A Novel Mutation of the KAL1 Gene in Kallmann Syndrome

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Abstract. Kallmann syndrome is defined by the association of hypogonadotropic hypogonadism and anosmia, for which three modes of transmission have been described: X-linked, autosomal recessive and autosomal dominant. The KAL1 gene, responsible for the X-linked form of the disease, has been isolated and its intron-exon organization determined. We report sequence analysis using PCR-direct sequencing method of the entire coding region and splice site junctions of the KAL1 gene in three males with Kallmann syndrome. We found a novel mutation in one case and no mutation in the other two cases. The mutation consisted of a C to T substitution in exon 1 converting codon 66 (CAG) encoding glutamine into a termination codon (TAG)/(Q66X). As a consequence of this mutation, the function of the KAL1 protein consisting of 680 amino acids was severely truncated so as to be consistent with Kallmann syndrome. As only this patient had unilateral renal hypoplasia among the three cases, this would suggest the existence of KAL1 gene mutation in this abnormality.

Key words: Kallmann syndrome, KAL1, Renal hypoplasia

KALLMANN syndrome is defined by the association of hypogonadotropic hypogonadism and anosmia. The first case was described by Maestre de San Juan [1]. Kallmann et al. described the first familial cases (12 cases, 3 pedigrees) in 1944 and showed the penetration of the X-linked form [2]. Although many cases have since been reported and their three modes of transmission described as X-linked, autosomal recessive, and autosomal dominant, many sporadic cases have also been reported. The incidence of this syndrome has been estimated to be in 1 per 10,000 males. It affects 5- to 7-fold more males than females, which suggests that the X-linked form is the most common. In 1989, Ballabio et al. assigned the locus for the X-linked form of this syndrome to Xp22.3, proximal to the steroid sulfatase locus [3]. In 1991, Franco et al. and Legouis et al. independently isolated the identical candidate gene, the KAL1 gene, by different positional cloning strategies [4, 5]. The KAL1 gene consists of 14 exons spanning approximately 210 kb on Xp22.3 [6, 7]. The gene has a close homologue on the Y chromosome, which is nonfunctioning. The predicted protein has been shown to have a similarity with neural migration factor [4, 5].

The pathogenetic association of hypogonadotropic hypogonadism and anosmia is based on embryologic study of olfactory nerve and Gn-RH secreting neuron development. These neurons originate in the olfactory placode which later forms the olfactory epithelium. From there, axons of olfactory neurons project to the olfactory bulb where they form synapses with the dendrites of mitral cells. Gn-RH neurons migrate along olfactory nerves to locate in the hypothalamus. Thus, Gn-RH neurons and olfactory axons share a common migration pathway [8, 9, 10]. Schwanzel-Fukuda et al. demonstrated deficiency in the migration of olfactory axons and
Gn-RH neurons by histopathological analysis of a 19-week-old human terminated fetus with X-linked Kallmann syndrome [11]. In this fetus, olfactory axons were arrested prematurely in the meninges between the cribriform plate and forebrain, and Gn-RH neurons ended their migration at the dorsal surface of the cribriform plate [10, 11]. Based on these findings, the KAL1 gene has been suggested to encode a migration factor for olfactory axons and Gn-RH neurons.

Since the KAL1 gene structure was clarified, some mutations of the KAL1 gene have been reported in many X-linked Kallmann syndrome patients [12-17]. To date, large deletions, including the entire KAL1 gene, have been reported in four families, a translocation in one family, a 3300-bp deletion in one family, small deletions in four families and point mutations in 13 families. So far, these studies have shown that point mutations are the most frequent type and that a great deal of heterogeneity in the KAL1 gene mutation in Kallmann syndrome exists. In addition, some Kallmann syndrome cases associated with the KAL1 mutation accompanied by unilateral renal aplasia, mirror movement or pes cavus deformity have been reported, thus it is likely that the KAL1 gene plays a role in various developmental systems. We report here the sequence analysis of the entire coding region and splice site junctions of the KAL1 gene in three males affected by Kallmann syndrome under our medical treatment.

Case 1

A 15-year-old boy was admitted to our hospital for delay of puberty. He was born at full term after a normal pregnancy. At the age of 2 years, he was diagnosed with bilateral retentio testes and treated with hCG for a short period. At the age of 11 years, he underwent surgery for the retentio testes. Anosmia had been apparent since infancy. The maternal grandmother had congenital dysosmia, but hypogonadism was not found among his family or relatives.

His height was 151.8 cm; weight was 50 kg. His penis was 3.0 cm in length, and his testes were both 2.0 ml in volume. Smell tests disclosed anosmia. Basal gonadotropin FSH and LH levels were within normal and below adult normal ranges, respectively, by immunoradiometric assay (FSH, SPAC-S FSH Kit, Daiichi RI, Tokyo, Japan: 7.0 mIU/ml, normal 2.9-8.2 mIU/ml; and LH, SPAC-S LH Kit, Daiichi RI: <0.5 mIU/ml, normal 1.8-5.2 mIU/ml). Serum testosterone level by radioimmunoassay was below the normal range (DPC Total Testosterone Kit, DPC, Tokyo, Japan: <0.2 ng/ml, normal 2.7-10.7 ng/ml). Serial LH-RH provocation tests showed stepwise LH and FSH elevations and then normal responses (peak LH and FSH, 7.5 mIU/ml and 9.2 mIU/ml, respectively) after 8 days of LH-RH stimulation. Evaluation of the other pituitary hormones showed normal basal levels and normal response to provocation tests. These findings were consistent with hypogonadotropic hypogonadism with anosmia, and the patient was diagnosed with Kallmann syndrome (Table 1).

Case 2

A 24-year-old man was admitted to our hospital with immature gonads. He was born at full term after a normal pregnancy. His parents noticed during his childhood that he did not recognize smells. At the age of 15 years, he was operated on for bilateral retentio testes. At the same time, he was diagnosed with hypogonadism due to his small penis and testes. Gonadotropin therapy was initiated but he discontinued it after 3 years. After discontinuation of the therapy, his gonadal development arrested at the pre-pubertal stage. Neither hypogonadism nor congenital dysosmia was found among his relatives.

On admission, his height was 169 cm, weight was 51 kg; arm span was 162.5 cm. Pubic hair was Tanner stage III, his penis was 3.2 cm in length and his testes were both 3.0 ml in volume. Smell tests disclosed anosmia.

Basal gonadotropin FSH and LH levels were both below adult normal ranges (FSH, 1.0 mIU/ml: and LH, <0.5 mIU/ml). Serum testosterone 0.8 ng/ml was low for his age. Serial LH-RH provocation tests showed stepwise LH and FSH elevations and then normal responses (peak LH and FSH, 18.3 mIU/ml and 12.5 mIU/ml, respectively) after 8 days of LH-RH stimulation. A clomiphene citrate provocation test (100 mg/day for 5 days) showed neither LH nor
FSH response. The other pituitary hormones showed normal basal levels and normal response to provocation tests. These findings were consistent with hypogonadotropic hypogonadism with anosmia, and the patient was diagnosed with Kallmann syndrome (Table 1).

**Case 3**

A 15-year-old boy was admitted to our hospital for delay of puberty. He was born at full term after a normal pregnancy. His parents were concerned about his delay in gonadal development, comparing him to his 12-year-old brother, who had already started puberty. Neither hypogonadism nor congenital dysosmia was found among his relatives.

His height was 165 cm, weight was 51 kg; arm span was 160 cm. He was muscularly weak compared with boys at the same age. His penis was 2.0 cm in length, and his testes were both 2.0 ml in volume. Smell tests disclosed anosmia.

Basal gonadotropin FSH and LH levels were below adult normal ranges and within normal, respectively, (FSH, 2.3 mIU/ml; LH, 4.9 mIU/ml). Serum testosterone 0.5 ng/ml was low for his age. Serial LH-RH provocation tests showed stepwise LH and FSH elevations and then normal responses (peak LH and FSH, 29.9 mIU/ml and 11.4 mIU/ml, respectively) after 8 days of LH-RH stimulation. A clomiphene citrate provocation test (100 mg/day for 5 days) showed neither LH nor FSH response. The other pituitary hormones showed normal basal levels and normal response to provocation tests. These findings were consistent with hypogonadotropic hypogonadism with anosmia, and the patient was diagnosed with Kallmann syndrome. Intravenous pyelography showed a filling defect in the bladder and no contrast shadows of the right ureter or kidney. Subsequent surgical findings included hypoplasia of the right kidney with an ectopic right ureteric orifice to the seminal vesicle (Table 1).

### Methods

**Genomic DNA isolation**

Genomic DNA was extracted from whole blood using cell lysis, proteinase K and NaCl (simple salting-out method) [18].

**PCR amplification**

Genomic DNA was used as a template for PCR
amplification. We used primers described by Hardelin et al. for amplification of each of the 14 exons of the KALI gene [15]. For exons 2 to 13, PCR products including the coding regions and the adjacent splice site junctions were amplified using intronic primers. For exons 1 and 14, which contained the 5′ and 3′ non-coding regions, respectively, we amplified each of the coding regions using an intronic primer and a second primer in the non-coding exonic sequence.

The PCR amplifications were performed on the genomic DNAs as follows: 94°C denaturation, 1 min; 55°C annealing (except 63°C for exons 1 and 2), 1 min; 72°C extension, 1 min; 30 cycles. For exons 1 and 10, the PCRs were carried out in 10% DMSO in order to avoid secondary structures (exon 1) or nonspecific annealing in the highly homologous Y chromosome pseudogene (exon 10) [15].

DNA sequencing

The PCR amplified fragments were gel-purified and submitted to direct sequencing. Dye-primer sequencing reactions were carried out using the Thermo Sequenase Core Sequencing kit (Amersham International plc, England). For all 14 exons, both strands were sequenced using the Hitachi sequencer SQ-3000 (Hitachi, Japan) and compared with their normal counterparts. One of three patients harbored a point mutation in exon 1. Exon 1 was then analyzed in his mother.

Restriction enzyme digestion analysis

The mutation in exon 1 created a novel Bfa I restriction site. To perform a pedigree analysis, PCR products of exon 1 of the patient, the patient’s mother, aunt and grandmother were digested overnight with Bfa I and analyzed on agarose gel.

Results

No mutation in the KALI gene was found in case 1 or case 2.

In case 3, only a single nonsense point mutation was identified in the entire protein coding sequences of the KALI gene. This was a novel mutation, a C to T transition in exon 1 causing a termination codon for glutamine at the 66th codon (Q66X) (Fig. 1). The patient’s unaffected mother was heterozygous for the mutation. The Q66X mutation was not detected in the patient’s aunt or grandmother (Fig. 2).

Discussion

The KALI gene encodes the putative KAL1 protein with 680 amino acids including a leader peptide. The mature protein consists of a cysteine-rich N-terminal region, including a whey acidic protein (WAP) four-disulphide-core motif, and four contiguous fibronectin type III repeats (Fig. 3) [4, 5]. Since the WAP motif has been described in several proteins with

![Fig. 1. Sequence analysis of exon 1 at the antisense site of the KALI gene. In case 3, a point mutation was identified in exon 1 of the KALI gene. This mutation consisted of a C to T substitution converting codon 66 (CAG) encoding glutamine into a termination codon (TAG). The patient’s unaffected mother was heterozygous for the mutation.](image-url)
anti-serine protease activity and fibronectin type III repeats are found in several extracellular matrix components involved in morphogenetic processes through cell adhesion properties, it is hypothesized that the KAL1 protein may be an extracellular matrix component with anti-protease and cell adhesion functions.

We found a KAL1 mutation in one Kallmann syndrome patient. This mutation consisted of a C to T substitution in exon 1 converting codon 66 (CAG) encoding glutamine into a termination codon (TAG) (Q66X). Since the patient's unaffected mother was heterozygous for the mutation and we did not find the mutation in the patient's aunt or grandmother, we determined this mutation to be a germline mutation starting de novo from the patient's mother.

Small deletions and point mutations in the KAL1 gene have been reported previously [15, 16, 17] (Fig. 2). The patient's pedigree analysis by restriction enzyme digestion analysis. The mutation created a novel BfaI restriction site. To perform a pedigree analysis, PCR products of exon 1 were digested overnight with BfaI and analyzed on an agarose gel. The Q66X mutation was not detected in the patient's aunt or grandmother.

Fig. 2. The patient's pedigree analysis by restriction enzyme digestion analysis. The mutation created a novel BfaI restriction site. To perform a pedigree analysis, PCR products of exon 1 were digested overnight with BfaI and analyzed on an agarose gel. The Q66X mutation was not detected in the patient's aunt or grandmother.

Fig. 3. Correlation of the KAL1 gene structure with the distribution of predicted domains in the KAL1 protein. The mature protein consists of a cysteine-rich N-terminal region, including a whey acidic protein (WAP) four-disulphide-core motif, and four contiguous fibronectin type III repeats.
The mutation sites thus far reported are between exon 5 and exon 12, and not in exon 1. Thus Q66X is the first mutation reported in exon 1 and the first mutation to be reported in Japan. Because Q66X was found in exon 1, the truncated KAL1 protein would be more severely affected than with other KAL1 gene mutations and would result in Kallmann syndrome.

Sequence analysis of the entire coding region and splice site junctions of the KAL1 gene failed to detect any mutations in the other two cases. There are four possible explanations. The first is the possibility of a different mode of inheritance, either autosomal dominant [19] or autosomal recessive [20], of the disease. The second is that according to Hardelin et al. [15], the mutation could lie in the non-coding part (150 bp) of exon 1 or in the large non-coding part (4120 bp) of exon 14. The latter might be essential for stability of the KAL1 transcript, as suggested by the recent molecular characterization of the Xp22.3/Yq11 translocation in a patient affected by Kallmann syndrome [13]. The mutation could also lie within an intron, creating a new splicing site, or the mutation could lie in the promoter region, resulting in an altered transcription rate. The third possibility is the existence of another X-linked gene responsible for Kallmann syndrome in the two cases. The existence of another gene mutation close to the KAL1 gene has not been excluded [15]. The fourth possibility is that the Kallmann syndrome observed

<table>
<thead>
<tr>
<th>Exon</th>
<th>KAL1 gene</th>
<th>Mutation site and Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td></td>
<td>Q66X→STOP</td>
<td>Izumi et al. (1999) (this report)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Trp237→STOP</td>
<td>Quinton et al. (1996)</td>
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<tr>
<td>5</td>
<td></td>
<td>Arg257→STOP</td>
<td>Hardelin et al. (1993)</td>
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<tr>
<td>5</td>
<td></td>
<td>Trp258→STOP</td>
<td>Georgopulos et al. (1997)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Asn267→Lys frameshift at 277</td>
<td>Hardelin et al. (1993)</td>
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<tr>
<td>5</td>
<td></td>
<td>Tyr278→STOP frameshift at 339</td>
<td>Georgopulos et al. (1997)</td>
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<tr>
<td>5</td>
<td></td>
<td>Gln417→STOP frameshift at 400</td>
<td>Georgopulos et al. (1997)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Arg423→STOP frameshift at 464</td>
<td>Hardelin et al. (1993)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Phe517→Leu</td>
<td>Georgopulos et al. (1997)</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>frameshift at 566</td>
<td>Quinton et al. (1996)</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>Splice mutation</td>
<td>Hardelin et al. (1993)</td>
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Fig. 4. Sites of small deletions and point mutations in the KAL1 gene. The mutation sites previously reported are between exon 5 and exon 12, and not in exon 1. (•: nonsense mutation, ◊: missense mutation, ↔: frameshift, ▲: splice mutation)
in the two cases was not a genetic disorder.

The three patients that we studied presented similar and typical manifestations of the illness, except that the case 3 patient also showed right renal hypoplasia (Table 1). This is the same patient in which we found the point mutation in the KAL1 gene. Previously reported point mutation cases have been associated with unilateral renal aplasia [15, 16]. Thus, the KAL1 gene may play a part in renal development. Conversely, if we observe Kallmann syndrome together with this abnormality, we must suspect that the etiology is genetic and caused by KAL1 gene mutation.

As Q66X was found in exon 1, the resulting KAL1 protein in this case would be expected to lose its function almost completely compared to other point mutations and selectively compared to gene deletions. However, this particular patient lacked the other symptoms of Kallmann syndrome associated with the KAL1 gene mutations such as pes cavus deformity and mirror movement. Moreover, in the one family reported with X-linked Kallmann syndrome, one case was accompanied by unilateral renal aplasia and the other was not [16]. This suggests that the gene associated with the accompanying symptoms such as pes cavus deformity, mirror movement or unilateral renal aplasia may be caused not only by the KAL1 gene but by other cooperative genes or factors.

Recently, the chicken KAL (KALc) homologue was isolated and studied [21-24]. Sequence comparison between the predicted human and chicken protein products revealed 77% amino acid identity. By overexpressing both the human and chick KALc DNAs in eukaryotic cells, the KAL1 protein has been elucidated as a glycosylated peripheral membrane protein with an apparent molecular weight of approximately 100 kDa [21]. In situ hybridization studies in chick embryos demonstrated that KALc is expressed at high levels at the mitral cells, which are the secondary sensory neurons forming synapses with the olfactory axons in the olfactory bulb [22-24]. Thus, it is suggested that a defect of neuronal interaction or synaptogenesis is the primary defect in Kallmann syndrome.

Analysis of missense mutations of the KAL1 gene may be a key to clarifying the function of the KAL1 protein and the etiology of Kallmann syndrome. We anticipate that new mutations in the KAL1 gene will be reported and the KAL1 protein functions will be made clear.

Note added in proof: After this manuscript was submitted, researchers reported a family with Kallmann syndrome that was a result of a KAL1 gene mutation in exon 1 [25].

References

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