IMPACT OF HIV-1 VIRAL LOAD ON GENOTYPIC CHARACTERISTICS AMONG PATIENTS FAILING NON-NUCLEOSIDE REVERSE TRANCRIPTASE INHIBITOR-BASED FIRST-LINE REGIMENS IN NORTHERN THAILAND

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Abstract. Widespread use of antiretroviral drugs has significantly increased drug resistance. In the resource limited countries, delayed detection of drug resistance may lead to accumulation of drug resistance mutations. We investigated the genotypic drug resistance mutation patterns in HIV-infected patients with various levels of plasma HIV RNA levels. Fifty-nine HIV-infected patients with antiviral therapy failure were recruited. Genotypic assays of HIV-1 protease and reverse transcriptase genes were analyzed. There was a significant difference in CD4 cell counts and percentage of CD4 ($p < 0.05$) between groups of patients with high and low viral load, who failed first-line non nucleoside reverse transcriptase inhibitor-based regimens. In addition, patients with HIV-1 viral load ≥ 4 $\log_{10}$ have a significantly higher likelihood of being infected with HIV-1 containing 3 to 5 resistance-associated mutations than those with HIV-1 viral load < 4 $\log_{10}$. Thus, delayed detection of increased HIV-1 viral load and antiretroviral drug-resistance may lead to accumulation of drug-resistant mutations and decreased CD4 cell count and percentage.

Keywords: HIV-1, viral load, antiretroviral drug resistance, Thailand

INTRODUCTION

Resistance to antiretroviral drugs is a major problem in the treatment of HIV-1 infection, which limits the efficacy of combination antiretroviral treatment (ART). The Thai government has provided free ART to all patients with HIV/AIDS. GPOvir, a combination of lamivudine, stavudine, and nevirapine, has been used as the first-line regimen in HIV-infected patients in Thailand since 2001 (Jenwitheesuk *et al*, 2003). However, widespread use of ART has significantly increased drug resistance and genotypic resistance testing is increasingly important and widely recommended as a standard care to evaluate drug resistant strains in clinical practice (Hammer *et al*, 2006, 2008; Hirsch *et al*, 2008).

HIV-1 antiretroviral resistance is a

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major cause of treatment failure (Kuritzkes, 2004; Gallant, 2005). Extensive mutation rate, prolonged use of antiretroviral agents and incomplete viral suppression lead to the development of drug resistant viruses and the management of treatment failure represents a serious challenge to the management of HIV-1 infected patients. In resource-limited country such as Thailand, there is usually a delay before the patient is prescribed a genotypic resistance test due to the cost of the test. This delay results in the patient having an elevated plasma HIV RNA level (viral load) at the time of the genotypic resistance testing (Sungkanuparph *et al*, 2007). In this study, we evaluated the correlation between different degrees of HIV RNA levels at the time of drug resistance testing and patterns of drug resistance mutations in HIV-infected patients, who had failed non-nucleoside reverse transcriptase inhibitor (NNRTI)-based first-line ART regimens.

MATERIALS AND METHODS

Clinical specimen and study design

This retrospective study was conducted among 59 HIV-infected patients ≥18 years of age. Inclusion criteria were as follows: 1) receiving NNRTI-based first-line ART for at least six months; 2) viral load undetectable at least once before the current genotypic resistance testing; and 3) viral load ≥ 1,000 copies/ml at the time of genotypic resistance testing. Demographic data of the patients were obtained from the medical records. Plasma specimens from these 59 patients were submitted to the HIV drug-resistance testing laboratory at the Faculty of Medicine, Chiang Mai University, Thailand between July 2007 and December 2009 for routine assessment of drug susceptibility.

The study was approved by the Ethics Committee of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

Extraction, amplification and sequencing of viral RNA

An in-house genotype assay of HIV-1 protease (PR) and reverse transcriptase (RT) genes was performed on the plasma specimens (Sugiura *et al*, 1999; Myint *et al*, 2002; Fujisaki *et al*, 2007). Viral RNA was extracted from 150 µl of individual plasma sample using a NucleoSpin® Viral Isolation kit (Macherey-Nagel, Düren, Germany). Single stranded HIV-1 RNA was reverse transcribed and amplified by a one step RT-PCR technique using Superscript™ III platinum *Taq* (Invitrogen, Carlsbad, CA). The outer primer pair used for amplification of the PR gene was DRPRO5 (5′-AGACAGGYTAATTTTTTAGGGA-3′), and DRPRO2L (5′-TATGGATTTTCAGGCCCAATTTTTGA-3′), and for RTgene, DRRT1L (5′-ATGATAGGGGGAATTGGAGGTTT-3′) and DRRT4L (5′-TACTTCTGTTAGTGCTTTGGTTCC-3′). Thermal cycling condition was as follows: 55°C for 20 minutes; 95°C for 2 minutes; 40 cycles of 94°C for 15 seconds, 52°C for 15 seconds, 72°C for 45 seconds; and 72°C for 5 minutes. DNA was then amplified by nested PCR using KOD DNA polymerase kit (Toyobo, Osaka, Japan). The inner primers, DRPRO1M (5′-AGAGCCAACAGCCCCACCAG-3′) and DRPRO6 (5′-ACTTTTGGGCCATCCATTCC-3′), were used for the PR amplicon and primers, DRRT6L (5′-TAATCCCTGCATAAATCTGACTTGC-3′) and DRRT7L (5′-GACCTACACCTGTCAACATAATTGG-3′), for RT amplicon. The thermal cycling conditions were as follows: 94°C for 15 seconds; 30 cycles of 94°C for 5 seconds; 60°C for 3 seconds

and 74°C for 30 seconds; and 72°C for 7 minutes.

The nested PCR amplicons were purified with NucleoSpin® extract II (Macherey-Nagel) and sequenced in both directions using BigDye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) in the ABI Prism 3100 Genetic Analyzer (Applied Biosystems) and 4 specific primers: two primers, DRPRO1M and DRPRO6, to analyze nucleotide sequence of PR gene; two primers, DRRT6L and DRRT7L, to analyze RT gene. The nucleotide sequences were edited using SeqScape software V2.6 (Applied Biosystems) and compared to the HIV-1 reference sequence strain HXB2. The edited sequences were interpreted for mutations related to drug resistance using Stanford Genotypic Resistance Interpretation Algorithm (http://hivdb.stanford.edu/pages/algs/HIVdb.html) and International AIDS Society-USA mutation panel (Johnson *et al*, 2009).

Phylogenetic analysis

The 59 sequences of HIV-1 *pol*-PR and *pol*-RT region (1,050 bp) were aligned using CLUSTAL X, version 2.0.10 software and compared to the HIV-1 reference sequences: subsubtype A1 (03AU-PS1044), subtype B (90TH-BK132), subtype C (92BR-BR025), subtype D (83CD-ELI), subsubtype F1 (93BE-VI850), subtype H (93BE-VI991), subtype J (97CD-J_97DC_KTB147), subtype K (97CD-EQTB11C), CRF01_AE (90TH-CM240). Artificial nucleotide bases and gaps were removed using BioEdit, version 7.0.9.0 software. Similarity and bootstrap value were identified with the number of bootstraps at 100 replicates. Genetic distance and bootstrap value were assessed and the phylogenetic tree was established with Neighbor-Joining method.

Statistical analysis

Statistical analysis was performed using SPSS for Windows, version 17.0. Frequencies and median of variables were expressed as percentages. Comparison of mean between two sample groups with different HIV-1 viral loads was performed using unpaired *t*-test. A Mann-Whitney *U* test was used to compare median between two sample groups with different HIV-1 viral load. A p-value of < 0.05 is considered statistically significant.

RESULTS

The 59 samples were divided into two groups: one group (27 samples; 46%) collected from patients with HIV-1 viral load $< 4 \log_{10}$ and the other group (32 samples; 54%) from patients with HIV-1 viral load $\geq 4 \log_{10}$. The two groups are not significantly different in demographic and clinical characteristics including age, sex, mode of transmission, and nucleoside reverse transcriptase inhibitor (NRTI) treatment (Table 1). However, the group with higher viral load has significantly lower CD4 cell counts and percentage than the other group.

Major mutations associated with resistance to NRTIs and NNRTIs were found in 54 patients (91%) and 55 patients (93%), respectively (Table 2). The prevalence of resistance mutations associated with any NRTIs or NNRTIs was not different between the two groups ($p > 0.05$). The prevalence of any of the point mutations associated with resistance to NRTIs or NNRTIs was also not significantly different between the two groups ($p > 0.05$). Twenty patients (62%) with HIV-1 viral load $\geq 4 \log_{10}$ were observed to have 3 to 5 resistance-associated mutations compared to 6 patients (22%) with HIV-1 viral load $< 4 \log_{10}$ ($p = 0.001$). The average

Table 1
Demographic and clinical characteristics of the study population.

Characteristics	Number of patient (n = 59) [a]	Patients with VL[b]< 4log (n = 27)	Patient with VL≥ 4log (n = 32)	p-value
Age, mean years ± SD[c]	39±1.095	40±1.751	38±1.361	0.203
Sex				0.290
Male	29 (49%)	15 (56%)	14 (44%)	
Female	29 (49%)	12 (44%)	17 (53%)	
Transgender	1 (2%)	-	1 (3%)	
Transmission group				0.098
Men who have sex with men	10 (17%)	3 (11%)	7 (22%)	
Heterosexual men	29 (49%)	12 (44%)	17 (53%)	
Heterosexual women	20 (34%)	12 (44%)	8 (25%)	
NRTI[d] backbone				0.118
Stavudine and lamivudine	47	23	24	
Zidovudine and lamivudine	8	4	4	
Didanosine and lamivudine	1	1	-	
Third drug				
Nevirapine	45	25	20	
Efavirenz	10	1	9	
PI[e]	1	1	-	
Time to viral load measurement, median months [IQR][f]	48 (18, 144)	56 (18, 144)	48 (20, 104)	0.217
Median plasma HIV-1 RNA, copies/ml [IQR]	11,974 (1,002, 398,000)	6,045 (1,002, 9,818)	65,840 (11,000, 398,000)	
Mean VL $\log_{10}$ copies/ml [IQR]	4.08 (3.0, 5.6)	3.78 (3.0, 3.99)	4.82 (4.04, 5.60)	
Median CD4+ cell count, cells/mm^3 [IQR]	145.5 (8, 458)	211.5 (19, 458)	110 (8, 348)	0.009
Median percentage of CD4+ cell count, cell/ mm^3 [IQR]	9.0 (1, 26)	10.5 (2, 26)	8.0 (1, 17)	0.012

[a]Number (%) of patients, unless otherwise indicated; [b]VL, viral load; [c]SD, standard deviation; [d]NRTI, nucleoside reverse transcriptase inhibitor; [e]PI, protease inhibitor; [f]IQR, interquartile range.

number of thymidine-associated mutations (TAMs) in the low viral load group was 1.0 [IQR 0, 4], whereas that in the high viral load group was 1.59 [IQR 0, 5]. The average number of major mutation to NNRTIs in low viral load group was 1.56 [IQR 0, 3], and that in the high viral load group was 1.72 [IQR 0, 4]. The number of patients with any TAMs in the low viral load group was 13 (48%), and that in the high viral load group was 19 (59%). The number of patients with any major mutation points to NNRTIs in the low viral load group was 25 (93%), and that in the high viral load group was 30 patients (94%). The most prevalent mutation associated with NRTIs resistance was M184V/I, which was found in 47 patients

Table 2
Development of nucleotide-associated mutations.

Mutation	Number of major RT mutation (%)[a]			*p*-value
	Number of patients (n = 59)	Patients with VL[b] < 4log (n = 27)	Patient with VL ≥ 4log (n = 32)	
NRTI[c] mutation				
M41L	10 (17)	2 (7)	8 (25)	0.075
A62V	4 (7)	2 (7)	2 (6)	0.863
K65R	8 (13)	4 (15)	4 (12)	0.800
D67N	18 (30)	9 (33)	9 (28)	0.672
T69N	22 (37)	8 (30)	14 (44)	0.272
K70R/E	16 (27)	6 (22)	10 (31)	0.446
L74V	2 (3)	1 (4)	1 (3)	0.905
V75I	3 (5)	0	3 (9)	0.106
F77L	2 (3)	1 (4)	1 (3)	0.905
F116Y	1 (2)	1 (4)	0	0.280
Q151M	4 (7)	1 (4)	3 (9)	0.397
M184V/I	47 (80)	23 (85)	24 (75)	0.341
L210W	5 (8)	2 (7)	3 (7)	0.791
T215Y/F	12 (20)	3 (11)	9 (28)	0.109
K219Q/E	16 (27)	5 (18)	11 (34)	0.178
Any NRTI mutation	54 (91)	26 (96)	28 (90)	0.063
1 or 2 NTRI resistance associated mutation	31 (36)	12 (44)	9 (28)	0.199
3 to 5 NTRI resistance associated mutation	26 (44)	6 (22)	20 (62)	0.001
Any TAMs[d]	32 (54)	13 (48)	19 (59)	0.397
NNRTI[e] mutation				
V90I	1 (2)	0	1 (3)	0.363
A98G	4 (7)	3 (11)	1 (3)	0.231
L100I	1 (2)	0	1 (3)	0.363
K101E/H/P	10 (17)	4 (15)	6 (19)	0.694
K103N	18 (30)	10 (37)	8 (25)	0.326
V106M/I/A	2 (3)	1 (4)	1 (3)	0.905
V108I	13 (22)	5 (18)	8 (25)	0.558
V179D/F/T	2 (3)	0	2 (6)	0.193
Y181C/I	34 (58)	16 (59)	18 (56)	0.820
Y188L/C/H	2 (3)	0	2 (6)	0.193
G190A/S	14 (24)	6 (22)	8 (25)	0.807
P225H	1 (2)	0	1 (3)	0.363
Any NNRTI mutation	55 (93)	25 (93)	30 (94)	0.863

[a]Number (%) of patients, unless otherwise indicated; [b]VL, viral load; [c]NRTI, nucleoside reverse transcriptase inhibitor, [d]TAMs, thimidine associated mutations; [e]NRTI, non-nucleoside reverse transcriptase inhibitor

Table 3
Development of HIV drug resistance.

Antiretroviral drug	Number of patient (%)[a]			*p*-value
	Number patients (n = 59)	Patients with VL[b]<4log (n = 27)	Patients with VL ≥4log (n = 32)	
Lamivudine	49 (83)	23 (85)	26 (81)	0.425
Zidovudine	15 (25)	5 (18)	10 (31)	0.266
Stavudine	13 (22)	3 (11)	10 (31)	0.093
Nevirapine	52 (88)	24 (89)	28 (87)	0.946
Efavirenz	30 (51)	16 (59)	14 (44)	0.590

[a]Number (%) of patients, unless otherwise indicated; [b]VL, viral load

(80%), followed by T69N in 22 (37%) and D67N in 18 (30%), respectively. The most prevalent mutation associated with NNRTIs resistance was Y181C/I, found in 34 patients (58%), followed by K103N in 18 (30%), G190A/S in 14 (24%), V108I in 13 (22%) and K101E/H/P in 10 (17%). Major mutations associated with resistance to protease inhibitors (PIs) were found in this study while minor mutations associated with resistance were detected at position L33F (1 patient, 2%), and A71T (1 patient, 2%).

Among the NRTIs, high-level resistance to lamivudine was the most frequent finding among the 59 samples (49 samples; 83%), followed by zidovudine resistance (15; 25%) and stavudine resistace (13; 22%) (Table 3). Among the NNRTIs, the most frequent high-level resistance was to nevirapine (52; 88%), followed by efavirenz resistance (30; 51%). There was no difference between the two groups in the proportion of samples with high-level resistance to lamivudine, zidovudine, stavudine, nevirapine, and efavirenze.

The HIV-1 *pol* [protease (PR) and RT] region of 59 strains was amplified, sequenced and subtyped. Phylogenetic analysis showed that 58 sequences (98%) belonged to HIV-1, CRF01_AE with a bootstrap value of 84% and the remaining sample (2%) belonged to HIV-1 subtype B with a bootstrap value of 77%. The percent similarity to HIV-1 subtype using Stanford HIV database showed the same result as the phylogenetic analysis. All of the percent similarity to HIV-1 subtypes analyzed by the Stanford HIV database was more than 93%.

DISCUSSION

Genotypic resistance of viral reverse transcriptase (RT) and protease (PR) was determined in 59 HIV-infected patients with treatment failure at Chiang Mai University, Thailand showing that 62% of the patients with HIV-1 viral load ≥ 4 $\log_{10}$ had 3 to 5 NRTI-resistance associated mutations compared to 22% of those with HIV-1 viral load < 4 $\log_{10}$ (p = 0.001). Comparison of the average number of TAMs and major resistance mutations to NNRTIs between the two groups also supported the findings that patients in the

high viral load group have accumulated a higher number of resistance mutations.

As HIV-1 resistance is affected by multiple factors, the genotypic resistance patterns cannot be accurately predicted. However, a few important observations were made in this study. Firstly, genotypic antiretroviral drug resistance testing should be considered to provide for better drug regimens to improve the management of dual NRTI failure (Durant *et al*, 1999). Secondly, there is a significant accumulation of nucleotide-associated mutations (NAMs) in the group with higher viral load. Thirdly, there is a significantly lower CD4 cell counts in the higher viral load group.

HIV can develop resistance rapidly after only a single mutation (Deeks *et al*, 1999; Engchanil *et al*, 2006). Therefore, detection of HIV-1 viral resistance at an early stage can prevent the accumulation of resistant mutations, especially NAMs. However, the barrier to the development of resistance to different antiretroviral drugs is different. A single mutation can confer high-level resistance to lamivudine, whereas for most other drugs, *eg*, zidovudine, stavudine, high-level resistance requires accumulation of three or more mutations (Larder and Kemp, 1989; Condra *et al*, 1996; Duwe *et al*, 2001). Although, comparison between the two groups of the frequency of NRTI resistance-associated mutations shows no significantly different frequency of M41L, T69, Q151M, T215Y/F, and K219Q/E mutations, these findings emphasize the need for larger studies to evaluate the resistance mutations and drug-resistant patterns in order to make recommendations on ART resistance monitoring.

Other important variables that are known or suspected to affect virological response to therapy, such as baseline viral load and CD4 count, drug phamacokinetics, drug binding to plasma proteins, concomitant drugs or drug-drug interactions, other substance, *eg*, herbs, or drug-food interactions, hepatic impairment, host genetic factors and medication adherence may have to be incorporated in future analysis of resistance factors in order to maximize the predictive value of resistance testing and ultimately to improve patients' outcome (DeGruttola *et al*, 2000; Ivanovic *et al*, 2008).

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