Two Cases of Thyroid Dysgenesis Caused by Different Novel *PAX8* Mutations in the DNA-Binding Region: *In Vitro* Studies Reveal Different Pathogenic Mechanisms

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Background: Mutations in *PAX8*, a transcription factor gene, cause thyroid dysgenesis (TD). The extreme variability of the thyroid phenotype makes it difficult to identify individuals harboring *PAX8* gene mutations. Here we describe two patients with TD and report two novel *PAX8* gene mutations (S54R and R133Q). We performed *in vitro* studies to functionally characterize these mutations.

Methods: Using *PAX8* expression vectors, we investigated whether the *PAX8* mutants localized correctly to the nucleus. To analyze the DNA-binding properties of S54R and R133Q, electrophoretic mobility shift assays were performed. Furthermore, we measured whether the mutant *PAX8* proteins were able to activate the *thyroglobulin* (*TG*)- and the *thyroperoxidase* (*TPO*)-promoters.

Results: S54R had an impaired binding to DNA and a negligible activity on the *TG*- and the *TPO*-promoters. The DNA-binding property of R133Q, which is located in the highly conserved terminal portion of the *PAX8* DNA-binding domain, was normal. Interestingly, it also exhibited dramatically impaired activation of the *TG*- and *TPO*-promoters. However, R133Q has no dominant negative effect on the WT protein *in vitro*. Thus, the underlying molecular mechanism by which the function of R133Q is impaired remains to be elucidated.

Conclusions: We identified and functionally characterized two novel mutations of the *PAX8* gene that lead to TD by distinct mechanisms. A structural defect of the mutant R133Q leading to a reduced capability for induced fit upon DNA interaction might explain the disparity between its apparently normal binding to DNA, but lack of promoter activation.

Introduction

CONGENITAL PRIMARY HYPOTHYROIDISM (CH) occurs in 1/3500 newborns. In the majority of cases (80%–85%) the cause is thyroid dysgenesis (TD), leading to absent, hypoplastic, or ectopic thyroid glands (1). The underlying molecular mechanisms causing TD are mostly unknown. Screening of candidate genes involved in thyroid organogenesis has revealed only a few mutations in the genes for *thyroid transcription factors 1 and 2* (*TTF1* and *TTF2*), the *thyrotropin-receptor* (*TSHR*), the *NK2 transcription factor-related locus 5* (*NKX2.5*), and the *paired box gene 8* (*PAX8*) (1). *PAX8* is a transcription factor, which is expressed in the thyroid, kidney, and central nervous system. It is important for initiation

of thyrocyte differentiation and maintenance of the follicular cell (2). *PAX8* regulates the expression of *thyroglobulin* (*TG*), *thyroperoxidase* (*TPO*), and the *sodium iodide symporter* (*NIS*) by binding to specific promoter regions. Furthermore, *PAX8* and *TTF1* synergistically activate the promoter of human *TG* (3). So far, 13 different mutations in the *PAX8* gene have been reported (3–17), and the phenotypes of affected individuals vary considerably. Even within the same family, heterozygous individuals with *PAX8* mutations can have no thyroid gland abnormality, have thyroid hypoplasia or TD (6), making it very difficult to identify individuals harboring a *PAX8* gene mutation (1). Here we report two affected individuals from unrelated families with CH who were identified to have *PAX8* gene mutations. Both mutations were located in the

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homeodomain of the *PAX8* gene. The S54R mutation was located in the middle of the DNA-binding domain, whereas the R133Q mutation was located in the last amino acid position of this region. *In vitro* studies of the two identified mutations were performed to understand the molecular mechanisms by which they cause CH. Our studies suggest that each of these mutations affects *PAX8* function via different mechanisms.

Materials and Methods

Subjects

Family 1. The probanda (patient II-1) was born after 42 weeks of gestation to consanguineous parents from Turkey. Her birth weight was 3230 g and length 52 cm. Hypothyroidism was diagnosed at neonatal screening (TSH spot test 169.3 μ U/mL, normal <15, total T₄ 24.2 nM, normal 77–205) and confirmation was obtained at the age of 6 days when the serum TSH was 714 μ U/mL and the serum level of free T₄ was low at 5.6 pM (normal 26–63). Because her serum TG at that time was low at 7 ng/mL (normal <30), TD was suspected and confirmed by ultrasound, which showed a severely hypoplastic thyroid gland. Since then, she has been treated with the thyroid hormone. With L-thyroxine (L-T₄) replacement therapy adjusted for age and weight, she developed normally and is currently doing well. Patient I-1, father of patient II-1, was born in Turkey where, at that time, neonatal screening was not available. When he moved to Germany he presented at the age of 2.5 years with psychomotor delay, failure to thrive, and an abnormal gait. His weight was 8800 g (below 3rd percentile) and his height was 71 cm (below 3rd percentile). On physical examination, he displayed typical signs of hypothyroidism with an enlarged tongue, dry skin, and reduced facial expression; no thyroid gland was palpable. He could not stand alone and his movements were slow. X-rays revealed that he had hip dysplasia with an abnormal acetabulum and a retarded maturation of the femoral head. Serum TSH was >80 μ U/mL (normal <5), total T₄ was 27 nM (normal 88–174), and FTI 1.5 (normal 3.8–14.3). He was treated with L-T₄, which resulted in normal physical and intellectual development. The patient's mother (patient I-2) is euthyroid.

Family 2. The probanda of family 2 (patient II-1) was born after 40 weeks of gestation with a birth weight of 3450 g and a length of 51 cm. Mild TSH elevation of 15 μ U/mL (normal <15) was found on neonatal screening and was 16 μ U/mL 6 days later. At the age of 6 weeks, laboratory evaluation revealed a serum TSH of 55 μ U/mL (normal 1.7–9.1), a free T₄ of 10.2 pM (normal 12–33), and a total T₃ of 3.5 nM (normal 1.2–4.0). She was first treated with 150 μ g iodine per day. Two weeks later she appeared to be euthyroid, but had still an elevated serum TSH of 51 μ U/mL. Her serum total T₃ and free T₄ levels did not significantly change and were 3.4 nM and 11.7 pM, respectively. Ultrasound revealed a small, normally located thyroid gland. Treatment with L-T₄ was initiated with a dose of 50 μ g per day, and she continued to develop normally. At the age of 3 years, treatment with L-T₄ was stopped. Three weeks later, her serum TSH was elevated at 54.5 mU (normal 0.7–6.4) and treatment with L-T₄ was restarted. At the age of 7 years, her ultrasound was repeated and showed a small thyroid gland. The size of the left thyroid lobe was

0.36 cm $\times$ 0.77 cm $\times$ 1.5 cm and that of the right lobe was 0.9 cm $\times$ 0.4 cm $\times$ 1.1 cm. Currently, she is receiving 75 μ g L-T₄ daily and is doing well.

Her younger sister (patient II-2) also had a mildly elevated serum TSH level at neonatal screening of 16.8 μ U/mL (normal <15). She was treated with L-T₄ immediately, but after a short time the mother stopped treatment because the girl developed diarrhea. Serum TSH showed a normal result, so she was without L-T₄ treatment when she presented at the age of 2 years with a mildly elevated TSH of 6.6 μ U/mL (normal 0.7–6.4) and the free T₄ was 14.6 pM (normal 12–33). An ultrasound of the thyroid showed a small gland with volumes of the right and left lobes of 0.8 mL each. L-T₄ substitution was recommended, but the referring doctor prescribed 150 μ g iodide instead, under which she continued to develop normally. Three years later, at the age of 5 years, her serum TSH was 11 μ U/mL and the free T₄ 14.3 pM (normal 10–28). Treatment with 50 μ g L-T₄ per day was started and her serum TSH decreased to 0.6 μ U/mL 12 months later. Her development is still normal and currently she is euthyroid (TSH 1.0 μ U/mL, free T₄ 23 pM). Both parents had a history of thyroid disease. Her mother (patient I-2) underwent subtotal thyroidectomy because of nodular goiter at the age of 25 years. Thereafter, she was treated with L-T₄ 150 μ g daily and was euthyroid when investigated in our clinic (Fig. 1). The father (patient I-1) had a unilateral goiter with a cold nodule (2.7 cm $\times$ 2.1 cm $\times$ 5 cm) so that hemithyroidectomy was performed when he was 31 years old. Thereafter, he was euthyroid under treatment with 50 μ g L-T₄ daily.

DNA amplification and DNA sequencing

The clinical and genetic studies were approved by the IRBs of the Johannes Gutenberg University of Mainz (Mainz, Germany) and the University of Chicago (Chicago, IL). After written informed consent was obtained from all patients and participating family members, genomic DNA from blood samples was isolated utilizing the QIAamp DNA Blood Kit (QIAGEN, Hilden, Germany). The coding sequences of the known candidate genes for TD (*TSHR*, *PAX8*, *TTF1*, *TTF2*, and *NKX2.5*) were amplified by PCR (conditions and primer sequences will be supplied upon request), purified with an

	Family 1			Family 2		
PAX8 mutation	S54R			R133Q		
Age at diagnosis	2 y	6 d		1 y	6 w	
TSH at diagnosis (μ g/mL)	>80	169	nd	nl	15	17
Thyroid morphology	HYPO	HYPO	NL	GOITER	HYPO	GOITER
Carrier of mutation	yes	yes	no	yes	yes	yes

HYPO=thyroid hypoplasia

FIG. 1. Pedigree of the two families. Individuals with thyroid dysgenesis are indicated with a filled black box.

Exonuclease I (New England Biolabs, Frankfurt, Germany) and a *Shrimp alkaline phosphatase* (GE Healthcare, München, Germany) treatment, and then sequenced using Big Dye Terminator v.3.1. The ethanol precipitated amplified products were loaded on a 3100 Avant Genetic Analyzer, and analyzed with the SeqScape software v.2.5 (Applied Biosystems, Darmstadt, Germany).

Constructs and mutagenesis

Construction of the expression vector encoding *PAX8* was described previously (17). The novel *PAX8* gene mutations S54R and R133Q, that are described in this report (see RESULTS) were introduced utilizing the QuikChange Mutagenesis XL kit (Agilent Technologies, La Jolla, CA) according to the manufacturer's protocol using the following primers: S54R_F: 5'-GCT CCG CGT CAG GCA TGG CTG CGT C-3', S54R_R: 5'-GAC GCA GCC ATG CCT GAC GCG GAG C-3', R133Q-F: 5'-CCA TTA ATA GAA TCA TCC AGA CCA AAG TGC AGC AAC C-3', R133Q_R: 5'-GGT TGC TGC ACT TTG GTC TGG ATG ATT CTA TTA ATG G-3', respectively.

Cloning of the *PAX8*-Enhanced Green Fluorescent Protein (EGFP) fusion construct has been described previously (9) and the two *PAX8* mutations were introduced again utilizing the QuikChange Mutagenesis XL kit (Agilent), and the same primers as for the mutagenesis mentioned above. The preparation of the reporter constructs *TG-luc*, *TPO-luc*, as well as the TTF1 expression construct was described previously (9,18). All constructs were verified by sequencing.

Cell culture and transfection

HeLa cells were grown in the PAN 401 medium, supplemented with 10% fetal bovine serum (PAN Biotech GmbH, Aidenbach, Germany) and 1% penicillin-streptomycin (PAN Biotech) at 37°C in a humidified incubator with 5% CO₂. Twelve hours before transfection, cells were trypsinized and plated in 24-well plates. When the cells reached 70% confluency, transfection with FuGene 6 (Roche, Mannheim, Germany) was carried out according to the manufacturer's instructions. 48 hours later, the cells were washed three times with ice cold phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA) and lysed in 150 µL of 1× passive lysis solution (Promega, Madison, WI). Ten microliters of the protein extract was used for the dual luciferase assay (Promega). The *luciferase* and *renilla luciferase* activities were measured according to the manufacturer's recommendations using the Lumat 9507 (Berthold Technologies, Bad Wildbad, Germany). All transfection studies were performed in triplicates and performed twice. Statistical significance of differences was analyzed by the Student's *t* test.

Immunofluorescence analysis

HeLa cells were plated in 12-well cell culture plates, where a round cover slip has been placed. The cells were transiently transfected with 200 ng *PAX8*-EGFP expression vector and 200 ng TTF1 expression cassette per well. Forty-eight hours after transfection, cells were washed twice with 1× PBS and, after 20 minutes, fixed with formalin (Fisher Scientific, Pittsburgh, PA). Afterward, the cells were permeabilized for 5 minutes with 0.2% Triton × 100 in 1× PBS and washed three times with 1× PBS. Blocking was performed with 1% bovine

serum albumin (BSA) in 1× PBS for 30 minutes. The monoclonal anti-TTF1-AB (clone 8G7G3/1; NeoMarkers/Lab Vision Corp., Fremont, CA) diluted 1:1000 was incubated for 30 minutes and washed three times for 5 minutes with 1× PBS. Again blocking was performed with 1% BSA in PBS for 30 minutes. As a secondary antibody, Alexa Fluor568-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) was used and incubated for 30 minutes. Counterstaining of the nucleus was done with Hoechst 33342 fluorochrome (Molecular Probes) at a dilution of 1:1000 for 1 minute. Before the cells were embedded, they were washed three times with 1× PBS.

Electrophoresis mobility shift assay

Recombinant proteins of the *PAX8* WT and mutants were generated using a coupled *in vitro* transcription/translation system (TNT[®]-T7 Coupled Reticulocyte Lysate System; Promega, Madison, WI). The *TPO* (TGA TGC CCA CTC AAG CTT AGA CAG) (19) and *TG* (CAC TGC CCA GTC AAG TGT TCT TGA) (3) oligo sequences were synthesized as described previously and were 5'-end-labelled with IRD700 (Metabion, Martinsried, Germany). Ten microliters binding reaction contained 1 µg of protein, 10–50 nM double-stranded oligo, 150 ng poly (dI-dC), 500 ng salmon sperm DNA, 5 mM DTT, 0.5% Tween20, 10 mM Tris, 50 mM NaCl, pH 7.5. The binding reaction was incubated for 30 minutes at room temperature. Afterward, 1 µL gel-loading buffer (containing 650 mg/mL sucrose, 0.3% OrangeG, 10 µM Tris pH 7.5, and 10 µM ethylenediaminetetraacetic acid (EDTA) pH 8.0) was added and the samples were loaded on a 4% native polyacrylamide gel (PAG). The PAG was supplemented with 50 mM Tris pH 7.5, 200 mM glycine, and 2 mM EDTA. The Towbin buffer (20) supplemented with 10 mM EDTA served as a running buffer. Thereafter, the PAG were scanned and visualized using the Odyssey Model 9120 and the Odyssey v2.1 software (Li-Cor Biosciences, Bad Homburg, Germany).

Results

Sequencing analysis revealed novel *PAX8* gene mutations in the two unrelated families. The index patient of family 1 (II-1) was found to have a heterozygous missense mutation in exon 3 of the *PAX8* gene, a C > G transversion at position 162, which results in the substitution of the normal serine with an arginine at codon 54 (S54R). S54 is highly conserved in the *PAX8* gene (data not shown) and was not found in 100 *PAX8* alleles from a general euthyroid population of a similar ethnic background ruling out a common polymorphism. The mutation was also found in the heterozygous state in the father (patient I-1), but not in the mother (Fig. 1).

The two affected children of family 2 (patients II-1 and II-2) were heterozygous for a G > A transition at position 398 in exon 5 of the *PAX8* gene. This substitution leads to the replacement of the normal arginine with a glutamine at position 133 (R133Q), which is the last amino acid of the DNA-binding domain in the *PAX8* protein and also highly conserved. One hundred *PAX8* alleles from a general euthyroid population of a similar ethnic background did not harbor the R133Q mutation. The mother (patient I-2), but not the father (patient I-1), was also heterozygous for R133Q.

The coding sequences of *TSHR*, *TTF1*, *TTF2*, and *NKX2.5* were normal in all members of both families.

FIG. 2. Nuclear localization of the two identified *PAX8* gene mutations. HeLa cells were transiently transfected with plasmids expressing a *PAX8*-EGFP-WT or *PAX8*-EGFP-mutant (S54R, R133Q) and TTF1-EGFP-WT as a control. The *PAX8* and TTF1 proteins were visualized by immunofluorescence microscopy. All proteins (WT and S54R, R133Q) are localized in the nucleus.

Since *PAX8* controls transcription through binding to specific DNA sequences, we first analyzed whether the mutant *PAX8* proteins localized correctly to the nucleus. For this purpose, the coding sequences of the WT *PAX8* and the mutants S55R, R133Q were cloned into EGFP expression constructs and transiently transfected into HeLa cells. Using a TTF1 expression construct as a control for nuclear localization, we found that the mutant *PAX8* proteins localized correctly into the nucleus (Fig. 2). To investigate the binding of the *PAX8* mutants to their target gene promoters, electrophoresis mobility shift assays were performed (Fig. 3A, B). Whereas WT and R133Q bound specifically and equally well to the target gene (*thyroid peroxidase*) promoter sequences, the mutant S54R did not bind (Fig. 3A). This was expected since 54R is located inside the DNA-binding domain of the *PAX8* protein. However, S54R was able to bind specifically to the *TG* promoter sequence (Fig. 3A, B). However, it showed a reduced binding affinity to the *TG* promoter sequence.

The functional relevance on downstream targets of the mutant *PAX8* proteins was evaluated by transient transfection in HeLa cells using expression vectors containing the WT or the mutant cDNAs. As shown in Figure 4, the intensity of a *luciferase* reporter gene expression under the control of the *TG* (or a *TPO* promoter, data not shown) was investigated. S54R did not activate the *TG* promoter-driven reporter gene and

FIG. 3. Electrophoresis mobility shift assay with the thyroid peroxidase (TPO) promoter-binding site and the thyroglobulin (TG) promoter-binding site. Oligonucleotides containing the *PAX8*-binding sequences of the *TPO* and *TG* gene promoters, respectively, were synthesized and labeled with an infrared dye (IRD700). The *in vitro* synthesized *PAX8* proteins were incubated with labeled oligonucleotides, separated via PAGE, and visualized (for details see Materials and Methods). **(A)** *PAX8* WT as well as the mutant R133Q binds normally to the promoter sequence. In contrast, S54R lost its binding ability to the *TPO* response element. Furthermore, the binding to the *TG* promoter sequence is shown. Compared to WT and R133Q, S54R binds weakly to the *PAX8*-binding sites of the *TG* promoter. **(B)** The DNA binding of WT, R133Q, and S54R is specific, since a decreased shift can be observed after adding increasing amounts of unlabeled oligos.

FIG. 4. Transient transfection of HeLa cells utilizing a *TG-luciferase* reporter gene. HeLa cells were transiently transfected with 0.25 µg *TG-luciferase* and various amounts of WT or mutant *PAX8* expression constructs. At all DNA concentrations tested, the mutations S54R (gray bars) and R133Q (white bars) have a significantly reduced transcriptional activity on the reporter gene.

exerted a dominant negative effect on the WT. The transcriptional activity of mutation R133Q was severely (80%–90%) reduced when compared to WT *PAX8*. This reduction in transactivation activity was thought not to be due to a dominant negative effect, since coexpression of R133Q did not impair WT transcriptional activity (Fig. 5).

Discussion

Due to the extreme variability of the thyroid phenotype, it is difficult to identify individuals harboring a *PAX8* gene mutation. We here report two new *PAX8* gene mutations found in individuals with TD. Both mutations are located in the segment of the gene encoding the homeodomain of the Pax8 protein, and have been characterized functionally *in vitro*.

FIG. 5. S54R exerts a dominant negative effect, whereas R133Q has no dominant negative effect. HeLa cells were transiently transfected with 0.25 µg *TG-luciferase* reporter constructs as well as 0.02 µg or 0.04 µg of *PAX8* WT or R133Q or S54R expression constructs, respectively. In addition, WT and mutants were transfected together (0.02 µg WT + 0.02 µg mutant, or 0.02 µg WT + 0.04 µg mutant). pcDNA was utilized to keep equal amounts of DNA in each transfection.

There is no question that the S54R mutation is causative for the phenotype. This mutation is located in a highly conserved area of the gene, which encodes the DNA-binding domain of the *PAX8* protein. Other mutations in this area of the gene have been described before and have been characterized at the molecular level (4,5,7,9–15,17). In agreement with previously reported mutations (R31C, R31H, Q40P, R52P, S54G, H55Q, C57Y, L62R), S54R has lost its ability to bind to *TPO* promoter-binding sites. Interestingly, the binding of S54R to *TG* promoter-binding sites is not abolished, but impaired. As a result, S54R is unable to activate *TG* promoter-driven reporter gene transcription and exerts a dominant negative effect on the WT.

The second mutation, R133Q, which is also located in a highly conserved area of the *PAX8* gene, alters the final amino acid of the DNA-binding domain in the *PAX8* protein. To our surprise, R133Q was able to bind to specific promoter DNA-sequences, but showed a severely impaired transactivation of the target gene reporter construct. A dominant-negative effect was excluded, and thus cannot explain the observed reduction in transactivation of the downstream targets. One can speculate that this phenomenon might be due to a structural defect of the mutant, which leads to a reduced capability for induced fit upon DNA interaction. Paired domain containing factors, such as *PAX8*, undergo a gain in alpha-helical content upon interaction with DNA, the so-called induced fit. It is possible that this is altered in the case of R133Q and, despite detectable protein–DNA interaction in the gel shift experiment, the mutation may have an altered DNA-binding property. Tell *et al.* have shown that this mechanism is causative for a *PAX8* gene mutation (L62R), which is located also in the homeodomain of the *PAX8* protein (21).

In conclusion, we describe and characterize two new *PAX8* gene mutations in patients with TD. The mutations have different pathogenic mechanisms by which they are causative. The variable phenotype in patients with *PAX8* gene mutations is likely caused by other mechanisms, which need to be investigated in further studies.

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Disclosure Statement

The authors have nothing to disclose.

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