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CLINICAL CASE SEMINAR

Two Novel Missense Mutations in G Protein-Coupled Receptor 54 in a Patient with Hypogonadotropic Hypogonadism

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It has recently been shown that loss-of-function mutations of the G protein-coupled receptor (GPR)54 lead to isolated hypogonadotropic hypogonadism (IHH) in mice and humans. Such mutations are thought to be rare, even within the clinical IHH population, and only a handful of alleles have been described, making further screening of IHH populations imperative. We examined the genes encoding GPR54 and its putative endogenous ligand, kisspeptin-1, for mutations in a cohort of 30 patients with normosmic HH or delayed puberty. One subject with HH, of mixed Turkish-Cypriot and Afro-Caribbean ancestry, was found to be a compound heterozygote for two previously undescribed missense mutations in GPR54: cysteine 223 to arginine (C223R) in the fifth transmembrane helix and arginine 297 to leucine (R297L) in the third extracellular loop. Assessed *in vitro* using a previously described sensitive signaling assay in cells stably expressing GPR54, the C223R variant was found to exhibit profoundly impaired signaling, whereas the R297L variant showed a mild reduction in ligand-stimulated activity across the ligand dose range. These novel mutations provide further evidence that human HH may be caused by loss-of-function mutations in GPR54. (*J Clin Endocrinol Metab* 90: 1849–1855, 2005)

HUMAN GONADAL FUNCTION is under the control of the anterior pituitary hormones LH and FSH, the release of which is, in turn, regulated by pulsatile secretion of hypothalamic GnRH. In the first few weeks after birth, this axis is functional, but by around 6 months of age, it becomes quiescent, thus entering the juvenile pause, which persists until puberty. The first detectable event at the initiation of puberty is an upsurge in the amplitude of GnRH pulses, leading to overnight LH pulsatility and subsequent maturation of the rest of the axis at puberty. The mechanisms of the childhood inhibition of GnRH release, and its eventual reactivation, are poorly understood but appear to include peripheral feedback suppression by sex steroids and the actions of ill-defined intrinsic central nervous system pathways (1).

Genetic defects in the hypothalamo-pituitary-gonadal axis are manifested clinically as hypogonadotropic hypogonadism (HH), with either primary failure to undergo puberty or severe delay in the process. Defects may broadly be categorized into those affecting embryonic migration of GnRH-secreting neurones, which lead to anosmia as well as HH (*e.g.* Kallmann syndrome due to *KAL*, or *FGFR1* mutations), and those affecting the pulsatile release or function of GnRH from neurones that have migrated to their normal position. Patients suffering from the latter are normosmic. Around 40% of patients with autosomal recessive normosmic familial HH in one series were found to have mutations in the GnRH receptor gene, whereas only around 10–17% of sporadic cases are thought to harbor such mutations in *GNRHR* (2, 3).

In the last year, the G protein-coupled receptor (GPR)54 has been shown to be a gatekeeper gene for activation of the GnRH axis based on loss-of-function mutations in mice and humans. In humans, loss-of-function mutations in GPR54 have been described in two consanguineous pedigrees and two isolated subjects with HH (4, 5) (OMIM 604161). In tandem, mice bearing homozygous targeted disruptions of the *Gpr54* gene have been found to exhibit a similar failure of sexual maturation (5). This shows that GPR54 is required for the normal function of this axis and suggests that the ligand, kisspeptin-1, may act as a novel neurohormonal regulator of the GnRH axis. The latter has recently received support from studies showing that peripheral and central administration of kisspeptin provokes GnRH release (6–8).

We now describe the genetic screening of the *GPR54* gene as well as the *KISS1* gene encoding its putative endogenous

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Abbreviations: CDP, Constitutional delay of puberty; GPR, G protein-coupled receptor; hCG, human chorionic gonadotropin; HH, hypogonadotropic hypogonadism; SNP, single nucleotide polymorphism.

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ligand, kisspeptin-1, in a cohort of 30 patients with normosmic HH, and report the clinical phenotype of a nonconsanguineous subject who shows compound heterozygosity for two novel missense mutations in the *GPR54* gene. We have also documented the prevalence of single nucleotide polymorphisms (SNPs) in the *GPR54* and *KISS1* genes, one previously undescribed, in both the study population and a Caucasian control population.

Subjects and Methods

Subjects

Subjects for genetic screening were recruited from the clinics at Great Ormond Street Hospital (London, UK) and Addenbrooke's Hospital (Cambridge, UK). Informed consent was obtained according to procedures approved by the local research ethics committee of each institution. Patient characteristics are outlined in Table 1. Isolated HH was defined as inappropriately low gonadotropin levels together with prepubertal concentrations of sex steroids. It was deemed idiopathic in which no family history of the condition was apparent, and congenital in which undescended testes with or without small penis were noted at birth. Constitutional delay of puberty (CDP) was defined as the absence of signs of puberty at 14 yr of age in boys, this being described as severe if signs were still not apparent at 15 yr. All patients were reportedly normosmic, had otherwise normal anterior pituitary function and no midline facial defects, and showed no evidence of structural brain pathology, where assessed. In all but three cases, the coding sequence of the *GnRHR* gene had been sequenced and found to be normal.

The Caucasian control population used was derived from the Medical Research Council Ely Study cohort, recruited from a population sampling frame with a high response rate (74%), making it representative of an ethnically homogeneous Caucasian population in this area of Eastern England (9, 10). In addition, 50 Afro-Caribbean control subjects were also selected from an independently assembled cohort used in a study of the genetics of obesity. This group was entirely unselected with respect to reproductive and gonadal function, as was a group of 45 Turkish control subjects recruited as part of studies into obesity and renal tubular disease.

Genetic screening

Genomic DNA was isolated from whole blood using a QIAamp blood kit (Qiagen, Crawley, UK), and amplified using a GenomiPhi DNA amplification kit (Amersham Biosciences, Chalfont, UK). PCR was performed using BioTaq (Bioline, London, UK) and carried out as recommended by the manufacturer, with addition of dimethylsulfoxide where indicated. Thirty-five cycles (60 sec at 95 C, 60 sec at the annealing temperature, and 60 sec at 72 C) were performed using a PTC-225 Peltier thermal cycler (MJ Research, Watertown, MA). PCR products were verified electrophoretically and sequenced using ABI BigDye Terminator (version 3.1) reagents with electrophoresis on an ABI Prism 3100-Avant genetic analyzer (PE Applied Biosystems, Foster City, CA). Subsequent sequence analysis was performed using Sequencher software (Gene Codes, Ann Arbor, MI). Primers, annealing temperatures, and concentrations of $MgCl_2$ and dimethylsulfoxide used for PCR are detailed in Table 2, together with sequencing primers where different.

TABLE 1. Description of cohort studied

Diagnosis	Sex	Ethnicity
Idiopathic IHH	11 Males[a] 3 Females	12 Caucasian 1 Turkish 1 Indian 1 Turkish/Jamaican
Familial IHH	1 Male 2 Females	1 Caucasian 1 Sri Lankan 1 Sudanese
Familial IHH or CDP	2 Males 0 Females	2 Caucasian
Familial CDP	8 Males 0 Females	8 Caucasian
Idiopathic severe CDP	3 Males 0 Females	3 Caucasian

[a] Includes proband described.

IHH, Isolated HH; Familial IHH or CDP, families where one sibling has HH and another has CDP within the same generation.

To distinguish compound heterozygosity for the two mutations described from heterozygosity for a double mutation, the *GPR54* exon 4 forward primer and exon 5 reverse primer (Table 2) were used to amplify exons 4 and 5 and the intervening intron using reaction conditions as for exon 5. The PCR product was then cloned into a pGEM-T Easy Vector (Promega, Southampton, UK), and 16 separate clones were sequenced.

Creation of GPR54 mutant alleles and in vitro functional assays

Wild-type human GPR54 was amplified from human brain cDNA (Origene Technologies, Rockville, MD) using the GC-Rich PCR kit (Roche Diagnostics, Lewes, UK). The 5′ primer TAATCAAAGCTTGCGATGCACACCGTGGCTACGTC and 3′ primer ACATTAGGATCCTCACTTATCGTCGTCATCCTTGTAATCGAGAGGGGCGTTGTCCTCC (incorporating a 3′ sequence encoding the FLAG epitope tag) were used. The resulting product was cloned into pCRBlunt (Invitrogen, Renfrew, UK) and sequence verified. Stable cell lines expressing the wild-type GPR54-FLAG or one of the two mutant species described were then created using the Flp-In system (Invitrogen). In brief, the GPR54-FLAG was subcloned into pcDNA5/FRT and the point mutations were introduced using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All the resulting constructs were verified by direct sequencing. The Flp-In-293 cell line (Invitrogen) was maintained in DMEM supplemented with 10% Fetal Clone III (Hyclone, Cramlington, UK) and 2 mM L-glutamine. To create stable GPR54-expressing cell lines, Flp-In-293 cells were cotransfected with the pcDNA5/FRT-GPR54-FLAG constructs and the Flp recombinase-expressing plasmid pOG44 using polyfect (Qiagen), with subsequent selection by hygromycin B. Transcription of the inserted alleles was confirmed by RT-PCR (data not shown).

To assess signal transduction by the wild-type and mutant receptors, a sensitive calcium mobilization signaling assay was used as previously described (11–13), with calcium flux in response to kisspeptin-1 112–121 (Phoenix Pharmaceuticals, Belmont, CA) measured on a FlexStation using a FLIPR calcium assay kit (both from Molecular Devices, Sunnyvale, CA) in response to Kisspeptin-1 112–121 (Phoenix Pharmaceuticals). Data were analyzed using SoftMax Pro software (Molecular Devices). Experiments were performed in duplicate on 2 consecutive days, and SDs are indicated.

Results

Genetic screening

Sequencing of the coding regions of the *GPR54* (GenBank accession no. AY253981) and *KISS1* (GenBank accession no. AY117143) genes in all 30 patients revealed the presence of two previously described SNPs in the *GPR54* gene, and three previously described SNPs, one novel SNP, and one previously described insertional polymorphism in the *KISS1* gene. The details of these common variants, and their frequency in both the study population and 180 Caucasian control alleles are shown in Table 3.

One boy with isolated HH was found to harbor two rare sequence variants in the GPR54 gene (Fig. 1): the first, a transversion c.667T>C in exon 4, results in substitution of a cysteine near the cytoplasmic end of the fifth transmembrane α-helix for arginine (C223R), whereas the second, a transversion c.891G>T in exon 5, leads to the substitution of an arginine in the third extracellular loop for leucine (R297L). Cloning a section of the GPR54 gene encompassing exons 4

TABLE 2. Primers and reaction conditions used for sequencing

Gene	Exon	Primers (5′–3′)	$[Mg^{2+}]$/mM	%DMSO	T_m
GPR54	1	F: TTC CTG AGT TCC ACA GGC GCA R[a]: AGG TTT CCA TGT GCC ACA CTC seq: AGG GAA AAG ATT CGA CGT TGG	1.5		66
	2	F[a]: GCC CAG CGC CCG CGC ATC R: GTC CCC AAG TGC GCC CTC TC	1.0	5	70
	3	F: CAG GCT CCC AAC CGC GCA G R[a]: CGT GTC CGC CTT CTC CCG TG	1.0	8	64
	4	F[a]: TGG AAA ATG GGC GCA ATA GCT R: TGA AGG TGG TTA GAC GAA AGG	1.5		66
	5	F[a]: GCC TTT CGT CTA ACC ACC TTC R[a]: GGA GCC GCT CGG ATT CCC AC seq1: TTA AGA CCT GGG CTC ACT GC seq2: AGC ACC AGG AAC AGC TGG ATG	1.5	5	66
KISS1	2	F[a]: TCT TGG AGG ACT GTC CCT TTT G R[a]: TTG CAA CAA CCC ACT TGC TCC C	1.5		66
	3	F[a]: ATG GGA TGA CAG GAG GTG TTG R: ACC ATC CAT TGA GGA TGG AAG seq: AGG AGT TCC AGT TGT AGT TCG	1.5		66

T_m, Annealing temperature (Centigrade); seq, sequencing primer only; DMSO, dimethyl sulfoxide.

[a] PCR primers also used for sequencing.

and 5 and the intervening intron from the genomic DNA of the proband established that he was a compound heterozygote for these two changes. The cysteine at position 223 is highly conserved in mouse, rat, zebrafish, and pufferfish, whereas R297 is conserved in mouse and rat but not in zebrafish or pufferfish. Neither variant was detected in 180 Caucasian control chromosomes. The proband's mother and father were of Turkish-Cypriot and Jamaican descent, respectively. Only his mother and younger brother were available for further study, and both were found to be heterozygous for the R297L variant but homozygous wild-type with respect to C223. The C223R variant was thus most likely inherited from his father, although *de novo* mutation cannot be ruled out. Screening of 100 control Afro-Caribbean alleles failed to reveal any further C223R changes, whereas R297L was not detected on screening 90 control Turkish alleles.

Case history

The proband (46, XY, birth weight 2.98 kg) was born at term after an uneventful pregnancy. He was noted to have a micropenis and undescended testes at birth, and serum gonadotropins were undetectable at 2 months of age (LH < 0.5 mIU/ml, FSH < 0.5 mIU/ml). His penis grew reasonably in response to three injections of depot testosterone, but whereas his right testis was identified in the scrotum, his left testis remained undescended.

TABLE 3. Prevalence of common sequence variants in *GPR54* and *KISS1*

dbSNP ID	SNP	AA change	Cohort frequency (%)	Control frequency (%)
A. *GPR54*				
rs8111938	615A>C	None	1.5	0
rs350132	1091T>A	H364L	23	28
rs3746147	1155G>A	None	0	0
B. *KISS1*				
rs12998	207G>A	E20K	4.7	4.2
	256A>G	Q36R	9.4	4.2
rs1132112	287C>G	None	0	0
rs4889	391C>G	P81R	36	13.9
	565_566insA	Omits 7AA	76	85

AA, Amino acid; Freq., frequency.

At 15 months of age, he was admitted for further investigation of anterior pituitary function. His height/length was progressing consistently along the 50th percentile and weight along the 25th percentile. He had a normal GH response to glucagon stimulation [2.1 (basal) to 30.5 ng/ml (peak) (6.2–91.5 mU/liter; North-East Thames Radio-Immuno Assay, cut-off for the diagnosis of GH deficiency, 11.7 ng/ml]. He had a normal cortisol response to glucagon stimulation [19.8–47.0 μg/dl (554–1317 nmol/liter)], normal free T_4 [1.37 μg/dl (17.5 nmol/liter)] and normal TSH response to TRH stimulation (0.5–7.3 mU/liter). He had normal basal prolactin, and undetectable basal gonadotrophins (LH < 0.5 mIU/ml, FSH < 0.5 mIU/ml). Results of a 3-d human chorionic gonadotropin (hCG) stimulation test (1000 IU daily) showed a limited testosterone response to stimulation (Table 4A). An ultrasound scan at this time identified both testes and bilateral hydroceles. The right testis was soft, measured 9 × 6 × 10 mm, and was within the scrotal sac. The left testis had a maximum diameter of 7 mm and was identified at the left inguinal ring. A prolonged course of hCG stimulation produced some increase in penile size but failed to stimulate descent of the left testis. Therefore, herniotomy and orchidopexy of a very small left testis were performed.

His progress during childhood was uneventful. However, he showed progressive weight gain and linear growth from the age of 3.5 yr so that by 9.7 yr of age, he was relatively tall (height 148.6 cm, > 97th percentile; parental target height 25th percentile) and heavy (weight 49.9 kg, ≫ 97th percentile) but with a small penis and testes. Repeat endocrine evaluation at 10 yr of age showed a poor gonadotrophin response to LHRH stimulation (Table 4B) and suboptimal testosterone response to 3 wk stimulation with hCG (1000 IU twice weekly) (Table 4C). He was therefore started electively on low-dose testosterone replacement to induce puberty. No neuroimaging was performed.

The proband's mother, who was heterozygous for R297L, had experienced menarche at 11 yr of age. She has regular

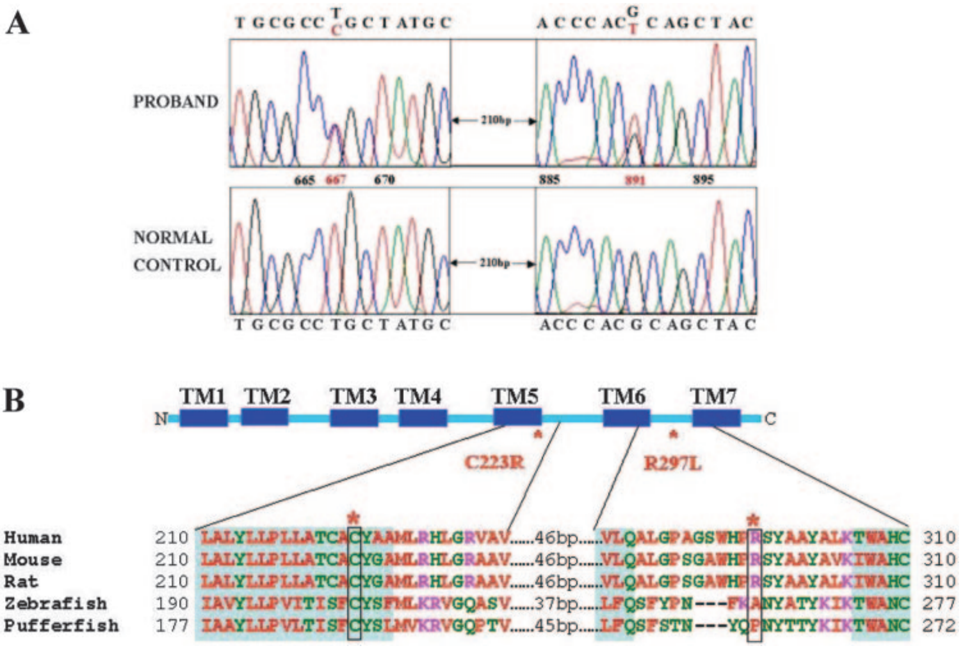

FIG. 1. Rare *GPR54* sequence variants found in propositus. TM, Transmembrane α helix.

menstrual cycles and detectable gonadotropins (LH 7.2 mIU/ml, FSH 8.3 mIU/ml). His father is believed to have normal reproductive function but was not available for testing. His younger brother, heterozygous for R297L but homozygous wild-type for C223 had normal genitalia and descended testes at birth. Of note, there was no known family history of hypogonadism or infertility in the father's eight siblings.

Characterization of mutant GPR54 function

Using the proprietary Flp-In system, isogenic cell lines stably expressing either wild-type GPR54 or one of the two mutant GPR54s at the same level of transcription were generated. A sensitive fluorometric calcium mobilization assay was then used as described to assess the response of each GPR54 species to the kisspeptin 112–121 decapeptide, previously established to be a potent agonist for GPR54 (12, 14). In our hands (Fig. 2), this assay is highly sensitive for detecting GPR54 activity: calcium flux above baseline for wild-type GPR54 is detectable at subnanomolar concentrations of ligand, with maximal flux achieved at around 100 nM.

The EC_{50} of the wild-type receptor was determined from 20 independent measurements. The (arithmetic) mean EC_{50} is 3.20 nM (lower 95% confidence interval 1.99 nM, upper 95% confidence interval 4.41 nM, SEM 0.56 nM). The range of EC50s was 0.2–9.8 nM. The C223R mutant was profoundly impaired in its signaling capability with an EC_{50} of 200 nM and maximal activity around 20% of wild-type GPR54. The function of R297L was only mildly affected, with a measured EC_{50} of 7 nM and a maximal activity of 85% of wild type (Fig. 2). A one-sample *t* test suggests this EC_{50} difference is significant, with $P < 0.0001$ and t = 6.6. Kolmogorov-Smirnoff deter-

TABLE 4. Results of dynamic endocrine testing

A. hCG stimulation test at 15 months		
	Pre-hCG	Day 3 post-hCG
DHEA-S [μg/liter (μmol/liter)]	<385 (<1.0)	<385 (<1.0)
Androstenedione [ng/ml (nmol/liter)]	0.17 (0.6)	0.17 (0.6)
Testosterone [ng/ml (nmol/liter)]	<0.15 (<0.5)	0.45 (1.5)

B. LHRH stimulation test at 10 yr			
	0 min	20 min	60 min
LH (IU/liter)	<0.7	1.0	<0.7
FSH (IU/liter)	<0.2	1.1	1.8

C. hCG stimulation test at 10 yr			
	Pre-hCG	Day 3 post-hCG	Day 21 post-hCG
DHEA-S [μg/liter (μmol/liter)]	1001 (2.6)	905 (2.35)	1220 (3.17)
Androstenedione [ng/ml (nmol/liter)]	1.0 (3.6)	1.2 (4.1)	1.7 (5.8)
Testosterone [ng/ml (nmol/liter)]	0.21 (0.7)	0.3 (1.0)	1.4 (4.6)
Dihydrotestosterone [ng/ml (nmol/liter)]	<0.04 (<0.12)	<0.04 (<0.12)	N/A

All injections were supplied by the hospital and administered under supervision. DHEA-S, Dehydroepiandrosterone sulfate; N/A, not available.

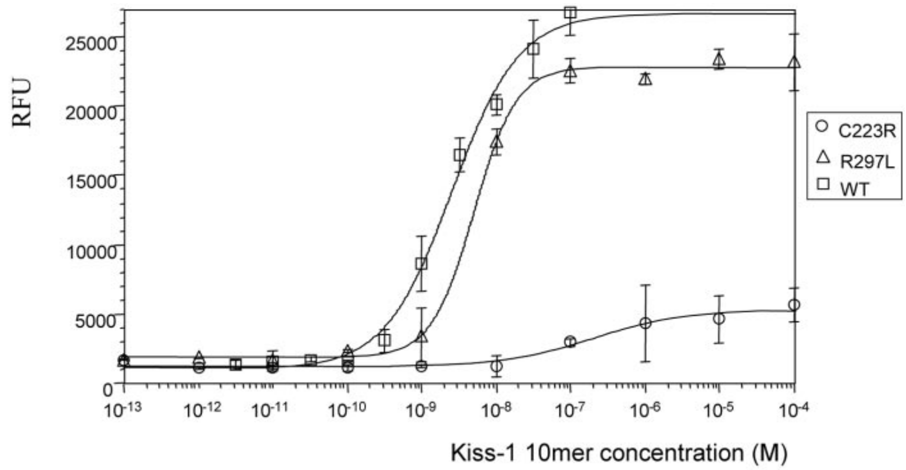

FIG. 2. Response of GPR54 variants to stimulation with kisspeptin 112–121 decapeptide. RFU, Relative fluorescence units. Experiments were performed in duplicate on 2 consecutive days, and SDs are indicated. Dose-response curves were generated using a four-parameter logistic equation. The resulting parameters and correlation coefficients are indicated.

y = ((A - D)/(1 + (x/C)^B)) + D:

	A	B	C	D	R^2
○ C223R	1169.893	0.711	2.04e-7	5304.917	0.969
△ R297L	1845.014	1.53	5.02e-9	22850.36	0.999
□ WT	997.683	0.965	2.54e-9	26693	0.994

mination of skewedness and kurtosis shows the control data fit a normal distribution, so a parametric *t* test is appropriate.

Discussion

Human GPR54 is a G protein-coupled receptor with homology to the family of galanin receptors, although galanin and galanin-like peptides do not bind the receptor with appreciable affinity. In the past year, homozygosity mapping and candidate gene analysis of large consanguineous pedigrees has led two groups to describe loss-of-function mutations in GPR54 in association with autosomal recessive HH (4, 5). In parallel, the independent generation and characterization of a *Gpr54* knockout mouse model, which shows a phenocopy of human isolated HH, has demonstrated that the function of GPR54 is conserved in mammals and that GPR54 is absolutely required for the normal function of this axis (5).

Mouse and human studies to date suggest GPR54 has a role in the release of GnRH (5, 6), further weight having recently been added to this hypothesis by the demonstration that peripherally and centrally administered kisspeptins can provoke robust LH and FSH release (6–8). However, it is not yet clear whether kisspeptins subserve primarily autocrine, paracrine, or endocrine function. Intriguingly, the putative endogenous GPR54 ligand, an amidated proteolytic product of full-length kisspeptin-1, sometimes called metastin (12–14), is normally present only at low picomolar concentrations in plasma but rises some 10,000-fold by the end of pregnancy (15). Both kisspeptin-1 and GPR54 are widely expressed, with the highest GPR54 expression being reported in placenta, brain, pituitary, pancreas, and spinal cord, whereas the highest kisspeptin-1 expression is seen in placenta, with significant levels also in pancreas and testis (12–14). This widespread expression implies a more complex role for GPR54/kisspeptin-1 than appreciated at present, and studies of humans harboring loss-of-function mutations are likely to make a valuable contribution to further elucidation of its biology.

The current report adds to the spectrum of loss-of-function mutations in GPR54. Previous studies reported five different mutations in four families or individuals with HH. Homozygosity for a 155-bp deletion spanning the splice acceptor site of the intron 4-exon 5 junction and part of exon 5 was detected in a consanguineous pedigree with five affected members (4). This homozygous deletion cosegregated with HH in this family and was inferred to result in loss of receptor function due to truncation of the coding sequence at residue 267. A second rare sequence variant, this time a missense mutation, L102P, immediately after the second transmembrane helix, was also found in one family on screening three other kindreds with familial HH. However, no cosegregation or functional studies of this mutant were reported (4). Another report of loss of function of GPR54 in patients with HH described both a homozygous L148S point mutation in a conserved region of the second intracellular loop in a consanguineous Saudi kindred and compound heterozygosity for two mutations, R331X and X399R, in an unrelated Afro-Caribbean patient, which were proven to result in significant loss of GPR54 signaling *in vitro* (5). In total, the published reports to date describe the screening of 68 unrelated probands with normosmic HH (five familial; 63 idiopathic) and normal GnRH receptor gene sequence, of whom three had two proven hypofunctional or nonfunctional GPR54 alleles (two familial; one idiopathic). This is consistent with our finding of one such patient in a further 17 studied (three familial; 14 idiopathic: Table 1). Cumulative results now suggest that, in those in which GnRHR coding sequence mutations have been ruled out, around 2–3% (two of 77 studied) of idiopathic normosmic HH may be accounted for by GPR54 loss-of-function mutations, whereas at present 25% (two of eight studied) of familial cases have been found to have such mutations. However, no GPR54 mutations were identified in patients or families with CDP alone.

It is highly likely, although not conclusively proven, that compound heterozygosity for the C223R and R297L variants is the cause of the idiopathic HH seen in our proband. C233R is severely defective in its signaling capability and is not seen as a common variant in the Afro-Caribbean population. In contrast, the effect of the R297L mutation on signaling is only modest, although highly statistically significant, when studied *in vitro*. However it is a highly conserved residue in mammalian GPR54 and was not found in an ethnically matched control population. It is likely that its effects in combination with the severe dysfunction of the C233R variant are sufficient to lead to a clinical phenotype.

Although the patient we describe is not yet beyond the age of normal puberty, he had sufficient clinical and biochemical evidence of HH to warrant induction of puberty at an age appropriate for his peer group. For example, bilateral cryptorchidism and micropenis were noted at birth; he had undetectable gonadotropins at 2 months of age [when the hypothalamic-pituitary-gonadal axis is usually relatively active (16)]; and his response to exogenous GnRH stimulation at the age of 10 yr was poor. In addition, his testosterone response to stimulation for 3 wk with hCG at this time was relatively low. Although this may reflect the tropic effect of prolonged gonadotropin insufficiency or delayed orchidopexy, it remains possible that the GPR54/kisspeptin-1 system could have a direct effect on testicular function, too. Of note, GPR54 mRNA has previously been detected, albeit at low levels, in human testis (13, 14). Thus, the effect of recombinant gonadotropin stimulation on the induction of fertility in patients with *GPR54* gene mutations remains to be seen.

The present report uses a GPR54 signaling assay that is substantially more sensitive than that used to evaluate previous pathogenic mutations. The assay reported originally was based on transient expression of GPR54, stimulation with kisspeptin-1 112–121 decapeptide, and determination of the generation of inositol triphosphate. Stimulation of inositol triphosphate levels was seen only at around 10 nM kisspeptin-1 112–121 for wild-type GPR54, with maximal activity around 100 μM (3). In contrast, this study employed real-time measurements of calcium flux, similar in principle to those previously used in the identification of kisspeptin as a *bona fide* GPR54 ligand (12–14). As seen in Fig. 2, this assay detects signaling at subnanomolar concentrations of the kisspeptin decapeptide, and activity is maximal at around 100 nM, some 2–3 orders of magnitude lower than the ligand concentration required to maximize inositol triphosphate generation in the previous assay.

In the absence of functional assessment of the GPR54 L102P variant, GPR54 C223R and R297L are the second- and third-point mutations after L148S reported to result in impaired GPR54 function because the 155-bp deletion, R331X, and X399R are all likely to have resulted in loss of function largely through impaired expression of mRNA and/or protein product. The new mutations may thus prove more informative with respect to understanding of the molecular determinants of GPR54 signaling. The dramatic loss of function of GPR54 C223R is unsurprising in view of likely disruption of the fifth transmembrane helix. Indeed, it will be of interest to establish whether receptor protein is expressed at wild-type level at the plasma membrane and, if so, whether ligand binding is preserved. R297L is situated in the final extracellular loop of GPR54 in a slightly less conserved region, and its effect on signaling is consequently relatively mild *in vitro*. It remains to be determined whether its additional loss of function *in vivo* is due to inefficient receptor synthesis or processing. Nevertheless, the dose-response characteristics of this mutation raise the possibility that supraphysiological doses of exogenous kisspeptin or an analog could be used to restore GnRH pulsatility and possibly fertility in this patient. A similar approach, using high doses of GnRH, has been used to induce ovulation in a woman with a partial loss-of-function mutation in the GnRH receptor (17). Furthermore, because we have not been able to establish whether the father of the propositus is indeed heterozygous for the C223R variant, we cannot conclude on phenotypic grounds that heterozygosity for this variant is not clinically expressed. Thus, dominant negativity of GPR54 C223R, or an interaction between C223R and R297L, cannot be formally excluded. However, although there are precedents for dominant-negative GPR mutations, often based on receptor misrouting or G protein sequestration, the dominant negativity is often more apparent *in vitro* than *in vivo* (18), and we believe it more likely in this case that it is the cumulative loss of function of the two variants that is responsible for the clinical phenotype.

In addition to identifying and characterizing two rare sequence variants in the *GPR54* gene, we have also confirmed two previously described SNPs (Table 3A). One of these is nonsynonymous, with an alternative histidine to leucine change encoded in the cytoplasmic tail. A third SNP recorded in the dbSNP database was not confirmed (1155G>A; rs3746147). Analysis of *KISS1* revealed four single nucleotide polymorphisms, three nonsynonymous, and one not previously recorded. In addition, an insertional polymorphism that leads to truncation of the kisspeptin-1 protein product by seven amino acids was also confirmed (Table 3B). Once again, a further SNP recorded in the dbSNP database was not confirmed (287C>G; rs1132112). All three nonsynonymous KISS1 sequence variants lead to amino acid changes in the amino-terminal part of the molecule. This is not directly involved in signaling but is likely to contribute to the regulation of processing and secretion of the active peptide. We have documented the frequency of occurrence of these variants in our study population and also in 180 Caucasian control chromosomes. This information could be used in future to guide case control studies looking at either indices of reproductive function or fertility or possibly indices of tumor invasion or metastasis in patients with malignancy, in view of the considerable interest in KISS1 as a metastasis-suppressing gene.

In summary, we have described two novel missense mutations in the G protein-coupled receptor GPR54 that result in impaired kisspeptin-1 stimulation of calcium flux. Compound heterozygosity for these mutations is associated with HH, detected at birth in the proband due to bilateral cryptorchidism and micropenis. Although not common, loss-of-function mutations in GPR54 appear to be a significant cause of HH in patients from diverse ethnic backgrounds.

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