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ORIGINAL ARTICLE

Identification and characterization of four *PAX8* rare sequence variants (p.T225M, p.L233L, p.G336S and p.A439A) in patients with congenital hypothyroidism and dysgenetic thyroid glands

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Summary

Context Thyroid dysgenesis may be associated with mutations in the paired box transcription factor 8 (*PAX8*) gene and is characterized by congenital hypothyroidism transmitted in an autosomal dominant mode.

Objectives The aim of this study was to identify new mutations in the *PAX8* gene. Sixty congenital hypothyroidism-affected individuals with dysgenetic (agenesis, ectopia and hypoplasia) and eutopic thyroid glands were studied.

Methods The 12 exons of the *PAX8* gene along with their exon–intron boundaries were amplified from genomic DNA and a mutational screening was performed by single-strand conformational polymorphism (SSCP) followed by direct sequencing of samples with abnormal migration patterns. The *PAX8* mutations were functionally characterized by transient transfection experiments.

Results Molecular analysis of the *PAX8* gene indicated that four affected individuals had four sequence differences: three novel variations [c.699C>T (p.L233L), c.1006G>A (p.G336S) and c.1317A>G (p.A439A)] and one recently reported [c.674C>T (p.T225M)], whereas the 56 remaining patients showed only wild-type alleles of *PAX8*. p.T225M, p.L233L and p.G336S variants were not detected in 530 chromosomes from 265 subjects randomly selected from the general population, whereas the p.A439A variant was identified in only one of the 530 chromosomes analysed. Functional analysis of the nonsynonymous substitutions showed that the p.T225M and p.G336S proteins had not lost their ability to bind a specific DNA sequence and to activate the transcription of the thyroglobulin (*TG*) promoter in synergy with thyroid transcription factor 1 (TTF1).

Conclusions We report the occurrence of two nonsynonymous substitutions, one recently reported (p.T225M) and one novel (p.G336S), and two novel synonymous substitutions (p.L233L and p.A439A) in the *PAX8* gene. p.T225M and p.G336S are rare sequence variants or may act by inhibiting an unknown particular function. Our study also confirms the very low prevalence of *PAX8* mutations in thyroid dysgenesis.

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Introduction

Congenital hypothyroidism affects about one in 3200 newborns and is characterized by elevated levels of TSH resulting from reduced thyroid function.[1] Patients with this disease can be divided into two groups: those with inborn errors of thyroid hormone biosynthesis (dyshormonogenesis) and those with thyroid developmental defects (dysembryogenesis or thyroid dysgenesis). The presence of dyshormonogenesis, which accounts for the 15% of the cases, has been linked to mutations in the Na^+/I^- symporter (*NIS*),[2] thyroglobulin (*TG*),[3] thyroperoxidase (*TPO*),[4] *DUOX2* (also known as *ThOX2* or *LNOX2*)[5] and *Pendrin* (*PDS*, also known as *SLC26A4*) genes.[6] These mutations produce a heterogeneous spectrum of congenital hypothyroidism with an autosomal recessive mode of inheritance. The thyroid dysgenesis group, which accounts for the remaining 85% of the cases, results from ectopic thyroid tissue at the base of the tongue or in any position along the thyroglossal tract, agenesis and hypoplasia.[7] In a minority of these patients, the congenital hypothyroidism is associated with mutations in the genes responsible for the development or growth of thyroid follicular cells: thyroid transcription factor 1 (*TTF1*, also known as *TITF1*, *NKX2.1* or *T/EBP*),[8–13] thyroid transcription factor 2 (*TTF-2*, also known as *TITF2*, *FOXE1* or *FKHL15*),[14–16] paired box transcription factor 8 (*PAX8*)[17–24] and TSH receptor genes.[25,26] More recently, mutations

in NKX2.5 were identified in patients with congenital cardiac defects and thyroid dysgenesis.[27] The majority of dysembryogenesis reported is sporadic. Vassart and Dumont,[1] considering knockout mice studies[28] and the discordance between monozygotic twins for thyroid dysgenesis,[29] suggested that epigenetic mechanisms leading to stochastic variations in the expression of multiple loci might be responsible for the sporadic character of thyroid dysgenesis.

PAX8 is a member of the mammalian PAX protein family that plays an important role in thyroid cell development, when the thyroid bud evaginates from the floor of the pharynx.[30] PAX8 recognizes specific DNA sequences through a highly conserved element, the 128-amino-acid paired box domain.[31] In addition to its role during embryogenesis, it is expressed in the adult thyroid as a transcription factor of the *TG*,[32] *TPO*[33] and *NIS*[34] genes by binding to their promoter regions. PAX8 is encoded by a single gene of 60 kb located on chromosome 2q12–q14 and contains a 4-kb transcript sequence divided into 12 exons.[35–37] To date, 10 inactivating mutations in the *PAX8* gene have been identified and characterized in patients with thyroid dysgenesis.[17–24] The autosomal dominant transmission is well established, with variable penetrance and expressivity between individuals. The p.R31H,[17] p.R31C,[20] p.Q40P,[19] p.R52P,[24] p.S54G,[21] p.C57Y,[18] p.L62R[17] and p.R108X[17] mutations are located in the paired box domain of PAX8 and result in severe reduction in DNA-binding activity *in vitro*. The remaining two mutations identified showed normal binding affinity but are transcriptionally inactive, the p.S48F[23] is localized in the paired box domain, whereas the deletion in exon 7, c.989–992delACCC,[22] is located outside of this domain.

The present study reports a mutational analysis of the *PAX8* gene in a large cohort of congenital hypothyroidism-affected individuals with dysgenetic (agenesis, ectopia and hypoplasia) and eutopic thyroid glands. Single-strand conformational polymorphism (SSCP) and direct sequencing analysis of the polymerase chain reaction (PCR) products demonstrated four rare sequence variants. Two result in nonsynonymous substitutions, one of which has been reported recently [c.674C>T (p.T225M)][38] and one of which is novel [c.1006G>A (p.G336S)], whereas the remaining two variants are synonymous substitutions [c.699C>T (p.L233L) and c.1317A>G (p.A439A)].

Materials and methods

Patients

The molecular study comprised 60 selected unrelated paediatric patients with nongoitre congenital hypothyroidism, 53 had thyroid dysgenesis (26 agenesis, 17 hypoplasia, 10 ectopy) and seven had orthotopic thyroid glands of normal size. The diagnosis of hypothyroidism was established during the neonatal screening or the first year of life by high TSH with low total T4 (TT4). Antithyroid antibodies were not detectable in any of the patients. Thyroid morphology was described according to thyroid echography studies performed before the start of L-thyroxine treatment at recommended doses.

Written informed consent was obtained from the parents of the children involved in this study and the research project was approved by the institutional review board.

Laboratory testing

Serum TT4 level was determined by FPIA-AxSYM (Abbott Laboratories, Abbott Park, IL). Serum TSH concentration was measured by MEIA-AxSYM (Abbott Laboratories). Serum TG level and anti-TPO and anti-TG antibodies were determined by the IMMULITE system (Lambers, Gwynedd, UK).

SSCP analysis

SSCP was used to screen for the presence of mutations in each exon of the *PAX8* gene. Genomic DNA was isolated from peripheral blood leucocytes by the standard cetyltrimethylammonium bromide (CTAB) method.[39]

The complete coding sequence of the human *PAX8* gene, along with the flanking intronic regions, was amplified using the primers reported previously.[17,18] PCR was performed in 100 μl, using a standard 5X Colorless Go Taq Buffer (Promega, Madison, WI), containing 200 ng of genomic DNA, 200 μM of each deoxy (d)-NTP (dATP, dCTP, dTTP and dGTP), 1·5 mM $MgCl_2$, 2·5 U Taq polymerase (Promega) and 50 pmol of each forward and reverse primer. Samples were denatured at 95 °C for 3 min followed by 35 cycles of amplification. Each cycle consisted of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and primer extension at 72 °C for 1 min. After the last cycle, the samples were incubated for an additional 10 min at 72 °C to ensure that the final extension step was complete. The amplified products were analysed in 2% agarose gel.

The gel matrix for SSCP contained 10% polyacrylamide (29 : 1), with or without 10% glycerol. Samples were electrophoresed during 24 h at a constant temperature (4 °C). DNA was visualized by silver staining.

PAX8 *DNA sequencing*

Samples showing an aberrant pattern in SSCP analysis were purified by the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and then sequenced by using the Taq polymerase-based chain terminator method (*f*mol, Promega) or an automated sequencing system (ABI Prism 3100 DNA sequencer; Applied Biosystems, Weiterstadt, Germany). Both the sense and antisense strands were sequenced using the same *PAX8*-specific primers that were used in the amplification. The results were analysed using the PC gene (Intelligenetics, Geneva, Switzerland), DNASTAR (DNASTAR Inc., University of California-SF, CA) and Nucleotide BLAST (www.ncbi.nlm.nih.gov/BLAST) software programs.

Sequence variants were numbered according to *PAX8* mRNA reference sequences reported in GenBank (NM_003466). The A of the ATG of the initiator methionine codon is denoted nucleotide +1. The codon for the initiator methionine is codon 1.

Data analysis

For the silent mutations, the possible effect on exonic splicing enhancers was assessed using the RESCUE-ESE (http://genes.mit.edu/burgelab/rescue-ese/) and ESEfinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process = home) programs.

Wild-type and mutated PAX8 *cDNA constructs*

The *PAX8* cDNA was produced by reverse transcriptase (RT)-PCR from human thyroid mRNA using 5′-CACCAAGCTTGGATCCAG-GATGCCTCACAACTCCATCAGA-3′ and 5′-GGAATTCTACA-GATGGTCAAAGGCCGT-3′ primers. The RT-PCR product was cloned into the pcDNA6B/V5-His expression vector using BamHI and EcoRI restriction sites introduced in the primers to obtain the *PAX8*WT-pcDNA6 construct. The mutant clones were generated using the Quick Change II Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The *PAX8*T225M-pcDNA6 construct was obtained with 5′-GAAAGCACCTTCGCATGGATGCCTTCAGCCAG-3′ and 5′-CTGGCTGAAGGCATCCATGCGAAGGTGCTTTC-3′ primers, and the *PAX8*G336S-pcDNA6 construct was obtained with 5′-CTGCAGCAAGTCAGCTCCGGGGTCCC-3′ and 5′-GGGAC-CCCGGAGCTGACTTGCTGCAG-3′ primers. All final constructs were verified by direct DNA sequencing.

h*TG*prom-SEAP (human *TG* promoter) and *TTF1*-pcDNA3 constructs were gifts from Gilbert Vassart.[21] The human *TG* promoter was subcloned into pGL3 Basic vector using KpnI and XhoI restriction sites to obtain a construct encoding a Firefly luciferase under control of the *TG* promoter (h*TG*prom-Luc).

Cell culture, transfection, and luciferase assays

HeLa cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% foetal bovine serum, glutamine (2 mM), penicillin (10 U/ml) and streptomycin (100 μg/ml). Cells were split into 12-well plates the day before transfection and grown up to 80% confluence. Transfections were carried out with Effectene transfection reagent (Promega) following the manufacturer's instructions. *PAX8*WT-pcDNA6, *PAX8*T225M-pcDNA6 and *PAX8*G336S-pcDNA6 constructs were cotransfected together with the h*TG*prom-Luc reporter construct (400 ng/well), *TTF1*-pcDNA3 (30 ng/well) and p*RL*-TK (20 ng/well; Promega) containing the Renilla luciferase gene as an internal control vector.

The total amount of DNA transfected was kept constant by adding empty pcDNA6B/V5-His vector. *TTF1*-pcDNA3 was able to activate the *TG* promoter in a dose-dependent manner while PAX8 alone was unable to activate the *TG* promoter at the concentration studied. The amount of TTF1 was optimized to evaluate only PAX8 activity. Cotransfection experiments with wild-type and mutant *PAX8* were performed with various ratios of plasmids (100, 200 and 400 ng/well). Each concentration was tested in triplicate in two different experiments. Cells were harvested 36 h after transfection and Firefly and Renilla luciferase activities were measured in cell extracts using the Dual-Luciferase Reporter Assay System (Promega).

Results

SSCP analysis and direct sequencing of PAX8

All 12 exons of the *PAX8* gene, including the flanking intronic sequences, were screened by SSCP from 60 unrelated patients with nongoitre congenital hypothyroidism. Initial analysis of PCR products showed four different patterns of migration that were not detected in 100 chromosomes from 50 subjects randomly selected from the general population (Fig. 1a). Sequence analysis of these variants revealed two nonsynonymous and two synonymous substitutions, all in a heterozygous state (Fig. 1b,c).

Nonsynonymous substitutions

A recently reported cytosine to thymine transition at nucleotide 674 in exon 7 (c.674C>T), which replaces the threonine at codon 225 with a methionine (p.T225M),[38] was detected in patient 17 and a novel guanine to adenine transition at nucleotide 1006 in exon 9 (c.1006G>A), which replaces the glycine at codon 336 with a serine (p.G336S), was identified in patient 60.

Synonymous substitutions

A novel cytosine to thymine transition at nucleotide 699 in exon 7 (c.699C>T), which does not change the lysine at codon 233 (p.L233L), was detected in patient 44 and a novel adenine to guanine at nucleotide 1317 in exon 12 (c.1317A>G), which does not modify the alanine at codon 439 (p.A439A), was identified in patient 38.

To evaluate whether these four changes were present in the population in low frequency we extended our initial screening. Therefore, 430 new chromosomes from 215 subjects randomly selected from the general population were additionally analysed using SSCP methodology. p.T225M, p.L233L and p.G336S variants were not detected in 530 (initial and extended population screening) chromosomes analysed, whereas the p.A439A variant was identified in only one of the 530 chromosomes. The RESCUE-ESE and ESEfinder computer programs showed that all basic exonic splicing enhancers elements accompanying the c.699C>T and c.1317A>G synonymous substitutions are conserved. In addition, SSCP and sequencing analysis of the *PAX8* gene revealed two known single nucleotide polymorphisms, g.IVS2+24T>C (rs1867763) and g.IVS6+51C>G (rs4849186), and two new polymorphisms, g.IVS10+15G>A and a promoter nucleotide change G>A located at 530 bp upstream from the A of the ATG of the initiator methionine codon (g.–530G>A).

Clinical findings and segregation analysis

The four patients with the identified *PAX8* mutations have marked hypothyroidism, three of them presenting hypoplastic glands (patients 17, 38 and 44) and one (patient 60) agenesis. The results of the thyroid function tests are shown in Table 1. The parents, brother and sister of patient 17 and the mother of patient 60 have clinical and biochemical euthyroidism.

SSCP and sequencing analysis of members of the family of patient 17 showed that p.T225M was also present in the father, brother and sister. This variation was not detected in the mother. Similarly, SSCP and sequencing evaluation of the mother of patient 60 showed that p.G336S was present in one allele. Unfortunately, the father was not available for segregation analysis.

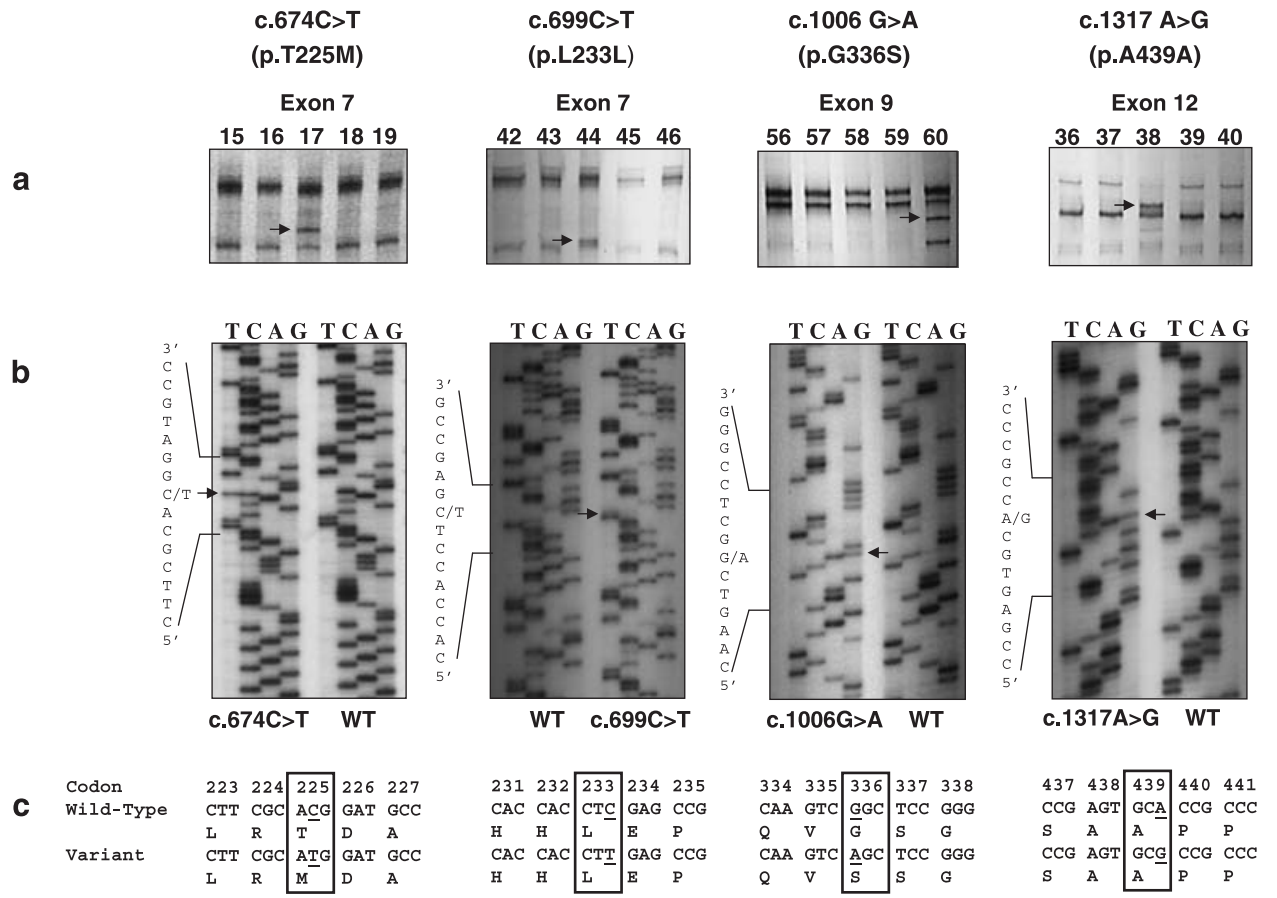

Fig. 1 SSCP screening and direct sequencing analysis. (a) SSCP screening of the *PAX8* mutations from 60 unrelated patients with nongoitre congenital hypothyroidism. Analysis of PCR products showed four different patterns of migration that were not detected in the control subjects. The arrows point to samples showing an aberrant pattern. (b) Partial *PAX8* DNA sequence (coding strands) from patients and control subjects (WT, wild-type). The arrows point to the nucleotide substitutions. (c) Partial nucleotide and deduced amino acid sequences from wild-type and variant *PAX8* gene. The nucleotide sequence is given in the upper line and the amino acid translation (represented by the single-letter code) is given below their respective codons. The nucleotide substitutions are underlined and the amino acids affected by the variations are boxed.

Table 1. Clinical, laboratory data and PAX8 variants identified in patients with congenital hypothyroidism and thyroid dysgenesis

Patient no.	Sex	Age at diagnosis	Serum TSH (mUI/l)	Serum TT4 (nmol/l)	Serum TT3 (nmol/l)	Serum TG (µg/l)	Thyroid echography	Variant	Exon
17	M	Neonatal screening	246	86·2	1·99	129	Hypoplasia	p.T225M	7
38	F	Neonatal screening	51·5	74·6	ND	57·9	Hypoplasia	p.A439A	12
44	M	Neonatal screening	371	38·6	ND	41	Hypoplasia	p.L233L	7
60	F	10 months	670	< 12·8	0·93	1·9	Agenesis	p.G336S	9
Reference range			2–5	132·5–200·7	2·45–3·07	11–92			

M, male; F, female; ND, not determined.
Conversion to conventional units: TT4, nmol/l ÷ 12·87 µg/dl; TT3, nmol/l ÷ 0·01536 ng/dl.

Transfection studies

To evaluate the functional relevance of the p.T225M and p.G336S mutations, we relied on the capacity of PAX8 to activate transcription. We investigated the ability of the p.T225M and p.G336S variants to activate transcription of a reporter gene under the control of the human TG promoter in synergy with TTF1.[40] HeLa cells were transfected with expression vector constructs encoding wild-type (*PAX8*WT-pcDNA6) or mutant *PAX8* (*PAX8*T225M-pcDNA6 and *PAX8*G336S-pcDNA6), together with TTF1-pcDNA3 and a reporter

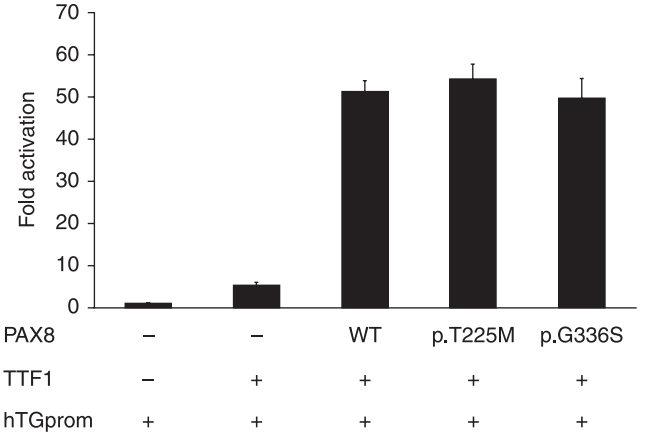

Fig. 2 Functional analysis of *PAX8*T225M-pcDNA6 and *PAX8*G336S-pcDNA6. HeLa cells were cotransfected with 200 ng of *PAX8* expression vector (*PAX8*WT-pcDNA6; *PAX8*T225M-pcDNA6; *PAX8*G336S-pcDNA6), along with 30 ng of *TTF1*-pcDNA3 and 400 ng of hTGprom-Luc. Promoter activity is shown as fold induction relative to the activity observed in the presence of reporter and empty vector. PAX8 alone was unable to activate TG promoter while TTF1 activated the promoter in a dose-dependent manner. The total amount of DNA for each transfection was kept constant adding pCDNA6B/V5-His. Values are the means of two independent experiments (in triplicate), SD values are shown. WT, wild-type.

gene construct containing the human *TG* promoter placed upstream of the coding sequence of Firefly luciferase (h*TG*prom-Luc) (Fig. 2).

Cotransfection of the *PAX8*WT expression vector produced up to 10-fold stimulation of luciferase activity compared to cotransfection with *TTF1*-pcDNA3 (Fig. 2). Cotransfection of *PAX8*T225M or *PAX8*G336S variants produced comparable stimulation of luciferase activity (Fig. 2). These observations led to the conclusion that the *PAX8*T225M and *PAX8*G336S proteins have not lost their ability to bind a specific DNA sequence and activate, in synergy with TTF1, transcription of the *TG* promoter.

Discussion

Differentiated thyroid follicular cells are characterized by the ability to synthesize the thyroid hormones, the iodothyronines T3 and T4, and by the expression of thyroid specific genes such as *TG* and *TPO*, which are exclusively expressed in this organ.[3] *TG* and *TPO* promoters are activated by three transcription factors, TITF1, FOXE1 and PAX8. Although none of these transcription factors are expressed only in the thyroid, their combination is unique to this gland.[41] Thyroid dysembryogenesis comprises a heterogeneous group of diseases with different pathogenesis. This type of primary congenital hypothyroidism is characterized by abnormal development of the gland.[7] The largest cohorts systematically screened for mutations in *PAX8* revealed that the mutation rate is very low.[17,18,22,24,42] In this study, all the 60 affected individuals had clinical and biochemical criteria suggestive for congenital hypothyroidism associated with dysgenetic (agenesis, hypoplasia or ectopy) or eutopic thyroid glands: hypothyroidism, elevated serum TSH with simultaneous low serum TT4 levels, inability to grow a goitre under strong stimulation by TSH and negative anti-TPO and anti-TG antibodies. Molecular analyses of the *PAX8* gene indicates that four affected individuals have four sequence differences, two nonsynonymous substitutions (c.674C>T, p.T225M; c.1006G>A, p.G336S) and two synonymous substitutions (c.699C>T, p.L233L; c.1317 A>G, p.A439A), whereas the 56 remaining patients show only wild-type alleles of *PAX8*.

Heterozygous inactivating mutations in the human *PAX8* gene cause congenital hypothyroidism with hypoplastic or ectopic hypoplastic glands, suggesting a dominant mode of inheritance. By contrast, heterozygous knockout mice with *PAX8* mutations do not exhibit a pathological phenotype whereas homozygous mice display severe thyroid hypoplasia.[43] The fact that the father, brother and sister of patient 17 are asymptomatic and carry the mutation p.T225M and that the unaffected mother of patient 60 carries the mutation p.G336S argues against the hypothesis that p.T225M and p.G336S participate in the pathogenesis of thyroid dysgenesis. However, it could be proposed, in agreement with previous studies,[1,17–24,29,42] that regulatory elements such as genetic and epigenetic factors could modulate the phenotypic expression in our families and be responsible for the variable penetrance or expressivity of the diseases. Pathogenic mechanisms that might be associated with thyroid development anomalies by heterozygous *PAX8* mutations and that might modulate the phenotypic expression in humans include: haploinsufficiency, monoallelic expression of either the normal or the mutant allele, maternal imprinting or dominant-negative mechanism.[17–19] Thyroid dysgenesis could be a multigenic condition. Alterations in other modifier-specific genes putatively involved in thyroid development and differentiation affecting the phenotype remain unknown. Of note, a deletion in the sonic hedgehog (*SHH*) gene was identified associated with hemiagenesis and ectopic development of the thyroid in mice.[44] Other possible explanations for intrafamilial discordance are epigenetic mechanisms that cause stochastic variations of gene expression at multiple loci or somatic mutations with a dominant effect in a thyroid development gene.[1]

PAX8 is a major gene in the regulation of the thyroid differentiated phenotype and plays an important role in the organogenesis of the thyroid. PAX8 contains a paired box domain (coded by exons 3 and 4), an octapeptide (coded by exon 5) and a residual paired type homeodomain (coded by exon 7).[45,46] The 128 amino acids between positions 9 and 137 show similarity to the Drosophila paired protein, a sequence-specific DNA-binding domain highly conserved in the human PAX protein family.[31] It consists of two structurally independent globular subdomains, each containing a helix–turn–helix motif joined by a linker region (Fig. 3). The N-terminal subdomain includes a short region of antiparallel β-sheet (β1 and β2 paired strands) followed by a type II β-turn, three α-helices and an extended C-terminal tail, connected with a linker to the C-terminal subdomain, which involves three α-helices.[31] An activating domain of the human PAX8 transcription factor is encoded by exons 10 and 11, whereas the sequence encoded by exon 9 has an antagonistic role for the activating domain.[45] The p.T225M and p.G336S substitutions are located in the C-terminal region of the *PAX8* gene outside of the paired domain (Fig. 3). p.T225M implies a neutral polar to neutral nonpolar amino acid change, whereas pG336S implies a neutral nonpolar to neutral polar amino acid change. The detrimental effect caused by the two synonymous substitutions identified in two

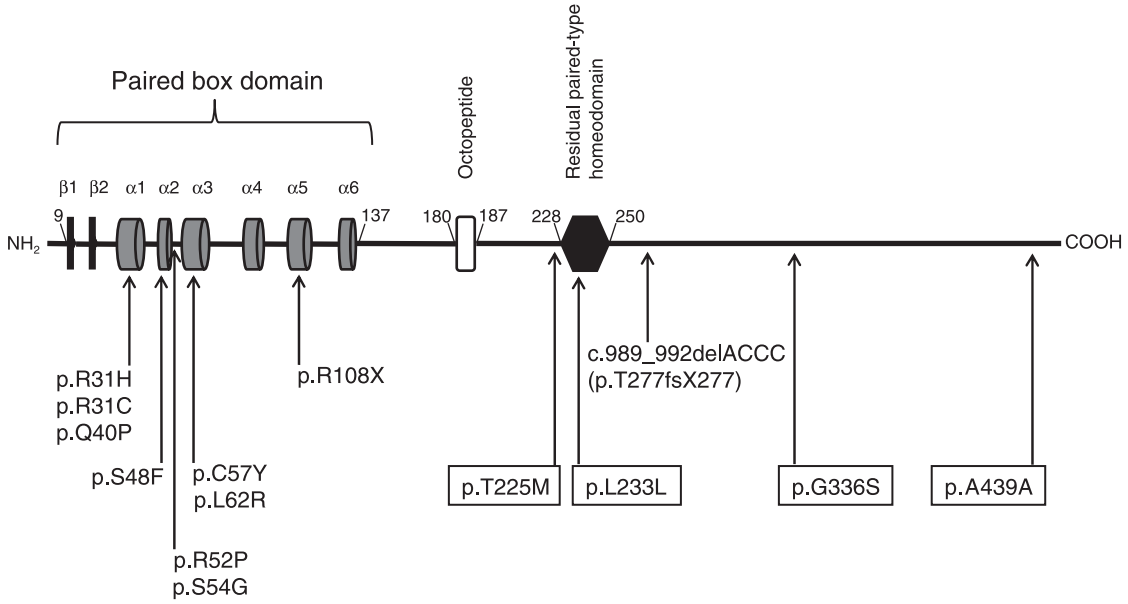

Fig. 3 Schematic representation of the human PAX8 protein domains and location of PAX8 inactivating mutations and nonsynonymous and synonymous substitutions. The paired box domain, octopeptide, residual paired-type homeodomain and the positions of the PAX8 inactivating mutations are shown.[17–24] The nonsynonymous and synonymous substitutions identified and characterized in this study are boxed.

congenital hypothyroidisms was not so significant. It has been reported that silent mutations located within *cis*-acting elements involved in splicing efficiency interfere with normal splice site selection.[47] However, all basic splicing *cis*-acting elements accompanying the c.699C>T and the c.1317A>G synonymous substitutions are conserved.

Except for one mutation, all *PAX8* inactivating mutations reported to date are located within the DNA-binding paired domain in the N-terminal subdomain (Fig. 3). The first *PAX8* gene mutation located outside the paired box domain was a heterozygous deletion, c.989–992delACCC, in exon 7, which causes a frameshift with a putative premature stop codon at amino acid 277.[22] The predicted mutant protein lacks the C-terminal region but contains the paired box domain, the octapeptide and the homeodomain. This protein retains the ability to bind a paired box domain sequence *in vitro* but is not capable of activating transcription from the reporter gene. These findings indicate that the C-terminal region is essential for transcriptional activity. The p.T225M and p.G336S variants, which are also located in the C-terminal region, were capable of exerting their normal transactivation effect when the mutant alleles were tested in cotransfection experiments with a *TG* promoter construct. During the completion of the present study, the same p.T225M variant was observed in a 15-year-old girl with thyroid ectopia and congenital hypothyroidism by Tonacchera *et al.*[38] Functional study of p.T225M in this patient also showed that the activation of the TG promoter is comparable with the activity of the wild-type protein. Based on the identification of the p.T225M substitution in two individuals with congenital hypothyroidism due to thyroid dysgenesis from two different cohorts, and the fact that the mutation has not been documented in any of 770 chromosomes of 385 normal subjects randomly selected from both studies, we hypothesized that the mutation is present at crucial positions of the *PAX8* gene resulting in a PAX8 protein that would not function normally. PAX8 protein interacts with several other transcription factors forming complexes on regulatory regions of target genes.[40] Consequently, it might be speculated that inactivating mutations, functionally relevant polymorphisms or rare variants located in the C-terminal region of the *PAX8*, such as p.T225M and p.G336S, could influence the normal thyroid development or the transcriptional control of several thyroid-specific genes, not tested by the present transient transfection assay with a *TG* promoter construct.

The occurrence in the same population of DNA sequence differences is traditionally described as a polymorphism, where more than one allele at a locus is present with a frequency of 1% or more. The structure, function and frequencies of DNA polymorphisms have been the subject of investigations for many years and several questions remain unanswered. The p.A439A variant was identified in only one of the 530 chromosomes analysed, whereas the p.T225M, p.L233L and p.G336S variants were not detected in the control population. According to the definition given above, these variations are not polymorphisms and could be called rare sequences variants.

In conclusion, we report the occurrence of two nonsynonymous substitutions, one recently reported (p.T225M) and one novel (p.G336S), and two novel synonymous substitutions (p.L233L and p.A439A) in the *PAX8* gene. p.T225M and p.G336S are rare sequence variants or, in a speculative fashion, may act in an inhibitory manner on an unknown particular function. The role of these variants remains to be investigated. Our study also confirms the very low prevalence of *PAX8* mutations in thyroid dysgenesis.

The identification and characterization of new inactivating mutations, polymorphisms and rare sequence variants in genes implicated in the organogenesis and regulation of the thyroid gland might be helpful for understanding the pathophysiology of this neonatal hypothyroidism.

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