	Histological variant	T	N	Tumor size (mm)	Tumor foci	Extrathyroid extension	ATA risk[a]	*BRAF* mutational status
1	M	58	PTC-fv	T1b	N0	18	Unifocal	None	Low	V600_K601delinsE
2	M	60	PTC-cl	T1b	N0	12	Unifocal	None	Low	V600E
3	F	61	PTC-cl	T2	N0	25	Unifocal	None	Low	V600E
4	F	66	PTC-cl	T1a	Nx	6	Multifocal	None	Low	V600E
5	M	27	PTC-cl	T1	N0	11	Multifocal	None	Low	V600E
6	F	23	PTC-cl	T1b	Nx	14	Multifocal	None	Low	V600E
7	M	45	PTC-cl	T1b	N0	18	Unifocal	None	Low	V600E
8	F	50	PTC-cl	T1b	N0	12	Multifocal	None	Low	wt
9	F	40	PTC-fv	T1a	Nx	4	Unifocal	None	Low	wt
10	F	35	PTC-cl	T1	N0	9	Multifocal	None	Low	wt
11	F	34	PTC-fv	T1b	Nx	20	Unifocal	None	Low	wt
12	M	51	PTC-fv	T1a	Nx	6	Unifocal	None	Low	wt
13	F	37	PTC-cl	T1b	Nx	20	Unifocal	None	Low	wt
14	F	33	PTC-cl	T1b	Nx	14	Unifocal	None	Low	wt
15	M	31	PTC-cl	T1b	Nx	11	Unifocal	None	Low	wt
16	M	58	PTC-cl	T1a	Nx	5	Unifocal	None	Low	wt
17	F	60	PTC-cl	T1a	Nx	5	Unifocal	None	Low	wt
18	F	64	PTC-cl	T3	N0	15	Unifocal	Minor	Intermediate	V600E
19	F	46	PTC-cl	T3	N1	30	Unifocal	Minor	Intermediate	V600E
20	F	46	PTC-cl	T3	N0	15	Unifocal	Minor	Intermediate	V600E
21	F	33	PTC-cl	T1b	N1a	11	Multifocal	None	Intermediate	V600E
22	F	42	PTC-cl	T1b	N1	15	Multifocal	None	Intermediate	V600E
23	F	34	PTC-cl	T1	N1	13	Unifocal	None	Intermediate	V600E
24	F	52	PTC-cl	T3	N0	8	Multifocal	Minor	Intermediate	V600E
25	F	62	PTC-cl	T3	N0	16	Unifocal	Minor	Intermediate	V600E
26	M	37	PTC-cl	T3	Nx	12	Unifocal	Minor	Intermediate	V600E
27	M	35	PTC-cl	T1b	N1b	20	Multifocal	None	Intermediate	V600E
28	M	42	PTC-cl	T3	N1b	14	Multifocal	Minor	Intermediate	V600E
29	F	65	PTC-cl	T3	N0	9	Unifocal	Minor	Intermediate	V600E
30	F	46	PTC-cl	T3	N0	12	Unifocal	Minor	Intermediate	V600E
31	F	50	PTC-cl	T3	N1	10	Unifocal	Minor	Intermediate	V600E
32	F	41	PTC-fv	T3	N1a	15	Unifocal	Minor	Intermediate	wt
33	F	19	PTC-cl	T1b	N1b	20	Unifocal	None	Intermediate	wt
34	F	34	PTC-cl	T3	N0	12	Unifocal	Minor	Intermediate	wt
35	M	27	PTC-cl	T3	N1	25	Multifocal	Minor	Intermediate	wt
36	F	42	PTC-cl	T3	N1	9	Unifocal	Minor	Intermediate	wt

All patients had no clinical evidence of distant metastases at the time of primary treatment

F female, *M* male, *PTC-cl* classical variant, *PTC-fv* follicular variant, *T* tumor, *N* node, *ATA* American Thyroid Association, *wt* wild type

[a] The 2009 American Thyroid Association risk stratification staging system

Recent investigations showed that alterations causing a marked impairment of the THR expression and function can influence the process of thyroid tumorigenesis [10, 32]. By analyzing the expression of *THRβ* in 17 PTCs, Kim et al. observed a significant reduction of its mRNA expression levels in cancer tissues [10]. A functional role of THRβ was further supported by in vitro data in which reactivation of the silenced *THRβ* expression delayed thyroid tumor progression [10]. Moreover, Zhu et al. have demonstrated in a mouse model of metastatic follicular thyroid cancer that functional loss of *THRβ* and *THRα* gene promoted the development of thyroid carcinomas and metastasis, suggesting that *THRs* could function as a tumor suppressor in this experimental model [11]. In our study, analysis of *THRβ*

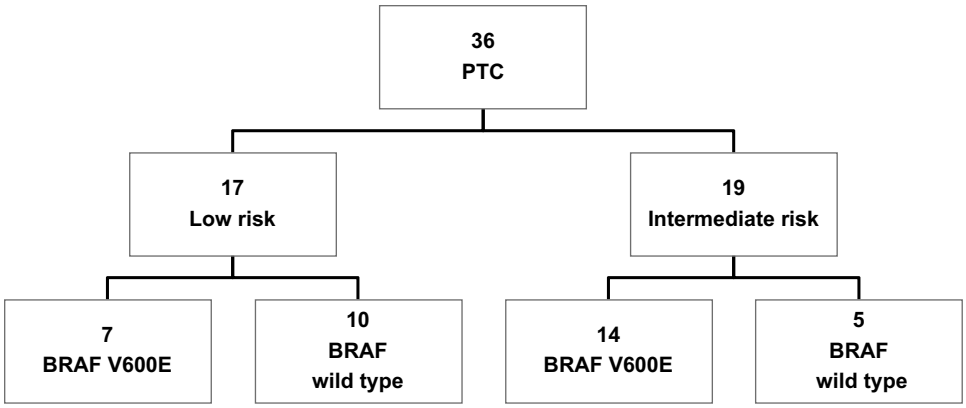

Fig. 1 Classification of human PTCs. Tumors were assigned to groups according to the 2009 ATA risk of recurrence staging system (intermediate and low risk) and the presence of *BRAF* V600E mutation

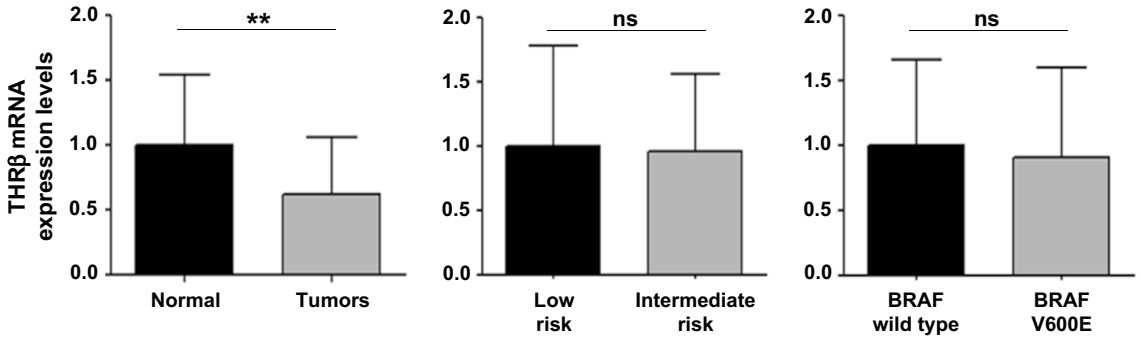

Fig. 2 Expression of *THRβ* in human PTCs. Expression levels of *THRβ* in tumor tissues are lower than corresponding normal. No significant differences were found between the PTCs at ATA intermediate risk vs low risk of recurrence, and *BRAF* V600E vs *BRAF* wild-type PTCs. Data represent the mean ± standard deviation. *p* value was obtained by Mann–Whitney test. $^{**}0.001 < p < 0.01$. *ns* not significant

Table 2 Correlation between *THRβ* expression and thyroid-specific genes (*NIS*, *TPO*, *Tg* and *TSH-R*)

Gene	r^a	*p* value
NIS	0.3771	0.0098
TPO	0.3917	0.0037
Tg	0.4504	0.0007
TSH-R	0.5049	0.0001

[a] Spearman rho rank correlation coefficient

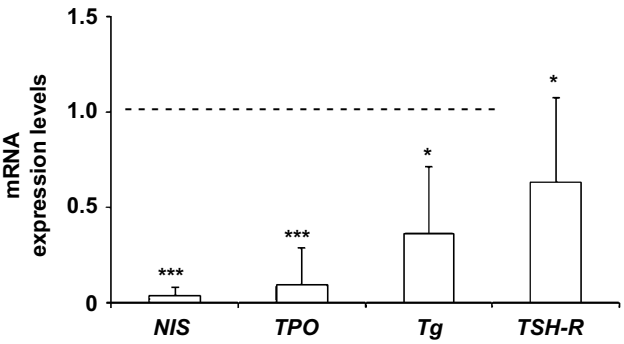

Fig. 3 Expression of thyroid-specific genes in human PTCs. Expression levels of NIS, *TPO*, Tg and TSH-R in tumor tissues are lower than corresponding normal, set equal to one. Data represent the mean ± standard deviation. *p* values were obtained by Mann-Whitney test. $^{*}0.01 < p < 0.05$; $^{***}p < 0.001$

expression was performed in a wider cohort of PTCs. In all 36 tumors examined, the transcript was down-regulated compared with the corresponding normal tissue. However, such as *NIS*, *Tg* and *TSH-R*, we did not find significant differences of *THRβ* mRNA levels by comparing the groups in terms of tumor extent and risk of having structural persistent/recurrent disease based on the 2009 ATA risk stratification staging system [19]. Thus, the reduced expression of *THRβ* may not be considered as a marker of aggressiveness, at least in this cohort of PTCs. This finding is also strengthened by the absence of significant differences in *THRβ* expression levels between the subgroups of tumors presenting the *BRAF* V600E mutation or not. *BRAF* V600E is the most common genetic alteration found in PTCs [33] and is associated with a reduced expression of the genes involved in iodide metabolism [34], the presence of worrisome clinicopathologic features, and a significantly higher risk of recurrence than *BRAF* wild-type tumors [30, 35].

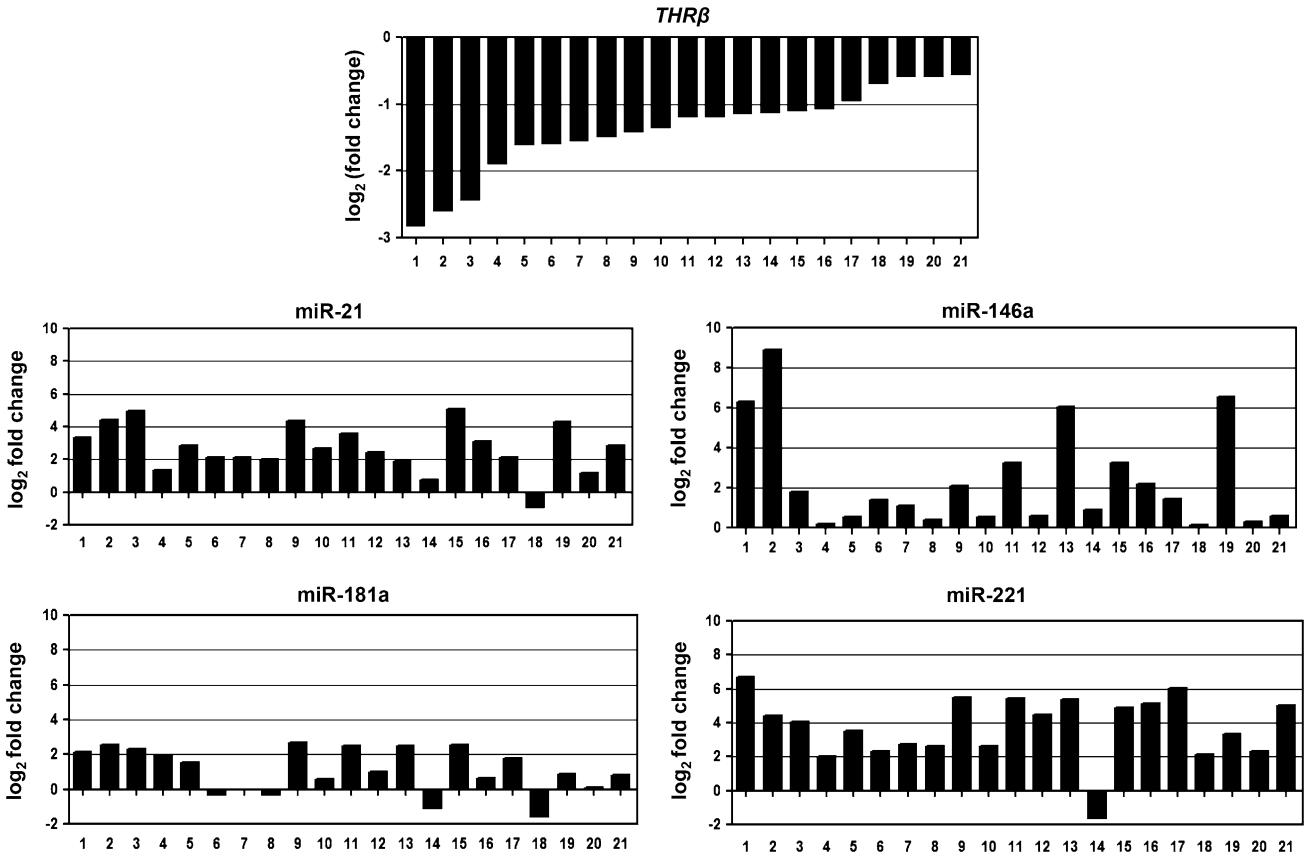

Fig. 4 Down-regulation of *THRβ* and up-regulation of miR-21, miR-146a, miR-181a and miR-221 in human PTCs. Levels of expression of miRNAs were examined in 21 PTCs. Results of relative quantification (tumor vs normal tissues) are shown as $\log_2$ fold change

In this series of PTCs, analysis of expression levels of thyroid-specific genes, which confirmed our previous finding regarding the presence of the lowest levels of *NIS* and the close to normal levels of *TSH-R* [21], revealed that *THRβ* expression was directly correlated with all the genes examined, suggesting for the loss of *THR* intra-thyroidal expression the behavior as a marker of differentiation.

It is now well established that disregulation of the profile of miRNAs expression occurs in a variety of malignancies, where they are emerging as oncogenes or tumor suppressor genes [12–14]. There are many reports about the expression of miRNAs in thyroid tumors [reviewed in 15, 16]. Interestingly, up-regulation of miR-21, miR-181a, miR-146a, and miR-221 has been described as an important mechanism of silencing of *THRβ* [17]. In our study, we found the upregulation of miR-21, miR-181a, miR-146a and miR-221 in all tumor tissues displaying low levels of *THRβ*. Although the correlation did not reach a statistical significance, the general trend confirms the results of Jazdzewski [17], suggesting that an up-regulation of these miRNAs might be responsible for the down-regulation of *THRβ* in PTCs. As for other thyroid-specific genes, a statistically significant and inverse correlation was found between expression levels of *Tg* and both miR-21 and miR-146a, and also between *TPO* and miR-21.

In conclusion, our findings demonstrate that a reduction of *THRβ* gene expression is a common feature of PTCs even if not associated with a more aggressive phenotype of the tumors. Moreover, it is directly correlated with the reduction of all the markers of differentiation and associated with overexpression of some miRNAs supposed to play a role in thyroid tumorigenesis. Overall, our results suggest that *THRβ* could represent an additional thyroid differentiation marker.

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

Ethical approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent The authors declare that informed consent was obtained from all individual participants included in the study.

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