Brief Genetics Report

Common Variants of the Hepatocyte Nuclear Factor-4α P2 Promoter Are Associated With Type 2 Diabetes in the U.K. Population

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Hepatocyte nuclear factor (HNF)-4α is part of a transcription factor network that is key for the development and function of the β-cell. Rare mutations in the *HNF4α* gene cause maturity-onset diabetes of the young. A number of type 2 diabetes linkage studies have found evidence of linkage to 20q12–13.1 where the *HNF4α* gene is located. Two recent studies have found an association between four common variants of the alternative P2 promoter region and type 2 diabetes. These variants are in strong linkage disequilibrium, and the minor alleles define one common risk haplotype. In both studies, the risk haplotype explained a large proportion of the evidence of linkage to 20q12–13.1. We aimed to assess this haplotype in a U.K. Caucasian study of 5,256 subjects. We typed two single nucleotide polymorphisms tagging the risk haplotype (rs4810424 and rs2144908) and found evidence of association in both case-control and family-based studies; rs4810424 marginally demonstrated the stronger association with an overall estimated odds ratio of 1.15 (95% CI 1.02–1.33) (P = 0.02). The effect of the P2 haplotype on type 2 diabetes risk is less than in the initial studies, probably reflecting that these studies used 20q12–13.1–linked cases. In conclusion, we have replicated the association of the HNF4α P2 promoter haplotype with type 2 diabetes in a U.K. Caucasian population where there is no evidence of linkage to 20q. *Diabetes* 53:3002–3006, 2004

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Received for publication 21 May 2004 and accepted in revised form 10 August 2004.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org.

FUSION, Finland-United States Investigation of NIDDM Genetics; HNF, hepatocyte nuclear factor; HWE, Hardy-Weinberg equilibrium; SNP, single nucleotide polymorphism; TDT, transmission disequilibrium test.

Hepatocyte nuclear factor (HNF)-4α is an excellent type 2 diabetes candidate gene. It is part of a transcription factor network that is key for the development, differentiation, and function of the pancreatic β-cell (1–4). Rare, severe mutations in the *HNF4α* gene cause maturity-onset diabetes of the young, a young-onset monogenic subtype of diabetes (5). A number of type 2 diabetes linkage studies have found evidence of linkage to 20q12–13.1, where the HNF4α gene is located (6–12). Several studies have shown the importance of a second promoter in the *HNF4α* gene (3,4,13). This promoter, P2, occurs 45 kb upstream of the liver-specific promoter and is the primary transcription start site in the β-cell. Two recent studies have found an association between four common variants of the P2 promoter region and type 2 diabetes (14,15). The four variants are in strong linkage disequilibrium (all pairwise r^2 >0.95) and form just one common risk haplotype. Of these single nucleotide polymorphisms (SNPs), rs2144908 marginally demonstrated the strongest association with type 2 diabetes with an odds ratio (OR) of 1.33 (95% CI 1.06–1.65) in the Finnish study and 1.46 (1.12–1.91) in the Ashkenazi Jewish study. In both studies, the risk haplotype explained a large proportion of the evidence of linkage to 20q12–13.1.

The effect of the HNF4α P2 haplotype on type 2 diabetes risk in the U.K. Caucasian population is not known. The associations described in the Finland-United States Investigation of NIDDM Genetics (FUSION) and Ashkenazi studies were identified using type 2 diabetic subjects from families with strong evidence for linkage to chromosome 20q12–13.1 (9,12). Although this strengthened the power to identify the initial association (16), it may have resulted in an overestimation of the population risk. We aimed to define the risk associated with this haplotype in a U.K. Caucasian study including type 2 diabetic subjects with no evidence of linkage to chromosome 20 (17,18). It is also important to replicate genetic associations in sufficiently powered follow-up studies because initial positive studies tend to overestimate the true risk associated with a variant (19,20).

To assess the role of the risk haplotype in our U.K.

TABLE 1
Clinical details of study subjects

	Case subjects	Control subjects	Family study probands
n	2,004	1,635	509
Male (%)	59	51	58
Age at diagnosis (years)*	51 (45–57)	31 (28–35)	41 (36–47)
BMI (kg/m^2)	30.1 (26.7–34.2)	26.3 (24.1–29.0)†	33.0 (28.9–37.4)
Treatment D/O/I (%)	11/63/26	‡	20/59/21

Continuous data are given as median (interquartile range). Only successfully genotyped subjects included. *Age at diagnosis for case subjects, age at study for control subjects. No clinical details were available for the ECCAC population control samples, so control characteristics are for the EFS samples only. †BMI measurement for men only, as women were pregnant at time of study. ‡Control subjects were not on treatment. D/O/I, diet/oral hypoglycemic agents/insulin.

study, we first confirmed that patterns of linkage disequilibrium across the P2 region are similar in the U.K., FUSION, and Ashkenazi populations (online appendix Fig. 1 [available at http://diabetes.diabetesjournals.org]). The minor alleles at four SNPs defined the associated haplotype in the FUSION and Ashkenazi studies. We genotyped three of these SNPs (rs2144908, rs4810424, and rs1884613) in 96 unrelated control samples. Genotype data from the fourth SNP, rs1884614, for the same 96 subjects were available from the recent extensive linkage disequilibrium scan of 20q12–13.1 (21). These SNPs have very similar minor allele frequencies (~15% in control subjects) and show almost identical patterns of linkage disequilibrium (all pairwise $r^2 > 0.95$) in the U.K. Caucasian, FUSION (15), and Ashkenazi Jewish (14) populations. The almost perfect linkage disequilibrium between these SNPs means that they define just two common haplotypes (frequency >0.05). We genotyped two of the haplotype tagging SNPs (rs2144908 and rs4810424) in a total of 5,256 subjects from a large type 2 diabetes genetics resource (2,004 cases vs. 1,635 control subjects and 509 families). Clinical details of these subjects are given in Table 1. This study had >99% power to detect the ORs suggested by the initial studies (1.33 and 1.46) and 92% power to detect an OR of 1.20 at $P < 0.05$.

TABLE 2
Case/control results

Genotypes/alleles	Case total (n = 2,004)	Control total (n = 1,635)	P
rs2144908			
AA	42 (0.02)	44 (0.03)	
AG	575 (0.29)	412 (0.25)	
GG	1,387 (0.69)	1,179 (0.72)	
Genotype			0.04
A	659 (0.16)	500 (0.15)	
G	3,349 (0.84)	2,770 (0.85)	
A allele carriers vs. noncarriers		1.15 (1.00–1.33)	0.06
Allelic		1.09 (0.96–1.24)	0.18
Combined allelic*		1.13 (1.00, 1.27)	0.04
rs4810424			
CC	41 (0.02)	38 (0.02)	
GC	563 (0.29)	396 (0.25)	
GG	1,382 (0.70)	1,166 (0.73)	
Genotype			0.05
C	645 (0.16)	480 (0.15)	
G	3,327 (0.84)	2,728 (0.85)	
C allele carriers vs. non-carriers		1.17 (1.01–1.36)	0.03
Allelic		1.12 (0.99–1.28)	0.08
Combined allelic*		1.15 (1.02, 1.29)	0.02

Data are genotype and allele numbers (frequency) with summary OR (95% CI). *Combined allelic OR and P values include data from family-based study (Table 3).

Table 2 presents the results of our case/control analysis. Online appendix Table 2 provides the data by cohort. Overall, the type 2 diabetic case group deviated mildly from Hardy-Weinberg equilibrium (HWE) ($P = 0.05$). Possible reasons for the apparent HWE deviation in the case group include: genotyping error, random sampling error, and because one genotype is predisposing to disease and therefore will be more frequent in subjects ascertained for that disease. Only genotyping errors will result in false association results (other than normal sampling variation that is reflected in the P value). We therefore took a number of steps to eliminate the possibility that we had made genotyping errors. These are fully described in "Genotyping and quality control" in the RESEARCH DESIGN AND METHODS section. Importantly, we saw similar results for both SNPs, which are in very tight linkage disequilibrium, and sequencing 15% of the samples identified no discrepancies. The difference in allele frequencies between case and control subjects did not reach significance (rs2144908 OR = 1.09 [95% CI 0.96–1.24], $P = 0.18$; rs4810424 1.12 [0.99–1.28], $P = 0.08$). However, there was nominal evidence of a difference in genotype frequencies ($P = 0.04$ and $P = 0.05$, respectively). The results also reached nominal significance when comparing carriers of at least one copy of the putative risk allele to subjects homozygous for the common allele (rs2144908 1.15 [1.00–1.33], $P = 0.06$; rs4810424 1.17 [1.02–1.36], $P = 0.03$). The pattern of deviation from HWE in the case group explains why the genotype and dominant analyses reached significance but the allelic association did not; the allelic association test is relatively conservative because the frequency of the high-risk homozygotes is less than expected under HWE (22).

TABLE 3
Results of TDT/sibTDT type 2 diabetes family-based association study for SNPs rs2144908 and rs4810424

SNP	Observed transmissions	Expected transmissions	Z	P	OR (95% CI)
rs2144908	132	115	2.257	0.02	1.32(1.00–1.75)
rs4810424	125	110	2.012	0.04	1.29(0.96–1.73)

Results are for the minor allele at each SNP. The Z score is the Z_{max} score from the TDT/sibTDT analysis; the P value is the associated two-tailed value. We used the discordant allele test ratio and the transmission disequilibrium testing transmission ratio to obtain an estimate of the family-based genotype relative risk.

Table 3 presents the results of our family-based analysis. All subjects were independent from the case/control study. Parents and probands were in HWE ($P = 0.73$ and $P = 0.81$, respectively). Using the transmission disequilibrium test (TDT)/SibTDT method of Spielman and Ewens (23) there was nominal evidence of overtransmission of the minor allele for both SNPs in 509 families (rs2144908 combined Z score = 2.26, $P = 0.02$, and rs4810424 2.01, $P = 0.04$). This equates to ORs of 1.32 (95% CI 1.00–1.75) and 1.29 (0.96–1.73), respectively (Table 3). Combining the case/control and family-based studies, the overall estimated OR for the minor alleles at rs2144908 and rs4810424 are 1.13 (1.00–1.27) ($P = 0.04$) and 1.15 (1.02–1.29) ($P = 0.02$).

By performing a large-scale, well-powered study, we have replicated the association of a common haplotype of the HNF4α P2 promoter region with type 2 diabetes. We found evidence of an association in both case/control and family-based studies. This is one of the largest type 2 diabetes genetic association studies, but despite this, individually our case/control and familial association studies do not reach strong levels of significance. However, both studies show nominal evidence for association, and in the context of the findings from the first two studies, our results provide strong evidence that variation around the HNF4α P2 promoter is predisposing to type 2 diabetes. Our result is important given that several recent reports have highlighted the need for positive genetic association studies to be replicated (19,20). These studies, including those of diabetes susceptibility variants (24–26), show that the evidence for or against a genetic association needs to be built up over many studies.

The OR associated with the P2 promoter variants is less in our U.K. Caucasian population (OR = 1.15 [95% CI 1.02–1.29]) than in the Finnish (1.33 [1.06–1.65]) or Ashkenazi Jewish (1.46 [1.12–1.91]) studies. This may represent a true population difference. It may also be due to chance variation (an OR of 1.15 is within the 95% CI of both previous studies). It may also, in part, be explained by the relatively young age of the control subjects in relation to the case subjects, as some of the control subjects will go on to develop type 2 diabetes. More likely, the use of 20q12–13.1–linked subjects in the initial studies may have led to an overestimation of the OR associated with the risk haplotype (9,12,16). In addition, initial positive genetic association studies often overestimate the size of effect for polygenic variation (19,20). Our study, which did not include any subjects with evidence of linkage to 20q12–13.1, may represent a more generally applicable type 2 diabetes OR. However, we note that most of our case subjects have been ascertained for young onset or family history (or both). This means that they may be enriched for genetic effects. The risk conferred by this haplotype in completely unselected type 2 diabetic patients may be less.

Our study has not helped detect which of the SNPs in the P2 region is the causal variant. We have shown that the four associated SNPs in the FUSION and Ashkenazi studies form the same haplotype in U.K. Caucasians. The recent extensive survey of the 20q12–13.1 region by Ke et al. (21) showed that patterns of linkage disequilibrium in the U.K. are very similar to those in FUSION subjects (15)

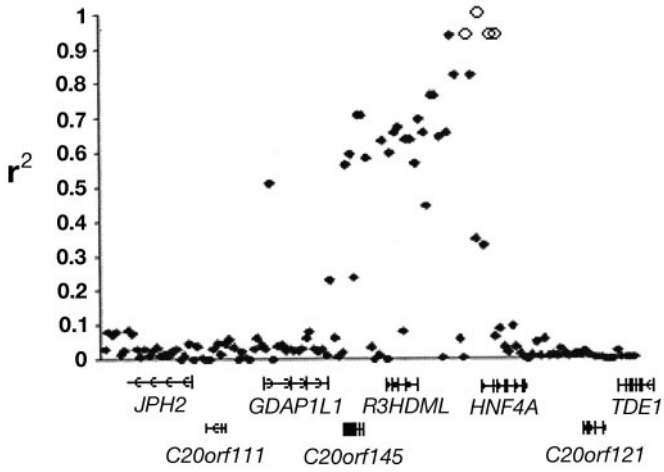

FIG. 1. Values (r^2) against rs2144908 for SNPs across 390 kb (from SNP rs6103644 to rs3091588) of chromosome 20 including the HNF4α gene region in the U.K. Caucasian population. The genotype data were mainly obtained from the extensive study of 20q12–13.1 recently published by Ke et al. (21); three of the type 2 diabetes–associated SNPs were typed in addition. The unfilled SNP with an r^2 of 1 is rs2144908. The other unfilled SNPs are the SNPs previously associated with type 2 diabetes.

(online appendix Fig. 1). Fig. 1 plots the pairwise r^2 values between rs2144908 and 132 SNPs across a 390-kb region of chromosome 20, including the HNF4A gene from the U.K. Caucasian population. Genotype data were obtained from supplementary information provided by Ke et al. (21). This shows the strong correlation between the at-risk haplotype and SNPs up to 145 kb upstream of rs2144908. Identification of the causal variant/s will therefore require functional work and further association studies in other populations (e.g., Africans). It should also be noted from Fig. 1 that variation of other genes 5′ of HNF4a may explain the type 2 diabetes association.

In conclusion, we have replicated the type 2 diabetes association of a haplotype describing common variation spanning the HNF4α P2 promoter in a U.K. Caucasian population. The type 2 diabetes OR associated with the minor allele is smaller than that observed in the initial studies, emphasizing the need for large follow-up studies, especially since the initial finding was in a dataset showing linkage to this region.

RESEARCH DESIGN AND METHODS

Linkage disequilibrium and haplotype analysis. Genotypes for three of the four SNPs (rs2144908, rs4810424, and rs1884613) that form the type 2 diabetes risk haplotype were genotyped by sequencing 96 U.K. Caucasian subjects from the European Cell Culture Collection (ECACC). Genotype data on the fourth SNP and for the 129 other SNPs shown in Fig. 1 from these same 96 subjects were available from the recent extensive haplotype and linkage disequilibrium study of the 20q12–13.1 region (21). Linkage disequilibrium statistics were obtained using the GOLD (graphical overview of linkage disequilibrium) program (27).

Case/control subjects. The clinical characteristics of the subjects in our case/control study are presented in Table 1. Informed consent was obtained from all participants. All type 2 diabetic subjects were unrelated U.K. Caucasians who had diabetes defined by either World Health Organization criteria or being treated with medication for diabetes. Known subtypes such as maturity-onset diabetes of the young or mitochondrial-inherited diabetes and deafness were excluded by clinical criteria and/or genetic testing. The type 2 diabetic case group was recruited from three sources: a collection of young-onset (defined as ≥18 and ≤45 years at age of diagnosis) type 2 diabetic subjects ($n = 277$), probands from type 2 diabetic sibships from the Diabetes U.K. Warren 2 repository ($n = 541$) described previously (17,28), and a new

collection of type 2 diabetic subjects from the Warren 2 repository (n = 1,186) with an age of diagnosis between 35 and 65 years but not selected for having a family history. The presence of GAD autoantibodies had been excluded for the first two groups of case subjects but not the new collection of case subjects (see online appendix Table 1 for full description of case subjects by group).

Population control subjects were all U.K. Caucasian. They were recruited from two sources: parents from a consecutive birth cohort (Exeter Family Study) with normal (<6.0 mmol/l) fasting glucose and/or normal HbA_{1c} levels (<6%, Diabetes Control and Complications Trial corrected) (28) and a nationally recruited population control sample of U.K. Caucasians obtained from the ECACC.

Family-based subjects. The clinical characteristics of the affected probands in our family-based study are presented in Table 1. Families fitting the following criteria formed our familial association study: an affected proband with both parents available or one parent and at least one unaffected sibling (89% of these families had >2 unaffected siblings). The characteristics of some of these families have been described previously (29).

Genotyping and quality control. Genotyping was performed by Kbiosciences (Herts, U.K.). They designed and used modified TaqMan assays, details of which are available on their website (http://www.kbioscience.co.uk). The genotyping was performed in 384-well plates. Each 384-well plate was made up of two 96-well case plates and two 96-well control plates. Ten percent of the genotyped samples were duplicates, and we included two negative controls per 96-well plate. Genotyping accuracy, as determined from the genotype concordance between duplicate samples, was 99.8%. The genotyping success rate was 98% for control and 97% for case samples. There were no Mendelian inheritance errors in the family-based samples after those with obvious relationship inconsistencies had been excluded (as determined by the genotyping of an additional 42 SNPs). In addition, rs2144908 and rs4810424 are in almost perfect linkage disequilibrium within the U.K. Caucasian population. The r^2 values were 0.983 and 0.978 and the D′ statistics 0.998 and 0.997 in case and control subjects, respectively, consistent with previous studies (14,15). In addition, we sequenced a random 15% of samples for both SNPs and found no discrepancies. All individual cohorts were in HWE (χ^2 $P > 0.05$), but the overall case group deviated mildly for rs2144908 ($P = 0.05$). The P value for HWE for rs4810424 is 0.06. These quality control measures and the similar results for the two SNPs, which are in very tight linkage disequilibrium, suggest that the deviation is due to chance variation rather than genotyping error.

Statistical analysis. ORs and P values were determined for our case/control analyses using χ^2 tests. All power calculations are for two-tailed P values <0.05, assuming a control allele frequency of 0.155. To analyze our family-based data we used the TDT/SibTDT method of Spielman and Ewens (23). We did not include families with two parents and one affected offspring ("trios") where the genotype of one parent was missing. We also analyzed the trios using TRANSMIT (http://archimedes.well.ox.ac.uk/pise/transmit), which allows for some missing data; the results were very similar (trios overtransmission $P = 0.05$). To estimate the OR for one-parent sibships we used the discordant-allele test (30). To estimate the ORs for the case/control and family-based combined analysis we used the following approach: each parental transmission (for transmission disequilibrium testing) or discordant allele sib pair (for discordant allele test) was considered as a single contingency table; our family-based study therefore produced a series of stratified contingency tables that could be combined using the Mantel-Haenszel method and produced our estimated combined familial OR; we added the allelic case/control contingency table to this family-based stratified series and again performed a Mantel-Haenszel analysis to obtain estimated familial case/control ORs, 95% CIs, and P values. All P values are two sided.

ACKNOWLEDGMENTS

We thank Diabetes U.K. for funding this research. A.T.H. is a Wellcome Trust Research Leave Fellow. T.M.F. is the European Association for the Study of Diabetes (EASD) 2003 Albert Renold award holder.

We thank Kaisa Silander, Karen Mohlke, Mike Boehnke, and colleagues who kindly gave us prepublication information about their findings.

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