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RESEARCH ARTICLE

Contribution of functionally assessed *GHRHR* mutations to idiopathic isolated growth hormone deficiency in patients without *GH1* mutations

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Abstract

Isolated growth hormone deficiency (IGHD) is a rare condition mainly caused by mutations in *GH1*. The aim of this study was to assess the contribution of *GHRHR* mutations to IGHD in an unusually large group of patients. All *GHRHR* coding exons and flanking intronic regions were sequenced in 312 unrelated patients with nonsyndromic IGHD. Functional consequences of all newly identified missense variants were assessed in vitro (i.e., study of the expression of recombinant GHRHRs and their ability to activate the cyclic adenosine monophosphate (cAMP) signaling pathway). Genotype-phenotype correlation analyses were performed according to the nature of the identified mutation. We identified 20 different disease-causing

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GHRHR mutations (truncating and missense loss-of-function mutations), among which 15 are novel, in 24 unrelated patients. Of note, about half (13/24) of those patients represent sporadic cases. The clinical phenotype of patients with at least one missense *GHRHR* mutation was found to be indistinguishable from that of patients with bi-allelic truncating mutations. This study, which unveils disease-causing *GHRHR* mutations in 8% (24/312) of IGHD cases, identifies *GHRHR* as the second IGHD gene most frequently involved after *GH1*. The finding that 8% of IGHD cases without *GH1* mutations are explained by *GHRHR* molecular defects (including missense mutations), together with the high proportion of sporadic cases among those patients, has important implications for genetic counseling.

KEYWORDS
functional studies, genotype-phenotype correlation, GHRHR, IGHD, molecular epidemiology

1 | INTRODUCTION

Congenital isolated growth hormone deficiency (IGHD), which is one of the rare causes of short stature, has an incidence that ranges from 1/10,000 to 1/4,000 births (Vimpani, Vimpani, Lidgard, Cameron, & Farquhar, 1977). This genetically heterogeneous condition can be explained by mutations within key genes of the somatotroph axis, namely *GH1* that encodes the growth hormone (GH), as well as *GHSR* and *GHRHR*, which encode the GH secretagogue receptor and the receptor of the GH releasing hormone (GHRH), respectively (Alatzoglou et al., 2009; Birla et al., 2016; Mullis, 2010; Pantel et al., 2006; Wajnrajch, Gertner, Harbison, Chua, & Leibel, 1996). Depending on studies, 5–30% of the cases for which a molecular cause has been found are familial (Alba et al., 2004; Bona, Paracchini, Giordano, & Momigliano-Richiardi, 2004; Lindsay, Feldkamp, Harris, Robertson, & Rallison, 1994). The *GHRHR* encodes a 423-amino-acid protein, which is a member of the secretin family of G-protein coupled receptors (GPCR). This seven transmembrane domain-containing receptor, which is expressed by somatotropic cells of the pituitary gland, is activated by the GHRH, a small peptide released by the hypothalamus. GHRHR activation will then result in both the release of the GH stored in secretory granules and GH production through cyclic adenosine monophosphate (cAMP)-dependent transcription of GH1 (Frohman & Kineman, 2002; Lee, Duan, Kotlar, & Jameson, 2001).

The contribution of *GH1* mutations to IGHD has been the subject of a number of studies (Alatzoglou & Dattani, 2010; Mullis, 2010; Procter, Phillips, & Cooper, 1998; Wagner, Eblé, Hindmarsh, & Mullis, 1998). This is, however, not the case for *GHRHR* mutations. Indeed, the 21 *GHRHR* disease-causing mutations identified worldwide correspond mainly to case reports (reviewed in Corazzini & Salvatori, 2013), and according to the very rare studies performed in cohorts of patients with IGHD (Alatzoglou et al., 2009; Graaff et al., 2009; Birla et al., 2016; Desai et al., 2013), the estimated contribution of *GHRHR* mutations to IGHD ranges from 0% to 15% (Graaff et al., 2009; (Desai et al., 2013). In addition, all (Desai et al., 2013) or several families with an identified *GHRHR* defect were found to carry the p.(E72*) mutation, which results from a founder effect (Wajnrajch et al., 2003). In an Indian study where *GHRHR* contribution was found to reach 15% of IGHD (Desai et al., 2013; 12 out of 80 independent probands), all affected individuals carried the p.(E72*) founder mutation. Of note, all the patients reported with *GHRHR* mutations had bi-allelic mutations, except one patient carrying a mono-allelic p.(V10G) a missense mutation in the signal peptide. This mutation is expected to impair GHRHR localization to the plasma membrane (Godi et al., 2009).

We performed the current study with the aim to assess the contribution of *GHRHR* mutations to IGHD in an exceptionally large group of reportedly unrelated patients from 312 families, without mutations of *GH1*. Among the new potentially disease-causing mutations were 10 *GHRHR* missense variants. All of them were subjected to a functional assay in transfected cells to assess their deleterious effect on the signaling activity of the protein, and to study phenotype-genotype correlation in the patients.

2 | PATIENTS AND METHODS

2.1 | Editorial policies and ethical considerations

This study was approved by the French ethics committee of Henri Mondor Hospital (Comité Consultatif de Protection des Personnes participant à une Recherche Biomédicale).

2.2 | Patients

This study focuses on a group of 312 reportedly unrelated patients diagnosed with inborn nonsyndromic IGHD and in whom no *GH1* mutation had been identified. A proportion of 38% of these patients were familial cases. *GH1* mutations were ruled out by Sanger

sequencing following a single long-range PCR amplifying the five exons from 2001 to 2014, and by an NGS targeted panel (custom Seqcap EZ choice capture, Roche; MiSeq sequencing machine, Illumina) from 2014. Both techniques explored full *GH1* intronic sequences to screen for *GH1* deep-intronic mutations. When whole gene deletions (6.7, 7, 7.6, and 45-kb deletions) were suspected following Sanger sequencing (absence of long-range PCR-amplification in the proband or absence of a *GH1* mutation in one of the parents suggesting hemizygosity in the proband), the PCR-RFLP technique developed by Vnencak-Jones, Phillips, & De-Fen (1990) was undertaken. The NGS panel explored deletions/duplications larger or equal to an exon by read-depth ratio calculation for each exon within each run. Samples were referred by Endocrinology Departments from University hospitals from France, Belgium, Algeria, Tunisia, Morocco, and Turkey. Detailed phenotypic data, including hormonal data and magnetic resonance imaging of the hypothalamic-pituitary area, were collected through a standardized form. All the patients included in the study displayed a eutopic posterior pituitary. The diagnosis of GH deficiency was based on the results of GH stimulation tests, with values under 10 ng/ml. Prior to being enrolled in this study, patients or parents of patients under 18 years of age gave their written informed consent to perform genetic investigations. Peripheral blood samples were collected from patients and, whenever possible, from their first-degree relatives.

2.3 | Screening for *GHRHR* mutations

For all 312 patients, genomic DNA was isolated from peripheral blood leukocytes, and the 13 coding exons of *GHRHR* (RefSeq NM_000823.3) and their flanking intronic regions were analyzed by Sanger sequencing or targeted parallel sequencing. For Sanger sequencing, PCR-amplified samples were purified by Exo-SAP (Affymetrix, Santa Clara, CA). Internal primers were used to sequence the corresponding PCR products with an ABI 3130XL automated capillary DNA sequencer (Applied Biosystems, Foster City, CA). *GHRHR* mutations were screened for by Sanger sequencing of all coding exons from 2001 to 2014, and by a targeted NGS panel (custom Seqcap EZ choice capture, Roche; MiSeq sequencing machine, Illumina) from 2014. When, by Sanger sequencing, a homozygous or heterozygous deletion (larger or equal to one exon) was suspected (i.e., absence of PCR-amplification of one or several exons in the proband or absence of a mutation in one parent, respectively), two techniques were applied to characterize the breakpoint: iterative long-range PCR and/or SNP array. Deletions/duplications were searched for by the NGS panel by read-depth analysis as described above. Sequences of all the primers used in this study are available upon request. Targeted parallel sequencing consisted of a capture enrichment (SeqCap EZ Choice Enrichment Kit (UTR); Roche, Basel, Switzerland) followed by parallel sequencing on a MiSeq system (Illumina, San Diego). Data were analyzed with an in-house double pipeline. All variants reported in the present manuscript have been submitted to the LOVD public database.

2.4 | Plasmid constructs

The entire human *GHRHR* complementary DNA was obtained from pituitary samples (Clontech, CA) and cloned into the pcDNA3.1/V5-His Topo TA expression vector (Invitrogen, Life Technologies Ltd, Paisley, UK) to generate the pcDNA3.1_GHRHR_WT plasmid encoding the wild-type GHRHR. All *GHRHR* missense variants were then generated from pcDNA3.1_GHRHR_WT through site-directed mutagenesis, using the Q5 Hot Start High Fidelity DNA polymerase (NEB, MA). The pCRE-luc reporter plasmid used to assess the activation of the cAMP signaling pathway, which contains 4 copies of the cAMP-responsive element, was purchased from Strategene (Santa Clara, CA). All the constructs were verified by sequencing the inserts and the vector-flanking fragments.

2.5 | Cell culture and transfection

Human embryonic kidney (HEK293T) cells were grown in dulbecco's modified eagle's medium (DMEM) (Gibco, Life Technologies Ltd, Paisley, UK) supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin at 37°C under 5% CO_2. Transfections were performed at approximately 80% confluence by the Fugene HD transfection reagent (Promega) as recommended by the manufacturer, with a 3:1 Fugene/DNA ratio.

2.6 | Immunofluorescence analyses

HEK293T transfectants expressing the different recombinant GHRHRs (normal or carrying a missense variant) were plated onto coated glass coverslips in 24-well culture plates coated with poly-L-lysine (Sigma-Aldrich, Saint-Louis, MO) and were subsequently fixed in 4% (v/v) paraformaldehyde/phosphate buffered saline (PBS) and then permeabilized in 0.25% Igepal/PBS. A 5% bovine serum albumin/5% milk/PBS solution was used to block nonspecific binding of antibodies for 1 hr at 37°C. Rabbit polyclonal anti-GHRHR antibody targeting the intracellular domain of the receptor (ab28692, Abcam) was used at 1:500 to detect GHRHR. The secondary antibody was a goat anti-rabbit immunoglobulin G, Alexa Fluor® 488 (Invitrogen) at 1:2,000. Nuclei were stained using ProLong® Diamond Antifade Mountant with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies). Cells were then examined with a Nikon 80i fluorescence microscope (Nikon, Tokyo, Japan).

2.7 | Assessment of the GHRHR-dependent cAMP signaling pathway

HEK293T cells were cotransfected with 400 ng of the pCRE-Luc reporter plasmid and either 40 ng of each *GHRHR* expression construct or the empty pcDNA3.1 expression vector. After transfection of 8 hr, HEK293T cells were activated with the human synthetic GHRH ligand (GHRH[1-44]NH_2, SC086, PolyPeptide Group) at 10^{-7} M. Twenty-four hours after transfection, cell lysates were assayed for luciferase activity using the Luciferase Assay System (Promega) on a TriStar LB 941 luminometer (Berthold Technologies, Munich, Germany). The total amount of proteins, as determined

through the Pierce Coomassie Plus Protein Assay Reagent (Thermo Fischer Scientific, Waltham, MA), was used to normalize luciferase activity. Results are shown as means ± standard deviation (SD) of three independent experiments performed in triplicate.

2.8 | Statistics

The difference between means was assessed by Student's *t* test ($p<.01$ was considered as significant) with the GraphPad Prism 5.0 software.

2.9 | Online tools

gnomAD: http://gnomad.broadinstitute.org/
LOVD: https://databases.lovd.nl/shared/variants/GHRHR/unique
MaxEntScan: http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html
Signal P4.1: http://www.cbs.dtu.dk/services/SignalP/

3 | RESULTS

3.1 | Spectrum of identified *GHRHR* sequence variants

The screening of the *GHRHR* gene in the group of 312 unrelated patients with nonsyndromic IGHD identified 22 different sequence variants in 26 probands (Figure 1a): one splice site mutation (c.161-1G>A), five frameshift mutations (p.[A8Dfs*22], p.[A91Gfs*13], p.[R156Afs*15], p.[V225Gfs*165], and p.[L364Ffs*21]), two intragenic deletions (c.[?_-48]_[57 + 1_58-1]del and p.[L201_V295del]), two nonsense mutations (p.[E72*] and p.[W243*]), and 12 missense variants (p.[R4W], p.[P16L], p.[R94Q], p.[L144H], p.[A153D], p.[R161W], p.[V164A], p.[F169L], p.[A176V], p.[T259K], p.[W283R], and p.[I387T]). Seventeen of those 22 *GHRHR* sequence variants are novel (in bold on Figure 1a). A total of 39 patients were identified with *GHRHR* sequence variants. Within all these families, the segregation analysis was compatible with the involvement of the identified *GHRHR* variants in IGHD. For further analyses, the patients were divided into two groups: patients with bi-allelic truncating loss-of-function mutations (deletions, splice site, frameshift, or nonsense mutations; $n = 21/39$; Table 1) and patients with at least one missense mutation ($n = 18/39$; Table 2). The main phenotypic features of the patients with identified *GHRHR* variants are summarized in Table 3: mean age at diagnosis was 8.9 years (range: 0.6–26) and mean height deficit SD score was −4.8 [range: −8–2]. The nonsense mutation p.(E72*), for which a founder effect was found (Wajnrajch et al., 2003), was present in four families. Two other variants were recurrent: the p.(L144H) found in patients from three families, and the p.(A8Dfs*22) in patients from two families.

All but one (p.[E72*]) truncating variants were absent from public variant databases single nucleotide polymorphism database (dbSNP,

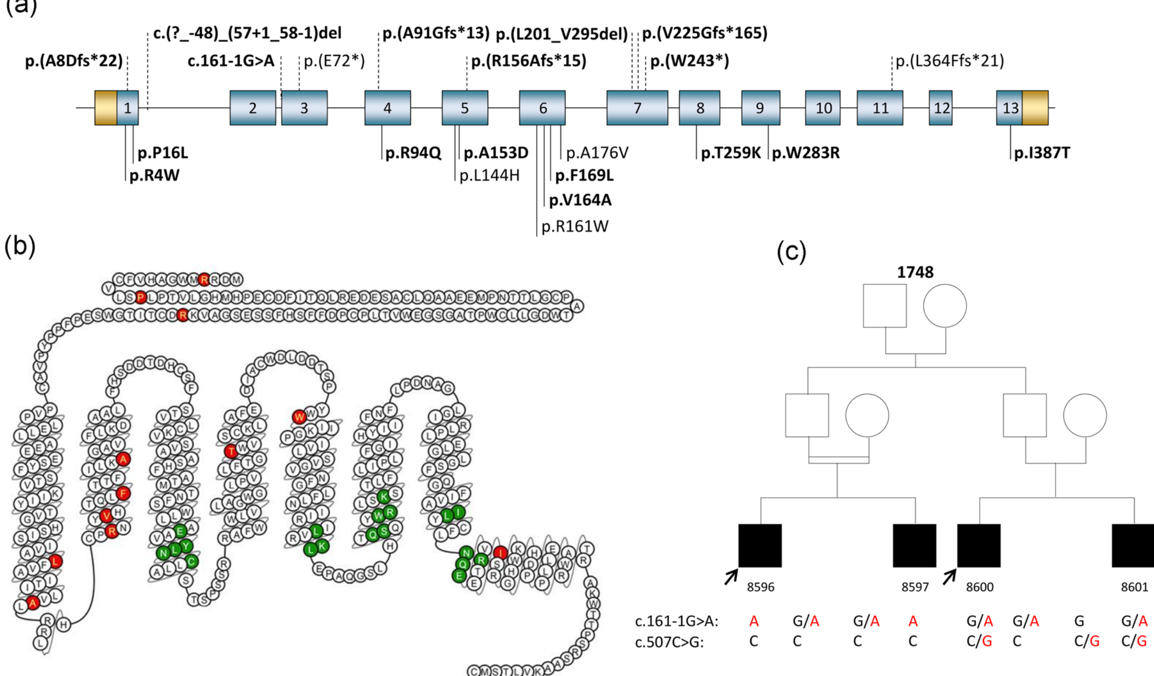

FIGURE 1 Schematic representation of the identified *GHRHR* variants at the genomic and protein levels. (a) The boxes represent the 13 *GHRHR* exons (coding regions in blue and noncoding regions in yellow). Top: 10 truncating mutations; bottom: 13 missense variants. Newly described variants are in bold characters. (b) Schematic representation of the GHRHR protein (modified from the GPCR database [Isberg et al., 2016]). In red: residues affected by missense variants; in green: potentially Gαs-interacting residues. (c) Family 1748: genealogical tree and genotypes

TABLE 1 Genotype of the patients with identified bi-allelic truncating mutations of *GHRHR*

Patient	Family	Variation (nucleotide)	Variation (protein)	Genotype	GnomAD: mutated alleles/total alleles (frequency %)	GnomAD: highest allele frequency % (population)
6791	645	c.22_23insA	p.(Ala8Aspfs*22)	Homozygous	NR	NR
7232	114	c.22_23insA	p.(Ala8Aspfs*22)	Homozygous	NR	NR
8807	1859	c.(?_-48)_(57 + 1_58-1)del	p.?	Homozygous	NR	NR
8596	1748	c.161-1G>A	p.?	Homozygous	NR	NR
8597		c.161-1G>A	p.?	Homozygous	NR	NR
6487	541	c.214G>T	p.(Glu72*)	Homozygous	35/18,6982 (0.02)	0.14 (South Asia)
6488		c.214G>T	p.(Glu72*)	Homozygous	35/18,6982 (0.02)	0.14 (South Asia)
5944	378	c.214G>T	p.(Glu72*)	Homozygous	35/18,6982 (0.02)	0.14 (South Asia)
5945		c.214G>T	p.(Glu72*)	Homozygous	35/18,6982 (0.02)	0.14 (South Asia)
8908	1912	c.214G>T	p.(Glu72*)	Homozygous	35/18,6982 (0.02)	0.14 (South Asia)
8909		c.214G>T	p.(Glu72*)	Homozygous	35/18,6982 (0.02)	0.14 (South Asia)
8382	1643	c.214G>T	p.(Glu72*)	Homozygous	35/18,6982 (0.02)	0.14 (South Asia)
8780	1842	c.271dupG c.674_677delinsGCTGTTGGCAGAAG	p.(Ala91Glyfs*13) p.(Val225Glyfs*165)	Compound heterozygous	NR NR	NR NR
8878		c.271dupG c.674_677delinsGCTGTTGGCAGAAG	p.(Ala91Glyfs*13) p.(Val225Glyfs*165)	Compound heterozygous	NR NR	NR NR
6765	637	c.465-91_1105-119del5291	p.(Arg156Alafs*15)	Homozygous	NR	NR
6766		c.465-91_1105-119del5291	p.(Arg156Alafs*15)	Homozygous	NR	NR
6767		c.465-91_1105-119del5291	p.(Arg156Alafs*15)	Homozygous	NR	NR
6768		c.465-91_1105-119del5291	p.(Arg156Alafs*15)	Homozygous	NR	NR
5091	94	c.597 + 153_883-273del	p.(Leu201_Val295-del)	Homozygous	NR	NR
6323	459	c.728G>A	p.(Trp243*)	Homozygous	NR	NR
6409	265	c.1089_1093del	p.(Leu364Phefs*21)	Homozygous	NR	NR

Abbreviations: C-Ter, C-terminal; NR, not reported; N-Ter, N-terminal; TM, transmembrane domain.

gnomAD) and all missense variants were either not described or had an allele frequency below 0.06% in control databases (Table 2). Beside the c.161-1G>A splice site mutation, which involves the invariant dinucleotide of a splice acceptor site which score falls from 7.9 to −0.8 (according to the MaxEntScan in silico tool for splicing defect), no other variant was predicted to result in a splicing defect. Among the 10 identified missense variants that were part of a bi-allelic mutant genotype, eight involve residues located in the highly hydrophobic transmembrane domains (p.[L144H], p.[A153D], p.[R161W], p.[V164A], p.[F169L], p.[A176V], p.[T259K], and p.[W283R]; Figure 1b). The remaining two missense variants (p.[R94Q] and p.[I387T]) were located in the extracellular N-terminal part and the intracellular C-terminal part of the receptor, respectively (Figure 1b). In addition, two missense variants, which were identified only in the heterozygous state (2/26 probands), involve residues located within the signal peptide of the protein (p.[R4W] and p.[P16L]). Signal peptide prediction scores for those variants range within normal values (around 70%; SignalP 4.1), thereby suggesting that they do not affect protein maturation.

3.2 | Functional consequences of the identified *GHRHR* missense variants on receptor expression and on the GHRHR-dependent cAMP signaling pathway

As mentioned above, 10 of the 22 identified *GHRHR* sequence variants are clearly disease-causing; these are the truncating loss-of-function mutations listed in Table 1. As for the identified missense variants, with the aim to assess their functional consequences, we first transiently expressed the corresponding GHRHR variants in HEK293T cells and analyzed their subcellular localization using an anti-GHRHR antibody targeting the intracellular end of the receptor; this was done for 11 of the 12 identified missense variants, the 12th one (p.[A176V]) having already been assessed as a loss-of-function mutation (Carakushansky et al., 2003). All GHRHR variants displayed a labeling pattern similar to that of the wild-type receptor (Figure 2a). A strong juxtanuclear signal, evocative of the Golgi apparatus, was also observed in most cells, an expression pattern typical of the overexpression of recombinant proteins in transient expression assays (e.g., Kremmidiotis et al., 1999). Overall, these experiments did not identify major alteration in protein production.

TABLE 2 Genotype of the patients with at least one identified *GHRHR* missense mutation

Patient	Family	Variation (nucleotide)	Variation (protein)	Genotype	GnomAD: mutated alleles/total alleles (frequency %)	GnomAD: highest allele frequency % (population)	Location	Conservation in vertebrates
6834	701	c.10C>T	p.(Arg4Trp)	Heterozygous	112/17,8960 (0.06)	0.14 (Ashkenazi)	Signal peptide	No
6705	610	c.47C>T	p.(Pro16Leu)	Heterozygous	75/17,7706 (0.04)	0.52 (East Asia)	Signal peptide	No
7276	997	c.281G>A	p.(Arg94Gln)	Homozygous	2/24,6268 (<0.01)	<0.01 (Africa)	N-Ter	Invariant
6763	638	c.431T>A	p.(Leu144His)	Homozygous	1/30,928 (<0.01)	<0.01 (Europe)	TM I	Invariant
6764		c.431T>A	p.(Leu144His)	Homozygous	1/30,928 (<0.01)	<0.01 (Europe)	TM I	Invariant
6775	6775	c.431T>A	p.(Leu144His)	Homozygous	1/30,928 (<0.01)	<0.01 (Europe)	TM I	Invariant
6776		c.431T>A	p.(Leu144His)	Homozygous	1/30,928 (<0.01)	<0.01 (Europe)	TM I	Invariant
7027	195	c.431T>A c.776C>A	p.(Leu144His) p.(Thr259Lys)	Compound heterozygous	1/30,928 (<0.01) NR	<0.01 (Europe) NR	TM I TM IV	Invariant No
8762	1834	c.458C>A	p.(Ala153Asp)	Homozygous	NR	NR	TM I	No
8763		c.458C>A	p.(Ala153Asp)	Homozygous	NR	NR	TM I	No
8761		c.458C>A	p.(Ala153Asp)	Homozygous	NR	NR	TM I	No
7410	1091	c.481C>T	p.(Arg161Trp)	Homozygous	2/24,6218 (<0.01)	<0.01 (South Asia)	TM II	Invariant
7102	60	c.491T>C	p.(Val164Ala)	Homozygous	NR	NR	TM II	Apolar (Val or Ile)
8600	1748	c.507C>G c.161-1G>A	p.(Phe169Leu) p.?	Compound heterozygous	30/24,6248 (0.01) NR	0.09 (South Asia) NR	TM II –	Invariant –
8601		c.507C>G c.161-1G>A	p.(Phe169Leu) p.?	Compound heterozygous	30/24,6248 (0.01) NR	0.09 (South Asia) NR	TM II –	Invariant –
5237	136	c.527C>T	p.(Ala176Val)	Homozygous	11/27,7180 (<0.01)	0.01 (East Asia)	TM II	In mammals
8552	1727	c.847T>C	p.(Trp283Arg)	Homozygous	NR	NR	TM V	Invariant
6466	1079	c.1160T>C	p.(Ile387Thr)	Homozygous	NR	NR	C-Ter	Apolar (Ile or Leu)

Abbreviations: C-Ter, C-terminal; NR, not reported; N-Ter, N-terminal; TM, transmembrane domain.

To further characterize the function of those GHRHR variants, we first assessed their ability to activate the cAMP signaling pathway in response to GHRH stimulation in HEK293T cells cotransfected with a pCRE reporter plasmid (Figure 2b). This study revealed a complete loss of function for eight of the 11 identified missense variants: p.(R94Q), p.(L144H), p.(A153D), p.(R161W), p.(V164A), p.(T259K), p.(W283R), and p.(I387T) missense variants. As for the p.(F169L) missense variant identified in the compound heterozygous state with the c.161-1G>A splice acceptor site mutation in two brothers (Family 1748, Figure 1c and Table 2), it resulted in a partial but significant loss of function (Figure 2b). Interestingly, the missense variants identified in the signal peptide (p.[R4W] and p.[P16L]) did not alter the cAMP-mediated transcriptional activity of the GHRHR.

3.3 | Phenotype-genotype correlation: bi-allelic truncating mutations versus at least one missense mutation

To perform genotype-phenotype correlations, we took into account the phenotypic features of all the patients with proven disease-causing mutations. These mutations include the 10 disease-causing truncating mutations, predicted to result in the absence of protein production due to nonsense-mediated messenger RNA decay or the production of a truncated protein, and the nine above-mentioned loss-of-function missense mutations. Two groups of patients were distinguished on the basis of their genotype: (a) the 21 patients with *GHRHR* bi-allelic truncating mutations, and (b) the 16 patients with a disease-causing missense mutation on at least one *GHRHR* allele (Table 2). In both groups, we analyzed the following phenotypic features: age and growth deficit at diagnosis (for probands only), length at birth, values of the GH dynamic tests and IGF-I levels. Length at birth (mean in cm ± SD) was found to be normal in both groups: 49.4 cm ± 2.3 in patients with bi-allelic truncating mutations ($n = 9$) versus 49.2 cm ± 1.0 in patients with at least one missense mutation ($n = 6$); a single patient (#6487) presented with intrauterine growth retardation (IUGR; 45 cm, −2.6 SD). The mean height of the fathers and the mothers with a heterozygous loss-of-function mutation is normal (fathers: 169.3 cm, range = 156.0–182.0, $n = 16$; mothers 159.0 cm, range = 145.2–175.0, $n = 16$). Probands with bi-allelic truncating mutations and those with at least one missense mutation were diagnosed around the same age (median age [interquartile range], respectively 7.7 yr [3.7–15.0] and 8.5 yr [3.2–14.2]; Figure 3a); mean age did not differ ($p = .8$, Student's *t* test). There is a trend for a more severe growth deficit at diagnosis (mean SD ± SEM) in probands with truncating mutations (−5.2 ± 0.7) than in those with at least one missense mutation (−4.4 ± 0.4); however, the difference (0.88 SD) is not statistically significant ($p = .3$; Student's *t* test; Figure 3b). Linear regression analysis of the growth

TABLE 3 Clinical and auxologic data of the patients with identified *GHRHR* variations

Patient	Sex	Birth length (cm)	Age (year)	Growth delay (SD)	Mean GH peak (ng/ml)	IGF-I (ng/ml)	MRI	Consanguinity	Geographic origin
Truncating mutations (21 patients)									
6791	F	48.0	12.0	−3.5	0.6	NA	HAP	Yes	Algeria
7232	M	NA	NA	NA	NA	NA	NA	Yes	Algeria
8807	M	NA	0.7	NA	1.6	<0.1	HAP	No	India/Sri Lanka
8596	M	NA	16.0	−4.5	0.1	<25.0	HAP	Yes	Turkey
8597	M	NA	12.0	−3.8	0.3	<25.0	HAP	Yes	Turkey
6487	M	45.0	3.7	−5.7	1.2	6.0	HAP	Yes	Sri Lanka
6488	F	47.5	2.5	−3.0	2.1	29.0	N	Yes	Sri Lanka
5944	F	NA	15.0	−6.7	0.1	4.2	HAP	Yes	Turkey
5945	F	NA	14.0	−8.6	0.1	7.5	HAP	Yes	Turkey
8908	M	NA	7.7	−4.0	0.7	<38.5	HAP	No	India
8909	M	NA	6.9	−5.0	0.7	<38.5	HAP	No	India
8382	F	NA	7.0	NA	<0.1[a]	25.0	N	NA	India/Sri Lanka
8780	M	53.0	1.0	−2.5	1.4	15.0	HAP	No	Spain/Belgium
8878	F	51.0	0.6	−3.4	1.9	47.0	HAP	No	Spain/Belgium
6765	F	50.0	NA	NA	0.3	NA	HAP	Yes	Algeria
6766	M	50.0	12.0	−6.0	0.1	NA	HAP	Yes	Algeria
6767	F	50.0	NA	NA	0.1	NA	NA	Yes	Algeria
6768	F	50.0	NA	NA	0.2	NA	N	Yes	Algeria
5091	M	NA	12.0	−6.5	0.6	NA	HAP	No	Turkey
6323	M	NA	16.0	−8.0	0.2[a]	NA	HAP	Yes	Tunisia
6409	F	NA	4.0	NA	<0.1	52.0	NA	NA	Romania
At least one missense variation (18 patients)									
6834[b]	M	NA	NA	NA	10.1	19.6	HAP	NA	Algeria
6705[b]	F	NA	3.8	−4.2	10.5	34.0	N	NA	China
7276	F	NA	8.6	−5.5	0[a]	<0.1	NA	Yes	Tunisia
6763	F	50.0	8.5	−5.5	0.3	NA	HAP	Yes	Algeria
6764	F	48.0	6.5	−5.0	0.1	NA	HAP	Yes	Algeria
6775	M	50.0	26.0	NA	0	NA	HAP	Yes	Algeria
6776	M	49.0	12.4	NA	0.3	NA	HAP	Yes	Algeria
7027	F	NA	1.2	−3.8	0.2[a]	15.0	N	NA	France
8763	F	NA	16.4	−6.4	<0.1	<25.0	HAP	Yes	Syria
8762	M	NA	14.4	−4.4	<0.1	<25.0	HAP	Yes	Syria
8761	M	NA	11.3	−6.2	0.2	<25.0	HAP	Yes	Syria
7410	M	NA	10.0	−4.8	0.1[a]	<30.0	AAP	Yes	Pakistan
7102	F	48.0	1.2	−3.6	0.9	45.0	HAP	Yes	Lebanon
8600	M	NA	14.2	−4.3	0.5	30.3	HAP	Yes	Turkey
8601	M	NA	9.7	−3.8	5.4	50.1	HAP	Yes	Turkey
5237	M	NA	4.2	−3.5	2.0	65.0	N	No	NA
8552	F	50.0	3.2	−2.0	1.0[a]	<10.0	HAP	NA	NA
6466	F	NA	8.0	NA	<0.1	39.0	N	Yes	NA

Abbreviations: AAP, aplastic anterior pituitary; F, female; HAP, hypoplastic anterior pituitary; M, male; N, normal; NA,: data not available.
[a]GH peak with one test.
[b]Patient heterozygous for a signal peptide variation. Patients with numbers in bold are the probands whose data are plotted in Figures 3A and 3B.

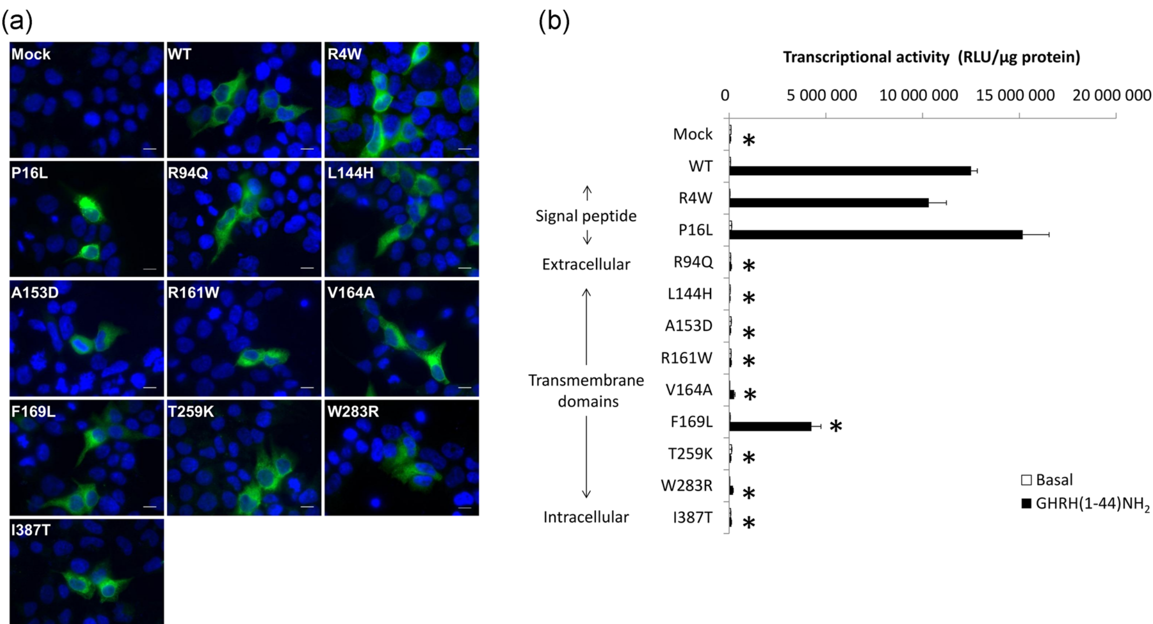

FIGURE 2 Functional characterization of the identified GHRHR missense variants. (a) Expression pattern of the recombinant human normal GHRHR and of the GHRHR variants carrying the missense variants identified in the study. Indirect immunofluorescence was performed on HEK293T cells transiently expressing the different recombinant GHRHR proteins revealed with an anti-GHRHR polyclonal antibody. HEK293T cells were permeabilized with 0.25% Igepal. All slides were scanned under ×100 magnification with the same contrast settings and are representative of two independent experiments. White bars indicate 10 µm. (b) cAMP-mediated transcriptional activity of the different recombinant GHRHR generated in this study. HEK293T cells were transiently cotransfected with 40 ng of expression vectors encoding the normal (WT) GHRHR or each of the GHRHR variants, and 400 ng of the pCRE-luciferase reporter plasmid. Basal activity levels were assessed under DMEM 10% FCS. After transfection of 6 hr, cells were activated with 10^{-7} M synthetic human GHRH-(1-44)-NH_2 peptide for 18 hr before luciferase activity assessment. Results are expressed in relative light units (RLU) normalized by the total amount of protein (in µg). Data are the mean ± *standard deviation* of one representative experiment out of three independent experiments, each performed in triplicate. *$p<.001$

deficit plotted as a function of age at diagnosis revealed that the growth deficit was proportionate to the age at diagnosis and showed a similar slope for patients with bi-allelic truncating mutations and those with at least one missense mutation (i.e.,−0.19 and −0.18 SD per year, respectively; Figure 3c). Analysis of auxologic data in all patients showed that mean GH dynamic tests are similar in both groups (GH in ng/ml ± SD): 1.9 ± 2.0 in patients with truncating mutations and 2.1 ± 4.1 in patients with at least one missense mutation ($p = .8$, Student's *t* test; Figure 3d). IGF-I levels (IGF-I in ng/ml ± SD) are slightly lower in the first group (19.2 ± 15.8) compared with the second one (25.2 ± 20.3); this difference does not reach significance ($p = .4$, Student's t test; Figure 3e).

4 | DISCUSSION

In this study of a large group of patients with IGHD and no *GH1* mutations (312 families), we identified 22 rare *GHRHR* variants, including 20 loss-of-function mutations (truncating or missense mutations), most likely to be disease-causing, and two signal peptide variants with undetectable effect on protein function. The 20 loss-of-function mutations were present in the homozygous or compound heterozygous state in 24 independent probands. Fifteen of those mutations are newly identified disease-causing defects: eight truncating mutations (c.161-1G>A, p.[A8Dfs*22], p.[A91Gfs*13], p.[R156Afs*15], p.[V225Gfs*165], c.[?_−48]_[57 + 1_58-1]del, p.[L201_V295del], p.[W243*]) and seven missense mutations (p.[R94Q], p.[A153D], p.[V164A], p.[F169L], p.[T259K], p.[W283R] and p.[I387T]). All those missense mutations showed a subcellular localization pattern similar to that of the normal GHRHR. However, given the technique used (i.e., immunofluorescence), a targeting defect cannot be excluded. Defective targeting to the plasma membrane is indeed the most common mechanism by which missense mutations located in transmembrane domains of GPCRs impede receptor signaling (Ulloa-Aguirre, Janovick, Miranda, & Conn, 2006). Noteworthy, all but one missense mutations involved in bi-allelic mutant genotypes have completely lost their ability to stimulate the cAMP signaling pathway. The loss of function resulting from the p.(A176V) and the p.(V164A) mutations reveal that even minor changes in the lateral chain of those residues can be deleterious.

The identification of heterozygous missense variants in the signal peptide of GHRHR in IGHD patients with no other *GHRHR* molecular defect has led to the idea that these *GHRHR* variants are associated with a dominant form of IGHD (Godi et al., 2009). Our data do not support this hypothesis. First, the p.(R4W) and p.(P16L) missense variants involve amino acids that are not conserved in vertebrates and are not predicted to disrupt signal peptide maturation. Second, as for the p.(R4W) and the p.(P16L) variants, they have been identified at relatively high allele frequencies in control populations

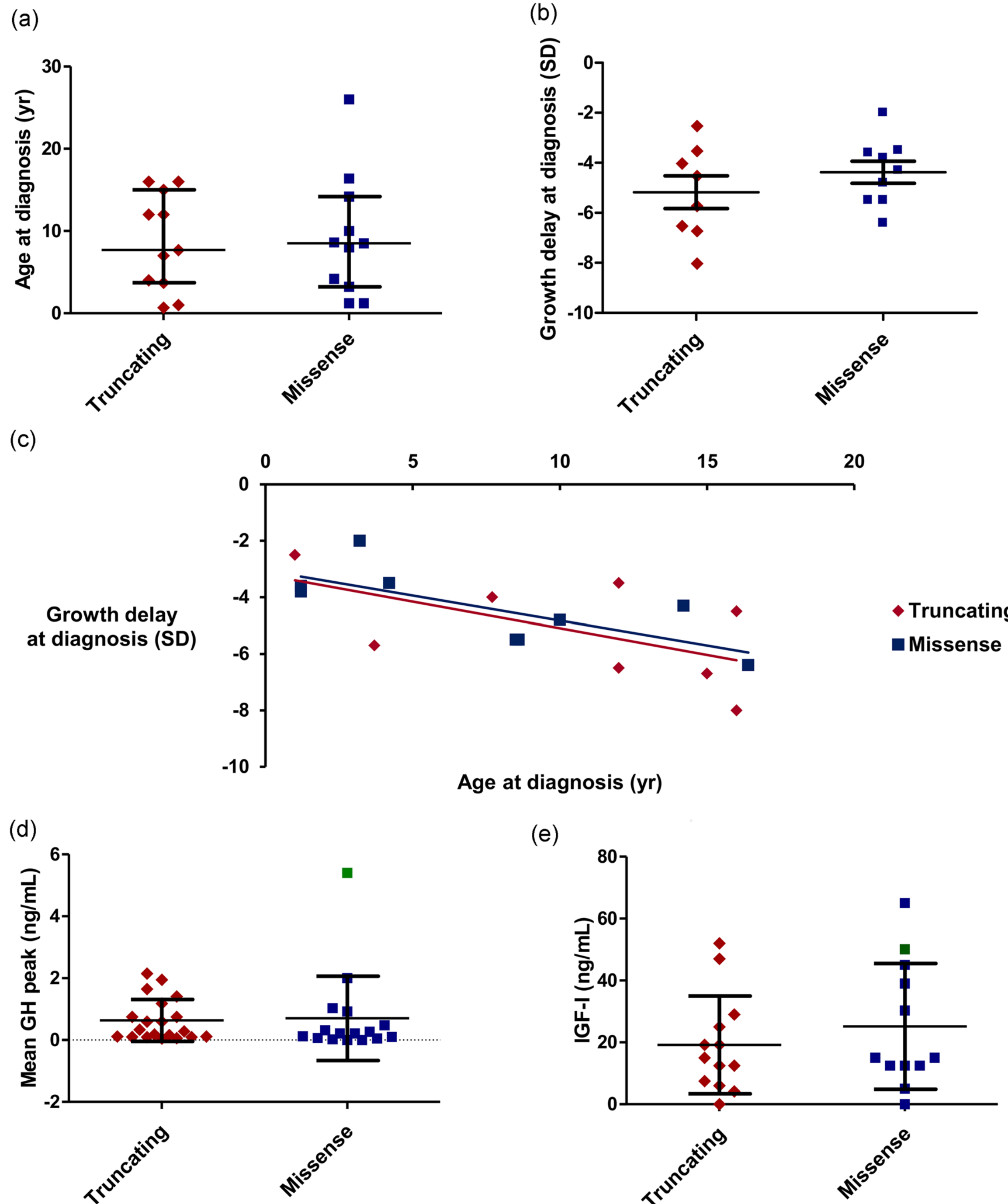

FIGURE 3 Phenotypic data scatter plots for probands with loss-of-function truncating or missense *GHRHR* mutations. (a) Age at diagnosis of the probands in years. Medians are shown with interquartile ranges (22 out of 24 probands). (b) Growth deficit at diagnosis in SDS for the probands carrying bi-allelic truncating mutations or bi-allelic mutations with at least one missense *GHRHR* mutation. Means are shown ± SEM (17 out of 24 probands). (c) Age at diagnosis (in year) and growth deficit (SDS) correlation plot (17 out of 24 probands). (d) GH peak values (in ng/ml) under the stimulation of the patients with *GHRHR* mutations (36 out of 37 patients). Each point represents the value of a single test or the mean of the 2–4 tests performed in one patient. Means are shown ± SD. (e) IGF-I levels in ng/ml for patients with *GHRHR* mutations (25 patients out of 37). The green dot indicates patient #8600 who is compound heterozygous for the missense mutation p.(F169L) and the c.161-1G > A splice acceptor site mutation. When GH or IGF-I values were below the detection limit, half the detection limit was taken as a value. Means are shown ± *standard deviation*

(0.88% [6/682] in Italian controls [Godi et al., 2009] and 0.52% [62/11,840] in the South Asian population from the gnomAD database, respectively). Under the hypothesis of a dominant transmission of the disease phenotype suggested for those variants, this would mean that 1.8% and 1% of subjects from these control populations may have IGHD, an estimate that largely overcomes the prevalence of the disease (1/10,000–1/4,000). In addition, as for the p.(R4W) the variant that involves the same residue as the p.(R4Q)

the variant, Gregory et al. (2016) showed in a recent report that the p.(R4Q) variant does not affect the response of HEK293 cells to GHRH. Third and most important, as shown here, the cAMP-mediated transcriptional activity of the receptor carrying those variants was found to be similar to that of the normal GHRHR. Overall, these data argue against the deleterious effect of all the heterozygous variants so far identified in the GHRHR signal peptide.

Among the 10 loss-of-function missense variants identified in this study, nine were found to be associated with a complete loss of function, while the remaining one (p.[F169L]) resulted in a partial loss of function in vitro. Interestingly, one of the two affected siblings (#8601) who is compound heterozygous for the p.(F169L) mutation and the c.161-1G>A splice acceptor site mutation, showed a normal GH test (9.6 ng/ml under L-DOPA) and another defective test (1.2/L under clonidine), ranging within the highest mean GH peak values at diagnosis. This patient also displayed the second highest IGF-I levels (50.1 ng/ml) among the patients with at least one missense mutation. His older brother (#8600) showed low GH dynamic test (mean: 0.5 ng/ml) and IGF-I values (30.3 ng/ml). Their cousins (#8596 and #8597), who are homozygous for the splice site mutation, showed very low auxologic data (mean GH peak values of, respectively, 0.1 and 0.34 ng/ml; IGF-I <25 ng/ml). However, from a clinical viewpoint, this difference in endocrine investigations and in vitro function does not seem to result in a less severe phenotype in the compound heterozygous patients #8600 and #8601, as the growth deficit at diagnosis (−4.3 and −3.8 SD) was comparable to that of their cousins homozygous for the splice site mutation (−4.5 and −3.8 SD). *GHRHR* screening should, therefore, be considered in patients whose subnormal GH tests suggest a partial IGHD.

Overall, our study shows that *GHRHR* missense loss-of-function mutations represent a major cause of IGHD (11 out of 24 families, 46%). Noteworthy, a majority of cases was sporadic (13 out of 24 independent patients, 54%). Although the proportion of patients born to a consanguineous union is high (15 out of 24 families, 62.5%), this study performed in a large group of patients also underlies the need to look for *GHRHR* mutations in nonconsanguineous patients. Except family #541, in which the father and the mother are, respectively 156 and 145.2 cm tall, the parents who are heterozygous for a loss-of-function mutation were all of the normal height, in keeping with the recessive transmission of the disease phenotype.

Patients with bi-allelic *GHRHR* mutations are diagnosed with IGHD around the same age as those with at least one missense mutation; they also have a similar growth deficit at diagnosis. Mean age at diagnosis (8.6 and 9.2 years) and growth deficit (−5.2 and −4.4 SD) were close to those observed in a previously described group of 15 patients with *GHRHR* mutations (Alatzoglou et al., 2009; respectively $p = .1$ and 0.2; Student's t test). Our study also shows that the growth deficit worsens with age at diagnosis in both groups. Anterior pituitary hypoplasia was observed in most patients (27/33, 82%). Except for patient #8601 (compound heterozygous for the p.[F169L] and c.161-1G>A mutations), GH secretion tests always showed an abnormal somatotropic function in both groups (means of 0.6 and 0.7 ng/ml); IGF-I levels were abnormally low in all cases (means of 19.2 and 25.2 ng/ml). The absence of major differences of the main phenotypic features between patients with bi-allelic truncating mutations and those carrying at least one missense mutation is consistent with the severe functional impairment of the missense mutations observed in vitro.

In the 15 patients for whom length at birth was available, only one presented with IUGR (patient #6487, born at 37.5 weeks of amenorrhea); this proportion (6%) is similar in the general population. This highlights that GHRHR is dispensable for fetal growth in humans, as previously shown in the little mouse that carries the p.(D60G) *Ghrhr* mutation (Eicher & Beamer, 1976; Lin et al., 1993).

In summary, the screening of an unusually large group of patients with nonsyndromic IGHD unveils a significant contribution of *GHRHR* mutations to this heterogeneous condition (8%, 24/312). The inheritance mode of IGHD due to *GHRHR* mutations is clearly recessive and as expected in such cases, the proportion of independent patients born to a consanguineous union is higher among patients with a *GHRHR* mutation than in the study cohort (62.5% vs. 16%). Lastly, this study also unveils that patients with a partial GH deficit could benefit from *GHRHR* screening.

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CONFLICT OF INTERESTS

The authors declared that there is no conflict of interests.

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REFERENCES

Alatzoglou, K. S., & Dattani, M. T. (2010). Genetic causes and treatment of isolated growth hormone deficiency—an update. *Nature Reviews Endocrinology*, *6*, 562–576.

Alatzoglou, K. S., Turton, J. P., Kelberman, D., Clayton, P. E., Mehta, A., Buchanan, C., … Savage, M. O. (2009). Expanding the spectrum of mutations in *GH1* and *GHRHR*: Genetic screening in a large cohort of patients with congenital isolated growth hormone deficiency. *Journal of Clinical Endocrinology and Metabolism*, *94*, 3191–3199.

Alba, M., Hall, C. M., Whatmore, A. J., Clayton, P. E., Price, D. A., & Salvatori, R. (2004). Variability in anterior pituitary size within members of a family with GH deficiency due to a new splice mutation in the GHRH receptor gene. *Clinical Endocrinology (Oxford)*, *60*, 470–475.

Birla, S., Khadgawat, R., Jyotsna, V. P., Jain, V., Garg, M. K., Bhalla, A. S., & Sharma, A. (2016). Identification of novel GHRHR and GH1 mutations in patients with isolated growth hormone deficiency. *Growth Hormone & IGF Research*, *29*, 50–56.

Bona, G., Paracchini, R., Giordano, M., & Momigliano-Richiardi, P. (2004). Genetic defects in GH synthesis and secretion. *European Journal of Endocrinology.*, *151*(Suppl1), S3–9.

Carakushansky, M., Whatmore, A. J., Clayton, P. E., Shalet, S. M., Gleeson, H. K., Price, D. A., … Salvatori, R. (2003). A new missense mutation in the growth hormone-releasing hormone receptor gene in familial isolated GH deficiency. *European Journal of Endocrinology*, *148*, 25–30.

Corazzini, V., & Salvatori, R. (2013). Molecular and clinical aspects of GHRHR receptor mutations. *Endocrine Development*, *24*, 106–117.

Desai, M. P., Mithbawkar, S. M., Upadhye, P. S., Rao, S. C., Bhatia, V., & Vijaykumar, M. (2013). Molecular genetic studies in isolated growth hormone deficiency (IGHD). *Indian Journal of Pediatrics*, *80*, 623–630.

Eicher, E. M., & Beamer, W. G. (1976). Inherited ateliotic dwarfism in mice. Characteristics of the mutation, little, on chromosome 6. *Journal of Heredity*, *67*, 87–91.

Frohman, L. A., & Kineman, R. D. (2002). Growth hormone-releasing hormone and pituitary development, hyperplasia, and tumorigenesis. *Trends in Endocrinology and Metabolism*, *13*, 299–303.

Godi, M., Mellone, S., Petri, A., Arrigo, T., Bardelli, C., Corrado, L., … Giordano, M. (2009). A recurrent signal peptide mutation in the growth hormone releasing hormone receptor with defective translocation to the cell surface and isolated growth hormone deficiency. *Journal of Clinical Endocrinology and Metabolism*, *94*, 3939–3947.

Graaff, L. C. G. De, Argente, J., Veenma, D. C. M., Herrebout, M. A. C., Friesema, E. C. H., Uitterlinden, A. G., … Hokken-Koelega, A. C. S. (2009). Genetic screening of a Dutch population with isolated GH deficiency (IGHD). *Clinical Endocrinology (Oxford)*, *70*, 742–750.

Gregory, L. C., Alatzoglou, K. S., Mccabe, M. J., Hindmarsh, P. C., Saldanha, J. W., Romano, N., … Dattani, M. T. (2016). Partial loss of function of the GHRH receptor leads to mild growth hormone deficiency. *Journal of Clinical Endocrinology and Metabolism*, *101*, 3608–3615.

Isberg, V., Mordalski, S., Munk, C., Rataj, K., Harpsøe, K., Hauser, A. S., … Gloriam, D. E. (2016). GPCRdb: An information system for G protein-coupled receptors. *Nucleic Acids Research*, *44*, D356–D364.

Kremmidiotis, G., Lensink, I. L., Bilton, R. L., Woollatt, E., Chataway, T. K., Sutherland, G. R., & Callen, D. F. (1999). The Batten disease gene product (CLN3p) is a Golgi integral membrane protein. *Human Molecular Genetics*, *8*, 523–531.

Lee, E. J., Duan, W. R., Kotlar, T. O. M., & Jameson, J. L. (2001). Restoration of growth hormone-releasing hormone (GHRH) responsiveness in pituitary GH3 cells by adenovirus-directed expression of the human GHRH receptor. *Endocrinology*, *142*, 414–420.

Lin, S. C., Lin, C. R., Gukovsky, I., Lusis, A. J., Sawchenko, P. E., & Rosenfeld, M. G. (1993). Molecular basis of the little mouse phenotype and implications for cell type-specific growth. *Nature*, *364*, 208–213.

Lindsay, R., Feldkamp, M., Harris, D., Robertson, J., & Rallison, M. (1994). Utah Growth Study: Growth standards and the prevalence of growth hormone deficiency. *Jornal de Pediatria*, *125*, 29–35.

Mullis, P. E. (2010). Genetics of isolated growth hormone deficiency. *Journal of Clinical Research in Pediatric Endocrinology*, *2*, 52–62.

Pantel, J., Legendre, M., Cabrol, S., Hilal, L., Hajaji, Y., Morisset, S., … Epelbaum, J. (2006). Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature. *Journal of Clinical Investigation*, *116*, 760–768.

Procter, A. M., Phillips, J. A., & Cooper, D. N. (1998). The molecular genetics of growth hormone deficiency. *Human Genetics*, *103*, 255–272.

Ulloa-Aguirre, A., Janovick, J. A., Miranda, A. L., & Conn, P. M. (2006). G-protein-coupled receptor trafficking: Understanding the chemical basis of health and disease. *ACS Chemical Biology*, *1*(10), 631–638.

Vimpani, G. V., Vimpani, A. F., Lidgard, G. P., Cameron, E. H., & Farquhar, J. W. (1977). Prevalence of severe growth hormone deficiency. *British Medical Journal*, *2*, 427–430.

Vnencak-Jones, C. L., Phillips, J. A., & De-Fen, W. (1990). Use of polymerase chain reaction in detection of growth hormone gene deletions. *Journal of Clinical Endocrinology and Metabolism*, *70*, 1550–1553.

Wagner, J. K., Eblé, A., Hindmarsh, P. C., & Mullis, P. E. (1998). Prevalence of human GH-1 gene alterations in patients with isolated growth hormone deficiency. *Pediatric Research*, *43*, 105–110.

Wajnrajch, M. P., Gertner, J. M., Harbison, M. D., Chua, S. C., & Leibel, R. L. (1996). Nonsense mutation in the human growth hormone-releasing hormone receptor causes growth failure analogous to the little (lit) mouse. *Nature Genetics*, *12*, 88–90.

Wajnrajch, M. P., Gertner, J. M., Sokoloff, A. S., Ten, I., Harbison, M. D., Netchine, I., … Leibel, R. L. (2003). Haplotype analysis of the growth hormone releasing hormone receptor locus in three apparently unrelated kindreds from the indian subcontinent with the identical mutation in the GHRH receptor. *American Journal Of Medical Genetics. Part A*, *120A*, 77–83.

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