TABLE 2
Haplotype analyses

Haplotype	Initial samples			Replication samples		
SNPs* 1 2 3 4 5 6	Case frequency	Control frequency	Permutation *P* value	Case frequency	Control frequency	Permutation *P* value
n	192	272		657	360	
G G T A A T	0.14	0.18	0.162	0.14	0.17	0.227
A G T A A T	0.38	0.29	**0.009**	0.36	0.32	**0.021**
G A C G G T	0.16	0.19	0.256	0.20	0.19	0.495
G A C A A C	0.08	0.11	0.096	0.12	0.12	0.481

Haplotype	Hiroshima samples			Joint (initial + replication+ Hiroshima) samples		
SNPs* 1 2 3 4 5 6	Case frequency	Control frequency	Permutation *P* value	Case frequency	Control frequency	Permutation *P* value
n	356	192		1,205	824	
G G T A A T	0.15	0.13	0.272	0.15	0.16	0.1175
A G T A A T	0.35	0.26	**0.007**	0.37	0.30	**0.0001**
G A C G G T	0.19	0.16	0.237	0.19	0.18	0.6447
G A C A A C	0.11	0.08	0.232	0.11	0.11	0.6643

*SNP1 is equivalent to rs2051040, SNP2 to rs2796495, SNP3 to rs2143754, SNP4 to rs1418442, SNP5 to rs932447, and SNP6 to rs3738568. The total of the frequencies of the common haplotypes does not reach 1.0 because rare haplotypes with frequencies <0.05 were excluded. *P* values <0.05 are shown in boldface. Given the conservative Bonferroni correction, a *P* value <0.0125 (0.05 divided by four common haplotypes obtained from haplotype frequency estimation) is considered significant in the initial samples. In the replication samples, a *P* value <0.05 is considered significant.

ably alter their insulin levels. A multiple regression analysis was used to test for associations between SNPs and insulin resistance after adjustment for age, sex, and BMI. The statistical analyses were performed using JMP for Windows version 4.00 software (SAS Institute, Cary, NC). *P* values were corrected by Bonferroni adjustment, and a *P* value <0.005 (i.e., 0.05 divided by the total number of SNPs) was considered significant in the initial study. The statistical power was calculated based on a test for differences in proportions of alleles between case and control subjects (described in detail by Ohashi et al. [12]).

Haplotype analysis. To examine the linkage disequilibrium (LD) structure, pairwise LD, D′, and r^2 between SNPs and haplotype frequencies were estimated via the method of maximum likelihood from two-locus genotype data using the E-M algorithm under the assumption of Hardy-Weinberg equilibrium (13). For the estimation of haplotype frequencies, we selected one of the SNPs as a tagging SNP from every set of SNPs with $r^2 > 0.80$. All haplotypes were jointly tested for association with disease status by performing a $2 \times n\chi^2$ test of independence in a permutation procedure, where n indicates the number of haplotypes with a frequency >0. Individual haplotypes were also tested for association with disease status with a $2 \times 2\chi^2$ test of independence in a permutation procedure. In the permutation procedure, to account for the variability introduced by the haplotype frequency estimation, significance was assessed by permuting case and control status and recalculating the test statistic 1,000 times for each of the sample sets and 10,000 times for the combined sample set. The above calculations were performed with SNPAlyze V3.2 Pro software (Dynacom, Yokohama, Japan).

RESULTS AND DISCUSSION

We identified four SNPs by screening *PRKAA2*, two SNPs of which were also reported in the public database (Fig. 1). Adding 6 more SNPs available in the JSNP (Japanese Single Nucleotide Polymorphisms) database (available at http://snp.ims.u-tokyo.ac.jp/), a total of 10 SNPs were genotyped in the initial samples. All the polymorphisms were in Hardy-Weinberg equilibrium and had a minor allele frequency >5%. None of the SNPs was associated with type 2 diabetes (Table 1 and online appendix Tables 1–3 [available at http://diabetes.diabetesjournals.org]). Neither the difference in genotype frequency nor that in allele frequency had any significant influence on susceptibility to type 2 diabetes.

The LD pattern and the D′ and r^2 values of the 192 diabetic patients providing the initial sample are shown in Fig. 2. The six groups of SNPs with r^2 values >0.8 and each of the tagging SNPs (SNPs 1–6) are shown in Figs. 1 and 2. All the haplotypes with frequencies >5% for the entire sample are shown in Table 2. A general $2 \times n$ test for independence revealed a significant difference ($P = 0.009$) in haplotype frequencies with a minor (*A*) allele for rs2051040 and a major allele for all the other SNPs (AGTAAT) between case and control samples. Other common haplotypes were not associated with type 2 diabetes. These results were confirmed in a larger replication sample with 657 diabetic patients and 360 nondiabetic subjects. The haplotype with the *A* (minor) allele for rs2051040 and a major allele for the other five SNPs was associated with type 2 diabetes ($P = 0.021$) (Table 2). Even a genetically homogenous population, such as that of Iceland, has been reported to show substantial divergence in allele frequencies among geographical areas (14). Therefore, we further confirmed the association between the haplotype AGTAAT and type 2 diabetes in a third panel ($P = 0.007$) (Table 2), for which we enrolled both 356 type 2 diabetic and *192* nondiabetic subjects from the same area of Japan (Hiroshima) to exclude the possibility that the associations with the haplotype were falsely obtained as a result of population stratification among subjects enrolled from different areas of Japan.

A joint analysis of the whole sample revealed a significant association between haplotype AGTAAT and type 2 diabetes ($P = 0.0001$) (Table 2). The total of the frequencies of the common hapolotypes is <1.0 because rare haplotypes with frequencies <0.05 were excluded. The complete set of all estimated haplotypes for the case and control subjects is shown in online appendix Table 4.

We next investigated whether the polymorphisms in *PRKAA2* were associated with insulin resistance, as assessed by HOMA of insulin resistance. The association was compared between subjects with and without the minor allele. Among the 10 SNPs investigated, only

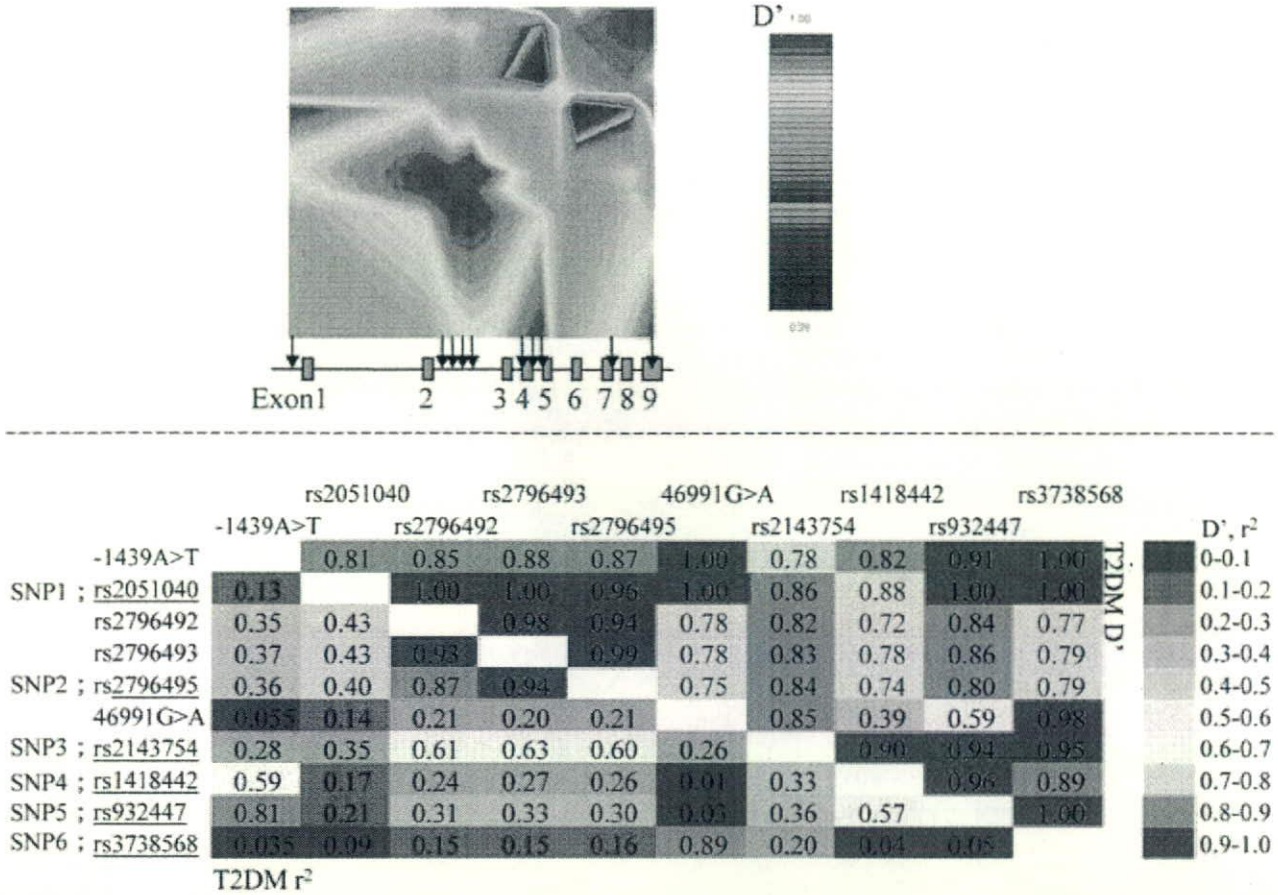

FIG. 2. Pairwise LD between SNPs. The *lower left* and *upper right* triangles indicate pairwise LD and r^2 and D' values, respectively, in 192 type 2 diabetic (T2DM) patients. Underlined SNPs are the tagging SNPs that represent all of the tested SNPs based on $r^2 > 0.8$.

rs2051040 was associated with insulin resistance. Subjects with the *A* (minor) allele for rs2051040 had a higher HOMA of insulin resistance than those without it (*AA/AG* vs. *GG* 1.95 ± 0.08 vs. 1.49 ± 0.10, regression coefficient = 0.18, $P = 0.002$). This finding was also confirmed in the joint replication samples (*AA/AG* vs. *GG* 1.83 ± 0.07 vs. 1.59 ± 0.09, regression coefficient = 0.099, $P = 0.037$). No other SNPs were associated with insulin resistance or any other diabetes-related quantitative traits, such as age, sex, BMI, fasting glucose, fasting insulin, HbA_{1c}, and HOMA-β.

PRKAA2 is a good candidate for the susceptibility gene to insulin resistance and type 2 diabetes. We therefore focused on *PRKAA2* and genotyped 10 SNPs spanning from the promoter region to the 3′ untranslated region. Even though alleles with an odds ratio of 1.2–1.3 could be detected with 80% power for the total sample for allele frequencies of 0.2 and 0.4, which correspond to the commonly observed allele frequencies in this study, we were unable to find associations between any single polymorphisms and type 2 diabetes. However, one common haplotype with the *A* (minor) allele for rs2051040 and a major allele for all the other SNPs was associated with type 2 diabetes. To jointly test all common haplotypes for associations with disease status, we also used PHASE version 2.1, performing a case-control permutation test with the default setting. In agreement with the haplotype analysis using SNPAlyze, haplotype analysis using PHASE software revealed a significant difference ($P = 0.01$) in haplotype frequencies between case and control subjects (data not shown). Likewise, SNPAlyze also revealed a significant difference (global $P = 0.004$) in a permutation test for differences in haplotype frequencies between case and control subjects. We therefore conclude that there is a significant difference in haplotype frequencies between case and control subjects and that this difference may be attributable to the haplotype AGTAAT, i.e., the haplotype with a minor (*A*) allele for rs2051040 and a major allele for all the other SNPs.

This intronic SNP rs2051040 alone was not associated with type 2 diabetes, but it was significantly associated with insulin resistance ($P = 0.002$), consistent with the known function of AMPK. The association was still significant even when the conservative Bonferroni adjustment was taken into account. Subjects with the *A* allele for rs2051040 had more marked insulin resistance, and a haplotype containing the *A* allele for rs2051040 was observed more frequently in case compared with control subjects. The initial results were further confirmed in the joint replication samples.

The risk haplotype frequency is quite close to the frequency of rs2051040 in the case subjects, for example 0.38 and 0.41 in the initial sample, but quite different, 0.29 and 0.37, respectively, in the control subjects. The A allele of rs2051040 is present on a rare haplotype, AGCAAT, whose frequency is higher in the control than in the case subjects, and the presence of this haplotype appears to be responsible for the discrepancy between the A allele of

rs2051040 and risk haplotype frequencies in the case and control subjects (online appendix Table 4).

We note two possible reasons why the association between rs2051040 and type 2 diabetes is evident in haplotypic, but not individual SNP, association analyses. One possibility is that an unidentified SNP, which is in LD with this risk haplotype but is in weaker LD with rs2051040, is the SNP actually causing type 2 diabetes. The other possibility is that rs2051040 and another SNP, contained in this risk haplotype, function in a coordinate manner to increase the risk of type 2 diabetes. The HapMap shows additional SNPs in the Chinese/Japanese sample between exon 1 and 2 of PRKAA2, where SNP finding was scarce in our study. An intronic SNP that is associated with type 2 diabetes in Japanese subjects may lie in this region. Further research to identify either the true causative SNP or as-yet-unidentified SNPs functioning in a coordinate manner with rs2051040 is needed.

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