Journal of Pediatric Endocrinology & Metabolism, 16, 719-726 (2003)

Frequencies of the D^{85} and Y^{85} Variants of *UGT2B15* in Children and Adolescent Girls with Hyperandrogenism

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ABSTRACT

Premature pubarehe (PP) appears to be a risk factor for the subsequent development of polycystic ovary syndrome (PCOS) during or after puberty. The clinical manifestations due to hyperandrogenism are influenced by androgen production, androgen metabolism, and androgen receptor activity. Glucuronidation by the UDP-glucuronyltransferase 2B (UGT2B) family of enzymes is one mechanism through which androgens are inactivated. Two variants differing by the amino acid at codon 85 have been described for UGT2B15, a member of this family. Both variants show similar substrate specificities. However, for the substrates α-androstanediol (α-diol) and dihydrotestostcrone (DHT), the D^{85} variant has a lower V_{max} than the Y^{85} variant. We compared the frequencies of these variants in 69 patients with PP, 46 adolescent girls with hyperandrogenism (HA), and 88 healthy controls to determine whether the frequency of the D^{85} variant was increased among patients with hyperandrogenism. Allele frequencies were comparable in children with PP, adolescent girls with HA, and healthy control subjects. Although D^{85} and Y^{85} appear to be common variants, we cannot exclude the possibility that the *UGT2B15* gene represents a minor modifying locus.

KEY WORDS

premature pubarche, premature adrenarche, polycystic ovary syndrome, hyperandrogenism, glucuronidation, UGT enzymes

INTRODUCTION

Premature pubarche (PP) is defined as the development of pubic hair prior to age 8 years in girls and age 9.5 years in boys[1]. It has been attributed to premature adrenal maturation among patients in whom other disorders associated with PP such as congenital adrenal hyperplasia and Cushing's syndrome have been excluded[2]. Some girls with PP develop hirsutism and olio/amenorrhea associated with persistent hyperandrogenism during the peri- and post-pubertal periods that evolves into the polycystic ovary syndrome (PCOS), a common heterogeneous disorder characterized by irregular menses, infertility, hyperandrogenism, and insulin resistance[3,4]. Familial clustering of PCOS suggests that genetic factors contribute to the development of this disorder[5-7].

Over the past decade, it has become apparent that PCOS may have long-term health implications. Specifically, some affected women show a greater propensity to develop impaired glucose tolerance, overt diabetes mellitus, coronary artery disease, or endometrial cancer[8-10]. Recently, lifestyle interventions have been demonstrated to modify the natural history of impaired glucose tolerance[11,12]. If the genetic markers indicative of greater susceptibility to develop PCOS could be characterized, girls at increased risk for PCOS and these other disorders could be identified so that preventative interventions could be initiated. To date, the majority of candidate genes investigated by mutation detec-

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tion, linkage, and case-control studies have failed to be consistently associated with PCOS[13-19].

Traditionally, hyperandrogenism associated with PP, HA, and PCOS has been attributed to excessive ovarian and/or adrenal androgen secretion. Indeed, we have found that the frequency of heterozygosity for mutations in the 21-hydroxylase (*CYP21*) and type 2 3β-hydroxysteroid dehydrogenase (*HSD3B2*) genes is increased in children with PP and adolescent girls with HA[20-22]. Since serum androgen concentrations reflect the balance between the rates of production and metabolism, the clinical manifestations of hyperandrogenism depend on the rate of androgen secretion, androgen receptor activity, and the rate of androgen metabolism in peripheral tissues[23]. Target tissue enzymes catalyze conversion of sex steroids to active and inactive forms. Sex steroids can be further metabolized by glucuronidation, the transfer of a glucuronic acid moiety to the steroid molecule by one of the uridine-diphosphate-glucuronyltransferase (UGT) enzymes leading to increased water solubility of the steroid molecule which facilitates its excretion in bile and urine[24,25]. Thus, decreased activity, i.e. loss-of-function mutations, of the enzymes that metabolize androgens could potentially lead to accumulation of these hormones resulting in hyperandrogenism. Serum concentrations of one glucuronidated metabolite, 3α-androstane-3α,17β-diol-glucuronide (3α-diol-G), appear to reflect target tissue androgen action and have been proposed as a measure of androgen status in children with disorders of puberty, disorders affecting sexual development, and congenital adrenal hyperplasia[26-28].

C19 androgens are substrates for UGT2B15, UGT2B17, and UGT2B7, members of the UGT2B family. UGT2B15 preferentially glucuronidates dihydrotestosterone (DHT) and 3α-diol. Two variants of *UGT2B15* have been described that differ at codon 85, coding for either aspartic acid (D) or tyrosine (Y)[29]. In expression studies, both variants show similar substrate specificities and K_m values, but the V_{max} for the D^{85} variant is slightly lower for the substrates DHT and 3α-diol, suggesting the possibility of slower glucuronidation. We hypothesized that because of the lower V_{max}, the D^{85} variant of *UGT2B15* might show decreased glucuronidation resulting in impaired excretion of C19 androgens and increased androgen concentrations. The net result would be hyperandrogenism with clinical manifestations of androgen excess. Hence, we speculated that the frequency of the D^{85} variants might be increased among patients with hyperandrogenism.

PATIENTS AND METHODS

Patients

Blood samples for extraction of genomic DNA were obtained from 62 girls and seven boys referred for evaluation of premature pubarche (PP) and 46 adolescent girls referred for the evaluation of hirsutism and/or oligomenorrhea (HA). For all patients with PP or HA, congenital adrenal hyperplasia, Cushing's syndrome, and androgen-secreting tumors were excluded as diagnoses on the basis of medical history, physical examination, and hormone determinations. Premature pubarche was defined as the development of pubic hair prior to 8 years of age in girls and 9.5 years in boys. Hirsutism was defined as a modified Ferriman-Gallwey score >8[30]. There were 106 whites, 11 blacks, one Oriental, two bi-racial black/white, and one bi-racial Oriental/white. Eleven healthy white control girls were included. There were two pairs of siblings among the patients (sister-sister and sister-brother) and three sisters in the healthy control group. Height, weight, pubertal stage, and clinical manifestations of hyperandrogenism were recorded for all children and adolescents. Body mass index (BMI) was calculated as wt (kg)/ht (m^2). Genomic DNA samples from 65 healthy adult white males, one Asian-Indian male, and 11 healthy white adult females were evaluated to determine the frequencies of the D^{85} and Y^{85} variants in a control population.

This protocol was approved by the Human Rights Committee of the Children's Hospital of Pittsburgh. Written informed consent was obtained from parents of all children and adolescents. Assent was obtained from all children >7 years of age. Written informed consent was obtained all from healthy control subjects.

Hormone determinations

Blood samples were obtained from PP and HA patients and the 11 healthy control subjects for hormone determinations. Androstenedione concentrations were measured by radioimmunoassay as previously described[31]. Testosterone, sex hormone binding globulin (SHBG), 3α-androstanediol glucuronide (3α-diol-G), and DHT were measured at Esoterix (Calabasas Hills, CA).

Mutation detection studies

Genomic DNA was extracted from peripheral blood leukocytes. To detect the two *UGT2B15* variants, restriction fragment length polymorphism analysis was performed. The G→T nucleotide change responsible for the D^{85} to Y^{85} polymorphism creates a *Tsp*5091 restriction site. Primers used for the PCR reaction were 5′-GTTACTTTAGCTCTGGAAGC-3′ and 5′-TAT ATCCATCTATCGAGACTTTTC-3′. The anti-sense primer was end-labeled with ^{32}P-ATP prior to PCR amplification. The annealing temperature used for the PCR reaction was 58°C. Each 12.5 µl PCR reaction product was subsequently incubated with 1.5 µl *Tsp*509I, 2.0 µl NE Buffer 1 (New England Biolabs, Beverly, MA), and 1.5 µl water, at 65°C for 2 hours. The digested PCR product was then electrophoresed on an 8% denaturing polyacrylamide gel. The gels were dried and autoradiography was performed. The size of the undigested PCR product was 245 base pairs (bp). Following restriction digestion, the product sizes were: 167 bp and 78 bp for the D^{85} variant; 167 bp, 46 bp and 32 bp for the Y^{85} variant.

Statistical analysis

AbSTAT statistical software (Release 1.94, Anderson-Bell, Boulder, CO) was used for statistical analysis. Only the eldest sibling of any sib pair was included for determination of allele frequency. Analysis of variance (ANOVA) was used to compare BMI and hormone concentrations between genotypes within each group (stage of pubic hair development). Post hoc power analysis was performed using Russ Lenth's power and sample size website (rlenth@stat.uiowa.edu).

RESULTS

Patients

Pubic hair development began prior to 8 years of age among the white girls and prior to 7 years among the black girls referred for evaluation. Mean chronological age at time of referral was 7.7 ± 1.5 years. Mean chronological age for the boys with PP was 9.3 ± 1.0 years; all had developed pubic hair prior to 9 years of age. The adolescent girls were referred for evaluation of hirsutism and/or oligomenorrhea. All had hyperandrogenism with elevated concentrations of either androstenedione and/or testosterone. Mean chronological age for the 46 adolescent girls with HA was 15.5 ± 1.9 years. Mean chronological age for the healthy control girls was 11.6 ± 2.1 years. Mean BMI at the time of referral was 20.0 ± 4.9 kg/m^2, 21.6 ± 6.1 kg/m^2, and 30.2 ± 8.1 kg/m^2 for the girls with PP, boys with PP, and adolescent girls, respectively. For the 11 healthy control girls, mean chronological age was 11.6 ± 2.1 years and mean BMI was 20.2 ± 3.4 kg/m^2.

Genotype analysis

Among the 69 children with PP, 17 were homozygous for the D^{85} variant, 20 were homozygous for the Y^{85} variant, and 32 were heterozygous. Among the 46 adolescent girls with HA, 12 were homozygous for the D^{85} variant, 10 were homozygous for the Y^{85} variant, and 24 were heterozygous (Table 1). Among the 88 healthy control subjects (Table 1), 22 were homozygous for the D^{85} variant, 25 were homozygous for the Y^{85} variant, and 41 were heterozygous for the two variants. Two sisters were heterozygous and one was homozygous for the Y^{85} variant. Excluding two younger siblings among the children with PP, D^{85} was present on 64 alleles and Y^{85} was present on 70 alleles.

Allele frequencies were 0.48 for the D^{85} variant and 0.52 for the Y^{85} variant in the children with PP. Among the adolescent girls with HA, D^{85} was present on 48 alleles and Y^{85} was present on 44 alleles. Allele frequencies were 0.52 for the D^{85} variant and 0.48 for the Y^{85} variant. There were no differences in allele frequencies between the children with PP and the adolescent girls with HA.

TABLE 1

Genotype results.

Group	D^{85}/D^{85}	Y^{85}/Y^{85}	D^{85}/Y^{85}
PP (n = 69)	17	20	32
Girls (n = 62)	14	19	29
Boys (n = 7)	3	1	3
HA (n = 46)	12	10	24
Controls (n = 88)	22	25	41
Children (n = 11)	1	3	7
Adults (n = 77)	21	22	34

The number of individuals heterozygous for both variants or homozygous for each of the variants is listed for patients with premature pubarche (PP) and hyperandrogenism (HA) and for healthy controls.

Among the healthy control subjects, D^{85} was present on 84 alleles and Y^{85} was present on 88 alleles when the two younger sisters were excluded from consideration. Allele frequencies were 0.49 for the D^{85} variant and 0.51 for the Y^{85} variant. Allele frequencies did not differ between the children with PP, patients with hyperandrogenism, and the healthy control subjects.

Hormone determinations

Among the girls with PP, mean androstenedione concentrations were 121 ± 47 ng/dl for those homozygous for D^{85}, 99 ± 40 ng/dl for those homozygous for Y^{85}, and 92 ± 52 ng/dl for those heterozygous for the variants (Table 2, Fig. 1). Mean androstenedione concentrations were 402 ± 157 ng/dl in adolescent girls homozygous for D^{85}, 517 ± 231 ng/dl in adolescent girls homozygous for Y^{85}, and 379 ± 126 ng/dl in the adolescent girls heterozygous for both variants (Fig. 1). There were no significant differences in androstenedione, DHT, α-AG, or SHBG concentrations or for α-AG/DHT ratios between carriers of the different variants (Table 2).

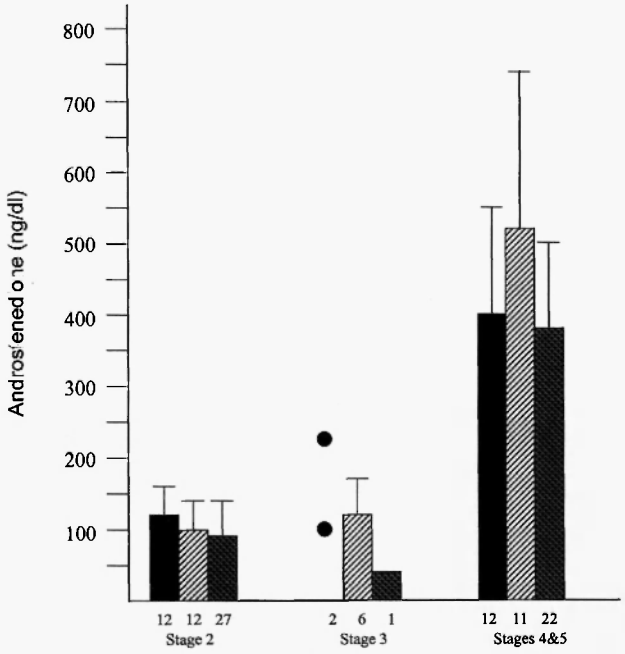

Fig. 1: Androstendione concentrations (ng/dl) are illustrated according to pubic hair Tanner stage and genotype. Solid bars (mean ± SD) indicate individuals homozygous for D^{85}, hatched bars indicate individuals homozygous for Y^{85}, and diamond bars indicate individuals heterozygous for the variants. The number of individuals in each group is shown under each bar. Values for the two patients who are homozygous for D^{85} and at pubic hair Tanner stage 3 are indicated by filled circles.

TABLE 2

Hormone concentrations

	D^{85}	Y^{85}	D^{85}/Y^{85}
STAGE 2			
Androstenedione (ng/dl)	121 ± 47 (12)	99 ± 40 (12)	92 ± 52 (27)
Testosterone (ng/dl)	16 ± 7 (7)	9v3 (6)	15.4 ± 12.1 (14)
DHT (ng/dl)	6.7 ± 3.2 (5)	6.2 ± 4.1 (4)	5.8 ± 5.1 (17)
α-AG (ng/dl)	99 ± 64 (5)	80 ± 94 (5)	60 ± 41 (18)
α-AG/DHT	16 ± 11 (4)	11 ± 8 (3)	19 ± 18 (16)
SHBG (μg/dl)	0.7 ± 0.5 (7)	0.8 ± 0.3 (6)	1.4 ± 0.7 (14)
STAGE 3			
Androstenedione (ng/dl)	107, 241 (2)	110 ± 57 (6)	43.0 (1)
Testosterone (ng/dl)	6.7 (1)	7.5 ± 3.1 (3)	6.2 (1)
DHT (ng/dl)	5 (1)	2 (1)	NA
α-AG (ng/dl)	38 (1)	NA	NA
α-AG/DHT	8.3 (1)	NA	NA
SHBG (μg/dl)	1.2 (1)	0.8 ± 0.6 (3)	1.6 (1)
STAGES 4 AND 5			
Androstenedione (ng/dl)	402 ± 157 (12)	517 ± 231 (11)	379 ± 126 (22)
Testosterone (ng/dl)	39 ± 20 (8)	51 ± 20 (3)	59 ± 37 (11)
DHT (ng/dl)	10.6 ± 5.5 (9)	10.6 ± 5.2 (6)	13.6 ± 5.5 (11)
α-AG (ng/dl)	261 ± 128 (9)	223 ± 63 (6)	347 ± 195 (14)
α-AG/DHT	27.0 ± 12.8 (9)	24.8 ± 19.9(5)	28.9 ± 17.1 (11)
SHBG (μg/dl)	1.3 ± 1.4 (8)	0.3 ± 0.10 (3)	0.6 ± 0.4 (13)

Hormone concentrations and hormone ratios (mean ± SD) for girls segregated according to pubic hair stage and genotype are listed. The number of patients in each category is indicated in parentheses following the hormone concentration or hormone ratio. Individual values are provided if there were fewer than three individuals in the subgroup.

Phenotype/genotype correlation

Among the girls with PP, BMI ranged from 13.5 to 37.2 kg/m^2. There were no differences in mean BMI among those homozygous or heterozygous for the D^{85} and Y^{85} variants. BMI ranged from 15.3 to 29.0 kg/m^2 among the boys with PP and from 18.8 to 54.2 kg/m^2 among the adolescent girls with HA. For the adolescent girls, no significant differences in BMI were present between those heterozygous or homozygous for the variants.

DISCUSSION

Glucuronidation is one mechanism through which endogenous, i.e. steroid hormones and bilirubin, and exogenous compounds, i.e. drugs, are metabolized. This process, catalyzed by the UDP-glucuronosyltransferase enzymes, renders the substrate more water-soluble to facilitate excretion in urine or bile. The highly homologous UGT2B15 and UGT2B17 enzymes glucuronidate C-19 steroids. UGT2B15 conjugates DHT and 3α-diol only at the 17β-OH position whereas UGT2B17 can conjugate many steroids, including dihydrotestosterone, 3α-diol, testosterone, and androsterone at both the 3α-OH and 17β-OH positions[32-34]. Here, because previous studies indicated that the D^{85} variant had a lower V_{max}, we speculated that the frequency of the D^{85} variant might be increased in patients with hyperandrogenism.

Precedent for this hypothesis is derived from the observation that polymorphic variants of the *UGT1A1* gene are associated with disorders of unconjugated hyperbilirubinemia including Crigler-Najjar and Gilbert syndromes[35,36]. The phenotypes associated with *UGT1A1* variants also include non-physiological neonatal hyperbilirubinemia, increased risk for gallstones in individuals with hereditary spherocytosis, and increased risk for hyperbilirubinemia in individuals with hereditary hemolytic anemias[37-42]. Risk for sporadic colorectal cancer has recently been associated with a variant of the *UGT1A7* gene[43].

We found that allele frequencies for the D^{85} and Y^{85} variants were comparable in both the children and adolescent girls with hyperandrogenism and the control subjects. Allele frequencies were consistent with Hardy-Weinberg equilibrium and with the frequencies reported by Lévesque *et al*[21]. Androstenedione concentrations were used as a surrogate measure because of the availability of hormone values for all PP and HA patients. No correlations were found between androstenedione concentration and genotype. However, since androstenedione has a keto-group at position 17, it is presumably not directly glucuronidated by UGT2B15. Thus, the lack of correlation between hormone concentrations and genotype is not surprising. In a smaller number of patients, no significant differences were found in DHT concentrations, α-diol-G concentrations, or α-diol-G/DHT ratios between the different *UGT2B15* genotypes. Hence, our results suggest that D^{85} and Y^{85} are common variants that do not have major differential effects on enzyme function.

However, the report of an increased frequency of homozygosity for D^{85} among men with prostate cancer suggests that this variant does influence local androgen concentrations and represents a weak modifier locus[44]. An alternative hypothesis is that other UGT enzymes, such as UGT2B7 and UGT2B17, modulate hormone concentrations[45]. Therefore, it is possible that a minor decrease in UGT2B15 enzyme activity is compensated by the presence of UGT2B17 and UGT2B7 with intact enzymatic activities.

In summary, for *UGT2B15*, the D^{85} and Y^{85} variants appear to be common alleles in our largely white population. Despite having sufficient statistical power to exclude a major gene effect, we cannot exclude the possibility that the D^{85} variant functions as a minor modifier locus[46-48].

ACKNOWLEDGEMENTS

We gratefully acknowledge the nursing assistance of Kristin Kolenc, Maryann Cwynar, Amy Gilliland, Janet Bell, and Tamara Johnston. We also appreciate the technical assistance of Carlie White and Debbie Cleary. We are grateful to Dean W. Hum, Ph.D., for helpful discussions and for providing nucleotide sequence information. We thank Candace Kammerer, Ph.D., for assistance with statistical analyses.

This study was supported in part by grants from the National Institutes of Health [R29-HD34808 (SFW), 5M01-RR-00084 (GCRC), and Training Grant DK07729 (MT)], the Genentech Foundation, the American Heart Association, and the Research Advisory Committee of the Children's Hospital of Pittsburgh.

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