

## Identification and functional characterization of a novel *PAX8* mutation (p.His39Pro) causing familial thyroid hypoplasia

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**Abstract.** Mutations in *PAX8*, the gene for a thyroid-specific transcription factor, causes congenital hypothyroidism (CH) with autosomal dominant inheritance. All previously detected *PAX8* mutations except one are located in the DNA-binding paired domain. The proband, a 1-yr-old boy, was diagnosed with CH in the frame of newborn screening. He had high serum TSH level (180 mU/L) and low serum free T<sub>4</sub> level (0.4 ng/dL). Ultrasonography revealed that the proband had thyroid hypoplasia. Importantly, he had a family history of CH, *i.e.*, his mother also had CH and hypoplasia. Next generation sequencing-based mutation screening revealed a novel heterozygous *PAX8* mutation (c.116A>C, p.His39Pro) that was transmitted to the proband from the mother. Expression experiments with HeLa cells confirmed that His39Pro-*PAX8* exhibited defective transactivation of the *TG* promoter–luciferase reporter. In conclusion, we identified and described a novel loss-of-function *PAX8* mutation in a family with thyroid hypoplasia. Patients with dominantly inherited CH and no extrathyroidal abnormalities could have *PAX8* mutations.

**Key words:** *PAX8*, mutation, genetics, congenital hypothyroidism

### Introduction

Molecular biology studies have revealed that organogenesis of the thyroid gland is regulated by several tissue-specific transcription factors [*i.e.*, thyroid transcription factors (TTFs)]. Three TTFs, namely, *PAX8* (1, 2), *NKX2-1* (formerly known as TTF-1) (3, 4), and *FOXE1* (formerly known as TTF-2) (5) have been known to play important roles in this process. *PAX8* is a member of the *PAX* gene family. Transcription factors of the *PAX* family are characterized by the presence of a DNA-binding paired domain at their N terminus. *PAX8* regulates the transcription of thyroid-specific genes, including thyroglobulin (Tg) and thyroid peroxidase, in cultured cell lines (6). *Pax8*-knockout mice have thyroid hypoplasia due to defective proliferation and survival of thyroid precursor cells (7). In humans, heterozygous *PAX8* mutations cause congenital hypothyroidism (CH) with autosomal dominant inheritance (8). The prevalence of *PAX8* mutations is 2.0% among Japanese patients with CH, 3.5% among German patients with

CH, and 1.7% among Chinese patients with CH (9–12).

To date, CH has been described in 77 patients belonging to 30 families (9, 10, 13–29). Clinical phenotypes of mutation carriers are variable, ranging from overt CH with severe thyroid hypoplasia to subclinical CH with a normal-sized thyroid. Of the previously reported and experimentally verified *PAX8* mutations, 95% have been identified in the paired domain. In the present study, we aimed to identify and functionally characterize a novel CH-associated *PAX8* mutation located in the paired domain.

### Patients and Methods

#### Patient report

The proband, a 1-yr-old boy, was born at 40 wk of gestation after an uneventful term pregnancy. The birth weight and birth length of the proband were 2,772 g and 48 cm, respectively. The proband was brought to medical attention due to high blood spot TSH level

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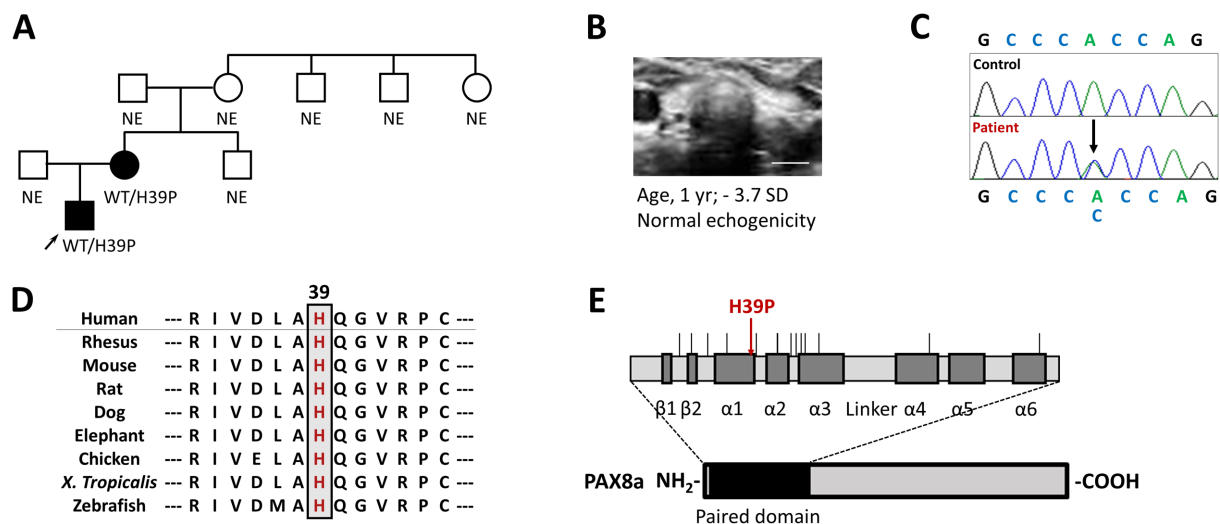
(78.1 mU/L; cutoff level, 9.5) at newborn screening for CH. His mother also suffered from CH and has been undergoing levothyroxine (L-T<sub>4</sub>) replacement therapy (Fig 1A). At 10 days of age, the proband had high serum TSH level (180 mU/L; reference 1.7–9.1), low serum free T<sub>4</sub> level (0.8 ng/dL; reference 0.9–2.3), and normal serum Tg level (32.8 ng/mL; reference 3.8–56) (Table 1). However, he did not have any CH-related symptoms, including constipation, jaundice, and poor weight gain; and his kidney ultrasound results were normal. He was diagnosed with primary CH, and L-T<sub>4</sub> replacement therapy (10 µg/kg/d) was started since then. When the proband was 1.7 yr old, his stature was short (74.5 cm; -2.5 SD), although his weight was normal (8.9 kg; -1.6 SD). He achieved developmental milestones normally, *i.e.*, walked independently, spoke a few words, and followed simple directions at 1.7 yr of age. At the

age of 1.9 yr, ultrasonography revealed normoechoic hypoplastic thyroid gland in the proband (-3.7 SD; Fig. 1B). At the last clinical visit (age, 1.9 yr), the proband was doing well with 4.5 µg/kg/d of L-T<sub>4</sub> treatment.

The mother of the proband, aged 32 yr, was diagnosed with CH within the frame of newborn screening for CH, and underwent L-T<sub>4</sub> replacement therapy since then. At the last clinical visit, she was doing well with 2.5 µg/kg/d of L-T<sub>4</sub> treatment. She reportedly had thyroid hypoplasia, which was detected by ultrasonography in the neonatal period; however, the ultrasound image is unavailable.

### Mutation detection

This study was approved by the ethics committee of National Center for Child Health and Development.



**Fig. 1.** Identification of a novel heterozygous *PAX8* mutation. (A) Pedigree of the mutation-carrying patients. Black symbols indicate individuals with congenital hypothyroidism. NE, not evaluated. (B) Ultrasonography of the mutation carrier showed normoechoic hypoplastic thyroid gland. The widths of the left and right lobes were 7.1 mm and 4.5 mm, respectively. Scale bar corresponds to 5 mm. (C) Electropherogram of the mutation carrier revealed the presence of a heterozygous mutation (c.116A>C, p.His39Pro). (D) Single-letter amino acid ClustalW alignments of residues surrounding His39. The mutated residue, which is conserved among vertebrate species, is shown in red. (E) A schematic diagram showing the secondary structure of the paired domain of *PAX8*. The two β-sheets and six α-helices are represented as boxes. The location of p.His39Pro is indicated with a red arrow. Black bars indicate the locations of previously reported missense *PAX8* mutations.

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

Variable	Proband	Proband's mother
Age (yr)	1	32
Sex	M	F
Age at diagnosis (yr)	0.1	0.1
Thyroid function		
Age at evaluation (yr)	0.1	NA
Serum TSH (mU/L)	180.1	NA
Serum free T <sub>4</sub> (ng/dL)	0.8	NA
Serum Tg (ng/mL)	32.8	NA
Thyroid ultrasonography	Hypoplasia	Hypoplasia

NA: not available.

Written informed consent for molecular analysis was obtained from the parents of the proband. Eleven known genes associated with primary CH (*DUOX2*, *DUOXA2*, *FOXE1*, *IYD*, *NKX2-1*, *PAX8*, *SLC5A5*, *SLC26A4*, *TG*, *TPO*, and *TSHR*) were analyzed using MiSeq next-generation sequencer (Illumina Inc., San Diego, CA, USA) as previously described (30). The variants in next generation sequencing were confirmed by PCR-based Sanger sequencing. The detected mutations were referred for 4.7KJPN (<https://jmorp.megabank.tohoku.ac.jp/202001/variants>; Japanese genetic variant database), 1000 Genomes Project (<http://www.1000genomes.org/>), and genome aggregation (<https://gnomad.broadinstitute.org/>) databases.

### Functional characterization of His39Pro-PAX8

Vectors expressing untagged human *PAX8* (*PAX8A*; NM\_003466) or human *NKX2-1* cDNA (NM\_004387), and luciferase reporter vector encoding the promoter sequence of the human *TG* gene (*TG-luc*) were constructed as described previously (9). An expression vector encoding N-terminal 3xFLAG-tagged human *PAX8* was constructed by introducing the 3xFLAG sequence using Gibson assembly technique (NEBuilder HiFi DNA Assembly Master Mix; New England Biolabs, Ipswich, MA, USA). The *PAX8* mutation His39Pro was introduced into these vectors using PrimeSTAR Mutagenesis Basal Kit (TaKaRa Bio Inc, Shiga, Japan) according to the manufacturer's protocol. HeLa cells were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Transient transfection was performed with Lipofectamine 3000 reagent (Thermo Fisher Scientific, Waltham, MA, USA) following to the manufacturer's protocol. For western blotting, cells transfected with 3xFLAG-tagged *PAX8* construct (WT or mutant) or empty vector were harvested at 48 h post transfection, and whole cell lysates were obtained by treating the cells with 1% Triton X-100/Tris buffered saline (TBS) supplemented with protease inhibitor cocktail. The lysates were resolved using 10% SDS-PAGE, and western blotting was performed using monoclonal anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MD, USA) as the primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich) as the secondary antibody. For visualization of subcellular localization, cells were fixed in 4% formaldehyde/PBS at room temperature for 10 min. Blocking and plasma membrane permeabilization were performed by incubating the cells with 5% bovine serum albumin (BSA) in TBS supplemented with 0.1% Triton X-100 at room temperature for 1 h. Immunofluorescence was performed using anti-FLAG M2 antibody as the primary antibody and Alexa Fluor 555-conjugated goat anti-mouse IgG antibody (Thermo Fisher Scientific) as the secondary antibody. For transactivation assays, cells were co-transfected with 90 ng of *TG-luc* and 10 ng of *PAX8* expression vector (WT or mutant) with or without 1 ng of *NKX2-1* expression vector. Luciferase activity

was measured 48 h post transfection using ONE-Glo Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Data are representative of four independent experiments with similar results.

## Results

### Mutation detection

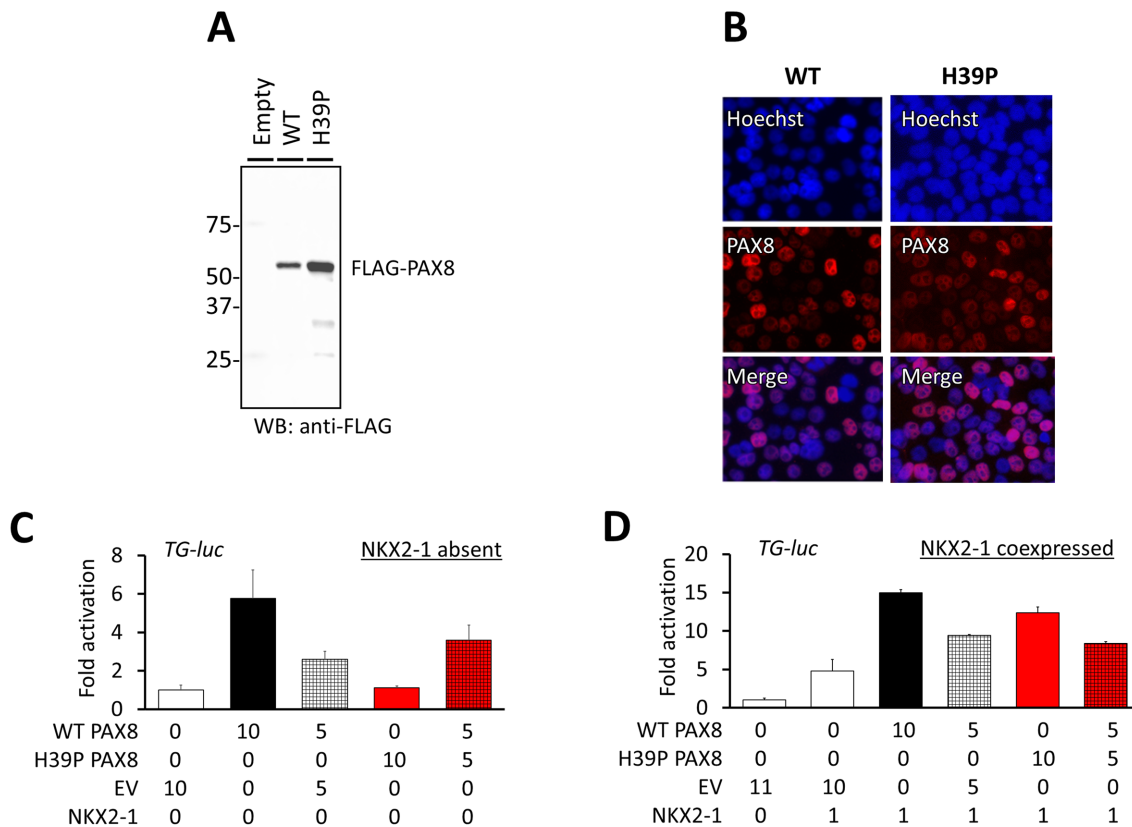
To clarify the molecular basis of familial thyroid hypoplasia of the pedigree, we conducted next generation sequencing-based mutation screening in the proband. As a result, we identified a novel heterozygous *PAX8* mutation (c.116A>C, p.His39Pro) (Fig. 1C). No mutation was found in any of the other sequenced genes. The His39 residue is conserved among vertebrate species with thyroid gland (Fig. 1D). The paired domain consists of two β-sheets (β1 and β2) and six α-helices (α1 to α6). His39 is located in the first α-helix of the paired domain (Fig. 1E). Family study revealed that the mutation was transmitted from the mother, who was also affected by CH, to the proband (Fig. 1A).

### In vitro functional analyses

To verify the pathogenicity of the identified *PAX8* mutation (His39Pro), we conducted a series of transient expression experiments in HeLa cells, a cell line derived from cervical cancer cells. Western blotting of 3xFLAG-tagged *PAX8* proteins showed that the protein expression level of His39Pro-*PAX8* was comparable with that of WT-*PAX8* (Fig. 2A). Visualization of subcellular localization of the 3xFLAG-tagged *PAX8* protein revealed that His39Pro-*PAX8* was localized in the nucleus (Fig. 2B). The transactivation capacity of His39Pro-*PAX8* was tested using *TG-luc*. To evaluate the interaction between *PAX8* and its transcriptional partner *NKX2-1*, different co-transfection patterns were tested: His39Pro-*PAX8* only or both WT- and His39Pro-*PAX8*, each with or without co-expressed *NKX2-1*. In the absence of *NKX2-1*, His39Pro-*PAX8* showed negligible transactivation of *TG-luc* (Fig. 2C, red bar). Co-transfection of WT-*PAX8* (5 ng) and His39Pro-*PAX8* (5 ng) showed no dominant negative effect as compared to transfection with WT only (5 ng) (Fig. 2C, red and grid bars). In the presence of *NKX2-1*, the transactivating capacity of His39Pro-*PAX8* (10 ng) was comparable with that of WT (10 ng) (81 ± 12% activity relative to WT-*PAX8*) (Fig. 2D, red bar). Moreover, when WT- and His39Pro-*PAX8* were co-transfected (WT 5 ng; mutant 5 ng), the transactivation level of *TG-luc* was significantly lower than that obtained with 10 ng of WT but was comparable to that obtained with 5 ng of WT (Fig. 2D, red and grid bars).

## Discussion

In the present study, we identified a novel *PAX8* mutation (p.His39Pro) causing familial thyroid



**Fig. 2.** Functional characterization of His39Pro-PAX8. (A) Western blotting showed that the protein expression level of His39Pro-PAX8 was comparable with that of WT. (B) Subcellular localization analysis showed comparable localization of WT-PAX8 and His39Pro-PAX8. The merged image revealed that His39Pro-PAX8 was localized in the nucleus. (C, D) Panel C shows transactivation assays in the absence of NKX2-1. His39Pro-PAX8 was nonfunctional, and co-transfection of WT-PAX8 and His39Pro-PAX8 showed no dominant negative effect. Panel D shows transactivation assays in the presence of NKX2-1. When His39Pro-PAX8 and WT-PAX8 were co-transfected, the transactivation activity was comparable with that obtained with WT only. The data are representative of three independent experiments with similar results. Bars indicate SEM.

hypoplasia. To date, 30 families of CH caused by *PAX8* mutations have been described (9, 10, 13–29). For 25 families, genetic analyses for family members were performed, showing parent-to-offspring transmission in 21 families (9, 10, 13–20, 22–25, 27, 28) and *de novo* occurrence of the mutation in five (13, 23, 26). Among the 21 families that displayed parent-to-offspring mutation transmission, 19 exhibited familial hypothyroidism, while two showed incomplete penetrance. These findings suggest the importance of family history of CH to suspect *PAX8* mutations on clinical grounds.

The p.His39Pro mutation exhibited negligible transactivation of *TG-luc* in the absence of NKX2-1, but showed significant transactivation in the presence of NKX2-1. This transcriptional “rescue” has been repeatedly reported in missense *PAX8* mutations located in the paired domain (9, 20, 23). Grasberger *et al.* pointed out that formation of PAX8–NKX2-1 complex in the *TG* promoter would be the molecular basis for the phenomenon (18). In this model, the PAX8 protein, the NKX2-1 protein, and the *TG* promoter bind each other, therefore, defective binding between PAX8 and the *TG* promoter can be rescued by NKX2-1 that bridges PAX8

and the *TG* promoter. In this study, we demonstrated that the rescue by NKX2-1 was abolished by co-transfection of WT- and His39Pro-PAX8. This observation implies that NKX2-1 binds more preferentially to WT-PAX8 than His39Pro-PAX8, therefore, His39Pro-PAX8 was no longer rescued by NKX2-1. In our expression experiments, the condition that best recapitulated the thyroid status of the patient was co-expression of WT-PAX8 and His39Pro-PAX8 in the presence of NKX2-1. However, this condition resulted in approximately 50% loss of transactivation capacity. Considering that CH is caused by haploinsufficiency of *PAX8*, we assume that this 50% reduction is sufficient to cause CH (23).

In our previous study, we performed functional analysis of His39Ala mutation, and found that it retains residual transcriptional capacity ( $43 \pm 3\%$ ) (31). The difference in the degrees of loss of function between His39Pro and His39Ala mutations can be explained by the nature of the substitutions: His to Ala mutation affects the side chain only, while His to Pro mutation affects both the main chain and the side chain. A similar discordance has also been observed in Gln40Pro and Gln40Ala mutations (31).

## Conclusions

We report clinical and molecular findings of a novel *PAX8* mutation (p.His39Pro) that caused CH in the proband and the mother. In dominantly-inherited thyroid hypoplasia patients with no extrathyroidal abnormalities, the *PAX8* defect should be suspected.

**Conflict of interest:** The authors declare no conflicts of interest.

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