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Identifying serum biomarkers for TACE therapy efficiency of hepatocellular carcinoma

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TABLE OF CONTENTS

1. ABSTRACT

Transcatheter Arterial Chemoembolization (TACE) is the first line of treatment in inoperable hepatocellular carcinoma. Magnetic affinity beads can be used to extract peptides from un-fractionated serum samples. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) can detect the presence and the molecular mass of peptides. In this study, we used a highly optimized ClinProt-matrix-assisted laser desorption/ionization time-of flight mass spectrometer (MALDI-TOF-MS) to screen hepatocellular carcinoma markers for TACE. 40 sera from 20 patients, including before and after TACE to explore those biomarkers, might be related with therapy efficiency, and some of the patients who received another therapy were analyzed as well. The spectra were analyzed statistically using FlexAnalysis™ and Clin-Prot™ bioinformatic software. The seven most significant differential peaks ($p<0.0.5$) were selected out by ClinProTool software to identify hepatocellular carcinoma markers for TACE therapy. Furthermore, the differential peptide of 3883Da was identified as plasma serine protease inhibitor precursor (Protein C inhibitor). This study provides a direct link between peptide marker profiles and TACE therapy, and the markers may have clinical utility for monitoring efficiency of therapy.

2. INTRODUCTION

Recent scientific progress of sequencing of the genome (1) and new approaches to modeling complex biological systems (2), may ultimately lead to improved anticancer therapy. A primary goal of proteomics is biomarker discovery for various human disease conditions, especially cancer treatments. In addition, plasma and serum are considered to be the source of choice in molecular diagnostics. However, although readily accessible from patients, plasma proteome analysis is influenced by a lot of major components at present condition, such as serum albumin, immunoglobulin, etc., the predominance of which raises the problem of detection of the less abundant marker species. Several researches have tried to provide early cancer detection or treatment efficiency via sophisticated software processing of serum/plasma mass spectrometry profiles, where the result is typically based on a pre-trained artificial intelligence system decision (3-5).

Hepatocellular carcinoma (HCC) is the fifth most common cancer (n = 564,336) and the third most frequent cause of cancer deaths (n = 548,554) in the world in 2000. It is largely a problem in developing countries, where 81% of the world's total cases occur. Areas of highest risk include West and Central Africa, Eastern and South-

Table 1. Characteristics of 20 serum samples

Characteristics	No. of samples
Male	18
Female	2
Mean age in years (range)	50.3.(33~75)
Clinical index	
Before therapy	
AFP(ng/mL)	8848.3.
CEA(ng/mL)	13.4.35
CA199(ng/mL)	36.5.03
Bilirubin(ng/mL)	15.2.81
Albumin(ng/mL)	37.2.94
After therapy	
AFP(ng/mL)	3942.1.09
CEA(ng/mL)	3.3.06
CA199(ng/mL)	35.8.21
Bilirubin(ng/mL)	14.7.75
Albumin(ng/mL)	37

Eastern Asia, and Melanesia (6). At present, China's rural cancer deaths in liver cancer mortality ranks first in the city and ranked second only to lung cancer. As the early symptoms of liver cancers were covered-up, patients were mostly during the middle and late stage when there was receipt of treatment. Meanwhile, as many patients combined with cirrhosis, the rate of surgical resection is very low (7). Therefore, most patients can only receive non-surgical treatment. In the current non-surgical therapy, interventional therapy is the most common method, often for those who had to go through vascular channels and percutaneous means of treatment. Practice has proved that selecting the appropriate interventional therapy prolongs life in patients with advanced liver cancer and alleviates their suffering. Transcatheter arterial chemoembolization (TACE) performed with a mixture of various anticancer agents and iodized oil has become widely accepted as a palliative procedure or even as an alternative to surgical resection for the treatment of HCC (8). When the hepatic artery becomes occluded as a consequence of performing repetitive TACE procedures on the hepatic artery, extra hepatic collateral pathways that supply the HCC can develop (9). Extra hepatic collateral pathways or parasitic blood supplies to the HCC can decrease the therapeutic efficacy of TACE of the hepatic artery, and additional TACE procedures to address these extrahepatic supplies to the HCC should be performed to effectively control the cancer. However, TACE often requires repeated cycles, and its treatment follow-up in addition to clinical examination indicators. Traditional ways to assess the efficacy of TACE are imaging studies, such as CT scan or functional MRI, which is too expensive for many patients in China. Therefore, to find biomarkers related with therapy for hepatocellular carcinoma patients is of great significance.

Currently, the screening of biomarkers for cancer treatment is a hot field in serous proteomics because serous proteins may often serve as indicators of disease recovery and are rich sources for biomarkers discovery. In addition, the affinity bead-based purification was developed to reduce costs and make proteomic procedures suitable for general mass spectrometry (MS) analysis. Proteins bound to the magnetic beads are then eluted, diluted, and directly analyzed by MALDI-TOF-MS. Bioinformatics algorithms are used to align and integrate hundreds of mass data points from large numbers of samples. So in this study, we compared serum proteomic profiles between before and after TACE for hepatocellular carcinoma patients to discover protein markers and establish the pattern for detecting efficiency of TACE. The patterns we described may have clinical utility as surrogate markers for therapy of hepatocellular carcinoma. Furthermore, the differential peptide of 3883Da was identified as plasma serine protease inhibitor precursor (Protein C inhibitor, PCI).

3. MATERIALS AND METHODS

3.1. Patients and blood sample preparation

Serum samples used in this experiment came from 20 consecutive HCC patients undergoing TACE by the Zhongshan Hospital of Fudan University Interventional radiology Department from November 2008 to June 2009 (Table 1). All of the subjects have signed informed consent. Patients were all in the early morning fasting state, and we got 5ml sera from them before TACE (t0). Then we performed TACE by selectively introducing a microcatheter into the right or the left hepatic artery or a segmental branch of the hepatic artery and injecting a mixture of iodized oil (Lipiodol; Andre Guerbet, Aulnaysous-Bois,France) and epirubicin hydrochloride (30~50mg per body surface). This was followed by the introduction of a gelatin sponge (1mm*1mm*1mm). After 45days (t1) we obtained another 5ml sera. Among them, 8 patients got the third blood drawings 45 days after they were treated with TACE for the second time (t2). Clinical parameters (etiology, tumor grading, and AFP levels) were recorded. The choice of TACE as treatment was based on the AASLD-EASL guidelines. All the serum samples were collected preoperatively in glass tubes without additive (BD Vacutainer™ Franklin Lakes, NJ) and were allowed to clot at room temperature for 40 min. Serum was separated by centrifugation at 2000 rpm for 15 min, immediately split into 200μL aliquots and frozen at -80°C until analysis. The time from collection to frozen storage was no more than 60 min. The processing, collection and storage protocols for all individuals were exactly the same.

3.2. Serum protein fractionation

The sera were left at 4~6ºC for 2h, centrifuged at 10000 rpm, left at 4 ºC for 10 min, and then aliquoted, and stored at -80 ºC. 10μL SPE-CM (Serum protein extracting-cational and magnetic) binding solution (Bioyong, Beijing, China) and 5μL serum sample were added to the SPE-CM beads and mixed completely, which was then placed on the magnetic beads separation device (MPC-auto96, Dynal, Oslo, Norway) The beads were pulled to the side by magnetic force, and the supernatant was removed and discarded. The magnetic beads were washed three times with SPE-CM washing solution by shaking the beads up and down as needed. The supernatant was removed and the beads remained in place. 5μL elution solvent was added to the bead pellet and mixed by pipeting up/down, and the beads were pulled to the side and a fraction of the elution was transferred to another tube. 10μL a-cyano-4-hydroxycinnamic acid (0.3. g/L in ethanol: acetone 2:1)

was added to the 1μL elution in a 348-well microtiter plate and mixed carefully. 1μL mixture was spotted in quadruplicate on a MALDI AnchorChip™ (Bruker Daltonics, Bremen, Germany).

3.3. Mass spectrometry analysis for profiling serum proteome

For MALDI-TOF-MS analysis, 1 μL of the above diluted purified serum was mixed with 0.5. μl of matrix solution and allowed to dry onto the MALDI sample plate (600 μM AnchorChip™, Bruker Daltonics Company). Two peptides were also included in the matrix solution for internal calibration: 10 pmol/mL angiotensin II and 10 pmol/mL ACTH18-39 (Bruker Daltonics). Laser desorption was targeted randomly on the sample plate and samples were measured using an Autoflex III MALDI-TOF mass spectrometer (Bruker Daltonics), operated in positive ion linear (reflection) mode. Ionization is achieved by irradiation with a 50 Hz nitrogen laser. Spectra are the mean of 100 ionizations with fixed laser power in linear geometry mode and mass maps are obtained in reflectron mode. The spectra were calibrated externally with a mixture of protein/peptide standards in the range of 1000 to 12 000 Da (Bruker Daltonics). The criteria for peak detection were: Signal/Noise (S/N) ratio > 5, 2 Da peak width filter, and maximum peak number of 200. For data bank analysis, all spectra were processed by automatic baseline subtraction, peak detection, recalibration, and peak area calculation according to the predefined parameter settings. The intensities of the peaks of interest were normalized against the peak intensity of the ACTH internal standard. These mass shifts are corrected by the FlexAnalysis™ software after alignment with the 2 internal standards.

3.4. Identification of differential peptides

Peptide extracts were dried and resuspended in 15–20 mL 5% formic acid for further MS/MS analysis by LTQ Orbitrap mass spectrometer (ThermoFisher, USA) and typically 5μL of peptide extracts were actually injected for analysis. Analysis steps by LTQ-Orbitrap were as follows: The peptide extracts were loaded at 15 mL.min-1 for 6 min on a nanoAcquity™Column, followed by eluting and separating on a nanoAcquity™UPLC™Column, using 90-min gradients with 95% water, 5% acetonitrile (ACN), 0.1.% formic acid (solvent A); and 95% ACN, 5% water, 0.1.% formic acid (solvent B) at a flow rate of 300 nL/min. The samples were run in data-dependent mode, where each full MS scanning was followed by three consecutive MS/MS scans of the 3 most abundant peptide molecular ions (typically doubly and triply charged ions), which were selected consecutively for CID. The MS survey scans (300-2,000Da) were carried out and the acquisition cycle consisted of a survey scan at the highest resolving power (100,000). Dynamic exclusion was used with a series of parameters and the acquired MS/MS data were processed using BioworksBrowser 3.3.1. A sequence database search was performed with the International Protein Index (IPI Human3.4.5).

3.5. Statistical methods, evaluation of marker efficacy

All MALDI-TOF-MS spectra were analyzed with FlexAnalysis™ to detect the peak intensities of interest and CLINPROT™ software to compile the peaks across the spectra obtained from all samples (Bruker Daltonics Company). We used SPSS software version 13.0. (SPSS INC., Chicago, IL, USA) to conduct all statistical comparisons. We employed T test for normal distribution of data and the Wilcoxon rank sum test for non-normal distribution of data of each protein peak. In order to measure the reliability of the markers selected out, the 8 patients that were followed up who received a good efficiency after one incidence of TACE, received another TACE, and the sera results were clustering with the before and after group for evaluation of the markers efficacy.

4. RESULTS

4.1. Standards and standard serum spectrum

In this study, we used standard products and standard serum to do quality control (QC). The external standard calibration standard contained 11 peptides (Table 2). Average molecular weight deviation was less than 100ppm for every eight samples of data collection before the first external standard calibration; we also added four standard serum as a quality control, mass spectrometry test results were shown in Figure 1A and 1B.

4.2. Analysis of peptide ion signals from MS-based serum profiling differentiates before and after TACE

All 20 patients after one TACE therapy were first checked by CT scan to make sure most of them received good therapy efficiency (Figure 2). Then the serum peptide profiles of a total of 40 sera samples, including before and after TACE were used with new high-resolution MALDI-TOF MS coupled with bead fractionation. Samples were then randomly distributed during processing and analysis. A total of 107 distinct m/z values were resolved in the 800–10000Da range. 7 peptides (3883, 5634, 6072, 4645, 8934, 6091, 6112) were detected out which were differently expressed in the sera of before and after therapy, and have significant confidence (P<0.0.5) in the identity of the above proteins by T test, and they were shown in Table 3. Within them, the top two discriminating peaks were 3883 Da and 5634 Da. Therefore they dramatically changed expression after TACE in hepatocellular carcinoma patients (Figure 3), and for most samples, the two peaks received relatively low intensity after TACE. As could be seen, the classification was achieved primarily through a contrast in peak intensities. This could be seen from the scatter plot shown. The combination of the top two discriminating peaks of 3883 Da and 5634Da got an accuracy of 100% (20/20) for the before group, and 80% (16/20) for the after group respectively. Therefore, their combination could separate after group sera from before groups (Figure 4).

4.3. Marker efficiency confirmed with twice treated sera

The two markers of 3883 and 5634 Da, which got the best discrimination between before and after one TACE therapy, were calculated at peak intensity of the 8 samples, which received another TACE. Then we compared the intensity with the mean and SD of the before and after one TACE therapy. For 3883 Da peak, only one sample belonged to the before group range, 2 samples belonged to the after group, and the other 5 received much

Table 2. The mass to charge ratio of the reference selected peaks in standards

Substance	Average mass(M+H)+
Angiotensin	1047.1.8
Angiotensin	1297.4.8
Substance P	1348.6.4
Bombesin	1620.8.6
ACTH clip 1-17	2094.4.2
ACTH clip 18-39	2466.6.8
Somatostatin 28	3149.5.7
Ubiquitin	4283.4.5
Insulin	5734.5.6
Cytochrome c	6181.0.5
Ubiquitin	8565.8.9

Table 3. The character of different peaks between before and after TACE

Peak mass/Da	Peak intensity		P value*
3883	58.9.4	42.4.8	0.0.02
5634	20.2.6	14.5.2	0.0.096
6072	12.2.2	17.0.1	0.0.317
4645	62.3.5	48.7.9	0.0.336
8934	136.0.1	216.3.1	0.0.341
6091	22.7.6	41.2.9	0.0.419
6112	27.4.9	39.2.6	0.0.486

*: Mann-Whitney test adjusted by the Benjamini and Hochberg method

lower intensity than the after group; for the 5634 Da peak, two samples belonged to the before group range, 2 samples belonged to the after group, and the other 4 received much lower intensity than the after group (Table 4). As the 8 samples were those who received good efficiency of TACE, these results might show the two markers' good relationship with TACE therapy, and can be used as an indicator for efficiency of TACE therapy.

4.4. Identification of peaks of proteins by MS/MS

In this study, we employed LTQ-Orbitrap analysis to identify 1 out of the above 7 peptides which were differently expressed between before and after therapy groups. After fractionation by nanoAquity UPLC (Waters Corporation, Milford, USA), the eluted plasma samples were further purified by C18 beads with 5μm and 3.5.μm, then serially eluted with 5% and 95% acetonitrile. Samples were then subjected to LTQ Orbitrap XL MS/MS analysis. MS/MS analysis of 3883Da detected most b and y ions (Figure 5A). The ions (peptide 3883Da) detected from serum of sample gave higher MS ion signals in the before patient sera than in the after sera. The peptide was R.SARLNSQRLVFNRPFLMFIVDNNILFLGKVNRP.-, corresponding to plasma serine protease inhibitor precursor (Figure 5B).

5. DISCUSSION

Hepatocellular carcinoma (HCC) ranks first among the complications of liver cirrhosis and is the major cause for increased mortality in these patients (10). In patients with early-stage tumors, curative therapies can be applied, including resection, liver transplantation and percutaneous ablation; however, these treatments are applicable in only 30-40% of patients with HCC (7, 11). Transarterial chemoembolization (TACE) is a regional therapy performed under radiological guidance by infusion of chemotherapeutic agents, lipiodol and gel foam particles by an interventional radiologist. TACE exploits the tumour vasculature of hepatomas which are preferentially supplied by the hepatic artery. Data from several recently published studies are more encouraging and suggesting that TACE is superior to conservative treatment for HCC patients (11, 12). As a form of targeted therapy, TACE has been effective in reducing tumor burden.

Assessment of response to treatment is necessary for clinical management and is critical for the evaluation of clinical trials. At present, CT is the standard imaging technique for monitoring the effectiveness of TACE and radiofrequency ablation. Contrast-enhanced sonography and MRI can complement CT in the evaluation of the therapeutic response. Both imaging techniques have limitations in the evaluation of the therapeutic effect of HCC, which is important in determining if the treated tumor is completely necrotic or requires additional treatment. Furthermore, both of these treatments cost too much for most patients in China. The discussed method is less expensive and much easier to handle, which is highly expected. Thus, in this study, we attempted to develop an alternative to CT and MRI, and this new technique is expected to be a cost-effective method to assess TACE efficacy.

In light of this, we directly profiled protein/peptide patterns from affinity bead-purified plasma samples with MALDI-TOF-MS and determined several markers that can assess the efficiency of TACE therapy for hepatocellular carcinoma patients. We found 107 distinguishable peaks in the 1,000 to 10,000 m/z range, with 7 peaks having statistically significant differential expression (P<0.0.5). These protein/peptide fragments with high specificity and sensitivity may be good serum biomarkers for assessing TACE efficiency. For the top two discriminating peaks, their peak intensity dramatically decreased in the 8 samples with two runs of TACE, and they received very good therapy efficacy. Therefore, the two markers could be used as indicators for TACE therapy efficacy. To the best of our knowledge, this is the first description of serum markers for TACE therapy of hepatocellular carcinoma patients using affinity bead and MALDI-TOF-MS based proteomic techniques. However, the number of specimens analyzed in our study was relatively small, and that may limit the validity of these markers and utility in clinic. Hence, further independent validation studies with a larger sample size are needed to determine the utility of these markers. Meanwhile, two general categories of cellular proteins are released into the circulation: large proteins that are actively secreted and low-molecular-weight (LMW) proteins that enter the blood passively from cellular degradation or cleavage (12, 13). There is great interest in the LMW region as a source of diagnostic information, particularly substances smaller than 20 kDa.

High-throughput serum proteomic techniques, including 2DE-PAGE, SELDI-MS, and HCLP have been

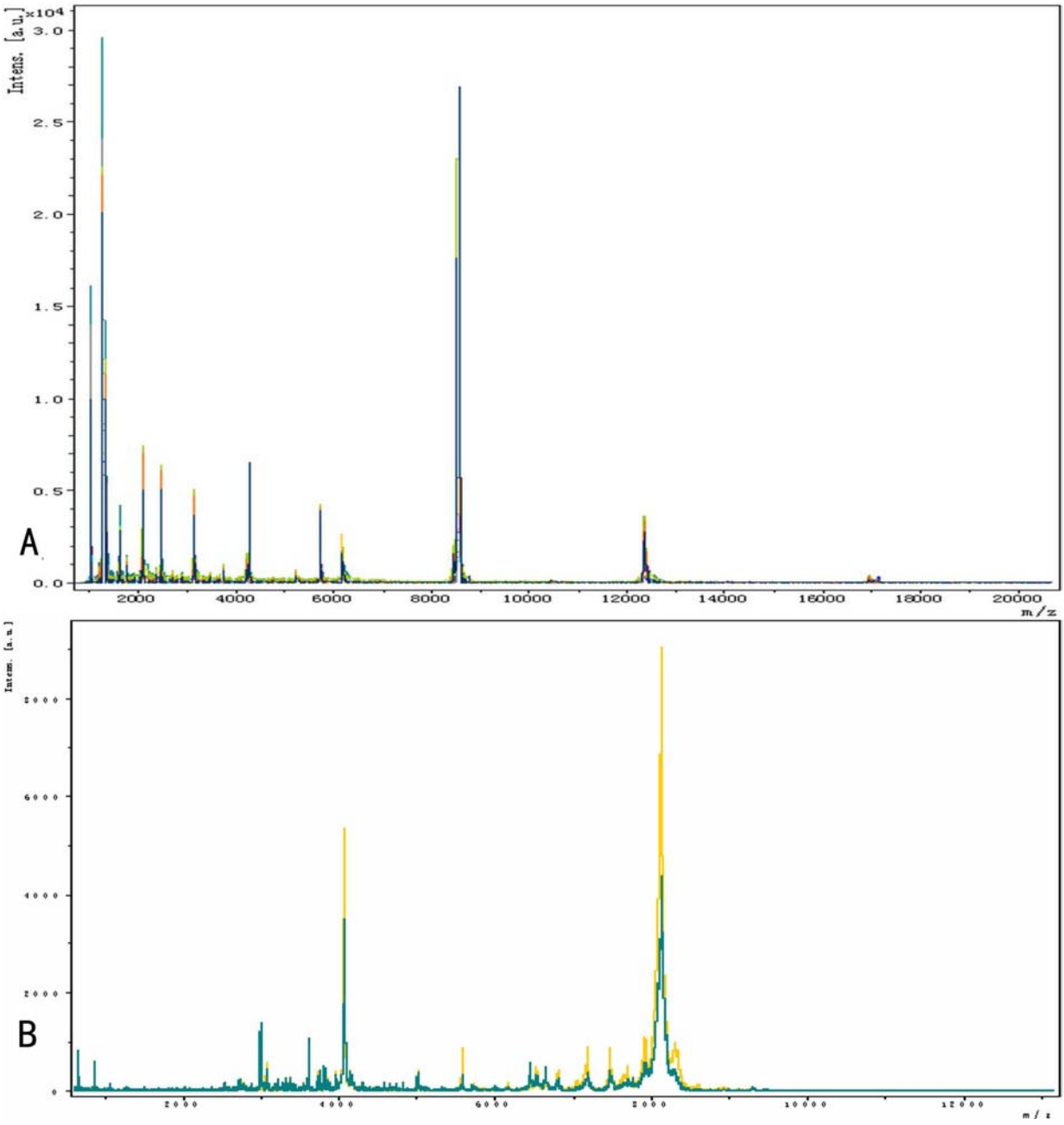

Figure 1. Mass spectrometry test: **(**A) Self-test for standards; (B) Self-test for standard serum.

used to study candidate serum biomarkers. 2D-PAGE enables simultaneous visualization of relatively large portions of the proteome and has been used to identify cancer markers. However, 2-DE-PAGE may not be clinically useful, due to its complexity. CLINPROT MALDI-TOF-MS, using magnetic beads affinity purification, is highly accurate and reproducible, with sensitivity which allows high throughput and is compatible with protein identification (14). It has been applied to the identification of many proteins associated with various solid organ malignancies, such as pancreatic cancer (15), and breast cancer (16). For gastric cancer, Ebert *et al* (15) used MALDI-TOF mass spectrometry after pre-fractionation of sera with magnetic hydrophobic C8 coated beads. A peptide fragment for m/z value 1465.6.4 Da was found to be highly elevated in cancer sera and was identified as fibrinopeptide A. As we know, identification of a biomarker is not only helpful for investigation of disease and prognosis, but also useful for therapy and drug development. In addition, if the markers could be identified and specific high affinity antibodies generated to them, then more direct and potentially less expensive methods for analysis could be developed. In this study, we compared serum proteomic profiles between before and after TACE

Table 4. The peak intensity of 3883 and 5634 Da for the 8 samples

Peak	Before	After	Case1	Case2	Case3	Case4	Case5	Case6	Case7	Case8
3883	58.9.4±26.3.6	42.4.8±15.4.8	12.2.0	16.8.	47.9.5	45.0.1	22.6.0	17.5.1	18.3.0	62.0.9
5643	20.2.6±8.3.	14.5.2±3.5.5	17.0.7	23.1.0	14.4.1	17.4.7	13.9.4	16.5.9	9.4.0	24.4.6

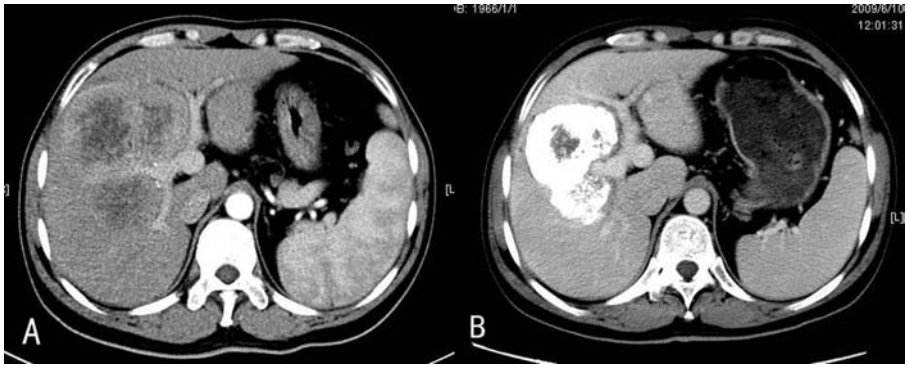

Figure 2. An example of CT scan figure for Hepatocellular Carcinoma Patients before and after TACE. Figure showed that the patients had received very good TACE therapy efficiency. (A): before TACE; (B): after TACE.

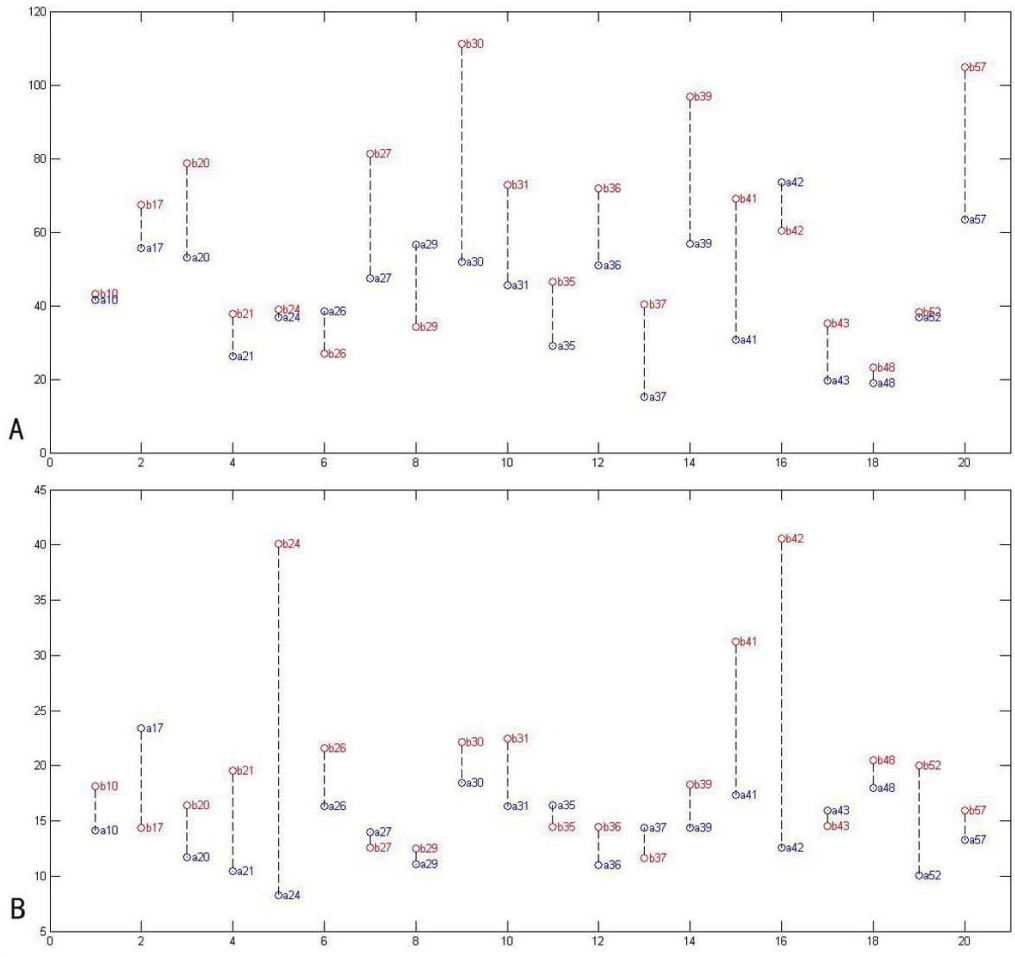

Figure 3. Mass spectra profiles of peak intensity with masses 3883.3.5 Da and 5633.9.5 Da. In both plots, the before group (b) was shown in red and the after group (a) in blue. In most cases, the peptide concentration (peak intensity) was much lower in the before group than in the after group.

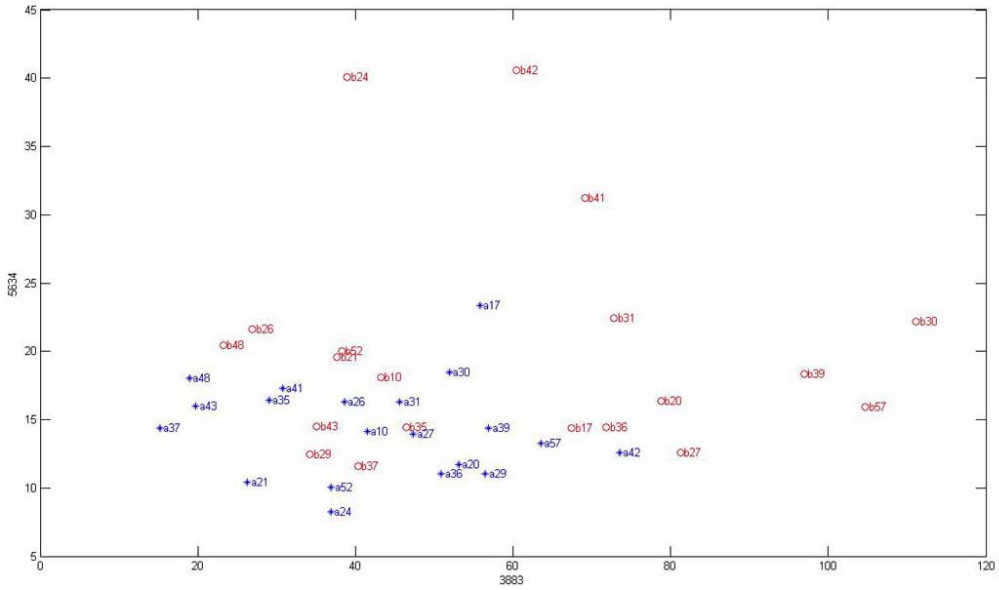

Figure 4. Bivariate plot of two protein peaks 3883 Da and 5634 Da showing significant distinction between distribution in before and after therapy data sets. Each of the green dots and the red crosses showed the sample of B group and A group for discovery set respectively. The x-axis represented 3883Da and the y-axis represented 5634Da in order to observe their ability of samples distribution.

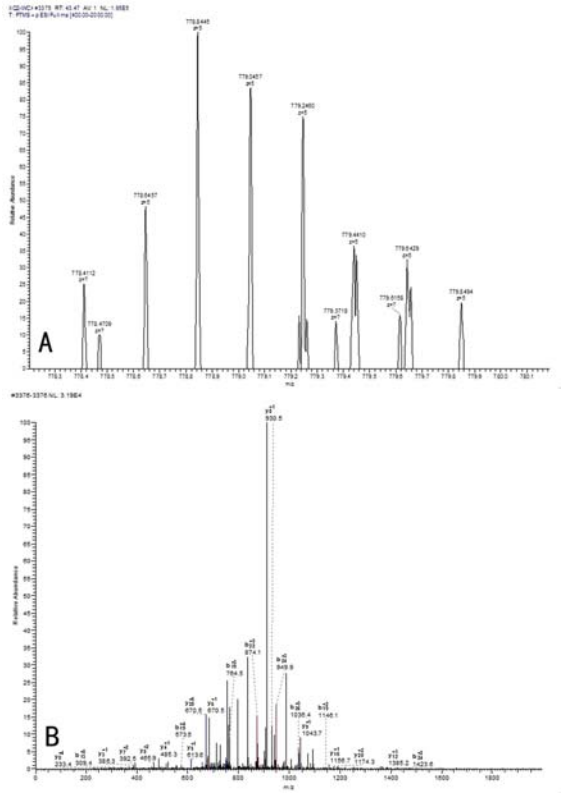

Figure 5. (A) MS/MS identification of serum peptides as fragment of plasma serine protease inhibitor precursor, single scan fragmentation spectrum of charge state of sera acquired in the orbitrap at 100,000 resolution (779.0.5 (5)); (B) MS/MS fragmentation spectrum analyzed in the orbitrap. The fragment ion spectrum shown here was taken for an MS/MS ion search of the International Protein Index (IPI Human3.4.5) database (http://www.ebi.ac.uk/IPI/IPIhelp.html). b and y fragment ion series are indicated together with the limited sequences.

for hepatocellular carcinoma patients to discover protein markers and establish the pattern for detecting efficiency of TACE. Furthermore, the differential peptide of 3883Da was identified as plasma serine protease inhibitor precursor (Protein C inhibitor). Protein C inhibitor (PCI) is a serine protease inhibitor (serpin) which limits the expression of protein C (17). It also efficiently inhibits coagulation factors such as factor Xa and thrombin. Protein C inhibitor has been shown to interact with Prostate specific antigen Protein C and PLAU (18). Recently it was found, using purified proteins, that the anticoagulant thrombin-thrombomodulin complex was also inhibited by PCI. The paradoxical inhibitory effect of PCI on both coagulant and anticoagulant proteases raised questions about the role of PCI in plasma. Reconstitution with a physiologic amount of PCI gave normal clotting times. Addition of PCI to normal plasma and protein C-deficient plasma resulted in a minor prolongation of the clotting time. This suggested that PCI can act as a weak coagulation inhibitor. This effect was less pronounced when protein C-deficient plasma was used, but could be restored by reconstitution with protein C. A concentration-dependent increase in clotting time was observed when PCI-deficient plasma was reconstituted with PCI. The combination of these results suggests that the major function of PCI in plasma during coagulation is the inhibition of thrombin. Although the physiologic roles played by PCI are unknown, research is underway to more clearly define the function(s) of PCI. Whatever answers are forthcoming from these studies, we predict that PCI may play an important role for TACE therapy efficiency of hepatocellular carcinoma.

In summary, we have described a novel serum proteomic technique using magnetic affinity beads to purify proteins before MALDI-TOF-MS analysis. This allowed identification of specific peaks within the protein spectra for comparison with control samples. As the 8 samples were those who received good efficiency of TACE, these results might show the two markers good relationship with TACE therapy, and can be used as indicator for efficiency of TACE therapy. The initial data is promising but a larger prospectively collected sample set, possibly from a multi-centre trial is necessary to validate and identify the differentially-expressed proteins we identified. So, our findings may have important implications for future peptide biomarker discovery for detecting efficiency therapy of hepatocellular carcinoma patients, and the identification of the candidate biomarkers and evaluation of their use as potential biomarkers will attract more attention.

6. ACKNOWLEDGEMENTS

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