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LIVER CANCER

New multi protein patterns differentiate liver fibrosis stages and hepatocellular carcinoma in chronic hepatitis C serum samples

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Abstract

AIM: To identify a multi serum protein pattern as well as single protein markers using surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF-MS) for detection and differentiation of liver fibrosis (F1-F2), liver cirrhosis (F4) and hepatocellular carcinoma (HCC) in patients with chronic hepatitis C virus (HCV).

METHODS: Serum samples of 39 patients with F1/F2 fibrosis, 44 patients with F4 fibrosis, 34 patients with HCC were applied to CM10 arrays and analyzed using the SELDI-TOF ProteinChip System (PBS-Ⅱc; Ciphergen Biosystems) after anion-exchange fractionation. All patients had chronic hepatitis C and histologically confirmed fibrosis stage/HCC. Data were analyzed for protein patterns by multivariate statistical techniques and artificial neural networks.

RESULTS: A 4 peptide/protein multimarker panel (7486, 12843, 44293 and 53598 Da) correctly identified HCCs with a sensitivity of 100% and specificity of 85% in a two way-comparison of HCV-cirrhosis versus HCV-HCC training samples (AUROC 0.943). Sensitivity and specificity for identification of HCC were 68% and 80% for random test samples. Cirrhotic patients could be discriminated against patients with F1 or F2 fibrosis using a 5 peptide/protein multimarker pattern (2873, 6646, 7775, 10525 and 67867 Da) with a specificity of 100% and a sensitivity of 85% in training samples (AUROC 0.976) and a sensitivity and specificity of 80% and 67% for random test samples. Combination of the biomarker classifiers with APRI score and alfa-fetopotein (AFP) improved the diagnostic performance. The 6646 Da marker protein for liver fibrosis was identified as apolipoprotein C-Ⅰ.

CONCLUSION: SELDI-TOF-MS technology combined with protein pattern analysis seems a valuable approach for the identification of liver cirrhosis and hepatocellular carcinoma in patients with chronic hepatitis C. Most probably a combination of different serum markers will help to identify liver cirrhosis and early-stage hepatocellular carcinomas in the future.

Key words: Hepatocellular carcinoma; Hepatitis C virus; Apolipoprotein C-Ⅰ; Proteomics; Surface-enhanced laser desorption/ionisation

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and ranks fifth in frequency of all malignancies in the world[1]. In the last decades incidence and mortality rates of HCC have increased significantly in Western countries[2] with hepatitis C virus (HCV) infection being the most important cause[3]. Liver cirrhosis is the most common complication of chronic HCV infection. Once cirrhosis is established, the risk of developing a HCC is 1%-5% a year[4]. In order to reduce HCC mortality, early identification of liver cirrhosis as well as early detection of hepatocellular carcinoma is needed.

However, prediction of liver cirrhosis and small HCC is often difficult in chronic hepatitis C. Several noninvasive tests like transient elastography (FibroScan®, Echosens, Paris, France), Fibrotest™ (Biopredictive, Paris, France; a serologic marker-based algorithm) and the APRI score (relation of AST to thrombocytes)[5] have been set up for discrimination of liver cirrhosis from lower fibrosis stages. In contrast to liver biopsy, these techniques are noninvasive, simple to use but either need further

evaluation or display insufficient predictive value [6].

Screening for HCC is generally recommended in patients with HCV cirrhosis[7]. Determination of alfa-fetopotein (AFP) and ultrasound every 3 to 6 mo has been proposed for early detection of HCC[7]. However, up to 44% of patients with HCC show normal levels of AFP, particularly during early stages[8]. Elevated serum levels of AFP may also be seen in patients with liver cirrhosis, acute and chronic hepatitis[9]. Low sensitivities of 39%-65% and positive predictive values of 9%-50% limit the use of AFP as a single marker for a cut off of 20 ng/mL[10]. Combination of AFP (cut-off level of 20 μg/L) with abdominal ultrasound improves sensitivity and specificity, but results of ultrasound scanning are influenced by the experience of the operator and the quality of the used equipment. Detection of HCC becomes even more difficult in cirrhotic livers since regenerative nodules may mimic tumors. Sensitivities of ultrasound as a screening tool for the detection of HCC vary from 50% up to 78%[11]. Contrast-enhanced ultrasound may improve sensitivity up to 89%, but specificity still remains rather low[12].

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) is one technology for serum protein profiling and identification of biomarkers. This method uses different chromatographic chip surfaces for binding peptides/proteins of biological samples (i.e. serum, urine, cerebrospinal fluid) and has lead to identification of novel biomarkers in prostate, bladder, ovarian, colorectal, or liver cancer[13-17].

The aim of the study was to identify serum protein patterns and single protein markers by SELDI-TOF mass spectrometry in patients with HCV cirrhosis and HCV associated hepatocellular carcinoma.

MATERIALS AND METHODS

Samples

A total number of 117 serum samples were collected from patients with chronic hepatitis C between 2001 and 2004. Three groups were enrolled: (1) patients with low grade fibrosis (F1/2, n = 39), (2) patients with cirrhosis but without hepatocellular carcinoma (n = 44) and (3) patients with cirrhosis and HCC (n = 34). All patients were anti-HCV and HCV RNA (bDNA Assay 3.0, Bayer, Leverkusen) positive. Patients with other liver diseases, HIV co-infections, other malignomas or antiviral treatment were excluded. Liver biopsy was available in all patients. Clinical data of the patients are shown in Table 1. Classification of the fibrosis stage was done according to Scheuer *et al*[18] . Blood samples were stored at -80°. No sample had been thawed more than once.

Protein profiling

Anion-exchange fractionation was used for serum preprocessing, to increase the number of protein peaks. To separate the serum samples into six different fractions (pH9 + flowthrough, pH7, pH5, pH4, pH3, organic elution), an Expression Difference Mapping Kit (Ciphergen Biosystems, Freemont, USA.) was used. Proteins were separated on the basis of their pI-values. Sample preparation and fractionation was done as described in the manufacturer's protocol. Fractions were stored at 4℃ overnight and analyzed the following day. Protein profiling was done using SELDI-TOF mass spectrometry (Ciphergen Biosystems). After testing several chromatographic chip surfaces and binding conditions the CM10 array were chosen for further experiments as they gave the highest number of discriminative peptide/protein peaks. The best results in terms of number of protein peaks and possibility to separate the three groups was found in fraction 1 (flow through + pH9).

Table 1 Clinical data of patients

	Fibrosis F1/F2 (n = 39)	Cirrhosis F4 (n = 44)	HCC (n = 34)
Age	44 ± 11	62 ± 8	67 ± 8
Gender (male/female)	29/10	22/22	25/9
ALT	57 ± 100	62 ± 43	55 ± 47
AST	28 ± 30	46 ± 30	52 ± 34
Bilirubin (mg/dL)	0.8 ± 0.5	1.2 ± 1.0	2.0 ± 4.0
Quick (%)	105 ± 7	85 ± 16	85 ± 17
AFP (μg/mL)[1]	3.4	6.2	39.95
Platelets (1000/μL)	214 ± 61	131 ± 62	145 ± 73
Albumin (g/dL)	4.5 ± 0.3	3.9 ± 0.6	3.5 ± 0.6
Child Pugh (A/B/C) (%)	NA	35/7/2 80/16/4	20/11/3 59/32/9
Okuda	NA	NA	18/2/4

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AFP: Alfa-fetoprotein; NA: Not available. [1]Median values are given.

The CM10 arrays were equilibrated twice with 150 μL of low stringency binding buffer (0.1 mol/L sodium acetate; pH4.0; Ciphergen Biosystems). Ten microliters of fraction 1 and 90 μL of binding buffer were applied to each spot of the ProteinChip Array and incubated for 30 min at room temperature. Spots were washed three times with binding buffer and rinsed twice with de-ionized water. Arrays were allowed to air dry and 1 μL of sinapinic acid in 50% acetonitrile (ACN) and 0.5% trifluoroacetic acid (TFA) were applied twice to each spot. Afterwards the protein chip arrays were analyzed using the SELDI ProteinChip Reader (PBS-Ⅱc; Ciphergen Biosystems).

Purification and identification of candidate biomarker

Anion Exchange Fractionation Selected sera (100 μL) containing biomarkers of interest were further fractionated with Q HyperD® F spin columns (Ciphergen Biosystems, Inc.). Each fraction was collected and analyzed again on a CM10 array with low stringency buffer. The candidate biomarkers were seen in the flow through and pH3 fraction. Both fractions were purified first with hydrophobic chromatography resin (BioSepra Q HyperD® F resins (BioSepra, Sergy Saint Christophe, France) then with RPC Poly-Bio beads (BioSepra).

Hydrophobic chromatography

Fifty microliters of RPC Poly-Bio beads (BioSepra) were equilibrated with 500 μL of 10% ACN/0.1% TFA. The full sample fraction was adjusted to a final concentration of 10% ACN/0.5% TFA and mixed with 50 μL of the

bead-material for 30 min at room temperature. Afterwards, the probe was centrifuged for 1 min at 5000 r/min. The supernatant was removed. Increasing concentrations of ACN (10%-60%) in 0.1% TFA were added in each step, mixed for 5 min and centrifuged for 1 min at 5000 rpm. Proteins in the eluted fractions were detected by profiling 1 μL of each fraction combined with 1 μL of SPA on a NP20 array.

Gel electrophoresis and passive elution

Fractions containing the candidate biomarkers eluted from the hydrophobic bead material were completely dried in a Speed-Vac, resolubilised in SDS-PAGE sample buffer and loaded onto different SDS-PAGE gels (Invitrogen, Carlsbad, USA). An 18% Tris-Glycine gel was used containing TrisGly SDS running buffer for the 6.6 kDa-marker. The gel was fixed and stained using Invitrogen Staining NuPage® Novex protocols. Bands of interest were excised from the gels and placed in tubes. Destaining was achieved by addition of 150 μL 50% ACN/50 mmol/L ammonium bicarbonate (3 times, 5 min at room temperature) and 200 μL of 50% ACN/100 mmol/L ammonium bicarbonate once. The gel pieces were then covered with 100 μL of 100% ACN for 10 min and dried in a Speed Vac.

For passive elution 100 μL of 45% formic acid, 30% ACN and 10% isopropanol was added. The tubes were sonicated for 30 min in a water bath at room temperature and incubated at room temperature for 4 h. One microliter of each sample was analysed on a NP20 array. Remainder of passive elution was incubated overnight and sonicated the next morning. Each supernatant was transferred to a new tube and dried in a Speed-Vac.

Sequencing grade modified porcine trypsin (Promega, Charbonnières-les-bains, France) with a concentration of 20 ng/μL in 25 mmol/L ammonium bicarbonate was added to each gel piece and incubated at 37℃ for 4 h. One microliter of the peptide digests was analysed on a NP20 array adding 1 μL of 20% CHCA (alpha-cyano-4-hydroxy-cinnamic acid) in 50% ACN/0.5% TFA. External calibration was performed using the All-In-One-Peptide Standard (Ciphergen Biosystems, Inc.). Peptides of the resulting tryptic digest were submitted to a database search with the Mascot search engine (http://www.matrixscience.com) using the Swiss-Prot and NCBI databases.

Additionally sequencing of the most important peptides in the tryptic digest was done using a Micromass Q quattro 2Q-TOF tandem quadruple TOF mass spectrometer equipped with a SELDI-TOF MS ProteinChip Interface PCI1000. The MS/MS data were exported as Sequest files and investigated with the Mascot search engine. Determination of Apo C- Ⅰ was furthermore confirmed by an immunoassay on protein A beads (Biosepra) using a specific rabbit anti-human apolipoprotein C- Ⅰ antibody (Academy Bio-Medical Company, Cambridge, UK) and a non-specific rabbit IgG control antibody. After loading of the beads with the antibody, subsequent washing with PBS (2 ×), followed by an incubation for 1 h with the flow through fraction of one serum sample, the beads were washed again with PBS (3 ×) and deionized water (1 ×). Finally, the captured proteins were eluted with 15 μL 100 mmol/L acetic acid and profiled on NP20 ProteinChip Arrays.

Statistical analysis

Data were analyzed using the ProteinChip Software package version 3.1 (Ciphergen Biosystems). For acquiring best results, two different protocols were established. The optimization range was set between 2.5 and 50 kDa for the first, 10 and 80 kDa for the second protocol. Mass spectra were generated using laser intensities of 167 and 190, detector sensitivities of 6 and 9, respectively. A total of 130 laser shots for each spot were collected. The protein masses were calibrated externally using the All-In-One Protein Standard (Ciphergen Biosystems.). All mass spectra were normalized to total ion current (TIC normalization), baseline was subtracted.

Protein peaks of all sample spectra were clustered with the Ciphergen Express software, version 3.0.1. The following clustering settings were used: auto-detect peaks to cluster; first pass 5.0 S/N (signal to noise) 3.0 valley depth; minimal peak threshold: 20% of all spectra. Cluster mass window: 0.2 peak width; second pass: 3.0 S/N; 3.0 valley depth; add estimated peaks to complete cluster; mass to charge ratio (M/z) range from 2.5 to 75 kDa. This cluster list was used to perform a decision tree classification with the Biomarker Patterns software, version 5.0.2 (Ciphergen Biosystems.). Initially, a training set of all serum samples was used for generating a decision tree model. The sample set was in addition taken for internal cross-validation (test set). The classification tree split the data into two nodes using one rule at a time in the form of peak intensity. The splitting decisions in this case were based on the normalized intensity levels of peaks from SELDI protein expression profiles. The process of splitting was continued until terminal nodes were created.

RESULTS

Biomarker pattern

The comparison between low fibrotic and cirrhotic patients revealed 22 significant peaks. For the differentiation of cirrhotic and HCC-patients 17 significant protein peaks with *P*-values less than 0.05 were found.

For differentiation between low fibrosis and cirrhosis a decision tree using 5 biomarkers with mass values of 2873, 6646, 7775, 10525 and 67867 Da was established (Figure 1). The algorithm correctly assigned 33 of 39 fibrotic (85%) and 44 of 44 (100%) cirrhotic patients in the training set. The ROC analysis gave an AUROC of 0.976. The algorithm for discrimination of cirrhosis versus low fibrosis based on the test data gave a sensitivity of 80%, a specificity of 67%, a positive predictive value (ppv) of 73% and a negative predictive value (npv) of 74% for liver cirrhosis. APRI score (cut-off 1.5) allowed determination of cirrhosis with a sensitivity of 75%, a specificity of 87%, a ppv of 86% and a npv of 76%. The combination of APRI-score with three biomarkers (2873, 6646 and 10525 kDa) resulted in a sensitivity of 93%, a specificity of 95%, a ppv of 95% and a npv of 93%. AUROC increased to 0.955 for the combination of APRI and biomarkers

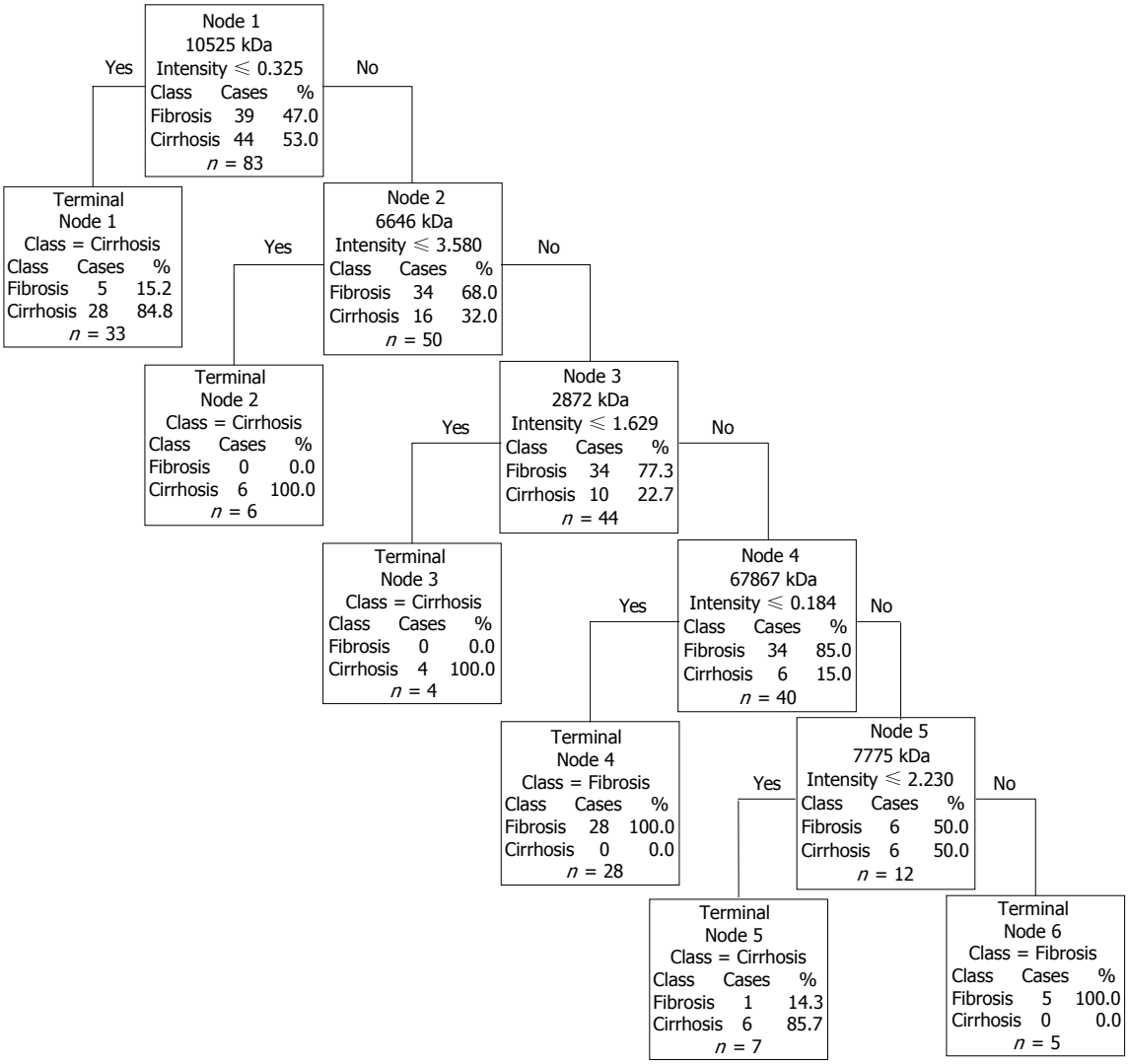


Figure 1 Decision tree for the differentiation of fibrosis versus cirrhosis**.** The root nodes contain the mass of the selected peak ("node") which is followed by the intensity value. Samples with intensities lower or equal to the intensity value go to the left terminal node, samples with higher intensities go to the next right descendant nodes.

compared APRI alone with an AUROC of 0.811.

Cirrhotic patients could be differentiated from HCC patients by creating a decision tree with 4 biomarkers with mean mass values of 7486, 12843, 44293 and 53598 Da (Figure 2). The training set allowed a correct classification of HCC in 34 of 34 (100%) and cirrhosis in 37 of 44 (84%) patients. The AUROC in the ROC plot was 0.943. The algorithm for discrimination of HCC versus cirrhosis in the test set revealed a sensitivity of 68% for HCC, a specificity of 80%, a ppv of 72% and a npv of 76%. AFP alone (cut-off 18 μg/L) achieved a sensitivity of 76%, a specificity of 82%, ppv of 76% and a npv of 82%. The AUROC was 0.791. Combination of AFP and the 12843 Da biomarker resulted in a sensitivity of 88%, a specificity of 82%, a ppv of 78% and a npv of 90%. The AUROC mounted to 0.861.

Identification of novel biomarkers

A biomarker of 6.6 kDa, used in the first decision tree, could be further characterized. Representative spectra views of the chosen protein are shown in Figure 3. SELDI analysis results of one sample expressing the 6.6 kDa marker after fractionation, purification and overnight passive elution as well as the separation within the stained gel are shown in Figure 4. The peptide mass fingerprinting of the 6.6 kDa biomarker after trypsin digestion is shown in Figure 5. By database search the tryptic digested 6.6 kDa protein was identified as apolipoprotein C-Ⅰ. Apolipoprotein C-Ⅰ was down-regulated in patients with liver cirrhosis (Figure 3) compared to patients with lower stages of liver fibrosis. Confirmation of the apolipoprotein C-Ⅰ identity was performed by an immunoassay using a rabbit apolipoprotein C-Ⅰ polyclonal antibody (Figure 6). The AUROC of apolipoprotein C-Ⅰ for differentiation of fibrosis from cirrhosis was 0.68.

DISCUSSION

Despite an increasing number of noninvasive tests and imaging techniques, detection of liver cirrhosis and hepatocellular cancer is often difficult in chronic hepatitis C patients. The present study therefore aimed at the identification of serum protein patterns and single protein markers by SELDI-TOF mass spectrometry to predict liver cirrhosis and hepatocellular carcinoma.

Using a weak cationic array (CM10) a 5 biomarker

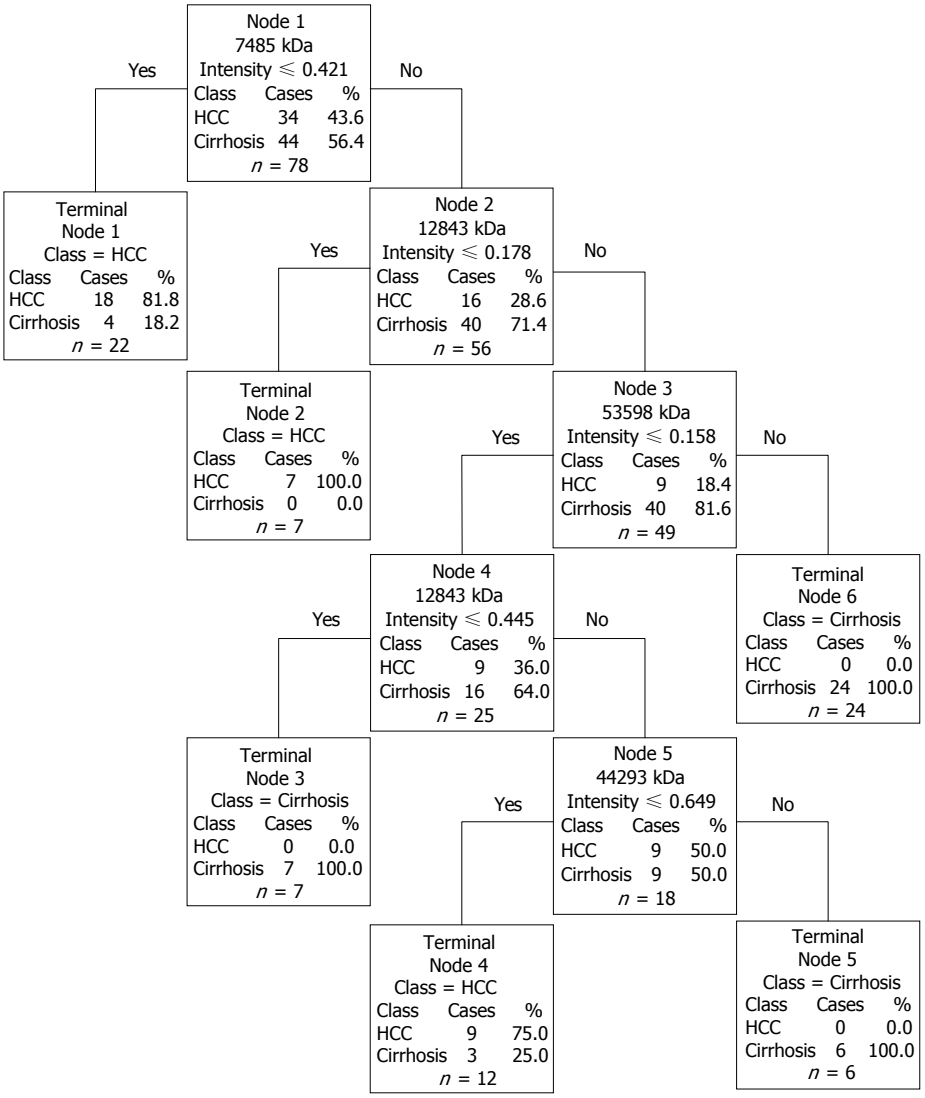


Figure 2 Decision tree for the differentiation of HCC versus cirrhosis.

pattern was identified, that allowed discrimination of liver cirrhosis from low fibrosis stages with a sensitivity of 80%, a specificity of 67% and a positive predictive value of 73%. APRI score in patients with hepatitis C gave, depending on the chosen cut-off, sensitivities of 57%-89%, specificities of 75%-93% and a ppv of 38%-57% for the identification of patients with cirrhosis compared to non-cirrhotics[5]. FibroTest is another non-invasive test, using a mathematical algorithm of 5 parameters (α2-macroglobulin, haptoglobin, γ-glutamyl transpeptidase, apolipoprotein A-Ⅰ, total bilirubin), to predict liver cirrhosis. Sensitivities and specificities vary significantly dependent on the chosen cut-off[19]. Fibroscan®, which determines liver stiffness by transient elastography, achieves a sensitivity of 87%, a specificity of 91% and a ppv of 77% using 12.5 kPa as the cutoff[6]. Correct prediction of an underlying HCC was possible from the same sample preparation using a 4 biomarker pattern with a sensitivity of 68%, a specificity of 80% and a ppv of 72% in patients with proven liver cirrhosis. Alfa-fetoprotein, which is the most widely used serum marker for diagnosis and surveillance of HCC, achieves sensitivities of 39%-65% and ppv of 9%-50%[7,10,20] according to literature but performed better in the present data set. Although proteomic patterns might not be superior to the mentioned noninvasive tests, they carry the advantage of being operator independent (compared to transient elastography) and allow both the discrimination of liver cirrhosis and hepatocellular carcinoma with one sample preparation.

Previous studies using SELDI-TOF mass spectrometry including only patients with hepatitis C liver cirrhosis have provided evidence that proteomic pattern can be used to discriminate cirrhosis from HCC yielding sensitivities of 85% to 94% and specificities of 86% to 91%[17,21]. The differences to the present study may be explained by the use of a different protein array (CM10 *vs* IMAC) and data analysis with different neuronal networks. A relative complex neuronal network was elaborated by Ward *et al*[21], using a majority vote of six committee models, which included between 4 and 17 protein peaks. Interpretation of the present results with the results of other studies testing proteomic patterns in patients with HCC were hampered by the fact that different HCC etiologies[22] or non-relevant controls like healthy volunteers were included in the other studies[23].

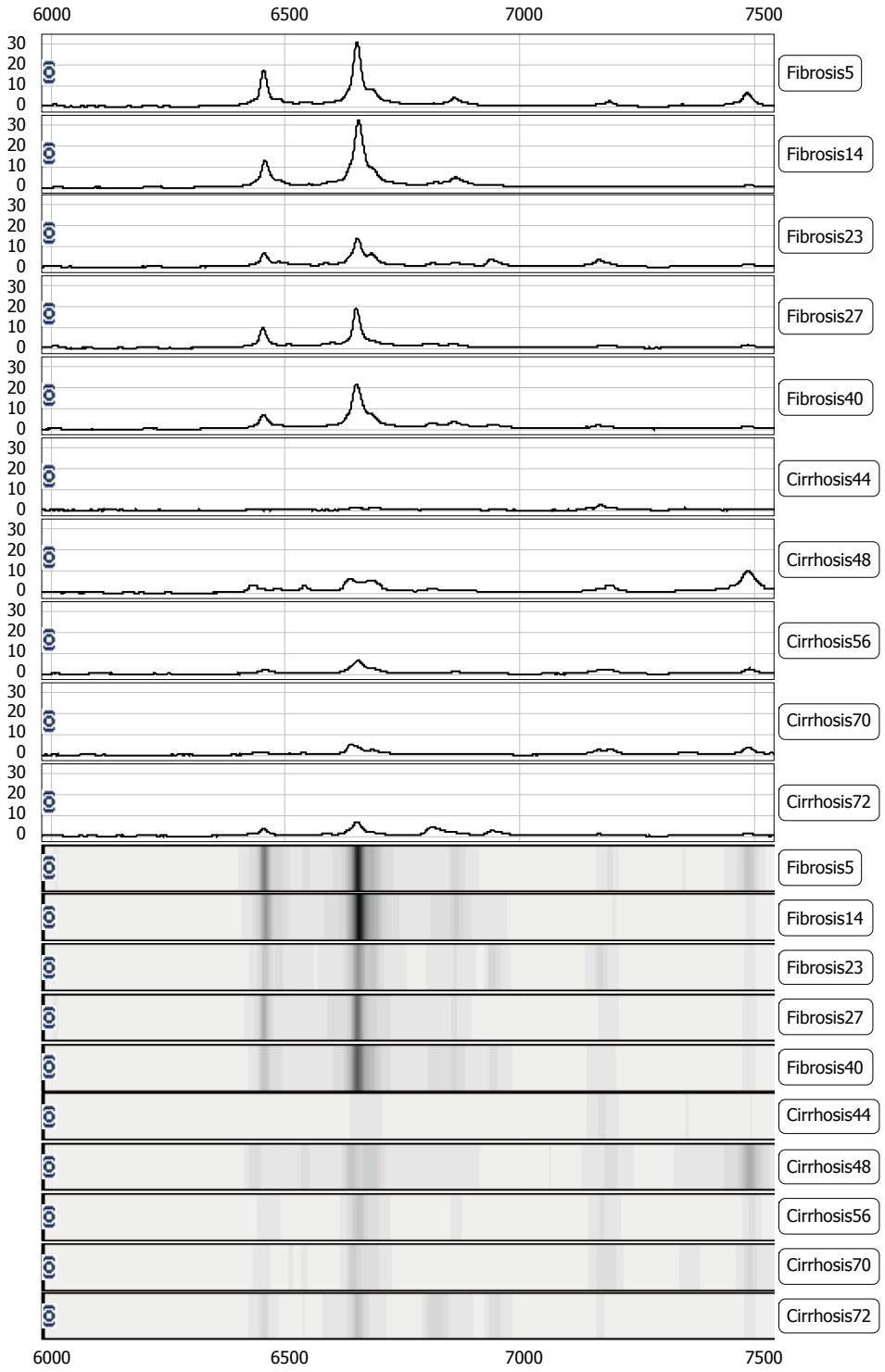


Figure 3 Representative protein spectra of the 6.6 kDa peak and gel view (below) for the differentiation of fibrosis versus cirrhosis patients. Numbering of patients is according to internally used data.

Apolipoprotein C-Ⅰ was identified as a marker for differentiation of liver fibrosis and cirrhosis. A role for apolipoproteins in liver fibrosis has been recognized earlier. Apolipoprotein A-Ⅰ is one of the parameters used for prediction of liver fibrosis within the Fibrotest-algorithm, in addition an index of prothrombin time γ-glutamyl transpeptidase and apolipoprotein A-Ⅰ has been proposed for identification of severe alcoholic liver disease[24]. However, apolipoproteins have not only been identified as a serum discriminator of fibrosis but also as a marker in different types of cancer[25]. Apolipoprotein C-Ⅰ down-regulation was detected to reliably distinguish colorectal cancer patients from healthy controls[26].

Apolipoprotein C-Ⅰ is primarily synthesized in the liver and only to a lesser degree in the small intestine. It is originally formed as a pro-peptide of 9.3 kDa which generates the mature protein upon cleavage during translation[27]. The observed decrease in serum levels of apolipoprotein C-Ⅰ in cirrhosis and HCC might be due to decreased synthesis rate or due to degradation by activated

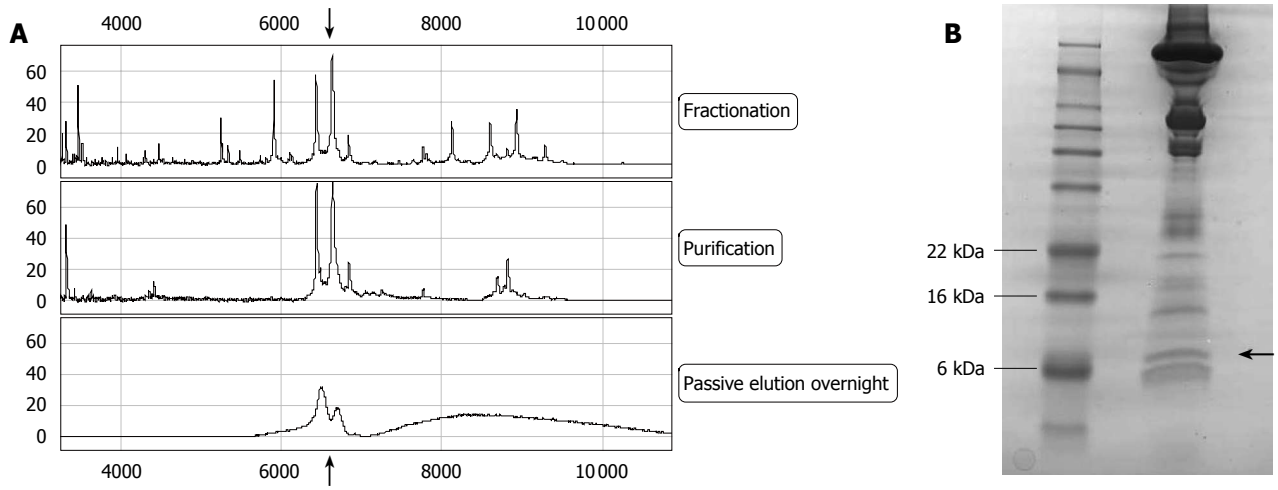
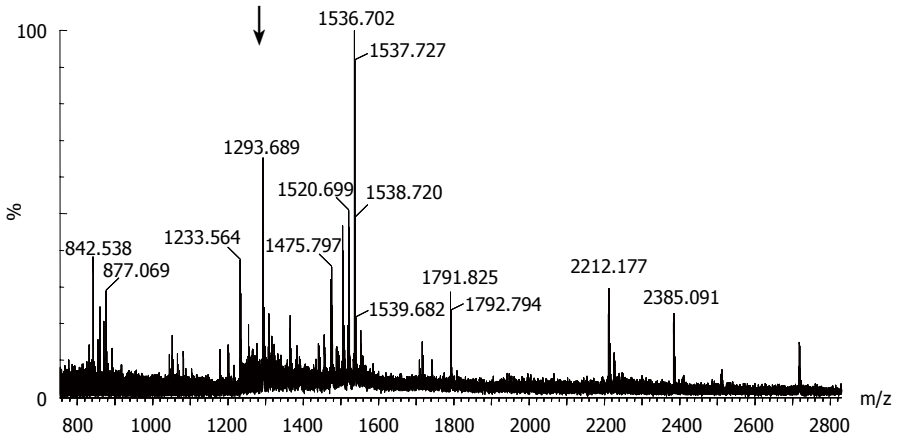


Figure 4 Isolation of the 6.6 kDa peak. First line shows spectrum after fractionation of the sample with Q Ceramic HyperD®F spin columns, the second line shows the spectra after purification using reverse phase chromatography. The last line spectra represent the purified biomarker on a NP20 array after SDS-gel purification and passive elution. The arrow indicates the 6.6 kDa peak (**A**); One-dimensional SDS polyacrylamide gel electrophoresis after staining of enriched sample (second lane). First lane: molecular marker proteins (See Blue® Plus2, Invitrogen, Carlsbad, USA). The arrow indicates the 6.6 kDa protein (**B**).



Mascot mapping result of marked peptide: Apolipoprotein C- I

Start-End	Observed	Mr (expct)	Mr (calc)	Delta	Score	Sequence
37-47	1293.68	1292.67	1292.66	0.01	86	LKEFGNTLEDK

Figure 5 Peptide mass fingerprint spectra of the 6.6 kDa biomarker after trypsin digestion using a Micomass Q quattro 2Q-TOF tandem quadruple TOF mass spectrometer equipped with a SELDI-TOF MS ProteinChip Interface PCI1000. The arrow indicates the peptide with the highest score in the database search. Mascot search results of the same peptide are shown below including start and end position of the found peptide sequence, the observed, experimental and calculated mass from the matched peptide sequence, the mass difference (delta), achieved ion score and the peptide sequence.

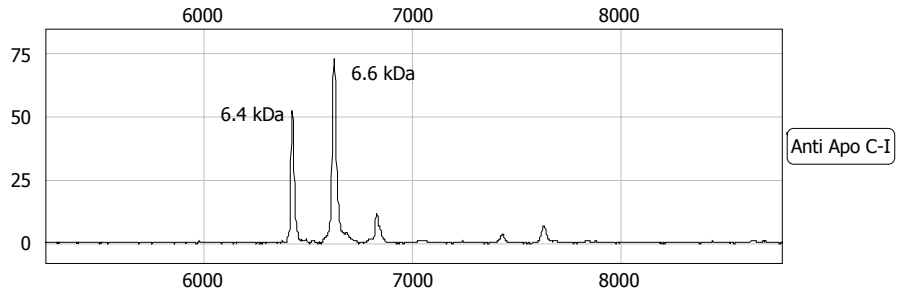


Figure 6 MS-spectrum of the immunoassay capture using a rabbit anti-human apolipoprotein C-I antibody on a NP20 array. Same peaks as seen in the passive elution are shown. The 6.6 kDa represents Apo C-I, the 6.4 kDa peak is Apolipoprotein C-I missing two N-terminal amino acids.

proteases. However, it has been previously demonstrated that multiple cellular genes involved in lipid metabolism are differentially regulated in chronic hepatitis C[28]. There is considerable evidence that cholesterol and fatty-acid-biosynthesis pathways play a role in HCV replication and infection. Liver steatosis is a typical feature of HCV infection[29], HCV core and NS5A proteins associated with lipid droplets and apolipoproteins A-Ⅰ and A-Ⅱ[30,31]. Interference with lipoprotein metabolism by the use of a HMG-CoA inhibitor pravasin, has been shown to improve survival in patients with hepatitis C associated hepatocellular carcinoma[32] and lovastatin inhibited HCV replication in the replicon model[33]. Thus, besides the function of apolipoprotein C-Ⅰ in lipid metabolism[34] an additional pathogenic role in liver fibrosis and cancerogenesis appears possible.

A number of other protein markers have been identified by mass spectrometry or 2D-gel electrophoresis

in HCV associated hepatocellular carcinoma. Among these are ferritin light chain[35-37], vitronectin[17], apolipoprotein E, chloride intracellular channel 1[37], liver aldolase, tropomyosin β-chain, ketohexokinase, enoyl-CoA hydratase, albumin, smoothelin, arginase-1[36], complement C3a[38] and brain derived neurotrophic factor (BDNF)[39]. Differences to the present study might result from the available samples (e.g. serum *vs* tissue), sample preparation (e.g. fractionation), applied methods (e.g. electrophoresis *vs* SELDI-TOF MS) and patient or control characteristics.

The present study indicates that SELDI-TOF MS is a suitable technique for identification of serum markers in HCV associated liver cirrhosis and hepatocellular carcinoma. Apolipoprotein C- Ⅰ appears to be a valuable marker, however larger studies will be needed to define exactly the role of the biomarker patterns and the single protein markers. Most probably a combination of different serum markers will help to identify liver cirrhosis and early-stage hepatocellular carcinomas in the future.

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