The Clinical and Genetic Characteristics of Chinese Male Pediatric Patients With Congenital Hypogonadotropic Hypogonadism

Yi Wang  
Beijing Children's Hospital Capital Medical University

Miao Qin  
Beijing Children's Hospital Capital Medical University

Lijun Fan  
Beijing Children's Hospital Capital Medical University

Beibei Zhang  
Beijing Children's Hospital Capital Medical University

Chunxiu Gong (chunxiugong@sina.com)  
Beijing Children's Hospital, Capital Medical University, National Center for Children's Health  
https://orcid.org/0000-0002-1262-7383

Research

Keywords: congenital hypogonadotropic hypogonadism, clinical and genetic characteristics, oligogenicity, dual CHH, family history

DOI: https://doi.org/10.21203/rs.3.rs-660182/v1

License: ©  This work is licensed under a Creative Commons Attribution 4.0 International License.  Read Full License
Abstract

Backgrounds

Congenital hypogonadotropic hypogonadism (CHH) are divided into Kallmann Syndrome (KS) and normosmic HH(nHH). The clinical and genetic characteristics of CHH are more studied in adults, but less in pre-adults.

Methods

Medical records of 126 patients with CHH at our hospital during 2008−2020 were evaluated.

Results

Totally, seven patients (5.6%) had hypospadias. Among 49 patients with positive family history, delayed puberty, KS/nHH and olfactory abnormalities accounted for 44.9%, 16.3%, and 12.2%, respectively. Sixty-five patients completed the hCG prolongation test, and T levels of 24 patients were lower than 100 ng/dl. 25 CHH-related genes were found in 78 patients, digenic mutations in 23 patients, and trigenic mutations in 3 patients. The most common pathogenic genes were FGFR1 (21.1%), PROKR2 (17.9%), ANOS1 (12.6%), and CHD7 (12.6%). The oligogenicity rate of common autosomal dominant heredity genes accounted for 50.0% (FGFR1, 10/20) and 33.3% (CHD7, 4/12), of autosomal recessive heredity gene PROKR2 accounted for 47.1% (8/17).

Conclusion

Micropenis and cryptorchidism are important cues for CHH in pre-adulthood; hypospadias is a rare phenotype of CHH. At least 22.9% of patients tested had testicular Leydig cell dysfunction (dual CHH). Oligogenic mutations were found in 27.4% of all patients with CHH.

Introduction

Congenital hypogonadotropic hypogonadism (CHH, MIM 615267) is a common cause of puberty absent, and adult infertility, with an incidence rate of 1 per 4000 new births [1]. When associated with anosmia or hyposmia, it is also known as Kallmann syndrome (KS, MIM 147950), and when associated with a normal sense of smell, it is termed normosmic CHH (nHH), with KS accounting for 50% of cases [2]. There are approximately 1200−1500 gonadotropin-releasing hormone (GnRH) neurons in the vertebrate hypothalamus, which can synthesize and release GnRH. CHH is caused by a deficiency in the synthesis, release, or action of GnRH, resulting in insufficient secretion of gonadotropins, followed by gonadal dysfunction. According to our previous research, a considerable number of patients may also have primary testicular Leydig cell dysplasia, namely dual CHH [3, 4].

According to its pathophysiology, CHH is mainly divided into two types: during the fetal period, neurodevelopmental gene mutations cause disorders in the development, differentiation, or migration of GnRH neurons, usually causing KS. Defects in GnRH synthesis, release, or action on pituitary gonadotropin cells caused by neuroendocrine gene mutations usually lead to nHH [1]. An increasing number of studies have found that CHH could be caused by gene defects that affect both neuronal development and the GnRH signaling pathway. Mutations in the same CHH-related pathogenic gene often cause phenotypic differences among patients or individuals in the same family, that is, the low penetrance of most genes suggests that CHH is not a strict monogenic disease [5, 6]. Studies including large CHH cohorts suggest that at least 20% of cases are oligogenic [6, 7]. However, our previous study of 64 patients indicated that oligogenic mutations accounted for only 9.8% [8].

Since the first KS-related pathogenic gene ANOS1 was cloned in 1991, an increasing number of CHH-related pathogenic genes have been identified. In 2015, the European CHH consensus summarized 31 pathogenic genes, including X-chromosome-linked recessive, autosomal recessive, and dominant genes [1]. At present, more than 90 candidate genes may be involved in the pathogenesis of CHH, and some newly reported genes have been confirmed in CHH patients; some genes involved in GnRH neuronal migration and axon formation in animal models have not been confirmed in CHH patients [7, 9-15]. In our previous study, only 10 pathogenic genes were confirmed in CHH patients [8].

Therefore, this study evaluated 126 male patients with CHH and aimed to further study and deepen the understanding of the relationship between genotype and phenotype, so as to provide a reference for early diagnosis and intervention of pediatric CHH.

Materials And Methods

Editorial policies and ethical considerations

The study was approved by the Ethics Committee of Beijing Children's Hospital, Capital Medical University, and written informed consent was obtained from the patients' parents or legal guardians. All data involved in the study could be available if necessary.

Subjects

A total of 126 male patients of Chinese Han nationality aged 0−18 years who were treated at the endocrine clinic of our hospital between 2008 and 2020 were enrolled. Except for one pair of siblings, none of the other patients were related to each other.

The diagnosis was made based on the phenotype, physical signs, chromosome karyotype, sex hormone levels including AMH and INHB, olfactory bulb MRI, hCG test, genetic test and etc.

Inclusion and exclusion criteria
Refer to previous literature reports [8].

**The diagnostic criteria of micropenis**

Refer to the literature of the Chinese Journal of Pediatric Surgery from 2010.

**Diagnostic criteria of dual CHH**

After the hCG prolongation test, the level of T still less than 100 ng/dl was diagnosed as dual CHH. If the hCG prolongation test was not performed, the level of T less than 100 ng/dl after treatment with GnRH (5–10 ug/90 min, 16 pulses/d) for half a year, was diagnosed as dual CHH. If the hCG prolongation test was not performed and the GnRH treatment was provided for less than half a year and the level of T was still greater than 100 ng/dl, it was considered that the testicular Leydig cells had a good response.

**HCG standard test, hCG prolonged test**

Please refer to a previously published article [8].

**Hormone detection**

LH, FSH, and T levels were detected using an enzyme-enhanced chemiluminescence immunoassay (Immulite 2000, Siemens Corporation, Munich, Germany).

**DNA sequence analysis**

A total of 51 patients underwent a gonadal panel, which included 164 genes, and 44 patients underwent whole exosome sequencing. DNA was extracted from peripheral blood leukocytes of patients and their parents and/or siblings, and the NEXTSEQ 500 sequencer (Illumina Corporation, San Diego, CA, USA) was used to filter out all possible pathogenic missense, frameshift, and splice site mutations. Design primers and Sanger sequencing were used to verify the mutations in the samples, and pathogenicity was judged according to ACMG rules; pathogenic, likely pathogenic, or uncertain mutations were considered to be possibly related to the disease.

**Statistical analysis**

Data analysis was performed using statistical product and service solutions 26.0 software (SPSS, International Business Machines Corporation, Armonk, NY, USA).

**Results**

**Clinical characteristics**

The chromosomes of all patients were 46 XY and SRY (+). Combined with the phenotypes, physical signs, hormone levels, presence of puberty, olfactory bulb imaging, and genetic test results, a total of 126 cases of CHH were diagnosed, including 88 cases of KS (69.8%), 37 cases of nHH (29.4%), and 1 case of CHARGE syndrome (Fig. 1A, supplementary table).

In total, 78.9% of the patients (101/126) had micropenis, including cases of micropenis (38.1%, 48/126), micropenis with cryptorchidism (35.2%, 45/126), micropenis with cryptorchidism and hypospadias (2.4%, 3/126), micropenis with hypospadias (3.2%, 4/126), cryptorchidism (8.7%, 11/126), and only absent puberty (11.9%, 15/126) (Fig. 1B and Table 1).

Two patients with KS had left renal agenesis (one with ANOS1 mutation and the other without gene detection). One patient with FGFR1 mutation showed a positive joint movement of the limbs. A total of 65 KS patients completed MRI examination of the olfactory bulb, and 7.7% (5/65) reported hyposmia, but no abnormal olfactory bulb, olfactory tract, or olfactory sulcus was found on MRI. A normal olfactory was reported in 30.8% of the cases (20/65), but structural abnormalities of the olfactory bulb, olfactory bundle, and/or olfactory sulcus could be seen on MRI, which was consistent with the results found in the literature and our previous reports.

**Genetic characteristics**

A total of 95 patients underwent genetic testing: 25 CHH-related pathogenic genes were found in 82.1% of cases (78/95) (Fig. 2A), digenic mutations in 24.2% of cases (23/95), and trigenic mutations in 3.2% of cases (3/95) (Table 2, Fig. 2B and 2C).

The most common gene mutations were FGFR1 (20/95, 21.1%), PROKR2 (17/95, 17.9%), ANOS1 (12/95, 12.6%), and CHD7 (12/95, 12.6%) mutations. Among the most common gene mutations, there was no significant difference in the proportion of FGFR1, PROKR2, and CHD7 mutations in KS or nHH (all p>0.05).

The majority of mutations in CHH probands were private, except for PROKR2 (p. W178S, n=5 in KS and nHH, respectively), ANOS1 (p. V560I, n=2 in KS), HS6ST1 (p. H63D, n=3 in KS), and IL17RD mutations (p. P191L and p. S671L, n=2 in KS, respectively) (Fig. 2A).

The oligogenicity of common autosomal dominant inherited pathogenic genes accounted for 50% (FGFR1, 10/20) and 33.3% (CHD7, 4/12), the oligogenicity of autosomal recessive inherited pathogenic genes accounted for 47.1% (PROKR2, 8/17, Fig. 2D), and the oligogenicity of X-linked genes accounted for 8.3% (ANOS1, 1/12). Among the 12 patients with CHD7 mutations, only one patient (8.3%) was diagnosed with CHARGE syndrome. Among the 12 patients with KS caused by ANOS1 mutation, two patients were brothers with deletion of exons 1 and 2, and all exon deletion was noted in one patient.
We further analyzed the common pathogenic gene mutation sites according to ACMG and found that 45.5%–75.0% of the mutation sites were pathogenic or likely pathogenic, except for one case of CHD7 mutation being likely benign, and the rest of these patients had either single-gene or oligogenic mutations (Fig. 2E).

**Dual CHH and genes**

In total, 105 patients completed the hCG standard test conducted to evaluate testicular Leydig cell function among 126 patients. Of these 105 patients, 68.6% (72/105) of patients had T < 100 ng/dl, of which 65 patients completed the HCG prolongation test, and 22.9% (24/105) of patients had T < 100 ng/dl, suggesting testicular Leydig cell dysfunction; therefore, at least 22.9% of cases could be diagnosed as dual CHH (Fig. 3).

**Family history and genes**

Among 49 patients with positive family histories, 44.9% (22/49) had a family history of delayed puberty (DP). In addition to the CHH-related genes (FGFR1, HS6ST1, IL17RD, and SEMA3A) reported in the literature in patients with DP [16, 17], two patients carried PROKR2 mutations from late-developing mothers (menarche at 16 years), and one patient carried KISS1R mutations from a late-developing father (first spermatorrhea at the age of 18–19 years). It is suggested that PROKR2 and KISS1R may also be shared genes of DP and CHH.

Six patients had a family history of hyposmia or anosmia (three cases were also associated with family history of DP). In this study, two patients with KS were siblings with deletion of exons 1 and 2 of ANOS1, and their elder brother who was diagnosed with KS carried the same mutation and received treatment with a GnRH pump in another hospital; the other two patients with KS both had an elder brother who were diagnosed with KS, and carried an ANOS1 missense mutation; and one patient with nHH had KISS1R compound heterozygous mutations and his elder sister carried the same mutation and had no breast development and menarche at the age of 17 years. Four patients had a family history of disorders of sex development (DSD), and three mothers were probands (Fig. 4). The three mothers were all treated with estrogen and progesterone to regulate their menstrual cycles. When they had fertility needs, they were administered GnRH pump therapy combined with assisted reproductive technology, and they carried three gene mutations (IL17RD mutation p. N503S; FGFR1 mutation p. P176S; PROKR2 mutation p. W178S).

**Discussion**

This study summarized the clinical and genetic characteristics of 126 patients, using CHH and DSD phenotypes (micropenis and cryptorchidism) and genetic testing as important cues for pediatric diagnosis of CHH. With an increase in the number of cases, more CHH candidate genes were confirmed in patients. We found that digenic and trigenic variants accounted for 24.2% (23/95) and 3.2% (3/95) of patients, respectively, who underwent genetic testing.

The proportion of gene mutations found in patients in this study was significantly higher than that in patients with CHH reported in the literature. The most common mutant genes were FGFR1 (21.1%), PROKR2 (17.9%), ANOS1 (12.6%), and CHD7 (12.6%) [6, 7, 18-20]. Among them, 10 cases (50.0%) of FGFR1 and 7 cases (41.2%) of PROKR2 mutations were oligogenic mutations. Previous studies reported that FGFR1 mutation may be related to hand and foot malformation in patients with CHH [19]; however, in this study, only one patient with hand and foot malformation had FGFR1 mutation, and there was no obvious phenotypic-genotypic correlation, which may be because more than half of the patients were oligogenic.

Previous literature on HH in adults reported that patients with FGFR1 mutations had a high incidence of cryptorchidism, small testicular size, long treatment time for spermatogenesis, and low sperm concentration [21]. However, in this study, compared with patients with non-FGFR1 mutations, the incidence of cryptorchidism in patients with FGFR1 mutation was not significantly higher, the testicular volume was not small, and the patients with FGFR1 mutations had mainly pure CHH. However, because the diagnosis of pure CHH in this study was based on the T level after the hCG prolongation test, which may be the cause of the difference, further follow-up studies will be conducted.

INHB is a marker of the number of Sertoli cells, and is usually lower than 30 pg/ml in male patients with complete CHH [1, 22]. In some patients with partial CHH, the level of INHB may overlap with that of DP and healthy controls [23, 24]. In this study, the INHB test was performed in 65 patients, including all age groups, of which 45 (69.2%) had an INHB level of > 30 pg/ml, suggesting that the function of testicular Sertoli cells in these patients was still good. After subsequent therapy with GnRH pump or HCG/HMG, it was more likely to promote spermatogenesis, which was consistent with the current spermatogenesis rate of 64%–80.3% in CHH patients after treatment [25, 26]. Among the 39 patients with T > 100 ng/dl after the hCG prolongation test, there were still 10 patients whose INHB level was less than 30 pg/ml, suggesting that some of the patients with good response to Leydig cells may still have had poor function of Sertoli cells. Therefore, the evaluation of testicular cell function in patients with HH requires a multi-faceted and multi-index comprehensive evaluation.

However, in this study, one patient with three gene mutations (SEMA3E/CHD7/NMF) was also diagnosed with dual CHH. The patient was treated with a standard GnRH pump for 6 months, the level of T was 112 ng/dl, and 12 months later, it was observed that the patient had spermatorrhea and good sperm motility and concentration. Therefore, the percentage of patients diagnosed with dual CHH in this study with restored fertility after treatment needs to be further studied. This phenomenon also suggests that there was a false negative response to short-term stimulation in the experiment.

In previous studies of adult cases, patients whose puberty was not induced by hormone therapy at the age of puberty usually showed infertility when GnRH pump therapy was used to stimulate spermatogenic potential in adults. Therefore, for CHH patients and their families, every gene mutation that causes GnRH deficiency should theoretically not be transmitted within the family. Recent studies have shown that a small number of gene mutation sites have strikingly high percentage, such as GNRHR Q106R (44%), GNRHR R262Q (29%) [6, 27-29] and TACR3 W275X (36%) [30-32]. The L173R of PROKR2 accounts for 40% of the CHH population in Europe and the United States, but it is rare in the Asian population [6, 33-36]. The recurrent mutation sites of several genes in this study were PROKR2 (p. W178S, n=5 in KS and nHH, respectively), ANOS1 (p. V560L, n=2 in KS), HS6ST1 (p. H63D, n=3 in KS), and IL17RD (p. P191L and p. S671L, n=2 in KS, respectively). It was proven that PROKR2 was one of the most common pathogenic genes in CHH, accounting for 17.9% (17/95) in this study, of
which W178S accounted for 58.8% (10/17). In another study of Chinese adult CHH patients, PROKR2 mutations accounted for 13.3% (18/135) and W178S accounted for 55.6% (10/18) [37]. Combined with two studies, W178S accounted for 57.1% of PROKR2 mutations in the Chinese CHH population (20/35).

Functional analysis showed that the mutant impeded receptor was expressed on the cell surface. The W178S of PROKR2 may be an ancient founder mutation, and it was not eliminated in the Chinese CHH population during evolution. The silencing of its effect on reproduction may be related to oligogenicity, or the mutant may revert during adulthood. However, in this study, the mother of one patient harboring W178S in PROKR2 was a proband, suggesting that the mutation had a wide spectrum and that the patient could undergo germ cell maturation after treatment. Therefore, the complex mechanism of its effect on reproduction needs to be further studied.

Of the five patients with single-gene mutations in PROKR2 (W178S), four were diagnosed with dual CHH. Some patients carried additional gene mutations. One patient with a repetitive mutation site HS6ST1 (H63D) and another with IL17RD (N503S) were also diagnosed with dual CHH, and PROKR2 (W178S) and IL17RD (N503S) were carried by the proband mother simultaneously; however, the results of the HCG test showed that the Leydig cells were dysfunctional. It has been suggested that some patients with dual CHH diagnosed by the HCG test in this study may still recover their reproductive function after standard treatment, and it is possible that Leydig cells had not been stimulated by GnRH for a long time, which may have led to the slow response of the receptor. The curative effects in the patients with the above mutations and the relationship between the curative effect and gene mutations will be further investigated. However, the FGFR1 mutation with the highest proportion of mutations was not found to be recurrent in patients with CHH, suggesting that the mutations of the gene had a greater impact on reproduction. Among the 126 patients in this study, three probands were mothers, and no probands were fathers. It has been reported that 64%–80.3% of male CHH patients could produce sperms after GnRH pump or HCG/HMG therapy, but the specific situation may need further observation.

Hypospadias is caused by an abnormal urethral opening closure in the early stage of embryonic development, including the early hormone-independent stage (5–8 weeks) and hormone-dependent stage (8–12 weeks) [38]. There was no significant intersection with the functional time of the HPG axis. Therefore, hypospadias and CHH may be two unrelated diseases. The 2015 CHH consensus also believes that the existence of the hypospadias phenotype can exclude the diagnosis of hypospadias [1].

In addition to our previous report, other HH patients with the hypospadias phenotype have also been reported; for example, Huseyin et al. reported that a 15-year-old boy had no puberty initiation, micropenis, cryptorchidism, and perineal scrotal hypospadias, and his elder sister had no secondary sexual development. Sequencing showed that they carried KISS1R homozygous mutations (p. Y323X) [39]. In 2017, Ji et al. reported that a 28-year-old KS patient presented with no signs of puberty, bilateral cryptorchidism, olfactory loss, and right deafness. Genetic tests found that in addition to nonpathogenic variants (rs808119, rs6185) of ANOS1 and GNRH1, SRD5A2 gene heterozygous variation (c.680G > A) was also present, and T/DHT was 26.40, after the hCG test; thus, it was considered that the patient had KS and 5α-RD [40]. In Indonesia, 11 patients with 46, XY DSDs were reported to carry pathogenic mutations, including PROKR2, PROK2, WDR11, FGFR1, and CHD7 mutations, and these patients had different degrees of hypospadias [41]. Other studies have found that FGFR8, GLI3, CHD7, and other CHH-related genes could cause hypospadias 38. These studies suggest that hypospadias and CHH are not completely separated, and the potential relationship between hypospadias and CHH needs to be further elucidated. There were seven patients with hypospadias in this study, but five carried no hypospadias related genes, one with KS had SALL1 gene mutation (p. M662V), and one with nHH had a SPECC1L mutation (p. M232V). SALL1 and SPECC1L gene mutations could cause hypospadias, suggesting that the combined gene mutation may be one of the causes of hypospadias in patients with CHH, but the mechanism of hypospadias in other patients is still not clear.

**Conclusion**

Microenesis, cryptorchidism, and molecular genetics are important cues for diagnosing CHH in pediatric patients. Hypospadias is a rare CHH phenotype. Oligogenic mutations accounted for 27.4% of all CHH patients, which may have been a mechanism of autosomal recessive heredity genes or incomplete gene penetrance pathogenicity. KS due to ANOS1 mutations was mostly caused by single genes, and CHD7 mutations led to isolated CHH. Approximately 25.0%–54.5% of the common pathogenic gene mutations are uncertain, and their roles in the pathogenesis of CHH require further study. PROKR2 and KISS1R may also be shared genes involved in DP and CHH. The FGF signaling pathway, represented by FGFR1, mainly causes pure CHH. The mothers of multiple probands carried the same mutation and multiple gene mutation sites appeared repeatedly, suggesting that the effect of these mutation sites on reproduction was relatively slight. In this study, T after short-term hCG stimulation as an indicator of testicular function may be a false negative. We will further monitor the levels of T and INHB in patients with CHH after GnRH pump or HCG/HMG treatment, and discuss dual CHH.

**Abbreviations**

CHH: Congenital hypogonadotropic hypogonadism; KS: Kallmann Syndrome; nHH: normosmic HH; GnRH: Gonadotropin-releasing hormone; DP: Delayed puberty; DSD: Disorders of sex development.

**Declarations**

**Acknowledgements**

We thank all the patients and their parents for participating in the study.

**Authors’ roles**

Miao Qin, Lijun Fan and Beibei Zhang collected the data. Yi Wang analyzed the data and wrote the manuscript. Chunxiu Gong revised the manuscript. All authors approved the final version of the manuscript.
Funding
The Pediatric Medical Coordinated Development Center of Beijing Hospitals Authority (XTYB201808), the Public Health Project for Residents in Beijing (Z151100003915103), and the National Key Research and Development Program of China (2016YFC0901505).

Conflict of interests
The authors declare that there is no conflict of interests.

Data availability statement
All relevant data are within the paper, any additional information about the cohorts may be made available upon request addressed to the corresponding author, pending the approval of the Institutional Review Board of the Beijing Children's Hospital, Capital Medical University.

References
1. Boehm U, Bouloux PM, Dattani MT, de Roux N, Dode C, Dunkel L, et al. Expert consensus document: European Consensus Statement on congenital hypogonadotropic hypogonadism–pathogenesis, diagnosis and treatment. Nat Rev Endocrinol. 2015; 11 (9): 547-564. doi: 10.1038/nrendo.2015.112.
2. Mitchell AL, Dwyer A, Pitteloud N, Quinton R. Genetic basis and variable phenotypic expression of Kallmann syndrome: towards a unifying theory. Trends Endocrinol Metab. 2011; 22 (7): 249-258. doi: 10.1016/j.tem.2011.03.002.
3. Grinspon RP, Loretif N, Braslavsky D, Valeri C, Schteingart H, Ballerini MG, et al. Spreading the clinical window for diagnosing fetal-onset hypogonadism in boys. Front Endocrinol (Lausanne). 2014; 5 51. doi: 10.3389/fendo.2014.00051.
4. Sykiotis GP, Hoang XH, Avbelj M, Hayes FJ, Thambundit A, Dwyer A, et al. Congenital idiopathic hypogonadotropic hypogonadism: evidence of defects in the hypothalamus, pituitary, and testes. J Clin Endocrinol Metab. 2010; 95 (6): 3019-3027. doi: 10.1210/jc.2009-2582.
5. Pitteloud N, Quinton R, Pearce S, Raivoi T, Aciero J, Dwyer A, et al. Digenic mutations account for variable phenotypes in idiopathic hypogonadotropic hypogonadism. J Clin Invest. 2007; 117 (2): 457-463. doi: 10.1172/JCI29884.
6. Sykiotis GP, Plummer L, Hughes VA, Mu M, Durrani S, Nayak-Young S, et al. Oligogenic basis of isolated gonadotropin-releasing hormone deficiency. Proc Natl Acad Sci USA. 2010; 107 (34): 15140-15144. doi: 10.1073/pnas.1009622107.
7. Miraoui H, Dwyer AA, Sykiotis GP, Plummer L, Chung W, Feng B, et al. Mutations in FGF17, IL17RD, DUSP6, SPRY4, and FLRT3 are identified in individuals with congenital hypogonadotropic hypogonadism. Am J Hum Genet. 2013; 92 (5): 725-743. doi: 10.1016/j.ajhg.2013.04.008.
8. Wang Y, Gong C, Qin M, Liu Y, Tian Y. Clinical and genetic features of 64 young male paediatric patients with congenital hypogonadotropic hypogonadism. Clin Endocrinol (Oxf). 2017; 87 (6): 757-766. doi: 10.1111/cen.13451.
9. Bouilly J, Messina A, Papadakis G, Cassatella D, Xu C, Aciero JS, et al. DCC/NTN1 complex mutations in patients with congenital hypogonadotropic hypogonadism impair GnRH neuron development. Hum Mol Genet. 2018; 27 (2): 359-372. doi: 10.1093/hmg/ddx408.
10. Quaynor SD, Bosley ME, Duckworth CG, Porter KR, Kim SH, Kim HG, et al. Targeted next generation sequencing approach identifies eighteen new candidate genes in normosmic hypogonadotropic hypogonadism and Kallmann syndrome. Mol Cell Endocrinol. 2016; 437 86-96. doi: 10.1016/j.mce.2016.08.007.
11. Turan I, Hutchins BI, Hackamdioglu B, Kotan LD, Gurbuz F, Ulubay A, et al. CCDC141 Mutations in Idiopathic Hypogonadotropic Hypogonadism. J Clin Endocrinol Metab. 2017; 102 (6): 1816-1825. doi: 10.1210/jc2016-3391.
12. Suter T, Jaworski A. Cell migration and axon guidance at the border between central and peripheral nervous system. Science. 2019; 365 (6456): doi: 10.1126/science.aaw8231.
13. Zhu Z, Han X, Li Y, Han C, Deng M, Zhang Y, et al. Identification of ROBO1/2 and SCEL as candidate genes in Kallmann syndrome with emerging bioinformatic analysis. Endocrine. 2020; 67 (1): 224-232. doi: 10.1007/s12020-019-02010-y.
14. Barraud S, Delember B, Poirsier-Violle C, Bouilang J, Merol JC, Grange F, et al. Congenital Hypogonadotropic Hypogonadism with Anosmia and Gorlin Features Caused by a PTCH1 Mutation Reveals a New Candidate Gene for Kallmann Syndrome. Neuroendocrinology. 2021; 111 (1-2): 99-114. doi: 10.1159/000506640.
15. Oleari R, Caramello A, Campinoti S, Lettieri A, Ioannou E, Paganoni A, et al. PLXNA1 and PLXNA3 cooperate to pattern the nasal axons that guide gonadotropin-releasing hormone neurons. Development. 2019; 146 (21): doi: 10.1242/dev.176461.
16. Howard SR. The Genetic Basis of Delayed Puberty. Front Endocrinol (Lausanne). 2019; 10 423. doi: 10.3389/fendo.2019.00423.
17. Zhu J, Choa RE, Guo MH, Plummer L, Buck C, Palmert MR, et al. A shared genetic basis for self-limited delayed puberty and idiopathic hypogonadotropic hypogonadism. J Clin Endocrinol Metab. 2015; 100 (4): E646-654. doi: 10.1210/jc.2015-1080.
18. Au MG, Crowley WF, Jr., Buck CL. Genetic counseling for isolated GnRH deficiency. Mol Cell Endocrinol. 2011; 346 (1-2): 102-109. doi: 10.1016/j.mce.2011.05.041.

19. Dode C, Levilliers J, Dupont JM, De Paep A, Le Du N, Soussi-Yanicostas N, et al. Loss-of-function mutations in FGFR1 cause autosomal dominant Kallmann syndrome. Nat Genet. 2003; 33 (4): 463-465. doi: 10.1038/ng1122.

20. Wang D, Niu Y, Tan J, Chen Y, Xu H, Ling Q, et al. Combined in vitro and in silico analyses of FGFR1 variants: genotype-phenotype study in idiopathic hypogonadotropic hypogonadism. Clin Genet. 2020; 98 (4): 341-352. doi: 10.1111/cge.13814.

21. Li S, Zhao Y, Nie M, Ma W, Wang X, Ji W, et al. Clinical Characteristics and Spermatogenesis in Patients with Congenital Hypogonadotropic Hypogonadism Caused by FGFR1 Mutations. Int J Endocrinol. 2020; 2020 8873532. doi: 10.1155/2020/8873532.

22. Pitteloud N, Hayes FJ, Boepple PA, DeCruz S, Seminara SB, MacLaughlin DT, et al. The role of prior pubertal development, biochemical markers of testicular maturation, and genetics in elucidating the phenotypic heterogeneity of idiopathic hypogonadotropic hypogonadism. J Clin Endocrinol Metab. 2002; 87 (1): 152-160. doi: 10.1210/jc.2001-0230.

23. Coutant R, Biette-Demeneix E, Bouvattier C, Bouhours-Nouet N, Gatelais F, Dufresne S, et al. Baseline inhibin B and antimullerian hormone measurements for diagnosis of hypogonadotropic hypogonadism (HH) in boys with delayed puberty. J Clin Endocrinol Metab. 2010; 95 (12): 5225-5232. doi: 10.1210/jc.2010-1535.

24. Adan L, Lechevalier P, Couto-Silva AC, Boissan M, Trivin C, Brailly-Tabard S, et al. Plasma inhibin B and antimullerian hormone concentrations in boys: discriminating between congenital hypogonadotropic hypogonadism and constitutional pubertal delay. Med Sci Monit. 2010; 16 (11): CR511-517.

25. Rastrelli G, Corona G, Mannucci E, Maggi M. Factors affecting spermatogenesis upon gonadotropin-replacement therapy: a meta-analytic study. Andrology. 2014; 2 (6): 794-808. doi: 10.1111/andr.262.

26. Hao M, Nie M, Yu BQ, Gao YJ, Wang X, Ma WL, et al. Gonadotropin treatment for male partial congenital hypogonadotropic hypogonadism in Chinese patients. Asian J Androl. 2020; 22 (4): 390-395. doi: 10.4103/aja.aja_88_19.

27. Cerrato F, Shagoury J, Kralickova M, Dwyer A, Falardeau J, Ozata M, et al. Coding sequence analysis of GNRHR and GPR54 in patients with congenital and adult-onset forms of hypogonadotropic hypogonadism. Eur J Endocrinol. 2006; 155 Suppl 1 S3-S10. doi: 10.1530/eje.1.02235.

28. Kim HG, Pedersen-White J, Bhagavath B, Layman LC. Genotype and phenotype of patients with gonadotropin-releasing hormone receptor mutations. Front Horm Res. 2010; 39 94-110. doi: 10.1159/000312696.

29. Bhagavath B, Ozata M, Ozdemir IC, Bolu E, Bick DR, Sherins RJ, et al. The prevalence of gonadotropin-releasing hormone receptor mutations in a large cohort of patients with hypogonadotropic hypogonadism. J Clin Endocrinol Metab. 2008; 93 (9): 3551-3559. doi: 10.1210/jc.2007-2654.

30. Topaloglu AK, Reimann F, Guclu M, Yalin AS, Kotan LD, Porter KM, et al. TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for Neurokinin B in the central control of reproduction. Nat Genet. 2009; 41 (3): 354-358. doi: 10.1038/ng.306.

31. Gianetti E, Tusset C, Noel SD, Au MG, Dwyer AA, Hughes VA, et al. TAC3/TACR3 mutations reveal preferential activation of gonadotropin-releasing hormone receptor 2 genes in neonatal life followed by reversal in adulthood. J Clin Endocrinol Metab. 2010; 95 (6): 2857-2867. doi: 10.1210/jc.2009-2320.

32. Avbelj Stefanija M, Jeanpierre M, Sykiotis GP, Young J, Quinton R, Abreu AP, et al. An ancient founder mutation in PROKR2 impairs human reproduction. Hum Mol Genet. 2012; 21 (19): 4314-4324. doi: 10.1093/hmg/dd264.

33. Sarfati J, Guiochon-Mantel A, Rondard P, Amulf I, Garcia-Pinero A, Wolczynski S, et al. A comparative phenotypic study of kallmann syndrome patients carrying monoallelic and biallelic mutations in the prokineticin 2 or prokineticin receptor 2 genes. J Clin Endocrinol Metab. 2008; 93 (9): 3551-3559. doi: 10.1210/jc.2007-2654.

34. Cole LW, Sidis Y, Zhang C, Quinton R, Plummer L, Pignatelli D, et al. Mutations in prokineticin 2 and prokineticin receptor 2 genes in human gonadotrophin-releasing hormone deficiency: molecular genetics and clinical spectrum. J Clin Endocrinol Metab. 2008; 93 (9): 3551-3559. doi: 10.1210/jc.2007-2654.

35. Zhao Y, Wu J, Jia H, Wang X, Zheng R, Jiang F, et al. PROKR2 mutations in idiopathic hypogonadotropic hypogonadism: selective disruption of the binding to a Galpha-protein leads to biased signaling. FASEB J. 2019; 33 (3): 4538-4546. doi: 10.1096/fj.201801575R.

36. Bouty A, Ayers KL, Pask A, Heloury Y, Sinclair AH. The Genetic and Environmental Factors Underlying Hypospadias. Sex Dev. 2015; 9 (5): 239-259. doi: 10.1159/000441988.
Table 1 Genetic results of 7 patients with hypospadias

| No. | Date of birth | Diagnosis | Age* (yrs) | First gene | Nucleotide | Amino acid | Second gene | Nucleotide | Amino acid | Third gene | Nucleotide | Amino acid |
|-----|---------------|-----------|------------|------------|------------|------------|-------------|------------|------------|------------|------------|------------|
| 1   | 2012/3/15     | KS        | 0.42       | HS6ST1     | c.187C>G   | p.H63D     | SALL1       | c.1984A>G  | p.M662V    |
| 2   | 2003/7/3      | KS        | 3.42       | ANOS1      | c.958G>A   | p.E320K    |
| 3   | 2000/9/11     | KS        | 9          | FGF17      | c.359C>T   | p.P120L    |
| 4   | 2005/8/30     | KS        | 10.17      | CHD7       | c.3247A>G  | p.T1083A   | CHD7        | c.6379G>A  | p.A2127T   | HS6ST1     | c.1177G>A  | p.D39:     |
| 5   | 2003/9/26     | KS        | 12         | ANOS1      | c.1678G>A  | p.V560I    |
| 6   | 2017/5/6      | nHH       | 3.25       | PROKR2     | c.472G>A   | p.V158I    | SPECC1L     | c.694A>G   | p.M232V    |
| 7   | 2009/6/4      | nHH       | 14         | Negative   |

*: Age at diagnosis.

Table 2 Oligogenic mutations of 26 patients
| Case | Diagnosis | Number of gene | Number of Mutant Alleles | Gene | Nucleotide Change | Amino Acid Change | Mutation Type |
|------|-----------|----------------|--------------------------|------|-------------------|-------------------|--------------|
| 1    | KS        | 3              | 3                        | SEMA3E| c.760G>C          | p.E254Q          | missense     |
|      |           |                |                          | CHD7 | c.2824A>G         | p.T942A          | missense     |
|      |           |                |                          | NSMF | c.188C>T          | p.P63L           | missense     |
| 2    | KS        | 3              | 3                        | FGFR1| c.340-344delTTTTC | p.F114fs13       | frameshift   |
|      |           |                |                          | FEZF1| c.614C>T          | p.A205V          | missense     |
|      |           |                |                          | FLRT3| m.2556G>A         |                   | noncoding region |
| 3    | nHH       | 3              | 4                        | PROKR2| c.533G>C          | p.W178S          | missense     |
|      |           |                |                          | CHD7 | c.*480_*481insAGGC|                   | UTR          |
|      |           |                |                          | CHD7 | c.*480_*481insCAGTATGCT| CGGGACGCCCTGGCTAAGAA|                   | UTR          |
|      |           |                |                          | FGF8 | c.-72A>G          |                   | UTR          |
| 4    | KS        | 2              | 3                        | FGFR1| c.801C>G          | p.Y2677Ter       | missense     |
|      |           |                |                          | PROKR2| c.743G>A         | p.R248Q          | missense     |
|      |           |                |                          | PROKR2| c.533G>C         | p.W178S          | missense     |
| 5    | KS        | 2              | 2                        | FGFR1| c.1034_c.1035del  | p.S345Cfs54*     | frameshift   |
|      |           |                |                          | ANOS1| c.907G>A         | p.V303I          | missense     |
| 6    | KS        | 2              | 2                        | FGFR1| c.736C>T         | p.R246W          | missense     |
|      |           |                |                          | CHD7 | c.8250T>G        | p.F2750L         | missense     |
| 7    | KS        | 2              | 2                        | FGFR1| c.1704+1G>A      |                   | splicing site|
|      |           |                |                          | SPRY4| c.88C>T          | p.R30W           | missense     |
| 8    | KS        | 2              | 2                        | PROKR2| c.533G>C        | p.W178S          | missense     |
|      |           |                |                          | PROK2 | c.301C>T        | p.R101W          | missense     |
| 9    | KS        | 2              | 2                        | PROKR2| c.691G>A        | p.E231K          | missense     |
|      |           |                |                          | IL17RD| c.192A>G       | p.M658V          | missense     |
| 10   | KS        | 2              | 3                        | PROKR2| c.239G>A        | p.R80H           | missense     |
|      |           |                |                          | PROKR2| c.169G>T        | p.G57C           | missense     |
|      |           |                |                          | SEMA3A| c.1453-9delG    |                   | frameshift   |
| 11   | KS        | 2              | 2                        | IL17RD| c.1319G>T       | p.G440V          | missense     |
|      |           |                |                          | GLI3  | c.1930G>A       | p.G644R          | missense     |
| 12   | KS        | 2              | 3                        | IL17RD| c.572C>T       | p.P191L          | missense     |
|      |           |                |                          | ANOS1| c.1654G>A      | p.E552K          | missense     |
|      |           |                |                          | ANOS1| c.1062+1G>A   |                   | splicing site|
| 13   | KS        | 2              | 2                        | KISS1R| c.149C>A       | p.A50E           | missense     |
|      |           |                |                          | CCKBR| c.1247G>A     | p.R416H          | missense     |
| 14   | KS        | 2              | 3                        | CHD7 | c.3247A>G       | p.T1083A         | missense     |
|      |           |                |                          | CHD7 | c.6379G>A       | p.A2127T         | missense     |
|      |           |                |                          | HS6ST1| c.1177G>A      | p.D393N          | missense     |
| 15   | KS        | 2              | 2                        | FGF17 | c.580C>G       | p.Q194E          | missense     |
|      |           |                |                          | CHD7 | c.7912A>G      | p.L480X          | truncation   |
| 16   | KS        | 2              | 2                        | FGF17 | c.1439T>G      | p.V436I          | missense     |
|   | nHH | 2   | 2   | FGFR1  | c.238C>T | p.R80C | missense |
|---|-----|-----|-----|--------|----------|--------|---------|
|   |     |     |     | SOX2   | c.695C>A | p.T232N | missense |
| 17| nHH | 2   | 2   |        |          |        |         |
|   |     |     |     | FGFR1  | c.142G>A | p.G48S  | missense |
|   |     |     |     | GLI3    | c.3286G>A | p.V1096M | missense |
| 18| nHH | 2   | 2   |        |          |        |         |
|   |     |     |     | PROKR2  | c.308C>T | p.A103V | missense |
|   |     |     |     | SEMA3E  | c.760G>C | p.E254Q | missense |
| 19| nHH | 2   | 2   |        |          |        |         |
|   |     |     |     | PROKR2  | c.533G>C | p.W178S | missense |
|   |     |     |     | CHD7    | c.6955C>T | p.R2319C | missense |
| 20| nHH | 2   | 2   |        |          |        |         |
|   |     |     |     | SOX2    | c.330C>A | p.Y110Ter | missense |
|   |     |     |     | SEMA3A  | c.1432G>A | p.E478K | missense |
| 21| nHH | 2   | 2   |        |          |        |         |
|   |     |     |     | SOX2    | c.6955C>A | p.T232N | missense |
|   |     |     |     | CHD7    | c.2656C>T | p.R886W | missense |
| 22| nHH | 2   | 2   |        |          |        |         |
|   |     |     |     | HS6ST1  | c.1177G>A | p.D393N | missense |
|   |     |     |     | TAC3    | c.107G>A | p.R36H  | missense |
| 23| nHH | 2   | 2   |        |          |        |         |
|   |     |     |     | KISS1R  | c.929G>A | p.C310Y | missense |
|   |     |     |     | NR0B1   | c.379G>A | p.A127T | missense |
| 24| nHH | 2   | 2   |        |          |        |         |
|   |     |     |     | FGFR1   | c.55A>G  |        | noncoding region |
|   |     |     |     | FGFR1   | c.1825-30G>A |        | intron |
|   |     |     |     | PROKR2  | c.533G>C | p.W178Se | missense |
| 25| nHH | 2   | 3   |        |          |        |         |
|   |     |     |     | WDR11   | c.386G>A | p.R129H | missense |
|   |     |     |     | SEMA3A  | c.2200C>T | p.R734W | missense |
| 26| nHH | 2   | 2   |        |          |        |         |

**Figures**
Figure 1

The chromosomes of all patients were 46 XY and SRY (+). Combined with the phenotypes, physical signs, hormone levels, presence of puberty, olfactory bulb imaging, and genetic test results, a total of 126 cases of CHH were diagnosed, including 88 cases of KS (69.8%), 37 cases of nHH (29.4%), and 1 case of CHARGE syndrome.
Figure 2

We further analyzed the common pathogenic gene mutation sites according to ACMG and found that 45.5%–75.0% of the mutation sites were pathogenic or likely pathogenic, except for one case of CHD7 mutation being likely benign, and the rest of these patients had either single-gene or oligogenic mutations.
In total, 105 patients completed the hCG standard test conducted to evaluate testicular Leydig cell function among 126 patients. Of these 105 patients, 68.6% (72/105) of patients had T < 100 ng/dl, of which 65 patients completed the HCG prolongation test, and 22.9% (24/105) of patients had T < 100 ng / dl, suggesting testicular Leydig cell dysfunction; therefore, at least 22.9% of cases could be diagnosed as dual CHH.
Figure 4

Four patients had a family history of disorders of sex development (DSD), and three mothers were probands.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytable1.xlsx