

Screening serum hepatocellular carcinoma-associated proteins by SELDI-based protein spectrum analysis

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Abstract

AIM: To find out potential serum hepatocellular carcinoma (HCC)-associated proteins with low molecular weight and low abundance by SELDI-based serum protein spectra analysis, that will have much application in the diagnosis or differentiated diagnosis of HCC, as well as giving a better understanding of the mechanism of hepato-carcinogenesis.

METHODS: Total serum samples were collected with informed consent from 81 HCC patients with HBV(+)/cirrhosis(+), 36 cirrhosis patients and 43 chronic hepatitis B patients. Serum protein fingerprint profiles were first generated by selected WCX2 protein chip capture integrating with SELDI-TOF-MS, then normalized and aligned by Ciphergen SELDI Software 3.1.1 with Biomarker Wizard. Comparative analysis of the intensity of corresponding protein fingerprint peaks in normalized protein spectra, some protein peaks with significant difference between HCC and cirrhosis or chronic hepatitis B were found.

RESULTS: One hundred and twenty-eight serum protein peaks between 2000 and 30 000Da were identified under the condition of signal-to-noise > 5 and minimum threshold for cluster > 20%. Eighty-seven of these proteins were showed significant differences in intensity between HCC and cirrhosis ($P < 0.05$). Of the above differential proteins, 45 proteins had changes greater than two-fold, including 15 upregulated proteins and 30 downregulated proteins in HCC serum. Between HCC and chronic hepatitis B, 9 of 52 differential proteins ($P < 0.05$) had intensities of more than two-fold, including 2 upregulated proteins and 7 downregulated proteins in HCC serum. Between cirrhosis and chronic

hepatitis B, 28 of 79 significant differential proteins ($P < 0.05$) changes greater than two-fold in intensity, including 17 upregulated proteins and 11 downregulated proteins in cirrhosis serum. For the analysis of these leading differential proteins in subtraction difference mode among three diseases, the five common downregulated proteins in HCC serum (M/Z 2870, 3941, 2688, 3165, 5483) and two common upregulated proteins (M/Z 3588, 2017) in HCC and cirrhosis serum were screened.

CONCLUSION: Because the interference of unspecific secreted proteins from hepatitis B and cirrhosis could be eliminated partly in HCC serum under subtraction difference analysis, these seven common differential proteins have the obvious advantage of specificity for evaluating the pathological state of HCC and might become novel candidate biomarkers in the diagnosis of HCC.

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Key words: Hepatocellular carcinoma; SELDI-TOF-MS

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide, with the highest incidence being in east and southeast Asia and sub-Saharan Africa, and the estimated annual number of cases is > 500 000^[1,2]. In recent years, an increasing incidence and mortality rate of HCC have also been observed in western industrialized countries^[3,4]. Most studies on the etiology of HCC suggest that risk factors such as HBV/HCV, cirrhosis, dietary aflatoxin B1 (AFB1) intake, and alcohol abuse are responsible for almost all HCC development. Both HBV

and HCV are attributed to > 80% of HCC development worldwide^[5]. In high incidence areas of China, > 90% of HCC cases are reported to have an HBV background^[6]. Liver cirrhosis, regardless of its etiology, is recognized as the most powerful independent risk factor for HCC, and about 55%-85% of cases finally develop into HCC with poor prognosis^[7,8]. On the other hand, the other two known risk factors (AFB1 and alcohol) only appear as synergistic factors with pre-existing chronic hepatitis or cirrhosis during liver carcinogenesis^[9,10]. Although much is known about the effects of the above etiological agents on HCC development, the detailed pathological mechanisms of HCC remain to be unequivocally defined, mainly due to its complex multigene and multifactorial involvement, as well as strong associations with hepatitis or cirrhosis.

Presently, early surgical interventions, including hepatectomy or percutaneous radio frequency ablation, are still the best hope for a clinical cure of HCC. Unfortunately, many cases of HCC exhibit intrahepatic and distant metastasis at the time of diagnosis, which miss the optimal opportunity for surgical resection. This indirectly indicates no ideal tumor markers can be recommended for early detection or differential diagnosis of HCC. Thus, discovering more HCC-associated proteins and identifying a single or a set of reliable, non-invasive new biomarkers is one of the best options for improving the diagnostic efficiency of HCC.

Alteration of serum protein profiles can effectively reflect the pathological states of liver injury. It seems much more challengeable to screen specific HCC-associated proteins in serum of HCC patients with the background of HBV/HCV or cirrhosis because hepatitis and cirrhosis can also cause great changes in the serum protein profile. Moreover, separation and identification of disease-associated proteins is laborious and difficult, relying on low-technology approaches and screening strategies. While some serum tumor markers, including enzymes (GGT (gamma-glutamyl transferase), AFU (alpha-1-fucosidase), DCP (des-gamma-carboxyprothrombin), and oncofetal and glycoprotein antigens [AFP (alpha fetoprotein), AFP-L3 (alpha fetoprotein-L3), GPC3 (glypican-3)], have been widely applied to the detection of HCC, the diagnostic accuracy for HCC is still unsatisfactory, especially in early and differential diagnosis. Even though AFP is a well-known biomarker for HCC, its sensitivity has been reported at 39%-64% and its specificity as 76%-91%, it has been shown to be particularly useful in detecting HCC with non-viral etiology^[11,12]. Unlike conventional serological biomarkers with high molecular weight (MW) and high abundance, a lot of uncharacterized serum proteins with low MW and low abundance, which promise to contain important disease information, have attracted much attention in the diagnosis of HCC^[13,14]. A promising high-throughput proteomics approach, on-chip protein fractionation coupled to SELDI-TOF-MS (surface enhanced laser desorption ionization-time of flight-mass spectrometry), provides a novel pathway for rapid screening and identification of specific tumor markers with low MW in the proteomes of body fluids. To date, a number of single or combined SELDI spectra peaks have been successfully identified by means of this technology

and used to discriminate differential diseases as disease related biomarkers^[11,15,16].

In the present study, by comparative analysis of SELDI serum spectra between HCC with HBV(+)/cirrhosis(+) and cirrhosis or HBV, using Ciphergen SELDI Software 3.1.1 with Biomarker Wizard and subtraction difference analysis, some potential serum common HCC-associated proteins with low MW were discovered, which might be new candidate biomarkers for HCC diagnosis.

MATERIALS AND METHODS

Study population and sample preparation

With patient consent, serum samples were obtained from 81 HCC patients with HBV/cirrhosis from the Liver Cancer Institute of Zhongshan Hospital, Fudan University, China. All HCC patients were diagnosed according to combined clinical criteria, including imaging data and serum tumor markers, and further confirmed by histopathological analysis. Samples from 36 patients with liver cirrhosis were kindly provided by the Department of Infection of Shanghai East Hospital, Shanghai, China. Diagnosis of liver cirrhosis was mainly dependent on clinical history, physical examination, laboratory findings, ultrasonography and/or computed tomography, with or without liver biopsy. Serum samples of 43 HBV-infected patients were collected based on the presence of HBsAg (hepatitis B surface antigen), HBeAg (hepatitis B e antigen) and HBV DNA, from Shandong Provincial Hospital, China. All serum samples were separated and divided into 10-μL aliquots, then stored at -80°C until assayed.

Ciphergen protein chip SELDI-TOF-MS analysis

Compared with other protein chips (anionic, copper mental binding and hydrophobic), the WCX2 protein chip (Ciphergen Biosystems, Fremont, CA, USA) was selected for further serum differential protein spectrum analysis because it generates the best serum profile with the largest number and resolution of protein fingerprint peaks. Serum samples were first diluted 1:2 with U9 sample buffer containing 9 mol/L urea, 2% CHAPS, 50mmol/L Tris/HCl and 1% DTT (pH 9.0, Sigma, USA). Subsequently, 108 μL WCX2 binding buffer (50 mmol/L sodium acetate, pH 4.0; Sigma) was mixed with the above samples homogeneously. One hundred microliters of diluted serum samples was then applied to each spot on pre-equilibrated WCX2 protein chips, packed in a processor for 60 min incubation with shaking. After two washes with WCX2 binding buffer and one quick rinse with HPLC grade water, 1 μL of a saturated solution of EAM sinapinic acid (Ciphergen Biosystems) in 50% acetonitrile and 0.5% trifluoroacetic acid (Sigma) was loaded on each spot and air-dried for MS detection. Mass accuracy was calibrated externally through the use of the All-In-One peptide molecular mass standard (Ciphergen Biosystems). The protein chips were read using the PBSII MS system (Ciphergen Biosystems). Each spot on protein chips was scanned with a laser intensity of 185 and a detector sensitivity of 8, and data acquisition parameters were set as 22. delta to 4. transients per to 7 ending position to 82 by SELDI Quantitation. Assessment of the reproducibility

Table 1 Common significant differential protein peaks between HCC with cirrhosis(+) /HBV(+) and HBV or cirrhosis

Peak M/Z	P	Mean HBV	Mean HCC	HBV/HCC	Peak M/Z	P	Mean C	Mean HCC	C/HCC	Peak M/Z	P	Mean C	Mean HBV	C/HBV
2870	0	2.4084	0.5602	4.2992	2870	0	4.1231	0.5602	7.3599	2870	0.005179992	4.1231	2.4084	1.7119
3941	0	9.081	3.3872	2.681	3941	1.01E-08	8.5539	3.3872	2.5253					
2688	1.00E-10	1.5411	0.5736	2.6869	2688	0	1.489	0.5736	2.5961					
3165	7.51E-08	1.8627	0.9297	2.0036	3165	7.00E-10	2.7519	0.9297	2.9601					
5483	0.000000168	1.6028	0.7756	2.0665	5483	1.54E-08	1.9195	0.7756	2.4749					
3588	0.00012821	0.3225	0.7492	0.4305						3588	0.00006427	0.9533	0.3225	2.9555
2017	0.017271682	0.2451	0.5364	0.4569	2017	0.000903778	0.6541	0.5364	1.2196	2017	1.89E-06	0.6541	0.2451	2.6691

of the SELDI system was the same as in our previous study^[17]. The intra- and inter-assay CV for intensity and m/z in this SELDI system were 17.46% and 0.024%, and 17.74% and 0.0237%, respectively.

Bioinformatics and subtraction difference analysis

Protein spectra were automatically generated after all raw data were collected. All spectra were first normalized to a total ion current with an m/z of 2000 and 30000 and then aligned by baseline subtraction, mass accuracy calibration using Ciphergen SELDI Software 3.1.1 with Biomarker Wizard. Protein peaks ranging from 2 to 30 kDa were autodetected in term of an S/N > 5 and a minimum threshold for cluster > 20%, and were further checked and labeled to minimize mismatched peaks. The m/z value within the 0.3% mass accuracy windows was defined to be identical between replicates. By comparative analysis of the intensity of corresponding protein fingerprint peaks in normalized protein profiles with Biomarker Wizard analysis, some protein fingerprint peaks between HCC and hepatitis or cirrhosis were found to be significantly different ($P < 0.05$). The P value represented the power of each peak in discriminating HCC from hepatitis or cirrhosis. From these, some leading differential proteins between HCC and cirrhosis or chronic hepatitis B (average intensity ratio > 2 or < 0.5) can be selected for future identification. Analysis of these leading differential proteins in subtraction difference mode, common differential proteins among three diseases, which showed potential specificity and clinical significance in dictator for HCC, can be determined successfully.

RESULTS

All protein spectra were normalized and aligned under the same parameters using Ciphergen SELDI Software 3.1.1 with Biomarker Wizard, and then auto-identified protein peaks were visually examined to minimize mismatched peaks between HCC and hepatitis or cirrhosis. Under the condition of signal-to-noise > 5 and minimum threshold for cluster > 20%, the sum of 128 serum protein peaks between 2 and 30 kDa were identified.

Comparison of serum protein spectra between HCC with HBV/cirrhosis and cirrhosis

Eighty-seven of 128 protein peaks were shown to be significantly different in intensity between HCC and cirrhosis after Biomarker Wizard software analysis

($P < 0.05$). Of the above differential proteins, forty-five differential proteins had over two-fold changes, which were defined as leading differential proteins, including 15 up-regulated proteins (m/z 8696, 13769, 13969, 28060, 8772, 23415, 22857, 9354, 9730, 11702, 9422, 9406, 2143, 9195, 5058) and 30 down-regulated proteins (m/z 2688, 2870, 3246, 3450, 3493, 3654, 4178, 4965, 2546, 4160, 3165, 2530, 2026, 2361, 3378, 3941, 5483, 2892, 3063, 3714, 2513, 2904, 2615, 2674, 2590, 3513, 3091, 5341, 3356, 8143) in HCC serum. However, the expression of eight up-regulated proteins (m/z 13769, 13969, 28060, 9354, 9422, 9406, 2143, 9195, 5058) also increased in serum of HBV patients in this study, which suggested up-regulation of these eight common differential proteins might have reflected the pathological changes of hepatitis B, but were non-specific for HCC development.

Comparison of serum protein spectra between HCC with HBV/cirrhosis and chronic hepatitis B

Using Biomarker Wizard software analysis, nine of 52 differential proteins ($P < 0.05$), including two up-regulated proteins (m/z 3588, 2017) and seven down-regulated proteins (m/z 2870, 3654, 3941, 2688, 3450, 3165, 5483) in HCC serum, were found to be over twofold different in intensity between HCC and chronic hepatitis B. Compared with hepatitis patients, the expression of m/z 3588 and 2017 was also up-regulated in the serum of cirrhosis patients (Figure 1, Table 1). Therefore, these two common leading differential proteins may play a role in the progress of HBV-induced or cirrhosis-induced HCC, which merits further investigation. The other seven downregulated proteins in HCC serum were all detected to be downregulated in HCC serum as compared with serum protein spectra of cirrhosis (Figure 1, Table 1). This demonstrated that these common differential proteins possessed potential specificity and clinical significance in HCC diagnosis because they eliminate the influence of hepatitis and cirrhosis under subtraction difference analysis. However, expression of the two other leading differential proteins (m/z 3654, 3450) was higher in cirrhosis than in HCC and hepatitis, which indicated they were more specifically associated with cirrhosis.

Comparison of serum protein spectra between cirrhosis and chronic hepatitis B

Between cirrhosis and chronic hepatitis B, 28 of 79 significant differential proteins ($P < 0.05$) were found to have a greater than twofold change in intensity, including

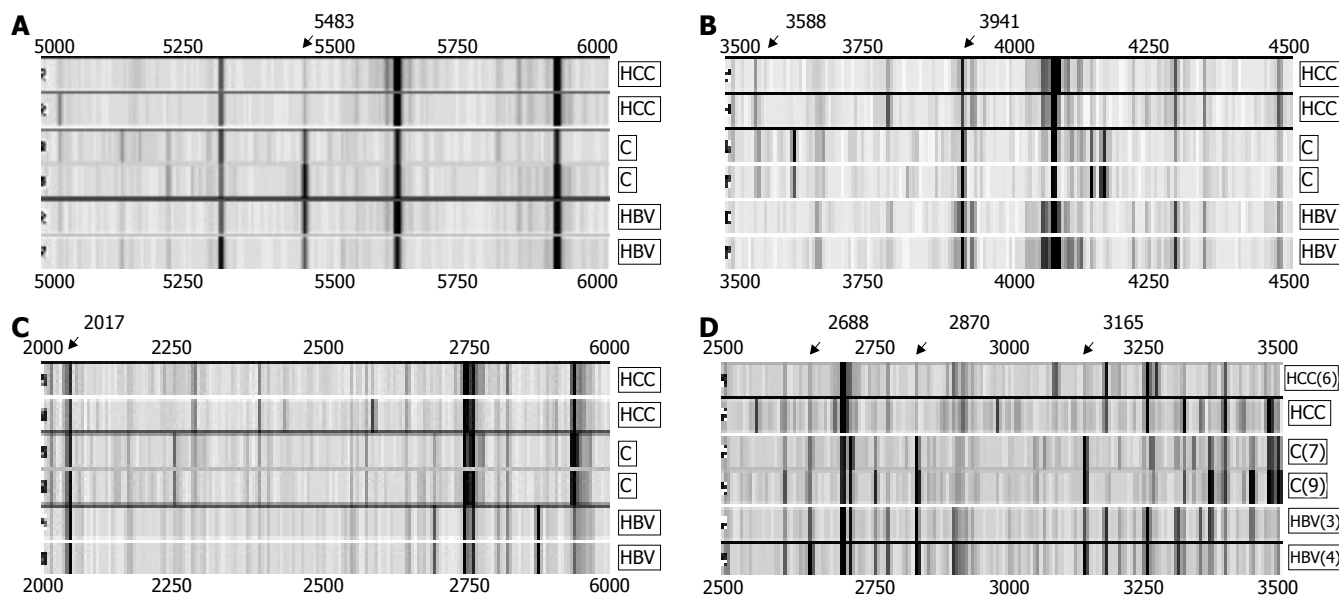


Figure 1 SELDI spectra of serum samples from patients with HCC with HBV/cirrhosis, cirrhosis and chronic hepatitis B. Arrows represent seven common leading differential proteins with low MW, including five common down-regulated proteins in HCC serum (m/z 2870, 3941, 2688, 3165, 5483) and two common up-regulated proteins (m/z 3588, 2017) in HCC and cirrhosis, using Ciphergen SELDI Software 3.1.1 with Biomarker Wizard and subtraction difference analysis. The intensity of each band in the gel view reflects the peak area in the original spectrum. HCC, HCC with HBV/cirrhosis; C, cirrhosis; HBV, hepatitis B virus.

17 up-regulated proteins (m/z 4178, 4965, 3493, 2892, 3476, 3246, 2017, 3714, 3279, 3450, 5003, 3588, 3513, 2513, 2904, 5810, 3654) and 11 down-regulated proteins (m/z 13769, 4075, 13969, 7772, 9291, 9422, 9354, 9406, 28060, 5058, 9195) in cirrhosis. Among 17 up-regulated differential proteins, expression of 11 (m/z 4178, 4965, 3493, 2892, 3246, 3714, 3450, 3513, 2513, 2904, 3654) also increased in cirrhosis, as compared to HCC, which indicated these proteins might have been specifically related to cirrhosis.

DISCUSSION

Several lines of evidence have shown that disease-related proteins or peptide fragments, produced by the tumor or tumor-host microenvironment, either at tumor onset or later, are readily released into the blood as it bathes or circulates through diseased tissue^[13,18,19]. Therefore, blood can be used as a rich source of tumor biomarkers for the clinical diagnosis of many tumors. Over the years, many clinical blood tests based on serum markers have been developed for the detection of cancer^[20-22]. For example, increased serum levels of CA125 (carbohydrate antigen 125), CA153 (carbohydrate antigen 153) and PSA (prostate specific antigen) are routinely used in clinics for detection of cancer in the ovary, breast and prostate, respectively. AFP, AFP-L3 and DCP are the most useful serum tumor markers for the diagnosis of HCC^[23-25], and combination of these three tumor markers can improve the diagnostic accuracy, especially in differentiating HCC from non-malignant liver disease^[12]. However, none of these biomarkers meets the goal of early diagnosis of HCC, or has satisfactory accuracy in detecting HCC and predicting prognosis when used alone. Recently, Wright^[26] *et al* have summarized traditional and novel serum markers for HCC by review of the latest literature (2001-2006). They

predicted that in the near future a new generation of HCC serum markers, including complex proteomics features and tumor specific autoantibodies, will have the potential to replace its flawed leader, AFP, after strict validation in properly controlled clinical studies.

In the past, 2DE coupled with MS was the major technology in biomarker screening of serum proteomes. Following this classic screening pathway, 11 serum proteins with differential expression that is increased or decreased in response to HCC treatment have been identified. These proteins might be involved in liver carcinogenesis or become candidate biomarkers for the development of diagnostic and therapeutic tools^[27]. In another recent study, two different screening strategies (2-DE combined with nano-HPLC-ESI-MS/MS or nano-HPLC-ESI-MS/MS) were simultaneously applied to discover more HCC-related proteins. This work means that scientists are beginning to address the shortcomings of 2DE in clinical proteomic studies of serum tumor marker screening^[28]. 2DE technology is labor intensive, time-consuming, and requires a large amount of sample, therefore, it is not suitable for large-scale serum screening programs and clinical testing^[29]. Furthermore, proteins with a low MW and abundance, and extreme pIs exceed the analytical capability of 2DE, although such proteins convey valuable information for disease assessment. As a result of its pre-fractionation, high resolution and powerful spectrum analysis software, application of SELDI-based systems can remedy some of the drawbacks of 2DE. They also have significant advantages over other proteomics technology for serum biomarkers screening.

For specific biomarkers discovery of HCC with HBV(+)/cirrhosis(+) in serum, two challenging aspects should be addressed (1) to eliminate the interference of most unspecific abundant proteins secreted by liver damage such as hepatitis, cirrhosis, etc., which were also 'reasonable'

differential proteins as compared with health controls (2) to reduce the complexity of serum proteome as more as possible so that most uncharacterized proteins with low abundance and low MW can be detected in serum. Serum proteins have a range of concentrations and are dominated by a handful of high-abundance proteins such as albumin, transferrin, haptoglobin, immunoglobulins and lipoproteins^[13]. Therefore, identification of low-abundance biomarkers is generally hampered by the presence of a few more abundant proteins. Pre-fractionation with simple chemical affinity beads or surface is an effective method to decrease the sample complexities. In this study, chip protein fractionation coupled to high resolution SELDI-TOF-MS not only reduced the complexity of serum proteomes prior to MS analysis, but also improved detection of uncharacterized proteins with low abundance and MW. On the other hand, subtraction difference analysis attenuated the influence of non-specific abundant proteins secreted by patients with hepatitis B or cirrhosis, and guaranteed that candidate tumor biomarkers screened were more specifically for HCC. Through mutually comparative analysis of serum SELDI spectra among three disease groups, the five common down-regulated proteins in HCC serum (*m/z* 2870, 3941, 2688, 3165, 5483) and another two common up-regulated proteins (*m/z* 3588, 2017) in HCC and cirrhosis serum were screened, and showed potential clinical significance for diagnosis of HCC (Figure 1, Table 1). Because most studies for HCC biomarker discovery mainly focused on the changes of its serum SELDI spectra in comparison with that of hepatitis B/C or cirrhosis alone, or between non-HCC and HCC patients^[16,17,30], some identified differential proteins probably reflect the pathological alteration of hepatitis or cirrhosis only, but unspecific for HCC development^[16,26]. Therefore, the above seven common differentially expressed proteins, screened in HCC serum, undoubtedly possessed an advantage in specificity for evaluating the pathological stage of HCC. More recently, applying combination technology of enrichment, SELDI and LC-MS/MS or SELDI, 2-DE as well as LC-MS/MS, some candidate biomarkers for HCV-related HCC such as complement C3a^[16], κ and λ immunoglobulin light chains^[30], *etc.* were identified in succession. These identification studies will offer a valuable reference to characterize these seven common differential proteins for HCC diagnosis in our proceeding study.

In summary, common differential candidate proteins screened here among three disease groups under SELDI system and subtract difference analysis not only show potential clinical significance in HCC indicator, but also provide a novel option to discover specific biomarkers in serum of HCC.

COMMENTS

Background

Although some serum tumor markers including enzymes (GGT, AFU, DCP), and oncofetal and glycoprotein antigens (AFP, AFP-L3, GPC3) have been widely used in the detection of HCC, the diagnostic accuracy for HCC is still unsatisfactory, especially for early and differential diagnosis. There is an urgent need to discover new specific HCC-associated proteins, using novel technology and screening strategies. For biomarkers for diagnosis of HCC with HBV/cirrhosis, two

challenging questions remain: (1) how to eliminate the interference of most non-specific abundant secreted proteins from hepatitis and cirrhosis; and (2) how to reduce the complexity of serum proteome, which hinders the discovery of specific HCC-associated serological proteins.

Research frontiers

A promising high throughput proteomics approach, on-chip protein fractionation coupled to SELDI-TOF-MS, provides a novel pathway for rapid screening and identification of specific tumor markers with low MW in the proteomes of body fluids.

Innovations and breakthroughs

Applying SELDI and subtraction difference analysis, we successfully discovered seven common differential candidate proteins with potential clinical significance for HCC indicator. This screening strategy provides a novel method for discovering specific biomarkers with low MW and low abundance in the serum of patients with HCC.

Applications

These seven common differential proteins with potential clinical significance for HCC diagnosis merit being further identified and validated in large numbers of HCC samples.

Terminology

SELDI-TOF-MS detects proteins affinity-bound to a protein chip array. This system is a novel, sensitive, rapid detection system for analyzing complex mixtures of proteins and peptides.

Peer review

This study investigated serum HCC-associated proteins with low MW and low abundance in patients with HCC with HBV/cirrhosis by SELDI-based serum protein spectrum analysis. Seven common differential candidate proteins with potential clinical significance in diagnosis of HCC were discovered by subtraction difference analysis. These preliminary results and subtraction screening strategy will provide a useful reference for the discovery of biomarkers for HCC.

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