

PREVALENCE OF HIV-1 POLYMERASE GENE MUTATIONS IN PRE-TREATED PATIENTS IN THAILAND

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Abstract. To determine the prevalence of drug resistance-conferring mutations in human immunodeficiency virus type 1 (HIV-1), 83 HIV-1 infected Thai patients who had been treated with any antiretroviral drug were studied. HIV-1 RNA was reverse transcribed and amplified by RT-PCR. The direct sequencing of HIV-1 reverse transcriptase (RT) and protease was then performed. Changes in nucleotide and amino acid sequences were determined by comparison with a pNL4-3 reference sequence. Data on mutations associated with resistance to antiretroviral drugs were obtained from literature. The mutations associated with lamivudine resistance (M184V/I) were found most often (in 45.7% of individuals). Zidovudine-resistant mutants: T215Y/F (36%), M41L (28%) and K70R (25.3%) were common; but mutations linked to didanosine (L74V) and multinucleoside-resistant genotypes (Q151M) were rarely recognized (2.4% and 3.6%, respectively). The stavudine-resistant mutant (V75T) and T69 insertions were not found. All subjects who had a significant exposure to antiretroviral drugs and current virological failure in the past carried drug-resistant genotypes. Genotypic resistance to zidovudine, lamivudine, zalcitabine, indinavir and ritonavir appeared in more than one third of the samples, which suggested that the prevalence of the HIV-1 resistance-conferring genotype resisting reverse transcriptase inhibitors and/or protease inhibitors was high in treatment experienced patients.

INTRODUCTION

The availability of new and more potent antiretroviral drugs has dramatically improved the life expectancy of HIV-infected patients. However, benefits seem to decline over time, as a result of the unfavorable impact of side-effects or the emergence of drug-resistant viruses (Hirsch *et al*, 1998; Carpenter *et al*, 2000). Salvage regimens, which include as many new compounds as possible, often provide a better outcome in individuals experiencing treatment failure (Murphy, 1999). However, in those who have already been exposed to almost all the available drugs, the chances of a virological

response are quite low (Murphy, 1999). Recent reports have suggested that information on the drug resistance profile in this situation can help in designing the best salvage intervention (Durant *et al*, 1999; Baxter *et al*, 1999; Cohen *et al*, 2000).

Laboratory data that derived from analyses of a large number of specimens from heavily pre-treated patients might provide the opportunity to recognize different genotypic behaviour for individual drugs (Yahi *et al*, 1999). It may also be possible to establish whether some compounds are more robust in the face of resistance than others, thereby, making them prime candidates for rescue interventions.

This study aimed to assess the prevalence and contribution of antiretroviral drug resistance mutations in the clinical failure of therapies such as the population consensus of the HIV-1 protease and reverse transcriptase gene,

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which was determined for HIV-1 isolates from HIV infected Thai patients experiencing treatment failure.

MATERIALS AND METHODS

The study population comprised 83 Thai subjects who met the following three criteria: (i) past exposure to one or more drugs belonging to all three different families (nucleosides, non-nucleosides and protease inhibitors); (ii) evidence of an acceptable compliance with current and past therapies; and (iii) a current plasma viral load of over 500 HIV RNA copies/ml. Samples from individuals on drug holidays for longer than 2 weeks before the time of the study were excluded, due to a possible reversion of circulating mutant genotypes (Devereux *et al*, 1999; Verhofstede *et al*, 1999). Quantification of plasma HIV RNA was performed using the Amplicor HIV-1 Monitor test (Roche Diagnostic Systems, Branchburg, New Jersey), by following the manufacturer's recommendation, and by the analysis of CD4 lymphocyte subsets, which was carried out using a dual-colored fluorescent-activated cell sorter analysis (FACScan, Becton Dickinson, Mount view, California).

For the genotypic analysis of HIV-1 protease and reverse transcriptase genes, the following method was adapted: viral RNA was isolated from plasma with a QIAamp Viral Extraction Kit (Qiagen, Chatsworth, California) according to the manufacturer's instructions; and a 2.2 kb fragment encompassing the protease and RT gene was generated by nested RT-PCR, as described by Hertogs *et al* (1998). PCR products were typically diluted to 1:4 with water and 10 μ l of each were placed into a sequencing reaction with dRhodamine dye-labeled dideoxyterminators, a sequencing module, and an HIV-1 Genotyping System Kit (PE Biosystems, Foster City, California). The sequencing reactions were analyzed on an ABI 310 instrument. Data were assembled, manually proofread and edited. Changes in nucleotide and amino acid sequence were determined by a comparison with the pNL4-3

reference sequence. The mutation score was calculated by using mutation-scoring tables, which contained penalties for drugs, based on the amino acid and position of the mutation. Mutation scores derived from published literature that linked mutations with antiretroviral drugs, including correlations between genotype and treatment history, genotype and phenotype, and genotype and clinical outcome. By adding together the scores of each mutation associated with resistance to a particular drug, the drug resistance interpretation was derived, as described by Schinazi *et al* (1999). Nucleotide sequence divergence was calculated for all sequences to rule out laboratory contamination or multiple sampling of the same patient at different testing sites. Phylogenetic analysis was performed using the distance matrix program DNADIST, with the stochastic model of Kimura-2 and the NEIGHBOR program, both of which were supplied in the PHYLIP package (distributed by Joseph Felsenstein, University of Washington, Seattle). Sequences were submitted to GenBank and assigned accession numbers: AF172706, AF187037-AF187039, AF191189-AF191210, AF193889-AF193896 and AF240377-AF240399.

RESULTS

Baseline characteristics of the patients.

All of the 83 plasma RNA samples could be amplified and analyzed by direct DNA sequencing. The median plasma HIV-1 RNA level was 308,075 copies/ml (range 10,978 - 750,000) and the median CD4 cell count was 170 cells/mm³ (range 4 - 303).

HIV-1 reverse transcriptase gene mutations.

Fifty-one patients (61.4%) had RT inhibitor-resistant strains. The median number of RT-resistance mutations per isolate was three (ranges zero to six). Virus from 48 patients (57.8%) carried a primary mutation that decreased drug susceptibility *in vitro* to nucleoside RT inhibitors. Eight isolates (9.6%) had one or more non-nucleoside RT inhibitor resistance mutation (K103N, Y181C, Y188L, and

Table 1
Prevalence of key drug-resistant mutations in 83 pre-treated HIV-infected patients with virological failure.

Nucleosides		Non-nucleosides		Protease inhibitors	
Mutation	Frequency %	Mutation	Frequency %	Mutation	Frequency %
M41L	27.7	K103N	1.2	D30N	0
T69D	4.8	Y181C	3.6	M46I/L	10.8
69 insertion	0	Y188L	1.2	G48V	9.6
K70R	25.3	G190A	7.2	I50V	0
L74I/V	10.8			V82A	8.4
V75T	0			I84V	7.2
Q151M	3.6			L90M	19.2
M184V/I	45.7				
T215Y/F	36.0				

G190A). Table 1 summarizes the frequency of drug-resistant mutations found at key positions for each of the three families of antiretroviral drugs in this study. The mutations associated with lamivudine resistance (M184V/I) were found most often (45.7%). Zidovudine-resistant mutants: T215Y/F (36%), M41L (28%), and K70R (25.3%) were common, whereas, mutations linked to didanosine (L74V) and multinucleoside-resistant genotypes (Q151M) were rarely recognized (2.4 and 3.6% respectively). The stavudine-resistant mutant (V75T) and T69 insertions were not found.

HIV-1 protease genes mutations

Twenty-nine patients (34.9%) had protease inhibitor-resistant strains. The median number of protease resistance mutations per isolate was one (range zero to three). Virus isolates from 1 patient (1.2%) had three primary mutations and 9 patients (10.8%) had viruses that carried two primary mutations in the protease gene. Nineteen (22.8%) and all 83 patients had a protease gene containing a viral isolate with only a single primary mutation and viruses with a secondary resistance mutation, respectively. The protease resistance mutations most commonly observed were L90M (19.2%), M46I/L (10.8%), G48V (9.6%) and V82A (8.4%).

The estimated minimum frequency of genotypic resistance was as follows: for nucleo-

side analogues, the resistance rates were 55.4% (zidovudine), 53.3% (zalcitabine), 45.7% (lamivudine), 2.4% (didanosine), and 0% (stavudine). Due to non-nucleosides showing some cases of extensive cross-resistance, resistance was shown in 9.6% of them (for either nevirapine or efavirenz). The frequency of genotypic resistance to protease inhibitors was 25.5% (saquinavir), 20.4% (indinavir), 19.2% (nelfinavir) and 13.2% (ritonavir).

Phylogenetic analysis

To ensure the integrity of the sequence data obtained, a phylogenetic analysis of the 83 sequences of both protease and reverse transcriptase was performed to exclude the laboratory strain and inter-sample contamination. The phylogenetic tree clearly demonstrated the independent segregation of sequences from the laboratory strain as well as from any other one. Most specimens were found to be HIV-1 subtype-E. Seventy-three (88%) sequences were clustered with CM240 subtype-E reference sequence, while 10 (12%) were clustered with pNL4-3 subtype-B reference sequence (data not shown).

DISCUSSION

It is important to monitor the prevalence of drug resistance for epidemiological reasons

and to assess the need for routine drug resistance testing to guide the clinical management of subjects with a history of extensive pre-treatment. This study clearly showed that reduced drug susceptibility was common in patients who experienced a virologic failure after starting potent combination therapy. Reduced susceptibility to a protease inhibitor was shown most often, especially in saquinavir, indinavir, and nelfinavir, and to nucleoside reverse transcriptase inhibitors: zidovudine, lamivudine and zalcitabine. At the same time, reduced susceptibility to stavudine and didanosine was extremely rare, which was in agreement with a previous report (Katzenstein *et al*, 1998). The mutation (Q151M) confers multinucleoside resistance, but has a low prevalence among multinucleoside experienced patients that raises concerns about the occurrence of multidrug resistance caused by a single amino acid change.

In this study, there were 45 patients treated with stavudine, but only 3 isolates with a mutation associated with stavudine resistance (Q151M). The genotypic basis for stavudine resistance *in vitro* is associated with a specific mutation at codon 75 (V75T) of the RT gene. These mutations are rare *in vivo* and their resistance to stavudine has often been assumed as uncommon. However, recent data indicated that the zidovudine-related mutation (codon 41, 67, 70, 215 and 219 of RT gene) confers a significant cross-resistance to stavudine. Furthermore, after prolonged exposure, stavudine may select these 'zidovudine - like' mutations (Pellegrin *et al*, 1999; Izopet *et al*, 1999; Coakley *et al*, 2000).

Virologic failure is commonly associated with the emergence of drug-specific resistance mutations. For many antiretroviral agents, the development of high-level resistance requires the accumulation of multiple mutations. Presumably, the specific pattern of mutations that emerges is determined by the effect of an individual mutation on both drug susceptibility and viral replicative capacity. A mutation that confers high-level resistance, but results in an unfit virus, will not emerge, or it will emerge

slowly. This probably explains why many of the protease mutations occur only after prolonged virologic failure such as D30N, which reduces viral fitness in the absence of compensatory mutations. By contrast, mutations that confer high level resistance without significant effects on viral fitness should emerge rapidly, as seen in resistance to lamivudine and the non-nucleoside analogues.

The lack of detectable resistance among some virologic failures may reflect a variety of factors, including insufficient drug exposure for the selection of resistance on account of non-adherence, and low plasma drug concentration due to enhanced drug metabolism, or other reasons.

In this study, all the subjects with a significant exposure to antiretroviral drugs and current virological failure in the past carried drug-resistant genotypes. Resistance to zidovudine, lamivudine, zalcitabine, indinavir or ritonavir appeared in more than one third of the samples, which is a finding that agrees with the results of a similar study conducted in France (Yahi *et al*, 1999).

Only a few years have passed since the introduction of protease and reverse transcriptase inhibitors to HIV-1 infected patients in Thailand, yet the prevalence of the HIV-1 resistance-conferring genotype to reverse transcriptase inhibitors and/or protease inhibitors is high in drug experienced patients. The transmission of resistant variants to uninfected individuals will cause serious clinical and public health concern. The widespread use of antiretroviral drugs will result in the increased transmission of drug-resistant viruses, resulting in an increased prevalence of resistant variants in newly infected patients.

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