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Research paper

Genetic association of *IL-6*, *TNF-α* and *SDF-1* polymorphisms with serum cytokine levels in diabetic foot ulcer

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ABSTRACT

The IL-6 −174G/C (*rs1800795*), TNF-α −308G/A (*rs1800629*) and −238 G/A (*rs361525*) and SDF-1 801 G/A (*rs1801157*) are well characterized SNPs which have previously been linked to various diabetic complications. However, the involvement of these SNPs in DFU remains poorly studied. In the present study we looked at the association of these SNPs with DFU (disease phenotype) and correlated it with the serum levels of cytokines (intermediate phenotype) along with other clinical risk factors of DFU (adiponectin, leptin and hsCRP). Genotyping was carried out in Normal glucose tolerance ((NGT) / Control = 106), T2DM without DFU (T2DM = 139), T2DM with neuropathy (DFU–DN = 191) and T2DM with PVD (DFU–PVD = 79) subjects by PCR–RFLP and the serum cytokine levels were determined by ELISA. *IL-6* −176 "C" allele conferred significant protection against T2DM but not against DFU. *TNF-α* −308 "A" allele (but not −238 SNP) conferred significant susceptibility towards both T2DM and DFU–DN. The *SDF-1* "A" allele conferred significant protection against both DM and DFU–DN but not against DFU–PVD. Further, these alleles were shown to influence the serum cytokine/chemokine levels under diabetic conditions. Thus SNPs in cytokine/chemokine genes serve as valuable biomarkers for DFU.

1. Introduction

Among the various complications associated with Type-2 Diabetes Mellitus (T2DM), Diabetic foot ulcer (DFU) represents a significant and often challenging clinical problem which is mainly due to either diabetic neuropathy (DN) and/or peripheral vascular disease (PVD) (Viswanathan and Rao, 2013). The etiology of DFU is multi-factorial and involves both genetic and environmental factors. A normal wound healing process progresses through a short inflammatory phase followed by proliferative phase and a remodeling phase which are required to provide sufficient wound strength and closure of wound at an appropriate time (Diegelmann and Evans, 2004). Chronic inflammation compounded with infections can lead to the formation of chronic wounds which can ultimately lead to amputation (Delbridge et al., 1985).

Compared to other diabetic complications like retinopathy and nephropathy, the genetics of DFU remains poorly studied. Single nucleotide polymorphisms (SNPs) in inflammatory genes serve as valuable candidates for DFU since the pro-inflammatory cytokines interleukin-6 (*IL-6*) and tumor necrosis factor-alpha (*TNF-α*) and the chemokine stromal cell-derived factor (*SDF-1*/*CXCL12*) have been shown to coordinate the three phases of wound healing (Kristiansen and Mandrup-Poulsen, 2005). The IL-6 −174G/C (*rs1800795*), TNF-α −308G/A (*rs1800629*) and −238 G/A (*rs361525*) and SDF-1 801 G/A (*rs1801157*) are well characterized SNPs which have previously been linked to various diabetic complications (Libra et al., 2006; Sikka et al., 2014; Djuric et al., 2010). However, the involvement of these SNPs in DFU remains poorly studied. Towards this end, we looked at the association of these SNPs with DFU (disease phenotype) and correlated it with the serum levels of these cytokines along with other serum biomarkers including adiponectin, leptin and high-sensitivity C-reactive protein (hsCRP) (intermediate phenotype).

Abbreviations: TNF-α, Tumor necrosis factor-alpha; IL-6, Interleukin-6; SDF-1, Stromal derived factor-1; hsCRP, High sensitive C-reactive protein; HOMA-IR, Homeostatic model assessment of insulin resistance; DFU, Diabetic foot ulcer; PVD, Perivascular disease; DN, Diabetic nephropathy; LDL, Low density lipoprotein; HDL, High density lipoprotein; VLDL, Very low density lipoprotein; TGL, Triglyceride lipid; FPG, Fasting plasma glucose; PPG, Post-prandial plasma glucose; SNP, Single nucleotide polymorphism.

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2. Materials and methods

2.1. Study population

A total of 515 subjects were recruited for this study and were divided into four study groups: Group-I normal glucose tolerance ((NGT) / Control; n = 106), Group-II Known T2DM without DFU (T2DM; n = 139), Group-III T2DM with neuropathic DFU (DFU–DN; n = 191) and Group-IV T2DM with PVD (DFU–PVD; n = 79). The patient and control groups were selected from the outpatient department of M.V. Hospital for Diabetes, Chennai, India. Diagnosis of diabetes was determined based on the WHO criteria. The study protocol was approved by the institutional ethics committee from Prof. M. Viswanathan Diabetes Research Centre (Ref No-MVH/IHEC/002/2011/17) and written informed consent was obtained from all the study participants. The study was conducted as per Helsinki's declaration.

2.2. Sample size calculation and power of study

A pilot study was first carried out using 50 subjects per group. Based on these preliminary results, with a confidence interval of 95%, an estimated p value < 0.05, and a power of 80%, the present sample size was derived. 10 subjects were added in each group to account for the high level of variation seen in the levels of serum biomarkers.

2.3. Inclusion and exclusion criteria

The inclusion criteria were adult subjects with T2DM and normal blood count and hemoglobin levels. The exclusion criteria were patients with type-1 diabetes and patients with a previous diagnosis of urolithiasis, liver cirrhosis, congestive heart failure, chronic lung diseases or viral hepatitis and subjects with gestational diabetes.

2.4. Anthropometric measurements and biochemical parameters

Anthropometric measurements including height and weight were obtained using standardized techniques. The body mass index (BMI) was calculated as the weight in kilograms divided by the square of height in meters. Fasting plasma glucose (FPG) (glucose oxidase–peroxidase method), serum cholesterol (cholesterol oxidase–peroxidase–amidopyrine method), serum triglycerides (glycerol phosphate oxidase–peroxidase–amidopyrine method), high density lipoprotein cholesterol (HDL-C) (direct method-polyethylene glycol-pretreated enzymes), and creatinine (Jaffe's method) were measured using a Hitachi-912 autoanalyzer (Hitachi, Mannheim, Germany). The intra- and inter assay coefficients of variation for the biochemical assays ranged between 4.5% and 6%. Glycated hemoglobin A1c (HbA1c) was estimated by high pressure liquid chromatography (Bio-Rad, Hercules, CA). The plasma concentrations of high-sensitivity C-reactive protein (hsCRP) were measured by high sensitive nephelometric assay. The intra- and the inter-assay coefficients of variation for hsCRP were 3% and 5.8%, respectively, and the detection limit was 0.15 mg/L.

2.5. Diagnosis of DN and PVD

DN was diagnosed using vibration perception threshold (VPT) which was measured using biothesiometer (Bio-medical Instruments Co., Newbury, OH, USA). Three readings were obtained on the first metatarsal at increasing voltage in both legs and the mean was taken. Patients with a VPT of >25 V were diagnosed as having neuropathy (Young et al., 1994) and those with 16–24 V were considered as having high risk for DN. PVD was diagnosed using ankle–brachial index (ABI) which is the ratio of the systolic blood pressure (SBP) measured at the ankle to that of brachial artery. Patients with ABI ≤ 0.9 were diagnosed with PVD. Severity of PVD was defined as per American College of Cardiology Foundation/American Heart Association Task Force (ACCF/AHA) 2011 Guidelines [ABI ≤ 0.4 — Severe, 0.41–0.7 — Moderate, 0.71–0.9 — Mild, 0.91–1.4 — Normal and >1.4 — non-compressible arteries] (Rooke et al., 2011).

2.6. Genotyping

Genomic DNA was extracted from peripheral blood using the standard phenol-chloroform extraction method (Libra et al., 2006). The SNPs were detected by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) as previously described (Libra et al., 2006). The primer sequences, amplicon sizes and the restriction enzymes used are given in Table 1.

2.7. Estimation of serum cytokines and biomarkers

The levels of IL-6, TNF-α, SDF-1, adiponectin and leptin in the serum were measured using ELISA following the manufacturer's instruction. The lower detection limits were: IL-6-1.9 pg/mL, TNF-α — 1.5 pg/mL, SDF-1 — 7.8 pg/mL, adiponectin — 0.01 pg/mL and leptin — 1.9 pg/mL. The intra- and inter-assay coefficients of variation for the assays were less than 5%. Fasting plasma samples were used for the estimation of insulin levels by chemiluminescence method (Roche Elecsys 2010, USA). Insulin resistance was assessed by the HOMA method (Matthews et al., 1985). Normal cut off value for insulin resistance was derived from normoglycemic control subjects. Subjects having HOMA IR > 2.4 were considered to have insulin resistance (Viswanathan et al., 2010).

2.8. Statistical analysis

Student *t*-test was used to compare groups for continuous variables, whereas $\chi 2$ test or Fisher exact test (as appropriate) was used to compare proportions. The Mann–Whitney *U* test was used in case of non-normally distributed parameters to compare medians. Spearman's correlation analysis was carried out to determine the association of serum cytokine levels with clinical parameters. Multivariate logistic regression analysis was used to determine the association of serum cytokines with T2DM–DFU. Kruskal–Wallis test was used for multiple parameters that did not show normal distribution. Multiple comparisons were corrected using the Holm's correction. All the analysis was done using SPSS statistical package (Version 20.0; SPSS, Chicago, IL) and p value less than 0.05 was considered significant.

3. Results

3.1. Clinical and biochemical characteristics of the study groups

Table 2 shows the clinical characteristics and serum biomarkers of the study subjects. Compared to NGT, the DM subjects (T2DM, DFU–DN and DFU–PVD) were significantly older and had higher BMI, fasting plasma glucose (FPG), postprandial plasma glucose (PPG), HbA1c, systolic (SBP) and diastolic blood pressures (DBP), triglycerides (TGL), urea, creatinine and HOMA-IR. The total serum cholesterol, HDL and LDL values were significantly lower in DFU–DN and DFU–PVD groups compared to NGT and T2DM subjects, since most of them were on statin therapy. With respect to diabetic complications, 16.2% of DFU–DN and 22.7% of DFU–PVD subjects had diabetic nephropathy (D.Nep). 12.6% of DFU–DN and DFU–PVD subjects had diabetic retinopathy (DR). 7.3% of DFU–DN and 11.3% of DFU–PVD subjects had both the complications (D.Nep + DR). With respect to treatment regimen, nearly 67% in DFU–DN and 85% in DFU–PVD were on a combination therapy for diabetes. The serum levels of IL-6 were significantly elevated in T2DM and DFU–DN groups in comparison with NGT group but not in the DFU–PVD group. The serum levels of TNF-α were significantly elevated in DFU–PVD groups (but not in the T2DM & DFU–DN group). The serum levels of SDF-1 were significantly elevated in DFU–DN groups while most subjects with DFU–PVD had undetectable levels. Leptin levels

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Table 1
Primer sequences, amplicon sizes and the restriction enzymes used in the study.

S. no.	Gene symbol	SNP position	Reference SNP number	Primer sequences	PCR product size (bp)	Restriction enzyme	Expected band patterns (bps)
1.	*IL-6*	−174G/C	*rs1800795*	F: 5′-TGACTTCAGCTTTACTCTTTGT-3′ R: 5′-CTGATTGGAAACCTTATTAGG-3′	198	Sty I	198, 140 & 58
2.	*TNF-α*	−308G/A	*rs1800629*	F: 5′-AGGCAATAGGTTTTGAGGGCC ATG-3′ R: 5′-ACACACAAGCATCAAGGATAC-3′	143	Nco I	143, 123 & 20
3.	*TNF-α*	−238G/A	*rs361525*	F: 5′-AAACAGACCACAGACCTGGTC-3′ R: 5′-CTCACACTCCCCATC CTCCCGGATC-3′	152	MspI	152, 132 & 20
4.	*SDF-1*	801G/A	*rs1801157*	F: 5′-CAG TCA ACC TGG GCA AAG CC-3′ R: 5′-AGC TTT GGT CCT GAG AGT CC-3′	302	MspI	302, 202 & 100

were not significantly different between the groups. The levels of adiponectin were significantly lower in the diabetic groups (T2DM, DFU–DN, and DFU–PVD) compared to the NGT group. The levels of hsCRP were significantly elevated in diabetic subjects compared to NGT.

3.2. Genetic association of IL-6, TNF-α and SDF-1 SNPs with disease phenotype-T2DM, DFU–DN and DFU–PVD

The genotype and allele frequencies of −174G/C (*IL-6*), −308G/A and −238G/A (*TNF-α*) and 801G/A (*SDF-1*) SNPs in the study groups were shown in Tables 3 and 4. The genotypic distribution of these SNPs was found to be in Hardy–Weinberg equilibrium. The frequency of GC and CC genotypes and the "C" allele of *IL-6* −174G/C SNP was found to be significantly lower in subjects with diabetes compared to NGT ($p < 0.001$). Multiple logistic regression analysis showed that both GC and CC genotypes conferred significant protection against diabetes when compared to the GG genotype, after adjusting for age and gender. With respect to DFU, none of the genotypes conferred any significant protection after adjusting for confounding factors. Overall the "C" allele showed significant protection against diabetes but not against DFU. With respect to *TNF-α* −308G/A SNP, the frequency of GA and AA genotypes was found to be significantly higher in the T2DM compared to the NGT group ($p < 0.05$). Multiple logistic regression analysis showed that both GA and AA genotypes were significant risk factors for both diabetes and DN. However, no such association was evident with respect to DFU–PVD. Overall the "A" allele was found to be a significant risk factor for the development of diabetes as well as DFU–DN. With respect to *TNF-α* −238G/A SNP, no significant association was seen either with diabetes or with DFU. With respect to SDF-1 801 SNP, the frequency of GA genotype was found to be significantly higher in T2DM and DFU–DN compared to the NGT group ($p < 0.05$). However the frequency of AA genotype was found to be significantly lower in T2DM, DFU–DN and DFU–PVD compared to the NGT group ($p < 0.05$). Multiple logistic regression analysis showed both GA and AA genotypes

Table 2
Clinical characteristics and serum biomarkers of the study subjects.

Clinical parameters	NGT (n = 106)	T2DM (n = 139)	DFU–DN (n = 191)	DFU–PVD (n = 79)
Gender (M/F)	56/50	80/59	125/66	56/23
Age (years)	39.7 ± 8.4	49.0 ± 9.9***	58.6 ± 8.5***	59.5 ± 8.5***
Body mass index (kg/m^2)	22.6 ± 2.2	27.4 ± 4.4**	26.8 ± 4.7*	26.6 ± 4.4*
Systolic BP (mm Hg)	114.2 ± 13.5	126.8 ± 17.6***	129.1 ± 15.9*	129.9 ± 17.3*
Diastolic BP (mm Hg)	76.4 ± 8.7	81.2 ± 9.0***	79.9 ± 7.2	80.0 ± 9.0
Fasting plasma glucose (mg/dL)	96.2 ± 8.5	164.5 ± 71.9***	177.1 ± 77.7	174.8 ± 72.2*
Postprandial plasma glucose (mg/dL)	109.0 ± 18.6	257.6 ± 99.0***	258.9 ± 82.2	247.3 ± 75.9
Glycated hemoglobin (%)	5.2 ± 0.2	8.6 ± 5.4***	9.7 ± 2.4***	9.5 ± 2.4***
Total serum cholesterol (mg/dL)	175.0 ± 33.6	185.2 ± 43.0*	156.5 ± 51.4***	157.8 ± 51.5***
Serum triglycerides (mg/dL)	109.1 ± 45.1	152.4 ± 76.7***	150.8 ± 117.2	158.0 ± 113.4
HDL-cholesterol (mg/dL)	44.4 ± 8.8	43.9 ± 9.0	33.5 ± 9.9***	33.7 ± 10.9***
LDL-cholesterol (mg/dL)	99.2 ± 26.6	115.2 ± 36.3***	93.6 ± 38.9***	92.0 ± 35.1***
VLDL-cholesterol (mg/dL)	30.8 ± 13.9	26.2 ± 13.7**	30.6 ± 22.6	31.3 ± 20.8**
Urea (mg/dL)	20.9 ± 6.4	23.9 ± 12.4**	38.2 ± 19.1***	37.2 ± 19.2***
Creatinine (mg/dL)	0.8 ± 0.1	0.9 ± 0.1	1.7 ± 1.3***	1.3 ± 0.5***
HOMA-IR	1.4 (0.4–3.2)	5.2 (1.3–31.8)***	4.8 (0.5–54.4)***	7.2 (1.2–229.5)***
Other complications (%)				
Diabetic nephropathy	nil	nil	31 (16.2)	18 (22.7)
Diabetic retinopathy	nil	nil	24 (12.6)	10 (12.6)
Nephropathy & retinopathy	nil	nil	14 (7.3)	9 (11.3)
Medication n (%)				
Oral agents	nil	74 (53.2)	2 (1.0)	nil
Insulin	nil	19 (13.6)	61 (31.9)	12 (15.1)
Oral agents + insulin	nil	46 (33.0)	128 (67.0)	67 (84.8)
Serum biomarkers [median (range)]				
IL-6 (pg/mL)	42.4 (1.9–95.3)	46.2 (7.0–584.3)*	44.6 (10.6–618.7)*	37.8 (1.9–168.9)
TNF-α (pg/mL)	146.2 (1.5–939.5)	140.1 (14.6–794.2)	143.6 (7.5–2802)	359.3 (138.4–2556)*
SDF-1 (pg/mL)	1558 (7.8–5435)	2683 (7.8–8980)	3122 (7.8–8956)***	Undetectable
Leptin (pg/mL)	3.0 (1.0–166.5)	3.1 (1.0–185.3)	3.1 (1.0–111.0)	3.9 (1.0–50.0)
Adiponectin (ng/mL)	536.0 (0.1–1787)	528.6 (6.2–1255)*	524.0 (63.3–1641)*	453.5 (164.9–1078)*
HS-CRP (mg/L)	0.9 (0.1–13.4)	4.2 (0.2–43.2)***	26.4 (0.4–50.6)***	19.4 (1.8–55.2)***

All data are reported as mean ± S.D. unless specified.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

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Table 3
Genotypic frequencies of IL-6 (−174 G/C), TNF-α (−308G/A) &(−238 G/A) and SDF-1 (801 G/A) SNPs.

	NGT (n = 106)	T2DM (n = 139)	OR (95% CI)[a]	DFU–DN (n = 191)	OR (95% CI)[a]	DFU–PVD (n = 79)	OR (95% CI)[a]
IL-6 −174G/C genotype							
GG	50	92	Ref	101	Ref	43	Ref
	47.20%	66.20%		52.90%		54.40%	
GC	44	46	**0.4****	85	0.9	34	1.01
	41.50%	33.10%	**(0.25–0.75)**	44.50%	(0.44–2.14)	43.10%	(0.35–2.87)
CC	12	1	**0.03****	5	0.24	2	0.74
	11.30%	0.70%	**(0.005–0.31)**	2.60%	(0.04–1.30)	2.50%	(0.06–8.63)
TNF −308G/A							
GG	77	78	Ref	77	Ref	63	Ref
	72.6%	56.1%		40.3%		79.8%	
GA	14	36	**2.7***	68	**5.9*****	13	1.44
	13.2%	25.9%	(1.25–5.86)	35.6%	(2.19–16.0)	16.4%	(0.39–5.35)
AA	15	25	**2.5***	46	**8.46****	3	1.38
	14.2%	18.0%	(1.05–5.96)	24.1%	(2.39–29.93)	3.8%	(0.13–13.7)
TNF −238G/A							
GG	81	106	Ref	140	Ref	59	Ref
	76.4%	76.2%		73.3%		74.7%	
GA	23	29	1.45	48	1.6	19	2.59
	21.7%	20.9%	(0.70–2.99)	25.1%	(0.63–4.0)	24.0%	(0.78–8.57)
AA	2	4	1.37	3	1.7	1	1.34
	1.9%	2.9%	(0.19–9.57)	1.6%	(0.10–26.8)	1.3%	(0.03–59.3)
SDF-1 801G/A							
GG	10	43	Ref	50	Ref	40	Ref
	9.4%	30.9%		26.2%		50.6%	
GA	86	87	**0.3*****	135	0.39	36	0.32
	81.2%	62.6%	(0.13–0.71)	70.7%	(0.13–1.17)	45.6%	(0.10–1.0)
AA	10	9	**0.23****	6	**0.11****	3	0.25
	9.4%	6.5%	(0.07–0.82)	3.1%	(0.02–0.63)	3.8%	(0.02–2.26)

Figures in bold were significant ($P < 0.05$).
[a] Odds ratio (OR) adjusted for confounding factor (age & gender).
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

to confer significant protection against T2DM while the AA genotype alone was found to confer protection against DFU–DN.

3.3. Genetic association of IL-6, TNF-α and SDF-1 SNPs with intermediate phenotype-serum cytokine levels and other biomarkers

Next, we estimated the clinical parameters and serum biomarkers within the three genotypes of IL-6, TNF-α and SDF-1 under baseline (NGT subjects) and diabetic conditions (DM subjects — T2DM, DFU–DN and DFU–PVD). In the *TNF-α* −238G/A SNP, there was no significant associations between these SNPs and disease phenotype (data not shown). In NGT subjects, the CC genotype of *IL-6* had significantly higher levels of serum VLDL and adiponectin compared to other genotypes (S. Table 1). For *TNF-α* −308G/A, the AA genotype had significantly higher levels of FBS and GG genotype had significantly lower levels of serum leptin compared (S. Table 2). For *SDF-1* 801G/A, the GG genotype had significantly lower levels of VLDL compared (S. Table 3). In DM subjects, the CC genotype of *IL-6* had significantly higher levels of serum triglycerides, urea and creatinine while the GG genotype had significantly higher levels of serum IL-6 (S. Table 4). For *TNF-α* −308G/A, the GA genotype had significantly lower levels of serum TNF-α and higher levels of serum adiponectin, while the AA genotype had significantly lower levels of FBS and PPBS (S. Table 5). For *SDF-1* 801G/A, the AA genotype had significantly lower levels of HbA1c and increased levels of HDL-cholesterol (S. Table 6).

Table 4
Allelic frequencies of IL-6 (−174 G/C), TNF-α (−308G/A and −238 G/A) and SDF-1 (801 G/A) SNPs in DFU.

	NGT (n = 106)	T2DM (n = 139)	DFU–DN (n = 191)	DFU–PVD
IL-6 −174G/C				
G	144 (67.9%)	230 (82.7%)	287 (75.1%)	120 (75.9%)
C	68 (32.10%)	48 (17.30%)	95 (24.90%)	38 (24.10%)
TNF −308G/A				
G	168 (79.2%)	192 (69.1%)	222 (58.1%)	139 (88.0%)
A	44 (20.8%)	86 (30.9%)	160 (41.9%)	19 (12.0%)
TNF 238G/A				
G	185 (87.3%)	241 (86.7%)	328 (85.9%)	137 (86.7%)
A	27 (12.7%)	37 (13.3%)	54 (14.1%)	21 (13.3%)
SDF-1 801G/A				
G	106 (50.0%)	173 (62.2%)	235 (61.5%)	116 (73.4%)
A	106 (50.0%)	105 (37.8%)	147 (38.5%)	42 (26.6%)

MAF minor allele frequency.

4. Discussion

Foot ulcer is a multi-factorial disorder wherein both genetic and environmental factors play an essential role. However, the pathophysiology of the disease is still not fully understood. In a physiological wound healing process, immune cells clear the bacteria, keratinocytes perform re-epithelialization, fibroblasts restore dermal matrix and endothelial cells ensure angiogenesis. These events are orchestrated perfectly by the cytokines and chemokines. In diabetic foot ulcers, immune cells over-express pro-inflammatory cytokines (*IL-1*, *IL-6* and *TNF-α*) and chemokines (*CCL2*, *SDF-1*/*CXCL12*, etc) and are not able to clear infections thereby extending the inflammatory phase. SNPs in the promoter/intronic regions of cytokine/chemokine genes alter transcript levels and are of functional significance. Towards this end, we investigated the role played by *IL-6*, *TNF-α* and *SDF-1* SNPs in DFU and correlated it with serum levels of IL-6, TNF-α and SDF-1 and other inflammatory/diabetogenic markers. The current study provides three novel findings: 1) *IL-6*-174 CC and GC genotypes conferred significant protection against DM but not against DFU, 2) *TNF-α*-308 AA and GA genotypes

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conferred significant susceptibility towards DM and DFU–DN and 3) *SDF-1* 801 AA and GA genotypes conferred significant protection against both DM and DFU–DN.

In the *IL-6* −174 SNP, we observed that the C allele under both homo (CC) and heterozygotic (GC) conditions conferred significant protection against DM but not against DFU. This SNP has previously been reported to be linked with obesity, insulin resistance and T2DM with conflicting results (Kubaszek et al., 2003; Wernstedt et al., 2004; Hamid et al., 2005; Bennermo et al., 2004). Our results are in agreement with Vozarova et al., who reported genetic association of the GG genotype with T2DM in the high risk Pima Indian population (Vozarova et al., 2003). However Chang et al., have reported the absence of association between this SNP and T2DM in the Taiwanese population (Chang et al., 2004). In their study, none of the participants carried a "C" allele (Chang et al., 2004). It is important to note that the "C" allele is a minor allele and might not be present in certain ethnic populations. No association was found between any of the genotypes and serum IL-6 levels in NGT subjects which is in accordance with the report of Huang et al. (2013). However, in the presence of diabetes, significantly elevated levels of serum IL-6 were noted in the GG genotype which is also in accordance with that of Bennermo et al. report (Bennermo et al., 2004). Libra et al., had shown an association between the GG genotype and increased susceptibility to peripheral arterial disease (PAD) which was associated with elevated levels of IL-6, fibrinogen and hsCRP in diabetic subjects (Libra et al., 2006).

The *TNF-α* −308G/A SNP was strongly associated with both diabetes and DN, but not with PVD. The "A" allele was found to confer significant risk to both diabetes and DN in both homozygous and heterozygous conditions. Our results are in agreement with previous reports which showed a strong association between this SNP and foot ulcer in non-diabetic subjects (Nagy et al., 2007; Wallace et al., 2006). However Sikka et al., had reported absence of association of this SNP with microvascular complication in north west Indian population (Sikka et al., 2014). Our results are in contrast with a recent large scale association analysis of TNF/LTA polymorphisms with T2DM wherein no association was seen between this SNP (along with 11 SNPs in this region) with T2DM ((Boraska et al., 2010). The apparent disparity between our results and the other reports mentioned before could be due to ethnic differences which have been well documented among several population based genetic studies. With respect to the effect of this SNP on serum cytokine levels, in diabetic subjects, the GA genotype had the lowest levels of TNF-α. Altarescu et al., showed a significant correlation between serum TNF-α levels and *TNF-α* AA genotypes in patients with type-I Gaucher's disease (Altarescu et al., 2005). At baseline GG genotype had significantly lower levels of serum leptin and in the presence of diabetes the AA genotype had significantly reduced levels of both fasting and post-prandial plasma glucose. It seems that apart from serum TNF-α, the −308 SNP seems to affect blood glucose and leptin levels. Our results on leptin levels are in agreement with Canhao et al. report (Canhao et al., 2008). Compared to −308 SNP, there have been relatively fewer studies available on the −238G/A SNP with diabetes and/or foot ulcer. Recently, Feng et al., published a meta-analysis in which they reported a lack of association between this SNP and T2DM (Feng et al., 2009). However in a Chinese population, this SNP was shown to be strongly associated with insulin resistance (Hu et al., 2009). With respect to the *SDF-1* 801G/A SNP, the "A" allele conferred significant protection to both DM and DFU–DN in homozygous condition. Djuric et al., had reported that the "A" allele was a susceptibility allele to proliferative diabetic retinopathy (Djuric et al., 2010).

5. Conclusion

From the present study and other available data, it appears that the *IL-6* −174 "C" allele and *SDF-1* 801 "A" allele seems to confer some amount of protection against diabetes but not against DFU, while the *TNF-α* −308 "A" allele (but not −238 SNP) seems to confer genetic susceptibility towards both conditions. At baseline, none of these alleles seem to control serum cytokine levels. However, in the presence of diabetes, both IL-6 −174G/C and TNF-α −308 G/A SNPs seem to control their respective serum cytokine levels. Interestingly these SNPs apart from having an effect on inflammation also seem to influence various risk factors of DFU including glycemic control, serum lipid profile, kidney function, vascular health and obesity. In these aspects, the present study gains importance since the effect of these SNPs on intermediate phenotype (serum levels) under the given disease phenotype (DFU) along with major risk factors of DFU was evaluated in a large cohort of ethnically high-risk individuals. However, the major limitation of our study is its cross-sectional nature, which means that no cause and effect relationship can be drawn from the present study. Overall the *IL-6*, *TNF-α* and *SDF-1* SNPs apart from controlling serum cytokine levels affected various risk factors of DFU.

Conflict of interest

None declared.

Author contributions

VA, RR and VV conceived and designed the experiment. UD performed the experiment and drafted the manuscript. EK helped in the collection of the samples. UD and VA analyzed the data. VA, RR and VV contributed to the discussion and reviewed the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2015.03.063.

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