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Uromodulin rs4293393 T>C variation is associated with kidney disease in patients with type 2 diabetes

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***Background & objectives*: Uromodulin, a *UMOD* gene encoded glycoprotein is synthesized exclusively in renal tubular cells and released into urine. Mutations lead to uromodulin misfolding and retention in the kidney, where it might stimulate cells of immune system to cause inflammation and progression of kidney disease. Genome-wide association studies (GWAS) have identified *UMOD* locus to be associated with hypertension and diabetic nephropathy (DN). In this study, we investigated the association between rs4293393 variation in *UMOD* gene and susceptibility to kidney disease in individuals with type 2 diabetes mellitus (T2DM).**

***Methods*: A total of 646 individuals, 208 with T2DM without evidence of kidney disease (DM), 221 with DN and 217 healthy controls (HC) were genotyped for *UMOD* variant rs4293393T>C by restriction fragment length polymorphism. Serum uromodulin levels were quantified by enzyme-linked immunosorbent assay.**

***Results*: A significant difference was found in genotype and allelic frequency among DM, DN and HC. TC+CC genotype and C allele were found more frequently in DN compared to HC (33.9 vs 23.0%, *P*=0.011 and 20.1 vs 12.9%, *P*=0.004, respectively). Compared to DM, C allele was found to be more frequent in individuals with DN (20.1 vs 14.7%, *P*=0.034). Those with DN had higher serum uromodulin levels compared to those with DM (*P*=0.001). Serum uromodulin levels showed a positive correlation with serum creatinine (r=0.431; *P*<0.001) and negative correlation with estimated glomerular filtration rate (r=−0.423; *P*<0.001).**

***Interpretation & conclusions*: The frequency of *UMOD* rs4293393 variant with C allele was significantly higher in individuals with DN. *UMOD* rs4293393 T>C variation might have a bearing on susceptibility to nephropathy in north Indian individuals with type 2 diabetes.**

Key words Diabetic nephropathy - polymorphism - uromodulin

Uromodulin, a glycosylphosphatidylinositol-anchored glycoprotein, is exclusively expressed in the thick ascending loop of Henle[1] and distal convoluted tubule[2] of the mammalian kidney. It is exclusively produced in the kidney and secreted into the urine[3]. The biological function of uromodulin remains elusive. It can bind with immunoglobulin G, complement 1q and tumor necrosis factor-alpha (TNF-α) suggesting a role in innate immunity[4-6]. In animal studies, immunization with homologous urine or purified uromodulin resulted in cellular immune response and tubulointerstitial nephritis[7]. This led to the suggestion that interstitial release of uromodulin after tubular damage can act as a signal to recruit immune cells[7]. Moonen *et al*[8] demonstrated the inability of uromodulin to bind with native cytokines *in vitro*. In contrast to these studies, El-Achkar *et al*[9] proposed a renoprotective role of uromodulin in ischaemia-reperfusion injury, using Tamm-Horsfall protein knockout mouse model.

Uromodulin-induced renal inflammation and damage may be due to intracellular retention or delayed translocation to outer membrane. Variations can cause delay in protein export by increasing retention time in the endoplasmic reticulum[10-12]. *UMOD* mutations have been shown to be associated with urinary concentration defect, salt wasting, hyperuricaemia, gout, hypertension and end-stage renal disease (ESRD)[13]. Uromodulin has been linked to medullary cystic kidney disease, glomerulocystic kidney disease, urinary tract infections, nephrolithiasis and acute kidney injury[9,11,13,14].

Genome-wide association studies (GWAS) have shown that single-nucleotide polymorphisms (SNPs) in *UMOD* gene (rs12917707 and rs42993393) were associated with chronic kidney disease (CKD)[15-17]. The BPGen consortium identified an association of the rs13333226 minor allele with higher estimated glomerular filtration rate (eGFR) and reduced risk of hypertension and cardiovascular disease[18]. *UMOD* gene missense mutation p.V458L was associated with reduced glomerular filtration rate in healthy individuals[19]. Another study[20] showed an association between *UMOD* SNP rs13333226 and hypertension and CKD in Swedish individuals with type 2 diabetes. This study was undertaken to evaluate the frequency of *UMOD* rs4293393 T>C in north Indian individuals with type 2 diabetes and to examine its association with kidney disease.

Material & Methods

This study was done in the department of Nephrology, Postgraduate Institute of Medical Education and Research, Chandigarh, a large tertiary care hospital in north India during July 2011 to December 2014. The study was approved by the Institute Ethics Committee, and all individuals provided written informed consent. A total of 429 (290 male and 139 female) patients with type 2 diabetes diagnosed according to the World Health Organization criteria[21] were recruited consecutively from the Nephrology and Endocrinology Clinic. Inclusion criteria for diabetic nephropathy (DN) (n=221) were individuals with diabetic retinopathy, eGFR <60 ml/min and/or proteinuria >500 mg/day, sustained for more than or equal to three months in the absence of another cause, and inclusion criteria for diabetic without nephropathy (n=208) were individuals with disease duration greater than five years, normal blood pressure, eGFR >60 ml/min and urinary albumin <150 mg/day or negative on dipstick urinary analysis. Also, 217 healthy individuals were also included with no diabetes or kidney disease. These healthy individuals were healthy prospective voluntary kidney donors.

Determination of UMOD rs42993393 T>C genotype: Peripheral leucocytes were isolated[22] from ethylenediaminetetraacetic acid (EDTA)-treated whole blood obtained from each patient, and genomic DNA was extracted using Qiagen DNA Isolation Kit (Qiagen, Hilden, Germany) for polymerase chain reaction (PCR) amplification of *UMOD* gene. The primer set used for the PCR amplification was: forward 5’ - GTGCAAATTTATTTCGCCTCCA -3’ and reverse 5’ - GGACTACCTTCTGGTTCTGACTTTCA -3’. Amplification was done for 30 cycles with the following cycle parameters: 95°C for 1 min, annealing at 59°C for 30 sec, followed by extension at 72°C for 30 sec and final elongation at 72°C for 10 min. SNP was analyzed with restriction fragment length polymorphism (RFLP) using *Msp1* restriction enzyme (New England BioLab Inc., USA). *Msp1* specifically cut at the CˇCGG to produce two products of size 87 base pair (bp) and 27 bp, which were resolved in 2 per cent agarose gel along with 100 bp DNA ladder and visualized by ethidium bromide staining.

The data obtained from RFLP were further confirmed by nucleotide sequencing (Applied Biosciences, Germany) of gene fragment (167 bp), which was amplified using specific primer set: forward 5’ -GGACCTCCCAGTCATCAGAC-3’ and reverse 5’ -GGCACCCTTCTGAAACACCC-3’. All primers were designed using *https://www.ncbi.nlm.nih.gov/tools/primer-blast/* and synthesized by Sigma-Aldrich, USA.

Serum level of uromodulin was measured in 40 DM, 80 DN and 40 healthy control (HC) individuals by enzyme immunoassay using a commercial kit (USCN Life Science, USA) as per the manufacturer’s instructions. This kit detected <5.8 pg/ml without any cross-reactivity.

Statistical analysis: Assuming difference in minor allele frequency of 12 per cent between controls and individuals with DN, a sample size of 209 individuals in each group was required to achieve power of 80 per cent at alpha of 5 per cent. Data are presented as mean±standard deviation unless indicated otherwise. Hardy-Weinberg equilibrium was calculated for SNP in each group using Michael H. Court's (2005–2008) online calculator (*http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20-%20HW%20calculator.xls*). Difference between groups were tested using Student's *t* test and Chi square test for continuous and nominal variables, respectively, while skewed distributed parameters were analyzed with Mann–Whitney U-test. The allelic and genotype association of SNP were evaluated by Pearson's Chi-square test; and odds ratio (OR) and 95 per cent confidence intervals were determined. For comparison of more than two groups, one-way ANOVA was used. Correlation analysis was done using Spearman's rank correlation. Two-tailed $P<0.05$ was considered significant. All analyses were performed using SPSS 16.0 (SPSS, Chicago IL, USA).

Results

Table I describes the clinical features of study individuals. There was no difference in age and gender distribution between groups. The duration of diabetes was longer and the prevalence of neuropathy and retinopathy was higher in individuals with kidney disease.

UMOD rs42993393 T>C and risk of kidney disease: Studied SNP followed Hardy–Weinberg equilibrium in healthy individuals (P=0.15, χ^2=2.08) and all individuals with diabetes (P=0.10, χ^2=2.68). Compared to healthy individuals, those with diabetes had a higher frequency of CC+TC genotypes (P=0.013; OR=1.59) and C allele (P=0.033, OR=1.43). This was primarily driven by individuals with kidney disease. The C allele

Table I. Demographic and clinical characteristics of the study subjects

Characteristics	DM (n=208)	DN (n=221)	Healthy controls (n=217)
Age (yr)	51.9±9.4	52.4±8.9	53.9±11.3
Number of men/women	140/68	150/71	133/84
BMI (kg/m^2)	26.1±4.5	25.39±3.9	22.5±3.6
Duration of diabetes (yr)	9.9±4.4	13.9±7.0***	-
SBP (mm Hg)	135.1±18.7	142.2±21.0***	113.6±12.6
DBP (mm Hg)	82.6±10.6	85.7±11.3**	81.2±9.1
Fasting blood sugar (mg/dl)	131.2±62.5	137.4±51.9	76.5±12.3
Post-prandial blood sugar (mg/dl)	192.0±70.8	196.6±73.4	ND
Albuminuria (mg/24 h)	96.6±65.2	1623.1±1833.0***	ND
Total cholesterol (mg/dl)	170.9±42.2	178.8±55.6	ND
HDL (mg/dl)	45.8±11.6	45.02±15.1	ND
LDL (mg/dl)	95.3±35.1	99.7±45.3	ND
Triglyceride (mg/dl)	154.5±74.0	169.4±93.1	ND
HbA_{1c} (%)	7.5±1.5	8.0±2.0*	ND
Neuropathy, n (%)	104 (50.0)	135 (61.1)***	0
Retinopathy, n (%)	59 (28.4)	221 (100.0)***	0
CAD, n (%)	39 (19.1)	41 (16.6)	0
Serum creatinine (mg/dl)	0.98±0.20	2.09±1.57***	1.01±0.06
Serum uromodulin (pg/ml)	49.3±25.6	72.3±37.7***	51.18±19.97
eGFR (min/ml/1.73m^2)	78.9±14.0	56.7±28.0***	-
eGFR (<60 min/ml/1.73m^2), n (%)	0	101 (46.5)	-

P *<0.05, **<0.01, ***<0.001 compared to DM group. Unless indicated otherwise, data are given as the mean±SD.
DM, Type 2 diabetes mellitus but no nephropathy; DN, diabetic nephropathy; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CAD, coronary artery disease; eGFR, estimated glomerular filtration rate; ND, not done; SD, standard deviation; HbA_{1c}, glycated haemoglobin

Table II. Genotype and allele frequencies of rs42993393 T > C genetic variations in the *UMOD* genes in the diabetic with and without nephropathy and healthy control groups

Groups (n)	Genotype, n (%)			OR (95% CI)	*P* (CC+TC vs TT)	Allele, n (%)		OR (95% CI)	*P* (C vs T)
	TT	TC	CC			T	C		
HC (217)	167 (76.9)	44 (20.3)	6 (2.8)			378 (87.1)	56 (12.9)		
Diabetic (429)	297 (69.2)	114 (26.6)	18 (4.2)	1.59 (1.09-2.32)[A]	0.013[A]	708 (82.5)	150 (17.5)	1.43 (1.01-1.99)[A]	0.033[A]
DM (208)	151 (72.5)	53 (25.5)	4 (2)	1.26 (0.81-1.95)[A]	0.300[A]	355 (85.3)	61 (14.7)	1.66 (0.75-1.64)[A]	0.456[A]
DN (221)	146 (66)	61 (27.7)	14 (6.3)	1.71 (1.12-2.61)[A] 1.36 (0.9-2.05)[B]	0.011[A] 0.143[B]	353 (79.8)	89 (20.2)	1.70 (1.18-2.45)[A] 1.46 (1.02-2.09)[B]	0.004[A] 0.034[B]

[A]OR and *P* value for DN/DM/diabetic vs HC; [B]OR and *P* value for DN vs DM. HC, healthy control; OR, odds ratio; CI, confidence interval

Table III. Clinical characteristics of diabetic subjects; with and without diabetic nephropathy (CC+CT vs TT)

Characteristics	DM (n=208)		DN (n=221)	
	CC+CT	TT	CC+TC	TT
Age (yr)	51.9±8.7	52.2±9.6	52.3±8.2	53.2±9.2
BMI (kg/m^2)	25.8±3.6	26.2±4.8	25.4±4.2	25.1±3.7
Duration of diabetes (yr)	10.4±6.3	9.6±6.4	13.7±7.8	13.9±6.5
SBP (mm Hg)	135.6±18.7*	133.2±18.1	140.7±22.1	143.4±20.4
DBP (mm Hg)	81.5±10.3	82.9±10.1	85.1±10.3	86.2±11.7
Fasting blood sugar (mg/dl)	130.6±49.1	130.0±67.1	139.2±50.1	136.4±52.6
Post-prandial blood sugar (mg/dl)	196.8±76.7	189.7±68.1	205.8±75.4	201.7±73.3
Total cholesterol (mg/dl)	166.2±77.3	150.1±72.1	184.4±57.5	175.5±54.4
HDL (mg/dl)	47.1±11.6	45.3±11.7	44.51±13.5	45.4±16.1
LDL (mg/dl)	102.5±38.2	92.5±33.2	104.8±48.9	96.6±42.9
Triglyceride (mg/dl)	166.2±77.8	150.1±72.1	160.2±77.1	174.7±89.7
HbA_{1c} (%)	7.7±1.6	7.5±1.4	8.3±1.9	7.8±1.7
Serum creatinine (mg/dl)	0.9±0.2	1.1±0.2	2.1±1.8	2.3±1.1
eGFR (min/ml/1.73 m^2)	77.65±13.89	78.86±14.0	59.3±27.7	55.40±28.2
eGFR (<60 min/ml/1.73 m^2), n (%)	0	0	30 (40.0)	71 (48.6)
Serum uromodulin# (pg/ml)	46.2±25.6	53.6±26.4	70.3±40.4	73.7±36.08

*P<0.05 compared to TT in DM group. Data are given as the mean±SD. #Serum uromodulin level of 40 DM samples and 80 DN samples; Student's *t* test and Chi-square test were performed wherever appropriate.

and CC+TC genotype frequency in DN individuals were significantly higher (*P*=0.003; OR=1.70 and *P*=0.01; OR=1.71, respectively) compared to HC, whereas those with diabetes but no kidney disease showed a similar genotype distribution as HC (*P*=0.30; OR=1.26 and C: *P*=0.57; OR=1.11, respectively). Upon comparison of DM and DN, those with DN showed a significantly higher frequency of C allele (*P*=0.03; OR=1.46) (Table II).

Serum uromodulin: Individuals with DN exhibited elevated serum uromodulin level compared to those with DM (72.32±37.76 pg/ml vs 49.32±25.61 pg/ml, *P*=0.001) and HC (51.18±19.91, *P*=0.001). Serum uromodulin levels showed a positive correlation with serum creatinine (r=0.431, *P*<0.001) and inverse correlation with eGFR (r=−0.423, *P*<0.001) in diabetic individuals.

Genotype-phenotype and UMOD rs42993393 T>C: The biochemical and clinical parameters and serum uromodulin were compared based on genotype distribution. None of the clinical parameters defining the renal complications or serum uromodulin level showed significant association with C allele (Table III).

Discussion

In this study an attempt was made to link *UMOD* gene variant rs42993393 with kidney disease among north Indian individuals with type 2 diabetes. This SNP, located 550 bp upstream to uromodulin transcription site, has been linked to kidney disease in a couple of studies[16,23,24]. The frequency of C allele and TC+CC genotype was found to be different in the overall population of the individuals with diabetes compared to HCs. Further, the frequency of C allele was higher in DN compared to HC and DM individuals, whereas there was no difference between HC and DM. Our results indicate that C allele and genotype with C allele may confer the risk of kidney disease in individuals with diabetes.

Gudbjartsson *et al*[16] found an association of T allele with elevated serum creatinine, uric acid and lower risk of calcium-containing kidney stone formation. They also demonstrated that hypertensive and type 2 diabetes patients carrying T allele had higher serum creatinine after the age of 50 yr compared to those without this variant. Köttgen *et al*[23] investigated the functional link between this SNP and uromodulin secretion. They found that increased secretion of uromodulin preceded the development of CKD. A study showed that rs4293393 TT genotype was independently associated with reduced eGFR[24]. The genotype and allelic frequency distribution of this SNP in our population were in contrast with previous reports[16,23,24]. However, other studies have shown either no difference in frequency of rs42993393 genotype/allele in patients with urinary tract infection in multi-centric cohort study[25] or protection against kidney stone[16].

Apart from rs42993393 variation in UMOD, some other variations have also been studied in diseases with renal impairment. Gómez *et al*[19] found a missense mutation p.V458L in which leucine variant was more frequent in individuals with reduced GFR as compared to healthy individuals with normal GFR. Associations of *UMOD* rs13333226 G allele with hypertension, CKD[20] and ESRD[26] have been reported. However, Cui *et al*[27] reported association of rs13333226G allele with slower decline in renal function in individuals with CKD. A study of *UMOD* variant rs12917707 in Italian diabetic cohort, no association was found with renal function[28]. In another study, there was no association between rs12917707 and IgA nephropathy or progression to ESRD[29]. Observations from these studies suggest that UMOD might be a strong genetic determinant of kidney function in some diseases such as diabetes and hypertension, but this association is modified by heterogeneity in populations.

In the present study it was found that the level of serum uromodulin in individuals with DN was raised compared to DM and HC individuals. However, the level was not affected by the distribution of rs4293393 genotype. An earlier study in non-diabetic individuals suggested that lower urinary and higher serum levels of uromodulin were associated with kidney disease[30]. Prajczer *et al*[30] investigated the serum and urinary uromodulin levels in 77 CKD patients and found a significant association of eGFR with urinary uromodulin and a trend showing inverse correlation with serum uromodulin. Other studies[24,31] showed an association of eGFR with plasma/urine uromodulin level. Our results were consistent with these studies.

The inverse relationship between serum uromodulin and eGFR suggests that uromodulin may accumulate as the GFR goes down. Alteration in the accumulation of uromodulin in the tubulointerstitial compartment can also affect serum uromodulin levels. High interstitial uromodulin concentrations can induce inflammation. In one study, serum uromodulin concentrations correlated with levels of proinflammatory cytokines, *viz.* TNF-α, IL-6 and IL-8[30].

The limitations of our study were lack of treatment data and cross-sectional design of the study. Caution is needed while attributing causality to the relationship between uromodulin and development of kidney disease as our study infers just association. Although we did not find an association between the presence of C/T allele and uromodulin levels in those with diabetes but no kidney disease at the time of the study, it would be interesting to follow these individuals to see if they develop kidney disease with increasing duration of diabetes.

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***Conflicts of Interest*:** None.

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