SRY-negative 46,XX male with normal genitals, complete masculinization and infertility

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XX maleness is a rare syndrome with a frequency of 1 in 20 000–25 000 males. XX males exist in different clinical categories with ambiguous genitalia or partially to fully mature male genitalia, in combination with complete or incomplete masculinization. In this study, we report a case of SRY-negative XX male with complete masculinization but infertility. The patient had fully mature male genitalia with descended but small testes and no signs of undervirilization. PCR analysis for SRY gene, ZFY, Amelogenin, AZFa, AZFb, AZFc genes, a pair of primers from heterochromatic region and six Y-STRs showed the absence of any Y-chromosome-derived material. Absence of SRY gene was confirmed by three independent PCRs for each of two sets of primers covering an increasing length of the gene. Sequence analysis of the coding regions of SOX9 and DAX1 genes did not reveal any mutation. Real-time PCR assay revealed normal copy number for SOX9 gene. Microsatellite analysis showed no evidence of 17q (SOX9 gene) or 22q duplication. Genotyping with X-STRs ruled out the possibility of any deletion on X chromosome. Development of the male phenotype in the absence of SRY probably resulted from the loss of function mutation in some unknown sex-determining gene, which normally inhibits the male pathway, or from a gain of function mutation in a gene downstream to SRY in male pathway.

Key words: 46,XX male/infertility/real-time PCR/sex determination/sex reversal/SRY gene

Introduction

It is well-known fact that the presence or absence of Y chromosome (SRY gene in particular) determines the sex in mammals. SRY is thought to direct the sex-determination pathway towards male development (Sinclair et al., 1990; Brennan and Capel, 2004). The fortuitous finding of chromosomal rearrangements in association with a sex-reversed phenotype has led to the isolation of SRY gene (Sinclair et al., 1990). Careful genetic analysis of cases with abnormal sexual development, presented with chromosomal translocations or deletions/duplications, has resulted in the identification of many genes playing role in sex determination (Call et al., 1990; Sinclair et al., 1990; Bennett et al., 1993). Despite the identification of SRY almost 15 years ago, the pathway downstream to SRY remains largely unknown, although SOX9 and DAX1 have recently been proposed to function downstream to SRY gene in male sex-determination pathway (Meeks et al., 2003; Brennan and Capel, 2004).

An increasing number of reports suggest that the male phenotype can develop even in the absence of SRY gene. Till date, many cases of XX males with or without SRY and apparently with no other Y-chromosome sequences have been reported (Zenteno et al., 1997; Abusheikha et al., 2001; Valetto et al., 2005). XX male syndrome is a rare disorder with a frequency of 1 in 20 000–25 000 males (de la Chapelle, 1981). These exist in three clinical categories: XX males with normal genitalia; XX males with ambiguous genitalia; and XX true hermaphrodites with ovarian and testicular tissues (de la Chapelle, 1987). According to the presence or absence of the Y-chromosome sequences, XX males can be divided into two categories. Approximately 90% of the cases carry varying amount of the Y sequences due to an illegitimate recombination between X and Y chromosomes, whereas 10% do not have any Y-chromosome sequences. Most of the XX males with SRY have normal genitalia, whereas most SRY-negative cases have ambiguous genitalia (Zenteno et al., 1997).

It was believed that testes differentiation pathway is the only active pathway in sex determination and the absence of testes differentiation signals results in ovary development, but Eicher and Washburn (1986) have emphasized that ovarian pathway also must be an active pathway. Sex reversal cases with delayed or sub-minimal SRY expression showed that the proper and timely expression of SRY gene was necessary to overcome the active ovarian signals (Brennan and Capel, 2004). Given that SRY behaves dominantly over the ovary differentiation signals (Swain and Lovell-Badge, 1999), the development of male phenotype in SRY-positive 46,XX individuals is not surprising. But except a few studies (Aleck et al., 1999; Huang et al., 1999; Seeherunvong et al., 2004), the aetiology of development of male phenotype in most of the SRY-negative 46,XX males remains unexplained.

Development of the testis and normal male genitals in a significant number of SRY-negative 46,XX males gives clue to the existence of other autosomal or X-linked genes in the sex-determining pathway. Comprehensive genetic analysis of these cases may help to decipher new gene(s) involved in the sex-determining pathway. In this study, we report a case of 46,XX infertile male with complete masculinization and descended but small testes, without SRY gene and all other Y-chromosome sequences.
Materials and methods

Case history and clinical details
A 34-year-old man attended the genetic clinic of the Institute of Reproductive Medicine (IRM), Kolkata, with complaints of infertility. His height was 156 cm and weight 64 kg. The patient had fully mature normal male genitalia with no symptoms of under virilization. The testicles were descended in the scrotum but small in size with volumes 4.8 ml and 5.1 ml (normal range 18–30 ml). Axillary and pubic hairs were of normal pattern and density. Serum concentrations of LH and FSH were elevated at 15.8 mIU/ml (normal range 2.0–14.0 mIU/ml) and 25.8 mIU/ml (normal range 1.5–12.0 mIU/ml), respectively. Testosterone hormones level was normal at 80 ng/dl (normal adult male range, 437–707 ng/dl, National Institutes of Health).

Cytogenetic analysis
Peripheral blood lymphocyte cultures were set up in duplicate in 5 ml of culture vials using Roswell Park Memorial Institute (RPMI) media supplemented with 10% fetal calf serum (FCS). Cells were grown in the presence of penicillin/streptomycin/gentamycin. Phytohemagglutinin (PHA) was added to stimulate cell division. Dividing cells were arrested at metaphase stage with colchicine and fixed in methanol : acetic acid (3:1). Fixed cells were dropped onto glass slides and allowed to air dry. Chromosomes were G-banded by the preparations with trypsin followed by staining with giemsa. A total of 100 metaphases were analysed to look at any heterozygosity among the cell populations.

DNA isolation and quantification
DNA was extracted from peripheral blood leukocytes of the patient, a normal fertile male and a female using the protocol described in our earlier study (Thangaraj et al., 2002b). DNA samples were quantified spectrophotometrically by measuring the absorbance at 260 nm. Serial dilutions of DNA were made up to 100 ng/μl of DNA solution was checked on 0.8% agarose gel. For real-time PCR assay, the 10 ng/μl of DNA solution was further serially diluted to 5.0, 2.5 and 1.25 ng/μl concentrations.

PCR and sequence analysis
We selected genes residing in different regions of Y chromosome to look at the presence or absence of Y-chromosome-derived sequences. SRY gene was amplified with two pairs of primers, covering 600 and 868 bp around HMG box of the gene (Table I). Similarly, ZFY gene was amplified with a pair of primers covering one of the zinc-finger regions. PCR primers for SRY, ZFY, SOX9 and DAX1 genes were designed using Genetool software and synthesized using 394 DNA/RNA oligosynthesizer (Applied Biosystems, Foster City, California, USA). Full coding regions of DAX1 and SOX9 genes along with exon–intron junctions were amplified and sequenced using BiDye™ chain terminator chemistry on ABI 3730 DNA analyzer (Applied Biosystems, Foster City, California), as described in our earlier study (Thangaraj et al., 2002a). Spermatogenic genes AZFa (sY84, sY66), AZFb (sY127, sY134), AZFc (sY254, sY255), DAZ (sY581, sY586, sY587) and a part of heterochromatic region of Y chromosome (sY160) were amplified by PCR to look for the presence/absence of these genes. Primers specific for the spermatogenic genes were taken from our earlier studies (Thangaraj et al., 2003a). The 10 μl PCR for all the genes consisted of 1.0 μl of PCR Gold buffer (10x), 1.0 μl of MgCl2 (25 mM), 0.8 μl of dNTP (10 mM), 2 pM of each primer, 1.0 unit of AmpliTaq Gold™ DNA polymerase (Applied Biosystems, Foster City, California) and 40 ng of DNA template. All the amplifications were performed in three independent PCRs each time with positive and negative controls using AmpliTaq Gold™ DNA polymerase (Applied Biosystems, Foster City, California) to enhance the amplification efficiency.

Genotyping
To further confirm the presence or absence of other Y-chromosome-derived sequences, we performed analysis with six STR markers (DYS19, DYS389I and II, DYS390, DYS391, DYS393) spanning the male-specific region of Y chromosome (MSY region). Primers for Y-STR markers were taken from our earlier study (Thangaraj et al., 1999). We sought genotyping with a total of 53 STR markers specific for X chromosome (Linkage Mapping Set, V 2.5, Applied Biosystems) to confirm the presence of two full-length X chromosomes. Six STR markers (D17S787, D17S944, D17S949, D17S785, D17S784, D17S928) were genotyped for the patient and three control samples to look at 17q (SOX9) duplication. Similarly, to look at the duplication of 22q region, three STR markers (D22S283, D22S423, D22S274) were selected from the long arm of chromosome 22 and genotyped for the patient and three control samples. The 10 μl Genotyping PCR consisted of 1.0 μl PCR Gold buffer (10x), 1.0 μl of MgCl2 (25 mM), 0.8 μl of dNTP (10 mM), 5 pM of each primer, 1.0 unit of AmpliTaq Gold™ DNA polymerase (Applied Biosystems) and 20 ng of DNA template. All the genotyping reactions were performed with PCR conditions consisting of initial denaturation at 94°C for 12 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 15 s and polymerization at 72°C for 30 s with a final extension at 72°C for 20 min. For genescan analysis, 3.0 μl of the PCR product was mixed with 0.2 μl of LIZ™ size standard (Applied Biosystems) and 6.8 μl of Hi-Di formamide (Applied Biosystems). After denaturation at 95°C for 5 min followed by cooling on ice for 5 min, the samples were run on 3730 DNA Analyzer (Applied Biosystems). The raw data were further analysed using GeneMapper software (Applied Biosystems).

Real-time PCR assay
Real-time PCR assay was performed to assess the copy number of SOX9 gene in the patient. For real-time PCR, primers specific to SOX9 gene were designed

Table 1. Primer sequences and the PCR conditions used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer symbol</th>
<th>Primer sequences (5’–3’)</th>
<th>PCR conditions</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRY</td>
<td>SRY 1</td>
<td>CCAGGATTCGACATGCAACATCATATGCTTTCTGC</td>
<td>94°C-12 min (94°C-1 min, 65°C-1 min, 72°C-2 min)</td>
<td>609</td>
</tr>
<tr>
<td></td>
<td>SRY 2</td>
<td>CTTGACGCGGTCCCCGCCCCCGGGGGGTGTC</td>
<td>×35 cycles, 72°C-10 min</td>
<td>868</td>
</tr>
<tr>
<td>ZFY</td>
<td>ZFY 1</td>
<td>GGCTGTCGCGACTCTTCTTCAAC</td>
<td>94°C-12 min (94°C-1 min, 58°C-1 min, 72°C-2 min)</td>
<td>603</td>
</tr>
<tr>
<td>SOX9</td>
<td>SOX9 1</td>
<td>CCGGACCGCGAGGGGGGAAGGGAGAGGAGG</td>
<td>×30 cycles, 72°C-10 min</td>
<td>719</td>
</tr>
<tr>
<td></td>
<td>SOX9 2</td>
<td>CCGCAGCGCGAGGGGAGGGAGGAGG</td>
<td>94°C-12 min (94°C-1 min, 58°C-1 min, 72°C-1 min)</td>
<td>464</td>
</tr>
<tr>
<td>DAX1</td>
<td>DAX1 1</td>
<td>CGCGCGCGCGCGCGCGCGCGCGCGC</td>
<td>94°C-12 min (94°C-1 min, 58°C-1 min, 72°C-1.5 min)</td>
<td>786</td>
</tr>
<tr>
<td></td>
<td>DAX1 2</td>
<td>CGCGCGCGCGCGCGCGCGCGCGCGC</td>
<td>×30 cycles, 72°C-10 min</td>
<td>750</td>
</tr>
<tr>
<td></td>
<td>DAX1 3</td>
<td>CGCGCGCGCGCGCGCGCGCGCGCGC</td>
<td>94°C-12 min (94°C-1 min, 58°C-1 min, 72°C-1.5 min)</td>
<td>644</td>
</tr>
</tbody>
</table>

342
using Primer Express software (Applied Biosystems). The primers used were forward primer, 5′-TGGTCTTTAATACACTGACCGTACCT-3′ and reverse primer, 5′-TATTCCGATCTTAAATCAGAGAAAGTG-3′ and gave a product of 102 bp upon PCR. The primers were standardized by gradient PCR (Mastercycler Gradient, Eppendorf, Germany) using different concentration of both forward and reverse primers at different annealing temperatures. Annealing temperature and primer concentration with no leftover primer in the standardization reaction were considered as the optimum. A normal fertile 46,XY male DNA was used as a standard for real-time PCR assay. The 10 μl PCR mixture consisted of 5.0 μl of SYBR® Green, 2.5 pM of each forward and reverse primer with different quantities of DNA and Mili-Q water to make up the volume. Three reactions were setup independently for each of the two concentrations of 2.5 and 5.0 ng. Real-time PCR was performed using Sequence Detection System HT7900 (Applied Biosystems). The raw data were analysed using Sequence Detection System software (Applied Biosystems). The test samples (2.5 and 5.0 ng). Real-time PCR was performed using Sequence Detection System HT7900 (Applied Biosystems). The raw data were analysed using Sequence Detection System software (Applied Biosystems).

**Results**

**Cytogenetic analysis**

Karyotype of the patient showed 46,XY chromosome complement (Figure 1). Analysis of 100 metaphases showed no evidence of mosaicism in peripheral blood cells and structural or numerical chromosomal abnormality.

**PCR and sequence analysis**

Molecular analysis of Y-chromosome-specific markers SRY, ZFY, Amelogenin, AZFa, AZFb, AZFc, DAZ and heterochromatic region revealed their absence in patient’s DNA (Figure 2). Possibility of PCR inhibitor in the DNA sample was excluded because amplification for the patient DNA was seen with SOX9, DAX1 and 53 X-chromosome-specific STR markers. All the above-mentioned markers showed consistent amplification in positive control and no amplification in the negative control. Sequence analysis of the coding regions of DAX1 and SOX9 genes showed no mutation in these genes.

**Genotyping**

Absence of PCR amplification of Y-STR markers further confirmed the lack of Y-chromosome sequences in the patient DNA. X-STR analysis showed heterozygous alleles for 42 of 53 markers, suggesting the presence of two X chromosomes. No more than two adjacent X-STRs were homozygous in the patient’s sample, excluding the possibility of any gross deletion on X chromosome. Microsatellite marker analysis for 17q and 22q arms showed no evidence of duplication of any of these regions in the patient.

**Real-time PCR assay**

The test sample was used in two different concentrations (2.5 and 5.0 ng) and defined as unknown for real-time PCR analysis. The proportionate variation in the number of PCR cycles to achieve the threshold absorbance in all the triplicates with each DNA concentration used showed that the DNA quantification was accurate for both the standards (Figure 3) and the test sample (Figure 4). Plotting the Ct value for patient samples with 2.5 and 5.0 ng DNA concentrations matched with the standard samples with 2.5 and 5.0 ng concentrations, showing the normal copy number of SOX9 gene in the patient DNA (Figure 5). The denaturation curve for all the standard and the patient samples showed that the primer was specific to the target site and no primer dimer was formed in the real-time PCR (data not shown), excluding the possibility of any gross error in the results.

**Discussion**

Majority of the XX males carry SRY gene translocated to the X chromosome due to an illegitimate recombination between X and Y chromosomes. These patients are sterile males and usually have normal male genitalia. XX males without SRY gene have ambiguous to normal genitalia, show incomplete to complete masculinization and are infertile. The existence of SRY-negative males ruled out the prevailing notion that the mere presence of SRY determines maleness. The most common observation that the individuals with SRY are male shows that it is the presence or absence of a normal SRY gene which determines maleness, provided all downstream genes are functionally intact.

In this study, we report a case of SRY-negative XX male having fully mature normal male genitalia with infertility as the main anomaly. Analysis of 100 metaphases showed only 46,XX cell populations without any numerical or structural chromosomal aberrations. Peripheral blood DNA was negative for SRY gene and other Y-chromosome sequences. STR analysis for the X chromosome ruled out the possibility of any major deletion on X chromosome to be the cause for the anomaly. The patient had short stature, which could be attributed

![Figure 1. Karyotype of the patient showing 46,XX chromosome complement.](image-url)
Rarely, hidden gonadal mosaicism for \textit{SRY} gene has been found to be the reason for the development of testicular tissue and male phenotype in 46,XX true hermaphrodites (Inoue \textit{et al.}, 1998; Jimenez \textit{et al.}, 2000) and 46,XX males (Dardis \textit{et al.}, 1997). The possibility of the \textit{SRY} gene mosaicism in the gonadal tissue, however, could not be ruled out in this case because of reluctance of the patient to provide gonadal biopsy.

Till date, a few \textit{SRY}-negative XX males have been reported. All these cases had immature to fully mature male genitalia, mostly with descended testes. Different hypotheses have been put forward to explain the occurrence of the \textit{SRY}-negative XX males. McElreavey \textit{et al.} (1993) proposed that \textit{SRY} acts by inhibiting a regulatory autosomal recessive gene, termed ‘\textit{Z}’, whose product normally inhibits the male pathway. Accordingly, XX males with complete male phenotype would be homozygous for null mutation in ‘\textit{Z}’ gene, and the cases with
SOX9 gene in a 46,XX male and hypothesized that SOX9 duplication underlies 46,XX female-to-male sex reversal. The above hypothesis was confirmed on the basis of development of male phenotype in XX mice transgenic for SOX9 gene (Vidal et al., 2001). Most of the other studies on SRY-negative 46,XX males have not addressed the issue of SOX9 duplication (Zenteno et al., 1997; Domenice et al., 2001). Real-time assay showed normal number of SOX9 copies in the present cases. Alternatively, microsatellite marker analysis for 17q also showed the absence of any duplication in this region, ruling out the possibility of SOX9 duplication to be the cause for XX maleness.

Similar to 17q duplication, two different studies have shown the duplication of a region of the long arm of chromosome 22 in a XX true hermaphrodite (Aleck et al., 1999) and one XX male with first degree of hypospadias (Seeherunvong et al., 2004). Other SRY-negative XX males in the later study did not show any duplication of this region of chromosome 22. Although duplication of SOX10 gene from this region of chromosome 22 has been sought as a candidate for XX sex reversal, but unlike SOX9 duplication, there has been no transgenic study to confirm that duplication of 22q/SOX10 can result in 46,XX maleness. We analysed three STR markers from 22q for the patient and three control samples but found no evidence of duplication of this region in the present case. Failure to find duplication of this region in the present study along with the previous reports shows that the 22q duplication in XX maleness is either a coincidence or contributes to the disorder very rarely.

In conclusion, evidences from the present and previous studies suggest that SRY-negative XX maleness largely remains unexplained, except few cases with SOX9 or 22q duplication. It needs to be determined by transgenic methods whether duplication of 22q really results in SRY-negative XX maleness. In majority of the cases, XX maleness should result either from the loss of function mutations in a gene normally inhibiting testes formation in genotypic females or from the gain of function mutations in a gene downstream to SRY in testis determining pathway. The hypothetical gene may be X-linked or autosomal. If the gene is autosomal, the degree of the male phenotype will be dependent on the extent of the loss or gain of function in the mutant gene. The phenotype in the heterozygotic mutants for X-linked gene will be determined by the ratio of the active and inactive copies of the gene. Because the present case had normal male phenotype, it should either be homozygous mutant for this hypothetical autosomal gene or a result of preferential inactivation of the normal copy of the X-linked heterozygous mutant gene.

References

Figure 5. Standard plot for real-time PCR assay of SOX9 gene copy number. Ct represents the number of the PCR cycle to achieve the threshold fluorescence in the real-time PCR. S1, S2 and S3 represent the triplicate for each of the four concentrations used for the standard DNA sample. Similar triplicates for each concentration of the patient’s sample are represented by U1, U2 and U3.


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