Expression of *tpo* mRNA in thyroid tumors: quantitative PCR analysis and correlation with alterations of *ret*, *Braf*, *ras* and *pax8* genes

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Abstract

Immunocytochemistry (ICC) of thyroid peroxidase (TPO) using the monoclonal antibody MoAb47 has been used as malignancy marker on thyroid fine needle aspiration. However, little is known about the fate of TPO in thyroid carcinoma. We performed a qualitative PCR (Q-PCR) analysis to measure the expression of variants of *tpo* mRNA in 13 normal tissue samples, 30 benign tumors (BT), 21 follicular carcinomas (FC), 20 classical papillary carcinomas (PCc), 12 follicular variants of papillary carcinomas (PCfv) and nine oncocytic carcinomas (OC). We also studied mutations involving the *ras*, *Braf*, *ret* or *pax8* genes. Results of Q-PCR were closely correlated with those of ICC ($P < 0.0001$; $R = 0.59$) and showed that overall *tpo* expression was lower in all carcinomas than in normal and BT ($P < 0.05$). The ratio *tpo2* or *tpo3* to *tpo1* was inverted in follicular tumors. Genetic mutations were observed in 90% of PCc, 61.9% of FC, 41.7% of PCfv, 0% of OC and 10% in BT. *pax8-ppar* $\gamma 1$ rearrangement was correlated with qualitative changes in *tpo* mRNA ($P < 0.01$). These results confirmed the decrease of TPO expression in 97% of thyroid carcinomas regardless of histological type and the overexpression of shorter splice variants in follicular tumors. Both reduction in quantity of TPO and impairment of its maturation process could account for the atypical immunohistochemical reaction of MoAb47 with TPO.

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Introduction

Thyroid peroxidase (TPO) is an essential membrane-bound enzyme in thyroid hormone synthesis. The full-length transcript codes for the 933 amino acid TPO1 protein (Kimura *et al.* 1987). Differential splicing produces shorter transcripts: *tpo2*, *tpo3* (*tpo_{zanelli}*), *tpo4* and *tpo5* lacking exon 10, 16, 14 and 8 respectively (Kimura *et al.* 1987, Zanelli *et al.* 1990, Elisei *et al.* 1991, Ferrand *et al.* 2003). Variants spliced on several exons have also been described: *tpo2/3* (10 and 16), *tpo2/4* (10 and 14) and *tpo6* (10, 12–14 and 16) (Ferrand *et al.* 2003).

In 1991 we discovered that the anti-TPO monoclonal antibody MoAb47 reacted with less than

30% of thyroid carcinomas (De Micco *et al.* 1991). When present in malignant cells, immunocytochemical (ICC) staining showed an abnormal pattern. Because these differences were so pronounced in comparison with other anti-TPO antibodies (De Micco *et al.* 1994a, Mizukami *et al.* 1994), MoAb47 was investigated as a malignancy marker on thyroid fine needle aspiration (FNA) samples. Several independent studies reached similar conclusions. The sensitivity and specificity of this method was close to 100% in initial short series (De Micco *et al.* 1994a, Christensen *et al.* 2000) and around 98% and 80% respectively in larger series (Faroux *et al.* 1997, De Micco *et al.* 1999). Based on these findings, staining with MoAb47 has been routinely used in several

Table 1 Relationship between mean *tpo* mRNA expression (measured by Q-PCR) and pTNM tumor stage

Tumor	pT1		pT2		pT3		pT4		pN0		pN1	
	No.	<i>tpo</i>	No.	<i>tpo</i>	No.	<i>tpo</i>	No.	<i>tpo</i>	No.	<i>tpo</i>	No.	<i>tpo</i>
PCc	7	4.59	1	0.44	11	5.75	1	2.82	6	5.22	11	4.91
PCfv	8	24.44	4	29.27	0		0		11	28.3	1	1.32
FCmi	1	67.36	9	55.83	1	96.93	0		x		x	
FCwi	1	55.67	1	9.12	7	37.04	1	47.14	x		x	
OC	2	14.11	4	34.57	2	39.02	1	83.51	x		x	

x, node status not determined (no surgery on nodes)

laboratories and has proven to be particularly useful in the ‘grey area’ of follicular tumors (De Micco *et al.* 1994b, 1999).

The mechanisms underlying TPO anomalies in thyroid tumors remain obscure. Northern blot analysis of *tpo* mRNA in carcinoma has yielded discrepant results (Hoang-Vu *et al.* 1992, Elisei *et al.* 1994) and the hypothesis that an immunological alteration accounts for the variable affinity of TPO for different antibodies (Mizukami *et al.* 1994, Tanaka *et al.* 1996) has been questioned (Czarnocka *et al.* 2001). In pilot studies using immunohistochemistry and *in situ* hybridization, we observed more heterogeneity in TPO and *tpo* mRNA in malignant tumors (De Micco *et al.* 2000). RT-PCR showed that modifications of alternative splicing could account for these variations (Ferrand *et al.* 2001, Le Fourn *et al.* 2004).

In this study we measured the expression of complete and spliced variants of *tpo* mRNA in thyroid tumors using quantitative PCR (Q-PCR). We also studied alterations involving the *ras*, *Braf*, *ret*, or *pax8* genes since they might be correlated with the modification in *tpo* mRNA expression.

Materials and methods

Patients and samples

Patients were treated in the Department of Endocrine Surgery at the Timone University Hospital in Marseilles. Patient work-up included FNA biopsy. Tissues taken during surgery were kept frozen. Histological diagnosis was established according to the World Health Organization classification (De Lellis *et al.* 2004). Pieces of normal tissues (NT=13) were taken near colloid nodules (CN). The benign tumors (BT) (*n* = 30) included seven CN and 23 follicular adenomas (FA). The sex ratio (women/men) was 1.31/1 and the mean age was 50.0 years (range, 23–74 years). The carcinomas

(*n* = 62) included 11 minimally invasive follicular carcinomas (FCmi), ten widely invasive follicular carcinomas (FCwi), 20 classical papillary carcinomas (PCc) (13 with purely papillary architecture and seven with mixed, papillary and follicular architecture), 12 follicular variants of papillary carcinomas (PCfv) and nine oncocytic carcinomas (OC). The sex ratio (women/men) was 2.44/1 and the mean age was 46.4 years (range, 16–79 years). Tumor stage according to Pathological Tumor Node Metastasis (pTNM) classification is shown in Table 1. The study protocol was approved by the Clinical Research Committee of Marseilles Public Hospital System. Informed individual consent to use residual pieces of tissues for research purpose was obtained.

ICC

ICC for TPO was routinely performed concurrently with Giemsa staining on one or two slides of FNA biopsy. Smears for ICC were air-dried and immediately stored at 4 °C until staining. ICC consisted of overnight incubation at 4 °C with MoAb47 (1/100) (DAKO, Glostrup, Denmark), incubation with a streptavidin–biotin–peroxidase kit (LSAB; DAKO) and revelation of peroxidase activity with a 3,3′diaminobenzidine–H₂O₂ kit. FNA samples from surgically removed benign tissue was used as a positive control. The primary antibody was omitted from negative controls. The percentage of positive cells was recorded, excluding cells exhibiting abnormal staining with fading of perinuclear staining (De Micco *et al.* 1994a).

RNA isolation and cDNA synthesis

RNA isolation was performed using Trizol (Invitrogen, Cergy-Pontoise, France). RNA was extracted with 200 µl chloroform and precipitated with 350 µl

Figure 1 Schematic representation of *tpo* variants showing deleted exons. Black boxes indicate the positions of Q-PCR primer pairs P1, P2, P3, P6 and TPO. F, forward; R, reverse.

isopropanol overnight at -207°C . After centrifugation and washing, pellets were diluted in 20 μl diethyl pyrocarbonate-treated water. RNA concentration was estimated with an optical density absorption setting of 260 nm. One microgram of RNA was reverse-transcribed in a volume of 20 μl using random priming and Superscript II (Invitrogen).

Q-PCR

A two-step relative method was used to quantify *tpo* mRNA. First, expression of total *tpo* mRNA in each sample was normalized to expression of the tata box binding protein gene (*tbp*) and compared with the median of values from NT (Picard *et al.* 2006). Secondly, the ratios of *tpo* variants to total *tpo* were determined in each sample.

Primers were designed using Primer Express software (Fig. 1) (Applied Biosystems, Courtabœuf, France). Their characteristics are given in Table 2. Analysis of total *tpo* mRNA expression was performed with TPO primers amplifying a sequence in exons 5–6. Primers spanning the banks of deleted exons were designed for separate quantification of

tpo variants (Vandenbroucke *et al.* 2001). The P1 primer pair amplified all variants, except those lacking exon 16 (*tpo1*, *tpo2*, *tpo4*, *tpo5* and *tpo2/4*). The P2 primer pair amplified variants lacking exon 10 (*tpo2*, *tpo6*, *tpo2/3* and *tpo2/4*). The P3 primer pair amplified variants lacking exon 16 (*tpo3*, *tpo6* and *tpo2/3*). The P6 primer pair amplified only *tpo6*.

Q-PCR experiments were performed on an ABI Prism-7700 system (Perkin-Elmer/Applied Biosystems). The amplification reaction was performed using 1 $\times$ SYBR GreenI PCR Mastermix (Eurogentec, Angers, France), 5 μl cDNA and 300 nM of each primer. The PCR program consisted of an initial step at 95°C for 5 min followed by a 40-cycle melting step at 95°C for 15 s and an annealing–elongation step at 59°C for 1 min. In each experiment, a standard curve was used to estimate PCR efficiency and dissociation curves were performed to check the absence of non-specific amplification and primer–dimer formation. PCR products were also checked on agarose gel. Non-template controls were systematically performed. At least two replicates were performed for each experiment. Reproducibility of Q-PCR experiments for P1, P2 and P6 was

Table 2 Characteristics of primers used for PCR and Q-PCR analysis of *tpo* mRNA variants and oncogenes

Primer pair		Sequence (5'-3')	Localization (exon)	Accession number	Size (bp)	Tm (°C)
<i>tpb</i>	F	CAC GAA CCA CGG CAC TGA TT	4	BT019657	89	59
	R	TTT TCT TGC TGC CAG TCT GGA C	5			59
<i>TPO</i>	F	AAC AAC AGA GAC CAC CCC AGA T	5/6	BC095448	86	59
	R	TGA CTG AAG CCG TCC TCA TAG AC	6			59
<i>P1</i>	F	CGG AGG CTT CGC AGG TC	15	BC095448	68	59
	R	ACG CAC TGG CAC TAA ATC CAC AC	16			59
<i>P2</i>	F	CGT GGA GGT TAC AAT GAG TGG A	9/11	BC095448	138	59
	R	CGA TGT TGT CAG GAT GCT TGT AC	11			59
<i>P3</i>	F	TTT GCA GGT GGA GAG TGC TG	15/17	BC095448	78	59
	R	CTG CCA CTT TGC CCT CAG GA	17			59
<i>P6</i>	F	GCT GGG AGG CTT AGT TGA AA	11	BC095448	112	59
	R	CCC GGA GTA CCA GTC ACC AT	11/15			59
<i>Braf</i>	F	GCA CAG GGC ATG GAT TAC TT	13/14	M95712	195	59
	R	GAT GAC TTC TGG TGC CAT CC	15/16			59
<i>Nras</i>	F	ATG ACT GAG TAC AAA CTG GT	1	X02751	288	54
	R	GTA GAG GTT AAT ATC CGC AA	2			54
<i>pax8-ppar γ1</i>	F 1	AAC CTC TCG ACT CAC CAG AC	<i>pax8</i> ex 7	X69699	362/68	60
	F 2	CCC TTC AAT GCC TTT CCC C	<i>pax8</i> ex 8		172/67	60
	F 3	CTA TGC CTC CTC TGC CAT C	<i>pax8</i> ex 9		69	60
	R	AGA ATG GCA TCT CTG TGT CAA C	<i>ppar γ</i> ex 1		NM_138712	60
<i>ret/PTC1</i>	F	ATT GTC ATC TCG CCG TTC	<i>H4</i> ex 1	M31213	306	54
	R	CTT TCA GCA TCT TCA CGG	<i>ret</i> ex 12			54
<i>ret/PTC3</i>	F	AAG CAA ACC TGC CAG TGG	<i>Ele1</i> ex 7	S71225	244	56
	R	CTT TCA GCA TCT TCA CGG	<i>ret</i> ex 12			54

F, forward; R, reverse; ex, exon; Tm, melting temperature.

evaluated in 25 samples by calculating the coefficient of variation (CV) using four measures on each sample. The mean CV values were 1.7% (± 0.74), 2.5% (± 1.27) and 1.9% (± 1.04) for *P1*, *P2* and *P6* respectively. The *tpb* CV for all samples was 3.3%.

Oncogene analysis

Primers used to amplify *ret*/papillary thyroid carcinoma (PTC)1, *ret*/PTC3 (Santoro *et al.* 2000), exon 15 of *Braf* (Nikiforova *et al.* 2004), exons 1 and 2 of *pax8/ppar γ 1* (Nikiforova *et al.* 2002) and *Nras* (Vasko *et al.* 2003) have been described previously. Reactions were performed in 25 μ l of a PCR solution containing 4 μ l cDNA, 5 mM MgCl₂, 1 $\times$ buffer, 4 ng/ μ l primer and 0.25 U Taq polymerase (Promega, Charbonnières, France). The amplification process included the following steps: denaturation at 94 °C for 5 min, amplification by 35 cycles of denaturation at 94 °C for 30 s, hybridization with Tm primers for 1 min and elongation at 72 °C for 1 min. PCR products were visualized by electrophoresis in 1.5% agarose gel. Products were submitted to direct sequencing (Genome Express, Grenoble, France). Mutations were confirmed by sequencing of antisense strands.

Statistical analysis

Frequencies of single variables were compared using the χ^2 or Fisher exact test. Oncogene mutations and patient age were analysed by Student's *t*-test. After checking for normal distribution of individual *tpo* mRNA values, mean values were analyzed by ANOVA and the Bonferroni test. *P* values less than 0.05 were considered as significant. Linear regression was studied to compare Q-PCR and ICC TPO values. All statistical tests were performed using the Statistical Package for Social Sciences (SPSS, Inc., Chicago, IL, USA).

Results

ICC

All follicular cells from BT and NT showed diffuse, cytoplasmic labeling of TPO that was more pronounced in the perinuclear area, thus giving a characteristic ring-shaped pattern (Fig. 2). More than 80% positive cells were found in six of seven CN and 21 of 23 FA and mean values were significantly higher in FA and CN than all histological types of carcinoma ($P < 0.0001$) (Table 3). All carcinomas except two FC contained less than 80% positive

Figure 2 ICC staining of TPO in thyroid cells obtained by FNA ($\times 400$). (A) FA: a homogeneous dark brown reaction is present in the cytoplasm and the perinuclear area (arrows) in all cells. (B) Papillary carcinoma: absence of immunostaining in the cytoplasm and around nuclei (arrow shows a nucleus: the light grey color of the nuclei is due to hematoxylin counterstaining). (C) FC: a heterogeneous coarse granular positive reaction is present in some cells on the edge of the cytoplasm (broken arrow); perinuclear staining is absent (arrow).

Table 3 Analysis of TPO expression by ICC and Q-PCR of overall *tpo* expression

Tissue type	ICC*		Q-PCR**	
	Mean	Range	Mean	Range
NT	100.0	100–100	111.1	46.9–221.9
FC	42.6	0–100	49.4	0.3–120.1
FCmi	48.2	10–80	60.6	25.6–120.1
FCwi	36.5	0–100	37.1	0.3–65.5
PCfv	30.0	0–60	26.0	1.3–60.7
PCc	9.7	0–60	4.9	0.1–29.1
OC	37.8	0–60	36.4	2.9–118.1
BT	90.6	60–100	126.0	14.9–456.3
FA	90	60–100	111.4	17.8–342.3
CN	91.6	70–100	173.6	14.9–456.3

*Results of ICC on FNA samples are expressed as the percentage of positive cells.

**Results of Q-PCR analysis are expressed as the percentage of the median value found in normal tissues.

cells. TPO expression was very low in PCc with a mean value significantly lower than in OC and FC (9.7% vs 37.8 and 42.9%; $P = 0.015$ and $P < 0.001$ respectively). PCfv (30%) and OC displayed intermediate mean values between PCc and FC. When present in malignant cells, staining was coarse and located at the periphery of the cytoplasm. The perinuclear ring was missing (Fig. 2).

Q-PCR

Mean total *tpo* mRNA was significantly lower in all histological types of carcinomas than in NT (111.2%) and BT (126.0%) ($P < 0.01$) (Table 3). Expression in PCc was the lowest (4.9%), always below 30% of the normal value (Fig. 3) and significantly lower than in OC and FC ($P = 0.015$ and $P < 0.001$ respectively). Intermediate values were found in OC (36.4%) and PCfv (26%). The highest

Figure 3 Q-PCR analysis of total *tpo* expression. Distribution in individual cases. The horizontal line shows the cut-off value (73%).

Table 4 Expression of *tpo* variants amplified with *P1*, *P2*, *P3* and *P6* primer pairs. Results are expressed as percentage of total *tpo* mRNA

Tissue type	<i>P1</i>	<i>P2</i>	<i>P3</i>	<i>P6</i>
NT	71.0 ± 14.8	49.9 ± 8.8	14.8 ± 4.2	4.5 ± 4.1
FC	89.3 ± 24.1	84.1 ± 26.2	20.5 ± 7.8	3.0 ± 4.6
FCmi	101.4 ± 23.4	92.9 ± 19.7	22.9 ± 6.9	3.5 ± 5.8
FCwi	75.9 ± 17.5	74.3 ± 29.8	17.8 ± 8.1	2.4 ± 2.8
PCfv	87.0 ± 20.8	72.6 ± 31.1	18.2 ± 4.4	3.9 ± 3.9
PCc	84.3 ± 23.5	74.6 ± 23.2	15.1 ± 4.6	6.5 ± 5.5
OC	74.9 ± 32.4	74.9 ± 15.7	25.6 ± 18.1	6.5 ± 9.0
BT	71.2 ± 46.4	53.1 ± 30.4	8.3 ± 7.6	3.9 ± 3.8
FA	70.6 ± 50.6	47.0 ± 26.7	8.1 ± 8.2	3.5 ± 3.8
CN	73.2 ± 31.8	73.3 ± 35.1	9.0 ± 5.6	5.2 ± 3.7

values in carcinoma were observed in FC (49.3%). Mean content in FCmi (60.6%) was significantly higher than in FCwi (37.1%; $P = 0.04$) and significantly lower than in CN and NT ($P = 0.005$ and $P = 0.01$ respectively). Mean FCmi content was also lower than in FA but this difference was not statistically significant ($P = 0.6$). Only one OC and one FCmi expressed *tpo* mRNA values in the normal range (118.1% and 120.2% respectively). Mean *tpo* expression was similar in FA and NT. It was higher in CN than NT but this difference was not statistically significant ($P = 0.09$).

Q-PCR analysis of *tpo* mRNA allowed accurate discrimination of BT and cancer. A receiver operating characteristic (ROC) curve showed that the best *tpo* mRNA threshold value for discrimination of malignancy was 73%. Values less than 73% were observed in eight of 23 FA and one of seven CN and values greater than 73% were observed in three of 11 FCmi and two of nine OC. Using this cut-off value, the sensitivity and specificity of *tpo* mRNA Q-PCR analysis for diagnosis of malignancy were 92 and 70% respectively. However, the mean values of total *tpo* mRNA were not correlated with the pTNM stage of the tumors (Table 1). Several PCc at stage T1N0 expressed less than 1% of normal values whereas the two tumors at stage T4 (one FC and one OC) expressed 47.14 and 83.51% of normal values respectively.

For each sample, we calculated the ratio of variants amplified with the *P1*, *P2*, *P3* and *P6* primers to the total *tpo* amplified with the *TPO* primers (Table 4).

P1 variant ratios were higher in all malignant tumors than in NT. The mean ratio in FA was close to normal. The highest ratio observed in carcinoma was in FCmi and was significantly higher than in NT ($P = 0.001$) and than in FCwi ($P = 0.01$).

P2 variant expression in malignant tumors was higher than in NT. The mean ratio in FA was close to normal. Mean ratio in FC was significantly different from NT ($P = 0.01$). FCmi presented the highest mean *P2* value that was significantly different from the mean *P2* value in NT and BT ($P = 0.003$ and $P = 0.001$ respectively). The only FCmi expressing normal total *tpo* mRNA (120.2%) also expressed 105.3% of *P2* variant.

All carcinomas had higher mean *P3* ratios than NT. BT had a lower mean *P3* ratio than NT. Ratios in PCc were close to normal. The highest rates were observed in FCmi and OC. The mean ratio in FCmi was significantly higher than in NT ($P = 0.02$) and BT ($P = 0.001$). The ratio in OC was significantly higher than in both BT ($P = 0.001$) and PCc ($P = 0.01$). The ratio in PCfv was intermediate between the ratios in FC and PCc.

Mean *P6* variant expression in relation to NT was higher in OC and PCc and lower in FC, PCfv and BT. PCfv presented intermediate values between FC and PCc. However, the amount of *P6* variant was highly variable from case to case and these differences were not significant.

ICC and Q-PCR correlation

The correlation was determined between TPO expression studied by ICC with MoAb47 and total *tpo* mRNA expression measured by Q-PCR. A linear regression curve was drawn using data from all samples. Findings showed a highly significant correlation ($P < 0.0001$) with a coefficient (R) equal to 0.59 (Fig. 4).

Oncogene defects

Oncogene mutations and rearrangements are summarized in Table 5. All *Nras* mutations occurred

Figure 4 TPO expression: comparison of ICC on FNA and Q-PCR analysis by linear regression shows a significant correlation ($R = 0.59$ and $P < 0.0001$). *ICC of TPO on FNA samples is expressed as the percentage of positive cells. Q-PCR of *tpo* mRNA is expressed as the percentage of the value in normal tissues.

at codon 61 (exon 2), with equal incidence of transition and transversion. Mutations were present in 38.1% of FC, 25% of PCfv, 10% of TB and no OC. *Nras* mutation was not correlated with *tpo* mRNA expression or clinical features, but was significantly more frequent in FC than in FA ($P = 0.02$).

pax8/ppar $\gamma 1$ rearrangements were present in 42.8% of FC, 8.3% of PCfv and no OC, CN and FA. Eight out of ten rearrangements involved fusion between exon 9 of *pax8* and exon 1 of *ppar $\gamma 1$* , with or without exon 8 of *pax8*. Two rearrangements involved fusion between exon 7 of *pax8* and exon 1 of *ppar $\gamma 1$* .

Patients presenting FC with *pax8/ppar $\gamma 1$* were significantly younger than patients presenting FC without the rearrangement (43.3 vs 58.1 years; $P = 0.04$). This difference was also observed in patients presenting PCfv with or without *pax8/ppar $\gamma 1$* (32 vs. 43.8 years; $P = 0.04$). The incidence of *pax8/ppar $\gamma 1$* was higher in FCmi (six of 11) than FCwi (three of ten) but this difference was not statistically significant. *pax8/ppar $\gamma 1$* was not correlated with gender.

A significant increase in mean *P1* variant expression was found in FC with *pax8/ppar $\gamma 1$* as compared with FC without *pax8/ppar $\gamma 1$* ($104.1 \pm 26.5\%$ vs $78.1 \pm 15.4\%$, $P = 0.01$).

Braf mutations were present in 75% of PCc and 8.3% of PCfv ($P < 0.001$). They were never observed in BT and OC. All *Braf* mutations involved T to A missense transversion at nucleotide 1799. One PCc presented a previously unreported mutation at nucleotide 1799-1800 (TG>AA) that also led to valine-to-glutamate substitution. No K601E mutation was found. *Braf* mutation was not correlated with gender but it was correlated with older age in patients with PCc (46.3 vs 29.8 years, $P = 0.02$). It was more frequent in PCc with pure papillary architecture (12 of 13) than in PCc with mixed papillary and follicular architecture (three of seven), but the difference was not statistically significant. Total *tpo* mRNA expression was lower in PCc with as opposed to without *Braf* mutation (3.4% vs 13.5%), but this difference was not statistically significant.

A *ret/PTC* rearrangement was found in five PCc, three PTC1 and three PTC3. One tumor exhibited both PTC1 and PTC3; it was at stage T3N1 with extra-glandular extension and multiple lymph-node metastasis. No other tumor type presented *ret/PTC* rearrangements. No correlation was found between *ret/PTC* and *tpo* expression or clinical features.

Table 5 Clinicopathological data and oncogene defects in 92 thyroid tumors

Histology	No. of cases	Female/male	Mean age (range)	<i>Nras</i> *	<i>pax8-ppar $\gamma 1$</i> *	<i>Braf</i> *	<i>ret/PTC1</i> *	<i>ret/PTC3</i> *
Carcinomas	62							
PCc	20	2.5/1	41 (16–70)	–	–	15 (75)	3 (15)	3 (15)
PCfv	12	1.75/1	43 (29–77)	3 (25.0)	1 (8.3)	1 (8.3)	0	0
FCmi	11	2.7/1	51 (31–73)	4 (36.4)	6 (54.5)	–	–	–
FCwi	10	2.3/1	53 (20–79)	4 (40)	3 (30)	–	–	–
OC	9	8/1	48 (26–71)	0	0	0	–	–
Benign tumors	30			3 (10)				
FA	23	1.55/1	53 (23–74)	2 (8.7)	0/9	0/9	0/9	0/9
CN	7	0.75/1	39 (23–58)	1 (14.3)	0/4	0/4	0/4	0/4

*Number of mutations or rearrangements (%).

Several tumors displayed more than one molecular alteration. Four FC (19%) exhibited both *Nras* mutations and *pax8/ppar γ 1* rearrangements. These tumors displayed no special histological or clinical features. Two PCc exhibited both *ret*/PTC (one PTC1 and one PTC3) and *Braf* mutations. Both were at stage T3 and were characterized by multiple intra-thyroid tumor foci and extra-glandular extension.

Discussion

In this study we used Q-PCR to measure total *tpo* mRNA expression and determine the relative proportion of the main spliced variants in normal and neoplastic thyroid tissue specimens. We also studied the presence *ret*/PTC rearrangements, *pax8/ppar γ 1* rearrangements, *Nras* mutations and *Braf* mutations.

Results of Q-PCR analysis of total *tpo* mRNA correlated closely with those of ICC using MoAb47 and confirmed a decrease in TPO expression in 97% of thyroid carcinomas, regardless of histological type. Reduction in TPO expression by malignant thyroid tumors has already been reported but previous studies concerned mainly PC (Hoang-Vu *et al.* 1992, Tanaka *et al.* 1996, Lazar *et al.* 1999, Huang *et al.* 2001). This reduction stems from a decrease in PAX8 expression and parallels the decrease of many other differentiated functions such as sodium iodide symporter expression (Fabbro *et al.* 1994, Lazar *et al.* 1999). Our study also includes a large series of benign and malignant follicular tumors. We found that mean *tpo* expression was significantly lower in PCc than in NT, FA and FC but also significantly lower in FC, OC and PCfv than in FA. In agreement with Lazar *et al.* (1999) we found similar *tpo* expression in FA and NT. Overlapping values were found in some individual cases of FA and FCmi exhibiting unusually low and high levels of *tpo* expression respectively. This overlapping was consistent with recent data suggesting the existence of an adenoma–carcinoma sequence in which a subset of FA might predispose to malignant transformation (Vasko *et al.* 2004, Sarquis *et al.* 2006). The finding that *tpo* is significantly lower in FCwi than FCmi and higher in PCfv than PCc confirms the strong correlation between *tpo* expression and follicular differentiation. The correlation with histological differentiation also appears in PCc since tumors with mixed papillary and follicular architecture expressed more TPO than tumors with pure papillary architecture.

Conversely, clinical pTNM staging based mainly on tumor size and extra-thyroid invasiveness has little correlation with *tpo* expression as long as the tumor tissue remains differentiated. Regarding differentiation, it should be noted that dedifferentiation of FC is often correlated with invasiveness, which would explain why *tpo* expression was usually lower in highly invasive than encapsulated tumors.

We found that the relative proportion of spliced *tpo* variants was enhanced in carcinomas, especially FC and OC. The increase was significant for *P2* variants in FC and *P3* variants in OC. Involvement of the whole spectrum of differentiated follicular tumors but not of PCc suggests that disruption of *tpo* mRNA maturation is a characteristic feature of follicular thyroid carcinogenesis.

The increase in shorter TPO isoforms could account for several features of follicular tumors and explain the variations observed in ICC experiments using various anti-TPO antibodies. Since it has been shown that TPO2 is folded incorrectly, unable to bind to heme and enzymatically inactive (Niccoli *et al.* 1997), predominance of TPO2 over TPO1 might reduce iodine concentration activity in tumor tissue. In addition, TPO2 and TPO3 have a shorter half-life than TPO 1 (3 and 7 h vs 11 h respectively), and remain almost exclusively in the intracellular compartment instead of reaching the cell surface (Niccoli *et al.* 1997, Niccoli-Sire *et al.* 2001). Immunological profiles cannot explain the differences in reactivity between MoAb47 and other monoclonal antibodies since MoAb47 recognized both TPO2 and TPO3 under experimental conditions (Niccoli *et al.* 1997, Niccoli-Sire *et al.* 2001). Rapid turnover of TPO2 and TPO3 might provide a more plausible explanation for the weak reaction obtained with MoAb47 on malignant follicular tumors. Indeed, a special property of MoAb47 in comparison with other antibodies is to react with a native, unglycosylated TPO, having a short half-life, rather than with mature forms of the protein (Chazenbalk *et al.* 1993, Fayadat *et al.* 1998). In NT, the concentration of native TPO and *tpo* mRNA is higher in the nuclear envelope than in the endoplasmic reticulum (Pohl *et al.* 1993). In tumor tissue, overproduction of mistargeted variants with a short half-life and the quantitative decrease of TPO expression could explain both the reduction of immunostaining obtained with MoAb47 and its location in the cytoplasm rather than in the perinuclear area. The presence of abnormally located TPO in malignant follicular tumors still expressing a significant amount of the protein

lowers the risk of overlooking the malignancy with ICC. For this reason, ICC appears to be more accurate for diagnosing malignancy than Q-PCR which only measures quantitative variations of *tpo* expression.

The high versus low incidence of *Braf* mutations and *ret*/PTC respectively found in PCc can be explained by the clinico-pathological features, i.e. patient age over 35 years in most cases, higher frequency of advanced tumor stage and purely papillary histology. These three features have been correlated with *Braf* mutations and *ret*/PTC previously (Cohen *et al.* 2003, Kim *et al.* 2004, Nikiforova *et al.* 2004, Trovisco *et al.* 2004, Di Cristofaro *et al.* 2005, Xing *et al.* 2004). The antagonism between oncogene activation of the ERK signaling cascade and expression of differentiated functions by thyroid cells has been confirmed by expression array analysis and may explain the inhibition of *tpo* expression in PC (Melillo *et al.* 2005). In our study, all PCc exhibited low *tpo* expression and PCc harboring *Braf*^{T1799A} mutation were associated with even lower *tpo* values. This difference was not statistically significant probably because the number of PCc without *Braf*^{T1799A} mutation was too small.

In FC, the rates of *pax8-ppar* $\gamma 1$ and *Nras* mutation were similar and within the same range as previously reported (Nikiforova *et al.* 2002, 2003, Dwight *et al.* 2003, Vasko *et al.* 2003). Unlike Nikiforova *et al.* (2003), we found overlapping in 19% (four of 21) of FC. This suggests that the two events are not mutually exclusive and that concurrence might contribute to the emergence of different phenotypes.

We found that *pax8-ppar* $\gamma 1$ has a direct impact on *tpo* maturation since a significant predominance of variants amplified by *P1* was observed in tumors exhibiting *pax8-ppar* $\gamma 1$. Expression profile analysis of FC displaying *pax8-ppar* $\gamma 1$ fusion oncogene demonstrated impairment of ribosomal proteins and of translation-associated genes that could explain disruption of the protein maturation process (Lui *et al.* 2005). Further study will be needed to determine the exact effect of *pax8-ppar* $\gamma 1$ on *tpo* mRNA maturation.

Unlike the *pax8-ppar* $\gamma 1$ rearrangement, *ras* mutation was not correlated with *tpo* expression. This unexpected finding supports recent speculation that FC with *pax8-ppar* $\gamma 1$ and FC with *ras* mutation constitute distinct biological entities (Nikiforova *et al.* 2003, Lui *et al.* 2005). Experimental data in human thyroid epithelial cells show that mutant

RAS induces self-limited proliferation without loss of differentiation (Gire & Wynford-Thomas 2000). This finding fits well with our observation that *Nras* mutation has a weak effect on *tpo* expression and that another more determinant event is necessary to explain its downregulation in FC.

None of the molecular mutations investigated here was found in OC. This suggests that the oncogenic process of OC and follicular tumors is different and that other molecular mutations remain to be discovered. In view of the similarities between OC and FC with regard to expression of differentiated function and splicing anomalies of *tpo*, it can be assumed that such mutations have overlapping pathways.

ICC of TPO using MoAb47 was the first marker procedure proposed to assist diagnosis of malignancy on thyroid FNA (De Micco *et al.* 1994a). Its value has been confirmed by several independent studies with sensitivity and specificity up to 97.4% and 82% respectively (De Micco *et al.* 1994a, 1999, Faroux *et al.* 1997, Christensen *et al.* 2000). To our knowledge, no other malignancy marker investigated in a large series of patients has presented sensitivity over 92% (Saggiorato *et al.* 2005). This quantitative analysis of *tpo* mRNA confirmed impairment of TPO expression in all histological thyroid carcinoma subtypes. In addition, we demonstrated that disruption of *tpo* mRNA maturation in follicular malignant thyroid tumors leads to overproduction of shorter TPO isoforms with different biological properties. This finding not only provides a plausible explanation for the TPO immunostaining anomalies observed using MoAb47 antibody in carcinomas but also lends further support to the diagnostic value of this marker in thyroid FNA.

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