



***IL-1 β* , *IL-1Ra* and *IL-18* gene variants in type 2 diabetes**

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is a complex disease characterized by elevated systemic glucose concentrations. Multiple loci pre-disposing to T2DM have emerged from genome-wide association studies. The *IL-1* family consists of two pro-inflammatory cytokines viz. *IL-1 α* , *IL-1 β* and a naturally occurring anti-inflammatory agent, *IL-1* receptor antagonist (*IL-1Ra* or *IL-1RN*). It is known fact that prolonged exposure of human islets to high glucose trigger *IL-1 β* production by β -cells themselves leading to nuclear factor activation and upregulation of *Fas* signaling thus triggering "autocrine apoptosis". *IL-18* is closely related to *IL-1 β* in structure and in the requirement of *caspase-1* to cleave its precursor into an active cytokine. DNA was isolated from venous blood samples, quantified and subjected to Polymerase Chain Reaction-Restriction Fragment Length Polymorphism using suitable primers and restriction endonucleases. The genotypic, allelic and carriage rate frequencies were analyzed for three genetic variants *IL-1 β* (-511 C/T), *IL-18* (-607 A/C) and *IL-1Ra* VNTR (intron 2) in patients and controls using SPSS software. Odd ratios (OR) with 95% confidence interval (CI) was determined to describe the strength of association by logistic regression model. All studied SNPs showed significant association ($P < 0.001$) with occurrence of T2DM and biochemical parameters as well. However, in haplotypic analysis, none of the combinations of alleles showed significant association. Interestingly, it was observed that alleles I and II of *IL-1Ra* might be involved in T2DM predisposition. The study will probably increase our insight into the proposed impact of *IL-1* in the development and/or progression of T2DM and its susceptibility in North-Indian population.

Key words: *IL-1 β* , *IL-1Ra*, *IL-18*, SNP, Type 2 Diabetes, PCR- RFLP, North Indian Population

INTRODUCTION

Multiple loci pre-disposing to Type 2 diabetes mellitus (T2DM) have emerged from genome-wide association studies [1]. The *IL-1* family consists of two pro-inflammatory cytokines viz. *IL-1 α* , *IL-1 β* and a naturally occurring anti-inflammatory agent, the *IL-1* receptor antagonist (*IL-1Ra* or *IL-1RN*). The genes encoding these cytokines comprise a cluster spanning 10.9 Mb on 2q13-14 [2]. The two *IL-1* isoforms (α and β) are encoded by distinct genes, are structurally related and bind to the same receptor [3]. All *IL-1* family genes are polymorphic and are associated with either susceptibility or severity of inflammatory diseases [4, 5]. The cytokines *IL-1 β* and *IL-1Ra* are produced by a variety of cell types, for example, monocytes, macrophages and keratinocytes. They are potent mediators of inflammatory response and play important roles in tissue remodeling during chronic inflammation [6]. Therefore,

cytokine genes may be important candidates with implications in pathogenesis of T2DM and associated complications.

IL-18 is a unique member of IL-1 family encoded by a gene at the diabetes susceptibility locus, *Idd2* located on chromosome 11q22.2-22.3 [7]. It is closely related to IL-1 β in structure and in the requirement of caspase-1 to cleave its precursor into an active cytokine. Insulin-producing islet β -cells secrete IL-18 and induce IFN γ in T cells [8]. Plasma IL-18 levels have been reported to be elevated in T2DM patients and children [9-11] and is independent of the major determinants of metabolic syndrome *viz.* obesity and insulin resistance [12]. IL-18 is a potent pro-inflammatory cytokine highly expressed in atherosclerotic plaques and play a role early in the inflammatory cascade and also in plaque destabilization [13]. The study will probably increase our insight into the proposed impact of IL-1 β , *IL-1Ra* and *IL-18* gene variants in the development and/or progression of T2DM.

EXPERIMENTAL SECTION

Patient selection and clinical evaluation

Type 2 diabetes patients were enrolled from the outpatient Diabetes Clinic of King George's Medical University (KGMU), Lucknow, India under the supervision of expert clinicians. Age/sex-matched normal controls were screened from healthy staff members of both universities. The study was conducted after due approval of Institutional Ethics Committee and written consent from all subjects. Controls showing a normal oral glucose tolerance test were included in the study whereas those having a history of coronary artery disease or other metabolic disorders were excluded. Subjects with fasting glucose (F) concentrations ≥ 126 mg/dl or 2-h post prandial (PP) glucose concentrations ≥ 200 mg/dl were categorized in the diabetes group [14]. Estimations of plasma glucose (mg/dl), serum insulin (mg/dl) and lipid profile *viz.* Total Cholesterol (TC), Triglycerides (TGL), High Density Lipoproteins (HDL) and Serum Creatinine (SCRT) were done using commercially available *Ecoline* kits from Merck by UV-vis double beam spectrophotometer (Shimadzu, Japan). Low Density Lipoproteins (LDL) and Very Low Density Lipoproteins (VLDL) were calculated by known formulae [15]. Height, weight and waist circumference were measured to calculate body mass index (BMI) and waist hip ratio (WHR). Clinical details of patients and controls were recorded.

DNA extraction and genotyping

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using salting out method with slight modifications [14, 16]. Variable Number of Tandem Repeat (VNTR) of *IL-1Ra* (Intron 2) were genotyped by polymerase chain reaction (PCR). Polymerase chain reaction (PCR) was performed with forward 5'-CTCAGCAACTCCTAT-3' and reverse 5'-TCCTGGTCTGCAGGTAA-3' primers at 60°C annealing temperature and 35 cycles. The VNTRs were analyzed in 2.0% agarose gels. The two SNPs, *IL-1 β* -511 C/T (rs16944) and *IL-18*-607 A/C (rs1946518) were amplified and genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The primers for rs16944 and rs1946518 were forward 5'-TGGCATTGATCTGGTTCATC-3'/reverse 5'-GTTTAGGAATCTTCCCACTT-3' and forward 5'-CTTTGCTATCATTCCAGGAA-3'/reverse 5'-TAACCTCATTCCAGGACTTCC-3' respectively. The 15 μ l reaction mixture contained 100 ng of template DNA, buffer (100 mM Tris, pH 9.0; 500 mM KCl; 15 mM MgCl₂; 0.1% gelatin), 200 μ M dNTP, 10 pmol of each primer and 1.0 unit Taq DNA polymerase. The PCR products were digested with restriction enzymes *SacI* and *MseI* (Fermentas, USA) respectively and electrophoresed on a 12.5% polyacrylamide gel (**Figure 1**). To ensure the quality of genotyping, random duplicates of 20% samples were performed and 98% concordance was found.

Statistical analysis

Allele frequencies and carriage rates in all groups were compared using 2x2 contingency table while genotype frequencies were compared using 2x3 contingency table by Fisher's exact test. The Hardy-Weinberg equilibrium at individual loci was assessed by chi-square (χ^2) statistics using SPSS software (ver. 17.0). Haplotype analysis was performed and odd's ratios were computed using multiple regression analysis. Linkage disequilibrium and haplotype analysis was performed by SHEsis software [17]. Student's t test was used to compare age, BMI, WHR, biochemical parameters *viz.* glucose (F and PP), TC, TGL, HDL, LDL, VLDL and SCRT of diabetic subjects with controls.

RESULTS

In case of *IL-1β* polymorphism, allele ‘C’ corresponded to 304 bp and allele ‘T’ to 189, 116 bp products. However, in *IL-18*, allele ‘C’ corresponded to 199, 70 bp and allele ‘A’ to 101/98, 70 bp products. Four alleles were observed in our population in case of *IL-1Ra* polymorphism having 4, 2, 5 and 3 repeats of 86 bp with product sizes of 410, 240, 500 and 325 bp designated as alleles I, II, III and IV. The gel pictures showing genotypes of *IL-1β*, *IL-1Ra* and *IL-18* are shown in **Figure 1**. *IL-1β*-511 C/T polymorphism showed genotypic association (P<0.001) but no allelic association. Most subjects were homozygous wild type ‘TT’, while ‘CC’ genotype was rare (14.81 and 4.12% in controls and T2DM patients respectively). Allele frequencies of ‘C’ and ‘T’ were almost same in controls and T2DM patients (**Table 1**). The carriage rate of C (+), C (-) and T (+), T (-) showed significant association with T2DM when compared to controls (P=0.047 and P=0.001 respectively) (**Table 1**). All allele and genotype frequencies were found to be in Hardy-Weinberg equilibrium.

Table 1: Genotypic, allelic and carriage rate frequencies of *IL-1β*-511 C/T, *IL-1Ra* and *IL-18*-607 A/C gene polymorphisms in healthy controls and T2DM cases.

Genotype frequencies					
	Genotypes	Controls (n=189)	Patients (n=194, *248)	P-value	
<i>IL-1β</i> -511 C/T	CC	28 (14.81)	8 (4.12)	<0.001	
	CT	37 (19.58)	78 (40.21)		
	TT	124 (65.61)	108 (55.67)		
<i>IL-1Ra</i> (Intron 2)*	I/I	124 (65.61)	156 (62.9)	0.003	
	II/II	7 (3.7)	37 (14.92)		
	IV/IV	1 (0.53)	1 (0.4)		
	I/II	53 (28.04)	49 (19.76)		
	II/III	2 (1.06)	1 (0.4)		
	I/III	2 (1.06)	4 (1.62)		
<i>IL-18</i> -607 A/C	AA	3 (1.59)	11 (5.67)	0.002	
	AC	142 (75.13)	115 (59.28)		
	CC	44 (23.28)	68 (35.05)		
Allele Frequencies					
	Alleles	Controls (n=378)	Patients (n=388, *496)	P-value	Odd's Ratio CI (95%)
<i>IL-1β</i> -511 C/T	C	93 (24.6)	94 (24.23)	0.904	1.021 (0.734-1.419)
	T	285 (75.4)	294 (75.77)		
<i>IL-1Ra</i> (Intron 2)*	I	303 (80.16)	365 (73.59)	0.061	1.311 (0.987-1.740)
	II	69 (18.25)	124 (25)		
	III	4 (1.06)	5 (1)		
	IV	2 (0.53)	2 (0.41)		
<i>IL-18</i> -607 A/C	A	148 (39.15)	137 (35.31)	0.271	1.179 (0.879-1.581)
	C	230 (60.85)	251 (64.69)		
Carriage Rate					
		Controls (n=189)	Patients (n=194, *248)	P-value	Odd's Ratio CI (95%)
<i>IL-1β</i> -511 C/T	C (+)	65 (34.39)	86 (44.33)	0.047	0.658 (0.436-0.995)
	C (-)	124 (65.61)	108 (55.67)		
	T (+)	161 (85.19)	186 (95.88)	0.001	0.247 (0.110-0.558)
	T (-)	28 (14.81)	8 (4.12)		
<i>IL-1Ra</i> (Intron 2)*	I (+)	179 (94.71)	209 (84.27)	0.001	3.340 (1.621-6.881)
	I (-)	10 (5.29)	39 (15.73)		
	II (+)	62 (32.8)	87 (35.08)	0.619	0.903 (0.603-1.348)
	II (-)	127 (67.2)	161 (64.92)		
	III (+)	4 (2.12)	5 (2.02)	0.942	1.051 (0.278-3.968)
	III (-)	185 (97.88)	243 (97.98)		
	IV (+)	1 (0.53)	1 (0.4)	0.847	1.314 (0.082-21.141)
	IV (-)	188 (99.47)	247 (99.6)		
<i>IL-18</i> -607 A/C	A (+)	145 (76.72)	126 (64.95)	0.012	1.778 (1.136-2.784)
	A (-)	44 (23.28)	68 (35.05)		
	C (+)	186 (98.41)	183 (94.33)	0.046	3.727 (1.023-13.579)
	C (-)	3 (1.59)	11 (5.67)		

Table 2: Association of anthropometrical/biochemical parameters with *IL-1β*-511 C/T, *IL-1Ra* and *IL-18*-607 A/C genotypes in controls and T2DM cases

Gene Genotypes ↓		p-values of anthropometrical/biochemical data										
Gene Genotypes ↓		AGE (Years)	BMI (Kg/m ²)	WHR	F (mg/dl)	PP (mg/dl)	TC (mg/dl)	TGL (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	SCRT (mg/dl)
<i>IL-1β</i> -511	C/C	0.189	0.126	0.332	0.001	0.001	0.063	0.296	0.065	< 0.001	0.901	0.303
	C/T	0.001	0.106	0.585	< 0.001	< 0.001	< 0.001	0.678	< 0.001	< 0.001	0.588	0.357
	T/T	< 0.001	0.076	0.322	< 0.001	< 0.001	< 0.001	0.015	0.093	< 0.001	0.024	0.567
<i>IL-1Ra</i>	I	< 0.001	0.192	0.451	< 0.001	< 0.001	< 0.001	0.028	0.026	< 0.001	0.041	0.413
	II	0.007	0.043	0.292	0.008	0.033	0.037	0.024	0.034	< 0.001	0.044	1.000
	I/II	0.007	0.043	0.292	0.008	0.033	0.037	0.024	0.034	< 0.001	0.044	1.000
	I/III	0.525	0.526	0.488	0.107	0.436	0.066	0.755	0.969	0.001	0.754	0.129
<i>IL-18</i> -607	A/A	0.062	0.933	0.214	0.297	0.366	0.055	0.662	0.695	0.008	0.854	0.062
	A/C	< 0.001	0.034	0.426	< 0.001	< 0.001	< 0.001	0.254	0.007	< 0.001	0.297	0.781
	C/C	< 0.001	0.056	0.219	< 0.001	< 0.001	< 0.001	0.016	0.010	< 0.001	0.024	0.714

Table 3: Association of SNPs in different candidate genes (*IL-1β*-511 C/T, *IL-1Ra* and *IL-18*-607 A/C) with T2DM in North Indian population

	T2DM (freq)	Control (freq)	χ ²	Pearson's P	Odds Ratio [95% CI]
C I C*	10.62(0.027)	14.96(0.040)	0.842	0.358	0.689 [0.310-1.533]
C II A*	22.42(0.058)	32.57(0.086)	2.214	0.136	0.656 [0.375-1.147]
C II C*	53.42(0.138)	39.58(0.105)	2.092	0.148	1.381 [0.890-2.142]
T I A*	16.89(0.044)	23.82(0.063)	1.375	0.240	0.683 [0.360-1.296]
T II C*	40.94(0.106)	27.32(0.072)	2.740	0.097	1.531 [0.922-2.542]
T II A*	90.13(0.232)	85.72(0.227)	0.063	0.801	1.044 [0.745-1.465]
T II C*	141.03(0.363)	145.14(0.384)	0.243	0.622	0.928 [0.691-1.247]
Global	-----	-----	8.681	0.192	-----

Multivariate logistic regression analysis for association of genotypes with biochemical parameters brought forth some interesting results. We found that the subjects with 'CC' genotype showed significant association with F, PP and LDL (P=0.001; 0.001 and <0.001 respectively). In case of 'CT' and 'TT' subjects significant association was observed with age, F, PP, TC, HDL and LDL (P<0.001). In addition, 'TT' genotypes also showed significant association with TGL and VLDL (P<0.001) (**Table 2**). No significant association was observed with BMI, WHR and SCRT (P>0.05).

Genotypic association of *IL-1Ra* was observed in the present study (P=0.003) but no allelic association. Genotypes II/III and IV were found to be rare in both controls and T2DM patients. Most subjects were homozygous I/I in the study population. Allele frequencies of I and II were slightly different in controls and T2DM patients but not significant (**Table 1**). The carriage rate of allele I (+) and (-) showed significant association (P=0.001; OR 3.340; CI 1.621-6.881) with T2DM when compared to controls (**Table 1**).

The association of genotypes with biochemical parameters using multivariate logistic regression showed that subjects with I/I genotype showed significant association with age, F, PP, TC, TGL, HDL, LDL and VLDL (P<0.05). In case of II/II and I/I genotypes significant association was observed with all clinical parameters except SCRT (P<0.05). Genotype I/II also showed significant association with all clinical parameters except BMI and SCRT (P<0.05). However, in case of genotype I/III only LDL showed significant association (P=0.001). No genotypic condition showed significant association with SCRT (P>0.05) (**Table 2**).

Likewise, the *IL-18*-607 A/C polymorphism also showed genotypic association (P=0.002). While genotype 'AA' was rare in controls and T2DM patients, 'CC' genotype was present in 23.28 % controls and 35.05 % patients. Most subjects were found to be heterozygous 'AC' in the study population (75.13% controls and 59.28 % cases). The difference in allele frequencies of 'A' and 'C' in controls and patients was not significant. However, the carriage rate of both 'A' and 'C' showed significant association with T2DM when compared to controls (P=0.012 and 0.046 respectively) (**Table 1**). The results showed that presence of 'C' increases the risk of diabetes upto 3.7 times while allele 'A' increases the risk only upto 1.7 times.

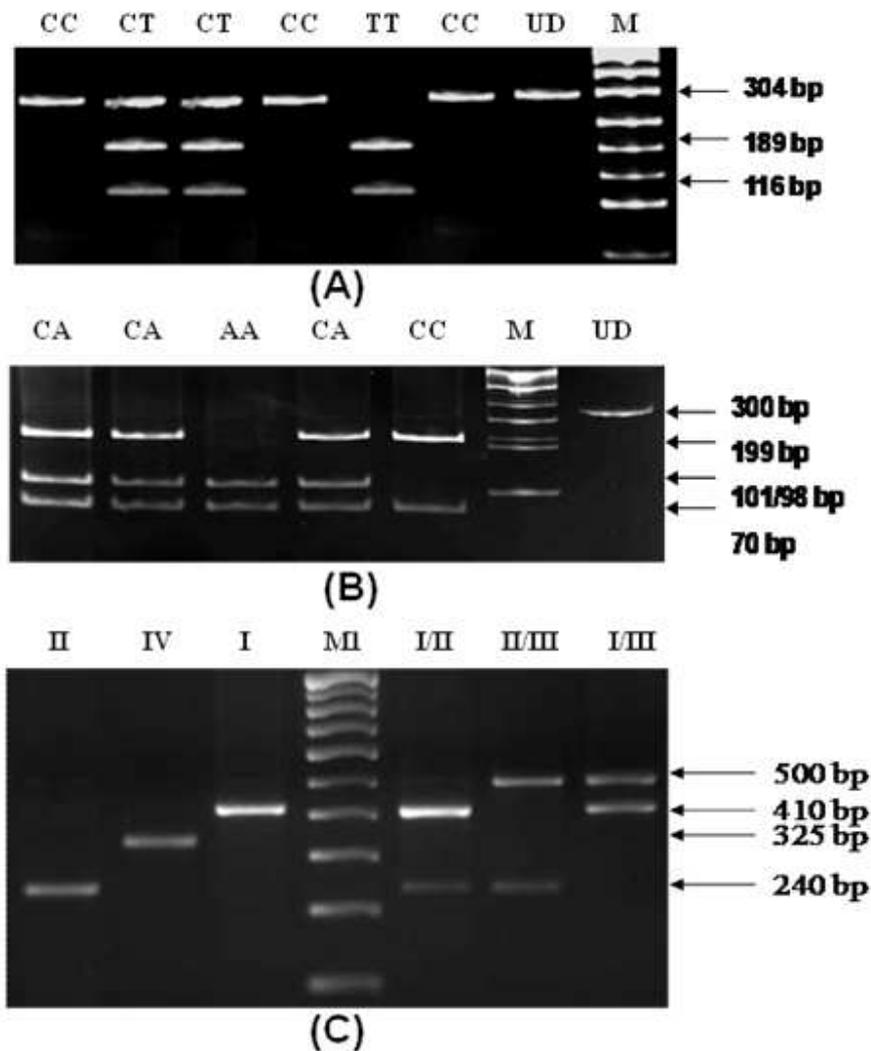


Figure 1: A 12.5% polyacrylamide gel showing genotypes of (A) *IL-1β* -511 C/T using SacI; (B) *IL-18* -607 A/C using MseI. M: pUC19/MspI Digest, UD: undigested; (C) A 2% agarose gel showing *IL-1Ra* VNTRs, M1: 100 bp Ladder

Association of genotypes with biochemical parameters using multivariate logistic regression showed that subjects with ‘AA’ genotype showed significant association with LDL alone (P=0.008). In case of ‘AC’ genotype significant association was observed with age, BMI, F, PP, TC, HDL and LDL (P<0.05) while ‘CC’ showed significant association with all clinical parameters except BMI, WHR and SCRT (P<0.05) (**Table 2**). None of the genotypic conditions showed significant association with WHR and SCRT (P>0.05).

Gene-gene interaction studies showed that none of the combinations of *IL-1β* [rs16944], *IL-1Ra* and *IL-18* [rs1946518] alleles showed significant association with the disease. The LD plots showing pairwise LD between the studied SNPs are shown in **Figure 2**. However, it was observed that alleles ‘I’ and ‘II’ of *IL-1Ra* may be involved in the T2DM predisposition (**Table 3**).

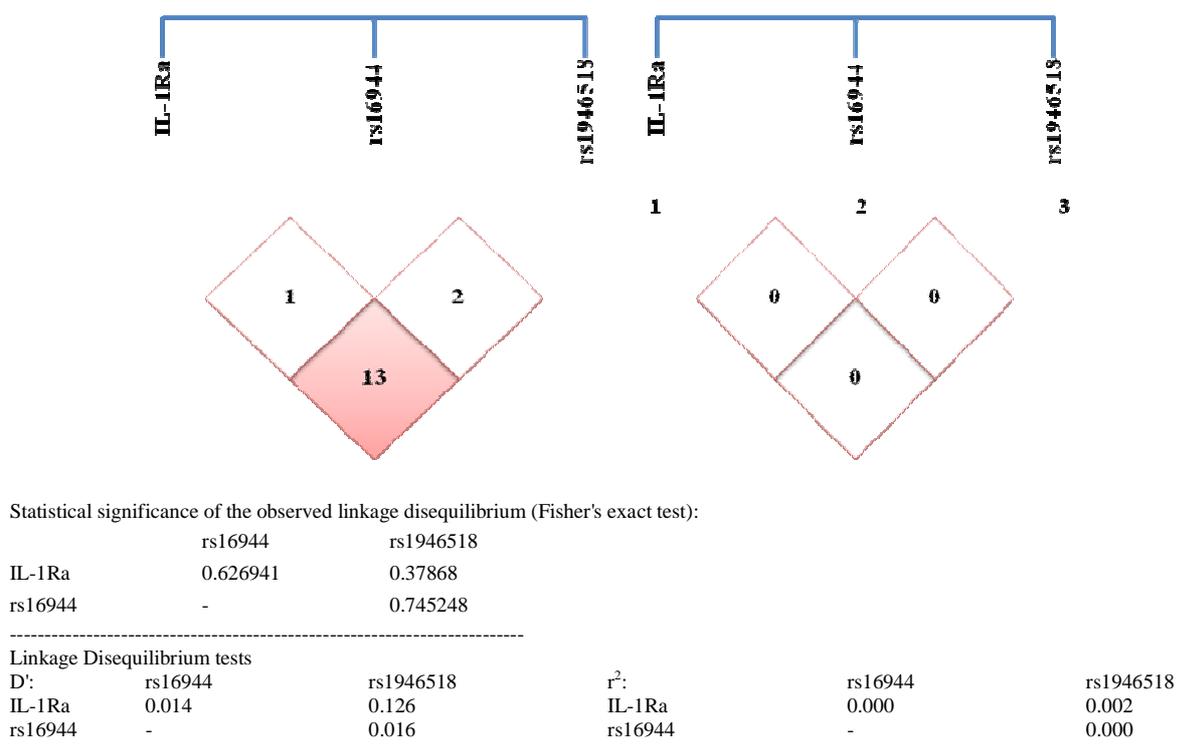


Figure 2: Linkage Disequilibrium (D') and Correlation coefficient (r²) of three SNPs viz. *IL-1Ra*, rs16944 and rs1946518 with T2DM in North Indian population. Pairwise LD in subjects is represented as pink

DISCUSSION

All *IL-1* family genes (*IL-1 α* , *IL-1 β* , *IL-1Ra* and *IL-18*) are polymorphic and several of these polymorphisms have been shown to be associated with either susceptibility or severity of inflammatory conditions and diseases [4, 5, 18, 19]. It has been recently suggested that prolonged exposure of human islets to high glucose trigger *IL-1 β* production by β -cells themselves leading to nuclear factor activation and upregulation of Fas signaling thus triggering "autocrine apoptosis" [20]. The cytokine proteins *IL-1 β* and *IL-1Ra* are potent mediators of inflammatory response and play important roles in tissue remodeling in chronic inflammation [6]. *IL-1* gene polymorphisms have been studied by various groups in T2DM and related complications [5, 19-25].

IL-18 expression has been reported in macrophages, dendritic cells (DC), Kupffer cells, keratinocytes, osteoblasts, adrenal cortex cells, intestinal epithelial cells, microglial cells, and synovial fibroblasts [26-30].

IL-18 levels have been associated with adiposity and insulin resistance in obese premenopausal women. It has been reported that *IL-18* concentration is increased by acute hyperglycemia in humans [31]. *IL-18* gene polymorphisms have been studied by various groups in T2DM and related complications [32-36].

In the present case-control study of *IL-1 β* gene polymorphism, significant association was observed in genotypic frequency distribution ($P < 0.0001$) while none was observed in allele frequencies. However, carriage rate of -511*C and -511*T were involved in T2DM manifestation (Table 1). The biochemical parameters brought forth some interesting results with *IL-1 β* gene polymorphism showing no significant association with BMI, WHR and SCRT ($P > 0.05$). However, the 'CC' genotype showed significant association with F, PP and LDL ($P < 0.001$), while 'CT' and 'TT' subjects with age, F, PP, TC, HDL and LDL ($P < 0.001$) and 'TT' genotype with TGL and VLDL ($P < 0.001$) (Table 2).

In *IL-1Ra* we found four different alleles in our population unlike previous reports which showed two alleles [22]. Our study also found positive association of *IL-Ra* genotype with T2DM. Most subjects in the study population were homozygous genotype I (65.61 and 62.90 % controls and T2DM patients respectively) as previously reported [22]. The biochemical parameters showed significant association with I, II and I/II genotypes of *IL-1Ra*. However, in case of genotype I/III, only LDL showed significant association ($P=0.001$) (**Table 1**). No genotypic condition showed significant association with SCRT ($P>0.05$). The *IL-Ra* gene did not show any association with myocardial infarction which is a severe diabetic complication [37]. The IL-1Ra is a major modulator of the IL-1 pro-inflammatory pathway. The relationship between a variable number tandem repeat (VNTR) polymorphism in intron 2 of the *IL-1Ra* gene and coronary artery disease (CAD) in patients with and without T2DM was studied by Marculescu *et al*, 2002 [38]. Our results showed that allele I of *IL-1Ra* gene polymorphism increases the risk of T2DM upto 3.4 times in the study population.

Opstad *et al*, 2011 [32] studied +183 A/G, -137 G/C and -607 C/A *IL-18* gene polymorphisms in T2DM subjects and showed that only +183 A/G was significantly associated with the disease. However, in Chinese population -607 C/A polymorphism was significantly associated with T2DM [33]. European researchers studied many *IL-18* gene polymorphisms with T2DM, metabolic syndrome, cardiovascular diseases and presented controversial results [34-36]. In our population, -607 C/A polymorphism showed genotypic association ($P=0.002$) (**Table 1**) and most subjects were found to be heterozygous. Biochemical analysis showed that 'AA' genotype showed significant association with LDL alone ($P=0.008$), 'AC' genotype with age, BMI, F, PP, TC, HDL, LDL ($P<0.05$) and 'CC' genotype with all clinical parameters except BMI, WHR and SCRT ($P<0.05$) while, no significant association was observed with WHR and SCRT ($P >0.05$) (**Table 2**). The result showed that -607*C of *IL-18* gene increases the risk of diabetes upto 3.7 times while -607*A increases the risk upto 1.7 times (**Table 1**). Individuals with 'AC' genotype of *IL-18* -607 C/A may be more susceptible to T2DM.

IL-1Ra, *IL-1 β* -511 C/T and *IL-18*-607 A/C gene-gene interaction analyses showed that none of the combinations of alleles had significant association with T2DM (**Table 3**) but in *IL-1Ra* only I and II alleles are involved in T2DM predisposition. T2DM is a combined metabolic disorder which includes hyperglycemia, dyslipidemia, stroke *etc.* being the most common causes of death in diabetic patients. Inflammation has been an important outcome of any kind of imbalance in the body and is therefore an indicator of several diseases including T2DM. Various groups all over the world are relentlessly working out the possible role of a vast number of genes associated with T2DM.

CONCLUSION

The present study was undertaken to explore the association of cytokine gene polymorphisms with T2DM and a sincere effort was made to determine whether specific genotypes/haplotypes of SNPs or gene-gene interactions could actually be useful in predicting the susceptibility risk of individuals. Individuals with 'AC' genotype of *IL-18* -607 C/A were observed to be more susceptible to T2DM. SNP studies have shown a considerable level of variation amongst various ethnic populations around the world. Therefore, it is essential to perform the association studies/SNP analyses in individual populations so that they can be benefitted. Individuals at risk will be able to take prior precautionary measures and avoid or delay the onset of the disease. These genetic studies will lead to the identification of risk haplotype(s) for disease susceptibility, development of prognostic regimens and alter treatment strategies for T2DM and related complications. The challenge for the future will be to understand the genotypic interactions between SNPs in the same gene or genes at different loci.

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