CCN2 (CTGF) Gene Polymorphism Is a Novel Prognostic Risk Factor for Cardiovascular Outcomes in Hemodialysis Patients

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Key Words

CCN2 • Connective tissue growth factor • Dialysis • Genetic polymorphism • Atherosclerosis, survival

Abstract

Background: The very high cardiovascular (CV) mortality and morbidity rates in hemodialysis (HD) patients are greatly related to atherosclerosis. CCN2 (connective tissue growth factor/CTGF) is a profibrotic factor that is secreted by endothelial cells, involved in atherogenesis, promoting fibroblast proliferation and matrix production. CCN2 protein is significantly increased in complicated fibrous plaques and enhances monocyte migration into atherosclerotic lesions. The aim of this study was to investigate a possible association between CCN2 gene polymorphism and CV morbidity and mortality in HD patients. **Methods:** 98 HD patients, followed for 24 months, were genotyped for the common polymorphism on the CCN2 gene (G-945C). HD patient characteristics were: age 64 ± 13 years, males 64%, diabetes 24%, hypertension 62%, smokers 38%, dyslipidemia 28%, all undergoing standard HD three times weekly. **Results:** All-cause mortality was not associated with CCN2 polymorphism (G-945C). In contrast, however, the GG genotype was strongly associated with CV mortality: OR 13 (1.49–155), $p = 0.0048$. Interestingly, the GG genotype was also greatly associated with the

serious CV events of stroke and myocardial infarction in surviving HD patients: OR 13.3 (2.5–87.08), $p = 0.0001$. **Conclusions:** We demonstrate for the first time that CCN2 gene polymorphism is a prognostic risk factor for CV morbidity and mortality in HD patients. These data may have important implications for better understanding the link between accelerated atherosclerosis and increased mortality in HD population.

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Introduction

Cardiovascular (CV) disease is the leading cause of mortality in dialysis patients [1]. The very high CV mortality and morbidity rates in this population are only partially explained by the high prevalence of traditional CV risk factors [2], which are classically related to atherosclerosis. The vascular changes observed in chronic kidney disease (CKD) patients consist not only of atherosclerosis but also of arteriosclerosis associated with both medial and intimal vascular calcification [3]. The degree of arterial stiffening and the extent of calcification are closely related [4], and both of these variables are strong and independent prognostic markers of all-cause and CV mortality in patients on hemodialysis (HD) [5, 6].

The CCN family of genes was named after a number of gene products (Connective tissue growth factor, Cyr61/Cef10, and Neuroblastoma overexpressed gene). CCN2 (formerly CTGF, or connective tissue growth factor) [7] is a profibrotic cytokine, discovered more than 15 years ago as a protein secreted by human endothelial cells [8]. Although strongly induced by transforming growth factor- β (TGF- β) in many cell types and considered to be a downstream mediator of renal fibrosis, CCN2 can also be independently upregulated by TGF- β [9–11]. Moreover, CCN2 promotes fibroblast proliferation and matrix production [12, 13].

Over the last 10 years, CCN2 has been shown to be involved in atherogenesis [14]. In fact, CCN2 mRNA is greatly expressed in vascular smooth muscle cells (VSMCs) of atherosclerotic blood vessels, but not in homologous normal arteries [15]. Furthermore, CCN2 protein expression is significantly increased in complicated atherosclerotic plaques compared with fibrous and more stable plaques and may enhance monocyte migration into atherosclerotic lesions, thus contributing to atherogenesis [16]. In addition, the finding of a strong chemotactic effect of CCN2 on bovine VSMCs in vitro may support the hypothesis that CCN2 plays an active role in the atherosclerotic process, by both promoting monocyte migration into the damaged vessels and inducing intimal angiogenesis [16].

Recently, Blom et al. [17] identified human CCN2 promoter polymorphisms. The expression of the gene encoding CCN2 has been found to be greatly upregulated in gene-expression-profiling studies of skin-biopsy specimens and of fibroblast cultures from skin from patients with systemic sclerosis. Fonseca et al. [18] demonstrated that the C allele at position -945 of the CCN2 promoter is critical for the normal repression of CCN2 transcription. However, a single substitution at this site in the G allele (G-945) appears to result in increased transcription and expression of CCN2, and is significantly associated with susceptibility to systemic sclerosis. Moreover, the CCN2 gene polymorphism (G-945) was associated with higher degree of calcification and fibrosis in 187 stenotic aortic valves excised from normal renal function patients [19].

Others and we have also shown that renal CCN2 mRNA and urinary secreted protein is upregulated in both animal models of diabetic nephropathy and in CKD patients, indicating a role for abnormal production in early progression [20, 21]. Collectively, this suggested to us that an abnormal CCN2 activity, driven at least in part by a CCN2 polymorphism, might be critically important

to the increased CV morbidity and mortality observed in dialysis patients. We investigated this possibility in the present prospective study of patients receiving dialysis.

Materials and Methods

Patients

Patients were recruited among the prevalent HD population at San Paolo Hospital, Milan, Italy. All the 98 recruited patients were Caucasian adults aged 64 ± 13 years, 64% were male, with a median dialysis vintage of 85 months (range 9–454 months). All patients were treated by standard bicarbonate dialysis for 4 h, 3 times weekly. Information on concomitant hypertension, diabetes mellitus and dyslipidemia was obtained from hospital charts; patients were investigated concerning their smoking status and previous CV events (myocardial infarction, angina, stroke, transient ischemic attack, pulmonary thromboembolism). Once enrolled, patients were followed up prospectively for 2 years.

Blood pressure was measured at the beginning of the dialysis session. Hypertension was defined as systolic blood pressure (SBP) ≥ 140 mm Hg and diastolic blood pressure (DBP) ≥ 80 mm Hg, or normal blood pressure in patients treated with one or more antihypertensive drugs. Patients were treated with a variety of such drugs, including β -blockers, ACE inhibitors, angiotensin receptor antagonists, diuretics, and calcium-channel blockers. Data were collected about concomitant pharmacological therapy.

Diabetes was defined as fasting blood glucose levels ≥ 126 mg/dl. Dyslipidemia was defined as serum LDL cholesterol levels ≥ 110 mg/dl in at least three measurements within 1 year. Smoking was defined as current use of cigarettes, with >10 cigarettes daily, for more than 10 years.

Blood samples for measurement of routine biochemical parameters were drawn from each patient before the beginning of the first HD treatment of the week. Serum levels are expressed as the mean of the available regular tests, at least three measurements in 1 year.

Identification of CCN2 Gene Polymorphisms

Patients were genotyped for the common polymorphism on the CCN2 gene (G-945C). Whole blood (3 ml) from patients and healthy volunteers was collected into potassium EDTA-containing tubes. DNA was prepared with the Istage Matrix extraction kit (Bio-Rad Laboratories). The polymerase chain reaction (PCR) for CCN2 was performed in a total volume of 25 μ l with 5 μ l of extracted genomic DNA, 100 μ M of dATP, dGTP, dTTP, and dCTP, 1.5 mmol/l of $MgCl_2$, and 1 U of *Taq* polymerase. The two primers, forward and reverse, were each used at a concentration of 80 nM, and were designed with Primer Express software. The CCN2 primer sequence was: forward: 5'-ATTGATGGCCACTCCTCCCTGTGCTTGCC-3'; reverse: 5'-GGCAAGGACAAGGGAGAGTGGCCATCAAT-3'. The PCR was started within 5 min of incubation at 94°C to activate the enzyme, and was followed by 35 cycles (20 s each) at 94°C, 55°C, then 30 s at 72°C. The amplification was verified on an agarose gel (2%) followed directly by sequencing with an automatic sequencer in fluorescent DNA capillary electrophoresis (ABI Prism 310; Applied Biosystems).

Fig. 1. CCN2 gene polymorphism does not associate with all-cause mortality at 2 years of follow-up. In both alive and dead HD patients, the distribution of CCN2 gene did not show a significant association with mortality rate.

Fig. 2. CCN2 gene polymorphism (G/G) is associated with CV mortality.

Statistical Analyses and Ethics

Differences between groups were examined by χ^2 test. Odds ratios (approximate relative risk) were calculated as an index of the association of the G-945C genotype with each phenotype (GG, GC, CC). For each odds ratio, two-tailed probability values and 95% confidence intervals were calculated.

The study was approved by the Ethics Committee of San Paolo Hospital, Milan, Italy. All patients gave their informed consent in writing before they were submitted to any procedures related to the study.

Results

A total of 98 HD patients were included in the study. Their main characteristics are summarized in table 1. No patients were lost or transplanted during follow-up. We first examined the relationship between all-cause mortality and CCN2 polymorphism (G-945C) (table 2; fig. 1). The presence of the CCN2 polymorphism (GG, or GC) was not different between survivors and expired patients when considering death by any cause. In contrast, however, the GG genotype was associated with CV mortality: OR 13 (1.49–155), $p = 0.0048$ (table 2; fig. 2). Most patients dying from CV-related causes were from the GG group ($p < 0.05$). In comparison, only a small proportion of C/C genotype patients died from CV-related death. The non-CV-related death group was made up mostly of a combination of patients bearing a CCN2 CC or GC genotype.

Interestingly, the GG genotype was also greatly associated with the serious CV events of stroke and myocardial infarction in surviving HD patients: OR 13.3 (2.5–87.08),

Table 1. Characteristics of the study population

Characteristic	HD patients (n = 99)
Male gender, %	64
Age (mean $\pm$ SD), years	64 $\pm$ 13
Diabetes mellitus type 2, %	24
Hypertension ^a , %	62
Smokers, %	38
Dyslipidemia ^b , %	28
C-reactive protein (mean $\pm$ SD), mg/dl	7.54 $\pm$ 9.36
Albumin (mean $\pm$ SD), g/dl	4.09 $\pm$ 0.31
History of CV events, %	62
iPTH (mean $\pm$ SD), pg/ml	274 $\pm$ 254
URR, %	78 $\pm$ 1
HD vintage (mean $\pm$ SD), months	85 $\pm$ 65

^a Hypertension was defined as SBP ≥ 140 mm Hg or DBP ≥ 80 mm Hg, or normal blood pressure in patients treated with one or more antihypertensive drugs including β -blockers, ACE inhibitors, angiotensin receptor antagonists, diuretics, and calcium-channel blockers. ^b Dyslipidemia was defined by serum LDL cholesterol levels ≥ 110 mg/dl in at least three measurements in 1 year.

$p = 0.0001$ (table 2; fig. 3). Among the group experiencing these serious CV-related events, the vast majority had the GG genotype (8/11 or 73%, compared to 10/60 or 17% of GG genotype patients in the group with no CV events). Conversely, only 3/11 (27%) patients with a major CV event had the CG or CC genotypes, compared to 50/60 or 83% in the group with no CV events (table 2).

Table 2. CCN2 polymorphism distribution in dialysis patients according to overall survival, CV mortality and occurrence of major CV events (stroke and myocardial infarction)

CCN2 polymorphism	All-cause mortality (p = n.s.)		CV mortality (p < 0.01)		CV events (p = 0.0001)	
	alive (71)	dead (27)	CV death (12)	non-CV death (15)	yes (11)	no (60)
G/G	17	7	8	2	8	10
G/C	38	14	1	9	2	35
C/C	16	6	3	4	1	15

The C allele at position -945 of the CCN2 promoter is critical for the normal repression of CCN2 transcription; a single substitution at this site in the G allele (G-945) appears to result in increased transcription and expression of CCN2, and is significantly associated with susceptibility to systemic sclerosis.

Discussion

In this study, we demonstrate for the first time that CCN2 gene polymorphism might be a novel prognostic risk factor for CV morbidity and mortality in HD patients.

CCN2 (CTGF) is a matricellular protein characterized by a cysteine-rich, heparin-binding structure. Extensive studies have shown that CCN2 mirrors many of the effects of TGF- β on skin fibroblasts, and other cells, such as stimulation of extracellular matrix, cell proliferation, and integrin expression, likely as a downstream modulator [8, 9]. Moreover, it has been demonstrated that CCN2 can promote endothelial cell growth and adhesion through a stimulation of macrophage migration and survival, suggesting an implication in endothelial cell function, angiogenesis and atherosclerosis progression [22]. Interestingly, CCN2 was initially identified in the culture supernatant of vascular endothelial cells [8] and it is an important inducer of development and regeneration of mesenchymal tissues including bone, cartilage and blood vessels.

Since its initial discovery, CCN2 has been widely known as a profibrotic factor that is involved in a variety of fibrotic disorders. CCN2 is associated with systemic sclerosis, pulmonary fibrosis, diabetic renal fibrosis, liver cirrhosis, pancreatic fibrosis, biliary atresia, atherosclerosis, and myocardial fibrosis [8]. Since CCN2 has a positive role in wound healing and mesenchymal tissue regeneration, the fibrotic changes observed in those diseases may be regarded as a result of deregulated regeneration of corresponding tissues [8]. VSMCs greatly ex-

Fig. 3. CCN2 gene polymorphism (G/G) is associated with major CV events.

press both CCN2 mRNA and protein in atherosclerotic conditions and calcified plaques, compared to normal arterial vessels [15, 16]. For this reason, over the last decade a central role in the pathogenesis of atherogenesis has been recognized for CCN2 [14].

It should be borne in mind that numerous factors contribute toward the marked arterial calcification observed in HD patients: all the 'classic' risk factors for atherosclerosis plus CKD-specific risk factors, such as duration of dialysis, uremic toxins, inflammation, and increased serum levels of calcium, phosphate and PTH [5].

In our study, we first examined the relationship between all-cause mortality and CCN2 polymorphism (G-945C) (fig. 1). The presence of the CCN2 polymorphism (GG, or GC) was not different between survivors and expired patients when considering death by any cause. Interestingly, only the GG genotype was strongly associated with CV mortality and with higher incidence of strokes and myocardial infarction in surviving HD patients (fig. 2, 3). No other data have been published on the human CCN2 promoter polymorphisms in either CKD or HD populations.

In previous studies we reported that other genotypes of inhibitory proteins involved in the pathogenesis of vascular calcification do (matrix Gla protein) or do not (fetuin-A) associate with mortality risk in HD patients [23, 24]. More recently, we demonstrated that HD patients have a different distribution of matrix metalloproteinases (MMPs) 1 and 3 gene polymorphisms as compared to the normal population [25]. MMPs are a family of en-

zymes involved in the biology of extracellular matrix and in atherogenesis. Consequently, altered polymorphisms of the MMP1 and MMP3 genes might be considered a negative prognostic factor for CV morbidity and mortality in HD population [25].

Thus, the finding of a new gene product influencing CV morbidity and mortality in the dialysis population is clinically relevant [26], as it adds useful information for understanding the complex interplay of genetic and environmental factors that ultimately determine occurrence of CV disease. Specifically, our data may have important implications for better understanding the link between accelerated atherosclerosis and increased mortality in HD

population, justifying the design of a prospective study on the role of CCN2 gene polymorphism in determining CV outcomes in HD patients. This might help to clarify the pathogenesis of increased risk of ectopic calcification and CV events in patients with renal failure.

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