國科會研究計畫成果報告

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愛滋病毒抗藥性突變基因之判讀

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Abstract

Suboptimal highly active anti-retroviral treatment is mainly due to poor adherence and will lead to emergence of drug resistance mutations. For the choice of salvage therapy and the study of rising spread of HIV resistance, drug resistance tests are needed. We use genotyping to test the drug resistance mutations in the past two years. We first determined the consensus sequence of reverse transcriptase (RT) and protease gene of Taiwan isolates. In patients with virological failure, both primary mutations (mutations known to confer drug resistance) and secondary mutations (mutations needed to improve the fitness of mutant viruses) can be detected. However, background polymorphisms do exist and it is necessary to compare the pre-treatment strains and drug resistant mutants obtained from the same patients in order to differentiate between secondary mutation and polymorphism. Both genetic changes may theoretically contribute to the drug resistance, but not proved. Moreover, the significance of mutations in RT codons from 300-440 also are not clear. At present, the number of mutations seems to correlate best with the treatment outcome, but more data are needed to establish the cut-off point.

Keywords **antiretroviral therapy** **drug resistance mutation** **genotype**

Introduction:

The highly active antiretroviral therapy (HAART) can suppress the replication of human immunodeficiency virus (HIV-1) to a very low level, however, does not eliminate them [1]. Not every patient had successful outcome, the failure rate of HAART is around 40% in Taiwan (Chen M.Y., personal observation). The current problems associated with HAART are long-term side effects and the development of resistance [2,3]. The emergence of drug resistance mutations is caused by suboptimal suppression of viral replication owing to poor adherence, inadequate drug regimens and variability in pharmacokinetics and pharmacodynamics in infected individuals [4]. The occurrence of primary resistance to antiretrovirals is rising, due to the widespread use of these drugs [5]. More recent data indicate that the likelihood of being infected by persons who have taken antiretroviral drugs is increasing over time [6]. Therefore, the drug resistance testing is recommended by the International AIDS Society-USA Panel among symptomatic seroconverters and recently infected subjects [7].

The three basic approaches to access the antiviral drug resistance are: phenotypic resistance testing, genotyping of the viral sequences and *Virtual*/Phenotype which combines knowledge of genotypic pattern and the corresponding phenotypic profile to arrive at a prediction of drug resistance. There are commercial kits available for these approaches. However, resistance testing and the interpretation system required to generate drug resistance profiles are still evolving [8]. Furthermore, resistance research has been virtually universally undertaken on viruses belonging to subgroup B, but study has demonstrated a high prevalence of so-called "secondary" resistance mutations in drug-naïve patients with non-subtype B viruses [9]. Our previous study showed that about one fifth of HIV-1 infected patients in Taiwan harbored subtype E [10]. Clearly, we need to establish our own database. In the past two years, we have

studied RT and protease genes and established the consensus sequence of these genes in Taiwan. Indeed, Taiwan specific and subtype E specific polymorphism was found during the process. For examples, the amino acid 63 in protease gene is pro in most viral strains isolated in Taiwan and amino acid 36 is Ile instead of Met in subtype E.It has been shown that mutant virus is less fit tan wild type [11]. Therefore, wild type virus predominated again when HAART was discontinued because of treatment failure. Secondary mutations must accumulate during the evolution of drug resistant mutants in order to achieve better fitness after the development of key mutations. But, HIV is highly mutable and the differentiation between secondary mutations and background polymorphisms will be difficult. Furthermore, it is possible that background polymorphisms may also influence the resistance profile. Therefore, we suggest that the analysis of drug resistance genotyping data should use the pre-treatment genetic sequences as reference.

MATERIALS AND METHODS

PATIENTS

Patients treated with combination therapy at National Taiwan University Hospital who experienced virological failure were enrolled. Plasma obtained before treatment, if available, was stored at -70°C. The 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 every four months. All the follow-up plasma samples were stored at -70°C. Patients who had initial response to combination therapy (plasma viral load reached undetectable level), but later had

detectable viral load over 3000 copies and had persistent detectable viremia after repeated test was studied.

SEQUENTIAL MUTATIONS IN HIV PROTEASE and RT GENES

The plasma obtained before the combination therapy and during rebound of viral load were used to study the drug resistance mutations. 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 primers used to amplify the HIV-1 reverse transcriptase and protease gene by nested PCR are listed below in the table. The reaction condition is similar to that described above. The PCR products are then purified and directly sequenced by dye-labeled terminator on autosequencer.

Primers for reverse transcriptase reaction

Protease 5'- TTCTCTTCTGTGTCAATGGCCATTGTTT

Reverse transcriptase 5'TTTTCCCACTAACTTCTgTATGTCATTGACA

Polymerase chain reaction

Protease Outer sense:5'-GCTAATTTTTTAGGGAA

Antisense:5'- TTCTCTTCTGTGTCAATGGCCATTGTTT

Inner sense:5'-CAGACCAGAGCCAACAGCCCCACCA

Antisense-5' gggCCATCCATTCCTggCTTTAATTT

Reverse transcriptase

SET I Outer sense: 5' CAGACCAGAGCCAACAGCCCCACCA

Antisense:5' TTTTCCCACTAACTTCTgTATGTCATTGACA

Inner sense:5'GTATTAGTAGGACCTACACCT

Antisense:5'CATTTATCAGGATGGAGTTCA

SETII Outer sense:5'CCATACCTAGTATAAACAATG

Antisense:5'CAGTCTTCTGATTTGTTGT

Inner sense:5'GGCAGCATAGAACAAAAATAG

Antisense:5'GTCTTCCTCTGTCAGTAACAT

Analysis of drug resistance data

The consensus sequences of subtype B and E *pol* genes were derived from the pretreatment HIV strains circulating in Taiwan. Background polymorphism and pre-existing drug resistance mutations can be established at this point. The mutations develop after treatment failure will be categorized according to published reference [16].

Results

Difference between amino acid sequence of subtype E and B

Protease gene	amino acid position	
	13	69
B	I	H
E	V	K

Reverse transcriptase	amino acid position												
	11	35	43	123	174	177	245	312	326	329	357	359	366
B	K	V	K	D	Q	D	V	E	I	I	M	G	K
E	T	T	E	S	K	E	E	T	V	V	K	S	R
	371	390	395	403	432								
B	A	K	K	T	E								
E	V	R	R	M	D								

The hot spot of mutations

Protease gene L10I/V I15V M36I M46I/L I72T/V/I V75I/M V82I/A L89M/T L90M I93L/V

Reverse transcriptase gene E6D V35I/L/T M41L D67N K103N/R/I V118I I135V/T K166R K173I/T V179I/D M184V I202V Q207E H208Y L210W

R211S/K F214L T215F/Y V245M/K P272A R277K T286A E291D E297A/K E312V/A/Q I326V Q334L G335D R356K R358K A371V T400A K431Q/T

Differentiate background polymorphisms from secondary mutations

As shown in table I, the non-primary mutations noted when treatment failure occurred might be the background polymorphisms instead of secondary mutations.

Table I. Background Polymorphisms and primary, secondary mutations.

Date

8 May 97' K10I M36I K41R K45R Q61H K70T

16 Apr 01' K10I ~~K20R~~ M36I K41R K45R ~~I54V~~ ~~Q61N~~ K70T ~~A71I~~ ~~I72T~~ ~~G73T~~ V82A L90M

the bold letter represents primary mutation. The secondary mutations are underlined.

Discussion:

The longer duration of suboptimal HAART, the drug resistant mutants are assumed to accumulate more mutation. The sequential genotyping of protease gene in one of our patient proved the theory (table II). Resistance to more than one class of antiretrovirals (multidrug resistance, MDR) may therefore emerge [12].

Table II. The accumulation of mutations over time

1 June 98'	I13V	G17D		M36I	M46I		H69K		L89T
22 Apr 99'	I13V	G17D	K20K/I	M36I	M46I	I62V/I	H69K A71V		L89T
11 Apr 00'	I13V	G17D	~~K20I~~	M36I	M46I		H69K ~~A71V~~	~~I84V~~	L89T

Interestingly, when Indinavir 800 mg plus ritonavir 200mg were used as salvage therapy, patient in table I failed while patient in Table II had successful control of viral replication. The patient who regained control of viral replication had one primary mutation and 8 other mutations versus two primary and 11 other mutations in the other patient who failed

salvage therapy. Patients who have higher number of mutations are expected to have higher level of resistance, however, the impact factor of primary or secondary mutations or background polymorphism is not known. Theoretically, the level of drug resistance can be calculated as the following equation:

Drug resistance index = A x primary mutations + B x secondary mutations + C x background polymorphisms

At present, no standard rule have been set for the interpretation of genotyping. We try to use the number of mutations to predict the outcome of salvage therapy retrospectively. In doing so, the background polymorphism should be determined. We plan to enroll 100 patients who have good adherence to salvage therapy and analyze their genetic sequences of reverse transcriptase and protease genes. By correlation with the outcome of treatment, we wish to establish the rule of predicted drug resistance index. Unfortunately, the project we proposed to NSC was not accepted. Now, we are seeking financial support from other source.

The mutation at RT codon 333 has been associated with resistance to zidovudine and lamivudine [13]. But we did not find mutations at this position, instead, other mutations were noted in codons 300-440. In our study, most of the mutations in C terminal half of RT proved to be background polymorphism, but interestingly some patients infected with subtype B converted to subtype-E specific polymorphisms. Further works are required to support our findings.

In conclusion, for the interpretation of genotyping test, the number of mutations accumulated after the initiation of treatment is important. The data of resistance mutations in Taiwan will be useful for the choice of salvage regimen. The pre-treatment prevalence of HIV-1 drug resistance mutation in Taiwan is important because the transmission of drug

resistant strains is a major public health concern [14]. HIV-1 subtype can also be ascertained from the sequence of the *pol* gene [15].

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