Brief Report

Evidence That Rho Guanine Nucleotide Exchange Factor 11 (ARHGEF11) on 1q21 is a Type 2 Diabetes Susceptibility Gene in the Old Order Amish

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Rho guanine nucleotide exchange factor 11 (ARHGEF11), located on chromosome 1q21, is involved in G protein signaling and is a pathway known to play a role in both insulin secretion and action. We genotyped 52 single nucleotide polymorphims (SNPs) in ARHGEF11 and compared the genotype frequencies of subjects with type 2 diabetes (n = 145) or type 2 diabetes/impaired glucose tolerance (IGT) (n = 293) with those of control subjects with normal glucose tolerance (NGT) (n = 358). Thirty SNPs, spanning the entire gene, were significantly associated with type 2 diabetes or type 2 diabetes/IGT. The most significantly associated SNP was rs6427340 (intron 2), in which the less common allele was the risk allele (odds ratio [OR] 1.82 [95% CI 1.20–2.70], P = 0.005 for type 2 diabetes vs. NGT and 1.79 [1.27–2.50], P = 0.0008 for type 2 diabetes/IGT vs. NGT). In an expanded set of nondiabetic subjects (n = 754), most of the type 2 diabetes– and IGT-associated SNPs were significantly associated with glucose levels during an oral glucose tolerance test, with the same SNP (rs6427340) showing the most significant associations (P = 0.007). All type 2 diabetes– and IGT-associated SNPs were in high linkage disequilibrium and constitute a single 133-kb haplotype block. These results, coupled with similar findings in Pima Indians, suggest that sequence variation in ARHGEF11 may influence risk of type 2 diabetes. *Diabetes* 56:1363–1368, 2007

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AUC, area under the curve; AFDS, Amish Family Diabetes Study; IGT, impaired glucose tolerance; LD, linkage disequilibrium; NGT, normal glucose tolerance; SNP, single nucleotide polymorphism.

Type 2 diabetes has a substantial genetic component, but the polygenic nature of this disease has complicated the identification of susceptibility genes (1). We previously reported (2) evidence for linkage of type 2 diabetes and impaired glucose tolerance (IGT) to chromosome 1q21-q24 (logarithm of odds 2.35, P = 0.0008) in the Old Order Amish that has also been reported in several other populations (3–8). Genotyping of multiple single nucleotide polymorphisms (SNPs) within the region of linkage as part of our linkage disequilibrium (LD) mapping studies in the Amish pointed to a region containing Rho guanine nucleotide exchange factor 11 (ARHGEF11). ARHGEF11 is 1 of 85 activators of Rho GTPases that play a fundamental role in G protein signaling and therefore many aspects of cellular regulation (9,10), including β-cell apoptosis, insulin secretion (11–13), insulin signaling (14–18), and lipid metabolism (19,20).

We hypothesized that variation in ARHGEF11 may affect insulin secretion and/or action and, as a result, type 2 diabetes susceptibility. We sequenced the 41 exons, intron-exon boundaries, and 1.5 kb of the putative proximal promoter region of ARHGEF11. A total of 39 variants were identified including 2 nonsynonymous (G1456S and R1467H), 2 synonymous (S694S and N1207N), 3 in the 5′ flanking region, 4 in the 5′-untranslated region, and 1 in the 3′-untranslated region; all other variants were in introns and did not predict any obvious alterations in RNA splicing.

Fifty-two SNPs among the 39 SNPs identified from sequencing and among validated SNPs in the SNP database (dbSNP, available at http://www.ncbi.nlm.nih.gov/projects/SNP/) covering the entire 132.8-kb region comprising ARHGEF11 and immediately flanking the gene from 22.4 kb upstream of the adenine thymine guanine start site to 1.0 kb downstream of the polyadenylation signal (average density 1 SNP per 2.7 kb) were genotyped in 145 type 2 diabetic, 148 IGT, and 358 normal glucose tolerant (NGT) individuals. These individuals were also tested for association with type 2 diabetes and type 2 diabetes/IGT (Fig. 1). All SNPs conformed to Hardy-Weinberg expectations. Using an additive model, 30 of the 52 SNPs spanning the entire ARHGEF11 gene were significantly associated with type 2 diabetes or type 2 diabetes/IGT (P = 0.0008–0.05) (Table 1 and Fig. 1). The two SNPs most strongly associated with type 2 diabetes and type 2 diabetes/IGT were rs6427340 in intron 2 (P = 0.005 and

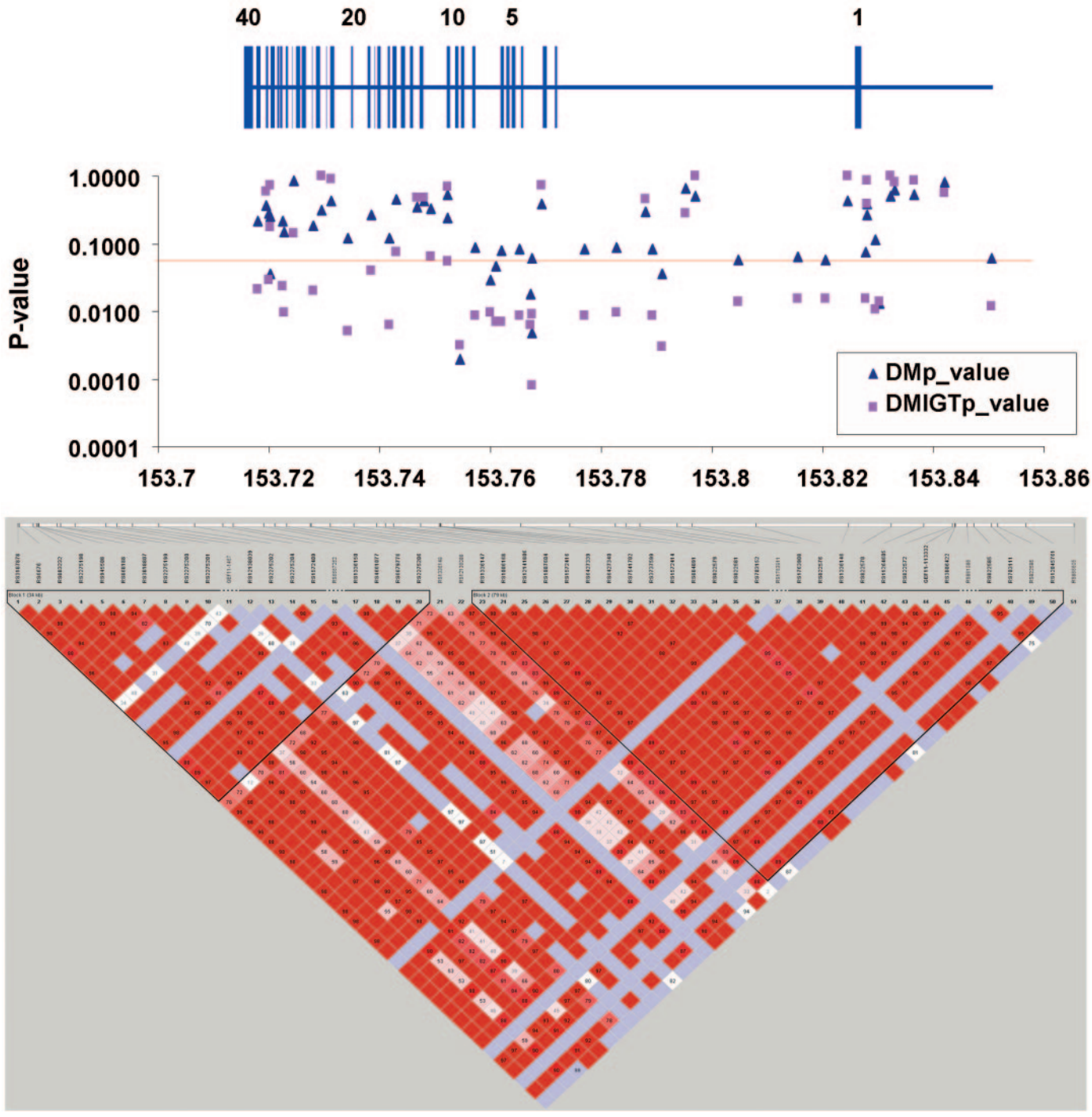

FIG. 1. Association analysis of 52 SNPs in ARHGEF11 with type 2 diabetes cases (DM; $n = 145$) and combined type 2 diabetes/IGT cases (DMIGT; $n = 293$) compared with NGT control subjects ($n = 358$). ARHGEF11 gene structure is shown above the graph, which plots the P values as a function of physical location on the chromosome. Below the graph is an LD plot of the 52 SNPs in ARHGEF11. D′ values are depicted as a color scale with darker shades of orange representing higher values. LD plot was generated using Haploview version 3.2.

0.0008, respectively) and rs12136088 in intron 8 ($P = 0.002$ and 0.003, respectively). The R1467H variant in exon 39 was more weakly associated with type 2 diabetes ($P = 0.04$, OR 0.66 [95% CI 0.44–0.98]), with the more common allele (C allele encoding arginine at codon 1467) being the "risk" allele for type 2 diabetes. The other two coding region variants (D1456S and S694S) did not show significant association with type 2 diabetes or type 2 diabetes/IGT.

The relationship between ARHGEF11 SNPs and quantitative traits was assessed in 754 nondiabetic members of the Amish Family Diabetes Study (AFDS). Eighteen of the 30 type 2 diabetes– and IGT-associated SNPs located from the 5′ flanking region to intron 8 were significantly associated with glucose area under the curve (AUC) during a 3-h oral glucose tolerance test ($P < 0.05$) (Fig. 2). These findings in nondiabetic subjects provide additional support for an effect of ARHGEF11 polymorphism on glucose homeostasis. The R1467H was not associated with glucose AUC among nondiabetic subjects. There was no evidence

TABLE 1
ARHGEF11 SNPs significantly associated with type 2 diabetes and type 2 diabetes/IGT in the Amish

SNP ID	NCBI location	SNP type	Minor allele	Minor allele frequency	Location (SNP type)	T2D vs. NGT		T2D/IGT vs. NGT	
						P	OR (95% CI)	*P*	OR (95% CI)
RS3187878	153717717	A/G	G	0.19	Exon 41 (3′ UTR)	0.09	1.52 (0.94–2.47)	0.01	1.61 (1.09–2.38)
RS6676	153717891	C/T	T	0.20	Exon 41 (3′ UTR)	0.21	1.35 (0.14–12.5)	0.02	1.57 (1.06–2.32)
RS2275198	153719954	A/G	A	0.19	Intron 39	0.28	1.31 (0.79–2.16)	0.03	1.54 (1.03–2.30)
RS945508	153720154	T/C	T	0.47	Exon 39 (R1467H)	0.04	0.66 (0.44–0.98)	0.17	0.81 (0.59–1.10)
RS3818807	153722376	A/T	T	0.20	Intron37	0.22	1.35 (0.17–11.0)	0.02	1.56 (1.06–2.31)
RS2275199	153722768	C/T	T	0.20	Exon 36 (N1207N)	0.15	1.44 (0.87–2.37)	0.01	1.69 (1.15–2.50)
RS2275201	153728111	C/T	T	0.20	Intron 29	0.18	1.39 (0.85–2.27)	0.02	1.60 (1.07–2.38)
RS2275202	153734293	C/T	T	0.05	Intron 22	0.12	0.47 (0.18–1.25)	0.005	0.33 (0.15–0.75)
RS2275204	153738508	A/G	G	0.19	Intron 20	0.26	1.34 (1.03–1.74)	0.04	1.52 (1.02–2.28)
RS1572409	153741849	A/T	T	0.20	Intron 17	0.12	1.34 (0.83–2.17)	0.03	1.54 (1.04–2.30)
RS12136088	153754485	G/T	T	0.23	Intron 8	0.002	1.56 (0.97–2.52)	0.003	1.77 (1.21–2.58)
RS1336147	153757272	A/G	G	0.24	Intron 8	0.09	1.48 (0.94–2.34)	0.009	1.63 (1.13–2.37)
RS1006168	153759971	A/C	C	0.24	Intron 6	0.03	1.67 (1.05–2.66)	0.01	1.63 (1.13–2.37)
RS12141806	153761026	A/T	T	0.23	Intron 6	0.05	1.60 (1.01–2.54)	0.007	1.68 (1.14–2.46)
RS1007604	153761953	G/T	T	0.23	Intron 5	0.15	1.39 (0.89–2.17)	0.02	1.54 (1.08–2.22)
RS1572416	153765127	A/C	A	0.24	Intron 3	0.08	1.49 (0.93–2.38)	0.009	1.64 (1.14–2.38)
RS6427339	153767340	C/T	T	0.42	Intron 2	0.02	1.66 (1.08–2.55)	0.006	1.60 (1.14–2.25)
RS6427340	153767457	C/T	C	0.41	Intron 2	0.005	1.82 (1.20–2.70)	0.0008	1.79 (1.27–2.50)
RS7541702	153767506	C/T	C	0.23	Intron 2	0.06	1.56 (0.98–2.50)	0.009	1.67 (1.12–2.44)
RS1572414	153776947	C/T	C	0.24	Intron 1	0.08	1.49 (0.93–2.38)	0.009	1.64 (1.14–2.38)
RS884891	153782676	C/T	C	0.24	Intron 1	0.09	1.47 (0.93–2.38)	0.009	1.61 (1.12–2.33)
RS822581	153789298	A/G	G	0.24	Intron 1	0.08	1.49 (0.94–2.34)	0.009	1.63 (1.13–2.37)
RS703152	153790978	A/G	A	0.24	Intron 1	0.09	1.48 (0.82–2.67)	0.01	1.61 (1.12–2.32)
RS822576	153804644	C/T	C	0.47	Intron 1	0.06	1.45 (0.01–100)	0.01	1.47 (1.08–2.00)
RS1336146	153815516	C/T	T	0.47	Intron 1	0.06	1.43 (0.97–2.10)	0.02	1.46 (1.07–1.99)
RS822570	153820575	A/G	G	0.47	Intron 1	0.06	1.44 (0.02–127)	0.02	1.46 (1.07–1.99)
RS822572	153827775	A/G	G	0.47	Exon 1 (5′ UTR)	0.08	1.40 (0.96–2.06)	0.02	1.45 (1.07–1.97)
RS861086	153829497	A/G	G	0.007	5′ Flank	0.113	12.5 (11.1–16.7)	0.01	12.5 (10.0–14.3)
RS822585	153830278	A/T	A	0.41	5′ Flank	0.01	1.69 (0.36–8.33)	0.01	1.52 (1.09–2.08)
RS1750810	153850563	C/G	C	0.47	5′ Flank	0.07	1.43 (0.005–500)	0.01	1.47 (1.09–2.00)

NCBI location is based on Build 35.1. OR > 1.00 denotes that the minor allele is the risk allele, while OR < 1.00 denotes that the major allele is the risk allele. T2D, type 2 diabetes; UTR, untranslated region.

for association of any SNPs with BMI, insulin level, insulin secretion index, or homeostasis model assessment of insulin resistance.

The SNPs within the ARHGEF11 region were in high linkage disequilibrium (LD) (Fig. 1). The nine SNPs that were most significantly associated with type 2 diabetes or type 2 diabetes/IGT defined five haplotypes (three with frequencies >0.05), accounting for >99% of chromosomes in the sample (Table 2). The TTGTTACCG haplotype was associated with a significantly higher risk of type 2 diabetes/IGT ($P = 0.010$), while the AGAAGCTTA haplotype was associated with a significantly lower risk of type 2 diabetes/IGT ($P = 0.0002$) (global P value for test = 0.006). These same haplotypes were also significantly associated with glucose AUC in the expanded set of nondiabetic subjects (global P value for test = 0.008) (Table 2).

To explore whether SNPs in ARHGEF11 were in LD with a potentially functional polymorphism in a neighboring gene, 96 additional SNPs flanking ARHGEF11 in the 153.4- to 154.0-Mb region (NCBI build 35.1) were also genotyped (supplementary Table 1 [found in an online appendix at http://dx.doi.org/10.2337/db06-1421]). The region is bound by ETV3 and HAPLN2 5′ and 3′ to ARHGEF11, respectively. Among these SNPs that flanked the ARHGEF11 gene, 12 SNPs 3′ of ARHGEF11 were also significantly associated with type 2 diabetes and/or type 2 diabetes/IGT. Seven of these SNPs were positioned within a predicted gene (FLJ328884) immediately 3′ to ARHGEF11, while the remaining five SNPs (of which four had an allele frequency <0.02) were each positioned within different genes (BCAN, CRABP2, INSRR, NTRK1, and FLJ00193). None of these SNPs were more strongly associated with type 2 diabetes or type 2 diabetes/IGT than rs6427340 or rs12136088, and all were in strong LD with the SNPs in ARHGEF11. No SNP centromeric to AGHGEF11 within the 153.4- to 154.0-Mb region was associated with type 2 diabetes or type 2 diabetes/IGT.

By quantitative real-time PCR, we determined that ARHGEF11 is ubiquitously expressed in various tissues, including that of the liver, muscle, and pancreas (online appendix supplementary Fig. 1).

Our region of linkage on chromosome 1q21-q24 spans ~30 Mb and contains at least 450 genes, including many excellent biological candidates. Our initial hypothesis-free LD mapping with >500 SNPs across the region led us to ARHGEF11, an activator of Rho GTPases. Although ARHGEF11 itself is not known to play a role in glucose homeostasis, previous studies implicate Rho subfamily G proteins (e.g., Cdc42 and Rac) in physiological insulin secretion (11–13). Furthermore, several recent studies implicated Rho GTPases in insulin signaling through activation of the Jun NH_2-terminal kinase and p38 mitogen-activated protein kinase pathways (10,14,15). The dynamic actin rearrangement required for insulin-stimulated translocation of GLUT4 is regulated by at least two distinct signals, one leading to the activation of phosphatidylino-

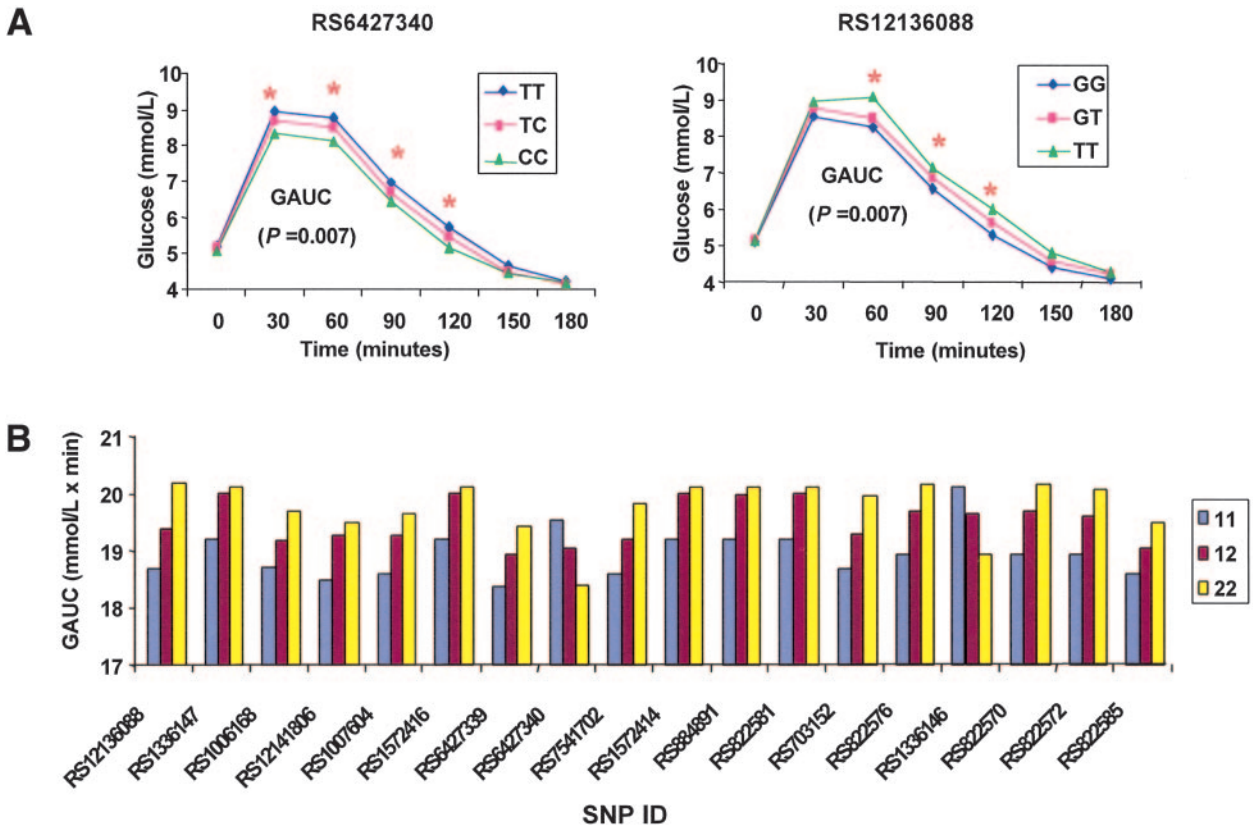

FIG. 2. ***A*: Examples of two ARHGEF11 SNPs associated with glucose levels during a 3-h oral glucose tolerance test in nondiabetic subjects. *B*: Eighteen type 2 diabetes–associated SNPs were significantly associated with oral glucose tolerance test glucose AUC (GAUC) and post-challenge glucose levels in nondiabetic subjects. *$P < 0.05$ (1, common allele; 2, minor allele).**

sitol 3-kinase (16) and the other to the activation of Rho family small GTP-binding protein TC10 (17). Finally, a number of enzymes involved in lipid metabolism are influenced by GTPases including phosphatidylinositol 4-phosphate 5-kinase, diacylglycerol kinase, and phospholipase D (19,20). ARHGEF11 is ubiquitously expressed in various tissues, including liver, muscle, and adipose tissue. Together, these data suggest that proteins involved in G protein signaling such as ARHGEF11 may play an important role in glucose homeostasis.

Our current study provides evidence that variation within ARHGEF11 influences risk of type 2 diabetes/IGT in the Amish. Given the strong LD across ARHGEF11, it is difficult to know which SNPs are the causal genetic variants. In this study, rs6427340 in intron 2 and rs12136088 in intron 8 were most significantly associated with type 2 diabetes and glucose traits; however, the functional consequences of these variants are unclear. Because we sequenced all exons, it is unlikely that we missed common coding variants, although it is possible that other potentially functional variants, perhaps in more distal regulatory regions or within regions of introns not sequenced, may have been missed. R1467H was the only variant encoding an alteration in amino acid sequence that was significantly associated with type 2 diabetes. However, this variant was not the most significantly associated variant and was not associated with any of the diabetes-related quantitative traits. Interesting, the R1467H substitution was significantly associated with type 2 diabetes and insulin resistance among the Pima Indians who also have linkage to type 2 diabetes on chromosome 1q21-q25 (22). However, contrary to our findings in the Amish, in

TABLE 2
Association analysis of *ARHGEF11* haplotypes with type 2 diabetes and type 2 diabetes/IGT and glucose AUC during an oral glucose tolerance test

	Haplotype frequency			T2D vs. NGT		T2D/IGT vs. NGT		Glucose AUC	
Haplotype	T2D	IGT	NGT	Haploscore	*P*	Haploscore	*P*	Haploscore	*P*
AGAAGCTTA	0.71	0.67	0.79	−2.35	**0.019**	−3.71	**0.0002**	−3.20	**0.001**
TGAAGCTTA	0.008	0.011	0.003	0.84	0.40	1.36	0.18	0.54	0.59
ATAAGCTTA	0.004	0.026	0.003	0.15	0.88	1.74	0.082	1.69	0.091
ATGTTACCG	0.050	0.044	0.028	1.73	0.083	1.83	0.068	1.62	0.11
TTGTTACCG	0.22	0.25	0.17	1.55	0.12	2.56	**0.010**	2.52	**0.012**

Haplotype frequencies were estimated with Haploscore for the nine positively associated *ARHGEF11* SNPs by case-control analysis and transmissional disequilibrium test (RS1572409, RS12136088, RS1336147, RS12141806, RS1007604, RS1572416, RS1572414, RS884891, and RS822581). Global *P* value for test = 0.28, 0.006, and 0.008 for type 2 diabetes vs. NGT, type 2 diabetes/IGT vs. NGT, and glucose AUC, respectively. Bold face data denote statistical significance. T2D, type 2 diabetes.

which the 1467R allele was the risk allele for type 2 diabetes, the 1467H allele was the risk allele for insulin resistance and type 2 diabetes in Pima Indians. Associations with opposite risk alleles in the two populations may suggest that R1467H is not the functional variant but rather is in LD with a true functional variant. Less likely, differences in genetic background or environmental exposures between these populations could modulate the phenotypic expression of the R1467H variant to increase or decrease type 2 diabetes risk in subjects carrying the same allele. Finally, it is also possible that one or both associations are false-positives due to multiple comparisons.

Type 2 diabetes–associated SNPs in ARHGEF11 did not provide evidence for linkage to type 2 diabetes/IGT using the same family structures as in our original linkage analysis (2) (data not shown). These findings are consistent with the prevailing idea that there is more than one type 2 diabetes susceptibility gene on chromosome 1q21-q24 (21).

Additional genotyping of SNPs in neighboring genes and LD analysis revealed that the type 2 diabetes/IGT–associated SNPs were in strong LD and constitute a large haplotype block that include one gene and two predicted genes. Indeed, several SNPs in the predicted gene FLJ32884 immediately 3′ to ARHGEF11 were associated with type 2 diabetes and/or type 2 diabetes/IGT. Thus we cannot rule out the possibility that the functional variant or haplotype is in this gene or even possibly other nearby genes.

In summary, we identified sequence variation in ARHGEF11 that may influence risk of type 2 diabetes. Further investigation of the role of ARHGEF11 in insulin secretion and signaling and functional studies of the type 2 diabetes–associated sequence variants will be necessary to more fully understand the mechanisms underlying these associations.

RESEARCH DESIGN AND METHODS

The AFDS was founded in 1995 to identify susceptibility genes for type 2 diabetes and related traits. Details of the AFDS design, recruitment, phenotyping, and pedigree structure have been previously described (23). Our initial case-control analysis included 145 subjects with type 2 diabetes, 148 subjects with IGT, and 358 subjects with NGT. NGT subjects included in this analysis were required to be at least 38 years of age at the time of examination to increase the probability of their capacity for diabetes resistance. For analysis of diabetes-related quantitative traits, an expanded set on 754 nondiabetic subjects (with NGT and IGT) was used. Glucose and insulin AUC during the oral glucose tolerance test were calculated using the trapezoidal rule. Homeostasis model assessment of insulin resistance was calculated as fasting insulin (μU/ml) × fasting glucose (mmol/l)/22.5, and insulin secretion index was calculated as ln[(insulin at 30 min − insulin at 0 min)/(glucose at 30 min − glucose at 0 min)]. Informed consent was obtained from all AFDS participants, and the study protocol was approved by the institutional review board at the University of Maryland School of Medicine.

Sequence analysis and genotyping. Direct sequencing was used to screen the 41 exons, exon-intron boundaries, and 1.5 kb of the proximal 5′ regulatory region of ARHGEF11 for genetic variation in 24 AFDS participants (18 type 2 diabetic subjects from non–first-degree relatives of families providing evidence for linkage of type 2 diabetes to chromosome 1q21-q24 and 6 NGT subjects). This sequencing set (48 chromosomes) provides 91% power to detect at least one copy of the minor alleles for SNPs with allele frequencies ≥0.05. Both strands were sequenced on an ABI 3730xl DNA sequencer (Applied Biosystems) and analyzed using Sequencher software (GeneCodes). From SNPs identified by sequencing and from dbSNP, 52 SNPs in ARHGEF11 were genotyped in the full sample. Another 22 SNPs upstream and 74 SNPs downstream of ARHGEF11 spanning from 153.4 to 154.0 Mb were also genotyped (supplementary Table 1). Genotyping was performed using the SNPstream Ultra High Throughput genotyping platform (Beckman Coulter), SNPlex (Applied Biosystems), TaqMan Allelic Discrimination Assay (Applied Biosystems), Pyrosequencing PSQ HS 96A, or Illumina Golden Gate assay (Illumina). Details regarding assays for specific SNPs are available from the authors upon request. The error rate, based on blind replicates for the SNPs examined, was 0–3%.

Real time PCR. RNA was obtained from 12 normal human tissues (Ambion). Real-time RT-PCR was performed using TaqMan primers and probe (assay ID: Hs00207600_m1; Applied Biosystems) on an ABI 7700 Sequence Detection System using standard conditions and cycle times/temperatures. The Taqman endogenous control was human cyclophilin A (PPIA), and data are shown as relative expression units.

Statistical analysis. Because of the extensive relatedness of study subjects in our Amish pedigrees, we tested for associations of SNP genotypes while accounting for the pedigree structure. We evaluated the association between SNP genotype and disease status (type 2 diabetes vs. NGT and type 2 diabetes/IGT vs. NGT) under the additive genetic model using a variance component approach, in which we modeled the probability that the subject was a case or control as a function of the individual's age, sex, and genotype, conditional on the correlations in phenotype among relative pairs. Statistical testing was accomplished using the likelihood ratio test. The OR was computed by comparing the odds of disease between subjects carrying one copy of the minor allele and subjects not carrying any copies of the minor allele. The association analyses were carried out using SOLAR as previously described (21).

In an expanded set of 754 nondiabetic subjects (including 606 NGT and 148 IGT subjects), we also evaluated the effect of genotype on levels of quantitative traits (e.g., BMI, glucose level, and insulin level) by comparing mean trait levels across genotypes as previously described (21). We compared the likelihood of a model in which the trait values were allowed to vary by genotype (unconstrained model) to that in which the genotype effects were constrained to be zero using the likelihoods ratio test. Within each model, we simultaneously estimated the effects of age, sex, and family relationship.

Pairwise LD and haplotype structure were determined using Haploview (24). Haploscore was used to estimate and compare haplotype frequencies between case and control groups (25).

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