Mutation analyses in pedigrees and sporadic cases of ethnic Han Chinese Kallmann syndrome patients

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Abstract

Kallmann syndrome, a form of idiopathic hypogonadotropic hypogonadism, is characterized by developmental abnormalities of the reproductive system and abnormal olfaction. Despite association of certain genes with idiopathic hypogonadotropic hypogonadism, the genetic inheritance and expression are complex and incompletely known. In the present study, seven Kallmann syndrome pedigrees in an ethnic Han Chinese population were screened for genetic mutations. The exons and intron–exon boundaries of 19 idiopathic hypogonadotropic hypogonadism (idiopathic hypogonadotropic hypogonadism)-related genes in seven Chinese Kallmann syndrome pedigrees were sequenced. Detected mutations were also tested in 70 sporadic Kallmann syndrome cases and 200 Chinese healthy controls. In pedigrees 1, 2, and 7, the secondary sex characteristics were poorly developed and the patients' sense of smell was severely or completely lost. We detected a genetic mutation in five of the seven pedigrees: homozygous *KAL1* p.R191ter (pedigree 1); homozygous *KAL1* p.C13ter (pedigree 2; a novel mutation); heterozygous *FGFR1* p.R250W (pedigree 3); and homozygous *PROKR2* p.Y113H (pedigrees 4 and 5). No genetic change of the assayed genes was detected in pedigrees 6 and 7. Among the 70 sporadic cases, we detected one homozygous and one heterozygous *PROKR2* p.Y113H mutation. This mutation was also detected heterozygously in 2/200 normal controls and its pathogenicity is likely questionable. The genetics and genotype–phenotype relationships in Kallmann syndrome are complicated. Classical monogenic inheritance does not explain the full range of genetic inheritance of Kallmann syndrome patients. Because of stochastic nature of genetic mutations, exome analyses of Kallmann syndrome patients may provide novel insights.

Keywords: Kallmann syndrome, idiopathic hypogonadotropic hypogonadism, *KAL1*, *FGFR1*, *PROKR2*, polygenic inheritance, complex disease

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Introduction

Idiopathic hypogonadotropic hypogonadism (IHH) is a congenital condition characterized by a lack of or incomplete pubertal development in individuals 18 years or older because of varying degrees of GnRH secretory defects.¹ Among IHH cases, nearly half of the patients have varying degrees of olfactory abnormalities (olfactory bulb, olfactory tract absence or hypoplasia) and are referred to as Kallmann syndrome (KS).² The remainder are termed normal-olfactory IHH (nIHH).

GnRH neurons originate outside the central nervous system (CNS) in the medial-olfactory placode and thereafter

migrate along the olfactory epithelium to the olfactory bulb, and thence to the olfactory tract.^{3,4} Ultimately, the GnRH neurons dissociate from their olfactory guiding fibers to reach the hypothalamus.⁵ These complex development defects are tightly regulated by specific spatiotemporal expression patterns of growth factors, adhesion molecules and/or diffusible attractants, and repellents.⁶

The etiology of KS is either spontaneous or hereditary. Its incidence is estimated as 1 in 8000 males and 1 in 40,000 females.⁷ The majority are sporadic cases, but approximately one-third have a family history and genetic mutations can be detected in about 30% of all KS individuals.⁸

The severity of the KS clinical phenotype is heterogeneous in its presentation. Some patients present with atypical KS, such as reversible KS⁹ (patients experience a degree of spontaneous pubertal development after intermittent exogenous hormone replacement therapy) or adult-onset KS (patients have normal pubertal development, but KS symptoms occur during adulthood).¹⁰ In addition, particular gene mutations are often associated with specific non-olfactory, non-reproductive system clinical manifestations, such as renal hypoplasia or cleft lip/palate.

The heterogeneous presentations of KS-related hypogonadotropic hypogonadism likely result from varied contributions of the genes.^{11–21} For example, the same mutation in fibroblast growth factor receptor (FGFR) can cause manifestations of disease, as well as a cohort of patients can undergo reversal of phenotype with the trophic hormone replacement. Even two or more gene–gene interactions can actually manifest the disease process. Furthermore, in the same gene involved, diseases may be manifested with or without olfactory involvement (KS vs. nIHH). In addition, disease heterogeneity is manifested in ethnic groups, like for example, nIHH was first identified in an isolated Bedouin population.²² Thus, the rationale may be expanded that it merits testing whether the reported genes or its variations can be identified across different ethnic groups across the globe because of the stochastic nature of genotype–phenotype presentations in KS.

KS and nIHH manifestations in an ethnic Han Chinese population have only been scantily reported.^{23,24} We have our own large case series of independent pedigrees, as well as isolated presentations, in which we examined these relationships between KS gene mutations and the clinical phenotype, we evaluated the clinical phenotypes in seven KS pedigrees and screened the patients for mutations in the known IHH-related genes. We also screened for the detected mutations in 70 sporadic KS cases and 200 healthy controls.

Materials and methods

Ethical statement

All participating subjects signed an informed consent form, and the research was approved by the Institutional Review Board of Chinese PLA General Hospital. All experiments were performed in accordance with the Declaration of Helsinki.

Patients

We diagnosed seven Chinese KS pedigrees, containing 19 patients in total, 17 of whom agreed to genetic testing (Figure 1). We also collected 70 sporadic KS patients and 200 Chinese healthy community volunteers ($n=400$ chromosomes) for use as the controls. Patient diagnostic criteria were based on the following: (1) age greater 18 years or older with absent or incomplete pubertal development; (2) male serum testosterone (T) ≤ 100 ng/dL, female serum estradiol (E2) ≤ 20 pg/mL; (3) normal adrenal axis and growth hormone axis (assessed by insulin

hypoglycemia stimulation test); (4) normal levels of serum thyroid hormone, prolactin, and ferritin; (5) hypothalamic–pituitary region magnetic resonance imaging (MRI) normal; (6) reported features of anosmia or hyposmia. Control populations included subjects with normal sexual development, without any endocrine or systemic diseases.

Genes examined in the current study

Currently, several genes have been causally related to pathogenesis of IHH. The genes that have been examined in KS include: *KAL1*, *FGFR1*, *FGF8*, *PROKR2*, *PROK2*, *CHD7*, *NELF*, *WDR11*, *HS6ST1*, *KISS1R*, *KISS1*, *TAC3*, *TACR3*, *LEPR*, *LEP*, *PCSK1*, *GNRHR*, *GNRH1*, *SEMA3A*, and *NDN7*. These genes are essential for the correct differentiation, migration, upstream signal regulation, and action of embryonic GnRH neurons. Among these genes, those that are clearly related to KS are *KAL1*, *FGFR1*, *PROKR2*, *PROK2*, *CHD7*, and *FGF8*. *CHD7* has been related to CHARGE syndrome and *NELF*,²⁵ *HS6ST1*,²⁶ and *WDR11*²⁷ may also be related to KS. The observed patterns of inheritance include all three classical Mendelian models: X-linked (OMIM#308700; *KAL1*); autosomal dominant (OMIM#147950; *FGFR1*, *FGF8*, and *CHD7*), and autosomal recessive (OMIM#244200; *PROKR2* and *PROK2*). In addition, some cases of two gene mutations have been reported, with either both being KS-related genes, (e.g. *FGFR1* + *PROKR2*) or one being a KS-related gene and the other an IHH-related gene (e.g. *PROKR2* + *GNRHR*).²⁸

Genetic mutational analysis

We obtained 5 mL blood samples from all subjects and extracted genomic DNA using a BloodGen Midi Kit (CWBIO, Beijing, China). Primers were designed to amplify the exons and exon–intron boundaries of 19 of the reported 20 IHH-related genes listed (supplementary Table 1). We did not analyze *CHD7*. Genomic DNA was PCR amplified in 50 μ L reactions using ExTaq polymerase (Takara, Dalian, China) with the following cycling conditions: predenaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 55–65°C for 30 s, and extension at 72°C for 1 min; followed by a final extension at 72°C for 10 min. The PCR products were directly sequenced in both directions using the original PCR primers with a BigDye v. 3.1 kit on an ABI 3100 (Applied Biosystems, Foster City, CA). Sequences were analyzed using CodonCode Aligner (<http://www.codoncode.com/index.htm>) and DNASTAR (<http://www.dnastar.com/>). If a mutation was found, we tested for the presence of this mutation in all other patients and controls, including unaffected family members.

Nonsense changes resulting in either a truncated protein, frameshift insertion, or deletion were categorized as definite mutations. Nucleotide changes that met the following criteria were also classified as disease-causing mutations, vide earlier studies:²⁹ (1) absent from single-nucleotide polymorphism (dbSNP) (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) and expressed sequence tag databases; (2) absent in ≥ 200 ethnically matched subjects; (3) evolutionarily conserved across species; and (4) segregated appropriately in the family

Figure 1 Pedigree charts of the seven pedigrees and sequencing chromatograms showing corresponding genetic mutations. The arrow indicates the proband in each pedigree. Individuals with half-filled symbols had abnormal olfaction but a normal reproductive system. Genetic testing results for each individual are shown. Genetic abnormalities were not found in the proband of pedigrees 6 or 7 WT, wild type. (A color version of this figure is available in the online journal.)

Clinical assessment of patients

Patients' clinical characteristics and phenotypic features like appearance, presence of pubic hair, penile length measurement, presence or absence of testicles, development of gynecomastia, and BMI assessment were initially documented. Blood samples were collected for hormone status (FSH, LH, and testosterone) assessment by routine biochemical methods.

Briefly, pubic hair was staged according to the standard Tanner staging (P1–P6). Testicle volume was measured with a Prader orchidometer. Male patients were divided into groups with or without breast development. The reference value of the male's luteinizing hormone (LH) was

1.5–9.3 IU/L; follicle stimulating hormone (FSH) was 1.4–18.1 IU/L; testosterone was 8.4–28.7 nmol/L. In the GnRH stimulating test, the normal male LH reflection peak was more than five times of the basic value and the peak value is ideally greater than 9 IU/L, which occurred at 30–60 min during the test. In the human chorionic gonadotropin (hCG) stimulating testosterone test, the testosterone peak of normal males occurred at 48–72 h and the peak value increased at least two times of the control. The patients were not treated with any medication before the above examination.

For the olfactory tests, the olfactory sensation scoring scale was: < 1.0, normal; 1.1–2.5, slightly damaged;

2.6–4.0, moderately damaged; 4.1–5.5, heavily damaged; > 5.5, complete loss. Other anomalies examined included non-reproductive system and non-olfactory system symptoms, such as craniofacial malformations (cleft lip/palate, high-arched palate, eye symptoms), deafness, abnormal fingers, tooth agenesis, unilateral renal hypoplasia, nervous system defects (oculomotor nerve abnormalities, movement associated with both hands), sleep disorders, and mood disorders. Patients' MRI were obtained by standard imaging.

Results

Variants detected in the pedigrees

The clinical presentations and genetic testing results of the proband and other family members in each pedigree are shown in Table 1 and gene analyses for KS pedigrees are shown in Table 2 and are summarized below. The hormonal assays conformed to the diagnosis of KS, with many patients with undetectable levels of testosterone and varying levels of evidence for central hypogonadotropism (Table 1).

Pedigree 1. There were three male patients in this pedigree (III-17 and III-18 were twins) and their clinical symptoms were severe. They did not have unilateral renal hypoplasia or synkinesia. A homozygous *KAL1* nonsense mutation c.C571T (p.R191ter) was detected in the proband (III-17) and both other patients (III-16, III-18). Individual III-15 was a heterozygous carrier of this X-linked mutation and had a normal phenotype. Individual III-13 had no genetic abnormality.

Pedigree 2. There were three male patients in this pedigree. Only the proband had severe clinical symptoms. There was no unilateral renal hypoplasia or synkinesia. A homozygous *KAL1* nonsense mutation c.C39A (p.C13ter) was detected in the proband (III-17) and the two other patients (III-5, III-15). Individuals II-6, II-8, and III-8 with a normal phenotype were found to be heterozygous carriers of this X-linked mutation. Individuals II-9 and III-6 were genetically normal. In both pedigrees 1 and 2, the differential mutation involving *KAL1* gene produced consistency in the phenotypic manifestation of the hypogonadotropic hypogonadism.

Pedigree 3. There were three male patients and one female patient in this pedigree. The reproductive system, olfactory phenotypes, and olfactory bulb MRI findings of the female were milder than those of the males. There was no cleft lip/palate, abnormal tooth development, or hearing impairment. A heterozygous *FGFR1* missense mutation c.C748T (p.R250W) was detected in all three patients. Individual III-6 also carried the mutation but had a normal phenotype. Imperfect penetrance and heterozygosity of the mutational status may potentially explain the milder or apparently normal phenotype in this individual. Individual III-3 was phenotypically and genetically normal.

Pedigree 4. There were one male patient and two female patients in this pedigree. The patients in this family declined clinical examination, but II-5 had self-reported hyposmia. Individual III-4 received sequential therapy with hCG and human menopausal gonadotropin, and after one year he had spontaneous pubertal development. He now has one child and is considered to have reversible KS. Individuals III-1 and III-2 did not have KS symptoms, but both had vision loss since birth. There were no sleep disorders, mood disorders, obesity or other non-reproductive, non-olfactory symptoms. A homozygous *PROKR2* missense mutation c.T337C (p.Y113H) was detected in the proband (III-3). Individual II-5 was genetically normal and individual III-4 refused genetic testing. However, individual II-6, with a normal phenotype, was heterozygous for this mutation.

Pedigree 5. There were two patients in this pedigree. The proband had typical KS symptoms; the mother had self-reported dysosmia, and her MRI findings of the olfactory bulb and olfactory groove showed dysplasia. A homozygous *PROKR2* missense mutation c.T337C (p.Y113H) was detected in both. Individual II-6, with a normal phenotype, was a heterozygous carrier of this mutation. In the current study, we have not examined the genotypic contributions of the parents of the individual II-6, but propose to pursue this test in future studies.

Pedigree 6. There were two patients in this pedigree. The proband had classical KS symptoms; the proband's mother had a severe olfactory deficit based on a test of olfaction, and MRI demonstrated that the olfactory bulb and olfactory tract were not developed. Interestingly, no mutation was found in the proband in any of the 19 genes tested.

Pedigree 7. The proband (III-1) and his sister (III-2) had typical KS symptoms. Individuals II-1 and II-2 had perished; they had no diagnosis, but the other family members reported that they had abnormal olfaction, were unmarried, and had no children. No mutation was found in the proband in any of the 19 genes tested.

In addition to the above-mentioned mutations, we also detected several SNPs that were present in dbSNP. SNPs in *LEPR* and *KISS1* (*LEPR*: c.A326G [p.K109R; rs1137100] and c.A668G [p.Q223R; rs1137101]; *KISS1*: c.C242G [p.P81R; rs4889]) were found in all seven pedigrees; an SNP in *PCSK1* (c.G239A [p.R80Q; rs1799904]) was found in pedigrees 1 and 3. According to SIFT (<http://sift.jcvi.org/>), all four of these SNPs are potentially harmful. However, the three *LEPR/KISS1* SNPs were present in the 1000 genomes database (<http://browser.1000genomes.org/index.html>) and can therefore be considered non-pathogenic.

Mutations detected in the sporadic KS patients and controls

We screened for the four mutations described above in the 70 sporadic KS patients with no reported or documented antecedent family history and 200 control subjects. The only gene identified in sporadic KS was *PROKR2* p.Y113H, for

Table 1 The clinical assessment of the KS pedigrees

General condition of patients				Development			Hormone levels						Olfactory				
Pedigree	gender	age	BMI (Kg/m2)	Pubic hair	Cryptorchidism	Penis length (cm)	Testis volume (ml)	Male breast development	LH(IU/L)		FSH(IU/L)		T(nmol/L)		Olfactory bulb MRI	other anomalies	
									Basis	Peak value	Basis	Peak value	Basis	Peak value			
1	III17	M	18	23.5	P1	N	4	Left:1 Right: <1	<0.07	0.63†	0.35	2.3\$	0.89	ND	4.8	agenesis of olfactory bulb and olfactory tract	None
	III18	M	18	23	P1	N	6	Left:2 Right:1	<0.07	0.75†	1.50	5.53\$	0.76	ND	4.6	agenesis of olfactory bulb and olfactory tract	None
	III16	M	28	29.2	P1	Y	2	NT	<0.07	0.47*	1.63	3.10\$	0.68	ND	5.8	ND	None
2	III5	M	17	19.1	P1	N	3	Left: <1 Right:1	0.36	2.96*	1.20	4.62‡	1.34	6.39	ND	agenesis of olfactory bulb and olfactory tract	None
	III7	M	19	19.3	P1	N	4	Left:1 Right:1	0.39	3.17*	0.39	1.91‡	1.47	6.55	ND	ND	None
3	III1	M	42	29.4	P2	N	4	Left:6 Right:5	0.04	3.87†	0.12	2.0\$	1.46	5.33	6	agenesis of olfactory bulb and olfactory tract	None
	III5	M	29	28.4	P1	N	3.4	Left:2 Right:3	0.23	1.48†	0.97	2.86\$	0.63	1.25	5.8	agenesis of olfactory bulb and olfactory tract	None
	III2	F	36	25.3	P2	F	F	B2	0.71	5.23†	2.71	6.02\$	F	F	4.4	hypogenesis of olfactory bulb, Olfactory tract	None
(continued)																	

(continued)

Table 1 Continued

General condition of patients			Development				Hormone levels						Olfactory				
							LH(IU/L)		FSH(IU/L)		T(nmol/L)						
Pedigree	gender	age	BMI (Kg/m2)	Pubic hair	Cryptorchidism	Penis length (cm)	Testis volume (ml)	Male breast development	Basis	Peak value	Basis	Peak value	scoring	Olfactory bulb MRI	other anomalies		
5	III3	M	12	ND	P1	N	ND	Left:1 Right: <1	0.1	ND	0.23	ND	0.7	ND	4.9	agenesis of olfactory bulb and olfactory tract	None
6	III1	M	25	24.3	P2	N	4	Left:2 Right:3	<0.07	0.48*	1.91	2.14‡	6.17	ND	5.8	agenesis of olfactory bulb and olfactory tract	None
II6	F	49	26.8	P4	F	F	F	B4	21.1	140.84 *	29.5	67.58‡	F	F	5.8	agenesis of olfactory bulb and olfactory tract	None
7	III1	M	31	ND	P2	N	3	Left:2 Right:2	0.19	1.72†	1	3.59§	1.54	3.08	ND	agenesis of olfactory bulb and olfactory tract	None
III2+	F	39	21.2	P3	F	F	F	B3	0.2	ND	0.9	ND	F	F	ND	agenesis of olfactory bulb and olfactory tract	None

Individuals III-2 in Pedigree 3, II-6 in Pedigree 6, and III-2 in Pedigree 7 were female. The proband in each family was: Pedigree 1: III-17; Pedigree 2: III-7; Pedigree 3: III-1; Pedigree 4: III-3; Pedigree 5: III-3. Y: yes; N: No; ND: not detected; NT: not touched; T: testosterone; * indicates LH peak value in GnRH stimulation test at 30 min; † indicates LH peak value in GnRH stimulation test at 60 min; ‡ indicates FSH peak value in GnRH stimulation test at 60 min; § indicates LH peak value in GnRH stimulation test at 120 min.

Table 2 The gene analysis of the KS pedigrees

						Changes in nucleotides and amino acid	New Mutation
Pedigree		Gene (OMIM)	Locate	Mode of inheritance	Type of mutation		
1	III17 III18 III16	KAL-1 (308700)	Xp22.32	X-linked recessive inheritance	homozygous and non-sense mutation	c.C571T p.R191X	N
2	III5 III7	KAL-1 (308700)	Xp22.32	X-linked recessive inheritance	homozygous and non-sense mutation	c.C39A p.C13X	Y
3	III1 III5 III2	FGFR1 (147950)	8p11.2-p11.1	autosomal dominant inheritance	Heterozygous missense mutation	c.C748T p.R250W	N
4	III3	PROKR2 (244200)	20p13	autosomal recessive inheritance	Heterozygous missense mutation	c.T337C p.Y113H	N

Figure 2 Representation of *KAL1* gene and domain structure of its encoded protein anosmin-1, showing the mutation sites in pedigrees 1 and 2. The long bar represents *KAL1* gene and the 14 boxes represent its 14 exons. The five blue semicircles indicate heparan sulphate-binding sites. Green boxes above and below the gene schematic indicate the nucleotide and amino acid changes, respectively, detected in pedigrees 1 and 2. (A color version of this figure is available in the online journal.)

which we detected one homozygous mutation and one heterozygous mutation. Among the normal controls, we detected a heterozygous *PROKR2* p.Y113H variant in 2/200 of the current case series. The *PCSK1* SNP p.R80Q was detected in 8/200 normal control subjects, which suggests that this is likely non-pathogenic, despite its absence in the 1000 genomes database.

Discussion

It has been previously reported that genetic abnormalities can be detected in about 30% of KS patients.⁸ Here, we detected a genetic mutation of the currently reported repertoire of involved genes in five of the seven pedigrees in this study. For individual genes, it has been reported that *KAL1*, *FGFR1*, and *PROK2/PROKR2* mutations account for 10–14%,³⁰ 10%,³¹ and 9%³² of all KS, respectively.

The present study is a report of the largest cohort of Han Chinese ethnic patients (an ethnic group that comprises more than 90% of the population of mainland China) with inherited KS. Interestingly, of the four mutations detected in the pedigrees, only the *PROKR2* p.Y113H mutation was detected among the 70 sporadic KS patients (1.4%

heterozygous and 1.4% homozygous), and this was also the only mutation detected among the normal controls (1% heterozygous). The sporadic cases and normal controls were screened only for the four mutations detected rather than for the 19 candidate genes; so it would be erroneous to assume that the mutation rate in our KS pedigrees is higher than that in the sporadic cases.

All our detected mutations have been described before,^{8,33,34} except *KAL1* p.C13ter (pedigree 2). *KAL1* encodes the extracellular matrix protein KS 1 sequence (also known as anosmin-1) (Figure 2). In this protein, the Whey acidic protein (WAP) domain and the first fibronectin III domain are conserved across species and interact directly with *FGFR1*.³⁵ Furthermore, anosmin-1 has five heparan sulfate (HS)-binding sites with very strong HS-recruiting capability, which are essential for its function. We speculate that in pedigree 2, the anosmin-1 protein would be truncated after the nonsense mutation, resulting in the absence of all these functional domains.

There are several possible reasons why we did not detect a mutation in pedigrees 6 or 7: the patients may have had an intronic mutation or intron copy number variation.³⁶ It is also likely that the mutation in these families is in a novel

KS-related gene. Epigenetic variation such as gene methylation might also play a pathogenic role. Further analysis of these patients in these pedigrees is necessary.

The genotype-phenotype relationships among the different mutations are not fully understood. For instance, patients in pedigrees 1 and 2, carrying a *KAL1* mutation, had severe symptoms, but pedigree 3 patients with an *FGFR1* mutation had variable clinical symptoms. The *FGFR1* gene, encoding the fibroblast growth factor receptor 1 protein, is a member of the receptor tyrosine kinase superfamily.⁸ The combination of fibroblast growth factor (FGF) with *FGFR1* results in receptor dimerization, tyrosine kinase domain phosphorylation, and the activation of a downstream pathway. HS strengthens the weak *FGFR*-FGF interaction,³⁷ and the HS is recruited by anosmin-1.³⁸ The resulting anosmin-1/*FGFR1*/FGF/HS complex can activate the *FGFR1* pathway.^{33,39} This pathway would be ablated in pedigrees 1 and 2, who carry a truncating anosmin-1 mutation. This could explain the relatively severe symptoms.

In contrast, the *FGFR1* missense mutation in pedigree 3 might affect the binding of ligand and anosmin-1 to some extent, but would not completely ablate the pathway, thus resulting in relatively mild symptoms. The milder symptoms in the female patients compared with the male patients in this family may be explained by an increased dosage of the X-chromosome-encoded anosmin-1.²⁹ The incomplete penetrance observed for individual III-6 in this family might be an example of maturity-onset KS and could be explained by delayed dominance (a phenomenon in which a dominant virulence gene in the heterozygous state only exerts its function after a certain age is reached); follow-up studies of this individual could confirm this.

The pathogenicity of the *PROKR2* p.Y113H heterozygous mutation is questionable. We detected this mutation heterozygously in 1% of our normal controls. Moreover, mice with gene knockout of *Prokr2* show a phenotype only in the homozygous state.⁴⁰ However, one sporadic patient had a heterozygous mutation, and this phenomenon has been reported previously.⁴¹ Patient III-4 in pedigree 4 was a reversible KS case and patients III-3 and III-4 responded well to treatment. Patient II-5 showed a phenotype but was genetically normal. It is possible that this individual carried a microdeletion that spans the mutation site and would not have been detected by our methods. We deduced that individual III-3 inherited one chromosome with a microdeletion (from II-5) and one with a mutation (from II-6; because II-6, with a normal phenotype, was a carrier of this mutation), so her genotype was detected as homozygous. Quantitative PCR is needed to confirm this speculation.

We detected four SNPs in *LEPR*, *KISS1*, and *PCSK1*. All were present in the 1000 Genomes database or were found among normal controls and are thus probably non-pathogenic. However, functional tests are needed to exclude their involvement.

It is clear that the genetic origins of KS/IHH are complex, and it is widely accepted that the most reasonable explanation for the various genotype-phenotype relationships is digenic or oligogenic inheritance,⁴² together with

complex interactions between the gene products.²⁹ Moreover, these interactions may be affected by environmental factors, modifier genes, and epigenetics.⁴³ In this study, we examined all genes (except *CHD7*) currently related to KS and nIHH but digenic or oligogenic inheritance mode was not found. Recent studies have shown that *CHD7* mutation is only present in KS patients with CHARGE syndrome phenotype characteristics. If the patient was diagnosed with hypogonadism and anosmia, attention merits to be paid to the presence of clinical characteristics of CHARGE syndrome.¹² *CHD7* needs to be detected only in case of deafness, ear morphological abnormalities, and/or hypoplasia or aplasia of the semicircular canals of the inner ear. Our patients had no clinical characteristics of CHARGE syndrome after evaluation, so *CHD7* was not examined. Therefore, we speculate that the other genetic factors involved in these pedigrees and sporadic cases are as-yet-undiscovered KS/nIHH-related genes or mutations within introns of the 19 known genes.

It has been reported that particular genetic mutations are often associated with a specific non-reproductive system, non-olfactory phenotype; for example, *KAL1* mutation is associated with unilateral renal hypoplasia and synkinesia;³⁷ *FGFR1* mutation is often accompanied by cleft lip/palate,⁴⁴ dental hypoplasia,⁴⁴ and abnormal hearing; *PROKR2* mutation is often associated with mood disorders,⁴⁵ obesity,⁴⁶ and abnormal circadian endocrine rhythms.⁴⁷ However, we did not find any of these abnormalities in our seven pedigrees by questioning, renal ultrasound, and pure-tone audiometry examination of some patients. It is possible that these associations vary by ethnicity and are uncommon in the Han Chinese population.

In conclusion, we detected diverse genetic mutations in five of our seven pedigrees, including the novel nonsense mutation *KAL1* p.C13ter. There were complicated genotype-phenotype relationships in the families, which might be due to skewed X-inactivation, delayed dominance, and microdeletion. Moreover, some relationships could not be explained by monogenic inheritance, and they might result from multiple or novel KS/nIHH gene inheritance, with modification by environmental and/or epigenetic factors. Gene-chip exome mutation analysis should be used to identify new candidate genes that may be important in pedigrees 6 and 7 in which we found no mutations. In addition, further genetic (intron analysis), gene methylation, and other epigenetic analyses, and then in vitro and in vivo functional studies, are needed.

Authors' contributions: YMM participated in the design of the study, performed data analysis, and helped to revise the manuscript. YQW carried out Data collection, Genetic mutational analysis, performed data analysis and Statistical analysis, and helped to draft the manuscript. WJG, ZHL, JTD performed Genetic mutational analysis and data analysis. JMB, XLW, APW, TPH, DLZ, JKY, JD, LJY, GQY, NJ, KC, LZ, GN carried out Data collection, Genetic mutational analysis. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

None declared.

REFERENCES

- Topaloglu AK, Kotan LD. Molecular causes of hypogonadotropic hypogonadism. *Curr Opin Obstet Gynecol* 2010;**22**:264–70
- Bianco SD, Kaiser UB. The genetic and molecular basis of idiopathic hypogonadotropic hypogonadism. *Nat Rev Endocrinol* 2009;**5**:569–76
- Schwanzel-Fukuda M, Pfaff DW. Origin of luteinizing hormone-releasing hormone neurons. *Nature* 1989;**338**:161–4
- Wray S, Nieburgs A, Elkabes S. Spatiotemporal cell expression of luteinizing hormone-releasing hormone in the prenatal mouse: evidence for an embryonic origin in the olfactory placode. *Brain Res Dev Brain Res* 1989;**46**:309–18
- Tobet SA, Bless EP, Schwarting GA. Developmental aspect of the gonadotropin-releasing hormone system. *Mol Cell Endocrinol* 2001;**185**:173–84
- Cadman SM, Kim SH, Hu Y, González-Martínez D, Bouloux PM. Molecular pathogenesis of Kallmann's syndrome. *Horm Res* 2007;**67**:231–42
- Bonomi M, Libri DV, Guizzardi F, Guarducci E, Maiolo E, Pignatti E, Asci R, Persani L. Idiopathic Central Hypogonadism Study Group of the Italian Societies of Endocrinology and Pediatric Endocrinology and Diabetes. New understandings of the genetic basis of isolated idiopathic central hypogonadism. *Asian J Androl* 2012;**14**:49–56
- Pitteloud N, Acierno JS Jr, Meysing A. Mutations in fibroblast growth factor receptor 1 cause both Kallmann syndrome and normosmic idiopathic hypogonadotropic hypogonadism. *Proc Natl Acad Sci USA* 2006;**103**:6281–6
- Raivio T, Falardeau J, Dwyer A, Quinton R, Hayes FJ, Hughes VA, Cole LW, Pearce SH, Lee H, Boepple P, Crowley WF Jr, Pitteloud N. Reversal of idiopathic hypogonadotropic hypogonadism. *N Engl J Med* 2007;**357**:863–73
- Mitchell AL, Dwyer A, Pitteloud N, Quinton R. Genetic basis and variable phenotypic expression of Kallmann syndrome: towards a unifying theory. *Trends Endocrinol Metab* 2011;**22**:249–58
- Meczekalski B, Podfigurna-Stopa A, Smolarczyk R, Katulski K, Genazzani AR. Kallmann syndrome in women: from genes to diagnosis and treatment. *Gynecol Endocrinol* 2013;**29**:296–300
- Dauber A, Hirschhorn JN, Picker J, Maher TA, Milunsky A. Delayed puberty due to a novel mutation in CHD7 causing CHARGE syndrome. *Pediatrics* 2010;**126**:e1594–8
- Balasubramanian R, Dwyer A, Seminara SB, Pitteloud N, Kaiser UB, Crowley WF Jr. Human GnRH deficiency: a unique disease model to unravel the ontogeny of GnRH neurons. *Neuroendocrinology* 2010;**92**:81–99
- Topaloglu AK, Kotan LD. Molecular causes of hypogonadotropic hypogonadism. *Curr Opin Obstet Gynecol* 2010;**22**:264–70
- Pitteloud N, Durrani S, Raivio T, Sykiotis GP. Complex genetics in idiopathic hypogonadotropic hypogonadism. *Front Horm Res* 2010;**39**:142–53
- Dodé C, Hardelin JP. Clinical genetics of Kallmann syndrome. *Ann Endocrinol (Paris)* 2010;**71**:149–57
- Silveira LF, Trarbach EB, Latronico AC. Genetics basis for GnRH-dependent pubertal disorders in humans. *Mol Cell Endocrinol* 2010;**324**:30–8
- Semple RK, Topaloglu AK. The recent genetics of hypogonadotropic hypogonadism – novel insights and new questions. *Clin Endocrinol (Oxf)* 2010;**72**:427–35
- Valdes-Socin H, Rubio Almanza M, Tomé Fernández-Ladreda M, Debray FG, Bours V, Beckers A. Reproduction, smell, and neurodevelopmental disorders: genetic defects in different hypogonadotropic hypogonadal syndromes. *Front Endocrinol (Lausanne)* 2014;**5**:109
- Hardelin JP, Dodé C. The complex genetics of Kallmann syndrome: KAL1, FGFR1, FGF8, PROKR2, PROK2, et al. *Sex Dev* 2008;**2**:181–93
- Fechner A, Fong S, McGovern P. A review of Kallmann syndrome: genetics, pathophysiology, and clinical management. *Obstet Gynecol Surv* 2008;**63**:189–94
- Crowley WF Jr, Pitteloud N, Seminara S. New genes controlling human reproduction and how you find them. *Trans Am Clin Climatol Assoc* 2008;**119**:29–37; discussion 37–8
- Qu Q, Liu J, Ni D, Zhang Q, Yang D, Wang N, Wu X, Han H. Diagnosis and clinical characteristics of congenital anosmia: case series report. *J Otolaryngol Head Neck Surg* 2010;**39**:723–31
- Zhang SL, Tang YP, Wang T, Yang J, Rao K, Zhao LY, Zhu WZ, Meng XH, Wang SG, Liu JH, Yang WM, Ye ZQ. Clinical assessment and genomic landscape of a consanguineous family with three Kallmann syndrome descendants. *Asian J Androl* 2011;**13**:166–71
- Xu N, Kim HG, Bhagavath B, Cho SG, Lee JH, Ha K, Melicani I, Wenzel W, Podolsky RH, Chorich LP, Stackhouse KA, Grove AM, Odom LN, Ozata M, Bick DP, Sherins RJ, Kim SH, Cameron RS, Layman LC. Nasal embryonic LHRH factor (NELF) mutations in patients with normosmic hypogonadotropic hypogonadism and Kallmann syndrome. *Fertil Steril* 2011;**95**:1613–20
- Tornberg J, Sykiotis GP, Keefe K, Plummer L, Hoang X, Hall JE, Quinton R, Seminara SB, Hughes V, Van Vliet G, Van Uum S, Crowley WF, Habuchi H, Kimata K, Pitteloud N, Bülow HE. Heparan sulfate 6-O-sulfotransferase 1, a gene involved in extracellular sugar modifications, is mutated in patients with idiopathic hypogonadotropic hypogonadism. *Proc Natl Acad Sci USA* 2011;**108**:11524–9
- Kim HG, Layman LC. The role of CHD7 and the newly identified WDR11 gene in patients with idiopathic hypogonadotropic hypogonadism and Kallmann syndrome. *Mol Cell Endocrinol* 2011;**346**:74–83
- Sarfati J, Guiochon-Mantel A, Rondard P, Arnulf I, Garcia-Piñero A, Wolczynski S, Brailly-Tabard S, Bidet M, Ramos-Arroyo M, Mathieu M, Lienhardt-Roussie A, Morgan G, Turki Z, Bremont C, Lespinasse J, Du Boullay H, Chabbert-Buffet N, Jacquemont S, Reach G, De Talence N, Tonella P, Conrad B, Despert F, Delobel B, Brue T, Bouvattier C, Cabrol S, Pugeat M, Murat A, Bouchard P, Hardelin JP, Dodé C, Young J. A comparative phenotypic study of Kallmann syndrome patients carrying monoallelic and biallelic mutations in the prokineticin 2 or prokineticin receptor 2 genes. *J Clin Endocrinol Metab* 2010;**95**:659–69
- Pitteloud N, Meysing A, Quinton R, Acierno JS Jr, Dwyer AA, Plummer L, Fliers E, Boepple P, Hayes F, Seminara S, Hughes VA, Ma J, Bouloux P, Mohammadi M, Crowley WF Jr. Mutations in fibroblast growth factor receptor 1 cause Kallmann syndrome with a wide spectrum of reproductive phenotypes. *Mol Cell Endocrinol* 2006;**254–255**:60–9
- Sato N, Katsumata N, Kagami M, Hasegawa T, Hori N, Kawakita S, Minowada S, Shimotsuka A, Shishiba Y, Yokozawa M, Yasuda T, Nagasaki K, Hasegawa D, Hasegawa Y, Tachibana K, Naiki Y, Horikawa R, Tanaka T, Ogata T. Clinical assessment and mutation analysis of Kallmann syndrome 1 (KAL1) and fibroblast growth factor receptor 1 (FGFR1, or KAL2) in five families and 18 sporadic patients. *J Clin Endocrinol Metab* 2004;**89**:1079–88
- Villanueva C, de Roux N. FGFR1 mutations in Kallmann syndrome. *Front Horm Res* 2010;**39**:51–61
- Sarfati J, Dodé C, Young J. Kallmann syndrome caused by mutations in the PROK2 and PROKR2 genes: pathophysiology and genotype-phenotype correlations. *Front Horm Res* 2010;**39**:121–32

33. Pitteloud N, Zhang C, Pignatelli D, Li JD, Raivio T, Cole LW, Plummer L, Jacobson-Dickman EE, Mellon PL, Zhou QY, Crowley WF Jr. Loss-of-function mutation in the prokineticin 2 gene causes Kallmann syndrome and normosmic idiopathic hypogonadotropic hypogonadism. *Proc Natl Acad Sci USA* 2007;**104**:17447–52
34. Cole LW, Sidis Y, Zhang C, Quinton R, Plummer L, Pignatelli D, Hughes VA, Dwyer AA, Raivio T, Hayes FJ, Seminara SB, Huot C, Alos N, Speiser P, Takeshita A, Van Vliet G, Pearce S, Crowley WF Jr, Zhou QY, Pitteloud N. Mutations in prokineticin 2 and prokineticin receptor 2 genes in human gonadotrophin-releasing hormone deficiency: molecular genetics and clinical spectrum. *J Clin Endocrinol Metab* 2008;**93**:3551–9
35. Hu Y, Guimond SE, Travers P, Cadman S, Hohenester E, Turnbull JE, Kim SH, Bouloux PM. Novel mechanisms of fibroblast growth factor receptor 1 regulation by extracellular matrix protein anosmin-1. *J Biol Chem* 2009;**284**:29905–20
36. Zhang SL, Tang YP, Wang T, Yang J, Rao K, Zhao LY, Zhu WZ, Meng XH, Wang SG, Liu JH, Yang WM, Ye ZQ. Clinical assessment and genomic landscape of a consanguineous family with three Kallmann syndrome descendants. *Asian J Androl* 2011;**13**:166–71
37. Choy C, Kim SH. Biological actions and interactions of anosmin-1. *Front Horm Res* 2010;**39**:78–93
38. Hu Y, Sun Z, Eaton JT, Bouloux PM, Perkins SJ. Extended and flexible domain solution structure of the extracellular matrix protein anosmin-1 by X-ray scattering, analytical ultracentrifugation and constrained modelling. *J Mol Biol* 2005;**350**:553–70
39. González-Martínez D, Kim SH, Hu Y, Guimond S, Schofield J, Winyard P, Vannelli GB, Turnbull J, Bouloux PM. Anosmin-1 modulates fibroblast growth factor receptor 1 signaling in human gonadotropin-releasing hormone olfactory neuroblasts through a heparan sulfate-dependent mechanism. *J Neurosci* 2004;**24**:10384–92
40. Matsumoto S, Yamazaki C, Masumoto KH, Nagano M, Naito M, Soga T, Hiyama H, Matsumoto M, Takasaki J, Kamohara M, Matsuo A, Ishii H, Kobori M, Katoh M, Matsushima H, Furuichi K, Shigeyoshi Y. Abnormal development of the olfactory bulb and reproductive system in mice lacking prokineticin receptor PKR2. *Proc Natl Acad Sci USA* 2006;**103**:4140–45
41. Dodé C, Teixeira L, Levilliers J, Fouveaut C, Bouchard P, Kottler ML, Lespinasse J, Lienhardt-Roussie A, Mathieu M, Moerman A, Morgan G, Murat A, Toubanc JE, Wolczynski S, Delpech M, Petit C, Young J, Hardelin JP. Kallmann syndrome: mutations in the genes encoding prokineticin-2 and prokineticin receptor-2. *PLoS Genet* 2006;**2**:e175
42. Sykiotis GP, Plummer L, Hughes VA, Au M, Durrani S, Nayak-Young S, Dwyer AA, Quinton R, Hall JE, Gusella JF, Seminara SB, Crowley WF Jr, Pitteloud N. Oligogenic basis of isolated gonadotropin-releasing hormone deficiency. *Proc Natl Acad Sci USA* 2010;**107**:15140–4
43. Martin C, Balasubramanian R, Dwyer AA, Au MG, Sidis Y, Kaiser UB, Seminara SB, Pitteloud N, Zhou QY, Crowley WF Jr. The role of the prokineticin 2 pathway in human reproduction: evidence from the study of human and murine gene mutations. *Endocr Rev* 2011;**32**:225–46
44. Dodé C, Hardelin JP. Kallmann syndrome. *Eur J Hum Genet* 2009;**17**:139–46
45. Kishi T, Kitajima T, Tsunoka T, Okumura T, Ikeda M, Okochi T, Kinoshita Y, Kawashima K, Yamanouchi Y, Ozaki N, Iwata N. Possible association of prokineticin 2 receptor gene (PROKR2) with mood disorders in the Japanese population. *Neuromolecular Med* 2009;**11**:114–22
46. Abreu AP, Trarbach EB, de Castro M, Frade Costa EM, Versiani B, Matias Baptista MT, Garmes HM, Mendonça BB, Latronico AC. Loss-of-function mutations in the genes encoding prokineticin-2 or prokineticin receptor-2 cause autosomal recessive Kallmann syndrome. *J Clin Endocrinol Metab* 2008;**93**:4113–8
47. Ozata M, Bulur M, Bingol N, Beyhan Z, Corakci A, Bolu E, Gundogan MA. Daytime plasma melatonin levels in male hypogonadism. *J Clin Endocrinol Metab* 1996;**81**:1877–81

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