The role of TLR4, TNF-α and IL-1β in type 2 diabetes mellitus development within a North Indian Population.

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Short running head: Inflammatory gene polymorphisms and type 2 diabetes mellitus.

Key words: Type 2 diabetes mellitus; TLR4 896; TNF-α -308; IL-1β -511; Inflammatory gene polymorphisms; North Indian population

SUMMARY

This study investigated the role of IL-1β-511 (rs16944), TLR4-896 (rs4986790) and TNF-α-308 (rs1800629) polymorphisms in type 2 diabetes mellitus (T2DM) among an endogamous Northern Indian population. 414 participants (204 T2DM patients and 210 non-diabetic controls) were genotyped for IL-1β-511, TLR4-896 and TNF-α-308 loci. The C allele of IL-1β-511 was shown to increase T2DM susceptibility by 75% (OR: 1.75 [CI 1.32-2.33]). Having two parents affected by T2DM increased susceptibility by 5.7 times (OR: 5.693 [CI 1.431-22.648]). In this study, we have demonstrated a conclusive association with IL-1β-511 locus and IL1B-511-TLR4-896 diplotype (CC-AA) and T2DM, which warrants further comprehensive analyses in larger cohorts.

INTRODUCTION

South Asians' are approximately five times more likely to develop T2DM, and its comorbidities, at an earlier age than white Europeans (Ramachandran et al., 2013). There are estimated 65 million cases of T2DM in India, which will double within the next two decades (Ramachandran et al., 2013).

T2DM is a complex metabolic condition characterised by an individual's inability to regulate blood sugar levels resulting in hyperglycaemia. T2DM is caused due to either a lack of insulin production from the pancreas or the body's tissues developing a resistance to insulin; and is normally preceded by low-grade inflammation and the release of inflammatory cytokines (Cruz et al., 2013). Inflammation and its pathways have been targeted in recent research on pathology and treatment of the disease among other gene targets (Brunetti et al., 2014; Goldfine and Shoelson, 2017).

Toll like receptor 4 (TLR4) protein is responsible for the mediation of immune responses and inflammation activation (Takeda et al., 2003). Interleukin-1β (IL-1β), a pro-inflammatory cytokine, protects against infection and stimulates neutrophils and macrophages to initiate phagocytosis (Netea et al., 2010). Tumor necrosis factor alpha (TNF-α) is another pro-inflammatory cytokine involved in the recovery from tissue damage, infection, and facilitates the movement of immune cells to defective tissues (Barbara et al., 1996).

Preclinical studies indicate TNF-α induces insulin resistance (Hotamisligil et al., 1993) and there is some evidence that TNF-α inhibitors reduce glycaemia, fasting glucose levels and diabetes incidence (Stanley et al., 2011; Goldfine and Shoelson, 2017). Preclinical and clinical studies also report IL-1β involvement in T2DM pathogenesis (Koenen et al., 2011; Sloan-Lancaster et al., 2013). The antagonist IL-1Ra inhibits IL-1β signalling and alleviated systemic inflammation and glycaemia in T2DM patients (Larsen et al., 2007). The expression of TLR4, IL-1β and TNF-α is also modulated by glucagon-like peptide-1 receptor agonist (Exenatide) among diabetics (Chaudhuri et al., 2011). This provides a rationale for the candidate genes featuring in this study to be investigated as they have the potential to form future drug targets for T2DM therapy.

IL-1β-511 (rs16944:T>C), TLR4-896 (rs4986790:A>G) and TNF-α-308 (rs1800629:G>A) polymorphisms are associated with T2DM in European, Mexican, Moroccan and Indian populations (Achyut et al., 2007; Assman et al., 2014; Sefri et al., 2014; Saxena et al., 2015 and Tripathi et al., 2015), although studies on Indian populations are limited. Genetic analyses of inflammatory genes contribute to the development of prognostic markers which could be used to distinguish individuals at risk. Therefore, it will be beneficial to conduct comprehensive studies on Indian subpopulations to disentangle the genetic burden of T2DM.

This study aims to investigate the association of IL-1β-511, TLR4-896 and TNF-α-308 polymorphisms with T2DM in an endogamous Northern Indian population.

MATERIALS AND METHODS

Study Subjects

This study consisted of 204 T2DM patients (104M;100F) and 210 non-diabetic unrelated controls (87M;123F). Participants belong to the endogamous group of Lobana Sikhs-an agriculturist population from Punjab, North India and were sampled in early 1990s as part of ongoing genetic and disease studies (Mastana et al., 2013). Samples were collected from unrelated patients and controls after obtaining written informed consent. T2DM status was diagnosed by either a medical record or tests showing fasting glucose levels [7.0 mmol/l or [126 mg/dl after a minimum 12-h fast or 2-h post glucose level (oral glucose tolerance test or 2-h OGTT) [11.1 mmol/l or 200 mg/dl] on more than one occasion. Control participants were free of diabetes symptoms and were matched to the patients for age, gender and geographical location. The current study was approved by the ethics committees of local medical hospitals and colleges and Loughborough University. Further details of clinical/diagnostic and demographic features of these samples are given in a previous study (Mastana et al., 2013).

Genotyping

IL-1β-511 (rs16944) polymorphism was amplified and analysed using PCR- restriction fragment length polymorphism method (PCR-RFLP) (Mirowska-Guzel et al., 2011). TLR4-896 (rs4986790) and TNF-α-308 (rs1800629) polymorphisms were genotyped using TaqMan® assays (Assay ID C__11722238_20 and C___7514879_10, respectively). 10% of samples were repeated to check genotyping reliability.

Statistical Analysis

Continuous variables were compared using means and standard deviations (unpaired t-tests). Chi-square analysis (χ^2) was performed to assess the difference in genotypic and allele frequencies and to test for Hardy Weinberg equilibrium (HWE). Odds ratios (ORs) and 95% confidence intervals (CI) were calculated to assess the risk of T2DM with differing genotypes and alleles. Synergistic analysis was carried out for different genotype combinations (diplotypes and triplotypes). Binary logistic regression was performed to determine if any genetic, demographic or biochemical covariates were independent predictors of T2DM. P-values of <0.05 were considered statistically significant. Bonferroni corrections were applied to control for multiple comparisons. Statistical tests were conducted using the Statistical Package of Social Sciences (SPSS) for Windows, version 23.0 (SPSS, Inc., Chicago, IL).

RESULTS

Demographics

The anthropometrical, biochemical, and clinical characteristics of the patient and control populations are summarised in Table 1. Patients, on average, were taller and heavier than controls, with larger waist and hip measurements and a larger waist hip ratio (WHR). Systolic blood pressure (SBP) was shown to be significantly higher in patients. High-density lipoprotein (HDL) levels were lower in patients than the controls (P<0.005).

Genotype and allele distribution

The distribution of different genotypes and alleles within patients and controls are presented in Table 2, along with synergistic effects at diplotype and triplotype combinations. Both patients and controls were within HWE for IL-1β-511 and TLR4-896. At TNF-α-308 locus,

only the patients were in HWE (χ^2= 3.539), and controls violated the HWE even after Bonferroni correction (χ^2 = 8.328).

The frequency of the IL-1β-511 CC genotype was higher in patients (19.8%) than in controls (10.4%). The C allele of IL-1β-511 increases the risk of T2DM by about 75% (OR: 1.75 (CI 1.32-2.33)) while T allele exerted a protective effect (OR: 0.57 (CI 0.43-0.76)).

TLR4-896 genotype distribution was similar between patients and controls. Neither A or G allele of TLR4-896 had a statistically significant association with the disease.

At TNF-α -308 locus, 89.9% of patients and 92.6% of controls possessed the most common GG genotype, whereas the frequency of the rare AA genotype was similar in both groups (1%). TNF-α-308 also showed no statistically significant allelic or genotypic association with T2DM, though heterozygote GA genotype increased the risk by 47% (OR: 1.47 [CI 0.70-3.09]).

Synergistic Effect

The synergistic effect of genotypic combinations is presented in Table 2. Four diplotype and 3 triplotype combinations were statistically significant before the Bonferroni correction. After the correction, only IL-1β-511-TLR4-896 diplotype combination CC-AA remained significantly associated with T2DM, although mean glucose levels did not present a statistically significant difference when compared to the reference genotype (TT-AA).

Binary logistic regression

Binary logistic regression analysis was carried out at two levels; in genotype only analysis (Table 3), ORs and associated significance values were broadly similar to the analyses given in Table 2. Table 4 gives the results of a logistic regression analysis including selected demographic and clinical variables, where the IL-1β (CC genotype) remains a significantly

susceptible genotype. TLR4 genotypes AG and GG change the direction of the effect and become susceptible. The regression analysis showed that having both parents affected by T2DM increases individual's risk by 5.7 times. BMI, SBP, DBP and HDL levels are all independent predictors of T2DM in this population.

DISCUSSION

This is the first study on a well characterised endogamous sample, analysing inflammatory genes for their association with T2DM. In this study, C allele of IL-1β-511 increased the likelihood of T2DM (OR: 1.75 [CI = 1.32-2.33]) while T allele exerted a protective effect (OR: 0.57 [CI = 0.43-0.76]), and the TC and CC genotypes increased T2DM risk when compared to TT (OR: 1.89 [CI = 1.23-2.92] and OR: 3.10 [CI = 1.66-5.78], respectively). Mojtaba et al. (2011) reported a significant association between increased IL-1β levels and decreased insulin concentration; decreased β cell function and increased fasting glucose. Further research should be conducted to decipher which allele/genotype of IL-1β induces these effects.

The present results contradict some previous studies where the T allele was associated with T2DM. (Achyut et al., 2007; Saxena et al., 2015 and Tripathi et al., 2015). The previous studies are difficult to interpret due to ambiguity in genotyping methods. Two studies report the C allele to be 304bp and T allele 189 and 116bp (Saxena et al., 2015 and Tripathi et al., 2015). This study coded the alleles in the same manner as Mirowska-Guzel et al. (2011) and Achyut et al. (2007) (T:304bp; C:190+114bp). Genotyping calls in this study were also confirmed using a TaqMan® assay which provides confidence in these results. The aforementioned publications analysed heterogeneous Indian populations recruited from outpatient clinics and hospitals, however, the present sample is from a field based study on a homogenous and endogamous Sikh population (Mastana et al 2013).

Although statistically insignificant trends were observed for the A allele of the TNF-α-308 locus and G allele of TLR4-896 locus, further studies are warranted with larger samples to confirm/evaluate these associations (Sefri et al., 2014; Assman et al., 2014).

The inconsistent results of IL-1β-511, TLR4-896 and TNF-α-308 association with T2DM across a range of populations suggests that ethnicity may play a role in how these genes interact with the disease. Populations from different geographical regions are exposed to different environmental risk factors, thus different gene-environment interactions are formed. This can alter how the genes influence T2DM in different populations, therefore producing inconclusive results.

IL-1β-511 seems to be the main contributor to susceptibility in the diplotype IL-1β-511-TLR4-896 (Table 2), due to the increased risk associated with the C allele. Synergistic effect is not a purely additive model and the increased susceptibility is attributable to the combination of independent risk alleles at both loci. IL-1β and TLR4 activity in macrophages can be suppressed by Dipeptidyl peptidase-4 inhibitors, thus potentially having an antihyperglycaemic effect (Dai et al., 2014). It is of great importance to conduct further preclinical/clinical studies on these candidate genes in order to develop drug therapies for T2DM (Chaudhuri et al., 2011; Goldfine and Shoelson, 2017).

The regression analysis confirmed significant independent association between T2DM and the IL-1β-511 CC genotype, family history of T2DM, BMI, SBP, DBP, and HDL levels. In this study, positive family history presents the largest risk to offspring when both parents have T2DM (OR = 5.693, CI 1.431-22.648). Maternal diabetes alone increased T2DM risk by 5.086 times, suggesting that maternal diabetes exerts a higher risk to offspring than paternal diabetes.

In this study, we have demonstrated a conclusive association with IL-1β and T2DM. IL-1β and TLR4 polymorphisms conclusively contribute to T2DM in a synergistic fashion. Research has also shown that above polymorphisms have been targeted in drug development to reduce inflammation in diabetics; further population focused interventions are required to improve gene based treatments. Maternal diabetes is a strong independent determinant of T2DM in this population. Further comprehensive studies on different geographical populations are warranted with larger samples to clarify the role of inflammatory gene polymorphisms in T2DM in Indian population.

Authors would like to thank all participants who provided samples and clinical colleagues for diagnostic help. Contributions to the manuscript are as follows; Study Design: SSM; Data Collection and Analysis: NED, MD, ECA, NJC, JSB, PS, SSM; Manuscript Drafting: NED; Manuscript Revisions: All authors.

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Table 1. Anthropometrical, biochemical and clinical characteristics of participants.

		N	Mean	Std. Deviation	Std. Error Mean	*T* test *P* value
Age (years)	Patients	204	61.374	10.538	0.738	0.446
	Controls	210	60.465	13.480	0.930	
Height (cm)	Patients	204	160.758	9.103	0.637	0.0191*
	Controls	210	158.660	9.037	0.624	
Weight (kg)	Patients	204	70.967	12.763	0.894	0.0089*
	Controls	210	67.597	13.312	0.919	
BMI	Patients	204	27.488	4.623	0.324	0.173
	Controls	210	26.852	4.850	0.335	
Waist (in.)	Patients	204	37.049	4.168	0.292	0.0001*
	Controls	210	35.195	4.482	0.309	
Hip (in.)	Patients	204	38.414	3.771	0.264	0.0121*
	Controls	210	37.513	3.497	0.241	
WHR	Patients	204	0.965	0.065	0.005	0.0004*
	Controls	210	0.939	0.082	0.006	
Body fat %	Patients	204	35.889	10.475	0.733	0.481
	Controls	210	35.210	9.083	0.627	
SBP (mmHg)	Patients	204	148.800	21.738	1.522	0.0015*
	Controls	210	141.100	26.869	1.854	
DBP (mmHg)	Patients	204	84.270	10.959	0.767	0.191
	Controls	210	82.730	12.846	0.886	
Cholesterol (mg/dl)	Patients	194	187.650	48.340	3.471	0.829
	Controls	130	186.470	47.680	4.182	
Triglycerides (mg/dl)	Patients	194	184.884	114.195	8.199	0.435
	Controls	130	175.614	88.602	7.771	
HDL (mg/dl)	Patients	193	39.218	12.545	0.903	0.0017*
	Controls	130	43.585	11.570	1.015	
LDL (mg/dl)	Patients	191	109.444	44.915	3.250	0.529
	Controls	130	106.432	37.294	3.271	
VLDL (mg/dl)	Patients	191	39.612	29.732	2.151	0.111
	Controls	130	34.966	17.625	1.546	

*Significance at P<0.05

BMI – body mass index, WHR – waist hip ratio, SBP – systolic blood pressure, DBP – diastolic blood pressure, HDL – high-density lipoprotein, LDL – low-density lipoprotein, VLDL – very low-density lipoprotein.

Table 2. Genotype, allele, diplotype and triplotype frequencies

Locus	Genotype	Patients (%)	Controls (%)	Odds Ratio (95% CI)	P value
IL1- β -511	TT	56 (27.7)	91 (44.8)	1.00 (Ref)	
	TC	106 (52.5)	91 (44.8)	1.89 (1.23-2.92)	0.005*
	CC	40 (19.8)	21 (10.4)	3.10 (1.66-5.78)	0.001*
HWE X^2 value		0.636	0.062		
Allele	T	218 (54.0%)	273 (67.2%)	0.57 (0.43-0.76)	<0.005*
	C	186 (46.0%)	133 (32.8%)	1.75 (1.32-2.33)	<0.005*
TLR4 896	AA	153 (76.9)	147 (72.4)	1.00 (Ref)	
	AG	40 (20.1)	48 (23.6)	0.80 (0.50-1.29)	0.427
	GG	6 (3.0)	8 (3.9)	0.72 (0.24-2.13)	0.747
HWE X^2 value		2.639	2.441		
Allele	A	346 (87.0)	342 (84.2)	1.25 (0.84-1.85)	0.323
	G	52 (13.0)	64 (15.8)	0.80 (0.54-1.19)	0.323
TNF-α -308	GG	178 (89.9)	189 (92.6)	1.00 (Ref)	
	GA	18 (9.1)	13 (6.4)	1.47 (0.70-3.09)	0.403
	AA	2 (1.0)	2 (1.0)	1.06 (0.15-7.62)	0.658
HWE X^2 value		3.539	8.328**		
Allele	G	374 (94.4)	391 (95.8)	0.74 (0.39-1.14)	0.452
	A	22 (5.6)	17 (4.2)	1.35 (0.71-2.59)	0.452
Two Loci Genotypes					
IL1- β -511 and TLR4 896	TT-AA	33	57	1.00 (Ref)	
	TT-GG	3	5	1.04 (0.23-4.62)	0.737
	TT-AG	16	25	1.11 (0.52-2.36)	0.949
	TC-AA	87	69	2.18 (1.28-3.71)	0.006
	TC-AG	16	16	1.73 (0.76-3.90)	0.266
	TC-GG	2	3	1.15 (0.18-7.25)	0.620
	CC-AA	32	15	3.68 (1.74-7.79)	0.001***
	CC-AG	7	6	2.02 (0.62-16.50)	0.377
TNF-α -308 and IL1- β -511	GG-TT	50	82	1.00 (Ref)	
	GG-TC	93	83	1.84 (1.16-2.91)	0.013
	GG-CC	33	19	2.85 (1.46-5.54)	0.003
	GA-TT	4	4	1.64 (0.39-6.85)	0.757
	GA-TC	7	5	2.30 (0.69-7.63)	0.281
	GA-CC	7	2	5.74 (1.15-28.73)	0.045
TLR4 896 and TNF-α -308	AA-GG	132	137	1.00 (Ref)	
	GG-GG	4	8	0.52 (0.15-1.76)	0.440
	AA-GA	14	9	1.61 (0.68-3.86)	0.385
	AA-AA	1	1	1.04 (0.06-16.77)	0.494
	AG-GG	37	42	0.91 (0.55-1.51)	0.825
	AG-GA	2	4	0.52 (0.09-2.88)	0.726
	AG-AA	1	1	1.04 (0.06-16.77)	0.494
Three Loci Genotypes IL1- β -511, TLR4 896 And TNF-α -308	TT-AA-GG	29	53	1.00 (Ref)	
	TT-GG-GG	2	5	0.73 (0.13-4.01)	0.959
	TT-AG-GG	15	22	1.25(0.56-2.27)	0.737
	TT-AA-GA	2	3	1.22 (0.19-7.71)	0.786
	TT-AG-GA	1	1	1.83 (0.11-30.31)	0.749
	TC-AA-GG	77	66	2.13 (1.22-3.73)	0.011
	TC-AG-GG	14	14	1.83 (0.77-4.35)	0.252
	TC-GG-GG	1	3	0.61 (0.06-6.13)	0.910
	TC-AA-GA	5	3	3.05 (0.68-13.67)	0.259
	TC-AG-GA	1	2	0.91 (0.08-10.51)	0.587
	CC-AA-GG	25	14	3.26 (1.47-7.23)	0.005
	CC-AG-GG	7	5	2.56 (0.75-8.79)	0.226
	CC-AA-GA	7	1	12.79 (1.50-109.13)	0.013

* - Statistically significant at $P<0.05$, ** - Population not within Hardy Weinberg Equilibrium, ***- Statistically significant at $P\leq0.001$ (after Bonferroni correction)

Table 3. Genotype only binary logistic regression

Significant predictors of T2DM are indicated in bold.

Predictor Variable	Regression Coefficient	Sig.	Exp(B)	95% C.I for Exp(B)	
				Lower	Upper
IL1β -511		0.001			
(TC)	**0.646**	**0.006**	**1.907**	**1.209**	**3.009**
(CC)	**1.064**	**0.001**	**2.897**	**1.546**	**5.431**
TLR4 896		0.957			
(AG)	-0.064	0.803	0.938	0.568	1.548
(GG)	-0.104	0.854	0.901	0.297	2.735
TNF-α -308		0.753			
(GA)	0.280	0.473	1.323	0.616	2.842
(AA)	0.247	0.809	1.281	0.173	9.502

Significant predictors of T2DM are indicated in bold.

Table 4. Binary logistic regression of genotypes, demographic and clinical variables

Predictor Variable	Regression Coefficient	Sig.	Exp(B)	95% C.I for Exp(B)	
				Lower	Upper
IL1β -511		0.107			
(TC)	0.256	0.415	1.292	0.698	2.393
(CC)	**0.916**	**0.035**	**2.498**	**1.066**	**5.855**
TLR4 896		0.171			
(AG)	0.622	0.085	1.864	0.917	3.787
(GG)	0.868	0.368	2.381	0.360	15.745
TNF-α -308		0.750			
(GA)	0.061	0.906	1.063	0.385	2.938
(AA)	-1.000	0.455	0.368	0.027	5.075
Gender (Male)	-4.037	0.134	0.018	0.000	3.488
AGE (Years)	0.014	0.622	1.014	0.960	1.071
PARENT AFFECTED		0.000			
(Father affected)	**0.819**	**0.039**	**2.268**	**1.042**	**4.939**
(Mother affected)	**1.629**	**0.000**	**5.086**	**2.231**	**11.593**
(Both parents affected)	**1.739**	**0.014**	**5.693**	**1.431**	**22.648**
BMI	**-0.157**	**0.011**	**0.855**	**0.758**	**0.964**
WAIST	0.375	0.062	1.454	0.981	2.156
HIP	0.139	0.103	1.149	0.972	1.358
BODYFAT (%)	-0.204	0.165	0.816	0.612	1.087
SBP	**0.035**	**0.000**	**1.036**	**1.019**	**1.053**
DBP	**-0.044**	**0.007**	**0.957**	**0.927**	**0.988**
CHOLESTEROL	-0.002	0.774	0.998	0.988	1.009
TRIGYCERIDES	-0.047	0.095	0.954	0.903	1.008
HDL	**-0.039**	**0.002**	**0.961**	**0.938**	**0.986**
LDL	0.003	0.610	1.003	0.991	1.015
VLDL	0.241	0.089	1.272	0.964	1.679
Constant	-6.839	0 .020	0.001		

Significant predictors of T2DM are indicated in bold.