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***CHD7* mutations in patients initially diagnosed with Kallmann syndrome – the clinical overlap with CHARGE syndrome**

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Abstract

Kallmann syndrome (KS) is the combination of hypogonadotropic hypogonadism and anosmia or hyposmia, two features that are also frequently present in CHARGE syndrome. CHARGE syndrome is caused by mutations in the *CHD7* gene. We performed analysis of *CHD7* in 36 patients with KS and 20 patients with normosmic idiopathic hypogonadotropic hypogonadism (nIHH) in whom mutations in *KALI*, *FGFR1*, *PROK2* and *PROKR2* genes were excluded. Three of 56 KS/nIHH patients had *de novo* mutations in *CHD7*. In retrospect, these three *CHD7*-positive patients showed additional features that are seen in CHARGE syndrome. *CHD7* mutations can be present in KS patients who have additional features that are part of the CHARGE syndrome phenotype. We did not find mutations in patients with isolated KS. These findings imply that patients diagnosed with hypogonadotropic hypogonadism and anosmia should be screened for clinical features consistent with CHARGE syndrome. If such features are present, particularly deafness, dysmorphic ears and/or hypoplasia or aplasia of the semicircular canals, *CHD7* sequencing is recommended.

Keywords

anosmia; CHARGE syndrome; *CHD7* gene; hypogonadotropic hypogonadism; Kallmann syndrome

Kallmann syndrome (KS) is a congenital disorder that combines hypogonadotropic hypogonadism and anosmia (1). Three modes of inheritance have been described: X-linked recessive, autosomal dominant and more rarely autosomal recessive. To date, several genes have been identified to cause KS, either alone or in combination. Mutations in these genes together account for approximately 30% of all cases. *KALI* encodes the protein anosmin and

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Informed consent

All patients or their legal representatives gave informed consent for the DNA studies and the collection of clinical data. The studies were approved by the institutional review boards. Additional informed consent for publication was obtained of the patient represented by his photograph in this manuscript.

is involved in the X-linked form of KS (KAL1, OMIM #308700) (2,3). Loss-of-function mutations in the fibroblast growth factor receptor-1 gene (*FGFR1*) cause a form of KS (KAL2, OMIM #147950) that is generally inherited in an autosomal dominant way (4,5). Dodé et al. reported in a further 10% of patients mutations in the prokineticin receptor-2 (*PROKR2*, KAL3, OMIM #607123) and prokineticin-2 (*PROK2*, KAL4, OMIM #607002) genes, encoding a cell surface receptor and one of its ligands, respectively (6). Mutations of the ligand, *PROK2*, can cause KS as well as normosmic idiopathic hypogonadotropic hypogonadism (nIHH) within the same family (6,7). The same intrafamilial phenotypic variability is seen in patients with *FGFR1* mutations (4). Thus, KS is a phenotypically and genotypically heterogeneous disorder. Not only the degree of hypogonadism and anosmia may vary significantly but also other symptoms including bimanual synkinesia and dental agenesis (*KAL1* and *FGFR1*), renal anomalies (*KAL1*) and cleft lip/palate (*FGFR1*) occur with variable frequency (8).

CHARGE syndrome (OMIM #214800) is an autosomal dominant condition characterized by a variety of congenital anomalies including coloboma, heart defects, choanal atresia, retarded growth and development, genital hypoplasia, ear anomalies and deafness. Other commonly observed congenital defects are semicircular canal hypoplasia, facial nerve palsy, cleft lip/palate and tracheo-esophageal fistula (9). Our group has discovered *CHD7* as the causative gene in CHARGE syndrome (10). Since this discovery, several authors have reported on the phenotypic spectrum of *CHD7*-positive patients, including patients without typical CHARGE syndrome (11-13). Therefore, we presume that the mild end of the phenotypic spectrum of *CHD7* mutations is not yet completely explored.

Recent studies revealed that anosmia and abnormal olfactory bulb development, as well as hypogonadotropic hypogonadism, are almost consistent findings in CHARGE syndrome, indicating that the key features of KS are also present in CHARGE syndrome (14-16). For this reason, it has been suggested by others that *CHD7* may be considered a candidate locus in suspected KS cases without known mutations (8). This hypothesis is worthwhile exploring, also because mutations in *CHD7* can result in a much milder phenotype than the classical CHARGE syndrome phenotype. Therefore, we sequenced *CHD7* in a large group of patients diagnosed as KS or nIHH but without mutations in *KAL1*, *FGFR1*, *PROK2* and *PROKR2*.

Materials and methods

Patients

A cohort of seven Japanese patients with a clinical diagnosis of KS, without mutations in *KAL1*, *FGFR1*, *PROK2* and *PROKR2*, was screened for *CHD7* mutations (17). The diagnosis KS in this cohort was based on an underdevelopment of secondary sexual characteristics in combination with anosmia or hyposmia. Subsequently, the cohort was enlarged by 49 *KAL1*, *FGFR1*, *PROK2* and *PROKR2* negative North American patients with KS or nIHH. GnRH deficiency in this cohort was defined by (a) absent/incomplete puberty by age 18 year; (b) serum testosterone <100 ng/dl in men or estradiol <20 pg/ml in women in association with low or normal levels of serum gonadotropins; (c) otherwise normal pituitary function; (d) normal serum ferritin concentrations; and (e) normal magnetic resonance imaging (MRI) of the hypothalamic-pituitary region (5).

The patients in whom *CHD7* mutations were identified were carefully evaluated for clinical features of CHARGE syndrome. The *CHD7* gene was analyzed in the parents. The patients or their legal representatives gave informed consent for the DNA studies and the collection of clinical data. The studies were approved by the institutional review boards.

Mutation screening

DNA was isolated according to standard procedures. The 37 coding exons of the *CHD7* gene (exon 2–38, accession number NM_017780, NCBI) and their flanking intron sequences were amplified by polymerase chain reaction (PCR). Subsequently, sequence analysis was performed using a 3730 automated sequencer (Applied Biosystems, Foster City, CA). Primer information and PCR conditions are given in a previous report of our group (11).

The DNA samples of 11 mutation-negative patients were subsequently screened for exon deletions and/or duplications of the *CHD7* gene by multiplex ligation probe dependent amplification (MLPA) analysis (Table 1). We used a commercially available set of probes, the SALSA P201 kit (MRC-Holland, Amsterdam, The Netherlands; <http://www.mrc-holland.com>). Further details are described in our recent report on MLPA analysis of the *CHD7* gene (18).

Results

The *CHD7* gene was first screened in a cohort of seven *KAL1*, *FGFR1*, *PROK2* and *PROKR2* negative patients of Japanese descent (five males, two females). All had hypogonadotropic hypogonadism and anosmia, whereas some had additional symptoms. Their clinical features are summarized in Table 1, and patient 2 is shown in Fig. 1.

In two of the seven patients, a heterozygous mutation in *CHD7* was identified: one nonsense mutation (c.8803G>T; p.Glu2935X) and one missense mutation (c.6347T>A; p.Ile2116Asn). The mutations were proven to be *de novo* in both patients and were not present in 600 alleles of healthy controls.

The study cohort was extended by 49 North American patients (28 males, 21 females), including 29 patients with KS and 20 with nHH of whom three had a positive family history for KS. Some of these patients had additional phenotypic features (Table 1). In one of the patients (patient 8), a *de novo* pathogenic nonsense mutation in *CHD7* was found (c.6070C>T; p.Arg2935X).

As whole exon deletions or duplications will be missed by sequence analysis, we performed MLPA analysis. Due to a limited amount of available DNA, we were only able to finish this analysis in 11 patients. Two patients with a relatively high suspicion for CHARGE syndrome based on the features choanal atresia and multiple cranial nerve anomalies (respectively, patient 15 and 23; Table 1) were among those 11 patients. No exon copy number alterations were found.

The main features of the three patients carrying a mutation in *CHD7* are given in Table 1. All three patients were proven to be anosmic by formal smell tests. Audiometry revealed a left-sided hearing impairment of 70 dB in patient 1, a bilateral hearing impairment of 60–90 dB in patient 2 and left-sided complete sensorineural deafness and right-sided partial conductive hearing impairment in patient 8. Patient 1 had agenesis of four permanent teeth, the first upper and lower molars. No choanal atresia or heart defects were present in patients 1, 2 and 8. Colobomas were present in patient 8 but excluded by fundoscopy in patients 1 and 2. Patient 2 experienced feeding difficulties during infancy, but these were ascribed to the cleft palate. The dysmorphisms of the ears of patient 2 are very characteristic for CHARGE syndrome with absence of the earlobe and the lower helical fold, and a typical triangular concha (Fig. 1). After identification of the *CHD7* mutation, a CT scan of the os petrosum showed bilateral hypoplasia of the semicircular canals. In patients 1 and 8, imaging studies of the temporal bones were not possible. Upon re-evaluation, patient 8 has not only deafness and bilateral colobomas but also left-sided facial nerve palsy, cleft lip and palate, short stature and developmental delay.

In retrospect, patients 2 and 8 have typical CHARGE syndrome according to the commonly used clinical criteria (9), while patient 1 has only some features of this syndrome.

Discussion

Hypogonadotropic hypogonadism is a frequent feature in CHARGE syndrome. Chalouhi et al. tested the olfactory function of 14 children with CHARGE syndrome and showed that all children had some degree of olfactory deficiency (14). Pinto et al. showed that olfactory deficiency and abnormal olfactory bulbs were present in all 18 CHARGE syndrome patients in their cohort (15).

These observations prompted us to analyze the *CHD7* gene in 36 patients with KS and 20 patients with nIHH lacking mutations in *KALI*, *FGFR1*, *PROK2* and *PROKR2*. *CHD7* mutations were identified by sequence analysis in 2 of 7 Japanese KS patients and in 1 of 49 KS/nIHH North American patients. By routine sequencing of the *CHD7* gene, we may have missed mutations located deep in introns or in the promoter region. Furthermore, MLPA analysis could not be performed in all patients.

Hypogonadism in KS is caused by GnRH deficiency. GnRH neurons of the forebrain are thought to originate from the nasal placode. During embryonic development, they migrate alongside the olfactory axons toward the hypothalamus. Mutations in *KALI*, *FGFR1*, *PROKR2* and *PROK2* can result in hypogonadotropic hypogonadism and anosmia. Therefore, the protein products of these genes are thought to be involved in this combined migration process (8,19). Because hypogonadotropic hypogonadism and anosmia are frequently present in CHARGE syndrome as well, it is possible that the same embryonic migration process is disturbed in CHARGE syndrome. *CHD7* encodes a protein of the chromodomain (chromatin organization modifier) family. This family shares a unique combination of functional domains consisting of two N-terminal chromodomains, followed by a SWI2/SNF2-like ATPase/-helicase domain and a DNA-binding domain. It is assumed that *CHD* protein complexes affect chromatin structure and gene expression and thereby play important roles in regulating embryonic development (20). Therefore, one might speculate that *CHD7* has a possible influence on the expression or actions of *KALI*, *FGFR1*, *PROK2* and/or *PROKR2* during development. However, because mutations in these genes account for only 30% of all KS cases, it is possible that *CHD7* impacts on other yet undiscovered, KS genes.

We identified a *de novo* *CHD7* mutation in three patients initially diagnosed with KS. The two nonsense mutations are known to be pathogenic. The missense mutation p.Ile2116Asn is not located in one of the known protein domains of the *CHD7* protein, but it concerns a drastic amino acid change that has not been detected in over 600 control alleles. Furthermore, the p.Gly2108Arg mutation has been shown to be associated with CHARGE syndrome in two families with a variable phenotype, indicating that this part of the protein probably has an important function (12). This indicates that the p.Ile2116Asn mutation is possibly pathogenic.

In retrospect, two of the three *CHD7*-positive patients (patients 2 and 8) had typical CHARGE syndrome with the presence of at least three major features (9). Patient 1 presented with only two additional CHARGE features (short stature and unilateral hearing impairment), although one should notice that vestibular function was not tested in this patient.

From this study, we conclude that it is important to evaluate patients with hypogonadotropic hypogonadism and anosmia for clinical features characteristic of CHARGE syndrome. All three patients were proven to be anosmic. Therefore, the chance to find a *CHD7* mutation seems higher in anosmic patients although the study group is too small to conclude that *CHD7* mutations cannot occur in patients with normosmic IHH. Indeed some patients with CHARGE syndrome are able to smell (personal observations). Because all three patients suffered from

hearing impairment, it is tempting to regard this feature as discriminating. However, sensorineural hearing impairment is also an associated feature in males with *KALI* mutations. Thus, hearing abnormalities may be a sensitive but not very specific symptom of *CHD7* mutations. Hypoplasia or aplasia of the semicircular canals is a much more consistent feature in CHARGE syndrome, even in mildly affected patients (9,12). Therefore, history taking regarding balance disturbances and gross motor development might reveal indicative information for the presence of a *CHD7* mutation. Abadie et al. have described a specific pattern of postural behavior related to vestibular anomalies in CHARGE syndrome. They noticed a frequent inability to crawl on all fours without resting the head on the floor (5-point crawl), a prolonged duration of standing with support stage and an inability to ride a bike without stabilizers (21). After the first years of life, balance disturbances may not be unequivocally present as a result of visual compensation. In these patients, disequilibrium in the dark is a helpful indication of vestibular deficit. If there is doubt about the vestibular function, screening for vestibular areflexia or imaging of the semicircular canals will be helpful. In the newborn, agenesis of the semicircular canals can be visualized on plain profile X-ray of the skull (9). In older patients, computerized tomography or MRI is necessary.

Finally, dilated fundus examination can be performed to reveal an optic disc coloboma. A less invasive, but of course also less accurate method, would be to ask for the presence of an optic field defect.

CHD7 screening in the large North American cohort revealed only one mutation. In general, these patients underwent a more extensive clinical work-up (5). From this cohort, we learned that it is not useful to screen the *CHD7* gene in each patient diagnosed with KS or nIHH; additional CHARGE features should be present. Such additional features do not imply that a *CHD7* mutation will be present as has been demonstrated by patient 15 who has choanal atresia but no *CHD7* mutation.

The patients carrying a mutation in *CHD7* in this cohort and the mild *CHD7*-positive patients reported by us in a previous study (12) show that the current diagnostic criteria cannot always discriminate between patients with and without a mutation in *CHD7* (9,12).

We conclude that it is useful to screen patients with hypogonadotropic hypogonadism and anosmia for clinical features consistent with CHARGE syndrome, particularly hearing impairment, vestibular dysfunction and dysmorphisms of the ears. If additional features of CHARGE syndrome are present, *CHD7* sequencing is recommended.

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Fig. 1.
Lateral view of patient 2. Note the dysmorphic ears with absence of the earlobe and the lower helical fold, and a triangular concha. These dysmorphism are typical for CHARGE syndrome.

Table 1

Clinical characteristics of all patients and results of *CHD7* analysis^a

No.	Sex	Diagnosis	Additional features	Family	Mutation <i>CHD7</i>	Parents	MLPA performed
1	M	KS	Dental agenesis, high-arched palate, unilateral perceptive deafness and short stature	Sp	c.8803G>T; p.Glu2935X; exon 38	<i>De novo</i>	–
2	M	KS	Cleft palate, auricular dysplasia, nystagmus, bilateral perceptive deafness and hypoplasia of semicircular canals	Sp	c.6347T>A; p.Ile2116Asn; exon 31	<i>De novo</i>	–
3	F	KS		Sp	–		–
4	F	KS		Sp	–		–
5	M	KS	High-arched palate	Sp	–		–
6	M	KS	Prosis	Sp	–		–
7	M	KS		Sp	–		–
8	F	KS	Facial nerve palsy, bilateral colobomas, cleft lip/palate, deafness, short stature and developmental delay	Sp	c.6070C>T; p.Arg2935X; exon 30	<i>De novo</i>	–
9	F	KS		Fam	–		+
10	M	KS		Fam	–		–
11	F	KS	Crohn's disease, syndactyly	Fam	–		–
12	M	KS		Fam	–		–
13	M	KS		Sp	–		+
14	F	KS		Sp	–		–
15	F	KS,	Choanal atresia	Fam	–		+
16	M	KS		Fam	–		–
17	M	KS	Congenital deafness and Hirschsprung's disease	Sp	–		–
18	F	KS		Fam	–		–
19	M	KS		Fam	–		–
20	F	KS		Fam	–		+
21	F	KS	Hearing impairment	Fam	–		–
22	M	KS	Deafness	Sp	–		–
23	F	KS	Multiple cranial nerve abnormalities	Sp	–		+
24	F	KS		Fam	–		–

No.	Sex	Diagnosis	Additional features	Family	Mutation <i>CHD7</i>	Parents	MLPA performed
25	F	KS		Sp	-		+
26	M	KS		Fam	-		-
27	M	KS	Hearing impairment	Sp	-		-
28	M	KS		Fam	-		-
29	M	KS		Fam	-		-
30	M	KS	Cryptorchidism	Fam	-		-
31	M	KS		Fam	-		-
32	F	KS	Narrow palate	Fam	-		-
33	F	KS	High-arched palate and hyperlaxity of hand joints	Fam	-		-
34	M	KS	Macrocephaly, hypertelorism, high-arched palate, ataxia, Dandy Walker malformation and developmental delay	U	-		-
35	M	KS		Fam	-		-
36	M	Partial KS	Spinal muscular atrophy	Sp	-		-
37	M	IHH, KS in family	Cardiac septum defect	Fam	-		+
38	M	IHH, KS in family	Hearing impairment	Fam	-		+
39	M	IHH, KS in family		Fam	-		-
40	F	IHH		Fam	-		+
41	M	IHH		Sp	-		+
42	F	IHH	Cardiac septum defect	Sp	-		+
43	M	IHH	Cryptorchidism	Fam	-		-
44	M	IHH	Growth hormone deficient	Fam	-		-
45	F	IHH		Fam	-		-
46	F	IHH		Fam	-		-
47	M	IHH	Cryptorchidism, blind, seizures, mental retardation and short stature	Sp	-		-
48	F	IHH		Fam	-		-
49	M	IHH	Ataxia	Sp	-		-
50	F	IHH		Fam	-		-
51	F	IHH		Fam	-		-
52	M	IHH		Fam	-		-

No.	Sex	Diagnosis	Additional features	Family	Mutation <i>CHD7</i>	Parents	MLPA performed
53	M	IHH		Fam	–		–
54	M	IHH	Developmental delay and high-arched palate	Fam	–		–
55	M	IHH		Fam	–		–
56	F	IHH		Fam	–		–

F, female; Fam, familial; IHH, idiopathic hypogonadotropic hypogonadism; KS, Kallmann syndrome (IHH + anosmia); M, male; MLPA, multiplex ligation probe amplification; partial KS, patient with IHH and anosmia, with some degree of spontaneous pubertal development; Sp, sporadic; U, unknown.

^aPatients 1–7 are of Japanese descent and patients 8–56 are from North America.