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RESEARCH ARTICLE

Association analysis of *TNFRSF1B* polymorphisms with type 2 diabetes and its related traits in North India

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Abstract Inflammation plays a crucial role in the pathogenesis of type 2 diabetes and various lines of evidences suggest an important contribution of type 2 receptor for TNFα (TNFR2), a mediator of inflammatory responses. Though genetic association of *TNFRSF1B* (encoding TNFR2) polymorphisms have been investigated in various studies, their involvement is not clear because of inconsistent findings. Because of high susceptibility of Indian population to type 2 diabetes and its complications, we evaluated the association of *TNFRSF1B* polymorphisms-rs1061622 (M196R; exon6) and rs3397 (3′UTR) and $(CA)_n$ repeat (intron 4) in 1,852 subjects including 1,040 cases and 812 controls with type 2 diabetes and its associated peripheral neuropathy and hypertension in North Indians of Indo-European ethnicity. The allelic and genotypic distributions of these polymorphisms were comparable among healthy control vs. type 2 diabetes, peripheral neuropathy vs. non-neuropathy and hypertensive vs. normotensive groups. $(CA)_n$ polymorphism has been shown to be associated with diabetic neuropathy in Caucasians, however, this could not be replicated in our study ($P = 0.27$). None of the polymorphisms were found to influence the 14 anthropometric and biochemical traits related to type 2 diabetes studied here. Thus, we conclude that *TNFRSF1B* is not a major contributing factor to the genetic risk of type 2 diabetes, its associated peripheral neuropathy and hypertension and related metabolic traits in North Indians.

Keywords *TNFRSF1B* · Polymorphisms · Type 2 diabetes · North India

Abbreviations

TNFRSF1B	Tumor necrosis factor receptor superfamily 1B gene
BP	Blood pressure
LDL-C	Low density lipoprotein cholesterol
HDL-C	High density lipoprotein cholesterol
HbA1c	Glycated hemoglobin
hsCRP	High sensitivity C-reactive protein

Introduction

Type 2 diabetes is defined as metabolic disorder that results from impaired insulin secretion and action. However, it is now believed to be a manifestation of ongoing sub-clinical inflammatory response (Schmidt et al. 1999), the molecular origin of which is unknown, probably involving cross-talks between metabolic and inflammatory pathways. One such response linking metabolic and inflammatory pathways is the activation of TNF-TNFR axis through an adipokine, Tumor Necrosis Factor α (TNFα) (Hotamisligil et al. 1993; Uysal et al. 1997). The most crucial player of this pathway

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is TNFR2, type 2 receptor for TNFα because of its involvement in lipid and glucose metabolism in addition to its role in inflammation and apoptosis. TNFR2 is also an important contributor to insulin resistance (Liu et al. 1998). Increased TNFR2 expression and plasma soluble TNFR2 levels have been found in various pathological conditions including obesity, insulin resistance and cardiovascular diseases (Hotamisligil et al. 1997; Fernandez-Real et al. 1998; Shai et al. 2005).

Various reports suggest a significant pathological role of TNFR2 in the manifestation of obesity, insulin resistance, inflammation and vascular complications. These phenotypes constitute the spectrum of sub-phenotypes associated with type 2 diabetes. Hence, the involvement of TNFR2 in these pathological conditions and strong links between obesity, inflammation, and type 2 diabetes implicates TNFR2 as an important biological candidate for type 2 diabetes. With this background, it can be speculated that genetic variants in *TNFRSF1B*, gene encoding TNFR2, might be associated with the development of type 2 diabetes and its related traits. The $(CA)_n$ repeat polymorphism in intron 4 and M196R (rs1061622) non-synonymous variation in exon 6 are the most studied variations in this gene and have been extensively investigated for association with various metabolic and inflammatory disorders. The $(CA)_n$ repeat has been found to be associated with essential hypertension, hypercholesterolemia, coronary artery disease, familial combined hyperlipidemia and diabetic neuropathy (Glenn et al. 2000; Benjafield et al. 2001a, b; Geurts et al. 2000). M196R is postulated to affect the proteolytic cleavage of the membrane bound TNFR2 to soluble form, TNF binding and/or TNF induced apoptosis by impaired NF-κB signaling (Stark et al. 2003; Till et al. 2005). Also, a haplotype including rs3397 in 3′UTR which alters TNFR2 stability and activity is associated with insulin resistance in young diabetic subjects (Puga et al. 2005; Fernandez-Real et al. 2000).

Indians have a high prevalence of insulin resistance, body fat and abdominal obesity, making them a high risk group for type 2 diabetes and its complications (McKeigue et al. 1991). In addition to highest prevalence of diabetes, India also has a large pool of individuals with impaired glucose tolerance which is projected to result in a significant increase in disease incidence in the next two decades (Ramachandran et al. 2001; Wild et al. 2004). However, the exact cause of susceptibility to diabetes and its associated complications are not clearly understood. Since it is postulated that pro-inflammatory state might be one of the major contributing factors, it is highly desirable to evaluate the role of such an important candidate as *TNFRSF1B* which provides the link between overlapping phenotypes related to type 2 diabetes, in this high risk group. Though *TNFRSF1B* is a strong biological candidate, its association with metabolic disorders including type 2 diabetes has been contradictory so far. In the present study we examined the association of SNPs rs1061622 (M196R), rs3397 and $(CA)_n$ repeat polymorphism with type 2 diabetes. We also investigated their association with type 2 diabetes associated peripheral neuropathy and hypertension on the basis of earlier associations of *TNFRSF1B* variant with these conditions in Caucasian population (Benjafield et al. 2001a; Glenn et al. 2000).

Research design and methods

Subjects

We recruited 1,852 subjects comprising 1,040 cases and 812 controls based on the criteria described previously (Tabassum et al. 2008). Briefly, cases included patients with type 2 diabetes who attended Endocrinology clinic of All India Institute of Medical Sciences, New Delhi and Guru Teg Bahadur Hospital, Delhi. Both case and control subjects were unrelated individuals of Indo-European ethnicity residing in the urban region of North India. Type 2 diabetes was diagnosed based on World Health Organization criteria (WHO Expert Committee 2003). Type 2 diabetic patients with systolic pressure ≥140 mmHg and/or diastolic pressure ≥90 mmHg or if undergoing antihypertensive treatment were diagnosed hypertensive (DPH-diabetic patients with hypertension). Type 2 diabetic patients with either diminution of pin-prick sensation or loss of perception of 10-g monofilament pressure sensation at the plantar aspect of great toes and metatarsal joints were defined to have sensory peripheral neuropathy; and subjects were classified as DPN (diabetic patients with neuropathy). Type 2 diabetic patients were categorized as diabetic patients without hypertension (DPWH) and diabetic patients without neuropathy (DPWN) if they had not developed hypertension and peripheral neuropathy, respectively after 10 years or more of diagnosis of type 2 diabetes. Control group constituted of healthy individuals of ≥40 years of age with glycosylated hemoglobin level (HbA1c) ≤6.0% and fasting plasma glucose level <110 mg/dl. Control subjects had no family history of type 2 diabetes in first and/or second degree relatives. In addition, at the time of enrolment 30% of the subjects were selected to undergo a 75 g oral glucose tolerance test (OGTT) at 0 and 120 min to confirm their glucose tolerance status. The subjects were considered for sampling under control group only if they had no personal or family history of diabetes or glucose intolerance and OGTT was performed only for those subjects who had no symptoms suggestive of uncontrolled hyperglycemia such as excessive thirst, urination and hunger. We observed that of the

total subjects with normal levels of HbA1c and fasting plasma glucose that underwent OGTT, only 8% of the individuals had impaired glucose tolerance (IGT) that were excluded from the study and none met diagnostic criteria for type 2 diabetes mellitus. Written informed consent was obtained from all the participants. The study was approved by the Ethics Committees of the participating institutions and was in accordance with the principles of the Helsinki Declaration.

Clinical studies

Anthropometric parameters like height, weight, waist and hip circumferences were measured in light clothes and without shoes as per standard guidelines. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Waist to hip ratio (WHR) was calculated using waist and hip circumferences. Systolic and diastolic blood pressures were measured twice in the right arm in sitting position after resting for at least 5 min using a standard sphygmomanometer and the average of two readings was used.

Venous blood samples were obtained from the subjects after 12 hours of overnight fasting. Levels of glucose, total cholesterol, HDL-C, LDL-C, triglycerides, urea, uric acid and creatinine were measured using Cobas Integra 400 Plus (Roche Diagnostics, Mannheim, Germany). HbA1c was determined by low-pressure liquid chromatography (LPLC) on DiaSTAT Analyzer (Bio-Rad Laboratories, Richmond, CA, USA). Plasma levels of hsCRP were measured using ELISA kit (Biocheck Inc., CA, USA).

Genotyping

Genomic DNA was isolated from leukocytes by salting out method. For genotyping $(CA)_n$ repeat locus, PCR was carried out with FAM labeled forward primer followed by electrophoresis on ABI PRISM 3100 Genetic analyzer (Applied Biosystems, Foster City, CA, USA). SNPs were genotyped using SNaPshot ddNTP Primer Extension Kit (Applied Biosystems). Genotypes were determined using GeneMapper Software v4.0. The repeat length was determined by sequencing the homozygous samples using CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA) and alleles were termed as $(CA)_{11}$ through $(CA)_{17}$ to indicate the number of CA repeats. For quality control, 10% of the samples were genotyped in duplicates. For SNPs, genotype calls were validated by sequencing at least five samples for each genotype. PCR primers used for genotyping were designed by Primer 3 (Rozen and Skaletsky 2000) as it is shown to provide better primer design for amplification (Chavali et al. 2005).

Statistical analysis

Each polymorphism was tested for Hardy–Weinberg equilibrium using GENEPOP program (http://wbiomed.curtin.edu.au/genepop). Linkage disequilibrium (LD) between polymorphisms was determined and haplotype analysis was carried out using Haploview 4.0 software (Barrett et al. 2005). Baseline clinical characteristics of patients with type 2 diabetes and control subjects were compared using Mann–Whitney U test. Fisher's exact test and χ^2 analyses were employed as appropriate to determine the differences in allelic and genotypic frequencies among different groups. Logistic regression analysis was carried out to calculate the odds ratio adjusted for age, sex and BMI. A P value of <0.003 was considered significant for allelic and genotypic comparison after Bonferroni correction for multiple testing performed for each group ($n = 5$) and the number of alleles tested ($n = 3$) (Bonferroni adjusted $\alpha = 0.05/15 = 0.003$). The uncorrected P values are provided in the text. To determine the genotype relationships with various clinical variables, median values were compared using Mann–Whitney U test or Kruskal Wallis test as appropriate. Statistical power of the study was estimated using PS power and sample size program (Dupont and Plummer 1997). The statistical analyses were performed using statistical package SPSS version 15.0 (SPSS, Chicago, IL, USA).

Results

Anthropometric and biochemical parameters related to the disease phenotype were compared among the patients with type 2 diabetic and control subjects. The descriptive data and their comparisons are provided in Table 1. Of the total type 2 diabetic subjects ($n = 1{,}040$) recruited in the study, 40.1% ($n = 417$), 11.3% ($n = 118$), 12.7% ($n = 132$) and 2.8% ($n = 29$) of patients were diagnosed to have hypertension, peripheral neuropathy, retinopathy and nephropathy, respectively.

Patients with type 2 diabetes had elevated BMI, WHR and blood pressure compared to control subjects. Significantly higher levels of hsCRP, triglycerides, urea, creatinine and uric acid were observed among patients compared to controls (all $P < 0.005$). We observed lower levels of total cholesterol and LDL-C in patients compared to control subjects. This lowering in cholesterol levels can be attributed to patients undergoing drug treatment especially statins at the time of recruitment.

The genotypic distributions of the polymorphisms were in Hardy–Weinberg equilibrium both among patients and controls ($P > 0.02$). The allelic and genotypic distributions of polymorphisms in different study groups are provided in

Table 1 Anthropometric and clinical characteristics of the study groups

Characteristics	Control subjects	Type 2 diabetic patients	*P* value
N (men/women)	812 (427/385)	1,040 (577/463)	
Age (years)	50 (45–61)	54 (45–63)	0.004
BMI (kg/m^2)			
Men	23.1 (19.5–25.6)	23.8 (23.0–25.8)	3.6×10^{-6}
Women	24.8 (20.8–28.3)	26.7 (24.2–29.4)	9.0×10^{-10}
WHR			
Men	0.95 (0.90–0.98)	1.0 (0.97–1.03)	1.2×10^{-26}
Women	0.86 (0.82–0.92)	1.0 (0.97–1.03)	6.7×10^{-5}
Systolic BP (mmHg)	124 (114–136)	130 (130–140)	3.8×10^{-24}
Diastolic BP (mmHg)	80 (70–86)	80 (76–87)	4.9×10^{-6}
HbA1c (%)	5.3 (4.9–5.6)	7.7 (6.5–9.2)	1.9×10^{-221}
Fasting glucose (mmoles/l)	4.9 (4.4–5.3)	7.7 (6.3–10.0)	2.2×10^{-203}
hsCRP (mg/l)	1.2 (0.6–2.8)	2.1 (0.9–4.4)	4.8×10^{-14}
Total cholesterol (mg/dl)	172 (147–201)	165 (139–195)	0.004
LDL-C (mg/dl)	109 (91–134)	101 (80–129)	1.8×10^{-5}
HDL-C (mg/dl)	43 (35–51)	42 (36–48)	0.153
Triglycerides (mg/dl)	116 (86–159)	137 (96–196)	4.3×10^{-8}
Urea (mg/dl)	24.1 (19.2–29.2)	27.0 (20.9–34.3)	1.2×10^{-9}
Uric acid (mg/dl)	4.7 (3.8–5.6)	4.9 (3.9–6.0)	0.033
Creatinine (mg/dl)	0.74 (0.65–0.88)	0.90 (0.72–1.10)	6.4×10^{-28}

N represents the number of individuals. Data are represented as median (interquartile ranges)

P values were calculated using Mann–Whitney *U* test

Table 2. At $(CA)_n$ locus, we identified seven alleles ranging from $(CA)_{11}$ to $(CA)_{17}$, with $(CA)_{15}$ being the major allele. Comparison of allele frequencies for $(CA)_n$ repeat polymorphism among type 2 diabetic patients and control subjects did not show any significant difference ($P = 0.92$). We examined the association of $(CA)_n$ repeat polymorphism with type 2 diabetes in accordance with the presence or absence of the three most common alleles-$(CA)_{15}$, $(CA)_{13}$ and $(CA)_{16}$. However, none of the genotypes investigated was found to be associated with type 2 diabetes. The distributions of alleles at rs1061622 and rs3397 loci did not differ significantly among patients and controls ($P = 0.16$ and 0.53, respectively). The genotypic comparison after adjusting for age, sex and BMI using logistic regression did not reveal any significant association with type 2 diabetes.

Obesity is a well known risk factor of type 2 diabetes and influence of BMI on the risk of development of type 2 diabetes has been consistently shown in number of studies. Recently, we also showed variability in the risk of type 2 diabetes among normal-weight and over-weight/obese individuals in North Indian population (Tabassum et al. 2008). This implies that the etiology of type 2 diabetes might be different in normal-weight individuals and overweight/obese individuals. Therefore, we segregated the subjects into two groups based on BMI: normal-weight (BMI <23 kg/m^2) and overweight/obese (BMI ≥23 kg/m^2) based on WHO criteria for Asian population (WHO Expert Consultation 2004) and compared the allelic and genotypic distributions among normal-weight cases and controls; and overweight/obese cases and controls (Table 3). We observed over-representation of $(CA)_{16}$ allele in normal-weight control groups (17.1%) compared to normal-weight diabetic patients (13.4%), however, the difference was not statistically significant [OR 0.75 (95% CI 0.55–1.02), $P = 0.07$]. Slightly higher representation of TT homozygotes of rs1061622 was found in overweight/obese control subjects compared to overweight/obese diabetic patients, though not significant (56.8 vs. 52.9%, $P = 0.21$). Moreover, a suggestive association of TT homozygotes of SNP rs3397 was observed when we compared overweight/obese diabetic subjects with overweight/obese control subjects [OR 0.85 (95% CI 0.72–1.00), $P = 0.06$].

Association of *TNFRSF1B* variant with diabetic neuropathy and hypertension in other populations instigated us to evaluate the same in our study population (Benjafield et al. 2001a; Glenn et al. 2000). Though there was a slight difference in allelic (17.8 vs. 14.9%, $P = 0.33$) and genotypic distribution of $(CA)_{16}$ allele (30.5 vs. 27.5% for carriers, $P = 0.64$) between DPN and DPWN groups, this did not reach the level of statistical significance (Table 2). Also, we found that SNPs rs1061622 and rs3397 were not associated with either of the diabetes associated clinical traits investigated here-peripheral neuropathy ($P = 0.54$ and 0.49, respectively) and hypertension ($P = 0.58$ and 0.77, respectively) among diabetic patients. We also performed the association analysis by comparing DPH and DPN with diabetic patients without any complication; however, this analysis also provided the same results.

Table 2 Allelic and genotypic distribution of *TNFRSF1B* polymorphisms among different study groups in North Indian population

Polymorphism		Control	Patients	DPN	DPWN	DPH	DPWH
$(CA)_n$	Allele	$2n$ = 1,624	$2n$ = 2,080	$2n$ = 236	$2n$ = 530	$2n$ = 818	$2n$ = 264
	$(CA)_{11}$	96 (5.9)	155 (7.4)	15 (6.4)	39 (7.3)	62 (7.7)	16 (6.1)
	$(CA)_{12}$	14 (0.9)	27 (1.3)	1 (0.4)	3 (0.6)	10 (1.2)	2 (0.7)
	$(CA)_{13}$	304 (18.7)	390 (18.8)	41 (17.4)	99 (18.7)	164 (20.0)	46 (17.4)
	$(CA)_{14}$	41 (2.5)	41 (2.0)	9 (3.8)	7 (1.3)	15 (1.8)	8 (3.0)
	$(CA)_{15}$	885 (54.5)	1,129 (54.3)	128 (54.2)	301 (56.8)	436 (53.3)	147 (55.7)
	$(CA)_{16}$	270 (16.6)	314 (15.1)	42 (17.8)	79 (14.9)	125 (15.3)	44 (16.7)
	$(CA)_{17}$	14 (0.9)	24 (1.1)	0 (0.0)	2 (0.4)	6 (0.7)	1 (0.4)
	OR (95% CI)*	1.01 (0.88–1.15)		0.90 (0.66–1.23)		0.90 (0.68–1.19)	
	P value	0.92		0.56		0.51	
rs1061622	MAF, G	25.6	27.7	26.4	26.0	28.0	25.4
	Genotype	n = 812	n = 1,026	n = 106	n = 265	n = 409	n = 130
	GG	47 (5.8)	76 (7.4)	4 (3.8)	19 (7.2)	33 (8.1)	8 (6.1)
	GT	321 (39.5)	417 (40.6)	48 (45.3)	100 (37.7)	163 (39.8)	50 (38.5)
	TT	444 (54.7)	533 (51.9)	54 (50.9)	146 (55.1)	213 (52.1)	72 (55.4)
	OR (95% CI)**	1.12 (0.93–1.34)		1.18 (0.75–1.85)		1.14 (0.77–1.70)	
	P value	0.25		0.54		0.58	
rs3397	MAF, T	42.3	41.3	38.6	40.0	41.4	39.7
	Genotype	n = 813	n = 1,030	n = 110	n = 265	n = 412	n = 131
	TT	138 (17.0)	167 (16.2)	10 (9.1)	43 (16.2)	66 (16.0)	19 (14.5)
	CT	412 (50.7)	516 (50.1)	65 (59.1)	126 (47.6)	209 (50.7)	66 (50.4)
	CC	263 (32.3)	347 (33.7)	35 (31.8)	96 (36.2)	137 (33.3)	46 (35.1)
	OR (95% CI)**	1.06 (0.87–1.29)		1.22 (0.75–1.95)		1.09 (0.72–1.64)	
	P value	0.61		0.49		0.77	

n and $2n$ represents the number of individuals and alleles, respectively

DPN, Diabetic patients with neuropathy; *DPWN*, Diabetic patients without neuropathy; *DPH*, Diabetic patients with hypertension; *DPWH*, Diabetic patients without hypertension

In allele section data are number of alleles (%) and in genotype section data are number of individuals with given genotype (%)

P values presented are uncorrected for multiple testing (Bonferroni adjusted $P = 0.003$)

* Odds ratio (95% confidence interval) of common allele compared with other alleles

** Odds ratio (95% confidence interval) of homozygotes of common allele compared with all other genotypes

Further, we investigated the effect of *TNFRSF1B* polymorphisms on quantitative metabolic traits. For repeat locus, we examined the genotype relationships with various clinical variables of patients with type 2 diabetes and control subjects according to the presence or absence of the three most common alleles-$(CA)_{15}$, $(CA)_{13}$ and $(CA)_{16}$. The analysis did not reveal any significant difference in the clinical parameters across the different genotypes both among cases and controls. Moreover, we did not find any association of SNPs rs1061622 and rs3397 with clinical parameters among patients and control subjects.

For LD analysis between the two SNPs and $(CA)_n$ repeat locus, we divided the $(CA)_n$ alleles into two groups designated as $(CA)_{15}$ and non-$(CA)_{15}$ alleles [any other allele except $(CA)_{15}$]. We observed that these loci were not in LD. However, in previous studies, 196R allele was found to be in LD with $(CA)_{13}$ and $(CA)_{14}$; and 196 M was in LD with $(CA)_{15}$ and $(CA)_{16}$ (Geurts et al. 2000; Peral et al. 2002). Haplotype analysis did not reveal association of any of the haplotypes with type 2 diabetes.

Discussion

Type 2 diabetes is a state of hyperglycemia that results from impaired insulin secretion and action. The major risk factors of type 2 diabetes include obesity and insulin resistance with associated impaired secretion of various adipokines (Youn et al. 2004; Weyer et al. 2001). A number of molecules involved in inflammatory processes have been investigated for their role in type 2 diabetes (Duncan et al. 2003; Schmidt et al. 1999; Pradhan et al. 2001). Recent studies also indicate type 2 diabetes to be a condition of low grade systemic inflammation. Hence,

Table 3 Allelic and genotypic distribution of *TNFRSF1B* polymorphisms among normal-weight and over-weight/obese individuals in North Indian population

Polymorphism		NW control	NW patients	OW control	OW patients
$(CA)_n$	Allele	$2n = 712$	$2n = 568$	$2n = 894$	$2n = 1{,}448$
	$(CA)_{11}$	43 (6.1)	50 (8.8)	52 (5.8)	97 (6.7)
	$(CA)_{12}$	6 (0.8)	7 (1.2)	8 (0.9)	16 (1.1)
	$(CA)_{13}$	126 (17.7)	111 (19.5)	174 (19.5)	270 (18.6)
	$(CA)_{14}$	14 (2.0)	9 (1.6)	25 (2.8)	31 (2.1)
	$(CA)_{15}$	398 (55.9)	309 (54.4)	479 (53.6)	789 (54.5)
	$(CA)_{16}$	122 (17.1)	76 (13.4)	145 (16.2)	228 (15.8)
	$(CA)_{17}$	3 (0.4)	6 (1.1)	11 (1.2)	17 (1.2)
	OR (95% CI)*	0.94 (0.75–1.17)		1.04 (0.88–1.23)	
	P value	0.63		0.70	
rs1061622	MAF, G	26.5	28.8	26.4	26.0
	Genotype	$n = 356$	$n = 283$	$n = 447$	$n = 713$
	GG	21 (5.9)	26 (9.2)	25 (5.6)	48 (6.7)
	GT	147 (41.3)	111 (39.2)	168 (37.6)	288 (40.4)
	TT	188 (52.8)	146 (51.6)	254 (56.8)	377 (52.9)
	OR (95% CI)**	1.05 (0.77–1.43)		1.17 (0.92–1.49)	
	P value	0.82		0.21	
rs3397	MAF, T	39.9	42.9	44.4	40.4
	Genotype	$n = 356$	$n = 282$	$n = 447$	$n = 718$
	TT	50 (14.0)	51 (18.1)	87 (19.5)	106 (14.8)
	CT	184 (51.7)	140 (49.6)	223 (49.9)	368 (51.2)
	CC	122 (34.3)	91 (32.3)	137 (30.6)	244 (34.0)
	OR (95% CI)**	1.09 (0.78–1.52)		0.85 (0.66–1.10)	
	P value	0.65		0.25	

n and 2*n* represents the number of individuals and alleles, respectively with MAF and frequency given in parantheses as percentages; NW and OW stand for normal-weight and over-weight/obese, respectively

P values presented are uncorrected for multiple testing (Bonferroni adjusted $P = 0.003$)

* Odds ratio (95% confidence interval) of common allele compared with other alleles

** Odds ratio (95% confidence interval) assuming dominant model for minor alleles

TNFR2 which is involved both in inflammation and insulin resistance is an important biological candidate for type 2 diabetes.

The polymorphisms of *TNFRSF1B* gene that encodes for TNFR2 have been associated with several phenotypes that constitute the spectrum of sub-phenotypes associated with type 2 diabetes and its complications as highlighted earlier. Therefore, here we examined the association of well studied polymorphisms of *TNFRSF1B* with type 2 diabetes and its associated peripheral neuropathy and hypertension in North Indian population. Keeping in mind the diversity and heterogeneity of Indian population and to avoid the plausible population stratification, we recruited case and control subjects residing in the urban region of North India belonging to Indo-European ethnicity forming the homogenous cluster in accordance with a recent report of genetic landscape of the people of India (Indian Genome Variation Consortium 2008). The report suggested that the effects of population stratification in disease association studies may be small, if cases and controls are both drawn from the same cluster within the Indian subcontinent.

We found that $(CA)_n$ repeat polymorphism in intron 4 of *TNFRSF1B* is not associated with type 2 diabetes in North Indian population. Based on the previous observation of association of $(CA)_{16}$ allele of $(CA)_n$ repeat polymorphism with peripheral neuropathy in diabetic patients in Caucasian population (Benjafield et al. 2001a), we examined the effect of *TNFRSF1B* polymorphisms including $(CA)_n$ repeat on susceptibility to develop complications among diabetic patients. In contrast to the observations of Benjafield et al., we found that $(CA)_n$ repeat polymorphism is not associated with peripheral neuropathy among diabetic patients in the North Indian population. We emphasize that we have adopted a stringent criteria for selection of the patients without peripheral neuropathy and hypertension (DPWN and DPWH groups) which includes absence of

these conditions after ten or more years of diabetes from the time of diagnosis. This categorically rules out any possibility of false negative association. Also, our sample size was much larger than that of the Caucasian study (Benjafield et al. 2001a). Assuming a risk allele frequency, heterozygote relative risk and homozygote relative risk similar to Caucasian study, our study had the power of $\geq$99% to detect the association. Hence, our study was sufficiently-powered to detect the association of $(CA)_n$ repeat polymorphism with type 2 diabetes. Though initially $(CA)_{16}$ was shown to be associated with hypertension in an Eastern Australian cohort (Glenn et al. 2000), this association could not be replicated in Anglo-Celtic whites (Speirs et al. 2005). The authors of the latter study attributed the observed association in the former case to lower sample size. Consistent with these observations, we show here that this polymorphism is not associated with hypertension in North Indian diabetic patients, thus refuting the role of TNFR2, if any, in hypertension.

The non-synonymous SNP rs1061622 which results in the substitution of methionine to arginine at position 196, is postulated to affect the proteolytic cleavage of TNFR2 which might affect the shedding of the receptor into the soluble form. It can be speculated that this may alter the spectrum of the receptor activity, and hence might lead to pathological conditions. However, our investigation showed that M196R polymorphism is not associated with type 2 diabetes and its associated vascular complications of peripheral neuropathy and hypertension in the North Indian population. The SNP rs3397 which is located in the 3′UTR of *TNFRSF1B* was found to be associated with insulin resistance in young diabetic subjects in haplotype combination with two other SNPs (Fernandez-Real et al. 2000). Also, this haplotype combination is shown to alter the stability and activity of TNFR2 and might result in increased expression of TNFR2 (Puga et al. 2005). Because of the functional significance of locus, we investigated SNP rs3397 for association with type 2 diabetes in North Indian population, however, it was not found to be associated with the disease.

Here, we explicitly demonstrate that the $(CA)_n$ repeat polymorphism and SNPs rs1061622 and rs3397 in *TNFRSF1B* gene are not associated with type 2 diabetes and with its associated peripheral neuropathy and hypertension in North Indian population. Moreover, these polymorphisms were found to have no effect on the quantitative metabolic traits related to type 2 diabetes. Hence, we conclude that though *TNFRSF1B* gene is an important biological candidate, the polymorphisms studied here are not the major contributing factors to the genetic risk of type 2 diabetes and its related traits in North India.

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