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Metagenomic study of single-nucleotide polymorphism within candidate genes associated with type 2 diabetes in an Indian population

P.N. Mukhopadhyaya[1], A. Acharya[1], Y. Chavan[1], S.S. Purohit[1] and A. Mutha[2]

[1]Medical Genetics Division, geneOmbio Technologies, Pashan, Pune, Maharashtra, India
[2]Diabetes Care and Research Foundation, Rasta Peth, Pune, Maharashtra, India

Corresponding author: P.N. Mukhopadhyaya
E-mail: pnm6619@gmail.com

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ABSTRACT. A population-based study was undertaken to evaluate linkage between single-nucleotide polymorphisms known as risk factors and type 2 diabetes in an Indian population. The study population was comprised of 40 normal glucose-tolerant individuals (21 males and 19 females) and 40 type 2 diabetes patients (21 males and 19 females). The genes and their corresponding single-nucleotide polymorphisms that we screened were VDR (rs 731236 and rs 1544410), IL-6 (rs 1800795), TCF7L2 (rs 7903146) and TNF-α (rs 1800629). The risk alleles were more frequent in the subjects with type 2 diabetes, except for the TNF-α gene, which was very infrequent in the population; the normal allele occurred at high and similar frequencies in both normal and diabetic individuals.

Key words: Insulin resistance; SNP; VDR; IL-6; TNF; TCF7L2

INTRODUCTION

Type 2 diabetes (T2D) is one of the most important life style diseases in the modern world. It is characterized by the presence of a high level of glucose in the blood due to sub-optimal physiological functioning of the body. From a pathophysiological as well as genetic point of view, type 1 and type 2 diabetes are different. However, clinically it is difficult to differentiate them because both are linked with conditions of deficient or absent endogenous insulin and evidence exists that there are also common etiological characteristics (Mathis et al., 2001; Wilkin, 2001; Donath et al., 2003).

The type 2 form of diabetes (also known as diabetes mellitus type 2) is identified as a complex metabolic disorder that is caused due to resistance of the body to insulin and defects in β-cell function. With the conclusion of the human genome mapping project and subsequent research worldwide to link genes with disease conditions, the past decade has seen significant research across the world for identification of T2D-related risk alleles of candidate genes. This was further facilitated by rapid evolution in technology by way of microarray, automated DNA sequencing and real-time polymerase chain reaction (PCR) platforms.

Rapid advances in clinical and genetic research on T2D are because of the significance that this disease assumed in the past few decades as evidenced by its alarming rate of growth (Huizinga and Rothman, 2006). From a disease previously associated more with older people, it is now seen as a common disease amongst the youth and the middle-aged population and, furthermore, this phenomenon is uniform across all six continents throughout the world (Wild et al., 2004).

India is the diabetes capital of the world. Declared as an epidemic by the World Health Organization (WHO), its report indicates that in the year 2000, around 32 million people in India suffered from diabetes. The International Diabetes Federation estimated the total number of diabetic patients in India now to be around 40.9 million, which is slated to rise to 69.9 million by the year 2025 (Sicree et al., 2006).

There are several genes that are implicated for their role in the metabolic pathway leading to conditions of T2D (Hitman and Niven, 1989). In this study, 5 prominent mutations spanning across 4 genes were investigated for their link with diabetic condition in an Indian resource population. The genes include the ligand inducible transcription factor-coding vitamin D receptor (VDR) gene (Ogunkolade et al., 2002), interleukin-6 (IL-6) gene (Vozarova et al., 2001), tumor necrosis factor-alpha (TNF-α) gene (Hotamisligil, 1999), and transcription factor 7-like 2 (TCF7L2) gene, a member of the T-cell-specific high-mobility group box-containing family of transcription factors (da Silva et al., 2009).

MATERIAL AND METHODS

Human subjects

The study subjects were a part of an ongoing insulin resistance study being undertaken by geneOmbio Technologies in association with Diabetes Care and Research Foundation (DCRF), India. The study was directed towards a sub-population living in the State of Maharashtra (India) and suffering from T2D. Blood, serum and DNA samples of 40 T2D cases (21 males, 19 females) and 40 normal glucose tolerant (NGT) (21 males, 19 females) individuals were studied. Of the 40 T2D cases, 30 were from family material (one index

case from each family) and the remaining 10 were unrelated T2D cases from the same resource population. All blood samples were obtained at the baseline visit and all participants provided a written informed consent for investigations.

The members of the resource population were 25 years of age or older with a mean age at the time of recruitment (mean ± SD) of 47.25 ± 12.24 years. The diagnosis of T2D was confirmed by analyzing medical records for symptoms, use of medication, and measuring of fasting glucose levels following the guidelines of American Diabetes Association (Diabetes Care, December 29, 2009; January 2010, Supplement).

Primary inclusion criteria comprised a medical record indicating either 1) a fasting plasma glucose level of ≥126 mg/dL or ≥7.0 mM after a minimum of 12 h fasting or 2) a 2-h post-glucose level [2-h oral glucose tolerance test (OGTT)] of ≥200 mg/dL or ≥11.1 mM on more than one occasion with symptoms of diabetes.

Impaired glucose tolerance was defined as a fasting plasma glucose level of ≥100 mg/dL (5.6 mM) but ≤126 mg/dL (7.0 mM) or a 2-h OGTT of ≥140 mg/dL (7.8 mM) but ≤200 mg/dL (11.1 mM).

In cases where a medical report was not readily available, self-reported T2D cases were confirmed by performing a 2-h OGTT. The 2-h OGTTs were performed following World Health WHO criteria (75 g oral load of glucose). Body mass index (BMI) was computed as weight (kg)/height (meter), and waist-to-hip ratio (WHR) was calculated as the ratio of abdomen or waist circumference to hip circumference.

The NGT subjects that participated in this study were from the same subpopulation group from Maharashtra (Table 1).

Table 1. Clinical characteristics of study population stratified by gender (mean ± SD).

	T2D	NGT	P**
Gender	40 (21M/19F)	40 (21M/19F)	Not applicable
Age (years)			
M	46.0 ± 14.6	42.29 ± 13.41	0.0080
F	48.6 ± 9.0	45 ± 8.83	0.020
Age at diagnosis (years)			
M	39.2 ± 13.3	Not applicable	Not applicable
F	41.1 ± 7.7	Not applicable	Not applicable
Duration of diabetes (years)			
M	6.8 ± 2.2	Not applicable	Not applicable
F	7.4 ± 2.5	Not applicable	Not applicable
BMI			
M	31.9 ± 5.0	27.9 ± 4.9	0.0005
F	31.9 ± 4.0	28.4 ± 4.3	0.0035
WHR			
M	0.99 ± 0.06	0.97 ± 0.06	0.9259
F	1.01 ± 0.08	0.99 ± 0.08	0.9305
Fasting glucose (mM)			
M	10.25 ± 1.88	5.12 ± 0.94	0.0233
F	10.28 ± 1.47	5.14 ± 0.73	0.023
Fasting			
Insulin (IU/mL)			
M	9.67 ± 0.41	11.7 ± 0.4	0.0073
F	9.5 ± 0.65	11.5 ± 0.7	0.0101
HbA1c (%)			
M	8.67 ± 0.41	5.98 ± 041	0.2713
F	8.5 ± 0.65	5.80 ± 0.65	0.2622

T2D = type 2 diabetes; NGT = normal glucose tolerance; M = male; F = female; BMI = body mass index; WHR = waist-to-hip ratio. **Difference between T2D cases and NGT controls.

All protocols were reviewed and approved by the project authorities at DCRF and geneOmbio Technologies in Pune, respectively, and a memorandum of understanding and material transfer agreements for sample sharing were signed between the two collaborating institutes.

Metabolic assays

Quantitation of lipids [total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, very low-density lipoprotein (VLDL) cholesterol, triglycerides (TG), insulin, and creatinine levels were estimated from serum samples. Quantification of HbA1c was done from whole blood. Quantification of lipids was done using standard enzymatic methods (Roche, Basel, Switzerland). HbA1c levels were determined by turbidometric inhibition immunoassay (Tina Quant; Roche). Creatinine was quantified by kinetic colorimetric assay (Roche). Insulin was measured by radioimmunoassay (Diagnostic Products, Cypress, USA). All quantitative parameters were determined following manufacturer instructions using a Hitachi 902 auto-analyzer (Roche).

SNP genotyping

DNA was extracted from blood cells using a column-based DNA extraction protocol using a geneOmbio Blood DNA extraction kit. Genotyping of samples for single-nucleotide polymorphisms (SNPs) within VDR (rs 731236 and rs 1544410), IL-6 (rs 1800795), TCF7L2 (rs 7903146), and TNF-α (rs 1800629) were done according to Halsall et al. (2004), Bennermo et al. (2004), Marquezine et al. (2008), and Romeo et al. (2001) respectively. For quality control, 2 replicates of positive controls and 1 replicate of negative controls were included in each PCR run to match the concordance. The discrepancy in the concordance was <0.01%. Genotyping success rate was 100% for all the investigated SNPs.

Statistical analysis

The Hardy-Weinberg equilibrium was used with a one-degree of freedom goodness-of-fit test separately among cases and controls with the help of the Pearson chi-square test. Allelic frequencies between test and control samples were done using the chi-square test or the Fisher exact probability test, wherever appropriate.

Unconditional logistic regression was used, before and after adjusting for gender, age and other variants for statistical analysis of genetic effects measured by the odds ratio (OR) and its corresponding 95% confidence limits.

Association analyses were performed for each polymorphism using the 'SNPassoc' software (Gonzalez et al., 2007). The common homozygote genotype that appeared in the control population was identified as the reference category. For testing the effect of each SNP at the 5% significance level, the likelihood ratio test (McGee, 2002) was used.

Akaike's information criterion (Akaike, 1974) was used to select the best genetic model for each SNP. Multiple linear regression analysis (Jolliffe, 1982) was performed to examine the effect of each of these variants on quantitative risk variables of T2D, which include fasting insulin, glucose and lipid levels. Skewed variables for the continuous traits such as TG, total cholesterol, LDL, and glucose were log-transformed before statistical

comparisons. Significant covariates for each dependent trait were identified by using Spearman's correlation (Corder and Foreman, 2009) and step-wise multiple linear regression with an overall 5% level of significance.

RESULTS AND DISCUSSION

All samples, including those with T2D (N = 40) and normal glucose tolerant (N = 40), were genotyped for 5 SNP within 4 genes of interest. Details of the resource population are shown in Table 1.

A total of 4 genes and 5 SNPs were identified for genotyping analysis within each of the samples from the resource population. Of these, 2 SNPs were in the VDR gene and one each in TNF-α, IL-6 and TCF7L2 genes, respectively. The details of the gene name, SNP identification number (reference SNP or the 'rs' number), position of the SNP on the chromosome as indicated by Genome Build version 37.1 (the FASTA sequence of the human chromosomes; Build 37; National Council of Biological Information, USA), and frequency of occurrence of each of the SNPs in the resource population are summarized in Table 2.

Table 2. Candidate gene polymorphisms and risk of type 2 diabetes in a section of Indian population comparing NGT *vs* T2D.

Gene/SNP	Chromosome	Position[a]	Genotype	NGT (%)	T2D (%)	Dominant OR (95%CI)
VDR	12	48238757				
rs 731236			TT	7 (17.5)	23 (57.5)	
			CT	25 (62.5)	12 (30.0)	
			CC	8 (20)	5 (12.5)	
Risk allele			Allele T	0.49	0.73	1.5
			Allele C	0.51	0.27	
VDR	12	48239835				
rs 1544410			GG	26 (65.0)	17 (42.5)	
			AA	4 (10.0)	14 (35.0)	
			GA	10 (25.0)	9 (22.5)	
			Allele G	0.78	0.54	
Risk allele			Allele A	0.22	0.46	2.1
TNF-α	6	31525175				
rs 1800629			GG	37 (92.5)	35 (87.5)	
			AA	0 (0)	2 (5.0)	
			AG	3 (7.5)	3 (7.5)	
			Allele G	0.96	0.91	
Risk allele			Allele A	0.04	0.09	2.2
IL-6	7	22766645				
rs 1800795			GG	12 (30.0)	23 (57.5)	
			CC	15 (37.5)	6 (15.0)	
			GC	13 (32.5)	11 (27.5)	
Risk allele			Allele G	0.46	0.71	1.5
			Allele C	0.54	0.29	
TCF7L2	10	114758349				
rs 7903146			CC	5 (12.5)	17 (42.5)	
			TT	16 (40.0)	2 (5.0)	
			CT	19 (47.5)	21 (52.5)	
Risk allele			Allele C	0.36	0.69	1.9
			Allele T	0.64	0.31	

Odds ratio (OR) were adjusted for age, gender, and BMI. [a]Position of single-nucleotide polymorphisms (SNPs) on chromosome was taken from NCBI (Genome Build 37.1). NGT = normal glucose tolerance; T2D = type 2 diabetes; CI = confidence interval; VDR = vitamin D receptor; TNF-α = tumor necrosis factor-alpha; IL-6 = interleukin-6; TCF7L2 = transcription factor 7-like 2.

The genes and SNPs that indicated strong association with conditions of T2D were VDR: rs 731236 and rs 1544410 with risk alleles = 'T' and 'A', respectively (Bid et al., 2009); IL-6: rs 1800795 with risk allele = 'G' (Vozarova et al., 2003); TNF-α: rs 1800629 with risk allele = 'A' (Day et al., 1998), and TCF7L2: rs 7903146 with risk allele = 'C' (Florez et al., 2006). However, in the TNF-α gene the risk allele 'A' had very low frequency in the population (0.09 in T2D and 0.04 in NGT) thus indicating that it may not be significant in predicting cases of T2D at least in this resource population.

Multiple linear regression was used to analyze data from the NGT group after adjusting for the impact of age, gender, BMI, disease status, and medication and test association of these SNPs with T2D-related parameters such as height, weight, BMI, WHR, total cholesterol, creatinine, insulin, VLDL cholesterol, LDL cholesterol, HDL cholesterol, and TG.

As shown in Table 3, the 'T' (risk) and 'A' (risk) alleles of the VDR gene (rs731236 and rs1544410, respectively) occurred at a frequency of 73 and 46% in T2D compared to 49 and 22% in the NGT group (OR = 1.5 and 2.0, respectively), thus favoring the risk allele significantly towards association with decreased insulin levels in our T2D resource population. For the IL-6 gene, the (risk) allele 'G' was found in 71% of T2D cases (OR = 1.5) compared to 46% in the NGT group thus showing a strong link with decreased insulin level. The 'C' (risk) allele of the TCF7L2 gene was encountered in 69% of T2D cases (OR = 1.9) compared to 36% of NGT cases. This again showing significant association with decreased levels of fasting insulin in T2D cases (Table 3). However, although the OR for risk allele 'A' in the TNF-α gene was computed at 2.2, the low frequency of occurrence of this allele in both genotypic conditions (homozygous: 5% in T2D *vs* 0% in NGT and heterozygous: 7.5% in each T2D and NGT cases) indicated that the association was not significant.

Table 3. Association of risk allele with type 2 diabetes trait.

Gene/SNP	Risk allele	Mean fasting insulin NGT (IU/mL)	Frequency of risk allele in NGT	Mean fasting insulin T2D (IU/mL)	Frequency of risk allele in T2D
VDR					
rs 731236	T	11.6	0.49	9.585	0.73
rs 1544410	A	11.6	0.22	9.585	0.46
IL-6					
rs 1800795	G	11.6	0.46	9.585	0.71
TCF7L2					
rs 7903146	C	11.6	0.36	9.585	0.69

For abbreviations, see legend to Table 2.

The primary role of vitamin D includes maintenance of mineral homeostasis by controlling calcium absorption in the gut and re-absorption by the kidney (DeLuca, 1984). However, although best known for their role in calcium uptake there are a host of other non-calcemic activities that vitamin D performs, primary among them being the induction of differentiation in peripheral blood mononuclear cells (PBMCs), and anti-proliferative effects in many forms of cancer. However, one of the important roles is as a necessary adjunct for insulin secretion (Holick, 1995). A study by Ogunkolade et al. (2002) confirmed an association between VDR polymorphisms and insulin secretion capacity and

demonstrated that the VDR genotype is a significant determinant of VDR mRNA and VDR protein levels in PBMCs, providing functional support to previously described genetic associations with the VDR gene. Furthermore, VDR expression was also shown to be a determinant of insulin secretion capacity. The association of the risk alleles for both targeted SNPs (rs 731236 and rs 1544410) with decreased insulin in the T2D cases in our study supports the observation by Holick (1995).

In recent years, a large amount of evidence has accumulated indicating that insulin resistance and T2D is closely related to a chronic, low-grade inflammatory state of the body. This is the reason why circulating levels of IL-6 molecules remain elevated in people with T2D (Pickup et al., 1997) and, furthermore, this value serves as an indirect measure for conditions of insulin resistance (Kern et al., 2001; Vozarova et al., 2001). However, in our study, we failed to come across patients with mutation only in the IL-6 gene. Therefore, the occurrence of insulin resistance rather than impairment could not be established. Nevertheless, the other parameters of T2D aligned with increased frequency of the risk allele as indicated in Table 3.

TCF7L2 is one of the members of the T-cell-specific high-mobility group box-containing family of transcription factors. These molecules bind to β-catenin and transduce signals generated by Wnt receptors at the cell surface thereby modifying the expression of multiple genes, many of which are associated with the cell cycle (Moon et al., 2004). Mutations in TCF7L2 are also implicated in certain types of cancer (Slattery et al., 2008). Polymorphisms in the human TCF7L2 gene have recently been associated with reduced insulin secretion and an increased risk of T2D (da Silva et al., 2009). It was further established that TCF7L2 controls the expression of genes involved in insulin granule fusion at the plasma membrane. These changes may underlie defective insulin secretion in β-cells lacking TCF7L2. The high frequency of the risk allele in our study endorses the observation of its increased link to conditions of T2D.

Tumor necrosis factor is a cytokine that is involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction (Locksley et al., 2001). It has been shown that endothelial dysfunction occurring in T2D is the result of the effects of this inflammatory cytokine TNF-α, mainly through the overexpression of TNF-R1, which subsequently activates NAD(P)H oxidase and hydrogen peroxide (Gao et al., 2007). However, in our study, we failed to establish a good correlation between the risk allele and conditions of T2D. The 'at risk' allele of 'A' occurred in homozygous condition in 0 and 5% in NGT and T2D populations, respectively. In heterozygous condition it occurred in 7.5% each in NGT and T2D populations thus failing to align in a statistically significant way with conditions of T2D.

The present study provided insight into the association of SNPs within genes linked to type 2 diabetes and further establishes the findings that are published in literature within an Indian resource population. The data raise prospects of developing an SNP-based genetic prediction test for detecting genetic predisposition towards this important lifestyle disease. With numerous supplements now known to fight Advanced Glycated Endproducts (AGE) thus ameliorating conditions related to type 2 diabetes such as benfotiamine (Stracke et al., 1996) coupled with better management ideas to defer or prevent the onset of this disease, development of a predictive test seems to be likely as well as beneficial.

This study forms part of a larger research program to validate a software program

(Dia*P*redict V: 1.3) developed at our center that can effectively be used as a clinician's tool for acquiring a quantitative value on the overall predisposition of a person towards type 2 diabetes after factoring related phenotypic characters as well as mutation profile of major pathway genes that contribute to this disease.

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