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Candidate gene analysis in premature pubarche and adolescent hyperandrogenism

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Objective: To identify genetic markers associated with premature pubarche in children and hyperandrogenism in adolescent girls.

Design: Association study.

Setting: Academic research environment.

Patient(s): Forty children with premature pubarche (PP), 29 adolescent girls with hyperandrogenism (HA), and 15 healthy control women.

Intervention(s): None.

Main Outcome Measure(s): Genetic variations at five loci selected because of known associations with hyperandrogenism, insulin resistance, hyperinsulinemia, or obesity.

Result(s): Heterozygosity for *CYP21* mutations was identified in 14 of 40 (35%) PP, 8 of 29 (28%) HA, and 1 of 30 (3%) controls. Heterozygosity for *HSD3B2* variants was identified in 3 of 40 (7.5%) PP, 5 of 29 (17%) HA, and 0/15 controls. Among the PP, 11 of 80 (14%), 5 of 80 (6%), and 7 of 80 (9%) alleles showed the *IRS-1*, *GRL*, and *ADRB3* variants, respectively. Among the HA, 5 of 58 (8.6%), 3 of 58 (5%), and 6 of 58 (10%) alleles showed the *IRS-1*, *GRL*, and *ADRB3* variants, respectively. Among the control participants, variant allele frequency was 1 of 30 (3.3%) for *IRS-1*, 2 of 30 (6.6%) for *GRL*, and 2 of 30 (6.6%) for *ADRB3*.

Conclusion(s): Our findings suggest that the development of PP and HA can be associated with the occurrence of multiple sequence variants at five susceptibility loci, especially steroidogenic enzyme genes. This approach offers a novel paradigm to investigate and identify the genetic factors relevant to polycystic ovary syndrome. (Fertil Steril® 2001;75:724–30. ©2001 by American Society for Reproductive Medicine.)

Key Words: Premature pubarche, polycystic ovary syndrome, hyperandrogenism, precocious puberty, genetic variation, hyperinsulinemia/insulin resistance

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Premature pubarche (PP) has been historically defined as the development of pubic hair before age 8 years in girls and age 9 years in boys. Following the exclusion of congenital adrenal hyperplasia and androgen-secreting tumors, PP is typically attributed to increased adrenal androgen secretion secondary to premature adrenal maturation. Indicators of hyperinsulinemia/insulin resistance such as increased mean insulin concentrations during oral glucose tolerance tests and decreased concentrations of sex hormone binding globulin (SHBG) and IGF-binding protein-1 (IGF-BP1) have been recognized in girls with PP (1). During gonadotropin-dependent puberty, the frequency of oligomenorrhea/amenorrhea and hyperandrogenism is increased in girls who have a history of PP compared to healthy control girls (2). Some adolescent girls develop hirsutism, oligomenorrhea/amenorrhea, hyperandrogenism, increased LH/FSH ratios, and hyperinsulinemia/insulin resistance, all of which are characteristic clinical, hormonal, and metabolic features of polycystic ovary syndrome (PCOS) (3–5). Thus, evidence continues to accumulate indicating that PP and adolescent hyperandrogenism (HA) in some, but not all, girls are early manifestations of PCOS (2, 6, 7).

PCOS is a common disorder characterized by irregular menses, chronic anovulation, infertility, hyperandrogenism, and insulin resistance/hyperinsulinemia (8). Hirsutism may be present. Familial clustering of women with

TABLE 1

Children with premature pubarche and adolescent girls with hyperandrogenism.

	Chronologic age (y)	Body mass index (kg/m^2)	Androstenedione (ng/dL)[a]	Testosterone (ng/dL)[a]
Premature pubarche girls	7.0 ± 1.4 (34)	20.5 ± 7.0 (34)	120.0 ± 72.0 (33)	20.3 ± 16.5 (8)
Premature pubarche boys	9.2 ± 1.0 (6)	20.4 ± 5.6 (6)	78.0 ± 36.3 (6)	ND
Hyperandrogenic girls	15.4 ± 2.0 (29)	29.5 ± 7.1 (28[b])	393.1 ± 177.4 (28)	95.2 ± 49.2 (23)

Note: Values are mean ± SD.

[a] Concentration at initial visit.

[b] One girl was wheelchair bound, so an accurate height could not be obtained.

Witchel. Candidate gene analysis. Fertil Steril 2001.

PCOS suggests the importance of genetic factors in the pathogenesis of this common disorder (9–11). However, the extreme phenotypic heterogeneity, even within a single family, has hampered traditional genetic approaches. Further, as laboratory testing of asymptomatic sisters of affected women often indicates biochemical evidence of hyperandrogenism, clinical phenotype alone may be insufficient to accurately assign disease status (12).

To date, candidate genetic loci, including *CYP17*, *CYP11A*, *CYP19*, follistatin, insulin receptor, insulin, and GnRH receptor, have been investigated by mutation detection, linkage, and case-control association studies (13–19). Yet despite the efforts of many investigators, the "PCOS genes" remain elusive. Available evidence supports the hypothesis that PCOS is a complex genetic trait or polygenic disorder (20). However, the limited ability of traditional methods of linkage analysis to detect small gene effects confounds identification of susceptibility loci. As the genes associated with PCOS are identified, predicting the risk for progression from PP and HA to PCOS and propensity for diabetes may become possible. Thus, elucidating predictive genetic and hormonal factors could lead to development of effective interventions.

The clinical features typical of PCOS direct the search for susceptibility loci to genes that influence androgen metabolism, insulin action, or body composition. Just as genotyping at several susceptibility loci has been helpful in the analysis of genetic risk factors associated with coronary artery disease (21), we selected five candidate loci that appear to be associated with the PCOS-related phenotypes of hyperandrogenemia, insulin resistance/hyperinsulinemia, and obesity. To determine whether genotyping at these loci would provide insight into the genes relevant to PP, HA, and possibly PCOS, we compared frequencies of these variants in children with PP, in adolescent girls with HA, and in healthy control women.

The rationale for choosing the 21-hydroxylase (*CYP21*) and type 2 3β-hydroxysteroid dehydrogenase (*HSD3B2*) genes was our previous findings that heterozygosity for mutations in these genes occurs more often in hyperandrogenic patients compared to healthy controls (22, 23). We chose the G972R variant of insulin receptor substrate-1 (*IRS-1*), the W64R variant of β_3-adrenergic receptor (*ADRB3*), and the N363S variant of glucocorticoid receptor (*GRL*) genes because these specific allelic variants have been reported to influence insulin resistance/hyperinsulinemia and body composition (24–26).

MATERIALS AND METHODS

Patients

Forty children with premature pubarche (PP) and 29 adolescent girls with hyperandrogenism (HA) were referred to the Children's Hospital of Pittsburgh by their primary care physicians (Tables 1 and 2). Among the children with PP, there were 6 boys and 34 girls. Their ages ranged from 4.6 to 10.75 years. All of the girls had developed pubic hair before age 8 years and all boys before 9 years. Among the PP group, 5 were black, 1 was white-black, 1 was Asian-white, and 33 were white. The 2 black girls had onset of pubic hair before age 7 years.

TABLE 2

Normal values.

	Androstenedione (ng/dL)		Testosterone (ng/dL)
	Female	Male	Female
Tanner I	<50	<50	<22
Tanner II	40–112	18–89	10–29
Tanner III	55–190	42–150	10–40
Tanner IV	70–245	60–198	24–62
Tanner V	74–284	79–245	27–70

Witchel. Candidate gene analysis. Fertil Steril 2001.

The adolescent girls had been referred for evaluation of oligomenorrhea/amenorrhea and/or hirsutism (HA). All were documented to have elevated androstenedione and/or testosterone concentrations. Among the HA girls, 3 were black, 1 was Asian, and 25 were white. Chronologic ages ranged from 10.4 to 18.0 years. For the children and the adolescent girls, congenital adrenal hyperplasia, Cushing's syndrome, and hyperprolactinemia had been excluded from diagnostic consideration by past medical history, physical examination, and laboratory studies. The control subjects consisted of 15 healthy white adult women. All 15 women had regular menstrual cycles and no evidence of hirsutism.

This protocol was approved by the Human Rights Committee (institutional review board) of the Children's Hospital of Pittsburgh. Informed parental consent and patient assent (for children older than age 7 years) was obtained for all of the children and adolescents. Informed consent was obtained from all adult participants.

Genotype Analyses

Genomic DNA was extracted from peripheral blood mononuclear cells. Molecular genetic analysis of *CYP21* was performed as described elsewhere (27, 28). Briefly, restriction fragment length polymorphism (RFLP), single strand conformational polymorphism (SSCP), allele specific oligonucleotide hybridization, and heteroduplex analyses were used to assay for nine common *CYP21* mutations, large deletions/gene conversion events involving the promoter region, P30L, intron 2 splicing mutation, I172N, V281L, T insertion, Q318X, R356W, and P453S.

SSCP analysis was used to detect sequence variants of the *HSD3B2* gene, as described elsewhere (23). With the use of three different gel conditions, we typically identify 85% of sequence variants.

Presence of the *IRS-1* variant was determined by RFLP analysis as described elsewhere (24). The N363S variant of the glucocorticoid receptor gene was readily recognized on SSCP analysis (29). The presence of the *ADRB3* variant was determined by RFLP analysis (25). For all three genes, homozygosity for either variant or heterozygosity was readily differentiated and a positive control was included in all analyses.

Statistical Analysis

Descriptive statistics, cross tabulation, the Fisher exact test, and independent *t* tests were performed using AbSTAT (Anderson-Bell, Boulder, CO).

RESULTS

Genetic Analyses

CYP21

Fourteen of 40 (35%) PP children were heterozygous for *CYP21* mutations. Mutations detected were intron 2 splicing mutation (n = 2), V281L (n = 5), I172N (n = 2), P30L (n = 1), P453S (n = 1), Q318X (n = 1), and large deletion/gene conversion events (n = 2). Eight of 29 (28%) HA girls were heterozygous for *CYP21* mutations. Seven girls were heterozygous for the V281L mutation and 1 was heterozygous for I172N. One of 15 (6.6%) healthy adult women was heterozygous for the intron 2 splicing mutation in *CYP21*. For PP and HA groups, mean basal androstenedione concentrations and mean body mass index (BMI) values did not differ between mutation carriers and those heterozygous for the wild type allele ($P > .05$).

HSD3B2

Three of 40 (7.5%) PP children were found to have variants of the *HSD3B2* gene. Two children were each found to have two linked nucleotide changes in the 3′-untranslated region (3′-UTR) [C8815→G; A8921→G]. Both variants altered restriction sites. By RFLP analysis, we confirmed that these variants were located on the same allele (23). One girl was identified to be heterozygous for T8307→C generating a missense mutation, L236S. Five of 29 (17%) HA adolescent girls were heterozygous for variants of the *HSD3B2* gene. In addition to 1 girl with L236S and 3 girls with the 3′-UTR variant, 1 girl was found to be heterozygous for C8100→T, which created a missense mutation, A167V. None of the healthy controls showed variants in this gene. For PP and HA groups, mean basal androstenedione concentrations and mean BMI values did not differ between mutation carriers and those heterozygous for the wild type allele ($P > .05$).

IRS-1

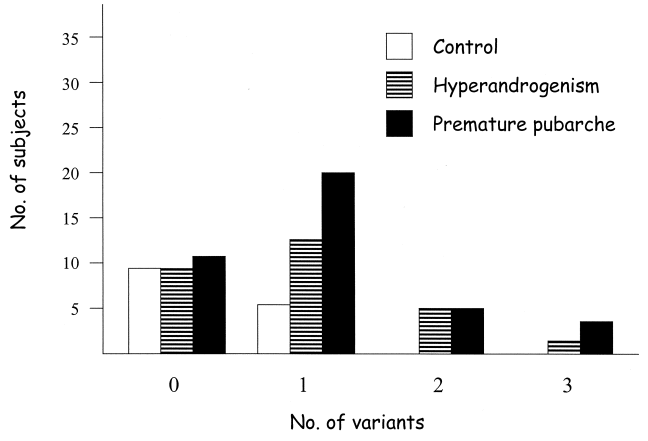
Eleven of 40 (27.5%) PP children were heterozygous for the G972R variant of *IRS-1*. Five of the 29 (17%) HA adolescent girls were heterozygous for the G972R variant. One of 15 (6.6%) healthy control women was heterozygous for the G972R variant. For the PP and HA subjects, mean BMI and mean basal androstenedione concentrations did not differ between the heterozygous carriers of the G972R variant and those homozygous for the wild type allele G972 ($P > .05$).

GRL

Five of 40 (12.5%) PP children were heterozygous for the *GRL* variant N363S. Three of 29 HA adolescent girls (10%) were heterozygous carriers of this glucocorticoid receptor variant. Two of 15 (13.3%) healthy controls were heterozygous for this variant. There was no difference in the frequency of heterozygosity for the N363 allele among the three groups. Among the patients, there was no difference in either mean BMI or mean basal androstenedione concentrations between the heterozygotic carriers of N363S variant and those homozygous for the wild type allele N363 ($P > .05$).

FIGURE 1

Number of susceptibility markers. The number of subjects with 0, 1, 2, or 3 variants in each group is shown.



Witchel. Candidate gene analysis. Fertil Steril 2001.

ADRB3

Seven of 40 (17.5%) PP children were heterozygous for the W64R variant of *ADRB3*. Four of 29 (13.7%) HA adolescent girls were heterozygous and 1 was homozygous for this variant. Two of the 15 (13.3%) healthy women were heterozygous for this variant. For the two patient groups, mean BMI and mean basal androstenedione concentrations were comparable between the heterozygous carriers of W64R and those homozygous for the wild type allele W64 ($P>.05$).

Multiple Variations

Eleven of 40 (27.5%) PP children showed no variants for any of the loci examined (Fig. 1 and Table 3). One variant was detected in 21 of 40 (52.5%) PP children. Five (12.5%) showed two variants, and 3 (7.5%) showed three variants. At least one variant involving a steroidogenic enzyme gene was found in all 8 PP children identified to carry two or more variants.

No variants in the selected candidate genes were identified in 9 of 29 (31%) HA adolescent girls (see Fig. 1 and Table 3). One variant was identified in 13 (44.8%) HA girls. Two variants were detected in 5 (17%), and three variants were detected in 1 girl (3.4%). At least one steroidogenic enzyme variant was found in 5 of 6 patients carrying two or more variants.

No variants were identified in 9 of the 15 (60%) healthy adult women controls (see Fig. 1 and Table 3). Six (40%) showed a single variant. None had two or three variants. The frequency of a steroidogenic enzyme variant was greater in the PP group than the control women, $P=.012$. The frequency of a steroidogenic enzyme variant was greater in the HA group than the control women, $P=.015$.

TABLE 3

Distribution of mutations in each group.

	Premature pubarche (n = 40)	Hyperandrogenic (n = 29)	Controls (n = 15)
No variants	11 (27.5)	9 (31)	9 (60)
CYP21	6 (15)	5 (17)	1 (6.6)
3β-HSD2	2 (5)	3 (10.3)	0 (0)
IRS-1	8 (20)	1 (3.4)	1 (6.6)
GRL	2 (5)	1 (3.4)	2 (13.3)
ADRB3	3 (7.5)	3 (10.3)	2 (13.3)
CYP21 + 3β-HSD2	0 (0)	0 (0)	0 (0)
CYP21 + IRS-1	2 (5)	2 (6.9)	0 (0)
CYP21 + GRL	1 (2.5)	0 (0)	0 (0)
CYP21 + ADRB3	2 (5)	1 (3.4)	0 (0)
3β-HSD2 + IRS-1	0 (0)	1 (3.4)	0 (0)
3β-HSD2 + GRL	0 (0)	1 (3.4)	0 (0)
3β-HSD2 + ADRB3	0 (0)	0 (0)	0 (0)
IRS-1 + GRL	0 (0)	1 (3.4)	0 (0)
IRS-1 + ADRB3	0 (0)	0 (0)	0 (0)
GRL + ADRB3	0 (0)	0 (0)	0 (0)
CYP21 + ADRB3 + ADRB3	0 (0)	1 (3.4)	0 (0)
CYP21 + 3β-HSD2 + ADRB3	1 (2.5)	0 (0)	0 (0)
CYP21 + IRS-1 + GRL	1 (2.5)	0 (0)	0 (0)
CYP21 + GRL + ADRB3	1 (2.5)	0 (0)	0 (0)

Note: The numbers of patients with mutations in a specific gene (left hand column) are listed (% total).

Witchel. Candidate gene analysis. Fertil Steril 2001.

PP Group Divided into Girls and Boys

As the major manifestations of PCOS are limited to women, the PP group was reanalyzed separately for girls and boys. Of the 34 girls, 8 (23.5%) were heterozygous carriers of *CYP21* mutations. Three of 34 (8.8%) were heterozygous for *HSD3B2* variants. Among the girls with PP, 9 of 34 (13.2%), 4 of 34 (5.8%), and 6 of 34 (8.8%) were heterozygous for the *IRS-1*, *GRL*, and *ADRB3* variants, respectively. Of the 6 boys, 5 (83%) were heterozygous carriers of *CYP21* mutations and none carried *HSD3B2* variants. Among the boys with PP, 2 of 6 (33.3%), 1 of 6 (16.7%), and 1 of 6 (16.7%) were heterozygous for the *IRS-1*, *GRL*, and *ADRB3* variants, respectively. Variant allele frequencies were comparable for the original 40 children with PP, the PP girls alone, and PP boys alone.

DISCUSSION

Genetic factors have been strongly implicated in the pathogenesis of PCOS. The association of PCOS with impaired glucose tolerance, and overt diabetes mellitus has generated great interest in understanding the pathophysiology and identifying the causative genes (30). However, traditional linkage analyses have been confounded by the

extreme phenotypic heterogeneity, even in a single family, and the imperfect definition of the male phenotype. In the search for the PCOS genes, several candidate genetic loci have been evaluated (13–19). However, to date no single gene has been identified as the PCOS susceptibility gene for the majority of cases. As PP and HA appear to be early manifestations of PCOS for some girls and to begin to characterize genetic markers associated with PCOS, we compared allelic variation at five distinct loci that influence androgen secretion, insulin action, and body composition.

The *CYP21* mutations identified in our subjects were loss of function mutations associated with congenital adrenal hyperplasia. Our data show that *CYP21* mutations were more common in PP children and HA adolescent girls (35% and 28%, respectively), compared to 6.6% in the healthy control group. Although these values were not statistically significant because of the small sample size, the allele frequencies are comparable to our prior results showing that heterozygosity for *CYP21* mutations was greater in PP and HA subjects than healthy controls (22). Comparable frequencies of heterozygosity for *CYP21* mutations among women and children with hyperandrogenism have been reported from England, France, Spain, and Greece (31–34). Differences in clinical features between symptomatic and asymptomatic obligate heterozygotic carriers may reflect influences of other genetic or environmental factors such as variations in the *IRS-1*, *GRL*, and *ADRB3* genes (35).

Three PP children and five HA adolescent girls were found to be heterozygous carriers of *HSD3B2* sequence variants. Expression studies have been recently reported for the A167V and L236S variants. The A167V variant shows 81.5% activity in a transient transfection assay (36). Although 3β-HSD2 activity was not decreased when the L236S variant was expressed in a heterologous system, this variant likely has deleterious effects because it occurs in less than 1% of the normal population, affects a conserved amino acid, and appears to segregate with clinical symptoms in our two patients and in a third undervirilized 46,XY patient reported by Moisan et al. (36, 37).

Notably, 17 (42%) children with PP and 13 (41%) adolescent girls with HA carry sequence variants involving steroidogenic enzyme genes compared to 1 (6.6%) healthy control women, $P=.012$ and $P=.015$, respectively. Thus, heterozygous loss of function variations in steroidogenic enzyme genes appear to be one risk factor or susceptibility marker for PP and HA.

The insulin receptor substrate proteins function immediately downstream of the insulin receptor. Although studies assessing the functional significance of the R972 variant of IRS-1 have been inconsistent (24, 38–43), recent investigations have demonstrated that the R972 variant is associated with impaired glucose-stimulated insulin secretion by the pancreatic beta cells and decreased insulin-stimulated glucose transport in skeletal muscle cells (44–46). Thus, this variant may play a contributory role and could be considered to be a minor susceptibility locus.

The N363S variant of the glucocorticoid receptor appears to be associated with increased sensitivity to glucocorticoids, decreased sensitivity to insulin, and perhaps obesity (26, 47). However, we did not find any association between heterozygosity for N363S and BMI in our population of children and adolescent girls. The frequency of the N363S variant ranges from 6% to 14% with comparable frequencies in the PP, HA, and control subjects. Considered as a single factor, the N363S variant does not appear to be a PCOS susceptibility locus. However, we cannot exclude the possibility that it acts as a weak modifier gene.

In different populations, the W64R variant of the β3-adrenoreceptor has been inconsistently associated with insulin resistance, diabetes, or obesity. Conflicting studies, including meta-analyses with opposite results, have failed to clarify the importance of the W64R variant as an obesity gene (48–51). We found no association of this variant with BMI in our patient population, but cannot exclude an effect that becomes apparent only in later adulthood (52).

We hypothesized that the concurrent presence of multiple genetic factors would be more likely in the PP and HA patients. In fact, for the PP and HA groups, we found that 14 of 69 (20%) showed the concurrent presence of two or more allelic variants. Of the 14 patients with two or more variants, at least one mutation was detected in a steroidogenic enzyme gene for 13 patients. On the other hand, none of the control group was found to have more than one variant. Only a single control showed a variant of a steroidogenic enzyme gene.

Although the clinical manifestations of PCOS appear to be limited to women, genetic variants associated with hyperandrogenism and insulin resistance are not limited to women. Thus, although the natural history of PP in boys appears to be relatively benign (53), attention to the genetic factors associated with PP in boys may help identify genes relevant to PCOS (54, 55). Curiously, five of (83%) six boys with PP were heterozygous for *CYP21* mutations.

Thus, we have found that the presence of polymorphisms/mutations at multiple candidate loci, especially steroidogenic enzyme genes, occurs more often in PP children and in HA adolescent girls than among healthy adult women. Our findings are consistent with the hypothesis that PP and HA reflect the effects of multiple susceptibility genes, epigenetic influences, and environmental factors. Similar studies in women with PCOS are necessary to confirm our findings. Importantly, this approach provides a novel paradigm to investigate and identify genetic factors relevant to PCOS.

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