**Brief Genetics Report**

**Adiponectin Receptor 1 Gene (ADIPOR1) as a Candidate for Type 2 Diabetes and Insulin Resistance**

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Considerable data support adiponectin as an important adipose-derived insulin sensitizer that enhances fatty acid oxidation and alters hepatic gluconeogenesis. Adiponectin acts by way of two receptors, ADIPOR1 and ADIPOR2. ADIPOR1 is widely expressed in tissues, including muscle, liver, and pancreas, and binds the globular form of adiponectin with high affinity. To test the hypothesis that sequence variations in or near the ADIPOR1 gene contribute to the risk of developing type 2 diabetes and the metabolic syndrome, we screened the eight exons (including the untranslated exon 1) of the ADIPOR1 gene with flanking intronic sequences and the 5' and 3' flanking sequences. We identified 22 single nucleotide polymorphisms (SNPs) in Caucasian and African-American subjects, of which a single nonsynonymous SNP (N44K) in exon 2 was present only in African-American subjects. We typed 14 sequence variants that had minor allele frequencies >5%. No SNP was associated with type 2 diabetes in Caucasians or African Americans, and no SNP was a determinant of insulin sensitivity or insulin secretion among nondiabetic members of high-risk Caucasian families. However, the two alleles of a SNP in the 3' untranslated region were expressed unequally, and ADIPOR1 mRNA levels were significantly lower among transformed lymphocytes from diabetic African-American individuals than among control cell lines. This altered gene expression might suggest a role for ADIPOR1 in the metabolic syndrome. *Diabetes* 53:2132–2136, 2004

Type 2 diabetes is characterized by obesity-related insulin resistance. Although most glucose uptake occurs in skeletal muscle in the postabsorptive state (1), considerable evidence (2) suggests a role in this uptake for factors secreted from adipose tissue. Adiponectin (also known as AdipoQ, APM1, and Acrp30) (3) has emerged as one such factor. Low adiponectin levels precede and predict type 2 diabetes (4), and increasing levels of plasma adiponectin improve insulin sensitivity (5), probably by acting through AMP kinase to increase fatty acid oxidation (6,7). Consistent with these roles, plasma adiponectin levels are decreased with obesity (3), despite increased adipose mass.

Recently, two related but distinct receptors for adiponectin were identified and the genes cloned from a human skeletal muscle expression library by binding to globular adiponectin (8). The two receptors, ADIPOR1 and ADIPOR2, were predicted to contain seven transmembrane domains, share 67% identity with the mouse gene, and show marked conservation of the membrane-spanning domains from yeast to mammals (8). Although expressed ubiquitously, ADIPOR1 showed the highest expression in skeletal muscle, whereas ADIPOR2 was found most abundantly in liver (8). ADIPOR1 appears to primarily bind the globular form of adiponectin, in contrast to ADIPOR2, which primarily binds the full-length form (8). More recently, both ADIPOR1 and ADIPOR2 were found to be abundantly expressed in human and rat pancreatic β cells (9), where expression was increased by exposure to the free fatty acid oleate (9).

Genetic variation in the adiponectin gene has been reported to be associated with obesity, insulin resistance, type 2 diabetes, and adiponectin levels in multiple studies (10). We hypothesized that variation in the adiponectin receptor genes would likewise contribute to the risk of type 2 diabetes by reducing insulin sensitivity. We selected the ADIPOR1 gene because of its ubiquitous expression, including high levels in skeletal muscle and pancreatic β-cells, and the evidence of an important role of the globular subunit in mediating adiponectin action (6,11,12). ADIPOR1 is located at 1q32.1 and is significantly telomeric to the 1q21-q24 linkage signals. We screened the entire ADIPOR1 gene for mutations and investigated the relationship of this variation to insulin sensitivity and type 2 diabetes. To further explore the role of genetic variation of ADIPOR1 on adiponectin levels, we examined allele-specific expression in transformed lymphocytes and compared total mRNA expression among individuals.

**RESEARCH DESIGN AND METHODS**

Caucasian individuals were ascertained in Utah for Northern European ancestry, as previously described (13). Additional Caucasian and African-American individuals were ascertained from Arkansas for similar criteria. All

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LD, linkage disequilibrium; SNP, single nucleotide polymorphism.

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individuals with type 2 diabetes had at least one other first-degree relative with type 2 diabetes. Nondiabetic control individuals had no known family history of diabetes and had either a normal 75-g oral glucose tolerance test result or a fasting glucose level <5.5 mmol/l (100 mg/dl). All subjects provided written informed consent under protocols approved by either the University of Utah Institutional Review Board or the University of Arkansas for Medical Sciences Human Research Advisory Committee.

Mutations were detected in 40 Caucasians (24 with type 2 diabetes and 16 glucose-tolerant control subjects) and 24 African Americans (16 with type 2 diabetes and 8 glucose-tolerant control subjects). Case-control association studies with type 2 diabetes were conducted in 192 Caucasian subjects with type 2 diabetes and 192 Caucasian control subjects and in 269 African-American subjects with type 2 diabetes and 136 nondiabetic African-American subjects. To reduce costs associated with typing large numbers of samples in large numbers of single nucleotide polymorphisms (SNPs), we constructed pooled DNA samples from each population. The pools equally represented each DNA sample and were constructed in triplicate from the concentration-adjusted DNA for each individual sample. We tested five pools: Caucasian control subjects (n = 192), Caucasian case subjects (n = 192), African-American control subjects (n = 130), African Americans with type 2 diabetes and diabetic nephropathy (n = 150) (14), and African Americans with type 2 diabetes and normal urine tests (n = 125) (14). As a result of new recruitment and necessary sample substitutions after pools were constructed, the individual samples included in the pooled and individual typing were slightly different, as reflected in slightly different sample sizes.

Insulin sensitivity was tested in 126 Caucasian nondiabetic members from 26 families for whom measurements of S were available (15). Allele-specific expression and mRNA expression levels were compared among 25 Caucasian (10 nondiabetic and 15 with type 2 diabetes) and 25 African-American (8 nondiabetic and 17 with type 2 diabetes) individuals.

**SNP detection and typing.** We designed 16 sets of primers for amplicons of 300–550 bp using Primer 3 and WAVEMAKER software version 4.0 (Transgenomic, Omaha, NE) to screen the eight exons (including the untranslated exon 1), 1,390 bp of the 5’ flanking region, and the 960-bp 3’ untranslated region, spanning 18,826 bp of genomic DNA. We screened all putative functional regions of the gene, including each exon and 100–200 bp of the flanking intronic sequence, 1,200 bp of the 5’ flanking sequence, and 960 bp of the 3’ flanking sequence. Mutations were detected using the Transgenomic WAVE HT DNA Fragment Analysis System (Transgenomic), and altered migration was confirmed by bidirectional sequence analysis (15) using infrared dye–labeled primers and GR4200 Sequencers (LI-COR Biotech, Lincoln, NE). SNPs were typed by Pyrosequencing (PSQ96; Pyrosequencing, Uppsala, Sweden) using the manufacturer’s protocols, except that a universal sequence was appended to one sequence–specific primer and amplification was performed in the presence of the universal biotinylated primer. Typing of pooled DNA samples was performed by Pyrosequencing using Allele Quantification software (Pyrosequencing) as described by others (16,17). Each pool was constructed in triplicate and analyzed in duplicate. Additionally, at least 92 individual samples were tested for each ethnic group to determine linkage disequilibrium (LD) and Hardy Weinberg equilibrium. If the SD of six measures was not <2%, if the SNP was nonanonymous, or if the difference in pooled frequencies exceeded 5%, individual samples were typed instead. The 5-bp insertion/deletion variant was typed using infrared fluorescent primers and separated on acrylamide gels using the LICOR GR4200 sequencers and read with SAGA VT software (LICOR Biotech). All SNPs were in Hardy-Weinberg equilibrium (P > 0.05).

**Analysis of mRNA expression in transformed lymphocytes.** Total RNA was isolated from Epstein-Barr virus–transformed lymphocytes grown to 0.5–1.0 × 10⁶ cells/ml as described previously (18). Allele-specific expression of SNP16 in the 3’ untranslated region was quantified using allele quantification software (SNP Software AQ; Pyrosequencing), the RT-PCR product, and the DNA primers for SNP16. Ratios were compared to DNA from the same individuals. ADIPOR1 mRNA levels were measured using total transformed lymphocyte RNA, primers designed using Primer Express software (Applied Biosystems, Foster City, CA), and real-time PCR performed using the SYBR green real-time PCR reagents kit according to the manufacturer’s protocol (Applied Biosystems). Reactions were performed in triplicate and detected on a Rotor-Gene RG 3000 (Corbett Research, Sydney, AU) and standardized to 18 S RNA.

**Statistical analysis.** Allelic association was tested separately for each ethnic group using the Fisher’s exact test. Significance in pooled DNA samples was judged using the statistics described by Risch and Teng (19). The role of ADIPOR1 SNPs in family members was tested using general linear models as described previously (20). Pairwise linkage (D’ and r2) was calculated from combined case and control population data using the expectation maximization algorithm separately for each ethnic group. Allele-specific expression was compared to DNA in the same individuals using the same primers by paired Wilcoxon’s signed-rank test to correct for unequal amplification, and expression ratios were corrected by dividing the observed RNA ratio by the observed DNA ratio. Individual variation in gene expression was compared using the unpaired t test in ln-transformed ADIPOR1–to–18 S mRNA ratios or by Mann-Whitney U test on nontransformed data. Analyses were performed in SPSS for Windows version 11.0 or 12.1 (SPSS, Chicago, IL).

**RESULTS**

We identified 19 SNPs by screening ADIPOR1 in both ethnic groups (Fig. 1), of which 3 SNPs were also reported in the public database (Table 1). Of 10 additional SNPs chosen from the public database for regions not included in our screening, I could not be successfully typed and 6 were not polymorphic in our populations, leaving 3 additional SNPs (see online appendix, Table 2 [available at http://diabetes.diabetesjournals.org]). Of the SNPs discover...
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TABLE 1
ADIPOR1 sequence variants and association with type 2 diabetes

<table>
<thead>
<tr>
<th>SNP ID no.</th>
<th>Position relative to ATG start</th>
<th>Location</th>
<th>Population</th>
<th>Variation (major/minor)</th>
<th>Rs no.</th>
<th>Frequency (diabetes/control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP28</td>
<td>−8503</td>
<td>Promoter</td>
<td>CAU/AA</td>
<td>C/T</td>
<td>rs66666089</td>
<td>0.326/0.340* 0.156/0.200†</td>
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<tr>
<td>SNP26</td>
<td>−7308</td>
<td>Promoter</td>
<td>CAU/AA</td>
<td>G/A</td>
<td>rs75172865</td>
<td>ND</td>
</tr>
<tr>
<td>SNP27</td>
<td>−7302</td>
<td>Promoter</td>
<td>CAU/AA</td>
<td>G/A</td>
<td>Novel</td>
<td>0.132/0.164* 0.322/0.325†</td>
</tr>
<tr>
<td>SNP23</td>
<td>−3881</td>
<td>Intron1</td>
<td>CAU/AA</td>
<td>T/C</td>
<td>rs1539555</td>
<td>0.330/0.335* 0.452/0.432†</td>
</tr>
<tr>
<td>SNP1</td>
<td>−1308</td>
<td>Intron1</td>
<td>AA</td>
<td>T/C</td>
<td>Novel</td>
<td>— 0.148/0.134†</td>
</tr>
<tr>
<td>SNP2</td>
<td>−383</td>
<td>Intron1</td>
<td>CAU</td>
<td>G/A</td>
<td>Novel</td>
<td>0.037/0.042* NP</td>
</tr>
<tr>
<td>5-bp I/D</td>
<td>−147</td>
<td>Intron1</td>
<td>AA</td>
<td>5-bp insertion/deletion</td>
<td>Novel</td>
<td>— 0.077/0.104*</td>
</tr>
<tr>
<td>SNP5</td>
<td>−105</td>
<td>Intron1</td>
<td>CAU/AA</td>
<td>G/A</td>
<td>rs2275738</td>
<td>0.432/0.405* 0.432/0.404†</td>
</tr>
<tr>
<td>SNP4</td>
<td>−101</td>
<td>Intron1</td>
<td>CAU/AA</td>
<td>T/G</td>
<td>rs2275737</td>
<td>0.470/0.497* 0.480/0.485†</td>
</tr>
<tr>
<td>SNP5</td>
<td>233</td>
<td>Exon2</td>
<td>CAU/AA</td>
<td>A/T</td>
<td>Novel</td>
<td>&lt;0.01;&lt;0.01†</td>
</tr>
<tr>
<td>SNP6</td>
<td>2502</td>
<td>Intron2</td>
<td>CAU/AA</td>
<td>A/G</td>
<td>Novel</td>
<td>0.038/0.046† NP</td>
</tr>
<tr>
<td>SNP7</td>
<td>4644</td>
<td>Intron4</td>
<td>CAU</td>
<td>A/G</td>
<td>rs2275735</td>
<td>0.463/0.427* 0.372/0.420</td>
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<tr>
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<td>4663</td>
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<td>AA</td>
<td>T/C</td>
<td>Novel</td>
<td>— 0.054/0.068†</td>
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<tr>
<td>SNP20</td>
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<td>CAU/AA</td>
<td>A/G</td>
<td>rs1342386</td>
<td>ND§</td>
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<td>SNP9</td>
<td>5843</td>
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<td>CAU/AA</td>
<td>A/G</td>
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</tr>
<tr>
<td>SNP10</td>
<td>7162</td>
<td>Exon6</td>
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<td>GGG(Gly)/GGA(Gly)</td>
<td>Novel</td>
<td>ND</td>
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<td>SNP11</td>
<td>7369</td>
<td>Intron6</td>
<td>AA</td>
<td>G/A</td>
<td>Novel</td>
<td>NP &lt;0.01†</td>
</tr>
<tr>
<td>SNP12</td>
<td>9512</td>
<td>3′ UTR</td>
<td>CAU</td>
<td>C/G</td>
<td>Novel</td>
<td>&lt;0.01† NP</td>
</tr>
<tr>
<td>SNP13</td>
<td>9881</td>
<td>3′ UTR</td>
<td>CAU</td>
<td>C/T</td>
<td>rs10581</td>
<td>0.053/0.057† NP</td>
</tr>
<tr>
<td>SNP14</td>
<td>10021</td>
<td>3′ UTR</td>
<td>AA</td>
<td>T/C</td>
<td>Novel</td>
<td>&lt;0.01†</td>
</tr>
<tr>
<td>SNP15</td>
<td>10990</td>
<td>3′ UTR</td>
<td>CAU/AA</td>
<td>T/C</td>
<td>Novel</td>
<td>ND</td>
</tr>
<tr>
<td>SNP16</td>
<td>10225</td>
<td>3′ UTR</td>
<td>CAU/AA</td>
<td>C/G</td>
<td>rs1139646</td>
<td>0.297/0.287* 0.36/0.42*</td>
</tr>
</tbody>
</table>

All data are based on the sequence NT_004671. *Typed in individual samples; †typed in pooled samples; ‡frequency determined in 96 individuals and not typed further; §not determined due to strong LD with SNP9; ‖not determined (ND) due to technical problems with the assay (homopolymer or repetitive sequences). AA, African American; CAU, Caucasian; NP, not present in population (allele frequency <0.005); UTR, untranslated region.

erated by screening and resequencing, three were not amenable to assay using available methods due to repetitive sequences. Four SNPs did not alter an amino acid and had minor allele frequencies <5% and hence were not typed due to low power to detect an association. The single nonsynonymous SNP in the first translated exon (exon 2; N44K) was typed in individual African-American samples, where it was not associated with type 2 diabetes. We selected 13 additional SNPs for analysis and one 5-bp insertion/deletion variant that together spanned the gene (boldface SNPs on Fig. 1 and Table 1), including four variants that were unique to African Americans and 3 SNPs that were observed only in Caucasians. For each of the 15 selected variants, we typed 96 Caucasian and 96 African-American subjects to determine the underlying gene structure and LD. Association with type 2 diabetes was tested in pooled samples if possible; otherwise individual samples were typed unless the SNP was in strong LD by 5% or 10% (online appendix, Table 3). Among African Americans, SNPs 28 through 9, spanning 14.3 kb, showed little recombination (D’ >0.8), whereas SNP16 in the 3′ untranslated region showed much lower pairwise LD (online appendix, Table 4). In contrast to LD by D’, the only usable LD by r2 was between SNPs 9 and 20 and SNPs 3 and 4, where both pairs were separated by <200 bp. Thus, nearly all Caucasian information could be captured by 5 SNPs, whereas 10 SNPs were required to capture most information in African-American subjects. Despite the relatively small number of “tagSNPs” in Caucasians, 22 haplotypes had a frequency >1%, and only 1 haplotype had a frequency >10% (online appendix, Table 5). This diversity likely reflects the low levels of LD by r2 among the five tagSNPs. Predicted haplotype frequencies for the most common haplotypes derived from those SNPs typed in individuals did not differ between case and control subjects for either population (online appendix, Table 5).

To test a role of ADIPOR1 in insulin sensitivity, we selected six common SNPs and one rare SNP to type in individuals of Northern European descent who had undergone evaluation of insulin sensitivity (S_I) using frequently sampled intravenous glucose tolerance tests. Under a general linear model with S_I as the dependent variable, BMI and age as covariates, and controlling for family membership, sex, and glucose tolerance status, no SNP had a significant impact on either S_I or insulin secretion measured as disposition index. In an exploratory study, SNPs 28, 23, 3, and 9 were associated with BMI as the
dependent variable in interaction with pedigree membership \((P = 0.018\) to \(P = 0.003\)) when age, sex, and diagnosis were included in the model.

SNP16 was both common and located in the 3’ untranslated region and thus ideal to examine allele-specific expression. Among 16 heterozygous individuals, the G (major) allele was slightly but significantly overexpressed relative to DNA, independent of ethnicity and diagnosis (ratio 1.35, \(P = 0.001\)). Overall, ADIPOR1 mRNA levels (expressed as the ratio of ADIPOR1 mRNA to 18S RNA) varied widely, from 0.5 to 21, among individuals. Although expression in cell lines from 10 Caucasian control and 10 Caucasian diabetic individuals did not differ, cell lines from 20 African-American individuals with diabetes showed a 45% decrease in ADIPOR1 mRNA levels when compared with cell lines from 10 African-American control subjects without considering the underlying genotype (geometric mean ratio 8.1 vs. 4.5, \(P = 0.028\) by Mann-Whitney U; ln-transformed ratios 2.10 ± 0.52 vs. 1.50 ± 0.82 [means ± SD] in control and diabetic samples, respectively).

**DISCUSSION**

Considerable data support a role for adiponectin in insulin action, glucose homeostasis, and possibly type 2 diabetes (3). Furthermore, many although not all studies (22) have shown an association of variation at the adiponectin gene with type 2 diabetes. Consequently, variation in expression or function of the adiponectin receptors must be considered strong candidates for impaired glucose homeostasis and the insulin resistance that accompanies and often precedes type 2 diabetes. Two related but distinct adiponectin receptors have been identified (8); we chose to study ADIPOR1 because it is most highly expressed in muscle, which is the tissue most responsible for postprandial glucose uptake, and because it appears to primarily bind the globular form of adiponectin, which in some studies (6,23) appeared to be the form most active in increasing fat oxidation and reducing free fatty acid levels.

Among the two ethnic groups studied and using both our own screening and public databases, we were able to confirm 22 sequence variants. Only one SNP altered an amino acid (N44K in exon 2), but was not associated with type 2 diabetes. No other SNP was associated with type 2 diabetes or altered insulin sensitivity. We have >80% power to detect an 8–10% difference in allele frequency over the range of minor frequencies examined in this study among either Caucasian or African-American case-control studies. This difference is equivalent to an odds ratio of ~1.7. Thus, we do not have adequate power to detect an association with odds ratios of 1.2–1.4, as observed in some confirmed diabetes genes, although no trend in this direction was observed. Surprisingly, four of seven SNPs tested showed an interaction with family membership to alter BMI. Although SNPs 23 and 28 are in strong LD (\(r^2 >0.95\)), SNPs 3 and 9 have low levels of LD with each other and with SNPs 23 and 28 and thus are independent observations. A biological explanation for this observation is not obvious, and without the other factors in our model, we could not demonstrate a difference in BMI between genotypes among the 126 subjects studied.

Although we found no association of any variant with type 2 diabetes, we did find two lines of support for altered ADIPOR1 gene expression. We used transformed lymphocytes for these studies, a tissue that has been increasingly used as a surrogate for tissues not easily obtained, and for which levels of gene expression appear to be stable and heritable (24). SNP16 (rs1139646) in the 3’ untranslated region showed a 35% increase in expression of the major allele (C) after correcting for unequal amplification. The 3’ untranslated region may play a role in posttranscriptional regulation of gene expression, and a search for functional elements placed SNP16 in the consensus sequence for the internal ribosome entry site. These sites generally control translation in the 5’ untranslated regions. That the altered allele ratios were seen in both Caucasian and African-American populations, despite much lower LD among African Americans, would support a direct role for SNP16 in the allele-specific expression. However, SNP16 was not associated with detectable physiologic effects, perhaps because of tissue-specific differences, inadequate power to detect subtle changes, or effects on a parameter that we did not measure. We also found that among African-American individuals, ADIPOR1 mRNA was reduced by 45% in cell lines from diabetic subjects compared with control subjects. Within each group, we observed large variations among individual levels of expression. We have observed similarly large differences for other genes (S.C.E. and H.W., unpublished data) and thus believe this is a biological property rather than a technical artifact. Nonetheless, these findings will require confirmation. Furthermore, we cannot determine from this study whether that decrease resulted from a cis-acting element in a regulatory region of the ADIPOR1 gene such as SNP16 or a promoter element well upstream of the gene or from a trans-acting variant in these individuals. However, the decrease is biologically plausible and might suggest a role for this gene or a gene regulating ADIPOR1 in the metabolic syndrome among African-American individuals. Studies of ADIPOR1 gene expression in muscle from African-American diabetic and control subjects might be particularly informative, as would an analysis of the 10 ADIPOR1 tagSNPs in nondiabetic African-American individuals characterized for insulin sensitivity.

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