行政院國家科學委員會專題研究計畫　成果報告

以 SELDI 來尋找人類肝細胞癌的生物標記

計畫類別： 個別型計畫
計畫編號： NSC93-2314-B-002-227-
執行期間： 93 年 08 月 01 日至 94 年 07 月 31 日
執行單位： 國立臺灣大學醫學院內科

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報告類型： 精簡報告

處理方式： 本計畫可公開查詢

中　華　民　國 94 年 10 月 24 日

(一) 研究計劃中文摘要：

肝細胞癌（簡稱肝癌）是全世界常見的癌之一，自從 1984 至今，肝癌就高居台灣十大癌症死因的首位，每年大約有 6000-8000 國人死於肝癌，然而肝癌的治療仍不十分的理想。肝癌治療的成效不佳的原因之一是發現太晚。用胎兒蛋白及 DCP 來早期診斷肝癌仍然不足，所以開發肝癌新的腫瘤標記仍然是需要的。基於此目的，本計劃將研發新的肝癌標記，以提高肝癌早期發現的比例。

SELDI-TOF MS，是美國CIPHERGEN公司根據MALDI-TOF MS為基礎改良出的產品，其主要特點為樣品可直接在晶片上進行樣品前處理，先在晶片上設計不同特性的化合物，可與特定種類蛋白質結合，去除非專一性結合蛋白質之後，直接串聯飛行時間-質譜儀進行分析，而得到一系列的圖譜。SELDI適合大量的蛋白質表現的分析，它適合不同來源的樣品比較，可將病人血液、尿液、組織萃取物等直接點在晶片上而不需破壞蛋白質，分析出來的圖譜與正常人圖譜比較，可得到蛋白質表現圖譜(protein expression profile)的差異，即可用來當作疾病的生物標誌(圖二)，而應用於臨床診斷上，甚至可進一步分析以找到新的生物標誌。目前已經成功的應用SELDI技術，在攝護腺癌、膀胱癌、卵巢癌、肺癌、大腸癌等，找到可能的癌症初期的生物標誌，如此便可早期治療以提高存活率。

在此計畫中，我們收集肝癌病人及對照組的血清，然後用 SEDLI 技術來分析。我們發現 C3a 在 C 型肝炎病毒感染的病人(尤其在 C 肝相關的肝癌病人)，有較高的表現。因此 C3a 有希望可以成為早期診斷肝癌的腫瘤標記。

(二) 研究計劃英文摘要：

Although the significant risk factors for hepatocellular carcinoma (HCC) are well known from epidemiological studies, diagnosis of this disease at an early stage is difficult and HCC remains one of the leading causes of cancer death worldwide. Thus, to identify any useful HCC-related biomarkers is still a need. We performed surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) to identify differentially expressed proteins in HCC serum using weak cation exchange (WCX2) protein chips. Protein characterization was performed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) separation and nano flow liquid chromatography tandem mass spectrometry (nano LC MS/MS). A total of fifty-six sera were collected from HCC patients and compared with forty-eight patients with chronic hepatitis and nine healthy individuals. A candidate marker with molecular weight about 8900 Da was detected as differentially expressed in patients with chronic hepatitis C and hepatitis C virus-related HCC. We identified this differentially expressed protein as complement C3a. The expression of C3a in HCC sera was further validated by PS20 chip immunoassay and Western blotting. Complement C3a was found to be elevated in patients with chronic hepatitis C and HCV-related HCC. Combination of SELDI-TOF MS and 2D-PAGE provide a solution to identify disease-associated serum biomarkers.

(三) 成果內容:

1 Introduction

Hepatocellular carcinoma (HCC) represents the fourth most common malignant tumor worldwide. Since 1984, it has been the leading cause of cancer death in Taiwan. About 6000-8000 people died of this cancer every year in Taiwan [1]. Alpha-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) are both used as the tumor markers for diagnosis of HCCs. However, both AFP and DCP are not ideal biomarkers for the early diagnosis of HCCs. To improve survival, further identification of useful diagnostic markers and the mechanisms of

hepatocarcinogenesis are very important. As advances in proteomics have occurred, a combination of protein separation technologies and mass spectrometry has provided opportunities for disease-associated marker identification and characterization. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) is a modification of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in which small amounts of protein are directly applied to a chip [2]. Recently, the SELDI approach has been successfully used to identify biomarkers for various cancers. Therefore, we applied the SELDI-TOF MS to globally analyze the low-molecular weight serum biomarkers that are associated with HCC.

2 Materials and Methods

2.1 Patients and serum samples

Total of 113 serum samples selected, thirty from patients with HBV-related HCC, twenty-six from patients with HCV-related HCC, eighteen from patients with chronic hepatitis B, thirty from patients with chronic hepatitis C and nine from normal controls were enrolled from National Taiwan University Hospital. The baseline characteristics of these patients were shown in table 1.

Table 1 Study population used in the SELDI experiment

group	Case no	Age	Gender (M/F)	Cirrhosis (%)	ALT (IU/ml)	AFP (ng/ml)	TNM staging no. (I, II, IIIA/IIIB, IVA/IVB)
HBV-HCC	30	53±12	26/4	63% (19/30)	79.0±92.2	3312.0±5922.1	(9, 16, 5/0, 0/1)
HCV- HCC	26	66±8	16/10	66% (17/26)	99.4±124.6	6928±18893.3	(3, 9, 10/0, 0/2)
CH-B	18	49±12	10/8	11% (2/18)	36.3±26.8	3.1±0.9	
CH-C	30	58±13	15/15	16% (5/30)	84.3±62.1	7.4±9.3	
Normal control	9	45±15	5/4	0% (0/9)			

2.2 ProteinChip analysis

Each serum sample was diluted and then ready to hybridize with Weak Cation Exchange 2 (WCX2) ProteinChip array. After the array surface was air-dried, 1 μL of saturated matrix α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile and 0.5% trifluoroacetic acid was applied and allowed to dry. Mass spectrometry analysis was performed on a PBS-II ProteinChip reader (Ciphergen Biosystems).

2.3 Data analysis

Mass peak detection was analyzed using ProteinChip Biomarker Software version 3.0.2 (Ciphergen Biosystems).

2.4 Protein identification by in-gel digestion and mass spectrometry

Proteins of interests were separated by 2D electrophoresis. The Coomassie blue-stained protein spots corresponding to candidate biomarker was excised and digested with sequencing-grade trypsin. Protein identification was carried out by nanoLC-ESI-MS/MS analysis. Peptides were characterized using a Qstar XL *Q-TOF* mass spectrometer (Applied Biosystems, Foster city, CA). Proteins were identified by an automated searching algorithm against the NCBI protein database using Mascot software (Matrix Science Inc., Boston, MA).

2.5 SELDI immunoassay

To confirm the identity of candidate biomarkers, 0.2-0.5 μg of C3a monoclonal antibody was applied on each spot of a PS20 ProteinChip and incubated for 16 h at 4 ℃ in a humidity chamber.

2.6 C3a Western blotting

To further confirm the protein chip immunoassay result, C3a Western blotting was done.

3 Results

3.1 HCC serum protein profiling by SELDI-TOF MS

Using a weak cation exchange (WCX2) chip, we identified proteins differentially expressed between sera from normal individuals, sera from chronic hepatitis patients and sera from HCC patients. From the result of SELDI-TOF MS analysis, 77 peak clusters were generated and one-way ANOVA statistical method was used to test if any peak cluster intensity was significantly different.

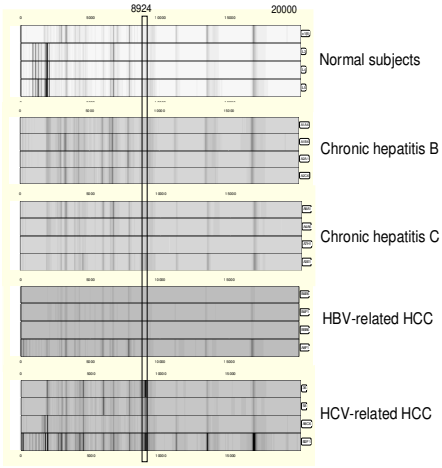

Figure 1

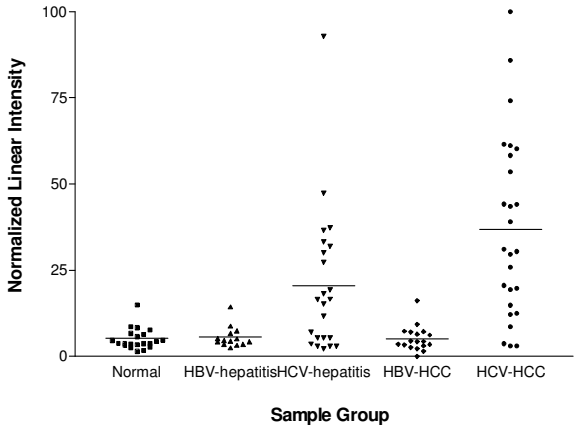

Figure 2

As shown in Fig. 1, we identified a peak with a molecular weight of about 8920 Da that was differentially expressed in the sera from the HCV-related liver disease patients. A scatter plot of the normalized linear intensity of the candidate 8.9 kDa marker in all of the study cases is shown in Fig. 2, and the mean intensities of cases within each group are indicated by the bar. The mean intensities ± standard deviation are as follows: 2.44±1.68 (normal), 2.55± 1.61 (chronic hepatitis B), 10.05±9.09 (chronic hepatitis C), 2.15±1.47 (HBV-related HCC) and 18.41±19.55 (HCV-related HCC), respectively. As shown in Table 2, The mean intensity of the 8.9 kDa marker was higher in patients with chronic hepatitis C than that in the normal controls (p=0.0009, Table 2) as well as higher in patients with HCV-HCC than that in patients with chronic hepatitis C (p=0.03). The mean intensities between normal, chronic hepatitis B and HBV-related HCC were not statistically significant. These results suggest that the appearance of the serum 8.9 kDa marker may have a correlation with HCV-infection hepatitis and/or HCV-related HCC.

Table 2

Group pairs	*p* value
Normal vs. Chronic hepatitis B	0.87
Normal vs. Chronic hepatitis C	0.0009^{*}
Normal vs. HBV-HCC	0.34
Normal vs. HCV-HCC	$< 1\times10^{-5*}$

Chronic hepatitis B vs. Chronic hepatitis C	0.001*
Chronic hepatitis C vs. HCV-HCC	0.03*
HBV-HCC vs. HCV-HCC	$< 1\times10^{-5}$*

*, statistically significant

3.2 Identification of candidate 8.9 kDa marker using 2D-PAGE and mass spectrometry

Following the determination of the range of the 8.9 kDa protein's pI, 2D-PAGE was applied to separate this protein from the whole serum. After comparing the two gels images from a normal individual and patient No. 36 (HCV-HCC) for the serum proteins, we identified a differentially expressed protein with molecular weight located between 6.5 kDa and 14 kDa and pI value near 9 (Fig. 3). This protein was excised and subjected to in-gel trypsin digestion, and the internal peptide sequences were determined by nano LC MS/MS. The database search result showed two of the differentially expressed protein internal sequences matched that of human complement C3a (Fig. 4).

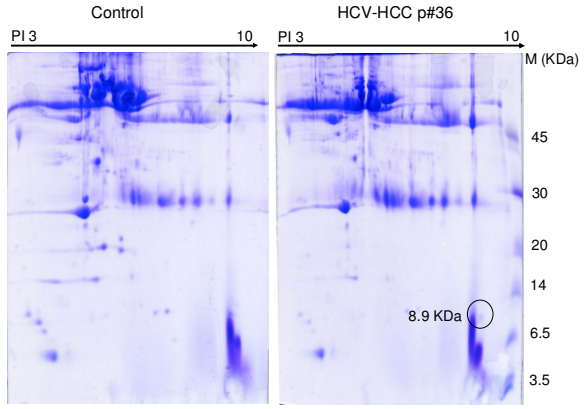

Figure 3

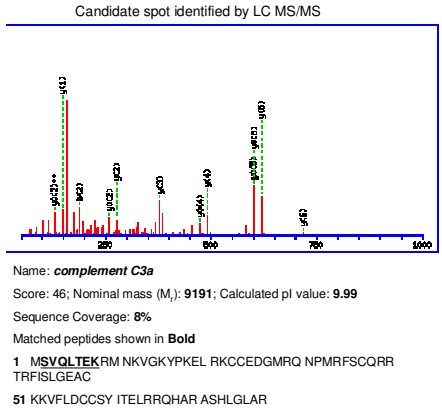

Figure 4

3.4 Validation of complement C3a expression

To confirm that the 8920 Da protein identified by nano LC MS/MS was C3a, we performed SELDI immunoassay with immobilization of a specific mouse anti-human C3a monoclonal antibody on a PS20 ProteinChip. The result showed that C3a was captured and detected for a HCV-related HCC (patient No. 36) serum, but not from a normal serum.

3.5 Complement C3a is elevated in sera from patients with chronic hepatitis C and HCV-related HCC

In order to characterize the role of C3a role in liver inflammation and carcinogenesis, we correlated C3a intensity with alanine aminotransferase (ALT) values in chronic hepatitis C and HCV-related HCC groups. The results showed no relationship between C3a intensities and ALT levels in either of the two groups (data not shown). In addition, the C3a did not correlate with tumor size, AFP level or cirrhosis in the HCV-related HCC group (data not shown). Because C3a appeared to be up-regulated in the serum of patients with chronic hepatitis C and HCV-related HCC from SELDI analysis, it deserved to be validated the level of expression in the subset sera of HCV-related disease. A higher percentage and intensity of the C3a signal was found in the sera of HCV-related disease groups (Fig. 5A). We also performed a semi-quantitative method of Western blot to detect serum C3a level from some patients with HCV carrier, HCV cirrhosis, HCV-HCC with or without cirrhosis. Fig 6 shows quantification of the data represented in Fig. 5B. The results from Western blot are consistent with SELDI data. Although C3a did not correlate with known clinical parameters, it may be an independent marker for chronic hepatitis C and HCV-related HCC.

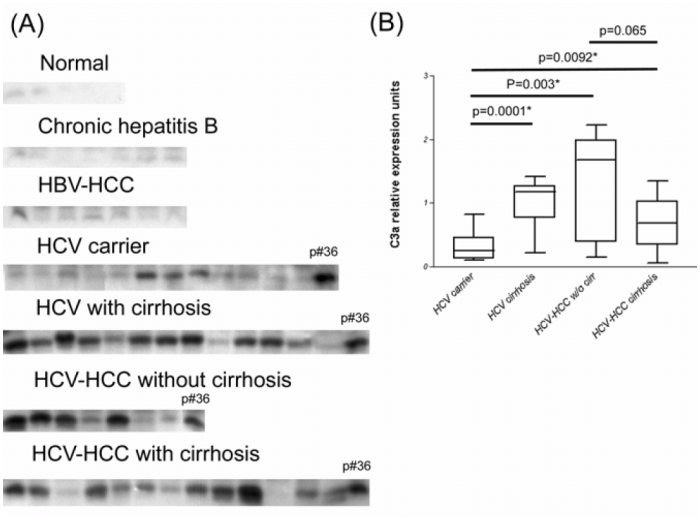

Figure 5

4 Discussion

Though several studies have applied proteomic methods to investigate the biomarkers of HCC, most of them have used traditional 2D electrophoresis methods [3-9]. Only limited studies have used SELDI-TOF MS to explore biomarkers in human HCC. Poon *et al*. used IMAC3 Cu and WCX2 chips to identify tumor-specific proteomic signatures associated with the differentiation of HCC from chronic liver disease without HCC [10]. The results indicated a 8944 m/z polypeptide with the higher intensities in the serum proteomes of HCC cases compared with chronic liver disease cases. Paradis *et al*. used IMAC-Zn chips to identify serum biomarkers between cirrhotic patients with and without HCC, and found the highest discriminating peak at 8900 Da [11]. The protein was purified further and was characterized as the C-terminal part of the V10 fragment of vitronectin. An *in vitro* study suggested that the increase in the 8900-Da fragment among the serum of patients with HCC may proceed from the cleavage of native vitronectin by metalloprotease. Schwegler *et al*. used IMAC-Cu chips and found that 38 differentially expressed proteins could be used to generate multiple decision classification trees to distinguish known liver disease states [12]. For distinguishing chronic HCV from HCV-HCC, a sensitivity of 61% and a specificity of 76% were obtained. Interestingly, an 8,939 m/z protein was found to have increased following HCV infection and was actually highest in the sera from HCV-associated cirrhosis subjects. However, they did not identify the 8.9 kDa candidate.

We adopted a similar strategy to identify HCC-related biomarkers, however, different chip surface (WCX2), serum fractionation and 2D-PAGE were performed. We also found that increased expression of 8.9 kDa protein was detected in the sera of chronic hepatitis C patients and HCV-related HCC patients. Interestingly, all the above mentioned studies found serum protein(s) with a molecular weight near 8.9 kDa that correlated with HCC. In this study, we demonstrated that C3a could be a potential marker in chronic hepatitis C and HCV-related HCC patients.

Complement components are important mediators of inflammation and contribute to the regulation of the immune response. In cancer patients, complement activation with subsequent deposition of complement components on tumor tissue has been demonstrated [13]. Human C3, the most abundant complement protein in serum (about 1.2 mg/ml), is made up of α and β chains [14]. Proteolytic activation of native C3 occurs by either the classical/lectin (C4b,2a) or alternative (C3b,Bb) pathway. C3 convertase leads to cleavage C3 between residues 726 and 727 (Arg–Ser) and generates C3b (Mr 176,000) and an N-terminal

fragment, C3a (Mr 9,000) [14]. C3a has been reported as a potent inflammatory mediator of innate immune response, and contributes essentially to the early priming stages of hepatocyte regeneration after toxic injury and partial hepatectomy [15]. A previous study has also demonstrated that complement activation products (C3a, C3b/iC3b) are generated in the serum of experimental mice after CCl_4 injection and that complement activation is required for normal liver regeneration [16]. In this regards, C3a might represent a marker of inflammation.

A recent report has also shown that elevated levels of C3a were found in the ascitic fluids of ovarian cancer patients [17]. Thus, C3a could be a marker related to carcinogenesis. Steel *et al*. used 2D-PAGE and MALDI-TOF MS to search HCC biomarkers and found that a carboxy terminal fragment of complement C3 was down-regulated in HBV-related HCC [18]. The C3 fragment was not identical to C3a based on differences in the molecular weight and pI.

Another interesting point in this study is that C3a was upregulated only in HCV-related HCC, but not in HBV-related HCC. The difference was not related to the serum ALT level. The exact explanation of this paradox is not known. It has been, however, well-documented that there are many differences between HBV-related HCC and HCV-related HCC. The molecular hepatocarcinogenesis might be different between HBV-related and HCV-related HCC and therefore the effect on the expression levels of C3a might also be different. However, this needs further investigation.

In conclusion, we applied SELDI and 2D technologies and identify complement C3a as a potential low-molecular weight serum biomarker associated with chronic hepatitis C and HCV-related HCC.

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(五) 計劃成果自評

In this study, we finished all the goals we set and we demonstrated that C3a could be a potential markers relating to HCC-HCC. The revised manuscript of this study has been sent to the journal of Proteomics. Whether C3a could be a potential marker for early diagnosis of HCC needs further confirmation.