CLINICAL STUDY

Screening for mutations in transcription factors in a Czech cohort of 170 patients with congenital and early-onset hypothyroidism: identification of a novel PAX8 mutation in dominantly inherited early-onset non-autoimmune hypothyroidism

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Abstract

Objective: Mutations in *NKX2.1*, *NKX2.5*, *FOXE1* and *PAX8* genes, encoding for transcription factors involved in the development of the thyroid gland, have been identified in a minority of patients with syndromic and non-syndromic congenital hypothyroidism (CH).

Design: In a phenotype-selected cohort of 170 Czech paediatric and adolescent patients with non-goitre CH, including thyroid dysgenesis, or non-goitre early-onset hypothyroidism, *PAX8*, *NKX2.1*, *NKX2.5*, *FOXE1* and *HHEX* genes were analysed for mutations.

Methods: *NKX2.1*, *NKX2.5*, *FOXE1* and *HHEX* genes were directly sequenced in patients with syndromic CH. *PAX8* mutational screening was performed in all 170 patients by single-stranded conformation polymorphism, followed by direct sequencing of samples with abnormal findings. The R52P *PAX8* mutation was functionally characterized by DNA binding studies.

Results: We identified a novel *PAX8* mutation R52P, dominantly inherited in a three-generation pedigree and leading to non-congenital, early-onset, non-goitre, non-autoimmune hypothyroidism with gradual postnatal regression of the thyroid size and function. The R52P *PAX8* mutation results in the substitution of a highly conserved residue of the DNA-binding domain with a loss-of-function effect.

Conclusions: The very low frequency of genetic defects in a population-based cohort of children affected by non-goitre congenital and early-onset hypothyroidism, even in a phenotype-focussed screening study, suggests the pathogenetic role of either non-classic genetic mechanisms or the involvement of genes unknown so far. Identification of a novel *PAX8* mutation in a particular variant of non-congenital early-onset hypothyroidism indicates a key function of *PAX8* in the postnatal growth and functional maintenance of the thyroid gland.

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Introduction

Primary congenital hypothyroidism (CH) is the most frequent inborn endocrine disorder (1). It can be caused by impaired thyroid development leading to a variable degree of thyroid dysgenesis (TD; OMIM 218700) or by dyshormonogenesis (OMIM 274400–274900) when any step of thyroid hormone biosynthesis can be affected. While dyshormonogenesis has been recognized as a genetic disorder mostly with autosomal recessive inheritance due to mutations in genes critical for thyroid hormone synthesis, the molecular pathogenesis of TD representing about 80% of CH cases (2) is still

unsolved (reviewed by (3)). In general, TD might be considered as a non-genetic condition due to its mainly sporadic occurrence and only 2% of familial cases (4). In addition, a significant female predominance (5) as well as the discordance of monozygotic twins (6) argues against a classic Mendelian inheritance of TD. However, in few cases, a recessive inheritance of thyroid hypoplasia and CH was described due to mutations in the thyroid-stimulating hormone (TSH)-receptor, which proves, in general, the potential of genetic alterations in thyroid development. Apart from mutations in the TSH-receptor that is expressed during later steps of thyroid development, transcription factors expressed during

early steps of thyroid budding and migration are other likely candidate genes for TD, in particular *Nkx2.1/Ttf1* (7), *Foxe1/Ttf2* (8), *Pax8* (9) and *Hhex* (10). TD in homozygous knock-out mice for *Nkx2.1/Ttf1* (11), *Foxe1/Ttf2* (12), *Pax8* (13) and *Hhex* (14) revealed the critical role of these genes for normal thyroid development. Interestingly, except in *Pax8*^{-/-} mice, the rest of the null mutations had major malformations of other organs representing the extrathyroidal expression pattern of the respective transcription factors.

Based on the phenotype of the knock-out mice, mutations of *FOXE1* (15–17) and *NKX2.1* (18) were identified in several patients with syndromic forms of CH. While patients with *FOXE1* mutations are affected by cleft palate in addition to TD (Bamforth–Lazarus syndrome (19), OMIM 241850), patients with *NKX2.1* mutations suffer from choreoathetosis. Mutations of *FOXE1* (OMIM 602617) and *NKX2.1* (OMIM 600635) in non-syndromic CH have not been reported yet (e.g. (20)). More recently, mutations in *NKX2.5* (OMIM 600584), that is expressed both in the developing thyroid and heart (21) and which was found to be mutated in various congenital heart defects (e.g. (22)), were described in four patients with CH and TD, of whom one was also affected by heart defect (23). Most mutations in patients with TD were found in the *PAX8* gene (OMIM 167415) so far with some familial dominant or sporadic cases of mainly non-syndromic, non-goitre CH (24–31). Although *PAX8* is also expressed during renal development, knock-out mice do not manifest an overt kidney phenotype and an additional renal malformation has been described only in two patients (28, 31). Mutations in the human *HHEX* gene (OMIM 604420) have not yet been identified.

Taken together, these data imply that some genetic defects in transcription factors expressed during early steps of thyroid development can be present in a given cohort of patients with TD, besides the majority of cases with a non-Mendelian genetic defect. To describe the incidence of these mutations, especially in the case of

thyroid transcription factors within a population-based context, we have screened a cohort of 170 patients mostly diagnosed by the Czech nationwide neonatal screening for CH. The screening was focussed on those phenotypes that had been described previously in other large studies investigating each candidate gene separately. In our phenotype-based mutation screening, we investigated *PAX8* in all patients with non-goitre congenital or early-onset hypothyroidism, as well as *NKX2.1*, *NKX2.5*, *FOXE1* and *HHEX* in several particular patients with CH and associated malformations. We have identified a novel loss-of-function mutation of the *PAX8* gene (R52P) causing non-congenital early-onset hypothyroidism dominantly inherited in three generations of one family.

Patients and methods

Patients

One hundred and seventy Czech Caucasian paediatric and adolescent patients with permanent primary non-autoimmune, non-goitre hypothyroidism diagnosed in infancy or early childhood were included in this study. One hundred and sixty-three probands were identified within the frame of the Czech nationwide neonatal screening programme for CH during the period from 1985 to 2002 (the assessment of total thyroxine (tT₄) by RIA during 1985–1995, since 1996 TSH levels measured by DELPHIA on dry blood spots from a heel prick, TSH cut-off 15 mIU/l). Except for six children with compensated hypothyroidism, all the cases were characterized by high TSH and low fT₄ and/or tT₄ levels. Five patients were born before routine neonatal screening for CH was established. Two patients had negative screening results but developed early-onset, non-goitre hypothyroidism. With regard to the thyroid morphology determined before the initiation of T₄ treatment, the cohort was composed as shown in Table 1. The classification is primarily based on

Table 1 Thyroid morphology and sex ratios in the examined group of patients.

Thyroid dysgenesis ^a					Normal thyroid ^b	N.A. ^c
Athyreosis	Ectopy	Hemithyroid	Hypoplasia	Rudiment		
26	8	1	36	39		
		110			39	21
		F/M=2.5			F/M=1.4	F/M=4.2
Total 170 (F/M=2.3)						

Thyroid morphology was described according to thyroid imaging studies (ultrasonography or scintigraphy) performed before the beginning of T₄ treatment. ^aThe distribution of various forms of TD and low number of ectopies can be attributed to the image technique used – in most of the patients the description was based solely on ultrasound imaging. Scintigraphy was performed just in the early beginning of CH screening and it was done only in a minority of patients (all cases of ectopy were diagnosed by scintigraphy). According to ultrasound findings, a eutopic thyroid gland with a size under normal age and sex limits but with normal structure was described as ‘hypoplasia’. The term ‘rudiment’ was used if only eutopic remnants of thyroid tissue with a high degree of fibrous rearrangement were visible by ultrasound (corresponding to hyperechogenic ultrasound signals). ^bIn the group of patients with a eutopic thyroid gland of a normal size and structure, gradual postnatal regression of the thyroid size and function developed in four patients with initially compensated hypothyroidism and in one patient with negative screening results. ^cThyroid imaging before the beginning of T₄ treatment was not done or results are not available. No palpable goitre was observed in physical examination, but the thyroid morphology was not further characterized. N.A., not available; F/M, female-to-male ratio.

ultrasound studies, since ^{99}Tc scintigraphy was provided only for a small number of patients in the early phase of the screening programme.

One hundred and sixty-five patients were unrelated individuals. Additionally, the study cohort included one pair of dizygotic male twins and one pair of dizygotic female twins with their sibling (whose mother had adult-onset hypothyroidism; Fig. 1A and B). Another 22 individuals had a positive family history of variable thyroid disorders: one boy with early-onset hypothyroidism had a hypothyroid mother and grandmother, three girls with TD and CH in older brothers, one boy with TD and CH in his father, one boy with hemithyroid and euthyroid had a mother with hemithyroid, two boys and four girls with TD and mothers with adult-onset hypothyroidism, and 10 girls with TD and hypothyroidism in grandparents or siblings of parents (Fig. 1C–E).

The occurrence of associated malformations (Table 2) was obtained from medical records or referring physicians. Moreover, screening for kidney malformation by ultrasound was performed in 57 children with TD (hitherto, unknown renal malformations were described in three of them).

The study was approved by the institutional ethics committee of the 3rd Faculty of Medicine, Charles University, Prague. Informed consent had been obtained from all subjects or their parents.

Methods

DNA extraction

Gene analysis was performed with genomic DNA extracted from leukocytes taken from peripheral blood using a modified salting out method (32).

PCR conditions

PCR were performed using the Expand High Fidelity PCR System (Roche Diagnostics). PCRs were run in a Gene Amp PCR system 9700 cycler (PE Applied

Biosystems, Foster City, CA, USA). Negative controls were always included. PCR products mixed with a loading dye were run on a 1–1.5% agarose gel (Ultra Pure Agarose Gel, Life Technologies) stained with ethidium bromide and visualized under u.v. light (all primers and PCR conditions are available upon request).

Direct sequencing

For direct sequencing, PCR products were purified with the QIAquick PCR Purification Kit (Qiagen). Sequencing reactions were prepared using appropriate primers and the DNA sequencing kit – BigDye Terminator Ready Reaction Cycle Sequencing Kit (PE Applied Biosystems, Warrington, UK) – in a 10 μl volume under standard conditions. Sequencing was performed in both directions on an ABI PRISM 377 sequencer (PE Applied Biosystems). Sequence variations were nomenclatured and numbered according to Ref. (33).

Single-stranded conformation polymorphism (SSCP)

Two microlitres of PCR product were mixed with a 14 μl formamide stop solution (95% formamide, 10 mM NaOH, 0.1% bromophenol blue, 0.1% xylene cyanol, 10% DMSO), denatured for 5 min and immediately cooled on ice. PCR products were loaded onto a denaturing formamide gel (2.5 ml 95% formamide, 2.5 ml $2\times$ MDE, 250 μl $10\times$ TBE, 15 μl TEMED, 80 μl 10% APS) and run on a non-denaturing $1\times$ MDE gel (1.75 ml $10\times$ TBE, 17.5 ml $2\times$ MDE, 14 μl TEMED, 140 μl 10% APS, 16 ml H_2O) using mutation detection enhancement gel solution (BioWhittaker Molecular Applications, Rockland, ME, USA). Electrophoresis was performed in $0.5\times$ TBE at 2 W constant power at room temperature for 20–30 h and at 4°C for 50–60 h. SSCP bands were detected with the silver staining method using 0.1% silver nitrate according to the standardized protocol, and gels were dried in vacuum. Samples

Figure 1 Selected familial cases of congenital hypothyroidism and/or thyroid dysgenesis. (A) Dizygotic male twins: CH diagnosed by neonatal screening (TSH 33.5 mIU/l and 23 mIU/l), mild thyroid hypoplasia. Parents euthyroid. (B) Dizygotic female twins: CH diagnosed by neonatal screening (TSH 125.6 mIU/l, tT_4 55.2 nmol/l and TSH 127.5 mIU/l, tT_4 25 nmol/l), normal thyroid. Older brother: CH diagnosed by neonatal screening (TSH 116 mIU/l, tT_4 23 nmol/l), severe thyroid hypoplasia. Mother: mild adult-onset hypothyroidism, normal thyroid. (C) Female patient: CH diagnosed by neonatal screening (TSH 192.8 mIU/l, tT_4 47 nmol/l), mild thyroid hypoplasia. Older brother: CH diagnosed by neonatal screening (TSH 150 mIU/l, tT_4 36 nmol/l), thyroid morphology not known. Parents euthyroid. (D) Male patient: compensated CH diagnosed by neonatal screening (TSH 21 mIU/l, tT_4 159 nmol/l), hemithyroid (severely hypoplastic left thyroid lobe), gradually decreasing thyroid function, substitution treatment since 4 months of age. Afterwards mother diagnosed with asymptomatic hypoplastic right thyroid lobe. (E) Male patient: compensated CH diagnosed by neonatal screening (TSH 43 mIU/l, fT_4 36.5 pmol/l), thyroid hypoplasia. Father treated with T_4 first 3 years of life, clinical data not available. Normal ranges: fT_4 10–26 pmol/l, tT_4 60–160 nmol/l, TSH 0.25–5 mIU/l, neonatal screening TSH cut-off 15 mIU/l. Thyroid morphology was assessed by ultrasound prior to substitution treatment.

Table 2 Associated cardiac and renal malformations in the examined group of patients.

Associated malformations (n=19)					
Cardiac (n=12)		Renal (n=4)		Renal + cardiac (n=3)	
PDA	1	Polycystosis	1	Congenital hydronephrosis + ASD + VSD ^d	1
PFO	3				
PDA + PFO	1	Unilateral agenesis	1		
PDA + ASD	1			Horseshoe kidney + PFO	
VSD	2	Congenital			1
AVC ^b	1	Hydronephrosis ^c	1		
MVP	1			Mild renal abnormalities + PFO	
SPA ^d	2	Cortical cyst	1		1
NKX2.5		PAX8		PAX8 + NKX2.5	

^aKaryotype 47, XY, +21.
^bKaryotype 46, XY, 15p+ in one patient.
^cMale patient with CH (TD) associated with the neonatal respiratory distress and perinatal asphyxia, severe neurological impairment and congenital hydronephrosis (analysed also for *NKX2.1*).
^dFemale patient with CH (TD), multiple congenital anomalies (renal and cardiac malformations, cleft palate) and severe psychomotor retardation (analysed also for *FOXE1*). Investigated candidate genes were proposed due to the spectrum of associated congenital malformations; moreover, all patients were screened for *PAX8* mutations due to their non-goitre phenotype. One female patient with mild forms of cerebral and thyroid dysgenesis investigated for *HHEX* was not included in the table. ASD, atrial septal defect; AVC, atrioventricular channel; PFO, patent foramen ovale; MVP, mitral valve prolaps; PDA, patent ductus arteriosus; SPA, stenotic pulmonary artery; VSD, ventricular septal defect.

showing an abnormal mobility pattern within the matrix when compared with the wild-type control were submitted to direct sequencing.

Synthesis of wild-type PAX8 and mutant PAX8 R52P proteins

For functional characterization, human PAX8 cDNA was cloned into the ClaI and EcoRI cloning sites of the pCS2+ expression vector (constructed by Dave Turner). Mutant R52P PAX8 was obtained by standard mutagenesis procedure. Wild-type and mutant proteins for shift assay were generated using the *in vitro* cell-free transcription/translation (TnT system, Promega).

Electrophoretic mobility shift assay (EMSA)

EMSA was prepared as described previously (34). Four micrograms of human wild-type PAX8 or mutant PAX8 R52P proteins were incubated with 1 µg BSA, 0.1 µg herring sperm DNA and 0.5 µg poly[dI-dC] in the binding buffer (10 mmol/l Tris-HCl pH 7.5, 1 mmol/l EDTA, 4% Ficoll 1 mmol/l dithiothreitol and 2 mmol/l phenylmethylsulphonyl fluoride). In the competition experiment, an 800-fold excess of cold wild-type competitor was added. Reactions were incubated for 20 min at 30 °C. Then, 100 fmol of an end-radiolabelled 24 bp double-stranded oligonucleotide containing the rat PAX8 binding site, located at -72 nt to -66 nt relative to the transcription start site of the thyroglobulin gene (35), was been added to the reaction and incubated for an additional 45 min at 30 °C. Immediately after incubation, samples were loaded onto a 5% polyacrylamide gel in 0.5× TBE buffer (pH 8.3). Electrophoresis was performed at 34 mA at 4 °C for ~2.5 h. After drying of the gel, complex formation was visualized by overnight autoradiography. The following

double-stranded oligonucleotide was used in the experiment: 5'-CACTGCCCCAGTCAAGTGTCTTGA-3' (only the non-coding strand is shown and the consensus PAX8 binding site is underlined; National Center for Biotechnology Information (NCBI) accession no. X06162; (29)).

Results

The aim of the study was to describe the prevalence of transcription factor gene mutations in TD patients based on previously described phenotypes. Those patients with associated malformations already described as a part of the phenotype in NKX2.1-, FOXE1- and NKX2.5-gene mutation carriers were primarily analysed for mutations in these respective candidate genes. One patient with a CN midline defect was screened for HHEX gene mutations based on the knock-out mice phenotype, although no mutation has been found in TD up to now. In addition, all patients were screened for mutations in the PAX8 gene, since most of the patients with identified PAX8 gene mutations so far had isolated TD.

NKX2.5, NKX2.1, FOXE1 and HHEX genes

Fifteen patients with heart defects were investigated for NKX2.5 mutations. A male patient with CH due to TD (eutopic hypoplastic thyroid gland), associated with neonatal respiratory distress and perinatal asphyxia, severe neurological impairment (central muscular hypotony, movement disorder, paroxysms, psychomotor retardation) and congenital hydronephrosis, was analysed for NKX2.1 mutations. A female patient with CH due to TD (eutopic hypoplastic thyroid gland), associated with multiple congenital anomalies including cleft

palate and severe psychomotor retardation, was tested for *FOXE1*. One female patient with mild forms of cerebral (cavum septum pellucidum) and thyroid dysgenesis (mild hypoplasia) was investigated for *HHEX* mutations, since the complex phenotype of the *Hhex*^{-/-} mouse includes a brain midline structure defect in addition to TD.

No significant sequence variations were observed in all four genes. Among the 15 patients with CH and associated congenital cardiac malformations, two known sequence variations of the *NKX2.5* gene were found: c.63A>G (p.E21E) in seven heterozygotes and one homozygote, and c.*61T>G in eight heterozygotes and four homozygotes. Direct sequencing of *FOXE1* revealed the heterozygous polymorphisms c.819 C>T (p.S273S) and c.510C>A (p.A170A) in a 14 residue alanine stretch.

PAX8 gene

Based on the thyroid phenotype described so far in PAX8 mutation carriers, we screened all patients with CH due to TD for mutations in the PAX8 gene as well as all patients with congenital or early-onset non-goitre hypothyroidism, including familial cases with dominant inheritance. Exon 2 with the ATG initiation codon and exons 3–4 encoding for the DNA-binding paired domain were screened by SSCP in all 170 individuals. In 34 of these, suffering either from renal malformations or with positive family history, all coding exons 2–12 were screened by SSCP. In addition, exons 3–4 have also been sequenced.

In a boy with unsuspicious neonatal screening for CH but early postnatal regression of the thyroid size and

function, we identified a novel heterozygous missense mutation in exon 3. The mutation changes a G in position 155 into a C (numbering starts with the adenine nucleotide at the ATG initiation codon in exon 2; Fig. 2B), leading to an amino acid exchange of a highly conserved arginine to proline at codon 52 (R52P) in the DNA-binding domain of PAX8. The same mutation was detected in his hypothyroid mother and maternal grandmother, both treated for early-onset, non-auto-immune hypothyroidism. Neither siblings of mother nor maternal great grandmother carried the mutation. The mutation has not been documented in any of 100 chromosomes of 50 subjects randomly selected from Czech Caucasian non-CH paediatric patients.

Since the R52P mutation cosegregates with a hypothyroid phenotype in a three-generation pedigree (Fig. 2A), affects a highly conserved amino acid and is absent from normal DNA samples, we hypothesized a loss-of-function effect. After cloning of the mutation into the wild-type PAX8 expression vector and production of wild-type and R52P mutant PAX8 proteins in a transcription/translation system, we found a complete loss of binding of the PAX8 R52P mutant to the thyroglobulin promoter oligonucleotide C, which indicates a loss-of-function effect of the mutation (Fig. 3).

Detailed clinical presentation of R52P-PAX8-gene mutation carriers

The index patient was born at term (birth weight 3500 g, length 51 cm) as the first son and only child of non-consanguineous parents after an apparently normal

Figure 2 Identification of the R52P PAX8 mutation in a three-generation pedigree. (A) Pedigree: I.1, dead of cardiac failure at 62 years, no obvious thyroid abnormalities; I.2 and III.4, normal thyroid morphology and function, R52P mutation not present; II.4, refused further investigation; III.3, eutopic thyroid gland, asymptomatic thyroid nodule, R52P mutation not present; II.2, III.2, IV.1, early-onset non-goitre non-autoimmune hypothyroidism, heterozygous carriers of the R52P PAX8 mutation. (B) Identification of the mutation: SSCP of exon 3 (left; 1× MDE gel, room temperature, power 2 W, run time 20 h) – abnormal bands in the index patient compared with the wild-type control, part of the direct sequencing of exon 3 (right): heterozygous c.155G>C transition leading to the amino acid exchange p.R52P. (C) Thyroid rudiment (transversal ultrasound imaging) in the index patient at the age of 3 years. SSCP, single-stranded conformation polymorphism; wt, wild-type allele; mut, allele carrying mutation.

Figure 3 DNA binding analysis. *In vitro* translated wild-type (wt) PAX8 or mutant PAX8 R52P protein binding to an oligonucleotide probe corresponding to the thyroglobulin promoter. The specific complex was identified by competition with unlabelled wild-type oligonucleotide (>). The novel PAX8 R52P mutation results in a loss of binding to the thyroglobulin promoter element.

pregnancy (mother was treated with combined T_4 and T_3 substitution for non-autoimmune permanent hypothyroidism) in 1999. Neonatal screening for CH was negative (TSH 9.49 mIU/l, cut-off 15 mIU/l), and therefore no further detailed thyroid functional data are available. The diagnosis of hypothyroidism was established when he was still asymptomatic at the age of 18 months due to the positive family history of non-autoimmune permanent hypothyroidism in his mother and grandmother. TSH was remarkably elevated (183.9 mIU/l, normal range 0.25–5 mIU/l), fT_4 and fT_3 were low (fT_4 7.19 pmol/l, normal range 10–26 pmol/l, fT_3 3.32 pmol/l, normal range 4.2–8.1 pmol/l). His body length was 87 cm. Ultrasound examination showed a eutopic thyroid gland of a normal structure but at a lower end of a normal size (total volume 1 ml, own normative data for this age 1.39 ± 0.31 ml). Subsequently, under T_4 substitution, the thyroid gland did not grow properly and even got reduced in its size. The tissue structure changed into the hyperechogenic fibrous rudiment (total volume 0.45 ml in 3 years, own normative data 1.82 ± 0.53 ml) (Fig. 2C). At the age of 3 years, blood samples taken after 4 weeks of the withdrawal of treatment confirmed permanent hypothyroidism (TSH 100 mIU/l, fT_4 4.74 pmol/l) with low thyroglobulin levels, relative to elevated TSH (57–33 μ g/l, normal range 30–85 μ g/l). His psychomotor and physical development is normal. His mother, born in 1974 before the initiation of screening programme for CH, was diagnosed at the age of 6 months due to the diagnosis of permanent hypothyroidism of her mother, born in 1953 and diagnosed at the age of 3 years because of her short stature and obesity. No thyroid imaging study was performed prior to treatment, but recent ultrasound examinations showed a eutopic rudimental thyroid gland in mother and grandmother. Antithyroid antibodies were not detectable in all the three carriers of the PAX8 R52P mutation. There were no detectable abnormalities of the structure or function of the kidneys.

Discussion

To our knowledge, this investigation of 170 patients, mostly diagnosed by the Czech nationwide neonatal

screening for CH, represents one of the largest cohorts systematically screened for defects in thyroid transcription factors so far.

However, despite the fact that we focussed the analysis of *NKX2.5*, *NKX2.1* and *FOXE1* genes on TD patients with associated malformations or complications that have been previously reported, such as movement defects (*NKX2.1*), heart defects (*NKX2.5*) or cleft palate (*FOXE1*), we did not find any mutations in these particular subgroups of patients. Our data confirm that the mutation rate of the three candidate genes *NKX2.1*, *NKX2.5* and *FOXE1* is very low, even in a phenotype-focussed study. Other mechanisms, such as epigenetic or somatic changes that are not inherited, could cause the inactivation of these three respective candidate genes. Alternatively, unknown genes, functionally similar to *NKX2.1*, *NKX2.5* and *FOXE1*, might be involved in the pathogenesis of these particular cases with associated malformations.

Searching for PAX8 gene mutations in this large cohort of 170 Czech patients revealed only one novel loss-of-function mutation leading to non-congenital, non-autoimmune but early-onset hypothyroidism dominantly inherited in three generations (Fig. 2). All the three affected members of the index family were diagnosed as heterozygous carriers of a novel R52P mutation in the DNA-binding paired domain (Fig. 4C and D). The PAX8 paired domain is a 128 amino acid DNA-binding domain highly conserved in the human PAX protein family, showing sequence similarity to the *Drosophila* paired protein (36). It consists of two structurally independent subdomains, each containing a helix-turn-helix motif joined by a linker region ((37); Fig. 4D). Except for two mutations (24, 29), all PAX8 mutations published so far (24–28, 30) are located within the N-terminal subdomain (Fig. 4C and D). At the structural level, these mutations affect either residues directly contacting DNA at the protein–DNA interface or residues involved in the folding and stability of the protein. At the functional level, most of the heterozygous mutations located in the N-terminal subdomain are known to reduce binding affinity to a specific DNA sequence (24, 25, 27, 28).

The alignment of *Drosophila* and nine human PAX proteins paired domains (Fig. 4B) indicates that the arginine at codon 52 of the N-terminal part of the PAX8 paired domain is highly conserved in PAX proteins 1–3, 5, 7–9. It is also a homologue of the arginine residue at position 44 of *Drosophila* paired protein, located between the second and third α -helix (37). It interacts with the loop between two strands of the β -sheet and stabilizes the docking of the β -turn together with other protein–protein and protein–DNA contacts, making a critical base that allows contacts in the minor groove of the DNA (37). Taken together, these structural and evolutionary considerations suggest that the R52P mutation leads to the loss of DNA binding, confirmed by our DNA binding studies (Fig. 3).

Figure 4 Position of R52P in the PAX8 paired domain. (A) Partial schematic presentation of human *PAX8* gene. (B) Alignment of *Drosophila* paired domain (prd) and nine human PAX paired domains (sequences composing $\alpha 1$ – $\alpha 3$ -helices) according to the sequence similarity and common structural features of PAX proteins (37). Amino acids affected by mutations in human PAX8 and corresponding amino acids of other PAX proteins are boxed. (C) PAX8 mutations in the N-terminal subdomain of the paired domain detected so far (numbering corresponding to human PAX8 numbering): R31H (24), R31C (26), Q40P (27), S48F (30), R52P (present study), S54G (28), C57Y (25), L62R (24), numbering corresponding to *Drosophila* paired protein above. (D) Schematic presentation of the secondary structure of the paired domain (37) with marked positions of PAX8 mutations: the N-terminal part (PAI subdomain) composed of a β -sheet ($\beta 1$ - and $\beta 2$ -strand), a type II β -turn (thickened line), three α -helices and a C-terminal tail, connected with a linker to the C-terminal subdomain (RED subdomain) containing three α -helices.

The phenotypical expression of the R52P PAX8 mutation seems to be unique, because the index patient was shown to be normal in the newborn screening for CH, but later on at the age of 18 months diagnosed with severe hypothyroidism. Obviously, the R52P mutation carrier, identified in our study, would have been missed in all other cohorts screened so far for *PAX8* gene mutations and focussed solely on CH patients identified in screening programmes. However, the mild phenotype in the index patient of one previously described familial case of *PAX8* gene mutation (24), characterized by a borderline elevated screening TSH and normal T_4 in the newborn period but aggravating hypothyroidism during the first month of life, resembles the findings in our R52P mutation index case. Taken together, the findings in the R52P mutation carriers in our study and the familial case presented before (24) argue that PAX8 might not only play a role during early thyroid

organogenesis, but also participate in the postnatal maintenance of thyroid function. Thus, our findings suggest that even if the results of neonatal screening for CH are negative and thyroid gland is correctly developed and apparently normal, *PAX8* gene deficiency can cause hypothyroidism in the early postnatal life due to the gradual growth and functional impairment. Therefore, the search for *PAX8* gene mutations might be extended to those rare patients with early-onset hypothyroidism who were negative in CH screening.

The exact molecular mechanism responsible for the insufficient postnatal growth and functional regression of the thyroid gland in PAX8 deficiency is still unclear. However, Pax8 is essential for the formation of thyroid follicular structures (13) and has a fundamental role in the initiation of the thyroid cell differentiation and in the maintenance of the differentiated state (38). Thereby, PAX8 deficiency may also result in postnatal

dysregulation of the proliferation of thyroid cells and follicles or alternatively in a reduced survival due to an increased apoptotic degradation of thyroid follicular structures. Thus, identification of the new R52P PAX8 mutation leading to a hitherto undescribed dominantly inherited form of hypothyroidism with an early non-congenital and non-autoimmune onset implies an additional role of PAX8 in the postnatal maintenance of thyroid function.

Overall, our study confirms the very low prevalence of mutations in the known thyroid transcription factor genes in TD. Taking into account the rare occurrence of TSH-receptor gene mutations described earlier, it seems obvious that even the search in a phenotype-focussed strategy will not reveal a significant number of additional genetic defects in the known candidate genes for TD. More efforts to identify non-classical genetic defects like an epigenetic silencing of critical candidate genes in TD are mandatory to unravel the pathogenesis of TD.

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