Case Control Study

Association of NFKB1 gene polymorphism (rs28362491) with levels of inflammatory biomarkers and susceptibility to diabetic nephropathy in Asian Indians

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Aim
To investigate the association of NFKB1 gene -94 ATTG insertion/deletion (rs28362491) polymorphism with inflammatory markers and risk of diabetic nephropathy in Asian Indians.

Methods
A total of 300 subjects were recruited (100 each), normoglycemic, (NG); type 2 diabetes mellitus (T2DM) without any complications (DM) and T2DM with diabetic nephropathy [DM-chronic renal disease (CRD)]. Analysis was carried out by polymerase chain reaction-restriction fragment length polymorphism and ELISA. Pearson's correlation, analysis of variance and logistic regression were
used for statistical analysis.

RESULTS
The allelic frequencies of -94 ATTG insertion/deletion were 0.655/0.345 (NG), 0.62/0.38 (DM) and 0.775/0.225 (DM-CRD). The -94 ATTG ins allele was associated with significantly increased levels of urinary monocyte chemoattractant protein-1 (uMCP-1); uMCP-1 (P = 0.026) and plasma tumor necrosis factor-alpha (TNF-α); TNF-α (P = 0.030) and almost doubled the risk of diabetic nephropathy (OR = 1.91, 95%CI: 1.080-3.386, P = 0.025).

CONCLUSION
-94 ATTG ins/ins polymorphism might be associated with increased risk of developing nephropathy in Asian Indian subjects with diabetes mellitus.

Key words: Diabetic nephropathy; Inflammation; NFKB1 -94 ATTG ins/del polymorphism; Urinary monocyte chemoattractant protein-1; Tumor necrosis factor-alpha

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Core tip: Type 2 diabetes mellitus (T2DM) is considered as long standing inflammatory disease. Diabetic nephropathy (DN) is the most common micro-vascular complication of T2DM. Pro-inflammatory cytokines like Monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-alpha (TNF-α) plays a crucial role in the pathogenesis of DN. Therefore we investigated -94 ins/del ATTG polymorphism in NFKB1 gene and its association with the risk of DN in Asian Indians. -94 ins/del ATTG single nucleotide polymorphism was found to increase the urinary MCP-1 and plasma TNF-α levels. Our findings open a new area of research to explore that -94 ins/del ATTG may be considered as genetic markers for early detection of diabetic patients who are at greater risk of development of nephropathy.

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INTRODUCTION
Chronic renal disease (CRD) is an intricate pathological process, often leading to end stage renal disease. The causes of CRD are quite multi-factorial ranging from infections to heredity, but type 2 diabetes mellitus (T2DM) is the major culprit amongst them[1]. In spite of the improvement in our knowledge about the etiopathogenesis of diabetic nephropathy (DN), the intricate mechanisms leading to the development of renal injury from chronic hyperglycemia are not yet fully understood. DN has been considered a micro-vascular complication of hyperglycemia, but various clinical and experimental studies have observed that there is a close link between hyperglycemia, inflammation and oxidative stress (OS)[2]. OS may also be involved in promoting a low grade systemic inflammation in patients with T2DM and vice versa[3]. Nuclear factor-kappa B (NF-κB) activation through hyperglycemia induced OS may lead to increased concentration of inflammatory cytokines[4].

NF-κB was identified as a transcription factor which controls the expression of numerous genes affecting immune response, inflammation, cell-growth control, apoptosis and therefore, is an emerging candidate for studies on the pathogenesis of inflammatory diseases including DN. There are five members of the NF-κB family in mammals: NF-κB1: p105/p50, NF-κB2: p52/p100, RelA: p65, RelB, and c-Rel. The chief form of NF-κB is a hetero-dimer of the p50 and p65/RelA subunits, encoded by the NFKB1 and RelA gene. Normally, inactive NF-κB is found in the cytoplasm bound to IκBα, which are specific inhibitor proteins in cytoplasm. Cell when exposed to a variety of proinflammatory stimuli leads to the quick phosphorylation followed by ubiquitinylation, and finally proteolytic breakdown of IκB. This causes transfer of NF-κB in nucleus and thus leading to increased transcription of gene[5]. NF-κB transcriptionally regulates many downstream proinflammatory genes, mainly including monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-alpha (TNF-α)[6].

MCP-1 is an important proinflammatory chemokine which affects the recruitment and function of monocyte[7]. MCP-1 is synthesized in response to a various proinflammatory stimuli by kidney cell[8]. A study done by Wada et al[9] in 2000 has shown that expression of MCP-1 increases in inflammation induced kidney diseases including DN. Urinary MCP-1 (uMCP-1) is a potential biomarker for renal damage[10]. Hyperglycemia induced secretion of abundant MCP-1 from renal parenchymal cells, attract monocytes into the kidney stimulating myofibroblast-like properties in mesangial cells. Kidney macrophages when exposed to MCP-1 in diabetic milieu promotes activation of macrophage. Thus, leading to release of reactive oxygen species (ROS), various pro-inflammatory cytokines and profibrotic growth factors[11,12]. Thus, resulting in exaggerated inflammation that leads to renal injury through proliferation of myofibroblast, augmented production of extracellular matrix by mesangial cells and fibroblasts.

TNF-α is a well known proinflammatory cytokine associated with systemic inflammation[13,14]. It is produced predominantly by macrophages and monocytes[13,14]. TNF-α acts via NF-κB signaling and mediates the transcription of various cytokines performing roles in cell survival, proliferation, inflammatory responses, cell adhesion and inflammation[15]. A study has shown that
there is upregulation of TNF-α expression in glomeruli of diabetic rats[16]. TNF-α is well acknowledged to cause damage to renal cells by enhancing renal hypertrophy, hemodynamic imbalance, albumin permeability[17]. The harmful effects of these responses lead to the development of renal disease in patients with T2DM, hence resulting in the progression of renal failure.

In addition to poor glycemic control, OS and inflammation; genetic factors seem to be main determinants of DN in terms of both occurrence and severity[18]; however the genetic mechanism causing DN is still unexplored. In our knowledge, there is no study available regarding the polymorphisms of NFKB1 and their correlation with levels of uMCP-1 and plasma TNF-α. We have reported[19] increased uMCP-1, plasma TNF-α levels in subjects with DN when compared to subjects with T2DM without nephropathy and observed a positive correlation between uMCP-1 and plasma TNF-α[20]. We have also highlighted that DN is associated with TNFA gene single nucleotide polymorphism (SNP)[20]. In recent times, a new functional NFKB1 promoter SNP consisting of a insertion/deletion (-94ins/del ATTG) (rs28362491) has been identified which can elicit a regulatory effect on the NFKB1 gene[21]. Since above mentioned polymorphism has been associated with various inflammatory diseases, autoimmune diseases and cancers[22], therefore, it is worthwhile to further investigate the association of -94 ins/del ATTG NFKB1 gene SNP with levels of uMCP-1, plasma TNF-α and nephropathy risk in subjects with T2DM.

MATERIALS AND METHODS

Study design

The present study comprises of total 300 subjects visiting Nephrology Outpatient Clinic and Medicine OPD at University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi. Subjects were divided into three groups of 100 each namely; Group 1: Normoglycemic (NG), Group 2: Subjects with T2DM for ≥ 10 years without nephropathy (DM), Group 3: Subjects with T2DM for ≥ 5 years with nephropathy (DM-CRD). T2DM was diagnosed according to revised ADA criteria[23]. Detailed clinical history and physical examination were recorded. Blood pressure (BP) of subjects was estimated using sphygmomanometer in the sitting position after a resting period of 10 min. The estimated glomerular filtration rate (eGFR) was measured by Modification of Diet in Renal Disease Abbreviated Equation (MDRD)[24].

The presence of micro-albuminuria in T2DM subjects was detected by Urine Test 11 MAU dipstick (Piramal Diagnostic, sensitivity: 10-15 mg/dL) and all participants having proteinuria and micro-albuminuria were clubbed in Group 3. All participants with nephropathy were in pre-dialysis stage. Normoglycemic (Group 1) subjects were recruited from employees of UCMS and GTB Hospital with the following criteria: (1) they did not have of diabetes mellitus (fasting plasma glucose < 100 mg% or postprandial glucose < 140 mg% or HbA1c < 5.7%) according to ADA criteria; (2) there was no presence of diabetes in their first or second degree relatives; and (3) they had normal BP, with systolic and diastolic BP not > 120 mmHg and 80 mmHg[25].

To circumvent any possible confounding factors, patients having renal disorders (hypertensive nephropathy, chronic glomerular nephritis, chronic interstitial disease, ischemic nephropathy, obstructive nephropathy), acute and chronic infections, congestive heart failure, malignancy and liver disorder were not included into the study. All subjects in Group 3 had retinopathy; but participants with macro-vascular complications like coronary artery disease and stroke were not included into the study. Patients taking renin-angiotension aldosterone system inhibitors, aspirin and vitamin D analogues were advised to discontinue these drugs for a period of a week before inclusion in the study since they have been found to influence the synthesis of uMCP-1 and TNF-α. However, patients were prescribed beta-blockers to control BP in that duration of one week. The Institutional Ethics Committee for Human Research approved the protocol of this study (approval number-UCMS/IEC-HR/2010/10). Prior to the inclusion into the present study, informed written consent was taken from all participants.

Biochemical parameters

Under aseptic conditions fasting venous blood samples were withdrawn and collected into EDTA and fluoride vials. For glycosylated hemoglobin (HbA1c) 200 µL whole blood was preserved at 4 °C-8 °C and processed within one week of collection. Blood samples collected in EDTA vial was subjected to centrifugation at 3000 rpm for 10 min in order to separate the plasma. Early morning first mid-stream urine sample was collected and stored in aliquots at -20 °C for estimation of MCP-1, albumin and creatinine.

Routine investigations such as fasting and post-prandial plasma glucose, urea, creatinine and uric acid were carried out using commercially available kits on autoanalyser (Olympus AU-400). HbA1c was estimated by ion-exchange resin chromatography using commercially available kits (Fortress, United Kingdom). Urinary protein excretion was expressed as albumin to creatinine ratio.

Markers of inflammation

uMCP-1 (Weldon, California; sensitivity less than 7.8 pg/mL) and plasma TNF-α (Diaclone, France; sensitivity less than 8 pg/mL) were estimated by commercially available ELISA kit.

DNA extraction and polymorphism genotyping

Cellular DNA of every individual was extracted from 200 µL EDTA-anticoagulated peripheral blood sample by means of DNA isolation kit (Zymo research, United States). The polymerase chain reaction was carried out in Thermocycler (Eppendorf Mastercyler Gradient-5331). In brief, 0.1 µg of DNA was amplified in a reaction mixture...
of 20 µL containing 0.5 µmol/L each of the following primer pairs (Forward 5'-TGGGCAACAAGTCGTTTATGA-3' and Reverse 5'-CTGGAGCCGGTAGGGAAG-3'). The reaction mixture also contained 0.5 mmol/L (dNTP mix), 2 µL (10 × PCR buffer) and 2.0 units Taq DNA polymerase, 2 mmol/L MgCl₂. The PCR protocol consisted an initial temperature of 94 °C (5 min) followed by 35 cycles of amplification (30 s at 94 °C, 45 s at 59 °C, and extension for 1 min at 72 °C). Final extension step was carried out for 2 min at 72 °C.[22]

For the study of the -94 insertion/deletion ATTG SNP in NFKB1, PCR product (281/285 bp) was subjected to fast digestion with restriction enzyme PstI. PCR products were treated with enzyme PstI in at 37 °C for 1 h and inactivated at 65 °C for 20 min. The insertion allele (ins) was cut down into two fragments of 45 bp and 240 bp by PstI restriction enzyme. But, there was no cleavage at the deletion allele (del) that has only one ATTG at its promoter.[22] The bands of digested products were visualized in 2% agarose gel electrophoresis stained with ethidium bromide.

### Statistical analysis

Demographic profiles and routine investigation was compared by $\chi^2$ and Student's t test and one-way ANOVA was used. To associate all the study groups with genotype two-way ANOVA followed by post-hoc Tukey's test was used. For association of genotypes with uMCP-1 and plasma TNF-α levels, analysis of variance was used. Logistic regressions was used to evaluate the risk of development of DN at the single SNP level. Power of sample size keeping 5% significance level and 80% power was calculated by genetic power calculator. A P value < 0.05 was considered statistically significant.

### RESULTS

**Characteristics of the study population**

Biochemical and demographic parameters of the various study groups are shown in Table 1. There was no difference in sex distribution and BMI within all the three study groups. The subjects of Group 2 (DM) and Group 3 (DM-CRD) were older than Group 1 (NG) subjects; however the period of diabetes was more in Group 2 (DM) than Group 3 (DM-CRD) which was as per our selection criteria. Incidence of hypertension was significantly higher in Group 2 (DM) and Group 3 (DM-CRD) participants as suggested by raised SBP and DBP (P < 0.001) when compared to NG. Poor glucose control was observed in DM-CRD as compared to DM as suggested by significantly higher (P < 0.001) fasting, postprandial plasma glucose and HbA1c. Renal function tests suggested that blood urea, plasma creatinine, and uric acid were significantly higher (P < 0.001) and eGFR was decreased (P < 0.001) in Group 3 (DM-CRD) as compared to Group 2 (DM).

### Distribution of ins/del in study population

The allele frequencies and genotype of the NFKB1 gene for -94 insertion/deletion ATTG SNP in various study groups are shown in Table 2. The distribution percentage of ins/ins, ins/del, del/del genotypes in Group 1 (NG), Group 2 (DM) and Group 3 (DM-CRD) (expressed in percentage) were 41%, 49% and 10%; 38%, 48% and 14%; and 61%, 33% and 6% respectively. The frequency of del/del genotype was significantly lower (P < 0.001) in Group 3 (DM-CRD) as compared to Group 2 (DM). However, allele frequencies of -94 insertion/deletion ATTG were 65.5%/34.5% in Group 1 (NG), 62%/38% in Group 2 (DM) and 77.5%/22.5% in Group 3 (DM-CRD).

### Relationship between the -94 ins/del AGGT SNP with inflammatory markers and disease risk

Correlation of -94 ins/del AGGT SNP with levels of...
**Table 3** Interaction analysis of -94 ins/del ATTG polymorphism with inflammatory markers

| Inflammatory marker | Groups | NG (n = 100) | DM (n = 100) | DM-CRD (n = 100) | P value |
|---------------------|--------|--------------|--------------|------------------|---------|
| uMCP-1 (pg/mg creatinine) | Total | 130.00 ± 42.22 | 271.00 ± 120.01 | 563.70 ± 100.1 | 0.030 |
| del/del | 85.1 ± 9.2 | 200.6 ± 66.5 | 460.9 ± 900.6 | 0.022 |
| ins/del | 110.9 ± 15.6 | 278.9 ± 103.9 | 507.9 ± 1016.3 | 0.026 |
| Plasma TNF-α (pg/mL) | Total | 15.55 ± 2.22 | 16.51 ± 3.75 | 21.38 ± 3.67 | <0.001 |
| del/del | 8.27 ± 1.06 | 10.21 ± 1.32 | 17.31 ± 1.17 | 0.022 |
| ins/del | 11.55 ± 0.05 | 14.05 ± 0.18 | 19.31 ± 0.44 | 0.000 |

*Significantly different from Normoglycemic at P < 0.001; †Significantly different from diabetic patients without nephropathy at P < 0.001.

uMCP-1 and plasma TNF-α have been studied and the results are shown in Table 3. The -94 ins allele were associated with increased levels of uMCP-1 (P = 0.026) and plasma TNF-α (P = 0.030) in the disease study groups, i.e., Group 2 (DM), Group 3 (DM-CRD).

The associations at the level of genotype is shown in Table 4. Highly significant association was observed for -94 ins/del AGGT polymorphism in subjects with Group 3 (DM-CRD) in comparison to Group 1 (NG); P = 0.022. In our present study, -94 ins SNP was found to increase risk for the development of DN by 1.91-fold in subjects with diabetes (OR = 1.91, 95%CI: 1.080-3.386, P = 0.025).

**DISCUSSION**

Polymorphism in the NFKB1 promoter region at position -94 ins/del AGGT has been correlated with many long standing inflammatory diseases like autoimmune diseases such as rheumatoid arthritis, asthma, AIDS, cancers and various diabetic complications. Genetic variations are known to play a vital role in determining risk of DN. A number of studies have investigated the relationship of ins allele of -94 ins/del AGGT polymorphism with various inflammatory diseases. Till date not a single study has tried to evaluate the association between this polymorphism and DN risk. Our study is first to document that patients with T2DM having ins/ins genotype were found to have increased risk of developing nephropathy. Latest studies have reported that p50 null mice have a significantly reduced inflammatory response in various models of inflammation such as asthma, arthritis, and autoimmune encephalomyelitis. A similar study conducted in sporadic colorectal cancer (CRC) and epithelial ovarian cancer (EOC) has supported results were in contrast with a genomic study on cancer conducted by Yang et al. in 2014. The dissimilarity of results could be due to diverse geographical distribution and ethnicity between our study and theirs was different, which could result in diverse genetic background.

A recent study has shown that TNF-α stimulates the MCP-1 production via NF-κB signalling pathway in rat astrocyte cultures. TNF-α was found to increase p65 and phosphorylated p65 levels in nuclear extracts of rat astrocytes, hence augmenting MCP-1 levels. This supports our finding that increased levels of TNF-α are associated with increased levels of uMCP-1.

**Table 4** Association between -94 ins/del ATTG polymorphism in the NFKB1 gene and diabetic nephropathy at the genotype level

| Genotype          | OR      | 95%CI     | P value |
|-------------------|---------|-----------|---------|
| DM vs NG ref      | 1.04    | 0.607-1.987 | 0.887  |
| DM-CRD vs NG ref  | 1.95    | 1.01-3.467  | 0.022  |
| DM-CRD vs DM ref  | 1.91    | 1.080-3.386 | 0.025  |

Ref: Reference group.
our findings which suggested that ins/ins genotype contribute to significantly increased risk of CRC and EOC. The probable mechanism of -94 ins/del AGGT polymorphism leading to increased risk of developing DN is explained in Figure 1. In almost all cell types, NF-κB complexes are typically localized in the cytoplasm where they bind to IKB inhibitory proteins. However, stimulation with hyperglycemia induced ROS and TNF-α leads to activation of IKK. IKK causes phosphorylation of IκBα bound to p50/p65. Phosphorylated IκBα dissociate from p50/p65 leading to nuclear translocation of unbound heterodimer p50/p65 (NF-κB). Binding of NF-κB to promoter gene causes translation of p65. Ins allele, rs28362491 NFKB1 gene, if present, causes increase expression of p50. Hence there is increased production of p50/p65 heterodimer complex. This heterodimer acts on its downstream proinflammatory targets viz: MCP-1 and TNF-α leading to its synthesis. MCP-1 is a positive regulator of TNF-α and vice versa. Both MCP-1 and TNF-α causes renal damage leading to development of Diabetic nephropathy. ROS: Reactive oxygen species; TNF-α: Tumor necrosis factor-alpha; IKK: IκB kinase complex; MCP-1: Monocyte chemoattractant protein-1.

The above mentioned probable hypothesis might lead to increased risk of developing renal damage in T2DM. However results of a recent study from China[38] in bladder cancer is in contradiction to our findings which could be due to ethnic and geographical differences. Furthermore, the sample size of our study was fairly small than aforementioned bladder cancer study.

The results of the current study suggest that the NFKB1 promoter -94 ins/del AGGT SNP is associated with increased possibility of developing nephropathy in patients with diabetes. This SNP may be considered as genetic markers for susceptibility to develop nephropathy in patients with T2DM. The limitation of the study is the small sample size. Therefore, further evaluation is necessary in big sample size to look for the possibility of this polymorphisms as potential genetic markers in the near future. This would help to identify patients with type 2 diabetics who may be at higher risk of developing nephropathy.
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