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ORIGINAL ARTICLE

Male Infertility

# Whole exome sequencing and trio analysis to broaden the variant spectrum of genes in idiopathic hypogonadotropic hypogonadism

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**Dozens of genes are associated with idiopathic hypogonadotropic hypogonadism (IHH) and an oligogenic etiology has been suggested. However, the associated genes may account for only approximately 50% cases. In addition, a genomic systematic pedigree analysis is still lacking. Here, we conducted whole exome sequencing (WES) on 18 unrelated men affected by IHH and their corresponding parents. Notably, one reported and 10 novel variants in eight known IHH causative genes (*AXL*, *CCDC141*, *CHD7*, *DMXL2*, *FGFR1*, *PNPLA6*, *POLR3A*, and *PROKR2*), nine variants in nine recently reported candidate genes (*DCAF17*, *DCC*, *EGF*, *IGSF10*, *NOTCH1*, *PDE3A*, *RELN*, *SLIT2*, and *TRAPPC9*), and four variants in four novel candidate genes for IHH (*CCDC88C*, *CDON*, *GADL1*, and *SPRED3*) were identified in 77.8% (14/18) of IHH cases. Among them, eight (8/18, 44.4%) cases carried more than one variant in IHH-related genes, supporting the oligogenic model. Interestingly, we found that those variants tended to be maternally inherited (maternal with $n = 17$ *vs* paternal with $n = 7$; $P = 0.028$). Our further retrospective investigation of published reports replicated the maternal bias (maternal with $n = 46$ *vs* paternal with $n = 28$; $P = 0.024$). Our study extended a variant spectrum for IHH and provided the first evidence that women are probably more tolerant to variants of IHH-related genes than men.**

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## INTRODUCTION

Idiopathic hypogonadotropic hypogonadism (IHH) is a rare genetic disorder that leads to delayed or absent puberty and infertility due to gonadotropin-releasing hormone (GnRH) insufficiency or deficiency.[1–3] IHH accompanied by anosmia or hyposmia is defined as Kallmann syndrome (KS), whereas IHH with normal olfaction is called normosmic IHH (nIHH). IHH is highly genetically heterogeneous, and more than 50 genes have been found to be related to IHH. However, only approximately 50% of cases were accounted for and presented all forms of classical Mendelian inheritance and oligogenicity.[4,5]

With the increasing use of next-generation sequencing (NGS), a variety of IHH-related genes have been explored in recent years.[6–9] However, the majority of genetic studies applied screening strategies that listed limited genes associated with the GnRH pathway or relevant function of IHH for screening, which cannot take full advantage of NGS and may miss potential causative genes beyond the gene lists. Previous studies were conducted mainly in sporadic cases, and little parental information was available for further investigations on pathogenicity.[7,8]

In this study, we first conducted a systematic genomic analysis using whole exome sequencing (WES) to screen for disease-causing variants in all known IHH genes and then identified potentially contributory variants in novel candidate genes by analyzing the remaining genes. We also investigated parental origins of those probably disease-causing variants by trio analysis and literatures mining.

## PARTICIPANTS AND METHODS

### *Study participants*

A total of 18 Han Chinese men with IHH and their families were recruited at Shanghai General Hospital (Shanghai, China). Their whole blood samples were obtained with informed consent. All of procedures involving human participants in this study were approved by the Ethics Committee of Shanghai General Hospital (2016KY196). Parenthood was confirmed by an EX20 kit (AGCU ScienTech Incorporation, Wuxi, China). The criteria for the diagnosis of IHH included: (1) absent or incomplete pubertal development by the age of 18 years in men; (2) clinical signs or symptoms of hypogonadism; (3) serum testosterone levels of <100 ng $dl^{-1}$ (3.5 nmol $l^{-1}$) in males with low or normal levels of gonadotropin; (4) normal thyroid, adrenal, and growth hormone axes; (5) normal magnetic resonance images of the hypothalamic and pituitary areas; and (6) the absence of sex chromosome abnormalities. Olfactory function was categorized into three groups: normosmia, hyposmia, and anosmia, based on self-reporting and test with familiar

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odors.[10] Clinical information of probands and their families can be found in **Supplementary Table 1**.

***Whole exome sequencing and data processing***

Genomic DNAs were extracted from the participants' peripheral blood samples using a DNeasy Blood and Tissue Kit (QIAGEN, Dusseldorf, Germany). The NimbleGen SeqCap EZ Exome Library SR version 3.0 (Roche, Basel, Switzerland) and the HiSeq X-TEN platform (Illumina, San Diego, CA, USA) were employed to enrich the human exome and sequencing, respectively. The average read depth was not less than 100× and more than 95% of the targeted region was covered over 20×.

Reads were aligned to the human genome reference assembly (UCSC Genome Browser hg19) with the Burrows-Wheeler Aligner (BWA).[11] The Genome Analysis Toolkit version 4.0 (GATK4.0, Broad Institute, Cambridge, MA, USA) was employed to remove PCR duplicates and evaluate the quality of variants by attaining effective reads, effective base, average coverage depth and coverage ratio.[12] Single-nucleotide variants (SNVs) and short insertions and deletions (indels) were also called by GATK4.0. ANNOVAR was used to functionally annotate variants with Sorting Tolerant From Intolerant (SIFT), PolyPhen-2, MutationTaster, Mendelian Clinically Applicable Pathogenicity (M-CAP), the 1000 Genomes Project, and the Genome Aggregation Database (gnomAD).[13–18] Online Mendelian Inheritance in Man (OMIM), Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway and Reactome were also annotated to the genes.[19–21]

All of the final identified variants were confirmed by Sanger sequencing and their pathogenicity classifications were evaluated according to American College of Medical Genetics and Genomics and Association for Molecular Pathology guidelines (ACMG/AMP).[22]

***Genes selected for screening***

A total of 106 genes (**Supplementary Table 2**) collected from reports in the literatures or databases were classified into two categories as follows: (a) 57 IHH-causative genes associated in human IHH cases with evidence such as functional assays and their mouse models manifesting IHH phenotype and (b) 49 IHH-reported candidate genes that were reported only recently in IHH patients through screening strategies without any functional characterization.

## RESULTS

We applied WES in 18 trios. For quality control, the GATK hard filter criteria and read depth $<10$ were used to remove low-quality variants. Considering common variants with less functionality[23] and the scarce incidence of IHH, we filtered out the variants with a minor allele frequency (MAF) higher than 0.01. Only loss-of-function (LoF) variants including frameshift indels, canonical splice-site, nonsense and start-loss, and missense variants predicted as damaging by at least two of four algorithms were considered in further analyses (**Supplementary Figure 1**).

***Variant spectrum in IHH genes***

The analysis initially screened two groups of all known genes described in the participants and methods section.

Thirteen (72.2%) of 18 patients were identified eleven variants in eight known causative genes, namely AXL receptor tyrosine kinase (*AXL*), coiled-coil domain containing 141 (*CCDC141*), chromodomain helicase DNA binding protein 7 (*CHD7*), Dmx like 2 (*DMXL2*), fibroblast growth factor receptor 1 (*FGFR1*), patatin like phospholipase domain containing 6 (*PNPLA6*), RNA polymerase III subunit A (*POLR3A*) and prokineticin receptor 2 (*PROKR2*), see **Table 1** and **2**. The *PROKR2* missense variant p.Trp178Ser was recurrently observed in three patients, and was statistically enriched in our IHH cohort compared to the population (3/18 in the IHH cases *vs* 56/9976 in the gnomAD East Asians, $P = 0.00015$, one-tailed Fisher's exact test). Furthermore, another *PROKR2* missense variant p.Ala103Val was identified in case P15. All of the remaining deleterious variants in the other seven known IHH genes were novel and unreported. Notably, the novel LoF variant *FGFR1* c.1664-2A>C in case P05 is *de novo* and evaluated as pathogenic.

In nine genes of the second group that were reported in recent NGS studies, we identified one nonsense, one frameshift deletion, and seven missense variants in 9 (50.0%) of 18 families (**Table 1**). The epidermal growth factor (*EGF*) nonsense variant c.3487C>T (p.Arg163*) predicted to attain a truncated protein was also previously detected in a target sequencing study of IHH.[7] Distribution of all the above identified variants in proteins is shown in **Figure 1**.

***Potential novel candidate genes***

Our subsequent analysis applied stricter criteria to screen for probably deleterious missense variants predicted as damaging by all of the algorithms. We first searched the remaining genes using annotation keywords (olfactory bulb, brain/nervous system development, hypothalamus, pituitary, and gonadotropin) in the OMIM, GO, KEGG pathway and Reactome pathway. Then we used genes as novel candidate genes if their functions were related to IHH through literatures mining. We finally found evidence of four potential novel candidate genes contributing to IHH: coiled-coil domain containing 88C (*CCDC88C*), cell adhesion associated, oncogene regulated (*CDON*), glutamate decarboxylase like 1 (*GADL1*), and sprouty related EVH1 domain containing 3 (*SPRED3*).

The *CCDC88C* missense variant p.Arg1299Cys was heterozygous in case P05. CCDC88C is a negative regulator of the Wnt signaling pathway, and bi-allelic mutations in *CCDC88C* were linked to midline brain malformation. Of note, the same variant p.Arg1299Cys was previously reported in a patient affected with pituitary stalk interruption syndrome (PSIS) with an etiologic overlap of IHH, who carried a mutation in an IHH-causative gene, tachykinin receptor 3 (*TACR3*).[24] Similarly, the *CCDC88C*-mutated case P05 in our study carried additional variants in DCC netrin 1 receptor (*DCC*) p.Gln91Arg, and *FGFR1* c.1664-2A>C, implying that the deleterious variants in *CCDC88C* act together with other variants to cause IHH through a digenic/oligogenic model.

One unreported and probably deleterious missense variant p.Val969Ile of another PSIS gene, *CDON*,[25] was also found in case P17 who carried a missense variant in *CHD7*, a causative gene of IHH. *CDON* seems to act similarly as *CCDC88C* through a digenic/oligogenic model to contribute to IHH.

Case P06 had a missense variant in *GADL1* (p.Ser221Cys), predicted as probably damaging. *GADL1* expression is present during early brain development and is higher in olfactory bulb than that in other tissues,[26] where is an active area for regeneration and migration of GnRH neurons. Consistent with this observation, case P06 was affected by anosmia, indicating that the function of *GADL1* might be involved in the etiology of IHH (**Table 2**).

A *de novo SPRED3* frameshift deletion (p.Gly52Asnfs*14) resulting in truncation of the protein was detected in case P09. SPRED3 is expressed exclusively in the brain.[25] *SPRED3* and sprouty RTK signaling antagonist 4 (*SPRY4*) are family members of SPROUTY (SPRY). The SPRED family functions as inhibitors of fibroblast growth factor (FGF) signaling cascades,[6] while *SPRY4* is a known IHH-causative gene, suggesting that *SPRED3* may also play a potential role in IHH.

**Table 1: Variant information of known and candidate idiopathic hypogonadotropic hypogonadism genes and novel candidate genes**

| *Gene* | *Transcript ID* | *Variant type* | *HGVS* | *ACMG classification* | *gnomAD_all* | *gnomAD_EAS* | *MutTaster* | *SIFT* | *PolyPhen-2* | *M-CAP* |
|---|---|---|---|---|---|---|---|---|---|---|
| IHH-causative genes | | | | | | | | | | |
| *AXL* | NM_021913.5 | Missense | c.1579C>T (p.Arg527Trp) | VUS | 0.000012 | 0 | D | D | D | D |
| *CCDC141* | NM_173648.4 | Missense | c.1951A>T (p.Asn651Tyr) | VUS | 0 | 0 | N | D | D | T |
| *CHD7* | NM_017780.4 | Missense | c.7235A>T (p.Glu2412Val) | VUS | 0 | 0 | D | D | D | D |
| | | Missense | c.5980T>G (p.Trp1994Gly) | VUS | 0 | 0 | D | D | D | D |
| *DMXL2* | NM_001174116.2 | Missense | c.4878G>C (p.Gln1626His) | VUS | 0.000020 | 0.00005809 | D | T | D | T |
| *FGFR1* | NM_023110.3 | Splice site | c.1664-2A>C | Pathogenic | 0 | 0 | D | NA | NA | NA |
| *PNPLA6* | NM_006702.5 | Missense | c.3422A>G (p.Asp1141Gly) | VUS | 0.000049 | 0.0007 | D | T | B | D |
| | | Missense | c.1244G>A (p.Arg415Gln) | VUS | 0.000008 | 0.00005835 | D | T | B | D |
| *POLR3A* | NM_007055.4 | Missense | c.2686G>A (p.Asp896Asn) | VUS | 0.000024 | 0 | D | T | D | D |
| *PROKR2* | NM_144773.3 | Missense | c.533G>C (p.Trp178Ser) | VUS | 0.0002 | 0.0027 | D | D | D | D |
| | | Missense | c.308C>T (p.Ala103Val) | VUS | 0.000073 | 0.001 | D | D | D | D |
| IHH-reported candidate genes | | | | | | | | | | |
| *DCAF17* | NM_025000.3 | Missense | c.1507T>C (p.Tyr503His) | VUS | 0 | 0 | D | D | D | D |
| *DCC* | NM_005215.3 | Missense | c.272A>G (p.Gln91Arg) | VUS | 0 | 0 | D | D | D | T |
| *EGF* | NM_001963.5 | Nonsense | c.3487C>T (p.Arg1163*) | VUS | 0.0002 | 0.0024 | D | NA | NA | NA |
| *IGSF10* | NM_178822.4 | Frameshift | c.1751_1752del (p.Thr584Serfs*5) | VUS | 0.0002 | 0.003 | D | NA | NA | NA |
| *NOTCH1* | NM_017617.4 | Missense | c.3679C>T (p.Pro1227Ser) | VUS | 0.000071 | 0.0006 | D | D | D | D |
| *PDE3A* | NM_000921.4 | Missense | c.3038G>T (p.Cys1013Phe) | VUS | 0.000057 | 0.0008 | D | T | P | D |
| *RELN* | NM_005045.3 | Missense | c.491G>A (p.Arg164Gln) | VUS | 0.000020 | 0.00005799 | D | D | B | T |
| *SLIT2* | NM_004787.3 | Missense | c.3076C>T (p.Pro1026Ser) | VUS | 0 | 0 | D | T | B | D |
| *TRAPPC9* | NM_031466.7 | Missense | c.3121G>C (p.Asp1041His) | VUS | 0.0002 | 0.0014 | D | D | D | T |
| Novel candidate genes | | | | | | | | | | |
| *CCDC88C* | NM_001080414.3 | Missense | c.3895C>T (p.Arg1299Cys) | VUS | 0.0045 | 0.0045 | D | D | D | D |
| *CDON* | NM_016952.5 | Missense | c.2905G>A (p.Val969Ile) | VUS | 0.000045 | 0.0002 | D | D | D | D |
| *GADL1* | NM_207359.2 | Missense | c.662C>G (p.Ser221Cys) | VUS | 0.000049 | 0.0007 | D | D | D | D |
| *SPRED3* | NM_001042522.2 | Frameshift | c.154delG (p.Gly52Asnfs*14) | VUS | 0 | 0 | D | NA | NA | NA |

B: benign; D: deleterious; gnomAD_all: allele frequency of all population in gnomAD; gnomAD_EAS: allele frequency of East Asian population in gnomAD; MutTaster: MutationTaster; N: neutral; NA: not available; P: possibly damaging; T: tolerated; VUS: variant of uncertain significance; ACMG: American College of Medical Genetics and Genomics; *AXL*: AXL receptor tyrosine kinase; *CCDC141*: coiled-coil domain containing 141; *CCDC88C:* coiled-coil domain containing 88C; *CDON*: cell adhesion associated, oncogene regulated; *CHD7*: chromodomain helicase DNA binding protein 7; *DCAF17*: DDB1 and CUL4 associated factor 17; *DCC*: DCC netrin 1 receptor; *DMXL2*: Dmx like 2; *EGF*: epidermal growth factor; *FGFR1*: fibroblast growth factor receptor 1; *GADL1*: glutamate decarboxylase like 1; *IGSF10*: immunoglobulin superfamily member 10; *NOTCH1*: notch receptor 1; *PDE3A*: phosphodiesterase 3A; *PNPLA6*: patatin like phospholipase domain containing 6; *POLR3A*: RNA polymerase III subunit A; *PROKR2*: prokineticin receptor 2; *RELN*: reelin; *SLIT2*: slit guidance ligand 2; *SPRED3*: sprouty related EVH1 domain containing 3; *TRAPPC9*: trafficking protein particle complex 9; IHH: idiopathic hypogonadotropic hypogonadism

### *Potential oligogenic inheritance in IHH*

Oligogenic inheritance with at least two potential pathogenic variants was observed in nearly half (8/18, 44.4%) of the families in this IHH cohort. Remarkably, one proband even had four potential pathogenic variants (**Table 2**). Our findings suggested that the oligogenic inheritance could be common in IHH (**Supplementary Figure 2**).

### *Maternal inherent bias of IHH*

Of 26 individual variants identified on autosomes in this study, 17 (65.4%) variants were maternally inherited, more than twice the paternal (7/26, 26.9%) variants, and two (7.7%) variants were *de novo* (**Table 2**). The inheritance of IHH-related variants had a preference on maternal origins ($P = 0.028$, one-tailed Fisher's exact test) compared to the potential deleterious variants of all of the genes except IHH genes in the cases (maternal with $n = 1495$ *vs* paternal with $n = 1535$; **Supplementary Table 3**).

Considering our study's limited sample size, we searched for all of the autosomal variants of IHH genes with pedigree information in the literatures for further validation. Genes with fewer than 9 variants were filtered out. Finally, we ascertained the qualified data of four genes, including *CHD7*, *FGFR1*, *PROKR2*, and gonadotropin releasing hormone receptor (*GNRHR*). The results consistently supported maternal bias (maternal with $n = 46$ *vs* paternal with $n = 28$; $P = 0.024$, binomial one-tailed test; **Supplementary Table 4**).

## DISCUSSION

The results of our study extended the variant spectrum of IHH and revealed three reported and 17 novel variants in the known IHH genes. Except for the *FGFR1* c.1664-2A>C variant, all of the variants were classified as uncertain significance of pathogenicity, implying that functional studies should be conducted in the future to provide additional evidence for the pathogenicity of those novel variants in IHH.

A total of 24 rare variants were identified in 77.8% (14/18) of the IHH-affected cases in this study. All of those variants were heterozygous, and most were missense and dispersedly distributed in cases, indicating strong complexity and heterogeneity of IHH. *PROKR2* had the highest variant frequency (4/18, 22.2%) in our study. Although *PROKR2* variant p.Trp178Ser has a relatively high allele population frequency (0.002–0.003) in East Asians, It was proven in previous studies to be functional damaging[27,28] using *in vitro* functional assays and was enriched in our IHH cohort.

**Table 2: Variant Inheritance of the mutated men with idiopathic hypogonadotropic hypogonadism**

| *Sample ID* | *Sex* | *Olfactory* | *Variant* | *Zygosity* | *Parental origin* | *Previously reported* |
|---|---|---|---|---|---|---|
| P01 | Male | Normosmia | NM_178822.4(*IGSF10*):c.1751_1752del (p.Thr584Serfs*5) | Heterozygous | Maternal | |
| P04 | Male | Normosmia | NM_017617.4(*NOTCH1*):c.3679C>T (p.Pro1227Ser) | Heterozygous | Paternal | |
| | | | NM_004787.3(*SLIT2*):c.3076C>T (p.Pro1026Ser) | Heterozygous | Paternal | |
| P05 | Male | Anosmia | NM_023110.3(*FGFR1*):c.1664-2A>C | Heterozygous | *De novo* | |
| | | | NM_005215.3(*DCC*):c.272A>G (p.Gln91Arg) | Heterozygous | Paternal | |
| | | | NM_001080414.3(*CCDC88C*):c.3895C>T (p.Arg1299Cys) | Heterozygous | Maternal | PSIS[24] |
| P06 | Male | Anosmia | NM_207359.2(*GADL1*):c.662C>G (p.Ser221Cys) | Heterozygous | Maternal | |
| P07 | Male | Anosmia | NM_021913.5(*AXL*):c.1579C>T (p.Arg527Trp) | Heterozygous | Maternal | |
| | | | NM_001963.5(*EGF*):c.3487C>T (p.Arg1163*) | Heterozygous | Maternal | IHH[7] |
| P09 | Male | Normosmia | NM_001042522.2(*SPRED3*):c.154delG (p.Gly52Asnfs*14) | Heterozygous | *De novo* | |
| | | | NM_005045.3(*RELN*):c.491G>A (p.Arg164Gln) | Heterozygous | Maternal | |
| P10 | Male | Anosmia | NM_006702.5(*PNPLA6*):c.3422A>G (p.Asp1141Gly) | Heterozygous | Maternal | |
| P11 | Male | Hyposmia | NM_173648.4(*CCDC141*):c.1951A>T (p.Asn651Tyr) | Heterozygous | Maternal | |
| P12 | Male | Hyposmia | NM_144773.3(*PROKR2*):c.533G>C (p.Trp178Ser) | Heterozygous | Maternal | KS, nIHH[7,27,28] |
| | | | NM_017780.4(*CHD7*):c.7235A>T (p.Glu2412Val) | Heterozygous | Maternal | |
| P14 | Male | Normosmia | NM_144773.3(*PROKR2*):c.533G>C (p.Trp178Ser) | Heterozygous | Maternal | KS, nIHH[7,27,28] |
| | | | NM_007055.4(*POLR3A*):c.2686G>A (p.Asp896Asn) | Heterozygous | Maternal | |
| | | | NM_006702.5(*PNPLA6*):c.1244G>A (p.Arg415Gln) | Heterozygous | Maternal | |
| | | | NM_031466.7(*TRAPPC9*):c.3121G>C (p.Asp1041His) | Heterozygous | Paternal | |
| P15 | Male | Anosmia | NM_144773.3(*PROKR2*):c.308C>T (p.Ala103Val) | Heterozygous | Paternal | KS, nIHH[7] |
| | | | NM_001174116.2(*DMXL2*):c.4878G>C (p.Gln1626His) | Heterozygous | Maternal | |
| | | | NM_025000.3(*DCAF17*):c.1507T>C (p.Tyr503His) | Heterozygous | Paternal | |
| P16 | Male | Normosmia | NM_000921.4(*PDE3A*):c.3038G>T (p.Cys1013Phe) | Heterozygous | Maternal | |
| P17 | Male | Anosmia | NM_017780.4(*CHD7*):c.5980T>G (p.Trp1994Gly) | Heterozygous | Paternal | |
| | | | NM_016952.5(*CDON*):c.2905G>A (p.Val969Ile) | Heterozygous | Maternal | |
| P18 | Male | Anosmia | NM_144773.3(*PROKR2*):c.533G>C (p.Trp178Ser) | Heterozygous | Maternal | KS, nIHH[7,27,28] |

IHH: idiopathic hypogonadotropic hypogonadism without smell information; KS: Kallmann Syndrome; nIHH: normosmic idiopathic hypogonadotropic hypogonadism; PSIS: pituitary stalk interruption syndrome. The definitions of the genes have been shown in **Table 1**

A recent study indicated that *PLXNA1* has an oligogenic inheritance rate of 77.7% in IHH,[29] while our results indicated an overall oligogenic inheritance rate of 57.1% (8/14) among the detected families by simultaneously screening all causative and candidate genes. The results of our study and the *PLXNA1* study consistently suggested that the rate of oligogenic inheritance of IHH genes varies and maintains at high levels.

According to our data, eight patients had at least two IHH gene variants. Two patients carried three variants and one patient even carried four variants. Our data supported "additive effect" and "cumulative mutation burden" that were proposed in studies related to IHH.[8,29] For example, two variants in proband P15, p.Ala103Val in *PROKR2* and p.Tyr503His in DDB1 and CUL4 associated factor 17 (*DCAF17*), were inherited from unaffected father, while *DMXL2* p.Gln1626His variant was inherited from unaffected mother. Proband 17 inherited *CHD7* p.Trp1994Gly and *CDON* p.Val969Ile variants from his unaffected father and mother, respectively. Notably, proband P05 in family 05 harbored a *de novo FGFR1* c.1664-2A>C variant. Since the *FGFR1* c.1664-2A>C variant was evaluated as pathogenic according to the ACMG guideline, this family might be considered as a case of monogenic inheritance. However, proband P05 also carried a paternal variant (*DCC* p.Gln91Arg) and a maternal variant (*CCDC88C* p.Arg1299Cys). Considering the facts that the loss-of-function mutations in *FGFR1* were identified to act in concert with other gene defects[8,30] and the *CCDC88C* p.Arg1299Cys variant was reported in a PSIS patient with an IHH-causative gene in a digenic manner,[24] the possibility of oligogenic inheritance in family 05 cannot be ruled out.

Six families harbored only one variant of IHH genes, but none had sufficient evidence to be identified as monogenic models. Among these variants, one was frameshift variant, immunoglobulin superfamily member 10 (*IGSF10*) p.Thr584Serfs*5, and the rest were missense variants. However, the possibility of being loss-of-function intolerant (pLI) value of *IGSF10* is zero, which means that single heterozygous LoF variant of *IGSF10* is not sufficient to cause disease. Furthermore, proband P18 was only detected one heterozygous variant, *PROKR2* p.Trp178Ser, whereas probands P12 and P14 carried the same *PROKR2* variant and additional variants in other candidate genes. The families' results consistently support the digenic/oligogenic inheritance in IHH, and novel IHH-associated genes and variants may be elucidated with advances in genetic knowledge (for example, noncoding variants) and genomic technologies (for example, those for detecting complex structural variations).

We also found four novel potential candidate genes for future investigations. Of interest, *CCDC88C* and *CDON* are known causative genes of PSIS, one of congenital hypopituitarism. PSIS has similar clinical phenotypes and shares some causative genes with IHH,[31] such as *PROKR2*, GLI family zinc finger 2 (*GLI2*), and WD repeat domain 11 (*WDR11*). Most IHH-causative genes are involved in early brain development, which may affect multiple organs and be associated with more than one disorder or syndrome.[32] In addition, the pathophysiology of IHH sometimes involves a combination of genetic variants that affect both neuronal development and the gonadotropic cascade.[4,33] Therefore, we suggest that the causative genes of associated development disorders should also be studied, such as abnormalities of the hypothalamus, pituitary, or midline brain, and the remaining genes except for known genes should not be ignored.

The prevalence of IHH is 4–5 times more common in men than women, whereas its genetic causes remain elusive.[34] It used to be thought

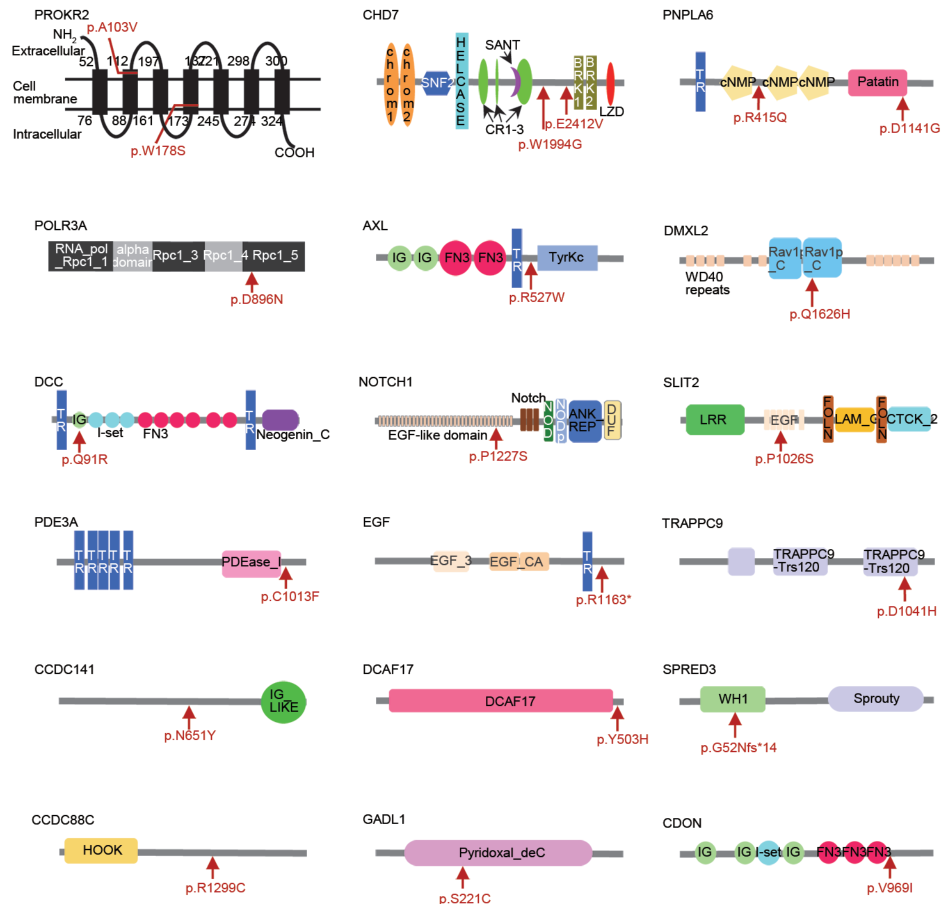


**Figure 1:** Distribution of identified variants in the IHH-related proteins. Domains and motifs were predicted by the SMART and InterPro tools. The variants identified in this study are indicated with red arrows. ANK_REP: ankyrin repeat-containing domain; cNMP: cyclic nucleotide-monophosphate binding domain; CTCK_2: cystine knot, C-terminal; DUF3454: domain of unknown function, notch; FN3: fibronectin type III domain; FOLN: follistatin-N-terminal domain-like; HOOK: hook-like protein family; IG: immunoglobulin; I-set: immunoglobulin I-set domain; IHH: idiopathic hypogonadotropic hypogonadism; LAM_G: laminin G domain; LRR: leucine-rich repeat; Neogenin_C: neogenin C-terminus; Patatin: patatin-like phospholipases domain; Pyridoxal_deC: group II pyridoxal-dependent decarboxylases; Rav1p_C: RAVE complex protein Rav1 C-terminal; SAP: a putative DNA/RNA binding domain found in diverse nuclear and cytoplasmic proteins; Sprouty: Sprouty protein; TR: transmembrane region; TRAPPC9-Trs120: transport protein Trs120 or TRAPPC9, TRAPP II complex subunit; TyrKc: tyrosine-protein kinase, catalytic domain; WH1: WH1 domain.

that X-linked inheritance contributes this sex-biased distribution, while there are only a small fraction of genetic findings in previous studies supporting this X-linked hypothesis. Intriguingly, it was previously reported that *FGFR1* deleterious variant caused milder phenotypes in female carriers than male carriers.[8] Interestingly, the damaging variants of *PROKR2* p.Trp178Ser in three unrelated male cases in this study were all inherited from their unaffected mothers. Furthermore, even if only IHH causative genes are considered, variants also tend to be maternally inherited in our study and previous reports ($P = 0.018$ and $P = 0.024$, respectively; **Supplementary Table 3** and **4**). Therefore, we proposed that females may be more tolerant to deleterious variants in IHH genes than males, which is an essential implication in the marked male preponderance of IHH. For stronger statistical significance and confirmation, more pedigrees should be enrolled and analyzed in the future. Our study provided new insights into the molecular variant spectrum and mechanism underlying male preponderance of IHH.

## AUTHOR CONTRIBUTIONS

ZL conceived and supervised the whole research. JZ conducted the whole exome sequencing. SYT designed the study and did the data processing and analysis. SYT and JZ wrote the manuscript. XBZ and PL contributed to patient recruitment, clinical information collection and follow-up. JQL organized the materials and conducted DNA extraction. JSC collected literatures and data of IHH genes with pedigree information. LBW participated in preparing and revising the manuscript. FZ supervised this investigation and prepared and revised the manuscript. All authors read and approved the final manuscript.

## COMPETING INTERESTS

All authors declared no competing interests.

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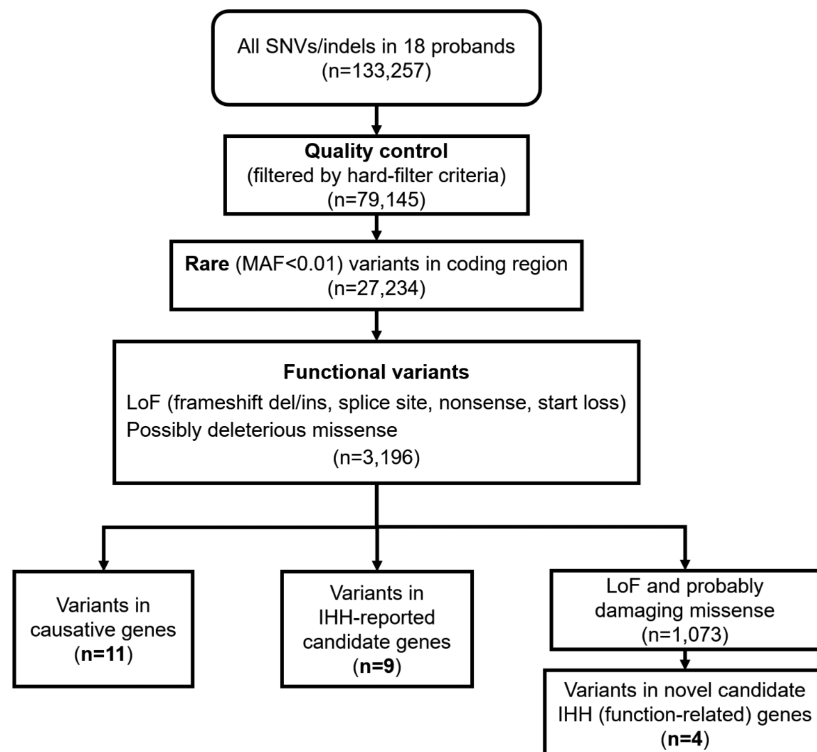
**Supplementary Table 1: Clinical information of individuals with heterozygous variants in idiopathic hypogonadotropic hypogonadism genes and their families**

| *Sample ID* | *Age* | *Height (cm)/ weight (kg)* | *FSH (mIU/mL)* | *LH (mIU/ mL)* | *T (ng/dL)* | *Olfactory* | *Other phenotype* | *Families* |
|---|---|---|---|---|---|---|---|---|
| P01 | 30 | 162/50 | 0.84 | 1.08 | 0.54 | Normosmia | No | Father: 56 yo, 173 cm/85 kg, no abnormalities<br>Mother: 56 yo, 159 cm/63 kg, no abnormalities |
| P04 | 28 | 176/65 | 0.74 | 0.18 | 0.37 | Normosmia | No | Father: age, height/weight are NA, no abnormalities<br>Mother: age, height/weight are NA, no abnormalities |
| P05 | 27 | 172/74 | 0.82 | 0.42 | 0.3 | Anosmia | No | Father: 55 yo, 165 cm/62 kg, no abnormalities<br>Mother: 54 yo, 157 cm/53 kg, no abnormalities |
| P06 | 30 | 178/70 | 1.08 | 0.21 | 0.17 | Anosmia | Hearing impairment | Father: age, height/weight are NA, no abnormalities<br>Mother: 55 yo, 161 cm/63 kg, no abnormalities |
| P07 | 28 | 178/80 | 0.86 | 0.18 | 0.45 | Anosmia | No | Father: 60 yo, 169 cm/74 kg, no abnormalities<br>Mother: 58 yo, 158 cm/60 kg, no abnormalities |
| P09 | 31 | 173/71 | 0.72 | 0.3 | 0.58 | Normosmia | No | Father: 56 yo, 169 cm/75 kg, no abnormalities<br>Mother: age, height/weight are NA, no abnormalities |
| P10 | 20 | 173/69 | 0.92 | 0.48 | 0.13 | Anosmia | No | Father: 45 yo, 169 cm/74 kg, no abnormalities<br>Mother: 45 yo, 158 cm/60 kg, no abnormalities |
| P11 | 21 | 180/55 | 0.17 | 0.1 | 0.43 | Hyposmia | No | Father: 47 yo, 175 cm/75 kg, no abnormalities<br>Mother: 44 yo, 161 cm/63 kg, no abnormalities |
| P12 | 33 | 176/77 | 0.58 | 0.21 | 0.08 | Hyposmia | No | Father: 59 yo, 163 cm/65 kg, no abnormalities<br>Mother: 59 yo, 161 cm/57 kg, no abnormalities |
| P14 | 35 | 187/92 | 0.94 | 0.31 | 1.65 | Normal | No | Father: 59 yo, 168 cm/67 kg, no abnormalities<br>Mother: 60 yo, 153 cm/51 kg, no abnormalities |
| P15 | 28 | 170/70 | 1.04 | 0.93 | 0.114 | Anosmia | No | Father: 53 yo, 172 cm/63 kg, no abnormalities<br>Mother: 54 yo, 153 cm/59 kg, no abnormalities |
| P16 | 27 | 174/70 | 1.02 | 0.76 | 0.15 | Normosmia | No | Father: 53 yo, 169 cm/68 kg, no abnormalities<br>Mother: 50 yo, 160 cm/63 kg, no abnormalities<br>Brother: 22 yo, 175 cm/73 kg, no abnormalities |
| P17 | 18 | 165/70 | 0.12 | 0.07 | 0.21 | Anosmia | No | Father: 48 yo, 171 cm/78 kg, no abnormalities<br>Mother: 46 yo, 161 cm/56 kg, no abnormalities |
| P18 | 23 | 181/75 | 0.56 | 0.75 | 0.24 | Anosmia | No | Father: 48 yo, 175 cm/78 kg, no abnormalities<br>Mother: 46 yo, 165 cm/55 kg, no abnormalities |

Age, height and weight are at the time of diagnosis. FSH: follicle-stimulating hormone; LH: luteinizing hormone; T: testosterone; yo: years old




**Supplementary Figure 1:** An overview of variant filtering workflow. MAF: minor allele frequency; LoF: loss-of function; SNV: single nucleotide variant.

**Supplementary Table 2: List of idiopathic hypogonadotropic hypogonadism related genes**

| *IHH-causative genes*[1–33] | | *IHH-reported candidate genes*[31,32,34–40] | |
|---|---|---|---|
| *ANOS1* | *OTUD4* | *B4GAT1* | *NOS1* |
| *AXL* | *PAX6* | *CASR* | *NOTCH1* |
| *CCDC141* | *PCSK1* | *CCKAR* | *NR5A1* |
| *CHD7* | *PHIP* | *CCKBR* | *NRP1* |
| *DMXL2* | *PLXNA1* | *CNTN2* | *NRP2* |
| *DUSP6* | *PNPLA6* | *CRY1* | *NTN1* |
| *EBF2* | *POLR3A* | *CXCR4* | *PALM2* |
| *EMX1* | *POLR3B* | *DCAF17* | *PDE3A* |
| *FEZF1* | *PROK2* | *DCC* | *PIN1* |
| *FGF8* | *PROKR2* | *DLX5* | *PLXNB1* |
| *FGF17* | *PROP1* | *EDNRB* | *RD3* |
| *FGFR1* | *RAB3GAP1* | *EGF* | *RELN* |
| *FLRT3* | *RAB3GAP2* | *EGFR* | *ROBO3* |
| *FSHB* | *RAB18* | *EPHA5* | *SEMA4D* |
| *GNRH1* | *RNF216* | *ERBB4* | *SLIT2* |
| *GNRHR* | *SEMA3A* | *FEZ1* | *SRA1* |
| *HESX1* | *SEMA3C* | *FGF13* | *STS* |
| *HS6ST1* | *SEMA3E* | *GAP43* | *TRAPPC9* |
| *IL17RD* | *SEMA7A* | *GH1* | *TSPAN11* |
| *KISS1* | *SOX2* | *GHR* | *TYRO3* |
| *KISS1R* | *SOX3* | *GLI2* | |
| *LEP* | *SOX10* | *GLI3* | |
| *LEPR* | *SPRY4* | *HGF* | |
| *LHB* | *STUB1* | *IGSF10* | |
| *LHX3* | *TAC3* | *JAG1* | |
| *NELFCD* | *TACR3* | *KLB* | |
| *NEMF* | *TBC1D20* | *LIF* | |
| *NR0B1* | *TUBB3* | *MET* | |
| *NSMF* | | *MTOR* | |

IHH: idiopathic hypogonadotropic hypogonadism

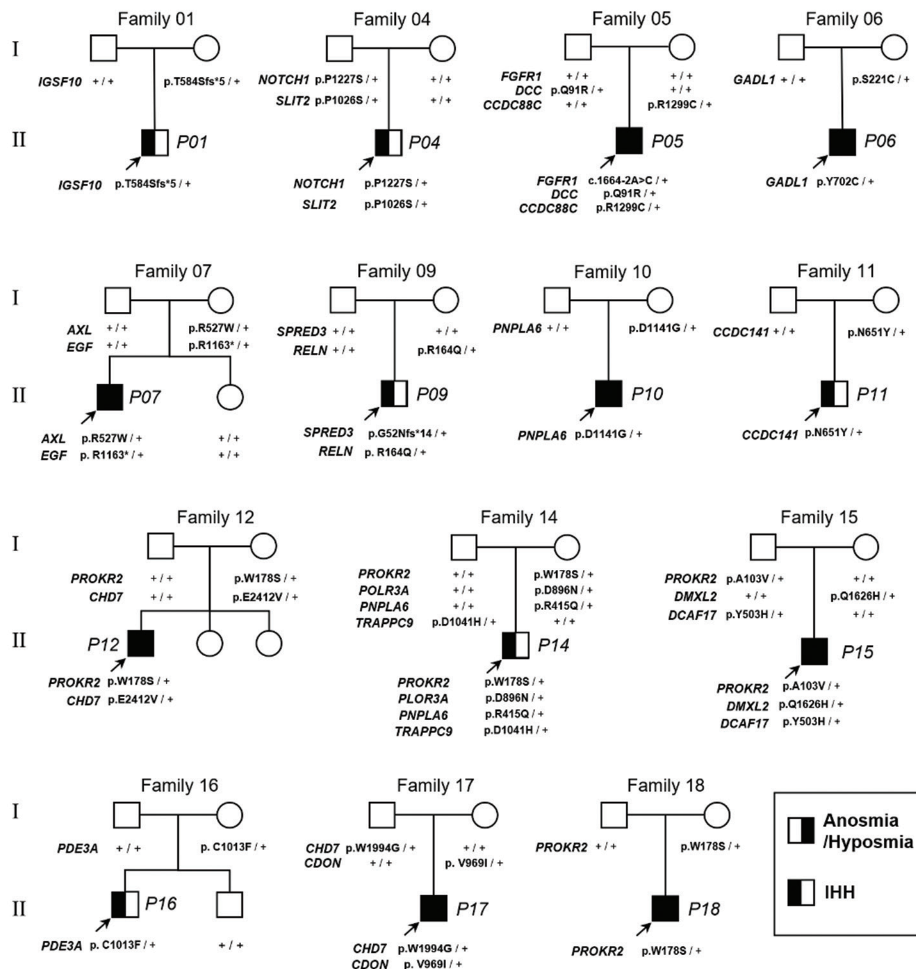
**Supplementary Table 3: Inheritance origin and statistical test of idiopathic hypogonadotropic hypogonadism genes and all genes in 18 affected individuals**

| *Gene list* | *Maternal* | *Paternal* | *P (Fisher's exact test, one-tailed)* | *Odds ratio* |
|---|---|---|---|---|
| Variants of IHH-causative genes | 10 | 2 | 0.018 | 5.12 |
| Plus variants of IHH-reported candidate genes | 13 | 7 | 0.122 | 1.90 |
| Plus variants of novel candidate genes | 17 | 7 | 0.028 | 2.49 |
| Variants of all genes | 1512 | 1542 | | |

IHH: idiopathic hypogonadotropic hypogonadism

**Supplementary Table 4: Inheritance origin and statistical test of idiopathic hypogonadotropic hypogonadism genes in literatures**

| *Gene* | *Chromosome* | *Maternal* | *Paternal* | *Total* | *P (Binomial test, one-tailed)* |
|---|---|---|---|---|---|
| *FGFR1* | chr8 | 25 | 12 | 37 | 0.024 |
| *GNRHR* | chr4 | 7 | 5 | 12 | 0.387 |
| *PROKR2* | chr20 | 9 | 7 | 16 | 0.402 |
| *CHD7* | chr8 | 5 | 4 | 9 | 0.500 |
| Total | | 46 | 28 | 74 | 0.024 |




**Supplementary Figure 2:** Pedigrees of IHH-affected cases and the inheritance of genetic variants. All variants were verified by Sanger sequencing in participants. Roman numerals denote generations. The probands are indicated by black arrows.

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