Meta Gene 7 (2016) 64–69

Contents lists available at ScienceDirect

Meta Gene

journal homepage: www.elsevier.com/locate/mgene

Clinical characteristics and follow-up of 5 young Chinese males with gonadotropin-releasing hormone deficiency caused by mutations in the *KAL1* gene

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ARTICLE INFO

Article history:
Received 26 August 2015
Revised 28 November 2015
Accepted 1 December 2015
Available online 3 December 2015

Keywords:
Isolated gonadotropin-releasing hormone deficiency
Kallmann syndrome
KAL1 mutations
Clinical phenotype

ABSTRACT

Isolated gonadotropin-releasing hormone (GnRH) deficiency (IGD) pertains to a group of genetic disorders consisting of anosmic hypogonadotropic hypogonadism (Kallmann syndrome, KS) and normosmic idiopathic hypogonadotropic hypogonadism (nIHH). KS is genetically heterogeneous. We hereby present 5 young male patients with GnRH deficiency caused by mutations in the *KAL1* gene. Their ages ranged from 9 months to 16 years. They were referred to our department for an endocrine consultation for micropenis. Hormone assays showed low circulating gonadotropins and testosterone. Molecular studies revealed *KAL1* mutations in all cases, three reported nonsense sequence variants in the *KAL1* gene were detected in 4 patients, respectively (c.784C > T (p.Arg 262*), c.1267C > T (p.Arg423*), and c.1270C > T (p.Arg424*)), and one patient harbored a novel hemizygous sequence variant [c.227G > A (p.Trp76*)]. Only one patient presented short stature without growth hormone deficiency and anosmia. Another patient had bilateral eyelid ptosis, trichiasis, and refractive error. This is the first report on the co-occurrence of a *KAL1* gene mutation and tent-like upper lip in four patients. All of our cases had normal olfactory bulbs and showed no renal agenesis, cleft lip/palate, and hearing impairment. These cases expand our knowledge of the phenotype associated with *KAL1* sequence variations, although the precise mechanism by which *KAL1* gene influences the development of this phenotype is still unknown.

1. Introduction

The hypothalamic–pituitary–gonadal (HPG) axis plays a crucial role in the development and progression of puberty. The pulsatile secretion of gonadotropin-releasing hormone (GnRH) into the hypophyseal-portal vessels controls the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the anterior pituitary gland, which then stimulates the gonads to produce sex steroids and gametes. Isolated GnRH deficiency (IGD) is a rare disorder with an estimated incidence of one case per 48,000 births. It is caused by a defect in the HPG axis, resulting in low levels of sex steroids and a delay or absence of puberty. IGD is divided into anosmic hypogonadotropic hypogonadism (Kallmann syndrome, KS) and normosmic idiopathic hypogonadotropic hypogonadism (nIHH) (Fathi and Luo, 2013). About 60% of patients with IGD present with anosmia or hyposmia (KS) by total or partial defects of the olfactory bulb due to the fact that the GnRH-releasing neurons are primarily derived from progenitor cells in the nasal compartment and migrate along the fibers derived from the olfactory system across the cribriform plate to the forebrain (Sykiotis et al., 2010).

KS is the most frequent cause of congenital hypogonadism. It is characterized by the association of optic atrophy, deafness, a cleft lip, renal malformations, cryptorchidism, and neurological anomalies. This disorder is a form of hypogonadotropic hypogonadism (HH), which is a condition affecting the production of hormones that facilitate sexual development. Males with hypogonadotropic hypogonadism are often born with an unusually small penis (micropenis) and undescended testes (cryptorchidism). They also present with delayed or incomplete puberty. KS and other syndromes causing a congenital deficiency of GnRH are characterized by low levels of LH and FSH with low levels of sexual steroids (testosterone and estradiol). In some sporadic cases of hypogonadotropic hypogonadism, etiologies that may disrupt the communication pathway between the hypothalamus and pituitary should be excluded. Mutations in at least 19 genes contribute to the molecular basis of IGD. The genetic defects are classified according to pathophysiology: defects in the neurodevelopmental pathway (*KAL1*, *NELF*, and

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http://dx.doi.org/10.1016/j.mgene.2015.12.001

SOX10), neuroendocrine pathway (*GNRH1*, *GNRHR*, *KISS1*, *KISS1R*, *TACR3*, and *TAC3*), and both (*PROKR2*, *PROK2*,*FGFR1*, *FGF8*, *CHD7*, and *HS6ST1*)(Ghervan and Young, 2014). Defects in the *KAL1* gene disrupt the migration of embryonic olfactory nerve cells. In at least one genetic form of the disease, deficiency in human gonadotropin results from impeding the migration of GnRH-releasing neurons from the olfactory placode to the hypothalamus during embryonic development. Disruption of olfactory nerve cells from extending to the olfactory bulb results in an impairment or loss of sense of smell. KS is genetically heterogeneous, and most KS cases occur sporadically (Hu et al., 2003). Given the success of these treatments, there is a growing need for genetic counseling. In addition, early diagnosis of KS would enable more timely management of affected children.

We hereby present 5 young males with GnRH deficiency caused by point variants in the *KAL1* gene. One patient presented with short stature and anosmia without growth hormone deficiency; another patient had bilateral eyelid ptosis, trichiasis, and refractive error. This is the first report on the co-occurrence of *KAL1* gene mutations and tent-like upper lip in four young male patients.

2. Subjects and methods

2.1. Clinical description of the patients

All the participants or parents gave their written informed consent for hormonal, morphological, and genetic analyses, which were conducted as part of routine patient care.

Case 1. A 5-month-old infant was referred to our department for an endocrine consultation for a micropenis. Fetal ultrasound examination performed at 12 and 30 weeks of gestational age showed no abnormalities. Initial hormonal evaluation of the gonadotropic axis showed an estradiol level of 43 pg/mL (N = 25–90). Basal LH and FSH levels were respectively 2.7 IU/L (normal range at the early follicular phase = 3.0–7.0) and 4.6 IU/L (normal range at the early follicular phase = 2.7–7.0). Other antepituitary functions (free thyroxine (FT4), thyroid stimulating hormone (TSH), peak cortisol) were normal, as was prolactin (Table 1). Magnetic resonance imaging (MRI) showed a normal antepituitary size and a normal pituitary stalk and normal olfactory bulbs.

Case 2. The patient was an 18-month-old male. Ultrasound examination during the 24th week of gestation showed a normal fetus. Normal delivery took place at the 39th week of gestation. At delivery, the newborn's weight and length were normal [weight: 3430 g (more than 50th percentile); length: 50 cm (more than 50th percentile)], except for the presentation of a micropenis (15 mm; −2SD) (Fig. 1A), undescended testes, and bilateral testicular hypoplasia (mean testicular volume: 0.33 m: (sonography); −3SD). The patient showed no other congenital anomalies (anosmia, hearing loss, missing teeth). Hormone assays conducted at age 18 months showed low circulating gonadotropins [FSH: 0.18 (normal range: 0.2–3.5); LH: 0.04 IU/L (0.5–6.5)], testosterone: 0.1 (normal range: 0.5–4.8), and low testicular peptide levels (AMH: 69 ng/mL; normal range: 80–154) (Table 1). Sense of smell was not assessed because of his young age. MRI performed at the age of 19 months revealed normal olfactory bulbs. His parent declined KAL1 carrier testing of the patient's younger brother.

Case 3. The patient was first referred to our clinic at the age of 15.6 years old. He was born by spontaneous delivery after an uneventful pregnancy (G1P1) at 35 weeks of preterm gestation to a nonconsanguineous and healthy 23-year-old mother and a 25-year-old father of Han ethnicity. The height of father and mother was 158 cm and 157 cm, respectively. At birth, his weight was 2450 g (about 3rd–10th percentile), length 48 cm (50th percentile), and his Apgar score was 7 and 10 at 1 and 5 min, respectively. Physical examination after birth revealed a micropenis (Fig. 1B); no other physical malformation was detected. Hearing tests conducted at 2 months of age showed normal hearing. His motor development was normal, with independent sitting and walking achieved on time (6 and 12 months, respectively). At the age of 12 months, the patient developed cryptorchidism of the left testis. He was treated with HCG (500 U i.m., twice a week), and after 4 weeks, the testes descended into the scrotums. Throughout the childhood, the patient manifested a slower growth rate. The patient was referred to local endocrinologist because of short stature at the age of 14. At that time, his height was 142.5 cm (less than 3rd percentile) and his weight was 30 kg (less than 3rd percentile). The case was referred for a conventional chromosomal analysis, which showed normal male karyotype (46, XY). His brain MRI scans was unremarkable. Bone age assessment based on left carpal X-ray was delayed in terms of chronological age. A stimulated growth hormone test was performed; the results showed that his growth hormone peak value was 16.2 ng/mL. The patient was diagnosed to have idiopathic short stature (ISS) with familial short stature. He was administered growth hormone (1.5 U/kg/d) for 3 months, and then his parents decided to stop the growth hormone therapy.

Physical examination showed a height of 146.2 cm, and weight of 32 kg at the age of 15.6 years old. The mean arterial pressure (MAP) of the patient was 115/65 mm Hg. He presented with a small penis, male phenotype at Tanner I stage, and no goiter. He did not tolerate exposure to different scents or aromas during childhood compared to the other family members. Due to the reported decreased sensitivity to smell, objective olfactory analyses were performed, the registration of olfactory-evoked potentials (OEPs) showed a considerable decrease in the amplitudes that were symmetrical on both sides. Endocrinological tests done at the age of 15.6 years old showed normal results for TSH, free triiodothyronine (FT3), FT4, prolactin (PRL), and Adrenocorticotropic Hormone (ACTH), However, FSH, LH and testosterone levels were lower than normal (Table 1). After being diagnosed with Kallmann Syndrome, the patient was first treated with HCG (2000 U i.m., twice a week for 4 weeks). A second HCG injection (2000 U i.m, twice a week for 4 weeks) was given 3 months later. Then he was started on hormone replacement with intramuscular injections of testosterone derivatives for 12 months after 6 months of being diagnosed. His secondary sexual characteristics developed satisfactorily after treatment for 1.5 years. His penis became larger and his pubic hair appeared. His height increased by near 12 cm within 1.5 years, and his height at the last follow-up measured 158 cm when he was 17.1 years old.

Case 4 and Case 5. Case 4 (14 years old) and Case 5 (16 years and 7 months old) were referred to our hospital based on the observation of a micropenis right after birth. Physical examination showed micropenis measuring <2.0 cm with no other malformations (Figs. 1C and D). No clinically apparent malformations of the midline were discovered in Case 4. Case 5 presented with bilateral eyelid ptosis, trichiasis, and refractive error from the age of 2-year-old till now. Testing of pulsatile LH secretion showed no significant pulses in Cases 4 and 5. Other antepituitary functions (FT4, FT3, TSH, ACTH, and peak cortisol) were normal, as was prolactin (Table 1). Testis B ultrasound results showed less than 1 mL of testes volume (normal volume: >10 mL). Olfactometry confirmed that the patient had normosmia. Brain MRI showed no agenesis of the olfactory bulbs, a normal antepituitary size, and a normal pituitary stalk. Audiometry showed normal hearing. Micropenis was not corrected by testosterone treatment in Cases 4 and 5. Combination therapy with recombinant human pituitary gonadotropins (LH and FSH) was therefore recommended, and was initiated 3 months ago for Case 4. This treatment resulted in a marked increase in testicle size (from 0.9 to 2.3 mL upon sonography) and penis length (from 2 to 4 cm). Case 5 refused to receive gonadotropin therapy.

Patients with KS often showed a cleft lip and cleft palate. However, our cases did not present a cleft lip/palate. The tent-like lips were evident in the four cases, their upper lips were thicker than normal, and the angles of their mouths were oriented downward (Fig. 1).

Table 1
Summary of clinical findings in Kallmann syndrome patients.

Patients	NO.1	NO.2	NO.3	NO.4	NO.5
Sex	Male	Male	Male	Male	Male
Age of first administration	5 months	18 months	15.6 years	14 years	16.6 years
Height (cm) (percentile) /weight (kg) (percentile)	67(50th) /7.5(50th)	83(50th–75th) /12.5(50th–75th)	146.2(1st) /32(1st)	153(25th–50th) /42(25th–50th)	167.8(25th–50th) /67(75th–95th)
Sense of smell	No data available	No data available	Hyposmia	Normosmic	Normosmic
Sexual maturation* (Tanner stage)	G1P1B1	G1P1B1	G1P1B1	G1P1B1	G1P1B1
Testicular volume and cryptorchidism** right/left	6 × 4 × 5 mm/8 × 5 × 6 mm	10 × 5 × 5 mm/7 × 4 × 4 mm **	14 × 5 × 7 mm/13 × 9 × 5 mm**;	No data available	14 × 5 × 7 mm; 13 × 9 × 5 mm;
Renal malformation, dental agenesis, synkinesis, short fourth metacarpal, hearing loss.	No	No	No	No	No
Other signs	No	No	No	No	Bilateral eyelid ptosis, trichiasis and refractive error
			Summary of laboratorial tests in the first assessment		
FSH (1.24–7.8 IU/L)	<0.03	2.11	<0.3	0.9	0.3
LH (6–23 IU/L)	<0.01	0.44	0.07	0.5	0.06
Testosterone ng/nl (2.41–8.27)	<0.1	<0.01	0.53	0.07	0.31
TSH, ACTH,COR,	Normal	Normal	Normal	No	Normal
MRI brain	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL
Karyotype	46, XY	46, XY	46, XY	46, XY	46, XY
KAL1 gene mutation (NM000216.2)	c.1270C > T, p.Arg424*	c.227G > A, p.Trp76*	c.1267C > T, p. Arg 423*	c.784C > T, p.Arg 262*	c.1270C > T, p. Arg 424*
Follow-up	He was given HCG and Dihydrotestoster and then went back to hometown.	HCG 500 U; im q5d 10 times, His penis became enlarged. After 2 years, his little brother was born and had similar small penis. The parents declined to do genetic analysis of the younger brother till now.	He was treated with HCG (2000 U im, twice a week for 4 weeks). A second HCG injection treatment was given 3 months later. Then he was started with testosterone derivatives (im) for 12 months, the total duration (HCG + testosterone derivatives) till the last follow up was 18 months. His penis became larger. His pubic hair appeared, his height was 158 cm at the last follow-up when he was 17.1 years old.	He was treated with HCG (1500 U im, twice a week), each treatment duration continued to 5 weeks, and he had finished 3 durations.	Declined to answer

Notes: *G, external genitalia; P, pubic hair; B, breast development; ** cryptorchidism.

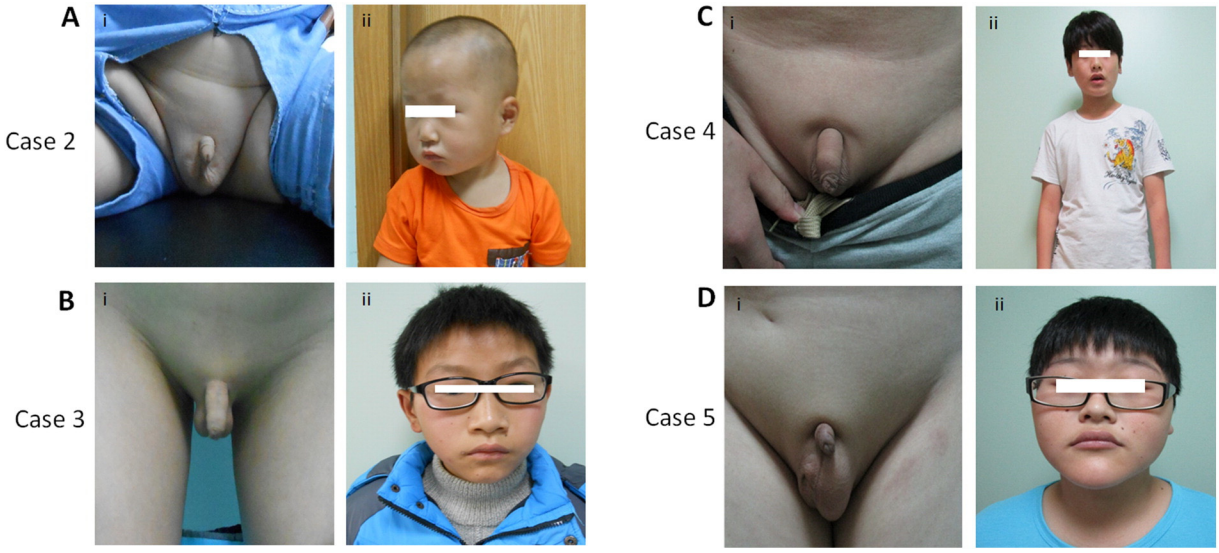

Fig. 1. Penis and testis abnormalities. A–D. Cases 2–5have micropenis. Small testis, and thicker upper lip. The parents of Case 1 refused to provide photos of their child.

2.2. Sanger sequencing of the KAL1 gene

The genomic DNA of the patient and parents was isolated from peripheral blood samples using a QIAamp Blood DNA Mini kit® (Qiagen GMBH, Hilden, Germany).All of the exons and exon–intron boundaries of the *KAL1* (GenBank accession number: NM_000216.2) gene were amplified by PCR (TaKaRa, Dalian, China) using primers listed in Table 2. The primers were designed using the UCSC ExonPrimer online software (http://genome.ucsc.edu/index.html). The products were examined on a 1% agarose gel and purified with a QIAquick Gel Extraction Kit (Qiagen GMBH, Hilden, Germany). The resulting DNA was sequenced via the ABI3730XL sequencer (Applied Biosystems, Foster City, CA, U.S.) with both forward and backward primers. The sequence data were analyzed with Mutation Surveyor DNA Variant Analysis Software (SoftGenetics, PA, USA).

3. Results

3.1. Sequencing of KAL1 gene

All the patients were evaluated by direct nucleotide sequencing. Cases 1 and 5 harbored a documented hemizygous nonsense mutation c.1270C > T (p.Arg424*) (Sato et al., 2004), Case 3 had another classical nonsense mutation, c.1267C > T (p.Arg423*) (Hardelin et al., 1993r), and Case 4 carried a reported nonsense mutation, c.784C > T (p.Arg262*) (Söderlund et al., 2002). On the other hand, in Case 2, we found a novel mutation in the *KAL1* gene that contributed to the patient's condition. The patient was hemizygous for a mutation designated as c.227G > A in exon 2. Sequence analysis indicated that the c.227G > A mutation at codon 76 of exon 2 was a nonsense mutation that led to the early termination of the protein translation process (p.Trp76*). Targeted testing showed that his mother was heterozygous for this same sequence variant (Fig. 2). All detected sequence variants in the *KAL1* gene of these cases were nonsense.

Table 2
Primers used to amplify exons and the boundary sequences of *KAL1*.

Exon	Primer-forward (5′-3′)	Primer-reverse(5′-3′)	Product size
1	gattgaactttccggctcag	gagttggggcaagatgtctc	554
2	tcattggaagggaaggacag	gcatcttgatggcagtggta	454
3	ggtccgcgttctgtaatgat	taatgcaagcagtgggtagc	454
4	aaggtttggtggggaaaaat	ctgccccatgtcgagttaat	525
5	cctgtatggagaggccacttt	gtttccgagcacattcgttt	522
6	aaatagcccaagctcttgtca	aaaacagcaaagccacctgt	501
7	actatgttgcccaggctgtc	cccctgcattctgtgaactt	595
8	gcacctggcctgaagtttat	acaacaccaaaattgcacct	558
9	cggtacctccgttggaaata	agccctctgggaaagaaatg	531
10	atctcacctccttttggctca	agtgcaatggtgtgaatgga	430
11	agccatgggagtgtttcaca	cacattgggccatcataaca	509
12	tccccaaaagactggaagaa	gagttggctctagccatcaaa	690
13	gagaaccccacaaatgaga	ggagggagggagaaaaagaa	555
14	tgggaagacatcaaagaggaa	gtgctccaaattcagggaaa	590

4. Discussion

HH is characterized by a defective development of the GnRH pulse generator (Grumbach, 2005). KS and normosmic HH (nHH) share anatomical and genetic etiologies with common features. Mutations in genes such as *FGFR1*, *FGF8*, *PROK2*, and *PROKR2* have been shown both in KS and in nHH, confirming that KS and nHH are two different phenotypes of the same pathology (Lewkowitz-Shpuntoff et al., 2012). Mutations in several genes/pathways affecting GnRH neuronal migration (neurodevelopmental genes) have been identified in KS patients.

The *KAL1* gene is one of the main genes that cause KS. We have described five cases of HH in which the diagnoses were strongly suspected during childhood. The molecular identification of mutations in the *KAL1* gene corroborated the diagnosis. To date, nearly 161 mutations have been identified in the *KAL1* gene, including missense/nonsense mutations, splicing mutations, small insertions/deletions, gross deletions, and complex rearrangements (data from HGMD). The proportion of missense/nonsense mutations accounted for nearly 40% of the total number of reported sequence variants. Sequencing all exons of *KAL1* gene of all our patients was performed. In our 5 patients, 4 variations were identified, and three variants were causative for KS in the previous studies (p.Arg424*, p.Arg423*, p.Arg262*) (Sato et al., 2004; Hardelin et al., 1993r; Söderlund et al., 2002). In Case 2, we identified a novel hemizygous mutation in exon 2, namely, c.227G > A (p.Trp76*) that is predicted to result in a truncated protein that results from the formation of a premature stop codon or nonsense-mediated RNA decay (NMD). Interestingly, all the detected variations were nonsense.

Only less than 20% of KS patients display a mutation in *KAL1*, indicating that the *KAL1* gene may be not the only disease causative gene. Hemizygous sequence variants in the *KAL1* gene, p.Arg191* and p.Cys13*; a heterozygous *FGFR1* variant, p.Arg250Trp; and a homozygous *PROKR2* variant, p.Tyr113His were previously detected in five out of seven Chinese KS pedigrees (Gu et al., 2015). Classical monogenic

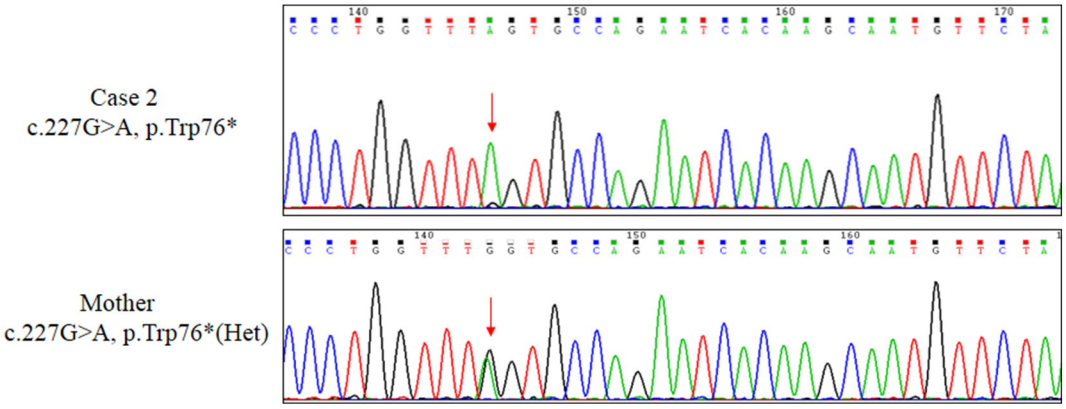

Fig. 2. A novel mutation was identified in the *KAL1* gene of Case 2. Sequences show a hemizygous nonsense mutation, c.227G > A (p.Trp76*) in exon 2 of the *KAL1* gene of the patient, whereas his mother was heterozygous for the same mutation.

inheritance does not explain the full range of genetic inheritance patterns for KS and HH, which in turn suggests that KS is not only a monogenic Mendelian disease, but rather a digenic or potentially oligogenic condition.

KS patients with *KAL1* mutations may have a variety of associated disorders of neurological or urogenital nature, and the most frequent are mirror movements, renal anomalies, neurogenic deafness, midline anomalies (cleft lip or palate), skeletal anomalies of the hands or feet, dental abnormalities, deafness, and schizophrenia (Verhoeven et al., 2013). A previous epidemiological study has reported that KS patients present a wide range of clinical features (Bonomi et al., 2012). Four of our cases showed thicker upper lips without a cleft lip/palate. All of our cases showed no renal agenesis. This was in agreement with the findings of Costa-Barbosa et al., who observed that renal agenesis and cleft lip/palate were not statistically significant phenotypic predictors (Costa-Barbosa et al., 2013). Furthermore, the present study did not detect mirror movements in the KS patients. Only one patient had bilateral eyelid ptosis. Synkinesia is often thought to be observed in *KAL1* genotypes with different prevalence rates ranging from 4.1% to 31% (Bonomi et al., 2012; Maione et al., 2013). No synkinesia was detected in our cases. These cases expand our knowledge of the phenotype caused by *KAL1* mutations, but the precise mechanism by which the *KAL1* gene contributes to this phenotype is still unknown.

Approximately 40% of patients with idiopathic HH have normal sense of smell (Fathi and Luo, 2013; Lewkowitz-Shpuntoff et al., 2012). However, only one adolescent had hyposmia, there were 2 patients who were too young to participate in the collection of smell data in our patients. MRI of olfactory structures of our HH patients confirmed normal olfactory bulbs. Koenigkam-Santos et al. showed that the olfactory bulb and sulcus aplasia were the most common findings in KS patients and demonstrated agreement between MRI findings and the smell test, especially the presence of bulb aplasia and anosmia (Koenigkam-Santos et al., 2011). However, there are also other reports that describe normosmic subjects with hypoplastic left olfactory bulbs (Hardelin et al., 1993r). Our MRI findings and the results of our smell test were not in agreement with above reports. KS subjects with olfactory bulb agenesis on MRI or harbored *KAL1* mutations had the most significant changes in olfactory fossa measurements and angles (Maione et al., 2013). The precise mechanism underlying this disorder is unclear; however, oligogenicity might be a plausible reason.

The prevalence of cryptorchidism was 35% in KS patients and 10% in nHH patients (Ghervan and Young, 2014). The presence of micropenis and/or cryptorchidism strongly argues for HH. Costa-Barbosa et al. performed a detailed comparative phenotypic evaluation between a group of KS subjects harboring known rare sequence variations (RSVs) in 8 genes (*KAL1*, *NELF*, *CHD7*, *HS6ST1*, *FGF8*/*FGFR1*, or *PROK2*/*PROKR2*) and a cohort of KS patients without RSVs, They found testicular volumes of male KS subjects with *KAL1* RSVs were smaller than KS patients without RSVs ((1.5 ± 0.1 mL vs 3.7 ± 0.3 mL, $P < .05$)(Costa-Barbosa et al., 2013). Our cases had similar smaller-sized testes, and we found cryptorchidism in two patients.

The selection for the appropriate treatment for KS should therefore be individualized. Treatment protocols are basically a choice between androgen replacement to virilise, gonadotropin therapy to induce fertility, and luteinizing hormone releasing hormone (LHRH) analog administration for most physiological replacements. Considering the age of the patients, our therapeutic aim was to restore normal development of the genitals and secondary sex characteristics. In KS with cryptorchidism, chorionic gonadotropin therapy results in the elimination of cryptorchidism without surgery. An attempt to "milk" the testes downwards should also be conducted. The cryptorchidism in Cases 2 and 3 was resolved after HCG therapy. Cases 2 and 3 served as examples where meticulous clinical examination saved the patients from unnecessary surgical intervention. It is also appropriate to treat HH patients with micropenis with androgens to enlarge the penis into that of the normal childhood range. Our experience with testosterone replacement in these patients was overwhelming in this regard.

5. Conclusions

In the present study, we identified four mutations in the *KAL1* gene in five KS patients. Three variations had been reported in the literature that could lead to KS. In addition, we identified a novel nonsense mutation in one patient, namely, c.227G > A, which is predicted to result in a premature stop codon (p.Trp76*). This finding could be used in the development of a database of *KAL1* gene mutations that disturb the development and function of the hypothalamic–pituitary–gonadal (HPG) axis. Unidentified gene mutations could partially account for the phenotypes of our patients with tent-like lips and eyelid ptosis.

Conflict of interest statement

The authors declare no conflict of interest related to this study.

Acknowledgments

This research was supported by the Shanghai Municipal Science and Technology Commission (Grant No. 12411950402). We are deeply grateful to the patients and their families for participating in this study.

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