CLINICAL STUDY

Functional characterization of four novel *PAX8* mutations causing congenital hypothyroidism: new evidence for haploinsufficiency as a disease mechanism

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Abstract

Background: Individuals carrying a heterozygous inactivating *PAX8* mutation are affected by congenital hypothyroidism (CH), although heterozygous *Pax8* knockout mice are not. It has remained unclear whether CH in *PAX8* mutation carriers is caused by haploinsufficiency or a dominant negative mechanism.

Objective: To report clinical and molecular findings of four novel *PAX8* mutations, including one early-truncating frameshift mutation.

Subjects and methods: Four probands were CH patients. Two had family history of congenital or childhood hypothyroidism. Three probands were diagnosed in the frame of newborn screening for CH, while one had a negative result in screening but was diagnosed subsequently. Three had thyroid hypoplasia and one had a slightly small thyroid with low echogenicity. For these probands and their family members, we sequenced *PAX8* using a standard PCR-based method. Pathogenicity of identified mutations was verified *in vitro*.

Results: We found four novel heterozygous *PAX8* mutations in the four probands: L16P, F20S, D46SfsX24, and R133Q. Family studies showed four additional mutation carriers, who were confirmed to have high serum TSH levels. Expression experiments revealed that three mutations (L16P, F20S, and R133Q) had defects in target DNA binding, while D46fs had protein instability that was rescued by the proteasome inhibitor MG132. All four mutations had reduced transactivation on the thyroglobulin promoter, supporting that they were inactivating mutations.

Conclusion: D46fs is the first *PAX8* mutation with confirmed protein instability. Our clinical and *in vitro* findings together suggest that pure *PAX8* haploinsufficiency can cause CH in humans.

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Introduction

PAX8, a member of the Pax gene family, plays pivotal roles in thyroid development and physiology. PAX8 is expressed in the developing thyroid to adulthood (1). PAX8 directly regulates transcription of thyroid-specific genes, such as thyroglobulin (Tg), in cultured cell lines (2). *Pax8* knockout mice have thyroid aplasia due to defective proliferation and survival of thyroid precursor cells (3). In humans, a heterozygous *PAX8* mutation causes congenital hypothyroidism (CH). To date, 32 mutation carriers belonging to 13 families have been described (4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14). Clinical phenotypes of mutation carriers are variable, ranging from overt CH with severe thyroid hypoplasia to subclinical CH with a morphologically normal gland. Detected mutations include ten amino acid-altering mutations in the DNA-binding paired domain (R31C, R31H, Q40P, S48F, R52P, S54G, H55Q, C57Y, L62R, and K80_A84dup) and two protein-truncating mutations (R108X and T277X). No consistent genotype–phenotype correlation has been suggested.

Several *PAX* genes other than *PAX8* have been implicated in Mendelian disorders, including *PAX2* (papillorenal syndrome; OMIM*167409), *PAX3* (Waardenburg syndrome; OMIM *606597), *PAX6* (aniridia; OMIM *607108), and *PAX9* (tooth agenesis; OMIM *167416). All four genes, when mutated, are assumed to cause a human disease via haploinsufficiency because both entire gene deletion(s) and nucleotide-level mutation(s) with early protein truncation produce disease phenotypes (Supplementary Table 1, see section on supplementary data given at the end of this article). This assumption is also supported by observations of

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mutant mice with inactivating *Pax* allele(s), showing the gene dosage effect (e.g. disease phenotype seen in heterozygotes; Supplementary Table 1). Contrastingly, the mechanism linking heterozygous *PAX8* mutations and CH in humans has remained obscure. To date, neither entire *PAX8* deletion nor early truncation mutation has been reported. Moreover, heterozygous *Pax8*-knockout mice are not affected by CH (15). Based on the phenotypic difference between mutation-carrying human patients and mice, a dominant negative effect in the former has been proposed (7), although most previous *in vitro* studies have failed to recapitulate the effect.

Here, we report the identification and functional characterization of four novel CH-associated *PAX8* mutations. Among the four mutations, one was a frameshift mutation (D46fs) causing protein instability *in vitro*. Our clinical and molecular findings about the first experimentally confirmed null *PAX8* mutation provides new evidence, indicating that *PAX8* haploinsufficiency can cause CH in humans.

Materials and methods

Mutation detection

This study was approved by the Institutional Review Board of Keio University School of Medicine. We obtained written informed consent for molecular studies from the study subjects or his/her parents. Leukocytic DNAs were extracted from the four probands and their family members with the Gentra Puregene Blood Kit (Qiagen). Coding exons and flanking introns of *PAX8* (transcript variant PAX8A; GenBank NM_003466.3) were analyzed by standard PCR-based sequencing as described previously (12). Detected mutations were tested in 100 control Japanese individuals.

Three-dimensional modeling

Three-dimensional structures of three missense mutants (L16P, F20S, and R133Q) were modeled with 3D-JIGSAW (http://bmm.cancerresearchuk.org/~3djigsaw/). The structure data of PAX6–DNA complex (protein data bank ID 6PAX; http://www.rcsb.org/pdb) were used as a template. The pictures of the modeled structures were produced with PyMOL (http://www.pymol.org).

Plasmids, cell culture, and transfection

Vectors encoding human PAX8 cDNA (untagged, myc tagged, or enhanced green fluorescent protein (EGFP) tagged) have been described previously (12). The four mutations (L16P, F20S, D46fs, and R133Q) were introduced into these vectors by site-directed mutagenesis (QuikChange XL Site-Directed Mutagenesis Kit; Agilent Technologies, Santa Clara, CA, USA). All final constructs were verified by direct sequencing. HeLa cells were maintained in DMEM supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum. For functional assays, cells were transfected with DNA using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol.

Western blotting

Cells transfected with each of myc-tagged PAX8 constructs (wild type (WT) or mutant) or the empty vector were harvested at 24 h after transfection. Crude cell lysate was obtained with the M-PER protein extraction reagent (Pierce, Rockford, IL, USA). Samples containing 20 μg protein were separated on 10% SDS–PAGE, and western blotting was performed with a mouse anti-myc MAB (Life Technologies) and a HRP-conjugated rabbit anti-mouse IgG polyclonal antibody (Sigma–Aldrich) as a second antibody. Bound antibody was revealed with a chemiluminescence kit (GE Healthcare, Buckinghamshire, UK).

Cells transfected with the myc-tagged D46fs mutant or the empty vector were treated with dimethyl sulfoxide (DMSO) or DMSO containing 1 μM MG132 (Sigma–Aldrich) for an additional 12 h. Western blotting analyses were performed as described earlier.

Visualization of subcellular localization

Cells grown on sterile glass coverslips were cotransfected with each PAX8–EGFP fusion construct (WT or mutant) and the vector encoding red fluorescent protein-tagged thyroid transcription factor-1 (TTF1). Twenty-four hours after transfection, cells were fixed in 2% formaldehyde/PBS at room temperature for 10 min. Then, coverslips were mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and were observed under a TCS-SP5 confocal microscope (Leica Microsystems, Mannheim, Germany).

Electrophoretic mobility shift assay

The two band shift probes, oligo-CT (TGA TGC CCA CTC AAG CTT AGA CAG) and oligo-C (CAC TGC CCA GTC AAG TGT TCT TGA), were prepared by annealing of 3′-biotin-labeled complementary oligonucleotides (purchased from BEX Co., Ltd, Tokyo, Japan). Five micrograms of nuclear protein extraction (prepared with the NE-PER nuclear extraction reagent (Pierce)) were incubated at room temperature in 20 μl binding reaction mixture containing 20 fmol probe, 50 mM KCl, 5 mM $MgCl_2$, 2.5% glycerol, 0.05% NP-40, and 1 μg poly (dI-dC) for 20 min. For competition experiments, a large excess (200×) of unlabeled competitor oligonucleotides was included in the binding reactions. The protein–DNA complexes were subject to gel

Table 1 Clinical summary of eight *PAX8* mutation carriers.

Variable	Family 1 L16P			Family 2 F20S		Family 3 D46fs		Family 4 R133Q	Reference
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	
Age (years), sex	11, F	8, M	41, F	5, F	38, F	10, M	39, M	10, F	
Newborn screening	Negative	Negative	Not tested	Positive	Not tested	Positive	Not tested	Positive	
Age at diagnosis (years)	0.5	4	12	0.1	34	0.1	39	0.1	
Thyroid function									
Age at evaluation (years)	3	4	12	0.1	34	6	39	5	
Serum TSH (mU/l)	**19.7**	**16.3**	**710**	**183**	**161**	**13.8**	**5.6**	**18.0**	0.5–5.0
Serum-free T_4 (ng/dl)	NA[a]	1.0	NA	1.1	**0.3**	0.9	**0.8**	NA	0.9–1.8
Serum Tg (ng/ml)	13	24	NA	NA	**360**	28	**32**	NA	<30
Thyroid ultrasonography									
Age at evaluation	9	4	NA	3	34	6	39	13	
Size (SDS)	**−3.1**	−1.7	NA	**−3.5**	**−3.3**	−1.3	**−3.0**	**−3.5**	−2 to +2
Echogenicity	Normal	Normal	NA	Normal	Normal	**Low**	**Low**	**Low**	Normal
Thyroid growth	**Absent**	NA	NA	**Absent**	NA	Present	NA	NA	Present
^{123}I uptake at 24 h (%)	12.3	10.8	NA	NA	NA	22.6	NA	20.6	8–40
$KClO_4$ discharge rate (%)	8.3	0.0	NA	NA	NA	**12.8**	NA	NA	<10

NA, not available; T_4, thyroxine; Tg, thyroglobulin. Abnormal values/findings are given in boldface.
[a]Total thyroxine was 7.6 μg/dl.

electrophoresis and transferred to a nylon membrane. The biotin-labeled probe was detected with the Light-shift chemiluminescent EMSA kit (Pierce).

Transactivation assays

Cells grown in 96-well plates with about 80% confluence were cotransfected with 50 ng of the luciferase reporter driven by the *TG* promoter (*TG*-luc (12)), and various doses of the effector plasmids or the empty vector. The amount of transfected plasmid was kept constant by adding the empty vector. Twenty-four hours after transfection, we measured firefly luciferase activities using ONE-Glo Luciferase Assay System (Promega). Luciferase activities were represented relative to the activity obtained by transfection of empty vector and were expressed as mean ± S.E.M. Welch's *t*-test was used for statistical comparisons with significance at $P<0.05$.

Results

Clinical histories

Clinical phenotypes of eight mutation carriers (Patients 1–8) belonging to four families (Families 1–4) are summarized in Table 1. The pedigrees are shown in Fig. 1.

Family 1 The proband (Patient 1; a girl) had a normal blood spot TSH level (9 mU/l; cutoff level, 10) at newborn screening for CH. Her first thyroid function test was conducted at age 6 months because her mother (Patient 3) had childhood hypothyroidism. At this point, she had a high serum TSH level (14 mU/l; reference, 0.5–5.0) with a normal free thyroxine (T_4) level (1.1 ng/dl; reference, 0.9–1.8). She had no CH-related symptom and was growing and developing normally. At age 9 months, levothyroxine (L-T_4) replacement was started because her serum TSH level increased to 22.3 mU/l. At age 3 years, we reassessed her thyroid status with transient discontinuation of therapy and confirmed permanent CH (TSH, 19.7 mU/l; T_4, 7.6 μg/dl (reference, 9.3–17.1); Tg, 13 ng/ml). Ultrasonography showed a normoechoic hypoplastic thyroid (−2.5 S.D. (16); Supplementary Figure 1, see section on supplementary data given at the end of this article). ^{123}I uptake at 24 h was 12.3% (reference, 8–40), and perchlorate discharge rate was 8.3% (reference, 0–10). Repeated ultrasonography at age 9 years showed thyroid hypoplasia (−3.1 S.D.; Supplementary Figure 1).

Patient 2, a younger brother of the proband, also had a negative result in newborn screening. At age 2 months, his first thyroid function test showed a slightly high serum TSH level (7.3 mU/l) with a normal T_4 level (11.2 μg/dl). At age 4 years, his second thyroid function test revealed subclinical hypothyroidism (TSH, 16.3 mU/l; free T_4, 1.0 ng/dl; Tg, 24 ng/ml).

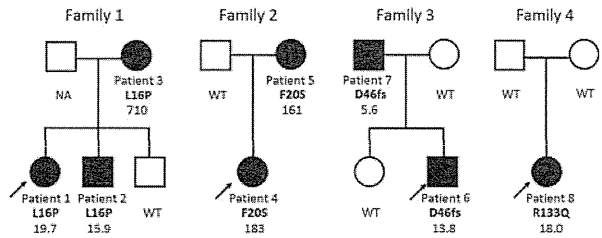

Figure 1 Pedigrees of four families with *PAX8* mutations are shown. Values presented below each symbol indicate TSH levels as expressed in milliunits per liter (reference, 0.5–5.0). Family studies showed that all mutation carriers had high serum TSH levels. Squares, men; circles, women; solid symbols, affected by hypothyroidism; open symbols, unaffected.

Ultrasonography showed a slightly hypoplastic thyroid (−1.7 S.D.; Supplementary Figure 1). ^{123}I uptake was normal (10.8% at 24 h), and no discharge after perchlorate challenge was observed. At this point, L-T_4 replacement was started.

Patient 3, a mother of the proband, was born before the implementation of newborn screening for CH. She was first evaluated for her thyroid function at age 12 years due to mild mental retardation and short stature. She had a markedly high serum TSH level (710 mU/l) with no thyroid autoantibodies. Thyroid scintigraphy showed a normally located thyroid gland with reduced radioiodine uptake (detailed data were unavailable). She has been receiving L-T_4 replacement therapy after the diagnosis.

Family 2 The proband (Patient 4; a girl) was born by cesarean section due to fetal distress. Newborn screening for CH revealed a high blood spot TSH level (79.6 mU/l). At age 9 days, she had a high serum TSH level (183 mU/l) with a normal serum-free T_4 level (1.1 ng/dl). Although her primary physician noted no CH-related symptoms, her distal femoral epiphyseal ossification center was absent, suggesting her hypothyroid status. Ultrasonography showed hypoplastic thyroid (−2.5 S.D.; Supplementary Figure 1). Immediately after the evaluation, L-T_4 replacement was started. At age 3 years, repeated ultrasonography showed hypoplastic thyroid (−3.5 S.D.) with normal echogenicity (Supplementary Figure 1).

Patient 5, a mother of the proband, was born before the implementation of newborn screening for CH. She had normal height (153 cm), had no obvious intellectual problem, and had no apparent symptoms of hypothyroidism. We evaluated her thyroid function at age 34 years as a family study of the mutation. She had an elevated serum TSH level (161 mU/l), a low free T_4 level (0.3 ng/dl), and a high Tg level (360 ng/ml). A hypoplastic normoechoic thyroid (volume, 1.4 ml) was shown by ultrasonography (Supplementary Figure 1). Thereafter, she has been receiving L-T_4 replacement therapy.

Family 3 The proband (Patient 6; a boy) had a high blood spot TSH level (17.5 mU/l) at newborn screening. At age 41 days, thyroid function test showed a slightly high serum TSH level (15.6 mU/l) with a marginally low free T_4 level (0.9 ng/dl). He did not have CH-related symptoms. Ultrasonography revealed a hypoplastic hypoechoic thyroid (−2.5 S.D.; Supplementary Figure 1). L-T_4 replacement was started. At age 6 years, we reevaluated his thyroid status with transient discontinuation of treatment. He had permanent CH with serum TSH 13.8 mU/l, free T_4 0.9 ng/dl, and Tg 28 ng/ml. He had normal ^{123}I uptake (19.7% at 24 h), although perchlorate discharge rate was slightly high (12.8%). Ultrasonography showed a normal-sized hypoechoic gland (−1.3 S.D.; Supplementary Figure 1).

Patient 7, a father of the proband, was born before the implementation of newborn screening. He had normal height (171 cm), had no intellectual problems, and was euthyroid physically. We evaluated his thyroid status at age 39 years as a family study of the mutation. He had a slightly high serum TSH level (5.6 mU/l), a slightly

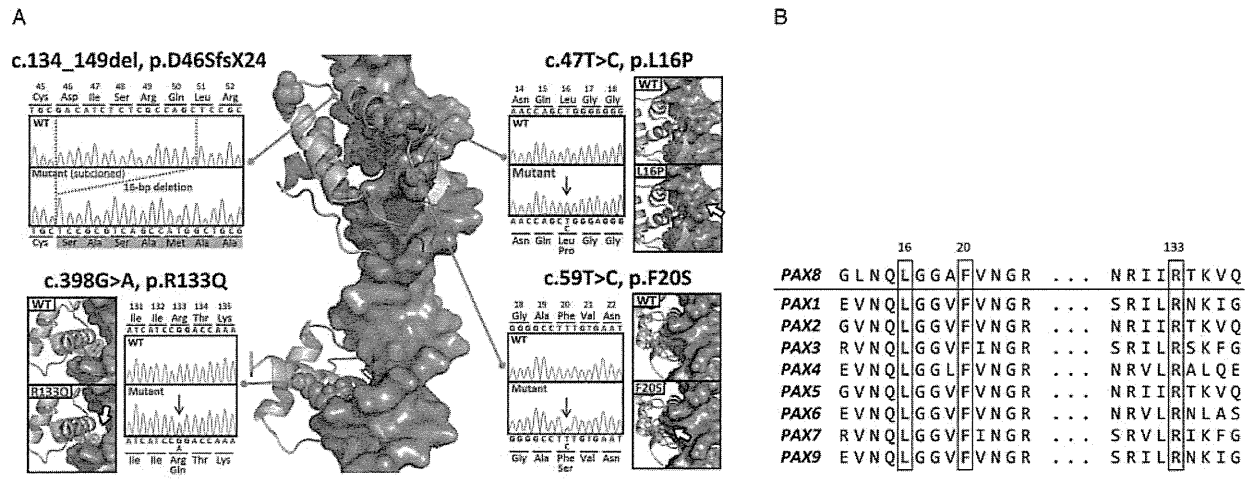

Figure 2 Location and impact of four novel *PAX8* mutations. (A) Three-dimensional structure of the DNA-binding paired domain (colored in gold) and its target DNA (colored in silver) based on the crystal structure data of PAX6–DNA complex. The domain consists of two β-sheets and six α-helices. Side chains of residues corresponding to 11 missense mutations (previously reported ones in turquoise (n=8) and novel ones found in this study in orange (n=3)) are shown as spheres. The eight previous mutations are located among three α-helices (α1–α3), whereas the three novel ones (L16P, F20S, and R133Q) are located outside the region. The D46fs mutation deletes normal protein sequence after the α1 helix. Representative chromatograms of each novel mutation are shown, along with the results of computational modeling of mutant structures. Note that the three missense mutations are predicted to affect protein–DNA interaction (indicated by white arrows). (B) Single-letter amino acid ClustalW alignments of residues surrounding Leu16, Phe20, and Arg133. The mutated residues, which are conserved in the Pax gene family, are colored in red.

low free T_4 level (0.8 ng/dl), and a slightly high Tg level (32 ng/ml). Ultrasonography showed a hypoplastic thyroid (volume, 5.4 ml) with low echogenicity (Supplementary Figure 1).

Family 4 The proband (Patient 8; a girl) had a high blood spot TSH level (40.6 mU/l) at newborn screening. She had no CH-related symptoms. The size of distal femoral epiphyseal ossification center was normal. Thyroid function test showed a high serum TSH level (33.2 mU/l) accompanied by a normal free T_4 level (1.4 ng/dl). She had been followed without treatment because her free T_4 levels were normal but was finally started on L-T_4 replacement at age 10 months (TSH, 13.9 mU/l; free T_4, 1.1 ng/dl). Reassessment of her thyroid status with discontinuing therapy at age 5 years confirmed permanent CH (TSH, 18.0 mU/l; free T_4, 1.2 ng/dl). ^{123}I uptake was normal (20.6% at 24 h). Perchlorate discharge test was not performed. Thyroid ultrasonography performed at age 13 years showed a hypoplastic gland (−3.5 S.D.) with low echogenicity (Supplementary Figure 1).

Mutation detection

We identified four novel heterozygous *PAX8* mutations in the four probands, including three missense mutations (c.47T>C, p.L16P in Family 1; c.59T>C, p.F20S in Family 2; c.398G>A, p.R133Q in Family 4) and one frameshift mutation (c.134_149del, p.D46SfsX24 in Family 3) (Fig. 2A). The four mutations were located in the paired domain (Fig. 2A) and were absent in 100 control individuals. Leu16, Phe20, and Arg133 are conserved among the PAX family genes (Fig. 2B). Computational modeling of the missense mutants predicted that they cause loss of contacts between the paired domain and its target DNA (Fig. 2A). Family studies revealed that the mutation was transmitted by either father or mother in three families (Families 1–3) and occurred *de novo* in Family 4 (Fig. 1).

In vitro *functional analyses*

To clarify the molecular pathogenesis of each mutant *PAX8*, we conducted a series of *in vitro* functional analyses using the human cervical cancer-derived HeLa cell line. Western blotting of myc-tagged PAX8 proteins showed that the protein expression level of D46fs was negligible, whereas those of the remaining three (L16P, F20S, and R133Q) were comparable with that of WT (Fig. 3A, left panel). When we treated transfected cells with the proteasome inhibitor MG132, we could detect the myc-tagged D46fs mutant, of which molecular weight was about 10 kDa, suggesting that the mutant protein was degraded via the proteasome-dependent pathway (Fig. 3A, right panel). Visualization of subcellular localization of the EGFP-tagged PAX8 proteins revealed that L16P, F20S, and R133Q were localized in the nucleus and were colocalized normally with TTF1, another thyroid-specific transcription factor (Fig. 3B). Electrophoretic mobility shift assay (EMSA) with two PAX8 response elements (oligo-CT and oligo-C) showed abrogated DNA-binding abilities of L16P, F20S, and R133Q on the two elements (Fig. 3C).

We assessed the effect of each mutation on target gene transactivation using a luciferase reporter driven by the *TG* promoter. To recapitulate interaction between WT and mutant PAX8, and interaction between PAX8 and TTF1 (i.e. synergistic transactivation (17)), various patterns of cotransfection were tested: mutant PAX8 only or WT-mutant cotransfection, each with or without coexpressed TTF1. In the absence of TTF1, the four mutants alone showed negligible transactivation (Fig. 3D, gray bars). Transactivating capacities measured by WT-mutant cotransfection (WT 5 ng; mutant 5 ng) were significantly lower than that derived from 10 ng of WT and were comparable with that derived from 5 ng of WT (Fig. 3D, stripe bars). This indicates that each mutant did not interfere with the transactivation of WT in WT-mutant cotransfection. In the presence of coexpressed TTF1, L16P, F20S, and R133Q had partial transactivating capacities, while D46fs did not (Fig. 3E, gray bars). However, when the four mutants were further cotransfected with WT PAX8 (WT 5 ng; mutant 5 ng), the transactivation levels of *TG*-luc were significantly lower than that derived from 10 ng of WT and were again comparable with that derived from 5 ng of WT (Fig. 3E, stripe bars).

Discussion

The thyroid phenotypes of the eight mutation carriers were considerably variable, regarding i) thyroid function (overt to subclinical hypothyroidism), ii) gland size (small to normal), and iii) gland echogenicity (low to normal). It is noteworthy that chronological changes were observed in several cases. Patients 1 and 2 had normal blood spot TSH levels in newborn screening but developed hypothyroidism thereafter. One similar screening-negative case has been described in the literature (10). As for Patient 1, thyroid ultrasonography was performed sequentially at ages 3 and 9 years and showed that her thyroid grew only minimally. This finding is consistent with our previous clinical observation suggesting that thyroid follicular growth is sensitive to a *PAX8* mutation (18). Another observation implying the chronological phenotypic changes in mutation carriers is the paradoxical clinical history of Patient 5. She presumably had compensated hypothyroidism in her childhood because she had apparently normal adult height and intelligence. Thus, her overt hypothyroidism, which was confirmed at age 34 years, should have developed in adulthood. Collectively, we presume that deleterious effects of *PAX8*

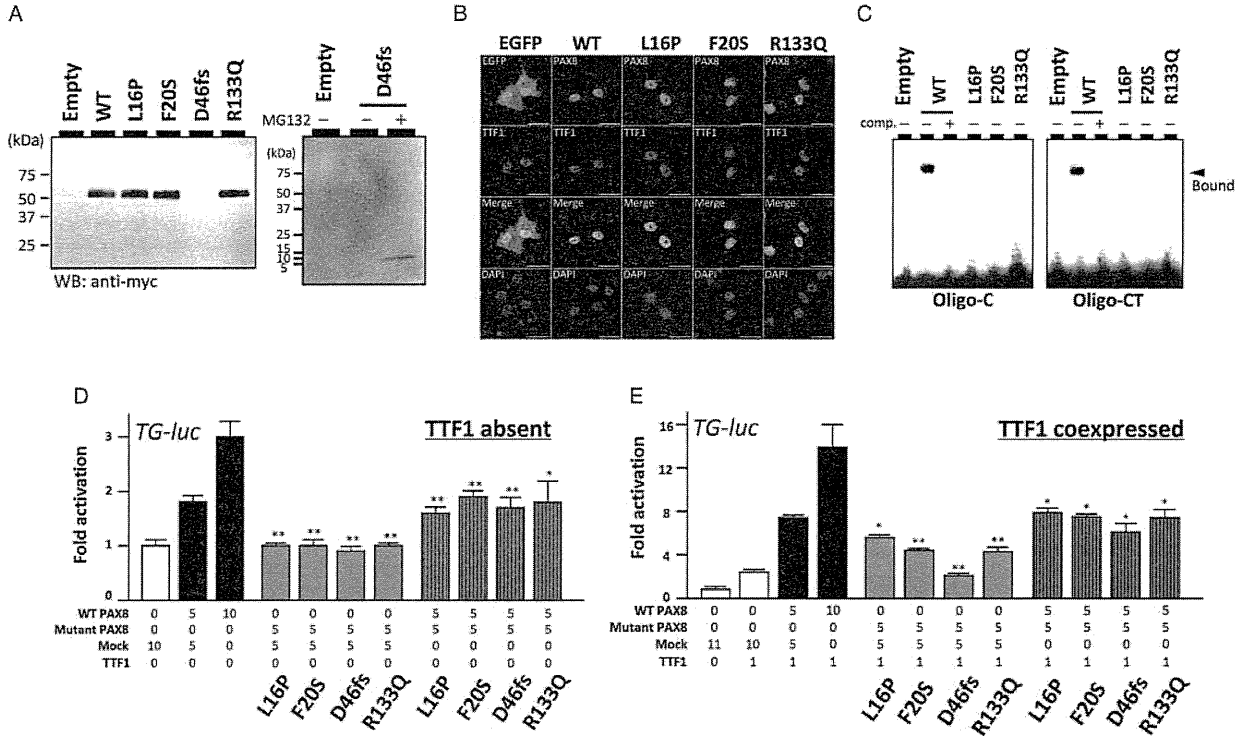

Figure 3 Functional characterization of four novel *PAX8* mutations. (A) Protein expression levels of myc-tagged PAX8 (wild type (WT) or mutant) were assessed by western blotting using a monoclonal anti-myc antibody. The protein expression level of D46fs was negligible, while those of the remaining three were comparable with that of WT (left panel). The D46fs mutation could be detected by western blotting with use of cell lysate extracted from MG132-treated HeLa cells (right panel), indicating that the mutation was degraded via proteasome-dependent pathway. (B) Subcellular localization analyses. Each enhanced green fluorescent protein-tagged PAX8 construct (WT or mutant) was cotransfected with red fluorescent protein-tagged thyroid transcription factor-1 (TTF1). Merged images show colocalization of TTF1 and each PAX8 protein. Bars indicate 50 μm. (C) DNA binding abilities of each PAX8 protein on two PAX8 response elements (oligo-CT and oligo-C) were tested by electrophoretic mobility shift assays. WT showed specific binding to the elements, which was competed by excess amount of cold competitors. Each mutant showed no binding on these two elements. (D and E) Transactivation activities of each PAX8 protein were assessed with use of the *TG*-luc reporter. HeLa cells grown in a 96-well plate were transfected with indicated amount (in nanogram) of the effector plasmid(s). Firefly luciferase activities were represented relative to the activity of the empty vector. Panel D displays the results comparing WT (black) and the four mutants (gray) in the absence of TTF1. WT transactivated the *TG*-luc in a dose-dependent manner. The four mutants showed negligible transactivating capacities. WT-mutant contransfection experiments showed no dominant negative effect. Panel E shows the results obtained in the presence of coexpressed TTF1 (1 ng). In this condition, three missense mutants showed various levels of transactivation, whereas D46fs remained nonfunctional. However, the transactivation levels derived from WT-mutant cotransfection (5 ng each) were comparable to that of WT only (5 ng). Data are representative of three independent experiments (each performed in quadricate) with similar results. Values are mean ± S.E.M. The results of mutant-only transfection (5 ng) were compared with that of equimolar WT (5 ng), while those of WT-mutant cotransfection (total amount 10 ng) were compared with that of equimolar WT (10 ng). $^{*}P<0.05$, $^{**}P<0.01$.

mutations can exacerbate over time, resulting in significant chronological changes in disease phenotypes.

In this study, two types of *PAX8* mutations were found: mutations with defective DNA binding (L16P, F20S, and R133Q) and a mutation with defective protein stability (D46fs). The former type seems to be the predominant mechanism of *PAX8* mutations: ten out of 12 previously reported mutations are amino acid-altering ones located in the paired domain (4, 7, 8, 9, 10, 11, 12, 13). The paired domain consists of two β-turns (β1 and β2) and six α-helices (α1–α6). All previously reported missense mutations are confined between α1 and α3 helices (i.e. mutational hotspot; Fig. 2A). L16P (between β1 and β2), F20S (β2), and R133Q (α6) are the first mutations that are located outside this hotspot. These data imply the importance of the β-turns and α6-helix of PAX8 in target DNA binding. As for the latter type, the D46fs mutation lacks 90% of normal protein sequence and acquires extra 23 frameshifted sequence. The results of western blotting with use of MG132 implied that the mutant was degraded via the proteasome-dependent pathway. Considering that the D46fs mutant consists of about 30% abnormal protein sequence, we speculate that the mutant was misfolded and was subject to endoplasmic reticulum-associated degradation. The D46fs mutation showed abrogated transactivation on the *TG* promoter irrespective of experimental conditions, supporting that it is actually a 'null' mutation. Our clinical and molecular data clearly demonstrate that one 'null'

PAX8 allele is enough to cause CH in humans (i.e. haploinsufficiency).

L16P, F20S, and R133Q had negligible transactivating capacities on the *TG* promoter in the TTF1 absent condition but showed significant transactivation when TTF1 is coexpressed. This 'rescue', which has been observed in three mutant PAX8 (S48F (11), H55Q (13), and K80_A84dup (12)) and one mutant TTF1 (P210L (19)), is likely based on the formation of PAX8–TTF1 complex on the *TG* promoter (20). However, no previous reports have tested whether those mutants could form the transcriptional complex. We suppose that demonstration of formation of the transcriptional complex containing the mutants, such as co-immunoprecipitation studies, will be required to verify the model.

In conclusion, we report clinical and molecular findings of four novel *PAX8* mutations, including three missense mutations located outside the previously recognized mutational hotspot and the first experimentally confirmed mutation with protein instability (D46fs). Our data imply that *PAX8* haploinsufficiency is enough to cause CH in humans.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-12-0410.

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.

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Gradual Loss of ACTH Due to a Novel Mutation in *LHX4*: Comprehensive Mutation Screening in Japanese Patients with Congenital Hypopituitarism

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Abstract

Mutations in transcription factors genes, which are well regulated spatially and temporally in the pituitary gland, result in congenital hypopituitarism (CH) in humans. The prevalence of CH attributable to transcription factor mutations appears to be rare and varies among populations. This study aimed to define the prevalence of CH in terms of nine CH-associated genes among Japanese patients. We enrolled 91 Japanese CH patients for DNA sequencing of *POU1F1*, *PROP1*, *HESX1*, *LHX3*, *LHX4*, *SOX2*, *SOX3*, *OTX2*, and *GLI2*. Additionally, gene copy numbers for *POU1F1*, *PROP1*, *HESX1*, *LHX3*, and *LHX4* were examined by multiplex ligation-dependent probe amplification. The gene regulatory properties of mutant LHX4 proteins were characterized *in vitro*. We identified two novel heterozygous *LHX4* mutations, namely c.249-1G>A, p.V75I, and one common *POU1F1* mutation, p.R271W. The patient harboring the c.249-1G>A mutation exhibited isolated growth hormone deficiency at diagnosis and a gradual loss of ACTH, whereas the patient with the p.V75I mutation exhibited multiple pituitary hormone deficiency. *In vitro* experiments showed that both *LHX4* mutations were associated with an impairment of the transactivation capacities of *POU1F1* and*αGSU*, without any dominant-negative effects. The total mutation prevalence in Japanese CH patients was 3.3%. This study is the first to describe, a gradual loss of ACTH in a patient carrying an *LHX4* mutation. Careful monitoring of hypothalamic–pituitary -adrenal function is recommended for CH patients with *LHX4* mutations.

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Introduction

The proliferation and terminal differentiation of the anterior pituitary gland is strongly influenced by the precise spatial and temporal expression of transcription factors [1–3]. Mutations in these transcription factors often result in various types of congenital hypopituitarism (CH) [1–3]. Although previous studies have shown that these transcriptional factor mutations are rare among CH patients and that the mutation prevalence varies among populations, only a few genetic screening studies have been conducted. Graaff *et al.* identified a single patient with a *POU1F1* mutation from a study population of 79 multiple pituitary hormone deficiency (MPHD) patients (1.2%) in The Netherlands [4], and Dateki *et al.* reported one patient harboring an *LHX4* gross deletion from a cohort of 71 MPHD patients (1.4%) in Japan [5]. On the other hand, Reynaud *et al.* reported a mutation prevalence of 13.3% in a study population of 165 MPHD patients from the international GENHYPOPIT network [6]. Approximately 90% of the mutations identified in this report were *PROP1* common mutations (149delGA and 296delGA). Although the 296delGA mutation represents a mutational hot spot within the *PROP1* gene rather than a common founder mutation [7], studies from other ethnic groups often report a low prevalence of *PROP1* mutations [8,9].

This study aimed to determine the prevalence of transcription factor mutations in Japanese CH patients with PCR-based sequencing of nine CH-associated genes, namely *POU1F1*, *PROP1*, *HESX1*, *LHX3*, *LHX4*, *SOX2*, *SOX3*, *OTX2*, and *GLI2*. Additionally, we examined the gene copy numbers of *POU1F1*, *PROP1*, *HESX1*, *LHX3*, and *LHX4* by multiplex ligation-dependent probe amplification (MLPA).

Materials and Methods

Subjects

This study population consisted of 91 patients with GH-treated CH. The inclusion criteria were as follows: 1) short statue with severe GH deficiency (GH peak < 3 ng/mL) confirmed by hypoglycemic provocation test, and 2) anterior pituitary hypoplasia as detected by brain magnetic resonance imaging (MRI). We excluded any CH patients of known cause, such as a brain tumor or brain surgery from this study. Patients or parents of patients under 18 years of age gave their written informed consent to

Table 1. Endocrine phenotype of 91 probands screened for 9 genes.

	No. (%) with deficiencies of			
	GH	TSH	ACTH	LH/FSH
IGHD (n = 14)	14(100)			
MPHD (n = 77)	77(100)	61(79)	34(44)	19(24)

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participate in this study, which was approved by the Institutional Review Board of Keio University School of Medicine and the Institutional Review Board of Kanagawa Children's Medical Center.

Endocrinological investigations

Hormonal assays were performed using several commercial RIA kits, and normal values for each center were taken into account. The results of biochemical investigations at diagnosis were recorded including basal free thyroxine (fT4), TSH, cortisol and ACTH levels, their peaks in response to pituitary stimulation tests. The patients were evaluated for serum GH level after two consecutive classical provocative tests (with arginine or insulin). GH peaks <6 ng/mL after stimuli support a diagnosis of GHD. GH peak < 3 ng/mL by hypoglycemic provocation test define severe GHD. A diagnosis of TSH deficiency was made if serum fT4 concentration was under the normal level (fT4 < 1.0 ng/dL) with inadequate low serum TSH concentration. Cortisol peaks <17 μg/dL by hypoglycemic provocation tests define ACTH deficiency. FSH–LH deficiency was diagnosed on the basis of delayed or absent pubertal development and inadequate increase in serum FSH and LH in response to LHRH.

Imaging investigations

MRI included T1 and T2 weighted high-resolution pituitary imaging through the hypothalamo-pituitary axis (T1 sagittal 3-mm slices, T1 and T2 coronal 3-mm slices). Details noted included the size of the anterior pituitary, position of the posterior pituitary signal, presence and morphology of the optic nerves, optic chiasm, pituitary stalk, septum pellucidum, and corpus callosum.

Mutation screening

For all patients, regardless the phenotype/pituitary MRI findings, we analyzed all coding exons and flanking introns of *POU1F1, PROP1, HESX1, LHX3, LHX4, OTX2, SOX2, SOX3*, and *GLI2* by PCR-based sequencing. We screened for deletion/duplication involving *POU1F1, PROP1, HESX1, LHX3*, and *LHX4* by MLPA analyses (SALSA MLPA KIT P216; MRC-Holland, Amsterdam, The Netherlands). We tested any detected sequence variations against 150 Japanese control subjects.

RT-PCR

For mRNA analysis of the *LHX4* c.249-1G>A mutation, total RNA was extracted from Epstein-Barr virus-transformed lymphocytes derived from the propositus of pedigree 1. The cDNA produced from reverse transcription of RNA was subjected to PCR amplification using primers encompassing exons 2 to 4, and were subsequently processed for direct sequencing.

Functional studies

We performed functional studies on the two novel *LHX4* mutations (p.R84X and p.V75I). To generate LHX4 expression vectors, LHX4 cDNA was cloned into pCMV-myc and pEGFP-N1 (Clontech, Palo Alto, CA). We introduced the two mutations by site-directed mutagenesis, using the PrimeSTAR Mutagenesis Basal Kit (TaKaRa, Otsu, Japan). The luciferase reporter vectors were constructed by inserting the promoter sequences of *POU1F1 (PIT1)*,*αGSU* into a pGL3 basic vector (Promega, Madison, WI). A transactivation assay was performed using dual-luciferase reporter assay system (Promega) on COS7 and GH3 cells. For western blot analyses, we harvested COS7 cells transfected with the myc-tagged LHX4. Western blotting was performed with a mouse anti-myc monoclonal antibody (Invitrogen, Carlsbad, CA). For subcellular localization analyses, we visualized and photographed COS7 cells transfected with GFP-tagged LHX4 using a Leica TCS-SP5 laser scanning confocal microscope (Leica, Exton, PA). The sequences of the biotin-labeled doublestranded oligonucleotide used as probe in the EMSA experiment was 5′-GTATGAATCATTAATTGA-CAACATATTTTC-3′, as described previously [10]. The probes were detected with the Lightshift chemiluminescent EMSA kit (Pierce) according to the manufacturer's instruction.

Results

Patient details

Of the 91 patients, on the basis of hormonal deficiencies, 14 were determined to have isolated GH deficiency (IGHD), whereas 77 were MPHD. Detailed endocrine phenotype was available in all of the 91patients (Table 1). Results of the MRI scans were available in all patients with IGHD and MPHD. Details regarding the structural abnormalities of the hypothalamo-pituitary axis on neuroimaging in the probands are shown in Table 2. Among 77 MPHD patients, 12 were diagnosed as Septo-optic dysplasia.

Mutation screening

We identified two novel heterozygous *LHX4* mutations, namely c.249-1G>A, expected to cause exon skipping, and c.223G>A (p.V75I), and one common heterozygous *POU1F1* mutation,

Table 2. Results of MR scans of probands screened for 9 genes.

	Morphology of						
	Anterior pituitary	Posterior pituitary			Stalk		
	Hypoplasia	Normal	Ectopic	Absent	Normal	Invisible	Thin
IGHD (n = 14)	14	5	9	0	4	5	5
MPHD (n = 77)	77	24	51	2	23	25	29
Total (n = 91)	91	29	60	2	27	30	34

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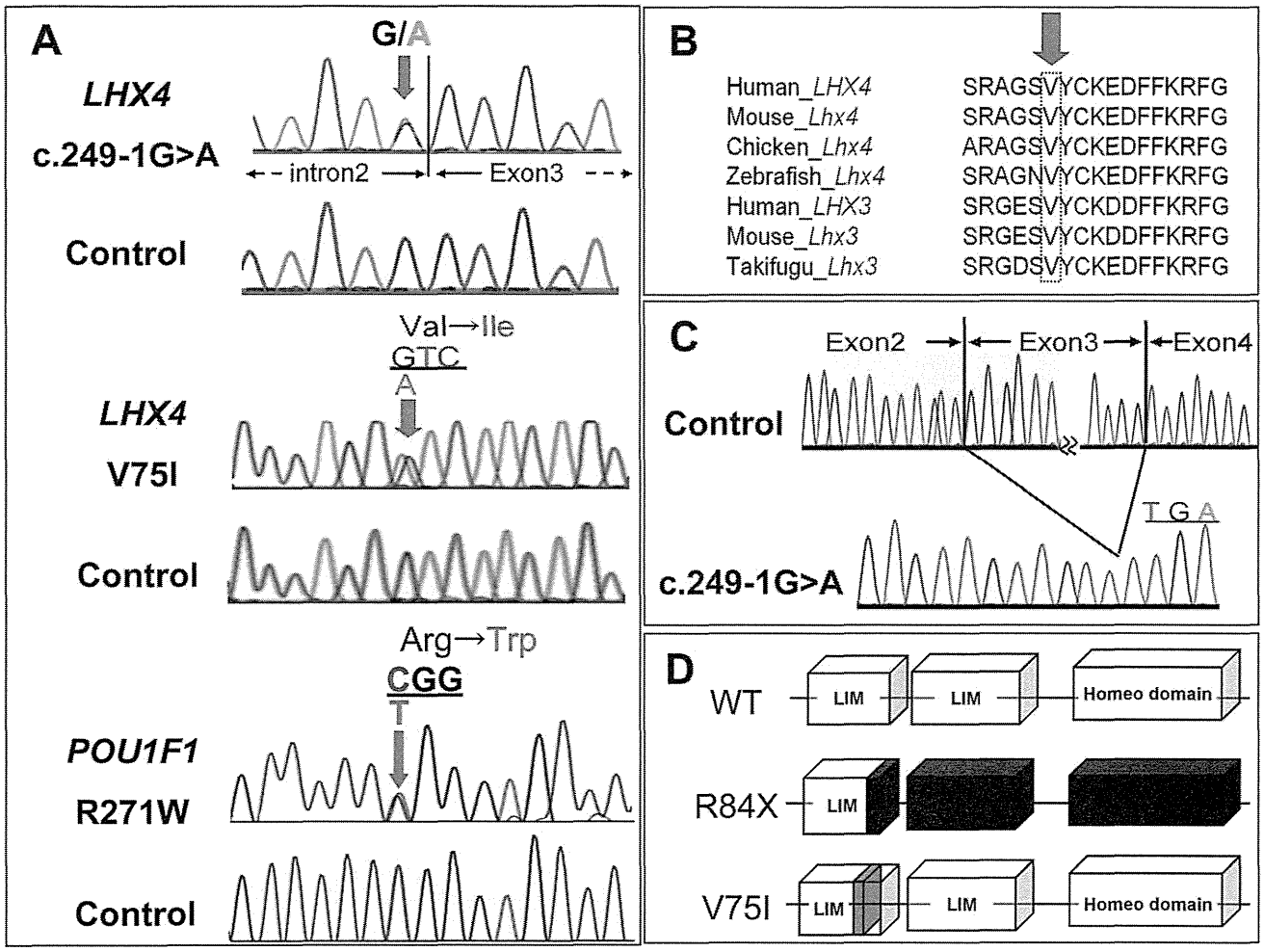

Figure 1. Identification of sequence variations of *LHX4* and *POU1F1*. *A*, Partial sequences of PCR products of the patients are shown. The upper chromatogram represents a heterozygous G to A substitution in the splice acceptor site of exon3. The middle chromatogram represents a heterozygous substitution of isoleucine (ATC) in place of valine (GTC) at codon 75. The arrow indicates the mutated nucleotide. The lower chromatogram represents a heterozygous substitution of tryptophan (TGG) in place of arginine (CGG) at codon 271. The arrow indicates the mutated nucleotide. *B*, Homology study showed valine at codon 75 is highly conserved through species in LHX4 and LHX3. C, Identification of exon3 skipping in the LHX4 cDNA derived from propositus of pedigree 1. LHX4 transcript with a deleted exon 3 creates a premature stop codon at the beginning of the remaining exon 4 (p.R84X). *D*, Schematic diagrams of the LHX4 protein. LHX4 cDNA encodes two LIM domains and one homeodomain. LHX4 with a p.R84X mutation results in the deletion of one of the two LIM domains and the entire homeodomain. Val75 is located within the first LIM domain.
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c.811C>T (p.R271W) [11] (FIG. 1A). The V75 in LHX4 is evolutionarily highly conserved (FIG. 1B), and these two *LHX4* mutations were not detected in any of the 150 healthy Japanese controls. We detected no gross or exon-level deletions/duplications using the MLPA analyses. For 14 IGHD patients, we additionally analyzed all coding exons and flanking introns of *GH1*, and *GHRHR* by PCR-based sequencing and MLPA (SALSA MLPA KIT P216 included all exons of *GH1* and *GHRHR*), failing to detect any sequence variation.

RT-PCR

The RT-PCR generated a product of smaller size than that obtained from a control sample. Sequencing revealed that it corresponded to a LHX4 transcript skipping exon 3 (FIG. 1C). If translated, this abnormal transcript would generate a protein lacking one of the two LIM domains (LD) and the entire homeodomain (HD), p.R84X (FIG. 1D).

Clinical phenotypes of the mutation carriers

Pedigree 1: *LHX4* c.249-1G>A (FIG. 2A). The propositus was a 16-year-old Japanese female, who was born at 39 weeks of gestation after an uncomplicated pregnancy and delivery. At birth, her length was 51.0 cm (1.2 SD) and weight 3.3 kg (0.6 SD). She was referred to us at 5 years of age because of short stature. Her height was 92.4 cm (-3.6 SD). Endocrine studies indicated that the patient had IGHD (Table 3). Brain MRI showed anterior pituitary hypoplasia, with a visible but thin stalk, and an ectopic posterior pituitary gland (EPP). No other central nervous system abnormalities were visualized. Recombinant human GH therapy was started at age 6. Her growth was responded well to GH replacement. Although she had no definite episode of adrenal insufficiency, longitudinal data showed that her blood cortisol peak, after stimulation by hypoglycemia with insulin tolerance tests, decreased gradually with age (20.5, 17.5, 16.4, and 10.0 μg/dL, at ages of 5, 13, 14, and 15 years, respectively, Ref. >17 μg/

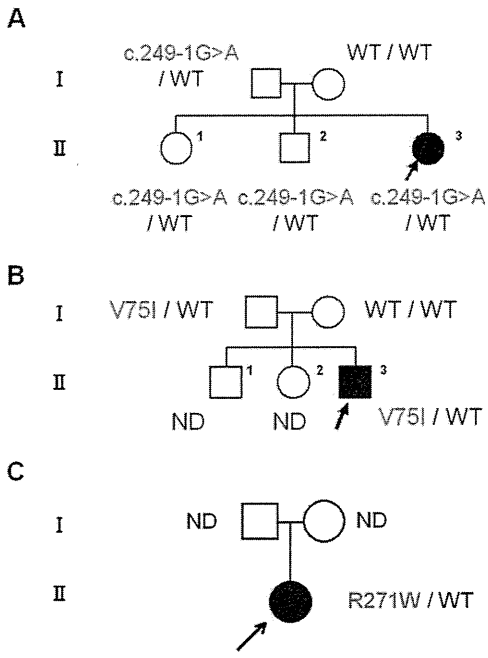

Figure 2. The pedigrees of the affected families. *A–C*, Pedigrees of families 1–3. Arrow indicates the propositus. ND: not determined.
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dL [12]), indicating of a gradual loss of ACTH. Follow-up MRI showed no changes as compared with the initial finding.

The father of the patient was 153.0 cm (-2.9 SD) tall, and the mother was 160.8 cm (0.5SD) tall. The elder brother and sister of the patient, both reached normal adult heights of 171.7 cm (0.2 SD) and 152.1 cm (-1.3 SD), respectively. Genetic analyses showed that the propositus, siblings and father carried the heterozygous *LHX4* c.249-1G>A mutation. No family members had any baseline hormonal abnormalities (Table 4).

Pedigree 2: *LHX4* p.V75I (FIG. 2B). The propositus was a 13-year-old Japanese male born at 41 weeks of gestation after an uncomplicated pregnancy and delivery. At birth, his length was 51.0 cm (1.0 SD) and weight 3.3 kg (0.7 SD). He was referred to us at 3 months of age because of a micropenis and bilateral cryptorchidism. He had undetectable plasma testosterone and LH levels, indicating hypogonadotropic hypogonadism. Severe growth failure was observed at the age of 11 months. Hormonal data revealed GH and TSH deficiencies in addition to tentative gonadotropin deficiency (Table 5). Brain MRI exhibited anterior pituitary hypoplasia, poorly developed sella turcica, visible but thin stalk, and EPP. No other central nervous system abnormalities were visualized. Replacement therapy with thyroxine and recombinant human GH was started at the age of 1 year. The patient responded well to GH replacement. At the age of 13 years, he showed small intrascrotal testes (1 ml), no pubic hair (P1), and a microphallus with low concentration of basal testosterone (0.05 ng/mL Ref: 2.0–7.5).

Table 3. Endocrinological findings in Propositus of pedigree 1.

		5yr			15yr			Reference	
	Stimulus	**Basal**		**Peak**	**Basal**		**Peak**	**Basal**	**Peak**
GH (ng/ml)	Insulin	2.7	→	2.9	1.8	→	2.6		>6
TSH (mIU/ml)	TRH	2.88	→	10.01	0.78	→	7.42		10–35
LH (mIU/ml)	LHRH	<0.2	→	2.8	6.7	→	21.2	<0.1[a]	1.93–4.73 [a]
								<0.10–2.65[b]	6.69–22.51 [b]
FSH (mIU/ml)	LHRH	0.5	→	15.5	7.0	→	9.6	0.64–3.03[a]	13.15–46.95[a]
								1.81–7.31[b]	8.58–17.62[b]
PRL (ng/ml)	TRH	10.4	→	19.7	5.7	→	28.1	1.7–15.4	increase 2 times
ACTH (pg/ml)	Insulin	44	→	46	7.3	→	14.9	9.8–27.3	28–130.5
Cortisol (μg/dl)	Insulin	19.1	→	20.5	7.5	→	10.0		>19.8[c]
									>17.0[d]
IGF-1 (ng/ml)		70.1			241			74–230[e]	
								262–510[f]	
Free T4 (ng/dl)		1.1			1.0			1.0–1.95	
Free T3 (pg/ml)		4.2			2.1			2.23–5.30	
Estradiol (pg/ml)					28			12.3–170[g]	

The conversion factors to the SI unit are as follows: GH 1.0 (μg/liter), LH 1.0 (IU/liter), FSH 1.0 (IU/liter), TSH 1.0 (mIU/liter), prolactin 1.0 (μg/liter), ACTH 0.22 (pmol/liter), cortisol 27.59 (nmol/liter), IGF-I 0.131 (nmol/liter), free T4 12.87 (pmol/liter), free T3, 1.54 (pmol/liter), and estradiol 3.671 (pmol/liter).
[a]Reference data of pre-pubertal Japanese girls [22]
[b]Reference data of pubertal (Tanner 2–3) Japanese girls [22]
[c]Reference data of UK children (younger than 10 years) [23]
[d]Reference data of UK children (older than 10 years) [23]
[e]Reference data of Japanese girls (5–7 years old) [24]
[f]Reference data of Japanese girls (15–17 years old) [24]
[g]Reference data of Japanese girls (15 years old) [25]
doi:10.1371/journal.pone.0046008.t003

Table 4. Endocrinological findings (baseline) in Family members of pedigree 1.

	Father	Mother	Brother	Sister	Reference (Adult)
GH (ng/ml)	0.7	3.2	0.5	0.4	0–23
IGF-1 (ng/ml)	110.0	156.0	357.0	276.0	Male: 41–369
					Female: 73–542
TSH (μU/ml)	0.77	1.60	0.50	0.94	0.3–3.50
Free T4 (ng/dl)	1.1	1.1	1.4	1.3	1.09–2.55
Free T3 (pg/ml)	2.5	2.6	3.1	3.1	3.23–5.11
LH (mIU/ml)	4.8	7.4	2.1	6.9	Male: 2.2–8.4
					Female: 1.4–15[a]
FSH (mIU/ml)	2.9	4.3	2.3	7.9	Male: 1.8–12
					Female: 3–10[a]
PRL (ng/ml)	11.2	11.2	7.8	5.5	Male: 1.5–9.7
					Female: 1.4–14.6
ACTH (pg/ml)	14	12	15	20	7.2–63.3
Cortisol (μg/dl)	8.2	6.3	10.3	10.3	7.6–21.4
Estradiol (pg/ml)		397		23	Female: 11–230[a]
Testosterone (ng/ml)	5.19		5.56		Male: 2.01–7.50

[a]Follicular phase

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The patient's father was 160.5 cm (-1.8 SD) tall. Genetic analyses showed that the propositus and father carried the same heterozygous *LHX4* p.V75I mutation. No other family member was available for genetic studies. Evaluation of the hormonal data for the father was refused.

Pedigree 3: *POU1F1* p.R271W (FIG. 2C). The propositus was a 28-year-old Japanese female, who was born at 37 weeks of gestation after an uncomplicated pregnancy and delivery. At birth, her length was 48.0 cm (-0.2 SD) and weight 2.6 kg (-1.0 SD). She was referred to us at 2 years of age because of severe short stature (-4.5 SD). Endocrine studies indicated that the patient had complete GH and PRL deficiencies and partial TSH deficiency (free T4 0.8 ng/dl, Ref. >1.0, with inadequate low TSH). Brain MRI at the age of 7 years exhibited anterior pituitary hypoplasia, normal stalk, and normal posterior pituitary gland. No other

Table 5. Endocrinological findings in Propositus of pedigree 2.

		11month			8yr			Reference	
	Stimulus	Basal		Peak	Basal		Peak	Basal	Peak
GH (ng/ml)	Insulin	1.1	→	0.9	0.6	→	0.6		>6
TSH (mIU/ml)	TRH	0.56	→	6.81	2.00	→	10.81		10-35
LH (mIU/ml)	LHRH	0.3	→	0.8	0.2	→	2.3	<0.1[a]	<0.10-4.29 [a]
FSH (mIU/ml)	LHRH	2.1	→	2.6	1.5	→	7.4	0.46-1.43[a]	5.38-11.67[a]
Testosterone (ng/ml)	HCG				<0.05		0.17		>1.2[a]
PRL (ng/ml)	TRH	5.6	→	10.1	7.7	→	13.0	1.7-15.4	increase 2 times
ACTH (pg/ml)	Insulin	44	→	170	44	→	50	9.8-27.3	28-130.5
Cortisol (μg/dl)	Insulin	31.0	→	38.4	13.4	→	17.2	5-20	>19.8[b]
IGF-1 (ng/ml)		6.9			157			18-150 [c]	
								50-356[d]	
Free T4 (ng/dl)		1.1			1.1			1.01-1.95	
Free T3 (pg/ml)		4.4			3.9			2.23-5.30	

The conversion factors to the SI unit are as follows: GH 1.0 (μg/liter), TSH 1.0 (mIU/liter), LH 1.0 (IU/liter), FSH 1.0 (IU/liter), testosterone, 0.035 (nmol/liter), prolactin 1.0 (μg/liter), ACTH 0.22 (pmol/liter), cortisol 27.59 (nmol/liter), IGF-I 0.131 (nmol/liter), free T4 12.87 (pmol/liter), and free T3, 1.54 (pmol/liter).

[a]Reference data of pre-pubertal Japanese boys (younger than 10 years) [22]

[b]Reference data of UK children (younger than 10 years) [23]

[c]Reference data of Japanese boys (younger than 1 years old) [24]

[d]Reference data of Japanese boys (7-9 years old) [24]

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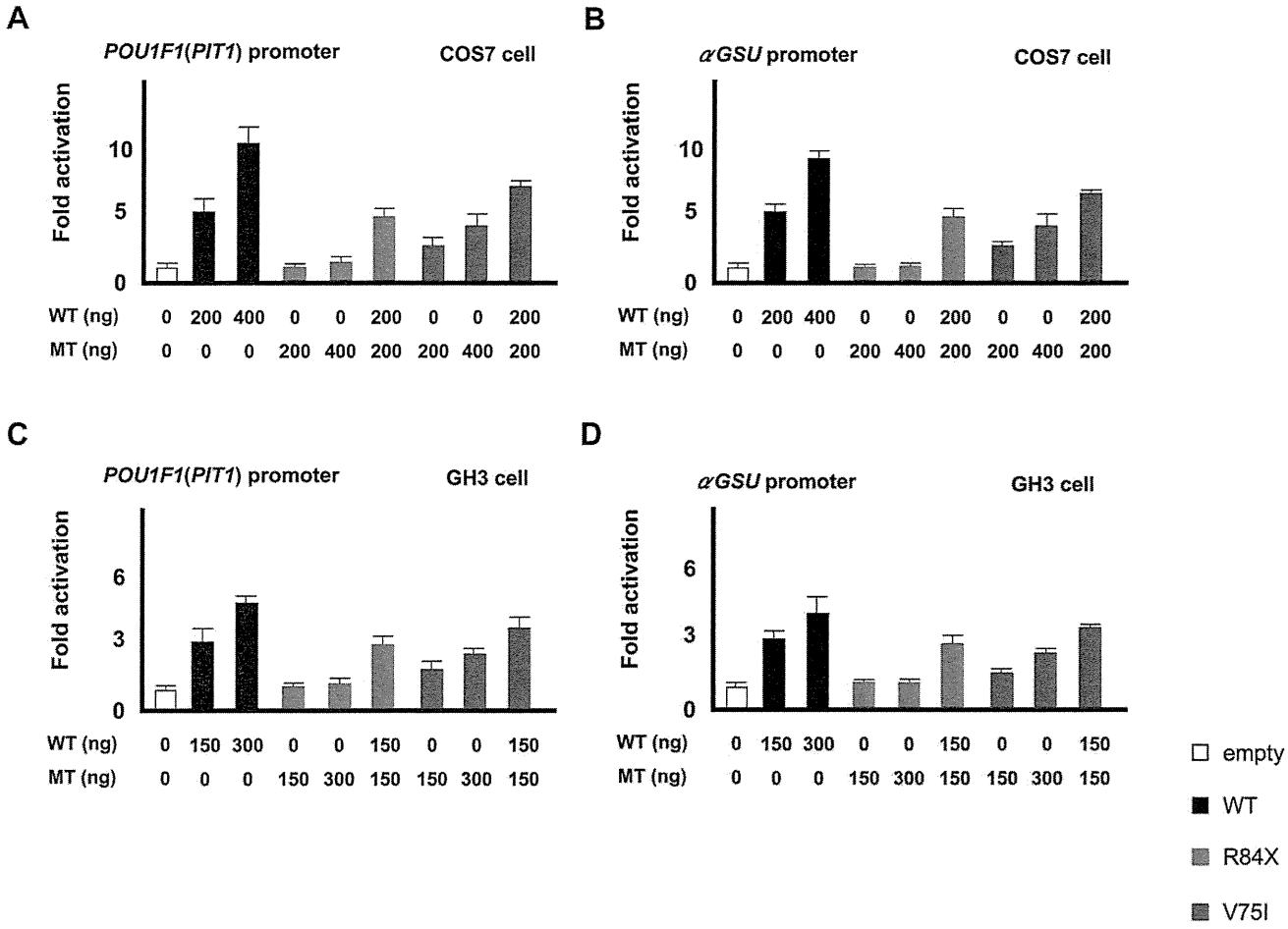

Figure 3. Transactivation assays of R84X and V75I LHX4 using *POU1F1*(*PIT1*) and*αGSU* reporter. *A and B*: COS7 cells were cotransfected with the pRL-CMV internal control vector, indicated amount (nanograms) of the effector plasmids, and the *POU1F1*(A) or*αGSU* (B) reporter. The data are the mean ± s.e.m. of at least three independent experiments performed in triplicate transfections. The white, black, red, and blue bars indicate the data of the empty expression vectors, expression vectors with wild type (WT) LHX4, expression vectors with R84X LHX4, and V75I LHX4, respectively. R84X LHX4 exhibited markedly reduced transactivation, whereas V75I LHX4 retained partial activity. The two mutants did not exhibit any dominant negative effect. The data are mean ± SEM of at least three independent experiments performed in triplicate transfections. *C and D*: GH3 cells were cotransfected with the pRL-CMV internal control vector, indicated amount (nanograms) of the effector plasmids, and the *POU1F1*(C) or*αGSU* (D) reporter.
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central nervous system abnormalities were visualized. The patient responded well to GH replacement.

Functional studies

Both in COS7 and GH3 cells, wild type LHX4 stimulated transcription of the *POU1F1* and*αGSU* reporters in a dose-dependent manner. R84X LHX4 had markedly reduced transactivation, whereas V75I LHX4 retained partial activity (FIG. 3A-D). The two mutants had no dominant negative effect. Western blot analysis showed that the expression of V75I LHX4 was comparable to that of the wild type, whereas R84X LHX4 was not detected (FIG. 4A). The V75I LHX4 mutant localized to the nucleus (FIG. 4B). WT LHX4 showed specific binding to the elements, which were competed by excess amount of (200 times) cold competitors. The V75I LHX4, which has an intact HD, bound with similar or slightly high efficiency to the WT LHX4 (FIG. 4C).

Discussion

In the present study, our mutation prevalence data (three mutation carriers in a total of 91 CH patients: 3.3%) is comparable with earlier report of Graaff *et al.* (1.2%) [4] or Dateki *et al* (1.4%) [5]. This study enrolled CH patients that fulfilled two definite inclusion criteria: 1) severe GH deficiency (GH peak < 3 ng/mL) confirmed by hypoglycemic provocation tests, which included IGHD and MPHD, and 2) anterior pituitary hypoplasia based on brain MRI. The subjects included in the two previous reports were diagnosed with MPHD and the reports of Dateki *et al.* did not describe any specific inclusion criteria. As *PROP1* common mutations (149delGA and 296delGA) are rare in Japan, our prevalence data were lower than that of Reynaud *et al.* [6]. These previous studies did not include screening for *SOX2*, *SOX3*, *OTX2* and *GLI2* (although the study by Dateki *et al.* included *SOX3* and *OTX2*), thus this study serves as the first report to include these genes. Despite extending the range of our genetic screening, our results imply the rarity of pathological abnormalities in the

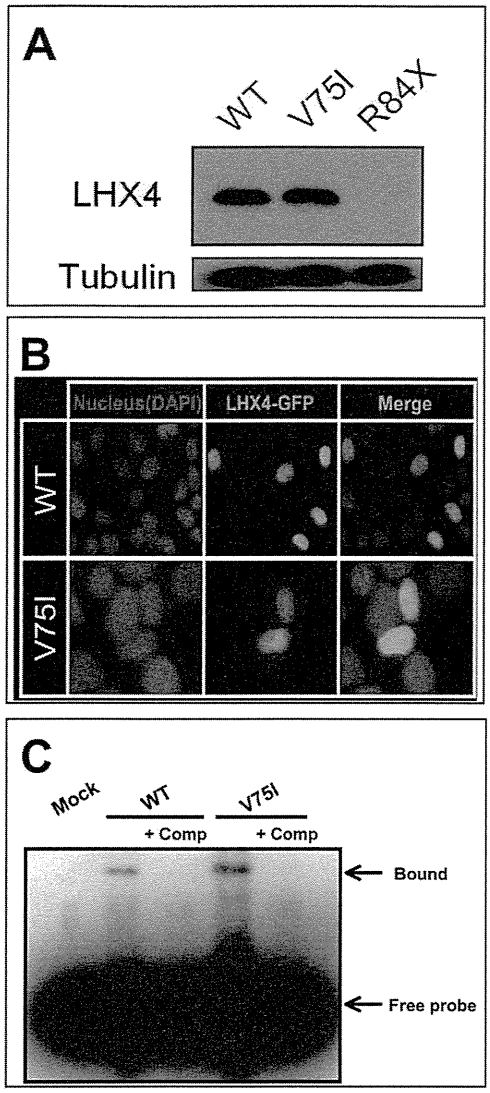

Figure 4. Functional characterization of two mutant LHX4. *A*, Protein expression level of myc-tagged WT and two LHX4 mutants was assessed by western blot using a monoclonal anti-myc antibody. The expression of V75I LHX4 was comparable to that of WT, whereas R84X LHX4 was not detected. Tubulin was used as a control. *B*, Subcellular localization analysis. For subcellular localization analyses, we visualized and photographed COS7 cells transfected with GFP-tagged LHX4 using a Leica TCS-SP5 laser scanning confocal microscope, after mounting the cells in Vectashield-DAPI solution. The WT and V75I LHX4 are localized to the nucleus. *C*, EMSA experiments. WT LHX4 showed specific binding to the elements, which was competed by excess amount of (200 times) cold competitors. The V75I LHX4, which has an intact HD, bound with similar or slightly high efficiency to the WT LHX4.
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currently known genes responsible for CH. Further studies are required to understand the pathogenesis of CH.

To date, eight families carrying a *LHX4* mutation have been reported [5,13–17]. We identified two novel mutations in *LHX4* (c.249-1G>A, p.V75I). Although both mutations were associated with impaired transactivation of *POU1F1* and*αGSU* without dominant-negative effects, indicating haploinsufficiency, the mechanism behind the loss of function resulting from these two mutations seems to be different. We did not detect R84X LHX4 on western blotting, indicating that the protein expression is markedly reduced due to the protein's instability. On the other hand, western blotting, visualization of subcellular localization, and DNA binding test revealed no significant difference between the wild type and V75I LHX4 variant. Val75 is a highly conserved amino acid located in the LD (FIG. 1C), which is important for protein-protein interaction, suggesting that substitution of Val75 to Ile results in defective interactions with transcriptional cofactors.

A striking finding of our report is that the propositus, who carried the c.249-1G>A *LHX4* mutation, exhibited a gradual loss of ACTH. Although late onset ACTH deficiency is well known in CH patients with *PROP1* mutations [18–20] and LHX3 [21], our study showed, for the first time, that a gradual loss of ACTH should be a point of concern among CH patients with *LHX4* mutations. Thus, this study suggests careful follow-up monitoring of the hypothalamic-pituitary-adrenal function in CH patients with *LHX4* mutations even if ACTH deficiency is not apparent at first evaluation. The patient's elder brother and sister were of normal adult height and had normal baseline hormonal levels. Even though this report is not the first description of the wide phenotypic spectrum in *LHX4* mutation carriers [13–17], it is noteworthy that *LHX4* mutation carriers can clinically and endocrinologically present as normal, even though the mutation is nonfunctional. The phenotypical variation documented in this study for patients with MPHD with mutations in *LHX4*, including dissimilarity within probands from the same pedigree, is likely partly due to the impact of other genes that are important but have not been recognized in pituitary development.

In summary, we found that only 3.3% of Japanese patients had mutation. *LHX4* mutation carriers exhibit wide phenotypic variability and can present as normal clinically and endocrinologically, even though they had a nonfunctional mutation. Gradual loss of ACTH should be monitored in CH patients with *LHX4* mutations.

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Author Contributions

Conceived and designed the experiments: MT SN TH. Performed the experiments: MT. Analyzed the data: MT. Contributed reagents/materials/analysis tools: TI MI NA SN YH YA KM MA. Wrote the paper: MT TH.

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CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

Delayed Onset Congenital Hypothyroidism in a Patient With *DUOX2* Mutations and Maternal Iodine Excess

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Congenital hypothyroidism (CH), one of the most common congenital endocrine disorders, causes irreversible intellectual disability in untreated patients. Today, the vast majority of patients receive early diagnosis and treatment in the context of newborn screening for CH, and achieve satisfactory cognitive development. However, a subset of patients with delayed onset are undetectable by newborn screening, and miss benefit from early intervention. Here, we report on a delayed-onset CH patient that had two contributing factors in the pathogenesis of CH simultaneously, i.e., a genetic defect and iodine excess. The patient was exposed to excessive iodine in utero because her mother consumed massive amounts of seaweed during pregnancy. Surprisingly, the patient had a negative result in newborn screening, but developed overt CH at age 3 months. She received thyroxine supplementation until when normalization of the thyroid function was confirmed at age 3 years (i.e., transient CH). Mutation screening for *DUOX2*, a causative gene for transient CH, showed biallelic mutations (p.[E327X] + [H678R]). This report provides a new example of environmental modification of phenotypes of CH due to a genetic defect, which can potentially distort screening results.

Key words: congenital hypothyroidism; neonatal screening; false negative reactions; iodine; DUOX2

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INTRODUCTION

Congenital hypothyroidism (CH) is the most common congenital endocrine disorder, affecting about 1 in 3,000 newborns worldwide. In developed countries, majority of patients are detected through newborn screening, in which blood samples on filter paper obtained at age 2–5 days are analyzed. Owing to early diagnosis and treatment, most patients can achieve normal or near normal intellectual outcome today. However, a subset of patients with delayed onset are undetectable by screening, and miss benefit from early intervention.

Inborn errors of thyroid hormone production are collectively referred to as thyroid dyshormonogenesis. Thyroid dyshormonogenesis is a relatively infrequent form of CH, accounting for about 15–20% of cases. Patients with thyroid dyshormonogenesis have a goiter, which results from hyperstimulation of the thyroid by thyroid stimulating hormone (TSH). Genetic defects of a molecule within the thyroid hormone synthesis pathway, such as sodium-iodine symporter (NIS) (*SLC5A5*, OMIM*601843), pendrin (*SLC26A4*, OMIM*605646), dual oxidase 2 (*DUOX2*, OMIM-*606759), thyroid peroxidase (*TPO*, OMIM*606765), and thyroglobulin (*TG*, OMIM*188450), cause thyroid dyshormonogenesis that is inherited as an autosomal recessive trait [Park and Chatterjee, 2005]. These genetic defects account for at least 70% of

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thyroid dyshormonogenesis [Narumi et al., 2011], while inappropriate iodine status (i.e., deficiency and excess) can cause transient CH resembling thyroid dyshormonogenesis. In this article, we report a patient with CH due to *DUOX2* mutations, the most common form of thyroid dyshormonogenesis [Narumi et al., 2011], with a delayed onset. Of interest, the patient was compromised by extreme iodine excess during pregnancy, and had a negative result in newborn screening for CH.

CLINICAL REPORT

The patient (a 6-year-old girl) was the first child of healthy nonconsanguineous Japanese parents. Reportedly, the mother of the patient consumed as much as 50–100 g of several kinds of seaweed everyday from the third trimester of pregnancy to 3 months after delivery, in order to "maintain good health". The estimated iodine intake level was 20–40 mg/day, which greatly exceeds the recommended dietary allowance (0.24 mg/day) and tolerable upper intake level (2.2 mg/day) for pregnant women in Japan according to the Dietary Reference Intakes 2010 by Ministry of Health, Labour and Welfare. During the period of seaweed overconsumption, she had neither goiter nor symptoms suggesting hypothyroidism, although we did not test her thyroid function. She did not overconsume seaweeds during the other pregnancy.

The patient was born at 38 weeks with a weight of 2,580 g, and was breastfed. Dried blood samples obtained at age 5 days were subject to newborn screening, in which blood-spot TSH level was measured. The result was negative (screening cutoff TSH level, 10 mU/L). At age 3 months, she had persistent jaundice and poor weight gain (weight, 5,210 g; −2.0 SD). Routine blood tests revealed hyperbilirubinemia (total bilirubin, 6.7 mg/dl) accompanied by a slightly elevated serum aspartate aminotransferase level (105 U/L; ref, 5–45). The thyroid function test showed that she had overt hypothyroidism: TSH, 492 mU/L (ref, 0.3–4.2) and free thyroxine, 0.2 ng/dl (ref, 1.0–1.8). No thyroid autoantibodies were detected. Urinary iodine level was not measured. Thyroid ultrasonography showed a slightly enlarged gland (+1.8 SD [Yasumoto et al., 2004]). Replacement therapy with levothyroxine was initiated, and hyperbilirubinemia and hypertransaminasemia improved subsequently. We reevaluated her thyroid status at age 3 years with discontinuation of therapy. She had normal thyroid function (TSH, 2.3 mU/L; free thyroxine, 1.7 ng/dl), with a normal-sized gland (0.0 SD) on ultrasonography. Thyroidal ^{123}I uptake was normal (24.9% at 24 hr; ref 8–40), with normal perchlorate discharge rate (8.3%; ref <10). The Wechsler Preschool and Primary Scale of Intelligence score evaluated at age 4 years was 93. At last clinical visit, she maintained a normal TSH level without therapy, was growing normally and was developing satisfactory.

Mutation Analysis

DNA samples were collected with written informed consent from the proband, her parents, and her sister. Coding exons and flanking introns of *DUOX2* were analyzed by standard PCR-based sequencing as previously described [Narumi et al., 2011]. The patient was compound heterozygous for a novel nonsense mutation (c.978G>T; c.979G>T, p.E327X) and a known functional single nucleotide polymorphism (c.2033A>G, p.H678R) [Narumi et al., 2011] (Fig. 1A). E327X is a null mutation lacking all functional domains of the DUOX2 protein (Fig. 1A). The DUOX2 allele harboring H678R was shown to have about 60% of residual function in our previous study [Narumi et al., 2011]. The mother and younger sister of the patient were heterozygous for E327X, while the father was heterozygous for H678R (Fig. 1B). These three individuals had normal thyroid function.

FIG. 1. A: Partial sequences of polymerase chain reaction products of patients are shown along with a schematic diagram of the dual oxidase 2 (DUOX2) protein. Arrows indicate mutated nucleotides. Note that all functional domains of DUOX2 are deleted by the E327X mutation. FAD-bd, flavin adenine dinucleotide binding domain; NADPH-bd, nicotinamide adenine dinucleotide phosphate binding domain; TM, transmembrane segment. B: A pedigree of the patient is shown with clinical parameters, which are aligned with each symbol. Values outside the normal range are typed in boldface.

DISCUSSION

The Japanese consume 1–3 mg of iodine per day [Zava and Zava, 2011], which is one of the highest iodine intake in the world. Perinatal exposure to large amounts of iodine, e.g., maternal ingestion of an iodine-rich drug, is an established cause of transient CH [Theodoropoulos et al., 1979]. The role of milder iodine excess in the pathogenesis of CH remains to be clarified. Nishiyama et al. [2004] studied urine iodine (UI) levels of 34 newborns that were positive at screening for CH in Kumamoto prefecture, Japan, and found that 15 babies had UI levels of greater than 20 μg/dl (a value corresponding to +2 SD of control newborns). Those 15 patients had only slightly high UI level (i.e., mild iodine excess), indicating

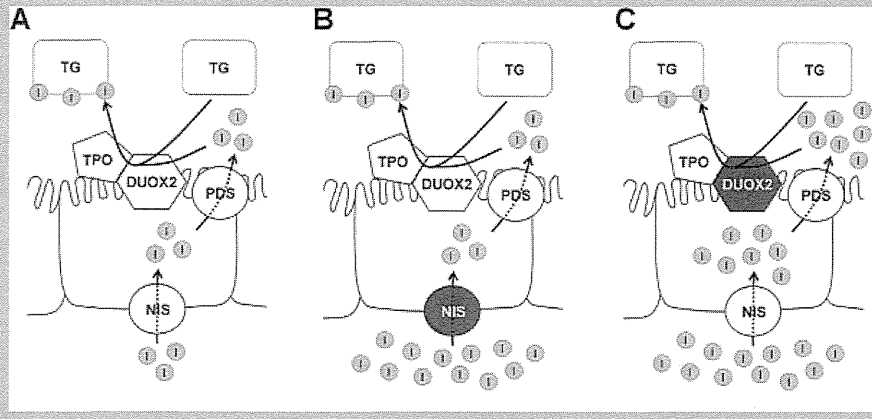

FIG. 2. Schematic diagrams showing a part of the thyroid hormone synthesis pathway. A: Iodine in blood (shown in lower part) is transported into thyroid cells through sodium-iodine symporter (NIS), and then transported into follicular lumen through another transporter named pendrin (PDS). At the apical surface of thyroid cells, iodide is incorporated to thyroglobulin (TG) by thyroid peroxidase (TPO). This reaction requires hydrogen peroxide, which is produced by dual oxidases 2 (DUOX2). B: In a thyroid with the NIS defect, excessive amount of iodine normalizes the defective iodine transportation. C: In a thyroid with the DUOX2 defect, iodine excess presumably compensates the defective iodination of TG.

that mild iodine excess can increase risk of CH. Considering that most newborns with a UI level above +2 SD (constituting 2.3% of population by definition) were assumed to have normal thyroid function, those 15 patients would be affected by other genetic and/or environmental factors making them susceptible to mild iodine excess. In any case, it has been widely accepted that iodine overload in the fetus and newborn negatively affects thyroid function. Therefore, it is notable that thyroid function was not impaired but rather restored by iodine excess in the report of patient with a *DUOX2* defect.

The thyroid hormone-producing capacity varies dramatically in the neonatal period. Several factors influence neonatal TSH and thyroid hormone levels, e.g., TSH surge at birth (followed by transient elevation of thyroid hormone), maturity of hypothalamus-pituitary-thyroid axis, iodine status (deficiency/excess), etc. Timing of onset of TSH rise can be affected by these factors, resulting in negative newborn screen in a subset of patients. Importantly, such false negatives are not rare: A study by Northwest Regional Newborn Screening Program, where second screening at age 2–6 weeks is routinely implemented, showed that the false negative rate of first screening was 7.6% [Hunter et al., 1998]. The public health impact of these false negatives remains to be clarified.

Mechanisms underlying delayed TSH rise are largely unknown except for premature birth. One important model is the genetic defect of NIS, which transports iodine from blood to thyroid cells. The NIS defect, a form of thyroid dyshormonogenesis, has high variability in age at disease onset, ranging from neonatal to adult age [Spitzweg and Morris, 2010]. Of interest, phenotypic expression of the defect is influenced by iodine status: Iodine excess alleviates defective hormone production (Fig. 2) [Matsuda and Kosugi, 1997]. Correspondingly, in our patient with the *DUOX2* mutations, large amount of transplacentally transferred iodine likely prevented her from developing hypothyroidism immediately after birth. A similar mutation-carrying case with a negative screening result has been reported by Vigone et al. [2005], although the level of iodine excess and severity of hypothyroidism differs considerably. The mechanism of alleviation of the *DUOX2* defect by iodine excess is unclear. Considering that thyroid peroxidase and *DUOX2* coordinately incorporate iodine into thyroglobulin at the apical membrane of the thyroid cells, excessive amount of iodine might compensate the defective iodination process (Fig. 2). Alleviation of hypothyroidism by iodine excess has also been reported in a patient carrying mutated dehalogenase 1 [Moreno et al., 2008], the molecule involving in intrathyroidal recycling of iodine. We speculate that similar phenotypic modification could be observed in other forms of thyroid dyshormonogenesis, such as the pendrin defect and the DUOX maturation factor 2 defect.

The main limitation of the present study is the lack of data about urine iodine determination of the proband. In this case, magnitude of iodine excess cannot be discussed in a quantitative manner.

We suspect that not only the NIS defect but also the *DUOX2* defect can have a delayed onset, probably associated with individual iodine status. Because *DUOX2* mutations are the most frequent genetic cause of CH, the defect could be an important source of false negative screen, especially in areas where baseline iodine intake level is high. Future studies targeting screening-negative CH cases will be required to develop more effective screening programs.

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