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# Role of variants rs5030717 and rs5030718 of *TLR4* in the risk prediction of nephropathy, hypertension and dyslipidaemia in type 2 diabetes mellitus

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**ABSTRACT**

**Background:** Type 2 diabetes mellitus describes a metabolic disorder characterised by prolonged elevated blood glucose that brings a risk of developing microvascular and macrovascular disease. Several factors, such as dysregulation of the Toll-like receptor 4 (TLR-4), are reputed to contribute to the multiple pathophysiological disturbances responsible for impaired glucose homeostasis. We hypothesised that variants rs5030717 and rs5030718 of *TLR4* are associated with diabetic nephropathy, hypertension and dyslipidaemia.

**Material & methods:** We recruited 370 diabetics (122 with nephropathy, 119 with hypertension and 129 with dyslipidaemia) and 120 ethnicity matched healthy controls. *TLR4* polymorphisms were evaluated using polymerase chain reaction followed by restriction fragment length polymorphism analysis. The genotyping data were compared between cases and controls using chi-square test and logistic regression analysis.

**Results:** Although there was no overall difference in the genotype frequencies of *TLR4* rs5030717 in diabetes v controls, the genotype frequencies of diabetic dyslipidaemia cases compared with controls were different ($p$ = 0.001). Overall, the rs5030718 GA and GG genotype frequencies in the entire diabetes cohort were different from those of the controls ($p$ = 0.037), and the frequencies of diabetic nephropathy cases ($p$ = 0.03) and diabetic dyslipidaemia cases were different ($p$ = 0.001) compared with controls. There were no links with diabetic hypertension.

**Conclusion:** *TLR4* polymorphisms rs5030717 and rs5030718 may be useful in predicting those type 2 diabetics who are at risk of hypertension, nephropathy and/or dyslipidaemia.

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*TLR4*; gene polymorphism; T2DM with Dyslipidaemia; T2DM with Hypertension; T2DM with Nephropathy

## Introduction

Type 2 diabetes mellitus is a metabolic disorder characterised by prolonged elevated blood glucose levels which bring a risk of developing microvascular (retinopathy, nephropathy, neuropathy) and macrovascular (coronary artery disease, peripheral arterial disease, stroke) complications [1,2]. According to the official WHO data, India is top of the list of countries, with 69 million persons affected by diabetics, a figure expected to rise to more than 123.5 million by 2040 [3,4]. Diabetes is an established risk factor for comorbid chronic conditions such as cardiovascular diseases, musculoskeletal diseases, and mental diseases [5–7]. Co-morbidity and complications (macrovascular and microvascular complications) among patients with diabetes is associated with considerable consequences for health care and related costs [8,9]. Several environmental factors and genetic factors contribute to the multiple pathophysiological disturbances that are responsible for impaired glucose homeostasis in diabetes. Early screening and prevention programme can reduce the risk of developing type 2 diabetes and its coexisting severe conditions [10].

Toll-like receptors (TLRs) are transmembrane proteins constituting an important group of pattern recognition receptors present on the cell surface or within the endosomes [11]. So far, 10 functional TLRs have been identified in humans [12], which serve as a surface receptor for lipopolysaccharides, the main endotoxins derived from Gram-negative bacteria [13]. TLRs play an important role in the activation of immune system by regulating the production of antiviral peptides and inflammatory cytokines and which leads to the development of an adaptive immune response [14–16]. Studies have suggested that low-grade inflammation, characterized by pro-inflammatory cytokine production, is associated with the pathogenic processes responsible for the development of type 2 diabetes. Furthermore, excessive production of pro-inflammatory cytokines in diabetes has been associated with the development of microvascular and macrovascular complications [17,18].

TLR-4 (CD284) is coded for by *TLR4*, located on chromosome 9q33.1. Previous studies have shown that *TLR4* is a potentially important gene linked with susceptibility to type 2 diabetes, and other features of

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the disease [19–21]. Several single nucleotide polymorphisms (SNPs) in *TLR4* (such as Asp299Gly and Thr399Ile) have been investigated for their association with diabetes and insulin resistance, although results are inconsistent [22–26]. We hypothesised that there are roles for the rs5030717 and rs5030718 variants in *TLR4* in type 2 diabetes and in three of its clinical consequences of nephropathy, hypertension and dyslipidaemia.

## Materials and methods

We tested our hypothesis in 490 subjects: 370 patients with type 2 diabetes (122 with nephropathy, 119 with hypertension, 129 with dyslipidaemia) and 120 controls, recruited from the diabetic clinic of the Department of Medicine at Era's Lucknow Medical College & Hospital, Lucknow, India. Data collection for each subject included age, sex, and body mass index, blood pressure and routine biochemical indices. Patients with overnight fasting plasma glucose > 6.99 mmol/L on two consecutive events were defined as diabetic. Cases with 24 hours urine albumin excretion rate of 30–300 mg/day (microalbuminuria) and > 300 mg/day (macroalbuminuria) were defined as diabetic nephropathy cases, cases with a mean systolic blood pressure > 140 mmHg and mean diastolic blood pressure > 90 mmHg or taking antihypertensive medications were defined as diabetic hypertensive cases, and cases with one or more lipid values increased (total cholesterol [TC], LDL and triglycerides [TG]) or decreased (high-density lipoprotein cholesterol [HDL]), alone or in combination were defined as diabetic dyslipidaemia. Control samples were defined as those with fasting blood sugar level below < 6.1 mmol/L without family history of diabetes and its complication, and none was receiving medications at the time of participation. Exclusion criteria were type 1 diabetes, gestational diabetes, maturity-onset diabetes of the young, coronary artery diseases and stroke. The project (Ref no. ELMC/R-Cell/EC/2014/100) was approved by the Ethics Committee of the Era's Lucknow Medical College and Hospital, Lucknow, India. Written informed consent was taken from all participants.

Serum creatinine levels were measured using the kinetic Jaffe method. Fasting blood sugar (glucose oxidase–peroxidase method), serum cholesterol (cholesterol oxidase–peroxidase), serum triglyceride (glycerol phosphate oxidase–peroxidase amidopyrine method), and HDL cholesterol were assessed on an XL-300 Transasia Auto-analyzer (Transasia, Mannheim, Germany). Low-density lipoprotein (LDL) was calculated by Friedewald's formula. HbA1C was measured using a semiautoanalyser (Transasia, Mannheim, Germany). For HbA1c estimation we used Gen X haemoglobin A1c-Direct kit of Gen X special live series (Proton Biologicals, India Pvt. Ltd). The kit for calculation of results apply IFCC calibrated values by using the following equation NGSP=(0.0915 X IFCC) + 2.15 expected values (NGSP units in % while IFCC units were in mmol/molHb). All the assays were performed following the standard manufacturer's protocols. All experiments were performed in accordance with the ethical standards of the Helsinki Declaration.

Genomic DNA was isolated from whole blood using DNA extraction kit (Macherey-Nage, Germany) following the manufacturers protocol. The DNA concentration was determined by a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, UK) and samples were stored at -20 °C. *TLR4* rs5030717 and rs5030718 polymorphisms were determined by polymerase chain reaction and restriction fragment length polymorphism. The primers for *TLR4* (rs5030717) were: forward 5′-CATTGGCTTGCTGTTTGCTGG -3′ and reverse 5′- GGAGGAATCATGACAAATAGCTTCC -3′ (R). The primers for *TLR4* (rs5030718) were: forward 5′-CATGAGTTCAAACTTCTTGGG -3′ and reverse 5′ -GTCAAGTTTCTCAGCTCTGTGAAG -3′. The 20 µl PCR reaction mixture had approximately 100-150 ng of genomic DNA, 10 pmol/l of each primer, 200 µmol/l of dNTPs, 20 mmol/l of TrisHCl, 50 mM of KCl, 2.5 mmol/l of $MgCl_2$, 1 U of Taq DNA polymerase and nuclease free water. The PCR Cycling Conditions include, initial denaturation at 94 °C for 5 min, followed by 33 cycles at 94 °C for 32 s, 64 °C [for rs5030717 (12375A > G)]/59 °C [for rs5030718 (14367G > A)] for30 sec, at 72 °C for 32 s, and a final extension at 72 °C for 6 min. PCR products were incubated for 10 hr at 37 °C with 5 U restriction enzyme (*MluC1* for rs5030717and *TaqI* for rs5030718 (Fermantas, Germany)), in a 20 mL reaction volume and separated by 3% agarose gel electrophoresis (Figures 1 and 2).

Data are presented as mean with standard deviation or median with interquartile range for continuous variables and proportion/percentages for categorical variables. The genotyping data were compared between cases and controls using chi-square test and logistic regression analysis. All statistical tests were performed using SPSS (Statistical Package for the Social Sciences) version 17 software.

## Results

Clinical, demographic and biochemical parameters of cases and controls are shown in Table 1. Compared to the controls, and as expected, patients with nephropathy has higher creatinine, patients with hypertension has higher SBP and DBP, and patients with dyslipidaemia has higher cholesterol and tryglycerides and lower HDL (all $p < 0.001$). The mean urine albumin level was 12.0 (27–44.5) mmol/l in diabetic

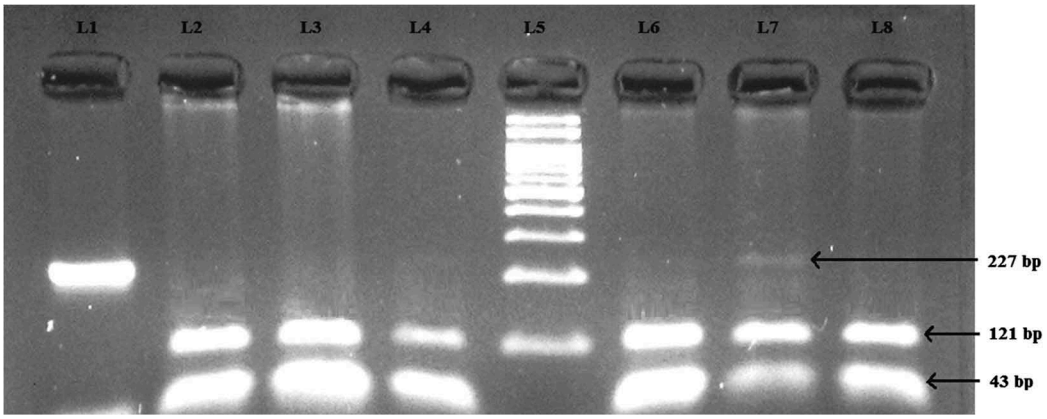


Figure 1. The 3% Agarose gel picture of *MluC1* digested products of *TLR4* 12375A>G. Lane 1 shows undigested PCR product corresponding to a band of 227 bp, Lane 7 shows AG genotype corresponding to bands of 227, 121 and 43 bp, Lane 2, 3, 4, 6, 8 shows AA genotype corresponding to band of 121 and 43 bp, whereas Lane 5 shows a 100 bp ladder.

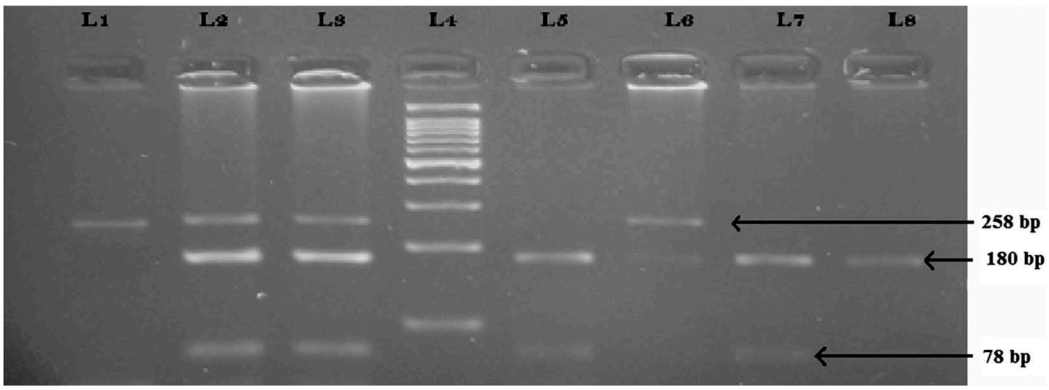


Figure 2. The 3% Agarose gel picture of *Taq1* digested products of *TLR4* 14367G>A. Lane 1 shows undigested PCR product corresponding to a band of 258 bp, Lane 2, 3, 6 shows GA genotype corresponding to bands of 258, 180 and 78 bp, Lane 5, 7, 8 shows the GG genotype corresponding to band sizes of 180 and 78 bp, whereas Lane 4 shows a 100 bp ladder.

Table 1. Clinical and biochemical parameters in controls and diabetics.

| Parameter | Controls ($n$ = 120) | Diabetic nephropathy ($n$ = 122) | Diabetic hypertension ($n$ = 119) | Diabetic dyslipidaemia ($n$ = 129) |
|---|---|---|---|---|
| Age (years) | 49 (10.2) | 51 (10.7) | 51 (11.3) | 50 (11.4) |
| Sex (M/F) | 64/56 | 57/65 | 58/61 | 59/70 |
| BMI (kg/m$^2$) | 24 (2.6) | 25 (4.8) | 28 (12.4) | 28 (8.7) |
| SBP (mm Hg) | 129 (10) | 137 (16) | 147 (17) | 130 (13) |
| DBP (mm Hg) | 83 (5) | 84 (12) | 91 (11) | 85 (8) |
| RBS (mmol/l) | 6.5 (1.1) | 12.9 (5.2) | 11.7(5.2) | 12.4(5.0) |
| Creatinine (μmol/l) | 81 (78–97) | 167 (134–186) | 106 (80–124) | 99 (80–115) |
| HbA1c (%) | 5.6 (0.4) | 8.1 (1.6) | 8.1(1.5) | 7.5 (1.7) |
| Cholesterol (mmol/l) | 9.4 (1.5) | 10.1 (1.5) | 9.3(1.6) | 11.2 (2.8) |
| Triglyceride (mmol/l) | 8.1 (6.1–10.3) | 8.3 (8.0–15.3) | 8.2 (7.3–8.9) | 9.5 (8.2–13.8) |
| HDL(mmol/l) | 3.1 (0.6) | 2.5 (0.4) | 2.5 (0.3) | 2.2 (0.4) |
| VLDL(mmol/l) | 1.8 (0.6) | 1.8 (0.5) | 1.7 (0.5) | 2.0 (0.4) |
| LDL(mmol/l) | 5.2 (1.2) | 5.5 (4.03) | 4.7 (1.7) | 5.8 (3.1) |

Notes: Data presented as mean (SD) or median (IQR), sex as number of subjects. SBP: systolic blood pressure; DBP: diastolic blood pressure; RBS: random blood sugar; Hb A1C: glycated hemoglobin; HDL: high-density lipoprotein; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein.

nephropathy cases, and 1.2 (1.05–1.33) mmol/L in the controls ($p < 0.001$).

The genotype distribution of both variants of *TLR4* were in good agreement with the predicted Hardy-Weinberg equilibrium rs5030717 $p = 0.5$ in cases and $p = 0.58$ in the controls: rs5030718 $p = 0.26$ in the cases and 0.32 in the controls). Overall, the rs5030717 AG, AA and GG genotype frequencies of the entire diabetes cohort ($n$ = 147, 206 and 17 patients respectively) was no different from those of the controls ($n$ = 52, 60 and 8 respectively) ($p = 0.452$). However, in sub-group analysis, the frequencies in diabetic dyslipidaemia cases differed from those of the control group, whereas in the diabetic nephropathy cases and diabetic hypertensive cases were no different from those in the controls (Table 2a). Overall the

**Table 2a.** Genotype frequencies of *TLR4* rs5030717 polymorphisms.

| Genotype | *N* (%) | *N* (%) |
|---|---|---|
| | Controls | Diabetes + nephropathy |
| AG | 52 (43.3) | 52 (42.7) |
| AA | 60 (50.0) | 68 (55.7) |
| GG | 8 (6.7) | 2 (1.6) |
| | | $p = 0.130$ |
| | Controls | Diabetes + hypertension |
| AG | 52 (43.3) | 65 (54.6) |
| AA | 60 (50.0) | 43 (36.2) |
| GG | 8 (6.7) | 11 (9.2) |
| | | $p = 0.094$ |
| | Controls | Diabetes + dyslipidaemia |
| AG | 52 (43.3) | 30 (23.3) |
| AA | 60 (50.0) | 95 (73.6) |
| GG | 8 (6.7) | 4 (3.1) |
| | | $p = 0.001$ |

Note: *N* = no. of subjects.

**Table 2b.** Genotype frequencies of *TLR4* rs5030718 polymorphisms.

| Genotype | *N* (%) | *N* (%) |
|---|---|---|
| | Controls | Diabetes + nephropathy |
| AG | 21 (17.5) | 10 (8.2) |
| GG | 99 (82.5) | 112 (91.8) |
| AA | 0 | 0 |
| | | $p = 0.03$ |
| | Controls | Diabetes + hypertension |
| AG | 21 (17.5) | 22 (18.5) |
| GG | 94 (82.5) | 95 (79.8) |
| AA | 0 | 2 (1.7) |
| | | $p = 0.350$ |
| | Controls | Diabetes + dyslipidaemia |
| AG | 21 (17.5) | 6 (5.0) |
| GG | 99 (82.5) | 122 (95.0) |
| AA | 0 | 0 |
| | | $p = 0.001$ |

Note: *N* = no. of subjects.

**Table 3.** The genotypes frequency of *TLR4* rs5030717 and rs5030718 polymorphisms in diabetics with different clinical conditions.

| Gene | Genotype | *N* (%) | *N* (%) |
|---|---|---|---|
| | | Nephropathy | Hypertension |
| rs5030717 | AG | 52 (42.7) | 65 (54.6) |
| | AA | 68 (55.7) | 43 (36.2) |
| | GG | 2 (1.6) | 11 (9.2) |
| | | | $p = 0.001$ |
| rs5030718 | GA | 10 (8.2) | 22 (18.5) |
| | GG | 112 (91.8) | 95 (79.8) |
| | AA | 0 | 2 (1.7) |
| | | | $p = 0.020$ |
| | | Hypertension | Dyslipidaemia |
| rs5030717 | AG | 65 (54.6) | 30 (23.3) |
| | AA | 43 (36.2) | 95 (73.6) |
| | GG | 11 (9.2) | 4 (3.1) |
| | | | $p < 0.001$ |
| rs5030718 | GA | 22 (18.5) | 6 (5) |
| | GG | 95 (79.8) | 122 (95) |
| | AA | 2 (1.7) | 0 |
| | | | $p = 0.001$ |
| | | Nephropathy | Dyslipidaemia |
| rs5030717 | AG | 52 (42.7) | 30 (23.3) |
| | AA | 68 (55.7) | 95 (73.6) |
| | GG | 2 (1.6) | 4 (3.1) |
| | | | $p = 0.004$ |
| rs5030718 | GA | 10 (8.2) | 6 (5) |
| | GG | 112 (91.8) | 122 (95) |
| | AA | 0 | 0 |
| | | | $p = 0.257$ |

rs5030718 GA and GG genotype frequencies in the entire diabetes cohort ($n = 38, 329$ respectively) was different from those of the controls ($n = 21, 99$ respectively) ($p = 0.037$). The AA analysis was excluded as numbers are too small. In sub-group analysis, the frequencies of the diabetic hypertension cases were no different from those of the controls, but the frequencies of the nephropathy and dyslipidaemia cases did differ from those of the controls (Table 2b).

Table 3 shows genotype frequencies between the three groups of diabetics. Frequencies in both *TLR4* variants differed between nephropathy and hypertension cases. Frequencies in both *TLR4* variants differed between dyslipidaemia and hypertension cases. However, in comparing frequencies between nephropathy and dyslipidaemia, there was a difference in the rs5030717 variant, but not in the rs5030718 variant.

## Discussion

Type 2 diabetes mellitus (formerly non-insulin-dependent diabetes) is a complex metabolic disease due to hyperglycemia and the product of peripheral insulin resistance and reduced insulin secretion [27]. Pathophysiology describes a complex interplay between genetic, epigenetic and environmental factors. Obesity and physical inactivity are considered the major environmental risk factors and 80–90% of diabetics are overweight or obese [28]. Previous studies have genotyped the common gene variants associated with diabetes in different populations, and point to the susceptibility genes such as *PPARG, IGF2BP2, KCNJ11,* SDF-1β, *ADAMTS9, NOTCH2, CDKAL1 CDC123/CAMK1D, TSPAN8/ LGR5, KCNQ1* and *TLR4* [21,22,29,30]. *TLR4* codes for a cell surface receptor that plays an important role in the activation of innate immune response to pathogens, upon activation it triggers the signaling cascade leading to the generation of pro-inflammatory cytokines and chemokines, many being involved in the development of type 2 diabetes and its complications [11,12,31].

There are a growing number of studies seeking to associate various *TLR4* gene polymorphisms with diabetes and cardiovascular disease in different ethnic and racial populations [25,32–38]. Peng et al., in a well-powered study, reported no association in any of seven *TLR4* SNPs (but not those we studied) with diabetic nephropathy [35], whereas Kuwabara et al. observed that renal *TLR4* expression was significantly higher in a murine model of diabetic nephropathy [36]. Our contribution is firstly that there is no difference in the genotype frequencies of rs5030717 in type 2 diabetes, but that the frequencies of rs5030718 are different. Secondly, we report clinical links. We observed no significant association of *TLR4* rs5030717 with diabetic nephropathy, while *TLR4* rs5030718 did show a significant association with nephropathy. A potential role of

*TLR4* rs4986790 in the risk of metabolic syndrome has been reported [37], whilst Schneider et al reported that the *TLR4* rs4986790 is associated with age-dependent blood pressure increase in patients with coronary artery disease [38]. Schneider et al. also [39] found that, in patients about to undergo coronary artery angiography, systolic blood pressure increase with obesity was blunted in cases with *TLR4* SNP rs4986790. In contrast with these results, we observed no significant association between *TLR4* rs5030717 and rs5030718 variants and hypertension in diabetes.

We acknowledge a number of limitations of our study. The sample size is modest, and for this reason we are not over-interpreting our data (such as sub-analysis for the effect of BMI [39]), and that the classification of patients may be flawed in that it is influenced by the effects of medications. Although the leading cause of diabetes is obesity and lack of exercise [28], these do not account for all disease [40], especially in co-morbidities, and genetic factors are being increasingly recognised as having a role. This work represents an advance in biomedical science because it shows that *TLR4* rs5030717 variant is associated with dyslipidaemia in diabetes whereas the rs5030718 variant is associated with nephropathy and dyslipidaemia.

## Summary table

*What is known about this subject*

- Macrovascular and microvascular complications among patients with diabetes is associated with considerable consequences for health care and related costs.
- Genetic linkage analyses and candidate gene approaches have implicated several loci and candidate genes, such as *TLR-4*, for predisposition to diabetes, and it comorbidities and complications.

*What this paper adds*

- There is no difference in the genotype frequencies of *TLR4* rs5030717 in diabetes, but the rs5030718 genotype frequencies is different.
- The genotype frequencies of rs5030717 in diabetic dyslipidaemia are altered.
- The genotype frequencies of rs5030718 in diabetic dyslipidaemia and nephropathy are altered compared to healthy controls, and those in dyslipidaemia are different from those of nephropathy.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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