

Extreme phenotypic variability of thyroid dysgenesis in six new cases of congenital hypothyroidism due to *PAX8* gene loss-of-function mutations

H E Ramos^{1,2,4,5,6}, A Carré^{1,2,3}, L Chevrier⁷, G Szinnai⁸, E Tron^{1,2,3}, T L O Cerqueira^{4,5}, J Léger⁹, S Cabrol⁹, O Puel¹⁰, C Queinnec¹¹, N De Roux⁷, L Guillot¹², M Castanet^{1,2,13} and M Polak^{1,2,3}

¹INSERM U1016, Université Paris Descartes, Sorbonne Paris Cité, Paris, France, ²Pediatric Endocrine, Gynecology and Diabetes Unit, Centre des Maladies Endocriniennes Rares de la Croissance, Hôpital Necker Enfants-Malades, AP-HP, Paris, France, ³IMAGINE Institute, Paris, France, ⁴Laboratório de Estudo da Tireoide (LET), Departamento de Biorregulação, Instituto de Ciências da Saúde, Universidade Federal da Bahia, Salvador, Bahia, Brazil, ⁵Curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa, Centro de Pesquisa Gonçalo Moniz – FIOCRUZ/BA, Salvador, Bahia, Brazil, ⁶Curso de Pós-Graduação em Processos Interativos de Órgãos e Sistemas, Instituto de Ciências da Saúde, Universidade Federal da Bahia, Salvador, Bahia, Brazil, ⁷INSERM U676, Paris Diderot University, Robert Debré Hospital, Paris, France, ⁸Pediatric Endocrinology, University Children's Hospital Basel, University Basel, Basel, Switzerland, ⁹Pediatric Endocrine Unit, Hôpital Armand Trousseau, AP-HP, Paris, France, ¹⁰Pediatrics Department, CHU, Bordeaux, France, ¹¹Pediatrics Department, CH de Cornouailles-Hopital Laennec, Quimper, France, ¹²Saint-Antoine Research Center, INSERM UMRS 938, Saint-Antoine Hospital, Université Pierre-et-Marie-Curie, Paris, France and ¹³Pediatrics Department, CH Charles Nicolle, University Hospital of Rouen, Rouen, France

Correspondence should be addressed to M Polak
Email
michel.polak@nck.aphp.fr

Abstract

Context: Within the last two decades, heterozygous loss-of-function *PAX8* mutations have been reported in patients with a wide degree of thyroid gland dysfunction and growth despite the presence of identical mutations.

Objectives: To search for *PAX8* mutations in a cohort of patients with congenital hypothyroidism (CH) and various types of thyroid gland defects.

Design: A cross-sectional study was conducted in a cohort of patients.

Setting: The French neonatal screening program was used for recruiting patients.

Patients: A total of 118 patients with CH, including 45 with familial and 73 with sporadic diseases, were included in this study. The thyroid gland was normal in 23 patients had hypoplasia, 25 had hemithyroid agenesis, 21 had athyreosis, and 21 had ectopy.

Results: We found four different *PAX8* mutations (p.R31C, p.R31H, p.R108X, and p.I47T) in ten patients (six patients with CH and four family members), two with sporadic and eight with familial diseases. Imaging studies performed in the index cases showed ectopic thyroid gland ($n=2$), hypoplasia ($n=2$), eutopic lobar asymmetry ($n=1$), and eutopic gland compatible with dysmorphogenesis ($n=1$). The previously reported p.R31C and the novel p.I47T *PAX8* mutations are devoid of activity.

Conclusion: Four different *PAX8* mutations were detected in six index patients with CH (ten total subjects). The p.R31C, p.R31H, and p.R108X mutations have been reported. The novel p.I47T *PAX8* mutation presented loss of function leading to CH. Thyroid ectopy was observed in two cases of *PAX8* (p.R31H) mutation, a finding that has not been reported previously. We observed a high inter-individual and intra-familial variability of the phenotype in *PAX8* mutations, underlining that population genetic studies for CH should include patients with various clinical presentations.

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Introduction

Over the last two decades, numerous heterozygous loss-of-function mutations affecting the gene for PAX8 have been identified in patients with congenital hypothyroidism (CH) (1, 2, 3). The PAX8 gene at 2q12–q14 is among the genes involved in thyroid gland development and, more specifically, in the proliferation and differentiation of thyroxine (T₄)-producing follicular cells (4, 5). PAX8 protein expression starts at the third gestational week in the human embryo and continues until adulthood (6, 7, 8, 9, 10). In thyrocytes, PAX8 contributes to proper timing of the switch from progenitor cell proliferation to the initiation of terminal differentiation programs marked by the expression of genes such as those encoding thyroglobulin, thyroperoxidase (TPO), and sodium/iodide symporter (8, 11).

Most patients with a PAX8 mutation demonstrate early thyroid growth defects. However, the onset and extent of thyroid dysfunction vary widely in these patients. Furthermore, thyroid gland ectopy, the most common cause of CH (12), has been reported in a single patient with a PAX8 mutation (1), suggesting that PAX8 may not play a crucial role in thyroid gland migration.

PAX8 mutations are usually located within the paired domain with only few mutants located outside (2). According to experimental studies that used reporter constructs containing consensus paired homeodomain-binding sites, some PAX8 mutations result in impaired DNA binding and loss of transcriptional activity (2). However, none of the mutation types or specific positions of the amino acid changes within the protein are associated with a specific clinical phenotype, and genotype–phenotype relationships have therefore been proved to be difficult to establish (2, 5). In addition, within some families having a single PAX8 mutation type, the thyroid gland features vary across patients (1, 10, 13, 14, 15, 16, 17, 18). Thus, the marked phenotypic variability among patients with CH may result, at least in part, from the effects of modifier genes, although environmental factors, epigenetic factors, or stochastic effects may also be important (19).

We conducted a cross-sectional study in 118 patients with CH, including 45 with familial and 73 with sporadic diseases. Thyroid morphology varied widely in these patients. We found four different PAX8 mutations in ten patients, two with sporadic and eight with familial diseases (two cases in each of four families).

Materials and methods

Patients

We enrolled 118 patients with CH, including 45 with familial and 73 with sporadic diseases (Supplementary Table 1, see section on supplementary data given at the end of this article), recruited from the French neonatal screening program and their family members. The thyroid gland was normal in size and position in 23 of these patients; 92 patients had hypoplasia ($n=25$), hemithyroid agenesis ($n=25$), athyreosis ($n=21$), or ectopy ($n=21$); and thyroid gland morphology was unknown in three patients. Two patients (A and C) were older at diagnosis and their phenotype in the neonatal period is unknown (Table 1). Informed consent was obtained from all patients or their families, and blood samples were collected. The study was approved by the Local Ethical Committee.

PAX8 sequencing

Genomic DNA was extracted from whole blood using the standard cetyltrimethylammonium bromide (CTAB) method. The coding region of the PAX8 gene, including exon/intron boundaries, was amplified by PCR, using the primers and conditions described previously (1, 20). PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen) and sequenced using the ABI PRISM Dye Terminator cycle sequencing Ready Reaction Kit (PE Applied Biosystems) according to the manufacturers' instructions. Sequences were analyzed using the Sequence Navigator Software (PE Applied Biosystems). Bidirectional sequencing was performed using an automated cycle sequencer (ABI Prism 3100, PE Applied Biosystems). Sequence alterations were examined in the context of the open reading frame to determine whether the alteration changed the corresponding amino acid. One hundred normal individuals were screened for the identified sequence alterations.

Mutagenesis

The mutations were introduced by site-directed mutagenesis (QuickChange, Stratagene, La Jolla, CA, USA) in hPAX8-pcDNA3 (given by G Vassart) using the sense primer 5'-GCCGGAAGTGGTCTGCCAGCGCATCGTAGACCTGGC-3' and antisense primer 5'-GCCAGGTC-TACGATGCGCTGGCAGACCACTTCCGGC-3' for the PAX8-p.R31C mutation and the sense primer 5'-AAGGCCCTGCGACACCTCTCGCCAGCTCCG-3' and

Table 1 Clinical features, laboratory data, and *PAX8* and *TSHR* gene mutations identified in ten patients belonging to a cohort of 118 patients with congenital hypothyroidism.

Patient	Sex	Age at diagnosis	Serum TSH ^a (mIU/l)	Thyroid ultrasound	Scintigraphy	PAX8 mutation
A	F	11 years	40	Hypoplasia	Hypoplasia with high cervical location	p.R31H
B	M	Neonatal screening	152	Agenesis	Dual thyroid ectopy	p.R31H
C	M	2 years		Agenesis	Hypoplasia	p.R31H
D	F	Neonatal screening	191	ND	Ectopy	p.R31H
E	M	Neonatal screening	236	Normal gland size and position, with lobar asymmetry	Hypoplasia	p.R31C
F	M	Neonatal screening	160	Hypoplasia	Orthotopic but dysplastic	p.R31C
G	F	ND	58	Normal gland size and position	ND	p.I47T
H	M	Neonatal screening	288	Hypoplasia	Normal	p.I47T
I	F	Neonatal screening	120	Hypoplasia	Normal	p.R108X
J	M	Neonatal screening	88	Hypoplasia	Normal	p.R108X

M, male; F, female; ND, not determined.

^aTSH at diagnosis; normal range for TSH: 2–5 mIU/l.

antisense primer 5'-CGGAGCTGGCGAGAGGTGTCG-CAGGGCCTT-3' for the *PAX8*-p.I47T mutation.

Cell culture and transactivation experiments

Human kidney epithelial cells (HEK293, ATCC (Manassas, VA, USA), and CRL-1573) were grown in DMEM supplemented with 10% fetal bovine serum (Biowest, Nuaille, France) and 1% penicillin and streptomycin (Invitrogen). For luciferase assays, we used a reporter gene construct containing *PAX8*-binding sites for the human *TPO* promoter (donated by G Vassart) upstream of the luciferase gene. We plated the HEK293 cells at a density of 2×10^5 cells/well in 24-well plates at 24 h before transfection. We transiently cotransfected cells with 520 ng of the reporter gene and 130 ng of h*PAX8*-pcDNA3, via 6 h of exposure to 3 μ l of lipofectamine in Opti-Mem medium (Invitrogen). After 48 h, the cells were harvested and the luciferase assay was performed. The mean \pm s.e.m. of four independent experiments, each performed in triplicate, was computed.

Western blot analysis

For western blotting, HEK293 cells (5×10^6) were plated on 100-mm diameter culture dishes at 24 h before transfection. Cells were transfected with 4 μ g of h*PAX8*-pcDNA3 with 20 μ l of lipofectamine. Supernatant proteins were

quantified at 48 h after transfection using the DC Protein Assay (Bio-Rad). Total protein, 15 μ g, was loaded on 10% SDS-PAGE and electroblotted onto a Hybond ECL membrane (Amersham Bioscience). The membranes were probed with an anti-*PAX8* antibody diluted 1:2000 (donated by G Vassart). The HRP-conjugated swine anti-rabbit antibody (Dako-Cytomation, Glostrup, Denmark) was used as the secondary antibody. Bound antibodies were revealed using a chemiluminescence kit (Amersham).

DNA-binding assay

Electrophoretic mobility shift assay (EMSA) was performed as described previously using a biotinylated labeled *TPO* (5'-CTGTCTAAGCTTGAGTGGGCATCA-3') probe derived from the corresponding promoter, as recommended by the manufacturer (21). For competition incubations, a 100-fold excess of cold probe was used. An unrelated protein (EBNA, Pierce, Rockford, IL, USA) was used to show specificity of the *TPO* DNA sequence.

Results

Genetic screening

Genetic screening identified ten patients (patients A–J) belonging to six families (families 1–6) exhibiting four

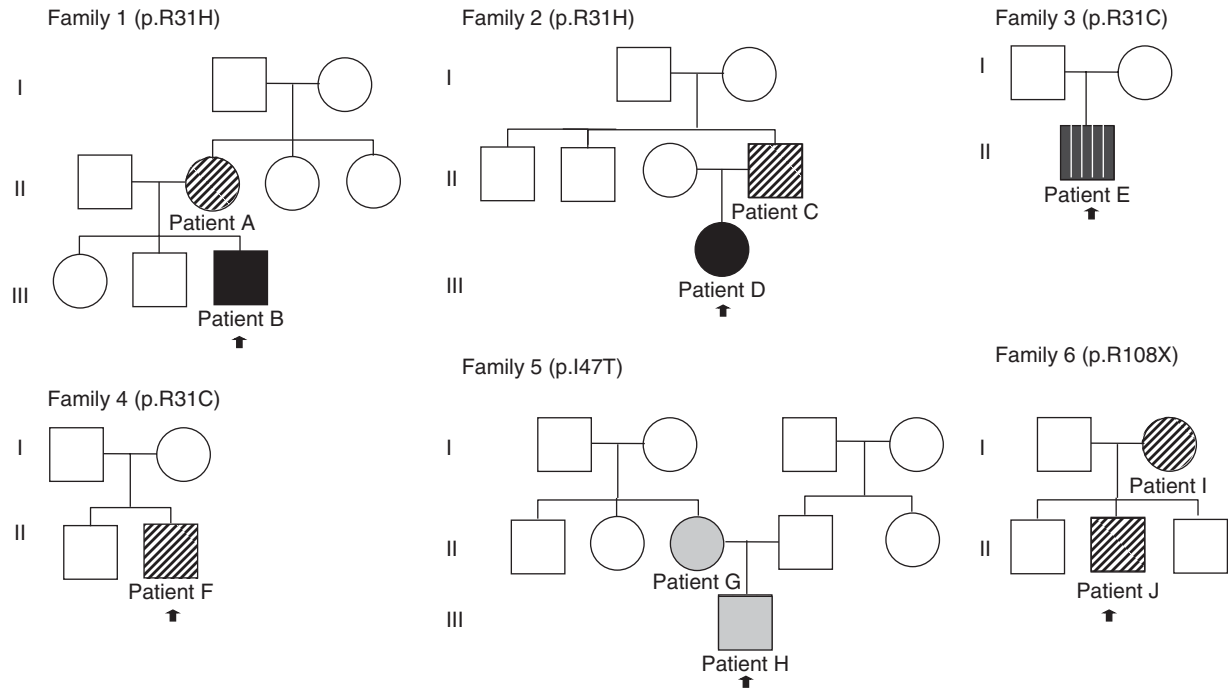


Figure 1

Pedigrees of the six families with *PAX8* mutations. All family members were analyzed. Squares, men; circles, women; open symbols, clinically unaffected individuals; solid black symbols, affected by hypothyroidism with ectopy or dual ectopy; solid hatched symbols; affected by hypothyroidism with thyroid

hypoplasia; solid gray symbol with vertical lines, affected by hypothyroidism with normal gland size and position but asymmetric lobes; solid gray symbol, normal size and orthotopic gland.

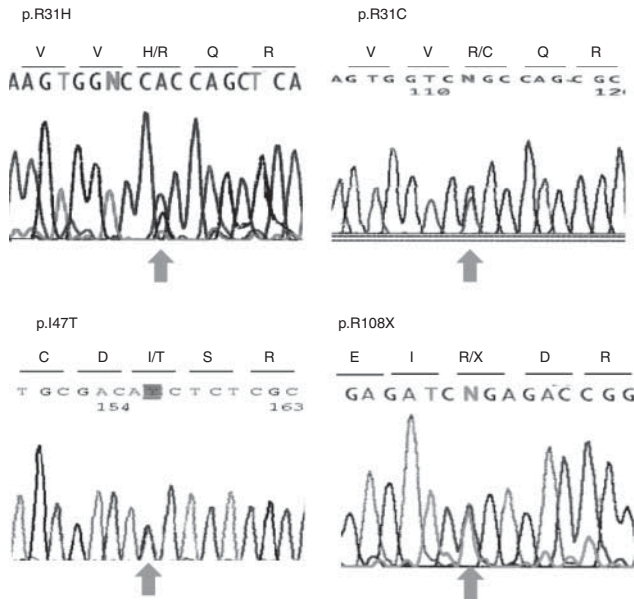
different *PAX8* mutations (one novel, p.I47T; and three known, p.R31H, p.R31C, and p.R108X) (Fig. 1) (1, 17). The novel heterozygous T-to-C transition at codon 47 of the *PAX8* gene-paired domain, detected in a boy and his mother (family 5), caused substitution of threonine for isoleucine (p.I47T) (Fig. 2). Of the ten patients, two had normal-sized thyroid glands in the normal position (G and H), five had thyroid gland hypoplasia (A, C, F, I and J), one (E) had lobar asymmetry with normal gland position, and two had thyroid ectopy or dual thyroid ectopy (B and D respectively) (Figs 1 and 3). All patients were also studied for mutation in known and novel candidate genes for thyroid dysgenesis (*TSHR*, *NKX2-1*, *NKX2-5*, and *HES1*).

Clinical phenotypes of patients with *PAX8* mutations

Family 1 ► In family 1, the mother (Fig. 1, patient A) was diagnosed with hypothyroidism at 11 years of age when she was evaluated for short stature. Her affected first son (Fig. 1, patient B) was detected by neonatal screening,

when the thyroid-stimulating hormone (TSH) level was 182 μ U/ml and the free T_4 (FT_4) level was 0.7 ng/dl. Both showed low I^{123} uptake by thyroid scintigraphy but had a severely hypoplastic gland (mother) or an undetected gland (son) by ultrasonography at diagnosis (Table 1). Re-evaluation at 3 weeks after withdrawal of levothyroxine replacement therapy showed abnormal high cervical position of the thyroid gland in the mother (Fig. 3A) and severe left lobar hypoplasia associated with high cervical ectopic right lobe in her affected son (Fig. 3B). Ultrasonography performed in the son to evaluate the kidneys was normal.

Family 2 ► In family 2, the proband (Fig. 1, patient D) was identified by neonatal screening. At the time of diagnosis, TSH was 291 μ U/ml (normal <20 μ U/ml) and FT_4 was 0.53 ng/dl (normal 0.8–1.9 ng/dl). A rudimentary sub-glossal ectopic thyroid gland was visualized along the thyroglossal tract by scintigraphy at 18 days of age (Fig. 3C). Ultrasonography of the kidneys performed

**Figure 2**

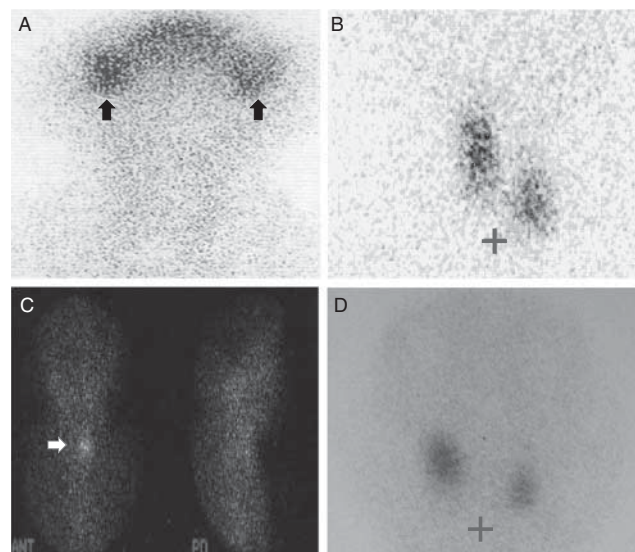
Chromatograms showing the mutation found in exon 3 of *PAX8* in the proband for each mutation. (Top left) Heterozygous A-to-G transition at codon 31 of the *PAX8* gene-paired domain causing substitution of a histidine residue with an arginine residue (p.R31H); (top right) heterozygous C-to-T transversion at codon 31 of the *PAX8* gene-paired domain causing substitution of a cysteine residue for an arginine residue (p.R31C); (bottom left) novel heterozygous T-to-C transition at codon 47 of the *PAX8* gene, causing substitution of threonine for isoleucine (p.I47T); (bottom right) heterozygous C-to-T substitution in the first position of arginine codon 108 of exon 3 leading to creation of a TGA stop codon predicting synthesis of an incomplete protein. Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-13-1006>.

subsequently was normal. The father (Fig. 1, patient C) had been diagnosed with CH at 2 years of age upon evaluation for short stature; no thyroid gland was visible by ultrasonography, but scintigraphy showed a severely hypoplastic thyroid gland in the normal position.

Family 3 ▶ Patient E (Fig. 1) was identified by neonatal screening, when the TSH level was 236 μ U/ml (normal <20 μ U/ml). Ultrasonography showed a normally sized and located thyroid gland and scintigraphy an asymmetric shape with the right lobe located cranially to the rest of the gland and a normal perchlorate discharge test (Fig. 3D). The kidneys were normal by ultrasonography and investigations for thyroid disease were normal in both parents.

Family 4 ▶ In family 4, the affected boy is the second child of healthy, nonconsanguineous parents. Neonatal TSH screening showed a TSH level of 155 μ U/ml (normal <20 μ U/ml) and an FT₄ level of 0.8 ng/dl. The child had jaundice and mild hypotonia. The parents were clinically unaffected, but biological investigations showed moderate TSH elevation in the father (TSH, 6.2 μ U/ml and FT₄, 1.0 ng/dl). Thyroid hormone replacement therapy was started at 13 days of age.

Family 5 ▶ In family 5, the proband (Fig. 1, patient H) was identified by neonatal screening, when the TSH level was 288 μ U/ml (normal <20 μ U/ml) and the FT₄ level was 0.5 ng/dl. Ultrasonograms showed thyroid hypoplasia and agenesis of the left kidney. The perchlorate discharge test was normal. Familial investigations found that the mother (patient G) had asymptomatic nonautoimmune hypothyroidism (TSH, 57.9 μ U/ml and FT₄, 0.6 ng/dl) and harbored the same novel mutation (p.I47T) but no

**Figure 3**

Phenotype variability demonstrated by scintigraphy in four patients with *PAX8* mutations. (A) Patient A: thyroid gland located high in the neck. Black arrows showing the salivary glands; (B) patient B: dual thyroid ectopy with hypoplastic orthotopic tissue; (C) patient C: rudimentary ectopic thyroid gland located along the thyroglossal tract (white arrow); and (D) patient D: normal gland size and position but asymmetric shape with the right lobe located cranial to the rest of the gland. +, sternal furcula. Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-13-1006>.

unilateral kidney agenesis on ultrasound. Thyroid hormone replacement was started.

Family 6 ▶ In family 6, both affected patients (Fig. 1, patients I and J) were identified by neonatal screening and had thyroid gland hypoplasia by ultrasonography.

Transactivation studies

To evaluate the functional relevance of the mutations, we relied on the capacity of *PAX8* to activate transcription from the *TPO* promoter (10). First, we used EMSA to assess the ability of the mutated proteins to bind to target DNA sequences. Both mutated proteins exhibited binding to the *TPO* promoter such as WT *PAX8* (Fig. 4A). Binding was abolished by an excess of unlabeled target DNA sequence (cold probes), indicating that the interactions were specific. The DNA sequences did not bind unrelated protein extracts (EBNA) (Fig. 4B). We tested the ability of the R31C and I47T *PAX8* mutants to activate transcription of a reporter gene under the control of the human *TPO* promoter. WT *PAX8* exhibited a greater activity than the empty vector (2.0-fold, $P=0.01$). By contrast, transcription was not significantly induced by transfection with the R31C or I47T mutated *PAX8* genes (Fig. 4D). These results were confirmed through cotransfection of WT or mutated *PAX8* gene with *NKX2-1* (Fig. 4D). The previously studied missense mutation p.R31H had demonstrated a failure to activate reporter gene transcription and was therefore not included in our functional studies (1). Western blotting of WT and mutated proteins transiently expressed in HEK293 cells revealed no difference in the synthesis or efficiency, indicating that the amino acid substitution does not cause destabilization of the mutant proteins (Fig. 4C).

Discussion

We report the results of *PAX8* mutation screening in 118 patients with CH and their family members. To date, 16 *PAX8* mutations and four *PAX8* variants have been described in familial and sporadic forms of thyroid dysgenesis (1, 2, 3, 9, 10, 13, 14, 15, 16, 17, 18, 22, 23, 24, 25, 26, 27, 28). Mutation frequencies differ markedly among affected transcription factor genes, suggesting regional and/or ethnic differences. In China, a recent population-based study has identified a *PAX8* mutation in only one of 300 tested patients (29). The fact that this study has essentially analyzed cases of normal-sized or confirmed thyroid dysgenesis and preferentially recruited

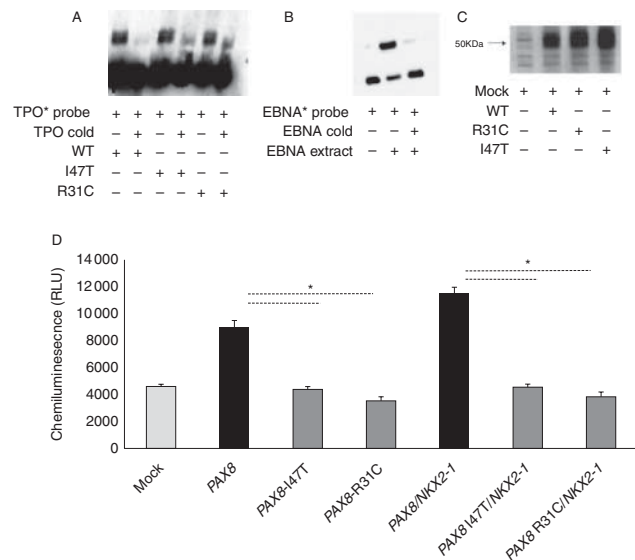


Figure 4

Functional study of the p.R31C and p.I47T *PAX8* mutations. (A) DNA-binding capacities of *PAX8*-p.R31C and *PAX8*-p.I47T on the *TPO* promoter. Nuclear extract of 2 μ g from cells transfected with WT or mutated *PAX8* were incubated with biotinylated *TPO* probes with (+) or without (-) a competitor (cold probe). (B) Unrelated protein extract (EBNA) from HEK293 cells was used as an internal control in the experiment. The nuclear extracts used together with EBNA* probe, EBNA cold, and EBNA extract were also *PAX8*-transfected cells. (C) The protein levels of WT *PAX8*, p.R31C *PAX8*, and p.I47T *PAX8* after transfection were measured by immunoblotting. Expression of the mutants was comparable with that of the WT gene. (D) Transfection experiments. Cotransfection of a human *TPO* luciferase reporter gene with WT *PAX8* consistently resulted in greater than twofold activation. By contrast, no significant activation occurred in cotransfection experiments with genes harboring the p.R31C or p.I47T *PAX8* mutation. These results were confirmed by cotransfection of *PAX8* with *NKX2-1* where there is no more synergy with *PAX8* mutants. Values are expressed as means of independent experiments (in triplicate) \pm s.d. Statistical analysis was performed using unpaired two-tailed Student's *t*-test to obtain the *P* value associated with the observed fold of activation differences ($P < 0.05$).

familial cases could have motivated a higher frequency of mutations.

Many types of *PAX8* mutations have been reported including nucleotide substitutions, deletions, and duplication (2, 23). Nonsynonymous mutations that affect the peptide code of a given mRNA contribute a minority of

reported sequence aberrations and only one of those with proven functional relevance, namely, the *PAX8*-T225M variant, which seems to impair coactivator recruitment (27, 30). Most patients with inactivating *PAX8* mutations had thyroid gland hypoplasia, although a minority had athyreosis or a normal-sized thyroid gland with normal function or compensated hypothyroidism (1, 2, 15, 31). This broad spectrum of expression, even within families, strongly suggests an influence on the phenotype of other factors such as the genetic background or stochastic phenomena, as well as a polygenic disease mechanism in some cases.

During development, the function of *PAX8* and its contribution to fine-tuning thyroid gland growth are regulated by alternative splicing, which generates isoforms that exhibit different transactivation properties but conserve regular DNA-binding characteristics (32). One patient harboring the p.R31H mutation had dual thyroid ectopy, indicating partial tissue migration (1, 17). The p.R31H mutation has been found in four other patients who had hypoplasia by ultrasonography and scintigraphy (one Italian, two Japanese, and one Chinese) (1, 17, 25, 29). Indeed, we found the same p.R31H mutation in four other patients with a variable phenotype (Table 1), including a dual thyroid ectopy along the thyroglossal tract (Fig. 3C). Another mutation, the p.R31C, has also been described previously in a kindred with apparent athyreosis (17). We have found this mutation in two unrelated families (3 and 4), each of which had a single affected individual; one of these two patients had a normal-sized gland with lobar asymmetry and the other had hypoplasia. We provide the first data on p.R31C function, which was characterized by impaired transactivation but normal DNA binding (Fig. 4A). The functional analyses for p.R31H and p.R108X have been published previously (1).

We identified a new mutation (p.I47T) in a kindred in which the proband had thyroid hypoplasia and left kidney agenesis. The *PAX8*/I47T mutated protein clearly retains the ability to bind DNA but is not able to efficiently activate transcription. This mutation within helix 2 of the paired domain might not be involved in contacts with the DNA but could affect the conformation of crucial C-terminal region known to be important for physical interactions with the basal transcriptional machinery (16, 33). The mutation was inherited from the mother, in line with the dominant model of inheritance proposed for the rare cases of familial thyroid dysgenesis due to *PAX8* mutations (2). This same loss-of-function mutation, in his mother, was presented as a nonautoimmune

hypothyroidism with a normal-sized thyroid. Although the mother was not screened for CH, her signs and symptoms of hypothyroidism were delayed until adulthood and the observed intra-familial variability included the kidney phenotype also. Possible explanations to this variability include incomplete penetrance of the p.I47T mutation, a polygenic etiology, or stochastic expression of *PAX8* alleles as documented for *PAX5* (34). Thyroid hypoplasia may be masked before replacement therapy initiation, as long-standing stimulation by high levels of serum TSH may increase the size of the gland. *PAX8* is expressed in the developing kidney, and our cohort included seven patients with unilateral kidney agenesis. However, among those with *PAX8* mutations, only patient H, harboring the p.I47T mutation, had unilateral kidney agenesis. The findings reported in this study support the idea that haploinsufficiency of *PAX8* can be marked by a high phenotypic variability, even within families.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-13-1006>.

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. Informed consent was obtained from the patient (or patient's guardian) for publication of the case report and accompanying images.

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