Clinical and genetic characterization of 6 cases with complete androgen insensitivity syndrome in China

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Running title: 6 cases of Chinese CAIS

Introduction

The mutations of androgen receptor (AR) gene are the most common cause for complete androgen insensitivity syndrome (CAIS). In the present study, 6 Chinese
patients were admitted in our hospital for the agenesis of secondary sexual characteristics between 2010 and 2013. We characterized the AR gene of 6 suspected CAIS cases, and found that the 6 cases with 46, XY karyotype were diagnosed with complete CAIS. In addition, 4 novel AR mutations were discovered, which were responsible for Chinese CAIS. We expected that the molecular study of the AR gene could facilitate the understanding of the mechanism of CAIS and provided the genetic counseling clinically.

Androgen insensitivity syndrome (AIS) is an X-linked recessive genetic disease, which is characterized by partial or complete cell resistance to androgens (Galani et al., 2008). Patients with AIS are present with male 46, XY karyotypes and exhibit different phenotypes ranging from a normal male habitus with the defect of male secondary sex characters (partial AIS) to a complete AIS (CAIS) (Ferlin et al., 2006; Zuccarello et al., 2008).

The AR gene mutation is the most common cause of AIS and currently, more than 400 mutations of AR gene have been reported, such as point mutations, nucleotide insertions, deletions, and complete or partial gene deletion (Gottlieb et al., 2012). It is reported that the AR gene mutation of Arg840Cys substitution is found in a Chinese family with AIS, which may be associated with infertility of male with hypospadias (Chu et al., 2002). In another report, two missense mutations of AR gene (such as GAC732ACC and GCC765ACC) have been firstly detected in two Chinese cases with CAIS (Ko et al., 1997). However, the AR mutations in Chinese CAIS patients have not been fully characterized.
In this paper, we reported 6 Chinese CAIS patients with different AR mutations and expected that our findings could be adapted for genetic counseling in clinic.

**Methods**

**Patients**

This study was approved by the Ethics Committee of the First People's Hospital of Yunnan Province and the study procedures were performed in accordance with the ethical standards. The patients or their parents provided the informed consents before this clinical research.

Six of eight patients aging from 12 to 22 years old were diagnosed with CAIS in our hospital between 2010 and 2013. All the patients were raised as girls and there were no family histories for these cases. Blood samples were collected for karyotype analysis and Y chromosome SRY gene detection. The physical examination and the basic information of the six patients were listed in Table 1.

**PCR amplification of AR gene**

The primers of AR gene were designed by Primer 5 and synthesized by Shanghai Invitrogen Corporation (Table 2). PCR reaction system (50 μl), contained genomic DNA 30 ng, deoxyonucleoside triphosphates (dNTP) 200 μmol/L, 10 × PCR buffer 5 μl, MgCl2 15 mmol/L and thermostable DNA polymerase (Taq enzyme) 1U. PCR was performed as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, annealing (according to Table 1) for 30 s, 72°C for 1 min, and a final elongation step at 94°C for 10 min. The PCR amplification reagents were purchased from the treasure Biotechnology (Dalian) Co., Ltd.
After amplification, the PCR products were evaluated by using 1% agarose gel electrophoresis and sequenced by Beijing Genomics Institute (Beijing, China).

**Histopathologic examination**

The gonadal tissues were isolated under surgery for pathological examination. The tissues were fixed in 10% formalin and embedded by paraffin. The serial sections (3 μm thickness) were cut and stained with hematoxylin eosin.

**Results**

A peripheral chromosome analysis showed a 46, XY karyotype for all the 6 patients, which was determined by SRY gene amplification. The PCR amplification and sequencing of AR gene exons showed that case 1 had a single nucleotide T-to-C transition in exon 7, which resulted in an amino acid substitution from arginine to cysteine at 856 site (Figure 1 A). In case 2, 1666C nucleotide deletion was detected in the exon 2 of AR gene, leading to frame shift mutation and the terminate of 560th amino acid (Figure 1 B). In addition, there was a missense mutation of C2076A in exon 4 of case 3, resulting in 692th amino acid residue substitution from asparaginate to lysine (N692K) (Figure 1 C). In case 4, the nonsense mutation of C1477T was detected in exon 1 of AR gene, which eventually led to the termination of 492th amino acid (Figure 1 D). In case 5, a frame shift mutation of 2301delT was detected, which resulted in the termination of the 787th amino acid (Figure 1 E). In case 6, 2068-2071AGG nucleotide deletion was detected in exon 4 (Figure 1 F). Although this mutation was in the 2 codons, the variation led to the loss of 691th amino acid (aspartic acid).
Pathological examination

Four of 6 patients (case 3, 4, 5 and 6) received gonad removal and vulva anaplasty in our hospital. The gonadal tissues were subjected to pathological examination. As shown in Figure 2, the gonadal tissues were confirmed to be the dysgenetic testicular tissues. After the operation, the four patients maintained female sexual characteristics by the hormone replacement.

Discussion

AIS, also served as "testicular feminization syndrome" is referred to the patients with inability to response to circulating androgens (Morris, 1953). Androgen plays a regulatory role in the differentiation of male sex and the reproductive function maintenance by binding to AR. The most common cause of AIS is the mutations of AR gene.

The mutations in case 1, 3 and 5 were detected in the LBD region. According to the AR Gene Mutations Database, the most mutations of AR were located in LBD. LBD plays a key role in the interactions of AR protein with androgen hormone. Androgen mediates the activation of target genes and induced the AR migration to the cell nucleus by binding LBD (Sack et al., 2001). Loss or mutation of the LBD region leads to inactivation of the receptor and the dysfunction of AR. In our paper, the mutation in case 2 was located in the DBD region. DBD is a relatively small and conserved protein domain, which is composed of two zinc finger structures. Mutations located in the DBD region can cause changes in the zinc finger structure and result in CAIS (Hughes et al., 1986; Quigley et al., 1992). In case 4, the
mutation was detected in NTD that was encoded by exon 1. The percentage of mutations in exon 1 for AIS is only 25%, while it is 70% for CAIS (Eisermann et al., 2013). AFl (function activation 1) is an important active region in NTD, which is involved in the interaction of N-/C-terminal regions. The mutation in case 4 is located in AFl. The mutation in case 6 was detected in the HR region which was associated with phosphorylation, acetylation, and degradation of AR protein (Eisermann et al., 2013). In the present study, there were different mutations of AR gene for the 6 cases, which induced the variation in the structure of AR and inhibited the interaction of AR protein and androgenic hormone. Among the 6 cases, the mutations in case 1 (C2566T) (Ahmed et al., 2000; Melo et al., 2003; Ledig et al., 2005) and case 5 (2301delT) (Hannema et al., 2004; Cheikhelard et al., 2008; Hoebek et al., 2011) have been reported in previous studies. To our knowledge, the mutations of AR gene in the remaining 4 cases have been firstly reported in Chinese CAIS patients and fully characterized by DNA sequencing. Furthermore, ectopic testis increases the risk for tumor by the long-term effect of the high body temperature in the abdominal cavity (Taplin et al., 1995). The incidence of testicular cancer is low before puberty, while it increases to 8% after puberty. In addition, the testosterone secretion shows adverse effects on the height growth and female secondary sexual syndrome development. Thus, orchiectomy surgery is recommended for patients with AIS post puberty. In our study, case 6 (12 years old) underwent gonadal resection and genital plastic surgery after genetic counseling. The appropriate treatment were expected to adjust the state of mind and reduce the risk of psychological problems of patients.
Besides, the LH levels were higher than normal level in all cases except for the case 6. The level of testosterone was in the normal level for case 1, 2, 4 and slightly increased in case 5. In addition, the level of testosterone was lower in case 3 and 6, compared with normal level. The age of case 3 and 6 was 17 and 12 respectively, which was younger than the others in the present study. Thus, we speculated that the hormonal level of CAIS patients might be related with the age.

Although the novel mutations of AR in the 4 cases have been discovered in Chinese patients, lack of functional analysis is a limitation in our study. Besides, the mutation frequency in an unaffected male population has not been investigated in our work. Further studies focusing on the causal mutations are warranted in the near future.

In conclusion, the CAIS was diagnosed based on the clinical examination and other molecular studies and 4 novel AR mutations were discovered in Chinese CAIS. Our findings of AR mutations pave the way to verify the mechanism of CAIS at molecular level and enriched the data for AR Gene Mutations Database.

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Competing Interests

The authors declare that they have no competing interests.
References


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Table 1  The clinical information of 6 cases with CAIS

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (year)</th>
<th>Height (cm)</th>
<th>Clinical features</th>
<th>Sex hormone</th>
<th>B-ultrasound</th>
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<tr>
<td>1</td>
<td>19</td>
<td>162</td>
<td>normal female external genitalia, normal breast development, absence of pubic hair and blind-ending vagina</td>
<td>hFSH: 8.5 (1.5-11.5 mU/mL); LH: 22.24 (1.1-8.2 mU/mL); Prol: 11.15 (2.7-17 ng/mL); E: 21.58 (0-60 pg/mL); Testo: 6.41 (2.5-10.51 ng/mL)</td>
<td>uterus and bilateral ovaries</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>160</td>
<td>normal female external genitalia, normal breast development, absence of pubic hair and blind-ending vagina</td>
<td>hFSH: 22.47 (1.5-12.4 mU/mL); LH: 32.89 (1.7-8.6 mU/mL); Prol: 20.4 (4.1-10.4 ng/mL); E: 18.4 (28-156 pmol/L); Testo: 11.2 (6.68-25.7 nmol/L)</td>
<td>primordial uterus and left side of ovary</td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>161</td>
<td>normal breast development, male breast development, normal female external genitalia</td>
<td>hFSH: 46.9 (0.95-11.95 mU/mL); LH: 19.13 (1.14-8.75 mU/mL); Prol: 40.53 (3.69-19.4 ng/mL); E: 10 (11-44 pg/mL); Testo: 0.6 (1.66-8.77 ng/mL)</td>
<td>infantile uterus without bilateral ovaries</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>163</td>
<td>normal female external genitalia, poor breast development, sparse pubic hair</td>
<td>hFSH: 19.44 (1.27-19.26 mU/mL); LH: 65.2 (6.24-8.64 mU/mL); Prol: 13.94 (2.64-13.13 ng/mL); E: 39 (20-70 pg/mL); Testo: 2.58 (1.75-7.81 ng/mL)</td>
<td>without obvious uterus, ovary or testis</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>165</td>
<td>normal female external genitalia, poor breast development</td>
<td>hFSH: 3.68 (1.27-19.26 mU/mL); LH: 36.4 (1.24-8.64 mU/mL); Prol: 25.48 (2.64-13.13 ng/mL); E: 66 (20-70 pg/mL); Testo: 8.08 (1.75-7.81 ng/mL)</td>
<td>without obvious uterus, ovary or testis</td>
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<tr>
<td>6</td>
<td>12</td>
<td>160</td>
<td>normal female external genitalia, blind-ending vagina</td>
<td>hFSH: 3.72 (0.95-11.95 mU/mL); LH: 0.64 (1.14-8.75 mU/mL); Prol: 15.41 (3.69-19.4 ng/mL); E: 10 (11-44 pg/mL); Testo: 0.47 (1.66-8.77 ng/mL)</td>
<td>bilateral inguinal hernia</td>
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</table>

hFSH: human pituitary follicle stimulating hormone, LH: luteinizing hormone Prol: proligestone; E: estradiol, Testo: testosterone.
<table>
<thead>
<tr>
<th>No.</th>
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<th>Sequence</th>
<th>Fragment Length (bp)</th>
<th>T_m (°C)</th>
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<td>F GAACTCTTCTGAGCAAGAGAAGG</td>
<td>683</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>R CTTCGGGATACTGCTTCCTGCTG</td>
<td></td>
<td></td>
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<tr>
<td>2</td>
<td>1</td>
<td>F CTTAAGGCTGCTCCGCTG</td>
<td>462</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R CAGCCTAGGCTCTCGCCTTC</td>
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<tr>
<td>3</td>
<td>1</td>
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<td></td>
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<td>R TAGGAGCCGCTAGATACCCCA</td>
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<tr>
<td>4</td>
<td>2</td>
<td>F TCACACCCTACAAGCAGCTC</td>
<td>532</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R CCTGTACAAAACAGGCTGCTG</td>
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<td></td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>F CAAATTGTGTGGGCGCAACT</td>
<td>402</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R TATGAAAGCGCTAGCGCTGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>F GAGTCTGTGACCGGGAGAATG</td>
<td>533</td>
<td>60</td>
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<td>R CAGGCCATGTGAGAAGACTGCT</td>
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<tr>
<td>7</td>
<td>5</td>
<td>F CTCCATCATCATCTCATTGAG</td>
<td>532</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R AGCCGCCTCATACTCGATTG</td>
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<tr>
<td>8</td>
<td>6</td>
<td>F CTGGAGCCACCAGCGAGA</td>
<td>368</td>
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<tr>
<td></td>
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<td>R ATGTCCAGGAGCTGGCTTTTC</td>
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<tr>
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<td>7</td>
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<tr>
<td></td>
<td></td>
<td>R GACCACACTCAAAGCCAGAG</td>
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**Figure 1** Sequencing diagram of the exons of the AR gene of different patients
A, B, C, D, E and F represent the sequencing of AR gene mutations for the cases 1 to 6, respectively. The mutation site is indicated by arrow.

**Figure 2** Pathological examination of the gonadal tissues
A: images for case 3. The tissues are tested to be testicular tissue with less spermatogenic cells and mesenchyme proliferation.
B: images for case 4. The tissues are considered to be the poorly differentiated epididymis tissues.
C: images for case 5. The tissues are stunted testicular tissue with no clear malignant lesions. A small number of spermatogenic cells and fibrous tissue proliferation are observed with no mature sperm.
D: images for case 6. There is no obvious special lesions. Small spermaduct, primordial germ cell and a small number of mesenchymal cells were observed.