Molecular analysis of *KAL-1*, *GnRH-R*, *NELF* and *EBF2* genes in a series of Kallmann syndrome and normosmic hypogonadotropic hypogonadism patients

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Abstract

We report the results of molecular analysis in a series of twelve Kallmann syndrome (KS) and five normosmic hypogonadotropic hypogonadism (nHH) Brazilian patients. Kallman syndrome 1 (*KAL-1*) gene analysis was performed in all patients and the gonadotrophin releasing hormone receptor (*GnRH-R*) gene was investigated in nHH patients using PCR analysis with exon-flanking primers followed by automated sequencing techniques. Two-point mutations at the *KAL-1* locus were found in two KS patients. One case exhibited a novel C deletion (del1956C) in exon 12 leading to a premature stop codon at position 617. The second case, a C to T transition at exon 5, showed a stop codon at aminoacid 191 (Arg191X). Renal agenesis and bimanual synkinesis, which are frequently found in patients with the *KAL-1* mutation, were observed in these cases. Among the KS patients, two previously reported cases had intragenic deletions of exons 5–10, while a third patient had a

KAL-1 gene microdeletion detected by fluorescence *in situ* hybridization. For the nHH patients, no abnormalities were observed at the exonic and flanking sequences of the *KAL-1* or *GnRH-R* genes. Nasal embryonic LHRH factor (*NELF*) and early B-cell factor 2 (*EBF2*) exons were evaluated in *KAL-1*/*GnRH-R* mutation-negative cases (seven KS and five nHH) by sequence analysis but no mutations were identified in the coding regions in these patients. In conclusion, this report includes the description of a novel point mutation of the *KAL-1* gene and suggests that the *KAL-1* mutations and deletions might be more prevalent in KS Brazilian patients than previously described in other series. *NELF* and *EBF2* genes have been considered good candidates for HH and a large number of patients need to be studied to assess their contribution to reproductive function.

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Introduction

Hypogonadotropic hypogonadism (HH) is a failure of sexual development or reproductive function due to abnormalities in the pituitary secretion of gonadotropins, follicle-stimulation hormone (FSH) and luteinizing hormone (LH). This profile can result from deficiencies in gonadotropin-releasing hormone (GnRH) production by the hypothalamus or by defects in the GnRH receptor function at the pituitary level. Congenital HH can be associated with anosmia (Kallmann syndrome (KS)) or is apparently isolated, without anosmia (normosmic HH (nHH)). Although the vast majority of KS and nHH cases are sporadic, recessive-X-linked, autosomal dominant and autosomal recessive modes of inheritance have been described (Quinton *et al.* 1996).

The discovery of the Kallman syndrome 1 (*KAL-1*) gene has led to a pathophysiological model correlating GnRH deficiency with abnormal olfactory bulb development in X-linked KS. This gene comprises 14 exons spanning approximately 210 kb on Xp22.3, escapes X-inactivation, and encodes a protein (anosmin)-sharing homology with molecules involved in neuronal migration and axonal pathfinding (Franco *et al.* 1991, Legouis *et al.* 1991). Several mutations in the *KAL-1* gene have been identified in patients with KS (Hardelin *et al.* 1993, Quinton *et al.* 1996). However, in a large number of patients, no *KAL-1* gene mutations have been found, suggesting that autosomal genes are most probably responsible for the majority of both familial and sporadic KS cases (Oliveira *et al.* 2001). Indeed, recent evidence points to loss-of-function mutations in the fibroblast growth factor

Table 1 Clinical characteristics and genotype of evaluated patients with KS and nHH

	Anosmia/ hyposmia	Genotype (<i>KAL-1</i>)	Clinical Phenotype	Inheritance
Patient				
KS1*	+	Complete deletion of <i>KAL-1</i> locus	High-arched palate	Sporadic
KS2*	+	Exons 5–10 deleted	Renal agenesis Mental retardation	Deletion inherited from mother; brother affected
KS3	+	Exon 12: deletion of 1956C creating frameshift and premature STOP codon	Bimanual synkinesis	Sporadic
KS4	+	No coding sequence mutation	Horseshoe kidney	Sporadic
KS5	+	No coding sequence mutation	—	Paternal aunt and cousin affected
KS6	+	No coding sequence mutation	Cubitus valgus	Sister affected and mother anosmic
KS7*	+	Exons 5–10 deleted	Renal agenesis High-arched palate Bimanual synkinesis	Clear X-linked
KS8	+	No coding sequence mutation	Mild facial anomalies	Sporadic
KS9	+	No coding sequence mutation	Hypertelorism Epicanthal folds Hypoplasia of 4 and 5 metacarpis	Sporadic
KS10	+	No sequence coding mutation	—	Sporadic
KS11	+	Exon 5: 721C to T base substitution creating premature STOP codon	Renal agenesis <i>Pes cavus</i>	Clear X-linked
KS12	+	No coding sequence mutation	—	Sporadic
nHH1	—	No coding sequence mutation	—	Sporadic
nHH2	—	No coding sequence mutation	—	Sporadic
nHH3	—	No coding sequence mutation	—	Sporadic
nHH4	—	No coding sequence mutation	—	Sporadic
nHH5	—	No coding sequence mutation	—	Brother affected

*Patients previously described in Trarbach *et al.* (2001) (KS1) and Trarbach *et al.* (2004) (KS2 and KS7).

receptor 1 (*FGFR-1*) gene underlying an autosomal dominant form of KS (Dodé *et al.* 2003, Sato *et al.* 2004).

Inactivating mutations in the gonadotropin release hormone receptor (*GnRH-R*) represent the first identifiable cause of autosomal recessive nHH in humans (de Roux *et al.* 1997, Layman *et al.* 1998). The *GnRH-R* gene is localized on 4q13 and consists of three exons (Kakar *et al.* 1992). So far, more than 16 natural point mutations have been described in this gene (reviewed in Karges *et al.* (2003)). In addition, a second locus has been mapped on 19p13 in a large nHH consanguineous family, leading to the identification of a loss-of-function mutation in the G protein-coupled receptor 54 (*GPR54*) gene (Acierno *et al.* 2003, de Roux *et al.* 2003). Furthermore, a short duplication of the coding sequence of the metastasis suppressor (*KiSS-1*) gene, which encodes a GPR54 ligand, was identified in one sporadic case of nHH, suggesting that this peptide can also play a role in the physiology of the gonadotropic axis (de Roux *et al.* 2004).

However, despite this genetic heterogeneity, only 10–20% of all patients with HH have their genetic basis elucidated (Oliveira *et al.* 2001, Layman 2002). New promising candidate loci for human HH include genes with potential influence on migration of GnRH neurons. GnRH neurons arise in medial olfactory placode epithelium, migrating along the nasal septum across the

cribriform plate to reach the hypothalamus (Schwanzel-Fukuda & Pfaff 1989). Recently, two genes, *NELF* and *EBF2*, have been implicated in this process. The Nelf (nasal embryonic LH releasing hormone factor) protein was first isolated in mouse and the expression patterns of the *Nelf* gene in the olfactory axons and GnRH cells during development are consistent with its proposed function as a migratory factor for GnRH neurons (Kramer & Wray 2000, 2001). The *Ebf2* gene has a key role in the neuroendocrine axis as proposed by Corradi *et al.* (2003). These authors described *Ebf2*-null mice in which the migration of GnRH neurons is defective leading to HH.

In this paper, we report the molecular findings regarding the *KAL-1*, *GnRH-R*, *NELF* and *EBF2* genes in a group of 17 patients with HH in order to verify the relevance of these genes in the pathogenesis of HH.

Subjects and methods

Patients

The subjects were 17 unrelated males, 12 diagnosed with KS (numbered KS1 to KS12) and 5 with nHH (numbered nHH1 to nHH5) (Table 1). In all patients, the HH was documented based on the following criteria: clinical signs

Table 2 PCR primers used for amplification of the coding sequence of the *NELF* gene, and their annealing temperature and product size

Exon	Forward and reverse primers 5' → 3'	Annealing temperature (°C)	Product size (bp)
1	CCTGACGTACGGTAGGTG ACACACCCTTGGTCCTGGT	60 (10% DMSO)	725
2	CCGACCCTCCTCCAGAC GAGGCAGGGATCAGCATC	60 (10% DMSO)	250
3a	CCTTCGGCCTGCTATGAAAC GCTGGCTGTGATGGTGAG	60	352
3b	CACCCCTACCATCACAGC CACCCCCAAACCTGTCTATG	60	278
4	CACCGCTCCTTTTGTCTCT ATAGGCACGTGGGTCTGTTC	60 (10% DMSO)	228
5	ACTGTCCCGACGTCTGTGT CAGCACAGACCAGAGATGGA	60	187
6	ACCGAAGGGGTGAGAGTAGA TCTTATCGCAGCTAGCAGCA	60	198
7	GCTAATGCGGGTTTTGCTC GGTCTAGGGGAGGCTCTGG	60 (10% DMSO)	167
8–9	TGGCCAGGATAGGAGGTG CGCAAGAGGGCATCTGTT	60 (10% DMSO)	547
10–11	CTCTGACGCAGCCTTGATGT CTTCTTCCCCTTGGTCTTCC	59	341
12–13	AAACTACCTCCACGCATGT AAGGCTCTGCCCTGTCTGT	60	491
14–15	TCTCTCTGCCTCGGACTCAT AGTGGCCTGATGGTGACTG	60 (10% DMSO)	357

DMSO, dimethyl sulfoxide.

and symptoms of hypogonadism; prepubertal testosterone (<100 ng/dl); 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 (1997). The protocol was approved by the Ethics Committee of the Faculdade de Ciências Médicas da Universidade Estadual de Campinas (UNICAMP) and all participants provided written informed consent.

Mode of inheritance In two patients with KS (KS11 and KS7) a recessive X-linked mode of inheritance was detected by the presence of asymptomatic females carriers, the presence of another affected male in the maternal family or among male siblings, the absence of affected females and the absence of male-to-male transmission. In another case (KS2), X-linkage was suspected because of the presence of retinal abnormalities, which are typically observed in X-linked KS. Two cases of KS (KS5 and KS6), have a familial history of HH suggestive of autosomal dominant inheritance based on the direct transmission of the phenotype across generations and the presence of at least one affected female. In the nHH probands, one case (nHH5) presented familial recurrence of hypogonadism (one affected brother) but the mode of transmission could not be ascertained.

Methods

Molecular analysis of the *KAL-1* gene was initially carried out both in KS and nHH patients; exceptions were made for the three KS patients (KS1, KS2 and KS7) in which genotypes have been previously described (Trarbach *et al.* 2001, 2004). The five nHH patients were further screened for mutations in the *GnRH-R* gene. Subsequently, *NELF* and *EBF2* sequencing analysis was performed in the *KAL-1/GnRH-R* mutation-negative cases (seven KS and five nHH). Genomic DNA was obtained from whole-blood leukocytes using a routine technique protocol based on cell lysis, proteinase K digestion and phenol/chloroform extraction (Sambrook *et al.* 1989). PCR was performed with 100–200 ng of DNA samples, 0.2 mM dNTP, 1.5 mM MgCl₂, 1 × PCR buffer (20 mM Tris–HCl pH 8.4 and 50 mM KCl), 1 U Taq polymerase (Invitrogen) and 0.6 pmol of each specific set of primers. The primer sequences corresponding to the flanking regions of the *KAL-1* and *GnRH-R* exons, sizes of the amplified products and amplification conditions were as reported by Hardelin *et al.* (1993) and Beranova *et al.* (2001) respectively, and those for *NELF* (NM_015537) and *EBF2* (NM_022659) are shown in Tables 2 and 3 respectively. Thirty cycles of PCR amplifications were performed in a thermal cycler (Gene Amp PCR System 9700, Applied Biosystems) with denaturation at 94 °C for 1 min,

Table 3 PCR primers used for amplification of coding sequence of *EBF2* gene and their annealing temperature and product size

	Forward and reverse primers 5' → 3'	Annealing temperature (°C)	Product size (bp)
Exon			
1	GTCAACAACGGTGAATGTGG AGCAGGCTGGAGTCTGTGT CCCATGAAAGAACAAACCTGA	56	283
2	CCCATGCCTGATTACTATGA GTCCTTGGTGATGGCTTGTG	58	295
3	GAAGTAGCGCAAAGGCAAAC AGGTTTGCTTTATAGCCAGGA	58	243
4	GCCTGCATTGTCTAAGTTTCC TTTGCCATCTTGGAATTTGC	56	124
5–6	CCCAACCTCTGACTCTGTCC GCACGCCTGTGATTACCT	56	460
7	TCCCAAAAGGCCCAATAGTA TGTGCCAAATGTGGAAGTCTG	56	300
8	TTTGCTTTACTTTGCCAACC CCCTCATTCTGTGCATAGCC	56	298
9	CAGTTTGTGTAGCCACCATCA	56	297

annealing at 55–63 °C for 1 min and extension at 72 °C for 1 min.

PCR products from all the exons of the *KAL-1*, *GnRH-R*, *NELF* and *EBF2* genes were purified by Wizard SV gel and PCR clean-up system (Promega). These products were sequenced for both DNA strands using the BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA, USA) in an ABI 377 Automated DNA Sequencer (PE Applied Biosystems) and, where a mutation was apparent, confirmed in two independent PCR analyses and sequencing.

Results

KS patients

Two-point mutations in the *KAL-1* gene were found in two patients with KS. Patient KS3, a sporadic case, exhibited a single base deletion 1956C in exon 12 (Fig. 1). This as-yet undescribed frameshift mutation leads to the introduction of a TGA termination signal 16 codons after

the deletion. This patient had a familial history of X-linked KS and the same mutation was observed in his brother. The second point mutation was found in exon 5 of patient KS11, an already known 721C to T transition changing codon 191 from CGA (arginine) to TGA (premature termination codon). In the remaining seven cases, no mutations were detected for either *NELF* or *EBF2* genes.

nHH patients

No abnormalities were found in the nHH patients for the *KAL-1*, *GnRH-R*, *NELF* and *EBF2* genes.

Polymorphisms

We found two polymorphic changes in the *KAL-1* gene in KS and nHH cases. These polymorphisms were: an A to G transition in exon 11 leading to amino acid substitution Ile534 Val (KS4, KS6 and KS8; nHH1) and a neutral nucleotide substitution in exon 12 (ATT>ATC;

Figure 1 Sequence analysis of exon 12 of the *KAL-1* gene. The mutation in patient KS3 was a deletion of one base, 1956C, at codon 602. The deletion causes a frameshift and a premature termination codon TGA, sixteen codons after the deletion. The base deleted is indicated with an asterisk and the normal sequence can be seen on the left.

Table 4 Summary of reported mutations in the *KAL-1* gene

	Nucleotide change	Amino acid change	Protein (domain)	References
Exon				
5	861G>A	W237X	FNIII-1	Hardelin <i>et al.</i> 1992
6	919C>T	R257X	FNIII-1	Hardelin <i>et al.</i> 1992
6	924G>A	W258X	FNIII-1	Hardelin <i>et al.</i> 1992
9	1411C>T	Q421X	FNIII-3	Hardelin <i>et al.</i> 1993
9	1417C>T	R423 X	FNIII-3	Hardelin <i>et al.</i> 1993
7	328C>G	Y328X	FNIII-2	Georgopoulos <i>et al.</i> 1997
1	346C>T	E66X	Cys	Izumi <i>et al.</i> 1999
6	934C>T	R262X	FNIII-1	Söderlund <i>et al.</i> 2002
13	2041C>T	R631X	FNIII-4	Jansen <i>et al.</i> 2000
4	664T>C	C172X	WAP	Oliveira <i>et al.</i> 2001
5	721C>T	R191X	FNIII-1	Oliveira <i>et al.</i> 2001
10	1519T>C	R457X	FNIII-3	Oliveira <i>et al.</i> 2001
9	1270C>T	R424X	FNIII-3	Sato <i>et al.</i> 2004
7	958G>T	E320X	FNIII-2	Albuisson <i>et al.</i> 2005
6	981delC	—	FNIII-1	Hardelin <i>et al.</i> 1992
7	1166insA	—	FNIII-2	Hardelin <i>et al.</i> 1993
12	1847delC	—	FNIII-4	Quinton <i>et al.</i> 1996
10	1542_1555delTGAAGCGTGTGCCC	—	FNIII-3	Georgopoulos <i>et al.</i> 1997
1	242_252delCTGCGCGGCGG	—	Cys-rich	Gu <i>et al.</i> 1998
12	1951delC	—	FNIII-4	Oliveira <i>et al.</i> 2001
9	1407_1420delTTTCAAAGACGAC	—	FNIII-3	Matsuo <i>et al.</i> 2000
12	1956delC	—	FNIII-4	This paper.
1	100_101delCG	—	Cys-rich	Sato <i>et al.</i> 2004
3	262_269delGAGCCCTG	—	Cys-rich	Sato <i>et al.</i> 2004
5	714_715delGA	—	FNIII-1	Sato <i>et al.</i> 2004
1	53_63dupCGCGGCGGCTG	—	Cys-rich	Söderlund <i>et al.</i> 2002
4	570_571insA	—	WAP	Albuisson <i>et al.</i> 2005
10	1651_1654delinsAGCT	—	FNIII-3	Albuisson <i>et al.</i> 2005
6	951T>A	N267K	FNIII-1	Hardelin <i>et al.</i> 1993
11	1701C>G	F517L	FNIII-3	Georgopoulos <i>et al.</i> 1997
11	1690G>A	E514K	FNIII-3	Maya-Nunez <i>et al.</i> 1998
4	488G>A	C163Y	WAP	Sato <i>et al.</i> 2004
1	3G>A	M1?	Cys	Albuisson <i>et al.</i> 2005
6	784G>C	R262P	FNIII-1	Albuisson <i>et al.</i> 2005
12	1711T>A	W571R	FNIII-4	Albuisson <i>et al.</i> 2005
Intron 12	IVS12+1G>A	—	—	Hardelin <i>et al.</i> 1993
8	1351_1357delAACAACA IVS9+1_2delGT	—	FNIII-2	Georgopoulos <i>et al.</i> 1997
Intron 4	IVS4+1G>T	—	—	Sato <i>et al.</i> 2004
Intron 1	IVS1+1G>t	—	—	Albuisson <i>et al.</i> 2005
Deletion of exon 2	—	—	—	Parenti <i>et al.</i> 1995
Deletion of exon 11	—	—	—	Quinton <i>et al.</i> 1996
Deletion of exon 1	—	—	—	Quinton <i>et al.</i> 1996
Deletion of exon 5	—	—	—	Söderlund <i>et al.</i> 2002
Deletion of exon 13–14	—	—	—	Bick <i>et al.</i> 1992
Deletion of exon 3–5	—	—	—	Maya-Nunez <i>et al.</i> 1998
Deletion of exon 5–10	—	—	—	Nagata <i>et al.</i> 2000
Deletion of exon 3–13	—	—	—	Massin <i>et al.</i> 2003
Whole gene deletion	—	—	—	Hardelin <i>et al.</i> 1993

FNIII, fibronectin III domain like; Cys, domain rich in cysteine; WAP, whey acid protein domain.

Ile611 Ile) (KS6 and KS9; nHH1). Both variations have been previously described by other groups (Hardelin *et al.* 1993, Georgopoulos *et al.* 1997). For the *EBF2* gene a new polymorphism 843 A to G was observed in exon 8 in KS and nHH patients: four were found to be heterozygous (KS5 and KS9; nHH1 and nHH2) and three homozygous (KS3 and KS4; nHH5) for this variation.

However, this coding change was conservative with the amino acid serine remaining at position 245.

Discussion

To date, several mutations in the *KAL-1* gene have been published (Table 4). Most of these mutations are located in

Figure 2 Sites of small deletions (horizontal bars) and point mutations (arrows) identified in the *KAL-1* gene to date. The distribution of *KAL* protein domains is indicated below. The location of the novel mutation identified in the present study is indicated by the asterisk.

the exon 5–14 region encoding the fibronectin III domain (FNIII)-like repeats (Fig. 2). Mutations causing defects in the amino-terminal of the protein are perhaps a cause of high antenatal mortality rate (Gu *et al.* 1998). In our series, two point mutations within the coding regions of the *KAL-1* gene were identified in two KS patients. One mutation was detected in a sporadic case (KS3) and consists of a novel one-base deletion 1956C leading to a premature stop codon at 617. The second was a familial case (KS11) with X-linked inheritance where a nonsense mutation Arg191X was found. This mutation has been previously reported by Oliveira *et al.* (2001) in a sporadic case. Both mutations are located in the region encoding FNIII-like repeats and presumably result in a non-functional truncated *KAL* protein or even in its complete absence, if we consider the degradation of anomalous mRNA by nonsense-mediated RNA decay (Culbertson *et al.* 1999).

We have previously identified three other *KAL-1* abnormalities in this series of Brazilian KS patients: in a sporadic case (KS1) a *KAL-1* gene microdeletion was detected by fluorescence *in situ* hybridization, while in two families with X-linked inheritance (patients KS2 and KS7) similar intragenic deletions of exons 5–10 were found after PCR analysis (Trarbach *et al.* 2001, 2004). Thus, the prevalence of *KAL1* mutations in these Brazilian patients was 100% in the familial X-linked KS (three of three families) and 30% in sporadic cases (two of seven). Although the frequency of *KAL-1* mutations in this study is higher than that reported by other authors (Georgopoulos *et al.* 1997, Oliveira *et al.* 2001), Sato *et al.* (2004) observed similar frequencies in familial X-linked KS (100%) and in sporadic cases (33%). However, these investigators acknowledge that there may be an ascertainment bias in the collection of patients, such as features indicative of contiguous gene syndrome and renal aplasia. This did not occur in the present study, and the reason for the high prevalence of *KAL-1* mutations in our series remains unknown.

In the remainder of our KS cases, no abnormalities in the coding exons of the *KAL-1* gene were found. In fact, Oliveira *et al.* (2001) concluded that autosomal genes are clearly responsible for the majority of both familial and

sporadic KS. To date, only loss-of-function mutations in the *FGFR1* gene, located in 8p11.2, were associated with KS (Dodé *et al.* 2003, Sato *et al.* 2004). Therefore, formal possibilities of the existence of a second X-linked gene causing KS or of molecular alterations located in the regulatory regions of the *KAL-1* gene promoter, in the untranslated regions of exons 1 and 14, or within introns creating new splicing sites, can not be excluded.

The clinical phenotype of our KS patients carrying *KAL-1* mutations, includes renal abnormalities and bimanual synkinesis. Both features had been exclusively associated with X-linked KS. For instance, approximately 40% X-linked KS patients have renal abnormalities (Kirk *et al.* 1994), but this symptom has recently been reported in a female patient who does not carry a mutation in the *KAL-1* gene (Sato *et al.* 2004). Similarly, bimanual synkinesis is present in over 75% of X-linked KS patients (Quinton *et al.* 1996, Mayston *et al.* 1997), nevertheless this anomaly was also observed in an autosomal form of KS associated with loss-of-function of *FGFR1* (Dode *et al.* 2003).

Although *GnRH-R* gene mutations have usually been detected in 40% of autosomal recessive and 16% of sporadic nHH patients (Beranova *et al.* 2001), no mutations were found in our five nHH patients. This is not unexpected due to the small number of cases. No mutations were identified in the coding sequences of the *NELF* or *EBF2* genes in our patients (seven KS and five nHH). To date, only one *NELF* heterozygous missense mutation 1438A>G, resulting in a Thr480Ala, has been reported in a sporadic case of HH. This mutation was not found in 100 control individuals and the observation that the Thr480 is highly conserved among mouse, rat, and human suggested that this amino acid substitution can be associated with the pathogenesis of HH (Miura *et al.* 2004). To our knowledge, this is the first report of molecular studies of the *EBF2* gene in a small series of KS and nHH patients. The above negative results clearly indicate that other genes (e.g. *FGFR-1*, *GPR54*, *KISS-1*) should be screened in these cases.

However, it should be noted that the present study is based on genomic DNA analysis and does not rule out localized embryological somatic mutations which would appear as sporadic cases. The clinical relevance of somatic mutations in endocrine diseases, as well as in different endocrine tumors, is becoming increasingly recognized (Bertherat *et al.* 2005). For instance, in McCune Albright's syndrome, early embryological activating somatic mutations of the *G-s-alpha* gene are clearly related to sporadic disease (Shenker *et al.* 1994). A similar scenario might exist in the GnRH-producing neurons failing to appropriately populate the hypothalamus of patients with KS. Unfortunately, no hypothalamic specimens were available in our patient series to investigate this question.

In conclusion, this report includes the description of a novel point mutation of the *KAL-1* gene and suggests that

the *KAL-1* mutations and deletions might be more prevalent in KS Brazilian patients than previously described in other series. Moreover, although renal agenesis and bimanual synkinesis can not be further considered exclusive for the X-linked KS form, the presence of these features is strongly indicative for the occurrence of *KAL-1* abnormalities in patients with HH and anosmia. Attempts to identify mutations within the coding region of *NELF* and *EBF2* genes failed in our series of KS and nHH patients with sporadic and familial cases of GnRH deficiency. However, these genes have been considered good candidates for HH and a large number of patients need to be studied to assess the contribution of *NELF* and *EBF2* genes to reproductive function.

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References

- Acierno JS, Shagoury JK, Bo-Abbas Y, Crowley WF Jr & Seminara SB 2003 A locus for autosomal recessive idiopathic hypogonadotropic hypogonadism on chromosome 19p13.3. *Journal of Clinical Endocrinology and Metabolism* **88** 2947–2950.
- Albuisson J, Pecheux C, Carel JC, Lacombe D, Leheup B, Lapuzina P, Bouchard P, Legius E, Matthijs G, Wasniewska M, Delpech M, Young J, Hardelin JP & Dode C 2005 Kallmann syndrome: 14 novel mutations in *KAL1* and *FGFR1* (*KAL2*). *Human Mutation* **25** 98–99.
- Beranov M, Oliveira LM, Bedecarrats GY, Schipani E, Vallejo M, Ammini AC, Quintos JB, Hall JE, Martin KA, Hayes FJ, Pitteloud N, Kaiser UB, Crowley WF Jr & Seminara SB 2001 Prevalence, phenotypic spectrum, and modes of inheritance of gonadotropin-releasing hormone receptor mutations in idiopathic hypogonadotropic hypogonadism. *Journal of Clinical Endocrinology and Metabolism* **86** 1580–1588.
- Bertherat J & Gimenez-Roqueplo AP 2005 New insights in the genetics of adrenocortical tumors, pheochromocytomas and paragangliomas. *Hormone and Metabolism Research* **37** 384–390.
- Bick D, Franco B, Sherins RJ, Heye B, Pike L, Crawford J, Maddalena A, Incerti B, Pragliola A, Meitinger T & Ballabio A 1992 Intragenic deletion of the *KALIG-1* gene in Kallmann's syndrome. *New England Journal of Medicine* **25** 1752–1755.
- Corradi A, Croci L, Broccoli V, Zecchini S, Previtali S, Wurst W, Amadio S, Maggi R, Quattrini A & Consalez GG 2003 Hypogonadotropic hypogonadism and peripheral neuropathy in *Ebf2*-null mice. *Development* **130** 401–410.
- Culbertson R 1999 RNA surveillance – unforeseen consequences for gene expression, inherited genetic disorders and cancer. *Trends in Genetics* **15** 74–80.
- Davidson TM & Murphy C 1997 Rapid clinical evaluation of anosmia. *Archives of Otolaryngology – Head and Neck Surgery* **123** 591–594.
- de Roux N, Young J, Misrahi M, Genet R, Chanson P, Schaison G & Milgrom E 1997 A family with hypogonadotropic hypogonadism and mutations in the gonadotropin releasing hormone receptor. *New England Journal of Medicine* **337** 1597–1602.
- de Roux N, Acierno JS, Houang M, Derbois D, Matsuda F, Meysing AU, Pugeat M, Le Bouc Y, Crowley WF Jr, Kelly P & Seminara S 2004 A unique short 3' duplication of the coding sequence of the GPR54 ligand identified in a large cohort of isolated hypogonadotropic hypogonadism patients. *ENDO 2004*, Endocrine Society's 86th Annual Meeting, New Orleans, LA, USA. <http://www.endo-society.org/scimeetings/endo2004/index.cfm>.
- de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL & Milgrom E 2003 Hypogonadotropic hypogonadism due to loss of function of the *Kiss1*-derived peptide receptor GPR54. *PNAS* **100** 10972–10976.
- Dodé C, Leveilliers J, Dupont J-M, De Paepe A, Dû NL, Soussi-Yanicostas N, Coimbra RS, Delmaghani S, Compain-Nouaille S, Baverel F, Pêcheaux C, Le Tessier D, Cruaud C, Delpech M, Speleman F, Vermeulen S, Amalfitano A, Bachelot Y, Bouchard P, Cabrol S, Carel J-C, Deleamarre-van der Waal H, Goulet-Salmon B, Kottler A-L & Richard O 2003 Loss-of-function mutations in *FGFR1* cause autosomal dominant Kallmann syndrome. *Nature Genetics* **33** 463–465.
- Franco B, Guioli S, Pragliola A, Incerti B, Bardoni B, Tonlorenzi R, Carozzo R, Maestrini E, Pieretti M, Taillon-Miller P, Brown CJ, Willard HF, Lawrence C, Persico MG, Camerino G & Ballabio A 1991 A gene deleted in Kallmann's syndrome shares homology with neural cell adhesion and axonal path-finding molecules. *Nature* **353** 529–536.
- Georgopoulos NA, Pralong FP, Seidman CE, Seidman JG, Crowley WF Jr & Vallejo M 1997 Genetic heterogeneity evidenced by low incidence of *KAL-1* gene mutations in sporadic cases of gonadotropin-releasing hormone deficiency. *Journal of Clinical Endocrinology and Metabolism* **82** 213–217.
- Gu WX, Colquhoun-Kerr JS, Kopp P, Bode HH & Jameson JL 1998 A novel aminoterminal mutation in the *KAL-1* gene in a large pedigree with X-linked Kallmann syndrome. *Molecular Genetics and Metabolism* **6** 59–61.
- Hardelin J-P, Leveilliers J, del Castillo I, Cohen-Salmon M, Legouis R, Blanchard S, Compain S, Bouloux P, Kirk J, Morainet C, Chaussain JL, Weissenbach J & Petit C 1992 X chromosome-linked Kallmann syndrome: stop mutations validate the candidate gene. *PNAS* **89** 8190–8194.
- Hardelin J-P, Leveilliers J, Blanchard S, Carel JC, Leutenegger M, Pinard-Bertellettom JP, Bouloux P & Petit C 1993 Heterogeneity in the mutations responsible for X chromosome-linked Kallmann syndrome. *Human Molecular Genetics* **2** 373–377.
- Izumi Y, Tatsumi K, Okamoto S, Hosokawa A, Ueno S, Fukui H & Amino N 1999 A novel mutation of the *KAL-1* gene in Kallmann syndrome. *Endocrinology Journal* **46** 651–658.
- Jansen C, Hendriks-Stegeman BI & Jansen M 2000 A novel nonsense mutation of the *KAL* gene in two brothers with Kallmann syndrome. *Hormone Research* **53** 207–212.
- Kakar SS, Musgrove LC, Devor DC, Sellers JC & Neill JD 1992 Cloning, sequencing, and expression of human gonadotropin releasing hormone (GnRH) receptor. *Biochemical and Biophysical Research Communications* **30** 289–395.
- Karges B, Karges W & de Roux N 2003 Clinical and molecular genetics of the human GnRH receptor. *Human Reproduction Update* **6** 523–530.
- Kirk JM, Grant DB, Besser GM, Shalet S, Quinton R, Smith CS, White M, Edwards O & Bouloux PM 1994 Unilateral renal aplasia in X-linked Kallmann's syndrome. *Clinical Genetics* **46** 260–262.
- Kramer PR & Wray S 2000 Novel gene expressed in nasal region influences outgrowth of olfactory axons and migration of luteinizing hormone-releasing hormone (LHRH) neurons. *Genes and Development* **14** 1824–1834.
- Kramer PR & Wray S 2001 Nasal embryonic LHRH factor (NELF) expression within the CNS and PNS of the rodent. *Gene Expression Patterns* **1** 23–26.

- Layman LC 2002 Human gene mutations causing infertility. *Journal of Medical Genetics* **39** 153–161.
- Layman LC, Cohen DP, Jin M, Xie J, Li Z, Reindollar RH, Bolbolan S, Bick DP, Sherins RR, Duck LW, Musgrove LC, Sellers JC & Neill JD 1998 Mutations in gonadotropin releasing hormone receptor gene cause hypogonadotropic hypogonadism. *Nature Genetics* **18** 14–15.
- Legouis R, Hardelin JP, Levilliers J, Claverie J-M, Compain S, Wunderle V, Millasseau P, Le Paslier D, Cohen D, Caterina D, Bougueleret L, Dleamarre-van der waal H, Lutfalla G, Weissenbach J & Petit C 1991 The candidate gene for the X-linked Kallmann syndrome encodes a protein related to adhesion molecules. *Cell* **67** 423–435.
- Massin N, Pêcheux C, Eliot C, Bensinmon J-L, Galey J, Kuttenn F, Hardelin J-P, Dodé C & Touraine P 2003 X chromosome-linked Kallmann syndrome: clinical heterogeneity in three siblings carrying an intragenic deletion of *KAL-1* gene. *Journal of Clinical Endocrinology and Metabolism* **88** 2003–2008.
- Matsuo T, Okamoto S, Izumi Y, Hosokawa A, Takegawa T, Fukui H, Tun Z, Honda K, Matoba R, Tatsumi K & Amino N 2000 A novel mutation of the *KAL 1* gene in monozygotic twins with Kallmann syndrome. *European Journal of Endocrinology* **143** 783–787.
- Maya-Nunez G, Zenteno JC, Ulloa-Aguirre A, Kofman-Alfaro S & Mendez JP 1998 A recurrent missense mutation in the *KAL-1* gene in patients with X-linked Kallmann's syndrome. *Journal of Clinical Endocrinology and Metabolism* **83** 1650–1653.
- Mayston MJ, Harrison LM, Quinton R, Stephens JA, Krams M & Bouloux PM 1997 Mirror movements in X-linked Kallmann's syndrome. I. A neurophysiological study. *Brain* **120** 1199–1216.
- Miura K, Acierno JS Jr & Seminara SB 2004 Characterization of the human nasal embryonic LHRH factor gene, NELF, and a mutation screening among 65 patients with idiopathic hypogonadotropic hypogonadism (IHH). *Journal of Human Genetics* **49** 265–268.
- Nagata K, Yamamoto T, Chikumi H, Ikeda T, Yamamoto H, Hashimoto K, Yoneda K, Nanba E, Ninomiya H & Ishitobi K 2000 A novel interstitial deletion of *KAL-1* in a Japanese family with Kallmann syndrome. *Journal of Human Genetics* **45** 237–240.
- Oliveira LM, Seminara SB, Beranova M, Hayes FJ, Valkenburgh SB, Schipani E, Costa EM, Latronico AC, Crowley WF Jr & Vallejo M 2001 The importance of autosomal genes in Kallmann syndrome: genotype-phenotype correlations and neuroendocrine characteristics. *Journal of Clinical Endocrinology and Metabolism* **86** 1532–1538.
- Parenti G, Rizzolo MG, Ghezzi M, Di Maio S, Sperandeo MP, Incerti B, Franco B, Ballabio A & Andria G 1995 Variable penetrance of hypogonadism in a sibship with Kallmann syndrome due to a deletion of the *KAL* gene. *American Journal of Medical Genetics* **57** 476–478.
- Quinton R, Duke VM, Priyal A, Zoysa PA, Platis AD, Valentine A, Kendall B, Pickman S, Kirk JMW, Besser GM, Jacobs HS & Bouloux PMG 1996 The neuroradiology of Kallmann's syndrome: a genotypic and phenotypic analysis. *Journal of Clinical Endocrinology and Metabolism* **81** 3010–3017.
- Sambrook J, Fritsch EF & Maniatis T 1989 *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- 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 2004 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. *Journal of Clinical Endocrinology and Metabolism* **89** 1079–1088.
- Schwanzel-Fukuda M & Pfaff DW 1989 Origin of luteinizing hormone-releasing hormone neurons. *Nature* **338** 161–164.
- Shenker A, Weinstein LS, Sweet DE & Spiegel AM 1994 An activating *Gs* alpha mutation is present in fibrous dysplasia of bone in the McCune-Albright syndrome. *Journal of Clinical Endocrinology and Metabolism* **79** 750–755.
- Söderlund D, Canto P & Méndez JP 2002 Identification of three novel mutations in the *KAL1* gene in patients with Kallmann syndrome. *Journal of Clinical Endocrinology and Metabolism* **87** 2579–2582.
- Trarbach EB, Baptista MTM, Maciel-Guerra A & Hackel C 2001 Cytogenetics analysis and detection of *KAL-1* gene deletion with fluorescence *in situ* hybridization. *Arquivos Brasileiros de Endocrinologia e Metabolismo* **45** 552–557.
- Trarbach EB, Monlleo IL, Porciuncula CGG, Fontes MIB, Baptista MTM & Hackel C 2004 Similar interstitial deletions of the *KAL-1* gene in two Brazilian families with X-linked Kallmann syndrome. *Genetics and Molecular Biology* **27** 337–341.

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