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

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Abstract

Our main objective was to search for mutations in candidate genes and for paired box gene 8–peroxisome proliferator-activated receptor gamma (PAX8–PPARγ) 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–PPARγ 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)–PPARγ was found in all tested patient’s tissues. A second rearrangement, PAX8 (exons 1–8)–PPARγ, was detected in the patient’s normal thyroid tissue. Under deprived medium condition, co-transfection of PAX8–PPARγ and TSHR–M453T dramatically increased the number of thyrocytes, an effect that was not observed with TSHR wild-type (WT); under complete medium conditions, co-transfection of PAX8–PPARγ 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–PPARγ rearrangements. PAX8–PPARγ was present as a mosaicism affecting tissues from endodermal and mesodermal origin. PAX8–PPARγ and TSHR–M453T
inhibited or promoted thyrocyte proliferation depending on medium conditions. The activating TSHR mutation could promote \textit{in vivo} FTC development in PAX8–PPAR\gamma-positive thyrocytes under poor blood supply with deprivation of growth factors but restrain 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, Niepomnisszce 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, Niepomnisszce 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 (Niepomnisszce et al. 2006).

Paired box gene 8 (PAX8)–peroxisome proliferator-activated receptor gamma (PPAR\gamma) rearrangement (Kroll et al. 2005) is highly prevalent in follicular thyroid carcinoma (FTC), correlating with an angioinvasive phenotype (Marques et al. 2002, Nikiforova et al. 2002). PAX8–PPAR\gamma rearrangement creates fusion transcripts, wherein the 5’ region of the PAX8 gene is fused in frame with exon 1 of the PPAR\gamma gene; the fusion protein resulting from PAX8–PPAR\gamma transcripts is believed to play a role in thyroid oncogenesis (Reddi et al. 2007). PAX8–PPAR\gamma 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\gamma rearrangement, PAX8 (exons 1–8+10)–PPAR\gamma (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\gamma (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).

\textit{In vitro}, co-transfection of PAX8–PPAR\gamma 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 was not observed in cell co-transfected with PAX8–PPAR\gamma 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\gamma and TSHR–M453T or TSHR–WT blocked cell proliferation. These results indicate that the effect of PAX8–PPAR\gamma 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 μU/ml (normal range 0.41–4.94 μU/ml), free thyroxine (T₄) 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 endotheoracic 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 ⁹⁹m⁹Tc-percetnephate thyroid scan showed an area of hyperfunctioning tissue in the left thyroid lobe with endotheoracic 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 ¹³¹I 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-T₄ treatment; two years after diagnosis and initial treatment, she is doing well, with negative ¹³¹I total body scans, performed after withdrawal of L-T₄, 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–PPARγ 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–PPARγ**

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,
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 PPARγ (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×SSC was co-denatured with the nuclear DNA at 80°C for 2 min. Detection of digoxigenin-labeled PPARγ probe was accomplished using an anti-digoxigenin fluorescein antibody (Roche Diagnostics GMbH) and a biotin-labeled PAX8 probe with CY 3–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–PPARγ 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 μg/ml insulin, 1 nM hydrocortisone, and 5 μg/ml transferrin) and 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 ng/ml amphotericin B).

pCR3.1 PAX8 (exons 1–8+10)–PPARγ 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

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

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody clone and source</th>
<th>Dilution</th>
<th>Pretreatment</th>
<th>Results thyroid tumor</th>
<th>Results normal thyroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid transcription factor-1</td>
<td>8G7G3/1, Dako</td>
<td>1:20</td>
<td>Water bath, 20 min</td>
<td>+n</td>
<td>+n</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>DAK-Tg6, Dako</td>
<td>1:2000</td>
<td>None</td>
<td>+c</td>
<td>+c</td>
</tr>
<tr>
<td>Thyroperoxidase</td>
<td>MoAb47, Dako</td>
<td>1:50</td>
<td>Microwave, 20 min</td>
<td>+c</td>
<td>+c</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>Polyclonal/BioGenex</td>
<td>1:5000</td>
<td>None</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Chromogranin A</td>
<td>DAK-A3, Dako</td>
<td>1:200</td>
<td>Microwave, 20 min</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Synaptophysin</td>
<td>SY38/BioGenex</td>
<td>1:1000</td>
<td>Microwave, 20 min</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cytokeratins 1, 2, 10, 11, 14, 15, 16, and 19</td>
<td>AE1/AE3, Dako</td>
<td>1:20</td>
<td>Microwave, 20 min</td>
<td>+c</td>
<td>+c</td>
</tr>
<tr>
<td>Cytokeratin 19</td>
<td>RCK108, Dako</td>
<td>1:100</td>
<td>Microwave, 20 min</td>
<td>+c*</td>
<td>+c*</td>
</tr>
<tr>
<td>Hector Battifora mesothelial cell</td>
<td>HBME-1, Dako</td>
<td>1:200</td>
<td>Water bath, 20 min</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Vimentin</td>
<td>V9, BioGenex</td>
<td>1:10 000</td>
<td>Microwave, 20 min</td>
<td>+c</td>
<td>+c</td>
</tr>
<tr>
<td>α-Estrogen receptor</td>
<td>6F11, Novocastra</td>
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<td>Water bath, 20 min</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>β-Estrogen receptor</td>
<td>Polyclonal, Santa Cruz, Biotechnology</td>
<td>1:50</td>
<td>Water bath, 20 min</td>
<td>+n*</td>
<td>+n*</td>
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<tr>
<td>Progesterone receptor</td>
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<td>Water bath, 20 min</td>
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<td>–</td>
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<tr>
<td>E-Cadherin</td>
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<td>Microwave, 20 min</td>
<td>+m</td>
<td>+m</td>
</tr>
<tr>
<td>β-Catenin</td>
<td>β-Catenin-1, Dako</td>
<td>1:300</td>
<td>Microwave, 20 min</td>
<td>+c</td>
<td>+c</td>
</tr>
<tr>
<td>Bcl-2 oncoprotein</td>
<td>124, Dako</td>
<td>1:5</td>
<td>Water bath, 20 min</td>
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<td>+m</td>
</tr>
<tr>
<td>Galectin-3</td>
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<td>+c, n</td>
<td>–</td>
</tr>
<tr>
<td>EGFR</td>
<td>EGFR pharmDx, Dako</td>
<td>Prediluted</td>
<td>Protease</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>SP4, Master Diagnostica</td>
<td>Prediluted</td>
<td>Microwave, 20 min</td>
<td>+n</td>
<td>–</td>
</tr>
<tr>
<td>P63 protein</td>
<td>4A4, Dako</td>
<td>1:10</td>
<td>Water bath, 20 min</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>p53 protein</td>
<td>DO-7, Novocastra</td>
<td>1:20</td>
<td>Microwave, 20 min</td>
<td>+n</td>
<td>–</td>
</tr>
<tr>
<td>P27</td>
<td>1B4, Novocastra</td>
<td>1:20</td>
<td>Water bath, 20 min</td>
<td>+n</td>
<td>n</td>
</tr>
<tr>
<td>PTEN protein</td>
<td>6H2.1, Dako</td>
<td>1:50</td>
<td>Water bath, 20 min</td>
<td>+c, n</td>
<td>+c, n</td>
</tr>
<tr>
<td>Ki-67</td>
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<td>Water bath, 20 min</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

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.
QuickChange II kit (Stratagene, La Jolla, CA, USA); the oligonucleotide used for the site-directed mutagenesis was 5'-GTCCTCCCCGTTTCTCAGTGCAACC-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 glutamate 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 trysinizing 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 PPARγ (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 ± 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...
BRAF mutations were found in the thyroid carcinoma nor in normal thyroid tissue.

A PAX8 (exons 1–8+10)–PPARγ (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)–PPARγ (exons 1–6) and PAX8 (exons 1–8+10)–PPARγ (exons 1–6), were found in the patient’s normal thyroid tissue, and one of these, PAX8 (exons 1–8+10)–PPARγ (exons 1–6), was also found in samples obtained from the patient’s PBL. FISH analysis further confirmed the presence of the PAX8–PPARγ 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–PPARγ 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–PPARγ rearrangement in nuclei extracted from the control thyroid specimens was 3.25% (range 1.50–5.37%). No PAX8–PPARγ 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–PPARγ, TSHR–WT, and TSHR–M453T on normal human thyroid cell proliferation in vitro are shown in Fig. 4. NT-1 cells express endogenous PPARγ (55 kDa), and additional expression of the chimeric PAX8–PPARγ 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–PPARγ had a strong
dose-related effect on NT-1 cell proliferation (Fig. 4B); 0.8 µg/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 µg/well of TSHR vectors, and *TSHR–M453T* had higher effect than *TSHR–WT* (Fig. 4B). However, co-transfection of *PAX8–PPARγ* (0.8 µg/well) with *TSHR–WT* or *TSHR–M453T* caused a dose-dependent inhibition of cell proliferation: cells co-transfected with 2 µg/well of *TSHR* vectors showed a proliferation similar to non-transfected cells, and those co-transfected with 0.8 µg/well of *TSHR* vectors had lower proliferation than cells transfected with *TSHR* vectors alone (Fig. 4B).

In deprived medium without TSH and growth factors, *PAX8–PPARγ*, *TSHR–WT*, or *TSHR–M453T* alone did not induce significant thyrocyte proliferation (Fig. 4C). However, co-transfection of *PAX8–PPARγ* with *TSHR–M453T* at 2 or 0.8 µg/well nearly replicates or duplicates respectively the number of NT-1 cells by day 4 (Fig. 4C). Co-transfection of *PAX8–PPARγ* and *TSHR–WT* induced an increase in cell number with the smaller amount of *TSHR–WT* (0.8 µg/well) but not with the higher amount (2 µg/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–PPARγ* 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, Spambalgi et al. 1996), and patients with McCune–Albright syndrome, a disease caused by activating mutations...
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 (Niepomniszcze et al. 2006). These findings led us to sequence RAS oncogenes, although without any positively correlating results.

We identified a PAX8–PPARγ rearrangement in our patient’s FTC. The PAX8–PPARγ 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–PPARγ rearrangement is highly prevalent in FTC (Suarez 2000, Nikiforova et al. 2002, Kroll et al. 2005), although the clinical importance of PAX8–PPARγ 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–PPARγ 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–PPARγ-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 3](https://www.endocrinology-journals.org/fig3.png)

**Figure 3** Representative results obtained in the FISH analysis for PPARγ and PAX8. Green dots are the BAC probes for PPARγ, 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–PPARγ rearrangement; B, nucleus extracted from tumoral tissue positive for the PAX8–PPARγ rearrangement; C, nuclei extracted from adjacent normal tissue negative for the PAX8–PPARγ rearrangement; D, nuclei extracted from PBL positive for the PAX8–PPARγ rearrangement.
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–PPARγ rearrangement and angioinvasiveness in follicular carcinomas (Nikiforova et al. 2002).

PAX8–PPARγ 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–PPARγ rearrangement in samples from the uterine leiomyoma surgically removed from the patient 15 years prior to her hospital admission reported here. These facts raise the question of the role of PAX8–PPARγ rearrangement in thyroid carcinoma development. Our results indicate that the presence of a PAX8–PPARγ 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–PPARγ 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–PPARγ showed a strong proliferative response in complete medium conditions but not in deprived medium conditions; accordingly, co-transfection of PAX8–PPARγ 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–PPARγ and TSHR–WT or TSHR–M453T under complete medium conditions or co-transfected with PAX8–PPARγ 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–PPARγ 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–PPARγ and TSHR–WT or TSHR–M543T 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–PPARγ transcripts were found in the patient’s normal thyroid tissue, PAX8 (exons 1–8)–PPARγ and PAX8 (exons 1–8+10)–PPARγ, whereas samples taken from the FTC and PBL showed only one transcript, PAX8 (exons 1–8+10)–PPARγ. The presence in normal thyroid tissue of two PAX8–PPARγ 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 (Youssoufian & Pyeritz 2002), and the transcript PAX8 (exons 1–8)–PPARγ, 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)–PPARγ rearrangement.

No genetic material was available from the patient’s parents, making it impossible for us to test for PAX8–PPARγ 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–PPARγ 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–PPARγ 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–PPARγ 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–PPARγ rearrangement caused the uterine leiomyoma. Pax8-null mice lack a functional uterus (Mittag et al. 2007), and PPARγ 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–PPARγ 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–PPARγ rearrangement. The PAX8–PPARγ rearrangement showed a mosaic distribution in tissues from both endodermal and mesodermal origins, which may indicate either a vertical transmission of the PAX8–PPARγ rearrangement or one or more early embryonic mutation events. Since PAX8–PPARγ 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–PPARγ-positive thyrocytes under poor blood supply and restrain 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–PPARγ 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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References


Camacho P, Gordon D, Chieffari E, Yong S, De Jong S, Pitale S, Russo D & Filetti S 2000 A Phe 486 thyrotropin receptor mutation in an autonomously functioning follicular carcinoma that was causing hyperthyroidism. Thyroid 10 1009–1012.


Niepomnisszche H, Suárez HG, Pitoia F, Danilowicz M, Manavela M, Elsner B & Bruno OD 2006 Follicular carcinoma presenting as autonomous functioning thyroid nodule and containing an activating mutation of the TSH receptor (T620I) and a mutation of the Ki-RAS (G12C) genes. *Thyroid* 16 497–503.


