

# Prevalence, Phenotypic Spectrum, and Modes of Inheritance of Gonadotropin-Releasing Hormone Receptor Mutations in Idiopathic Hypogonadotropic Hypogonadism\*

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## ABSTRACT

Mutations in the GnRH receptor (*GNRHR*) have been described as a cause of reproductive failure in a subset of patients with idiopathic hypogonadotropic hypogonadism (IHH). Given the apparent rarity of these mutations, we set out to determine the frequency and distribution of *GNRHR* mutations in a heterogeneous population of patients with IHH who were well characterized with respect to diagnosis, phenotype, and mode of inheritance and to define their distribution within the receptor protein.

One hundred and eight probands with IHH were screened for mutations in the coding sequence of *GNRHR*. Forty-eight of the 108 patients had a normal sense of smell, whereas the remaining 60 had anosmia or hyposmia (Kallmann syndrome). Exon segments in the *GNRHR* were screened for mutations using temperature gradient gel electrophoresis, and all mutations were confirmed by direct sequencing.

Five unrelated probands (3 men and 2 women), all normosmic, were documented to have changes in the coding sequence of the *GNRHR*. Two of these probands were from a subgroup of 5 kindreds consistent with a recessive mode of inheritance, establishing a *GNRHR* mutation frequency of 2 of 5 (40%) in patients with normos-

mic, autosomal recessive IHH. The remaining 3 probands with *GNRHR* mutations were from a subgroup of 18 patients without evidence of familial involvement, indicating a prevalence of 3 of 18 (16.7%) in patients with sporadic IHH and a normal sense of smell.

Among the five individuals bearing *GNRHR* mutations, a broad spectrum of phenotypes was noted, including testicular sizes in the male that varied from prepubertal to the normal adult male range. Three probands had compound heterozygous mutations, and two had homozygous mutations. Of the eight DNA sequence changes identified, four were novel: Thr<sup>32</sup>Ile, Cys<sup>200</sup>Tyr, Leu<sup>266</sup>Arg, and Cys<sup>279</sup>Tyr. COS-7 cells transiently transfected with complementary DNAs encoding the human *GNRHR* containing each of these four novel mutations failed to respond to GnRH agonist stimulation.

We conclude that 1) the spectrum of phenotypes in patients with *GNRHR* mutations is much broader than originally anticipated; 2) the frequency of *GNRHR* mutations may be more common than previously appreciated in familial cases of normosmic IHH and infrequent in sporadic cases; and 3) functional mutations of the *GNRHR* are distributed widely throughout the protein. (*J Clin Endocrinol Metab* 86: 1580–1588, 2001)

**I**N THE CLINICAL syndrome of idiopathic hypogonadotropic hypogonadism (IHH), affected individuals present with delayed sexual development and inappropriately low gonadotropin and sex steroid levels in the absence of anatomical

or functional abnormalities of the hypothalamic-pituitary axis. There are many subtypes of IHH, including congenital IHH with anosmia [Kallmann syndrome (KS)] and IHH with adrenal insufficiency (adrenal hypoplasia congenita). Isolation of the gene responsible for X-linked KS (*KAL*) has led to a pathophysiological model correlating GnRH deficiency with abnormal olfactory bulb development (1–3). Mutations in the X-linked gene for adrenal hypoplasia congenita, *DAX1*, have also uncovered the crucial role of this orphan nuclear hormone receptor at multiple levels of the reproductive axis (4–6). Despite these discoveries, until recently little has been known about the genes that cause isolated IHH without anosmia or adrenal insufficiency.

The GnRH receptor gene (*GNRHR*, 4q21.2), encodes a seven-transmembrane domain G protein-coupled receptor,

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but lacks the intracellular carboxyl-terminus typically seen in other members of this family (7, 8). Although an obvious candidate gene for IHH, the nearly universal response of such patients to exogenous pulsatile GnRH initially made the candidacy of this gene seem unlikely (9). However, 3 yr ago, compound heterozygous mutations in the *GNRHR* were identified in a family with IHH (10). Now, numerous compound heterozygotes (10–14) and one homozygous (15) *GNRHR* mutation(s) have been described, causing subtypes of IHH varying from complete to partial resistance to GnRH.

The purpose of this study was to determine the prevalence of *GNRHR* mutations in a large population of well phenotyped patients with IHH (classified according to mode of inheritance) and establish genotype/phenotype correlations where possible. These studies demonstrate that *GNRHR* mutations can cause a broad range of reproductive phenotypes. *GNRHR* mutations, which appear to be distributed throughout the receptor, account for a larger proportion of both familial and sporadic cases of IHH than previously appreciated.

### Experimental Subjects

#### *In vivo studies*

The diagnosis of IHH was based on the absence of spontaneous puberty by age 18 yr and hypogonadal sex steroid levels (testosterone,  $\leq 3.5$  nmol/L; estradiol,  $\leq 73$  pmol/L) in the setting of inappropriately normal or low gonadotropin levels. In 65 of our 108 patients (60%), additional evidence for a diagnosis of IHH was provided by 1) the absence of normal pulsatile gonadotropin secretion during 12–24 h of frequent (every 10 min) blood sampling (16, 17); 2) normal basal and stimulated levels of TSH, PRL, GH, and cortisol on baseline and stimulation testing; and 3) no evidence of a mass lesion on imaging of the hypothalamic-pituitary region.

One hundred and eight unrelated probands from well characterized pedigrees were screened for mutations of the *GNRHR*. Patients were divided into three diagnostic categories: 1) idiopathic, congenital IHH/KS ( $n = 87$ ), 2) partial IHH/KS ( $n = 14$ ), and 3) acquired or adult-onset IHH ( $n = 7$ ). Patients were categorized as having partial IHH if low amplitude LH pulses were detectable during every 10 min blood sampling using modified Santen and Bardin pulse analysis or if the clinical history was consistent with partial pubertal development. Patients were categorized as having adult-onset IHH if they had undergone age-appropriate spontaneous sexual maturation by history (with proven paternity in some cases), but subsequently developed isolated IHH without an identifiable cause (18).

Fifty healthy volunteers (consecutive donors to the Massachusetts General Hospital blood bank) were also screened for mutations of the *GNRHR*.

#### *Modes of inheritance*

Genetic criteria were used to establish the likely mode of disease transmission as outlined in previous analyses (19). A family was classified as X-linked if only males were affected and unaffected females could be considered carriers. There could be no male to male transmission. A family was classified as autosomal recessive if all affected individuals were members of the same generation and included at least one female. Consanguinity provided additional support for this designation. A family was classified as autosomal dominant if direct transmission of the phenotype was demonstrable across generations, even if incomplete expressivity was present. Male to male transmission was considered definitive evidence for dominant inheritance. Delayed puberty and isolated anosmia were used as surrogate markers of the phenotype (19).

#### *Clinical phenotyping: baseline frequent sampling*

When possible, patients were admitted to the General Clinical Research Center of the Massachusetts General Hospital for phenotyping studies. Blood samples were collected every 10 min for 12–24 h for measurement of LH in 65 of the 108 patients. Pulsatile hormone secretion was assessed using the modified version of the Santen and Bardin method as previously described (20, 21). Gonadotropin responses to a single bolus of 100  $\mu\text{g}$  GnRH were also assessed. This study was approved by the subcommittee on human studies of the Massachusetts General Hospital; all subjects gave informed consent before participating.

### Materials and Methods

#### *In vivo studies*

The serum LH and FSH concentrations of patients 1, 2, 3, and 5 were determined by one of two methods. 1) Immunoassays were calibrated against the Second International Reference Preparation of human menopausal gonadotropin (WHO 71/223) (22–24), with a minimal detectable dose of 0.8 IU (WHO 71/223)/L. Inter- and intraassay coefficients of variation were less than 10%. Free- $\alpha$  subunit was measured by a monoclonal antibody RIA, using a highly purified  $\alpha$ -subunit of hCG as the standard (25, 26). 2) A microparticle enzyme immunoassay was performed using the automated Abbott AxSYM system, with the Second International Reference Preparation as the reference standard. The assay sensitivity for both LH and FSH was 1.6 mIU/mL. The intraassay coefficients of variation (CVs) for LH and FSH were less than 7% and 6%, respectively, with interassay CVs for both hormones of less than 7.4%. Serum testosterone concentrations were measured using the Coat-A-Count RIA kit (Diagnostic Products, Los Angeles, CA), which had intra- and interassay CVs of less than 10%. Estradiol ( $E_2$ ) was measured by the AxSYM system (Abbott Laboratories, Chicago, IL), which had an analytical sensitivity of 36.7 pmol/L and a functional sensitivity of 73.4 pmol/L. The intraassay CV was less than 6.4% with an interassay CV less than 10.6%.

#### *In vitro studies*

*Mutational analysis.* Genomic DNA was extracted from peripheral blood leukocytes or cultured white blood cells. Melting map analysis was performed for each exon fragment using WinMelt software (Bio-Rad Laboratories, Inc., Hercules, CA). When necessary, exon fragments were split, and primers for exon amplification were selected using Lasergene software (DNASTAR, Inc., Madison, WI). A 40-mer GC clamp [(GC) $n$ CGCCCGCCGCCCCGCGCCCGCCCGCCCGCCCCGCCCC] was attached to the 5'-end of either the forward or the reverse primer to prevent strand melting. Three sets of primers were used to amplify exon 1: sense, 5'-ACACAAGGCTTGAAGCTCTGTCCT-3'; antisense, 5'-(GC) $n$ TCTTCTCTGTGTCTCACTTCTGA-3'; sense, 5'-CCACTCTGACCTGTCTGGAAAG-3'; antisense, 5'-(GC) $n$ AGCTTTAGATAACTGAGAACITTCG-3'; and sense, 5'-AACATCTGACCTAGC-CAACCTG-3'; antisense, 5'-(GC) $n$ CAAGGTAACAGAACAGAG-CCAGAA-3'. One primer pair was used to amplify exon 2: sense, 5'-(GC) $n$ AGGAGCTTAGAAATTGCTTTAGGTA-3'; antisense, 5'-TGC-TATTTAAAAACTGCCACAA-3'. Two primer pairs were used to amplify exon 3: sense, 5'-(GC) $n$ GCTGTCTTCTTTTGTCCACTTTG-3'; antisense, 5'-CAATACCAAATCCTAGGACATAG-3'; and sense, 5'-CACTTCATTTACTGTCTGCTGGAC-3'; antisense, 5'-(GC) $n$ ATTTCATACCTTACCCTTCTCATA-3'. Reactions were performed in a final volume of 100  $\mu\text{L}$  containing 50 pmol of each PCR primer, 200  $\mu\text{mol}$  of each deoxy-NTP, 2.5 U *Taq* polymerase, and 10 mmol/L Tris-HCl (pH 8.3). PCR reactions were carried out for 35 cycles: denaturing at 94 C for 30 s, annealing at 53–58 C for 30 s, and elongation at 72 C for 30 s. The quality and predicted molecular size of each PCR product were assessed by electrophoresis through a 1% agarose gel.

#### *Temperature gradient gel electrophoresis (TGGE)*

Given the large number of individuals screened for mutations, TGGE, a mutation detection method that can separate two DNA fragments that differ in sequence by as little as a single base substitution, was employed (27, 28). The TGGE apparatus is a horizontal acrylamide slab gel (Diagen, Dusseldorf, Germany) placed on a horizontal aluminum platform per-

fused with water at a specified temperature gradient maintained by a digitally controlled heater bath (NESLAB Instruments, Portsmouth, NH). Using the denaturation information obtained from the Winmelt profile analysis software, the optimal temperature gradient and running time for each amplified exon of the *GNRHR* were determined using a diagonal TGGE. Once the conditions for each exon were optimized, each PCR product was denatured and renatured in the absence or presence of the comparable PCR product from genomic DNA of a healthy volunteer to examine homo- or heterozygotic mutations. Samples with a final volume of 10  $\mu$ L were denatured (95 C, 5 min), allowed to reanneal (55 C, 10 min), and analyzed in parallel on a polyacrylamide gel. Electrophoresis was performed at 280 V. Gels were then silver stained to allow the identification of DNA. PCR products that revealed an abnormal pattern, *i.e.* the presence of heteroduplexes in addition to the homoduplexes, were subjected to bidirectional DNA sequence analysis using either the same primers as those used for PCR amplification or nested primers.

### Functional studies of the GnRH receptor

All cell culture reagents were supplied by Life Technologies, Inc. (Gaithersburg, MD). GenePorter reagent was obtained from Gene Therapy System (San Diego, CA), myo-[2-<sup>3</sup>H]inositol was purchased from NEN Life Science Products (Boston, MA), GnRH agonist (des-Gly<sup>10</sup>[D-Ala<sup>6</sup>]GnRH ethylamide) was obtained from Sigma (St. Louis, MO), and AG-X8 resin was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA).

### Site-directed mutagenesis

A hemagglutinin protein (HA)-tagged human *GNRHR* (*hGNRHR*) complementary DNA clone provided by Dr. Thomas Gudermann (29) was used as a template for generating *hGNRHR* mutants. To generate the Thr<sup>32</sup>Ile *hGNRHR* mutant, the Thr codon ACC was replaced with the Ile codon ATC using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and a pair of complementary mutagenic primers (sense, 5'-CAACCTCCCCACTCTGATCTTGCTGGAAAAGATCCG-3'; antisense, 5'-CGGATCTTTCCAGACAAGATCAGAGTGGGGA-GGTTG-3') according to the manufacturer's protocol. To generate the other three mutants, two-stage PCR-based mutagenesis was performed. To generate the Cys<sup>200</sup>Tyr mutant, the Cys codon TGC was replaced with the Tyr codon TAC using the following primers: sense, 5'-TAACACAC-TACAGTTTTTACAAT-3'; and antisense, 5'-ATTGTGAAAAACTG-TAGTGTGTTA-3'. To generate Leu<sup>266</sup>Arg, the Leu codon CTA was replaced with the Arg codon CGA using the following primers: sense, 5'-CTGAAGACTCGAAAAATGACGGTT-3'; and antisense, 5'-AAC-CGTCATTTTTCGAGTCTTCAG-3'. To generate Cys<sup>279</sup>Tyr, Cys (TGC) was replaced with Arg (TAC) with the following primers: sense, 5'-ATTTACTGTCTACTGGACTCCCTA-3'; and antisense, 5'-TAGGG-AGTCCAGTAGACAGTAAAT-3'. The sequence of the mutated *hGNRHR* was confirmed by bidirectional sequence analysis.

### Immunofluorescence detection of tagged GnRH receptors

To determine the expression of the natural and mutant GnRH receptors, COS-7 cells were plated on glass-bottom 35-mm tissue culture dishes (MatTek Corp., Ashland, MA) and transiently transfected with 2  $\mu$ g wild-type or mutant *hGNRHR* constructs using GenePorter. After 48 h, cells were rinsed with PBS, fixed for 30 min at room temperature with 4% formaldehyde, washed with PBS, and blocked with 1% BSA-PBS for 30 min at room temperature. After blocking, cells were washed with PBS, incubated overnight at 37 C with 5  $\mu$ g/ml anti-HA-fluorescein antibody (clone 12CA5, Roche Molecular Biochemicals, Indianapolis, IN), washed four times with phosphate-buffered saline, and examined using a Bio-Rad Laboratories, Inc. (MRC-1024 multiphoton system), confocal laser microscope.

### Inositol phosphate (IP) assay

The protocol used for measurement of total IP accumulation was an adaptation of that described by Panchenko *et al.* (30). COS-7 cells were transiently transfected by electroporation with 2  $\mu$ g/well wild-type or

mutant *hGNRHR* constructs and seeded into six-well tissue culture plates. After 24 h, cells were incubated in inositol-free DMEM for 2 h, with subsequent addition of 2  $\mu$ Ci/well myo-[2-<sup>3</sup>H]inositol, followed by the addition of 10 mmol/L LiCl 15 min later. Cells were further incubated for 14 h and stimulated with 100 nmol/L GnRH agonist for 45 min. Cells were extracted on ice twice with 20 mmol/L formic acid. Lysates were neutralized to pH 7.5 with 7.5 mmol/L HEPES and 150 mmol/L KOH and centrifuged at 14,000  $\times$  g for 2 min. After the protein content was measured (Coomassie Plus protein assay reagent, Pierce Chemical Co., Rockford, IL), supernatants were loaded onto a 0.5-mL AG-X8 resin anion exchange column previously equilibrated with 2 mL 1 mol/L NaOH, 2 mL 1 mol/L formic acid, and 5  $\times$  5 mL ddH<sub>2</sub>O. The columns were then washed with 5 mL ddH<sub>2</sub>O followed by 5 mL 5 mmol/L borax and 60 mmol/L sodium formate, and IP was eluted with 3 mL 0.9 mol/L ammonium formate and 0.1 mol/L formic acid. A 500- $\mu$ L aliquot was counted, the counts were corrected for protein content, and results were expressed as the fold increase compared with the control plasmid (pcDNA3).

## Results

### *In vivo* studies: subjects and mode of inheritance

A total of 108 probands were examined for mutations in the *GNRHR*. Table 1 shows the breakdown of the individuals into diagnostic subcategories. Forty-eight patients had IHH with normal olfaction. Sixty patients had IHH and anosmia/hyposmia (Kallmann syndrome). Although the vast majority of patients in each subcategory had congenital disease, seven patients had adult-onset IHH. Fourteen patients across both subgroups had partial variants of the condition.

The same 108 patients were also classified according to their presumed mode of inheritance, maintaining the dis-

**TABLE 1.** Breakdown of HH patients according to diagnosis

	n	<i>GNRHR</i> mutants
Hypogonadotropic hypogonadism		
Congenital HH		
Autosomal recessive	5	2
Autosomal dominant	8	0
X-Linked	0	0
Unknown (either autosomal or X-linked)	1	0
Sporadic	18	2
Adult onset		
Sporadic	4	0
Partial variants		
Partial HH-sporadic	1	0
Fertile eunuch-sporadic	3	1
No pedigree information	8	0
Total	48	5
Hypogonadotropic hypogonadism + anosmia		
Kallmann syndrome		
Autosomal recessive	4	0
Autosomal dominant	11	0
X-Linked	3	0
Unknown (either autosomal or X-linked)	1	0
Sporadic	29	0
IHH but in a family with KS-dominant	1	0
IHH but in a family with KS-recessive	2	0
Delayed puberty + anosmia-dominant	1	0
Delayed puberty + anosmia-sporadic	1	0
Partial variants		
Partial KS-dominant	1	0
Partial KS-sporadic	2	0
Fertile eunuch variant-sporadic	3	0
No pedigree information	1	0
Total	60	0

inction between normosmic and anosmic individuals. No pedigree information was available for a total of 9 individuals.

**Familial cases.** Of the 48 patients with congenital hypogonadotropic hypogonadism and normal olfaction, 5 were classified as autosomal recessive. *GNRHR* mutations were identified in 2 of these 5 families (patients 1 and 4; Table 1). Stated alternatively, 40% of normosmic hypogonadotropic hypogonadism probands whose pedigrees were consistent with an autosomal recessive mode of inheritance were found to have a mutated GnRH receptor. In 2 other autosomal recessive families, parents of the affected individuals were members of a consanguineous union, but there were no females among the affected subjects; both probands were negative for mutations in *GNRHR*. Six probands with hypogonadotropic hypogonadism and anosmia (in either the proband or family member) were also classified with an autosomal recessive mode of inheritance. However, no *GNRHR* mutations were identified in these individuals. No *GNRHR* mutation was identified in any other familial case of IHH.

**Nonfamilial cases.** Of the nonfamilial cases (*i.e.* sporadic), a total of 18 individuals with normosmic IHH were studied. Two patients (no. 2 and 3) were found to have mutations. One patient (no. 5) with a partial variant of the condition was also found to have a mutation. Twenty-nine patients with sporadic KS were also studied; no mutations were identified. In total, the percentage of mutations in all patients with non-anosmic IHH was 5 of 48, or 10.4%. If noncongenital cases are excluded (*i.e.* adult onset), the percentage rises to 5 of 41, or 12.2%.

**Clinical phenotype.** Table 2 summarizes the clinical phenotypes of the five patients found to have *GNRHR* mutations. Three of the five patients were male. Patients 2 and 5 were evaluated at the Reproductive Endocrine Unit of Massachusetts General Hospital, whereas patient 4 was evaluated off-site. Frequent blood sampling in the General Clinical Research Center in both patients 2 and 5 revealed an apulsatile LH pattern. However, as summarized in Fig. 1, there were striking differences between the two men on gonadal examination. Patient 2 had testicular volumes of 1.4 and 2 cc, whereas patient 5 had testicular volumes of 15 and 17 cc. The testicular size of patient 4 at the time of presentation is unknown.

Differences between the female patients affected with *GNRHR* mutations were also apparent (Table 2). Although patient 1 presented with small breasts, patient 3 was noted to have Tanner III breasts at initial examination. Patient 1 had low gonadotropin and  $E_2$  levels. However, patient 3 had LH of 6.9 mIU/mL, FSH of 8.9 mIU/mL, and  $E_2$  of 139.5 pmol/L. In contrast to the male patients, patients 1 and 3 had low amplitude pulses on baseline frequent sampling.

#### *In vitro studies*

**Mutational analysis.** Six nucleotide sequence abnormalities were described in five patients. Four changes were novel. Figure 2 diagrams the corresponding amino acid changes against the other mutations previously reported in the literature.

In patient 2 a cytosine in exon 1 was changed to thymidine at position 95, resulting in a threonine being replaced by isoleucine at residue 32 (Thr<sup>32</sup>Ile) near the junction of the N-terminus and the first transmembrane domain. In the same patient exon 2 contained a guanine to adenine mutation at position 599, resulting in a cysteine being replaced by a tyrosine at amino acid 200 (Cys<sup>200</sup>Tyr) in the second extracellular loop.

In patient 3 a thymidine in exon 3 was changed to a guanine at nucleotide 797, causing a leucine to be replaced by an arginine at residue 266 (Leu<sup>266</sup>Arg) in the third intracellular loop. In exon 1 a mutation of an adenine to a guanine changed glutamine at residue 106 into an arginine (Gln<sup>106</sup>Arg) in the first extracellular loop. This mutation has been previously described (10).

Patient 4 was one of two patients to have a homozygous mutation. A guanine was changed to an adenine at position 836, resulting in a cysteine being replaced by a tyrosine (Cys<sup>279</sup>Tyr) in the sixth transmembrane domain.

Patient 5 contained the homozygous mutation Gln<sup>106</sup>Arg. Patient 1 contained the compound heterozygous mutations Gln<sup>106</sup>Arg and Arg<sup>262</sup>Gln, both of which have been previously described (10, 31).

**Polymorphisms.** Some patients were found to have changes in the coding sequence of *GNRHR* of unknown significance. One individual with congenital IHH was found to be heterozygous for a *GNRHR* mutation (Arg<sup>262</sup>Gln). TGGE and sequencing of the remaining exons failed to identify a second coding sequence abnormality. One individual was found to have a change in the last base pair of exon 2 (nucleotide 741), changing a cytosine to a thymidine; however, this coding change was conservative with the amino acid histidine remaining at position 247. Again, no coding sequence abnormality was found in the remaining exons. In 3 additional cases, individuals were positive on TGGE, but subsequent sequencing localized the base pair change to the intron between exons 1 and 2. Because this change occurred in 2 of the 50 healthy volunteers, it would appear that this change has no influence on the IHH/KS phenotype. One normal volunteer was also found to be heterozygous for the Gln<sup>106</sup>Arg mutation.

**Functional studies of the mutant receptors.** The presence of *hGNRHR* proteins at the cell surface of COS-7 cells transiently transfected with plasmids encoding wild-type and mutant *hGNRHR* constructs was determined by immunocytochemistry. Confocal fluorescence microscopy revealed a similar cellular presence of wild-type and mutant receptors in each case (Table 2).<sup>1</sup>

The ability of the receptors to transduce a signal after GnRH agonist stimulation was assessed by measuring IP accumulation, an indicator of phospholipase C activity. Stimulation of COS-7 cells transiently transfected with wild-type *hGNRHR* construct resulted in a 5.2-fold increase in intracellular IP levels, whereas all four of the novel mutant *hGNRHRs* failed to respond to GnRH agonist stimulation (Table 2 and Footnote 1).

<sup>1</sup> Bédécarrats, G. Y., M. Beranova, F. Kada, S. B. Seminara, P. M. Corin, and U. B. Kaiser, manuscript in preparation.

TABLE 2. Clinical characteristics of patients with *GNRHR* mutations

Patient no.	Sex	Mode of inheritance	Physical exam	Lab evaluation	100 µg GnRH stimulation				Frequent blood sample	Mutation	Cell surface	IP
					LH BL	LH PK	FSH BL	FSH PK				
1	F	Recessive	Eunuchoid "Small breasts" Normal external genitalia	LH 0.3 IU/L FSH 0.5 IU/L E <sub>2</sub> <73 pmol/L	0.3	1.3	0.5	1.6	5 LH pulses in 12 h Mean LH 2.0 IU/L Mean LH amp 1.2 IU/L	Gln <sup>106</sup> Arg Arg <sup>268</sup> Gln		
2	M	Sporadic	Not eunuchoid Gynecomastia Female escutcheon Left testicular vol: 2 cc Right testicular vol: 1.4 cc by ultrasound	LH <0.5 IU/L FSH 0.3 IU/L T <9.4 nmol/L	0.5	0.7	0.3	1.2	0 LH pulses in 12 h Mean LH 0.8 IU/L	Thr <sup>32</sup> Phe Cys <sup>200</sup> Tyr	+	-
3	F	Sporadic	Eunuchoid Tanner III breasts	LH 6.9 IU/L FSH 8.9 IU/L E <sub>2</sub> 139.5 pmol/L	1.2	10.2	5.5	9.6	3 LH pulses in 12 h Mean LH 1.5 IU/L Mean LH amp 1.1 IU/L	Gln <sup>106</sup> Arg Leu <sup>266</sup> Arg	+	-
4	M	Recessive		LH 2.71 IU/L FSH 1.7 IU/L		No response			Not performed	Cys <sup>279</sup> Tyr Cys <sup>279</sup> Tyr	+	-
5	M	Sporadic	Eunuchoid Left testicular vol: 15 cc Right testicular vol: 17 cc	LH 3.5 IU/L FSH 2.4 IU/L T 2.5 nmol/L	6.1	14.5	3.6	4.6	0 LH pulses in 12 h Mean LH 3.6 IU/L	Gln <sup>106</sup> Arg Gln <sup>106</sup> Arg	+	-

BL, Baseline; PK, peak; amp, amplitude.

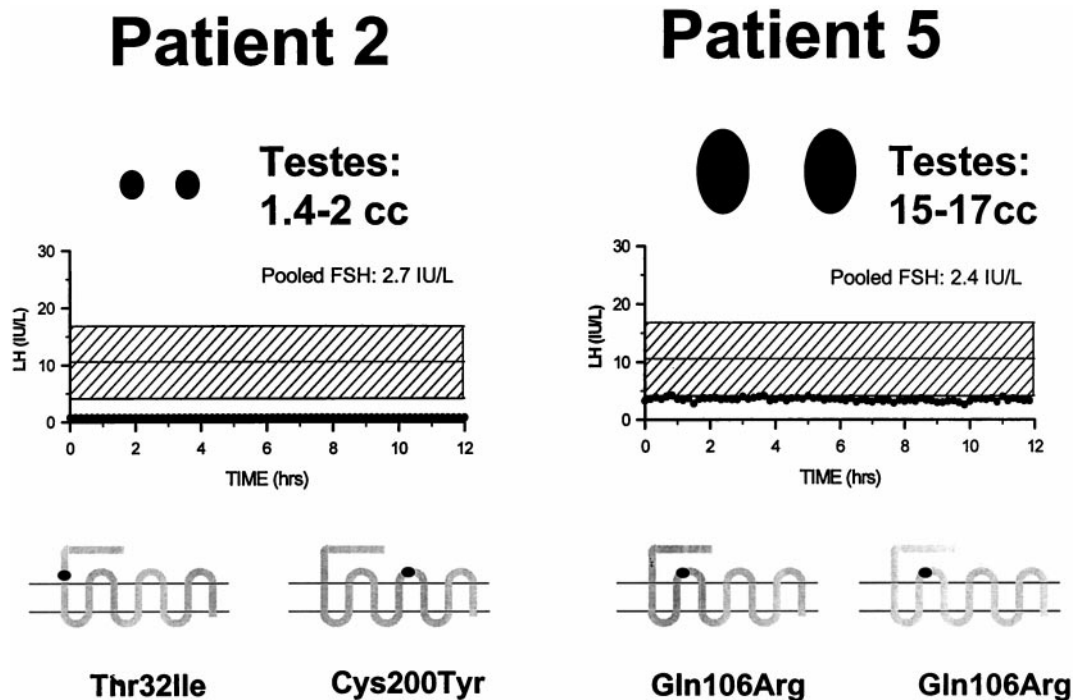


FIG. 1. Comparison of 2 patients (no. 2 and 5) with respect to testicular volume (TV), neuroendocrine profile, and *GNRHR* mutations. The graphs represent serum LH levels drawn every 10 min for 12 h. The shaded area represents the normal range for LH levels as defined in 20 normal men (16).

### Discussion

This screening study of a large and well characterized population of hypogonadotropic patients for mutations in the coding sequence of the GnRH receptor demonstrates that 1) patients with *GNRHR* mutations manifest a broad spectrum of phenotypes; 2) *GNRHR* mutations account for 40% of autosomal recessive IHH in subjects with a normal sense of smell; 3) *GNRHR* mutations also occur in individuals with sporadic disease, but with a lower frequency; and 4) *GNRHR* mutations do not occur in patients with IHH and defects in olfaction. Four novel mutations in the receptor were also identified in this study. Despite cellular expression and presence on the cell surface, Thr<sup>32</sup>Ile, Cys<sup>200</sup>Tyr, Leu<sup>266</sup>Arg, and Cys<sup>279</sup>Tyr *hGNRHR* mutants were unable to respond to GnRH stimulation *in vitro* and resulted in loss of receptor function. Given the breadth of phenotypes described in this study, this report raises questions about the role of GnRH induced gonadotropin stimulation.

The GnRH receptor is a member of the rhodopsin-like G protein-coupled receptor family. Activation of these receptors is associated with conformational changes that facilitate coupling with G proteins and allow the receptor to transition between inactive and active states (32). Site-directed mutagenesis and conformational modeling have been used to study the effects of specific changes within the receptor; however, correlating mutations within the *GNRHR*-coding sequence to changes in structural conformation and to specific clinical phenotypes has been challenging.

Within the receptor protein, mutations have been described in the amino-terminus, first extracellular loop, transmembrane domains 3–7, and third intracellular loop (10–15). This study is the first to describe a mutation, Cys<sup>200</sup>Tyr, in the

second extracellular loop. All of the mutations described in this study resulted in a loss of receptor function. Moreover, an HA tag was used on the mutant constructs to demonstrate that the decreases in IP accumulation were not due to loss of *hGNRHR* cell surface expression.

An important finding of this study is that mutations that cause the same functional changes in the *GNRHR* can lead to strikingly different clinical phenotypes. Nowhere is the breadth of phenotypes more dramatic than in two of the male patients described in this study (patients 2 and 5). Both patients have IHH and an apulsatile LH secretory pattern. Patient 2 has prepubertal testes, whereas patient 5 has a testicular size in the normal male range. Individuals such as patient 5 with hypogonadism but normal sized testes (with or without spermatogenesis) have been termed fertile eunuchs, a phrase originally coined to reflect a primary pituitary deficiency of LH, but normal FSH secretion (33, 34). Although patient 2 is a novel compound heterozygote (Thr<sup>32</sup>Ile, Cys<sup>200</sup>Tyr), patient 5 has a homozygous *GNRHR* mutation (Gln<sup>106</sup>Arg) that has been described previously to reduce GnRH binding and IP signaling (10). Therefore, Gln<sup>106</sup>Arg mutants may be capable of transmitting GnRH signal, albeit in a reduced fashion. How this signal results in normal testicular growth is not clear.

Pooled FSH samples during patient 5's frequent sampling study revealed a level of 2.4 IU/L. Could this FSH be sufficient to account for patient 5's normal testicular size? Patient 2 had a similar pooled FSH level of 2.7 IU/L and yet had prepubertal-sized testes. Caron reported a patient with a *GNRHR* mutation and an FSH level of 3.3 IU/L (normal range, 1.2–11) with bilateral cryptorchidism and a testicular volume less than 3 (12). As several assays of unknown com-

# hGnRH Receptor

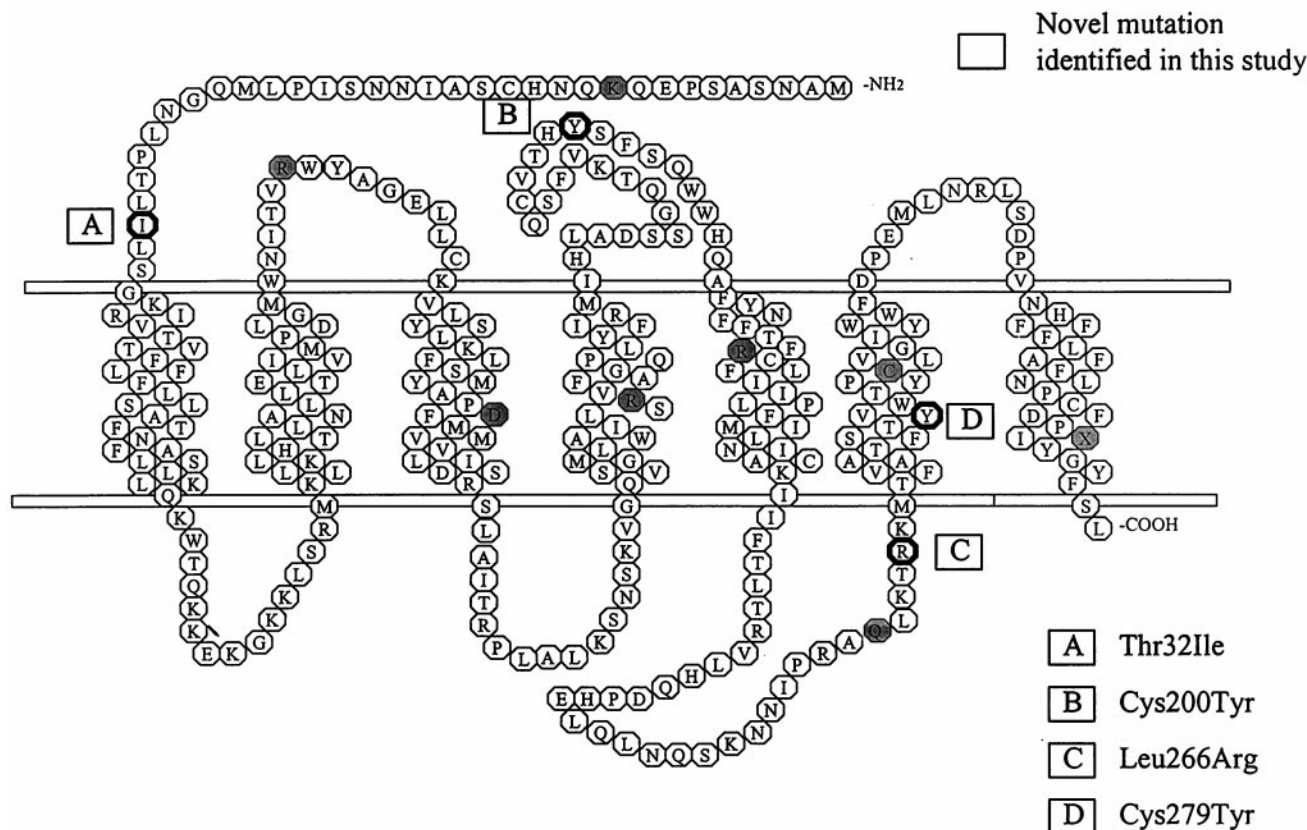


FIG. 2. Schematic of the GnRH receptor, adapted from Ref. 12. Mutations previously described in the literature are shaded in *dark gray*. Novel mutations identified in this study are outlined with a *thick black line*.

parability are involved, quantitative comparisons cannot be made. However, the presence of a normal serum level of FSH *a priori* cannot, by itself, account for the normal testicular growth of patient 5. What is even more difficult to reconcile in patient 5 is his level of testicular growth in the face of hypogonadal testosterone levels, suggesting that other factors, perhaps gonadotropin independent, may also play an important role in gonadal development. Further data from our group has demonstrated the subsequent development of gonadotropin pulsatility in patient 5 after hCG stimulation, suggesting an effect of testosterone, whether direct or indirect, in modulating pituitary responsiveness to GnRH (Pitteloud N., P. A. Boepple, S. DeCruz, S. B. Valkenburgh, W. F. Crowley, and F. J. Hayes, submitted for publication).

Studying patients with shared *GNRHR* mutations also provides some important insights. Patient 5 with his homozygous Gln<sup>106</sup>Arg mutation demonstrated no LH pulses on frequent blood sampling, yet he appears to be the least affected proband phenotypically in this patient cohort. Patient 3 shares one mutation (Gln<sup>106</sup>Arg) with patient 5, whereas her other allele contains the novel missense mutation, Leu<sup>266</sup>Arg, in the third intracellular loop. She presented with breast development, measurable gonadotropins and E<sub>2</sub>, and three LH pulses in 12 h. Patient 1 also has the Gln<sup>106</sup>Arg

mutation, whereas her second allele contains the mutation Arg<sup>262</sup>Gln, also located in the third intracellular loop.

Although patient 1 presented with lower baseline gonadotropins and E<sub>2</sub>, she did have pulsatile LH activity (five pulses over 12 h). Although patients 3 and 5 had modest responses to a single bolus of 100  $\mu$ g GnRH iv, patient 1 had no gonadotropin response. Therefore, in patients with *GNRHR* mutations, a gonadotropin response, albeit small, to a single pharmacological GnRH stimulation test seems to bear a loose correlation to phenotype, whereas pulsatile LH activity (as obtained in these 12-h windows) does not bear a clear relationship to the clinical presentation.

Although studying patients with identical *GNRHR* mutations is important, genotype/phenotype correlations are not easy to make, whether comparing identical genotypes across sexes or within a family. For example, patient 1 has the identical genotype (Gln<sup>106</sup>Arg, Arg<sup>262</sup>Gln) as the first patient reported with a *GNRHR* mutation, a male with partial puberty, 8-cc testes, spermatogenesis, normal gonadotropin increments to 100  $\mu$ g GnRH administered iv, and low amplitude LH pulsations every 2 h (10). Yet, patient 1 had absence of breast development, one nonestrogen-induced menstrual bleed, no gonadotropin responses to 100  $\mu$ g GnRH, iv, and erratic low amplitude LH pulsations, suggesting that patient

1 had a more severe phenotype, at least from a neuroendocrine perspective (31). Other reports in the literature describe different gender discrepancies. For example, two sisters with breast development had gonadotropin levels that responded to a single injection of GnRH, whereas their brother with complete IHH had no gonadotropin response (13). Therefore, there may be sexually dimorphic responses to *GNRHR* mutations at both pituitary and gonadal levels. Although continued studies may help to elucidate further correlations, it is clear that no single clinical trait can be used to include or exclude the presence of a *GNRHR* mutation except the presence of anosmia.

In a previous screening study without subclassification of phenotype or mode of inheritance (11), *GNRHR* mutations were identified in just 1 of 46 patients with normosmic IHH, yielding a frequency of *GNRHR* mutations of 2.2%. The current report not only classifies patients on the basis of smell, but also on the complexity of phenotype (*i.e.* partial *vs.* complete hypogonadotropic hypogonadism) and the presumed mode of inheritance (*i.e.* dominant, recessive, X-linked, sporadic). In this cohort of 108 patients, 5 individuals had *GNRHR* mutations. When these patients were rephenotyped on the basis of smell, all 5 patients with *GNRHR* mutations were in the normosmic group (5 of 48, or 10.4%). When those 48 normosmic IHH probands were recategorized according to the mode of inheritance, 5 appeared to be from autosomal recessive kindreds, whereas 18 had no familial involvement. Two of the 5 probands with *GNRHR* mutations (patients 1 and 4) were from an autosomal recessive subgroup ( $n = 5$ ), yielding a frequency of 2 of 5, or 40%. Therefore, although the number of cases with recessive inheritance was small ( $n = 5$ ), the proportion of cases with *GNRHR* mutations within that subgroup was high (40%). Viewed from another perspective, it is likely that another gene(s) accounts for autosomal recessive IHH. The remaining 3 probands with *GNRHR* mutations (2, 3, and 5) were sporadic, yielding a frequency of 3 of 18, or 16.7%, in normosmic congenital IHH.

That the subtype of IHH (congenital *vs.* adult onset, IHH *vs.* KS) and mode of inheritance may serve as important guideposts for genetic testing has important implications for clinical practice. For example, the presence of a *GNRHR* mutation is not necessarily a contraindication to the use of exogenous, pulsatile GnRH, albeit at higher doses (31). Such therapy reduces the risk of multiple gestation in individuals seeking ovulation induction (35). In addition, patients with *GNRHR* mutations are unlikely to pass IHH onto their children, an increasingly important consideration for couples pursuing fertility therapy.

In summary, this study examines the frequency and distribution of *GNRHR* mutations in a large, heterogeneous population of patients with IHH. Patients bearing mutations demonstrate a broad range of IHH, from complete to partial defects. Receptor mutations may account for a larger proportion of cases of IHH than previously appreciated. Patients with autosomal recessive or sporadic normosmic IHH should be considered candidates for screening.

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