Original Article

Clinical and molecular features of 40 Chinese patients with idiopathic hypogonadotropic hypogonadism

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Background: Male idiopathic hypogonadotropic hypogonadism (IHH) is a heterogeneous clinical rare genetic disorder that can be divided into two forms: Kallmann syndrome (KS) and olfactory normal IHH (nIHH). Nearly half of unknown pathogenic genes and related pathogenic mechanisms have yet to be explored.

Methods: Clinical data of 40 IHH patients (22 KS and 18 nIHH) were retrospectively recorded. All patients were diagnosed at the Department of Endocrinology of Jinling Hospital, Jiangsu Provincial People's Hospital, and the First Affiliated Hospital of the University of Science and Technology of China from 2014 to 2021. The proband genomic DNA (gDNA) was confirmed by whole exome sequencing (WES) and Sanger sequencing.

Results: Ten new genetic mutations related to IHH in four families and eight sporadic unrelated IHH patients were identified. The total positive detection rate of 40 patients was 30% (nIHH 8/18 + KS 4/22), and the FGFR1 mutation rate accounted for 7.5% (3/40). Mutation rates of ANOS1, CHD7, and KISS1R were 5% (2/40), respectively. The mutation rates of SEMA3E, PROKR2, and SOX10 were 2.5% (1/40), respectively. After analysis by SIFT and PolyPhen-2 software, all missense mutation sites, such as *SEMA3E* (p.P323S), *CHD7* (p.W1785C), *PROKR2* (p.Y223D and p.R298C), were harmful; all nonsense mutation sites, such as *FGFR1* (p.R661X) and *KISS1R* (p.R331X, p.Y103X), analyzed were pathogenic by Mutation Taster software. The comparison of MEGA5 software showed that all the variants had extremely high homology among different species and were extremely conservative in evolution.

Conclusions: The study aims to expand the genotype mutation spectrum of IHH and provide evidence for the follow-up clinical treatment and genetic counseling of the disease.

Keywords: Idiopathic hypogonadotropic hypogonadism (IHH); Kallmann syndrome (KS); whole exome sequencing (WES); Sanger sequencing

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Introduction

Idiopathic/isolated hypogonadotropic hypogonadism (IHH) is a rare disease caused by impaired congenital hypothalamic gonadotropin-releasing hormone (GnRH) neuronal function, impaired synthesis, secretion, or function of GnRH results in decreased secretion of gonadotropin by the pituitary gland, also known as congenital hypogonadotropic hypogonadism (CHH) (1). Delayed puberty is the most typical manifestation of IHH disease is a clinical and genetically heterogeneous disease with sporadic and familial characteristics. It has been reported that a single gene mutation does not only cause IHH disease; some are also caused by multiple gene mutations (2). At present, the known gene mutations can only explain 30–50% of the pathogenic causes, and there are still primarily unknown pathogenic genes and related pathogenic mechanisms to be explored.

Slight and transient reactivation of the Hypothalamus-Pituitary-Gonad (HPG) axis in early infancy (approximately 4 to 16 weeks) provides a unique opportunity to diagnose congenital IHH in both male and female infants (3). GnRH neuron originates from the basal olfactory plate of the olfactory system and moves to the hypothalamus. The hypothalamus secretes GnRH, which is transported to the anterior pituitary through the capillary network, stimulating follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion to control reproduction (4). Traditionally, congenital IHH can be divided into two categories: (I) Kallmann syndrome (KS) is caused by developmental defects of GnRH neurons and olfactory bulbs, with varying degrees of loss or reduction of smell (5); (II) nIHH is usually caused by lack of FSH and LH due to GnRH secretion or interruption of function (6). The incidence of IHH is 1–10:100,000; about 60% of patients with IHH have complete or partial loss of smell (KS), and the ratio of men to women is as high as 5:1 (7). In addition, compared with affected female patients, IHH male patients show a more severe phenotype in the family (1).

Applying new technologies such as whole exome sequencing (WES) has increased the molecular diagnosis rate of IHH patients from approximately 30% to 50% (8). So far, the genes closely related to the molecular pathogenesis of the disease are *ANOS1* (*KAL1*), *LHB*, *FSHB*, *GNRHR*, *FGFR1*, *KISS1R*, *SOX2*, *PROKR2*, *CHD7*, *FGF8*, *GNRH1*, *KISS1*, *SEMA3A*, *FGF17*, *SOX10*, *DMXL2*, *SEMA3E*, *AMH*, *AMHR2*, *SPRY4*, *SEMA7A*, etc. The proteins encoded by these genes are involved in developing and migrating GnRH neurons or controlling different stages of GnRH function (9). It has been reported in the literature that mutations in genes related to GnRH secretion (*GNRH1*, *GNRHR*, KISS1/*KISS1R*, *TAC3*/*TACR3*, etc.) generally cause nIHH (6).

To better understand the molecular genetic basis of IHH, we retrospectively recorded 40 unrelated IHH patients for a series of Sanger sequence, WES, and bioinformatics analyses. Our data provide an essential foundation for patients' follow-up clinical diagnosis, treatment, and genetic counseling and expand the disease's genotype-phenotype spectrum. We present this article in accordance with the MDAR reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-23-225/rc).

Highlight box

Key findings

- Ten new genetic mutations related to IHH in four families and eight sporadic unrelated IHH patients were identified.

What is known and what is new?

- The incidence of IHH is 1–10:100,000; about 60% of patients with IHH have complete or partial loss of smell (KS), and the ratio of men to women is as high as 5:1.
- The total positive detection rate of 40 patients was 30% (nIHH 8/18 + KS 4/22), and the FGFR1 mutation rate accounted for 7.5% (3/40). Mutation rates of ANOS1, CHD7, and KISS1R were 5% (2/40), respectively. The mutation rates of SEMA3E, PROKR2, and SOX10 were 2.5% (1/40), respectively.

What is the implication, and what should change now?

- The study aims to expand the genotype mutation spectrum of IHH and provide evidence for the follow-up clinical treatment and genetic counseling of the disease.

Methods

Clinical research of samples

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics board of the Jinling Hospital, Nanjing University School of Medicine (No. 2018NZGKJ-003) and informed consent was taken from the patients or legal guardians. We diagnosed 40 IHH patients (22 KS and 18 nIHH) from 2014 to 2021 in Nanjing Jinling Hospital. All the patients were Han Chinese males who came to our hospital for gonadal dysplasia or fertility problems.

Sample collection criteria: (I) reproductive phenotype:

small penis, small testis or cryptorchidism or penis testis were childish, secondary sex characteristics were not prominent, etc. Patients with or without anosmia; a family history of related diseases; (II) sex hormone tests: low testosterone (T), very low FSH and LH levels or below the upper limit of normal [(in general: T <3.47 nmol/L, LH <0.7 IU/L), while prolactin (PRL) values were average (to exclude Prolactinoma)]; (III) brain MRL/CT showing normal or underdeveloped olfactory lamina and/or olfactory sulci (to exclude pituitary tumors); (IV) chromosome karyotype 46, XY without Y chromosome microdeletion; (V) other related hormone tests showed no abnormalities.

We screened olfactory using 75% medical alcohol, distilled water, and acetic acid; abnormal olfactory was defined as KS and normal olfactory was nIHH. We examined the age and weight of the patients, surgical history, PRL, FSH, LH, and T. We assessed the left and right testicular volumes and performed semen density analysis according to WHO criteria.

We collected the peripheral blood of patients and some family members to extract genomic DNA (gDNA) from blood. The quality of DNA samples was tested as follows: the total amount of DNA samples should be ≥200 ng, and the concentration should be ≥40 ng/μL. We detected the purity of DNA samples by NanoPhotometerR spectrophotometer, and Qubit 8.3.0 Fluorometer was utilized to detect the concentration of DNA samples.

WES, Sanger sequencing, and bioinformatics analysis

We performed the WES to 40 IHH probands, and the sequencing company assisted with WES. WES experiments for this study were conducted with the assistance of Beijing Nuohe Zhiyuan BioInformation Technology Co., Ltd. Specific steps: first, we extracted 3mL of peripheral blood from all participants to extract gDNA. We tested the sample quality again; the total amount of DNA was ≥2 ng, and the concentration was ≥40 ng/μL. Then the library construction and quality inspection. Then the samples were sequenced on the machine, and we analyzed the sequencing results informatically.

We verified all gene rare sequencing variants (RSVs) by Sanger sequencing. In only four patients, we conducted the pedigree investigation and related clinical examination for objective or family reasons. We designed primer using primer 5.0 software and amplified the exome regions of possible disease gene mutation sites analyzed by the proband and some families from the WES results.

We verified the success of polymerase chain reaction (PCR) by agarose gel electrophoresis and the gel imaging system. If the position of the PCR product band coincides with the target fragment, we performed the Sanger sequencing map with Chromas software. Check whether the peak map of the mutation site of the proband is consistent with that of the control group, and if not, verify whether the sequencing result is consistent with the WES consequence. Then, observe whether the family members also appear in this phenomenon, analyze whether it is compatible with the sense of co-separation of the mutation site and the disease, and finally, query whether the mutation site is novel.

Mutations detected in the study are based on the rules for naming mutations established by the Human Genome Variation Society (HGVS). According to the American College of Medical Genetics and Genomics (ACMG) guidelines, the biological information of rare mutation sites, including the correlation of mutations and diseases, mutation pathogenicity, minimum allele frequency of the human genome database, etc. We utilized MEGA5 software to analyze the homology of amino acids encoded by gene mutation sites between different species to determine this mutation site's evolutionary conservation. The variants are separated into five categories: pathogenic, possibly pathogenic, of unknown significance, perhaps benign, and benign. In addition, based on SIFT and PolyPhen-2 software to classify missense mutations, if the mutation site is likely to be harmful by SIFT or PolyPhen-2, this variant can be classified as pathogenic (10,11). Based on mutation Taster software analysis of nonsense mutations, if the prediction is harmful, this variant can be classified as pathogenic (12). Because their highly destructive effects on protein structure or expression are pathogenic, frameshift mutations have not been confirmed further.

Statistical analysis

The data were analyzed using SPSS software (version 23.0; IBM Corp., Armonk, NY, USA). Mean values were compared between groups using the Maine-Whitney U test for normally distributed data and the Wilcoxon test for paired data. A P value <0.05 was considered statistically significant.

Results

Clinical data of all patients

Our routine clinical examination of 4 patients with family

history and 8 patients with sporadic IHH found that all patients had low hormone levels. Probands 1, 4, 5, and 6 were associated with olfactory disorders, and all the remaining patients had the usual olfactory sense. Patient 4 examined non-reproductive phenotype with sensorineural hearing loss and Iris heterochromatic. The clinical characteristics of patients carrying disease-causing genes are described in *Table 1*. We carried out a pedigree investigation on four patients (*Figure 1*). Proband 1's father and brother had hyposmia and were diagnosed with KS. We examined the brother of proband 3 with an abnormal penis, left and right testicular volume 2 mL, and diagnosed with nIHH. The sister of proband 4 was concerned by sensorineural hearing and olfactory impairment.

WES and Sanger sequencing results of all patients

We filtered the WES data and analyzed it to identify important mutation sites. WES results showed that 12 cases (4 families and 8 sporadic cases) carried gene mutations. Sanger sequencing was consistent with WES results; members of four families were sequenced by Sanger sequencing (Table S1), confirming that the mutated sites were co-isolated with genotype and disease (*Figure 2* and *Figure 3*). Two patients carried the known mutation site of KISS1R. The remaining 10 patients took new mutation sites of knowing disease-causing genes. We determined that a gene mutation was the leading cause of IHH in 4 cases of pedigree and 8 cases of sporadic IHH patients. Four mutations were found to cause IHH in the corresponding pedigree. Except for KISS1R, the remaining mutations are absent in the Human Gene Mutation Database, HPSD, and the dbSNP.

Bioinformatics and mutation rate analysis

The overall positive detection rate of 40 patients was 30% (nIHH 8/18 + KS 4/22), with the FGFR1 mutation rate accounting for 7.5% (3/40). ANOS1, CHD7, and KISS1R mutation rates were 5% (2/40), respectively. The mutation rate of SEMA3E, PROKR2, and SOX10 was 2.5% (1/40), respectively. We found that all missense mutation sites SEMA3E (p.P323S), CHD7 (p.W1785C), PROKR2 (p.Y223D and p.R298C) were harmful; all nonsense mutation sites FGFR1 (p.R661X), KISS1R (p.R331X, p.Y103X) are pathogenic (*Table 1*). The comparison of MEGA5 software shows that all the variants have extremely high homology among different species and are strongly conservative in evolution (*Figure 4*).

Discussion

SEMA3E gene mutations are primarily related to KS and affect the survival of GnRH neurons. Cariboni *et al.* (13) demonstrated that during hypothalamic migration, SEMA3E and the receptor PLXND1 are essential factors for the survival of GnRH neurons in brain development. In addition, through WES and computer model analysis, *SEMA3E* (p.R619C) and *CHD*7 (p.F1019C) mutations were identified among the two brothers with KS (14). The specific R619C missense mutation caused selective death and apoptosis in central GnRH neurons. Both mutations are inherited from the parents, and we studied the family I patients in this chapter similarly. It further illustrates the clinical heterogeneity of KS caused by *SEMA3E* gene mutation.

FGFR1, a loss of function mutation in approximately 10% of patients with IHH, is also required for the migration, differentiation, survival, and secretion of normal GnRH neurons (8). Xu *et al.* (15) reported a case of a 2.5 years old male patient with low serum hormones (T, FSH, LH, etc.), cryptorchidism, and normal olfaction. Genetic testing revealed that the boy carried a novel heterozygous mutation of *FGFR1* (c.1889T>C, p.L630P). In family II, only the proband carries the *FGFR1*, c.1981C>T mutation, which is a new mutation and is not inherited from the parents. We predict that the proband *FGFR1*, p.R661X, may also disrupt FGF8/FGFR1 signaling, the main pathway for GnRH neuron development (8,16). In 07 and 08 nIHH patients, the amino acid residues of E707 and F747 are also located in the highly conserved tyrosine kinase domain of FGFR1, which plays a catalytic role. We predict that *FGFR1*, p.E705Gfs*16, and p.F745Rfs*45 will decrease tyrosine kinase activity, resulting in downstream MAPK signaling failure and affecting GnRH neuron secretion.

Seminara *et al.* (17) reported that one IHH patient carried *KISS1R* p.R331X and p.X399R mutations. The *KISS1R* mutation causes hypogonadism and delays puberty development in humans and mice, which can be improved by administering exogenous GnRH (18). Both brothers in Family III have nIHH disease, and the parents are consanguineous in marriage. Both the proband and the younger brother are homozygous carriers of known *KISS1R*, c.991C>T mutation, and both parents carry the heterozygous mutation site of the gene. The 12th nIHH patient carries a known homozygous mutation of *KISS1R*,

Table 1 Genotype-phenotype and gene mutation pathogenicity of IHH patients

Pt.	Age at diagnosis (years)	Clinical diagnosis	Olfaction	Reproductive phenotype	Basal hormone levels (T nmol/L; FSH IU/L; LH IU/L)	Mutation	Hem	SIFT_ score/ pred	Polyphen2_ HVAR_ score/pred	Mutation Taster_ score/ pred
1	29	KS	Anosmia	Penis: 5 cm, testicular volume: left and right sides 2 mL	T 0.54; FSH 0.69; LH 0.15	NG_021242.2: c.967C>T (SEMA3E: Exon9, c.967C>T, p.P323S)	1	0.005/D	0.998/D	1/D
2	16	nIHH	Normal	External genital dysplasia	T 0.18; FSH 0.2; LH 0.3	NG_007729.1: c.1981C>T (FGFR1: Exon15, c.1981C>T, p.R661X)	1	/	/	1/A
3	26	nIHH	Normal	Penis abnormal, testicular volume: left and right sides 5 mL	T 0.2; FSH 1.67; LH 0.67	NG_008277.1: c.991C>T (KISS1R: Exon5, c.991C>T, p.R331X) (reported)	0	/	/	1/A
4	22	KS	Anosmia	Delayed puberty, penile, testicular dysplasia	T 0.69; FSH 0.4; LH2.2	NG_007948.1: c.1287delG (SOX10: Exon4, c.1287delG, p.S431Rfs*69)	1	/	/	/
5	31	KS	Hyposmia	Penis:8 cm, testicular volume: left and right sides 2 mL	T 1.14; FSH 1.6; LH 1.1	NG_007088.2: c.844delC (ANOS1: Exon6, c.844delC, p.R282Vfs*26)	0	/	/	/
6	25	KS	Anosmia	Penis normal, testicular volume: left 2 mL, right 3 mL, had history of cryptorchidism	T 23; FSH 1.45; LH 0.45	NG_007088.2: c.1886_1887insCTACTCT (ANOS1: Exon13, c.1886_1887insCTACTCT, p.R631Sfs*36)	0	/	/	/
7	27	nIHH	Normal	Left testicular volume 2 mL, right cryptorchidism	T 5.86; FSH 0.51; LH 0.37	NG_007729.1: c.2117_2118insT (FGFR1: Exon16, c.2117_2118insT, p.E705Gfs*16)	1	/	/	/
8	27	nIHH	Normal	Left testicular volume 1 mL, right cryptorchidism	T 0.55; FSH 1.84; LH 0.44	NG_007729.1: c.2239_2255delTTCAAGCAGCTGGTGGA (FGFR1: Exon17, c.2239_2255delTTCAAGCAGCTGGTGGA, p.F745Rfs*45)	1	/	/	/
9	23	nIHH	Normal	Penis abnormal, left and right testicular volume: abnormal	T 0.59; FSH 0.1; LH 0.1	NG_007009.1: c.5695_5698delGGCC NG_007009.1: c.5700_5707delACTTTACT (CHD7: Exon29, c.5695_5698delGGCC, c.5700_5707delACTTTACT, p.G1899R, Q1900_W1903del)	1	/	/	/
10	26	nIHH	Normal	Penis normal, testicular volume: left 2 mL, right 3 mL, right had history of cryptorchidism	T 2.87; FSH 0.2; LH 0.6	NG_007009.1: c.5355G>T (CHD7: Exon25, c.5355G>T, p.W1785C)	1	0/D	0.993/D	1/D
11	30	nIHH	Normal	Left and right testicular volume: 6–8 mL	T 1.52; FSH 1.7; LH 1.3	NG_008132.2: c.667T>G NG_008132.2: c.892C>T (PROKR2: Exon2, c.667T>G, p.Y223D c.892C>T, p.R298C)	1	0/D	1/D	1/D
12	24	nIHH	Normal	Penis normal, left and right testicular volume: 1–2 mL	T 0.76; FSH 0.98; LH <0.1	NG_008277.1: c.309C>A (KISS1R: Exon2, c.309C>A, p.Y103X) (reported)	0	/	/	1/A

The testicular volume of Chinese men is about 15–25 mL. Normal levels of male sex hormone: T, 9.4–37 nmol/L; FSH, 1.2–5.5 IU/L; LH, 1.3–6.3 IU/L. Hem_1: heterozygous mutation; Hem_0: homogeneous mutation; A: disease_causing_automatic; D: disease_causing. IHH, idiopathic hypogonadotropic hypogonadism; T, testosterone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; KS, Kallmann syndrome; nIHH, normal IHH.

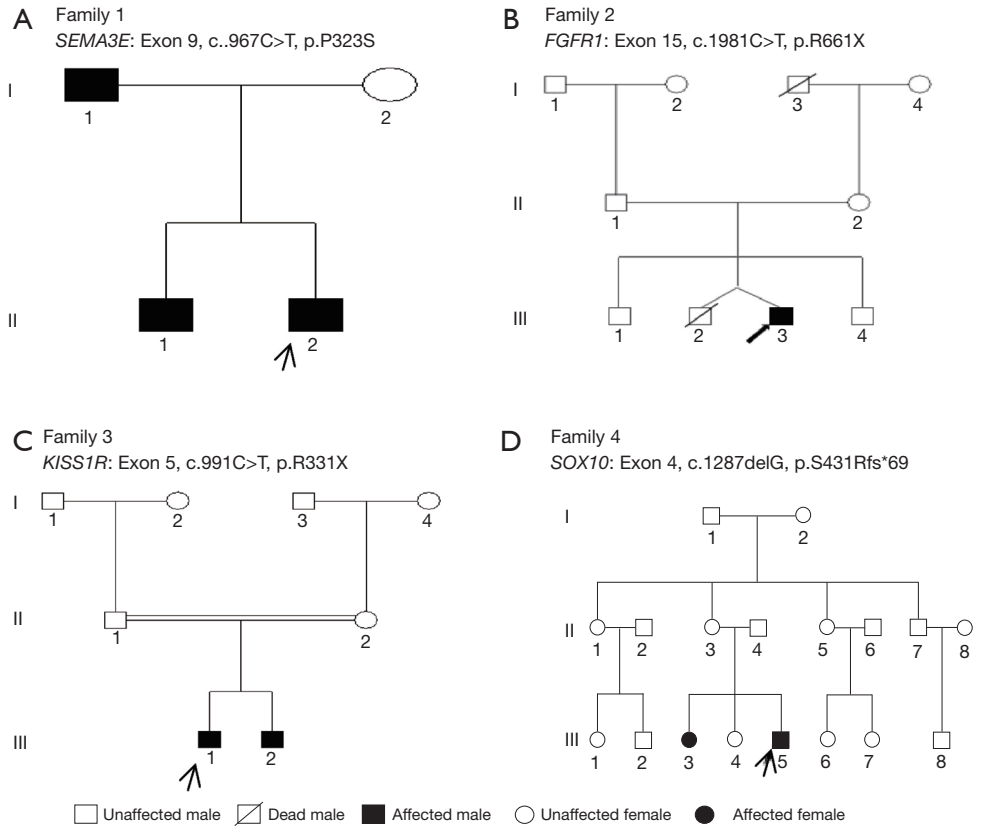

Figure 1 The pedigree charts of IHH carrying variants. Two pedigrees with KS (A,D) and two pedigrees (B,C) with nIHH were gathered to analyze genotype. The proband is shown as the arrow. KS, Kallmann syndrome; nIHH, normal idiopathic hypogonadotropic hypogonadism.

c.309C> A, p. Y103X. Y103X completely switched the receptor's structure by deleting five transmembrane domains, and MAPK/ ERK/SRE luciferase detection did not respond (19).

SOX10 contains a highly conserved HMG (high mobility group) structure that can precisely determine and bind DNA sequences domain (20). According to a literature review, *SOX10* mutations account for 4.4% of IHH patients studied (21). SOX10 is an essential transcription factor during neural crest development and can interact with other proteins (such as PAX3) to regulate neural crest cell development (22,23). The transcription factor SOX10 is an essential regulator of the neural crest and the central nervous system (CNS) in all vertebrates, so mutation or deletion of the *SOX10* gene leads to severe developmental disorders (20).

For unrelated men with sporadic KS, the incidence of *ANOS1* mutations may range from 3.5% to 10% (24,25). In sporadic unrelated IHH patients 05 and 06, R282 and L629 amino acid residues are located in or near the fibronectin type III domain. We predict that *ANOS1* mutation in these 2 KS patients will cause defects in kinase activity, likely disrupting neuronal migration and axon orientation (26).

About 6% of IHH patients (with or without olfactory impairments) had the *CHD*7 mutation (7). *CHD*7 mutation is also the primary pathogenic gene causing CHARGE syndrome, so we need to explore further the CHARGE syndrome in patients 09 and 10 (27). Recently, a study has demonstrated that CHD7 is pivotal in developing GnRH neurons and the olfactory system (28). Studies have revealed that *PROKR2* knockout gene mice exhibit dysplasia of the olfactory bulb, and migration of GnRH neurons fails (29).

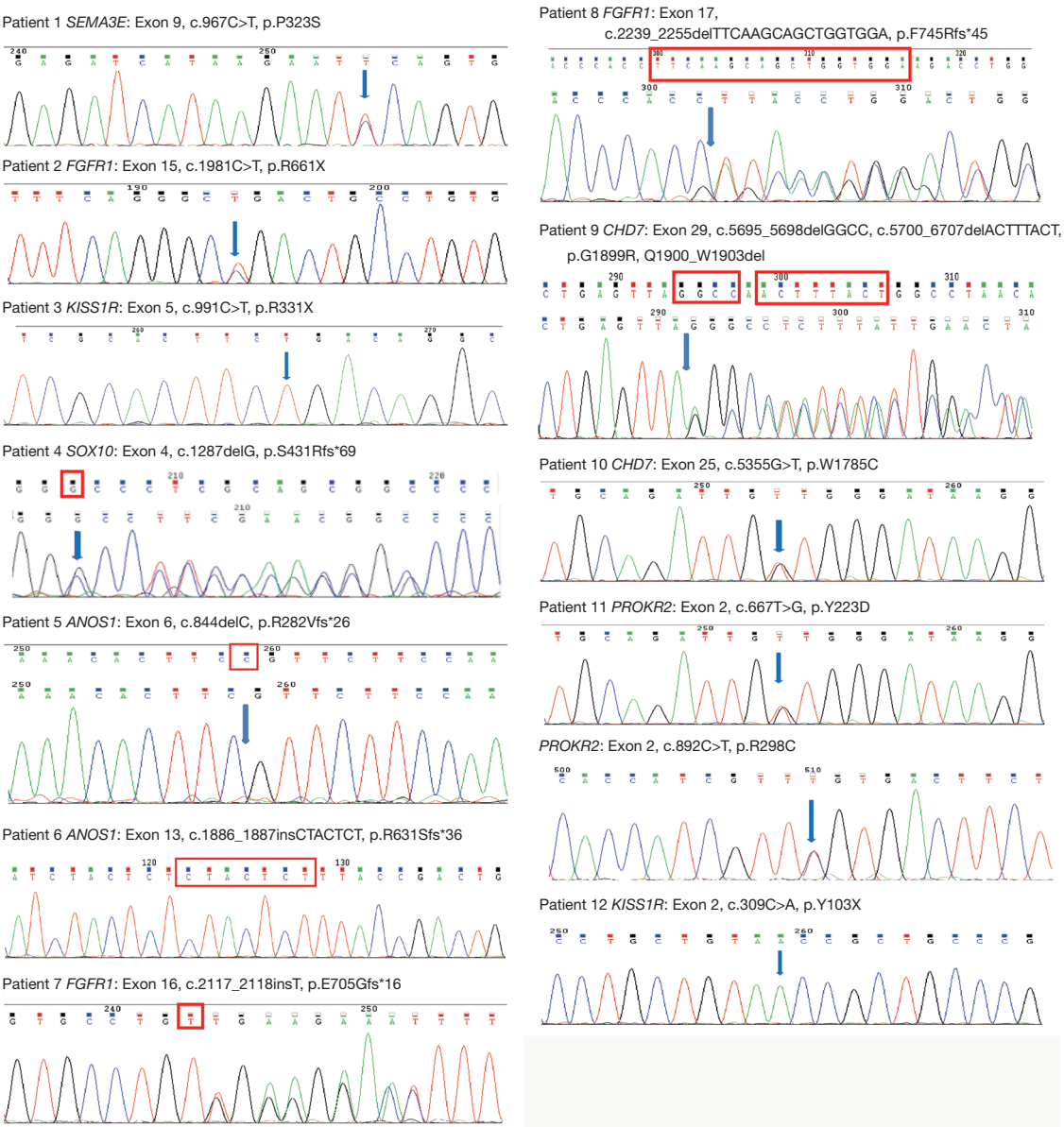

Figure 2 The result of variants with Sanger sequencing of 12 patients with IHH. Red boxes set out the mutation sequence and blue arrows indicate the mutation site. IHH, idiopathic hypogonadotropic hypogonadism.

Functional loss mutations in *PROKR2* have been reported in approximately 5.6% of IHH patients (30). The 11th nIHH patient carries both c.667T>G and c.892C>T mutations on *PROKR2*.

In a clinical sense, recognizing those IHH genes and associated phenotypes may improve our diagnostic capabilities by enabling us to prioritize the screening of particular genes such as synkinesia (ANOS1), dental agenesis (FGF8/FGFR1) and hearing loss (CHD7) (26). The doctor may be translated IHH-associated gene studies into novel treatments (31).

Conclusions

The study applied WES and PCR techniques to the screening diagnosis of pathogenic genes of IHH, which

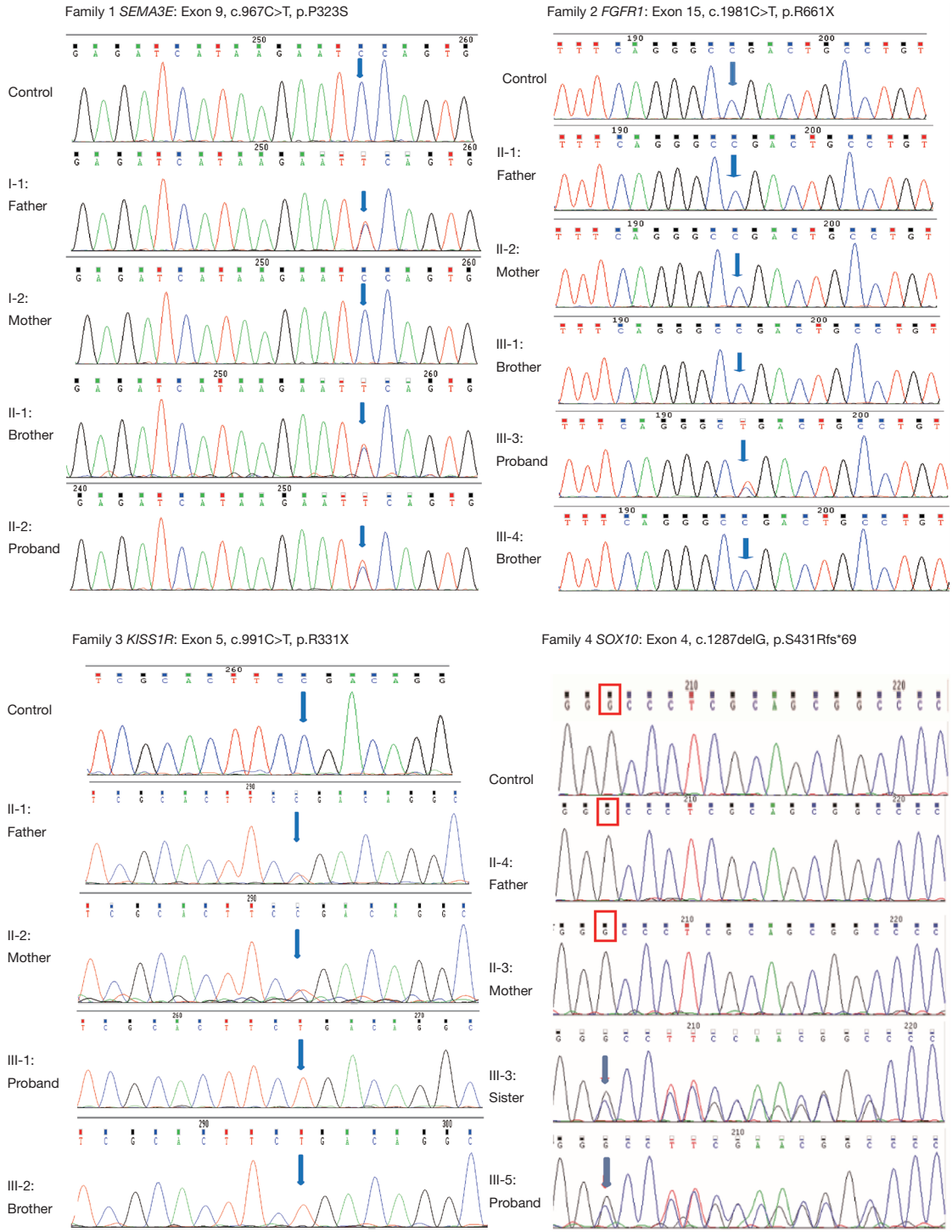

Figure 3 Sanger sequencing maps of 4 families with IHH. Identification of gene mutations in affected families. Blue arrows show the mutation site. IHH, idiopathic hypogonadotropic hypogonadism.

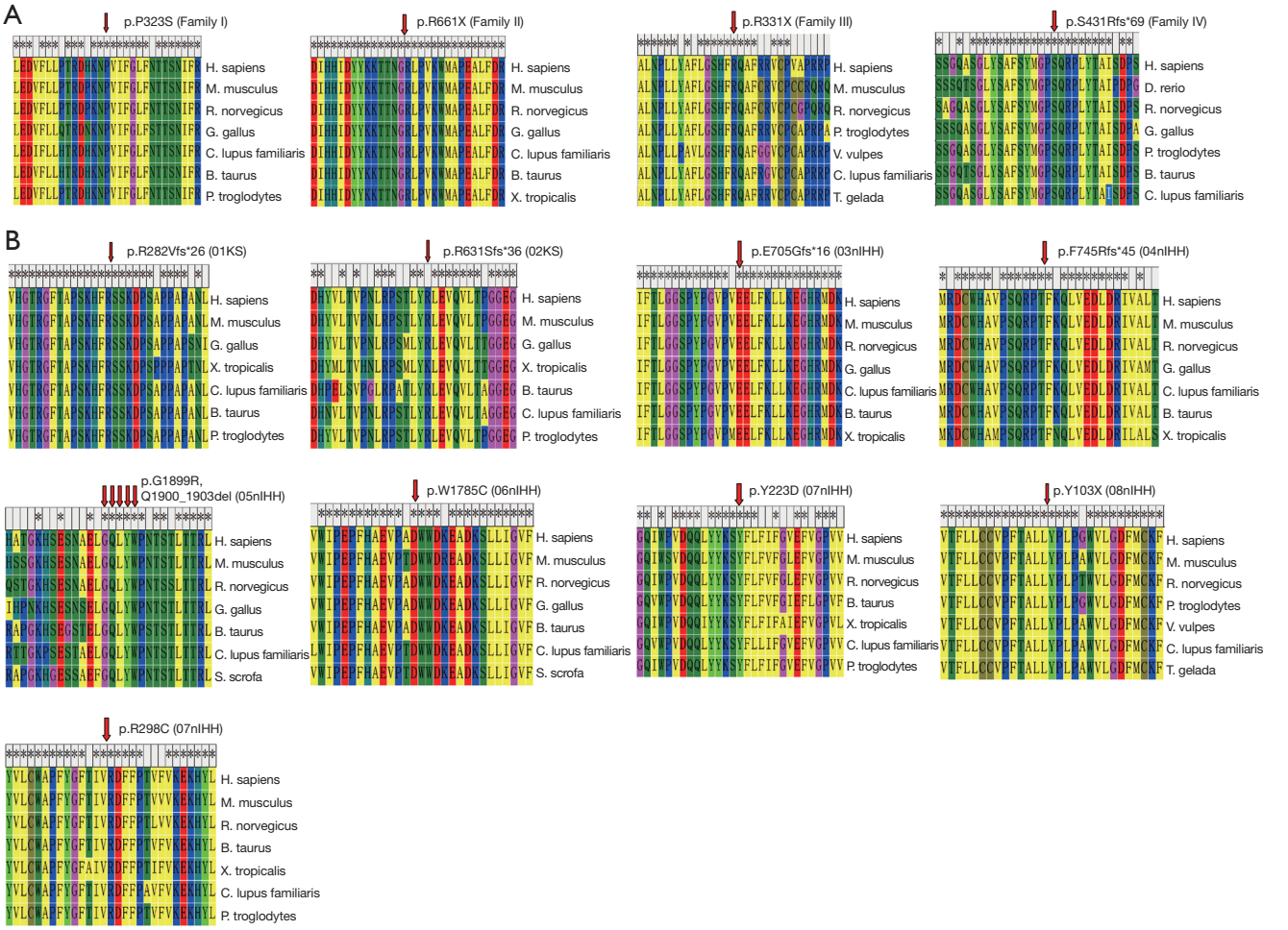

Figure 4 The result of conservation analysis of mutational amino acids. (A) Mutational amino acid homology 4 IHH families. (B) Mutational amino acid homology 8 patients with sporadic unrelated IHH. The red arrows indicate the position of the mutated amino acid residues mutated in the present study in the protein sequence and the homology of the mutation site encoding amino acids between the seven different species. The asterisk (*) indicate the homology of the mutation site encoding amino acids between the seven different species. IHH, idiopathic hypogonadotropic hypogonadism.

provided an indispensable basis for the follow-up clinical treatment of IHH patients and genetic counseling during assisted reproduction. We will further increase the samples and collect the peripheral blood of members to facilitate the discovery of more disease-causing genes.

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Footnote

Reporting Checklist: The authors have completed the MDAR

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Data Sharing Statement: Available at https://tau.amegroups.com/article/view/10.21037/tau-23-225/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-23-225/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics board of the Jinling Hospital, Nanjing University School of Medicine (No. 2018NZGKJ-003) and informed consent was taken from all the patients.

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