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Short Communication

Similar interstitial deletions of the *KAL-1* gene in two Brazilian families with X-linked Kallmann Syndrome

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Abstract

Mutations in the *KAL-1* gene localized at Xp22.3 have been shown to be responsible for the X-linked Kallmann syndrome (KS), a disorder characterized by the association of hypogonadotropic hypogonadism and anosmia. In this paper, we describe the investigation of two families with X-linked KS, in which similar interstitial deletions spanning exons 5 to 10 of the *KAL-1* gene were identified. The presence of interspersed repetitive DNA sequences within the *KAL-1* gene might have predisposed to this type of mutation.

Key words: X-linked KS, intragenic deletions, *KAL-1* gene, PCR, phenotypic variability.

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Introduction

Kallmann syndrome (KS) is a disorder defined by the association of hypogonadotropic hypogonadism with anosmia or hyposmia (Kallmann *et al.*, 1944) that is due to a neuronal migration defect involving both the gonadotropin-releasing hormone (GnRH) and the olfactory-producing neurons (Schwanzel-Fukuda *et al*., 1989). Although the majority of KS cases are sporadic, segregation analysis in familial cases revealed X-linked, as well as autosomal recessive and autosomal dominant modes of transmission, indicating genetic heterogeneity (Hermanussen and Sippell, 1985; Chaussain *et al.*, 1988; Waldstreicher *et al.*, 1996).

The *KAL-1* gene responsible for the X-linked form of KS (MIM 308700) was mapped to Xp22.3 by linkage analysis and deletion studies (Ballabio *et al.*, 1989; Petit *et al.*, 1990) and was subsequently isolated by different positional cloning strategies (Franco *et al.*, 1991; Legouis *et al.*, 1991). This gene consists of 14 exons spanning approximately 210 kb (Del Castillo *et al*., 1992) and encodes a protein, anosmin-1, that contains four fibronectin type III repeats and a putative protease inhibitor domain, structural features found in several cell and substrate adhesion molecules (Franco *et al.*, 1991; Legouis *et al.*, 1991). This raises the possibility that anosmin-1 could function in the migration of GnRH-secreting neurons and olfactory axons, during embryonic development (Franco *et al.*, 1991; Legouis *et al.*, 1991; Rugarli *et al.*, 1996).

In addition to hypogonadism and anosmia, a variety of other defects occur in patients with X-linked KS. They include neurological deficits such as bimanual synkinesis (Kallmann *et al.,* 1944; Sunohara *et al.*, 1986), cerebellar dysfunction, nystagmus (Sunohara *et al.,* 1986; Schwankhaus *et al.,* 1989), mental retardation (Kallmann *et al.,* 1944; Wegenke *et al.,* 1975), hearing loss (White *et al.,* 1983), and somatic defects, such as unilateral renal agenesis, *pes cavus* and high-arched palate (White *et al.,*

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1983; Schwankhaus *et al.,* 1989; Zenteno *et al.,* 1999). Some of these symptoms (e.g. synkinesis and renal agenesis) are more frequently observed in patients with X-linked KS, indicating that these features represent pleiotropic effects of mutations in the *KAL-1* gene (Hardelin *et al.,* 1993) and suggesting a more generalized role of this gene during development, involving the nervous system and non-neuronal tissues (Hardelin *et al.*, 1992,1993).

In this paper, we report two unrelated Brazilian families with X-linked KS and similar interstitial deletions spanning exons 5 to 10 of the *KAL-1* gene.

Subjects and Methods

Patient reports

We studied two unrelated families with KS-affected males. In family 1 (Figure 1A), from Arapiraca, state of Alagoas, northeastern Brazil, five males were clinically evaluated (II-6, III-4, III-7, III-12, and IV-6), and in family 2 (Figure 1B), from Campinas, state of São Paulo, southeastern Brazil, two affected brothers (II-4 and II-6) were examined. In family 1, X-linked inheritance of KS was assumed, based on the presence of asymptomatic female carriers, the presence of at least two affected males in the maternal family or among male siblings, the absence of affected females and the absence of male-to-male transmission. In family 2, X-linked inheritance of KS was suspected because one of the affected patients had a renal abnormality.

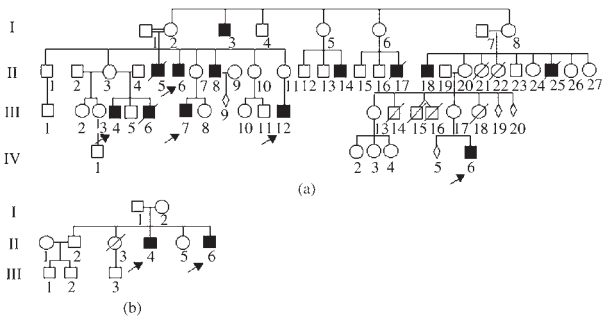

Figure 1 - Heredograms of (**a**) family 1 and (**b**) family 2 with X-linked KS. Affected males. Arrows indicate patients clinically examined in this study.

Laboratory testing was performed in all patients, revealing low levels of testosterone, FSH (follicle-stimulating hormone) and LH (luteinizing hormone), consistent with hypogonadism. Formal olfactory testing was not performed, but anosmia was detected in the individuals II-6, III-4 (Family 1) and II-4 (Family 2) by direct inquiry. Clinical evaluation details of all patients are given in Table 1. The protocol was approved by the Ethics Committee of the State University of Campinas (UNICAMP) School of Medicine. Informed consent was obtained from all subjects included in this study.

Molecular analysis of the *KAL-1* gene

Genomic DNA of individuals II-11, III-4 and III-17 (Family 1), I-2, II-4 and II-6 (Family 2) was extracted from peripheral leukocytes, using the phenol/chloroform method. The 14 coding exons of the *KAL-1* gene were amplified by PCR from DNA of the affected males, patients III-4 (Family 1), II-4 and II-6 (Family 2). The sequences of primers and the size of the amplified products were as previously described by Hardelin *et al.* (1993). PCR amplifications were performed in 50 µL reaction mixes containing 200-500 ng of genomic DNA, 0.2mM dNTPs, 1.5 mM of $MgCl_2$, 0.6 pmol of each of the primers, 1x PCR buffer, and 1 U Taq polymerase. After a first denaturation step (10 min,

Table 1 - Clinical features of evaluated patients from families 1 and 2 with X-linked KS.

Patient	Age	Anosmia/ hyposmia[a]	Hypogonadotropic hypogonadism	Micropenis/ Cryptorchidism	Bimanual synkinesis	Renal abnormality[b]	Other characteristics	MRI of olfactory structures
II-6*	32 y	Yes	Yes	Yes	Yes	No	High-arched palate Genu valgum Eunuchoid habitus	Not performed
III-4*	20 y	Yes	Yes	Yes	Yes	Agenesis at left side	High-arched palate Genu valgum Eunuchoid habitus	Bilateral aplasia of sulcus and rudimentary bulbs
III-7*	6 y	?	Yes	Yes	Yes	No	High-arched palate	Not performed
III-12*	4 mo	?	Yes	Yes	?	No		Not performed
IV-6*	4 mo	?	Yes	Yes	?	No	Epicanthal folds Bilateral ptosis of eyelids Asymmetric ears	Not performed
II-4#	35 y	Yes	Yes	Yes	No	No	Gynecomastia	Not performed
II-6#	28 y	No	Yes	Yes	No	Horseshoe kidney on right side	Mental retardation	Not performed

*patients from family 1; # patients from family 2. y = year. mo = months. [a]detected by direct inquiry. [b]determined by abdominal ultrasonography. ? features not evaluated due to patient age.

95 °C), 30 PCR amplification cycles of 1 min at 95 °C, 1 min at 57 °C (except for exon 1, 60 °C), and 2 min at 72 °C were carried out, followed by a final extension of 10 min at 72 °C. The PCR products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide and photographed. If no amplification product of *KAL-1* exons was detected, PCR was repeated with the addition of primers SRY1 and SRY4 for the *SRY* gene (Assumpção *et al*., 2002) as internal positive control.

Comparative duplex PCR for carrier female status determination was performed, following the procedure described by Nagata *et al.* (2000), in subjects II-11 and III-17 (Family 1) and I-2 (Family 2), mothers of affected males. The reactions included primers for exon 20 of the autosomal *NPC1* gene as internal standard to quantify the dosage of *KAL-1* exon 7 PCR products. The amplified products were analyzed by electrophoresis on 2.6% agarose gel stained with ethidium bromide, and the gel image was captured with a Kodak Digital Science DC120 camera. The intensity of each band was measured using 1D IMAGE ANALYSIS software, and the gene dosage was evaluated by the *KAL-1/NPC1* ratio. Samples from normal males (n = 10) and females (n = 10) were also run as controls. The expected values were 0.0, 0.5, and 1.0 for affected males, carrier females and normal males, and normal females, respectively.

Results

The genomic DNA from patients III-4 (Family 1), II-4 and II-6 (Family 2) did not yield PCR products from exon 5 to exon 10, while the remaining exons were amplified. These findings indicate the presence of an interstitial deletion encompassing exons 5-10 in the *KAL-1* gene of these patients (Figure 2).

By duplex PCR, the status of obligatory carrier females was confirmed in subjects II-11 and III-17 (Family 1) and I-2 (Family 2). In these females, the *KAL-1*/*NPC1* ratios were 0.53, 0.54 and 0.47, respectively, indicating the presence of only one normal *KAL-1* allele. In the control subjects, the *KAL-1*/*NPC1* ratios were next to 0.5 for males and 1.0 for females (Figure 3).

Discussion

We describe here two interstitial deletions of the *KAL-1* gene encompassing exons 5-10 in two unrelated and geographically distant Brazilian families. Although molecular analysis by PCR was performed in only one patient from Family 1, the causative role of the *KAL-1* gene for KS in this family was confirmed by the demonstration of the carrier status of the mothers of patients III-12 and IV-6. Additional symptoms found in the five clinically examined affected males of this family included bimanual synkinesis (3/5), renal agenesis (1/5), high-arched palate (3/5), and dysmorphic facial features (1/5). In Family 2, molecular investigation of the *KAL-1* gene was performed in both patients, and the carrier status of their mother was also demonstrated. One of these patients had mental retardation and a horseshoe right kidney in association with KS.

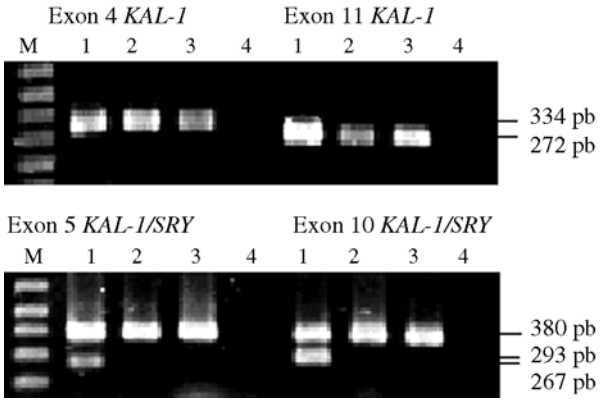

Figure 2 - PCR amplification of exons 4 (334 bp), 5 (267 bp), 10 (293 bp), and 11 (272 bp) of the *KAL-1* gene in two patients affected by X-linked KS. Lanes 1-4 correspond, respectively, to a normal 46,XY male, patient III-4 (Family 1), patient II-6 (Family 2), and a negative control with omission of the genomic DNA in the PCR. Lane M: molecular weight marker. Exons 4 and 11 (**a**) may be amplified in the three males (lanes 1-3), whereas exons 5 and 10 are amplified only in the control male (lane 1). For exons 5 and 10 (**b**), a product of the *SRY* gene (internal positive control, 380 bp) can also be visualized.

Nagata *et al.* (2000) described two brothers with X-linked KS due to a deletion of exons 5-10 of the *KAL-1* gene, but neither mental retardation nor bimanual synkinesis were reported to be present. In fact, mental retardation is rarely observed in patients with isolated KS, but has been described in patients affected by associations of several diseases linked to Xp22.3, as contiguous gene syndromes (Meindl *et al*., 1993; Weissortel *et al*., 1998). Bimanual synkinesis is one of the most frequent findings and is considered a marker for X-linked KS (Krams *et al.,* 1999; Quinton *et al.*, 2001), with an estimated prevalence

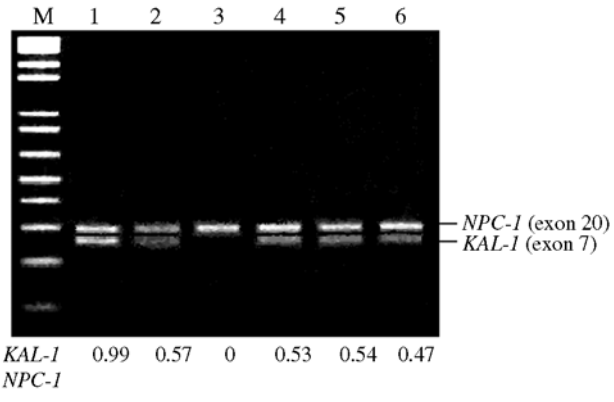

Figure 3 - Comparative duplex PCR for identification of carrier females. Lanes: 1 - control female; 2 - control male; 3-5 - individuals from family 1, respectively, III-4 (affected male), II-11 and III-17 (mothers of patients III-12 and IV-6); and 6 - individual I-2 from family 2 (mother of patients II-4 and II-6). M - molecular weight marker. Values for the *KAL-1*/*NPCI* ratio are given below each lane.

of 85% among X-KS patients (MacColl *et al.,* 2002). However, in a recent report on a family with a mutation in the *FRGR1* gene (*KAL-2*), mapped to 8p, bimanual synkinesis was observed, suggesting that this feature should not be considered specific to the X-linked form (Dodé *et al*., 2003).

Several small deletions, point mutations and a few single-exon deletions have been identified in the *KAL-1* gene in patients with KS (Hardelin *et al.*, 1992; Parenti *et al.*, 1995; Georgopoulus *et al.,* 1997; Quinton *et al.,* 1996; Söderlund *et al.,* 2002). Large deletions involving more than one exon of *KAL-1* have been reported for exons 13-14 (Bick *et al*. (1992), 3 to 5 (Maya-Nunez *et al.*, 1998), 5 to 10 (Nagata *et al.,* 2000), and 3 to 13 (Massin *et al*., 2003). Although the breakpoints were not determined in most cases, it is possible that intronic regions flanking these deletions contain repeated elements that might promote nonallelic recombination. We used the RepeatMasker software (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker", accessed 9/16/2003) for repeat identification in the intronic sequences between exons 4-5 and 10-11 of *KAL-1*. Elements of a FLAM_A repeat of the *Alu* family were found in both introns on opposite strands (Figure 4a). Although no similarity was found between these two elements using BLAST (, accessed 10/7/2003), a significant similarity score was detected for a short sequence of 51 nucleotides when the whole intronic sequences were compared (Figure 4b). This short sequence is contained in the FLAM_A repeat in intron 10 and is adjacent to the FLAM_A repeat in intron 4. These analyses failed to detect other repeats with remarkable sequence identity that could mediate illegitimate recombination events.

In the deletion of exons 13-14 described by Bick *et al.* (1992), sequencing of the junction fragments revealed a 6 bp homology motif (CAAATT) at the deletion breakpoints. These short stretches of sequence homology have been suggested to facilitate end-joining in recombination events in several regions of the genome (Krawczak and Cooper, 1991; Woods-Samuels *et al.,* 1991). It is noteworthy that copies of this 6bp motif are present in the intronic sequences flanking the 5-10 deletion (Figure 4a). Thus, at least two different molecular mechanisms may be responsible for the observed intragenic deletion. Together with the similar deletion previously described by Nagata *et al*. (2000), our data suggest that a recurrent mechanism could predispose to this specific large exon-type deletion.

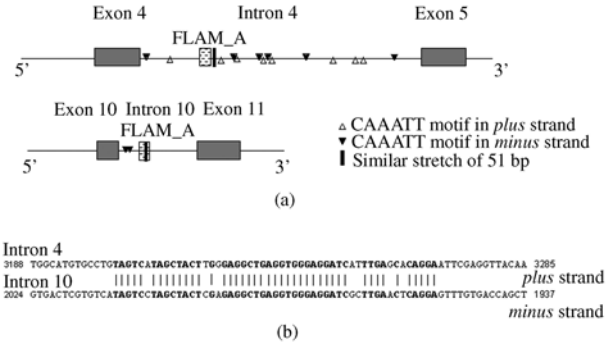

Figure 4 - (**a**) Relative positions of FLAM_A *Alu* repeats and CAAATT motifs on introns 4 and 10 of the *KAL-1* gene, and (**b**) alignment of the similar 51 bp stretch (E value: 2e-04).

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