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Sex Determination in Children with Ambiguous Genitalia by Polymerase Chain Reaction (PCR)

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ABSTRACT Sex determination in infants and children with ambiguous genitalia usually necessitates time-consuming and costly karyotyping .We have evaluated a simple,rapid and reliable method of postnatal sex determination by amplification of X and Y specific microsatellite markers DXS6797 and SRY respectively by polymerase chain reaction(PCR). Three probands M78, M59 and M61 with ambiguous genitalia were investigated.M78 showed a female complement of 46,XX while M59 and M61 have male complement of 46,XY.M78 was diagnosed as female pseudohermaphrodite while M59 and M61 were identified as male pseudohermaphrodite.Except M78, where a SRY sequence was identified by polymerase chain reaction (PCR) , the results of PCR were in agreement with those of cytogenetic analysis .The present study reaffirm the fact that the polymerase chain reaction (PCR) based sex determination is more sensitive, rapid and reliable than classical cytogenetic method for the detection of cryptic Y-specific SRY sequence in patients with ambiguous genitalia.

INTRODUCTION

Sex chromosomes play a crucial role in the etiology of normal sexual development .The bipotential gonad requires at least two X chromosomes to develop into an ovary.Alternatively,in the presence of SRY gene located on the Y chromosome, a testis develops.Discordance between chromosomal sex and the appearance of the external genitalia can lead to sexual ambiguity.In case of sexual ambiguity ,the patient may be either true hermaphrodite or pseudohermaphrodite.Both ovary and testes are present in the former while both the gonads are either absent or only one of them is present in the latter (Mueller and Young 1995).Ambiguous genitalia in the newborn and children need immediate and rational management and assignment of sex for rearing should be guided by the etiology of the genital malformation (Sultan et al. 2002).This complex situation requires a strategy of clinical, hormonal,genetic,molecular and radiographic investigation to determine the etiology of the intersex state.The choice of gender must take into account both the chromosomal and gonadal sex.However, the karyotyping from whole blood lymphocyte culture is costly and time consuming .On the other hand, polymerase chain reaction (PCR) based sex determination is rapid, reliable and economic and provides an accurate means of determining sex of an individual,including the detection of hidden Y sequence(Nakahori et al.1991; Caenazzo et al.1998).

The present study was initiated to evaluate a simple PCR based DNA diagnostic method (Ghosh and Dey 2003) by using polymorphic microsatellite markers of X and Y chromosomes for sex determination of individuals with ambiguous genitalia and to acquire more knowledge on the molecular mechanism of sex determination.

MATERIALS AND METHODS

The patient with ambiguous genitalia were referred to our laboratory for the determination of sex chromosome constitution.The case histories of the patients were as follows:

Case M78: The proband first came to medical attention at the age of 10 months because of ambiguous external genitalia.The patient was the third child born to a 32 years old father and 28 years old mother .The proband was reared as male child and had eight years old brother.A fetus was aborted spnotaneously at third trimester before the birth of the proband.The parents were healthy and unrelated.Clinical examination of the proband revealed micropenis,female like external genitalia,poorly developed scrotum and testes were not palpable.Pelvic ultrasound did not reveal any ovaries or testes. Testosterone was

undetected.

Case M59: The proband first came to medical attention at the age of 9 years because of ambiguous external genitalia.The patient was phenotypically male and was the third child born to a 28 years old father and 23 years old mother.The marriage was cosanguineous.The proband had two normal sisters 10 and 6 years old.Clinical examination revealed female like external genitalia with small and palpable testes.Pelvic ultrasound did not reveal any ovaries. No hormonal assay was made.

Case M61: The proband first came to medical attention at the age of 2 years because of ambiguous external genitalia and hernia.The patient was reared as male and the only child born to a 22 years old mother and 33 years old father.Parents were healthy and unrelated.Clinical examination revealed phallus like organ with no palpable testes.Pelvic ultrasound did not reveal any ovaries,uterus or testicles.Testosterone level was low.

Chromosome preparation was made from lymphocyte culture by following the coventional air drying method.Diploid count and sex chromosome constitution of each patient was established from G-banded karyotype.

Genomic DNA was isolated from uncoagulated blood samples of probands by using salting out procedure of Miller et al.(1988).Polymerase chain reaction was carried out by using two tetranucleotide microsatellite markers of X and Y chromosomes DXS6797 and SRY respectively.The sequences of two primers were as follows:

DXS6797:

FP-5^{I}- TTCCCTCTCTCCCTCTGTCT-3^{I}

RP-5^{I}- ACACACACCCAAAACCAGAT-3^{I}

SRY:

FP-5^{I}- CAATTCTTCGGCAAGCATCTT-3^{I}

RP-5^{I}- TACAGGCCATGCACAGAGAG-3^{I}

The PCR conditions for the first primer consisted of initial denaturation at 94^{0}C for 3 min followed by 27 cycles of PCR amplification at 94^{0}C for 30 sec; 55^{0}C for 75 sec; 72^{0}C for 15 sec and final extension for 6 min at 72^{0} C.For the second primer,the initial denaturation at 94^{0}C for 4 min followed by 35 cycles of amplification at 94^{0}C for 30 sec;64^{0}C for 2 min;72^{0}C for 2 min and final extension for 7 min at 72^{0}C in a perkin-Elmer 2400 Gene Amp. Thermal Cycler. The amplified PCR products were resolved in 6% polyacrylamide gel by following the method described earlier by Ghosh and Dey (2003). Alleles of X and Y markers were analysed and sex of each proband was determined.

RESULTS AND DISCUSSION

Cytogenetic Analysis: Chromosome analysis and karyotyping revealed normal female complement of 2n=46,XX in proband M78 while probands M59 and M61 have typical male complement of 2n=46,XY.

Molecular Analysis: Analysis of DNA polymorphism in three probands M78, M61 and M59 by using X and Y markers was shown in figures 1 and 2 respectively. In figure 1, lane 1 contains size marker pBR322/Alu I digestion .Lane 2 shows female control with two X alleles. The following lane 3 contains two X alleles of proband M78.On the other hand, proband M61 and M59 showed single X allele each in lane 4 and 6 respectively. In figure 2, lanes 1,3 and 4 contain SRY positive bands of probands M59, M61 and M78 respectively. The lane 4 shows SRY positive band of male control while last lane contains size marker.The presence of two X alleles indicate the presence of two X chromosomes while the presence of SRY positive band indicates the presence of SRY sequence (sex determining region of Y) of Y chromosome. The X and Y alleles were scored visually. The X alleles were lies within the size range of 257-403 base pairs (Fig.1) while the size of Y allele was around 79 base pairs (Fig. 2).

The results of the present study demonstrate the usefulness of microsatellite markers of X and Y chromosomes in determining the sex of individuals with ambiguous genitalia. M78 revealed female complement of 46,XX and without any cytogenetic evidence of Y chromosome. However, PCR analysis clearly showed the presence of Y specific SRY sequence along with two X alleles. Although majority of 46,XX true hermaphrodites were negative for the Y-DNA sequence, including SRY sequence (Ranie et al.1989; Ramos et al.1997), a minority of individuals were found to be positive for SRY sequence (Kojima et al.1998). However, there was no report of Y specific SRY sequence in female pseudohermaphrodites. Although it is now clear that the SRY gene plays a central role in triggering the formation of the testis from undifferentiated gonad, it has been suggested that other genes located either on the X or autosome may be

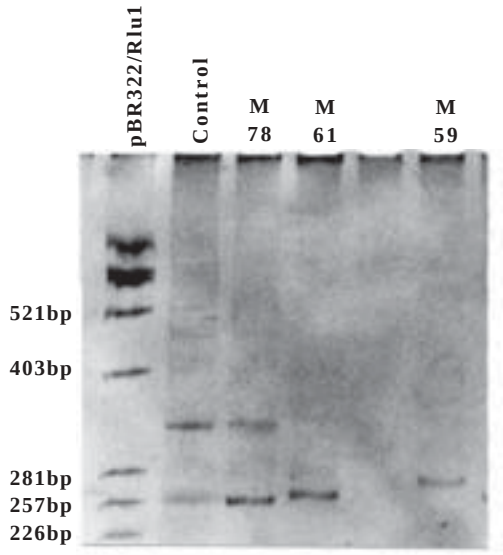

Fig. 1. The first lane contains a size marker pBR 322/Alu I digestion. Lane 2 shows female control with two X alleles. In the following lane 3 the proband M78 shows two X alleles.The probands M 61 and M 59 each shows single X allele in lanes 4 and 6 respectively.

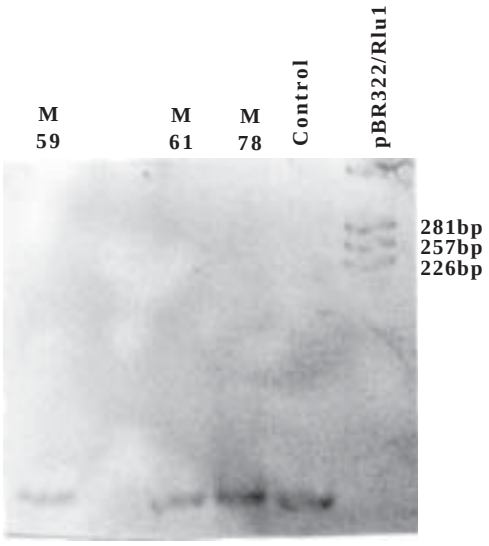

Fig. 2. Lanes 1,3 and 4 contain single SRY positive bands of probands M59, M61 and M78 respectively.Lane 5 shows single SRY positive band of male control.The last lane contains size marker pBR 322/Alu I digestion.

involved in testicular differentiation(Abusheikha et al.2001).In M78, though there were two X chromosomes and a SRY sequence ,there were no evidence of either testes or ovaries, indicating the disturbances in the cascades of gonadal differentiation. Inspite of all the investigations, no gonads were detected in M61.However,both cytogenetic and molecular study revealed XY constitution and SRY sequence in M61. Nevertheless,only in M59 testes were palpable and SRY sequence was also detected. These findings indicate the possibility of a mutation or deletion in SRY sequence that might have failed to trigger the development of testes in M78 and M61.It has also been proposed that abnormalities in the differentiation of bipotential gonad is responsible for sexual ambiguity (Mueller and Young 1995). We therefore conclude that pseudohermaphroditism like hermaphroditism (Torres et al.1996, Abuseikha et al. 2001) is also genetically and phenotypically heterogeneous condition.

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