The *CYP3A4*1B* Variant Is Related to the Onset of Puberty, A Known Risk Factor for the Development of Breast Cancer[1]

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Abstract

Breast development, one of the first signs of puberty, is closely associated with age at menarche; and early menarche is in turn a well-established risk factor for female breast cancer. We examined the relationships between the onset of puberty and gene variants for certain enzymes that regulate hormone metabolism among 137 healthy nine-year-old girls from two pediatric clinics. High-activity *CYP17* alleles, involved in estrogen formation, and high-activity *CYP1A2* and *CYP1B1* alleles, whose gene products metabolize estradiol, were not associated with pubertal stage. High activity *CYP3A4*, but not *CYP3A5*, which primarily metabolizes testosterone, showed a striking association with the onset of puberty (adjusted odds ratio, 3.21; 95% confidence interval, 1.62–6.89 for the genotype 0-1-2 rapid alleles). Of the homozygous *CYP3A4*1B/1B* girls, 90% had reached puberty; whereas, for the low-activity homozygous *CYP3A4*1A/1A* individuals, only 40% had done so. In heterozygotes, 56% had reached puberty. *CYP1B1*, *CYP3A4*, and *CYP3A5* rapid variants were more common in African-American than in Hispanic or Caucasian girls.

Introduction

Age at first breast development is one of the earliest signs of puberty and is associated with age at menarche. Early menarche is a well-established risk factor for female breast cancer. It has long been hypothesized that these associations are attributable to longer lifetime exposure to estrogens, especially during the critical period of breast development; however, factors such as height, weight, low physical activity, and decreased energy intake during and before puberty also appear to play a role (1, 2).

Genetic variation in certain enzymes involved in steroidogenesis has also been implicated in risk for breast cancer and for age at menarche (3, 4). In this study, we sought to examine the effects of genetic polymorphisms in cytochrome P450 (CYP)17, CYP1A2, CYP1B1, CYP3A4, and CYP3A5 on the onset of puberty. *CYP17*, which is involved in estradiol formation, exhibits two common alleles: a low-activity allele designated *CYP17*A1* and a high-activity allele designated *CYP17*A2* (5); CYP1A2, which plays a major role in the catabolism of estradiol, has several genetic variants, but the most common is located in intron 1 (designated *CYP1A2*1F*) and is associated with increased inducibility, resulting in about a 2-fold decrease in the level of the enzyme (6). CYP1B1 also has several genetic variants, but again the most common is *CYP1B1*3*, a high-activity variant that converts estradiol predominantly to 4-hydroxy-estradiol, a more potent estrogen (7).

CYP3A4, on the other hand, plays a major role in the catabolism of testosterone through 6-hydroxylation and, to a minor extent, estrogen metabolism through 16α-hydroxylation (8). It exhibits a common variant in the 5′-flanking region designated *CYP3A4*1B*. Although its function has not yet been established *in vivo*, it has been shown *in vitro* to exhibit about a 2-fold increase in activity over the wild-type *CYP3A4*1A* (9). *CYP3A5* confers a large proportion of CYP3A activity in the kidney and in some liver samples, and the *CYP3A5*1* variant exhibits higher phenotypic expression (10).

Materials and Methods

Study Subjects. Eligible girls were identified from appointment registers in a private pediatric practice in Manhattan and in the pediatric clinic of the Mount Sinai Hospital (New York City). Informed consent was obtained from a parent or guardian of each girl. Two hundred girls were recruited (response rate, 89%). There were 137 girls with complete information. The 137 girls had a mean age of 9.5 $\pm$ 0.3 years and consisted of 39 African Americans, 57 Hispanics, and 41 Caucasians. Breast development was assessed using Tanner breast stages determined by the attending physician(s) based on standardized methods (11). Reliability of the breast staging was similar to that reported in other studies (12). Personal characteristics and sociodemographic data were recorded. Pediatric nurses determined BMI[3] and also obtained blood samples. Details on this approach are reported elsewhere (12).

Received 5/10/02; revised 10/18/02; accepted 12/11/02.
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[1] Supported by EPA Grants R825816/R827039, National Institute of Environmental Health Sciences Grant ES 09584, and CDC Grant CCU300860 (to G. B. and M. W.); and by National Institute of General Medical Sciences Grant ES08658, NIH Grant P30 CA21765, Pharmacogenetics Research Network and Database Grant U01GM61374/93 (to E. S.); and by the American Lebanese Syrian Associated Charities.
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[3] The abbreviations used are: BMI, body mass index; OR, odds ratio; CI, confidence interval.

Genotyping. The DNA samples were purified from EDTA-preserved whole blood using the Qiagen QIAamp whole Blood Extraction kit (Valencia, CA). *CYP17* and *CYP1B1* genotyping were carried out as described by Carey *et al.* (13) and by Tang *et al.* (7). *CYP1A2* genotyping was accomplished from human genomic DNA using the primer pair CYP1A2 (forward): 5′-GGAAGGTATCAGCAGAAAGC-3′, and CYP1A2 (reverse): 5′-GAATACCAGGCAGAAGATGG-3′). The reaction mixture (50 μl) contained 100 ng DNA, 1 μM each primer, 10 mM Tris-HCl buffer (pH 8.3), 2% DMSO, 2.5 mM $MgCl_2$, 200 μM each 2-deoxynucleoside 3′-triphosphate (PE Biosystems Inc., Foster City, CA), 2 units of Taq DNA polymerase (PE Biosystems Inc.). PCR amplification was carried out for 35 cycles by heat-denaturing at a temperature of 94°C for 30 s, annealing at a temperature of 56°C for 30 s, and using a primer extension temperature of 72°C for 2 min, with a Perkin-Elmer DNA Model 9600 thermal cycler (Foster City, CA). The PCR products (502 bp) were digested with *Bsp*120I (MBI Fermentas, Hanover, MD) for 3 h without further purification and were analyzed by gel electrophoresis (2.0% Metaphor Agarose, FMC BioProducts, Rockland ME) to detect the (C to A) *CYP1A2*1F* variant of the *CYP1A2* gene. The wild-type *CYP1A2*1F* showed a 493-bp product, and the homozygous variant exhibited 251-bp and 243-bp fragments.

***CYP3A4* Genotyping.** A two-step PCR-based restriction fragment length polymorphism assay was used to determine the genotype of a *CYP3A4*1A/B* single nucleotide polymorphism at the promoter region (−290) on the nifedipine-specific response element (A to G). The first PCR reaction was done using a set of primers [CYP3A4 (forward): 5′-CTGGAGCTGTGGCTTGTTGG-3′, CYP3A4 (reverse): 5′-CGAAGCAGGGCTGGAGCTGC-3′] to generate a 319-bp fragment covering the nifedipine-specific response element. The first PCR reaction was carried out using 100 ng of genomic DNA, 1× AdvanTaq Plus buffer, 1 μM each primer, 250 μM 2′-deoxynucleoside 3′-triphosphate, and 1 μl (10 units/μl) of AdvanTaq Plus DNA polymerase (ClonTech) in a final 50-μl volume. PCR amplification was carried out for 30 cycles by heat denaturing at a temperature of 95°C for 90 s, annealing at a temperature of 65°C for 30 s, and primer extension at 70°C for 30 s, with a final step at 70°C for 3 min. PCR products verified by agarose gel electrophoresis were then diluted (1:500). One μl of this dilution was used for a secondary PCR reaction using a second set of nested primers to amplify a 168-bp fragment. Following are the nested primers: CYP3A4 (F3)-*Scr*FI: 5′-GGACAGCCATAGAGACAAGG**C**CA-3′; CYP3A4 (R2): 5′-CACTCACTGACCTCCTTTGAGTTCA-3′. A mismatch (underlined) was introduced in the forward primer to generate a restriction site (*Scr*FI) in the variant allele (CCNGG, −288 to −292), but not in the wild type. Nested PCR reactions were done using 1 μl of the first PCR dilution, 1× AdvanTaq Plus buffer, 1 μM each primer, 250 μM 2-deoxynucleoside 3′-triphosphate, and 1 μl (10 units/μl) of AdvanTaq Plus DNA polymerase (ClonTech) in 50 μl of volume, with the following temperature profile: (*a*) 95°C for 1 min; (*b*) 95°C for 30 s; (*c*) 70°C for 1 min; (*d*) 5 cycles from (*b*) to (*c*); (*e*) 95°C for 30 s; (*f*) 66°C for 1 min; (*g*) 25 cycles from (*e*) to (*f*); and (*h*) 70°C for 3 min. Ten μl of the nested PCR products were digested with 0.5 units of *Scr*FI (New England Biolabs) in 3.5 μl H_2O and 1.5 μl of 10× NEBuffer no. 4 [50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT (pH 7.9)] at 37°C for 2 h. Then, the digests were electrophoresed on a 3% agarose gel and stained with ethidium bromide. *CYP3A4*1A* homozygotes exhibited a 168-bp fragment, and *CYP3A4*1B* homozygotes showed 146-bp and 22-bp fragments.

CYP3A5 genotypes were determined as reported previously (10). The *CYP3A5*6* variant frequency was 0.034 in our population, with no **6/*6* genotypes, and this allele was treated as a low-activity genotype for purposes of statistical analyses.

Statistical Analysis. Associations between breast development stage (Tanner breast stage 2 or higher *versus* stage 1) and genotypes were estimated by logistic regression. We used the first principal component to adjust all logistic regressions for the explanatory value of height, weight, age, and BMI. This principal component is referred to as PRINC1 hereafter. PRINC1 captures 90.7% of the deviance in the Tanner breast score 2 or higher (any breast), which can be explained by height, weight, age, and BMI. The change in deviance for PRINC1 is 22.4 ($P < 0.0001$). In logistic regression models, we examined associations of T2B with genotype adjusted for race/ethnicity and PRINC1. Genotype was coded as an ordinal variable (0, 1, or 2 rapid alleles) and associations with breast development were reported as ORs and 95% CIs. The *P* representing the effect of increasing gene dose was used to assess trend (*P*-trend).

Table 1 Association of breast pubertal stage T2B ≥2 with the number of high-activity alleles among 137 girls 9.5 years of age[a]

Reference allele	Odds trend	95% CI	*P* trend
*CYP17*A1*	1.44	0.80–2.63	0.2253
*CYP1A2*1F WT*	1.07	0.55–2.08	0.8444
*CYP1B1*1*	0.98	0.55–1.72	0.9830
CYP3A4*1A	**3.21**	**1.62–6.89**	**0.0007**
*CYP3A5*3*	1.45	0.75–2.87	0.2687

[a] ORs are for increasing number of gene variants (0, 1, 2) with trend indicated by the *P*. The test for lack-of-fit was not significant for any models.

Results

Study Population. We undertook genotyping for enzymes regulating hormone metabolism in a study of pubertal development among 137 healthy girls, including 39 African Americans, 57 Hispanics, and 41 Caucasians. Breast development (T2B, also called Tanner breast stage) was categorized as positive for stage 2 or higher and negative for stage 1, *i.e.*, no development. Height, weight, age, and BMI were positively associated with T2B. A larger proportion of African-American girls had T2B scores above 1 (adjusted OR, 6.3; 95% CI, 2.3–19.5) relative to Hispanic girls. Caucasian girls did not differ from Hispanic girls* (adjusted OR, 1.1; 95% CI, 0.4–2.6). The effect of enzyme polymorphisms on the frequency of girls with T2B >1 was examined using ORs and 95% CIs after adjusting for race/ethnicity and PRINC1. Table 1 summarizes the effect of genotype after adjusting for race and the first principle component of height, weight, age, and BMI. The trend reflects the change in the odds with the number of high-activity alleles comprising the genotype. The lack-of-fit test evaluates whether or not the trend adequately describes the differences among genotypes. Specific outcomes are detailed below.

***CYP17*.** The *CYP17* genetic polymorphism did not show any association with the onset of breast development. As depicted in Fig. 1, the proportion of girls who had reached T2B (puberty) was similar for each genotype: *CYP17*A1/*A1* (low activity), *CYP17*A1/*A2* (intermediate activity), and *CYP17*A2/*A2* (high activity).

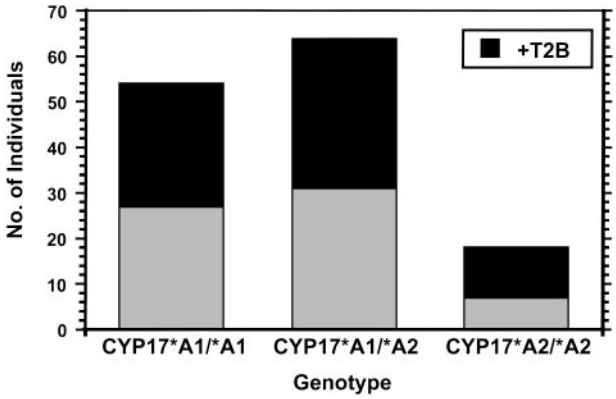

Fig. 1. *CYP17* genotype distribution at the onset of puberty. The proportion of girls with positive Tanner 2B scores (*T2B*) are shown on the top portion of each histogram depicting the number of girls carrying each genotype.

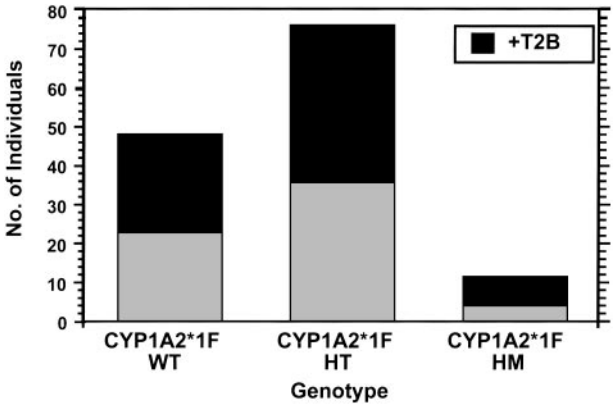

Fig. 2. *CYP1A2* genotype distribution at the onset of puberty. The proportion of girls with positive Tanner 2B scores (*T2B*) are shown on the top portion of each histogram depicting the number of girls carrying each genotype.

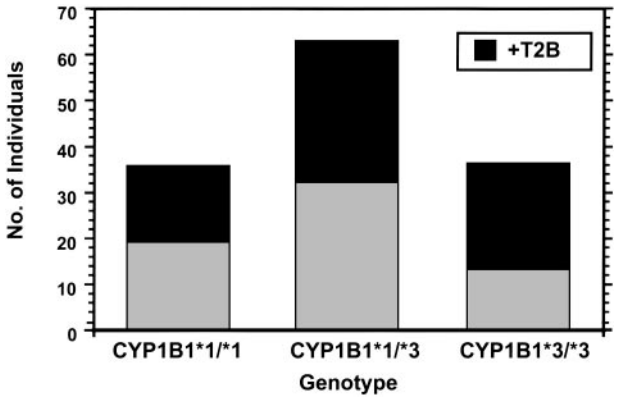

Fig. 3. *CYP1B1* genotype distribution at the onset of puberty. The proportion of girls with positive Tanner 2B scores (*T2B*) are shown on the top portion of each histogram depicting the number of girls carrying each genotype.

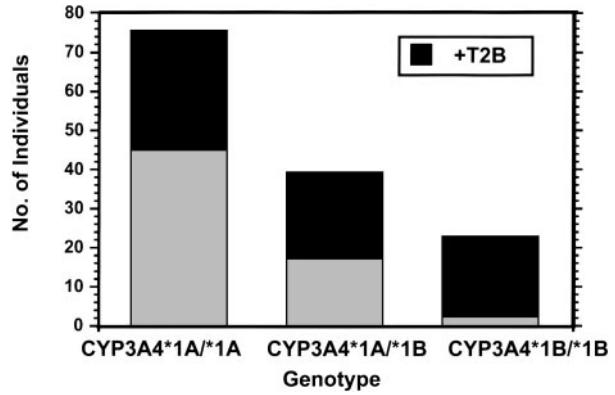

Fig. 4. *CYP3A4* genotype distribution at the onset of puberty. The proportion of girls with positive Tanner 2B scores (*T2B*) are shown on the top portion of each histogram depicting the number of girls carrying each genotype.

CYP1A2. The intron 1 polymorphism that allows inducibility of CYP1A2, *CYP1A2*1F*, also did not exhibit any disproportionate distribution of girls who had reached T2B (puberty) *versus* those who had not, as shown in Fig. 2.

CYP1B1. Similarly, the high-activity *CYP1B1*3/*3* genotype compared with that of the heterozygous and homozygous *CYP1B1*1/*1* (low-activity) genotypes (Fig. 3) was not significantly associated with the onset of puberty.

CYP3A4. The genetic polymorphism in the 5′-flanking region of *CYP3A4*, which results in the variant *CYP3A4*1B*, was significantly associated with the onset of puberty (adjusted OR, 3.21; CI, 1.62–6.89, for the genotypes as 0-1-2 rapid alleles). *CYP3A4*1B* alleles; *P*-trend = 0.0007). As depicted in Fig. 4, 90% of girls with the *CYP3A4*1B/*1B* had Tanner breast stage 2 or higher, compared with 56% of the heterozygotes and 41% of those with wild-type homozygous.

CYP3A5. The *CYP3A5*1*, the rapid polymorphism in CYP3A5, was not significantly associated with the onset of breast development (adjusted OR, 1.45; CI, 0.75–2.87, for 0-1-2 rapid alleles; *P*-trend = 0.27), although the variant was more common in girls with T2B (Fig. 5).

Racial/Ethnic Distribution. As shown in Fig. 6, the *CYP3A4*1B* allele frequency was highest in African Americans (0.62), followed by Hispanics (0.52), and lowest in Caucasians (0.17). Race/ethnicity was not significantly associated with T2B when added to a logistic regression after adjusting for PRINC1 and the *CYP3A4* genotype ($P = 0.066$). This test of race/ethnicity has 2 degrees of freedom and can be subdivided into 1 degree of freedom contrasts. The contrast between African Americans and Hispanics remains significant having an adjusted OR of 3.8 and CI, 1.2–12.5. In models assessing race/ethnicity and puberty, adjusting for *CYP3A5*1*, and other covariates, the African-American race remained significant.

Discussion

None of the genetic polymorphisms involving enzymes that control estrogen formation or metabolism examined in this study seemed to play a major role in affecting the onset of puberty. This was unexpected, inasmuch as CYP17 plays a major role in estradiol formation and, possibly, age at menarche (reviewed in Ref. 5). CYP1A2 appears to dominate the control of serum estradiol levels (14, 15), and CYP1B1 increases active estrogen species by formation of the highly estrogenic 4-hydroxy-estradiol (16). Instead, the *CYP3A4*1B* variant predicted the onset of breast development. *In vitro* studies have found a 1.5–2.0-fold increase in CYP3A4 activity with dexamethasone

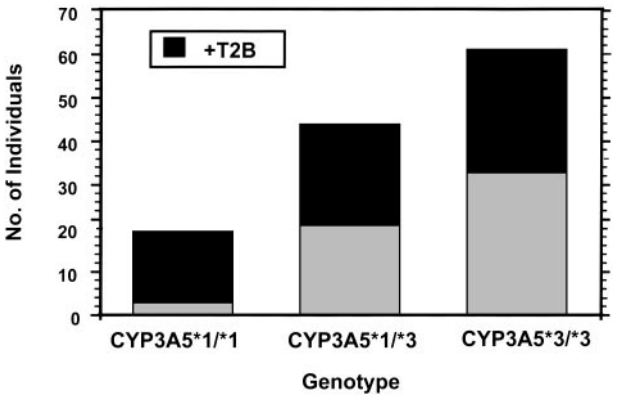

Fig. 5. *CYP3A5* genotype distribution at the onset of puberty. The proportion of girls with positive Tanner 2B scores (*T2B*) are shown on the top portion of each histogram depicting the number of girls carrying each genotype.

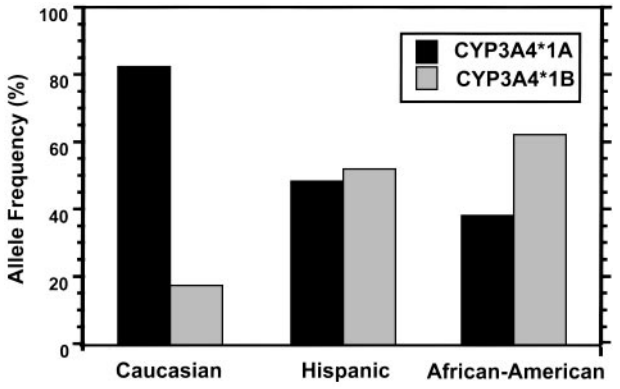

Fig. 6. Ethnic distribution of *CYP3A4*1* genotypes in different ethnic groups. Shown are the distribution of *CYP3A4*1A* and *CYP3A4*1B* genotype among 41 Caucasian, 57 Hispanic, and 39 African-American girls.

enhancement (9). *In vivo* studies have not yet shown the *CYP3A4*1B* variant to be a high-activity allele (17, 18), but proper studies with a cross-over design have not been carried out. This variant may also be linked with known high-activity variants in CYP3A5 (10). However, because *CYP3A4*1B* is an inducible gene, additional studies will be necessary to establish the relationship between *CYP3A4* genotype and phenotype to better understand the effect of this gene on early development.

CYP3A4 is the major CYP in human hepatic tissue and plays a major role in testosterone metabolism, catalyzing its 6β, 2β, and 15β hydroxylation (19, 20). A hypothesis that could explain these findings is that CYP3A4 causes changes in the estradiol:testosterone ratio as these hormone levels rise at the onset of puberty. Like estradiol, serum testosterone concentrations in prepubertal girls are significantly lower than those in pubertal girls (21). Thus, high-activity *CYP3A4*1B* may cause a disproportionate drop in testosterone levels, which may increase the estradiol:testosterone ratio and result in the hormonal cascade that accompanies puberty (luteinizing hormone/follicle stimulating hormone ratio increase, luteinizing hormone hyperpulsatility, insulin secretion, increase in BMI).

Early puberty and the interval until menarche may be a critical period in breast development, when the breast is susceptible to environmental carcinogens. Thus, it would be of interest to investigate whether the *CYP3A4*1B* genotype and the CYP3A phenotype are risk factors for breast cancer. Interestingly, this variant has been shown to be associated with an increased risk for prostate cancer in African Americans (22), and higher-grade tumors (23), and possibly childhood leukemias (24). In addition, androgens may have a role in insulin resistance and related growth factors that are being widely investigated as risk factors for breast cancer (25).

The racial/ethnic distribution reported here for the *CYP3A4*1B*, with an allele frequency of 0.62 in African Americans, 0.52 in Hispanics, and 0.17 in Caucasians, is similar to that previously reported for African Americans (0.50–0.80; Refs. 26–28) but is much higher than for other Hispanics (0.09–0.20) and somewhat higher than for Caucasians (0.04–0.09). However, the Hispanics in this study are primarily of Puerto Rican ancestry and, thus, may differ from the other Hispanic populations reported. The *CYP3A5*3* allele frequencies in our study were similar to those reported elsewhere (10), *e.g.*, African Americans 0.60, Hispanics 0.30, and Caucasians 0.12 in these girls. The high prevalence of the *CYP3A* rapid genotypes in African-Americans compared with Caucasians is consistent with the much earlier average age [by 9 months (29)] of puberty among African-American girls compared with Caucasians and with our hypothesis involving testosterone metabolism (30). Furthermore, African-American women have a much higher incidence of premenopausal breast cancer and much more aggressive disease (31, 32), which could stem from initiating events occurring during development of the breast (33). Onset of puberty in our study was similar for Hispanics and Caucasians, although in other studies, age at menarche was earlier in Mexican Hispanics than in Caucasians (34), but breast cancer risk in the United States among Hispanics is lower by 30–50% (28, 35). Yet, the *CYP3A4*1B* genotype appears to predict breast development, regardless of race/ethnicity, and these effects may be mediated through the role of the *CYP3A4* gene in testosterone catabolism.

The stages of puberty (2 through 5) are about 1 year apart, with menarche occurring between stages 3 and 4, or 2–3 years after first breast development (12). The ages at breast stage 2 (T2B) and menarche are only modestly correlated ($r = 0.4$–0.5) in longitudinal studies (36, 37). Recently, Lai *et al.* (38) did not detect an association between the *CYP3A4*1B* genotype and menarche, as recalled in adulthood. One explanation is that different hormones trigger early puberty and menarche, with the latter involving the growth hormone pathway and internal feedback loops that mature after breast development. Our study was based on physician assessment of breast stage, which may be more objective than recall, and we were able to adjust for other variables such as age at puberty and body size, and so forth. Limitations of our study include its cross-sectional design, the clinic-based sample, and the relatively small sample size, which may have limited our ability to detect small effects of the less prevalent polymorphisms. However, the sample is similar to the population in the neighboring area, and the prevalence of breast development in our study is similar to a large national study of African-American and Caucasian girls (12, 39).

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Cancer Epidemiol Biomarkers Prev 2003;12:327-331.