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Molecular analysis of familial androgen insensitivity syndrome due to replacement of glutamic acid 802 by lysine

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Abstract We studied a Japanese family presenting at least two cases of complete androgen insensitivity syndrome (CAIS) and negative androgen receptor binding. The index subject showed a 46, XY karyotype and a complete female phenotype. For the purpose of further diagnosis and genetic counseling, molecular analysis of the androgen receptor (AR) gene was performed. Direct sequencing of the AR gene identified a mutation at nucleotide 2935 (A → G). This replacement was a novel missense mutation, resulting in the substitution of glutamic acid 802 by lysine which deleted a recognition site for *EcoRI* in exon 6 of the AR gene. We identified another affected individual, using chromosome and molecular analysis of the AR gene at exon 6. Furthermore, although heterozygote carriers could not be identified on clinical grounds, molecular identification of healthy individuals and heterozygote carriers in the family members provided definitive information for genetic counseling. We believe that the molecular analysis of familial CAIS is very informative for both the affected individuals and other family members.

Key words Familial complete androgen insensitivity syndrome · Testicular feminization · Androgen receptor gene · Missense mutation · X-linked recessive inheritance

Introduction

Androgens are responsible for the growth and maturation of male reproductive systems into functional adult sexual organs (Wiener et al. 1997). Testosterone and its derivative 5 α -dihydrotestosterone (5 α -DHT) are major androgens that play crucial roles in male development. Androgen action occurs only in target cells containing androgen receptors (ARs) that regulate the transcription of other androgen-regulated specific genes. Androgen insensitivity syndrome (AIS) (MIM3000681/312300) is a masculinization disorder of the male urogenital tract caused by a defective or impaired AR protein. AIS can be divided into two classes according to phenotype: complete androgen insensitivity syndrome (CAIS) and partial androgen insensitivity syndrome (PAIS). Individuals with CAIS are characterized by a normal female phenotype lacking pubic and axillary hair; PAIS presents at birth with a wide variety of incomplete masculinization of male genital organs. The AR protein is composed of 918 amino acids encoded by an AR gene that has been mapped to the X chromosome and consists of eight exons and intervening introns (Chang et al. 1988a,b; Lubahn et al. 1988). Sequence analysis of the AR gene derived from AIS individuals indicated that a mutation of the AR gene, resulting in replacement of a single amino acid, is the cause of feminization in AIS individuals (Lubahn et al. 1989; Quigley et al. 1995). The frequently observed mutations are nonsense or missense point mutations resulting in the substitution of a single amino acid of the AR (Quigley et al. 1995).

Because children with CAIS have almost normal female phenotype and external genitalia, the diagnosis of AIS is rarely made before the children reach puberty. Rather, CAIS is usually diagnosed in post-pubertal females with primary amenorrhea (Wiener et al. 1997). When AIS is

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suspected, chromosomal sex determination should be performed. Following confirmation of 46, XY, it is necessary to distinguish AIS from other male pseudohermaphroditisms. Definitive diagnosis of AIS was traditionally made using a binding assay, i.e., demonstration of absence or impairment of the patient's AR (Griffin and Wilson 1989). However, binding assays are very laborious and time-consuming. It is now possible to examine the DNA sequence by amplifying each exon of the *AR* gene by polymerase chain reaction (PCR), although a specific phenotype cannot be predicted by detected gene mutations (Lubahn et al. 1989; Hiort et al. 1996). Hormonal findings from AIS individuals, although dependent on age, are also useful for diagnosis. Elevated, male, levels of testosterone are usually observed in postpubertal individuals with AIS.

We report here a family with receptor-negative CAIS caused by a novel missense mutation of the *AR* gene. We describe an approach for the molecular diagnosis of androgen insensitivity and the detection of affected and carrier individuals.

Subjects and methods

Family

The pedigree of the family with AIS is shown in Fig. 1A. The index subject, III-2 (see Fig. 1) was referred by a physician at age 18 years. Clinical and biochemical data concerning the patient showed a 46, XY karyotype, but a female habitus with complete female external genitalia, short vagina ending in a blind pouch, and normal breast development. Serum concentrations of testosterone and 5 α -DHT were 4.7 ng/ml and 0.48 ng/ml, respectively, both within the normal range for males. Laboratory data are summarized in Table 1.

The sister, III-1, and the mother, II-2, of the index subject III-2 had a normal 46, XX karyotype. The mother, II-2, had two sisters: II-3 with primary amenorrhea, suggesting a diagnosis of CAIS, and II-4, who lacked these symptoms and had three children. After the clinical diagnosis of AIS, a laparotomy was performed on the index subject, III-2, and the bilateral intraabdominal gonads were removed. Histological examination showed small but almost normal seminiferous tubes with Sertoli cells and few germinal cells.

Interstitial tissue was hyperplastic and abundant, and was composed of fetal type Leydig cells.

For the purpose of definite diagnosis and genetic counseling of this family, we carried out DNA analysis of the *AR* gene.

Fig. 1A,B. Pedigree and restriction enzyme analysis of a family with androgen insensitivity syndrome (AIS). **A** Individuals in the region enclosed by the dotted line were subjected to molecular analysis. The index subject, III-2, is indicated by the arrow. Black squares indicate affected 46, XY individuals (II-3 and III-2) and a potentially affected individual (III-4). Circles with black dots indicate carriers (I-1 and II-2) and a potential carrier (II-4). The carrier status of individual III-5 (circle with question mark) was uncertain. **B** Polymerase chain reaction (PCR) products, obtained by amplification of genomic DNA by exon 6 primers, were digested by *EcoRI* and then resolved on 2% NuSieve GTG agarose/1% SeaKem GTG agarose. PCR products amplified from a normal allele have an *EcoRI* site that cleaves the 294-bp fragment into 152-bp and 142-bp products. PCR products amplified from a mutant allele are not digested by *EcoRI*. Lanes 3–8 correspond to those individuals enclosed by the dotted line in **A**. Lane 1, Size marker (ϕ X174/*Hae*III) digest; lane 2, normal female control; lane 3, 46, XY father of index subject; lane 4, 46, XX carrier mother; lane 5, 46, XX carrier grandmother; lane 6, 46, XX normal sister; lane 7, 46, XY index subject; lane 8, 46, XY affected aunt

Table 1. Endocrinological data of index subject, III-2^a

Hormone	Laboratory data		Normal range	
	Before gonadectomy	After gonadectomy	Male	Female
Testosterone (ng/ml)	4.7	0.1	2.7–10.7	0.1–0.9
5 α -DHT (ng/ml)	0.48	0.02	0.2–2	0.05–0.3
Estradiol (pg/ml) ^b	44	29	≤50	—
LH (mIU/ml) ^b	24.0	46.0	2.5–13.0	—
FSH (mIU/ml) ^b	4.1	38.2	2.0–12.0	—

LH, Luteinizing hormone; FSH, follicle-stimulating hormone; DHT, dihydrotestosterone

^a Subject III-2, as in Fig. 1

^b The normal female ranges depend on the menstrual cycle

PCR and DNA sequencing

DNA was isolated from the peripheral blood leukocytes of the index subject, III-2, using a QiaAmp DNA blood kit (Qiagen, Hilden, Germany). Exons 2–8 of the *AR* gene were amplified by PCR, using a programmable thermal cycler, 9700 (PE Applied Biosystems, Foster City, CA, USA). The primer sets and cycling conditions were as described previously (Kasumi et al. 1993; Komori et al. 1997). Sequencing analyses were performed using an ABI 373A DNA sequencer (PE Applied Biosystems). Each reaction was repeated at least twice to avoid misreading. Genomic DNA of the family members was also isolated from peripheral leukocytes, and exon 6 was amplified by PCR.

Digestion of exon 6 DNA with the restriction enzyme *EcoRI*

Amplified PCR products of exon 6 were digested with the restriction enzyme *EcoRI* (GIBCO BRL, Rockville, MD, USA) at 37°C for 180 min. The reaction products were electrophoresed on a 2% NuSieve GTG agarose/1% SeaKem GTG agarose (FMC, Rockland, ME, USA) gel, stained in ethidium bromide solution, and photographed.

Hormone-binding assay

Genital skin biopsies of the index subject, III-2, and subject II-3 were performed to obtain fibroblasts for hormone-binding assay. Androgen-binding ability was measured in cultured genital skin fibroblasts according to the method described previously by Kasumi et al. (1993).

Results

Analysis of each exon sequence

The predicted size of PCR products of each exon was 384 bp (exon 2), 413 bp (exon 3), 418 bp (exon 4), 285 bp (exon 5), 295 bp (exon 6), 416 bp (exon 7), and 347 bp (exon 8). The amplified products of the DNA of the index subject, III-2, were checked by electrophoresis. A novel missense mutation at nucleotide 2935 (A → G) in exon 6 was identified by sequencing (Fig. 2). This mutation resulted in the substitution of glutamic acid 802 by lysine and loss of the *EcoRI* restriction site around nucleotide 2935 (Fig. 2).

Restriction enzyme analysis

The PCR products of the sister, III-1; mother, II-2; father, II-1; individual II-3; and the index subject, III-2 in exon 6 were subjected to restriction enzyme analysis by *EcoRI* (Fig. 1B). The PCR products of the sister, III-1, were digested by *EcoRI* and showed that she is homozygous for the normal *AR* gene. Restriction enzyme analysis of the exon 6

Fig. 2. Partial sequence of androgen receptor (*AR*) gene exon 6 of index subject, III-2. A single nucleotide mutation (G → A) is shown at nucleotide 2935, resulting in the substitution of glutamic acid by lysine. This mutation deletes an *EcoRI* site that cleaves the 294-bp fragment into 152-bp and 142-bp fragments. Nucleotide numbering was as described by Chang et al. (1988b)

PCR products of the mother, II-2, demonstrated the presence of both canonical and mutated bases, which indicated that she is a heterozygous carrier for the mutant gene. Individual II-3, with a 46, XY karyotype, had one mutant *AR* gene, and the father, II-1, with a 46, XY karyotype, had one normal *AR* gene. The relationship between the pedigree of the family and restriction analysis results is presented in Fig. 1A,B.

Hormone-binding activity

Androgen binding activity was measured using a whole cell binding assay. In the genital skin fibroblasts of the index subject, III-2, and individual II-3 no androgen binding was detected; the dissociation constant (*K_d*) for both individuals was undetectable, whereas normal skin fibroblasts showed an androgen-binding capacity (*B_{max}*) of 5000 sites/cell and a *K_d* of 5×10^{-10} nM.

Discussion

It is well known that AIS is caused by mutations in the *AR* gene and is inherited as an X-linked recessive trait (Quigley et al. 1995). To date, 188 cases of CAIS caused by *AR* gene defects have been reported. In this study we identified a novel missense mutation at nucleotide 2935 (A → G), which resulted in the substitution of glutamic acid 802 by lysine in exon 6 (Gottlieb et al. 1999). Exon 6 is composed of 131 bp and encodes the steroid-binding domain (Lubahn et al. 1989). Because the principal function of the steroid-binding domain is the specific and high-affinity binding of androgen, amino acid substitutions in this domain can cause complete absence of androgen binding (Quigley et al. 1995). These findings account for the patients in this study showing no

androgen binding. Although the phenotypes of both affected individuals, III-2 and II-3, were identical in this family, other studies have shown that two unrelated individuals with the identical single nucleotide mutation had markedly different phenotypes (McPhaul et al. 1992). This finding indicates that other factors influence the phenotype of AIS individuals.

As the diagnosis of AIS is possible by chromosomal analysis, laboratory tests, and clinical phenotype, molecular analysis of the AIS individual is not always performed. The management of individuals with various clinical forms of AIS has been based primarily on analysis of the phenotype of the affected individual and the likelihood of a successful psychosexual outcome (Quigley et al. 1995). However, molecular analysis is necessary to reveal the distribution of mutant genes in the family and to detect heterozygous carriers of the 46, XX karyotype. In the family reported here, the index subject, III-2, showed a typical AIS phenotype. Molecular analysis was planned because determination of the carrier status of her sister, III-1, and her mother, II-2, was requested. When the index subject, III-2, was diagnosed as having AIS, her sister, III-1, was engaged to be married, and was deeply concerned about the possible effect on children (for any carrier female there is a 1:2 chance for a child with 46, XY to develop AIS). In the absence of reliable clinical signs, detection of female carriers in an AIS-affected family is most accurately determined by molecular analysis of the *AR* gene (Quigley et al. 1995). Molecular analysis showed that the sister, III-1, was homozygous for the normal *AR* gene, which alleviated her concern. Although AIS is inherited as an X-linked recessive trait, there is the possibility that the original mutation in this family had occurred as a de-novo mutation in the index subject, III-2. If the mother, II-2, had been homozygous for the normal *AR* gene, further examination of the family members would not have been necessary. Unfortunately, test results indicated that the mother, II-2, and the grandmother, I-1, were possible carriers, and we decided to extend the investigation to other family members. Individual II-3 was diagnosed with CAIS by phenotype and by molecular and cytogenetic analyses. She also had a gonadectomy soon after the diagnosis. Our findings lead us to suspect another affected individual, III-4, and a carrier, II-4, although this has been difficult to confirm, because neither of these subjects have agreed, as yet, to undergo genetic testing. Individual III-4 has a typical CAIS phenotype similar to that of the index subject, III-2.

The identification of a mutation provides precise information that allows for genetic counseling of families affected by AIS. However, genetic counseling and explanation of the condition to AIS individuals involves very delicate problems and varies with each individual situation. The index subject, III-2, was under age and we explained her condition to her in agreement with the wishes of her parents (II-1 and II-2). The affected individuals, II-3 and III-2, were informed that their gonads had functional abnormality caused by hormonal insufficiency, that pregnancy could not be expected, and that gonadectomy was

necessary to prevent malignant change. However, we did not refer to the sex chromosome abnormality, because the fact that they had XY chromosomes may have caused a serious mental shock.

Once a precise *AR* gene mutation is defined in an affected family, it is also possible to perform prenatal diagnosis by analyzing the appropriate region of the *AR* gene in DNA obtained by chorionic villus sampling or amniocentesis (Quigley et al. 1995). Fortunately, in the family reported here, the sister, III-1, was not a carrier, and prenatal diagnosis was not considered. Although there are ethical issues involved in the prenatal diagnosis of AIS, *EcoRI* digestion analysis made prenatal diagnosis technically possible in this family.

We conclude that the molecular analysis of familial CAIS is very informative for both the affected individuals and for other family members.

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