Identification of a paired box gene 8–peroxisome proliferator-activated receptor gamma (*PAX8–PPAR γ*) rearrangement mosaicism in a patient with an autonomous functioning follicular thyroid carcinoma bearing an activating mutation in the TSH receptor

J Lado-Abeal^{1,2}, R Celestino⁴, S B Bravo³, M E R Garcia-Rendueles³, J de la Calzada⁵, I Castro¹, P Castro⁴, C Espadinha⁶, F Palos¹, P Soares^{4,7}, C V Alvarez³, M Sobrinho-Simões^{4,7} and J Cameselle-Teijeiro⁸

¹UETeM (Unidade de Enfermidades Tiroideas e Metabólicas), Department of Medicine, School of Medicine, University of Santiago de Compostela, Santiago de Compostela 15705, Spain

²Endocrinology Division, Department of Internal Medicine, School of Medicine, TexasTech University Health Sciences Center, 3601 4th Street STOP 9410, Lubbock, Texas 79430-9410, USA

³Department of Physiology, School of Medicine, University of Santiago de Compostela, Santiago de Compostela 15705, Spain

⁴IPATIMUP (Institute of Molecular Pathology and Immunology, University of Porto), Porto 4200-465, Portugal

⁵Endocrinology Section, Hospital do Meixoeiro, Complexo Hospitalario Universitario de Vigo, Servizo Galego de Saude (SERGAS), Vigo 36200, Spain

⁶Molecular Endocrinology Group, Centro de Investigação de Patobiologia Molecular (CIPM), Instituto Português de Oncologia de Lisboa Francisco Gentil E.P.E., Lisboa 1099-023, Portugal

⁷Department of Pathology, School of Medicine, University of Porto, Porto 4200-319, Portugal

⁸Department of Pathology, School of Medicine, University of Santiago de Compostela. Complexo Hospitalario Universitario de Santiago (CHUS), Servizo Galego de Saude (SERGAS), Santiago de Compostela 15706, Spain

(Correspondence should be addressed to J Lado-Abeal at TexasTech University Health Sciences Center;

Email: joaquin.lado@ttuhsc.edu)

Abstract

Our main objective was to search for mutations in candidate genes and for paired box gene 8–peroxisome proliferator-activated receptor gamma (*PAX8–PPAR γ*) rearrangement in a well-differentiated angioinvasive follicular thyroid carcinoma (FTC) causing hyperthyroidism. DNA and RNA were extracted from the patient's thyroid tumor, as well as 'normal' thyroid tissue, and from peripheral blood lymphocytes (PBLs) of the patient, her daughter, and two siblings. Nuclear isolation was extracted from the patient's tumor, 'normal' thyroid tissue, PBLs, and uterine leiomyoma tissue. TSH receptor (*TSHR*), *RAS*, and *BRAF* genes were sequenced. We searched for *PAX8–PPAR γ* in thyroid, PBL, and uterine leiomyoma samples from the patient and family members. Proliferative effects of detected mutants on non-transformed human thyrocytes cultures. An activating *TSHR* mutation, M453T, was detected in the tumor. *PAX8* (exons 1–8+ 10)–*PPAR γ* was found in all tested patient's tissues. A second rearrangement, *PAX8* (exons 1–8)–*PPAR γ* , was detected in the patient's normal thyroid tissue. Under deprived medium condition, co-transfection of *PAX8–PPAR γ* and *TSHR–M453T* dramatically increased the number of thyrocytes, an effect that it was not observed with *TSHR* wild-type (WT); under complete medium conditions, co-transfection of *PAX8–PPAR γ* with either *TSHR–M453T* or *TSHR–WT* inhibited cell proliferation. We report a patient with hyperthyroidism due to a FTC bearing an activating *TSHR* mutation and *PAX8–PPAR γ* rearrangements. *PAX8–PPAR γ* was present as a mosaicism affecting tissues from endodermal and mesodermal origin. *PAX8–PPAR γ* and *TSHR–M453T*

inhibited or promoted thyrocyte proliferation depending on medium conditions. The activating *TSHR* mutation could promote *in vivo* FTC development in *PAX8-PPAR γ* -positive thyrocytes under poor blood supply with deprivation of growth factors but restraint the tumor growth when growth factors are supplied.

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Introduction

Well-differentiated thyroid carcinomas causing thyrotoxicosis are a rare event (Paul & Sisson 1990), and mostly result from bulky metastases that produce an excess of thyroid hormone (Paul & Sisson 1990). Nevertheless, some cases of thyroid carcinoma presenting as ‘hot’ thyroid nodules have been described (Russo *et al.* 1997, Camacho *et al.* 2000, Mircescu *et al.* 2000, Gozu *et al.* 2004, Majima *et al.* 2005, Niepomniszcz *et al.* 2006).

The molecular basis of ‘hot’ thyroid carcinoma remains unknown. As in benign autonomously functioning thyroid nodules, mutations in either the TSH receptor (*TSHR*) gene, or the adenylate cyclase-stimulating G alpha protein (*GNAS1*) gene that activates the cAMP cascade, have been detected in a small number of thyroid carcinomas (Russo *et al.* 1995, Spambalg *et al.* 1996, Collins *et al.* 2003), with a handful of those being autonomously hyperfunctioning thyroid carcinomas (Russo *et al.* 1997, Camacho *et al.* 2000, Mircescu *et al.* 2000, Collins *et al.* 2003, Fuhrer *et al.* 2003, Gozu *et al.* 2004, Niepomniszcz *et al.* 2006). Chronic cAMP stimulation may contribute, under certain conditions, to tumor progression (Ludgate *et al.* 1999), although it does not seem sufficient for malignant transformation of thyroid follicular cells, and other genetic alterations are probably required to achieve cancer development (Matsuo *et al.* 1993, Russo *et al.* 1995, Spambalg *et al.* 1996, Ludgate *et al.* 1999, Collins *et al.* 2003, Sobrinho-Simões *et al.* 2008).

Mutations of the *RAS* proto-oncogene family are highly prevalent in follicular carcinomas (Kondo *et al.* 2006, Sobrinho-Simões *et al.* 2008) and are considered as an important step in thyroid tumorigenesis. Concurrent mutations in *RAS* and *TSHR* or *GNAS1* genes have been found in some cases of non-functioning differentiated thyroid carcinomas and high adenyl cyclase activity (Russo *et al.* 1995, Suarez 2000). However, we are aware only of one case of an autonomously hyperfunctioning thyroid follicular carcinoma with concurrent mutations in *TSHR* and *RAS* genes having been reported to date (Niepomniszcz *et al.* 2006).

Paired box gene 8 (*PAX8*)-peroxisome proliferator-activated receptor gamma (*PPAR γ*) rearrangement (Kroll *et al.* 2005) is highly prevalent in follicular

thyroid carcinoma (FTC), correlating with an angiogenic phenotype (Marques *et al.* 2002, Nikiforova *et al.* 2002). *PAX8-PPAR γ* rearrangement creates fusion transcripts, wherein the 5' region of the *PAX8* gene is fused in frame with exon 1 of the *PPAR γ* gene; the fusion protein resulting from *PAX8-PPAR γ* transcripts is believed to play a role in thyroid oncogenesis (Reddi *et al.* 2007). *PAX8-PPAR γ* rearrangements have been also detected in 38% of the follicular variant of papillary carcinoma and 33% of follicular adenomas (Castro *et al.* 2006).

Here, we report the case of a patient presenting with hyperthyroidism caused by an autonomously hyperfunctioning FTC. The tumor harbored a *TSHR* gene mutation and a *PAX8-PPAR γ* rearrangement, *PAX8* (exons 1–8+10)-*PPAR γ* (exons 1–6). The *TSHR* gene mutation, a base change at cDNA position c.1358T>C causing the replacement of methionine by threonine at position 453, produces a mutant *TSHR*, M453T, with higher basal constitutive cAMP activity than the WT receptor. Surprisingly, *PAX8* (exons 1–8+10)-*PPAR γ* (exons 1–6) rearrangement was also present in the patient's normal thyroid tissue, peripheral blood lymphocytes (PBLs), and uterine leiomyoma (surgically removed 15 years before the date of the study). *In vitro*, co-transfection of *PAX8-PPAR γ* and *TSHR-M453T* vectors had a strong proliferative effect on normal human thyroid cells grown in a medium deprived of TSH and growth factors, an effect that it was not observed in cell co-transfected with *PAX8-PPAR γ* and *TSHR-WT* vector; however, cells cultured in a medium enriched with TSH and growth factors and newborn calf serum (NCS); co-transfection of *PAX8-PPAR γ* and *TSHR-M453T* or *TSHR-WT* blocked cell proliferation. These results indicate that the effect of *PAX8-PPAR γ* rearrangement and *TSHR-M453T* activating mutation on thyrocyte proliferation depend on the cell context.

Materials and methods

Clinical case

A 55-year-old Spanish woman was referred to us for goiter and hyperthyroidism. Goiter had been found when she was in her 30s but she did not have a clinical follow-up. None of her parents, two children, or four

siblings had goiter. Her past medical history included a hysterectomy due to a leiomyoma at the age of 40 years, type II diabetes mellitus, currently being treated with a sulfonylurea, and arterial hypertension currently being treated with an angiotensin-converting enzyme inhibitor. The patient worked for 14 years in a company that manufactured latex gloves, and believes that she was exposed to high levels of chemical inhalation since no proper protection was taken. Upon neck examination, a nodule of ~5 cm was evident in the left thyroid lobe, which extended caudally into the thorax; her right thyroid lobe presented a 1 cm nodule. Serum TSH was <0.02 $\mu\text{U/ml}$ (normal range 0.41–4.94 $\mu\text{U/ml}$), free thyroxine (T_4) was 1.62 ng/dl, 20.9 pmol/l (normal range 0.85–1.69 ng/dl), and free tri-iodothyronine was 6.25 pg/ml, 96.25 nmol/l (normal range 2.30–4.30 pg/ml). A thorax X-ray showed an enlargement of the mediastinum with tracheal deviation to the right side, suggesting endothoracic goiter. A thyroid ultrasonography showed a nodule of 4.3 cm with multiple calcifications in the left thyroid lobe, and a nodule of 0.8 cm in the right thyroid lobe. No kidney abnormalities were detected by abdominal ultrasound. A $^{99\text{m}}\text{Tc}$ -pertechnetate thyroid scan showed an area of hyperfunctioning tissue in the left thyroid lobe with endothoracic extension and suppression of the rest of the gland. A clinical diagnosis of multinodular goiter with a ‘hot’ autonomously functioning thyroid nodule was made, and surgical removal was indicated. The patient underwent total thyroidectomy and, since histopathology evidenced an encapsulated FTC with vascular invasion (see below), 100 mCi of ^{131}I was administered for ablation. A post-therapeutic total body scan did not show uptake of radioiodine outside the thyroid bed. The patient was discharged from hospital with a TSH-suppressive L- T_4 treatment; two years after diagnosis and initial treatment, she is doing well, with negative ^{131}I total body scans, performed after withdrawal of L- T_4 , and maintaining serum levels of thyroglobulin under 0.2 ng/dl.

Histopathology, *in situ* hybridization, and immunohistochemical analyses

The thyroid specimen was processed routinely. Immunohistochemical studies were performed on 4 μm thick paraffin sections using a peroxidase-conjugated dextran-labeled polymer (Dako EnVision Peroxidase/DAB; Dako, Glostrup, Denmark), in order to avoid misinterpreting endogenous biotin or biotin-like activity in cell cytoplasm or in nuclei as positive staining, with 3,3'-diaminobenzidine as

the chromogen. Antibodies, dilutions, suppliers, pre-treatment, and immunostaining results are listed in Table 1. *In situ* hybridization for thyroglobulin was also performed on 4 μm thick paraffin sections using a commercial single-stranded DNA probe with a length of 552 nucleotides situated in the mRNA coding region of the thyroglobulin gene (CEM-00 16-20, Thyroglobulin histosonda, Cenbimo, Lugo, Spain) following the manufacturer's protocol.

Genetic studies

Genomic DNA and total RNA were extracted from the patient's FTC, adjacent morphologically normal thyroid tissue, and PBL; no samples were extracted from the right thyroid lobe. Equivalent extractions were also made from the PBL of two siblings and the patient's daughter. A RealPure Extraction DNA Genomic kit (Durviz SL, Valencia, Spain) was used for DNA extraction, and TRIzol reagent (Invitrogen) was used for total RNA extraction, as per the manufacturer's instructions. The study was approved by the Institutional Review Board of the University of Santiago de Compostela School of Medicine, and informed consents were obtained.

Exons 8–10 of the *TSHR* gene, exons 2 and 3 of *HRAS*, *KRAS* and *NRAS* genes, and exons 11 and 15 of the *BRAF* gene were amplified from DNA samples by PCR using Ecotaq polymerase (Ecogen, Barcelona, Spain) and in-house designed oligonucleotide primers as previously described (Palos-Paz *et al.* 2008, Cameselle-Teijeiro *et al.* 2009). PCR products were purified with a High Pure PCR Product Purification kit (Roche Diagnostic) and confirmed by direct sequencing (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems, PE Corporation, Foster City, CA, USA).

RNA was reverse transcribed (RT) using M-MLV reverse transcriptase (Invitrogen), 1 μg total RNA, 5 μM random hexamers, and 2 mM deoxynucleotides (Ecogen) in a 20- μl reaction volume. The presence of *PAX8*–*PPAR* γ rearrangements was analyzed by RT-PCR as previously described (Marques *et al.* 2002). PCR products were analyzed and purified by electrophoresis in a 2% agarose gel, and then submitted to sequencing as described above.

Fluorescent *in situ* hybridization of *PAX8*–*PPAR* γ

Fluorescent *in situ* hybridization (FISH) was performed on isolated nuclei extracted from 50- μm paraffin-embedded sections of the patient's thyroid tumor, ‘normal’ thyroid tissue, and uterine leiomyoma. FISH was also performed on the patient's PBL, that of her daughter, the PBL of two of her four siblings,

Table 1 Antibodies, dilutions, suppliers, and results of immunohistochemical staining

Antigen	Antibody clone and source	Dilution	Pretreatment	Results thyroid tumor	Results normal thyroid
Thyroid transcription factor-1	8G7G3/1, Dako	1:20	Water bath, 20 min	+n	+n
Thyroglobulin	DAK-Tg6, Dako	1:2000	None	+c	+c
Thyroperoxidase	MoAb47, Dako	1:50	Microwave, 20 min	+c	+c
Calcitonin	Polyclonal/BioGenex	1:5000	None	–	–
Chromogranin A	DAK-A3, Dako	1:200	Microwave, 20 min	–	–
Synaptophysin	SY38/BioGenex	1:1000	Microwave, 20 min	–	–
Cytokeratins 1, 2, 10, 11, 14, 15, 16, and 19	AE1/AE3, Dako	1:20	Microwave, 20 min	+c	+c
Cytokeratin 19	RCK108, Dako	1:100	Microwave, 20 min	+c*	+c*
Hector Battifora mesothelial cell	HBME-1, Dako	1:200	Water bath, 20 min	–	–
Vimentin	V9, BioGenex	1:10 000	Microwave, 20 min	+c	+c
α -Estrogen receptor	6F11, Novocastra	1:5	Water bath, 20 min	–	–
β -Estrogen receptor	Polyclonal, Santa Cruz Biotechnology	1:50	Water bath, 20 min	+n*	+n*
Progesterone receptor	PgR 636, Dako	1:50	Water bath, 20 min	–	–
E-Cadherin	36, Transduction Lab	1:2000	Microwave, 20 min	+m	+m
β -Catenin	β -Catenin-1, Dako	1:300	Microwave, 20 min	+m	+m
Bcl-2 oncoprotein	124, Dako	1:5	Water bath, 20 min	+c	+c
Galectin-3	9C4, Novocastra	1:200	None	+c, n	–
EGFR	EGFR pharmDx, Dako	Prediluted	Protease	–	–
Cyclin D1	SP4, Master Diagnostica	Prediluted	Microwave, 20 min	+n	–
P63 protein	4A4, Dako	1:10	Water bath, 20 min	–	–
p53 protein	DO-7, Novocastra	1:20	Microwave, 20 min	+n	–
P27	1B4, Novocastra	1:20	Water bath, 20 min	+n	+n
PTEN protein	6H2.1, Dako	1:50	Water bath, 20 min	+c, n	+c, n
Ki-67	MIB-1, Dako	1:200	Water bath, 20 min	<2%	<1%

m, membranous; c, cytoplasmic, or (n) nuclear staining pattern; (*) weak and/or focal staining. Dako, Glostrup, Denmark; BioGenex, San Ramon, CA, USA; Novocastra, Newcastle upon Tyne, UK; Santa Cruz Biotechnology, Santa Cruz, CA, USA; Transduction Lab, Lexington, KY, USA; Master Diagnostica, Granada, Spain.

a brother and a sister, and on 12 normal thyroid specimens obtained from the tissue bank of IPA-TIMUP, University of Porto. BAC probes for *PAX8* (RPCI 1165 I12, BAC PAC Resources, Oakland, CA, USA) and *PPAR* γ (RPCI1130 G23, BAC PAC Resources) were used following the procedure previously described (Marques *et al.* 2004). Nuclear suspensions were spotted on SuperFrost Slides (Menzel-Glaser, GmbH, Memmert, Germany) and pre-treated with 0.1% pepsin (Sigma–Aldrich) in 0.2% HCl at 37 °C. Probe mixture in 50% formamide in 2 $\times$ SSC was co-denatured with the nuclear DNA at 80 °C for 2 min. Detection of digoxigenin-labeled *PPAR* γ probe was accomplished using an anti-digoxigenin fluorescein antibody (Roche Diagnostics GmbH) and a biotin-labeled *PAX8* probe with CY₃–avidin (Jackson ImmunoResearch Lab, West Grove, PA, USA). Nuclei were counterstained with DAPI-Vectashield mounting solution (Vector, Burlingame, CA, USA). Fluorescence hybridization signals were then analyzed and recorded with a Cytovision System (Applied Imaging, Newcastle, UK). In each case, 200

intact non-overlapping nuclei were counted. Nuclei in which the two probes were fused or touching were scored as positive for the fusion gene.

Cell culture, mutant vector construction, and transient expression studies

NT-1 cells (Bravo *et al.* 2003) were used to investigate the effects of *PAX8*–*PPAR* γ rearrangement and *TSHR*–*M453T* on cell proliferation and apoptosis. NT-1 cells were grown in complete medium (Coon’s modified Ham’s F-12 medium supplemented with 5% NCS, 2 mM glutamine, a five-hormone mixture, 5H (1 mIU/ml TSH, 10 ng/ml somatostatin, 10 μ g/ml insulin, 1 nM hydrocortisone, and 5 μ g/ml transferrin) and 100 IU/ml penicillin, 100 μ g/ml streptomycin and 2.5 ng/ml amphotericin B).

pCR3.1 *PAX8* (exons 1–8+10)–*PPAR* γ vector was a gift from Dr T G Kroll; pSVL *TSHR*–*WT* vector was a gift from Dr G Vassart; *TSHR*–*M453T* mutant was introduced into a pSVL *TSHR*–*WT* vector by site-directed mutagenesis using a

QuickChange II kit (Stratagene, La Jolla, CA, USA); the oligonucleotide used for the site-directed mutagenesis was 5'-GTCCCCCGCTTTCTCACGTGCAACC-TGGCC-3'. All vectors were verified by direct sequencing as described above.

Transfections were performed as described (Bravo *et al.* 2010) with nucleofection (Amaxa, Lonza, Spain) using 4.25 µg/well of vectors (0.25 µg of pCmax-GFP (Amaxa) plus 2 or 0.8 µg of each plasmid and empty vectors). After transfection, cells were seeded in 24-well dishes at two densities: 10 000 cell/well (complete medium conditions) and 30 000 cell/well (deprived medium conditions); the next day, some replicates were counted (Time 0), and medium was changed to either complete medium (see above) or deprived medium (Coon's modified Ham's F-12 medium supplemented with 0.1% NCS, 2 mM glutamine and 100 IU/ml penicillin, 100 µg/ml streptomycin and 2.5 ng/ml amphotericin B). Cells were counted at 24 h or 4 days. In deprived conditions, the medium was changed every 2 days. Before trypsinizing and counting, apoptosis was evaluated in live cells staining with Hoescht (Bravo *et al.* 2003).

Immunoblotting

Cytoplasmic low detergent extracts with Hepes lysis buffer were used to detect the TSHR receptors (5 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton, 1.5 mM MgCl₂, and 5 mM EGTA) as described (Bravo *et al.* 2003). Total cell extracts, including nuclear proteins, were obtained by adding 20 µl of boiling 1% SDS per 60-mm dish as described (Palos *et al.* 2008). After scraping, lysates were cooked at 95 °C for 5 min in a thermoblocker, and diluted 1:5 with Hepes lysates buffer. Human thyroid tissue extract was loaded as a control. Western blotting was performed with 50 µg of protein extracts in 10% SDS-PAGE. Immunodetection was carried out with antibodies against PPAR γ (H-100, 1:1000) and TSHR (2C11, 1:1000) from Santa Cruz Biotechnology Inc. (Heidelberg, Germany).

Statistical analysis

All transient transfection experiments were repeated at least three times with four replicates per condition. Statistical analyses of the data were performed using the Wilcoxon test. Data are presented as mean $\pm$ S.E.M. with statistically significance set at $P < 0.05$ and were evaluated using GraphPad Prism 5.00 (GraphPad Software, La Jolla, CA, USA).

Results

Histopathology, *in situ* hybridization, and immunohistochemical analyses

The thyroidectomy specimen showed an encapsulated, solid, reddish yellow tumor measuring 5 cm in maximal dimension in the left lobe and a well circumscribed, unencapsulated nodule of 0.8 cm in the right lobe. The tumor combined well-formed colloid-containing follicles and solid/trabecular patterns of growth, with cuboidal cells showing round pale vesicular nuclei and moderate amount of cytoplasm (Fig. 1A). No nuclear overlapping, irregularity of nuclear contour, grooves, or nuclear pseudoinclusions were observed. The mitotic index was < 1 mitosis per 10 high-power fields. Foci of hyalinization and irregular calcifications were found, but no psammoma bodies or necrosis was detected. The tumor was encapsulated and displayed two areas, in which there was complete penetration of the capsule by neoplastic tissue (Fig. 1C and D) as well as multiple foci of angioinvasion (Fig. 1B and E–G). The nodule in the right lobe was an adenomatous lesion.

Strong, diffuse positivity was found in tumor cells for thyroglobulin both at protein and mRNA levels (Fig. 1H). The neoplastic cells also showed reactivity for thyroid transcription factor-1, thyroperoxidase, cytokeratins (clone AE1/AE3), vimentin, E-cadherin, β -catenin, bcl-2, galectin-3, cyclin D1 (Fig. 1I), PTEN protein, p27, and p53 (Fig. 1J). Very focal immunoreactivity for cytokeratin 19 was found; and fewer than 10% of the nuclei were positive for the β -estrogen receptor. No immunoreaction was observed for calcitonin, chromogranin, synaptophysin, Hector Battifora mesothelial cell antigen, α -estrogen receptor, progesterone receptor, EGFR, or p63 (Table 1). The intravascular tumor cells showed an immunohistochemical profile identical to that of the neoplastic nodule. The proliferative index in the neoplasia, evaluated by MIB-1, was $< 2\%$.

Genetic, FISH, and *in vitro* functional studies

In DNA samples from the FTC, a heterozygous mutation in the *TSHR* gene was detected at position c.1358T $>$ C, corresponding to exon 10. The base change caused amino acid methionine (ATG) to be substituted by threonine (ACG) at codon 453, M453T (Fig. 2A). No *TSHR* gene mutation was found in DNA samples from the patient's normal thyroid tissue specimen or from her PBL. *TSHR*-M453T mutation has higher basal cAMP constitutive activity than *TSHR*-WT (de Roux *et al.* 1996). Neither RAS nor

Figure 1 Follicular carcinoma with TSH receptor mutation and *PAX8-PPAR γ* rearrangement. The tumor (A) contained a mixture of solid/trabecular (top) and follicular (bottom) patterns of growth. Multifocal capsular invasion (C and D), and massive vascular invasion (B and E–G) were found; the asterisk indicates the tumor thrombus. Strong positivity for thyroglobulin using *in situ* hybridization for mRNA (H), as well as nuclear immunoreactivity for cyclin D1 (I) and p53 (J) was detected. (A and B, hematoxylin and eosin, $\times 100$; C–G, hematoxylin and eosin, $\times 200$; H–J, $\times 400$).

BRAF mutations were found in the thyroid carcinoma nor in normal thyroid tissue.

A *PAX8* (exons 1–8+10)–*PPAR γ* (exons 1–6) rearrangement was detected in the FTC by RT-PCR (Fig. 2B) and confirmed by direct sequencing (Fig. 2C). Surprisingly, two different transcripts, *PAX8* (exons 1–8)–*PPAR γ* (exons 1–6) and *PAX8* (exons 1–8+10)–*PPAR γ* (exons 1–6), were found in the patient's normal thyroid tissue, and one of these, *PAX8* (exons 1–8+10)–*PPAR γ* (exons 1–6), was also found in samples obtained from the patient's PBL. FISH analysis further confirmed the presence of the *PAX8-PPAR γ* rearrangement in all the patient's analyzed tissue samples, i.e. in the FTC, adjacent normal thyroid tissue, PBL, and uterine leiomyoma (Fig. 3). FISH showed a similar percentage of *PAX8-PPAR γ* rearrangement in nuclei extracted from the patient tumoral and normal thyroid tissue and in PBL (16.5, 16.4, and 14% respectively).

No quantification was possible in the leiomyoma tissue due to the low quantity of nuclei obtained. The average percentage of *PAX8-PPAR γ* rearrangement in nuclei extracted from the control thyroid specimens was 3.25% (range 1.50–5.37%). No *PAX8-PPAR γ* rearrangement was detected in PBL samples, either from the patient's daughter or from her two siblings when screened by RT-PCR or by FISH.

The effects of *PAX8-PPAR γ* , *TSHR-WT*, and *TSHR-M453T* on normal human thyroid cell proliferation *in vitro* are shown in Fig. 4. NT-1 cells express endogenous *PPAR γ* (55 kDa), and additional expression of the chimeric *PAX8-PPAR γ* fusion protein (around 95 kDa) can be seen in the corresponding transfected cells (Fig. 4A). *TSHR* protein was detected as a smaller band around 65 kDa (immature, non-glycosylated) and as a fully glycosylated band around 120 kDa (Fig. 4A). In complete medium with TSH and growth factors, *PAX8-PPAR γ* had a strong

Figure 2 (A) Electropherogram showing a change from thymine to cytosine in *TSHR* gene exon 10 in DNA samples from the follicular thyroid carcinoma. This base substitution produced the change from methionine to threonine at amino acid position 453 of the TSH receptor. (B) RT-PCR analysis for the *PAX8-PPAR γ* fusion gene. The 281-bp band corresponds to the *PAX8 (exons 1–8+10)-PPAR γ* fusion transcript, and the 178-bp band corresponds to the *PAX8 (exons 1–8)-PPAR γ* fusion transcript. Sample 1 (corresponding to a sample from apparently normal tissue adjacent to the tumor) presented the two different transcripts, and sample 2 (corresponding to a sample from tumor tissue) presented only one aberrant transcript. The negative controls (3, 4) are from a PCR performed either with the product of the negative control of the first round of amplification (3) or without template (4). PCR products were electrophoresed on a 2% agarose gel stained with ethidium bromide. M, molecular weight marker; 1, sample 1 (normal thyroid tissue); 2, sample 2 (tumor tissue); 3 and 4: negative controls. (C) Electropherograms of the two *PAX8-PPAR γ* transcripts detected by RT-PCR in the patient's tissues samples (follicular thyroid carcinoma and adjacent normal thyroid tissue) and PBL. *PAX8 (exons 1–8)-PPAR γ* , detected in adjacent normal thyroid tissue, had the breakpoint at the end of *PAX8* exon 8 (upper). *PAX8 (exons 1–8+10)-PPAR γ* , detected in follicular thyroid carcinoma, adjacent normal thyroid tissue, and PBL, had the breakpoint at the end of *PAX8* exon 10 with complete deletion of *PAX8* exon 9 (lower). The breakpoint in *PPAR γ* was always detected at the beginning of exon 1.

dose-related effect on NT-1 cell proliferation (Fig. 4B); 0.8 $\mu\text{g}/\text{well}$ of vector was used in subsequent experiments to prevent artifact results due to overexpression of the fusion protein. *TSHR-WT* and *TSHR-M453T* induced also proliferation of NT-1 cells; a higher effect was observed when transfecting 0.8 $\mu\text{g}/\text{well}$ than 2 $\mu\text{g}/\text{well}$ of *TSHR* vectors, and *TSHR-M453T* had higher effect than *TSHR-WT* (Fig. 4B). However, co-transfection of *PAX8-PPAR γ* (0.8 $\mu\text{g}/\text{well}$) with *TSHR-WT* or *TSHR-M453T* caused a dose-dependent inhibition of cell proliferation: cells co-transfected with 2 $\mu\text{g}/\text{well}$ of *TSHR* vectors showed a proliferation similar to non-transfected cells, and those co-transfected with 0.8 $\mu\text{g}/\text{well}$ of *TSHR* vectors had lower proliferation than cells transfected with *TSHR* vectors alone (Fig. 4B). In deprived medium condition without TSH and growth factors, *PAX8-PPAR γ* , *TSHR-WT*, or *TSHR-M453T* alone did not induce significant thyrocyte proliferation (Fig. 4C). However, co-transfection of *PAX8-PPAR γ* with *TSHR-M453T* at 2 or 0.8 $\mu\text{g}/\text{well}$ nearly triplicates or duplicates respectively the number of NT-1 cells by day 4 (Fig. 4C). Co-transfection of *PAX8-PPAR γ* and *TSHR-WT* induced an increase in cell number with the smaller amount of *TSHR-WT* (0.8 $\mu\text{g}/\text{well}$) but not with the higher amount (2 $\mu\text{g}/\text{well}$), suggesting the presence of an inhibitory feedback that was not observed with *TSHR-M453T*. For each experimental condition, apoptosis was lower than 0.5%.

Discussion

To our knowledge, this is the first description of a patient with hyperthyroidism caused by an autonomously hyperfunctioning FTC harboring a somatic *TSHR* activating mutation and a *PAX8-PPAR γ* rearrangement.

TSHR activating mutations are a well-known cause of toxic thyroid adenomas (Vassar & Dumont 1992, Palos-Paz *et al.* 2008), and have also been found in a handful of the rare 'hot' FTCs (Russo *et al.* 1997, Camacho *et al.* 2000, Mircescu *et al.* 2000, Collins *et al.* 2003, Fuhrer *et al.* 2003, Gozu *et al.* 2004, Niepomniszcze *et al.* 2006). These mutations activate the *TSHR* in the absence of ligand binding, increasing intracellular cAMP levels and activating the phospholipase C-diacylglycerol-inositol phosphate cascade, thus stimulating thyrocyte proliferation and thyroid hormone secretion (Vassar & Dumont 1992). *TSHR* and *GNAS1* gene activating mutations are rarely found in thyroid carcinomas (Russo *et al.* 1995, Spambalg *et al.* 1996), and patients with McCune-Albright syndrome, a disease caused by activating mutations

Figure 3 Representative results obtained in the FISH analysis for $PPAR\gamma$ and PAX8. Green dots are the BAC probes for $PPAR\gamma$, red dots are BAC probes for PAX8, and the nuclei are stained with DAPI. Nuclei in which the two probes were fused or touching were scored as positive for the fusion gene. A, nucleus extracted from adjacent normal tissue positive for the $PAX8-PPAR\gamma$ rearrangement; B, nucleus extracted from tumoral tissue positive for the $PAX8-PPAR\gamma$ rearrangement; C, nuclei extracted from adjacent normal tissue negative for the $PAX8-PPAR\gamma$ rearrangement; D, nuclei extracted from PBL positive for the $PAX8-PPAR\gamma$ rearrangement.

in the *GNAS1* gene, do not usually develop thyroid cancer (Collins *et al.* 2003). These findings suggest that autonomously functioning thyroid carcinomas require additional mutational and/or epigenetic events.

Well-differentiated thyroid carcinomas often present mutations in genes involving mitogen-activated protein kinase signaling pathway (Kondo *et al.* 2006, Sobrinho-Simões *et al.* 2008). RAS genes (*HRAS*, *KRAS*, and *NRAS*) encode membrane-associated guanine nucleotide-binding proteins implicated in thyroid carcinomas. Point mutations affecting the GTP-binding domain (codons 12/13) and the GTPase domain (codon 61) result in constitutive activation of the protein and tumor development. Somatic missense mutations of RAS genes have been found in FTCs and adenomas (Nikiforova *et al.* 2003, Sobrinho-Simões *et al.* 2008). The concurrent presence of *TSHR* or *GNAS1* activating mutations and RAS mutations have been previously reported in two differentiated thyroid carcinomas which were cold on scintiscan (Russo *et al.* 1995). More recently, an autonomous functioning thyroid follicular carcinoma bearing a *TSHR* gene activating mutation, T620I, and a mutation in *K-RAS* codon 12, G12C, has been reported (Niepomniszcz *et al.* 2006). These findings led us to sequence RAS oncogenes, although without any positively correlating results.

We identified a $PAX8-PPAR\gamma$ rearrangement in our patient's FTC. The $PAX8-PPAR\gamma$ fusion protein is a putative oncogene capable of increasing cell growth and inducing loss of differentiation in immortalized human thyrocyte cultures (Au *et al.* 2006, Reddi *et al.* 2007). Accordingly, $PAX8-PPAR\gamma$ rearrangement is highly prevalent in FTC (Suarez 2000, Nikiforova *et al.* 2002, Kroll *et al.* 2005), although the clinical importance of $PAX8-PPAR\gamma$ rearrangement remains disputable (Sobrinho-Simões *et al.* 2008). Such rearrangements have been associated with younger age in patients with follicular adenomas and follicular carcinomas, as well as with overtly invasive features in the follicular carcinomas (Nikiforova *et al.* 2003). The association between younger age of patients and tumors displaying the rearrangement has been more recently confirmed (Castro *et al.* 2006); however, in this study, there were few cases showing invasive characteristics to allow accurate evaluation of the relationship between $PAX8-PPAR\gamma$ rearrangement and prominent vascular invasiveness. In two other series (Marques *et al.* 2004, Sahin *et al.* 2005), it has been reported that patients with $PAX8-PPAR\gamma$ -negative tumors have less well-differentiated tumors, and are more likely to have metastatic disease. The tumor in the current study was a well-differentiated, overtly

Figure 4 Counteracting or additive effects of *PAX8-PPAR γ* and *TSHR-M453T* on proliferation of normal human thyrocytes depending on the cell culture medium conditions. (A) Western blot of cell extracts from NT-1 human thyrocytes transiently transfected with *PAX8-PPAR γ* or *TSHR* vectors. All cells expressed endogenous *PPAR γ* (55 kDa, white arrow) and *PAX8-PPAR γ* fusion protein (around 95 kDa, black arrow) were detected only in the corresponding transfected cells. Human thyroid tissue expressed such a big amount of *PPAR γ* in comparison with tubulin that there was an alteration in the running of the lane, although the size was identical to NT-1 when smaller amounts were loaded (data not shown). *TSHR* was detected as a smaller band around 65 kDa (immature, non-glycosylated) and a fully glycosylated band around 120 kDa. (B) Transfection of *PAX8-PPAR γ* , *TSHR-WT*, or *TSHR-M453T* induced significant proliferation in the NT-1 thyrocytes after 24 h of culture in complete medium (5% NCS+5H). Lower amounts of *TSHR* vectors induced higher cell proliferation, independently of the amount transfected. (C) In deprived medium (0.5% NCS), NT-1 thyrocytes transfected separately with *PAX8-PPAR γ* , *TSHR-WT*, or *TSHR-M453T* did not proliferate. However, co-transfection of *PAX8-PPAR γ* (0.8 μ g/well) and *TSHR-M453T* (2 μ g/well) triplicated the cells after 4 days. This effect was also seen when a lower amount (0.8 μ g/well) of mutated or normal *TSHR* vector was co-transfected with *PAX8-PPAR γ* , although to a smaller degree, suggesting that *TSHR-WT*, but not *TSHR-M453T*, maintained an inhibitory feedback under these conditions.

angioinvasive follicular carcinoma. In an attempt to explain the prominent vascular invasiveness of our case, we screened for *BRAF* mutations, a frequent genetic event in papillary thyroid carcinomas associated with vascular invasion (Elisei *et al.* 2008), and found negative results. For the same reasons, we also looked for epigenetic dysregulation using an extensive panel of antibodies: overexpression of cyclin D1 and p53 was the only positive markers; we found

that might be associated with tumor progression. Taken together, our results in the present case are consistent with the putative association between *PAX8-PPAR γ* rearrangement and angioinvasiveness in follicular carcinomas (Nikiforova *et al.* 2002).

PAX8-PPAR γ rearrangement was unexpectedly observed in nearly 20% of cells analyzed from the otherwise apparently normal thyroid tissue and PBL of our patient. Moreover, and although not quantified, we

also identified a *PAX8-PPAR γ* rearrangement in samples from the uterine leiomyoma surgically removed from the patient 15 years previous to her hospital admission reported here. These facts raise the question of the role of *PAX8-PPAR γ* rearrangement in thyroid carcinoma development. Our results indicate that the presence of a *PAX8-PPAR γ* rearrangement affecting <20% of the thyrocytes is not sufficient to induce malignant cell transformation. From our *in vitro* studies, it is tempting to speculate that *PAX8-PPAR γ* effects in follicular cells need an activation of TSHR signaling to contribute to FTC development. NT-1 thyrocytes express endogenous TSHR, and when NT-1 cells are grown in complete medium, which has growth factors and TSH, an activation of the endogenous TSHR is expected. Activation of the cAMP pathway is also a well-described consequence of *TSHR-WT* overexpression in cellular transfection experiments. NT-1 cells transfected with *PAX8-PPAR γ* showed a strong proliferative response in complete medium conditions but not in deprived medium conditions; accordingly, co-transfection of *PAX8-PPAR γ* and *TSHR-WT* in deprived medium conditions was associated with an increase in cell proliferation at lower concentrations of *TSHR-WT* plasmid. However, NT-1 cell proliferation was blocked when the cells were co-transfected with *PAX8-PPAR γ* and *TSHR-WT* or *TSHR-M453T* under complete medium conditions or co-transfected with *PAX8-PPAR γ* and higher concentrations of *TSHR-WT* in Deprived Medium, an effect that was not observed with *TSHR-M453T*. These results suggest that some activation of TSHR is required for *PAX8-PPAR γ* proliferative effects on NT-1 cells but if the TSHR activation exceeds a threshold then the cellular proliferation is blocked. This finding argues for the presence of an inhibitory mechanism in cells expressing both *PAX8-PPAR γ* and *TSHR-WT* or *TSHR-M453T* under TSH stimulation, but the inhibitory mechanism is not activated in the absence of TSH. The nature of such inhibitory mechanism remains unknown and deserved further studies.

Two distinct *PAX8-PPAR γ* transcripts were found in the patient's normal thyroid tissue, *PAX8 (exons 1-8)-PPAR γ* and *PAX8 (exons 1-8+10)-PPAR γ* , whereas samples taken from the FTC and PBL showed only one transcript, *PAX8 (exons 1-8+10)-PPAR γ* . The presence in normal thyroid tissue of two *PAX8-PPAR γ* transcripts could be the result of different splice variants of the same rearrangement, although to our knowledge *PAX8 (exons 1-8+10)* does not match with *PAX8* splice variants described so far. The cellular milieu generated by chromosomal

instability allows for the acquisition of new mutations (Yousoufian & Pyeritz 2002), and the transcript *PAX8 (exons 1-8)-PPAR γ* , presented in normal thyroid tissue but not in PBL or thyroid carcinoma could also have arisen after exon 10 was lost from the *PAX8 (exons 1-8+10)-PPAR γ* rearrangement.

No genetic material was available from the patient's parents, making it impossible for us to test for *PAX8-PPAR γ* rearrangement and any potential transmission of the rearrangement from parents to patient. However, the lack of a family history of goiter or thyroid carcinoma and the fact that <20% of the FISH-analyzed nuclei bore a *PAX8-PPAR γ* rearrangement indicate that the mutation must be associated with a somatic mosaicism. If revertant mosaicism is disregarded, mosaicism can be associated only with a *de novo* mutation after fertilization in one of the cells of the embryo (Bernards & Gusella 1994). The presence of *PAX8-PPAR γ* rearrangement in tissues of endodermal and mesodermal origin such as those in the thyroid, PBL, and uterus indicates that the mutation occurred at an early post-zygotic stage.

Uterine leiomyomas occur frequently; however, the presence of a *PAX8-PPAR γ* rearrangement in the uterine leiomyoma of the patient in the current study raises the question of whether the two rearrangement events could be related, such that the *PAX8-PPAR γ* rearrangement caused the uterine leiomyoma. Pax8-null mice lack a functional uterus (Mittag et al. 2007), and PPAR γ activation inhibits the growth of primary cultures of human leiomyoma cells (Houston et al. 2003), indicating that both factors apparently play a role in uterine development and function. To date, the putative role of *PAX8-PPAR γ* rearrangement as a uterine tumor initiator has not been studied, however, and therefore remains hypothetical.

In conclusion, we have presented the first description of a patient with hyperthyroidism due to an angioinvasive FTC bearing the TSHR activating mutation, M453T, and a *PAX8-PPAR γ* rearrangement. The *PAX8-PPAR γ* rearrangement showed a mosaic distribution in tissues from both endodermal and mesodermal origins, which may indicate either a vertical transmission of the *PAX8-PPAR γ* rearrangement or one or more early embryonic mutation events. Since *PAX8-PPAR γ* and *TSHR-M453T* increased thyrocyte proliferation in cultures deprived of serum and growth factors but inhibited it when serum and growth factors are added, the activating TSHR mutation *in vivo* could promote FTC development in some *PAX8-PPAR γ* -positive thyrocytes under poor blood supply and restraint the tumor growth as soon as blood and growth factors are provided.

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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Author contribution statement

J Lado-Abeal and J de la Calzada attended the patient, and J Lado-Abeal designed the laboratory strategy to search the mutations and acted as a coordinator of the research. R Celestino, P Soares, and M Sobrinho Simoes designed the strategy, and performed the assay for searching the PAX8–PPARG rearrangement by FISH. I Castro and F Palos found the TSHR mutation, cloned the mutant and performed the functional analysis with TSHR mutant. M Sobrinho Simoes and J Cameselle Teijeiro performed the pathological analyses of the tumor. S A Bravo, M E R Garcia-Rendueles, and C V Alvarez performed transfection and western blot of NT-1 cells.

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