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 (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\(\rightarrow\)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.245K\(\rightarrow\)G\(\rightarrow\)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.

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...
hypothalamus as well as within other neuronal tissues (3), and KISS1, its 54-amino acid peptide ligand (19, 20, 21), is found in the anterior pituitary, pancreas, small intestine, testis, and ovary (3, 19, 22, 23). Several reports on patients with KISS1R mutations showed variable response to GNRH stimulation (1, 2, 13, 14) and low testicular response to hCG therapy (14, 23), but the effect of K–KR-a on the pituitary and the gonads has not yet been fully elucidated.

We present a novel homozygous splice site mutation in the KISS1R gene in three siblings, a possible phenotype in heterozygote carriers, and a subnormal gonadal response to hCG providing further support for a peripheral KISS1R role in adult gonadal function.

**Patients and methods**

Three siblings with complete IHH from a Palestinian family were identified. The index case is a healthy 16-year-old female, born to first-cousin parents, presented with delayed puberty and normal olfaction (Fig. 1A). Her height was 154.2 cm with a BMI of 17.6 kg/m². Breast Tanner stage was 1 and pubic hair was at stages 2–3. Bone age was delayed at 12 years. Laboratory tests revealed low basal LH, FSH, and estradiol (E2) levels (0.7, 2.8 IU/l, and 13.6 pg/ml respectively) with only a low peak response of LH (4.8 IU/l) and FSH (9.4) to GNRH stimulation. Serum androgens and other hormonal axes were within normal ranges. β-HCG was zero and karyotype was 46,XX. A 3-day hCG stimulation test (1500 IU on alternate days) did not increase the low E₂ level (27.2 pg/ml). Pelvic ultrasonography revealed infantile uterus and the ovaries could not be visualized. Brain CT scan was normal. Secondary sexual characteristics, menstruation, and final height (164.8 cm; target 160–167 cm) were achieved by hormone replacement therapy (ethinyl E₂ and medroxyprogesterone acetate) followed by oral contraceptive pills.

An elder 19.8-year-old healthy brother (#2) presented later with normosmic delayed puberty without apparent history of cryptorchidism or micropenis. His prepubertal, pretreatment penile length was documented as 6–7 cm and at presentation his pubertal penile length following earlier short courses of testosterone (total of 6–9 monthly injections of 200 mg testosterone enanthate) replacement was on the upper normal range, while his testes were 1–2 cc in size. Testosterone levels were low at 53.9 ng/dl (adult normal 300–1200 ng/dl). GNRH stimulation test revealed low basal and peak gonadotropin levels of 0.3 and 2.2 IU/l for LH and 1.2 and 3.6 IU/l for FSH respectively. Acquisition of full secondary sexual features and final height (180.7 cm) was induced by monthly i.m. injections of testosterone enanthate. At 25 years of age, his height was 180.7 cm (target 173–180 cm), testes 3–4 cc, and pubic hair and penis at Tanner 5. An elder sister with primary amenorrhea (not evaluated due to health insurance limitations) received therapy similar to her younger sister. The menarche of the mother and two older sisters was delayed compared with other family members and appeared at 14, 15, and 16 years old respectively. One of these sisters required gonadotropin injections to achieve pregnancy.

**Homzygosity mapping**

Genomic DNA was extracted from peripheral blood mononuclear cells of family members (24). Homozygosity mapping was performed using Affymetrix SNP6.0 arrays. The array was hybridized according to the manufacturer’s directions. Briefly, 250 ng genomic DNA was digested with either NspI or StyI. A universal adaptor was then ligated to the digested DNA and PCR amplified. The resulting PCR reactions, from both digests, were combined, purified, fragmented, end-labeled, and finally hybridized overnight to the SNP6.0 array. The array was washed and stained with SAPE before being scanned using Affymetrix’s 7G GeneChip scanner. The quality of hybridization was assayed visually and using Genotyping Console (GTC; Affymetrix, Santa Clara, CA, USA). Data analysis was performed using GTC and KinSNP (25).

**KISS1R sequencing**

PCR amplification was performed using the following primers (5’–3’): exon 1: F, gctgggtgaatagagggc and R, ggagttgcgcactctgcag; exon 2: F, cactctgtgtgcactcg and R, cactgcccggagcactc; exon 3: F, gcctagtgttgccacag and R, gcctactttcatgcagct; exon 4: F, gcattg-gaaatggcgcag and R, gcagggctagcagc; exon 5: F, gcagtgacacgagctgg and R, aaactgaccaacgctca.
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′-gggaactcgctggtcatcta-3′, exon 3_cDNA_R: 5′-agagccc-taccagagcgtga-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](https://www.eje-online.org)

**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 samples 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.
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 exoloop 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.

<table>
<thead>
<tr>
<th>DNA mutation</th>
<th>Protein mutation</th>
<th>Sex</th>
<th>Clinical endocrine manifestations</th>
<th>Gonadotropin Tx and fertility</th>
<th>Heterozygote state (parents or siblings)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.245→1G&gt;A (exons 1-2)</td>
<td>G82AsX151</td>
<td>M</td>
<td>Testes 1–2 ml, normal neonatal and prepubertal penile length 6–7 cm</td>
<td>Testosterone ↑</td>
<td>Delayed puberty</td>
<td>Present report</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>No spontaneous puberty and azoosperma</td>
<td>Testicular volume ↔</td>
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<td></td>
<td></td>
<td></td>
<td>Primary amenorrhea, P2–3, B1</td>
<td>Azoosperma</td>
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<td></td>
<td></td>
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<td>Bilateral cryptorchidism and microphallus at 21–32 years: testes 3–4 ml, P3–5,</td>
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<td></td>
<td></td>
<td></td>
<td>and penis length 5–7.2 cm</td>
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<tr>
<td>c.305C&gt;T (exon 2)</td>
<td>p.L102P</td>
<td>F (2)</td>
<td>Micropenis and cryptorchidism</td>
<td>Fertility achieved with pulsatile GNRH Tx</td>
<td>NA</td>
<td>(14)</td>
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<tr>
<td></td>
<td></td>
<td>M (2)</td>
<td>No spontaneous puberty</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Primary amenorrhea, P3–5, B3–4</td>
<td></td>
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<tr>
<td>IVS2–4→−2del GCAins ACCGGCT (exons 3–4)</td>
<td>Truncated protein</td>
<td>M (2)</td>
<td>Micropenis and cryptorchidism</td>
<td>Fertility achieved with pulsatile GNRH Tx</td>
<td>Normal</td>
<td>(17)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>No spontaneous puberty</td>
<td></td>
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<tr>
<td>c.443T&gt;C (exon 3)</td>
<td>p.L148S</td>
<td>M (4)</td>
<td>Delayed puberty, testes 1–5 ml, P1–3, and ‘short penis’</td>
<td>Fertility achieved with gonadotropin Tx</td>
<td>NA</td>
<td>(2)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Primary amenorrhea, P4, B3</td>
<td></td>
<td></td>
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<tr>
<td>c.667T&gt;C (exon 4)/c.891G&gt;T (exon 5)b</td>
<td>p.C223R/p.R297Lb</td>
<td>F</td>
<td>Micropenis and bilateral cryptorchidism</td>
<td>M, testosterone ↑</td>
<td>Mother – normal</td>
<td>(15)</td>
</tr>
<tr>
<td>c.667T&gt;C (exon 4)/c.891G&gt;T (exon 5)b</td>
<td>p.C223R/p.R297Lb</td>
<td>M</td>
<td>No spontaneous puberty</td>
<td>Mother – delayed puberty</td>
<td></td>
<td>(1)</td>
</tr>
<tr>
<td>c.915delT&gt; C (exon 5)</td>
<td>p.F272S</td>
<td>M (5)</td>
<td>Small penis, cryptorchidism, and no spontaneous puberty</td>
<td>NA</td>
<td>Brother – at 24 years fully pubertal</td>
<td>(13)</td>
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<td></td>
<td></td>
<td></td>
<td>No spontaneous puberty</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>c.1001–1002insC (exon 5)</td>
<td>p.334fsinC</td>
<td>M</td>
<td>Cryptorchidism and mild hypospadias</td>
<td>Menarche and ovulation with gonadotropin Tx and IVF</td>
<td>Fertility achieved with pulsatile GNRH Tx</td>
<td>(16)</td>
</tr>
<tr>
<td>c.991C&gt;T (exon 5)/c.1195T&gt;A (exon 5)b</td>
<td>p.R331X/p.X399Rb</td>
<td>M</td>
<td>At 17 years: testes 1 ml and penis 5×1.75 cm</td>
<td>Fertility achieved with pulsatile GNRH Tx</td>
<td>NA</td>
<td>(2)</td>
</tr>
</tbody>
</table>

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

Number in parenthesis refers to number of patients with similar clinical findings.

Compound 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 genitilia 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 KISS1R 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 microopenis (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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