行政院國家科學委員會專題研究計畫 成果報告

C 型肝炎病毒套膜蛋白 E2 導致肝臟纖維化的機制探討
研究成果報告(精簡版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 96-2320-B-040-005-
執 行 期 間 ：96 年 08 月 01 日至 97 年 07 月 31 日
執 行 單 位 ：中山醫學大學醫學檢驗暨生物技術學系(所)

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報 告 附 件 ：出席國際會議研究心得報告及發表論文

處 理 方 式 ：本計畫可公開查詢

中 華 民 國 97 年 10 月 31 日

國科會專題研究計畫成果報告撰寫格式

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報告之正文以中英文撰寫均可。在字體之使用方面，英文使用 Times New Roman Font，中文使用標楷體，字體大小請以 12 號為主。

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

C 型肝炎病毒套膜蛋白 E2 導致肝臟纖維化的機制探討

計畫類別：☑個別型計畫　　□ 整合型計畫
計畫編號：NSC96－2320－**B**－040－005－
執行期間：2007 年 08 月 01 日至 2008 年 07 月 31 日

計畫主持人：邱慧玲
共同主持人：
計畫參與人員：謝明儒、陳承龍

成果報告類型(依經費核定清單規定繳交)：☑精簡報告　□完整報告

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□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
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處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：中山醫學大學醫學檢驗暨生物技術學系

中　華　民　國　97 年　10 月 27 日

中文摘要及關鍵詞

關鍵詞：C 型肝炎病毒、套膜蛋白 E2、纖維化、transforming growth factor β1

C 型肝炎病毒（hepatitis C virus）的長期慢性感染會導致肝臟纖維化，進而造成肝硬化。研究指出此病毒之套膜蛋白 E2 與細胞膜上的 CD81 結合後，會藉由 ERK/MAPK 的途徑活化轉錄因子 AP-2，進而導致 MMP-2 的蛋白表現量以及活性上升，顯示 E2 可能與 HCV 導致的纖維化有關。因此本計畫將 E2 基因送入 HSC 中，利用 RT-PCR 以及西方墨點法來進一步分析，證實 E2 可導致 HSC 活化，並影響若干纖維化相關因子的 mRNA 以及蛋白表現量，包括 α-SMA、Collagen α (I)、interleukin (IL)-6、IL-10、IL-1β、TGF-β1、connective tissue growth factor (CTGF)、MMP-2 等。最後再利用 E2 的 siRNA 處理後，觀察纖維化相關因子的影響，更進一步證實之前的研究成果。

Abstract

Keywords: hepatitis C virus, E2, fibrogenesis, transforming growth factor β1

Chronic infection of hepatitis C virus (HCV) may lead to hepatic fibrosis and subsequently cirrhosis, however, the underlying mechanisms have not been established. Previous studies have indicated that the binding of HCV E2 protein and CD81 on the surface of HSC may activate ERK/MAPK pathway to enhance the expression of the transcriptional factor, AP-2, which subsequently lead to the increased protein level and activity of MMP-2. Therefore in this study, full-length E2 gene was transiently transfected into HSC cells and the mRNA and protein levels of fibrosis-related molecules, including α-SMA, Collagen α (I), IL-6, IL-10, IL-1β, TGF-β1, CTGF, MMP-2, and TIMP-1 were analyzed to evaluate the impact of E2 on fibrosis. Furthermore, a pre-treatment with siRNA for E2, was employed to see if it may abolish the E2-induced fibrosis by determining the mRNA and protein levels of studied fibrosis-related molecules. Results from this study soundly proved that the expression of HCV E2 may induce fibrosis of HSC cells.

Pathogenesis of liver fibrosis

Liver fibrosis results from chronic damage to the liver, causing excessive accumulation of extracellular matrix (ECM). During this process, collagens represent the major matrix proteins and are responsible for forming important mechanical scaffolds. Furthermore, their proteolysis by specific proteases appears to be rate-limiting for ECM removal. In liver, the fibril forming interstitial collagens type I and III, and the sheet-forming basement membrane collagen type IV are the most abundant ECM components. In the case of cirrhosis, the content of collagens increases up to 10-fold (Schuppan et al., 2001). Fibrogenesis is characterised by an up-regulation of ECM synthesis, a down-regulation of matrix metalloproteinases (MMP) secretion and activity, and by an increase of the physiological inhibitors of the MMPs, the tissue inhibitors of MMPs (TIMPs) (Benyon and Arthur, 2001). Among the four known TIMPs, the universal MMP-inhibitor TIMP-1 is most important (Iredale, 2001). However, an increase of certain MMPs may also be detrimental. Thus activation of MMPs at the wrong place and time can lead to removal of the regular, differentiation-inducing ECM, such as basement membranes, with subsequent unfavourable tissue remodeling, architectural distortion and a fibrogenic response. For example, MMP-2 mainly degrades basement membrane collagen and denatured collagens and which is up-regulated during fibrogenesis. Collagens, MMPs and TIMPs are mainly produced by myofibroblastic cells (MF) which either derive from activated HSC or from activated fibroblasts (Knittel et al., 1999; Friedman, 2000). Activated liver macrophages, i.e. Kupffer cells, or proliferating bile ductular epithelia, but also endothelia, other mononuclear cells and myofibroblasts themselves are sources of fibrogenic cytokines and growth factors that can stimulate HSC and perivascular fibroblasts to become MF. Following chronic injury, HSCs activate or transdifferentiate into myofibroblast-like cells, acquiring contractile, proinflammatory, and fibrogenic properties. Activated HSCs migrate and accumulate at the sites of tissue repair, secreting large amounts of ECM and regulating ECM degradation. Quiescent HSCs express markers that are characteristic of adipocytes (PPARγ, SREBP-1c, and leptin), while activated HSCs express myogenic markers (α smooth muscle actin, c-myb, and myocyte enhancer factor–2). Hepatic cell types other than HSCs may also have fibrogenic potential. Myofibroblasts derived from small portal vessels proliferate around biliary tracts in cholestasis-induced liver fibrosis to initiate collagen deposition. HSCs and portal myofibroblasts differ in specific cell markers and response to apoptotic stimuli. Culture of $CD34^{+}CD38^{-}$ hematopoietic stem cells with various growth factors has been shown to generate HSCs and myofibroblasts of bone marrow origin that infiltrate human livers undergoing tissue remodeling. These data suggest that cells originating in bone marrow can be a source of fibrogenic cells in the injured liver. The relative importance of each cell type in liver fibrogenesis may depend on the origin of the liver injury. While HSCs are the main fibrogenic cell type in pericentral areas, portal myofibroblasts may predominate when liver injury occurs around portal tracts.

During hepatic fibrogenesis, a complicated interaction between different hepatic cell types takes place. Hepatocytes are targets for most hepatotoxic agents, including hepatitis viruses, alcohol metabolites, and bile acids. Damaged hepatocytes release ROS and fibrogenic mediators and induce the recruitment of white blood cells by inflammatory cells. Apoptosis of damaged

hepatocytes stimulates the fibrogenic actions of liver myofibroblasts while inflammatory cells, such as lymphocytes, activate HSCs to secrete collagen. Activated HSCs also secrete inflammatory chemokines, express cell adhesion molecules, and modulate the activation of lymphocytes. Therefore, a vicious circle of mutual stimulation occur between inflammatory and fibrogenic cells. Furthermore, Th2 response has been associated with more active fibrogenesis while Kupffer cells may release ROS and cytokine to involve in liver inflammation. In chronic cholestatic disorders, epithelial cells stimulate the accumulated portal myofibroblasts to initiate collagen deposition around damaged bile ducts. Finally, changes in the composition of the ECM can directly stimulate fibrogenesis. Moreover, the altered ECM can serve as a reservoir for growth factors and MMPs. Type IV collagen, fibrinogen, and urokinase type plasminogen activator stimulate resident HSCs by activating latent cytokines such as TGF-β1, which is the prominent profibrogenic cytokine and is released from almost any cell during inflammation, tissue regeneration and fibrogenesis. In most cell types, TGF-β1 strongly upregulate the production and deposition of major ECM molecules (Friedman, 2000; Bissell et al., 2001; Gressner et al., 2002). Therefore, TGF-β1 and HSC, the major effector of fibrogenesis, are the prime targets for antifibrotic therapies.

The possible pathogenesis of HCV-associated liver fibrosis

In HCV-related hepatic disease, the virus causes the injury of hepatocytes, followed by inflammation, which in turn leads to activation of HSC. Once activated, HSCs produce various types of compounds, including growth factors, cytokines, chemokines, and vasoactive peptides that have pleotrophic effects in the local environment, including some of which have autocrine effects on stellate cells themselves. Activated HSCs produce massive amounts of extracellular matrix proteins, as well as matrix-degrading enzymes including over 15 MMPs and their inhibitors. The degradation of matrix then in turn stimulates HSC activation and production of interstitial collagens, creating a viscous circle.

HCV is an RNA virus, the biology of which is extremely complex. HCV is made up of a variety of different proteins including core (C), envelope (E1, E2), and nonstructural proteins. The E2 envelope protein is presumed to be involved in virus-host cell interaction and appears to bind to the major loop of CD81, the cell surface receptor for HCV (Pileri et al., 1998). It was demonstrated that CD81 is expressed in the liver, and moreover is increased in the injured liver (Mazzocca et al., 2005). Subsequently, it was demonstrated that incubation of stellate cells with HCV E2 glycoprotein induced a time-dependent increase of MMP-2 gelatinolytic activity. In addition, interaction of E2/CD81 in stellate cells induced the up-regulation of MMP-2 itself by activation of ERK/MAPK phosphorylation (and activator protein-2/DNA binding). Finally, knockdown of CD81 using siRNA abolished the effects of E2 on stellate cells, confirming the role of CD81-E2 interaction in upregulation of MMP-2. These data suggested that stellate cells are a direct target for HCV proteins and HCV itself directly activates production of MMP-2 by stellate cells. The implication is that once produced, MMP-2 disrupts the normal basement membrane and thus facilitates stellate cell activation. Additionally, ECM degradation by MMP-2 may allow other cells to migrate into the injury milieu. Since chemokines and other factors that stimulate movement of cells to areas of injury are present in the wounding environment, it is

possible that up-regulation of MMP-2 by HCV E2 in stellate cells could lead to enhanced penetration of inflammatory cells to sites of injury.

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Research Design and Methods

During this proposal, the impact of E2 on the mRNA and protein levels of fibrosis-related

molecules, including α-SMA, Collagen α (I), IL-6, IL-10, IL-1β, TGF-β1, CTGF, MMP-2, and TIMP-1 was studied. Furthermore, a pre-treatment with siRNA for E2 was employed to see if these treatments may abolish the E2-induced fibrosis by determining the mRNA and protein levels of studied fibrosis-related molecules.

The construction of a recombinant plasmid containing HCV E2 and a cell line expressing HCV E2

A DNA fragment containing full-length E2 coding region (aa 498-777 of the HCV polyprotein) was obtained from a PCR amplification with primers 5'-GCGAATTCACCCACACGACGGGGAGGGT -3' (EcoRI site underlined) and 5'-GCGGATCCGGCTGAGTTCTGACCTATCC -3' (BamHI site underlined) and then cloned into a expression vector pEGFP-N1 (Beckton-Dickinson) to yield a E2 expression vector, pEGFP-E2.At the day prior to transfection, cells were plated on 6-cm tissue culture dishes at a density of 4 x 10^5 cells/dish and cultured for 18 hours. A lipofectamine reagent (Invitrogen, Life Technologies) was used to carry out transfection according to the manufacturer's instructions. Each plate received a mixture of 6 μg of pEGFP-N1 or pEGFP-E2 and 6 μl Lipofectamine reagent in 2.0 ml DMEM (without serum). After the cells exposed to the DNAs for 6 hours, an additional 4.0 ml of fresh DMEM was added to each plate. A mock transfection with pEGFP-N1 only will be simultaneously performed to act as a mock control and transfection efficiency was monitored by pEGFP-N1 which encodes an enhanced green fluorescent protein. At 24 and 48 hours post-transfection, cells were harvested and analyzed for Western blot.

Reverse transcription-PCR (RT-PCR)

One microgram of the total RNA was reverse transcribed by using oligo (dT) primers (New England Biolabs, Beverly, Mass.) and Superscript II enzyme (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. The cDNA was diluted fivefold with water sequentially two times; 1 μl each from these dilutions was used in a PCR. *Taq* polymerase (Roche Diagnostics, Indianapolis, Ind.) was used for amplification with primers specific for α-SMA, Collagen α (I), IL-6, IL-10, IL-1β, TGF-β1, CTGF, MMP-2 and TIMP-1. Total RNA was extracted as recommended by the manufacturer and quantitated by spectrophotometry. The thermal cycling conditions comprised 5 min at 94ºC, and 20 cycles of 1 min denaturation at 55ºC, and 2 min annealing/extension at 72ºC. The PCR products were analyzed by electrophoresis on 2% agarose gel. Gels were scanned, calibrated, and quantified by using ChemiImager 4400 software (Alpha Innotech, San Leandro, Calif.). Each dilution at each time point was normalized by using GAPDH amplified from the cDNA at that dilution.

Western blot analysis

Total cellular proteins extracted from transient expression cells were fractionated by SDS-polyacrylamide gel electrophoresis and blotted onto a membrane. After blocking with milk, the membrane was incubated with antibodies specific against fibrosis factor, α-SMA, Collagen α (I) and MMP-2, followed by further incubation with a secondary antibody. Finally, the immune complex was detected by an enhanced chemiluminescent assay.

Gelatinolytic Zymography

The culture media from cell-to-cell non-contacted co-culture was mixed with non-denatured 5 X sample buffer (1 M Tris-Cl pH 6.8, 1% Bromophenol Blue, 20% SDS). Media mixture was electrophoresed in 8% polyacrylamide gel containing 1 mg/mL gelatin (Sigma), and the gel was firstly rinsed twice in distilled water with 2.5% Triton X-100 for 10 minutes and then secondly incubated in the activation buffer (50 mM Tris buffer, pH 7.4 containing 5 mM $CaCl_2$ and 1 µM $ZnCl_2$) at 4oC for 12 h. In the gel stained with Coomassie brilliant blue R-250, MMP-2, appeared as a clear area and the band density was measured using TINA image software (Raytest).

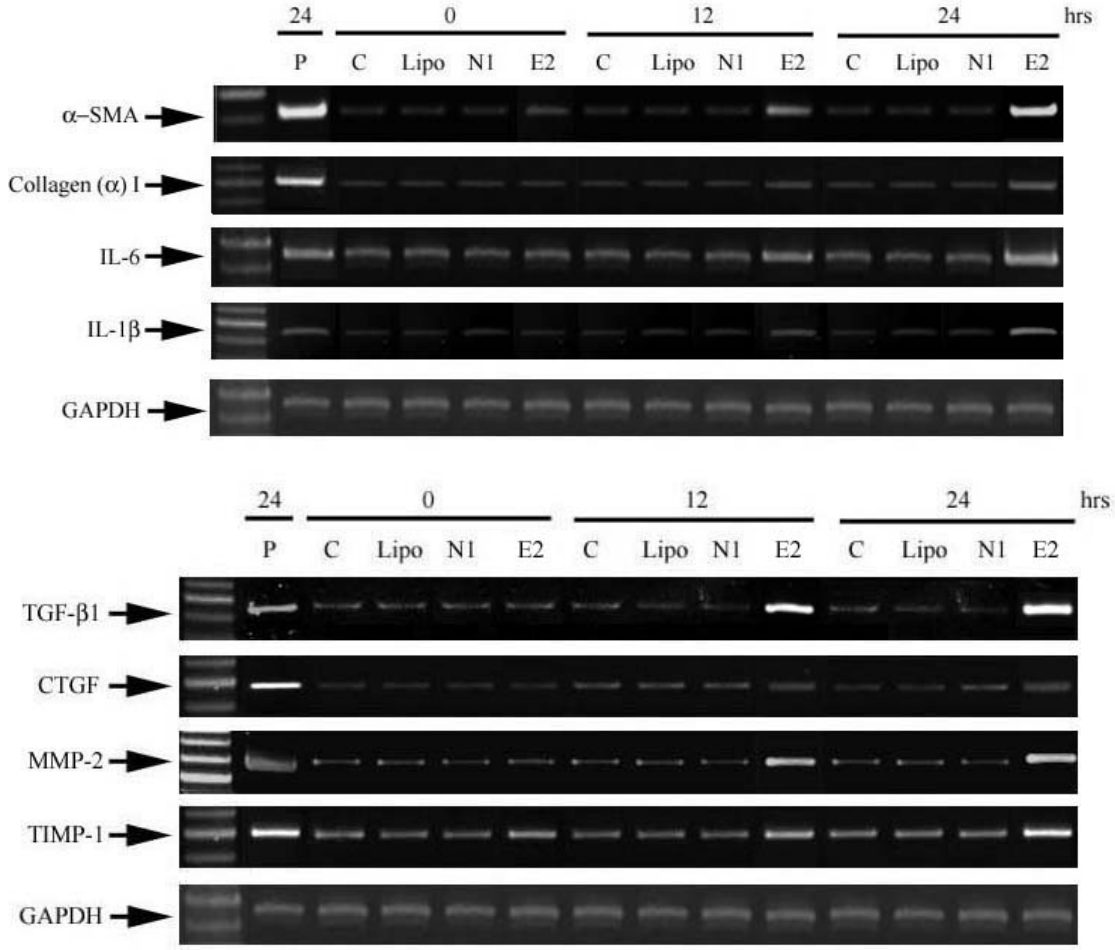
HCV E2 gene silencing

The small interfering RNAs (siRNAs) used for HCV E2 (synthesized by the USC Microchemical Core, Los Angeles, Calif.) were designed according to the guidelines from Elbashir et al. (Elbashir et al., 2001) based on the reported sequences (Machida et al., 2004). HSC cells were transfected with the siRNAs and incubated as previously described (Surabhi et al., 2002). Briefly, 5 x 10^5 cells were suspended in 50 µl of serum- and antibiotic-free DMEM medium and then cultured in 6 cm tissue culture dish. A preincubated solution of lipofectamine 2000 reagent (Invitrogen) containing 100 pmol of siRNA (50 µl in total volume) was added to HSC cells, followed by incubation overnight at 37°C. Cells were transfected with the same siRNA again on day. Nonfunctional siRNA (Ambion) was used as a control. Transfection efficiency was determined by using a control (nonsilencing) siRNA labeled with rhodamine (Qiagen, Valencia, Calif.) to be >70%.

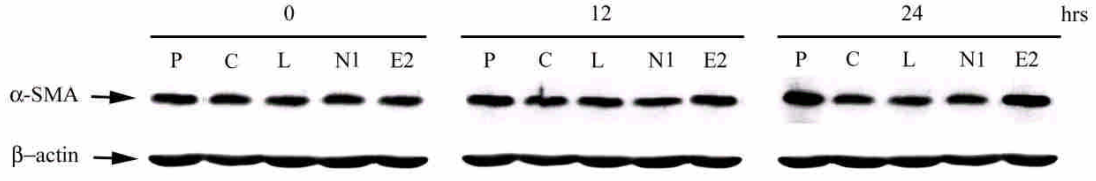
Results

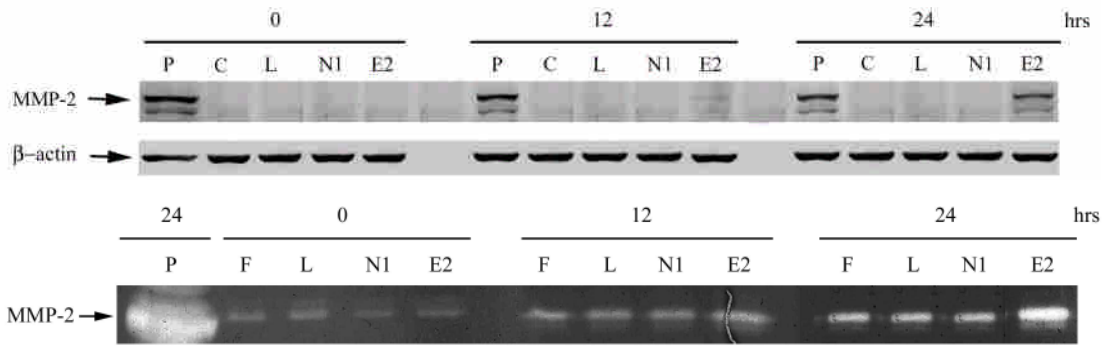
The up-regulation of several fibrosis markers by HCV E2 expression

In this year's project, HSC, a mouse hepatic stellate cell line, was transiently transfected with a recombinant plasmid containing the entire coding region for HCV E2 gene. The mRNA levels of several well-established fibrosis markers (including α-SMA, CTGF, and Collagen (α) I), inflammatory cytokines (including IL-6, IL-10, IL-1β, and TGF-β1), and certain collagenase (MMPs and their inhibitors, TIMPs) were semi-quantitated by RT-PCR with 20 cycles of amplification. Results shown in the following figure revealed that these analyzed molecules were all up-regulated by E2 expression in a time-dependent fashion, indicating that E2 protein may cause fibrosis of HSC cells.

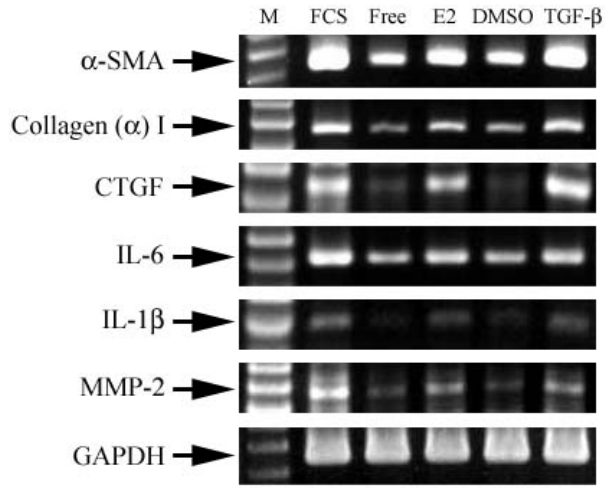


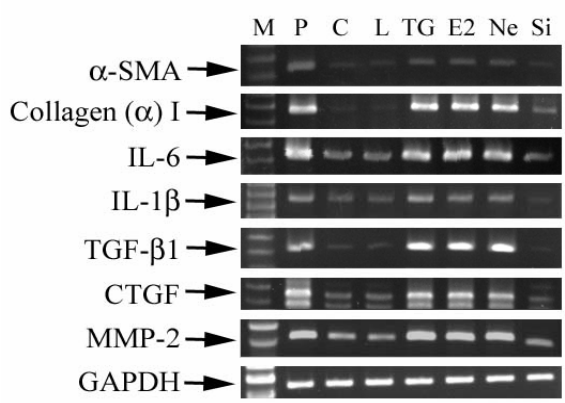
As shown in the following figure, the protein levels of α-SMA and MMP-2, as well as the activity of MMP-2, were also found to be increased by E2 expression in a time-dependent fashion. Altogether, these results preliminarily proved that E2 may initialize the hepatic fibrogenesis.





To further prove this finding, a treatment with TGF-β and E2-siRNA were conducted since TGF-β is an important "coalescent" mediator in the progression to fibrosis and may serve as a positive control in following experiments. Western blot analysis revealed that E2 protein may enhance the expression of fibrosis-related molecules to levels similar to that being treated with TGF-β. Meanwhile, such enhanced expressions were all abolished by a introduction of siRNA specific for E2, proving that such enhanced expression was indeed E2-involved. Therefore, this 3-year project is proposed to further explore the underlying molecular mechanism of this pathological event.





出席國際學術會議心得報告

計畫編號	NSC96-2320-B-040-005-
計畫名稱	C 型肝炎病毒套膜蛋白 E2 導致肝臟纖維化的機制探討
出國人員姓名 服務機關及職稱	邱慧玲 中山醫學大學醫學檢驗暨生物技術學系(所)教授
會議時間地點	97 年 6 月 19-22 日 馬來西亞 吉隆坡
會議名稱	13th International Congress on Infectious Diseases 第十三屆流行病學國際會議
發表論文題目	Silibinin may abolish the enhanced expression of fibrosis-related molecules cause by hepatitis C virus E2 protein

一、參加會議經過

為了讓研究生增加國際交流的機會，本次會議特別與一位博士班學生一起參加此次會議。我們於6月19日抵達馬來西亞首都吉隆坡之後，即前往會議地點（KUALA LUMPUR CONVENTION CENTRE）進行報到手續，領取大會議程及摘要手冊後即前往會場聆聽多場特別演講，於大會指定之22日將準備好的論文海報張貼於指定位置，並在展出海報處說明研究成果，與多位學者進行深入討論。

二、與會心得

本次大會的主題涵蓋傳染性疾病的基礎及臨床各方面，舉凡與傳染性疾病有關的致病機轉、藥物研發、免疫學、治療等等均涵蓋在內，因此藉由此次會議讓我有機會獲得不少新觀念及之前未曾有過的一些想法。與其他相關研究人員的諸多討論，也獲得很多寶貴的意見及肯定。會中聆聽許多大師級的演講，受益良多。此次經驗讓我深深覺得在埋頭研究之餘，多與外界溝通亦是很重要的。另外也對國外的團隊研究精神有深刻體驗，國內應多加舉辦如此大型會議、增加補助出國額度、或盡量補助博士班學生出國開會或短期研究之經費，讓我們有機會多參與國際會議以及多見識國際研究成果