國科會專題研究計劃成果報告

計劃名稱:臺灣愛滋病毒亞型對合併療法療效之影響
The clinical study of combination therapy of HIV infection in Taiwan-The impact of HIV-1 subtype on treatment outcome

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ABSTRACT

More than one hundred and sixty patients with advanced stage of HIV-1 infection had been treated in National Taiwan University Hospital after the availability of highly active antiretroviral therapy (HAART). Undetectable viral load (less than 400 copy/ml) was achieved in 58% of our patients who had been treated for more than 20 months. The study of circulating HIV-1 subtypes showed that 27% of our patients were infected with HIV-1 subtype E. The preliminary result of HAART revealed similar rate of treatment failure in both patient groups infected with subtype E and non-E. We detected multiple mutations in reverse transcriptase and protease gene which conferred drug resistance in patients with treatment failure. In addition, there were other mutations of unknown significance. More data of mutations will be accumulated in the following years which may help us to clarify the association of mutations and drug resistance. The genotype of drug resistance is less well characterized in patients infected with subtype E. In our study, we found some potential subtype-specific mutation sites. Future study may provide answer to the question.

Keywords: AIDS combination therapy HIV-1 subtype
protease inhibitor drug resistance

摘　　要

國內開始以併用療法治療愛滋病已有三十個月.台大醫院治療之病患絕大多數爲已發病而 CD4 細胞數目較低者,若療效以血清病毒量查不到 (每毫升血清病毒數<400) 爲治療目標,則治療期已達二十個月者有 58%爲治療成功. 台大醫院治療之病患中 E 與非 E 亞型之比率爲 27%與 73%,若考慮病毒亞型之影響則 E 與非 E 亞型之治療成功率分別爲 60%與 53.8%,並無有意義之差別.由於抗藥性病毒株的出現是治療失敗的主要原因之一,故監控蛋白分解脢與反轉錄脢基因之突變可及早因應. 我們發現抗藥性病毒基因之突變在初期只有一個,但久的了以後則累積多個. 我們也發現有些突變過去並未曾被報告與抗藥性相關,其意義爲何有待發掘更多同樣之突變來判斷.另外病毒亞型 E 之抗藥行性突變目前並未完全清楚,我們的初步結果顯示亞型特異性突變存在之可能.這些均需長期臨床療效之研究分析後再研究抗藥性病毒基因之突變方能得到答案.

關鍵詞:　愛滋病　　併用療法　　病毒亞型　　蛋白分解　抑制劑
抗藥性突變

Introduction

In the era of highly active antiretroviral therapy, the principles of antiretroviral treatment[1-5] are (1) potent regimens in which three reverse transcriptase inhibitors (RTI) or two RTIs and one potent protease inhibitor (PI) are included should be used. (2) the goal of treatment is to reach undetectable level of viral load by using a sensitive test. (3) measurement of viral load and CD4 is essential[6,7]. (4) original HAART must be replaced promptly in the presence of treatment failure. The detection of mutations associated with drug resistance is of great help in deciding which salvage drug should be used. The success of HAART is based on the fact that by limiting the viral replication to a very low level, immune system will not be damaged and the drug resistant viral mutants are less likely to develop in low productive state. Besides, more mutations are required to confer drug resistance in the presence of triple therapy which results in slower development of resistance[8].

Mutants resistant to antiretroviral therapy may exist prior to initiation of treatment[9,10] , but combinations of multiple mutations associated with drug resistance are highly unlikely to occur naturally. In the presence of selective pressure derived from antiretroviral therapy, drug resistant mutants may have competitive advantage and eventually come to represent the dominant quasi-species[11]. Stepwise accumulation of multiple mutations then adds to the fitness of the drug resistant mutant and may later confer resistance to other antiviral agents[12-14]. Therefore, routine monitoring of drug resistance mutations had been proposed for design of salvaging therapy[15,16]. Our clinical study of antiretroviral treatment tried to answer if patient groups infected with different HIV-1 subtypes had different treatment outcome and different genotype of drug resistance.

MATERIALS AND METHODS

PATIENTS

Patients treated at National Taiwan University Hospital with combination therapy were followed up monthly. Before treatment, baseline data including CD4 cell count, hemogram, blood chemistry, urinalysis, viral load, past medical record and complete physical examination was collected. The laboratory tests were repeated one month later and then every four months. Plasma samples were stored at -70°C. Patients were randomly assigned to different treatment regiments (3RTIs or 2RTIs+PI). Combination therapy including 2 NRTIs and 2 PIs are allowed to be used as salvaging regimens. During each visit, physical examination and drug side effect will be checked.

QUANTITATION OF VIRAL LOAD

The assay for HIV RNA was done by a quantitative reverse-transcriptase polymerase-chain-reaction assay (Amplicor HIV Monitor, Roche). The lower limit of quantification was 50 RNA copies per milliliter. We had compared RT-PCR and DNA signal amplification method (Chiron) in patients with subtype E infection. No significant difference in sensitivity was noted as compared with subtype B.

HIV-1 SUBTYPING

A rapid and simple method for subtyping HIV-1 developed by us had been proved to be useful [17]. The principle is to use a pair of subtype E specific vpu primers during the second round of nested PCR for vpu gene. Only patients infected with HIV subtype E have band of PCR product visible on agarose gel in the presence of ethidium bromide. No band is seen with subtype B and faint band is noted in the case

of subtype C and A. Because only a few case of subtype A and C are present in Taiwan, our method can satisfy present protocol.

Genomic DNA was extracted from PBMC by conventional phenol/ chloroform method. In PCR reaction, 1-2 ug of genomic DNA is added to 50ul first round reaction mixture containing 50mM KCl, 10mM Tris pH 8.3, 1.5 mM $MgCl_2$, o.1% Triton X-100, 5 pmol of each primers and o.2 mM of each dNTPs. The reaction condition is 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for one minute. For second round, the reaction is the same except that the annealing temperature is 55°C. The PCR products are then analyzed on agarose gel electrophoresis. The outer primers are S 5'-CTTAGGCATCTCCTATGCA and AS 5'-TCTGATGCACAAAATAG AGT .The inner E-specific primers are S 5'-GTGTTCAAGCGGTTAAGTAGACA and AS 5'-ATCACGTTATCATCCTGACT TCATCGC.

SEQUENTIAL MUTATIONS IN HIV PROTEASE GENE

Patients who had initial response to combination therapy (plasma viral load reached undetectable level or decreased more than 10 fold), but later had detectable viral load again or 5 fold increase from the nadir which was proved by repeated quantification of viral load will be enrolled in this study. HIV RNA was extracted from 200 ul plasma by using RNA isolation and purification kit. HIV-1 RNA was then reverse transcribed to cDNA by using reverse transcriptase M-MLV (Promega). The primer was 5'-TTGTTTTACATCATTAGTGTGGGC. The HIV protease gene was be amplified with nested PCR. The primers used in first PCR are: S 5'-GAAGCAATGAGCCAAGTACAAAT and anti-sense 5'-GATATGTCCATTGGCC

TTGCCCCT. The inner primers are S 5'-TTCAATTGTGGCAAAGAAGGGCAC and anti-sense 5'-TAAGTCTTTTGATGGGTCATAATA. The PCR products are then purified and directly sequenced by dye-labeled terminator on autosequencer.

Results:

Patients with HIV infection were treated with highly active antiretroviral therapy (HAART) in Taiwan since April 1997. Over one hundred and sixty patients had been treated in National Taiwan Hospital in the past 30 months. The majority of our patients were referred to us for treatment of acquired immunodeficiency syndrome (AIDS) - related diseases such as pneumocystic carinii pneumonia, extrapulmonary tuberculosis, cryptococcal meningitis, amebic liver abscess, B cell lymphoma and Kaposi's sarcoma. Most of them had CD4 cell count less than 200/mm^3. The principle of combination therapy is two RTIs plus one or two PIs. Various combinations of RTIs and PIs were used based on the consideration of toxicity, concomitant therapy of AIDS - related diseases and treatment results. Therefore, we did not intend to compare the efficacy of various combinations. The goal of HAART is to lower the viral load to less than 500 copy/ml. As shown in figure 1, the rate of successful treatment was between 50-60 % after treatment for more than 3-5 months.

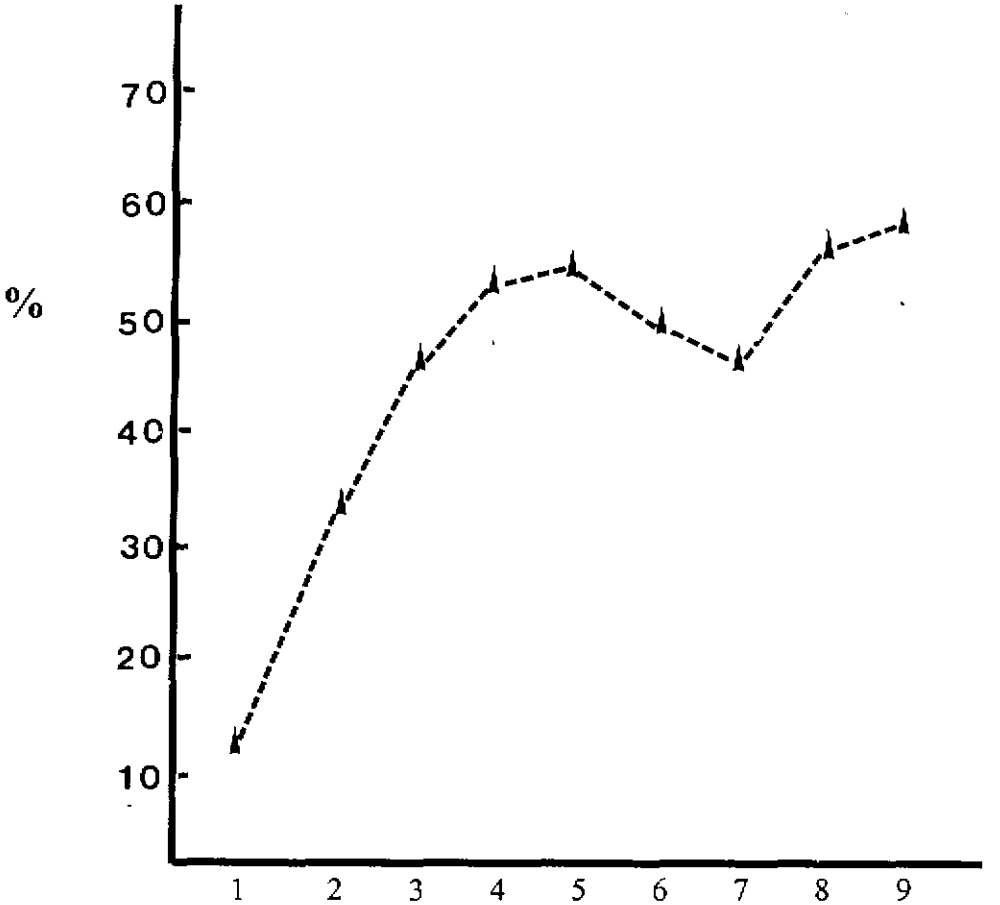
HIV-1 subtype had been determined in 161 patients by using subtype E specific nested PCR. Fourty-three (27%) patients were found to have subtype E infection (28 men and 15 women) and 118 (73%) patients had non-E infection (115 men and 3 women). Almost all the non-E HIV-1 subtypes in Taiwan were subtype B[17]. The original object of our study was to find the impact of HIV-1 subtype on successful rate of HAART. Among 62 patients who had been treated for more than 20 months, 15 and 39 patients were infected with subtype E and non-E (all were presumed to be subtype B) respectively. No significant difference was found between patients infected with subtype E and non-E in the successful rate of treatment with HAART (60% *vs* 53.8%)

Drug- resistant mutations were studied in patients who had good compliance

but their viral loads were greater than 10^3 (Table 1). Reverse transcriptase and protease mutations found in patients with had subtype E (6, 58, 76, 200) and subtype B (17, 73, 71) infection were listed in table 2. The subtype specific amino acids were marked with shaded background. As expected , patients who had been treated for more than two years but failed to achieve undetectable viral load showed multiple mutation sites in reverse transcriptase. On the other hand, patient 200 who was treated for only 10 months had only one mutation which conferred drug resistance. Study of protease gene also revealed drug resistance mutations. However, primary and secondary drug resistant mutations were not found in patient 73, although he had multiple RT- resistant mutations. Differences were noted between subtype B and E at some sporadic sites in reverse transcriptase and protease gene. The significance of the difference is not known at present.

Table 1 The level of viral load when drug-resistant mutations were detected

Patients	Viral load x10^3	Subtype
200	5.014	E
6	2.31	E
58	28.2	E
76(5-11)	112	E
76(9-29)	29.52	
17	63.7	B
73	255	B
71	10.75	B



	1	2	3	4	5	6	7	8	9
Total number	148	119	98	83.	83	77	65	62	40
<500copy/ml	18	41	44	45	47	38	30	36	25

Figure 1. Percentage of viral load less than 500 copy/ml after antiretroviral treatment. Time of viral load detection: 1. before treatment, 2. between 1-2monhts, 3. between 3-5 months, 4. between 6-8 months, 5. between 9-11 months, 6. between 12-14 months, 7. between 15-18 months, 8. Between 19-22 months, 9. Between 23-25 months after treatment.

Table 2

Protease mutations

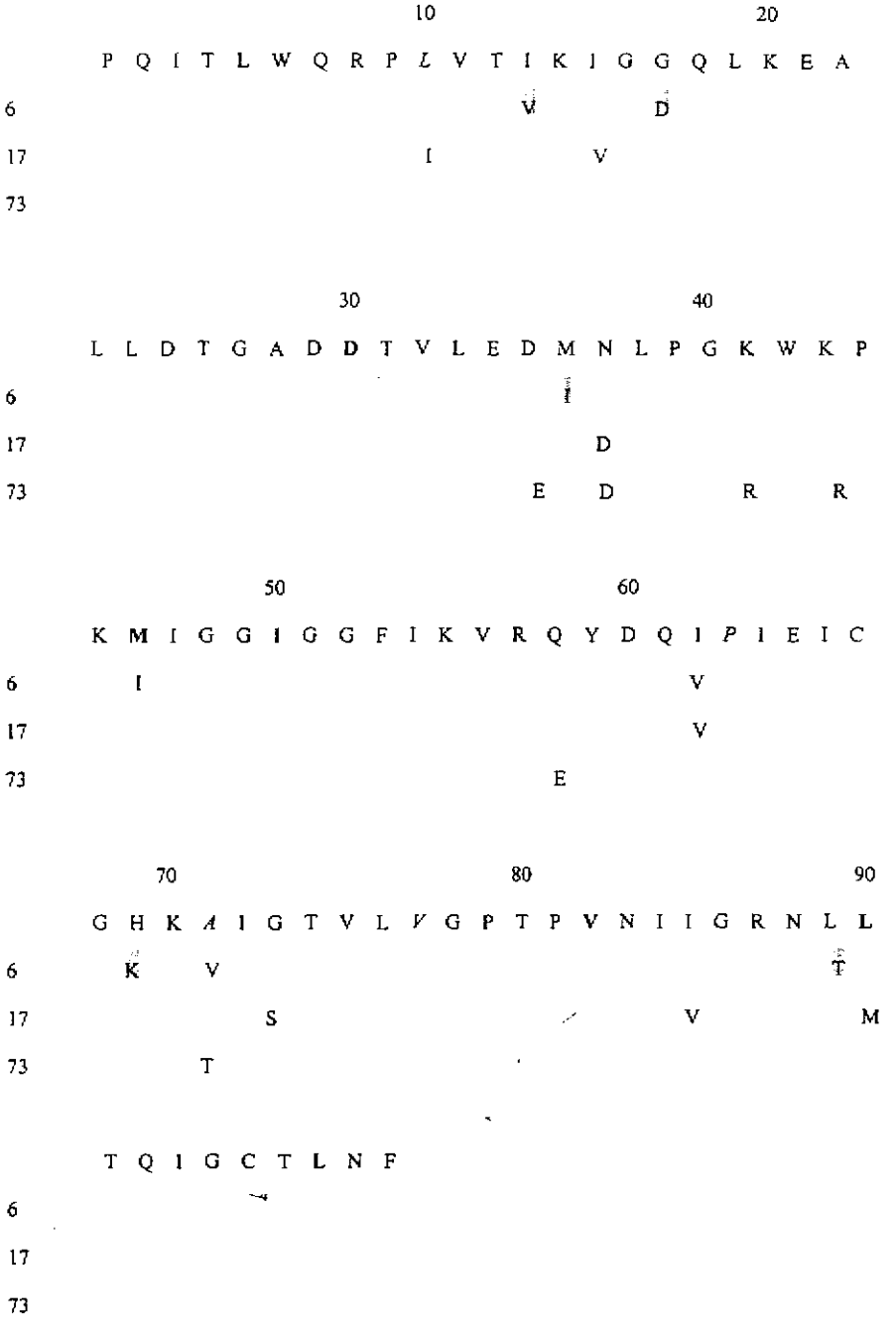


Table 2
Reverse transcriptase mutations

	10										20									
	P	I	S	P	I	E	T	V	P	V	K	L	K	P	G	M	D	G	P	K	V	K
200						D					T											
6						N		I			T									R		
58						D					T											
76(5-11)						D					T											
76(9-29)						D					T											
17						D																
73						D																
71					V																	

	30												40									
	Q	W	P	L	T	E	E	K	I	K	A	L	V	E	I	C	T	E	*M*	E	K	E
200													T				R				E	
6													T				K				E	
58													T				K		L		E	
76(5-11)													T				G				E	
76(9-29)													T				G		L		E	
17																			L			
73													M				A	F	L		E	
71																						

	50										60									
	G	K	I	S	K	I	G	P	E	N	P	Y	N	T	P	V	F	A	I	K	K	K	*D*
200																							
6																I							N
58																							
76(5-11)																							
76(9-29)																							
17																							
73																							N
71																							

	70											80								
	S	T	K	W	R	K	L	V	D	F	R	E	L	N	K	R	T	Q	D	F	W	E
200																					
6			R																		
58	G																				
76(5-11)																					
76(9-29)	S																				
17																					
73																				C	
71		S	R												K						

	90								100									110			
	V	Q	L	G	I	P	H	P	A	G	L	K	K	K	K	S	V	T	V	L	D	V	G
200																						
6																						
58																						
76(5-11)																						
76(9-29)																						
17																						
73									S													
71														N								

	120											130									
	D	A	Y	F	S	V	P	L	D	E	D	F	R	K	Y	T	A	F	T	I	P	S
200									H		S											
6											S											
58											S					R						
76(5-11)											S											
76(9-29)											S											
17										K	E											
73							I			K												
71										K												

	140												150								
	I	N	N	E	T	P	G	I	R	Y	Q	Y	N	V	L	P	Q	G	W	K	G	S	P
200																							
6	V														A								
58	V																						
76(5-11)	T																						
76(9-29)	T																						
17																							
73																							
71		V													E								

	160												170								180	
	A	I	F	Q	S	S	M	T	K	I	L	E	P	F	R	K	Q	N	P	D	I	V	I
200																I	K			E		I	
6																I	K			E			
58					C																		
76(5-11)																	K			E	M		
76(9-29)																I	K			E			
17									R														
73																							
71						T			R														

										190										200			
	Y	Q	Y	M	D	D	L	Y	V	G	S	D	L	E	I	G	Q	H	R	T	K	I	
200				V																I		A	
6				V																			
58																				K			
76(5-11)																							
76(9-29)				V																A			
17																							
73				V													E			I			
71				V																			

	210															220
	E	E	L	R	Q	H	L	L	R	W	G	F	T	T	P	D	K	K
200					A													
6					A													
58					A			W					Y					
76(5-11)					A				S									
76(9-29)					A				S				F					
17													F					
73					E	Y		W	K				Y				N	
71					E				K									

Discussion

The successful control of HIV-1 infection is not achievable unless viral replication can be almost totally suppressed. At present, available therapeutic regimens are very potent and the focus of HAART has turned to the long-term toxicity, long-term efficacy and dosing frequency of antiretroviral drugs. The treatment outcome in our patients with advanced stage of HIV-1 infection showed that successful control of HIV-1 infection could last longer than 2 years in some patients who had very low CD4 cell count. However, only about 60% of patients who had been followed for more than 20 months still had undetectable viral load. The major cause of treatment failure was non-adherence. HAART is less well-tolerated in advanced stage of HIV-1 infection and treatment of concurrent opportunistic infections causes more adverse effect as well as impedes the efficacy of antiretroviral drugs through drug-drug interaction. All the above factors contributed to the low rate of successful treatment.

In our proposal, we tried to answer whether patients infected with different HIV-1 subtypes responded differently to antiretroviral treatment? The analysis of reverse transcriptase gene did show some subtype-specific amino acid sequence (Table 2), it is reasonable to hypothesize that these differences may influence the emergence of resistant mutants. However, our preliminary result did not show significant difference in rate of undetectable viral load between long-treated patients who were infected with subtype E or non-E. Since these two groups do not match in CD4 cell counts, AIDS-related diseases and regimen of HAART, it is too early to draw final conclusion.

The study of mutations in RT and protease gene showed existence of multiple mutations that had been associated with drug resistance. These patients usually had

rebound in viral load for a long period. In contrast, only one major mutation that conferred drug resistance was detected in a patient treated for 10 months (patient 200). Our results emphasized the importance of early detection of mutations associated with drug resistance. We also found that there were other accessory mutations with unknown significance. For example, patient 73 had no mutation associated with drug resistance in protease gene but had several mutations of unknown significance. The possibility of finding some new mutations associated with drug resistance can be achieved by gathering more genetic mutations in our patients with treatment failure. Most of the present studies of drug resistance are done in patients infected with subtype B, the drug resistance genotype is less well characterized. It is interesting that subtype-specific mutations were noted at position 122, 173, 174 and 176 of RT in our patients with treatment failure. More data are needed to provide evidence for the existence of subtype-specific genotype of drug resistance. Patient 76 had different pattern of mutations at two different time point. This could be the result of evolution of the virus to have better fitness or poor compliance[18]. At present only the 220 amino acid in amino terminus of reverse transcriptase was studied, we plan to analyze the whole gene in the future. Moreover, presence of PI-resistant variants in untreated subjects[19,20] will be checked in future study.

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