**KAL1** mutations are not a common cause of idiopathic hypogonadotrophic hypogonadism in humans

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Hypogonadotrophic hypogonadism results in the absence of puberty and if left untreated leads to infertility. Mutations in **KAL1** are known to account for some of the cases of Kallmann syndrome. The aim of this study was to determine the prevalence of **KAL1** mutations in a large number of patients with idiopathic hypogonadotrophic hypogonadism (IHH). One hundred and thirty eight patients (109 males and 29 females) with IHH were studied for mutations in **KAL1**. DNA from these patients was subjected to denaturing gradient gel electrophoresis or single strand conformation polymorphism to identify mutations. Sequencing was performed to confirm mutations detected. Four mutations were found in 109 males (3.7%). All four mutations were in anosmic/hyposmic men making the prevalence 4/63 (6.3%) in this group of patients. No mutations were found in the 29 female patients. **KAL1** mutations are an uncommon cause of Kallmann syndrome.

**Key words:** gene mutation/hypogonadotrophic hypogonadism/idiopathic hypogonadotrophic hypogonadism/KAL1 gene/Kallmann syndrome

**Introduction**

Kallmann syndrome consists of the combination of hypogonadotrophic hypogonadism and anosmia. Associated clinical features may include neurologic abnormalities (synkinesia, oculomotor deficits, deafness, mental retardation and cerebellar defects), unilateral renal agenesis, midline facial defects and pes cavus. The hypogonadotrophic hypogonadism usually presents as absent puberty in males with small testes, low serum levels of testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) and azoospermia/oligospermia. Female patients present with absent puberty and absent breast development. If untreated, infertility results. Replacement of gonadotrophin releasing hormone (GnRH) or gonadotrophins, improves fecundity. The hypogonadotrophic hypogonadism appears to be related to impaired GnRH neuron migration or reduced GnRH secretion or pulsatility, or impaired GnRH action at the pituitary, which then results in low gonadotrophin secretion (MacColl et al., 2002).

Kallmann syndrome was the first inherited cause of human idiopathic hypogonadotrophic hypogonadism (IHH), for which the molecular basis was characterized (Franco et al., 1991; Legouis et al., 1991). Kallmann syndrome is caused by mutations in the **KAL1** gene localized to chromosome Xp22.3, which encodes anosmin-1, a secreted protein that has neural cell adhesion molecule properties. The precise function of anosmin-1 remains unknown, but the protein contains a signal peptide, a cysteine rich region, a highly conserved four-disulfide core whey acid protein (WAP) domain, four fibronectin type III (FNIII) repeats, and a C-terminal histidine-rich region (Franco et al., 1991; Legouis et al., 1991). The WAP domain is found in protease inhibitors, which have been shown to play a role in axonogenesis and neuron migration. The FNIII domains are present in extracellular matrix proteins, cellular adhesion molecules and protein tyrosine kinases and phosphatases that have been implicated in neuronal migration and in axon targeting (Franco et al., 1991; Legouis et al., 1991).

Anosmin-1 appears to be involved in the migration of olfactory and GnRH neurons from the olfactory placode region across the cribriform...
plate to synapse with the mitral cells of the olfactory bulb (Gonzalez-Martinez et al., 2004). It has been suggested that this neural network provides a scaffold for GnRH neurons, which then arrive at their normal position in the arcuate nucleus of the hypothalamus. In vitro evidence in support of effects on both olfactory and neuroendocrine development include: (i) anosmin-1 initiates neurite outgrowth from olfactory bulb neurons in C. elegans (Rugarli et al., 2002) and rat (Soussi-Yanicostas et al., 2002), as well as in primary human olfactory neuroblasts (Gonzalez-Martinez et al., 2004), but does so in a cell-type specific manner (Soussi-Yanicostas et al., 1998); (ii) anosmin-1 directly increases GnRH neuron migration in immortalized mouse GN11 cells (Cariboni et al., 2004); (iii) disruption of the kal1.1 in zebrafish also interferes with GnRH neuron migration (Whitlock et al., 2006). It is possible that anosmin-1 interacts with FGFR1 to effect GnRH neuronal migration (Gonzalez-Martinez et al., 2004).

A variety of KAL1 mutations in hypogonadotropic hypogonadal males have been described (Legouis et al., 1991; Bick et al., 1992; Hardelin et al., 1992, 1993; Wulfsberg et al., 1992; Bouloux et al., 1993; Martul et al., 1995; Parenti et al., 1995; Quinton et al., 1996; Georgopoulos et al., 1997; Gu et al., 1998; Maya-Nunez et al., 1998; O’Neill et al., 1998; Weisssert et al., 1998; Persson et al., 1999; Jansen et al., 2000; Matsuo et al., 2000; Izumi et al., 2001; Oliveira et al., 2001; Soderlund et al., 2002; Massin et al., 2003; Albuissson et al., 2005; Trarbach et al., 2005; Chocholska et al., 2006). Initially, gene deletions were identified by Southern blot analysis (Bick et al., 1992), but a variety of point mutations were later characterized (Hardelin et al., 1993). KAL1 mutations were initially proposed to be the most common cause of inherited human hypogonadotropic hypogonadism. Although common in clearly recognized X-linked recessive families, the prevalence of KAL1 mutations varied between 3.1% and 27.8% in families where there was hypogonadotrophic hypogonadism. Although common in clearly

DNA Analysis

DNA was extracted from each patient using standard methodology and subjected to polymerase chain reaction (PCR) for each of the 14 exons of the KAL1 gene, along with their splice junctions. Primer sequences have been previously published by Hardelin et al. (1993) and conditions for PCR were 94°C (60 s), 55°C (45 s), 72°C (60 s) for exon 1 and 94°C (60 s), 55°C (45 s), 72°C (45 s) for exons 2–14. Each PCR included a negative control, which contained all reagents except DNA. For denaturing gradient gel electrophoresis (DGGE), a 40 bp GC-clamp was added to one of the primers of a primer pair to increase the detection of mutations (Sheffield et al., 1989).

PCR products were first run on ethidium containing 1.2% agarose gels in the presence of a molecular weight marker and photographed under ultraviolet light to confirm presence of the fragment of interest. PCR products were then subjected to DGGE or single strand conformation polymorphism (SSCP).

Denaturing gradient gels were 22 cm vertical 6.5% polyacrylamide gels containing 20–80% gradients (100% = 7 M urea and 40% formamide). Gels were electrophoresed at 400–1200 volt-hours, as determined by test gels, in E buffer at 60°C (Layman et al., 1997, 1998). SSCP analysis was performed as described by Nishimura et al. (1998). Briefly, PCR products were mixed with formamide dye, heat-denatured and then were electrophoresed on 6% denaturing acrylamide gels (5 ml glycerol, 5 ml 5X TBE, 12.5 ml 37 : 5 : 1 acrylamide/bis and 77.5 ml ddH2O) for 3–4 h at room temperature, silver stained and photographed.

Variant fragments identified by DGGE/SSCP were subjected to DNA sequencing using the Big Dye Terminator kit on an ABI 310 Automated DNA sequencer according to the manufacturer’s instructions. Each sequencing reaction was confirmed by repeat analysis in both forward and reverse directions. Putative mutations were confirmed at least six times.

For the patient with exon 1 to exon 13 deletion, PCR for the deleted exons was repeated multiple times with positive controls to confirm the deletion. To ensure that the deletion was not due to poor DNA quality, several exons from additional genes (FGFR1 exon 9; TNR exons 8 and 10; DCC exons 15, 16 and 29) were subjected to PCR.

Analysis of sequence variations for possible polymorphism was performed using Sorting Intolerant From Tolerant (SIFT) (Ng and Henikoff, 2001; Raveaara et al., 2005), with a P < 0.05 being considered as intolerant. Species included in this analysis are: human (gi#4557683), zebrafish (gi#6708054), jungle fowl (gi# 45382209), chick (gi#50403735), puffer fish (gi#6708052), Japanese quail (gi#50401099), Chimpanzee (gi#55662534), cat (74007097), fruitfly (gi#45580809), mosquito (gi#58382167), C. elegans (gi#25149859), moth (gi#14878825), Japanese rice fish (gi#88595354), freshwater pufferfish (gi#47226778), domestic cow (gi#7669851), red flour beetle (gi#10768220), Bornean orangutan (gi#5572158), bushrat possum (gi#14582214), honey bee (gi#6650567), mouse (gi#1094622), purple sea urchin (gi#72095991), pig (gi#14722324), wild sheep (gi#78126182). Functional domains in the anosmin-1 protein were identified using the Human Protein Reference Database (Peri et al., 2003).

Results

For 136 of the 138 IHH patients, agarose gel electrophoresis demonstrated the appropriate sized bands for each of the 14 exons of KAL1, documenting the lack of deletions. However, two patients had intragenic deletions of KAL1. One patient, had a deletion of exons 1–13, which were present in controls (exon 14 was present). This patient had complete IHH, anosmia and bilateral cryptorchidism and a maternal uncle and maternal cousin with Kallmann syndrome.

Materials and methods

Patients

One hundred and thirty eight (109 males and 29 females) consecutive, unrelated probands with IHH were studied for mutations in the KAL1 gene. IHH was defined as irreversible pubertal delay at an age ≥17 years for females and ≥18 years for males accompanied by low serum gonadotrophins and normal prolactin and thyroid stimulating hormone (Bhagavath et al., 2006). Males had serum testosterone < 100 ng dl–1 (normal 300–1100 ng dl–1), and females had irreversible hypoestrogenic amenorrhoea, as demonstrated by an estradiol <30 pg ml–1 or a vaginal smear containing parabasal cells. No pituitary tumour was demonstrated by sellar X-ray films, CT or MRI. Since the full spectrum of KAL1 mutations is not known, IHH patients with and without anosmia were studied. Anosmia was present in 50 males and 4 females, whereas hyposmia was present in an additional 13 males and 3 females.

IHH was also sub-classified according to the severity of hormone deficiency into a completely deficient endocrine state (complete IHH) or a partially deficient state (incomplete IHH). Complete IHH was defined as the complete lack of sex steroid manifestations of puberty. In females, absent breast development (Tanner stage 1) and primary amenorrhoea constituted complete IHH, whereas in males, testicular size <3 ml (the largest size found in normal prepubertal males) constituted complete IHH (Burris et al., 1988). Incomplete IHH was defined as the presence of any breast development in females and as testicular volume ≥4 ml in males. This study was approved by the Human Assurance Committee at the Medical College of Georgia.
This patient’s pedigree corresponds to family 2 in Bhagavath et al. (2006). The other patient with deletions of exons 13 and 14 (confirming the findings previously described by Bick et al.) had unilateral cryptorchidism, micropenis and anosmia (Bick et al., 1992). His brother also had Kallmann syndrome. Using DGGE and SSCP, 5 DNA sequence differences were identified. Subsequent DNA sequencing and analysis revealed five different mutations in five unrelated probands listed in Table I and diagrammatically represented in Figure 1. These putative mutations include: 487–489delTGT (C163del), 769C>T (R257X), 1997A>T (K666M)/2003G>A (R668H) and 768G>A (S206S).

Two of the point mutations are highly likely to cause Kallmann syndrome. In one patient with incomplete IHH, anosmia and unilateral renal agenesis, a C163del was observed. This C163del removes a Cys residue in the WAP domain. Since codons 163 and 164 are both Cys of the exact same sequence, the deletion could be of either codon.

In another unrelated IHH proband with complete IHH, anosmia and cryptorchidism, a R257X nonsense mutation was identified. This mutation would be predicted to result in a truncated protein lacking three of the four FNIII domains. A double mutant (K666M/R668H) was found in two unrelated IHH patients, both of whom were anosmic (Table I). The silent mutation (S206S) is a polymorphism. SIFT has been used for assessing the significance of missense mutations (Raevaara et al., 2005); however, the deletion of a Cys residue C163del, is predicted to be deleterious as expected since Cys residues participate in disulfide bond formation. This cysteine is conserved 88% among 26 organisms analysed and no substitutions are tolerated. Amino acids K at position 666 and R at position 668 are less well conserved at 31% and 15%, respectively. These substitutions are predicted to be tolerated with scores of 0.05 and 1.00, respectively. The median sequence conservation is 3.63 for K666M and 3.70 for R668H, making it unlikely that K666M is a mutation even though the tolerated score is 0.05 (a score <0.05 is considered not tolerated).

Discussion

Kallmann syndrome is a common X-linked recessive form of human hypogonadotropic hypogonadism. KAL1 gene mutations account for 33–70% of the cases of familial X-linked forms of Kallmann syndrome (Maya-Nunez et al., 1998; Sato et al., 2004; Albuisson et al., 2005). The prevalence of KAL1 mutations in apparent sporadic forms of IHH, with or without anosmia, has been variably reported between 3.1% and 27.8% (Sato et al., 2004; Albuisson et al., 2005). However, most of these are small studies.

In the present study, we screened 138 IHH patients (109 males and 29 females) for KAL1 mutations. No KAL1 mutations were identified in the 29 females. Of 109 males, 50 had anosmia, 13 had hyposmia and cryptorchidism, maternal uncle and maternal cousins. One affected brother.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Protein domain affected</th>
<th>Amino acid change</th>
<th>Nucleotide change</th>
<th>Other anomalies</th>
<th>Sense of smell</th>
<th>Other affected family members</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 4</td>
<td>WAP</td>
<td>487–489delTGT</td>
<td>C163del</td>
<td>Deletion of exons 1–13</td>
<td>Complete</td>
<td>One affected brother</td>
</tr>
<tr>
<td>Exon 5</td>
<td>WAP</td>
<td>768G&gt;A</td>
<td>S206S</td>
<td>Deletion of IVS12, exon 13, IVS13, exon14</td>
<td>Complete</td>
<td>One affected brother</td>
</tr>
<tr>
<td>Exon 6</td>
<td>FNIII (part of 1; 2–4 deleted)</td>
<td>1997A&gt;T</td>
<td>R668H</td>
<td>Deletion of exons 1–13</td>
<td>Complete</td>
<td>One affected brother</td>
</tr>
<tr>
<td>Exon 14a</td>
<td>FNIII (4th)</td>
<td>2003G&gt;A</td>
<td>K666M</td>
<td>Deletion of IVS12, exon 13, IVS13, exon14</td>
<td>Complete</td>
<td>One affected brother</td>
</tr>
</tbody>
</table>

WAP, Whey Acidic Protein; FNIII, Fibronectin type III domain.
had normal sense of smell and data were not available in 16 patients. Of 109 males, 4 had at least one other affected family member. Among the 105 apparently sporadic male patients, 2 (1.9%) had KAL1 mutations. Of the 4 familial cases, 2 (50%) had KAL1 mutations/del-
etions. The overall prevalence of KAL1 mutations in males with IHH was 4/109 (3.7%), but for anosmic/hyposmic males, the prevalence was 4/63 (6.3%). These figures correspond to those of Albuisson et al. (2005).

Four of the mutations, including three deletions (C163del, del exons 1–13 and the previously described deletion of exons 13 and 14) and one point mutation (R257X) are highly likely to be causative. Two unrelated probands had double variants (K666M/R668H). However, it is uncertain if these represent true mutations or polymorphisms. They will be tested in vitro to determine their effects upon cell secretion and GnRH neuron migration.

In this study, we screened both normosmic and anosmic/hyposmic IHH patients for mutations in KAL1 since variable expressivity with KAL1 mutations has been reported. Siblings with the same intragenic deletion (exons 3–13) in KAL1 have been reported to have very different clinical presentations including varying degrees of anosmia (Massin et al., 2003). For these reasons, we studied both normosmic and anosmic/hyposmic IHH patients, but found mutations only in those with an abnormal sense of smell (Table I).

Initially DGGE and SSCP were used to detect mutations, which would be expected to determine 80–90% of the mutations (Sheffield et al., 1989; Nishimura et al., 1998). Using DGGE with GC-clamped PCR products for subsequent analysis should permit the detection of 95% of the mutations (Sheffield et al., 1989). This makes it very unli-

kely that many mutations were missed by these gene screening methods.

The R257X mutation has been described by others (Hardelin et al., 1992), and this truncating mutant is predicted to remove three FNIII domains, which are important in neuron migration (Gonzalez-Martinez et al., 2004; Hu et al., 2004). Our patient has complete IHH with bilateral cryptorchidism and anosmia, reflecting the severity of this mutation. In comparison, the patient reported by Hardelin et al. (1992) had more severe disease: micropenis and bilateral cryptorchid-i

sm at birth; by 11 years age he had bimanual synkinesia and mild bilateral pes cavus.

The IVS12-exon14 deletion has been reported previously (Bick et al., 1992) in a male with complete IHH, hyposmia, visual field abnormalities and an affected brother. The deletion of exons 1–13 and Cys 163 (C163del) is reported here for the first time. This patient, with a nearly complete KAL1 gene deletion, presented with complete IHH, anosmia and cryptorchidism and had an affected maternal uncle and a maternal cousin. The precise breakpoints of the deletion are currently being mapped.

Interestingly, the patient with the C163del mutation, which occurs within the WAP domain known to be important in neuron migration, had incomplete IHH, anosmia and unilateral renal agenesis. As the deletion involves the sixth cysteine in the WAP domain, it is expected to result in protein misfolding by disrupting disulfide bond formation. This mutation is the only deletion identified in a patient with some evidence of sex steroid production, as indicated by partial testicular growth. None of these mutations were identified in 50 controls, thereby reducing the probability that these were polymorphisms.

The double variants of K666M and R668H are likely to be poly-

morphisms. SIFT analysis shows low levels of conservation at these positions across different organisms. Even at these low levels of conservation, the substitutions K666M and R668H are predicted to be tolerated with scores of 0.05 and 1.00, respectively. Even though the score is quite low at 0.05 for K666M, the median sequence conservation is 3.63, making it less likely to be a mutation even if the score was lower than 0.05 (Ng et al., 2001).

These findings suggest that mutations in KAL1 account for a small minority of cases of hypogonadotropic hypogonadism in humans, indicating that other genes play an important role in the pathogenesis of IHH. Mutations in the FGFR1, GNRRH, NR0B1 (DAX1), GPR54, LEP and LEPR genes have been shown to cause IHH (Grumbach, 2005). Anosmia has only been reported in patients with KAL1 and FGFR1 gene mutations. Three genes (KAL1, FGFR1 and GNRRH) constitute the most frequent molecular etiologies of IHH (6.3%, 7.1% and 3.6%, respectively), but the molecular basis for most cases of IHH remains unknown (this article; Sato et al., 2004; Bhagavath et al., 2005). It is however possible that there might be un-

identified mutations in the 5′ untranslated region of KAL1.

No mutations were identified in females with IHH, suggesting that KAL1 rarely, if ever, causes phenotypic effects in females. However, it is clear that anosmia occurs in females, suggesting that other genes that affect olfactory development and GnRH function must be involved. FGFR1 is one such gene that has been shown to cause an autosomal dominant form of Kallmann syndrome (Dode et al., 2003). As mentioned earlier, FGFR1 has been postulated to interact with the anosmin-1 in effecting olfactory neuronal migration since it has similar patterns of expression during development and utilizes heparan sulfate proteoglycans (Gonzalez-Martinez et al., 2004). We are currently screening these patients for mutations in FGFR1. Another gene that has been implicated in GnRH neuron migration is NELF (Kramer and Wray, 2000), but definitive mutations have not yet been described (Miura et al., 2004).

In summary, KAL1 mutations are an uncommon cause for male Kallmann syndrome patients, occurring in about 3.7% of all IHH males and 6.3% of anosmic/hyposmic IHH males in our study popu-

lation. Female Kallmann syndrome patients are unlikely to be affected by KAL1 mutations, although it is still possible if skewed X inactiva-
tion, a coexistent X chromosome deletion or two mutant alleles are also present. There is considerable phenotypic variation among patients with mutations in KAL1 gene, although most of the patients affected have complete IHH (Oliveira et al., 2001), which our data also supports. How exactly these mutations cause Kallmann syndrome is the subject for new investigation into the molecular and cellular actions of the anosmin-1 protein.

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