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CLINICAL RESEARCH

A specific gene-expression signature quantifies the degree of hepatic fibrosis in patients with chronic liver disease

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Abstract

AIM: To study a more accurate quantification of hepatic fibrosis which would provide clinically useful information for monitoring the progression of chronic liver disease.

METHODS: Using a cDNA microarray containing over 22000 clones, we analyzed the gene-expression profiles of non-cancerous liver in 74 patients who underwent hepatic resection. We calculated the ratio of azan-stained: total area, and determined the morphologic fibrosis index (MFI), as a mean of 9 section-images. We used the MFI as a reference standard to evaluate our method for assessing liver fibrosis.

RESULTS: We identified 39 genes that collectively showed a good correlation (r > 0.50) between gene-expression and the severity of liver fibrosis. Many of the identified genes were involved in immune responses and cell signaling. To quantify the extent of liver fibrosis, we developed a new genetic fibrosis index (GFI) based on gene-expression profiling of 4 clones using a linear support vector regression analysis. This technique, based on a supervised learning analysis, correctly quantified the various degrees of fibrosis in both 74 training samples (r = 0.76, 2.2% *vs* 2.8%, P < 0.0001) and 12 independent additional test samples (r = 0.75, 9.8% *vs* 8.6%, P < 0.005). It was far better in assessing liver fibrosis than blood markers such as prothrombin time (r = -0.53), type Ⅳ collagen 7s (r = 0.48), hyaluronic acid (r = 0.41), and aspartate aminotransferase to platelets ratio index (APRI) (r = 0.38).

CONCLUSION: Our cDNA microarray-based strategy may help clinicians to precisely and objectively monitor the severity of liver fibrosis.

Key words: Liver fibrosis; Hepatitis virus; DNA microarray; Supervised learning analysis; Scoring system

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INTRODUCTION

The prognosis and management of chronic liver diseases often depends heavily on the degree of liver fibrosis. This is particularly true of viral hepatitis, where chronic hepatitis B and C infections affect as many as 370 and 130 million persons respectively worldwide[1,2]. Liver histology has been the gold standard for monitoring hepatic fibrosis. In response to a need for critical evaluation of fibrosis in controlled therapeutic trials, a variety of staging systems have been developed[3,4]. However, intra- and inter-observer variability has resulted in discrepant staging, even in studies involving expert hepatopathologists[5]. Alternatively, several serum markers, such as alpha2 macroglobulin

and hyaluronic acid, have substantial predictive value for the diagnosis of cirrhosis, but not the important earlier stages of liver fibrosis[6]. Therefore development of a new comprehensive and objective evaluation system to quantify the extent of liver fibrosis is important. More recently, the value of noninvasive assessments of liver fibrosis, such as liver stiffness (Fibroscan) and METAVIR fibrosis score have been studied[7-10]. However, no standard diagnostic modality is currently established for assessing liver fibrosis.

To date, there are a few DNA microarray studies investigating gene-expression patterns of non-cancerous liver from patients with chronic liver disease[11-15]. These studies successfully identified differentially regulated genes in liver fibrosis, but none investigated any correlation between gene-expression profiles and the extent of liver fibrosis. In our recent report[16], we demonstrated that a specific gene-expression signature can objectively and accurately quantify the variable degrees of hepatic fibrosis in a rat model. Here, we focused on identifying whether our molecular based strategy precisely quantifies the severities of liver fibrosis in humans as well.

MATERIALS AND METHODS

Liver samples

Seventy-four patients, including 67 with hepatocellular carcinoma (HCC) and 7 with colorectal liver metastasis who had undergone an initial hepatectomy at our institute and affiliated hospitals were entered in this study. The 67 included 11 positive for hepatitis B surface antigen (HBsAg), 47 positive for hepatitis C virus (HCV) antibody, and 9 negative for either HBsAg or HCV antibody. The 7 metastatic patients were seronegative for either HBsAg or HCV antibody, and they all had liver function values within the normal limits and histologically normal liver was analyzed. The resected non-cancerous liver specimens were meticulously taken as far from the tumor as possible, and were immediately frozen in liquid nitrogen and kept at -80℃ until subsequent RNA preparation. Remaining liver was soaked in 10% formaldehyde then embedded in paraffin for azan staining. Written informed consent was obtained from all patients according to the guidelines approved by the Institutional Research Board at each hospital.

Preparation of sections and measurement of the area of liver fibrosis

Five thick fixed sections were stained with azan for collagen fibers as described previously[16]. The section images (× 40) were randomly selected and captured using the Sony DXC S500/OL digital camera. These captured images were entered into a personal computer as PICT files (Adobe Photoshop version 7.0). To determine the morphologic fibrosis index (MFI), the ratio of azan-stained:total area was calculated as described[16]. We examined how many or few section-images would be sufficient to determine the MFI as a reference for the degree of liver fibrosis (Figure 1). The fluctuation rate of the MFI in each liver specimen was calculated as follows:

Fluctuation rate (%) = $(n - n\text{-}1)/n\text{-}1 \times 100$

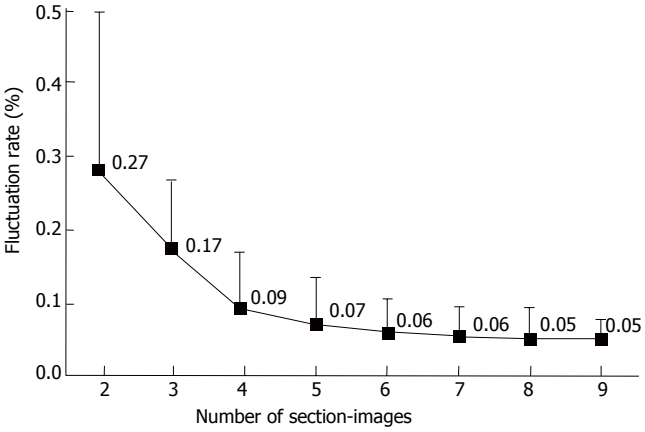


Figure 1 The number of section-images used to calculate the morphological fibrosis index (MFI) and the fluctuation rates of the MFI in the 12 randomly selected liver samples (mean ± SD).

where, n represents the mean MFI value for n section-images. We calculated the fluctuation rates in 12 randomly selected liver tissue specimens and plotted the mean of these 12 samples according to the number of section-images. As expected, when the number of images was small, the mean MFI value fluctuated markedly. As the number of section-images increased, the fluctuation attenuated and a plateau was subsequently reached at 9 images. In this study, we thus determined the MFI value as a mean of 9 section-images. The mean MFI value (1.13%) of the 7 patients with colorectal liver metastasis but normal liver histology was considered as background and subtracted to determine the MFI values for the 67 patients with HCC.

All non-cancerous liver specimens were independently examined by 2 experienced hepatopathologists (A.S. and Y.Y.) blinded to the results of the DNA microarray analysis, MFI values, and clinical data. The severity of liver fibrosis (0-4) was evaluated semi-quantitatively according to the METAVIR scoring system[5]: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis. Quadratic weighted kappa was calculated to estimate the reproducibility of the staging between the 2 pathologists.

Liver function tests

We calculated the Pearson correlation coefficient between MFI values and 10 preoperative clinical parameters that have been associated with liver function or disease: indocyanine green dye retention at 15 min, prothrombin time (as a% of normal time), hepaplastin, platelet count, albumin, total bilirubin, aspartate aminotransferase, alanine aminotransferase, type Ⅳ collagen 7 s and hyaluronic acid. The correlation coefficient between the MFI and aspartate aminotransferase to platelets ratio index (APRI)[17] was also calculated.

RNA preparation

Total liver RNA was isolated from frozen tissue by a guanidium/cesium trifluoroacetate extraction method

using a Quick Prep total RNA extraction kit (Amersham Pharmacia Biotech, Little Chalfont, England)[18]. To ensure the use of only high quality RNA, the concentration and purity were determined by an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA)[19].

Oligonucleotide microarray

We used the commercially available Human 1A Oligo Microarray (Agilent) containing over 22 000 unique 60-mer oligonucleotides representing over 17 000 unique human genes, listed http://www.chem.agilent.com/scripts/generic.asp?lpage=5175&indcol=Y&prodcol = Y. Cyanine-labeled cRNA was prepared using a T7 linear amplification as described in the Agilent Low RNA Input Fluorescent Linear Amplification Kit Manual (Agilent). Briefly, 50 ng of purified total RNA was reverse transcribed to generate double-stranded cDNA using an oligo dT T7 promoter primer and MMLV reverse transcriptase. Next, cRNA was synthesized using T7 RNA polymerase, which simultaneously incorporated Cy3 or Cy5 labeled CTP. During this process, experimental samples from potentially fibrotic liver specimens were labeled with Cy5 whereas a control sample from a mixture of the 7 normal liver specimens was labeled with Cy3. The quality of the cRNA was again checked using the Agilent 2100 Bioanalyzer. One microgram aliquots each of Cy3 and Cy5 labeled cRNA were combined, and fragmented in a hybridization cocktail (Agilent). The labeled cRNAs were then hybridized to 60-mer probe oligonucleotide microarrays and incubated for 17 h at 60℃. The fluorescent intensities were determined by an Agilent DNA Microarray Scanner and were analyzed using G2567AA Feature Extraction Software Version A.7.5.1 (Agilent), which used the LOWESS (locally weighted linear regression curve fit) normalization method. The gene-expression values were calculated as the log ratio of the Cy5 to Cy3 channel signals. All calculated data were imported into the Rosetta Luminator system v2.0. (Rosetta Biosoftware, Kirkland, WA) and all intensity data were plotted as log ratios. The original data will be available at URL supplemental website at http://www.mib-beppu.kyushu-u.ac.jp/MIB_res/clin_surg/MA/MA_data.html. This microarray study followed MIAME guidelines issued by the Microarray Gene Expression Data group[20].

Identification of genes associated with liver fibrosis

We first excluded genes which had missing values in > 20% of samples. We thus analyzed 16 990 genes, missing values in each sample were estimated using the 10-nearest neighbors' method based on the Euclid distance[21]. The 67 samples whose MFI values were measured, and the 7 normal liver samples (MFI = 0%), were used for this analysis. The correlation coefficient between MFI values and the expression of each gene in the 74 samples was calculated.

Development of quantification scores for liver fibrosis: Genetic fibrosis index

Next, we determined the parameters in the estimation equation using a linear support vector regression (L-SVR) analysis[22]. The estimation equation based on the L-SVR was expressed in linear equation form, namely "the estimated MFI value = parameter 1 × the expression value of gene a1 + parameter 2 × the expression value of gene a2 + … + constant b". These parameters and the constant were determined according to Cherkassky's method[23]. Here, we define the estimated MFI value based on the gene-expression values as the genetic fibrosis index (GFI)[16]. To select the optimal set of marker genes that best correlate GFI to MFI, we used a forward-backward stepwise selection method. In this method, the correlation coefficient between the expression value of each gene listed in Table 1 and the MFI value for the 74 samples was calculated and genes with the highest correlation coefficients were selected. Using these selected genes, the estimation equation parameters and constant were determined, and the correlation coefficient between the GFI and MFI values was calculated one at a time by the L-SVR based system. We repeated these steps with the addition or elimination of genes to improve the correlation coefficient, and we subsequently determined the 4 optimized marker clones. For independent validation, the GFI of 12 additional test samples (one normal and 11 chronically damaged livers) was calculated using these 4 marker clones.

RESULTS

MFI and the histological stage of liver fibrosis

The MFI of the 67 patients with HCC ranged from 0.2% to 18.8% (6.8% ± 3.5%). To understand the distribution of MFI values in a clinical setting, we first examined the correlation between the MFI and the stage of liver fibrosis in the METAVIR scoring system (Figure 2). The correlation coefficients between the MFI and the stages of liver fibrosis by Pathologists A and B were $r = 0.62$ and $r = 0.61$, respectively. The reproducibility of the METAVIR scoring between the 2 pathologists was good (quadratic weighted kappa = 0.86). The mean MFI value for liver cirrhosis, stage F4, was approximately 10%.

MFI and liver function tests

We next determined the correlation between the MFI and the results of so-called liver function tests in the 74 samples. The best correlation coefficient ($r = -0.53$) was observed between the MFI and the prothrombin time. The platelet count ($r = -0.52$), indocyanine green dye retention test at 15 min ($r = 0.49$), and type IV collagen 7s ($r = 0.48$) also had good correlation coefficients (Figure 3). Conversely, APRI did not correlate well with the MFI ($r = 0.38$).

Identification of differentially regulated genes during liver fibrogenesis

We identified differentially regulated genes associated with the degree of liver fibrosis in the 74 samples. Thirty-nine genes showed a good correlation ($r > 0.50$) between gene-expression and MFI (Table 1). There were no genes with a correlation coefficient more negative than -0.5. The 39 genes included 24 named genes 18 of which (75%) were associated with immune functions. Specifically, 9 genes

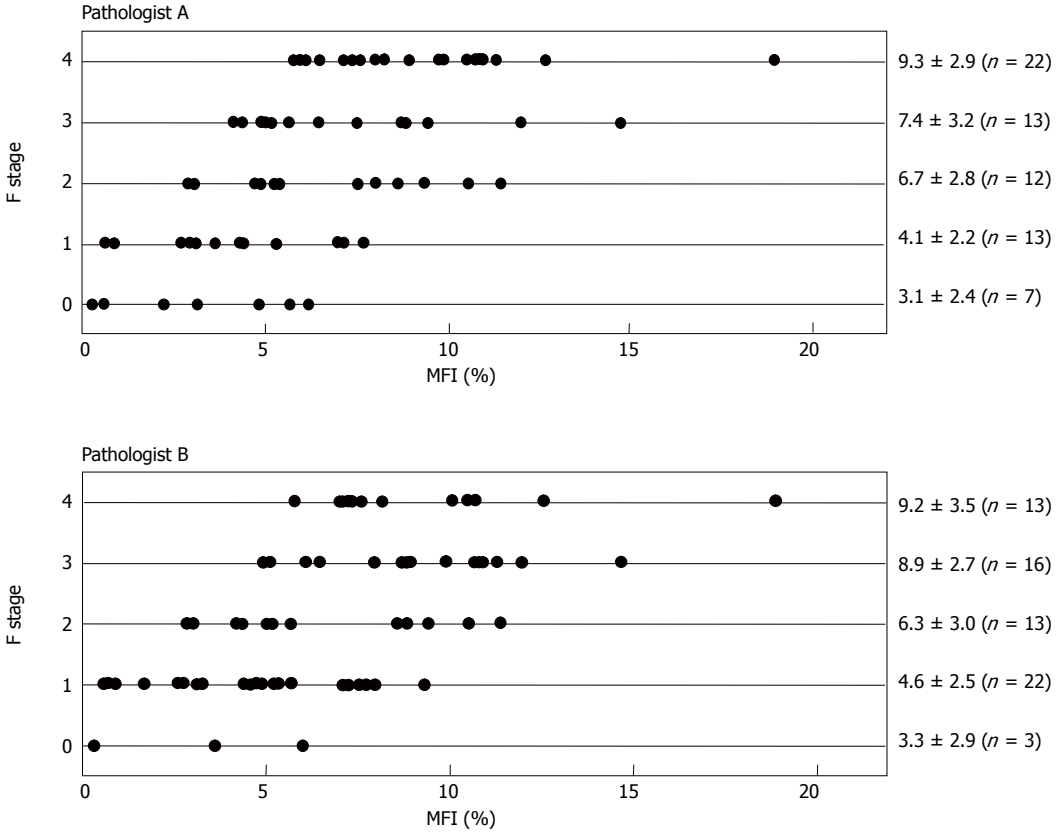
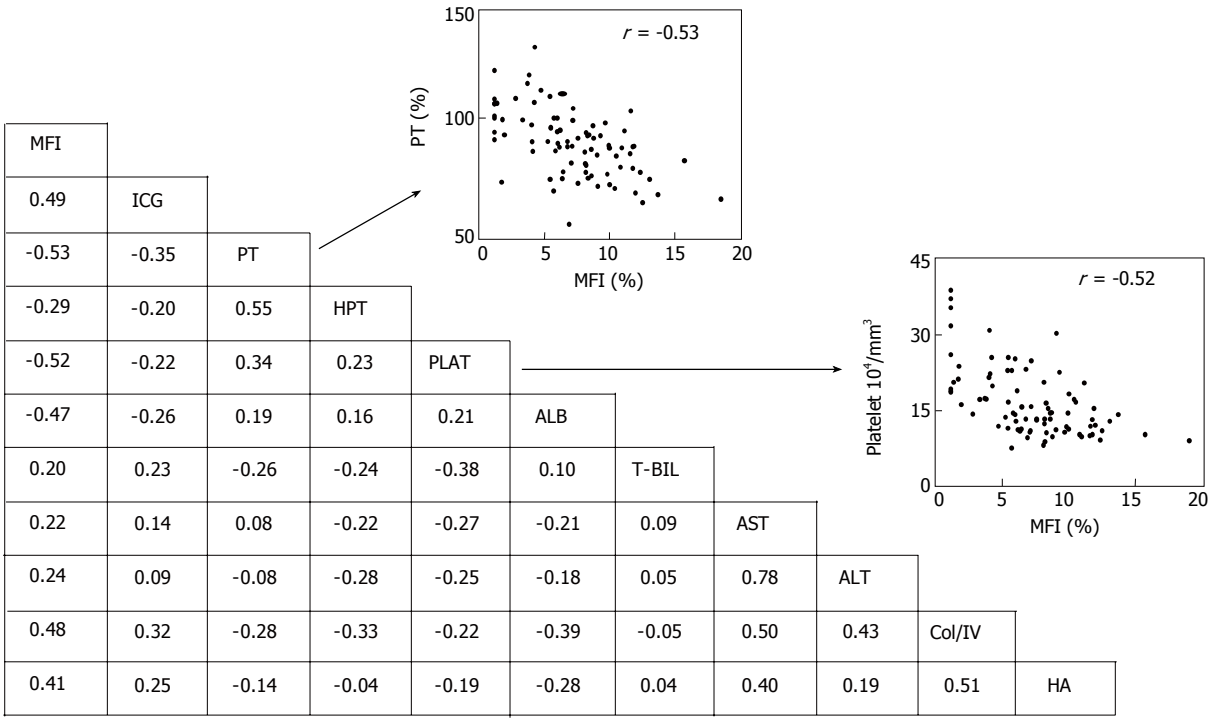


Figure 2 Relationship between the morphological fibrosis index (MFI) and the histological stage of liver fibrosis. METAVIR scores were independently determined by two experienced pathologists.



MFI										
0.49	ICG									
-0.53	-0.35	PT								
-0.29	-0.20	0.55	HPT							
-0.52	-0.22	0.34	0.23	PLAT						
-0.47	-0.26	0.19	0.16	0.21	ALB					
0.20	0.23	-0.26	-0.24	-0.38	0.10	T-BIL				
0.22	0.14	0.08	-0.22	-0.27	-0.21	0.09	AST			
0.24	0.09	-0.08	-0.28	-0.25	-0.18	0.05	0.78	ALT		
0.48	0.32	-0.28	-0.33	-0.22	-0.39	-0.05	0.50	0.43	Col/IV	
0.41	0.25	-0.14	-0.04	-0.19	-0.28	0.04	0.40	0.19	0.51	HA

Figure 3 Correlation coefficient among parameters for the assessment of liver damage. The left column represents the correlation coefficient between the morphological fibrosis index (MFI) values and clinical parameters of liver function tests. ICG: Indocyanine green dye retention test at 15 min; PT: Prothrombin time (as a % of normal); HPT: Hepaplastin test; PLAT: Platelet count; ALB: Albumin; T-Bil: Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; Col/IV: Type Ⅳ collagen 7s; HA: Hyaluronic acid.

encoded major histocompatibility complex (MHC) class Ⅱ molecules, such as *HLA-DR4* and *HLA-DRB5*. The most highly correlated gene was *PIK3C2B*, involved in intracellular signaling pathways. *PRKCB1*, *PTPRC*, *UNC5B*, and *ARHGDIB* are also associated with intracellular signaling pathways.

Table 1 Thirty-nine genes associated ($r > 0.50$) with the degree of liver fibrosis (MFI value)

Accession No.	Gene Name	Description	CC[1]
NM_002646	PIK3C2B[2]	Phosphoinositide-3-kinase, class 2, beta polypeptide	0.60
NM_152270	NM_152270[2]	Hypothetical protein FLJ34922 (FLJ34922)	0.59
NP113470	NP113470	Unknown	0.58
AK024488	AK024488	FLJ00087 protein	0.57
AB064167	AB064167	IGL mRNA for immunoglobulin lambda light chain VLJ region	0.57
BC015833	BC015833	cDNA clone MGC:27152 IMAGE:4691630	0.57
A_23_P32661	A_23_P32661	Unknown	0.57
M35730	M35730	MHC class Ⅱ DQ3.1ER (DR4)	0.56
NP077661	NP077661	Unknown	0.56
D29642	D29642	KIAA0053	0.56
NM_001778	CD48	CD48 antigen (B-cell membrane protein)	0.56
NM_006144	GZMA	Granzyme A (granzyme 1, cytotoxic) T-lymphocyte-associated serine esterase 3)	0.56
NM_002125	HLA-DRB5	MHC class Ⅱ, DR beta 5	0.55
NM_001803	CDW52	CDW52 antigen (CAMPATH-1 antigen)	0.55
NP649772	NP649772	Unknown	0.54
THC1889877	THC1889877	Unknown	0.54
AX721203	AX721203	Sequence 163 from Patent WO0220754	0.54
BC036926	BC0369262	cDNA clone MGC:46491 IMAGE:5225843	0.54
M13975	M13975	Protein kinase C beta-Ⅱ type (PRKCB1)	0.53
AF035024	AF035024	MCE11H myosin-reactive immunoglobulin heavy chain variable region	0.53
NM_033503	BMF	Bcl2 modifying factor (BMF), mRNA	0.53
A_23_P124264	A_23_P124264	Unknown	0.53
U96396	U96396	Anti-streptococcal/anti-myosin immunoglobulin kappa light chain variable region	0.53
A_23_P435390	A_23_P435390	Unknown	0.53
A_23_P9854	A_23_P9854	Unknown	0.52
AF490771	AF490771	MHC class Ⅱ antigen (HLA-DRB1) mRNA, HLA-DRB1*1401 allele	0.52
NM_002121	HLA-DPB1	MHC class Ⅱ, DP beta 1	0.52
L03178	L03178	Cell-type T-cell immunoglobulin gamma chain, V region (IGHV@)	0.52
NM_170744	UNC5B	Unc-5 homolog B (C. elegans)	0.51
S65186	S65186[2]	EMT=T-cell-specific tyrosine kinase	0.51
NM_022555	HLA-DRB3	MHC class Ⅱ, DR beta 3	0.51
NM_001175	ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	0.51
NM_002118	HLA-DMB	MHC class Ⅱ, DM beta	0.51
NM_002838	PTPRC	Protein tyrosine phosphatase, receptor type, C	0.51
L34102	L34102	MHC class Ⅱ HLA-DQB1*0502	0.51
AB020689	AB020689	KIAA0882 protein	0.51
NM_002341	LTB	Lymphotoxin beta (TNF superfamily, member 3)	0.50
AJ297586	AJ297586	MHC class Ⅱ antigen (HLA-DRB1 gene), DRB1*0402 allele	0.50
NM_033554	HLA-DPA1	MHC class Ⅱ, DP alpha 1	0.50

[1]For each clone, the correlation coefficient (CC) between the gene expression level and the morphological fibrosis index (MFI) was determined; [2]These 4 clones were used to calculate the genetic fibrosis index (GFI).

Development of a genetic fibrotic index to quantify the degree of liver fibrosis

We selected 4 clones, namely *PIK3C2B*, *NM_152270*, *BC036926*, and *S65186*, as the optimal marker set using the supervised learning method, L-SVR analysis. Based on the expression profiles of only these 4 clones, the GFI of each sample was then calculated, and the correlation between the GFI and the MFI was determined (Figure 4A). Our method correctly quantified the degree of liver fibrosis in the 74 training samples (Figure 4A, $r = 0.76$, $P < 0.0001$). Furthermore, an almost identical quantification was successfully achieved in the 12 additional independent test samples (one normal and 11 chronically damaged livers) (Figure 4B, $r = 0.75$, $P < 0.005$), supporting the usefulness of our quantifying system for liver fibrosis. Two representative azan-stained images of test samples with a good correlation between GFI and MFI are shown in Figure 4C. In addition, we showed that good correlations were obtained both in livers positive for HBsAg and those positive for HCV antibody (Figure 5).

DISCUSSION

To assess the severity of hepatic fibrosis using newer methods, such as Fibroscan and Fibrotest[7-10], most recent studies use the liver biopsy as a reference standard. However, studies using the Fibrotest have suggested that most errors are due to the histological staging itself[24], particularly, the difficulty in distinguishing F2 from F1 or F3 seems to be the main cause of misclassification. Histological scoring system, such as the METAVIR fibrosis score, is a categorical assessment and not a numerical

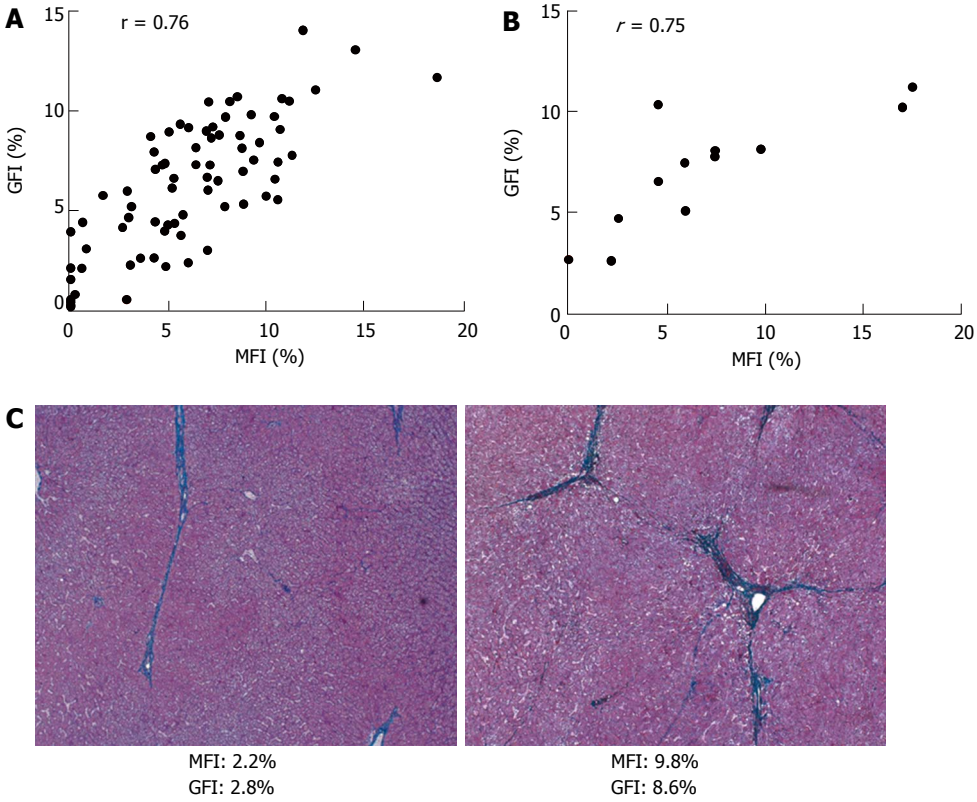


Figure 4 Correlation between the morphologic MFI values and the genetic GFI values in the 74 training samples (**A**) and the 12 test samples (**B**). (**C**) Two representative sections after azan staining (× 40) and their corresponding MFI and the GFI values.

measurement along a continuum in a mathematical sense[25]. In the current study, we therefore used as our reference the MFI, which is a mean of 9 surgical section-images, and subsequently investigated the value of our cDNA microarray-based strategy for the assessment of liver fibrosis. Although the MFI correlates well with the METAVIR scoring system (Figure 2), there was overlap among the different stages, indicating a limitation of such a histological staging system. The MFI can represent a linear score from mild fibrosis to overt cirrhotic livers. It has been reported that the area of fibrosis determined by image analysis is a reliable morphological method and was superior to histological staging, even if only small pieces of liver biopsy specimens were examined[26,27]. To the best of our knowledge, this is the first study to use the area of fibrosis (MFI) of human surgical specimens, which provide relatively large amounts of liver tissue, as a reference standard to evaluate new strategies for assessing hepatic fibrosis.

Using a cDNA microarray containing over 22000 clones, we analyzed the gene-expression profiles of liver tissue specimens and correlated them to the MFI values. We identified 39 differentially regulated genes, including 24 named genes, associated with the degree of liver fibrosis in 74 liver samples. Both the number of genes and the number of patients examined in this study were larger than those of previous reports investigating gene expression profiles of human livers with fibrosis[11-15]. Many of the genes we identified are involved in immune responses and cell signaling, which is consistent with a previous report investigating HCV-infected livers[14,15]. Among these, genes encoding MHC class II molecules were correlated most predominantly to the severity of liver fibrosis. Because MHC gene products are critical in regulating antiviral

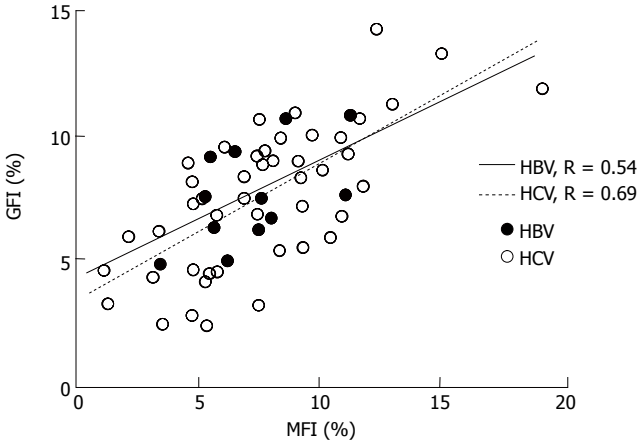


Figure 5 Correlation between the morphologic MFI values and the genetic GFI values in livers infected with hepatitis B virus (HBV) and hepatitis C virus (HCV).

immune reactions against both HBV and HCV, genetic factors controlling the host's immune response might also play an important role in determining the disease severity in patients with viral hepatitis[28-30]. In addition, we identified several genes, such as *PIK3C2B* and *ARHGDIB*, which regulate the activation of hepatic stellate cells[31,32], which might contribute to the molecular pathogenesis of liver fibrosis.

To examine possible advantages of our method over the use of blood markers of liver damage, we calculated the correlation coefficient between the MFI and 10 clinical parameters of so-called "liver function tests" (Figure 3) to include some of the large number of promising serum markers of hepatic fibrosis, e.g. collagen IV and hyaluronic acid. Prothrombin time correlated best with the MFI (r = 0.53). The correlation

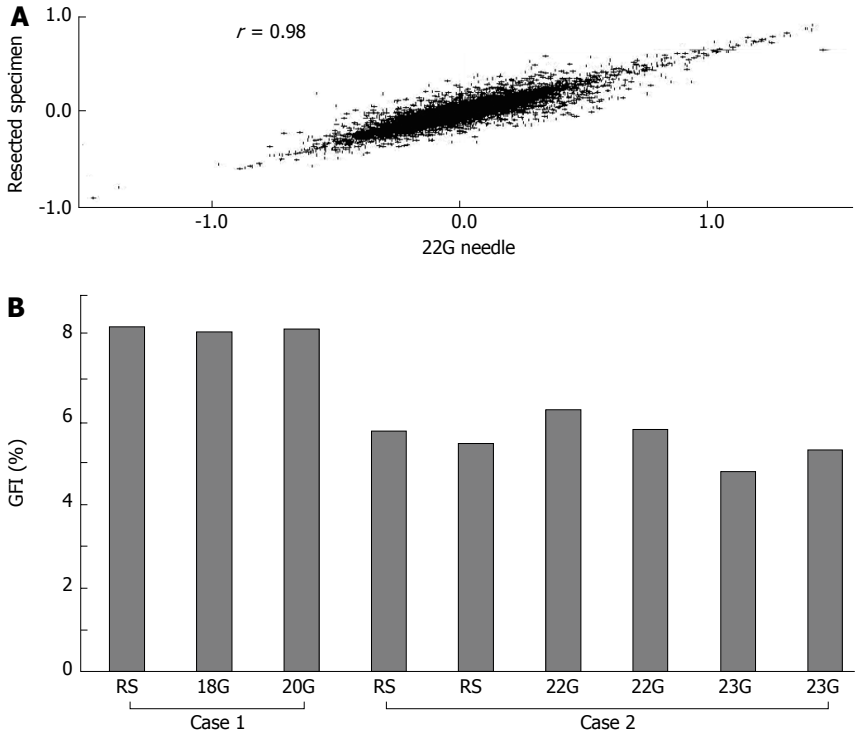


Figure 6 Gene-expression correlation between surgical resection and 22-guage needle liver biopsy specimens from the same patient. Intensity data were plotted and the correlation coefficient calculated by the Rosetta Luminator system v2.0 (**A**). The GFI values of resected and biopsy specimens were determined using the same formula based on the 4 marker clones (**B**). RS: Resected specimen.

coefficient (r = 0.38) between the MFI and the APRI[17] was inferior to that of the prothrombin time alone. It may well be that a panel of serum markers may prove more accurate than any individual marker. We next developed quantified scores for human liver fibrosis based on a specific gene-expression signature or index (GFI), which was validated by a supervised learning analysis. Our data showed that the GFI in the 74 training samples correlated with the MFI markedly better (r = 0.76) than any biochemical or hematological markers examined in this study. Furthermore, a similar correlation (r = 0.75) was confirmed in the 12 test samples as well. Therefore, our current study demonstrates for the first time that a single test based on gene-expression profiling accurately quantifies the variable extent of liver fibrosis in a clinical setting.

Marker genes involved in the pathogenesis of liver fibrosis might differ depending upon the etiology of the chronic liver damage. We therefore examined whether a different correlation with the MFI was observed between different etiologies (Figure 5). The correlation between MFI and GFI did not apparently differ markedly between HBV and HCV infected livers. This is reasonable because we selected our marker genes that were correlated to the degree of liver fibrosis regardless of etiology. One might expect a better performance if we analyzed samples with a single cause of liver fibrosis. However, we did not obtain better results even when we examined only livers infected with HCV (n = 54) using the same statistical method (data not shown).

Although the MFI may reliably determine the extent of fibrosis, a distinct advantage of the GFI is the potential for measuring a fibrosis index using very small liver biopsies. For example, in preliminary studies there was a very high correlation (r = 0.98, P < 0.01) between the levels of gene expression comparing genetic profiles of resected liver tissue and very small 22-guage liver biopsy specimens from the same patient (Figure 6A). Regardless of the sample size, the GFI values were quite reproducible from the same patient (Figure 6B). We extracted on average 3.4 ± 0.5 g of high quality total RNA using the 23-guage Surecut needle (TSK Laboratories), while only 0.2 g total RNA is sufficient for microarray analysis in our system. This will need to be validated with a larger number of patients, and might even include sampling more than two liver areas to reduce sampling error with minimal risk using the 23-guage needle.

Finally, we successfully created a scoring system to accurately and objectively quantify the degree of liver fibrosis in humans, based on the gene-expression signatures. This genome-wide information contributes to an improved understanding of molecular alterations during the development of liver cirrhosis, and could potentially become a powerful tool for monitoring the stage of liver fibrosis before and after treatment, such as with interferon/ribavirin therapy. This may be particularly important given recent exciting evidence on the potential reversibility of hepatic fibrosis in some patients[33]. Although our method still relies on invasively obtained liver tissue, however small, our data does draw attention to potentially helpful novel diagnostic and therapeutic targets, some of which may even have serum-measurable correlates based on future research.

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