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Association Study of AMP-Activated Protein Kinase (AMPK) Subunit Genes in Polycystic Ovary Syndrome

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Abstract

Objective—To examine the genes for AMP-activated protein kinase (AMPK) subunits $\alpha 2$ (*PRKAA2*) and $\gamma 3$ (*PRKAG3*) as candidates for polycystic ovary syndrome (PCOS) and its component traits.

Design and methods—287 White PCOS women were recruited from the reproductive endocrinology clinic at the University of Alabama at Birmingham and 187 White control subjects were recruited from the surrounding community. Seven *PRKAA2* single nucleotide polymorphisms (SNPs) and four *PRKAG3* SNPs were genotyped in PCOS cases and controls. Genotyping and association analysis were performed at Cedars-Sinai Medical Center.

Results—Nominal associations of *PRKAA2* variants with insulin-related traits and the *PRKAG3* Pro71Ala variant with PCOS were not statistically significant after multiple testing correction. Among PCOS patients, there were no associations between variants in AMPK subunit genes and androgenic or reproductive traits.

Conclusions—Variants in genes for AMPK $\alpha 2$ and AMPK $\gamma 3$ were not associated with PCOS or its component traits. Our evidence does not demonstrate AMPK is a major genetic risk factor for PCOS.

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Introduction

Polycystic ovary syndrome (PCOS) is a common complex genetic disorder, affecting ~6-8% of reproductive age women (1,2). PCOS is characterized by hyperandrogenism, ovulatory dysfunction, polycystic ovarian morphology, and frequently insulin resistance.

Adenosine monophosphate-activated protein kinase (AMPK) is a cellular energy sensor, acting to maintain ATP levels in the cell by sensing the AMP/ATP ratio and then phosphorylating metabolic enzymes and regulating gene expression with the net result of activating processes that generate ATP and inhibiting processes that consume ATP. AMPK influences food intake, fatty acid and glucose uptake in skeletal and cardiac muscle, insulin secretion, and hepatic fatty acid synthesis and gluconeogenesis (3). Consequently, AMPK subunit genes have been examined as candidates for type 2 diabetes susceptibility, with conflicting results (4,5).

As PCOS is a metabolic disorder associated with an increased risk of diabetes, AMPK may play a role in this association. Metformin, commonly prescribed for PCOS patients, activates AMPK activity in skeletal muscle (6). In addition, AMPK activation appears to be decreased in PCOS ovarian theca cells, suggesting that metformin stimulation of AMPK in the ovary itself may promote ovulation (7). We therefore examined the genes for AMPK α 2 (*PRKAA2*) and AMPK γ 3 (*PRKAG3*) as candidate genes for PCOS and its component traits.

Subjects and Methods

Subjects and Phenotyping

A total of 287 consecutive unrelated White patients with PCOS, aged 13-47 years, and 187 healthy unrelated White control women, aged 14-60, were recruited from the Birmingham, Alabama, USA area. PCOS cases were recruited from the reproductive endocrine practice of one of the investigators (RA) at the University of Alabama at Birmingham (UAB). All subjects were non-Hispanic Caucasians. Participation in research studies was offered to patients meeting inclusion criteria (premenopausal, non-pregnant, on no hormonal therapy for at least three months, and meeting diagnostic criteria for PCOS). PCOS was diagnosed following the 1990 NIH consensus criteria (8).

Controls were healthy women, with a history of regular menstrual cycles and no family history of hirsutism. These women had no evidence of hirsutism, acne, or alopecia, or endocrine dysfunction and had not taken hormonal therapy (including oral contraceptives) for at least 3 months prior to testing. Controls were recruited by word of mouth and advertisements in the Birmingham, Alabama area, through a call for “healthy women” without detailing further the nature of the studies.

Subjects underwent a physical examination and serum androgen measurement, per a previously described protocol (1). Fasting glucose and insulin were also obtained in a subset of the cohort (~70%). This subset did not differ demographically or hormonally from the study subjects overall. The computer-based homeostasis model assessment (HOMA, www.dtu.ox.ac.uk/homa) utilizes fasting glucose and insulin to calculate indices of insulin resistance (HOMA-IR) and insulin secretion (HOMA-%B) (9). For the insulin-related traits only, six PCOS subjects with diabetes were excluded because the hyperglycemia of diabetes may induce secondary changes in insulin-related traits that reduce their utility for genetic analysis. In addition, in PCOS cases only, we evaluated the following reproductive traits: severe oligomenorrhea (defined as >3 months between menses), infertility (yes/no), and parity (yes/no). Clinical characteristics of the subjects are given in Supplemental Table 1.

All subjects gave written informed consent, and the study was performed according to the guidelines of the Institutional Review Boards of UAB and Cedars-Sinai Medical Center, Los Angeles, CA, USA.

Genetic Analysis

We selected six *PRKAA2* single nucleotide polymorphisms (SNPs) (rs11206887, rs2143749, rs2746349, rs12749128, rs857155, rs3738568) using genotype data of the Caucasian population of the HapMap database (release 24, phase II) (10). These SNPs capture 28 of 35 (80%) of the Caucasian HapMap SNP alleles at $r^2 > 0.8$. An additional *PRKAA2* SNP (rs2051040) was selected because it was associated with type 2 diabetes in a Japanese cohort (4). Four validated *PRKAG3* SNPs (rs6436094, rs16859382, rs692243, rs650898) were genotyped, selected based on frequency $> 5\%$ because only two SNPs in HapMap (rs6436094, rs692243) are polymorphic in Caucasians. SNP rs692243 is a non-synonymous variant (proline-to-alanine at codon 71). The eleven SNPs were genotyped in the 474 subjects using the 5'-exonuclease assay (TaqMan MGB, Applied Biosystems, Foster City, CA, USA). Supplementary Table 2 lists PCR primers and TaqMan MGB probes.

Statistical Analysis

Unpaired T-tests and chi-square tests were used to compare clinical characteristics between women with and without PCOS; quantitative trait values were log- or square root-transformed as appropriate to reduce non-normality.

Association with presence/absence of PCOS and qualitative traits was evaluated using logistic regression, adjusting for age and body mass index (BMI) by including both as independent variables. Association with quantitative traits was evaluated using analysis of covariance (ANCOVA), again adjusting for age and BMI. Recognizing that insulin secretion responds to ambient insulin resistance, HOMA-%B associations were also calculated with HOMA-IR as a covariate in addition to age and BMI. Quantitative trait associations were conducted in the PCOS and control groups separately. To handle multiple testing, significance was taken as $P < 0.006$, considering that we analyzed two genes against four families of traits (PCOS diagnosis, androgens, metabolic traits, reproductive endpoints), yielding a Bonferroni correction factor of 8 (i.e. 8 independent comparisons). Association analyses excluding 26 post-menopausal subjects from the control group yielded the same results as analyses conducted in the entire cohort; therefore, the latter results are reported.

Results

PRKAA2

We genotyped 7 SNPs spanning the *PRKAA2* gene (Fig. 1A). SNP frequencies are shown in Table 1. All markers were in Hardy-Weinberg equilibrium. Linkage disequilibrium among these markers (D') ranged from 0.45 to 1.0 (average pairwise D' of 0.90). The r^2 values ranged from 0 to 0.98 (average 0.23).

Among the women with PCOS, carriers of the minor alleles of SNPs rs2143749, rs2746349 or rs857155 had higher fasting insulin levels than non-carriers ($P=0.044$, 0.021, 0.027, respectively). Carriers of the minor allele of SNP rs2143749 also had increased HOMA-%B ($P=0.030$), which was not significant when HOMA-IR was also included as a covariate ($P=0.26$). Lastly, PCOS women who were carriers of the minor allele of SNP rs2746349 had increased HOMA-IR compared to non-carriers ($P=0.039$). Trait values for PCOS women are shown in Supplemental Table 3. None of these *PRKAA2* associations were significant after considering multiple testing. We did not find significant associations between *PRKAA2*

variants and PCOS itself, BMI, androgens, or reproductive traits. No significant associations were observed with quantitative traits in control subjects.

PRKAG3

We genotyped 4 SNPs spanning the *PRKAG3* gene (Fig. 1B). The genotype frequencies (Table 1) for all markers were in Hardy-Weinberg equilibrium. Linkage disequilibrium among the 4 markers (D') ranged from 0.61 to 1.0 (average 0.91); r^2 values ranged from 0.001 to 0.55 (average 0.10).

Carriers of the minor allele of SNP rs692243 (Pro71Ala) had an increased likelihood of PCOS, with an age- and BMI-adjusted odds ratio of 1.82 (95% CI, 1.07 to 3.10, $P=0.028$), which did not meet the multiple-testing corrected P -value cutoff of 0.006. We found no associations in PCOS or control women between the *PRKAG3* variants and any of the quantitative traits, including BMI.

Discussion

This is the first study evaluating association of variants in the *PRKAA2* and *PRKAG3* genes with PCOS. Nominal associations of *PRKAA2* variants with insulin-related traits and of the *PRKAG3* Pro71Ala variant with PCOS were not statistically significant after correcting for multiple testing,

AMPK is a heterotrimeric enzyme composed of a catalytic alpha subunit (α) and two non-catalytic regulatory (β and γ) subunits. Seven genes code for the possible components of AMPK ($\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, $\gamma 1$, $\gamma 2$ and $\gamma 3$). We considered *PRKAA2* and *PRKAG3*, which code for the $\alpha 2$ and $\gamma 3$ subunits, as the highest priority candidate genes given prior physiologic evidence implicating their proteins in metformin action (6) and insulin sensitivity (11,12).

No other human genetic association studies of AMPK in PCOS have been published. A few human genetic studies examined AMPK in non-PCOS cohorts with conflicting results. Some demonstrated associations between *PRKAA2* variants and type 2 diabetes, HOMA-IR and cholesterol fractions (4,13,14). One study found no association of variation in *PRKAA2* with type 2 diabetes, fasting glucose, insulin resistance, insulin secretion or body fat distribution (5). Our *PRKAA2* results are consistent with the latter study.

PRKAG3 variants were not associated with insulin sensitivity or secretion in prior studies (15), similar to our current results in PCOS women. Previously, the minor allele of SNP rs692243, which is a non-synonymous variant (proline-to-alanine at codon 71), was shown to be significantly associated with higher serum LDL-cholesterol and apolipoprotein B-100 levels, supporting the functional significance of this SNP (15). We do not have cholesterol levels for our patients, but we did observe that this same SNP, rs692243, was marginally associated with PCOS itself.

Studies conducted primarily in animals documented AMPK expression in the ovary, suggesting a role for this fuel sensor in fertility (16). Recent work has found expression of AMPK in human ovaries, with AMPK activation decreased in PCOS thecal cells, and that metformin treatment of these cells increased AMPK activity (7). Thus, activation of AMPK in the ovary itself could be a reason why patients with PCOS who are treated with metformin experience amelioration of ovulatory dysfunction. However, variants in the AMPK subunit genes we analyzed were not associated with ovulatory dysfunction in PCOS patients as assessed by severe oligomenorrhea, infertility and/or parity. As reproductive traits are interrelated with metabolic traits in PCOS, the association of reproductive traits with AMPK SNPs was predictably lacking in the absence of any association of metabolic traits with these SNPs.

The value of our study is that it reports on genes that have not yet been evaluated in PCOS patients, in a homogeneous and well-characterized case/control cohort. We have performed a number of genetic association studies in this cohort from Alabama. While this may seem duplicative, it is critical to completely assess a well-defined population, minimizing the variability brought on by analyzing different genes in different populations. Thus, it is common in genetic epidemiology to use a single cohort for ongoing investigation and to report each investigation as a separate experiment, when that is how the work was performed. For example, many genetic association publications have resulted from the Insulin Resistance Atherosclerosis Family Study (IRAS-FS) and the Multi-ethnic Study of Atherosclerosis (MESA).

A limitation of the current study is that insulin resistance and insulin secretion were measured by HOMA indices, which may be insufficiently reflective of these traits to allow detection of association with genetic variants, particularly in the setting of PCOS (17). This may have hindered our ability to discover robust associations of AMPK subunit genetic variants with insulin-related traits. Analysis in a cohort characterized by physiologic studies (e.g. euglycemic clamp) would be necessary to definitively rule out an association of *PRKAA2* or *PRKAG3* variants with insulin resistance.

In conclusion, variants in the genes for AMPK α 2 and AMPK γ 3 were not associated with PCOS susceptibility or the component metabolic, reproductive, or androgenic traits in PCOS patients, suggesting that *PRKAA2* and *PRKAG3* are not major genetic risk factors for PCOS. Whether genetic variants in other AMPK subunit genes play a role in PCOS susceptibility remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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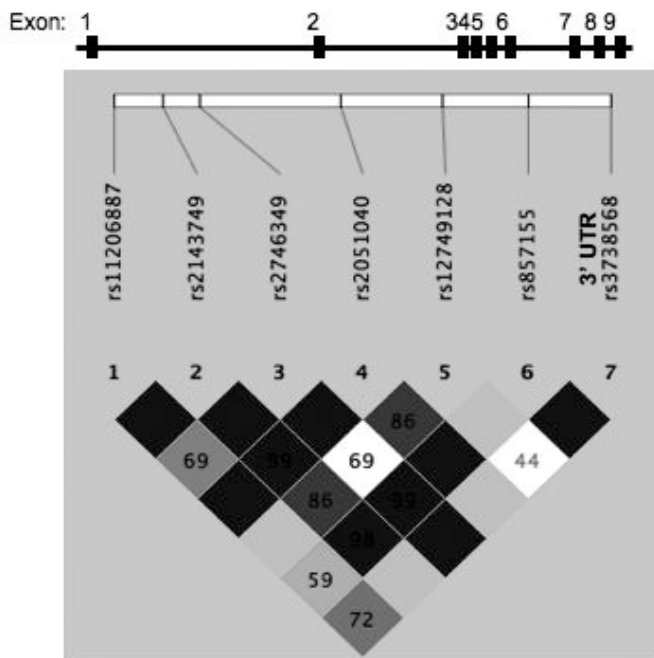
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A. *PRKAA2*



B. *PRKAG3*

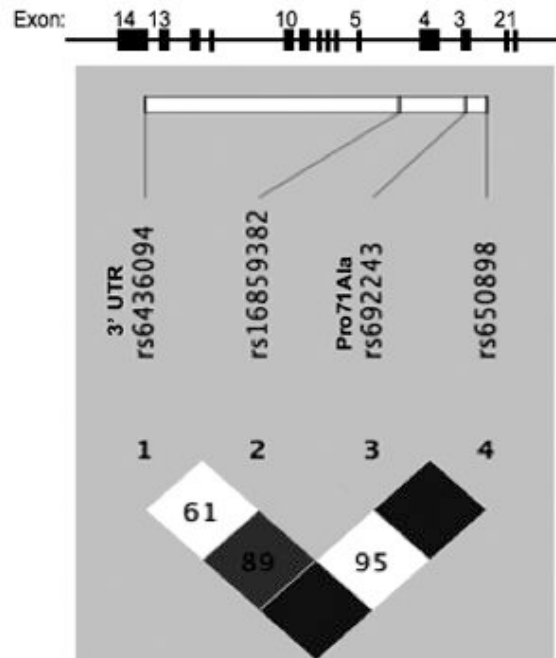


Figure 1. Gene structure and linkage disequilibrium plots for *PRKAA2* (A) and *PRKAG3* (B). Gene structure is shown at top. *PRKAA2* has 9 exons and is located on chromosome 1 (1p31). *PRKAG3* has 14 exons and is located on the reverse (minus) strand of chromosome 2 (2q35). The locations of the genotyped SNPs relative to the exons are indicated. The linkage disequilibrium plots at bottom display D' values (%) for each pair of SNPs in the box at the intersection of the diagonals from each SNP. The dark solid blocks indicate $D' = 1$ (100%) for the corresponding pair of variants. The lighter solid boxes also indicate $D' = 1$, but with a low confidence score. 3' UTR indicates SNP located in the 3' untranslated region.

Table 1
Frequency and position information on *PRKAA2* and *PRKAG3* SNPs

| SNP Designation | Alleles | Location in gene | Overall | PCOS | Control |
|----------------------|---------|-------------------|---------|-------|---------|
| <i>PRKAA2</i> | | | | | |
| rs11206887 | A/G | Intron 1 | 0.166 | 0.154 | 0.185 |
| rs2143749 | C/G | Intron 1 | 0.345 | 0.352 | 0.333 |
| rs2746349 | A/G | Intron 1 | 0.486 | 0.478 | 0.497 |
| rs2051040 | G/A | Intron 2 | 0.346 | 0.348 | 0.343 |
| rs12749128 | A/G | Intron 2 | 0.024 | 0.016 | 0.038 |
| rs857155 | A/C | Intron 6 | 0.475 | 0.467 | 0.488 |
| rs3738568 | T/C | Exon 9 (3' UTR) | 0.030 | 0.029 | 0.032 |
| <i>PRKAG3</i> | | | | | |
| rs6436094 | A/G | Exon 14 (3' UTR) | 0.271 | 0.291 | 0.235 |
| rs16859382 | C/T | Intron 4 | 0.011 | 0.012 | 0.009 |
| rs692243 | C/G | Exon 3 (Pro71Ala) | 0.195 | 0.233 | 0.135 |
| rs650898 | T/C | Intron 2 | 0.087 | 0.093 | 0.076 |