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Mutation analysis of the KAL Gene in Female Patients with Gonadotropin-Releasing Hormone Deficiency

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Isolated gonadotropin-releasing hormone (GnRH) deficiency, including Kallmann's syndrome (KS) and idiopathic hypogonadotropic hypogonadism (IHH), is a congenital disorder, which is characterized by a functional deficit in hypothalamic GnRH secretion. Despite recent advances in the understanding of the pathogenesis of the X-linked form of KS as the identification of the KAL gene (Xp22.3), the genetic basis of the sporadic form in female patients remains unclear. Although most searches for mutations in X chromosome have been reported in males, the newly recognized phenomenon of inheritance, such as genomic imprinting and uniparental disomy, raises the possibility of a female phenotype in the X-linked genetic defect. Here, the molecular study of the coding region of the KAL gene (exon 5 to 14) in 10 unrelated females with KS (n=6) or IHH (n=4) is reported. None of the subjects had familial histories of delayed puberty or hypogonadism. Samples from 4 healthy, unrelated female volunteers were used for identification of polymorphisms. PCR of the 10 exons of the KAL gene was performed on genomic DNA. The PCR products of the 10 exons were subject to single strand conformation polymorphism (SSCP) analysis to identify possible mutations. In an SSCP analysis of the amplified fragments (fragment size: 147 to 302bp), no mutations or polymorphisms were found in any of the 10 patients and 4 controls.

In conclusion, it is unlikely that KAL gene mutations are a clinically significant cause of sporadic GnRH deficiency in female patients, indicating the existence of defects in unidentified genes that result in the expression of the phenotypes in females.

Key Words: Genetics, gonadotropin-releasing hormone deficiency, KAL gene, Kallmann's syndrome, SSCP analysis

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INTRODUCTION

Isolated GnRH deficiency, including Kallmann's syndrome (KS) and idiopathic hypogonadotropic hypogonadism (IHH), is a well recognized congenital disorder, characterized by a functional deficit in hypothalamic GnRH secretion.[1-3] In females, this rare condition has the typical clinical features of sexual infantilism, primary amenorrhea and the impact of estrogen deprivation, such as osteoporosis and cardiovascular disease. Isolated GnRH deficiency is diagnosed through extremely low plasma levels of gonadal steroids, inappropriately low/normal gonadotropins and irreversible features of clinical hypogonadism. A hypogonadotropic state results from a deficient hypothalamic secretion of GnRH, which may be explained by a defect in the migration of GnRH neurons to the hypothalamus.[4]

Because several mode of inheritance, including X-linked, autosomal recessive and autosomal dominant patterns, have been documented in segregation analyses of familial cases,[5-9] it has been suggested that there are multiple genes responsible for the pathogenesis of this disease. In recent years, a candidate gene for the X-linked Kallmann's syndrome was mapped to the Xp22.3 region,[10,11] known as the KAL gene, whose predicted protein has homology with neural cell adhesion mole-

cules, which may be involved in the process of GnRH neurons migration from their origin in the olfactory placode to the hypothalamus.[12-15]

Although the findings of mutations and genetic deletions in the KAL gene have provided some explanations of the pathogenesis of the X-linked form of Kallmann's syndrome, the precise gene alterations of most patients with the sporadic form of GnRH deficiency, especially in females, remains unclear. Recent studies found the incidences of genetic defects within the coding region of the KAL gene in male patients with sporadic and familial GnRH deficiency were considerably low, and suggested that the X-linked form of inheritance represented the least common form of this disease.[16,17]

Over the past years, X-linked genetic disorders, characterized clinically by a predominance of affected males, the presence of unaffected female carriers and an absence of male to male transmission, were hardly considered to be the cause of the phenotypic manifestations in females. However, the newly recognized phenomenon of inheritance, such as uniparental disomy and genomic imprinting, have allowed for the possibility of the expression of the X-linked genetic disorders in female patients.[18,19] For this reason, the frequencies of the mutations and genetic deletions in the KAL gene in female patients with the sporadic form of KS or IHH were studied using single strand conformation polymorphism (SSCP) analysis of the PCR amplified DNA to elucidate their clinical significance.

MATERIALS AND METHODS

Patients

Ten unrelated females with isolated GnRH deficiency, aged 17-35 yr, were included in this study. All patients were determined to be sporadic from their detailed family histories that showed the absence of hypogonadism, delayed puberty or anosmia in any known relative.

The patients were evaluated for isolated GnRH deficiency, according to criteria previously described.[3,20] All 10 patients showed long-standing clinical hypogonadism, with no evident cause, gonadal steroid levels in the hypogonadal range (serum estradiol < 20 pg/ml), despite an age of 18 yr or greater, normal or low gonadotropin range, normal baseline levels of TSH and prolactin and normal radiological imaging of the hypothalamo-pituitary region. Although one patient with IHH was 17 yr and 3 months of age when her blood test was taken, the absence of further pubertal development at the age of 18 yr was confirmed through a follow up examination. To define any abnormality of the hypothalamo-pituitary and olfactory areas sella MRI was performed in nine patients and sella CT in the other one.

The clinical, laboratory and radiological features of the 10 patients are summarized in Table 1.

To distinguish isolated GnRH deficiency from the physiological delay of puberty, a GnRH stimulation test (single 2.5μg/kg IV dose of native GnRH) was performed in all of the 4 patients with IHH, with the results shown in Table 1.[21,22] The results of the insulin-induced hypoglycemia test were only available for three patients (patient No. 7, 8 and 10) with IHH, and showed normal levels of GH and cortisol.

Five patients with primary amenorrhea and anosmia, who were defined as having Kallmann's syndrome, did not have a GnRH stimulation test, and the diagnosis of GnRH deficiency was made on the basis of their delayed puberty and primary amenorrhea at the age of 20 - 35 yr, low basal gonadotropins (LH range, 0.1 - 2.5 mIU/ml), undetectable estradiols (< 13 pg/ml) and clinical hypogonadism. Although the results of the formal olfactory function test were only available for 2 individuals (patient No. 3 and 5), 4 of the 5 patients with clinical anosmia showed developmental defects in the olfactory sulcus or bulb in the sella imaging study (Fig. 1).[23]

One female (patient No. 6) was compatible with Kallmann's syndrome, based on the result of the sella MR imaging (bilateral agenesis of the olfactory bulbs and hypoplastic olfactory sulci), but the presence of anosmia could not be defined due to her mental retarded clinical status.

Methods

Genomic DNA extraction

Blood was collected in EDTA coated tubes.

Table 1. Clinical, Radiological and Laboratory Characteristics of the 10 Female Patients with Isolated GnRH Deficiency

Patient No	Age (yr)	Diagnosis	Olfactory status	Olfactory sulcus	E_2 (pg/ml)	Stimulated(60')* LH/FSH(mIU/ml)	Affected family
1	24	KS	Anosmic	Hypoplastic	< 13.0	Not performed	None
2	30	KS	Anosmic	Normal	< 13.0	Not performed	None
3	35	KS	Anosmic	Hypoplastic	< 13.0	Not performed	None
4	31	KS	Anosmic	Hypoplastic	< 13.0	Not performed	None
5	18	KS	Anosmic	Hypoplastic	< 13.0	Not performed	None
6	17	KS(?)†	Unknown	Hypoplastic	< 13.0	Not performed	None
7	21	IHH	Normosmic	Normal	< 13.0	2.9/3.1	None
8	18	IHH	Normosmic	Normal	< 13.0	2.4/2.7	None
9	20	IHH	Normosmic	Normal	< 13.0	1.2/3.0	None
10	19	IHH	Normosmic	Normal	15.8	0.2/1.8	None

KS, Kallmann's syndrome; IHH, idiopathic hypogonadotropic hypogonadism.
*Single 2.5 μg/kg IV dose of native GnRH (FACTREL, gonadrelin hydrochloride).
†The presence of anosmia could not be defined due to patient's mentally retarded clinical status.

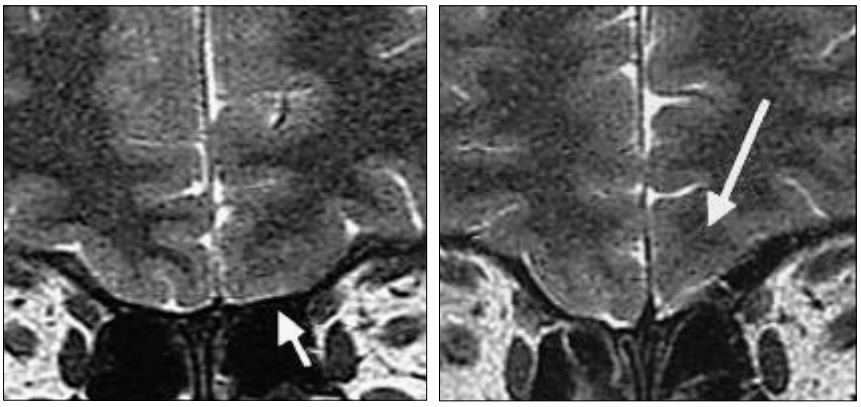

Fig. 1. Coronal MR images show the absence of the normal olfactory sulcus (short arrow) and bulb (long arrow) (patient No.3).

Total genomic DNA was isolated from the blood (6 ml) by the standard method.[24] The genomic DNA was suspended in TE buffer, pH 8, and stored at -20℃. Genomic DNA from healthy, unrelated female volunteers was used to identify any polymorphisms and mutations.

PCR amplification

Genomic DNA (100 ng) was used as a template for the amplification of each exon. Of the 14 exons of the KAL gene, 10 (exon 5-14, known locations of mutations and polymorphism in the previous studies) encoding the fibronectin type III repeats were examined in this study.[16,25] The primers used for the PCR amplifications were identical to those described by Hardelin et al.[25]

The PCR reactions were carried out in a total reaction mixture volume of 50 μl, containing 100 ng genomic DNA, 0.5 unit *Taq*, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 100 pM of the primers. PCR amplifications were performed for 30 cycles on a thermal cycler (Perkin Elmer Instrument, Gene Amp PCR System 2400), with denaturation at 94℃ for 30 s, annealing at 56℃ for 30 s and extension at 72℃ for 1 min. The PCR products of the patients and controls were then screened by single strand conformation polymorphism (SSCP).

SSCP analysis

For the single strand conformational polymorphism, the polymerase chain reaction products (fragment size 147-302 bp) of the patients and

controls were diluted, 1:1, in a dye solution, containing 20 mM EDTA, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol, and then denatured at 99℃ for 10 min, and immediately chilled on ice. The reaction products were loaded onto 1X MDE gel and underwent electrophoresis in 1X TBE buffer for 5 hours.

RESULTS

Each of the 10 exons of the coding region of the KAL gene was separately amplified by PCR, and then subject to the SSCP analysis. No mutations were identified in any of the 10 patients screened by SSCP. No polymorphisms were found in 10 patients and 4 controls. One of the representative autoradiogram is shown in Fig. 2.

DISCUSSION

Since the first identification, in 1992, of genetic defects in the KAL gene,[26] the search for mutations has been performed in various groups of KS and IHH patients. The KAL gene, which spans a 210 kb region of the genomic DNA in Xp 22.3, has 14 coding exons and encodes a protein, anosmin, and shares homology with molecules involved in neuronal migration and axonal pathfinding.[12,13]

Although the finding of KAL mutations in patients with KS has demonstrated that this gene

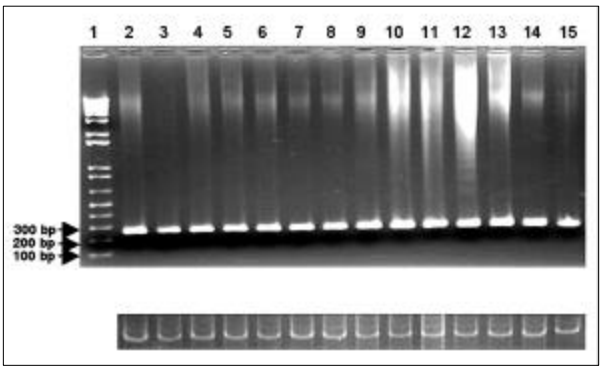

Fig. 2. A. PCR amplification of exon 5 (267 bp) of the KAL gene (Xp22.3) in 10 patients with isolated GnRH deficiency (lanes 6-15) and 4 controls (lanes 2-5). Lane 1: DNA ladder (multiples of 100 bp). B. SSCP picture of exon 6 (218 bp) of the KAL gene. Two single-stranded bands are normally apparent in all of the 10 patients and 4 controls.

is responsible for the X-linked form of the disease, the genetic basis of the majority of patients with sporadic GnRH deficiency remains unclear. Most cases of GnRH deficiency in humans are sporadic, and the incidence of genetic defects within the coding region of the KAL gene in patients with sporadic GnRH deficiency is low (5-8%).[5,16,27] A recent report by Oliveira et al. strongly indicated that the majority of both familial and sporadic KS were either autosomal dominant or recessive in their etiology, and caused by defects in at least two, currently unknown, autosomal genes.[17] Nevertheless, the strong suspicion of genetic defects of the autosomal genes in GnRH deficiency, the autosomal KS (and IHH) loci, remain to be identified.

In our series of 10 patients with GnRH deficiency, there were no identifiable familial members with delayed puberty, hypogonadism or anosmia. Because of the rarity of the disease, and the universal clinical features of infertility, a lack of a documented familial history of GnRH deficiency is no guarantee of 'genuine' sporadic. However, it can be expected that the frequency of spontaneous mutations that account for this syndrome could be fairly high in these non-familial cases.

There is little information on the percentage of spontaneous mutations that lie on the X chromosome, including the KAL gene, for the female patients with KS or IHH. Previous studies have never clearly addressed if KAL mutations are present in female patients without a familial history. Since the KAL gene escapes X inactivation, obligate KS patient carriers harbor both normal and mutated alleles, and are hardly considered to have a phenotypic expression. However, the partial expression of X-linked diseases in female carriers has been described in numerous conditions, and the disease can manifest in the hemizygous state,[28,29] perhaps because of genomic imprinting with the differential expression of genes inherited on the paternal and maternal chromosomes.[18] Furthermore, the rare genetic phenomenon, such as uniparental disomy can lead to the expression of a recessive disease in patients.[30]

In this series, PCR-SSCP analysis was used, but failed to identify any genetic defects in the 10 exons (exon 5 to 14) of the KAL gene. Because most of the mutations and polymorphisms iden-

tified in previous studies were located in these axons,[16,25] this result precludes genetic defects in the KAL gene from being the common cause of sporadic GnRH deficiency in female patients. However, in patients with familial KS, and proven X-linked kindred, no mutations have been documented throughout the coding sequence of the KAL gene in an important percentage of the patients, suggesting that mutations may occur in the noncoding regions of this gene, or in other X-linked genes.[25,31,32]

In this study, no polymorphic changes, previously described by another group, were observed.[32] For the rapid detection of sequence variations of the DNA fragments amplified by PCR, SSCP analysis was used. The SSCP technique is a method capable of identifying most sequence variations in a single strand of DNA, typically between 150 - 250 nucleotides in length. The fragment sizes of the 10 exons in this study ranged from 147 to 302 bp. Although it is known that the optimum DNA fragment size for SSCP analysis is approximately 200 bp or less, no restriction enzymes were used for the digestion of the larger fragments. Theoretically, SSCP analysis is capable of identifying point mutations and polymorphisms. However, the failure to detect novel polymorphisms in this study limits the accuracy of this method in the identification of point mutations in the coding region of the KAL gene.

In conclusion, attempts to identify any genetic defects, including gene deletions, major rearrangement or point mutations, within the coding region of the KAL gene (exon 5 to 14), using PCR-SSCP analysis, failed in our series of female patients with sporadic GnRH deficiency. Considering the sensitivity of PCR-SSCP analysis, the alteration of the KAL gene has been eliminated as the common basis for female GnRH deficiency. However, the definitive exclusion of point mutations in female patients with GnRH deficiency will require the cloning and sequencing of the KAL gene from larger numbers of affected individuals.

REFERENCES

1. Naftolin F, Harris GW, Bobrow M. Effect of purified luteinizing hormone releasing factor of normal and hypogonadotropic anosmic men. Nature 1971;232:496-7.
2. Hoffman AR, Crowley Jr WF. Induction of puberty in men by long-term pulsatile administration of low-dose gonadotropin-releasing hormone. N Engl J Med 1982; 307:1237-41.
3. Spratt DI, Carr DB, Merriam GR, Scully RE, Rao PN, Crowley Jr WF. The spectrum of abnormal patterns of gonadotropin-releasing hormone secretion in men with idiopathic hypogonadotropic hypogonadism: clinical and laboratory correlations. J Clin Endocrinol Metab 1987;64:283-91.
4. Schwanzel-Fukuda M, Bick D, Pfaff DW. Luteinizing hormone-releasing hormone (LHRH) expressing cells do not migrate normally in an inherited hypogonadal (Kallmann) syndrome. Mol Brain Res 1989;6:311-26.
5. Waldstreicher J, Seminara SB, Jameson JL, Geyer A, Nachtigall LB, Boepple PA, et al. The genetic and clinical heterogeneity of gonadotropin-releasing hormone deficiency in the human. J Clin Endocrinol Metab 1996;81:4388-95.
6. Chaussain JL, Toublanc JE, Feingold J, Naud C, Vassal J, Job JC. Mode of inheritance in familial cases of primary gonadotropin deficiency. Horm Res 1988;29:202-6.
7. Dean JCS, Johnston AW, Klopper AI. Isolated hypogonadotropic hypogonadism: a family with autosomal dominant inheritance. Clin Endocrinol 1990;32:341-7.
8. Santen RJ, Paulsen CA. Hypogonadotropic eunuchoidism. I. Clinical study of the mode of inheritance. J Clin Endocrinol Metab 1972:36:47-54.
9. White BJ, Rogol AD, Brown KS, Lieblich JM, Rosen SW. The syndrome of anosmia with hypogonadotropic hypogonadism: a genetic study of 18 new families and a review. Am J Med Genet 1983;15:417-35.
10. Petit C, Levilliers J, Weissenbach J. Long-range restriction map of the terminal part of the short arm of the human X chromosome. Proc Natl Acad Sci USA 1990; 87:3680-4.
11. Ballabio A, Bordoni B, Guioli S, Basler E, Camerino G. Two families of low-copy number repeats are interspersed on Xp22.3: implications for the high frequency of deletions in this region. Genomics 1990;8:263-70.
12. Franco B, Guioli S, Pragliola A, Inceti B, Bardoni B, Tonlorenzi R, et al. A gene deleted in Kallmann's syndrome shares homology with neural cell adhesion and axonal path-finding molecules. Nature 1991;353:529-36.
13. Legouis R, Hardelin JP, Levilliers J, Claverie JM, Compain S, Wunderle V, et al. The candidate gene for the X-linked Kallmann syndrome encodes a protein related to adhesion molecules. Cell 1991;67:423-35.
14. Schwanzel-Fukuda M, Abraham S, Crossin KL, Edelman GM, Pfaff DW. Immunocytochemical demonstration of neural cell adhesion molecule(NCAM) along the migration route of luteinizing hormone-releasing hormone(LHRH) neurons in mice. J Comp Neurol 1992; 321:1-18.
15. Nadia SY, Castro F, Julliard AK, Perfettini I, Chedotal A, Petit C. Anosmin-1, defective in X-linked form of

Kallmann syndrome, promotes axonal branch formation from olfactory bulb output neurons. Cell 2002;109: 217-28.
16. Georgopoulos NA, Pralong FP, Seidman CE, Seidman JG, Crowley Jr WF, Vallejo M. Genetic heterogeneity evidenced by low incidence of KAL-1 gene mutations in sporadic cases of gonadotropin-releasing hormone deficiency. J Clin Endocrinol Metab 1997;82:213-7.
17. Oliveira LMB, Seminara SB, Beranova M, Hayes FJ, Valkenburgh SB, Schipani E, et al. The importance of autosomal genes in Kallmann syndrome: genotype-phenotype correlations and neuroendocrine characteristics. J Clin Endocrinol Metab 2001;86:1532-8.
18. Hall JG. Genomic imprinting and its clinical implications. N Engl J Med 1992;326:827-9.
19. Crowley Jr WF, Jameson JL. Clinical counterpoint: Gonadotropin-releasing hormone deficiency: perspectives from clinical investigation. Endocr Rev 1992;13: 635-40.
20. Ibanez L, Potau N, Zampolli M, Virdis R, Gussinye M, Carrascosa A, et al. Use of leuprolide acetate response patterns in the early diagnosis of pubertal disorders: comparison with the gonadotropin-releasing hormone test. J Clin Endocrinol Metab 1994;78:30-5.
21. Bessar GM, McNeilly AS, Anderson DC, Marshall JC, Harsoulis P, Hall R, et al. Hormone responses to synthetic luteinizing hormone and follicle stimulating hormone in man. Br Med J 1972;3:267-71.
22. Rosenfield RL. Puberty and its disorders in girls. Endocrinol Metab Clin North Am 1991;20:15-42.
23. Knorr JR, Ragland RL, Brown RS, Gelber N. Kallmann's syndrome: MR findings. Am J Neuroradiol 1993;14:845-51.
24. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual, ed 2. New York: Cold Spring Harbor Laboratory Press; 1989. p.14.7
25. Hardelin J-P, Levilliers J, Blanchard S, Carel J-C, Leutenegger M, Pinard-Bertelletto J-P, et al. Heterogeneity in the mutations responsible for X chromosome-linked Kallmann syndrome. Hum Mol Genet 1993;4:373-7.
26. Bick D, Franco B, Sherins RJ, Heye B, Pike L, Crawford J, et al. Brief report: intragenic deletion of the KALIG-1 gene in Kallmann's syndrome. N Engl J Med 1992;326: 1752-5.
27. Quinton R, Duke VM, Robertson A, Kirk JMW, Matfin G, de Zoysa PA, et al. Idiopathic gonadotrophin deficiency: genetic questions addressed through phenotype characterization. Clin Endocrinol 2001;55:163-74.
28. Moser H, Emery AE. The manifesting carrier in Duchenne muscular dystrophy. Clin Genet 1974;5:271-84.
29. Rousseau F, Heitz D, Oberle I, Mandel JL. 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 1991;28:830-6.
30. Voss R, Ben-Simon E, Avital A, Godfrey S, Zlotogora J, Dagan J, et al. Isodisomy of chromosome 7 in a patient with cystic fibrosis: could uniparental disomy be common in humans? Am J Hum Genet 1989;45: 373-80.
31. Quinton R, Duke VM, de Zoysa PA, Platts AD, Valentine A, Kendall B, et al. The neuroradiology of Kallmann's syndrome: a genotypic and phenotypic analysis. J Clin Endocrinol Metab 1996;81:3010-7.
32. Maya-N G, Zenteno JC, Ulloa-Aguirre A, Kofman-Alfaro S, Mendez JP. A recurrent missense mutation in the KAL gene in patients with X-linked Kallmann's syndrome. J Clin Endocrinol Metab 1998;83:1650-3.