Elevated interleukin-4 levels predicted advanced fibrosis in chronic hepatitis C

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Abstract

Background: Cytokine imbalance has been associated with chronic hepatitis C virus (HCV) infection. We hypothesized that cytokines have an important role in fibrosis development in HCV infection.

Methods: Data of 92 patients were analyzed retrospectively. Fluorescent Bead immunoassay was used to measure the following serum cytokine levels: Interferon γ, tumor necrosis factor α, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and IL-12. Various statistical analyses were used as appropriate.

Results: Of the 92 HCV-infected patients, 49 (53.3%) were male, 23 (25%) patients had advanced (fibrosis grades 3-4) fibrosis, and the mean age of the study population was 51.9 ± 9.4 years. Elevation of baseline IL-4 level (>490 pg/mL) was associated with liver fibrosis grade by χ^2 test (odds ratio [OR] = 2.99; 95%, CI = 1.02-8.78; p = 0.042) and multivariate logistic regression (OR = 4.26; 95% CI = 1.13-16.02; p = 0.032). Also, IL-4 had strong diagnostic value in advanced liver fibrosis by using area under receiver operating characteristics curve analysis. Assessment of fibrosis score was consequently developed from our findings and compared with other noninvasive serum markers to assess liver fibrosis.

Conclusion: This study provides evidence that increased IL-4 expression predicted advanced liver fibrosis in treatment of naive HCV-infected patients. The newly developed "FIL4" score had good predictive value for advanced fibrosis before treatment and this value was even strong in HCV-genotype 1b patients.

Keywords: Cytokines; HCV; Interleukin 4; Liver fibrosis; Liver fibrosis indexes

1. INTRODUCTION

More than 100 cytokines have been described as regulating the stability of human immunity. Cytokines are released by many different cells including hepatic epithelial cells, and play various important roles in host responses to virus infection, immunologic responses, cell inflammation, cancer, etc.[1] T-helper cell (Th) 1 cytokines (interferon gamma [IFN-γ], tumor necrosis factor alpha [TNF-α], and interleukin [IL]-2) are responsible for host immune responses; however, Th2 cytokines (IL-4, IL-5, IL-6, and IL-10) are responsible for inhibition of development of inflammation responses. Serum Th2 cytokines are significantly higher in chronic hepatitis C (CHC) patients compared with non-CHC patients and are decreased during interferon therapy.[2] Elevated levels of Th2 cytokines, especially IL-4, have been found in fibrotic skin tissues and IL-4 is crucial in the process of collagen production in human conjunctival fibroblasts. IL-4 triggers the activation of human stellate cells (HSCs) and increases the collagen production in HSCs by signal transduction and activation of the transcription 6-related pathway.[3] Also, IL-4 activates the synthesis of collagen by nonparenchymal liver cells.[4]

Globally, 2.5% of the population (more than 177.5 million people) are infected with Hepatitis C virus (HCV) and it is a serious burden of global health.[5] CHC is a major cause of liver damage and also the cause of long-term sequelae including liver fibrosis, liver cirrhosis, and primary hepatocellular carcinoma.

The definition of liver fibrosis is described as the gathering of extracellular matrix and its increased expression of proteins such as collagens, elastin, laminin, and fibronectin. Liver fibrosis is also considered as a wound healing process for continuing chronic liver injury.[6] Currently, a number of noninvasive methods such as serum-based markers or technique-based assessments are preferred rather than liver biopsy for diagnosis of liver fibrosis.[7] An appropriate combination of serum markers or combinations of commonly obtained laboratory tests are used for prediction of liver fibrosis in HCV-infected patients. HSC is the main collagen-producing cell and is crucial in fibrogenesis. The

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HCV core and also nonstructural proteins directly induce HSC activation and activate HSCs differentiating into myofibroblastic cells to develop extracellular matrix.[8] As fibrosis occurs, there is an accumulation of inflammatory cells that release cytokines; correspondingly, immune cells can activate the HSC and then influence the infiltration of lymphocytes. The activation and proliferation of HSC is triggered by the secretion of numerous proinflammatory cytokines.[9]

We hypothesized that the cytokines play an important role in fibrosis development in HCV infection. This is a novel study that analyzes cytokines in HCV infection, attempts to determine the potential mechanism of interactions between host and virus, and identify the predictors of liver fibrosis development in HCV infection. The aim of this study is to clarify the relationship between on-treatment inflammatory cytokine variations and the development of liver fibrosis in HCV-infected patients. The factors that could predict clinical outcomes and severity of liver fibrosis in HCV-infected patients were also identified.

2. METHODS

2.1. Subjects

This retrospective study was performed in Kaohsiung Medical University Hospital (Kaohsiung, Taiwan). Ninety-two HCV-infected patients were included in this study. The inclusion criteria in this study were as follows: (a) adult patients over 18 years of age and detectable serum HCV RNA for more than 6 months, and (b) liver biopsy-proven patients. The exclusion criteria were the following conditions: (1) patients coinfected with hepatitis B, hepatitis D, or human immunodeficiency virus; (2) decompensated liver cirrhosis; (3) overt hepatic failure; (4) renal function impairment (estimated glomerular filtration rate <50 mL/min); (5) primary biliary cirrhosis; (6) autoimmune hepatitis; (7) Wilson disease or hemochromatosis; (8) sclerosing cholangitis; (9) α_1-antitrypsin deficiency; (10) current or history of alcohol consumption (≥20 g daily); (11) liver transplantation; (12) presence of hepatocellular carcinoma and other malignancy; and (13) preexisting psychiatric disorder.

2.2. Treatment regimen

Either PegIFN α-2a (Pegasys, Hoffmann-La Roche, Basal, Switzerland) 180 μg/wk or Peg-IFN α-2b (PEG-Intron, Schering-Plough Inc., Kenilworth, NJ, USA) 1.5 μg/kg/wk plus weight-based RBV (1000 mg/d for <75 kg patients and 1200 mg/d for ≥75 kg, respectively) were used for antiviral treatment. All patients were treated for a duration of 24 to 48 weeks according to a response-guided therapy, based on the HCV genotype, viral loads, and viral response.

2.3. Liver histology

All patients received liver biopsies to validate the severity of chronic hepatitis when enrolled in this study. Liver histology was graded and staged on the basis of the scoring system proposed by Knodell and Scheuer.[10] A single pathologist was blinded to make the diagnosis for each sample. Written informed consent was obtained from each participant and the study design was in concordance with ethical guidelines as approved by the Ethics Committee of Kaohsiung Medical University Hospital (no. KMUHIRB-G(II)-20170020; August 29, 2017). All clinical investigations were conducted according to the principles laid down in the Declaration of Helsinki.

2.4. Laboratory tests

HCV antibodies were detected by using a third generation, commercialized enzyme-linked immunosorbent assay kit (Abbot Laboratories, Chicago, IL, USA). HCV RNA was quantified by a real-time polymerase chain reaction assay (detection limit: 50 IU/mL; Real time HCV; Abbot Molecular, Des Plaines IL, USA). HCV genotypes were classified by the method proposed by Okamoto et al.[11]

2.5. Cytokine assessment

We tested the serum of the patients with complete antiviral treatment. A Fluorescent Bead immunoassay (Bio-Rad Laboratories, Hercules, CA, USA) was used to measure the serum cytokine levels, according to the manufacturer's recommendations. Cytokine concentrations were calculated by using a reference standard curve made with various concentrations of the standards. Each sample was tested in triplicate and the average was calculated. Serum samples were collected from the participants at baseline, second, fourth, and 12th week of the treatment, end-of-treatment, and at 3-month follow-up. The following cytokines were analyzed: Th1-mediated cytokines (IL-2, TNF-α, and IFN-γ), Th2-mediated cytokines (IL-4, IL-5, IL-6, and IL-10), and immune-modulatory cytokines (IL-1β, IL-8, and IL-12).

2.6. Statistical analyses

The Student's *t* test was used for comparison analysis of continuous variables. The χ^2 test or Fisher's exact test was used to assess categorical variables. Multivariate logistic regression test was further performed to identify the independent factors predicting liver fibrosis. The area under curve (AUC) was calculated using receiver operating characteristics (ROC) analysis. The optimum cut-off value of IL-4 concentration to divide the risk strata was calculated by using the Youden index. ROC and R software (R 3.3.1; pROC package) were used for comparing the diagnostic tests for fibrosis. A two-tailed $p < 0.05$ was considered statistically significant. All statistical analyses were performed using the Statistic Packages for Social Sciences Program (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.).

3. RESULTS

The demographic characteristics and comparisons in mild or severe fibrosis are shown in Table 1. Out of the 92 HCV-infected patients, 23 (25%) patients had advanced (fibrosis grade 3-4) fibrosis. Age (mean ± SD of 56.3 ± 7.4 vs 50.4 ± 9.7 years, $p = 0.009$), GOT level (mean ± SD of 120.8 ± 63.2 vs 92.4 ± 55.5 U/L, $p = 0.048$), and ferritin level (mean ± SD of 641.0 ± 548.0 vs 384.1 ± 363.6 ng/mL, $p = 0.013$) were significantly higher in the advanced fibrosis group than in the mild fibrosis group, while platelet level (mean ± SD of 153.2 ± 48.5 vs 184.5 ± 61.6 × 10^9/L, $p = 0.030$) was lower in the advanced fibrosis group.

3.1. Assessment and association of cytokines with liver fibrosis

We examined cytokines that were Th1-mediated cytokines (IL-2, TNF-α, and IFN-γ), Th2-mediated cytokines (IL-4, IL-5, IL-6, and IL-10), and immune-modulatory cytokines (IL-1β, IL-8, and IL-12) in this study. We found that baseline IL-4 level was significantly elevated in the advanced fibrosis group compared with the mild fibrosis group (mean ± SE of 309.6 ± 41.5 vs 527.6 ± 99.7 pg/mL, $p = 0.019$). The rest of the baseline features were similar between the groups (Figure 1). Serial serum levels of the IL-4 cytokines during and after HCV treatment between groups were determined. The IL-4 level was higher in the advanced fibrosis group during therapy but only baseline level was significantly higher in the advanced fibrosis group compared with the mild fibrosis group. Thus, we chose baseline IL-4 level for further analysis. However, the dynamic change of serum IL-4 level during PegIFN/RBV therapy was not significantly associated with either liver fibrosis stage or viral response.

3.2. Predictive value of IL-4 in advanced liver fibrosis in HCV-infected patients

To evaluate the impact of IL-4 concentration on the fibrosis grade, we divided the subjects into high or low IL-4 groups. We setup the baseline IL-4 cut-off level as 490 pg/mL by using the area under the ROC curve and AUC was 0.659 (95% CI = 0.512-0.806, $p = 0.041$) (data not shown). For the same

Table 1

Demographic comparison in severe and mild fibrosis group

Characteristics	Total	Fibrosis 3-4	Fibrosis 0-2	*p*
N	92	23	67	
Age (mean ± SD)	51.9 ± 9.4	56.3 ± 7.4	50.4 ± 9.7	**0.009**
Sex (male), %	49(53.3%)	13(56.5%)	35(52.2%)	0.722
GOT, U/L, (mean ± SD)	99.0 ± 58.0	120.8 ± 63.2	92.4 ± 55.5	**0.048**
GPT, U/L (mean ± SD)	157.3 ± 102.0	178.5 ± 110.5	151.1 ± 100.5	0.282
WBC, 10^9/dL, (mean ± SD)	5677.0 ± 1700.3	5381.3 ± 1713.4	5797.7 ± 1712.7	0.317
Hemoglobin, g/dL, (mean ± SD)	14.1 ± 1.5	14.1 ± 1.1	14.1 ± 1.6	0.922
Platelet, 10^9/L, (mean ± SD)	175.7 ± 59.5	153.2 ± 48.5	184.5 ± 61.6	**0.030**
Ferritin, ng/mL, (mean ± SD)	450.2 ± 427.0	641.0 ± 548.0	384.1 ± 363.6	**0.013**
Log HCV RNA, IU, (mean ± SD)	5.2 ± 1.0	5.2 ± 1.0	5.2 ± 0.9	0.877
HCV genotype				0.117*
1b	44(47.8%)	8(34.8%)	36(53.7%)	
Non-1b	48(52.2%)	15(65.2%)	31(46.3%)	

HCV=hepatitis C virus; N=Number of patients; WBC=white blood cell.

*p value of χ^2 test.

analysis of AUC, we set the cut-off values for age (≥ or ≤45 years), GOT level (≥ or ≤80 U/L), ferritin level (≥ or ≤450 ng/mL), and platelet count (≥ or ≤ 100 × 10^9/L) as shown. Patients with higher IL-4 concentrations (≥490 pg/mL) were more likely to have advanced fibrosis (57.9% vs 31.5%, p = 0.042) than those with lower IL-4 concentrations. Multivariate analysis revealed that baseline IL-4 level was an independent factor for predicting advanced fibrosis (adjusted odds ratio [OR] = 4.34, 95% CI = 1.11-16.8, p = 0.034). However, we found significant differences of GOT in both mild and advanced fibrosis groups with significant association in χ^2 (OR = 3.83, 95% CI = 1.26-11.62; p = 0.015) and multivariate logistic regression analysis (adjusted OR = 6.39, 95% CI = 1.13-36.1; p = 0.036). There were no significant associations for age, platelet count level and ferritin level groups (Table 2).

We stratified IL-4 concentration in patients according to the presence of high viral load, high GOT, GPT, ferritin level, and HCV genotype 1b. Among the patients with a high viral load (≥4 × 10^5 IU/mL), the IL-4 level was higher in the advanced fibrosis group (626.5 ± 340.9 pg/mL) than in the mild fibrosis group (371.0 ± 329.8 pg/mL). Interestingly, IL-4 level was significantly different in advanced and mild fibrosis groups with HCV genotype 1b (706.8 ± 519.5 pg/mL vs 285.1 ± 303.3 pg/mL; p = 0.007). A similar result occurred in patients with high ferritin level (≥336 ng/mL) (Supplementary Figure 1). Patients with advanced fibrosis also had significantly higher IL-4 level in the high GOT (≥40 U/L) group (554.2 ± 431.4 pg/mL vs 298.2 ± 298.2 pg/mL; p = 0.009) compared to the mild fibrosis group (Data not shown).

3.3. Developing new assessment score to predict advanced fibrosis

On the basis of the high predictive results of the four variables, the following fibrosis and IL-4 score (FIL4) was derived: FIL4 = Age + GOT + ferritin + IL-4

The cut-off point:

1. Age 45 y/0
2. GOT 80 IU/L
3. Ferritin 450 ng/mL
4. IL-4 490 pg/mL

We simplified the scoring as 1 point for each elevated variable mentioned above. The comparison of our FIL4 with AST/ALT ratio, AST to platelet ratio index (APRI), and FIB4 index by AUC analysis are shown in Table 3 and Figure 2. The FIL4 gave slightly higher AUC (0.833) than the other indexes. We also used R software to compare FIL4 with the other indexes. There were statistically significant differences in FIL4 compared to APRI (p = 0.005), FIB4 (p = 0.03), AST/ALT ratio (p = 0.002), respectively (Table 3).

HCV genotype 1b is acknowledged as a difficult-to-treat condition using pegylated interferon and ribavirin treatment, so we made AUC comparisons between our "FIL4" and other fibrosis indexes (AST/ALT ratio, APRI and FIB4). In HCV genotype 1b patients, the AUC for "FIL4" was the only significant variable for diagnosing advanced fibrosis (AUC = 0.818; p = 0.009), while the other fibrosis indexes (AUC for AST/ALT ratio – 0.553, p = 0.665; AUC for APRI – 0.636, p = 0.267; AUC for FIB4 – 0.631, p = 0.283) were not significant at all. In addition, there

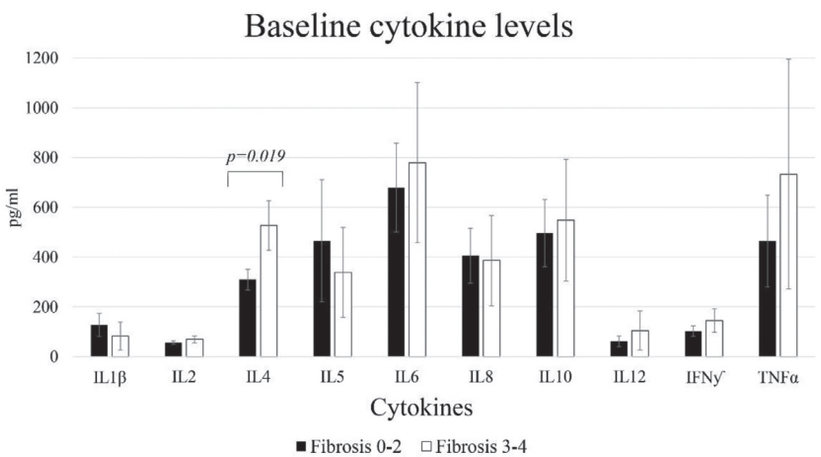

Fig. 1 Cytokine expression levels between fibrosis 0-2 and fibrosis 3-4 groups.

Table 2

The association among IL-4, age, GOT, and ferritin with liver fibrosis in hepatitis C virus patients

					Multivariate Logistic Regression	
	Fibrosis 0-2	Fibrosis 3-4	χ^2 Test *p*	χ^2 or Fisher's Exact Test OR, 95% CI	OR, 95% CI	*p*
IL-4 baseline (<490)	37 (68.5%)	8 (42.1%)	0.042	2.99 (1.02-8.78)	4.34 (1.11-16.8)	0.034
IL-4 baseline (≥490)	17 (31.5%)	11 (57.9%)				
Age (<45)	16 (23.9%)	0 (0%)	0.009	1.451 (1.24-1.69)	1.07 (0.98-1.16)	0.113
Age (≥45)	51 (76.1%)	23 (100%)				
GOT (<80)	35 (53%)	5 (22.7%)	0.015	3.83 (1.26-11.62)	6.39 (1.13-36.1)	0.036
GOT (≥80)	31 (47%)	17 (77.3%)				
Ferritin (<450)	46 (69.7%)	8 (34.8%)	0.003	4.31 (1.57-11.79)	2.95 (0.79-11.0)	0.108
Ferritin (≥450)	20 (30.3%)	15 (65.2%)				
Platelet (<100)	63 (94%)	21 (91.3%)	0.643	1.50 (0.25-8.78)	0.36 (0.02-4.59)	0.432
Platelet (≥100)	4 (6%)	2 (8.7%)				

Table 3

Area under the curve of AST/ALT ratio, APRI, FIB4 score, and FIL4

Characteristic	Area	Std. Error	*P*	95% CI	*p* for Comparison*
AST/ALT ratio	0.599	0.076	0.212	0.45-0.748	0.00218
APRI	0.689	0.063	0.017	0.565-0.812	0.00599
FIB4	0.710	0.063	0.008	0.586-0.833	0.03117
FIL4	0.833	0.051	0.0001	0.733-0.934	

**p* value of statistical comparison of FIL4 to the other indexes for predicting liver fibrosis.

were statistically significant differences in FIL4 compared to APRI (p = 0.029) and AST/ALT ratios (p = 0.047), respectively, but not in FIB4 (p = 0.096) in statistical comparison analysis. In HCV genotype non-1b patients, all indexes were significant but "FIL4" was highest in AUC (0.857) and the p value is 0.001 (Table 4). The statistical comparison analysis showed no difference in predicting advanced fibrosis of HCV-infected patients.

4. DISCUSSION

Our study demonstrated that IL-4 is an important cytokine in predicting advanced liver fibrosis in HCV-infected patients. We also found that the combination of IL-4 level with known liver fibrosis-associated factors such as age, GOT, and ferritin level improved the diagnostic and predictive results for advanced fibrosis. From this, we demonstrated the "FIL4" and analyzed the outcome to predict fibrosis grade in HCV-infected patients.

Liver biopsy is still the gold standard to assess the fibrosis stage in CHC patients. However, there are a number of noninvasive markers that are a combination of direct or indirect markers of hepatic function.[7] In the current study, we developed a simple score to predict liver fibrosis from our novel findings that included IL-4 in fibrosis generation. It was found that age, serum GOT level, serum ferritin level, and serum IL-4 level were independent predictors of fibrosis, and that the score had predictive power. Age and high GOT level has been associated with advanced fibrosis.[12] An elevated serum ferritin level has been shown to have independent predictive value in severe liver fibrosis in CHC patients.[13] Other factors such as serum ALT level, γ-glutamyl transferase, HCV RNA, or HCV genotype were not associated with advanced fibrosis in our study.

Many studies have discussed noninvasive markers of hepatic fibrosis in HCV-infected patients. We compared our score with well-known markers such as AST/ALT ratio,[14] APRI,[15] and FIB4 index,[16] and determined that our score held significantly higher

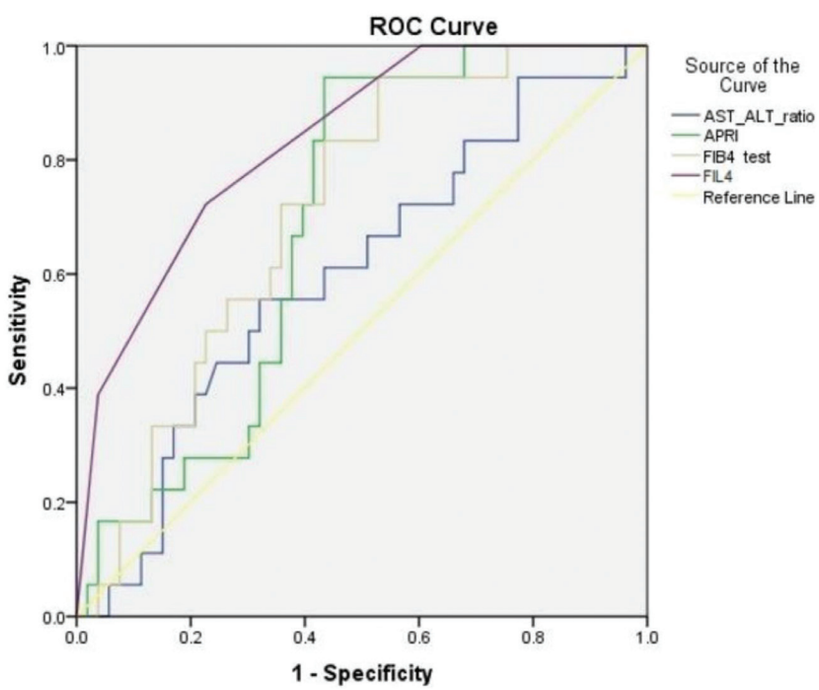

Fig. 2 Receiver operating characteristics curve comparison for AST/ALT ratio, APRI, FIB4 score, and FIL4.

Table 4

Area under the curve of AST/ALT ratio, APRI, FIB4 score, and FIL4 in hepatitis C virus genotype 1b and non-1b

Genotype	Characteristics	Area	Std. Error	*P*	95% CI	*p* for Comparison*
1b	APRI	0.636	0.104	0.267	0.433-0.839	0.029
	FIB4	0.631	0.111	0.283	0.414-0.849	0.096
	AST/ALT ratio	0.553	0.130	0.665	0.299-0.807	0.047
	FIL4	0.818	0.087	0.009	0.648-0.988	
Non-1b	APRI	0.769	0.085	0.013	0.602-0.935	0.131
	FIB4	0.851	0.066	0.001	0.722-0.980	0.663
	AST/ALT ratio	0.711	0.092	0.051	0.531-0.891	0.115
	FIL4	0.857	0.065	0.001	0.731-0.984	

*p value of statistical comparison of FIL4 to the other indexes for predicting liver fibrosis.

value in predicting liver fibrosis by using statistical comparison method of R software. The prediction value was strengthened in HCV-genotype 1b patients, better than the other indexes.

IL-4 is a multifunctional cytokine that controls cell growth, including the immune system, and maintains antiinflammatory effects by inhibiting Th1-cell activation.[17] IL-4 has also previously been reported as supporting T cell differentiation due to Th2[18] and enhances collagen synthesis by nonparenchymal liver cells in vitro.[4] IL-4 was increased in severe recurrent HCV infection[19] and also induces apoptosis of human hepatocyte (HepG2) cell lines.[20] Most researchers have considered that activated HSC was the primary fibrogenic cell to contribute to extracellular matrix accumulation, which is crucial in liver fibrogenesis.[21] Although serum IL-4 level was higher in CHC patients compared to healthy controls,[22] we found that IL-4 level was higher in the advanced fibrosis group. These results were also replicated in the subgroup analyses as stratified by viral load, HCV genotypes (1b), high GOT level, high GPT level, and high ferritin level.

In the present study, we recruited a low number (92 patients) in the study population and prospective studies with larger numbers of patients are required to validate our findings. Additionally, the long-term outcome of the newly developed FIL4 score for predicting advanced fibrosis needs to be investigated.

In conclusion, this study provides evidence that increased IL-4 expression and the "FIL4" score are linked to prediction of advanced liver fibrosis in treatment of naive HCV-infected patients. Additionally, we suggest that serum IL-4 level could be included in noninvasive markers to predict advanced fibrosis.

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Supplementary data related to this article can be found at http://links.lww.com/JCMA/A14.

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