Analysis of genetic and clinical characteristics of a Chinese Kallmann syndrome cohort with ANOS1 mutations

Min Nie¹, Hongli Xu¹, Rongrong Chen², Jiangfeng Mao¹, Xi Wang¹, Shuyu Xiong¹, Junjie Zheng¹, Bingqing Yu¹, Mingxuan Cui¹, Wanlu Ma¹, Qibin Huang¹, Hongbing Zhang² and Xueyan Wu¹

¹Department of Endocrinology, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Key laboratory of Endocrine, Ministry of Health, Beijing, China and ²Department of Physiology, State Key Laboratory of Medical Molecular Biology, School of Basic Medicine, Graduate School of Peking Union Medical College, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China

Abstract

Objective: To analyze ANOS1 gene mutations in a large Chinese Kallmann syndrome (KS) cohort and to characterize the clinical presentation of the disease in patients with ANOS1 mutations.

Patients and methods: Chinese patients with KS, including 187 sporadic and 23 pedigree cases were recruited. Patients’ ANOS1 gene sequences were analyzed by direct sequencing of PCR-amplified products. In silico analysis was used to assess functional relevance of newly identified missense mutations. Patients’ clinical characteristics were analyzed retrospectively.

Result(s): Fifteen nonsynonymous rare ANOS1 variants were found in 13 out of 187 sporadic and 8 out of 23 familial IHH probands. Seven novel (C86F, C90Y, C151W, Y379X, c.1062 + 1G > A, Y579L fs 591X, R597X) and eight recurrent ANOS1 mutations (S38X, R257X, R262X, R423X, R424X, V560I, c.1843-1G > A, p.R631X) were identified. All the novel mutations were predicted to be pathogenic. The prevalence of cryptorchidism was high (38.1%) and occurred in patients with different kind of ANOS1 mutations, while the patients with the same mutation did not present with cryptorchidism uniformly.

Conclusion(s): The prevalence of ANOS1 gene mutations is low in sporadic KS patients, but is much higher in familial KS patients. In the present study, we identify seven novel ANOS1 mutations, including two mutations in the CR domain, which are probably pathogenic. These mutations expand the ANOS1 mutation spectrum and provide a foundation for prenatal diagnosis and genetic counseling.

Introduction

The hypothalamic–pituitary–gonadal (HPG) axis plays a crucial role in the development, progression and maintenance of normal reproductive function (1). The pulsatile secretion of gonadotropin-releasing hormone (GnRH) regulates the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the pituitary, which subsequently stimulate the gonads to produce sex steroids and gametes. Isolated hypogonadotropic hypogonadism (IHH) is caused by defects in the HPG axis, resulting in low levels of sex steroids, delayed puberty or absence of puberty and sterility. IHH is divided into Kallmann syndrome (KS) and normosmic idiopathic hypogonadotropic hypogonadism (nIHH). Approximately 50% of all IHH cases are due to KS (2).

KS is a congenital disorder characterized by IHH and hyposmia/anosmia. Both GnRH neurons and olfactory neurons originate from the nasal placode. During normal
development, GnRH neurons migrate along olfactory neuron axons to the hypothalamus. In KS, defects in GnRH neuron migration lead to IHH, and the absence or hypoplasia of the olfactory bulb and its axonal tracts results in hyposmia/anosmia. Some KS patients may also have other associated abnormalities, such as renal agenesis, synkinesis, cleft lip, cleft palate, dental agenesis, shortening of metacarpals, sensory neural hearing loss and seizures (3). The etiology of KS is not quite clear, but mutations in genes that regulate GnRH neuron development, migration and function are important causative factors (4).

The ANOS1 gene is the pathogenic gene found to cause X-linked KS. It is located on the X chromosome (Xp22.3) adjacent to pseudoautosomal region 1 (PAR1), a highly variable and unstable region of the chromosome. ANOS1 encodes the protein anosmin-1, an extracellular matrix protein. Anosmin-1 comprises an N-terminal cysteine-rich domain (Cys-box), followed by a whey acidic protein (WAP) domain, four fibronectin type III (FnIII) domains and a histidine-rich C terminal region (5). Anosmin-1 promotes neuronal cell adhesion, neurite outgrowth, axonal guidance and CNS projection neuron branching. Additionally, it plays a role in the migration of multiple types of neuronal precursors, including GnRH-producing neurons and oligodendrocyte precursors. ANOS1 mutations are found in approximately 10–20% of familial and sporadic KS patients. Nearly seventy mutations have, hitherto, been identified in the ANOS1 gene, which are spread widely throughout the entire gene with no mutation ‘hot spots’ in the affected regions.

In this study, we sequenced the ANOS1 gene in 187 sporadic and 23 familial cases of KS from the Chinese patient population to determine the prevalence of ANOS1 mutations in a large Chinese KS cohort and to analyze the clinical characteristics of KS patients with ANOS1 mutations.

**Subjects and methods**

**Subjects**

Twenty-three familial and 187 unrelated sporadic Chinese patients with KS were recruited from Peking Union Medical hospital between January 2009 and December 2016. All patients were male and diagnosed preliminarily with KS based on clinical manifestation and sex hormone data. The study was approved by the PUMCH’s Ethics Committee for Human Research and complies with the Declaration of Helsinki. Inclusion criteria were (1) absence of pubertal development by 18 years of age, (2) low concentration of sex steroids, (3) low or inappropriately normal gonadotropin levels at hypogonadal levels, (4) documented absence of sense of smell (anosmia) or deficient sense of smell (hyposmia), (5) normal function of growth hormone-IGF1 (insulin-like growth factor 1) axis, pituitary–adrenal axis and pituitary–thyroid axis and (6) other conditions leading to secondary hypogonadism were excluded from the study.

After obtaining informed consent, blood samples from all patients and some parents were collected for genetic testing. The clinical manifestations, including synkinesis, cleft lip, cleft palate, dental agenesis, shortening of metacarpals, renal agenesis, sensory neural hearing loss and seizures, testis size, the sperm counting and medication history, were collected and analyzed retrospectively.

**Hormonal assays**

Basal serum FSH, LH and testosterone were measured using chemiluminescent immunoassays (Bayor Diagnostics Corporation, USA). The intra- and inter-assay variation coefficients were 3.9% and 4.5% for FSH, 2.3% and 2.8% for LH and 5.6% and 6.6% for total testosterone respectively. The lowest measurable limits were 0.23 IU/L, 0.07 IU/L and 5.2 ng/dL for FSH, LH and total testosterone respectively.

**Mutation screening**

Genomic DNA was extracted from leukocytes of peripheral blood using a QIAGEN Mini Blood kit according to the manufacturer’s instructions. Fourteen exons and the exon–intron boundaries of the ANOS1 gene were amplified by PCR. The PCR products were purified with QIAquick PCR Purification Kit, and subsequently sequenced using a Taq big dye terminator sequencing kit and an ABI3730 automated sequencer (Applied Biosystems). The entire coding region, including the exon–intron boundaries of the ANOS1 gene, was sequenced in both forward and reverse directions in all patients. All sequencing primers are available on request.

The resulting sequences were analyzed using Chromas pro V1.7 (Applied Biosystems) and a BLAST search was performed with the reference sequences GenBank NG_007088.1 (ANOS1, g.DNA), GenBank NM_000216.2 (ANOS1, c.DNA) and GenBank NP_000207.2 (ANOS1, p.protein). For genomic DNA and cDNA numbering, the
A nucleotide of the ATG translation initiation codon was designated +1. The variants were identified as mutations if they were not found in 100 control subjects, dbSNP database in NCBI (http://www.ncbi.nlm.nih.gov/snp/) or exome variant sever (http://evs.gs.washington.edu/EVS/).

Patients with identified ANOS1 mutations were screened for digenic/oligogenic mutations by sequencing additional genes related to the hypothalamic–pituitary–gonadal axis (FGF8, FGFR1, GNRH1, GNRHR, KISS1, KISS1R, NELF, PROK2, PROKR2, TAC3, TACR3, LEP, LEPR, WDR11, HS6ST1, CHD7 and SEMA3A) (all primer sequences and PCR conditions are available upon request) to try to explain the reason why the patients with the same mutation presented with not exact the same clinical manifestations.

Bioinformatics analysis of novel missense variants

Evolutionary conservation at the affected amino acids was assessed by a multiple sequence alignment of anosmin-1 orthologs (MSA ORTHO). The human anosmin-1 sequence, as annotated in the UniProt database (http://www.uniprot.org/), was aligned with 26 orthologous sequences within the same database (May 2013) from closely and distantly related species (6). MSA ORTHO was built using the Bioedit program (https://www.bioedit.com/).

Pathogenic effects of missense mutations identified in this study were also analyzed using an extensive set of prediction tools since different principles and algorithms were used in different in silico analysis tools, and the prediction result is not exactly same for a specific missense mutation. The following in silico analysis tools were included in this study. (1) MutationTaster. It assesses the functional impact based on evolutionary conservation of the affected amino acid in protein homologs (7) (http://www.mutationtaster.org/). (2) SIFT. Its prediction is based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences (8) (http://sift.jcvi.org/). (3) PolyPhen. It predicts possible impact of an amino acid substitution on the structure and function using straightforward physical and comparative considerations (9) (http://genetics.bwh.harvard.edu/pph2/). (4) Mutation assessor. It uses the evolutionary information deriving from protein family alignments of large numbers of homologous sequences and 3D structures of sequence homologs to assess the function of missense variant (10) (http://mutationassessor.org/). (5) Panther. It evaluates a single substitution at a specific amino acid position in a protein based on the information about evolutionarily related proteins (11) (http://www.pantherdb.org/tools/csnpscoreForm.jsp). (6) SNAP. The prediction is based on the evolutionary information taken from an automatically generated multiple sequence alignment, structural features such as predicted secondary structure and solvent accessibility (12) (https://rostlab.org/services/snap). (7) SNPs & Go. It utilizes sequence information, evolutionary information derived in different ways and the defined functional GO score to estimate possible impact of an amino acid replacement (13) (http://snps-and-go.biocomp.unibo.it/). (8) MutPred. It evaluates the influence of an amino acid displacement upon protein sequence and molecular models change of structural features and functional sites between wild-type and mutant sequences (14) (http://mutpred.mutdb.org/). (9) Provean. Its prediction is based on an alignment-based score, which measures the change in sequence similarity of a query sequence to a protein sequence homolog before and after the introduction of an amino acid variation to the query sequence (15) (http://provean.jcvi.org/index.php) and (10) Sapred. It assesses the effect of missense variant through the structural neighbor profiles, nearby functional sites, aggregation properties and disordered regions (16) (http://sapred.cbi.pku.edu.cn/).

Results

Mutation screening and oligogenetic analysis

Overall, our mutation screening of ANOS1 revealed fifteen mutations in 8 probands out of 23 KS pedigree and 13 out of 187 sporadic IHH cases. Among 21 KS patients, four missense, eight nonsense, two splicing site and one insertion mutations were identified (Fig. 1), of which eight mutations (S38X, R257X, R262X, R423X, R424X, V560I, c.1843+1G>A and R631X) were reported previously. Three missense substitutions (c.257 G>T/p.C86F, c.269 G>A/p. C90Y, c.453 C>G/p.C151W), one splice-site mutation (c.1062+1G>A), two nonsense mutations (Y379X, R597X) and one insertion (c.1736 ins T, Y579L) were novel (i.e. absent from the queried databases and the 100 controls). Each mutation was detected only in a single pedigree or case, with the exception of c.1678 G>A/p.V560I (three sporadic patients), c.1270 C>T/p. R424X (one sporadic patient and one pedigree) and c.784 C>T/p.R262X (two sporadic patients). These mutations are widely distributed in nine different exons or at intron–exon boundaries; that is, we found no mutation ‘hot spot’ region in ANOS1 (Fig. 2A). All the mutations we
Clinical Study

M Nie and others Genetic and clinical characteristics of KS patients with ANOS1 mutation

Figure 1
The sequencing chromatogram of the mutation in ANOS1 gene. Dash indicates mutated nucleotide. Novel mutations were shown in red.

Figure 2
Distribution of mutations in ANOS1 gene and anosmin1 protein. (A) Blue box stands for the exons. (B) Recurrent mutations are in the upper of the box, novel mutations found in this study are in the below of the box and shown in red. (C) Distribution of missense or nonsense mutations in relation to the anosmin1 protein. Novel mutations were shown in red.
identified locate in different functional domains, except FnIII-3 (Fig. 2B).

ANOS1 mutations including C90Y, R257X, R262X, R424X, R597X, Y579Lfs591X and R631X were found in familial patients, and their mother was found to be a carrier. The detailed pedigree chart of the familial patients is shown in Fig. 3.

One patient was found to harbor not only two ANOS1 mutations (c.1062+1G>A and E552K) but also heterozygotic KISS1R L200V mutation. Two patients possessed both ANOS1 C86F and KISS1R Tyr323His (Het) mutations and both ANOS1 V560I and FGF8 E176K mutations respectively.

Bioinformatics analysis of novel missense variants

The cysteine residue at the position 86th, 90th and 151st of anosmin-1 protein is conserved across twenty-six different species (Fig. 4). Ten in silico analysis tools for missense variants classified C86F, C90Y and C151W as pathogenic variants (Table 1).

Patient characteristics

Data collected from the 21 patients who harbored ANOS1 gene mutations are summarized in Table 2. All these patients have low LH, FSH and testosterone. Gynecomastia and agenesis of right kidney occurred in one patient. Thirteen out of 21 patients’ medical histories included MRI test results and all of them (100.0%) had absence/hypoplasia of olfactory bulbs and olfactory tracts. The prevalence of cryptorchidism was high among ANOS1 mutation-harboring patients (8 out of 21, 38.1%) and was found in patients with nonsense mutations (S38X, R257X, R262X, R424X, R597X, R631X), frameshift mutations (Y579Lfs 591X) and missense mutations (C90Y, V560I). However, we also found that patients with the same mutation (V560I, R424X, R597X) do not uniformly present with cryptorchidism. Only one patient displayed agenesis of right kidney, and no patients showed synkinesis.

All patients accepted the treatment of testosterone or HCG/HMG. HCG/HMG therapy markedly increased the testicular sizes, except the patients with history

---

**Figure 3**

KS pedigree (1–6) with ANOS1 mutation are shown. Affected individuals with KS are shown as completely shaded. The arrow indicates the proband of each pedigree. All individuals marked numbers were screened for the mutation of ANOS1 gene.
of cryptorchid. Spermatogenesis was evaluated in ten patients and sperm appeared in seven patients (70%).

**Discussion**

In this study, we analyzed the ANOS1 gene variants in a large Chinese cohort of KS patients from a single medical center and found eight recurrent and seven novel mutations. The clinical characteristics of the KS patients with ANOS1 mutation (the prevalence of cryptorchidism and olfactory bulb/sulci) were also investigated.

The prevalence of ANOS1 mutations is very low in sporadic patients (13 out of 187, 6.95%); however, it is much higher in familial patients (6 out of 23, 26.1%). This result is similar with previous reports. In those studies, the incidence of the ANOS1 mutations was reported to be 3–8% in sporadic IHH (17, 18) and about 30% in familial IHH (19, 20). Of the fifteen mutations found in this study, four are missense mutations. Two naturally occurring mutations (C86F, C90Y) located in the Cys-box domain of anosmin-1 were identified for the first time. These novel missense mutants will provide us a valuable

**Table 1** Functional prediction of missense mutation found in KS patients.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sift</th>
<th>Polyphen</th>
<th>Panther</th>
<th>SNAP</th>
<th>Mutation assessor</th>
<th>Mutation tasting</th>
<th>SNPs and Go</th>
<th>MutPred</th>
<th>Provean</th>
<th>Sapred</th>
</tr>
</thead>
<tbody>
<tr>
<td>C86F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C90Y</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C151W</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+: pathogenic.
Table 2  Clinical and laboratory finding of the KS patients bearing ANOS1 mutations.

<table>
<thead>
<tr>
<th>No.</th>
<th>Family members with KS</th>
<th>Other clinical features</th>
<th>MRI$^a$</th>
<th>LH (IU/L) (basal)</th>
<th>FSH (IU/L) (basal)</th>
<th>TS (nmol/L)</th>
<th>Nucleotide change</th>
<th>Protein change</th>
<th>Age at the treatment initiation (years)</th>
<th>Treatment</th>
<th>Duration</th>
<th>Testis size (mL) (LR)→after treated</th>
<th>Sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0001</td>
<td>−</td>
<td>Cryptorchid (right), maldescent (left)</td>
<td>nt</td>
<td>0.1</td>
<td>1.1</td>
<td>1.01</td>
<td>c.113 C&gt;A</td>
<td>p.S38X</td>
<td>21</td>
<td>TS</td>
<td>2 years</td>
<td>1.0→nt</td>
<td>nt</td>
</tr>
<tr>
<td>S0002</td>
<td>−</td>
<td>−</td>
<td>2</td>
<td>1.3</td>
<td>2.8</td>
<td>1.18</td>
<td>c.1678 G&gt;A</td>
<td>p.V560I FGF8, E176K(Het)</td>
<td>31</td>
<td>HCG + HMG</td>
<td>6.5 years</td>
<td>5.5→12,12</td>
<td>2.63*10^6/mL</td>
</tr>
<tr>
<td>S0003</td>
<td>−</td>
<td>Cryptorchid (left)</td>
<td>nt</td>
<td>0.1</td>
<td>0.3</td>
<td>0.69</td>
<td>c.1678 G&gt;A</td>
<td>p.V560I</td>
<td>20</td>
<td>HCG + HMG</td>
<td>3 years</td>
<td>0.1→8,8</td>
<td>6.79*10^6/mL</td>
</tr>
<tr>
<td>S0004</td>
<td>−</td>
<td>−</td>
<td>nt</td>
<td>0</td>
<td>0.1</td>
<td>0.50</td>
<td>c.1678 G&gt;A</td>
<td>p.V560I</td>
<td>30</td>
<td>HCG + HMG</td>
<td>19 months</td>
<td>3,3→10,10</td>
<td>6→8/HP</td>
</tr>
<tr>
<td>S0005</td>
<td>−</td>
<td>−</td>
<td>nt</td>
<td>0</td>
<td>1.0</td>
<td>0.40</td>
<td>c.1267 C&gt;T</td>
<td>p.R423X</td>
<td>25</td>
<td>HCG + HMG</td>
<td>2 months</td>
<td>1.1→nt</td>
<td>nt</td>
</tr>
<tr>
<td>S0006</td>
<td>−</td>
<td>−</td>
<td>1</td>
<td>0.1</td>
<td>0.79</td>
<td>0.80</td>
<td>c.453 C&gt;G</td>
<td>p.C151W</td>
<td>20</td>
<td>HCG + HMG</td>
<td>3 years</td>
<td>1.1→4,4</td>
<td>nt</td>
</tr>
<tr>
<td>S0007</td>
<td>−</td>
<td>−</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.64</td>
<td>c.1062+1G&gt;A</td>
<td>p.E552K FGF8, L200V(Het)</td>
<td>20</td>
<td>TS</td>
<td>5 years</td>
<td>1.1→8,8</td>
<td>0→2/HP</td>
</tr>
<tr>
<td>S0008</td>
<td>−</td>
<td>−</td>
<td>3</td>
<td>0.3</td>
<td>1.3</td>
<td>0.90</td>
<td>c.1137C&gt;G</td>
<td>p.Y379X</td>
<td>20</td>
<td>TS</td>
<td>8 months</td>
<td>1.1→16,16</td>
<td>94.4*10^6/mL</td>
</tr>
<tr>
<td>S0009</td>
<td>−</td>
<td>−</td>
<td>1</td>
<td>2.2</td>
<td>2.5</td>
<td>3.31</td>
<td>c.1843-1G&gt;A</td>
<td>p.Y379X</td>
<td>21</td>
<td>TS</td>
<td>4 years</td>
<td>2.2→8,8</td>
<td>nt</td>
</tr>
<tr>
<td>S0010</td>
<td>−</td>
<td>−</td>
<td>1</td>
<td>0.0</td>
<td>0.9</td>
<td>0.86</td>
<td>c.784 C&gt;T</td>
<td>p.R262X</td>
<td>34</td>
<td>HCG + HMG</td>
<td>11 years</td>
<td>1.1→1,1</td>
<td>nt</td>
</tr>
<tr>
<td>S0011</td>
<td>−</td>
<td>Cryptorchid (both) + gynecomastia</td>
<td>nt</td>
<td>0.2</td>
<td>0.7</td>
<td>nt</td>
<td>c.784 G&gt;T</td>
<td>p.R262X</td>
<td>34</td>
<td>HCG + HMG</td>
<td>4 years</td>
<td>2,2→8,8</td>
<td>13.05*10^6/mL</td>
</tr>
<tr>
<td>S0012</td>
<td>−</td>
<td>−</td>
<td>1</td>
<td>0</td>
<td>0.2</td>
<td>1.10</td>
<td>c.1270 C&gt;T</td>
<td>p.R424X</td>
<td>19</td>
<td>TS</td>
<td>10 months</td>
<td>1.1→2,2</td>
<td>0</td>
</tr>
<tr>
<td>S0013</td>
<td>−</td>
<td>−</td>
<td>nt</td>
<td>0.1s</td>
<td>0.8</td>
<td>1.00</td>
<td>c.257 G&gt;T</td>
<td>p.C86F KISS1R, Y323H(Het)</td>
<td>20</td>
<td>TS</td>
<td>11 years</td>
<td>2,2→4,4</td>
<td>nt</td>
</tr>
<tr>
<td>F0001</td>
<td>Cousin, maternal uncle</td>
<td>Cryptorchid (both)</td>
<td>nt</td>
<td>0</td>
<td>0.2</td>
<td>0.56</td>
<td>c.269 G&gt;A</td>
<td>p.C90Y</td>
<td>19</td>
<td>TS</td>
<td>1.5 years</td>
<td>1.1→2,8</td>
<td>nt</td>
</tr>
<tr>
<td>F0002-10 Brother</td>
<td>Cryptorchid (both)</td>
<td>1</td>
<td>0</td>
<td>0.5</td>
<td>0.45</td>
<td>c.1789A&gt;T</td>
<td>p.R597X</td>
<td>19</td>
<td>TS</td>
<td>HCG + HMG</td>
<td>2.2→4,4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>F0003-3 Brother</td>
<td>Cryptorchid (both)</td>
<td>1</td>
<td>0</td>
<td>0.3</td>
<td>0.0</td>
<td>c.1789A&gt;T</td>
<td>p.R597X</td>
<td>18</td>
<td>TS</td>
<td>HCG + HMG</td>
<td>2.2→4,4</td>
<td>0.7*10^6/mL</td>
<td></td>
</tr>
<tr>
<td>F0003-1 Brother</td>
<td>−</td>
<td>nt</td>
<td>0.29</td>
<td>1.7</td>
<td>0.66</td>
<td>c.1270 C&gt;T</td>
<td>p.R424X</td>
<td>19</td>
<td>TS</td>
<td>HCG</td>
<td>1.5,1.5→2,2</td>
<td>nt</td>
<td></td>
</tr>
</tbody>
</table>

(Continued)
Clinical Study

M Nie and others

Genetic and clinical characteristics of KS patients with ANOS1 mutation

We have developed a molecular model to investigate the key functional sites of the anosmin-1 protein. Novel mutations including C86F, C90Y, C151W, c.1062 + 1G>T, Y379X, R597X, Y579L were found in this study. Although we did not perform functional studies of the effects of these mutations, evidence from bioinformatics analysis supports the hypothesis that these mutations are probably pathogenic. Four of the identified mutations (c.1062 + 1G>T, Y379X, R597X, Y579L) could disrupt the protein structure, result in loss of anosmin-1 function by changing splice sites, form a truncated protein or cause a frame-shift respectively. For the other three missense mutations we identified (C86F, C90Y, C151W): first, the cysteine residue is conserved across twenty-six different species. Second, all the prediction programs we employed classified these mutations as pathogenic variants. Third, two of these cysteine residues (C86 and C90) are located at the Cys-box domain and C151 is located at the WAP domain. The Cys-box contains a core of five disulphide bridges, which form between residues 49–83, 53–77, 86–105, 90–101 and 116–120. All ten cysteine residues that create the five core disulfide bonds are conserved (21). The WAP domain contains eight conserved cysteine residues that form four intramolecular disulphide bonds including 134–164, 147–168, 151–163 and 157–172. Similarly, the positions of these eight cysteine residues are conserved (22). Previous studies identified five mutations in patients with KS that affect cysteine residues that form disulphide bonds in the WAP domain of anosmin-1 (C134G, C163R, C163Y and C172R) (23, 24, 25), but no mutations that affect cysteine residues in the cysteine-rich N-terminal region of the protein had been reported. In this study, two mutations in the cysteine-rich region and one mutation in the WAP domain are described for the first time. If the cysteines at position 86, 90 or 151 were converted to other amino acids, it would disrupt the highly conserved Cys86–Cys105, Cys90–Cys101 and Cys151–Cys163 intramolecular disulphide bonds, and probably affect the folding or stability of this region.

The prevalence of cryptorchidism in this cohort with ANOS1 mutations is 38.1%. Many studies have explored the incidence of cryptorchidism in KS, with conflicting results. One study of a large cohort of 124 patients with the complete form of IHH reported that 15.3% had cryptorchidism (18), but in KS patients with ANOS1 mutations, 40% had a family history of cryptorchidism (26). Renal agenesis presented in 35–40% of X-linked KS cases and an even greater incidence of right renal agenesis occurred in KS patients with ANOS1 mutations (27, 28). Only one patient in this study presented a left kidney agenesis and the prevalence of right kidney agenesis in this cohort with ANOS1 mutations is 38.1%.

Table 2

<table>
<thead>
<tr>
<th>No.</th>
<th>Family members with KS</th>
<th>Other clinical features</th>
<th>LH (IU/L) (basal)</th>
<th>FSH (IU/L) (basal)</th>
<th>TS (nmol/L)</th>
<th>Nucleotide change</th>
<th>Protein change</th>
<th>Age at the treatment initiation (years)</th>
<th>Treatment</th>
<th>Duration</th>
<th>Testis size (mL) (L,R) → after treated</th>
<th>Sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>F0004</td>
<td>Maternal uncle, cousin</td>
<td>Cryptorchid (right), agenesis of right kidney</td>
<td>1</td>
<td>0</td>
<td>0.74</td>
<td>c.769C&gt;T</td>
<td>p.R257X</td>
<td>20</td>
<td>TS</td>
<td>2 years</td>
<td>9 months, 3 years</td>
<td>2,2→8.8</td>
</tr>
<tr>
<td>F0005</td>
<td>Maternal uncle</td>
<td></td>
<td>1</td>
<td>0</td>
<td>0.34</td>
<td>c.1736 ins T</td>
<td>Y579Lfs591X</td>
<td>20</td>
<td>TS</td>
<td>5 years</td>
<td>5 months, 46 months</td>
<td>1,1→2,5,2,5</td>
</tr>
<tr>
<td>F0006</td>
<td>Maternal uncle, cousin</td>
<td>Cryptorchid (both)</td>
<td>1</td>
<td>0</td>
<td>1.35</td>
<td>c.1891 C&gt;T</td>
<td>p.R631X</td>
<td>18</td>
<td>TS</td>
<td>4 years</td>
<td>5 months, 4 years</td>
<td>1,1→nt</td>
</tr>
</tbody>
</table>

*1: absence of olfactory bulbs and olfactory tracts; 2: absence of olfactory bulbs and hypoplasia of olfactory tracts; 3: hypoplasia of olfactory bulbs and absence of olfactory tracts. –, negative; GnRH, gonadotropin-releasing hormone; HCG, human chorionic gonadotropin; HMG, human menopausal gonadotropin; nt, not test; TS, testosterone.
in our cohort showed right renal agenesis; therefore, the frequency was lower than previously described (28), possibly due to the different ethnic background of the patients. In two other studies from China (20, 26) and one study from Korea (29), no patients were reported to have renal agenesis. Absence or hypoplasia of the olfactory bulb/tract was found in 100% of patients in this cohort, which is similar to previous studies (84.62%) (29). This result showed that there is a strong correlation between sense of smell and MRI images of olfactory bulb/sulci.

There are not many patients (70%) who have been detected with sperm in this study, which is similar with the results of a meta-analysis about 48 studies of HCG/HMG therapy and 16 studies of pulsatile GnRH therapy for HH patients (30). In that study, the rate of successful spermatogenesis in HH patients treated with HCG/HMG was 68% (95% CI: 58–77%). The reason why spermatogenesis failed may be related with following factors, such as cryptorchidism (31, 32) and the short duration of HCG+HMG treatment (33).

In conclusion, the prevalence of ANOS1 gene mutations is low in sporadic KS patients, but is much higher in familial KS patients. Seven novel ANOS1 mutations that are probably pathogenic were identified in the present study. Two mutations located in the CR domain of anosmin-1 protein were found for the first time. These mutations expand the ANOS1 mutation spectrum and provide information that could be useful for prenatal diagnosis of KS and genetic counseling. Cryptorchidism and the absence or hypoplasia of the olfactory bulb/tract is very high in KS patients with ANOS1 mutations.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

Funding

This work was supported by National Key Research and Development Program of China (2016YFC0905100), CAMS Innovation Fund for Medical Sciences (2016-I2M-1-002) and the National Key Program of Clinical Science (WBZ2011-873).

Author contribution statement

X W and H Z: conceived and designed the experiments; X W, H X, J M, X W, S X, J Z, W M and Q H: collected blood samples and clinical data; M N, R C, B Y and M C: performed the experiments; M N: wrote the paper.

Acknowledgement

The authors thank the subjects and their family members for their participation in the research.

References

4. Topaloglu AK & Kotan LD. Genetics of hypogonadotropic hypogonadism. Endocrine Development 2016 29 36–49. (doi:10.1159/000438841)
M Nie and others

Genetic and clinical characteristics of KS patients with ANOS1 mutation

Received 26 April 2017
Revised version received 20 July 2017
Accepted 31 July 2017


