Two somatic TSH receptor mutations in a patient with toxic metastasising follicular thyroid carcinoma and non-functional lung metastases

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Abstract

In a 59-year-old patient, thyroid follicular cancer was diagnosed in two right-sided toxic thyroid nodules, which had presented clinically as unilateral thyroid autonomy. In addition, the patient had histologically proven lung metastases of thyroid cancer; however, these failed to exhibit iodine uptake and were resistant to radioiodine treatment. The functional activity of the thyroid nodules prompted us to screen for TSH receptor (TSHR) mutations, and the histological diagnosis of follicular carcinoma led us to search for the PAX8-PPARγ¹ rearrangement and mutations in the ras genes. Each thyroid nodule harboured a different TSHR mutation (large nodule, Asp633Tyr; small nodule, Phe631Ile). Presence of both mutations in one sample suggestive of local invasion of a thyroid carcinoma could not be demonstrated, although several specimens from different nodule locations were screened. Only the wild-type TSHR sequence was identified in the histologically normal left thyroid lobe, and no genetic alterations were found in the other investigated genes. No TSHR mutations were detected in the pulmonary metastases.

This is the first case report of a patient with toxic follicular thyroid carcinoma harbouring two different TSHR mutations and presenting with non-functional lung metastases.

Introduction

Gain-of-function mutations in the thyrotropin receptor gene (TSHR) have been identified in various clinical conditions, namely toxic thyroid nodules and subsets of toxic multinodular goitres caused by somatic TSHR mutations, in addition to autosomal dominant non-autoimmune hyperthyroidism and gestational thyrotoxicosis attributable to germline TSHR mutations (Parma et al. 1993, Paschke & Ludgate 1997). Constitutive thyrotropin receptor activation by these mutations confers TSH-independent thyroid hormone production and thyroid growth, resulting in hyperthyroidism and thyroid hyperplasia.

In addition, to date, somatic TSHR mutations have been reported in nine differentiated thyroid carcinomas, five of which mimicked the phenotype of toxic thyroid nodules (Russo et al. 1995, 1997, 1999, Spambalg et al. 1996, Camacho et al. 2000, Mircescu et al. 2000). We describe the first patient with metastasising toxic follicular thyroid cancer, harbouring two different somatic TSHR mutations.

Case report

A 59-year-old patient presented with a 4-year history of a toxic right-sided goitre and a recent event of cardiac failure and atrial fibrillation. His thyroid function tests showed subclinical hyperthyroidism (TSH < 0.01 mU/l, free triiodothyronine and free thyroxine (T4) in the upper normal range) while he was receiving carbimazole 20 mg/day. Ultrasonography demonstrated two thyroid nodules (3.5 · 2 · 5 cm, solid with calcifications, and 1.5 · 2 · 2 cm, solid) in the right lobe (34 ml) and a normal left thyroid lobe (2 ml). Increased uptake of technetium on scintiscan (6.76%) in the entire right lobe, with complete suppression of the left lobe, was compatible with two right-sided toxic nodules (Fig. 1). After recompensation of cardiac failure, thyroid surgery was planned.

However, a left-sided pulmonary lesion was noted on the preoperative chest X-ray and confirmed on computed tomography (CT) scan (Fig. 2). Because the patient had a long-standing history of heavy smoking, bronchoscopy was...
performed, whereby an additional left-sided endobronchial tumour (2 cm) and several suspicious mucosal lesions were found and harvested for biopsy. Histopathological analysis of these specimens gave the unexpected diagnosis of multiple metastases of follicular thyroid carcinoma.

The patient underwent total thyroidectomy and received ablative radioiodine therapy with 5.698 GBq (154 mCi) iodine-131 (TSH concentration 21.07 mU/l; serum thyroglobulin concentration 10.3 ng/ml; thyroglobulin antibodies 27 IU/l (normal range < 60 IU/l)). At day 5, a post-therapeutic total-body scan was performed, which showed uptake of radioiodine in the thyroid bed, but not over the lungs.

The macroscopic appearance of the thyroid specimen was that of a calcified nodule filling the right thyroid lobe, with compression of normal thyroid tissue at the margins and a separate right-sided small solid thyroid nodule. The isthmus and the left lobe were of normal size and appearance (Fig. 3).

Histological analysis of the thyroid specimen showed follicular neoplasia with capsule invasion, in addition to vascular invasion being present in both nodules in several locations, suggesting a diagnosis of follicular carcinoma in both nodules (Figs 3 and 4). Apart from the two nodules, only normal thyroid tissue was found in the remainder of the right lobe and the entire left thyroid lobe.

At 4 months follow-up, a diagnostic $^{131}$I total-body scan was performed (647.5 MBq (17.5 mCi) $^{131}$I) after withdrawal of L-T4 treatment (TSH concentration 26.5 mU/l). The scan was completely negative, as were serum thyroglobulin concentrations (< 0.3 ng/ml) measured at the same time.

At 8 months follow-up, the patient was generally well, with undetectable serum thyroglobulin concentrations while receiving TSH-suppressive L-T4 treatment. However, a repeat CT scan revealed persistence of the pulmonary lesions, with an unchanged appearance on bronchoscopy and the consistent finding of histologically thyroglobulin-positive pulmonary metastasis (Fig. 5). Subsequently, the patient underwent extensive thoracic surgery to remove all macroscopically visible thyroid metastases.

**Materials and methods**

**DNA extraction**

Genomic DNA was isolated from the two thyroid nodules, from surrounding normal thyroid tissue of the right lobe, and left lobe normal thyroid tissue, using a Qiagen Tissue Kit according to the manufacturers’ instructions. In addition, DNA was extracted from the paraffin-embedded tissue sec-
tion of the lung metastases (Fig. 5). Thus paraffin-embedded tissue sections 20 µm thick were deparaffinized in xylol, rehydrated in an alcohol series, air-dried, and resuspended in 100 µl lysis buffer (500 mM Tris-HCl 10 mM NaCl, 20 mM EDTA, 1% SDS, pH 8.9). DNA extraction was performed at 56 °C by the addition of 1 µl proteinase K (50 mg/ml) and then following the Qiagen Tissue Kit procedure.

RNA extraction
Total RNA was extracted from the two nodules and normal tissue using the method of Chomczynski & Sacchi (1987). Complementary DNAs were prepared by reverse transcription of 1 µg total RNA in a 20 µl reaction mixture containing 5× first-strand buffer (50 mM Tris-HCl (pH 8.3), 375 mM KCl and 15 mM MgCl₂, 0.5 mM dNTPs, 5 mM dithiothreitol), 0.15 U RNase inhibitor, 2.5 µM oligo dT primer and 2 U MMLV reverse transcriptase (all reagents from Promega). cDNA synthesis was performed at 42 °C for 1 h, followed by an incubation at 95 °C for 10 min (MG Research Cycler).

PCR, denaturing gradient gel electrophoresis and sequencing
Exons 9 and 10 of the TSHR gene and exons 1 and 2 of the H- and K-ras genes were amplified from genomic DNA by PCR (Führer et al. 1997, Krohn et al. 2001). Screening for TSHR mutations was performed by denaturing gradient gel electrophoresis (DGGE) as described previously (Trulzsch et al. 2001). TSHR PCR fragments with abnormal gel shifting patterns and ras PCR products were sequenced on both strands using the respective PCR primers as sequencing primers.
primers and the BIGDye terminator sequencing chemistry (PE Applied Biosystems, Foster City, USA) according to the manufacturer’s instructions. Analysis of the sequencing reactions was performed on an automatic 377 ABI sequencer.

Screening for the presence of the PAX-8/PPARγ fusion gene in the thyroid nodules was carried out by PCR (initial denaturation at 95 °C for 3 min; 30 cycles of 30 s at 95 °C, 30 s at 56 °C, 1 min at 72 °C; final elongation step at 72 °C for 6 min) using primers that span all four known PAX8-PPARγ rearrangements (Kroll et al. 2000): PAX8 forward primer: 5′-GCA ACC TCT CGA CTC ACC AG-3′ and PPARγ reverse primer: 5′-CAA AGG AGT GGG AGT GGT CT-3′. PCR reactions were performed in a 50 µl reaction mixture containing 1 µl cDNA, 10 mmol/l Tris-HCl (pH 8.3), 1.5 mmol/l MgCl2, 50 mmol/l KCl, 0.01% gelatin, 200 µmol/l deoxy-NTP and 1 U Taq Polymerase (InVitrogen) and were analysed by standard 1.5% agarose gel electrophoresis. A positive control (fusion of PAX8 exons 1–7 to PPARγ exons 1–6), which, using the same set of primers, yields a 238 bp fragment, was kindly provided by Professor T Kroll (Emory University School of Medicine, Atlanta, USA). Informed consent was obtained from the patient before analysis of the tissue samples.

The studies were approved by the local ethics committee at the University of Leipzig.

Results

The highly unusual finding of two functionally toxic thyroid nodules, both with a histology of thyroid follicular carcinoma, in one patient prompted us to screen for a number of possible genetic alterations.

First, the nodules were investigated for presence of gain-of-function TSHR mutations using DGGE (Fig. 6a) and sequencing. Each nodule was found to harbour a different somatic TSHR mutation: TSHR mutation Asp633Tyr (GAC/L50478TAC) was detected in the large nodule. This mutation, previously identified in toxic thyroid nodules, has been demonstrated to be constitutively active in vitro transfection experiments (Porcellini et al. 1994). In the small nodule, a novel amino acid exchange of phenylalanine for isoleucine (TTC/L50478ATC) was detected in residue 631 (Fig. 6b). Two other amino acid exchanges resulting in constitutive TSHR activation have previously been described in this codon in toxic thyroid nodules (F631C, F631L) and a patient with hereditary non-
Figure 4 Histology of the thyroid specimen. (a) Large nodule; (b) small nodule; (c) small nodule. Original magnification ×10 (haematoxylin & eosin stain). Invasion of the capsule is demonstrated in both nodules, consistent with the diagnosis of follicular carcinoma. Only normal thyroid tissue was found in the left thyroid lobe (not shown).
autoimmune hyperthyroidism (F631L) (Porcellini et al. 1994, Kopp et al. 1995). In contrast, only the wild-type (wt TSHR) sequence was found in both the right and left lobe normal thyroid tissue (not shown).

Secondly, we hypothesised that only one of the 'toxic thyroid nodules' was the follicular carcinoma exhibiting local invasion of the neighbouring toxic follicular adenoma. However, serial sections of the right thyroid lobe showed that the two nodules were completely separated from each other, without evidence of invasion. In addition, genomic DNAs extracted from five different locations within each nodule were investigated. DGGE was used for TSHR mutation screening because of its superior sensitivity for detection of heterozygous mutations in samples with varying degrees of normal and diseased tissue components (Trultzsch et al. 2001). However, although one or other of the two TSHR mutations was detected in every sample, the presence of both mutations in one sample could not be demonstrated.

Thirdly, we screened for mutations in the H- and K-ras genes, implicated in various thyroid pathologies and associated in vitro with a follicular phenotype (Bond et al. 1994, Wynford-Thomas et al. 1997, Gire & Wynford-Thomas 2000). However, no ras mutations were identified in either of the two nodules.

Fourthly, we investigated the presence of a PAX8-PPARγ1 fusion gene, previously reported as specific for follicular thyroid carcinoma (Kroll et al. 2000). Using primers, which allow detection of all four known PAX8-PPARγ1 rearrangements, we were unable to identify a PAX8-PPARγ1 fusion gene in the cDNAs prepared from both nodules.

Fifthly, we aimed to clarify the molecular origin of the lung metastases. Thus we extracted and sequenced genomic DNA from the initial transbronchial biopsy specimen that led to the diagnosis of metastasising thyroid cancer, but found only wt TSHR sequences. The same results were obtained when we later screened surgically removed lung tissue with macroscopically visible tumour infiltration.

**Discussion**

In this paper we present a patient with unilateral thyroid autonomy attributable to two right-sided toxic nodules, both
Figure 6 Molecular analysis. (a) Screening for TSHR mutation in exon 10 by DGGE. Heteroduplex formation was observed for the PCR products amplified from the two nodules (lane 1: small nodule; lane 2: large nodule) and a positive control with the T632I TSHR mutation (lane 6), but not for the PCR product amplified from left lobe normal thyroid tissue (lane 3) and the wt TSHR controls (lanes 4 and 5). (b) Subsequent sequencing of the PCR products led to the identification of TSHR mutation D633Y in the large nodule and TSHR mutation F631I in the small nodule. Only the wt TSHR sequence was found in the normal left lobe thyroid tissue (not shown).
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of which exhibited the histological features of thyroid follicular carcinomas (Fig. 4) and were first diagnosed through their lung metastases (Figs 2 and 5). Consistent with their functional activity, somatic TSHR mutations (D633Y and F631I) were identified in each nodule (Fig. 6b). Other genetic alterations, e.g. ras mutations and PAX8-PPARγ1 rearrangement, that could be expected to contribute to the phenotype of follicular thyroid carcinoma were not found.

To our knowledge, this is the first patient with differentiated toxic thyroid cancer harbouring two different TSHR mutations. In view of the unusual clinical, pathological and molecular findings, we were highly critical of this diagnosis. However, the diagnosis was established by: (i) demonstration of both capsular (Fig. 4) and vascular invasion in both nodules; (ii) repeated finding of lung metastases (Fig. 5) of thyroid follicular cancer, which exhibited weak thyroglobulin staining; (iii) failure to demonstrate local invasion of a follicular carcinoma into a hot thyroid nodule, by the absence of co-localisation of both TSHR mutations in one DNA sample, even though different locations within the nodules were investigated and DGGE was used as a highly sensitive method of screening (Fig. 6a). Because the patient is male, clonality analysis of the thyroid tumours was not possible; (iv) failure to detect, other than in the two nodules, any other lesions on either macroscopic inspection or histological analysis of the patient’s thyroid specimen.

The association of TSHR mutations with thyroid malignancy, described to date in nine thyroid carcinomas involving different histologies such as follicular, papillary and insular thyroid cancer, remains an unresolved molecular puzzle. Thus it is unclear whether (certain) TSHR mutations might hasten the onset of thyroid malignancy or, perhaps more likely, whether the occurrence of an activating TSHR mutation in a thyroid cancer could be the incidental result of an increased mutation rate resulting from another molecular alteration.

Activating TSHR mutations, which have been reported in 57–82% of hyperfunctioning thyroid nodules, are, according to general perception, invariably linked to benign thyroid disease (Parma et al. 1993, 1997, Krohn & Paschke 2001, Trulutzsch et al. 2001). In agreement with this, continuous cAMP activation by thyroid-specific expression of either the A2 adenosine receptor, gsp or cholera toxin A1 in transgenic mice has been shown to cause stimulation of differentiated thyroid growth and hyperthyroidism (Ledent et al. 1992, Michiels et al. 1994, Zeiger et al. 1997). However, foci of transformed thyrocytes have been described in old A2 adenosine transgensics (Van Sande et al. 1995) and, interestingly, transgensics with a mutant α2B-adrenergic receptor exhibited an aggressive phenotype of rapidly developing hyperfunctioning thyroid nodules, with frequent evolution towards metastasising thyroid malignancy, most probably as a result of dual pathway (cAMP and phosphokinase C) activation (Ledent et al. 1997). Constitutive stimulation of the phosphokinase C pathway has been described for a small number of TSHR mutations in vitro; however, these included only two of the nine TSHR mutations identified in ‘hot’ thyroid cancers (M486F and T632I; Van Sande et al. 1995). Finally, possibly the strongest argument against a constitutive TSHR mutation propagating evolution of thyroid cancer in humans is the fact that thyroid malignancy has been reported in only one among more than 150 patients of the 10 families and nine individuals with activating TSHR germline mutations described to date (Leclere et al. 1997, www.uni-leipzig.de/innerc/thsr).

Which hypotheses could then be established on the basis of the molecular findings in our patient, in particular for the recurrent lung metastases with absence of TSHR mutations, which are difficult to reconcile with TSHR mutation bearing primary thyroid malignancies?

First, there may initially have been one primary thyroid tumour, which did not harbour a TSHR mutation and for which we were unable to demonstrate a specific genetic alteration – for example, we did not identify ras mutations or a PAX8-PPARγ1 rearrangement. Hence, the occurrence of two different somatic TSHR mutations would represent secondary events conferring autonomous function exclusively to the two thyroid nodules. Besides the known genetic heterogeneity of primary tumours, a very high degree of genetic divergence in early disseminated tumour cells has recently been reported (Klein et al. 2002). Subsequently, this seems to be reduced with the emergence of clinically evident metastases, suggesting that clonal selection of cells leading to metastases takes place after dissemination. This remains speculative, but one could thus hypothesise that occurrence of activating TSHR mutations in such tumour cells provides a further growth advantage driving the manifestation of a ‘hot’ cancerous nodule.

Secondly, the patient could harbour a combination of benign and malignant lesions in his right thyroid lobe – that is, two (one hot, one benign) thyroid nodules, each with a different somatic mutation, and a third lesion representing the metastasising thyroid follicular cancer (with no TSHR mutation). However, on serial sections we did not observe invasion of a thyroid cancer into the two (hot) nodules.

Thirdly, this patient might have the unique characteristic of having possessed three different thyroid cancer cell lines (one without a TSHR mutation) from the outset. In this case, one can again only speculate as to the localisation of the thyroid cancer without a TSHR mutation. Interestingly, the small nodule, which presented as a homogenous solid tumour (Fig. 3), showed a predominance of wt TSHR over the F631I allele in all sequencing reactions (Fig. 6b), as opposed to the larger, macroscopically heterogeneous thyroid nodule with an almost equal distribution of wt TSHR and D633Y alleles (Fig. 6b).

Although we were not able to resolve the molecular puzzle in this patient, it is noteworthy that his other medical
history has, apart from arterial hypertension, been uneventful, and that there was no increased prevalence of cancer in the family history. We have since followed up this patient closely; at 24 months after initial diagnosis and 12 months after extensive lung surgery, he has remained well, with no signs of recurrent thyroid malignancy.

In summary, we describe the first patient with metastasising toxic follicular carcinoma harbouring two different somatic \textit{TSHR} mutations in the \textit{TSHR} gene and presenting with non-functional pulmonary metastases.

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