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Association of the vitamin D metabolism gene *CYP27B1* with type 1 diabetes

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Abstract

Objective—Epidemiological studies have linked vitamin D deficiency with the susceptibility to type 1 diabetes. Higher levels of the active metabolite, 1α,25-dihydroxyvitamin D, could protect from immune destruction of the pancreatic β cells. 1α,25-dihydroxyvitamin D is derived from its precursor 25-hydroxyvitamin D by the enzyme 1α-hydroxylase encoded by the CYP27B1 gene, and is inactivated by 24-hydroxylase encoded by the CYP24A1 gene. Our aim was to study the association between the CYP27B1 and CYP24A1 gene polymorphisms and type 1 diabetes.

Research Design and Methods—We studied 7,854 patients with type 1 diabetes and 8,758 controls from Great Britain and 2,774 affected families. We studied four *CYP27B1* variants, including common polymorphisms −1260C>A (rs10877012) and +2838T>C (rs4646536), and 16 tag polymorphisms in the CYP24A1 gene.

Results—We found evidence of association with type 1 diabetes for *CYP27B1* −1260 and +2838 polymorphisms, which are in perfect linkage disequilibrium. The common C allele of *CYP27B1* −1260 was associated with an increased disease risk in the case-control analysis (OR = 1.07, $P = 2.9 \times 10^{-3}$), and in the fully independent collection of families (RR = 1.11, $P = 6.4 \times 10^{-3}$). The combined support of an association for *CYP27B1* −1260 is $P = 3.8 \times 10^{-6}$. For the CYP24A1 gene we found no evidence of association with type 1 diabetes (multilocus test $P = 0.23$).

Conclusions—The present data provides evidence that common inherited variation in the vitamin D metabolism affects susceptibility to type 1 diabetes.

Type 1 diabetes is strongly inherited and yet exhibits striking epidemiological features such as seasonality in diagnosis, with more cases diagnosed in the autumn and winter months, and a north-south geographical gradient, suggesting inverse correlation between the amount of sunshine and type 1 diabetes incidence (1;2). Lower serum concentrations of 1α,25-dihydroxyvitamin D [1α,25$(OH)_2$D], the hormonally active form of vitamin D, and of its precursor 25-hydroxyvitamin D [25(OH)D] have been reported at the diagnosis of type 1

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diabetes compared to normal controls (3-5). Epidemiological studies indicate that vitamin D supplementation in early childhood is associated with decreased type 1 diabetes incidence (6-8). However, a direct role of impaired vitamin D metabolism in the etiology of type 1 diabetes has not been proven. If vitamin D is a significant factor in type 1 diabetes, then it might be expected that common functional sequence polymorphisms in the genes that influence vitamin D action could predispose to the disease. We have previously studied the gene of the vitamin D receptor (VDR), which binds 1α,25$(OH)_2$D and mediates the effects of vitamin D. We found no association between *VDR* sequence variants and type 1 diabetes, in contrast to some other studies with smaller sample sizes (9), and a recently conducted meta-analysis also found no evidence of association (10).

Several studies have reported associations of type 1 diabetes and other autoimmune diseases with polymorphisms in the CYP27B1 gene on chromosome 12q13.1-q13.3 (11-14), which encodes 1α-hydroxylase, the enzyme that converts 25(OH)D into 1α,25$(OH)_2$D. However, these results have not been verified. In the present study we have investigated the association between type 1 diabetes and sequence variants in the CYP27B1 gene. Circulating 1α,25$(OH)_2$D is biologically inactivated through a series of reactions beginning with 24-hydroxylation. Vitamin D 24-hydroxylase is encoded by the CYP24A1 gene located on chromosome 20q13.2-q13.3. Here, we have for the first time also studied the association between type 1 diabetes and *CYP24A1* polymorphisms.

METHODS

Subjects

We studied a case-control collection comprising 7,854 patients with type 1 diabetes and 8,758 healthy controls from Great Britain. The recruitment of these subjects and sample processing have been described elsewhere (15). We also studied *CYP27B1* polymorphisms in a family collection including 2,774 type 1 diabetes families with one or two affected offspring (815 from Great Britain and Northern Ireland, 841 from Finland, 335 from the USA, 360 from Norway and 423 from Romania), providing 3,081 parent-child trio genotypes for *CYP27B1* −1260 and 2,198 trio genotypes for *CYP27B1* +2838. The collection of all DNA samples has been approved by relevant ethical committees. We obtained written informed consent from all participants.

Genotyping

In the CYP27B1 gene we genotyped three single nucleotide polymorphisms (SNPs), *CYP27B1* −1260C>A (rs10877012, located in the 5' region), *CYP27B1* +2838T>C (rs4646536, located in intron 6) that were previously reported (11-14) and rs8176345, a synonymous SNP in exon 5 that we found by sequencing. We used HapMap data (16) to select tag SNPs that capture common variants in the CYP24A1 gene. Of the 111 HapMap SNPs located in the region (NCBI build 34, coordinates chr 20: 53,450,894..53,482,103), 54 SNPs had minor allele frequency (MAF) > 0.05, and 16 were chosen as tag SNPs that capture association of other common variants with $r^2 > 0.8$. *CYP24A1* SNPs were genotyped in up to 5,239 cases and 5,539 controls (exact numbers for each SNP are shown in Table 3). Genotyping was done using TaqMan (Assay-by-design, Applied Biosystems, Warrington, UK; see Supplementary note). All genotypes were scored by two researchers independently to minimize error. Genotypes of controls and parents did not deviate from Hardy-Weinberg equilibrium above that expected at random ($P > 0.05$).

DNA sequencing

Direct sequencing of nested PCR products from 32 healthy controls from Great Britain was performed using an Applied Biosystems (ABI) 3700 capillary sequencer (Foster City, CA,

USA). Polymorphisms were identified using the Staden Package (http://www.mrc-lmb.cam.ac.uk/pubseq/) and mapped to the NCBI human genome build 35.

Statistical analyses

All statistical analyses were performed within Stata statistical package (http://www.stata.com), using additional Stata routines (http://www-gene.cimr.cam.ac.uk/clayton/software/). We analyzed cases and controls using logistic regression models (17), adjusting for 12 broad geographical regions, to allow for geographic variation in allele frequencies across Great Britain (18). The families were analyzed using the transmission disequilibrium test (TDT) (19) and conditional logistic regression (17). A score test was used to combine tests from family and case-control studies as described previously (15). We used htstep, htsearch and haptag programs within Stata 8.2 to select tag SNPs in the CYP24A1 gene. For these SNPs we performed a multilocus test using mlpop program in Stata 8.2, which tests for association between disease and the tag SNPs due to linkage disequilibrium with one or more causal variants in the region. It contrasts allele frequencies of a non-redundant set of tag SNPs between cases and controls by use of Hotelling's T^2 test (20;21). We did not apply correction for multiple testing.

RESULTS

Association analysis of the *CYP27B1* polymorphisms

We found evidence that the promoter polymorphism *CYP27B1* −1260 is associated with type 1 diabetes in both the case-control ($P = 2.9 \times 10^{-3}$; odds ratio [OR] for C allele = 1.07, 95% confidence interval [CI] = 1.02 - 1.13; Table 1) and the family ($P = 6.4 \times 10^{-3}$; relative risk [RR] for C allele = 1.11, 95% CI = 1.03 - 1.20; Table 1) collections. Consequently, when we combined evidence from both collections, which are fully independent from each other, the combined test provided statistical support for an association between type 1 diabetes and *CYP27B1* −1260 at $P = 9.08 \times 10^{-5}$ (1 df) and 3.8×10^{-6} (2 df). There was no evidence of regional heterogeneity in the allele frequencies of *CYP27B1* −1260 ($F_{11,7261} = 0.86$, $P = 0.58$) in controls from different parts of Great Britain, while we found evidence of population heterogeneity ($F_{3,3486} = 3.44$, $P = 0.016$) in allele frequencies among parents from various countries studied.

In contrast to other previously published studies (11-14), we found that intronic SNP *CYP27B1* +2838 was also associated with type 1 diabetes in both collections. The major allele T was associated with increased type 1 diabetes risk in both the case-control ($P = 7.1 \times 10^{-3}$; OR = 1.08, 95% CI = 1.02 - 1.14; Table 2) and the family ($P = 2.2 \times 10^{-3}$; RR = 1.15, 95% CI = 1.05 - 1.26; Table 2) collections. A combined test provided *P*-values of 2.52×10^{-3} (1 df) and 8.47×10^{-5} (2 df).

We noted that in all population samples that we studied, including controls from Great Britain and parents of the patients from Great Britain and Northern Ireland, Norway or Romania there is almost perfect linkage disequilibrium between SNPs *CYP27B1* −1260 and +2838 with D' = 1.0 and $r^2 = 0.99$ (we obtained lower *P*-values for *CYP27B1* −1260 because more samples were genotyped for this SNP than for +2838). To verify genotyping of *CYP27B1* −1260 and +2838 we directly sequenced 376 cases and 533 controls and found complete concordance in the results. This raised the possibility that in the German and Polish population samples studied previously (11-14) there may have been genotyping error in the analysis of *CYP27B1* −1260 polymorphism. Therefore, in Cambridge we regenotyped 120 DNA samples from 36 type 1 diabetes families from the original German laboratory for the two SNPs, obtaining only 88.2% concordance between the two genotype datasets for *CYP27B1* −1260, and this problem was compounded by evidence of data handling errors. Contrary to previous analyses (11;12;14), in

these German samples we found the near-perfect LD between the two SNPs (*CYP27B1* −1260 and +2838 SNPs: D' = 1.00 and r^2 = 0.99) as we report here for all other populations studied, indicative of past genotyping and data analysis errors.

Resequencing of the *CYP27B1* gene

We then resequenced 8 kb of the CYP27B1 gene, including all exons, introns and 2 kb 5' and 3' of the gene, using DNA samples of 32 healthy subjects from Great Britain, in order to test for the presence of an obvious candidate for a causal variant, such as an amino-acid changing polymorphism or a splice mutation. We discovered two novel rare SNPs with MAFs < 0.01, one in the promoter at position −1138 and one in the 3' untranslated region (ss67078180 and ss67078183, respectively; http://www.ncbi.nlm.nih.gov/SNP/). We did not genotype these SNPs because even large samples that we studied here were underpowered to demonstrate association of such rare variants. We also found a synonymous SNP rs8176345 in exon 5 with MAF = 0.03 that was not in linkage disequilibrium with the common CYP27B1 SNPs at positions −1260 and +2838 (r^2 = 0.06 and 0.06, respectively). We genotyped rs8176345 in a subset of the case-control collection comprising 3,040 type 1 diabetes patients and 3,349 controls but obtained no evidence of an association (*P* = 0.23; OR = 0.87; 95% CI = 0.71 - 1.09). We also identified a common promoter SNP rs3782130 at position −1074 with MAF = 0.33. Since we were unable to develop a working high throughput genotyping assay for this SNP, we sequenced it in 376 cases and 533 controls, and found that it was also in complete linkage disequilibrium with SNPs at positions −1260 and +2838 (r^2 = 0.99 and 0.97, respectively).

Interaction analyses

We performed case-only gene-gene interaction tests (15) between known type 1 diabetes susceptibility loci and *CYP27B1* −1260. We did not undertake the same analyses for *CYP27B1* −2838 because these SNPs are in perfect linkage disequilibrium. There was no consistent evidence of an interaction (that is the deviation from a multiplicative model) for the joint effects of *CYP27B1* −1260 and *INS* rs689 (−23*Hph*I), *PTPN22* rs2476601 (Arg620Trp) or *CTLA4* rs3087243 (Supplementary Table 1). However, there was some evidence for an interaction with *HLA-DRB1* (Supplementary Table 1), but when we analyzed *CYP27B1* −1260 stratified by specific *HLA-DRB1* genotypes, we found that risk ratios were not consistent between the case-control and family collections (Supplementary Table 2). Therefore, we conclude that in conferring risk of type 1 diabetes *CYP27B1* does not interact with the previously known disease genes. We conducted a similar interaction analysis for *CYP27B1* −1260 and seven *VDR* SNPs (*Fok*I, *Apa*I, *Bsm*I, *Taq*I, rs2544043, rs12721366 and rs4303288), which previously showed marginal or little evidence of an association with type 1 diabetes (9;10). However, we found no evidence of an interaction (Supplementary Table 1). We compared the relative risk of type 1 diabetes conferred by the *CYP27B1* −1260 in the different populations that we studied here, but found no evidence of heterogeneity above that expected at random (χ_6^2 = 3.11, *P* = 0.79). We also tested *CYP27B1* −1260 for age-at-diagnosis and sex effects in a case-only analysis but did not find evidence for these (Supplementary Table 1), or for parent-of-origin effect in the affected families (*P* = 0.76).

Analysis of the CYP24A1 gene

In the case-control collection we tested 16 tag SNPs that capture association of the common variants that were present in the CYP24A1 gene in HapMap (Table 3). A multilocus test revealed no evidence of association between *CYP24A1* polymorphisms and type 1 diabetes (*P* = 0.23). Therefore, we did not undertake follow-up genotyping of any of the *CYP24A1* polymorphisms in additional cases and controls or families.

DISCUSSION

The present study provides the first evidence of association between *CYP27B1* polymorphisms and type 1 diabetes in a fully validated analysis. Our results in the present report indicate what appears to have been technical and analytical errors in the previous studies (11-14). Nevertheless, these initial reports did contribute to our motivation to carry out the current analysis of *CYP27B1* in type 1 diabetes.

Taking into account prior epidemiological and experimental links between vitamin D and type 1 diabetes (3-8;22-27) and the association between *CYP27B1* and type 1 diabetes that we established here, we suggest that common inherited variation in the CYP27B1 gene affects vitamin D metabolism and is an etiological factor predisposing to type 1 diabetes. Rare *CYP27B1* mutations that completely inactivate 1α-hydroxylase are known to cause vitamin D-dependent rickets type I (OMIM 264700), characterized by low concentrations of 1α,25 $(OH)_2D$ (28;29). We hypothesize that the presence of the *CYP27B1* −1260 C allele, or another variant in linkage disequilibrium with it (such as two that we have studied here, *CYP27B1* +2838 in intron 6 and rs3782130 in the 5' region) reduces the level of the active 1α-hydroxylase and conversion of 25(OH)D to 1α,25$(OH)_2D$, leading to increased predisposition to type 1 diabetes. Recently, preliminary data have suggested that type 1 diabetes patients carrying at *CYP27B1* −1260 risk genotype CC had lower CYP27B1 mRNA levels in the peripheral blood mononuclear cells compared to healthy controls with the CC genotype (30). Functional roles of the *CYP27B1* polymorphisms should be investigated in further experiments, evaluating their effects on 1α-hydroxylase activity and 1α,25$(OH)_2D$ concentration, in particular, in the immune cells, such as dendritic cells and monocytes, that underpin immune responses (31; 32).

Given our evidence that variation in the CYP27B1 gene etiologically contributes to type 1 diabetes risk, other genes that control vitamin D metabolism are also biologically plausible candidates and studies of their association with type 1 diabetes are required. Here we investigated the CYP24A1 gene that encodes vitamin D 24-hydroxylase, an enzyme that inactivates 1α,25$(OH)_2D$, and found no evidence of association. Studies of *CYP27A1* or *CYP2R1* that encode vitamin D 25-hydroxylases, and of the vitamin D-binding protein gene (33;34) are also needed.

In the immune system 1α,25$(OH)_2D$ has been shown to suppress production of the IL-12, IL-2, TNFα and IFNγ cytokines and activate expression of TGFβ1 and IL-4 cytokines, thereby inhibiting Th1-type responses, and to induce regulatory T cells (35). It can also regulate differentiation and maturation of dendritic cells critical in induction of T-cell mediated immune responses (36). These immunomodulatory effects may explain the reported protective effects of vitamin D in type 1 diabetes (37). In the animal models 1α,25$(OH)_2D_3$ and its analogues have been effective in prevention of autoimmune diabetes (23-27), as well as of other autoimmune diseases (38-42). Epidemiological studies in humans also indicate that intake of vitamin D and its high circulating levels are associated with a lower risk of rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus (43-45). Genetic studies reported association of the *CYP27B1* polymorphisms with Addison's disease, Hashimoto's thyroiditis and Graves' disease (12;13), but these results await confirmation. The possibility that *CYP27B1* and 1α,25$(OH)_2D$ may be involved in multiple autoimmune diseases suggests that effects of vitamin D on type 1 diabetes involve immune regulation, but this does not rule out additional effects, such as protection of pancreatic beta cells and their functions.

Our study indicates that genetic variation in the vitamin D metabolism is an etiological factor in type 1 diabetes. This evidence justifies further experiments investigating molecular and cellular actions of vitamin D and mechanisms of its protective effect in type 1 diabetes.

Epidemiological studies indicate that vitamin D supplementation in early childhood may reduce type 1 diabetes risk (6-8). Given that vitamin D insufficiency is more common among children and young adults than was previously thought (46), its correction may be a viable approach to prevent type 1 diabetes or delay its development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

CYP27B1 −1260 allele and genotype frequencies and association test results in 7,854 case and 8,758 control genotypes and 3,081 parent-child trio genotypes.

	Cases, n (%)	Controls, n (%)	OR (95% CI)	*P*-value
Allele				
A	4,999 (31.8)	5,836 (33.3)	1.00 (reference)	
C	10,709 (68.2)	11,680 (66.7)	1.07 (1.02-1.13)	2.9×10^{-3} *
Genotype				
A/A	767 (9.8)	999 (11.4)	1.00 (reference)	
C/A	3,465 (44.1)	3,838 (43.8)	1.20 (1.08-1.33)	
C/C	3,622 (46.1)	3,921 (44.8)	1.22 (1.10-1.36)	9.6×10^{-4} †
	Transmitted, n (%)	**Untransmitted, n (%)**	**RR (95% CI)**	***P*-value**
Allele C	1,405 (52.6)	1,264 (47.4)	1.11 (1.03-1.20)	6.4×10^{-3} ‡
Genotype				
A/A	274 (8.9)	989 (10.7)	1.00 (reference)	
C/A	1,369 (44.4)	4,055 (43.9)	1.27 (1.08-1.48)	
C/C	1,438 (46.7)	4,199 (45.4)	1.33 (1.12-1.58)	3.9×10^{-3} §

For the case-control collection a full genotype model was significantly different from the model of multiplicative allelic effects (likelihood-ratio test, $\chi_1^2 = 5.0$, $P = 0.025$).

*1 df likelihood ratio test for multiplicative allelic effects

†2 df likelihood ratio test for genotype effects

‡Transmission Disequilibrium Test

§2 df likelihood-ratio test for the full genotype model; genotypes for the family-based pseudo-controls were estimated as described previously (17)

Table 2

CYP27B1 +2838 allele and genotype frequencies and association test results in 5,552 case and 7,435 control genotypes and 2,198 parent-child trio genotypes.

	Cases, n (%)	Controls, n (%)	OR (95% CI)	*P*-value
Allele				
C	3,576 (32.2)	5,031 (33.8)	1.00 (reference)	
T	7,528 (67.8)	9,839 (66.2)	1.08 (1.02-1.14)	7.1×10^{-3} *
Genotype				
C/C	573 (10.3)	877(11.8)	1.00 (reference)	
T/C	2,430 (43.8)	3,277 (44.1)	1.16 (1.03-1.31)	
T/T	2,549 (45.9)	3,281 (44.1)	1.20 (1.07-1.36)	0.010†
	Transmitted, n (%)	**Untransmitted, n (%)**	**RR (95% CI)**	***P*-value**
Allele T	995 (53.6)	863 (46.4)	1.15 (1.05-1.26)	2.2×10^{-3} ‡
Genotype				
C/C	182 (8.3)	709 (10.8)	1.00 (reference)	
T/C	996 (45.3)	2,926 (44.4)	1.27 (1.06-1.53)	
T/T	1,020 (46.4)	2,959 (44.9)	1.36 (1.11-1.67)	6.1×10^{-4} §

For the case-control collection a model of multiplicative allelic effects was not significantly different from the full genotype model (likelihood-ratio test, $\chi_1^2 = 1.9$, $P = 0.17$).

* 1 df likelihood ratio test for multiplicative allelic effects

† 2 df likelihood ratio test for genotype effects

‡ Transmission Disequilibrium Test

§ 2 df likelihood-ratio test for the full genotype model; genotypes for the family-based pseudo-controls were estimated as described previously (17)

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Table 3

Analysis of 16 tag polymorphisms of the CYP24A1 gene in type 1diabetes cases and controls.

CYP24A1 polymorphism	Alleles 1/2	Minor allele	Cases 11 (%)	Cases 12 (%)	Cases 22 (%)	Cases Total	Controls 11 (%)	Controls 12 (%)	Controls 22 (%)	Controls Total	MAF	OR	(95% CI)
rs2762928	T/A	T	92 (1.8)	1,187 (23.5)	3,775 (74.7)	5,054	106 (1.9)	1,283 (23.2)	4,150 (74.9)	5,539	0.13	1.01	(0.93 - 1.09)
rs2585428	G/A	A	1,453 (29.1)	2,441 (48.9)	1,101 (22.0)	4,995	1,552 (28.0)	2,739 (49.5)	1,247 (22.5)	5,538	0.47	0.98	(0.92 - 1.03)
rs912505	G/A	G	205 (4.1)	1,735 (34.7)	3,055 (61.2)	4,995	227 (4.2)	1,829 (33.4)	3,413 (62.4)	5,469	0.21	1.04	(0.97 - 1.11)
rs8124792	G/A	A	4,616 (90.1)	492 (9.6)	14 (0.27)	5,122	4,680 (89.6)	523 (10.0)	21 (0.40)	5,224	0.05	0.95	(0.84 - 1.08)
rs4809956	T/C	T	175 (3.5)	1,613 (31.8)	3,289 (64.8)	5,077	180 (3.4)	1,707 (32.2)	3,417 (64.4)	5,304	0.19	1.00	(0.93 - 1.07)
rs2426498	C/G	G	3,816 (75.0)	1,182 (23.2)	87 (1.7)	5,085	4,124 (75.1)	1,258 (22.9)	107 (2.0)	5,489	0.13	0.99	(0.91 - 1.07)
rs13038432	G/A	G	29 (0.64)	668 (14.7)	3,854 (84.7)	4,551	30 (0.59)	690 (13.5)	4,404 (86.0)	5,124	0.07	1.10	(0.98 - 1.22)
rs2245153	T/C	C	3,066 (64.3)	1,493 (31.3)	207 (4.3)	4,766	3,274 (65.4)	1,533 (30.6)	198 (4.0)	5,005	0.19	1.05	(0.98 - 1.13)
rs6022999	G/A	G	258 (5.2)	1,719 (34.8)	2,970 (60.0)	4,947	259 (4.8)	1,956 (36.2)	3,188 (59.0)	5,403	0.23	0.98	(0.92 - 1.05)
rs6127118	G/A	A	2,670 (56.7)	1,769 (37.5)	273 (5.8)	4,712	3,022 (59.7)	1,761 (34.8)	277 (5.5)	5,060	0.23	1.10	(1.03 - 1.18)
rs3787557	T/C	C	3,522 (74.4)	1,135 (24.0)	80 (1.7)	4,737	3,772 (74.6)	1,175 (23.2)	108 (2.1)	5,055	0.14	1.00	(0.92 - 1.09)
rs2762939	C/G	C	258 (5.6)	1,788 (38.5)	2,596 (55.9)	4,642	309 (6.1)	1,909 (37.4)	2,888 (56.6)	5,106	0.25	1.01	(0.94 - 1.08)
rs6068810	T/G	T	8 (0.17)	297 (6.3)	4,447 (93.6)	4,752	6 (0.12)	349 (7.2)	4,489 (92.7)	4,844	0.04	0.87	(0.75 - 1.02)
rs2181874	G/A	A	2,848 (57.5)	1,819 (36.7)	289 (5.8)	4,956	3,102 (57.0)	1,994 (36.6)	346 (6.4)	5,442	0.25	0.97	(0.91 - 1.03)
rs2244719	T/C	C	1,361 (26.9)	2,629 (51.9)	1,074 (21.2)	5,064	1,506 (27.5)	2,734 (49.8)	1,246 (22.7)	5,486	0.48	0.97	(0.92 - 1.03)
rs2248359	T/C	T	919 (17.54)	2,430 (46.4)	1,890 (36.1)	5,239	817 (15.3)	2,592 (48.7)	1,918 (36.0)	5,327	0.40	1.05	(0.99 - 1.11)

Multilocus test (20;21), $F_{16,11183} = 1.24$, $P = 0.23$.

MAF, minor allele frequency; OR, odds ratio for a minor allele; 95% CI, 95% confidence interval.