

An ancient founder mutation in *PROKR2* impairs human reproduction

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Received January 30, 2012; Revised June 1, 2012; Accepted June 28, 2012

Congenital gonadotropin-releasing hormone (GnRH) deficiency manifests as absent or incomplete sexual maturation and infertility. Although the disease exhibits marked locus and allelic heterogeneity, with the causal mutations being both rare and private, one causal mutation in the prokineticin receptor, *PROKR2* L173R, appears unusually prevalent among GnRH-deficient patients of diverse geographic and ethnic origins. To track the genetic ancestry of *PROKR2* L173R, haplotype mapping was performed in 22 unrelated patients with GnRH deficiency carrying L173R and their 30 first-degree relatives. The mutation's age was estimated using a haplotype-decay model. Thirteen subjects were informative and in all of them the mutation was present on the same ~123 kb haplotype whose population frequency is ≤10%. Thus, *PROKR2* L173R represents a founder mutation whose age is estimated at approximately 9000 years. Inheritance of *PROKR2* L173R-associated GnRH deficiency was complex with highly variable penetrance among carriers, influenced by

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additional mutations in the other *PROKR2* allele (recessive inheritance) or another gene (digenicity). The paradoxical identification of an ancient founder mutation that impairs reproduction has intriguing implications for the inheritance mechanisms of *PROKR2* L173R-associated GnRH deficiency and for the relevant processes of evolutionary selection, including potential selective advantages of mutation carriers in genes affecting reproduction.

INTRODUCTION

Mammalian reproduction is controlled at the neuroendocrine level by the tightly coordinated pulsatile secretion of gonadotropin-releasing hormone (GnRH) from a small network of approximately 1200 specialized hypothalamic neurons (1,2). GnRH secretion stimulates the anterior pituitary to release gonadotropins, which, in turn, act on the gonads to regulate steroidogenesis and gametogenesis. Corresponding to the dominating role of this single hormone in the hypothalamic–pituitary–gonadal axis, the developmental ontogeny and homeostatic regulation of the GnRH neuronal network are highly complex and tightly linked to evolutionary fitness. Unique insights into the genetic networks and signaling pathways involved in GnRH neuronal ontogeny and hormonal action have been derived from the study of the human disease model of congenital isolated GnRH deficiency (3). This genetic disease manifests as incomplete or absent sexual maturation and infertility, and it is clinically diagnosed as idiopathic hypogonadotropic hypogonadism [IHH (MIM 146110)] with either normal sense of smell (normosmic IHH, nIHH) or anosmia [Kallmann syndrome, KS (MIM 147950)] (4). Although the current knowledge of GnRH biology is incomplete, it is evident that multiple developmental and neuroendocrine pathways are involved in the ontogeny and homeostasis of GnRH neurons, the secretion of GnRH and its action on the pituitary. This complex regulation likely facilitates adaptation to diverse environmental pressures on reproduction to ensure survival and evolution of the species.

The chemokine prokineticin 2 (PROK2) and its cognate G protein-coupled receptor (PROKR2) comprise one of the major signaling systems involved in GnRH ontogeny. The mammalian prokineticin family has been implicated in diverse biologic processes, including neurogenesis, angiogenesis, carcinogenesis, circadian rhythm regulation, inflammation, immune system modulation and pain perception (5). In the mouse brain, *Prokr2* is the predominant prokineticin receptor, with high expression in the olfactory bulb and hypothalamus (6–8). During embryonic development, *Prok2*–*Prokr2* signaling is essential for neurogenesis in the olfactory bulb and for the migration of GnRH neurons from their developmental origin in the olfactory placode to the hypothalamus (8–10). *Prok2*^{−/−} and *Prokr2*^{−/−} mice display hypoplastic olfactory bulbs, disrupted GnRH neuronal migration and hypoplastic reproductive organs due to hypogonadotropic hypogonadism (9,10). Consistently, germline loss-of-function mutations in *PROK2* (MIM 607002) and *PROKR2* (MIM 607123) have been identified in patients with isolated GnRH deficiency (11–17).

Without hormonal treatments to induce puberty and confer reproductive capacity, the large majority of IHH patients are

infertile. Therefore, the causative mutations must have been subjected to strong purifying (i.e. negative) selection during evolution. In accordance with this notion, almost every mutation that has been shown to underlie GnRH deficiency is rare among patients, and many mutations are even unique to specific patients or families (17). However, a few mutations occur in strikingly high percentages of GnRH-deficient patients who harbor mutations in the respective gene, including *GNRHR* (MIM 138850) Q106R (44%), *GNRHR* R262Q (29%) (17–23) and *TACR3* (MIM 162332) W275X (36%) (24–26). In the prokineticin pathway, a commonly reported mutation *PROKR2* L173R (40%) has emerged (11–13,15–17). In addition to its implication in congenital isolated GnRH deficiency, the same mutation was also associated with hypothalamic amenorrhea (HA) (27), a common reproductive disorder in females characterized by a reversible form of GnRH deficiency triggered by stressors.

The causal role of *PROKR2* L173R in GnRH deficiency is strongly supported by the demonstration of dramatic reduction of the mutant receptor's activity in cell-based functional assays (12,28). Furthermore, homozygous patients phenocopy *Prokr2*^{−/−} mice by presenting with severe KS (10,11,16). Notably, the *PROKR2* L173R mutation has been identified in numerous patients with congenital GnRH deficiency from diverse geographic locations and ethnic backgrounds (11–13,15–17). This could be either because the mutation has arisen *de novo* multiple times during human evolution (i.e. it is a 'hot spot' mutation) or because it has been inherited from a common ancestor (i.e. it is a 'founder' mutation) despite being associated with infertility. To distinguish between these possibilities, this study tracked the genetic ancestry of *PROKR2* L173R; it also established the mutation's frequency among large cohorts of patients with congenital GnRH deficiency and unaffected controls from various geographic and ethnic populations, and determined the inheritance patterns of GnRH deficiency in mutation carriers. Having documented that it is a founder mutation, its age was estimated; and lastly, a plausible explanation for the persistence of the mutation in the human gene pool is offered.

RESULTS

***PROKR2* L173R is a founder mutation of ancient origin**

The *PROKR2* haplotype was evaluated in 23 probands (including 22 patients with GnRH deficiency and 1 proband with HA) carrying the *PROKR2* L173R mutation and 33 available family members by genotyping 5 flanking short tandem repeats (STRs) and 93 single-nucleotide polymorphisms (SNPs). In 13 probands (11 heterozygotes and 2 homozygotes), the haplotypes at the *PROKR2* locus could be

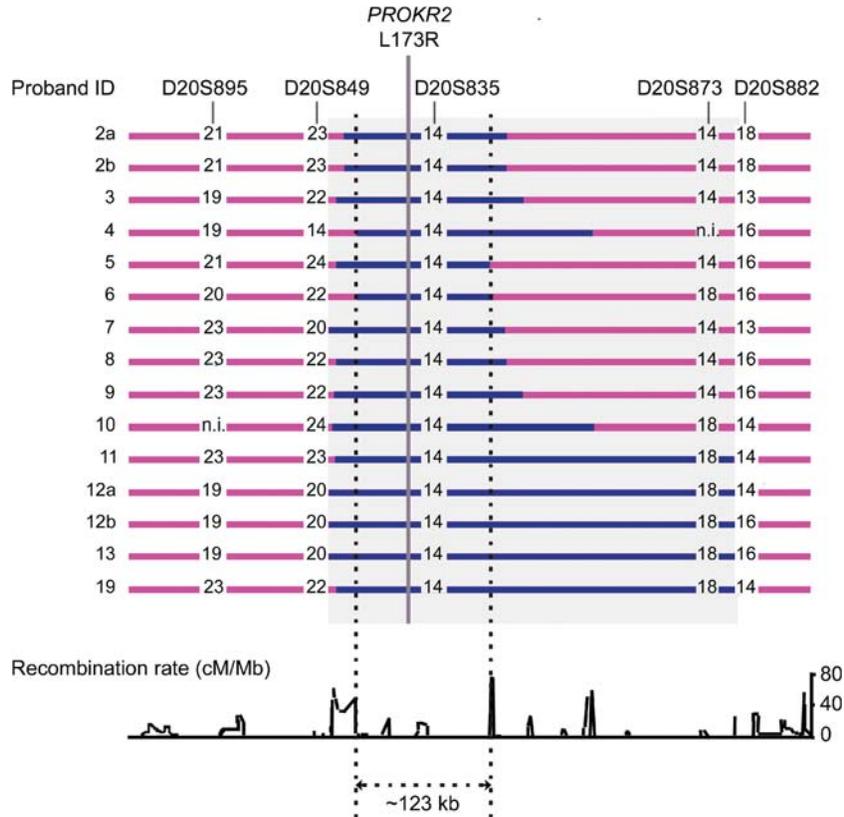


Figure 1. Haplotypes of informative probands. For each STR marker, the number of dinucleotide repeats is shown. n.i. (not informative): alleles that cannot be unambiguously attributed to the L173R haplotype. Gray rectangle: region genotyped via SNP array analysis. Pink horizontal lines: haplotypes. Blue horizontal lines: haplotype regions identical to each other. In each of the homozygous probands (#2 and #12), the two haplotypes are labeled 'a' and 'b'. The haplotype shared by all probands is marked with vertical dotted lines and is delimited by two major recombination hot spots on chromosome 20 (genomic region: NCBI build 36.1; recombination rates: HapMap, CEU population, public release #27).

unambiguously determined (Supplementary Material, Table S1). In all of these probands, *PROKR2* L173R resided on the same core haplotype which has a size of ~ 123 kb (Fig. 1). In the remaining 10 probands, the haplotypes at the *PROKR2* locus could not be unambiguously determined; nevertheless, the presence of *PROKR2* L173R on the same haplotype was plausible (Supplementary Material, Table S1). This shared haplotype does not extend beyond major recombination hot spots flanking *PROKR2* and does not include adjacent genes, making their contribution to this disease highly unlikely. According to HapMap Project data, the frequency of the shared haplotype is 6.7% in Europeans (Utah residents with northern and western European ancestry), $< 1\%$ in Africans (Yoruba from Ibadan, Nigeria) and 2.2% in East Asians (Han Chinese from Beijing and Japanese from Tokyo) (29). Thus, on the assumption that the frequency of the haplotype in the general population is not higher than 10%, the probability that *PROKR2* L173R is a hot spot mutation that arose *de novo* 15 independent times (including 11 possibly independent events in 11 heterozygotes and 4 in 2 homozygotes) on the same haplotype is $\leq 10^{-15}$. Therefore, *PROKR2* L173R in all likelihood represents a founder mutation. The small size of the shared haplotype and the evidence of multiple recombination events on each flank (Fig. 1) indicate that the genotyped families are branches of a very ancient genealogy. Based upon the size of the shared haplotype and the shape of the genetic tree,

the age of the *PROKR2* L173R mutation is estimated by an algorithm (30) to be approximately 300 generations (approximately 9000 years) with a 95% confidence interval of approximately 150–400 generations. Because nearly all branches of the genetic tree have distinct ends, a biased result from a putative recombination hot spot can be ruled out, and the estimation of the mutation's age is robust. The mutation thus likely arose during the neolithic expansion of the human population that happened approximately 5500–12 000 years ago.

HA-associated *PROKR2* L173R shares the same ancient haplotype

The *PROKR2* locus haplotype of the Caucasian proband (#6) with HA, a milder form of GnRH deficiency, and three of her first-degree relatives were studied. The mutation was found to reside on the same core haplotype as in the probands with congenital GnRH deficiency (Fig. 1). The patient's affected son with delayed puberty and her asymptomatic daughter had both inherited the disease haplotype (Fig. 2).

PROKR2 L173R is one of the most common mutations in congenital GnRH deficiency

As an indicator of the strength of evolutionary selective pressure on an ancient founder mutation, the frequency of the

Table 2. Demographic and phenotypic characteristics of GnRH-deficient patients carrying the *PROKR2* L173R mutation

Subject (Ref.)	Sex	Ethnicity (origin)	Dx	Inheritance	Puberty	C/M	MRI	Additional phenotypes	<i>PROKR2</i> and other gene defects ^a
1 (16)	M	Maghrebian (Morocco)	KS	Familial	Absent	-/-	Hypoplastic OB	/	L173R/WT
2 (16)	M	Maghrebian (Morocco)	KS	Sporadic	Absent	+/+	Aplastic OB	↑BW	L173R/L173R
3 (17)	F	Caucasian (Lebanon)	nIHH	Familial	Partial	/	NA	Aortic insufficiency	L173R/WT
4 (17)	F	Caucasian (Greece)	KS	Sporadic	Partial	/	Aplastic OB, partial empty sella	High arched palate, scoliosis	(M64V + L173R)/WT
5 (36)	M	Caucasian (Spain)	KS	Sporadic	Absent	+/-	Aplastic L OB, hypoplastic R OB	High arched palate, pes planus	L173R/WT der(1), t(1;10)(q44;q26) translocation ^b
6 (27)	F	Caucasian (UK)	HA	Familial	Normal	/	NA	Eating disorder	L173R/WT
7 (12)	M	Caucasian (UK)	KS	Sporadic	Absent	+/-	NA	Two supernumerary teeth	L173R/WT
8 (17)	M	Caucasian (UK)	KS	Sporadic	Absent	+/+	NA	Synkinesia, hypoplastic R ear, delayed motor development, delayed permanent tooth eruption	L173R/WT
9 (11,16)	M	Caucasian (Europe)	KS	Familial	Absent	-/-	Aplastic OB	High arched palate, psychomotor problems, ↓BW	L173R/WT
10 (11,16)	M	Caucasian (Europe)	KS	Familial	Absent	+/+	NA	/	L173R/Q210R
11 (34)	F	Caucasian (Europe)	KS	Familial	Absent	/	Aplastic R OB, hypoplastic L OB & sulcus	Diastema, cleft uvula, scoliosis, learning disabilities	L173R/WT <i>FGFR1</i> C55fsX45/WT
12 (11,16)	M	Caucasian (Europe)	KS	Sporadic	Absent	+/+	Aplastic OB	/	L173R/L173R
13 (12)	M	Ashkenazi Jewish (Poland)	KS	Sporadic	Absent	-/-	Hypoplastic R olfactory sulcus, atrophic infundibulum	Synkinesia, pectus excavatum, pes planus, ↑BW	L173R/WT
14 (16)	M	Caucasian (Europe)	KS	Familial	Absent	+/-	Hypoplastic OB	Brachidactyly (hands), polydactyly (R foot)	L173R/WT
15	F	Caucasian (Europe)	nIHH	Sporadic	Absent	/	Partial empty sella	/	L173R/WT
16 (11,16)	M	Caucasian (Europe)	KS	Sporadic	Absent	-/+	NA	↑BW	L173R/WT
17 (11,16)	M	Caucasian (Europe)	KS	Sporadic	Absent	-/-	NA	Severe depression	L173R/WT
18 (16)	M	Caucasian (Europe)	KS	Sporadic	Absent	-/+	Normal OB	↓BW	L173R/WT
19 (17)	M	Caucasian (French Canada)	KS	Sporadic	Absent	-/-	Hypoplastic R OB, pituitary hypoplasia	Night blindness, ↑BW	L173R/WT
20 (13)	F	Brazilian (Brazil)	KS	Sporadic	Absent	/	Hypoplastic OB and sulci	↑BW	L173R/WT
21 (13)	M	Brazilian (Brazil)	KS	Sporadic	Absent	-/-	Aplastic OB and sulci	↑BW	L173R/WT
22	M	Brazilian (Brazil)	AHH	Sporadic	Normal	-/-	Normal OB	/	L173R/WT
23	M	Brazilian (Brazil)	nIHH	Sporadic	NA	-/-	NA	/	L173R/WT

KS, Kallmann syndrome; nIHH, normosmic hypogonadotropic hypogonadism; HA, hypothalamic amenorrhea; AHH, adult onset GnRH deficiency; C, cryptorchidism; M, micropallus; +, present; -, absent; OB, olfactory bulb; R, right; L, left; NA, not assessed; BW, body weight; WT, wild type. The origin describes the ethnic background of the parents.

^a*KALI*, *GNRHR*, *GNRH1*, *FGFR1*, *FGF8*, *PROK2*, *KISS1R*, *TAC3* and *TACR3* were sequenced in all probands except #5, #8 and #19 (all genes except *TAC3*, *TACR3* and *GNRH1*), #6 and #7 (all genes except *TAC3*, *TACR3*, *GNRH1* and *GNRHR*), #13 (all genes except *TAC3* and *TACR3*), #20 (only *FGFR1*), #21 (only *KALI* and *FGFR1*), #22 (no additional genes), #23 (only *KALI*, *FGFR1* and *GPR54*).

^bA *de novo* chromosomal rearrangement (36) whose implication for GnRH deficiency or prokineticin signaling is unclear.

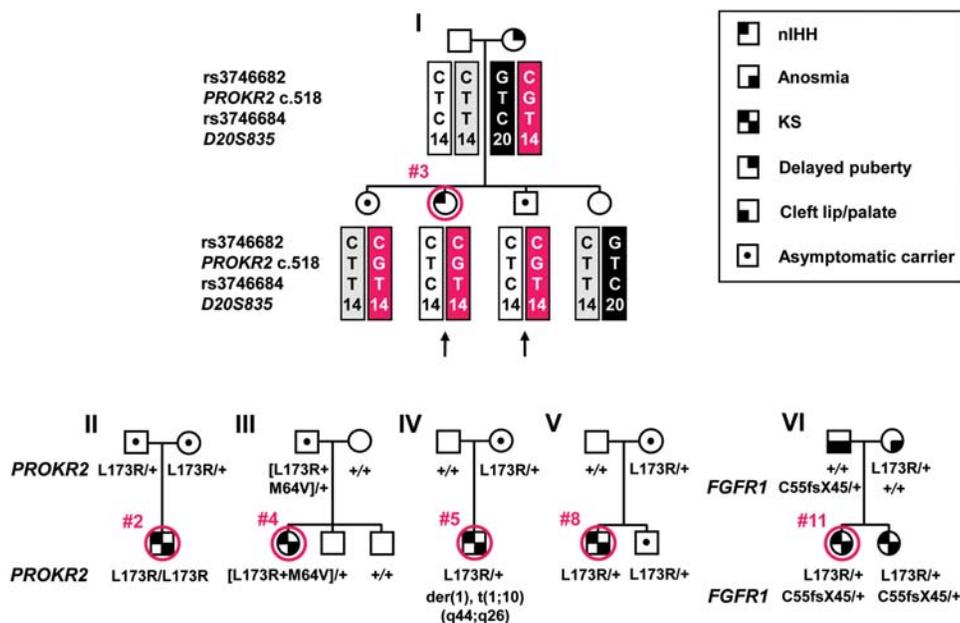


Figure 3. Informative previously unpublished pedigrees. Red circles: probands. Red numbers: probands' IDs. Haplotypes in Pedigree #3 are presented with representative polymorphisms and the *PROKR2* L173R mutation (c.518T>G). The mutation-bearing L173R haplotype is boxed red. Black arrows indicate two siblings that have the same paternal and maternal *PROKR2* haplotypes, yet are discordant for the disease. SNPs and the mutation are stated according to the cDNA sequence, which is reverse and complementary to the genomic.

at the same locus on the non-L173R chromosome and arguing against a recessive mode of disease inheritance due to compound heterozygosity.

Thus far, four cases of digenic inheritance of isolated GnRH deficiency associated with *PROKR2* L173R mutations have been reported, three in combination with *KAL1* mutations (15,16) and one with an *FGFR1* mutation (34), thus demonstrating that a variety of GnRH deficiency associated loci may influence the penetrance of L173R-associated congenital GnRH deficiency. An additional novel *PROKR2* mutation (M64V) was identified on the same L173R haplotype in proband #4, and a *de novo* chromosomal translocation was found in proband #5 (36); the functional significance of these two alterations is presently unknown. Most probands harboring the heterozygous *PROKR2* L173R mutation exhibit no additional mutations in either *PROKR2* or other known disease genes. This complex inheritance of GnRH deficiency associated with *PROKR2* L173R suggests a role of yet unknown genetic and/or environmental factors in the pathogenesis and clinical manifestation of the disease.

DISCUSSION

The paradox: a mutation that hinders puberty and reproduction is inherited over thousands of years

Whereas detrimental mutations are progressively eliminated from the human gene pool (37), founder mutations persist and are transmitted to offspring. Because variation in reproductive success is the fundament of natural selection, the existence of founder mutations that compromise pathways involved in the neuroendocrine control of reproduction is at first glance counterintuitive. Nevertheless, such founder

mutations have been recently identified in *TAC3* (c.209-1G>C) and *GNRHI* (c.18-19insA) (25,38). Both of these mutations are exceedingly rare among GnRH-deficient patients (only three probands and one sibling pair, respectively); have been identified only in specific ethnic groups (Africans from Congo and Haiti; and Romanians, respectively); and have occurred relatively recently (approximately 600 and 240–1500 years ago, respectively) (25,38). In contrast, *PROKR2* L173R is one of the most commonly identified causative mutations among patients with congenital GnRH deficiency (found in 2.4%) (11–13,15–17) (Table 1); has been found in patients from diverse ethnic and geographic origins (11–13,15,16) (Table 1); and is the oldest known mutation associated with isolated GnRH deficiency with an estimated age of approximately 9000 years. Moreover, *PROKR2* L173R is one of the oldest founder mutations impairing reproductive fitness in general, with an age comparable with *CFTR* (MIM 602421) F508del, which causes cystic fibrosis (MIM 219700) and arose approximately 10 000 years ago (39). This highly paradoxical identification of *PROKR2* L173R as an ancient founder mutation has intriguing implications for the relevant processes of evolutionary selection and for the mechanisms of inheritance of *PROKR2* L173R-associated GnRH deficiency.

The enigma: how can a founder mutation that impairs the neuroendocrine control of reproduction survive natural selection?

The persistence of founder mutations in the human gene pool despite their deleterious effects in the homozygous state is considered to be due to 'overdominant' selection of heterozygous carriers over homozygous carriers and non-carriers. For

Table 3. Potential selective advantages of *PROKR2* L173R heterozygosity according to the non-reproductive functions of prokineticin signaling in model organisms

Function of Prok2/Prokr2 signaling	Ref.	Potential heterozygote advantage
Pro-inflammatory signaling that promotes tumor angiogenesis	(59,60)	Decreased cancer risk
Modulation of immunity by induction of granulocyte and monocyte proliferation, chemotaxis, differentiation, survival and activation	(61)	Resistance to infection and/or autoimmunity
Regulation of anxiety and depression-related behaviors	(62)	Improved stress-coping abilities
Hypothalamic regulation of appetite and energy expenditure	(43,44)	Adaptation to caloric restriction during famine

example, *CFTR* F508del heterozygotes may be relatively protected compared with non-carriers against typhoid fever (40) and cholera (41). Interestingly, *PROKR2* L173R has a much lower carrier rate among unaffected subjects (0.16%) compared with other ancient founder mutations, such as *CFTR* F508del [cystic fibrosis; 4% in northern Europe (39)] or *PAH* R408W (MIM 612349) [phenylketonuria (MIM 261600); neolithic era; 1% in Ireland (42)]. The carrier rate of a founder mutation depends on its geographic and temporal origin, migration patterns, population bottlenecks, genetic drift and particularly the strength of positive selective pressure. Whether *PROKR2* L173R heterozygotes have a selective advantage (when not manifesting GnRH deficiency-associated infertility) is presently unknown. Nevertheless, since prokineticins have been associated with several physiologic processes in addition to neurogenesis and reproduction in animal models, it is tempting to speculate that *PROKR2* heterozygotes have a selective advantage related to one or more of those functions (Table 3). However, evidence supporting such functions of prokineticins in humans is currently lacking. For example, whereas Prok2/Prokr2 signaling was shown to be involved in the regulation of energy balance in mice (43,44), obesity was equally prevalent in GnRH-deficient patients with or without *PROKR2* mutations (16).

On the other hand, we recently reported *PROKR2* L173R mutation in a proband with HA (27), a reversible form of GnRH deficiency triggered by stressors, including energy deficits. We show here that this patient carries the founder mutation. Thus, the persistence of an allele that impairs reproduction may in fact relate to its value in suppressing fertility under certain conditions. During negative environmental circumstances of malnutrition such as famine, excessive energy expenditures during migration and social stress during high predator density, being genetically predisposed to postpone the energetically costly process of reproduction could have been advantageous to both the female and her future offspring. Thus, the *PROKR2* L173R mutation may have conferred protection on female carriers by setting a lower threshold for the inhibition of the reproductive neuroendocrine axis during periods when pregnancy would drain the bearer's nutritional and/or energy resources (27). The presented hypothesis on survival value of the mutation is

currently lacking confirmation data as it targets genetic influences on survival in environmental conditions of ancient past. Identification of the same mutation in particular population subgroups with known history of migration would permit further study of the hypothesis. Other mean of testing our speculation would be by using 'knock-in' experiments in animal models exposed to environmental pressures, with the limitation of interspecies variation.

An exegesis: congenital GnRH deficiency associated with *PROKR2* L173R is inherited in an oligogenic manner

The disease phenotype associated with *TAC3* c.209-1G>C, *GNRHI* c.18-19insA and most other known founder mutations impairing reproduction, such as *CFTR* F508del, is inherited as a recessive trait (24,25,38,45,46). In contrast, congenital GnRH deficiency associated with *PROKR2* L173R does not appear to be inherited in a recessive fashion, as *PROKR2* L173R heterozygotes are likely to be GnRH-deficient and thus infertile (Table 1). Recent studies have shown that inheritance of GnRH deficiency associated with *PROKR2* L173R and other *PROKR2* mutations is complex and does not consistently conform to a monogenic pattern of inheritance (16,17). Whereas biallelic *PROKR2* mutations show complete penetrance (16), most of the patients in this cohort have monoallelic mutations which display incomplete penetrance (Fig. 3). Additional mutations in other genes associated with isolated GnRH deficiency, such as *PROK2*, *KAL1* and *FGFR1*, have been documented to coexist in several patients with *PROKR2* mutations (including L173R) (11,12,15–17,34). These findings indicate that GnRH deficiency associated with *PROKR2* L173R is inherited in an oligogenic manner and thus suggest it is unlikely that heterozygosity for the L173R mutation alone causes the full syndrome of severe GnRH deficiency and infertility. Rather, it appears that the spectrum of reproductive phenotypes associated with *PROKR2* L173R, ranging from the common and reversible HA to the rare and severe GnRH deficiency, results from the interaction of the mutation with either environmental (HA) or additional, yet to be identified, genetic defects.

In conclusion, *PROKR2* L173R is an ancient founder mutation that was not eliminated during evolution despite being associated with GnRH deficiency and failure of reproductive competency. Future studies should elucidate the factors involved in the selection process of the mutation which may include those affecting the penetrance of GnRH deficiency in heterozygotes, such as oligogenicity (16,17), the predisposition of carriers to become reproductively quiescent where such conservation could assist their survival (HA), and/or potential non-reproductive roles of the prokineticin system.

MATERIALS AND METHODS

Subjects

Haplotype mapping was performed in 22 unrelated probands with congenital GnRH deficiency carrying the *PROKR2* L173R mutation and 30 first-degree family members who were available from 12 pedigrees. The carrier rate and MAF of *PROKR2* L173R in GnRH-deficient patients and ethnically matched unaffected

volunteers with normal puberty and reproductive function were evaluated in a total of 1299 GnRH-deficient patients and 1214 unaffected controls: 1048 patients and 729 controls from six published studies (11–13,15–17) and an additional 251 patients and 485 controls genotyped in this study. In addition, haplotype mapping was performed in one patient with HA carrying the *PROKR2* L173R (27) and her three first-degree family members. The ethics committees of participating institutions approved the study and informed consent was obtained from all subjects before enrollment.

Subject phenotyping

IHH was diagnosed according to standard criteria: (i) serum testosterone (T) ≤ 100 ng/dl in men or estradiol (E2) ≤ 20 pg/ml in women, with low or normal serum gonadotropins; (ii) otherwise normal anterior pituitary function; and (iii) normal serum ferritin. A detailed individual and family history included pubertal development and associated reproductive and non-reproductive phenotypes, i.e. delayed puberty, anosmia, cryptorchidism, microphallus, synkinesia, obesity, hearing loss, ocular disorders, skeletal and renal malformations, and midline defects (cleft lip or palate and bifid uvula). A complete physical examination included Tanner staging and testicular volume measurement using a Prader orchidometer. The degree of pubertal development was based on clinical history and/or testicular size (47). Olfactory acuity was assessed by clinical history and, when possible, by olfactory smell testing (48). Imaging studies included renal ultrasound and MRI of the hypothalamo-pituitary region and olfactory bulbs.

The proband with HA has been described in detail in a prior report (27).

Gene sequencing

Genetic analysis of 10 genes underlying GnRH deficiency was performed using previously described gene sequencing methods. Exons and intron–exon junctions were analyzed for alterations in *PROKR2* (11) (in 23 *PROKR2* L173R carrying probands, additional 247 GnRH-deficient probands and 185 controls) and other genes underlying GnRH deficiency: *KALI* (49) (MIM 308700), *GNRHR* (18), *GNRH1* (38) (MIM 152760), *FGFR1* (50) (MIM 136350), *FGF8* (51) (MIM 600483), *PROK2* (9,11), *KISS1R* (52) (MIM 604161), *TACR3* and *TAC3* (24) (MIM 162330)—all genes were sequenced in 13 probands; in 9 probands a subset of genes was sequenced and in 1 proband no additional gene besides *PROKR2* was analyzed. All sequence variations were identified on both strands, and mutations were confirmed in independent PCRs. Nucleotide and amino acid variations are described using standard nomenclature (53). To determine the familial segregation of identified mutations and delineate genotype–phenotype correlations, first-degree relatives available in the pedigrees of 13 out of 23 haplotyped probands were genotyped; 4 of these pedigrees were previously reported (11,27,34).

Haplotype mapping

Two sets of polymorphic markers were genotyped in each proband and family member: STRs and SNPs. (i) Five STR markers flanking *PROKR2* were genotyped (Supplementary Material, Table S1): *D20S849* and *D20S895* are, respectively, 89 and 196 kb telomeric to the L173R mutation, whereas *D20S835*, *D20S873* and *D20S882* are, respectively, 32, 313 and 351 kb centromeric. PCR amplification conditions and primer sequences used for STR marker amplification are available upon request at the corresponding author. Forward primers were labeled with fluorescein amidite. PCR product sizes were analyzed at the MGH DNA Core Lab using capillary electrophoresis on an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA), and amplicon sizes were determined using the ABI GeneMapper 4.0 (Applied Biosystems). (ii) Three exonic SNPs were genotyped by sequencing (11). In addition, 95 tag SNPs in the region flanking *PROKR2* between STRs *D20S849* and *D20S882* (Supplementary Material, Table S1) were selected from the HapMap CEU reference panel using the *Tagger* function in Haploview 4.1 (<http://www.broadinstitute.org/haploview/haploview>; Broad Institute, Cambridge, MA, USA) (29). Tag SNP selection algorithm ‘pairwise tagging’ (54) was used to capture SNPs of an MAF > 0.001 with a correlation coefficient (r^2) higher than 0.6. SNP genotyping was performed in multiplex by The Broad Institute Center for Genotyping and Analysis, using Sequenom MassARRAY iPLEX Gold (Sequenom, San Diego, CA, USA) (55). A bead-less and label-free primer-extension chemistry was used with a high-fidelity polymerase to generate allele-specific products whose distinct masses were detected by MALDI-TOF MS (56). The genotypes of the multiplexed SNPs were differentiated using extension primers with unique masses. Two SNPs had extremely low call rate and were therefore removed from further evaluation (rs7267662 and rs11699382). Five HapMap DNA samples were used as controls for the accuracy of genotyping. The haplotypes of the 13 probands with available first-degree relatives were determined from the genotypes of the SNPs and STR markers by considering the parental genotypes. Comparing these haplotypes, the presence of a shared haplotype was established and its size determined. The frequency of the shared haplotype in the CEU population of the HapMap Project (<http://hapmap.ncbi.nlm.nih.gov/>) was obtained using Haploview 4.1 (29).

Nature and chronology of *PROKR2* L173R

The probability that *PROKR2* L173R is a hot spot mutation that arose *de novo* multiple independent times on the same haplotype was calculated using the binomial probability formula. The age of the mutation was estimated from 13 informative probands’ mutation-carrying haplotypes by a previously described algorithm based on haplotype-decay models (30). The rationale is that the age of the mutation is inversely proportional to the size of the shared haplotype, because meiotic recombination over consecutive generations degrades the ancestral haplotype on which the mutation initially occurred (57). To decipher the history of mutation ancestry,

the shape of the genetic tree was considered, taking into account the density, length and position of branches on both sides of the mutation. The positions of branches in a mutation genetic tree are a reflection of both mutation-specific natural selection and background population history. Because various genetic trees could be behind the haplotypes obtained, specific algebraic expressions associated with any possible genealogical framework were constructed. Haplotype decay in any branch or stem is a power function or a compound of power functions, an example for a one-node five-branch tree being the following (n stands for the number of generations; s stands for the genetic interval):

$$2n^4(1 - s_1)^{n-1}(1 - s_2)^{n-1}(1 - s_3)^{n-1}(1 - s_4)^{2n-1}.$$

For a two-node three-branch tree, the two complementary paths of construction are represented by the alternative equations, where n is the number of generations for the root node and u the number of generations for the branch node:

$$2nu(1 - s_1)^{u-1}(1 - s_2)^{2n-1},$$

$$2u(2n - u)(1 - s_1)^{2n-u-1}(1 - s_2)^{2u-1}.$$

The method is recursive and the possible construction paths reflect the hierarchical organization of genealogical trees, because there are always several possible ways to break down a complex genealogy into subtrees.

For the estimation of age, an intergenerational time interval of 30 years was used (58).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We are grateful to all patients and families for their kind participation.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the National Institutes of Health (U54 HD028138 and R01 HD015788 to W.F.C., R01 HD056264 to N.P., P01 GM061354 to J.F.G.); the Agence Nationale de la Recherche (ANR-09-GENO-017 to C.D.); and the Slovene National Research Agency (P3-0343 to M.A.S.).

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