行政院國家科學委員會專題研究計畫　成果報告

以 PROTEOMEX 來尋找人類肝細胞癌的生物標記

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(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，雖然目前以超音波及甲種胎兒蛋白定期追蹤檢查，可以早期篩檢出肝癌，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。雖然胎兒蛋白及 DCP 都被用來做來診斷肝癌的腫瘤標記，可惜的是大約在 1/3 的小型肝癌病人，其血中的胎兒蛋白仍是正常的，因此用胎兒蛋白及 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。

最近幾年對於癌症的基因研究，主要是用比較性雜交法、微小衛星分析、以及微陣列來分析基因表現。雖然這些都是分析基因表現很好的工具，主要的缺點就是，所分析出來的東西是 mRNA，而 mRNA 的表現不一定與蛋白質的表現平行。生物功能真正執行者為蛋白質，它的很多特性不是由 DNA 或 RNA 分析就可得知，比如近年來最熱門的訊息傳導，涉及蛋白質的磷酸化，必需靠直接蛋白質分析，因此蛋白質的實驗分析，在功能上有絕對的需要。蛋白質體研究，包括了細胞內蛋白質的定性跟定量變化以及蛋白質間交互作用等，可以說是在後基因體時代的新型態研究工作，蛋白質體學可以用來研發癌症新的腫瘤標記。PROTEOMEX 或稱為 SERPA 是一種結合傳統蛋白質體分析與血清學的新的方法，此方法利用是先用二次元電泳將蛋白質分離，將已分離的蛋白質轉移到膜上，然後再以病人或對照組的血清來做西方點墨，此方法可以用來找尋癌症病人新的診斷及治療腫瘤標記。

在此計畫中我們利用 PROTEOMEX 來尋找肝癌的腫瘤標記。我們萃取肝癌細胞的蛋白質，用二次元電泳將蛋白質分離，將已分離的蛋白質轉移到膜上，然後再以肝癌病人或慢性 B 型肝炎但無肝癌的病人，以及無肝病的正常者的血清來做西方點墨。依西方點墨結果選出有興趣的點，再用質譜儀或胺基酸定序儀分析其胺基酸序列，即可判別原蛋白質的身分。我們總共找到 27 個可能與肝癌相關的腫瘤標記，如果這些腫瘤標記能進一步被證實的確能做為肝癌的早期標記進，我們將可提升肝癌治療的成績。

(二) 研究計劃英文摘要：

Hepatocellular carcinoma (HCC) is a major cause of cancer-related death in Taiwan. The major risk factors identified in the development of HCC are persistent hepatitis caused by infection of hepatitis B virus (HBV) or hepatitis C virus (HCV). Hepatocarcinogenesis was preceded by a long period of subclinical stage, and diagnosis of this disease was often at late stage. The 5-year survival rate for HCC patients is below 50%. Current methods for the diagnosis of HCC main rely on serological markers such as alpha-fetoprotein (AFP) and certain liver enzymes, together with ultrasonography. However, AFP index has poor specificity. Searching new markers for HCC diagnosis and monitoring this disease is urgent. To identify tumor antigens presented by HCC, we used a proteomic approach combined serological and 2D-SDS/PAGE techniques. HCC patient sera serve as antibodies, and tumor tissue protein as antigens. Numerous circulating anti-tumor antibodies have been described in the serum of patients for a variety of cancers. In this study, 20 HCC serum samples and 10 HCC surgical tumor tissues were collected. Tumor proteins were extracted and separated by 2D-SDS/PAGE, and then were transferred to PVDF membranes for immunodetection. Comparison of autoantibody responses generated by HCC patients and normal subjects made us identify the differentially HCC candidate antigen spots. Following in-gel digestion and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), we have identified 27 candidate antigens that may be involved in chronic hepatitis B and HBV-related HCC. Characterization of these candidate antigens may provide insight the carcinogenesis of HCC and improve diagnosis.

(三) 成果內容:

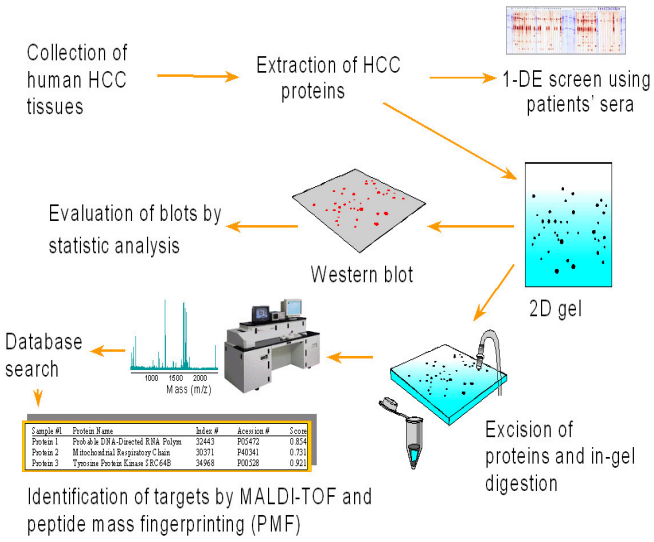
INTRODUCTION

Hepatocellular carcinoma (HCC) is one the most common malignancies in the world. 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). However, the therapeutic results remain unsatisfactory. Alpha-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) are used as the tumor markers for diagnosis of HCCs. However, both the AFP and DCP thus are not ideal biomarkers for the early diagnosis of HCCs. To improve the survival, further investigations of the early diagnostic markers is very important.

The global analysis of cellular proteins has recently been termed proteomics and is a key area of research that is developing in the postgenomic era (2, 3). With respect to cancer, proteomics has the potential to identify novel targets for therapy or markers for diagnosis. The proteome reflects both the intrinsic genetic programme of the cell and the impact of its immediate environment and is therefore valuable in biomarker discovery (4).The proteomic profiling techniques in biomarker discovery include (1). 2-D PAGE / MALDI-MS (two-dimensional gel electrophoresis, polyacrylamide gel electrophoresis) / matrix assisted laser desorption/ionization-mass spectrometry), (2). LC/MS/MS (liquid chromatography/MS/MS), (3). SELDI-TOF (surface-enhanced laser desorption ionization time-of-flight) (5). These techniques have their own advantages and shortcomings. They are complementary to each others in the identification of biomarkers. Proteomic approaches are likely to discover a wide range of new cancer biomarkers that can be used for the early diagnosis of cancers (6).

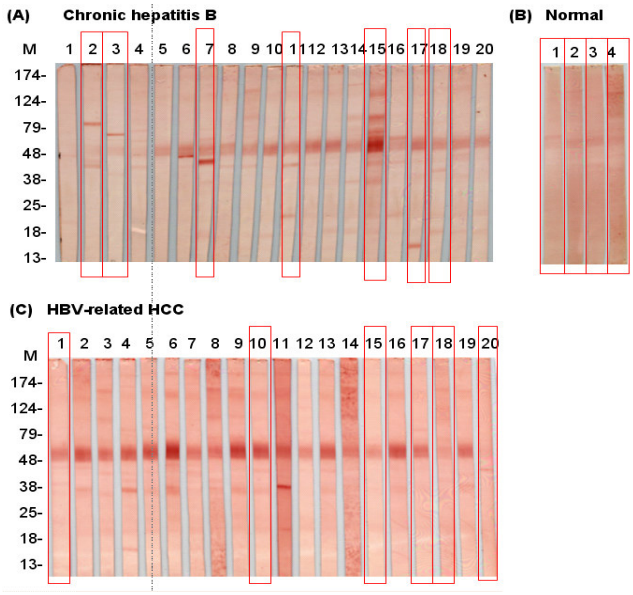
PROTEOMEX or SERPA (serological proteome analysis) is a combination of conventional proteome analysis with serology. This approach employs 2-D PAGE allowing the separation of individual cellular proteins from both tumor as well as corresponding normal tissue. The separated proteins were then transferred onto membranes which were subsequently incubated with sera from cancer patients or normal healthy volunteers (7, 8). PROTEOMEX can be used for the identification of diagnostic and prognostic markers in cancers. PROTEOMEX has been successfully applied to find serum markers / tumor associated antigens (9-12). In this current project, we applied the PROTEOMEX to identify the HCC biomarkers.

MATERIALS AND METHODS

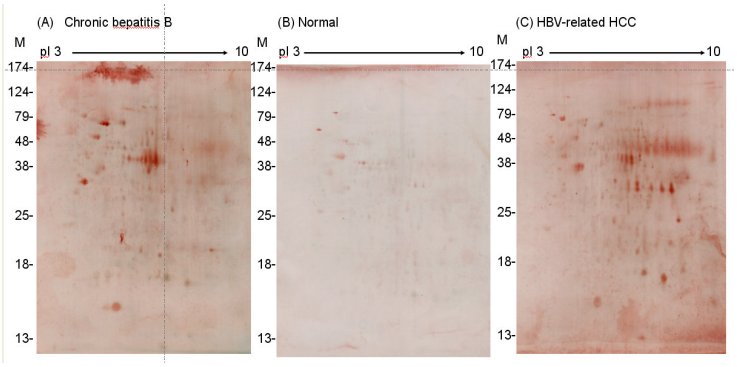


In this study, 20 HCC serum samples and 10 HCC surgical tumor tissues were collected. The experimental strategy was illustrated in the figure. Tumor proteins were extracted and separated by 2D-SDS/PAGE, and then were transferred to PVDF membranes for immunodetection. Comparison of autoantibody responses generated by HCC patients and normal subjects made us identify the differentially HCC candidate antigen spots. Following in-gel digestion and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF).

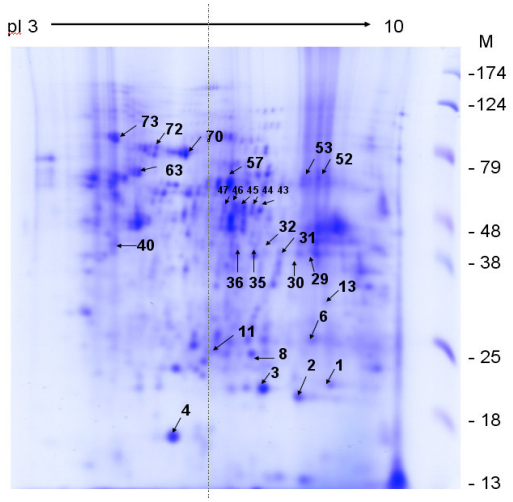
RESULTS



Screening of HCC antigen-antibody response by Western blottting. Crude HCC proteins were transferred onto PVDF membrane, and cut into strips as antigens for separate serum IgG hybridization. We obtained 20 sera from (A) chronic hepatitis B patients, 4 sera from (B) healthy volunteers and 20 sera from (C) HBV-related HCC patients. Western blot was detected by a colorimetric method, and 3-amino-9-ethyl carbazole was used as developing substrate. Red-boxed serum were pooled for further 2D-western blotting.



Isolation of candidate antigens by 2D-western blotting. Crude HCC proteins were separated by 1D-IEF(isoelectric focusing) according to protein pI, and 11% SDS-PAGE in second dimension. 13 cm IPG strip, pH3-10 was used. (A) 7 pooled sera form chronic hepatitis B patients, (B) 4 pooled sera from healthy volunteers and (C) 6 pooled sera from HBV-related HCC patients.



Identification of candidate antigens by MALDI-TOF mass spectrometry. 1 mg HCC proteins were resolved by 2D-SDS/PAGE, and the gel was stained with Coomassie blue. Protein spots were excised from the gel, and subjected to in-gel digestion and mass spectrometric analysis. The identified proteins were shown in table 1.

Table 1. Identification of candidate autoantigens in HBV-related HCC by MALDI-TOF mass spectrometry

Spot no	Protein name	M.W./pI
1	MYEF2 protein	22.44/9.4
2	proliferation-inducing gene 17	20.09/8.9
3	manganese-containing superoxide dismutase	23.67/6.9
4	antigen MLAA-35	16.43/6.8
6	tyrosyl-DNA phosphodiesterase protein	21.27/9.4
8	MYL3 protein	21.93/5.0
11	peroxiredoxin 6	25.04/6.0
13	MTO1 isoform 2	34.29/9.1
29	HSPC235	40.15/8.9
30	LACTB protein	36.49/8.7
31	atopy related autoantigen	35.98/7.2
32	MSTP003	38.21/8.0
35	unnamed protein product	39.90/8.3
36	transcription elongation factor A-like 4	40.58/7.0
40	LOC493860 protein	45.48/5.4
43	HNRPM protein	44.44/8.7
44	ZNFN1A1 protein	52.71/5.9
45	LRRFIP1 protein	58.24/4.8
46	LOC90379 protein	57.89/5.2
47	caspase 10	54.52/6.6
52	chronic myelogeneous leukemia tumor antigen	63.52/5.0
53	aldehyde dehydrogenase	51.84/7.6
57	KIAA1389 protein	61.28/8.6
63	heat shock protein 60	61.06/5.7
70	acyl-CoA oxidase 3	77.55/7.3
72	heat shock protein 70 kDa	73.68/5.9
73	glucose-regulated protein, 78 kDa	72.33/5.1

DISCUSSION

In this study, we have successfully identified 27 candidate antigens that may be involved in HBV-related HCC. Some of them, such heat shock protein families, have been demonstrated to be associated with HCC. Characterization of these candidate antigens may provide insight the carcinogenesis of HCC and improve diagnosis. Confirmations of these potential biomarkers are in progress.

(四) 參考文獻

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(五) 計劃成果自評

In this study, we finished most of all the goals we set. We found 27 potential biomarkers associated with HCCs. The confirmation of these biomarkers will be finished soon in the near future.