Tohoku J. Exp. Med., 2010, **222**, 319-327

Increased Urinary Lipocalin-2 Reflects Matrix Metalloproteinase-9 Activity in Chronic Hepatitis C with Hepatic Fibrosis

Jin-Wook Kim,[1] Sang Hyub Lee,[1] Sook-Hyang Jeong,[1] Haeryoung Kim,[2] Keun Soo Ahn,[3] Jai Young Cho,[3] Yoo-Seok Yoon[3] and Ho-Seong Han[3]

[1]Department of Internal Medicine, Seoul National University College of Medicine, Seoul, South Korea
[2]Department of Pathology, Seoul National University College of Medicine, Seoul, South Korea
[3]Department of Surgery, Seoul National University College of Medicine, Seoul, South Korea

Hepatic fibrosis is characterized by excessive accumulation of extracellular matrix. Matrix metalloproteinases (MMPs) play an important role in the remodeling of the extracellular matrix during hepatic fibrosis. Lipocalin-2 (LCN2) forms complexes with MMP-9 and can be detected in the urine of patients with several types of cancers. The objective of this study was to examine the relationship between urinary LCN2 levels and MMP-9 activity with respect to the stage of liver fibrosis in patients with chronic hepatitis C (CHC), and to assess the utility of urine LCN2 as a non-invasive marker of hepatic fibrosis. Fresh spot urine samples were prospectively collected from forty-two interferon-naive CHC patients who underwent liver biopsy. The stage of hepatic fibrosis was assessed according to the METAVIR fibrosis score; 18 patients had no or mild fibrosis (stages F0 and F1) and 24 patients showed significant fibrosis (stages F2-F4). Immunoblot analyses demonstrated co-migration of urine LCN2 and MMP-9. Gelatin zymography showed that urinary MMP-9/MMP-2 activity ratios were higher in patients with significant fibrosis (F2-F4) than in patients no or mild fibrosis (F0-F1). Urine LCN2 levels which were normalized to urine creatinine concentration (urine LCN2-to-creatinine ratio; ULCR) were higher in F2-F4 patients compared to F0-F1 patients. There was a positive correlation between ULCR and urine MMP-9/MMP-2 activity ratios (r = 0.735). ULCR and AST-to-platelet ratio index were independent predictors of significant fibrosis by multivariate analysis. The present study suggests that urinary LCN2 is a novel marker of hepatic fibrosis by reflecting urine MMP-9 activity in CHC.

Key words: fibrosis; hepatitis C virus; lipocalin-2; matrix metalloproteinase-9; urine
Tohoku J. Exp. Med., 2010, **222** (4), 319-327.

Hepatic fibrosis results from any kind of chronic liver injury and characterized by excessive accumulation of extracellular matrix (ECM), which eventually leads to liver cirrhosis. Correct evaluation of hepatic fibrosis is important in the management of chronic hepatitis C virus (HCV) infection because disease progression and decision to start antiviral therapy depend on the status of hepatic fibrosis (Ghany et al. 2009). Currently, liver biopsy is the gold standard for the assessment of hepatic fibrosis in CHC (Ghany et al. 2009). However, due to the invasiveness and potential sampling errors of liver biopsy, the identification of non-invasive blood markers as possible alternative diagnostic tools has received considerable attention (Bravo et al. 2001; Bedossa et al. 2003; Manning and Afdhal 2008). Non-invasive markers can be conceptually classified as indirect and direct. Indirect markers reflect changes in liver function, and they do not necessarily have a mechanistic relationship with fibrogenesis [eg. AST-to-platelet ratio index (Wai et al. 2003) and Fibrotest (Imbert-Bismut et al. 2001)]. In contrast, direct markers reflect the metabolism of hepatic ECM proteins (Friedman 2008): hyaluronic acid (McHutchison et al. 2000; Mehta et al. 2008), type IV collagen (Murawaki et al. 2001), laminin (Walsh et al. 2000), and matrix metalloproteinases. Despite the potential advantage of representing ongoing fibrogenic activity (Gressner et al. 2009), direct markers have been less well validated than direct markers.

Matrix metalloproteinases (MMPs) degrade hepatic ECM proteins in chronic liver injury/fibrosis models (Benyon and Arthur 2001; Iredale 2007). Within the MMP family, MMP-9 (gelatinase B) is a 92-kDa protein that is secreted and activated by hepatic stellate cells during their trans-differetiation process (Takahara et al. 2003; Han et al. 2004, 2007). Activated MMP-9 can degrade type IV

Received September 28, 2010; revision accepted for publication November 21, 2010. doi: 10.1620/tjem.222.319
Correspondence: Jin-Wook Kim, Department of Internal Medicine, Seoul National University Bundang Hospital, Gumi-dong 300, Seongnam, Kyonggi-Do 463-802, South Korea.
e-mail: kimjw@snubh.org

collagen, which is a major component of the basement membrane and normal hepatic ECM (Benyon and Arthur 2001; Iredale 2007; Friedman 2008). Several previous reports showed increased hepatic expression of MMP-9 in experimental cirrhosis (Kossakowska et al. 1998; Reif et al. 2004) and CHC (Lichtinghagen et al. 2001; Nunez et al. 2004). These findings raise the possibility that MMP-9 may serve as a direct marker of hepatic fibrosis in CHC.

Lipocalin-2 (LCN2), also known as NGAL (neutrophil gelatinase-associated lipocalin), is a 25-kDa transporter belonging to the lipocalin superfamily. LCN2 binds MMP-9 and forms a 115-135-kDa MMP-9-LCN2 complex (Kjeldsen et al. 1993; Yan et al. 2001), preventing the degradation of MMP-9 (Yan et al. 2001; Fernandez et al. 2005; Roy et al. 2008). The MMP-9/LCN2 complex is present in the urine of breast cancer patients (Yan et al. 2001) and is believed to promote MMP-mediated matrix degradation and tumor progression (Fernandez et al. 2005). LCN2 is over-expressed from hepatocytes in a hepatic fibrosis model (Takahara et al. 2006), and we have reported that LCN2 is expressed in cirrhotic human liver tissues as well as in HBV-associated hepatocellular carcinoma (Kim et al. 2009). From these observations, we hypothesized that urinary LCN2 may reflect MMP-9 activity in CHC. In this study, we sought to elucidate the relationship between urinary LCN2 and MMP-9 activity and to examine whether urine LCN2 and MMP-9 are associated with the stage of liver fibrosis in patients with CHC.

Methods

Patients

This prospective study was approved by the institutional review board of our hospital (B-0608/036-025), and written informed consent was obtained from each subject, in accordance with the Declaration of Helsinki. Forty-two consecutive Korean patients with CHC who were admitted to the Seoul National University Bundang Hospital for percutaneous liver biopsy before interferon treatment were enrolled in this study (Table 1). Patients were excluded if they had any of the following: 1) active or recent bacterial infection 2) combined hepatocellular carcinoma 3) serum creatinine > 1.2 mg/dL 4) urine WBC > 5 per high power field 5) co-morbidity such as malignancy, chronic obstructive lung disease, asthma, rheumatologic disease or inflammatory bowel disease and 6) clinically evident ascites. The stage of hepatic fibrosis was assessed according to the METAVIR fibrosis score (Bedossa and Poynard 1996): F0 = no fibrosis; F1 = portal fibrosis without septa; F2 = portal fibrosis with rare septa; F3 = numerous septa without cirrhosis, and F4 = cirrhosis. Among the forty-two patients, eighteen patients had no or mild fibrosis (METAVIR stage F0, F1) and twenty-four patients showed significant fibrosis (METAVIR stage F2-F4). When demographic and laboratory variables were compared, F2-F4 fibrosis group showed significantly higher age, higher prothrombin time, lower serum albumin levels and lower platelet counts compared to the F0-F1 group (Table 1).

All liver biopsies with at least 6 or more portal tracts were reviewed by one pathologist (H.K.). Mean biopsy length (± S.D.) was 15.6 ± 2.8 mm. The degree of liver fibrosis was assessed using the METAVIR group scoring system (Bedossa and Poynard 1996) as follows: F0 = no fibrosis; F1= portal fibrosis without septa; F2= portal fibrosis with rare septa; F3 = numerous septa without cirrhosis; F4 = cirrhosis. For immunoblot and zymography, frozen liver tissue was homogenized in RIPA buffer.

Urine collection and processing

Early morning urine samples were collected on the day of liver biopsy, immediately stored at 4°C, and centrifuged at 200 g for 5 min within 1 hr from collection. The supernatant was snap-frozen and stored at –70°C until further analysis.

Table 1. Demographic and laboratory features of enrolled patients based on fibrosis staging.

	Overall	METAVIR Stage		
		F0-F1	F2-F4	*P* value
N	42	18 (F0/F1 2/16)	24 (F2/F3/F4 12/3/9)	
Age (y)	56.2 ± 10.9	50.5 ± 9.9	60.5 ± 9.7	0.002
Sex (M/F)	28/14	13/5	15/9	0.508
HCV genotype (1/2/unknown)	19/21/2	7/11/0	12/10/2	0.439
AST (IU/L)	87 ± 86	60 ± 35	107 ± 106	0.051
ALT (IU/L)	113 ± 92	97 ± 62	124 ± 109	0.306
Bilirubin (mg/dL)	0.91 ± 0.48	0.82 ± 0.35	0.98 ± 0.56	0.315
Albumin (g/dL)	4.6 ± 0.4	4.3 ± 0.3	4.0 ± 0.4	0.013
Prothrombin time (INR)	1.05 ± 0.09	1.01 ± 0.05	1.08 ± 0.11	0.018
Platelet (10^9/L)	159 ± 53	190 ± 48	136 ± 45	0.000
Hyaluronic acid (ng/ml)	63.8 ± 77.5	40.4 ± 47.5	83.8 ± 92.6	0.112
HCV RNA (log10 copies/mL)	5.41 ± 0.79	5.38 ± 0.90	5.45 ± 0.71	0.784

Results are shown as mean ± standard deviation or number; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus.

Measurement of urinary lipocalin-2 by enzyme-linked immunosorbant assay (ELISA)

The urine samples were diluted 50 times to measure the urinary LCN2 concentration by ELISA according to the manufacturer's instructions (NGAL ELISA kit, BioPorto, Gentofte, Denmark). To adjust for variations in the dilution of spot urine samples, the ELISA result for LCN2 was divided by the simultaneously measured urinary creatinine concentration (Quantichrome DICT-500, BioAssays Systems, Hayward, CA) to obtain the urine LCN2-to-creatinine ratio (ULCR, expressed in ng/mg) (Trevisan 1990).

Immunoblot analysis

Monoclonal antibodies against human LCN2 (MAB1757) and human MMP-9 (MAB911) were purchased from R&D Sytstems and used at dilutions of 1:250 and 1: 100, respectively. The urine samples were concentrated by using a Centricon Plus-20 centrifugal filter device according to the manufacturer's instruction (Millipore, Billerica, MA) before loading. Concentrated urine samples (20 μl) were mixed with the non-denaturating buffer used for zymography and separated by 10%-polyacrylamide gel electrophoresis. Purified MMP-9 (R&D Systems) and LCN-2 (BioPorto) were run in parallel as standards. Resolved proteins were transferred to Hybond-P membranes (GE Healthcare, Buckinghamshire, UK). The membranes were blocked with 5% low fat dry milk in Tris buffered saline-Tween 20 (10 mM Tris, pH 7.2, 50 mM NaCl, 0.5% Tween 20) for 1h at room temperature, followed by incubation with primary antibody at 4°C for 16h. Blots were washed with Tris buffered saline-Tween 20 and incubated with horseradish peroxidase conjugated secondary antibodies at a dilution of 1:10,000 (Santa Cruz Biotechnology, Santa Cruz, CA) for 1h at room temperature. Labeled proteins were visualized with enhanced chemiluminescence reagent (Luminol Reagent, Santa Cruz Biotechnology).

Substrate gel electrophoresis (gelatin zymography)

The MMP-9 activity in urine was detected by gelatin zymography as reported previously (Yan et al. 2001), with a few modifications (Birkedal-Hansen et al. 2008). In brief, urine samples (20 μl) were mixed with 5 μl of non-reducing sample buffer (5% SDS, 0.2 M Tris, pH 6.8, 20% v/v glycerol, and 0.1% w/v bromophenol blue) and were separated on a 10% polyacrylamide gel containing 0.1% gelatin (Sigma-Aldrich G8150, St. Louis, MO). Liver homogenate was diluted with RIPA buffer and 500 ng of protein was loaded per lane. The plasma samples were diluted 1:100 with RIPA buffer and 10 μl was loaded per lane. Purified MMP-2 (72 kDa, Enzo Life Sciences, Plymouth Meeting, PA) and MMP-9 (GE Healthcare, Buckinghamshire, UK) were run in parallel as standards. Activation of latent MMP-2 and MMP-9 was performed by incubation in 2 mM aminophenyl mercuric acetate at 37°C for 1 h before electrophoresis. After electrophoresis, gels were washed four times successively in washing buffers and incubated for 16 h as described (Birkedal-Hansen et al. 2008). The gels were stained with 0.5% Coomassie Brilliant Blue R250 and destained with gel destaining solution (30% methanol, 10% acetic acid) until the bands were clear on the background of a uniform blue stain. The gels were directly scanned by a Hewlett-Packard Scanjet scanner, and each band was quantified using the TotalLab TL100 software (Nonlinear Dynamics, Newcastle upon Tyne, UK). The urinary MMP-9/MMP-2 activity ratio was obtained by dividing the densitometric measurement of MMP-9-LCN2 complexes by the corresponding densitometric measurement of MMP-2 for each sample.

Statistical analysis

Continuous variables are expressed as the mean ± S.D. unless stated otherwise. Comparison of means was performed by Student's *t*-test or Mann-Whitney *U* test. The Chi-square test was used to compare categorical data. Multiple logistic regression was applied to identify predictors of significant hepatic fibrosis. Statistical calculations were done by using SPSS software package version 15 (SPSS Inc., Chicago, IL). The diagnostic accuracies of fibrosis markers were evaluated by measuring the area under the receiver operating characteristics (AUROC) using Medcalc version 11 (Medcalc software, Mariakerke, Belgium). Comparison of ROC curves were done as previously reported (DeLong et al. 1988).

Results

When the urine samples from CHC patients with cirrhosis (METAVIR F4) were subjected to Western blot analysis using an antibody against human LCN2, two protein bands were detected: LCN2 monomer and larger (~150 kDa) protein (Fig. 1A). Previous reports identified the larger band to be the MMP-9/LCN2 complex in cancer patients (Yan et al. 2001; Roy et al. 2008). The immunoblot for human MMP-9 using the same urine sample also revealed ~150 kDa protein (Fig. 1B), suggesting that the high molecular weight urinary LCN2 represents MMP-9/LCN2 complex(es) in CHC with cirrhosis. The MMP-9 bands were not detectable in F0 fibrosis.

Next, we assessed the urinary MMP-9 activity and its possible association with hepatic fibrosis in CHC by gelatin zymography (the representative results are presented in Fig. 2). MMP-9 and MMP-2 activities were discernible in the urine, plasma and liver tissue from CHC patients. Urinary MMP-9 activities represented MMP-9 monomer, MMP-9/LCN2 complex and MMP-9 dimer (Fig. 2A) (Moses et al. 1998; Yan et al. 2001; Fernandez et al. 2005; Roy et al. 2008). All of the MMP-9 activities were present in the F4 urine and F2 liver tissue, whereas F0 sample gave very weak MMP-9 activity.

The MMP-2 activity was readily visible in the urine samples, but was very faint compared to the MMP-9 activity in the CHC liver sample. The urinary MMP-2 activity was present regardless of the stage of hepatic fibrosis (Fig. 2B). However, the MMP-9 activities were readily detectable only in CHC patients with F2-F4 fibrosis (Fig. 2B). The urinary MMP-9 activities adjusted to MMP-2 activity (MMP-9/MMP-2 ratio) were also significantly higher in CHC patients with F2-F4 compared F0-F1 fibrosis (Fig. 3). These findings suggest that the urinary MMP-9 activities, but not MMP-2 activity, may serve as a marker for significant hepatic fibrosis in CHC.

However, zymographic measurement of urinary MMP-9 activities may be impractical for clinical application because it is semi-quantitative and laborious. As urine MMP-9 forms complex(es) with LCN2 in CHC, we wanted to know whether urinary LCN2 could be used as a surrogate

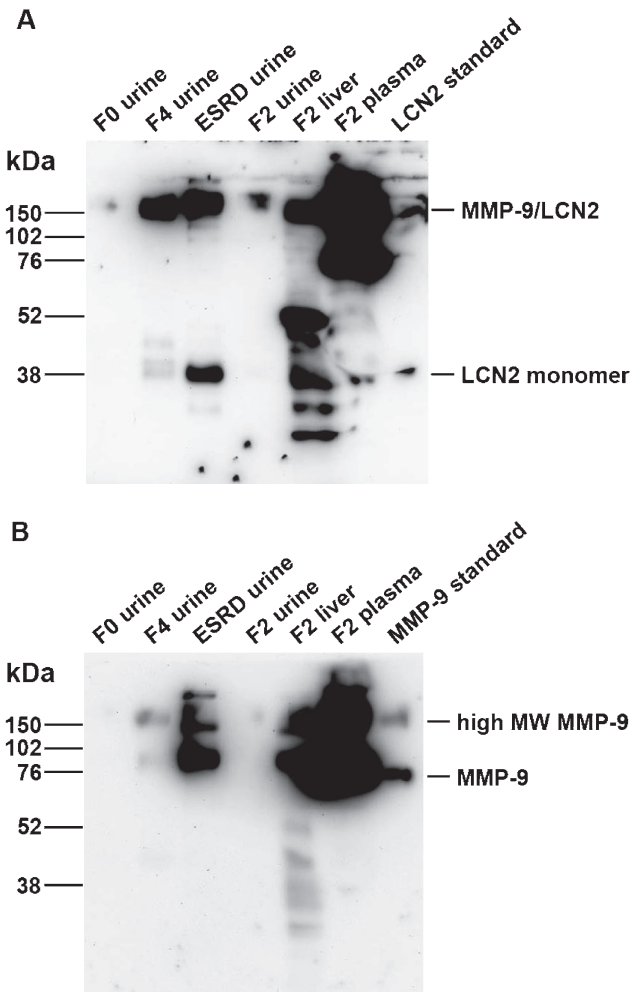

Fig. 1. Urinary LCN2 and MMP-9 in CHC patients with cirrhosis.
(A) Western blot analysis of urinary LCN2 in CHC with F4 fibrosis shows two major forms: high molecular weight bands (~150 kDa) that are the dominant form of LCN2, whereas the smaller band represents free LCN2 monomer. The F2 urine sample shows weaker signal and F0 sample shows lowest signal of high molecular weight LCN2. The liver tissue and plasma sample from the same F2 patient also revealed the high molecular weight LCN2. The urine sample from a patient with end-stage renal disease (ESRD) was run as a positive control. Liver tissue and plasma were used at 10-fold dilution. (B) Immunoblot analysis of urinary MMP-9 using concentrated urine samples shows high molecular weight bands (~150 kDa) that co-migrate with the high molecular weight form of LCN2, as well as smaller MMP-9 monomer (92 kDa) in F4 patients. Positive control and liver tissue/plasma of a F2 patient also shows the MMP-9 signals, whereas F0 sample gives no definite signal for MMP-9. Liver tissue and plasma were used at 10-fold dilution.

marker for MMP-9 activity and hepatic fibrosis. As expected, the adjusted urinary LCN2 levels (urine LCN2-to-creatinine ratio; ULCR) correlated significantly with the MMP-9/MMP-2 ratio ($r = 0.735$, $p < 0.0001$; Fig. 4), and the ULCR was significantly increased in F2-F4 group compared to F0-F1 group ($p < 0.001$, Fig. 5). By multivariate analysis, AST-to-platelet ratio index (APRI), a well-known indirect serum marker of hepatic fibrosis (Wai et al. 2003), and ULCR were independent discriminative factors for the prediction of F2-F4 fibrosis (Table 2). Serum hyaluronic acid, currently accepted as a direct marker of hepatic fibrosis (Mehta et al. 2008), was not a significant factor in this model. Regression formula was constructed from the multivariate model as follows:

$$\text{ULCR/APRI combination score} = 0.217 \cdot \text{ULCR} + 3.264 \cdot \text{Ln (APRI)} - 1.522.$$

The performances of APRI, hyaluronic acid, ULCR and the ULCR/APRI combination score for the correct diagnosis of significant hepatic fibrosis were summarized in Table 3. The ULCR/APRI combination score showed the highest AUROC value (0.87) which is significantly better than hyaluronic acid and similar to APRI or ULCR. The ULCR/APRI combination score was able to classify the presence or absence of significant fibrosis correctly in 81% of CHC at the cut-off value of 0.4.

Taken together, these observations suggest that urine LCN2 represents MMP-9 activities, and that adjusted urinary LCN2 levels can predict significant hepatic fibrosis in CHC patients.

Discussion

It has been reported that LCN2 is synthesized in the cirrhotic liver (Takahara et al. 2006; Kim et al. 2009), and that LCN2 forms a complex with 92-kDa gelatinase (MMP-9) in the urine of certain cancer patients (Kjeldsen et al. 1993; Yan et al. 2001). Urine samples have been scarcely studied to identify liver fibrosis markers, and we were able to identify only one experimental report that analyzed urine samples with respect to hepatic fibrosis (Smyth et al. 2009). In that study, LCN2 was identified as a marker of carbon tetrachloride-induced liver fibrosis (Smyth et al. 2009). Our study has shown for the first time that urinary LCN2 levels (ULCR) are elevated in CHC patients with significant fibrosis compared to patients with no or minimal fibrosis. We believe this association is due to the fact that urinary LCN2 forms complexes with MMP-9, because 1) our immunoblot analysis and zymography data indicate that LCN2/MMP-9 complex is present in the urine of CHC patients; 2) there is a positive correlation between urine LCN2 and urinary MMP-9 activities; and 3) urinary MMP-9 activities were significantly higher in F2-4 fibrosis.

To our knowledge, this is the first report demonstrating the relationship between urinary MMP-9 activity and the degree of hepatic fibrosis in CHC. Currently hepatic fibrosis is viewed as a dynamic reversible process, in which an imbalance between fibrogenesis and fibrolysis leads to an accumulation of ECM. Since matrix degradation begins in the early phase of liver fibrosis (Han 2006; Friedman 2008), it is not surprising that MMP-9, which degrade normal

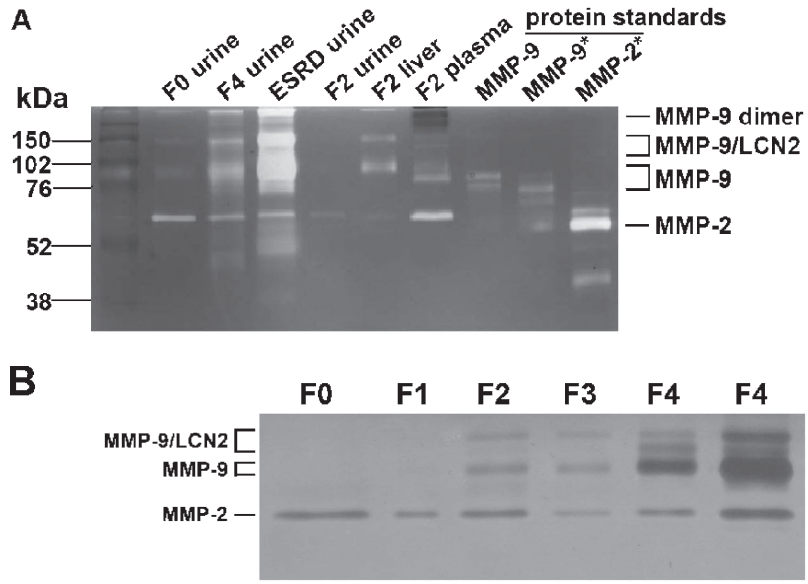

Fig. 2. Detection of urinary MMP-9 activities in CHC patients by gelatin zymography.
(A) Urinary MMP-9 and MMP-2 activities were identified in representative CHC patients and in a patient with end-stage renal disease (ESRD) as a positive control (Bolignano et al. 2009). Urinary MMP-9 activities represented MMP-9 monomer, MMP-9/LCN2 complex and MMP-9 dimer in F4 fibrosis. Positive control (ESRD) and liver tissue/plasma of a F2 patient also shows the MMP-9 activities, whereas F0 sample gives very weak MMP-9 activity. MMP-2 activity was readily detectable in all urine samples and plasma, whereas it was relatively weak in the liver tissue. Liver tissue and plasma were used at 100-fold dilution. *: MMP standards that were activated by aminophenyl mercuric acetate.
(B) Urinary MMP-9 activities were readily visible in CHC patients with significant hepatic fibrosis (F2-F4), but not in F0/F1 patients (B). The MMP-2 activity was detectable in all urine samples regardless of the degree of hepatic fibrosis.

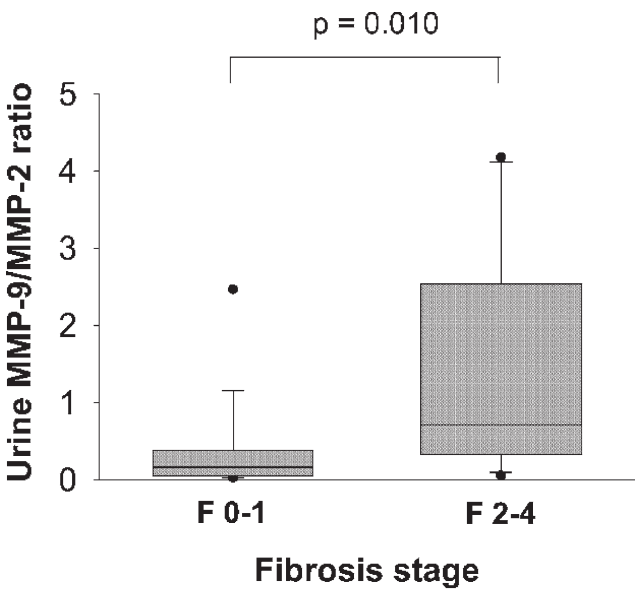

Fig. 3. Urinary MMP-9 activities elevate in CHC with significant fibrosis.
Urinary MMP-9 activities were measured by gelatin zymography, quantified by densitometric software, and adjusted to the MMP-2 activity of the same sample (urine MMP-9/MMP-2 activity ratio). The ratio was significantly higher in the F2-F4 fibrosis group compared to the F0-F1 group.

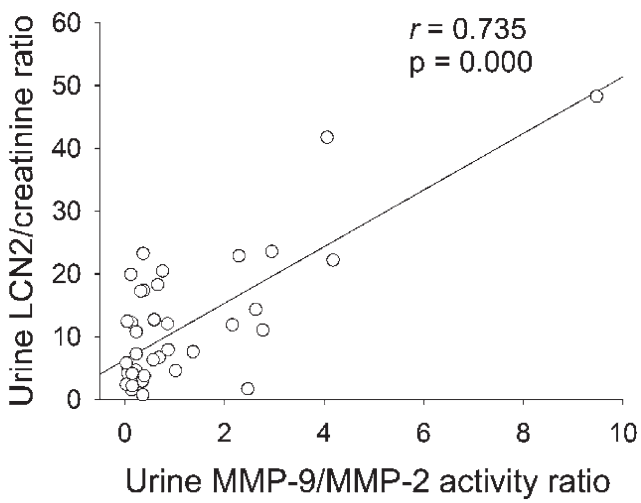

Fig. 4. Correlation between urine LCN2-to-creatinine ratio (ULCR) and urine MMP-9/MMP-2 activity ratio.
Urinary LCN2 levels were measured by ELISA, adjusted by concomitant urine creatinine concentration, and correlated to the urinary MMP-9 / MMP-2 activity ratios.

hepatic ECM, is expressed in the liver injury/fibrosis models (Kossakowska et al. 1998; Reif et al. 2004) and CHC (Lichtinghagen et al. 2001, 2003; Nunez et al. 2004). However, previous studies have reported controversial associations between hepatic fibrosis and blood MMP-9 levels in CHC patients (Hayasaka et al. 1996; Kuo et al. 2000; Lichtinghagen et al. 2000, 2003; Leroy et al. 2004; Reif et

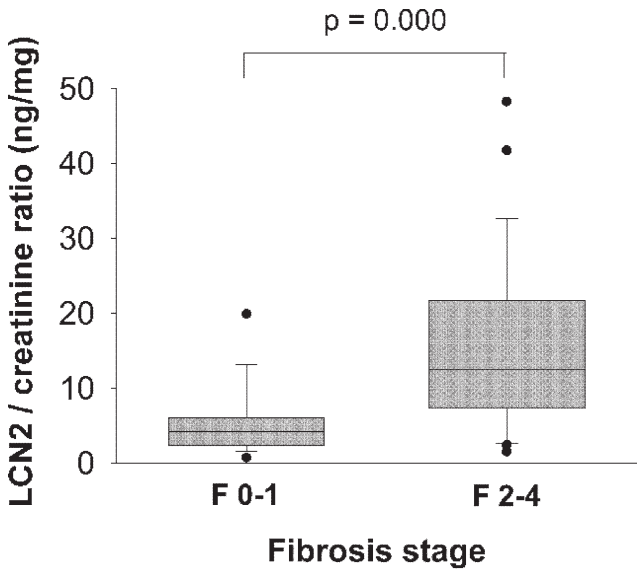

Fig. 5. Comparison of urine LCN2 between chronic hepatitis C patients with or without significant fibrosis (F2-F4). Adjusted urinary LCN2 levels (urine LCN2-to-creatinine ratio; ULCR) were significantly higher in the F2-F4 fibrosis group compared to the F0-F1 group. Each box represents the 25th and 75th percentiles, and the median is indicated with the solid line in each box.

Table 2. Variables associated with the presence of significant fibrosis (F2-F4) by multivariate analysis.

Factor	Odds ratio (95% CI)	*P* value
Age (y)	1.019 (0.916, 1.133)	0.731
ALT (IU/L)	0.994 (0.975, 1.012)	0.496
Albumin (g/L)	0.278 (0.011, 7.213)	0.278
Prothrombine time (%)	0.905 (0.754, 1.088)	0.288
Hyaluronic acid	0.991 (0.973, 1.011)	0.376
Ln (APRI)	26.156 (1.251, 546.987)	0.035
ULCR	1.242 (1.049, 1.470)	0.012

ALT, alanine aminotransferase; APRI, AST-to-platelet ratio index (AST/40 × platelets [10^9/L]); ULCR, urine LCN2-to-creatinine ratio.

al. 2005; Trocme et al. 2006). These inconsistent results may be explained by the fact that the serum levels of MMPs might be influenced by proteolytic clearance and release from extra-hepatic sources, especially from circulating blood cells during handling of samples (Lichtinghagen et al. 2000; Jung et al. 2001, 2002; Gerlach et al. 2007). Moreover, some evidence suggests that blood levels of MMPs do not reflect intra-hepatic activity (Kossakowska et al. 1998; Lichtinghagen et al. 2003). Because the binding of LCN2 prevents the degradation of MMP-9 (Yan et al. 2001), it can be postulated that hepatic MMP-9 forms complexes with LCN2 and can be detected in urine without proteolysis. Also, urine samples will not be contaminated by MMP-9 from white blood cells as long as pyuria is absent.

We concentrated urine samples for Western blot of MMP-9 because the signal was too weak, probably due to very low urinary MMP-9 levels. Our pilot study also showed that ELISA was not able to detect sufficient amount of urinary MMP-9 to differentiate fibrosis stages (data not shown). In contrast, gelatin zymography is a highly sensitive technique that detects as little as 32 pg of MMP-9 and shows a linear relationship between the band density and the amount of MMP-9 in the range below 1,000 pg (Leber and Balkwill 1997). Indeed, our zymography data also shows increased MMP-9 activity according to the degree of hepatic fibrosis.

Surprisingly, the MMP-2 activity was constantly present in all of the urine samples regardless of fibrosis stage (Fig. 2B). The hepatic expression MMP-2 is believed to increase as fibrosis advances (Milani et al. 1994; Lichtinghagen et al. 2001; Nunez et al. 2004). This discrepancy might be explained if urinary MMP-2 activity is mainly contributed by extrahepatic sources. This hypothesis is concordant with the findings that the correlation between circulating MMP-2 levels and hepatic fibrosis is absent (Walsh et al. 1999; Boeker et al. 2002; Reif et al. 2005) or very weak (Ebata et al. 1997; Leroy et al. 2004). We reasoned that if the urine MMP-2 activity was not affected by the degree of hepatic fibrosis, normalization of MMP-9 activities to concomitant MMP-2 activity may

Table 3. Performance characteristics of APRI, HA, ULCR and fibrosis score for prediction of significant hepatic fibrosis (METAVIR F2-F4).

Markers	Cut-off	Sensitivity (95% CI)	Specificity (95% CI)	AUROC (95% CI)	Accuracy (%)
Ln (APRI)	> –0.0083	75.0 (53.3, 90.2)	72.2 (46.5, 90.3)	0.764 (0.608, 0.881)	73.8
HA	> 24.5	66.7 (43.0, 85.4)	72.2 (46.5, 90.3)	0.651 (0.482, 0.796)	69.2
ULCR	> 6.69	79.2 (57.8,92.9)	83.3 (58.6, 96.4)	0.822 (0.673,0.922)	78.6
ULCR/APRI combination score	> 0.399	75.0 (53.3, 90.2)	94.4 (72.7, 99.9)	0.870* (0.731, 0.954)	81.0

Abbreviations: APRI, AST-to-platelet ratio index; HA, hyaluronic acid; ULCR, urine lipocalin 2-to-creatinine ratio; AUROC, area under the receiver operating characteristics.
*$P < 0.05$ vs. HA.

ensure the comparability by minimizing the effect of gel-to-gel variation in zymographic measurements. As expected, the urine MMP-9/MMP-2 activity ratios are correlated to ULCR and degree of hepatic fibrosis. Because quantification of urine MMP-9/MMP-2 activity by zymography is not readily available in hospital laboratory settings, urinary LCN2 may be a practical surrogate marker of the urinary MMP-9 activity in CHC patients.

It is an interesting finding that APRI score and ULCR, but not hyaluronic acid, were independent predictors of significant fibrosis by multivariate analysis. This result is in accord with the finding that the regression model combining the ULCR and APRI score (ULCR/APRI combination score) shows the highest performance (AUROC) (Table 3). We speculate that combination of direct (ULCR) and indirect (APRI) markers may improve the prediction of significant hepatic fibrosis in CHC. However, the performance of ULCR/APRI combination was not significantly better than ULCR or APRI alone, although it is significantly superior to hyaluronic acid. The AURUC of ULCR is also higher than that of APRI or HA, but again the differences were not significant. As this study has enrolled limited number of patients, further studies with larger sample size may be able to elucidate whether ULCR, either alone or in combination with APRI, is superior to conventional serum markers in predicting significant hepatic fibrosis in CHC.

There are caveats in the interpretation of our results. Elevated LCN2 is not liver disease-specific: LCN2 is elevated in various clinical conditions such as bacterial infection and other co-morbidities that can elicit a systemic inflammatory response (Xu and Venge 2000). Moreover, LCN2 is elevated in several cancers including hepatocellular carcinoma (Bratt 2000; Kim et al. 2009) and acute or chronic kidney injury (Mishra et al. 2004; Mishra et al. 2006; Bolignano et al. 2009). Although we excluded these possibilities in our subjects, these are limitations for the use of LCN2 as a potential marker for hepatic fibrosis. In addition, there is a possibility that elevated urine LCN2 may be due to elevated renal tubular excretion which represents subclinical renal injury in cirrhotic patients. The significance of urinary LCN2 with respect to renal dysfunction has not yet been studied in liver cirrhosis. Although renal function is believed to be relatively well preserved in compensated cirrhosis (Wong et al. 1993; Woitas et al. 1997), further studies are warranted to elucidate the possible correlation between urine LCN2 and the glomerular filtration rate in patients with HCV-associated liver cirrhosis.

In conclusion, elevated urine LCN2 levels reflect increased urine MMP-9 activity, which is associated with significant hepatic fibrosis in CHC patients.

Acknowledgments

We thank So Hyun Shin, Seung Hee Shim, Yu Rim Kim, and Ji Hye Lee for technical assistance.

This work was supported in part by a grant from the Seoul National University Bundang Hospital (No 02-2007-034) to J-W Kim. The authors declare that we have no competing interests.

References

Bedossa, P., Dargere, D. & Paradis, V. (2003) Sampling variability of liver fibrosis in chronic hepatitis C. *Hepatology,* **38**, 1449-1457.

Bedossa, P. & Poynard, T. (1996) An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology,* **24**, 289-293.

Benyon, R.C. & Arthur, M.J. (2001) Extracellular matrix degradation and the role of hepatic stellate cells. *Semin. Liver Dis.,* **21**, 373-384.

Birkedal-Hansen, H., Yamada, S., Windsor, J., Pollard, A.H., Lyons, G., Stetler-Stevenson, W. & Birkedal-Hansen, B. (2008) Matrix Metalloproteinases. *Curr. Protoc. Cell Biol.,* **40**, 10.8.1-10.8.23.

Boeker, K.H., Haberkorn, C.I., Michels, D., Flemming, P., Manns, M.P. & Lichtinghagen, R. (2002) Diagnostic potential of circulating TIMP-1 and MMP-2 as markers of liver fibrosis in patients with chronic hepatitis C. *Clin. Chim. Acta,* **316**, 71-81.

Bolignano, D., Lacquaniti, A., Coppolino, G., Donato, V., Campo, S., Fazio, M.R., Nicocia, G. & Buemi, M. (2009) Neutrophil gelatinase-associated lipocalin (NGAL) and progression of chronic kidney disease. *Clin. J. Am. Soc. Nephrol.,* **4**, 337-344.

Bratt, T. (2000) Lipocalins and cancer. *Biochim Biophys Acta,* **1482**, 318-326.

Bravo, A.A., Sheth, S.G. & Chopra, S. (2001) Liver biopsy. *N. Engl. J. Med.,* **344**, 495-500.

DeLong, E.R., DeLong, D.M. & Clarke-Pearson, D.L. (1988) Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics,* **44**, 837-845.

Ebata, M., Fukuda, Y., Nakano, I., Katano, Y., Fujimoto, N. & Hayakawa, T. (1997) Serum levels of tissue inhibitor of metalloproteinases-2 and of precursor form of matrix metalloproteinase-2 in patients with liver disease. *Liver,* **17**, 293-299.

Fernandez, C.A., Yan, L., Louis, G., Yang, J., Kutok, J.L. & Moses, M.A. (2005) The matrix metalloproteinase-9/neutrophil gelatinase-associated lipocalin complex plays a role in breast tumor growth and is present in the urine of breast cancer patients. *Clin. Cancer Res.,* **11**, 5390-5395.

Friedman, S.L. (2008) Mechanisms of hepatic fibrogenesis. *Gastroenterology,* **134**, 1655-1669.

Gerlach, R.F., Demacq, C., Jung, K. & Tanus-Santos, J.E. (2007) Rapid separation of serum does not avoid artificially higher matrix metalloproteinase (MMP)-9 levels in serum versus plasma. *Clin. Biochem.,* **40**, 119-123.

Ghany, M.G., Strader, D.B., Thomas, D.L. & Seeff, L.B. (2009) Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology,* **49**, 1335-1374.

Gressner, A.M., Gao, C.F. & Gressner, O.A. (2009) Non-invasive biomarkers for monitoring the fibrogenic process in liver: a short survey. *World J. Gastroenterol.,* **15**, 2433-2440.

Han, Y.P. (2006) Matrix metalloproteinases, the pros and cons, in liver fibrosis. *J. Gastroenterol. Hepatol.,* **21 Suppl 3**, S88-91.

Han, Y.P., Yan, C., Zhou, L., Qin, L. & Tsukamoto, H. (2007) A matrix metalloproteinase-9 activation cascade by hepatic stellate cells in trans-differentiation in the three-dimensional extracellular matrix. *J. Biol. Chem.,* **282**, 12928-12939.

Han, Y.P., Zhou, L., Wang, J., Xiong, S., Garner, W.L., French, S.W. & Tsukamoto, H. (2004) Essential role of matrix metalloproteinases in interleukin-1-induced myofibroblastic activation of hepatic stellate cell in collagen. *J. Biol. Chem.,* **279**, 4820-4828.

Hayasaka, A., Suzuki, N., Fujimoto, N., Iwama, S., Fukuyama, E., Kanda, Y. & Saisho, H. (1996) Elevated plasma levels of matrix metalloproteinase-9 (92-kd type IV collagenase/gelati-

nase B) in hepatocellular carcinoma. *Hepatology,* **24**, 1058-1062.

Imbert-Bismut, F., Ratziu, V., Pieroni, L., Charlotte, F., Benhamou, Y. & Poynard, T. (2001) Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. *Lancet,* **357**, 1069-1075.

Iredale, J.P. (2007) Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *J. Clin. Invest.,* **117**, 539-548.

Jung, K., Lein, M., Laube, C. & Lichtinghagen, R. (2001) Blood specimen collection methods influence the concentration and the diagnostic validity of matrix metalloproteinase 9 in blood. *Clin. Chim. Acta,* **314**, 241-244.

Jung, K., Lein, M., Roemer, A. & Lichtinghagen, R. (2002) Circulating gelatinase B (MMP-9)—the impact of the preanalytical step of blood collection. *Matrix Biol.,* **21**, 381-382.

Kim, J.W., Lee, S.H., Park, Y.S., Jeong, S.H., Kim, N., Lee, D.H. & Lee, H.S. (2009) Transcriptome analysis of hepatitis B virus-associated small hepatocellular carcinoma by serial analysis of gene expression. *Int. J. Oncol.,* **35**, 129-137.

Kjeldsen, L., Johnsen, A.H., Sengelov, H. & Borregaard, N. (1993) Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. *J. Biol. Chem.,* **268**, 10425-10432.

Kossakowska, A.E., Edwards, D.R., Lee, S.S., Urbanski, L.S., Stabbler, A.L., Zhang, C.L., Phillips, B.W., Zhang, Y. & Urbanski, S.J. (1998) Altered balance between matrix metalloproteinases and their inhibitors in experimental biliary fibrosis. *Am. J. Pathol.,* **153**, 1895-1902.

Kuo, W.H., Chou, F.P., Lu, S.C., Chu, S.C. & Hsieh, Y.S. (2000) Significant differences in serum activities of matrix metalloproteinase-2 and -9 between HCV- and HBV-infected patients and carriers. *Clin. Chim. Acta,* **294**, 157-168.

Leber, T.M. & Balkwill, F.R. (1997) Zymography: a single-step staining method for quantitation of proteolytic activity on substrate gels. *Anal. Biochem.,* **249**, 24-28.

Leroy, V., Monier, F., Bottari, S., Trocme, C., Sturm, N., Hilleret, M.N., Morel, F. & Zarski, J.P. (2004) Circulating matrix metalloproteinases 1, 2, 9 and their inhibitors TIMP-1 and TIMP-2 as serum markers of liver fibrosis in patients with chronic hepatitis C: comparison with PIIINP and hyaluronic acid. *Am. J. Gastroenterol.,* **99**, 271-279.

Lichtinghagen, R., Bahr, M.J., Wehmeier, M., Michels, D., Haberkorn, C.I., Arndt, B., Flemming, P., Manns, M.P. & Boeker, K.H. (2003) Expression and coordinated regulation of matrix metalloproteinases in chronic hepatitis C and hepatitis C virus-induced liver cirrhosis. *Clin. Sci. (Lond),* **105**, 373-382.

Lichtinghagen, R., Huegel, O., Seifert, T., Haberkorn, C.I., Michels, D., Flemming, P., Bahr, M. & Boeker, K.H. (2000) Expression of matrix metalloproteinase-2 and -9 and their inhibitors in peripheral blood cells of patients with chronic hepatitis C. *Clin. Chem.,* **46**, 183-192.

Lichtinghagen, R., Michels, D., Haberkorn, C.I., Arndt, B., Bahr, M., Flemming, P., Manns, M.P. & Boeker, K.H. (2001) Matrix metalloproteinase (MMP)-2, MMP-7, and tissue inhibitor of metalloproteinase-1 are closely related to the fibroproliferative process in the liver during chronic hepatitis C. *J. Hepatol.,* **34**, 239-247.

Manning, D.S. & Afdhal, N.H. (2008) Diagnosis and quantitation of fibrosis. *Gastroenterology*, **134**, 1670-1681.

McHutchison, J.G., Blatt, L.M., de Medina, M., Craig, J.R., Conrad, A., Schiff, E.R. & Tong, M.J. (2000) Measurement of serum hyaluronic acid in patients with chronic hepatitis C and its relationship to liver histology. Consensus Interferon Study Group. *J. Gastroenterol. Hepatol.,* **15**, 945-951.

Mehta, P., Ploutz-Snyder, R., Nandi, J., Rawlins, S.R., Sanderson, S.O. & Levine, R.A. (2008) Diagnostic accuracy of serum hyaluronic acid, FIBROSpect II, and YKL-40 for discriminating fibrosis stages in chronic hepatitis C. *Am. J. Gastroenterol.,* **103**, 928-936.

Milani, S., Herbst, H., Schuppan, D., Grappone, C., Pellegrini, G., Pinzani, M., Casini, A., Calabro, A., Ciancio, G., Stefanini, F., Ciancio, A.K. & Surrenti, C. (1994) Differential expression of matrix-metalloproteinase-1 and -2 genes in normal and fibrotic human liver. *Am. J. Pathol.,* **144**, 528-537.

Mishra, J., Ma, Q., Kelly, C., Mitsnefes, M., Mori, K., Barasch, J. & Devarajan, P. (2006) Kidney NGAL is a novel early marker of acute injury following transplantation. *Pediatr. Nephrol.,* **21**, 856-863.

Mishra, J., Mori, K., Ma, Q., Kelly, C., Yang, J., Mitsnefes, M., Barasch, J. & Devarajan, P. (2004) Amelioration of ischemic acute renal injury by neutrophil gelatinase-associated lipocalin. *J. Am. Soc. Nephrol.,* **15**, 3073-3082.

Moses, M.A., Wiederschain, D., Loughlin, K.R., Zurakowski, D., Lamb, C.C. & Freeman, M.R. (1998) Increased incidence of matrix metalloproteinases in urine of cancer patients. *Cancer Res.,* **58**, 1395-1399.

Murawaki, Y., Koda, M., Okamoto, K., Mimura, K. & Kawasaki, H. (2001) Diagnostic value of serum type IV collagen test in comparison with platelet count for predicting the fibrotic stage in patients with chronic hepatitis C. *J. Gastroenterol. Hepatol.,* **16**, 777-781.

Nunez, O., Fernandez-Martinez, A., Majano, P.L., Apolinario, A., Gomez-Gonzalo, M., Benedicto, I., Lopez-Cabrera, M., Bosca, L., Clemente, G., Garcia-Monzon, C. & Martin-Sanz, P. (2004) Increased intrahepatic cyclooxygenase 2, matrix metalloproteinase 2, and matrix metalloproteinase 9 expression is associated with progressive liver disease in chronic hepatitis C virus infection: role of viral core and NS5A proteins. *Gut*, **53**, 1665-1672.

Reif, S., Aeed, H., Shilo, Y., Reich, R., Kloog, Y., Kweon, Y.O. & Bruck, R. (2004) Treatment of thioacetamide-induced liver cirrhosis by the Ras antagonist, farnesylthiosalicylic acid. *J. Hepatol.,* **41**, 235-241.

Reif, S., Somech, R., Brazovski, E., Reich, R., Belson, A., Konikoff, F.M. & Kessler, A. (2005) Matrix metalloproteinases 2 and 9 are markers of inflammation but not of the degree of fibrosis in chronic hepatitis C. *Digestion,* **71**, 124-130.

Roy, R., Louis, G., Loughlin, K.R., Wiederschain, D., Kilroy, S.M., Lamb, C.C., Zurakowski, D. & Moses, M.A. (2008) Tumor-specific urinary matrix metalloproteinase fingerprinting: identification of high molecular weight urinary matrix metalloproteinase species. *Clin. Cancer Res.,* **14**, 6610-6617.

Smyth, R., Lane, C.S., Ashiq, R., Turton, J.A., Clarke, C.J., Dare, T.O., York, M.J., Griffiths, W. & Munday, M.R. (2009) Proteomic investigation of urinary markers of carbon-tetrachloride-induced hepatic fibrosis in the Hanover Wistar rat. *Cell Biol. Toxicol.,* **25**, 499-512.

Takahara, T., Zhang, L.P., Yata, Y., Xue, F., Minemura, M., Sato, H. & Watanabe, A. (2003) Modulation of matrix metalloproteinase-9 in hepatic stellate cells by three-dimensional type I collagen: its activation and signaling pathway. *Hepatol. Res.,* **26**, 318-326.

Takahara, Y., Takahashi, M., Wagatsuma, H., Yokoya, F., Zhang, Q.W., Yamaguchi, M., Aburatani, H. & Kawada, N. (2006) Gene expression profiles of hepatic cell-type specific marker genes in progression of liver fibrosis. *World J. Gastroenterol.,* **12**, 6473-6499.

Trevisan, A. (1990) Concentration adjustment of spot samples in analysis of urinary xenobiotic metabolites. *Am. J. Ind. Med.,* **17**, 637-642.

Trocme, C., Leroy, V., Sturm, N., Hilleret, M.N., Bottari, S., Morel, F. & Zarski, J.P. (2006) Longitudinal evaluation of a fibrosis index combining MMP-1 and PIIINP compared with MMP-9, TIMP-1 and hyaluronic acid in patients with chronic hepatitis C treated by interferon-alpha and ribavirin. *J. Viral Hepat.,* **13**, 643-651.

Wai, C.T., Greenson, J.K., Fontana, R.J., Kalbfleisch, J.D., Marrero, J.A., Conjeevaram, H.S. & Lok, A.S. (2003) A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology,* **38**, 518-526.

Walsh, K.M., Fletcher, A., MacSween, R.N. & Morris, A.J. (2000) Basement membrane peptides as markers of liver disease in chronic hepatitis C. *J. Hepatol.,* **32**, 325-330.

Walsh, K.M., Timms, P., Campbell, S., MacSween, R.N. & Morris, A.J. (1999) Plasma levels of matrix metalloproteinase-2 (MMP-2) and tissue inhibitors of metalloproteinases -1 and -2 (TIMP-1 and TIMP-2) as noninvasive markers of liver disease in chronic hepatitis C: comparison using ROC analysis. *Dig. Dis. Sci.,* **44**, 624-630.

Woitas, R.P., Heller, J., Stoffel-Wagner, B., Spengler, U. & Sauerbruch, T. (1997) Renal functional reserve and nitric oxide in patients with compensated liver cirrhosis. *Hepatology,* **26**, 858-864.

Wong, F., Massie, D., Colman, J. & Dudley, F. (1993) Glomerular hyperfiltration in patients with well-compensated alcoholic cirrhosis. *Gastroenterology,* **104**, 884-889.

Xu, S. & Venge, P. (2000) Lipocalins as biochemical markers of disease. *Biochim. Biophys. Acta,* **1482**, 298-307.

Yan, L., Borregaard, N., Kjeldsen, L. & Moses, M.A. (2001) The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL). Modulation of MMP-9 activity by NGAL. *J. Biol. Chem.,* **276**, 37258-37265.