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Clinical Characteristics and Genetic Analyses of Patients with Idiopathic Hypogonadotropic Hypogonadism

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What is already known on this topic?

Approximately 50% of all normosmic idiopathic hypogonadotropic hypogonadism (nIHH)/Kalman syndrome cases can be explained by genetic variations reported in more than 50 genes. It has been suggested that gonadotropin releasing hormone receptor variations account for approximately 40-50% of familial, autosomal recessive nIHH and approximately 17% of sporadic nIHH.

What this study adds?

Many variants of uncertain significance (VUS) were obtained in children with idiopathic hypogonadotropic hypogonadism. In this study protein models showed that variants interpreted as VUS according to American College of Medical Genetics and Genomics guidelines could account for the clinical IHH.

Abstract

Objective: Idiopathic hypogonadotropic hypogonadism (IHH) is classified into two groups-Kalman syndrome and normosmic IHH (nIHH). Half of all cases can be explained by mutations in >50 genes. Targeted gene panel testing with nexrt generation sequencing (NGS) is required for patients without typical phenotypic findings. The aim was to determine the genetic etiologies of patients with IHH using NGS, including 54 IHH-associated genes, and to present protein homology modeling and protein stability analyzes of the detected variations.

Methods: Clinical and demographic data of 16 patients (eight female), aged between 11.6-17.8 years, from different families were assessed. All patients were followed up for a diagnosis of nIHH, had normal cranial imaging, were without anterior pituitary hormone deficiency other than gonadotropins, had no sex chromosome anomaly, had no additional disease, and underwent genetic analysis with NGS between the years 2008-2021. Rare variants were classified according to the variant interpretation framework of the American College of Medical Genetics and Genomics (ACMG)/Association for Molecular Pathology. Changes in protein structure caused by variations were modeled using RoseTTAFold and changes in protein stability resulting from variation were analyzed.

Results: Half of the 16 had no detectable variation. Three (18.75%) had a homozygous (pathogenic) variant in the *GNRHR* gene, one (6.25%) had a compound heterozygous [likely pathogenic-variants of uncertain significance (VUS)] variant in *PROK2* and four (25%) each had a heterozygous (VUS) variant in *HESX1, FGF8, FLRT3* and *DMXL2*. Protein models showed that variants interpreted as VUS according to ACMG could account for the clinical IHH.

Conclusion: The frequency of variation detection was similar to the literature. Modelling showed that the variant in five different genes, interpreted as VUS according to ACMG, could explain the clinical IHH.

Keywords: Protein modelling, hypogonadotropic hypogonadism, genetic analyses

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Introduction

Normal pubertal development depends on the production and appropriate activity of gonadotropin-releasing hormone (GnRH) produced by neurons in the ventromedial hypothalamus (1,2). Isolated GnRH deficiency, also called idiopathic hypogonadotropic hypogonadism (IHH), is a group of genetic disorders associated with defects in the production and/or action of this hypothalamic peptide that controls human reproduction (3). IHH is divided into two main groups: Kalman syndrome (KS) and normosmic IHH (nIHH). IHH can be congenital (congenital heart disease) or acquired. The majority of hereditary causes of IHH are congenital (4).

Recently, with advances in genetic techniques, such as next generation sequencing (NGS), approximately 50 % of all nIHH/KS cases can be explained by genetic variations reported in more than 50 genes (4,5,6). Since the identification of the role of *ANOS1* (formerly *KAL1*) in the pathogenesis of X-linked KS, variants in *ANOS1, FGFR1, GNRH/GNRHR* and *PROK2/PROKR2* associated with IHH have been reported in several studies in the Human Gene Mutation Database as "disease-causing" (7). *GNRHR* is the first gene found to be responsible for isolated nIHH with deficiencies in follicle stimulating hormone (FSH) and luteinizing hormone (LH) (8,9,10). It has been suggested that *GNRHR* variations account for approximately 40-50 % of familial, autosomal recessive nIHH and approximately 17 % of sporadic nIHH (11). As a result of genetic studies performed in the last two decades, it has been found that many genes are associated with IHH (4). Genetic heterogeneity, variable expression and incomplete penetrance make it difficult to correlate the genotype-phenotype of IHH (12,13,14). Genetic tests are recommended for diagnosis of IHH and are necessary to determine the prognosis of IHH and to provide relevant genetic counseling (15).

Despite these recent advances in our understanding of the pathogenesis of IHH, it is likely that many more pathogenic genes remain to be discovered. While Sanger sequencing analyzes may be indicative for patients with specific findings or a family history, multi-gene panel testing NGS is required for patients who do not have typical phenotypic findings and/or no family history (16). Perhaps the most current challenge in the molecular genetic diagnosis of nIHH is the evaluation of variants of unknown clinical significance (VUS). Segregation analysis of family members is very important to reveal the genetic etiology. In addition, comprehensive *in silico* analyzes to assess the structural and functional impact of each genetic change on the protein product may be useful.

Mutations can cause changes in protein functional properties and protein-protein interactions by triggering changes in protein structure and stability. These changes are the basis of the development mechanism of many diseases (17,18,19). It should be kept in mind that the changes caused by mutations will trigger changes not only in the mutant protein but also in other proteins and structures with which it interacts. Therefore, elucidating the molecular mechanism of diseases is a complex and heterogeneous process. In recent years, *in silico* tools have significantly contributed to making many data and findings meaningful in this complex problem. In particular, computational studies that reduce the experimental processes that can take years to brief periods in the development of drugs and vaccines that can be the solution to global health problems come to the fore with their high reliability. It has been confirmed by numerous scientific studies that artificial intelligence-supported applications that use technical scientific data in the analysis of protein structure and stability provide high-reliability data. It has also been shown that computational tools used in protein homology modeling and stability analysis produce results that are equal to the data obtained by experimental methods, and some applications even produce better results than experimental data (20,21).

This study was conducted to determine the genetic etiology of patients with IHH by targeted gene panel including 54 genes known to cause IHH and to present protein homology modeling and protein stability analyzes of any detected variations.

Methods

Clinical and demographic data of patients followed up with the diagnosis of nIHH in the Pediatric Endocrine Departments of İnönü University Faculty of Medicine and Malatya Training and Research Hospital between the years of 2008 and 2021 were analyzed.

The diagnosis of nIHH was made according to the following criteria:

1) Absence or insufficient development of secondary sexual characteristics after the age of 13 in girls and after the age of 14 in boys;

2) Clinical signs or symptoms of hypogonadism;

3) Insufficient (low) sex steroid concentrations [testosterone or estradiol (E2)], and LH and FSH concentrations during the GnRH test;

4) Normal levels of free thyroxine, thyroid stimulating hormone, prolactin, insulin-like growth factor-1, adrencorticotropic hormone, and cortisol;

5) No evidence of structural lesions on imaging of the hypothalamic-pituitary region;

6) No evidence of chronic systemic diseases (such as uremia, thalassemia, poorly controlled diabetes mellitus), eating disorders (such as anorexia nervosa, bulimia), or protein energy malnutrition;

7) No patients reported olfactory problems;

8) None had features typical of Bardet-Biedl, Biemond, or Prader-Willi syndrome;

9) Absence of sex chromosome abnormalities (6,22,23,24).

GnRH test was done at 08:00 in the morning. Blood samples for FSH, LH, E2 or testosterone were taken. Then 100 mcg of GnRH was administered intravenously. Blood samples were taken for FSH and LH levels at 20, 40, 60 and 90 minutes after drug administration.

The study was approved by the Ethics Committee of İnönü University Faculty of Medicine (approval number: 2022/2650, date: 11.01.2022). Written consent was obtained from all patients or their legal guardians, if under eighteen years.

Clinical and Endocrinological Evaluation

Medical records including, clinical features, sense of smell, family history, associated anomalies, micropenis-cryptorchidism history, and laboratory-radiological findings were retrospectively reviewed. Pubertal development was graded according to the guidelines recommended by Marshall and Tanner (11). Testicular volume was measured with a Prader orchidometer. Olfactory function of the patients was evaluated by anamnesis, olfactory function test could not be used to diagnose olfactory abnormalities.

Statistical Analysis

Descriptive statistical method was used in this study. Data were summarized as count (percentage).

Next Generation Sequencing and Bioinformatics Analysis

Genetic Analyses

Genomic DNA was extracted from peripheral blood and NGS was performed by capture of the coding regions and splice sites of the following target genes: *ANOS1, CHD7, CYP19A1, DUSP6, DMXL2, DUSP6, ESR1, FEZF1, FGF8, FGFR1, FSHB, FGF17, FLRT3, GH1, GLCE, GLI2, GNRH1, GNRHR, HESX1, HS6ST1, IHX3, IL17RD, KISS1, KISS1R, LEP, LEPR, LHX3, LHB, LHX4, LHCGR, NROB1, NR5A1, NSMF, OTX2, OTUD4, PNPLA6, POLR3A, POLR3B, POU1F1, PROK2, PROKR2, PROP1, RNF216, SEMA3A, SEMA3E, SOX2, SOX3, SOX10, SPRY4, STUB1, TACR3, TUBB3, TAC3, WDR11*. An Illumina custom enrichment panel was used for this (Illumina, San Diego, CA, USA).

After library enrichment and quality control, the samples were sequenced on the Illumina MiSeq platform (San Diego, CA, USA) with 100-bp paired-end reads at an average sequencing depth of 100×.

The sequencing reads were aligned to the human reference genome assembly (GRCh37: Genome Reference Consortium Human Build 37) using BWA. Then, BAM files were sorted, indexed and de-duplicated using SAMtools and Picard. For the filtering process, exonic and splicing variants, including missense/nonsense variants, and indels were selected. Annotation of detected variants was performed using Illumina BaseSpace Variant Interpreter, InterVar, Franklin, VarSome, ClinVar, OMIM, and Pubmed. Variants with a frequency higher than 0.1% were filtered out. dbNSFP, which contains SIFT, PolyPhen-2, LRT, and Mutation Taster, was used to predict the pathogenicity of variants. Rare variants were classified according to the American College of Medical Genetics and Genomics (ACMG)/Association for Molecular Pathology variant interpretation framework (25).

All variants identified by NGS were confirmed by Sanger sequencing. Sanger sequencing was performed using the Applied Biosystems 3130 Genetic Analyzer (Foster City, CA, USA). Detected variants were classified as "pathogenic", "likely pathogenic (LP)", or "variant of uncertain significance (VUS)" according to the international guidelines of the ACMG. To assess the association between any identified genetic variants and IHH, hypothetical protein structures were constructed and analyzed *in silico* (see below).

Protein Homology Modeling

Modeling of changes in protein structure caused by variations was performed with RoseTTAFold, which uses deep learning-based, three-track neural network algorithms. Rosetta provides both *ab initio* and comparative models of protein domains. Comparative models are built from structures detected and aligned by HHSEARCH, SPARKS, and Raptor. Loop regions are assembled from fragments and optimized to fit the aligned template structures. *De novo* models are built using the Rosetta *de novo* protocol (26). Since the protein structures of some of the IHH-related genes (*PROK2, DMXL2* and *PROP1*) examined in this study were not previously defined, wild-type protein structures were also modeled in this study for the first time. Identification of the reference sequence data for the variants is given in Table 1. The *GNRHR*, *FLRT3* and *FGF8* homology models were created using templates from the Protein Data Bank: 7BR3, 5CMP and 2FDB. Protein model quality analyzes were performed with ProSA and QMEANDisco (27,28).

Topological differences between wild-type and mutant protein were analyzed by TM-score (29). Superimpositional and conformational analysis of proteins were performed with DDS and PyMOL (ver2.4.1).

Protein Stability Analyzes

Changes in protein stability after variation was analyzed with mCSMstability (30), DUET (31), SDM (32), and DynaMut2 (33) bioinformatics tools. All interatomic contacts calculated with Arpeggio were displayed using NGL viewer (34,35).

Results

Of 39 patients with IHH whose file data were available, 18 (46%) were male and 21 (54%) were female. Of these, 16 unrelated patients (eight female) with the diagnosis of IHH and whose genetic panel had been performed were included in the study. Mean age of the patients at presentation was 14.8 years. All of the patients presented with delayed puberty. None of the patients reported problems with sense of smell. There was a history of delayed puberty in the family of six (37.5%) patients.

Six (75%) male patients had micropenis. A patient with a normal penis size (patient number F15P15) had received six doses of intramuscular testosterone therapy in an external center before attending our clinic. Three patients (F1P1, F2P2 and F3P3) had a history of unilateral cryptorchidism. No patient had a history of bilateral cryptorchidism. The pubertal stage of 14 patients (87.5%; seven girls and seven boys), was evaluated as Tanner stage 1. One male patient (6.25%) was Tanner stage 2, and one female patient (6.25%) was at Tanner stage 4 of puberty. Both patients (patients F15P15 and F12P12) who had started puberty had received sex steroid replacement therapy in an external center before attending our clinic. GnRH stimulation test was performed in all patients. The clinical and laboratory findings of the patients at presentation are summarized in Table 2.

Molecular Findings

Eight (50%) had a variation in one of the genes included in the panel while eight had no detectable variant in the gene panel used. Three (18.75%) had a pathogenic, homozygous variant in the *GNRHR* gene, one (6.25%) had LP, compound heterozygous variant in *PROK2*, and four (25%) had a VUS in one each of four different genes, *HESX1*, *FGF8*, *FLRT3* and *DMXL2* (Table 1). The variants detected in the study and the assessment of pathogenicity are shown in Table 1 (25).

The previously reported hot spot pathogenic variant c.415C>T in the *GNRHR* gene, was detected homozygously in our three index cases. Parents were shown to be carriers by segregation analyzes, and parents had a history of delayed puberty. The same variation was present in a homozygous fashion in two siblings of P1 and the twin of P2. The three siblings were being followed in our clinic due to delayed

Table 1. The pathogenicity assessment of the detected variants

Patient number	Gene	Transcript number	Nucleotide change	AA change	MAF by gnomAD	Zyg	Variant location	Variant type	ClinVar	ACMG class	ACMG pat crit
F1P1	*GNRHR*	NM_000406.3	c.415C>T	p.Arg139Cys	-	Hom	Exon 1	Mis	NP	Pat	PS3, PM1, PM2, PM5, PP3
F2P2	*GNRHR*	NM_000406.3	c.415C>T	p.Arg139Cys	-	Hom	Exon 1	Mis	NP	Pat	PS3, PM1, PM2, PM5, PP3
F3P3	*GNRHR*	NM_000406.3	c.415C>T	p.Arg139Cys	-	Hom	Exon 1	Mis	NP	Pat	PS3, PM1, PM2, PM5, PP3
F4P4	*PROK2*	NM_001126128.2	c.217C>T	p.Arg73Cys	0.0000716	Het	Exon 2	Mis	Pat	LP	PM2, PP3, PP5
	PROK2	NM_001126128.2	c.1A>C	p.Met1Leu	-	Het	Exon 1	Mis	NP	VUS	PVS1, PM2
F5P5	*HESX1*	NM_003865.3	c.18G>C	p.Gln6His	-	Het	Exon 1	Mis	VUS	VUS	PM2, PM6, PP2
F6P6	*FGF8*	NM_033163.5	c.476C>T	p.Thr159Met	-	Het	Exon 6	Mis	NP	VUS	PP2, PP3, BS2, PM6
F7P7	*FLRT3*	NM_013281.3	c.1541A>G	p.Asn514Ser	0.00000798	Het	Exon 2	Mis	NP	VUS	PM1, PM2
F8P8	*DMXL2*	NM_001174116.3	c.5915A>T	p.Glu1972Val	-	Het	Exon 24	Mis	NP	VUS	PM2

AA: amino acid, MAF: minor allele frequency, Zyg: zygosity, ACMG Class: The American College of Medical Genetics and Genomics Classification, ACMG Pat Crit: ACMG Pathogenicity Criteria, Het: heterozygous, Hom: homozygous, Del: deletion, Frms: frameshift, Mis: missense, Splc: splicing, NP: not provided, Pat: pathogenic, LP: likely pathogenic, VUS: variant of uncertain significance

Table 2. Age, clinical presentation, family history, Tanner stage and GnRH test findings of all patients

	Sex	Age at diagnosis (years)	Clinical presentation	Family history	Stretched penile length (cm)	Tanner stage of gonads at diagnosis	GnRH test peak	
							FSH (mIU/mL)	LH (mIU/mL)
F1P1	M	11y 9/12	Cryptorchidism, micropenis	Pubertal delay in brother, dad and grandfather. Sister has no menstruation	4.5	1	1.88	0.2
F2P2	M	11y 7/12	Cryptorchidism micropenis	Pubertal delay in twins. Parents had a child with *in vitro* fertilization.	3.7	1	1.74	0.66
F3P3	M	13y 7/12	Cryptorchidism, micropenis	Micropenis in brother	3	1	0.45	0.24
F4P4	F	14y 9/12	No menstruation	-		1	3.56	1.28
F5P5*	M	16y 10/12	Pubertal delay	Pubertal delay in father and brother.	8	1	5.3	7.1
F6P6	F	14y 3/12	No menstruation	-		1	9.54	2.03
F7P7	M	14y 7/12	Micropenis	-	4	1	5.98	0.93
F8P8	F	14y 11/12	No menstruation	Late menstruation in mother		1	1.66	1.89
F9P9	F	15y 7/12	No menstruation	-		1	3.64	1.02
F10P10	F	15y 5/12	No menstruation	-		1	3.28	1
F11P11	F	15y 2/12	No menstruation	-		1	2.52	0.77
F12P12	F	16y 6/12	No menstruation	-		4	4.1	3.2
F13P13	M	10y 4/12	Micropenis	-	4	1	5.89	0.8
F14P14	F	16y 10/12	No menstruation	-		1	5.2	1.75
F15P15	M	17y 10/12	Pubertal delay	Pubertal delay in uncle		2	1.14	0
F16P16*	M	16y 8/12	Pubertal delay micropenis	-	6	1	3.9	8.4

NB brain magnetic resonance imaging of all patients was evaluated as normal.
*The 5th and 16th patients had late puberty. Pubertal induction therapy was given to them. They were followed up for one year at pediatric endocrinology department and their testicular volumes remained < 4 mL.
y: year, F: family, P: patient, F: female, M: male, N: not detected, GnRH: gonadotropin-releasing hormone, FSH: follicle stimulating hormone, LH: luteinizing hormone

puberty and were receiving pubertal induction therapy.

In one patient, c.1A>C and c.217C>T variants in the *PROK2* gene were detected in a compound heterozygous fashion. According to the ACMG classification, these variants are interpreted as VUS/LP. As a result of segregation analysis, the heterozygous c.271C>T variant was found in the mother of the patient, and the heterozygous c.1A>C variant was found in the father. There was no history suggestive of hypogonadism in the parents.

In one patient, a heterozygous variation, c.18G>C, was detected in *HESX1*, and this was found to be a *de novo* mutation.

A c.476C>T, heterozygous variant was detected in *FGF8* in one patient. The segregation analysis showed no such variant in the mother, while heterozygous variation was found in the same gene in the father. It was learned that the father had puberty tarda and had children without any therapy.

A c.1541A>G heterozygous variation was detected in *FLRT3* in one patient. Genetic analysis could not be performed in the parents of this patient.

A heterozygous variation, c.5915A>T was detected in *DMXL2* in one patient. While heterozygous variation was detected in the same gene in the mother, no variation was found in the father. It was learned that the mother had late menstruation but had children spontaneously.

Protein Structural Analysis

In this study, the relationship between the changes in protein structure caused by seven variations in six different genes (*GNRHR, PROK2, HESX1, FGF8*, *FLRT3* and *DMXL2*)

and IHH was investigated. Tertiary models of proteins containing mutant residues were created using deep learning algorithms. The protein tertiary models created were within the quality limits of X-ray and NMR. QMEAN scores ranged from -2.06 to 0.66. The *GNRHR*.p.Arg139Cys variation is associated with HH disease. *GNRHR* is a G-protein-coupled GnRH receptor, regulates LH and FSH secretion and has seven transmembrane segments and an extracellular amino terminus (36). *GNRHR*.p.Arg139Cys variations were noted for their highly destabilizing effects (-2.35 and -2.086 kcal. mol^{-1}, respectively) and increased solvent accessibility. The *GNRHR*.p.Arg139Cys variation changed the protein topology (rmsd 0.157 Å). The Arg139Cys variation in the cytoplasmic region may affect the coupling of the G protein with the receptor. The Arg139 residue in wild-type *GNRHR* contributes to cytoplasmic region stability with twenty-one weak bond interactions (Figure 1a). It was observed that the number of these interactions decreased to thirteen due to the changed conformation in the mutant protein, and the two hydrophobic and one polar interaction with Met76 was abolished (Figure 1b). Solvent accessibility of residue 139 increased approximately 3-fold after variation.

The *PROK2*.p.Met1Leu variation resulted in a possible 43 amino acid shortening of the mature protein length and changed topology (Figure 2a, 2b). The rmsd was 0.930 Å in superimpose. The *PROK2*.p.Met1Leu variation may have shifted the start signal to the methionine codon at the 44th codon. Therefore, stability assessment of the *PROK2*.p.Met1Leu variation was performed at the conformational level, since the mutant protein did not contain mutant residue. The rmsd was 0.582 Å for the *PROK2*.p.Arg73Cys variation at superimpose. The variation caused a change in conformation (Figure 1c, 1d) and topology (Figure 2c, 2d) of the protein product.

In this paper a three-dimensional model of *HESX1* is presented for the first time. The model developed was within the NMR quality limitations (Z score -4.1). The Gln6His variation caused limited change in protein structure. The -NE2 group 5.2 Å moved away from the main backbone (Figure 3a) as a result of the variation, increasing exposure to solvent accessibility (Table 3). After the variation, the two hydrophobic and one polar contact created between residue-1 and residue-6 were abolished (Figure 3b, 3c). The interaction between residue-3 and -6 with two polar and three hydrogen bonds was reduced to three polar interactions after the variation. The conformational change induced by the variation revealed one van der Waals (vdw) and one polar interaction between residue-6 and residue-10 that was not present in the wild type. *HESX1*.p.Gln6His variation caused a decrease in protein stability (-0.732 kcal. mol^{-1}).

FGF8.p.Thr159Met variation increased protein instability (-0.444 kcal.mol^{-1}). An increase in the solvent accessibility of the 159th residue after the variation was identified (Table 3). The variation detected in our patient in protein modeling caused a putative change in the conformational structure of *FGF8* (rmsd 0.184 Å) (Figure 4a). Changes in the conformation and topology of two consecutive heterodimer helix-turn-helix motifs located in the N-terminal domain of the FGF8 protein may result in changes in protein functional

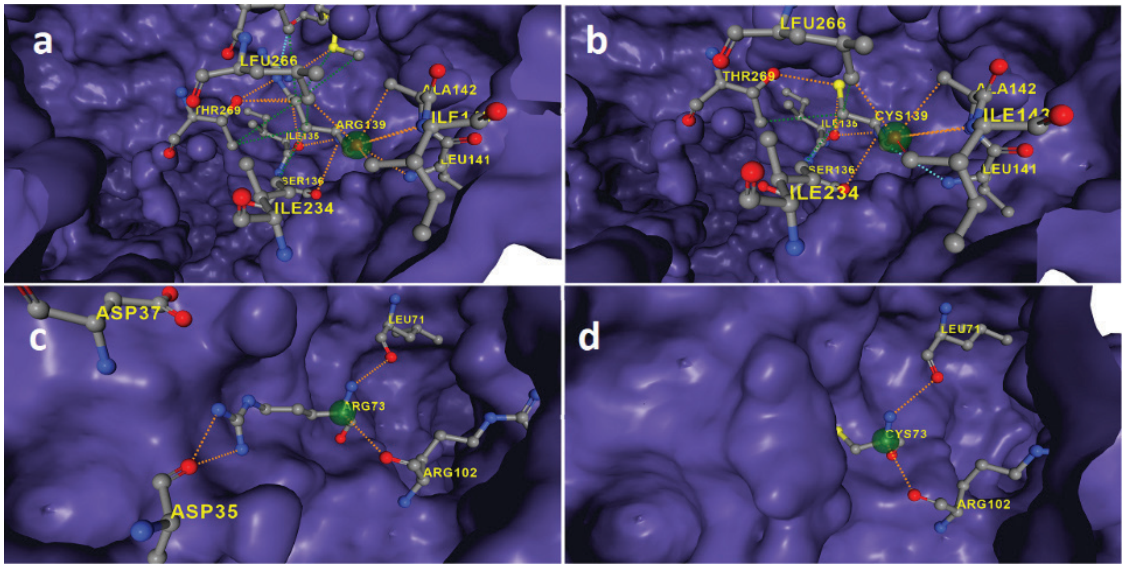

Figure 1. Surface/stick representation of changes in protein stability and bond formation caused by variations. Green transparent sphere indicates mutant position. Colors in dashed lines represent-green: hydrophobic, red: hydrogen bond, blue: Van der Waals, navy blue: carbonyl, orange: polar. a) *GNRHR* wild-type, b) *GNRHR* mutant, c) *PROK2* wild-type, d) *PROK2* mutant

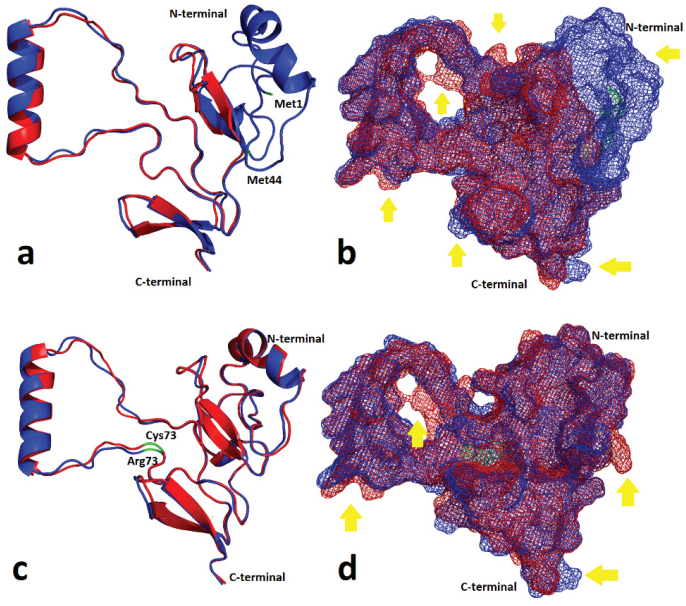

Figure 2. Superimpose representation of the changes in protein conformation and topology caused by *PROK2* variations

Blue: wild-type *PROK2*, red: mutant *PROK2*, yellow arrow: indicates change, green: mutant residue. a) Cartoon representation of the *PROK2*.p.Met1Leu variation, b) Mesh topological representation of the *PROK2*.p.Met1Leu variation, c) Cartoon representation of the *PROK2*.p.Arg73Cys variation, d) Mesh topological representation of the *PROK2*.p.Arg73Cys variation

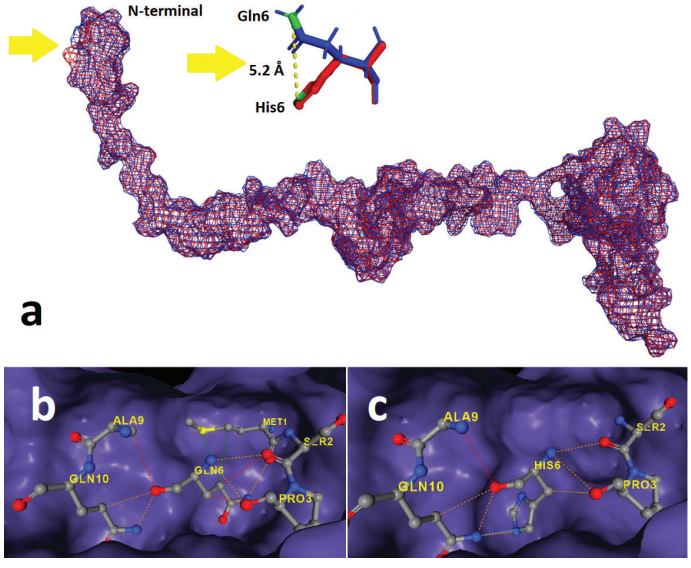

Figure 3. Representation of the changes in protein congormation and topology caused by HESX1 variation

a) Superimpose (blue: wild-type, red: mutant, yellow arrow: indicates change), b) Surface/stick representation of wild-type *HESX1*, c) Surface/stick representation of mutant *HESX1* (colors in dashed lines represent-green: hydrophobic, red: hydrogen bond, blue: Van der Waals, navy blue: carbonyl, orange: polar)

properties, protein-protein/DNA, and receptor interaction (Figure 4b). The interaction between Thr159 and Arg125 in wild-type FGF8 was abolished in the mutant protein (Figure 4c, 4d).

The *FLRT3*.p.Asn514Ser variation changed the topology of the tunnel formation located near the transmembrane domain (residue 529-549) (Figure 5a, 5b). The two polar interactions between Asn514 and Glu516 in the wild-type FLRT3 protein were abolished in the mutant FLRT3 (Figure 5c, 5d). It was observed that the interaction between wild-type Asn514 and Gln517 with a hydrophobic force of one hydrogen was provided by two hydrogen bonds and a polar interaction between Ser514 and Gln517 in the mutant protein. The interaction between residue 514 and residue 517 was achieved with one hydrogen bond and one hydrophobic force in wild-type FLRT3, while in mutant FLRT3 this was changed to two hydrogen bonds and one polar interaction. The *FLRT3*.p.Asn514Ser variation decreased protein stability and solvent accessibility (-0.188 kcal.mol^{-1}) (Table 3).

The *DMXL2*.p.Glu1972Val variation increased protein stability (0.139 kcal.mol^{-1}). The p.Glu1972Val variation abolished the two polar interactions between residue 1973 and Lys2013 (Table 3).

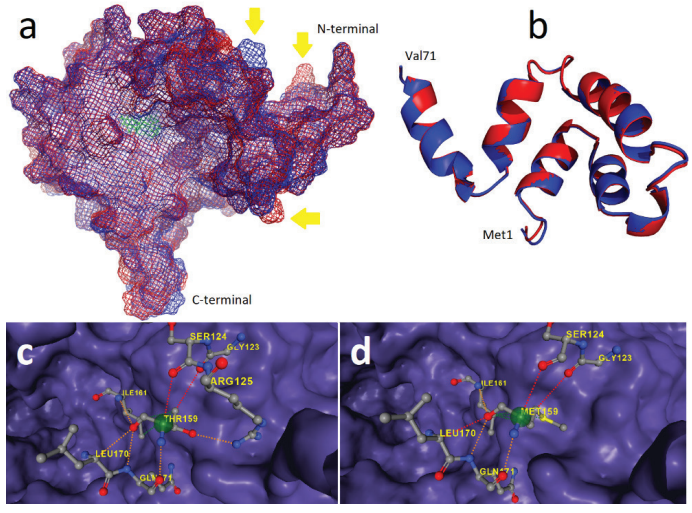

Figure 4. Illustration of variation-induced change in *FGF8*

a) Mesh topological representation of topological changes in *FGF8*, b) Cartoon representation of N-terminal domain of *FGF8* (blue: wild-type *FGF8*, red: mutant *FGF8*, yellow arrow: indicates change, green: mutant residue), c) Surface/stick presentation of residue interactions of wild-type *FGF8*, d) Surface/stick presentation of residue interactions of mutant *FGF8* (colors in dashed lines represent- green: hydrophobic, red: hydrogen bond, blue: Van der Waals, navy blue: carbonyl, orange: polar)

Table 3. Effects of mutant residues on protein stability

Protein . Mutation	Protein stability (ΔΔG kcal.mol^{-1})				Output	%RSA exchange
	mCSMstability	DUET	SDM	DynaMut2		
GNRHR.p.Arg139Cys	-2.086	-2.267	-1.57	-1.61	Highly destabilizing	0.9 → 2.4
PROK2.p.Arg73Cys	0.13	0.104	-0.18	0.56	Stabilizing	45.4 → 68.1
HESX1.pGln6His	-0.732	-0.466	-0.33	-0.26	Destabilizing	60.2 → 74.3
FGF8.p.Thr159Met	-0.444	-0.283	0.08	-0.3	Destabilizing	26.2 → 35.4
FLRT3.p.Asn514Ser	-0.188	-0.002	-0.33	0.29	Destabilizing	99.6 → 81.7
DMXL2.p.Glu1972Val	0.139	0.539	0.78	0.3	Stabilizing	70.7 → 72.3

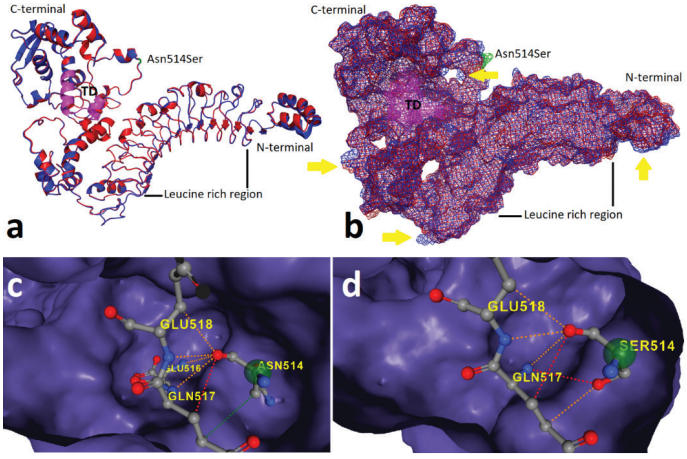

Figure 5. Representation of the changes caused by the *FLRT3* variation

a) Superimpose cartoon representation of the FLRT3.p.Asn514Ser variation, b) Superimpose mesh topological representation of the FLRT3.p.Asn514Ser variation (blue: wild-type FLRT3, red: mutant FLRT3, yellow arrow: indicates topological change, green: mutant residue, magenta: transmembrane domain (TD), c) Surface/stick presentation of residue interactions of wild-type FLRT3, d) Surface/stick presentation of residue interactions of mutant *FLRT3* (colors in dashed lines represent-green: hydrophobic, red: hydrogen bond, blue: Van der Waals, navy blue: carbonyl, orange: polar)

Discussion

In this study targeted NGS analysis was used in patients with nIHH of unknown genetic etiology. We found a genetic etiology in 50% (8/16) of cases. The most common variation was the C.415C>T homozygous variation in the *GNRHR* gene, which was interpreted as pathogenic according to the ACMG Classification. The c.415C>T (p.Arg139Cys) variant, which was present homozygously in our patients, is a known hot spot variation in the *GNRHR* gene. This variant was first reported by Topaloglu et al. (37) in 2009 and it was found in two Turkish sisters (aged 16 and 23), whose parents were first-degree cousins, who presented with delayed puberty. *GNRHR* variations are known to account for approximately 40-50% of familial nIHH (11) and our results were compatible with this. Protein models showed that the *GNRHR*.p.Arg139Cys variation was highly destabilizing and increased solvent accessibility. The *GNRHR*.p.Arg139Cys variation also changed the protein topology on *in silico* modeling. It is possible that these variational changes decrease intracellular signaling mechanism effectiveness and lead to reduced activation of phospholipase-C, rather than receptor binding affinity. De Roux et al. (8) revealed that variations in the cytoplasmic loop did not change the binding of GnRH to the receptor, but decreased activation of the effector macromolecule phospholipase-C.

The *PROK2* gene encodes prokinetecin 2, an 81 amino acid peptide that signals through the G protein-linked product of the *PROKR2* gene (38). Variations in *PROKR2* and *PROK2* are generally seen in combination with other variations with oligogenic inheritance in IHH (4). In our study, c.217C>T (p.Arg73Cys), interpreted as LP according to ACMG classification, and c.1A>C (p.Met1Leu), interpreted as VUS, were found to be combined in a heterozygous fashion in one patient in *PROK2*. Protein models showed that the *PROK2*.p.Met1Leu variation resulted in a possible 43 amino acid shortening of the mature protein length and changed topology. The absence of the -AVITGA- sequence, which is highly conserved across species and thought to be important for the functional properties of *PROK2*, may result in impaired protein function (39,40,41). We hypothesize that this variation, which is currently interpreted as VUS according to the ACMG classification, may be associated with HH. The *PROK2*.p.Arg73Cys variation caused a putative change in conformation and topology of the protein product. The cysteine residue introduced by the p.Arg73Cys variation is likely to affect the formation of disulfide bonds in the protein (42). The decrease in receptor affinity caused by the changed protein structure with these identified *PROK2* variations may be the reason for the decrease in receptor signaling, intracellular calcium mobilization, and MAPK signaling that will result in the HH phenotype and lack of GnRH (43,44). The patient's mother was carrying the c.271C>T variant, and her father was heterozygous

for the c.1A > C variant. There was no history of delayed puberty in the parents. It was thought that the compound heterozygous variation in our patient may have caused thier clinical findings.

The *HESX1* gene is part of a family of homeobox genes that act during early embryonic development to control the formation of many body structures. HESX1 protein is a transcription factor that plays an important role in early-stage brain development. The HESX1 protein is required for the structural development of the forebrain and pituitary. HESX1 exerts its effects in combination with PROP1 and many other proteins during embryonic development to coordinate the formation of different parts of the brain through the control of gene expression (45,46,47). It is not clear whether *HESX1* variations cause mild forms of IHH, or partial or complete absence of puberty due to GnRH deficiency/impaired gonadotropins (48). Newbern et al. (48) investigated the presence of *HESX1* variation in 217 patients, followed up with the diagnosis of KS or IHH and in whom other anterior pituitary deficiencies were excluded and a control group of 192 patients. They detected a *HESX1* heterozygous variant in three patients, two of whom were Turkish. In the control group, no variation was detected and no variation was found in the 1,000 genomes database. In our study, one patient was heterozygous for *HESX1*, which was interpreted as VUS according to ACMG classification. Segregation analysis confirmed that the variant was *de novo*. We evaluated this change, which we believe may explain the patient's clinical picture. Protein models showed that *HESX1*.p.Gln6His variation caused a decrease in protein stability. We suggest that heterozygous variations of the *HESX1* gene, whose homozygous variations lead to severe phenotypes, such as septo-optic dysplasia, may cause IHH. However, further studies are needed to confirm this hypothesis.

Studies have shown that there is a 30-50 % decrease in total GnRH neurons in mice harboring heterozygous *FGF8* gene variations, while a greater reduction in GnRH neurons is seen in mice with co-variation in *FGFR1* and *FGF8* genes (32). Olsen et al. (49) showed that variation of Phe32Ala in the N-terminal region of *FGF8*b, the isomer of *FGF8*, resulted in decreased receptor affinity and changes in protein functional properties. In the presence of other gene variations accompanying p.Glu176Lys and p.Arg184Cys variations in *FGF8*, in addition to HH, some clinical problems reflected in the phenotype, such as dental agenesis, hearing loss and hand malformation, have been reported (32). In our study, a heterozygous variation of c.476C > T, interpreted as VUS according to the ACMG classification, was detected in *FGF8* in one patient. While no variation was detected in the mother of the patient, the same heterozygous variation was found in her father. It was learned that her father had delayed puberty but had children spontaneously. It was thought that this variant may explain the patient's clinical picture, but more studies are needed.

The *FGF8*.p.Thr159Met variation, detected in our patient, caused a change in the conformational structure of *FGF8* (rmsd 0.184 Å) on protein modelling. The fact that the patient and her father had a history of delayed puberty together with the predicted decreased protein stability of the detected variant suggest that this variant may explain the HH in the patient. In this study, we report the association of the *FGF8*.p.Thr159Met variation with HH for the first time.

A heterozygous variation of c.1541A > G, interpreted as VUS according to ACMG classification, was detected in *FLRT3* in one patient. Genetic analysis could not be performed in the parents of this patient. The variation changed the putative protein conformation and decreased protein stability in protein modelling. We suggest that this variation, which is currently interpreted as VUS according to the ACMG classification, may be associated with HH.

A heterozygous variation of c.5915A > T, was detected in *DMXL2*, which was interpreted as VUS according to the ACMG Classification, in one patient. While heterozygous variation was detected in the same gene in the mother of the patient, no variation was found in the father. The mother had a history of late menstruation but had children spontaneously. The *DMXL2*.p.Glu1972Val variation increased protein stability (0.139 kcal.mol^{-1}) in protein modeling. We hypothesize that this variation, which caused delayed puberty in both mother and the patient, and abolished two polar interactions on protein modeling, could be the etiology of our patient's HH.

Amato et al. (50) performed genetic analyzes of 130 CHH patients using NGS (including 29 known and seven candidate genes) and detected pathogenic/LP variations in 43 (33 %). In this study, as in our study, the most common variation detected in nIHH patients was in the *GNRHR* gene.

Study Limitations

The number of our patients was small as we were working with a rare genetic disease group. Genetic analysis could not be performed on the parents of a patient whose genetic variation was determined as VUS according to ACGM. Olfactory function test were not performed because they are not available at our hospital. Olfactory function of the patients was evaluated by anamnesis and this may be unreliable. Finally, functional analysis was not performed in variations classified as VUS by the ACGM.

Conclusion

In this study, pathogenic/LP variation was detected in 25% of 16 patients and VUS in a further 25%, while no variation was detected in 50% using a panel containing 54 genes associated with IHH. The frequency of detection of variants is similar to the literature. The most frequently detected variation was in the *GNRHR* gene, a finding consistent with several previous reports. Protein models showed that variants interpreted as VUS (*PROK2*, *HESX1*, *FGF8*, *FLRT3* and *DMXL2*) according to ACMG could account for the clinical IHH. Association of the *FGF8*.p.Thr159Met variation with HH was reported for the first time in this study. Large-scale genetic studies are needed to understand the genetic aspects of nIHH in Turkey and in other populations. Overall, the practical yield of this study is considerable because it reflects professional experience gained in a single center and represents one of the first studies in Turkish children including molecular analysis of 54 causal IHH-related genes. Confirmatory genetic testing in patients with suspected nIHH allows for definitive diagnoses, which may guide management and provide rationales for screening other family members presymptomatically. In studies conducted with NGS, as in our study, through advancing molecular testing and identification of new genes, the number of patients with nIHH may be expected to rise rapidly. It is reasonable and appropriate to conclude here that verification of these candidate genes would not only help treatment plans for these patients, but would also facilitate further research into GnRH neuronal migration.

Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee of İnönü University Faculty of Medicine (approval number: 2022/2650, date: 11.01.2022).

Informed Consent: Written consent was obtained from all patients or their legal guardians, if under eighteen years.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Nurdan Çiftci, Ayşehan Akıncı, Ekrem Akbulut, Emine Çamtosun, İsmail Dündar, Mustafa Doğan, Leman Kayaş, Concept: Nurdan Çiftci, Ayşehan Akıncı, Ekrem Akbulut, Emine Çamtosun, İsmail Dündar, Design: Nurdan Çiftci, Ayşehan Akıncı, Ekrem Akbulut, Emine Çamtosun, Data Collection or Processing: Nurdan Çiftci, Ayşehan Akıncı, Ekrem Akbulut, Emine Çamtosun, Mustafa Doğan, Leman Kayaş, Analysis or Interpretation: Nurdan Çiftci, Ayşehan Akıncı, Ekrem Akbulut, Emine Çamtosun, İsmail Dündar, Literature Search: Nurdan Çiftci, Ayşehan Akıncı, Ekrem Akbulut, Emine Çamtosun, Writing: Nurdan Çiftci, Ayşehan Akıncı, Ekrem Akbulut, Emine Çamtosun, Leman Kayaş.

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