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# *ANOS1* variants in a large cohort of Chinese patients with congenital hypogonadotropic hypogonadism

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**ABSTRACT**

**Objective:** Congenital hypogonadotropic hypogonadism (CHH) is a rare congenital gonadal dysplasia caused by defects in the synthesis, secretion or signal transduction of hypothalamic gonadotropin releasing hormone. The main manifestations of CHH are delayed or lack puberty, low levels of sex hormones and gonadotropins, and may be accompanied with other clinical phenotypes. Some patients with CHH are also accompanied with anosmia or hyposmia, which is called Kalman syndrome (KS). *ANOS1*, located on X chromosome, is the first gene associated with CHH in an X-linked recessive manner. This study aims to provide a basis for the genetic diagnosis of CHH by analyzing the gene variant spectrum of *ANOS1* in CHH and the relationship between clinical phenotype and genotype.

**Methods:** In this study, whole exome sequencing (WES) was used to screen rare sequencing variants (RSVs) of *ANOS1* in a Chinese cohort of 165 male CHH patients. Four commonly used *in silico* tools were used to predict the function of the identified RSVs in coding region, including Polyphen2, Mutation Taster, SIFT, and Combined Annotation Dependent Depletion (CADD). Splice Site Prediction by Neural Network (NNSPLICE) was employed to predict possibilities of intronic RSVs to disrupt splicing. American College of Medical Genetics and Genomics (ACMG) guidelines was used to assess the pathogenicity of the detected RSVs. The *ANOS1* genetic variant spectrum of CHH patients in Chinese population was established. The relationship between clinical phenotype and genotype was analyzed by collecting detailed clinical data.

**Results:** Through WES analysis for 165 CHH patients, *ANOS1* RSVs were detected in 17 of them, with the frequency of 10.3%. A total of 13 RSVs were detected in the 17 probands, including 5 nonsense variants (p.T76X, p.R191X, p.W257X, p.R262X, and p.W589X), 2

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splicing site variants (c.318+3A>C, c.1063-1G>C), and 6 missense variants (p.N402S, p.N155D, p.P504L, p.C157R, p.Q635P, and p.V560I). In these 17 CHH probands with *ANOS1* RSVs, many were accompanied with other clinical phenotypes. The most common associated phenotype was cryptorchidism (10/17), followed by unilateral renal agenesis (3/17), dental agenesis (3/17), and synkinesia (3/17). Eight RSVs, including p.T76X, p.R191X, p.W257X, p.R262X, p.W589X, c.318+3A>C, c.1063-1G>C, and p.C157R, were predicted to be pathogenic or likely pathogenic *ANOS1* RSVs by ACMG. Eight CHH patients with pathogenic or likely pathogenic *ANOS1* variants had additional features. In contrast, only one out of nine CHH patients with non-pathogenic (likely benign or uncertain of significance) *ANOS1* variants according to ACMG exhibited additional features. And function of the non-pathogenic ANOS1 variants accompanied with other CHH-associated RSVs.

**Conclusion:** The *ANOS1* genetic spectrum of CHH patients in Chinese population is established. Some of the correlations between clinical phenotype and genotype are also established. Our study indicates that CHH patients with pathogenic or likely pathogenic *ANOS1* RSVs tend to exhibit additional phenotypes. Although non-pathogenic *ANOS1* variants only may not be sufficient to cause CHH, they may function together with other CHH-associated RSVs to cause the disease.

**KEY WORDS** congenital hypogonadotropic hypogonadism; *ANOS1*; Kallmann syndrome; cryptorchidism

# 中国先天性低促性腺激素性性腺功能减退症患者*ANOS1*的突变

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**［摘 要］目的：**先天性低促性腺激素性性腺功能减退症(congenital hypogonadotropic hypogonadism，CHH)是一种罕见的先天性疾病，由于下丘脑促性腺激素释放激素的合成、分泌或信号转导缺陷引起先天性性腺发育不良。CHH以青春期发育延迟或缺乏、性激素及促性腺激素水平低下为主要表现，同时可能伴有其他临床表型。一部分CHH患者伴有嗅觉丧失或低下，被称为卡尔曼综合征(Kallmann syndrome，KS)。*ANOS1*基因是第一个被发现的CHH致病基因，位于X染色体上，其突变可导致X-连锁隐性遗传的CHH。本研究拟通过分析CHH患者中*ANOS1*的基因突变图谱以及临床表型和基因型的关系，为CHH的遗传学诊断奠定基础。**方法：**利用全外显子组测序(whole exome sequencing，WES)的方法筛选来自中国的165名男性CHH患者中*ANOS1*基因的罕见变异(rare sequencing variants，RSVs)。利用Polyphen2、Mutation tastaster、SIFT和CADD(Combined Annotation Dependent Depletion)4种常见的生物信息学工具预测编码区变异的功能，基于神经网络的剪接位点预测(Splice Site Prediction by Neural Network，NNSPLICE)软件对检测到的内含子区的RSVs进行注释，并利用美国医学遗传学和基因组学学院(American College of Medical Genetics and Genomics，ACMG)遗传变异分类标准与指南判断*ANOS1* RSVs是否具有致病性。初步建立中国人群CHH患者*ANOS1*的遗传突变谱，通过收集部分患者详细的临床资料，建立临床表型和基因型的相关性。**结果：**WES分析显示165名CHH患者中17例患者发生了*ANOS1*突变，突变频率为10.3%。在17名CHH患者中共检测到13个*ANOS1* RSVs，包括5个无义突变(p.T76X、p.R191X、p.W257X、p.R262X和p.W589X)，2个剪切位点突变(c.318+

3A>C 和 c.1063-1G>C)和 6 个错义突变(p.N402S、p.N155D、p.P504L、p.C157R、p.Q635P 及 p.V560I)。在 17 名携带 *ANOS1* RSVs 的患者中，很多同时伴有其他临床表型，其中最常见的为隐睾(10/17)，其次是单侧肾脏发育不全(3/17)，牙齿发育不全(3/17)和联带运动(3/17)。利用 ACMG 对上述检测到的 *ANOS1* RSVs 进行分析，8 个 RSVs 包括 p.T76X、p.R191X、p.W257X、p.R262X、p.W589X、c.318+3A>C、c.1063-1G>C 和 p.C157R 被预测为致病性或可能致病性的罕见突变，且携带这些突变的患者经过临床检查全部伴随其他临床表型；然而，在携带其他被预测为非致病性(不确定性或可能良性)的 *ANOS1* RSVs 患者中，只有 1 例患者同时伴随其他临床表型，且这些患者中大部分同时伴有其他 CHH 致病基因的 RSVs。**结论：** 初步建立了中国人群 CHH 患者的 *ANOS1* 基因突变谱及基因型-表型相关性。携带致病性或可能致病性 *ANOS1* RSV 的 CHH 患者往往伴随其他表型。单纯非致病性 *ANOS1* RSV 可能不足以引起 CHH，但它们可能与其他 CHH 致病基因突变一起发挥作用，导致该疾病的发生。

**[关键词]** 先天性低促性腺激素性性腺功能减退症；*ANOS1*；卡尔曼综合征；隐睾

The impulse secretion of gonadotropin releasing hormone (GnRH) in the hypothalamus plays a critical role in the initiation and maintenance of normal puberty and reproductive function in human. GnRH stimulates the pituitary to secrete gonadotropins of luteinizing hormone (LH) and follicle stimulating hormone (FSH), which lead to the production of sex hormones[1-2]. Deficient in the synthesis, secretion, or action of GnRH leads to congenital hypogonadism hypogonadism (CHH, OMIM 140116), a rare disorder characterized by delayed or absent puberty. CHH was first reported in association with olfactory defects and named Kallmann syndrome (KS, OMIM 300836) [3]. Subsequently, a number of CHH patients with normal olfaction were defined as normosmic CHH (nCHH). Additional abnormalities, such as midline defects, ataxia, renal agenesis, synkinesia, and digital anomalies, have also been reported as variable characteristics of this disease[4-5].

*ANOS1*, the first gene associated with KS, was surfaced during the investigation of KS brothers with a large contiguous gene deletion on the X chromosome[6]. Then *ANOS1* part deletion was detected in a male infant with abnormal genitalia, hypogonadotropic hypogonadism, agenesis of the olfactory bulbs and tracts (i. e., KS) associated with chondrodysplasia puncta and ichthyosis[7]. *ANOS1*, located on chromosome Xp22.31, comprises 14 exons and encodes a 680-amino acid extracellular cell adhesion protein. The anosmin-1 protein encoded by *ANOS1* is comprised of an N-terminal cysteine-enriched Cys-box domain, a whey acidic protein (WAP) domain, 4 fibronectin type III (FnIII) domains, and a histidine-rich C terminal region (Figure 1)[8].

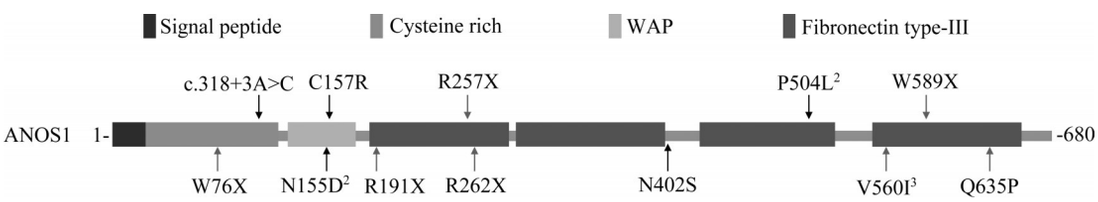


**Figure 1 Distribution of RSVs in *ANOS1***

*ANOS1* promotes neuronal cell adhesion, neurite outgrowth, axonal guidance, and central nervous system (CNS) projection neuron branching. The expression of *ANOS1* during embryonic development has been detected at various brain sites including the olfactory bulbs and the cerebellum, as well as other sites such as spinal cord, inner ear, and kidney[6, 9-10]. Accordingly, defects including renal agenesis, cleft palate, mirror movements, and hearing loss were often associated with KS caused by *ANOS1*[11-15]. About 160 variants have been identified in the *ANOS1* gene, and most of them are loss-of-function variants (frameshift, nonsense and splice site) presumed to be pathogenic. However, the contribution of missense and likely benign *ANOS1*

variants to the CHH is largely unknown.

In this study, we aim to investigate the prevalence of *ANOS1* in a large cohort of male CHH patients from China, and evaluate the contribution of *ANOS1* variants to CHH by using detailed phenotyping and co-segregation analysis.

# 1 Patients and methods

## 1.1 Patients and clinical evaluation

A total of 165 male CHH probands from China (115 KS and 50 nCHH) were recruited at Xiangya Hospital (Changsha, China) and the People's Hospital of Henan Province (Zhengzhou, China). All patients or their guardians gave written informed consents for hormonal, anthropometric, and genetic detection. The studies involving human samples were approved by the Ethics Committee of School of Life Sciences, Central South University (No. 2017030801). This study also consisted of a control group of 450 unrelated, ethically matched male Chinese participants from Xiangya Hospital.

The diagnosis of CHH was defined by: absence of pubertal development by 18 years of age, or previously medical induced puberty under this age, or neonatal micropenis with cryptorchidism; low sex-steroid levels in association with inappropriately low/normal gonadotropin levels and an abnormal/normal response to a GnRH stimulation test; inappropriately low/normal gonadotropin levels; otherwise normal anterior pituitary anatomy and function; excluded secondary causes of hypogonadism. Olfactory function was assessed by self-reported for KS or interpreted by University of Pennsylvania Smell Identification Test (UPSIT score < 5th percentile of age) for nCHH/KS. The brain and olfactory bulb structure were examined using magnetic resonance imaging (MRI) if available. Comprehensive auditory functions were assessed by a pure tone test if suspicious. Other clinical manifestations often seen in CHH, such as cleft lip, synkinesis, cleft palate, dental agenesis, renal agenesis, skeletal deformities, and seizures were carefully analyzed. Additionally, bone densitometry, weight, height, skin texture, testis size, sperm count and medication history for phenotype-genotype analysis were also registered. Familial co-segregation was performed whenever available.

## 1.2 Whole exome sequencing

Whole exome sequencing (WES) in CHH and control cohorts was performed using previously described methods[16]. Non-synonymous rare sequencing variants (RSVs) with minor allele frequency (MAF)<1% in the dbSNP, ExAC, Genome Aggregation Database (gnomAD), Chinese Millionome Database (CMDB), and 1000 Genomes in *ANOS1* as well as a panel of genes involved in CHH, including *CCDC141, AXL, DUSP6, FEZF1, FGF8, FGF17, FGFR1, FLRT3, GNRH1, GNRHR, HESX1, HS6ST1, IL17RD, KISS1, KISS1R, NSMF, PROK2, PROKR2, SEMA3A, SEMA3E, PLXNA1, SEMA7A, SPRY4, TAC3, TACR3, WDR11, LEP, LEPR, NR0B1, PCSK1, STUB1, CHD7, DMXL2, SOX2, PNPLA6, RNF216, SOX10,* and *OTUD4*.

## 1.3 Gross or partial deletions sequencing

Copy number variations (CNV) analysis was performed using ExomeDepth algorithm as described previously[17].

## 1.4 Bioinformatics analysis

RSVs were interpreted according to American College of Medical Genetics and Genomics (ACMG) guidelines[18]. Missense variants were automated in InterVar (http://wintervar. wglab. org/) and re-interpretation was made through manual adjustment according to phenotypes, segregation, inheritance, and statistical difference compared with control. RSVs were divided into 5 types: Pathogenic (P), likely pathogenic (LP), of uncertain significance (U), likely benign (LB), and benign (B). Furthermore, 4 commonly used *in silico* tools were used to predict the function of the identified missense variants, including Polyphen2, MutationTaster, SIFT, and Combined Annotation Dependent Depletion (CADD). Splice Site Prediction by Neural Network (NNSPLICE)[19] was employed to predict possibilities of intronic RSVs to disrupt splicing.

## 1.5 Statistical analysis

A gene-collapsed RSV association test on CHH, versus 450 controls, was performed to compare rare-variant allele frequencies by means of a two-tailed Fisher's exact test. A Fisher's exact test was also used to compare the percentages of patients with different groups as appropriate. The significance level was set at $P<0.05$.

## 2 Results

### 2.1 Enrichment of *ANOS1* RSVs in CHH patients

Overall, we identified 13 non-synonymous *ANOS1* RSVs in 17 CHH patients (Table 1 and Figure 1). Among them, 5 nonsense RSVs, p.T76X, p.R191X, p.W257X, p.R262X, and p.W589X, have been previously reported. Two novel splicing variants (c.318+3A>C and c. 1063-1G>C) were predicted to disrupt the normal splicing. Additionally, 4 novel missense RSVs (p.N402S, p. N155D, p. P504L, and p. C157R) and 2 previously reported missense variants (p.Q635P and p.V560I) were identified in 10 unrelated patients. No CNV or intragenic deletions of *ANOS1* were found in this study. For the patients with nonsense and splicing *ANOS1* RSVs, only proband 4 carried p.E2009K *CHD7* RSV and p.R191X *ANOS1* RSV, whereas other patients only carried the *ANOS1* RSVs. In contrast, most patients (proband 11, 13, 14, 15, 16, and 17, Table 1) with missense RSVs also carried additional RSVs in another CHH associated genes.

Most RSVs were detected only in a single pedigree or case, except 3 missenses (p.N155D, p.P504L, and p.V560I). It should be noted that p.N155D and p.V560I were also found in one out of 450 ethnically matched control. The prevalence of *ANOS1* RSVs was significantly higher in CHH cohort compared to ethnically matched controls (10.3%, 17/165 vs 0.4%, 2/450; $P<0.001$).

Co-segregation was analyzed in 14 available pedigrees. As shown in Figure 2 and Table 1, probands 2, 6, and 17 carried *de novo* RSVs, whereas other probands inherited *ANOS1* variants from their mothers (Figure 2). According to the ACMG guidelines and NNSPLICE, 8 variants were pathogenic or likely pathogenic, 3 variants were of uncertain significance, and 2 were likely benign (Table 1).

### 2.2 Genotype-phenotype correlation

Clinical data from the 17 probands with *ANOS1* RSVs are summarized in Table 2. The mean age at diagnosis was 18.2 years. Sixteen of them were affected with KS while proband 4 showed normal olfaction on formal test and alleged normal sense of smell in daily life. However, he was not available for MRI examination. All of the 8 probands who harbored pathogenic or likely pathogenic *ANOS1* variants exhibited additional features, including unilateral or bilateral cryptorchidism, dental agenesis, unilateral renal agenesis, synkinesia, and hearing loss (Table 2).

Proband 1, a four-years-old boy, inherited a p.T76X *ANOS1* variant from his unaffected mother. He had strabismus, synkinesia, optic nerve dysplasia, and bilateral cryptorchidism as well as KS. Proband 2 carried a *de novo* c.318+3A>C *ANOS1* RSV and showed bilateral cryptorchidism and right renal deficiency other than KS.

Proband 3 inherited a c.1063-1G>C *ANOS1* RSV from his unaffected mother and manifested bilateral cryptorchidism and developmental delay. Proband 4 carrying p.E2009K *CHD7* RSV and p.R191X *ANOS1* RSV showed dental agenesis, potosis, and microphthalmia besides CHH. He admitted normal sense of smell, but unfortunately refused to perform olfactory bulb MRI. His mother (II:2 in Family 4, Figure 2) with p.E2009K *CHD7* and p.R191X *ANOS1* RSVs manifested scarce pubic hair and bicornate uterus but showed normal puberty and fertility. His maternal uncle (II: 5 in Family 4, Figure 2) who carried p.E2009K *CHD7* RSV is unaffected. Proband 5, an eight-years-old boy with p.R262X *ANOS1* RSV, showed cryptorchidism, dental agenesis, and KS. The individual II:3 in family 5 also carried p.R262X *ANOS1* RSV and was diagnosed with KS without associated phenotypes, whereas 2 women (I: 2 and II: 2, Figure 2) with this RSV were unaffected.

Proband 6 with a *de novo* p.W589X *ANOS1* RSV showed KS and right cryptorchidism. Proband 7 carrying a p.R257X *ANOS1* RSV was diagnosed as KS with synkinesia, bilateral cryptorchidism, and scoliosis. His maternal cousin (III: 2 in Family 7, Figure 2) with the same RSV had KS and synkinesia. Proband 8 with a p. C157R *ANOS1* RSV showed mild hearing loss, cryptorchidism, and left renal agenesis along with KS. And his nephew (III: 1 in Family 8, Figure 2) with the same RSV was diagnosed as KS along with cryptorchidism. Two women (I: 2 and II: 2, Figure 2) with this RSV were unaffected. Proband 9 with a p.Q635P *ANOS1* RSV displayed right cryptorchidism and synkinesia in addition to KS.

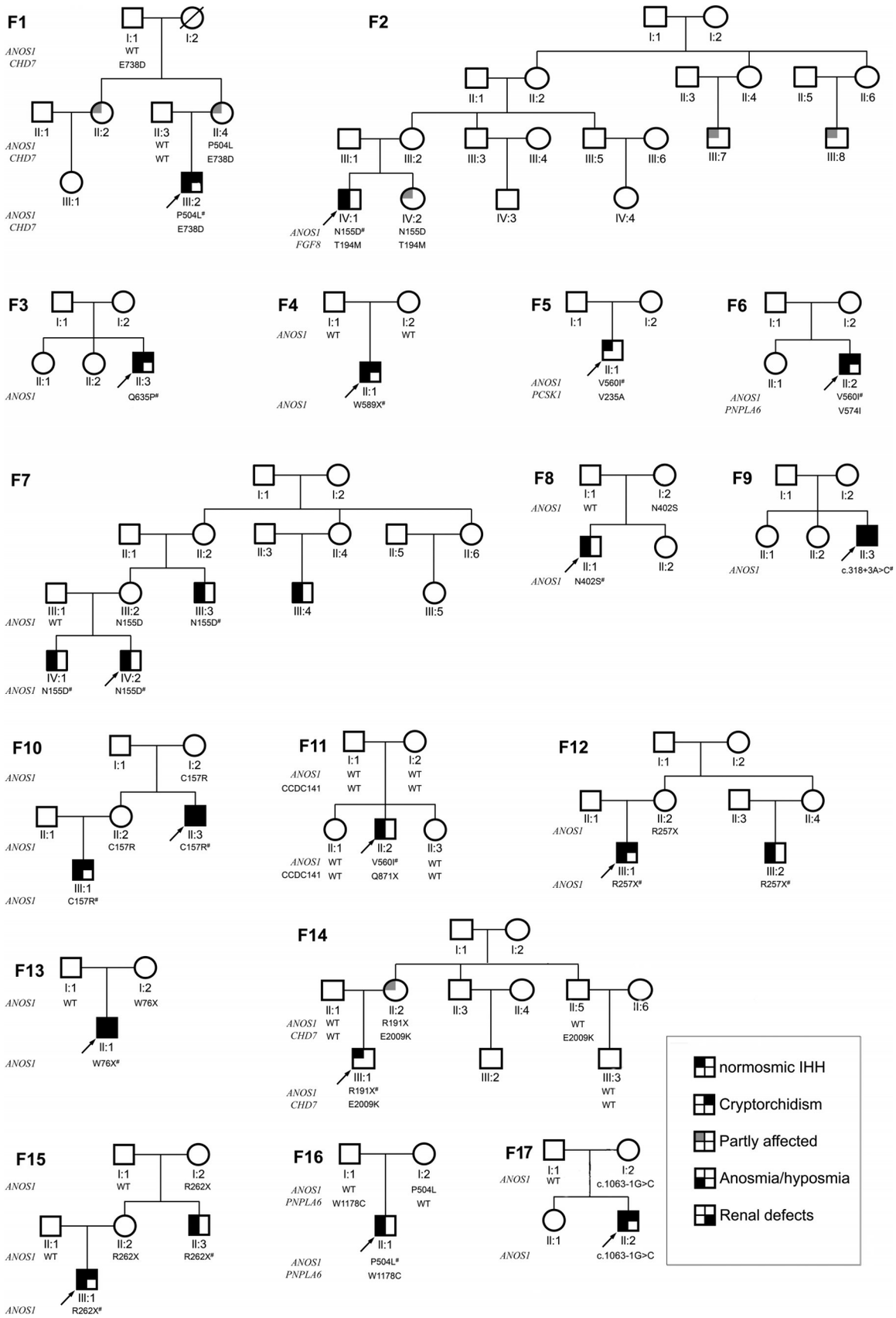


**Figure 2 Informative pedigrees of CHH probands carrying *ANOS1* RSVs identified in this study**

Proband of each family is indicated by an arrow. The available genotypes are indicated below each individual. Squares depict males and circles depict females. F: Family; CHH: Congenital hypogonadism hypogonadism; RSVs: Rare sequencing variants; WT: Wild type.

**Table 1 Molecular genetic characteristics of the CHH probands with identified *ANOS1* variants**

| ID | Dx | c.HGVS | p.HGVS | Domain | MAF | | NNSPLICE | ACMG criteria | *In silico* prediction | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | matched | all | | | CADD | SIFT | Polyphen2 | MutationTaster |
| 1 | KS | ANOS1 c.C1511T | p.P504L | FnIII | 0.002718 | 0.0002901 | | U | 11.3 | T | B | N |
| | | CHD7 c.2214A>C | p.E738D | | 0 | 0 | | U | 14.7 | T | B | D |
| 2 | KS | ANOS1 463A>G | p.N155D | WAP | 0.008177 | 0.000676 | | LB | 8.8 | T | B | N |
| | | FGF8 c.581C>T | p.T194M | | 0 | 0 | | U | 28.8 | D | D | D |
| 3 | KS | ANOS1 1904A>C | p.Q635P | FnIII | 0 | 0 | | U | 24.8 | D | D | D |
| 4 | KS | ANOS1 1766G>A | p.W589X | FnIII | 0 | 0 | | P | 39 | T | . | A |
| 5 | KS | ANOS1 1678 G>A | p.V560I | FnIII | 0.009956 | 0.007762 | | LB | 2.6 | T | B | N |
| | | PCSK1 704T>C | p.V235A | | 0.002199 | 0.0001566 | | LP | 24.4 | D | D | D |
| 6 | KS | ANOS1 1678 G>A | p.V560I | FnIII | 0.009956 | 0.007762 | | LB | 2.6 | T | B | N |
| | | PNPLA6 1720G>A | p.V574I | | 0 | 0.00000827 | | U | 13.2 | T | B | D |
| 7 | KS | ANOS1 463A>G | p.N155D | WAP | 0.008177 | 0.000676 | | LB | 8.8 | T | B | N |
| 8 | KS | ANOS1 1205A>G | p.N402S | FnIII | 0 | 0 | | U | 4.3 | T | B | N |
| 9 | KS | ANOS1: c.318+3A>C | | Cysteine | 0 | 0 | 0.89 | | | | | A |
| 10 | KS | ANOS1 469T>C | p.C157R | WAP | 0 | 0 | | P | 26.3 | D | D | D |
| 11 | KS | ANOS1 1678 G>A | p.V560I | FnIII | 0.009956 | 0.007762 | | LB | 2.6 | T | B | N |
| | | CCDC141 c.2611C>T | p.Q871X | | 0 | 0 | | P | 35 | T | | A |
| 12 | KS | ANOS1 769C>T | p.R257X | FnIII | 0 | 0 | | P | 35 | T | | A |
| 13 | KS | ANOS1 C228C>A | p.T76X | Cysteine | 0 | 0 | | P | 35 | T | | A |
| 14 | nCHH? | ANOS1 C571C>T | p.R191X | FnIII | 0 | 0 | | P | 35 | T | | A |
| | | CHD7 6025G>A | p.E2009K | | 0 | 0 | | U | 28 | D | D | D |
| 15 | KS | ANOS1 784C>T | p.R262X | FnIII | 0 | 0 | | P | 35 | T | | A |
| 16 | KS | ANOS1 c.C1511T | p.P504L | FnIII | 0.002718 | 0.0002901 | | U | 11.3 | T | B | N |
| | | PNPLA6 c.3534G>C | p.W1178C | | 0 | 0 | | U | 31 | D | D | D |
| 17 | KS | ANOS1 c.1063-1G>C | | FnIII | 0 | 0 | 0.98 | LP | | | | A |

Dx: Diagnosis; KS: Kallmann syndrome; nCHH: Normosmic congenital hypogonadotropic hypogonadism; HGVS: Human Genome Variation Society; Homo: Homozygous; Het: Heterozygous; N/A: Not available; WAP: Whey acidic protein domain; FnIII: Four fibronectin type III domains; Cysteine: N-terminalCys-box domain rich of cysteine; MAF: Minor allele frequency in ExAC database; matched: Ethnically-matched population in ExAC; all: All populations in ExAC; NNSPLICE: Splice Site Prediction by Neural Network; P: Pathogenic; B: Benign; D: Damaging; U: Uncertain significance; LP: Likely pathogenic; LB: Likely benign; CADD: Combined Annotation Dependent Depletion; SIFT: T, Tolerated; D, Deleterious; Polyphen2: B, Benign; D, Damaging; Mutation Taster: N, polymorphism; D, Disease_causing; A, Disease_causing_automatic.

**Table 2 Clinical and laboratory findings of the CHH patients carrying *ANOS1* mutations**

| No. | Age/ years | Gender | Dx | Other features | At diagnosis | | | | OB | Sperm |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | $E_2$/ ($pg·mL^{-1}$) | T/ ($ng·mL^{-1}$) | FSH/ ($mIU·mL^{-1}$) | LH/ ($mIU·mL^{-1}$) | | |
| 1 | 12 | M | KS | Cryptorchidism, dental agenesis, kidney agenesis, hypospadias | 9.9 | 0.4 | 0.2 | 0.1 | NA | — |
| 2 | 25 | M | KS | — | 28 | 0.2 | 0.3 | 0.3 | NA | $5.9×10^6$/mL |
| 3 | 17 | M | KS | Cryptorchidism, synkinesia | 13 | 0.9 | 0.5 | 0.4 | Anosmia | 2−6 |
| 4 | 18 | M | KS | Right cryptorchidism | 5 | 1.1 | 0.3 | 0.2 | Anosmia | $4×10^6$/mL |
| 5 | 20 | M | KS | Blue color blindness | 17 | 0.4 | 1.3 | 0.9 | NA | $5.2×10^6$/mL |
| 6 | 17 | M | KS | Right cryptorchidism | 15 | 0.3 | 0.4 | 0.4 | Hyposmia | NA |
| 7 | 16 | M | KS | — | 17 | 0.3 | 1.8 | 0.8 | Hyposmia | $6.9×10^6$/mL |
| 8 | 19 | M | KS | — | 1.7 | 0.3 | 0.6 | 0.3 | Hyposmia | $3.0×10^6$/mL |
| 9 | 18 | M | KS | Cryptorchidism Right kidney deficiency | 5.6 | 0.5 | 1.8 | 0.2 | Anosmia | 0 |
| 10 | 22 | M | KS | Left renal agenesis, cryptorchidism, left hearing loss | 16 | 1 | 0.3 | 0.4 | Aosmia | $0.9×10^6$/mL |
| 11 | 22 | M | KS | — | 20 | 1 | 0.3 | 0.4 | Hyposmia | $5.6×10^6$/mL |
| 12 | 23 | M | KS | Scoliosis, synkinesia cryptorchidism | 18 | 0.1 | 0.3 | 0.2 | Aosmia | 0 |
| 13 | 4 | M | KS | Strabismus, synkinesia optic nerve dysplasia cryptorchidism | 14 | 0.04 | 0.4 | <0.1 | NA | — |
| 14 | 28 | M | nCHH? | Dental agenesis, ptosis, microphthalmos | 14 | 0.57 | 2.26 | 0.52 | NA | $4.9×10^6$/mL |
| 15 | 8 | M | KS | Dental agenesis, cryptorchidism | 20 | 0.02 | 0.3 | 0.1 | NA | — |
| 16 | 36 | M | KS | — | 11 | 0.1 | 2.2 | 0.7 | Aosmia | NA |
| 17 | 2 | M | KS | Umbilical fistula, cryptorchidism, development delay | 16 | 0.82 | 0 | 0.07 | Aosmia | — |

Age means the age of diagnosis. M: Male; Dx: Diagnosis; KS: Kallmann syndrome; nCHH: Normosmic congenital hypogonadotropic hypogonadism; OB: Olfactory bulb; NA: Not applicable. Reference range for testosterone (T) in normal adult men is 1.75−7.81 ng/mL, for estradiol ($E_2$) <53 pg/mL, for luteinizing hormone (LH) 1.2−8.6 mIU/mL, and for follicle-stimulating hormone (FSH) 1.3−19.3 mIU/mL.

We also identified 5 non-pathogenic *ANOS1* RSVs in 8 CHH patients. Six patients also carried RSVs in other CHH-related genes. And only one showed additional symptoms along with KS. A significantly higher proportion of CHH patients with pathogenic or likely pathogenic *ANOS1* RSVs showed additional phenotypes, compared with those with non-pathogenic *ANOS1* variants (8/8 vs 1/9, $P$<0.01). Proband 13, with p.P504L *ANOS1* and p.E738D *CHD7* RSVs, showed KS along with dental agenesis, unilateral renal agenesis, strabismus, and hypospadias. His mother (II:4) and aunt (II:2) who also carried these RSVs manifested no armpit and pubic hair but showed normal puberty and fertility. And his grandfather (I:2) with a p.E738D *CHD7* variant was unaffected (Figure 2).

### 2.3 Treatment evaluation

The male patients except proband 1, 3, 5, and 13 accepted the treatment with testosterone (T) or human

chorionic gonadotropin/human menopausal gonadotropin treatment (HCG/HMG). All of the treatments obviously increased the testicular sizes in males. Spermatogenesis was evaluated in 11 male patients (Table 2), and sperm appeared in 9 patients (at least 24 months of continuous HCG/HMG therapy). As regards to the 2 patients (proband 2 and 7) with failure of spermatogenesis, they were both found with bilateral cryptorchidism and several neurologic defects.

## 3 Discussion

In this study, we systematically analyzed the *ANOS1* RSVs in a large Chinese cohort of male CHH patients. As a result, we identified 13 *ANOS1* RSVs in 17 probands, with the frequency of 10.3%. Besides the common concomitant symptoms of cryptorchidism, synkinesia, and unilateral renal agenesis, dental agenesis was also frequently found in CHH patients with *ANOS1* RSVs. The prevalence (10/17) of cryptorchidism in this cohort is higher than that in previous reports[15, 20-22].

CHH patients with RSVs in *FGFR1* signaling pathways are prone to have additional skeletal phenotypes, such as cleft lip/palate, dental agenesis, syndactyly, and clinodactyly[12, 23-27]. A previous study[4] found that dental agenesis is significantly enriched in *FGFR1* signaling group and is useful for prioritizing genetic screening. Intriguingly, Another study[28] found that anosmin-1, encoded by *ANOS1*, interacted with *FGFR1-FGF-HS6ST1* complex and enhances *FGFR1* signaling. In this study, 3 probands (4, 5 and 13) had dental agenesis, reinforcing the potential interaction between anosmin-1 and *FGFR1* signaling[29-30].

*ANOS1* RSVs are linked to KS in almost all cases except one study. In this circumstance, 2 patients who carry truncating or frameshift *ANOS1* RSVs show normosmia or borderline olfactory function[12]. In the current study, 16 out of 17 patients with *ANOS1* RSVs showed complete absent or decreased olfaction. However, proband 4 with a *p.R191X ANOS1* RSV and a *p.E2009K CHD7* RSV showed borderline olfaction test and alleged normal olfactory function in daily life. Nevertheless, we cannot rule out the possible slightly hypoplasia of olfactory bulb in this patient without MRI analysis.

*ANOS1* is one of the main causative genes for CHH and loss-of-function *ANOS1* RSVs are likely sufficient to cause CHH in an X-linked recessive manner. Indeed, 5 nonsense and 2 frameshift RSVs ( p.T76X, p.R191X, p. W257X, p. R262X, p. W589X, c. 318+3A>C, and c. 1063-1G>C) predicted to be loss of function led to CHH only in males. The missense C157R in Family 3 predicted to be pathogenic by all the *in silico* tools was located in the N-terminal WAP domain of *ANOS1*. The WAP domain contains 8 conserved cysteine residues forming 4 intramolecular disulphide bonds, which is composed of C134-C164, C15-C163, C147-C168, and C157-C172 forming the structural core of the protein and locating at the putative protease binding site of *FGFR1-HS6ST1* complex[31]. Substitutions are predicted to disrupt WAP domain core structure formation, leading to protein instability and loss of interaction with serine proteases. Accordingly, 2 male individuals in Family 8 (II: 3 and III: 1) carrying the C157R RSV which is predicted to destroy the C157-C172 binding site exhibited bilateral cryptorchidism, sensorineural hearing loss, and KS. Additionally, only II:3 displayed left renal agenesis.

Furthermore, our data suggest that those non-pathogenic *ANOS1* RSVs are not the major contributor for CHH per se. Indeed, 6 out of 8 probands (proband 11, 13, 14, 15, 16, 17) with non-pathogenic *ANOS1* RSVs also carried RSVs in another CHH gene. However, some non-pathogenic *ANOS1* RSVs may play a synergetic role with RSVs in other CHH-related genes in the pathogenesis of CHH. For instance, proband 13 with p.P504L *ANOS1* and p.E738D *CHD7* RSVs was affected with KS. However, his mother and aunt (II:4, II: 2) who also carried these 2 RSVs and his grandfather (I: 2) who only had p.E738D *CHD7* did not show CHH. Besides, proband 17 carried a second heterozygous p. Q871X *CCDC141* RSV, which alone seems not sufficient to cause the disease[32-33].

There is another thing worth discussing for the RSV p.N155D *ANOS1*. In Family 10, all men with p. N155D *ANOS1* RSV (II: 3, III: 1, and III: 2) had KS whereas the woman (II:2) with this RSV was unaffected. In Family 11, the proband (IV:1) with p.N155D *ANOS1* and T194M *FGF8* RSVs was diagnosed as KS, whereas his sister (IV: 2) with these 2 RSVs was unaffected. Furthermore, 2 male individuals (III: 3 and III: 4) in Family 11 were also diagnosed as KS, implying an X-

linked recessive inheritance although we have no genetic information for these 2 individuals. However, the p. N155D *ANOS1* RSV was predicted to be likely benign by all *in silico* tools and had a relatively high frequency (0.008177) in the ethnically-matched population in public database. Whether this RSV co-segregating with the CHH phenotype in an X-linked recessive manner or just a coincidence required further analysis in other population or functional experiments. Indeed, although *in silico* predictions of some RSVs are shown to be benign, the lack of specificity of the *in silico* programs and the rarity of these RSVs in extensive normative databases indicate that these RSVs must undergo further biological evaluation prior to discounting their pathogenicity.

Taken together, our study indicates that CHH patients with pathogenic or likely pathogenic *ANOS1* RSVs tend to exhibit additional phenotypes. Although non-pathogenic *ANOS1* RSVs only may not be sufficient to cause CHH, they may function together with other CHH-associated RSVs to cause the disease.

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