

Assessment of the Role of Common Genetic Variation in the Transient Neonatal Diabetes Mellitus (TNDM) Region in Type 2 Diabetes

A Comparative Genomic and Tagging Single Nucleotide Polymorphism Approach

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Recent evidence supports the strong overlap between genes implicated in monogenic diabetes and susceptibility to type 2 diabetes. Transient neonatal diabetes mellitus (TNDM) is a rare disorder associated with overexpression of genes at a paternally expressed imprinted locus on chromosome 6q24. There are two overlapping genes in this region: the transcription factor zinc finger protein associated with cell cycle control and apoptosis (*ZAC* also known as *PLAGL1*) and *HYMAI*, which encodes an untranslated mRNA. Several type 2 diabetes linkage studies have reported linkage to chromosome 6q22–25. We hypothesized that common genetic variation at this TNDM region influences type 2 diabetes susceptibility. In addition to the coding regions, we used comparative genomic analysis to identify conserved noncoding regions, which were resequenced for single nucleotide polymorphism (SNP) discovery in 47 individuals. Twenty-six SNPs were identified. Fifteen tag SNPs (tSNPs) were successfully genotyped in a large case-control ($n = 3,594$) and family-based ($n = 1,654$) study. We did not find any evidence of association or overtransmission of any tSNP to affected offspring or of a parent-of-origin effect. Using a study sufficiently powered to detect odds ratios of <1.2 , we conclude that common variation in the TNDM region does not play an important role in the genetic susceptibility to type 2 diabetes. *Diabetes* 55:2272–2276, 2006

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HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency; SNP, single nucleotide polymorphism; TNDM, transient neonatal diabetes mellitus; tSNP, tag SNP.

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Type 2 diabetes is a polygenic disorder, and progress in unraveling its underlying molecular genetics has so far been modest. However, two types of study have yielded valuable insights. First, >30 genome-wide scans for linkage to type 2 diabetes have now been performed, and these have yielded some chromosome regions that have been replicated in more than one scan (1). Second, there is increasing evidence that common variation in the genes associated with rare monogenic conditions is important for disease susceptibility in common polygenic disorders (1). We have shown that heterozygous activating mutations in *KCNJ11*, which encodes the Kir6.2 subunit of the ATP-sensitive K^+ channel, are a common cause of permanent neonatal diabetes (2), while the common variant in this gene (E23K), with an allele frequency of ~ 0.35 , has been reproducibly associated with type 2 diabetes (3–6). This overlap between mono- and polygenic disease susceptibility is not restricted to neonatal diabetes genes but is also seen in the *PPARG*, *HNF4A*, and *GCK* genes (7,8,9,10).

A chromosomal region between 6q22 and 6q25 is associated with type 2 diabetes in several studies (11–16). In one study (13), evidence for linkage of diabetes to chromosome 6q was observed from paternally derived genes. Among the genes in this region are the two genes of which one or both are paternally overexpressed in 80% of cases of transient neonatal diabetes mellitus (TNDM) (17,18). These genes are the transcription factor zinc finger protein associated with cell cycle control and apoptosis (*ZAC*; also known as pleomorphic adenoma gene 1 [*PLAGL1*]), and *HYMAI*, which encodes an untranslated mRNA; both are located at chr6q24 (17,19). The primary defect in TNDM due to chromosome 6q abnormalities is β -cell dysfunction (20). This presents around birth with marked hyperglycemia, which then remits during infancy, allowing discontinuation of insulin treatment, only to relapse in late childhood or early adulthood (20). The role of common genetic variation in the TNDM region is not known but could potentially disrupt normal β -cell function and predispose to type 2 diabetes.

Since the TNDM locus lies within a chromosomal region

TABLE 1
Clinical details of study subjects

	Case subjects	Control subjects	Family study probands	Unaffected siblings
<i>n</i>	1,988	1,606	536	208
Male (%)	59	51	58	44
Age at diagnosis or testing (years)*	51 (45–57)	31 (28–35)	41 (36–47)	49 (43–55)
BMI (kg/m ²)	30.1 (26.7–34.2)	24.8 (22.2–27.8)	33.0 (28.9–37.4)	28.1 (25.4–31.2)
Treatment D/O/I (%)	11/63/26	†	20/59/21	†

Data are median (interquartile range) unless otherwise indicated. Only successfully genotyped subjects were included. No clinical details were available for the European Cell Culture Collection population control samples, so control characteristics are for the Exeter Family Study samples only. *Age at diagnosis for case subjects; age at study for control subjects. †Control subjects and unaffected siblings were not on treatment. D/O/I, diet/oral hypoglycemic agent/insulin.

associated with type 2 diabetes in several studies, it represents an excellent candidate gene for type 2 diabetes susceptibility. Therefore, we performed a large-scale case-control ($n = 3,594$) association study of the region. As the locus is imprinted, we also examined the parent of origin of transmitted alleles in a large family study ($n = 1,654$). Details of the cohorts studied are given in Table 1. Inclusion of other known types of diabetes in the type 2 diabetes probands were previously minimized through clinical, immunological, and genetic testing, as previously described (21).

Single nucleotide polymorphisms (SNPs) were extracted from GoldenPath (<http://genome.ucsc.edu>) and were also sought by resequencing the nine exons of *ZAC*, its splice junctions, putative promoter region, and noncoding regions of high homology (>75% homology over >100 bp) between human and mouse in 47 individuals. This gave >99% power to detect alleles of >5% frequency. In total, 26 SNPs were identified; this represented an average coverage of one SNP per 3.1 kb of genomic sequence (Fig. 1). The linkage disequilibrium pattern across the region was assessed using both r^2 and D' (Fig. 2). We identified tag SNPs (tSNPs) using both pairwise and aggressive tagging approaches (22). The multimarker approach did not improve the tSNP efficiency; thus, SNPs were chosen based on pairwise r^2 statistics. We successfully typed 15 tSNPs in 1,988 case and 1,606 control subjects and in a type 2 diabetes family-association study (1,654 individuals from 536 nuclear families). The 15 tSNPs captured 88% of SNPs in our study with an $r^2 > 0.7$ and 92% of the 26 SNPs with

an $r^2 > 0.5$. The results of our tSNP analysis for the case-control study are shown in Table 2 and supplementary Table 1 (online appendix [available at <http://diabetes.diabetesjournals.org>]). There was no association of any of the tSNPs with type 2 diabetes, nor were there significant deviations from expected transmission rates in our family-based study (Table 3 and supplementary Table 2). Only one of the SNPs (rs2076684) reached nominal significance ($P = 0.05$) in the family study. There was no association of any of the SNPs with diabetes when the family-based study was combined with the case-control study (data not shown).

Given the imprinted status of the *TNDM* locus, we used our family data to test the hypothesis that variants were overtransmitted by parent of origin. There was no evidence for a parent-of-origin effect (data not shown).

This study is well designed to have sufficient power to detect moderate diabetes susceptibility polymorphisms. The case-control component of our study was designed to have >80% power to detect ORs of 1.14 (minor allele frequency [MAF] 0.1 [0.50–1.24]) at $P < 0.05$. The coverage of the *ZAC* locus and surrounding sequence was good, with an average density of one SNP per 3 kb, and we have captured the majority of common variants with a mean $r^2 > 0.7$. Of the 26 SNPs in our study, 14 are also in HapMap 2. These 14 SNPs captured 58% (42 of 73) of the 73 HapMap SNPs (>5% MAF in Caucasians) with an $r^2 > 0.7$ and 86% (63 of 73) with an $r^2 > 0.5$. A comparison of our data with HapMap 2 is shown in supplementary Fig. 2. As with any large association study, we cannot exclude the possibility

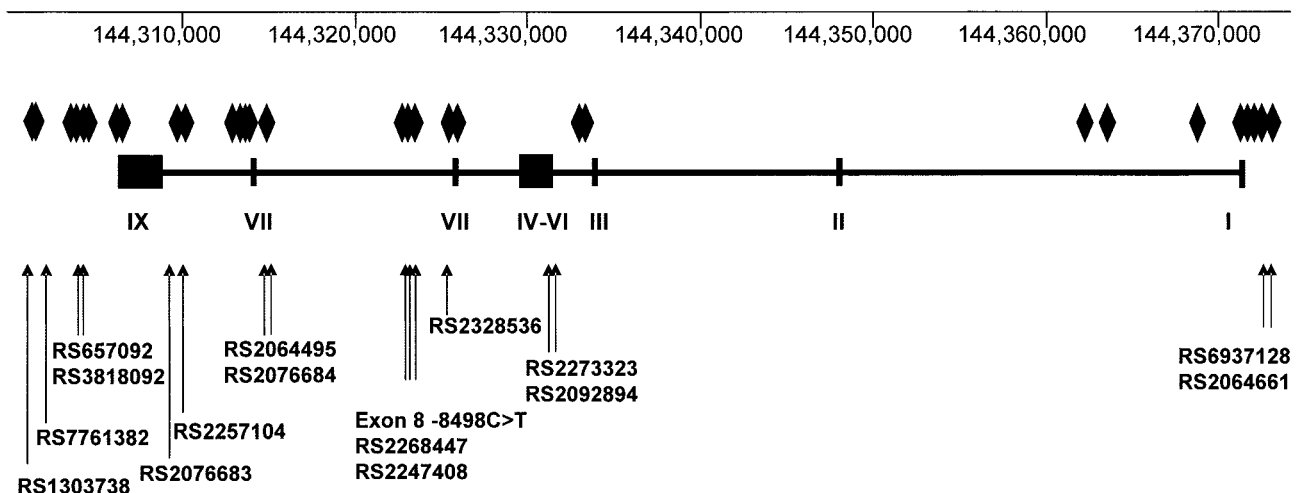


FIG. 1. Schematic representation of the *ZAC/PLAGL1* gene. Schematic representation of the *ZAC/PLAGL1* gene region illustrating the nine exons of *ZAC* and the position of the 26 SNPs identified. tSNPs are indicated by arrows, and the 26 SNPs identified are shown as diamonds.

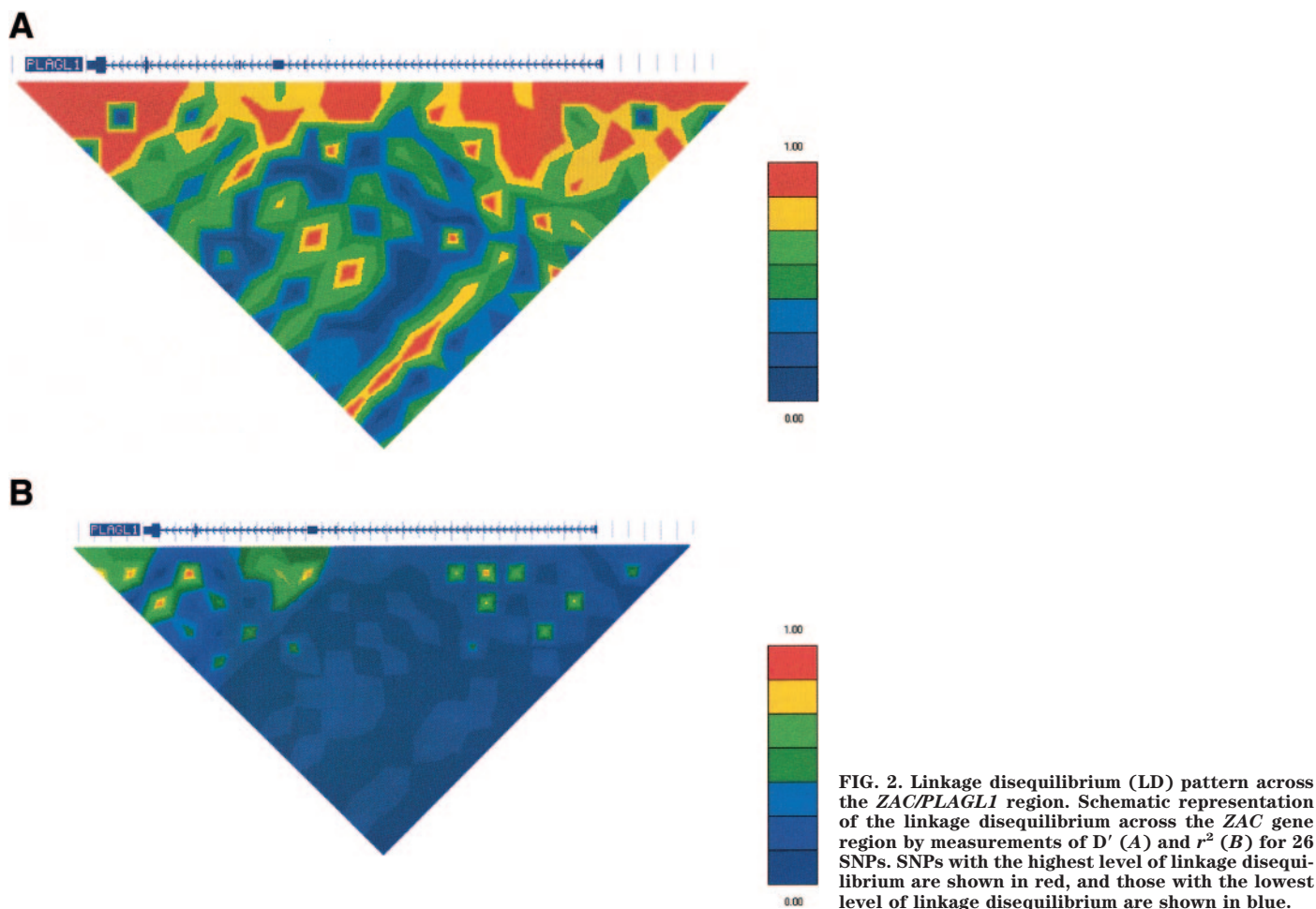


FIG. 2. Linkage disequilibrium (LD) pattern across the ZAC/PLAGL1 region. Schematic representation of the linkage disequilibrium across the ZAC gene region by measurements of D' (A) and r² (B) for 26 SNPs. SNPs with the highest level of linkage disequilibrium are shown in red, and those with the lowest level of linkage disequilibrium are shown in blue.

that multiple rare alleles (<5%) at this locus are associated with type 2 diabetes. We are aware that the control subjects used are a younger, population-based resource rather than age matched to our cases. As ~5% of the control subjects will go on to develop diabetes, this would

slightly reduce the power of our study compared with the use of the same number of age-matched control subjects. However, using the same case-control and family-based samples, associations have recently been shown between

TABLE 2
Case and control subjects' tSNP analysis

SNP name	Alleles (1/2)	Allele 1 frequency in control subjects	OR (95% CI) for allele 1	P
rs7761382	C/T	0.57	0.98 (0.89–1.08)	0.71
rs6570592	C/T	0.46	1.01 (0.92–1.11)	0.89
rs3818092	G/C	0.23	0.94 (0.84–1.05)	0.25
rs2076683	T/G	0.44	1.04 (0.95–1.15)	0.42
rs2257104	C/A	0.57	1.01 (0.92–1.11)	0.86
rs2076684	C/G	0.09	0.95 (0.80–1.14)	0.58
rs2064495	T/C	0.79	1.08 (0.96–1.21)	0.20
Exon8-8498C>T	T/C	0.15	1.06 (0.93–1.21)	0.36
rs2268447	T/C	0.31	0.98 (0.89–1.08)	0.68
rs2247408	T/C	0.31	0.98 (0.89–1.09)	0.73
rs2328536	T/C	0.69	1.02 (0.92–1.13)	0.68
rs2273323	T/C	0.06	1.02 (0.84–1.25)	0.84
rs2092894	G/A	0.55	1.02 (0.93–1.12)	0.71
rs6937128	G/A	0.25	0.98 (0.88–1.10)	0.77
rs2064661	C/T	0.82	1.00 (0.88–1.13)	0.99

The case-control study had 80% power to detect an OR >1.2–1.4. The simple allelic OR model is presented. Multiplicative, recessive, and dominant models were all tested and were also negative.

TABLE 3
Family-based tSNP analysis

SNP name	Alleles (1/2)	Overall observed allele 1 transmission	Overall expected allele 1 transmission	Overall P
rs7761382	C/T	269	267	0.87
rs6570592	C/T	270	254	0.14
rs3818092	G/C	141	148	0.47
rs2076683	T/G	248	253	0.70
rs2257104	C/A	263	258	0.66
rs2076684	C/G	40	50	0.05
rs2064495	T/C	196	189	0.46
Exon8-8498C>T	T/C	114	111	0.70
rs2268447	T/C	185	195	0.32
rs2247408	T/C	176	188	0.20
rs2328536	T/C	226	217	0.34
rs2273323	T/C	41	49	0.12
rs2092894	G/A	242	239	0.81
rs6937128	G/A	172	165	0.47
rs2064661	C/T	139	154	0.07

Results were analysed using the transmission-disequilibrium test/sib transmission-disequilibrium test methods. The family study had 80% power to detect ORs of 1.4 and 1.68 for MAFs of 50% and 10%, respectively.

the *KCNJ11*, the K23 allele (3), the *HNF4A* P2 promoter haplotype (9), and the *PPARG* P12 allele (23) and type 2 diabetes, with ORs consistent with other large type 2 diabetes case-control studies and meta-analyses of multiple studies. Conversely, the large negative studies we have performed in *HNF1A* (24) also have not been found to be positive in similar-sized studies (25), suggesting that our results are representative.

We have the largest set, internationally, of type 2 diabetic nuclear families to assess parent-of-origin effects. These resources are difficult to obtain due to the late age of onset of most patients with type 2 diabetes (26). Even though our cohort is relatively large, we acknowledge that our parent-of-origin study was of limited power to rule out a moderate susceptibility variant, but we had power to detect ORs of 1.4 and 1.68 for MAF of 0.5 and 0.1, respectively.

In conclusion, using a comparative genomic and tSNP approach, we have comprehensively assessed the role of common genetic variation in susceptibility to type 2 diabetes. We have found no evidence in a large-scale study in a U.K. Caucasian population that common genetic variation at this locus influences type 2 diabetes susceptibility. TNDM is unusual among genetic disorders in that the disease is caused by an overabundance of a protein, rather than its absence, and occurs chiefly by epigenetic rather than genetic change. This study suggests that it is unlikely that a possible epigenetic difference at the TNDM locus is inherited from this locus. However, the degree of epigenetic variation at the TNDM locus in critical tissues for glucose homeostasis has not been investigated and thus cannot be excluded.

RESEARCH DESIGN AND METHODS

Variant detection and assessment of linkage disequilibrium in subjects. SNPs were identified by sequencing, on an ABI 3100 platform, using standard protocols, 1) the coding sequence, splice junctions, and promoter of *ZAC* and 2) regions of high (>75% over 100 bp) conservation between the genomic sequences of human and mouse *ZAC* and 20-kb flanking sequence (Santa Cruz build of Feb 2003; <http://genome.ucsc.edu>). Many of the SNPs thus identified were represented in Goldenpath (<http://genome.ucsc.edu>), from which additional SNPs were identified. SNPs were initially genotyped by sequencing 47 anonymized, unrelated, control individuals from the Wessex Regional Genetics Laboratory research archives, in order to select those in Hardy-Weinberg equilibrium (HWE) and with MAF >0.05. Twenty-six SNPs met these criteria. This study was started before HapMap 2 data were available.

Pairwise linkage disequilibrium estimates were calculated using GOLD (27). A tagger was used to select tSNPs (<http://www.broad.mit.edu/mpg/tagger/>) (22).

Case-control and family-based study subjects. The clinical characteristics of the case-control subjects are presented in Table 1. Informed consent was obtained from all subjects. Only samples that we attempted to genotype for all SNPs were included in the analyses. All type 2 diabetic participants were unrelated U.K. Caucasians with diabetes defined either by World Health Organization criteria or empirically by treatment for diabetes, and we excluded patients with type 1 diabetes and rare diabetic subtypes, such as maturity-onset diabetes of the young and late autoimmune diabetes in adults, by means of genetic or clinical testing. The type 2 diabetes case group was assembled from three sources: a collection of early-onset (aged 18–45 years) type 2 diabetic patients, probands from type 2 diabetes sibships from the Warren 2 repository (both described previously [21]), and a group of type 2 diabetic subjects from the Warren repository aged 35–65 years at diagnosis with no family history (9). Control subjects were derived from two sources: individuals from the Exeter Family Study (21) and a nationally recruited collection of U.K. Caucasians obtained from the European Cell Culture Collection. The clinical characteristics of the probands from the family-based sample are presented in Table 1, and for some subjects, clinical characteristics have been previously described (26). Families comprised an affected proband with either both parents or one parent with at least one unaffected sibling.

Genotyping and quality control. Genotyping was performed by KBioSciences (Herts, U.K.) by means of modified TAQMAN assays (www.kbio

[science.co.uk](http://www.kbio)). Calculation of genotype concordance between duplicate samples showed an overall genotyping accuracy of 99.9% (10 errors out of 8,770 informative duplicates [9.5% of the sample]). The minimum duplicate concordance rate was 99.2% for rs3818092. The overall genotype success rates were as follows: 96% for cases, 96% for control subjects, and 95% for families. All individual SNPs had a genotype success rate >90% in cases, control subjects, and families. After excluding families with obvious relationship inconsistencies (as determined by the genotyping of an additional 77 SNPs), the Mendelian inconsistency error rate was 0.0004. There was no evidence of heterogeneity between the case and control subjects (all $P > 0.01$), except for the comparison between the W2 cases and W2 probands for SNP rs2064661 (OR 1.35, $P = 0.001$). This comparison is not significant when we adjust for multiple hypothesis testing. All cohorts were in HWE in both case and control subjects (all $P > 0.01$), except for one SNP (rs2257104) that deviated mildly in the control cohort only ($P = 0.006$). For the 77 SNPs that have been genotyped in this and other studies in these control groups, 3 SNPs (0.039) had a P value of <0.05; 2 SNPs (0.026) had a P value of <0.01 (including rs2257104). The results are therefore as expected given the number of SNPs genotyped. However, to rule of genotyping quality, we resequenced two 96-well plates of control subjects and found two discrepancies in this subset of 172 (1%) control subjects, which is insufficient to explain the deviation from HWE. Given the absence of other quality-control errors for this SNP, we suggest that this is due to multiple testing rather than genotyping error.

Statistical analysis. ORs and P values for the case-control study were calculated using χ^2 tests. For the family studies, the expected transmissions and P values were calculated using the sibTDT program Discordant Alleles Sib Test and transmission-disequilibrium test (28). Similar results were obtained using the Family Based Association Test program (29). Parent-of-origin data were obtained using the Parent of Origin Association Test of Weinberg (30). To estimate the ORs for the combined analysis of case-control and family-based subjects, we combined ORs from the discordant allele, transmission-disequilibrium test, and case-control studies using Mantel-Haenszel meta-analysis.

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