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Oxidative stress pathway genes and chronic renal insufficiency in Asian Indians with Type 2 diabetes☆

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Abstract

Background: There are significant regional variations in prevalence of diabetes and diabetic chronic renal insufficiency (CRI) in India. Oxidative stress plays an important role in the development of diabetic complications. To determine the importance of the polymorphisms in the genes involved in maintenance of cellular redox balance, we performed a case control study in subjects from south and north India. **Methods:** Successive cases presenting to the study centers with Type 2 diabetes of >2 years duration and moderate CRI (n=194, south India 104, north India 90) diagnosed by serum creatinine ≥2 mg/dl after exclusion of nondiabetic causes of CRI were compared with diabetes subjects with no evidence of renal disease (n=224, south India 149, north India 75). Twenty-six polymorphisms from 13 genes from the oxidative stress pathway were analyzed using polymerase chain reaction–restriction fragment length polymorphism. Genes included were superoxide dismutases (SOD1, 2, 3), uncoupling proteins (UCP1, 2), endothelial nitric oxide synthase (NOS3), glutathione-*S*-transferases (GST) (M1, T1, P1), vascular endothelial growth factor (VEGF), paraoxonase (PON) 1 and 2, and nicotinamide adenine dinucleotide phosphate reduced, oxidase $p22^{phox}$. Genes were tested for their association with CRI using χ^2 test. **Results:** In south Indian (SI) subjects there was significant allelic and genotypic association of the wild-type allele in SOD2 (Ala9Val; P=.002 and P=.013, respectively), UCP1 (−112 T>G, P=.012 and P=.009; Ala64Thr, P=.015 and P=.004), NOS3 (Glu298Asp, P=.002 and P=.009) and GSTP1 (Ile105Val, P=.003 and P=.004) genes with development of CRI. None of these observations were replicated in the north Indian (NI) subjects. A genotypic but not allelic association was observed for two markers, VEGF (−460 T>C) and PON1 (Arg192Gly) among NI diabetic CRI subjects. **Conclusion:** The nonreplication of association suggests differential genetic susceptibility of the two populations to diabetic chronic renal insufficiency. In the SI diabetic subjects, oxidative stress pathway genes might be an important predictor for the development of diabetic complications. Further, the association of wild-type alleles may suggest that they confer greater survival ability to comorbid complications and may be nephroprotective.

Keywords: Oxidative stress; Candidate genes; Single nucleotide polymorphisms; Association; Chronic renal insufficiency; India

1. Introduction

Diabetic nephropathy (DN) with diabetic chronic renal insufficiency (CRI) is a leading cause of end stage renal disease worldwide (ADA, 2002). The genesis of DN involves myriad of factors including older age, male sex, hyperglycaemia, and hyperlipidaemia. Ethnicity is the other major risk factor, with African Americans, Asians,

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and native Americans being more prone to develop DN than Caucasians (Gross et al., 2005; van Dijk & Berl, 2004; Young, Maynard, & Boyko, 2003). In south Asia, there are significant differences in prevalence of diabetes with high prevalence in southern and western Indian populations, as compared to northern and eastern Indians (Gupta & Misra, 2007). The regional variation in prevalence of diabetes and variable propensity for renal disease along with reports of familial clustering of nephropathy, suggest a possible genetic basis (Viswanatham, Snehalatha, Mathai, Jayaraman, & Ramachandran, 1998). Clinical as well as experimental evidences suggest that four pathways are involved in the development of diabetic microvascular complications (for reviews, see Brownlee, 2001; King & Loeken, 2004; Leon & Raij, 2005; Sheetz & King, 2002). These pathways activated by chronic hyperglycaemia include the polyol, advanced glycation end products, protein kinase C, and hexoseamine pathways. Inability of the inhibitors of these single pathways to block all the downstream events suggested that all the pathways could be possibly linked to a common upstream event and overproduction of superoxide by electron transport chain was proposed to be this single unifying mechanism (Brownlee, 2001). The excess of superoxide produced activates the four pathways through DNA damage-mediated activation of polyadenosine diphosphate (ADP) ribose polymerase-1 (PARP-1); PARP-1 transfers polyADP-ribose units to several nuclear proteins including glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH is a multi-functional enzyme, which, besides functioning as a glycolytic enzyme in the cytoplasm, plays an important role in DNA repair in the nucleus. The transfer of polyADP-ribose units prevents translocation of GAPDH from the nucleus to cytoplasm. This prevents its function as a glycolytic enzyme, leading to the accumulation of the upstream intermediate compounds of the glycolytic pathway. These intermediates such as fructose-6-phosphate, dihydroxyacetone phosphate along with glucose are substrates/activators of the above-mentioned four pathways (Brownlee, 2005). The activation of these pathways, in turn, leads to the secondary production of reactive oxygen species (ROS).

Several genes that detoxify or reduce the production of superoxide and other free radicals have been identified. Superoxide dismutase (SOD) is a family of enzymes involved in the conversion of superoxide to H_2O_2. Uncoupling proteins (UCP) cause a leakage in the proton gradient. The importance of these enzymes in diabetic complications is underscored by the observations made under hyperglycaemic conditions. During such condition, increased flux of glucose through the glycolytic and tricarboxylic acid (TCA) cycles leads to increase in the production of electron donors (Nicotinamide Adenine Dinucleotide, reduced [NADH] and Flavin Adenine Dinucleotide, reduced [$FADH_2$]). This leads to increased passage of electrons through the electron transport chain, causing an increase in the potential gradient across the inner mitochondrial membrane. When the potential gradient increases above a threshold value, the production of superoxide, primarily at the ubiquinone–semiubiquinone step, is increased. This effect of hyperglycaemia is abolished by overexpression of mitochondrial SOD (SOD2 or MnSOD), which converts harmful superoxide radical to H_2O_2, or overexpression of UCP-1, which causes a basal leak in the proton gradient reducing superoxide production (Nishikawa et al., 2000). Despite the demonstrated implication of ROS in the etiology of microvascular complications (Brownlee, 2001, 2005), a comprehensive analysis of the genetic susceptibility conferred by these groups of genes has not been carried out. A few reports on the associations of SOD2, glutathione-*S*-transferase (GST)-T1 (GSTT1) and GSTMI, nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase $p22^{phox}$, paraoxonase (PON) 2, and endothelial nitric oxide synthase (NOS3) among type 2 diabetes patients with diabetic nephropathy from a Japanese population are available (Doney, Lee, Leese, Morris, & Palmer, 2005; Fujita, Narita, Meguro, Ishii et al., 2000; Fujita, Narita, Meguro, Shimotomai et al., 2000; Ksiazek, Wojewoda, Muc, & Buraczynska, 2003; Matsunaga-Irie et al., 2004; Neugebauer, Baba, & Watanabe, 2000; Nomiyama et al., 2003; Pinizzotto et al., 2001; Santos et al., 2005; Shimizu, Onuma, Kawamori, Makita, & Tomino, 2002). However, no such reports on these genes from the Indian population are available.

Nearly 30% of the cases of end-stage renal disease in India are due to diabetes, and this group is more likely to develop this complication than the Caucasians (Earle, Porter, Ostberg, & Yudkin, 2001; Viswanatham et al., 1998; Young et al., 2003). India has the unfortunate distinction of being the country with the maximum number of diabetics world-wide; 31.7 million people were estimated to be affected with diabetes in the year 2000, with the projection for the year 2030 being 79.4 million people (Wild, Roglic, Green, Sicree, & King, 2004). With such drastic increase, the risk of these patients developing diabetes specific complications will also rise enormously. Therefore, it is essential to identify the risk factors for the development and progression for such complications. Considering the central role of superoxide radical in the genesis of microvascular complications mentioned above, we analysed, in a pilot study, the role of 26 polymorphisms from 13 major genes involved in the maintenance of cellular redox balance and important physiological functions in the development of CRI in two geographically distinct populations, one from south India and another from north India.

2. Methods

2.1. Subjects

In this case control association study, consecutive south Indian (SI) samples were recruited from M.S. Ramiah Medical College, Bangalore, and the north Indian (NI) samples from All India Institute of Medical Sciences, New

Table 1
List of primers, annealing temperature and allelic profiles of the polymorphisms analysed

Gene	Marker details	Primer sequence	Annealing temperature/ time, product size	Enzyme used & expected allele sizes (bp)
SOD1	rs2070424 A>G	F-5′-AGTACTGTCAACCACTAGCA-3′ R-5′-CCAGTGTGCGGCCAATGATG-3′	64°C/30 s, 570 bp	MspI *A*=570 *G*=369+201
SOD2	Ala9Val rs1799725 C>T	F-5′-CCAGCAGGCAGCTGGCACCG-3′ R-5′-TCCAGGGCGCCGTAGTCGTAGG-3′ (Chistyakov et al., 2001)	64°C/30 s, 91 bp	Age1 *C*=91 *T*=74+17
SOD3	rs699473 T>C	F-5′-CTGCTTCAGGCGTACACAGA-3′ R-5′-AATTGCTTCGCCTCCACCTC-3′	58°C/30 s, 396 bp	NlaIII *C*=243+153 *T*=243+126+27
	Ala58Thr rs2536512 A>G	F-5′-ACTGTGTTCCTGCCTGCT-3′ R-5′-AAGCTGCCGGAAGAGGAC-3′	62°C/30 s, 250 bp	BstUI *A*=209+41 *G*=161+48+41
UCP1	−3826 C>T	F-5′-AGTATGAGCAAGGGCAACTGA-3′ R-5′-TCAAATGCAAAGCACTGTATGA-3′	62°C/30 s, 197 bp	BclI *T*=117+80 *C*=197
	−112 T>G	F-5′-GGCAGCAAACCCGATTCCTG-3′ R-5′-GGAAGACCGGAACGCAGAGG-3′	62°C/30 s, 155 bp	BstNI *T*=155 *G*=138+17
	Ala64Thr C>T	F-5′-GCCCGCTGTAGAGTTTCATC-3′ R-5′-GAACTCAATTTCGCTCCCTTC-3′	60°C/30 s, 152 bp	MspAI *T*=147+5 *C*=110+37+5
UCP2	−866 G>A	F-5′-GCCTGAACCAGGGTGTAAGT-3′ R-5′-TTCGCCTTTAATTGGCTGAC-3′	59°C/30 s, 205 bp	MluI *A*=205 *G*=160+45
	45-bp duplication del>ins	F-5′-GATGTTCGTCACCTATGAGCAG-3′ R-5′-CACCAGCACTGAGACAATGAG T-3′	61°C/30 s, 263 bp	Del=263 Ins=308
NOS3	−786 T>C	F-5′-CAGCTAGTGGCCTTTCTCCA-3′ R-5′-CGCAGGTCAGCAGAGAGACT-3′	61°C/30 s, 211 bp	MspI *T*=211 *C*=168+43
	27-bp dup 4b>4a	F-5′-TGGTTATCAGGCCCTATGGT-3′ R-5′-GGAGGCTGCTCCTGCTACT-3′	61°C/30 s, 235 bp	4b, ins=235 4a, del=208
	Glu298Asp G>T	F-5′-AAGGCAGGAGACAGTGGATG-3′ R-5′-CAGTCAATCCCTTTGGTGCT-3′	61°C/30 s, 246 bp	BstKTI *G*=246 *T*=160+86
VEGF	−634 G>C	F-5′-GAGGTAGCAAGAGCTCCAGA-3′ R-5′-CTGTCTGTCTGTCCGTCAGC-3′	60°C/30 s, 195 bp	BsmFI *C*=195 *G*=115+80
	−460 T>C	F-5′-TGTGCGTGTGGGGTTGAGCG-3′ R-5′-TACGTGCGGACAGGGCCTGA-3′ Lin et al., 2003	62°C/30 s, 175 bp	BstUI *T*=175 *C*=155+20
	18 bp del>ins	F-5′-GAGGCTATGCCAGCTGTAG-3′ R-5′-TGCATATAGGAAGCAGCTTG-3′	62°C/30 s, 154 bp	Ins=154 Del=136
	936 C>T	F-5′-AAGGAAGAGGAGACTCTGCGC AGAGC-3′ R-5′-TAAATGTATGTATGTGGGTGGG TGTGTCTACAG-3′ Renner et al., 2000	65.4°C/30 s, 208 bp	NlaIII *C*=208 *T*=122+86
GSTT1 *	GSTT1 presence/null	F-5′-GGTCATTCTGAAGGCCAAGG-3′ R-5′-TTTGTGGACTGCTGAGGACG-3′	60°C/45 s, 131 bp	131
GSTM1*	GSTM1 presence/null	F-5′-TGCTTCACGTGTTATGGAGGTT-3′ R-5′-ATACGGTGGAGGTCAAGGACA-3′	60°C/45 s, 101 bp	101
ESR-1 Control*		F-5′-ACTCCCCACTGCCATTCATC-3′ R-5′-GTGCGAGTGGCTCAGTGTGT-3′	60°C/45 s, 168 bp	168
GSTPI	Ile105Val rs947894 A>G	F-5′-GAATGACGGCGTGGAGGAC-3′ R-5′-CCAAGCCACCTGAGGGGTAA-3′	60°C/30 s, 149 bp	HpyCH4 IV *A*=149 *G*=116+33

(*continued on next page*)

Table 1 (*continued*)

Gene	Marker details	Primer sequence	Annealing temperature/ time, product size	Enzyme used & expected allele sizes (bp)
PON1	rs854573 A>G	F-5′-CAATGTGAGGCCAAAGATGC-3′ R-5′-TCAAGGCTTTTGGTCCCAGT-3′	60°C/30 s, 143 bp	AluI *A*=143 *G*=111+32
	Met55Leu rs854560 A>T	F-5′-TGAAAGCCAGTCCATTAGGC-3′ R-5′-CCCAGTTTCAAGTGAGGTGTG-3′	58°C/30 s, 138 bp	NlaIII *A*=138 *T*=107+31
	Arg192Gly rs662 A>G	F-5′-CTGTGGGACCTGAGCACTTT-3′ R-5′-CCATCGGGTGAAATGTTGAT-3′	60°C/30 s, 181 bp	AlwI *A*=181 *G*=120+61
PON2	Cys311Ser rs6954345 G>C	F-5′-CAGACCCATTGTTGGCATAA-3′ R-5′-GGGCTTATTGATGATTGAGTGA-3′	58°C/30 s, 140 bp	DdeI *C*=140 *G*=96+44
NADPH oxidase $p22^{phox}$ (CYBA)	−930 G>A	F-5′-GGGTGTGGCTGGAATGGT-3′ R-5′-GCACACCTGGACTCCCTGA-3′	60°C/30 s, 169 bp	BbvI *A*=169 *G*=110+59
	His72Tyr rs4673 C>T	F-5′-CCTTTGGTGCTTGTGGGTAA-3′ R-5′-CGGCCCGAACATAGTAATTC-3′	60°C/30 s, 227 bp	RsaI *C*=227 *T*=167+60
	640 rs1049255 A>G	F5′-ACCGACGAGGTCGTGTGAC-3′ R5′-AGAAGGAAGGCGATGCTGAT-3′	60°C/30 s, 215 bp	AdeI *G*=215 *A*=169+46

CYBA, Cytochrome b-245, alpha polypeptide.

* Genotyping of GSTM1 and GSTT1 was carried out in a multiplex reaction using estrogen receptor-1 (ESR-1) amplicon as a control for amplification.

Delhi, Jaipur Diabetes and Research Center, Jaipur, and Monilek Hospital and Research Centre, Jaipur, following clearance from ethical committees of respective institutions. Written informed consent was obtained from the participating subjects before sample collection. The samples were classified as SI or NI based on their ethnicity, mother tongue, ancestral history, and marital relationships; SIs are primarily of Dravidian origin, and the NI, of Indo-European origin (Basu et al., 2003). Type 2 diabetes mellitus (T2DM) subjects having diabetes for ≥10 years and serum creatinine <2 mg/dl, with no history of kidney diseases, were included as controls. The cases of T2DM with CRI were those with persistently elevated serum creatinine ≥2 mg/dl, diabetes of ≥2 year duration, and presence of diabetic retinopathy. All patients underwent either a fundoscopic examination or fluoroangiographic study for diagnosis of retinopathy. Patients with drug-induced nephrotoxic damage or secondary causes of renal insufficiency such as obstructive renal disease, renal stone disease, and acute urinary tract infection were excluded.

From south India, a total of 149 cases with T2DM and 106 cases with CRI were evaluated, and NI centers evaluated 75 cases of T2DM and 90 cases of diabetic CRI. Clinical data included information on duration of diabetes, presence of any complication, history of other disorders, weight (kg), height (cm), body mass index (kg/m^2), and systolic and diastolic blood pressure. Reliable documentation of antidiabetic and antihypertensive medication could not be obtained for the large majority of patients and hence these data are not reported. Ten milliliters of venous blood was collected from each individual included in the study for biochemical and genetic analysis. Biochemical analyses to determine fasting glucose, glycated hemoglobin, serum creatinine, triglycerides, total cholesterol, and albumin were carried out at the respective centers using automated analyzers and similar protocols to ensure uniformity. Using serum creatinine as a surrogate marker, we carried out a retrospective calculation of glomerular filtration rate (GFR) by the online modified diet in renal disease (MDRD) calculator (http://www.nephron.com/cgi-bin/MDRD_GFR.cgi; Levey et al., 2003). An aliquot of blood from the four centeres was transported to the genetics laboratory for DNA isolation.

3. Genetic analysis

Genomic DNA was isolated from whole blood using the conventional phenol chloroform extraction method and used for polymerase chain reaction (PCR)-based genotyping. A total of 26 polymorphisms from 13 genes were genotyped using the PCR–restriction fragment length polymorphism method. The details of primers used and expected allele profiles are provided in Table 1. Briefly, the PCR was performed in a total reaction volume of 20 μl containing 100 ng of DNA template; 200 μM deoxyribonucleotide triphosphates (dNTPs), 5 pmol of each primer, 1.5 mM $MgCl_2$, 0.5 U of Taq polymerase (Biotools, Madrid, Spain), and 1× PCR reaction buffer. For digestion, PCR products were incubated with 1 U of restriction enzyme overnight. Depending on the size, the digestion products were resolved on a 2.5–3.5% ethidium bromide-containing agarose gel. Samples with known genotypes were included in each set of digestion to ensure that the observed genotypes were not due to partial/incomplete digestion. Some samples could not be

Table 2
Clinical characteristics of north India and south India sample sets

	North India			South India		
Characteristics	DM (n=75)	CRI (n=90)	*P*	DM (n=149)	CRI (n=106)	*P*
Gender (M/F)	40/35	78/12	≪.05	102/47	81/25	.164
Age (years)	61.03±8.88	53.56±10.99	≪.05	60.45±11.472	55.97±11.502	.003
Duration of diabetes (years)	15.39±8.168	9.62±6.841	≪.05	15.45±6.913	13.97±6.452	.171
Hb1Ac	7.3±1.0	10.4±7.7	<.05	7.2±1.4	7.3±1.1	.539
Systolic pressure (mm Hg)	142.89 (106–190)	148.73 (110–210)	.023	137.11 (116–180)	151.14 (90–220)	.021
Diastolic pressure (mm Hg)	86.14 (80–104)	90.76 (70–110)	≪.05	82.39 (62–100)	87.24 (60–130)	≪.05
Serum creatinine (mg/dl)	1.162±0.486	4.567±2.281	≪.05	0.957±0.273	6.87±3.75	≪.05
Serum triglyceride (mmol/l)	1.635±0.568	1.873±0.518	.001	2.636±1.449	1.786±0.623	.012
Serum cholesterol (mmol/l)	4.791±0.874	4.832±0.911	.886	5.189±1.053	4.508±1.051	.004
GFR (ml/min per 1.73 m^2)	66.46±17.511	17.05±8.347	≪.05	86.84±27.45	17.705±19.125	≪.05
Retinopathy	64.4%	100%	≪.05	30%	100%	≪.05

genotyped due to failure of repeated PCRs due to unknown technical reasons.

4. Statistical analysis

All statistical tests were done using SPSS version 11.0. Categorical variables such as gender were compared using χ^2 test. Normally distributed continuous variables were compared using *t* test. Continuous variables where skewed distribution was observed were compared by Mann–Whitney *U* test, and values are reported as mean and range. Individual single nucleotide polymorphism (SNP) association (allelic and genotypic) was tested using χ^2 test (for contingency table with low cell values, CLUMP was used; Sham and Curtis, 1995). For SNPs that were found to be significant, strength of association was tested by odds ratio estimates at 95% confidence interval. Haplotypes were reconstructed using PHASE version 2.0.2 (Stephens & Donnelly, 2003; Stephens, Smith, & Donnelly, 2001). Power calculations were carried out using the Power and Precision Software (www.power-analysis.com).

5. Results

The clinical and demographic characteristics of the two sample sets (SI and NI) are provided in Table 2. Amongst the

Table 3
Distribution of allelic and genotypic frequencies among patients with T2DM without CRI (coded as 1) and T2DM with CRI (coded as 2) among SI patients

			Allele			Genotype			
Gene	Polymorphism[a]	DM/CRI	1	2	*P*	11	12	22	*P*
SOD2	Ala9Val	1	162 (0.544)	136 (0.456)	.002	47 (31.5)	68 (45.6)	34 (22.8)	.013
	C>T	2	144 (0.679)	68 (0.321)		51 (48.1)	42 (39.6)	13 (12.3)	
UCP1	−112	1	215 (0.773)	63 (0.227)	.012	83 (59.7)	49 (35.3)	7 (5.0)	.0096[b]
	T>G	2	183 (0.863)	29 (0.137)		80 (75.5)	23 (21.7)	3 (2.8)	
	Ala64Thr	1	44 (0.162)	228 (0.838)	.015	2 (1.5)	40 (29.4)	94 (69.1)	.0048[b]
	T>C	2	16 (0.084)	174 (0.916)		2 (2.1)	12 (12.6)	81 (85.3)	
UCP2	−866	1	76 (0.260)	216 (0.740)	.031	10 (6.8)	56 (38.4)	80 (54.8)	.0780
	A>G	2	74 (0.349)	138 (0.651)		11 (10.4)	52 (49.1)	43 (40.5)	
NOS3	Glu298Asp	1	225 (0.765)	69 (0.235)	.002	91 (61.9)	43 (29.3)	13 (8.8)	.009[b]
	G>T	2	185 (0.873)	27 (0.127)		82 (77.4)	21 (19.8)	3 (2.8)	
GSTPI	Ile105Val	1	211 (0.708)	87 (0.292)	.003	73 (49.0)	65 (43.6)	11 (7.4)	.004
	A>G	2	174 (0.820)	38 (0.180)		74 (69.8)	26 (24.5)	6 (5.7)	
VEGF	−460	1	166 (0.560)	130 (0.440)	.349	46 (31.1)	74 (50.0)	28 (18.9)	.603
	T>C	2	110 (0.520)	102 (0.480)		27 (25.5)	56 (52.8)	23 (21.7)	
PON1	Arg192Gly	1	187 (0.654)	99 (0.346)	.655	62 (43.3)	63 (44.1)	18 (12.6)	.236
	A>G	2	140 (0.673)	68 (0.327)		43 (41.3)	54 (51.9)	7 (6.7)	

[a] The 11 genotype corresponds to the first allele e.g. Ala9Val C>T 11=CC, 12=CT, 22=TT genotypes.
[b] Calculated by 2×2 contingency, table cells with cell values less than 5 were merged with the preceding column.

Table 4
Distribution of haplotype(s) carrying the risk allele(s) among patients with T2DM without CRI (DM) and T2DM with CRI (CRI) in SI and NI sample sets

Gene	Haplotype	CRI (%)	DM (%)	OR (CI); *P*
South India				
UCP1 (−T3826C-T112G-Ala64Thr)	C−T−C+T−T−C	183 (86.32)	220 (78.01)	1.778 (1.09–2.88); .018
	Rest	29 (13.68)	62 (21.99)	
UCP2 (−866–45 bp)	A-del	40 (18.87)	35 (11.74)	1.747 (1.067–2.860); .025
	Rest	172 (81.13)	263 (88.26)	
NOS3 (T-786C-27bp–Glu298Asp)	T-4b-Glu	144 (67.92)	175 (58.33)	1.513 (1.046–2.187); .027
	Rest	68 (32.08)	125 (41.67)	
North India				
UCP1 (−3826-112-MspAI)	T−T−C+C−T−T	144 (80.00)	117 (78.00)	1.128 (0.663–1.912); .656
	Rest	36 (20.00)	33 (22.00)	
UCP2 (−866–45 bp)	A-del	27 (15.00)	19 (12.67)	1.2167 (0.647–2.288); .542
	Rest	153 (85.00)	131 (87.33)	
NOS3 (T-786C-27 bp–Glu298Asp)	T-4b-Glu	121 (67.22)	114 (76.00)	0.648 (0.398–1.054); .07
	Rest	59 (32.78)	36 (24.00)	

SI samples, no significant difference in the distribution of gender, duration of diabetes, and glycated hemoglobin was observed between cases and controls. The other clinical characteristics were significantly different between the two groups. On the other hand, among the NI case-control group, except for serum cholesterol, all the other clinical variables were significantly different. Hardy–Weinberg equilibrium (HWE) was estimated for both diabetes mellitus (DM) and CRI groups in both the sample sets. In the SI sample set, NOS3 Glu298Asp (only in DM) and NADPH oxidase $p22^{phox}$ −930 A>G (both in DM and CRI) were not in HWE. In the NI sample set, SOD2 Ala9Val (in DM), VEGF −460 C>T and 936C>T, and PON1 rs662 (only in CRI) were not in HWE ($P<.05$).

6. SI subjects (cases 106, controls 149)

Of the 26 polymorphisms in 13 genes analyzed in the SI subjects, significant association of SOD2 (Ala9Val), UCP1 −112 T>G; Ala64Thr, C>T), UCP2 (−866A>G), NOS3 (Glu298Asp, G>T), and GSTP1 (Ile105Val) with CRI was

Table 5
Distribution of allelic and genotypic frequencies among patients with T2DM without CRI (coded as 1) and T2DM with CRI (coded as 2) from North India

Gene	Polymorphism[a]	DM/ CRI/ controls	Allele			Genotype			
			1	2	*P*	11	12	22	*P*
SOD2	Ala9Val	1	84 (0.560)	66 (0.440)	.825	28 (37.3)	28 (37.3)	19 (25.3)	.910
	C>T	2	92 (0.548)	76 (0.452)		29 (34.5)	34 (40.5)	21 (25.0)	
UCP1	−112	1	117 (0.780)	33 (0.220)	.656	45 (60.0)	27 (36.0)	3 (4.0)	.461[b]
	T>G	2	144 (0.800)	36 (0.200)		59 (65.5)	26 (28.9)	5 (5.6)	
	Ala64Thr	1	21 (0.140)	129 (0.860)	.782	1 (1.3)	19 (25.3)	55 (73.3)	.647[b]
	T>C	2	22 (0.130)	148 (0.870)		2 (2.3)	18 (21.2)	65 (76.5)	
UCP2	−866	1	54 (0.360)	96 (0.640)	.407	9 (12.0)	36 (48.0)	30 (40.0)	.599
	A>G	2	57 (0.317)	123 (0.683)		10 (11.1)	37 (41.1)	43 (47.8)	
NOS3	Glu298Asp	1	128 (0.877)	18 (0.123)	.174	56 (76.7)	16 (21.9)	1 (1.4)	.267[b]
	G>T	2	148 (0.820)	32 (0.180)		62 (68.9)	24 (26.7)	4 (4.4)	
GSTPI	Ile105Val	1	109 (0.727)	41 (0.273)	.368	40 (53.3)	29 (38.7)	6 (8.0)	.499[b]
	A>G	2	134 (0.770)	40 (0.230)		51 (58.6)	32 (36.8)	4 (4.6)	
VEGF	−460	1	81 (0.540)	69 (0.460)	.914	18 (24.0)	45 (60.0)	12 (16.0)	.042
	T>C	2	95 (0.546)	79 (0.454)		30 (34.5)	35 (40.2)	22 (25.3)	
PON1	Arg192Gly	1	69 (0.540)	59 (0.460)	.507	16 (25.0)	37 (57.8)	11 (17.2)	.013
	A>G	2	82 (0.500)	82 (0.500)		27 (32.9)	28 (34.1)	27 (32.9)	

[a] The 11 genotype corresponds to the first allele e.g. Ala9Val C>T 11=CC, 12=CT, 22=TT genotypes.
[b] Calculated by 2×2 contingency table cells with cell values less than 5 was merged with the preceding column.

observed (Table 3). Significant observations in all these markers except UCP2 were the excess of wild-type allele and wild-type genotype and their association with CRI.

The distribution of SOD2 Ala9Val genotypes was found to be significantly different between DM and CRI groups (Table 3). There was an excess of the wild-type allele Ala (OR=1.780, 95%CI=1.233–2.569; *P*=.001) and Ala/Ala genotype (OR=1.927, 95%CI=1.157–3.210; *P*=.011) in the CRI category. Similar association and excess of the wild-type allele and genotype in CRI category was observed for UCP1 −112 T>G [OR=1.849, 95% CI=1.142–2.994 for T allele, *P*=.012; OR=2.076, 95%CI=1.189–3.625, *P*=.0089 for TT) and UCP1 Ala64Thr (OR=2.099 95%CI=1.146–3.844, *P*=.015 for Ala, C allele; OR=2.585, 95%CI=1.318–5.072; *P*=.0048 for CC (Ala/Ala) genotype] (Table 3). Excess of the wild-type allele and genotype was seen for NOS3 Glu298Asp in CRI category [OR=2.101, 95% CI=1.293–3.415, *P*=.002 for allele G (Glu); OR=2.103, 95%CI=1.197–3.695; *P*=.009 for GG genotype) (Table 3). For GSTP1 (Ile105Val, A>G), significant excess of wild-type Ile (A allele, OR=1.888, 95%CI=1.227–2.904; *P*=.003) and Ile/Ile genotype was observed in CRI category (OR=2.407, 95%CI=1.425–4.068; *P*=.0008). A trend towards significance was observed for UCP2 −866 A>G, and unlike the other markers, an excess of the rarer allele was seen in the CRI category (OR=1.524, 95%CI=1.037–2.239, *P*=.031 for allele A), and the wild-type genotype was found to be protective (OR=0.563, 95%CI=0.339–0.934, *P*=.026 for GG genotype) (Table 3).

Other polymorphisms investigated in SOD1 (rs2070424), SOD3 (rs699473 and Ala58Thr), UCP1 (−3826 T>C), UCP2 (45-bp duplication), NOS3 (T −786C, 27 bp duplication), VEGF (18-bp ins/del, −460C>T, C −634G, and 936C>T), PON1 (rs854573, rs854560, rs662), PON2 (Cys311-Ser), GSTMI (null), GSTTI (null), and NADPH oxidase $p22^{phox}$ (Cytochrome b-245, alpha polypeptide [CYBA], −930 A>G, C242T, A640G) genes were not associated with the development of the disease in this sample set (data not shown).

Targeted analysis for distribution of haplotypes carrying the risk alleles identified in the above allelic associations was carried out. In agreement with the observation of allelic as well as genotypic associations, the haplotypes carrying the risk alleles (of UCP1, UCP2, and NOS3) were significantly in excess in the CRI patients (Table 4).

7. NI subjects (cases, 90; controls, 75)

Genotypic distributions of VEGF-460 and PON1 Arg192Gly polymorphisms were significantly different between DM and CRI categories (Table 5). This significance could be attributed to the presence of an excess of heterozygote in the patients with DM in VEGF −460 [for CT genotype, OR=2.23 (1.87–4.18), *P*=.012] and PON1 [Arg192Gly, A>G (for AG) genotype, OR=2.643 (1.347–5.186); *P*=.004]. However, no allelic associations were observed for these two polymorphisms (*P*>.05, Table 5). The distribution of haplotypes in VEGF gene was not significantly different between DM and CRI categories. A haplotype in PON1 was found to be protective to CRI development [A-A-A; OR=0.512 (0.261–1.005), *P*=.048].

8. Discussion

Hyperglycaemia-induced oxidative stress is a crucial factor in the development of diabetic complications (Brownlee, 2001; King & Loeken, 2004). In this study, we investigated the possible role of oxidative stress gene polymorphisms in the progression of diabetes related complications, using CRI as a phenotype. Few markers namely, NOS3 Glu298Asp (only in SI-DM) and NADPH oxidase $p22^{phox}$ −930 A>G (both in SI-DM and CRI); SOD2 Ala9Val (in NI-DM); VEGF −460 C>T & 936C>T; and PON1 rs662 (only in NI-CRI) were not in HWE in the study populations. Such deviations, in literature, have been attributed primarily to a possibility of genotyping error (Salanti, Amountza, Ntzani, & Ioannidis, 2005). We have ruled out this possibility by inclusion of samples of known genotypes, as positive Restriction Fragment Length Polymorphism (RFLP) controls and two independent workers confirmed genotypes. The other possible reason for the observed deviations could be either genetic drift, recent origin/introduction of the polymorphism, absence of random mating, and/or a stratification bias. The last two possibilities can be ruled out as our sample collection consisted of randomly collected unrelated individuals of known ethnicity. However, the other factors cannot be ruled out, as our current sample set is not appropriate to comment on the selection forces operating on the population.

The two major observations in this study are (*a*) the excess of the wild-type/functionally efficient alleles of all the associated markers with CRI in SI subjects and (*b*) the significant association of a few genes with CRI among the SI subjects which was not replicated in the NI cohort.

9. Association of the wild-type alleles with diabetic renal insufficiency in SI subjects

We observed a significant association of polymorphisms in SOD2 (Ala9Val), UCP1 (−112 T>G and Ala64Thr), NOS3 (Glu298Asp), and GSTP1 (Ile105Val) genes with the development of CRI in the SI population. A common feature in these observations was the excess of the wild-type allele amongst the patients with CRI (Table 3). These observed associations may be a distinct risk factor for SI population and are discussed further. Overexpression of SOD2 (MnSOD) and UCP1 prevent hyperglycaemia induced superoxide overproduction (Brownlee, 2001; Nishikawa et al., 2000). Functional polymorphisms altering expression levels are known in these, as well as other genes found associated with CRI in the SI population. The presence of the variant allele of

any of these polymorphisms can potentially lead to lowered level of gene product, finally leading to reduced activity of the specific enzyme and consequently reduced detoxification capacity. Logically, these variants should be the predisposing factors to the development of CRI. On the contrary, we have observed an excess of the wild-type or the functionally efficient allele predisposing to CRI. This is intriguing considering the well-defined and important role of these genes in the maintenance of cellular homeostasis. Though, it is often difficult to correlate the association of the wild-type alleles with a disease phenotype, a possible explanation for the current observation could be attributed to lower survival of CRI subjects carrying the variant allele.

Both diabetic nephropathy and CRI, as determined by elevated creatinine, are risk factors for development of cardiovascular diseases (Czekalski, 2005; Mann, 2005; Mann, Gerstein, Pogue, Bosch, & Yusuf, 2001; Retnakaran, Cull, Thorne, Adler, & Holman, 2006; Ritz & Orth, 1999; Ruggenenti & Remuzzi, 1998). Elevated creatinine (≥1.4 mg/dl) has been reported to be an independent risk factor for the incidence of cardiovascular death, myocardial infarction, and stroke (Ruggenenti & Remuzzi, 1998). Additionally, decreased GFR has been independently associated with risk of death and cardiovascular events (Go, Chertow, Fan, McCulloch, & Hsu, 2004). Further, increased incidence of end-stage renal disease has been attributed to enhanced survival of T2DM, DN, and CRI patients, living long enough to develop such complications, as a consequence of intensive therapy (Hayden, Whaley-Connell, & Sowers, 2005; Ritz & Stefanski, 1996). In the SI population, a threefold higher risk for the development of cardiovascular disease in patients with diabetic nephropathy compared to normoalbuminuric T2DM patients has been reported (Viswanatham et al., 1998).

Thus, we may hypothesize that in our study, the patients carrying the variant alleles, associated with reduced function, might not have survived to reach the stage of CRI, possibly due to premature mortality caused by cardiovascular events. Consequently, enrichment of the wild-type allele may be due to higher survival rates of carriers of the wild-type allele. Support for this hypothesis comes from the observation of association of NOS3 Glu298Asp polymorphism, the T allele (Asp) of which has been reported to be associated with hypertension, coronary artery disease and myocardial infarction in the Japanese population (Miyamoto et al., 1998; Shimasaki, Yasue, Yoshimura, Nakayama, & Kugiyama, 1998; Yoshimura et al., 1998). We observed an excess of the wild-type [Glu, G] allele in the CRI patients from south India. Thus, patients carrying the mutant allele (less efficient allele) might not have survived the comorbid complications (cardiovascular death, myocardial infarction and stroke). The observation of excess of the haplotype carrying the Glu allele (T-4b-Glu) and deficit of the haplotype carrying the Asp (T-4b-Asp) allele among CRI patients provides additional support to the hypothesis. Further, in case of SOD2 Ala9*Val* or UCP1 −112T>*G* or UCP2 −866 A>*G* or GSTP1 Ile105*Val* SNPs, decreased activity of the variant allele has been reported (allele in italics; Esterbauer et al., 2001; Herrmann et al., 2003; Hu et al., 1998; Mori et al., 2001; Nagai, Sakane, Ueno, Hamada, & Moritani, 2003; Rosenblum, Gilula, & Lerner, 1996; Shimoda-Matsubayashi et al., 1996). In addition, the haplotypes carrying the wild-type alleles were in excess in CRI patients (UCP1, T-*T*-*C* and C-*T*-*C*; UCP2 A-*del*; Table 4). The possibility of the wild-type allele/haplotype in each of these situations leading to better survival of the CRI patients seems most likely and thus supportive of our hypothesis.

10. Nonreplication of association across SI and NI subjects

All the above-discussed associations were observed in SI population, but none of these were replicated in the NI population. However, only a genotypic but not allelic association was observed for two other markers (VEGF −460 T>C and PON1 Arg192Gly) among NIs. Further, no association was observed for haplotypes carrying the VEGF −460 T>C polymorphism, and only marginal association of a haplotype in PON1 [A-A-A; uninformative OR; OR=0.512 (0.261−1.005)] was observed in our study sample. This makes it difficult to correlate the relevance of these SNPs in CRI manifestation for NI population. It is relevant to mention here that prior to the initiation of this study, allelic and genotypic frequencies of all the markers tested were analysed in 96 individuals each from the two populations (SI and NI). We observed similar allelic and genotypic frequencies for majority of the markers (except SOD3 Ala58Thr and UCP1 −3826 C/T; data not shown). Therefore, the differences observed between the two populations may reflect a differential susceptibility of the two groups to the development of this disorder [SI being at higher risk, (Viswanatham et al., 1998); but with no comparable data on NI population]. This may be mediated by gene environment interactions such as exposure of the two populations to different kinds of environments and lifestyle [including physical activity (Mohan, 2004), food habits, etc.]. The SI sample set is predominantly of rural and semiurban origin, whereas the NI centers receive a very heterogeneous population (predominantly urban). Thus genetic, environmental, as well as sample heterogeneity might be responsible for the nonreplication of associations observed in the SI population.

Alternatively, the nonreplication of associations between NI and SI populations could be due to the sample size. Power calculations for the SI sample set revealed that only 35% of the studies using a similar sample size would be able to detect similar differences of small effect size (w=0.10). However, this study has adequate power to detect a medium effect size (w=0.25, 98% power in SI and 90% in NI population; Power and Precision software). These are limitations of the present study, and it is essential to replicate these observations in a prospective study and/or also in independent sample sets from the same populations.

In conclusion, oxidative stress might be an important predictor of development of complications in type 2 diabetes subjects. The carriers of wild-type and efficient alleles of oxidative stress genes are more likely to survive the comorbid complications and these alleles may be nephroprotective.

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