Association of Common Variants of CDKN2A/2B Rs10811661 (C/T) and WFS1 Rs6446482 (C/G) to Type 2 Diabetes Mellitus in the Indian Population of Eastern Uttar Pradesh

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Abstract

Aim: Recent genome-wide association studies (GWAS) have identified several unsuspected genes that significantly increase the risk of type 2 diabetes mellitus (T2DM). We aimed to replicate the association of a common variant in CDKN2A/2B (rs10811661) and WFS1 (rs6446482) in the population of Eastern Uttar Pradesh, India. These variants have been identified to be differentially associated with T2DM in different ethnic groups in previous GWAS.

Results: We found SNP rs10811661 of CDKN2A/2B (OR 1.50, 95% CI 1.109-2.032, P=0.009) and SNP rs6446482 of WFS1 (OR 1.43, 95% CI 1.074-1.896, P=0.014) as significant risk factors for T2DM in Eastern Uttar Pradesh population. Normal Glucose-Tolerant (NGT) subjects carrying risk allele of rs10811661 (C/T) and rs6446482 (C/G) polymorphisms had significantly higher Fasting Plasma Glucose (FPG) and 2-hour Postprandial Plasma Glucose (2h-PPGG) levels compared to non-carriers.

Conclusion: Our study replicates the association of well established common variants of CDKN2A/2B rs10811661 (C/T) and WFS1 rs6446482 (C/G) with type 2 diabetes in the population of Eastern Uttar Pradesh, India. Interestingly, our data show larger effect size for both of the SNPs than those reported in European populations.

Keywords: Association studies; Body mass index; CDKN2A/2B; WFS1; Ethnicity; Single Nucleotide Polymorphism (SNP); Type 2 diabetes mellitus

Introduction

Type 2 Diabetes Mellitus (T2DM) is a substantial health issue worldwide with increasing prevalence at alarming rate [1]. India leads the world with largest number of diabetic patients (around 40.9 million in 2006 which is expected to rise to ~70 million by 2025) and is therefore termed as "Diabetes capital of the world" [2]. T2DM is characterized by pancreatic beta cell dysfunction and insulin resistance as a result of the interaction of genetic and environmental factors. Association of a number of genes with T2DM has been shown in the recent past [3]. Recent GWAS have identified several unsuspected genes (with previously unknown functions in pathology of T2DM) associated with T2DM [4-8]. However, the underlying molecular mechanisms in the development of diabetes remain poorly understood. In the present study, we have examined the association of the most significant genetic variant of loci CDKN2A/2B (rs10811661) and WFS1 (rs6446482) with diabetes in the population of Eastern Uttar Pradesh, India. These variants have been found to be convincingly associated with T2DM in Caucasian populations [6-14]. However, recently Nemr et al. [15] have found that CDKN2A/2B rs10811661 is not associated with T2DM in Lebanese.

CDKN2A and CDKN2B are adjacent Cyclin dependent kinase inhibitor genes on chromosome 9p. SNP rs10811661 located upstream of CDKN2A/2B showed genome-wide significant association to T2DM (OR 1.20, P=7.8×10^-14) in all data meta-analysis. SNP rs10811661 may have a long-range effect on one of the genes, or may influence a gene not yet annotated [6]. The region of association is limited to a 9 kb region flanked by strong recombination hot-spots, in which there are multiple conserved non-coding sequences but no known genes or microRNAs [9]. CDKN2A and CDKN2B encode p16INK4a and p15INK4b respectively. p16INK4a inhibits CDK4, a powerful regulator of pancreatic beta cell replication. In mice, Cdkn2a over-expression leads to islet hypoplasia and diabetes [8].

WFS1 encodes wolframin, a transmembrane glycoprotein that maintains calcium homeostasis of the endoplasmic reticulum. Mutations in this gene causes Wolfram syndrome (OMIM 222300), characterized by diabetes insipidus, juvenile-onset non-autoimmune diabetes mellitus, optic atrophy and deafness. Disruption of WFS1 gene in mice causes overt diabetes or impaired glucose tolerance depending on genetic background. Both humans and mice deficient in wolframin show pancreatic beta cell loss. Thus, WFS1 is critical for survival and function of insulin-producing pancreatic beta cells. Four of the SNPs rs10010131, rs6446482, rs752854 and rs734312 (H611R) in the WFS1 locus have been convincingly shown to be associated with T2DM in Caucasians, having odds ratios (ORs) 0.90–0.92 and P values 1.3×10^-4 – 1.4×10^-7 [10]. Replication of the association between variants in WFS1 and risk of type 2 diabetes was also shown by Franks et al. [16] in European population. Statistically significant associations between

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the major alleles of three variants of WFS1 rs10811661, rs6446482 and prevalent T2D in the DESIR cohort at baseline was observed by Cheurfa et al. [17]. Also, the most frequent haplotype at the haplotype block containing the WFS1 gene modulated insulin secretion and was associated with an increased risk of type 2 diabetes.

Material and Methods

Sample collection

Samples were collected from Eastern Uttar Pradesh in this case-control study. Blood from diabetic patients and normal healthy controls (>35 years) was collected after informed consent according to the approved protocol by the Institutional Ethical Committee of Banaras Hindu University from the patients attending out-patient departments of Institute of Medical Sciences, Banaras Hindu University, Heritage Hospital and Prakash Pathology, Varanasi.

Screening of the study subjects

We have genotyped two single nucleotide polymorphisms, CDKN2A rs10811661 and WFS1 rs6446482 in 517 unrelated individuals from Eastern Uttar Pradesh, India, including 271 type 2 diabetic patients and 246 ethnically matched control subjects. Subjects were diagnosed diabetic according to WHO criteria (1999). Subjects were included in the diabetes group if they had fasting glucose concentrations ≥ 126 mg/dl or 2-hour glucose concentrations ≥ 200 mg/dl after a 75 g Oral Glucose Tolerance Test (OGTT). Clinical history of diabetes and associated complications as well as the family history were recorded. Non-diabetic control subjects were chosen based on the absence of a history of diabetes in the subject and among first-degree relatives, as well as normal glucose tolerance, confirmed by a 75 g oral glucose tolerance test. After screening with standard OGTT, age, gender and Body Mass Index (BMI) matched 246 normal healthy controls were enrolled from the population undergoing routine health check-up.

Anthropometric and biochemical evaluation

Anthropometric measurements including weight, height, and waist were obtained using standard protocol. The BMI was calculated as the weight in kilograms divided by the square of height in meters. Clinical and biochemical data (fasting plasma glucose (FPG) and 2 hour postprandial plasma glucose (PPPG)) were obtained as part of our study protocol.

DNA analysis and genotyping

Blood sample (4-5 ml) was taken in 0.5 M EDTA (Sigma, USA) vials. Genomic DNA was extracted from peripheral blood leucocytes using the standard salting-out method.

CDKN2A/2B: We genotyped 271 diabetic subjects and 246 healthy controls. Polymorphic region of CDKN2A/2B (rs10811661) was PCR amplified using a forward primer: 5'-ATAAGGGTCTTGCCCTGT-C-3' and reverse: 5'-GTCAAAAACCTTCCCATCC-3'. The cycling conditions were 94°C for 5 minutes, followed by 32 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds and final extension at 72°C for 7 minutes. The PCR products of 121 bp were digested with BspHI (Bangalore Genie, India) for the rs10811661 (C/T) polymorphism. The resulting products were electrophoresed on a 3% agarose gel.

WFS1: We genotyped 234 diabetic subjects and 234 normal healthy controls (smaller sample size than CDKN2A/2B is due to the genotyping failure in some of the samples). A 136 bp polymorphic region of rs6446482 was PCR-amplified using forward primer: 5'-TGTTCCAATCTACGTCAGT-3' and reverse primer: 5'-TGCAAAGGAGAGAGTGCG-3' followed by Rsal (Bangalore Genie, India) digestion for the rs6446482 (C/G) polymorphism. The cycling conditions were 94°C for 5 minutes, 35 cycles at 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds and final extension at 72°C for 7 minutes. The resulting products were electrophoresed on a 3% agarose gel.

Statistical analysis

Clinical information of the T2DM patients and healthy controls was recorded as shown in table 1. Data on quantitative characteristics are expressed as mean ± SD. Allele and genotype frequencies were compared between T2DM patients and controls using 2×2 and 2×3 contingency tables respectively with χ² test. Odds ratio (OR) with 95% Confidence Interval (CIs) was determined to describe the strength of association. P values of less than 0.05 were considered statistically significant.

Results

The study group cases had significantly higher values of BMI (P=0.024 for CDKN2A/2B; P=0.0027 for WFS1); waist circumference (P=0.0128 for CDKN2A/2B; P < 0.0001 for WFS1); FPG (P<0.0001 for both the SNPs) and 2h-PPPG (P<0.0001 for both the SNPs) compared to controls. The cases were also found to be of significantly higher age group than healthy controls (Table 1). CDKN2A/2B (rs10811661) and WFS1 (rs6446482) polymorphisms show Hardy-Weinberg distributions in our study group. The genotype and allele distributions of the SNPs, rs10811661 and rs6446482 are significantly different between the T2DM and control groups. The major allele T of rs10811661 of CDKN2A/2B (OR 1.50, 95% CI 1.109-2.032; P=0.009) shows modest effect size and the major allele C of rs6446482 of WFS1 also shows modest effect size (OR 1.43, 95% CI 1.074-1.896; P=0.014) (Tables 2 and 3) indicating that these variants are risk factors associated to type 2 diabetes in this population. Our data support the association of rs10811661 of CDKN2A/2B to T2DM under both dominant (P=0.0270;
OR 1.50, 95% CI 1.05 - 2.14) and recessive (P=0.0308; OR 2.62, 95% CI 1.05 - 2.14) compared to non-carriers (genotype GG) (Table 6).

Further, in Normal Glucose-Tolerant (NGT) subjects, FPG and 2h-PPPG levels were found to be significantly higher in carriers of at risk allele (genotypes TT+CT) of rs1081166 of CDKN2A/2B (C/T) polymorphism (P=0.0031 and P=0.049 for FPG and 2h-PPPG, respectively) compared to non-carriers (genotype CC) (Table 5). Similarly, carriers of at risk allele of rs6446482 of WFS1(C/G) polymorphism (genotypes CC+CG) also showed significantly higher FPG and 2h-PPPG levels (P=0.007 and P=0.029 for FPG and 2h-PPPG, respectively) compared to non-carriers (genotype GG) (Table 6). Compared to the carriers of the risk genotypes of both rs1081166 of CDKN2A/2B and rs6446482 of WFS1 together in T2DM group, the FPG and 2h-PPG levels were not significantly different from the carriers of risk genotypes of either CDKN2A/2B (rs1081166) or WFS1 (rs6446482) separately (Table 7).

Discussion

Asian Indians have an increased risk of developing T2DM. Characteristic “Asian Indian Phenotype” (higher body fat percent but a lower lean mass for a given BMI, central obesity leading to high insulin resistance) make them more susceptible to the disease [2]. Most of the studies related to search for T2DM susceptibility loci have been conducted in populations of European decent. Since different gene-environment interactions operate in different populations to increase risk of developing diabetes, the association studies of Western populations are required to be replicated in different Asian Indian populations.

Table 2: Genotype /Allele frequency distribution of CDKN2A/2B rs10811661(C/T) variant among control subjects and type 2 diabetes patients and their Odds Ratio (OR).

| Genotype/Allele | T2DM frequency (%) | Control Frequency (%) | χ² | P-value | OR (95% CI) |
|-----------------|---------------------|-----------------------|-----|---------|-------------|
| CDKN2A/2B rs10811661 |                     |                       |     |         |             |
|                 | n=271               | n=246                 |     |         |             |
| T/T             | 183 (0.675)         | 143 (0.581)           | 5.721 | 0.028   | 2.925 (1.201 - 7.110) |
| C/T             | 81 (0.30)           | 87 (0.354)            | 2.574 | 0.123   | 2.128 (0.852 - 5.298) |
| C/C             | 7 (0.025)           | 16 (0.065)            |      |         |             |
| C               | 95 (0.175)          | 119 (0.420)           |      |         |             |
| T               | 447 (0.825)         | 373 (0.760)           | 6.968 | 0.009   | 1.50 (1.109-2.032) |

Table 3: Genotype /Allele frequency distribution of WFS1 rs6446482 (C/G) variant among control subjects and type 2 diabetes patients and their Odds Ratio (OR).

| Genotype/Allele | T2DM frequency (%) | Control Frequency (%) | χ² | P-value | OR (95% CI) |
|-----------------|---------------------|-----------------------|-----|---------|-------------|
| WFS1 rs6446482  |                     |                       |     |         |             |
|                 | n=234               | n=234                 |     |         |             |
| C/C             | 135 (0.58)          | 110 (0.47)            | 3.408 | 0.065   | 1.809 (0.965-3.389) |
| G/G             | 80 (0.34)           | 96 (0.41)             | 0.380 | 0.538   | 1.228 (0.642-2.346) |
| G/G             | 19 (0.08)           | 28 (0.12)             |      |         |             |
| C               | 350 (0.73)          | 316 (0.67)            | 6.017 | 0.014   | 1.43 (1.074-1.896) |
| G               | 118 (0.27)          | 152 (0.33)            |      |         |             |

Table 4: Comparison of dominant and recessive models for CDKN2A/2B and WFS1.

| Characteristics | Type 2 diabetics (n=271) | Control (n=246) | P-value |
|-----------------|--------------------------|-----------------|---------|
| Fasting plasma glucose | CC (n=264) (Risk group)   | 154.03 ± 53.09  | 0.045  | 80.38 ± 05.72  | 86.34 ± 10.36  | 0.003 |
| 2h Plasma glucose | TT+CT (n=264) (Risk group) | 240.59 ± 74.21  | 0.439  | 117.22 ± 11.16 | 124.44 ± 19.83 | 0.049 |

Table 5: Correlation of the CDKN2A/2B rs10811661(C/T) genotype with FPG and 2h-PPPG.

| Characteristics | Type 2 diabetics (n=234) | Control (n=234) | P-value |
|-----------------|--------------------------|-----------------|---------|
| Fasting plasma glucose | GG (n=19) (Non-risk group) | 151.12 ± 66.17 | 0.047  | 83.53 ± 12.84  | 88.46 ± 21.21  | 0.007 |
| 2h Plasma glucose | CC+CG (n=215) (Risk group) | 223.84 ± 86.26 | 0.256  | 117.37 ± 13.57 | 126.87 ± 19.83 | 0.029 |

Table 6: Correlation of the WFS1 rs6446482 (C/G) genotype with FPG and 2h-PPPG.

| CDKN2A/2B rs10811661 (TT+CT) + WFS1 rs6446482 (CC+CG) | CDKN2A/2B rs10811661 (TT+CT) | WFS1 rs6446482 (CC+CG) |
|------------------------------------------------------|-------------------------------|------------------------|
| FPG (mg/dl)                                          | 143.536 ± 62.087             | 143.006 ± 59.355 (P=0.467) | 145.480 ± 64.206 (P=0.396) |
| 2h-PPG (mg/dl)                                       | 216.784 ± 84.286             | 216.699 ± 86.448 (P=0.497) | 219.996 ± 89.921 (P=0.378) |

Table 7: Combined effect of risk genotypes of CDKN2A/2B and WFS1 in T2DM group on FPG and 2h-PPG compared with the risk genotypes of CDKN2A/2B and WFS1 separately (Mean ± S.D.).
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Recent GWAS and meta-analysis provide convincing evidence for CDKN2A/2B gene region to be involved in T2DM [6-9,11,13,14]. Meta-analysis of genotype data from GWAS in northern Europeans have confirmed that SNPs rs10811661 and rs564398 in the CDKN2A/2B region are T2DM susceptibility variants, although the combined evidence for rs10811661 is far stronger than that for rs564398 [6,8,9]. GWAS in French-Canadian obtained nominal association signals for proxies (r² ≥ 0.9) of rs10811661 (rs2383208, P=2×10⁻⁴) and rs564398 (rs1063192, P=2×10⁻²) [7]. A strong association between the major allele of rs10811661 and T2DM was reported in French Europids [19], Chinese Huns population [20], and Korean population [21]. In a Danish population, variants of CDKN2A/2B was found to be highly associated with T2DM with an OR of 1.30 per risk allele and the SNPs within CDKN2A/2B loci impaired glucose induced insulin release in healthy Danes [22]. Association of variant of CDKN2A/2B was modestly replicated in Asians but not replicated in African Americans and Pima Indians [23]. Recent GWAS in Diabetes Prevention Program (DPP) have shown CDKN2A/2B (rs10811661) as a potential intervention-interaction site showing response to treatment with Troglitazone by improving insulin sensitivity [24]. An intervention-site showing response to treatment with Program (DPP) have shown and Pima Indians [23]. Recent GWAS in Diabetes Prevention associated with T2DM with an OR of 1.30 per risk allele and the SNPs

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