

Variants in the Ca_v2.3 (α1E) Subunit of Voltage-Activated Ca²⁺ Channels are Associated with Insulin Resistance and Type 2 Diabetes in Pima Indians

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Running Title: Association of CACNA1E with type 2 diabetes

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ABSTRACT

Objective: Linkage to type 2 diabetes mellitus (T2DM) has been reported on chromosome 1q21-25 in Pima Indians. Fine mapping identified SNPs near the *CACNA1E* gene associated with this disease. *CACNA1E* encodes the voltage-dependent calcium channel $Ca_v2.3 Ca^{2+}$, and mice lacking this channel exhibit impaired glucose tolerance and insulin secretion. Therefore, *CACNA1E* was investigated as a positional candidate gene.

Research Design and Methods: *CACNA1E* was sequenced and 28 SNPs were genotyped in the same group of Pima subjects who had been analyzed in the linkage study. Allele specific expression was used to functionally evaluate a variant in the 3'UTR.

Results: A novel G/A variant in the 3'UTR was associated with young-onset T2DM (odds ratio=2.09 per copy of the G allele; 95% CI=1.31-3.33, adjusted p=0.001) and had an effect upon the evidence for linkage at chromosome 1q21-25 (p=0.004). Among 372 non-diabetic Pima subjects who had undergone metabolic testing, the risk allele was associated with reduced insulin action that included increased fasting, 30, 60 and 120 minute plasma glucose concentrations and increased fasting plasma insulin during an oral glucose tolerance test (all p<0.01) as well as a decreased rate of insulin-stimulated glucose disposal at both physiologically and maximally stimulated insulin concentrations (both p<0.002). Functional analysis of this variant showed that the non-risk allele had a 2.3 fold higher expression as compared to the risk allele.

Conclusion: A functional variant in *CACNA1E* contributes to T2DM susceptibility by affecting insulin action. This variant partially explains the linkage to T2DM on chromosome 1q21-25 in Pima Indians.

INTRODUCTION

Voltage-gated Ca^{2+} channels mediate Ca^{2+} entry into cells in response to membrane depolarization. These channels are composed of a major pore-forming subunit ($\alpha 1$ subunit) and multiple auxiliary subunits. The human $\text{Ca}_v2.3$ subunit is expressed in neuronal cells (2) and pancreatic β -cells (3). In β -cells, this subunit has an important role in coordinating Ca^{2+} -dependent processes such as excitation secretion of insulin. Recent studies in $\text{Ca}_v2.3$ -deficient mice showed that disruption of $\text{Ca}_v2.3$ Ca^{2+} channels impaired glucose tolerance and insulin secretion (4-5), while insulin tolerance tests in $\text{Ca}_v2.3$ knockout mice indicated reduced insulin sensitivity (5). $\text{Ca}_v2.3$ $-/-$ mice were also significantly heavier than wild-type mice, and their basal levels of plasma glucose and insulin tended to be higher, suggesting that the cause of hyperglycemia in $\text{Ca}_v2.3$ $-/-$ mice may be insulin resistance (5).

Human $\text{Ca}_v2.3$ is encoded by the *CACNA1E* gene, which maps to a region on chromosome 1q21-25. Linkage to T2DM at chromosome 1q21-25 has been reported in diverse populations with European, East Asian, Native American and African American ancestries (6-12). In a genome-wide linkage scan in Pima Indians, this region was linked to young-onset T2DM (12), and follow-up studies in the Pima Indians included association mapping with SNPs at ~ 10 kb density spanning the 25 Mb region of linkage. Results from association mapping identified several variants in/near *CACNA1E* that were modestly associated with T2DM. Therefore, *CACNA1E* was directly analyzed as a positional candidate gene for linkage to T2DM in Pima Indians.

METHODS

Subjects:

Twenty-four non-first degree related Pima Indians were selected for sequencing of the *CACNA1E* coding and promoter regions. Among these 24 subjects, 12 developed T2DM before the age of 25 years, and 12 were non-diabetic after 45 years of age. Variants were genotyped for association analyses in 1037 Pima Indians (from 332 nuclear families) who were participants in our prior genome-wide linkage study for determinants of T2DM (12). Among these 1037 subjects, 680 contributed to the original linkage analysis on chromosome 1q and were analyzed in the present study for association with young-onset diabetes. These 680 subjects included 578 with T2DM with an age of onset of <45 years (mean \pm SD age onset=31.0 \pm 7.8 years; 62.1% female; maximum BMI=37.9 \pm 8.0 kg/m²) and 102 who were non-diabetic and ≥ 45 years old (mean age=53.7 \pm 6.3 years; 60.8% female; maximum BMI=34.6 \pm 6.3 kg/m²). The remaining genotyped subjects were analyzed for quantitative metabolic traits (see below). Diabetes was diagnosed by an oral glucose tolerance test (OGTT) according to the World Health Organization criteria (13). Subjects provided written consent prior to participation. This study was approved by the Tribal Council of the Gila River Indian Community and the Institutional review Board of NIDDK.

Metabolic Phenotyping:

Among the 1037 subjects genotyped, 372 were non-diabetic, full heritage Pima Indians who had also undergone metabolic testing. Glucose tolerance was determined by a 75g OGTT with measurements of fasting, 30, 60, 120, and 180-min plasma glucose and insulin

concentrations (14). To measure the acute insulin response, blood samples were collected prior to a 25 g glucose bolus infusion and at 3, 4, 5, 6, 8, and 10-min after infusion. The acute insulin response was calculated as the mean increment in plasma insulin concentrations from 3-5 min (15). Insulin action *in vivo* was assessed using a two-step hyperinsulinemic-euglycemic clamp (14-15). Body composition was estimated by underwater weighing until January, 1996 and by dual energy x-ray absorptiometry (DPX-1, Lunar Radiation Corp.) thereafter.

Sequencing and Genotyping:

Sequencing was done using Big Dye terminator (Applied BioSystems) on an automated DNA capillary sequencer (model 3730; Applied Biosystems). Variants were genotyped using the TaqMan Allelic Discrimination Assay (Applied BioSystems) on an ABI Prism 7700 (Applied BioSystems) or SNPlex genotyping System 48-plex (Applied BioSystems) on an automated DNA capillary sequencer.

Allelic-Specific Gene Expression:

Subcutaneous abdominal adipose tissue biopsies were obtained from 11 subjects known to be heterozygous (G/A) for the 3'UTR SNP +8130G/A. Biopsies were performed in our Clinical Research Center as previously described (16). Total RNA was extracted from the adipose tissue using an RNAeasy Mini Kit (Qiagen, Valencia, CA), and cDNA was synthesized by reverse-transcription (Clone-tech). Allelic-specific gene expression was assessed by real-time PCR using TaqMan allele-specific probes (TGCAGCTGGTGTGGG-VIC; TGCAGCTGATGTGGG-FAM; Applied BioSystems) on an ABI Prism 7700 (Applied BioSystems) (17). Triplicate

experiments were performed. Data were presented as a ratio of allelic expression (A/G). Background was calculated as allelic ratio of genomic DNA (assumed ratio=1).

Statistical Analysis:

Associations were assessed using programs of the SAS institute (Cary, NC). The relationship between trait and marker was generally assessed via a numeric variable representing genotype (i.e., an additive model). For continuous variables, linear regression was used to adjust for covariates. The association with young-onset diabetes was assessed by logistic regression and was controlled for sex and heritage. Both linear and logistic models were fit with generalized estimating equations to account for the correlation among siblings (18). This method provides a general test of association that accounts for the non-independence among siblings, but, as it is not a test of within-family association, it is not robust to population stratification. Therefore, to control for potential population stratification, the association with diabetes was also analyzed with a within-family association test using the method of Abecasis et al (19). To assess the impact of the observed association on the linkage, LOD scores were recalculated with and without adjustment for the marker and the statistical significance of the difference was assessed with a modified bivariate Haseman-Elston model (20). Haplotype frequencies for pairs of SNPs were calculated in all 1037 individuals with the Estimating Haplotypes (EH) program. D' was calculated as a measure of allelic association, and r^2 as a measure of concordance. For haplotype analyses SNPs were placed in "blocks", which were defined as consecutive SNPs with $D' > |0.9|$. By this definition blocks can

overlap; for the sake of thoroughness we analyzed all blocks, regardless of overlap, and made no attempt to place each SNP in a unique block. Association between traits and individual haplotypes were examined with a modification of zero-recombinant haplotyping procedure, as described previously (21).

RESULTS AND DISCUSSION

Sequencing of 49 exons and ~2kb of the upstream region of *CACNA1E* in 24 Pima Indians identified 14 variants. Two variants were novel, a -1039G/T (1039 bp upstream of the ATG site) and a +8130G/A in the 3'UTR (8130bp downstream from ATG in the cDNA), while the remaining 12 SNPs could be found in public databases. Sixteen additional SNPs, positioned within introns and more distal regions flanking the *CACNA1E* locus that were not sequenced, were selected from public databases to provide a denser coverage of genetic information across this region. All 28 SNPs (online Figure 1, Table 1 [available at <http://diabetes.diabetesjournals.org>]) were genotyped in 1037 Pima Indians for association analyses.

The frequencies of the 28 variants in subjects with young-onset T2DM compared with the older (age>45 years) non-diabetic subjects, are given in Table 1. All SNPs were in Hardy-Weinberg equilibrium. Five of the variants, rs625226, rs3753737, rs798209, rs473200 and +8130G/A, were nominally associated with young-onset T2DM in a general association test. Among these SNPs, rs3753737 and +8130G/A were also nominally associated with T2DM using a within family test. In simulation studies, we estimate that the power of the present sample is > 70% to detect a significant ($p < 0.05$) association with a causal variant with minor allele frequency > 0.05 accounting for 2% of the variance in age of

onset of diabetes. Given the previous physiological implication of *CACNA1E* in diabetes in mice, we chose to preserve statistical power and did not correct for multiple comparisons. Therefore, it remains possible that these nominal associations may represent false positive findings.

To help address whether these associations may be false positives, we further investigated whether any of the 28 variants were associated with pre-diabetic traits among 372 non-diabetic, full-heritage Pima Indians who had undergone metabolic testing. Among these non-diabetic individuals, subjects with the G/G genotype at +8130G/A, which was the risk genotype for young-onset T2DM, had significantly higher mean fasting, 30-min, 60-min and 120 min plasma glucose concentrations and higher mean fasting plasma insulin concentrations during an OGTT, as well as a lower mean rate of insulin-stimulated glucose disposal at both physiologically and maximally stimulated insulin concentrations during a hyperinsulinemic, euglycemic clamp (Table 2). These results in humans are consistent with the finding of increased post-load plasma glucose levels and decreased insulin sensitivity in *CACNA1E* knockout mice (5). Prior *in vitro* studies using the insulinoma cell line INS-1 suggested a role of *CACNA1E* in insulin secretion (22), but our *in vivo* study did not detect an association between SNPs in this gene and acute insulin secretion using a 25g glucose bolus infusion.

The degree of linkage disequilibrium (LD; summarized by D' and r^2) between the 28 variants in 1037 subjects is shown in Figure 1. The common variants (minor allele frequency > 0.05) fell into 13 haplotype "blocks." Each block, ranging in size from 0.6-93.6 kb, contained on average 2- 3 SNPs, except for one block located in 3'UTR which extended across 5

SNPs. Five haplotypes were associated with T2DM ($p \leq 0.05$) (Table 3). The strongest association was with haplotype AA for +8130G/A and rs16858150, where its frequency was lower in cases compared to controls (frequency= 0.09 in diabetic vs. 0.17 non-diabetic subjects, OR=0.44, CI 0.28-0.70, $p=0.001$). Conversely the frequency of haplotype GA for +8130G/A and rs16858150 was higher in the cases compared to the controls (frequency= 0.85 in diabetic vs. 0.74 in non-diabetic subjects; OR=1.82, CI 1.23-2.69, $p=0.005$). This finding largely reflects the single marker associations of +8130G/A. Additional analyses examined pairs of SNPs or haplotypes such that +8130G/A was analyzed in conjunction with each additional significant variant to determine their potential independent contributions to the association. The effect of +8130G/A was statistically significant ($p < 0.05$) conditional on each of these other variants, but none of the other variants was significant conditional on +8130G/A; the association of the GAG haplotype in block 2 was of marginal significance controlled for +8130G/A ($p=0.07$). Thus +8130G/A represents the major associated variant in the present study.

Since SNP +8130G/A provided the strongest evidence for disease association using either a single SNP or haplotype analysis, this variant was functionally analyzed. The location of this variant in the 3'UTR led to the hypothesis that it could affect mRNA expression/stability. Allelic-specific expression in adipose tissue mRNA isolated from 11 subjects heterozygous for the G/A showed that the ratio of A (non-risk) allele vs. G (risk) allele (A/G) was 2.37 ± 0.30 (mean \pm SEM), which was significantly different from the background ratio of 0.97 ± 0.09 (t-test $p=0.008$).

To assess whether the +8130G/A contributed to the evidence for linkage to young-onset T2DM on chromosome 1 in Pima Indians, LOD scores were recalculated with and without adjustment for this marker. A reduction in linkage of 0.6 LOD (from 2.6 to 2.0, $p=0.004$) was observed after adjustment for +8130G/A. No reduction, or a smaller reduction, was observed when the linkage was adjusted for the other SNPs genotyped in/near this gene.

Although the +8130G/A variant appears to underlie a portion of the linkage on chromosome 1q21-25 among families selected for having a high prevalence of young-onset T2DM, the effect of this variant on diabetes risk in the general Pima population appears to be minimal. The association of this variant with T2DM in a population-based sample of Pima Indian subjects (after excluding all participants of the linkage study, $N=2427$; 862 with diabetes, 1565 without diabetes) was marginal (OR=1.17 one-sided $p=0.08$). Thus, it appears that this variant has its greatest impact in families with a high prevalence of young-onset diabetes, such as those selected for the linkage study and the present follow-up study.

Linkage to T2DM on chromosome 1q21-25 has also been observed in 7 other populations in the International Type 2 Diabetes Chromosome 1q Consortium (6-14). As part of this consortium, SNPs are being genotyped in subjects from all of these populations across a region that spans 148-170 Mb on chromosome 1 (23). *CACNA1E* is positioned slightly telomeric to this region at 179.5 Mb. However, SNP +8130G/A was specifically selected to attempt replication in the consortium samples (1540 cases with type 2 diabetes and 1512 controls without diabetes). The frequency of the G allele was ~ 0.70 among the Chinese and ~ 0.95 for most of the European-derived populations and for

African-Americans; the one exception was the Amish in whom the frequency was 0.83. This SNP was not associated with T2DM in the combined samples from the consortium (Odds ratio = 1.06 per copy of the G allele; 95% CI= 0.90-1.27, $p=0.46$, adjusted for population), nor was this SNP significantly associated ($p<0.05$) with diabetes among any of the individual populations. Two additional SNPs in *CACNA1E* (rs473200 and rs704326) were also genotyped in the consortium samples, but neither replicated. Further detailed mapping in the consortium samples or other populations with a high prevalence of young-onset T2DM is required to determine the role of other variants in or near *CACNA1E* in susceptibility to T2DM.

It is possible that the present findings represent a false positive, however, this SNP has the second strongest evidence for association with young-onset T2DM in Pima Indians among the 5150 SNPs typed to date across the region of linkage. The SNP with the strongest evidence for association maps to an intergenic region, and this SNP is similarly not associated with T2DM in the combined consortium samples. The third strongest association maps to *ARHGEF11*, where variants in this gene are also associated with T2DM in the Amish population, but not in the combined consortium samples (24, 25). Therefore, an alternative explanation for the lack of replication in other ethnic groups is that multiple variants underlie the various linkage observations on chromosome 1q21-25, and these variants have different effect sizes among

the different ethnic groups. The observed difference between populations for the effect of this variant could also be explained by clinical heterogeneity. Pima Indians, in particular those selected for the linkage study and the current follow-up study, have a much earlier age of onset of diabetes as compared with the other populations. Pima Indians, on average, also have a much high BMI. Studies of *CACNA1E* in other groups of subjects with young-onset diabetes and/or high BMI are required to examine this possibility.

Our allele specific expression study suggests that +8130G/A may affect diabetes susceptibility via reduction of *CACNA1E* expression. Since the risk allele was the common allele (frequency =0.89), reduced *CACNA1E* expression, reduced *in vivo* insulin action, and a modest increase in diabetes risk is the common phenotype among Pima Indians.

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