

行政院國家科學委員會專題研究計畫 成果報告

利用基因晶片探討活血化瘀中藥改善氣球擴張所引起血管  
再阻塞的作用(2/2)

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# 行政院國家科學委員會專題研究計畫期中報告

## 利用基因晶片探討活血化癥中藥改善氣球擴張所引起血管再阻塞的作用

Microarray analysis of the preventive effects of Chinese Herbal Medicines on balloon injury-induced restenosis

計畫編號：NSC 91-2320-B-039-011

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### 摘要

關鍵詞：氣球擴張術，血管再阻塞，生物基因晶片

心血管疾病一直以來均高居國人死亡原因的前幾名，其中心肌梗塞更是佔了相當高的比例。目前臨床最常施行的急救方法之一，是利用氣球擴張術使阻塞之血管恢復血流供應。然而，在美國每年大約有百分之 30 至 50 (約二十五萬) 的心肌梗塞患者，在接受冠狀動脈氣球擴張術的治療之後，六個月之內有血管再阻塞的現象。本計畫之研究目標希望藉由目前已發展出來的生物基因晶片作一系統性的探討，以進一步了解眾多引起血管再阻塞之基因協同作用。再探討具有活血化癥功效的中藥包括 *Salvia miltiorrhiz* (丹參)、*Magnolia officinalis* (厚朴)，以口服餵藥的方式給予二週，藉其作用在氣球擴張的大鼠動物模型，評估藥物改善血管再阻塞的分子機轉。

### Abstract

Keywords : angioplasty , restenosis , cDNA microarray

Cardiovascular diseases have been the major leading cause of death in recent years. Among which myocardial infarction was accounted for the great proportion. Percutaneous transluminal coronary angioplasty (PTCA) is one of the most effective approaches in rescuing people from myocardial infarction. However, there are about 30 to 50% of people receiving PTCA in the United States (about 250,000 people) each year suffered from balloon injury-induced restenosis within six months of surgery. The primary goal of this 2-year proposal is to explore the overall gene expression involved in restenosis formation by the newly developed microarray analysis.

We have established the gene expression pattern for restenosis by cDNA microarray analysis. Two major circulation activators of Chinese herbal medicine, *Salvia miltiorrhiz* (丹參), *Magnolia officinalis* (厚朴), have been selected to study their anti-restenosis effects. These circulation activators was administered P.O. for two weeks after balloon injury. The pharmacological mechanism of circulation activators of Chinese herbal medicine against restenosis formation can then be elucidated.

### II、Background and Specific Aims

Coronary heart disease has been rated as one of the leading causes of death worldwide. Percutaneous Transluminal Coronary Angioplasty (PTCA) has been extensively applied as a clinical approach to treat coronary heart disease. However, the injury denudes the vascular endothelial cells and traumatizes the media. This damage results in activation of multiple signal transduction pathways leading to restenosis. The affected genes involved in the process of restenosis include transforming growth factor  $\beta$ -1 (Hartsough et al., 1996), intercellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) (Walpola et al., 1995), endothelin-1 (Malek et al., 1993; Morita et al., 1993), heparin-binding epidermal growth factor-like growth factor (Biro et al., 1992), the proto-oncogenes c-fos, c-jun, and c-myc, and platelet-derived growth factor (PDGF) (Benett et al., 1994), etc. These molecules have been attributed to the formation of restenosis at the site of angioplasty in 30-50% of patients within 6 months after PTCA (Nikol and Hofling, 1995). Therefore, even though PTCA is a very

effective remedy in saving people's lives from myocardial infarction, restenosis has significantly limited its clinical application. Although the deployment of endovascular stents at the time of angioplasty improve the short-term outcome, "in-stent" stenosis remains an enduring problem.

Together with the newly innovated tool provided by recent advances in molecular biology and catheter design, the systematic investigation of restenosis in detail can then be made possible. However, most of these approaches are quite laborious and limited by the number of samples and genes that can be compared simultaneously. In this proposal, we have applied a complementary DNA (cDNA) microarray analysis as an efficient tool for solving the difficulties in quantifying expression of a large number of genes involved in the balloon injury-induced restenosis. The merit of this technique is due to its high throughput identification of the cDNA library from the known human genes. In contrast to the conventional approaches in elucidating the genetic regulation for each disease, which was mostly working on one or two genes at a time, cDNA microarray analysis is dealing with thousands of genes simultaneously. Due to the high degree of homology between human and mammalian genes, it is plausible to study gene expression in rat model subjected to balloon injury using human cDNA microarray under lower stringency of hybridizing conditions (Huang et al., 2000). These results can provide a general insight into the interrelation of those genes previously reported to be responsible for restenosis. The molecular basis of genes responsible for neointimal formation can then be used to elucidate the mechanisms of Chinese herbal medicines in preventing restenosis.

### **III、 Results and Discussion**

In this grant proposal, we aimed to explore the genomic profiles of those genes elicited by balloon injury in rat carotid arteries via cDNA microarray analysis. Description of the materials and methods are as follows.

#### **Administration of Chinese herbal medicine to balloon injury rat models**

Two pure compounds, salvianolic acid B and magnolol extracted from *Salvia miltiorrhiza*(丹参) and *Magnolia officinalis*(厚朴), respectively, were tested in the rat balloon injury model. After establishing the genomic profiles of restenosis induced by balloon injury in rat carotid arteries by cDNA microarray analysis, the aim of this proposal was to explore the preventive effects of two Chinese herbal medicines on restenosis at genomic level. Three concentrations ranging from 100 nM to 100  $\mu$ M for each compound were given to determine the most effective dose in preventing restenosis. SD Rats with 300-400g of body weight were subjected to balloon injury as described (Wu et al., 2001). The rat common carotid arteries receiving balloon injury were rendered for mRNA extraction for the subsequent RT-PCR analysis. One cm of arteries was collected for paraffin sectioning at the thickness of 10  $\mu$ m to determine the vascular morphology. The measurements of the neointimal thickness or vascular remodeling are then processed with the computer program Matrox Inspector (Matrox Electronic Systems Ltd., USA). Our results demonstrated a significant neointimal formation on common carotid arteries 14 days after balloon injury.

#### **Constant drug delivery by osmotic pump**

In the conventional drug delivering system, both I.P. injection or P.O. feeding can not ensure a steady blood concentration. In this proposal, we will use ALZET osmotic pumps (ALZA Corp. Palo Alto, CA, USA) for direct drug delivery into blood circulation to maintain a constant plasma level. These pumps were embedded subcutaneously and cannulated into the right jugular veins. Drugs were delivered via such devices one week prior to balloon injury and continued thereafter for two weeks

#### **Analysis and confirmation of drug effects on restenosis by RT-PCR**

The changes of gene expression by salvianolic acid B and magnolol in balloon

injury-induced restenosis were analyzed according to the microarray analysis of differential gene expression by balloon injury. F635 represents the Cy5 (red fluorescence) Twenty of the most up-regulated and most down-regulated genes were chosen from the cDNA microarray results to decipher the changes of mRNA levels throughout the time course of drug treatment. Since primers specific to those 7680 clones of rat cDNAs spotted onto microarray membrane have been identified, those twenty genes affected by salvianolic acid B and magnolol can be confirmed by RT-PCR. These results explore the genetic profiles of restenosis affected by salvianolic acid B and magnolol.

### Analysis of genes involved in balloon injury-induced restenosis

The scanning signals of each corresponding gene from control and balloon injured arteries were presented as scattered plot using KaleidaGraph of ScanAlyze (Michael Eisen, Stanford University). A dispersed distribution of genes on the scattered plot from a 45-degree diagonal line as shown in Figure 1 indicated a greater difference in gene expression profile between F635 and F532, representing the experimental and control groups, respectively. The ratio of means between balloon injured and normal group was expressed as histogram analysis in figure 2.

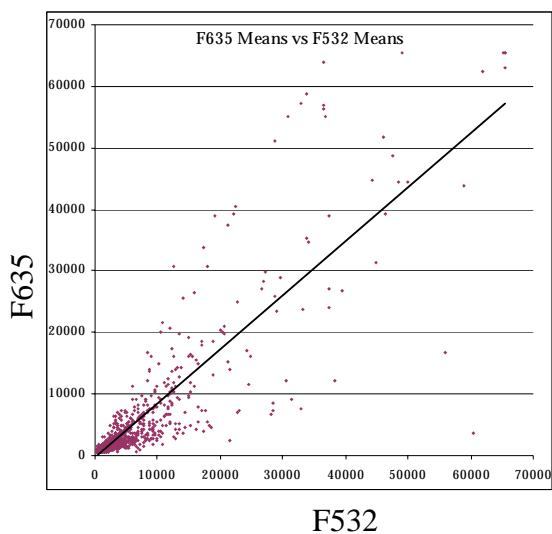


Figure 1. The differential gene expression between balloon-injured rat carotid artery (F635) vs normal control (F532). F635 at Y-axis represents the balloon injured genes while F532 at X-axis represents the normal control genes.

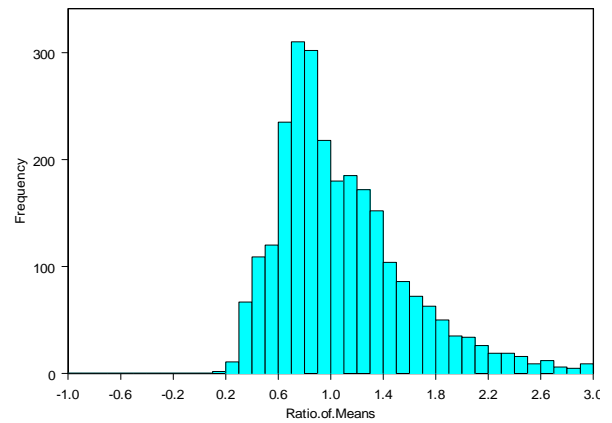


Figure 2. Histogram analysis of the ratio of means for F635 over F532. The number of gene expression ratio at each fold were demonstrated as frequency at Y-axis

### Analysis of gene expression affected by Chinese Herbal medicine involved in restenosis by semi-quantitative RT-PCR

Among the 7680 genes tested by rat cDNA microarray analysis between balloon injured and normal group, 20 most affected genes were selected to evaluate the molecular mechanisms of *Salvia miltiorrhiz* and *Magnolia officinalis* on prevention of restenosis. Figure 3 shows the RT-PCR results on the effects of these two herbal medicines on up-regulated gene expression by injury.

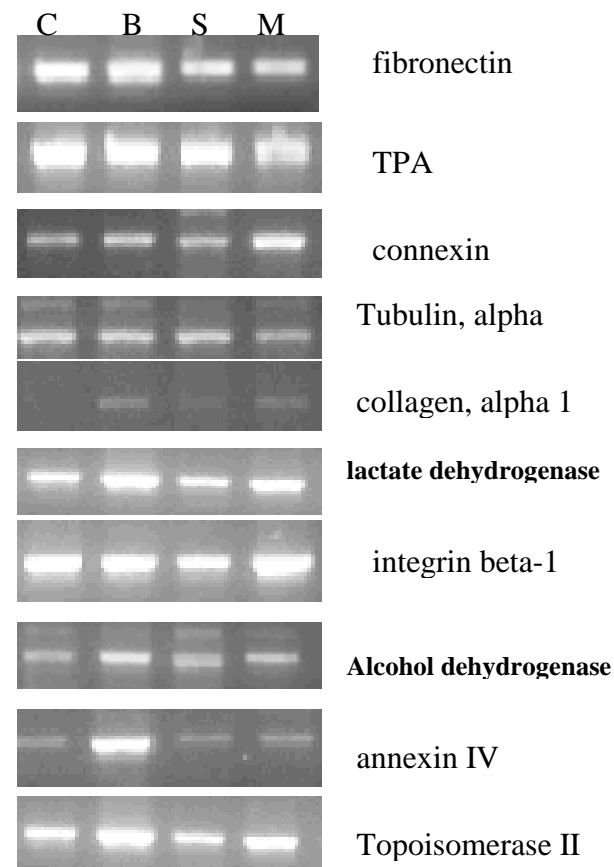


Figure 3. Effects of *Salvia miltiorrhiz* (S) and *Magnolia officinalis* (M) on various up-regulated genes induced by balloon injury. Most genes up-regulated by balloon injury were found to be suppressed by these two drugs. C: control; B: Balloon injury.

Figure 4 shows the RT-PCR results on the effects of these two herbal medicines on down-regulated gene expression by injury.

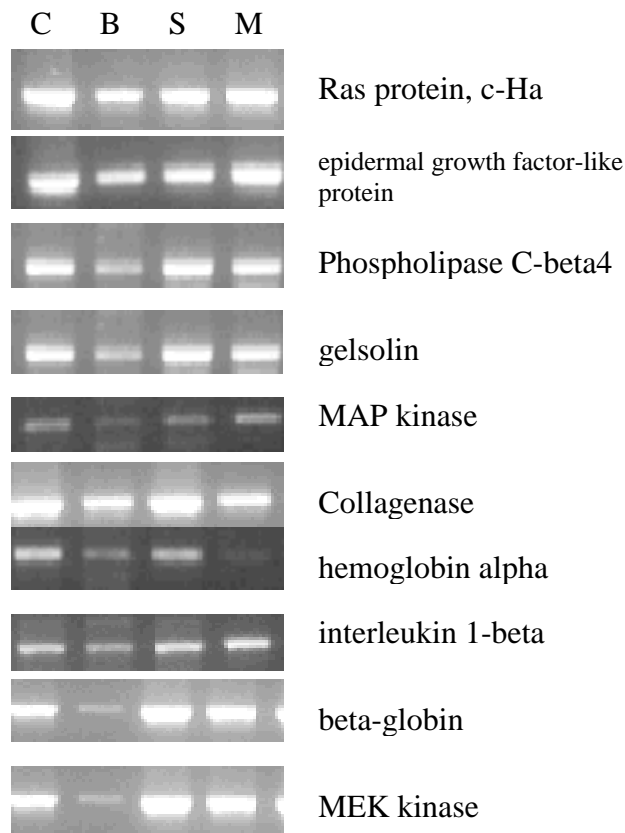


Figure 4. Effects of *Salvia miltiorrhiz* (S) and *Magnolia officinalis* (M) on various down-regulated genes induced by balloon injury. Most genes down-regulated by balloon injury were found to be activated by these two herbs. C: control; B: Balloon injury.

#### IV. Self Evaluation

The aim of this study is to elucidate the balloon injury-induced gene profile in rat carotid arteries. With microarray analysis using 7680 rat cDNAs to screen rat genes affected by balloon injury, we found that there were more than 500 genes with differential expression levels. Twenty genes with most significant differences in expression levels were tested to evaluate the effects of *Salvia miltiorrhiz* and *Magnolia officinalis* on balloon injury-induced neointima formation. Together with microarray and RT-PCR results, we found quite a few interesting genes affected by balloon injury, including collagenase, Ras Protein c-Ha,

Phospholipase C, Tissue plasminogen activator, MAP kinase, fibronectin. Those genes constructed a genetic profile responsible for balloon injury-mediated neointimal formation. In this grant proposal, we have elucidated the molecular mechanism of *Salvia miltiorrhiz* and *Magnolia officinalis* in preventing restenosis through affecting such genetic profile. These interesting data may provide the insight into the potential application of *Salvia miltiorrhiz* and *Magnolia officinalis* for clinical prevention on PTCA-induced restenosis.

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