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Case Report

Compound Heterozygous Mutations (PHE53/54DEL and HIS373LEU) of the P450c17 Gene Result in a 17α-Hydroxylase/17,20-Lyase Deficient Male Pseudohermaphrodite with Unambiguous External Genitalia

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Uehara, S., Sato, J., Nishiyama, Y., Matsuzaki, S., Funato, T., Murotsuki, J., Yaegashi, N., Okamura, K. and Yajima, A. *Compound Heterozygous Mutations (PHE53/54DEL and HIS373LEU) of the P450c17 Gene Result in a 17α-Hydroxylase/17, 20-Lyase Deficient Male Pseudohermaphrodite with Unambiguous External Genitalia.* Tohoku J. Exp. Med., 2000, **190** (4), 279–287 —— The autosomal recessive disease 17α-hydroxylase/17,20-lyase deficiency is characterized by mutation of the P450c17 enzyme, which catalyzes 17α-hydroxylation and 17, 20-lysis in the steroidogenic pathways. Although 17 mutations of this enzyme have been reported, only a few of them resulted in a completely unambiguous phenotype of female external genitalia in 46, XY individuals. We report here a Japanese patient with a 46,XY karyotype, who showed such a unambiguous female external genitalia. Nucleotide sequencing of the P450c17 gene revealed the patient to be a compound heterozygote carrying two different mutations (PHE53/54DEL in exon 1 and HIS373LEU in exon 6). As these mutations have been previously detected in unrelated Japanese patients, it is confirmed that these mutations accumulate regionally. Since these mutations could be screened by a multiple genotyping method, the method is applicable when 17α-hydroxylase/17, 20-lyase deficiency is suspected in Japanese patients. ———— congenital adrenal hyperplasia; CYP17; regional accumulation of the same mutations; mutation screening; XY sex reversal

Congenital adrenal hyperplasia, an autosomal recessive hereditary disease frequently diagnosed in male pseudohermaphrodites, includes five types of steroid biosynthetic enzyme deficiency: type I, 20, 22-desmolase deficiency; type II, 3β-hydroxysteroid dehydrogenase deficiency; type III, 21-hydroxylase deficiency;

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type IV, 11β-hydroxylase deficiency; and type V, 17α-hydroxylase deficiency (McKusick 1994). Deficiency of 17α-hydroxylase was first reported by Biglieri et al. (1966) in an affected female, while New (1970) reported the first affected male. Because 17α-hydroxylase and 17,20-lase activities reside within the same polypeptide chain (cytochrome P450c17), 17α-hydroxylase deficiency is a complicating factor of 17, 20-lase deficiency. In the steroidogenic pathways, P450c17 catalyzes 17α-hydroxylation of pregnenolone and progesterone, and 17, 20-lysis of 17α-hydroxypregnenolone and 17α-hydroxyprogesterone. Consequently, 17α-hydroxylase deficiency results in excessive synthesis of deoxycorticosterone and corticosterone and deficient synthesis of estrogen and androgen. Excessive mineralocorticoids generally induce hypertension and hypokalemia. Moreover, in female patients, deficient estrogen production induces primary and secondary amenorrhea and prevents sexual development. In genotypically male patients, deficient androgen production leads to male pseudohermaphroditism. Abnormal patient phenotypes are characterized by defects in either the 17α-hydroxylase or 17,20-lase activities, or both, with any residual activities dependent on the particular P450c17 mutation(s) encoded by the CYP17, the gene map locus of which is 10q24.3 (Fan et al. 1992). Thus, in male pseudohermaphroditism due to 17α-hydroxylase/17, 20-lase deficiency, large phenotypic variation is observed such that patients with unambiguous external genitalia (complete female external genitalia) are rare.

We recently discovered a male pseudohermaphrodite whose external genitalia were completely female. After hormonal examinations, the patient was diagnosed as having 17α-hydroxylase/17,20-lase deficiency. Based on the clinical diagnosis of the patient, we analyzed the nucleotide sequence of CYP17 in order to investigate the relationship between the genotype and disease phenotype. In order to establish a screening system for the detection of mutations, the patient's DNA was analyzed by a multiplex genotyping method, and compared with DNA obtained from normal individuals.

Methods

Case report

A 23-year-old patient presented to our clinic at the Tohoku University Hospital for evaluation of bilateral inguinal tumors. The patient was born as the second daughter of non-consanguineous parents without specific medical histories.

At age of 16 the patient visited another hospital due to delay of menarche, and after being karyotyped as 46, XY, was diagnosed as having complete-type androgen insensitivity syndrome. The patient subsequently received a laparotomy for castration of bilateral gonads. Surgical evaluation revealed that the gonads were located in the abdominal cavity and no development of Müllerian or Wolffian derivatives were identified. The resected gonads were diagnosed histopathologically to be normal testes with normal seminiferous tubules and stroma.

Thereafter the patient received Kaufmann therapy; conjugated estrogen 0.625 mg/day for 11 days, and then ethinylestradiol 0.05 mg/day and norgestrel 0.5 mg/day for 10 days. At 23 years of age, the patient became aware of gradually enlarging bilateral tumors in the inguinal region.

At the first visit to our clinic, the patient's height was 174 cm and body weight 73 kg. The patient lacked breast development (Tanner grade II under Kaufmann therapy). External genitalia showed an immature female appearance with no pubic hair development (Tanner grade I). Vaginal speculum examination revealed a narrow vagina lacking the uterine cervix. Pelvic examination, ultrasonography, and magnetic resonance imaging revealed the absence of the uterus. GTG-banded chromosomal analysis performed on peripheral blood lymphocytes and skin fibroblasts revealed a non-mosaic 46, XY karyotype. Since serum endocrine studies revealed the relatively high progesterone level, relatively low estradiol level, very low androgen level, very high corticosterone level, low cortisol level and the high ACTH level, we diagnosed the patient to be a male pseudohermaphrodite with 17α-hydroxylase/17,20-lyase deficiency. The inguinal tumors were surgically resected. Pathological examination revealed that the tumors were the ductus deferens with inflammatory changes. The patient's elder sister, the karyotype of which was 46, XX, suffered from secondary amenorrhea and primary sterility.

Preparation of genomic DNA

Peripheral blood was collected from the patient. Genomic DNA was extracted using the SepaGene kit (Sanko Junyaku, Tokyo) according to the manufacturer's instructions.

Nucleotide sequencing

Exonic sequence analysis of CYP17 was carried out using the method of Monno et al. (1993). Oligonucleotide polymerase chain reaction (PCR) primers were designed, according to the published nucleotide sequence (Picado-Leonard and Miller 1987), to flank the regions that contained each exon (exons 1 to 8). Primer sequences are shown in Table 1. Each PCR reaction mixture (50 μl) contained 0.5 μg genomic DNA, 5 μl 10x PCR buffer including 15 mM $MgCl_2$ (Takara Shuzo, Otsu), 0.4 mM each primer, 200 mM dNTPs, and 0.25 U Taq DNA polymerase (Takara Shuzo). All amplifications were performed in a TaKaRa PCR Thermal Cycler MP (Takara Shuzo). After initial incubation of 5 minutes at 94°C, reactions were cycled for 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C for 35 cycles, followed by a final extension of 10 minutes at 72°C. PCR products were electrophoresed on 2% agarose gels, purified and concentrated using a QIAEX II apparatus (Qiagen, Tokyo), and subcloned into the pGEM-T plasmid (Promega, Madison, WI, USA). Plasmids were then transformed into *Eschericia Coli* (DH-5α, Toyobo, Tokyo), and bacterial colonies that contained the correct

TABLE 1. *Primer and probe sequences*

Primers	Forward (5′-3′)	Reverse (5′-3′)
Exon 1	TTGCCACAGCTCTTCTACTC	TCTGAAGACCTGAACCAATC
Exon 2	TGGGTGTGAGATTCCTACAG	TCCTAACCCCTTACCCCTG
Exon 3	TGGTACAGAGAGGGGGTAAG	GGGACAATGTCAGGGTCTAC
Exon 4	AGCTAAGATCCGCCTCCAG	TCCACCCTGCTCTTGTGATT
Exon 5	GGCAGGAGTGTCACAGATG	TGGGGTCTAGGATCAATGAG
Exon 6	ACACACTAGTCACCTCCAAC	TGAATGCATCATGGGGCTAG
Exon 7	ACTTTTCCTCTTCCACTCTG	TTGCAGAGGTGAAGGGGTA
Exon 8	TCAACCAGGGCAGAACCATG	TGTGTTGTGGGGCCACATAG
Fluorescent hybridization probes		
Mutation probes (5′-3′)		
Exon 1	CCATACGAACAGAATAGATGGGGCCATATTTTTT-fluorescein[a]	
Exon 6	CCTCAGGCCCGTGGCCCCTATG-fluorescein[a]	
Anchor probes (5′-3′)		
Exon 1	LC red640[a]-CTGCAGCTTGAAGAAGTTGTTATG[b]	
Exon 6	LC red640[a]-TCATCCCCCACAAGGCC[b]	

[a] Fluorescein and LC red640 are fluorescent dyes.
[b] 3′-End nucleotides were phosphorylated.

insert were identified by direct colony PCR using the same primers as described above. After preparation of plasmid DNA using the Mini-Prep kit (Qiagen), nucleotide sequencing analysis was performed on each product by the dideoxynucleotide method on a DSQ-1000L automated DNA sequencer (Shimazu, Tokyo). To distinguish mutations from randomly misincorporated nucleotides, sequences from six clones were examined.

Multiplex genotyping

To confirm the results of exon 1 and 6 nucleotide sequencing, DNA samples from the patient and control individuals were analyzed by multiplex genotyping. Mutation probes were designed to cover the mutated sites in exons 1 and 6, and adjacent anchor probes designed between the primer sites. The probe sequences are shown in Table 1. Each PCR reaction mixture (20 μl) contained 0.5 μg genomic DNA, 1 μM each primer, 0.2 μM site-specific 3'-fluorescein-labeled probe, 0.4 μM site-specific 5'-LC red-labeled probe, 2 μM $MgCl_2$, and 10x ready-to-use reaction mix (LightCycler DNA Master Hybridization Probes, Roche Diagnostics, Mannheim, Germany). Samples were then loaded into separate glass capillaries. PCR subsequent melting curve acquisitions were performed on a rapid fluorescent thermal cycler (LightCycler System, Roche Diagnostics). The PCR protocols for both exons included 45 cycles of denaturation at 95°C in transition (i.e., samples were heated to, but not held at 95°C), annealing at 60°C for 10 seconds and

extension at 72°C for 15 seconds. High-resolution melting transitions from 40 to 85°C were carried out at a rate of 0.2°C/second. Fluorescein fluorescence (520 to 560 nm) was monitored for 50 milliseconds per sample at each 0.2°C increment.

The multiplex genotyping method uses fluorescently labeled hybridization probes and PCR, a technique that provides simultaneous amplification and genotyping (Bernard et al. 1998). Adjacent 3'-fluorescein-labeled and 5'-LC red-labeled probes are designed as a mutation probe and an anchor probe, respectively, in order to hybridize to the PCR products amplified by the unlabeled primers. Probe hybridization to wild type-derived PCR products (without an allelic mutation) is stable, whereas mismatches between probe and PCR product due to mutations lead to unstable probe binding. This hybridization instability results in a reduced melting temperature of the PCR products. As melting temperature can be monitored as a decline of fluorescence energy under gradual temperature change, the melting curve profile allows detection of the different genotypes.

Results

Nucleotide sequence analysis revealed that the patient was a compound

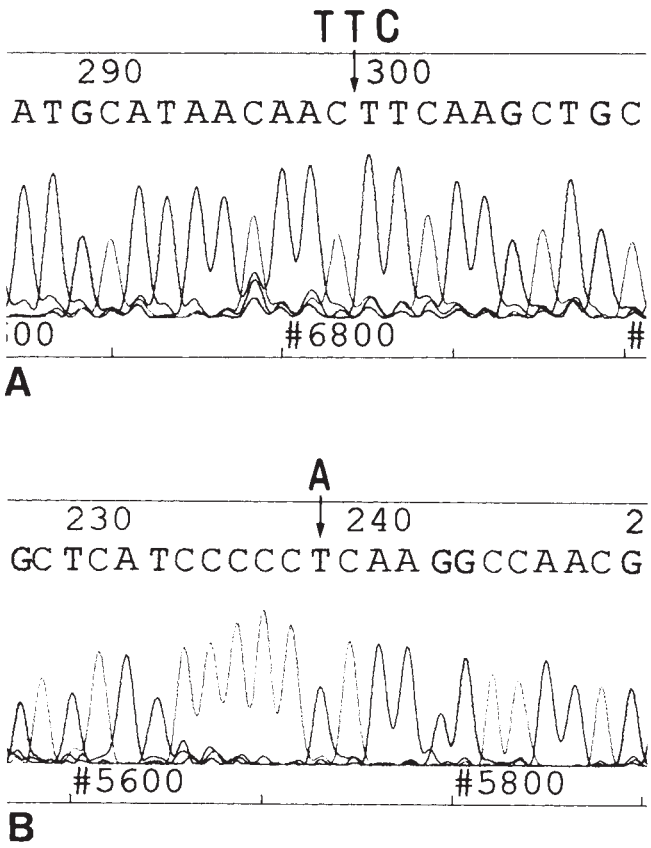

Fig. 1. Partial sequences of patient CYP17 alleles. A: deletion of the phenylalanine codon (TTC) at amino acid position 53 or 54 in exon 1. B: missense mutation due to the replacement of histidine (CAC) by leucine (CTC) at position 373 in exon 6. The two different mutations indicated the patient to be a compound heterozygote.

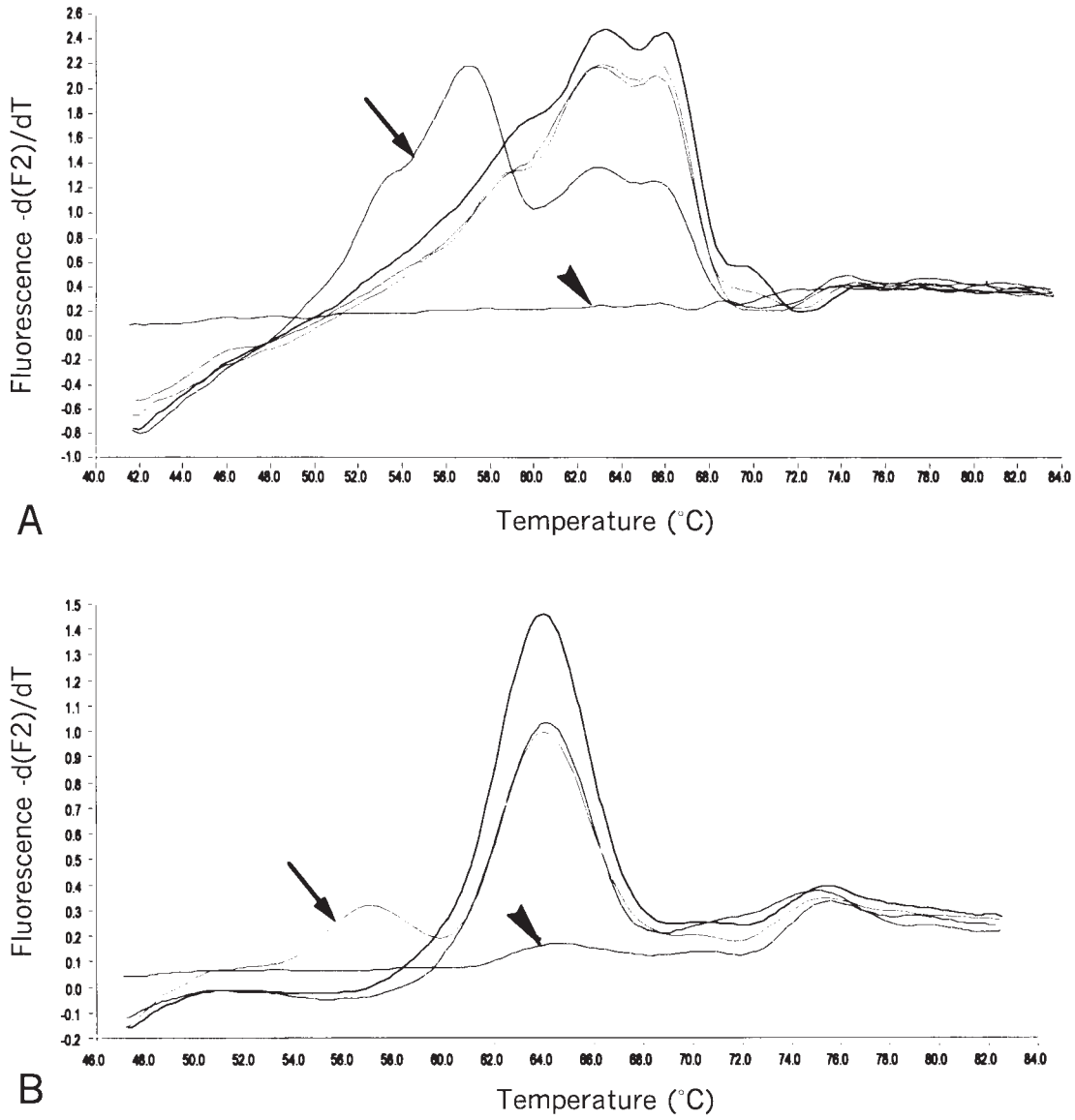

Fig. 2. Melting temperature curves obtained from homogenous multiplex genotyping. A: curves for exon 1. B: curves for exon 6. Arrows indicate PCR product curves from the patient's DNA, which show two melting peaks (one being the same as that observed in PCR products from normal individuals, and the other an extra peak due to the mutation). Arrowheads indicate the PCR product curves from negative controls.

heterozygote carrying two different CYP17 mutations. The first mutation consisted of a deletion of a phenylalanine codon (TTC) at either amino acid position 53 or 54 in exon 1 (abbreviated as PHE53/54DEL). The second consisted of a missense mutation due to the substitution of a leucine (CTC) for histidine (CAC) at position 373 in exon 6 (abbreviated as HIS373LEU). Partial sequences containing each mutation are shown in Fig. 1.

Melting curves, obtained by analysis of the multiplex genotyping products, are shown in Fig. 2. For both mutations, different melting curves were observed in PCR products from normal individuals and the patient. Whereas PCR products derived from normal individual DNA samples showed only one peak at

63°C, those derived from the patient samples showed two peaks, at 63 and 57°C.

Discussion

In our investigation of a patient with male pseudohermaphroditism due to 17α-hydroxylase/17,20-lyase deficiency, we detected two different CYP17 mutations; PHE53/54DEL in exon 1 and HIS373LEU in exon 6. Genotyping results suggested that the patient was a compound heterozygote such that each CYP17 allele carried either a PHE53/54DEL or a HIS373Leu mutation. PHE53/54DEL has been detected previously in some Japanese women unrelated to the patient (Yanase et al. 1989; Miura et al. 1996). This supports findings, as reported by Miura et al. (1996), that the prevalence of the PHE53/54DEL mutation was relatively high in Japanese patients. The HIS373Leu mutation has also been detected in Japanese woman unrelated to the patient (Monno et al. 1993). There have been other reports of CYP17 mutations detected both in Japanese and foreign patients, and the regional accumulation of some mutations has been noted (Kagimoto et al. 1988, 1989; Yanase et al. 1990; Biason et al. 1991; Lin et al. 1991; Ahlgren et al. 1992; Imai et al. 1992; Jones et al. 1992; Oshiro et al. 1995; Laflamme et al. 1996; Biason-Lauber et al. 1997; Geller et al. 1997; Yamaguchi et al. 1997).

The two mutations could be screened by a multiplex genotyping method in the present study. Melting temperature curves of the PCR products that contained the PHE53/54DEL or HIS373Leu mutations showed an extra peak at lower temperatures than the normal peak, due to unstable probe hybridization to the mutated sequences. Therefore, we suggest that this multiplex genotyping protocol is a useful method for the screening of PHE53/54DEL or HIS373Leu mutations that have regionally accumulated in Japan.

The external genitalia of our patient with a 46, XY karyotype showed as completely and unambiguously female without pubic hair development. The patient had been mis-diagnosed as having androgen insensitivity syndrome, and underwent bilateral gonadectomies. In approximately half of the previous reports describing 46, XY patients with 17α-hydroxylase/17,20-lyase deficiency, the external genitalia of patients were ambiguous (New 1970; Ahlgren et al. 1992; Geller et al. 1997; Biason-Lauber et al. 1997). This phenotypic difference in external genitalia of genotypically male patients can be explained by residual 17α-hydroxylase/17,20-lyase activities. The activity of 17α-hydroxylase in COS-1 cells transfected with CYP17 containing the exon 1 PHE53/54DEL mutation was less than 23%, while 17, 20-lyase activity was less than 5% (Yanase et al. 1989). In contrast, COS-1 cells transfected with CYP17 containing the exon 6 HIS373Leu mutation lacked both enzyme activities (Monno et al. 1993). This indicated that only the PHE53/54DEL allele was responsible for the 17α-hydroxylase/17,20-lyase activities in our patient. Assuming that the total enzyme activities were dependent on the sum of the activities of the two alleles

(Ahlgren et al. 1992), we can estimate that in our genotypically male patient, the relative activity of 17α-hydroxylase was approximately 10% of the normal range, and 17,20-lyase approximately 2%. Thus, androgen production was probably 2% of that observed in the normal male. Androgen is an essential hormone for male sexual characterization. In genotypic males, the threshold activity of 17, 20-lyase for ambiguous external genitalia is thought to be less than 20% of the normal range (Ahlgren et al. 1992). Given the estimated residual activity of 17, 20-lyase in our patient, we speculate that where residual 17,20-lyase activity is approximately 2% or less, an unambiguous female-type external genitalia develops in a genotypic male with 17α-hydroxylase/17,20-lyase deficiency.

It is well-known that androgen bioactivity deficiency can result from androgen receptor deficiency (androgen insensitivity) syndrome or Leydig cell hypoplasia (LH receptor deficiency) syndrome. However, few clinicians have knowledge of the 17α-hydroxylase/17,20-lyase deficiency syndrome. Because these three syndromes all show very similar phenotypes of male pseudohermaphroditism in genital sex, clinicians will need to differentiate between them using endocrinological and molecular analyses.

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