The Importance of Autosomal Genes in Kallmann Syndrome: Genotype-Phenotype Correlations and Neuroendocrine Characteristics*

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ABSTRACT

Kallmann syndrome (KS) consists of congenital, isolated, idiopathic hypogonadotropic hypogonadism (IHH) and anosmia. The gene responsible for the X-linked form of KS, *KAL,* encodes a protein, anosmin, that plays a key role in the migration of GnRH neurons and olfactory nerves to the hypothalamus. In addition to X-linked pedigrees, autosomal dominant and recessive kindreds with KS have been reported. The relative importance of these autosomal *vs.* X-linked genes in producing KS, and the frequency of *KAL* mutations, are currently unknown because these are rare disorders and large series are unusual.

We examined 101 individuals with IHH (± anosmia) and their families to determine their modes of inheritance, incidence of mutations in the coding sequence of *KAL,* genotype-phenotype correlations, and [in a subset (n = 38)] their neuroendocrine phenotype.

Of the 101 patients, 59 had true KS (IHH + anosmia/hyposmia); whereas, in the remaining 42, no anosmia was evident in the patients or their families. Of the 59 KS patients, 21 were familial, whereas 38 were sporadic cases. Mutations in the coding sequence of *KAL* were identified in only 3 of 21 familial cases (14%) and 4 of 38 (11%) of the sporadic cases. Of the X-linked cases confirmed by mutational analysis, only 1 of 3 pedigrees appeared X-linked by inspection whereas the other 2 contained only affected brothers. Female members of known *KAL* mutation families (n = 3) exhibited no reproductive phenotype and were not anosmic, whereas families with anosmic women (n = 3) were not found to carry mutations in *KAL*. Mutations were uniformly absent in nonanosmic IHH probands (n = 42), as well as in families with both anosmic and nonanosmic members (n = 2). Overall, 4 novel mutations were identified (C172R, R191×, R457×, and delC@L600).

With respect to neuroendocrine phenotype, KS men with documented *KAL* mutations (n = 8) had completely apulsatile LH secretion, whereas those with autosomal modes of inheritance demonstrated a more variable spectrum with evidence of enfeebled (but present) GnRH-induced LH pulses.

Our conclusions are: 1) Confirmed mutations in the coding sequence of the *KAL* gene occur in the minority of KS cases, *i.e.* only 14% of familial and 11% of sporadic cases; 2) The majority of familial (and presumably sporadic) cases of KS are caused by defects in at least two autosomal genes that are currently unknown; 3) Obligate female carriers in families with *KAL* mutations have no discernible phenotype; 4) *KAL* mutations are uniformly absent in patients with either normosmic IHH or in families with both anosmic and nonanosmic individuals; and 5) Patients with *KAL* mutations have apulsatile LH secretion consistent with a complete absence of GnRH migration of GnRH cells into the hypothalamus, whereas evidence of present (but enfeebled) GnRH-induced LH pulses may be present in autosomal KS cases. Taken together, these findings suggest that autosomal genes account for the majority of familial cases of KS, and that unique neuroendocrine phenotypes consistent with some GnRH neuronal migration may exist in these patients. (*J Clin Endocrinol Metab* **86:** 1532–1538, 2001)

I N 1944, KALLMANN first described the clinical association of hypogonadotropic hypogonadism and anosmia (1) that has been classified as Kallmann syndrome (KS) ever since. In 1992, the first genetic defect in the *KAL* gene in Xp 22.3 was described (2); and the physiology and developmental biology of the protein it encodes, anosmin, is undergoing characterization (3). In the X-linked form, KS is characterized by a failure of hypothalamic GnRH secretion caused by a migratory defect of GnRH neurons from the olfactory placode to the hypothalamus (4). In addition, the associated anomalies of unilateral renal agenesis and synkinesia have been suggested to be specific to the X-linked form (5–7). These phenotypic abnormalities seem to have correlates in the developmental pattern of *KAL* gene transcripts in the chick embryo (8).

However, in the human, KS is characterized by considerable clinical and genetic heterogeneity. Families have been documented with individuals having full KS, normosmic idiopathic hypogonadotropic hypogonadism (IHH), and isolated anosmia within a single sibship (9). To shed further light on this heterogeneity, we examined a broad spectrum of patients with IHH, with and without anosmia, to deter-

mine their clinical spectrum, modes of inheritance, incidence of mutations in the coding sequence of *KAL*, and neuroendocrine phenotypes.

Our results demonstrate that autosomal genes are clearly responsible for the majority of both familial and sporadic KS and that these cases have clinical and neuroendocrine phenotypes that distinguish them from the X-linked form.

Subjects and Methods

Experimental subjects

One hundred and one probands with IHH were evaluated. In all patients, complete IHH was documented based on the following previously published criteria: age more than 18 yr; clinical signs and symptoms of hypogonadism; prepubertal testosterone (<100 ng/dL) and estradiol levels (<20 pg/mL); 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 (10). Patients were questioned as to their ability to smell, because the subjective reporting of anosmia has proven a reliable marker of this symptom (9).

Modes of inheritance. Genetic criteria were used to establish the likely mode of disease transmission as outlined in previous analyses (9). A family was classified as X-linked if only males were affected and unaffected females could be considered carriers. There could be no male-to-male transmission. A family was considered autosomal dominant if direct transmission of the phenotype was demonstrable across generations. Male-to-male transmission was considered definitive evidence for dominant inheritance. A family was classified as autosomal recessive if all affected individuals were members of the same generation and included at least one female. Consanguinity provided additional support for this designation. Delayed puberty and isolated anosmia were used as surrogate markers of the phenotype (9).

Despite these basic genetic criteria, it is not always possible to correctly distinguish between different modes of genetic transmission. For example, a nonpenetrant, dominant genetic trait may be expressed in a male grandparent and a male child but not in a male parent, thereby appearing X-linked. In addition, heterozygous female carriers with partial expression have been documented in numerous X-linked disorders. Finally, kindreds with only affected brothers within a single sibship may represent any of the major modes of genetic inheritance, including dominant, recessive, and X-linked forms (for the purposes of this study, these latter kindreds were labeled as indeterminate). Therefore, recognizing these ambiguities, the best effort was made to classify families appropriately, with the knowledge that the *KAL* mutation analysis performed in this study would provide additional clarification.

Methods and materials

Mutation detection. Mutation detection was carried out by multiple methods of analysis. In the first method, a sequence polymorphism, located 8.7 kb downstream from the 3′ end of the *KAL* gene, was used (11) to examine the possibility of association of this locus with IHH in affected individuals of four families. Other DNA samples were analyzed using temperature gradient gel electrophoresis (TGGE) or single-stranded conformational polymorphism (SSCP) on PCR products generated with exon-specific primers based on adjacent intronic sequences. All abnormalities discovered by TGGE and SSCP screening were confirmed by direct sequencing.

Genomic DNA was extracted from peripheral blood leukocytes using standard procedures (12). PCR oligonucleotide primers were based on those published by Hardelin *et al.* (5) or designed using Lasergene software (DNASTAR Inc., Madison, WI). The latter primers are: exon 1: 5′CGCGGCGGCTGCCTGGTCCTC3′ (forward) and 1: 5′GGCCGCAG-CCCC-AGAAAGAACC3′ (reverse); exon 2: 5′TTAAAGTAGAAATCT-GTGTATGTG3′ (forward); exon 7.1: 5′ATGCACAGGGATGTCCG-GCTC3′ (reverse); exon 7.2: 5′CAACAGTGATGGGAGT GTGAC3′ (forward); exon 8: 5′TCTCCCTCCATTGTGCCTTGTTGTGAT3′ (reverse); exon 9.1: 5′ACTTGCAGTTGGCCATCCTGA3′ (reverse); exon 9.2: 5′AGAAAAGGTGGAATTCAAACA3′ (forward); exon 11.1: 5′TTGCT-AGCATAATTCTCTTTCTCTTG3′ (forward); exon 11.1: 5′TTCCCCT-TAAGAGCAGAGCAT3′ (reverse); exon 11.2: 5′TCCTGCAAGTAT-AAG G T GACTGTCCA3′ (forward); exon 13: 5′GCCTGGAATA-GAATCTAGTAAGTC3′ (reverse); exon 14: 5′AGGGTCTCGTGCTTTT-TCTACAAAT3′ (forward). A 40-base GC-clamp was incorporated on the 5′-end of either the forward or reverse primer to increase the sensitivity of the TGGE technique (13, 14).

Before TGGE, the theoretical melting temperature of the predicted PCR product corresponding to each pair of primers was determined using WinMelt software (Bio-Rad Laboratories, Inc., Hercules, CA). These predictions allowed the optimization of primer design for each particular exon. Exons 7, 9, and 11 were each divided into two overlapping segments (*i.e.* exons 7.1 and 7.2) that were independently amplified in different reactions because computer analyses indicated that these full-length exons would not yield the uniform melting curves required for TGGE.

PCR reactions were performed in a vol of 100 μL containing 50 pmol of each PCR primer, 200 μmol of each deoxynucleotide triphosphate, 2.5U *Taq* polymerase, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl2, 50 mmol/L KCl, 0.01% gelatin, and 200 ng genomic DNA. Amplification of exon 1 was carried out in the presence of 8% dimethylsulfoxide. PCR was performed in a Gene Amp 9600 system (Perkin-Elmer Corp., Foster City, CA) using 35 cycles. DNA was denatured at 95 C for 5 min in the first cycle and for 30 sec in all subsequent cycles. Annealing was performed at 53–69.7 C for 30 sec, depending on the exon. Elongation was performed at 72 C for 30 sec, with a final elongation of 10 min. Amplification was confirmed by agarose gel electrophoresis.

TGGE. Samples were analyzed, as previously described, using a TGGE apparatus (Diagen, Düsseldorf, Germany) (15). The temperature gradient, running time, position of the loading wells for parallel TGGE, and gel concentration were established by diagonal TGGE of each PCR product. Each product was examined in the absence and presence of a similar amplified segment from a healthy volunteer to look for abnormal patterns. PCR products that revealed a heteroduplex or mutant homoduplex were ethanol-precipitated and sequenced (500-ng DNA template/reaction) on an ABI200 377 automated DNA sequencer (Perkin-

TABLE 1. Patient population by diagnosis, mode of inheritance, and neuroendocrine studies

	101 Probands with hypogonadotropic hypogonadism screened for *KAL* mutations					
Anosmia	Yes; n = 59 with KS				No; n = 42 with IHH	
Inheritance	Sporadic n = 38	Familial n = 21			Sporadic n = 23	Familial n = 13
KAL Mutations	4	1/1 X-L			0	0/1 X-L
		2/6 Indeterminate				0/1 Indeterminate
		0/8 AD				0/4 AR
		0/6 AR				0/7 AD
Neuroendocrine studies	KAL gene		KAL gene			
	NL	Mutant	NL	Mutant[a]		
	n = 10	n = 2	n = 2	n = 7	n = 16	n = 0
Apulsatile LH	7	2	1	7	10	0
Low Amplitude LH	3	0	1	0	6	0

[a] Patients with novel mutations as described in this study (n = 3) and patients previously described by our group (n = 4) (19).

Elmer Corp.). Positive controls created by PCR-directed mutagenesis (16, 17) were included on each gel to optimize the TGGE gel conditions and check the sensitivity.

SSCP. SSCP was performed, as previously described (18), for exon 4 because of difficulties in optimizing the condition for this exon on the TGGE apparatus. PCR was performed with the inclusion of ^{32}P deoxy-ATP. Twenty microliters of PCR product was mixed with 0.06% SDS, heated at 90 C for 3 min, and rapidly chilled on ice. Four microliters of each sample was loaded onto a 6% acrylamide gel with 10% glycerol (49:1 acrylamide to bisacrylamide) and run at 4 C for 14 h at 6 V. The gel was dried and exposed to x-ray film (Biomax MS, Eastman Kodak Co., Rochester, NY) at −80 C for 1 h.

Pulsatile LH secretion. Thirty-seven patients were admitted to the General Clinical Research Center of the Massachusetts General Hospital for overnight blood sampling (q 10 min), for 12–24 h, to assess LH levels. The presence of LH pulses was determined by a modification of the method of Santen and Bardin (16, 17), as previously described. Individuals were considered as having apulsatile LH secretion if they had 1 peak or less in 24 h, and low amplitude LH secretion if the mean amplitude was less than 2 sds of mean LH amplitude of 10.2 ± 1.8 IU/L, as identified in 20 normal men (10). All immunoassays were performed according to previously published methods (10, 16).

Results

Pedigree characteristics

Of the 101 IHH probands evaluated, 59 were anosmic (KS), whereas 42 had a normal sense of smell (IHH) (Table 1). Of the 59 KS cases, 21 were familial. By pedigree inspection, only a single family seemed X-linked (*i.e.* only affected males in multiple generations and females as obligate carriers). Eight families had vertical transmission of partial or complete KS phenotypes, suggesting dominant transmission. In 6 of 8 pedigrees, probands with complete KS had family members in preceding generations with only isolated anosmia but normal reproductive function. In another 6 families, affected individuals included both affected males and females within sibships; 2 of these families were also notable for consanguinity, suggesting an autosomal recessive mode of inheritance. An additional 6 families consisted of 2 or more affected brothers in a single sibship without affected females; these families were labeled as being indeterminate. Of the 42 IHH cases with normal olfaction, 13 were familial. Pedigree information was not available for 6 of the 42 cases.

### Correlations of genotype to pedigree characteristics

Using the dinucleotide repeat polymorphism downstream from *KAL*, allele patterns eliminated linkage between *KAL* and KS in 4 kindreds. Using TGGE and direct sequencing, mutations in the *KAL* gene were found in only 2 subgroups: 11% (4 of 38) of sporadic KS cases and in 14% (3 of 21) of KS familial cases (the single X-linked family and in 2 of 6 families with only affected brothers). Stated alternatively, all families with vertical transmission of a partial or complete KS phenotype and all families with affected males and females in a single sibship were free of detectable *KAL* mutations. Similarly, no mutations in the coding sequence of the *KAL* gene were identified in any of the 42 nonanosmic IHH probands nor in families in which both KS and normosmic IHH patients coexisted (n = 2). Genetically proven female carriers of *KAL* mutations in 3 families all had normal pubertal development, with regular menstrual cycles and normal olfaction.

TABLE 2. Genotype-phenotype characteristics of 9 probands with Kallmann syndrome due to *KAL* mutations

Genotype				Phenotype					
Exon	Nucleotide & AA change	PT	Mode of inheritance	Cryptorchidism	Microphallus	UL Renal agenesis	Testicular volume/testosterone	Other	LH Pattern
4	TGT to CGT Cys[172] to Arg	1	3 Brothers affected	No (BL in both brothers)	No	No (+ in one brother)	TV = 1–2 mL; T = not available	Synkinesia	Apulsatile
5	CGA to TGA Arg[191] to Stop	2	Sporadic	UL	No	No	TV = 1–2 ml; T = 143 ng/dl		Apulsatile
		3	Sporadic	BL	Yes	No	TV = 1 ml; T = 48 ng/dl		N/A
7[a]	TAC to TAG Tyr[328] to Stop	4[a]	X-linked	No	No	N/A	TV = 1–2 ml; T = 62 ng/dL	High-arched palate; Color blindness	Apulsatile
8[a]	11 Base deletion Splice at Asn[400]	5[a]	X-linked	BL	No	N/A	TV = 1–2 ml; T = 95 ng/dl		Apulsatile
10[a]	14 Base deletion Frameshift at Pro[164]	6[a]	Sporadic	No	No	N/A	TV = 1 ml; T = not available	Color blindness	Apulsatile
10	CGA to TGA Arg[457] to Stop	7	X-linked	UL	No	N/A	TV = 8 ml (R); T = 10 ng/dl		Apulsatile
		8	Sporadic	UL	Yes	Yes	TV = 1–2 ml; T = 1 ng/dl		Apulsatile
12	1 Base deletion (C) Frameshift at Leu[600]	9	2 Brothers affected	No	No	N/A	TV = 2 ml; T = 95 ng/dl	Synkinesia	Apulsatile

[a] Patients previously described (19). UL/BL, Uni/bilateral; N/A, not accessed; TV, testicular volume; T, testosterone.

FIG. 1. *KAL* gene mutations and anosmin. Schematic representation of the *KAL* gene, indicating the mutations described in the literature (*arrows above the gene*) and the novel mutations reported in the present study (*arrows below the gene*), in relation to anosmin structure.
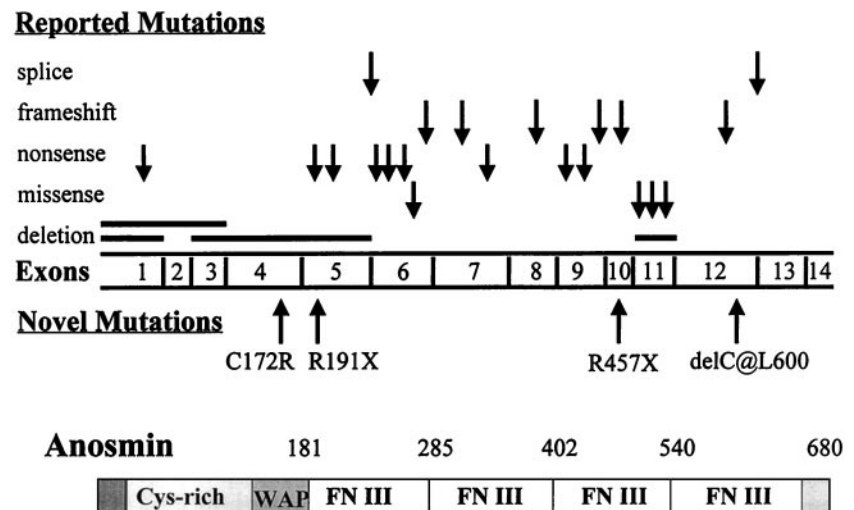
Conversely, kindreds with anosmic women who gave birth to sons with KS did not harbor *KAL* mutations (n = 3).

Mutations, polymorphisms, and genotype/phenotype correlations

The four novel mutations in the *KAL* gene detected in the present study (C172R, R191×, R457×, and delC@L600) are depicted in Table 2 along with their phenotype, in addition to mutations published previously by our group (19). All were found in patients in whom hypogonadotropic hypogonadism was accompanied by anosmia (Fig. 1). One mutation corresponds to a T-to-C substitution at nucleotide 664 (TGT to CGT) in exon 4 (the WAP region), changing cysteine into arginine at codon 172 (C172R). This mutation was found in each of three brothers with KS. The second novel mutation consists of a C-to-T substitution at nucleotide 721 in exon 5 (CGA to TGA) that changes an arginine into a stop at codon 191 (R191×) (fibronectin III repeat domain). This mutation was detected in two cases of sporadic KS. In the third novel mutation, a T substitutes for a C at nucleotide 1519, changing codon 457 from an arginine to a stop codon (CGA to TGA, R457×) (fibronectin III repeat domain). This mutation was detected in two unrelated patients, one with sporadic KS and the other with X-linked KS. The final mutation consists of a single base deletion of a C at nucleotide 1951 (codon 600), causing a frameshift in exon 12, and a stop codon (TAA) to be introduced at position 618 (exon 13). This mutation was identified in two brothers.

We also found two previously described polymorphisms (20). The first is a substitution of an A for a G at codon 534, changing valine into isoleucine. This change was identified in 67% of our population, a somewhat higher percentage, compared with previous reports of 48% in normals (20). The second polymorphism is a T-to-C substitution in exon 12 at codon 611 that was detected in 32% of our patients. This conservative base pair change (isoleucine) has been previously identified in 24% of controls (20). Polymorphisms previously described in exons 2, 13, and 14 (19, 20) were not detected in our population.

The clinical characteristics of the patients with *KAL* mu-

tations are described in Table 2. Individuals with the same mutation presented with different phenotypes, even within the same family. For example, two unrelated KS individuals presented with the same mutation in exon 5 (R191×) but different phenotypes. One patient was born with microphallus and bilateral cryptorchidism (patient 3), whereas the other individual had only right cryptorchidism (patient 2). Lack of concordance between genotype and phenotype was also observed within families. In the KS family with three affected brothers all harboring the C172R mutation, synkinesia was present in all three, bilateral cryptorchidism was present in two, but only one brother had unilateral renal aplasia.

Neuroendocrine patterns of secretion

Thirty-seven individuals who underwent mutation analysis, as described here and in a previous report by our group (19), underwent frequent blood sampling (21 KS and 16 IHH). Seventy-three percent (n = 27) exhibited an apulsatile pattern of LH secretion (≤1 LH peak in 24 h), and 27% (n = 10) presented with low amplitude pulses (three or more pulses in 24 h) (Table 1). All individuals bearing *KAL* mutations that were studied with frequent blood sampling (n = 8) demonstrated an apulsatile pattern of LH secretion. Three of these eight individuals were members of the same family. Figure 2 shows the data for three representative individuals. A shows a proband (patient 5 in Table 1) from an X-linked pedigree with a documented *KAL* mutation (splice mutation at Asn[400]) and with an apulsatile pattern of LH secretion during a q 10-min blood sampling study. B shows a proband from a familial KS pedigree, albeit indeterminate in its mode of inheritance, with no *KAL* coding sequence mutation. The pattern of LH secretion for this proband is again apulsatile but with some possible evidence of LH secretion. C shows a male proband from an autosomal dominant pedigree with no detectable *KAL* coding sequence mutation. However, the neuroendocrine profile of this proband demonstrates not only measurable LH levels but also two LH pulses.
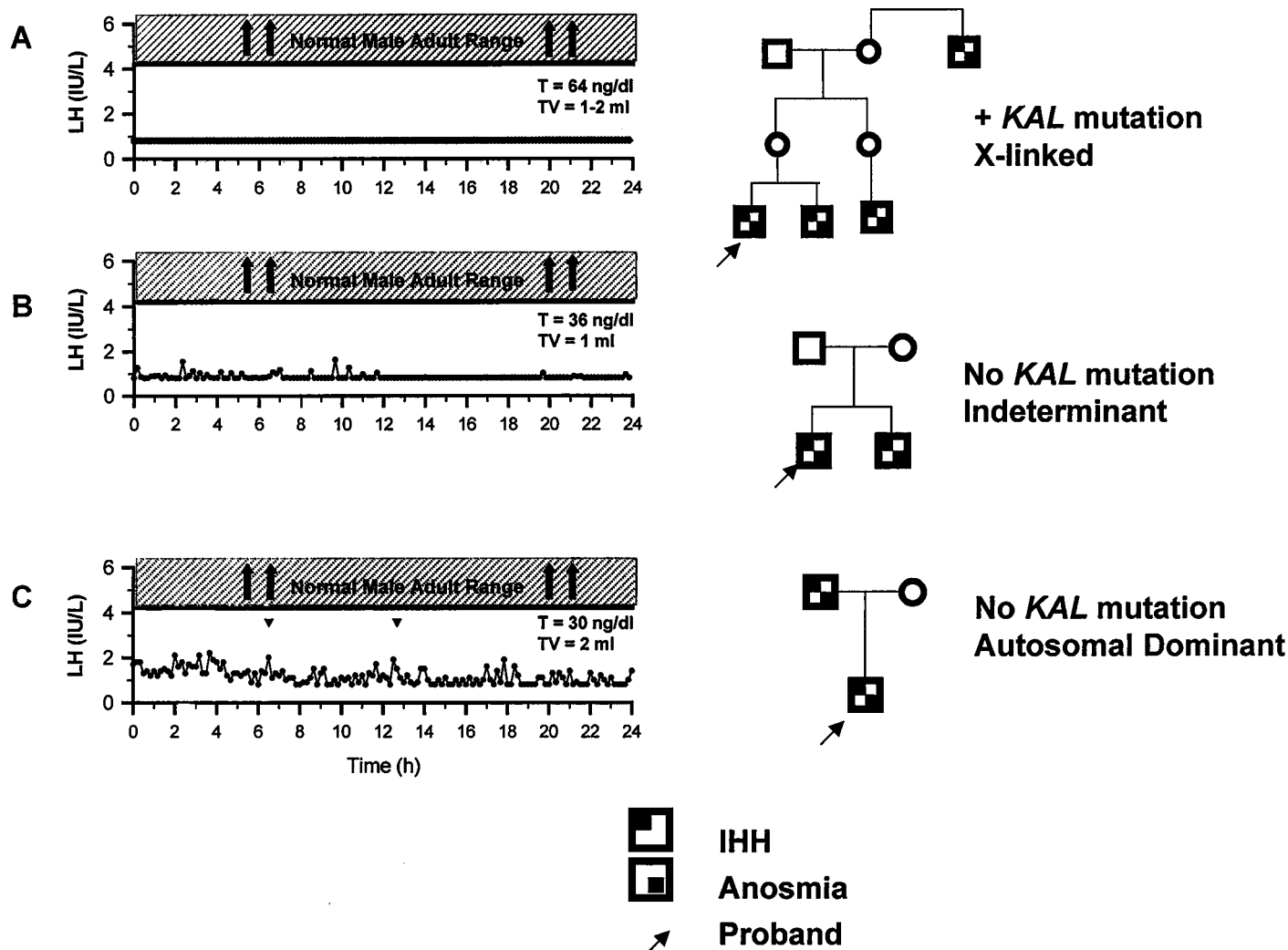
FIG. 2. LH secretion in KS. A, B, and C, Baseline secretory pattern of LH in three individuals with KS (*arrow*) assessed at 10-min intervals; *shaded area*, lower limit of range of serum LH concentrations defined in normal men (35); T, serum testosterone measured in pooled samples constituted from equal aliquots of the frequent blood samples; TV, testicular volumes recorded using a Prader orchidometer; *inverted triangles*, LH pulses.

Discussion

This study supports and quantifies the importance of autosomal genes in producing KS. Whereas much attention has been focused upon the discovery, analysis, and clinical features of cases with the X-linked form of KS, this study makes it clear that most cases of familial (and presumably sporadic) KS are either autosomal dominant or recessive in their etiology. It is also clear that there are striking phenotypic differences between those with mutations in the *KAL* gene and those with autosomal modes of inheritance.

Of the 101 probands, 59 of which were anosmic and 21 of 59 which were familial, only a minority of the kindreds had a pedigree consistent with X-linkage. However, it was theoretically possible that KS caused by *KAL* mutations might include a more variable phenotype; hence, a comprehensive mutational screening approach was taken to address this issue. However, this study does not support the expansion of the X-linked KS phenotype to include patients with IHH with normal olfaction, families in which individuals with

normal and impaired sense of smell coexist, nor a female carrier state in which anosmia/hyposmia or reproductive abnormalities occur. However, X-linked cases with confirmed mutations in *KAL* did exhibit a spectrum of phenotypes both within and across families with identical genotypes. In addition, they all demonstrated a complete absence of LH pulsations during frequent blood sampling. Given the evidence of failure of GnRH neurons to migrate beyond the cribiform plate in a single case of KS in whom a *KAL* gene defect was confirmed (4), this complete absence of GnRH-induced LH pulses fits with the failure of these neurons to arrive in the medial basal hypothalamus and thus participate in the induction of pulsatile LH secretion. Conversely, the presence of low-amplitude LH pulses in autosomal cases documents some degree of GnRH secretion. Thus, the neuroendocrine profiles should be considered as an additional intermediary phenotype in this complex disorder. For the moment, the genes underlying the autosomal cases of KS remain to be discovered.

Our findings demonstrate that the frequency of mutations in the coding sequence of the *KAL* gene is low in both familial and sporadic Kallmann patients. Whereas coding sequence mutations in the *KAL* gene have previously been reported in as many as 50–60% of familial cases (5, 6, 20), these investigators have largely focused upon X-linked kindreds. However, the mode of inheritance of pedigrees included in these prior studies has occasionally been ambiguous (5). In addition, at least three families with apparent X-linked inheritance have been reported with no *KAL* coding sequence mutations, suggesting that mutations may occur in noncoding regions of the *KAL* gene (5, 6). However, polymorphic allele patterns of these pedigrees were not examined to determine that these kindreds were, in fact, linked to the *KAL* locus. Therefore, the formal possibilities remain that another X-linked gene causes KS or that these families were not truly X-linked. Nevertheless, our finding of only three *KAL* mutations in the entire cohort of anosmic familial cases (n = 21), combined with other pedigree features, certainly demonstrates that the majority of familial KS is attributable to autosomal (and not X-linked) genes.

Since the *KAL* gene escapes X inactivation, obligate carrier mothers of KS patients harbor both normal and mutated alleles. The presumption that these women have a decrease in anosmin levels raises the possibility of a partial phenotype in either reproductive or olfactory function. Partial expression of X-linked diseases in female carriers has been described in numerous conditions (21–24). In fact, our group has described female carriers in another X-linked form of hypogonadotropic hypogonadism (congenital adrenal hypoplasia) with a reproductive phenotype (25). Previous studies have never clearly addressed whether *KAL* mutations may be present in normosomic patients. Kirk *et al.* (26) performed olfactory testing for KS in eight female carriers who were identified on the basis of the birth of an affected son. No genetic data were available on these patients except for a single family that was known to harbor a 7Mb interstitial deletion on Xp. As a group, the female KS carriers had a lower median threshold for detection of all odorant classes, compared with the control groups. However, considerable overlap of the KS carriers with unaffected relatives was apparent. Nonetheless, these authors concluded that obligate female carriers were hyposmic, but with considerable overlap with normals, making olfactory testing unsatisfactory for detection of carrier status.

Therefore, this study sought to examine whether anosmic women with normal reproductive function might actually have *KAL* mutations. We evaluated three families in which anosmic women gave birth to sons with KS, and none had any mutation in the coding sequence of the *KAL* gene. Conversely, three obligate female *KAL* mutant carriers, identified in our screening program, had no olfactory defect by history (although formal testing was not performed). The absence of documented mutations in the *KAL* coding sequence in women with anosmia in KS families and the apparently normal olfactory function in female carriers with documented *KAL* mutations provide additional evidence against isolated anosmia being a partial phenotype of *KAL* mutations. The occurrence of isolated anosmia (without IHH) as an alternative phenotypic manifestation of the same genetic

defect within a given KS family thus seems to be a feature of the autosomal dominant cases that demonstrates variable expressivity.

In addition to examining isolated anosmia as a possible surrogate marker for *KAL* mutations, we also examined the possibility that normosmic IHH might be a partial phenotype for KS. However, two pedigrees in which siblings had both IHH and KS also did not exhibit a *KAL* mutation.

Variable expression of other phenotypic features associated with KS (*i.e.* synkinesia, renal agenesis, and cryptorchidism) has been well documented. However, neuroendocrine profiles of LH secretion have not been reported in detail for this condition. Previous studies have shown that the GnRH neurons originate in the olfactory placode and migrate into the brain along branches of the terminalis and vomeronasal nerves (27). Schwanzel-Fukuda *et al.* (4) studied a KS fetus with a deletion from Xp 22.31 to Xpter, *i.e.* encompassing the entire *KAL* gene, and demonstrated that migration of the GnRH and olfactory neurons was arrested just below the telencephalon at the cribiform plate. No GnRH expression in cells or fibers was detected in the brain, yet dense clusters of GnRH cells and thick fascicles of GnRH fibers remained in the nose. Mutations in *KAL* (and by extension, anosmin) seem to cause premature failure of migration of the olfactory neurons and, consequently, of the GnRH neurons to the brain. We demonstrated that eight patients with mutations in the coding sequence of the *KAL* gene that were studied with 10-min blood sampling exhibited uniformly apulsatile patterns of LH secretion and, by extension, a complete absence of GnRH secretion. Though other familial cases may also demonstrate an apulsatile pattern, we identified a familial, non-X-linked case that had evidence of low-amplitude LH pulses (Fig. 1). Thus, it seems that some non-X-linked KS cases may not be attributable to abnormalities in GnRH migration, having some GnRH-induced LH signaling, albeit attenuated, present. Obviously, additional neuroendocrine studies are required to address this issue. Interestingly, however, at least by the data presented in this study, apulsatile LH secretion may be one of the more consistent phenotypic features of KS attributable to *KAL* mutations.

Previously described mutations in the *KAL* gene are distributed throughout the gene, although most point mutations cluster in the four fibronectin type III repeat (FNIII) domains (Fig. 1) (5, 6, 19, 20, 28–31). However, mutations in the cysteine-rich region were also published in KS patients presenting with synkinesia and renal malformations, the most common nonreproductive abnormalities associated with this syndrome. The C172R mutation described in the WAP-region was identified in a family with three KS brothers, all of whom presented with synkinesia in addition to their reproductive disorder. One brother in this family also had unilateral renal aplasia, as described previously for mutations in the FNIII region. Two unrelated patients with the *KAL* mutation, W258×, have been reported (6); one had bimanual synkinesia, left renal aplasia, and cryptorchidism whereas the other had no such phenotypic anomalies. Hardelin *et al.* (5) described another patient with the same W258× mutation, presenting with synkinesia and unilateral renal aplasia but no cryptorchidism. Thus, there seems to be extensive variability in the phenotypic expression of mutations

both within and between families (2, 5, 6, 19, 20, 28, 30–34). Even though the mutations are more frequently located in the FNIII region, there do not seem to be hot spots in the KAL gene. There is also no correlation between phenotype and location of the mutation (5).

In summary, a small proportion of both familial and sporadic cases of KS carry *KAL* mutations. Considering just the familial cases, *KAL* mutations were identified only in families that were clearly X-linked or in which only brothers were affected. No mutations were found in nonanosmic IHH patients in families with both IHH and KS patients or in families with anosmic women with normal reproductive function and KS males. In our small population of genetically proven female carriers, anosmia does not seem to represent a partial phenotype. Affected individuals with *KAL* gene mutations have apulsatile LH secretion. Autosomal genes causing KS exist, seem to account for the majority of cases, and may have unique neuroendocrine phenotypes.

Acknowledgments

References

1. **Kallmann FJ, Schoenfeld WA.** 1944 The genetic aspects of primary eunuchoidism. Am J Ment Def. 158:203–236.
2. **Bick D, Franco B, Sherins RJ, et al.** 1992 Brief report: intragenic deletion of the KALIG-1 gene in Kallmann's syndrome. N Engl J Med. 326:1752–1755.
3. **Hardelin JP, Julliard AK, Moniot B, et al.** 1999 Anosmin^{-1} is a regionally restricted component of basement membranes and interstitial matrices during organogenesis: implications for the developmental anomalies of X chromosome-linked Kallmann syndrome. Dev Dyn. 215:26–44.
4. **Schwanzel-Fukuda M, Bick D, Pfaff DW.** 1989 Luteinizing hormone-releasing hormone (LHRH)-expressing cells do not migrate normally in an inherited hypogonadal (Kallmann) syndrome. Mol Brain Res. 6:311–326.
5. **Hardelin JP, Levilliers J, Blanchard S, et al.** 1993 Heterogeneity in the mutations responsible for X chromosome- linked Kallmann syndrome. Hum Mol Genet. 2:373–377.
6. **Quinton R, Duke VM, de Zoysa PA, et al.** 1996 The neuroradiology of Kallmann's syndrome: a genotypic and phenotypic analysis. J Clin Endocrinol Metab. 81:3010–3017.
7. **Quinton R, Duke VM, de Zoysa PA, Bouloux P-MG.** 1996 The neurobiology of Kallmann's syndrome. Hum Reprod. 11:121–127.
8. **Legouis R, Lievre CA, Leibovici M, Lapointe F, Petit C.** 1993 Expression of the KAL gene in multiple neuronal sites during chicken development. Proc Natl Acad Sci USA. 90:2461–2465.
9. **Waldstreicher J, Seminara SB, Jameson JL, et al.** 1996 The genetic and clinical heterogeneity of gonadotropin-releasing hormone deficiency in the human. J Clin Endocrinol Metab. 81:4388–4395.
10. **Spratt DI, Carr DB, Merriam GR, Scully RE, Rao PN, Crowley Jr WF.** 1987 The spectrum of abnormal patterns of gonadotropin-releasing hormone secretion in men with idiopathic hypogonadotropic hypogonadism: clinical and laboratory correlations. J Clin Endocrinol Metab. 64:283–291.
11. **Bouloux P-MG, Hardelin JP, Munroe P, et al.** 1991 A dinucleotide repeat polymorphism at the Kallmann locus (Xp22.3). Nucleic Acids Res. 19:5453.
12. **Sambrock J, Fritsch EF, Maniatis T.** 1989 Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press.
13. **Abrams ES, Murdaugh SE, Lerman LS.** 1990 Comprehensive detection of single base changes in human genomic DNA using denaturing gradient gel electrophoresis and a GC clamp. Genomics. 7:463–475.
14. **Sheffield VC, Cox DR, Lerman LS, Myers RM.** 1989 Attachment of a 40-base-pair G+C-rich sequence (GC clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. Proc Natl Acad Sci USA. 86:232–236.
15. **Schipani E, Weinstein LS, Bergwitz C, et al.** 1995 Pseudohypothyroidism type 1b is not caused by mutations in the coding exons of the human parathyroid hormone (PTH)/PTH-related peptide receptor gene. J Clin Endocrinol Metab. 80:1611–1621.
16. **Hayes FJ, McNicholl DJ, Schoenfeld D, Marsh EE, Hall JE.** 1999 Free alpha-subunit is superior to luteinizing hormone as a marker of gonadotropin-releasing hormone despite desensitization at fast pulse frequencies. J Clin Endocrinol Metab. 84:1028–1036.
17. **Santen RJ, Bardin CW.** 1973 Episodic luteinizing hormone secretion in man. Pulse analysis, clinical interpretation, physiologic mechanisms. J Clin Invest. 52:2617–2628.
18. **Hayashi K.** 1991 PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. PCR Methods Appl. 1:34–38.
19. **Georgopoulos NA, Pralong FP, Seidman CE, Seidman JG, Crowley Jr WF, Vallejo M.** 1997 Genetic heterogeneity evidenced by low incidence of KAL-1 gene mutations in sporadic cases of gonadotropin-releasing hormone deficiency. J Clin Endocrinol Metab. 82:213–217.
20. **Maya-Nunes G, Zenteno JC, Ulloa-Aguirre A, Kofman-Alfaro S, Mendez JP.** 1998 A recurrent missense mutation in the KAL gene in patients with X-linked Kallmann's syndrome. J Clin Endocrinol Metab. 83:1650–1653.
21. **Moser H, Emery AE.** 1974 The manifesting carrier in Duchenne muscular dystrophy. Clin Genet. 5:271–284.
22. **Kaladhar Reddy B, Anandavalli TE, Reddi OS.** 1984 X-linked Duchenne muscular dystrophy in an unusual family with manifesting carriers. Hum Genet. 67:460–462.
23. **Rousseau F, Heitz D, Oberle I, Mandel JL.** 1991 Selection in blood cells from female carriers of the fragile X syndrome: inverse correlation between age and proportion of active X chromosomes carrying the full mutation. J Med Genet. 28:830–836.
24. **Kling S, Coffey AJ, Ljung R, et al.** 1991 Moderate haemophilia B in a female carrier caused by preferential inactivation of the paternal X chromosome. Eur J Haematol. 47:257–261.
25. **Seminara SB, Achermann JC, Genel M, Jameson JL, Crowley Jr WF.** 1999 X-linked adrenal hypoplasia congenita: a mutation in DAX1 expands the phenotypic spectrum in males and females. J Clin Endocrinol Metab. 84:4501–4509.
26. **Kirk JMW, Grant DB, Savage MO, Besser GM, Bouloux P-MG.** 1994 Identification of olfactory dysfunction in carriers of X-linked Kallmann's syndrome. Clin Endocrinol (Oxf). 41:577–580.
27. **Legouis R, Cohen-Salmon M, del Castillo I, et al.** 1993 Characterization of the chicken and quail homologues of the human gene responsible for the X-linked Kallmann syndrome. Genomics. 17:516–518.
28. **del Castillo I, Cohen-Salmon M, Blanchard S, Lutfalla G, Petit C.** 1992 Structure of the X-linked Kallmann syndrome gene and its homologous pseudogene on the Y chromosome. Nat Genet. 2:305–310.
29. **Hardelin JP, Levilliers J, Young J, et al.** 1993 Xp22.3 deletions in isolated familial Kallmann's syndrome. J Clin Endocrinol Metab. 76:827–831.
30. **Ballabio A, Zoghbi H.** 1995 Kallmann syndrome. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill; 4549–4557.
31. **O'Neill MJ, Tridjaja B, Smith MJ, Bell KM, Warne GL, Sinclair AH.** 1998 Familial Kallmann syndrome: a novel splice acceptor mutation in the KAL gene. Hum Mutat. 11:340–342.
32. **Parenti G, Rizzolo MG, Ghezzi M, et al.** 1995 Variable penetrance of hypogonadism in a sibship with Kallmann syndrome due to a deletion of the KAL gene. Am J Med Genet. 57:476–478.
33. **Maya-Nunes G, Cuevas-Covarrubias S, Zenteno JC, Ulloa-Aguirre A, Kofman-Alfaro S, Mendez JP.** 1998 Contiguous gene syndrome due to deletion of the first three exons of the Kallmann gene and complete deletion of the steroid sulphatase gene. Clin Endocrinol (Oxf). 48:713–718.
34. **Colquhoun-Kerr JS, Gu W-X, Jameson JL, Withers S, Bode HH.** 1999 X-linked Kallmann syndrome and renal agenesis occurring together and independently in a large Australian family. Am J Med Genet. 83:23–27.
35. **Spratt DI, O'Dea LStL, Schoenfeld D, Butler J, Rao PN, Crowley Jr WF.** 1988 Neuroendocrine-gonadal axis in men: frequent sampling of LH, FSH, and testosterone. Am J Physiol. 254:E658–E666.