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Homozygous mutation in the prokineticin-receptor2 gene (Val274Asp) presenting as reversible Kallmann syndrome and persistent oligozoospermia: Case Report

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Prokineticin 2 (Prok2) or prokineticin-receptor2 (Prok-R2) gene mutations are associated with Kallmann syndrome (KS). We describe a new homozygous mutation of Prok-R2 gene in a man displaying KS with an apparent reversal of hypogonadism. The proband, offspring of consanguineous parents, presented at age 19 years with absent puberty, no sense of smell, low testosterone and gonadotrophin levels. Magnetic resonance imaging showed olfactory bulb absence. The patient achieved virilization and spermatogenesis with gonadotrophin administration. Two years after discontinuing hormonal therapy, he maintained moderate oligozoospermia and normal testosterone levels. Prok2 and Prok-R2 gene sequence analyses were performed. The proband had a homozygous mutation in Prok-R2 exon 2 that harbours the c.T820>A base substitution, causing the introduction of an aspartic acid in place of valine at position 274 (Val274Asp). His mother had the same mutation in heterozygous state. This report describes a novel homozygous mutation of Prok-R2 gene in a man with variant KS, underlying the role of Prok-R2 gene in the olfactory and reproductive system development in humans. Present findings indicate that markedly delayed activation of gonadotrophin secretion may occur in some KS cases with definite gene defects, and that oligozoospermia might result from a variant form of reversible hypogonadotrophic hypogonadism.

Keywords: hypogonadotrophic hypogonadism; prokineticin-receptor2; anosmia; reversible Kallmann syndrome

Kallmann syndrome (KS) is a congenital form of idiopathic hypogonadotrophic hypogonadism (iHH) in which gonadotrophin deficiency is associated with defective sense of smell (anosmia or hyposmia) (Seminara *et al*., 2003). These alterations are thought to be secondary to impaired migration of GnRH-secreting and olfactory neurons from the olfactory placode region to the hypothalamus (Schwanzel-Fukuda *et al*., 1989; Gonzales-Martines *et al*., 2004; Cariboni and Maggi, 2006). KS is a heterogeneous disease in terms of variable reproductive phenotype and complex genetic transmission (Quinton *et al*., 1999, 2001; Seminara *et al*., 2003). Several monogenic defects have been described for KS, including X-linked KAL-1 gene mutations (Franco *et al*., 1991; Legouis *et al*., 1991) and autosomal-dominant fibroblast growth factor receptor 1 (FGFR1) gene (or KAL2) mutations (Dodé *et al*., 2003; Pitteloud *et al*., 2006). Recently, the role of two genes encoding prokineticin 2 (Prok2) and its receptor (Prok-R2) in the neurogenesis of olfactory bulbs and GnRH neuron migration has been suggested by evidence of KS phenotype in KO mice (Ng *et al*., 2005; Matsumoto *et al*., 2006; Pitteloud *et al*., 2007a, b). Moreover, mutations of either Prok2 or Prok-R2 human orthologs have been described in some KS patients (Dodé *et al*., 2006; Pitteloud *et al*., 2007a, b; Leroy *et al*., 2008). Variable degrees of olfactory and reproductive dysfunctions have been reported in subjects across and within families carrying the same mutation, suggesting incomplete penetrance and an influence of modifier genes or environmental factors (Quinton *et al*., 1999, 2001; Seminara *et al*., 2003; Pitteloud *et al*., 2007a, b). Although impaired sexual maturation with absent, partial or delayed puberty is the main reproductive phenotype in KS, a variant form known as reversible KS has been described in several cases, in which gonadotrophins, testosterone and fertility recover spontaneously (Quinton *et al*., 1999; Pitteloud *et al*., 2005; Ribeiro *et al*., 2007; Raivio *et al*., 2007). Mutations of known genes responsible of iHH [KAL1, FGFR1, GnRH receptor (GnRHR)]

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have been described in some KS variant (Pitteloud *et al*., 2001, 2005; Lin *et al*., 2006; Raivio *et al*., 2007; Ribeiro *et al*., 2007). In this report we describe a man with a variant KS carrying a new homozygous mutation of Prok-R2 gene displaying an apparent reversal of his reproductive condition following 5 year hormone replacement therapy. This study extends our understanding about the role of Prok2–Prok-R2 gene pathways in the regulation of olfactory and gonadal system development and function in humans.

Case presentation

Written informed consent was obtained from the proband, mother and normal individuals used as controls. The study was performed under ethical approval of the Institutional Review Board. The proband, a 19-year-old male presented to the Endocrine Unit of Second University Hospital with absent puberty. Physical examination revealed eunuchoidal body proportions with a height of 169 cm, an arm span of 182 cm, an upper to lower segment ratio of 0.8, a weight of 57 kg and a BMI of 20 kg/m^2. He had no beard, puberal Tanner stage 1, a stretched phallus length of 3.5 cm, scrotal testes of 3 ml bilaterally, absence of ginecomastia, synkinesis, hearing loss, dental agenesis and midline defects. He had anosmia, confirmed by modified formal smelling test (Doty *et al*., 1984), and normal colour perception at Ishihara tests (Kanehara Shuppan Co., Tokyo, Japan). Family history, obtained by proband and his mother revealed parental consanguinity (Fig. 1). He was an only child of first-cousin parents. The 39-year-old mother had had a delayed menarche at 15 year, followed by regular menstrual cycles, and had always had a normal sense of smell. The father was not available for clinical and genetic examination.

Clinical study

Clinical, hormonal and seminal data at first observation and during follow-up are summarized in Table I. At diagnosis FSH, LH, testosterone and inhibin B levels were low. After a GnRH stimulation test, FSH and LH peaked to 2.3 and 1.2 mIU/ml, respectively. Baseline prolactin, thyroid and adrenal hormone levels were normal. The growth hormone-insulin-like growth factor 1 axis was also normal, basally and after stimulation testing. Magnetic resonance imaging showed absence of olfactory bulbs and a normal hypothalamic pituitary region. Diagnosis of KS was made and replacement therapy with hCG alone (Gonasi HP, AMSA, Italy, 1000–2000 IU i.m. twice a week) was started. After 1 year of therapy, the patient achieved puberal Tanner stage 3 and a testis volume of 5 ml. Semen analysis showed azoospermia. Recombinant FSH (Gonal F, Serono, Swizereland 75 IU s.c. twice a week) was added to hCG. After 1 year of combined therapy, serum testosterone level was 21 nmol/l, and seminal analysis, performed according World Health Organization guidelines (1992), showed moderate oligozoospermia. Then the patient continued with hCG alone (2000 IU two times a week). A re-evaluation after 6 months showed a normal testosterone value and oligozoospermia. On patient demand testosterone replacement therapy (testosterone enanthate, TE, 250 mg i.m. every 3 weeks) was prescribed instead of

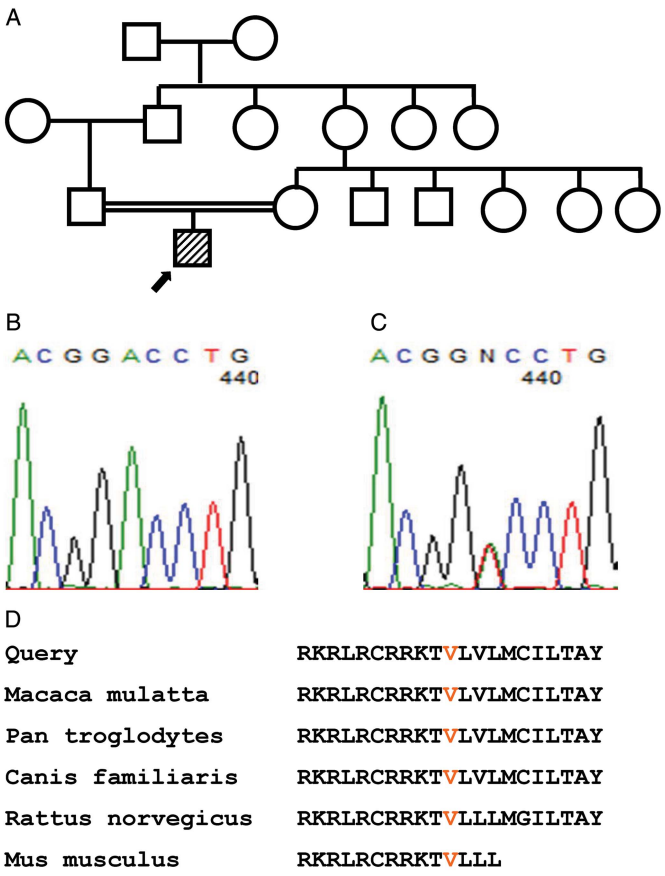

Figure 1: Family pedigree.
The arrow indicates the proband (**A**). DNA sequence electrophoretograms showing the prokineticin-receptor2 (Prok-R2) homozygous c.T830>A base substitutation (p.Val274Arg) in the proband with Kallmann Syndrome (**B**) and the heterozygous mutation in the mother (**C**). Interspecies comparison of the amino acid sequences of Prok-R2 around the mutated residue (**D**) demonstrating high conservation in primates, dog and rodents.

gonadotrophins, to maintain virilization and sexual activity. After 3 years of TE therapy, the patient presented increased testis size, supranormal testosterone levels and persistent spermatogenesis. He referred spontaneously interrupted pregnancy in his partner. TE administration was discontinued and re-examination recommended. Six months after discontinuing therapy he had preserved sexual activity, normal testosterone, FSH and LH levels, and mild oligozoospermia. The patient was discharged without therapy and re-evaluated after 2 years. At this time, gonadotrophin levels were normal, testosterone was in the low level of adult range (11.45 nmol/l) and seminal analysis revealed persistence of moderate oligozoospermia (Table I).

Genetic study

Genomic DNA was obtained from peripheral blood samples by using Wizard Genomic DNA Purification Kit (Promega). Primers used for amplification and sequencing of Prok2 (NCBI Gene ID: 60675) and Prok-R2 (NCBI Gene ID: 128674) coding exons were designed on the basis of reported sequences (Dodé *et al*., 2006). PCRs were performed using

Table I. Clinical, endocrine and seminal data in a man displaying Kallmann syndrome.

Observation	1 (diagnosis)	2	3	4	5	6	7	
Age (years)	19	20	21	21.5	24.5	25	27	
Therapy	None	hCG	hCG + rFSH	hCG	TE	None	None	
Mean testicular volume (ml)	3	5	11	11	18	18	20	Normal values
FSH (mIU/ml)	0.1	–	–	–	–	5.1	4.9	1.5–9.0
LH (mIU/ml)	0.5	–	–	–	–	7	5.8	2.0–10
Testosterone (nmol/l)	0.52	9.69	21.11	25.6	32.87	15.92	11.42	11.07–31.1
Inhibin B (pg/ml)	70	125	144	156	–	–	–	50–250
TSC (millions)	–	0	28	24	37.5	27	18	>40[a]
SC (mil/ml)	–	0	14	12	16	18	9	>20[a]
Progressive motility, %	–	–	40	10	25	20	30	>50[a]

[a]World Health Organization manual 1992. TSC, total sperm count; SC, sperm concentration; TE, testosterone enanthate.

15 pmol of each primer and 20 ng of genomic DNA in a final volume of 25 μl. Thirty-five cycles of PCR were carried out to amplify all exons, with 5 min of initial denaturation at 94°C followed by 30 s denaturation at 94°C, 30 s annealing at 55°C, 30 s extension at 72°C, and a final 7 min extension at 72°C to ensure that all amplicons were completely extended. The primers and programmes used for KAL1, FGFR1 and GnRHR coding exon analysis have been reported elsewhere (Hardelin *et al*., 1993; Sato *et al*., 2004; Antelli *et al*., 2006).

Results

In the proband, sequence analysis did not identify any mutation in the exons of Prok2 gene. In contrast, analysis of Prok-R2 showed a homozygous mutation in the second exon of the gene that harbours the c.T820>A base substitution (Fig. 1B). This mutation was absent in our 100 control chromosomes. This mutation causes the introduction of an aspartic acid at the place of a valine in position 274 (Val274Asp). The proband mother sequence analysis showed the same heterozygous mutation (Fig. 1C). This amino acid is localized in a highly conserved domain of the protein (Fig. 1D), likely corresponding to the third intracellular loop. This domain is probably involved in transduction-signal pathway. We submitted the wild type and the substituted amino acid sequence to bioinformatic tools (SwissModel and EsyPred3D) to create a computational three-dimensional model. The overlap between wild-type and mutated sequence model obtained with SwissModel shows a mismatch in the mutated region suggesting that this amino acid substitution probably modified protein three-dimensional structure (data not shown). The same result was obtained using EsyPred3D tool. Sequence analysis excluded KAL1, FGFR1 and GnRHR gene mutations in the proband.

Discussion

This report describes reversal of hypogonadotrophic hypogonadism in a patient with KS carrying a novel homozygous mutation in Prok-R2 gene. The patient harbours a T/A base substitution at codon 274 of Prok-R2 gene, leading to a Val274Asp mutation in the receptor protein, associated with hypogonadotrophic hypogonadism and anosmia due to olfactory system anomalies. This base substitution does not represent a common polymorphic variant in the population of our country, as it was absent in a series of 100 alleles from 50 ethnically matched controls (10 women and 40 men). The proband inherited the trait from consanguineous parents. His mother, who had had a delayed menarche but a normal sense of smell, showed the same heterozygous mutation in Prok-R2 gene. His father was unavailable for clinical and genetic

Table II. Cases of reversible iHH associated with gene mutations.

References	At diagnosis					At reversal							Gene
	Age, Years	Mean TS, ml	FSH, mIU/ml	LH, mIU/ml	Testosterone, nmol/l	Age, Years	Mean TS, ml	FSH, mIU/ml	LH, mIU/ml	Testosterone, nmol/l	SC, mil/ml	TR, Years	
Pitteloud *et al.* (2005), Raivio *et al.* (2007)	18	6	1.6	1.6	0.35	24	9.5	11.7	11.5	11.76	4	5	FGFR1
Raivio *et al.* (2007)	20	11	2.7	6.8	2.94	27	14	6.3	14.6	53.51	0.1	1.9	FGFR1
Ribeiro *et al.* (2007)	22	3			7.27	42	16			16.61			KAL1
Pitteloud *et al.* (2001)	30	17	2.4	3.7	3.1	26.7	20	3.9	7.1	12.5	91.4	0.5	GnRHR
Lin *et al.* (2006)	15.9					32		8.5	5.9	12.5	18		GnRHR
Present case	19	3	0.1	0.5	0.52	27	20	4.9	5.8	11.42	9	5.5	Prok-R2

TS, testicular size; TR, time to reversal; FGFR, fibroblast growth factor receptor 1; GnRHR, gnRH receptor; Prok-R2, prokineticin-receptor2.

studies, but the state of consanguinity suggests a possible heterozygous condition too. Studies on mutant mice lacking Prok2 or Prok-R2 genes indicated that Prok2/Prok-R2 signalling is critical for neurogenesis of olfactory bulbs and GnRH neuron migration (Ng *et al*., 2005; Matsumoto *et al*., 2006; Pitteloud *et al*., 2007a, b). The phenotype in our patient resembles that of mice with homozygous Prok-R2 mutations, which had abnormal development of the olfactory bulb and reproductive system atrophy (Ng *et al*., 2005). Dodé *et al*. (2006) first described mutations in Prok-R2 gene in 10% of a cohort of 192 KS male and female patients. Mutations in Prok2 gene, coding for a ligand of Prok-R2, were also described (Dodé *et al*., 2006; Pitteloud *et al*., 2007a, b; Leroy *et al*., 2008). Such findings and the present report indicate that Prok2 and Prok-R2 genes, as well as KAL1 and FGFR1, may be involved in olfactory bulb development and GnRH neuron migration in humans too. Both Prok2 and Prok-R2 mutations do not appear to be associated with clinical anomalies (bimanual synkinesis, renal agenesis and midline defects) reported in other genetic forms of the disease. The homozygous mutation in our proband is located in the second exon, in a highly conserved region of Prok-R2 gene that probably encodes the third intracellular loop, suggesting a critical role of this residue in receptor function. It is noteworthy that 8 out 10 mutations described by Dodé *et al*. (2006) have been found in exon 2 of the gene. However, the exact biological role of the mutation is still unclear, in absence of functional studies. The substitution of a neutral amino acid, such as valine, with the polar aminoacid asparagine can induce modifications in the three-dimensional assembling of the Prok-R2 protein, causing changes in protein–protein interactions, as suggested by computational analysis. This structural change can lead to abnormal receptor dimerization or impaired intracellular signalling.

Prok-R2 mutations were found in the homozygous state only in two cases, whereas in the others they appear in heterozygous or in compound heterozygous state (Dodé *et al*., 2006). Moreover, these patients presented variable degree of olfactory and reproductive dysfunction (anosmia, hyposmia, delayed puberty or hypogonadotrophic hypogonadism). In our family, the homozygous proband had a more severe phenotype than his heterozygous mother who presented only with delayed menarche. The presence of complete phenotype in Prok-R2 heterozygous patients might be explained by the co-existence of additional inherited alterations in other genes involved in reproductive and olfactory system development and function (Dodé *et al*., 2006; Pitteloud *et al*., 2007a, b; Leroy *et al*., 2008). In fact, heterozygous FGFR1 mutations have been found associated with additional mutations in nasal embryonic LHRH factor (NELF) and GnRHR genes in two families, indicating that, at least in some pedigree, KS may have a digenic inheritance (Pitteloud *et al*., 2007a, b). Moreover, Dodé *et al*. (2006) reported a subject with KS who exhibited combined mutation of PKR2 and KAL1, indicating that in some instances the syndrome may be an oligogenic trait. However, mutations in Prok2, KAL1, FGFR1 and GnRHR genes were not detected in our family.

The patient achieved complete virilization and fertility under gonadotrophin substitution therapy. Reversal of his hypogonadism was suggested by maintenance of spermatogenesis and fertility under long-term TE administration. Persistent normalization of pituitary–gonadal hormones after a further 2 years without TE replacement therapy indicates a reversal of hypogonadotrophic hypogonadism state. This is in accordance with recently established criteria (Raivio *et al*., 2007). KS reversible phenotype is a variant that may be found more frequently than suspected. In fact, Raivio *et al*. (2007) found hypogonadotrophic hypogonadism reversal in 10% of cases. Definite gene alterations have been reported in three out of 13 subjects examined by Raivio *et al*. (2007) and in single cases by others (Pitteloud *et al*., 2001, 2005; Lin *et al*., 2006; Ribeiro *et al*., 2007) (Table II). Reversible KS has been reported in patients carrying FGFR1 and KAL1 gene mutations (Pitteloud *et al*., 2005; Ribeiro *et al*., 2007; Raivio *et al*., 2007). Moreover, alterations in GnRHR gene have been found in two patients with mild forms of iHH: one with fertile eunuch syndrome and another two with a history of delayed puberty (Pitteloud *et al*., 2001; Lin *et al*., 2006). In the present study, we have demonstrated for the first time that reversible KS may be associated with a Prok-R2 mutation. The pathophysiology of hypogonadotrophic hypogonadism reversal and the interplay between different genes involved in the ontogeny and growth of GnRH system still remain unclear. We can hypothize that some gene mutations may be associated with a partial migration defect of GnRH neurons, resulting in variable degrees of impaired gonadotrophin secretion, but supportive evidence is still lacking. GnRH neuron migration and growth may be stimulated after the embryonic period by exogenous or endogenous sex steroids, contributing to the recovery of pituitary–gonadal function. Present and previous findings (Pitteloud *et al*., 2005; Ribeiro *et al*., 2007; Raivio *et al*., 2007) indicate that hypogonadotrophic hypogonadism cannot be considered an irreversible state in all the subjects harbouring genetic mutations in known genes involved in the control of gonadotrophin secretion pathway. Thus, the markedly delayed activation of gonadotrophin secretion in a subgroup of KS patients might be one extreme on a phenotypic spectrum of disorders characterized by altered regulation of the GnRH pulse generator, including constitutional delay of puberty and functional hypogonadism (Bhasin, 2007). From a clinical perspective, a periodic re-examination is advisable after discontinuing therapy to evaluate a possible reversal of hypogonadotrophic hypogonadism. Furthermore, these subjects with variant KS may provide the evidence that oligozoospermia in a subset of infertile men can result from partial and reversible hypogonadotrophic hypogonadism. Thus, the identification of such variant iHH may offer new therapeutic approaches to male infertility.

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