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# Replication of recently described type 2 diabetes gene variants in a South Indian population

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**Abstract**

Recent genomewide association studies have identified several new gene variants associated with type 2 diabetes mellitus (T2D) mostly in European populations. These need to be replicated in other populations. We studied 926 unrelated T2D and 812 normal glucose-tolerant subjects randomly selected from the Chennai Urban Rural Epidemiology Study in Southern India. A total of 45 single nucleotide polymorphisms (SNPs) from 15 genes and 13 unannotated loci identified from recent genomewide association T2D studies were genotyped. Only 6 of 45 SNPs studied were replicated in this South Indian population. Three SNPs—rs7756992 ($P = .007$), rs7754840 ($P = .015$), and rs6931514 ($P = .029$)—of the *CDKAL1*, rs7020996 ($P = .003$) of the *CDKN2A/B* gene, rs7923837 ($P = .038$) of the *HHEX* gene, and rs12056034 ($P = .033$) of the *BAZ1B* gene were associated with T2D in our population. Large-scale studies are needed in our population to validate our findings.
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## 1. Introduction

The precise mechanisms underlying the development of type 2 diabetes mellitus (T2D) have not been elucidated, but a combination of multiple genetic and environmental factors appears to play important roles in the pathogenesis of the disease [1]. That genetic factors also affect the predisposition to T2D is shown by heritability studies [2]. Candidate genes and positional cloning efforts have suggested many putative susceptibility variants, but unequivocal replications are so far limited to variants in just 3 genes: *PPARG*, *KCNJII*, and *TCF7L2* [3-6].

Recent genomewide association studies (GWASs) conducted by several independent European and American groups have identified multiple susceptibility loci for T2D in populations of European descent [7-11]. Some of these loci have been shown to be associated with T2D in other ethnic populations also [12-16]. Therefore, it is imperative to evaluate the contributions of these loci in conferring susceptibility to T2D in other ethnic populations. This is particularly so because there are differences in the frequencies of some genetic associations in different ethnic groups [17]. It is well known that Asian Indians have greater insulin resistance [18], greater waist to hip ratio [19], and increased susceptibility to diabetes [20-22] compared with Europeans; and this is referred to as the *Asian Indian Phenotype* [23]. With this background, the aim of the present study was to determine whether the gene variants identified by the recent T2D GWASs in other populations are associated with susceptibility to T2D in an Asian Indian (South Indian) population.

## 2. Subjects and methods

### 2.1. Subject

The study subjects were recruited from the Chennai Urban Rural Epidemiology Study (CURES), an ongoing epidemiology study conducted on a representative popula-

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tion (aged ≥20 years) of Chennai (formerly Madras), the fourth largest city in India with a population of about 5 million people. The methodology of the study has been published elsewhere [24]. In Phase I of CURES, 26 001 individuals were recruited based on a systematic random sampling technique. Self-reported diabetic subjects on drug treatment of diabetes were classified as "known diabetic subjects." Fasting capillary blood glucose was determined using a One Touch Basic glucometer (Life scan Johnson & Johnson, Milpitas, CA) in all subjects. In Phase 2 of CURES, the known diabetic subjects (n = 1529) were invited to visit the center for detailed studies. In Phase 3, every 10th subject without known diabetes (n = 2600) was recruited to undergo oral glucose tolerance tests using 75-g oral glucose load (dissolved in 250 mL of water); and the response rate was 90% [25]. Subjects who had fasting plasma glucose less than 6.1 mmol/L (110 mg/dL) and 2-hour plasma glucose value less than 7.8 mmol/L (140 mg/dL) were categorized as normal glucose tolerant (NGT). Those who were confirmed by oral glucose tolerance test to have 2-hour plasma glucose greater than or equal to 11.1 mmol/L (200 mg/dL) based on World Health Organization consulting group criteria were labeled as "newly detected diabetic subjects."

Type 1 diabetes mellitus is diagnosed at our center in those who require insulin right from diagnosis or if they have ketosis or documented evidence of ketoacidosis or poor insulin reserve as shown by C-peptide. None of the diabetic subjects in this study satisfied the criteria for type 1 diabetes mellitus, namely, requirement of insulin from the time of diagnosis for the control of hyperglycemia or evidence of ketosis or ketoacidosis or absent $\beta$-cell reserve as tested by C-peptide levels [26,27].

For the present study, we randomly selected (using randomly generated numbers) unrelated 926 T2D and 812 NGT subjects from Phase 3 of CURES. In this study, 135 subjects were newly detected for diabetes, whereas 791 subjects were already known diabetic subjects. Written informed consent was obtained from all study subjects, and the protocol was approved by the Institutional Ethics Committee of the Madras Diabetes Research Foundation.

### 2.2. Measurement of clinical and biochemical variables

Anthropometric measurements including weight, height, and waist measurements were obtained using standardized techniques. The body mass index (BMI) was calculated using the formula weight (in kilograms)/height (in square meters). Blood pressure (BP) was measured with a mercury sphygmomanometer (Diamond Deluxe BP apparatus, Pune, India) from the left arm in supine position after a 30-minute rest in bed. Two readings were taken 5 minutes apart, and the mean of the 2 was taken as the BP. Fasting plasma glucose (glucose oxidase-peroxidase method), serum cholesterol (cholesterol oxidase-peroxidase-amidopyrine method), serum triglycerides (glycerol phosphate oxidase-peroxidase-amidopyrine method), and high-density lipoprotein cholesterol (direct method polyethylene glycol–pretreated enzymes) were measured using Hitachi-912 Autoanalyzer (Hitachi, Mannheim, Germany). The intra- and interassay coefficient of variation for the biochemical assays ranged between 0.04 to 0.08. Low-density lipoprotein cholesterol was calculated using the Friedewald formula. Glycated hemoglobin was estimated by high-pressure liquid chromatography using the Variant machine (Bio-Rad, Hercules, CA). The intra- and interassay coefficient of variation of glycated hemoglobin was less than 10%.

### 2.3. Genotyping

DNA was isolated from whole blood using the phenol chloroform method, and quantity and purity of each DNA sample were determined by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). We selected 45 single nucleotide polymorphisms (SNPs) for genotyping at each locus (AHI1, BAZ1B, CDKAL1, EXT2, HHEX, IGF2BP2, LOC387761, LOC441171, MC4R, MLXIPL, SLC30A8, STK32C, PPARG, WFS1, and 12 unannotated loci) on the basis that they were previously reported to be associated with T2D by at least one of the GWASs. All SNPs were genotyped using MassARRAY system (Sequenom, San Diego, CA). Briefly, polymerase chain reaction amplification and primer extension were performed according to the manufacturer's instructions and as published elsewhere [28]. After desalting of the reaction products, approximately 15 nL was loaded into a SpectroCHIP (Sequenom) by using Nanodispenser-1000 and analyzed in the fully automated mode with the MALDI TOF MassARRAY system (Sequenom). SpectroTYPER software (Sequenom) automatically called genotypes, and only conservative and moderate calls were accepted for this study. Ten percent of the samples were replicated, and discordances in replicate samples were less than 0.4%. The call rate for each assay was set at greater than or equal to 85%.

### 2.4. Statistical analysis

Hardy-Weinberg equilibrium (HWE) was performed by using Pearson $\chi^2$ statistics in cases and controls separately for each and every SNP including the 6 associated SNPs. Single nucleotide polymorphisms with HWE $P < .05$ or $\chi^2$ value greater than 3.84 were excluded to ensure that SNPs are in HWE. Statistical analyses were performed in SPSS (Chicago, IL) version 15.0, and the allelic frequencies between the diabetic patients and controls were compared using $\chi^2$ tests. The odds ratios (ORs) with 95% confidence interval (CIs) are presented for each SNP. Power was estimated using PS Power and Sample Size Calculations (Nashville, TN).

## 3. Results

Table 1 shows the clinical and biochemical characteristics of the study group. Compared with NGT subjects, the

Table 1
Clinical and biochemical characteristics of the study groups

| Parameters | NGT (n = 812) | DM (n = 926) | *P* value |
|---|---|---|---|
| Age (y) | 38 ± 12 | 52 ± 11 | <.001 |
| BMI (kg/m$^2$) | 23 ± 4 | 25 ± 4 | <.001 |
| Systolic BP (mm Hg) | 117 ± 17 | 131 ± 23[a] | <.001 |
| Diastolic BP (mm Hg) | 73 ± 11 | 78 ± 12 | <.001 |
| Fasting Insulin ($\mu$IU/mL)[a] | 8.5 ± 5.5 | 12.2 ± 9.6 | <.001 |
| Fasting plasma glucose (mg/dL) | 85 ± 8 | 163 ± 75 | <.001 |
| 2-h post plasma glucose (mg/dL)[a] | 101 ± 20 | 281 ± 64 | <.001 |
| Glycosylated hemoglobin (%) | 5.5 ± 0.47 | 8.6 ± 2.3 | <.001 |
| Total cholesterol (mg/dL) | 176 ± 37 | 202 ± 42 | <.001 |
| Serum triglycerides (mg/dL) | 113 ± 63 | 181 ± 135 | <.001 |
| LDL cholesterol (mg/dL) | 111 ± 31 | 123 ± 38 | <.001 |
| HDL cholesterol (mg/dL) | 43 ± 10 | 42 ± 9 | .243 |

LDL indicates low-density lipoprotein; HDL, high-density lipoprotein.
[a] Computed only for NGT and newly diagnosed diabetes.

diabetic subjects were older ($P < .001$) and had significantly higher BMI ($P < .001$), systolic and diastolic BP ($P < .001$), serum cholesterol ($P < .001$), and triglycerides ($P < .001$).

Results of all the 45 SNPs studied are given in Appendix Table 1. All the 45 SNPs passed quality control filters. None of the SNPs showed monomorphic condition in this population.

A separate analysis comparing the newly diagnosed (135) and known diabetes (761) subjects showed that age, fasting plasma glucose, and glycated hemoglobin were significantly increased in subjects with known diabetes compared with newly diagnosed diabetic subjects($P < .05$); but there were no significant differences in the genotype frequencies between known diabetic and newly diagnosed diabetic groups.

Table 2 summarizes the genotyping results obtained in the present study. Out of the 45 SNPs of the AHI1, BAZ1B, CDKAL1, EXT2, HHEX, IGF2BP2, LOC387761, LOC441171, MC4R, MLXIPL, SLC30A8, STK32C, PPARG, and WFS1 genes tested, significant association with T2D was seen with 6 SNPs from 4 genes, namely, 3 SNPs—rs7756992, rs7754840, and rs6931514—of CDKAL1 gene, rs7020996 of CDKN2A/B gene, rs7923837 of HHEX gene, and rs12056034 of BAZ1B gene. The distribution of allelic and genotypic frequencies of the 6 SNPs was compared between the 2 study groups. Genotype distributions of all the 6 SNPs were in HWE. No significant associations with T2D were observed in the other 39 SNPs. We further stratified the subjects based on different genetic models, such as additive, dominant, and recessive models. Under the dominant model, all the 6 SNPs showed significance initially; but the significance was lost in the case of rs6931514 (CDKAL1) and rs7923837 (HHEX) when adjusted for age, sex, and BMI (Table 3).The power was computed for an OR of about 1.2 for all the 6 associated SNPs, and the power ranged from 20.0% to 50.4%

## 4. Discussion

In the present study, we have replicated the association of 6 SNPs in 4 candidate genes derived from recent GWASs with T2D in this South Indian population [7-11]. In addition to these genes, the TCF7L2 gene has already been demonstrated earlier to be associated with T2D in the same study subjects [29]. Among all the genes that were investigated, 3 SNPs, namely, rs7756992, rs7754840, and rs6931514, of the CDKAL1 gene showed an association with T2D in the present study. These SNPs showed an association with T2D in a number of previous European and American GWASs [15]. The rs7020996 of CDKN2A/B gene [13], the SNP rs7923837 of HHEX gene [7], and the SNP rs12056034 of the BAZ1B gene [30] have all been shown to

Table 2
Association of SNPs with T2D

| Serial no. | Gene | SNP | Risk allele | Minor allele freq | | Allelic *P* value | Genotype | Genotype freq | | *P* value |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Cases | control | | | Cases | Control | |
| 1 | CDKAL1 | rs7756992 A/*G* | G | 0.267 | 0.218 | .002 | AA | 414 | 432 | .007 |
| | | | | | | | AG | 312 | 248 | |
| | | | | | | | GG | 52 | 31 | |
| 2 | CDKAL1 | rs7754840 G/*C* | C | 0.252 | 0.207 | .005 | GG | 408 | 420 | .015 |
| | | | | | | | GC | 279 | 229 | |
| | | | | | | | CC | 45 | 25 | |
| 3 | CDKAL1 | rs6931514 A/*G* | G | 0.263 | 0.215 | .040 | AA | 209 | 188 | .029 |
| | | | | | | | AG | 154 | 89 | |
| | | | | | | | GG | 25 | 19 | |
| 4 | CDKN2A/B | rs7020996 C/*T* | C | 0.088 | 0.130 | .020 | CC | 570 | 487 | .003 |
| | | | | | | | CT | 107 | 102 | |
| | | | | | | | TT | 7 | 19 | |
| 5 | HHEX | rs7923837 A/*G* | G | 0.411 | 0.458 | .002 | AA | 258 | 225 | .038 |
| | | | | | | | AG | 345 | 354 | |
| | | | | | | | GG | 128 | 164 | |
| 6 | BAZ1B | rs12056034 A/*G* | A | 0.062 | 0.088 | .008 | AA | 669 | 568 | .033 |
| | | | | | | | AG | 73 | 93 | |
| | | | | | | | GG | 10 | 13 | |

Table 3
Association of SNPs with T2D under different genetic models

| Serial no. | Gene | SNP | Genotypes | *P* value | *P* value[a] | Additive model | | | Dominant model | | | | | Recessive model | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | OR[a] | 95% CI | | *P* value | *P* value[a] | OR[a] | 95% CI | | *P* value | *P* value[a] | OR[a] | 95% CI | |
| | | | | | | | Lower | Upper | | | | Lower | Upper | | | | Lower | Upper |
| 1 | CDKAL1 | rs7756992 | AA vs AG | **.01** | **.03** | 1.311 | 1.01 | 1.69 | **.003** | **.01** | 1.373 | 1.072 | 1.75 | **.05** | **.05** | 1.671 | 0.98 | 2.84 |
| | | | AA vs GG | **.01** | **.02** | 1.86 | 1.08 | 3.2 | | | | | | | | | | |
| | | | AG vs GG | .23 | .21 | 1.426 | 0.81 | 2.49 | | | | | | | | | | |
| 2 | CDKAL1 | rs7754840 | GG vs GC | **.04** | .16 | 1.209 | 0.92 | 1.57 | **.01** | **.04** | 1.304 | 1.01 | 1.68 | **.03** | **.01** | 2.05 | 1.14 | 3.67 |
| | | | GG vs CC | **.01** | **.009** | 2.204 | 1.22 | 3.97 | | | | | | | | | | |
| | | | GC vs CC | .14 | **.05** | 1.805 | 0.98 | 3.29 | | | | | | | | | | |
| 3 | CDKAL1 | rs6931514 | AA vs AG | **.008** | .12 | 1.365 | 0.92 | 2.02 | **.01** | .14 | 1.318 | 0.9 | 1.91 | .99 | .95 | 0.98 | 0.47 | 2.03 |
| | | | AA vs GG | .59 | .79 | 1.102 | 0.52 | 2.32 | | | | | | | | | | |
| | | | AG vs GG | .41 | .52 | 0.777 | 0.34 | 1.71 | | | | | | | | | | |
| 4 | CDKN2A/B | rs7020996 | CC vs CT | **.007** | **.007** | 0.675 | 0.5 | 0.89 | **.002** | **.03** | 0.703 | 0.5 | 0.98 | .05 | .41 | 0.629 | 0.2 | 1.89 |
| | | | CC vs TT | **.03** | **.03** | 0.38 | 0.15 | 0.93 | | | | | | | | | | |
| | | | CT vs TT | .22 | .86 | 0.898 | 0.27 | 2.96 | | | | | | | | | | |
| 5 | HHEX | rs7923837 | AA vs AG | .11 | .62 | 1.086 | 0.78 | 1.5 | **.02** | .49 | 1.114 | 0.81 | 1.51 | .04 | .52 | 1.09 | 0.83 | 1.41 |
| | | | AA vs GG | **.01** | .44 | 1.148 | 0.8 | 1.63 | | | | | | | | | | |
| | | | AG vs GG | .18 | .67 | 1.061 | 0.8 | 1.4 | | | | | | | | | | |
| 6 | BAZ1B | rs12056034 | AA vs AG | **.01** | **.04** | 0.665 | 0.44 | 0.99 | **.009** | **.02** | 0.649 | 0.44 | 0.95 | .37 | .28 | 0.576 | 0.2 | 1.58 |
| | | | AA vs GG | .31 | .24 | 0.551 | 0.2 | 1.51 | | | | | | | | | | |
| | | | AG vs GG | .96 | .58 | 0.753 | 0.26 | 2.11 | | | | | | | | | | |

*P* values ≤.05 are given in bold.
[a] Adjusted for age, sex, and BMI.

be associated with T2D. In the case of CDKAL 1 gene, all the 3 minor alleles are the risk alleles. Analysis of the different genetic models showed that the association fits greater with additive and dominant models with an effect size of 1.3. Similarly, in the case of the other 3 SNPs, the association showed greater fit with the additive and the dominant models.

Overall, among the genes that were investigated in the present study, CDKAL1 appears to have the strongest association with T2D in the population. Although the function of the CDKAL1 gene product is unknown, one study suggested that CDKAL1 has a role in the inhibition of cyclin-dependent kinase 5 activity in pancreatic $\beta$-cells, which prevents a decrease in insulin gene expression resulting from glucotoxicity [31]. The same study also observed reduced insulin secretion in response to glucose loading in homozygous carriers of the CDKAL1 rs7756992 polymorphism risk allele.

Earlier study by Sanghera et al [32] in a diabetic case-control cohort of Khatri Sikhs (Asian Indian Sikhs) examined the role of the 9 most significant loci previously reported to be associated with T2D in white populations, of which 5 SNPs, namely, rs4402960, rs7754840, rs13266634, rs10811661, and rs1111875, were included in the present study. The SNP rs4402960 showed association with T2D in the Khatri Sikh population, whereas no association was seen in our population. Whereas we have shown an association of SNP rs 7754840 with T2D, an association with decreased high-density lipoprotein cholesterol was shown in the study by Sanghera et al, but not with T2D. However, our study concurred with their study in that no association was observed with the other 3 SNPS and T2D.

There are several limitations to our study. The relatively small sample size limits our ability to detect gene variants of modest effect size. A true susceptibility gene in a population might not be readily discernable in other populations because of inadequate sample sizes as well as differences in the genetic background, linkage disequilibrium (LD), and environmental exposures. Findings from previous GWASs cannot always be extrapolated to other populations with different lifestyles and environmental backgrounds. In particular, the genetic background for T2D development in Asian Indians appears to be different from white people of European origin as shown by some of our earlier studies [33]. Furthermore, the SNP frequency differences are additional factors, influencing T2D susceptibility. In the present study, the directionality of association of SNPs between case and control is similar to that observed in other studies. However, there are differences in the frequencies of the SNPs between the various populations (Table 4). In the case of CDKAL1: rs7756992, the frequency in our study compares well with the European populations but is lower compared with Hong Kong and West Africa. In the case of CDKAL1: rs7754840, the frequency of SNP is lower compared with all the other populations such as in the Wellcome Trust Case Control Consortium (WTCCC) study

Table 4
Allele frequency in different population

| Serial no. | Gene | SNP | Risk allele | Ours | | Hong Kong | | West Africa | | French | | FUSION | | WTCCC | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Cases | Control | Cases | Control | Cases | Control | Cases | Control | Cases | Control | Cases | Control |
| 1 | CDKAL1 | rs7756992 A/G | G | 0.267 | 0.218 | 0.517 (15) | 0.462 | 0.612 (15) | 0.625 | 0.295 (15) | 0.258 | | | | |
| 2 | CDKAL1 | rs7754840 G/C | C | 0.252 | 0.207 | 0.42 (6) | 0.358 | | | | | 0.406 (8) | 0.372 | 0.35 (8) | 0.31 |
| 3 | CDKAL1 | rs6931514 A/G | G | 0.263 | 0.215 | | | | | | | | | | |
| 4 | CDKN2A/B | rs7020996 C/T | C | 0.088 | 0.13 | | | | | | | | | | |
| 5 | HHEX | rs7923837 A/G | G | 0.411 | 0.458 | 0.205 (6) | 0.176 | | | 0.335 (15) | 0.377 | | | | |
| 6 | BAZ1B | rs12056034 A/G | A | 0.062 | 0.088 | | | | | | | | | | |

and the Finland-United States Investigation of NIDDM Genetics (FUSION) study. However, with regard to the frequency of HHEX: rs7923837, the French population has a lower frequency compared with our study.

Finally, the average age of the control population is lower than that of the subjects with T2D, which might also contribute to the lack of power in the study; and this is another limitation of this study. However, the OR has been computed after adjusting for age.

In conclusion, in our replication study of recently discovered genetic variants, we report that in addition to our previous finding of association of TCF7L2 gene [29], the CDKAL1 gene, CDKN2A/2B gene, HHEX gene, and BAZ1B gene are associated with T2D in this South Indian population.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.metabol.2010.04.024.

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