行政院國家科學委員會專題研究計畫　成果報告

利用 AFLP 技術探究缺氧對肝臟部份切除後再生之影響及基因表現之變化(3/3)

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計畫主持人： 李伯皇

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一、中文摘要

已發表的報告中已指出，缺氧後的肝臟會有促進肝再生的現象，而且兩者成正相關的關係；另有報告發現在肝臟切除手術前先給予刺激（如剖腹探查）同樣的可以增進肝臟的再生，但確切的機制及基因被誘發表現或抑制表現的程度其增減上並沒有研究報告得說明之。

本研究將以大鼠肝臟缺氧後（各處理為：缺血零分鐘，缺血三十分鐘，缺血六十分鐘）再行切除的模式，收集術後 6 小時及 24 小時的肝臟檢體研究缺氧對肝臟切除後肝再生的影響，並將收集的檢體儲存於-70℃。實驗時將檢體取出部份以液態氮及研缽研磨後萃取 mRNA 反轉錄成第一股 cDNA 後再合成第二股 cDNA，合成的 cDNA 以兩種不同切位的限制胺切割酵素切割後，接上特殊人為設計的轉接器即形成兩端具已知序列的 cDNA 片段。

進行 AFLP 分析時則以外加 2~3 個氨基酸的引子數對來進行聚合胺放大反應放大 cDNA 片段以供定序電泳分析片段差異，此法可取得不同處理間的具相異性片段，回收這些片段作序列分析及相似性比對，即可知道這些相異片段為哪些基因的部分序列並推得受處理的檢體中基因層面所受的影響。

在此研究中,我們針對這不同處理的 7 個檢體以兩端各八個引子(共 16 個引子)，進行排列組合進行片段放大工作,獲得 64 種組合結果，再接者將放大反應得到的產物以電泳膠分析後，選取具差異性意義之片段，並切下、給予編號保留此片段，然後對照以其引子對再次放大進行序列分析，此階段共獲得 126 個具處理間差異的片段進行定序工作。

經過 DNA 定序以及比對的結果，總共獲得 68 個在不同的處理組合中有不同表現量的基因，所包含的基因種類有：代謝，生長相關，肝臟再生，結構性蛋白質，神經生成，凝血機制相關，訊息傳導，細胞連結，原致癌基因，基因表現調控，發炎反應，血液生成相關。

這些基因隨著不同的處理方法，呈現出了不同的 RNA 表現，可見其中應有與肝臟術後再生有關的基因表現物。

關鍵詞：引子、片段多型性分析、肝臟、缺血/灌流

Abstract

In this project, liver resection after a period of ischemia in rats will be used as a model to study the relation between ischemia before hepatectomy and liver regeneration after hepatectomy. And mRNA from the samples will be extracted which stored in -70 ℃ and double stranded cDNA will be synthesized for amplified fragment length polymorphism (AFLP) analysis.

To prepare an AFLP template, the restriction fragments for amplifications are generated by two different restriction endonucleases. When used together, these enzymes generate small cDNA fragments that will be amplified well and are in the optimal size range (<1Kb) for separation on denaturing polyacrylamide gels. Following heat inactivation of the restriction endonucleases, the cDNA fragments are ligated to adapters to generate template DNA for amplification. PCR is performed in two consecutive reactions.

In the first reaction, called preamplification, cDNAs are amplified with AFLP primers, each having one selective nucleotide. The PCR products of the preamplification reaction are diluted and used as a template for the selective amplification using two AFLP primers, each containing three selective nucleotides. This two-step amplification strategy results in consistently cleaner and more reproducible

fingerprints with the added benefit of generating enough templates DNA for thousands of AFLP reactions. Products from the selective amplification are separated on a denaturing polyacrylamide (sequencing) gel.

This technique would be taken dissimilarity fragments among the samples that could be reclaimed to sequence their DNA sequences and similar comparing, so we may understand how the genes expression during these treatment.

Keywords：AFLP. liver. ischemia/reperfusion

二、緣由與目的

AFLP (amplified fragment length polymorphism) is a new DNA finger printing technique, it combined restriction enzymes digestion and PCR (polymerase chain reaction), so was named SRFA (selective restriction fragment amplification) or AFLP (Zabeau, 1993). It consists of three main parts (1) restriction enzyme digestion and adaptors ligation, (2) selective amplified digested fragments and (3) electrophoresis gel analysis of amplified fragments.

Two different restriction enzymes were used to digest DNA and then ligated adaptors to the end. Evidence adaptors and digestion site contiguous sequences and prolongation 3' end several bases to excogitating primers. Finally, to amplify selectivity partial fragments and analyzed by sequencing gel electrophoresis (Vos et al., 1995).

Two restriction enzymes, a rare cutter and a frequent cutter generate the restriction fragments for amplification. The AFLP procedure results predominantly in amplification of those restriction fragments, which have a rare cutter sequence on one end and a frequent cutter sequence on the other end. The rationale for using two restriction enzymes is the following: The frequent cutter will generate small DNA fragments, which will amplify well and are in the optimal size range for separation on denaturing gels (sequence gels).

So, that only the rare cutter/frequent cutter fragments are amplified. This limits the number of selective nucleotides needed for the AFLP reaction. For example, EcoRI (G/AATTC) is used as the standard rare-cutting enzyme, and MseI (T/TAA) is used as the frequent cutter. The majority of DNA fragments should be <500 bp for high quality AFLP fingerprints. MseI (T/TAA) gives small DNA fragments in many species, because they have A-T-rich genomes. Sometimes MSEI will not yield sufficiently small DNA fragments, and generally other 4 base cutter enzymes will also fail to do so.In that case a second 4 base cutter should be added to reduce the size of DNA fragments.

In the subsequent ligation reaction, double strand adapters for the second 4 base cutters should be included. The use of two restriction enzymes makes it possible to label only one strand of the double stranded PCR products, which prevents the occurrence of "doublets" on the gels due to unequal mobility of the two strands of the amplified fragments. Using two different restriction enzymes gives the greatest flexibility in:" tuning" the amount of fragments to be amplified. Apart from these technical considerations, the rare cutter sites may serve as landmarks in physical mapping studies.

After restriction enzyme digestion, adaptors were ligated to the DNA end that comprised two parts: core sequence and enzyme specific sequence (ENZ). Core sequence was factitious excogitating just a binding site for primer and ENZ could make core sequence to ligate with the cohesive end of digestion fragments because it is restriction site sequence.

The adapters are designed in such a way that the restriction sites are not restored after ligation. During the ligation reaction the restriction enzymes are still active. In this way fragment-to-fragment ligation is prevented, since fragment concatamers are restricted. Adapter-to-adapter ligation is not possible because the adapters are not phosphorylated.

AFLP primers are including three parts: core sequence, ENZ and selective extension (EXT). EXT was added one to three bases for different species, to purpose reducing the

number of amplified fragments. As a rule, three bases are usual used to analyze genomic DNA but when size smaller than 5×10^8 Kb it might be adding one or two bases. AFLP was used in high and fixed temperature that prevent the result from loss of fragments specific according to temperature change.

三、結果與討論

After PCR, add an equal volume of formamide dye (98% formamide, 10mM EDTA, bromophenol blue, xylene cyanol) to each reaction. Heat the samples for 3 min at 90℃ and immediately place on ice. Pour a 5% denatured polyacrylamide gel (20:1 acrylamide: bis; 7.5 M urea; 1X TBE buffer) and electrophoresis at constant power until xylene cyanol is two-thirds down the length of the gel.

Then silver staining, Purification of oligonucleotides from denaturing polyacrylamide gel, cut out the bands directly with a clean scalpel or razor blade. Chop the gel slabs into fine particles by forcing the gel through a small-bore syringe to aid the diffusion of the olignucleotides from the matrix. Place the crushed gel slab in a 15-ml centrifuge tube capable of withstanding high temperatures. Add 3 ml TE buffer for every 0.5 ml of gel slab. Freeze the sample for 30 min at -80℃ or until frozen solid. Quickly thaw it in a hot water bath (~50℃) and let soak 5 min at 90℃. Elute on a rotary shaker overnight at room temperature. Centrifuge the tube 2 min at 1000×g, room temperature, to pellet gel fragments.

在我們的研究中經聚合反應、電泳分析及片段擷取的結果，我們對照電泳影像中發現電泳的結果大致可分為 3 大類：

1.當引子尾端的 A-T 比例較高時（例如 E-aac）所聚合的產物很難去區分檢體之間的差異，因為這樣的引子與模板結合能力較弱，篩選能力較差，其聚合得到的片段多為模糊而不集中，以至於在電泳膠中無法分辨不同之處。

2.另一類電泳的結果, 則表現出細胞在維持生命時所必須轉譯的基因，電泳圖上所顯現的片段幾乎每一個不同處理的檢體都具有，且片段明顯聚合大量，這類多為一般的代謝作用所需之基因。

3.此類型之電泳圖則為最理想之片段擷取電泳圖，這類聚合放大所得之產物經電泳分析，不但各檢體之間有明顯差異之片段，而且每一個片段均為清晰完整的顯影，這代表處理的方式誘發的基因表現為大量且易見的，擷取此種片段經定序必具有其意義。

在切取表現量有所不同的片段進行核酸定序時，發現檢體中含有數種核酸片段，推論應為引子之專一性不足所致。於是提高引子黏和溫度至攝氏五十八度之後，將核酸檢體進行第二聚合胺鍊鎖反應，之後進行電泳，再將與原目標核酸長度一致的片段粹取出來，進行核酸定序以及比對。

比對結果（見表一）顯示，有 68 個在各處理組合表現不同之基因，可見缺血確實會對某些基因的表現造成影響，包括：細胞代謝，訊息傳導，結構蛋白。並且會誘發某些基因的表現，包括：肝臟再生，細胞生長，血液生成，凝血反應，神經生成，發炎反應。

若再進一步比較某些基因在經過不同處理後的表現量（見表二），可以發現缺血時間較久的組別，有關肝臟再生的基因會被開啟並且表現。特別是 liver annexin-like protein (LAL)，在術後二十四小時所收取的樣本中，有持續的表現，可見缺血確實會開啟此基因，而且是在手術後一天左右表現量會大幅增加。

訊息傳導的基因, 則多在缺血後 24 小時以後才被開啟，推測這些基因多為與細胞修復，再生相關的，在肝臟受傷害的早期並不會，而是要等到緊急的狀況過去後，再開始進行修補的工作。

有關轉錄及轉譯的基因，則並沒有大變化，應是細胞維持基本生理作用所需。造血相關的基因 hematopoietic lineage switch 2 related protein 則有被抑制的傾向，因為手術過程中血液流失，生物體應該會進行造血，推測這一個基因應該具有

抑制造血作用的功能。

四、計畫成果自評

本實驗方法 AFLP 是在基因微陣列（microarray）方法成熟前所發展出來的，用以探測細胞內多種基因的表現，雖然方法較 microarray 慢，但是好處在於，許多未知功能的基因可能可以經由這種方法被發現，相對來說，microarray 可以點上去的偵測點就大部分都是已知功能的，許多重要但未知的基因就有可能被忽略，所以仍然有他不可替代的價值存在。

經由本實驗的執行，我們找到許多被肝臟手術時阻斷血流的時間長短所影響的基因，可以進一步再進行實驗印證。可以做的實驗包括 western blot, in-situ hybridization, real-time RT-PCR, 甚至做 knock-out gene 的測試。以我們的廣泛尋找肝臟手術相關基因的成果為基礎，我們可以進一步檢測這些表現量有差異的目標基因，其結果可望為基因功能描繪更完整的圖像，進而研發具體方法降低肝臟手術風險以及提高存活率，在醫學的演進上可以說具有極大的價值。

五、參考文獻

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Number	Annotation/Description	Accession	Classification
29=2	alpha-2-HS-glycoprotein; alpha 2 HS-glycoprotein alpha 2 (fetuin)	NM012898	Metabolism
38=1	Rat phosphorylated N-glycoprotein (pp63) mRNA, complete cds.	M29758	Metabolism
41=1	genes for 18S, 5.8S, and 28S ribosomal RNAs.	V01270	rRNA
45=3	genes for 18S, 5.8S, and 28S ribosomal RNAs	V01270	rRNA
47=1	Rat phosphorylated N-glycoprotein (pp63) mRNA	M29758	Metabolism
48=2	alpha-2-HS-glycoprotein (Ahsg), mRNA.	NM_012898	Metabolism
49=1	mRNA for small androgen receptor-interacting protein	AJ132390	Growth-related
56=1	genes for 18S, 5.8S, and 28S ribosomal RNAs	V01270	rRNA
63=1	SLIT1 mRNA for SLIT1-La splicing product	AB073215	Intron
64=1	5.8S ribosomal RNA gene and internal transcribed spacer 2	AF061799	rRNA
69=1	genes for 18S, 5.8S, and 28S ribosomal RNAs	V01270	rRNA
70=1	5' non-coding region of S-adenosylmethionine synthetase	X60822	Metabolism
97=1	liver regeneration protein lrryan mRNA,	AY298742	Liver regeneration
100=3	Rat mRNA for mitochondrial 3-2trans-enoyl-CoA isomerase	X61184	Metabolism
136=3	Rattus norvegicus 18 BAC CH230-8D7	AC095558	?
140=2	similar to fibrillin3 (LOC363853), mRNA	XM_344062	?
143=1	similar to brain carcinoembryonic antigen (LOC292664), mRNA.	XM_214842	?
157=1	partial gfap gene for glial fibrillary acidic protein	Z48978	Struct protein
157=2	carbamoyl-phosphate synthetase 1 (Cps1), mRNA	NM_017072	Metabolism
159=3	proline rich synapse associated protein 1 (Shank2), mRNA	NM_133441	Neurogenesis
173=1	BAC CH230-159G8 complete sequence	AC099476	?
173=3	2 BAC CH230-149H21 complete sequence	AC099304	?
174=1	R.norvegicus FGR mRNA	X57018	?
174=2	similar to Retinoic acid receptor responder protein 1	XM_227232	Struct protein
174=3	FGR (Fgr), mRNA	NM_024145	?
177=1	similar to T3 olfactory receptor (LOC293362), mRNA	XM_219147	Neurogenesis
180=1	ectonucleoside triphosphate diphosphohydrolase 5(Entpd5)	NM_199394	Thromboregulatory
185=2	mRNA for mitochondrial transmembrane GTPase FZO1B	AB084166	Signal transd
188=1	UDP-galactose:ceramide galactosyltransferase mRNA	U07683	Neurogenesis
188=2	similar to enthoprotin; epsin 4; clathrin interacting protein	XM_340788	Cell adhesion
197=1	liver regeneration-related protein LRRG03 mRNA, complete cds.	AY327504	Liver regeneration
199=1	mitochondrial inner membrane translocase component Tim17b	XM_228758	Cell adhesion
199=2	mRNA for plasminogen protein	AJ242649	Struct protein
209=4	18 BAC CH230-178G23 complete sequence	AC109037	?
210=1	Rat glutathione S-transferase mRNA, complete cds.	J03752	Oncogenesis, membrane trafficking
216=1	Ab1-233 mRNA, complete cds	AY325155	Liver regeneration
216=7	Ab1-233 mRNA, complete cds	AY325155	Liver regeneration
227=1	liver annexin-like protein (LAL) mRNA, complete cds.	AF131077	Liver regeneration
228=1	similar to eukaryotic translation initiation factor 2, subunit 3	XM_216704	Trans Factor
228=2	1 BAC CH230-132J4	AC105631	?
231=1	developmentally regulated RNA-binding protein 1 (Drb1)	NM153306	Neurogenesis
231=2	cytochrome P450 4F5 gene, complete cds	AF288818	Metabolism
232=1	5' non-coding region of S-adenosylmethionine synthetase	X60822	Intron
236=1	Rat liver alpha-2-macroglobulin mRNA, complete cds	J02635	Inflammation
236=2	similar to Lipin 2 (LOC316737), mRNA	XM_237521	Metabolism
236=3	p53 (PG-III) pseudogene, partial ORF	L12046	Protooncogene
252=3	similar to putative matrix metalloproteinase (LOC300338), mRNA	XM235849	Viral Protein
269=1	Rat delta-3,delta-2-enoyl-CoA isomerase mRNA, complete cds	M61112	Metabolism
276=1	SEC23A (S. cerevisiae) (Sec23a), mRNA	XM_234203	Transport protein
279=1	G protein-binding protein CRFG (Crfg), mRNA	NM_053689	Signal transd
281=1	Hematopoietic lineage switch 2 related protein (Hls2-rp)	AF097723	Hematopoiesis

283=1	nephrin Mrna, complete cds	AF161715	Cell adhesion
285=3	liver annexin-like protein (LAL) mRNA, complete cds.	AF131077	Liver regeneration
290=1	Rat liver alpha-2-macroglobulin mRNA, complete cds	J02635	Inflammation
292=1	R.norvegicus mRNA for ras-related GTPase, ragB	X85184	Signal transd
295=4	ADP-ribosylation factor 4 mRNA, complete cds	L12383	Metabolism
297=1	similar to pyruvate dehydrogenase (lipoamide) beta	XM_214142	Metabolism
302=1	plasma glutamate carboxypeptidase (Pgcp), mRNA	NM_031640	Liver regeneration
351=1	Rat alpha-2-u globulin mRNA	M27434	Trans Factor
358=1	estrogen sulfotransferase mRNA, complete cds	M86758	Trans Factor
397=1	dystrophin-related protein 2 B-form splice variant (Drp2)	AF195788	Neurogenesis
397=2	similar to hypothetical protein D15Wsu59e (LOC294810), mRNA	XM_215506	?
397=3	similar to hypothetical protein D15Wsu59e (LOC294810), mRNA	XM_215506	?
407=1	peroxiredoxin 4 (Prdx4), mRNA	NM_053512	Metabolism
408=2	similar to Hypothetical protein KIAA0141, mRNA	BC064660	?
420=1	peroxiredoxin 4 (Prdx4), mRNA	NM_053512	Metabolism
436=1	similar to Hypothetical protein KIAA0141, mRNA	BC064660	?
437=1	liver UDP-glucuronosyltransferase, phenobarbital-inducible form	NM_173295	Metabolism

表一。大鼠肝臟經缺血灌流不同處理後表現量不同之基因統計表。

第一欄之數字表示不同的核酸檢體。編號方式為：將七個處理組別所得之檢體抽取 RNA 後，以不同引子組合作聚合胺鍊鎖反應。因為 AFLP 的實驗設計，使得’端及’端各有八個引子，即六十四種引子組合（8*8），再配合不同的處理後，共得四百四十八個核酸樣本（8*8*7）。等號後之數字則表示同一個核酸樣本裡，若判斷出各處理組間亮度不同的位置不只一個，由分子量大的依序編號 1,2,3…。

第一組為對照組：不處理。第二組：缺血零分鐘，六小時後收檢體。第三組：缺血零分鐘，二十四小時後收檢體。第四組：缺血三十分鐘，六小時後收檢體。第五組：缺血三十分鐘，二十四小時後收檢體。第六組：缺血六十分鐘，六小時後收檢體。第七組：缺血六十分鐘，二十四小時後收檢體。

Number	Annotation/Description	對照組	第二組	第三組	第四組	第五組	第六組	第七組
Liver regeneration								
97=1	liver regeneration protein lrryan mRNA	3	2	2	2	3	3	0
197=1	liver regeneration-related protein LRRG03 mRNA	5	4	5	2	3	1	0
216=1	Ab1-233 mRNA	2	2	2	2	2	3	1
227=1	liver annexin-like protein (LAL) mRNA	0	3	5	1	3	2	1
302=1	plasma glutamate carboxypeptidase (Pgcp)	4	0	0	1	1	1	1
Signal transduction								
185=2	mitochondrial transmembrane GTPase FZO1B	1	3	5	0	5	1	1
279=1	G protein-binding protein CRFG (Crfg), mRNA	1	3	2	0	2	5	0
292=1	mRNA for ras-related GTPase, ragB	2	3	3	0	5	0	1
Trans Factor								
228=1	similar to eukaryotic translation initiation factor 2	0	0	0	2	0	0	1
351=1	Rat alpha-2-u globulin mRNA	3	0	2	1	2	0	1
358=1	estrogen sulfotransferase mRNA, complete cds	5	0	0	1	0	2	0
Hematopoiesis								
281=1	hematopoietic lineage switch 2 related protein	5	3	0	2	2	1	1
Oncogenesis, membrane trafficking								
210=1	Rat glutathione S-transferase mRNA, complete cds.	5	2	2	3	2	2	5
Thromboregulatory								
180=1	ectonucleoside triphosphate diphosphohydrolase 5	0	0	1	2	3	0	0

表二，各基因在不同處理組合中的表現量。跑電泳膠時，固定 marker DNA 的量，以 500 bp 的亮度當作比較基準 5，完全沒表現的為 0。如此，可以得到 0,1,2,3,4,5 六個表現量等級。