Heterozygous defects in *PAX6* gene and congenital hypopituitarism

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Abstract

Background: The prevalence of congenital hypopituitarism (CH) attributable to known transcription factor mutations appears to be rare and other causative genes for CH remain to be identified. Due to the sporadic occurrence of CH, *de novo* chromosomal rearrangements could be one of the molecular mechanisms participating in its etiology, especially in syndromic cases.

Objective: To identify the role of copy number variations (CNVs) in the etiology of CH and to identify novel genes implicated in CH.

Subjects and methods: We enrolled 88 (syndromic: 30; non-syndromic: 58) Japanese CH patients. We performed an array comparative genomic hybridization screening in the 30 syndromic CH patients. For all the 88 patients, we analyzed *PAX6* by PCR-based sequencing.

Results: We identified one heterozygous 310-kb deletion of the *PAX6* enhancer region in one patient showing isolated GH deficiency (IGHD), cleft palate, and optic disc cupping. We also identified one heterozygous 6.5-Mb deletion encompassing *OTX2* in a patient with bilateral anophthalmia and multiple pituitary hormone deficiency. We identified a novel *PAX6* mutation, namely p.N116S in one non-syndromic CH patient showing IGHD. The p.N116S *PAX6* was associated with an impairment of the transactivation capacities of the PAX6-binding elements.

Conclusions: This study showed that heterozygous *PAX6* mutations are associated with CH patients. *PAX6* mutations may be associated with diverse clinical features ranging from severely impaired ocular and pituitary development to apparently normal phenotype. Overall, this study identified causative CNVs with a possible role in the etiology of CH in $<10\%$ of syndromic CH patients.

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Introduction

The proliferation and terminal differentiation of the anterior pituitary gland are strongly influenced by the precise spatial and temporal expression of transcription factors (1, 2, 3). Mutations in these transcription factors result in various types of congenital hypopituitarism (CH), ranging from isolated growth hormone deficiency (IGHD) to multiple pituitary hormone deficiency (MPHD) (1, 2, 3). Previous studies have shown that these transcriptional factor mutations are rare among CH patients. In Japan, Takagi *et al.* (4) identified three mutation carriers (two *LHX4* mutations and one *POU1F1* mutation) in a total of 91 CH (14 patients had IGHD, whereas 77 patients had MPHD) patients (3.3%). Reynaud *et al.* (5) reported a mutation prevalence of 13.3% in a study population of

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165 MPHD patients from the international GENHYPOPIT network. These results imply the rarity of pathological abnormalities in the previously known genes in CH patients and strongly suggest that other causative genes for CH remain to be identified in most patients.

Several cases of CH with submicroscopic chromosomal rearrangements, such as 20p11 deletions and 22q12.3q13.3 duplication, whose definitive causative genes responsible for CH are not fully captured, have been reported (6, 7, 8). These patients were syndromic and showed extra pituitary clinical spectra, including dysmorphic faces, midline defects, and intellectual disability. Because most cases of CH are sporadic, we hypothesized that *de novo* chromosomal rearrangements could be a certain fraction of the molecular mechanisms participating in the etiology, especially in syndromic cases. Therefore, we decided to perform a genome-wide screening for submicroscopic anomalies in our syndromic CH cohort to determine the role of copy number variations (CNVs) in the etiology of CH, and to identify new candidate loci and thus novel candidate genes for CH.

Subjects and methods

Subjects

This study population consisted of 88 patients with GH-treated CH. The inclusion criteria were as follows: i) severe GH deficiency (GH peak <3 ng/ml) confirmed by hypoglycemic provocation tests and ii) anterior pituitary hypoplasia as detected by a brain magnetic resonance imaging (MRI). Of the 88 patients, 30 were syndromic (CH with extra-pituitary abnormalities such as midline defects, intellectual disability, and cardiac defects), whereas 58 were non-syndromic. The details of 30 syndromic patients are summarized in Table 1. In all the patients, mutations in *POU1F1, PROP1, HESX1, LHX3, LHX4, OTX2, SOX2, SOX3,* and *GLI2* have been excluded by PCR-based sequencing (4). Gene copy number aberrations in *POU1F1, PROP1, HESX1, LHX3,* and *LHX4* were also excluded by multiplex ligation-dependent probe amplification (MLPA) analyses (4). Approval for this study was obtained from the Institutional Review Board of Keio University School of Medicine. We obtained written informed consent for molecular studies from the patient or the patient's parents.

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 (fT_4), thyroid-stimulating hormone (TSH), cortisol, adrenocorticotropin (ACTH) levels, and 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 peaks, 3 ng/ml by hypoglycemic provocation test, define severe GHD. A diagnosis of TSH deficiency was made if serum fT_4 concentration was under the normal level (fT_4, 1.0 ng/dl) with inadequate low to normal serum TSH concentration. Cortisol peaks, 17 mg/dl by hypoglycemic provocation tests, define ACTH deficiency. Follicle-stimulating hormone (FSH)–luteinizing hormone (LH) deficiency was diagnosed on the basis of delayed or absent pubertal development at the age of 16 years and inadequate increase in serum FSH and LH in response to LH-releasing hormone.

Of the 88 patients, on the basis of hormonal deficiencies, 13 were determined to have IGHD, whereas 75 had MPHD (Supplementary Table 1, see section on supplementary data given at the end of this article).

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.

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 given in Supplementary Table 2, see section on supplementary data given at the end of this article.

Comparative genomic hybridization array screening

We performed an array-comparative genomic hybridization (CGH) screening in 30 syndromic CH patients, whose karyotypes were confirmed to be normal by G-banding. CGH was performed using the Agilent SurePrint G3 Human CGH Microarray kit, 4×180K, 13 kb spatial resolution, or 1×1 M, 2.1 kb spatial resolution (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's protocol.

Table 1 The details of 30 syndromic patients.

Patient ID	Age (years)	Sex	Affected pituitary hormones	MRI findings	Clinical findings (extra-pituitary abnormalities)
1 (pedigree 1)	6	M	GH	APH, EPP, TS	Cleft palate, optic disk cupping
2 (pedigree 2)	6	M	GH, TSH	APH, EPP, TS	Bilateral anophthalmia, PDA, dysmorphic face, MR
3	15	F	GH, ACTH	APH, NPP, TS	SOD (ONH+APH)
4	20	M	GH, TSH	APH, NPP, ACC, IS	SOD (ONH+APH+ACC)
5	28	M	GH, TSH, ACTH	APH, EPP, IS	Cleft palate, MR
6	18	M	GH	APH, EPP, IS	MR
7	14	F	GH	APH, EPP, ASP, TS	SOD (ONH+APH+ASP)
8	8	M	GH, TSH	APH, EPP, ASP, TS	SOD (ONH+APH+ASP)
9	10	F	GH	APH, NPP, TS	SOD (ONH+APH)
10	5	F	GH, TSH, ACTH	APH, NPP, IS	Congenital absence of the scalp, ear anomaly
11	24	F	GH, LH/FSH	APH, NPP, NS	Hearing impairment, PDA
12	8	M	GH	APH, NPP, NS	Cleft palate
13	16	F	GH	APH, NPP, IS	Cleft palate, hearing impairment
14	5	F	GH, TSH, ACTH	APH, EPP, IS	SOD (ONH+APH)
15	4	M	GH	APH, EPP, IS	SOD (ONH+APH)
16	7	M	GH, TSH	APH, EPP, IS	Dysmorphic face, MR
17	9	M	GH, TSH, ACTH	APH, EPP, TS	Cleft palate, VSD
18	10	F	GH, ACTH	APH, EPP, ACC, TS	SOD (ONH+APH+ACC)
19	14	M	GH, TSH	APH, EPP, ASP, TS	SOD (ONH+APH+ASP)
20	3	M	GH, TSH, ACTH	APH, EPP, IS	SOD (ONH+APH)
21	8	M	GH, TSH	APH, EPP, ACC, IS	SOD (ONH+APH+ACC)
22	8	M	GH, TSH, ACTH	APH, EPP, IS	SOD (ONH+APH)
23	19	F	GH, TSH	APH, EPP, IS	SOD (ONH+APH)
24	14	M	GH, TSH	APH, NPP, NS	Cleft palate
25	10	F	GH, TSH	APH, EPP, ACC, IS	SOD (ONH+APH+ACC)
26	11	F	GH, TSH, ACTH	APH, NPP, IS	Cleft palate, PDA
27	23	F	GH, LH/FSH	APH, NPP, NS	Morning glory syndrome
28	3	M	GH, TSH	APH, EPP, IS	SOD (ONH+APH)
29	6	M	GH, TSH, ACTH	APH, EPP, IS	SOD (ONH+APH)
30	20	M	GH, TSH, ACTH, LH/FSH	APH, EPP, IS	Hearing impairment, right microphthalmia, MR

ACC, agenesis of the corpus callosum; APH, anterior pituitary hypoplasia; ASP, absence of septum pellucidum; EPP, ectopic posterior pituitary; F, female; IS, invisible stalk; M, male; MR, mental retardation; NPP, normal posterior pituitary; NS, normal stalk; ONH, optic nerve hypoplasia; PDA, patent ductus arteriosus; SOD, septo-optic dysplasia; TS, thin stalk; VSD, ventriculoseptal defect.

PAX6 mutation screening

For all the 88 patients, we analyzed all coding exons and flanking introns of *PAX6* by PCR-based sequencing. For detailed protein sequence information, see EMBL database accession numbers ENSP00000368418 (*PAX6*). We screened for deletion/duplication involving *PAX6* by MLPA analyses (SALSA MLPA KIT P219; MRC-Holland, Amsterdam, The Netherlands). We tested for any detected sequence variations against 150 Japanese control subjects.

Functional studies on the novel *PAX6* mutation (p.N116S)

The details of the experimental procedures are described in Supplementary methods, see section on supplementary data given at the end of this article. In brief, *PAX6* cDNA was cloned into pCMV-myc and pEGFP-N1 (Clontech) to generate PAX6 expression vectors. We introduced the mutation by site-directed mutagenesis using the Prime STAR Mutagenesis Basal kit (TaKaRa, Otsu, Japan). The primer pairs, forward (F) and reverse (R), used for mutagenesis were as follows: N116S (F)-5′-AACGATAGCA-TACCAAGCGTGTCATCAA-3′, N116S (R)-5′ -TGGTATGC-TATCGTTGGTACAGACCC-3′. Transactivation assay was performed using P6CON luciferase reporter vector, which was constructed by inserting the six copies of *PAX6* consensus binding elements, and dual-luciferase reporter assay system (Promega) on COS1 and GH3 cells. For subcellular localization analyses, we visualized and photographed COS1 cells transfected with GFP-tagged *PAX6*. For western blotting analyses, we harvested COS1 cells

transfected with the myc-tagged PAX6. Western blotting analysis was performed using a mouse anti-myc MAB (Invitrogen). EMSA experiment was performed using the Lightshift chemiluminescent EMSA kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Crystal structure modeling

The crystal structure of the PAX6 paired domain (protein data bank ID 6PAX; http://www.rcsb.org/pdb/) was used as a reference WT structure for modeling the structure of p.N116S *PAX6* by using the PyMOL Molecular Graphics System (http://www.pymol.org).

Results

CGH array screening

Among the 30 syndromic CH patients, we identified one heterozygous deletion, which was of estimated minimum extent 308 kb, extending from bp 31 474 276 to bp 31 783 119 on chromosome 11q13 (NCBI Build 37/hg 19) (Fig. 1A). Maximum size was 311 kb, from bp 31 471 923 to bp 31 785 385. The deletion included two genes (*ELP4* and *IMMP1L*) and *PAX6* putative enhancer region, with the coding region of *PAX6* being unaffected, in one patient with IGHD, cleft palate, and optic disc cupping. The deletion of the *PAX6* putative enhancer, or other deletions encompassing the one we identified, was not registered as frequent, non-pathogenic CNVs in existing database, including the database of genomic variants, DECIPHER, and UCSC. To confirm the deletion, fluorescence *in situ* hybridization (FISH) was performed on the metaphase slides from peripheral blood lymphocytes using the RP11-151O13 clones (Fig. 1A). Parental analysis revealed that the clinically normal father possessed the deletion in somatic mosaicism (number of cells with P*AX6* haploinsufficiency was eight, out of 20 counted cells), confirmed by FISH, whereas the mother did not. Second, we also identified one heterozygous 6.5-Mb deletion encompassing *OTX2* (Fig. 1B) in a MPHD patient with bilateral anophthalmia, dysmorphic facies, severe developmental delay, and a patent ductus arteriosus. No other syndromic CH patients had pathological CNVs.

PAX6 mutation screening

We identified one novel heterozygous *PAX6* mutation, namely p.N116S (Fig. 1C). The p.N116 in *PAX6* is an evolutionarily highly conserved amino acid located in the

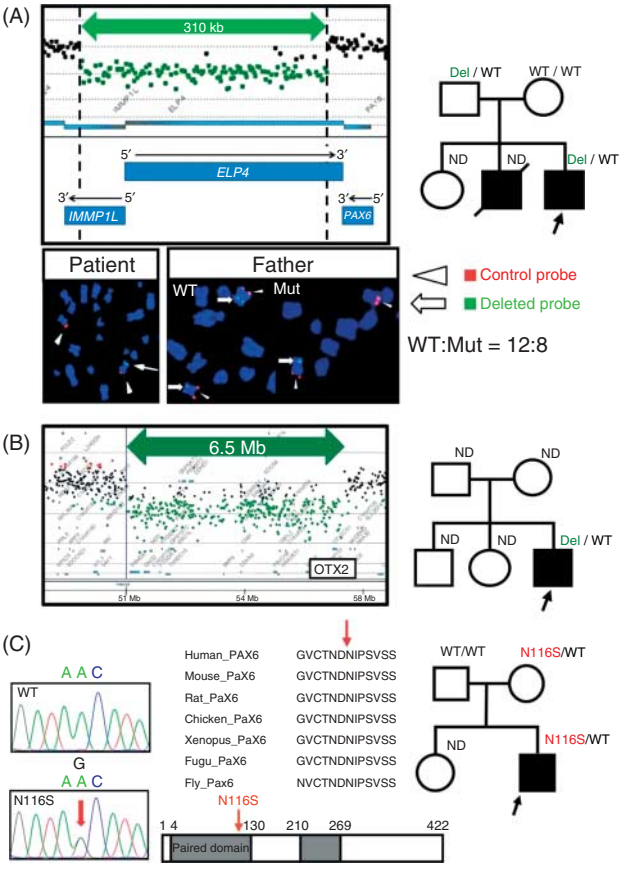

Figure 1

Identification of deletions and sequence variation.
(A) Graphical representation of the results of the array CGH analysis (Agilent 1×1M SurePrint G3 Human CGH Microarray) shows one heterozygous 310-kb deletion of *PAX6* putative enhancer region, including two neighboring genes: *ELP4*, and *IMMP1L*, with the coding region of *PAX6* being unaffected. The pedigree of this family is shown in the right side. Arrow indicates the propositus. The deletion was confirmed by FISH, using the RP11-151O13 clones. The clinically normal father possessed the deletion in somatic mosaicism (number of cells with P*AX6* haploinsufficiency was eight, out of 20 counted cells).
(B) Graphical representation of the results of the array CGH analysis (Agilent 4×180K SurePrint G3 Human CGH Microarray) shows one heterozygous 6.5-Mb deletion encompassing *OTX2.* The pedigree of this family is shown in the right side. Arrow indicates the propositus. (C) Partial sequences of PCR products of the patients are shown, and the chromatogram represents a heterozygous substitution of serine (AGC) in place of asparagine (AAC) at codon 116. Asn 116 is highly conserved through species, and is located within the paired domain. Genetic analyses showed that the propositus and his clinically normal mother carried the same heterozygous *PAX6* p.N116S mutation.

paired domain (Fig. 1C). The p.N116S was not detected in any of the 150 healthy controls and was absent from extant database, including dbSNP, the 1000 Genomes Project, Exome Variant Server, and NHLBI Exome Sequencing Project. We detected no gross or exon-level deletions/duplications on performing the MLPA analyses.

Clinical phenotypes of the mutation carriers

Pedigree 1: *PAX6* enhancer deletion ▸ The propositus was a 6-year-old Japanese boy, who was born at 37 weeks of gestation after an uncomplicated pregnancy and delivery (Fig. 1A). At birth, his length was 48.0 cm (−0.5 S.D.) and weight 2.98 kg (−0.1 S.D.). He had cleft palate that was surgically repaired. He was referred to us at 3 years of age because of his short stature. His height was 85.2 cm (−3.0 S.D.). Endocrine studies indicated that he had IGHD (Supplementary Table 3, see section on supplementary data given at the end of this article). A brain MRI showed anterior pituitary hypoplasia, with a visible but thin stalk, and an ectopic posterior pituitary gland (EPP) (Fig. 2A). No other CNS abnormalities were visualized. Recombinant human GH therapy was started at an age of 3 years. He responded well to GH (0.175 mg/kg per week) replacement therapy. Examination by experienced ophthalmologists revealed a bilateral optic disc cupping. His father was 173.0 cm (0.4 S.D.) tall, and his mother was 153.0 cm (−1.0 S.D.) tall. His older brother was diagnosed to have holoprosencephaly by a brain MRI (Fig. 2B), and he died at the age of 1 year. The older sister did not have a short stature. Hormonal data of the father, harboring the deletion in somatic mosaicism, was not evaluated.

Pedigree 2: *OTX2* deletion ▸ The propositus was a 6-year-old Japanese boy, who was born at 40 weeks of gestation after an uncomplicated pregnancy and delivery (Fig. 1B). The patient was the third child of non-consanguineous healthy parents. His older brother and sister were clinically normal. At birth, his length was 51.0 cm (1.0 S.D.) and weight 3.05 kg (0.1 S.D.). A constellation of malformations was noticed, including bilateral anophthalmia, and small and uplifted earlobes with very small external auditory canals. The testes were undescended, the scrotum small, and the foreskin hypoplastic. Echocardiogram revealed a patent ductus arteriosus, which was surgically corrected at 1 month of age. At the age of 1 year, he was diagnosed with central hypothyroidism with a low fT_4 (0.44 ng/dl: ref. 0.99–1.91) and an inadequately increased TSH level of 11.58 mU/l. Further endocrine studies indicated that the patient also had

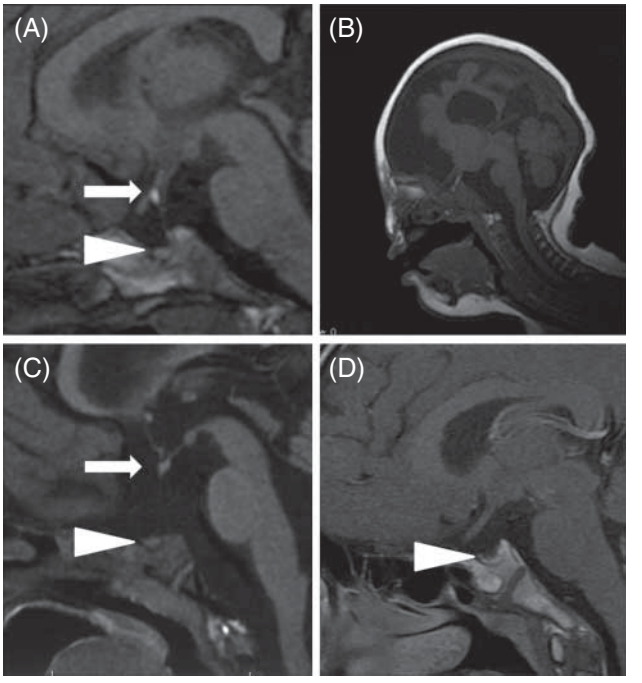

Figure 2
Pituitary structures on T1-weighted MRI scans of patients in pedigrees 1–3. Arrows indicate ectopic posterior pituitary and arrow heads indicate anterior pituitary hypoplasia. (A and B) Propositus in pedigree 1 (A) and his elder brother, who was diagnosed as holoprosencephaly (B), (C) propositus in pedigree 2, and (D) propositus in pedigree 3.

GHD (Supplementary Table 4, see section on supplementary data given at the end of this article). The brain MRI exhibited anterior pituitary hypoplasia, visible but thin stalk, and EPP (Fig. 2C). Replacement therapy with thyroxine and recombinant human GH was started at the ages of 1 and 3 years respectively. No other family members were available for genetic studies or evaluation of hormonal data.

Pedigree 3: *PAX6* p.N116S ▸ The propositus was a 15-year-old Japanese male, who was born at 40 weeks of gestation after an uncomplicated pregnancy and delivery (Fig. 1C). At birth, his length was 44.0 cm (−2.4 S.D.) and weight 2.67 kg (−0.8 S.D.). He was referred to us at the age of 3 years because of short stature and bilateral cryptorchidism. His hormonal data revealed IGHD (Supplementary Table 5, see section on supplementary data given at the end of this article). A brain MRI exhibited anterior pituitary hypoplasia, visible but thin

stalk, poorly developed sella turcica, and eutopic posterior pituitary (Fig. 2D). He showed no ocular malformation. Replacement therapy with recombinant human GH was started at the age of 4 years. The patient responded well to GH replacement therapy. At the age of 15 years, he showed no pubic hair; his penile length was 9 cm, and his testicular volume was 6 ml bilaterally, with normal concentration of basal testosterone (3.98 ng/ml ref: 2.0–7.5). The results of his gonadotropin-releasing hormone and human chorionic gonadotropin test were normal (Supplementary Table 5). His father was 170.0 cm (−0.1 S.D.) tall, and mother was 158.0 cm (0.1 S.D.) tall. Genetic analyses showed that the propositus and his mother carried the same heterozygous *PAX6* p.N116S mutation. Hormonal data of the mother were normal (Supplementary Table 6, see section on supplementary data given at the end of this article).

Functional studies

Both in COS1 and GH3 cells, WT *PAX6* stimulated the transcription of the P6CON reporter in a dose-dependent manner. The p.N116S retained partial activity without any dominant negative effect (Fig. 3A). The p.N116S mutant localized to the nucleus (Fig. 3B). The western blot analysis showed that the expression of the p.N116S was comparable with that of the WT (Fig. 3C). The WT *PAX6* showed specific binding to the oligonucleotide elements,

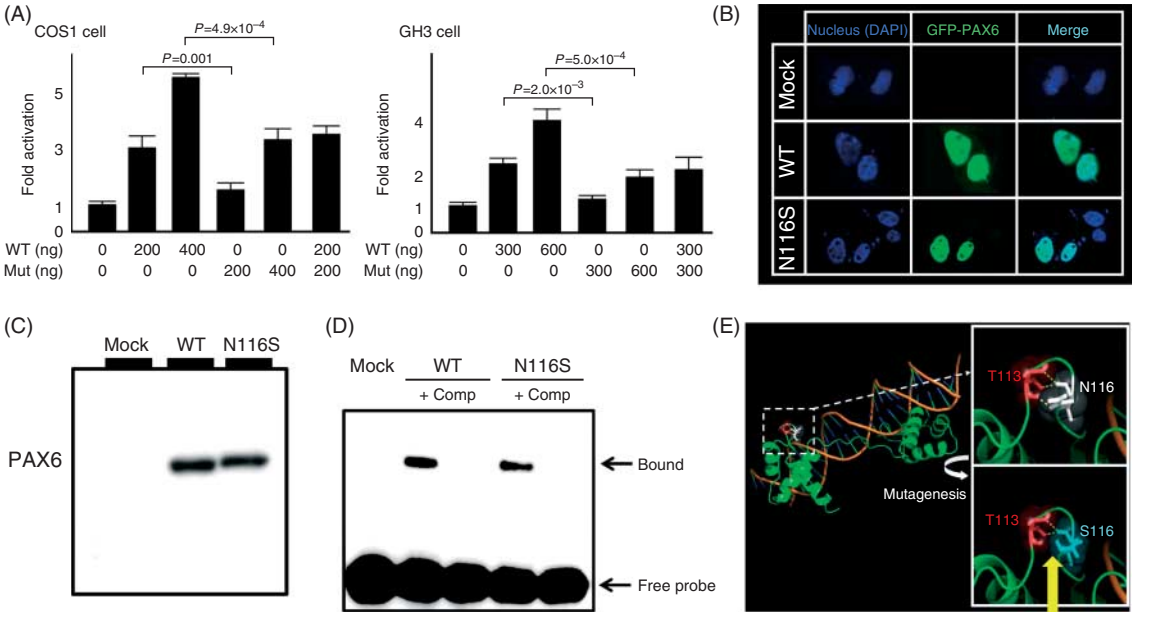

Figure 3

Functional characterization and modeled structure of N116S PAX6. (A) Transactivation assays of the p.N116S PAX6 using P6CON reporter in COS1 and GH3 cells. COS1 or GH3 cells were cotransfected with the pRL-CMV internal control vector, indicated amount (nanograms) of the effector plasmids, and the P6CON reporter. The data are the mean ± S.E.M. of at least three independent experiments performed in triplicate transfections. The p.N116S PAX6 retained partial activity and did not exhibit any dominant negative effect. (B) Subcellular localization analysis. For subcellular localization analyses, we visualized and photographed COS1 cells transfected with GFP-tagged *PAX6* using a Leica TCS-SP5 laser scanning confocal microscope, after mounting the cells in Vectashield-DAPI solution. The WT and p.N116S *PAX6* are localized to the nucleus. (C) Western blotting analysis. Western blotting analysis showed that the expression of p.N116S *PAX6* was comparable with that of the WT. (D) EMSA experiments. WT *PAX6* showed specific binding to the elements, which was competed by excess amount (200 times) of cold competitors. The p.N116S *PAX6*, which has an intact HD, bound with similar efficiency to the WT *PAX6*. (E) Modeled structure of the p.N116S *PAX6* in comparison with the WT structure (upper panels). Modeling of mutant was performed using a built-in mutagenesis function of the PyMOL Molecular Graphics System. Crystal structural modeling showed that the p.N116S *PAX6* was predicted to lose the hydrogen bond between N116 and T113 (arrow).

which was competed for by an excess amount of cold competitors (200 times). The p.N116S bound with similar efficiency to the WT *PAX6* (Fig. 3D).

Crystal structure modeling

The p.N116S was predicted to lose the hydrogen bond between N116 and T113 (Fig. 3E).

Discussion

This study describes the first series of CH patients to be screened for chromosomal rearrangements with high-resolution oligonucleotide microarrays. Overall, this study identified two different CNVs with a possible role in the etiology of CH in two patients among 30 syndromic CH patients (6.6%). Thus, the causes of CH remain elusive in most patients, and further studies are required to understand the pathogenesis of CH.

PAX6 is a well-known regulator of eye development, and its heterozygous mutations in humans cause congenital eye anomalies such as aniridia, foveal hypoplasia, optic-nerve malformations, and Peters' anomaly. Kioussi *et al*. (9) showed that *PAX6* is an early dorsal marker of early anterior pituitary gland, and its expression controls the established boundaries of somatotrope, lactotrope, and thyrotrope cell types. Dorsal expression of *PAX6* is essential for establishing a sharp boundary between dorsal and ventral cell types, and the absence of *PAX6* leads to a marked increase in the thyrotrope cell lineage, whereas the somatotrope and lactotrope cell lineage changes are very much diminished (9). These data strongly support our findings that *PAX6* mutations are associated with CH.

Recently two papers have been published, showing that *PAX6* mutations may be associated with impaired pituitary function (10, 11). Hergott *et al*. reported data on detailed pituitary studies using the stimulation testing of five *PAX6* mutation carriers (10). In this paper, they found no clear pituitary deficiencies other than subtle corticotrope deficiency. Shimo *et al*. (11) reported one case of *PAX6* mutation with subtle hypogonadotropic hypogonadism and borderline GH deficiency. However, this particular case is unconvincing because the subject was obese (which blunts GH response to GH stimulation testing), had normal adult stature of 165 cm, normal menses and pregnancies. Therefore, we showed, for the first time, that heterozygous *PAX6* mutations are associated with definite CH patients with or without ocular malformation, and the frequency of *PAX6* mutations in CH was 2.3%. To date, more than 350 mutations or variants in *PAX6* have been reported (12), and almost all were found in the heterozygous state. Although extremely rare, at least four patients harboring biallelic mutations in *PAX6* have been reported (13, 14, 15). Three patients were stillborn or died in early infancy with severe CNS anomalies, but one long surviving patient among them showed MPHD and anterior pituitary hypoplasia (13), further supporting a role for PAX6 in the pituitary development in human.

In pedigree 1, we identified a heterozygous 310 kb deletion of the downstream flanking region of *PAX6* in an IGHD patient with cleft palate and bilateral optic disc cupping. The known control that *PAX6* exerts over the temporospatial expression of Sonic hedhehog (*SHH*) (16), the critical importance of *PAX6* on pituitary development through *SHH* (9), and the critical importance of *SHH* on palate development (17) strongly support the argument for *PAX6* defects being pathogenic events responsible for the phenotype. Previously, several authors described submicroscopic deletions downstream of *PAX6*, with the coding region of *PAX6* being unaffected (18, 19, 20, 21, 22). Davis *et al*. (18) identified a 1.3 Mb deletion, including six neighboring genes: *ELP4, DPH4, DCDC1, DCDC5, MPPED2,* and *IMMP1L*, localized ~35 kb downstream of *PAX6* in a patient with aniridia, autism, and intellectual disability. Analysis of the region with a breakpoint located 124 kb downstream of the *PAX6* polyadenylation site using YAC transgenic mice, DNase I hypersensitivity mapping and reporter transgenic assays, showed the presence of several *cis*-regulatory elements, including a lens-specific and a retina-specific element. These elements are located within the introns of the *ELP4* gene and are *PAX6*-specific long-range control elements (22, 23). ELP4, which is ubiquitous but with variable expression in human tissues, acts as subunit of the RNA polymerase II elongator complex, which is a histone acetyltransferase component of the RNA polymerase II holoenzyme and is involved in transcriptional elongation (24). IMMP1L, which is located at the mitochondrion inner membrane, catalyzes the removal of transit peptides required for the targeting of proteins from the mitochondrial matrix across the inner membrane into the inter-membrane space (25). The deletion, we identified, includes *ELP4* and *IMMP1L*. While deletions in the *ELP4* gene have been shown to suppress *PAX6* gene expression and cause aniridia (19), implications of *IMMP1L* in the described phenotype are not clear. Although pituitary-specific elements are not well studied, we believe that our findings will shed light on the role for *PAX6* in the pituitary development in humans.

Wide phenotypic spectrum in *PAX6* mutation carriers is noteworthy. In pedigree 1, the proband's older brother was diagnosed to have holoprosencephaly, the most common forebrain developmental anomaly in humans. Unfortunately gene analysis data for the brother were not available. However, the mosaicism of the deletion in his father strongly suggested that the most severe phenotype was due to the same deletion and the variable penetrance of *PAX6* mutations. Of course, the effect of environmental factors, second hit in *PAX6*, or mutation in other genes for holoprosencephaly, was not excluded. In pedigree 3, the patient's mother was of normal adult height and had normal baseline hormonal levels. The p.N116S was associated with impaired transactivation without dominant-negative effects, indicating haploinsufficiency. The western blotting analysis, visualization of subcellular localization, and a DNA-binding test revealed no significant difference between the WT and p.N116S *PAX6*. Asn 116 is a highly conserved amino acid located in the paired domain, suggesting that substitution of Asn 116 to Ser, which is predicted to lose the hydrogen bond between N116 and T113, results in defective interactions with transcriptional cofactors. *PAX6* mutations may be associated with diverse clinical features ranging from severely impaired ocular and pituitary development to apparently normal phenotype. PAX genes may be sometimes monoallelicaly expressed and the individual factors governing such monoalleic expression are not understood. Since the found mutation is heterozygous, theoretically a favorable monoallelic expression from the correct allele in the mother might cause the lack of phenotype, and the expression from the mutated allele in the patient might cause the phenotype (26, 27, 28). The phenotypical variation within the same pedigree could also be partly due to the impact of other genes that are important but have not been recognized in pituitary development. Of course, the possibility that p.N116S *PAX6* could have no significance *in vivo* effect in human, and that he had a *de novo* mutation in some other yet-to-be- identified gene causative for CH, should also be considered.

In summary, we showed, for the first time, that heterozygous *PAX6* mutations are associated with definite CH patients with or without ocular malformation. The frequency of *PAX6* mutations in CH was 2.3%. *PAX6* mutations may be associated with diverse clinical features ranging from severely impaired ocular and pituitary development to apparently normal phenotype. Overall, this study identified causative CNVs with a possible role in CH in $<10\%$ of the syndromic CH patients.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-14-0255.

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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Author contribution statement
M Takagi and T Hasegawa conceived and designed the experiments, M Takagi performed the experiments, M Takagi analyzed the data: K Nagasaki, I Fujiwara, T Ishii, N Amano, Y Asakura, K Muroya, Y Hasegawa, M Adachi contributed for reagents/materials/analysis tools, and M Takagi and T Hasegawa wrote the paper.

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Supplemental Table 1 Endocrine phenotype of 88 CH patients

	No. (%) with deficiencies of			
	GH	**TSH**	**ACTH**	**LH/FSH**
IGHD (n=13)				
Syndromic	**7 (100)**			
Non-syndromic	**6 (100)**			
Total	**13(100)**			
MPHD (n=75)				
Syndromic	**23 (100)**	**19 (83)**	**11 (48)**	**3 (13)**
Non-syndromic	**49 (100)**	**40 (82)**	**23 (47)**	**16 (33)**
Total	**75(100)**	**59(79)**	**34(45)**	**19(25)**

Supplemental Table 2 Results of MRI scans of 88 CH patients

	Morphology of						
	Anterior pituitary	Posterior pituitary			Stalk		
	Hypoplasia	Normal	Ectopic	Absent	Normal	Invisible	Thin
IGHD (n=13)							
Syndromic	7	3	4	0	1	3	3
Non-syndromic	6	2	4	0	3	1	2
Total	13	5	8	0	4	4	5
MPHD (n=75)							
Syndromic	23	7	16	0	3	14	6
Non-syndromic	52	16	34	2	20	25	7
Total	75	23	50	2	23	39	13
Total (n=88)	88	28	58	2	27	43	18

Supplemental Table 3 Endocrinological findings in Propositus of pedigree 1 (*PAX6 enhancer deletion*)

		3yr			Reference		
	Stimulus	Basal		Peak	Basal		Peak
GH (ng/ml)	Insulin	0.6	→	1.7			>6
TSH (mIU/ml)	TRH	1.79	→	16.68			10-35
LH (mIU/ml)	LHRH	< 0.2	→	2.5	<0.1[a]		<0.1-4.29 [a]
FSH (mIU/ml)	LHRH	1.1	→	5.2	0.46-1.43[a]		5.38-11.67[a]
PRL (ng/ml)	TRH	11.3	→	60.9	1.7-15.4		increase 2 times
ACTH (pg/ml)	Insulin	31.1	→	186	9.8-27.3		28-130.5
Cortisol (μg/dl)	Insulin	13.4	→	29.6			>17.0
IGF-1 (ng/ml)		< 4			29-173		
Free T4 (ng/dl)		1.3			1.0-1.95		
Free T3 (pg/ml)		3.4			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), 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 [1]

Supplemental Table 4 Endocrinological findings in Propositus of pedigree 2 (*OTX2 deletion*)

		1yr			Reference		
	Stimulus	Basal		Peak	Basal		Peak
GH (ng/ml)	Arginine	0.8	→	1.5			>6
ACTH (pg/ml)	CRF	11.5	→	47.0	9.8-27.3		28-130.5
Cortisol (μg/dl)	CRF	6.1	→	17.6			>17.0
IGF-1 (ng/ml)		< 4			11-172[a]		
TSH (mIU/ml)		11.58					
Free T4 (ng/dl)		0.44			1.0-1.95		
Free T3 (pg/ml)		2.23			2.23-5.30		

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

[a] Reference data of Japanese boys (1-2 years old) [2]

Supplemental Table 5 Endocrinological findings in Propositus of pedigree 3 (*PAX6* p.N116S)

		9 yr			15 yr			Reference		
	Stimulus	Basal		Peak	Basal		Peak	Basal		Peak
GH (ng/ml)	Insulin	<0.1	→	<0.1						>6
TSH (mIU/ml)	TRH	3.02	→	15.46	2.89					10-35
LH (mIU/ml)	LHRH				2.6	→	19.1	0.17-1.63[a]		13.11-25.15[a]
FSH (mIU/ml)	LHRH				4.1	→	6.8	2.12-5.24[a]		5.75-13.25[a]
Testosterone (ng/ml)	HCG	<0.05			3.98	→	9.64			>1.2[a]
PRL (ng/ml)	TRH	5.2	→	76.3	6.3	→	82.7	1.7-15.4		increase 2 times
ACTH (pg/ml)	Insulin	10	→	347	124	→	291	9.8-27.3		28-130.5
Cortisol (μg/dl)	Insulin	7.1	→	30	7.1	→	25.3	5-20		>17.0
IGF-1 (ng/ml)		6.9			93.0			87-405[b]		
								287-555[c]		
Free T4 (ng/dl)		1.0			1.3			1.01-1.95		
Free T3 (pg/ml)		3.1			3.5			2.23-5.30		

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[a] Reference data of pubertal Japanese boys [1]

[b] Reference data of Japanese boys (9-10 years old) [2]

[c] Reference data of Japanese boys (15-16 years old) [2]

Supplemental Table 6 Endocrinological findings (baseline) in unaffected mother of pedigree 3 (*PAX6* p.N116S)

		Mother		Reference (Adult)
GH (ng/ml)		0.8		0-23
IGF-1 (ng/ml)		230		Female: 73-542
TSH (µU/ml)		2.95		0.3-3.50
Free T4 (ng/dl)		1.2		1.09-2.55
Free T3 (pg/ml)		2.4		3.23-5.11
LH (mIU/ml)		4.7		Female: 1.4-15[a]
FSH (mIU/ml)		3.4		Female: 3-10[a]
PRL (ng/ml)		19.4		Female: 1.4-14.6
ACTH (pg/ml)		9.3		7.2-63.3
Cortisol (µg/dl)		7.2		7.6-21.4
Estradiol (pg/ml)		143		Female: 11-230[a]

[a]Follicular phase

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		1yr			Reference		
	Stimulus	Basal		Peak	Basal		Peak
GH (ng/ml)	Arginine	0.8	→	1.5			>6
ACTH (pg/ml)	CRF	11.5	→	47.0	9.8-27.3		28-130.5
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Supplemental Table 5 Endocrinological findings in Propositus of pedigree 3 (*PAX6* p.N116S)

		9 yr			15 yr			Reference		
	Stimulus	Basal		Peak	Basal		Peak	Basal		Peak
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[b] Reference data of Japanese boys (9-10 years old) [2]

[c] Reference data of Japanese boys (15-16 years old) [2]

Supplemental Table 6 Endocrinological findings (baseline) in unaffected mother of pedigree 3 (*PAX6* p.N116S)

		Mother		Reference (Adult)
GH (ng/ml)		0.8		0-23
IGF-1 (ng/ml)		230		Female: 73-542
TSH (µU/ml)		2.95		0.3-3.50
Free T4 (ng/dl)		1.2		1.09-2.55
Free T3 (pg/ml)		2.4		3.23-5.11
LH (mIU/ml)		4.7		Female: 1.4-15[a]
FSH (mIU/ml)		3.4		Female: 3-10[a]
PRL (ng/ml)		19.4		Female: 1.4-14.6
ACTH (pg/ml)		9.3		7.2-63.3
Cortisol (µg/dl)		7.2		7.6-21.4
Estradiol (pg/ml)		143		Female: 11-230[a]

[a]Follicular phase

References

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