Urinary connective tissue growth factor level in relation to nephropathy and retinopathy in patients with type 2 diabetes

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Introduction
Connective tissue growth factor (CTGF), a major promoter of fibrosis, has been linked to diabetic complications. The aim of this observational case–control study was to investigate urinary connective tissue growth factor (uCTGF) as a marker of diabetic nephropathy and diabetic retinopathy (DR) in a cohort of patients with type 2 diabetes mellitus (T2DM).

Patients and methods
The study included 60 patients with T2DM (group A: 20 patients with normoalbuminuria; group B: 20 patients with microalbuminuria, and group C: 20 patients with macroalbuminuria) and 20 age-matched and sex-matched healthy participants as controls. Thorough clinical evaluation and fundus examination were done to determine the presence of DR. Laboratory workup included glycated hemoglobin, serum creatinine level, estimated glomerular filtration rate (eGFR) using modification of diet in renal disease (MDRD) formula, urine albumin to creatinine ratio (UACR), and uCTGF measurements.

Results
uCTGF levels showed a stepwise increase in group A (116.2±33.4), group B (197.9±75.7), and group C (365.5±197.3) ($P<0.001$). In patients with T2DM, uCTGF showed significant positive correlation with UACR ($r=0.731$, $P<0.001$) and HA1c ($r=0.230$, $P=0.038$) but negative correlation with eGFR ($r=-0.421$, $P<0.001$). uCTGF was an independent predictor of eGFR (β coefficient=–0.042, $P=0.017$) and UACR (β coefficient=0.720; $P<0.001$). In contrast, patients with DR had no significantly different uCTGF level from patients without retinopathy (329.18±47.149 vs. 252.02±27.3, $P=0.067$).

Conclusion
In the present study, uCTGF was significantly associated with markers of nephropathy but not with retinopathy in patients with T2DM.

Keywords:
nephropathy, retinopathy, type 2 diabetes mellitus, urinary connective tissue growth factor

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Introduction

Microvascular complications are the leading cause of multiple disabling conditions in diabetic patients. Diabetic nephropathy (DN) is one of the leading causes of end-stage renal disease (ESRD) worldwide [1]. Moreover, diabetic retinopathy (DR), the most common diabetic microvascular complication, is a leading cause of blindness globally [2].

Tissue fibrosis characterized by extracellular matrix (ECM) accumulation and angiogenesis is one of the important pathogenic mechanisms in diabetic complications [3]. It is induced by hyperglycemia and other metabolic disturbances associated with diabetes causing tissue injury and induction of profibrotic cytokines [4].

Connective tissue growth factor (CTGF) is a matricellular protein which has a potent profibrotic effect implicated in ECM formation [5]. It stimulates ECM synthesis directly or acts as a downstream mediator or as a co-factor for TGF-β signaling [6]. Plasma levels of CTGF are increased in nondiabetic and diabetic states including cardiomyopathy, liver fibrosis, and nephropathy [7].

CTGF is expressed in the kidney by mesangial cells, tubular cells, and podocytes [4]. Both plasma and urinary connective tissue growth factor (uCTGF) levels were elevated in type 1 diabetic patients with DN [8,9]. Moreover, plasma CTGF was shown to predict ESRD and mortality in macroalbuminuric patients [10]. In addition, uCTGF was found to be a prognostic indicator of progression of DN in patients with type 2 diabetes mellitus (T2DM) [11]. Increasing data indicate

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significant role of CTGF in the pathogenesis of DR [12,13]. CTGF was found to be expressed in the retina of patients with early DR [12]. In vitreous body of diabetic patients without clinical signs of DR, CTGF levels are increased [14]. Furthermore, plasma CTGF was found to be higher in type 1 diabetic patients with retinopathy than those with normal fundi [15]. However, uCTGF relation to DR needs further evaluation in patients with T2DM.

Although ACR is the standard method to assess severity of DN, recent studies are conflicting regarding the sensitivity and the specificity of urinary albumin [3,16]. New markers accurately categorizing patients with DN according to disease severity will help in disease monitoring and applying appropriate therapy. The aim of the current study was to evaluate uCTGF as a potential marker of diabetic nephropathy and to assess its relation to the presence of DR in T2DM patients.

Patients and methods

This cross-section study included 60 patients with T2DM and 20 healthy participants selected as controls. Patients were classified into three groups: 20 patients with normoalbuminuria as group A, 20 patients with microalbuminuria as group B, and 20 patients with macroalbuminuria as group C. Exclusion criteria included patients with ESRD, urinary tract infection, liver cirrhosis, congestive heart failure, malignancy, connective tissue disease, interstitial lung disease, acute kidney injury, and any type of diabetes other than T2DM.

All patients were subjected to thorough medical evaluation including determination of duration of diabetes, medications, cardiovascular risk factors, history of coronary heart disease, stroke, or atherosclerosis, BMI, and blood pressure measurement. Biochemical tests including fasting blood glucose, glycosylated hemoglobin (HbA1c), total cholesterol, complete blood count, serum creatinine, estimated glomerular filtration rate (eGFR) using modification of diet in renal disease (MDRD) formula, complete urine analysis, urine albumin to creatinine ratio (UACR), and uCTGF measurement. Pelvi-abdominal ultrasound was done to determine the kidney size and echogenicity. Fundus examination was done by a single ophthalmologist to determine the presence of DR.

The participants were recruited from tertiary care hospital outpatient clinics after approval of the institutional ethical committee. All patients provided informed consent to participate in this study. The study protocol and procedures conform to the ethical guidelines of the 1975 declaration of Helsinki.

Urinary connective tissue growth factor assay principle

Urine samples were centrifuged for 20 min at ~1000×g and stored at −80°C. Human CTGF ELISA kit (Elabscience Biotechnology Co. Ltd, Wuhan, Hubei, China) was used to quantify uCTGF level in pg/ml that employs the quantitative sandwich enzyme immunoassay technique using a biotinylated detection antibody specific for CTGF. The test was performed according to manufacturer's instructions.

Statistical analyses

Data were coded and entered using the statistical package SPSS version 15 (SPSS Inc., Chicago, Illinois, USA). The data were summarized using descriptive statistics: mean, SD, minimal and maximum values for quantitative variables and number and percentage for qualitative values. Statistical differences between groups were tested using χ^2-test for qualitative variables, independent sample t-test for quantitative normally distributed variables, whereas nonparametric Mann–Whitney test was used for quantitative variables that are not normally distributed. Spearman's correlation was done to test for linear relations between variables. Linear regression analysis was done to detect predictors of eGFR and UACR. Full receiver operating characteristic analyses were made to determine the sensitivity, specificity, and positive and negative predictive values of uCTGF as marker of albuminuria. P values less than or equal to 0.05 were considered statistically significant.

Results

Characteristics of patients and controls are shown in Table 1. Patients in groups B and C had significantly higher uCTGF levels than control group ($P<0.001$). Patients in group A had significantly lower uCTGF level than groups B and C ($P<0.001$). Moreover, uCTGF was higher in group C than group B ($P=0.002$) (Fig. 1). Higher percentage of patients in group C had DR (65%) versus those in group B (15%) ($P=0.002$), and none of group A patients had DR.

Spearman's correlation analysis of uCTGF level with different parameters in T2DM patients showed significant positive correlation with age ($r=0.469$, $P<0.001$), duration of diabetes ($r=0.471$, $P<0.001$), cholesterol level ($r=0.293$, $P=0.007$), HbA1c ($r=0.230$, $P=0.038$), serum creatinine level ($r=0.421$, $P<0.001$), and UACR ($r=0.731$, $P<0.001$). However, uCTGF had

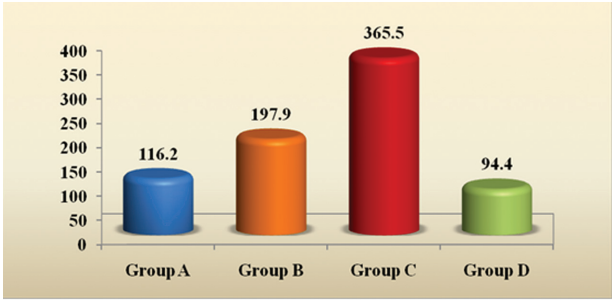
negative correlation with eGFR (r=–0.421, P<0.001). uCTGF had no significant correlation with hypertension (P=0.858) and BMI (P=0.068) (Table 2).

Multivariate regression analysis was performed to investigate predictors of UACR and eGFR in diabetic patients (Table 3). uCTGF was the only significant independent predictor of UACR in diabetic patients (P<0.001). However, UACR but not uCTGF was the significant predictor of eGFR.

The area under the receiver operating characteristic curve of uCTGF for the identification of microalbuminuria was 0.892 (95% CI: 0.804–0.950) (P<0.001) (Fig. 2). uCTGF level greater than or equal to 166.3 pg/ml had high sensitivity (95.65%), a specificity of 72.88%, a positive predictive value of 57.9%, a negative predictive value of 97.7%, and an accuracy of 62.7% for the diagnosis of microalbuminuria.

DR was detected in higher percentage of group C diabetic patients (60%) compared with 15% of group B. None of group A patients had evidence of DR on fundus examination. Patients with DR had no significantly different uCTGF level from patients without retinopathy (329.18±47.149 vs. 252.02±27.3, P=0.067).

Figure 1



uCTGF levels (pg/ml) in different groups. The highest uCTGF level was in group C followed by group B and lastly group A. The difference was significant between group A and B (P<0.001); group A and C (P<0.001); group B and C (P<.002); but not significant between group A and D (P=0.156).

Discussion

The main finding of this study was the rise of uCTGF in diabetic patients in a stepwise manner according to the degree of nephropathy. This confirms previous studies in patients with T1DM that reported elevation of uCTGF in patients with diabetic nephropathy [8,17]. Moreover, in participants with T2DM, CTGF level was higher in patients with DN than those without DN [18]. However, this was not constant among different populations. In one study in white patients with T2DM, *CTGF* polymorphism was not related to diabetic nephropathy [19].

In the present study, uCTGF level had significant positive correlation with UACR and negative correlation with eGFR. This goes with the result obtained by Nguyen *et al.* [8] that uCTGF levels correlate with markers of severity of DR. Moreover, plasma CTGF was found to be elevated in individuals with diabetic nephropathy, and it was a predictor of ESRD and deterioration of kidney function in patients with DN [10].

We performed regression analysis to identify variables that predict eGFR and UACR in diabetic patients. uCTGF was the only predictor of UACR; however, it was not significantly related to eGFR after adjustment of other confounders. In a prospective study, uCTGF was reported as predictor of progression of microalbuminuria but not eGFR in patients with early diabetic nephropathy [11]. Early increase in uCTGF in DN points to its crucial role in the development fibrosis which is the initial pathogenic mechanism in DN [11].

Table 1 Characteristics of patients and controls

	Group A (n=20)	Group B (n=20)	Group C (n=20)	Group D (n=20)
Age (years)	51.3±9.2	56.4±5.3	54.8±4.9	50.5±10.7
Diabetes duration (years)	3.1±2.2	6.8±5.3	13.0±5.3	–
Hypertension	6 (30)	10 (50)	16 (80)	–
BMI (kg/m^2)	30.2±6.6	31.1±5.2	29.1±4.7	25.9±4.4
HbA1c (%)	8.3±1.8	7.4±1.3	7.9±2.0	5.3±0.3
Cholesterol (mg/dl)	194.0±58.0	190.2±52.1	169.3±32.1	109.9±18.5
Serum creatinine level (mg/dl)	1.1±0.3	1.1±0.4	1.7±0.6	0.9±0.1
eGFR (ml/min/1.73 m^2)	82.2±25.1	76.4±28.7	50.0±17.1	91.2±25.5
UACR (mg/g)	17.0±6.1	111.0±59.6	365.5±197.3	14.7±4.7
uCTGF (pg/ml)	116.2±33.4	197.9±75.7	365.5±197.3	94.4±34.7
Diabetic retinopathy	0 (0)	3 (15)	12 (60)	–

Values are represented as mean±SD or number (%). eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; UACR, urinary albumin–creatinine ratio; uCTGF, urinary connective tissue growth factor.

Table 2 Correlations of urinary connective tissue growth factor level with different variables in diabetic patients

Variables	uCTGF (pg/ml)	
	r	*P* value
Age (years)	0.469	<0.001
Duration of DM (years)	0.471	<0.001
[b]BMI (kg/m^2)	0.203	0.068
Cholesterol level (mg/dl)	0.293	0.007
HbA1c (%)	0.230	0.038
Serum creatinine level (mg/dl)	0.421	<0.001
eGFR (ml/min/1.73 m^2)	–0.421	<0.001
UACR (mg/g)	0.731	<0.001

DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; UACR, urinary albumin–creatinine ratio; uCTGF, urinary connective tissue growth factor.

Table 3 Multivariate regression analysis to predict estimated glomerular filtration rate and urinary albumin–creatinine ratio

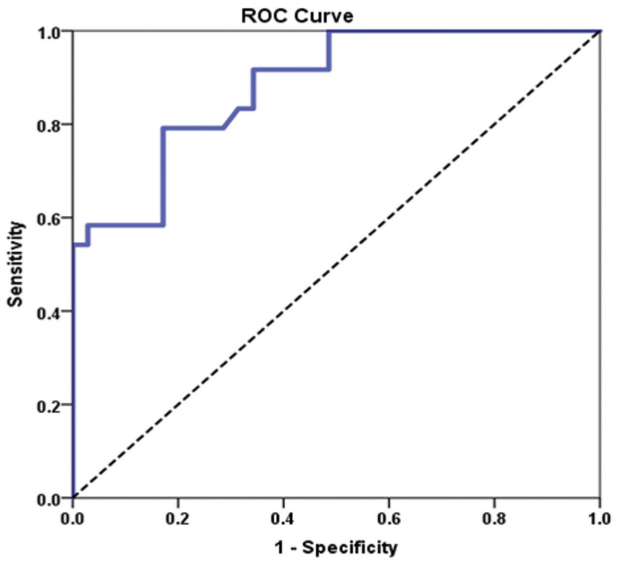
	β coefficients	*P* value	95% CI of β
eGFR (ml/min/1.73 m^2)			
UACR (mg/g)	–0.042	0.017	–0.076 to –0.008
uCTGF (pg/ml)	0.006	0.804	–0.043 to 0.055
Age (years)	–0.827	0.002	–1.342 to –0.312
Duration of DM (years)	–0.468	0.447	–1.688 to 0.753
UACR (mg/g)			
uCTGF (pg/ml)	0.720	<0.001	0.409
Age (years)	2.184	0.252	–1.588
Duration of diabetes (years)	8.090	0.071	–0.714

DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; UACR, urinary albumin–creatinine ratio; uCTGF, urinary connective tissue growth factor.

In experimental type 1 diabetic mice, there was early increase in uGTGF level during development of DN; moreover, it peaked with progression of DN and decreased with development of proteinuria [20]. However, in our study, uCTGF level was elevated in patients with macroalbuminuria than those with microalbuminuria. This goes with the results of one study that demonstrated that participants with both microalbuminuria and macroalbuminuria had increase in glomerular CTGF mRNA [21].

Tubular injury was found to occur early in the pathogenesis of DN [22]. Recently, it has been reported that tubular damage might be responsible for the early rise in uCTGF by increased production of tubular cells or by decreased tubular reabsorption [23]. Moreover, CTGF production is increased in glomeruli in DN with progression of kidney disease when both chronic glomerular and tubular damages are present [24]. This could explain the higher levels of uCTGF in patients with macroalbuminuria.

Figure 2



ROC curve of uCTGF for prediction of microalbuminuria. Receiver operator characteristic (ROC) curve of uCTGF for prediction of microalbuminuria at cutoff value ≥166.3 pg/ml had 95.65% sensitivity and 72.88 % specificity. Area under the curve was 0.892 and *P*<0.001.

High glucose levels and advanced glycation end products were found to have promoting effect on CTGF expression and stimulating production of CTGF in renal mesangial cells leading to glomerular sclerosis [25,26]. This could explain the existence of significant correlation between uCTGF and HbA1c in the current study.

The presence of DR is strictly linked to the existence of DN. CTGF was reported in some studies as stimulator of angiogenesis, whereas other researchers reported that CTGF is an antiangiogenic agent [27,28]. Previous studies reported elevation of CTGF expression in both the vitreous humor and the retina of participants with DR [12,29]. Furthermore, plasma CTGF level was found to be elevated in patients with T1DM with DR. However, in the current study, uCTGF level was not significantly different in patients with DR and those without. Same results were obtained by Roestenberg *et al.* [15] in patients with T1DM. This could be explained by the finding of recent experimental study that higher percentage of uCTGF originates from the kidney rather than from plasma CTGF [23].

Potential limitations of our study should also be mentioned. First, the study included small number of cases. Second, it was a case–control cross-sectional study that cannot identify causal relation,

so the effect of CTGF gene polymorphism on diabetic nephropathy needs further evaluation.

In conclusion, this study confirms the significant elevation of uCTGF in patients with diabetic nephropathy and its correlation with eGFR and UACR as markers of disease severity. Furthermore, uCTGF was a sensitive marker for the presence microalbuminuria in patients with T2DM. However, it was not significantly related to the presence of DR in those patients.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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