

Association of IL-4 and IL-1RN (receptor antagonist) gene variants and the risk of type 2 diabetes mellitus: a study in the north Indian population

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ABSTRACT

Background: Inflammation is a key event closely associated with the pathophysiology of type 2 diabetes mellitus (T2DM). Association of genetic polymorphisms of inflammatory cytokines with T2DM is largely unknown. Our objective was to investigate the relationship of polymorphism of IL-1RN and IL-4, two important biomarkers of inflammation, with the risk of T2DM.

Setting and Design: We recruited 120 clinically diagnosed T2DM patients and 150 normal healthy controls for this study in order to evaluate the nature of polymorphisms of IL-1RN and IL-4.

Material and Methods: Genomic DNA was isolated from the blood of all subjects, and the variable number of tandem repeat (VNTR) polymorphisms of IL-1RN and IL-4 genes was identified by polymerase chain reaction.

Statistical Analysis Used: Genotype distribution and allelic frequencies were compared between patients and control group. Means, as well as odds ratios (ORs) with 95% confidence intervals (CI), were calculated using SPSS software (version 11.5).

Result: Our study revealed that distribution of both IL-4 and IL-1RN (VNTR) gene polymorphisms was significantly associated with T2DM subjects. We, however, failed to find any association of gene-gene (IL-4 and IL-1RN) interaction with T2DM.

Conclusions: Both IL-4 and IL-1RN (VNTR) gene polymorphisms were significantly associated with T2DM subjects. This may suggest that the genetic polymorphisms of IL-4 and IL-1RN genes could serve as susceptibility indicators for T2DM in the Indian population, but the actual mechanism of these associations will require more elaborate

investigations. Lack of association of gene-gene (IL-4 and IL-1RN) interaction with T2DM may indicate the independent nature of influence of both these genes on the risk of T2DM.

Keywords: IL-4; IL-1RN; polymorphism; T2DM

Introduction

T2DM is manifested by hyperglycemia resulted from resistance to insulin in fat, muscle, and other key target tissues of insulin; and decreased insulin secretion by beta cells of islets of Langerhans. Chronic low-grade inflammation and activation of the innate immune system are closely involved in the pathogenesis of T2DM.^[1] Recently, T2DM has also been recognized as an immune-mediated disease leading to impaired insulin signaling and selective destruction of insulin-beta-producing cells in which cytokines play an important role.^[2-4] Cross-sectional studies have provided support for the hypothesis that chronic subclinical inflammation may be associated with insulin resistance and may precede the development of clinically overt T2DM.^[5]

IL-4 is an important anti-inflammatory cytokine that directs macrophages toward a phenotype that is characterized by the elaboration of other anti-inflammatory molecules, like IL-10, IL-1RN, and IL-1R2. IL-4 shifts the inflammatory balance by inhibiting the secretion of the pro-inflammatory cytokines IL-1beta, TNF-alpha, and IL-6 from macrophages. Disturbance of the anti-inflammatory response could be a critical component of the chronic inflammation found in T2DM. Type 2 diabetes mellitus disrupts anti-inflammatory cytokine function of IL-4 as a mediator of production of antagonist of interleukin-1 (IL-1RN).^[6]

IL-1RN, a naturally occurring competitive inhibitor of IL-1, binds to the type I receptor and protects human pancreatic cells from IL-1beta-induced functional impairment and apoptosis.^[7-8] Claus *et al.* proposed that IL-1RN has possible therapeutic potential in the treatment of T2DM.^[9] Recently, it was indeed revealed that administration of IL-1RN in the form of Anakinra has been successfully shown to improve beta cell function in patients with T2DM.^[10]

The nature of association of IL-4 and IL-1RN polymorphisms with, and their combined effect on, Indian T2DM patients is not known. As a first step to evaluate whether these cytokines' polymorphisms have functional influence on the susceptibility to T2DM, we conducted a

study of the association of the gene polymorphisms of IL-4 and IL-1RN with diabetes in a population-based study in the Indian population.

Material and methods

Patients and clinical evaluation

The current study was carried out with prior approval from the institutional ethical committee. Patients with T2DM were enrolled from among the outpatients attending the diabetes clinics of a medical university from March to October 2007.

Screening and management of patients was done as per American Diabetes Association guidelines.^[11] 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 with all clinical details.^[12] A total of 120 subjects after screening were included in this study. A questionnaire was used to record clinical history of diabetes and associated complications, hypertension; as well as family history.

After screening with standard oral glucose tolerance test, a total of 150 age- and BMI-matched normal healthy controls were enrolled from among the healthy staff members of the institute and university for this study. Subjects having history of coronary artery disease or other metabolic disorders were excluded from the control group.

Sample processing and genotyping

After obtaining an informed consent from both the groups, 5 mL of blood sample was taken in EDTA and plain vials. Genomic DNA was extracted from peripheral blood leucocytes pellet using the standard salting-out method.^[13]

IL-4 intron-3 VNTR

The region that contains the VNTR polymorphism of 70 bp within the IL-4 intron-3 was amplified by using the following PCR primer pairs: forward, 5'-TAGGCTGAAAGGGGAAAGC-3'; and reverse, 5'-CTGTTACCTCAACTGCTCC-3'.^[14] The reaction was carried out in a total volume of 10 μ L, containing genomic DNA (100 ng);

10 pmol of each primer, 1X Taq polymerase buffer and 0.5U of Taq DNA polymerase (Bangalore Genei, India). The cycling conditions were 95°C for 10 minutes, followed by 32 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and final extension at 72°C for 8 minutes. Alleles of 183 bp (two repeats) and 253 bp were designated as B1 and B2 respectively and were analyzed by electrophoresis on a 2% agarose gel.

IL-1RN exon-2 VNTR

Genotyping of IL-1RN gene polymorphism (VNTR containing 86 bp within the intron-2) was amplified by using the following primer pairs: forward, 5'-CTCAGCAACACTCCTAT-3'; and reverse, 5'-TCCTGGTCTGCAGGTAA-3'. The reaction was carried out in a total volume of 10 µL, containing genomic DNA (100 ng); 10 pmol of each primer. PCR amplification was performed in programmed thermal cycler (Master cycler gradient, Eppendorf, USA) under the following conditions: 95°C for 5 minutes, 30 cycles at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, and one cycle of extension at 72°C for 10 minutes. The PCR products of 240 bp (allele A = two repeats), 410 bp (allele B = four repeats), 500 bp (allele C = five repeats), 325 bp (allele D = three repeats), 595 bp (allele E = six repeats), and 86 bp (allele F = one repeat) were analyzed by electrophoresis on a 2% agarose gel.^[15]

The molecular weight of each band was determined by using the software in Biovis Gel Software, version 4 (Expert Vision, Mumbai), and the unknown samples were compared with the 100 bp DNA ladder (MBI-Fermentas, USA). To improve the genotyping quality and validation, 20% of samples were re-genotyped by other laboratory personnel, and the results were found to be reproducible with no discrepancy noticed in genotyping. Genotyping of 10% of samples was confirmed by DNA sequencing

Statistical analysis

Allele frequencies, genotype frequencies, and carriage rates of the alleles in all the groups were compared with Fisher exact test using the program SPSS software (version 11.5, SPSS Inc., Chicago, IL). Data on quantitative characteristics were expressed as means ± SD. Comparisons between groups were made with the χ^2 test (nominal data) or Student *t* test (interval data). Allele frequency was calculated as the number of occurrences of the

test allele in the population divided by the total number of alleles. The carriage rate was calculated as the number of individuals carrying at least 1 copy of the test allele divided by the total number of individuals. All P values were two sided, and differences were considered statistically significant for $P < .05$. Hardy-Weinberg equilibrium was tested by the χ^2 method. Odds ratio (OR) at 95% confidence interval (CI) was determined to describe the strength of association by logistic regression model. The sample size was calculated using the QUANTO ver. 1 program (<http://hydra.use.edu/gxe>). Calculated sample size was adequate to study both polymorphisms. Log additive inheritance model was used for T2DM as a polygenic disease.

Results

A total of 120 T2DM patients (mean age, 48.41 ± 10.16 years) and 150 healthy controls (mean age, 50 ± 11.28 years) were evaluated for the IL-1RN and IL-4 gene polymorphism study. The mean blood pressure, plasma glucose, cholesterol (LDL/HDL/VLDL/total), serum creatinine, and other parameters are listed in Table 1.

The comparative distribution of genotype in patients versus controls showed that overall genotype distribution for both IL-4 ($P = .002$) and IL-1RN ($P < .001$) gene polymorphisms was significantly associated with T2DM patients [Table 2]. In regard to allele frequency and carriage rate, there was lack of association in case of IL-4 ($P = .209$ and $.210$ respectively) among patients and controls. However, a significant difference in both allele frequency and carriage rate ($P < .001$ and $P = .041$ respectively) was observed for IL-1RN gene polymorphism [Table 2]. The genotype frequency of BB (63.3% vs. 30.0%) and allele frequency of B (75.83% vs. 55.0%) of IL-1RN were significantly higher in patients than in controls. Similarly, in case of IL-4 the frequency of B2B2 genotype (65.0% vs. 50.67%) and that of B2 allele (77.08% vs. 72.33.0%) of were significantly higher in patients than in controls [Table 2].

In order to investigate gene-gene interaction, we analyzed the combined effect of IL-4 and IL-1RN on, and their possible association with, T2DM. Genotypes were grouped into high- or low-producer phenotypes — IL-4, B1B1, or B1B2 = high-producer (HP) phenotypes; B2B2 = low-producer (LP) phenotypes. Similarly in case of IL-1RN, AA = HP phenotypes, and all others were considered LP phenotypes. Comparative analysis of combinations of IL-4 and IL-1RN (IL-4^{low}-IL-1RN^{low} / IL-4^{high}-IL-1RN^{low} / IL-4^{low}-IL-1RN^{high}) showed that all these combinations lacked significant difference ($P = .56$, $.99$, and $.84$ respectively)

between controls and T2DM patients [Table 2]. IL-4 and IL-1RN high-producing genotypes were taken as reference for this analysis.

We also analyzed the association of different clinical parameters of T2DM patients with IL-4 and IL-1RN genotypes; there was lack of association with any of these parameters [Table 3].

Discussion

Earlier studies have reported that hyperglycemia associated with T2DM acutely increases peripheral cytokines like IL-6 and TNF- α and IL-1 proteins.^[16] *In vitro* studies revealed that IL-6 and TNF- α can impair insulin-signaling pathway, resulting in insulin resistance.^[17-18] It is proposed that cytokines might normally function in a feedback pathway to limit the number of adipocytes or lipid storage by exerting catabolic effects on the adipocyte, blocking lipid synthesis and lipoprotein lipase expression, while activating lipolysis, and can block or reverse differentiation of fibroblastic precursors into adipocytes.^[19] However, *in vivo* results till date are largely inconsistent. Identification of appropriate markers of T2DM for recognizing genetic influence upon initiation and progression of the disease might assist the clinicians in adopting a more precise approach for the identification of 'high-risk' T2DM patients and in the development of personal medicine strategies for targeting inflammatory components; thus meeting a crucial medical need, as well as enabling planning therapeutic interventions. Genetic polymorphisms studied so far in T2DM with TNF- α and IL-6 have also revealed no or only marginal association.^[20-21]

Lisa *et al.* reported lack of association of IL-4 with type 1 diabetes mellitus, but the nature of association of IL-4 with T2DM is unknown.^[22] Recently, Achut *et al.* demonstrated a significant association of VNTR polymorphism of IL-4 with increased risk of T2DM, as well as its associated complications in the north Indian population.^[23] In our study, we have also found positive association of not only IL-4 but also of IL-1RN genotype with T2DM. It is already showed that T2DM inhibits IL-4-mediated IL-1RN production, and administration of IL-1RN improves T2DM. Thus our current findings suggest that genetic polymorphism of both IL-4 and IL-1RN may finally influence initiation and progression of T2DM as already reported mechanistic pathways. Measurement of the IL-4 and IL-1RN levels in serum, along with the association studies of polymorphism, would further strengthen the predicted functional implications; however, was one important limitation of this study. Besides, like

many other significantly associated cytokine polymorphisms in T2DM, how these cytokines induce insulin resistance and help in progression of the vicious cycle of insulin resistance and beta cell degradation is largely unknown.

Since both IL-1RN and IL-4 have been found to be associated with risk of T2DM, it was imperative to investigate if combination of their particular polymorphic variants could specifically influence the susceptibility further. Therefore, we also tried to find out the combined effect of IL-1RN and IL-4 on T2DM and observed IL-4^{low}-IL-1RN^{low} genotype showed 1.26 times risk in patients, although the *P* value was not significant.

In conclusion, this study supports that genetic variation in IL-4 and IL-1RN cytokine genes may predispose to the development of T2DM in the Indian population. The combined effect of IL-4 and IL-1RN revealed a further increase in risk of T2DM.

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Table 1: Clinical characteristics of patients with T2DM

Clinical characteristics	Patients
Age (Years)	48.41 \pm 10.16
Duration of Diabetes (Years)	4.64 \pm 5.05
BMI (Kg/m ²)	24.44 \pm 4.62
WHR (Waist hip ratio)	0.92 \pm 0.07
Fasting Plasma Glucose (Mg/dl)	196.34 \pm 77.80
Post Prandial Plasma Glucose (Mg/dl)	261.18 \pm 104.24
Total-Cholesterol (Mg/dl)	228.17 \pm 31.35
LDL-Cholesterol (Mg/dl)	162.83 \pm 30.41
HDL-Cholesterol (Mg/dl)	43.04 \pm 3.64
VLDL-Cholesterol (Mg/dl)	22.56 \pm 3.12
Triglyceride (Mg/dl)	112.71 \pm 13.69
Blood pressure (Systolic)(mmHg)	135.00 \pm 17.49
Blood pressure (Diastolic)(mmHg)	83.24 \pm 13.22

Serum Cretenine (MGM%)	1.03 ± 0.09
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Data are mean ± standard deviation (SD) or frequencies (%).

Table 2: Distribution of genotypes, allele frequency, and carriage rate of IL-4 and IL-1RN gene polymorphisms and the risk of T2DM

Genotypes	IL-4 Gene Polymorphism				IL-1RN Gene Polymorphism			
	B1B1	B1B2	B2B2	P value, OR (95%CI)	AA (240)	BB (410)	A/B (240/410)	P value, OR (95%CI)
Patients (n=120)	13 (10.83%)	29 (24.17%)	78 (65.0%)	0.002 , 2.30 (1.34-3.95)	14 (11.67%)	76 (63.33%)	30 (25.0%)	<0.001 4.22 (2.41-7.40)
Control (n=150)	9 (6.0%)	65 (43.33%)	76 (50.67%)		30 (20.0%)	45 (30.0%)	75(50.0%)	
Allele Frequency	B1	B2	0.209 1.06 (0.96-1.18)	A (240)	B (410)	P value, OR (95%CI)		
Patients (n=120)	55 (22.92%)	185 (77.08%)		58 (24.17%)	182 (75.83%)	<0.001 1.38 (1.22-1.56)		
Control (n=150)	83 (27.67%)	217 (72.33%)		135 (45.0%)	165 (55.0%)			
Carriage Rate	B1	B2	0.210 1.10 (0.95-1.26)	A (240)	B (410)	P value, OR (95%CI)		
Patients (n=120)	42 (35.0%)	107 (89.17%)	44 (36.67%)	106 (88.34%)	0.041 1.19 (1.01-1.39)			
Control (n=150)	74 (49.33%)	141 (94.0%)	105 (70.0%)	120 (80.0%)				
Combined genotype								
Combined genotype	IL-4 ^{low} -IL-1Ra ^{low}		IL-4 ^{high} -IL-1Ra ^{low}	IL-4 ^{low} -IL-1Ra ^{high}	IL-4 ^{high} -IL-1Ra ^{high}			
Control (n=150); (%)	68 (56.67%)		50 (33.33%)	14 (9.33%)	18 (12.0%)			
Patients (n=120); (%)	62 (51.67%)		36 (30.0%)	9 (7.5%)	13 (10.83%)			
P, OR at 95% CI	0.56, 1.26 (0.57-2.79)		0.99, 0.99 (0.43-2.29)	0.84, 0.89 (0.29-2.67)	1.0 (Ref)			

Table 3: Association of various clinical parameters with IL-4 and IL-1RN genotypes in T2DM patients

Clinical Parameters.	IL-4		P value	IL-1Ra		P value
	(B1B1)	(B2B2) + (B1B2)		(A/A)	(B/B) + (A/B)	
Age (years)	45.38 ± 8.69	48.78 ± 10.31	0.257	49.38 ± 12.55	48.07 ± 9.85	0.662
Duration of diabetes (years)	5.33 ± 5.54	4.57 ± 5.04	0.708	4.20 ± 3.56	5.22 ± 5.34	0.677
BMI (kg/m ²)	22.77 ± 2.25	24.60 ± 4.76	0.259	24.69 ± 4.88	24.30 ± 4.32	0.782
WHR(WaistHipRatio)	0.94 ± 0.04	0.92 ± 0.07	0.464	0.90 ± 0.06	1.02 ± 0.09	0.674
Fasting Plasma Glucose (mg/dl)	167.36 ± 97.11	169.58 ± 75.73	0.929	191.57 ± 53.38	174.35 ± 79.79	0.489
Post Prandial Plasma Glucose (mg/dl)	286.55 ± 160.99	258.50 ± 97.39	0.445	274.10 ± 50.30	268.77 ± 99.56	0.868
Total-cholesterol (mg/dl)	227.33 ± 24.23	228.28 ± 32.21	0.922	231.27 ± 31.07	226.91 ± 32.09	0.668
LDL-cholesterol (mg/dl)	161.71 ± 24.58	162.97 ± 31.16	0.893	163.16 ± 31.84	161.09 ± 30.09	0.830
HDL-cholesterol (mg/dl)	43.00 ± 2.33	43.04 ± 3.78	0.969	44.25 ± 3.88	42.98 ± 3.91	0.318
VLDL-cholesterol (mg/dl)	21.86 ± 2.19	22.65 ± 3.22	0.415	22.95 ± 3.15	22.70 ± 3.48	0.823
Triglyceride (mg/dl)	109.33 ± 10.96	113.13 ± 13.98	0.367	117.09 ± 12.53	112.46 ± 14.68	0.318
Blood pressure (systolic)(mmHg)	128.00 ± 23.87	135.66 ± 16.92	0.353	139.00 ± 23.46	132.84 ± 16.51	0.492
Blood pressure (diastolic)(mmHg)	76.89 ± 8.94	83.92 ± 13.41	0.203	85.00 ± 15.19	80.58 ± 10.38	0.489
Serum Creatinine (mg/dl)	1.02 ± 0.13	1.04 ± 0.13	0.73	1.08 ± 0.13	1.02 ± 0.13	0.13

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