

## **AKT2: First Evidence of Genetic Association with Polycystic Ovary Syndrome**

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*Objective:* Insulin resistance has been reported in up to 70% of women with polycystic ovary syndrome (PCOS). Physiologic and genetic data currently implicate post-insulin receptor signaling defects in substrates such as GSK3 $\beta$ . The *AKT2* gene was chosen as a candidate for PCOS because its product affects glucose metabolism and mitogenic signaling, interacts with GSK3 $\beta$ , and mediates cell survival in the ovary.

*Research Design and Methods:* Subjects were recruited from the reproductive endocrinology clinic at the University of Alabama at Birmingham; controls were recruited from the surrounding community. 287 White women with PCOS and 187 White controls were genotyped for 4 SNPs in *AKT2*. Genotyping took place at Cedars-Sinai Medical Center in Los Angeles. Single nucleotide polymorphisms (SNPs) and haplotypes were tested for association with PCOS risk and phenotypic markers of PCOS.

*Results:* Minor allele carriers of SNPs rs3730051 and rs8100018 had increased odds of PCOS (OR=2.2, p=0.004 and OR=2.4, p=0.001, respectively). The haplotype T-G-C-T was significantly associated with PCOS (OR=2.0, p=0.01). Carriers of the risk haplotypes for both *AKT2* and *GSK3B* had a further increased odds of PCOS (OR=3.1, p=0.005).

*Conclusions:* These data suggest that polymorphisms in two components of the insulin signaling pathway, *AKT2* and *GSK3B*, are associated with PCOS. Presence of multiple lesions in a single pathway may confer increased risk.

**P**olycystic ovary syndrome (PCOS), characterized by hyperandrogenism, oligo-ovulation, and polycystic ovarian morphology, affects 7% of women (1). Inherent to PCOS is a profound peripheral insulin resistance, observed in up to 70% of PCOS subjects (2). The molecular basis of this intrinsic resistance remains unknown; post-insulin receptor defects appear to contribute to the pathophysiology of both insulin resistance and hyperandrogenemia (2).

The insulin signaling pathway includes two main pathways; a metabolic arm through the activation of phosphatidylinositol-3-kinase (PI3K) and a mitogenic arm acting via the mitogen-activated protein kinase (MAPK) (3). The expression and activity of downstream targets of PI3K have been investigated in PCOS tissues, with distinct molecular defects in post-insulin receptor signaling identified in fibroblasts and adipose tissues of PCOS subjects (4).

Akt, also known as protein kinase B, participates in insulin signaling via activation by 3'-phosphoinositide-dependant kinase, which is activated by the product of PI3K, Ptd (Ins)3,4,5P<sub>3</sub> (5). Three highly conserved genes encode the forms of Akt, Akt1, Akt2 and Akt3 (5). Akt2 is the major form involved in insulin signaling in adipose tissue and is required for net gain in surface glucose transporter-4 (GLUT4) in response to insulin signaling (6).

Akt2 is widely expressed and participates in insulin metabolism, mitogenic signaling, and apoptosis (5). Activation of Akt by insulin in adipocytes is reduced in type 2 diabetes (5) and experimental reduction of Akt2 leads to decreased insulin sensitivity and reduced glucose disposal (3), both of which are features of PCOS. Insulin-stimulated Akt phosphorylation is reduced by

40-60% in PCOS skeletal muscle compared to matched controls (7).

In response to insulin signaling, Akt2 phosphorylates and inhibits glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), which facilitates glycogen synthesis (8). Physiologic data indicate that insulin-stimulated glycogen synthesis is impaired in PCOS fibroblasts (9) and cultured ovarian granulosa cells (10). In PCOS adipocytes GSK3 $\beta$  tyrosine phosphorylation is increased and insulin-stimulated serine phosphorylation is decreased (11) which could result in an overactive GSK3 $\beta$  that is less able to be suppressed by insulin, possibly explaining impaired insulin-stimulated glycogen synthesis. Aberrant GSK3 $\beta$  expression and activity has also been reported in PCOS ovarian theca, with overexpression of GSK3 $\beta$  stimulating 17 $\alpha$ -hydroxylase activity of P450c17 (12). Based on this physiologic data we previously genotyped *GSK3B* for association between polymorphisms in the gene and PCOS and identified a risk haplotype (13).

In the current study, we continued this line of investigation by examining whether variants in *AKT2* were associated with susceptibility to PCOS or the biochemical features of PCOS. Analysis revealed associations between the minor allele of two SNPs from *AKT2* and PCOS susceptibility. Presence of these alleles in a haplotype spanning the *AKT2* gene region was also associated with PCOS.

## **RESEARCH DESIGN AND METHODS**

**Subjects and phenotyping**—A total of 287 consecutive unrelated White patients with PCOS and 187 unrelated White control women were recruited from the Birmingham, Alabama area. Cases were recruited from the reproductive endocrine practice of one of the investigators (RA) at the University of Alabama at Birmingham (UAB). Participation

in research was offered to patients meeting inclusion criteria (premenopausal, non-pregnant, on no hormonal therapy, including oral contraceptives, for at least three months, and meeting 1990 NIH criteria (14). The comprehensive physical examination and hormonal evaluation used in our research has been previously described in detail (15).

Controls were healthy women, with regular menstrual cycles and no family history of hirsutism. These women had no hirsutism, acne, alopecia, or endocrine dysfunction and had not taken hormonal therapy (including oral contraceptives) for at least three 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 the nature of the studies.

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.

**SNP genotyping and haplotype determination**—We selected four single nucleotide polymorphisms (SNPs), rs11671439, rs8100018, rs3730051 and rs2304188, which span the 51.5 kb genomic length of *AKT2* at chromosome 19q13.1-13.2. These were selected because they are predicted to tag the haplotypes (across the entire gene, plus 10 kb upstream and 10 kb downstream) occurring at >1% frequency in the Caucasian population of the HapMap database (16). The four SNPs were genotyped using the 5'-exonuclease assay (TaqMan MGB, Applied Biosystems, Foster City, CA) (17); duplicate genotyping of 96 samples for one SNP yielded 100% concordance. The genotyping success rate was 92.5%.

Haploview 3 (18) was used to determine haplotypes as well as haplotype blocks, using an accelerated expectation maximization algorithm. Haploview was also used to calculate linkage disequilibrium (LD,

the  $D'$  statistic) between each pairwise combination of all the SNPs. Haplotypes were assigned to individual subjects only when the assignment could be made with a greater than 95% certainty.

**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 of SNPs or haplotypes with presence/absence of PCOS was evaluated using logistic regression, adjusting for BMI and age in every analysis. Association between genotype and quantitative traits utilized analysis of covariance (ANCOVA), again adjusting for age and BMI. Significance was taken at  $P < 0.017$  to account for the effects of multiple testing, considering that we analyzed one linkage disequilibrium group of SNPs against three families of traits (PCOS diagnosis, androgens, metabolic traits), yielding a correction factor of three (i.e. three independent comparisons).

In exploratory analyses, various multiple logistic regression models were utilized to explore the contribution of *AKT2* haplotype to PCOS risk while simultaneously considering the risk haplotype from *GSK3B* (13). Analyses were carried out using Statview 5.0 (SAS Institute, Cary, NC).

## RESULTS

Clinical features of the cohort are given in Table 1. Strong LD ( $D' \geq 0.95$ ) was observed between each of the four *AKT2* SNPs (Table 2, Figure 1). Minor allele carriers of two SNPs had an increased frequency of PCOS. Presence of the G allele at rs8100018 conferred an odds ratio (OR) of 2.4, (95% confidence interval (CI)=1.4-4.1,  $p=0.001$ ), while presence of the G allele at rs3730051 conferred an OR of 2.2, (95% CI=1.3-3.7,  $p=0.004$ ). There were no

significant associations with any quantitative traits.

Five haplotypes, comprised of SNPs rs11671439, rs8100018, rs3730051 and rs2304188, accounted for >99% of the population (Table 3). There was a significant association between haplotype T-G-C-T and PCOS (OR=2.0, 95% CI=1.1-3.4, P=0.01). This haplotype includes the minor alleles of both rs8100018 and rs3730051, the two SNPs associated with PCOS. There were no significant associations between haplotypes and any quantitative traits.

*GSK3B* was previously analyzed for association with susceptibility to PCOS (13). The nine-SNP haplotype C-A-C-C-G-G-A-G-G was significantly associated with PCOS (OR=1.7, 95% CI=1.03-2.85, P=0.028) and was the most common haplotype among the White PCOS subjects but the second most common in controls. Taking into account the previous association between *GSK3B* haplotype and PCOS, we conducted exploratory analyses with a set of logistic regression models that simultaneously considered *AKT2* and *GSK3B* (Table 4). The first model was designed to evaluate the independence of the risk haplotypes of both *AKT2* and *GSK3B* on PCOS susceptibility. In this model, each risk haplotype retained a significant association with PCOS (*AKT2* risk haplotype carriers, OR=2.1, P=0.01; *GSK3B* risk haplotype carriers OR=1.8, P=0.036), demonstrating independence. In the next logistic regression with PCOS as the dependent variable, the independent variables were age, BMI, and number of risk haplotypes (for each subject, ranging from 0 to 4). This revealed that each additional risk haplotype conferred a 58% increase in the odds of PCOS (P=0.01). In the final combined model, carriers of both risk haplotypes had an increased odds of PCOS when compared to subjects with neither haplotype (OR of 3.1, P=0.005).

## CONCLUSIONS

Our data demonstrate that polymorphisms in the *AKT2* gene are significantly associated with PCOS. The minor alleles of rs3730051 and rs8100018 were associated with PCOS, and the corresponding haplotype was also associated with PCOS. We used independent, additive and combined logistic regression models to demonstrate that the association between *AKT2* haplotype T-G-C-T and PCOS was independent of the *GSK3B* risk haplotype, but PCOS risk was increased when both were present. These data offer two potential susceptibility loci from the insulin signaling pathway that may confer increased PCOS risk and suggest that the presence of multiple lesions in a single pathway confer increased risk.

The significant association of rs3735001 and rs8100018 with PCOS extends to a haplotype that includes the minor alleles of these two *AKT2* SNPs. Carriers of both minor alleles, in a haplotype, had the same OR as carriers of either risk allele alone, as a SNP. This suggests that these alleles are markers tagging the same variant in the gene region. The unknown functional variant can be a coding variant or a non-coding variant that alters *AKT2* promoter activity or mRNA transport and stability, resulting in changes in protein expression.

Our analyses did not report any significant associations with quantitative traits related to glucose homeostasis. This was also the case for *GSK3B* (13). As analyses to detect association between quantitative traits and genotype are conducted only within PCOS cases, the reduced sample may have decreased power to detect an effect. Also, in our cohort insulin resistance was quantified via homeostatic model assessment, which may be insufficiently reflective of insulin resistance to allow detection of association with genetic variants. Analysis in a larger cohort or one characterized by physiologic

studies (e.g. euglycemic clamp) would be necessary to definitively rule out an association of *AKT2* variants with insulin resistance, and whether such a relationship mediates the effect of genetic variants on PCOS risk. Alternatively, while Akt2 and GSK3 $\beta$  are best known for their role in glucose uptake (5), it is possible that their role in PCOS does not manifest as alteration in androgen levels or metabolic disturbance, but in other pathways not reflected in typical quantitative traits. Akt is known to participate in several pathways including inflammation, lipogenesis, and endothelial function (5) and it is possible that aberrant function in these pathways contributes to the pathophysiology of PCOS.

Akt is expressed in granulosa and theca cells of primordial follicles, and is involved in signal transduction, cell cycle and cell fate (19,20). Recent studies have shown altered rates of apoptosis in PCOS ovarian tissue including granulosa (21) and theca (22). Overexpression of Akt2 has been shown to result in a reduced rate of apoptosis in a number of cell types including ovary (5) and is a potential causal factor in the increased follicular growth and lack of atresia associated with PCOS. Aberrant Akt2 and GSK3 $\beta$  expression and activation could result in increased cell survival in PCOS follicles and contribute to the theca cell hypertrophy, abnormal follicular development, and promote the proliferation of granulosa. An increase in the latter less proliferative and highly steroidogenic cell type (23) could potentially increase androgen production by the ovary, a characteristic of PCOS. Increased basal, LH- and insulin-stimulated Akt phosphorylation has been reported in PCOS ovarian theca (22), with a subsequent increase in levels of aromatase and LH receptor mRNA expression (20) resulting in stimulation of granulosa cell proliferation. Increased serine and tyrosine phosphorylation of Akt in the ovary has been demonstrated in

an animal model exhibiting PCOS-like symptoms (24).

We have performed a number of genetic association studies in this cohort. Each report represented a separate genotyping experiment performed over the past several years, each testing an independent hypothesis; therefore, each was analyzed separately. We have published positive association of variants in six genes with PCOS (Table A1 is available in the online appendix at <http://care.diabetesjournals.org>). To assess the independence of these associations and their relative significance, we conducted a logistic regression simultaneously analyzing all of the associated variants, with PCOS status as the dependent variable (Appendix Table A2). This joint analysis revealed that the variants most significantly associated with PCOS were the *AKT2* and *GSK3B* risk haplotypes.

Two *AKT2* SNPs and the haplotype characterized by them were significantly associated with an increased odds of PCOS. Genes with pleiotropic effects are sensible candidates for PCOS, a syndrome characterized by dysfunction in multiple systems. *AKT2* is expressed in several key PCOS tissues including adipose, pancreas and ovary and has a potential role in the regulation of insulin signaling and glucose uptake, insulin secretion, and cell proliferation in the ovary, all of which may be deranged in PCOS (1). By examining multiple members of a pathway that has been previously implicated in both the genetics (13) and physiology (11) of PCOS, we have demonstrated that presence of variants in multiple members of a single pathway may increase PCOS susceptibility.

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**Table 1.** Clinical features of PCOS and Control Subjects.

	Control (n=187)	PCOS (n=287)
Age (yr)	33.0 (17.0)	27.5 (11.5)*
BMI (kg/m <sup>2</sup> )	24.1 (6.4)	34.7 (13.5)*
WHR	0.78 (0.08)	0.83 (0.10)*
mFG score	0 (0)	7.0 (5.0)*
Hirsute (%)	0	73.9*
Total testosterone (nmol/l)	1.42 (0.92)	2.77 (1.07)*
Free testosterone (pmol/l)	12.1 (9.0)	29.1 (16.3)*
DHEAS (μmol/l)	2.58 (2.03)	5.66 (4.61)*
SHBG (nmol/l) <sup>†</sup>	220.0 (120.0)	150.0 (70.0)*
Insulin (pmol/l)	49.5 (45.9)	129.15 (129.2)*
Glucose (mmol/l)	4.77 (0.56)	4.77 (0.72)
HOMA-IR	0.92 (0.83)	2.29 (1.93)*
HOMA-%B	103.9 (59.5)	175.3 (99.3)*

Data are median (interquartile range).

Abbreviations: BMI is body mass index, WHR is waist to hip ratio; mFG is the modified Ferriman-Gallwey hirsutism score; SHBG is sex hormone-binding globulin; HOMA-IR is insulin resistance estimated by the homeostatic model assessment; and HOMA-%B is beta-cell function estimated by the homeostatic model assessment.

\*P < 0.001 compared to control group

<sup>†</sup>SHBG activity was measured by competitive binding analysis, using Sephadex G-25 and [<sup>3</sup>H]T as the ligand; this assay gives values of approximately 100-300 nmol/l in normal adult women.

**Table 2.** SNP location and frequency data.

Variant	Location	Alleles (Major/Minor)	Overall MAF	PCOS MAF	Control MAF
rs11671439	Intron 1	C/T	0.078	0.068	0.088
rs8100018	Intron 4	C/G	0.262	0.296	0.216
rs3730051	Intron 8	A/G	0.221	0.247	0.180
rs2304188	Intron 10	C/T	0.153	0.141	0.178

MAF=Minor allele frequency.

**Table 3.** Haplotype frequency data in the PCOS and control subjects.

Haplotype	Overall Frequency	PCOS Frequency	Control Frequency
T-A-G-T	0.504	0.497	0.517
T-G-C-T	0.216	0.240	0.176
C-A-G-T	0.154	0.138	0.181
T-A-G-C	0.075	0.067	0.088
T-A-C-T	0.047	0.053	0.037

**Table 4.** Logistic regression models simultaneously considering *AKT2* and *GSK3B* in PCOS risk.

Model	Independent Variables	Odds Ratio	95% CI	P Value
Independence	Carrier state (Y/N) for <i>AKT2</i> risk haplotype*	2.10	1.2 – 3.7	0.010
	Carrier state (Y/N) for <i>GSK3B</i> risk haplotype*	1.80	1.0 – 3.1	0.036
Additive	Number of <i>AKT2</i> or <i>GSK3B</i> risk haplotypes†	1.58	1.1 – 2.2	0.010
Combined	Carrier state (Y/N) for both risk haplotypes in <i>AKT2</i> and <i>GSK3B</i> ‡	3.11	1.4 – 6.9	0.0050

PCOS diagnosis was the dependent variable in all models. Each analysis also included age and BMI as independent variables.

\*Carriers are homozygous or heterozygous for risk haplotype (T-G-C-T in *AKT2*, C-A-C-C-G-G-A-G-G in *GSK3B*)

†Number of risk haplotypes per individual is 0-4.

‡Compares subjects who are carriers of at least one *AKT2* risk haplotype and at least 1 *GSK3B* risk haplotype to all other subjects.

**FIGURE LEGEND**

Figure 1. Gene structure and linkage disequilibrium plot for *AKT2*. The gene structure of *AKT2* is shown at top; the gene has 14 exons and is located on the reverse strand of chromosome 19q13. The locations of the genotyped SNPs relative to the exons are indicated. The linkage disequilibrium (LD) plot at the bottom displays  $D'$  values (%) for each pair of SNPs in the box at the intersection of the diagonals from each SNP.  $D'$  between each pair of SNPs was 1.0 (solid boxes), and was 0.98 between rs3730051 and rs8100018, indicating strong LD across the region.

