When Genetic Load Does Not Correlate with Phenotypic Spectrum: Lessons from the GnRH Receptor (*GNRHR*)

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Context: A broad spectrum of GnRH-deficient phenotypes has been identified in individuals with both mono- and biallelic *GNRHR* mutations.

Objective: The objective of the study was to determine the correlation between the severity of the reproductive phenotype(s) and the number and functional severity of rare sequence variants in *GNRHR*.

Subjects: Eight hundred sixty-three probands with different forms of GnRH deficiency, 46 family members and 422 controls were screened for *GNRHR* mutations. The 70 subjects (32 patients and 38 family members) harboring mutations were divided into four groups (G1-G4) based on the functional severity of the mutations (complete or partial loss of function) and the number of affected alleles (monoallelic or biallelic) with mutations, and these classes were mapped on their clinical phenotypes.

Results: The prevalence of heterozygous rare sequence variants in *GNRHR* was significantly higher in probands *vs.* controls ($P < 0.01$). Among the G1-G3 groups (homozygous subjects with successively decreasing severity and number of mutations), the hypogonadotropic phenotype related to their genetic load. In contrast, subjects in G4, with only monoallelic mutations, demonstrated a greater diversity of clinical phenotypes.

Conclusions: In patients with GnRH deficiency and biallelic mutations in *GNRHR*, genetic burden defined by severity and dose is associated with clinical phenotype. In contrast, for patients with monoallelic *GNRHR* mutations this correlation does not hold. Taken together, these data indicate that as-yet-unidentified genetic and/or environmental factors may combine with singly mutated *GNRHR* alleles to produce reproductive phenotypes. **(*J Clin Endocrinol Metab* 97: E1798–E1807, 2012)**

Isolated GnRH deficiency (IGD) in humans is a clinically and genetically heterogenous condition (1–16). Increasingly, reports of the range and number of nucleotide variants identified in patients with IGD across a variety of genes raise new questions about the precision of genotype-phenotype correlations (17, 18). While some patients harbor homozygous or compound heterozygous mutations, increasing numbers of patients with monoallelic heterozygous changes are being reported (13, 19). There is an expanding body of data that indicates that heterozygote mutations may lead to reductions in total gene product, whether through gene deletion, degradation of an unsta-

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Abbreviations: AOIHH, Adult-onset idiopathic hypogonadotropic hypogonadism; CDP, constitutional delay of puberty; cLOF, complete loss of function; dbEST, Database for Expressed Sequence Tags; dbSNP, Database for Single Nucleotide Polymorphisms; HA, hypothalamic amenorrhea; hCG, human chorionic gonadotropin; IGD, isolated GnRH deficiency; IHH, idiopathic hypogonadotropic hypogonadism; KS, Kallmann syndrome; pLOF, partial loss of function; nIHH, normosmic IHH; NL, normal; RSV, rare sequence variant; SNP, single-nucleotide polymorphism.

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ble mRNA, or the creation of a poorly functioning protein. Some proteins, such as transcription factors, are quite sensitive to such reductions in gene dosage, whereas others, *i.e.* G protein-coupled receptors have traditionally been associated with recessive traits wherein heterozygous are typically asymptomatic. However increasing numbers of heterozygote mutations in patients with reproductive phenotypes are being discovered in genes that encode G protein-coupled receptors including *GNRHR* (GnRH receptor), *PROKR2* (prokineticin 2 receptor), *KISS1R* (kisspeptin receptor), and *TACR3* (neurokinin B receptor) (11–13, 15, 20).

Functional determinations of total mutation burden can improve the predictive power of genotypic information in certain rare diseases such as the ciliopathies (21). Applying this concept, this study focuses on the role of gene dosage and severity of loss of function in a large cohort of individuals bearing either monoallelic or biallelic mutations in *GNRHR*, one of the first genes to be identified as a cause of GnRH deficiency (6, 22). In addition to examining gene dosage effects, this study also integrates the homo/heterozygosity of each variant with *in vitro* and *in silico* information regarding its functional severity and thus expands the genotypic profiling of GnRH deficiency.

The following hypotheses are specifically examined: 1) both homozygous and heterozygous variants in *GNRHR* occur more commonly in patients with GnRH deficient states than in healthy controls, 2) the reproductive phenotype of patients harboring biallelic mutations is correlated with the functional severity of the *GNRHR* variants, and 3) in contrast, the reproductive phenotype of subjects harboring monoallelic mutations does not correlate with mutation severity.

In the course of examining these hypotheses, unique reproductive phenotypes were uncovered, suggesting that *GNRHR* mutation may also exert direct effects on gonadal function.

Patients and Methods

All activities were approved by the Massachusetts General Hospital Institutional Review Board. All subjects provided written informed consent.

Patient cohorts

GNRHR (accession no. NM_010323) was screened in 863 probands with IGD [n = 375, 280 males, 95 females; 23 of which had adult-onset idiopathic hypogonadotropic hypogonadism (AOIHH), Kallmann syndrome (KS) (n = 360, 272 males, 88 females), hypothalamic amenorrhea (HA) (n = 77 females), and constitutional delay of puberty (CDP) (n = 51, 29 males, 22 females)].

Controls

GNRHR was screened in volunteers with normal reproductive function by history and physical examination (252 Caucasians, 50 African-Americans). Sequence data from 120 subjects from the 1000 Genomes Project (http://www.1000genomes.org) was also used to expand this data set.

GnRH deficiency

The diagnosis of GnRH deficiency was based on previously published criteria (23). Olfactory testing was performed using a smell identification test (University of Pennsylvania Smell Identification Test) (24). A score of the fifth percentile or greater based on sex/age was deemed normal [normosmic (n = 130); anosmic (n = 292)]. Patients with reduced olfaction were diagnosed with KS. For the remaining patients, the assignment of diagnosis was informed by self-reported sense of smell.

Hypothalamic amenorrhea

Women had HA if they were between 18 and 40 yr old and had secondary amenorrhea for 6 months or longer with low/normal gonadotropins and low estradiol levels in the presence of weight loss (>15% of body weight), more than 15 h/wk exercise (25), or an eating disorder (26). The Eating Attitudes Test was administered to exclude clinical eating disorders (27).

Adult-onset idiopathic hypogonadotropic hypogonadism

Participants were diagnosed with AOHH based on previously published criteria (28).

Constitutional delay of puberty

The diagnosis of CDP was based on initiation of puberty at an age greater than 2 SD later than the general population without apparent pathology followed by eventual completion of pubertal development [females: no thelarche by 13 yr and/or no menarche by 15 yr; males: testes <4 cc and/or no growth spurt by age 14 yr (29, 30)].

Ethnicity/inheritance

The 863 probands were Caucasian (n = 646), Asian (n = 47), Black/African-American (n = 19), Native Hawaiian/Pacific Is-

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landers (n = 2), American Indian or Alaska Natives (n = 2), and mixed ancestry (n = 11), with the remainder of unknown race. Five hundred eighty-eight probands were male and 275 were female. One hundred eighty-eight probands had one or more family member with some form of GnRH deficiency or associated phenotype, 267 had no family history of GnRH deficiency, and the remaining were unknown as to their mode of inheritance. Of the familial cases, 148 appeared to be autosomal dominant; 40 autosomal recessive; 18 X-linked recessive; and 23 had affected brothers in a single sibling pair. Subjects were designated as monoallelic (one or more changes on the same allele) or biallelic (homozygous or compound heterozygous) on the basis of the pedigree.

Neuroendocrine studies

Two hundred fifty-two of 863 probands (170 males, 82 females) were admitted to the Clinical Research Center of Massachusetts General Hospital for blood sampling every 10 min for 12–24 h to assess LH secretion (31).

Assays

Samples were assayed for LH by RIA (32) or an automated microparticle enzyme immunoassay (AxSYM System; Abbott Laboratories, Abbott Park, IL). The second assay was calibrated using the same reference preparations as the RIA to make results comparable across data sets. Data regarding LH pulses are based on the limits of detection of each system and integrated by virtue of a common standard in both assays that permitted interconversion.

Mutation analysis

Exon segments of genomic DNA were sequenced and all sequence variants were confirmed (33) in all probands and control subjects. Nucleotide changes were assessed in dbSNP (Database for Single Nucleotide Polymorphisms), dbEST (Database for Expressed Sequence Tags), and among control alleles. Data for heterozygous patients were analyzed for single nucleotide polymorphism (SNP) heterozygosity using 13 different SNP to ensure that intragenic deletions were not missed.

The 863 probands were also screened for mutations in other genes involved in idiopathic hypogonadotropic hypogonadism (IHH), including *FGFR1* (n = 818), *KISS1R* (n = 769), *NELF* (n = 743), *TAC3* (n = 456), *TACR3* (n = 459), *FGF8* (n = 818), *GNRH1* (n = 475), *KAL1* (n = 807), *PROK2* (n = 840), *PROKR2* (n = 839), and *CHD7* (n = 188).

Functional studies

The severity of *GNRHR* variants was determined by *in vitro* studies (6, 34–36) or prediction programs [PolyPhen (http://genetics.bwh.harvard.edu/pph/) (37), Mutation Taster (http://www.mutationtaster.org), and Panther (http://www.pantherdb.org/tools/csnpScoreForm.jsp) (38) for nonsynonymous changes; and Mutation Taster and NNSPLICE 0.9 version (http://www.fruitfly.org/seq_tools/splice.html) for synonymous changes]. Changes leading to a frame shift, a loss of the first methionine, or *in vitro* studies showing the abolition of receptor function were considered to be a complete loss of function (cLOF) mutations (n = 7). Six variants were categorized as a partial loss of function (pLOF) mutations. Variants that were not studied *in vitro* but were predicted to impair GnRH receptor function *in silico* across all programs (n = 4) were categorized as presumed pLOF mutations. Three variants were either cLOF (A171T) or

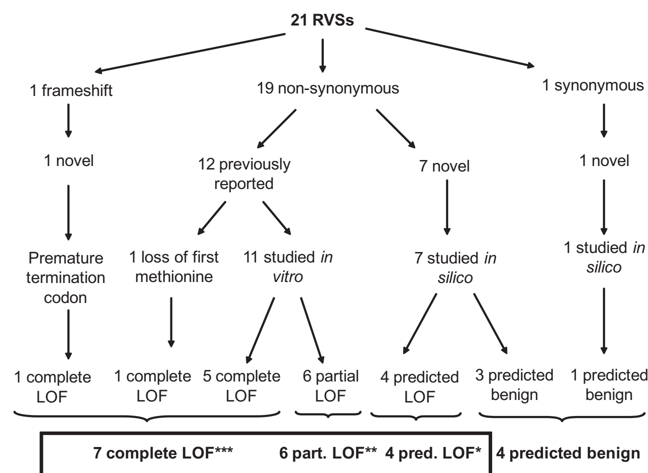

FIG. 1. Functional assessment of RSV.

pLOF (N10K, Q11K) *in vitro* but were classified as benign by at least one prediction program and were included among cLOF/pLOF mutations.

Probands and family members (the latter including both affected and unaffected subjects) carrying *GNRHR* mutations were divided into four groups: G1, subjects with both alleles carrying cLOF mutations (cLOF/cLOF); G2, subjects with one allele harboring a cLOF mutation and the other having a pLOF mutation (cLOF/pLOF); G3, subjects with both alleles having pLOF mutations (pLOF/pLOF); and G4, subjects with only one allele harboring a mutation (cLOF/NL or pLOF/NL) [Figs. 1 and 2and Supplemental Tables 1–3, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org, showing that family members carrying rare sequence variants (RSV) were given the same identification number as the proband followed by a unique letter designation].

LH secretion patterns were compared between groups. When a subject had more than one sampling study, the mean number of LH pulses and mean LH levels were used in analyses. Values were compared by one-way ANOVA.

Results

Identification of variants and analysis of pathogenicity

Twenty-one RSV in *GNRHR* (*i.e.* ≤1% of control allele) were identified in 35 of 863 probands (4.0%) (Tables 1 and 2 and Supplemental Fig. 1), none of which have been reported in dbSNP or double-banded expressed sequence tag. Figure 1 outlines these RSV, their amino acid changes, *in vitro* studies, and predictions of pathogenicity. Seven of the 21 variants (33%) were cLOF (M1T, Q11fsX23, R139H, A171T, L266R, C279Y, P320L), six of 21 (29%) were pLOF (N10K, Q11K, T32I, Q106R, S217R, R262Q), four of 21 (19%) were predicted to be deleterious [and considered pLOF for the purpose of this study (P96S, L117P, P146S, L166P)], and four of 21 (19%) were predicted to be benign (A50V, L83V, S168A, F216F) (Table 1). The three subjects with only presumed benign RSV

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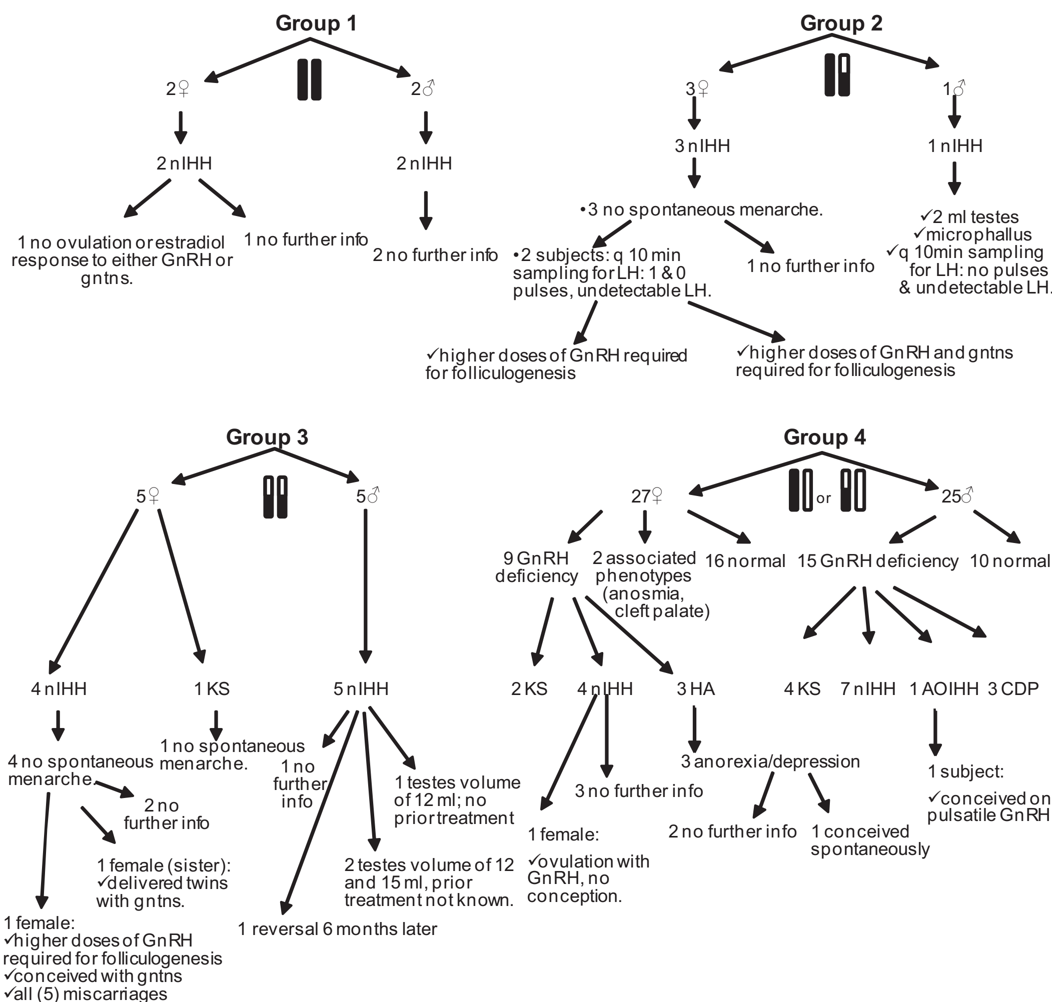

FIG. 2. Phenotype of individuals included in the four groups. *White bar*, normal allele; *black bar*, complete loss of function mutation(s); *white and black bar*, partial loss of function mutation(s).

were not placed into any of the aforementioned groups (Supplemental Tables 1 and 2).

Although 20 of 21 RSV were not observed in controls, one variant (Q106R) was heterozygous in two controls (Table 1). The frequency of heterozygous *GNRHR* RSVs in probands (22 of 863 individuals, 2.5%) was significantly higher than that of controls (2 of 422 individuals, 0.5%) (χ^2, $P < 0.01$).

Inheritance and segregation

None of the identified variants arose *de novo* (Supplemental Fig. 2). Of the 35 probands bearing variants, 12 came from pedigrees in which the disease phenotype appeared to be familial [dominant (n = 5), recessive (n = 6), or brother-brother (n = 1)]. Nine were sporadic and 14 had an unknown mode of inheritance (Table 2). Of the 46 family members who provided DNA, 38 carried *GNRHR* RSV. Ten of 38 had IGD, two had isolated anosmia or cleft lip/palate, and the remaining 26 had no known reproductive or other abnormalities (Supplemental Tables 1–3). Although variants were frequently identified in reproductively normal family members, all the family members with a GnRH-deficient phenotype carried the same *GNRHR* variant(s) as the proband.

In G1 (cLOF/cLOF, n = 4, two males, two females), all individuals had normosmic GnRH deficiency [three homozygous C279Y (no. 2, no. 2b, no. 2c), and one homozygous Q11fsX23 (no. 1)] (Table 2 and Supplemental Tables 1 and 2). Baseline LH pulsatility was not ascertained in any of these individuals. Proband no. 1, after receiving estrogen/progestin supplementation, was treated with pulsatile GnRH (one cycle, 250 ng/kg) and exogenous FSH (dose escalations of 75–225 IU/d over three cycles) from ages 34 to 35 yr. However, her estradiol levels remained low and she did not ovulate in response to either therapy (Supplemental Tables 1 and 2 and Fig. 2).

In G2 (cLOF/pLOF, n = 4, one male, three females), all individuals had normosmic IGD [compound heterozygous for M1T/Q106R/R139H (no. 3); N10K/Q11K/P320L (no. 4); T32I/R139H (no. 5), and Q106R/L266R (no. 6)] (Table 2 and Supplemental Tables 1 and 2). None

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TABLE 1. Studied and predicted functional consequences of rare variants in *GNRHR*

Change (AA)	Functional studies	PolyPhen	Mutation Taster	Panther (score)	NNSPLICE9.0	Classification for this study	In controls?	Reference
M1T[a]	Complete LOF	LOF	LOF	—	—	Complete LOF	No	(55)
N10K[b]	Partial LOF	Benign	LOF	−7.1	—	Partial LOF	No	(34)
Q11K[b]	Partial LOF	Benign	LOF	−4.8	—	Partial LOF	No	(34)
Q11fsX23[a]	Complete LOF	—	—	—	—	Complete LOF	No	
T32I[b]	Partial LOF	LOF	LOF	−5.1	—	Partial LOF	No	(35)
A50V	Not published	Benign	Benign	−1.4	—	Benign	No	
L83V	Not published	Benign	LOF	−7.7	—	Benign	No	
P96S[c]	Not published	LOF	LOF	−8.0	—	Predicted LOF	No	
Q106R[b]	Partial LOF	LOF	LOF	−4.9	—	Partial LOF	2[d]	(6)
L117P[c]	Not published	LOF	LOF	−7.2	—	Predicted LOF	No	
R139H[a]	Complete LOF	LOF	LOF	−9.6	—	Complete LOF	No	(56)
P146S[c]	Not published	LOF	LOF	−6.9	—	Predicted LOF	No	
L166P[c]	Not published	LOF	LOF	−7.7	—	Predicted LOF	No	
S168A	Not published	Benign	LOF	−5.1	—	Benign	No	
A171T[a]	Complete LOF	Benign	LOF	−5.3	—	Complete LOF	No	(35)
F216F	Not published	—	Benign	—	Benign	Benign	No	
S217R[b]	Partial LOF	LOF	LOF	−7.8	—	Partial LOF	No	(36)
R262Q[b]	Partial LOF	LOF	LOF	−6.1	—	Partial LOF	No	(6)
L266R[a]	Complete LOF	LOF	LOF	−6.9	—	Complete LOF	No	(35)
C279Y[a]	Complete LOF	LOF	LOF	−9.9	—	Complete LOF	No	(35)
P320 liter[a]	Complete LOF	LOF	LOF	−9.5	—	Complete LOF	No	(34)

Panther uses the score 0, neutral to −10, deleterious. LOF, Loss of function; —, not assessable.

[a] Complete loss of function.

[b] Partial loss of function.

[c] Predicted to be pathologic; no asterisk: predicted to be benign.

[d] Found in two controls in a heterozygous state.

of the females had spontaneous menarche. Two females (no. 3 and no. 4) demonstrated no and one low-amplitude LH pulse(s) in 12 h, respectively, with the mean LH levels below the limit of detection of the assay. Both patients received estrogen/progestin supplementation before initiating fertility treatments. These patients developed follicles with increasing doses of pulsatile GnRH, but their cycles had abnormal dynamics. Subject no. 4 (N10K/Q11K/P320L) required GnRH dose escalations from the usually effective dose of 75 ng/kg, iv, to 500 ng/kg to achieve folliculogenesis with appropriate estradiol secretion. Despite estradiol levels of 126–256 pg/ml, she failed to mount an induced LH surge. She was subsequently treated with gonadotropins to which she was normally responsive, eventually delivering twins on her fourth treatment cycle (34).

Subject no. 3 (M1T/Q106R/R139H) also required higher-than-normal doses of GnRH (100–250 ng/kg) to achieve folliculogenesis. She ovulated spontaneously on 250 ng/kg GnRH, iv, with a transiently positive human chorionic gonadotropin (hCG), but folliculogenesis was disordered in two further cycles with early luteinization and persistent progesterone secretion. This cycle was followed by two gonadotropin cycles in which she achieved poor folliculogenesis with higher-than-usual doses. At age 35 yr, she underwent *in vitro* fertilization and conceived using a protocol of high-dose recombinant FSH in addition to a small daily dose of hCG. Although she demonstrated a good follicular response by ultrasound, estradiol secretion remained low, requiring follicular phase supplementation with estradiol, and fertilization rates were poor. However, she delivered a healthy son (no. 3d) without cryptorchidism or micropenis (Supplemental Table 2 and Fig. 2).

The one male subject in group 2 (no. 5, T32I/R139H) presented with a severe GnRH deficiency [2 ml testes and microphallus (stretched penile length $<$10.5 cm [39])] and no LH pulses over 12 h (mean LH $<$1.6 IU/liter). He declined treatment with pulsatile GnRH (Supplemental Table 1 and Fig. 2).

G3 contained 10 individuals (five males, five females) classified as pLOF/pLOF [two homozygous Q106R (no. 8, no. 9), one homozygous R262Q (no. 13), and seven compound heterozygous (P96S/Q106R (no. 7), Q106R/S217R (no. 10, no. 10c), Q106R/R262Q (no. 11, no. 11b), L166P/R262Q (no. 12, no. 12b)]. All had normosmic GnRH deficiency except for one female (no. 9) with KS. None of the females had spontaneous menarche. Although the size of the groups is too small to allow statistical comparisons, in contrast to G1 and G2, individuals in G3 presented a greater evidence for endogenous GnRH induced LH pulsatility (n = 3, mean number of LH

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TABLE 2. Probands harboring rare variants in *GNRHR*

Proband (and reference)	Gender	Diagnosis	Smell test (centile)	Ethnicity	Change (bp)	Change (AA)	Inheritance	Coding mutations in other genes
Complete loss of *GNRHR*, group 1, cLOF/cLOF								
1	F	nIHH	—	A	[c.32delA] + [c.32delA]	Q11fsX23 (homo)	NA	None[a]
2	M	nIHH	—	A	[c.836 G>A] + [c.836 G>A]	C279Y	AR	None[b]
Severe loss of *GNRHR*, group 2, cLOF/pLOF								
3	F	nIHH	82	C	[c.2 T>C] + [=]; [c.317 A>G] + [=]; [c.416 G>A] + [=]	M1T, Q106R, R139H	AR	None[c]
4 (35)	F	nIHH	60	C	[c.30 T>A] + [=]; [c.31 C>A] + [=]; [=] + [c.959 C>T]	N10K, Q11K, P320L	S	None[c]
5	M	nIHH	50	C	[c.95 C>T] + [=]; [c.416 G>A] + [=]	T32I, R139H	AD	None[d]
6	F	nIHH	—	AA	[c.317 A>G] + [=]; [c.797 T>G] + [=]	Q106R, L266R	S	None[c]
Severe loss of *GNRHR*, group 3, pLOF/pLOF								
7	M	Fertile eunuch	41	C	[c.286 C>T] + [=]; [c.317 A>G] + [=]	P96S, Q106R	S	None[c]
8 (42)	M	nIHH reversal	—	C	[c.317 A>G] + [c.317 A>G]	Q106R (homo)	S	None[c]
9	F	KS	<5	C	[c.317 A>G] + [c.317 A>G]	Q106R (homo)	S	None[d]
10	M	nIHH/fertile eunuch	—	C	[c.317 A>G] + [c.317 A>G]; [c.651 C>A] + [=]	Q106R, S217R	AD	None[c]
11 (41)	F	nIHH	12	C	[c. 317 A>G] + [=]; [c.785 G>A] + [=]	Q106R, R262Q	AD	*FGFR1*: R470L[c]
12	M	nIHH/fertile eunuch	—	A	[c.497 T>C] + [=]; [c.785 G>A] + [=]	L166P, R262Q	AD	None[c]
13	F	nIHH	—	C	[c.785 G>A] + [c.785 G>A]	R262Q (homo)	NA	None[c]
Moderate loss of *GNRHR*, group 4, cLOF/NL or pLOF/NL								
14	F	nIHH	—	UK (Hispanic)	[c.247 C>G] + [=]; [c.317 A>G] + [=]	L83V, Q106R	AR	*FGFR1*: N117S[e]
15	M	nIHH	—	UK	[c.317 A>G] + [=]	Q106R	NA	None[f]
16	M	nIHH	25	A	[c.317 A>G] + [=]	Q106R	NA	*PROKR2*: V331M[c]
17	M	nIHH	—	UK	[c.317 A>G] + [=]	Q106R	NA (Brother/Brother)	None[d]
18	M	AOIHH	14	C	[c.317 A>G] + [=]	Q106R	S	None[c]
19	M	KS	—	C	[c.317 A>G] + [=]	Q106R	NA	None[d]
20	F	KS	—	C	[c.317 A>G] + [=]	Q106R	AR	None[d]
21	M	KS	<5	C	[c.317 A>G] + [=]	Q106R	AD	None[d]
22	M	nIHH	30	NA	[c.317 A>G] + [=]	Q106R	NA	None[g]
23	M	nIHH	50	C	[c.317 A>G] + [=]	Q106R	S	None[g]
24	F	nIHH	—	C	[c.317 A>G] + [=]; [c.742-132 A>G] + [=]	Q106R	AR	None[c]
25	F	HA	32	C	[c.317 A>G] + [=]; [c.504 T>A] + [=]	Q106R, S168A	S	None[d]
26	M	CDP	59	C	[c.350 T>C] + [=]	L117P	S	None[h]
27	F	nIHH	—	C	[c.436 C>T] + [=]	P146S	NA	None[c]
28	M	KS	<5	C	[c.436 C>T] + [=]	P146S	AD	None[i]
29	M	KS	<5	C	[c.511 G>A] + [=]	A171T	NA	None[d]
30	M	nIHH	—	C	[c.785 G>A] + [=]	R262Q	S	*FGFR1*: K618N[j]
31	F	KS	—	C	[c.785 G>A] + [=]	R262Q	NA	None[c]
32	F	HA	—	UK (Hispanic)	[c.785 G>A] + [=]	R262Q	NA	None[d]
Normal GNRHR (not included in the four groups)								
33	F	CDP	<5	C	[c.149 C>T] + [=]	A50V	NA	*PROKR2*: N325K[e]
34	M	nIHH	25	UK	[c.648 C>T] + [=]	F216F	NA	None[c]
35	M	nIHH	—	C	[c.649 C>T] + [=]	F216F	NA	None[c]

C, Caucasian; A, Asian; AA, African-American; UK, unknown. AR, autosomal recessive; AD, autosomal dominant; S, sporadic; NA, not assessable; M, male; F, female.

[a] Screened for *FGF8*, *FGFR1*, *KAL1*, *NELF*, *PROK2*, *PROKR2*, *TAC3*, and *TACR3*.

[b] Screened for *FGF8*, *FGFR1*, *KAL1*, *PROK2*, and *PROKR2*.

[c] Screened for *FGF8*, *FGFR1*, *GNRH1*, *KAL1*, *KISS1*, *KISS1R*, *NELF*, *PROK2*, *PROKR2*, *TAC3*, and *TACR3*.

[d] Screened for *FGF8*, *FGFR1*, *KAL1*, *KISS1*, *KISS1R*, *NELF*, *PROK2*, and *PROKR2*.

[e] Screened for *FGF8*, *FGFR1*, *GNRH1*, *KAL1*, *KISS1R*, *KISS1*, *PROK2*, *PROKR2*, *TAC3*, and *TACR3*.

[f] Screened for *GF8*, *FGFR1*, *GNRH1*, *KISS1*, and *KISS1R*.

[g] Screened for *KISS1R*, *TAC3*, *TACR3*, *GnRH1*, *PROK2*, and *PROKR2*.

[h] Screened for *FGF8*, *FGFR1*, *GNRH1*, *KAL1*, *KISS1R*, *NELF*, *PROK2*, *PROKR2*, *TAC3*, and *TACR3*.

[i] Screened for *FGF8*, *FGFR1*, *KAL1*, *KISS1*, *PROK2*, and *PROKR2*.

[j] Screened for *FGF8*, *FGFR1*, *KAL1*, *KISS1R*, *KISS1*, *NELF*, *PROK2*, *PROKR2*, *TAC3*, and *TACR3*.

pulses = 3.5 ± 1.3 over 12 h, mean LH concentration = 3.6 ± 1.6 IU/liter) (Supplemental Table 2 and Fig. 2). One female (no. 11, Q106R/R262Q) was initially treated with pulsatile GnRH (75 ng/kg, one cycle) but had no follicular development. During 100 ng/kg treatments (three cycles), she had an endogenous LH surge but inadequate progesterone levels. Finally, at 250 ng/kg (eight cycles), monofolliculogenesis and normal neuroendocrine dynamics

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were documented. This patient conceived three times on pulsatile GnRH (250 ng/kg) and then twice with exogenous gonadotropins, but all five conceptions resulted in miscarriages (40). However, her sister (no. 11b, same mutations) bore three children after exogenous gonadotropins. Both sisters were also heterozygous for a deleterious *FGFR1* mutation [R470L (17)] (Supplemental Table 2 and Fig. 2). In summary, female patients in G3 were able to achieve follicular development with increasing doses of either GnRH or gonadotropins, but in one case multiple miscarriages occurred.

G3 (pLOF/pLOF) also contained two males with attenuated forms of GnRH deficiency (Table 2, Supplemental Table 1, Fig. 2). Patient no. 8 (homozygous Q106R) presented with microphallus but after fathering a baby after 4 months of hCG monotherapy (1000 IU sc every other day) (41), he demonstrated spontaneous recovery (five LH pulses over 12 h, testosterone 271 ng/dl, testes 17 ml bilaterally, and sperm count 42 million/ml 6 months after discontinuation of hCG) (23). Subject no. 7 (P96S/Q106R) presented with the fertile eunuch syndrome, with 12 ml testes before treatment initiation. Two other males (no. 10, Q106R/S217R; no. 12, L166P/R262Q) in this group also had normosmic IHH (nIHH) with normal-sized testes (12 and 15 ml), but no information is available regarding prior treatment (Supplemental Table 1 and Fig. 2).

G4 [cLOF/normal (NL) or pLOF/NL] contained 52 individuals with only one impaired allele, including 26 patients (15 males, 11 females) (no. 5b, 10b, 10d, 12c, 14, 14b, 15, 16, 17, 18, 19, 20, 21, 21b, 22, 23, 24, 24b, 25, 26, 27, 28, 29, 30, 31, 32) and 26 normal family members (10 males, 16 females) (no. 3b, 3c, 3d, 4b, 4c, 4d, 6b, 6c, 8b, 8c, 8d, 8e, 11c, 11d, 11e, 11f, 11 g, 12d, 12e, 14c, 14d, 18b, 24c, 29b, 30b, 32b) (Supplemental Tables 1–3). Of 36 individuals in whom genomic sequence data were available, 33 of 36 (91.7%) were heterozygous by SNP analysis (Supplemental Table 4). Six had KS (no. 19, 20, 21, 28, 29, and 31) and 11 had nIHH (no. 14, 14b, 15, 16, 17, 22, 23, 24, 24b, 27, 30). Others had milder forms of GnRH deficiency: three females had HA (no. 25, 10d, and 32); one male had AOHH (no. 18); and three males had CDP (no. 10b, 12c, 26). Two females presented with isolated anosmia or cleft lip/palate (no. 5b and 21b, respectively) (Supplemental Tables 1 and 2 and Fig. 2). Five individuals underwent blood sampling every 10 min and, similar to group 3 (pLOF/pLOF), had discernible pulses: mean of 3.4 ± 2.3 LH pulses over 12 h and a mean LH concentration of 2.8 ± 2.0 IU/liter. Patient no. 30 (heterozygous R262Q) responded well to increasing doses of GnRH (ED_{50} = 13 and 23 ng/kg in two different studies), and patient no. 18 (heterozygous Q106R) fathered a baby on pulsatile GnRH treatment (5–25 ng/kg dose escalation over 1 yr).

The number of LH pulses and mean LH levels were not statistically different among G2, G3, and G4.

Probands were screened for mutations in genes implicated in the pathogenesis of GnRH deficiency. RSV were identified in only five of 33 probands [heterozygous *FGFR1* in three (no. 14, 11, 30) and heterozygous *PROKR2* in two (no. 33, no. 16)] and one of 36 family members (no. 11b, heterozygous *FGFR1*).

Discussion

The central aim of this study was to examine the spectrum of reproductive phenotypes in a large cohort of individuals bearing mutations in *GNRHR* and to relate this to the apparent genotypic burden. Although the number of individuals in groups 1 and 2 are modest, the phenotypes of the patients are consistent with severe GnRH deficiency. More attenuated forms of GnRH deficiency, such as the fertile eunuch syndrome and reversible hypogonadotropism, were identified in group 3 (pLOF/pLOF) but not in groups 1 or 2.

In contrast, patients in G4 (pLOF/NL or cLOF/NL) do not follow the trend established by patients in G1-G3. Patients in G4 demonstrated a broad phenotypic spectrum, ranging from severe GnRH-deficient states such as KS (12%) and IHH (21%), to attenuated GnRH deficiency (13% with HA, AOIHH, or CDP), to seemingly normal GnRH neuronal function (normal puberty and sexual function (50%) (Fig. 3). The possibility that patients in G4 had a gene deletion on their nonmutated allele was excluded as the vast majority of patients were clearly heterozygous for common polymorphisms. In addition,

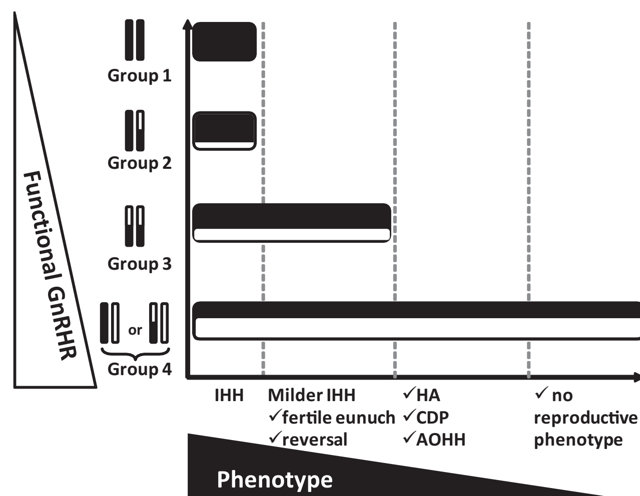

FIG. 3. Relationship between the phenotype of patients harboring loss-of-function mutations and the number/severity of GnRH mutations.

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the prevalence of heterozygous RSV in *GNRHR* was significantly higher in probands compared with controls ($P < 0.01$), supporting the concept that these monoallelic changes do contribute to abnormal reproductive function.

Several possibilities exist to explain how monoallelic changes in *GNRHR* contribute to the abnormal reproductive phenotypes. Dominant-negative effects of *GNRHR* mutations have been described *in vitro* (35, 42). However, if that was the mechanism of all heterozygote mutations, a greater proportion of individuals in G4 would be expected to have GnRH deficiency. Instead, 50% of the individuals carrying heterozygote *GNRHR* mutations had seemingly normal reproductive function.

Given that GnRH deficiency has been established to be an oligogenic disease in a subset of patients (17, 18), a second possibility is that patients with *GNRHR* mutations may have additional genetic defects yet to be discovered. Indeed, the presence in G4 of seven individuals with abnormal olfaction as well as the subject who presented with only cleft lip/palate strongly suggests the presence of additional genetic defects in pathways that affect olfactory bulb and facial development/function. As recently shown by Sarfati *et al.* (19), the overwhelming majority of GnRH-deficient patients with mutations in *PROKR2* have monoallelic mutations (11, 13) without dominant-negative effects (43), suggesting that these individuals carry mutations in other genes for GnRH deficiency (18, 19). Although only six of 70 patients actually had a second genetic abnormality identified in the current study, it is likely that increasingly availability of sequencing will accelerate the pace of gene discovery and the occurrence of demonstrable second mutations.

Finally, it is possible that heterozygote *GNRHR* mutations, through modest reductions in gene dosage, may confer susceptibility to environmental, behavioral, or psychosocial constraints on GnRH secretion (44). This hypothesis may be exemplified in two families in this study, each with biallelic siblings with nIHH (families of no. 10 and no. 12). Both sets of fathers (no. 10b and no. 12c) harbored monoallelic changes and presented with CDP (Supplemental Fig. 2 and Supplemental Table 1). One of the mothers (no. 10d) had a normal puberty but subsequently developed HA triggered by anorexia (Supplemental Table 2); the other mother (no. 12e) had normal puberty and sexual function, but it is not known whether she ever experienced excessive exercise or food deprivation (20) (Supplemental Table 3). In this study, the frequency of heterozygous changes in *GNRHR* was significantly more common in probands than in controls, suggesting that *GNRHR* variants are not merely innocent bystanders but may contribute to reproductive pathogenicity. Understanding how environmental inputs interact with genetic variation to modify phenotypic expression remains a great challenge in contemporary genetics.

Although the association of *GNRHR* mutations with decreased responsiveness to exogenous GnRH is expected, the impaired response to gonadotropin treatments reported in one female in G1 (no. 1) and another in G2 (no. 3) suggests additional gonadal defects. Both *GNRH* and *GNRHR* are expressed in the ovary of several species (45) including humans (46). *GNRHR* mRNA expression in granulosa-luteal cells increases during follicular development (47), and *GNRHR* binding has been demonstrated in granulosa cells of preovulatory follicles and corpora lutea, although it is lacking in primordial and early antral follicles (48, 49). This stage-specific expression and function raises the hypothesis that *GNRHR* mutations may have a role in folliculogenesis and contribute to abnormal gonadal responses to fertility treatments.

In G3, a female with a compound heterozygous *GNRHR* mutation and a *FGFR1* mutation conceived five times on pulsatile GnRH but had five miscarriages (40). Both *GNRHR* and *FGFR1* are expressed in the placenta and have been proposed as regulators of placental function through hCG secretion and angiogenesis, respectively (50–54). Although fetal *gnrhr* is not thought to be essential for maintenance of early pregnancy in mice (53), the potential significance of GnRH and its receptor on the maternal side of the placenta is still being investigated.

In conclusion, mutations in *GNRHR* are relatively common causes of hypogonadotropic hypogonadism, occurring in approximately 4% of a large population of patients with GnRH deficiency (5.6% of normosmic patients and 1.9% of KS). Receptor function in patients harboring biallelic mutations in *GNRHR* appears to correlate with the phenotypic spectrum of GnRH deficiency. However, patients harboring monoallelic mutations in *GNRHR* demonstrate a wider spectrum of GnRH-deficient states, suggesting the presence of yet-to-be-identified genetic and/or nongenetic factors that work in combination with the mutated *GNRHR* allele to produce reproductive phenotypes.

Acknowledgments

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