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Interleukin-6 *(IL-6)*-597 A/G (rs1800797) & -174 G/C (rs1800795) gene polymorphisms in type 2 diabetes

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***Background & objectives*: Diabetes is a metabolic pro-inflammatory disorder characterized by chronic hyperglycaemia and increased levels of circulating cytokines suggesting a causal role for inflammation in its aetiology. In order to decipher the role of interleukin-6 (IL-6) in type 2 diabetes mellitus (T2DM) we analyzed two promoter polymorphisms -597 A/G (rs1800797) and -174 G/C (rs1800795) in T2DM cases from north India, and in healthy controls.**

***Methods*: DNA was isolated from venous blood samples of T2DM patients (n=213) and normal healthy controls (n=145). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed after biochemical analysis. The genotypic and allelic frequency distributions were analyzed.**

***Results*: The clinical/biochemical parameters of T2DM cases when compared to controls showed a significant difference. No significant association was observed with -597A/G polymorphism while, -174 G/C showed a highly significant association (P<0.001). In haplotypic analysis, combination of -597G*/-174C* showed significant association (P=0.010).**

***Interpretation & conclusions*: Our data suggest that *IL-6* gene polymorphisms play a prominent role in T2DM disease susceptibility in population from north India.**

Key words Gene polymorphism - interleukin-6 - PCR-RFLP - SNP - T2DM

Type 2 diabetes mellitus (T2DM) accounts for 90 per cent of diabetes cases worldwide. Over the past 30 years, the status of diabetes has changed from being considered as a mild disorder of old age to one of the major causes of morbidity and mortality affecting the young and middle-aged people. It has been estimated that in India 101.3 million people would be suffering from diabetes by 2030[1].

In recent years, evidences have shown that T2DM is associated with a subclinical systemic inflammation attributable to the dysregulation of innate immune system[2]. This immune response is characterized by elevated blood levels of certain acute phase markers, the principal mediator being interleukin-6(IL-6)[2]. Augmented levels of IL-6 are associated not only with T2DM but also with impaired glucose tolerance

(IGT), indicating a potential role of this cytokine in its aetiology[3]. A high rate of plasma clearance of IL-6 suggested that concentration is regulated at the levels of transcription and translation[4]. Studies on single nucleotide polymorphisms (SNPs) in the promoter region of *IL-6* gene in different populations worldwide suggested its possible role in T2DM susceptibility (Table I). Therefore, we attempted to analyze the association of two *IL-6* promoter polymorphisms *viz*. -597 A/G (rs1800797) and -174 G/C (rs1800795) with T2DM patients from north India.

Material & Methods

Patient selection and clinical evaluation: T2DM cases (n=213; 128 males; 85 females) with an age ranging from 22-76 yr were randomly enrolled from the outpatient Diabetes Clinic of King George's Medical University (KGMU), Lucknow, India, according to the National Diabetes Data Group (NDDG) and World Health Organization (WHO) inclusion-exclusion criteria[18]. Age/sex-matched normal controls (n=145; 96 males; 49 females) were screened from healthy staff members of both KGMU and University of Lucknow during January 2011 to January 2012. Sample size was calculated using QUANTO software, USA using 2 tailed analysis (Power: 90; Disease prevalence: 3%; error: 5%). The study was approved by the Institutional Ethical Committee of KGMU and a written informed consent was taken from all subjects enrolled in the study. Plasma glucose (Fasting blood sugar; FBS and Post-prandial; PPBS) (mg/dl) and lipid profile *viz*. total cholesterol (TC), triglycerides (TGL), high density lipoproteins (HDL) and serum creatinine (SCRT) were estimated using commercially available Ecoline kits (Merck, Germany) by UV-Vis spectrophotometer followed by low density lipoproteins (LDL) and very low density lipoproteins (VLDL) LDL and VLDL were calculated by known formulae. Height, weight and waist circumference were measured to calculate body mass index (BMI) and waist hip ratio (WHR).

DNA extraction and genotyping by PCR-RFLP: Blood samples (5 ml) were collected in 0.5M ethylene diamine tetra acetic acid (EDTA) and genomic DNA was extracted using the salting out method with slight modifications[19,20]. DNA was amplified with primers specific for -597 A/G (rs1800797) and -174 G/C (rs1800795) (Fig. 1) in a 15µl reaction mixture containing 100 ng of template DNA, buffer (100 mM Tris, *p*H 9.0; 500 mM KCl; 15 mM $MgCl_2$; 0.1% gelatin), 200µM deoxynucleotide triphosphate (dNTP), 10pmol of each primer (Integrated DNA Technologies, USA) and 1.5 units Taq DNA polymerase (Banglore Genei, India). PCR for -597 A/G (rs1800797) was

Table I. Variants of *IL-6* gene and their association with T2DM in different populations

Polymorphism	Morbidities	Ethnic group	Sample size	Association	Reference
	T2DM	Finnish	390	NS	4
	T2DM	Indian population	40	S	5
	T2DM	Caucasian subjects	7398	S	6
	T2DM	Germany	60	S	7
	T2DM	KORA Survey	704	S	8
	T2DM	Framingham Heart Study population	1428	S	9
	T2DM	Taiwanese	101	S	10
	T2DM	Nutrition-Potsdam cohort	188	S	11
	T2DM	Finnish	490	S	12
-174 G/C	T2DM	Native Americans and Spanish Caucasians	463 and 329	S	13
	T2DM	KORA Survey	704	S	14
	T2DM	21 studies	>20,000	S	15
-174G/C -597 A/G	T2DM	Boston	2691	NS	16
Genome wide association scan (18 SNPs)	T2DM	Canadian	6720	S with fasting	17

S, significant association; NS, non significant association

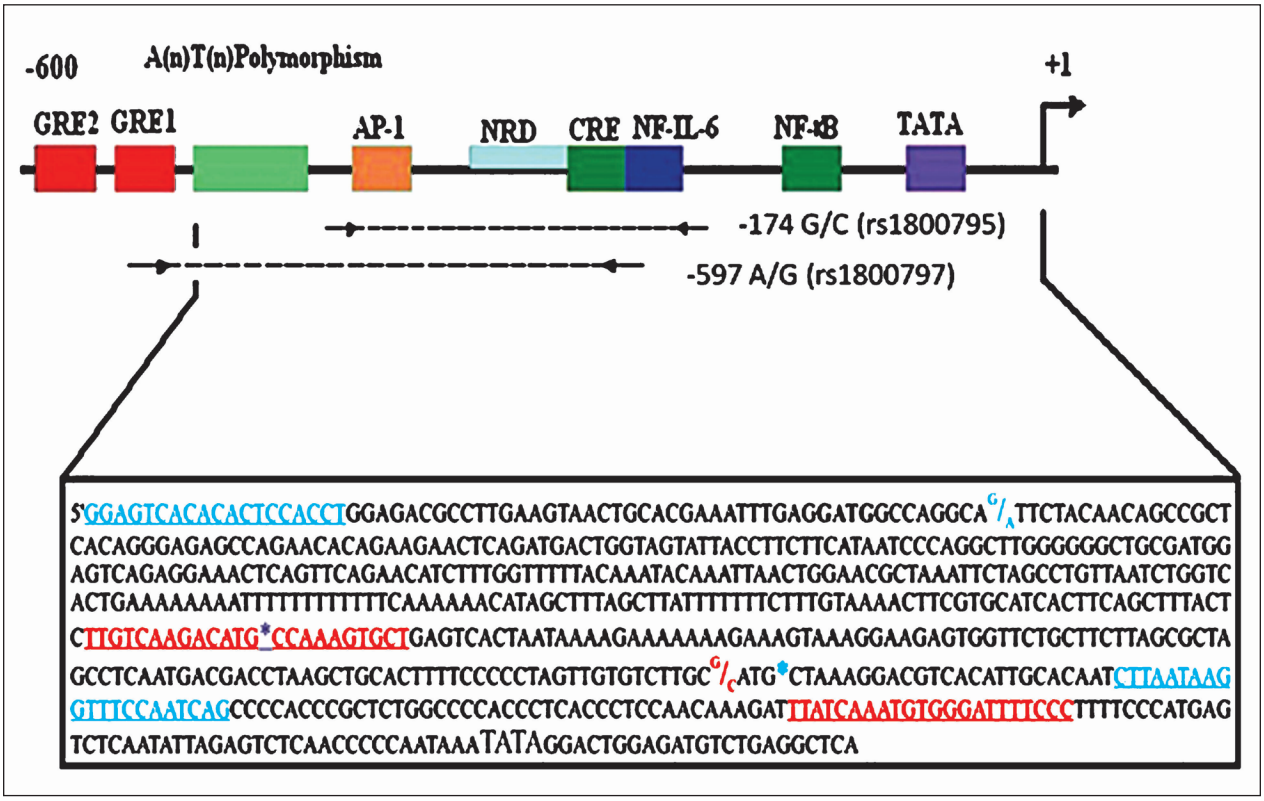

Fig. 1. Schematic representation of promoter region of *IL-6* gene from -600 to +1. Nucleotide sequence shown in text box, the 5' and 3' primers for -597A/G SNP are shown in blue while those for -174G/C are shown in red. GATG, FokI recognition site; *, Nla III restriction site; AP-1, activator protein-1; NRD, negative regulatory domain; CRE, cAM*P* responsive element; GRE, glucocorticoid responsive element; TATA, TATA box; NF-kB, nuclear factor - kB.

performed in 35 cycles (95°C for 30 sec; 61.5°C for 40 sec and 72°C for 50 sec) and -174 G/C (rs1800795) in 44 cycles (95°C for 30 sec; 57°C for 30 sec and 72°C for 60 sec) with an initial denaturation at 95°C for 5 min and a final extension at 72°C for 10 min. The PCR products were digested with respective restriction enzymes (FokI and NlaIII, Fermentas, USA) and electrophoresed on 12 per cent polyacrylamide gel. To ensure the quality of genotyping, random duplicates were performed in 20 per cent of the samples and 98 per cent concordance was found.

Statistical analysis: Allele frequencies and carriage rates were compared in controls and T2DM patients using 2x2 contingency table. Genotype frequencies were compared using 2x3 contingency table by Fisher's exact test. Carriage reates were calculated to know the inheritance frequency of individual alleles involved in disease manifestation. The Hardy-Weinberg equilibrium at individual locus was assessed by chi-square ($\chi 2$) statistics using SPSS (v15.0) Inc., USA. Student t-test and multivariate logistic regression analysis were performed for anthropometric and biochemical parameters of each genotype. Bonferroni correction was carried out for multiple testing camparisons. Haplotypes were analyzed by SHEsis software[21].

Results

The association between clinical/biochemical parameters of controls and T2DM patients showed a significant difference in FBS, PPBS, TC, TGL, HDL, LDL and VLDL (P<0.001) (Table II). The *IL-6* -597 A/G (rs1800797) and -174 G/C (rs1800795) polymorphisms were successfully genotyped in 213 T2DM cases and 145 healthy controls. Results of genotypic patterns by PCR-RFL*P* are shown in Fig. 2. The allele and genotype frequency distributions and carriage rates of both polymorphisms are shown in Table III. All frequencies were found to be in Hardy-Weinberg equilibrium (HWE).

In case of *IL-6* -597 A/G (rs1800797), no genotypic and allelic association was observed in the present study. Genotype GG of -597 A/G was rare in both controls and T2DM cases (2.86 and 4.70 %, respectively) while,

Table II. Clinical characteristics of controls and T2DM cases

Clinical characterstics	Controls (n=145)	Cases (n=213)
Age (yr)	47.82 ± 10.16	49.15 ± 10.36
Waist hip ratio (WHR)	0.94 ± 0.03	0.97 ± 0.66
Body mass index (BMI) kg/m^2	23.33 ± 1.85	24.05 ± 4.46
Fasting blood glucose (FBS) mg/dl	83.87 ± 7.20	173.81 ± 72.73***
Post-prandial blood glucose (PPBS) mg/dl	139.65 ± 10.16	272.75 ± 101.07***
Total cholesterol (TC) mg/dl	184.30 ± 28.15	222.03 ±41.51***
Triglycerides (TGL) mg/dl	126.01 ± 40.52	112.74 ±18.52***
High density lipoproteins (HDL) mg/dl	48.32 ± 11.45	45.28 ± 7.46**
Low density lipoproteins (LDL) mg/dl	64.33 ± 27.31	153.00 ± 49.05***
Very low density lipoproteins (VLDL) mg/dl	25.20 ± 8.10	22.59 ± 3.87***
Serum creatinine (SCRT) mg/dl	1.03 ± 0.13	1.05 ± 0.11

Values are mean ± SD
P^{**}<0.01 ***<0.001 compared to control

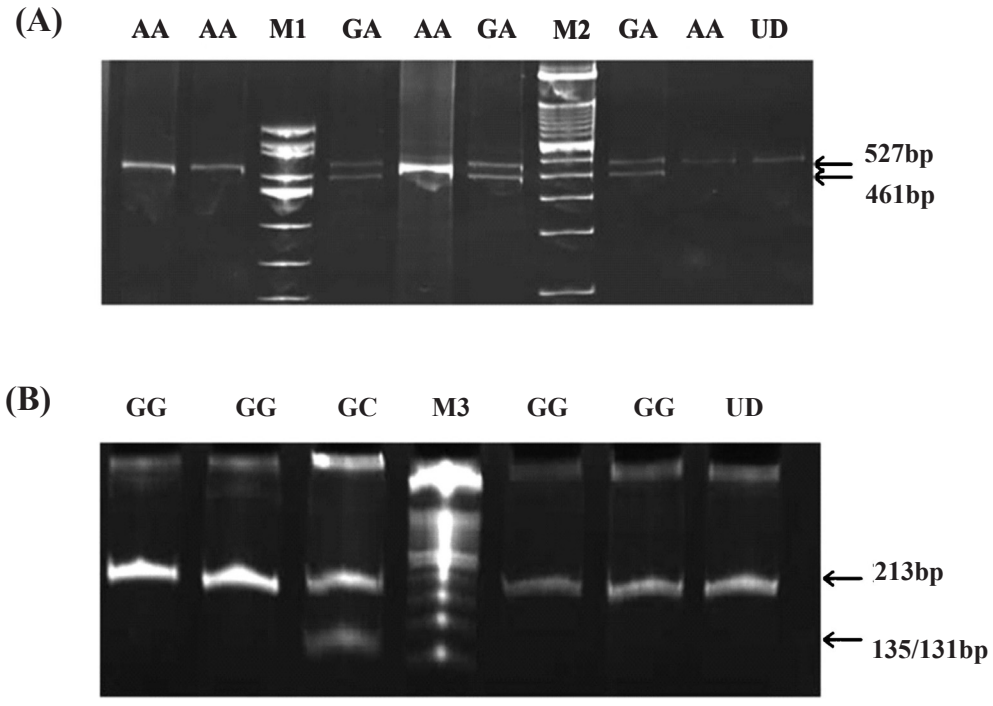

Fig. 2. Polyacrylamide gel (12%) photographs of PCR-RFLP products (amplicons digested with restriction enzymes) depicting the genotypes of IL-6 polymorphisms. **A**. PCR products with IL-6 -597 A/G SNP digested with FokI showing the genotypes AA (527 bp) and GA (527 and 461 bps). **B** PCR products with IL-6-174 G/C SNP digested with NlaIII showing the genotypes GG (213 bp) and GC (213 and 135/131 bps). M1, ΦX174/HinfI digest; M2, 100bp Ladder; M3, 50bp Ladder; UD, Undigested.

GA accounted for 20.00 and 16.43 per cent in controls and T2DM cases, respectively. Individuals with AA genotype were major in our population. No association was observed in carriage rates of 'A' and 'G' alleles in controls and T2DM cases (Table III).

IL-6 -174 G/C (rs1800795) polymorphism showed significant genotypic and allelic associations (P<0.001; 0.006) while, CC genotype was rare and GG was most prevalent in our population. GC genotype was present in 14.48 per cent controls and 21.60 per cent T2DM cases (Table III). The carriage rate of 'G' showed significant association (P<0.001; OR 0.127; CI 0.042-0.384) with T2DM when compared to controls but 'C' allele showed no association (Table III).

Analysis of genotypic associations with biochemical parameters using multivariate logistic regression showed that subjects with GG genotype had significant association with TC, TGL, LDL and VLDL (P=0.005, 0.021, <0.001 and 0.021 respectively). In case of 'GA' of -597 A/G polymorphism, significant association was observed with age, BMI, FBS, PPBS, TC, TGL, LDL and VLDL while AA showed significant association with all clinical parameters except age, BMI, WHR and SCRT (Table IV). None of the genotypes showed association with WHR and SCRT. GG genotype of *IL-6*-174 G/C polymorphism showed significant association with FBS, PPBS, TC, TGL, HDL, LDL and VLDL. While GC showed significant association with all biochemical parameters except total cholesterol and CC showed significant association with FBS, PPBS and LDL (Table IV). In haplotypic analysis, -597G* and -174C* allele combination showed significant association (P=0.010). The 'D' and r^2 values were calculated in haplotypic analysis and found to be 0.070 and 0.004, respectively. Another important observation was that the combination of -597G* and -174G* alleles also showed upto 1.5 times increased risk of developing T2DM (Table V).

Discussion

Earlier studies on the association of -597 A/G (rs1800797) and -174 G/C (rs1800795) SNPs with T2DM and insulin resistance (IR) have demonstrated

Table III. Distribution of genotype, allele frequencies and carriage rates of *IL-6* -597 A/G and -174 G/C polymorphisms among controls and T2DM cases

	IL-6-597 A/G			*IL-6*-174 G/C	
	Genotype frequencies				
	Controls Count (%) (n=140)	Cases Count (%) (n=213)		Controls Count (%) (n=145)	Cases Count (%) (n=213)
AA	108 (77.14)	168 (78.87)	GG	105 (72.41)	163 (76.52)
GA	28 (20.00)	35 (16.43)	GC	21 (14.48)	46 (21.60)
GG	4 (2.86)	10 (4.70)	CC	19 (13.11)	4 (1.88)
P value	0.508			<0.001	
Allele frequencies					
A	244 (87.14)	371 (87.09)	G	231 (79.65)	372 (87.32)
G	36 (12.86)	55 (8.21)	C	59 (20.35)	54 (12.68)
P value	0.983			<0.001	
Odd's ratio (95% CI)	1.005 (0.641-1.576)			0.568 (0.379-0.851)	
Carriage rates					
A (+)	136 (97.14)	203 (95.30)	G (+)	126 (86.89)	209 (98.12)
A (-)	4 (2.86)	10 (4.70)	G (-)	19 (13.11)	4 (1.88)
P value	0.392			<0.001	
Odd's ratio (95% CI)	1.674 (0.515-5.444)			0.127 (0.042-0.384)	
G (+)	32 (22.86)	45 (21.13)	C (+)	40 (27.59)	50 (23.48)
G (-)	108 (77.14)	168 (78.87)	C (-)	105 (72.41)	163 (76.52)
P value	0.700			0.379	
Odd's ratio (95% CI)	1.106 (0.662-1.849)			1.242 (0.766-2.012)	

Table IV. Association of anthropometric/biochemical parameters with *IL-6* -597 A/G and -174 G/C genotypes

Anthropometric/Biochemical data → SNPs ↓	Genotypes ↓		Age	BMI	WHR	FBS	PPBS	TC	TGL	HDL	LDL	VLDL	SCRT
-597 A/G	G/G	Controls (n=4)	53.250 ± 5.737	24.053 ± 1.294	0.955 ± 0.017	81.000 ± 5.292	141.500 ± 12.689	180.108 ± 37.451	120.110 ± 6.536	47.906 ± 8.992	51.380 ± 8.851	24.022 ± 1.307	1.095 ± 0.090
		Cases (n=10)	51.143 ± 12.941	21.614 ± 3.412	0.960 ± 0.046	158.629 ± 78.711	248.186 ± 98.688	242.400 ± 28.516	100.800 ± 13.702	44.080 ± 5.289	178.160 ± 24.818	20.160 ± 2.740	1.040 ± 0.117
	P value		0.768	0.210	0.843	0.086	0.065	0.005	0.021	0.334	<0.001	0.021	0.419
	G/A	Controls (n=28)	45.120 ± 9.960	22.670 ± 1.921	0.935 ± 0.042	92.160 ± 36.511	139.900 ± 10.672	191.610 ± 27.558	122.496 ± 27.629	47.591 ± 11.130	69.182 ± 28.175	24.499 ± 5.526	1.061 ± 0.206
		Cases (n=35)	50.412 ± 9.699	24.531 ± 3.898	0.942 ± 0.074	165.088 ± 69.527	264.839 ± 102.133	241.943 ± 18.752	104.743 ± 13.600	45.989 ± 4.719	175.234 ± 20.935	20.949 ± 2.720	1.026 ± 0.119
	P value		0.045	0.033	0.682	<0.001	<0.001	<0.001	0.002	0.449	<0.001	0.002	0.423
	A/A	Controls (n=108)	48.156 ± 10.283	23.480 ± 1.866	0.947 ± 0.032	84.646 ± 19.680	139.519 ± 9.757	182.579 ± 28.438	127.952 ± 44.796	48.551 ± 11.894	62.801 ± 27.766	25.590 ± 8.959	1.018 ± 0.112
		Cases (n=168)	49.042 ± 10.358	24.255 ± 4.531	0.985 ± 0.757	172.652 ± 73.947	266.311 ± 101.040	229.051 ± 30.772	114.568 ± 13.944	42.932 ± 3.656	163.506 ± 29.542	23.014 ± 3.177	1.043 ± 0.085
	P value		0.504	0.114	0.616	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	0.001	0.068
-174 G/C	G/G	Controls (n=105)	47.315 ± 10.533	23.274 ± 1.975	0.942 ± 0.036	84.935 ± 23.054	139.787 ± 9.801	184.344 ± 28.566	128.517 ± 45.104	47.800 ± 11.157	62.809 ± 26.722	25.704 ± 9.021	1.023 ± 0.140
		Cases (n=163)	49.786 ± 10.486	24.008 ± 4.509	0.935 ± 0.070	169.473 ± 72.017	268.001 ± 100.349	240.994 ± 24.973	112.088 ± 15.031	43.996 ± 3.968	175.138 ± 24.034	22.498 ± 3.212	1.039 ± 0.095
	P value		0.074	0.143	0.347	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.277
	G/C	Controls (n=21)	46.450 ± 11.185	23.115 ± 1.776	0.948 ± 0.033	93.100 ± 32.211	139.000 ± 11.361	183.039 ± 36.093	125.317 ± 34.195	54.468 ± 14.471	74.808 ± 33.557	25.063 ± 6.839	1.088 ± 0.106
		Cases (n=46)	47.667 ± 9.742	24.884 ± 4.133	1.116 ± 1.389	173.434 ± 75.161	250.137 ± 91.393	195.111 ± 12.137	112.056 ± 12.361	41.773 ± 4.238	130.550 ± 12.126	22.267 ± 2.627	1.088 ± 0.104
	P value		0.659	0.072	0.591	<0.001	<0.001	0.071	0.040	<0.001	<0.001	0.033	1.000
	C/C	Controls (n=19)	51.944 ± 5.341	23.899 ± 1.106	0.946 ± 0.024	83.444 ± 8.024	139.647 ± 11.186	185.493 ± 13.626	113.996 ± 8.496	44.144 ± 5.449	60.448 ± 20.562	22.798 ± 1.699	0.994 ± 0.119
		Cases (n=4)	50.250 ± 9.743	23.490 ± 2.589	0.900 ± 0.141	182.500 ± 103.648	325.000 ± 209.616	200.000 ± 17.282	116.000 ± 15.319	40.700 ± 2.600	134.100 ± 14.297	25.200 ± 6.835	0.938 ± 0.052
	P value		0.627	0.630	0.147	<0.001	0.001	0.080	0.716	0.238	<0.001	0.173	0.370

Age (yr), WHR; waist hip ratio, BMI, body mass index (kg/m^2); FBS, fasting blood glucose (mg/dl); PPBS, post-prandial blood glucose (mg/dl); TC, total cholesterol (mg/dl); TGL, triglycerides (mg/dl); HDL, high density lipoproteins (mg/dl); LDL, low density lipoproteins (mg/dl); VLDL, very low density lipoproteins (mg/dl); SCRT, serum creatinine (mg/dl)

Table V. Distribution of haplotypes of *IL-6* genotypes in controls and T2DM cases

rs1800797/ rs1800795	T2DM (freq)	Control (freq)	χ^2	Pearson's *P*	Odds ratio (95%CI)
AC*	47.86(0.112)	45.00(0.161)	3.462	0.062	0.661 (0.426~1.024)
GC*	6.14(0.014)	13.00(0.046)	6.561	0.010	0.300 (0.114~0.794)
AG*	323.14(0.759)	199.00(0.711)	2.008	0.156	1.279 (0.910~1.798)
GG*	48.86(0.115)	23.00(0.082)	1.956	0.161	1.447 (0.860~2.435)

*Allele combination of *IL-6*-597 A/G (rs1800797) and -174 G/C (rs1800795) gene polymorphisms. Figures in parantheses indicate the frequency of respective genotype in each group *i.e.* T2DM and controls.

varied results among different populations. Analyses of small cohorts of native Americans and Spanish Caucasians showed the 'G' allele of -174 G/C SNP to be associated with higher risk of T2DM[22], but this SNP was not linked with diabetes in the Finnish Diabetes Prevention Study (DPS)[23]. In another study, non diabetic subjects showed an association of *IL-6*-174 C/C genotype with higher insulin sensitivity[14,24]. However, neither this nor -597 A/G polymorphism was investigated in detail with respect to diabetes status, parameters of metabolic syndrome and subclinical systemic inflammation in comprehensive, population-based studies.

A Korean population was screened for *IL-6* gene promoter polymorphisms *viz.* -174G/C, -572C/G, -597G/A and -1363G/T and correlated with serum concentrations of IL-6 and high-sensitivity C-reactive protein (hs-CRP)[25]. The study suggested that the *IL-6* -572G/G genotype was associated with higher serum IL-6 and hs-CRP concentrations and increased risk for T2DM. A significant interaction has been identified between body mass index (BMI) and rs1800795 polymorphism of the *IL-6* gene that influences both insulin resistance and onset of T2DM. The obese individuals homozygous for the 'C' allele demonstrated the highest level of IR and greatest risk of T2DM[3]. Moreover, FBS and PPBS showed significant association with all genotypes except GG of -597 A/G (rs1800797). However, genotypes of -597 A/G (rs1800797) showed significant associations with TC and only GG genotype of -174 G/C (rs1800795) showed significant association with TC. In case of HDL only AA of -597 A/G (rs1800797) and GG/GC showed significant association. GA genotype of -597 A/G (rs1800797) showed significant associations with age and BMI and no other genotypes of -174 G/C (rs1800795) and -597 A/G (rs1800797) showed any significant association with age & BMI. The similar negative association of WHR and SCRT was observed with genotypes of -597 A/G (rs1800797) and -174 G/C (rs1800795). The prominent finding in respect to biochemical parameters was that LDL showed significant association with all genotypes of both -597 A/G (rs1800797) and -174 G/C (rs1800795) gene polymorphisms while TGL and VLDL showed association with all genotypes except CC genotype of -174 G/C (rs1800795).

Genotyping in the present study showed that unlike -597 A/G, the -174 G/C SNP was significantly associated with T2DM in north Indian population. Our -174 G/C (rs1800795) results were in support of the metagenomic study in an Indian population[26] which showed the prevalence of GG genotype. 'C' allele and CC genotype at -174 G/C SNP were found to be protective for diabetes as frequency of this genotype was more in control. Haplotype analysis showed that in combination, the -174C* with -597G* allele increased the risk and susceptibility of developing T2DM in the north Indian population. The results showed that the allele 'G' was involved in disease prevalence and manifestation. No significant association of genotypes, allele and carriage rates was observed in -597 A/G (rs1800797) polymorphism. However, in case of -174 G/C (rs1800795) genotypic and allelic frequencies showed significant association. The carriage rate of 'A' allele of -174 G/C (rs1800795) also showed significant association. However, it is important that the study groups are matched with respect to age, degree of obesity, glucose tolerance and sex distribution[5,27].

The power for analyzing binary traits of *IL-6* -597 A/G (rs1800797) polymorphism associated with T2DM in this study was the major limitation of our study. *IL-6* -174 G/C (rs1800795) polymorphism has been studied in T2DM and other diseases as well as with

pre and post disease complications in almost all ethnic groups and has shown controversial results. Moreover, till now only a few studies on -597 A/G (rs1800797) gene polymorphism have been reported and showed negative correlation with T2DM[28].

Our data suggest that *IL-6* gene polymorphisms play a prominent role in determining susceptibility to T2DM. The present study provides a lead to the contribution of cytokine gene heterogeneity to the susceptibility and development of T2DM but it is essential to find out the degree of association in an individual population.

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