

Cytogenetic and Molecular Evaluation of Ambiguous Genitalia In Pediatric Patients

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ABSTRACT

Objective: A newborn with ambiguous genitalia needs prompt evaluation to detect life-threatening conditions such as salt-losing crisis in congenital adrenal hyperplasia (CAH) and gender assignment. Sex assignment in these newborn continues to be a challenging diagnostic and therapeutic problem. In our study, we aimed to investigate the causes and characteristics of ambiguous genitalia in 21 newborn who were referred to a cytogenetic laboratory.

Materials and Methods: Cytogenetic analysis was performed for each case. The cases were analysed by also molecular genetic and interphase FISH technique to exhibit exist Y. Chromosome molecular genetic analysis was performed for Y chromosomal loci (SRY, ZFY, SY84, SY86, SY127, SY134, SY254, SY255). Genomic DNA was extracted from peripheral blood.

Results: In all of patient were detected 46,XX and 46,XY karyotype by cytogenetic analysis. 21 cases were successfully analyzed by interphase-FISH. Some individuals carry a Y chromosome but are phenotypically female or one of cases have a female karyotype but are phenotypically male.

Conclusion: The correlation between genotype (SRY+/-) and phenotype is still unclear. The etiology of ambiguous genitalia is variable. The physician managing these families could minimize the trauma of having a child with unidentified sex by providing appropriate genetic counseling so that the parents can make an early decision. ©2008, Fırat University, Medical Faculty

Key words: Ambiguous genitalia, SRY, cytogenetic analysis, intersex disorders

ÖZET

Pedatrik Hastalarda Ambiguous Genitalyanın Sitogenetik ve Moleküler Değerlendirmesi

Amaç: Ambiguous genitalyalı yeni doğanın, cinsiyet belirlenmesi ve konjenital adrenal hiperplaziye (KAH) tuz kaybetme krizi gibi yaşamı tehlikeye sokan durumların tespit edilmesi için değerlendirilmesi gerekir. Bu yeni doğanlarda cinsiyet belirlenmesi, tanı koyma ve tedaviyle ilgili problemlerin üstesinden gelmeyi sağlar. Bu çalışmamızda, sitogenetik laboratuvarına gönderilen 21 yeni doğanda ambiguous genitalyanın özellikleri ve bu duruma yol açan nedenleri araştırmayı amaçladık.

Gereç ve Yöntem: Her bir vaka için sitogenetik analiz yapıldı. Y kromozomunun varlığını göstermek için vakalar moleküler genetik ve interfaz FISH tekniğiyle analiz edildi. Y kromozom bölgeleri (SRY, ZFY, SY84, SY86, SY127, SY134, SY254, SY255) için moleküler genetik analiz yapıldı. Genomik DNA periferik kandan izole edildi.

Bulgular: Hastaların hepsinde sitogenetik analizle 46,XX ve 46,XY karyotipi tespit edildi. 21 olgu interfaz FISH tekniğiyle başarılı bir şekilde analiz edildi. Bazı bireyler Y kromozomu taşımaktadır ama fenotipik olarak dişidir ya da bir olgu dişi karyotipe sahiptir ama fenotipik olarak erkektir. Genotip (SRY+/-) ve fenotip arasındaki korelasyon hala net değildir.

Sonuç: Ambiguous genitalyanın etiyolojisi değişkenlik göstermektedir. Hekimlerin, ebeveynlere erken karar verebilmesi açısından uygun genetik danışmanlık vermesiyle cinsiyet belirsizliği bir çocuğa sahip olma travmasını hafifletebilir. ©2008, Fırat Üniversitesi, Tıp Fakültesi

Anahtar kelimeler: Ambiguous genitalya, SRY, sitogenetik analiz, interseks bozukluklar

Intersex disorders are rare congenital malformations and the reported incidences of the different malformations with over 80% being diagnosed with congenital adrenal hyperplasia (CAH) (1). 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 (2). The importance of the Y chromosome in male determination has been well established since the 1950's (3, 4). The main factor influencing the sex determination of an embryo is the genetic sex determined by the presence or absence of the Y chromosome (5). As a general rule, XY embryos become males and XX embryos become females. However, some individuals carry a Y chromosome but are phenotypically female (46,XY females) or have a female karyotype but are phenotypically male (46,XX males). 46,XX

maleness is a rare sex reversal syndrome affecting 1 in 20,000 newborn males. Molecular analysis of sex-reversed patients led to the discovery of the SRY gene (7).

In this article was initiated to evaluate a simple (Polimerase chain raction) PCR based DNA diagnostic method by using pseudoautosomal region (PAR) 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. Thus, we evaluate ten 46,XX and eleven 46,XY sex-reversed patients with various phenotypes. In addition to we discuss the syndromes that result from development of testes in subjects with a 46,XX karyotype: 46,XX maleness, 46,XY androgen insensitivity syndrome (AIS) and ambigious genitalia with other syndromic situation.

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MATERIAL AND METHODS

Cases were identified from searching databases held in the department of medical Biology and Genetic of the Firat Medical Center, Elazig, during the study period, from 1998 to 2006. Twenty-one patients were seen in the neonatal period. All children, who were either born with ambiguous genitalia at the Firat Medical Center, Elazig or were referred from other hospitals for investigation were included in this study. We studied the causes and characteristics of ambiguous genitalia in 21 newborn who were referred to a cytogenetic laboratory in our department. All had chromosomal analyses. Karyotyping was performed in 50 metaphases by conventional and G banding techniques. The cases were analysed by also interphase FISH technique using dual prob (Vysis, Ullinois, USA) to exhibit exist Y chromosome. Hybridization was performed in according to the manufacturer's instructions. Dual-labeling hybridization was performed using 10µl of the hybridization mixture containing fluorescein direct labeled chromosome Y alpha-satellite probe and rhodamine direct-labelled SRY gene probe. Genomic DNA was extracted from peripheral leukocytes collected from a venous blood sample. Genomic DNA was extracted by standard methods from peripheral blood of all patients (6). DNA was amplified by PCR using oligonucleotide primers (X specific primer 5'-CTG CAG AAA CAA GCT CAT CAG CGT GAC TAT-3', Y specific primer 5'-GTA CTA CCT TTA GAA AAC TAG TAT TTT CCC-3' ve both of X ve Y specific 5'-GAA TTC TTA ACA GGA CCC ATT TAG-3' and 5'-GAA TTC TTA ACA GGA CCC ATT TAG GAAT TAA-3). The DNA was amplified for 30 cycles with denaturation at 94°C for 5 min, at 94°C for 1 min, at 54°C 1 min, at 72°C for 2 min and extension at 72°C for 6 min using a eppendorf thermal cycler. The PCR products were separated by electrophoresis on 2% per cent agarose gel containing ethidium bromide and photographed using Gel Doc system (Hero Lab, Germany). Product was obtained 771 bp for X and 947 bp for Y. DNA obtained from a men with 46,XY karyotype was used as control. Molecular analysis of sex-reversed patients led to the discovery of the SRY gene. We performed molecular genetic analysis for Y chromosomal loci (SRY, ZFY, SY84, SY86, SY127, SY134, SY254, SY255) blood leukocytes with Y Chromosoma Deletion Kit (Dr.Zeydanlı Life Science, Ankara, Turkey).

RESULTS

All patients presented 46,XX and 46,XY karyotype at cytogenetic analysis. Clinical and cytogenetic findings of the patients are shown in Table1. In eight (38%) of cases have a 46,XX karyotype and eleven (62%) have a 46,XY or Y-containing karyotype (46,XX). Eight patients with 46,XX karyotype were detected CAH (38%). Three patients with female phenotype and 46,XY karyotype were founded AIS (14.2%). 46,XX maleness were defined 4.7 % of all patients. One syndromic children was diagnosed with Edward's Syndrome.

Total of 21 cases were successfully analyzed by interphase-FISH. Some individuals (cases 1, 11, 21) carry a Y chromosome but are phenotypically female or one of cases (cases 6) have a female karyotype but are phenotypically male. It was detected SRY gene and centromeric signals in patients with 46,XX karyotype (Figure 1). The presence of SRY causes the bipotential gonad to develop into a testis. SRY-positive XX males have normal genitalia; in contrast SRY-negative XX

males usually have genital ambiguity. A small number of SRY positive XX males also present with ambiguous genitalia. We obtained 771 bp product for eight patients with 46,XX karyotype, 771 bp and 947 bp products for thirteen patients with 46,XY karyotype. One syndromic children were diagnosed with Edward's Syndrome. It was obtained 648 bp for SRY product. Case 6 was not determined examined other Y chromosomal sequences (Figure 2). This case was sporadic. We weren't found other Y chromosomal sequences in patient with 46,XX karyotype.



Figure 1. The existence of SRY gene was determined by interphase FISH

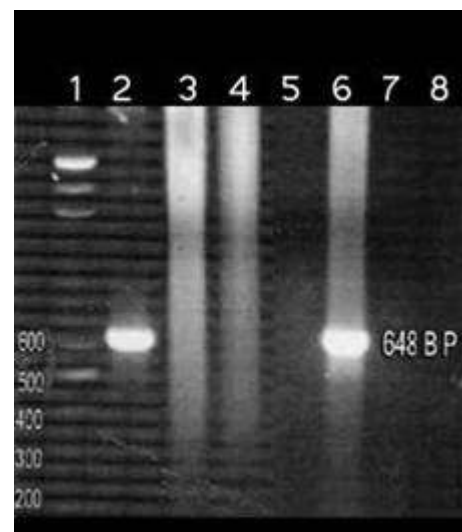


Figure 2. Result of PCR using SRY specific primers. Lane 1: Gene Ruler100 bp DNA ladder plus (Fermentas, USA); lane 2: health males control, lane 3, lane 4, lane 5: SRY negative lanes 6; SRY positive for case 6.

Table 1. Clinical and cytogenetic findings of the patients.

Case	Age(Day)	Social sex	External Genitalia	SRY	Cytogenetic Diagnosis
1	25	F	Ambiguous	+	46,XY (ADS)
2	10	F	Ambiguous	-	46,XX
3	8	F	Ambiguous	-	46,XX
4	2	M	Ambiguous	+	46,XY
5	3	M	Ambiguous	+	46,XY
6	18	M	Ambiguous	+	46,XX
7	7	M	Ambiguous	+	46,XY
8	10	M	Ambiguous	+	46,XY
9	5	F	Ambiguous	-	46,XX
10	2	M	Ambiguous	+	46,XY
11	1	F	Ambiguous	+	46,XY (ADS)
12	8	F	Ambiguous	-	46,XX
13	9	M	Ambiguous	+	46,XY
14	28	M	Ambiguous	+	46,XY
15	7	M	Ambiguous	+	46,XY
16	15	F	Ambiguous	-	46,XX
17	3	F	Ambiguous	-	46,XX
18	19	F	Ambiguous	-	47,XX,+18
19	6	M	Ambiguous	+	46,XY
20	8	M	Ambiguous	+	46,XY
21	12	F	Ambiguous	+	46,XY(ADS)

DISCUSSION

Human sexual differentiation is a highly complex process under the control of multiple genes and hormones. Abnormalities in normal sexual differentiation are relatively common and occur in approximately 1 per 4500 live births (8). Human males with a 46,XX karyotype were first described in 1964 by three different groups of investigators (9, 10). The frequency of this syndrome has been estimated to be 1 in 20,000-25,000 newborn males, although there are considerable geographic variations (11). Molecular analyses have demonstrated that approximately 90% of patients with 46,XX karyotype carry a variable amount of Y material due to a Y-to-X interchange originated by an illegitimate recombination during paternal meiosis (12). In 1999, Kusz et al. demonstrated that in XX males with Y-to-X translocations, preferential inactivation of the Y-bearing X chromosome could be the major mechanism causing a sexually ambiguous phenotype (13). The origin of male phenotype in XX males could be the result of at least three different mechanisms: 1) translocation of Y sequences, including the SRY gene, to an X chromosome or to an autosome; 2) a mutation in a yet unknown X-linked or autosomal gene in the testis-determining pathway, and 3) cryptic Y chromosome mosaicism (14). XX males can be classified as Y-positive or Y-negative depending on the presence or absence of Y-derived specific sequences. Most 46,XX males fail to undergo normal pubertal development; axillary and body hair are generally scant, while pubic hair shows a female pattern of distribution. Gynecomastia occurs in approximately one third of cases and lack of spermatogenesis is always present. External genitalia are underdeveloped and the presence of small testes is a distinctive trait of the syndrome (11). In approximately 15% of XX males hypospadias, cryptorchidism, or severe genital ambiguity is observed (15). We detect presence of SRY gene in our patient (case 6) with male phenotype and 46,XX karyotype. It is a possible mechanism that this patient carry SRY gene due to X to Y translocation. 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 involved in testicular differentiation (16,17).

There was no report of Y specific SRY sequence in female pseudohermaphrodites. We not detected Y chromosomal sequences in female hermaphrodites with 46,XX karyotype and ambiguous genitalia. Our study consistent with previous studies.

Al Agha were determined five syndromic child with ambiguous genitalia. These were: Cardiogenital syndrome (2 cases); Denys-Drash syndrome (1 case); Miller-Dieker syndrome (1 case); and Klinefelter syndrome (1 case) (2). Of all 51 children, 12 (23.5%) had no definite final diagnoses (8). We determined one syndromic patient with ambiguous genitalia and 47,XX,+18. These patients is determined syndromic cases.

Incomplete forms of AIS (PAIS) on the other hand rather present with an incomplete masculinization than a female phenotype and can have a varying range of internal male structures. Frequent features include phenotypic males with a penoscrotal hypospadias, often containing small testes. Additionally a small, blind-ending vaginal pouch without evidence of other female structures can be found. The clinical manifestation of all CAH forms is characterized by the virilization of the outer genitalia. It can be mild with a clitoral hypertrophy or a fusion of the posterior labial folds only, but also as severe as a male phenotype with bilateral undescended testes. On further examination, all patients with a regular karyotype of 46,XX have regular ovaries, Mullerian structures and regressed Mullerian duct syndrome, Wolfman ducts, since the missing SRY prevented the development of Sertoli cells and MIS, respectively (1). Our patients with AIS detected complete AIS syndrome phenotype and 14.3% incidence in all patients. Al Agha were determined 17.2% of ambiguous patients (8).

The birth of a newborn with ambiguous genitalia frequently comes as a surprise for the parents, therapist. Although some authors report that 60% of affected children are diagnosed prenatally, many parents are faced with the situation at birth (1). Physical examination is key to diagnosis. Careful palpation to locate gonads at the genital folds or in the inguinal region provides the first element for diagnostic orientation. If gonads are absent, a diagnosis of female pseudohermaphroditism seems advisable; if gonads are palpated, a diagnosis of male pseudohermaphroditism is more appropriate. Karyotyping is systematic while PCR analysis of the SRY gene provides information about the presence of a Y chromosome within 1 day (2). However, 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 (18,19). The presence of a translocation of chromosomal material encoding the TDF from Y to X chromosome or to an autosome would explain testicular

development in XX sex-reversed PCR techniques of eight 46,XX and eleven 46,XY patients with ambiguous genitalia.

In conclusion, medical management of XX males includes androgen replacement when needed, psychologic orientation to prevent abnormal social and sexual behavior in cases of impaired body image, and reconstructive surgery as soon as the diagnosis is established in individuals with genital ambiguity. Unfortunately, in our retrospective study provides no information concerning the outcomes of psychological and sexual function following the initial decision of sex of rearing. The etiology of ambiguous genitalia is variable. The physician managing these families could minimize the trauma of having a child with unidentified sex by providing appropriate genetic counseling so that the parents can make an early decision. Prenatal DNA testing in at-risk families should be considered and appropriate therapy offered to minimize or prevent genital ambiguity.

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