

Polymorphisms in Both Promoters of Hepatocyte Nuclear Factor 4- α Are Associated With Type 2 Diabetes in the Amish

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Hepatocyte nuclear factor 4- α (*HNF4A*) is a transcription factor located on chromosome 20q13 that regulates expression of genes involved in glucose metabolism and homeostasis. Recently, two groups independently identified single nucleotide polymorphism (SNPs) in an alternate upstream promoter (P2) of *HNF4A* that were associated with type 2 diabetes in Ashkenazi Jews and Finns. We genotyped haplotype-tagging SNPs (htSNPs) across the two promoter regions and the coding region of *HNF4A* in individuals with type 2 diabetes ($n = 137$), impaired glucose tolerance (IGT) ($n = 139$), and normal glucose tolerance ($n = 342$) from the Amish Family Diabetes Study (AFDS) to test for association with type 2 diabetes. In the P1 promoter region, we observed a significant association between the A allele of rs2425640 and type 2 diabetes (odds ratio [OR] 1.60, $P = 0.03$). Furthermore, the mean age of type 2 diabetes onset was, on average, 5.1 years earlier in those with the AA or GA genotype at SNP rs2425640 than in those with the GG genotype (57.8 vs. 62.9 years, $P = 0.011$). In the P2 promoter, the htSNP rs1884614 showed borderline association with both type 2 diabetes (OR 1.40, $P = 0.09$) and the combined type 2 diabetes/IGT trait (1.35, $P = 0.07$). In an expanded set of 698 nondiabetic AFDS subjects, we found association between rs1884614 and glucose area under the curve during an oral glucose tolerance test (additive model, $P = 0.022$; dominant model, $P = 0.010$). The results of this study provide evidence that variants in both the P1 and P2 promoters of *HNF4A* increase risk for typical type 2 diabetes. *Diabetes* 53:3337–3341, 2004

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AFDS, Amish Family Diabetes Study; HNF, hepatocyte nuclear factor; htSNP, haplotype-tagging single nucleotide polymorphism; IGT, impaired glucose tolerance; LD, linkage disequilibrium; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; SNP, single nucleotide polymorphism.

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Hepatocyte nuclear factor 4- α (*HNF4A*) is a transcription factor that is expressed in several tissues, including liver and pancreas, where it regulates expression of genes involved in gluconeogenesis and glucose-stimulated insulin secretion, respectively (1–4). Relatively rare mutations in *HNF4A* have been identified that cause maturity-onset diabetes of the young type 1 (rev. in 5), a dominantly inherited, early-onset form of type 2 diabetes characterized by impaired glucose-induced insulin secretion due to pancreatic β -cell dysfunction (6–9). *HNF4A* expression patterns are complex as a result of alternative splicing and transcription from two different promoters, the proximal P1 promoter and the P2 promoter, which lies ~ 45 kb upstream of the P1 promoter (10–13).

The 12 coding exons of *HNF4A* span ~ 29 kb on chromosome 20q13, a region of overlapping linkage to type 2 diabetes in several Caucasian (14–19) and Asian (20,21) populations. Recently, through fine-mapping efforts in this region of chromosome 20q, two groups concurrently identified single nucleotide polymorphisms (SNPs) in the P2 and P1 promoter regions and coding exons of *HNF4A* that are associated with type 2 diabetes in the Ashkenazi Jews (22) and Finns (FUSION 1 [Finland-United States Investigation of NIDDM Genetics 1]) (23). Silander et al. (23) identified 10 SNPs across a 64-kb region spanning the P2 and P1 promoter regions and exons 1–3 of *HNF4A* that were associated with type 2 diabetes in the FUSION 1 population. In the Ashkenazi Jewish cohort, the SNPs closer to the P1 promoter and coding exons were not associated with type 2 diabetes (22); however, four SNPs spanning a ~ 10 -kb region encompassing the P2 promoter were associated with type 2 diabetes (rs4810424, rs1884613, rs1884614, and rs2144908). These four SNPs are located in a 177-kb region of strong linkage disequilibrium (LD), including >140 kb upstream of the P2 promoter (23). A second haplotype block was observed within the *HNF4A* coding region, while LD tended to decay across the ~ 45 -kb gap separating *HNF4A* from P2 (22,23). In addition to the observed association with type 2 diabetes, these P2 SNPs appeared to explain a significant portion of the linkage to chromosome 20q12-q13 observed in both the Ashkenazi Jews and Finns. The replicating evidence pre-

TABLE 1

Allele frequencies and results of association analysis in subjects with type 2 diabetes, IGT, and NGT for SNPs in the *HNF4A* region

SNP location (kb)†	SNP name	Major/minor allele	Minor allele frequency			Diabetes vs. NGT*		Diabetes + IGT vs. NGT*	
			Type 2 diabetes (n = 137)	IGT (n = 139)	NGT (n = 342)	OR	P	OR	P
-3.926	rs1884614	G/A	0.208	0.180	0.139	1.40	0.09	1.35	0.07
39.604	rs2425637	G/T	0.380	0.368	0.372	1.00	0.99	1.04	0.82
43.592	rs2425640	G/A	0.414	0.363	0.363	1.60	0.03	1.26	0.16
50.693	rs3212183	T/C	0.377	0.374	0.368	0.97	0.88	0.98	0.89
66.316	rs1028583	G/T	0.411	0.358	0.382	0.83	0.35	0.94	0.67
73.035	rs3818247	G/T	0.255	0.281	0.265	0.93	0.82	0.87	0.53

*P values are based on genotype frequencies, and ORs reflect the odds of disease associated with having two copies of the minor allele versus the odds of disease associated with having two copies of the major allele and were adjusted for age, sex, and pedigree structure. Reported P values are not adjusted for multiple comparisons. P values <0.05 are shown in bold.

sented by these studies suggests that SNPs near the P2 promoter of *HNF4A* increase susceptibility to type 2 diabetes.

Although no evidence for linkage to type 2 diabetes was detected on chromosome 20q12-q13 in our genome-wide scan (average marker density = 9.7 cM) in the Old Order Amish (logarithm of odds = 0.00 between markers D20S107 and D20S119, which are ~6 cM apart) (24), we tested whether SNPs in *HNF4A* and its promoters were associated with type 2 diabetes in the Amish. We selected six haplotype-tagging SNPs (htSNPs) spanning the P2 and P1 promoters and the *HNF4A* coding region from the LD blocks defined in the Ashkenazi Jews (22) and Finns (23). Given that the Amish are a young founder population, we hypothesized that haplotype blocks would be as large as or larger than those in the other populations, thus allowing us to capture most or all of the variation across the gene with these SNPs. These SNPs were genotyped in 618 individuals enrolled in the Amish Family Diabetes Study (AFDS), which included 137 subjects with type 2 diabetes, 139 individuals with impaired glucose tolerance (IGT), and 342 control subjects with normal glucose tolerance (NGT). The NGT control subjects selected were ≥38 years of age in order to increase the probability of their capacity for diabetes resistance. Table 1 summarizes the allele frequencies in individuals with type 2 diabetes, IGT, and NGT and the results of genotypic association analysis for each SNP. All SNPs conformed to Hardy-Weinberg expectations. For rs2425640, one of the SNPs located in the P1 promoter region, the frequency of the A allele was significantly higher in the type 2 diabetic group than in the control group in the Amish (genotypic odds ratio [OR] 1.60, P = 0.030). Furthermore, the mean age of diabetes onset was

58.0 years in subjects with the AA genotype for SNP rs2425640, 57.8 years in those with the GA genotype, and 62.9 years in those with the GG genotype. The mean age of type 2 diabetes onset was, on average, 5.1 years earlier in those with the AA or GA genotype at SNP rs2425640 than in those with the GG genotype (57.8 vs. 62.9 years, P = 0.011).

We genotyped one (rs1884614) of the four P2 promoter SNPs reported to be associated with type 2 diabetes and in near-perfect LD with each other in both the Ashkenazi Jewish and Finnish populations. In the Amish, the frequency of the A allele at the rs1884614 SNP was lower in control subjects with NGT than in both the type 2 diabetic group (genotypic OR 1.40, P = 0.09) and the combined type 2 diabetic/IGT group (genotypic OR 1.35, P = 0.07), although these differences did not achieve statistical significance, as observed in the Ashkenazi Jews and Finns. None of the other SNPs in the P1 promoter region or the coding region were associated with type 2 diabetes in the Amish, including the other SNPs observed to be associated with type 2 diabetes in the Finns (rs2425637 and rs3212183) and Ashkenazi Jews (rs3818247). Haplotype analysis revealed that only those haplotypes containing the rs2425640 A allele and rs1884614 A allele were associated with increased type 2 diabetes prevalence (results not shown).

Table 2 shows the pairwise LD ($|D' |$ and r^2) among the genotyped SNPs in the Amish. The haplotype block structure in the Amish appears very similar to that reported in Finns and Ashkenazi Jews, suggesting that the SNP density we chose is adequate for the detection of most of the common variation in *HNF4A*. As shown in the Ashkenazi Jews and the Finns, the SNP representing the P2 haplotype block that was genotyped in the Amish (rs1884614) was

TABLE 2

Pairwise LD among *HNF4A* SNPs in the Amish*

	rs1884614	rs2425637	rs2425640	rs3212183	rs1028583	rs3818247
rs1884614	—	0.40	0.12	0.13	0.38	0.52
rs2425637	0.05	—	0.81	0.83	0.06	0.11
rs2425640	0	0.28	—	0.65	0.33	0.35
rs3212183	0.01	0.65	0.17	—	0.12	0.21
rs1028583	0.04	0	0.09	0.01	—	0.63
rs3818247	0.13	0.01	0.07	0.01	0.27	—

*Values in the upper right represent $|D' |$, while values in the bottom left represent r^2 . Shown in bold are the two SNPs, rs1884614 and rs2425640, in the P2 and P1 promoters, respectively, that were associated with type 2 diabetes and glucose traits.

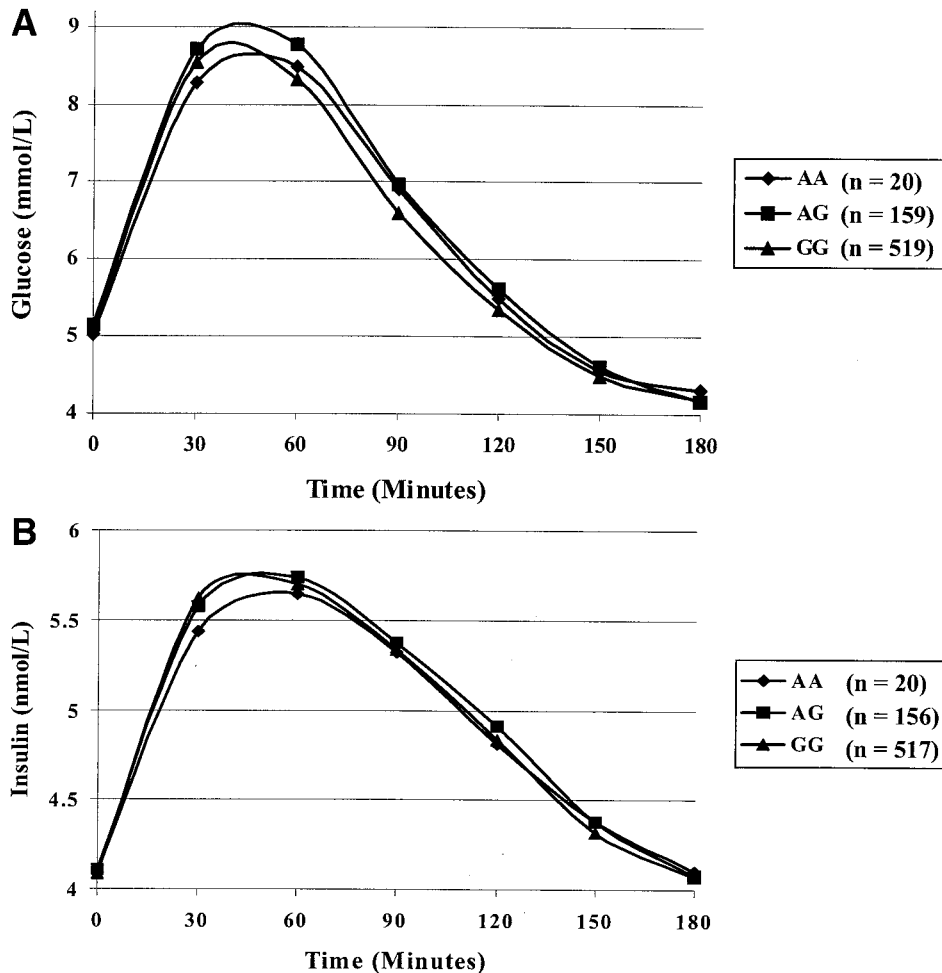


FIG. 1. Mean plasma glucose (A) and insulin (B) levels at 30-min intervals during a 3-h 75-g OGTT according to SNP rs1884614 genotype groups. Carriers of the A “risk” allele exhibited higher total glucose area under the curve during the OGTT (additive model, $P = 0.022$; dominant model, $P = 0.010$). There was no association with total insulin area under the curve during the OGTT (additive model, $P = 0.919$; dominant model, $P = 0.919$).

clearly not in LD with rs2425640 in the P1 promoter or with the other *HNF4A* SNPs.

In addition to the case/control analysis, we genotyped rs1884614 and rs2425640 in an additional 217 nondiabetic Amish subjects to create an expanded set of 698 nondiabetic individuals (NGT [$n = 568$] and IGT [$n = 130$]) and performed an association analysis with diabetes-related quantitative traits. Figure 1 shows the mean plasma glucose levels at 30-min intervals during a 3-h oral glucose tolerance test (OGTT) according to genotype at the rs1884614 SNP. Carriers of the A “risk” allele for the rs1884614 SNP exhibited significantly higher total glucose area under the curve during the OGTT (additive model, $P = 0.022$; dominant model, $P = 0.010$). Higher glucose levels in nondiabetic A carriers provides additional evidence that the A allele of rs1884614 (or a haplotype marked by this allele) influences glucose homeostasis and type 2 diabetes risk. However, presence of the A allele was not associated with either fasting insulin or total insulin area under the OGTT curve (Fig. 1). Although we do not have direct measures of insulin secretion, the finding of increased OGTT glucose levels without differences in OGTT insulin levels in carriers of the A “risk” allele suggests a relative deficiency in insulin secretion in these

individuals. This interpretation is consistent with the hypothesis that the A allele, present in the islet-specific P2 promoter, may decrease expression of *HNF4A* in insulin-secreting β -cells, thus affecting β -cell function. The quantitative trait analysis for rs2425640 in the P1 promoter showed no association with glucose- or insulin-related traits.

In conclusion, we found that htSNPs in the P1 and P2 regions of *HNF4A* are associated with type 2 diabetes and diabetes-related traits in the Amish. Rs2425640 in the P1 region was also associated with type 2 diabetes in the Finns; however, contrary to our findings in the Amish, in which the A allele was the at-risk allele for both type 2 diabetes risk and an earlier onset of diabetes, the frequency of the G allele was significantly higher in Finnish subjects with type 2 diabetes. This discrepancy between the two populations may indicate that this SNP is not the functional SNP but is marking an at-risk haplotype that differs between the Amish and Finns. Of note, this SNP was not associated with type 2 diabetes in the Ashkenazi Jews, but others in the region were associated with type 2 diabetes, suggesting again that the SNPs thus far examined may be marking at-risk haplotypes in the different populations. Alternatively these discrepancies between popula-

tions could represent false-positive or false-negative results. Rs1884614, an htSNP in the P2 region of *HNF4A* was associated with glucose levels during an OGTT in the Amish and was also associated with type 2 diabetes in both the Ashkenazi Jews and the Finns. In all populations studied to date, the P1 and P2 SNPs reside in different haplotype blocks, suggesting the presence of two independent variants influencing type 2 diabetes risk. This replication across several studies lends further support to the possibility that variation in the P1 and P2 regions of *HNF4A*, or SNPs in strong LD with these regions, contributes to the pathogenesis of type 2 diabetes. Of note, our genome scan did not provide any evidence for linkage to type 2 diabetes or related traits to this region of chromosome 20 in the Amish (24). This observation is likely due to the relative insensitivity of linkage analysis compared with association analysis and suggests that this allele may also influence type 2 diabetes risk more broadly in other populations. Although *HNF4A* is the strongest candidate gene for type 2 diabetes in this region, the P2 SNPs reside in a large haplotype block that contains several other known and predicted genes and expressed sequence tags; therefore, the possibility must be considered that the pathogenic SNP(s) may reside in another gene. Further studies in other populations, as well as functional analysis, will be required to further define the role of variation in *HNF4A* in type 2 diabetes pathogenesis.

RESEARCH DESIGN AND METHODS

The AFDS was initiated in 1995 with the goal of identifying susceptibility genes for type 2 diabetes and related traits in a cohort of individuals from the Old Order Amish population in Lancaster County, Pennsylvania. Details of the AFDS design, recruitment, phenotyping, and pedigree structure have been described previously (25). Briefly, probands with previously diagnosed type 2 diabetes (onset between 35 and 65 years of age) and all first- and second-degree relatives of probands and spouses over the age of 18 were recruited. Phenotypic characterization of study participants included medical and family history, anthropometry, and a 3-h 75-g OGTT with insulin levels. The diagnosis of type 2 diabetes was defined on the basis of the OGTT using criteria of the American Diabetes Association (2-h glucose >11.1 mmol/l or fasting glucose >7 mmol/l), by current treatment with diabetes medications, or by a previous physician-documented diagnosis of diabetes. IGT was defined by a 2-h OGTT glucose between 7.8 and 11.1 mmol/l. NGT was defined by a fasting glucose <6.1 mmol/l and a 2-h OGTT glucose <7.8 mmol/l. The total glucose and insulin areas under the curve during the 3-h OGTT were calculated using the trapezoid method. BMI was calculated as weight (in kilograms) divided by height (in meters) squared. The mean age of diagnosis of diabetes in the AFDS cohort was 57.8 ± 5.7 years, and the mean BMI was 27.2 ± 5.0 kg/m² (range 22.4–38.3). Informed consent was obtained from all study subjects, and the Institutional Review Board at the University of Maryland School of Medicine approved the study protocol.

Genotyping. Genotyping was completed using the Orchid/Beckman SNPstream Ultra High Throughput genotyping platform. This genotyping method is described in detail elsewhere (26). Briefly, the protocol involved PCR amplification of target sequences surrounding the SNPs to be assayed in panels of 12-plex reactions. Following enzymatic purification, the PCR products were subjected to single-base primer extension with fluorescent-labeled dye terminators. Each extension primer contained a unique 20-nucleotide tail at its 5' end whose sequence was designed to hybridize to its complementary probe immobilized in a mini-array within each well of a 384-well SNP-IT plate (Beckman Coulter, Fullerton, CA). The microarray plate was imaged by the SNPscope reader (Beckman Coulter). The two-color system allowed the detection of the SNP by comparing signals from the two fluorescent dyes. The image signals were then transferred to genotyping software that translated the images of the arrays into genotype calls. The error rate based upon blind replicates for the SNPs examined in the present study was 0–1.2%.

Statistical analysis. Before analysis, genotypes were checked for Mendelian consistency using the pedigree information and inconsistencies ($<.5\%$ of genotypes) were resolved or removed before analysis. Allele frequencies were calculated for each SNP by gene counting, and observed genotypes were

tested for fit to the expectations of Hardy-Weinberg using the χ^2 test. Pairwise LD was computed between the SNPs using the two most commonly used statistics $|D'|$ and r^2 , and haplotypes were inferred for each individual using an expectation maximization algorithm implemented in the ZAPLO software program (27).

We evaluated the association between SNP genotype and disease status (type 2 diabetes versus NGT and type 2 diabetes/IGT versus NGT) using a variance component approach, in which we modeled the probability that the subject was a case or control subject, as a function of the individual's age, sex, and genotype, conditional on the correlations in phenotype among relative pairs. For the primary analysis, we considered an additive genetic model in which the genotype was coded as 0, 1, or 2, depending on whether the subject was homozygous for the minor allele (genotype = 2), heterozygous (genotype = 1), or homozygous for the major allele (genotype = 0). Statistical testing was accomplished using the likelihood ratio test, in which we compared the likelihood of the data under a model in which the genotype effect was estimated against the likelihood of a nested model in which the genotype effect was constrained to be zero. Secondary analyses were carried out under the dominant and recessive genetic models by imposing appropriate constraints on the genotypic effects. Parameter estimates (i.e., β coefficients) were obtained by maximum likelihood and ORs by taking the inverse log of the β coefficient. The OR for the additive model was scaled to reflect the odds that a case was homozygous for the minor allele versus the odds that the case was homozygous for the major allele. The variance components analysis was carried out using the SOLAR software program (28).

Finally, mean levels of glucose (fasting and glucose area under the curve during a 3-h OGTT) and insulin (fasting and insulin area under the curve during a 3-h OGTT) were estimated according to *HNF4A* genotypes in an expanded set of nondiabetic AFDS subjects ($n = 698$). To account for the relatedness among family members, the measured genotype approach was used (29), in which we estimated the likelihood of specific genetic models given the pedigree structure. Parameter estimates were obtained by maximum likelihood methods, and the significance of association was tested by likelihood ratio tests. Within each model, we simultaneously estimated the effects of age and sex. Insulin values were transformed by their natural logarithms (\ln) to reduce skewness. Quantitative trait analyses were conducted using the SOLAR program (28).

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