Expanding the Phenotype and Genotype of Female GnRH Deficiency

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Context: GnRH deficiency is a rare genetic disorder of absent or partial pubertal development. The clinical and genetic characteristics of GnRH-deficient women have not been well-described.

Objective: To determine the phenotypic and genotypic spectrum of a large series of GnRH-deficient women.

Design, Setting, and Subjects: Retrospective study of 248 females with GnRH deficiency evaluated at an academic medical center between 1980 and 2010.

Main Outcome Measures: Clinical presentation, baseline endogenous GnRH secretory activity, and DNA sequence variants in 11 genes associated with GnRH deficiency.

Results: Eighty-eight percent had undergone pubarche, 51% had spontaneous thelarche, and 10% had 1–2 menses. Women with spontaneous thelarche were more likely to demonstrate normal pubarche ($P = 0.04$). In 27% of women, neuroendocrine studies demonstrated evidence of some endogenous GnRH secretory activity. Thirty-six percent (a large excess relative to controls) harbored a rare sequence variant in a gene associated with GnRH deficiency (87% heterozygous and 13% biallelic), with variants in *FGFR1* (15%), *GNRHR* (6.6%), and *PROKR2* (6.6%) being most prevalent. One woman had a biallelic variant in the X-linked gene, *KAL1*, and nine women had heterozygous variants.

Conclusions: The clinical presentation of female GnRH deficiency varies from primary amenorrhea and absence of any secondary sexual characteristics to spontaneous breast development and occasional menses. In this cohort, rare sequence variants were present in all of the known genes associated with GnRH deficiency, including the novel identification of GnRH-deficient women with *KAL1* variants. The pathogenic mechanism through which *KAL1* variants disrupt female reproductive development requires further investigation. **(*J Clin Endocrinol Metab* 96: E566–E576, 2011)**

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Abbreviations: E_2, Estradiol; EFP, early follicular phase; KS, Kallmann's syndrome; nIHH, normosmic idiopathic hypogonadotropic hypogonadism; RSV, rare sequence variant.

Isolated GnRH deficiency is a disorder of hypogonadism attributable to low or inappropriately normal gonadotropins resulting in absent or incomplete puberty, often seen in association with nonreproductive phenotypes such as craniofacial, skeletal, neurologic, renal, and olfactory abnormalities (1). The olfactory phenotype has traditionally been used to subdivide these patients into normosmic idiopathic hypogonadotropic hypogonadism (nIHH) and anosmic [Kallmann's syndrome (KS)] variants. Although females with GnRH deficiency were included in Kallmann's original report of the familial nature of this disorder, men have been the focus of much of the subsequent scientific literature because of the significant excess of males to females reported with this condition (1).

Rare sequence variants (RSVs) in genes involved in GnRH neuronal migration (*FGF8*, *FGFR1*, *KAL1*, *PROK2*, *PROKR2*, and *NELF*), secretion (*GNRH1*, *GPR54*, *TAC3*, and *TACR3*) and receptivity (*GNRHR*) have been reported to contribute to GnRH deficiency in both men and women (reviewed in Ref. 1), although the relative frequency of the RSVs in each gene has not been investigated in a large female cohort. An important exception is the X-linked gene, *KAL1*, in which RSVs have only been found in GnRH-deficient men. However, because of the assumption that female GnRH deficiency could not be explained by a RSV in a gene associated with an X-linked recessive disorder and the absence of a reproductive phenotype in a small number of obligate *KAL1* female carriers (2–4), there are fewer than 100 published cases in which *KAL1* has been screened in GnRH-deficient women (3–6).

Systematic clinical investigation has broadened the phenotype of male GnRH deficiency to include not only severe congenital hypogonadism but also late pubertal arrest (7), adult-onset disease (8), and even adult reversal (9). Recent genetic studies of male probands and their families suggest that a broader phenotypic spectrum may also exist in women (10–13), but this hypothesis has not been addressed systematically.

Through detailed phenotypic and genotypic profiling of a large cohort of females with isolated GnRH deficiency, the current study demonstrates a clinical spectrum in both breast development and menses. It also reveals the relative frequency of RSVs in the genes implicated in normal GnRH function, including the unexpected finding of *KAL1* RSVs in 6.2% of this entirely female cohort.

Subjects and Methods

Patient population

The cohort comprised 248 females referred to an academic medical center for presumed isolated GnRH deficiency between 1980 and 2010. Ninety-six were patients of physicians in the Reproductive Endocrine Unit (REU) of Massachusetts General Hospital. The remainder were self-referred or referred by physicians from around the world in response to a clinical trial posting and completed testing by mail (questionnaire, smell testing, blood samples for DNA isolation). All women were ≥16 yr old at the time of evaluation, had low estradiol (E_2) levels in the face of low or inappropriately normal gonadotropins, no other pituitary hormone deficiencies, and no neuroanatomic or functional cause of hypogonadotropic hypogonadism. None of the women had a known eating disorder, each had achieved the minimum weight for height necessary for the onset of menstrual cycles (14), and none exercised excessively [defined as greater than 20 miles per week of running or its equivalent (15)].

Both phenotypic and genotypic information was available in 207 women, of whom 62 had baseline neuroendocrine sampling. The majority (61%) was tested for all 11 genes, while 85% were tested for ≥5 genes. The remaining women had detailed phenotypic information, but either DNA was not available or they were not included in RSV frequency calculations because they were female relatives of the proband who harbored the same RSV. Complete DNA sequencing of the 11 genes was performed in 80–160 or 200–350 alleles from female or male controls, respectively, who had normal reproductive function by history and physical examination. Each *KAL1* RSV identified in GnRH-deficient women was tested in 870 X-chromosomes (from male and female controls).

This study was approved by the Massachusetts General Hospital Human Research Committee, and signed informed consent was obtained from each subject before participation.

Phenotyping

Clinical assessment

A detailed questionnaire was administered to all subjects to assess family history, ethnicity, height, weight, eating attitudes and behaviors, dysmorphic features, pubertal development, and results of brain imaging. Categorization of anosmia was based on history alone or on the results of olfactory testing (40-item University of Pennsylvania Smell Identification Test) (16). For statistical purposes, women scoring ≥5th% based on age were coded as normosmic and all others were coded as anosmic. Ovarian volumes were determined by transvaginal or transabdominal ultrasound and calculated with the formula for an ellipse ($V = 0.52 \times$ maximal longitudinal $\times$ antero-posterior $\times$ transverse diameters).

Neuroendocrine evaluation

Hormone replacement was discontinued for at least one month before initial REU evaluation. Frequent blood sampling (every 10 min overnight for 12 h) was performed to assess endogenous GnRH secretion as manifested by LH pulsatility. FSH and E_2 were assayed from pools from these frequent sampling studies. Pulsatile LH was analyzed using a validated modification of the Santen and Bardin method (17, 18). Results were compared with those previously reported for 17 normally cycling women studied during the early follicular phase (EFP) of an ovulatory cycle (19, 20). Women with GnRH deficiency were classified as having low amplitude or low frequency LH pulse patterns if their levels were more than two SD's below the normal range [LH amplitude 2.3 ± 1.0, frequency 7 ± 1.8 pulses per

12-hour (mean ± SD)]. Women whose pulse frequency and amplitude were indistinguishable from controls were further evaluated for a pattern of sleep augmentation, as previously described (21).

Detection of DNA sequence variants

Genomic DNA was obtained from peripheral blood samples by standard phenol-chloroform extraction. Exonic and proximal intronic (≤15 bp from splice sites) DNA sequences of 11 genes implicated in the etiology of GnRH deficiency were amplified by PCR and determined by direct sequencing. These genes include *KAL1* (anosmin-1, OMIM 308700), *GNRH1* (gonadotropin-releasing hormone 1, OMIM 152760) *GNRHR* (GnRH receptor, OMIM 138850), *GPR54* (KISS1 receptor, OMIM 604161), *NELF* (nasal embryonic LHRH factor, OMIM 608137), *FGF8* (fibroblast growth factor 8, OMIM 600483), *FGFR1* (fibroblast growth factor receptor 1, OMIM 136350), *PROK2* (prokineticin 2, OMIM 607002), *PROKR2* (prokineticin receptor 2, OMIM 607212), *TAC3* (tachykinin 3, OMIM 162330), and *TACR3* (tachykinin receptor 3, OMIM 162332). One KS woman with features of CHARGE syndrome was also tested for *CHD7* (chromodomain helicase DNA-binding protein 7, OMIM 608892). PCR primers and amplification conditions for each gene have been described previously (3, 22–30). All sequence variations were observed on both DNA strands and were confirmed in a separate PCR. Homozygosity for the *KAL1* Q131H variant was confirmed by multiplex ligation-dependent probe amplification (MLPA, MRC-Holland, The Netherlands). Genes and proteins are described using standard nomenclature (31). Presentation of results is restricted to sequence variants that were 1) at splice junctions within 5 bp of coding sequence, or 2) in coding sequence and nonsynonymous; and 3) present in <1% of control alleles. Rare synonymous changes were also compared between cases and controls for internal validation.

Functional analysis

The *in vitro* functional data reported has been previously described (10, 12, 23–26, 30, 32–36). Where *in vitro* data were not available, five different prediction programs were used to determine the potential significance of missense variants and one prediction program was used for intronic changes. These included PolyPhen (37), Mutation Taster (38), Panther (39), SIFT (40), pMUT (41), and Human Splicing Finder (42). *In vitro* data and prediction program results are presented in Supplemental Table 1 (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org/).

Mode of inheritance

Phenotypic characterizations of the proband and family members were used to determine the mode of inheritance as previously described (7). No families in this cohort met the definition of X-linked recessive or X-linked dominant inheritance, although the latter could not always be distinguished from autosomal dominant inheritance (*i.e.*, in cases of small pedigrees with transmission of the trait through the female). A family was classified as sporadic if no other relatives were affected and as unknown if no pedigree information was available.

Assays

Serum LH and FSH were measured using a two-site monoclonal nonisotopic system (Axsym; Abbott Laboratories, Abbott Park, IL) as previously described (43–46), and expressed in international units per liter (IU/liter) of the Pituitary 2nd International Standard 80/552. Estradiol was measured by two different RIAs using highly specific antisera with a functional sensitivity of ≤20 pg/ml (73.4 pmol/liter) which were cross-referenced (47, 48).

Statistical methods

Data are expressed as the mean ± SE unless otherwise indicated. Because of the association of a more severe reproductive phenotype with anosmia in men with GnRH deficiency, comparisons were performed between nIHH and KS women using independent samples *t* tests (for parametric data) and Wilcoxon Rank Sum tests (for nonparametric data) for continuous variables. χ^2 or Fischer's exact test were used as tests of association and to compare categorical variables between nIHH and KS women. A *P* value of <0.05 was considered to be statistically significant.

Results

Phenotype studies

Clinical presentation

The clinical and biochemical features of the 248 women are summarized in Table 1. Forty-seven percent of women were anosmic. As the women presented for evaluation at a relatively advanced age (mean 28.5 yr), nearly all had undergone at least some treatment with hormone replacement before presentation. However, before hormone replacement, 88% had undergone normal pubarche, 51% had some degree of breast development, and 10% had one or two spontaneous menses with no difference noted between nIHH and KS women. Women with thelarche were more likely to demonstrate pubarche than those without (97% *vs.* 77%, $P = 0.04$) but did not have higher FSH ($P = 0.3$) or E_2 levels ($P = 0.09$), or larger ovarian volumes ($P = 0.6$).

Neuroendocrine and ultrasonographic studies

E_2 levels were low, nearly all being undetectable [<20 pg/ml (<73.4 pmol/liter)], with normal to low gonadotropins (Table 1). Mean ovarian volumes were smaller than in normal adult women [mean 9.5 cc; 95% confidence interval (3.9–15.9 cc)] (49), and nIHH women had larger ovarian volumes than KS women ($P = 0.02$) with a tendency for higher gonadotropin levels (Table 1). Normosmic and KS women had similar patterns of LH secretion with the majority (75 and 70%, respectively) having an apulsatile pattern (Fig. 1A) and a smaller number demonstrating a low amplitude and/or low frequency pattern. In 8% of women (n = 6), the mean frequency and amplitude of LH pulses was within 2 SD of the EFP mean in normal women. Two of these women with evidence of pulsatile GnRH release exhibited a pattern of sleep augmentation that is characteristic of children in early puberty

TABLE 1. Clinical presentation of GnRH-deficient women

	Full cohort (n = 248)	KS women (n = 116)	nIHH women (n = 132)	*P* value
Age, yr	28.5 ± 0.9	27.2 ± 1.3	29.8 ± 1.3	0.16
BMI, kg/m^2	25.4 ± 2	26.5 ± 2.6	23.1 ± 2.4	0.43
Thelarche, %	51% (44/86)	50% (20/40)	52% (24/46)	1
Menarche, %	10% (10/98)	8% (4/48)	12% (6/50)	0.7
Pubarche, %	88% (49/56)	84% (21/25)	90% (28/31)	0.7
Ovarian volume, cc	2.8 ± 0.3 (n = 39)	2.0 ± 0.4 (n = 18)	3.4 ± 0.4 (n = 21)	**0.02**
LH, IU/liter	0.6 ± 0.1 (n = 85)	0.5 ± 0.1 (n = 44)	0.7 ± 0.2 (n = 41)	0.13
FSH, IU/liter	1.5 ± 0.2 (n = 85)	1.2 ± 0.3 (n = 44)	1.7 ± 0.2 (n = 41)	0.12
Baseline study				
Apulsatile, %	73% (51/70)	70% (24/34)	75% (27/36)	0.8[a]
Pulsatile, %	27% (19/70)	30% (10/34)	25% (9/36)	
Low frequency and/or amplitude, %	19% (13/70)	21% (7/34)	17% (6/36)	
Normal frequency and amplitude, %	8% (6/70)	9% (3/34)	8% (3/36)	

[a] Represents pulsatile *vs.* apulsatile patterns in KS *vs.* nIHH.

(Fig. 1B). The six women with LH pulse frequency and amplitude within the EFP range demonstrated a similar spectrum of clinical reproductive phenotypes, nonreproductive phenotypes [olfaction, age, body mass index (range 19–23)], and genetic variation as the overall cohort, but had higher gonadotropin levels (LH 2.0 ± 0.6 *vs.* 0.3 ± 0.07, $P < 0.001$; and FSH 4.2 ± 0.4 *vs.* 1.0 ± 0.14, $P < 0.001$) compared with women with absent pulses. Despite their mild phenotype, none of the six women with a more robust pattern of pulsatile LH secretion has developed menstrual cycles after up to twenty years of follow-up (Table 2). The overall group of women with evidence of some underlying GnRH secretion was no more likely to have experienced thelarche or menarche than women without LH pulses ($P = 0.8$ and $P = 0.07$, respectively). Women with menses (n = 10) were also not different from the overall cohort in terms of reproductive and nonreproductive phenotypes, body mass index, age, or LH secretory pattern.

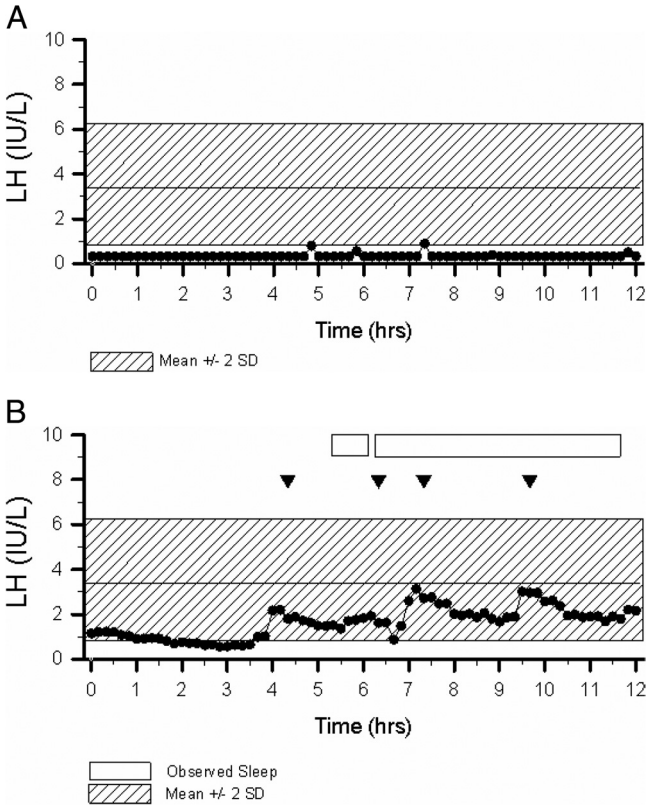

FIG. 1. A, Representative 12-h pattern of LH secretion in a 25-year-old woman with KS (Subject 17) who harbors two *KAL1* RSVs demonstrates an absence of LH pulses. *Shaded region* represents the normal range for LH in healthy women with normal menstrual cycles in the EFP of their ovulatory menstrual cycles (19, 20). B, Representative 12-h pattern of LH secretion in a 27-year-old woman with nIHH (Subject 5) who carries a *TACR3* RSV demonstrates pulses of similar amplitude and frequency to that seen in normal women during the EFP but with amplified pulses during sleep. *Shaded region* represents the normal range for LH in healthy women with normal ovulatory menstrual cycles in the EFP (19, 20). *Arrowheads* signify LH pulses, and *boxes* represent periods of observed sleep.

Genetic studies

Detailed family histories were available in 148 women, revealing a familial pattern of GnRH deficiency in 66% (64% autosomal dominant, 36% autosomal recessive, 0% X-linked). Normosmic IHH cases were more likely to be familial (and autosomal recessive), whereas KS cases were more likely to be sporadic ($P < 0.005$; Supplemental Table 2).

Thirty-six percent of GnRH-deficient women harbored at least one RSV in a gene known to be associated with GnRH deficiency. This was significantly more than in control men and women (14%; $P < 0.001$) and suggests that most of the RSVs detected confer susceptibility to the GnRH deficiency phenotype. This difference was in large part attributable to an increased frequency of RSVs in *FGFR1*, *GNRHR*, *PROKR2*, and *KAL1* in GnRH-deficient women compared with controls (Supplemental Table 3). As expected, the frequency of rare synonymous variants was not different between GnRH-deficient women and controls (8% and 12%, respectively, $P = 0.1$).

TABLE 2. Phenotypic and genotypic characteristics of the six GnRH-deficient women with LH pulse patterns indistinguishable from normal early follicular phase women [previously shown to have a mean LH pulse amplitude of 2.3 IU/liter (95% CI 0.3–4.3), mean pulse frequency of 7 pulses/12 h (95% CI 3.4–10.6), and mean ovarian volume of 9.5 cc (95% CI 3.9–15.9)] (19, 20, 49)

Subject	KS/ nIHH	Thelarche/ menarche	LH (IU/liter)	FSH (IU/liter)	Mean LH pulse amplitude (IU/liter)	LH pulse frequency (pulses/12 h)	Ovarian vol (cc)	Nonreproductive phenotype	Rare sequence variant	Follow-up (yrs)
1	KS	+/+	0.6	5.2	0.6	5	N.A.		N.A.	16
2	KS	−/−	3.8	5.3	0.5	9	8.8	Agenesis of the CC, short 4th MC, high-arched palate	NEG	6
3	nIHH	+/−	3.5	4.7	1.5	10	8.8		*PROKR2* het (R85C[a])	4
4	KS	+/−	2.6	4.3	0.4	5	1.4	Hypoplastic olfactory bulbs and sulci, hearing loss	*KAL1* het (K185N)	20, pregnant with GnRH pump
5	nIHH	+/+	1.0	3.2	1.1	4	8	3 frontal incisors	*TACR3* het (A449T)	2
6	nIHH	+/−	0.6	2.8	2.7	5	4	Gap between teeth, otosclerosis	NEG	2, pregnant with gonadotropins

CC, corpus callosum; MC, metacarpals; het, heterozygous; N.A., not assessed. Subject numbers are consistent across tables.

[a] Loss of function variant.

Seventy-six different RSVs were identified in GnRH-deficient women compared with 23 in controls (data not shown). Sixty-five of the GnRH-deficient women had an alteration in a single gene (86% heterozygous, 14% biallelic), whereas 10 had alterations in more than one gene (nine digenic, one trigenic) (Table 3). Fifty percent of RSVs were loss of function [frameshift or by previously reported *in vitro* testing (Supplemental Table 1)]. The remainder were either missense variants that have not been tested *in vitro* or intronic changes within 5 bps of the exon. Sixty-nine percent of these missense variants are predicted to be deleterious by two or more prediction programs. RSVs were most frequent in *FGFR1*, *GNRHR*, and *PROKR2* (Fig. 2, Supplemental Table 3), and these genes also harbored the greatest number of unique RSVs. RSVs were also identified in *FGF8* (1.5%), *GNRH1* (0.7%), *GPR54* (2%), *NELF* (1%), *PROK2* (2%), *TAC3* (2%), and *TACR3* (3.6%). Although not every woman was sequenced for all 11 genes, the RSV frequency remained unchanged when limiting the analysis to the 120 women who were completely assessed.

Notably, we have also identified *KAL1* RSVs in 10 women with GnRH deficiency (6.2%; nine heterozygous, one biallelic) at a frequency that is similar to that of *PROKR2*. Seven *KAL1* missense variants were identified, one of which is novel (T649M). The remaining RSVs in *KAL1* were identified in a mixed cohort of men and women with GnRH deficiency, however detailed phenotypic information was not provided (50) (Tables 4 and 5, Supplemental Table 1). To date there is no validated functional *in vitro* assay for *KAL1*, and none of the women with *KAL1* RSVs had affected male relatives. However, as in males, the majority of RSVs identified in this cohort fall within the fibronectin III domains (51). In addition, five of the seven RSVs (Q131H, K185N, P277T, V587L, and T659M) are predicted to be deleterious by at least one

TABLE 3. Women with rare sequence variants in more than one gene associated with GnRH deficiency

Subject	Variant gene #1	Nucleotide change	Amino acid change	Homo/ het	Variant gene #2	Nucleotide change	Amino acid change	Homo/ het	Variant gene #3	Nucleotide change	Amino acid change	Homo/ het
7	*FGFR1*	c.716T>C	I239T[a]	Het	*GNRH1*	c.91C>T	R31C[a] frame-shift	Het	*PROKR2*	c.604A>G	S202G	Het
8	*FGFR1*	c.1549-2A>G	Intronic	Het	*KAL1*	c.1759G>T	V587L	Het				
9	*FGFR1*	c.1409G>T	R470L[a]	Het	*GNRHR*	c.317A>G	Q106R[a]	Het				
					GNRHR	c.785C>A	R262Q[a]	Het				
10	*FGFR1*	c.350A>G	N117S[a]	Het	*GNRHR*	c.247C>G	L83V	Het				
					GNRHR	c.317A>G	Q106R[a]	Het				
11	*KAL1*	c.1464A>G	T472A	Het	*PROKR2*	c.151G>A	A51T	Het				
12	*FGFR1*	c.682T>G	Y228D[a]	Het	*GPR54*	c.565G>A	A189T	Het				
13	*PROK2*	c.70G>C	A24P[a]	Het	*PROKR2*	c.343G>A	V115M[a]	Het				
14	*FGFR1*	c.854C>G	P285R	Het	*KAL1*	c.1464A>G	T472A	Het				
15	*KAL1*	c.1627G>A	V543I	Het	*CHD7*	c.2440C>T	Q814X[a]	Het				
16	*FGFR1*	c165_171 deletion	Frame-shift	Het	*PROKR2*	c.518T>G	L173R[a]	Het				

Het, heterozygous. Subject numbers are consistent across tables.

[a] Loss-of-function variant.

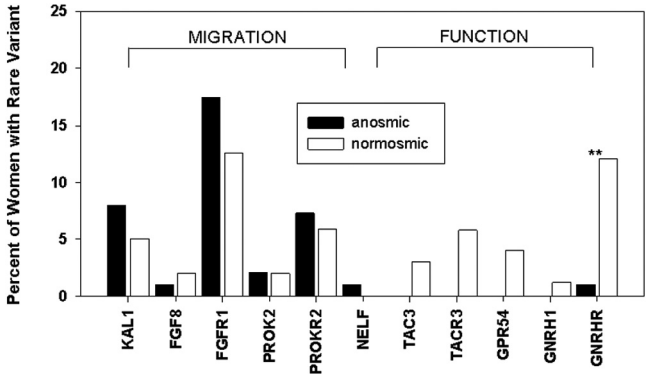

FIG. 2. Frequency of rare sequence variants in genes involved in neuronal migration and function of GnRH in KS *vs.* nIHH women. **, *GnRHR* variants were more common in nIHH women than in KS women ($P < 0.01$). Variants in *NELF* were only identified in KS women, and variants in *GNRH1*, *GPR54*, *TAC3*, and *TACR3* were only identified in nIHH women.

prediction program (Table 4). While not predicted to be deleterious, one of the RSVs (V543I) has previously been described in an unrelated man with severe KS in the absence of other genetic variants (50). Like the KS woman with this RSV (subject 15, Tables 4 and 5), this patient did not have synkinesia or renal agenesis. T472A was present in five GnRH-deficient women. T472A and V587L were present in 0.30% and 0.47%, respectively, of 870 control X-chromosomes; however, neither was present in any of the genomes screened thus far in the 1000 Genomes Project (www.1000genomes.org), arguing that each may be a susceptibility factor for GnRH deficiency that is not fully penetrant. Men with RSVs in *KAL1* are more likely to demonstrate a more severe reproductive phenotype compared with other GnRH-deficient men (3, 7), however female *KAL1* carriers displayed a similar spectrum of reproductive features to those not harboring these RSVs. Sixty percent of women with *KAL1* RSVs were anosmic, whereas none demonstrated synkinesia or renal agenesis. Six women had a RSV only in *KAL1*, whereas four also harbored RSVs in other genes associated with GnRH deficiency (*PROKR2*, *FGFR1*, and *CHD7*).

Also of note, one woman with anosmia had a heterozygous *GNRHR* missense variant (Q106R) which has been identified in nIHH patients and shown to be pathogenic in *in vitro* studies (32, 24). While this woman had no additional defects in genes known to be associated with anosmia including *FGF8*, *FGFR1*, *KAL1*, *PROK2*, *PROKR2*, and *NELF*, her presentation suggests digenicity with an as yet undiscovered gene involved in neuronal migration.

Genotype/phenotype correlations

RSVs in *GNRHR* were more prevalent in nIHH compared with KS women ($P < 0.01$), and RSVs in *GPR54*, *TAC3*, and *TACR3* were *only* present in nIHH women. Women with thelarche, isolated menses, or endogenous LH pulses did not exhibit a unique genetic signature, although these analyses are limited by the relatively small number of subjects with each RSV. Similarly, there was no specific phenotypic signature for any of the individual genes assessed.

The 10 women with RSVs in more than one gene did not appear to be more severely affected than those with a single gene RSV [50% *vs.* 62% with thelarche ($P = 0.2$), 11% *vs.* 16% with isolated menses ($P = 1$), respectively]. Although only three of the women with RSVs in more than one gene underwent frequent sampling to assess LH secretion, a spectrum of GnRH deficiency was observed (one low amplitude and low frequency pattern of LH secretion and two apulsatile). In digenic pedigrees, individuals with a larger number of affected genes were more likely to manifest GnRH deficiency as opposed to milder defects such as delayed puberty, anosmia, or cleft lip/palate as has been noted previously (50). For example, in one pedigree, the father carried a *FGFR1* RSV (C55fsX45) and had anos-

TABLE 4. Genotypic characterization of women with *KAL1* variants

Subject	Gene	Exon and domain	Nucleotide change	Amino acid change	Homo/ het	Mono/ oligogenic	Inheritance	Polyphen	SIFT	pMUT	Panther	Mutation taster	Control frequency
4	*KAL1*	5 (FNIII)	c.555G>C	K185N	Het	Mono	Sporadic	+	−	−	+	+	0%
8	*KAL1*	12 (FNIII)	c.1759G>T	V587L	Het	*FGFR1*	Autosomal dominant (surrogate marker)[a]	−	−	+	−	−	0.47%
11	*KAL1*	10 (FNIII)	c.1464A>G	T472A	Het	*PROKR2*	Unknown	+	−	−	−	−	0.3%
14	*KAL1*	10 (FNIII)	c.1464A>G	T472A	Het	*FGFR1*	Unknown	+	−	−	−	−	0.3%
15	*KAL1*	12 (FNIII)	c.1627G>A	V543I	Het	*CHD7*	Sporadic	−	−	−	−	−	0%
17	*KAL1*	4 (WAP)	c.393G>T	Q131H	Homo	Mono	Sporadic	+	+	−	+	+	0%
	KAL1	6 (FNIII)	c.829C>A	P277T	Het			+	+	−	+	+	0%
18	*KAL1*	10 (FNIII)	c.1464A>G	T472A	Het	Mono	Unknown	+	−	−	−	−	0.3%
19	*KAL1*	10 (FNIII)	c.1464A>G	T472A	Het	Mono	Unknown	+	−	−	−	−	0.3%
20	*KAL1*	10 (FNIII)	c.1464A>G	T472A	Het	Mono	Unknown	+	−	−	−	−	0.3%
21	*KAL1*	13 (FNIII)	c.1946C>T	T649 M	Het	Mono	Sporadic	+	−	+	−	−	0%

Homo, Homozygous; Het, heterozygous; WAP, whey acidic protein; FNIII, fibronectin type III; +, not tolerated, pathological, probable, possible, deleterious, or disease causing. −, tolerated, neutral, benign, not deleterious. Subject numbers are consistent across tables.

[a] Subject 8's mother harbored the same *KAL1* variant and had delayed puberty, a validated surrogate marker of inheritance of GnRH deficiency.

TABLE 5. Phenotypic characterization of women with *KAL1* variants

Subject	Reproductive phenotype	Nonreproductive phenotype	Olfaction
4	Tanner II breasts, normal pubarche, 1° amenorrhea, pulses of normal frequency and amplitude on baseline	Normal head CT	KS
8	No thelarche, 1° amenorrhea	Dental abnormalities, short fourth metacarpals	nlHH
11	Spontaneous thelarche, 1° amenorrhea	Retinitis pigmentosa	nlHH (not formally tested)
14	1° amenorrhea		KS
15	Spontaneous thelarche, 1° amenorrhea	CHARGE association: bilateral coloboma, ASD, developmental delay, hearing loss, ataxia, cleft lip (negative for 22q deletion)	KS
17	Tanner III breasts, 1° amenorrhea, apulsatile baseline	clinodactyly, flat nasal bridge, cannot fully extend elbows, normal sella turcica film	KS
18	1° amenorrhea		nlHH (not formally tested)
19	1° amenorrhea		nlHH (not formally tested)
20	1° amenorrhea		KS
21			KS

mia, cleft lip/palate, and missing teeth, whereas his two daughters, who had RSVs in both *FGFR1* (C55fsX45) and *PROKR2* (L173R), were GnRH-deficient.

Discussion

Recent studies in patients with GnRH deficiency have provided remarkable insight into the genes that control GnRH neuronal development and function and have suggested that the clinical phenotype of GnRH deficiency may be broader than previously thought. Because of their minority status among GnRH-deficient patients, women have often been overlooked in focused studies of the genetics and clinical presentation of GnRH deficiency. In the current study, RSVs in all of the genes known to be associated with GnRH ontogeny and function, including *KAL1*, were identified in a large cohort of GnRH-deficient women. The large excess of RSVs in cases relative to controls argues strongly that the majority of these RSVs contribute to the clinical GnRH deficiency phenotype, which has been found to include both thelarche and occasional menses.

The traditional clinical description of the reproductive phenotype of female GnRH-deficiency has included absent thelarche and primary amenorrhea. In the current series of 248 women with GnRH-deficiency, the majority of women exhibited some degree of breast development and a small percent experienced isolated menses. As thelarche and menses are signs of early and prolonged estrogen production, respectively, neither would be expected to be highly prevalent in women with GnRH deficiency. While adrenarche and gonadarche are thought to proceed independently, it is noteworthy that women with spontaneous thelarche were more likely to have undergone pubarche, perhaps suggesting that aromatization of adrenal androgens contributes to early breast development in these patients. Alternatively, the association of thelarche and pubarche may reflect a permissive role of estrogen on pubic hair development (52).

There was no association between a history of thelarche and/or isolated menses and either FSH levels, estradiol levels, or evidence of pulsatile LH secretion. Our ability to ascertain an association between estradiol levels and thelarche may be limited by the sensitivity of the assay. Furthermore, frequent sampling studies were performed at the time of initial presentation which in most cases occurred several years after breast development had occurred by history. Finally, the possibility that this phenotypic discordance reflects a temporal decline in GnRH activity from the initial time of thelarche to the time of evaluation, as reported in GnRH-deficient men (7, 8), cannot be excluded.

Frequent sampling studies were consistent with absent pulsatile GnRH secretion in the majority of GnRH-deficient women, whereas some suggested enfeebled or more robust GnRH secretion. Of the six women with an LH pulse frequency and amplitude that was indistinguishable from EFP control women, none met criteria for functional hypothalamic amenorrhea, three were anosmic, four had primary amenorrhea, four had associated phenotypes, and three had RSVs in the genes associated with GnRH deficiency, suggesting that they should not be excluded

from this cohort. Two of these women had augmentation of LH pulse amplitude during sleep, which is a feature of early puberty (53) that has also been observed in GnRH-deficient men (54). While it is possible that these women were assessed in the early stages of a reversal from a state of GnRH deficiency to normal GnRH production, none subsequently manifested any clinical signs of reproductive axis recovery in follow-up, arguing against this possibility. It has also been shown that genes implicated in GnRH deficiency may predispose to functional hypothalamic amenorrhea (55), raising the possibility that this subset of GnRH-deficient women with a more robust LH pulse pattern may bridge the gap between hypothalamic amenorrhea and more profound GnRH deficiency.

Genotypic analysis of this large female GnRH-deficient cohort identified a RSV in one or more of the genes known to be involved in GnRH neuron migration or function in more than a third of patients. To achieve an unbiased assessment of the phenotypic spectrum of female GnRH deficiency, we excluded women who appeared to be GnRH-deficient but in whom a diagnosis of functional hypothalamic amenorrhea could not be discounted, and thus this is likely an underestimate. *FGFR1*, *GNRHR*, and *PROKR2* are the most commonly altered genes in GnRH-deficient women. RSVs in more than one gene were identified in 13% of women, providing further support for the importance of gene–gene interactions in the pathogenesis of GnRH deficiency (50). Interestingly, women with digenic or trigenic RSVs did not appear to be more severely affected than those with monogenic RSVs, although this conclusion is preliminary as not every woman has been screened for all 11 genes. This finding contrasts with that of a recent small study which reported a more severe phenotype among women with biallelic compared with monoallelic mutations in *PROK2/PROKR2* (13). Further studies which include larger groups of digenic GnRH-deficient men and women will be necessary to determine how these genes interact to produce a given phenotype.

Our analysis also led to the unexpected identification of 10 GnRH-deficient women who harbor RSVs in *KAL1*. One KS woman (subject 17, Tables 4 and 5) was identified with a heterozygous and a homozygous *KAL1* RSV, where the presence of two copies was confirmed using MLPA. Neither of these RSVs was present in 870 X-chromosomes from control men and women, and both are predicted to be deleterious by four prediction programs. In addition, no other genetic defects were identified in this patient. Taken together, these findings provide strong evidence that the *KAL1* RSVs are pathogenic in this patient.

The heterozygous *KAL1* RSVs found in the nine other women may also be pathogenic. Four of the five RSVs are predicted to be deleterious by at least one prediction program, an additional RSV has previously been identified as the sole genetic defect in a man with a severe form of KS (50), and all RSVs were seen in <1% of 870 control X-chromosomes. In addition, five of these women tested negative for RSVs in all other genes.

These findings raise the question of the mechanism through which a heterozygous variant on the X-chromosome can cause GnRH deficiency in women, because the *KAL1* gene has been thought to escape X-inactivation in females. This supposition has been based upon the location of the *KAL1* gene in the pseudoautosomal region of the X chromosome, the absence of a reproductive phenotype in a small number of obligate *KAL1* female carriers (2–4), and the ability of oligonucleotide primers to amplify anosmin mRNA transcripts in mouse/human hybrid cell lines containing either the active or inactive X-chromosome (57). *KAL1* mutations in males are then thought to involve functional inactivation of *KAL1* and the failure of *KAL1*-related sequences on the Y chromosome to compensate for this loss of function.

One possibility is that *KAL1* RSVs in these females may act in a dominant negative fashion rather than through the simple loss of function usually associated with recessive inheritance. However, the hypothesis of X-linked dominant inheritance runs counter to the X-linked recessive inheritance pattern observed in published *KAL1* pedigrees. A second possibility is that *KAL1* may undergo X-inactivation that varies with developmental stage or by tissue, in line with recent reports that have suggested that a gene's ability to escape inactivation is not "all-or-none" but may instead be incomplete and may vary between women and across tissues (56–58). In this scenario, the reproductive phenotype of *KAL1* female carriers could be attributable to skewed (nonrandom) X-inactivation in these particular individuals, whereas the previously reported absence of such a phenotype in female *KAL1* carriers (2–4) might reflect nonskewed (random) inactivation or inactivation skewed to favor continued expression of the normal allele. A final possibility is that GnRH-deficient women with *KAL1* heterozygous variants carry additional genetic defects. While digenicity was confirmed in only four of the nine heterozygotes, additional, as yet undiscovered genetic defects may exist in the other five women. Further work will be required to distinguish these possibilities.

In summary, the phenotypic spectrum of isolated GnRH deficiency in women is broader than previously appreciated and does not differ between nIHH and KS women. In light of this phenotypic variability, dismissing the diagnosis of GnRH deficiency in women with spontaneous thelarche and isolated menses is not appropriate, as GnRH function may change over time and/or adre-

narche may provide the substrates for early breast and endometrial development. Women with GnRH deficiency harbor RSVs in all of the genes implicated in this disorder, including *KAL1*. Further studies of *KAL1* X-inactivation are necessary to fully understand the role of *KAL1* in GnRH and olfactory neuron development in women.

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