CLINICAL STUDY

A novel severe N-terminal splice site *KISS1R* gene mutation causes hypogonadotropic hypogonadism but enables a normal development of neonatal external genitalia

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Abstract

Background: Kisspeptin 1 receptor (*KISS1R*) gene mutations are rare but have recently become an important etiology of normosmic isolated hypogonadotropic hypogonadism (IHH).

Objectives: To characterize the genetic defect, the phenotype, and response to therapy of three IHH siblings with a novel severe *KISS1R* mutation.

Patients and methods: Three siblings (16- and 22-year-old sisters and their 20-year-old brother) born to consanguineous parents with normal neonatal external genitalia presented with no pubertal development, normosmia, and a low response to GnRH stimulation. Homozygosity mapping, *KISS1R* gene sequencing, and RNA expression were performed.

Results: The females' basal low estradiol level (50 pmol/l) failed to rise in response to human chorionic gonadotropin (hCG). The brother's low testosterone (1.87 nmol/l) responded to combined hCG and human menopausal gonadotropin (hCG) and HMG therapies, but the testes remained small (1–2 ml). Secondary sexual characteristics were attained by exogenous sex steroid replacement. SNP array studies revealed shared homozygosity for a chromosome 19 region encompassing *KISS1R*. Sequencing revealed a novel homozygous *KISS1R* mutation at the nt-1 canonical acceptor splice site of intron 1 in affected siblings. The mother (menarche at 14 years) was heterozygous. cDNA sequencing showed that the G>A mutation results in skipping of exon 2 and a premature stop codon at residue 151.

Conclusions: The novel severe N-terminal *KISS1R* splice site (c.245–1G>A) mutation results in IHH. Heterozygous female carriers may manifest a subtle fertile phenotype. The subnormal gonadal response to hCG in patients may implicate a direct role of *KISS1R* in gonadal function. The normal neonatal virilization in a male homozygous to this severe mutation challenges the hypothesis that *KISS1R* is required for fetal development of male external genitalia.

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Introduction

Studies on disorders of pubertal development have identified neuroendocrine genes involved in the regulation of puberty initiation. Since 2003, loss-of-function mutations of the kisspeptin 1 receptor (*KISS1R*, also known as *GPR54*) gene were described in patients with normosmic isolated hypogonadotropic hypogonadism (IHH) (1, 2). Subsequently, knockout mice experiments of either *Kiss1r* or its ligand, *Kiss1*, showed a phenotype of IHH (3, 4), suggesting that the *Kiss1*–*Kiss1r* axis (K–KR-a) is a key player in pubertal development (5). The stimulative effect of kisspeptin 1 administration on GnRH-dependent gonadotropin secretion (6, 7, 8, 9, 10) established kisspeptin 1 as a

potent regulator of GnRH secretion in humans and animal models (3). Further support for the role of *KISS1R* in the onset of puberty was the finding of a gain-of-function mutation in *KISS1R* causing central precocious puberty (11) and the increased expression of both *KISS1* and its receptor in the hypothalamus during sexual maturation (8, 10, 12). Curiously, despite the obvious significance of K–KR-a in sexual maturation, to date relatively few loss-of-function mutations in *KISS1R* (1, 2, 11, 13, 14, 15, 16, 17) and only one inactivating mutation in *KISS1* (18) causing IHH have been reported.

Although the hypothalamic role of the K–KR-a has been established, its role in other tissues has not been intensively studied. *KISS1R* is expressed within the

PCR products were purified using NucleoSpin Extract II (Machery-Nagel, Bethlehem, PA, USA), cleaned, and sequenced for *KISS1R* using BigDye Terminator v3.1. Mutation testing in controls was performed by amplifying genomic DNA with the above-mentioned *KISS1R* primers. PCR products were digested with the BglI restriction enzyme and run on agarose gels.

RNA studies

RNA was extracted from Epstein–Barr virus-transformed B cell lines using Tri-Reagent (MRC, Cincinnati, OH, USA) and reverse transcribed with Improm II reverse transcriptase (Promega) using random hexamers in the presence of RNase inhibitor (rRNasin, Promega) at 50 °C for 60 min. The cDNA was amplified by PCR with primers in the *KISS1R* coding region: exon 1_cDNA_F: 5'-gggaactcgtggtcatcta-3', exon 3_cDNA_R: 5'-agagcc-taccagatgtga-3'. Informed consent was obtained from study participants, and studies were approved by the Institutional Review Board of Shaare Zedek Medical Center.

Results

Hormonal treatment

At the age of 22 and 23, our male patient underwent two 6-month trials of gonadotropin replacement therapy using chorionic gonadotropin, 3000 U twice a week, and lyophilized human postmenopausal gonadotropin, 150 U three times/week. This treatment resulted in an increase in the testosterone levels, 908 ng/dl, but it failed to significantly increase the testicular size (3–4 ml) or reverse the azoospermia.

Genomic analysis

Homozygosity mapping using an SNP array was performed on the three affected siblings. Genotyping data was analyzed to determine the regions of homozygosity of at least 2 Mb present in all three affected patients, and the genes in these regions were examined. The *KISS1R* gene (*KISS1* receptor; MIM 604161; GPR54), present in one of the homozygous regions identified, was an obvious candidate gene (Fig. 2A).

Figure 2 Homozygosity mapping and identification of a *KISS1R* mutation (c.245–1G>A). (A) Homozygosity mapping results. Top: DNA samples from the three affected siblings (and other family members – not shown) were genotyped using the Affymetrix SNP 6.0 array and results shown are for data analysis using GTC Software (25). The bar above is an ideogram of chromosome 19, with cytogenetic bands indicated in shades of gray, the centromere in red, and centromeric heterochromatin in black. Every horizontal row represents the results of one patient. Homozygous regions are indicated by a vertical line and heterozygous regions by dots. Colored blocks represent multiple vertical lines, i.e. regions of homozygosity along the chromosome. Bottom: the homozygous area shared by all three affected siblings is magnified with lines representing individual SNPs homozygous in all three affected samples. The red arrow indicates the location of the *KISS1R* gene. (B) *KISS1R* sequencing. A novel homozygous G>A mutation at the nt-1 canonical acceptor splice site of intron 1 was found in all three affected siblings (right). The mother was heterozygous (middle), while a healthy sister and five normal Jerusalem Palestinians had the normal sequence (left). Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-12-0127>.

Figure 3 The *KISS1R* (c.245–1G>A) mutation results in exon 2 skipping, leading to an aberrant and truncated mRNA and protein (p.A82Gfs*151). (A). Predicted effect of exon 2 skipping. A schematic illustration of the expected length of a PCR product, in wild-type and mutant *KISS1R* cDNA, using primers flanking exon 2. Black arrows indicate primer locations and red hexagons indicate stop codon location. (B) *KISS1R* cDNA – PCR products. *KISS1R* cDNA fragments were amplified using the primers shown in A and run on an agarose gel. M–pUC19 *MspI* DNA marker (Fermentas, Vilnius, Lithuania); 1 and 2 – affected individuals, PCR product size is only 211 bp. 3 – Unaffected individual, PCR product size is 336 bp. Lack of additional transcripts was confirmed by amplification of exons 1–5 (data not shown). (C) Mutant cDNA and protein sequence showing the skipping of exon 2 (end of exon 1 shadowed in blue, beginning of exon 3 in yellow) and a premature stop codon at amino acid 151. (D) Detection of the c.245–1G>A mutation by restriction enzyme analysis. The mutation abolishes a *BglI* site. Lane M, pUC mix DNA marker (Fermentas). Lanes 1–3, affected individuals; lane 4, parent of an affected child (obligate carrier); lanes 5 and 6, normal controls. Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-12-0127>.

Sequencing revealed a novel homozygous *KISS1R* mutation: an IVS1–1G→A transition (c.245–1G>A, chr19, nt 918543; Feb. 2009 GRCh37/hg19 assembly) at the canonical acceptor splice site of intron 1, in the three affected siblings. This mutation was predicted to cause skipping of exon 2 and create an early stop codon at nt 610 in the cDNA (predicted to result in a mutated translated protein, p.A82Gfs*151). The mother was heterozygous for the mutation, while a healthy sister and five normal individuals from the same ethnicity were homozygous for the normal sequence (Fig. 2B).

To determine whether the mutation indeed affects splicing, *KISS1R* cDNA from the lymphoblastoid cell lines of affected patients was amplified and sequenced. Exon 2 skipping was expected to result in a cDNA PCR product shorter by 125 bp than the wild-type product (Fig. 3A), and this was indeed the result, whereby no RT-PCR fragment including exon 2 was observed (Fig. 3B). Direct sequencing of the mutated cDNA confirmed exon 2 skipping in these cells. The reading frameshift caused by the mutation predicts both an aberrant protein amino acid sequence following the end of exon 1 and an early truncation already at the 151st codon as the frameshift causes this codon to become a stop codon (Fig. 3C). As the c.245–1G>A mutation eliminates a *BglI* site from the DNA sequence, we were

able to distinguish healthy controls from affected individuals using a simple restriction fragment length polymorphism (RFLP) assay (Fig. 3D).

Discussion

In three Palestinian siblings with IHH, we identified a novel homozygous *KISS1R* splice site mutation (c.245–1G>A) in the canonical acceptor site of intron 1. This mutation results in skipping of exon 2, which encodes the entire second transmembrane and third exolooop domains (11), and in a frameshift that creates an altered protein from the end of exon 1 (i.e. residue 82) to a premature stop codon at protein residue 151. Absence of a normal second intracellular loop and transmembrane domains 3, 6, and 7 has previously been shown to severely damage G-protein-coupled receptors/G-protein coupling (26) and activation (27) respectively. Thus, the severely aberrant transcript identified is expected to either result in a nonfunctioning protein product or undergo nonsense mediated decay.

Previously reported *KISS1R* mutations include nine point mutations (2, 11, 13, 14, 15), one 155 bp deletion (1), one insertion (16), and one acceptor splice site mutation (17). All are located closer to the C-terminal and are expected to result in the formation of a more

Table 1 Loss-of-function recessive *KISS1R* mutations: N- to C-terminal.

DNA mutation	Protein mutation	Sex ^a	Clinical endocrine manifestations	Gonadotropin Tx and fertility	Heterozygote state (parents or siblings)	Reference
c.245–1G>A (exons 1–2)	G82AfsX151	M	Testes 1–2 ml, normal neonatal and prepubertal penile length 6–7 cm No spontaneous puberty and azoospermia	Testosterone ↑ Testicular volume ↔ Azoospermia NA	Delayed puberty	Present report
c.305C>T (exon 2)	p.L102P	F (2) M (2)	Primary amenorrhea, P2–3 B1 Bilateral cryptorchidism and micropenis At 21–32 years: testes 3–4 ml, P3–5, and penis length 5–7.2 cm Primary amenorrhea, P3–5 B3–4	Testicular volume ↔ Fertility achieved with pulsatile GnRH Tx NA	NA	(14)
IVS2–4–2del GCA ins ACCGGCT (exons 3–4) c.443T>C (exon 3)	Truncated protein p.L148S	F (3) M (2)	Micropenis and cryptorchidism No spontaneous puberty Delayed puberty, testes 1–5 ml, P1–3, and 'short penis' Primary amenorrhea, P4 B3 Micropenis and bilateral cryptorchidism No spontaneous puberty Testes 4 ml, penis 7 cm, and P3	Fertility achieved with pulsatile GnRH Tx NA	Normal	(17)
c.667T>C (exon 4)/ c.891G>T (exon 5) ^b IVS4–13–42del155 (intron 4–exon 5)	p.C223R/p.R297 L ^b Truncated protein p.247X	F M M M (3) F	No pubertal development Partial breast development and single uterine bleeding Small penis, cryptorchidism, and no spontaneous puberty No spontaneous puberty Primary amenorrhea Cryptorchidism and mild hypospadias At 32 years: testes 3–5 ml Oligoasthenozoospermia At 17 years: testes 1 ml and penis 5 × 1.75 cm	Fertility achieved with gonadotropin Tx NA M, testosterone ↑ NA NA NA NA	NA Mother – normal Mother – delayed puberty Father – normal	(2) (15) (1)
c.T815>C (exon 5)	p.F272S	M (5)	Menarche and ovulation with gonadotropin Tx Fertility achieved with 2 years pulsatile GnRH Tx and IVF Fertility achieved with pulsatile GnRH Tx	NA	Brother – at 24 years fully pubertal	(13)
c.1001–1002insC (exon 5)	p.334fsinC	F M	Menarche and ovulation with gonadotropin Tx Fertility achieved with 2 years pulsatile GnRH Tx and IVF Fertility achieved with pulsatile GnRH Tx	Menarche and ovulation with gonadotropin Tx Fertility achieved with 2 years pulsatile GnRH Tx and IVF Fertility achieved with pulsatile GnRH Tx	NA	(16)
c.991C>T (exon 5)/ c.1195T>A (exon 5) ^b	p.R331X/p.X399R ^b	M			NA	(2)

M, male; F, female; NA, not assessed or no information; P, pubic hair Tanner stage; B, breast Tanner stage.

^aNumber in parenthesis refers to number of patients with similar clinical findings.^bCompound heterozygote.

functional residual protein (Table 1) than that produced by the mutation in our report. Consistent with this observation, the clinical phenotype in our cases was indeed severe, with complete absence of pubertal initiation. However, notably, neonatal genitalia appeared normal for both genders.

So far, the K–KR-a has been established to be a critical factor in GNRH release from hypothalamic neurons, serving as a gatekeeper for the initiation of puberty and sexual maturation (1, 2, 11). Nevertheless, additional findings might suggest a more complex role of the K–KR-a pathway. Dose-dependent release of LH from pituitary cells exposed *in vitro* to *KISS1* (19), and evidence that circulating sex steroids are needed for pituitary *KISS1* expression (22, 28), suggest a direct effect of *KISS1R* on the pituitary. Indeed, a variable pituitary response to GNRH stimulation that may correlate with mutation severity has been reported in patients carrying *KISS1R* mutations (1, 2, 13, 14, 17). The hormonal profile of our two homozygote patients showed low (but not absent) response to GNRH stimulation. As the mutation is severe and expected to result in a complete loss of function, this response, albeit small, suggests that the pituitary response to GNRH is affected but is not totally dependent on the K–KR-a signaling pathway. Alternatively, the low response to GNRH may also be an indirect consequence of low pituitary priming due to both initial low hypothalamic stimulation by *KISS1* and different treatment protocols that were given to these patients to initiate puberty. These hypotheses and the variable response to GNRH stimulation reported in patients with *KISS1R* mutations, even in the same family (as in our case) (14), suggest that together with the K–KR-a signaling pathway, other cofactors and the variable degree of previous pituitary priming codetermine the pituitary response to GNRH.

Less is known about the function of K–KR-a in the gonads (14, 15, 19, 20). A limited number of studies reported variable gonadal response to gonadotropin replacement therapy in patients with *KISS1R* mutations (Table 1). While nearly normal ovulation (13) and spermatogenesis (16) were achieved in patients with the F272S and insC 1001–1002 mutations respectively, only a low to totally absent testicular response with no sperm maturation or hormonal synthesis was reported in patients with the L102P and R297L/C223R mutations (14, 15). In our patients, only the married male was interested in a trial of gonadotropin replacement for the induction of spermatogenesis (so induction of ovulation was not studied). Two courses of 6-month treatment with gonadotropin replacement therapy did induce adequate testosterone levels but failed to increase the testicular size or reverse his azoospermia. It is reasonable that additional and longer periods of pulsatile gonadotropins are indicated for successful priming following years of gonadal under-stimulation. Alternatively, it may be considered that the poor gonadal response to gonadotropins may reflect the

absence of the direct regulatory effect of the K–KR-a on gonadal function in a case of a severely truncated and dysfunctional *KISS1R* protein. Interestingly, unlike some reports of undervirilization in male infants with *KISS1R* gene mutation (13, 14, 15, 16, 17), our male patient, as described in other reports (1, 2), although carrying a severe mutation, had no history of cryptorchidism or micropenis (Table 1), challenging the assumption that K–KR-a is required for normal male fetal genital development (13, 17).

The role of *KISS1R* mutations has also been examined in constitutional delay of puberty (CDP) (15, 17). One study reported delayed puberty in a heterozygote mother of a patient with a *KISS1R* deletion mutation (1). In the family we describe, the mother (heterozygote) and two other ‘healthy’ sisters (Fig. 1) had significantly delayed menarche while the noncarrier unaffected sister had menarche at 11 years of age (average age for the extended family; Table 1). This might be viewed as further evidence for a role of the *KISS1R* gene in patients with CDP, although larger genotype–phenotype studies are needed to validate this hypothesis.

In conclusion, we describe a novel severe homozygous *KISS1R* splice site mutation in a consanguineous Palestinian family with IHH. mRNA analysis verified skipping of exon 2 and formation of an aberrant protein, which prematurely terminates at residue 151. The blunted response to GNRH stimulation and the inadequate testicular response to gonadotropins strengthen the hypothesis that *KISS1R* may have a direct role in both the pituitary and the gonads. The presence of normal male genitalia in spite of the severe mutation challenges the concept that K–KR-a is required for normal male fetal virilization. Finally, significantly delayed puberty in heterozygous female patients suggests that further genotype–phenotype surveys are needed to determine the possible role of *KISS1R* mutations in CDP.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

O Breuer researched the data and wrote the manuscript; M Abdulhadi-Atwan wrote the manuscript and researched the data; S L researched the data; H Fridman researched the data; P Renbaum researched the data and reviewed/edited the manuscript; E Levy-Lahad researched the data, wrote the manuscript, and reviewed/edited the manuscript; D H Zangen researched the data, wrote the manuscript, and reviewed/edited the manuscript.

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