行政院國家科學委員會專題研究計畫　成果報告

比較 PCR-RFLP, microarray 及 MALDI-TOFMS 三種方法分析血小板抗元的精確性

計畫類別：個別型計畫
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計畫主持人：施木青
共同主持人：張建國

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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

(計畫名稱)

比較 PCR-RFLP, Microarray 及 MALDI-TOFMS 三種方法分析血小板抗原的精確性

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計畫參與人員：

成果報告類型(依經費核定清單規定繳交)： ◎完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□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
□國際合作研究計畫國外研究報告書一份

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：中國醫藥大學醫事技術學系

中　華　民　國 九十三　年 九 月 二十七 日

摘要

血小板抗原從 HPA-1 至 HPA-13,Oe 和 Gov 至少有十五種多。分別表現在血小板膜上 GPⅢa, GP Ⅱb, GPIb, GPIa 和 GPIbβ的蛋白質。單一基因的變化造成血小板基因型的多形性變化。本研究收集中國人(69 人)，印尼人(89 人)，泰國人(27 人)和菲律賓人(95 人)的 DNA，以 PCR-RFLP, Microarray 和 MALDI-TOFMS 三種方法偵測這四個國家人種的血小板基因型變化。結果 HPA-1a, HPA-2a, HPA-4a, HPA-5a, HPA-8a 和 HPA-9a 的盛行率分別為 97.9~100%, 86.4~97.8%, 99.2~100%, 96.3~100%, 100%和 100%, 六種血小板抗原的基因型的盛行率無顯著差異，大多集中在 a 的表型，而且結果的一致性很高。PCR-RFLP 方法作血小板抗原的 SNP 簡便、經濟，但速度慢、費時、費人力，不適合作大量篩檢，Microarray 方法則需要較成熟的技術，否則難以消除非特異雜合反應，干擾結果判讀；MALDI-TOFMS 方法速度快、準確度高，很適合作大量篩檢，設備雖昂貴，但操作成本相對低。HLA 抗原和 HPA 抗原引起的血小板抗體是導致血小板輸血無效的主因。輸血前選擇 HLA 和 HPA 同型的供血者是改善目前血小板輸血無效的最佳方法。

關鍵詞：人類血小板抗原、基因型

Abstract

There are at least 15 kinds of platelet antigens which are HPA-1 to HPA-13, Oe and Gov. These antigens demostrate their antigenesity on platelet menbrane's proteins: GPIIIa,GPIIb,GPIa,GPIb,GPIb β .The SNP of platelet antigen was due to single gene mutation on these GP proteins.

In these study we collect leucocyte's DNA from 69 Chinese,89 Indonesian, 27 Tailand, and 95 Philipinos for detecting the genotypes of platelet antigens.Three methods were used, there were PCR-RFLP, Microarray and MALDI-TOFMS.The frequency of platelet genotypes for HPA-1a,,HPA-2a,HPA-4a,HPA-5a,HPA-8a and HPA-9a were 97.9-100%,86.4-97.8%,99.2-100%,96.3-100%,100% and 100% respectively.The genotype frequency of the six platelet antigens were no significant difference among the four nation's people.Most of them were HPA-a phenotypes. PCR-RFLP was a easy and economic method when used to detect platelet genotype. However the low speed, time comsuming , and labor loading made it was not suitable for mast screening. Although the Microarry method can be used for mast screening, it need high technique to eliminate non-specific hybridization. In conclusion, MALDI-TOFMS method was the method for choice to detect SNP of platelet antigens

. HLA and HPA antibodies were the two major causes for ineffective platelet transfusion. Selection of the same HLA and HPA genotypes or phenotypes will improve the medical effect of platelet transfusion.

Keywords：Human Platelet Antigen, genotype

研究計畫內容：

(一) 前言(Introduction)：

Thrombocytopenia is principally a disorder of increased platelet destruction mediated by autoantibodies , isoimmune alloantibodies or bone marrow failure.These disorders include : idiopathic thrombocytopenic purpura(ITP), post-transfusional purpura, and neonatal alloimmune thrombocytopenia(NAIT). Antibodies to specifc platelet membrane glycoproteins can be detected in most patients with thrombocytopenia. These antibodies are directed against epitopes on glycoprotein IIb/IIIa, the most abundant and immunologenic platelet-surface glycoprotein which determined the human platelet antigens(HPA).There are two clinical application of HPA, one for determination of specific antibodies in patient's serum and the other for selection of compatible donor platelet for transfusion.

HPA typing is essential for the diagnosis and treatment of a variety of diseases. More than thirteen HPA genotypes have been described from HPA-1 to HPT-13,Oe and Gov. PCR method is the best choice for the determination of specific HPA genotypes because most of the DNA sequence of HPA have been cloned and published in Gene Bank. Various PCR-based HPA typing technique have been developed.We modified the PCR-RFLP method and used it to analyze the gene frequency in four nations. Our purpose was to estabilish the genetic basis of HPA and compare the difference between them. Our study is the frist complete analysis of HPA-1 to HPA-13,Oe and Gov in four different populations. Our results offer a economic laboratory method for typing HPA and it colud have some impact on the clinical management of thrombocytopenia.

Varierty of HPA system belongs to single nucleotide polymorphism(SNP).There are many techniques can be used to detect SNP such as ACRS,ARMS,ASO,SSCP,DGGE,SBE, Microarray,and MALDI-TOF….etc..In these methods, Microarray and MALDI-TOF mass spectrophotometry are the two rapid and economic methods that can be used for mass survey. Now we are developing these methods and evaluating their accuracy.This proposal has got the grant support from National Science Council(NSC 92-2314-B-039-031) .Our efforts will try to estabilish a technique plateform for HPA genotyping.

(二) 研究計畫背景及目的：

血小板抗原(Human platelet antigen, 簡稱 HPA)是由血小板表面細胞膜上的蛋白質來決定。目前已超過十三種以上的變異型。一般而言。較常見的以"a", 而罕見的則以"b"表示。血小板抗原的分子基礎。已相當清楚, 絕大部分是因單一鹼基的改變。因而造成血小板細胞膜上的蛋白質上的胺基酸產生改變。主要發生在五種血小板表面的蛋白質上。包括 GPIIb (HPA-3.9), GPIIIa (HPA-1,4,5,7,8,10,11 及 Oe), GPIb (HPA-2), GPIa (HPA-5 及 13) 及 GPIbβ (HPA-12)。也有少數在其它的膜蛋白質上。如 CD109 (Gov) [1-10]。最近的研究發現。這些變異型的血小板抗原與新生兒免疫性血小板過少症, 輸血後紫斑, 輸血小板無效症, 冠狀動脈阻塞症。及腦血管病變有關(11-22)。因此。診斷這些變異型。對於以上疾病的預防及治療有非常重要的意義。

偵測各種變異型血小板抗原的抗體尚未商品化。因此想利用血清學的方法找出各種變異型。仍然相當困難。由於這些 HPA 變異型的基因突變所在，已相當清楚(1-10)。因此直接偵測基因變異所在。將是更方便可行之方法。在過去二、三年。我們利用 PCR-RFLP 的方法分析了 8 種血小板抗原在東南亞四族群

的變化情形(23), 最近我們用同樣的方法分析 15 種 HPA 抗原在東南亞四族群的分佈情形。結果顯示: HPA3 及 GOV 中"a"及"b" alleles 的發生率。在此四族群皆相當高。而其它 HPA 抗原主要以"a" allele 為主。"b" allele 很少超過 5%者 (24)。

偵測 single nucleotide polymorphism 的方法很多。但所有的方法。幾乎均以 PCR 方法為基礎發展出來的。如 PCR-RFLP (涵蓋 ACRS), ARMS, ASO (allele specific oligonucleotide hybridization), SSCP, DGGE, SBE (single base extension), chemical 或 RNAase cleavage-based ligase-based detection, duplex-specific nuclease preference….等方法用於偵測 SNP (23-39)。最近幾年更因為新的儀器的使用。 將這些方法發展成自動化及大量篩選的方法 (40-51)。一般而言。這些方法可歸納成三類: 第一類可偵測單一 SNP, 或幾個 SNP, 上述方法均可完成此目的 (23-39); 第二類可偵測幾十或幾百個 SNP, 如 Microarray or oligochip (46-49)；第三類可快速大量偵測單一或幾個 SNP (44, 51,52), 這一類的方法往往要利用新的儀器才能達到, 如 MALDI-TOF instrument…等 (50,54)。這些方法各有其優缺點。有的方法較不方便。有的需要特殊儀器。而精確性方面也不相同。不同的基因偵測的結果精確度也各不相同 (40-52)。

血小板抗原的變異系統也屬於 SNP 的範圍。在過去已有相當多 PCR-based 的方法應用在此領域。如 PCR-RFLP, ASO, PCR and reverse dot-blot hybridization, SSCP, allelic specific PCR, PCR homoduplex formation assay and multiplex PCR and ligation-based type (23-33, 53). 但尚未有用新的儀器發展出來的方法。或偵測目前所有已知 HPA antigens 的方法。因此。本研究將比較 PCR-RFLP, Microarray 及 MALDI-TOF mass spectrometry 等三種方法用於 HPA 抗原偵測的精確性，並比較其經濟效益。

(三) 研究方法：

1. 淬取血液中白血球之 DNA: 在過去我們已淬取了 566 例台灣人。100 例菲律賓人。107 例印尼人及 137 例泰國人的 DNA (23,54). 這些個案已完成 PCR-RFLP 分析所有的 HAP antigens 的發生率。本研究計畫再利用這些個案用於做 Microarray 及 MADI-TOF 的分析。

2. PCR-RFLP 的分析: 此方面的研究已完成, 請參考 preliminary results。

3. Microarray 分析: 首先我們要做涵蓋所有已知的 HPA antigens wild type 及 variants 的 oligochip, 其 sequences 如（表一）

4. MALDI-TOF mass spectrometry analysis:

   (1) 取 2λ的溶液內含 2.5U exonuclease I 及 2.5U shrimp alkaline phosphatase 加入 PCR 產物中。在 37℃作用 30 分鐘。以除 dNTP 及 PCR primers, 然後加熱以不活化上述酵素。

   (2) Mini-sequencing reactions: 加入 oligonucleotide primers, dideoxynucleotides 及 thermostable polymerase to extend the PCR product by a single base in a total reaction volume of 10ul (the primers as below):

   (3) Extension reaction products will be purified with Poros 50-R1 reversed phase chromatography matrix and centifuged.

   (4) DNA was bound to the reverse phase media in the presence of triethylammonium acetate, rinsed with aqueous buffer, and eluted with either 25% acetonitrile or MALDI matrix

consisting either trihydroxyaceto phenone (THAP) or 3-hydroxypicolinic acid.

(5) Masses of the mini-sequencing primers and extension products will be assessed using linear delayed extraction mass spectrometry and a Voyager-DEMALDI-TOF instrument. Genotypes for the samples will be resolved with the aid of the Genespectrometer software package.

(6) 操作流程圖如（圖一）

5．Direct sequencing PCR products。

6．比較三種方法的經濟效益、精確性及便利性。

(四) 預期完成之工作項目及成果

1. 完成利用 PCR-RFLP, Microarray 及 MALDI-TOF 的方法分析東南亞四種族 HPA 變異的發生情形。
2. 完成上述三種方法之比較。
3. 所建立的方法可臨床應用, 以診斷及預防因變異血小板抗原所引起的相關疾病。
4. 參予的工作人員, 將可獲得基因偵測法的設計及應用方面的技能。

(五) **Preliminary results**:

我們已完成的研究：

1．M. C. Shih, T. C. Liu, I. L. Lin, S. F. Lin, C. M. Chen, J. G. Chang (2003). Gene Frequencies of the HPA-1 to HPA-13, Oe and Gov Platelet Antigen Alleles in Taiwanese, Indonesian, Filipino and Thai populations. INTERNATIONAL JOURNAL of Molecular MEDICINE 12: 609-614, 2003.

2．Liu TC, Shih MC, Lin CL, Lin SF, Chen CM, Chang JG. Gene frequencies of the HPA-1 to HPA-8w platelet antigene alleles in Taiwanese, Indonesian, and Thai. Ann Hematol. 81:244-248, 2002.

(六) 結果與討論：

本研究共收集中國(69 人)、印尼(89 人)、泰國(27 人)、菲律賓(95 人)的 DNA 檢體進行三種方法的測試與結果比對。PCR-RFLP 方法，本實驗室均已測試完成並作成報告(23,24)，MALDI-TOFMS 與 PCR-RFLP 結果的比較如表(二)所示. 比對血小板 6 個抗原中僅泰國人的 HPA-2 發現率有較大差異, 其它都很相近.檢討原因,可能與樣本母數差異太大有關. MALDI-TOFMS 方法是，依 primer 延伸一個 Base 或 2 個 Base 來加以區別和鑑定其基因的 SNP 位置，其結果與 PCR-RFLP 的結果的一致性很高。有差異的結果用傳統 DNA sequence 的方法加以驗證，原有方法再重作一次，結果就趨於一致。由此可見以基因為基礎的各種檢測方法可信度都很高，不同方法結果略有差異，關鍵不在方法本身，而是在於技術的熟練度。傳統 PCR-RFLP 的方法仰賴手工技術太多，操作過程中的品質控制非常重要，若有二重做的複驗機制，其作出的結果與 MALDI-TOFMS 的結果幾乎完全一致。至於 MALDI-TOFMS 方法也並非完全可靠： 在所有測試件中有一例在 HPA-5 的結果上有差異，MALDI-TOFMS 呈現 wild type 而 PCR-RFLP 呈現 hetero type ，兩者經 sequence 再次確認是屬 hetero type（圖二）.到目前為止，尚未見有

MALDI-TOFMS 來分析血小板抗原的基因分型，本研究屬首例，研究結果顯示：

1. MALDI-TOFMS 方法很適合做 HPA-SNP 的研究。
2. MALDI-TOFMS 方法速度快，準確度很高，適合作大量篩檢。
3. PCR-RFLP 方法，若有二重作複驗機制，也有很精確的結果，只是速度慢、耗時、費人力，不適合作大量篩檢。
4. MALDI-TOFMS 設備昂貴，但操作成本相對便宜，未來應用於基因 SNP 的研究應多加鼓勵。
5. Microarray 方法，由於技術不熟練及 non-specific hybrid reaction，雖嘗試各種溫度的改變，但效果還是不好，不列入比較與探討。
6. 血小板抗體是導致血小板輸血無效的主要原因，引發血小板抗體的兩大主要抗原一是血小板抗原，另一是人類白血球抗原(HLA)，HLA 抗原一抗體的作用機制已很清楚，血小板抗原則是近年來才有比較多的研究報告。國際血液及輸血協會(ICSH/ISBT)在 1990 年才組成工作小組統合血小板抗原血清型的名稱，至今 15 種以上的血小板抗原的血清型和基因型都已被證實。血小板抗原一抗體反應導致輸血小板無效的個案也一一被證實。本研究成果有助於大規模篩檢供血者血小板的抗原的基因型作為血小板輸血的配對基礎。理論上，輸血小板前選擇 HLA 和 HPA 同型的供血者是最佳選擇，但礙於檢驗步驟的煩瑣和成本過高的考量，未能付之於實際應用，本研究成果可讓中華民國捐血基金會及血液專家重新思考其可行性。

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（表一）Primer and probe sequence used in Microarray Oligochip

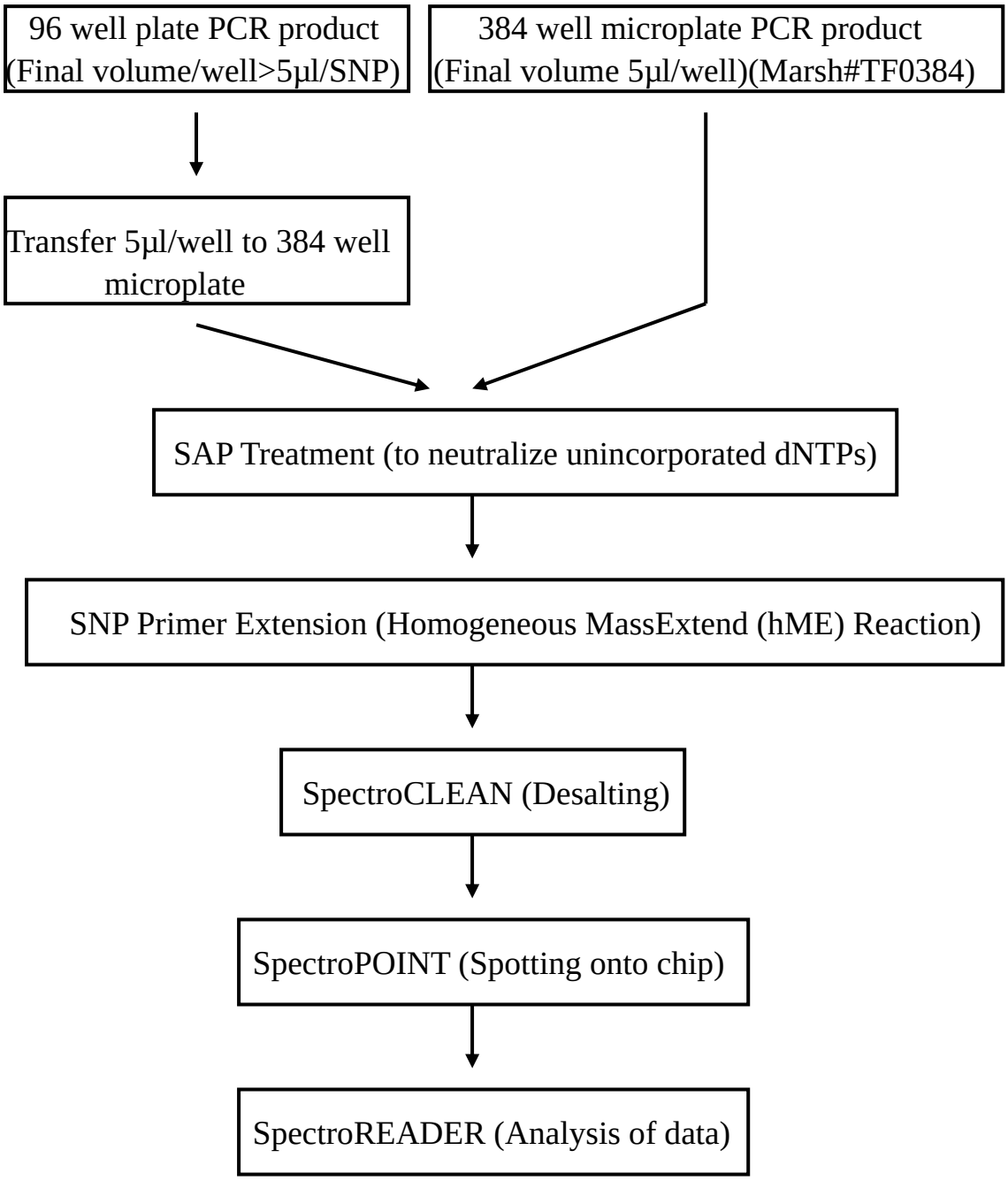
Gene Name	Mutation	Sense seq	Antisense Seq	Probe-W Seq
GOV	AGG->TGG	tcagttcttggttttgtgatgt	aaaccagtagccacccaaga	ttcagttacaggatttacc
GOV	TAC->TCC	tcagttcttggttttgtgatgt	aaaccagtagccacccaaga	acttcagttacaggattta
GP1ba-HPA-2	ACG->ATG	acctgaaaggcaatgagctg	gggagctcagtcaagttgttg	ggctcctgacgcccacacc
GPIa HPA-5	GAG->AAG	tgacctaaagaaagaggaaggaa	tttttctttccaaatgcaagtt	actatcaaagaggtaaaaa
GPIIb-HPA-3	ATC->AGC	gggcctgaccactccttt	ggaagatctgtctgcgatcc	ggctgcccatccccagccc
GPIIb-HPA-9	GTG->ATG	gggcctgaccactccttt	ggaagatctgtctgcgatcc	ccccccccaggtggactggg
GPIIIa Oe		atggggacacctgtgagaag	atcctctcctacctcccagc	gcacctttaagaagtgagt
GPIIIa-HPA-1	CTG->CCG	ttgctggacttctctttggg	tctggggcacagttatcctt	ccctgcctctgggctcacc
GPIIIa-HPA10	G->A	aggataactgtgccccagaa	gagtgacctgggagctgtct	gtgaggcccgagtactaga
GPIIIa-HPA11	CGT->CAT	ggagccctacatgacgaaaa	tagaacctgggtgtgtgcaa	cctgcaaccgttactgccg
GPIIIa-HPA-4	CGA->CAA	ctgtggagcatccagaacct	acaggcttgtccacaaatgc	cccagatgcgaaagctcac
GPIIIa-HPA-6	CGA->CAA	tgtgagtgctcagaggagga	gtggcagacacattgaccac	gcagcccccgagagggtca
GPIIIa-HPA-7w	CCC->GCC	caggtgagcttcagcattga	acgatcaggctgtccttgaa	cgaggctgtccccaggaga
GPIIIa-HPA-8w	CGT->TGT	ggagccctacatgacgaaaa	tagaacctgggtgtgtgcaa	cgttactgccgtgacgaga

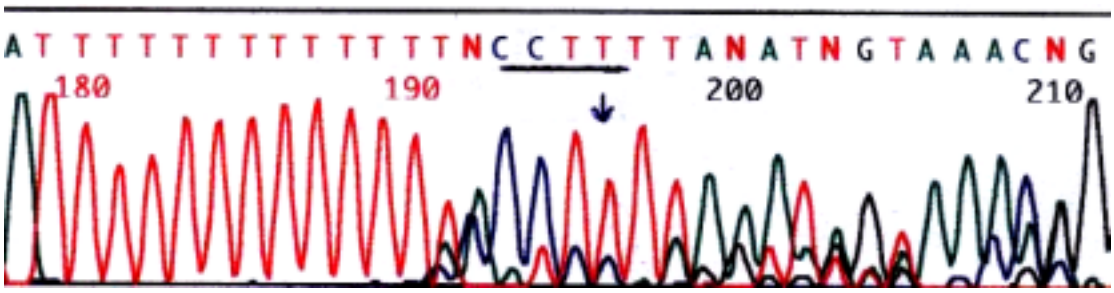
（表二）

MALDI-TOFMS 與傳統 PCR-RFLP 測血小板抗原的結果比較表									
		MALDI-TOFMS				PCR-RFLP			
血小板抗原		印尼 n=89	泰國 n=27	菲律賓 n=95	中國 n=69	印尼 n=107	泰國 n=137	菲律賓 n=100	中國 n=566
HPA-1	1a(%)	99.4	100	97.9	100	99.1	98.5	98.0	99.8
	1b(%)	0.6	0	2.1	0	0.9	1.5	2.0	0.2
HPA-2	2a(%)	97.8	86.4	94.6	93.5	93.9	93.8	97.5	94.7
	2b(%)	2.2	13.6	5.4	6.5	6.1	6.2	2.5	5.3
HPA-4	4a(%)	100	100	99.3	99.2	100	100	99.5	99.8
	4b(%)	0	0	0.7	0.8	0	0	0.5	0.2
HPA-5	5a(%)	96.3	97.5	97.1	100	99.5	96.4	96.5	99.0
	5b(%)	3.8	2.5	2.9	0	0.5	3.6	3.5	1.0
HPA-8	8a(%)	100	100	100	100	100	100	100	100
	8b(%)	0	0	0	0	0	0	0	0
HPA-9	9a(%)	100	100	100	100	100	100	100	100
	9b(%)	0	0	0	0	0	0	0	0

(圖一)

Genotyping-SNP Detection Procedure of MALDI-TOFMS method





（圖二）DNA sequence of HPA-5,Code192 presents CTT/CTC heterotype

計畫成果自評：

本計畫的目的在於尋找一個適合偵測血小板抗原基因型且可應用於實驗室的方法，作為輸血小板前選擇合適的供血者，用以發揮最好的血小板輸血療效。Microarray 和 MALDI-TOFMS 二種方法都很適合用來偵測 HPA 的 SNP. 但查閱歷年來的研究報告，卻沒有人作此嚐試。本計畫屬第一次嚐試。

MALDI-TOFMS 方法在精確度、速度、人力…等各方面都優於傳統的 PCR-RFLP 方法，這是本計畫的最大發現。至於 Microarray 方法是否優於 MALDI-TOFMS 方法，由於實驗的不成功，無法做一精確比較。但從本計畫執行中的經驗，只要 Microarray chip 設計妥適，其精確度與方便性可能更優於 MALDI-TOFMS。本研究室會繼續這方面的努力。

選擇合適的 HLA 和 HPA 抗原型作為血小板輸血之用，可提昇血小板之療效已很清楚；至今各醫療院所未做此篩檢主要在於個案數不多，操作的技術困難度高，人力和試藥成本高，阻礙了它的普遍化。本研究成果可提供中華民國捐血中心建立 MALDI-TOFMS 方法，集中處理全台灣 HPA 基因型的篩檢。

附件二

可供推廣之研發成果資料表

□ 可申請專利　□ 可技術移轉　日期 93 年 9 月 14 日

國科會補助計畫	計畫名稱：比較 PCR-RFLP, Microarray 及 MALDI-TOFMS 三種方法分析血小板抗原的精確性 計畫主持人：施木青 計畫編號：NSC 92－2314－B－039－031 學門領域：醫技領域
技術/名稱	MALDI-TOFMS 方法應用於 HPA 基因的分析
發明人/創作人	施木青
技術說明	中文：血小板抗原至少有 15 種，在輸血療效上，有其重要性，若能利用 MALDI-TOFMS 方法鑑定供血人與受血人的血小板抗原基因型，選擇最合適的血小板作為輸血之用，可改善目前血小板輸血的缺失。 英文：There are at least 15 genotype of HPA, it is important in platelet transfusion. MALDI-TOFMS method can detect HPA genotype for the donor and recipient. It can improve the platelet transfusion efficacy, if HPA genotype can matched before transfusion.
可利用之產業及可開發之產品	生技公司可開發套組試劑
技術特點	一次測試就能定出 HPA genotype，若在輔助於電腦配對，就可能選出最合適的血小板作為輸血之用.
推廣及運用的價值	台灣捐血基金會，若能建立此項技術，對血小板輸血療效一定有很大的貢獻。

※ 1.每項研發成果請填寫一式二份，一份隨成果報告送繳本會，一份送　貴單位研發成果推廣單位（如技術移轉中心）。

※ **2.本項研發成果若尚未申請專利，請勿揭露可申請專利之主要內容。**