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共同主持人：謝易修

計畫參與人員：張宏榮,謝易修,林秋菊,楊順發

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Circulating matrix metalloproteinase-2 is associated with cystatin C level, post-transplant duration, and diabetes mellitus in kidney transplant recipients

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Keywords:	matrix metalloproteinase, cystatin C, renal transplantation, chronic renal allograft dysfunction, chronic allograft nephropathy



Circulating matrix metalloproteinase-2 is associated with cystatin C level,

post-transplant duration, and diabetes mellitus in kidney transplant recipients

Hong-Rong Chang^{1,2}, Shun-Fa Yang³, Chiu-Chu Lin^{1,4}, Mei-Lan Lee³, Jong-Da Lian^{1,2},

Yih-Shou Hsieh⁴

¹Division of Nephrology, Department of Internal Medicine, Chung Shan Medical

University Hospital, Taichung 402, Taiwan

²School of Medicine, Chung Shan Medical University, Taichung 402, Taiwan

³Institute of Medicine, Chung Shan Medical University, Taichung 402, Taiwan

⁴Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung

402, Taiwan

Corresponding authors:

1. Yih-Shou Hsieh, Ph.D.

Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung
402, Taiwan

Tel.: +886-4-24730022 ext 11678; Fax: +886-4-23248195

E-mail: csmcysh@csmu.edu.tw

2. Jong-Da Lian, MD

Division of Nephrology, Department of Internal Medicine,
Chung Shan Medical University Hospital, Taichung 402, Taiwan

Tel: +886-4-24739595 Ext 32630

Fax: +886-424739220

E-mail: cshy590@csh.org.tw

Short title: MMP-2 in kidney transplant recipients

Abstract:

Serum matrix metalloproteinase-2 (MMP-2) has been reported to be higher in patients with chronic transplant nephropathy than in patients with acute rejection, stable graft function and healthy donors. However, further clarification of the relationships between serum MMP-2 and various clinical parameters is needed. Gelatin zymography and ELISA were employed to measure MMP-2 activity and level, respectively, in the plasma of 152 kidney transplant recipients and 50 healthy control subjects. Associations of MMP-2 with treatment (cyclosporine [CsA] [n=28], tacrolimus [TAC] [n=71], statins [n=32], or steroids [n=64]), hypertension (n=74), coronary artery disease (n=13), preexisting diabetes mellitus (n=25), and posttransplant diabetes mellitus (n=26) were examined. The serum creatinine (SCr) and the MMP-2 levels in the CsA group (1.11±0.34 mg/dl and 705.94±142.12 ng/ml, respectively) and TAC group (SCr, 1.17±0.42 mg/dl; MMP-2, 636.17±175.99 ng/ml) were significantly higher ($p<0.001$) than those of control subjects (SCr, 0.76±0.17 mg/dl; MMP-2, 517.79±220.86 ng/ml). Univariate analysis demonstrated the MMP-2 level was positively associated with cystatin C level ($r=0.38$, $p<0.001$), SCr level ($r=0.25$, $p=0.014$), and proteinuria ($r=0.22$, $p=0.029$), and negatively associated with creatinine clearance ($r=-0.26$, $p=0.009$). Stepwise regression analysis demonstrated the MMP-2 level was associated with cystatin C level ($p<0.001$), posttransplant days ($p=0.025$), and posttransplant diabetes mellitus ($p=0.03$). We conclude

1
2 that circulating MMP-2 is associated with cystatin C, posttransplant duration, and diabetes
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5 mellitus in kidney transplant recipients.

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7 Keywords: matrix metalloproteinase; cystatin C; renal transplantation; chronic renal
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10 allograft dysfunction; chronic allograft nephropathy
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Introduction

Chronic renal allograft dysfunction precedes most graft failures. Chronic allograft dysfunction is associated with donor and recipient factors, renal function, renal events, and renal histology in the first year posttransplantation. Differential diagnoses include ureteric obstruction, renal artery stenosis, glomerulonephritis, infection, nephrotoxic agents, late or recurrent acute rejection, and chronic allograft nephropathy [1]. Glomerulosclerosis represents the final and irreversible destruction of functioning nephrons and could be seen as occurring in two phases [2]. Glomerulosclerosis is a time-dependent response to glomerular injury from early ischemia, immune-mediated tubular loss, and late calcineurin nephrotoxicity [3]. It is well known that extracellular matrix (ECM) turnover plays a critical role in the process of glomerulosclerosis, and remodeling of ECM is an important physiologic feature of normal growth and development. Many diseases, including chronic allograft nephropathy, have been associated with an imbalance between ECM synthesis and degradation, which may result in an accumulation of ECM molecules [3, 4]. The major regulators of ECM degradation in the glomerulus are matrix metalloproteinases (MMPs) [5]. Thus, changes in MMPs expression or activity may directly alter ECM turnover, which may lead to glomerular scarring and a decline in allograft function [4, 5].

MMPs are a large family of zinc-requiring enzymes, which include interstitial collagenases, stromelysins, gelatinases, elastases, and membrane-type matrix metalloproteinases (MT-MMP) [6]. They synergistically degrade ECM components, and

1
2 as such, are involved in embryonic development and a variety of pathophysiological tissue
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4 remodeling processes (including angiogenesis, invasive cell behavior, inflammation,
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7 wound healing, and fibrosis).
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10 The gelatinases (MMP-2 and MMP-9) are a subfamily of MMPs that share the ability
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12 to degrade basement membrane types IV and V collagens, aggrecan, elastin, and gelatins
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14 [6]. Their involvement in the pathogenesis of chronic kidney disease (CKD) was
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16 suggested. Various studies have shown that MMP-2 plays a role in both normal renal
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18 development and glomerular diseases [4, 7]. In the developing kidney, MMP-2 mRNA
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20 expression is limited to the mesenchyme. However, MMP-2 protein has been found in
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22 immature nephron structures undergoing epithelial differentiation [8, 9]. Accumulating
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24 evidence has established that MMPs play a role in the development of glomerulosclerosis
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26 [4, 9], a number of glomerulonephritides [10, 11], and interstitial renal fibrosis [12, 13].
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28 Regarding diabetes mellitus, a marked decrease in MMP-2 mRNA expression was
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30 detected in glomeruli of diabetic patients [14].
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38 We have recently reported a correlation between plasma MMP-2 level and serum
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40 creatinine (SCr) level in non-diabetic patients with CKD [15]. The present study increases
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42 the enrolled patient number and determines whether similar correlations are also present in
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44 kidney transplant recipients.
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48 **Subjects and methods**

49 *Subjects and specimen collection*

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2 A total of 303 subjects, including 50 healthy control subjects and 253 kidney
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4 transplant recipients from Chung Shan Medical University Hospital, Taichung, Taiwan,
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6 were enrolled. Patients were excluded if they were infected with viral hepatitis B (n=30),
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8 C (n=27), or B+C (n=5) [16], or had abnormal liver function (n=14) [17], malignancy
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10 (n=11; urothelial carcinoma [n=8], hepatocellular carcinoma [n=1], lung cancer [n=1], and
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12 adrenal tumor [n=1]) [18, 19], active infection (n=3; pneumonia [n=1], cytomegalovirus
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14 infection [n=1], and herpes zoster [n=1]) [20], subclinical leukocytosis (n=18) defined as
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16 white blood cell count $>11,000 /\text{mm}^3$ without clinical symptoms, sirolimus treatment
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18 (n=44)[21, 22], angiographically defined coronary artery diseases (n=13, the presence of
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20 one or more stenoses greater than 50% in at least one major artery or a left main stem
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22 stenosis greater than 30%), diabetes mellitus (n=23) or posttransplant diabetes mellitus
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24 (n=26) [23]. A total of 99 kidney transplant recipients and 50 healthy control subjects were
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26 recruited for analysis. Venous blood samples were obtained from all subjects and placed
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28 in tubes containing EDTA, immediately centrifuged, and stored at -80°C . Biochemical
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30 markers of renal function, including SCr and calculated creatinine clearance (CCr)
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32 estimated by the Cockcroft-Gault equation, were obtained for each sample, respectively.
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34 Institutional review board approval was obtained for this study protocol, and each subject
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36 provided written informed consent.
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49 *Gelatin zymography and ELISA determination of activities and levels of MMP-2*

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51 Activity of MMP-2 was determined by gelatin zymography according to a protocol
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1 developed by Kleiner *et al* [24]. About 20 μl of each sample containing a total of 20 μg of
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4 protein was loaded onto a precast sodium dodecyl sulfate-polyacrylamide gel containing
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7 0.1% gelatin and subjected to electrophoresis. The gels were then processed as described
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10 by Yang *et al* [25], and the densities of non-stained bands represented the levels of latent
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12 forms of MMP-2 [26]. MMP-2 level in plasma samples was analyzed using a human
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14 MMP-2 ELISA kit (R&D Systems, Abingdon, UK). Each sample was assayed in duplicate
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17 and samples with a reading higher than the linear range of the standard curve were
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20 appropriately diluted and tested again.
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22 *Determination of serum cystatin C level*

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25 A fully automated commercial particle-enhanced nephelometric immunoassay was
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27 used in this study (N Latex Cystatin C kit, Dade Behring Diagnostics, Marburg, Germany).
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30 The kit consisted of reagents for determination of cystatin C in serum, freeze-dried
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32 polystyrene particles coated with rabbit antibodies to cystatin C, a cystatin C control of
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34 human origin, and a cystatin C calibrator consisting of purified cystatin C of human origin.
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37 Within a fixed time period, the rate of antigen–antibody complex formation was
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40 determined from the scatter of a beam of incident light (840 nm), the intensity of the
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43 scattered light being proportional to the concentration of cystatin C in the sample. The
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46 assay utilized a stored six-point calibration curve generated from multiple dilutions of a
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49 human cystatin C calibrator. The assay time was 6 min with a throughput of 75 samples
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52 per hour. The sample (volume, 40 μL ; range of concentration measurement, 0.23–8.0
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2 mg/L; sample dilution, 1:100) was measured on a BN II nephelometer. The lower
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4 quantitation limit of this assay was 0.0156 mg/dL while accuracy (expressed as the
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6 difference between the expected and the measured values of cystatin C controls) was
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8 9.53%, and the intra- and inter-assay imprecision averaged 3.66% and 4.6%, respectively.
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11 12 *Statistical analysis*

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15 Values were expressed as means \pm S.D. The statistical significance of difference
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17 between groups was determined by one-way analysis of variance (ANOVA) or
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19 Mann-Whitney rank sum test. Linear regression analysis was applied to assess the
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21 correlation between two variables. Logistic regression models were used to assess the
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23 an individual parameter was associated with the serum MMP-2 level. Multivariate
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25 analysis with stepwise regression was applied to detect independent variables associated
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27 with the circulating level of MMP-2. A *p* value of less than 0.05 was considered
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29 statistically significant.
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Results

Characteristics of healthy controls

The characteristics of the healthy controls (20 male and 30 female; age, 40.1 ± 16.5 years) are summarized in Table 1. The SCr level and the CCr were 0.8 ± 0.2 mg/dl and 107.7 ± 35.7 μ mol/l, respectively. The level of MMP-2 was 517.8 ± 220.9 ng/ml and was not associated with age, sex, body weight, SCr level, CCr, and cystatin C level.

Characteristics of kidney transplant recipients

Comparisons between the healthy subjects and kidney transplant recipients are summarized in Table 2. The age of the CsA group (53.9 ± 11.8 years) and the TAC group (46.6 ± 12.6 years) were both older than the control group (40.1 ± 16.5 years, $p < 0.001$). The body weight and sex were comparable between all groups. The CCr of the CsA group (61.1 ± 19.8 ml/min) and TAC group (65.4 ± 19.3 ml/min) were both lower than that of the control group (107.7 ± 35.7 ml/min, $p < 0.001$). The serum creatinine level in the CsA group (SCr: 1.11 ± 0.34 mg/dl) and TAC group (1.17 ± 0.42 mg/dl) were comparable and significantly higher ($p < 0.001$) than that in the control group (SCr: 0.76 ± 0.17 mg/dl).

Gelatin zymographic and ELISA analysis for MMP-2

Plasma activities of MMP-2 of the control subjects and kidney transplant recipients were assayed by gelatin zymography. The intensity of the 72-kDa MMP-2 band (Figure 1) indicated that MMP-2 activity was higher in kidney transplant recipients than control subjects.

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2 To quantitatively determine the MMP-2 level, an ELISA was performed and showed
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4 that the MMP-2 levels in the CsA (705.94 ± 142.12 ng/ml) and TAC (636.17 ± 175.99 ng/ml)
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6 groups were comparable and significantly higher ($p < 0.001$) than that in the control
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8 (517.79 ± 220.86 ng/ml) group.
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12 Multiple linear regression demonstrated that serum MMP-2 level was positively
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14 associated with cystatin C level ($r = 0.38$, $p < 0.001$), SCr level ($r = 0.25$, $p = 0.014$),
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16 proteinuria ($r = 0.22$, $p = 0.029$) and negatively associated with CCr ($r = -0.26$, $p = 0.009$)
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18 (Table 3).
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22 *MMP-2 level associated with cystatin C level, post-transplant days, and diabetes mellitus*

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24 To determine whether MMP-2 was related to various clinical parameters and
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26 whether the previously reported relationship between MMP-2 and diabetes mellitus [23]
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28 existed in our kidney transplant recipients, the patients with pre-existing diabetes mellitus,
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30 posttransplant diabetes mellitus, and angiographically defined coronary artery disease
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32 were included for analyses. Table 4 lists the associations between MMP-2 level and
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34 various parameters including statin therapy ($n = 32$), hypertension ($n = 74$), steroid therapy
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36 ($n = 64$), coronary artery disease ($n = 13$), preexisting diabetes mellitus ($n = 25$), and
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38 posttransplant diabetes mellitus ($n = 26$). Circulating MMP-2 level was significantly higher
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40 in patients with diabetes mellitus (including preexisting and posttransplant diabetes
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42 mellitus) (731.5 ± 204.6 ng/ml) than in patients without diabetes (656.3 ± 167.7 ng/ml)
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44 ($p = 0.017$). No association of MMP-2 level with sex, statin therapy, hypertension, steroid
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2 therapy, or coronary artery disease was demonstrated. Logistic regression models (Table
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4 4a) and stepwise regression analysis (Table 4b) demonstrated that MMP-2 level was
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6 independently associated with cystatin C level ($p<0.001$), posttransplant days ($p=0.025$),
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8 and diabetes mellitus ($p=0.033$).
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Discussion

In the glomerulus, epithelial cells produce both MMP-2 and MMP-9 [27, 28], whereas mesangial cells only produce MMP-2 [29]. MMPs play a role in glomerulosclerosis [9, 30] and interstitial kidney fibrosis [13]. Change in MMP-2 expression or activity in the glomerulus will change ECM turnover, which may lead to glomerular scarring and a decline in renal function. Since MMPs are rapidly released into the extracellular space after matrix synthesis, MMP-2 may easily diffuse into the blood and serve as an indicator of collagen turnover. Thus, hepatic [16, 17] and renal diseases (such as glomerulonephritis [10, 11, 31], chronic transplant nephropathy [32], CKD [15] or hemodialysis [33]) can affect the level of circulating MMP-2.

In the present study, levels of circulating MMP-2 and SCr were significantly higher in the CsA- or TAC-treated kidney transplant recipients than in controls. These results are consistent with our previously reported finding of higher circulating MMP-2 in patients with nondiabetic CKD (SCr: 6.58 ± 0.38 mg/dl or 581.7 ± 33.6 μ mol/l, CCr: 11.28 ± 0.75 mg/dl) before dialytic therapy than in normal controls (SCr: 0.77 ± 0.03 mg/dl or 68.1 ± 2.7 μ mol/l, CCr: 105.1 ± 5.66 mg/dl) [15].

Chronic allograft nephropathy (CAN) is one of the underlying causes of chronic renal allograft dysfunction. The histological hallmark of CAN is progressive fibrosis in which ECM turnover and accumulation of collagen in the kidney play an important role [34]. In

1
2 a rat model of CAN [35], MMP-2, MMP-9, and TIMP-3 were considered to play a critical
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4 role in the development of fibrosis in the renal allograft. The MMP-2 and MMP-9 were
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6 upregulated and the inhibitory effect of TIMP-3 was downregulated in kidney allografts.
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10 In human kidney transplant recipients, Rodrigo et al. reported that serum MMP-2 and
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12 MMP-3 were significantly higher in patients with chronic transplant nephropathy than in
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14 healthy donors or patients with acute rejection or stable graft function [32]. Interestingly,
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16 in the same study, plasma activities of MMP-2 correlated with proteinuria ($r=0.48$), and
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18 plasma activities of MMP-3 correlated with SCr ($r=0.44$).
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23 Recently Lutz et al. reported that inhibition of MMPs early after transplantation
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25 reduced the development and progression of CAN but promoted CAN if initiated at later
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27 stages [36]. The early MMP-inhibition in a rat model resulted in significantly reduced
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29 24-hour protein excretion that was paralleled by a lower grade of CAN after 20 weeks.
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33 However, late MMP inhibition starting at week 12 after transplantation resulted in
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35 significantly higher proteinuria and a higher grade of CAN as compared with controls.
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38 Therefore, a pathogenic, time-course-dependent, differential role of MMPs was suggested
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40 in the development and progression of CAN. Our clinical results also suggested a
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42 time-dependent role of MMP-2. Post-transplant duration was correlated with plasma level
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44 of MMP-2, which might indirectly indicate the relationship between posttransplant
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46 duration *versus* progression of CAN (Figure 3) [1].
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2 Because of the strict exclusion of patients with multiple co-morbidities in this study,
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4 most patients with severe graft dysfunction and all patients with CAN treated with
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6 sirolimus therapy were excluded. Therefore, the SCr level of the enrolled patients was
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8 only 1.11 ± 0.34 mg/dl in the CsA group and 1.17 ± 0.42 mg/dl in the TAC group. Similar
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10 with our previous observation that the MMP-2 level is correlated with SCr in patients with
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12 nondiabetic CKD [15], the MMP-2 level is correlated with cystatin C which is as good as
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14 SCr to estimate GFR and is less sensible to changes in body mass [37]. In addition,
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16 recently Lee and his colleagues reported plasma MMP-2, TIMP-1 and hs-CRP
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18 concentrations were significantly increased in type-2 diabetic patients. Our study also
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20 demonstrated an increased circulating level of MMP-2 in the kidney transplant recipients
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22 with pre-existing and posttransplant diabetes mellitus.
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30 In conclusion, plasma level of MMP-2 in patients with chronic renal allograft
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32 dysfunction who received kidney transplants was increased and associated with cystatin C
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34 level, posttransplant duration, and diabetes mellitus. Further studies are needed to assess
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36 the exact role of MMPs in the pathologic mechanism predisposing patients to chronic
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38 renal allograft dysfunction.
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Table 1. Analysis of variables in the healthy controls.

		MMP-2: 517.8 ± 220.9 (ng/ml)		
	Mean±SD	N	r	p
Age	40.1 ± 16.5	50	-0.06	0.675
Body weight	61.9 ± 10.5	50	0.09	0.514
Ccr	107.7 ± 35.7	50	0.00	0.995
Cr	0.8 ± 0.2	50	0.16	0.260
Cystatin C	1.01 ± 0.56	17	0.33	0.196
Male	544.7±221.6	20	-	0.488
Female	499.9±222.3	30	-	

NOTE. To convert serum creatinine in mg/dl to $\mu\text{mol/l}$, multiply by 88.4.

Table 2. Comparisons between the healthy controls and the kidney transplant recipients.

Variables	Group	N ^a	Mean	SD	F value	p value
Age	Control	50	40.06	16.54	9.11	<0.001*** Age _{CsA} , Age _{TAC} > Age _{Control}
	CsA	28	53.89	11.84		
	TAC	71	46.55	12.55		
Body weight	Control	50	61.86	10.53	1.63	0.200
	CsA	28	57.61	10.60		
	TAC	71	59.26	10.72		
Ccr	Control	50	107.68	35.65	46.61	<0.001*** CCr _{Control} > CCr _{CsA} , CCr _{TAC}
	CsA	28	61.07	19.77		
	TAC	71	65.42	19.33		
SCr	Control	50	0.76	0.17	22.25	<0.001*** SCr _{CsA} , SCr _{TAC} > SCr _{Control}
	CsA	28	1.11	0.34		
	TAC	71	1.17	0.42		
MMP-2 (ng/ml)	Control	50	517.79	220.86	10.46	<0.001*** MMP-2 _{CsA} , MMP-2 _{TAC} > MMP-2 _{Control}
	CsA	28	705.94	142.12		
	TAC	71	636.17	175.99		
Sex	Control	50	Male (20; 40.0%)			0.893
	CsA	28	Male (10; 35.7%)			
	TAC	71	Male (29; 40.8%)			

^a The patients with diabetes mellitus or coronary artery disease were excluded.

Table 3. Correlations between serum MMP-2 level and various clinical parameters.

	MMP-2 (ng/ml)		
	N ^a	r	p
Age	99	0.13	0.188
Body weight	99	0.02	0.823
Posttransplant days	99	0.06	0.585
CCr (cc/min)	99	-0.26	0.009
SCr (mg/dl)	99	0.25	0.014
Cystatin C (mg/dl)	99	0.38	<0.001
MMF dose (mg/day)	99	0.06	0.552
Steroid dose (mg/day)	99	0.18	0.068
WBC	99	0.01	0.893
Proteinuria (semiquantitative)	99	0.22	0.029
GPT	99	-0.07	0.509
GOT	99	-0.04	0.767

^a The patients with diabetes mellitus or coronary artery disease were excluded.

Table 4a. Associations between MMP-2 level and clinical variables revealed by logistic regression.^a

Variable	Items	MMP-2 (ng/ml)			t value	p value
		N ^a	Mean	SD		
Sex	Male	77	679.89	197.38	-0.11	0.911
	Female	75	683.23	169.94		
Statin	None	120	681.80	196.06	0.03	0.973
	Yes	32	680.57	130.04		
HTN	None	78	653.50	172.39	-1.95	0.053
	Yes	74	711.09	191.75		
Steroid	Withdrawal	88	673.19	199.23	-0.66	0.513
	Use	64	693.02	160.88		
CAD	None	139	679.71	186.35	-0.40	0.690
	Yes	13	701.10	158.38		
DM	None	101	656.29	167.72	-2.42	0.017*
	Yes	51	731.54	204.63		
DM	None	101	656.29	167.72	2.92	0.057
	Pre-DM	25	733.19	195.29		
	PTDM	26	729.95	217.09		

^a The patients with diabetes mellitus and coronary artery disease were included for analysis.

Table 4b. Associations between MMP-2 level and clinical variables revealed by stepwise regression.^a

Step	Model	Unstandardized		Standardized	t	Sig.
		coefficients	coefficients	Beta		
		B	Std. error	Beta		
1	Cystatin C	153.53	45.32	0.264	3.39	<0.001
2	Posttransplant days	0.05	0.02	0.175	2.26	0.025
3	DM	64.72	30.13	0.167	2.15	0.033
	Constant	438.56	59.09		7.42	<0.001
ANOVA		F=7.16	P<0.001***	R ² =0.127		

^aThe patients with diabetes mellitus and coronary artery disease were included for analysis.

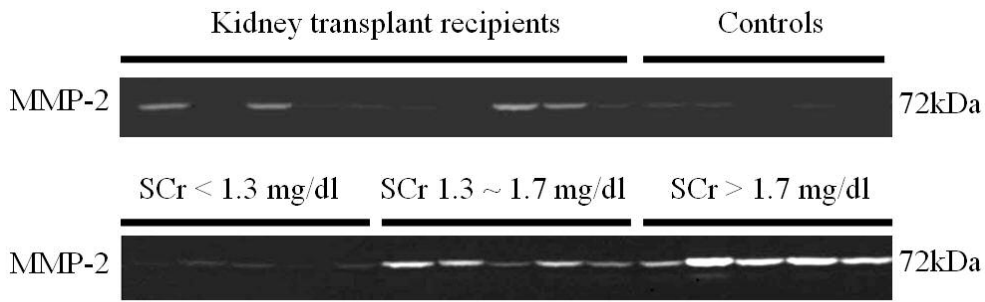
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6 Fig. 1. MMP-2 activity analyzed by gelatin zymography. The upper panel is a
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8 representative zymograph of plasma samples from the kidney transplant recipients
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10 and control subjects, and the lower panel, a representative zymograph of plasma
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12 samples from kidney transplant recipients with different levels of serum creatinine
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14 < 1.3, 1.3-1.7 and >1.7 mg/dl.
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18 Fig. 2. MMP-2 level and serum creatinine level in the kidney transplant recipients and 50
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20 control subjects determined using an ELISA kit following the manufacturer's
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22 instructions. MMP-2 levels were significantly higher in the CsA- or TAC-treated
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24 kidney transplant recipients than in control subjects.
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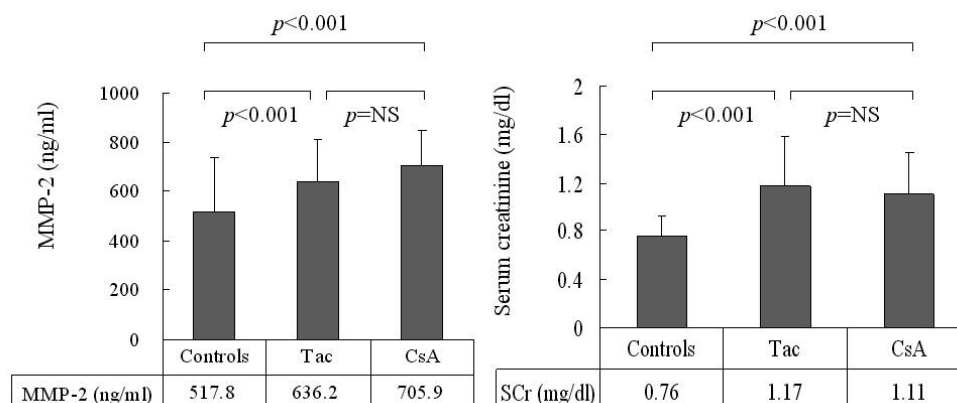
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29 Fig. 3. Linear regression analysis showing the relationship between the ELISA-determined
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31 plasma level of MMP-2 and serum cystatin C level or posttransplant days.
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MMP-2 activity analyzed by gelatin zymography. The upper panel is a representative zymograph of plasma samples from the kidney transplant recipients and control subjects, and the lower panel, a representative zymograph of plasma samples from kidney transplant recipients with different levels of serum creatinine < 1.3, 1.3-1.7 and >1.7 mg/dl.

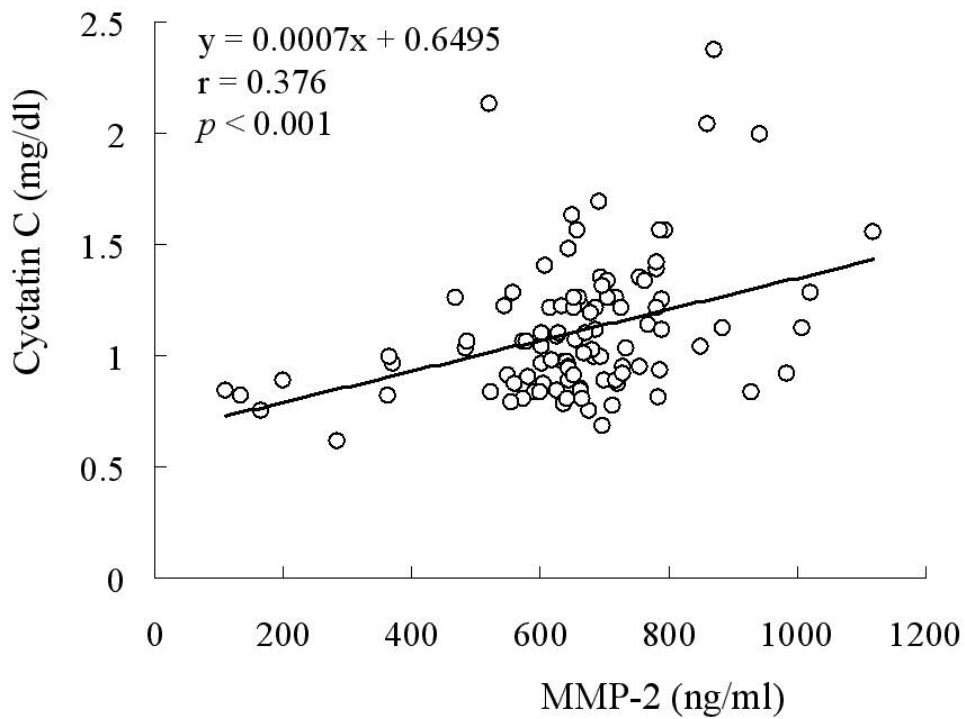
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MMP-2 level and serum creatinine level in the kidney transplant recipients and 50 control subjects determined using an ELISA kit following the manufacturer's instructions. MMP-2 levels were significantly higher in the CsA- or TAC-treated kidney transplant recipients than in control subjects.

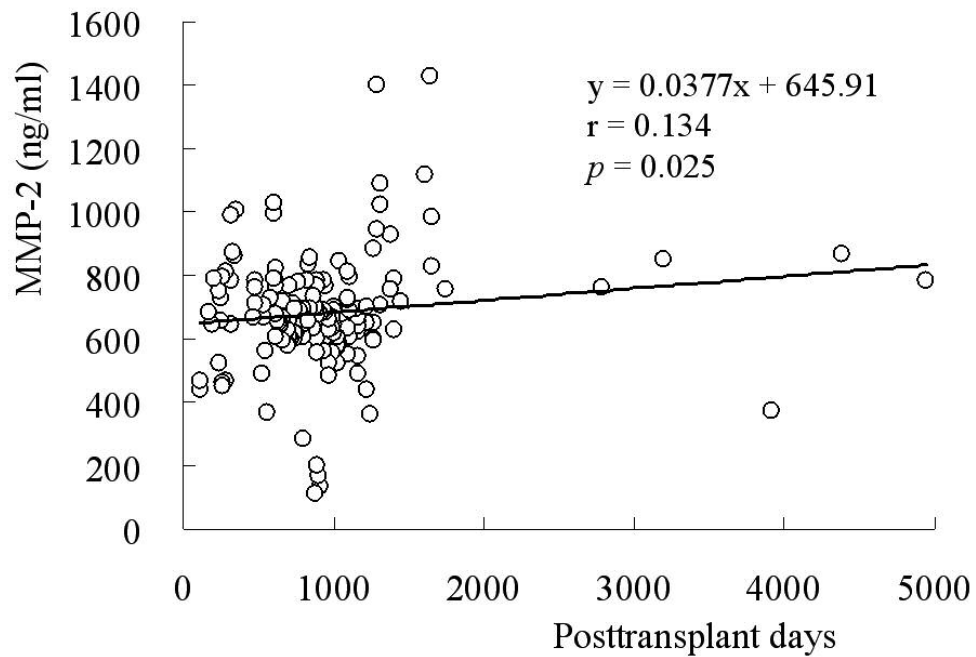
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Linear regression analysis showing the relationship between the ELISA-determined plasma level of MMP-2 and serum cystatin C level or posttransplant days.

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