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## Original Article

# Molecular analysis of *KAL-1* in a series of Kallmann syndrome and normosmic idiopathic hypogonadotropic hypogonadism patients from Northwestern China

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## Abstract

We conducted an analysis of the Kallmann syndrome 1 (KAL-1) genotype in 17 patients with Kallmann syndrome (KS), 9 patients with normosmic idiopathic hypogonadotropic hypogonadism (nIHH) and 20 age-matched normal men in Northwestern China.  To do this, we used multiplex PCR analysis with exon-flanking primers and automated sequencing techniques with peripheral blood DNA samples.  Intragenic deletions were found at the *KAL-1* locus in two KS patients.  One case with an atrial septal defect exhibited an intragenic deletion of exon 6.  Another KS patient with cryptorchidism had intragenic deletions of exons 5 and 6.  For the nIHH patients, no abnormalities were observed in the exonic and flanking sequences of *KAL-1*.  This report describes two intragenic deletions of *KAL-1* in two KS patients and suggests that *KAL-1* deletion might be more prevalent in KS patients with other congenital organ abnormalities than those described previously in other series from Northwestern China.

## 1   Introduction

Idiopathic hypogonadotropic hypogonadism (IHH) is a failure of sexual development or reproductive function due to abnormalities in the pituitary secretion of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH).  These abnormalities can result from deficiencies in the production of gonadotropin-releasing hormone (GnRH) by the hypothalamus or by defects in GnRH function at the pituitary level.  IHH can be associated with anosmia in Kallmann Syndrome (KS) or can be apparently isolated, without anosmia (normosmic IHH, nIHH).  Although the vast majority of KS and nIHH cases are sporadic, recessive X-linked, autosomal dominant and autosomal recessive modes of inheritance have been described [1–3].

The Kallmann syndrome 1 (*KAL-1*), a candidate gene for X-linked KS, was cloned in 1991 by Legouis *et al.* [4].  The discovery of the *KAL-1* has led to a pathophysiological model correlating GnRH deficiency with defective migration of GnRH neurons due to abnormal olfactory bulb development in X-linked KS.

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This gene comprises 14 exons spanning approximately 210 kb on Xp22.3, escapes X-inactivation and encodes a protein (anosmin) that shares homology with molecules involved in neuronal migration and axonal pathfinding. *KAL-1* encodes an extracellular matrix glycoprotein of approximately 100 kDa termed anosmin-1, and is expressed in multiple embryonic tissues and organs, including the primitive olfactory bulb and kidney [5, 6]. In addition, it has a nonfunctional homolog at Yq11.2. Numerous *KAL-1* gene mutations responsible for KS have been reported in X-linked familial and sporadic cases [5–7].

Here, we report a molecular analysis of the *KAL-1* gene in 17 unrelated males with KS and 9 males with nIHH in Northwestern China.

## 2 Materials and methods

### 2.1 Patients

Between March 2006 and November 2008, 17 unrelated males with KS were diagnosed according to the clinical signs and symptoms of hypogonadism.

The olfactory bulb structures of KS patients were examined by brain magnetic resonance imaging. The other nine unrelated males with IHH were identified based on clinical signs and symptoms of hypogonadism, prepubertal testosterone (< 1.6 nmol L$^{-1}$), low or inappropriately normal gonadotropin levels, normal baseline and reserve testing of other anterior pituitary hormones and normal radiological imaging of the hypothalamic–pituitary region. Anosmia/hyposmia was evaluated using the olfactory test described by Davidson and Murphy [8]. All study participants came from Northwestern China, and were diagnosed at the First Hospital of Xi'an Jiaotong University (Xi'an, China). The control group comprised 20 healthy volunteers. The protocol was approved by the Ethics Committee of the First Hospital of Xi'an Jiaotong University, and all participants provided a written informed consent.

The ages of the 26 participants ranged from 13 to 37 years. All of them had had uneventful pregnancies, and anosmia or hyposmia had been present since early childhood. In adolescence, there was an absence of subnormal pubertal development. On physical examination, certain clinical characteristics were found in all individuals: normal stature, infantile genitalia and scant pubic hair. The high-resolution G-banded karyotype was 46, XY, and computed axial tomography of the hypothalamic-pituitary region did not show any

disorder in any of the patients.

### 2.2 Blood sampling and DNA extraction

Blood samples (3 mL taken into EDTA by venipuncture) were obtained from all subjects. Immediately after collection, whole blood was stored at −80ºC until use. Genomic DNA for PCR analysis was isolated from thawed whole blood using Axypre, a whole blood genomic DNA miniprep kit (Axygen biosciences, Union City, CA, USA).

### 2.3 Mutation analysis

*KAL-1* genotypes were identified by multiplex PCR. Primer sequences corresponding to the flanking regions of the *KAL-1* exons, sizes of the amplified products and amplification conditions were as reported by Hardelin *et al.* [9]. For exons 2–13 of the *KAL-1*, PCR products corresponded to a segment of DNA spanning the coding region and the adjacent splice site junctions. For exons 1 and 14 of the *KAL-1*, which contain the 5′ and the 3′ untranslated regions, respectively, only the coding fragment was amplified using an intronic primer and a second primer annealing to the corresponding untranslated region [10, 11]. PCR was performed in a 25-µL reaction buffer containing 200 µmol L$^{-1}$dNTPs, 1.5 mmol L$^{-1}$ MgCl$_2$, 10 pmol of each primer, approximately 200 ng of template DNA and 2U of thermostable Taq DNA polymerase (MBI, Vilnius, Lithuania). After a 5-min pretreatment at 95ºC, the specific conditions used for all PCR amplifications (35 cycles) were denaturation for 45 seconds at 94ºC; annealing for 45 seconds at 63°C for exon 1, 57ºC for exon 2 and at 55ºC for exons 3–14 of the *KAL-1*; fragment extension for 45 seconds at 72ºC; and a final elongation step for 7 min at 72ºC. For the amplification of exons 1, 4, 7 and 14 of the *KAL-1*, 10% dimethylsulfoxide was included. PCR products were electrophoresed on a 2% agarose gel stained with ethidium bromide to verify the correct size of the expected fragments. Subsequently, all PCR fragments were purified by Nucleospin Extract (Machery-Nagel, DÜren, Germany) and sequenced for both DNA strands using the BigDye terminator cycle sequencing ready reaction kit (Sangon, Shanghai, China) in an ABI 3730 Automated DNA Sequencer (Sangon). Each potential mutation or deletion was confirmed by a second independent PCR amplification and sequencing. The primers for *β-actin* were 5′-ACTCCCCATCCCAAGACC-3′ and 5′-CCTTAATGTCACGCACGAT-3′. A 400-bp fragment of the *β-actin* gene was amplified in all samples as

an internal standard.

## 2.4 Statistical analysis

Exact 95% confidence intervals (CIs) were calculated as suggested by Agresti and Coull [12] and Brown *et al*. [13]. This approach can be substantiated on the grounds that it is the exact algebraic counterpart to the large-sample hypothesis test and that its worth does not strongly depend on the sample size. All analyses were performed using SPSS version 13.0 statistical software (SPSS, Chicago, IL, USA).

## 3 Results

Hypogonadotropic hypogonadism was documented in all patients. Basal testosterone concentrations were consistently in the prepubertal range (1.6 nmol L$^{-1}$). Meanwhile, gonadotropin levels were always below the normal adult male range (FSH, 3.0–30.0 IU/L; LH, 5.0–28.0 IU/L) and normal prepubertal male range (FSH, 1.0–9.0 IU/L; LH, 3.0–7.0 IU/L).

### 3.1 KS patients

Genetic defects were found in only two patients out of 17 participants with KS ($P = 0.118$; 95% CI, 0.0353–0.2710). One KS patient with an atrial septal defect had an intragenic deletion of exon 6, whereas the remaining exons of the gene amplified normally (Figure 1). Another KS patient with cryptorchidism had intragenic deletion of exons 5 and 6, whereas the other exons of the gene amplified normally (Figure 2). In the remaining 15 KS cases, no mutations or deletion were detected in *KAL-1*.
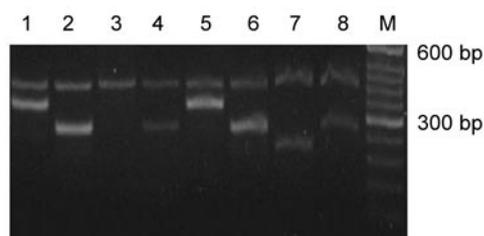
### 3.2 nIHH patients

No abnormalities were found in *KAL-1* among the nIHH patients.

## 4 Discussion

Various types of *KAL-1* abnormalities have been reported in patients with KS. These include missense and nonsense mutations, splice site mutations, intragenic deletions and submicroscopic chromosomal deletions involving the entire *KAL-1*. To date, a search for mutations in the *KAL-1* has been performed in various groups of patients with X-linked KS or sporadic KS. Defects have been found to be widely distributed throughout this gene in ~14% and 11% of patients with familial X-linked KS and males with sporadic KS, respectively [14, 15].

In our series, only two complete intragenic deletions within the coding region of the *KAL-1* were identified in two KS patients. One patient, who had an atrial septal defect, showed an intragenic deletion of exon 6. The other, who had cryptorchidism, showed intragenic deletion of exons 5 and 6. No mutations or deletions were detected in the *KAL-1* among the remaining KS patients from Northwestern China. In fact, Oliveira [16] concluded that autosomal genes are clearly responsible for the majority of both familial and sporadic KS cases. To date, only loss-of-function mutations in the *FGFR1* gene, located at 8p11.2, have been associated with KS [17]. Therefore, the possibility of the existence of a second X-linked gene causing KS or of molecular alterations located in the regulatory regions of the *KAL-1*



Figure 1. Electrophoresis pattern for exons 4–7 of *KAL-1* using DNA from the KS patient with an atrial septal defect (1–4) and a normal control (5–8) based on multiplex PCR. A 400-bp fragment of the *β-actin* gene was used as an internal standard using primers (5′-ACTCCCCATCCCAAGACC-3′ and 5′-CCTTAATGTCACGCA CGAT-3′). M: 50 bp DNA ladder (TaKaRa Bio, Otsu, Japan); deletion of exon 6 is observed in the patient's DNA.



Figure 2. Electrophoresis pattern for exons 4–7 of *KAL-1* using DNA from the KS patient with cryptorchidism (1–4) and a normal control (5–8) based on multiplex PCR. A 400-bp fragment of the *β-actin* gene was used as an internal standard using primers (5′-ACTCCCCATCCCAAGACC-3′ and 5′-CCTTAATGTCACGCACGAT-3′). M: 50 bp DNA ladder (TaKaRa Bio, Otsu, Japan); deletion of exons 5–6 is observed in the patient's DNA.

promoter, in the untranslated regions of exons 1 and 14 or within introns creating new splicing sites, cannot be excluded.

A deletion of exons in *KAL-1* has been reported previously. These mutations involved entire *KAL-1* deletions and interstitial deletions encoding the fibronectin III domain (FNIII)-like repeats [18]. As the mutation reported by Gu *et al.* [19] causes a premature stop codon, it can be considered to be an extended deletion rather than a mutation that causes a defect in the functionality of the protein. This deletion comprises only part (exon 5) of the coding region of the first FNIII-like repeat of the anosmin-1 protein. The rest of the deletion comprises part of the conserved cysteine-rich N-terminal region, which corresponds to the whey acidic protein motif. In this region, several mutations have been reported to date. The patient who presented with this deletion exhibited a severe phenotype that included facial asymmetry, abnormal eye movements, bimanual synkinesis and the absence of the right kidney, in addition to other congenital malformations [20, 21].

At present, we cannot establish a correlation between the location and number of polymorphisms, and the occurrence of the mutation or the severity of the disease in our patients. Five causal genes have been identified to date for KS, namely (by chronological order of discovery), *KAL-1*, *FGFR1*, *PROKR2* and *PROK2*, and *FGF8* [22]. Advanced analysis has been done for the remaining KS patients in our recent work.

No mutations or deletions were detected in *KAL-1* among the nIHH patients in our study. Nearly all mutations have been identified in patients with both HH and olfactory dysfunction of variable extent. In particular, there has been no report documenting a *KAL-1* mutation-positive patient with normal olfactory function, although two males with apparently normal gonadal function have been identified through familial studies of their brothers with the typical KS phenotype [23, 24]. This study supports this observation as well.

New promising candidate loci for human HH include genes with potential influence on the migration of GnRH neurons. GnRH neurons arise in the medial olfactory placode epithelium, migrating along the nasal septum and across the cribiform plate to reach the hypothalamus [25]. Recently, two genes, nasal embryonic LH-releasing hormone factor (NELF) and early B-cell factor 2 (EBF2), were implicated in this process. NELF was first isolated in mouse, and its expression patterns in olfactory axons and GnRH cells during development

are consistent with its proposed function as a migratory factor for GnRH neurons [26, 27]. Therefore, these genes are good candidate genes to underlie HH, and HH patients should be studied in the future.

In conclusion, this report includes descriptions of two intragenic deletions in the *KAL-1* in two KS patients and suggests that deletions in *KAL-1* might be more prevalent in Chinese KS patients with other congenital organ abnormalities than that previously described in other series from Northwestern China. More candidate genes for HH and a large number of HH patients should be identified for future studies.

## Acknowledgment

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