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Research paper

Genetic variations in key inflammatory cytokines exacerbates the risk of diabetic nephropathy by influencing the gene expression

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ABSTRACT

Background: Diabetic nephropathy is the single strongest predictor of mortality in patients with diabetes. The development of overt nephropathy involves important inter-individual variations, even after adjusting for potential confounding influences of modifiable and non-modifiable risk factors. Genome-wide transcriptome studies have reported the activation of inflammatory signaling pathways and there is mounting indication of the role of genetic factors.

Methods: We screened nine genetic variations in three cytokine genes (*TNF-α*, *IL-6* and *IL-β*) in 1326 unrelated subjects comprising of healthy controls (n = 464), type 2 diabetics with nephropathy (DN, n = 448) and type 2 diabetes without nephropathy (T2D, n = 414) by sequence-specific amplification. Functional implication of SNPs was elucidated by correlation studies and relative gene expression using Realtime-Quantitative PCR (RT-qPCR).

Results: Individual SNP analysis showed highest association of *IL-1β* rs16944-TT genotype (OR = 3.51, 95%CI = 2.36–5.21, P = 0.001) and *TNF-α* rs1800629-AA genotype (OR = 2.75, 95% CI = 1.64–4.59, P = 0.001) with T2D and DN respectively. The haplotype frequency showed significant risk of seven combinations among T2D and four combinations among DN subjects. The highest risk of T2D and DN was associated with GGTGAGTTT (OR = 4.25, 95%CI = 3.3–14.20, P = 0.0016) and GACGACCTT (OR = 21.3, 95%CI = 15.1–28.33, P = 0.026) haplotypes respectively. Relative expression by RT-qPCR showed increased cytokine expression in cases as compared to controls. TNF-α expression was increased by more than four-folds (n-fold = 4.43 ± 1.11) in DN. TNF-α, IL-6 and IL-1β transcript levels were significantly modulated by promoter region SNPs.

Conclusions: The present study implicates a strong association between cytokine *TNF-α*, *IL-6* and *IL-1β* gene promoter polymorphisms and modulation of transcript levels with susceptibility to nephropathy in diabetes subjects.

Abbreviations: AER, Albumin excretion rate; BMI, Body mass index; bp, Base pair; BUN, Blood Urea Nitrogen; CKD, Chronic kidney disease; Ct, Cross threshold; DBP, Diastolic blood pressure; DN, Diabetic nephropathy; DNA, Deoxyribonucleic acid; DNase, Deoxyribonuclease; ECM, Extracellular matrix; eGFR, Estimated Glomerular filtration rate; ESRD, End stage renal disease; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GBM, Glomerular basement membrane; HDL, High density lipoprotein; IL, Interleukin; LD, Linkage disequilibrium; LDL, Low density lipoprotein; PBMNCs, Peripheral blood mononuclear cells; PCR, Polymerase chain reaction; RNA, Ribonucleic acid; RNase, Ribonuclease; RT-qPCR, Realtime-Quantitative polymerase chain reaction; SBP, Systolic blood pressure; SD, Standard deviation; SNP, Single nucleotide polymorphism; T2D, Type 2 diabetes; TG, Triglyceride; TNF, Tumor necrotic factor

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1. Introduction

Diabetes-related complications represent one of the most important health problems worldwide with dire social and economic projections (Cooper, 2012). One of the most important medical concerns of the diabetes epidemic is diabetic nephropathy (DN). Diabetic nephropathy is regarded as a prototypical disease of gene and environmental interactions because not all diabetic subjects with traditional risk factors develop clinically evident nephropathy, indicating a role for individual susceptibility. The majority (> 85%) of GWAS-identified single nucleotide polymorphisms (SNPs) are located in the non-coding regions of the genome and thus their functional implication lies in identifying the target genes, cell types, and the mode of dysregulation caused by these non-coding SNPs (Maurano et al., 2012). Recent studies indicate that complex trait-causing variants localize to cell-type-specific, functionally important gene regulatory regions where they can disrupt or create transcription factor binding sites to alter transcript levels only in disease-target cell types (Ko and Susztak, 2013; Susztak, 2014). Several elements of the immune system including cytokines and resident chemokines, macrophage recruitment, T lymphocytes, and immune complex deposition have recently been associated with DN (Navarro-González and Mora-Fernández, 2008; Gaballa and Farag, 2013). Since renal cells are also capable of synthesizing pro-inflammatory cytokines such as tumor necrotic factor-alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6), therefore, these cytokines acting in a paracrine or autocrine manner may induce significant effects leading to the development and progression of several renal disorders (Matoba et al., 2010; Pruijm et al., 2012; Shankar et al., 2011). The rationale of this study involved a concerted effort of genotyping, correlation and gene expression techniques involving three pro-inflammatory cytokine genes (*TNF-α*, *IL-6* and *IL-1β*) in the development and progression of DN as well as identification of high risk patients involving susceptibility or poor clinical outcome.

2. Materials and methods

2.1. Collection and processing of samples

This prospective, case control study was carried out in the period between March 2013 and June 2016. Study subjects were recruited consecutively from patients who came to the Endocrinology and Nephrology Departments of the Sher-i-Kashmir Institute of Medical Sciences (outpatient clinic and inpatient ward) and who gave consent to participate in the study.

The study population comprised of 464 healthy individuals (control group), 414 type 2 diabetes cases without nephropathy (T2D Group) with no known prior history of albuminuria or overt nephropathy, and 448 cases of type 2 diabetes with nephropathy (DN Group) having persistent proteinuria/albuminuria and (eGFR) < 60 mL/min/1.73 m^2 at the time of referral. The clinical status of subjects was recorded on a pre-designed proforma. 1–2 mL whole blood sample was drawn from each subject in sterile polypropylene vials containing EDTA as anticoagulant. Genomic DNA was isolated using QIAamp® DNA Blood Mini Kit (Qiagen) as per the manufacturers' instructions. Peripheral blood mononuclear cells (PMNCs) were isolated from whole blood using Lymphoprep™ (Axis-Shield, Norway). Total cellular RNA was extracted using TRIzol® Reagent (Life Technologies) and purified using Rneasy® Mini Kit (Qiagen). The purity and quantification of both DNA and RNA was determined spectrophotometrically using Nanodrop (Thermo Scientific). For gene expression RNA was transcribed into cDNA using Maxima® First Strand cDNA synthesis Kit (Thermo Scientific).

2.2. Genotyping and relative gene expression

Nine genetic variations in *TNF-α* (rs361525, rs1800629, rs1799964), *IL-6* (rs1800795, rs1800796, rs1800797) and *IL-1β* (rs1143627, rs1143634, rs16944) were genotyped using sequence-specific PCR and direct sequencing methods. SNP information, primer sequence and amplicon size is given in table (Supplementary Table 1). Relative expression of *TNF-α*, *IL-6* and *IL-1β* genes was determined by Realtime-Quantitative PCR (RT-qPCR) using SYBR green fluorescence. The RT-qPCR was performed using Maxima SYBR Green qPCR Master Mix (Thermo Scientific) on LightCycler® 480 (Roche). Normalization of target gene expression was performed by the reference Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene. The primer sequence and amplicon size for each gene is given in table (Supplementary Table 2).

2.3. Statistical analyses

Categorical variables and continuous variables were tested and compared for significant differences using *t*-tests. Quantitative trait association for SNPs was performed using a series of analysis of variance (ANOVA) tests. Hardy-Weinberg equilibrium (HWE) was assessed using the χ^2 goodness-of-fit statistic at a significance level of < 0.05. To test all SNPs for genotypic association, adjusted odds ratios (ORs) and 95% confidence intervals (CI) were computed using Vassar statistic (*http://vassarstats.net/*) and SNPStats (*http://bioinfo.iconcologia.net/SNPstats*) softwares. All analyses were adjusted using linear regression. Correlation between risk factors was determined using Pearson's correlation coefficient. Logistic regression analysis was then employed to study the genotype-phenotype association. Linkage disequilibrium plot and haplotype frequency for multiple SNPs was calculated using Iconcologia software (Catalan Institute of Oncology©). Expression data was analyzed by LightCycler® 480 software (version 2.0). Relative gene expression levels were calculated by comparative Ct ($2^{-\Delta\Delta CT}$) and the data was expressed in terms of fold change using Livak method (Livak and Schmittgen, 2001).

3. Results

The clinical, anthropometric and systemic observations of cases are shown in Table 1. The most common co-morbidity found in both T2D and DN cases was hypertension. 67.6% (303/448) of DN cases and 42.75% (177/414) T2D patients were observed to be hypertensive. The albumin excretion rate (AER mg/24 h) showed that 67.85% (304/448) of DN patients were macroalbuminuric. Mean eGFR of all DN patients was 22.5 ± 13.58 mL/min/1.73 m^2. None of our patients had received renal transplant during the course of study.

3.1. Genotyping results

Sequencing results showed rs361525 G ↔ A, rs1800629 G ↔ A, rs1799964 T ↔ C), *IL-6* (rs1800795 G ↔ C, rs1800796 A ↔ G, rs1800797 G ↔ C, rs1143627 C ↔ T, rs1143634 C ↔ T and rs16944 C ↔ T genotypic variants in our study subjects. The genotype/allele frequencies of all SNPs are given in table (Supplementary Table 3).The SNPs followed the Hardy-Weinberg equilibrium (χ^2 < 3.84, P > 0.05). Genotypic association of SNPs with disease is illustrated in Tables 2–4 for *TNF-α*, *IL-6* and *IL-1β* respectively. The genotype analyses of individual SNPs showed highest association of *IL-1β* rs16944-TT and *TNF-α* rs1800629-AA genotypes with the risk for T2D and DN respectively.

The correlation between individual genotypes and disease parameters for *TNF-α*, *IL-6* and *IL-1β* SNPs is shown in Tables 5–7 respectively. The risk genotypes almost invariably showed association with traditional risk factors for T2D and DN. These included higher glycemic index, dyslipidemia, hypertension, increased albumin excretion, proteinuria and decreased eGFR. Risk conferring genotypes for DN also showed correlation with shorter duration of diabetes (rs361525-AA, rs1800629-AA, rs1799964-CC, rs1800797-GG, and rs16944-TT) and lower age of onset (rs1800629-AA, rs1799964-CC, rs1800797-GG and rs16944-TT).

Table 1
Clinical, anthropometric and systemic parameters of cases.

Parameter	T2D (414)	DN (448)
Age (years)	54.05 ± 8.79	57.91 ± 8.70
Duration of diabetes (years)	7.08 ± 4.54	10.08 ± 5.81
BMI (kg/m^2)	26.88 ± 4.40	26.55 ± 4.44
Systolic BP (mm Hg)	126.45 ± 11.61	138.70 ± 15.15
Diastolic BP (mm Hg)	79.83 ± 7.53	86.02 ± 9.71
Blood sugar (F) mg/dl	143.06 ± 38.99	154.31 ± 55.02
Blood sugar (PP) mg/dl	199.71 ± 48.39	215.16 ± 69.70
HB_{AIC} (%)	7.59 ± 1.49	8.52 ± 1.61
Urea mg/dl	28.90 ± 8.99	81.71 ± 55.04
Creatinine mg/dl	0.88 ± 0.26	3.68 ± 2.37
BUN	13.50 ± 4.20	38.18 ± 25.72
Alkaline phosphatase	127.69 ± 63.30	113.54 ± 43.13
Alanine transaminase U/l	52.98 ± 34.66	39.90 ± 25.04
Aspartate transaminase U/l	50.87 ± 32.32	38.93 ± 24.07
T. protein g/dl	7.43 ± 0.54	6.52 ± 0.92
Albumin g/dl	3.94 ± 0.57	3.39 ± 0.67
T. bilirubin mg/dl	1.17 ± 1.08	0.88 ± 0.40
Calcium mg/dl	8.48 ± 1.0	7.80 ± 1.41
Phosphorus mg/dl	3.70 ± 1.08	5.49 ± 2.44
Uric acid mg/dl	5.44 ± 1.31	7.19 ± 1.56
T. cholesterol (mg/dl)	188.71 ± 57.6	172.31 ± 52.84
LDL (mg/dl)	104.21 ± 28.19	92.71 ± 24.38
HDL (mg/dl)	43.26 ± 7.99	37.42 ± 6.52
Triglycerides (mg/dl)	212.05 ± 79.23	196.56 ± 75.08

Data expressed as mean ± SD.
Distribution of the data was checked by Q-Q-Plots and tested using the Shapiro–Wilk test. An appropriate nonparametric test was conducted for data showing deviation from normal distribution.
BUN: Blood Urea Nitrogen, LDL: Low density lipoprotein, HDL: High density lipoprotein, BMI: Body mass index, BP: Blood pressure, F: Fasting, PP: Post prandial.

3.2. Haplotype frequency and linkage analysis

Haplotype frequency for multiple SNPs was measured by Expectation Maximization (EM) method using SNPstats software. The order of genotypes for haplotype frequency estimation is *TNF-α* rs361525, *TNF-α* rs1800629, *TNF-α* rs1799964, *IL-6* rs1800795, *IL-6* rs1800796, *IL-6* rs1800797 and *IL-1β* rs1143627, *IL-1β* rs1143634 and *IL-1β* rs16944. Frequency threshold for rare haplotypes was set at 0.01. We observed 14 haplotypes with frequency > 0.01 among which GGT GAGCCC was the most common haplotype. The relative risk was determined by comparing the global haplotype (GGTGAGCCC) with other haplotypes. Table 8 shows the risk of haplotypes with T2D as well as DN. The risk of seven haplotype combinations showed significant association with respect to the global haplotype among T2D cases. The highest risk of T2D was associated with GGTGAGTTT haplotype (OR = 4.25, 95%CI = 3.3–14.20, $P = 0.0016$). Upon comparing the haplotypes, four combinations showed significant association with DN. The highest risk conferring haplotype for DN was GACGACCTT (OR = 21.3, 95%CI = 15.1–28.33, $P = 0.026$). The haplotypes showed increased risk in several folds as compared to the individual SNPs.

The linkage disequilibrium (LD) plot for all SNPs is shown in Fig. 1. We observed nominal linkage between rs1143627 and rs1143634. The values of coefficient of LD and significance were $D' = 0.50$ and $P = 0.011$ respectively.

3.3. Realtime-quantitative PCR (RT-qPCR) expression results

The relative expression of *TNF-α*, *IL-6* and *IL-1β* genes was quantitatively expressed as an *n*-fold difference relative to the reference gene (*GAPDH*). The fold expression of target genes across study groups is shown in Fig. 2. The relative expression of TNF-α showed more than two fold increase in T2D cases (*n*-fold = 2.6 ± 0.66) and four folds increase in DN cases (*n*-fold = 4.43 ± 1.11) when compared with the healthy controls ($P = 0.007$). IL-6 expression was also increased by more than two folds in T2D (*n*-fold = 2.3 ± 0.23) and DN cases (*n*-fold = 2.8 ± 0.44); however the difference in expression between T2D and DN was not significant ($P = 0.089$). IL-1β expression on the other hand was relatively higher in T2D cases (*n*-fold = 2.2 ± 0.62) as compared to DN cases (*n*-fold = 1.85 ± 0.58). The significance was determined after adjusting for clinical confounders, smoking status and use of statins among cases.

Independent association of the genotypes on expression levels was demonstrated by comparing the *n*-fold change in expression. The relative expression of TNF-α across different genotypes is shown in Fig. 3. *TNF-α* rs1800629-AA genotype was associated with a four-fold increased expression (*n*-fold = 4.01 ± 0.66, $P = 0.012$). *IL-6* rs1800795 and rs1800796 did not show any significant association with the expression of IL-6. However rs1800797-GG genotype was associated with higher expression of IL-6 (*n*-fold = 2.8 ± 0.58, $P = 0.023$). The relative expression of IL-6 across the genotypes of specific SNPs is shown in Fig. 4. The Higher expression of IL-1β was associated with rs1143627-CC (*n*-fold = 2.1 ± 0.46) and rs16944-TT (*n*-fold = 2.8 ± 0.59) genotype. The difference however was significant only with respect to the rs16944-TT genotype ($P = 0.036$). Fig. 5 shows the relative expression of IL-1β across the genotypes. Comparative analysis showed significant modulation of TNF-α, IL-6 and IL-β expression by rs1800629-AA and rs1799964-CC, 1800797-GG and 16,944-TT genotypes respectively.

4. Discussion

This study was conducted to screen variations in multiple pro-inflammatory cytokine genes and ascertain the functional relevance of these SNPs by comparative expression analysis across the genotypes. The study comprised of 1326 unrelated subjects and exclusively from ethnic population of Kashmir valley in northern region of India. Subjects were matched for gender however DN subjects were relatively older due to the longer duration of diabetes before clinically overt presentation. This discrepancy was overcome by adjusting for the age

Table 2
Genotype association of *TNF-α* rs361525, rs1800629 and rs1799964 SNPs with T2D and DN.

SNP	Model	Genotype	Controls $N = 464$	T2D $N = 414$	DN $N = 448$	T2D + DN $N = 862$	OR (95% CI)	*P* value	OR* (95% CI)	*P** value
rs361525	Dominant	G/G	360 (77.6%)	288 (69.6%)	272 (60.7%)	560 (65%)	1		1	
		G/A-A/A	104 (22.4%)	126 (30.4%)	176 (39.3%)	302 (35%)	1.86 (1.44–2.41)	0.001	1.82 (1.43–2.32)	0.001
rs1800629	Codominant	G/G	356 (76.7%)	300 (72.5%)	228 (50.9%)	528 (61.2%)	1		1	
		G/A	96 (20.7%)	99 (23.9%)	184 (41.1%)	283 (32.8%)	1.87 (1.43–2.44)	0.001	2.44 (1.90–3.12)	0.001
		A/A	12 (2.6%)	15 (3.6%)	36 (8%)	51 (5.9%)	2.36 (1.25–4.48)	0.006	2.754 (1.64–4.59)	0.001
rs1799964	Codominant	T/T	224 (48.3%)	240 (58%)	168 (37.5%)	408 (47.3%)	1		1	
		T/C	192 (41.4%)	141 (34.1%)	216 (48.2%)	357 (41.4%)	1.001 (0.79–1.25)	1	1.52 (1.21–1.91)	0.01
		C/C	48 (10.3%)	33 (8%)	64 (14.3%)	97 (11.2%)	1.099 (0.76–1.58)	0.645	1.64 (1.15–2.32)	0.007

P, OR: Significance and Odds Ratio between all cases and controls.
*P**, OR*: Significance and Odds Ratio for presence and absence of nephropathy.

Table 3
Genotype association of *IL-6* rs1800795, rs1800796 and rs1800797 SNPs with T2D and DN.

SNP	Model	Genotype	Controls $N = 464$	T2D $N = 414$	DN $N = 448$	T2D + DN $N = 862$	OR (95% CI)	*P* value	OR* (95% CI)	*P** value
rs1800795	Codominant	G/G	280 (60.3%)	213 (51.5%)	248 (55.4%)	461 (53.5%)	1		1	
		G/C	152 (32.8%)	156 (37.7%)	160 (35.7%)	316 (36.7%)	0.75 (0.60–0.95)	0.032	0.96 (0.77–1.21)	0.81
		C/C	32 (6.9%)	45 (10.9%)	40 (8.9%)	85 (9.9%)	1.47 (0.96–2.25)	0.084	1.02 (0.68–1.52)	0.91
rs1800796	Dominant	A/A	336 (72.4%)	255 (61.6%)	252 (56.2%)	507 (58.8%)	1		1	
		A/G-G/G	128 (27.6%)	159 (38.4%)	196 (43.8%)	355 (41.2%)	1.83 (1.43–2.34)	0.001	1.16 (0.96–2.02)	0.19
rs1800797	Codominant	G/G	224 (48.3%)	168 (40.6%)	144 (32.1%)	312 (36.2%)	1		1	
		G/C	184 (39.7%)	183 (44.2%)	224 (50%)	407 (47.2%)	0.63 (0.28–0.71)	0.039	0.79 (0.35–0.95)	0.025
		C/C	56 (12.1%)	63 (15.2%)	80 (17.9%)	143 (16.6%)	0.74 (0.14–0.92)	0.029	0.81 (0.31–0.89)	0.042

P, OR: Significance and Odds Ratio between all cases and controls.
*P**, OR*: Significance and Odds Ratio for presence and absence of nephropathy.

and duration during the data analyses. In our study mean systolic and diastolic blood pressure was higher in nephropathy subjects. In fact, hypertension was found to be the most common comorbidity. Hypertension has been shown to be nearly twice as prevalent in patients with diabetes and generally exists prior to kidney disease (Van Buren and Toto, 2011). The BMI and lipid panel of T2D and DN subjects were comparable except for HDL levels that were relatively lower in DN subjects. Our results are in conformity with the single largest study examining the association between HDL levels and the development of new or worsening microvascular complications of diabetes, defined prospectively by renal and retinal events (Morton et al., 2012). The study showed that lower baseline HDL level is a significant and independent predictor of the development and progression of diabetic nephropathy.

Among the *TNF-α* SNPs, significant association was observed for rs361525 for both T2D and DN cases using dominant model. However, the SNP was not functionally associated with the risk factors of disease. Similar results were obtained by a recent study on Mexican population (Vázquez-Huerta et al., 2014). Significant association with T2D and DN was observed for rs1800629, the risk being nearly two fold for the carriers of AA genotype. The association studies on some Asian populations show positive significance of rs1800629 with T2D as well as DN (Manchanda et al., 2006; Singh et al., 2015), however other studies and a meta-analysis on Caucasian subjects did not demonstrate any association (Vázquez-Huerta et al., 2014; Lee et al., 2005; Buraczynska et al., 2007; Bouhaha et al., 2010; Feng et al., 2011). In case of rs1799964, we found only a single published study and the results related to the findings of our study (Gupta et al., 2015). The genotypic results of *IL-6* gene were similar to a recent study on north Indian population but with different ethnicity (Saxena et al., 2013). A relatively stronger association was demonstrated by one study investigating the rs1800795 in T2D patients though the association of rs1800796 was insignificant (Saxena et al., 2014). However in conformity with our results, the association of rs1800795 with nephropathy was not established (Ryu and Kim, 2012). Unlike these results one study on Turkish population demonstrated that rs1800795-GG genotype was an independent risk factor for DN (Karadeniz et al., 2014). Another study on Greek subjects with T2D showed higher risk with rs1800795-GC genotypes however another study on Tunisian population showed no effects (Papaoikonomou et al., 2013; Bouhaha et al., 2010). The GC and CC genotypes of rs1800797 were protected against both T2D and DN when compared to GG genotype in our study. We observed higher risk of T2D and DN among the carriers of rs1800797-GG genotype. These results are in conformity with other studies that elucidate a higher risk

Table 4
Genotype association of *IL-1β* rs1143627, rs1143634 and rs16944 SNPs with T2D and DN.

SNP	Model	Genotype	Controls $N = 464$	T2D $N = 414$	DN $N = 448$	T2D + DN $N = 862$	OR (95% CI)	*P* value	OR* (95% CI)	*P** value
rs1143627	Codominant	C/C	220 (47.4%)	174 (42%)	196 (43.8%)	370 (42.9%)	1		1	
		C/T	192 (41.4%)	177 (42.8%)	200 (44.6%)	377 (43.7%)	1.10 (0.87–1.38)	0.416	1.11 (0.88–1.39)	0.37
		T/T	52 (11.2%)	63 (15.2%)	52 (11.6%)	115 (13.3%)	1.22 (0.86–1.72)	0.29	0.871 (0.61–1.23)	0.48
rs1143634	Codominant	C/C	256 (55.2%)	147 (35.5%)	144 (32.1%)	291 (33.8%)	1		1	
		C/T	176 (37.9%)	189 (45.6%)	204 (45.5%)	393 (45.6%)	0.98 (0.78–1.23)	0.907	0.94 (0.75–1.19)	0.68
		T/T	32 (6.9%)	78 (18.8%)	100 (22.3%)	178 (20.6%)	1.31 (0.83–2.07)	0.265	1.46 (0.96–2.24)	0.91
rs16944	Codominant	C/C	236 (50.9%)	207 (50%)	220 (49.1%)	427 (49.5%)	1		1	
		C/T	200 (43.1%)	180 (43.5%)	188 (42%)	368 (42.7%)	1.37 (1.08–1.72)	0.007	1.17 (0.93–1.47)	0.17
		T/T	28 (6%)	27 (6.5%)	40 (8.9%)	67 (7.8%)	3.51 (2.36–5.21)	0.001	2.00 (1.48–2.70)	0.001

P, OR: Significance and Odds Ratio between all cases and controls.
*P**, OR*: Significance and Odds Ratio for presence and absence of nephropathy.

Table 5
Correlation between individual genotypes of *TNF-α* rs361525, rs1800629 and rs1799964 SNPs with disease parameters.

Parameter	rs361525 GG	rs361525 GA	rs361525 AA	rs1800629 GG	rs1800629 GA	rs1800629 AA	rs1799964 TT	rs1799964 TC	rs1799964 CC
Age	0.158*	−0.157	−0.170	0.011	−0.180	−0.185**	0.110**	−0.029	−0.130**
Gender	−0.024	0.002	0.058	0.060	0.045	0.040	0.032	−0.011	−0.034
Dyslipidemia	0.118	0.131	0.121	0.104	0.102	0.134*	0.082	0.076	0.074
BMI	−0.065	−0.115	0.111*	0.022	−0.065	−0.065	0.021	0.050	−0.112**
Hypertension	−0.030	−0.510	0.046	−0.053	−0.063	−0.063	−0.151**	0.072*	0.126**
HBAIC	−0.100	−0.161	0.610	−0.140**	0.103*	0.133**	0.030	0.079*	0.082*
Urea	−0.090	0.095	0.085	−0.069*	0.035	0.135**	−0.035	−0.011	0.073*
Creatinine	−0.107*	0.103*	0.018	−0.102**	−0.014	0.140**	−0.090**	0.047	0.069*
T. protein	0.007	0.062	−0.127*	0.050	0.008	0.009	0.033	0.012	−0.071*
Albumin	0.084	0.126	−0.090	0.001	−0.054	−0.050	0.036	−0.007	−0.046
Calcium	−0.013	−0.014	−0.003	0.170	0.120	0.126	0.013	−0.063	0.077*
Phosphorus	−0.038	0.051	−0.028	−0.007	0.004	0.004	−0.077*	0.037	0.065
Uric acid	0.108	0.106	0.014	−0.045	−0.004	−0.004	0.034	0.042	0.099*
Albuminuria	−0.113	−0.107	0.079*	−0.216**	0.040	0.042	−0.200**	0.133**	0.109**
24 h UP	−0.195*	−0.089	−0.086	−0.116*	−0.122**	0.152**	0.018	−0.045	0.041
eGFR	0.013	0.015	−0.062	0.202**	0.112**	−0.137**	0.015	−0.052	−0.053*
Duration	0.176**	−0.133*	−0.119*	0.160**	−0.202**	−0.280**	0.184**	−0.054	−0.207**

* Correlation is significant at 0.05 level.
** Correlation is significant at 0.01 level.

of developing nephropathy and faster progression to end-stage renal disease (ESRD) (Kitamura et al., 2002; Ryu and Kim, 2012; Buraczynska et al., 2007). In case of *IL-1β* gene, all three SNPs involved base transition from C ↔ T however one of the SNPs (rs1143634) was present in the coding region of gene in exon 5. In our study we found significant association between rs16944 and the risk of T2D and DN, whereas rs1143627 and rs1143634 showed insignificant association. Studies examining polymorphisms within the *IL-1* gene cluster showed strong association with ESRD independent of race (Tripathi et al., 2015). Patients homozygous for rs16944-T genotype were found to have higher risk of renal failure (Lee et al., 2004). The risk of developing DN has been shown to be significantly enhanced in rs16944-T allele carriers (Stefanidis et al., 2014). The association of individual genotypes with risk factors was further analyzed by correlation studies. The striking observation was the lower age of patients and shorter duration of diabetes demonstrating either early onset or rapid progression to nephropathy and ESRD. Age has been reported as an important effect modifier in nephropathy as studies show different prognostic implications in renal diseases with respect to age (Eriksen and Ingebretsen, 2006; O'Hare et al., 2007). In older patients, age-related co-morbidities tend to predict global outcomes like mortality whereas in younger patients specific renal outcomes can be predicted (Eriksen and Ingebretsen, 2006). The *TNF-α* rs361525 showed positive association individually but did not feature in the haplotype combinations or with risk factors owing to its relatively smaller frequency. The positive association obtained can be attributed to the proxy effects of *TNF-α* rs1800629. Combinations harboring *IL-6* rs1800797-G and *IL-1β* rs16944-T alleles showed increase risk of T2D and combinations harboring *TNF-α* rs1800629-A, *TNF-α* rs1799964-C and *IL-1β* rs16944-T alleles showed nearly 20-fold increased risk of DN. The haplotypes harboring risk alleles of individual genotypes showed additive effects indicating the increased risk of disease in proportion to mutational load. The increased risk can also be explained by gene-gene or SNP-SNP interactions that further augment the risk by influencing the signaling pathways leading to amplified cytokine expression.

Increased expression of cytokines has been noted in DN. Marked activation of key pathways influencing transcriptional regulation of cytokine production has been observed in kidneys from human nephropathy and mouse disease models (Berthier et al., 2009; Sanchez-Nino et al., 2009; Lee et al., 2013). DN cases exhibited increased transcript levels of both TNF-α and IL-6 as compared to T2D subjects. However IL-1β transcript levels were relatively increased in T2D cases.

Table 6
Correlation between individual genotypes of *IL-6* rs1800795, rs1800796 and rs1800797 SNPs with disease parameters.

Parameter	rs1800795 GG	rs1800795 GC	rs1800795 CC	rs1800796 AA	rs1800796 AG	rs1800796 GG	rs1800797 GG	rs1800797 GC	rs1800797 CC
Age	0.108**	−0.078**	−0.057*	−0.046	0.035	0.023	−0.068*	0.039	0.010
Gender	−0.022	−0.009	0.053	0.021	0.035	0.041	0.034	−0.045	0.016
Duration	0.079	−0.017	0.106	0.013	−0.038	0.045	−0.111**	0.094*	−0.007
BMI	0.057	−0.017	−0.067*	0.070*	−0.055	−0.034	0.019	0.085	−0.139**
Hypertension	0.034	−0.034	−0.003	−0.005	−0.023	0.057	0.161**	−0.067	−0.115**
HBAIC	0.031	0.000	−0.052	−0.048	0.019	0.068*	−0.043	0.035	0.008
Urea	−0.004	−0.021	0.040	−0.103**	0.053	0.105**	0.222**	−0.112**	−0.058
Creatinine	−0.006	−0.023	0.049	−0.071*	0.065	0.017	0.225**	−0.073*	−0.098**
T. protein	−0.061	0.053	0.015	0.099**	−0.024	−0.161**	−0.108**	0.038	0.045
Albumin	−0.051	0.071*	−0.031	0.142**	−0.037	−0.220**	−0.146**	0.055	0.054
Calcium	−0.113**	0.096**	0.033	0.027	0.063	−0.175**	−0.129**	0.060	0.038
Phosphorus	0.022	−0.014	−0.014	−0.084*	−0.025	0.221**	0.356**	−0.131**	−0.139**
Uric acid	0.135**	−0.136**	−0.006	−0.045	0.001	0.091**	0.155**	−0.039	−0.078*
Albuminuria	0.031	−0.027	−0.008	−0.029	−0.003	0.069	0.079**	0.059	−0.103**
24 h UP	−0.033	0.046	−0.019	−0.108	0.142**	−0.055	0.222**	−0.047	−0.131**
eGFR	0.071	−0.002	−0.120*	0.046	−0.026	−0.040	−0.219**	0.128**	0.043
Dyslipidemia	−0.120*	−0.077*	−0.106**	0.033	−0.061	0.041	0.086*	0.009	−0.127**

* Correlation is significant at 0.05 level.
** Correlation is significant at 0.01 level.

Table 7
Correlation between individual genotypes of *IL-1β* rs1143627, rs1143634 and rs16944 SNPs with disease parameters.

Parameter	rs1143627 CC	rs1143627 CT	rs1143627 TT	rs1143634 CC	rs1143634 CT	rs1143634 TT	rs16944 CC	rs16944 CT	rs16944 TT
Age	0.072	−0.030	−0.062	0.171	−0.177	0.007	0.085**	0.021	−0.085**
Gender	0.039	−0.011	−0.042	−0.018	0.033	−0.028	0.090	0.078	0.016
Duration	0.030	0.016	−0.066	0.130**	−0.084	−0.088	−0.079*	0.155**	−0.099*
BMI	0.017	−0.013	−0.006	0.024	−0.036	0.021	0.096	−0.058	−0.041
Hypertension	0.052	−0.042	−0.014	0.052	−0.066	0.023	−0.018	−0.065	0.083*
HBAIC	−0.024	0.015	0.013	−0.033	−0.004	0.069*	−0.055	0.080*	0.200*
Urea	−0.038	−0.002	0.058	0.017	−0.054	0.068	−0.041	−0.006	0.054
Creatinine	−0.042	−0.009	0.074	−0.013	−0.012	0.047	−0.038	−0.023	0.073*
T. protein	0.000	0.003	−0.004	−0.059	0.081*	−0.041	0.069	0.111**	−0.057*
Albumin	−0.004	−0.024	0.040	−0.001	0.038	−0.068*	−0.052	0.093**	−0.055
Calcium	0.118	−0.050	−0.098**	−0.008	0.054	−0.085*	−0.095	0.088*	−0.162**
Phosphorus	−0.076	0.045	0.046	0.000	−0.084*	0.154**	−0.003	−0.076*	0.098**
Uric acid	0.042	−0.044	0.003	0.054	−0.060	0.011	−0.044	0.004	0.047
Albuminuria	−0.003	0.013	−0.014	0.014	−0.029	0.028	−0.045	−0.003	0.056
24 h UP	−0.133	0.015	0.183	0.037	−0.054	0.030	−0.015	0.051	−0.044
eGFR	0.053	0.022	−0.115	−0.086	0.088	−0.001	0.022	0.074	−0.113*
Dyslipidemia	0.000	0.006	−0.009	0.031	−0.018	−0.023	0.010	0.010	−0.024

* Correlation is significant at 0.05 level.
** Correlation is significant at 0.01 level.

Table 8
Haplotype association risk in T2D and DN cases.

S. no	Haplotype	T2D		DN	
		OR (95% CI)	*P* value	OR (95% CI)	*P* value
1	GGTGAGCCC	1	–	1	–
2	GGTGACCCC	0.60 (0.31–0.81)	0.04	0.79 (0.40–0.96)	0.031
3	GGTCAGCCC	0.52 (0.25–1.27)	0.077	0.91 (0.30–2.77)	0.87
4	GGTGAGCTC	3.9 (1.12–6.06)	0.005	1.96 (0.89–4.32)	0.095
5	GGTGAGTCC	0.63 (0.15–1.72)	0.06	0.94 (0.38–2.32)	0.9
6	GGCGAGCTC	2.74 (1.55–5.54)	0.035	7.2 (3.04–11.48)	0.0041
7	GGTGACCTC	1.3 (1.1–3.5)	0.034	0.59 (0.26–1.29)	0.19
8	GGTGGGCCC	0.09 (0.02–1.35)	0.08	1.15 (0.49–2.71)	0.74
9	GACGAGCCC	2.96 (1.08–4.03)	0.015	10.1 (2.01–17.7)	0.001
10	GGTCAGCTC	3.1 (2.01–7.7)	0.01	0.72 (0.23–2.25)	0.58
11	GGTGAGTTT	4.25 (1.79–8.32)	0.04	6.28 (2.07–16.11)	0.012
12	GGTCGCTTC	3.98 (3.3–14.20)	0.0016	*	*
13	GACGACCTT	*	*	21.3 (15.1–28.33)	0.026

P-value is adjusted for confounding factors.
* Frequency very low for calculation.

The relative expression of TNF-α was enhanced in rs1800629-AA genotype. These results show that the promoter region SNP rs1800629 affects the transcription of *TNF-α* and is augmented by rs1799964. Studies investigating the function of promoter region polymorphisms of *TNF-α* gene show significant differences of TNF-α plasma level in DN patients carrying rs1800629-A and rs1799964-C alleles however data on expression studies is not available (Vázquez-Huerta et al., 2014; Singh et al., 2015; Qidwai and Khan, 2011). *TNF-α* signaling has recently received significant attention because activation of this pathway predicted progressive nephropathy in a large patient population of T2D subjects (Niewczas et al., 2012). We also observed increased expression of IL-6 in DN subjects. We did not observe significant changes in IL-6 expression across the genotypes of rs1800795 and rs1800796 but IL-6 expression was significantly increased in carriers of rs1800797-GG genotype. Earlier studies showed that subjects carrying rs1800795-G allele had higher plasma IL-6 levels (Terry et al., 2000; Hulkkonen et al., 2001; Vickers et al., 2002). However, recent studies have shown correlation between rs1800795-C allele with increased circulatory levels of IL-6 (Kiszel et al., 2007; Kamyshova et al., 2016). These seemingly conflicting results point to variability in *IL-6* regulation at transcriptional and translational levels across different cellular sources. The association of rs1800797-G allele and increased secretion of IL-6 capacity by PBMNCs as observed in several studies are in conformity with our results (Buraczynska et al., 2007; Kitamura et al., 2002). These studies have suggested that rs1800797-G allele is an aggravating factor in the progression of nephropathy. Unlike TNF-α and IL-6, the expression of IL-1β was higher in T2D cases. Unlike other cytokines, the localized pancreatic secretion of IL-1β is mediated by inflammasome activation that has been shown to affect islet function independently leading to the development of β-cell dysfunction in T2D (Masters et al., 2010; Dunmore and Brown, 2013; O'Neill et al., 2013). We observed higher levels of IL-1β expression for rs1143627-CC and rs16944-TT genotypes. Similar results were obtained by one study that showed higher transcription efficiency of rs1143627 owing to its location in a TATA box (Kimura et al., 2004). The same study also reported no significance in expression of IL-1β with respect to rs1143627 (Kimura et al., 2004). A higher transcript level of IL-1β was observed among T2D patients by a recent study but the association with rs16944 was not established (Patel et al., 2016). Comparative genotype-phenotype analysis showed modulation of transcript levels by promoter region *TNF-α* rs1800629, *TNF-α* rs1799964, *IL-6* rs1800797, *IL-1β* rs1143627, and *IL-1β* rs16944 SNPs. The expression results are concomitant to the SNP and haplotype association studies.

5. Limitations of study

The main limitation of the study is that the diagnosis of DN is highly subjective and is often based on exclusion of other causes, depending on the physician's judgment and experience. Whereas the diagnosis is made on the presence of albuminuria in a patient with a history of diabetes of at least 5 years, the gold standard for definitive diagnosis is a kidney biopsy. However the biopsy is not justified in absence of treatment options available beyond the current application of optimal control of diabetes, hypertension and dyslipidemia and lifestyle. The other limitation of our study is that we didn't examine relationship of

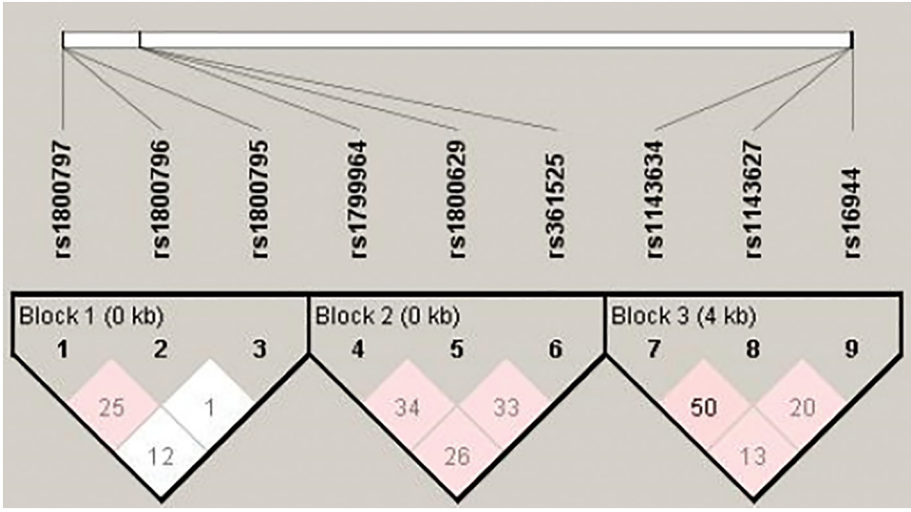

Fig. 1. Linkage Disequilibrium plot of *TNF-α*, *IL-6* and *IL-1β* SNPs.

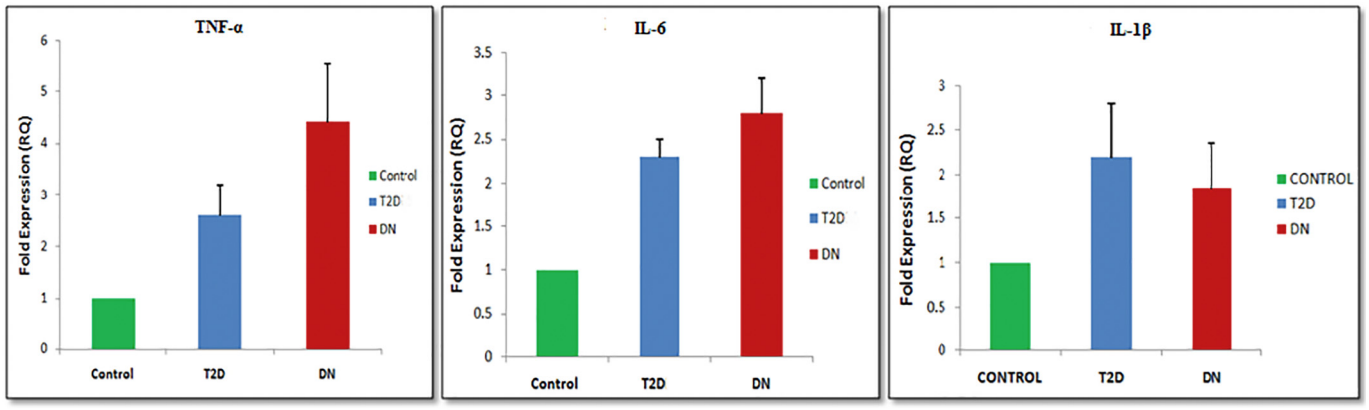

Fig. 2. Relative expression of TNF-α, IL-6 and IL-1β in study subjects.

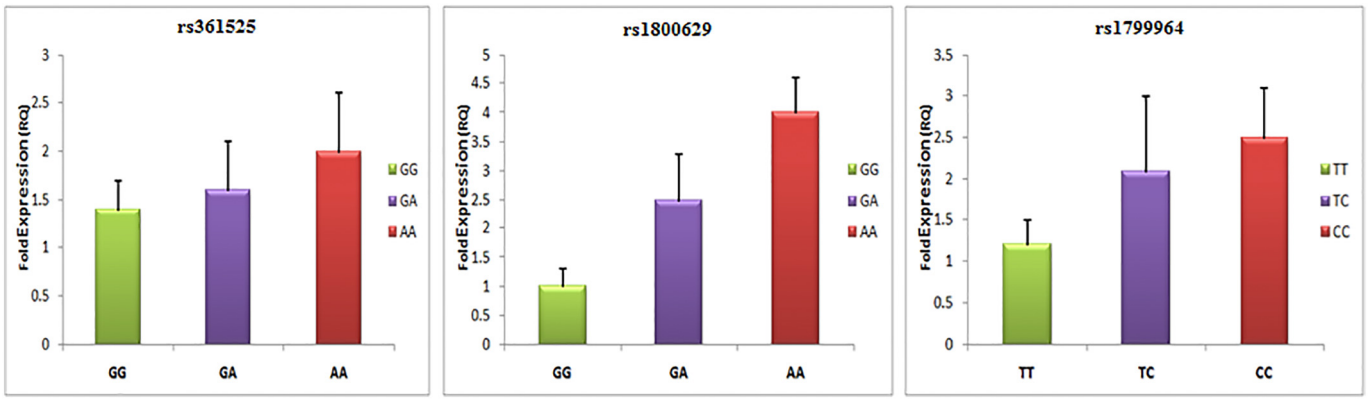

Fig. 3. Comparative expression of *TNF-α* (rs351525, rs1800629 and rs1799964) SNPs across genotypes.

nephropathy with TGF-β which many researchers consider as a key driver of DN.

6. Conclusion

DN is a disease with temporal and individual heterogeneity. This study elucidates that genetic changes in pro-inflammatory cytokines are associated with the pathogenesis and/or progression of DN. The amplified upshot of multiple genotypes influences the inflammatory response of cytokines by augmenting their transcript levels leading to exacerbation of nephropathy among diabetic subjects. The elementary inferences of this study provide an avenue for future perspectives and interventions involving individually and temporally targeted approaches for development of molecular diagnostics and effective therapeutics in DN.

Compliance with ethical standards

Informed consent was obtained from all participants included in the study. All procedures performed in this study were in accordance with

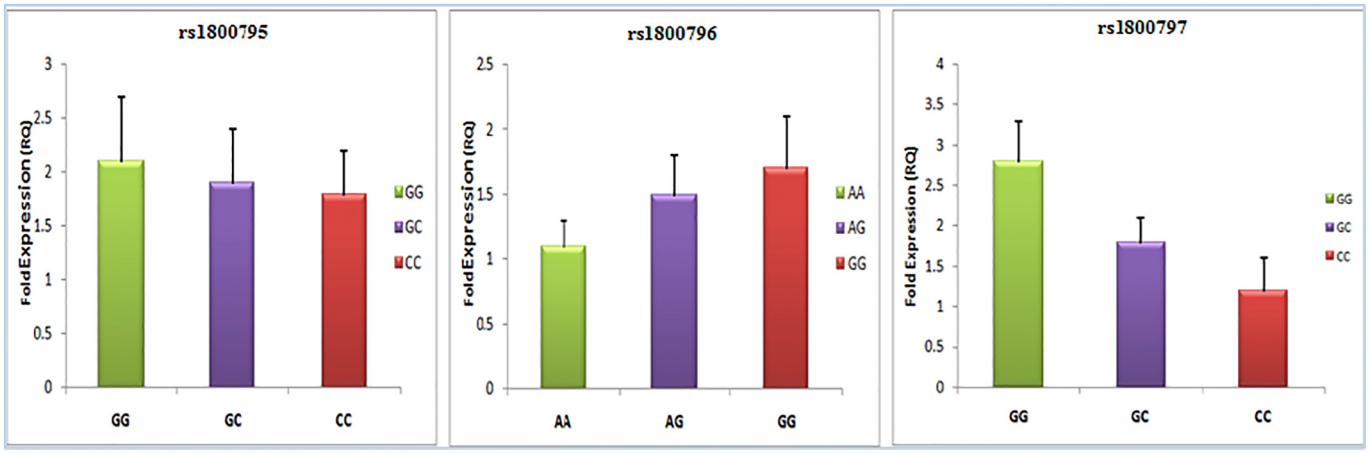

Fig. 4. Comparative expression of *IL-6* (rs1800795, rs1800796 and rs1800797) SNPs across genotypes.

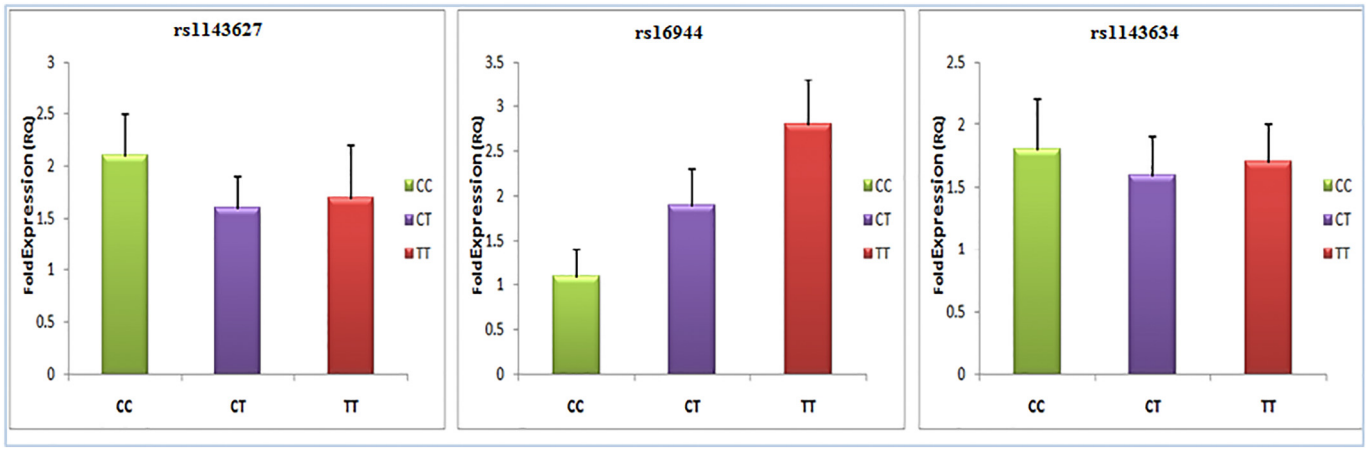

Fig. 5. Comparative expression of *IL-1β* (rs1143627, rs16944 and rs1143634) SNPs across genotypes.

the ethical standards of the institutional ethics committee (SKIMS-IEC, Protocol No. 48/2012). The study protocol conformed to the ethical guidelines of the 2013 Declaration of Helsinki developed by World Medical Association (WMA).

Conflict of interest

The authors declared that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2018.03.095.

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