

Variation in Resistin Gene Promoter Not Associated With Polycystic Ovary Syndrome

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Polycystic ovary syndrome (PCOS) is a leading cause of anovulatory infertility and affects ~4–7% of reproductive age women in the U.S. It is characterized by hyperandrogenemia and chronic anovulation and is associated with insulin resistance, obesity, and increased risk for type 2 diabetes. In a screen of candidate genes, a region on chromosome 19p13.3 was identified that shows significant evidence for both linkage and association with PCOS. A promising candidate gene for PCOS, resistin, maps to exactly this region. Resistin is a protein hormone thought to modulate glucose tolerance and insulin action. We tested for association between a single nucleotide polymorphism in the promoter region of the resistin gene and three phenotypes: PCOS, obesity, and insulin resistance. We did not find evidence for association with any of the phenotypes. It is therefore unlikely that variation in the resistin gene accounts for the strong association that we observe between chromosome 19p13.3 and PCOS. Instead, this association is most likely due to a gene or genetic element in this region that has not been identified. *Diabetes* 52: 214–217, 2003

Polycystic ovary syndrome (PCOS) is the leading cause of anovulatory infertility in women (1) and is characterized by hyperandrogenemia (HA) and chronic anovulation. PCOS affects 4–7% of reproductive age women in the U.S. (2) and is associated with a

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HA, hyperandrogenemia; PCOS, polycystic ovary syndrome; PPAR γ , peroxisome proliferator-activated receptor- γ ; SNP, single nucleotide polymorphism; TDT, transmission/disequilibrium test.

high incidence of insulin resistance and the sequelae of insulin resistance, including type 2 diabetes (2) and premature arteriosclerosis (3,4). A large number of genes in multiple metabolic pathways have been put forth as candidate susceptibility genes for PCOS (5).

In an analysis of 37 candidate susceptibility genes for PCOS (6,7), we found evidence for linkage and association with only one region: the insulin receptor gene (*INSR*) region at chromosome 19p13.3 (6,8 and M.U., A. Woodroffe, R.S.L., J.F.S., A.D., R.S.S., unpublished observations). The evidence for linkage, an excess (>50%) of identity by descent in affected sisters, was found for a 14-Mb region flanking the *INSR* and was strongest at the STRP marker, D19S884 (identity by descent = 0.63, $\chi^2 = 8.79$; nominal $P = 0.003$). The same marker also shows strong evidence for association with PCOS; this was detected by the transmission/disequilibrium test (TDT) (6,8,9) with allele 8 of the marker (135 transmissions, 83 nontransmissions, $\chi^2 = 12.95$; nominal $P < 0.00032$). Our findings in the *INSR* region are supported by a case-control study (10) that also found evidence for association between an allele of D19S884 and PCOS. The evidence for this region is stronger than that for any other regions that we tested.

If the causal element in this region is the *INSR* gene, the strong association observed between PCOS and D19S884 is surprising because of the large chromosomal distance involved: D19S884 is located ~900 kb centromeric to the *INSR* gene. The effect detected by the TDT depends on disequilibrium between the phenotype and marker allele tested, and disequilibrium generally does not extend over such distances in large modern populations (11–14). It is therefore unlikely that the observed association is due to a variant at the *INSR* gene itself. More likely, the association is due to a variant in another gene in the region, or in a very distant regulatory element of *INSR*.

In the interval between the *INSR* gene and D19S884, there is a plausible candidate gene for PCOS (Fig. 1). This gene encodes resistin, a protein hormone that was discovered in mice by a screen for genes that are induced during adipocyte differentiation and downregulated in mature adipocytes treated with thiazolidinediones (15,16). Thiazolidinediones are insulin-sensitizing drugs that are high-affinity ligands for peroxisome proliferator-activated receptor- γ (PPAR γ), an adipogenic determination factor (16). A large-scale study (17) of PPAR γ in type 2 diabetes

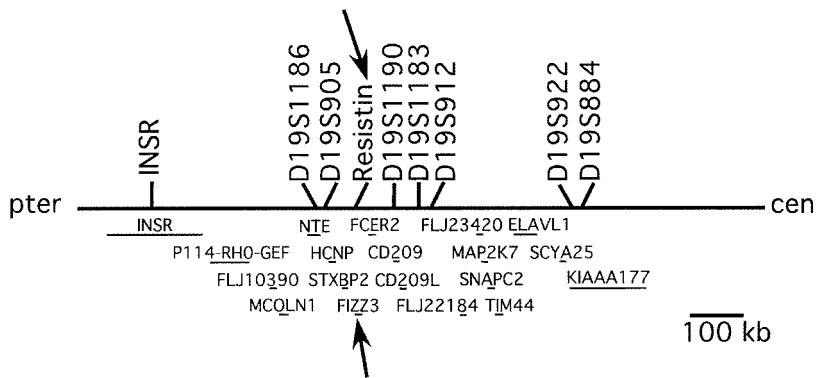


FIG. 1. Map of D19S884 region of chromosome 19p13.3. Locations of the short tandem repeat polymorphisms are indicated above the line. The locations and names of genes mapping to the INSR/D19S884 region are shown below the markers. The resistin gene (FIZZ3) and the resistin SNP are indicated by arrows.

provided evidence that variation in PPAR γ contributes to susceptibility to the disease.

We consider resistin an excellent candidate gene for PCOS for two reasons. First, it maps ~420 kb from D19S884, the marker that shows linkage and linkage disequilibrium with PCOS in our families as well as association in an independent case-control sample. Second, the role of the resistin molecule in insulin sensitivity makes it a plausible candidate gene for PCOS. We determined genotype frequencies for a single nucleotide polymorphism (SNP) in the promoter of the resistin gene in a sample of 500 unrelated individuals of European descent and used the TDT to test for linkage and association of this SNP with PCOS and the related phenotypes, insulin resistance and obesity.

RESEARCH DESIGN AND METHODS

Family ascertainment and phenotypes. We studied 258 families with 323 affected offspring. Both parents were available in 256 families, and 2 families had one parent available. A total of 253 families (310 daughters) were of Caucasian origin, and 5 families (9 daughters) were of Hispanic origin (6,7). Informed consent was obtained from every subject. Criteria for diagnosis of PCOS/HA were as previously described by Legro et al. (18) and by Urbanek and colleagues (6,7). Both male and female subjects were considered obese if they had a BMI ≥ 30 kg/m 2 (19). By this criterion, 206 offspring of 173 families were considered obese. A woman was given the diagnosis of insulin resistance if her fasting glucose-to-insulin ratio was <4.5 (20). All individuals taking confounding medication (e.g., insulin, nicotinic acid, prednisone, metformin, troglitazone, sulfonylureas, and thiazide diuretic) or not satisfying the above criteria were given the diagnosis "unknown." There were 159 daughters in 140 families that were considered insulin resistant by our criteria.

Genotyping. The resistin gene was screened by denaturing high-performance liquid chromatography (dHPLC) for mutations in 94 Finnish individuals: 64 with non-insulin-dependent diabetes, 16 normoglycemic elderly control subjects, and 14 obese insulin-sensitive probands. The resistin gene was screened in seven different fragments: three overlapping fragments in the promoter region (~760 bp upstream of exon 1) and the four exons that included some intron sequence. Putative variants were screened to determine the nucleotide change (K.S., F.S.C., manuscript in preparation). A C/G variation located 420 bp upstream to the initiation codon in the putative promoter region was the only variant with a frequency $>10\%$. We genotyped the C/G variant in 854 individuals in 258 families using Pyrosequencing technology (Uppsala, Sweden). PCR products were generated by amplifying 10 ng genomic DNA (forward primer: 5'-GAGAAGTGGTCTTGCTCTGT-3'; reverse primer: 5'-biotin-TGAGCAGACAGTAAGGGC-3') in 50 μ l reaction volume containing 1 \times

PCR buffer II (PE Biosystems), 1.5 mmol/l MgCl $_2$, 125 μ mol/l dNTPs, 0.2 μ mol/l primers, and 1.5 units of AmpliTaq Gold $^{\text{TM}}$ (PE Biosystems). Cycling conditions were 1 cycle at 95°C for 5 min followed by 45 cycles at 95°C for 15 s, 57°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 10 min. SNP genotyping was carried out on the Pyrosequencing PSQ96 system in the presence of 333 nmol/l of the sequencing primer (5'-CAGTCTCTGGACATGAAG-3') and enzymes and reagents provided in the SNP Reagent Kit (Pyrosequencing AB) according to manufacturer protocols.

Statistical analysis. We used the TDT to test for linkage disequilibrium (presence of both linkage and association) between the SNP in the promoter of the resistin gene and the phenotypes, PCOS/HA, obesity, and insulin resistance. These analyses were carried out as described previously (6,9).

RESULTS

We genotyped the C/G polymorphism in the promoter of the resistin gene in 258 PCOS families using pyrosequencing. The more common allele (C) has a frequency of 0.70 in the parents ($n = 500$) of our data, and the genotypes of the parents were in Hardy-Weinberg equilibrium (Table 1). Table 2 shows the results of the TDT for this SNP and three phenotypes: PCOS/HA, obesity, and insulin resistance. We observed 249 informative transmissions to daughters with the PCOS/HA phenotype, but there was no significant departure from random expectation (134 transmissions of allele C, 115 nontransmissions; $\chi^2 = 1.45$, $P > 0.2$). There were 175 informative transmissions to obese offspring, but there was no evidence of association between the promoter variant and obesity (94 transmissions of allele C, 81 nontransmissions; $\chi^2 = 0.97$, $P > 0.3$). There was also no evidence for association between the promoter variant and insulin resistance in our families. We observed a total of 139 informative transmissions, but neither allele was preferentially transmitted to insulin-resistant individuals (76 allele C, 63 non-allele C; $\chi^2 = 1.22$, $P > 0.26$). By our power analysis (see below), the probability of detecting an association as strong as that observed at D19S884, if it were present, was 98%. This high power confirms that if there were an effect of the kind for which we tested, we would have been very likely to detect it in this study.

TABLE 1
Parental allele and genotype frequencies

	Allele C	Allele G	Genotype CC	Genotype CG	Genotype GG
Observed frequency (n)	0.702 (702)	0.298 (298)	0.490 (245)	0.424 (212)	0.086 (43)
Expected (n)			245	210	45

TABLE 2
TDT analysis for PCOS/HA and associated phenotypes

	Transmissions (<i>n</i>)	Allele C transmitted (<i>n</i>)	Allele C not transmitted (<i>n</i>)	χ^2	<i>P</i>
PCOS/HA	249	134	115	1.450	>0.2
Obesity	175	94	81	.097	>0.3
Insulin resistance	139	76	63	1.22	>0.2

DISCUSSION

Resistin, a protein hormone that is believed to modulate glucose tolerance and insulin action (15), is a plausible candidate susceptibility gene for PCOS because of both its function and map location. Resistin mRNA levels are increased in both diet-induced and genetically obese mice (16). Resistin impairs glucose tolerance *in vivo*, and neutralization of resistin by anti-resistin IgG in mice results in improved insulin sensitivity compared with control subjects. Conversely, administration of resistin to mice reduces insulin-stimulated glucose uptake (15). Since many PCOS patients, in addition to being hyperandrogenic, are also insulin resistant and/or obese, resistin might be expected to play a role in PCOS.

The human resistin gene maps to chromosome 19p13.3 and is located between *INSR* and D19S884, ~470 kb from *INSR* (Fig. 1). The location of the human resistin gene strongly supported its possible importance in the etiology of PCOS, since it maps ~420 kb from D19S884, an anonymous marker that showed the strongest evidence for association with PCOS of any of the 54 markers that were tested in our families.

In our families, however, we find no evidence for association between a SNP in the promoter of the resistin gene and any of the three phenotypes we tested in our families (PCOS/HA, insulin resistance, and obesity). At D19S884, instead of the expected (random) transmission ratio of 50%, we found a transmission ratio of 62% for allele 8. If resistin were closer than D19S884 to the site responsible for association with PCOS, we would expect (other things being equal) that the association would be stronger than that at D19S884. We carried out standard calculations to determine the power of our analysis. We found that with our sample size of 249, the probability is >98% that we would obtain a result significant at or beyond the $P < 0.05$ significance level if the true transmission ratio were ≥ 0.62 . Thus, if the resistin promoter region had, or was associated with, an effect as large as that at D19S884, we would have been very likely to detect it. We conclude that the association that we detected in the *INSR* region with the STRP D19S884 is probably not due to a variant in the resistin gene.

With our present data, we cannot rule out the possibility that some other variant in the resistin gene contributes to PCOS. However, we consider this explanation unlikely for two reasons: 1) the resistin gene is relatively small, so such a putative site would not be much closer to D19S884, and therefore would not be expected to show much greater association; 2) other short tandem repeat polymorphisms (D19S1190, D19S1183, and D19S912), located between resistin and D19S884 but closer to resistin (Fig. 1), also fail to show association with PCOS (M.U., A. Woodroffe, R.S.L., J.F.S., A.D., R.S.S., data not shown).

The possible role of resistin in diabetes or insulin

resistance has recently been investigated in humans in other studies, but no strong evidence has been found. Resistin expression was low and not significantly different among human fat cells from unaffected, insulin-resistant, and type 2 diabetic patients (21), and mRNA levels in human adipocytes were not correlated with BMI (22). No significant associations have been found between resistin gene variants and type 2 diabetes (23,24).

Although we have shown that variation in the resistin gene is unlikely to be responsible for the association and linkage with PCOS that we find in this region, these findings do not eliminate the possible importance of resistin in the etiology of PCOS, especially in view of the insulin sensitivity defects. Variation in the function or expression of resistin molecule might play a role in the etiology of PCOS, but the determinants of that variation might be located in genes that do not map to resistin. Thus, variants in proteins acting functionally upstream of the resistin protein could modulate the expression levels of resistin and thereby affect the phenotype.

In addition to testing for association between the resistin promoter variant and PCOS, we also tested for association between the variant and two subphenotypes of PCOS, obesity and insulin resistance. These characteristics are very commonly seen in PCOS patients, and by analyzing these phenotypes, we might have enriched for a subcategory of PCOS where resistin plays a role in the etiology of the disease. However, we found no evidence for association between the promoter variant and either phenotype.

We have carried out an analysis of a large sample of family data using the TDT. Our results make it very unlikely that the promoter region variant we tested plays a role in PCOS, insulin resistance, or obesity in PCOS families. By extension, it is also unlikely that variation in the resistin gene accounts for the strong association that we observed between allele 8 of D19S884 and PCOS. Instead, this association is more likely due to some other gene or genetic element in the region of D19S884.

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