Molecular and cytogenetic findings in 46,XX males

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This paper reports 3 cases with 46,XX sex reversed male. Three 46,XX hypogonadal subjects showed complete sex reversal and had normal phallus and azoospermia. We studied them under clinical, cytogenetic and molecular aspects to find out the origin of the sex reversal. Patients had markedly elevated serum follicle-stimulating hormone (FSH) and lutenizing hormone (LH) and decreased or normal range of serum testosterone. The testicular volumes were small (3-8ml). Testicular biopsy showed Leydig cell hyperplasia and atrophy of seminiferous tubules. We obtained a normal female karyotype with 46,XX, and the presence of Y chromosome mosaicism was ruled out through XY dual fluorescent *in situ* hybridization (FISH). By using polymerase chain reaction (PCR), we amplified short arm (SRY, PABY, ZFY and DYS14), centromere (DYZ3), and heterochromatin (DYZ1) region of the Y chromosome. PCR amplification of DNA from these patients showed the presence of the sex-determining region of the Y chromosome (SRY) but didn't show the centromere and heterochromatin region sequence. The SRY gene was detected in all the three patients. Amplification patterns of the other regions were different in these patients; one had four amplified loci (PABY+, SRY+, ZFY+, DYS14+), another had two loci (SRY+, ZFY+) and the other had two loci (PABY+, SRY+). We have found that each patient's translocation elements had different breakpoints at upstream and downstream of the SRY gene region. We conclude that the testicular development in 46,XX male patients were due to insertion or translocation of SRY gene into X chromosome or autosomes.

Keywords: 46,XX sex reversed male, SRY gene, Testis development

INTRODUCTION

SRY (sex-determining region Y) gene on the Y chromosome induces testicular development, and subsequent male sexual differentiation is a consequence of the hormonal products of the testis (Jost *et al.*, 1973). The Y-encoded testis-determining gene has been named TDF (testis-determining factor) in humans. Although many different genes are required for both male and female sex determination, understanding the role of TDF gene may provide a general model for the genetic control of developmental decisions in mammals (Sinclair *et al.*, 1990).

46,XX maleness is a rare sex chromosomal constitution characterized by the testicular development in persons who lack a Y chromosome (Chapelle *et al.*, 1964). 46,XX males have a normal male phenotype and external genitalia but their height and tooth size are in the female range.

They are shorter and some patients may have genital abnormalities (Shah *et al.*, 1961). 46,XX males occur approximately at the rate of one in every 20,000 newborn males. All 46,XX males are azoospermic because of gonadal dysgenesis. Most patients have markedly elevated serum FSH and LH, and about half of these patients have decreased serum testosterone. It is reported that approximately 90% of the XX males are SRY-positive (McElreavey *et al.*, 1993). Most cases are attributable to an interchange of a fragment of the short arm on the Y chromosome containing the SRY gene with the X chromosome (Terkelsen, 1964;

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Ferguson-Smith, 1966). Other mechanisms have been proposed such as autosomal or X-linked inheritances (Chapelle 1987; Skordis *et al.* 1987), which don't exclude a regulatory cascade hypothesis (McElreavey *et al.* 1993).

In this study, three patients referred due to azoospermia were studied under cytogenetic, clinical and molecular aspects to find out the mechanism of sex reversal.

MATERIALS AND METHODS

The subjects

The three patients were referred for clinical and genetic studies because of azoospermia and small testicular volume below 10 ml. Physical examinations didn't reveal any distinguishable abnormalities. None of the patients had any family history related to the pathological entity. Ages ranged from 29 to 37 years. The height, weight, testicular volume and penile size were measured. Immunoreactive serum FSH, LH, testosterone, and estradiol levels were measured. Only two patients undertook testicle biopsy. The clinical characteristics are shown in table 1.

Chromosome study and fluorescence *in situ* hybridization (FISH)

A cytogenetic analysis on peripheral blood cells and DNA extraction were performed according to standard methods (Verma and Babu. 1995). We counted 70-80 metaphase cells to rule out mosaicism which may have aneuploidy of X and Y chromosome. And FISH analysis using X and Y dual centromeric probe (Cytocell, Applied Imaging co.) was conducted according to cytocell protocol. Using an image analyzer (Cytovision, applied imaging co.) after the FISH experiment, we could confirm whether or not there was the presence of aneuploid of X & Y chromosome.

Table 2. PCR conditions (annealing temperature, annealing time and product size) in Y chromosome specific regions.

Region	Annealing Temp. (°C)	Annealing Time (sec)	Size(bp)
PABY	55	120	1100
SRY	63	10	609
ZFY	58	30	735
DYS14	55	60	198
DYZ3	61	120	170
DYZ1	55	30	149

Polymerase chain reaction (PCR) analysis

Genomic DNA was isolated from the peripheral blood of the three patients, normal male control and normal female control by standard techniques (Sambrook *et al.* 1989). PCR was performed to detect the breakpoints and presence of the SRY gene. The following six Y-specific sequence primers were used for PCR analysis (Fig. 1): PABY (pseudoautosomal boundary region, Ellis *et al.*, 1990), SRY (sex-related Y, Berta *et al.*, 1990), ZFY (Zinc finger Y, Sorgo *et al.*, 1991), DYS14 (Y-specific single copy, Lo *et al.*, 1990), DYZ3 (centromeric region, Witt and Erickson, 1989, 1991) and DYZ1 (Y-specific repeat sequence of heterochromatin region, Lo *et al.*, 1989). 50-100 ng from DNA of the three patients, normal male and normal female, were used for the PCR and conditions were followed according to. The amplified products were detected by electrophoresis with a 2.0% agarose gel containing ethidium bromide and directly visualized by UV transilluminator.

Table 1. Clinical characteristics and karyotypes of three sex-reversed patients.

	Case 1	Case 2	Case 3
Karyotype	46,XX	46,XX	46,XX
Age	37	36	29
LH (NL: 1.5-7.0 mIU/ml)	18	22	15
FSH (NL: 1.2-10 mIU/ml)	42	64	43
Testosterone (3.0-10ng/ml)	1.9	3.6	6.4
Height (cm)	170	165	171
Weight (kg)	60	55	61
Penis (cm)	8	7	7.5
Testes vol. (Rt/Lt, cc)	8/8	4/4	3/3
Testicle Biopsy	Not done	Leydig cell hyperplasia	Leydig cell hyperplasia

NL: Normal limited

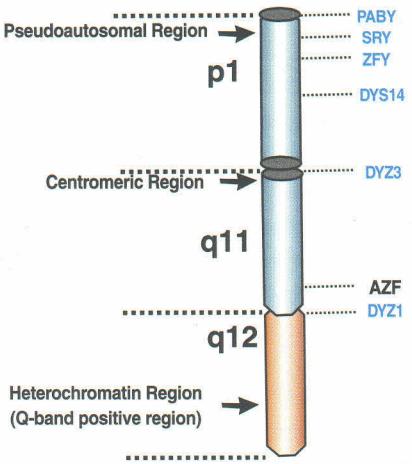

Fig. 1. Location of genes on the Y chromosome.

RESULTS

On physical examination of the three patients, external genitalia and general phenotypes were within the normal range (Table 1). The size of testes were smaller than normal in case 2 and 3, while case 1 had normal size. Pathological examinations were conducted for case 2 and 3 only, confirming Leydig cell hyperplasia. All three patients had high level of serum LH; 18 mIU/ml, 22 mIU/ml and 15 mIU/ml. FSH was also high; 42 mIU/ml, 64 mIU/ml and 43 mIU/ml. However, testosterone level was lower than normal in case 1, and normal in case 2 and 3.

Through the GTG-banding, we observed that the three patients had 46,XX, sex-reversal karyotypes (Fig. 2). But we weren't able to find any case of mosaicism which was done by counting 70-80 metaphase cells. In addition, with FISH using X and Y dual probe, we observed two X signals of centromere region only (Fig. 3). The results of DNA analysis using PCR showed various breakpoints in all the patients. Case 1 had PABY, SRY, ZFY and DYS14 genes, but had deletions of DYZ3 and DYZ1 genes. Case 2, the patient had SRY, ZFY genes, but had deletions of PABY, DYS14, DYZ3 and DYZ1 genes. Case 3 had PABY, SRY genes, but had deletions of ZFY, DYS14, DYZ3 and DYZ1 genes (Table 3). Therefore, although the SRY gene was found in all three patients, each patient had various deletions on both upstream and downstream regions of SRY gene.

Table 3. PCR amplification results of specific regions according to physical order on the Y chromosome. Each locus is represented by + (presence) or - (absence).

	Case 1	Case 2	Case 3	Control male	Control female
PABY	+	-	+	+	-
SRY	+	+	+	+	-
ZFY	+	+	-	+	-
DYS14	+	-	-	+	-
DYZ3	-	-	-	+	-
DYZ1	-	-	-	+	-

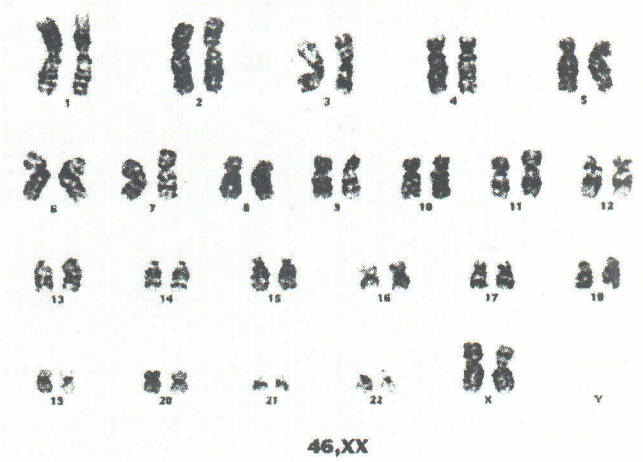

Fig. 2. The karyotype showed a normal 46,XX of sex reversed patients.

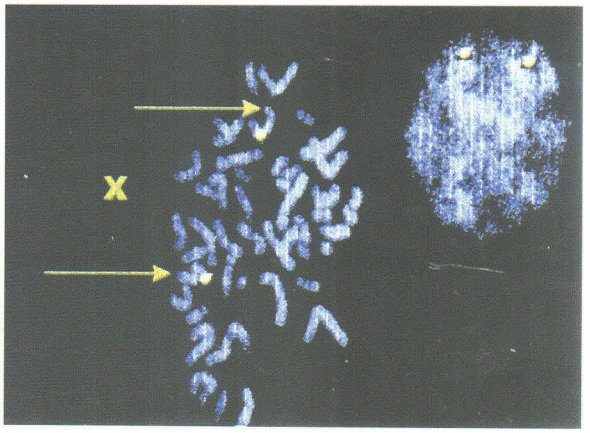

Fig. 3. FISH signals were obtained with X and Y dual probes in 46,XX male patients. Arrows point to centromeric regions of the two X chromosomes.

DISCUSSION

These cases demonstrate abnormal separation of genes responsible for testicular development from those for spermatogenesis. This testis-determining factor, which may be equivalent to the SRY gene, is located in the distal part of the short arm on the Y chromosome. This region causes the mesenchyma to differentiate into seminiferous tubules and Sertoli cells. The Sertoli cells secrete mullerian-

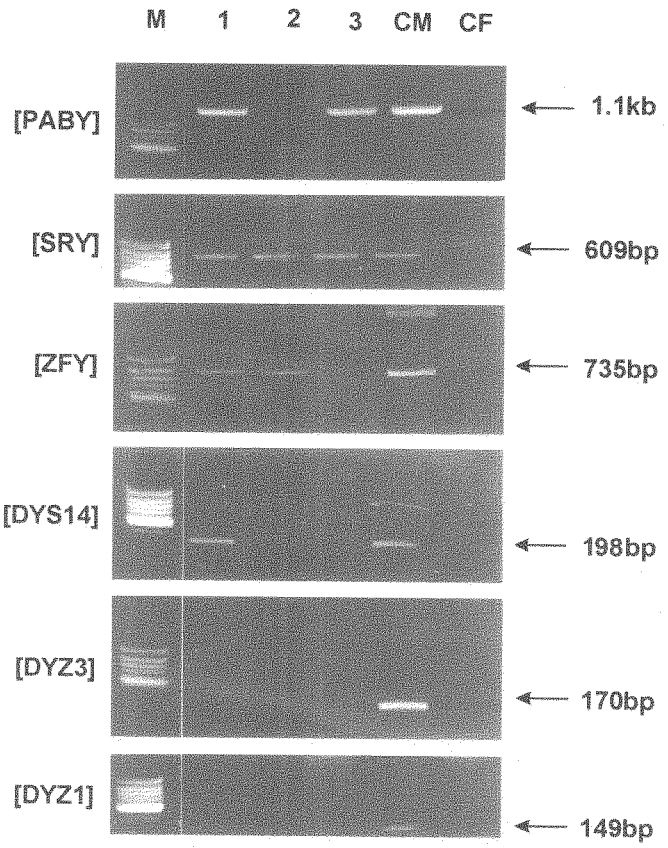

Fig. 3. Electrophoretic analysis of PCR products with specific regions (PABY, SRY, ZFY, DYS14, DYZ3 and DYZ1). The absence of a band indicates a deletion. The arrows denote the sizes of amplification in each region. M: marker (ψX174-HincII), 1-3: cases, CM: control male, CF: control female.

inhibiting factors which are responsible for the regression of female internal genitalia. The fetal testis secretes testosterone required for the development of the male internal and external genitalia. The clinical presentation of the 46,XX male ranges from complete-masculinization to men with feminization with gynecomastia, small phallus, hypospadias or ambiguous genitalia; Despite the variable phenotypes, the lack of transfer of the SRY gene results in azoospermia. Hormonal analysis reflects this primary gonadal dysfunction with elevated FSH and LH levels, estradial levels may be high and testosterone levels is below than normal. It is believed that the presence of the SRY gene in 80-90% among 46,XX sex-reversal patients resulted from translocation and insertion of the SRY gene to the X chromosome or autosome during parental meiosis (Berkovitz *et al.*, 1992, Chapelle, 1972). But, in about 10% of the 46,XX sex-reversal patients, true hermaphroditism is observed along with hypospadia or external ambiguous genitalia. The three subjects in this study were referred to a genetic research laboratory with non-obstructive azoospermia. They had normal external genitalia, two rather small testis and normal male phenotype with non-observable hypospadias. In this study, we obtained 46,XX by chromosome analysis. To rule out mosaicism, we applied the FISH analysis confirming true 46,XX female karyotypes. For the PCR, we chose the six specific regions in the short arm, centromere and long arm on the Y chromosome. The results showed that each patient had deleted breakpoints on different regions of the Y chromosome. The SRY gene which is known to play an important role in determining male sex were found in all three patients. These accounts for the development of a male phenotype while karyotype is 46,XX female. There is also a possibility of the SRY gene being inserted or translocated to the X chromosome or autosome. We suspect the SRY gene could have been inserted or translocated causing a loss of a gene or mutation. Therefore, if the SRY gene had translocated to the X chromosome, there is a possibility that the X chromosome may be inactive.

In addition, we must also consider other possible deletions of genes which may affect testicular development. Most of 46,XX males with normal male phenotype were found to have the SRY gene, and only 5% did not. However, the fact that there are 46,XX males with male phenotype shows that SRY gene is not the only gene responsible for male sex determination (McElreavey *et al.*, 1992).

Also, mutation had been detected in the open reading frame of the SRY gene (Hawkins 1993), McElreavey *et al.* (1993) reported the deleted region of 5' within HMG box. Therefore, it is certain that the SRY gene plays an important role in the testis differentiation process and sex determination in mammals.

In this study, the SRY gene were found in all the three patients, but each patient had different breakpoints in the Y chromosome. However, we couldn't find out the clinical differences resulting from the different breakpoints. Futhermore, by observing that the three patients had normal phenotype with normal external genitalia, we believe that the SRY gene plays an important role in normal male sexual differentiation. We suggest that the testicular development in three patients was due to inserted or translocated SRY gene.

REFERENCES

Berkovitz G. D., Fechner P. Y., Marcantonio S. M., Bland G., Stetten G., Goodfellow P. N., Smith K. D. and Migeon C. J. (1992) The role of sex-determining region of the Y chromosome (SRY) in the etiology of 46,XX true hermaphroditism. *Hum Genet* 88: 411-416

Berta, P., Hawkins, J. R., Sinclair, A. H., Griffiths, B. L., Goodfellow,

P. N. and Fellows, M. (1990) Genetic evidence equating SRY and the testis-determining factor. *Nature* 348: 448-450

Chapelle, A., Hortling, H., Niemi, M. and Wennstrom, J. (1964) XX sex chromosomes in a human male. First case. *Acta Med Scand* [Suppl] 412: 25-38

Chapelle, A. (1972) Nature and origin of males with XX sex chromosomes. *Am J Hum Genet* 24: 71-105

Chapelle, A. (1987) The Y-chromosomal and autosomal testis-determining genes. *Deveolpment* [suppl] 101: 33-38

Ellis, N., Taylor, A., Bengtsson, B. O., Kidd, J., Rogers, J. and Goodfellow, P. (1990) Population structure of the human pseudoautosomal boundary. *Nature* 344: 663-665

Ferguson-Smith, M. A. (1966) X-Y chromosomal interchange in the aetiology of true hermaphroditism and of XX Klinefelter's syndrome. *Lancet* II: 475-476

Hawkins, J. R. (1993) Mutational analysis of SRY in XY females. *Hum Mutat* 2: 347-350

Jost, A., Vigier, B., Prepin, J. and Perchellet, J. P. (1973) Studies on sex differentiation in mammals. *Recent Prog Horm Res* 29: 1-14

Lo, Y.-M. D., Patel, P., Wainscoat, J. S., Sampietro, M., Gillmer, M., D. G. and Fleming, K. A. (1989) Prenatal sex determination by DNA amplification from maternal peripheral blood. *Lancet* II: 1363-1365

Lo, Y.-M. D., Patel, P., Sampietro, M., Gillmer, M. D. G., Fleming, K. A. and Wainscoat, J. S. (1990) Detection of single-copy fetal DNA sequence from maternal blood. *Lancet* 335: 1463-1464

McElreavy, K., Vilain, E., Nacer, A., Costa, J.-M., Souleyreau, N., Kucheria, K., Boucekkine, C., Brauner, R., Flamant, F. and Fellous, M. (1992) XY sex reversal associated with a deletion 5' to the SRY "HMG box" in the testis-determining region. *Proc Natl Acad Sci USA* 89: 11016-11020

McElreavey, K., Vilain, E., Abbas, N., Hersowitz, I. and Fellous, M. (1993) A regulatory cascade hypothesis for mammalian sex determination: SRY represses a negative regulator for male development. *Proc Natl Acad Sci USA* 90: 3368-3372

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd Edn., Cold spring Harbor Laboratory Press, Cold Spring Harbor, NY

Shah, P. N., Naik, S. N., Mahasan, D. K., Dave, M. J. and Paymaster, J. C. (1961) A new variant of human intersex with discussion on the deveolpmental aspects. *Br Med J* 2: 474-477

Sinclair, A. H., Berta, P., Palmer, M. S., Hawkins, J. R., Griffiths, B. L., Smith, M. J., Foster, J. W., Frischauf, A., Lovell-Badge, R. and Goodfellow, P. N. (1990) A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* 346: 240-244

Skordis, N. A., Stetka, D. G., MacGillivray, M. H. and Greenfield, S. P. (1987) Familial XX males coexisting with familial XX true hermaphrodites in same pedigree. *J Pediatr* 110: 244-248

Sorgo, W., Gortner, L., Bartmann, P., Streb, H. P., Berhoffer, R., Teller, W. M., Zachmann, M., Heymer, B., Graf, M., Lattermann, U., Jager, R. J. and Ebensperger, C. (1991) Gonadal agenesis in a 46, XY female with multiple malformations and positive testing for the sex-determining region on the Y chromosome. *Horm Res* 35: 124-131

Therkelsen A. J. (1964) Sterile man with chromosomal constitution, 46,XX. *Cytogenetics* 3: 207-218

Verma, R. S. and Babu, A. (1995) Human chromosome, In *Principles and technique*. 2nd Edn., pp.134-137, McGraw-Hill

Witt, N. and Erickson, R. P. (1989) A rapid method for detection of Y-chromosomal DNA from dried blood specimens by the polymerase chain reaction. *Hum Genet* 86: 271-274

Witt N. and Erickson R. P. (1991) A rapid method for detection of Y-chromosomal DNA from dried blood specimens by the polymerase chain reaction (erratum). *Hum Genet* 86: 540