

RESEARCH ARTICLE

Association of *IL-10* gene (–1082A>G, –819C>T and –592C>A) polymorphism and its serum level with metabolic syndrome of north Indian subjects

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Abstract

Metabolic syndrome (MetS) is an inflammatory disorder, in which various cytokines play important role in tilting balance towards disease state. Interleukin-10 (*IL-10*) is an important antiinflammatory cytokine, but its genetic polymorphisms and serum levels in Indian MetS subjects are unknown. Three *IL-10* gene polymorphisms (–1082A>G (rs1800896), –819C>T (rs1800872) and –592C>A (rs1800871)) were genotyped with PCR-RFLP in MetS subjects ($n = 384$) and age/sex matched control subjects ($n = 386$). Serum *IL-10* was measured using enzyme-linked immunosorbent assay. Serum *IL-10* level was significantly low in MetS subject and significantly correlated with clinicobiochemical parameters of MetS. Of three investigated promoter polymorphisms, *IL-10* –819C>T and –592C>A were significantly associated with risk of MetS. The mutant alleles –819T and –592A of *IL-10* gene polymorphism were significantly higher in MetS subjects compared to controls. Of the four different haplotypes obtained, common ACC haplotype and rare GTA haplotype of *IL-10* polymorphisms were associated with MetS. The mean of fasting insulin and HOMA-IR were significantly different between the genotypes of both –819C>T and –592C>A polymorphisms of *IL-10* in MetS subjects. These results suggested that polymorphisms in *IL-10* gene (–819C>T and –592C>A), haplotypes (ACC and GTA) and serum level are significantly associated with risk of MetS. *IL-10* –819C>T and –592C>A polymorphic variants are also significantly associated with insulin level and homeostasis model assessment-insulin resistance in north Indian MetS subjects.

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Introduction

Metabolic syndrome (MetS) is estimated to affect about one third of urban population in India (Vikram *et al.* 2006). Further, several studies point to a rising prevalence of MetS in urban Asian Indian adults (41.1–49.2%) (Ramachandran *et al.* 2003; Wasir *et al.* 2008). MetS is described as clusters of cardiovascular risk factors including abdominal obesity, insulin resistance, hypertension, hyperglycaemia, increased triglycerides and decreased high-density lipoprotein cholesterol (Grundey 1999). Immune system response and (HDL-C) metabolic regulation are physiologically integrated and

the proper function of each is dependent as well as maintains the central homeostatic mechanism. Its deregulation leads to the cluster of chronic metabolic disorders (Hotamisligil 2006). Abdominal obesity is considered as a marker of dysfunctional adipose tissue and the most prevalent manifestation of MetS (Després and Lemieux 2006). Chronic inflammation is invariably a common feature of the MetS, where inflammatory signals originate from within visceral adipose tissue in addition to the peripheral system (Wisse 2004). Adipocytes and macrophages within the adipose tissue secrete numerous cytokines and other factors that are suggested to contribute to the characteristic pathophysiological changes which are seen in the MetS. However, several aspects of the role of inflammation in MetS

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are still unknown or controversial, which may have great implications in estimating risk and devising new therapy for MetS.

Interleukin-10 (*IL-10*) is a Th2 cytokine that plays a major antiinflammatory role in the regulation of the immune system. *IL-10* potently inhibits expression and/or production of proinflammatory cytokines. Type 2 diabetes is considered as an inflammatory disorder (Donath and Shoelson 2011) and has been suggested that Th2 induced component of anti- β cell immunity is mediated principally by *IL-10* (Lee et al. 1994, 1996; Pakala et al. 1997). Previous study has already reported direct association of low *IL-10* production capacity with the MetS and type 2 diabetes, and it is thought to be due to failure of *IL-10* to oppose the proinflammatory environment (van Exel et al. 2002a). It is well known that obesity in MetS is characterized by a state of mild inflammation associated with the expression and release of proinflammatory adipokines (Trayhurn 2005).

IL-10 encoding gene consists of five exons, mapped on chromosome 1q31-32 (Eskdale et al. 1997). Several polymorphic sites within the promoter region have been described, including two microsatellite and three biallelic polymorphisms at positions -1082, -819 and -592 from the transcription start site (Turner et al. 1997; Eskdale et al. 1998). *IL-10* gene polymorphisms (-1082A>G, -819C>T and -592C>A) have been found to be associated with several pathological conditions, such as diabetes mellitus (Mohebbatikaljahi et al. 2009; Saxena et al. 2012), diabetic nephropathy (Kung et al. 2010; Erdogan et al. 2012), acute coronary syndrome (Babu et al. 2012), obesity and insulin resistance (Scarpelli et al. 2006) in different populations. Serum *IL-10* level was reported to be altered in several inflammatory diseases such as myocardial ischemia / reperfusion injury (Shibata et al. 1997), inflammatory bowel disease (Autschbach et al. 1998), atherosclerosis (Pinderski Oslund et al. 1999), type 2 diabetes (van Exel et al. 2002b; Straczowski et al. 2005) as well as obesity (Manigrasso et al. 2005; Calcaterra et al. 2009; Chou et al. 2013). *IL-10* polymorphisms can directly influence serum *IL-10* level (Chen et al. 2012).

Studies on association of *IL-10* gene polymorphisms (Lu et al. 2011) and its protein level (Esposito et al. 2003) with MetS are limited. The reported studies were conducted with small sample sizes. Interestingly, there is no report of *IL-10* gene polymorphisms in MetS of Indian population. The relationship between the polymorphisms in *IL-10* gene and their plasma levels are clinically important due to their pivotal role in stimulation or regulation of inflammatory and immune responses (Howard and O'Garra 1992). Critical role of *IL-10* in pathophysiology of a number of specific diseases including MetS are recently emerging. Therefore, we undertook the present study with an objective to investigate the nature of association of *IL-10* gene polymorphisms and its serum level with the risk of MetS as well as their relationship with clinicobiochemical risk factors in a large sample of north Indian subjects.

Material and methods

Ethical statements

This is a hospital-based case-control study and conducted with prior ethical approval from the Institution, the Department of Physiology, King George's Medical University, Lucknow, with the collaboration CSIR-Central Drug Research Institute, Lucknow. All subjects were recruited after obtaining written informed consent from the outdoor patient, Department of Cardiology, King George's Medical University, Lucknow.

Study subjects

MetS subjects ($n = 384$) aged 18–60 years were recruited on the basis of inclusion and exclusion criteria. The modified criteria of National Cholesterol Education Programme, Adult Treatment Panel III (NCEP, ATP III) were used for inclusion of MetS; waist circumference, males >90 cm, females >80 cm, fasting plasma glucose >100 mg/dL, triglycerides (TG) >150 mg/dL, blood pressure >130/85 mmHg and HDL-C; males <40 mg/dL, and females <50 mg/dL. As per the recommendation (NCEP, ATP III), subjects matching any three of the risk factors were included as MetS (Grundy et al. 2005) and subjects not matching minimum criteria were excluded from the study. Age/sex matched controls ($n = 386$) were recruited from those coming for minor complaints in out patient departments, King George's Medical University, Lucknow. Subjects with conditions, which may affect metabolic parameters, such as polycystic ovarian syndrome, thyroid dysfunctions, rheumatoid arthritis, inflammatory bowel disease, pregnancy, infection, coronary artery disease and cancer were excluded from this study. Total 5 mL fasting venous blood sample (>8 h) was collected from each subject and of which 1 mL blood was collected in fluoride vial, 2 mL in EDTA vial and remaining 2 mL in plain vial. Serum and plasma were separated immediately, aliquot prepared and stored at -80°C until further analysis.

Clinical data including history, diagnosis and anthropometrical data were collected in a predesigned study proforma. Body weight of subjects were measured with the help of digital weighing machine. Height was measured using stadiometer with the help of a fixed scale. Body mass index was calculated by the formula; weight (kg) / height (m^2). Waist circumference (WC) was measured mid-way between iliac crest and lowermost margin of the ribs, in quiet breathing. Hip circumference (HC) was measured at the maximum protruding part of buttocks at the level of the greater trochanter, while keeping the feet together with the subjects wearing minimal clothing. Waist hip ratio was calculated with the help of the formula WC (cm) / HC (cm). Blood Pressure was measured by a manual mercury sphygmomanometer (Diamond) at the time of subject recruitment, as previously described (Madeshiya et al. 2015). For all the biochemical

estimations, subjects were asked in advance for an overnight fasting of at least 8 h.

Plasma glucose and insulin estimation

Fasting plasma glucose was estimated by using Merckotest glucose kit based on the glucose oxidase–peroxidase method (Merk Specialities, Mumbai, India) (Varely *et al.* 1980). Plasma insulin was estimated by using a radioimmunoassay kit (Immunotech, Prague, Czech Republic) with the help of a gamma counter. Insulin resistance (IR) was quantified by using homeostasis model assessment (HOMA). It was calculated using the formula (HOMA-IR = fasting insulin (μ U/mL) \times fasting plasma glucose (mmol/L)/22.5). HOMA-IR index is most widely used in Asian population for quantifying insulin resistance and β -cell function (Matthews *et al.* 1985).

Blood lipid profiling

Lipid profiling (total cholesterol, TC; triglycerides, TG; HDL-C) was carried out using commercially available kits (Merck Specialities) with the help of a semiautomated analyzer (Microlab 300, Merck) on the same day of sample collection. Low-density lipoprotein (LDL-C) and very low-density lipoprotein (VLDL-C) were calculated by using the Friedewald formula of ‘LDL-C=TC–(HDL-C + VLDL-C)’ and ‘VLDL-C= TG/5’ (Friedewald *et al.* 1972).

Serum IL-10 level estimation

Circulating serum IL-10 level was estimated by using enzyme-linked immune sorbent assay (ELISA) kit (R&D Systems) with the help of ELISA reader (Biorad, Gurgaon, India) as per the manufacturer’s instructions.

Genotyping of –1082A>G, –819C>T and –592C>A polymorphisms of IL-10 gene

Genomic DNA was prepared from whole peripheral blood collected in EDTA vial using Quick-gDNA™ Blood MiniPrep kits (Zymo Research Corp, Irvine, USA) according to the manufacturer’s instructions. For the analysis of IL-10 gene polymorphisms (–1082A>G, –819 C>T and –592C>A), 100 η g DNA was amplified using a thermal cycler (Eppendorf thermal cycler) and 2 \times Dream Taq Green PCR Master Mix (Thermo Scientific). The specific primers, restriction endonuclease enzyme (RE), cycling conditions used in the polymerase chain reactions (PCR) and restriction fragment length polymorphism (RFLP) are listed in table 1. The digested PCR products of every single-nucleotide polymorphism (SNP) were analysed by gel electrophoresis using 4% agarose gel (figure 1). For quality control assessment, one sample with known genotype and a reagent blank were included after every 20 samples in the PCR. About 20% samples were randomly regentyped by other personnel of laboratory for validation of genotyping accuracy and no discrepancy was found.

Table 1. List of primers with thermocycling conditions, restriction endonuclease enzyme and product sizes for genotyping of polymorphisms (Seo *et al.* 2005).

SNP	Primer	Cycling condition	PCR product size (bp)	RE with incubation temperature and time	RE digested product size
–1082A>G (rs1800896)	F5'-CTCGCTGCAAC CCA AC TGGC-3' R5'-TCTTACCTAT CCC TACTTCC-3'	Initial denaturation 94°C for 7 min, denaturation 94°C for 45 s, annealing 55°C for 1 min, elongation 72°C for 1 min, final extension 72°C for 10 min, 35 cycles	136	<i>MnII</i> Incubation at 37°C for 2 h	<i>Aa</i> : 139 bp <i>GG</i> : 106 bp and 33 bp <i>AG</i> : 139 bp, 106 bp and 33 bp
–819C>T (rs1800872)	F5'-TCATTCTATGT GTG GAGATGG-3' R5'-TGGGGGAAG T GGG TAAGAGT-3'	Initial denaturation 94°C for 5 min, denaturation 94°C for 30 s, annealing 62°C for 45 s, elongation 72°C for 45 s, final extension 72°C for 7 min 35, cycles	209	<i>MaeIII</i> Incubation at 55°C for 2 h	<i>CC</i> : 209 bp <i>TT</i> : 152 bp and 84 bp <i>TC</i> : 209 bp, 152 bp and 84 bp
–592C>A (rs1800871)	F5'-CCTAGGTGAC AGTG AC GTGG-3' R5'-GGTGAGCACT ACC TGACTAGC-3'	Initial denaturation 94°C for 5 min, denaturation 94°C for 30 s, annealing 65°C for 45 s, elongation 72°C for 45 s, final extension 72°C for 7 min, 35 cycles	412	<i>RsaI</i> Incubation at 37°C for 2 h	<i>CC</i> : 412 bp <i>AA</i> : 236 bp and 176 bp <i>CA</i> : 412 bp, 236 bp and 176 bp

RE, restriction endonuclease.

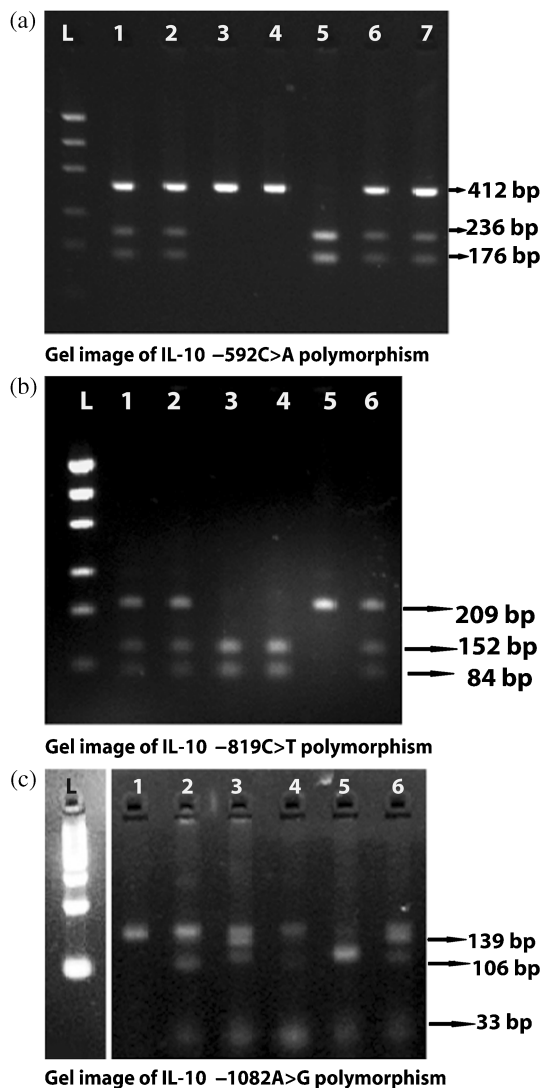


Figure 1. Agarose gel image (Biorad Gel Documentation System); (a) restriction fragment length polymorphism analysis of *IL-10* (-592C>A) gene: L, 100 bp DNA molecular weight marker (Thermo Scientific); lanes 1, 2, 6 and 7, CA heterozygous mutant; lanes 3 and 4, CC homozygous wild type; lane 5, AA homozygous mutant. (b) Restriction fragment length polymorphism analysis of *IL-10* (-819C>T) gene: L, 100 bp DNA molecular weight marker (Thermo Scientific); lanes 1, 2 and 6 TC heterozygous mutant; lanes 3 and 4, TT homozygous mutant; lane 5, CC homozygous wild type. (c) Restriction fragment length polymorphism analysis of *IL-10* (-1082A>G) gene: L, 100 bp DNA molecular weight marker (Thermo Scientific); lane 1, AA homozygous wild type; lanes 2, 3, 4 and 6, AG heterozygous mutant; lane 5, GG homozygous mutant.

Statistical analysis

The results are presented in mean \pm SD and percentages. Sample size and statistical power calculation prior to the study were estimated by using n-Masters 1.0 sample size calculation software developed by CMC, Vellore, India (Jeyaseelan and Rao 1989). The study sample size yielded statistical power of 80%. The gender difference,

Hardy–Weinberg equilibrium testing, genotypes frequency and allele frequency association between the groups were compared by χ^2 test. The unpaired *t*-test was used to compare the continuous parameters between case and control. The one way analysis of variance (ANOVA) was used to compare the continuous parameters among the different genotypes in cases and controls followed by Tukey's multiple comparison test. The Pearson correlation coefficient was calculated between *IL-10* levels and study parameters. All statistical tests were two-tailed and *P* value < 0.05 was considered as significant. All statistical analyses were carried out by using SPSS software (ver. 16.0, SPSS, Chicago). Haplotype analysis was carried out using Haploview software, Broad Institute, USA (Barrett et al. 2005).

Results

The clinicobiochemical characteristics of MetS subjects (*n* = 384) and healthy controls (*n* = 386) are shown in table 2. As the study was designed to match the age and gender between cases and controls, no statistically significant difference was observed for these parameters (*P* > 0.005). The mean body mass index was significantly (*P* = 0.0001) higher among the cases (28.19 ± 4.53) compared with healthy controls (23.19 ± 4.20). Similarly, waist circumference, hip circumference and waist hip ratio were also significantly (*P* = 0.0001) higher among the cases compared with the control subjects. Statistically significant increase in the systolic and diastolic blood pressures was also observed among the MetS subjects as compared to controls (*P* = 0.0001). Fasting plasma glucose was also significantly higher (*P* = 0.0001) among the cases (122.07 ± 33.78) than the controls (91.99 ± 16.65). All the estimated lipid levels, except HDL-C were higher among the cases compared with the controls (table 2). The fasting insulin level was almost two times higher in MetS subjects than the controls (10.40 ± 7.00 versus 5.77 ± 3.54 ; *P* = 0.0001) and hence, the HOMA-IR was significantly higher (*P* = 0.0001) in MetS subjects. Thus, excepted age, gender and height, all other parameters were significantly altered in MetS subjects.

Serum *IL-10* level was significantly lower (*P* = 0.0001) in the cases compared with controls (table 2). Upon analysis for correlation of *IL-10* serum level with clinicobiochemical characteristics of cases and controls, a significant negative correlation of *IL-10* was found in both systolic and diastolic blood pressures; triglycerides; LDL-C; VLDL-C; fasting insulin; HOMA-IR in both cases and controls (table 3). However, total cholesterol was negatively correlated with *IL-10* only among cases but not in controls. HDL-C was found to be positively correlated with *IL-10* in both cases and controls. None of the anthropological parameters were found to be significantly correlated with serum *IL-10* level. Strangely, a marginally significant positive correlation of height with *IL-10* level was observed in only MetS case group.

Table 2. Anthropometrical and biochemical characteristics of cases and controls.

Parameter	Control (<i>n</i> = 386) (mean ± SD)	Case (<i>n</i> = 384) (mean ± SD)
Age (yr)	40.45 ± 10.29	40.25 ± 9.61
Male gender, no. (%)	241 (62.4%)	256 (66.7%)
Weight (kg)	59.43 ± 11.46	72.55 ± 13.42***
Height (cm)	160.10 ± 8.89	160.34 ± 9.23
Body mass index (kg/m ²)	23.19 ± 4.20	28.19 ± 4.53***
Waist circumference (cm)	83.24 ± 10.59	97.45 ± 11.47***
Hip circumference (cm)	91.98 ± 8.63	101.09 ± 10.49***
Waist hip ratio	0.90 ± 0.07	0.97 ± 0.08***
Systolic blood pressure (mm/Hg)	120.98 ± 10.04	134.87 ± 15.23***
Diastolic blood pressure (mm/Hg)	81.34 ± 7.07	90.02 ± 9.09***
Fasting plasma glucose (mg/dL)	91.99 ± 16.65	122.07 ± 33.78***
Total cholesterol (mg/dL)	162.21 ± 43.12	185.03 ± 51.04***
Triglycerides (mg/dL)	113.72 ± 48.52	182.18 ± 69.98***
HDL-C (mg/dL)	44.12 ± 12.08	33.13 ± 9.51***
LDL-C (mg/dL)	96.49 ± 43.85	115.46 ± 49.60***
VLDL-C (mg/dL)	22.74 ± 9.70	36.44 ± 14.00***
Fasting insulin (U/mL)	5.77 ± 3.54	10.40 ± 7.00***
HOMA-IR	1.35 ± 1.15	3.22 ± 2.84***
IL-10 (pg/mL)	3.86 ± 2.64	2.66 ± 1.97***

Level of significance indicated as ****P* < 0.0001.

Table 3. Correlation of IL-10 serum level with anthropometrical and biochemical characteristics of cases and controls.

Parameter	Control (<i>n</i> = 386)		Case (<i>n</i> = 384)	
	<i>r</i> value	<i>P</i> value	<i>r</i> value	<i>P</i> value
Weight (kg)	0.031	0.545	0.032	0.528
Height (cm)	0.022	0.668	0.103	0.045*
Body mass index (kg/m ²)	0.021	0.680	−0.036	0.485
Waist circumference (cm)	−0.045	0.374	−0.058	0.256
Hip circumference (cm)	−0.040	0.431	−0.031	0.548
Waist hip ratio	−0.035	0.495	−0.052	0.306
Systolic blood pressure (mm/Hg)	−0.247	0.0001***	−0.213	0.0001***
Diastolic blood pressure (mm/Hg)	−0.275	0.0001***	−0.173	0.001**
Fasting plasma glucose (mg/dL)	−0.160	0.002**	−0.199	0.0001***
Total cholesterol (mg/dL)	−0.074	0.147	−0.125	0.014*
Triglycerides (mg/dL)	−0.172	0.001**	−0.120	0.019*
HDL-C (mg/dL)	0.323	0.0001***	0.172	0.001**
LDL-C (mg/dL)	−0.127	0.012*	−0.128	0.012*
VLDL-C (mg/dL)	−0.172	0.001*	−0.120	0.019*
Fasting insulin (U/mL)	−0.240	0.0001***	−0.192	0.0001***
HOMA-IR	−0.195	0.0001***	−0.208	0.0001***

Level of significance indicated as **P* < 0.05, ***P* < 0.001, ****P* < 0.0001.

The genotype frequencies of *IL-10* gene polymorphisms in control subjects were consistent with Hardy–Weinberg equilibrium (*P* = 0.57 for −1082A>G and *P* = 0.38 for −819C>T and −592C>A). The distribution of alleles and genotypes of the three *IL-10* gene polymorphisms in cases and controls are enumerated in table 4. The frequency of *TT* genotype of *IL-10* −819C>T gene polymorphism (codominant model) was significantly higher among the cases (17.2%) than controls (12.7%) compared with *CC* wild genotype of *IL-10* −819C>T gene polymorphism. In dominant

model (*CC* versus *TC+TT*), the frequency of *TC+TT* genotype was also higher among the cases than controls, compared with *CC* wild genotype of *IL-10* −819C>T gene polymorphism. In the recessive model (*TT* versus *CC+TC*) analysis, there was no significant difference in genotype frequency of *IL-10* −819C>T gene polymorphism. The frequency of mutant allele −819T was significantly higher and wild allele −819C was significantly lower among cases compared with controls. The polymorphism at −819C>T and −592C>A variants of *IL-10* gene were found to be in perfect linkage

Table 4. Distribution of alleles and genotypes of *IL-10* gene polymorphism in cases and controls.

SNP	Genotype	Control (n = 386)	Case (n = 384)	OR (95% CI)	P value	Allele	Control (2n = 772)	Case (2n = 768)	OR (95% CI)	P value
IL-10 -1082A>G	Codominant model (AA vs GG and AG)									
	AA ^a	229 (55.7%)	214 (55.7%)	1.00		A ^c	592	563	1.20	0.14
	GG ^b	23 (9.1%)	35 (9.1%)	1.62 (0.93–2.84)	0.11		(76.68%)	(73.30%)	(0.95–1.51)	
	AG	134 (35.2%)	135 (35.2%)	1.07 (0.79–1.46)	0.68					
	Dominant model (AA vs AG + GG)					G	180	205		
	AG + GG	157 (40.7%)	170 (44.3%)	1.16 (0.87–1.54)	0.34		(23.32%)	(26.70%)		
IL-10 -819C>T	Codominant model (CC vs TT and TC)									
	CC ^a	171 (44.3%)	142 (37.0%)	1.00		C ^c	508	460	1.29	0.02*
	TT ^b	49 (12.7%)	66 (17.2%)	1.62 (1.05–2.49)	0.03*		(65.8%)	(59.9%)	(1.05–1.59)	
	TC	166 (43.0%)	176 (45.8%)	1.27 (0.93–1.73)	0.13					
	Dominant model (CC vs TC + TT)					T	264	308		
	TC + TT	215 (55.7%)	242 (63.0%)	1.35 (1.01–1.80)	0.04*		(34.2%)	(40.1%)		
IL-10 -592C>A	Codominant model (CC vs AA and CA)									
	CC ^a	171 (44.3%)	142 (37.0%)	1.00		C ^c	508	460	1.29	0.02*
	AA ^b	49 (12.7%)	66 (17.2%)	1.62 (1.05–2.49)	0.03*		(65.8%)	(59.9%)	(1.05–1.59)	
	CA	166 (43.0%)	176 (45.8%)	1.27 (0.93–1.73)	0.13					
	Dominant model (CC vs CA + AA)					A	264	308		
	CA + AA	215 (55.7%)	242 (63.0%)	1.35 (1.01–1.80)	0.04*		(34.2%)	(40.1%)		

^aConsidered as reference wild genotype for comparison with codominant model and dominant model; ^bconsidered as reference mutant genotype for comparison with recessive model; ^cconsidered as reference wild allele for comparison; level of significance indicated as * $P < 0.05$.

disequilibrium (LD), i.e. the individual with A allele at –592 bp also had T allele at –819 bp, whereas those with C allele at –592 bp also had C allele at –819 bp. Because of this LD, the similar observation was found for –592C>A variants of *IL-10* gene (table 4). There was no significant difference of IL-10 –1082A>G alleles and genotypes frequency between the cases and the controls in codominant, dominant as well as recessive models.

In our study population, we observed the presence of only four haplotypes (*ACC*, *ATA*, *CCG* and *GTA*) of the eight possible haplotypes. We calculated the frequency of these four haplotypes in study and control groups. We have found that the frequency of *ACC* haplotype was significantly lower ($P=0.01$) and *GTA* haplotype was significantly higher ($P=0.04$) among the cases in comparison with controls. On the other hand, the frequencies of *ATA* and *CCG* haplotypes were not significantly ($P=0.18$ and 0.78) different among the cases and controls (table 5). The highest LD value ($r^2 = 1.0$; $D' = 1.0$) corresponds to SNP's *IL-10* –592C>A–*IL-10* –819C>T. The lowest LD values were found between the SNP's *IL-10* –819C>T–*IL-10* 1082A>G ($r^2=0.002$; $D' = 0.09$) and between *IL-10* –592C>A–*IL-10* 1082A>G ($r^2 = 0.002$; $D' = 0.09$).

We carried out a sub-group analysis based on the *IL-10* genotypes of all the three polymorphisms for all the clinicobiochemical parameters and serum *IL-10* level in the

Table 5. Haplotype frequencies of *IL-10* gene polymorphism among cases and controls.

Haplotype	Control (n = 386)	Case (n = 384)	χ^2 value	P value
<i>ACC</i>	0.495	0.431	6.386	0.01*
<i>ATA</i>	0.272	0.302	1.744	0.18
<i>GCC</i>	0.163	0.168	0.074	0.78
<i>GTA</i>	0.070	0.099	4.088	0.04*

Level of significance indicated as * $P < 0.05$.

MetS subjects and controls (tables 6 and 7). The mean height of MetS subject was associated with genotypes of only –1082A>G polymorphism, while fasting insulin and HOMA-IR were associated with both –819C>T and –592C>A polymorphism of *IL-10*. However, there was no significant difference in *IL-10* serum level between the genotypes of *IL-10* gene polymorphism. The post-hoc comparison test showed the highest significant mean±SD value for fasting insulin and HOMA-IR in MetS subjects carrying homozygous mutant genotype *TT* of –819C>T and *AA* of –592C>A *IL-10* gene polymorphism in comparison with wild type and heterozygous mutant genotype of both the SNPs. In the control group, none of the genotypes of all the three *IL-10* gene polymorphisms were significantly

Table 6. Anthropometrical and biochemical characteristics of the cases according to IL-10 genotype.

Parameter	IL-10 -1082A>G				IL-10 -819C>T				IL-10 -592C>A			
	AA	GG	AG	P value	CC	TT	TC	P value	CC	AA	CA	P value
Age (yr)	40.64 ± 9.46	40.77 ± 8.81	39.49 ± 10.05	0.52	39.48 ± 9.11	40.94 ± 10.32	40.61 ± 9.74	0.47	39.48 ± 9.11	40.94 ± 10.32	40.61 ± 9.74	0.47
Weight (kg)	72.58 ± 12.98	68.39 ± 10.19	73.58 ± 14.68	0.12	72.03 ± 13.29	73.49 ± 15.86	72.61 ± 12.57	0.76	72.03 ± 13.29	73.49 ± 15.86	72.61 ± 12.57	0.76
Height (cm)	159.81 ± 8.96	157.57 ± 8.99 ¹	161.90 ± 9.51 ¹	0.02*	160.74 ± 9.92	160.03 ± 9.28	160.14 ± 8.66	0.81	160.74 ± 9.92	160.03 ± 9.28	160.14 ± 8.66	0.81
Body mass index (kg/m ²)	28.43 ± 4.75	27.65 ± 4.19	27.94 ± 4.25	0.47	27.86 ± 4.45	28.62 ± 5.33	28.29 ± 4.27	0.48	27.86 ± 4.45	28.62 ± 5.33	28.29 ± 4.27	0.48
Waist circumference (cm)	97.64 ± 11.73	94.00 ± 10.73	98.05 ± 11.16	0.16	96.54 ± 12.23	98.30 ± 11.51	97.87 ± 10.82	0.47	96.54 ± 12.23	98.30 ± 11.51	97.87 ± 10.82	0.64
Hip circumference (cm)	101.33 ± 10.29	100.74 ± 15.74	100.81 ± 9.13	0.88	100.43 ± 9.68	101.48 ± 12.38	101.48 ± 10.39	0.64	100.43 ± 9.68	101.48 ± 12.38	101.48 ± 10.39	0.47
Waist hip ratio	0.96 ± 0.08	0.94 ± 0.10	0.97 ± 0.07	0.11	0.96 ± 0.08	0.97 ± 0.07	0.97 ± 0.07	0.66	0.96 ± 0.08	0.97 ± 0.07	0.97 ± 0.07	0.66
Systolic blood pressure (mm/Hg)	133.40 ± 14.06	138.66 ± 16.52	136.22 ± 16.44	0.07	132.92 ± 13.40	137.21 ± 17.04	135.56 ± 15.79	0.12	132.92 ± 13.40	137.21 ± 17.04	135.56 ± 15.79	0.12
Diastolic blood pressure (mm/Hg)	89.91 ± 9.62	90.63 ± 6.68	90.04 ± 8.83	0.91	89.13 ± 8.19	91.03 ± 10.32	90.37 ± 9.29	0.29	89.13 ± 8.19	91.03 ± 10.32	90.37 ± 9.29	0.29
Fasting plasma glucose (mg/dL)	122.63 ± 33.07	116.05 ± 34.29	122.75 ± 34.84	0.54	121.75 ± 33.60	128.01 ± 39.87	120.10 ± 31.31	0.26	121.75 ± 33.60	128.01 ± 39.87	120.10 ± 31.31	0.26
Total cholesterol (mg/dL)	186.50 ± 51.74	187.02 ± 54.96	182.18 ± 49.12	0.72	183.27 ± 47.22	183.48 ± 56.78	187.03 ± 51.97	0.77	183.27 ± 47.22	183.48 ± 56.78	187.03 ± 51.97	0.77
Triglycerides (mg/dL)	185.35 ± 71.29	172.13 ± 83.02	179.76 ± 64.20	0.51	178.35 ± 79.94	186.96 ± 55.21	183.48 ± 66.45	0.67	178.35 ± 79.94	186.96 ± 55.21	183.48 ± 66.45	0.67
HDL-C (mg/dL)	32.94 ± 9.65	32.62 ± 9.74	33.56 ± 9.29	0.79	33.01 ± 9.81	32.07 ± 9.94	33.63 ± 9.12	0.51	33.01 ± 9.81	32.07 ± 9.94	33.63 ± 9.12	0.51
lipoprotein (mg/dL)	116.49 ± 50.52	119.98 ± 54.22	112.67 ± 47.04	0.66	114.58 ± 44.69	114.02 ± 57.88	116.71 ± 50.27	0.90	114.58 ± 44.69	114.02 ± 57.88	116.71 ± 50.27	0.90
LDL-C (mg/dL)	37.07 ± 14.26	34.43 ± 16.60	35.95 ± 12.84	0.51	35.67 ± 15.99	37.39 ± 11.04	36.70 ± 13.29	0.67	35.67 ± 15.99	37.39 ± 11.04	36.70 ± 13.29	0.67
VLDL-C (mg/dL)	10.52 ± 6.11	10.72 ± 10.35	10.12 ± 7.30	0.84	9.16 ± 5.14 ²	12.79 ± 10.88 ²	10.50 ± 6.21	0.002*	9.16 ± 5.14 ²	12.79 ± 10.88 ²	10.50 ± 6.21	0.002*
Fasting insulin (U/mL)	3.26 ± 2.41	3.10 ± 3.21	3.20 ± 3.35	0.94	2.83 ± 2.05 ³	4.30 ± 4.77 ^{3,4}	3.14 ± 2.30 ⁴	0.002*	2.83 ± 2.05 ³	4.30 ± 4.77 ^{3,4}	3.14 ± 2.30 ⁴	0.002*
HOMA-IR	2.68 ± 1.96	2.72 ± 2.11	2.60 ± 1.95	0.35	2.81 ± 2.08	2.32 ± 1.88	2.66 ± 1.90	0.24	2.81 ± 2.08	2.32 ± 1.88	2.66 ± 1.90	0.24
IL-10 (pg/mL)												

Data are presented as mean ± SD. Level of significance indicated as * $P < 0.05$, post-hoc comparison test: ¹ $P = 0.03$, ^{2,3,4} $P = 0.001$.

Table 7. Anthropometrical and biochemical characteristics of the controls according to the IL-10 genotype.

Parameter	IL-10 -1082A>G				IL-10 -819C>T				IL-10 -592A>C			
	AA	GG	AG	P value	CC	TT	TC	P value	CC	AA	CA	P value
Age (yr)	40.19 ± 10.23	38.13 ± 10.45	41.30 ± 10.37	0.33	40.49 ± 10.48	39.12 ± 10.60	40.80 ± 10.03	0.60	40.49 ± 10.48	39.12 ± 10.60	40.80 ± 10.03	0.60
Weight (kg)	59.42 ± 11.76	58.27 ± 1.61	59.64 ± 1.00	0.87	59.21 ± 11.13	57.92 ± 11.55	60.11 ± 11.79	0.47	59.21 ± 11.13	57.92 ± 11.55	60.11 ± 11.79	0.47
Height (cm)	160.22 ± 9.07	161.65 ± 9.26	159.62 ± 8.54	0.57	159.81 ± 9.26	157.65 ± 10.22 ¹	161.11 ± 7.93 ¹	0.04*	159.81 ± 9.26	157.65 ± 10.22 ¹	161.11 ± 7.93 ¹	0.04*
Body mass index (kg/m ²)	23.16 ± 4.32	22.19 ± 3.39	23.42 ± 4.11	0.42	23.17 ± 3.95	23.44 ± 5.05	23.14 ± 4.19	0.90	23.17 ± 3.95	23.44 ± 5.05	23.14 ± 4.19	0.90
Waist circumference (cm)	83.18 ± 10.79	83.07 ± 13.01	83.38 ± 9.85	0.98	83.29 ± 10.75	82.76 ± 11.73	83.34 ± 10.13	0.94	83.29 ± 10.75	82.76 ± 11.73	83.34 ± 10.13	0.94
Hip circumference (cm)	91.85 ± 9.06	90.43 ± 9.18	92.47 ± 7.77	0.55	92.15 ± 8.87	91.71 ± 8.99	91.89 ± 8.33	0.93	92.15 ± 8.87	91.71 ± 8.99	91.89 ± 8.33	0.93
Waist hip ratio	0.90 ± 0.07	0.92 ± 0.08	0.90 ± 0.07	0.64	0.90 ± 0.07	0.90 ± 0.09	0.91 ± 0.06	0.88	0.90 ± 0.07	0.90 ± 0.09	0.91 ± 0.06	0.88
Systolic blood pressure (mm/Hg)	120.83 ± 10.17	123.22 ± 12.12	120.87 ± 9.46	0.55	120.37 ± 9.62	121.92 ± 10.14	121.33 ± 10.46	0.53	120.37 ± 9.62	121.92 ± 10.14	121.33 ± 10.46	0.53
Diastolic blood pressure (mm/Hg)	81.07 ± 7.24	82.52 ± 6.30	81.59 ± 6.93	0.57	81.00 ± 6.96	81.59 ± 6.61	81.61 ± 7.34	0.70	81.00 ± 6.96	81.59 ± 6.61	81.61 ± 7.34	0.70
Fasting plasma glucose (mg/dL)	93.04 ± 16.98	91.11 ± 15.37	90.34 ± 16.27	0.32	92.06 ± 13.16	90.00 ± 12.00	92.51 ± 20.62	0.65	92.06 ± 13.16	90.00 ± 12.00	92.51 ± 20.62	0.65
Total cholesterol (mg/dL)	163.89 ± 44.25	164.11 ± 41.22	159.01 ± 41.59	0.57	162.93 ± 42.11	148.91 ± 33.89	165.40 ± 45.98	0.06	162.93 ± 42.11	148.91 ± 33.89	165.40 ± 45.98	0.06
Triglycerides (mg/dL)	114.93 ± 47.62	122.75 ± 77.16	110.11 ± 43.73	0.43	115.49 ± 52.14	99.74 ± 32.16	116.02 ± 48.21	0.09	115.49 ± 52.14	99.74 ± 32.16	116.02 ± 48.21	0.09
HDL-C (mg/dL)	43.99 ± 11.80	44.19 ± 9.29	44.31 ± 13.03	0.97	44.03 ± 11.70	44.76 ± 12.66	44.01 ± 12.37	0.92	44.03 ± 11.70	44.76 ± 12.66	44.01 ± 12.37	0.92
LDL-C (mg/dL)	98.26 ± 44.81	95.37 ± 39.00	93.66 ± 43.11	0.62	97.58 ± 43.96	84.20 ± 33.13	99.01 ± 46.07	0.10	97.58 ± 43.96	84.20 ± 33.13	99.01 ± 46.07	0.10
VLDL-C (mg/dL)	22.99 ± 9.52	24.55 ± 15.43	22.02 ± 8.75	0.43	23.10 ± 10.43	19.95 ± 6.43	23.20 ± 9.64	0.09	23.10 ± 10.43	19.95 ± 6.43	23.20 ± 9.64	0.09
Fasting insulin (U/mL)	5.98 ± 3.68	5.21 ± 2.27	5.51 ± 3.48	0.36	5.64 ± 3.43	5.62 ± 2.82	5.95 ± 3.85	0.69	5.64 ± 3.43	5.62 ± 2.82	5.95 ± 3.85	0.69
HOMA-IR	1.43 ± 1.33	1.19 ± 0.54	1.25 ± 0.87	0.28	1.30 ± 0.88	1.28 ± 0.72	1.43 ± 1.47	0.54	1.30 ± 0.88	1.28 ± 0.72	1.43 ± 1.47	0.54
IL-10 (pg/mL)	3.88 ± 2.72	2.92 ± 1.75	3.99 ± 2.62	0.20	3.88 ± 2.66	4.08 ± 2.86	3.77 ± 2.56	0.75	3.88 ± 2.66	4.08 ± 2.86	3.77 ± 2.56	0.75

Data are presented as mean ± SD. Level of significance indicated as * $P < 0.05$, post-hoc comparison test: ¹ $P = 0.03$.

different for any clinicobiochemical parameters except height. The genotypes of *IL-10* -819C>T and *IL-10* -592A>C polymorphisms of *IL-10* were found to be significantly different for height ($P=0.04$). The post-hoc comparison test showed the significant difference of height between the *TT* and *TC* genotypes of *IL-10* -819C>T as well as *AA* and *CA* of *IL-10* -592C>A gene polymorphism among the control subjects.

Discussion

IL-10 has multifaceted antiinflammatory properties that are known to regulate insulin sensitivity and atherosclerotic process (Han *et al.* 2010). Elevated glucose level suppresses *IL-10* (Reinhold *et al.* 1996). This in turn probably promotes proinflammatory environment and developing insulin resistance. *IL-10* also helps in cholesterol uptake and efflux in macrophages (Han *et al.* 2009). There are compelling evidences linking *IL-10* with MetS. Studies in adults showed circulating *IL-10* might exert some beneficial metabolic effects (van Exel *et al.* 2002a; Heeschen *et al.* 2003; Strackowski *et al.* 2005). Earlier studies suggest that circulating levels of the antiinflammatory cytokine *IL-10* are elevated in obese women without MetS and that low *IL-10* levels are associated with the MetS (Esposito *et al.* 2003). In early years, the role of *IL-10* gene polymorphisms in MetS related complications has been reported by few authors among different populations such as diabetes (Mohebbatikaljahi *et al.* 2009; Saxena *et al.* 2012), acute coronary syndrome (Babu *et al.* 2012), obesity and insulin resistance (Scarpelli *et al.* 2006).

In our study, the frequency of homozygous mutant *TT* genotype and mutant allele *T* of *IL-10* -819C>T (rs1800872) polymorphism was found to be significantly higher among the cases compared to controls. Similarly, in case of *IL-10* -592C>A (rs1800871) polymorphism, homozygous mutant *AA* genotype and mutant allele *A* of *IL-10* -592C>A polymorphism was found to be higher among the cases when compared to controls. The similar frequencies of *IL-10* -819C>T and *IL-10* -592C>A polymorphic sites were due to perfect LD between the SNPs. In our study, the mutant allele frequencies of *IL-10* -819T and *IL-10* -592A in controls were similar to the previously reported frequency in Turkish population (-819T, 31.0% and -592A, 31.0%) (Mohebbatikaljahi *et al.* 2009) and in Japanese population (-592A, 31.0% control) (Ide *et al.* 2003). However, some other studies reported high as 48.7% in Greek population (Tsiavou *et al.* 2004) and low as 25.6% (Saxena *et al.* 2012) in Indian population for allele frequency of mutant alleles of *IL-10* -819C>T and *IL-10* -592C>A gene polymorphism. Saxena *et al.* (2012) reported lower frequency of *IL-10* -592A allele could be due to comparatively lower sample size of the control subjects ($n=168$). Overall, the 592A-allele frequency is low in both the studies and corroborates with other reports from Indian population (Chatterjee *et al.* 2005). In the third polymorphism *IL-10* -1082A>G (rs1800896),

we did not observe any significant differences between cases and controls in either genotypes or alleles. This polymorphic site has not been investigated by any other group in MetS subjects.

To the best of our knowledge, the only study reported on *IL-10* gene polymorphisms in MetS did not find any association (Lu *et al.* 2011). The authors analysed the three polymorphisms of *IL-10* gene (one functional 819C>T (rs1800871) and two tagging SNPs rs3790622 and rs3021094) in a cohort of 260 MetS subjects from southern Taiwan and found none to be associated with MetS. Here, we investigated three functional SNPs of *IL-10* gene (-1082A>G (rs1800896), -819C>T (rs1800872) and -592C>A (rs1800871)) in a comparatively larger sample size ($n = 384$) in north Indian MetS subjects. In contrast to Lu *et al.* (2011), we observed significant association of 819C>T polymorphism with MetS. In case of -819C>T, rs1800872, there is significant variation in T-allele frequencies obtained by Lu *et al.* (2011) (67.6%) versus the present study (34.2%). In general, the T-allele frequency of -819C>T (rs1800872) polymorphism is reported to be high in Asian population (60.06%), whereas it is low in Caucasian populations (25.35%) (Yu *et al.* 2013). Racial and ethnic variability in polymorphic cytokine allele frequency are well reported (Van Dyke *et al.* 2009; Pooja *et al.* 2011) and this may be a plausible reason for contradictory finding from two different populations. Interestingly, majority of reports from Indian population suggest lower T-allele of -819C>T in control subjects similar to Caucasian population (Chatterjee *et al.* 2005) and our results corroborated with these earlier findings. Thus, this report further validates significance of investigating populationwise variation of candidate SNPs in healthy control subjects and their association with pathological conditions. In addition, the healthy control subjects in our study were devoid of any single-risk components of MetS unlike in the study by Lu *et al.* (2011), where control subjects with 0–1 risk factors were also included.

The three biallelic polymorphisms at positions -1082, -819 and -592 generally produce three core haplotypes: *GCC*, *ACC* and *ATA* (Turner *et al.* 1997; Singhal *et al.* 2015). In our haplotype analysis, we observed all three major haplotypes, *GCC*, *ACC*, *ATA* and a rare *GTA* haplotype. The *GTA* haplotype is extremely rare in Caucasians, but the authors have reported this haplotype in other populations including India (Mok *et al.* 1998; Eskdale *et al.* 1999; Kingo *et al.* 2003; Zammiti *et al.* 2006; Singhal *et al.* 2015). In this study, the frequency of *ACC* haplotype, which consisted of wild allele was significantly lower (0.431% versus 0.099%, $P = 0.01$) and *GTA* haplotype, which consisted of mutant allele was significantly higher (0.495% versus 0.07%, $P = 0.04$) among cases in comparison with controls. This is the first report of association of polymorphic variants and haplotype frequencies of *IL-10* in Indian MetS subjects. Therefore, unless replicated in other ethnic groups, it cannot be confirmed whether these observations are specific to north Indian population or that of entire Indian population.

Upon the subgroup analysis based on the *IL-10* genotypes of all the three polymorphisms, only fasting insulin and HOMA-IR were significantly associated with *IL-10* –819 and *IL-10* –592 genotypes among the cases and which is consistent with a previous study (Scarpelli et al. 2006). Likewise, the findings of study on Caucasian Italian subjects showed that the polymorphism in the promoter region of *IL-10* at positions –592, –819 and –1082 was associated with insulin resistance and obesity (Scarpelli et al. 2006). Several studies have demonstrated that *AA* genotype of *IL-10* –592C>A gene polymorphism associated with lower *IL-10* serum level (Scarpelli et al. 2006; Kung et al. 2010; Zheng et al. 2014). Some other study has reported *CC* genotype of *IL-10* –592C>A gene polymorphism to be associated with lower *IL-10* serum level in Brazilian population (Pereira et al. 2015). Similarly, another study from Chinese population reported *CC* genotype of *IL-10* –819C>T gene polymorphism to be associated with higher *IL-10* serum level (Sun et al. 2010), while another study reported that *CC* genotype of *IL-10* –819C>T gene polymorphism is associated with lower production of *IL-10* (Pereira et al. 2015). Moreover, in a study from Turkey, it was observed that the *GG* genotype of *IL-10* –1082A>G gene polymorphism is associated with higher production of *IL-10* (Aydingöz et al. 2015). However, in contrast, several studies reported that there was no effect of *IL-10* –1082A>G gene polymorphism on its expression level (Lech-Maranda et al. 2004; Scarpelli et al. 2006; Hussein et al. 2014), whereas some other studies reported lack of association of serum *IL-10* level with any of the three polymorphisms (Roh et al. 2002). In the present study, we could not find any significant difference in circulating serum level of *IL-10* between the genotypes of *IL-10* gene polymorphisms among both cases and controls. The lack of association between circulating serum level of *IL-10* and its gene polymorphisms could be due to the presence of other *IL-10* inhibiting factors in the inflammatory environment of MetS (Ji et al. 2003). Another possible reason for such lack of association could be due to the possibility that the *IL-10* level is not regulated by single allele or genotype (Lech-Maranda et al. 2004). Moreover, we assume that these polymorphic variants though influence gene expression; additional post-translational regulation could also have impact on its final protein expression which needs to be investigated.

Lower serum *IL-10* level is implicated in MetS and diabetes (Saxena et al. 2012). In our study also, the circulating serum level of *IL-10* was significantly lower in cases as compared to controls. The results were consistent with some previous reports (van Exel et al. 2002a; Esposito et al. 2003; Choi et al. 2007), where the MetS subjects were reported to be significantly associated with the low production of *IL-10*. However, some authors report contradictory findings (Calcaterra et al. 2009), which could be due to the use of different subject selection criteria and ethnicity. Correlation analysis of *IL-10* serum level and our study parameter reveals that the serum level of *IL-10* was negatively correlated with systolic blood pressure, diastolic blood pressure, fasting plasma

glucose, triglycerides, HDL-C, LDL-C, VLDL-C, fasting insulin, HOMA-IR in both cases and controls, while the total cholesterol was negatively correlated with serum level of *IL-10* in cases only. These findings were in agreement with previous study where significant association between low *IL-10* serum level and high serum glucose, high HbA1c, type 2 diabetes and dyslipidemia is reported (van Exel et al. 2002a).

In conclusion, serum *IL-10* level in MetS subject is significantly low and supports the existing view that higher proinflammatory state is responsible for MetS. As expected, serum *IL-10* level was correlated well with majority of clinicobiochemical parameters associated with MetS. Of the three investigated promoter polymorphisms, the genotypes and alleles of –819C>T and –592C>A polymorphic variants are significantly associated with risk of MetS. Of the four observed haplotypes, *ACC* and *GTA* haplotypes of *IL-10* polymorphisms are associated with MetS risk. *IL-10* polymorphic variants (–819C>T and –592C>A) are associated with insulin level and HOMA-IR of MetS subjects, but none of the polymorphic variants are associated with serum *IL-10* level. Overall, these results suggest that low serum *IL-10* level has significant role in MetS and could be an important predictor for early diagnosis of MetS. The homozygous mutant genotype and mutant allele of –819C>T and –592C>A polymorphic variants may predispose the individual towards developing MetS or related risk factor such as insulin resistance. However, functional studies would definitely help to understand the potential implications of this polymorphism on insulin resistance and other related risk factors of MetS. Therefore, a more comprehensive appraisal of genetic variation in *IL-10* gene would be essential to fully assess the role of this gene in MetS and other associated phenotypes like insulin resistance.

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