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Potential proteomic biomarkers in assessing liver fibrosis using SELDI-TOF MS

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Background/aims: *The accurate assessment of the severity of liver fibrosis is of paramount importance in determining treatment strategies, response to treatment and prognosis in patients with chronic liver disease. The aim of this study was to investigate potential proteomic biomarkers for assessing stages of hepatic fibrosis.* ***Methods:*** *Serum samples of 83 patients with chronic liver disease (using METAVIR index, 17 F0, 30 F1, 6 F2, 9 F3, and 21 F4 patients) and 29 healthy controls were analyzed using surface-enhanced laser desorption/ionization time-of- flight mass spectrometry on IMAC30 ProteinChip arrays. Discriminatory peaks between groups were identified using Mann–Whitney U non-parametric test. Comparison of mild (F0, F1) and severe fibrosis (F2, F3, F4) was performed using tree classification (cross-validation) with the Biomarker Patterns Software, version 5.0 (Ciphergen Biosystems, US).* ***Results:*** *No statistically significant discriminatory peak was evident between F0, F1 and F2 fibrosis. More than 30 peaks were found to be discriminatory between patients with cirrhosis (F4) and all other stages of liver fibrosis, including F2 and F3. Six surface-enhanced laser desorption/ionization proteomic features were found to be discriminative for mild (F0, F1) vs. advanced (F2, F3, F4) fibrosis (AUROC ≥0.8, p<0.05, Mann-Whitney test). The decision tree (m/z 4280, 10453 and 6376) yielded a sensitivity of 83.3% (30/36), a specificity of 85.1% (40/47), a positive predictive value of 81.1%, and a negative predictive value of 86.9%, with an AUROC of 0.94.* ***Conclusions:*** *The results of this study revealed discriminatory peaks between the protein profiles of patients with cirrhosis and other stages of liver fibrosis. Potential proteomic biomarkers can be notably determined for discriminating mild and advanced fibrosis using surface-enhanced laser desorption/ionization time-of- flight mass spectrometry.*

Key words: Biomarker, liver fibrosis, proteomics, SELDI-TOF MS

Karaciğer fibrozisinin değerlendirilmesinde SELDI-TOF MS ile potansiyel biyolojik belirteçlerin araştırılması

Amaç: *Kronik karaciğer hastalığı olan olgularda karaciğer dokusundaki fibrozis varlığının ve miktarının değerlendirilmesi; tedavi stratejisini, tedaviye yanıtı ve prognozu belirleyen en önemli etkendir. Bu çalışmanın amacı karaciğer fibrozisinin değerlendirilmesinde proteomik analiz ile yeni biyolojik belirteçlerin araştırılmasıdır.* ***Yöntem:*** *Kronik karaciğer hastalığı olan toplam 87 hasta (Metavir indeks ile, 17 F0, 30 F1, 6 F2, 9 F3 ve 21 F4) ve 29 sağlıklı kontrol serumu analiz edilmiştir. Serum proteinleri IMAC30 (bakır) ProteinÇip dizinlerinde çalışılmış ve SELDI-TOF kütle spektrometrisi ile analiz edilmiştir. İstatistiksel olarak anlamlı ayırt edici pikler parametrik olmayan Mann-Whitney U testi ile analiz edilmiştir. Hafif (F0,F1) ve ileri evre fibrozis (F2,F3,F4) karşılaştırması karar ağacı oluşturularak (çapraz doğrulama) "Biomarker Patterns" Yazılımı versiyon 5.0 (Ciphergen Biosystems, US) ile yapılmıştır.* ***Bulgular:*** *F0 ve F1 evreleri arasında ayırt edici proteomik özellik bulunamamıştır. F2 ve F3 evreleri dahil olmak üzere siroz (F4) grubu ve diğer fibrozis evreleri arasında 30'dan fazla ayırt edici pik bulunmuştur. 6 proteomik özellik hafif (F0,F1) ve ileri evre (F2,F3,F4) fibrozis için ayırt edici bulunmuştur (AUROC ≥0.8, p<0.05, Mann-Whitney test). Karar ağacı ile bulunan üçlü belirteç (4280 m/z, 10453 m/z ve 6376 m/z) 83.3% duyarlılık, 85.1% özgünlük, %81.1 pozitif prediktif değer ve %86.9 negatif prediktif değer (AUROC 0,94) sağlamıştır.* ***Sonuç:*** *Bu çalışmanın sonucu siroz hasta grubu ve karaciğer fibrozisinin diğer evreleri arasında ayırt edici proteomik özellikler ortaya çıkarmıştır. Ayrıca, SELDI-TOF kütle spektrometrisi ile hafif ve ileri evre fibrozisini ayırt edici potansiyel serum biyolojik belirteçleri belirlenmiştir.*

Anahtar kelimeler: Biyolojik belirteç, karaciğer fibrozisi, proteomiks, SELDI-TOF kütle spektrometrisi

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INTRODUCTION

Hepatic fibrosis is a common feature of chronic liver diseases of different etiologies. It is associated with progression of liver disease, and its increase can herald complications of liver disease, including cirrhosis, hepatocellular carcinoma (HCC), end-stage liver disease, and death. Chronic hepatitis C and chronic hepatitis B are important causes of chronic liver disease and cirrhosis worldwide. The accurate assessment of the presence and severity of liver fibrosis is of paramount importance in determining management strategies, response to treatment and prognosis in patients with chronic liver disease.

Histological diagnosis and assessment of the degree of fibrosis with liver biopsy have long been the gold standard. However, it is an invasive procedure with inherent risk. Approximately 20% of patients have pain after the procedure. The risk of severe complications ranges from 0.3% to 0.5% (1), and risk of mortality attributed to liver biopsy, although low (0.05%), exists (2). Furthermore, biopsy is prone to sampling error, leading to 20-33% of false histological allocations, especially in patients with advanced liver disease (stage ≥F2 in the METAVIR scale) (3-6). In addition, the use of suction needles is widespread and often results in tissue fragmentation, further complicating a reliable assessment (7).

The search for noninvasive assessment of liver fibrosis started in the 1970s, and several noninvasive markers to detect liver fibrosis have been developed throughout the years (8-14). Currently, noninvasive assessment of liver fibrosis involves not only serum markers but also a physical approach, transient elastography (TE), which measures stiffness of the liver (15). Among the several serum markers, the most investigated and validated ones are the aspartate to platelet ratio index (APRI) and the FibroTest (16). These tests and TE have been well validated in chronic hepatitis C but to a lesser extent in chronic hepatitis B. TE may be problematic in obese patients (17), and may give overestimated scores in chronic hepatitis patients with active necroinflammation (18). Serum levels of fibrosis markers related to the fibrogenic process, such as hyaluronic acid, may be confounded by associated diseases with fibrosis in other organs (16).

Advances in high throughput proteomics technology have driven proteomics research into identification of effective biomarkers. Surface-enhanced laser desorption/ionization time-of- flight mass spectrometry (SELDI-TOF MS) is a proteomic high-throughput technique, which has been improved with the progression of protein-chip systems. SELDI-based techniques together with bioinformatics have so far been successfully applied for early detection of several cancer types (19,20). In addition, SELDI-TOF MS has also been used to assess liver fibrosis and cirrhosis in patients with chronic hepatitis B virus (HBV) (21,22) and chronic hepatitis C virus (HCV) infection (23,24). In the present study, we aimed to discover potential biomarkers in the serum samples of patients with HBV or HCV infection for assessment of liver fibrosis using SELDI-TOF MS.

MATERIALS AND METHODS

Samples

From January 2007 to May 2008, serum samples of 90 patients with chronic liver disease with available liver biopsy and 29 healthy controls (HCs) (19 males, 10 females; mean±SD age: 46.2±6.6) were collected at the Gastroenterology Department of Ankara University Medical School. Healthy subjects with normal serum levels of aminotransferases and alkaline phosphatase (ALP) were included in the study. These persons had no history of chronic liver disease. The study protocol was approved by the Ethics Committee of the Medical School of Ankara University on 22 January 2007 (registration number 106-2759). All patients gave informed consent. Blood samples were collected before liver biopsy on the same day. Serum samples were aliquoted and stored at -80°C until testing. None of the samples was thawed more than once. An experienced pathologist (EE), unaware of clinical data, evaluated all biopsy samples. Only samples of patients for whom reproducibility of liver fibrosis staging was confirmed by another pathologist (BS) were included in the study. Thus, seven patients were excluded from the study because of different biopsy evaluation results. Serum samples of 83 patients (53 patients with chronic hepatitis C, 30 patients with chronic hepatitis B) with pathological agreement were used in the study (35 males, 48 females; mean±SD age: 49.2±8.5). Hepatic fibrosis was assessed using the METAVIR index, which revealed F0 fibrosis in 17, F1 in 30, F2 in 6, F3 in 9, and F4 in 21 patients.

Serum Proteomic Profiling

To determine the best serum profiles with regard to number and resolution of the protein peaks, four chips with different ProteinChip surfaces (cationic, anionic, hydrophobic, and Cu metal binding; Ciphergen Biosystems, Fremont, CA, USA) were tested. For further analysis, the IMAC30 (Cu metal binding surface) protein chip, which displayed the best serum profile, was selected. Serum samples were first thawed completely, vortexed briefly, and centrifuged at 3500 g for 10 minutes (min) at 4°C to remove debris. Each serum sample was then denatured by the addition of U9 solution (9M urea, 2% CHAPS and 150 mM Tris-HCl, pH 9) in a 2:5 ratio, and mixed on a platform shaker for 30 min at 4°C. IMAC30 protein chip arrays were pre-equilibrated twice with 100 mM CuSO4 for 5 min, and washed three times with 1 mM HEPES (pH 7) for 2 min. Arrays were then incubated four times with 150 µl of the binding buffer (PBS 1X, 0.3 M NaCl, 0.1% Triton X-100) for 5 min. Then, 100 µl binding buffer was added to 2.5 µl U9-treated serum sample and applied randomly to a pre-activated protein chip array in duplicate for an incubation for 1 hour (h) on a shaker at room temperature. After incubation, each array was washed three times with binding buffer (5 min for each), and then without Triton-binding buffer, and rinsed twice with 1 mM HEPES. Finally, after air drying, 1 µl of a saturated solution of sinapinic acid matrix (Ciphergen Biosystems, US) in 0.5% trifluoroacetic acid and 50% acetonitrile was added on each spot and air-dried for further analysis. The protein chips were analyzed using SELDI ProteinChip System 4000 Mass spectrometer (Ciphergen Biosystems, US) and the data were analyzed with Ciphergen Express software, version 3.0 (Ciphergen Biosystems, US). After collecting all the raw data automatically with protein peaks ranging from 2000 to 35000 Da, the data was further normalized to total ion current and aligned. These analyses included automatic peak detection, baseline subtraction and mass accuracy calibration.

To assess machine reproducibility, quality control (QC) serum sample, collected from a healthy volunteer (EK), was applied onto different spots on every chip run. In these spectra, several independent control peaks were identified to calculate the CV (coefficient of variance) of intensity and CV of m/z in intra-assay and inter-assay.

Statistical Analysis

Protein peaks of all sample spectra were clustered with the Ciphergen Express software, version 3.0, performing Expression Difference Mapping. Autodetect peaks labeling was achieved in all spectra with 5.0 S/N (signal to noise ratio), 3 valley depth for the first pass, minimal peak threshold: 20% of all spectra and 3.0 S/N, 1 valley depth for the second pass, with 0.3% cluster mass window, and the estimated peaks were added. Proteomic peak features that differed significantly between groups were identified using the Mann–Whitney nonparametric test. To determine the best discriminative proteomic index, area under receiver operating characteristics curves (AUROC) was used as a measurement. Statistically significant discriminatory peaks between groups were determined with AUROC ≥ 0.8 and $p<0.05$. Comparison between patients with advanced (F2, F3, F4) and low-grade fibrosis (F0, F1) was also performed using tree classification (cross-validation) depending on peak intensity with the Biomarker Patterns software (BPS), version 5.0 (Ciphergen Biosystems). Briefly, the classification tree split the data into two nodes using one rule at a time in the form of peak intensity. The splitting decisions were based on the normalized intensity levels of peaks. The process of splitting was continued until terminal nodes were reached.

RESULTS

Reproducibility of the SELDI system

The reproducibility of the SELDI system was tested using 4 peaks from a QC sample. For all peaks, the CV for mass accuracy was 0.07%. The CVs calculated for intra-assay and inter-assay intensities were 27% and 30%, respectively.

Identification of Fibrosis-Associated Proteomic Features

A total of 168 proteomic peaks were generated. Among them, 6 peaks had mean values of intensity that differed significantly between patients with advanced fibrosis and those with no or mild fibrosis, and 15 peaks differed significantly between patients with advanced fibrosis and HCs. The number of discriminatory peaks between different stages of liver fibrosis with AUROC ≥ 0.8 and $p<0.05$ are shown in Table 1. When analysis was performed between F0, F1 and F2 groups separately, no statistically discriminatory peak was evident (Table 1). There was also no significant difference observed in the analysis of the HC and low-grade fibrosis groups (F0 or F1). However, the

Table 1. Number of discriminatory peaks between groups

Compared Groups	Numbers of discriminatory* peaks
HC vs. F0	0
HC vs. F1	0
HC vs. F2	2
HC vs. F3	12
HC vs. F4	43
HC vs. F2, F3, F4	15
F0 vs. F1	0
F0 vs. F2	0
F0 vs. F3	11
F0 vs. F4	47
F1 vs. F2	0
F1 vs. F3	2
F1 vs. F4	39
F2 vs. F3	6
F2 vs. F4	34
F3 vs. F4	35
F0, F1 vs. F2, F3, F4	6

*Statistically discriminatory peaks were determined with AUROC ≥0.8 and p<0.05 (Mann–Whitney *U* non-parametric test).

HC: Healthy control.

Table 2. Discriminatory proteomic features between F2 and F3

Proteomic feature (mean m/z value)	AUROC	*Average intensity of F2 cases relative to F3 cases	*P value
5905	0.83	2.17	0.02
6112	0.83	2.05	0.02
5950	0.83	1.94	0.04
5335	0.83	2.01	0.03
6559	0.83	1.84	0.02
6184	0.80	1.80	0.03

AUROC: Area under receiver operating characteristics curve.

* Mann-Whitney test.

comparison between F3 and F2 or between F3 and F4 revealed 6 and 35 statistically significant proteomic features, respectively (Table 1). Among the former 6 proteomic features, which were discriminative between F3 and F2 (Table 2), 2 proteomic features (6112 m/z, 5950 m/z) were found to be discriminative also for F3 vs. F4, showing lower intensity in patients with F3 (data not shown). The other 4 protein peaks of 5905 m/z, 5335 m/z, 6184 m/z, and 6559 m/z were discriminatory for only F2 vs. F3, giving AUROCs between 0.80-0.83.

The number of discriminatory peaks between patients with cirrhosis and different stages of fibrosis, including mild fibrosis (F0 or F1) and F2 or F3 groups, are given in Table 3. A total of 26 statistically significant discriminatory proteomic features between patients with cirrhosis and F2 or F3 are listed in Table 4. In Figure 1, SELDI-TOF spectra of patients with cirrhosis (F4), F2 and F3 are shown for the most discriminatory peak of 9183 m/z, with an AUROC and p value of 0.92 and 1.54 x 10^{-5}, respectively.

The comparison between patients with low-grade fibrosis (F0, F1) and advanced fibrosis (F2, F3, F4) revealed 6 discriminatory proteomic features with m/z of 4280, 3768, 3952, 9495, 4642, and 11838 Da (Table 5). The expression levels of 3 of these highly significantly discriminatory proteomic features with m/z 4280, 3768 and 3952 are shown in Figure 2. Among these, the peaks with 4280 m/z and 3952 m/z were also found to be discriminatory for HC vs. F2, both giving AUROC value of 0.8 and p values of 0.01 and 0.02, respectively (data not shown). The visual comparison of intensities of these 3 peaks between fibrosis stages are shown as a box plot display in Figure 3.

Table 3. Number of discriminatory peaks between the cirrhosis and other groups

Compared groups	Number of discriminatory* peaks positively correlated with cirrhosis (F4)	Number of discriminatory* peaks negatively correlated with cirrhosis (F4)	Total number of discriminatory peaks
F4 vs. HC	16	27	43
F4 vs. F0	21	26	47
F4 vs. F1	12	27	39
F4 vs. F2	11	23	34
F4 vs. F3	10	25	35
F4 vs. F0, F1	15	31	46
F4 vs. F0, F1, F2	14	29	43
F4 vs. F0, F1, F2, F3	13	27	40
F4 vs. F2, F3	2	24	26

Statistically discriminatory peaks were determined with AUROC ≥0.8 and p<0.05 (Mann–Whitney *U* non-parametric test).
HC: Healthy control.

Table 4. Statistically significantly discriminatory proteomic features between patients with cirrhosis and F2 or F3

Proteomic feature (mean m/z value)	AUROC	*Average intensity of F2, F3 cases relative to F4 cases	*P value
9183	0.92	2.65	1.54×10^{-5}
4609	0.91	3.85	1.54×10^{-5}
9350	0.90	2.08	1.01×10^{-5}
9389	0.90	2.39	1.01×10^{-5}
9608	0.90	3.37	2.33×10^{-5}
9497	0.89	2.04	5.19×10^{-5}
6858	0.88	1.87	5.92×10^{-5}
4593	0.88	5.99	4.55×10^{-5}
6678	0.86	2.27	1.84×10^{-4}
9421	0.86	2.53	8.71×10^{-4}
5866	0.86	0.36	1.44×10^{-4}
4280	0.85	2.71	3.35×10^{-4}
9136	0.84	1.89	3.76×10^{-4}
3953	0.83	2.30	8.33×10^{-4}
8133	0.83	3.23	4.23×10^{-4}
9063	0.83	1.86	3.35×10^{-4}
8757	0.83	1.99	4.23×10^{-4}
8162	0.82	3.3	5.96×10^{-4}
5968	0.82	0.51	9.30×10^{-4}
4468	0.81	1.82	3.58×10^{-4}
8863	0.81	1.81	6.67×10^{-4}
7839	0.81	2.06	5.96×10^{-4}
8811	0.81	1.98	8.33×10^{-4}
8889	0.80	1.81	1.6×10^{-4}
9288	0.80	1.92	1.6×10^{-4}
3968	0.80	1.86	1.0×10^{-4}

AUROC: Area under receiver operating characteristics curve.
* Mann-Whitney test.

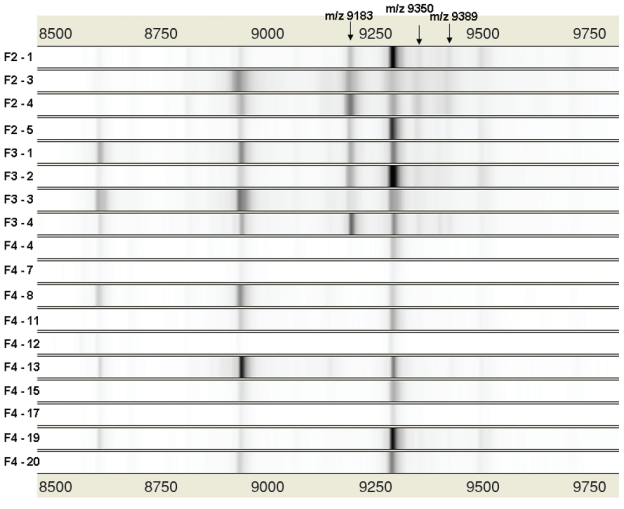

Figure 1. SELDI-TOF mass spectra. Protein peaks and gel views of the peaks 9183 m/z, 9350 m/z and 9389 m/z, which are negatively correlated with cirrhosis. Numbers represent cases used in the experiment.

Table 5. Discriminatory proteomic features between low-grade (F0, F1) and advanced fibrosis (F2, F3, F4)

Proteomic feature (mean m/z value)	*AUROC	**Average intensity of F0, F1 cases relative to F2, F3, F4 cases	**P value
4280	0.84	5.28	1.7×10^{-8}
3768	0.83	4.04	4.0×10^{-7}
3952	0.81	3.26	1.6×10^{-6}
9495	0.80	1.52	2.0×10^{-6}
4642	0.80	1.87	1.8×10^{-6}
11838	0.80	0.45	2.6×10^{-6}

*Area under the ROC curve. **Mann-Whitney test.

With the help of BPS, a decision tree classification with 4 terminal nodes was created to separate low-grade and advanced fibrosis (Figure 4). The root node of the decision tree includes the most important protein peak with an m/z of 4280 Da,

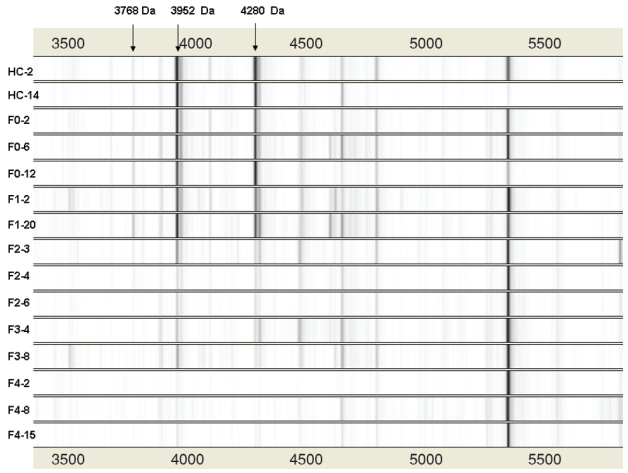

Figure 2. SELDI-TOF mass spectra. Protein peaks and gel views of the peaks 3768 m/z, 3952 m/z and 4280 m/z, which are positively correlated with low-grade fibrosis. Numbers represent cases used in the experiment.

which was also determined as statistically the most discriminatory single peak in the present study. If the intensity of the peak 4280 m/z was lower or equal to 18.086 and 10453 m/z was lower or equal to 0.667, these samples split into terminal node 2, which includes 93.3% F2, F3, F4 and 6.7% F0, F1 cases (Figure 4). If the intensity of peak 4280 m/z was higher than 18.086, samples split into terminal node 4, which includes 3.3% of samples with advanced fibrosis and 96.7% of samples with low-grade fibrosis. Subsequently, 15 samples with low-grade fibrosis entered terminal node 3, which includes a peak of an m/z of 10453 Da with intensity higher than 0.667 and peak of an m/z of 6376 Da with intensity higher than 0.787. This model correctly identified 44 of 47 F0, F1 (93.6%) and 33 of 36 F2, F3, F4 (91.7%) patients. The test data yielded a sensitivity of 83.3% (30/36), a specificity of 85.1% (40/47), a positive predictive value of 81.1%, and a negative predictive value of 86.9%, with an AUROC of 0.94.

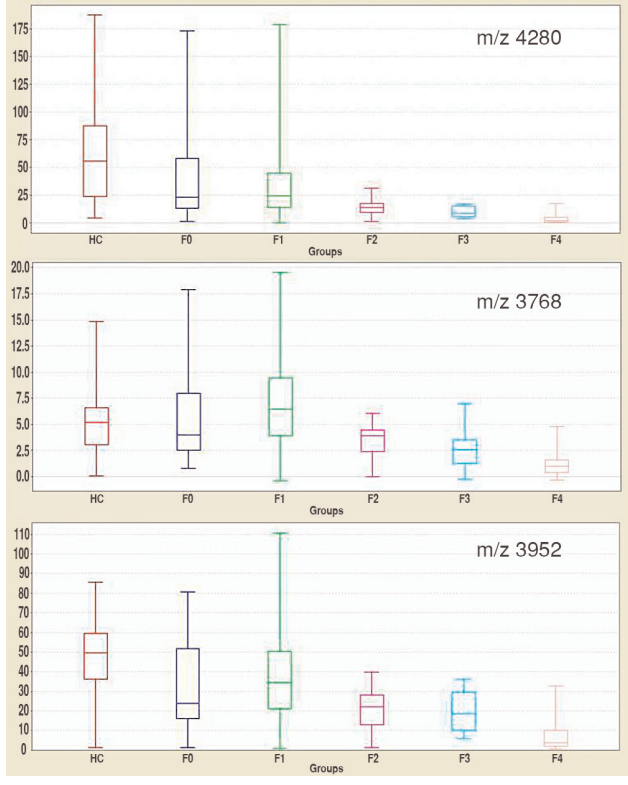

Figure 3. Box plot displays of intensity levels of 4280 m/z, 3768 m/z and 3952 m/z. The comparison of different fibrosis stages (F0, F1, F2, F3 and F4) and healthy control (HC) groups was performed by using non-parametric Kruskal-Wallis method with Ciphergen Express software, version 3.0.1.

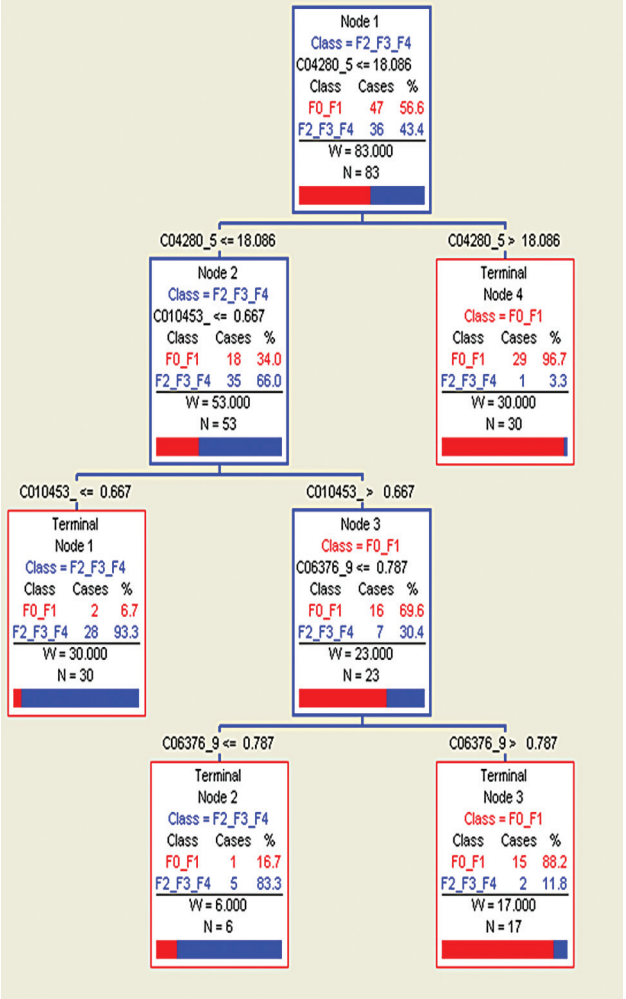

Figure 4. Decision tree for the differentiation of low-grade fibrosis versus advanced fibrosis. The root node contains the most discriminatory protein peak (4280). The next descendant nodes are created by the intensity value of the peaks. Samples with higher intensities go to the next right descendant nodes and samples with lower or equal intensities go to left terminal nodes.

DISCUSSION

In this study, serum proteome differences in different liver fibrosis stages were investigated. No statistically significant discriminatory peaks were found that would differentiate between F0, F1 and F2 fibrosis (Table 1), in line with numerous data on fibrosis markers in patients with chronic viral hepatitis. This is not surprising, since quantification of fibrosis by image analysis revealed that an increase in the area of fibrosis is rather subtle until F2 fibrosis (6). Thus, expectedly in both chronic hepatitic C and B, several biochemical and fibrosis markers could more or less reliably differentiate mild fibrosis from advanced fibrosis. However, the area of fibrosis detected by image analysis was found to increase by almost three-fold from F2 to F3 fibrosis and about five-fold from F2 to F4 fibrosis (6). Thus, aiming for a marker that would differentiate F1 fibrosis from F0 or F2 fibrosis may scientifically not be realistic. However, searching for a marker that would differentiate not only advanced fibrosis from mild fibrosis but also F3 fibrosis from F2 or F4 appears to be reasonable from a scientific perspective. Indeed, in this study, we were able to find such proteomic peaks. Comparison between F2 and F3 revealed six (5905 m/z, 5335 m/z, 5950 m/z, 6112 m/z, 6184 m/z, and 6559 m/z) statistically significantly discriminatory proteomic features, and two of them (6112 m/z and 5950 m/z) were found to be discriminative also for F3 vs. F4. The other four protein peaks of 5905 m/z, 5335 m/z, 6184 m/z, and 6559 m/z were discriminatory for F2 vs. F3. These data have practical value in the clinic and need confirmation with other studies, due to the low numbers of patients in the F2 and F3 groups in our study.

The comparison between cirrhosis and mild fibrosis revealed 46 discriminatory peaks. Of these 46 peaks, 15 showed higher and 31 showed lower intensity in patients with cirrhosis. The number of discriminatory peaks for cirrhosis and significant fibrosis of stage F2 or F3 was 26. These results showed that there are statistically significant differences even between serum proteomes of cirrhotic patients and significant fibrosis (F2 or F3).

A few other studies have looked at identifying serum proteomic signatures associated with liver fibrosis and cirrhosis using SELD-TOF MS. Zhu et al. (22) explored serum protein spectra of 25 patients with HBV-induced liver cirrhosis and 45 non-cirrhotic samples using CM10 protein chips (22). They reportedly were able to predict cirrhosis with 80% sensitivity and 82% specificity. Cui et al. (25) used a decision tree classification model analyzing test data from serum proteomes of 18 patients with HBV-induced liver cirrhosis and 52 HCs. This model yielded a sensitivity of 92% and specificity of 94%. Poon et al. (21) identified 30 SELDI proteomic features predictive for significant fibrosis (Ishak stage ≥3) and cirrhosis using serum samples of 46 patients with chronic hepatitis B. The artificial neural network (ANN) indices they developed from the proteomic fingerprint were correlated with the degree of Ishak score (r=0.831). Göbel and his colleagues (23) analyzed chronic hepatitis C serum samples of 39 patients with F1/F2 fibrosis, 44 patients with F4 fibrosis and 34 patients with HCC using CM10 protein chips. They found a five peptide/protein multimarker pattern (2873, 6646, 7775, 10 525 and 67 867 Da), which discriminated patients with cirrhosis from F1 or F2 fibrosis, with a specificity of 100% and a sensitivity of 85% in training samples (AUROC 0.976) and a sensitivity of 80% and specificity of 67% for random test samples. Morra and his colleagues (24) reported eight independent SELDI proteomic features to be discriminative between patients with no or mild fibrosis (F0, F1) and those of patients with advanced fibrosis (F2, F3, F4). In the same study, a proteomic index with the combination of these eight proteomic features was created using multivariate logistic regression. This index showed high accuracy for predicting advanced fibrosis, with an AUROC of 0.88. In our study, we generated a decision tree to identify multi serum protein pattern for differentiation of mild (F0, F1) and advanced fibrosis (F2, F3, F4) using cross-validation with the help of BPS. This model revealed triple proteomic features with 4280, 10453 and 6376 Da, yielding a sensitivity of 83.3%, a specificity of 85.1%, a positive predictive value of 81.1%, and a negative predictive value of 86.9%.

Comparing the results of the present study with the above-mentioned studies, there was no discriminatory peak with same molecular mass. This is also the case when comparing the results of the above-mentioned studies to each other. This contradiction may be explained by the use of different protein chips (CM10 vs. IMAC) for our study and one important caveat inherent to all studies, namely the small sample sizes investigated. This is no doubt also a drawback of the current study. Further, we included patients with both HBV- and HCV-induced liver disease. In addition, different methodologies of data analysis may explain the variability.

In conclusion, the results revealed discriminatory peaks between cirrhosis and other stages of liver fibrosis, but beyond that, peaks that were able to discriminate F2 from F3 and F3 from F4 fibrosis. The triple proteomic peaks consisting of 4280 Da, 10453 Da and 6376 Da reliably discriminate mild stage from advanced fibrosis with high sensitivity and specificity. These discriminating peaks are candidate biomarkers that need to be validated with a significant number of patients.

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