附件一

行政院國家科學委員會補助專題研究計畫 ☑成果報告 □期中進度報告

（計畫名稱）

慢性C型肝炎之藥物基因體學研究

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計畫參與人員：

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執行單位：國立台灣大學醫學院臨床醫學研究所

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中文摘要

慢性 C 型肝炎是一種全球性重要的肝病，病人可能因而死於肝硬化與肝細胞癌。干擾素與 ribavirin 是目前治療慢性 C 型肝炎的標準藥物，但持續性病毒清除率（治癒率）祇 50～60 %，同時藥物副作用的個體差異也很大。影響 C 型肝炎治療反應的病人個體差異因素除了年齡、性別、肝硬化外，基因差異(genetic variation)及其分子機轉並不清楚。釐清個體間對藥物治療之不同反應的基因差異，將有助於在治療病人前預測治療效果而設法加以改進，避免嚴重副作用，與發展新型藥物。此外，為何合併 ribavirin 與干擾素α能使持續病毒清除率由單用干擾素的 10～20 % 提高至之 50～60 %？此分子機轉迄今未明。是否 ribavirin 作用的與個體基因表現差異造成治療落差之分子機轉相關，值得探究。

本研究的長期目標在利用基因體層次的方法去尋找新的生物標記來預測干擾素與 ribavirin 治療後果，促使每一位慢性 C 型肝炎病人的治療效果能達到最高，並瞭解 ribavirin 大幅增強干擾素α治療慢性 C 型肝炎的效果的分子機轉。特定的目標在：（1）以 cDNA 微陣列(microarrays)分析慢性 C 型肝炎病人肝切片組織，找出病人對干擾素與 ribavirin 治療反應有個別差異的基因決定因素（genetic determinants）。（2）在對抗病毒治療有不同反應的病人群間，以蛋白質體學(proteomics)方法尋找有不同表現的蛋白質。（3）利用人類肝細胞、肝癌細胞株與動物，以微陣列分析與蛋白質體分析來全面性尋找 ribavirin 在合併干擾素α後大幅增強治療 C 型肝炎效果的分子機轉。所得的結論將與由病人研究得來的治療反應差異相關的基因相驗證。（4）由慢性 C 型肝炎病毒感染相關的肝癌開刀標本建立可支持 C 型肝炎病毒繁殖的細胞株，作為研究藥物基因體學及藥物作用分子機轉的體外模式，同時可用來測試 C 型肝炎新型藥物。

DNA 微陣列分析將使用具有 9,600 種類 cDNA 的薄片，以 colorimetric 方法偵測基因表現及各組間的差異。蛋白質體分析將利用二維電泳與質譜儀來找出與治療反應相關的特殊蛋白分子。慢性 C 型肝炎病人接受干擾素加 ribavirin 治療前與停藥後的肝切片與血清將分成持續反應者(sustained responder)、復發者(relapser)與無反應者(nonresponder)三組加以分析。經由微陣列與蛋白質體的分析可望偵測到肝細胞影響治療反應的 candidate markers。這些 markers 可藉以預測治療效果或可篩選找出病人的基因多形性(polymorphisms)。與預測藥物反應相關的蛋白也可藉以製造抗體，供臨床預測應用。為系統性探究 ribavirin 合併干擾素可顯著增強抗 C 型肝炎的分子機轉，我們擬分別先以干擾素，ribavirin，與合併兩者處理開刀標本分離出的肝細胞及肝癌細胞株，再用 DNA 微陣列與蛋白質體分析尋找特別表現的 RNA 與蛋白質。為進一步證明這些機轉的確與治療 C 型肝炎有關，我們擬由開刀切除的 C 型肝炎相關的肝癌標本建立可支持 C 型肝炎病毒繁殖的細胞株。此種細胞株亦可作為測試治療 C 型肝炎新藥之用。

以微陣列與蛋白質體分析來探討慢性 C 型肝炎的藥物基因體學，預期將可增加對此種病人藥物治療效果的瞭解，預測治療效果，改進目前藥物的療效，造福病人，而符合國家基因體醫學之目標。

目前 cDNA 微陣列分析正在進行中。在探討 ribavirin 的作用機轉實驗中，以二維電泳分析，我們發現有一特殊細胞蛋白存在於經 ribavirin 處理的人類成年人正常肝細胞（未轉形者）中有表現，但在未經 ribavirin 作用之肝細胞則無表現，我們暫以 RBV-30 稱之。以 liquid chromatography / mass spectrometry 分析，發現 RBV-30 是一種小鼠的生長因子，可支持 CD4 +與 CD8 + T 細胞的 proliferation。我們已製成可表現 RBV-30 的載體，正在進行其功能研究及表現與 C 型肝炎治療成敗之相關研究。

關鍵詞: 慢性 C 型肝炎，干擾素，ribavirin，藥物基因體學，微陣列分析

Abstract

Chronic hepatitis C is a global health problem caused by chronic infection with the hepatitis C virus (HCV), which may lead to liver cirrhosis and hepatocellular carcinoma. Interferon alfa monotherapy induces a sustained virological response (SVR) of 10-20%. Combination therapy of interferon and ribavirin can increase the SVR up to 40-60%, or even higher by using pegylated interferon. The genetic basis for the interindividual differences in response to the combination therapy is poorly understood. Clarification of the host genetic variations related to the drug response may help predict the treatment outcome, minimize severe adverse reaction and optimize therapeutic effect. Furthermore, the molecular mechanisms of the enhancing effect of ribavirin against chronic hepatitis C is unclear. Clarifying the mechanisms may also contribute to optimize the treatment outcome.

The specific aims are as follows: (1) To identify the genetic determinants of interindividual differences in response to interferon plus ribavirin by cDNA microarray analysis in liver biopsy samples from different patient groups based on their response to interferon plus ribavirin therapy. (2) To search for the proteins that are differentially expressed in the different patient groups. (3) To elucidate the molecular mechanisms of the remarkable enhancing effect of ribavirin against hepatitis C by applying genome-wide cDNA microarrays and proteomic analysis.

Microarrays consisting of 9,600 human cDNAs is currently used to examine gene expression profiles in liver biopsy samples from patients with chronic hepatitis C receiving interferon α plus ribavirin therapy. Groups of genes with distinct differences in gene expression among sustained responders, relapsers, and nonresponders will be searched for. For the proteomic approach to search for parameters that help predict the treatment outcome, we will first try to screen out the differential expressed proteins in liver specimens and serum samples from different patient groups. These protein samples will be subjected to two-dimensional gel electrophoresis and LC/MS/MS analysis for definite identification. The results will be verified using conventional western blot and ELISA analyses.

During waiting for the full function of the microarray core lab, by two-dimensional polyacrylamide gel electrophoresis, we have found a cellular protein induced by ribavirin in primary adult hepatocytes but not in hepatocytes untreated with ribavirin. We temporarily term it as RBV-30. By liquid chromatography mass spectrometry, RBV-30 was identified to be a homologue protein of mouse novel growth factor that supports proliferation of cells in the lymphoid lineage including CD4+ and CD8+ T cells. We has cloned the RBV-30` gene into the PET32b *E. coli* expression vector. We plan to have more studies to prove our hypothesis in the future grant period, in addition to the microarray and proteomic approaches.

Key words : Chronic hepatitis C, interferon, ribavirin, pharmacogenomics, microarray

Introduction

Chronic hepatitis C and treatment outcomes

Chronic infection with the hepatitis C virus (HCV) and its sequels is a common and serious health problem. Globally about 170 million people are infected. Chronic hepatitis C typically progresses slowly, and liver cirrhosis and hepatocellular carcinoma (HCC) may ensue (1). In the United States, hepatitis C is responsible for the increasing burden of liver transplantation and incidence of HCC. In Taiwan, 2-5% of the population are positive with anti-HCV antibody, and 20-30% of HCC are caused by chronic hepatitis C viral infection (2).

Early treatment and eradication of hepatitis C virus infection is important to prevent the disease progression. Interferon alfa monotherapy is the first available drug which can induce a sustained virologic response (SVR) of 10-20% after 6-12 months treatment (3). Two independent, randomized, controlled trials from Taiwan and Italy first showed that the combination of standard interferon alfa with ribavirin is remarkably efficacious (SVR 40-50%) as firstline treatment for chronic hepatitis C (4). Further trials have confirmed the result (5,6). Recently, the combination of long-acting, pegylated interferon and oral ribavirin can induce the virologic sustained response even higher (54-56%)(3). Because 40-50% of the patients fail to have sustained response to the combination therapy, we need to explore new strategies to improve the treatment. Moreover, the combination therapy is very expensive and associated with numerous side effects, searching for good predictors of drug response to optimize treatment efficacy and to minimize side effects is an important research goal.

Factors influencing response to antiviral therapy in chronic hepatitis C

The outcomes of the antiviral therapy for chronic hepatitis C are influenced by viral, host and interferon-related factors (7). Genotype 1, high viral load, slow HCV RNA clearance during interferon treatment of HCV, male gender, older age, and liver cirrhosis of the hosts are clearly associated with low sustained response with interferon therapy (8). A few studies on HLA alleles, immune responses and cytokine signal transduction have been reported as influencing factors of interferon response (9). However, the results of most studies remain controversial and inconclusive. A notable exception is that interleukin 10 promoter polymorphisms may significantly affect response to interferon plus ribavirin therapy (10).

Pharmacogenomics to search for genetic basis of drug response

Recently, molecular genetic tools have enable us to discover variations in the human genome and to correlate them with disease and treatment response. There are now many evidences that inherited differences (polymorphisms) in drug metabolism, drug targets and disease pathways of drug effects may have great consequences on the efficacy and toxicity of medication (11-14). Whether genetic heterogeneity plays an

important role in determining treatment outcomes and drug toxicity of interferon and ribavirin therapy, and whether significant drug response markers can be recognized and useful for clinical use, is not known.

Pharmacogenomics is the study that uses genome-wide approaches to elucidate the genetic basis for individual's differences in drug efficacy and toxicity. The ultimate goals is to optimize drug therapy and to minimize adverse drug reactions. It may also lead to mechanism-based approaches to the discovery and development of new medications (11-15). Studies using genome-wide approaches to identify significantly expressed genes in hepatitis C are very limited. McCaughan et al. used cDNA microarray analysis to study gene expression patterns in the liver during chronic HCV infection (16). Using the similar approach, Bigger et al. studied the changes of liver gene expression during the course of an acute resolving HCV infection in a chimpanzee (17), and Honda et al identified differential gene expression in the liver tissues between chronic hepatitis B and C (18). However, **pharmacogenomic studies of chronic hepatitis C based on genome-wide approaches have not yet been reported.**

To identify the genes or groups of genes in the genome that are important for determining the treatment outcomes, linkage studies involving genotyping families with microsatellite markers are impractical because drug response data can rarely be obtained from multiple members of a family (19). A genome-wide association study using single nucleotide polymorphisms (SNP) as markers is a possible way(20). However, 1.42 million SNPs have been found in the human genome in November, 2000 (1.69 million SNPs in June, 2001), providing an average density of one SNP per 1.9 kilobases (21). In order to perform each association study on a genome-wide basis using SNPs in an open population, hundreds to thousands of individuals must be genotyped with a large number of SNPs (30,000 to 500,000). It requires support from advances in genotyping methods and huge resources. Besides, allelic heterogeneity will be a potential hurdle and limitation (19).

The phenotypic consequences of genetic polymorphisms affecting drug response are presumably associated with changes at the RNA and protein level. Global analysis of gene expression by cDNA microarrays and proteomic methods thus provide other approaches for identifying pharmacological markers and candidate genes. These candidate genes could be further screened for polymorphisms. The cDNA microarray method, a powerful tool for genome-wide, parallel analysis of of gene expression, has been applied in various biological studies for identifying differentially expressed genes (22-25). By using the cDNA microarray with colorimetric detection system and arrays of 9600 features, one of our collaborator (JJW Chen) could identify metastasis-associated genes in model lung cancer cell lines (26,27). Proteomics is the large-scale study of gene expression at the protein level, which will ultimately provide direct measurement of protein expression levels and insight into the activity state of all relevant proteins (28). Proteomics have been

applied to the studies in cellular signaling pathways, disease pathogenesis and clinical trials (29). By using liquid chromatography/tandem mass spectrometry, one of our collaborator (YG Tsay) could identify and quantify phosphopeptides (30). Because we have collections of liver biopsy and serum samples from patients with chronic hepatitis C, before, after, or during interferon plus ribavirin therapy, we plan to use genomic approaches to test our hypothesis that there is a genetic basis for drug response in chronic hepatitis C. The knowledge will ultimately lead us to develop a better way to treament each chronic hepatitis C patient.

Mechanisms of action of ribavirin in combination therapy

The mechanisms by which combination thrapy with interferon plus ribavirin has greater efficacy than interferon monotherapy remain unclear. Interferon alfa has direct antviral effects and immunomodulatory activities. Ribavirin is a broad-spectrum antiviral nucleoside analogue. When used alone in patients with chronic hepatitis C, ribavirin has no significant effect on reducing serum HCV levels despite it can normalize serum aminotransferase level in 25% of patients. There is evidence that ribavirin can suppress HCV-specific IL-10 and enhance IFN-γ leading to increased antiviral immune response (31). Ribavirin may also be an RNA virus mutagen which can reduce replication of HCV (32). **Elucidating the mechanism of ribavirin action by cDNA microarray and proteomic approaches has not been reported.** We plan to study the effects of interferon and ribavirin to hepatocytes *in vitro* and *in vivo*. It may reaveal novel pathways of ribavirin action, and eventually leads to a better way of treatment. The search for ribavirin signaling pathway is complementary to the afore-mentioned search for the genetic basis of drug response.

Importance of hepatocyte culture system for HCV

A reliable cell culture system for HCV is mandatory to delineate molecular pathways of antiviral drug action and new drug development. In this study, we need to prove the activation of the genes which can predict treatment outcomes identified in this study after adding interferon and ribavirin, and correlate the gene change with the decline of the viral load in the cell line. Recently, a highly efficient cell culture model has been developed (33). This system is based on the self replication of engineered HCV minigenomes (replicon) in a transfected human hepatoma cell line (Huh-7). Nevertheless, this cell line can not produce infectious HCV. A lymphoma cell line capable of producing infectious HCV have been developed, but the cells are not hepatocytes. We will request these 2 cell lines for our study, and furthermore, we will try to cultivate HCV-infected hepatocytes from resected hepatoma for this proposal. Previous attempts have shown that HCV replication in these cells may be low. However, we can assay the viral load changes by quantitative RT-PCR.

Methods

Describe the research design and the procedures to be used to accomplish the specific aims of the project. Include how the data will be collected, analyzed, and interpreted. Describe any new methodology and its advantage over existing methodologies. Discuss the potential difficulties and limitations of the proposed procedures and alternative approaches to achieve the aims. Point out any procedures, situations, or materials that may be hazardous to personnel and the precautions to be exercised. If human subjects and vertebrate animals are involved, please identify the sources of these research materials and how these specimens will be safeguarded. This section should be written in such a way that original ideas, concepts, and hypotheses, innovative scientific strategies, the deployment of novel technologies, and any unusual aspects of your laboratory or work environment could be readily delineated.

Specific Aim 1. To identify the genetic determinants of interindividual differences in response to interferon plus ribavirin by cDNA microarray analysis in liver biopsy samples

Our overall goal is to use clinical samples to identify the genetic basis of individual differences in response to the combination therapy (interferon plus ribavirin) in patients with chronic hepatitis C. The logic of our strategy is to first identify the distinct gene expression changes either at the mRNA level or the protein level, and then correlate these changes to the clinical phenotypes of different treatment outcomes. We have about 200 patients who have completed 6-month treatment

with interferon and ribavirin. The sustained response rate is 60%. For the 40% patients who are non-sustained responders, about 30% are relapsers and 10% nonresponders. All of them received liver biopsy before treatment. More than 60% of patients had follow-up liver biopsy 6 months after stop of treatment. Most of the liver biopsy samples has been stored in the liquid nitrogen tanks in addition to the routine paraffin blocks. These patients will be divided into 3 groups, *i.e.*,

Sustained responders, relapsers, and nonresponders. We will extract RNA from pretreatment and posttreatment liver biospsy samples of these patients which were stored in the liquid nitrogen. Labeled mRNA probes will be made from these total cellular RNA and subjected to cDNA microarray analysis. We will compare the gene expression patterns among groups of patients with different responses and between the paired, pre- and post-treatment liver biopsy samples. Although cDNA microarrays can be expected to be very valuable for the study of the genetic basis of complex diseases and drug response, there are several possible pitfalls including cellular heterogeneity of tissues, comparability of arrays, data analysis, and need for confirmational studies to corroborate functional relevance of findings. To have strict comparable conditions between arrays, we will control some variables, such as HCV genotypes, viral loads, histological changes, gender and age. Normal tissue cells exist with a heterogeneous cell subpopulations. The gene expression presented by the whole cellular RNA may not represent the gene expression of hepatocyte. With the recent advent of Laser Capture Microdissection (LCM), it is possible to directly compare gene expression patterns of pure hepatocyte population among patient groups with different response to interferon plus ribavirin therapy. We will select 5

patients in each group to see whether there are significant differences of gene expression patterns between the results obtained by the liver biopsy samples and by the LCM method.

Experimental methods of microarray analysis

We will use microarrays containing 9,600 human cDNAs which were prepared on nylon membrane. A colorimetric detection method will be used to record the gene expressions. Total cellular RNAs will be extracted with RNAzol B, and mRNA isolated with olig-dT resin. Purified mRNA derived from liver tissue will be reversed transcribed and labeled with biotin. Southern hybridization process will then be performed. The level of gene expression will be presented by color intensities generated by colorimetric reactions. The quantitative information of gene expression will be retrived by image analysis software. Gene will be grouped on the basis of expression profiles by the self-organizing maps algorithm. The gene expression profiles will then be correlated with the drug response.

Specific Aim 2. To search for the proteins that are differentially expressed in different patient groups based on their response to anti-viral therapy

In order to search for the parameter that help us predict the outcome of antiviral therapy, we will take on a proteomic approach to investigate this question. We will first try to screen out the differential expressed proteins in various specimens from different patient groups. The proteins in different tissue specimens will be prepared using tissue-specific procedures. These protein samples will be resolved with a **two-dimensional gel electrophoresis** system. After **gel staining,** we will identify the proteins with distinct staining intensities in different patient groups. We will subject these proteins to **LC/MS/MS analysis** for definite identification. These results will be verified using conventional Western blot and ELISA analyses.

I. To search for the proteins in plasma that are differentially expressed in different
patient groups.

As the plasma sample can be more easily acquired for large-scale screening and eventual examination, we are very interested in whether there are any plasma proteins differentially expressed in different patient groups.

a) To screen out the plasma proteins that are differentially expressed in different patient groups.

We will first screen what proteins are expressed differently using a small number of samples from various patient groups using 2D gel electrophoresis and LC/MS/MS methods. Five to ten plasma samples from each patient group will be subjected to 2D

gel electrophoresis analysis. The protein spots whose staining intensity is significantly different will be excised out and subjected to LC/MS/MS experiment for protein identification.

b) To establish these plasma proteins as bona fide protein markers that help predict the outcome of antiviral therapy.

We will verify these results using a larger number of samples using Western blotting or ELISA methods.

II. II. To search for the proteins in liver tissue that are differentially expressed in different patient groups.

We will also examine whether expression of proteins is different among samples from different patient groups. Again, we will first screen the candidate marker proteins using the proteomic approach mentioned above. Then, The results will be verified using conventional biochemical methods.

a) To screen out liver proteins that are differentially expressed in different patient groups

We will first screen what proteins are expressed differently using a small number of samples from various patient groups using 2D gel electrophoresis and LC/MS/MS methods. Five to ten liver biopies from each patient group will be subjected to 2D gel electrophoresis analysis. We will analyze the protein spots whose staining intensity is significantly different among these patient groups using LC/MS/MS experiment.

b) To establish these liver proteins as bona fide protein markers that help predict the outcome of antiviral therapy

We will verify these results using a larger number of samples also using Western blotting or ELISA methods. Polyclonal antibodies against these proteins will be first generated by the methods just described. Western blotting and ELISA experiments will be employed similar to the scheme used, except the liver tissue lysates are tested. The readings from ELISA experiments will be compiled to demonstrate the difference of antigen amounts in the plasma of different patient groups.

III. Experimental methods

a) Preparation of samples for 2D gel electrophoresis and LC/MS/MS analysis

1. Plasma samples: An aliquot of 6.25 µl of human plasma is mixed with 10 µl of a solution containing SDS (10% w/v) and DTE (2.3% w/v). The sample is heated to 95°C for 5 minutes and then diluted to 500 µl with a solution containing urea (8 M), CHAPS (4% w/v), Tris (40 mM), DTE (65 mM) and a trace of bromophenol blue. Sixty µl (45 µg) of the final diluted plasma sample is subjected to 2D gel elctrophoresis (34).

2. Liver samples: liver biopsies will be mixed with 300 µl of a solution containing urea (8 M), CHAPS (4% w/v), Tris (40 mM), DTE (65 mM) and a trace of

bromophenol blue. Sixty µl (45 µg) of the final diluted liver sample was loaded on the first dimensional separation (35).

b) Two dimensional gel electrophoresis:

We will use the immobilized pH gradient (IPG) system as the first dimension as described in Bjellqvist et al. (36). A non-linear immobilized pH gradient gel strip (3.5-10.0 NL IPG 18 cm) (Pharmacia-Hoeffer Biotechnology) is used. The IPG gel strips are rehydrated in Pharmacia reswelling cassette with 25 µl of a solution containing urea (8 M), CHAPS (2% w/v), DTE (10 mM), IPG buffer pH 3.5-10 (2% v/v) and a trace of Bromophenol Blue. After placing IPG strips, humid electrode wicks, electrodes and sample cups in position, the strips and cups are covered with low viscosity paraffin oil. Samples are applied at the cathodic end of the IPG strips in a slow and continuous manner. The voltage is linearly increased from 300 to 3500 V during 3 hours, followed by 3 additional hours at 3500 V, whereupon the voltage is increased to 5000 V. A total of 100 kVH is used in an overnight run.

After the first dimension run, the strips are equilibrated within the strip tray with 100 ml of a solution containing Tris-HCl (50 mM) pH 8.4, urea (6 M), glycerol (30% v/v), SDS (2% w/v) and DTE (2% w/v) for 15 min. The cysteine residues are subsequently modified with 100 ml of a solution containing Tris-HCl (50 mM) pH 6.8, urea (6 M), glycerol (30% v/v), SDS (2% w/v), iodoacetamide (2.5% w/v) and a trace of Bromophenol Blue for 15 min.

The second dimension is a modified SDS-PAGE system without SDS in the gel. The gels are poured until 0.7 cm from the top of the plates and overlayered with sec-butanol for about two hours. After the removal of the overlay and its replacement with water the gels are left overnight. The second dimension gels are overlayered with a solution containing agarose (0.5% w/v) and Tris-glycine-SDS (25 mM-198 mM-0.1% w/v) pH 8.3 heated at about 70°C and the IPG gel strips are immediately loaded through it. The gels are run at a constant current of 40 mA/gel.

c) Silver stain:

The gels will be removed from the glass plates and washed in deionized water for 5 min. They are then soaked in ethanol: acetic acid: water (40: 10: 50) for 1 hour and then in ethanol: acetic acid: water (5: 5: 90) for 2 hours or overnight. The gels are washed in deionized water for 5 min and then soaked in a solution containing glutaraldehyde (1%) and sodium acetate (0.5 M) for 30 min. We will then wash the gels 3 times in deionized water for 10 min. In order to obtain homogeneous dark brown staining of proteins, gels are soaked twice in a 2,7 naphtalene-disulfonic acid solution (0.05% w/v) for 30 min. After rinsing 4 times in deionized water for 15 min, the gels are stained in a freshly made ammoniacal silver nitrate solution for 30 minutes, which are made by slowing mixing 6 g of silver nitrate into a solution containing 160 ml of water, 10 ml of concentrated ammonia (25%) and 1.5 ml of sodium hydroxide (10 N). After staining, the gels are washed 4 times in deionized

water for 4 min. The images are developed in a solution containing citric acid (0.01% w/v) and formaldehyde (0.1% v/v) for 5 to 10 min. When a slight background stain appears, development is stopped with a solution containing Tris (5% w/v) and acetic acid (2% v/v).

***d)* Liquid chromatography-tandem mass spectrometry**:

We will use the methods primarily described in Tsay et al. (30). The gel piece containing the studied protein is first soaked in 25 mM NH4HCO3 for 10 min and then in 25 mM NH4HCO3/50% acetonitrile for 10 min. After drying in a Speed-Vac (Savant), the gel is incubated in 100 ml of 2%b-mercaptoethanol/25 mM NH4HCO3 for 20 min at room temperature and at dark. The same volume of 10% 4-vinylpyridine in NH_4HCO_3/50% acetonitrile is added for cysteine alkylation. After a 20-min incubation, the gel is soaked in 1 ml of 25 mM NH_4HCO_3 for 10 min. After being washed in 25 mM NH_4HCO_3/50% acetonitrile for 10 min, the gel was dried and then incubated with 25 mM NH_4HCO_3 containing 50 ng of modified trypsin (Promega) overnight (~ 18 h). The tryptic digest is removed from the gel, which was extracted with 200 ml of 0.1% formic acid. These two fractions are combined together and then are dried in a Speed-Vac and then kept at -20°C for storage. It is resuspended in 0.1% formic acid immediately before use.

Electrospray mass spectrometry is performed using a Finnigan Mat LCQ ion trap mass spectrometer interfaced with an ABI 140D HPLC (Perkin-Elmer). A 150 3 0.5 mm PE Brownlee C18 column (Perkin-Elmer) (5 mm particle diameter, 300 Å pore size) with mobile phases of A (0.1% formic acid in water) and B (0.085% formic acid in acetonitrile) are used. The peptides will be eluted at a flow rate of 5 µl/min with an acetonitrile gradient, which consist of 5–16% B in 5 min, 16–20% B in 40 min, and 20–65% B in 40 min. The spectra for the eluate are acquired as successive sets of three scan modes. The MS scan determines the intensity of the ions in the m/z range of 395 to 1605, and a specific ion is selected for zoom scan and MS/MS scan. The former examines the charge number of the selected ion and the latter acquireds the spectrum (CID spectrum or MS/MS spectrum) for the fragment ions derived by collision-induced dissociation. In the first analysis, the most abundant ion in an MS spectrum is selected for CID experiment; in the second analysis, only the ions with *m/z* values corresponding the potential phosphopeptides are selected for CID experiment. The acquired CID spectra are interpreted using a Finnigan Corporation software package the SEQUEST Browser, which correlates the MS/MS spectra with the protein sequence database containing all human proteins.

e)Westen blotting and ELISA method

Polyclonal antibodies against plasm proteins or liver proteins will be first generated by repeated immunization of rabbits with GST-fusion recombinant proteins. Antiserum will be purified by running over an Affi-Gel (Bio-Rad) affinity column

containing the immobilized GST-fused proteins, followed by elution of bound antibody with 100 mM glycine, pH 2.8.

Total plasma (100 μg/lane) prepared from different partients will be electrophoresed on polyacrylamide gels under standard SDS-polyacrylamide gel electrophoresis conditions. Proteins were then transferred onto 0.45-μm nitrocellulose membrane and were probed for specific proteins using prepared specific antibodies. These will also confirm the specificity of these antiserums.

These samples will be also examines using ELISA methods. The 96-well plates will be coated with 50 μl of 10 μg/ml specific antibody in PBS and incubated at +4 °C overnight. Subsequently the mixture will be discarded, and 200 μl of incubation buffer (0.5% bovine serum albumin, 0.05% Tween 20, 0.02% NaN_3 in PBS) is added and incubated 2 h in room temperature to block unspecific protein binding sites. Plates are washed four times with washing buffer (0.05% Tween 20 in 0.9% saline). Standard dilutions of recombinant proteins, and plasma samples were prepared in incubation buffer, and 50 μl of standards or samples are added in duplicates and incubated at +4 °C overnight. Thereafter plates are washed four times with washing buffer and specific antibody is added at 2 μg/ml in 50 μl and incubated 2 h in room temperature. Subsequently plates are washed four times with washing buffer, and 50 μl of a biotinylated specific antiserum is added at 2 μg/ml in 50 μl and incubated 2 h in room temperature. Subsequently plates are washed four times with washing buffer, and 50 μl of alkaline phosphatase-conjugated streptavidin diluted 1:1000 in incubation buffer is added and incubated 1 h in room temperature. Plates are then washed six times in washing buffer and 50 μl of 1 mg/ml p-nitrophenyl phosphate dissolved in 10% diethanolamine, 0.02% NaN3, 0.5 mM MgCl2, pH 9.8, is added to each well, and absorbance is recorded at 405 nm by a microplate reader.

The readings from ELISA experiments will be compiled to demonstrate the difference of antigen amounts in the plasma of different patient groups.

Specific Aim 3. To elucidate the molecular mechanisms of the remarkable enhancing effect of ribavirin against hepatitis C by applying genome-wide cDNA microarrays and proteomic analysis in hepatocyte cultures and animal experiment

The mechanisms by which combination thrapy with interferon plus ribavirin has greater efficacy than interferon monotherapy remain unclear. Elucidating the mechanism of ribavirin action by cDNA microarray and proteomic approaches has not been reported. We plan to study the effects of interferon and ribavirin to hepatocytes *in vitro* and *in vivo*. It may reaveal novel pathways of ribavirin action, and eventually leads to a better way of treatment. The search for ribavirin signaling pathway is probably complementary to the afore-mentioned search for the genetic basis of drug response. Normal human hepatocytes isolated from surgical specimens as well as hepatoma cells (Huh-7) will be treated with interferon, ribavirin or both

drugs in different doses and various duration. Gene expression patterns at RNA level and protein level will be analyzed according to the methods described above. Because ribavirin may have actions other than the effects on hepatocytes, we will treat mice with interferon, ribavirin or both drugs, and compare the gene expression patterns using inhouse-made microarrays consisting of 6,144 mouse cDNA (made by our collaborator, Dr. JJW Chen).

Specific Aim 4. To establish a hepatoma cell line capable of supporting HCV replication

A reliable cell culture system for HCV is mandatory to delineate molecular pathways of antiviral drug action and new drug development. In this study, we need to prove the activation of the genes which can predict treatment outcomes identified in this study after adding interferon and ribavirin, and correlate the gene change with the decline of the viral load in the cell line. In addition to the request for the cell line with replicating HCV replicons and a. lymphoma cell line capable of producing infectious HCV as mentioned above, we will try to cultivate HCV-infected hepatocytes from resected hepatoma to establish a hepatocyte culture system with HCV replication oueselves. Previous attempts have shown that HCV replication in these cells may be low. However, we can assay the viral load changes by quantitative RT-PCR. Freshly resected hepatoma tissue from patients with chronic HCV infection will be immediately put into suitable culture media, perfused with collagenase solution, and the dissociated hepatocytes will be washed several times and put into long-term culture. The presence of negative-stranded HCV RNA in cells as well as the presence of HCV RNA in the culture media will be checked repeatedly.

Results

We have some exciting finding in the studies directed toward the specific aims 3 during the first year grant period. The following are the summaries of results in each specific aim :

(1) <u>Specific aim 1</u> :**To identify the genetic determinants of interindividual differences in response to interferon plus ribavirin by cDNA microarray analysis in liver biopsy samples from different patient groups based on their response to interferon plus ribavirin therapy.**

We have done cDNA microarray analysis of several liver samples. The results have been analyzed. Because our co-investigator, Dr. Jeremy Chen who runs the microarray core lab in NTUH, found contamination problems in some clones, the progress in microarray study has been slowed down. We do not wish to risk our precious liver samples before everything is reliable. A new, accurate system (the sequences in each clone has been re-confirmed) can be used soon. We will catch up the timeline. Meantime, we are trying Affimatrix gene chips for this purpose.

(2) <u>Specific aim 2</u> : **To search for the proteins that are differentially expressed in the patient groups with different response to interferon plus ribavirin.**

We are working on proteomic analysis of serum proteins from patient with chronic hepatitis C.

(3) <u>Specific aim 3</u> : **To elucidate the molecular mechanisms of the remarkable enhancing effect of ribavirin against hepatitis C by applying genome-wide cDNA microarrays and proteomic analysis in hepatocyte cultures and animal experiments.**

In three independent experiments, by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), we have found a cellular protein induced by ribavirin in the primary adult hepatocytes but not in the control hepatocytes untreated with ribavirin (**Please see the attached 2-D gel photographs. The arrowhead denotes the specific spot**). By liquid chromatography mass spectrometry (LC/MS)(Finnigan®), the protein was identified to be a homologue protein of mouse SF20/IL-25. We temporarily term it as RBV-30 (because it was induced by ribavirin 30 μ g/ml). The SF20/IL-25 is a novel bone marrow stroma-derived growth factor that binds to mouse thymic shared antigen-1 (TSA-1). SF20/IL-25 supports proliferation of cell in the lymphoid lineage, including CD4 + and CD8 + T cells. We has cloned the RBV30 (or human SF20) gene into the PET326 *E. coli* expression vector in frame with the C-terminal peptide containing the polyhistidine tag. To express the recombinant fusion protein, the constructed expression vector was transformed into BL21RIL *E. coli* strain. After induction with 1 mM IPIG for 3 hours, the transformed BL21 RIL were lysed. The lysate was applied to SDS-PAGE and subsequently detected with anti-His monoclonal antibody and rabbit anti-RBV-30 polyclonal antibody (Fig.1). We have cloned the RBV-30 gene into an expression rector. The vector is capable of expressing RBV-30. We are new elucidating its function as well as the correlation of its expression to the IFN plus ribavirin treatment outcomes of chronic hepatitis C patients.

(4) <u>Specific aim 4</u> : **To establish a hepatoma cell line capable of supporting HCV replication to help confirm the anti-HCV mechanisms of ribavirin revealed by microarrays and proteomic studies.**

We have not yet established a hepatoma cell line which can support HCV replication. The effort with a protocol provided by Professor M Lai at USC is still ongoing. Meantime, we have obtained a culture of G418-resistant Huh-7 cells supporting the replication of the Nneo/3-5B HCV replicon from Professor Stanley Lemon at UT. Currently we are using this HCV replicon system to test some of our hypothesis.

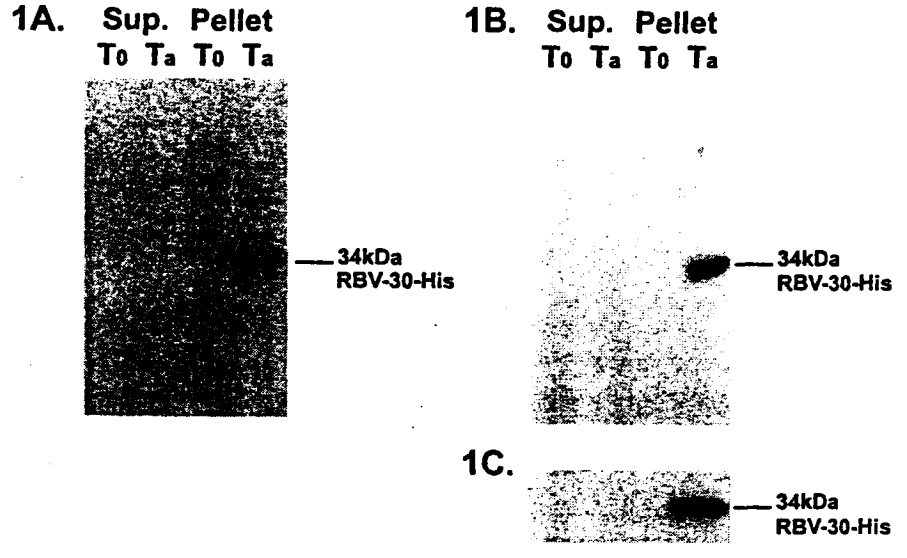

Fig. 1 Expression of human RBV-30 expression in *E. coli*.
A. SDS-PAGE of the supernatant and pellet forms of proteins, staining with Commassie Blue.
B. Western blot of the SDS-PAGE by anti-His antibody.
C. Western blot of the SDS-PAGE by anti-RBV-30 antibody.