Role of sequence variations of the GnRH receptor and G protein-coupled receptor 54 gene in male idiopathic hypogonadotropic hypogonadism

Fabio Lanfranco1,2, Jörg Gromoll1, Sigrid von Eckardstein1, Eva M Herding1, Eberhard Nieschlag1 and Manuela Simoni1

1Institute of Reproductive Medicine of the University, D-48129 Münster, Germany and 2Division of Endocrinology and Metabolism, Department of Internal Medicine, University of Torino, Italy

Abstract

Objective: To determine the frequency of mutations of the gonadotropin-releasing hormone receptor (GnRHR) and of the G protein-coupled receptor 54 (GPR54) genes in normosmic idiopathic hypogonadotropic hypogonadism (IHH).

Methods: In a retrospective study we analyzed the GnRHR and the GPR54 genes of 45 IHH patients and 50 controls. Genomic DNA was amplified by PCR to obtain partially overlapping amplicons encompassing the exon–intron boundaries of the GnRHR and GPR54 genes and analyzed by single-stranded conformation polymorphism gel electrophoresis and/or DNA sequencing.

Results: One heterozygous R262Q mutation of the GnRHR gene was identified in one patient with familial IHH. The silent single-nucleotide polymorphism (SNP) 453C>T occurred at the same frequency in patients and controls. One patient with sporadic IHH and consanguineous parents showed a novel homozygous sequence variation of the GPR54 gene (1001_1002insC) resulting in an open reading frame shift and elongation of 43 amino acids with an increased number of proline residues in the intracellular receptor domain. This patient had delayed puberty, low testosterone (3.4 nmol/l), and low-normal LH and FSH levels responsive to GnRH. Pulsatile GnRH administration normalized testosterone levels and induced spermatogenesis sufficiently to induce a pregnancy with assisted reproduction. Two common SNPs in exon 1 and exon 5 of the GPR54 gene showed similar frequency distribution and hormonal profiles in IHH and controls.

Conclusions: Mutations of the GnRHR and of the GPR54 gene are rare in IHH and should be investigated especially in cases with autosomal recessive transmission. Common SNPs of the GnRHR and GPR54 genes do not play any role in IHH.

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Introduction

Idiopathic hypogonadotropic hypogonadism (IHH) is a genetically heterogeneous condition characterized by a functional deficit in hypothalamic gonadotropin-releasing hormone (GnRH) production or action (1–3). Patients with IHH have been classically divided into those with anosmia or hyposmia (Kallmann syndrome) and those with normal olfaction (4, 5). Sporadic and familial cases with different modes of inheritance have been described. Mutations in two distinctive genes located at the short arm of the X-chromosome, KAL1 and DAX1, are responsible for the X-linked forms of IHH (2, 4–8). Mutations of the fibroblast growth factor receptor 1 gene were recently reported in patients with Kallmann syndrome (9, 10). These were the only identified causative genes involved in inherited human IHH, and they were dominant. The molecular basis of the first autosomal recessive form of IHH, affecting both women and men, was determined to be mutations of the GnRH receptor (GnRHR) gene (11, 12).

GnRHR is a G protein-coupled receptor with seven transmembrane segments and an extracellular amino terminus but no intracellular carboxyl terminus; it activates phospholipase C and mobilizes intracellular calcium via G proteins (13, 14). The GnRHR gene (NM_000406) is localized on chromosome 4 at band q13.1 (15). The coding sequence comprises three exons and spans over 20 kb. Numerous compound heterozygous (11, 12, 16–18) and four homozygous (19–22) GnRHR mutations have been described, causing subtypes of IHH varying from complete to partial resistance to GnRH.
GnRH receptor (GnRHR) mutations account for less than 50% of familial IHH cases and a small fraction of sporadic cases, indicating that IHH is a heterogeneous condition caused by various genetic defects. A new cause for IHH has been recently described: it consists of mutations of the G protein-coupled receptor 54 (GPR54) gene, which is localized on the short arm of chromosome 19 (19p13). GPR54 (NM_032551) is a G protein-coupled receptor expressed mainly in the brain, pituitary and placenta. GPR54 is formed of five exons and encodes a 398 amino acid protein. In the past 2 years homozygosity mapping and candidate gene analysis of large consanguineous pedigrees led two groups to describe loss-of-function mutations in GPR54 in association with autosomal recessive IHH (23, 24). In parallel, the independent generation and characterization of a Gpr54 knockout mouse model, which shows a phenocopy of human isolated IHH, demonstrated that the function of GPR54 is conserved in mammals and that GPR54 is necessary for the normal function of this axis (24, 25). A further case of GPR54 compound heterozygous mutation in a patient with IHH was described recently (26).

The aim of this study was to analyze the occurrence of sequence variants, mutations and single-nucleotide polymorphisms (SNPs) of the GnRHR and GPR54 gene in a cohort of 45 male patients with normosmic IHH. We report the clinical phenotype of a subject with consanguineous parents who showed a novel homozygous mutation in the GPR54 gene and the SNP analysis of the GnRH and GPR54 genes in hypogonadal patients and controls.

Subjects and methods

Subjects

In a retrospective study design, 45 hypogonadal male patients were selected from the database of the out-patient clinic of the Institute of Reproductive Medicine of the University in Münster, Germany. Six of the 45 patients reported at least another case of IHH, defined as lack of spontaneous pubertal maturation requiring medical intervention, in their family (familial cases). However, since detailed pedigrees were not available, the mode of inheritance could not be identified. The other 39 patients were sporadic cases. Hypogonadotropic hypogonadism was defined as testosterone concentrations below 12 nmol/l in the setting of inappropriately low gonadotropin levels (below or around the lowest limit of the normal range: 2 IU/l for luteinizing hormone (LH) and 1 IU/l for follicle-stimulating hormone (FSH)) upon repeated determinations. Additional elements for the diagnosis of IHH were: absence of spontaneous pubertal maturation, no evidence of a hypothalamic–pituitary anatomical lesion on imaging, and otherwise normal pituitary function as documented by normal basal and stimulated levels of prolactin, thyroid-stimulating hormone, growth hormone and cortisol. All patients were normosmic, as assessed by a smell test in which the subject was asked to identify odors produced by aromatic essences. Eight patients had bilateral and five patients monolateral undescended testes. Patients provided a blood sample for DNA analysis and clinical data were retrieved from the database and patient charts. The control group consisted of 50 eugonadal men with normal hormone levels and normal semen analysis recruited as volunteers to participate in a male contraception trial. All subjects were of Caucasian origin. Patients and controls gave written informed consent according to the protocol approved by the Ethics Committee of the Medical Faculty and State Medical Board.

Genetic screening

Genomic DNA was extracted from peripheral blood using the FlexiGene DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Mutation screening of the GnRH receptor and of the GPR54 gene were performed by single-stranded conformation polymorphism (SSCP) gel electrophoresis as previously described (27, 28). Genomic DNA was amplified by PCR to obtain partially overlapping amplicons encompassing the exon–intron boundaries. The primers used are shown in Tables 1 and 2. The amplicons were further analyzed by SSCP. Fragments showing an abnormal migration pattern were sequenced. In addition, about one-third of the amplicons randomly chosen among those with a normal migration pattern were directly sequenced in order to check the accuracy of the SSCP results. SSCP analysis and subsequent sequencing revealed common SNPs both in the GnRH receptor gene (453C>T, refSNP ID: rs4986942) and in the GPR54 gene (24A>G, refSNP ID: rs10407968 and 1091A>T, refSNP ID: rs350132). The Gnrhr 453C>T SNP was further screened by MaeIII restriction fragment length analysis. The SNPs of the GPR54 gene were analyzed by SSCP and sequencing.

Hormone measurements

Serum hormones were analyzed as described previously (27, 29). Serum LH, FSH, estradiol and sex hormone binding globulin (SHBG) were assayed by fluororimmunoassay using AutoDelfia equipment (Perkin Elmer, Freiburg, Germany). Testosterone levels were determined using a commercial ELISA (DRG, Marburg, Germany). Intra- and inter-assay coefficients of variation were below 10%. The normal range for unextracted serum testosterone is 12–35 nmol/l. All analytical methods were performed and documented in accordance with the ISO/9001 certified quality management system applied in our laboratory.

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Statistics

After exclusion of the two patients with mutations of the GnRHR and of the GPR54 gene, SNP data of 43 patients were considered. In addition, SNP data were obtained in 50 control subjects for the GnRHR and 48 control subjects for the GPR54 gene. Statistical analysis was performed by applying a commercially available software package (GraphPad Prism; GraphPad Software, Inc., San Diego, CA, USA). Data were analyzed for normal distribution. Data are presented as means ± S.E.M. Unpaired t-test, one-way ANOVA, Fisher’s exact test and a Chi-square test were used for the analysis of the data. P values < 0.05 were considered statistically significant.

Results

The basal hormone levels of patients and controls are shown in Table 3. Serum LH, FSH, testosterone and estradiol levels were significantly different between patients and controls (t-test; P < 0.001), while no significant differences were observed for SHBG.

GnRHR gene

One man with familial IHH was found to be heterozygous for a GnRHR mutation (c.785G > A) exchanging arginine for glutamine at position 262 (R262Q). No other mutation could be identified upon complete sequencing of the GnRHR gene. This patient (karyotype 46, XY) was the fourth son of a kindred of 17 siblings and was diagnosed with IHH at the age of 23 years. One of his sisters was reported to have primary amenorrhea and IHH was diagnosed. She was under hormonal treatment and adopted two children. The patient was previously treated with human chorionic gonadotropin (hCG)/human menopausal gonadotrophin (hMG), leading to an increase of testicular volume up to 11 ml and appearance of sperm in the ejaculate (maximal sperm concentration: 5 × 10⁶/ml). He is now 53 years old and is under testosterone replacement therapy. The GPR54 gene was completely sequenced and was normal in this patient. Neither the affected sister, nor other members of his family were available for genetic screening.

No other mutations of the GnRHR gene were found in the other patients. The occurrence of a previously described, synonymous polymorphism exchanging C to T...
at nucleotide position 453 was investigated by restriction analysis using MaeIII (30). This polymorphism was found to be mostly heterozygous, with the same allelic frequency in both patients and controls (Table 4). No significant differences in the hormonal levels depending on the 453C > T SNP were observed within the groups (data not shown).

**GPR54 gene**

In another patient with isolated IHH a homozygous sequence variant in the GPR54 gene was found (Fig. 1). This consisted of a homozygous insertion of a cytosine after nucleotide position 1001 (1001_1002insC). This insertion results in a frame shift in the open reading frame with elongation of the GPR54 protein from 398 to 441 amino acids.

The patient (karyotype 46, XY) was the only child of parents of German descent following an uneventful pregnancy. His parents were cousins of second degree. No known family history of hypogonadism or infertility was reported. He suffered from delayed puberty and bilateral undescended testes. Orchidopexy was performed at the age of 18. At the age of 20 he underwent hormonal evaluation, which showed moderately low LH (1.1 IU/l) and FSH (2.2 IU/l) levels along with reduced testosterone levels (3.6 nmol/l). A normal gonadotropin response to GnRH administration (100 µg i.v.) was recorded (LH peak: 10.5 IU/l; FSH peak 5.7 IU/l). He underwent testosterone enanthate replacement therapy, which induced complete virilization, with mild bilateral lipomastia. Testosterone levels were in the normal range (15.6 nmol/l) while gonadotropins were increased (LH: 11.5 IU/l; FSH 24.9 IU/l).

A mild hypospadias was present. Ultrasound scan showed both testes in the scrotal sac with reduced volume (right: 3 ml; left 5.3 ml). Semen analysis revealed a normal semen volume (5 ml) with oligoasthenozoospermia (concentration 0.4 x 10⁹/ml; A + B motility 34%; 30% normal forms). At the age of 33, having been under pulsatile GnRH therapy for 2 years, he fathered a healthy son following an IVF procedure. The patient is currently 46 years old and is under hCG and hMG therapy. The patient’s parents were not available for further examination.

No other mutation was found in the patient group. Two SNPs, in exon 1 and exon 5 were found. The 24G > A variant is a synonymous SNP, while the 1091A > T polymorphism exchanges the amino acid histidine for leucine at codon 364 (H364L) in the intracellular domain. The allelic frequency of both polymorphisms was the same in patients and controls (Table 4). No significant differences in the hormonal levels depending on the SNPs were observed within both groups of patients and controls by ANOVA (data not shown). The combination of the two SNPs gives rise to four haplotypes, the distribution pattern of which did not differ in patients and controls (Table 5).

Considering the three SNPs in the GnRHR and GPR54 genes together, 13 combinations were observed, again with similar frequency distribution in both groups (Table 6). No difference in hormone concentrations was found in correlation with each combination within both groups of patients and controls by ANOVA (not shown).

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**Table 3** Serum hormone values (means±S.E.M.) in the subjects investigated.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>LH (IU/l)</th>
<th>FSH (IU/l)</th>
<th>Testosterone (nmol/l)</th>
<th>Estradiol (pmol/l)</th>
<th>SHBG (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1HH, n = 45 (age: 21.7±1.2 years)</td>
<td>0.8±0.2</td>
<td>1.0±0.1</td>
<td>3.1±0.5</td>
<td>39.3±3.6</td>
<td>43.4±5.0</td>
</tr>
<tr>
<td>Controls, n = 50 (age: 25.2±1.8 years)</td>
<td>4.0±0.3</td>
<td>3.6±0.3</td>
<td>19.5±0.8</td>
<td>73.3±4.8</td>
<td>36.0±2.8</td>
</tr>
</tbody>
</table>

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**Table 5** GPR54 haplotype distribution in patients (n = 43) and controls (n = 48).

<table>
<thead>
<tr>
<th>Haplotype (nucleotide at position 24/1091)</th>
<th>Patients (no. of alleles)</th>
<th>Controls (no. of alleles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/A</td>
<td>48</td>
<td>59</td>
</tr>
<tr>
<td>A/T</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>G/A</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>G/T</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Undecided</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

P = 0.22 by Chi-square test.
Discussion

The pathogenesis of IHH has not yet been clarified in all cases. One possible mechanism responsible for the clinical picture of IHH is represented by mutations in the GnRHR gene. In the present study, screening for GnRHR mutations in a group of 45 hypogonadal IHH patients resulted in the detection of only one heterozygous R262Q mutation in one patient with familial IHH. From our data it is not possible to conclude that this heterozygous sequence variant plays any role in the observed phenotype. The same heterozygous mutation was reported in one patient with IHH by Beranova et al. (31) in the absence of other sequence abnormalities. However, the same heterozygous R262Q mutation was associated with normal gonadal function in other subjects (11).

The R262Q mutation in the third intracellular loop has been repeatedly described in IHH patients and, in vitro studies, results in only partial impairment of signal transduction with decreased activation of phospholipase C without modifying ligand binding (17, 32). In our patient other sequence variants were found neither in the GnRHR nor in the GPR54 gene, leaving the pathogenesis of normosmic IHH unresolved. The heterozygous R262Q mutation might be unrelated to the IHH of our patient. Alternatively, a second mutation occurring in genomic regions not analyzed in the present study, such as the promoter or the introns, perhaps influencing gene transcription and/or splicing, might be involved. Unfortunately, other members of the family were not available for the analysis. Sixteen different GnRHR gene mutations have been described in the literature so far (4, 11, 12, 15–17, 19, 20, 22, 31, 33–36). It is worth noting that there is a certain variability in the clinical manifestation, and identical GnRHR mutations do not lead to identical phenotypes (32, 37). In accord with the literature, our data suggest that mutations of the GnRHR gene are rare in non-familial cases of IHH and mutation analysis is principally indicated in patients with normosmic autosomal recessive familial IHH.

We describe the occurrence of a novel homozygous mutation of the GPR54 gene in a male patient with IHH. Three previous studies have reported seven different mutations in five families or individuals with IHH (23, 24, 26). The sequence variant 1001_1002insC described in the present study is the first case of an insertional mutation leading to a frame shift. The insertion is located immediately 3' of the seventh transmembrane segment, in the intracellular domain of the receptor, and results in a significant alteration of the protein structure with a remarkable stretch of proline residues.

### Table 6

<table>
<thead>
<tr>
<th>GnRHR 453C &gt; T</th>
<th>GPR54 24G &gt; A</th>
<th>GPR54 1091A &gt; T</th>
<th>Patients (n)</th>
<th>Controls (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>AA</td>
<td>AA</td>
<td>13 (30.2%)</td>
<td>16 (33.3%)</td>
</tr>
<tr>
<td>CC</td>
<td>AA</td>
<td>AT</td>
<td>7 (16.3%)</td>
<td>9 (18.8%)</td>
</tr>
<tr>
<td>CC</td>
<td>AA</td>
<td>TT</td>
<td>6 (13.9%)</td>
<td>3 (6.3%)</td>
</tr>
<tr>
<td>CC</td>
<td>AG</td>
<td>AA</td>
<td>7 (16.3%)</td>
<td>10 (20.8%)</td>
</tr>
<tr>
<td>CC</td>
<td>AG</td>
<td>AT</td>
<td>2 (4.7%)</td>
<td>0</td>
</tr>
<tr>
<td>CC</td>
<td>AG</td>
<td>TT</td>
<td>0</td>
<td>1 (2.1%)</td>
</tr>
<tr>
<td>CC</td>
<td>GG</td>
<td>AA</td>
<td>0</td>
<td>1 (2.1%)</td>
</tr>
<tr>
<td>CT</td>
<td>AA</td>
<td>AA</td>
<td>3 (6.9%)</td>
<td>1 (2.1%)</td>
</tr>
<tr>
<td>CT</td>
<td>AA</td>
<td>AT</td>
<td>1 (2.3%)</td>
<td>6 (12.5%)</td>
</tr>
<tr>
<td>CT</td>
<td>AA</td>
<td>TT</td>
<td>1 (2.3%)</td>
<td>1 (2.1%)</td>
</tr>
<tr>
<td>CT</td>
<td>AG</td>
<td>AA</td>
<td>1 (2.3%)</td>
<td>0</td>
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<tr>
<td>CT</td>
<td>AG</td>
<td>TT</td>
<td>1 (2.3%)</td>
<td>0</td>
</tr>
<tr>
<td>TT</td>
<td>AA</td>
<td>AT</td>
<td>1 (2.3%)</td>
<td>0</td>
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</tbody>
</table>

P = 0.3388 by Chi-square test.
residues conferring high hydrophobicity. Although functional studies were not performed, such a drastic structural change is compatible with impairment of signal transduction, most probably resulting in the clinical phenotype. Alternatively, mRNAs containing a premature stop codon or lacking an in-frame termination codon are thought to be eliminated by ad hoc surveillance cellular mechanisms called nonsense-mediated decay and non-stop decay (38, 39) and it is therefore possible that the aberrant mRNA originating from the mutation is eliminated before transcription. In any case, an important functional impairment of the GPR54 gene in our patient should be expected. Of particular clinical interest is the fact that the patient was successfully treated with pulsatile GnRH therapy, achieving normal virilization and sperm maturation sufficient to induce a pregnancy with assisted fertilization. This is a clear, direct demonstration in the human that the KISS1/GPR54 system is operative above the GnRH/GnRHR level, possibly stimulating GnRH processing or secretion, as shown previously in rodents (40–42) and non-human primates (43).

The previously described mutations of the GPR54 gene in patients with IHH were found in a family with a homozygous 155 bp deletion at the intron 4–exon 5 junction (23), two families with homozygous missense mutations, L102P and L148S respectively (23, 24) and two isolated, non-familial cases with compound heterozygosity, i.e. R331X plus X399 (24) and C223R plus R297L (26). Altogether, our data and that of the literature now report data on 128 patients with IHH without anosmia and non-homologous GnRHR. Although the methodological used in the different studies was different, these data suggest a higher prevalence of GPR54 mutations among familial (2 out of 13: 15.4%) than non-familial cases (3 out of 115: 2.6%). Our patient represents the first case of a homozygous sequence variant in a consanguineous family occurring as a non-familial IHH case.

Considering both genes together, according to the literature the probability of detecting mutations in either the GnRHR or the GPR54 gene in familial cases of IHH without anosmia can be as high as 50% (26, 31) so that genetic screening of these two genes is strongly indicated in such cases. The much lower incidence of the non-familial cases (<10% according to the literature, 1 case out of 39 (2.6%) detected in the present investigation) reflects the autosomal recessive nature of the disease. Unfortunately, the response to functional tests (GnRH stimulation test, monitoring of gonadotropin pulsatile secretion) in cases with mutations is rather heterogeneous and has no predictive value for identifying the carriers of mutations. The large majority of sporadic cases of IHH without anosmia remain idiopathic and other, still unrecognized factors are obviously involved in the pathogenesis of the disease. The KISS1 gene represents an obvious candidate to screen for, given its clear functional role in the GPR54–GnRHR axis (40, 41). No mutations of the KISS1 gene were found in 30 patients with IHH or constitutional delay of puberty so far (26) but more cases should be investigated in the future in order to understand the role of the putative GPR54 ligand in the activation of the hypothalamo–pituitary–gonadal axis and in the pathogenesis of IHH.

In this study we characterized SNPs in both the GnRHR and the GPR54 genes in hypogonadal and control subjects. The 453C > T synonymous SNP of the GnRHR gene showed an allele frequency of 0.080 and 0.092 in controls and patients respectively, similar to the estimated allele frequency available in the NCBI SNP database (0.091). The two other SNPs in the coding region of the GnRHR gene reported in the database, i.e. the 549G > C (rs13130501) and the 550C > G (rs13149772), were not observed in the present investigation and must have an allele frequency <0.005, since they were not found in 198 chromosomes analyzed. From our data it does not appear that common SNPs in the coding region of the GnRHR gene are associated with IHH, in agreement with a recent comprehensive study which characterized the role of GnRHR and the GnRHI haplotypes in pubertal timing (44).

As for the GPR54 gene, the synonymous 24A > G SNP was analyzed for the first time in this study and showed a similar allele frequency in patients and controls. Similarly, no difference in the frequency distribution of the non-synonymous SNP 1091A > T was observed between the two groups, in accord with the data previously presented by Semple et al. (26). Even when the GPR54 haplotypes or the genotype combinations of both genes were considered together, no significant difference was found between patients and controls. The two additional exonic SNPs of the GPR54 gene reported in the database, i.e. the 615C > A (rs8111938) and the 155A > G (rs3746147) were not found, confirming the very low allele frequency reported by Semple et al. (26). Our data suggest that SNPs of the GnRHR and the GPR54 gene are not associated with IHH.

In conclusion, we found a novel homozygous sequence variant of the GPR54 gene in a patient with consanguineous parents and non-familial IHH. The effective stimulation of gonadotropin secretion, testosterone and spermatogenesis achieved by pulsatile GnRH therapy, showed that the KISS1/GPR54 system is necessary to stimulate GnRH secretion and/or action. The role of GPR54 in the regulation of the release of GnRH, as recently investigated in various experimental models (40, 42, 43, 45) has implications for the understanding of puberty and its disorders, as well as for possible development of ligands to manipulate the function of this axis for therapeutic purposes, e.g. suppression of the gonadal axis in patients with metastatic prostate cancer. Mutations of the GnRHR and of the GPR54 gene should be searched for in patients with IHH, especially in familial cases and when an autosomal recessive mode of inheritance is suspected.
Acknowledgements

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