Premature Termination Mutation ($^{772}\text{Glu}\rightarrow\text{Stop}$) in the Hormone-Binding Domain of the Androgen Receptor in a Patient with the Receptor-Negative Form of Complete Androgen Insensitivity Syndrome

KYOSUKE IMASAKI, TAJIRO OKABE, HIROSHI MURAKAMI*, KEINOSUKE FUJITA**, RYOICHI TAKAYANAGI, AND HAJIME NAWATA

Third Department of Internal Medicine, Faculty of Medicine, Kyushu University, Fukuoka 812,

**Department of Pediatrics, Osaka City University Medical School, Osaka 545, and **Department of Pediatrics, Children's Medical Center, Osaka City General Hospital, Osaka 534, Japan*

Abstract. We have characterized the androgen receptor in a Japanese infant with complete androgen insensitivity syndrome (or androgen resistance), and have investigated the molecular basis. Androgen binding was undetectable in cultured genital skin fibroblasts from this patient by whole-cell androgen receptor binding assay. Sequence analysis of the entire coding region of the androgen receptor gene from this patient revealed a single nucleotide substitution (G→T) at nucleotide position 2676 in exon E (or 5), resulting in conversion of glutamine codon (GAG) to amber stop codon (TAG) at amino acid position 772 within the hormone-binding domain of the androgen receptor. This premature termination mutation (or nonsense mutation), introducing a truncated androgen receptor that lacks most of its androgen binding capacity, is thought to cause the receptor-negative form of complete androgen insensitivity syndrome in this patient.

Key words: Androgen receptor, Complete androgen insensitivity syndrome, Receptor-negative form, Premature termination mutation, Constitutive transcriptional activity

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THE HUMAN androgen receptor (AR) with a molecular weight of 110 kDa is a single polypeptide which consists of 919 amino acid residues, deduced from the structure of the human AR cDNA [1, 2]. The structure of the AR, like the other members of the superfamily of steroid/thyroid/vitamin D/retinoid receptors, includes three domains: a variable amino-terminal domain associated with the regulation of the target gene transcription, a central cysteine-rich hydrophilic DNA-binding domain, and a hydrophobic carboxyl-terminal hormone-

binding domain. The human AR gene, which is approximately 90 kilobases (exons and introns) in size, is located on the X chromosome at position Xq11–12 [2, 3] and is divided into eight exons [4]. The amino-terminal domain is encoded by exon A (or 1), the DNA-binding domain by exons B and C (or 2 and 3), and the hormone-binding domain by exons D–H (or 4–8).

Androgen, mediated by the AR, plays an indispensable role in the prenatal genital virilization in a 46, XY fetus. Genetic defects in the AR gene give rise to androgen insensitivity syndrome (AIS), in which the reduced androgen effect leads to various degrees of disturbance of genital virilization. AIS is the major cause of male pseudohermaphroditism. The genetic defects of the AR can cause a wide spectrum of AIS ranging from phenotypic

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Correspondence to: Dr. Kyosuke IMASAKI, Third Department of Internal Medicine, Faculty of Medicine, Kyushu University, 3–1–1 Maidashi, Higashi-ku, Fukuoka 812, Japan

females with complete or incomplete AIS to phenotypic males with Reifenstein syndrome or infertile male syndrome [5].

Herein, we report the analysis of the AR gene of a Japanese infant with the receptor-negative form of complete AIS. Our study revealed a premature termination mutation (⁷⁷²Glu→stop) in exon E within the hormone-binding domain of the AR, which may be responsible for the pathogenesis of complete AIS in this patient.

(The cDNA and deduced amino acid sequences of the human AR reported by several laboratories vary in the lengths of the glutamine and glycine homopolymeric segments in exon A. In this report, the nucleotide and amino acid numeration is according to the sequence of Lubahn *et al.* [6] to indicate the position of the mutation.)

Materials and Methods

A patient

The patient was identified at birth as female. This patient underwent the surgery for bilateral inguinal hernias at the age of 1 week, and then the hernias turned out to contain masses which were histologically diagnosed as testes by biopsy. Moreover, the karyotype of this patient turned out to be 46, XY. This patient was therefore referred to our hospital for evaluation of male pseudohermaphroditism at the age of 1 month. No clitoromegaly and no labial fusion were found in the external genitalia. No uterus was found. Endocrinological studies revealed that serum testosterone (T) and 5 α -dihydrotestosterone (DHT) levels before and af-

ter hCG stimulation test were within the normal range for this patient's age, and serum LH and FSH responses to GnRH stimulation test were higher than in normal infants (Table 1). This patient was therefore diagnosed as having complete AIS. A younger sibling of this patient is also affected with complete AIS.

Cell culture

Fibroblasts were obtained from punch biopsy of genital skin of the complete AIS patient and normal controls (46, XY, normal males). Informed consent for genital skin biopsy was obtained from each individual according to the Johns Hopkins Institutional Guidelines for Clinical Investigation. Genital skin fibroblasts were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 2 mmol/L L-glutamine and 10% fetal calf serum (FCS). Cultures were maintained in a humidified incubator at 37 °C in 5% CO₂.

Whole-cell binding assay

Androgen binding in cultured genital skin fibroblasts was measured by a method previously described [7]. Briefly, genital skin fibroblasts obtained from the patient and normal controls were grown to confluence in twelve 75 cm² culture flasks, and the medium was changed to FCS-free medium at 36 h before the assay. On the day of the assay, the cells were harvested with trypsin-EDTA and suspended in Medium C (FCS-free MEM with 2 mmol/L L-glutamine and 25 mmol/L tricin). For the binding assay, duplicate aliquots (0.5 ml) of well-mixed cell suspension were incubated for 1 h

Table 1. Endocrinological studies of the patient

	GnRH test		hCG test		
	LH (mIU/ml)	FSH (mIU/ml)	T (ng/ml)	DHT (ng/ml)	T/DHT ratio
Basal	0.5	1.9	1.054	0.06	17.6
Peak	47.5	9.7	21.17	4.02	5.27

After overnight fasting, GnRH (2 μ g/kg) was administered intravenously, and serum samples were obtained at 30-min intervals for 2 h. hCG (1000 U, once a day for 3 days) was administered intramuscularly and serum samples were obtained before the first injection and at 24, 48, 72 and 96 h after the last injection. GnRH test and hCG test were performed at the age of 7 months and 1 month, respectively. LH and FSH responses to GnRH test were within the normal range at the age of 1 month (data not shown), but were higher than in normal infants at the age of 7 months as shown in this table.

at room temperature with various concentrations (0.1–2.0 nmol/L) of 7 α , 17 α -dimethyl [17 α -methyl-³H]-19-nortestosterone ([³H]Mibolerone) in the presence or absence of a 1000-fold excess of unlabeled Mibolerone contained in 0.5 ml Medium C. After the incubation, the cells were washed three times with phosphate-buffered saline (PBS) and lysed with 1.0 ml ethanol. The lysates were transferred to a scintillation vial containing 10 ml aquasol to count radioactivity. Specific binding of [³H] Mibolerone was calculated by subtracting non-specific binding from total binding. The maximum binding capacity was derived from Scatchard analysis [8] by a computer-assisted linear regression method.

PCR amplification and direct sequencing of the AR gene

Genomic DNA for polymerase chain reaction (PCR) was prepared from cultured genital skin fibroblasts of the patient and normal controls as previously described [9]. Oligonucleotides flanking each exon of the AR gene used as PCR primers were the same as used in our previous study [10, 11]. PCR was carried out as previously described [10, 11]. The purified PCR products were directly sequenced with inner primers (sequences not shown).

Digestion of exon E PCR products with the restriction enzyme Tsp EI

The exon E fragments of the AR gene amplified by PCR were digested with the restriction enzyme of Tsp EI at 65 °C for 10 h, and electrophoresed on 2.0% agarose gel and visualized under ultraviolet light after being stained with ethidium bromide.

Results

Whole-cell binding assay

The saturation curve and Scatchard plot of [³H]Mibolerone binding to cultured pubic skin fibroblasts are shown in Fig. 1. Androgen binding was undetectable in fibroblasts from the patient compared with normal male controls. This patient is therefore affected with the receptor-negative form of AIS.

Fig. 1. Saturation curve and Scatchard plot (inset) of [³H] Mibolerone binding to cultured pubic skin fibroblasts in the patient. Open circles and shaded area: normal controls (46, XY, healthy males, age 23–41 years; N=5), closed circles: patient.

Sequence analysis of each exon of the AR gene

For further investigation, the molecular approach was carried out with genomic DNA extracted from the patient's fibroblasts. The sequence analysis revealed a single nucleotide substitution (G→T) in exon E within the hormone-binding domain of the AR. Glutamine codon (GAG) at amino acid position 772 was replaced by premature termination codon (TAG) (Fig. 2A.). No mutations were found in the other exons. The number of glutamine codons (CAG repeats) in the glutamine homopolymeric segment in exon A was 19, which is within the normal range (14–35 glutamine codons) of variations in the length of this segment [12].

Digestion of exon E PCR products with Tsp EI

In order to confirm the presence of this mutation in exon E of the patient, the digestion of PCR

Fig. 2. A: Nucleotide sequences of exons E and F and the flanking intron of the androgen receptor gene. The figure shows a single nucleotide substitution resulting in conversion of glutamine codon (GAG) to amber stop codon (TAG) at amino acid position 772 by direct sequencing. Lanes 1 and 2 indicate the patient and the normal control, respectively. B: Schematic presentation of the structure of the androgen receptor in the patient. The figure indicates the position of the premature termination mutation resulting in truncation of the androgen receptor protein.

products of exon E by the restriction enzyme of *Tsp* EI was performed. Because *Tsp* EI recognizes the 4-base pair (bp) sequence of "AATT", *Tsp* EI cannot digest the normal exon E DNA fragment; that is, the normal exon E fragment is still only one fragment after *Tsp* EI digestion. On the other hand, since the point mutation (G→T) occurred in the patient's AR gene, *Tsp* EI can digest and divide the patient's exon E DNA fragment (283 bp in length) into two fragments (189 bp and 90 bp in length, respectively) (Fig. 3A). As shown in Fig. 3, the result confirmed that the AR gene in the patient had the point mutation.

Discussion

Since the human AR gene was identified [4], a number of genetic studies of AIS have been reported. The elucidation of the molecular basis of AIS has greatly contributed to a general understanding of the structure-function relationship in the human AR. Furthermore, molecular analysis of the AR gene has revealed that there is a wide variety of molecular defects underlying the clinical and biochemical heterogeneity of AIS [13]. Several types of mutations of the AR are now recognized: complete or partial gene deletion, 1–4-base deletion or insertion, and 1-base substitution causing amino acid substitution, premature termination

Fig. 3. A: Agarose gel electrophoresis of exon E PCR products digested with *TspEI*. The left lane contains the DNA size standard Φ X174 digested with *HincII*. Lanes 1 and 2 indicate the patient and the normal control, respectively. B: The scheme of the *TspEI* site of exon E in the patient. The open box and the connecting lines with it indicate exon E and flanking regions of exon E, respectively. E1 and E2 indicate the positions of the PCR primers. The sign (—|) indicates the restriction site of *TspEI*.

mutation, or aberrant splicing [13]. All of these types of mutations have been reported to be associated with the receptor-negative form of complete AIS.

In the present study, we identified a single base substitution ($^{2676}\text{G} \rightarrow \text{T}$) in exon E of the AR gene in the patient with the receptor-negative form of complete AIS. This single nucleotide substitution introduces a premature termination codon ($^{772}\text{Gln} \rightarrow \text{stop}$) within the hormone-binding domain, which would remove 148 carboxy terminal amino acids of the AR protein. The truncated AR that lacks most of its androgen binding capacity is thought

to cause the receptor-negative form of complete AIS in this patient.

This mutation that we detected is a new one that has not been reported previously. Premature termination mutations of the AR gene have been reported thus far in all exons except for exon B in approximately 10 cases with complete AIS (^{60}Glu , ^{590}Lys , ^{718}Trp , ^{752}Arg , ^{786}Arg , ^{796}Trp , ^{831}Arg , ^{853}Ser , $^{883}\text{Lys} \rightarrow \text{stop}$) [14–19].

Premature termination mutations result in truncation of the AR protein to various degrees, depending on the location of the mutations. In each case, androgen binding was undetectable and, not surprisingly, all patients with this type of mutation reported so far have had the clinical phenotype of complete AIS. In most of these cases, the truncated AR protein was expressed at a very low level or not at all. Interestingly, however, in three of these cases, immunoblotting (Western blotting) or immunocytochemical studies have demonstrated that the truncated AR protein was expressed at the normal level [14, 16, 17]. Thus, the location of the premature termination mutation would determine its effect on the AR mRNA stability. *In vitro* mutagenesis studies have demonstrated that the deletion of the entire hormone-binding domain results in a nuclear AR with a low level of constitutive (hormone-independent) transcriptional activity [20]. Without exception, however, all patients with a premature termination mutation of the AR reported so far have had the clinical phenotype of complete AIS, and that indicated that such a truncated AR protein does not have constitutive (hormone-independent) transcriptional activity *in vivo*, in contrast with the above observations *in vitro*. The cause of the discrepancy between *in vivo* and *in vitro* is unclear at present. We are planning to investigate whether the above discrepancy exists or not in the patient reported in this paper. Moreover, the elucidation of the cause of the above discrepancy would greatly contribute to a further understanding of the mechanism of the AR function.

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