Investigation of mutations in the SRY, SOX9, and DAX1 genes in sex reversal patients from the Sichuan region of China

L. Chen, X.P. Ding, X. Wei and L.X. Li

Bio-resource Research and Utilization Joint Key Laboratory of Sichuan-Chongqing, Institute of Medical Genetics, College of Life Science, Sichuan University, Chengdu, China

Correspondence author: X. P. Ding
E-mail: Brainding@scu.edu.cn

Received December 10, 2012
Accepted June 28, 2013
Published March 12, 2014
DOI http://dx.doi.org/10.4238/2014.March.12.4

ABSTRACT. We investigated the molecular genetic mechanism of sex reversal by exploring the relationship between mutations in the sex-determining genes SRY, SOX9, and DAX1 with genetic sex reversal disease. Mutations in the three key genes were detected by polymerase chain reaction (PCR) and sequencing after karyotype analysis. The mutations detected were then aligned with a random sample of 100 normal sequences and the NCBI sequence database in order to confirm any new mutations. Furthermore, the copy number of SOX9 was measured by fluorescence quantitative PCR. Seven of the 10 male sex reversal patients (46, XX) contained an excess copy of the SRY gene, while one of the eight female sex reversal patients (46, XY) was lacking the SRY gene. Additionally, a new mutation (T-A, Asp24Lys) was detected in one female sex reversal patient (46, XY). No other mutation was detected in the analysis of SOX9 and DAX1, with the exception of an insertion mutation (c.35377791insG) found in the testicular-specific enhancer (TESCO) sequences in an SRY-positive
female sex reversal patient (46, XY). Eight of the 18 sex reversal cases (44.4%) showed obvious connections with SRY gene translocations, mutations, or deletions, which was significantly higher than that reported previously (33.3%), indicating a need to further expand the range of sample collection. Overall, these results indicated that the main mechanism of sex reversal are not associated with mutations in the coding regions of SOX9 and DAX1 or copy number variations of SOX9, which is consistent with results of previous studies.

**Key words:** Sex reversal; SRY; SOX9; DAX1; Gene mutation; Real-time fluorescence quantitative PCR

**INTRODUCTION**

Approximately 1.7% of newborns present varying degrees of disorders of sex development (DSD) on a global scale (Blackless et al., 2000; Hughes et al., 2006). DSDs are most often manifested when an individual’s sex chromosomes or gonads do not match their external gender, or when the anatomical gender is blurred to different degrees with respect to reproductive organs, such as two sets of reproductive organs. Complete reversal syndrome (sex reversal syndrome, SRS) can be divided into two categories: 46, XX male sex reversal and 46, XY female sex reversal, with probabilities of 1 in 20,000 to 1 in 25,000 and 1 in 10,000, respectively (Jiang et al., 1993; Rajender et al., 2006). Therefore, it is particularly important to study the potential genetic mechanisms contributing to human sexual development disorders.

Research into the molecular biological mechanism of DSDs can result in more accurate diagnoses of the disease, and help to determine more extensive and specific strategies for psychological, endocrinological, surgical, and other clinical treatments. Previous studies have revealed that mutations, deletions, or ectopic changes in several sex-determining genes, SRY, SOX9, DAX1, SF1, and WNT4, can result in sex reversal. Changes in gene copy numbers of SOX9, DAX1, and WNT4, followed by changes in the level of gene expression, have also been shown to cause sex reversal (Bardoni et al., 1994; Kwok et al., 1995; Jordan et al., 2001). In the present study, we studied three key sex-determining genes, SRY, SOX9, and DAX1, in 18 patients with sex reversal in Sichuan Province, China.

**MATERIAL AND METHODS**

**Study subjects**

The study subjects were 18 patients who were being primarily treated for infertility after marriage for several years at the Institute of Genetic Medicine of the College of Life Sciences, Sichuan University from January 2010 to October 2012. This study consisted of 10 cases of 46, XX male sex reversal (group A) and 8 cases of 46, XY female sex reversal (group B). Group A presented with the following characteristics: small testes, azoospermia, small penis, Adam’s apple, short stature, thyroid hormone levels of 0.56-0.90 ng/mL (mean value: 0.76 ng/mL), follicle-stimulating hormone (FSH) levels of
21.16–29.45 mIU/mL (mean value: 26.64 mIU/mL), and luteinizing hormone (LH) levels of 8.86–112.20 mIU/mL (mean value: 9.94 mIU/mL). Group B presented with the following characteristics: primary amenorrhea, immature vulva, immature ovary, immature uterus, clitoral enlargement, FSH levels of 58.98–117.9 mIU/mL (mean value: 89.23 mIU/mL), LH levels of 12.37–40.50 mIU/mL (mean value: 26.32 mIU/mL), and estradiol levels of 18.86–106.54 pM (mean: 67.54 pM). In addition, 100 samples of normal control peripheral blood were obtained from married, childbearing volunteers. All patients received the right to informed consent.

Cytogenetic analysis

Metaphase chromosome karyotype split phase was prepared after culturing the patients’ peripheral blood under sterile conditions, and were analyzed using the conventional G-banding technique. The karyotype analysis was based on the International System for Human Cytogenetic Nomenclature (ISCN); at least 20 karyotypes were observed in each specimen.

DNA sequence analysis

Five milliliters blood was extracted from patients and normal controls, placed in a vacuum ACD anticoagulant tube, and stored at -20°C. Whole blood genomic DNA was extracted with a DNA extraction kit (Anhui U-gene Biotechnology Co., Ltd), and the genomic DNA concentration was determined by the optical density method. Sequences of the entire coding regions of \( SRY \), \( DAX1 \), \( SOX9 \), and the upstream testis-specific enhancer element sequence of \( SOX9 \) (TESCO) were amplified with primers shown in Table 1 (Shanghai Invitrogen Biotechnology Co., Ltd); the housekeeping GAPDH gene was used as the internal reference. The PCR system was 25 µL in total, containing 1 U Taq DNA polymerase (Takara), 100 ng template DNA, 0.2 mM each upstream and downstream primer, 0.2 mM dNTPs, and 2.5 µL buffer. The PCR product was processed by 30 min of 2% agarose gel electrophoresis at a voltage of 100 V, and the amplification product was sent to the Shanghai Invitrogen Biotechnology Co., Ltd. for sequencing.

<table>
<thead>
<tr>
<th>gene</th>
<th>Primers sequences (5'→3')</th>
<th>PCR conditions</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( SRY )</td>
<td>GGTAGGGCGCGAGAAATG</td>
<td>95°C-4 min (95°C-30 s, 51°C-30 s, 72°C-30 s) x 35 cycles, 72°C -10 min</td>
<td>559</td>
</tr>
<tr>
<td>( SRY )</td>
<td>TTCTTGGCAGACATCTTC</td>
<td></td>
<td>497</td>
</tr>
<tr>
<td>( SOX9 )</td>
<td>CGGTTGCTCTCTAAAGGTG</td>
<td>95°C-4 min (95°C-30 s, 57°C-30 s, 72°C-30 s) x 35 cycles, 72°C-10 min</td>
<td>823</td>
</tr>
<tr>
<td>( SOX9 )</td>
<td>TTGTCAAGTGCGGTA</td>
<td></td>
<td>416</td>
</tr>
<tr>
<td>( SOX9 )</td>
<td>AGAGGGAGCGGAGGTG</td>
<td>95°C-4 min (95°C-30 s, 52°C-30 s, 72°C-30 s) x 35 cycles, 72°C-10 min</td>
<td>698</td>
</tr>
<tr>
<td>( SOX9 )</td>
<td>AGGGCGGAGCGAGTAG</td>
<td></td>
<td>967</td>
</tr>
<tr>
<td>( SOX9 )</td>
<td>ACCGACCCACAGACTTC</td>
<td>95°C-4 min (95°C-30 s, 52°C-30 s, 72°C-30 s) x 35 cycles, 72°C-10 min</td>
<td>698</td>
</tr>
</tbody>
</table>

**Table 1.** Primers sequences of the \( SRY \), \( SOX9 \), TESCO, \( DAX1 \) genes and PCR.
SOX9 copy number changes were detected with quantitative PCR on a SYBR Green dye I SLAN®-96P Automatic Medical PCR analysis platform. The third exon of the SOX9 sequence was selected for amplification, the primer sequences were: F 5′-TGGTCTTTAACACCGGTACCT-3′ and R 5′-TATTCCGGCATTTAATCAGAGAAAGTG-3′ (Bardoni et al., 1994), with a product length of 102 bp. The housekeeping GAPDH gene was selected as the internal reference; primer sequences: F 5′-ACCGATTTGGTCTGAT TGGGC-3′ and R 5′-CTCGCTCCTGGAAGATGGTGAT-3′, with a product length of 216 bp. Samples obtained from married, childbearing, normal men and women were used as the positive control. The PCR system was 20 μL, containing 10 μL 2X All-in-One qPCR Mix (Guangzhou Fulengen Co., Ltd), 100 ng DNA template, and 0.1 μM primers. The reaction conditions were as follows: 95°C denaturation for 10 min, 95°C denaturation for 20 s, annealing at 61°C for 20 s, 72°C extension for 20 s, 40 cycles, with a final extension at 72°C for 10 min. The optimum concentration of the primer was determined by the concentration gradient curve and the melting curve of the PCR in order to avoid experimental error due to primer dimerization and non-specific amplification. Each reaction was repeated three times to obtain average values.

RESULTS

SRY

Seven of the 10 male sex reversal patients (46, XX) carried an excess of SRY; only one of the eight female sex reversal patients (46, XY) was lacking SRY, while the rest were positive for this gene (Figure 1). Results of bidirectional sequencing and NCBI sequence alignment of SRY showed that T was replaced with an A at nucleotide 149 of the SRY gene [upstream of the high mobility group (HMG) box] in one female sex reversal patient (46, XY), as shown in Figure 2. This resulted in a mutation of aspartic acid 24 of the coding region to lysine (Asp24Lys), and clinical data are shown in Table 2. This mutation was not found in any of the 100 normal control samples, and has not been reported to date. Therefore, this result likely reflects a novel mutation.

Figure 1. Gel electrophoresis of SRY gene in 18 case sex reversal patients. Band 216bp is inner reference gene (housekeeping gene, GAPDH), band 559 and 497bp are SRY gene. Lane 1 = marker; lane 2, 3 = control, normal male, female; lane 4, 6, 8-10, 18-20 = 46, XY female sex reversal patients; lane 5, 7, 11-17, 21 = 46 XX male sex reversal patients.
**SOX9 and DAX1**

No mutations were found in the coding regions of *SOX9* or *DAX1* among the 18 sex reversal patients. The upstream TESCO sequence of *SOX9* was amplified by PCR and the product was directly sequenced. One insertion mutation (C. 35377791insG) was found in one *SRY*-positive sex reversal patient (46, XY), as shown in Figure 2; clinical data is shown in Table 2. This mutation was not found in any of the 100 normal control samples and has not been previously reported. Therefore, this mutation is also likely to be novel.

![Sequence result of SRY, TESCO gene.](image)

**Figure 2.** Sequence result of *SRY*, *TESCO* gene. **a.** Mutation in 46, XY female sex reversal patients (T→A, Asp24Lys); **b.** *SRY* gene sequence of normal control; **c.** mutation in *TESCO* region (35377791insG) in 46, XY female sex reversal patients with SRY gene positive; **d.** *TESCO* region sequence of normal control.

<table>
<thead>
<tr>
<th>Case</th>
<th>Social gender</th>
<th>Age</th>
<th>Chromosome type</th>
<th>Clinical symptom</th>
<th>Hormone detection</th>
<th>SRY</th>
<th>TESCO gene detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female</td>
<td>29</td>
<td>46, XY</td>
<td>Female appearance, primary amenorrhea, vaginal stenosis, infantile vulva, immature ovary and uterus</td>
<td>E2: &lt;8.0 pg/mL; FSH: 99.04 mIU/mL; LH: 47.71 mIU/mL</td>
<td>CD149T→A</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>33</td>
<td>46, XY</td>
<td>Female appearance, primary amenorrhea, clitoral enlargement; B ultrasound, no uterus, ovaries do not appear</td>
<td>E2: 11.8 pg/mL; FSH: 165.08 mIU/mL; Testosterone: 47.71 mIU/mL</td>
<td>ND</td>
<td>c.35377791insG</td>
</tr>
</tbody>
</table>

ND = no detected.

The sequencing detection results described above cannot clearly explain the cause of the 18 cases of sex reversal, as we speculate that the occurrence of sex reversal is instead determined by the expression level of the SOX9 protein, which likely changes depending on the change in the SOX9 copy number. We used the quantitative PCR technique to verify this hypothesis and detected SOX9 copy number changes in three *SRY*-negative 46, XX male sex reversal patients, and in seven *SRY*-positive 46, XY female sex reversal patients. No significant differences with the normal control group were found based on calculations using the 2^{-ΔΔCT} method. The PCR results are shown in Figure 3.
DISCUSSION

Several 46, XX individuals were found to carry an extra SRY gene, whereas 46, XY individuals were found to have mutations or deletions of this gene. These results confirmed that SRY is the best candidate sex determination gene (Berta et al., 1990; Abusheikha et al., 2001; Hersmus et al., 2009). However, results of previous studies have shown that mutations or deletions of the SRY gene accounts for only 1/3 of sex reversal cases, implying that 2/3 of the cause of sex reversal remains unclear (Singh and Vutukuri, 2006). Subsequently, genomics, gene structure, and functional analyses revealed that sex reversal was associated with mutations in some other key genes, such as SOX9, DAX1, SF1, and WNT4 (Kwok et al., 1995; Achermann et al., 1999; Hanley et al., 2000). Increases in the copy numbers of SOX9 and DAX1 result in high expression of their corresponding proteins, which in turn leads to sex reversal (Bardoni et al., 1994; Kwok et al., 1995; Jordan et al., 2001). Although the precise mechanism of the expression of these genes is currently unknown, one hypothesis states that in the XY embryo, SRY acts as the testicular differentiation switch, which drives the genital ridge to develop directly into the testis (Sekido and Lovell-Badge, 2008; Rey and Grinspon, 2011).

SRY and SF1 protein complexes bind to a 3.2 kb testis-specific enhancer upstream of SOX9 (TESCO), causing high expression of the SOX9 protein, which in turn stimulus anti-Müllerian hormone expression, and therefore inhibition of the development of the Müllerian ducts. Under the influence of high concentrations of SRY and SOX9 proteins, the genital ridge supporting cells differentiate into Sertoli cells by a self-regulating mechanism. Compared with knowledge of the genes involved in testicular development, ovarian developmental genes remain poorly understood. Regulatory roles in ovarian development have been demonstrated in only three candidate genes (WNT4, DAX1, and FOXL2). Of these genes, only DAX1 was found to be located on the X chromosome, and was once considered as an ovarian determining gene. However, although genetically modified (Dax1) male mice showed a reversal of gender when DAX1 expression was elevated, Dax1 mutant females (Dax1-/-) with reduced DAX1 expression levels showed normal sexual maturation, fertility, and ovulation (Swain et al., 1998). These results indicated that DAX1 is not an ovarian determining gene after all, and it is now generally considered to be an “anti-testis” gene.

The human SRY gene, which is located on the Y chromosome with a full-length of 897 bp, was the first gene to be recognized as a testis-determining factor gene. It contains only

Figure 3. Concentration gradient curve and melting curve of the Fluorescence quantitative PCR. a. PCR amplify curve, the left curve represents SOX9 gene, while the right one represents internal reference GAPDH gene. b. Melting curve of the Fluorescence quantitative PCR, the left curve represents SOX9 gene, while the right one represents internal reference GAPDH gene.
a single exon encoding 204 amino acids, which is a highly conserved sequence belonging to
the HMG box motif group of the high-speed mobility class of proteins. Numerous studies have
shown that translocations, deletions, and mutations of the SRY gene can cause sex reversal. In
the present study, eight of the 18 (44.4%) cases of sex reversal were found to be associated with
the SRY gene, which represents a slightly higher proportion than the 1/3 that has previously been
reported (Singh and Vutukuri, 2006). This result may be related to the following facts. First, dif-
ferences between study subjects; subjects in the present study were all adults as they were seek-
ing treatment for infertility, therefore pediatric data is lacking. Second, results might be biased
due to the limited number of specimens. Third, an increased amount of environmental radiation
materials may be affecting the human genome. Mutations in SRY were previously detected in
10-15% of female sex reversal patients (46, XY), including point mutations and deletions, which
were found mostly in the HMG sequence of the SRY gene (Salehi et al., 2006).

A new mutation was discovered in the present study in one female sex reversal patient
(46, XY). The site of the mutation lies in the HMG box upstream of the SRY gene, in which T was
converted to an A at nucleotide 149, causing mutations in the coding region so that aspartic acid
24 was changed to lysine (Asp24Lys). In this patient, no testes or other male genital organs were
found, female genitalia showed significant dysplasia, primary amenorrhea and vaginal stenosis
were observed, and the ovary and uterus were immature. Since there was no evidence of abnor-
malities in SOX9 and DAX1, it was suspected that the occurrence of these symptoms was related
to the mutation, pending further experimental verification of functional analysis of the mutant.

The SOX9 gene, which maps to chromosome 17 (17q24.3-25.1), is an important mem-
ber of the SOX protein family. Because the SOX9 gene is expressed in multiple tissues and since
it determines differentiation of the reproductive organs, along with a series of other genes in the
genital ridge, it is considered to be an autosomal sex-determining gene. Previous studies have
revealed that 2/3 of patients with varying degrees of female sex reversal (46, XY) were associ-
ated with cases of short limb dysplasia, and increases in the SOX9 gene copy number can also
lead to male sex reversal (46, XX) (Huang et al., 1999; Georg et al., 2010). In transgenic mice
with three SRY and SF1 binding sites containing sub TESCO testis-specific enhancements, only
full locus mutations resulted in the loss of enhancer activity (Sekido and Lovell-Badge, 2008).

In the present study, no mutation was found in the coding regions of the 18 sex reversal
patients and no differences in SOX9 gene copy number were found between the patient group
and the normal control group, based on quantitative PCR data, which is in accordance with re-
results of some previous work (Georg et al., 2010). TESCO sequencing results showed one insert
mutation (c.3537791insG) in one SRY-positive female sex reversal patient (46, XY). Clinical
data showed that this patient had a significant female appearance, no male reproductive organs
or glands, primary amenorrhea, and ovary and uterus disease. The occurrence of these traits may
be related to the insertion mutation affecting the TESCO enhancer, leading to sex reversal. The
functional effects of the mutations will be tested by further experimental validation.

The human DAX1 gene, which is located on the X chromosome (Xp21.1-21.2) with a
total length of 4908 bp, contains two exons and one intron. It is widely expressed in the adre-
nal gland and in the hypothalamus-pituitary-gonadal axis, and it plays an important role in the
regulation of the adrenal and gonadal systems. Because highly expressed DAX1 in mice has
been shown to result in the generation of female sex reversal as a result of gonadal develop-
ment regulation, it is considered to be an anti-testis gene. DAX1 mutations or deletions cause
human adrenal hypoplasia (AHC). Testicles can develop in patients with AHC, which is gen-
erally accompanied by confusion of testis cords, which promote low attenuation of gonadal sexual function disorders. No mutations were detected in the DAX1 coding region in the 18 patients with sex reversal analyzed in the present study, which confirmed results of a previous study (Hanley et al., 2000).

In summary, we found that complete sex reversal occurred mainly due to mutations of the SRY gene, which accounted for 44.4% of the 18 cases of sex reversal in patients from Sichuan province. Therefore, although mutations in SOX9 and DAX1 have previously been reported to be associated with sex reversal, these mutations do not appear to be the main cause, which is in accordance with previous work. In addition, two novel mutations were found in SRY and in the TESCO region.

ACKNOWLEDGMENTS

Research supported by the Bio-resource Research and Utilization Joint Key Laboratory of Sichuan-Chongqing, Institute of Medical Genetics, College of Life Science, Sichuan University. We are grateful to the patients and volunteers who participated in this study.

REFERENCES

