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Cytogenetic Analysis and Detection of KAL-1 Gene Deletion With Fluorescence In Situ Hybridization (FISH) in Patients With Kallmann Syndrome

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Recebido em 06/11/00
Revisado em 05/03/01
Aceito em 16/04/01

ABSTRACT

Kallmann syndrome (KS) is a disease clinically characterized by the association of hypogonadotropic hypogonadism and anosmia or hyposmia, for which three modes of transmission have been described: X-linked, autosomal recessive and autosomal dominant. The KAL-1 gene, responsible for the X-linked form of the disease, has been isolated and its intron-exon organization determined. In this study, two families with X-linked KS and four sporadic male patients with hypogonadotropic hypogonadism and anosmia were cytogenetically investigated with high-resolution techniques and FISH. Chromosomal analysis did not reveal any rearrangements or deletions. Deletion of the KAL-1 gene was detected by FISH in only one sporadic patient, with the typical features of KS and a high palate. Among the familial cases renal abnormalities and pes cavus deformity were observed. **(Arq Bras Endocrinol Metab 2001;45/6:552-557)**

Unitermos: Kallmann syndrome; Deletion KAL-1; FISH; Renal abnormalities.

RESUMO

A síndrome de Kallmann (SK) é caracterizada clinicamente pela associação de hipogonadismo hipogonadotrófico e anosmia ou hiposmia, para a qual três modos de herança foram descritos: ligada ao X, autossômica dominante e recessiva. O gene KAL-1, responsável pela forma da síndrome ligada ao X, foi isolado e sua organização éxon-íntron determinada. Neste estudo, duas famílias com síndrome de Kallmann ligada ao X e quatro indivíduos do sexo masculino com hipogonadismo hipogonadotrófico e anosmia foram citogeneticamente investigados por meio de técnicas de alta-resolução e FISH. A análise citogenética não revelou qualquer rearranjo cromossômico. A deleção do gene KAL-1 foi detectada por FISH em apenas um caso esporádico, em um paciente com sinais característicos de SK e palato alto. Entre os casos familiais foram observadas anomalias renais e pes cavus. **(Arq Bras Endocrinol Metab 2001;45/6:552-557)**

Keywords: Síndrome de Kallmann; Deleção KAL-1; FISH; Anomalias renais.

KALLMANN SYNDROME (KS) is a disease clinically characterized by the association of hypogonadotropic hypogonadism and anosmia or hyposmia. The hypogonadism is due to insufficient release of gonadotropin releasing hormone (GnRH) from the hypothalamus (1), while anosmia has been related to agenesis of the olfactory bulbs (2). Since this disorder was described, patients with hypogonadotropic hypogonadism have classically been divided into those with anosmia (KS) and those with normal olfaction [idiopathic hypogonadotropic hypogonadism (IHH)].

Olfactory testing is available to characterize sense of smell and differentiate the individuals with KS from IHH (3,4), although this differentiation may be difficult because of variability in expression of anosmia in KS (5). In addition, magnetic resonance imaging may also be helpful due

to the detection of olfactory anatomic abnormalities in most, but not all, KS patients (6).

Though mainly sporadic, familial cases were first reported by Kallmann in 1944 (7). Subsequent segregation analyses revealed X chromosome-linked, autosomal recessive and autosomal dominant modes of transmission indicating genetic heterogeneity (8-10). The incidence of KS has been estimated as one in 10,000 males and one in 50,000 females (11). The 5 to 7 fold excess of affected males versus females originally suggested that the X-linked mode (MIM 308700) of inheritance was the most frequent (12). However, it has recently been demonstrated that the X-linked form of the disease accounts for the minority of patients and that most affected subjects are due to mutations in autosomal genes (13,14).

The *KAL-1* gene for X-linked KS was isolated by two independent groups, using approaches typically employed in positional cloning. Characterization of the *KAL-1* gene structure revealed the presence of 14 exons spanning approximately 210kb on Xp22.3 and shown to encode a protein sharing homology with molecules involved in neuronal migration and axonal pathfinding (15,16). The gene escapes X-inactivation and has a closely related homologue on the Y chromosome, which is nonfunctional (17,18). The finding of mutations in patients affected by KS has demonstrated that the *KAL-1* gene is responsible for the X-linked form of the disease (19-21). Furthermore, some of the additional clinical anomalies occasionally observed in KS, such as mirror movements (21-23), *pes cavus* deformity (22), unilateral renal aplasia (24), and high-arched palate (25) could be ascribed to the altered *KAL-1* gene.

KS rarely occurs as the result of a deletion involving only the *KAL-1* gene (19,21). Deletions of this gene are most frequently observed in males with a contiguous gene syndrome, including the loss of genes for ichthyosis, chondrodysplasia punctata, mental retardation and short stature in the distal short arm of the human X chromosome (26,27).

The development of a fluorescence *in situ* hybridization (FISH) probe for the *KAL-1* gene and its use in complementary routine diagnostic procedures can contribute to the etiologic investigation of hypogonadotropic hypogonadism. In the present study, high-resolution chromosome technique and FISH analysis were used to evaluate the cytogenetic status of four males from two families with the X-linked form and four sporadic cases of KS.

SUBJECTS AND METHODS

Four sporadic cases of KS and four patients derived from two families were included in this study. In the first family (figure 1A) two members were affected by KS and the X-linked mode of transmission was determined according to the following criteria: presence of asymptomatic females carriers, presence of another affected male in the maternal family or among male siblings, absence of affected females, and absence of male to male transmission. In the second family (figure 1B), two males with hypogonadotropic hypogonadism were

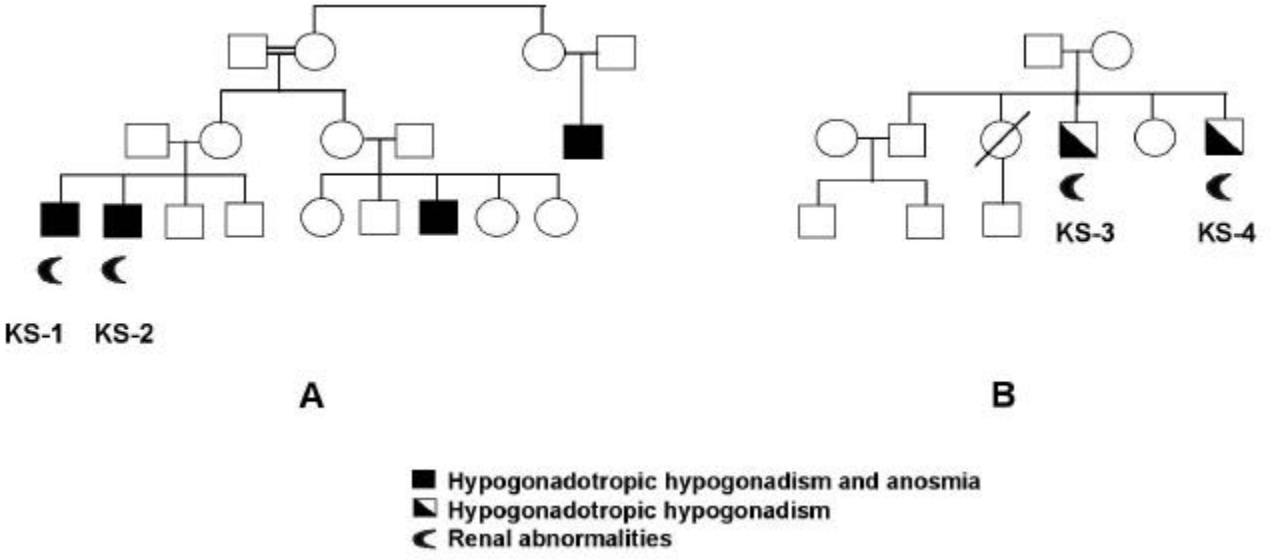

Figure 1. Heredograms of two families with X-linked KS.

investigated because of the presence of kidney abnormalities, which are typically observed in patients with *KAL-1* mutations. Hyposmia was referred for one of the brothers (KS-3).

Diagnostic criteria for KS were clinical signs and symptoms of hypogonadism, serum testosterone levels in the hypogonadal range (0.07-1.94ng/mL), gonadotropin levels below the normal adult male range (LH: 0.01-2.0mUI/mL / FSH: 0.01-1.8mUI/mL), normal baseline levels of other anterior pituitary hormones, and normal radiological imaging of the hypothalamic-pituitary region. Evaluation of olfactory function was not performed and the cases with anosmia/hyposmia were found on direct inquiry. Patients' clinical features are summarized in table 1. This study was approved by the Ethics Committee, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP).

Cytogenetic investigation: Peripheral blood lymphocytes (0.5mL) were cultured for 72 hours at 37°C in 10mL or RPMI-1640 (Nutricell) medium supplemented with 20 percent fetal calf serum (Nutricell) and 0.2mL of phytohemagglutinin P (Difco). After 70 hours of incubation, freshly dissolved ethidium bromide 10μg/mL (Sigma) was added to the cultures in order to obtain prometaphase chromosomes. Colquicine (0.05μg/mL - Sigma) was added in the last half-hour of incubation. The cells were spun down and suspended in 0.075M KCl for 50 min at 37°C and then fixed 3 times with 1:3 acetic acid-methanol. The cells were spread on clean slides by air-drying with flaming. G bands were obtained by mild treatment with 0.125% trypsin (Gibco) for 5-15s, and stained for 10 min in Giemsa. A minimum of 32 cells was analyzed for each patient.

Fluorescence *in situ* hybridization (28) for detection of the *KAL-1* gene *locus* was performed with Locus Specific Identifier-Kallmann/Chromosome Enumeration Probe X dual color DNA probes (LSI-Kallmann/CEPX, Vysis incorporation) following manufacturer's protocol. Cells were considered normal if they had red and green signals in the analysis of 30 metaphase and prometaphase chromosomes. Normal control specimens were incorporated into each FISH assay.

Digital images were obtained using an Olympus BX 60 epi-fluorescence microscope equipped with a Cyto Vision™ system (Applied Imaging Corporation), for capture and image analysis.

RESULTS

In all individuals, the examination of GTG-banded prometaphase chromosomes revealed normal male karyotypes (46,XY). FISH analysis with Kallmann probe revealed absence of *KAL-1* gene in the KS-5 case. Figure 2 shows the deletion detected in this patient and a normal male control. In all the other cases, both signals were detected on the X-chromosomes, like on the normal male control.

DISCUSSION

In the present study, we describe the finding of a deletion of *KAL-1* gene in one patient, among 4 sporadic cases of Kallmann syndrome. This deletion was detected only by FISH since the chromosomal analysis showed a normal karyotype. The patient exhibited the typical clinical features of KS and a high palate.

Tabela 1. Clinical features of patients with hypogonadotropic hypogonadism with or without anosmia.

Nº	Age at last visit	Clinical features associated with gonadotropin deficiency					Inheritance
		A	Mp	Cp	Gn	Other	
KS-1	32	+	+	-	+	unilateral renal agenesis, *pes cavus*	X-linked (brother of KS-2)
KS-2	28	+	+	+	-	unilateral renal agenesis	X-linked (brother of KS-1)
KS-3	35	-/+*	+	+	+	horseshoe kidney	X-linked (brother of KS-4)
KS-4	28	-	+	+	-	unilateral renal agenesis mental retardation	X-linked (brother of KS-3)
KS-5**	27	+	+	+	-	high palate	sporadic
KS-6	24	+	+	-	-	*pes cavus*	sporadic
KS-7	47	+	+	+	-	ataxia, hypertelorism	sporadic
KS-8	37	+	+	-	-		sporadic

A: anosmia; Mp: micropenis; Cp: cryptorchidism; Gn: gynecomastia
* hyposmia; ** patient with KAL-1 deletion detected by FISH

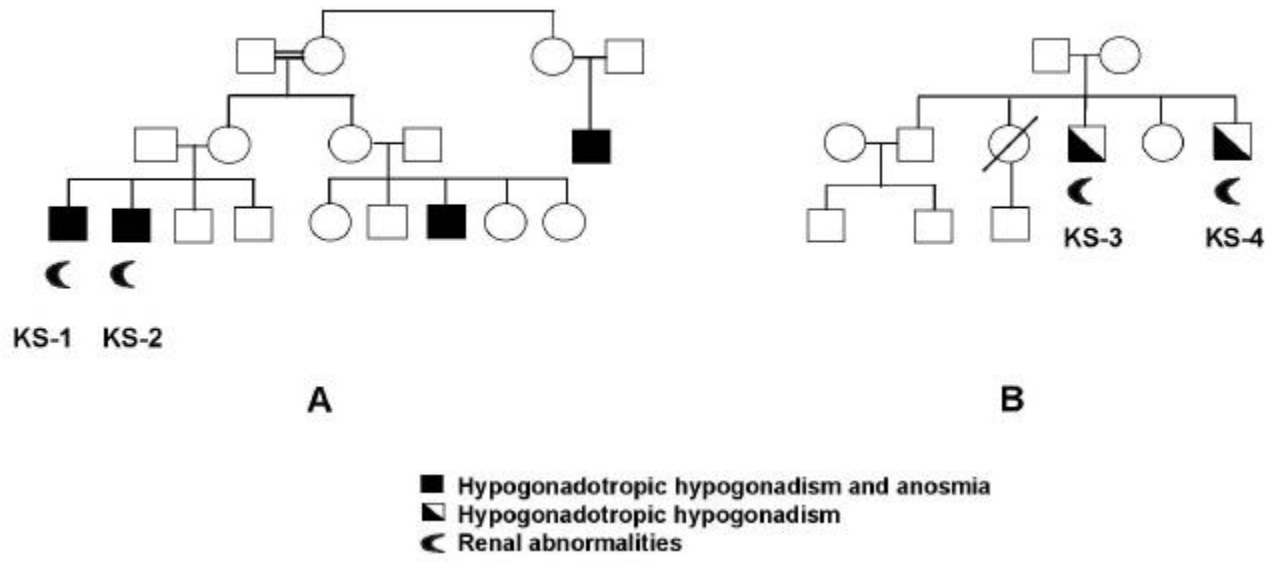

Figure 2. Hybridization of LSI-Kallmann/CEPX probes on human metaphase chromosomes. The LSI Kallmann probe hybridizes to the band Xp22.3 (red signal) and the CEPX to the centromere of the human X chromosome (green signal). In the left image (patient KS-5), only the centromeric region signal is visualized. At right a normal X-chromosome is shown where the marks of the *KAL-1 locus* and centromeric region can be seen.
Note: Pseudocoloring was performed for image reproduction in black and white.

Although no deletions were detected in the X-linked KS cases, it should be noted that these findings do not exclude the existence of point mutations or intragenic deletions of the *KAL-1* gene.

Most of the reported cases of deletions of the *KAL-1* gene have been associated to the contiguous genes syndrome and have been evidenced by Southern blotting techniques (26,27,29). The cases of deletion in Xp22.3 responsible for isolated Kallmann syndrome (i.e. which does not belong to a contiguous gene syndrome) are rare. In two studies, four KS individuals with single removal of *locus KAL-1* were identified by Southern-blot, among twenty-two males with X-linked familial KS (19,21). The first case of KS with deletion of *KAL-1* gene detected by FISH was reported by Hou et al. (30), in a patient with affected relatives. To our knowledge, the patient reported in the present study would be the first deletion detected by FISH in a sporadic case of KS.

It has been demonstrated that some genes may contain repetitive sequences that promote deletions. The human steroid sulfatase *locus* (STS) on the distal short arm of the X chromosome is characterized by a high frequency of deletion, caused by recombination between such elements flanking the gene (31). It is likely that entire *KAL-1* deletions are originated by repetitive sequences localized in both sides of the gene (32).

In cases of X-linked KS, approximately 50% of the families have molecular alterations in *KAL-1* gene and it is of interest that unilateral renal agenesis occurs in half of all males with mutations (19). In the present X-linked KS, all individuals exhibited renal abnormalities. These and other reports corroborated the hypothesis that *KAL-1* gene plays a yet undefined role in kidney development (33). Nevertheless, the mutation is not invariably associated with renal failure (34,35). In fact, the description of a large family with a high frequency of renal agenesis, either in the presence or in the absence of a *KAL-1* gene mutation suggests the existence of another gene that contributes to renal agenesis (36).

In conclusion, the cytogenetic evaluation in a series of KS patients indicates that FISH can be useful for the detection of complete *KAL-1* gene deletion in cases with features consistent with KS independently of its familial occurrence.

ACKNOWLEDGMENTS

We wish to express our gratitude to Dr. Nilma Lúcia Viguetti Campos (Laboratório de Citogenética Humana, Departamento de Genética Médica, FCM/UNICAMP) for cytogenetic assistance and Dr. Denise Engelbrecht Zantut Wittmann (Disciplina de Endocrinologia, Departamento de Clínica Médica, FCM/UNICAMP) for helpful suggestions. EBT was

supported by a fellowship from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). We also wish to thank FAPESP for the acquisition of the image analysis system (FAPESP no. 1996/ 12814-5).

REFERENCES

1. Sherins RJ, Howards SS. In: Walsh PC, ed. **Male Infertility**. Philadelphia: Campbell's Urology, **1986**:640-97.
2. De Morsier G. Etudes sur les dysraphies cranio-encephaliques. 1. Agenesie de lobes olfactifs (telencephaloshizis lateral) et des comissures calleuse et anterieure (telencephaloschizis median). La dysplasie olfacto-genitale. **Schweiz Arch Neuro Psychiat1954**;74: 309-61.
3. Rosen SW, Gann P, Rogol AD. Congenital anosmia: detection thresholds for seven odorant classes in hypogonadal and eugonadal patients. **Ann Otol Rhinol Laryngol 1979**;88:288-92.
4. Davidson TM, Murphy C. Rapid clinical evaluation of anosmia. The alcohol sniff test. **Arch Otolaryngol Head Neck Surg 1997**;123:591-4.
5. Spratt DI, Carr DB, Merriam GR, Scully RE, Rao PN, Crowley 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.
6. Fuerxer F, Carlier R, Iffenecker C, Schaison G, Doyon D. Magnetic resonance imaging of the olfactory pathways in Kallmann de Morsier syndrome. **J Neuroradiol 1996**;23:223-30.
7. Kallmann FJ, Scchoenffeld WA, Barrera SE. The genetic aspects of primary eunuchoidism. **Am J Ment Defic 1944**;48:203-36.
8. Chaussain JL, Toublanc JE, Feingold J, Naud C, Vassal J, Job JC. Mode of inheritance in familial cases of primary gonadotropic deficiency. **Horm Res 1988**;29:202-6.
9. Hermanussen M, Sippel WG. Heterogeneity of Kallmann syndrome. **Clin Genet 1985**;28:106-11.
10. Dean JSC, Johnston AW, Klopper AI. Isolated hypogonadotrophic hypogonadism: a family with autosomal dominant inheritance. **Clin Endocrinol 1990**;32:341-7.
11. Jones JR, Kemmann E. Olfacto-genital dysplasia in the female. **Obstet Gynecol Ann 1976**;5:443-6.
12. Pawlowtzki H, Diekstall P, Schadel A, Miny P. Estimating frequency of Kallmann syndrome among hypogonadic and among anosmic patients. **Am J Med Genet 1987**;26:473-9.
13. Georgopoulos NA, Pralong FP, Seidman CE, Crowley WF Jr, 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.
14. 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.
15. Legouis R, Hardelin JP, Levilliers J, Claverie JM, Compain S, Wunderie V, et al. The candidate gene for the X-linked Kallmann syndrome encodes a protein related to adhesion molecules. **Cell 1991**;67:423-35.
16. Franco B, Guioli S, Pragliola A, Incerti B, Bardoni B, Tonlorenzi R, et al. A gene deleted in Kallmann syndrome shares homology with neural cell adhesion and axonal path-finding molecules. **Nature 1991**;353:529-36.
17. Incerti B, Guioli S, Pragliola A, Zanaria E, Borsani G, Tonlorenzi R, et al. Kallmann syndrome gene on the X and Y chromosomes: implications for evolutionary divergence of human sex chromosomes. **Nat Genet 1992**;2:311-4.
18. Del Castillo I, Cohen-Salmon M, Blanchard S, Lutfalla G, Petit C. Structure of the X-linked Kallmann syndrome gene and its homologous pseudogene on the Y chromosome. **Nat Genet 1992**;2:305-10.
19. Hardelin JP, Levilliers J, Blanchard S, Carel JC, Leutenegger M, Pinard-Bertelletto JP, et al. Heterogeneity in the mutations responsible for X chromosome-linked Kallmann syndrome. **Hum Mol Genet 1993**;2:373-7.
20. Hardelin JP, Levilliers J, Del Castillo I, Cohen-Salmon M, Legouis R, Blanchard S, et al. X chromosome-linked Kallmann syndrome: stop mutations validate the candidate gene. **Proc Natl Acad Sci USA 1994**;41:577-80.
21. Quinton R, Duke VM, Zoisa PA, Platts AD, Valentine A, Kendall B, et al. The neuroradiology of Kallmann syndrome: a genotypic and phenotypic analysis. **J Clin Endocrinol Metab 1996**;81:3010-7.
22. Schwankhaus JD, Currie J, Jaffe MJ, Rose SR, Sherins RJ. Neurologic findings in men with isolated hypogonadotropic hypogonadism. **Neurology 1989**;39:223-6.
23. Danek A, Heye B, Schroedter R. Cortically evoked motor responses in patients with Xp22.3-linked Kallmann syndrome and in female gene carriers. **Ann Neurol 1992**;31:299-304.
24. Wegenke JD, Uehling DT, Wear JB, Gordon ES, Bargman JG, Deacon JS, et al. Familial Kallmann syndrome with unilateral renal aplasia. **Clin Genet 1975**;7:368-81.
25. Lieblich JM, Rogol AD, White BJ, Rosen SW. Syndrome of anosmia with hypogonadotropic hypogonadism (Kallmann syndrome): clinical and laboratory studies in 23 cases. **Am J Med 1982**;73:506-19.
26. Ballabio A, Sebastio G, Carrozzo R, Parenti G, Piccirillo A, Persico MG, et al. Contiguous gene syndrome due to deletions in the distal short arm of the human X chromosome. **Proc Natl Acad Sci USA 1989**;86:10001-5.
27. Bick D, Curry CJR, McGill JR, Schorderet DF, Bux RC, Moore CM. Male infant with ichthyosis, Kallmann syndrome, chondrodysplasia punctata, and a Xp chromosome deletion. **Am J Med Gen 1989**;33:100-7.
28. Pinkel D, Straume T, Gray JW. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. **Proc Natl Acad Sci USA 1986**;83:2934-8.
29. Weissörtel R, Strom TM, Dörr HG, Rauch A, Meitinger. Analysis of an interstitial deletion in a patient with Kallmann syndrome, X-linked ichthyosis and mental retardation. **Clin Genet 1998**;54:45-51.
30. Hou JW, Tsai WY, Wang TR. Detection of *KAL-1* gene deletion with fluorescense *in situ* hybridization. **J Formos Med Assoc 1999**;98:448-51.
31. Yen PH, Li X-M, Tsai SP, Johnson C, Mohandas T, Shapiro LJ.

Frequent deletion of the human X chromosome distal short arm result from recombination between low copy repetitive elements. **Cell 1990**;61:603-10.

32. Bardoni B, Guioli S, Raimondi E, Heilig R, Mandel JL, Ottolenghi S, et al. Isolation and characterization of a family of sequences dispersed on the human X chromosome. **Genomics 1988**;3:32-8.

33. Bouloux P. Congenital pituitary endocrine failure. In: Shaw RW, ed. **Hypothalamic Pituitary Dysfunction**. New York: Parthenon, **1994**:17-33.

34. Zenteno JC, Méndez JP, Maya-Nunez G, Ulloa-Aguirre A, Kofman-Alfaro S. Renal abnormalities in patients with Kallmann syndrome. **BJU Int 1999**;83:383-6.

35. Kirk JMW, Grant DB, Besser GM, Shalet S, Quinton R, Smith CS, et al. Unilateral renal aplasia in X-linked Kallmann syndrome. **Clin Genet 1994**;46:260-2.

36. Colquhoun-Kerr JS, Gu WX, Jameson L, Withers S, Bode HH. X-linked Kallmann syndrome and renal agenesis occurring together and independently in a large Australian family. **Am J Med Genet 1999**;83:23-7.

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