

Targeted Gene Panel Sequencing for Molecular Diagnosis of Kallmann Syndrome and Normosmic Idiopathic Hypogonadotropic Hypogonadism

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ABSTRACT

Background Isolated gonadotropin-releasing hormone (GnRH) deficiency (IGD) is classified either as Kallmann syndrome (KS) with anosmia or normosmic idiopathic hypogonadotropic hypogonadism (nIHH) and caused by mutations in more than 30 different genes. Recent advances in next-generation sequencing technologies have revolutionized the identification of causative genes by using massively parallel sequencing of multiple samples. This study was performed to establish the genetic etiology of IGD using a targeted gene panel sequencing of 69 known human IGD genes.

Methods This study included 28 patients with IGD from 27 independent families. Exomes were captured using customized SureSelect kit (Agilent Technologies) and sequenced on the Miseq platform (Illumina, Inc.), which includes a 163,269 bp region spanning 69 genes.

Results Four pathogenic and six likely pathogenic sequence variants were identified in 11 patients from 10 of the 27 families (37%) included in the study. We identified two known pathogenic mutations in CHD7 and PROKR2 from two male patients (7.4%). Novel sequence variants were also identified in 10 probands (37%) in CHD7, SOX3, ANOS1, FGFR1, and TACR3. Of these, while eight variants (29.6%) were presumed to be pathogenic or likely pathogenic, the remaining two were classified as variants of uncertain significance. Of the two pre-pubertal males with anosmia, one harbored a novel heterozygous splice site variant in FGFR1.

Conclusions The overall diagnostic yield was 37% of the patients who had undergone targeted gene panel sequencing. This approach enables rapid, cost-effective, and comprehensive genetic screening in patients with KS and nIHH.

*These two authors contributed equally.

Introduction

Isolated gonadotropin-releasing hormone (GnRH) deficiency (IGD) is caused by a defect in GnRH production, secretion, or action [1]. IGD is classified either as Kallmann syndrome (KS), which is associated with anosmia, or normosmic idiopathic hypogonadotropic hypogonadism (nIHH), wherein patients retain a normal sense of smell [1]. IGD is a genetically heterogeneous disorder that is associated with over 30 causative genes. Over the last 20 years, mutations in several genes and pathways that affect IGD have been identified [2]. In addition, studies have reported that 10–20% of patients have oligogenic defects in different genes that act synergistically to modify the severity of IGD [3, 4].

Certain non-reproductive features (e. g., synkinesia, renal agenesis, dental agenesis, hearing defects, and skeletal abnormalities) are highly linked to specific genetic defects, and these features can prove helpful during prioritization of genes for screening [5]. Traditional Sanger sequencing can provide a genetic diagnosis in cases with distinct phenotypes and the presence of family histories [6]. However, when a proband has no affected family members or associated non-reproductive phenotypic features, serial single-gene or multi-gene panel testing is required for genetic diagnosis [5–7].

Over the last 20 years, significant progress has been made in the understanding of the molecular genetics of IGD. Recent advances in next-generation sequencing (NGS) techniques have enabled massive parallel sequencing of multiple genes. Targeted enrichment techniques have been developed to reduce genome complexity via targeted sequence captures and optimized the coverage of targeted regions for high throughput deep sequencing rather than whole exome sequencing (WES) or whole genome sequencing (WGS) [8]. Therefore, NGS techniques have changed how genetic research assesses genetically heterogeneous diseases. As an example, recent studies that have used NGS technologies have demonstrated numerous candidate genes for IGD, such as OTUD4, FEZF1, STUB1, DMLX2, RNF216, PNPLR6, CCDC14, and SEMA3E11, 9, 10]. Furthermore, targeted NGS of 261 candidate genes in patients with IGD resulted in the identification of 18 new candidate genes and 2 pathogenic mutations in FGFR1 [11].

In spite of these studies, NGS techniques are not widely used in clinical practice due to the high cost and complexity of data analysis. We have therefore designed a targeted gene panel for the diagnosis of IGD using 69 known human IGD genes. In sum, this study was performed to apply targeted gene panel testing in clinical practice and to identify the genetic etiology of patients with IGD using targeted gene panel sequencing.

Materials and Methods

Subjects

This study involved 28 patients from 27 independent families, including 23 males with IGD (82.1%), three females with IGD (10.7%), and two pre-pubertal males with anosmia (7.1%). A diagnosis of IGD was made according to the following criteria: 1) absent or incomplete pubertal development by the age of 17 years in females and 18 years in males; 2) clinical signs or symptoms of hypogonadism; 3) serum testosterone levels of < 1.0 ng/mL in males or serum estradiol levels of < 20 pg/mL in females with low or normal

levels of gonadotropin; 4) normal thyroid, adrenal, and growth hormone axes; 5) normal magnetic resonance image (MRI) of the hypothalamic and pituitary areas; and 6) the absence of sex chromosome abnormalities [2, 5, 12]. This study was approved by the Institutional Review Board at Asan Medical Center, Seoul, Korea, and informed consent was obtained from all patients or their parents.

Clinical and endocrinological evaluation

Clinical features were obtained by retrospective review of the patients' medical records, which included data about sense of smell, family history, associated anomalies, history of micropenis, history of cryptorchidism, and laboratory and radiologic findings. Pubertal development was rated according to the guidelines recommended by Marshall and Tanner [13]. Testicular volume was measured with a Prader orchidometer. While olfactory function was evaluated in four patients using the 12 item smell identification test [14], the olfactory function of 24 patients was validated by self-reported history.

Targeted gene panel sequencing and bioinformatics analysis

Genomic DNA was extracted from peripheral blood leukocytes using the Gentra Puregene Blood Kit (Qiagen, Hilden, Germany). A targeted panel was designed using 69 genes that were known to be involved in hypothalamus/pituitary development [15–23], GnRH neuronal migration [10, 11, 24–34], synthesis and secretion of GnRH [35–42], IGD associated neurological disorders [43–48], and rare syndromes [26, 30, 49–57] associated with hypogonadism (**Table 1S**, Supporting Information). For comprehensive analysis of the causative genes in IGD, the previously reported IGD-associated genes in human study were selected from the online databases that follows: PubMed (<http://www.ncbi.nlm.nih.gov/pubmed/>), GeneTests (<http://www.genetests.org/>), and Online Mendelian Inheritance in Man (OMIM, <http://www.ncbi.nlm.nih.gov/omim/>).

We designed oligonucleotide bait tiles against exons and exon-intron boundaries within 20 base pairs of 69 genes that spanned a 163,269 bp region. Untranslated regions and promoter regions were not captured. Exomes were captured using a customized Target Enrichment System Kit (Celeomics, Seoul, Korea) and then sequenced on the Miseq Platform (Illumina Inc., San Diego, CA, USA). The mean depth of coverage was 163 reads per base. Sequence reads were aligned to the human reference genome (hg19) using the Burrow-Wheeler Alignment program (BWA version 0.7.12). SAMtools 0.1.19 and Genome Analysis Toolkits (GATK version 3.5) were used for single nucleotide polymorphism (SNP) variant calling from aligned sequence reads. GATK version 3.5, FreeBayes 0.9.2.1, and Scalpel-0.5.3 were used for insertion-deletion variant calling. After removing duplicates with PICARD (<https://broadinstitute.github.io/picard/>), annotation was performed with a Variant Effect Predictor [58].

To reduce the risk of false-positive findings, SNP Phred scores of ≥ 15 and percent of non-reference calls of ≥ 15 were analyzed. Variants with minor allele frequencies of > 1% in the 1000Genomes Browser (<http://browser.1000genomes.org/>), NHLBI ESP Exome Variant Server (<http://evs.gs.washington.edu/EVS/>), and genome Aggregation Database (gnomAD; <http://gnomad.broadinstitute.org/>) were excluded. Sequence variants were computationally annotated

using dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>)[59] for known polymorphisms, and ClinVar (<http://www.ncbi.nlm.nih.gov/clinvar/>)[60] and Human Gene Mutation Database (<http://www.hgmd.org/>)[61] to determine variant pathogenicity. The date of last access for online resources was January 10th, 2018. Rare sequence variants found in targeted gene panel sequencing were confirmed by Sanger sequencing using custom-designed primers. Our sequencing method and algorithm were validated using the NA12878 DNA from the Coriell Cell Repositories of the National Institute of Standards and Technology-led Genome in a Bottle Consortium. Analytic sensitivity, specificity, and reproducibility were 100%, respectively [62].

In silico prediction of the likely effects of novel sequence variants was performed using the following web-based programs: PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>), Sorting Intolerant From Tolerant (SIFT; <http://sift.jcvi.org/>), MutationTaster (<http://www.mutationtaster.org/>), Mutation Assessor (<http://www.mutationassessor.org/v1/>), and Protein Variation Effect Analyzer (PROVEAN; <http://provean.jcvi.org/>) for missense variants, and EX-SKIP (<http://ex-skip.img.cas.cz/>), Human Splicing Finder (<http://www.umd.be/HSF3/>), MaxEntScan (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), NNSPICE (http://www.fruitfly.org/seq_tools/splice.html), and SpliceSiteFinder-like (<http://www.umd.be/searchsplicesite.html>) for splice site variants. Novel sequence variants were classified as being pathogenic, likely pathogenic, a variant of uncertain significance (VUS), likely benign, or benign, in accordance with the guidelines of the American College of Medical Genetics Laboratory Practice Committee Working Group [63].

Results

Clinical and endocrinological characteristics

The patients' characteristics are summarized in **Table 2S**. Among the 26 patients with IGD, 12 (46.2%) were diagnosed with KS, and 14 (53.8%) were categorized with nIHH. The mean age at presentation in IGD patients was 16.5 ± 3.1 years (range, 9–21 years). A clinical diagnosis of IGD was made after follow up to the age of 17 years in females and 18 years in males; the mean patient age at the time of the study was 22.8 ± 4.3 years (range, 18–32 years). Most patients were sporadic, and all but two male siblings (Subjects 8 and 9), who had been diagnosed with nIHH, had no family history of delayed puberty of IGD. All parents of the patients with IGD had normal fertility.

Micropenis was present in 15 male patients (9 with KS and 6 with nIHH), and cryptorchidism was present in 3 males (2 with KS and 1 with nIHH). Hearing defects were apparent in five patients (four with KS and one with nIHH). Congenital heart disease was noted in three patients with KS (pulmonary atresia and ventricular septal defect). Among 25 IGD patients who underwent brain MRIs, aplasia or hypoplasia of the olfactory bulbs/sulci was detected in 6 of the 12 patients with KS (50%). The GnRH stimulation test was performed in 24 IGD patients, wherein peak luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels were 4.1 ± 3.4 mIU/mL and 3.5 ± 1.7 mIU/mL in the KS patients, and 5.6 ± 6.3 mIU/mL and 5 ± 4.3 mIU/mL in the nIHH patients, respectively (reference range at the onset of puberty: peak LH and FSH levels after GnRH injection, 12.8 ± 7.7 and 5.1 ± 2.8 mIU/mL in males; 4.9 ± 2.8 and 8.1 ± 2.6 in females)[64].

Two pre-pubertal males (Subjects 13 and 28) presented with anosmia at 8.1 and 5.8 years, respectively, and aplasia of the olfactory bulbs was seen in both patients. Micropenis and cryptorchidism was observed in Subject 13. While both patients underwent ANOS1 analyses prior to this study, no mutations were detected.

Molecular features of patients with pathogenic or likely pathogenic sequence variants

Four pathogenic and six likely pathogenic sequence variants were identified in 11 patients from 10 of the 27 families (37%) included in the study (**Table 1**, **Fig. 1S**). Among the patients with pathogenic mutations, Subject 1 was heterozygous for c.5405-7 G>A in CHD7[65], and Subject 3 was heterozygous for a novel nonsense variant of p.Q40* in CHD7 (**Table 1**). These patients were classified as KS with hearing defects. Subject 2 with KS and a history of cryptorchidism was heterozygous for PROKR2 p.W178S, which is known to be pathogenic [66]; brain MRI showed hypoplastic olfactory sulci and bulbs. Two male siblings (Subjects 8 and 9) with homozygous frameshift mutations (p.M176Tfs*14) in TACR3 exhibited delayed puberty without anosmia at the age of 16 and 20 years of age, respectively.

Likely pathogenic variants were identified in six patients (**Table 1**). Subject 4, with heterozygous p.Y339H mutation in FGFR1, presented with primary amenorrhea without skeletal and olfactory abnormalities. Subject 5, carrying p.N185Kfs*16 in FGFR1, showed KS with small olfactory bulbs on brain MRI. A novel hemizygous frameshift variant, p.Q421Kfs*61 in ANOS1, was identified in Subject 6. Hemizygous in-frame deletion of the polyalanine tract in SOX3 (p.A234_240del) was detected in Subject 7 with nIHH, who presented with delayed puberty, without other anomalies, at 18 years of age. Subject 10, who presented with nIHH and finger syndactyly, harbored a heterozygous variant of c.1855-1 G>A in FGFR1, which was predicted to be damaging by prediction programs (**Table 3S**). Subject 11 with a history of micropenis and cryptorchidism at birth presented with anosmia at the age of 8 years and was heterozygous for c.1663+2T>G in FGFR1. This variant was predicted to be deleterious by in silico programs (**Table 3S**).

Clinical and molecular findings in patients with variants of uncertain significance

VUS were identified in two patients with IGD (one with KS and one with nIHH) (**Table 2**, **Fig. 1S**). Subject 12 harbored a novel heterozygous VUS of c.3201+4A>G in CHD7. He presented with KS and aplastic olfactory bulbs, as seen on brain MRI. Subject 13 carried a novel heterozygous missense variant of p.S681I in FGFR1, which was not found in normative population databases and was predicted to be deleterious by in silico programs (**Table 4S**).

Discussion

This study demonstrated the clinical efficacy of targeted gene panel sequencing in patients with IGD of unknown genetic etiology. Targeted gene panel sequencing achieved genetic diagnosis in 37% of the patients. Genetic diagnosis using Sanger sequencing is challenging due to clinical and genetic heterogeneity, as well as complex genetic architecture such as oligogenicity. Therefore, target-

► **Table 1** Molecular genetic findings in patients harboring pathogenic or likely pathogenic variants.

Subject	Sex	Age at presentation (years)	Phenotype	Sequence variants			Interpretation
				Gene	Nucleotide change	Amino acid change	
1	M	14	Kallmann syndrome, hearing defect	<i>CHD7</i>	c.5405-7 G>A	Splice site	Pathogenic
2	M	21	Kallmann syndrome	<i>PROKR2</i>	c.533 G>C	p.W178S	Pathogenic
3	M	16	Kallmann syndrome, hearing defect	<i>CHD7</i>	c.118 C>T	p.Q40 *	Pathogenic
4	F	19	nIHH, primary amenorrhea	<i>FGFR1</i>	c.1015 T>C	p.Y339H	Likely pathogenic
5	M	20	Kallmann syndrome	<i>FGFR1</i>	c.551 dup	p.N185Kfs * 16	Likely pathogenic
6	M	16	nIHH	<i>ANOS1</i>	c.1260del	p.Q421Kfs * 61	Likely pathogenic
7	M	18	nIHH	<i>SOX3</i>	c.699_719del	p.A234_A240del	Likely pathogenic
8	M	20	nIHH	<i>TACR3</i>	c.527_533del/ c.527_533del	p.M176Tfs * 14/ p.M176Tfs * 14	Pathogenic
9	M	16	nIHH				
10	M	18	nIHH, finger syndactyly	<i>FGFR1</i>	c.1855-1 G>A	Splice site	Likely pathogenic
11	M	7	Anosmia, micropenis, cryptorchidism	<i>FGFR1</i>	c.1663+2T>G	Splice site	Likely pathogenic

F, female; M, male; nIHH, normosmic idiopathic hypogonadotropic hypogonadism

► **Table 2** Molecular genetic findings in patients with variants of uncertain significance.

Subject	Sex	Age at presentation (years)	Phenotype	Sequence variants		
				Gene	Nucleotide change	Amino acid change
12	M	19	KS	<i>CHD7</i>	c.3201+4A>G	Splice site
13	M	14	nIHH	<i>FGFR1</i>	c.2042 G>T	p.S681I

KS, Kallmann syndrome; M, male; nIHH, normosmic idiopathic hypogonadotropic hypogonadism

ed NGS can support the genetic diagnosis of IGD with genetic heterogeneity and oligogenic inheritance.

This study identified an in-frame deletion in the first polyalanine tract of *SOX3* in Subject 7, who had been diagnosed with nIHH. The *SOX3* gene has a single exon on chromosome Xq26–27 and encodes a transcription factor that is involved in pituitary morphogenesis [67]. The dosage of *SOX3* is critical for normal pituitary development. In-frame duplication or deletion of *SOX3* results in the expansion or deletion of the polyalanine tract and has been identified in patients with combined pituitary hormone or isolated growth hormone deficiencies [68–70]. Consistent with our study, in-frame deletion of the polyalanine tract in *SOX3* was recently identified in a patient with nIHH [23], suggesting a diverse phenotypic spectrum of polyalanine deletions in *SOX3*.

In the current study, variants in *CHD7* and *FGFR1* were found in three (3 of 12 variants, 25%) and five (5 of 12 variants, 41.7%) patients, respectively, which were representative of a relatively high proportion of our cohort. Hearing defects represent a significant non-reproductive feature in patients with *CHD7* mutations [5]. Midline facial defects, dental agenesis, and skeletal anomalies can indicate mutations in the *FGF8/FGFR1* pathway [2]. Bimanual synkinesia and renal agenesis are important clinical clues in *ANOS1* mutations [2]. However, in the present study's cohort, these non-reproductive features including hearing defects and syndactyly were noted in only three patients. Of these patients, two had pathogenic *CHD7* mutations (Subjects 1 and 3) and one had a novel

FGFR1 variant (Subject 10). The majority of the remaining patients shared a similar phenotype, making it difficult to prioritize genetic variants for screening. Therefore, the application of NGS-based panel testing is an effective diagnostic tool for detecting causative genes in IGD patients with genetic heterogeneity.

Congenital anosmia is a rare condition that can be categorized into isolated or syndromic [71], and KS is a well-described form of syndromic congenital anosmia. Several genetic defects involved in the development and migration of olfactory and GnRH neurons are overlapped by both congenital anosmia and KS [33, 71]. A previous study performed multi-gene panel sequencing including *ANOS1*, *FGF8*, *FGFR1*, *PROK2*, and *PROKR2* in 25 patients with isolated congenital anosmia [33]. As a result, genetic defects in *PROK2* and *PROKR2* were identified in four unrelated patients [33]. A recent NGS study has suggested that six syndromic KS genes, including *ANOS1*, *CHD7*, *FGFR1*, *PROK2*, *PROKR2*, and *SEMA3A*, are involved in isolated congenital anosmia [71]. In the current study, a pre-pubertal male (Subject 11) with anosmia, who had a history of micropenis and cryptorchidism, harbored a likely pathogenic variant in *FGFR1*, necessitating a follow-up assessment for pubertal progression.

This study has several limitations. For instance, molecular testing for family members was not performed to validate inheritance patterns and segregation, as the parents' blood samples were not available. In comparison with a previous targeted gene panel study of nIHH/KS [11], we designed a targeted panel that included all

known IGD-associated genes in humans to reduce the complexity of potential interpretations [11]. When compared to WES and WGS, targeted NGS reduces incidental findings and provides higher sequencing coverage and depth in the targeted regions.

Conclusions

We achieved a genetic diagnosis in 10 families (37%) and detected VUS in two patients (7.4%). In addition, novel pathogenic or likely pathogenic variants were identified in eight probands in ANOS1, CHD7, FGFR1, TACR3, and SOX3. This approach enables rapid, cost-effective, and comprehensive genetic screening in patients with IGD.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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