CASE REPORT

Two novel GnRHR gene mutations in two siblings with hypogonadotropic hypogonadism

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Abstract

Objective: Mutations in the gonadotropin-releasing hormone receptor (GnRHR) gene are the cause of isolated hypogonadotropic hypogonadism (HH). We describe the molecular investigations of the GnRHR gene in two siblings affected by HH and their clinical course.

Design: The female was referred at age 14 for pubertal delay with no secondary sexual signs, whereas the male had been followed since prepuberty. Hormonal evaluation showed very low levels of gonadotropins, luteinizing hormone-releasing hormone test (LHRH test) and sexual steroids in both patients, suggesting a possible defect in the mechanism of action of the GnRH gene on its receptor.

Methods: The GnRHR gene of the two siblings and their parents were analyzed by PCR followed by direct sequencing.

Results: Two new single nucleotide substitutions resulting in the T104I and the Y108C substitutions in the first extracellular loop (ECL1) were identified in both siblings. The molecular analysis confirmed the carrier status of the parents.

Conclusions: We identified two new missense mutations in the GnRHR gene in two siblings with HH. The nature of the substitutions lying in the ECL1 involved in the ligand–receptor interaction, as well as the high conservation of the two residues in all mammalian GnRHR, are suggestive of some implications in the phenotype observed.

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Introduction

Hypogonadotropic hypogonadism (HH) is a disorder characterized by the absence of, or incomplete, pubertal development and fertility caused by an alteration in gonadotropin secretion. In the last few years, several genes involved in the hypothalamic–pituitary–gonadal (HPG) axis development and function have been identified and mutations in these genes were associated with the pathogenesis of HH (1, 2).

Gonadotropin-releasing hormone (GnRH), produced in the hypothalamus and secreted into the portal circulation, exerts its function as the principal regulator of gonadotropin luteinizing hormone (LH) and follicle-stimulating hormone (FSH) synthesis and secretion, through binding to its specific receptor (GnRHR) on the surface of gonadotrope pituitary cells.

The alteration of this process leads to variable forms of HH – the response to pulsatile administration of GnRH and the association of this condition with other clinical features is often useful to distinguish defects in GnRH synthesis or action (2). In particular, in the isolated forms, male patients usually present with microphallus and low testicular volume with delayed

or absent virilization and in some cases, mild or severe gynecomastia, whereas female patients show delayed or absent pubertal development and hypogonadism features such as small ovaries and uterus (3–6).

The molecular study of GnRH in patients with isolated HH (IHH) did not reveal any alteration, whereas 18 mutations in the GnRHR gene have been identified until now (7).

The GnRHR gene, spanning about 20 kb, is located on chromosome 4q13. Its coding sequence comprises 3 exons and encodes a 328 amino acid protein expressed principally at the level of gonadotrope cells of the pituitary gland but also in the testis, ovary, placenta and prostate (8). The protein belongs to the seven transmembrane (TM) G-protein coupled receptor (GPCR) family, but shows some particular features such as the absence of the cytoplasmatic C-terminal tail and the TM2/TM7 reciprocal N/D substitutions.

Here, we describe a family with two siblings affected by IHH. The molecular study of the GnRHR gene revealed the presence of two nucleotide substitutions in both siblings, which is not previously described.

Patients and methods

Case report

The female propositus (patient 1), first of three children (1 female, 2 males) of non-consanguineous parents, was of South Italian origin. The pregnancy was uneventful with cesarean delivery at 43 weeks of gestation. The newborn was a normal female weighing 3300 g. She was referred at the age of 14.5 years for pubertal delay characterized by Tanner stage III pubic hair (PH) and axillary hair with no breast development. Her height, weight and body mass index (BMI) standard deviation scores (SDS) according to the Italian percentiles (9) were -0.3 , -1.8 and -2.2 respectively. Bone age (BA) was 14 years, pelvic ultrasound examination revealed a prepubertal uterine volume of 2.4 cm^3 without apparent ovaries. The karyotype was 46,XX. Her sense of smell and audiometry were normal and magnetic resonance imaging (MRI) excluded a lesion of the hypothalamic–pituitary area. The plasma ferritin concentration was normal. The hormonal diagnostic and follow-up evaluations are reported in Table 1. After the diagnosis of HH, GnRH pulsatile treatment with a synthetic deca-peptide (gonadorelin (Kryptocur, Aventis Pharma, Barcelona, Spain) 0.2 mg/puff – 1 intranasal puff every 2 h from 08 00 to 10 00 h and 0.2 mg/puff at 02 00 and 05 00 h) was started. After 3 months of treatment, both basal and stimulated gonadotropins increased slightly (Table 1). Pelvic

ultrasound examination showed a small increment of uterine volume (3.2 cm^3) with bilaterally appreciable ovaries (mean volume 1.2 cm^3) although no significant breast enlargement was noted. Further, after 3 months of GnRH treatment without any further clinical improvement, the same treatment was continued by adding etinil–estradiol at a dose of 0.005 mg/day for 3 months and 0.01 mg/day thereafter. This therapy was continued for 22 months and induced a progressive maturation of the secondary sexual characteristics (PH 5, B4, A×4) with ultrasound uterine and ovarian volumes of 15.5 and 3.9 cm^3 respectively. A progestin (Farlutal, Carlo Erba OTC) was then added from day 11 to day 21 at a dose of 5 mg/day for 6 months and of 10 mg/day thereafter, which induced regular menstruation. After 30 months, the treatment was interrupted and the pituitary–gonadal axis was reevaluated (Table 1) without significant changes when compared to the initial examination. Final height (165.5 cm; s.d.=0.78) was achieved at 17.9 years, when body weight was 48.6 kg (s.d.= -0.97) and BMI 17.7 (s.d.= -1.73). At 20 years of age, the patient was sent to the adult gynecologist for further follow up.

The proband's younger affected brother (patient 2) was the 3rd son from a term cesarean delivery with a birth weight of 3350 g. His clinical follow up started at 9.3 years of age when he had prepubertal testicular volume (2 ml), PH at Tanner stage II and his height, weight and BMI SDS were 0.34, -0.37 and 0.88 respectively. Spontaneous gonadarche (testicular

Table 1 Hormonal follow-up of the two affected siblings.

	GnRH test FSH, b/p/p [^] (mU/ml) LH, b/p/p [^] (mU/ml)		E2 b/p [^] (pg/ml)	P b/p [^] (ng/ml)	T b/p [^] (ng/ml)	PRL (ng/ml)
Patient 1						
Pretreatment (14.5 years)	0.6/0.6	1.3/2.1	4.0	1.5	0.3	5.0
After 3 days of GnRH treatment*	2.7/3.1	2.8/4.7	5.0	1.1	0.4	–
After 3 months of GnRH treatment*	<1.0/1.9	3.5/5.1	5.0	1.1	0.6	–
After 6 months of GnRH treatment	<1.0/1.8	1.0/3.5	3.0	0.8	<0.1	9.0
Last evaluation without treatment	0.3/2.8	0.5/5.9	<10	0.5	0.3	5.0
Patient 2						
Pretreatment (15.2 years)	0.4/1.6	1.6/11.1	–	–	1.3/7.0	–
Pretreatment (16.2 years)	0.5/1.3/2.3 [^]	1.1/9.6/9.0 [^]	10.0/10.0 [^]	0.5/0.8 [^]	1.2/2.8 [^]	5.0
After 6 months of treatment†	0.8/1.0	1.8/4.6	–	–	1.0	–
After 12 months of treatment†	0.8/1.4/2.3 [^]	2.0/11.0/9.0 [^]	76.0/64.0 [^]	1.1/2.0 [^]	8.3/9.9 [^]	10.0
After 4 months of stop therapy	2.6/4.3/5.5 [^]	5.1/23.4/15.6 [^]	11.0/49.0 [^]	0.6/1.2 [^]	4.0/6.7 [^]	8.0

b/p refers to basal/peak levels of FSH and LH after GnRH test; /p[^] refers to peak levels of FSH, LH, E₂ (estradiol), P (progesterone) and T (testosterone) after Leuporelin test (maximal LH and FSH stimulation occurred at 6 h after GnRH stimulation and maximal gonadal responses were elicited 24 h after the same agonist was administered); PRL, prolactin.

*Kryptocur (gonadorelin 0.2 mg/puff): 1 puff every 2 h in the first trimester; night time regimen was then simplified in 0.2 mg/puff at 0200 and 0500 h in the second trimester due to poor compliance. †hCG, 2000 IU weekly – hMG, 75 IU weekly (the last injections were performed 3–6 days before the GnRH tests).

volume of 4 ml) was started at the age of 11.9 years with subsequent slow progression of secondary sexual characteristics. The pretreatment evaluation of the HPG-axis was made at the age of 15.2 (BA 14.5 years, testicular volume of 9 ml and Tanner PH stage III) and 16.2 years (BA 15.0 years, testicular volume of 10 ml and Tanner PH stage III). On both occasions (Table 1, patient 2), the gonadotropin and testosterone basal levels were low, and also FSH and LH levels were low/low normal post GnRH stimulation test, as were FSH, LH and testosterone values post Leuprolide test. At the age of 16.3 years, treatment with i.m. exogenous gonadotropins (hCG, 2000 IU weekly; hMG, 75 IU weekly) was started and the HPG axis was examined during and after treatment (Table 1). Gonadotropins and testosterone levels rose significantly after 12 months of treatment and remained normal, although at a lower level, 4 months after its suspension. At that time (17.6 years of age) patients' height, weight and BMI were 177.6 cm (s.d.=0.61), 63.9 kg (s.d.=−0.41) and 20.2 (s.d.=−0.82) respectively (9); pubertal evaluation showed a Tanner stage IV PH and a mean testicular volume of 11 ml.

Hormonal evaluation

Informed parental consent was obtained both for hormonal evaluations and treatment according to the Declaration of Helsinki. FSH and LH were measured basally and after a GnRH test (samples taken before injection and 15, 30, 45, 60 and 90 min after 50 µg i.v. injection of Lutrelif, Ferring) and after a Leuprorelin test (samples taken before injection and 6, 12 and 24 h after 0.5 mg s.c. injection of Enantone Die, Takeda Italia Farmaceutici, Cerano, Italy) using an ICMA commercial method (Bayer). Commercial ICMA kits were used also for testosterone, progesterone, estradiol and prolactin (Bayer).

Molecular analysis

Informed consent was obtained before DNA analysis. Genomic DNA was isolated from leucocytes by Wizard Genomic DNA Purification Kit (Promega). The complete coding region of the GnRHR gene and its flanking regions were PCR amplified in three separate fragments by a couple of primers: 1F 5'-CATGGACTTTA-GATTCGGT-3'; 1R 5'-GTTAAGAAGTTTGCCCAAG-3'; 2F 5'-AGATCTATTGACACTTACCC-3'; 2R 5'-ATGAGC-TAATATATGCAAAGT-3'; 3F 5'-ATTTCTCCCATTTGAT-TAAGTTG-3'; 3R 5'-CATTTGTGTTAATCATTTCCC-3'. All PCRs were performed in a final volume of 50 µl containing 150 ng DNA. For exon 1, after the initial denaturation at 95 °C for 5 min, DNA was denatured at 95 °C for 50 s and the primers annealed for 20 s at 48 °C for 5 cycles and at 46 °C for 25 cycles, the extension was performed at 72 °C for 50 s. The reactions were terminated by a final extension at 72 °C for 10 min.

For PCRs of exons 2 and 3, after the initial denaturation at 95 °C for 5 min, 30 cycles at 95 °C for 40 s and 20 s at 48 °C (exon 2) or 45 °C (exon 3) were carried out. The extension was performed at 72 °C for 40 s. The reaction was terminated by a final extension at 72 °C for 5 min.

Direct sequencing of the entire coding regions of GnRHR gene was performed using the forward PCR primers and an additional one for the exon 1, 1SEQF 5'-CTCTCAA-GAATGAAGCTGCTC-3'. Cycle sequencing reactions were performed as follows: 1 µl sequencing primer, 5 µl PCR purified by Qiaquick PCR purification kit (Qiagen) and 4 µl of Dye Terminator Cycle Sequencing in a final volume of 20 µl 30 cycles of 30 s at 95 °C and 4 min at 60 °C were carried out. Cycling reactions were subjected to precipitation and capillary electrophoresis on a CEQ 2000 (Beckman-Coulter, Fullerton, CA, USA).

Results

The molecular analysis of the GnRHR gene entire coding and splicing regions (GenBank accession no. NT022778) revealed in both siblings the presence of two new single nucleotide substitutions in exon 1: the substitution C>T at nucleotide 311 resulting in the replacement of T104 by I, and the substitution 323 A>G resulting in the replacement of Y108 by a C. The two alterations detected were confirmed on both strands in two different PCRs. The molecular analysis confirmed the carrier status of the parents. In particular, the father was heterozygote for the Y108C and the mother for the T104I.

Discussion

HH is defined as a deficiency of the pituitary secretion of FSH and LH, which results in the impairment of pubertal maturation and reproductive function. The GnRHR gene is one of the genes whose alterations is a cause of HH. In this study, we have presented two siblings affected by HH in whom the same two new mutations were identified.

Several authors have reported the wide spectrum of clinical manifestations with GnRHR gene mutations (8, 4, 10–12). Even in the same family, it has been noted that siblings with the same GnRHR defect had different phenotypes (13).

Also in our two affected siblings, the clinical manifestations were apparently different: the brother, in fact, started spontaneous puberty, although with incomplete progression, whereas the sister did not. The different sensitivities of the hypothalamic–pituitary axis in the two siblings were also marked in the gonadotropin response to exogenous GnRH administration. In fact, although hormonal evaluation showed very low basal levels of gonadotropins for both of them, basal

sexual steroid levels and GnRH-stimulated LH responses were considerably different in the two siblings (Table 1). In fact, patient 2 showed an evident basal production of testosterone and about twice the LH-stimulated levels of his sister at every examination. The attempt of pulse therapy performed on patient 1 failed to produce significant changes in pituitary–gonadal function. The examination of the HPG axis through a GnRH test after discontinuation of treatment showed rather different results. The brother, in fact, maintained a nearly normal hormonal picture, whereas his sister did not. The results of the Leuporelin test in the brother confirmed the ability of this super-active analog to overcome the receptor defect and to induce testosterone production (Table 1); the levels of both basal and stimulated testosterone during the follow-up appeared to be a good indicator of pubertal progression. Unfortunately, this test was not performed in the sister and we cannot compare their sensitivity to this challenge. Both siblings are compound heterozygotes for T104I and Y108C. They affect two residues lying in the first extracellular loop (ECL1) of the protein involved in the ligand–receptor interaction. However, as in many other GPCRs, the absence of GnRHR's crystal structure has prompted the setting up of molecular models that have led to some progress only for the TM helices but not for the ECL, where T104 and Y108 are located (14,15). Although functional studies are necessary to verify the relative contribution of the two mutations on the phenotypes observed, it is possible to make some speculations based on the conservation degree of the affected residues as well as the type of substitutions. The T104I leads to the substitution of a polar residue that, as reported by Millar *et al.* (16), is perfectly conserved among the type I receptors from bony fish to mammals with the exception only of *Xenopus*, which has a hydrophobic residue; the Y108 carries an aromatic group and is less conserved among type I receptors, but this position is never occupied by a C, suggesting that this change could be dangerous for the structure/function of the receptor, and may be due to the presence of a sulphidrilic group. Thus, the substitution of these two residues for others with such different chemical features suggests that these alterations may be involved in the phenotypes shown by the two siblings. In addition, a position effect of these two substitutions (as the affected residues are linearly located near each other) as well as the synergistic effect of the two mutations must be taken into account and verified by functional studies. Since supraphysiological stimulation with GnRH analog elicits a response, it is possible that the mutations do not completely abolish the receptor function. Different reasons may be attributable to the different pattern of expression in the two siblings: one may be the contribution of other genes, particularly in this case, in which different sex-specific genes and patterns of expression and interaction are involved; however, the opposite sex expression of the GnRHR defect severity

observed in our cases compared to what reported by De Roux *et al.* (13), seems to exclude an univocal sex-related severity.

A second reason accounting for differences in the disease expression could be related to the low BMI of patient 1 that may cause a blunted ability to release GnRH, whereas the brother may have had a post-treatment sensitization of the 'gonadostat' with partial recovery of the ability to release GnRH in response to testosterone fluctuations. Finally, the unsuccessful response to intranasal GnRH pulsatile administration in the sister could also be partially due to the difficult compliance to the required treatment regimen. In conclusion, GnRH-R analysis should be considered particularly in patients with other family members affected by IHH and after the exclusion of other causes of central hypogonadism, including Kallman's syndrome, multiple pituitary insufficiency, brain tumors as well as constitutional pubertal retardation. The identification and functional characterization of GnRH-R mutations in patients with IHH may extend our understanding of gonadotropin deficiency. However, the clinical benefits of this knowledge are currently limited, since patients with GnRH-R mutations will be more easily treated with substitutive testosterone or estrogen/progestin, as confirmed also in the present study.

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