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PRELIMINARY EVIDENCE OF GLYCOGEN SYNTHASE KINASE 3 BETA AS A GENETIC DETERMINANT OF POLYCYSTIC OVARY SYNDROME

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Abstract

In this study of 352 women with PCOS and 289 controls, haplotypes spanning the gene for glycogen synthase kinase 3 β (*GSK3B*) were constructed based on nine single nucleotide polymorphisms in White and Black subjects separately. In each racial group we observed that a specific, although different, *GSK3B* haplotype was associated with increased frequency of PCOS, suggesting that *GSK3B* contributes to the pathophysiology and inherited basis of PCOS.

Keywords

Glycogen synthase kinase; Polycystic ovary syndrome; Genetic epidemiology

Polycystic ovary syndrome (PCOS) affects ~6.5% of reproductive-aged women (1-3). Most affected women have impaired insulin action and hyperinsulinism (4-7). Because family studies demonstrated that genetic factors contribute to PCOS (8,9), we hypothesize that inherited genomic variants affecting insulin signaling may increase the risk of developing PCOS. We have examined components of the insulin-signaling pathway in isolated adipocytes of PCOS women, and observed reduced insulin-stimulated glucose uptake in PCOS compared to controls. PCOS adipocytes displayed higher tyrosine phosphorylation and lower insulin-stimulated serine phosphorylation of glycogen synthase kinase 3 β (*GSK3B*), suggesting

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Capsule:

This cross-sectional genetic association study demonstrated that haplotypes in the gene for glycogen synthase kinase 3 beta are associated with an increased frequency of polycystic ovary syndrome.

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GSK3 β overactivity (10). This led us to examine the *GSK3B* gene as a candidate for PCOS susceptibility.

Haplotype-based genetic analysis was carried out in 352 consecutive patients with PCOS, recruited from the Reproductive Endocrinology and Infertility clinic at the University of Alabama at Birmingham (UAB; 308 White, 44 Black). The presence of PCOS was defined by the 1990 NIH criteria (11). Specific criteria for defining hirsutism, hyperandrogenemia, ovulatory dysfunction, and exclusion of related disorders were as previously reported (1). In addition, 289 healthy control women were recruited from the Birmingham area (187 White, 102 Black). Controls had regular menstrual cycles and no evidence of hirsutism, acne, alopecia, endocrine dysfunction, or family history of hirsutism. Controls responded to posted advertisements, and primarily did not include women affiliated with the clinic. No subject had used hormonal preparations, including oral contraceptives, for three months prior to study, and none were pregnant. Subjects gave written informed consent according to the guidelines of the Institutional Review Boards of UAB and Cedars-Sinai Medical Center (CSMC).

Hormonal measures were obtained between days 3 and 8 (follicular phase) following a spontaneous menstruation or progesterone-induced withdrawal bleed, including total testosterone (T), sex-hormone binding globulin (SHBG), calculated free T, DHEAS, and 17-hydroxyprogesterone (17-HP), as previously described (1). A modified Ferriman-Gallwey (mFG) scoring system was used to quantify hirsutism (12), and subjects were deemed hirsute if their mFG score was 6 or greater. Fasting glucose and insulin were also obtained, and a computer-based homeostasis model assessment (www.dtu.ox.ac.uk/homa) was used to estimate insulin resistance (HOMA-IR) and insulin secretion (HOMA-%B) (13,14).

We selected nine single nucleotide polymorphisms (SNPs; rs6805251, rs1719889, rs1719895, rs7624540, rs2319398, rs7620750, rs1381841, rs6770314, rs2199503) that span the 267 kilobase genomic length of *GSK3B*, including four (rs6805251, rs1719889, rs1719895, rs2199503) predicted to tag the haplotypes occurring at >1% frequency in the Caucasian population of the HapMap database (15). The nine SNPs were genotyped in the 641 subjects using the 5'-exonuclease assay (TaqMan MGB, Applied Biosystems, Foster City, CA) described previously (16,17). The PCR primers and TaqMan MGB probe sequences are available from the authors. Haploview 3 (18) was used to determine haplotypes by using an accelerated expectation maximization algorithm. Haplotypes were determined separately in Whites and Blacks. Haploview assigned a haplogenotype to 95% of the subjects and calculated linkage disequilibrium (LD, the D' statistic) between each pairwise combination of all 9 SNPs.

The primary phenotype for genetic association analysis was the presence/absence of PCOS, given that such analysis utilizes all available subjects to maximize power. Secondary analyses included androgen-related traits (total and free T, DHEAS, 17-HP, SHBG, mFG score) and insulin-related traits (fasting insulin, fasting glucose, HOMA-IR, HOMA%B) within the PCOS cohort. For insulin-related traits, subjects with diabetes (n=6) were excluded because hyperglycemia of diabetes may induce secondary changes in insulin-related traits that reduce their utility for genetic analyses. To minimize multiple testing, only haplotypes showing association with the primary phenotypes were examined for association with secondary phenotypes (rather than all haplotypes).

Haplotypes were the genotypic unit utilized in association analyses. Whites and Blacks were analyzed separately, given their different haplotype frequencies. Association of haplotypes with PCOS and presence/absence of hirsutism was evaluated using logistic regression. Association with total and free T, DHEAS, SHBG, 17-HP, mFG score, and insulin-related traits was evaluated using analysis of covariance (ANCOVA). All analyses were adjusted for age and body mass index (BMI) by inclusion of these parameters as covariates in the logistic

regression or ANCOVA analyses. Quantitative trait values were log- or square root-transformed to reduce skewness.

Given that multiple association tests were performed, we estimated empirical P values by permutation analysis to confirm any significant haplotypic associations. The samples were permuted by shuffling haplotypic data 1000 times, and subsequent association analyses were carried out to obtain the distribution of the test statistic under the null hypothesis of no association. The empiric P values were obtained as the proportion of the 1000 replicates that had a P value less than or equal to the nominal ones obtained from the actual (unshuffled) data. These empiric P values are reported below.

Linkage disequilibrium (D') among the nine SNPs in Whites ranged from 0.69 to 1.0 (average D' 0.96). In Blacks D' ranged from 0.05 to 1 (average D' 0.90). The overall high degree of linkage disequilibrium confirmed the possibility of constructing haplotypes made up of all nine variants. The common (overall frequency >5%) *GSK3B* haplotypes and their frequencies are displayed in Table 1. Whites and Blacks shared most haplotypes, but had dramatically different haplotype frequencies ($\chi^2 = 206.8$, $P < 0.0001$).

Among White subjects, haplotype CACCGGAGG was associated with an increased frequency of PCOS (age- and BMI-adjusted odds ratio 1.7, 95% confidence interval 1.03-2.85, empiric $P = 0.028$). This haplotype was the most common (32%) among Whites with PCOS but was the second most common (26%) haplotype among controls (Table 1). Among Black subjects, haplotype TTTATAAAG was associated with increased PCOS risk (age- and BMI-adjusted odds ratio 2.9, 95% confidence interval 1.0-8.0, empiric $P = 0.041$). This haplotype occurred with a 20% frequency in Blacks with PCOS and 15% in Black controls.

Given the association of the above haplotypes with PCOS, secondary analyses were carried out to evaluate whether these haplotypes were associated with androgen- or insulin-related quantitative traits in women with PCOS. Neither haplotype showed any associations with quantitative traits in their respective ethnic groups.

Our haplotype-based approach designed to capture common variation across the whole gene demonstrated that variation in *GSK3B* is associated with PCOS. GSK3 phosphorylates and inactivates glycogen synthase (GS). Insulin signaling leads to serine phosphorylation and inactivation of GSK3, promoting glycogen synthesis. GSK3 activity is increased by tyrosine phosphorylation by unknown kinases. In addition to its role in glycogen synthesis, GSK3 may inhibit insulin signaling in general by inducing serine phosphorylation of insulin receptor substrate-1 (IRS-1) (19). GSK3 protein levels and activity are increased in muscle from subjects with type 2 diabetes and are inversely correlated with both GS activity and insulin-stimulated glucose uptake (20,21).

Our preliminary data in adipocytes has suggested that intrinsic defects in GSK3 β phosphorylation are present in PCOS. Specifically, in PCOS tyrosine phosphorylation of GSK3 β was enhanced, and insulin-stimulated serine phosphorylation of GSK3 β was defective (10). These phosphorylation data are consistent with a constitutively hyperactivated GSK3 β that is resistant to suppression by insulin, a possible intrinsic (and likely genetic) defect in PCOS adipocytes. Given that GSK3 β inhibits GS, this data is consistent with other evidence demonstrating decreased glycogen synthesis in response to insulin in PCOS cultured ovarian granulosa cells (22) and skin fibroblasts (23). Genetically determined elevated GSK3 β activity may thus predispose to PCOS by promoting insulin resistance.

GSK3 may also influence PCOS by affecting androgen production and/or action. In human ovarian theca cells, over-expression of GSK3 β enhanced the 17-hydroxylase activity of P450c17; basal GSK3 β activity was found to be increased in theca cells from PCOS women

(24). In prostate cancer cell lines, GSK3 activity modulates androgen receptor function (25, 26). The androgen receptor may be a substrate of GSK3 (27).

The effects of the associated *GSK3B* haplotypes on PCOS risk were moderate. This is consistent with the notion that common, complex diseases are genetically influenced by variants in multiple genes, with each gene contributing a modest effect. For example, variation in validated type 2 diabetes genes, such as peroxisome proliferator-activated receptor-gamma (*PPARG*), only modestly alter risk for type 2 diabetes, with odds ratios ranging from 1.12 to 1.39 (28). Although our cohort is among the largest in PCOS genetics, our results must be replicated by others before *GSK3B* is firmly established as a gene for PCOS.

Black and White subjects had similar haplotypes, but with very different frequencies, a reflection of their separate genetic history. Within each group a particular *GSK3B* haplotype was associated with PCOS. Whether these haplotypes carry the same or different functional variants is unknown and cannot be inferred from the current data. That these were common haplotypes is consistent with the high prevalence (~6.5%) of PCOS (1-3). Because we did not observe association with quantitative traits, we cannot determine whether *GSK3B* variation influences PCOS risk by affecting insulin resistance and/or androgen action. We plan to perform sequencing in subjects with and without the risk haplotypes to identify functional variants in *GSK3B* and to expand our sample size to increase our ability to identify association with quantitative traits.

In conclusion, our haplotype-based approach demonstrated that variation in *GSK3B* is associated with PCOS. Inherited abnormalities in GSK3 action have the potential of influencing both insulin resistance and hyperandrogenism, two important factors in PCOS, and would be exciting evidence of a single molecular defect underlying these two prevalent abnormalities. Further molecular and genetic studies of GSK3 in PCOS are needed to confirm these preliminary findings.

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REFERENCES

1. Azziz R, Woods KS, Reyna R, Key TJ, Knochenhauer ES, Yildiz BO. The prevalence and features of the polycystic ovary syndrome in an unselected population. *J Clin Endocrinol Metab* 2004;89:2745–9. [PubMed: 15181052]
2. Asuncion M, Calvo RM, San Millan JL, Sancho J, Avila S, Escobar-Morreale HF. A prospective study of the prevalence of the polycystic ovary syndrome in unselected Caucasian women from Spain. *J Clin Endocrinol Metab* 2000;85:2434–8. [PubMed: 10902790]
3. Diamanti-Kandarakis E, Kouli CR, Bergiele AT, Filandra FA, Tsianateli TC, Spina GG, et al. A survey of the polycystic ovary syndrome in the Greek island of Lesbos: hormonal and metabolic profile. *J Clin Endocrinol Metab* 1999;84:4006–11. [PubMed: 10566641]
4. Dunaif A, Segal KR, Futterweit W, Dobrjansky A. Profound peripheral insulin resistance, independent of obesity, in polycystic ovary syndrome. *Diabetes* 1989;38:1165–74. [PubMed: 2670645]
5. Legro RS, Kunselman AR, Dodson WC, Dunaif A. Prevalence and predictors of risk for type 2 diabetes mellitus and impaired glucose tolerance in polycystic ovary syndrome: a prospective, controlled study in 254 affected women. *J Clin Endocrinol Metab* 1999;84:165–9. [PubMed: 9920077]
6. DeUgarte CM, Bartolucci AA, Azziz R. Prevalence of insulin resistance in the polycystic ovary syndrome using the homeostasis model assessment. *Fertil Steril* 2005;83:1454–60. [PubMed: 15866584]
7. Carmina E, Lobo RA. Use of fasting blood to assess the prevalence of insulin resistance in women with polycystic ovary syndrome. *Fertil Steril* 2004;82:661–5. [PubMed: 15374711]

8. Escobar-Morreale HF, Luque-Ramirez M, San Millan JL. The molecular-genetic basis of functional hyperandrogenism and the polycystic ovary syndrome. *Endocr Rev* 2005;26:251–82. [PubMed: 15561799]
9. Vink JM, Sadrzadeh S, Lambalk CB, Boomsma DI. Heritability of polycystic ovary syndrome in a Dutch twin-family study. *J Clin Endocrinol Metab* 2006;91:2100–4. [PubMed: 16219714]
10. Goodarzi, MO.; Williams, H.; D.A., M.; Pall, M.; Chang, W.; Antoine, HJ., et al. Role of abnormal glycogen synthase kinase 3 beta in the polycystic ovary syndrome: physiologic and genetic evidence. 88th Annual Meeting of the Endocrine Society; Boston, MA. 2006;
11. Zawadzki, JK.; Dunaif, A. Diagnostic criteria for polycystic ovary syndrome: towards a rational approach. In: Dunaif, A.; Givens, JR.; Haseltine, F.; Merriam, GR., editors. *Polycystic ovary syndrome*. Blackwell Scientific Publications; Cambridge: 1992. p. 377–84.
12. Hatch R, Rosenfield RL, Kim MH, Tredway D. Hirsutism: implications, etiology, and management. *Am J Obstet Gynecol* 1981;140:815–30. [PubMed: 7258262]
13. Wallace TM, Levy JC, Matthews DR. Use and abuse of HOMA modeling. *Diabetes Care* 2004;27:1487–95. [PubMed: 15161807]
14. Levy JC, Matthews DR, Hermans MP. Correct homeostasis model assessment (HOMA) evaluation uses the computer program. *Diabetes Care* 1998;21:2191–2. [PubMed: 9839117]
15. The International HapMap Consortium. The International HapMap Project. *Nature* 2003;426:789–96. [PubMed: 14685227]
16. Goodarzi MO, Guo X, Taylor KD, Quiñones MJ, Samayoa C, Yang H, et al. Determination and use of haplotypes: ethnic comparison and association of the lipoprotein lipase gene and coronary artery disease in Mexican-Americans. *Genet Med* 2003;5:322–7. [PubMed: 12865761]
17. Livak KJ. Allelic discrimination using fluorogenic probes and the 5' nuclease assay. *Genet Anal* 1999;14:143–9. [PubMed: 10084106]
18. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;21:263–5. [PubMed: 15297300]
19. Liberman Z, Eldar-Finkelman H. Serine 332 phosphorylation of insulin receptor substrate-1 by glycogen synthase kinase-3 attenuates insulin signaling. *J Biol Chem* 2005;280:4422–8. [PubMed: 15574412]
20. Nikoulina SE, Ciaraldi TP, Mudaliar S, Mohideen P, Carter L, Henry RR. Potential role of glycogen synthase kinase-3 in skeletal muscle insulin resistance of type 2 diabetes. *Diabetes* 2000;49:263–71. [PubMed: 10868943]
21. Eldar-Finkelman H. Glycogen synthase kinase 3: an emerging therapeutic target. *Trends Mol Med* 2002;8:126–32. [PubMed: 11879773]
22. Wu XK, Zhou SY, Liu JX, Pollanen P, Sallinen K, Makinen M, et al. Selective ovary resistance to insulin signaling in women with polycystic ovary syndrome. *Fertil Steril* 2003;80:954–65. [PubMed: 14556818]
23. Book CB, Dunaif A. Selective insulin resistance in the polycystic ovary syndrome. *J Clin Endocrinol Metab* 1999;84:3110–6. [PubMed: 10487672]
24. Munir, I.; Geller, DH.; deBruin, CE.; Magoffin, DA. Dysregulation of GSK3beta activity in polycystic ovarian syndrome (PCOS) theca: a mechanism to promote excessive androgen biosynthesis in PCOS. 88th Annual Meeting of the Endocrine Society; Boston, Massachusetts. 2006;
25. Mazor M, Kawano Y, Zhu H, Waxman J, Kypta RM. Inhibition of glycogen synthase kinase-3 represses androgen receptor activity and prostate cancer cell growth. *Oncogene* 2004;23:7882–92. [PubMed: 15361837]
26. Liao X, Thrasher JB, Holzbeierlein J, Stanley S, Li B. Glycogen synthase kinase-3beta activity is required for androgen-stimulated gene expression in prostate cancer. *Endocrinology* 2004;145:2941–9. [PubMed: 14988390]
27. Wang L, Lin HK, Hu YC, Xie S, Yang L, Chang C. Suppression of androgen receptor-mediated transactivation and cell growth by the glycogen synthase kinase 3 beta in prostate cells. *J Biol Chem* 2004;279:32444–52. [PubMed: 15178691]
28. Parikh H, Groop L. Candidate genes for type 2 diabetes. *Rev Endocr Metab Disord* 2004;5:151–76. [PubMed: 15041791]

Table 1

GSK3B haplotypes and haplotype frequencies

Haplotype	Whites			Blacks		
	PCOS Freq.	PCOS Count	Control Freq.	Control Count	PCOS Freq.	Control Count
CACCGGAGG	0.324	195	0.261	91	0.082	7
CACCGGAGA	0.271	163	0.277	96	0.036	3
TACCTGAGG	0.163	98	0.191	66	0.265	23
TTCATAGAG	0.113	68	0.135	47	0.057	5
TTTATAAAG	0.104	63	0.104	36	0.198	17
TTCCTAAAG	0	0	0	0	0.230	20
TTCATAAAG	0	0	0	0	0.048	4
Cumulative freq.	0.976		0.968		0.915	
					0.070	13
					0.910	
						18
						10
						54
						12
						27
						35