Genetics inMedicine | ORIGINAL RESEARCH ARTICLE

Evaluating CHARGE syndrome in congenital hypogonadotropic hypogonadism patients harboring *CHD7* variants

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Purpose: Congenital hypogonadotropic hypogonadism (CHH), a rare genetic disease caused by gonadotropin-releasing hormone deficiency, can also be part of complex syndromes (e.g., CHARGE syndrome). *CHD7* mutations were reported in 60% of patients with CHARGE syndrome, and in 6% of CHH patients. However, the definition of *CHD7* mutations was variable, and the associated CHARGE signs in CHH were not systematically examined.

Methods: Rare sequencing variants (RSVs) in *CHD7* were identified through exome sequencing in 116 CHH probands, and were interpreted according to American College of Medical Genetics and Genomics guidelines. Detailed phenotyping was performed in CHH probands who were positive for *CHD7* RSVs, and genotype–phenotype correlations were evaluated.

Results: Of the CHH probands, 16% (18/116) were found to harbor heterozygous *CHD7* RSVs, and detailed phenotyping was performed in 17 of them. Of CHH patients with pathogenic or likely pathogenic *CHD7* variants, 80% (4/5) were found to exhibit multiple CHARGE features, and 3 of these patients were reclassified as having CHARGE syndrome. In contrast, only 8% (1/12) of CHH patients with nonpathogenic *CHD7* variants exhibited multiple CHARGE features ($P = 0.01$).

Conclusion: Pathogenic or likely pathogenic *CHD7* variants rarely cause isolated CHH. Therefore a detailed clinical investigation is indicated to clarify the diagnosis (CHH versus CHARGE) and to optimize clinical management.

Genet Med advance online publication 16 November 2017

Key Words: CHARGE syndrome; chromodomain helicase DNA binding protein 7; congenital hypogonadotropic hypogonadism; Kallmann syndrome

INTRODUCTION

Congenital hypogonadotropic hypogonadism (CHH) is a rare genetic disorder (affecting 1 in 4,000 to 10,000)[1] caused by isolated gonadotropin-releasing hormone (GnRH) deficiency, leading to absent or incomplete puberty and infertility. Nonreproductive features such as anosmia, hearing impairment, cleft lip/palate, and scoliosis are often seen in patients with CHH, and are considered CHH-associated phenotypes.[2–4] In particular, anosmia is present in 50% of CHH patients and this co-occurrence is termed Kallmann syndrome (KS). CHH can be part of a complex syndrome such as Bardet-Biedl syndrome, septo-optic dysplasia, CHARGE syndrome, or several others.[5] CHARGE syndrome has a prevalence of 1 in 15,000 to 17,000.[6] The CHARGE acronym represents a nonrandom cluster of multiorgan malformations including coloboma, heart defects, choanal atresia, retardation of growth and development, genital hypoplasia, and ear anomalies.[6] Based on the clinical severity (i.e., the number of major and minor CHARGE signs), a patient can be diagnosed with typical, partial, or atypical CHARGE syndrome.[7] Despite the distinct diagnostic criteria, several phenotypes, such as hearing impairment and

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Submitted 4 October 2017; accepted 4 October 2017; advance online publication 16 November 2017. doi:10.1038/gim.2017.197

hypogonadotropic hypogonadism, overlap between CHH and CHARGE syndrome.

CHARGE syndrome is an autosomal dominant disease. *CHD7*, encoding chromodomain helicase DNA binding protein 7, is the major causative gene for CHARGE syndrome. CHD7 plays an important role in chromatin modeling and transcription regulation, and it regulates genes implicated in neural crest guidance.[8–10] Heterozygous mutations in *CHD7*, mainly nonsense or frameshift, were found in approximately 60% (range of 33% to 100%) of CHARGE cases.[6] Nearly all cases of CHARGE syndrome are sporadic (>97%), and most CHARGE patients harbor de novo *CHD7* mutations (when tested).[6]

In contrast, CHH is a genetically heterogeneous disease, with mutations observed in >20 genes exhibiting varying modes of inheritance.[5] Oligogenicity, defined as mutations in more than one causative gene, occurs in 7% of CHH patients.[11] Thirty percent of CHH patients are considered familial cases, as they have family members with CHH, delayed puberty, or anosmia. Variable expressivity is often observed in familial CHH.[5] Interestingly, *CHD7* mutations have been reported in 6% of CHH patients with or without additional CHARGE-like features.[5] Some have proposed that CHH is a mild allelic form of CHARGE syndrome.[12,13] Still, the correlation between CHARGE-like phenotypes and *CHD7* genotypes in CHH is unclear, owing to the lack of systematic phenotyping which specifically evaluates CHARGE-like features in CHH patients.

Identification of genotype–phenotype correlations is also hindered by the lack of a consistent definition of mutations across studies. With advanced sequencing technologies and the availability of genetic data in large populations (e.g., 135,000 subjects in the Genome Aggregation Database), previous criteria for mutations based on minor allele frequency and/or prediction algorithms do not appear to be sufficient to define a pathogenic variant. Indeed, the American College of Medical Genetics and Genomics (ACMG) recently updated the guidelines to standardize the interpretation of sequencing variants, outlining 28 different criteria which integrate data from population studies, computational and predictive algorithms, functional assays, segregation analysis, and others.[14] To minimize potential bias in data collection and evaluation, a Web-based tool (InterVar) has been developed and validated to facilitate the application of ACMG criteria.[15]

In this study, we aimed to refine the role of *CHD7* in CHH. We performed detailed phenotyping, focusing on CHARGE-like features in a large CHH cohort who were positive for rare variants in *CHD7*. Subsequently, we used the ACMG guidelines to classify these variants and performed phenotype–genotype correlation.

MATERIALS AND METHODS

Subjects and clinical studies

The clinical and genetic studies were approved by the ethics committee of the University of Lausanne, and all participants provided written informed consent before participating in the study. The clinical trial registry number is NCT01601171. The CHH cohort includes 116 unselected probands of non-Finnish European origin (61 KS and 55 normosmic CHH). The diagnosis of CHH is defined by (i) absent or incomplete puberty by age 16, (ii) low/normal gonadotropin levels in the context of low serum testosterone/estradiol levels, and (iii) otherwise normal anterior pituitary function and normal imaging of the hypothalamic–pituitary area. Olfaction was assessed by self-report and/or formal testing.[16] The 2005 Verloes criteria[7] were used to classify CHARGE-like features and diagnose CHARGE syndrome. In addition to a comprehensive medical exam and review of medical/surgical history, CHD7-positive probands underwent magnetic resonance imaging (MRI) or computerized tomography (CT) scans whenever possible, so that inner ear structures could be visualized. Audiometry/audiogram and cardiac ultrasound/MRI scans were performed in cases with clinical suspicion. Because a single CHARGE-like feature can be associated with CHH independent of mutations in *CHD7*,[4] only the presence of two or more additional CHARGE-like features was considered a significant clinical finding in this study. Both affected and unaffected family members were recruited for clinical characterization and genetic studies when available. The control group consists of 405 unrelated non-Finnish European participants from a population-based study, Cohorte Lausannoise (CoLaus).[17]

Genetic studies

Exome sequencing in CHH and control cohorts was performed using previously described methods.[18] Nonsynonymous rare sequencing variants (RSVs) with MAF <1% in non-Finnish European controls from the Genome Aggregation Database (gnomAD, http://gnomAD.broadinstitute.org/) in *CHD7* (NM_017780) and in 23 other known CHH genes were included in this study. The included CHH genes are *ANOS1* (NM_000216.2), *SEMA3A* (NM_006080), *FGF8* (NM_033163.3), *FGF17* (NM_003867.2), *SOX10* (NM_006941), *IL17RD* (NM_017563.3), *AXL* (NM_021913), *FGFR1* (NM_023110.2), *HS6ST1* (NM_004807.2), *PCSK1* (NM_000439), *LEP (*NM_000230), *LEPR* (NM_002303), *FEZF1 (*NM_001024613), *NSMF* (NM_001130969.1), *PROKR2* (NM_144773.2), *WDR11* (NM_018117), *PROK2* (NM_001126128.13), *GNRH1* (NM_000825.3), *GNRHR* (NM_000406.2), *KISS1* (NM_002256.3), *KISS1R* (NM_032551.4), *TAC3* (NM_013251.3), and *TACR3* (NM_001059.2). All *CHD7* variants were confirmed by Sanger sequencing, as well as any RSVs in the remaining 23 CHH genes in these patients. Variants are reported in agreement with Human Genome Variation Society nomenclature.[19]

CHD7 RSVs were interpreted according to the ACMG guidelines.[14] For missense variants, InterVar (http://wintervar.wglab.org/) was used for the automated interpretation, and included information on segregation, phenotype, and structural modeling data. Variants were classified into five categories: pathogenic, likely pathogenic, of uncertain significance, likely benign, and benign. Pathogenic or likely

pathogenic RSVs in other CHH genes were also reported in the study. In addition, the identified *CHD7* RSVs were also classified by the commonly used criteria based on SIFT and Polymorphism Phenotyping v2 (PolyPhen-2): a variant is categorized as pathogenic if predicted to be deleterious by either SIFT or PolyPhen-2. Finally, the Bergman system,[20] a classification system for missense *CHD7* variants in CHARGE syndrome, was also used to categorize identified *CHD7* RSVs.

Statistical analysis

A gene-collapsed rare variant association test on CHH, versus controls, was performed to compare rare-variant allele frequencies by means of a two-tailed Fisher's exact test. A Fisher's exact test was also used to compare the percentages of patients in different groups as appropriate. The significance level was set at $P < 0.05$ (two-sided).

RESULTS

CHD7 RSVs are significantly enriched in CHH versus controls

Seventeen heterozygous *CHD7* RSVs, including 2 protein-truncating variants and 15 missense variants, were identified in 18 of the 116 CHH probands (**Supplementary Table S1**, **Figure 1a**). Only 1 missense RSV (p.M340V) was found in the 405 controls from the CoLaus cohort. The allele frequency of *CHD7* RSVs was significantly higher in CHH probands relative to CoLaus controls (7.8%, 18/232, vs. 0.1%, 1/810; $P = 1.6 \times 10^{-11}$).

The protein-truncating variants included a splicing variant (c.2613+5G > A) that was previously shown to result in the skipping of exon 9 and found in an unrelated normosmic CHH patient,[12] and a nonsense variant (p.R2428*) previously reported in an unrelated CHARGE syndrome patient (http://www.chd7.org). Of the 15 missense RSVs, 5 were located in CHD7 functional domains (**Figure 1a,b**). Structural modeling predicted that all variants within the functional domains (except p.A1107V) were deleterious (**Figure 1b**). Segregation analysis was performed in 16/18 (89%) pedigrees. Three RSVs were de novo, including the nonsense and two missense, and the others RSVs were inherited (**Supplementary Table S1**, **Figure 2**). One affected family member was found to harbor compound heterozygous RSVs (**Figure 2**, family 4). Genetic data for family members of the remaining two probands were not available.

Further classifying the variants according to the ACMG guidelines, we found that five variants were pathogenic or likely pathogenic, seven variants were of uncertain significance, and five were benign or likely benign (**Supplementary Table S1**).

Genotype–phenotype correlation

Detailed phenotyping of additional CHARGE signs was performed in 17/18 CHH probands, and the data were utilized for subsequent genotype–phenotype correlation analysis (**Table 1**). The remaining proband was unavailable for clinical follow-up.

All five of the probands harboring pathogenic or likely pathogenic *CHD7* variants also presented with anosmia (i.e., KS). Further, four of the five probands also exhibited CHARGE-associated features.

Patient 1 is a female proband previously diagnosed with KS. Although she was also noted to have coloboma and pulmonary artery stenosis, she did not have further evaluation for CHARGE syndrome. Genetic testing revealed that she harbored a de novo nonsense *CHD7* variant (p.R2428*). Follow-up MRI showed bilateral hypoplasia of semicircular canals, confirming the diagnosis of typical CHARGE syndrome (i.e., two major and two minor CHARGE signs).

Patient 2 is a male patient born with bilateral choanal atresia that was subsequently surgically repaired. He had normal growth and development during childhood, without evident CHARGE signs. Further medical evaluation for absent puberty revealed isolated hypogonadotropic hypogonadism and anosmia (Sniffin' Sticks score 5/16, less than fifth percentiles), leading to the diagnosis of KS. He was found to harbor a de novo *CHD7* RSV (p.Y1412D). Additional detailed phenotyping revealed a mild external ear defect, an audiogram indicated mild bilateral conductive hearing loss, and MRI showed hypoplasia of olfactory bulbs, semicircular canal malformation, and VII nerve hypoplasia. No heart defects were identified in the cardiac evaluation. The patient exhibited two major and three minor CHARGE signs, consistent with the diagnosis of typical CHARGE syndrome.

Patient 3 is a male proband who was referred to our clinical service with a diagnosis of KS. He also presented with coloboma and facial palsy. He was found to harbor a de novo *CHD7* missense RSV (p.C989Y). Further detailed phenotyping revealed bilateral external ear malformation, and an audiogram indicated mild bilateral sensorineural hearing loss. Cardiac and inner-ear MRI scans were normal. Based on these findings (i.e., one major and three minor CHARGE signs), he was reclassified as having atypical CHARGE syndrome.

Patient 4 is a female proband diagnosed with KS with a history of congenital heart malformation (mitral valve prolapse), synkinesia, and scoliosis. She was found to harbor *CHD7* p.A1107V, and a subsequent inner-ear CT scan revealed semicircular canal hypoplasia. She exhibited one major and two minor CHARGE signs, but did not meet the diagnostic criteria for CHARGE syndrome. Expanding the genetic testing in this family, we found that both the proband's mother and sister harbored the same *CHD7* p.A1107V variant, while the proband's sister had inherited an additional benign variant (p.M340V) from their father (**Figure 2**). This affected sister also had KS and all of the CHARGE features present in the proband, as well as the additional features of unilateral sensorineural hearing loss and unilateral renal hypoplasia; this resulted in a diagnosis of atypical CHARGE syndrome (**Table 2**). Their mother, who carried the heterozygous p.A1107V variant, was reproductively normal but exhibited two CHARGE signs

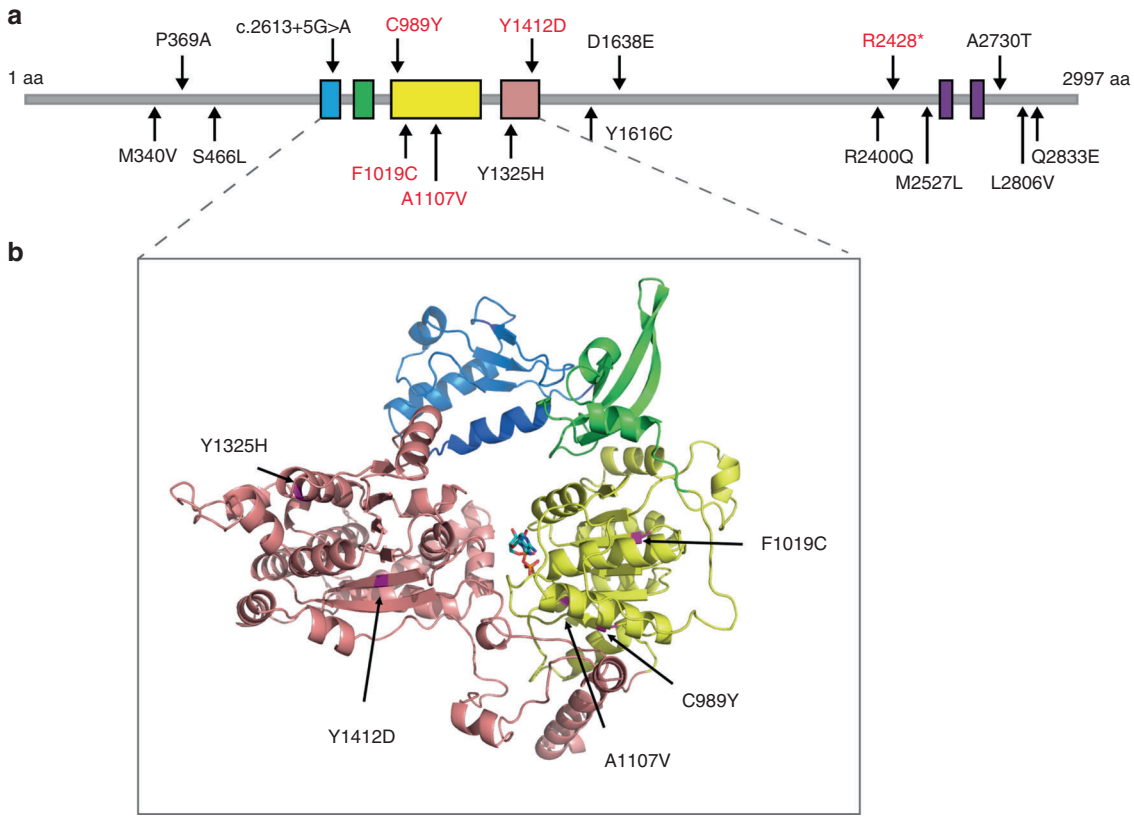

Figure 1 *CHD7* rare sequencing variants identified in congenital hypogonadotropic hypogonadism probands. (**a**) Schematic of the CHD7 protein (1-2997 amino acid) and locations of identified rare sequencing variants (RSVs). Pathogenic or likely pathogenic RSVs according to American College of Medical Genetics and Genomics guidelines are noted in red. The functional domains of CHD7 in both (**a**) and (**b**) are noted as follows: blue, chromodomain 1; green, chromodomain 2; yellow, helicase N; pink, helicase C; purple, BRK. (**b**) Structural model of CHD7 chromo- and helicase domains. The location of RSVs is indicated by arrows. The ATP-binding domain is depicted in cyan (located in the helicase N-lobe).

(semicircular canal hypoplasia and hearing impairment), while the father was phenotypically normal, without any signs of CHARGE or CHH (**Table 2**). The phenotypic difference between the two unaffected sisters can be the result of the variable expressivity of *CHD7* pathogenic variants.[21,22] However, it is also possible that the additional *CHD7* p.M340V variant, although predicted as benign, may contribute to the phenotypic difference observed between the two affected siblings.

Patient 5 is a male proband with isolated KS. Both he and his affected brother were found to harbor the *CHD7* p.F1019C variant. The patient exhibited no additional CHARGE features, although a radiological evaluation of the inner-ear structures was not performed. Interestingly, he was the only patient in this group to harbor an additional pathogenic variant in another CHH gene (*SEMA3E* p.R619C).[18]

Overall, the majority (80%, 4/5) of CHH probands with pathogenic or likely pathogenic *CHD7* RSVs exhibited at least two additional CHARGE features, and three of these patients met the diagnostic criteria for CHARGE syndrome (**Table 1**).

Nonpathogenic *CHD7* variants (of uncertain significance, likely benign, or benign) were found in 12 CHH patients. Notably, almost half (42%, 5/12) of these patients were found to harbor a pathogenic or likely pathogenic variant in other CHH genes (**Table 1**, **Figure 2**). Patient 6 was the only patient to exhibit two or more additional CHARGE features. She had KS with sensorineural hearing loss and intellectual disability, yet did not meet the diagnostic criteria for CHARGE syndrome. She was found to harbor an additional protein-truncating variant in *FGFR1* (p.R365Kfs*5). Three patients with CHH each exhibited a single additional CHARGE feature: sensorineural hearing loss in patient 7, mild heart defect (mild dilatation of right ventricle, aortic root, and ascending aorta) in patient 8, and intellectual disability in patient 12. The remaining seven patients did not exhibit any additional CHARGE features, although some had other CHH-associated phenotypes, such as synkinesia or dental or skeletal defects (**Table 1**).

In summary, a significantly higher proportion of CHH patients with pathogenic or likely pathogenic *CHD7* RSVs exhibited multiple CHARGE features, compared with those with nonpathogenic *CHD7* variants (80%, 4/5 vs. 8%, 1/12, $P = 0.01$). Indeed, three CHH patients with pathogenic or likely pathogenic *CHD7* RSVs were reclassified as having CHARGE syndrome following detailed clinical investigation, whereas none of the probands with nonpathogenic variants merited diagnostic reclassification (60%, 3/5, vs. 0%, 0/12, $P = 0.02$).

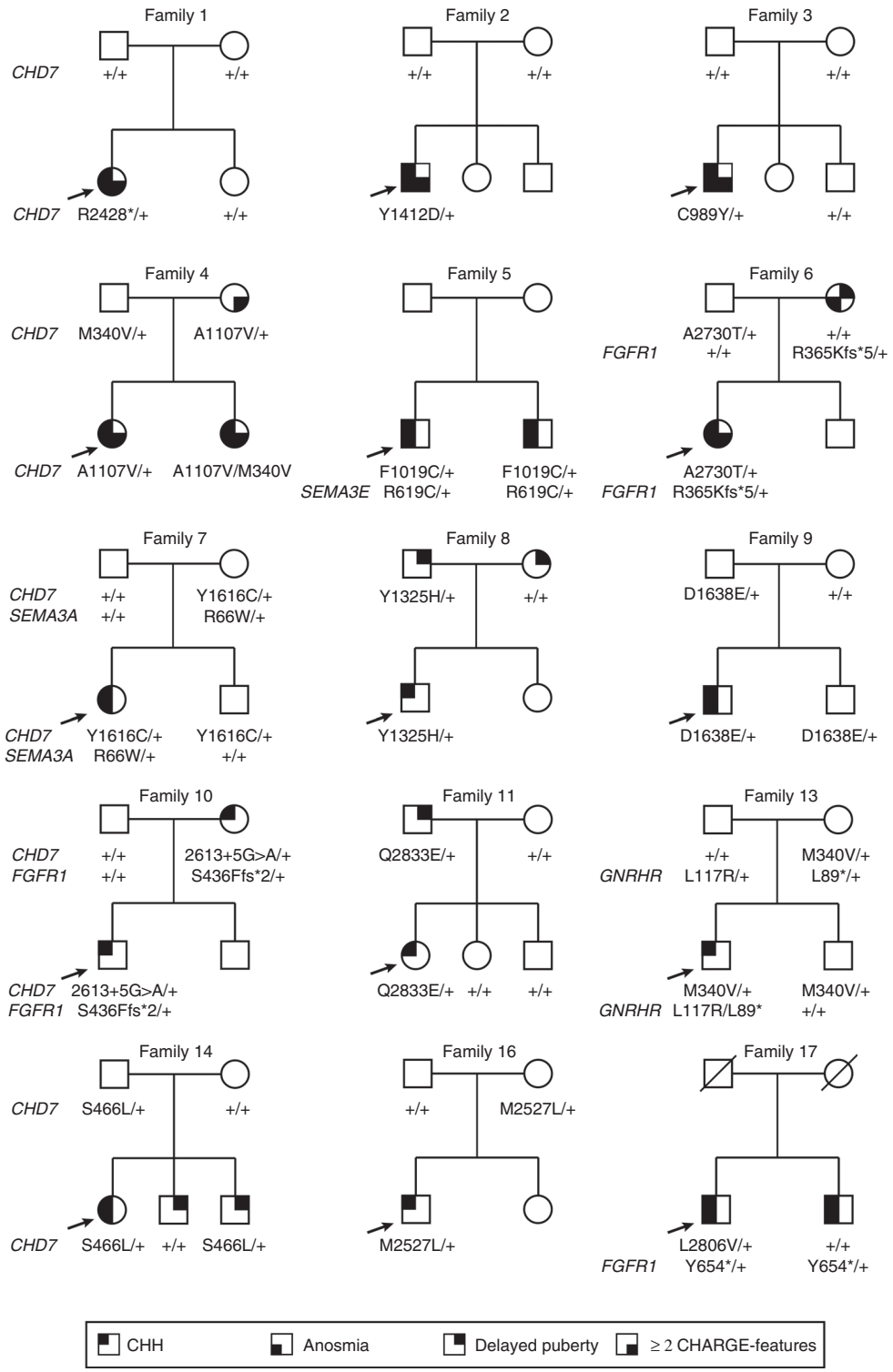

Figure 2 Informative pedigrees of congenital hypogonadotropic hypogonadism (CHH) probands. Symbols: +, wild type; arrow, proband; circle, female; square, male.

Genotype–phenotype correlation based on different classification of *CHD7* RSVs

The *CHD7* missense variants found in CHH probands were further classified using SIFT/PolyPhen-2 and the Bergman system (**Table 3**). The Bergman system integrates information from structural modeling, prediction algorithms (Polyphen-2 and Align-GVGD), segregation, and population data.[20] This system has been used to evaluate missense *CHD7* variants in

Table 1 Phenotype–genotype correlation in CHH probands with *CHD7* RSVs

Pt	Initial Dx	*CHD7* RSVs	Other pathogenic variants	CHARGE features								Other phenotypes	Final Dx	ACMG criteria
				Major			Minor							
				C	A	SCC	RED	E	ID	MO	HPD			
1	KS	p.R2428*	–	+	–	+	–	–	–	+	+	Nasal cartilage distortion	CHARGE	P
2	KS	p.Y1412D	–	–	+	+	+	+	–	–	+	Oval palate	CHARGE	LP
3	KS	p.C989Y	–	+	–	–	+	+	–	–	+	Atropy of optic chiasma	Atypical CHARGE	LP
4	KS	p.A1107V	–	–	–	+	–	–	–	+	+	See **Table 2**	KS	LP
5	KS	p.F1019C	*SEMA3E* p.R619C	–	–	U	–	–	–	–	+	Dental defects	KS	LP
6	KS	p.A2730T	*FGFR1* p.R365Kfs*5	–	–	–	+	–	+	–	+	Optic nerve atrophy, CLP, CC agenesis	KS	US
7	KS	p.Y1616C	*SEMA3A* p.R66W	–	–	–	+	–	–	–	+	–	KS	US
8	nCHH	p.Y1325H	–	–	–	–	–	–	–	+	+	–	nCHH	US
9[M]	KS	p.D1638E	–	–	–	–	–	–	–	M	+	Synkinesia, dental defect	KS	US
10	nCHH	c.2613+5G > A	*FGFR1* p.S436Ffs*3	–	–	–	–	–	–	–	+	Skeletal defect	nCHH	US
11	nCHH	p.Q2833E	–	–	–	U	–	–	–	–	+	–	nCHH	US
12	nCHH	p.M340V	–	–	–	–	–	–	+	–	+	–	nCHH	B
13	nCHH	p.M340V	*GNRHR* p.[L89*];[L117R]	–	–	–	–	–	–	–	+	Dental defect, astigmatism	nCHH	B
14	KS	p.S466L	–	–	–	U	–	–	–	–	+	–	KS	B
15	nCHH	p.R2400Q	–	–	–	–	–	–	–	–	+	–	nCHH	LB
16	nCHH	p.M2527L	–	–	–	–	–	–	–	–	+	Dental defect, astigmatism	nCHH	LB
17	KS	p.L2806V	*FGFR1* p.Tyr654*	–	–	–	–	–	–	–	+	–	KS	B

A, choanal atresia; ACMG, American College of Medical Genetics and Genomics; B, benign; C, coloboma; CC, corpus callosum; CHH, congenital hypogonadotropic hypogonadism; CLP, cleft lip and palate; Dx, diagnosis; E, ear anomalies; HPD, hypothalamic–pituitary dysfunction; ID, intellectual disability; KS, Kallmann syndrome; LB, likely benign; LP, likely pathogenic; M, Marfan syndrome (confirmed by the identification of *FBN1* p.R1692del and aorta root dilation in this proband); MO, mediastinal organ malformation (heart and esophagus); nCHH, normosmic CHH; P, pathogenic; Pt, patient; RED, rhemboencephalic dysfunction; RSV, rare sequencing variant; SCC, semicircular canals dysplasia; U, unknown; US, uncertain significance.
CHARGE features and CHARGE diagnosis are according to the 2005 Verloes criteria.[7]

Table 2 Detailed phenotype in the family of patient 4

CHD7 RSVs	Proband [A1107V]; [WT]	Sister [A1107V];[M340V]	Mother [A1107V];[WT]	Father [WT];[M340V]
CHARGE major signs				
Coloboma	–	–	–	–
Choanal atresia	–	–	–	–
Semicircular canal dysplasia	+	+	+	–
CHARGE minor signs				–
Rhombencephalic dysfunction	–	+	+	–
Ear anomalies	–	–	–	–
Intellectual disability	–	–	–	–
Hypothalamic–pituitary dysfunction	+	+	–	–
Malformation mediastinal organs	+	+	–	–
CHH associated phenotypes				–
Synkinesia	+	+	–	–
Scoliosis	+	+	+	–
Renal defect	–	+	–	–
Diagnosis summary	KS with multiple CHARGE features	Atypical CHARGE syndrome	Normal reproduction with CHARGE features	Unaffected

KS, Kallmann syndrome; RSV, rare sequencing variant; WT, wild type.

Table 3 Comparison of different classification systems for *CHD7* RSVs

Pt	*CHD7* RSVs	Other mutations	Final Dx	Multiple CHARGE features	Classification of *CHD7* RSVs		
					ACMG criteria[14]	Bergman[20]	SIFT/PPH2
1	p.R2428*	–	CHARGE	+	P	–	–
2	p.Y1412D	–	CHARGE	+	LP	P	P
3	p.C989Y	–	Atypical CHARGE	+	LP	P	P
4	p.A1107V	–	KS	+	LP	US	P
5	p.F1019C	*SEMA3E* p.R619C	KS	–	LP	US	P
6	p.A2730T	*FGFR1* p.R365Kfs*5	KS	+	US	B	P
7	p.Y1616C	*SEMA3A* p.R66W	KS	–	US	B	P
8	p.Y1325H	–	nCHH	–	US	US	P
9	p.D1638E	–	KS	–	US	B	P
10	c.2613+5G > A	*FGFR1* p.S436Ffs*3	nCHH	–	US	–	–
11	p.Q2833E	–	nCHH	–	US	B	B
12	p.M340V	–	nCHH	–	B	B	B
13	p.M340V	*GNRHR* p.[L89*];[L117R]	nCHH	–	B	B	B
14	p.S466L	–	KS	–	B	B	P
15	p.R2400Q	–	nCHH	–	LB	B	P
16	p.M2527L	–	nCHH	–	LB	B	B
17	p.L2806V	*FGFR1* p.Y654*	KS	–	B	B	P

ACMG, American College of Medical Genetics and Genomics; B, benign; Dx, diagnosis; KS, Kallmann syndrome; LB, likely benign; LP, likely pathogenic; nCHH, normosmic congenital hypogonadotropic hypogonadism; P, pathogenic; PPH, PolyPhen-2; Pt, patient; RSV, rare sequencing variant; US, uncertain significance.

CHARGE syndrome, but has not previously been applied in CHH. Of 15 *CHD7* RSVs, 11 (73%) were categorized by SIFT and/or PolyPhen-2 as damaging. Using the Bergman system, only 2 of the 15 missense RSVs were classified as likely pathogenic, 3 RSVs as of uncertain significance, and the remaining variants were likely benign. The Bergman system yielded results similar to the ACMG criteria, yet appeared to be more stringent, as it demonstrated 100% sensitivity and 100% specificity in predicting CHARGE syndrome resulting from *CHD7* RSVs (**Table 3**).

DISCUSSION

In this study, we identified 17 *CHD7* RSVs in 16% (18/116) of CHH probands. Overall, *CHD7* RSVs are significantly enriched in the CHH cohort versus controls (7.8% vs. 0.1%, $P = 1.6 \times 10^{-11}$), supporting the implication of *CHD7* in CHH. We demonstrated that applying the updated ACMG guidelines[14] revealed an excellent correlation between the pathogenicity of *CHD7* RSVs and the observed clinical severity in CHH (i.e., additional CHARGE-like features). In this study, four out of five *CHD7* RSVs classified as

pathogenic or likely pathogenic cause multiple CHARGE features among CHH probands, resulting in the reclassification of three patients as having CHARGE syndrome according to the Verloes criteria. These clinical findings are in sharp contrast with features found in the patients harboring the 12 *CHD7* RSVs classified as nonpathogenic (i.e., of uncertain significance, benign, or likely benign), as only one patient exhibited two or more additional CHARGE signs. Notably, this patient also harbors an additional pathogenic variant in *FGFR1*, a known CHH gene with pleiotropic effects. Further, these data raise the question of the role of nonpathogenic variants in CHH. These variants are probably not the major cause of CHH, and indeed pathogenic or likely pathogenic variants in another CHH gene were found in 5/12 (42%) of these patients. However, we cannot exclude the possibility that these seemingly benign *CHD7* variants act as genetic modulators of the overall CHH phenotype, as supported by the statistical enrichment of *CHD7* RSVs in CHH.

Our study is the first to systematically evaluate CHARGE features in a large cohort of CHH probands. Radiological examination of the inner ear to investigate semicircular canal hypoplasia (a major sign of CHARGE syndrome) is rarely performed in CHH patients; in previous studies only 0–38% of probands were thus tested.[12,13,23] In the present study, we systematically phenotyped the CHH probands and performed inner-ear MRI/CT in 77% (14/18) of them. Our data suggest that the ACMG criteria can identify a subset of CHH patients requiring a complete clinical evaluation for CHARGE signs (those with pathogenic or likely pathogenic *CHD7* variants) versus those who have a low risk of having additional CHARGE signs. We found that 75% (3/4) of probands with pathogenic or likely pathogenic variants in *CHD7* also had pathology of the inner ear, compared to 0% (0/10) in probands with nonpathogenic *CHD7* RSVs. Because detailed phenotyping demands enormous clinical effort and medical resources, the capacity to identify patients with a high risk of having additional CHARGE features is extremely important.

We classified the *CHD7* RSVs with commonly used criteria based on SIFT and/or PolyPhen-2, as well as the Bergman system, a prediction algorithm developed for evaluating missense variants in CHARGE syndrome. In contrast to the ACMG criteria and the Bergman system, SIFT and/or PolyPhen-2 classified > 70% of RSVs as being deleterious. It is well known that both SIFT and PolyPhen-2 have higher accuracy in predicting loss-of-function variants than in predicting benign variants, which has a higher false-positive rate.[24] To date, only one study has attempted to assess the functional impact of CHD7 mutants in a model system. Balasubramanian et al.[13] found that 75% (9/12) of CHH-associated and 100% (4/4) of CHARGE-associated *CHD7* mutations in a zebrafish model were loss-of-function mutations. However, it is not possible to differentiate the mutations resulting in CHH from those causing CHARGE syndrome. While Balasubramanian and colleagues' functional data are compelling, it is important to note that segregation data were presented for only three families. The advantage of the ACMG criteria and the Bergman system used in the present study is their ability to integrate a broad spectrum of evidence, including segregation data.

Although similar results were obtained using ACMG criteria and the Bergman system, it is important to note that the Bergman system weights heavily for de novo *CHD7* variants – a feature more commonly found in CHARGE syndrome than in CHH. This may explain the minor differences in the results of the two classifications. In our cohort, the Bergman system had 100% sensitivity and specificity for identifying causative variants for CHARGE syndrome (defined by the Verloes criteria). Recently, Hale et al.[25] proposed broadening CHARGE-syndrome clinical diagnostic criteria by including pathogenic *CHD7* variants as major criteria. While these proposed criteria are aimed at improving diagnoses in patients with atypical presentation by integrating molecular findings into the diagnostic process, the current study aimed to analyze the genotype–phenotype correlation of *CHD7* variants in CHH. Thus, the inclusion of genotype information within the phenotype classification (i.e., diagnosis of CHARGE syndrome) may lead to a bias in the results. Furthermore, the Hale proposal requires that *CHD7* missense variants be "de novo and recurrent" to be considered pathogenic. This is not consistent with the now widely accepted guidelines from ACMG. The extra level of stringency recommended by Hale et al. can lead to underclassification of truly pathogenic missense variants—an especially critical point for our study because most *CHD7* variants identified in CHH are missense rather than the predominant nonsense and frameshift variants seen in CHARGE syndrome.[6,13,23]

Interestingly, all five CHH patients found to harbor pathogenic or likely pathogenic *CHD7* RSVs in this study were anosmic (i.e., had Kallmann syndrome), consistent with three of five prior studies identifying *CHD7* mutations exclusively in KS patients.[12,13,23,26,27] Further, anosmia and hypogonadotropic hypogonadism were reported to be highly correlated in patients with CHARGE syndrome.[28] Indeed, murine studies showed a developmental role of *Chd7* in both GnRH neurons and the olfactory system: (i) the expression of *Chd7* in the embryonic olfactory placode at E10.5-E11 is temporally and spatially consistent with the genesis of GnRH neurons;[12] (ii) *Chd7*-haploinsufficiency is associated with decreased cellular proliferation in the olfactory placode, along with downregulation of expression of *Fgfr1* and *Bmp4*, two morphogens critical for GnRH early neuron development;[29] and (iii) *Chd7* heterozygous knockout mice exhibit reduced olfaction, delayed pubertal onset, and reduced GnRH neuron number, mimicking Kallmann syndrome.[29,30] Notably, a recent study showed that Chd7 regulates the embryonic expression and signaling of *Fgf8* in mice. Heterozygous defects in *Chd7* and *Fgf8* exhibit a synergistic effect in cerebellar vermis development.[31] Because Fgf8 is a critical morphogen for both GnRH neuron fate specification[32,33] and olfactory bulb development,[34] further studies are warranted to elucidate the epistatic interaction between *FGF8* and *CHD7* in GnRH neuron biology in both human and animal models.

In the era of Sanger sequencing, CHH probands were often not screened for variants in *CHD7*, in part because of its prohibitively large size. With the wide use of next-generation sequencing technologies in both diagnostic and research settings, *CHD7* RSVs are increasingly being found in CHH patients. Therefore, our study has implications for future clinical genetic practice when *CHD7* RSVs are identified in patients with CHH. It is important to study the genetic segregation and apply ACMG guidelines to classify the pathogenicity of the variant. If the variant is predicted to be pathogenic or likely pathogenic, there is a high risk of the patient's exhibiting additional CHARGE features, or even undiagnosed CHARGE syndrome. Furthermore, appropriate genetic counseling based on the presence of pathogenic *CHD7* variants in CHH families is critical, as a high degree of variable expressivity is often observed in CHARGE-syndrome families.[21,22] Thus even in CHH probands who have pathogenic *CHD7* variants and only minor CHARGE features, the same mutation in future generations may result in a more severe CHARGE phenotype. In conclusion, a comprehensive clinical screening of CHARGE-like features is indispensable in the patients with pathogenic *CHD7* variants in order to clarify the diagnosis (CHH versus CHARGE syndrome) and to provide an optimized clinical follow-up, as well as tailored genetic counseling.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

ACKNOWLEDGMENTS

This work was supported by Swiss National Science Foundation Project SNF 310030_173260 (to N.P.). We are grateful to all the CHH patients and their families who contributed their time, medical information, and DNA samples to this study. We thank all the clinical collaborators in the COST action GnRH network (http://www.gnrhnetwork.eu/). We thank Caroline Spaight, Emmanuelle Paccou, Caroline Chambion, and Jenny Meylan-Merlini for their help in patient recruitment, DNA extraction, and database maintenance. We thank Nicole Corsten-Janssen and Conny van Ravenswaaij-Arts (University of Groningen) for their assistance with the CHD7 database (http://www.chd7.org). We are also grateful for the access to exome sequence data from the CoLaus cohort, which was sequenced as part of a partnership between the Wellcome Trust Sanger Institute, the CoLaus principal investigators, and the Quantitative Sciences department of GlaxoSmithKline; in particular, we thank Peter Vollenweider for his support of this project.

DISCLOSURE

The authors declare no conflict of interest.

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