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COMMENT

An ATG Repeat in the 3′-Untranslated Region of the Human Resistin Gene Is Associated with a Decreased Risk of Insulin Resistance

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Resistin is overexpressed in human adipose tissue of obese individuals and is likely to modulate insulin sensitivity. Resistin is, therefore, a candidate gene for insulin resistance. We searched for polymorphisms in the resistin gene by single strand conformation polymorphism and direct sequencing. An ATG triplet repeat in the 3′-untranslated region was identified and considered for association with insulin resistance. Three alleles were identified (allele 1: 8 repeats, allele frequency, 0.3%; allele 2: 7 repeats; allele frequency, 94.5%; allele 3: 6 repeats; allele frequency, 5.2%). Two hundred and three unrelated white Caucasian nondiabetic subjects from Sicily and 456 from the Gargano area (center east coast of Italy) were analyzed. Among Sicilians, subjects carrying allele 3 had a lower fasting insulin and insulin resistance index (homeostasis model assessment of insulin resistance; $P < 0.001$ for both) and glucose ($P = 0.025$) and insulin ($P = 0.002$) levels during the oral glucose tolerance test. In subjects from Gargano, those carrying allele 3 had lower fasting plasma glucose levels and serum triglycerides ($P = 0.01$ for both). When the 2 populations were analyzed together, subjects carrying allele 3 had lower fasting insulin levels ($P < 0.005$), homeostasis model assessment of insulin resistance ($P < 0.005$), and serum triglycerides ($P = 0.01$).

In conclusion, our data suggest that subjects carrying allele 3 of the resistin gene are characterized by relatively high insulin sensitivity. (*J Clin Endocrinol Metab* 87: 4403–4406, 2002)

THE INSULIN resistance syndrome (*i.e.* the variable association among obesity, hyperinsulinemia, dyslipidemia, and high blood pressure) plays an important role in the pathogenesis of type 2 diabetes mellitus and cardiovascular diseases (1–4) and is due to both environmental and genetic factors. Although environmental determinants are well known, the genetic background is still poorly understood (5).

Recent advances have improved our understanding of the biology of adipose tissue, not any longer an inert storage compartment for excess energy in the form of triglycerides, but a major regulator of the metabolic flux adaptation to the availability of stored energy (6). Adipose tissue exerts this function through different chemical messengers secreted by adipocytes and acting in an autocrine, paracrine, or endocrine manner (6), including leptin, adipsin, TNFα, adiponectin, and resistin (7–10). Dysregulation of this network has been implicated in the etiology of insulin resistance (1, 6).

Resistin, a newly identified cystein-rich protein, was identified in mice by screening for genes induced during adipocyte differentiation (10). Resistin levels are regulated by the fasting/fed state and insulin, suggesting its involvement in sensing the nutritional status of the animal to affect adipogenesis (10, 11). In mature adipocytes resistin is downregulated by rosiglitazone, an insulin-sensitizing drug (10). White adipose tissue resistin expression was reported increased in obese rodent models in some (10, 11), but not all (12), studies. In these models resistin immunoneutralization improved insulin sensitivity (10).

In human adipose tissue, resistin gene expression is relatively low (13, 14), whereas it is increased in morbidly obese compared with lean subjects (13). Although the specific cellular component (*i.e.* adipocytes or monocytes/macrophages) overproducing resistin in adipose tissue of obese subjects has not yet been identified (13), it is possible that, in analogy to the rodent model, resistin might affect insulin sensitivity in humans also.

We have now evaluated, therefore, whether polymorphisms in the resistin gene may contribute to the genetic susceptibility to insulin resistance and type 2 diabetes in humans.

Subjects and Methods

Experimental subjects

To avoid the confounding effect of hyperglycemia and morbid obesity on insulin resistance-related abnormalities, we selected only nondiabetic (fasting plasma glucose, <126 mg/dl) and not severely obese

Abbreviations: AF, Allele frequency; BMI, body mass index; HOMA$_{IR}$, homeostasis model assessment of insulin resistance; OGTT, oral glucose tolerance test; SSCP, single strand conformation polymorphism; UTR, untranslated region.

[body mass index (BMI), <40 kg/m^2] subjects. Two separate series of unrelated Caucasian subjects were studied: 203 from Sicily and 456 from the Gargano area (center east coast of Italy).

The following parameters were measured in all subjects: BMI, mean blood pressure, fasting glucose and insulin, and lipid profile. The homeostasis model assessment of insulin resistance ($HOMA_{IR}$) (15) was calculated according to the formula: fasting glucose (mmol/liter) − fasting insulin (pmol/liter)/22.5. In individuals from Sicily, glucose and insulin levels were also measured before and 60 and 120 min after the 75-g oral glucose tolerance test (OGTT).

Serum cholesterol and triglycerides were evaluated by enzymatic methods (ILTest Cholesterol and ILTest Triglycerides, Instrumentation Laboratory, Lexington, MA). The high density lipoprotein fraction has been separated by use of Mg^{2+} and dextran sulfate method (CHOL-HDL reagent, Sclavo Diagnostics, Siena, Italy). Plasma glucose was measured by the glucose oxidase method on a Glucose Analyzer 2 (Beckman, Palo Alto, CA), and plasma insulin was measured by microparticle enzyme immunoassay (IMx insulin assay, Abbott, North Chicago, IL).

Overweight and obese subjects (BMI, ≥25) were recruited from the out-patient metabolic clinic of our institution. Normal weight subjects were recruited from the staff of our hospital. Informed consent was obtained from all participants before entry the study, which was approved by the local research ethic committee.

Polymorphism screening

To screen for sequence variations within the resistin gene, DNA from 65 unrelated blood donors was used. Ten sets of primers were designed to amplify all the exons and their flanking sequences. In addition, about 1 kb of the transcription start upstream region was covered by 8 overlapping PCR primer sets. Primers used are shown in Table 1.

Single strand conformation polymorphism (SSCP) was performed as follows. Amplification reactions were performed for 30 cycles in 25 μl containing 100 ng genomic DNA, 25 pmol of each nucleotide primer, and 0.5 U *Taq* polymerase (in 1.5 mmol/liter $MgCl_2$, 10 mmol/liter Tris-HCl, 50 mmol/liter KCl, and 0.1% Triton X-100, pH 8.8). PCR products were denatured for 5 min at 94 C in 90% formamide, 20 mmol/liter EDTA, and 10 mmol/liter NaOH. Samples were then chilled on ice, loaded on native 8–12% (according to amplimers size) acrylamide (optimized from 19:1 to 29:1 acrylamide-bisacrylamide) gel (0.04 × 20 × 42 cm; with or without 10% glycerol) in Tris-borate-EDTA buffer, and electrophoresed at 10 watts constant power for 16–28 h at room temperature. Products were colored with Cyber Gold (Amersham Pharmacia Biotech, Little Chalfont, UK), and gels were scanned on a Storm 860 apparatus (Molecular Dynamics, Inc., Little Chalfont, UK). PCR products showing different migration patterns at SSCP as well as 20 samples showing the most common pattern were directly sequenced in an ABI PRISM 310 automatic sequencer (PE Applied Biosystems, Foster City, CA). All genotyping was carried out in duplicate for each individual, and the investigator was unaware of the sample origin.

Microsatellite analysis

To analyze the 3′-untranslated region (3′UTR) ATG repeat, the RES-5 forward (5′-GGA GGC GGC TCC AGG TCC-3′) and RES-5 reverse (5′-GCA GTA GAA AGT CGC GGT GT-3′) oligonucleotide primers were used. PCR products were resolved in a 6% denaturing polyacrylamide gel for 3 h at 70 V. Products were then colored with Cyber Gold, and gels were scanned on a Storm 860 apparatus (Molecular Dynamics, Inc.).

Statistical analysis

Values are given as the mean ± SEM. The mean values of unrelated individuals from the two genotyped groups were compared by *t* test or Mann-Whitney *U* test, as appropriate. Allele frequencies were compared by the χ^2 test. Two-way ANOVA was applied to analyze glucose and insulin profiles during the OGTT. All differences were also tested after adjusting for gender or BMI by analysis of covariance.

Results

We searched for polymorphisms in both the regulatory and coding regions of the resistin gene by SSCP. Five variations were identified (Table 2), two of which have been previously described (16); their allele frequencies were in Hardy Weinberg equilibrium (data not shown). Because of the low allele frequency (AF) (*i.e.* <2%) the only exonic variation found (*i.e.* T233C) was not further considered for association with insulin resistance. It is, in fact, very unlikely that a variant with such a low AF may play a role in the genetic susceptibility of insulin resistance in the general population. Of the remaining four variations, only that found in the 3′UTR was associated with insulin resistance, as described below. No linkage disequilibrium was observed be-

TABLE 2. Single nucleotide polymorphisms in the *hResistin* gene

Designation	Location	Type	Allele frequency
−179C → G	Promoter	Noncoding	23.6%
Intron 2 C39T	Intron	Noncoding	28%
T233C	Exon	Missense[a]	1.9%
Intron 3 C30T	Intron	Noncoding	15%
ATG repeat	3′ UTR	Noncoding	1 = 0.3%, 2 = 94.5%, 3 = 5.2%[b]

[a] C63R.
[b] 1, eight repeats; 2, seven repeats; 3, six repeats.

TABLE 1. PCR amplification conditions

	Primers	Annealing temperature (C)	Amplimer type (bp)
Exon 1	5′-CAGGGACTTATTAGCCAAGC-3′		
	5′-AAGAGACCCACAGCTGGATC-3′	60	286
Exon 2	5′-CAGATCCTACTCCCTCCATG-3′		
	5′-TTGGAGTCAGGTCTGTGCCA-3′	62	288
Exon 3	5′-GACCGTTTGGTCTCACAGCT-3′		
	5′-AAGATCCTAGGGGAGTAGAGGCT-3′	58	198
Exon 4/1	5′-TCAGCCTCCCAGCTCAGAGT-3′		
	5′-CTCCAGGTTTATTTCCAGCTC-3′	62	261
Exon 4/2	5′-GGAGGCGGCTCCAGGTCC-3′		
	5′-GCAGTAGAAAGTCGCGGTGT-3′	62	203
−265 → −5	5′-CTCTGCTTGTCTACCTGTTCC-3′		
	5′-TCCTGCACCGCAGCTCTTTC-3′	64	260
−516 → −181	5′-TGAATGTGGTATGTCATTCTCAC-3′		
	5′-CTTCATGTCCAGAGACTGGTC-3′	64	335
−688 → −432	5′-GTATGTCTAATGGAGACAGGG-3′		
	5′-TCCAGTCTGGGCAACAGAGC-3′	60	257
−839 → −619	5′-ATGGTGCCCAAGCTAGTCATC-3′		
	5′-GATCACTTGAGGTCAGGAGCT-3′	64	221

tween this variation and any of the other three tested for association. In the 3′UTR polymorphism a total of three alleles were identified (allele 1: eight repeats; AF, 0.3%; allele 2: seven repeats; AF, 94.5%; allele 3: six repeats; AF, 5.2%). Due to the very low AF, allele 1 was not tested for association with insulin resistance.

Among the 203 unrelated individuals from Sicily (73 males and 130 females; age, 35.6 ± 0.8 yr), those carrying allele 3 in the 3′UTR (n = 20, with 1 homozygous) had a significantly lower fasting insulin (39.7 ± 3.5 *vs.* 68.4 ± 3.7 pmol/liter; $P < 0.001$) and insulin resistance index (16) $HOMA_{IR}$ (8.6 ± 0.8 *vs.* 15.1 ± 0.9; $P < 0.001$) compared with homozygotes for allele 2 (Table 3). These differences remained significant ($P < 0.05$) after adjusting for gender, but not for BMI. In subjects carrying allele 3, triglycerides and mean blood pressure were also lower than in the subjects homozygous for allele 2, although the differences were not statistically significant with the present sample size (Table 3). In individuals carrying allele 3, glucose and insulin levels during the OGTT were lower in respect to subjects homozygous for allele 2 ($P = 0.025$ and $P = 0.002$ respectively, by two-way ANOVA; Fig. 1) and remained significantly different ($P < 0.05$) when adjusted for gender or BMI. In addition, a significant interaction between resistin polymorphism and BMI was observed in both glucose ($P < 0.01$) and insulin ($P = 0.02$) profiles during the OGTT.

To minimize the risk of false positive results due to population stratification (17), 456 subjects from Gargano (159 males and 297 females; age, 38.1 ± 0.5 yr) were studied to replicate these results. The Gargano population, although geographically close to the Sicilian one, has a different ethnicity (18). Subjects carrying allele 3 (n = 50, no homozygous) had lower fasting plasma glucose levels (4.8 ± 0.7 *vs.* 5.0 ± 0.2 mmol/liter; $P = 0.01$) and serum triglycerides (0.89 ± 0.06 *vs.* 1.08 ± 0.04 mmol/liter; $P = 0.01$) compared with allele 2 homozygous subjects. Glucose levels remained significant ($P < 0.02$) after adjusting for gender or BMI. Subjects carrying allele 3 also tended to have lower mean levels of fasting insulin, $HOMA_{IR}$, and mean blood pressure, although differences were not statistically significant in the sample size examined.

Although the two populations studied were different with respect to BMI, a variable for which results may be easily adjusted, data were pooled and examined together to increase the sample size and statistical power. Subjects carrying allele 3 had lower fasting insulin levels ($P < 0.005$), $HOMA_{IR}$ ($P < 0.005$), and serum triglycerides ($P = 0.01$; Table 3). When adjusted for gender, these differences remained significant ($P < 0.05$); in addition, fasting plasma glucose difference across the two genotype groups became significant ($P < 0.05$). Differences in insulin and $HOMA_{IR}$, but not glucose and triglycerides, remained significant after adjusting for BMI. In addition, a significant interaction between resistin polymorphism and BMI was observed for insulin and $HOMA_{IR}$ levels ($P < 0.02$ for both). Subjects carrying allele 3 also had a significantly decreased risk (0.45; 95% confidence interval, 0.25–0.80) of being

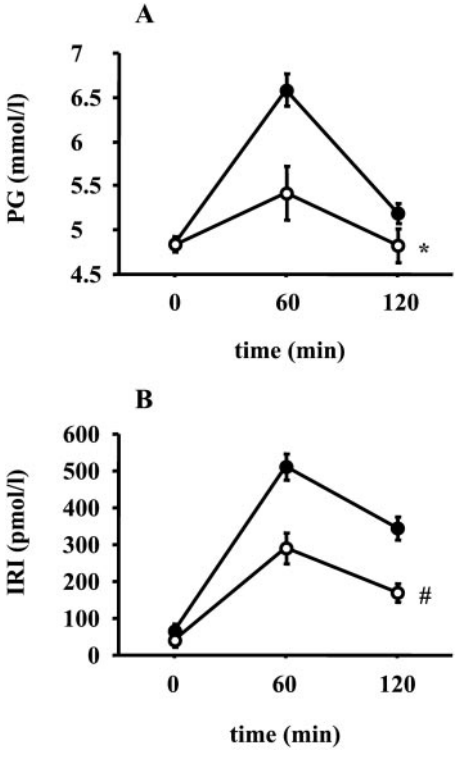

FIG. 1. Plasma glucose (PG; A) and immunoreactive insulin (IRI; B) levels before (time zero) and 60 and 120 min after a 75-g oral glucose load in 203 subjects from Sicily. ●, Subjects homozygous for allele 2; ○, subjects carrying allele 3. Data are the mean ± SEM. *, $P = 0.025$ *vs.* subjects homozygous for allele 2, by two-way ANOVA. #, $P = 0.002$ *vs.* subjects homozygous for allele 2, by two way ANOVA.

TABLE 3. Clinical characteristics of the subjects from the two populations studied

Genotype	Sicily		Gargano		All	
	2/2	*X/3*	*2/2*	*X/3*	*2/2*	*X/3*
No.	183	20	406	50	589	70
Males/females	63/120	10/10	142/264	17/33	205/384	27/43
Age (yr)	35.6 ± 0.8	35.5 ± 2.8	38.4 ± 0.6	35.4 ± 1.7	37.6 ± 0.5	35.4 ± 1.5
BMI (kg/m^2)	27.3 ± 0.4	25.9 ± 1.2	25.3 ± 0.2	24.7 ± 0.7	25.9 ± 0.2	25.1 ± 0.6
PG (mmol/liter)	4.8 ± 0.4	4.8 ± 0.9	5.0 ± 0.2	4.8 ± 0.7[a]	5.0 ± 0.0	4.8 ± 0.1[b]
IRI (pmol/liter)	68.4 ± 3.7	39.7 ± 3.5[c]	55.0 ± 1.5	50.9 ± 3.8	58.9 ± 1.5	48.0 ± 3.0[d]
$HOMA_{IR}$	15.1 ± 0.9	8.6 ± 0.8[c]	12.4 ± 0.4	11.0 ± 0.9	13.2 ± 0.4	10.4 ± 0.7[d]
Chol/HDL	4.3 ± 0.1	4.3 ± 0.3	3.9 ± 0.1	3.7 ± 0.2	3.9 ± 0.1	3.9 ± 0.1
Tg (mmol/liter)	1.1 ± 0.1	1.0 ± 0.2	1.1 ± 0.0	0.9 ± 0.1[a]	1.1 ± 0.0	0.9 ± 0.1[a]
MBP (mm Hg)	89.1 ± 0.8	86.9 ± 2.0	88.1 ± 0.5	86.2 ± 1.1	88.4 ± 0.4	86.4 ± 0.9

Data are mean ± SEM. Genotype: *2/2* are homozygous for allele 2 and *X/3* are carriers of allele 3 (*i.e.* 69 *2/3* and 1 *3/3*). PG, Fasting plasma glucose; IRI, fasting immunoreactive insulin; Chol/HDL, fasting total cholesterol/fasting high-density lipoprotein ratio; Tg, fasting triglycerides; MBP, mean blood pressure.

[a] $P = 0.01$, [b] $P < 0.05$, [c] $P < 0.001$, [d] $P < 0.005$, after adjusting for gender.

relatively insulin resistant (*i.e.* to have an individual $HOMA_{IR}$ value above the median value of the entire cohort). In subjects carrying allele 3 mean blood pressure values were lower, although differences were not statistically significant in the examined sample size.

Discussion

Together these data indicate that the ATG repeat in the 3′UTR of the human resistin gene associates with insulin resistance, and that subjects carrying the less frequent allele 3 are characterized by relatively high insulin sensitivity. Differences observed in variables associated with insulin resistance in the two studies may well be based upon interaction of the human resistin gene with other genetic and/or environmental determinants that are not equally distributed in the two different populations.

Recently, two different reports (19, 20) failed to find any association between the resistin gene and either obesity (19) or type 2 diabetes (19, 20). However, these studies have not described the ATG repeat at the 3′UTR that we found to be associated with different insulin sensitivity. In addition, although obesity and type 2 diabetes are certainly characterized by insulin resistance, both diseases are likely to recognize different and additional genetic backgrounds than insulin resistance *per se*. Finally, different population ethnicity (20) may also explain the different results obtained.

We believe it is unlikely that the association between the 3′UTR polymorphism and $HOMA_{IR}$ is simply due to chance, because the *P* value for the $HOMA_{IR}$ difference is highly significant. In addition, the difference in triglycerides observed in all subjects across the two genotype groups is coherent with $HOMA_{IR}$ data. Together these suggest high insulin sensitivity in subjects carrying the less frequent allele 3. This is also suggested by the lower glucose and insulin profiles during OGTT observed in individuals carrying this allele. Finally, although resistin polymorphism associates with different features of insulin resistance syndrome in the two populations studied, the replication of similar data in cohorts of different ethnicity makes it unlikely that there is a spurious association due to population stratification (17).

The cluster of metabolic abnormalities known as the insulin resistance syndrome is responsible for a large proportion of cardiovascular morbidity and mortality in the western world (1). Insulin resistance is likely to be polygenic, *i.e.* due to the simultaneous involvement of many genes, each having a small effect (5). The involved genes, however, are mostly unknown (5). Our data suggest that resistin may be one these genes, although the biological mechanism of this association is unclear. The 3′UTR may regulate gene expression by several mechanisms (21); therefore, although entirely speculative, one possibility is that subjects carrying allele 3 are more insulin sensitive because of a reduced expression and secretion of resistin. However, additional studies comparing resistin expression in adipose tissue across different genotypes are needed to test this hypothesis. Another possibility is that the ATG repeat in the 3′UTR of the resistin gene is not itself responsible for the association with insulin resistance, but, rather, is in linkage disequilibrium with an unidentified causal single nucleotide polymorphism impairing either gene function or expression, which, in turn, may affect insulin sensitivity. It is also possible that the casual single nucleotide polymorphism is located within a gene different from but close to the resistin gene.

In conclusion, we found an association between the ATG repeat in the 3′UTR of the resistin gene and insulin resistance. Our data suggest that this polymorphism may play a role in the individual susceptibility to insulin resistance and type 2 diabetes.

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