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FIGURE LEGENDS

Fig. 1. PCR amplification of all coding exons of TPMT under a single condition, as shown in Table 2. The leftmost lane (MW) represents the 1-kb ladder marker showing 100-, 200-, 300-, 400-, 500-, 650-, 850-, and 1000-bp fragments; the other 8 lanes were loaded with 3 μL of PCR products.

Fig. 2. DHPLC elution profiles previously reported TPMT polymorphisms within the coding region. The column temperatures were at 59.1°C, 56.2 °C, 57.0 °C, 56.4 °C, 55.8 °C, 56.5 °C, and 55.2° C for exons 3, 4, 5, 6, 7, 8, and 10, respectively. Exons 3, 4 and 10 were analyzed at two different temperatures (Table 2); only the chromatogram with the best resolution of the heteroduplex peaks was shown for each polymorphism. WT: wildtype homozygote.

Fig. 3. DHPLC elution profiles and sequencing chromatograms of heterozygotes for novel TPMT variants identified in the present study. (A) Arg163Cys substitution caused by a nucleotide substitution 487C>T in exon 7. The column temperatures were at 55.8 °C.
(B) Arg226Gln substitution caused by a nucleotide substitution 677C>A in exon 10. The column temperatures were at 55.2 °C. WT: wildtype homozygote.

Table 1 PCR primer sequences for site-directed mutagenesis of known TPMT polymorphisms

Known polymorphism	Outer forward primer*1	Inner reverse primer sequence (5'-3')*2	Inner forward primer sequence (5'-3')*2	Outer reverse primer*1
1A>G	3F	CCATCCACAGTTTCAGAGACACCT	CAAAGGTGTCTCTGAAACTGTGGA	3R
83A>T	3F	CTTGCCATACTTCCAGAGTTAGTA	TACTAACTCTGGAAGTATGGCAAG	3R
146T>C	4F	GTATCTAAATGCTTCTTTGATAGCCTGAAGAGG	CCTCTTCAGGCTATCAAAGAAGCATTTAGATAC	4R
238G>C	5F	CGGTCTGGAAACCTGCATAAAATCATAC	GTATGATTTTATGCAGGTTTCCAGACCG	5R
292G>T	5F	GCTCTGTAAAAAATTATTGTATCCC	GGGATACAATAATTTTTTACAGAGC	5R
374C>T	6F	AAATGTTCCCCAAAGAACTCTGT	ACAGAGTTCTTTGGGGAACATTT	6R
395G>A	6F	TCAAAAATACTGCAATAGTACAATGA	TCATTGTACTATTGCAGTATTTTTGA	6R
430G>C	7F	GTCAAATTTGCGAATATTTGTCCTACC	GGTAGGACAAATATTCGCAAATTTGAC	7R
460G>A	7F	GCAACTAATGTTCCTCTATCCCAAATC	GATTTGGGATAGAGGAACATTAGTTGC	7R
IVS7-1G>A	8F	GAAAACATTGTATCTGCATAGTTACAA	TTGTAACTATGCAGATACAATGTTTTC	8R
539A>T	8F	CACAGGAGAAACTGAAACTTCTTTCCC	GGGAAAGAAGTTTCAGTTTCTCCTGTG	8R
IVS9-1G>A	10F	TTGCATATTTTACTTGAAACAAGA	TCTTGTTTCAAGTAAAATATGCAA	10R
644G>A	10F	CTCAAGACAATGTATATTGCATATTT	AAATATGCAATATACATTGTCTTGAG	10R
681T>G	10F	CCAACTTTTCTGTCGTTCTTCAA	TTGAAGAACGACAGAAAAGTTGG	10R
719A>G	10F	GTAGACATAACTTTTCAAAAAGACAGTCAATTCCCC	GGGGAATTGACTGTCTTTTTGAAAAGTTATGTCTAC	10R

*1 See Table 2 for primer sequence

*2 Underlined nucleotide shows mismatch position

Table 2 PCR primer sequences, PCR annealing temperature, and DHPLC analysis conditions for TPMT mutation screening

Exon		Primer sequence (5'-3') *1	PCR annealing temperature (℃)	DHPLC temperature (℃)	DHPLC Gradient (%B*3/ 4.5min)
3	3F	GGCCCGCCGCCCCCGCCGcggaagacatatgcttgtgaga	Touchdown*2	54.7	54-63
	3R	tgtgaagaattaaatgtgcatcc	63-58	59.1	50-59
4	4F	taaaacctttttttttttcctcttc	Touchdown	56.2	49-58
	4R	GCCGCCCCCGCCCGttcatcattaaggcaagataattctg	63-58	57.8	48-57
5	5F	tctgctttcctgcatgttctt	Touchdown	57.0	53-62
	5R	GCCGCCCCCGCCCGcgtgctaaataggaaccatcg	63-58		
6	6F	cttggccctctttccttgac	Touchdown	56.4	54-63
	6R	cccatagtttgggagctaacc	63-58		
7	7F	aaacgcagacgtgagatcct	Touchdown	55.8	54-63
	7R	gccttacacccaggtctctg	63-58		
8	8F	tggaccaattcccagcttag	Touchdown	56.5	55-64
	8R	agaaaaaaaaaaaacccaacaact	63-58		
9	9F	aacatgccacatcatcacct	Touchdown	56.4	53-62
	9R	ggtgatctgcccaccttg	63-58		
10	10F	ccaccatacccagctcattt	Touchdown	53.3	57-66
	10R	cctcaaaaacatgtcagtgtga	63-58	55.2	56-65

*1 For exons 3, 4 and 5, non-template GC-clamp was added to the primers (shown in uppercase) to detect mutations within the higher melting temperature domain.

*2 Touchdown protocol: The annealing temperature was decreased by 0.5°C every second cycle beginning at 63°C and decreasing to a 'touchdown' (Don et al., 1991) annealing temperature of 58°C, which was then used for 30 cycles.

*3 Buffer A:0.1 mol/L triethylammonium acetate; buffer B:0.1 mol/L triethylammonium acetate containing 250 mL/L acetonitrile. "54-63" indicates that the gradient consists of 54% B: 46% A through to 63% B: 37% A.

Table 3. TPMT genptype among 288 control individuals

Genotype	Exon with polymorphism	Nucleotide substitution	Amino acid Substitution	No. of Subject
*1/*1	N.A.	N.A.	N.A.	240
*1/*1S	5	339C>T	Silent	1
*1/*1S	7	474T>C	Silent	4
*1S/*1S	7	474T>C	Silent	14
*1/*2	5	238G>C	Ala80Pro	1
*1S/*3A	7	460G>A,	Ala154Thr	2
	7	474T>C	Silent	
	10	719A>G	Tyr240Cys	
*3A/*3A	7	460G>A	Ala154Thr	1
	10	719A>G	Tyr240Cys	
*1/*3C	10	719A>G	Tyr240Cys	14
*1/*8	10	644G>A	Arg215His	9
Novel 1/*8	7	487C>T	Arg163Cys	1
	10	644G>A	Arg215His	
Novel 2/*1	10	677G>A	Arg226Gln	1

N.A.: Not applicable

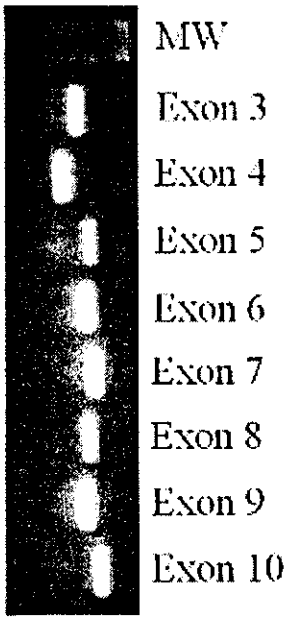

Fig. 1

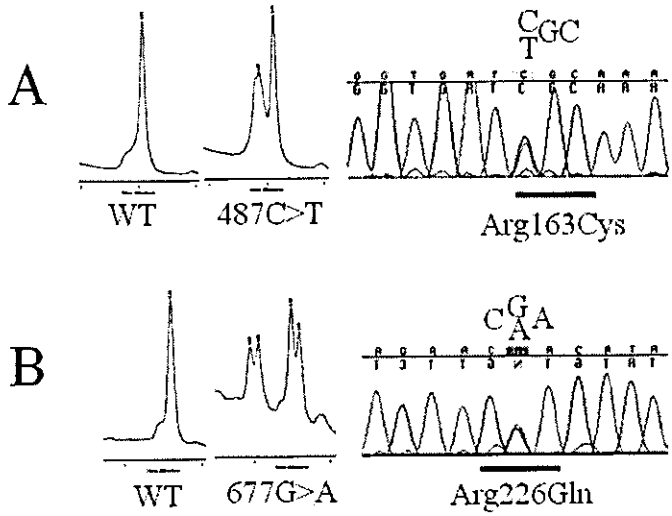

Fig. 3

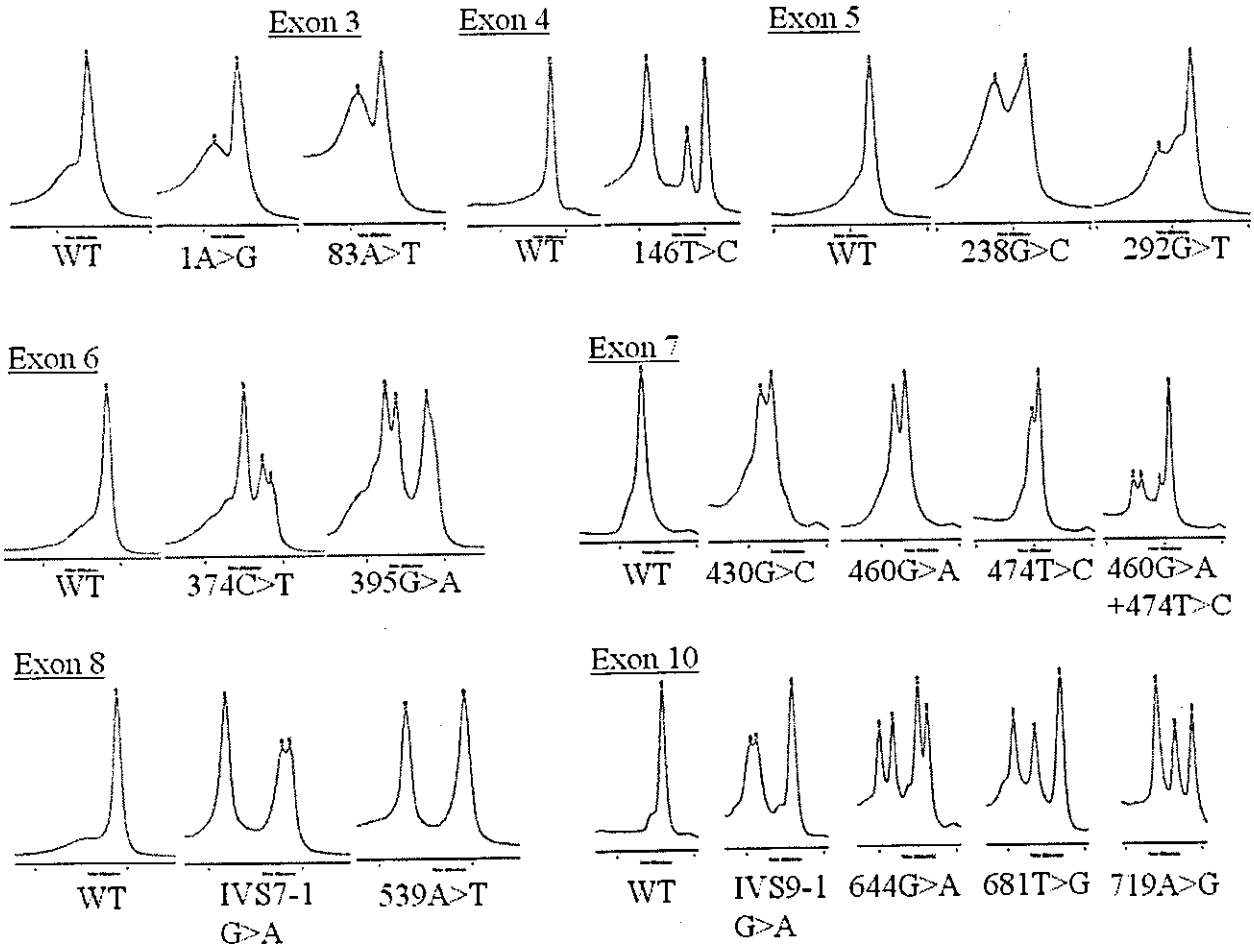

Fig. 2

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Original article

A major influence of CYP2C19 genotype on the steady-state concentration of *N*-desmethylclobazam

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Abstract

N-desmethylclobazam (N-CLB), the major metabolite of clobazam (CLB), exerts a large influence on therapeutic and adverse effects of CLB. A substantial inter-individual variability has been observed in the ratios of N-CLB concentration/CLB dose and of the N-CLB/CLB concentration. We document here a genotype–phenotype correlation between CYP2C19 polymorphisms and those ratios. Patients with two mutated CYP2C19 alleles show significantly higher ratios than those with the wild type genotype: patients with one mutated allele exhibited intermediate trait. That is, the degree of elevation in the ratios was dependent on the number of mutated alleles of CYP2C19 (gene-dose effect). The N-CLB concentration/CLB dose ratio of patients with two mutated alleles was more than six fold higher than that of wild type patients. Thus, the serum N-CLB/CLB concentration ratio may be a valuable parameter to screen for patients at risk for side effects. Such precautions may be clinically relevant in populations where the mutant allele frequency is high, such as in Asian populations (~35%). Patients co-medicated with CYP3A4 inducer showed lower CLB concentration/CLB dose ratios and higher N-CLB/CLB concentration ratios. The overall effect of CYP3A4 inducer on N-CLB metabolism, however, was small and, thus, we conclude that the CYP2C19 genotype is the major determinant of the N-CLB concentration. For this reason it is crucial for the better management of epilepsy and other chronic illnesses in general to establish the correlation of genotype of CYP enzymes and pharmacokinetics/dynamics of drugs.

Keywords: Clobazam; Pharmacogenetics; CYP; P450; Benzodiazepine; Poor metabolizer

1. Introduction

Clobazam (CLB) is a benzodiazepine analog and has been widely used as an anti-epileptic drug (AED), often in combination with other AEDs. *N*-desmethylclobazam (N-CLB), the major metabolite of CLB, demonstrates a longer half-life, and much higher steady-state plasma concentration as compared to the parent drug CLB [1,2], exerting a larger influence on therapeutic and adverse effects of treatment [2,3]. Hence, it has been strongly recommended that the serum concentrations of not only the parent drug, CLB, but also the metabolite, N-CLB, be routinely measured. In this regard it is of note that a substantial inter-individual variability in the ratio of the serum N-CLB concentration/weight-adjusted CLB dose [4] and in the ratio of the serum N-CLB/CLB concentration has been observed with therapeutic drug monitoring.

The variability in the serum N-CLB concentration/CLB dose ratio and in the serum N-CLB/CLB concentration ratio has been partly accounted for by concurrent medication [5,6], especially in patients receiving drugs with phenobarbital, carbamazepine, and phenytoin [7]. Co-medication with those drugs induces CYP3A4, the major isoenzyme of the cytochrome P450 (CYP) enzyme family which plays a dominant role in the biotransformation of diverse drugs. CYP3A4 metabolizes CLB to N-CLB, and leads to the increased serum N-CLB/CLB concentration ratio. However, in patients who are not receiving CYP3A4 inducers, the serum N-CLB concentration is still not predictable from the initial CLB dose alone.

CYP2C19, another member of the CYP family is involved in the metabolism of several commonly prescribed

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drugs, including mephenytoin [8] and omeprazole [9]. It was first implicated in the metabolism of N-CLB based on the observation that the serum N-CLB concentration/CLB dose ratio and the serum N-CLB/CLB concentration ratio were significantly increased in patient(s) concurrently medicated with felbamate, an inhibitor of CYP2C19 [6]. Recently, Contin et al. [10] documented two patients with elevated serum N-CLB concentration/CLB dose ratios and N-CLB/CLB concentration ratios; one of these patients was homozygous and the other was heterozygous for the mutated allele of CYP2C19. The heterozygous patient was further reported in the recent issue of this journal [11].

It is known that in patients with CYP2C19 mutated alleles the metabolism of such drugs as mephenytoin and omeprazole is reduced [12]. A single base pair substitution (guanine to adenine) in exon 5 of CYP2C19 (the *2 variant according to the international nomenclature for the P450 alleles [13]) creates an aberrant splice site that alters the reading frame of the mRNA, starting with amino acid 215, and produces a premature stop codon 20 amino acids downstream [14]. A single base pair mutation (guanine to adenine) at position 636 in exon 4 of CYP2C19 (the *3 variant according to the international nomenclature), also results in a premature stop codon [15]. Hence, both CYP2C19*2 and *3 alleles result in a truncated, non-functional protein.

There are inter-racial differences in the allele frequency of the *2 and *3 alleles of CYP2C19. The combined frequency of *2 and *3 alleles is much higher in the Japanese population (35%) [15–17] than in the Caucasian population (20%). Furthermore, the *2 and *3 alleles account for almost all of the individuals with the 'poor-metabolizer' phenotype in the Japanese population [15–17]. Giving careful consideration to such a unique population structure among Japanese, we tested whether the CYP2C19 genotype was associated with an altered metabolism of CLB and N-CLB among Japanese patients receiving CLB for the treatment of epilepsy.

2. Subjects and methods

A group of 16 patients, between the ages of 1.5 and 33 years (median, 7 years), were recruited from the Neuropediatrics Clinic at the Keio University Hospital (Table 1). All patients had received oral administration of tablets or granules of CLB (Mystan; Dainippon, Co. Ltd, Osaka, Japan) with the dose unchanged at least for 4 weeks at the time of study. In 6 out of 16 patients, the underlying neurological conditions were documented: these included subdural hematoma, intracerebral hemorrhage, meningoencephalitis, megalencephaly, neurofibromatosis, and brain abscess. All the patients were concomitantly receiving other AEDs. Seven patients were receiving at least one AED(s) with CYP3A4-inducing properties (inducers: carbamazepine, phenytoin, and phenobarbital), whereas 9 patients were receiving only anti-epileptic drug(s) without CYP3A4-inducing properties (non-inducers: valproic acid, ethosuximide, and zonisamide) [7]. Serum concentrations of CLB and N-CLB were measured using gas chromatography-mass spectrometry [16]. We assumed that the spot concentrations represented the steady-state concentrations [4] because the steady state is reached within about 4 days of repeated doses for CLB and within 10 days for N-CLB [1]. The study protocol was approved by the institutional review board, and all the participants or the parent(s) of the participants provided their written informed consent.

Genomic DNA was isolated from whole blood samples using the QIAamp Blood Kit (QIAGEN, Hilden, Germany), and CYP2C19*2 and CYP2C19*3 mutations were detected by polymerase chain reaction using previously described primers [17]. The amplified fragments were analyzed using either an autosequencer (ABI PRISM 3100, Applied Biosystems) or a denaturing high-performance liquid chromatography (DHPLC) system (Transgenomic, Omaha, NE) using a previously described method [18]. Since both the CYP2C19*2 and *3 alleles abolish protein

Table 1
Demographic characteristics of patients grouped according to CYP2C19 genotype

	Group 1 ($n = 7$)	Group 2 ($n = 6$)	Group 3 ($n = 3$)	Significance*
Age (year)				
Mean	6.79	12.2	14.3	
Range	1.5–14	4–22	5–33	
Male/female	4/3	0/6	2/1	NS
Body weight (kg)#	22.8 ± 10.7	33.7 ± 15.2	36.7 ± 32.5	NS
Dose (mg/kg per day)#	0.28 ± 0.19	0.32 ± 0.19	0.39 ± 0.26	NS
C_{ss} (ng/ml)#				
CLB	130 ± 130	219 ± 196	168 ± 96	NS
N-CLB	586 ± 459	2015 ± 1333	4806 ± 2575	$P < 0.05$†

Patients are classified according to the total count of mutated allele(s) into three groups (abscissa): group 1, CYP2C19 *1/*1; group 2, CYP2C19 *1/*2 or CYP2C19 *1/*3; group 3, CYP2C19 *2/*2, CYP2C19 *3/*3, or CYP2C19 *2/*3. C_{ss}, steady-state serum concentration; NS, not significant.

* Significance determined by Tukey-Kramer or Fisher exact test.

\# Data are expressed as mean ± standard deviation.

† Comparison between groups 1–2 and groups 1–3.

function [14,15], their contributions to the poor-metabolizer phenotype were expected to be comparable.

We classified the patients according to the total count of mutated allele(s): group 1, CYP2C19 *1/*1; group 2, CYP2C19 *1/*2 or CYP2C19 *1/*3; group 3, CYP2C19 *2/*2, CYP2C19 *3/*3, or CYP2C19 *2/*3. The patients were also cross-classified based on the presence or absence of a CYP3A4-inducer co-medication. Differences in the response variables including the CLB concentration/CLB dose ratio, the NCLB concentration/CLB dose ratio, and the N-CLB/CLB concentration ratio among the groups were then evaluated using a two-way ANOVA with more than one observation per cell (S-plus, Insightful, USA).

3. Results

The number of patients with each genotype was as follows: CYP2C19 *1/*1, $n = 7$; CYP2C19 *1/*2, $n = 4$; CYP2C19 *1/*3, $n = 2$; CYP2C19 *2/*2, $n = 2$; CYP2C19 *3/*3, $n = 0$; CYP2C19 *2/*3, $n = 1$. Hence, there were 7 patients in group 1 (*1 homozygotes), 6 patients in group 2 (heterozygotes for *2 or *3), and 3 patients in group 3 (homozygotes for *2 or compound heterozygotes for *2 and *3). The background characteristics (age, sex, weight, dose/weight) were comparable among the three groups (summarized in Table 1).

The serum concentrations of CLB and N-CLB (ng/ml) adjusted for the daily dose of CLB per body weight (mg/kg per day) were compared among the three groups (Fig. 1). The mean values for the CLB concentration/CLB dose ratios in groups 1, 2, and 3 were 443, 819, and 457 ng/ml per mg/kg/day, respectively (Fig. 1A). The mean values for the N-CLB concentration/CLB dose ratios in groups 1, 2, and 3 were 2111, 7156, and 13,504 ng/ml per mg/kg/day, respectively (Fig. 1B). Thus, the effect of the number of mutated allele(s) of CYP2C19 (a gene-dose effect) on the N-CLB concentration/CLB dose ratio was robust whereas such an effect was not observed with respect to the CLB concentration/CLB dose ratio. The N-CLB/CLB concentration ratio increased as the number of mutated allele(s) increased, again suggestive of a gene-dose effect (Fig. 2).

Within each of groups 1–3, patients receiving the CYP3A4-inducer co-medication tended to have a lower CLB concentration/CLB dose ratio (Fig. 1A), a lower N-CLB concentration/CLB dose ratio (Fig. 1B) and a higher N-CLB/CLB concentration ratio than those not receiving CYP3A4-inducer (Fig. 2). Thus, we have evaluated differences in the ratios (CLB concentration/CLB dose, N-CLB concentration/CLB dose, N-CLB/CLB concentration) between patients receiving inducer and those not: a two-way ANOVA was applied by cross-classifying each of the three groups in two-ways, based on the presence or absence of CYP3A4-inducer co-medication. The CLB concentration/CLB dose ratio was invariable and independent of the genotype category ($P = 0.63$) but significantly decreased in the presence of CYP3A4-inducing co-medication ($P = 0.005$). The N-CLB concentration/CLB dose ratio was strongly dependent upon the genotype category

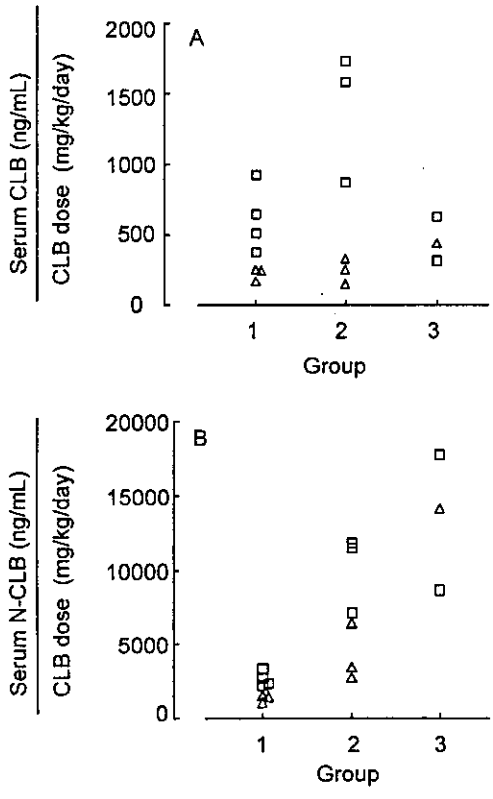

Fig. 1. Gene-dose effect of CYP2C19 polymorphism on the serum CLB concentration/CLB dose ratio (A) and on the serum N-CLB concentration/CLB dose ratio (B). Patients are classified according to the total count of mutated allele(s) into three groups (abscissa): group 1, CYP2C19 *1/*1; group 2, CYP2C19 *1/*2 or CYP2C19 *1/*3; group 3, CYP2C19 *2/*2, CYP2C19 *3/*3, or CYP2C19 *2/*3. △, Patients receiving CYP3A4-inducing co-medication(s); □, patients receiving CYP3A4-non-inducing co-medication(s).

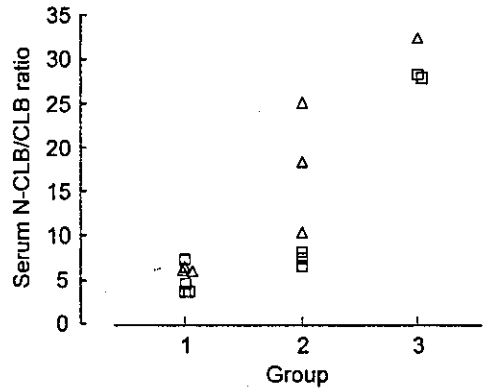

Fig. 2. Gene-dose effect of CYP2C19 polymorphism on the serum N-CLB/CLB concentration ratio. Patients are classified according to the total count of mutated allele(s) into three groups (abscissa): group 1, CYP2C19 *1/*1; group 2, CYP2C19 *1/*2 or CYP2C19 *1/*3; group 3, CYP2C19 *2/*2, CYP2C19 *3/*3, or CYP2C19 *2/*3. △, Patients receiving CYP3A4-inducing co-medication(s); □, patients receiving CYP3A4-non-inducing co-medication(s).

($P < 0.0001$) and significantly decreased in the presence of CYP3A4-inducing co-medication ($P = 0.01$). The N-CLB/CLB concentration ratio was strongly dependent upon the genotype category ($P < 0.0001$) and increased in the presence of CYP3A4-inducing co-medication ($P = 0.04$).

4. Discussion

In the present study, we documented a genotype–phenotype correlation between two mutated CYP2C19 polymorphisms and the N-CLB concentration/CLB dose ratio as well as the N-CLB/CLB concentration ratio. Those ratios in the patients with two mutated CYP2C19 alleles (group 3 in this study) were significantly higher than those in patients with the wild type genotype (group 1). Patients with one mutated allele (group 2) exhibited an intermediate trait. These observations give further credence to the notion that CYP2C19 is involved in the degradation of N-CLB [10].

When the patients in each group were cross-classified based on the presence or absence of CYP3A4-inducing co-medication, patients co-medicated with CYP3A4 inducer tended to have lower CLB concentration/CLB dose ratios and higher N-CLB/CLB concentration ratios. These changes in the two ratios are most likely to be due to elevated expression of CYP3A4 which converts CLB to N-CLB. However, the overall effect of a CYP3A4 inducer on N-CLB metabolism was smaller than that of CYP2C19 polymorphism. We conclude that CYP2C19 genotype is the major determinant of the steady-state concentration of N-CLB.

From a clinical standpoint, it is important to note that the mean values for the N-CLB concentration/CLB dose ratio of patients with two mutated alleles (group 3) were more than six fold higher than those of wild type patients (group 1). Although anti-epileptic potency of N-CLB has been reported to be one-fourth of that of CLB [19,20], it is highly likely that patients with two mutated alleles are susceptible to a higher incidence of side effects given a significant increase in the N-CLB concentration in group 3. Indeed, while receiving a standard dosage of CLB, one of the patients with two mutated alleles experienced excessive somnolence, one of the common adverse effects of CLB. Thus, a large-scale prospective study is warranted to determine whether homozygosity for a CYP2C19 mutation predisposes an individual to a higher incidence of side effects.

The serum N-CLB/CLB concentration ratio may be a valuable parameter for detecting patients with CYP2C19 polymorphisms: All three patients with two mutated CYP2C19 alleles (group 3) had N-CLB/CLB concentration ratios of 25 or more. We recommend that the N-CLB/CLB concentration ratio be closely monitored at the initial phase of CLB therapy in order to screen patients with two mutated alleles (group 3) who may be at risk for developing side effects if the standard dose of CLB is prescribed. Such precautions may be clinically relevant in populations where the mutated allele frequency is high, such as in Asian populations (15–20%). In interpreting the N-CLB/CLB concentration ratio, it should be noted that in those patients taking CYP3A4-inducing co-medication the ratio may be elevated in heterozygotes (group 2) as well.

The degree of elevation in the N-CLB concentration/CLB dose ratio and that in the N-CLB/CLB concentration ratio were dependent on the number of mutated alleles of CYP2C19. This gene-dose effect recapitulates observations for omperazole, a proton pump inhibitor which also is metabolized by CYP2C19 [12]. Omperazole produces a greater cure rate for gastric ulcers and accompanying *Helicobacter pylori* infections in patients with heterozygous as well as homozygous mutations of CYP2C19 because blood levels are higher in these individuals [21]. Therefore, it is likely that not only the blood concentration of N-CLB (established in this study) but also the efficacy of the drug for the treatment of epilepsy (both therapeutic and adverse effects) is gene-dose dependent. Studies with a more homogeneous patient population with a larger number of patients enrolled will allow such an assessment.

The limitations of the present study are two-fold. First, the CLB and N-CLB concentrations were measured only at a single time point, as opposed to multiple points. However, all the patients had been receiving CLB for much longer than the periods required to reach a steady-state levels of CLB and N-CLB [1]. Thus, it is assumed that a single data point is likely to represent the steady-state concentration. A formal population pharmacokinetic evaluation [22] would allow the N-CLB concentration to be predicted based on the patient's genotype. Second, the patients were only screened for the *2 and *3 alleles. Hence, some of the *2 or *3 heterozygotes who were not receiving CYP3A4 inducers but who had elevated N-CLB concentration/CLB concentration ratios may have actually been compound heterozygotes. However, the rarity of mutated alleles other than *2 and *3 in the Japanese population makes such concerns merely theoretical. When studying other ethnic groups, however, screening for other mutated alleles may be necessary, considering that several new alleles of CYP2C19 that induce coding changes were recently identified [23].

Our data indicate that three different patient groups (wild-type, heterozygotes for the mutated alleles, and mutation homozygotes or compound heterozygotes) may respond to CLB in distinctive manners. In future evaluations of new AEDs, the genotype of the subject should be considered as a critical confounding factor when the drug under development, or its active metabolite, is targeted by a highly polymorphic drug metabolizing enzyme, such as in the presently reported situation with CYP2C19. Furthermore, the results of clinical trials performed in one ethnic group or country should be carefully interpreted when

applied to another group or country because the gene frequency of the mutated allele(s) can vary significantly among different ethnic groups. Lastly we emphasize that it is of crucial clinical importance for the better management of epilepsy and other chronic illnesses in general to establish the correlation of genotype of CYP enzymes and pharmacokinetics/dynamics of drugs.

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Association of Micropenis with Pro185Ala Polymorphism of the Gene for Aryl Hydrocarbon Receptor Repressor Involved in Dioxin Signaling

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Abstract. The prevalence of undermasculinized external genitalia has increased in several countries including Japan, and this phenomenon has primarily been ascribed to the deleterious effects of environmental endocrine disruptors such as dioxins. To examine a possible role of the genetic susceptibility to dioxins in the development of micropenis (MP), we studied the Arg554Lys polymorphism of the gene for aryl hydrocarbon receptor (*AHR*) and the Pro185Ala polymorphism of the gene for aryl hydrocarbon receptor repressor (*AHRR*), in 73 boys with MP (34 boys with mild MP from –2.1 to –2.5 SD and 39 boys with severe MP below –2.5 SD) and 80 control males (50 boys and 30 fertile adult males). The allele and genotype frequencies of the *AHR* polymorphism were comparable between the two groups of males, but those of the *AHRR* polymorphism were significantly different, with the Pro allele and the Pro/Pro genotype being more frequent in boys with MP than in control males (*P*-value: 0.0029 for the allele frequency and 0.011 for the genotype frequency). In addition, both polymorphisms were comparable in the allele and genotype frequencies between boys with mild MP and those with severe MP and between control boys and control fertile adult males. The results suggest that the *AHRR* Pro185Ala polymorphism may constitute a susceptibility locus for the development of MP in response to dioxins.

Key words: micropenis, dioxin, aryl hydrocarbon receptor, aryl hydrocarbon receptor repressor, polymorphism

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MICROPENIS (MP) is a heterogeneous condition defined as significantly small penis without associated external genital ambiguity such as hypospadias [1, 2]. It is a common ailment and can take place as an isolated form or as a part of impaired male sex development [1, 2]. While MP with other genital features often results from single gene abnormalities, apparently isolated MP, though it still can be caused by single gene abnormalities in rare cases [3, 4], usually occurs as a multifactorial trait subject to various relatively minor genetic and environmental factors [1, 2].

The prevalence of undermasculinized external genitalia has increased during the last few decades at least in several countries including Japan [5, 6]. Similar tendencies have also been observed for spermatogenic failure and testicular cancer [5]. Furthermore, deterioration of male reproductive health is also identified in many wildlife species [7]. It has been hypothesized, therefore, that these adverse changes in males are interrelated events primarily caused by the deleterious effects of environmental endocrine disruptors (EEDs) [5]. Indeed, most EEDs are known to have estrogenic effects that disturb the endocrine balance and affect male reproductive function [5].

Dioxins are environmental contaminants primarily

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produced in the manufacture of chlorinated hydrocarbons. Experimental studies with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) have indicated that dioxins exert deleterious effects on male reproductive systems, in addition to other diverse disadvantageous biological effects [8]. For example, *in utero* and lactational exposure of male rats to TCDD disturbs development of male reproductive organs and affects spermatogenesis [8]. These findings imply that dioxins belong to EEDs that are disadvantageous to male reproductive health [5].

The detrimental effects of dioxins would depend on individual's susceptibility, in addition to the dosage and the developmental stage of exposure. In this context, the biological effects of dioxins are known to be mediated by several molecules including the aryl hydrocarbon receptor (AHR), the aryl hydrocarbon receptor nuclear translocator (ARNT), and the aryl hydrocarbon receptor repressor (AHRR) [9–12]. Dioxins bind to AHR in the cytoplasm, and the ligand-bound AHR is translocated into the nucleus and dimerizes with ARNT. The ligand-AHR-ARNT complex binds to the cognate xenobiotic responsive elements (XREs) in the promoter/enhancer regions of multiple genes including those involved in the xenobiotic metabolism, and regulates their transcription. AHRR is induced by the AHR-mediated signaling and constitutes a component of a negative feedback loop in the dioxin-related signal transduction pathway; AHRR also dimerizes with ARNT and the AHRR-ARNT complex binds to the XREs in competition with the ligand-AHR-ARNT complex, without altering expression of the target genes [12]. Thus, it is expected that polymorphisms of the genes for these molecules could be relevant to the individual's susceptibility to dioxins.

To date, two informative polymorphisms, Arg554Lys at exon 10 of *AHR* and Pro185Ala at exon 6 of *AHRR*, have been identified in the Japanese population, whereas no useful polymorphism has been detected for *ARNT* in the Japanese population, including the Asp511Asn and Asp517Glu at exon 16 that have been identified in non-Japanese populations [13–15]. In this context, Fujita *et al.* [14] have reported that the frequencies of the Pro allele and the Pro/Pro genotype of *AHRR* Pro185Ala polymorphism are significantly higher in 59 boys with severe MP (<–2.5 SD) than in 80 control males, whereas the prevalence of *AHR* Arg554Lys polymorphism is similar between the two groups of males. To further examine whether the *AHRR* Pro185Ala polymorphism constitutes a susceptibility locus for MP, we studied a different group of boys with MP.

Materials and Methods

Subjects

Seventy-three Japanese boys with MP (age, 0–14 yr; median, 7 yr) were studied. They satisfied the following selection criteria: (1) stretched penile length below –2.0 SD of the mean in age-matched normal Japanese boys [16]; (2) lack of other discernible genital and extragenital features, including hypospadias and gynecomastia; (3) 46,XY karyotype in all the ≥20 lymphocytes analyzed; (4) absence of demonstrable mutation of the genes for 5α-reductase-2 (*SRD5A2*) and androgen receptor (*AR*) that could cause MP [3, 4]; (5) no increase in the allele and genotype frequencies of Val89Leu polymorphism at exon 1 of *SRD5A2* that is known to decrease 5α-reductase-2 activity by ~30% and no expansion of CAG repeat lengths at exon 1 of *AR* that has been shown to be inversely correlated with transactivation function of the *AR* gene [3, 4]; and (6) apparently normal growth and development.

Since –2.0 SD has been regarded as the lower limit of normal variations for most quantitative traits and –2.5 SD has been used as the lower limit of normal penile lengths [1, 2], the 73 boys were divided into two groups: (1) 34 patients with mild MP from –2.1 to –2.5 SD below the mean (age, 0–13 yr; median, 8 yr); and (2) 39 patients with severe MP below –2.5 SD of the mean (age, 0–14 yr; median, 6 yr). Cryptorchidism was present in three boys with mild MP (two bilateral and one unilateral) and in two boys with severe MP (one bilateral and one unilateral), and testis was palpable in the inguinal region in all the five boys with cryptorchidism. Basal serum gonadotropin and testosterone values were within age- and pubertal tempo-matched Japanese reference data in most boys, except for low follicle stimulating hormone levels in a 9-year-old boy with mild MP (0.2 mIU/mL) and in a 7-year-old boy with severe MP (<0.2 mIU/mL).

For controls, the previous data of 50 Japanese boys with apparently normal external genitalia who were diagnosed as having idiopathic short stature (age, 3–16 yr; median, 8.5 yr) and 30 Japanese adult males with proven fertility (age, 25–48 yr; median, 38.0 yr)

were utilized [14]. All the boys with MP and the control males came from the urban or suburb area of Tokyo metropolis and Kawasaki City. They were free from particular residential environments such as the vicinity of chemical factories or farms, specific dietary habits such as vegetarianism or nearly pure meat or fish diet, and intake of drugs with hormonal effects. This study has been approved by the Institutional Review Board Committees at National Center for Child Health and Development. Informed consent was obtained from each subject and/or his parents.

Analysis of Arg554Lys polymorphism in AHR

Leukocyte genomic DNA was analyzed by the 5' nuclease assay with Taqman Minor Groove Binder (MGB) probes on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, www.appliedbiosystems.com) [17]. The Taqman MGB probe for the arginine allele was labeled with FAM, and that for the lysine allele was labeled with VIC. They were obtained from Applied Biosystems, together with the PCR primers for the amplification of a genomic region encompassing the MGB probe sequence (Assays-On-Demand SNP Genotyping Products; Assay ID: C_11170747_10).

Analysis of Pro185Ala polymorphism in AHRR

Genomic DNA was amplified by PCR with primers flanking exon 6, and the PCR products were digested with *Bbv*I. The methods were as described previously [14]. The PCR products harbor a naturally occurring *Bbv*I site and a polymorphism dependent *Bbv*I site where the proline allele is digested with *Bbv*I and the alanine allele is undigested with *Bbv*I. Thus, the *Bbv*I digestion yields two fragments for the alanine allele and three fragments for the proline allele. The presence of the naturally occurring *Bbv*I site served as an internal control in this method.

Statistical analysis

Statistical significance was examined by Pearson's χ^2 test. $P<0.05$ was considered significant.

Results

Arg554Lys polymorphism in AHR

The data are summarized in Table 1. There was no significant difference in the allele and the three types of genotype frequencies between boys with MP (mild, severe, and total) and control males, as well as between boys with mild MP and those with severe MP and between control boys and control adults (data not shown).

Pro185Ala polymorphism in AHRR

The data are summarized in Table 1. The allele frequencies were significantly different between boys with MP (mild, severe, and total) and control males, with the Pro allele being more prevalent in boys with MP than in control males. Furthermore, at least one of the three types of genotype frequencies was significantly different between boys with MP (mild, severe, and total) and control males, with the highest statistical significance being identified for different types of genotype comparisons. By contrast, no significant difference was identified for the allele and the three types of genotype frequencies between boys with mild MP and those with severe MP and between control boys and control adult males (data not shown).

Discussion

The results provide further support for the previously proposed notion that the Pro allele of *AHRR* Pro185Ala polymorphism raises the susceptibility to the development of severe MP [14], and suggest that the Pro allele is relevant to the occurrence of not only severe MP but also mild MP. Since the highest statistical significance was identified for different types of genotype comparisons between boys with MP (mild, severe, and total) and control males, the Pro allele may exert a co-dominant effect on the development of MP phenotype. By contrast, since no association was identified between MP and the Arg554Lys polymorphism, this polymorphism is unlikely to act as a modifier for the development of MP, as has been suggested previously [14].

The Pro allele of *AHRR* Pro185Ala polymorphism has also been suggested to increase the predisposition to male infertility. Watanabe *et al.* [18] reported that

Table 1. Summary of the association study

		Polymorphism analysis				*P*-value			
		Micropenis			Control (n=80)	M-MP vs. C	S-MP vs. C	T-MP vs. C	M-MP vs. S-MP
		Mild (n=34)	Severe (n=39)	Total (n=73)					
<*AHR*: Arg554Lys>									
Allele freq.	Arg	40	47	87	88	0.59	0.44	0.42	0.86
	Lys	28	31	59	72				
Genotype freq.	Arg/Arg	12	15	27	26	0.84	0.76	0.73	0.95
	Arg/Lys	16	17	33	36				
	Lys/Lys	6	7	13	18				
	Arg/Arg	12	15	27	26	0.77	0.52	0.56	0.78
	Arg/Lys+Lys/Lys	22	24	46	54				
	Arg/Arg+Arg/Lys	28	32	60	62	0.56	0.56	0.47	0.97
	Lys/Lys	6	7	13	18				
<*AHRR*: Pro185Ala>									
Allele freq.	Pro	49	52	101	84	**0.0061**	**0.038**	**0.0029**	0.48
	Ala	19	26	45	76				
Genotype freq.	Pro/Pro	18	16	34	22	**0.023**	0.092	**0.011**	0.53
	Pro/Ala	13	20	33	40				
	Ala/Ala	3	3	6	18				
	Pro/Pro	18	16	34	22	**0.0092**	0.14	**0.014**	0.31
	Pro/Ala+Ala/Ala	16	23	39	58				
	Pro/Pro+Pro/Ala	31	36	67	62	0.085	**0.047**	**0.015**	0.86
	Ala/Ala	3	3	6	18				

AHR: aryl hydrocarbon receptor; AHRR: aryl hydrocarbon receptor repressor; M-MP: mild micropenis; S-MP: severe micropenis; T-MP: total micropenis; and C: control.
Mild micropenis: penile length from –2.1 to –2.5 SD; and severe micropenis: penile length below –2.5 SD.

while the difference in the allele frequency of the *AHRR* polymorphism did not reach a significant level, the Pro/Pro genotype frequency was significantly higher in 123 infertile males than in 112 fertile males. The prevalence of the *AHR* Arg554Lys polymorphism was similar between infertile and fertile males. The results would also imply the relevance of the *AHRR* Pro185Ala polymorphism to the deterioration of male reproductive health.

The Pro allele may exert a weaker negative feedback effect on dioxin signaling than the Ala allele, leading to an enhanced dioxin action. In this regard, it has been shown that an agonist-activated AHR-ARNT heterodimer exerts estrogenic actions via a direct interaction with unliganded estrogen receptors (ERs), whereas the heterodimer exhibits anti-estrogenic activities in the presence of high doses of estrogens and represses estrogen-bound ER function [19]. It may be possible, therefore, that an enhanced dioxin effect results in an exaggerated estrogenic effect in males but not in females. Thus, an enhanced dioxin signaling may result in a disturbed endocrine status such as attenuated gonadotropin secretion in males by exaggerating estrogenic action [20], contributing to the development of MP as well as male infertility. Furthermore, such a sex dimorphism in a hormonal action of dioxins may possibly explain the previous finding that the frequency of *AHRR* Pro185Ala polymorphism as well as *AHR* Arg554Lys polymorphism is similar between 45 females with endometriosis, that is stimulated by estrogens and regarded as a target of EEDs [21, 22], and 108 control females [13].

Several points should be made with respect to the present study. First, the analyzed subjects are still too small in number to allow for a definitive conclusion. Second, there may be some unidentified underlying

genetic and/or environmental difference between the MP boys and the control males. Third, since functional studies have not been performed for the Pro185Ala polymorphism, it remains to be elucidated whether the polymorphism has a direct effect on the dioxin-related signal transductions. Indeed, it is possible that the polymorphism serves as a marker for a true hidden functional polymorphism, or that the positive association between MP and the polymorphism has been obtained just by chance. Lastly, it also remains to be determined whether similar results can be reproduced in other presumably EED-related male reproductive disorders such as cryptorchidism and hypospadias, and in other ethnic groups with an increased prevalence of such disorders. Indeed, although multiple studies have been performed for the two polymorphisms, most studies have focused on the susceptibility to cancers or the induction of *CYP1A1* [23, 24], and only three previous studies have shed a light on hormonal effects of dioxins [13, 14, 18].

Despite the above caveats, the present study suggests that the Pro185Ala polymorphism in *AHRR* may constitute a susceptibility locus for the development of mild and severe forms of MP. This notion awaits further case-control studies in undermasculinized disorders and functional studies of the Pro185Ala polymorphism.

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Micropenis and the 5α-Reductase-2 (SRD5A2) Gene: Mutation and V89L Polymorphism Analysis in 81 Japanese Patients

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The 5α-reductase-2 encoded by the SRD5A2 gene plays a critical role in male sex differentiation by converting testosterone into 5α dihydrotestosterone in the peripheral target tissues. In this study, we examined the SRD5A2 gene in 81 Japanese patients with micropenis (age, 0–14 yr; median, 7 yr) whose stretched penile lengths were between −2.5 SD and −2.0 SD in 39 patients (age, 0–13 yr; median, 8 yr) and below −2.5 SD in 42 patients (age, 0–14 yr; median, 6 yr), together with 100 control males (50 boys and 50 fertile adult males). Mutation analysis was performed for exons 1–5 and their flanking introns by denaturing HPLC and direct sequencing, revealing Y26X/R227Q in an 11-yr-old boy with a penile length of −2.6 SD, G34R/R227Q in a 9-yr-old boy with a penile length of −3.6 SD, and R227Q/R227Q in a 3-yr-old boy with a penile length of −2.4 SD, together with heterozygous R227Q in a control boy and a fertile adult male. Polymorphism analysis was carried out for the most frequent V89L known to reduce the enzyme activity by approximately 30% in 78 patients, except for the three patients with SRD5A2 mutations, and in the 100 control males by direct sequencing, showing that allele and genotype frequencies were similar between 78 patients with micropenis below −2.0 SD or 40 patients with micropenis below −2.5 SD and the 100 control males, the 50 boys, or the 50 fertile adult males, with no statistically significant differences.

The results suggest that, in Japanese patients, micropenis can be caused by SRD5A2 gene mutations, especially by R227Q which has been shown to retain approximately 3.2% of normal enzyme activity and appears relatively frequent in Asian populations, and that V89L polymorphism is unlikely to raise the susceptibility to the development of micropenis. (*J Clin Endocrinol Metab* 88: 3431–3436, 2003)

MICROPENIS IS A HETEROGENEOUS condition defined as significantly small penis that is free from associated external genital ambiguity such as hypospadias (1, 2). It is a quantitative character and can occur either as a single gene disorder or as a multifactorial disorder subject to various genetic and environmental factors (1, 2). Because the development of male external genitalia including penile growth is primarily caused by the biological effects of gonadal androgens, genes involved in the gonadal androgen production and in the peripheral androgen action could be relevant to the development of micropenis (1–3).

The 5α-reductase-2 plays a crucial role in male sex differentiation by converting testosterone (T) into 5α dihydrotestosterone (DHT) in the peripheral target tissues (3). It is known that masculinization of Wolffian ducts is primarily caused by T, whereas that of external genitalia, urethra, and prostate is primarily due to 5αDHT (3, 4). Thus, 5α-reductase-2 deficiency, although it permits Wolffian development, results in various degrees of male pseudohermaphroditism with undermasculinized external genitalia, primarily depending on the residual enzyme activity (5–7).

The 5α-reductase-2 is encoded by the SRD5A2 gene on chromosome 2p23 (8). The SRD5A2 gene consists of five exons and is expressed in the 5αDHT-dependent genital tissues as well as in other organs/tissues, including liver (9, 10). To date, multiple mutations distributed throughout the coding region of the SRD5A2 gene have been identified in patients with 5α-reductase-2 deficiency, and phenotypic spectrum in such patients is known to range widely from nearly female external genitalia to apparently male external genitalia (4, 9). Indeed, micropenis phenotype has been reported in a boy with a mutant SRD5A2 gene (11). Furthermore, a polymorphism in the SRD5A2 gene may also be relevant to the development of micropenis by raising the susceptibility to undermasculinization. In this regard, the most frequent polymorphism V89L (Val→Leu substitution at the 89th codon) at exon 1 has been shown to decrease 5α-reductase-2 activity by approximately 30% (12, 13), and previous studies have suggested that this polymorphism may reduce the susceptibility to androgen-dependent prostate cancer (13, 14). Thus, V89L polymorphism may be more prevalent in patients with micropenis than in normal males.

To our knowledge, however, there has been no report describing a systematic mutation or polymorphism analysis of the SRD5A2 gene in patients with micropenis. Thus, we performed mutation and V89L polymorphism analysis in patients with micropenis.

Abbreviations: DHPLC, Denaturing HPLC; DHT, dihydrotestosterone; hCG, human chorionic gonadotropin; T, testosterone; TE, T enanthate; THF, tetrahydrocortisol.

Subjects and Methods

Subjects

Eighty-one consecutive Japanese patients with micropenis (age, 0–14 yr; median, 7 yr) were studied, after obtaining written informed consent; 64 of the 81 patients have been described previously in our study of AR gene analysis (15). The selection criteria included: 1) stretched penile length below −2.0 SD of the mean in age-matched normal Japanese boys (16); 2) lack of hypospadias; 3) no gynecomastia; 4) age- and pubertal tempo-matched basal serum LH, FSH, and T levels; 5) 46,XY karyotype; 6) no definitive AR gene mutation indicated by a heteroduplex detection method and sequencing (15); 7) no recognizable malformation syndromes known to be associated with genital abnormalities; and 8) normal growth and development. Because −2.0 SD has been regarded as the lower limit of normal variations for most quantitative traits and −2.5 SD has been used as the lower limit of normal penile lengths (1, 2), the 81 patients were divided into two groups: group 1–39 patients with small penis between −2.0 and −2.5 SD below the mean (age, 0–13 yr; median, 8 yr); and group 2–42 patients with small penis below −2.5 SD of the mean (age, 0–14 yr; median, 6 yr; thus, the sum of groups 1 and 2 represents the total of 81 patients with micropenis below −2.0 SD). Cryptorchidism was bilaterally present in three patients aged 3, 8, and 13 yr in group 1 and in four patients aged 0, 5, 8, and 11 yr in group 2, and unilaterally present in two patients aged 6 and 9 yr in group 1 (right and left side, respectively) and in two patients aged 3 and 7 yr in group 2 (right and left side, respectively).

For controls, 50 Japanese boys with apparently normal external genitalia who were diagnosed as having idiopathic short stature (age, 3–16 yr; median, 8.5 yr) and 50 Japanese adult males with proven fertility (age, 25–48 yr; median, 38.5 yr) were similarly analyzed with permission. All of the 100 control males had a 46,XY karyotype.

Analysis of the SRD5A2 gene

Leukocyte genomic DNA was amplified for the 5 exons and their flanking introns of the SRD5A2 gene by PCR, using five sets of primers designed on the basis of the previous report (17) and the genomic sequence of the human SRD5A2 gene (GenBank accession no. L03843). The primer sequences and the annealing temperatures are shown in Table 1, together with the PCR product sizes. Subsequently, the PCR products for exon 1 were subjected to direct sequencing from both directions on an ABI PRISM 310 autosequencer (Applied Biosystems, Foster City, CA), to examine a mutation and the V89L (G265C) polymorphism. The PCR products for exons 2–5 were first screened for a mutation by a heteroduplex detection method with a proven sensitivity and specificity of more than 95% (18, 19), and when abnormal heteroduplex patterns were detected, corresponding PCR products were directly sequenced on the autosequencer. For the heteroduplex detection, the PCR products of each patient were mixed with those of a normal male known to have a wild-type sequence and subjected to denaturing HPLC (DHPLC) on an automated instrument (WAVE; Transgenomic, San Jose, CA). The DHPLC melting temperature was calculated by WAVE Maker software version 4.1, and several different temperatures (the calculated temperature and around that temperature) were used for the DHPLC analysis (Table 1).

Statistical analysis

The statistical significance of the allele and genotype frequencies of the V89L polymorphism was examined by the χ^2 test. P value less than 0.05 was considered significant.

Results

SRD5A2 gene mutations

SRD5A2 mutations were identified in three patients (Table 2, cases 1–3). Direct sequencing for exon 1 revealed a heterozygous C78G transversion resulting in a substitution of the 26th tryptophan codon by stop codon (Y26X) in case 1 (Fig. 1A) and a heterozygous G100C transversion leading to a substitution of the 34th glycine codon by arginine codon (G34R) in case 2 (Fig. 1B). Mutation screening for exon 4 detected abnormal chromatograms common to cases 1–3; subsequent direct sequencing revealed a heterozygous G680A transition causing a substitution of the 227th arginine codon by glutamine codon (R227Q) in cases 1 and 2 and a homozygous G680A transition (R227Q) in case 3 (Fig. 1C). The unrelated parents of case 2 and the consanguineous parents of case 3 were shown to be heterozygous for the mutations of cases 2 and 3, respectively (Table 3). In addition, a different type of aberrant chromotogram was found for exon 4 in two other cases, and sequencing analysis revealed a heterozygous silent substitution (T696C, H232H) in the two patients. No other abnormal chromatograms were found for exons 2–5. Y26X and G34R mutations were undetected in the 100 control males, whereas R227Q mutation was identified in a heterozygous status in two control males (a boy and an adult male).

Clinical findings of cases 1–3 are summarized in Table 2. Cases 1 and 2 had micropenis below −2.5 SD, and case 3 had mild micropenis of −2.4 SD together with bilateral undescended testes at the position of the external inguinal rings. The testes of case 3 could be manipulated to the upper scrotal regions but immediately ascended to their original positions. Human chorionic gonadotropin (hCG) tests (3000 IU/m^2 per dose im for 3 consecutive days; blood sampling on d 1 and 4) showed markedly elevated T/5αDHT ratios, together with poor T response in case 3. GnRH tests (100 μg/m^2 bolus iv; blood sampling at 0, 30, 60, 90, and 120 min) resulted in normal FSH and LH responses, except for a mild FSH hyperresponse in case 3. Analyses of steroid hormone metabolites for random urine samples by a gas chromatograph-mass spectrometry revealed markedly increased ratios of 5β to 5α metabolites, especially for tetrahydrocortisol (THF)

TABLE 1. The PCR primer sequences, the product sizes, and the PCR annealing and DHPLC melting temperatures

Exon	Forward primer Reverse primer	Product size (bp)	PCR annealing temp. (C)	DHPLC melting temp. (C)
1	5′-GCCGCGCTCTCTTCTGGGAG-3′ 5′-AGTGCGCTGCACTGGGCGCC-3′	371	60	Not performed
2	5′-AACAGTGAATCCTAACCTTTCCTCCC-3′ 5′-TTGTTAGCTGGGAAGTAGGTGAGAAG-3′	245	60	59, 60
3	5′-TGTGAAAAAAGCACCACAATCTGGA-3′ 5′-GCTCCAGGGAAGAGTGAGAGTCTGG-3′	212	60	56, 57, 61, 62
4	5′-TGCAATGATTGACCTTCCGATTCTTC-3′ 5′-TGTTTGGAGAAGAAGAAAGCTACGTG-3′	241	60	58, 59, 60
5	5′-TCAGCCACTGCTCCATTATATTTAC-3′ 5′-TTGACAGTTTTCATCAGCATTGTGG-3′	170	60	57, 58, 59

TABLE 2. Summary of clinical findings and laboratory data in cases 1–3

	Case 1	Case 2	Case 3
Mutations of SRD5A2	Y26X/R227Q	G34R/R227Q	R227Q/R227Q
V89L genotype	V/L	V/L	V/V
Age (yr)	11	9	3
Genital findings			
Penile length (cm)	2.8 (−2.6 SD)	2.0 (−3.6 SD)	2.2 (−2.4 SD)
Testis size (ml)	Right 3, left 4	Bilateral 2	Bilateral 1
Pubic hair stage	Tanner I	Tanner I	Tanner I
Others			Undescended testes
Serum hormone levels			
T (nmol/liter)			
Baseline	1.3 (<0.4–18.4)	<0.4 (<0.4)	<0.4 (<0.4)
hCG stimulated[a]	13.4 (4.0–15.0)	7.6 (4.0–15.0)	2.2 (4.0–15.0)
5αDHT (nmol/liter)			
Baseline	0.07 (0.10–0.59)	<0.07 (<0.07)	<0.07 (<0.07)
hCG stimulated[a]	0.21 (0.35–2.84)	0.28 (0.35–2.84)	0.07 (0.35–2.84)
T/5αDHT ratio	64 (8–21)[b]	27 (8–21)[b]	31 (8–21)[b]
FSH (IU/liter)			
Baseline	5.1 (0.6–5.2)	1.2 (0.5–4.7)	1.6 (0.2–2.3)
GnRH stimulated[c]	12.7 (4.7–16.6)	6.5 (4.7–9.5)	10.8 (4.4–9.5)
LH (IU/liter)			
Baseline	1.5 (0.5–2.5)	<0.2 (<0.2–1.8)	<0.2 (<0.2–0.2)
GnRH stimulated[c]	17.8 (10.9–20.6)	3.2 (1.7–3.8)	2.7 (1.7–3.8)
Urine steroid metabolites			
Et/An ratio	6.7 (0.4–1.1)	4.2 (0.5–0.8)	2.4 (0.5–1.0)
5βTHF/5αTHF ratio	33.0 (0.6–1.8)	32.0 (0.8–1.6)	24.0 (0.4–0.7)
5βTHB/5αTHB ratio	4.5 (0.2–0.6)	4.3 (0.3–0.6)	2.5 (0.2–0.5)
TE therapy (im)			
Dosage (mg/injection)	25	25	25
Injection number	2	2	3
Penile length increment (cm)	0.5	0.2	0.6
Increment (cm) per TE 25 mg	0.25 (0.3–1.0)[d]	0.1 (0.25–0.75)[d]	0.2 (0.25–0.75)[d]
5αDHT therapy (transdermal)			
Dosage (mg/d)[e]	25	25	12.5
Duration (wk)	8	16	8
Penile length increment (cm)	1.2	2.8	1.4
Final penile length (cm)	4.5 (−0.5 SD)	5.0 (−0.3 SD)	4.2 (±0 SD)

The values in *parentheses* indicate age-matched and pubertal tempo-matched Japanese reference hormone data (20–22, and our unpublished observation); the reference values for hCG stimulated T and 5αDHT have been obtained from boys who received hCG tests because of micropenis in childhood and subsequently showed normal pubertal development, and those for GnRH stimulated FSH and LH have been derived from boys who received triple tolerance tests (insulin, TRH, and GnRH) because of short stature. Et, Etiocholanolone; An, androsterone; THB, tetrahydrocorticosterone.

[a] hCG (3000 IU/m^2 im for 3 consecutive days, blood sampling on d 4).

[b] After hCG stimulation.

[c] Peak value during a GnRH test (100 μg/m^2 bolus iv; blood sampling at 0, 30, 60, 90, and 120 min).

[d] Range of penile length increment (cm per 25 mg of TE) in similarly treated prepubertal (n = 70) or early pubertal boys (n = 8) with no demonstrable mutation in the AR or SRD5A2 gene.

[e] One or 0.5 g of 2.5% 5αDHT gel was applied to the genitalia once per day (23).

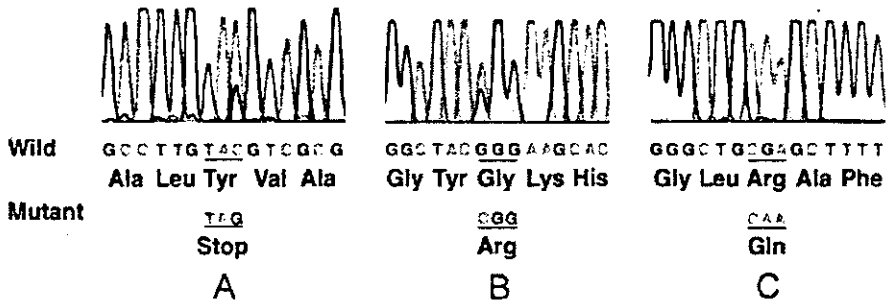

FIG. 1. Mutations of the SRD5A2 gene. A, A heterozygous C78G transversion at exon 1, resulting in Tyr26Stop (Y26X) in case 1. B, A heterozygous G100C transversion at exon 1, leading to Gly34Arg (G34R) in case 2. C, A homozygous G680A transition at exon 4, causing Arg227Gln (R227Q) in case 3.

derived from cortisol. Because cases 1–3 and/or their parents hoped to receive therapy immediately, 25 mg of testosterone enanthate (TE) was administrated im two or three times with an interval of more than 4 wk, resulting in subnormal penile length responses. After establishing the diagnosis of 5α-reductase-2 deficiency, 12.5 or 25 mg of 5αDHT (Andractim gel, Laboratories Besins Iscovesco, Paris, France) was transdermally applied to the genital region once per day for 8 or 16 wk according to the method of Choi *et al.* (23), increasing the penile length to nearly the average of age-matched Jap-

TABLE 3. Summary of clinical findings and laboratory data in parents of cases 2 and 3

	Case 2		Case 3	
	Father	Mother	Father	Mother
Mutations of SRD5A2	R227Q	G34R	R227Q	R227Q
V89L genotype	V/V	V/L	V/V	V/L
Age (yr)	47	40	41	36
Basal serum hormones				
T (nmol/liter)	19.7 (11.2–50.0)	1.8 (0.5–2.7)	16.5 (11.2–50.0)	1.8 (0.5–2.7)
5αDHT (nmol/liter)	1.2 (0.87–2.60)	0.2 (0.17–1.00)	1.1 (0.87–2.60)	0.2 (0.17–1.00)
T/5αDHT ratio	16 (16 ± 4)	9 (5 ± 2)	15 (16 ± 4)	9 (5 ± 2)
FSH (IU/liter)	4.5 (1.0–10.5)	4.5 (1.9–9.5)[a]	2.5 (1.0–10.5)	3.2 (0.5–6.4)[b]
LH (IU/liter)	3.8 (1.0–8.4)	4.6 (1.6–9.3)[a]	1.0 (1.0–8.4)	15.8 (0.5–64.0)[b]
Urine steroid metabolites				
Et/An ratio	1.2 (0.5–1.0)	1.2 (0.6–1.4)	0.8 (0.5–1.0)	1.5 (0.6–1.4)
5βTHF/5αTHF ratio	2.4 (0.6–1.7)	1.5 (0.9–2.6)	2.1 (0.6–1.7)	2.7 (0.9–2.6)
5βTHB/5αTHB ratio	0.5 (0.3–0.8)	0.4 (0.4–1.0)	0.4 (0.3–0.8)	0.4 (0.4–1.0)

The values in *parentheses* indicate adult Japanese reference hormone data (20). Et, Etiocholanolone; An, androsterone; THB, tetrahydrocorticosterone.

[a] At a follicular phase.

[b] At a luteal phase.

TABLE 4. Frequency of the V89L polymorphism

	Allele frequency		Genotype frequency		
	V	L	VV	VL	LL
Patient groups 1 and 2 (n = 78)[a]	93 (59.6%)	63 (40.4%)	28 (35.9%)	37 (47.4%)	13 (16.7%)
Patient group 2 (n = 40)[b]	43 (53.8%)	37 (46.2%)	9 (22.5%)	25 (62.5%)	6 (15.0%)
Control males (n = 100)	107 (53.5%)	93 (46.5%)	28 (28.0%)	51 (51.0%)	21 (21.0%)
Boys (n = 50)	49 (49.0%)	51 (51.0%)	12 (24.0%)	25 (50.0%)	13 (26.0%)
Adult males (n = 50)	58 (58.0%)	42 (42.0%)	16 (32.0%)	26 (52.0%)	8 (16.0%)

[a] Patients with micropenis below −2.0 SD.

[b] Patients with micropenis below −2.5 SD.

anese boys. In addition, the testes of case 3 were found to reside at the upper scrotal regions after the treatment.

Endocrine studies were also performed for the parents of cases 2 and 3 (Table 3). Basal serum T, 5αDHT, FSH, and LH levels were normal, and T/5αDHT ratio was normal in the two fathers and at the upper limit in the two mothers. Steroid hormone profile analysis for random urine samples indicated elevated 5βTHF/5αTHF ratios in the father of case 2 and the parents of case 3 and increased etiocholanolone/androsterone ratios in the father of case 2 and the mother of case 3.

V89L polymorphism

The V89L polymorphism was analyzed for 78 patients with no demonstrable SRD5A2 mutations (38 patients in group 1, and 40 patients in group 2) and for the 100 control males. The allele (V and L) and genotype (VV, VL, and LL) frequencies were similar between 78 patients with micropenis below −2.0 SD (groups 1 + 2) or 40 patients with micropenis below −2.5 SD (group 2) and the 100 control males, the 50 boys, or the 50 adult males, as well as between the 50 boys and the 50 adult males, with no statistically significant differences (Table 4).

The V89L polymorphism was also examined for cases 1–3, showing VL genotype in cases 1 and 2 and VV genotype in case 3 (Table 2). In addition, parental genotyping in cases 2 and 3 showed that case 2 inherited R227Q and V allele from the father and G34R and L allele from the mother, and that case 3 inherited R227Q and V allele from the parents (Tables 2 and 3). Thus, it was indicated that R227Q and V allele were linked in cases 2 and 3, the father of case 2, and the parents of case 3.

Discussion

Mutation analysis showed two missense mutations (G34R and R227Q) and one nonsense mutation (Y26X) of the SRD5A2 gene in three patients with micropenis. The two missense mutations have previously been reported in patients with 5α-reductase-2 deficiency (8, 11), and functional studies have indicated that both mutations severely compromise the enzyme activity (8, 12). The nonsense mutation is a novel one and should actually abolish the enzymatic activity, because it drastically truncates the enzyme. Furthermore, the results of parental studies are consistent with compound heterozygosity for G34R and R227Q in case 2 and homozygosity for R227Q in case 3. In addition, it is unlikely that Y26X and R227Q in case 1 resides on the same allele, because 5α-reductase-2 deficiency is an autosomal recessive disorder. Thus, although it might be possible that a different type of mutation(s) remained undetected by the DHPLC analysis or existed in an unexamined region such as the promoter and the intron sequences, our results imply that micropenis can be caused by SRD5A2 mutations, with an estimated prevalence of approximately 3.7% (3 of 81) in Japanese patients with micropenis below −2.0 SD and approximately 4.8% (2 of 42) in those with micropenis below −2.5 SD.

Cases 1–3 with SRD5A2 mutations shared R227Q in common. In this regard, several findings are noteworthy: 1) *in*