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Point Mutations in the Steroid-Binding Domain of the Androgen Receptor Gene of Five Japanese Patients with Androgen Insensitivity Syndrome

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Yaegashi, N., Uehara, S., Senoo, M., Sato, J., Fujiwara, J., Funato, T., Sasaki, T. and Yajima, A. *Point Mutations in the Steroid-Binding Domain of the Androgen Receptor Gene of Five Japanese Patients with Androgen Insensitivity Syndrome.* Tohoku J. Exp. Med., 1999, **187** (3), 263-272 —— We analyzed the androgen receptor (AR) gene in five Japanese patients diagnosed with androgen insensitivity syndrome (AIS). All AR genes from the five patients had single-nucleotide substitutions, which introduced a premature termination codon in three patients (Gln640, Arg752, and Gln640 and Trp751), and a single amino acid substitution in two patients (Arg831 to Gln, and Leu812 to Phe). All the mutations occurred in the steroid-binding domain, comprising exons D through G. The three patients with the premature termination codon(s) and the one patient with Arg831Gln were clinically diagnosed as having complete AIS, while the patient with Leu812Phe had a partial form of AIS. Pubic skin fibroblasts from four of the five patients did not show detectable androgen binding. These data on mutations that have not been reported previously, provide valuable information for the further characterization of structural and functional relationships in the steroid-binding domain of the AR protein. ———— androgen receptor; nucleotide substitution; nonsense mutation; missense mutation

Androgen insensitivity syndrome (AIS) is an X-linked hereditary disease marked by abnormal sex differentiation, with an estimated prevalence of 1 in 65 000 males (Jagiello and Atwell 1962). AIS is classified into complete and partial (incomplete) forms. The complete form of AIS (CAIS) is characterized by the presence of unambiguous female external genitalia without pubic hair deve-

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lopment, a blindly ending vagina, intra-abdominal or inguinal testes, and the absence of Wolffian and Mülerian derivatives. The phenotype of the partial form (PAIS) varies in the degree of the virilization of external genitalia: Some patients are phenotypically female with mild virilization, such as those having an enlarged clitoris and labial fusion, while others are phenotypically male with undervirilized external genitalia, such as those having micropenis, microscrotum and hypospadia. These abnormalities are caused by a lack or reduction of androgen bioactivity due to absence or malfunction of the androgen receptor (AR) protein (Griffin and Wilson 1989), because androgens play an improtant role in male sexual differentiation by stimulating the differentiation of male internal and external genitalia of 46, XY individuals.

The AR gene is located on Xq12, and was cloned and sequenced by Lubahn et al. (1988). The AR gene consists of eight exons (A-H). The full-length AR protein, like other steroid receptors, is a single polypeptide composed of relatively discrete functional domains: a transcriptional regulation amino-terminal domain, a DNA-binding domain, a hinge region, and a steroid (androgen)-binding domain (Quigley et al. 1995). The transcriptional regulation amino-terminal domain is encoded by exon A, and the DNA-binding domain by exons B and C. The steroid-binding domain is distributed over five exons, D-H (Lubahn et al. 1989). Androgens bind to the steroid-binding domain and change the allosteric constitution of the AR protein to allow the DNA-binding domain to bind to regulatory elements of specific target genes (Wahli and Martinez 1991), activating downstream androgen receptor-interacting proteins (Chang et al. 1995), and inducing virilization of the urogenital tract in a male embryo.

Since the cloning of the AR gene and the sequencing of its intron-exon boundaries (Lubahn et al. 1988, 1989), there has been an explosion of information regarding the molecular pathogenesis of AIS. A wide variety of defects in the AR gene associated with AIS have been identified and characterized in over 330 individuals with various forms of AIS (Gottlieb et al. 1998). At the clinical level, CAIS is associated with a broad range of molecular defects, which include complete or partial gene deletions, single base mutations that introduce premature termination codons or disrupt the splicing of AR mRNA, and missense mutations that cause single amino acid substitutions, almost exclusively within the steroid- and DNA-binding domains. On the other hand, it is postulated that PAIS patients show various degrees of virilization depending on the degree of androgen bioactivity. PAIS is almost invariably associated with amino acid substitutions in the steroid-binding domain. Single nucleotide mutations that cause amino acid substitutions in the AR protein have diverse effects upon androgen binding, associated with an equally wide range of phenotypes of external genitalia (Quigley et al. 1995). However, it has not been possible to establish any clear correlation between genotypes and phenotypes to date and more genetic analyses of the AR gene are necessary to obtain a sufficient understanding of the pathophysiology

of AIS and a further understanding of the relationship between the structure and function of the AR gene. Thus, we analyzed the AR gene in five Japanese patients diagnosed with AIS.

Materials and Methods

Subjects

Five patients with AIS were analyzed for mutations of the AR gene. The clinical characteristics and the familial history of each patient are summarized in Table 1. Peripheral lymphocyte karyotyping demonstrated that all the patients were 46, XY, except for patient 3 who showed a 47, XXY karyotype. The karyotypes were ascertained by repeated analyses of more than 30 metaphase cells. Four patients (patients 1-4 in Table 1) were clinically diagnosed as having CAIS because of the characteristic phenotype, and laparoscopic or laparotopic gonadectomy confirmed that their intra-abdominal gonads were testes and that Wolffian and Mülerian derivatives were absent. The gonads of patient 3 showed testis-like structures with atrophic seminiferous tubules, with fibrous degenerated stroma (Uehara et al. 1999). Seminoma lesions were histopathologically demonstrated in the gonads of patients 1 and 4. Patient 5 was diagnosed as having PAIS, that is, phenotypically male with micropenis, microscrotum, female-type distribution of pubic hair and gynecomastia; high dose androgen therapy improved his genital abnormalities. Endocrine examination of all the 46, XY patients showed that gonadotropin concentrations were higher than the normal range for males, and testosterone and dihydrotestosterone concentrations were within the normal range for males. In patient 3, who had a 47, XXY karyotype, high concentrations of gonadotropins also were observed but androgen concentrations were lower than the normal range for males.

Table 1. *Age at first visit, chief complaint, sex, phenotype, and familial history of each subject*

Patient (No.)	Age	Chief complaint	Sex[a]	Phenotype	Familial history
1	19	Primary amenorrhea	F	Complete AIS	−
2	12	Inguinal tumor (testes)	F	Complete AIS	+[b]
3	30	Primary amenorrhea and sterility	F	Complete AIS	+[c]
4	48	Abdominal tumor	F	Complete AIS	Unclear
5	13	Gynecomastia	M	Partial AIS	−

[a] Sex refers to the sex of upbringing and is indicated as M (male) or F (female).
[b] A cousin was suspected of having complete AIS due to primary amenorrhea.
[c] Two aunts had primary amenorrhea and sterility.
AIS; Androgen insensitivity syndrome.

Whole cell receptor assay of AR

One assay was performed on tissue from patients 1 and 4, and a second assay on tissue from patients 2 and 3. The method of Nakao et al. (1992) was followed for the AR radioreceptor assay. Briefly, pubic skin samples from patients and from a female control were minced and cultured in flasks containing 5 ml of Eagle's minimum essential medium (MEM; GIBCO BRL, Rockville, MD, USA) supplemented with 15% fetal calf serum until sufficient numbers of fibroblasts were obtained. Two days before the assay the medium was changed to serum-free MEM. For the assay the fibroblasts were harvested in Hank's balanced salt solution containing 0.01% trypsin and 0.02% EDTA, rinsed with phosphate-buffered saline (PBS) and suspended in MEM containing 25 mM tricine (Sigma-Aldrich Japan, Tokyo). Duplicate tubes of fibroblasts (3×10^5 cells/ml in the first assay and 7×10^5 cells/ml in the second assay) were incubated for 1 hour at room temperature with 0.16 to 5 nM of [^{3}H] methyltrienolone ([^{3}H] R1881; New England Nuclear Research Products, Boston, MA, USA) in the presence or absence of a 1000-fold excess concentration of unlabeled R1881. After incubation, cells were washed three times with PBS and then lysed with 1 ml of absolute ethanol. The aliquots were transferred to vials containing 10 ml of ACS-II cocktail (Amersham, Buckinghamshire, England) for liquid scintillation counting. The results were analyzed by the Scatchard method to quantify specific [^{3}H] R1881 binding.

Nucleotide sequencing

Sequence analysis was carried out for exons B through H of the AR gene, using the method of Nakao et al. (1992) and Lubahn et al. (1989); exon A was not examined because of the rarity of mutations in this region. Genomic DNA was extracted from peripheral blood samples. The primer sequences, product sizes, and annealing temperatures are shown in Table 2. PCR products were purified and concentrated with a QIAEX II apparatus (Qiagen, Tokyo) and subcloned into pGEM-T (Plasmid, Promega, Madison, WI, USA). *Escherichia coli* DH-5α was transformed with subcloned pGEM-T, and direct colony PCR using both forward and reverse M13 primers was carried out to screen for bacterial colonies containing the correct insert. Sequence analysis was performed for each product by the dideoxynucleotide method with a DSQ-1000L automated DNA sequencer (Shimazu, Tokyo). To distinguish mutations from random misincorporated nucleotides, PCR sequences were examined for six independent clones obtained from each exon.

Results

Androgen receptor assay

Pubic skin fibroblasts were obtained from four of the five patients (patients 1 through 4, all of whom had CAIS). Androgen binding was not detectable under

TABLE 2. *Primer sequences, product sizes and annealing temperatures*

Exon	Primer sequences (5'-3')	Product size (bp)	Annealing temperature (°C)
B	GCCTGCAGGTTAATGCTGAA	374	60
	GTTATTTGATAGGGCCTTGC		
C	GTTTGGTGCCATACTCTGTC	410	60
	ATGGCCACGTTGCCTATGAA		
D	GAGTTTAGAGTCTGTGACCA	456	60
	GATCCCCCTTATCTCATGCT		
E	AACCCGTCAGTACCCAGACT	283	60
	GCTTCACTGTCACCCCATCA		
F	GGGCTTATTGGTAAACTTCC	290	60
	GTCCAGGAGCTGGCTTTTCC		
G	TCAGATCGGATCCAGCTATC	412	60
	TCTATCAGGCTGTTCTCCCT		
H	GAGGCCACCTCCTTGTCAAC	302	60
	AAGGCACTGCAGAGGAGTAG		

bp, base pairs.

TABLE 3. *Androgen binding and mutations in AIS subjects*

Patient (No.)	Receptor assay	Mutation	
		Exon	Type
1	Negative[a]	D	Gln640CAG→stopTAG
2	Negative[b]	E	Arg752CGA→stopTGA
3	Negative[b]	D	Gln640CAG→stopTAG
		E	Trp751TGG→stopTGA
4	Negative[a]	G	Arg831CGA→GlnCAA
5	ND[c]	F	Leu812CTC→PheTTC

[a] Incontrol fibroblasts, K_d was 0.71 nM, and the binding capacity was 0.42 fmol/3×10^5 cells.

[b] In control fibroblasts, K_d was 0.60 nM, and the binding capacity was 0.64 fmol/7×10^5 cells.

[c] ND, not done.

room temperature in any of the patients examined (Table 3). In control fibroblasts in the first assay, K_d (dissociation constant) was 0.71 nM, and the binding capacity was 0.42 fmol/3×10^5 cells; in control fibroblasts in the second assay, K_d was 0.60 nM, and the binding capacity was 0.64 fmol/7×10^5 cells.

Nucleotide sequencing

All AR genes from the five patients had single nucleotide substitutions, which introduced a premature termination codon in three patients (patient 1, glutamine

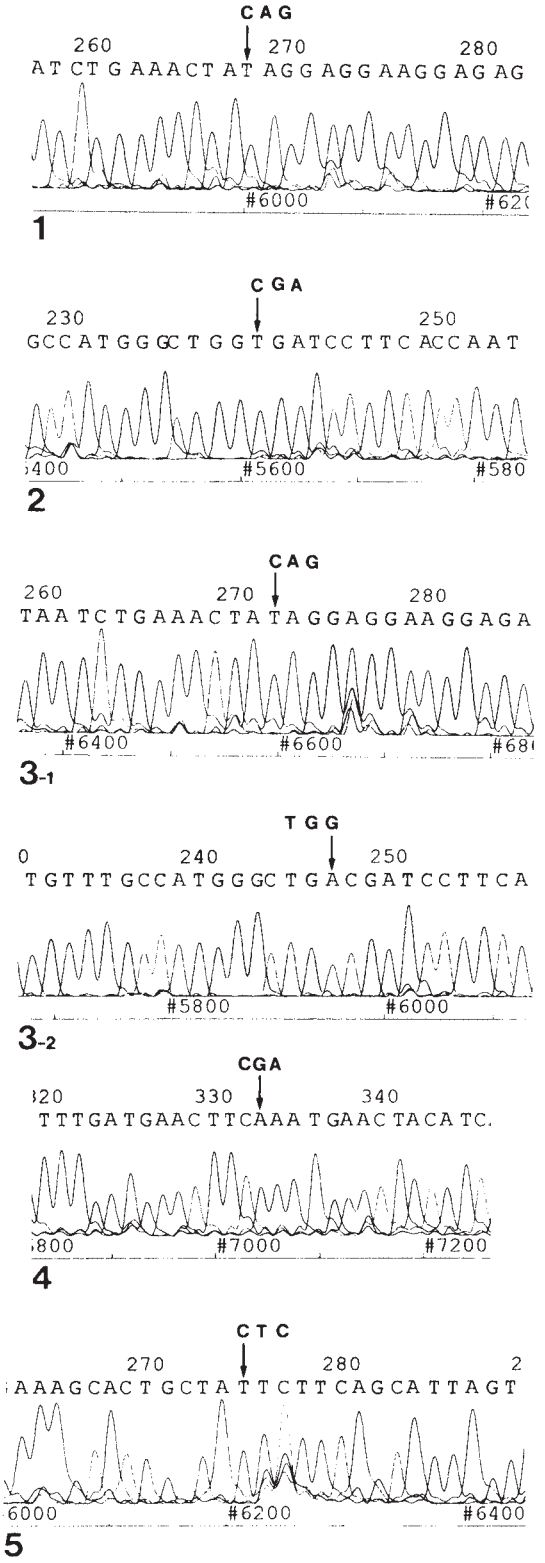

Fig. 1. Partial sequences of the AR gene in patients 1 through 5.
The two mutations detected in patient 3 are separately shown as 3-1 and 3-2. Arrows indicate substituted nucleotides; the nucleotides above the arrows indicate wild type sequences.

at position 640 to a stop codon in exon D; patient 2, arginine 752 to a stop codon in exon E; patient 3, glutamine 640 to a stop codon in exon D and tryptophan 751 to a stop codon in exon E), and a single amino acid substitution in two patients (patient 4, arginine 831 to glutamine in exon G; patient 5, leucine 812 to phenylalanine in exon F). These mutations were found in all six of the clones examined for each exon. The mutations are shown as partial sequences in Fig. 1. All of the three patients with a premature termination codon (patients 1, 2 and 3) and the one with a missense mutation (patient 4) were clinically diagnosed as having CAIS, while the remaining patient with a missense mutation (patient 5) had PAIS, that is, the patient was phenotypically male. The results are summarized in Table 3.

Discussion

All five of the AIS patients analyzed in this study had mutations in the AR gene. All mutations were single-base substitutions introduced a premature termination codon (nonsense mutation) or single amino acid substitution (missense mutation). In previous reports, including the AR gene mutation database (Gottlieb et al. 1998), the nonsense mutations Gln640stop (patients 1 and 3 in this study) and Trp751stop (patient 3) have not been reported, but Arg752stop (patient 2) has been reported in patients with CAIS (Pinsky et al. 1992; Brinkmann et al. 1995). The missense mutation Arg831Gln (patient 4) has been reported previously in patients with CAIS (Brown et al. 1990; McPhaul et al. 1992), but Leu812Phe (patient 5) has not been reported previously. Since single base mutations account for over 90% of the mutations in the AR gene mutation database (Gottlieb et al. 1998), the present data are consistent with mutations in the database. The mutations found in this study occurred in exons D, E, F, and G, which encode the steroid-binding domain. In the database, approximately 80% of missense and nonsense mutations occur in the steroid-binding domain, which consists of only 32% (291 amino acids) of the whole length (921 amino acids) of the AR gene. Mutations in this domain therefore appear to be the ones most often responsible for AIS.

Bourguet et al. (1995) determined the 3-dimensional structure of the ligand-binding domain of the human retinoid-X receptor, a member of the nuclear receptor superfamily that includes the AR, and reported that the ligand-binding domain forms a pocket; mutation of some residues surrounding the pocket can alter ligand binding without impairing other functions of the ligand-binding domain. Therefore, the degree of phenotypic abnormality of patients with a missense mutation in the steroid-binding domain of the AR gene may depend on the degree of alteration in protein structure. Moreover, recent studies have revealed that the AR can regulate the expression of androgen target genes through a complicated process requiring other adaptors or coactivators, that is, AR interacting proteins (Chang et al. 1995). Therefore, mutations of the AR must also be

considered in understanding the relationship between alternative structures of the AR and interacting proteins.

The nonsense mutations that were detected in this study produced CAIS. All nonsense mutations described in the AR mutation database result in CAIS, regardless of their location. Because the nonsense mutations detected in this study are assumed to yield trucated, nonfunctional AR without any appreciable nonsense-mediated mRNA decay or reinitiation at a downstream initiator methionine codon, it is not surprising that these mutations resulted in absence of androgen binding in vitro and could cause CAIS in vivo.

The missense mutation Arg831Gln detected in patient 4 resulted in CAIS, as was the case for CAIS patients described previously (Brown et al. 1990; McPhaul et al. 1992). Two CAIS patients having a related missense mutation, Arg831Leu, have been described by Shkolny et al. (1995). The arginine residue at position 831 in exon G is not contained within a region of amino acid homology conserved among the various steroid receptors (Hollenberg et al. 1985; Green et al. 1986; Weinberger et al. 1986; Misrahi et al. 1987), suggesting that the arginine residue does not directly contact the steroid molecule. Instead, since the arginine residue seems to be close to the AR ligand-binding pocket (Bourguet et al. 1995), and AR interacting proteins such as heat shock proteins (hsps), receptor interacting protein 140 (RIP140) and dnaJ bind to regions that include exon G (Gottlieb et al. 1998), the lack of steroid binding and consequent lack of transcription activation in this mutant is likely to be the result of an altered conformation of the AR protein.

Leu812Phe in exon F produced PAIS in patient 5. Although this mutation has not been reported previously, substitution of phenylalanine for leucine has been previously reported by Batch et al. (1992) and Tsukada et al. (1994) in the human AR gene. Batch et al. (1992) reported a Leu762Phe mutation in exon E that induced CAIS, while the case described by Tsukada et al. (1994) was one of PAIS with Leu789Phe in exon F. Leucine at position 812 is conserved among the various steroid receptors (Hollenberg et al. 1985; Green et al. 1986; Weinberger et al. 1986; Misrahi et al. 1987) and seems to be necessary for the normal conformation of the AR protein. In other steroid receptors, the residues flanking leucine 812 are leucine or isoleucine at position 811 and leucine or phenylalanine at position 813. Leucine, phenylalanine and isoleucine are neutral, nonpolar amino acids. Therefore, the substitution of phenylalanine for leucine at position 812 may not have caused a significant conformational change in the receptor protein or complete alteration in exon F. Instead, it may have only reduced transcriptional activity of the AR protein, resulting in PAIS in patient 5.

In summary, the data presented in this study provide valuable information for the further characterization of the structure and function of the steroid-binding domain of the AR and of the relationship of the AR to sex differentiation.

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