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Drug-resistant mutation patterns in CRF01_AE cases that failed d4T + 3TC + nevirapine fixed-dosed, combination treatment: Follow-up study from the Lampang cohort

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ABSTRACT

HIV/AIDS patients are treated in Thailand's national antiretroviral treatment (ART) program with a generic combination tablet of stavudine, lamivudine, and nevirapine (GPOvir). To determine GPOvir-resistant mutations, HIV-1 sequences of 59 GPOvir-failure cases from the Lampang cohort were compared with sequences from 76 randomly selected ART-naïve cases. The GPOvir-failure cases had not only known stavudine-, lamivudine- and nevirapine-resistant mutations, but also V118I, G196E, and H221Y. Among the 59 GPOvir-failure cases, 29 were ART-naïve prior to GPOvir (naïve group), and 30 had previous ART (exposed group). To clarify the effect of previous ART in drug-resistant acquisition pathways, naïve and exposed groups were compared. The exposed group had predominantly thymidine analogue-related mutations, whereas the naïve group had a higher prevalence of Q151M and K103N mutations. M184V lamivudine resistance was most frequent in both naïve and exposed groups. To identify which mutations in CRF01_AE pol were polymorphisms, the connection and RNase domains were also analyzed. CRF01_AE-specific polymorphisms were found in 19 residues, and GPOvir-failure cases had significantly higher frequency of N348I, E399D, P537S, and I542M. Our results expand identification of mutations in CRF01_AE pol that are polymorphisms by also analyzing the connection and RNase H domains.

1. Introduction

The number of people living with HIV/AIDS in Thailand at the end of 2008 was 532,500 (Ministry of Public Health, 2008). In resource-poor countries such as Thailand, the recommended first-line regimen for treating HIV/AIDS is a combination of two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTIs) (WHO, 2003). HIV/AIDS patients in Thailand have been treated since 2002 through the national antiretroviral treatment (ART) program with a generic, fixed-dosed single tablet (GPOvir) with 3 antiretroviral agents: stavudine (d4T), lamivudine (3TC) and nevirapine (NVP). The major reason for plasma viral load rebound and treatment failure remains the emergence of drug resistance. Therefore, HIV drug-resistance genotypic testing (HIV genotyping) has become an important tool in deciding about appropriate treatment regimens. HIV genotyping, i.e., the determination of mutations that confer drug resistance, is now widely established as the standard of care to guide treatment in the context of both primary infection and virological failure (Hirsch et al., 2003). To date, the design and development of antiretroviral drugs, research on drug resistance, and interpretation systems have been largely based on the HIV-1 subtype B virus, the major subtype in developed countries. However, the findings on subtype B may not always be

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Table 1
Demographic and clinical characteristics of GPOvir virologic-failure cases at 6 or 24 months ($n = 59$).

Variable	ART-naïve cases ($n = 29$) n (%)	ART-experienced cases ($n = 30$) n (%)	Odds ratio
Age (years)			
<35	19 (65.5)	22 (73.3)	0.70
≥35	10 (34.5)	8 (26.7)	
Gender			
Male	20 (69.0)	18 (60.0)	1.47
Female	9 (31.0)	12 (40.0)	
CD4 at baseline (cells/μl)			
<50	16 (55.2)	15 (50.0)	1.27
≥50	10 (34.5)	12 (40.0)	
Unknown	3 (10.3)	3 (10.0)	
AIDS symptoms			
Asymptomatic	3 (10.3)	2 (6.7)	1.67
AIDS/symptomatic	25 (86.2)	28 (93.3)	
Unknown	1 (3.5)	0 (0.0)	
Route of transmission			
Heterosexual	27 (93.1)	29 (96.7)	0.47
Homosexual	2 (6.9)	1 (3.3)	

applicable to other subtypes, and some minor mutations, which are recognized as drug-resistant mutations in subtype B, exist as natural variants in non-B subtypes (Kantor and Katzenstein, 2003).

Furthermore, under antiretroviral treatment certain subtypes select specific mutations that are different from those of subtype B (Brenner et al., 2003; Grossman et al., 2004; Loemba et al., 2002). For example, in data on GPOvir-failure cases collected from 7 hospitals in Thailand, where the most prevalent subtype is CRF01_AE, the most commonly reported drug-resistant mutations were G190S/A and Y181C/I; and K103N, Y181C/I, M184V/I were significantly associated with efavirenz, NVP, 3TC, respectively (Chetchotisakd et al., 2006). The reported pattern was identical to that of subtype B. Interestingly 26% of cases in that study had received dual-NRTI treatment before the GPOvir regimen, but ART-naïve and ART-experienced patients were not analyzed in detail for differences in resistance-acquisition patterns. However, another study of drug-resistance mutation patterns among GPOvir-failure cases in Thailand found that the most frequent resistance mutation was M184V, with higher frequencies of K65R (6%) and Q151M (8%) than for subtype B (Sungkanuparph et al., 2007). Thus, there are differences in the reported drug-resistance patterns after GPOvir treatment, indicating the need for further data on drug resistance of GPOvir-resistant cases to better understand drug-resistance acquisition patterns in CRF01_AE.

Therefore, the aim of this study was to clarify drug-resistance mutation pattern in GPOvir treatment-failure cases from the Lampang cohort (Tsuchiya et al., 2009). To understand the effect of previous antiretroviral exposure in GPOvir-resistance acquisition, we analyzed data not only from ART-naïve cases but also from those previously treated with mono- or dual therapies. Recently, several studies demonstrated that resistance to NRTI and/or NNRTI therapies is enhanced and the balance between nucleotide excision and template RNA degradation is affected by mutations in the connection domain and RNase H region (Brehm et al., 2007; Delviks-Frankenberry et al., 2007; Ehteshami et al., 2008; Julias et al., 2003; Nikolenko et al., 2007; Ntemgwa et al., 2007; Santos et al., 2008; Waters et al., 2009; Yap et al., 2007; Zelina et al., 2008). However, these studies were mostly on subtype B, with less information on non-B subtypes. Therefore, to clarify the effect of CRF01_AE mutations in the connection domain and RNase H region, we analyzed the sequences of these domains in GPOvir treatment-failure cases.

2. Materials and methods

2.1. Samples

Plasma samples were collected from patients in Lampang Hospital, a government referral hospital in Lampang province of northern Thailand. In total, 345 HIV-1-infected Thai patients agreed and started GPOvir therapy at the hospital's Day Care Center clinic between 1 April 2002 and 31 January 2004. Of these 345 cases, 244 cases were ART-naïve, and 101 cases had been exposed to ART before initiating GPOvir treatment (baseline). Their plasma samples were collected and analyzed for HIV-1 sequences at baseline and at different time points until the end-point of 24-month follow-up or a switch in therapy. Treatment-failure cases were defined as cases with a detectable viral load (>50 copies/ml) despite having received GPOvir therapy for at least 3 months; this criterion was met by 78 cases. However, 19 cases were excluded from the study for the following reasons: 9 changed to other treatment, 1 had poor adherence, 6 changed to undetectable viral load, and 3 had unknown treatment histories. Samples from the remaining 59 cases were sequenced. Their demographics and clinical variables are summarized in Table 1. These cases were separated into two groups: ART-naïve and ART-exposed. The ART-naïve cases ($n = 29$, 49.1%) had never been exposed to antiretrovirals prior to GPOvir treatment and the ART-exposed cases ($n = 30$, 50.9%) had been exposed to antiretrovirals. These groups did not differ significantly in terms of clinical variables. Most patients were infected with HIV-1 through heterosexual contact ($n = 56$, 94.9%), which did not differ from the HIV-1 transmission pattern in the whole GPOvir study population (Tsuchiya et al., 2009). In the ART-exposed group, the most common treatment regimen was dual therapy with AZT and ddC or ddI (Table 2); none were previously exposed to NNRTIs.

This study was conducted according to principles of the Declaration of Helsinki, the Lampang HIV study was approved by the Thai government ethics committee, and written informed consent was obtained from patients who agreed to join this study.

2.2. Sequencing of the RT and RNase H genes

All samples were determined for viral load, and when it exceeded 1000 copies/ml, the RT region (residues 1–240) was tested for drug resistance using an in-house genotyping protocol reported elsewhere (Myint et al., 2002; Saeng-Aroon et al., 2007).

Table 2
Previous antiretroviral treatment histories of drug-experienced cases ($n = 30$).

Treatment regimen	*n*
AZT/ddC	14
AZT/ddI	4
AZT	1
AZT/ddC/RTV	1
AZT/ddI/RTV	1
AZT/ddI/IDV	1
SQV/RTV	1
AZT/ddI–AZT/ddC	1
GPOvir	1
Unknown	5

Note: AZT: azidothymidine; ddC: dideoxycytidine; ddI: dideoxyinosine; RTV: ritonavir; IDV: indinavir; SQV: saquinavir; GPOvir: stavudine, lamivudine and nevirapine.

In brief, HIV RNA was extracted from 140 μl plasma using the NucleoSpin viral RNA extraction kit (NucleoSpin, Duren, Germany) following the Manufacturer's instructions. cDNA and PCR product were then obtained using the SuperScript III One Step RT-PCR kit (Invitrogen, Carlsbad, CA) and primers listed in Table 3.

For amplifying the extended RT regions, connection and RNase H domains, new primers were designed. Outer PCR was performed with RT1 and GPR2M, while nested PCR was performed with primers RT7L and GPR3L. The amplification profile for outer PCR was 40 min at 55 °C, 2 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 15 s at 55 °C and 1.30 min at 72 °C, and 7 min at 72 °C. The reaction mixture for the nested PCR contained 3.5 μl of the product from the first PCR. The amplification profile in the second PCR was 2 min at 92 °C followed by 30 cycles of 10 s at 94 °C, 4 s at 60 °C and 15 s at 74 °C, and 7 min at 72 °C. The amplicon (1700 bps) represented the HIV-1 pol region, spanning the RT region, connection domain, and RNase H domain. Both strands of the PCR product were sequenced using six different primers and BigDye® Terminator v3.1 chemistry on an ABI 3100 Genetic Analyzer. SeqScape software version 2.5 was used for editing and assembling sequence fragments, and the assembled sequences were compared with the reference strain HBX2 from the Los Alamos HIV sequence database. To obtain maximum prevalence of drug-resistance mutations, codons with wild type and resistant mixtures were counted as resistance positive.

In addition to sequencing and analyzing the 5′ 240-amino acid RT region, we clarified the substitution patterns of the connection and RNase H domains under GPOvir treatment by sequencing and analyzing 49 plasma samples of GPOvir-failure cases collected at their last visit. As naïve control sequences, the connection and RNase H domains were sequenced from 76 randomly selected HIV-1 CRF01_AE ART-naïve cases from the same hospital.

2.3. Data analysis, determination of subtypes, drug-resistant mutations and polymorphisms

To confirm that patients were infected with HIV-1 CRF01_AE, all nucleotide sequences were aligned using Clustal W, version 2.0.10 and BioEdit, version 7.0.9.0. Phylogenetic tree and bioinformatics analyses were conducted using MEGA, version 4 (Tamura et al., 2007). The genetic distances were calculated using Kimura's 2-parameter analysis (Kimura, 1981), and phylogenetic trees were constructed by the neighbor-joining method. The overall polymorphism of RT genes was analyzed by comparing 76 CRF01_AE sequences from our cohort and reference sequences, all treatment-naïve cases, from Los Alamos HIV sequence database (http://www.hiv.lanl.gov). The following sequences were obtained from the Los Alamos HIV sequence database: 42 subtype B (accession no. EF637056, DQ837381, DQ676874, EF637057, DQ676870, DQ676877, DQ676880, EF363122, DQ127537, BD455696, K03455, EF363124, U69584, EF637053, DQ487190, AY314044, EF363122, DQ007902, DQ007903, DQ990880, AY945710, EF363127, DQ396398, EF637054, DQ207940, EF637048, EF175209, EF637051, DQ853436, EF637050, EF637049, DQ676886, DQ127548, DQ207942, AB428551, AB428557, AB428556, AB428553, AB428554, AB428552, AB428555, AB428561), 26 CRF01_AE (DQ859178, DQ859179, DQ859180, EF036527, EF036528, EF036529, EF036530, EF036531, EF036532, EF036533, EF036534, AY945712, AY945713, DQ789392, AY945716, AY945717, AY945719, AY945720, AY945721, AY945722, AY945724, AY945727, AY945728, AY945730, AY945731, AY945732).

Resistance-related mutations were based on guidelines published by the International AIDS Society United States (IAS-USA) HIV Resistance Testing Guideline Panel 2008 (Johnson et al., 2008) according to the subtype B consensus strain. To compare the distribution of qualitative variables according to groups, χ^2-test was used or the Fisher exact test when the sample was too small. All statistical tests were interpreted at the 5% significance level.

Table 3
Primers used for amplification and sequencing.

Name	Region	Usage	Primer sequence 5′–3′	Position
RT1L	RT	Outer forward	ATGATAGGGGGAATTGGAGGTTT	2388–2410
RT4L	RT	Outer reverse	TACTTCTGTTAGTGCTTTGGTTCC	3402–3425
RT7L	RT	Inner forward	GACCTACACCTGTCAACATAATTGG	2485–2509
RT6L	RT	Inner reverse	TAATCCCTGCATAAATCTGACTTGC	3348–3372
RT7L	RT	Sequencing	GACCTACACCTGTCAACATAATTGG	2485–2509
RT26	RT	Sequencing	CAAAAATTGGGCCTGAAAATCC	2692–2713
RT28	RT	Sequencing	TGGAATATTGCTGGTGATCC	3012–3031
RT6L	RT	Sequencing	TAATCCCTGCATAAATCTGACTTGC	3348–3372
RT1L	RT	Outer forward	ATGATAGGGGGAATTGGAGGTTT	2388–2410
GPR2M	Connection and Rnase H	Outer reverse	GGACTACAGTCYACTTGTCCATG	4380–4402
RT7L	RT	Inner forward	GACCTACACCTGTCAACATAATTGG	2485–2509
GPR3L	Connection and Rnase H	Inner reverse	TTAAAATCACTARCCATTGYTCTCC	4285–4309
RT7L	RT	Sequencing	GACCTACACCTGTCAACATAATTGG	2485–2509
RT26	RT	Sequencing	CAAAAATTGGGCCTGAAAATCC	2692–2713
RT28	RT	Sequencing	TGGAATATTGCTGGTGATCC	3012–3031
RT31	RT	Sequencing	GAGCTCATCTATTGAGCTGG	3166–3185
RT32	RT	Sequencing	GAACCTCCATTCCTTTGGATGGG	3219–3241
RT6L	RT	Sequencing	TAATCCCTGCATAAATCTGACTTGC	3348–3372
RT35	Connection and Rnase H	Sequencing	GCAGAAGTACAGAAACAAGG	3528–3547
GPR3L	Connection and Rnase H	Sequencing	TTAAAATCACTARCCATTGYTCTCC	4285–4309

Table 4
Known mutations associated with drug resistance (residues 1–240).

HXB2	Residue	Mutation frequency		*p*
		Naïve group (*n* = 76)	Failure group (*n* = 49)	
M	41	M(76)	M(34),L(15)	<0.001
A	62	A(76)	A(47),V(2)	–
K	65	K(76)	K(47),R(2)	–
D	67	D(76)	N(26),D(22),G(1)	<0.001
K	70	K(76)	K(38),R(10),G(1)	<0.001
L	74	L(76)	L(47),I(2)	–
V	75	V(76)	V(46),I(3)	–
F	77	F(76)	F(48),L(1)	–
A	98	A(76)	A(45),G(3),S(1)	–
L	100	L(76)	L(49)	–
K	101	K(76)	K(36),E(12),H(1)	<0.001
K	103	K(76)	K(36),N(11),S(2)	<0.001
V	106	V(74),I(2)	V(45),I(4)	–
V	**108**	**V(76)**	**V(44),I(5)**	**<0.05**
Y	115	Y(76)	Y(49)	–
F	116	F(76)	F(44),Y(5)	<0.05
V	118	V(76)	V(43), I(6)	<0.05
Q	151	Q(76)	Q(43),M(6)	<0.05
Y	181	Y(76)	Y(22),C(23),V(4)	<0.001
V	179	V(68),I(6),IV(2)	V(35),I(10),D(1),IV(3)	–
M	184	M(76)	V(37),I(8),M(4)	<0.001
Y	188	Y(76)	Y(48),L(1)	–
G	190	G(76)	G(29),A(18),S(2)	<0.001
G	**196**	**G(76)**	**G(44),E(5)**	**<0.05**
L	210	L(76)	L(37),W(12)	<0.001
T	215	T(76)	T(27),F(12),Y(10)	<0.001
K	219	K(76)	K(42),Q(6),E(1)	<0.05
H	**221**	**H(76)**	**H(42),Y(7)**	**<0.05**
P	225	P(76)	P(48),H(1)	–

Subtype B consensus residues are displayed on the left side of each position.
Bold represents new substitutions not previously reported for CRF01_AE.

3. Results

3.1. New patterns of drug-resistance mutations emerge in CRF01_AE GPOvir-failure cases

Drug-resistance mutations related to GPOvir failures are summarized in Table 4. Sequences were compared between 49 samples of treatment-failure cases collected at their last visit and 76 randomly selected treatment-naïve samples at baseline. Almost all of the known mutations associated with d4T/3TC/nevirapine treatment were significantly higher in the GPOvir-failure cases, except the 4 following mutations: K65R, L100I, V106M/A, and Y188C/L/H. Other than known mutations, V118I and H221Y were observed in significantly higher prevalence in the GPOvir-failure group ($p < 0.05$). Interestingly, cases with the H221Y mutation all had Y181C, and the linkage of the two mutations was statistically significant ($p < 0.05$), as previously reported (Liu et al., 2007). A likely role for these mutations in resistance to NRTIs has been suggested by a report of pre- and post-treatment frequencies of H221Y (0–13.7%) in subtype C isolates from India (Deshpande et al., 2007). In subtype B isolates, H221Y and D223E/Q were associated with therapy only if individuals receiving both NRTI and NNRTI were included. G196E was also reported to be significantly different in subtype B ($p < 0.05$) (Gonzales et al., 2003).

3.2. Drug-resistance mutations, especially d4T resistance-related mutations, are more prevalent in the ART-exposed group than the naïve group

The frequencies of drug-resistance mutations in the ART-naïve and -exposed groups at baseline, 6, 12, 18, and 24 months are shown in Fig. 1. The two groups differed significantly in their resistance mutation-acquisition patterns. The most apparent difference was the frequency of d4T resistance-related mutations. The exposed group had significantly higher frequencies of mutations M41L, D67N, K70R, L210W, T215Y/F and K219Q/E, most of which already existed at baseline. As for K70R, T215Y/F and K219Q/E, their frequencies at difference time points did not change during the observation period, whereas the frequencies of M41L and D67N increased from 10% (4 of 40) to 20% (9 of 46) and from 10% (4 of 40) to 26% (12 of 46), respectively.

In contrast to the exposed group, a few cases in the naïve group acquired d4T resistance-related mutations, similar to a pattern previously reported (Arts et al., 1998; Lacey and Larder, 1994). Frequencies of d4T resistance-related mutations did not increase during the observation period in the naïve group, except for the D67N mutation. The prevalence of D67N in the naïve group increased from 0% (baseline) to 15% (7 of 46) at 24 months. Thus, GPOvir appears to be selecting the D67N mutation. As described above, the exposed group showed similar findings; D67N prevalence increased from 10% (4 of 40) at baseline to 26% (12 of 46) at 24 months. The 10% of cases at baseline in the exposed group can be explained by previous AZT exposure, and the additional 16% might result from induction and selection by d4T administration.

Another notable finding of our study is the detection of the Q151M multi-drug-resistant mutation. In the ART-naïve group, 5 cases acquired Q151M during the observation period. As Q151M prevalence increased over the treatment period, from 0% at baseline to 11% (5 of 46) at 24 months, it is clear that GPOvir treatment selected the Q151M mutation. Interestingly, the prevalence of Q151M in our study is similar to that of two previous reports on CRF01_AE, i.e., 8% (Sungkanuparph et al., 2007) and 11% (Chetchotisakd et al., 2006). As the CRF01_AE prevalence of Q151M is higher than that of subtype B, CRF01_AE appears to be more prone to acquire this mutation.

In both the ART-naïve and -exposed groups, the most frequently observed mutation was lamivudine-resistant M184V, suggesting that this mutation has a low genetic barrier. Comparing the two

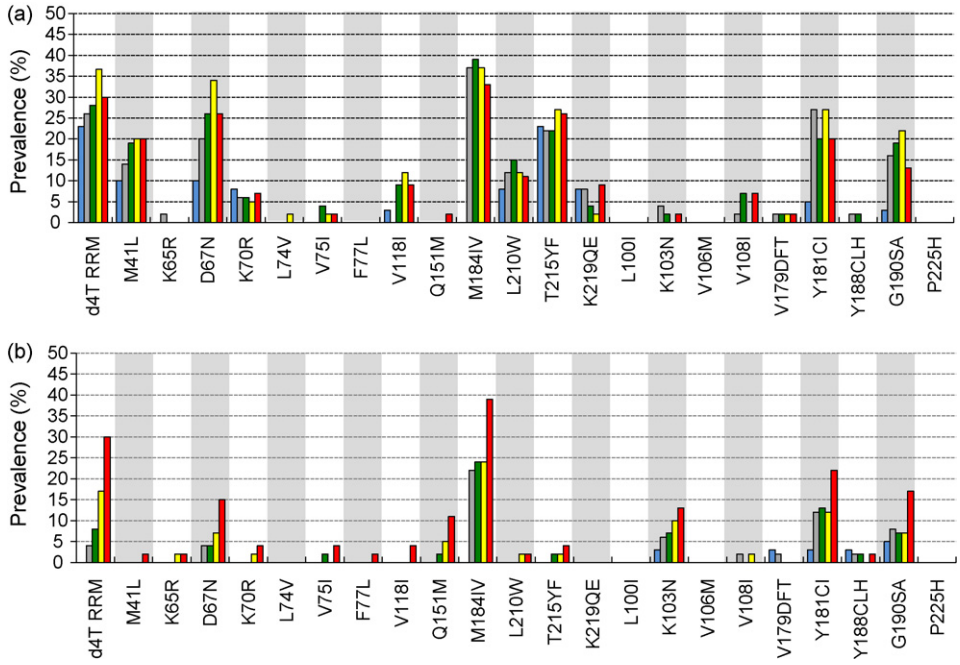

Fig. 1. Summary of drug resistant mutations detected in (a) antiretroviral exposed group, and (b) naive group. Light blue, gray, green, yellow and red bars indicate prevalence of mutation detected at baseline, 6, 12, 18 and 24 months after initiation of GPOvir treatment, respectively. d4T related resistance mutations (d4T RRM) include M41L, K65R, D67N, K70R, Q151M, L210W, T215YF and K219QE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

groups, M184V was detected earlier and at higher prevalence in the exposed group (0% at baseline and 37% [18 of 49] at 6 months) than in the ART-naïve group (0% at baseline and 22% [11 of 49] at 6 months).

The ART-naïve and -exposed groups also had an interesting difference in their patterns of NNRTI-resistance acquisition. The ART-naïve group had a higher frequency of K103N mutation (13% [6 of 46]) than the exposed group (2% [1 of 46]). Instead of acquiring K103N, the exposed group tended to develop Y181C/I and G190A mutations. Since neither group had a history of NNRTI treatment prior to GPOvir, this difference in drug-resistance acquisition patterns cannot be explained by previous NNRTI treatment or differences in NNRTI-mutation patterns at baseline.

3.3. *Polymorphisms and drug-resistant mutations in the connection domain and RNase H mutations in CRF01_AE*

To determine subtype-specific polymorphisms in CRF01_AE, we compared sequences from 76 randomly selected CRF01_AE ART-naïve cases at baseline from our cohort with 42 subtype B reference sequences from the Los Alamos database. CRF01_AE-specific polymorphisms were determined at 9 residues in the connection domain and at 10 residues in the RNase H domain (Table 5). Interestingly, G335D and A371V, which have been recognized as NRTI-related resistance mutations in subtype B (Brehm et al., 2007; Nikolenko et al., 2007), were observed as natural polymorphisms among CRF01_AE. However, the contribution of these mutations to GPOvir resistance is not yet clear. Further studies are needed to clarify their role in NRTI resistance.

To determine treatment-specific mutations related to GPOvir administration in the connection and RNase H domains, sequences were compared between 49 samples from the last visit of GPOvir-failure cases and 76 baseline samples. The results (Table 6) show 13 mutations in the connection and RNase H domains: 9 mutations in the connection domain (Y318F, G335C/D, N348I, A360I/V, V365I, T369I, A371V, T376S, and E399D) and 4 in the RNase H domain (N447S, Q509L, P537S, and I542M). Among these mutations, N348I ($p < 0.001$) and E399D ($p < 0.001$) in the connection domain, and P537S ($p < 0.05$) and I542M ($p < 0.001$) in the RNase H domain were observed at significantly higher prevalence in GPOvir-failure cases. E312Q, G335C/D, N348I, A360I/V, V365I, and A376S were previously reported to confer AZT resistance in subtype B (Nikolenko et al., 2007). In addition, A371V and Q509L were reported to be selected *in vitro* by AZT and to confer greater AZT resistance and cross-resistance to other nucleoside RT inhibitors in combination with thymidine analogue-related mutations (TAM) (Brehm et al., 2007). However, as the number of cases in our study is small, the significance of E312Q, Y318F, G333D, A360I/V, V365I, T376S, and Q509L in CRF01_AE drug resistance is not well understood.

4. Discussion

Here we compared two patient groups, ART-naïve or -exposed at baseline, and analyzed their differences in their responses to nevirapine + 3TC + d4T (GPOvir) and drug-resistance acquisition patterns. This drug combination is widely used in the developing world today, and drug-resistant mutation patterns induced by this combination have been described in different countries and subtypes (Chetchotisakd et al., 2006; Kumarasamy et al., 2003; Pujari et al., 2004; Sungkanuparph et al., 2007; Zhou et al., 2007), but most reports describe drug-resistance mutation patterns in naïve cases, and few in exposed cases.

By comparing ART-naïve and -exposed groups, we observed the following notable findings. First, our study demonstrated that GPOvir is effective in exposed cases as well as naïve cases. Of the 101 exposed cases in our study, many had previously been treated with several nucleoside analogue inhibitors. Nonetheless, 64.4% of cases were successfully treated with GPOvir at the 24-month readout time point, despite our earlier finding that previous exposure to antiretrovirals was associated with virological failure (Tsuchiya et al., 2009). This finding suggests that though the priority of antiretroviral usage in resource-limited settings should be considered, pre-exposure history may not be an excuse to limit use of nevirapine + 3TC + d4T.

Table 5
Frequency of polymorphisms in HIV-1 RT from treatment-naïve subtypes B and CRF01_AE.

HXB2	Residue	Subtype B ($n = 42$)	Subtype CRF01_AE ($n = 76$)	Region
E	6	E36,D6	**D74**,E2	DNA polymerase
K	11	K42	**T55**,K18,S3	
V	35	V33,I6,M1,R1,T1	**T75**,M1	
T	39	T41,A1	**K75**,E1	
K	43	K40,N1,R1	**E73**,K2,A1	
K	122	K31,E9,R1	**E74**,K2	
D	123	D28,E13,	**S68**,N6,D2	
Q	174	Q42	**K72**,N2,Q2	
D	177	D32,E9,N1	**E73**,D3	
I	178	I40,L1,M1	**M47**,I28,V1	
R	211	R19,K15,G7,S1	**S70**,K5,N1	
K	238	K41,R1	**R56**,K20	
V	245	V19,E11,M5,K3,T2,A1,I1	**E74**,Q1,V1	
T	286	T25,A14,V2,P1	**A61**,T15	
E	291	E40,D2	**D70**,E6	
V	292	V41,I1	**I70**,V6	
E	312	E41	**T73**,N3	
I	326	I38,V4	**V63**,I13	Connection domain
I	329	I34,L6,V2	**V73**,I3	
G	335	G41,N1	**D75**,G1	
M	357	M33,T5,V3,I1	**K75**,R1	
G	359	G39,S3	**S76**	
K	366	K38,R4	**R73**,K3	
A	371	A34,V7,T1	**V76**	
K	390	K24,R18	**R65**,K11	
T	403	T23,M13,I4,A1	**M63**,T13	
N	447	N39,S3	**S72**,N4	RNase H domain
N	460	N22,D19,S1	**D67**,N9	
D	471	D41,N1	**E76**	
Q	480	Q40,H2	**H75**,Y1	
L	491	L23,S12,P3,V3,A1	**S69**,P5,L2	
Q	512	K35,Q3,R2,T1,N1	**R72**,K4	
N	519	S31,N11	**N69**,S7	
A	534	A41,T1	**S76**	
V	536	T42	**V76**	
A	554	A28,T8,S4,N2	**S76**	

Amino acid substitutions in treatment-naïve B and CRF01_AE cases differ significantly ($p < 0.05$).

Second, our comparison of resistance patterns in naïve and exposed groups led to several interesting observations about differences in drug-resistance acquisition pathways. Among these differences, the d4T-resistance patterns were especially intriguing. The naïve group showed three patterns of d4T-resistance mutations. The first pathway was acquisition of the Q151M multiresistant mutation. Q151M is an alternative pathway of AZT resistance that does not depend on ATP binding and excision (Lennerstrand et al., 2001a, 2001b) and is known as the major AZT resistance pathway in HIV-2 (Boyer et al., 2006; Perach et al., 1997). The second pathway was acquisition of D67N, known as part of d4T resistance-related mutations. The third pathway could be the K65R acquisition route. Although few cases (2%) were found in our study population, K65R has been reported to be predominantly selected by GPOvir administration (Sungkanuparph et al., 2007, 2008) (6% and 7%). The mechanisms of the selection process for these three pathways, how they are selected, and how they evolved remain unclear. Other than these three types of resis-

Table 6
Mutations/polymorphisms outside known drug-resistance mutations.

HXB HXB2	Residue	Mutation frequency		p	Region
		Naïve group ($n = 76$)	Failure group ($n = 49$)		
E	312	T(73), N(3)	T(44),N(5)	–	RT/DNA polymerase
Y	318	Y(76)	Y(47),F(2)	–	Connection domain
G	333	G(76)	G(49)	–	
G	335	D(75), G(1)	D(48),G(1)	–	
N	348	N(76)	N(41), I(8)	<0.001	
A	360	A(76)	A(48), V(1)	–	
V	365	V(76)	V(48), I(1)	–	
A	371	V(76)	V(49)	–	
A	376	A(71), S(4), T(1)	A(43), S(4), T(2)	–	
E	399	E(75), D(1)	E(33), D(16)	<0.001	
N	447	S(72), N(4)	S(37), N(12)	–	RNase H domain
Q	509	Q(76)	Q(48), L(1)	–	
P	**537**	P(76)	P(44), S(5)	<0.05	
I	**542**	I(75), M(1)	I(40), M(9)	<0.001	

Subtype B consensus amino acid sequence is shown as a reference on the left side of each position. Bold represents new substitutions not previously reported.

tance mutation-acquisition pathways, it is possible that resistance mutations had been transmitted.

In contrast to multiple pathways for acquiring d4T resistance in the naïve group, d4T resistance acquisition in the exposed group was much simpler. d4T resistance-related mutations were the most frequently observed mutations, and few K65R or Q151M mutations were detected. As many cases had a history of AZT as mono- or dual therapy, d4T resistance-related mutations appear to have been induced during previous AZT exposure, and these mutations were re-selected by GPOvir treatment. We also observed different mutation patterns in NNRTI resistance. K103N was less prevalent in the exposed group. Some of the differences observed between our two study groups may be attributed to intra- or intermolecular interference, which has been reported to affect drug-resistant mutation-acquisition pathways (Parikh et al., 2006; Quan et al., 1998).

Regarding mutations in the connection and RNase H domains, these two domains are not usually analyzed in clinical samples since most RT inhibitor-resistance mutations map to the DNA polymerase domain of RT (Clavel and Hance, 2004). Thus, less information is available for these domains in CRF01_AE. Therefore, we collected information on these two domains from our cohort. We found that G335C/D and A371V, which have been reported to confer AZT resistance in subtype B (Brehm et al., 2007; Nikolenko et al., 2007), were natural polymorphisms of CRF01_AE, and N348I, E399D, P537S, and I542M appeared to be induced by GPOvir exposure. Among these last 4 mutations, N348I and E399D were reported to affect AZT and NNRTI resistance in subtype B (Hachiya et al., 2008; Poveda et al., 2008); P537S and I542M are two newly discovered mutations in our study.

In conclusion, our study shows the potential of GPOvir for antiretroviral treatment-naïve and -exposed groups and demonstrates differences in drug-resistance acquisition pathways. Selection of pre-existing mutations and different pathways was affected by interference with drug-resistance mutations. Although developing countries currently have no alternative treatment regimen to GPOvir, its usage could be detrimental to salvage regimens because (1) d4T selects the multi-drug-resistance mutations, Q151M and K65R, the latter conferring resistance to tenofovir, and (2) both 3TC and nevirapine have low genetic barriers to acquiring drug resistance. More studies are needed to provide a better basis for selecting second-line treatments after GPOvir failure.

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