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The identification of *HESX1* mutations in Kallmann syndrome

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Abstract

Objective—To determine if *HESX1* mutations are present in patients with idiopathic hypogonadotropic hypogonadism (IHH)/Kallmann syndrome (KS). *HESX1* mutations have previously been characterized in patients with septo-optic dysplasia (SOD), isolated growth hormone deficiency (IGHD), and combined pituitary hormone deficiency (CPHD). We hypothesized that IHH/KS represents a milder phenotypic variant of SOD.

Design—PCR-based DNA sequencing was performed on 217 well-characterized IHH/KS patients. Putative missense mutations were analyzed by sorting intolerant from tolerant (SIFT) and Clustal Ω.

Setting—An academic medical center

Patients—217 IHH/KS and 192 controls

Interventions—DNA was extracted from patients and controls; genotype/phenotype comparisons were made

Main Outcome Measures—DNA sequence of *HESX1*, SIFT analysis, and ortholog alignment

Results—Two novel heterozygous missense mutations (p.H42Y and p.V75L) and previously reported heterozygous missense mutation p.Q6H in *HESX1* were identified in 3/217 (1.4%) patients. All were males with KS. Both p.Q6H and p.H42Y were predicted to be deleterious by SIFT, while p.V75L was conserved in 8/9 species. No other IHH/KS gene mutations were present.

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Conclusions—*HESX1* mutations may cause KS in addition to more severe phenotypes. Our findings expand the phenotypic spectrum of *HESX1* mutations in humans, thereby broadening its role in development.

Keywords

HESX1; Kallmann syndrome; Hypogonadotropic hypogonadism; Delayed puberty; GnRH deficiency

Introduction

Hesx1 (homeobox gene expressed in embryonic stem cells 1) is a developmental gene identified in mouse that encodes an embryological transcription repressor important for organ commitment and cell differentiation and proliferation (1). HESX1 is a member of the *Paired* (*prd*) class of homeodomain proteins, a set of transcription factors characterized by a tripartite helical domain that binds specific palindromic DNA targets as a dimer (1). The human ortholog, *HESX1*, has extensive homology to mouse and maps to chromosome 3p14.3. The human gene, which contains four exons and has the same structural organization as in mouse, encodes a 558bp open reading frame resulting in a 185 amino acid protein. The protein contains two highly conserved functional domains. The first is an N-terminal 7 amino acid *engrailed* homology (eh1) domain that binds its co-repressor known as transducin-like enhancer of split-1 (TLE1), while the second consists of a 59 amino acid C-terminal homeodomain containing transcriptional repressor activity (1). As one of the earliest markers of the developing anterior pituitary, *Hesx1* transcripts become confined to Rathke's pouch, an invagination of oral ectoderm that gives rise to the anterior pituitary primordium. During pituitary cell differentiation, *Hesx1* expression disappears in a spatiotemporal pattern as one of its downstream targets, *Prop1*, rises (1–3).

In addition, HESX1 has an important function in the temporal and sequential development of the forebrain, hypothalamus, optic nerve, and posterior pituitary (1). This is further supported by mutations in both the human and mouse gene affecting these structures. The *Hesx1* knockout mouse displays a phenotype that strongly resembles human septo-optic dysplasia (SOD), a clinical triad characterized by agenesis of midline brain structures, optic nerve hypoplasia, and hypopituitarism (1). In fact, the first characterized human *HESX1* mutation in an SOD patient was a homozygous p.R160C point mutation resulting in the loss of HESX1 DNA binding (1). Additional human mutations have been described in other disorders such as isolated growth hormone deficiency (IGHD) (4–5) and combined pituitary hormone deficiency (CPHD) (6–7), which consists of a deficiency in GH and at least one additional pituitary hormone. At least 16 different human *HESX1* mutations, both homozygous (1, 3, 8–10) and heterozygous (4–7, 11–15) have been identified in humans with pituitary disorders.

It is not currently known if human *HESX1* mutations cause the less severe phenotype of idiopathic hypogonadotropic hypogonadism (IHH), a disorder of absent or incomplete pubertal development due to GnRH deficiency and subsequent impaired gonadotropin secretion. When IHH is accompanied by anosmia or hyposmia, it is termed Kallmann syndrome (KS). At least 18 different genes are known to cause IHH/KS, but the molecular basis is only known for approximately 30–40% of patients (16–21). We hypothesized that IHH/KS, a milder allelic variant of SOD, could be due to heterozygous HESX1 mutations.

Materials and Methods

Patients

IHH was defined as absent puberty in females age >17 and males age >18 with normal imaging of the pituitary and hypothalamus and low serum gonadotropins, as described previously (22). The remainder of pituitary function was normal as evidenced by serum TSH, T4, prolactin, and AM cortisol levels. All females had absent or arrested breast development and hypoestrogenic amenorrhea, while all males had a total testosterone < 100 ng/dL (normal 300–1100 ng/dL) (22). Patients were categorized as complete or incomplete IHH as data allowed. Complete IHH was defined as total lack of breast buds (Tanner stage 1) in females and testicular size of 3cc or less in males (23). Incomplete IHH indicates evidence of prior steroid production and was defined as breast development of Tanner 2 or greater in females and testes size ≥ 4cc in males (23). KS was defined IHH and anosmia or hyposmia using the University of Pennsylvania Smell Identification Tests, if available, or by history. A total of 217 IHH/KS patients were studied: complete IHH (n = 58), incomplete IHH (n = 51) and unknown (n=108) since they received prior treatment prior to their diagnosis. Sense of smell consisted of the following: anosmia (n =68), hyposmia (n =15), normosmia (n =70), and unavailable (n =64). This study was approved by the Human Assurance Committee of Georgia Health Sciences University, and each patient signed an informed consent.

Molecular Analysis

DNA was extracted from white blood cells in 217 patients with IHH/KS as described previously (24). The protein coding regions of all four exons and splice junctions of the *HESX1* gene were first amplified by PCR for 30 cycles, with each cycle consisting of 94°C for 1min, 55 °C for 45 seconds, and 72 °C for 45 seconds. Each PCR included a negative control containing all reagents except DNA. The PCR products were first analyzed by electrophoresis on 1.2% agarose gels, stained with ethidium bromide, and photographed.

Direct DNA sequencing of PCR products was performed to screen for putative *HESX1* mutations as described previously (16–19). PCR products were first ethanol precipitated, sequenced using the Big Dye Terminator Sequencing kits (ABI PRISM Foster City, CA), ethanol precipitated again, resuspended in HiDi Formamide, and placed on an ABI 377 automated DNA sequencer. Putative mutations were sequenced at least twice in both forward and reverse directions. Sequences were compared to published sequence reported in NCBI GenBank. To determine if identified sequence variants were polymorphisms, 192 ethnically matched control DNA samples were subjected to *HESX1* sequencing. In addition, the identified genetic variants were compared with known benign polymorphisms in the NCBI SNP database, and the 1,000 Genomes Database.

To exclude digenic disease, which has been reported to occur in about 10–12% of IHH/KS patients (21, 25) the coding regions and splice junctions for other known KS genes *KAL1* (26), *FGFR1*(27), *CHD7*(18), *NELF* (28), *SOX2* (29), and *WDR11*(19) were also sequenced in each person with a *HESX1* mutation, as described previously.

Comparative Sequence Analysis

Orthologs of human *HESX1* were obtained from NCBI GenBank. Full sequences for the following nine species were identified: human (NM_003865.2), rat (NM_001109106.1), chimpanzee (NM_001081570.1), cow (NM_001191140.1), dog (XM_541834.1), mouse (NM_010420.2), monkey (XM_001100209.2), rabbit (XM_002713403.1), and orangutan (XM_002813612.2). Comparison of conserved amino acids was performed using Clustal Omega for alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/).

SIFT (Sorting Intolerant From Tolerant)

To predict the significance of missense mutations, SIFT (Sorting intolerant from tolerant) analysis was performed to see if the mutation was likely to be deleterious (http://sift.bii.a-star.edu.sg/) (30). An intolerant amino acid change was defined by P <0.05. SIFT analysis was also performed for previously reported *HESX1* missense mutations.

Results

HESX1 Mutation Detection

The four exons of *HESX1* were screened for mutations in patients with IHH/KS by PCR-based DNA sequencing. No deletions or insertions of exons were present in any of the 217 IHH/KS patients studied. Three different heterozygous missense *HESX1* variants were found in 3/217 (1.4%) unrelated IHH/KS patients, but not in 192 ethnically-matched controls, the SNP database, or the 1,000 Genomes database (Table 1). Patient 1 had a c.18G>A, resulting in a non-synonymous p.Q6H substitution in exon 1, in which a CAG (Gln) was changed to a CAC (His) at codon 6. Patient #2 had a c.124C>T resulting in a p.H42Y missense variant in exon 1 in which a CAC (His) was altered to TAC (Tyr) at codon 42. Patient 3 had a c. 223G>T in exon 2 that resulted in a p.V75L missense variant. Of interest, all three patients were males with anosmia—one was Caucasian of Northern European descent and two were Turkish. All three were the only affected individuals in the family. Interestingly, none of these patients had additional anomalies of SOD or anomalies associated with KS (Table 1). Neither patient 1 or 2 with *HESX1* mutations had a mutation in *KAL1*, *FGFR1*, *CHD7*, *NELF*, *SOX2*, or *WDR11*. Patient 3, who had a missense mutation predicted to be tolerated by SIFT, was studied for three additional genes, and had no mutations in *KAL1*, *FGFR1*, or *SOX2*.

A fourth heterozygous c.385G>A nucleotide change resulting in a p.V129I missense variant within the homeodomain exon 3 was identified in a female with normosmic IHH. However, this same change was seen once in 192 controls and reported previously in controls (5), indicating it represents a polymorphism. Four additional patients contained a heterozygous c.374A>G in *HESX1* resulting in a p.N125S missense substitution, a previously reported benign Afro-Caribbean polymorphism (14).

Predicted functional results by comparative sequence analysis and SIFT

Alignment of *HESX1* orthologs from nine species was accomplished using Clustal Ω. The three missense variants (p.Q6H, p.H42Y, p.V75L) are not located within the co-repressor binding domain or the homeodomain of HESX1 (Figure 1). The Gln6 (Q6) residue is conserved in 6/8 species, while His42 (H42) is conserved in 8/8 species (*Pongo* does not contain the involved sequence regions so comparisons may not be made); Val75 is conserved in 8 of 9 species (Figure 1). SIFT analysis predicts that p.Q6H (P = 0.03) is deleterious, as a basic amino acid (histidine) substitutes for the uncharged polar amino acid (glutamine). Similarly, SIFT predicts that p.H42Y will be deleterious (P = 0), since a basic amino acid (histidine) is altered to an uncharged polar amino acid (tyrosine). However, p.V75L (P = 0.16) was predicted to be tolerated despite its conservation in 8/9 species. Of interest, the polymorphism p.V129I uniquely lies within the C–terminal homeodomain (Figure 1), but it is not predicted to be deleterious by SIFT (P = 0.1).

Discussion

Human *HESX1* mutations leading to clinical disease have thus far proven to be rare, being found in only ~1% of patients with pituitary deficiency (4, 6). Mutations in the *HESX1* gene were first identified in patients with SOD, a clinical disorder with a strong resemblance to

the phenotype of the *Hesx1* knockout mouse (1). Homozygous *HESX1* mutations generally result in more severe human phenotypes, such as SOD (1), life-threatening neonatal conditions (8) and panhypopituitarism (9–10, 15), whereas published heterozygous mutations seem to display variable expressivity and are more often associated with milder phenotypes such as IGHD (4–5, 12).

To date there have only been 16 reported *HESX1* mutations—six homozygous and 10 heterozygous (Table 2). The six homozygous *HESX1*mutations included three missense (1, 3, 10), two frameshift (8–9), and one splice mutant (8). All patients had panhypopituitarism, including one with SOD. The functional effects of the five (of six) mutations studied revealed that one impaired DNA binding, one lacked significant homeodomain sequences, and three lacked PROP1 repression activity. As can be seen Table 2, most homozygous mutations appear to affect either the repressor or the DNA binding domain; and result in autosomal recessive disease since parents are carriers.

The ten heterozygous mutations included six missense (4–5, 7, 12, 14–15), two frameshift (11, 13), one splice variant (5), and a nonsense mutation (15) (Table 2). The phenotypes consisted of CPHD (n = 6), IGHD (n = 2), or IGHD associated with optic nerve atrophy (n = 1) or SOD (n =1). Six of the 10 mutations demonstrated functional effects *in vitro*, but four were not located within either of the two functional domains. Some of the heterozygous mutations (p.S170L, p.T181A, p.Q6H, p.E149K) have all been seen in at least one unaffected parent, suggesting autosomal dominant inheritance with reduced penetrance (4–5, 7, 12).

IHH/KS is generally regarded to be due to GnRH deficiency, with at least 18 genes known to be etiologic in about 30–40% of patients (18–21). However, mutations in pituitary expressed genes, such as *GNRHR* (17, 31), also cause IHH. We hypothesized that IHH/KS would be a mild allelic variant of SOD, and determined the prevalence of *HESX1* mutations in a large sample of IHH/KS patients. Consistent with this hypothesis, 3/217 (1.4%) demonstrated heterozygous missense *HESX1* variants that were not seen in 192 controls, the SNP database, or 1,000 Genomes database. Since none of the missense variants were localized to the homeodomains, *in vitro* analysis was not possible. However, the p.Q6H was predicted by SIFT to be deleterious and the wild type Q6 residue is conserved in 6/8 orthologs. The p.H42Y was also predicted by SIFT to be deleterious; and the H42 was conserved in 8/8 species. These findings strongly implicate that these two missense variants likely are causative in the KS phenotype. Further supportive evidence that p.Q6H is causative comes from previous publications in which this mutation has been found in heterozygous form in CPHD (5, 7, 12).

A third heterozygous missense mutation p.V75L was also identified in a KS patient. Although the V75 residue was not predicted to be deleterious, it was conserved in 8/9 orthologs, and was absent in 192 controls, the SNP database, and 1,000 Genomes database. Therefore, it is possible that it could be involved in the pathogenesis of IHH/KS. Of the nine previously reported missense *HESX1* mutations that have been linked to various phenotypes including CPHD and IGHD, eight out of nine were predicted to be deleterious by SIFT with $P < 0.05$ (Table 2). Four of the mutations predicted to be intolerant by SIFT were confirmed by *in vitro* analysis, but five did not affect either homeodomain and could not be studied *in vitro*. Interestingly, all three of our individuals with *HESX1* mutations were males with KS (IHH with anosmia/hyposmia). Our patients included 83 with KS, 70 with normosmic IHH, and 64 whose sense of smell was unknown. Therefore, 3/83 (3.6%) of KS patients had *HESX1* missense mutations. Our sample size is too small to really determine if *HESX1* mutations occur more frequently in KS than in nIHH.

Unfortunately, no other family members were available for DNA analysis so the mode of inheritance is difficult to ascertain—autosomal dominant inheritance with reduced penetrance or sporadic. However, our findings are consistent with those in the literature. As reported by several investigators (4, 7, 12), a number of heterozygous *HESX1* mutations were all seen in at least one unaffected parent, which supports autosomal dominant inheritance with reduced penetrance.

We also identified four patients with heterozygous p.N125S, which is a previously reported benign polymorphism in the Afro-Caribbean population (14), and one patient with p.V129I, a missense variant identified in 1/192 controls in the present study and that was previously also found in controls (5). Therefore, these variants are unlikely to be involved in the pathogenesis of IHH/KS, suggesting that they are polymorphisms.

In summary, 3/217 (1.4%) of all IHH/KS patients tested had *HESX1* mutations. If only KS patients are considered, 3/83 (3.6%) had mutations. Our findings support that KS is a milder phenotypic manifestation of *HESX1* mutations, but without other more severe craniofacial, central nervous system, and ophthalamalogic abnormalities. If larger samples of IHH/KS support this data, *HESX1* may be one of the more common genes to cause KS, which include *KAL1*(32), *FGFR1* (33), and *CHD7* (18). The identification of mutations in KS expands the phenotypic spectrum of *HESX1* mutations in humans, thereby broadening its role in development.

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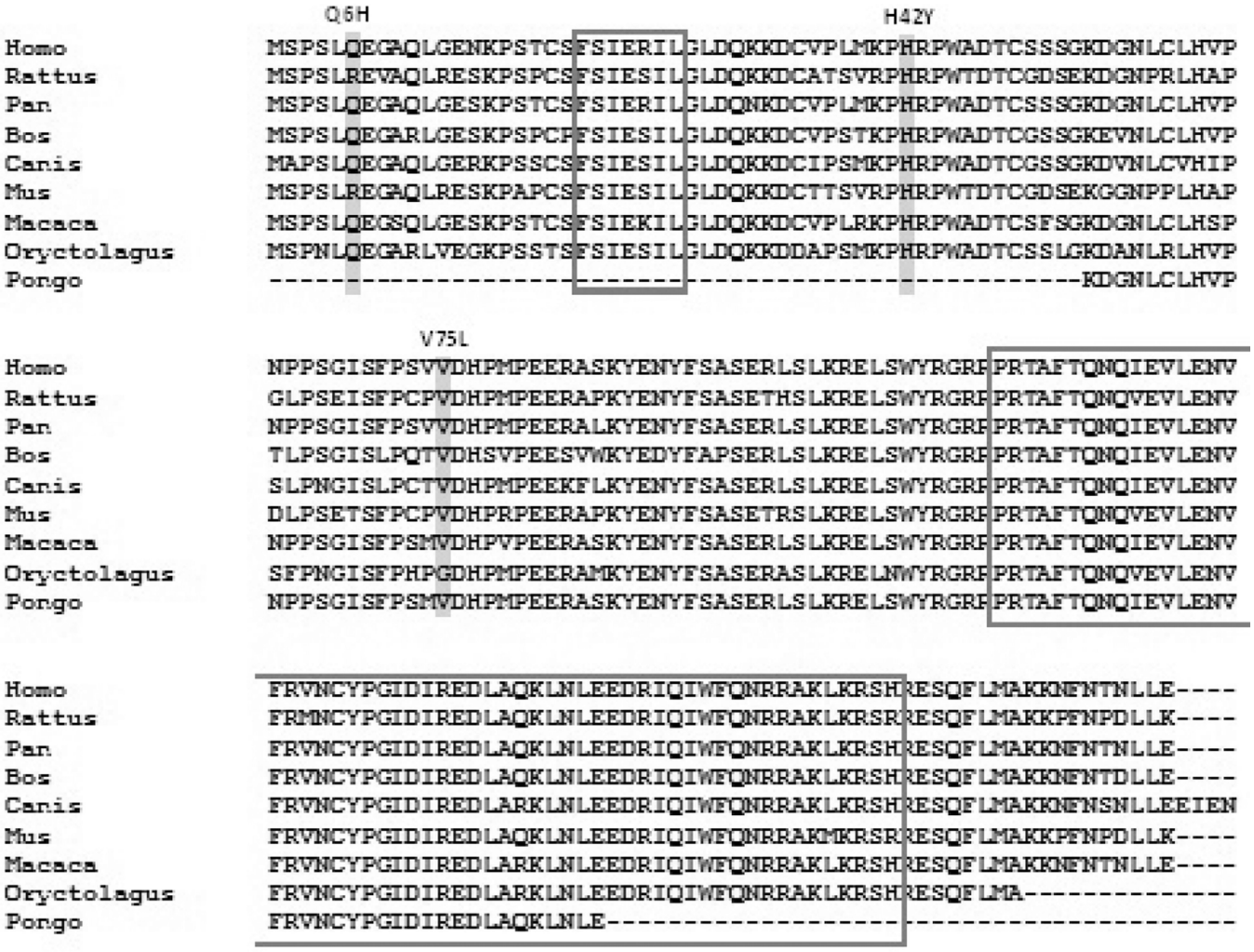

Figure 1.

Predicted open reading frame of human HESX1 compared with orthologous proteins of eight other higher order species. The two boxed regions represent the highly conserved smaller N-terminal co-repressor binding domain and the larger C-terminal homeodomain, respectively. The shaded regions indicate the three *HESX1* mutations as identified in the genetic screen of 217 IHH/KS patients.

Table 1

Summary of patient phenotypes and identified heterozygous variants in HESX1.

Patient	Sex	Ethnicity	Phenotype	Sequence Variant	Exon	Control Comparison	SNPdb/ 1000 Genomes	SIFT analysis (<0.05)
1	M	Northern European	Anosmic; IHH	p.Q6H (c.18G>A)	1	0/192	No match	**0.03**
2	M	Turkish	Anosmic; complete IHH Testes <3cc bilaterally T=45ng/dL, FSH=0.3mIU/mL, LH=0.1mIU/mL	p.H42Y (c.124C>T)	1	0/192	No match	**0.00**
3	M	Turkish	Anosmic; incomplete IHH Testes <3.5cc bilaterally FSH=0.3mIU/mL, LH=0.1mIU/mL	p.V75L (c. 223G>T)	2	0/192	No match	0.16

Numbers in boldface represent an amino acid non-synonymous substitution that is predicted to be deleterious by SIFT analysis and affect protein function. Each genetic variant was compared against known polymorphisms published in NCBI SNP Blast and 1000 Genomes Browser. M, male; F, female; HTZ, heterozygote; SNPdb, NCBI SNP Blast. T = testosterone (normal = 300-1100ng/dL).

Table 2

Summary of 16 previously reported human *HESX1* mutations.

Mutation	Exon	State	Phenotype	Functional Effect	SIFT* (<0.05)	Reference
p.R160C (c.478C>T)	4	HMZ	SOD	inability to bind DNA	0.00	Dattani (1)
p.I26T (c.77T>C)	1	HMZ	CPHD/EPP	impaired ability to recruit TLE1	0.00	Carvalho (3)
c.449_450delAC	1	HMZ	CPHD/AP aplasia/ life-threatening neonatal condition	truncated protein lacking PROP1 repression activity	-	Sobrier (8)
c.357 + 2T>C	2	HMZ	CPHD/AP aplasia/ life-threatening neonatal condition	truncated protein lacking PROP1 repression activity	-	Sobrier (8)
p.R160H	4	HMZ	Panhypopituitarism/ AP aplasia/ thin pituitary stalk	unknown	0.00	Durmaz (10)
Alu-insertion	3	HMZ	Panhypopituitarism/ complete absence of AP/ coloboma	severely truncated proteins lacking significant homeodomain sequences	-	Sobrier (9)
p.Arg109X (c.325C>T)	2	HTZ	PSIS/EPP/panhypopituitarism	premature stop codon leading to loss of homeodomain^{P}	-	Reynaud (15)
c.306/307insAG	2	HTZ	CPHD/ hypoplastic AP/ left ON hypoplasia/ EPP	premature stop codon; reduced functional dimers by binding wild-type^{P}	-	Tajima (11)
c.357+3G>A	2	HTZ	IGHD	alternative splicing product lost; decreased down-regulation	-	Vivenza (5)
p.S170L (c.509C>T)	4	HTZ	IGHD/ ON hypoplasia	impaired DNA binding activity	0.00	Thomas (12), Brickman (14)
p.T181A (c.541A>G)	4	HTZ	IGHD	unknown	0.28	Thomas (12)
p.Q6H (c.18G>C)	1	HTZ	CPHD	unknown	0.03	Vivenza (5), Corneli (7), Thomas (12)
c.1684delG	4	HTZ	IGHD/SOD	increased DNA binding activity/enhanced PROP1 repression	-	Cohen (13)
p.E149K	3	HTZ	IGHD/EPP/ supernumary digits	inability to repress PROP1	0.00	McNay (4)
p.Q117P	2	HTZ	CPHD/ micropenis/ hypoplastic AP/ EPP	unknown	0.00	Coya (6)
p.K176T	4	HTZ	CPHD/SOD on MRI	unknown	0.00	Coya (6)

HMZ, homozygous; HTZ, heterozygous; SOD, septo-optic dysplasia; CPHD, combined pituitary hormone deficiency; IGHD, isolated growth hormone deficiency; PSIS, pituitary stalk interruption syndrome; AP, anterior pituitary; EPP, ectopic posterior pituitary; ON, optic nerve;

^{P}predicted;

*only applicable where amino acid substitution.