Functional analysis of PAX8 variants identified in patients with congenital hypothyroidism in situ

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Abstract. Paired box transcription factor 8 (PAX8) is essential for thyroid organogenesis and development. Heterozygous pathogenic variants of PAX8 typically cause congenital hypothyroidism (CH) due to thyroid hypoplasia. Additionally, pathogenic PAX8 variants have been identified in patients with gland in situ (GIS). This study was conducted to analyze the in vitro functional consequences of four PAX8 variants (p.D94N, p.E90del, p.V58I, and p.L186Hfs*22) previously identified in patients with CH and GIS. The transcriptional activity of PAX8 variants on the thyroglobulin (TG) promoter was assessed in a luciferase reporter assay. The levels of transcriptional activity on the TG promoter of p.E90del and p.L186Hfs*22 were significantly reduced, whereas p.D94N and p.V58I showed residual activation. In addition, a dominant negative effect on the wild-type (WT) was not detected in any PAX8 variant using a luciferase reporter assay. Two PAX8 variants (p.E90del and p.L186Hfs*22) may be pathogenic causes of CH with GIS.

Key words: congenital hypothyroidism, PAX8, gland in situ

Highlights

- Two PAX8 variants (p.E90del and p.L186Hfs*22) showed loss of function.
- These two PAX8 variants (p.E90del and p.L186Hfs*22) retained partial transcriptional capacity, which may be sufficient to cause CH with GIS.
- Unlike PAX8, which is mostly localized in the nucleus, p.L186Hfs*22 is in the cytoplasm.
Introduction

Congenital hypothyroidism (CH) is the most frequent neonatal endocrine disorder, with an incidence of 1/2000–1/4000 (1). CH is caused by disorders in thyroid gland development (dysgenesis) or thyroid hormone biosynthesis (dyshormonogenesis). Thyroid dysgenesis (TD) accounts for 85% of CH cases, whereas dyshormonogenesis (DH) accounts for 10–15% of cases (2). Mutations in TSHR, NKX2-1, FOXE1 (encoding TTF2), NKX2-5, and PAX8 have been identified in some patients with various forms of TD (3–7). Pathogenic PAX8 variants are observed in 1.4%–3.3% of CH cases with TD (8). To date, more than 20 experimentally verified loss-of-function variants of PAX8 have been described in 30 unrelated families (7, 9–26). The clinical phenotypes of PAX8 mutation carriers are variable, ranging from overt CH with severe thyroid hypoplasia to subclinical CH with gland in situ (GIS) (27–31).

PAX8, a member of the PAX family, contains a 4-kb coding sequence divided into 12 exons and is located on chromosome 2q12. It regulates the transcription of thyroid-specific genes, including thyroperoxidase (TPO), thyroglobulin (TG), and sodium/iodide symporter (SLC5A5), by binding to specific promoter regions via the highly conserved 128 amino acid paired box domain (26, 32). Additionally, more than 90% of PAX8 variants were identified in the DNA-binding region of the paired domain (33). We previously analyzed the genetic causes of CH using next-generation sequencing (34). In this study, four PAX8 variants (p.D94N, p.E90del, p.V58I, and p.L186Hfs*22) were identified in patients with CH and GIS. This study was performed to determine the functional consequences of these PAX8 variants, located either in the paired domain or outside the domain.

Patient and Methods

Study participants

We enrolled patients with permanent primary CH diagnosed by neonatal screening who regularly visited Hokkaido University Hospital, Japan. Four PAX8 variants (p.D94N, p.E90del, p.V58I, and p.L186Hfs*22) have been identified in patients with CH and GIS (34). The Ethics Committees of the Hokkaido University Graduate School of Medicine approved the study, and written informed consent was obtained from patients or parents for genetic studies.

Cell culture and transfection

HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO2.

For functional assays, the cells were transfected with reporter plasmid (TG-luc) and WT or PAX8 variant (p.D94N, p.E90Del, p.V58I, p.L186Hfs*22, and p.Q40P) expression plasmid or empty vector (EV) together with 10.0 ng Renilla luciferase plasmid using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer’s protocol.

Site-directed mutagenesis and construction of expression vectors

The expression vectors containing PAX8, and TG-luc promoter were described previously (20). For plasmid construction, four variants (p.D94N, p.E90del, p.V58I, and p.Q40P) were introduced by site-directed mutagenesis as previously described (35). The fourth variant, p.L186Hfs*22 (c.535_556dup), was constructed by restriction enzyme digestion (BstXI, PstI) and the religation method using the 765 bp PAX8 synthesized sequence (Eurofins Genomics, Tokyo, Japan) pEX-A2J2-pL186Hfs*22 (c535_556dup) containing a 22 bp duplication. All final constructs were verified by direct sequencing prior to transfection. In addition, WT and PAX8 variant plasmids tagged with FLAG × 3 at the N-terminus were generated using restriction enzyme digestion (EcoRI, BglII) and the ligation method using the 116 bp FLAG-tagged PAX8 sequence (EcoRI-FLAG-PAX8-BglII) (Eurofins Genomics). These FLAG-tagged WT and PAX8 variants were confirmed using DNA sequencing before transfection. In addition, we constructed a PAX8 representative variant (p.Q40P) previously reported by Congdon et al. (2001) (11). For mutagenesis, the following primer sets were used for plasmid construction (Table 1).

Transcriptional activation of PAX8 variants on TG promoter

HeLa cells grown in 24-well plates to approximately 80% confluence were transfected with 500 ng reporter plasmid (TG-luc) and 40 ng of each WT or each PAX8 variant (p.D94N, p.E90del, p.V58I, and p.L186Hfs*22) and p.Q40P) expression plasmid or EV together with 10.0 ng Renilla luciferase plasmid. A constant amount of plasmid was transfected by adding EV. To determine the effect of the PAX8 variants on the WT, PAX8 variants were co-transfected in equal amounts with WT (WT 20 ng, PAX8 variant 20 ng) and reporter vectors. The cells were harvested 24 h later and lysed in 100 μL 1X Passive Lysis Buffer (Promega, Cat. # E1941).

Protein extracts (10 μL) were analyzed sequentially for firefly and Renilla luciferase activities using a dual-luciferase reporter assay system (Promega, Madison, WI, USA). Luciferase activity of each PAX8 variant was normalized to Renilla luciferase activity. Data are representative of three independent experiments with similar results.

Western blot analysis

The cells were transfected with 5.0 μg FLAG-tagged
WT or each PAX8 variant (p.D94N, p.E90Del, p.V58I, and p.L186Hfs*22) expression plasmid. After 40 h of transfection, the cells were lysed with ice-cold TNE buffer (20 mM Tris-HCl, 0.5% Triton X-100, and 1 mM EDTA [pH 7.4]) for 5 min on ice. The lysates were centrifuged at 15,000 × g for 30 min at 4°C. After centrifugation, the protein concentration was determined using a Qubit Protein Assay Kit (Invitrogen). Protein lysates were normalized to total protein (22 μg/lane) and resolved by 5–12.5% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by electrotransfer of the proteins onto a polyvinylidene fluoride membrane. The membrane was blocked with 5% skim milk in phosphate-buffered saline with Tween 20 (PBST) for 1 h at room temperature (around 20–22°C). The membrane was incubated with the following primary antibodies: anti-FLAG (1:1000; F3165; Sigma-Aldrich, St. Louis, MO) and mouse monoclonal anti-β-actin (1:5000; A1978; Sigma-Aldrich) in PBST overnight at 4°C. After washing three times with PBST for 10 min, anti-mouse IgG HRP (1:2000; SC-516102; Santa Cruz Biotechnology, Dallas, TX, USA) was applied as a secondary antibody for 1 h at room temperature (around 20–22°C). After washing three times with PBST for 15 min each time, the membrane was detected with HRP substrate (Takara Bio, Shiga, Japan) and imaged using Image Quant LAS 4000 (GE Healthcare, Little Chalfont, UK).

Fluorescence analysis and confocal microscopy

HeLa cells were plated on an 8-well chamber slide and transfected with 0.2 μg FLAG-tagged WT or PAX8 variant (p.D94N, p.E90Del, p.V58I, and p.L186Hfs*22) expression plasmids. At 24 h after transfection, the cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and washed three times with PBST for 15 min each time. Immunofluorescence was analyzed using anti-FLAG monoclonal antibody as the primary antibody (1:1000; F3165; Sigma-Aldrich) and Alexa Fluor 594-conjugated goat anti-mouse IgG antibody (Thermo Fisher Scientific) as the secondary antibody. The nuclei were stained as blue (Hoechst 33342; Takara Bio). Localization of each FLAG-tagged PAX8 was examined by using confocal microscopy (Leica TCS SP8 Laser Confocal Microscopy, Wetzlar, Germany).

Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). Multiple comparisons between groups were performed using a one-sample t-test (Wilcoxon test). All data were analyzed using GraphPad Prism software (version 8.0; GraphPad, Inc., San Diego, CA, USA). A value of P < 0.05 was considered as significant in all analyses.

Results

Clinical characteristics of patients carrying PAX8 variants

The genotypes and phenotypes of patients with CH and PAX8 variants are summarized in Table 2, which were reported in our previous study (34). All patients were identified through neonatal blood screening for CH, and L-thyroxine replacement was started immediately. Ultrasound scans showed that all patients had a normal-sized thyroid GIS. The locations of the PAX8 variants are shown in Fig. 1. The p.D94N, p.E90del, and p.V58I variants were in the paired box domain. The p.L186Hfs*22 variant was outside of the domain.

Transcriptional activation of PAX8 variants on TG promoter

We performed functional analysis of the PAX8 variants (p.D94N, p.E90Del, p.V58I, and p.L186Hfs*22) using the TG promoter. To better characterize the PAX8 variants (p.D94N, p.E90Del, p.V58I, and p.L186Hfs*22), a previously reported p.Q40P variant (with a DNA-binding defect) (11) was also analyzed. As shown in Fig. 2a, transactivating capacities were significantly lower in cells co-transfected with the TG promoter and PAX8 variants (p.E90del and p.L186Hfs*22); however, they showed partial transactivation (19.9 ± 3.1%; 30.6 ± 3.3% activity relative to WT-PAX8, respectively), whereas p.D94N exhibited residual activation (90.3 ± 2.7%) (Fig. 2a). Only one variant (p.V58I) showed elevated transactivating capacities (123.4 ± 3.8%). P.Q40P had significant activity (90.3 ± 2.7%).
A low transcriptional capacity (9.9 ± 2.1%) for the TG promoter. This result agrees with those of previous reports (8, 11, 14). Co-transfection of WT and PAX8 variants (WT 20 ng, PAX8 variant 20 ng) showed no dominant negative effect compared to co-transfection with EV and WT (total amount 40 ng) (Fig. 2b). Based on these results, p.E90del and p.L186Hfs*22 may be pathogenic, whereas p.D94N and p.V58I may not be pathogenic.

Protein expression of PAX8 variants

Western blotting of FLAG-tagged PAX8 proteins showed that the protein expression levels of p.D94N, p.E90del, and p.V58I were comparable to those of WT-PAX8, which is consistent with previous reports (8, 28) whereas the expression of p.L186Hfs*22 was lower than that of WT-PAX8; the protein size was smaller than that of other variants because of the premature stop codon (Fig. 3).

Subcellular localization of PAX8 variants

Visualization of the subcellular localization of FLAG-tagged PAX8 protein revealed that p.D94N, p.E90del, and p.V58I were localized in the nucleus, in agreement with previous studies (8, 15, 28); whereas p.L186Hfs*22 was outside the nucleus in cytosol (Fig. 4).

Discussion

We examined the functional consequences of four PAX8 variants (p.D94N, p.E90del, p.V58I, and p.L186Hfs*22) on the TG promoter, as previously reported in patients with CH and GIS (34). Pathogenic variants of patients with CH with GIS were most frequently identified in TG, TPO, DUOX2, and THSR (36). However, several PAX8 variants were reported in patients with CH and GIS (23, 29–31). In our study, p.E90del, and p.L186Hfs*22 were predicted as pathogenic, whereas p.D94N and p.V58I were not.

Similar to our study, Camats et al. (27) reported that patients with CH with GIS had PAX8 variants (p.S59R, p.Y66del, p.S70G, p.R133W, and p.R133Q), which were shown to cause loss of function. p.R242Gfs*37 was also identified in a patient with a GIS (31). It is well-known that pathogenic variants of PAX8 have broad phenotypic variability from TD to GIS, even in familial cases. Based on the transactivating capacities relative to WT, Iwahashi et al. (33) proposed the following terms: profound loss of function (LOF), less than 10.0% activity; moderate LOF-10.0–29.9% activity; minimal LOF-30.0–69.9% activity; functionally neutral-70.0–119.9% activity; hyperfunctioning-equal or more than 120% activity. The authors also assumed that a 50% reduction in the transactivation capacity is sufficient to cause CH. In our study, p.E90del showed moderate LOF (19.9 ± 3.1%), and p.L186Hfs*22 led to a minimal LOF (30.6 ± 3.3%). Thus, the two variants were sufficient to cause CH with GIS. Most PAX8 variants were in the nucleus (8, 15,

Table 2. Clinical characteristics of patients with CH with PAX8 variants

| Variants of PAX8 | Current age (yr) | Sex | NBS-TSH (mIU/L) | NBS-FT4 (ng/dL) | TSH level at the first visit (mIU/L) | FT4 level at the first visit (ng/dL) | Current dose of L-T4 (μg/d) | Thyroid ultrasound |
|------------------|----------------|-----|----------------|----------------|------------------------------------|---------------------------------|------------------|------------------|
| E90del (c.268_270delGAG) | 5 | F | 16.4 | 1.59 | 22.55 | 1.71 | 25 | Normal |
| V58I (c.172C>T) | 12 | F | 10.4 | 1.46 | 25 | 0.34 | 15 | Normal |
| D94N (c.280G>A) | 13 | F | 11.8 | 2.56 | 9.3 | 1.45 | 50 | Normal |
| L186Hfs*22 (c.535_556dup) | 18 | M | 37.1 | 1.58 | 120.9 | 0.68 | 50 | Normal |

NBS, neonatal blood screening. NA, not available. Cutoff values for neonatal blood screening were as follows: TSH 10 mIU/L and FT4 0.9 ng/dL.

Fig. 1. Schematic representation of human PAX8. The positions of the constructed PAX8 variants are shown.
and p.L186Hfs*22 was mainly located outside the nucleus (Fig. 4). PAX8 predicts several potential sites for phosphorylation by various kinases, two potential nuclear localization signals (NSL), and amino acid sequences that may determine protein stability (37). These results suggest that p.L186Hfs*22 does not have a partial second NSL or subsequent sequences, which may cause a local change in this variant.

Our results demonstrate that two variants of PAX8 (p.E90del and p.L186Hfs*22) may be the pathogenic causes of CH with GIS.

Conflict of interests: On behalf of all authors, the corresponding author states that there is no conflict of interest.
Fig. 3. Western blot analysis of PAX8 protein. Western blotting of FLAG-tagged PAX8 proteins showed that the protein expression levels of p.D94N, p.E90del, and p.V58I were comparable to those of WT-PAX8, whereas p.L186Hfs*22 was lower than that of WT-PAX8, and the protein size was smaller than those of other variants because of the presence of a premature stop codon.

Fig. 4. Immunofluorescence analysis. Visualization of the subcellular localization of the FLAG-tagged PAX8 protein revealed that p.D94N, p.E90del, and p.V58I were localized in the nucleus, whereas p.L186Hfs*22 was outside the nucleus in cytosol. Nuclei were stained with blue (Hoechst 33342). Scale bar 75 μm.
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