〔國科會計畫執行成果報告〕

肝硬化的嚴重度與肝組織間質金屬性蛋白分解酵素的關係

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肝臟纖維化及硬化常被認為是不可能回復的，大部分肝硬化的患者會逐漸有功能代償失調之現象，甚至出現一些併發症，如：食道靜脈曲張、腹水，最後可能發生肝衰竭。

已有不少的動物實驗及少數的人體報告顯示肝臟纖維化是可回復的。臨床研究證實，Ｃ型肝炎患者在接受干擾素治療後肝臟纖維化獲得改善。

臨床醫師的經驗常認為早期的硬化是可逆的，然而晚期的硬化則不可逆。但是此經驗缺乏適當的理由。另外也有研究者認為在纖維化過程中有一個「不回頭點」，但是此認知亦缺乏適當的描述。

間質金屬性蛋白分解酵素（Matrix metalloproteinase, MMPs）是一群可分解細胞外間質的酵素，動物實驗顯示它們對肝纖維化的回復具有絕對的重要性，更有研究顯示在初期的肝傷害導致纖維化時，間質金屬性蛋白分解酵素的合成增加，但是當纖維化越來越厲害時，其生成則逐漸減少。我們初步的動物研究亦顯示形成時間越久的肝纖維化越不容易回復，而且此不回復性與間質金屬性蛋白分解酵素的生成減少成正比。此外，我們也以四氯化碳來造成肝臟的損傷，經過四週到十六週四氯化碳的注射，MMP-2 和 MMP-9 的表現也逐漸降低，這些結果顯示，間質金屬蛋白分解酵素的表現降低，亦降低了肝臟纖維化回復的能力。此結果說明在纖維化形成過程的肝臟中，間質金屬性蛋白分解酵素的表現也許是一個代表此纖維化過程的持久性及可逆性的良好指標。

本計畫就是要用病人的肝組織來證明這個假說。這個結果將能增加我們對肝纖維化致病原理更深的了解，並希望能對激發出新的治療方法來促進肝硬化的回復上有所幫助。

實驗材料及方法

Materials

我們收集了30個因肝細胞癌或轉移性肝癌而開刀的病人之肝組織（non-tumor part），並立即將其保存在液態氮中，至開始做實驗時才取出。

RT-PCR

取大小約40 mg之肝組織homogenized 後，以Trizol將RNA萃取出來，並進行1% agarose gel electrophoresis確定RNA品質。接著以合成之primers (table 2)，進行RT-PCR (invitrogen, SuperScript III one-step RT-PCR kit)，以47℃ for 20 min 、94℃ for 2 分，1 cycle 以及 94℃ for 45 秒、60℃ for 45” and 72℃ for 90秒的條件，進行35 cycles。

One-step RT-PCR

1. Take 40 mg liver tissue with Trizol (invitrogen) 1 ml, homogenized.
2. Repetitive pipetting
3. 0.2 ml chloroform (no additive)/1 ml reagent
4. Shake by hand 15”, stand 2-3 min., RT
5. Centrifuge 12,000 rpm 15 min, 4℃
6. Repeat steps 2-5
7. Transfer aqueous phase (supernatant of up layer) to a new vial
8. 0.5 ml isopropanol/1 ml reagent, stand 10 min., RT
9. Centrifuge 12,000 rpm, 10 min., 4℃
10. Discard supernatant
11. Add 1 ml 75% EtOH, wash pellet 1X
12. Vortex
13. Centrifuge 7,500 g 5 min., 4℃
14. Discard EtOH
15. Dry pellet by speed Vac around 10 min.
16. Dissolved RNA with DEPC H_2O (take 1λ to 50X dil)
17. OD 260

18. Resuspend with TE buffer (1 μg/μl)

19. 1% agarose gel check (1 μg RNA)

20. Take 0.5-1 μg RNA to perform one-step RT-PCR

Components	**Volume/50 μl**	**Final Concentration**
2X Reaction Mix	25 μl	1X
50 mM $MgSO_4$	-----	1.2 mM
Template RNA		
Sense Primer (20 μM)	1 μl	0.4 μM
Anti-sense Primer (20 μM)	1 μl	0.4 μM
RT / Platinum *Taq* Mix (III)	2 μl	---------
Autoclaved dis. H_2O (DEPC H_2O)		
Final Volume	**50 μl**	

21. 35 cycles

MMPs and TIMPs 之Primers sequences

	Sense	**Anti-Sense**	**Amplicon**
MMP-1	5'-CAAAATCCTGTCCAGCCCATCG-3'	5'-CGGCAAATTCGTAAGCAGCTTC-3'	216 bp
MMP-2	5'-CTCTCCTGACATTGACCTTGGCAC-3'	5'-GTATTCATTCCCTGCAAAGAACAC-3'	279 bp
MMP-9	5'-GGCATCCGGCACCTCTATGGTCC-3'	5'-GCCACTTGTCGGCGATAAGGAAGG-3'	369 bp
TIMP-1	5'-CTGTTGGCTGTGAGGAATGCACAG-3'	5'-TTCAGAGCCTTGGAGGAGCTGGTC-3'	106 bp
TIMP-2	5'-ATGAGATCAAGCAGATAAAGATG-3'	5'-GGTCCTCGATGTCGAGAAACTC-3'	446 bp
GAPDH	5'-TTCACCACCATGGAGAAGGCTG-3'	5'-CTTCCACGATACCAAAGTTGTC-3'	226 bp

Quantitative real-time PCR

將total RNA以AMV-RT (Avian Myeloblastosis Virus reverse transcriptase) 在45℃反應60分鐘轉成cDNA，利用mutiplex quantitative real-time PCR的方法，以合成之probe 和primers (TIB MOLBIOL, Germany) 進行MMP-1, 2, 9 and TIMP-1 , TIMP-2 mRNA之定量（Roche Lightcycler，Taqman）[21]。

1. Take 1 μg tRNA, add Oligo(dT) 1 μl and add H_2O to 17 μl
2. 65℃, 15 min. then place it in RT 15 min.

3. Add RT buffer, 5 μl, AMV RT, 1 μl and dNTP 2 μl
4. Add H_2O to 25 μl
5. 45℃, 60 min., then 95℃, 5 min., keep in - 20℃ till to use.
6. Performed real-time PCR. Follows below:

Materials	volume	Final concentration
Template (cDNA : 40 ng/λ)	1 λ	2 ng/λ
Primer sense (5 μM/λ)	1 λ	0.25 μM/λ
Anti-sense (5 μM/λ)	1 λ	0.25 μM/λ
Taqman probe (4 μM/λ)	0.5 λ	0.1 μM/λ
$MgCl_2$ (25 mM/μl)	1 λ	1.25 mM/λ
Master Mix (take 1b 60 λ into 1a)	1 λ	
H_2O	14.5 λ	
Total volume	**20 μl**	

95^0C, 10 sec. 60^0C, 30 sec., 50 cycles

Primer and Probe (TM)

	Sense	Anti-sense
MMP1	gATATCTTTTgTCAggggAgATC	TCTCTgAAATTgTTggTCCAC A
MMP1 TM	TTCATCAAAATgAgCATCCCCTCCA q	
MMP2	CAgggCggCggTCACA	gTATCgAAggCAgTggAgAggA
MMP2 TM	AATCCgACTggCTAggCTgCTgAgC q	
MMP9	gCTggCAgAggAATACCTgTAC	CAgggACAgTTgCTTCTggA
MMP9 TM	6FAM- CACTCgggTggCAgAgATgCgT q	
TIMP1	gTTATgAgATCAAgATgACCAAgAT	TgTgggACCTgTggAAgTA
TIMP1 TM	CAAgCCTTAggggATgCCgCTgAC q	
TIMP-2	gCCCTgggACACCCTgAg	AgTCCATCCAgAggCACTCg
TIMP2 TM	F-CTgAACCACAggTACCAgATgggCT	
GAPDH	gAAggTgAAggTCggAgTC	gAAgATggTgATgggATTTC
GAPDH TM	6FAM-CAAgCTTCCCgTTCTCAgCCT q	

Histochemistry

我們以Masson Stain之方法，分析collagen在肝組織中沈積的程度。將肝組織

以 10% formalin固定，切成 5μm 之大小，再以Weigert's Iron Hematoxylin solution、Biebrich Scarlet-Acid Fuchsin、Phosphomolybdic-Phosphotungstic Acid、Aniline Blue and 1% Acetic Acid 做組織染色，根據 Metavir 分類系統，依組織纖維化的程度分為F0-F4。

Masson stain

1. Section with 10% formalin (5 μm)
2. Mordant in Bouin's solution (for fix), microwave 1 min, stand 15 min.
3. Wash in running tap water to remove the picric acid, 5 min
4. Weigert's working hematoxylin, 10 min
5. Biebrich scarlet for 5 min
6. Rinse in distilled water.
7. Phosphotungstic/phosphomolybdic acid for 5 min, discard solution
8. Transfer directly into Light green for 5 min.
9. Rinse in distilled water.
10. 1% Acetic acid for 1 min, discard solution.
11. Rinse in distilled water.
12. Dehydrate, clear, and coverslip

HydroxyProline

取大小約200 mg之肝組織，homogenized後，加入2 ml 6N HCl (1mg liver tissue/10 l HCl)，120 ℃，overnight (over 16 hr)，調整pH值至7.0 (4-9)，以DAB呈色，ELISA reader 540 nm 判讀結果。

1. Take 200 mg liver tissue
2. Add 2 ml 6N HCl (1mg liver tissue/10μl HCl) then homogenized
3. 120 ℃，overnight (over 16 hr)
4. Cool down then centrifuge 12,000 rpm for 10 min
5. Take 150 μl sup. to new eppendorff
6. Add 6N NaOH 150 μl , neutralization.
7. Adjust PH to 7.0 (4-9)

8. Take pre-treat sample or standard 40 μl
9. Add B solution 25 μl
10. Add D solution 150 μl
11. ELISA reader, 540 nm。

A solution : Add 35 mg Chloromine T into 500 μl H_2O
B solution : Take ***A solution*** 100 μl into acetate/citrate buffer 400 μl (1:4)
C solution : Take DAB 0.33 g into 500 μl 60% $HClO_4$
D solution : Take ***C solution*** 500 μl into 1.5 ml isopropanol (1:3 diluent)

Statistics
我們以SAS 統計軟體分析real-time PCR, hydroxyproline, fibrotic index and 組織切片染色之結果，並以student's *t*-test比較差異。

實驗結果

我們收集的30個開刀病人中，B型肝炎的患者有17個, **C**型肝炎的患者有7個，因轉移性肝癌而開刀的患者有6個, 其中依METAVIR score （F0 : no fibrosis, F1 : portal fibrosis without septa, F2 : few fibrotic septa, F3 : numerous septa without cirrhosis，F4 : cirrhosis）又分F0 = 4, F1 = 5, F2 = 5, F3 = 8, F4 = 8。

MMP-9與肝臟纖維化指數（F0-F1與F2-F4）顯示統計上的意義（$p = 0.02$）, Hydroxyproline 與F.I (Fibrotic Index)亦有統計上的意義（$p < 0.05$）。MMP-1, -2, TIMP-1, -2則在各組中皆無明顯統計上的意義。

在MMP-1, -2, -9, TIMP-1, -2, colleagen之相關性上，TIMP-2與MMP-2（$p = 0.03$）和GOT （$p = 0.02$）具有統計上的意義。

討論

有許多文獻報告在肝纖維化演變成肝硬化的過程中, MMP-1, -2, -9, TIMP-1 and TIMP-2在血清中其濃度皆會持續上升，但亦有研究發現，在肝硬化末期，血清中之MMP-9開始降低。肝臟發炎會誘發 cytokine、chemokine 的增加及改變了

matrix metalloproteinases（MMPs）和tissue inhibitors of metalloproteinases (TIMPs)之間的平衡，進而促使肝臟纖維化的進行。

本研究從肝臟組織中探討MMPs與TIMPs之間在肝臟纖維化中，對細胞外間質的影響及其在肝臟發炎中間質金屬蛋白分解酵素之表現。在MMP-1, -2, TIMP-1和TIMP-2的mRNA的表現與肝纖維化並無明顯差異，但MMP-9 mRNA的表現在肝纖維化指數F0-F1(n= 12)與F2-F4 (n=15)卻有顯著的差異（$p = 0.02$）。在細胞外間質中，hydroxylproline的含量與在各分組中有明顯統計上的差異（$p < 0.05$），這表示隨著肝纖維化嚴重程度，肝組織中hydroxylproline的含量亦增加。

我們在先前的動物研究顯示越持久的肝纖維化越不容易回復，而且此不回復性與間質金屬性蛋白分解酵素的生成減少成正比。但我們從人體肝臟組織中卻發現了除了MMP-9外，其餘之間質金屬蛋白分解酵素的生成並沒有因肝臟纖維化的嚴重程度而有明顯增加或減少。文獻中曾指出，在肝臟發炎期間，MMPs之生成會增加，其TIMPs之生成亦會隨之增加。在我們所收集的30個患者之肝臟組織中卻發現肝臟組織中MMP-1, -2, TIMP-1及TIMP-2與F.I並無顯著差異，這30個患者血清中之肝功能指數（GOT）並未明顯上升，顯示肝臟無明顯的發炎現象，或許這就是在我們實驗中，MMPs 與TIMPs在肝臟纖維化的過程中，與F.I並無明顯差異的原因之一。MMP-9屬於gelatinase B，會活化latent TGF-β，而TGF-β與Fibrogenesis有密切的關係，因此，MMP-9 mRNA expression 與F.I有明顯差異。MMP-2屬於gelatinase A，我們發現，其mRNA expression與TIMP-2之expression有相關係，可能是因為TIMP-2的生成增加了，抑制了MMP-2的生成，因為GOT與TIMP-2在統計上有明顯的差異。

就如先前的研究發現，在肝臟纖維化的發生過程中，會因肝臟的發炎而誘發cytokine, chemokine及活化一些signal transduction protein (如：MAPK, AkT, NFκB)，這些因子也會影響MMPs與肝臟纖維化的關係，亦有研究發現，在ECM中有一些醣蛋白分子與Fibrogensis有關，這也是我們接下來要探討在肝臟之ECM內，是否會因這些glycoproteins而使得ECM中一些components（如MMPs）失衡或改變了fibrogenesis pathway。

表一、t-TEST

	FI	N	Mean	STD	Range	*p*
MMP-1	0-2	2	2.77	1.85	(1.46-4.07)	*0.71*
	3-4	8	5.04	7.84	(0.63-23.87)	
	0-1	4	1.95	1.43	(1.01-4.07)	*0.28*
	2-4	6	6.35	8.83	(0.63-23.87)	
	0-3	7	5.42	8.24	(1.01-23.87)	*0.60*
	4	3	2.65	3.08	(0.63-6.19)	
MMP-2	0-2	14	0.58	0.51	(0.16-2.17)	*0.61*
	3-4	16	0.67	0.50	(0.23-2.13)	
	0-1	9	0.14	0.62	(0.23-2.17)	*0.95*
	2-4	21	0.43	0.45	(0.16-2.13)	
MMP-9	0-2	7	1.22	0.87	(0.47-3.05)	*0.21*
	3-4	20	2.01	1.68	(0.25-6.85)	
	0-1	12	1.16	0.72	(0.47-3.05)	***0.02***
	2-4	15	2.42	1.81	(0.25-6.85)	
	0-3	19	1.43	0.88	(0.47-3.28)	*0.12*
	4	8	2.88	2.23	(0.25-6.85)	
TIMP-1	0-2	14	0.98	0.61	(0.28-2.20)	*0.09*
	3-4	16	1.68	1.43	(0.47-5.29)	
	0-1	9	0.97	0.59	(0.28-2.14)	*0.13*
	2-4	21	1.52	1.31	(0.40-5.29)	
	0-3	22	1.27	1.08	(0.28-5.29)	*0.54*
	4	8	1.57	1.42	(0.64-4.96)	
TIMP-2	0-2	14	0.74	0.62	(0.08-2.46)	*0.49*
	3-4	16	0.92	0.79	(0.31-3.76)	
	0-1	9	0.63	0.39	(0.21-1.38)	*0.18*
	2-4	21	0.93	0.80	(0.08-3.76)	
HyP. conc.	0-2	14	4.29	1.27	(2.39-6.09)	***0.0479***
	3-4	16	5.82	2.60	(2.74-13.67)	
	0-3	22	4.39	1.36	(2.39-6.79)	***0.0346***
	4	8	7.08	2.89	(4.85-13.67)	
	0-1	9	4.15	1.20	(2.39-6.09)	***0.0483***
	2-4	21	5.52	2.41	(2.74-13.67)	
GOT (AST)	0-2	14	48.2	51.2	(12-211)	*0.58*
	3-4	15	58.7	60.3	(23-247)	
	0-1	9	59.89	60.96	(15-211)	*0.72*
	2-4	20	51.70	54.15	(12-247)	
GPT (ALT)	0-2	14	47.9	36.2	(13-141)	*0.44*
	3-4	15	69.8	101.6	(23-438)	
	0-1	8	56.88	40.97	(17-141)	*0.85*
	2-4	20	61.80	91.92	(13-438)	

表二、correlation of MMPs and HyP

	MMP-1	MMP-2	MMP-9	TIMP-1	TIMP-2	GOT	GPT
MMP-1							
MMP-2	*r* = -0.04 *p* = 0.91						
MMP-9	*r* = 0.56 *p* = 0.10	*r* = -0.22 *p* = 0.24					
TIMP-1	*r* = -0.26 *p* = 0.43	*r* = 0.15 *p* = 0.42	*r* = 0.14 *p* = 0.49				
TIMP-2	*r* = -0.18 *p* = 0.58	***r* = 0.39** ***p* = 0.03**	*r* = -0.02 *p* = 0.93	*r* = 0.27 *p* = 0.15			
GOT	*r* = -0.23 *p* = 0.49	*r* = 0.31 *p* = 0.09	*r* = -0.19 *p* = 0.42	*r* = 0.01 *p* = 0.96	***r* = 0.42** ***p* = 0.02**		
GPT	*r* = -0.21 *p* = 0.53	*r* = 0.18 *p* = 0.36	*r* = -0.18 *p* = 0.36	*r* = -0.08 *p* = 0.66	*r* = 0.18 *p* = 0.36	*r* = 0.87 *p* < 0.0001	
HyP	*r* = -0.35 *p* = 0.30	*r* = 0.31 *p* = 0.09	*r* = 0.25 *p* = 0.20	*r* = 0.15 *p* = 0.43	*r* = 0.09 *p* = 0.62	*r* = 0.03 *p* = 0.87	

參考文獻

1. Accelerated reversal of carbon tetrachloride-induced cirrhosis in rats by the endothelin receptor antagonist TAK-044. K Anselmi, VM Sbbotin and E Nemoto et al. J Gastroenterol and Hepatol 17:589-597, 2002.
2. Matrix as a Modulator or Hepatic Fibrogenesis. D Schuppan, M Ruehl, R Somasundaram, EG Hahn. Seminars in Liver Disease 21(3) :351-371, 2001.
3. Expression of Matrix Metalloproteinase-2 and -9 and Their Inhibitors in Peripheral Blood Cells of Patients with Chronic Hepatitis C. R Lichtinghagen, O Huegel, T Seifert, et al. Clin Chem 46:2, 183-192, 2000.
4. Comparison of matrix metalloproteinase expression in normal cirrhotic human liver. R Lichtinghagen, K Breitenstein, B Arndt. et al. Virchows Arch 432:153-158, 1998.
5. Differential Role of p38 in IL-1 Induction of MMP-9 and MMP-13 in an Established Liver Myofibroblast Cell Line. HS Lee, LH Miau, CH Chen, LL Chiou, GT Huang, PM Yang and JC Sheu. J Biomed Sci 10:757–765, 2003.
6. Extracellular Matrix Degradation and the Role of Hepatic Stellate Cells. R. Christopher Benyon, and Michael J.P. Arthur. Seminars in liver disease 21(3): 37-384, 2001.
7. Liver Fibrosis: Insights Into Migration of Hepatic Stellate Cells in Response to Extracellular Matrix and Growth Factors. C Yang, M Zeisberg, B Mosterman, A Sudhakar,U Yerramalla, K Holthous, L Xu, F Eng, N Afdhal and R Kalluri. Gastroenterol 124:147–159,2003.
8. Extracellular matrix remodelling: the role of matrix Metalloproteinases. Ivan Stamenkovic. *J Pathol* 200: 448–464, 2003.
9. Circulating matrix metalloproteinases 1, 2, 9 and their inhibitors TIMP-1 and

TIMP-2 as serum marker of liver fibrosis in patient with chronic hepaitits C : comparison with PIIINP and hyaluronic acid. V Leroy, F Monier, S Bottari et al. Am J Gastroenterol 99(2):271-279, 2004.

10. MMPs are required for recruitment of antigen-nonspecific mononuclear cells into the liver by CTLs. G Sitia, M Lsogawa, M Lannacone, et al. J Clin Invest 113(8):1158-1167, 2004.
11. Activity of the Matrix Metalloproteinase-9 Promoter in Human Normal and Tumor Cells. C Morelli, K Campion, C Parolin, G Palu, and M Tognon. J Cell physiol 199:126–133, 2004. (IF:5.463)
12. Hepatocyte growth factor induces collagenase (Matrix metalloproteinase-1) via the transcription factor Ets-1 in human hepatic stellate cell line. I Ozaki, G Ahao, T Mizuta et al. J hepatol 36:169-178, 2002.
13. An absolute role of the PKC-dependent NF- B activation for induction of MMP-9 in hepatocellular carcinoma cells. N hah and ST Lee. Biochem Biophy Res Comm 305:428-433, 2003.
14. CD40 activates NF- B and c-Jun N-terminal kinase and enhances chemokine secretion on activated human hepatic stellate cells. RF Schwabe, B Schnabl, YO Kweon et al. J Immunol 166:6812-6819, 2001.
15. Differential expression of toll-like receptors 2 and 4 in patients with liver cirrhosis. T Manigold, U Bocker, C Hanck et al. Eur J Gastroenterol Hepatol 15:275-282, 2003.
16. Reduced hepatic glycogen stores in patients with liver cirrhosis. L krähenbühl, C Lang, S Lüdes et al. Liver International 23:101-109, 2003.
17. Smad expression of hepatic stellate cells in liver cirrhosis in vivo and hepatic stellate cell line in vitro. Y kitamura and H Ninomiya. Pathol International 53:18-26, 2003.

18. Transduction of the liver with activated Akt normalizes portal pressure in cirrhotic rats. MR Manuel, CM Pilar, FV Guillermo et al. Gastroenterol 125:522-531, 2003.
19. Animal experiment and clinical study of effect of gamma-interferon on hepatic fibrosis. HL Weng, WM Cai and RH Liu. World J Gastroenterol 7(1):42-48, 2001.
20. Modifications of a specific assay for hydroxyproline in urine. Kivirikko KL, Laitinen O, Prockop DJ. Anal Biochem 19:249-255, 1967.
21. All trans-retinoic acid selectively down-regulates matrix metalloproteinase-9 (MMP-9) and up-regulates tissue inhibitor of metalloproteinase-1 (TIMP-1) in human bronchoalveolar lavage cells. M Frankenberger, RW. Hauck, B Frankenberger, et al. Molecular Medicine 7(4): 263–270, 2001.
22. Expression of matrix metalloproteinases, their inhibitors, and urokinase plasminogen activator in human meningiomas. K. Siddique, N Yanamandra, M Gujrati et al. Intern J Oncol 22:289-294, 2003.
23. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. DE Gomez, DF Alonso, H Yoshiji et al. Eur J Cell Biol 74: 111-122, 1997.
24. Inhibition of endothelial cell migration by gene transfer of tissue inhibitor of metalloproteinases-1. HA Fernandez, K Kallenbach, Seghezzi G, et al. J Surg Res 82: 156-162, 1999.
25. Regulatory role of extracellular matrix components in expression of matrix metalloproteinases in cultured hepatic stellate cells. YL Li, M Sato, N Kojima et al. Cell Structure and Function 24:255-261, 1999.
26. Apoptosis : The nexus of liver injury and fibrosis. A Canbay, S Friedman and GJ Gores. Hepatology 39(2):273-278, 2004.