Full Length Research Paper

Identification of overexpressed cytokines as serum biomarkers of hepatitis C virus-induced liver fibrosis using bead-based flexible multiple analyte profiling

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Hepatic inflammation is the stimulator to activate hepatic stellate cells (HSCs) and triggers fibrogenesis. Cytokines are produced during liver inflammation and maybe considered as liver fibrosis biomarker. The aim of this study was to investigate whether cytokines can be used as reliable biomarkers of liver fibrosis using flexible multi-analyte profiling (xMAP). A total of 61 chronic hepatitis C patients with different severity of liver fibrosis were enrolled. Liver biopsy was used as standard to assess the severity of fibrosis according to METAVIR classification. Afterward, 15 samples from healthy controls were analyzed and totally 50 cytokines were screened using flexible multi-analyte profiling to discover differential biomarkers. Finally, levels of protein expressions of individual stages of liver fibrosis were measured. In histological examination, the necroinflammatory score (histology activity index, HAI) was increased from F_1 to F_4 stage in hepatitis C virus (HCV) infected patients, indicating that inflammation was accompanied with the progression of liver fibrosis. Using flexible multi-analyte profiling, four serum cytokines, including IFN-α2 (p=0.023), GRO-α (p=0.013), SCF (p=0.047) and SDF-1α (p=0.024), were identified under antibody specific recognition and elevated with HAI score. This study reveals the relationship between cytokines and liver fibrosis, and demonstrated that IFN-α2, GRO-α, SCF and SDF-1α may be used as biomarkers to predict liver fibrosis. The overexpressed cytokines may play a role in the progression of liver fibrosis and deserves further investigation.

Key words: Cytokine, flexible multi-analyte profiling, hepatitis C virus, liver fibrosis.

INTRODUCTION

Reliable biomarkers of liver fibrosis are important to

grade the severity of liver disease in clinical practice. Although liver biopsy followed by histological examination is still the gold standard to diagnose liver fibrosis (Afdhal and Nunes, 2004), it is necessary to develop some other methods to improve diagnostic accuracy and reduce the disadvantages of biopsy such as invasive character and small sample size (Regev et al., 2002; Thampanitchawong and Piratvisuth, 1999). Nowadays,

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noninvasive diagnostic techniques such as ultra-sonography, computed tomography and magnetic resonance imaging have been used to detect the morphological changes in the hepatic parenchyma for advanced fibrosis (Hirata et al., 2001). As a reliable predictor, FibroScan has been used recently to diagnose liver fibrosis (Zhu et al., 2011). Due to the low cost and ready availability, ultrasonography is the most popular technique to detect liver disease. However, ultra-sonography is highly-operator dependent and liver echogenicity can not accurately differentiate hepatic fibrosis from steatosis (Bataller and Brenner, 2005). Therefore, noninvasive serological biomarkers become one of the solutions for complementing the diagnosis of liver fibrosis, indicating that the development of reliable noninvasive biomarkers for assessing hepatic fibrosis in therapeutic purpose is urgent.

Approximately 70% of the patients infected with heap-titis C virus (HCV) become chronic carriers (Marcellin, 1999) and chronic inflammation could lead to fibrosis, liver cirrhosis or even hepatocellular carcinoma. Liver fibrosis is the process of repair when liver is injured or in inflammation (Marcellin, 1999). In liver tissue, hepatic stellate cells (HSCs) are the major source of extracellular matrix proteins (ECMs) excretion in facing constant inflammation and eventually triggers fibrogenesis (Eng and Friedman, 2000; Friedman, 2000, 2003; Marcellin, 1999). Furthermore, cytokines that participate in the inflammatory process (Marra, 2002) are secreted into blood. For example, the level of interferon gamma inducible protein 10 (IP-10), increases in the serum of HCV-infected patients, and has significant correlations with poor response to anti-viral therapy (Reiberger et al., 2008). Therefore, serum cytokines may be used as biomarkers of hepatic inflammation or fibrosis.

Currently, many biomarkers have been reported to be correlated with liver fibrosis, including alpha-2 macroglobulin, vitamin D binding protein, apolipoprotein AI (Ho et al., 2010), tissue inhibitor of metalloproteinases-1 (TIMP-1), hyaluronic acid (HA), N-terminal propeptide of type III procollagen (PIIINP), and YKL-40 (Bataller and Brenner, 2005; Johansen et al., 2000; Poynard et al., 2002). Moreover, several biomarker panels such as FibroTest, Forns index, AST-to-platelet ratio index (APRI) and GlycoCirrhoTest have been reported to stage fibrosis (Callewaert et al., 2004; Le Calvez et al., 2004; Rossi et al., 2003; Thabut et al., 2003). In the past, the discovery of serological biomarkers was limited due to the abundant amount of albumin and IgG in serum, which would decrease the accuracy in analytical experiments. Therefore, the low abundant proteins such as cytokines in serum have not been analyzed. Regardless of two dimension electrophoresis (Gangadharan et al., 2007; White et al., 2007) or other existing technologies, analyzing proteins at micro- or pico-level quantity in serum has been difficult. Using specific antibodies to detect the known proteins based on flexible multi-analyte

profiling (xMAP) technology may be another solution to detect the low-abundant serum proteins. In this study, noninvasive serological biomarkers were discovered using Bio-Plex suspension array system based on xMAP technique. This method provided a broad screening for different proteins using specific antibody (Yurkovetsky et al., 2007). Due to the high reproducibility of robust assay and high precision of immunoassay, we used commercial cytokines 27- and 23-panels to search for putative serum biomarkers of liver fibrosis. Less total assay time, fewer progressive steps and smaller sample volume were needed using xMAP compared to conventional enzyme-linked immunosorbent assay (ELISA). It also provided multiple analyses at the same time and had higher reproducibility because the result was the mean value after multiple readings (Chowdhury et al., 2009; dupont et al., 2005; Prabhakar et al., 2002). By this way, com-parison of 50 cytokines between normal and disease group were completed, and candidate biomarkers were selected. This study aimed to investigate whether over-expressed cytokines could be used as serological biomarkers of liver fibrosis in patients with chronic hepatitis C.

MATERIALS AND METHODS

Serum sample

Serum samples (n = 61) from HCV-infected patients were obtained in Cheng Hsin general hospital in Taiwan (approval No. 97016). Firstly the blood samples were allowed to clot for 30 min at 4°C and then centrifuged at 1400 × g at 4°C. Collected serum was frozen at -80°C for store. The patients were diagnosed by a pathologist using hepatic biopsy procedure and followed in subsequent histological examinations. The serum samples were used to determine the stages of hepatic fibrosis according to METAVIR classification, including F_1, F_2, F_3 and F_4. On the other hand, we analyzed three individual HCV-infected samples in every stage of liver fibrosis and compared results to three healthy controls; liver biopsy in control group was not performed due to ethical issues.

Cytokine standard preparation

The cytokine 23-plex panel or 27-plex panel (Bio-Rad) were used in this study. Each 128 µL of cytokine stocks were added with 72 µL of serum standard diluent and continued to make four-fold serial dilution to eight standard samples totally.

Flexible multi-analyte profiling

The 25 µL of the serum samples was diluted with 75 µl of the appropriate human serum sample diluent (Bio-Rad). We used Bio-Plex™ Human Cytokine 27-Plex and 23-Plex Panel to search for putative biomarkers of hepatic fibrosis in serum. The experiment followed the instruction manual described briefly as below. First, 50 µL of anti-cytokine conjugated beads was incubated with 50 µL of diluted standard or sample in each well of a 96-well filter plate at room temperature for exactly 1 h on a shaker with 300 rpm. After removing the solution and washing, we added 25 µL of detective antibody conjugated with biotin to each well and incubate for 30 min

Table 1. Characteristic of 61 HCV-infected liver fibrosis patients

Characteristic	Stages of liver fibrosis				P value (ANOVA)
	F1 (n = 20)	F2 (n = 29)	F3 (n = 12)	F4 (n = 29)	
Gender (n)	Female: 11	Female: 11	Female: 5	Female: 17	
	Male: 9	Male: 18	Male: 7	Male: 12	
BMI	26.0±0.8	23.6±1.1	24.5±0.9	25.8±0.8	NS
AST (IU/L)	60.5±6.8	111.0±40.9	88.7±11.5	112.5±8.3	NS
ALT (IU/L)	143.6±18.2	131.1±17.5	141.2±21.9	143.0±13.8	NS
WBC (1000/µl)	6.5±0.3	5.7±0.3	6.2±0.4	5.0±0.3	0.016
Plt (1000/µl)	221.6±15.7	189.5±7.1	178.6±13.22	113.9±8.6	<0.001

BMI, Body mass index; AST, aspartate aminotransferase; ALT, alanine transaminase; WBC, white blood cell; Pit, platelet; NS: non-significant.

on a shaker with 200 rpm. Then 50 µL of streptavidin-Phycoerythrin (PE) was added in each well and incubated for 10 min on a shaker. Afterward, each well was added with 125 µL of assay buffer and it was ready for the measurement. A black was necessary for the measurement and all the incubations were performed in dark.

Statistical analysis

After fluorescent measurement by Bio-Plex 200 suspension array system, we used five-parameter logistic regression algorithms (5PL) to calculate the standard curve. This regression is commonly used in immunoassays and provides a large range of quantification than linear regression analysis. The concentrations of measured cytokine were calculated using Bio-Plex manager 4.1 software. The p value was calculated using one-way analysis of variance (ANOVA), and value less than 0.05 was considered to be statistically significant.

RESULTS

Patient profiles

A total of 61 patients with chronic hepatitis C were enrolled. The stage of liver fibrosis was determined by histological findings. The clinical characteristics of the samples were investigated by measuring several indicators, including aspartate aminotransferase (AST), alanine transaminase (ALT), white blood cells (WBCs), and platelet. The conventional indicators of liver function, AST and ALT, were not associated with the stage of liver fibrosis (Table 1). However, we previously demonstrate that AST and ALT increased in thioacetamide-induced liver fibrosis animal model as compared to healthy controls (Liu et al., 2011). Moreover, the levels of AST (> 60.5 IU/L) and ALT (> 131.1 IU/L) indeed were higher than healthy ones, demonstrating that AST and ALT were the markers of liver disease. WBC and platelet were decreased in advanced liver fibrosis (both $p < 0.05$) as previously reported (Wai et al., 2003). The APRI index was increased from F_1 to F_4 stage ($p < 0.0001$, Figure 1A). Moreover, histology activity index (HAI) score was increased with the progression of liver fibrosis (p = 0.003,

Figure 1B), indicating inflammation was accompanied with the progression of liver fibrosis.

Discovery of cytokine biomarker of liver fibrosis

To identify the putative cytokine biomarkers of liver fibrosis in this study, xMAP was used. A total of 15 serum samples from HCV-infected patients' triplicate in each stage of liver fibrosis and healthy controls were analyzed, which were selected in accordance with the level of HAI index in Figure 1B. Moreover, the samples were collected before the patients received any clinical treatment, such as anti-viral therapy, and liver transplantation. The total protein concentration was equivalent in each stage of the serum samples (data not shown). In the xMAP analysis, we totally analyzed 50 available cytokines using commercial 23- and 27-Plex panel (Bio-Rad). Due to the fact that the beads were stained with two different fluorescent dyes and formed 100 different types, it allowed us to analyze the protein level at the same time. After xMAP analysis, we finally found that four cytokines were increased gradually in serum from normal controls and patients with F_1 to F_4 stage of liver fibrosis, including interferon-α2 (IFN-α2), growth-related oncogene-α (GRO-α), stem cell factor (SCF), and stromal cell derived factor-1α (SDF-1α) (Figure 2). Other measured cytokines were irregular with the stages of liver fibrosis (data not shown).

ROC curves of four serum fibrosis indices

We used receiver operating characteristic (ROC) analysis to distinguish normal/F1 from F_2 to F_4. The area under curve (AUC) of serum fibrosis indices in ROC curves are shown in Table 2, which demonstrated that these four liver fibrosis markers had acceptable discrimination (all > 0.8, $p < 0.05$) for diagnosing liver fibrosis. Moreover, ROC analysis was also used to determine the sensitivity and 1-specificity of the assay in detecting the severity of liver fibrosis. On this account of taking higher sensitivity
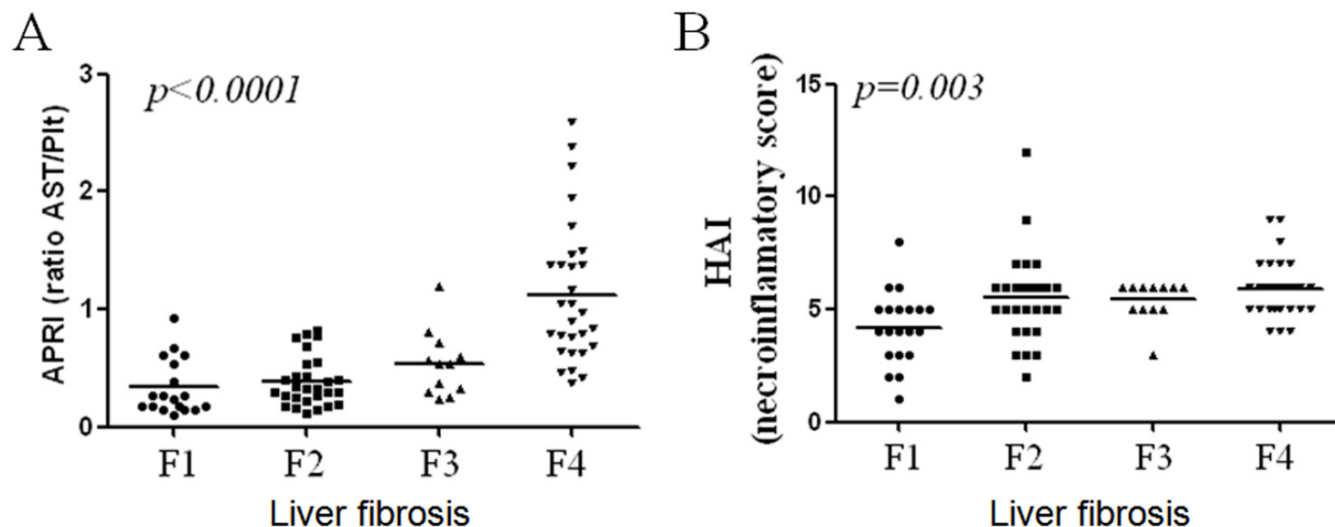
Figure 1. APRI and HAI levels in the stages of liver fibrosis. (A) APRI index was used to evaluate the enrolled patients with hepatitis C virus-induced liver fibrosis. The result shows that APRI increased gradually from F1 to F4, indicating APRI is a reliable index for predicting liver fibrosis. (B) HAI was also performed to measure the necroinflmmatory levels of the tissues with liver fibrosis, which demonstrated that HAI increased from F1 to F4, indicating inflammation was accompanied with liver fibrosis. AST, aspartate aminotransferase; Plt, platelet; HAI, histology activity index. APRI, AST-to-platelet ratio index.

and specificity, the cut-off points of the four indices were selected and shown in Table 2.

DISCUSSION

This study aimed to widely screen serum cytokines and evaluate their relationship with liver fibrosis using xMAP. Furthermore, four cytokines including IFN-α2, GRO-α, SCF and SDF-1α were identified to elevate in serum in parallel with severity of fibrosis in chronic hepatitis C patients. In addition, the concentrations of these cytokines were measured as references to predict the stage of liver fibrosis in patients with chronic hepatitis C.

Clinically, ELISA is a useful tool for determining serum antibody concentrations and is usually performed for disease diagnosis. However, Thierry's group reported that the results obtained by xMAP assay were not consistant with the data derived from ELISAs and a constant conversion factor (Reijn et al., 2007). Therefore, the absolute concentrations of the four candidate proteins may be different between these two assays and specific reference value must be defined for each type of analysis (Elshal and McCoy, 2006; Ray et al., 2005). For clinical application, it is necessary to measure the reference values for ELISA or xMAP assay; the later one can measure multi-markers at once and could be used to replace conventional ELISA assay in the future.

To our knowledge, xMAP assay was used to simultaneously evaluate the cytokine expressions in HCV-infected patients after anti-viral treatment (Wan et al., 2009). In this study, we used xMAP to evaluate the 50

kinds of cytokines in HCV-infected liver fibrosis. For the searched results, the level of SDF-1α was found to be increased in liver disease such as fibrosis or cirrhosis (Horani et al., 2007; Itoh et al., 1994; Panasiuk et al., 2007; Radaeva et al., 2006; Wald et al., 2004; Wald et al., 2007). However, IFN-α2 and GRO-α were reported to be associated with the stage of liver fibrosis for the first time. IFN-α2 was prioritized to treat chronic hepatitis B and C in many clinical trials (Buti and Esteban, 2005; Cooksley, 2005). Furthermore, interferon plus ribavirin has become the standard of care for the treatment of chronic hepatitis C. On the other hand, GRO-α (also called CXCL1), a chemokine of C-X-C family, significantly increased in liver tissue of alcoholic hepatitis (Maltby et al., 1996). To the best of our knowledge, this is the first report that IFN-α2, SCF and GRO-α increase in liver fibrosis, thus could be used as putative biomarkers for predicting the severity of liver fibrosis. In particular, GRO-α, expressed by macrophages, neutrophils and epithelial cells, with neutrophil chemoattractant activity involved in the accumulation of neutrophils, and thus mediated liver injury and inflammation (Ramaiah and Jaeschke, 2007). Different cytokines are secreted during inflammatory process under many causes of liver injury such as ethanol abuse, viral infection, cholestasis or metabolic syndrome; these four cytokines were confirmed to be specifically associated with the stage of liver fibrosis in histological examination in this study. However, further studies are still needed to reveal the role of the four discovered cytokines in the progression of liver fibrosis. In this study, we also found that other cytokines, including interleukin (IL)-2ra, IL-3, IL-12, IL-16, IL-18, Cutaneous T
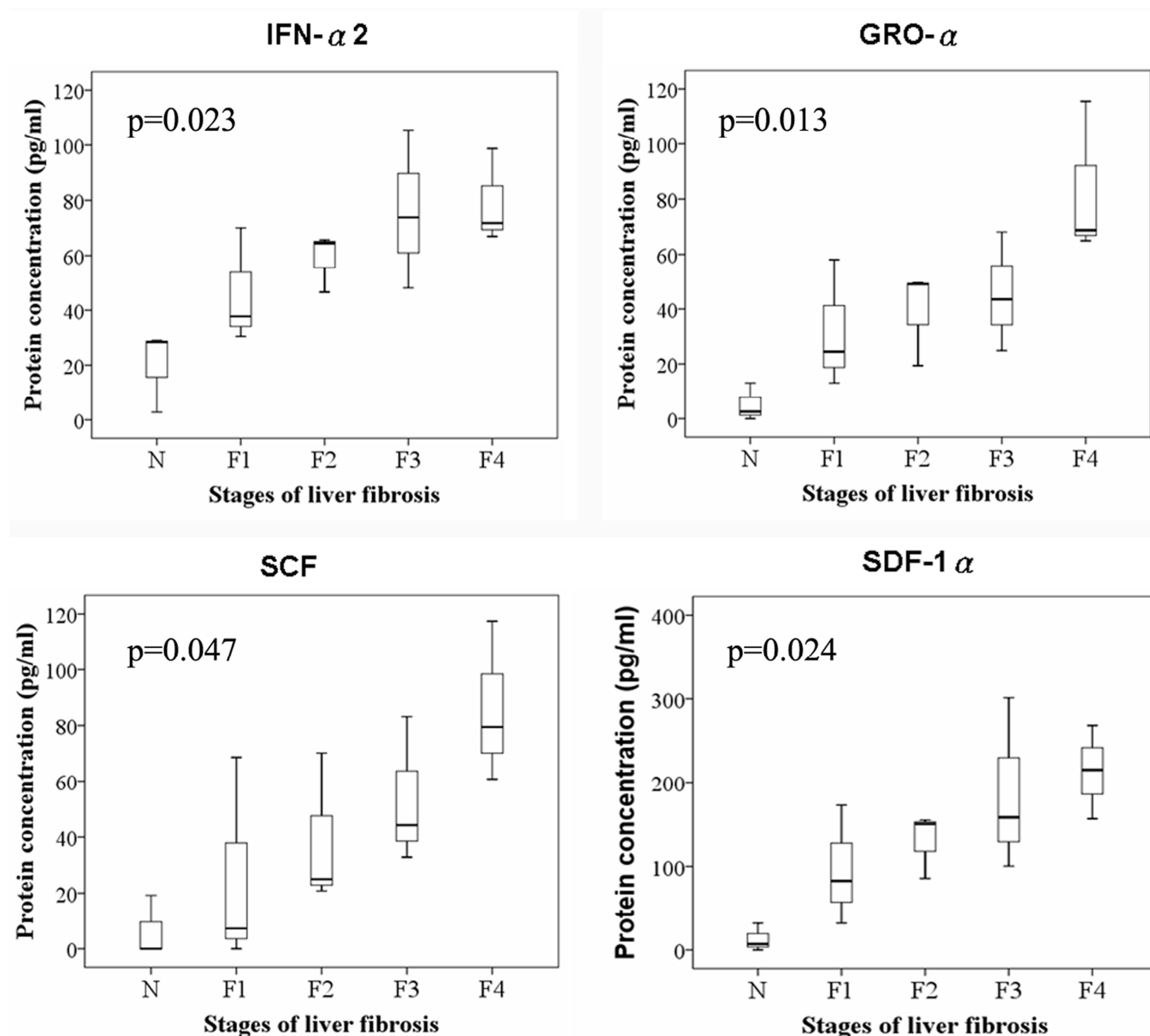
Figure 2. The overexpressed cytokine biomarkers of HCV-infected liver fibrosis. Totally we discovered four candidate biomarkers with increase trend from normal to advanced hepatic fibrosis by using box-whisker plot, including IFN-α2, SCF, GRO-α and SDF-1α. There were 15 samples analyzed, including the samples from normal controls (n=3), F1 (n=3), F2 (n=3), F3 (n=3) to F4 (n=3). IFN-α2, interferon-α2; GRO-α, growth-related oncogene-α; SCF, stem cell factor; SDF-1α, stromal cell derived factor-1α.

cell-attracting chemokine (CTACK), hepatocyte growth factor (HGF), intercellular adhesion molecule-1 (ICAM-1), leukemia inhibitory factor (LIF), macrophage colony-stimulating factor (M-CSF), tumor necrosis factor-related apoptosis inducing ligand (TRAIL), vascular cell adhesion molecule-1 (VCAM-1), IP-10, increased in HCV-infected liver fibrosis (F2 to F4) compared to normal or early stage (data not shown). In which, the elevated IL-18 was consistent with the previous study which revealed that higher serum level of IL-18 in HCV infection (Chattergoon et al., 2011). Unfortunately, the level of IL-18 did not

gradually increase with the stages of liver fibrosis ($p>0.05$, data not shown). Moreover, the elevated IP-10 level was consistent with the study reported previously for predicting the response of HCV viral load, the hepatic inflammatory activity, and fibrotic stage (Reiberger et al., 2008). Those cytokines seem to associate with the HCV infection or hepatic inflammation. Take together, serological biomarkers are one of the solutions to diagnose liver fibrosis in hepatitis C patients in order to decrease the use of invasive liver biopsy. Observably, the cytokines found in this study are in low abundance (<1

Table 2. ROC curve of the four liver fibrosis indexes.

Index	AUC	Cut-off point (pg/ml)	Sensitivity	1-Specificity	p
IFN-α2	0.907	56.30	0.778	0.167	0.010
GRO-α	0.889	46.45	0.667	0.167	0.013
SCF	0.907	28.95	0.778	0.167	0.010
SDF-1α	0.889	152.65	0.667	0.167	0.013

The cut-off point was selected to distinguish Normal/F1 (n=6) from F2 to F4 (n=9). AUC, Area under curve.

ng/mL). Due to the inference from highly abundant albumin and IgG in serum, the detection of some valuable yet less abundant proteins like cytokines that present in pico or micro levels requires highly sensitive technique. In this study, we found four putative bio-markers of liver fibrosis, including IFN-α2, SCF, GRO-α and SDF-1α, using flexible multi-analyte profiling and measured the protein concentrations in each stage of liver fibrosis.

In conclusion, this study was to assess the association of cytokines with liver fibrosis using xMAP. Four cytokines, IFN-α2, GRO-α, SCF and SDF-1α, were found to be increased in serum in parallel with the severity of liver fibrosis in patients with chronic hepatitis C, sug-gesting they could be used as markers for predicting the severity of liver fibrosis and reduce the usage of invasive liver biopsy in clinical practice. However, the role of these cytokines in the progression of liver fibrosis needs further study to confirm.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Shu-Lin Liu: Experimemtal design; Yang-Chih Cheng: Patient's screening and experimemtal design; Chun-Chia Cheng: Experimental operation and article writing; Ai-Sheng Ho: Patient's screening, tissue's collection, serum centrifugation and transportation, co-leader of the project, Jungshan Chang: Experimental design and corres-ponding author; Ling-Yun Chen: Manuscript revision; Chia-Chi Wang: Patient's screening and data analysis.

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Abbreviations

HSCs, Hepatic stellate cells; **xMAP,** flexible multi-analyte profiling; **HAI,** histology activity index; **HCV,** hepatitis C virus.

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