

行政院國家科學委員會專題研究計畫 成果報告

基因微陣列分析缺血/灌流對人類肝切除手術引發之相關細胞  
凋亡基因表現之研究(2/2)

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計畫主持人：李伯皇

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# 基因微陣列分析缺血 / 灌流對人類肝切除手術引發之相關細胞凋亡基因表現之研究

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## 一、摘要

缺血會引起實驗動物細胞凋亡 (apoptosis) 及萎縮 (atrophy) 是已知的。在缺血 / 灌流對實驗動物造成傷害的正確機制及媒介質迄今仍不十分清楚。在我們先前研究大鼠的肝臟缺血 60 分鐘再灌流 24 小時的過程中, 藉由 TUNEL 分析及配合清晰之 DNA ladder bands 的結果顯示當肝臟再灌流 6 小時之肝細胞的死亡達到最高峰。然而在缺血終止時 bax RNA、bcl-x RNA、bcl-xL 卻有表現, 此舉顯示此時相關之細胞凋亡基因可能已開始表現。我們更進一步地做了一個研究以便於了解間歇性缺血 (intermittent ischemia) 再灌流 6 小時對大鼠肝臟誘發細胞凋亡之影響, 並與連續性缺血 (continuous ischemia) 作一比較。結果顯示, 在再灌流 6 小時的時間點, 間歇性缺血對大鼠肝臟造成之傷害較連續性缺血嚴重, 而且當間歇性缺血時間越久則肝細胞受傷愈嚴重, 至於 bax、bcl-xL、fas 亦皆有表現。

我們強烈相信缺血 / 灌流在病人切除肝臟手術過程中誘發之細胞凋亡之分子機制是非常值得研究的。所以我們針對間歇性缺血及連續性缺血在病人切除肝臟手術過程中誘發的細胞凋亡做一研究。我們將利用基因微陣列更進一步分析相關之細胞凋亡基因的表現。故本計畫之焦點在於正確且客觀地探討間歇性缺血 vs. 連續性缺血對切肝手術病人傷害的程度, 以及被誘發之早期細胞凋亡之相關基因產生的傷害或保護之表現情形。

我們對 10 個因肝癌在臺大醫院做肝切除手術的病人設計了以下實驗: (1) 肝臟未缺血時 (即時間原點), (2) 當缺血的時間點為 15 及 30 分鐘, (3) 再灌流時間點分別為 5、15 及 30 分鐘時, 收集肝臟檢體及抽取病人血液並分離出血清。萃取肝臟檢體

的 RNA 進行 9600 點的基因微陣列實驗, 統計分析結果發現相對於時間原點有顯著差異表現的基因, 在缺血時有 13 個, 再灌流時有 31 個, 其中重複的有 5 個。

關鍵詞: 缺血, 灌流, 基因微陣列分析, 肝臟, 細胞凋亡。

## Abstract

Ischemia is known to be a cause of apoptosis and atrophy in experimental animals. Until today, the exact mechanisms and mediators involved in ischemia / reperfusion injury remains unclear. In our previous study on rats, liver was ischemia 60 minutes followed by reperfusion for 24 hours. Results showed that the death of hepatocytes is maximal after reperfusion for 6 hours by TUNEL assay and clear DNA ladder bands. Whereas the expressions of bax RNA, bcl-x RNA, and bcl-xL were not negative at the end of ischemia, which imply the regulated apoptosis related genes might have been expressed at this time. Furthermore, we made a study to recognize the effect of intermittent ischemia following reperfusion for 6 hours induction apoptosis on rats' liver, and the continuous ischemia was compared. The finding showed that the injury of intermittent ischemia was more serious than that of continuous ischemia after reperfusion for 6 hours. And the longer time of intermittent ischemia, the more hepatocytes were damaged. The expression of bax, bcl-xL and fas all were positive.

We strongly believe it is worth to investigate the molecular mechanisms of induced apoptosis by ischemia / reperfusion during liver resection operations in human patients. So we made a study to test the effects of continuous and intermittent ischemia on induction of hepatocytes death

during liver resection in human patients. In addition, we investigated the expression of related apoptotic genes by cDNA microarray. Therefore the focus of our project was exploring objectively and correctly the injury of intermittent vs. continuous ischemia on liver surgery in human patients, and the expression of apoptosis-related genes which makes induction of damage or protective effects during early state.

We designed the experiments on liver resection operations of 10 liver cancer patients in National Taiwan University Hospital. The liver specimens and serum were collected at the time points of 0, 15 and 30 minutes in ischemia condition, and following the reperfusion time points of 5, 15 and 30 minutes. We extracted the RNA of liver specimens and then performed the cDNA microarray experiments which used the 9600 -clones chips. After statistic analysis, we found out some genes significantly different from those expressed at the control time point, ischemia 0 minutes. There were 13 different expression genes in period of ischemia and 31 genes in period of reperfusion. Among them 5 genes were overlapping.

Keywords: ischemia, reperfusion, cDNA microarray, liver, apoptosis.

## 二、緣由與目的

Clamping of the portal triad is often used during liver surgery to minimize blood losses. Ischemia is known to be a cause of hepatocellular apoptosis and atrophy in experimental animals. Portal vein ligation is also well known to induce hepatic apoptosis in an experimental model. Until now, the exact mechanisms and mediators involved in ischemia / reperfusion injuries remain unclear. Although various etiologic factors have been identified. These factors include activation of protease and phospholipases, alteration in calcium concentration, ATP depletion, cell damage by free radicals, inhibition of nitric oxide synthesis, cytokines, chemokines, and endothelins. In addition, an active role of cells of the immune system, such as neutrophils, has been defined. There are several mediators regulate the pathway of

apoptosis, such as Bad, Bcl-2, Bcl-x, CAS, CPP32, Fas, Fas-I, ICH-I, TIAR, FADD, TNF, PARP, TRAIL... etc.

In our previous study on rats, liver was ischemia 60 minutes followed by reperfusion for 24 hours. Results showed that the death of hepatocytes is maximal after reperfusion for 6 hours by TUNEL assay and clear DNA ladder bands. Whereas the expressions of bax RNA, bcl-x RNA, and bcl-xL were not negative at the end of ischemia, which imply the regulated apoptosis related genes might have been expressed at this time. Furthermore, in order to recognize the effect of intermittent ischemia / reperfusion, hepatocytes apoptosis was studied on rat liver. The effect of continuous ischemia / reperfusion was also studied. Rats were divided into five groups. Group I was treated with sham-operation (as negative control), and group II and III were treated with ischemia for 30 minutes and 60 minutes respectively. And group IV and V were treated with intermittent ischemia with periods of 15 minutes and 30 minutes twice respectively, and between ischemia conditions unclamping with 5 minutes of reperfusion. All of 5 groups were reperfused for 6 hours. The results showed that the hepatocytes death of group V was maximal, and the other group III, IV, II and I were decreasingly. And group V, III and IV had clear DNA ladder bands. These results demonstrated the injury of intermittent ischemia was more serious than that of continuous ischemia. The longer the intermittent ischemia of the liver done, the more the hepatocytes were injured. Besides, the expression of bax, bcl-xL and fas all were positive.

We have noted there is very limited date available on the induction apoptosis for liver resection in human patients, so we especially focus on the expression of ischemia inducing apoptosis related genes. We strongly believe it is worth to investigate the molecular mechanisms of induced apoptosis by ischemia / reperfusion, which will be helpful for liver recovery from liver surgery in human patients. We have complete data and excellent techniques for ischemia /

reperfusion inducing apoptosis in rat model. Therefore it will be applied for study of continuous vs. intermittent ischemia effecting on induction apoptosis for hepatectomy in human patients. In addition, we will investigate the expression of related apoptosis genes by cDNA microarray. It is impossible to obtain the patients' liver specimens at 6 hours after resection. The focus of our project is to explore objectively and correctly the injury on intermittent vs. continuous ischemia in clinical liver surgery, and the expression of apoptosis-related genes which induce damage or protective effects during early state. On the other hand, the expression of fas in serum after reperfusion will be elucidated. We expect to realize that ischemia / reperfusion induced apoptosis molecular mechanisms, turn on protective effects genes and blocking out damage genes in order to minimize injury for liver surgery in human patients.

We designed the experiments on liver resection operations of 10 liver cancer patients in National Taiwan University Hospital. The liver specimens and serum were collected at the time points of 0, 15 and 30 minutes in ischemia condition, and following the reperfusion time points of 5, 15 and 30 minutes. The injury degree at the different time point for ischemia in liver patients will be distinguished individually and compared each other by measuring apoptotic bodies using of the TUNEL assay. The morphology of apoptotic body was identified with electric microscopy observation. DNA ladder agarose gel electrophoresis will also be performed to make sure the DNA fragmentation. Finally we extracted the RNA of liver specimens and then performed the cDNA microarray experiments which used the 9600-clones chips. After statistic analysis, we will understand the gene expression profiling of ischemia / reperfusion. And further, we can study particularly those genes related to apoptosis by other experiments.

### 三、結果與討論

本實驗由先前對大鼠的實驗結果中可以得知大鼠對缺血時間的忍受度為 60 分

鐘，然後其灌流時細胞凋亡的比例為一個先降再升的曲線，當灌流時間大於 6 小時時為細胞凋亡最嚴重之時間。

這次我們在臨床上收集的檢體時間分別如下：缺血 0 分鐘（正常之檢體）、缺血 15 分鐘、缺血 30 分鐘、灌流後 5 分鐘、灌流後 15 分鐘及灌流後 30 分鐘。這些檢體經由 TUNEL assay 的分析結果觀察可知各檢體之間有一個趨勢：正常的肝臟細胞本來就會有一定比例的細胞自體凋亡數目，當接受缺血達 15 分鐘時這個細胞自體凋亡的比例會有趨緩的現象，即細胞自體凋亡的數目明顯是減少的，而缺血時間達 30 分鐘時細胞自體凋亡的程度顯著提高，其比例甚至高於正常狀況之肝臟細胞自體凋亡數目。

接著我們將經缺血後灌流不同時間的檢體做相同的 TUNEL assay，綜合不同的樣本發現仍然有一個相似的趨勢再灌流 5 分鐘時細胞自體凋亡的數目相較於缺血 30 分鐘的檢體下降了一些，而再灌流時間達 15 分鐘時的檢體分析結果與再灌流 5 分鐘的檢體分析結果看不出有差異，然而當再灌流的時間長為 30 分鐘時所得到的檢體其細胞自體凋亡比例就明顯的較再灌流 5 及 15 分鐘的檢體為低，但這 3 個時間點取得的檢體之細胞自體凋亡比例與正常肝臟細胞自體凋亡的比例是沒有顯著差異的。

之後我們將這六個時間點的肝臟檢體研磨並萃取出 RNA，進行基因微陣列分析實驗，經由統計分析結果發現，這十個病人在晶片上所分析的 9600 個基因表現的模式頗為集中（見圖一），顯示大部分的基因都可列入實驗數據做統計。若以手術進行中收集的檢體順序來看，一開始正常時候的無缺血、缺血 15 分鐘、缺血 30 分鐘、灌流 5 分鐘、灌流 15 分鐘到最後灌流 30 分鐘，在各個時間點的基因表現模式並沒有戲劇性的變化（見圖二），大部分的基因並沒有因為缺血 / 灌流使得表現量有極大的變化。

我們特別挑出少數幾個相對於時間原點（缺血 0 分鐘）有 2 倍以上顯著差異的基因（見表一），分別在缺血 15 分鐘有 5 個，缺血 30 分鐘有 9 個，灌流 5 分鐘有 10 個，灌流 15 分鐘有 13 個，灌流 30 分鐘有

17 個，特別的是，所有基因相對於對照組都是上升表現。其中在缺血的 2 個時間點表現都有差異的基因是 EphA5，再灌流的 3 個時間點基因表現都有差異的是 clipin C (CORO2B)及 Homo sapiens clone 23765 mRNA sequence (F-box and leucine-rich repeat protein 5)，而 clipin C 甚至在缺血 30 分鐘時就已有差異，同樣在缺血及灌流 2 個時期相較於正常無缺血時，基因表現都有顯著差異的有：(1)Tax interaction protein 1(TIP-1)、(2)EST, Weakly similar to GLYCINE RECEPTOR ALPHA-1 CHAIN PRECURSOR、(3)HCG-1(ubiquitin-like 3)及(4)ribosomal protein S6(RPS6)。

在肝臟缺血 15 分鐘時，基因 KIAA0670 protein(apoptotic chromatin condensation inducer in the nucleus, ACINUS)比起無缺血時有明顯上升表現，顯示此時可能有細胞凋亡的現象發生，促使細胞的核染色質凝聚以形成凋亡小體 (apoptotic bodies)。之後的缺血 30 分鐘，V-myb avian myeloblastosis viral oncogene homolog-like 2(Myb-related protein B, B-Myb)表現量上升，這個基因主要用來調節細胞週期的 G1 後期及 S 期，但有研究指出此基因另有調節細胞凋亡的功能，而且是抑制細胞凋亡的發生，故有可能此時細胞正在進行回饋控制，因為先前的 TUNEL assay 結果顯示缺血 30 分鐘的細胞凋亡現象是最旺盛的。

再灌流 30 分鐘時，v-fos FBJ murine osteosarcoma viral oncogene homolog(c-fos)和 programmed cell death 6 都有明顯的上升，前者在許多研究報告中發現是動物缺血 / 灌流實驗的 immediate early gene，甚至有報告指出在灌流 15 分鐘時 c-fos 就已經被誘導表現，且此現象不僅在肝臟發生，其他器官例如小腸、腎臟也有發現 c-fos 的表現。而 programmed cell death 6 已知是細胞凋亡機制中的一員，且可經由 fas 啟動細胞凋亡的發生，和先前大鼠實驗得知灌流後 fas 有表現的結果相符合。

由於我們實驗所使用的檢體來自於病人臨床手術，故收取的缺血 / 灌流的時間點有所限制，我們只能針對早期基因的表現模式做一探究，不似腎臟可以進行長時間的缺血實驗，但肝臟是所有器官中再生

能力最強的，若能清楚瞭解缺血 / 灌流引發的細胞凋亡機制，進而研究肝臟再生的分子機制，將有助於病人的術後恢復情況。

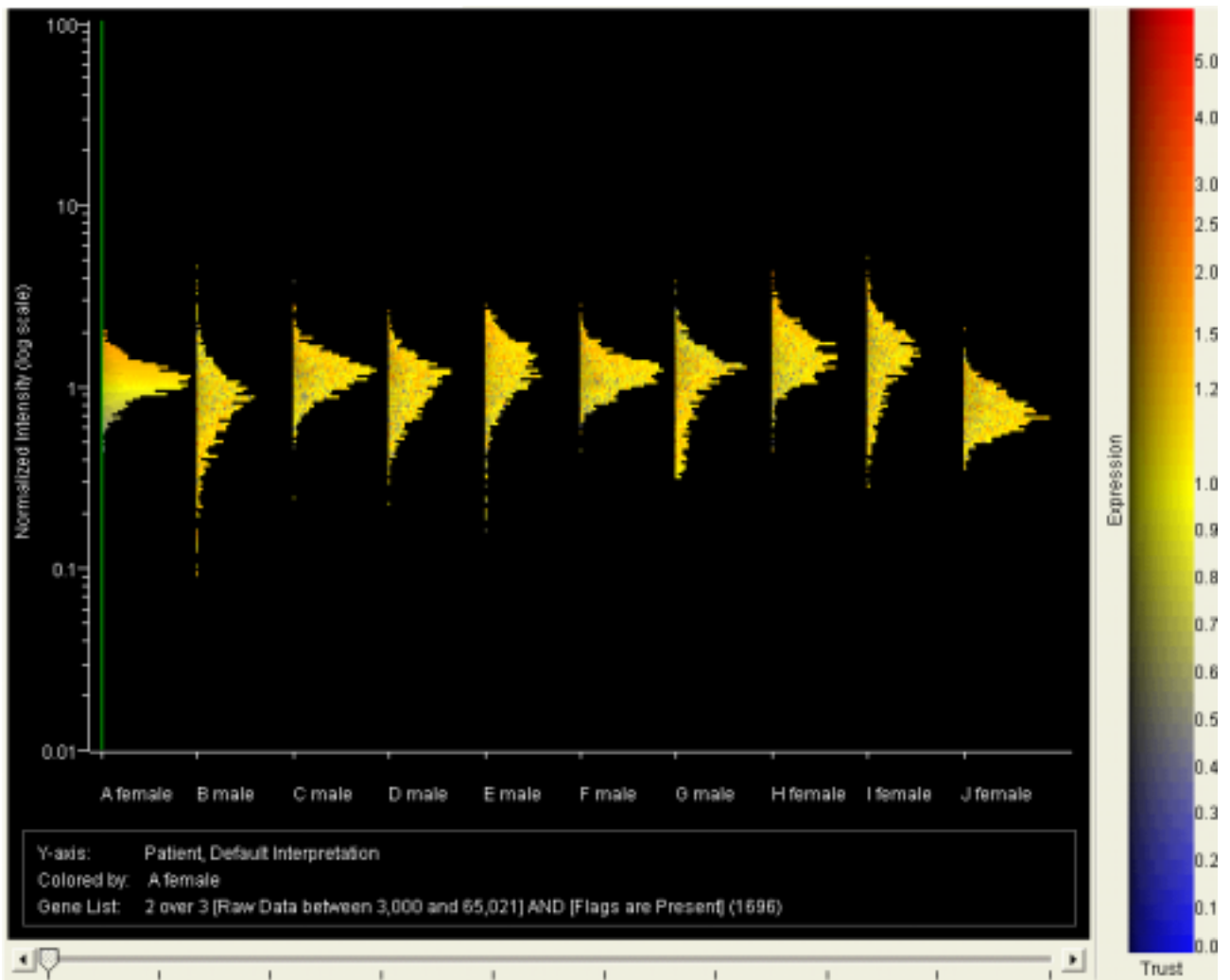
#### 四、計畫成果自評

本篇報告將肝臟在缺血 / 灌流時的基因表現模式，藉由基因微陣列分析實驗完整的呈現出來，其中關於細胞凋亡的基因還須經由其他實驗方法加以驗證，例如 Real-time RT-PCR 及 western blot。

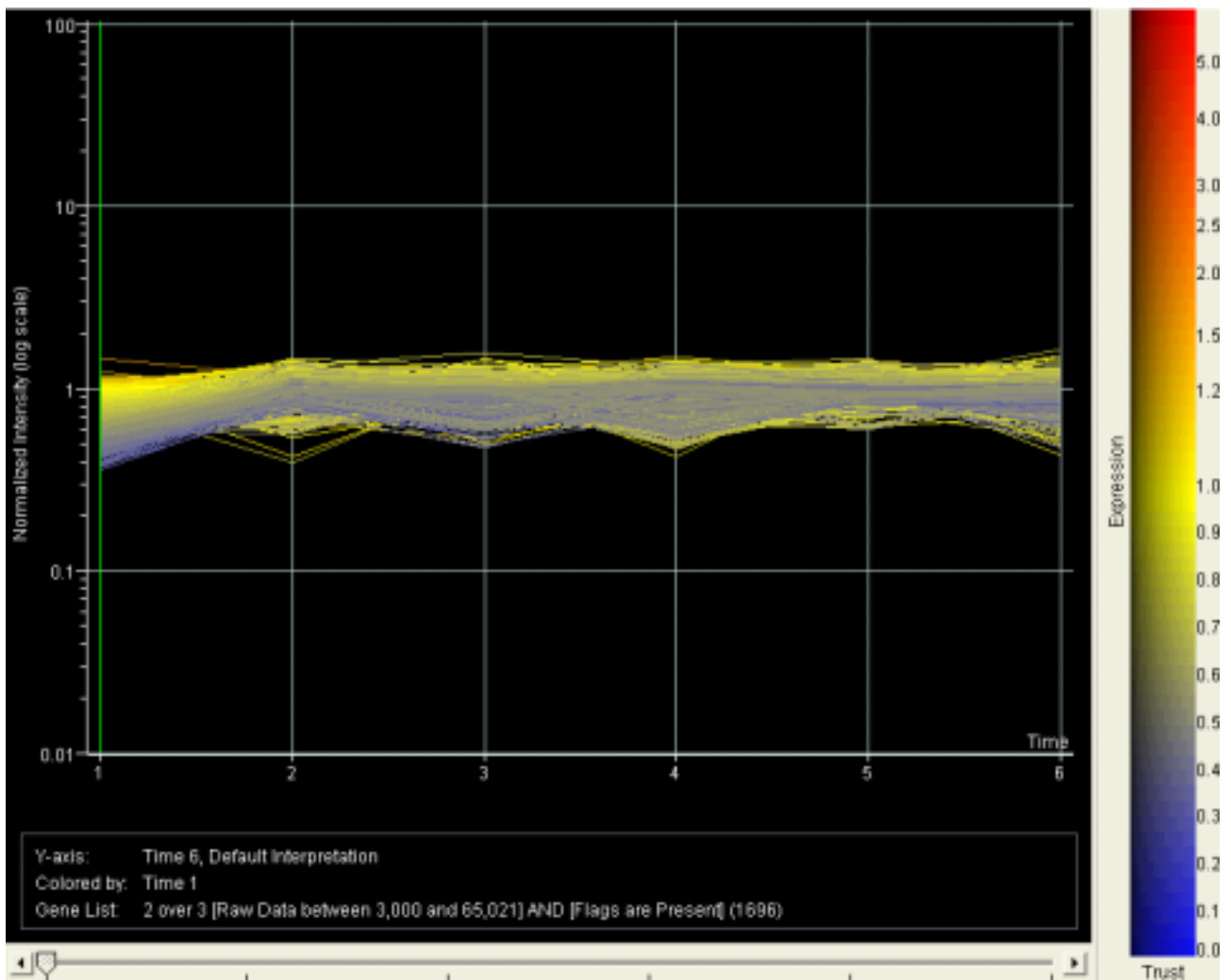
#### 五、參考文獻

1. Shimamatsu K. and Wanless I. R. Role of ischemia in causing apoptosis, atrophy, and nodular hyperplasia in human liver. *Hepatology* 26(2): 343-350. 1997.
2. Bilbao G., Contreras J. L., Eckhoff D. E., Mikheeva G., Krasnykh V., Douglas J. T., Thomas F. T., Thomas J. M. and Curiel D. T. Reduction of ischemia-reperfusion injury of the liver by in vivo adenovirus-mediated gene transfer of the antiapoptotic Bcl-2 gene. *Ann. Surg.* 230(2): 185-193. 1999.
3. Kohli V., Gao W., Camargo C. A. Jr. and Clavien P. A. Calpain is a mediator of preservation-reperfusion injury in rat liver transplantation. *Proc. Natl. Acad. Sci. USA* 94(17): 9354-9359. 1997.
4. Clavien P. A., Yadav S., Sindram D. and Bentley R. C. Protective effects of ischemic preconditioning for liver resection performed under inflow occlusion in humans. *Ann. Surg.* 232(2): 155-162. 2000.
5. Belghiti J., Noun R., Malafosse R., Jagot P., Sauvanet A., Pierangeli F., Marty J. and Farges O. Continuous versus intermittent portal triad clamping for liver resection: a controlled study. *Ann. Surg.* 229(3): 369-375. 1999.
6. Kelley-Loughnane N., Sabla G. E., Ley-Ebert C., Aronow B. J. and Bezerra J. A. Independent and overlapping transcriptional activation during liver development and regeneration in mice. *Hepatology* 35(3): 525-534. 2002.
7. Johnstone R. W., Ruefli A. A. and Lowe S. W. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 108: 153-164. 2002.
8. Supavekin S., Zhang W., Kucherlapati R., Kaskel F. J., Moore L. C. and Devarajan P. Differential gene expression following early

- renal ischemia/reperfusion. *Kidney Int.* 63(5): 1714-1724. 2003.
9. Ishii S., Abe T., Saito T., Tsuchiya T., Kanno H., Miyazawa M., Suzuki M., Motoki R. and Gotoh M. Effects of preconditioning on ischemia/reperfusion injury of hepatocytes determined by immediate early gene transcription. *J. Hepatobiliary Pancreat Surg.* 8(5): 461-468. 2001.
10. Itoh H., Yagi M., Fushida S., Tani T., Hashimoto T., Shimizu K. and Miwa K. Activation of immediate early gene, c-fos, and c-jun in the rat small intestine after ischemia/reperfusion. *Transplantation* 69(4): 598-604. 2000.
11. Le Moine O., Louis H., Stordeur P., Collet J. M., Goldman M. and Deviere J. Role of reactive oxygen intermediates in interleukin 10 release after cold liver ischemia and reperfusion in mice. *Gastroenterology* 113(5): 1701-1706. 1997.



圖一 A-J 十個病人做基因微陣列分析實驗，在 6 個不同時間點的 9600 個基因表現的模式都頗為集中，顯示大部分的數據都可列入統計分析。



圖二 在 6 個不同的時間點：(1)缺血 0 分鐘（正常對照組）(2)缺血 15 分鐘(3)缺血 30 分鐘(4)灌流 5 分鐘(5)灌流 15 分鐘(6)灌流 30 分鐘基因表現量的變化，顯示只有少數幾個基因表現有較大的起伏。



表一 相較無缺血時有 2 倍以上顯著差異表現的基因。

Cluster number	Description	Classification
Ischemia 15 min		
Hs.239147	ESTs, Highly similar to ARRESTIN-D [Rattus norvegicus]	
Hs.92511	EphA5	
Hs.98843	ESTs, Weakly similar to GLYCINE RECEPTOR ALPHA-1 CHAIN PRECURSOR	
Hs.12956	Homo sapiens Tax interaction protein 1 mRNA, partial cds	
Hs.227133	Homo sapiens mRNA for KIAA0670 protein, partial cds	
Ischemia 30 min		
Hs.24907	clipin C	
Hs.101047	Human (HeLa) helix-loop-helix protein HE47 (E2A) mRNA, 3' end	
Hs.92511	EphA5	
Hs.241507	ribosomal protein S6	
Hs.179718	V-myb avian myeloblastosis viral oncogene homolog-like 2	
Hs.267871	ATPase, H <sup>+</sup> transporting, lysosomal (vacuolar proton pump) non-catalytic accessory protein 1 (110/116kD)	
Hs.173091	Homo sapiens HCG-1 protein (HCG-1) mRNA, complete cds	
Hs.250595	Homo sapiens SPHAR gene for cyclin-related protein	
Hs.14376	actin, gamma 1	
Reperfusion 5 min		
Hs.194688	Homo sapiens BAC clone RG208H19 from 7q11.23	
Hs.24907	clipin C	
Hs.79018	chromatin assembly factor I (150 kDa)	
Hs.117367	solute carrier family 22 (organic cation transporter), member 1	
Hs.174220	cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase), polypeptide 8	
Hs.278500	KIAA0060 gene product	
Hs.173091	Homo sapiens HCG-1 protein (HCG-1) mRNA, complete cds	
Hs.5548	Homo sapiens clone 23765 mRNA sequence	
Hs.57600	clathrin-associated/assembly/adaptor protein, small 1 (19kD)	
Hs.20141	similar to <i>S. cerevisiae</i> SSM4	
Reperfusion 15 min		
Hs.194688	Homo sapiens BAC clone RG208H19 from 7q11.23	
Hs.24907	clipin C	
Hs.76698	ESTs, Weakly similar to F59F4.2 [ <i>C.elegans</i> ]	
Hs.117367	solute carrier family 22 (organic cation transporter), member 1	

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Hs.22466	Homo sapiens mRNA for KIAA0891 protein, partial cds
Hs.241507	ribosomal protein S6
Hs.11899	3-HYDROXY-3-METHYLGLUTARYL-COENZYME A REDUCTASE
Hs.98843	ESTs, Weakly similar to GLYCINE RECEPTOR ALPHA-1 CHAIN PRECURSOR
Hs.5548	Homo sapiens clone 23765 mRNA sequence
Hs.147065	EST
Hs.77694	KIAA0429 gene product
Hs.155247	Aldolase C, fructose-bisphosphate
Hs.181695	cholinergic receptor, nicotinic, alpha polypeptide 4

Reperfusion 30 min

Hs.11482	ESTs, Highly similar to F11 antigen [H.sapiens]
Hs.22559	Human mRNA for KIAA0197 gene, partial cds
Hs.24907	clipin C
Hs.76698	ESTs, Weakly similar to F59F4.2 [C.elegans]
Hs.98843	ESTs, Weakly similar to GLYCINE RECEPTOR ALPHA-1 CHAIN PRECURSOR
Hs.25647	v-fos FBJ murine osteosarcoma viral oncogene homolog
Hs.12956	Homo sapiens Tax interaction protein 1 mRNA, partial cds
Hs.8087	Homo sapiens clone 24778 unknown mRNA
Hs.8889	Human cytosolic serine hydroxymethyltransferase (SHMT) mRNA, complete cds
Hs.5548	Homo sapiens clone 23765 mRNA sequence
Hs.146602	EST
Hs.77961	Human RNA for HLA-Bw72 antigen
Hs.93379	eukaryotic translation initiation factor 4B
Hs.80019	programmed cell death 6
Hs.177461	Human mRNA for ribosomal protein L39, complete cds
Hs.585	Apolipoprotein B (including Ag(x) antigen)
Hs.181695	cholinergic receptor, nicotinic, alpha polypeptide 4

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