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RESEARCH

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Screening of GHSR, GHRHR, GH1 genes in isolated growth hormone deficiency disease in Egyptian patients

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Abstract

Background Isolated growth hormone deficiency (IGHD) is a hereditary disorder that causes significant short stature. GHD has a reported incidence of 1/4000–1/10,000 births. It is caused by mutations in the major somatotroph axis genes, involving GH1, codes for growth hormone, GHSR, and GHRHR, codes for growth hormone secretagogue receptor and growth hormone-releasing hormone receptor, respectively.

Aims of the study The present study aims to examine the clinical phenotype and investigate the genetic etiology of ten Egyptian patients with type I isolated growth hormone insufficiency.

Patients and methods Patients recruited for the study were clinically diagnosed by two provocation tests and were subjected to a thorough history, clinical examination, and anthropometric measurements. Sanger sequencing and mutational analysis of the three genes, GH1, GHSR, and GHRHR, was our approach, performed in all enrolled IGHD patients. The variants identified were analyzed using the biological, population, sequence variants, and clinical genetics databases. Prediction of the pathogenicity of the novel variants was done by in silico prediction tools following the American College of Medical Genetics and Genomics (ACMG) guidelines.

Results Sanger sequencing revealed a previously reported pathogenic mutation (NM_000823.4: c.1069C > T; p.Arg357Cys) in the GHRHR gene in one patient and a novel frameshift variant (NM_198407.2: c.1043dup; Ser349Leu fs*6) in the GHSR gene in another patient. This is the fourth report highlighting the autosomal dominant inheritance of the GHSR mutation as a cause of isolated growth hormone deficiency. A number of previously reported variants, but of rare frequency, were identified in this study. In our IGHD cases, 90% of the patients were underweight, 50% had anemia, and 80% showed hypovitaminosis D.

Conclusion Our findings broaden the mutational spectrum underlying the IGHD in Egyptian patients and point out the importance of mutation screening of the GHSR and GHRHR genes. This study also acknowledges the autosomal dominant mode of inheritance of the GHSR mutation as a cause for dwarfism.

Keywords Growth hormone deficiency, GH1 gene, GHRHR gene, GHSR gene, Sanger sequencing analysis

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Background

Human growth hormone (HGH), termed somatotropin, consists of one polypeptide chain, which is formed of 191 amino acids and is released from the somatotropic cells of the anterior pituitary gland [1]. HGH is developed under tight regulation by several complex feedback mechanisms, of which growth hormone-releasing hormone (GHRH), produced by the hypothalamus, has the main regulatory role. GH functions by regulating the growth of every tissue and organ in the body during childhood. GH mainly controls the growth of cartilage and bone in adolescents [2].

Growth hormone deficiency (GHD) is an endocrine disorder that occurs in children and adults. Congenital brain malformations, genetic defects in genes that are associated with the somatotropic axis, or pituitary development may lead to GHD. Surgery, infection, infiltrative disorders, midline tumors, cranial irradiation, and trauma are among the acquired causative conditions. GHD could be idiopathic without an obvious cause, isolated GHD, or in association with deficiencies of other pituitary hormones [3].

Isolated growth hormone deficiency (IGHD) is usually manifested by proportionate growth retardation and is accompanied by decreased growth velocity, delayed bone maturation, and dentition. Patients may have delayed puberty until their late teens, but they are usually fertile [4]. An estimated 1/4000 to 1/10,000 live babies are affected by IGHD [5]. IGHD frequently appears in families as a sporadic condition; however, familial cases with genetic etiology appear in about 30% of cases [6].

Familial IGHD has been subdivided into four types according to the mode of inheritance and severity of GH deficiency: Type IA (OMIM#262400), IB (OMIM#612781), and IV (OMIM #618157) (autosomal recessive inheritance), II (OMIM#173100) (autosomal dominant inheritance), and III (OMIM#307200) (X-linked inheritance). Type IA is characterized by the infantile onset of severe growth failure (SDS < −4.5), a complete absence of GH in the serum, and is caused by biallelic mutations in the Growth Hormone 1 (GH1) gene (OMIM1#39250). Type IB is a milder form with low but measurable quantities of GH, due to mutations in the GH1 gene. Type II is characterized by detectable low levels of GH; however, the age of onset and degrees of short stature vary. It is usually caused by splicing mutations in exon 3 of the GH1 gene that lead to exon skipping and the production of truncated proteins. Type III is characterized by agammaglobulinemia and is caused by mutations in the Bruton agammaglobulinemia Tyrosine Kinase (BTK) gene (OMIM#300300). Type IV is characterized by early-onset, severe growth hormone deficiency (SDS up to -7.4) and is caused by mutations in the growth hormone-releasing hormone receptor (GHRHR) gene (OMIM#139191). Another type is called growth hormone deficiency, which is an isolated partial (OMIM#615925) of autosomal dominant and recessive inheritance and is due to homozygous, heterozygous, or compound heterozygous mutations in the growth hormone secretagogue receptor (GHSR) gene (OMIM#601898).

Growth hormone 1 (UniProtKB/Swiss-Port: P01241), also called somatotropin, is encoded by the GH1 gene, located on chromosome 17q23.3. This gene spans 1640 bps and contains 5 exons. It is crucial for the regulation of growth. Its major role is to promote insulin growth factor-1 (IGF-1) secretion from hepatic and non-hepatic tissues. Growth hormone-releasing hormone receptor (UniProtKB/Swiss-Port: Q02643) is encoded by the GHRHR gene, located on chromosome 7p14.3. This gene spans 54,586 bps and contains 13 exons. The growth hormone secretagogue receptor (UniProtKB/Swiss-Port: Q92847) is encoded by the GHSR gene, located on chromosome 3q26.31. This gene spans 5,166 bps and contains 2 exons. It encodes a protein that has a role in body weight regulation and energy homeostasis. This protein is part of the G-protein-coupled receptor family.

GHD is a treatable condition, and GH therapy is strongly recommended for children and adolescents with GHD to normalize adult height and avoid significant short stature [7]. GHD diagnosis depends on the laboratory investigation of GH secretion in a stimulation test setting [8]. Due to the pulsatile secretion nature of GH, these test results should not be the sole diagnostic criterion [7]. A GHD diagnosis must carefully evaluate clinical history, growth measurements, and physical examination [3]. In the presence of parental consanguinity or a positive family history, a genetic diagnosis should be considered, though it is not always conclusive [9].

In the present research, we aimed to study the clinical phenotype of ten Egyptian patients with IGHD Type I. And to investigate the genetic etiology in these patients through mutational analysis of GH1, GHRHR, and GHSR genes that are known to play essential roles in the formation or activity of GH.

Patients and methods

Patients

The study includes ten unrelated Egyptian patients with Type I isolated growth hormone deficiency who were referred to the outpatient Clinical Genetics clinic of the National Research Centre's (NRC). The research was reviewed and approved by the Ethical Committee of the NRC. Written, informed consent was obtained from all participants.

The enrolled patients met the following criteria: age between 7 and 17 years old, decreased height (< −2.5

SD), low growth hormone level response (≤10 ng/ml), and absence of any associated congenital disorders. All patients were subjected to a thorough history and pedigree construction, clinical examination, anthropometric measurements, and investigation of related laboratory parameters. A thyroid function test with thyroid-stimulating hormone (TSH) and free thyroxine T4 (FT4) was performed to exclude hypothyroidism as a cause of short stature. Karyotype in all female patients was performed to exclude Turner syndrome. Two GH provocative tests assessed GH level response (Clonidine and Insulin Tolerance Test; ITT).

Mutational analysis

The extraction of genomic DNA from peripheral blood lymphocytes was done using the PAXgene Blood DNA Kit (Qiagen, Germany). Twenty pairs of unique and overlapping primers were designed by primer3 software to amplify the 5 coding exons of the GH1 (NM_000515.5), the 13 coding exons of the GHRHR (NM_000823.4), and the 2 coding exons of the GHSR (NM_198407.2) genes (Table 1). Purification and sequencing of the amplified fragments using the Exo-SAP PCR Clean-up Kit (Fermentas, Germany) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), respectively, were done. Sequencing results were aligned against the reference genomic (GRCh38) and transcript sequences. The analyses of the identified sequence variations and the prediction of the pathogenicity of the novel variants were done according to the guidelines of the American College of Medical Genetics and Genomics (ACMG) guidelines [10, 11]. The biological, population, and clinical genetics databases used involved VarSome [12], NCBI dbSNP [13], genome aggregation database (gnomAD) [14], combined annotation dependent depletion (CADD) [15], and NCBI ClinVar [16].

Statistical methods

We used the Microsoft Excel program version 2016 to make the statistical analysis. Minimum, maximum, mean, standard deviation (SD), and median were used to represent the data quantitatively.

Results

The study included ten unrelated Egyptian patients diagnosed to have IGHD Type I (seven males and three females) with a male-to-female ratio of 2.3:1. Their average age was 12.034 ± 3.4 years, within the range of 7–17 years. A positive family history was present in 8 of 10 patients. All the enrolled patients presented clinically with short stature. The mean height of patients was 129.68 ± 12.5 cm (males 132.59 ± 11, females 122.9 ± 15.4). Isolated growth hormone deficiency was established using a growth hormone stimulation test done by clonidine and an insulin tolerance test (ITT). The medical assessments and laboratory investigations of all patients are summarized in Table 2. The mean, median, and range of the anthropometric, hormonal,

Table 1 PCR primers for amplification of the coding exons of the GH1, GHRHR, and GHSR genes

Gene	Exon No	Primer sequence	Amplified fragment size (bp)
GH1	1	TAAAAAGGGCCCACAAGAGA GGCCAAATACTGGGCTTACA	238
	2	AAAGTCACCCCTTCCTGCC CCTCTGTTGCCCTCTGGTT	242
	3	CTAGGTTCTGCAGGGGAAGG CGCTGAGTGAGGTTCCCA	297
	4	AGGGCAGCAGTGTTTCTCTA CCGTGAGTGGATGCCTTCT	337
	5	GGAGGGGTCACAGGGATG TGAGAAAGGGAGGGAACAGT	290
GHRHR	1	CTGAGAAGGGGAAGCAGAGG GAGGGTCTCAGCTGGCTAC	194
	2 & 3	ATGAATCAGGCCTTGTCCCT TCCAGATGAAAGCACCTCCC	485
	4	CCACCCTCTCTGTTGCTCA GCACCCACCCGATACAAATG	289
	5	GCTTCACCTGCTTGATTGTCA ATGGGTATGGCGCCTAGATC	296
	6	GATTCGATTCACCTCCTGCC GGTGACATGGGAAAGGAGCA	392
	7	GGAGGTTCTGTATCTGAGTAGGG TCTGCATCCTTGACTCTGAGA	500
	8	CTACGTGGCTGATGGTGGT GCCTGACTGTCCACTCCAC	221
	9	GGAGGCATTGAACAGAGTTCA TGGTGGTAAATGATCTGCAACC	571
	10	CCATCTCCAGGCTACCAGTT GTATGGGGCTGAGGTCATGA	365
	11	TGAGAGGAGATGAAGTGCACA CCTCCAGCACCCTCAATGG	250
	12	TAGCAGAAAGACGGTGGACA ACAAGAGTGAAGGTGTGGC	235
	13	TGCCCCATGTCTCTGTTTCT AGCTGCCCAAATTCAAGTGT	286
GHSR	1	ACTGAAGAAAGAGGTAGCGACT AACTTCGGCGACCTCCTC	588
	1	GGCCCAGATGACGAAGATGA CGTCCCAGAGCCTGTTCA	539
	2	CAGCTTCCTCCCAAGTTCTG TGTTTCTCTGAAGTCAATGGTCA	500

Table 2 Clinical assessments and laboratory investigations of ten IGHD Type I Egyptian patients

Patients	Sex	Age	Hight (cm)	Weight (Kg)	Family history	GH clonidine	GH ITT	TSH	FT4	HB%	Ca^{+2}	Ph^{-3}	Alkaline Phosphatase	Vit.D
1	f	7y	106	15	+ 2 Sibs	7	4.8	2.1	1.3	11.2	11.5	5.1	138	33
2	f	11y 8 m	126.5	23	+ Grandmother	1.2	3.8	2.3	1.7	11.9	9.6	5.4	185	14
3	f	13y	136.2	31	+ 1 Sib	4.6	3.9	2.2	1.3	12.1	7	5	167	9
4	m	7y 8 m	120	17	+ Grandmother	4.2	1.3	1.5	1.3	10.6	10.2	5.2	172	22
5	m	9y 3 m	130	32	+ Grandmother	1.7	1.2	3.1	1.62	11.8	9.8	4.6	205	11
6	m	11y	123	20	No FH	7.6	3.9	1.3	1.4	11.1	9.9	5.6	201	11
7	m	12y 3 m	124	27	No FH	9.6	8.2	–	–	13.3	9.4	4.1	169	7
8	m	15y 6 m	139.3	42	+ Mother and aunt	4	2.2	–	–	11.6	9.6	4.9	254	8.5
9	m	16y	145.3	35	+ Fatherand 1 sib	4.5	2.4	–	–	12	9.5	5	160	15
10	m	17y	146.5	36	+ Grandmother	9.5	9.3	3.6	1.5	11.5	8.5	5.5	129	12

and laboratory parameters for all patients are represented in Table 3.

All our IGHD cases were underweight for their age, except P5, who has a normal weight. Anemia was found in 50% of the studied cases, while 80% of them have hypovitaminosis D. The studied patients had a normal total serum calcium concentration, except for P1 (hypercalcemia) and P3 (hypocalcemia). Normal levels of serum phosphate were seen in all patients except for P7 (hypophosphatemia) and P10 (hyperphosphatemia). The alkaline phosphatase level was normal in all cases. All our patients have normal thyroid function.

Normal ranges of the biological parameters: GH clonidine, GH ITT: > 10 ng/mL [17]; TSH: 0.5–4.0 µU/mL; FT4: 0.8–1.8 ng/dL; HB% (4 to < 14 years): 11.4–14.1 g/dL[18], HB% (14 to < 21 years: female): 11.3–14.9 g/dL, HB% (14 to < 21 years: male): 12.9–16.5 [19]; Ca^{+2} (4 to < 12 years): 8.7–10.7 mg/dL, Ca^{+2} (12–18 years): 8.5–10.7 mg/dL[20].; Ph^{-3} (5 to < 13 years): 4.3–6.3 mg/dL, Ph^{-3} (13 to < 16 years: female): 3.3–5.9 mg/dL, Ph^{-3} (13 to < 16 years: male):3.7–6.5 mg/dL, Ph^{-3} (16 to < 19 years): 3–5.3 mg/dL[21].; Alkaline Phosphatase (1 to < 10 years): 156–369 U/L, Alkaline Phosphatase (10 to < 13 years): 141–460 U/L, Alkaline Phosphatase (13 to < 15 years: female): 62–280 U/L, Alkaline Phosphatase (13 to < 15 years: male): 127–517 U/L, Alkaline Phosphatase (15 to < 17 years: female): 54–128 U/L, Alkaline Phosphatase (15 to < 17 years: male): 89–365 U/L, Alkaline Phosphatase (17 to < 19 years: female): 48–95 U/L, Alkaline Phosphatase (17 to < 19 years: male): 59–164 U/L[21].; Vit D (25-Hydroxyvitamin D) sufficiency: > 20 ng/mL, Vit D insufficiency: 12–20 ng/mL, Vit D deficiency < 12 ng/mL [22].

Table 3 The mean, median, and range of the anthropometric, hormonal, and laboratory parameters for all patients

Parameters	Mean ± SD	Median	Range	
			Min	Max
Age (year)	12.03 ± 3.44	11.96	7.00	17.00
Weight (Kg)	27.80 ± 8.90	29.00	15.00	42.00
Height (cm)	125.88 ± 16.05	125.25	102.00	146.50
Height, SDS	− 3.44 ± 0.63	− 3.39	− 4.40	− 2.60
GH clonidine (ng/mL)	5.39 ± 2.94	4.55	1.20	9.60
GH ITT (ng/mL)	4.1 ± 2.59	3.85	1.20	9.3
TSH (µU/mL)	2.30 ± 0.68	2.20	1.30	3.60
FT4 (ng/dL)	1.47 ± 0.16	1.45	1.30	1.70
HB% (g/dl)	11.71 ± 0.72	11.70	10.60	13.30
Ca^{+2} (mg/dL)	9.50 ± 1.16	9.60	7.00	11.50
Ph^{-3} (mg/dL)	5.04 ± 0.45	5.05	4.10	5.60
Alkaline phosphatase (U/L)	178.00 ± 35.97	170.50	129.00	254.00
Vit D (ng/mL)	14.25 ± 7.83	11.50	7.00	33.00

SDS: Standard deviation score

All children's heights in the study group were below − 2 SDS compared to normal children with the matched sex and age group; their mean was − 3.44 ± 0.63. Our IGHD children have hypovitaminosis D, with a mean of 14.25 ± 7.83.

The sequencing analysis of the three studied genes showed four novel variants: one in the GH1 and GHSR genes, two in the GHRHR gene, and three rare previously reported variants: one reported missense pathogenic mutation in the GHRHR gene and two reported variants with uncertain significance in the GHRHR and the GHSR genes. Table 4 summarizes the in silico analysis of the detected novel and rare previously reported variants.

Sequencing of the GH1 five exons revealed one novel splice site variant at intron 3 (c.292–8 G > T). This variant was heterozygous in 5 of 10 (50%) of patients. Varsome and gnomeAD showed that the c.292–8 G > T variant is located at a position with a negative phyloP score, indicating that it is fast evolving. The delta score of the SplicAI tool indicated a low probability of the variant altering splicing. The MaxEnScan tool also showed no abrogation of the potential splice site by the c.292–8G > T variant. The CADD and MutationTaster2 in silico prediction tools for pathogenicity showed it as "likely benign." No variants classified as likely pathogenic or pathogenic were reported.

Sequencing of the thirteen exons of the GHRHR gene detected a previously reported pathogenic missense mutation in exon 11 (c.1069 C > T; p.Arg357Cys) (Fig. 1). The c.1069 C > T was homozygous in one patient (P5). Segregation analysis showed that the parent has heterozygous alleles and a normal phenotype. Pedigree analysis (Fig. 1) revealed that the parent has a consanguineous marriage and that the family has a history of a short-statured grandmother. A previously reported variant (c.367–54C > T) located in intron 4 of the GHRHR gene was also detected in a homozygous state in all patients. This variant has no specific clinical significance reported on ClinVar. The bioinformatics analysis showed that it is fast-evolving, has a low probability of altering splicing, and is likely benign. In the GHRHR, two novel homozygous, intronic variants (c.367–56 C > T and c.367–49 C > T) were also identified in all of our patients; these variants also had low probabilities of altering splicing. They were predicted to be benign or likely benign.

Sequencing of the two exons of the GHSR gene identified a novel frameshift heterozygous variant in exon 2 (c.1043dup) (Fig. 2), in one patient (P4). Segregation analysis showed that the mother has a heterozygous variant and a normal phenotype, while the father has the wild alleles and a normal phenotype. A positive family history

Table 4 In silico analysis of the novel variants and previously reported VUS in the three genes

Gene	Variant		phyloP Score	SpliceAI	MaxEntScan	gnomAD	1KGP	CADD	MutationTaster2
GH1	c.292-8G > T	Novel	− 1.289	0.03 (Acceptor gain)	No abrogation of potential splice site, distance from splice site 8	–	–	0.522 (Likely benign)	Benign
GHRHR	c.367-56C > T	Novel	− 0.115		No abrogation of potential splice site	0.00000657	–	2.951 (Likely benign)	Benign
	c.367-49C > T	Novel	− 0.379		No abrogation of potential splice site	–	–	2.555 (Likely benign)	Benign
	c.367-54C > T	PR	− 0.061	0.02 (Acceptor gain)	–	0.00001314	0.0002	4.75 (Likely benign)	–
GHSR	c.1021G > A	PR	0.507	0 (No consequence)		0.00000658		11.8 (Likely benign)	–
	c.1043dup	Novel	1.138–3.268 (PhastCons: 1)		No abrogation of potential splice site	–	–		Deleterious Frameshift variant Amino acid changed (S349Lfs*6) Protein features might be affected

Results highlighted the benign nature of the novel variants and the low CADD score, which was less than 15 in all cases. The GHSR novel duplication variant was the only change that could be deleterious and affect protein stability or function

VUS: Variant with uncertain significance; 1KGP: 1000 genome project; CADD: combined annotation dependent depletion; gnomAD: the genome aggregation database; MaxEntScan: maximum entropy scan; PhyloP: phylogenetic *p*-values; PhastCons: phylogenetic analysis with space/time models-conservation; S349Lfs*6: a variant with Ser349Leu, frame-shift, terminating at position Ter6; PR: previously reported

of the grandmother is highlighted in the pedigree. Using the MutationTaster2 tool, this variant was predicted to be deleterious causing an amino acid sequence change (S349Lfs*6) that leads to protein truncation (− 13 AA, less than 10% of the reading frame is missing) and might lead to nonsense-mediated mRNA degradation. Protein sequence conservation analysis using PhyloP and PhastCons tools showed the wild-type nucleotide to be conserved. A previously reported missense variant (c.1021 C > T; p.Glu341Lys) was also identified in exon 2 of the GHSR gene in one of our patients (P10). It has no clinical report in the ClinVar database. Variant analysis showed the c.1021 C > T to have a less conservative position, no consequence on splicing, and to be "likely benign" (Additional file 1).

Discussion

Isolated growth hormone deficiency is a common condition that results from abnormalities in the synthesis or activity of GH. It is usually manifested by proportionately short stature, growth retardation, delayed bone maturation, and dentition [4]. The diagnosis of a GHD child involves many steps, including reporting the case clinical history and examination, testing biochemical variables, capturing x-ray images on the pituitary gland, and screening for gene mutations in patients with congenital GHD [23]. In our cohort of IGHD type I, 80% had a positive family history of short stature, pointing to a familial disease. This is the highest frequency ever published, where most of the GHD cases reported worldwide were sporadic and only 5–30% were familial [24–26].

All of our patients' cohorts were in the childhood age group, with a mean age of 12.03 ± 3.4 years. The dominance of males in our study agrees with the literature that boys treated for GH-short stature are two times that of treated girls. This sex ratio relation to short stature was proven in many healthcare systems and different countries [27], involving Japan, the USA, Europe, Australia, and New Zealand [28]. Several explanations of gender bias were hypothesized, including the social and cultural acceptance of girls being short, the delayed growth and puberty in boys, the lower biochemical indications in boys before puberty like serum growth hormone-binding protein (GHBP), serum IGF-1 (Insulin-like growth factor-1), IGF-binding protein-3, and the IGF1/IGFBP3 ratio [27]. The pulsatile character of GH concentrations in blood bias standard estimations of serum GH as environmental stress and condition affect GH levels. Therefore, we performed the GH stimulation tests with clonidine and ITT, guided by several studies [2, 29]. All of our patients' results showed low but detectable circulating GH.

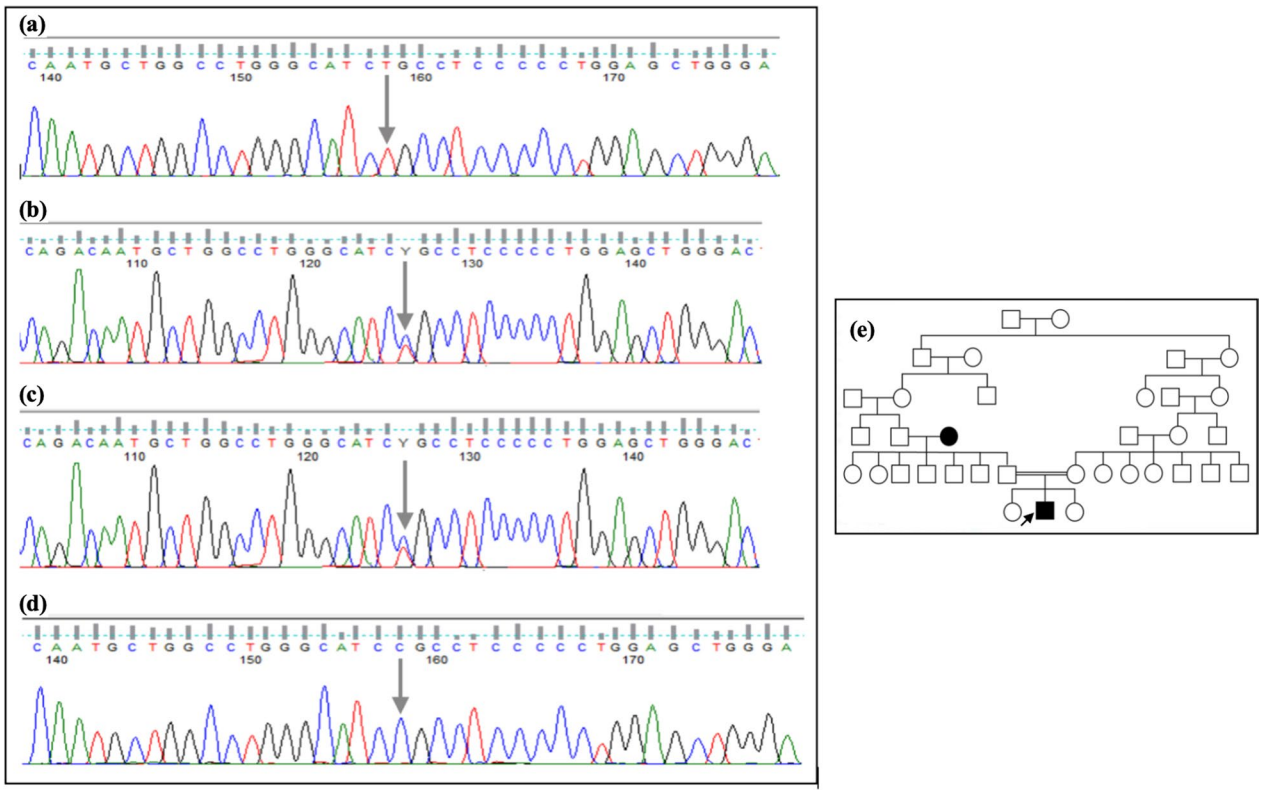

Fig. 1 Sanger sequencing results of the c.1069C > T missense variant in the GHRHR gene in Family 5. The sequencing results in **a** Patient5 (P5) shows a homozygous variant, **b** and **c** his mother and father show the heterozygous variant, **d** the sequencing result of a normal allele; **e** the pedigree of Family 5 shows the affected patient and his grandmother

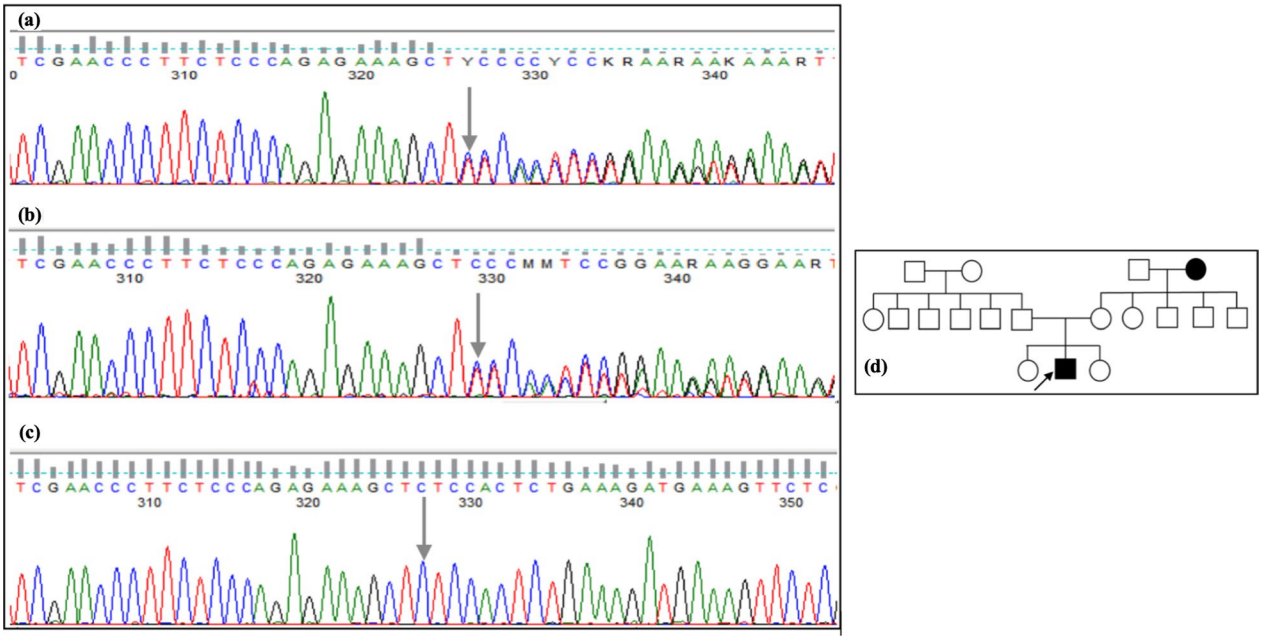

Fig. 2 Sanger sequencing results of the c.1043dup frame-shift variant in the GHSR gene for Family 4. The sequencing results in **a** and **b** patient (P4) and his mother show the heterozygous variant, respectively, **c** his father shows the wild alleles. **d** The pedigree of Family (4) shows the affected patient and his grandmother

In our study, 90% of IGHD cases were underweight, based on their matched age and gender. This was not in line with earlier studies [30, 31]. Growth hormone opposes the action of insulin and stimulates lipolysis; therefore, GHD is anticipated to promote the storage of fat [30].

Thyroid analysis revealed normal TSH and FT4 values in our IGHD cases. Examination of the thyroid function is crucial, as the presence of hypothyroidism accompanied by GHD may indicate combined pituitary hormone deficiency. In addition, isolated thyroid hormone deficiency is a prevalent cause of idiopathic low stature (ILS), reported in 16% of 181 ILS cases [32]. In 80% of our IGHD study participants, hypovitaminosis D was exhibited. Hamza et al. [33] reported the same in 50 GHD patients: that in 84% of them, decreased vitamin D levels were revealed. Moreover, they reported that substitutive rGH treatment normalizes the biological index in more than half of GHD cases with initial hypovitaminosis D.

In 50% of the IGHD cases that we evaluated, there was anemia. This may be explained in light of reports revealing that idiopathic GHD among kids is linked to reduced Hb levels [34]. Additionally, Esposito et al. [35] showed that Hb levels were raised in anemic GHD children taking GH therapy. Finally, serum calcium and phosphate levels were within the normal range in 80% of our IGHD cases. This contrasts with Klatka et al. [36], who recognized a decrease in calcium and phosphorus levels in GHD children.

From the genetic aspects, congenital IGHD is a heterogeneous disorder resulting from mutations in the main somatotroph axis genes, GH1 (that codes for growth hormone), GHSR, and GHRHR, that code for growth hormone secretagogue receptor and growth hormone-releasing hormone receptor, respectively [5, 37–40]. It was established that IGHD cases are caused by mutations in the GH1 gene and, to a lesser extent, mutations in the GHRHR or GHSR genes [5, 37, 41, 42].

Sequencing of the three genes in our group of Egyptian patients revealed previously reported pathogenic mutations (NM_000823.4: c.1069C > T; p.Arg357Cys) in the GHRHR gene and a novel frameshift variant (NM_198407.2: c.1043dup; Ser349Leu fs*6) in the GHSR gene with a high probability of being pathogenic, according to the in silico study using the MutationTaster2 tool and conservation analysis by PhyloP and PhastCons. No variants classified as likely pathogenic or pathogenic were found in the GH1 gene.

The c.1069C > T; p.Arg357Cys variation in exon 11 of the GHRHR gene was found to be homozygous in one of our patients (P5). This patient had a family history of a short grandmother, suggesting a familial disease. The consanguineous marriage of the parents and their heterozygous alleles confirmed the autosomal recessive inheritance. The only study that reported this variant was that of Haskin et al. [43], who found the variant to be homozygous in ten patients of two highly consanguineous families of Arab–Israeli origin. The in vitro functional assay demonstrated the complete inactivity of the mutant receptor. Haskin et al. [43] reported that the extracellular loops and the transmembrane domains provide critical information for achieving a specific interaction of GHRH with its receptor. Therefore, the substitution of Arg with Cys eliminates a basic moiety that might be critical for this interaction. Like in our group of patients, their patients have manifested low levels of GH, short stature, and growth retardation since early childhood. However, they also reported a good growth response to GH treatment, while we did not study this point. Interestingly, they found a relatively high prevalence of the mutant allele (2%) in their controls. They attributed that to the high consanguinity within their population. ClinVar dataset revised c.1069C > T variation as a germline pathogenic mutation in 2020 by PerkinElmer Genomics, which reported it in association with IGHD, Type IB.

In a previous study of an Egyptian family [44], a new biallelic frameshift mutation in exon 4 (c.391delG) of the GHRHR gene was detected in the father and three of his offspring with autosomal recessive IGHD. It was found that this mutation led to an in-frame termination codon (TAG) located 85 base pairs after the mutation, leading to a protein truncation lacking the whole transmembrane domains and intracellular terminus. In their study, there were no hotspot mutations reported in the GHRHR gene [44]. In the present study, the c.391delG mutation was not found in any of the patients.

Pantel et al. [38] reported a functionally significant GHSR mutation that was segregated in the heterozygous state with IGHD and short stature in two unrelated Moroccan families. They reported the GHSR gene as a new molecular etiology for IGHDs. Further genomic testing proved that the GHSR missense mutation causes isolated IGHD in unique ethnic categories like Moroccan with autosomal dominant [38] and autosomal recessive inheritance [45], Brazilian [46] (autosomal dominant inheriting), and Japanese [47] (autosomal dominant inheriting). Fritez et al. [48] showed that the contribution of GHSR mutations to the etiology of IGHD is 6% in the Moroccan population [48]. In our research, this is the fourth report proving the autosomal dominant inheritance of GHSR as a cause of IGHD.

The novel heterozygous c.1043dup frameshift variant in the GHSR gene in P4 has a positive family history of a short grandmother, suggesting a familial disease. The patient's mother was found to have the heterozygous variant but with a normal stature phenotype, suggesting

the possibility of a generation-skipping phenomenon due to incomplete penetrance. This IGHD transmitted in a dominant mode with incomplete penetrance of the GHSR gene mutation had been previously reported by Pantel et al. [38, 45]. Widespread variations, variations in regulatory areas, epigenetics, and environmental variables were among the explanations given for the incomplete penetrance of dominant mutations [49, 50]. This frameshift variant, c.1043dup, is predicted to be possibly deleterious, leading to a downstream stop codon (S349Lfs*6) and a prematurely truncated protein or causing mRNA-mediated decay.

Several previously reported benign or likely benign variants were identified in the three studied genes. In the GH1 gene, four of these variants, rs6171, rs695, rs6173, and rs200134, were detected at frequencies of 25%, 5%, 5%, and 5%, respectively. In the GHRHR gene, six variants, rs2302021, rs4988495, rs4988496, rs4988498, rs4988504, and rs2228078, were identified, with frequencies of 5%, 10%, 10%, 10%, 5%, and 5%, respectively. In the GHSR gene, four variants, rs2232165, rs495225, rs2232169, and rs572169, were identified at frequencies of 20%, 30%, 20%, and 10%, respectively.

Plachy et al. [51] revealed that 71% of GHD-diagnosed patients have not shown pathogenic mutations despite using the NGS panel, including 398 genes related to growth in molecular screening of vertically transmitted short stature cases. Yu et al. [52] also found no pathogenic mutations in 85.6% of 109 growth hormone-deficient patients screened by whole exome sequencing (WES). This may be due to the presence of yet-undiscovered genes related to IGHD pathogenesis.

In the present study, the absence of pathogenic mutations in the three studied genes in eight IGHD patients may also be explained by the presence of yet-uncovered genes associated with IGHD or non-coding regulatory mutations that were not covered in our Sanger sequencing approach. Moreover, Plachy et al. [51] revealed pathogenic or likely pathogenic mutations in several genes causing IGHD without any additional phenotypic features. The mutations were discovered in the following genes: IGFALS (OMIM#601489), ACAN (OMIM#155760), COL2A1 (OMIM#120140), COL11A2 (OMIM#120290), NPR2 (OMIM#607072), EXT2 (OMIM#60821), FGFR3 (OMIM#134934), and PTPN11 (OMIM #176876). These findings significantly impede the process of molecular screening for IGHD.

Limitations of the study

The limitations of our manuscript might be due to the lack of Egyptian population data that can be used as a reference for growth hormone deficiency or its associated genetic variations. Lack of follow-up figures for the measurement of biological values.

Conclusion and recommendation

Our findings broaden the mutational spectrum of Egyptian patients with isolated growth hormone insufficiency. We revealed a previously reported pathogenic mutation (NM_000823.4: c.1069C > T; p.Arg357Cys) in the GHRHR gene and a novel frameshift variant (NM_198407.2: c.1043dup; Ser349Leu fs*6) in the GHSR gene with a high probability to be pathogenic according to the guidelines of the ACMG for the interpretation of sequence variations. This is the fourth report to acknowledge the autosomal dominant inheritance of the GHSR mutation as a cause of IGHD. Further functional studies are mandatory and highly recommended to confirm the deleteriousness of the c.1043dup variant. Next-generation sequencing is recommended for the genetic assessment of a larger cohort of patients to better identify the genetic etiology of GHD in the Egyptian population.

Abbreviations

CADD	Combined annotation-dependent depletion
Camp	Cyclic adenosine monophosphate
FT4	Free thyroxine
GPCR	G-protein-coupled receptors
gnomAD	Genome aggregation database
GHBP	Growth hormone-binding protein
GHD	Growth hormone deficiency
GHRHR	Growth hormone-releasing hormone receptor
GHSR	Growth hormone secretagogue receptor
GHRH	Growth hormone-releasing hormone
HGH	Human growth hormone
IGF-1	Insulin growth factor
ITT	Insulin tolerance test
ACMG	American college of medical genetics and genomics
IGHD	Isolated growth hormone deficiency
SD	Standard division
TSH	Thyroid-stimulating hormone

Supplementary Information

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Additional file 1. Mutation t@sting.

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Author contributions

Study's conceptualization and design were done by [MT], [MA], [TA], and [TF]. Blood sample and clinical data were collected by [MT], [MA], and [TF]. Material preparing was done by [TA] [GA] and [AA]. [AA] made Biochemical tests. [TA] and [GA] performed data collecting and analysis and wrote manuscript's first draft. On earlier versions of the work, all of the authors offered suggestions. All authors examined and confirmed the last draft.

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Availability of data and materials
Complete information produced or analyzed in this investigation is present in published document.

Declarations

Ethics approval and consent to participate
This research was done as reported by the rules of the Declaration of Helsinki. Acceptance was awarded by Egyptian National Research Centre's Ethical Committee (Date: 05 January 2023 / No. 2421022023).

Consent for publication
Legal guardians' informed consent was acquired.

Competing interests
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