Progression of Fibrosis in Hepatitis C With and Without Schistosomiasis: Correlation with Serum Markers of Fibrosis

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Serial liver biopsies are the gold standard by which the progression of fibrosis is evaluated. This longitudinal cohort study assessed the different rates in the progression of fibrosis using serial liver biopsies and serum fibrosis markers YKL-40 and PIIINP and the cytokines, transforming growth factor beta (TGF-β) and tumor necrosis factor alpha (TNF$-\alpha$). A 10-year cohort study was performed in patients with hepatitis C virus (HCV) alone or HCV and schistosomiasis. Patients were enrolled at the time of acute HCV infection and prospectively evaluated with two liver biopsies (at entry and end of follow-up), and true rates in the progression of fibrosis were calculated per year. Serum YKL-40, N-terminal propeptide of collagen III (PIIINP), TGF-β, and TNF-α were measured, as well as the expression of TGF-β, TNF-α, and YKL-40 mRNA in liver tissue. A significant increase in the progression rates of fibrosis occurred in the coinfected group (0.61 ± 0.13) compared with the HCV monoinfection group (0.1 ± 0.06; $P < .001$)). The progression of fibrosis rate/year had a direct linear correlation for YKL-40 ($r = 0.892$, $P < .001$) and for PIIINP ($r = 0.577$, $P < .01$). YKL-40 showed a linear correlation with TGF-β ($r = 0.897$, $P < .001$). Hepatic mRNA levels of YKL-40 and TGF-β correlated with the serum levels, confirming a hepatic source for the elevated serum levels. In conclusion, serial cytokine and fibrosis markers can accurately determine the rate at which fibrosis is progressing, identifying both those with rapid fibrosis and those with stable disease. *Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).* (HEPATOLOGY 2006;43:771-779.)

Hepatitis C virus (HCV) infection is characterized by silent onset in most infected individuals, a high rate of viral persistence, and the potential for development of chronic liver disease, ranging from chronic hepatitis to cirrhosis and hepatocellular carcinoma.[1,2] However, the progression of fibrosis in chronic hepatitis C is highly variable, and the natural history of the disease usually extends over several decades.[3,4] In epidemiological studies of chronic HCV infection, age, duration of infection, alcohol consumption, male sex, and coinfection with HIV, hepatitis B virus, or schistosomiasis have been related to histological severity.[5-10] Key cytokines secreted in response to cell injury such as tumor necrosis factor-alpha (TNF-α) and transforming growth factor beta-1 (TGF-β1) have been implicated in the development of liver inflammation and fibrosis.[11-13] TNF-α has been shown to modulate hepatic stellate cell activation as well as synthesis of some extracellular matrix proteins and proteins involved in matrix degradation.[14]

Serial liver biopsies are the current gold standard to evaluate the progression of fibrosis.[15] A number of serological and urinary compounds such as procollagens, tissue inhibitors of metalloproteinases (TIMP), type IV S collagen, hyaluronic acid, and laminin and mediators of extracellular matrix production such as TGF-β have been

Abbreviations: HCV, hepatitis C virus; TNF-α, tumor necrosis factor alpha; TGF-β, transforming growth factor beta; TIMP, tissue inhibitors of metalloproteinases; ALT, alanine aminotransferase; PCR, polymerase chain reaction; PIIINP, aminoterminal propeptide of type III procollagen; AST, aspartate aminotransferase; ECM, extracellular matrix.

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Received April 22, 2005; accepted January 23, 2006.

Supported by Espinosa Fibrosis Fund, BIDMC to NHA and NIH grants NIAID R29A141563 and R21 A1054887 to MJK and SK respectively.

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.21117

Potential conflict of interest: Nothing to report.

evaluated as noninvasive markers of liver fibrosis.[16-26] Most of these studies have focused on using these markers in cross-sectional studies to diagnose the stage of liver fibrosis.

We recently proposed that YKL-40, also known as human cartilage glycoprotein 39 or CHONDREX is an excellent marker for staging fibrosis in the liver and differentiating cirrhosis from chronic hepatitis with stage 1 and 2 fibrosis in patients with HCV.[27-30]

Schistosomiasis is a chronic helminthic disease infecting more than 200 million people worldwide.[31] Concomitant schistosomiasis and HCV infection is common in many developing countries[32,33] and exhibits a unique clinical, virological, and histological pattern manifested by virus persistence with high HCV RNA titers, higher necroinflammatory and fibrosis scores in liver biopsies, and poor response to interferon therapy.[33-35] Patients with hepatitis C and *Schistosoma mansoni* coinfection show markedly accelerated hepatic fibrosis.[9,10]

Therefore, in this study, we used serum fibrosis markers and profibrogenic and pro-inflammatory cytokines to predict differences in the rate of progression of fibrosis in a rapidly progressive cohort versus a traditional HCV slowly progressive cohort. Our studies indicate that serum YKL-40 and TGF-β can accurately predict the progression of fibrosis over an 8- to 10-year period in patients with progressive HCV and Schistosomiasis coinfection and are also effective in identifying stable patients without progression of fibrosis.

Patients and Methods

Study Population. Patients were enrolled into this longitudinal cohort study from patients with acute HCV who failed to clear viremia within 6 months of initial infection. The diagnosis of acute HCV infection was based on the following criteria: elevated values of serum alanine aminotransferase (ALT) to more than 10 times above the upper limit of normal; seroconversion from negative to anti-HCV–positive antibody status assessed by second-generation enzyme-linked immunosorbent assay (Abbott Laboratories, Abbott Park; IL); positive polymerase chain reaction (PCR) for HCV RNA (Amplicor, Roche Diagnostics, Branchburg, NJ); with or without a history of sudden onset of malaise, jaundice, fever, and other symptoms related to liver disease in a previously healthy individual. Overall, 87 patients were enrolled and divided into two groups; HCV monoinfection (n = 39) and HCV coinfection with *Schistosoma mansoni* (n = 48). Shistosomiasis was diagnosed by history, detection of *S. mansoni* ova in stools (modified Kato test) or rectal biopsy; and seropositivity to schistosomal antibodies (indirect hemagglutination: Femouz laboratories, Cedex, France). No patient had clinically active schistosomiasis.). An initial experimental study cohort comprised 42 patients (M:F 26:16; mean age, 29.0 $\pm$ 8.3 years), and a second group of 45 patients were used as a validation cohort for the YKL-40 biomarker.

Patients were followed prospectively for 96 $\pm$ 4.6 months (range, 97-125 months). Patients were examined semi-annually until the end of study. All patients participating in the study presented oral and written informed consent. In the extremely rare case in which literacy was an issue, patients had the consent form read and carefully explained to them in the presence of a family member, both had to consent and the form was stamped, and both patient and family member made their mark. The study was approved by the Office for Human Protections Research Board of An Shams University (P-002104), and the protocol and all procedures of the study were conducted in conformity with the ethical guidelines of the Declaration of Helsinki and the human experimentation guidelines of the U.S. Department of Health and Human Services.

Laboratory Tests of Liver Disease and Virological Markers. Serum ALT, albumin and bilirubin concentrations, and prothrombin time were determined at entry and semi-annually until the end of follow-up. Serum HCV RNA was estimated by PCR, using a commercial kit (Amplicor HCV; Roche Diagnostics, Branchburg, NJ), and genotyping was performed using a second-generation reverse hybridization, line-probe assay (Inno-LiPA HCV II; Innogenetics, Zwijndrecht, Belgium). The entire cohort had ultrasonography and endoscopy, and the results are given for the end of the study procedures.

Histological Assessment. All patients were subjected to a baseline liver biopsy within 8 to 10 months after the onset of symptoms. Another liver biopsy was performed at the end of follow-up (mean of 96 $\pm$ 4.6 months after onset of symptoms). The study commenced in 1992, and interferon-based therapy became available in Egypt in 1999, but with limited access because of lack of national insurance and cost. The 2nd liver biopsy was performed in some patients before commencing interferon therapy and in the remainder to determine disease progression. A second biopsy after a minimum of 4 to 5 years is standard of care at many U.S. centers, including BIDMC, to evaluate disease progression and is clinically justified. Two passes were performed at each biopsy time point, one for histology and one for intrahepatic RNA studies. Liver biopsies were stained with hematoxylin-eosin and a connective tissue stain (chromotrope aniline blue). Liver biopsies were read by two pathologists in a blinded fashion, adopting the grading and scoring system proposed by

Ishak et al.[36] Moreover, biopsies were assessed for morphological features of schistosomiasis and graded as follows: 0: no evidence for schistosomiasis, 1: poor evidence, 2: suggestive of schistosomiasis, 3: strong evidence for schistosomiasis.

The progression rate of fibrosis per year was estimated as the difference between fibrosis scores of the baseline and follow-up biopsies divided by the interval between the two biopsies.

Serum TGF-β, TNF-α, YKL-40, Aminoterminal Propeptide of Type III Procollagen Measurement: Fasting serum TGF-β, TNF-α, YKL-40, and aminoterminal propeptide of type III procollagen (PIIINP) levels were quantitated at baseline and annually until the end of the study (96 $\pm$ 4.6 months) in the experimental study group. Serum TGF-β (BioSource -International Inc, Nivelles, Belgium), serum TNF-α (Boehringer Mannheim, Germany), and YKL-40 (Metra, Biosystems, Mountain View, CA) were measured by commercially available ELISA assay according to the manufacturer's instructions. PIIINP was measured by radioimmunoassay (Orion Diagnostica, Espoo, Finland) following the manufacturer's instructions.

RNA Studies. Intra-hepatic TGF-β and TNF-α transcript expression was assessed in baseline and follow-up biopsies using standard techniques. YKL-40 mRNA gene expression was measured using TaqMan quantitative PCR. (See supplemental data at the HEPATOLOGY website: http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).

YKL-40 Validation Cohort. The validation group consisted of 45 patients, 19 with HCV alone and 26 with HCV plus schistosomiasis coinfection. The validation cohort was used only to validate the YKL-40 serum marker. This cohort was derived from patients enrolled in a study of immune responses and progression of fibrosis in HCV and schistosomiasis and has been previously published.[10] The validation cohort again included patients with acute HCV who developed chronic hepatitis, and their clinical characteristics are given in Table 4. Patients from this cohort who had adequate serum stored for YKL analysis were included. Patients in this cohort were followed a mean of 114 $\pm$ 12 months, once again with a baseline liver biopsy 6 months after the onset of acute HCV and at the end of the follow-up. Serum tests for YKL-40 were performed on serum stored at the baseline biopsy, 5 years of follow-up, and at year 10, the end of the follow-up period when the second biopsy was performed. The validation cohort did not have any studies performed on liver tissue and was only used to confirm the serial changes in YKL-40 over time. No difference was found between the validation cohort and the initial experimental cohort with respect to clinical characteristics such as genotype (95% genotype 4), viral load, and ALT/aspartate aminotransferase (AST) at baseline. Analysis of fibrosis markers in this cohort was limited to only YKL-40 due to a limited supply of available serum.

Table 1. Demographic and Baseline Characteristics of Patients With Hepatitis C Virus (HCV) Monoinfection, and HCV/*S. mansoni* Coinfection in Experimental Group

Parameter	Group A HCV Monoinfection	Group B HCV & *S. mansoni* Co-infection
Number	20	22
M/F	12/8	13/9
Age(y):mean $\pm$ S.D	30.6 $\pm$ 5.1	29.2 $\pm$ 6.7
Risk factors		
i. Occupational exposure	10	15
ii. Blood transfusion	3	4
iii. Dental procedures	2	2
iv. Intravenous drug use	4	1
V. Surgery	1	0
Disease duration (mo)	7.4 $\pm$ 4.1	8.5 $\pm$ 3.9
ALT (U/mL) mean $\pm$ S.D	123.5 $\pm$ 31.1	108.2 $\pm$ 28.5
AST (U/mL) mean $\pm$ S.D	98.5 $\pm$ 27.3	113.5 $\pm$ 30.8
Albumin (g/dL) mean $\pm$ S.D	4.2 $\pm$ 0.3	4 $\pm$ 0.4
Platelets (per microliter) mean $\pm$ SD	198,000 $\pm$ 50,000	170,000 $\pm$ 38,000
RNA (cop $\times$105/mL) mean $\pm$ SD	16.5 $\pm$ 4.8*	38.8 $\pm$ 8.7*

NOTE. **Group A:** 20 patients with chronic hepatitis C, **Group B: 22** patients co-infected with HCV and *S. mansoni.*

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase.
*$P < .01$ between groups A and B.

Statistical Analysis. Results were expressed as mean $\pm$ SD and analyzed using paired and unpaired Student *t* test, chi squared, nonparametric Mann-Whitney *U* test, Wilcoxon rank sum test, or Fisher's exact test where appropriate. Correlation between different parameters was performed using Pearson or Spearman's rank test. *P* values of .05 or less were regarded as significant. All statistical procedures were performed using an SSPS for windows version 10 package (SPSS Inc., Chicago, IL).

Results

Baseline Clinical Characteristics of Patients. The clinical, virological, and histological profile of the experimental cohort patients is shown in Table 1. No statistically significant differences were found between the monoinfected and coinfected patients for age, sex, peak ALT at entry, or source of infection and HCV genotype (4a). HCV patients coinfected with *S. mansoni* had significantly higher HCV RNA titers ($P < .001$).

Histological Hepatic Inflammation. The clinical baseline biopsy features of the Schistosomiasis group are shown in Table 2. The total necroinflammatory scores

Table 2. Histological Evidence of Schistosomiasis at Baseline in Patients With HCV/*S. mansoni* Coinfection

Parameter	HCV & *S. mansoni* Co-infection (n = 22)
S. mansoni ova	17/22 (77.2%)
Eosinophils	16/22 (78%)
Granuloma	12/22 (54.4%)
Pigment	15/22 (68.2%)
Fibrosis of pipestem type	1/22 (4.5%)
Grading for schistosomiasis:	
• Grade 0	0
• Grade 1	0
• Grade 2	5 (22.7%)
• Grade 3	17 (77.2%)

Table 3. End of Follow-up Characteristics of Patients With Hepatitis C Virus (HCV) Monoinfection, and HCV/*S. mansoni* Coinfection in Experimental Group

Parameter	Group A HCV Monoinfection	Group B HCV & *S. mansoni* Co-infection
ALT (U/mL) mean ± SD	84.5 ± 24.5	93.1 ± 31.7
AST (U/mL) mean ± SD	77.9 ± 31.5	91.9 ± 40.2
Albumin (g/dL) mean ± SD	3.9 ± 0.7	2.8 ± 1.3*
Platelets (per microliter) mean ± SD	187,000 ± 54,000	121,000 ± 27,000
RNA (cop × 105/mL) mean ± SD	10.6 ± 2.3†	19.2 ± 2.1†
Splenomegaly: n (%)	1 (5)‡	20 (91)‡
Esophageal varices n (%)	1 (5)‡	21 (95)‡

*$P < .01$.
†$P < .05$.
‡$P < .001$.

were significantly higher at liver biopsy 1 (baseline biopsy) in coinfected patients ($P < .05$). Coinfected patients had significantly higher degrees of interface hepatitis (1.5 ± 0.7 vs. 0.6 ± 0.5; $P = .027$) and periportal necrosis (1.9 ± 0.9 vs. 1.1 ± 0.2; $P = .0016$). No significant difference was seen in necroinflammatory scores between monoinfected and coinfected patients in liver biopsy 2 (follow-up biopsy) (Fig. 1A).

In both monoinfected and coinfected patients, neither ALT levels nor viral load correlated with the necroinflammatory scores in baseline or follow-up biopsies (Wilcoxon's signed rank test $P = .5$, $P = .7$ respectively; data not shown).

Clinical Follow-up. The clinical and virological data of the experimental cohort patients is shown in Table 3. At baseline, only HCV RNA levels were significantly higher in coinfected patients. At the end of treatment, however, statistically significant differences were found between the mono-infected and coinfected patients for serum albumin levels, platelet counts, and HCV RNA titers. Unlike the biomarkers, reduction in platelets and albumin were only seen late in follow-up once cirrhosis had developed. There was no difference in ALT or AST at baseline, throughout the follow-up and at the end of the study. At the end of follow-up, almost all HCV patients coinfected with *S. mansoni* had splenomegaly and esophageal varices (see Table 3).

Histological Progression of Fibrosis. Initially at baseline biopsy, both monoinfected and coinfected patients had no fibrosis (stage: 0). Only one patient in the coinfected group had mild pipestem fibrosis. In the coinfected group, 2 of 22 (9.1%) progressed to stage 1 fibrosis, 2 of 22 (9.1%) progressed to stage 2 fibrosis, 4 of 22

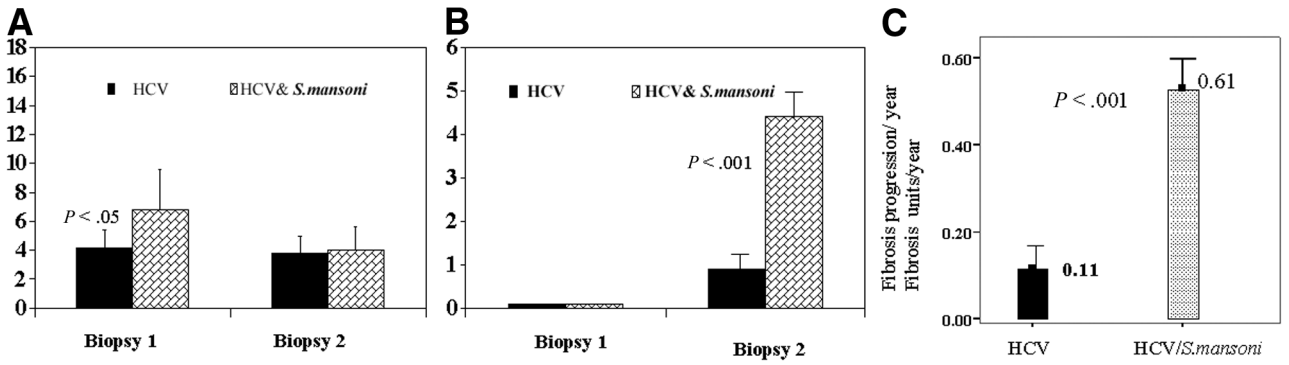

Fig. 1. (A) Comparison of the necroinflammatory scores at baseline biopsies (biopsy 1, performed 6-8 months after acute hepatitis) and follow-up biopsies (biopsy 2, performed at end of follow-up) in 20 monoinfected patients (black bars) and 22 coinfected patients (white bars). Bars represent means. There was significant difference in necroinflammatory scores between monoinfected and coinfected patients in baseline biopsies ($P < .05$) but not in follow-up biopsies. (B) Fibrosis scores at baseline biopsies and follow-up biopsies in 20 monoinfected patients (black bars) and 22 coinfected patients (white bars). Bars represent means. At liver biopsy 1, both monoinfected and coinfected patients had no fibrosis (stage: 0). Coinfected patients had significantly greater increase in fibrosis scores detected in biopsy 2 compared with monoinfected individuals (4.3 ± 0.9 vs. 0.8 ± 0.5, respectively; $P < .001$). (C) Fibrosis progression rates (fibrosis units per year) in monoinfected patients (black) versus coinfected patients (shaded).The rate of liver fibrosis progression was significantly higher in coinfected patients than in monoinfected patients (0.61 ± 0.13 in the coinfected group vs. 0.1 ± 0.06 in the monoinfected group; $P = .001$).

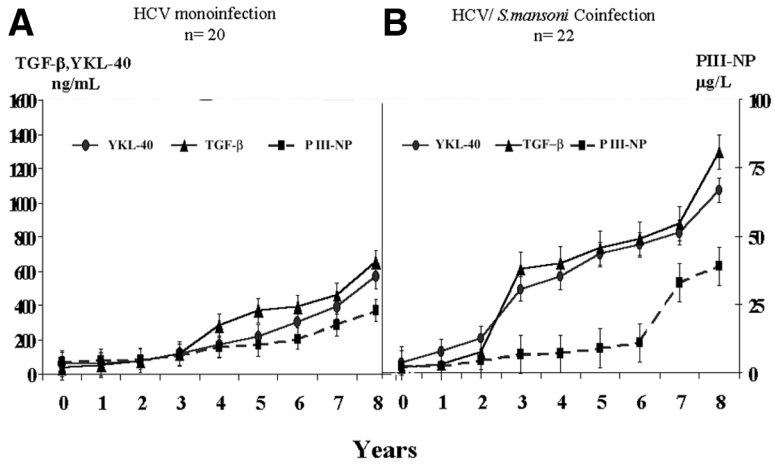

Fig. 2. Scattergrams showing the mean serum levels of each of the fibrosis markers (YKL-40, PIII-NP, and TGF-β) at different points in the monoinfected group and the coinfected group.

(18.2%) progressed to stage 3 fibrosis, 8 of 22 (36.4%) to stage 4, and 6 of 22 (27.3%) to stage 5 fibrosis.

In the HCV alone group, 2 of 20 (10%) progressed to stage 1 fibrosis, 1 of 20 (5%) progressed to stage 2 fibrosis, and 17 of 20 (85%) remained the same with stage 0 fibrosis. Overall, coinfected patients showed a striking increase in fibrosis scores detected in biopsy 2 compared with monoinfected individuals (4.4 ± 0.9 vs. 0.8 ± 0.5, respectively; $P < .001$) (Fig. 1B). The rate of progression of liver fibrosis (fibrosis units per year) was significantly accelerated in coinfected patients in comparison with monoinfected patients (0.61 ± 0.13 in the coinfected group versus 0.1 ± 0.06 in the monoinfected group; $P < .001$) (Fig. 1C). The increased fibrosis in the coinfected cohort was statistically significant by chi-square analysis ($P < .001$).

Fibrosis Markers. The mean baseline and follow-up values of YKL-40 and PIII-NP in both groups are shown in Fig. 2.

At baseline, no significant difference was seen in serum YKL-40, PIII-NP, and TGF-β in monoinfected and coinfected patients. The rate of increase during the first 2 years was comparable in the two groups. Coinfected patients showed a sharp increase in serum YKL-40 levels and serum TGF-β levels starting the 3rd to the 4th year of follow-up (Fig. 2B). The highest YKL-40 levels were de-

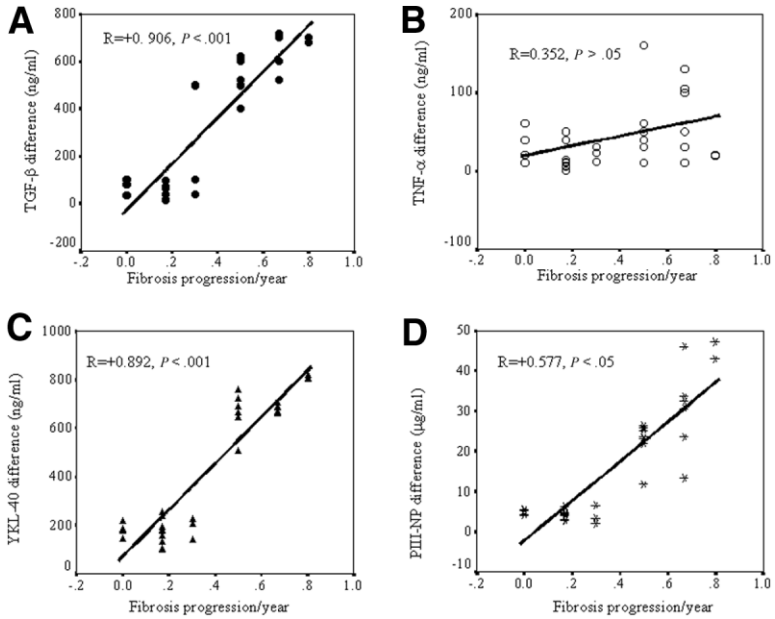

Fig. 3. Scattergrams showing the relationship between rate of fibrosis progression and TGF-β, TNF-α and the fibrosis markers (YKL-40, PIIINP). TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; PIIINP, aminoterminal propeptide of type III procollagen.

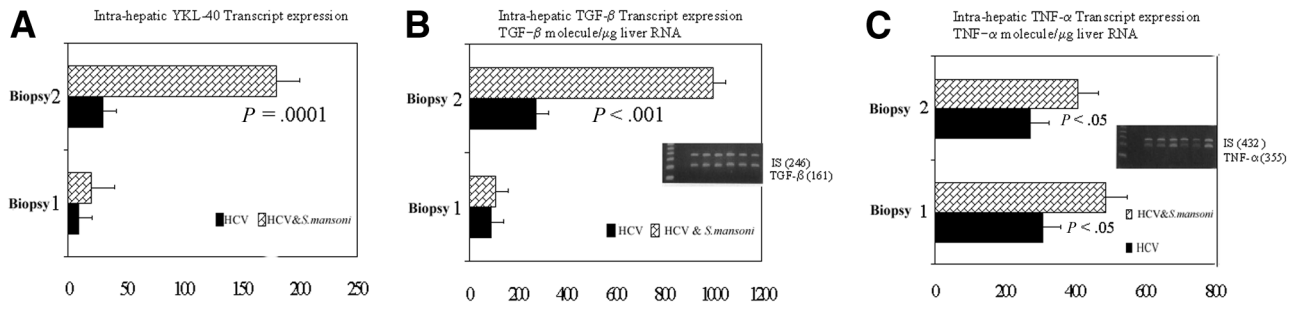

Fig. 4. Expression of transcripts specific for YKL-40 (A), TGF-β (B), and TNF-α (C) within the liver tissue from 20 HCV monoinfected patients and 22 HCV/*S. mansoni*–coinfected patients. RNA preparations from baseline liver biopsies and follow-up biopsies were analyzed for YKL-40 expression by TaqMan (TM) quantitative real-time RT-PCR. and for TGF-β and TNF-α by a competitive RT-PCR technique. TGF-β and TNF-α cDNA has been coamplified with an appropriate concentration of respective internal standard (SI). TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; RT-PCR, reverse transcription polymerase chain reaction.

tected in coinfected patients, who showed marked worsening of fibrosis (n = 17 with fibrosis progression rate > 0.3 fibrosis units/year) (data not shown). YKL-40 paralleled serum TGF-β levels at all times with a highly statistically significant relationship between YKL-40 and TGF-β ($r = +0.897$, $P < .001$).

The peak increase in PIII-NP levels in coinfected patients was detected at later times (years 7 and 8). A weaker correlation was detected between PIII-NP and TGF-β ($r = +0.403$, $P < .05$). Early on, serum TNF-α levels were higher in coinfected patients compared with levels in monoinfected patients; however, the levels were fluctuating over time and did not correlate with either YKL-40 or PIII-NP (data not shown).

Serum TGF-β levels increased in parallel with severity of liver damage and progression of fibrosis, which was markedly accelerated in coinfected patients. The association between serum TGF-β and rates of progression of fibrosis is shown in Fig. 3A. Patients who had fibrosis scores (>3) at the end of follow-up (17 coinfected patients) showed higher mean and median serum TGF-β levels starting year 3 ($R = +0.903$, $P < .001$).

We found no significant relationship between overall degree of fibrosis or progression rates of fibrosis and TNF-α (Fig. 3B). Serum TNF-α did, however, correlate at all points with the necroinflammatory score ($R = +0.4$, $P < .05$; data not shown).

To determine whether changes in serum fibrosis markers would parallel the changes in progression of fibrosis, we correlated serum YKL-40 and serum PIIINP change rate (difference between baseline and follow-up values) to the fibrosis progression rate (fibrosis unit/year) (Fig. 3C-D). A stronger direct linear correlation was observed between YKL-40 levels ($r = +0.892$, $P < .001$) and fibrosis progression rate when compared with PIIINP ($r = 0.577$, $P < .05$), suggesting that YKL-40 may be more efficient than PIII-NP in early detection of fibrosis and in monitoring progression of fibrosis.

Hepatic mRNA Expression. We then analyzed TGF-β TNF-α, and YKL-40 messenger RNA (mRNA) expression in liver tissue of baseline and follow-up biopsy specimen from the two groups of patients. Data have been normalized for β-actin transcript expression. The levels of both TGF-β and YKL-40 m RNA expression in the follow-up biopsies were 6-fold higher than the levels in baseline biopsies only for the coinfected patients (Fig. 4A and B) and were highest in those with the more advanced fibrosis stage. There was no significant correlation between mRNA levels of either YKL-40 or TGF-β and histological inflammatory index. These increases in hepatic message paralleled the changes seen in serum expression of both YKL-40 and TGF-β, confirming a hepatic source

Table 4. Clinical Characteristics, Fibrosis Progression and YKL-40 Levels in the Validation Cohort

Parameter	HCV Monoinfection	HCV & *S. mansoni* Co-infection
Number	19	26
M/F	11/8	17/9
Age (yrs):mean ± S.D	36.6 ± 8.1	34.2 ± 7.6
ALT/AST (U/mL)	74/88	68/75
Fibrosis score		
Baseline	0	0
Year 10	1.52 ± 1.3	5.0 ± 0.6
Fibrosis progression rate (U/yr)	0.16	0.56
YKL (ng/mL)		
Baseline	53 ± 35	80 ± 45
Year 5	110 ± 64	278 ± 92*
Year 10	172 ± 76	503 ± 106*
Change in YKL from baseline (ng/mL)		
Year 5	59 + 39	190 + 83*
Year 10	117 + 56	423 + 101*

*$P < .0001$ between groups, two-tailed t test.

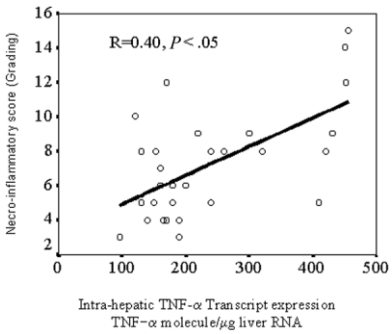

Fig. 5. Correlation between necroinflammatory score and intrahepatic TNF-α expression. The two parameters show a mildly significant positive correlation ($R = +0.40$, $P < .05$). TNF-α, tumor necrosis factor alpha.

of origin for these markers. In monoinfected patients, who did not show progression of fibrosis, there was no major change in hepatic expression of mRNA for either YKL-40 or TGF-β at the second biopsy (Fig. 4A-B).

TNF-α mRNA expression was not different between baseline and follow-up liver biopsy in either coinfected or HCV-alone patients. However, levels of TNF-α mRNA were significantly higher in coinfected patients than in HCV alone at both baseline and follow-up (Fig. 4C) and appeared to correlate best with degree of inflammation and necroinflammatory scores ($R = 0.40$, $P < .05$, Fig. 5) but not with fibrosis scores or the rate of progression of fibrosis.

Validation Cohort. Because YKL-40 is a relatively new marker for HCV-related fibrosis, we examined YKL-40 serum levels in a further cohort of 45 patients with matched liver biopsies. The baseline clinical and demographic data for the validation cohort is given in Table 4. The rate of disease progression in the validation cohort was identical to that seen in the experimental cohort. A very significant correlation with YKL levels and disease progression was seen in the HCV/Schistosomiasis coinfected group and with no disease progression in the mono-infected group (Table 4). All patients in the HCV and schistosomiasis group had significant increases in YKL compared with the HCV alone group, as shown in Fig. 6. Using an increase in YKL-40 of 100 ng/mL from baseline at year 5 and 200 ng/mL at year 10 to indicate disease progression was both highly specific and sensitive. In the entire combined cohort, only two patients with mild disease progression (<2 points increase over 10 years on Ishak) had increases in YKL-40 at years 5 or 10 as listed above (96% sensitivity). Similarly, only two patients with progressive disease failed to increase their YKL levels, giving a specificity of 96%.

Discussion

This unique cohort study clearly confirms previous reports showing the more rapid rate of progression of liver fibrosis in patients who have both schistosomiasis and HCV compared to HCV alone.[9,10,33] The rate of progression of fibrosis at 0.61 units per year has most coinfected patients developing cirrhosis within 10 years of exposure to HCV and was seen in both the experimental and validation cohorts. This rate of progression of fibrosis would be comparable to patients with HIV and HCV or HCV patients with significant alcohol consumption (>50 g/d). The fibrosis rate of 0.1 units per year seen in the HCV alone patient more closely resembles that proposed by Poynard et al.[37] for most studies of hepatic fibrosis in uncomplicated HCV, with cirrhosis occurring between 20 and 40 years. The study is clearly limited by the relatively small number of patients and the low rate of disease progression in the HCV monoinfection group. Combining both experimental and validation cohorts, the rate of progression in HCV monoinfected was only 0.15 U/year. This slow rate of progression can be best explained by the relatively few cofactors for disease progression, as patients had no alcohol consumption, no HIV or hepatitis B virus coinfection and are infected at a young age (mean age at infection, 30 years).

Examining the hepatic and serum levels of TGF-β and TNF-α gives us some insight into the potential mechanism for the more rapid fibrosis in patients with schistosomiasis. Initially, there is no difference in liver fibrosis; however, histological liver inflammation and TNF-α levels are higher in the coinfected group, suggesting these patients are primed by the schistosomal infection to a more aggressive level of inflammation. Within 2 years, we begin to see increases in serum pro-fibrogenic TGF-β levels and YKL-40 in the coinfected group, suggesting that the fibrotic process is progressing with changes in the extracellular matrix (ECM). These continue throughout the next 6 years of follow-up and, assuming some linearity to the progression of fibrosis, they strongly parallel the changes seen on the repeat liver biopsy. The serum levels

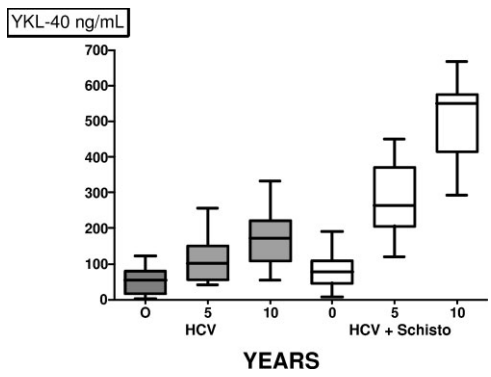

Fig. 6. Boxplot of YKL levels in both groups in the validation cohort at baseline, year 5 and year 10.

closely parallel the hepatic mRNA increases for both YKL-40 and TGF-β and suggest that the serum levels are a reflection of ECM modeling in the liver.

Perhaps the most important finding in this study is the very close clinical correlation of the panel of markers for fibrosis and cytokines with disease progression. Using YKL-40, PIINP, and TGF-β, we would have clearly been able to identify differences in progression rates of fibrosis between the two cohorts in the experimental study group. In the validation group, YKL-40, as a single serum marker for fibrosis, was able to differentiate progression rates between slow and rapid fibrosing patients. In our prior studies in a U.S. population, a YKL-40 level of >350ng/mL indicated stage 3 or above fibrosis,[27] and this was seen in all patients in the coinfected group with rapidly progressive disease to stage 4 or 5. The lack of a marker for a change of fibrosis in the HCV alone group also shows how useful serial markers can be to determine lack of disease progression. Interestingly, in patients with disease progression, changes also occur in standard clinical markers such as platelets and albumin but not in ALT or AST. However, because the study started approximately 12 years ago, we were unable to longitudinally evaluate other clinical markers such as platelet count or APRI, which may have performed equally well as the markers of fibrosis we measured.

In addition, patients with schistosomiasis developed evidence of portal hypertension with splenomegaly and esophageal varices. However, this was independent of liver fibrosis and reflects the underlying pre-hepatic portal hypertension associated with schistosomiasis. The associated hypersplenism is also a factor in the development of thrombocytopenia. However, these clinical changes tend to occur later in disease progression, whereas the markers of fibrosis start to rise when there is an estimated transition to Ishak stage 3 with bridging fibrosis.

Most studies of markers of fibrosis have been cross-sectional and focused on the ability of markers to diagnose cirrhosis. Some studies have also shown that successful therapy of HCV can be associated with a reduction in serum markers of fibrosis such as PIIINP,[38,39] but there are no long-term studies on the role of markers in predicting resolution or stabilization of fibrosis. This study represents a truly unique cohort of patients followed sequentially for almost 10 years and thus is an excellent model for fibrosis studies such as this one.

When examining liver ECM turnover, patients with more rapidly progressive diseases such as alcoholic hepatitis have the highest levels of ECM markers.[22,40] This has been shown for markers such as type IV collagen and hyaluronic acid, which correlate best with the degree of alcoholic hepatitis and perivenular fibrosis.[23,24] Rather than reflect the total collagen level, they accurately correlate with the degree of new collagen production and turnover and will also fall with abstinence from alcohol. In a similar fashion, the levels of YKL-40 were higher than that seen in some patients (>110 ng/mL) in the coinfected group, and these levels are reflecting the very active ECM with rapidly progressive liver diseases. In prior studies, YKL-40 has been shown to be an excellent marker in active alcoholic liver disease.[28,41] Because we are using the markers for monitoring disease, the absolute levels are not as important as the rate of increase over time, and certainly YKL-40 and TGF-β in individuals show excellent sensitivity to disease progression. In fact, in the validation cohort, the sensitivity and specificity of YKL-40 for predicting disease progression was greater than 95% and represents one of the first cohort studies to really use longitudinal markers of serum fibrosis.

This variation of markers of fibrosis in individuals with disease progression could have a strong potential clinical role in patients with HCV. Many HCV patients have slowly progressive disease and at initial diagnosis have only minor histological changes of fibrosis and inflammation and are not candidates or refuse interferon-based treatment. The standard of care has been to follow these patients and repeat liver biopsies in 4 to 5 years. However, as demonstrated by the HCV-alone group, who had a disease progression rate of 0.1 ± 0.06 fibrosis units per year, these markers of fibrosis can be used longitudinally to determine patients with slow rates of disease progression who do not need biopsy or therapy. Larger clinical cohorts need to verify these results in patients who are not treated for HCV but are followed clinically for disease progression before these biomarkers can be truly integrated into clinical practice.

Although this study demonstrates an important use for markers of fibrosis and their ability in serial analysis over time to predict progression of liver disease, an alternative important area for investigation is the role in predicting disease regression. Several ongoing large studies with both alpha and gamma interferons are looking at fibrosis as end points of therapy, serial measurements of markers of fibrosis can predict regression of fibrosis. These studies will potentially determine whether a clinically useful panel of markers could be used to replace or guide the use of liver biopsy.

In summary, this study shows the rapid rate of progression of fibrosis in patients with HCV and schistosomiasis compared to HCV alone. Progression of fibrosis may be mediated by an initially increased inflammatory response caused by elevated TNF-α and subsequent activation of hepatic TGF-β. The utilization of serum markers of fibrosis shows great potential in disease monitoring, and

larger studies will be required to confirm the findings of this initial cohort study.

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