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 gonadotropin 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.

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

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


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...
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 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.
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; Table 3).

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

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward and reverse primers 5’→3’</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GTCAACAACCGTGAATGTGG</td>
<td>56</td>
<td>283</td>
</tr>
<tr>
<td>2</td>
<td>AGCAGGCTGGAGTCGTTGTT</td>
<td>58</td>
<td>295</td>
</tr>
<tr>
<td>3</td>
<td>CCCATGAAAGAACAAGCTTGGA</td>
<td>58</td>
<td>243</td>
</tr>
<tr>
<td>4</td>
<td>GCCTGGATTTGTCAAAGTTTC</td>
<td>56</td>
<td>124</td>
</tr>
<tr>
<td>5-6</td>
<td>CCCAACCTGTGACTCTGTTCC</td>
<td>56</td>
<td>460</td>
</tr>
<tr>
<td>7</td>
<td>GCACGCTTTGTTAATTTACCT</td>
<td>56</td>
<td>300</td>
</tr>
<tr>
<td>8</td>
<td>TGTGCTTACTTCCCGAACC</td>
<td>56</td>
<td>298</td>
</tr>
<tr>
<td>9</td>
<td>CCCTCATCCTGTCCAGACC</td>
<td>56</td>
<td>297</td>
</tr>
</tbody>
</table>

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.
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
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


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