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Detection of aberrations in androgen receptor gene by analysis of single-stranded conformation polymorphisms in polymerase chain reaction products

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Abstract Analysis of single-stranded conformation polymorphisms in polymerase chain reaction (PCR) products (PCR-SSCP) is a sensitive method for detecting point mutations in genomic DNA. To investigate its utility in examining the androgen receptor gene, we analyzed data on a patient with the testicular feminization syndrome (TFS) with a known point mutation in exon C. We detected mobility shifts of fragments of the corresponding region. Since examination of the subject's brother (legally sister), who also has TFS, revealed an identical shift pattern, we sequenced the exon C of the sibling and detected a mutation identical to that in the former. We conclude that PCR-SSCP is available for screening mutations of the androgen receptor gene.

Key words Androgen receptor · Point mutation · PCR-SSCP · Testicular feminization syndrome

Androgens are crucial to the development of the normal male phenotype, including fertility, and bind to a specific androgen receptor (AR) protein in target cells. The receptor protein is thus transformed to the activated form and the androgen-AR complex is transferred to the nucleus, where it induces expression of the target gene. Some defects of androgen receptor (AR) lead to androgen resistance, which encompasses a broad spectrum of clinical disorders, ranging from mild undervirilization to genotypic males having a female phenotype, i.e., the testicular feminization syndrome (TFS). Another clinical problem is caused by the androgen resistance of prostatic carcinoma, since AR mutations are detected in 12.5% of endocrine therapy-resistant patients at autopsy [19].

AR is a member of the steroid receptor family, which is a part of a larger nuclear receptor superfamily. Cloning of the human AR [2, 4, 9, 20] has permitted an examination of the molecular defects responsible for the clinical disorders mentioned above. More than 70 mutations have been reported in patients with complete or partial TFS [18]. Mutations of the hormone-binding domain are most frequent and are approximately 3 times more common than those of the DNA-binding domain.

The recently developed PCR-SSCP analysis is a sensitive tool for detecting single-nucleotide substitution [16], for example, in the loci of tumor suppressor genes, candidate genes of inherited diseases [7, 15, 21]. To determine whether this technique may be applicable to the detection of mutations in the androgen receptor gene, we used analysis of single-stranded conformation polymorphisms in polymerase chain reaction products (PCR-SSCP) to examine the genomic DNAs of siblings with complete TFS.

Materials and methods

Subjects

A 21-year-old man (legally woman) with a history of amenorrhea was diagnosed as having testicular feminization syndrome (TFS) based on the result of chromosomal analysis, 46, XY, and a classic clinical/endocrine phenotype (Table 1). Examination of her younger brother (legally sister), performed later, revealed the same diagnosis. Their mother had a normal female genotype, 46, XX, and had a normal phenotype. Figure 1 represents the pedigree of this family.

Sequencing of AR gene

Genomic DNA was extracted from peripheral lymphocytes of both siblings. Twelve pairs of oligonucleotide primers flanking the individual coding exons were selected, according to the criteria of

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Table 1 Patient characteristics

Patient	Age (Years)	Karyotype	External genitalia	Internal genitalia	Ovary	Testis
1. NG	22	46, XY	Female type	None	None	Intra–peritoneal
2. KG	20	46, XY	Female type	None	None	Intra–peritoneal

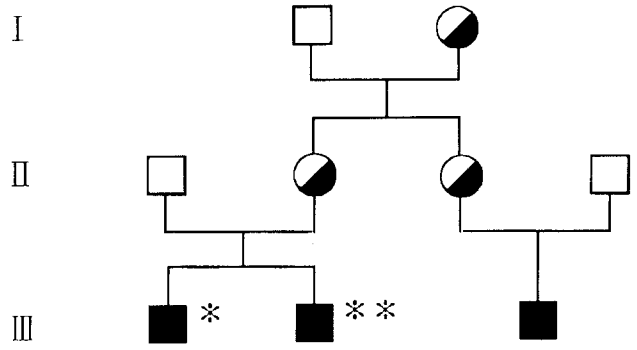


Fig. 1 Pedigree of the patients in the study. The cousin of the patients examined is also an affected hemizygous male. *Half-filled circles* represent obligate heterozygous carriers. *Patient 1, **Patient 2

Lubahn et al. [10]. They were designed to generate fragments of approximately 190–500 base pairs and were synthesized on a DNA synthesizer. Amplification of each coding region was performed by use of Ampli Taq DNA polymerase (Perkin-Elmer-Cetus, Norwalk, USA) according to the manufacturer's specifications. The incubation conditions were principally 30 cycles of a denaturing step at 94 °C for 1min, annealing at 55 °C for 1min, and polymerization at 72 °C for 1 min. When amplification was insufficient, appropriate modifications were made. After amplified fragments of the correct size had been collected and purified, they were ligated into PUC19 vectors, and transformation of *E. coli* JM109 (Toyobo, Tokyo, Japan) was carried out by Hanahan's method [6]. Insert DNA in white (non-blue) colony was confirmed by measuring the size of DNA fragment amplified with primers designed to flank the insertion point in *lacZ*. Then insert DNA was selected from plasmid DNA, purified and ligated into the cloning vector M13, and sequenced using Sequenase (USB, Cleveland, OH) for Sanger's dideoxy sequencing reactions [17].

SSCP method

PCR reactions were performed with the above-mentioned oligonucleotide primer pairs with 50 ng genomic DNA, 20 µmol each dNTP, 0.1 µmol each primer, 10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 0.125 u Taq DNA polymerase and 2 µCi [α-^{32}P] dCTP (10 mCi/ml, 3000 Ci/mmol, Amersham) in a final volume of 5 µl. Amplification conditions consisted of an initial step at 94 °C for 20 s, followed by annealing and extension at 60 °C for 2 min. The PCR product (5 µl) was mixed with 45 µl of a solution of 95% formamide, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.05% bromphenol blue, and 0.05% xylene cyanol and heated to 94 °C for 2 min. Then each denatured sample was applied (2 µl/lane) to 5% polyacrylamide gel containing 5% glycerol. Electrophoresis was performed at room temperature at 30 W constant power for 2–3 h.

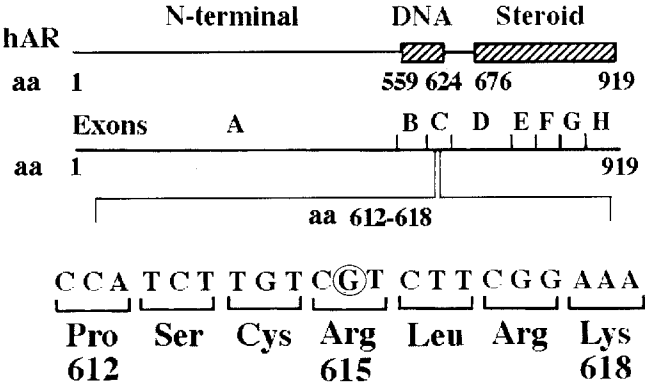
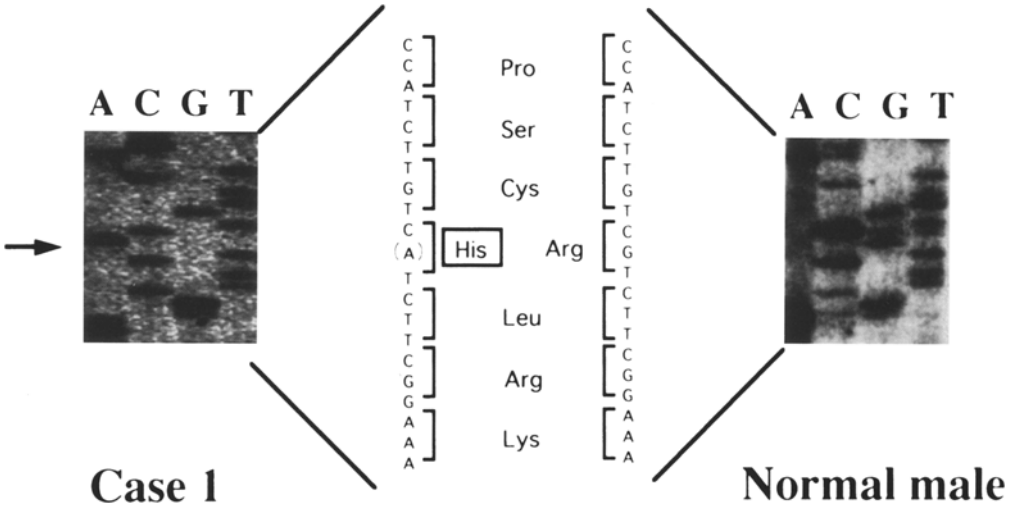


Fig. 2 Schematic presentation of human androgen receptor (*hAR*) gene and a single-nucleotide substitution of guanidine-to-adenine at amino acid residue 615 detected in the present patients. *aa* amino acid, *DNA* DNA-binding domain, *Steroid* steroid-binding domain

Fig. 3 Comparison of the mutated AR sequence found in patient 1 with normal AR sequences. A single-nucleotide substitution of guanine-to-adenine results in an arginine-to-histidine (*enclosed with square*) substitution at amino acid residue 615



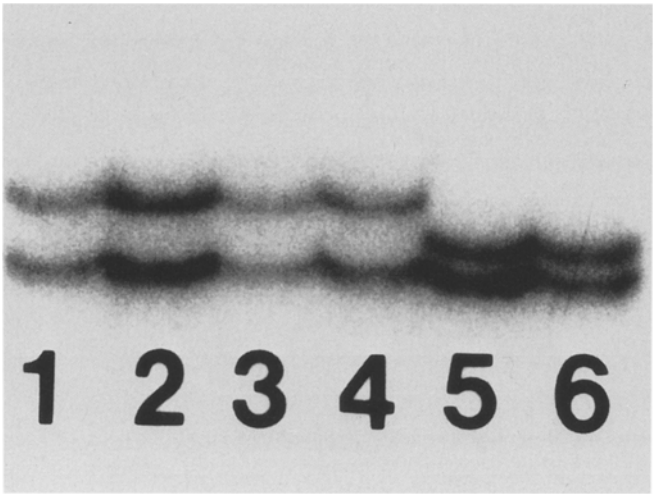


Fig. 4 PCR-SSCP analysis of AR gene (exon C). Samples were as follows: *lanes 1, 2* idiopathic infertile male, *lane 3* normal male, *lane 4*, TFS patient that proved to have no mutation in exon C, *lane 5*, patient 1, *lane 6* patient 2. *Lanes 5, 6* show the mobility shift of single-stranded DNA fragments due to a single-base substitution. The identical shift patterns of the two lanes reflect identical mutations in both siblings

The gel was dried on filter paper and exposed to Kodak XAR film for 2–24 h without use of intensifying screens.

Results

We sequenced the entire coding region of AR in the genomic DNA from patient 1, and detected a single nucleotide substitution of guanine-to-adenine that results in an arginine-to-histidine substitution at amino acid residue 615 (Figs. 2, 3). This substitution is located within the exon C that encodes the latter half of the DNA-binding domain. Figure 4 shows several examples of SSCP patterns of AR gene (exon C) seen in: idiopathic infertile males (lanes 1, 2), normal male (lane 3), TFS – patient 1 (lane 5), TFS – patient 2 (lane 6), and another TFS patient subsequently proved to have no mutation in exon C (lane 4), respectively. SSCP patterns for exon C in patients 1 and 2 (the younger sister of patient 1) were identical and different from those of other subjects, including the normal control. Believing that patient 2 had a mutation identical to that in patient 1, we sequenced her exon C and confirmed the mutation.

Discussion

TFS is broadly classified into two types, receptor-binding positive and receptor-binding negative, according to the results of ligand-binding studies. The receptor-binding positive type is supposedly caused by defects in the androgen receptor that are not detectable in a ligand-binding study; for example, defects that impair binding of the receptor-ligand complex to a target DNA sequence. Recent progress in the study of molecular mechanisms of steroid hormone receptors has enabled us to elucidate the structure-function relationship in AR of subjects with TFS. A defect in any of four events can cause androgen resistance: steroid hormone binding, activation of hormone-receptor complex, DNA binding, and transcription of a specific gene [3].

The DNA-binding domain of AR is composed of the most highly conserved exons, B and C, which encode the zinc finger motif and are supposed to bind the hormone-responsive element of the target gene. Any abnormalities in this domain may be correlated with a failure of DNA binding, which results in a reduced androgenic effect. Even a single nucleotide substitution, as well as a partial deletion, may impair the DNA-binding ability of AR if that nucleotide is included in a highly conserved and functional region in the other members of the steroid receptor superfamily.

Five different cases of complete TFS have been well described to date, each caused by defective DNA-binding domain. In 1990, Marcelli et al. [13] reported a case that was caused by the premature termination of the receptor protein at amino acid residue 588, namely, a single-base substitution (A → T) that resulted in the conversion of a lysine codon into a stop codon. They also demonstrated that the truncated receptor protein was not able to bind ligand or to activate the long terminal repeat of the mouse mammary tumor virus in cotransfection assays. The other cases involved the receptor-binding positive type of TFS. In 1992, Zoppi et al. [22] reported three cases of single-base substitution at amino acid residues 557 (G → A) and 574 (T → C) in the first zinc finger motif and 615 (G → C) in the second zinc finger motif, respectively.

In 1993, Mowszowicz et al. [14] demonstrated in two siblings an Arg → His substitution identical to that found in the patients in the present study at amino acid 614 (corresponding to position 615 based on our sequence). The AR in their cases had normal androgen-binding capacity and affinity, but reduced DNA-binding activity. They also detected an impairment of transcriptional activity of the mutated AR, reflected in the loss of induction of chloramphenicol acetyltransferase activity (CAT assay). Northern blot analysis of expression of AR mRNA in their cases showed decreased expression. Their explanation for the result is that, even though the possibility of additional mutations in the promoter region of the AR gene resulting in an alteration of AR mRNA level cannot be excluded, mutations of the AR alter the turnover rate of AR mRNA or protein. Regrettably, we did not perform a functional analysis of AR in the patients in the present study, but we can at least speculate that the causes of TFS in these two siblings may be similar or even identical. A defective DNA-binding domain may cause an intersex phenotype due to partial androgen insensitivity. Klocker et al. [12] reported a single-base substitution

(G → A) at amino acid residue 596 in patients with Reifenstein's syndrome, and showed that the residual transactivation activity of the mutant receptor was approximately one-third that of the wild-type receptor. Moreover, they identified an alanine residue at position 596 and/or 602 as being important for full AR function [11].

The pedigree of this family strongly suggests that the present mutation is not sporadic but hereditary, because their cousin also has TFS. Since examination of the mother was impossible in this family, we are now examining the cousin and his mother. Although a de novo AR mutation detected with SSCP analysis has already been reported [8], we believe we are the first to have applied SSCP analysis to detect identical AR aberrations reflected by an identical pattern of DNA mobility shift in siblings with TFS and to have shown it to be a sensitive tool for detecting sequence changes, including single-base substitutions in DNA fragments. This technique takes advantage of the fact that, under nondenaturing conditions, single-stranded DNA has a sequence-specific conformation and the mobility of DNA in polyacrylamide gel electrophoresis is affected by its conformation and temperature.

In this study, we demonstrated that PCR-SSCP analysis was useful for screening mutations in the AR gene of genomic DNA especially when genetic inheritance needs to be examined. We consider it likely that this technique will also prove useful for detecting other suspected AR genetic variants including somatic mutations, for example, in cases of idiopathic male infertility [1] and in hormone-independent prostatic carcinoma [5, 19].

References

1. Aiman J, Griffin JE (1982) The frequency of androgen receptor deficiency in infertile men. J Clin Endocrinol Metab 54:725
2. Chang C, Kokontis J, Liao S (1988) Structural analysis of complementary DNA and amino acid sequences of human and rat androgen receptors. Proc Natl Acad Sci USA 85:7211
3. Evans RM (1988) The steroid and thyroid hormone receptor superfamily. Science 240:889
4. Faber PW, Kuiper GGJM, van Roji HCJ, van der Korput JAGM, Brinkmann AO, Trapman J (1989) The N terminal domain of the human androgen receptor is encoded by one large exon. Mol Cell Endocrinol 61:257
5. Gaddipati JP, McLeod DG, Heidenberg HB, Sesterhenn IA, Finger MJ, Moul JW, Srivastava S (1994) Frequent detection of codon 877 mutation in the androgen receptor gene in advanced prostate cancers. Cancer Res 54:2861
6. Hanahan O (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166:557
7. Hensel CH, Xiang RH, Sakaguchi AY, Naylor SL (1991) Use of the single strand conformation polymorphism technique and PCR to detect p53 gene mutations in small cell lung cancer. Oncogene 6:1067
8. Lobaccaro J-M, Lumbroso S, Berta P, Chaussain J-L, Sultan C (1993) Complete androgen insensitivity syndrome associated with a de novo mutation of the androgen receptor gene detected by single strand conformation polymorphism. J Steroid Biochem Mol Biol 44:211
9. Lubahn DB, Joseph DR, Sar M, Tan J, Higgs HN, Larson RE, French FS, Wilson EM (1988) The human androgen receptor: complementary deoxyribonucleic acid cloning, sequence analysis and gene expression in prostate. Mol Endocrinol 2:1265
10. Lubahn DB, Brown TR, Simental JA, Higgs HN, Migeon CJ, Wilson EM, French FS (1989) Sequence of the intron/exon junctions of the coding region of the human androgen receptor gene and identification of a point mutation in a family with complete androgen insensitivity. Proc Natl Acad Sci USA 86:9534
11. Kaspar F, Klocker H, Denninger A, Cato ACB (1993) A mutant androgen receptor from patients with Reifenstein syndrome: identification of the function of a conserved alanine residue in the D box of steroid receptors. Mol Cell Biol 13:7850
12. Klocker H, Kaspar F, Eberle J, Überreiter S, Radmayr C, Bartsch G (1992) Point mutation in the DNA binding domain of the androgen receptor in two families with Reifenstein syndrome. Am J Hum Genet 50:1318
13. Marcelli M, Tilley WD, Wilson CM, Griffin JE, Wilson JD, McPhaul MJ (1990) Definition of the human androgen receptor gene structure permits the identification of mutations that cause androgen resistance: premature termination of the receptor protein at amino acid residue 588 causes complete androgen resistance. Mol Endocrinol 4:1105
14. Mowszowicz I, Lee HJ, Chen HT, Mestayer C, Portois M-C, Cabrol S, Mauvais-Jarvis P, Chang C (1993) A point mutation in the second zinc finger of the DNA-binding domain of the androgen receptor gene causes complete androgen insensitivity in two siblings with receptor-positive androgen resistance. Mol Endocrinol 7:861
15. O'Rahilly S, Choi WH, Patel P, Turner RC, Flier JS, Moller DE (1991) Detection of mutations in insulin-receptor gene in NIDDM patients by analysis of single-stranded conformation polymorphisms. Diabetes 40:777
16. Orita M, Suzuki Y, Sekiya T, Hayashi K (1989) Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 5:874
17. Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463
18. Sultan C, Lumbroso S, Poujol N, Belon C, Boudan C, Lobaccaro J-M (1993) Mutations of androgen receptor gene in androgen insensitivity syndromes. J Steroid Biochem Mol Biol 46:519
19. Suzuki H, Sato N, Watabe Y, Masai M, Seino S, Shimazaki J (1993) Androgen receptor gene mutations in human prostate cancer. J Steroid Biochem Mol Biol 46:759
20. Tilley WD, Marcelli M, Wilson JD, McPhaul MJ (1989) Characterization and expression of a cDNA encoding the human androgen receptor. Proc Natl Acad Sci USA 86:327
21. Triggs-Raine BL, Akerman BR, Clarke JTR, Gravel RA (1991) Sequence of DNA flanking the exons of the HEXA gene, and identification of mutations in Tay-Sachs disease. Am J Hum Genet 49:1041
22. Zoppi S, Marcelli M, Deslypere J-P, Griffin JE, Wilson JD, McPhaul MJ (1992) Amino acid substitutions in the DNA-binding domain of the human androgen receptor are a frequent cause of receptor-binding positive androgen resistance. Mol Endocrinol 6:409