Sporadic Reifenstein Syndrome due to a *De Novo* Mutation (⁷⁴⁶Val→Met) of the Androgen Receptor

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Abstract We have analyzed the androgen receptor (AR) of a young Japanese man with sporadic Reifenstein syndrome. Androgen binding assays in cultured genital skin fibroblasts showed a normal maximum binding capacity and a normal apparent dissociation constant. However, androgen binding in fibroblasts decreased to 37 % when the assay temperature was raised from 30 °C to 41 °C, indicating thermolability of the AR. Sequence analysis of the AR gene revealed a single nucleotide substitution (G→A) at nucleotide position 2,598 in exon E (or 5), resulting in conversion of valine (GTG) to methionine (ATG) at amino acid position 746 within the hormone-binding domain of the AR. This missense mutation may explain thermolability of the AR, which is thought to be the pathogenesis of Reifenstein syndrome in this patient. Sequence analysis also revealed that his mother was not a heterozygous carrier of this AR mutation. Moreover, sequence analysis of the D loop in the mitochondrial deoxyribonucleic acid (DNA) confirmed the familial relationship between this patient and his mother. These findings strongly suggest that this AR mutation is a *de novo* mutation newly occurring in this patient's generation.

Key words: androgen insensitivity syndrome, Reifenstein syndrome, androgen receptor, thermolability, missense mutation, *de novo* mutation

Introduction

The human androgen receptor (AR), with a molecular weight of 110 kDa, is a single polypeptide which consists of 919 amino acid components, deduced from the structure of

human AR complementary DNA (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

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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 of a 46, XY fetus. Genetic defects in the AR gene give rise to androgen insensitivity syndrome (AIS), in which a reduced androgen effect leads to various degrees of disturbance of genital virilization. These genetic defects can cause a wide spectrum of AIS ranging from phenotypic females with complete or incomplete AIS to phenotypic males with Reifenstein syndrome or infertile male syndrome (5). Reifenstein syndrome is one of the four phenotypes of AIS, and is characterized by male phenotype, hypospadias, micropenis, bifid scrotum, infertility, and gynecomastia.

Herein, we report the analysis of the AR gene of a young Japanese patient with sporadic Reifenstein syndrome associated with a thermolabile AR. Our study revealed a single amino acid substitution ($^{746}\text{Val} \rightarrow \text{Met}$) within the hormone-binding domain of the AR, which may explain thermolability of the AR.

(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 positions of the mutations.)

Materials and Methods

The patient

A 17-yr-old Japanese man was referred to our hospital for evaluation of gynecomastia, which had been present since the age of 14 years. He had undergone surgery for hypospadias at the age of 4 years. Micropenis (2.5 cm in length), bifid scrotum, no beard, decrease of pubic hair (Tanner stage III), and palpable mammary glands were noted. He had a normal male karyotype (46, XY). The testes were palpable (right 8 mL, left 10 mL in volume) in the bifid scrotum. Microscopic examination of biopsy samples obtained from the left testis showed a few spermatocytes (Fig. 1). Endocrinological examination revealed that serum luteinizing hormone (LH), testosterone (T), and 5α -dihydrotestosterone (DHT) levels were slightly high, serum follicle stimulating hormone (FSH) level

Fig. 1 Histological findings of the biopsy sample obtained from the left testis of the patient at the age of 17 years (Hematoxylin and eosin stain). The seminiferous tubules show hyalinization. A few spermatocytes and Sertoli cells are present. Thickness of the basement membranes can be seen. The stroma contains a few Leydig cells without cluster formation.

was slightly low, and the serum LH response to the FSH and LH-releasing hormone (GnRH) stimulation test was higher than in normal adult males (Table 1). There was no family history of similar disease. His elder brother was not affected with AIS. He was therefore diagnosed as a case of sporadic Reifenstein syndrome.

Cell culture

Fibroblasts were obtained from punch biopsies of the genital skin of the 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 in 5 % CO₂ at 37 °C.

Table 1 Endocrinological data of the patient (at the age of 17 years).

	GnRH test		hCG test		
	LH (mIU/ml)	FSH (mIU/ml)	T (ng/ml)	DHT (ng/ml)	T / DHT ratio
Basal	5.9 (0.3-3.5)	1.3 (1.7-7.4)	13.6 (5.5-8.9)	1.20 (0.20-0.75)	11.3
Peak	80.8 (8.6-32.1)	3.4 (5.4-16.3)	23.5 (11.1-13.7)	1.86	15.4

Serum LH and FSH were measured by IRMA. Serum T and DHT were measured by RIA. After overnight fasting, GnRH (100 µg) was administered intravenously, and serum samples were obtained at 30-min intervals for 2 h. hCG (4,000 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.

Whole-cell androgen binding assays

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 assays. On the day of the assays, the cells were harvested with trypsin-ethylenediaminetetraacetic acid (EDTA) and suspended in Medium C (FCS-free MEM with 2 mmol/L L-glutamine and 25 mmol/L tricin). For the binding assays, duplicate aliquots (0.5 mL) of well-mixed cell suspension were incubated for 1 h at room temperature with various concentrations (0.1-2.0 nmol/L) of 7α, 17α-dimethyl [17α-methyl-³H]-19-nortestosterone ([³H] Mibolerone) in the presence or absence of a 1,000-fold excess of non-labeled Mibolerone contained in 0.5 mL Medium C. After incubation, the cells were washed three times with phosphate-buffered saline (PBS) and lysed with 1.0 mL ethanol. The lysates were transferred to scintillation vials 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.

The effect of increase in incubation temperature from 30 °C to 41 °C was studied by measurement of specific [³H]Mibolerone binding only with a saturating concentration (2.0 nmol/L) of [³H]Mibolerone.

Polymerase chain reaction (PCR) amplification and direct sequencing of the AR gene

Genomic DNA for PCR was prepared from peripheral leukocytes of the patient, his mother, and normal controls as previously described (9). Oligonucleotides flanking each exon of the AR gene used as PCR primers were the same as those 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 *Bsp*HI

The exon E fragments of the AR gene amplified by PCR were digested with the restriction enzyme *Bsp*HI at 37 °C for 10 h, electrophoresed on 1.4 % agarose gel, and visualized under ultraviolet light after being stained with ethidium bromide.

PCR amplification and direct sequencing of the D loop in the mitochondrial DNA

The mitochondrial DNA (mtDNA) for PCR was prepared from peripheral leukocytes of the patient and his mother as previously described (9). PCR was carried out as previously described (12). The purified PCR products were directly sequenced with inner primers. PCR primers and sequencing inner primers were the same as those previously described (12).

Results

Androgen binding assays

The maximum binding capacity (Bmax) and the apparent dissociation constant (Kd) for

Fig. 2 Saturation curve and Scatchard plot (inset) of $[^3\text{H}]$ Mibolerone binding to cultured genital skin fibroblasts of the patient. Open circles and shaded area: normal controls; closed circles: patient. Normal controls: age 23-41 years, n=5.

the ARs in genital skin fibroblasts were 8,007 sites/cell and 0.41 nmol/L, respectively [Normal: Bmax 6,924 $\pm$ 2,040 sites/cell, Kd 0.56 $\pm$ 0.23 nmol/L (mean $\pm$ standard deviation (SD); n=5)], indicating normal capacity and affinity in the patient (Fig. 2). When the incubation temperature was increased from 30 °C to 41 °C, the amount of androgen binding in fibroblasts from the patient decreased to 37 % of that seen at 30 °C with $[^3\text{H}]$ Mibolerone as the binding ligand, indicating thermolability of ligand binding to the AR (Fig. 3). In contrast, binding in fibroblasts of normal controls decreased by less than 10 % under the same conditions. In other words,

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Fig. 3 Effect of temperature on [3 H]Mibolerone binding to cultured genital skin fibroblasts of the patient. Decline of specific binding was expressed as percentage of baseline calculated as $[100 \times (\text{specific binding at } 41^\circ\text{C})/(\text{specific binding at } 30^\circ\text{C})]$. Normal controls: age 1-30 years, $n=3$.

the patient was affected with the receptor-thermolabile type of Reifenshtein syndrome.

Sequence analysis of each exon of the AR gene

For further investigation, the molecular approach was carried out with genomic DNA extracted from the peripheral leukocytes. Sequence analysis of the entire coding regions of the AR gene revealed a single nucleotide substitution in exon E within the hormone-binding domain of the AR in the patient. Namely, valine codon (GTG) at amino acid position 746 was replaced by methionine codon (ATG) (Fig. 4.A).

Fig. 4

A Nucleotide sequences of exon E of the androgen receptor gene. The figure shows a single nucleotide substitution resulting in conversion of valine codon (GTG) to methionine codon (ATG) at amino acid position 746. Lanes 1, 2, and 3 indicate the patient, his mother, and a normal control, respectively.

B Schematic presentation of the structure of the androgen receptor in the patient. The figure indicates the position of the nucleotide substitution, which results in the amino acid alteration (valine to methionine).

No mutations were found in the other exons. The number of glutamine codons (CAG repeats) in the glutamine homopolymeric segment in exon A was 26, which was within the normal range (14-35 glutamine codons) of variations in the length of this segment (13). On the other hand, sequence analysis also revealed that the patient's mother did not have this point mutation; in other words, his mother was not a heterozygous carrier for this mutation (Fig. 4.A). Thus, this mutation was suspected to be a *de novo* mutation occurring in the patient's gener-

ation.

Digestion of exon E PCR products with *Bsp*HI

In order to confirm the presence or absence of this mutation in exon E of the patient and his mother, the digestion of PCR products of exon E by the restriction enzyme *Bsp*HI was performed. Because *Bsp*HI recognizes the 6-base pair (bp) sequence of "TCATGA", it could not digest the normal exon E DNA fragment; in other words, the normal exon E fragment was still only one

fragment after *Bsp*HI digestion. However, because the point mutation (G→A) occurred in the patient's exon E, *Bsp*HI could digest and divide the patient's exon E DNA fragment (283 bp in length) into two fragments (173 bp and 110 bp in length). As shown in Fig. 5.A, the result confirmed that the patient had this point mutation, but his mother did not have this one.

Sequence analysis of the D loop in the mtDNA

Because the animal mtDNA is maternally inherited, the examination of sequence polymorphism in the mtDNA has been reported to be very useful for testing whether individuals are maternally related (12). Therefore, in order to confirm the familial relationship between the patient and his mother, sequence analysis of a 347-bp section (nucleotide positions 16,042 to 16,388) at one end of the D loop in the mtDNA was performed. The result showed that nucleotide sequences of the 347-bp section of the D loop in the mtDNA of the patient and his mother were perfectly identical, which determined their familial relationship. This result confirmed that the mutation of the patient was certainly a *de novo* mutation newly occurring in the patient's generation.

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 the general understanding of the structure-function relation in the human AR. Furthermore, molecular analysis of the AR gene has revealed that there is a wide variety of molecular defects underlying

Fig. 5

A Agarose gel electrophoresis of exon E PCR products digested with *Bsp*HI. The left lane contains the DNA size standard Φ X174 digested with *Hinc*II. Lanes 1, 2, and 3 indicate the patient, his mother, and a normal control, respectively.

B The location of the *Bsp*HI 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 *Bsp*HI.

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the clinical and biochemical heterogeneity of AIS (14). 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 mutation, or aberrant splicing (14). Among these mutations of the AR, many are single amino acid substitutions in the hormone-binding domain. As far as Reifenstein syndrome is concerned, single amino acid substitutions in the DNA- and hormone-binding domain have been previously reported. In the present study, we identified a single amino acid change from valine to methionine at position 746 within the hormone-binding domain of the AR in a patient with sporadic Reifenstein syndrome. Moreover, androgen binding to the AR in this patient showed the qualitative abnormality of thermolability. These findings suggest that this mutation is responsible for the pathogenesis of Reifenstein syndrome in this patient, although an expression study using the mutant AR remains to be performed to prove it.

The mechanism by which such an amino acid substitution within the hormone-binding domain of the AR causes dysfunction of the AR and shows the characteristic of thermolability remains unclear at present. However, this mutation is located within the "hot spot" regions for mutation (approximately corresponding to exons E and G) which have been reported to be particularly important for the formation and function of the hormone-receptor complex (15). Therefore this mutation of the AR may cause a conformational change which may affect the stability of the hormone-receptor complex.

Moreover, the mutation (⁷⁴¹Trp→Arg) of the AR, which is located near the mutation reported

in this paper (⁷⁴⁶Val→Met), has been reported to be associated with the receptor-negative type of complete AIS (15). Thus, a slight difference in mutation sites (at a distance of 5 amino acid components) determines whether the mutant AR is the thermolabile type or the binding-negative type, suggesting that the conformation of the tertiary structure of the AR is very important for the binding between the AR and the androgen.

Interestingly, the patient's mother was not a heterozygous carrier for the AR mutation. The familial relationship between the patient and his mother was confirmed by direct sequencing of the D loop in the mtDNA. Thus, this mutation is certainly a *de novo* mutation occurring in the patient's generation, and is suspected to have occurred in the oocyte in his mother's ovary. To our knowledge, there have been only two previous reports about a *de novo* mutation of the AR gene, in which, however, the familial relationship between the AIS patient and the mother was not confirmed (16, 17). This report, therefore, is the first one about a *de novo* mutation of the AR gene in an AIS patient with confirmation of the familial relationship.

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