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Genetic variant in the promoter of connective tissue growth factor gene confers susceptibility to nephropathy in type 1 diabetes

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Abstract

Background—The evidence for genetic susceptibility in the pathogenesis of diabetic nephropathy is well recognised, but the genes involved remain to be identified. It is hypothesised that mutations within the gene encoding connective tissue growth factor (*CTGF/CCN2*) will increase the propensity of diabetic subjects to develop nephropathy.

Methods and results—Genomic screening was performed for single nucleotide polymorphisms (SNPs) within the *CTGF* gene in 862 subjects from the DCCT/EDIC cohort of type 1 diabetes. A novel SNP was identified in the promoter region that changes a C-G at the position –20. The frequency of GG genotype in microalbuminuric patients (albumin excretion rate (AER) >40 mg/24 h) is significantly greater than diabetics with AER <40 mg/24 h, $p < 0.0001$. The relative risk (RR) to develop microalbuminuria in diabetic subjects with the polymorphism is 3X higher than diabetic subjects without the polymorphism (RR 3.142, 95% CI 1.9238 to 5.1249; $p < 0.05$). Kaplan–Meier survival curves demonstrated that the GG genotype group developed microalbuminuria and macroalbuminuria at a more rapid rate than the GC or CC genotypes. Functional studies demonstrated that the basal activity of the substituted allele/promoter (–20 GG allele) was significantly greater than that of the wild type promoter (–20 CC genotype). This higher level of basal activity of substituted allele *CTGF/CCN2* promoter was abrogated upon suppression of Smad1 levels, indicating that SNP region in the *CTGF/CCN2* promoter plays a vital role in the gene expression.

Conclusions—These findings provide the first evidence that variants within the promoter region of the *CTGF/CCN2* gene predisposes diabetic subjects to develop albuminuria and demonstrate that Smad1 controls the expression of *CTGF/CCN2* promoter through this region.

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INTRODUCTION

Diabetic nephropathy is the leading cause of end stage renal failure, and is clinically manifested by albuminuria, hypertension and a progressive decline in glomerular filtration rate.^{1–4} About 30–40% of patients with type 1 diabetes develop progressive nephropathy.³ Although several factors such as hyperglycaemia, hypertension and duration of diabetes are known to increase the risk of nephropathy, there is growing evidence that genetic determinants also play a substantial role.^{5,6}

Although the association of hyperglycaemia and diabetic nephropathy is well established, the genetic risk factors and cellular signalling mechanisms that promote glomerulosclerosis in diabetes are still undefined. Recent evidence indicates that the profibrotic signals initiated by TGF- β are mediated via activation of CTGF.⁷ The human *CTGF/CCN2* gene is located on chromosome 6q23.1 and consists of five exons encoding a signal peptide and the four protein molecules that make up the 38 kDa cysteine-rich molecule.^{7–9} *CTGF/CCN2* gene is an immediate early gene that is involved in tissue response to injury, including matrix production and proliferation, and emerging evidence indicates that it plays a role in the pathogenesis of renal disease. Renal expression of CTGF is upregulated in diabetes and in other progressive renal diseases.^{10–13} In this regard, we reported that plasma CTGF N fragment levels in type 1 diabetes are associated with higher arterial pressure, albuminuria and greater carotid intima-media thickness (IMT).¹⁴

While genetic studies support the notion that inherited factors play a major role in the pathogenesis of diabetic nephropathy, the major genes and molecular mechanisms involved in predisposition to diabetic nephropathy are not defined. Although a number of candidate genes have been implicated, no studies have explored the role or contribution of genetic variants in the *CTGF/CCN2* gene. Therefore, we performed a screen for variations in the promoter region, all exons and introns of the *CTGF/CCN2* gene in the Diabetes Control and Complications Trial/Epidemiology and Diabetes Intervention and Complications Study (DCCT/EDIC) cohort of type 1 diabetic patients, and examined whether polymorphisms in the *CTGF/CCN2* gene predict development and/or accelerate the onset of nephropathy in patients with diabetes.

METHODS

Study population

The study population was a subgroup of the DCCT/EDIC cohort. The original DCCT cohort, which enrolled 1441 subjects between 1983 and 1989, consisted of men and women between the ages of 13–40 years with 1–15 years of diabetes at study entry.¹⁵ Half of the patient population was randomly assigned to conventional diabetes treatment and the other half was assigned to intensive diabetes treatment. In 1993, the DCCT study was stopped after an average follow-up time of 6.5 years, when intensive treatment was shown to reduce the development and progression of retinopathy, nephropathy and neuropathy by as much as 76%.¹⁵ The subjects were then invited to enrol in EDIC, a multicentre longitudinal observational study of the development of macrovascular complications and development and further progression of microvascular complications.¹⁶ At EDIC baseline in 1994, the average age of the DCCT/EDIC cohort was 35 years (range 19–50 years). Fifty-four per cent of the cohort were male, and the mean duration of diabetes was 12 ± 5 years. Fasting blood samples, collected in years 1997–1999 (EDIC years 3–5) from 1025 DCCT/EDIC subjects for DNA isolations, were shipped directly from participating EDIC clinics to the Medical University of South Carolina (MUSC). The Institutional Review Boards of MUSC and all participating DCCT/EDIC clinics approved the study, and written informed consent was obtained from each participant.

Genetic analysis

Genomic DNA isolated from peripheral blood collected from each patient using a DNA isolation kit (Gentra Systems, Minneapolis, Minnesota, USA) was used as template in PCR to screen for single nucleotide polymorphisms (SNP) within the coding region and the promoter region of the *CTGF* gene. Five pairs of sense and antisense primers that span the entire *CTGF* gene, from the 5' promoter region to the 3' of intron 5 (includes promoter region, 5 exons and 5 introns) were designed using a reference sequence from the National Centre for Biotechnology Information (Genbank accession no. AL354866 and X92511). Primers included 600bp fragment upstream of the transcription start site, all exons and introns (sense primers: tcgcactggctgtctct, agcagccgcagccg, ggcagacgaacgtccatg, ccctaagtgggtcctatcc, gtttgaattgggtgggaatc; antisense primers: ggtttacaggtaggcatcttg, ttgatgagcaggaagtg, gtgagcctcgtgctggac, gcagcatggacgttcg, gctcttgccacgctgttag). A new SNP (C → G) was found at position -20 in promoter region. For genotyping, new primers covering the SNP in promoter region was designed to amplify all the samples of DCCT/EDIC cohort (sense primer: tcgagctggaggtggagt, antisense primer: ggatcaatccggtgtgagttgatg). Genomic DNA was bidirectionally sequenced using amplification primers and sequences were detected on a Megabase N500 sequencer and results were analysed with sequencer software that can reliably identify heterozygotes (Gene Codes Corporation, Ann Arbor, Michigan, USA).

Promoter expression and activity analysis

The reporter plasmid encoding positions -823 to +74 region of the wild-type *CTGF/CCN2* promoter linked to firefly luciferase (CTGF-Luc) was provided by Dr Gary Grotendorst (University of Miami, Miami, Florida, USA). The C→G substitution allele at position -20 was introduced using Quick Change site directed mutagenesis kit (Stratagene, Cedar Creek, Texas, USA) using the following primers, gggcgcccgacgctttatagctc and gagcgataaaagcgtcggtgccgcc (substituted allele/promoter nucleotide in italic underlined), and confirmed by sequencing. For transient transfections, primary human mesangial cells (HMC) were transfected with 0.5 µg wild type and substituted allele/promoter plasmid DNA, respectively, using FuGENE6 transfection reagent (Roche Diagnostics, Indianapolis, Indiana, USA). Following transfection (16–19 h), HMC were stimulated with TGF-β (5 ng/ml) for 24 h. Luciferase activity was measured in different protein concentrations (2.5–30 µg) to determine the optimal protein concentration for the linear range of luciferase activity. Transfection efficiencies were normalised by co-transfection with pSV-β-galactosidase control vector. β-galactosidase was measured using Galacto-Light-Plus (Tropix).

Adenoviral smad 1 siRNA

An adenoviral siRNA vector targeted to the 5'-cacacaccttggaacata-3' region of Smad1 mRNA (AdsiSmad1) was generated as described earlier.¹⁷¹⁸ For transient adenoviral transfection, primary HMC were transduced with either AdsiSmad1 or AdsiSCR (scramble siRNA control) virus. After 48 h of infection, the cells were transiently transfected with either wild type CTGF-Luc promoter plasmid or the substituted allele/promoter using Fugene6 reagent (Roche Diagnostics). After 24 h, some cells were stimulated with TGF-β and further incubated for another 24 h. The cells were then harvested and assayed for luciferase activity using Promega's Luciferase assay kit. Transfection efficiencies were normalised by co-transfection with pSV-β-galactosidase control vector.

End point definitions

The primary clinical measurement of nephropathy used in this analysis is the albumin excretion rate. To examine the relationship of the *CTGF/CCN2* gene polymorphism with the development of diabetic nephropathy, the time to event for micro- and macroalbuminuria

events was constructed. For this outcome, time is defined to be the time from DCCT randomisation to the first renal exam in which the albumin excretion rate (AER) ≥ 40 mg/24 h or the first renal exam in which AER ≥ 300 mg/24 h for the time to microalbuminuria or macroalbuminuria, respectively.

Statistical analysis

The -20C/G polymorphism in the promoter region of *CTGF* gene was hypothesised to be associated with diabetic complications as observed according to the outcome definitions. To examine this hypothesis in the type 1 DCCT/EDIC cohorts, regression and survival analysis techniques were implemented. Before substantive analyses, descriptive comparisons were conducted. Kruskal–Wallis tests, a non-parametric alternative to a one-way analysis of variance (ANOVA) model, were used for comparisons of interval scaled values (eg, duration of diabetes) among the three polymorphism genotypes. χ^2 tests were used for nominal variables (eg, sex, race) to test the hypothesis that the polymorphism occurred at the same rate across classification variables. Simple and multivariable least squares regression models were estimated to examine the cross-sectional association of the log albumin excretion rate and clinical markers. The survival analyses extended this analysis to examine the rates of disease progression among the three levels of the *CTGF* genotypes. Cox proportional hazards models were used to adjust the relationship of the *CTGF* polymorphism on time to event for the effects of the DCCT treatment group, the primary versus secondary prevention DCCT strata, age of onset of diabetes, duration of diabetes at time of DCCT randomisation, and gender. A total of 43 DCCT participants had AER values >40 mg/24 h but <300 mg/24 h at time of randomisation (ie, left censored for microalbuminuria). These subjects were not included in the survival analysis for time to development of microalbuminuria. All available renal measurements (DCCT and EDIC) through EDIC year 9 were included in the survival analysis. The cross sectional analysis utilised AER measurements and clinical characteristics at the time of blood collection (1997–1999), a point in time where there was a higher incidence of nephropathy.

To measure the incremental increase in discrimination for the *CTGF* polymorphism, the concordance index (or C-statistic) was used. The value of the C-statistic is closely related to the area under a receiver operating characteristic (ROC) curve and is interpretable as the probability cases (ie, participants that develop nephropathy) have higher risks as measured by the linear component of the regression model. Accordingly, a value of 0.5 represents a chance prediction and the discrimination of the model is improved as the C-statistic approaches 1.0.¹⁹ The C-statistic was estimated for four multiple Cox models for each nephropathy definition. Model 1 consisted of only the main effects of DCCT randomised treatment and the primary prevention cohort indicator. The second model added age of onset of diabetes and duration of diabetes at time of randomisation to model 1. Model 3 added to two indicator variables for the polymorphism genotypes (GG vs CC; CG/GC vs CC). Finally, model 4 consisted of only the indicator variables for the polymorphism genotypes.

All reported p values are two-sided. The type I error rate (α) was set to be 0.05 for each statistical comparison. No correction for multiple testing across end points was implemented; however, post hoc comparisons of group means were conducted using the Tukey method. Regression model diagnostics were implemented for the final multiple linear regression model, and the residual analysis suggested the log transformation of AER was appropriate. The proportional hazards assumption was also graphically assessed and deemed justified. Analyses were conducted using the SAS System, version 9.1. STATA version 9.2 was used to compute the C-statistic.

RESULTS

We identified a novel SNP in the promoter region of the *CTGF/CCN2* gene that changes a C-G at the position -20. The SNP is located between the TATA box and the GC rich motif recently characterised as a Smad 1 binding site (figure 1). The frequency of this polymorphism is about 5.3%. No SNPs were identified in the coding region of the gene.

Clinical characteristics of the 862 patients with complete datasets and on whom the genetic analysis were performed are shown in table 1. The CC, CG, GG genotypes of the *CTGF/CCN2* gene polymorphism were noted in 541, 275, and 46 patients, respectively, and these values were consistent in Hardy–Weinberg equilibrium ($p=0.16$). Our analyses revealed that there is a significant relationship between duration of diabetes ($p<0.029$) and inclusion into the secondary prevention study group ($p=0.015$) and the GG genotype. Carriage of the GG genotype was also significantly greater in diabetic subjects with AER ≥ 40 mg/24 h than diabetic subjects with normal AER (<40 mg/24 h). Specifically, 26% (12 of 46), 12% (31 of 271), and 8% (43 of 532) of diabetic subjects had abnormal AER in the GG, CG, and CC genotype groups, respectively ($X^2=16.2$, $df=2$, $p<0.003$, N missing AER=13).

We next examined whether the -20 C/G SNP in the promoter of *CTGF/CCN2* gene associates with AER midway through the EDIC follow-up. The mean log AER (SD) in patients with the homozygous genotype GG is 3.5 ± 1.8 mg/24 h ($n=46$) compared to 2.6 ± 1.2 mg/24 h ($n=541$) in patients with the homozygous CC genotype ($p<0.001$). Furthermore, the relative risk (RR) to develop microalbuminuria in patients with the GG genotype is three times higher than patients with the CC genotype (RR 3.142, 95% CI 1.9238 to 5.1249; $p<0.05$).

Multiple linear regression models were further constructed to examine the relationship of the polymorphism with log AER after adjustments for clinical characteristics. The following candidate variables were selected for inclusion in the multiple linear regression model based on univariate associations: age, body mass index (BMI), duration of diabetes, HbA1c, systolic blood pressure (SBP), total cholesterol, ACE inhibitor usage, and previous DCCT randomised treatment. The multiple linear regression model fit the data well ($F=33.85$; $df=10$, 773; $p<0.001$) and explained 30% of the variation in the log AER (table 2). The -20C/G (at three levels) significantly improved the model fit ($F=6.85$; $df=2$, 783; $p=0.001$), even after adjusting for the set of adjustment variables identified in the simple linear regression models; however, post hoc comparisons of individual levels of the polymorphism suggested this overall difference was related to the GG genotype. Examination of the least squares means for the *CTGF/CCN2* gene polymorphism using Tukey's correction suggested that the GG statistically differed from either the CC or CG genotype combinations ($p<0.001$ and $p=0.006$, respectively), yet the mean levels of log AER did not differ statistically between the CG to CC genotype combination ($p=0.52$). The least squares estimated means (SE) of the GG, CG and CC are 3.33 (0.19), 2.71 (0.07), and 2.61 (0.05), respectively.

The time from DCCT randomisation to onset of micro-/macroalbuminuria is presented in figure 2 and tables 3 and 4. In an unadjusted analysis (ie, Kaplan–Meier), the three genotypes had different estimated survival functions for microalbuminuria ($p<0.001$) and macroalbuminuria ($p<0.001$). The median survival time was only estimable for the GG genotype combination and microalbuminuria (median=11.5 years after DCCT randomisation). The increased rate of nephropathy development is consistent with the cross sectional multiple linear regression results, namely that the GG genotype combination developed microalbuminuria (figure 2) and macroalbuminuria (figure not shown due to censoring rates of 93%, 88% and 78% for the CC, CG or GG genotype, respectively) faster

than the other two genotypes. Notably, there were differential rates of developing nephropathy among the *CTGF/CCN2* genotypes, and this observation was examined in more detail with a proportional hazards model.

Proportional hazard models for microalbuminuria and macroalbuminuria were constructed to test for the significance and discrimination of the *CTGF/CCN2* gene polymorphism in the presence of key DCCT study design variables. The likelihood ratio tests of the *CTGF/CCN2* polymorphism for the micro-/macroalbuminuria models were $p=0.001$ ($X^2=14.21$ on 2 degrees of freedom) and $p=0.013$ ($X^2=8.73$ on 2 degrees of freedom), respectively (note: this is a test of model 3 vs 4 as identified in table 4). The estimated instantaneous hazard ratios (HRs) for the GG versus CC were 2.0 (95% CI 1.3 to 3.2; $p=0.0024$) and 2.5 (95% CI 1.2 to 5.0; $p=0.013$), for micro- and macroalbuminuria respectively, and for the GC/CG versus CC comparison, these HRs were 1.5 (95% CI 1.2 to 2.0; $p=0.003$) and 1.8 (95% CI 1.1 to 2.9; $p=0.018$) (table 3).

The polymorphism demonstrated an ability to discriminate cases of microalbuminuria from those participants that did not develop microalbuminuria through EDIC year 9. Discrimination of the development of macroalbuminuria was better than that for the development of microalbuminuria, but a similar pattern was observed. These results clearly suggest that the novel polymorphism is associated with micro-macroalbuminuria as well as having predictive associations with the rate at which micro- and macroalbuminuria develops.

Effect of the *CTGF/CCN2* gene –20C/G variant on promoter activity in HMC

As shown in figure 3, the basal activity of the –20 G promoter variant was significantly greater than that of the –20C variant at every protein concentration tested. The basal promoter activity of the –20 G promoter was increased over twofold (436353 ± 45403 relative luciferase units (RLU)/s compared to 185190 ± 12053 RLU/s in the –20 CC genotype, $p<0.001$, $n=5$, 5 μ g protein, figure 3A), demonstrating that the –20 G/C SNP is functionally active and may contribute to the regulation of the basal promoter activity.

We next examined whether the *CTGF/CCN2* –20C and the –20 G promoter variants will respond differently to TGF- β stimulation. Stimulation of HMC expressing the *CTGF/CCN2* –20C promoter with TGF- β produced about a 1.8-fold increase in luciferase reporter activity compared with unstimulated cells ($p<0.003$, $n=5$). Strikingly, TGF- β stimulation of HMC expressing the *CTGF/CCN2* –20 G promoter produced about a 5.6-fold increase in luciferase reporter activity compared to unstimulated HMC expressing the –20C variant ($p<0.006$, $n=5$) and a 2.63-fold increase in luciferase reporter activity compared to unstimulated –20 G expressing cells ($p<0.007$, $n=5$).

To explore the mechanism underlying the increased basal and TGF- β stimulated activity of the *CTGF/CCN2* promoter –20C/G SNPs, we examined whether Smad 1 was involved in this process. Smad 1 was recently shown to activate *CTGF/CCN2* promoter through a response element adjacent to the –20 position, suggesting that the enhanced activity of the substituted allele/promoter may depend on Smad 1 (figure 1).

The results in figure 3C demonstrate that, following suppression of Smad1, the basal activity of the –20 G *CTGF/CCN2* promoter was reduced to levels below those observed in the –20C promoter. Treatment with TGF- β resulted in a significant increase in the activity of both *CTGF/CCN2* promoter variants, and downregulation of Smad1 abolished TGF- β -induced stimulation of both promoters. The effects were more pronounced in the substituted allele/promoter, suggesting a greater dependency of the substituted allele/promoter on Smad 1.

DISCUSSION

Microalbuminuria, an early marker of diabetic nephropathy, signifies high risk for progressive renal failure and cardiovascular disease. Identifying biomarkers and major risk factors that contribute to the development of microalbuminuria may provide insights into the mechanisms of diabetic renal injury. Although several established risk factors such as duration of diabetes, hyperglycaemia and hypertension have been shown to increase the risk of developing nephropathy, they do not solely account for the incidence and progression of diabetic nephropathy.⁵²⁰²¹ Rather, there is growing evidence that the risk of developing diabetic nephropathy is in large part genetically determined.^{22–24} Evidence for such a notion is derived from epidemiological studies demonstrating familial clustering of diabetic nephropathy.^{25–27} Further support for the genetic predisposition to diabetic nephropathy is derived from studies demonstrating that the probability of developing nephropathy is greater in subjects with parental history of hypertension and cardiovascular disease.²⁸

Our findings clearly demonstrate an association between a –20C/G SNP in the promoter region of the *CTGF/CCN2* gene and the risk of developing diabetic nephropathy. The frequency with which the –20 GG genotype in the *CTGF/CCN2* gene is found in diabetic subjects with albuminuria is significantly greater than in those with a normal albumin excretion rate. In addition, diabetic subjects with the homozygous GG genotype have significantly higher excretion rates of albumin than diabetic subjects with the more prevalent homozygous CC genotype. The RR to develop microalbuminuria in patients with the GG genotype is increased threefold. Furthermore, survival analysis demonstrates that the time to develop microalbuminuria is faster in subjects with the GG genotype than diabetic subjects with the CC genotype. While the GG genotype showed the greatest risk of diabetic nephropathy, there was evidence that the CG/GC genotype was also associated with increased risk. This may suggest that the G allele may play more of a dominant or codominant role in the development of diabetic nephropathy. Proportional hazard models demonstrate that the polymorphism in the *CTGF/CCN2* gene is associated with diabetic nephropathy and illustrate further that the GG genotype has predictive associations with the rate at which diabetic nephropathy develops. Although this finding requires confirmation in another cohort of diabetic populations, our analyses identify *CTGF/CCN2* as a susceptibility gene for diabetic renal disease.

In another study of an Irish type 1 diabetic population, 10 SNPs were identified in the *CTGF* gene, four of which were in the promoter region of the gene. No significant association was detected between the variants in the *CTGF* gene and diabetic nephropathy in this patient cohort.²⁹

The novel SNP we discovered at position –20 in the *CTGF/CCN2* promoter is located between the TATA box and the GC rich motif recently characterised as a Smad 1 binding site.¹³ Our functional analyses indicate that this region is critical for Smad1 dependent transcriptional regulation of the *CTGF* gene. The basal activity of the –20G variant was significantly greater than that of the –20C promoter in transiently transfected human mesangial cells, indicating that the –20C/G SNP is functionally active and contributes to the regulation of the basal promoter activity. Downregulation of Smad1 suppressed the basal of the –20 G variant to levels below those observed with the –20C promoter. Furthermore, knockdown of Smad1 abolished the TGF- β induced increase in the activity of both the –20C and –20 G promoter variants. Together, these data demonstrate that Smad 1 plays a critical role in mediating elevated levels of *CTGF/CCN2* gene expression and that the promoter region containing –20 C/G SNP in the *CTGF/CCN2* promoter plays a central role regulating the expression of this gene in response to inflammatory cytokines.

In summary, we have discovered a novel polymorphism in the promoter region of the *CTGF/CCN2* gene that is part of the Smad1 response element involved in the transcriptional expression of the CTGF gene. Our findings demonstrate a genetic link between CTGF and diabetic nephropathy, offer new insights into the pathogenesis of diabetic nephropathy, and identify CTGF as a susceptibility gene for increased risk of nephropathy in type 1 diabetic subjects.

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Figure 1. Location of the -20 C/G single nucleotide polymorphism (SNP) in the promoter region of the human *CTGF/CCN2* gene. The -20 G variant is located between the TATA box and the Smad1 binding site.

Figure 2. Kaplan–Meier survival probabilities of time to onset of microalbuminuria predicted by CTGF genotypes. CC (homozygous); CG or GC (heterozygous); GG (homozygous).

Figure 3.

(A) Significance of -20 G allele on CTGF promoter activity. Substitution mutation (C to G) at -20 was introduced into the CTGF/CCN2 promoter-luciferase reported construct. -20 C and -20 G promoter variants were transiently transfected into human mesangial cells using FuGENE6 transfection reagent. Luciferase activity was measured in cell lysates normalised for protein concentration and expressed as relative luciferase units per second (RLU/s). * $p < 0.001$ versus C allele, $n = 5$. (B) Effect of TGF- β on CTGF promoter activity. Human mesangial cells transiently transfected with -20C and -20G substituted allele/promoter (SA/P) were stimulated with TGF- β (5 ng/ml) for 24 h. Luciferase reporter activity was measured in cell lysates normalised for protein concentration and expressed as RLU/s. * $p < 0.008$

versus -20C variant; †p<0.003 versus -20C variant; #p<0.006 versus -20C variant; ‡p<0.0007 versus -20G variant. (C) Role of Smad1 in CTGF/CCN2 gene regulation. Human mesangial cells transduced with Smad1 siRNA (AdsiSmad1) or control non-silencing siRNA (AdsiSCR) adenoviruses followed by transient transfections with either the -20C or -20G promoter variants and stimulated with TGF- β (5 ng/ml) for 24 h. Luciferase reporter activity was measured in cell lysates normalised for protein concentration and expressed as RLU/s. The open bars represent unstimulated control cells and the closed bars represent TGF- β treated cells.

Table 1
Sample description by CTGF/CCN2 polymorphism at time of DCCT randomisation

Variable	Overall sample			CC			CG/GC			GG			Test statistic [†]	p Value
	(N=862)			(N=541)			(N=275)			(N=46)				
	N or mean	% or STD		N or mean	% or STD		N or mean	% or STD		N or mean	% or STD			
% Randomised intensive treatment	431	50.7%		287	53.1%		132	48.0%		18	39.1%		4.5	0.108
% DCCT primary prevention cohort	421	48.8%		257	47.5%		149	54.2%		15	32.6%		8.4	0.015
Age (years) *	27.1	7.1		27.1	7.0		27.0	7.1		27.5	6.9		0.3	0.85
% Male	483	56.0%		306	56.6%		152	55.3%		25	54.4%		0.2	0.91
Body mass index (kg/m ²)	23.4	2.8		23.5	2.9		23.3	2.7		22.7	2.5		3.8	0.150
Systolic BP (mm Hg) *	114.7	11.5		114.6	11.7		114.7	11.4		115.4	10.4		0.4	0.82
Diastolic blood pressure (mm Hg) *	73.2	8.7		73.4	8.7		72.6	8.6		74.5	9.3		1.6	0.45
HbA1c *	8.8	1.6		8.8	1.6		8.9	1.6		8.8	1.6		0.3	0.86
Duration of diabetes (months) *	67.3	49.7		68.0	49.6		63.3	48.9		83.6	53.3		7.1	0.029
Age of onset of diabetes (years)	22.0	8.1		21.9	8.1		22.3	8.1		21.0	8.2		1.0	0.59
% Family history of type 1 diabetes	121	14.0%		75	13.9%		41	14.9%		5	10.9%		0.6	0.75
Albumin excretion rate (mg/24 h) *	15.5	17.8		15.2	14.9		16.2	23.2		15.2	11.4		2.1	0.35

* At the time of Diabetes Control and Complications Trial (DCCT) randomisation.

[†] For categorical variables, the test statistic is a Pearson χ^2 statistic with 2 degrees of freedom. For other variables, Kruskal–Wallis tests, which are distributed χ^2 with 2 degrees of freedom, were computed.

Table 2

Log albumin excretion rate regressed on CTGF/CCN2 polymorphism and clinical characteristics at time of specimen collection using multiple linear regression (N=784^{*})

Variable	Parameter estimate	SE	t Value	p Value
Intercept	-2.060	0.550	-3.75	0.0002
DCCT intensive control relative to standard	-0.307	0.085	-3.60	0.0003
Age (years) [†]	-0.026	0.006	-4.05	<0.0001
Body mass index (kg/m ²) [†]	0.001	0.011	0.06	0.95
Duration of diabetes (years) [†]	0.030	0.009	3.43	0.0006
HbA1c [†]	0.196	0.032	6.08	<0.0001
Systolic blood pressure (mm Hg) [†]	0.022	0.003	6.66	<0.0001
Total cholesterol (mg/dl) [†]	0.005	0.001	3.91	<0.0001
ACE inhibitor use [†]	1.220	0.128	9.52	<0.0001
GG allele combination relative to CC	0.724	0.195	3.71	0.0002
GC allele combination relative to CC	0.101	0.092	1.10	0.27

^{*} N=78 observations had at least one missing data point and were deleted listwise from the regression model. The resulting regression model fit the data ($F=33.85$; $df = 10, 773$; $R^2=0.30$). The parameter estimates for GG versus CC and GC versus CC are the difference in mean log albumin excretion rate for the GG or GC allele combinations relative to the CC allele combination. Least squares means (SE) for the three allele combinations are: GG 3.3 (0.19); GC 2.7 (0.07); CC 2.6 (0.05) (or 28 mg/24 h; 15 mg/24 h; 14 mg/24 h).

[†] Clinical parameters as measured at time of specimen collection (1997–1999).

Hazard ratio (HR) estimates for the development of microalbuminuria and macroalbuminuria as a function of clinical characteristics and CTGF/CCN2 polymorphism genotypes

Table 3

Variable	HR	95% HR confidence limits	z	p Value
Development of microalbuminuria (n=819)*				
DCCT randomised treatment (intensive relative to standard)	0.61	0.47	0.79	14.4 <0.001
Primary prevention cohort (relative to secondary prevention)	0.67	0.47	0.96	4.9 0.027
Age at diabetes onset	0.96	0.95	0.98	16.7 <0.001
Duration of diabetes at DCCT randomisation	1.00	0.99	1.00	1.3 0.26
Sex (male relative to female)	1.01	0.78	1.30	<0.1 0.95
GG genotype relative to CC	2.03	1.29	3.21	9.3 0.002
GC/CG genotype(s) relative to CC	1.50	1.15	1.95	9.1 0.003
Development of macroalbuminuria (n=862)				
DCCT randomised treatment (intensive relative to standard)	0.32	0.20	0.54	18.7 <0.001
Primary prevention cohort (relative to secondary prevention)	0.64	0.34	1.22	1.8 0.18
Age at diabetes onset	0.98	0.95	1.01	1.5 0.23
Duration of diabetes at DCCT randomisation	1.00	1.00	1.01	0.1 0.72
Sex (male relative to female)	1.71	1.06	2.76	4.9 0.027
GG genotype relative to CC	2.46	1.21	5.00	6.2 0.013
GC/CG genotype(s) relative to CC	1.77	1.10	2.85	5.6 0.018

* N=43 subjects developed microalbuminuria before the start of DCCT (ie, left censored) and were excluded from the risk set.

Table 4

Concordance (C-statistic) values examining the discrimination of the CTGF/CCN2 polymorphism

Description of Cox model	Time to macroalbuminuria		Time to microalbuminuria	
	(AER 40 mg/24 h)		(AER 300 mg/24 h)	
	C-statistic	p Value [†]	C-statistic	p Value [†]
Model 1: DCCT randomised treatment, indicator variable for primary prevention cohort	0.5929	N/A	0.6745	N/A
Model 2: Model 1 + age of onset of diabetes, duration of diabetes at time of DCCT randomisation, sex	0.6323	N/A	0.7023	N/A
Model 3: Model 2 + CTGF/CCN2 polymorphism indicator variables	0.6539	<0.001	0.7295	0.013
Model 4: CTGF/CCN2 polymorphism indicator variables alone	0.5693	<0.001	0.6063	0.003
Number of events	253		79	
Number at risk [*]	819		862	

^{*} N=43 subjects developed microalbuminuria before the start of DCCT (ie, left censored) and were excluded from the risk set.

[†] p Values are for a 2 degree of freedom likelihood ratio test for the main effect of the CTGF/CCN2 polymorphism. The p value provided for model 3 is for the comparison of model 3 relative to model 2. The p value provided for model 4 is a test of the unadjusted effects of the polymorphism.