

Identification of Treatment Efficacy-Related Host Factors in Chronic Hepatitis C by ProteinChip Serum Analysis

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Recent development of proteomic array technology, including protein profiling coupling ProteinChip array with surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF/MS), provides a potentially powerful tool for discovery of new biomarkers by comparison of its profiles according to patient phenotypes. We used this approach to identify the host factors associated with treatment response in patients with chronic hepatitis C (CHC) receiving a 48-wk course of pegylated interferon (PEG-IFN) alpha 2b plus ribavirin (RBV). Protein profiles of pretreatment serum samples from 32 patients with genotype 1b and high viral load were conducted by SELDI-TOF/MS by using the three different ProteinChip arrays (CM10, Q10, IMAC30). Proteins showed significantly different peak intensities between sustained virological responders (SVRs), and non-SVRs were identified by chromatography, SDS-PAGE, TOF/MS and tandem mass spectrometry (MS/MS) assay. Eleven peak intensities were significantly different between SVRs and non-SVRs. The three SVR-increased peaks could be identified as two apolipoprotein (Apo) fragments and albumin and, among the eight non-SVR-increased proteins, four peaks identified as two iron-related and two fibrogenesis-related protein fragments, respectively. Multivariate analysis showed that the serum ferritin and three peak intensity values (Apo A1, hemopexin and transferrin) were independent variables associated with SVRs, and the area under the receiver operating characteristic (ROC) curves for SVR prediction by using the Apo A1/hemopexin and hemopexin/transferrin were 0.964 and 0.936. In conclusion, pretreatment serum protein profiling by SELDI-TOF/MS is variable for identification of response-related host factors, which are useful for treatment efficacy prediction in CHC receiving PEG-IFN plus RBV. Our data also may help us understand the mechanism for treatment resistance and development of more effective antiviral therapy targeted toward the modulation of lipogenesis or iron homeostasis in CHC patients.

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INTRODUCTION

Chronic hepatitis C (CHC) is among the leading causes of chronic liver disease worldwide, which afflicts approximately 170 million people (1). The severity of disease varies from asymptomatic chronic infection to cirrhosis and hepatocellular carcinoma (2). Although combination therapy with pegylated interferon (PEG-IFN) alpha and ribavirin (RBV) is

now established as the most effective treatment for chronic hepatitis C virus (HCV) infection with genotype 1b, the sustained virological response (SVR) rate in these patients is still around 50% (3,4). Moreover, physicians have also found that 20% of patients are nonvirological responders (NVRs; those whose HCV RNA does not become negative during 48 weeks of combination therapy). Pre-

diction of NVR status is of clinical importance because these patients have no chance of achieving a sustained virological response even after prolonged combination therapy (5). However, mechanisms involving resistance to PEG-IFN and RBV have not been fully elucidated, and it is difficult to predict treatment responses before initiation of PEG-IFN and RBV combination therapy. Considering side effects and treatment cost, prediction of treatment response before therapy with more reliable markers is mandatory.

The recent development of proteomic array technology, including protein profiling via coupling ProteinChip array with surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF/MS), provides a poten-

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tially powerful tool for global visualization of the proteome in a biological milieu (6–8). It enables the obtaining of spectra composed of hundreds of protein peaks, each characterized by its mass-to-charge ratio (m/z). Comparison of protein profiles according to patient phenotypes led to the identification of isolated or clustered peaks characteristic of pathologic conditions. This approach has led to the discovery of new biomarkers, as shown in patients with prostate, ovarian and liver malignancies (9–11).

The aim of this study was to investigate the global serum protein profile in patients with chronic HCV infection receiving the same complete treatment schedule consisting of PEG-IFN plus RBV treatment using ProteinChip array proteomic technology. Our results show that pretreatment serum profile variations differed substantially according to the response to antiviral treatment in CHC patients. Analysis of the protein profile is useful for predicting treatment response and understanding the mechanisms underlying resistance to antiviral therapy in the clinical setting, which may lead to the development of more efficacy treatment options for chronic HCV infection.

MATERIALS AND METHODS

Patients

Among patients with CHC treated at the Mie University Hospital between April 2007 to July 2008, 32 patients of HCV genotype 1b with a high viral load (>5.0 log IU/mL by real-time reverse-transcription polymerase chain reaction [RT-PCR] assay) (TaqMan HCV; Roche Diagnostics, Tokyo, Japan) were included in the present study. The inclusion criteria were as follows: (1) an established diagnosis of CHC with the findings consistent with chronic hepatitis on liver biopsy and (2) the absence of other causes of chronic liver disease (undetectable hepatitis B surface antigen, no excessive alcohol consumption [defined as intake >20 g/d], hemochromatosis, autoimmune hepatitis, Wilson's disease, α_1 -antitrypsin deficiency, primary biliary

cirrhosis or primary sclerosing cholangitis). Blood samples were drawn from all patients at enrollment and stored at -80°C until use for SELDI analysis. Liver biopsy was performed in all patients at enrollment. Liver tissue specimens were divided into two; one portion was fixed in buffered formalin and embedded in paraffin for histological examination and the other was immediately frozen and stored at -80°C for RNA extraction. Fixed hepatic tissues were stained with hematoxylin and eosin and Masson's trichrome and were graded for the degree of necroinflammatory activity and staged for the extent of fibrosis using the criteria of Desmet *et al.* (12).

All patients received the same complete treatment consisting of PEG-IFN alpha 2b (PegIntron; Schering-Plough, Kenilworth, NJ, USA) at a dose of 1.5 $\mu\text{g}/\text{kg}/\text{wk}$ in combination with RBV (Rebetol; Schering-Plough) at a dose adjusted according to body weight (<60 kg, 600 mg/d; 60 – 80 kg, 800 mg/d; >80 kg, $1,000$ mg/d) at the course of 48 wks. Only patients with good observance were selected (that is, patients who received $>80\%$ dose of each drug during the 48 wks). A patient negative for serum HCV RNA during the first 6 months after the completion of therapy was defined as an SVR, and a patient for whom HCV RNA became negative at the end of therapy and reappeared thereafter was defined as a transient responder (TR). A patient who was positive for HCV RNA even during the course of therapy was defined as an NVR.

Informed consent was obtained from each patient included in the study, and the study protocol conformed to the ethical guidelines of the 1983 Declaration of Helsinki as reflected in *a priori* approval by the Ethical Committee of Mie University.

Protein Profiling using SELDI-TOF/MS

Serum samples (20 μL) were denatured by adding 30 μL U9 buffer (50 mmol/L Tris-HCl [pH 9.0], 9 mol/L urea, 2% CHAPS) and agitated at 4°C for 30 min. Denatured samples were applied to

90 μL Q Ceramic Hyper D F[®] anion-exchanger resin (Pall Corporation, Port Washington, NY, USA), and 50 μL U1 buffer (nine-fold diluted U9 buffer with 50 mmol/L Tris-HCl [pH 9.0]) was added. Fractions were collected using 100 μL of 50 mmol/L Tris-HCl buffer (pH 9.0) containing 0.1% octyl α -D-glucopyranoside and six different buffers with stepwise decreasing pH values. As the last step of sample fractionation, the resin was washed with organic solution composed of 33.3% isopropanol, 16.7% acetonitrile and 0.1% trifluoroacetic acid to elute remaining proteins. Therefore, eight fractions (flow through + pH 9.0/pH 8.0/pH 7.0/pH 6.0/pH 5.0/pH 4.0/pH 3.0/organic) were finally obtained, and these were called Fr 1 to Fr 8. All fractionated samples were subjected to three different types of ProteinChip array: CM10 (weak cation exchange), Q10 (strong anion exchange) and IMAC30 (immobilized metal affinity capture). The binding buffer was 100 mmol/L sodium acetate buffer (pH 4.0) for CM10 arrays, 50 mmol/L Tris-HCl buffer (pH 9.0) for Q10 arrays and 100 mmol/L sodium phosphate buffer (pH 7.0) containing 500 mmol/L NaCl for IMAC30 arrays. Both α -cyano-4-hydroxycinnamic acid and sinapinic acid were used as energy-absorbing molecules. In the procedure from sample fractionation to applying an energy-absorbing molecule, the laboratory automation system Biomek[®]2000 was used (Beckman Coulter, Fullerton, CA, USA). All of the arrays were analyzed by a Protein Biological System IIc ProteinChip reader (Bio-Rad Laboratories, Hercules, CA, USA). The high mass setting was set to 100 kDa and the focus mass was set to 6.5 kDa. The arrays were analyzed in two different conditions when using sinapinic acid as an energy-absorbing molecule, the high mass setting was set to 200 kDa and the focus mass was set to 20 kDa in addition to the above. Mass spectrometric profiles were generated by averaging 130 laser shots at optimized analyzing conditions, which were determined individually for each condition (fraction, array, energy-

absorbing molecule) on the basis of maximum protein peak yield. To quantify the SELDI-MS data, we used the Biomarker Wizard System (CIPHERGEN ProteinChip Software 3.1.1; CIPHERGEN Biosystems, Fremont, CA, USA), which automatically calculated each protein peak intensity. The data from the peak intensities were normalized with total ion current to compensate for the variations in sample concentrations loaded onto a spot; the data were represented as arbitrary units, calculated using this software.

Protein Purification and Identification

We could assume an isoelectric point of protein biomarkers on the basis of peak detected fraction and on-chip characterization by using CM10 and Q10 arrays combined with various types of binding buffer. Thus, a target biomarker was separated according to isoelectric point value by ion exchange chromatography initially. Subsequently, further purification was performed using chromatography techniques such as affinity, size exclusion and reverse phase. Obtained fraction containing target biomarkers was subjected to SDS-PAGE, and the gel was stained by Coomassie Brilliant Blue. The isolated protein bands were excised and destained with 50% methanol containing 50 mmol/L ammonium bicarbonate. The gel piece was soaked in 50% formic acid–25% isopropanol–15% acetonitrile with shaking to extract proteins for at least 2 h, and extracts were applied to H50 ProteinChip array to measure the mass values. The rest of the corresponding bands were analyzed by peptide mass fingerprinting. The procedure of in-gel trypsin digestion and tryptic peptide extraction for peptide mass fingerprinting was according to Shevchenko *et al.* (13). TOF/MS spectra of tryptic peptides were acquired with a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA), and parts of intense peaks in TOF/MS spectra were analyzed in tandem mass spectrum mode to obtain tandem mass spectrometry (MS/MS) spectra. Obtained

TOF/MS and MS/MS spectra were analyzed using mass values of monoisotopic peaks for searches (MASCOT: <http://www.matrixscience.com>) against the Swiss-Prot and/or NCBI (National Center for Biotechnology Information) database.

mRNA Quantification in Liver Biopsy Samples

mRNA extraction and purification from liver biopsy samples was performed as previously described (14). Hepatic mRNA expression levels of hemopexin were quantified by TaqMan real-time RT-PCR using an ABI PRISM 7000 thermocycler (Applied Biosystems). Data were calculated by linearization of C_t values and corrected by the signal obtained in the same cDNA preparation for GAPDH. Primers and probes were designed using the Primer Express Software package (Applied Biosystems), which spanned intron to avoid coamplification of genomic DNA. The sequences were as follows: hemopexin, forward primer 5'-GAATG TTGCTGAAGGCGAGA-3', reverse primer 5'-GAACCTTCTTCTCCTGCGGA-3' and FAM/TAMURA probe 5'-TGCACCTGGGG TTGTGGAGCCTA-3'; and GAPDH, forward primer 5'-GAAGGTGAAG GTCGGAGTC-3', reverse primer 5'-GAAGATGGTGATGGGATTC-3' and FAM/TAMURA probe 5'-CAAGCTTCCC GTTCTCAGCC-3'.

Statistical Analysis

Data are expressed as the mean \pm SD. The baseline characteristics were compared using the unpaired Student *t* test, Mann-Whitney *U* test or one-way factorial analysis of variance; multiple comparison test was used for continuous variables; and χ^2 test was used for categorical variables. Spearman rank-order correlation was used to determine the correlation between different characteristics. We determined the area under the receiver operating characteristic (ROC) curve to assess the diagnostic value of protein peaks by the SELDI-TOF/MS method, individually or in combination. We used multivariate anal-

ysis to determine the independent variables associated with treatment response. Variables showing significance on univariate analysis were subjected to multiple logistic regression to determine significant independent factors. Two-sided *P* values of <0.05 were considered statistically significant. We performed all statistical analyses by using the commercially available SPSS 11.5 software (SPSS, Chicago, IL, USA).

RESULTS

Clinical Characteristics of the Patients

Table 1 shows demographic and laboratory and histological data at treatment initiation. Ten patients were included in the SVR group, 12 in the TR group, and the remaining 10 in the NVR group. TR and NVR groups were combined as non-SVR. Previous history for IFN-based therapy had 22 patients (68.8%). All patients had HCV genotype 1 with high viral load. Data were compared according to virological response by univariate analysis. As shown in Table 1, only serum hyaluronic acid and ferritin levels were significantly higher in non-SVR group, and no other clinical factors, including body mass index, HCV RNA titer and histological findings, were significantly different between SVR and non-SVR groups.

Serum Protein Profiling by SELDI-TOF/MS

Using the SELDI ProteinChip system, we analyzed whether the initial serum protein profile could help to predict antiviral treatment response in CHC patients treated with a 48-wk course of PEG-IFN plus RBV therapy. Therefore, the protein profiles of pretreatment sera were compared according to subsequent treatment response. Peaks were detected automatically after baseline subtraction using CIPHERGEN ProteinChip Software, version 3.1.1. Using three different ProteinChip arrays (CM10, Q10 and IMAC30), each serum yielded a mean of 857 peaks with *m/z* varying from 2.5 to 200 kDa. Comparing SVR and non-SVR

Table 1. Patients characteristics at baseline according to final virological response.

	CHC		P values
	SVR (n = 10)	Non-SVR (TR + NVR) (n = 22)	
Age (years)	51.6 ± 7.7 ^a	60.5 ± 7.1 ^a	NS
Gender (M/F)	5/5	11/11	NS
Body mass index (kg/m ²)	24.3 ± 4.3 ^a	23.2 ± 3.3 ^a	NS
Naive/relapser and nonresponder	4/6	6/16	NS
Laboratory data			
Alanine aminotransferase (IU/L)	119 ± 161 ^a	71.8 ± 53.1 ^a	NS
Aspartate aminotransferase (IU/L)	59.2 ± 70.0 ^a	61.5 ± 34.8 ^a	NS
Total cholesterol (mg/dL)	174 ± 31 ^a	168 ± 34 ^a	NS
LDL cholesterol (mg/dL)	104 ± 28 ^a	102 ± 28 ^a	NS
HDL cholesterol (mg/dL)	48.3 ± 16.4 ^a	48.5 ± 11.9 ^a	NS
Triglyceride (mg/dL)	96.7 ± 52.9 ^a	108 ± 35 ^a	NS
Hyaluronic acid (ng/mL)	52.4 ± 38.6 ^a	146 ± 133 ^a	0.0228
Serum ferritin (ng/mL)	160 ± 81 ^a	347 ± 336 ^a	0.0418
Hemoglobin (g/L)	13.6 ± 1.3 ^a	13.0 ± 1.3 ^a	NS
Platelet count (×10 ⁴ /mm ³)	15.8 ± 6.9 ^a	13.9 ± 4.0 ^a	NS
Serum HCV RNA (log IU/mL)	6.38 ± 0.90 ^a	6.56 ± 0.71 ^a	NS
Liver histology			
Inflammatory activity (0/1/2/3) ^b	0/8/1/1	0/10/10/2	NS
Fibrosis staging (0/1/2/3/4) ^c	0/4/4/0/2	0/3/7/7/5	NS

^aData are mean ± SD.

^bInflammatory activity was graded according to the intensity of necroinflammatory lesions: 0, no histological activity; 1, mild activity; 2, moderate activity; 3, severe activity.

^cFibrosis staging was scored as follows: 0, no fibrosis; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, numerous septa without cirrhosis; 4, cirrhosis.

serum profiles, we identified a set of 11 peaks, for which the levels of expression significantly differed between SVR and non-SVR patients (Table 2). Three of them were significantly higher in SVRs, whereas the remaining eight peaks were increased in the group of non-SVRs. Rep-

resentative peak spectra in SVR and non-SVR patients within the 3,000–10,000 *m/z* range, which included three statistically significant different peaks between SVRs and non-SVRs, are shown in Figure 1. The three SVR-increased serum proteins could be identified as two apolipoprotein

fragments (A1 and C1) and albumin, and among eight non-SVR-increased proteins, four proteins identified as two iron-related (hemopexin and transferrin) and two fibrogenesis-related (connective tissue-activating peptide III [CTAP-III] and platelet factor-4) protein fragments, respectively (Table 2). When the statistical analyses were performed at separation from non-SVR to TR (n = 12) and NVR (n = 10), serum peaks of apolipoprotein (Apo) A1 and C1 were significantly higher in the SVR group than in both the TR and NVR groups, but albumin fragments were only significantly higher in the SVR group compared with the NVR group (Figure 2). These three SVR-increased proteins were not significantly different when compared with the TR and NVR groups. Serum peaks of hemopexin and transferrin were significantly elevated in both TR and NVR groups than in the SVR group, but CTAP-III was significantly higher only in the TR group. Platelet factor-4 fragment peaks were not significantly different when the non-SVR groups were separately analyzed at the TR and NVR. These four non-SVR-increased proteins were not significantly different between the TR and NVR groups.

Multivariate Analysis

To identify the variables independently associated with SVR, multivariate

Table 2. Discriminatory peaks and mean values between groups (SVR and non-SVR group).

Peak <i>m/z</i>	SVR (n = 10) ^a	Non-SVR (n = 22) ^a	P value	Protein name	Accession number	Biological function
Overexpressed proteins						
3356	1.46 ± 0.17	1.18 ± 0.28	0.006	Apo A1	P02647	Lipid metabolism
66647	7.46 ± 0.36	7.07 ± 0.45	0.008	Albumin	P02652	Serum protein
6449	3.89 ± 1.75	2.78 ± 0.78	0.02	Apo C1	P02654	Lipid metabolism
Downregulated proteins						
59459	0.30 ± 0.05	0.37 ± 0.07	0.009	Hemopexin	P02790	Iron metabolism
79237	0.36 ± 0.15	0.51 ± 0.12	0.01	Transferrin	P02787	Iron metabolism
9304	0.10 ± 0.11	0.14 ± 0.07	0.023	CTAP-III	P02775	Fibrogenesis
8688	0.81 ± 0.20	1.09 ± 0.38	0.028	Not identified	—	—
17435	0.79 ± 0.26	0.98 ± 0.25	0.031	Not identified	—	—
8821	1.49 ± 0.42	1.93 ± 0.66	0.035	Not identified	—	—
7765	0.52 ± 0.43	0.77 ± 0.35	0.038	Platelet factor-4	P02776	Coagulation
6629	1.27 ± 0.51	1.82 ± 0.63	0.042	Not identified	—	—

^aData are mean ± SD.

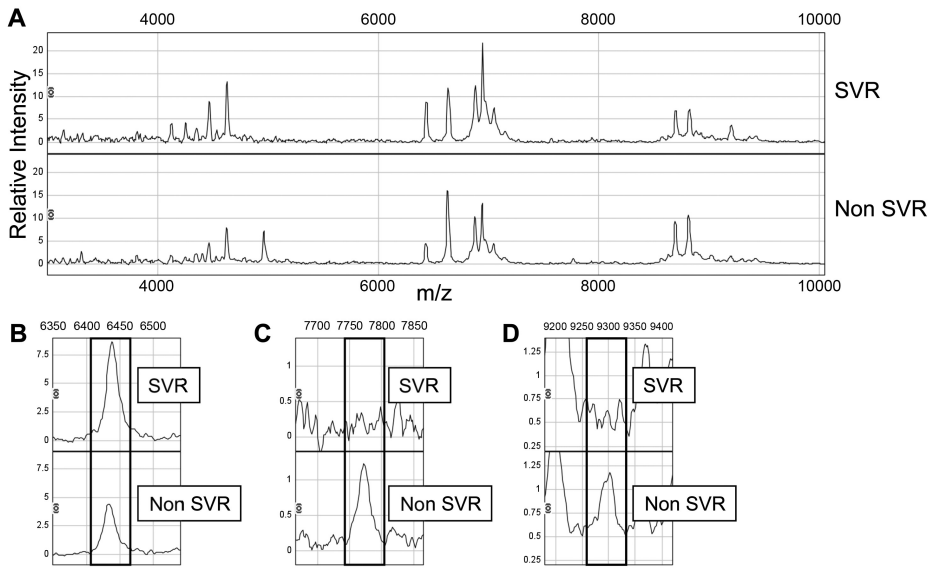


Figure 1. Representative protein profiles of pretreatment sera of SVRs and non-SVRs. Patients with CHC were treated with a 48-wk course of PEG-IFN alpha plus RBV therapy. Protein peak spectrum of pretreatment sera was analyzed by the SELDI-TOF/MS system, and representative protein peaks within 3,000–10,000 *m/z* are shown (A). Statistically significantly different peaks between SVRs and non-SVRs are shown in the enlarged view (6,449 *m/z* peak, identified as Apo C1 fragment (B); 7,765 *m/z* peak, identified as platelet factor-4 fragment (C); 9,304 *m/z* peak, identified as the CTAP III fragment (D)).

analysis was performed including 11 SELDI-TOF/MS-measured variables and two laboratorial serum markers (hyaluronic acid and ferritin) that were

significantly different between SVR and non-SVR groups by univariate analysis. As shown in Table 3, three protein peaks (Apo A1, hemopexin and transferrin) by

SELDI-TOF/MS and serum ferritin levels were independent factors that were associated with SVR for PEG-IFN plus RBV.

ROC Analysis

To determine the usefulness of these protein peak quantifications as predictors for PEG-IFN plus RBV treatment response, ROC analysis was conducted. To increase the performance of the prediction, the most efficient peak combination predictive of treatment response was determined using regression analysis, including the 11 peaks that significantly differed in univariate analysis. The area under the ROC curve for the combination of Apo A1 and hemopexin peak value was 0.964, and hemopexin and transferrin peak value was 0.936, suggesting that the quantification of these variables by SELDI-TOF/MS was useful for the prediction of SVR in CHC patients (Figure 3).

mRNA Levels of Hemopexin in the Liver

Because the above-mentioned results suggest that hemopexin, which is known as an acute-phase glycoprotein and behaves as a heme carrier protein (15), is

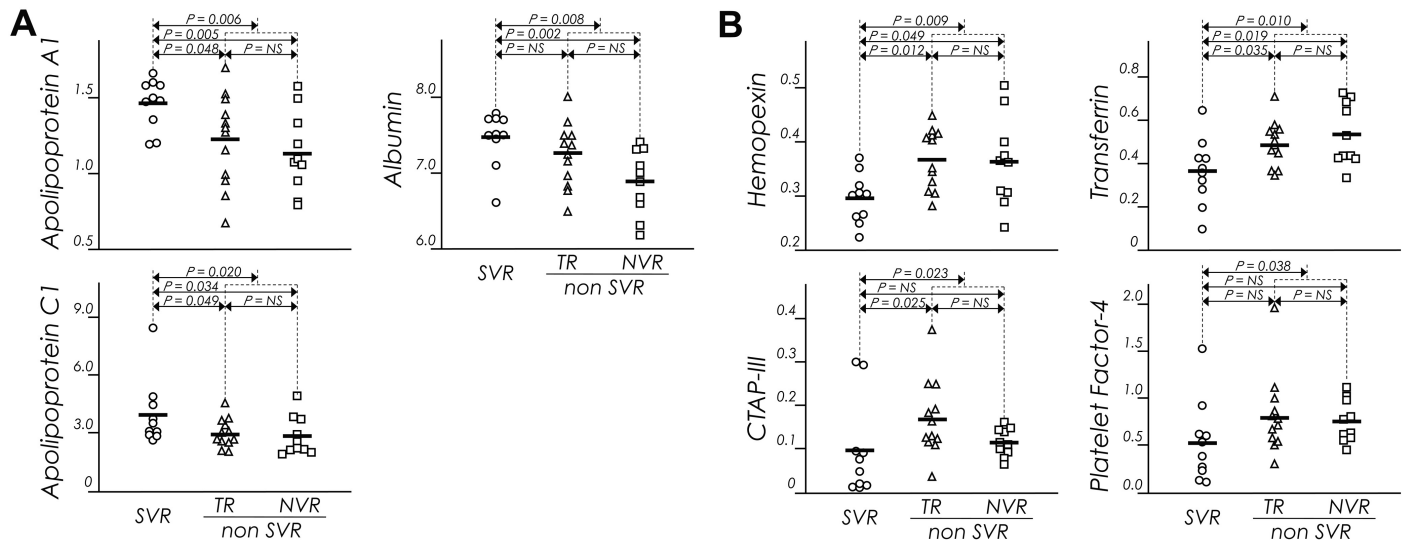


Figure 2. Protein peak comparison between SVRs, TRs and NVRs. CHC patients with genotype 1b were treated with a 48-wk course of PEG-IFN alpha plus RBV. Pretreatment serum samples were analyzed by SELDI-TOF/MS, and statistically different protein peaks were determined. (A) Protein peaks that were significantly higher in SVRs are shown. (B) Protein peaks that were significantly higher in non-SVRs are shown. Horizontal lines show the means.

significantly related to treatment resistance for PEG-IFN plus RBV, and because hemopexin is exclusively produced by hepatocytes (16), we decided to measure the hepatic hemopexin mRNA levels in the liver of patients with CHC. Hemopexin mRNA levels were measurable in all patients, and its levels were significantly correlated with serum hemopexin levels quantitated by the SELDI-TOF/MS system (Figure 4A). Hepatic mRNA levels were significantly higher in non-SVR patients than in SVR patients (Figure 4B). Statistical differences also existed when the non-SVR patients were analyzed separate from the TR and NVR groups.

DISCUSSION

PEG-IFN in combination with RBV is the most effective therapy for CHC (3,4). However, this treatment is not satisfactory, because many patients do not respond or relapse after treatment—especially patients infected with genotype 1b. Considering the length of the treatment period, as well as its high frequently observed side effects and costs, accurate prediction of treatment response before initiation of therapy is critical. Numerous viral and host factors have been reported to play a role in the IFN-based treatment response. For example, HCV genotype (3,4), baseline viral load (17), viral kinetics during treatment (18) and amino acid pattern in the interferon sensitivity-determining region in NS5A (19) and Core (20) have been reported to be significantly associated with treatment outcome in a number of independent studies. Several host factors, including age, sex, race, liver fibrosis, steatosis and iron overload, have also been reported to be associated with treatment response (21,22). Recently, several researchers identified the single-nucleotide polymorphisms (SNPs) located near the gene IL28B on chromosome 19 were associated with the null response for PEG-IFN plus RBV therapy (23,24). While these results are intriguing, these SNPs were not useful for predicting sustained viral clearance, and the measurement of human genomic SNPs requires

Table 3. Multivariate analysis for prediction of SVR in patients with CHC.

Factor	Odds ratio	95% CI	P value
SELDI-TOF/MS assay			
3356 m/z (Apo A1 fragment)	1.321	1.104–2.013	0.003
59459 m/z (Hemopexin)	0.93	0.892–0.963	0.005
79237 m/z (Transferrin)	0.779	0.524–0.904	0.014
Serum ferritin	0.922	0.836–0.956	0.034

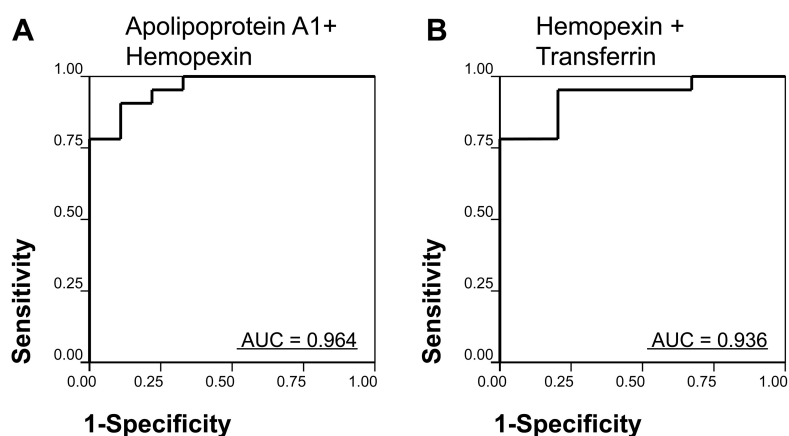


Figure 3. ROC curve for prediction of SVR in patients with CHC treated with a 48-wk course of PEG-IFN alpha plus RBV. ROC curves were generated combined the peak values of Apo A1 and hemopexin (A), and hemopexin and transferrin (B). AUC, area under the curve.

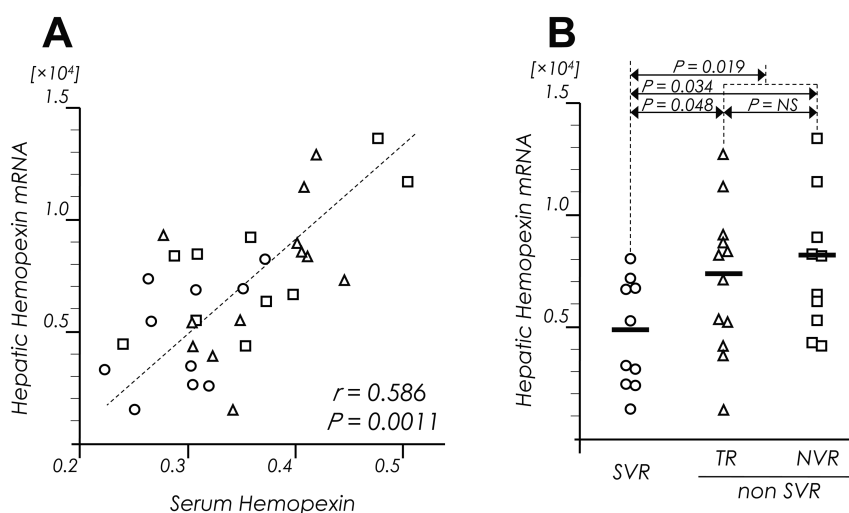


Figure 4. Hepatic mRNA levels of hemopexin in patients with CHC. Hepatic mRNA was extracted from the liver biopsy samples, and hemopexin mRNA levels were quantified by real-time PCR assay. (A) Correlation between hepatic mRNA levels of hemopexin and serum hemopexin levels determined by SELDI-TOF/MS methods. Open circles represent SVRs. Open triangles represent TRs. Open squares represent NVRs. (B) Comparison of hepatic mRNA levels of hemopexin between SVRs, TRs and NVRs.

time and cost. If a reliable, inexpensive and easily measurable predictor for SVR is identified for use in patients before treatment initiation, the clinical benefit is enormous.

The development of proteomic array technology for profiling, in which a ProteinChip Array is coupled with SELDI-TOF/MS, has created a powerful tool for discovering new biomarkers. The great advantages of this method are its speed and high-throughput capacity and the fact that it only requires a small amount of sample. Also, this tool is suitable for the analysis of both proteins and low-molecular-weight peptides (6–8). Comparison of proteome profiles in groups of patients with different phenotypes enables the identification of specific biomarkers (6–11). In this study, we used this ProteinChip system to analyze and compare the pretreatment serum protein profiling of CHC patients who achieved SVRs and non-SVRs (TRs and NVRs) by PEG-IFN plus RBV to define the new biomarker(s) for treatment outcome prediction. We selected the patients who fulfilled the following criteria: (1) infected with HCV genotype 1b and high viral load and (2) received the same complete treatment schedule of PEG-IFN plus RBV at the course of 48 weeks, to make the virological and treatment factors that influence treatment efficacy uniformed. We identified a set of 11 protein peaks in pretreatment serum that had significantly different values when both groups were compared: three were overexpressed in the SVR group and eight were increased in the group of non-SVRs. The three SVR-increased serum proteins were identified as two Apo fragments and albumin, and among eight non-SVR-increased proteins, four proteins were identified as two iron-related (hemopexin and transferrin) and two fibrogenesis-related (CTAP-III and platelet factor-4) protein fragments. As compared with TRs and NVRs, these 11 peak intensities were not significantly different, suggesting that these variables were not useful for null response prediction before the start of PEG-IFN plus RBV.

To determine the usefulness of these protein peak quantifications as predictors, we performed ROC curve analysis by using these 11 peak intensities. The area under the ROC curve for the combination of most significantly SVR-elevated protein peak (Apo A1) and most non-SVR-elevated protein peak (hemopexin) was 0.964, suggesting that the quantification of these variables by SELDI-TOF/MS was useful for the prediction of SVR. Multivariate analysis performed by using 11 significantly different protein peaks and two laboratorial serum markers (hyaluronic acid and ferritin) between SVR and non-SVR groups showed the usefulness of three protein peaks (Apo A1, hemopexin and transferrin) by SELDI-TOF/MS and serum ferritin levels for SVR prediction.

The most striking evidence of our study is that the significantly different protein peaks of SELDI-TOF/MS between SVR and non-SVR groups were Apo fragments of A1 and C1. It was reported that HCV biology is closely linked to serum apolipoprotein components, which act at several steps of viral replication, including assembly, cell entry and protection from the host immune response (25,26). Voisset *et al.* (27) reported that the HDL particles, that are mainly constructed by Apo A1, stimulate cell entry of HCV. Another significantly different Apo fragment between SVR and non-SVR groups, Apo C1, was also reported to affect the HCV replication by specifically recruiting HCV glycoproteins on the viral surface and intrinsically increasing the fusogenicity of HCV particles (28). It was also reported that high serum Apo A1 levels induced the disruption of the HCV particles and loss of infectivity (29). Although the precise mechanism underlying the association of serum Apo components and PEG-IFN and RBV treatment response in CHC is not well defined, serum Apo components may play a central role in treatment response by modulating the HCV infectivity to hepatocytes, which may affect the HCV dynamics after the start of PEG-IFN plus RBV. Further study is necessary.

Recently, the role of iron has been pointed out as an important element affecting the natural history of chronic HCV infection. Elevated iron-related serum markers and increased hepatic iron deposition are relatively common and correlate with severity of hepatic inflammation and fibrosis in patients with CHC. Elevations of serum transferrin saturation and ferritin level are reported to be present in 18% to 46% of HCV-infected cases and mild-to-moderate hepatic iron deposition is in 10% to 56% (22,30,31). Iron overload seems to be associated with a poor response to IFN therapy (22,32,33), and removal of excess iron by repeated phlebotomy in combination with IFN may be of therapeutic benefit for patients with CHC (34,35), although contradicting results have been reported (36,37). In this study, two iron-related protein peaks (hemopexin and transferrin) and serum ferritin levels were significantly elevated in the non-SVR group. Moreover, multivariate analysis demonstrated that these three variables were independently related factors to PEG-IFN and RBV treatment response. Hemopexin is an acute-phase serum glycoprotein with a high heme binding affinity (15). Hemopexin is mainly produced by the liver and released into the circulation, where it binds heme and delivers it to the liver (15,16). Thus, hemopexin provides protection against free heme-mediated oxidative stress (38). Consistently, hemopexin-null mice recover less efficiently than wild-type controls after acute hemolysis and suffer from severe renal damage due to iron overload and oxidative injury (39). Moreover, compound mutant mice for hemopexin showed marked liver inflammation and fibrosis after hemolytic stress (40). In patients with CHC, it was also reported that elevated oxidative stress markers are frequently observed in serum and liver (41,42). We also reported that hepatic 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidatively generated DNA damage, was significantly correlated with iron overload conditions

(serum ferritin levels and hepatic iron score) in chronic HCV patients (43). Considering this background, our results (i.e., increased hemopexin, transferrin and ferritin in the non-SVR group) may suggest that iron overload in CHC involves PEG-IFN plus RBV treatment resistance via oxidative stress formation. We evaluated hepatic mRNA expression of hemopexin by using real-time PCR of liver biopsy samples and confirmed the significant positive correlation between hepatic mRNA levels and its serum peak values by SELDI-TOF/MS. Hemopexin was significantly higher in non-SVR patients than in SVR patients at the mRNA levels of liver samples.

Although the 11 peak intensities of serum proteins were significantly different between SVR and non-SVR, the measured ranges overlapped each other (Figure 2). These results may be because most serum proteins (apolipoproteins, albumin and transferrin) are indispensable for human life, and their serum levels are regulated to be relatively constant.

In conclusion, comparison of pretreatment SELDI-TOF/MS protein profiling among different treatment responses for PEG-IFN plus RBV is useful for identification of predictive serum markers for treatment response in patients with CHC. The data from these assays can provide valuable information that may influence the decision about the treatment strategy in each individual patient. Finally, this clinical human study demonstrates that the apolipoproteins and iron-related molecules are the host factors influencing sustained viral clearance in CHC patients. Our data will help us to understand the mechanism for treatment resistance and to develop more effective antiviral therapy targeted toward the modulation of lipogenesis or iron homeostasis in chronic HCV infection.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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