行政院國家科學委員會專題研究計畫　成果報告

以螢光二次元差異性電泳及質譜儀來分析肝癌與其相對應的非癌肝組織，蛋白質表現的差異

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計畫主持人：許金川

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（一）研究計劃中文摘要：

關鍵詞：肝細胞癌，蛋白質體學，二次元差異性電泳，質譜儀

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。預後不良的原因，除了發現太晚之外，肝癌復發也是一個很重要的原因。因此如果想要提升肝癌的治療成績，進一步研究肝癌的制癌機轉及找尋更好的早期診斷標記仍是必須的。

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。

蛋白質體研究可以說是在後基因體時代的新型態研究工作。二次元電泳分析可以說是過去幾年蛋白質體研究的主流。從一個細胞樣本中，把總體蛋白質抽取出來，以二次元電泳分析之，定出所要找的目標蛋白質色點；切出此色點後，可直接在膠體中以專一性蛋白胺水解之，並以 HPLC 或毛細管電泳分離出單一胜汰片段，此一片段可用質譜儀或胺基酸定序儀分析其胺基酸序列，所得胺基酸序列由電腦資料庫中搜尋，即可判別原蛋白質的身分。雖然二次元電泳分析已被廣泛的應用在蛋白質體研究，然而沒有任何兩片的蛋白質膠體是完全一樣的，這使得不同膠片間與不同實驗室間的資料，很難做正確的比對。

螢光二次元差異性電泳可以彌補上述的缺點。這個技術主要是奠基於使用 Cy3 與 Cy5 這兩種不同的螢光物，來標示要比較的兩種蛋白質體，然後將這兩種蛋白質體在同一片膠上跑電泳，然後再用電腦軟體來分析這兩種螢光的強度差異，用此來反應蛋白質表現量的差異。將有差異的點切出，以專一性蛋白胺水解之，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。由於這兩個蛋白質體是在同一片膠上跑電泳，所以分析時可以避開膠與膠之間的差異性，使得分析的結果較可靠，同時也可以做跨膠之間的比較。

在此計畫中，我們用 8 對肝癌與其相對應的非癌肝組織，來做螢光二次元差異性電泳分析。我們發現，有 9 個蛋白質(例如 heat shock protein)在肝癌組織的表現相對較高，11 個蛋白質在肝癌組織的表現相對較低。我們目前正在進一步確定這些蛋白是否真正可以作為肝癌的腫瘤標記。

（二）研究計劃英文摘要：

Keywords：hepatocellular carcinoma, proteomics, differential gel electrophoresis, DIGE, MALDI, mass spectrometry

Hepatocellular carcinoma (HCC) has been the leading cause of cancer death in Taiwan. About 6000-8000 people died of this cancer every year in Taiwan. Though regular sonographic examination can early detect small HCC and there are many therapeutic modalities for HCC, the therapeutic results remains unsatisfactory. To improve the survival, further investigation of the early diagnostic markers and the mechanisms of hepatocarcinogenesis is very important.

In the recent years, investigating the genome-wide expression profiles of cancers has been the predominant method to identify cancer-related genes. Though using cDNA microarray for genome-wide expression profiling is a very powerful tool to clarify the genetic changes in cancers, the major pitfall of these methods is that the mRNA expression does not parallel protein expression in many cases.

The introduction of fluorescent 2D differential gel electrophoresis (DIGE) has now made it possible to detect and quantitate differences between experimental pairs of samples resolved on the same 2D gel. The basis of this technique is to use two fluorescent dyes (Cy3 and Cy5) to differentially label lysine residues of two protein samples for comparative analysis on a single gel. The ability to directly compare two samples on the same gel not only avoids the complications of gel-to-gel variation but also enables a more accurate and rapid analysis of differences and reduces the number of gels that need to be run. Following automated image analysis, using the novel and innovative software, spots of interest are selected for gel excision, subjected to in-gel enzymatic digestion, and mass spectrometry identification.

In this current project, we enrolled eight paired of HCC and the corresponding non-tumor liver tissues, and subjected to DIGE analysis. We found that 9 proteins (eg. heat shock protein) were upregulated in the HCC tissues, while 11 proteins were downregulated in the HCC tissues. We are currently to investigate whether these proteins could be used as the new HCC diagnostic markers.

（三）成果內容:

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide, mainly in South Africa and Southeast Asia. Major risk factors are chronic hepatitis resulting from infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) and exposure to various carcinogens including aflatoxin B1 (1). However, the molecular mechanisms underlying HCC in most patients remain unclear.

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. With respect to cancer, proteomics has the potential to identify novel targets for therapy or markers for diagnosis. Methods of proteomics at present involve combination of serial sophisticated techniques including two-dimensional gel electrophoresis (2-DE), image analysis, mass spectrometry, amino acid sequencing, and bioinformatics to resolve comprehensively, to quantify, and to characterize proteins. However, no two gel images are directly superimposable. This limitation makes image comparison complex and difficult to determine what are the true differences between these two gels.

The introduction of fluorescent 2D differential gel electrophoresis (DIGE) by Unlu et al. (17), which is further modified by Amersham Biosciences, Inc, has now made it possible to detect and quantitate differences between experimental pairs of samples resolved on the same 2D gel. The basis of this technique is to use two fluorescent dyes (Cy3 and Cy5) to differentially label lysine residues of two protein samples for comparative analysis on a single gel. A third fluorescent dye (Cy2) has also been introduced to label the pooling of aliquots of all biological samples in the experiment.

This Cy2-labeled proteins are used as the internal standard. The ability to directly compare two samples on the same gel not only avoids the complications of gel-to-gel variation but also enables a more accurate and rapid analysis of differences and reduces the number of gels that need to be run.

Materials and Methods

Protein extraction from liver tissues

A total of eight HCC and the corresponding non-tumor liver tissues were enrolled. The liver tissues were ground in a mortar filled with liquid nitrogen, then the protein were extracted. Adjust the pH to 8-9 to fit the condition for the subsequent dye labeling. The protein concentrations of the homogenates are determined using the Coomassie protein assay reagent (Pierce).

Protein labeling

Cell lysates are labeled with N-hydroxy succinimidyl ester-derivatives of the cyanine dyes Cy2, Cy3, and Cy5 (Amersham Biosciences, Inc.) according to the manufacture's protocol. Typically, 100 μg of lysate is minimally labeled with 400 pmol of either Cy3 or Cy5 for comparison on the same 2D gel. Labeling reactions are performed on ice in the dark for 30 min and then quenched with a 50-fold molar excess of free lysine to dye for 10 min on ice. Differentially labeled samples are mixed and reduced with 65 mM dithiothreitol for 15 min. Ampholines/pharmalytes, pH 3–10 (1% (v/v) each; Amersham Biosciences, Inc.), and bromphenol blue are added, and the final volume was adjusted to 350 μl with lysis buffer. A pool of all samples is also prepared and labeled with Cy2 to be used as a standard on all gels to aid image matching and cross-gel statistical analysis. The Cy3 and Cy5 labeling reactions (100 μg of each) from each time point are mixed and run on the same gels with an equal amount (100 μg) of Cy2-labeled standard.

Protein Separation by 2D Gel Electrophoresis

Immobilized non-linear pH gradient (IPG) strips, pH 3–10 (Amersham Biosciences, Inc.), are rehydrated with Cy-labeled samples in the dark at room temperature overnight, according to the manufacturers guidelines. Isoelectric focusing is performed using a IPGphor II apparatus (Amersham Biosciences, Inc.) for a total of 80 kV-h at 20 °C, 10 mA. Strips are equilibrated for 15 min in 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 1% (w/v) SDS containing 65 mM dithiothreitol and then for 15 min in the same buffer containing 240 mM iodoacetamide. Equilibrated IPG strips are transferred onto 18X20 cm 12% uniform polyacrylamide gels poured between low fluorescence glass plates. Strips are overlaid with 0.5% (w/v) low melting point agarose in running buffer containing bromphenol blue. Gels are run in Protean II gel tanks (Bio-Rad) at 30 mA per gel at 10 °C until the dye front had run off the bottom of the gels.

Gel Imaging

2D gels are scanned directly between glass plates using the highly sensitive Typhoon 9400 Variable Mode Imager (Amersham Biosciences, Inc.) according to the manufacture's protocol. Gels are fixed in 30% (v/v) methanol, 7.5% (v/v) acetic acid overnight and washed in water, and total protein is detected by post-staining with Sypro-Ruby dye (Molecular Probes) for 3 h at room temperature. Excess dye was removed by washing twice in water, and gels are imaged at the appropriate excitation and emission wavelengths for the stain.

Images analysis

The images are analyzed with DeCyder software (Amersham Biosciences, Inc.) according to the manufacture's protocol. The DeCyder software consists of two analysis modules: Differential In-gel Analysis and Biological Variation Analysis. DeCyder automatically detects, matches and analyzes protein spots in multiplexed fluorescent images, and is able to give routine detection of <10% differences with >95% confidence. Statistical analysis is carried out on each and every difference.

Protein Identification by delayed extraction matrix-assisted laser desorption ionization (MALDI) MS

Changes observed by 2D-DIGE analyses are aligned with Sypro-Ruby protein patterns, and spots are selected for picking according to this post-stained image. Spots of interest are excised from 2D gels using an automated spot picker (Amersham Biosciences, Inc.) following the manufacturer's instructions.

The excised spots are subjected to in-gel tryptic digestion. The digests are mixed with saturated α-cyano-4-hydroxycinnamic acid solution in acetonitrile/H2O and spotted onto a MALDI sample plate, then MALDI MS analysis is performed on a Voyager DE-STR workstation (PerSeptive Biosystems, Framingham, MA) equipped with a 337-nm nitrogen laser. The peptide spectra, acquired in reflectron mode at an accelerating voltage of 20 kV, were the sum of 50 laser shots. The mass spectra are externally calibrated using low mass peptide standards. This procedure typically results in mass accuracies of 50–100 ppm. The peptide mass fingerprint data are compared with those in the National Center for Biotechnology Information nonredundant protein database using the MS-Fit search tool (University of California San Francisco Mass Spectrometry Facility, San Francisco, CA).

Result

HCC and their corresponding nontumor tissue were obtained from eight patients (Table 1) who received surgical resection, and samples were homogenized and then total proteins were extracted as described in Materials and Methods. Protein expression was compared between each tumor/nontumor pair utilizing DIGE technology with a mixed-sample internal standard. As shown in Table 2, protein extracts were labeled with either Cy3 or Cy5 fluorescence dyes, and then each patient's Cy3/Cy5-labeled sample pair was mixed with a Cy2-labeled sample containing an equal mixture of all 16 samples prior to running all three samples together on each of 8 individual gels. In order to get more reliable data, we interchange the Cy3 or Cy5 for labeling each tumor/nontumor pair. After 2D gel electrophoresis, the Cy2, Cy3, and Cy5 channels were individually imaged from each of the eight gels using dye-specific excitation and emission wavelengths, and the images were analyzed using DeCyder software. Using the DIGE technique with the mixed-sample internal standard, protein spot-features were identified from over 2000 features as changing in abundance across the 8 patient samples. The Cy3 (tumor) and Cy5 (nontumor) spot maps from patient 1 (Fig. 1) are representative
of the spot maps from the other patients (data not shown). Proteins of interest were excised from a preparative gel for subsequent mass spectrometry and database interrogation.

Of the 278 features with statistical significance (paired *t*-test; $p<0.05$) in a DeCyder BVA analysis, 30 were targeted for protein identification. The proteins subsequently

identified from these features using MALDI-TOF mass spectrometry are listed in Table 3. Up-regulation protein spots include cGMP-specific 3', 5'-cyclic nucleotide phosphodiesterase, T cell receptor α chain, Tropomyosin, Heat shock protein 71 kD, Triosephosphate isomerase, DNA polymerase gamma, Lactate dehydrogenase-C, Inorganic pyrophosphatase , Actin capping protein α-subunit. Down-regulated protein spots include similar to transforming acidic-coil containing protein 2, carbonic anhydrase II, ferritin light chain, MHC class I antigen, high mobility group box 1 and fructose-1,6-biphosphatase. There are some proteins that can not be identified by MALDI-TOF will be subjected to LC MS/MS analysis.

Discussion

We used 2-D DIGE technology to simultaneously screen paired liver cancers and corresponding nontumor tissues in eight patients for protein abundance changes, followed by protein identification by mass spectrometry and database interrogation. Compared with the traditional 2-D electrophoresis, DIGE technology allows for each patient comparison to be performed on proteins resolved in the same 2-D gel separation, thereby removing error caused by gel-to-gel variation, and also allows for the quantification of the abundance change for each protein-pair. In this case, however, a direct Cy3/Cy5 comparison limits the detection of significant abundance changes to the 95^{th} percentile confidence level based on the variation present between the samples for each tumor/nontumor pair-wise analysis. Thus the greater the difference between the two samples, the higher the abundance change must be to fall within the 95th percent confidence level. Using the mixed-sample internal standard in this experimental design allowed for the detection of significant abundance changes based on the variance of the mean change within the cohort. It allowed for the detection of 278 statistically significant protein abundance changes across multiple paired samples that were not apparent from individual comparisons due to the large variation between samples. When applied to an expanded patient cohort, this approach may identify useful biomarkers for liver cancer, as well as correlate specific changes in protein expression with different disease stages. Future studies may benefit from the use of western blots and immunohistochemistry staining to confirm interest protein expression in HCC.

In this study, we found heat shock protein 71(HSP71) was up-regulated in HCC. HSP71 is a constitutively expressed chaperone and its function was related to protein stabilization and folding. Heat shock protein 70 family members showed a tendency toward overexpression in HCV-related HCC (1). The expression of ferritin light chain decreased in our HCC tissues, and this decrease has been reported in previously proteomic study of HCC (2). Ferritin plays an important role in iron homeostasis, and excess of iron was associated with increased risk for HCC.

Using 2D-DIGE made us to identify same protein targets with previously HCC proteomic studies(3,4,5), suggesting this is a reliable technique. The identification of differentially expressed proteins is still in progress. We hope this study can help to understand HCC carcinogenesis, and find biomarkers related to HCC.

Table 1 Clinical and pathologic data of patient tumor samples

Pt#	Age	Sex	AFP(ng/ml)	HBsAg	Anti-HCV	Tumor Size	Grade	Cirrhosis
1	52	M	10785	+	-	12*12*6	2-3	-
2	86	M	<20	-	-	10*8.2*2.7	2-3	-

3	66	M	<20	-	+	7*6*5	2	+
4	74	F	340	+	-	7*6*8	2	+
5	67	F	<20	+	+	4.5*4*4	2	+
6	81	M	2486.6	+	-	14*12*7.5	1-2	-
7	71	M	<20	+	+	15*13*12	3-4	+
8	65	M	<20	-	-	11*8.5*8	2	-

AFP, α-fetoprotein; HBsAg, hepatitis B virus surface antigen; Anti-HCV, anti-Hepatitis C virus antibody

Table 2 Experimental design for 2D-DIGE comparison of 9 HCCs and their corresponding nontumor tissues

GelNo.	Cy2	Cy3	Cy5
1	Pooled Std	Tumor	Nontumor
2	Pooled Std	Tumor	Nontumor
3	Pooled Std	Tumor	Nontumor
4	Pooled Std	Nontumor	Tumor
5	Pooled Std	Nontumor	Tumor
6	Pooled Std	Tumor	Nontumor
7	Pooled Std	Nontumor	Tumor
8	Pooled Std	Nontumor	Tumor

Std, standard

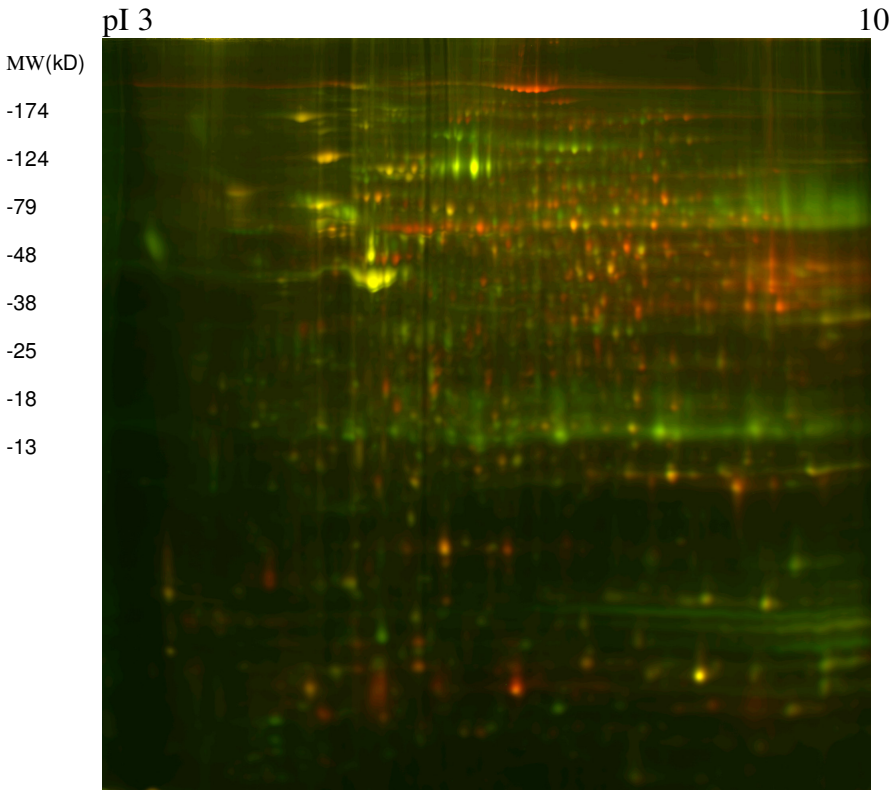

Figure 1 2-D DIGE analysis using the mixed-sample internal standard. Cy3 (green) and Cy5(red) pseudocolor DIGE spot map image from patient #1 was shown for comparison. p*I* range is from 3 to 10 (left to right), and molecular mass separation 174 kDa to 13 kDa (top to bottom).

Table 3 (A) Up-regulated and (B) Down-regulated proteins in human hepatocellular carcinoma

(A) Up-regulated proteins

Spot no.	Name	MW/pI	Function
3	cGMP-specific 3', 5'-cyclic	15.92 kD/6.14	Catalysis of the reaction: guanosine 3',5'-cyclic nucleotide phosphodiesterase phosphate + H2O = guanosine 5'-phospha
4	T cell receptor α chain	15.78 kD/6.4	Immune response
9, 10	Tropomyosin	32.99 kD/4.63	Cytoskeletal protein binding
11-14	Heat shock protein 71 kD	70.90 kD/5.38	Molecular chaperones
21	Triosephosphate isomerase	26.64 kD/6.45	Catalysis of the reaction: D-glyceraldehyde 3-phosphate = glycerone phosphate
23	DNA polymerase gamma	22.22 kD/6.21	Replication of mitochondrial DNA
25	Lactate dehydrogenase-C	36.31 KD/7.08	Catalysis of the oxidation of lactate to produce pyruvate
27	Inorganic pyrophosphatase	31.84 kD/5.42	Catalysis of the hydrolysis of a pyrophosphate bond between two phosphoric groups
28	Actin capping protein α-subunit	32.75 kD/5.58	Actin filaments assembly

(B) Down-regulated proteins

Spot no.	Name	MW/pI	Function
5	Similar to transforming, acidic-coil containing protein 2	22.52 kD/6.12	
7	Carbonic anhydrase II	29.25 kD/6.87	Catalysis of the reaction: H2CO3 = CO2 + H2O
8	Hypothetical protein	26.28 kD/5.7	
15	Ferritin light chain	20.02 kD/5.51	Iron-storing protein complex
16	MHC class I antigen	20.91 kD/5.53	Lymphocyte recognition and antigen
17	Unnamed protein	15.42 kD/5.73	
18	Unnamed protein	20.21 kD/4.09	
20	High mobility group box 1	24.99 kD/5.76	
24	Hypothetical protein	32.34 kD/8.17	
26	Fructose-1,6-biphosphatase	36.81 kD/6.54	Catalysis of the reaction: D-fructose 1,6-bisphosphate + H2O
30	Unnamed protein	34.73 kD/7.19	

(四) 參考文獻

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(五) 計劃成果自評

In this study, we have shown using of 2D-DIGE technology to search candidate proteins that are differentially expressed in HCC. Due to the advantages of pre-labeling technology and reduced inter-gel variance, it is suitable to apply this technology in comparing two proteomes from different physiological conditions. Because the identification protein step is important to decode DIGE data, we strongly recommend combination MALDI-TOF and other mass spectrometry (ex, LC MS/MS) to help this process.