SUSTAINED APPEARANCE OF DRUG RESISTANCE-ASSOCIATED MUTATIONS IN HIV-1 CRF01_AE PROTEASE AND REVERSE TRANSCRIPTASE DERIVED FROM PROTEASE INHIBITOR-NAIVE THAI PATIENTS

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Abstract. Previous studies revealed that HIV-1 CRF01_AE viruses derived from antiretroviral drug-naïve Thai patients contained several protease (PR) inhibitor (PI) resistance-associated mutations. In this report, we examined the sustained appearance of drug resistance-associated mutations in CRF01_AE PR and reverse transcriptase (RT). Peripheral blood samples were collected every 3 months from April 2008 to April 2009 from 39 HIV-1-infected Thai patients, including 17 drug-naïve and 22 RT inhibitors (RTIs)-treated individuals, and polymerase chain reaction-mediated amplification and sequencing analysis of the viral genome encoding PR and RT were performed. We successfully analyzed the deduced amino acid sequence of CRF01_AE PR and RT derived from samples continuously collected from 15 drug-naïve and 20 RTIs-treated patients. Drug resistance-associated mutations were continuously detected in CRF01_AE PR derived from most patients. The continuous appearance of such PR mutations was observed not only in the proviral DNA genome derived from peripheral blood mononuclear cells, but also in the viral RNA genome of plasma virus. In contrast, RTI resistance-associated mutations were only sporadically detected in samples derived from drug-naïve and RTIs-treated patients, except for the continuous appearance of two mutations in samples derived from two drug-naïve patients. Our results demonstrate that many PI resistance-associated mutations and only a few RTI resistance-associated mutations continuously appear in CRF01_AE viruses derived from PI-naïve patients residing in northern Thailand.

Key words: HIV-1 CRF01_AE virus, drug resistance-associated mutations, reverse transcriptase, protease

INTRODUCTION

Antiretroviral (ARV) therapy (ART) with two or more reverse transcriptase (RT) inhibitors (RTIs) and protease (PR) inhibitors (PIs) for human immunodefi-
ciency virus type 1 (HIV-1)-infected patients has achieved durable virological suppression as well as appreciably reducing HIV-1 transmission, morbidity and mortality associated with HIV-1 disease (Gulick et al., 1997; Hammer et al., 1997). The Thai government developed the ART program, which provides HIV-1-infected patients with a locally produced generic drug, the Government Pharmaceutical Organization produced GPOvir, which contains two nucleoside/nucleotide analogue RT inhibitors (NRTIs), stavudine (d4T) and lamivudine (3TC), and a non-nucleoside analogue RT inhibitor (NNRTI), nevirapine (NVP). GPOvir is currently the first-line regimen in Thailand. In addition, NRTIs, abacavir (ABC), didanosine (ddl), tenofovir disoproxil fumarate (TDF), zalcitabine (ddC) and zidovudine (AZT); an NNRTI, efavirenz (EFV); and PIs, atazanavir (ATV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV) and saquinavir (SQV) are available in this country. The treatment of HIV-1-infected patients with these ARV drugs has been successful, but the emergence of drug-resistant viruses with widespread drug use is presently one of the major obstacles associated with ART in Thailand (Sutthent et al., 2005), similar to many other countries described elsewhere (DeGruttola et al., 2000; Ross et al., 2000; Conway et al., 2001).

HIV-1 is subdivided into three groups, M (major), O (outlying) and N (new or non-M, non-O), and the major HIV-1 pandemic has been caused by group M viruses. The viruses in group M are further classified into subtypes and circulating recombinant forms (CRFs), which are prevalent in specific geographical regions. While subtype B of HIV-1 is the predominant subtype in the Americas, Europe and Australia, there is a growing epidemic of non-B subtypes and CRFs in Africa and Asia (Hemelaar et al., 2006; McCutchan, 2006). CRF01_AE is one of the major HIV-1 subtypes that dominates the global epidemic, and is prevalent throughout Southeast Asia (Hemelaar et al., 2006; McCutchan, 2006). In particular, CRF01_AE is responsible for more than 95% of infections in Thailand, Cambodia and Viet Nam (Hemelaar et al., 2006).

Amino acid variations in HIV-1 RT and PR affect the drug susceptibility of viruses and/or viral fitness (Johnson et al., 2008; Shafer and Schapiro, 2008). The currently available ARV drugs were designed against subtype B virus, but are believed to retain their activity against most of the other subtypes and CRFs; however, limited data are presently available as to how viral diversity among different subtypes and CRFs affects drug susceptibility and resistance. In addition, the drug-resistance database is well established for subtype B, but not for non-B subtype viruses. Recently, our study (Auwanit et al., 2009), as well as others (Sukasem et al., 2007, 2008), showed that PI resistance-associated mutations are detected in CRF01_AE viruses derived from drug-naïve patients residing in Thailand. As further surveillance studies on drug resistance-associated mutations among CRF01_AE viruses circulating in Thailand, we collected peripheral blood samples from 39 HIV-1-infected patients every 3 months for a year, and the sustained appearance of drug resistance-associated mutations in CRF01_AE PR and RT was examined.

MATERIALS AND METHODS

Specimens
Seventeen drug-naïve, HIV-1-infected patients with CD4 counts of more than 250 cells/mm³ and 22 RTIs-treated patients with CD4 counts of less than 250 cells/mm³
were enrolled in this project. All patients were negative for hepatitis B and C viruses at the time of enrollment. Peripheral blood samples derived from these patients were subjected to this study after approval from the ethics committee of the Department of Medical Sciences, Ministry of Public Health of Thailand and with written informed consent from all patients.

**Measurement of CD4 count and viral load**

As clinical markers, the CD4 count and viral load of the patients were monitored during the study period. The CD4 count was measured by flow cytometric analysis at the Chiang Rai Prachanukoh Hospital, according to the manufacturer’s protocol (Beckman Coulter, Fullerton, California, USA). The viral load was measured as follows. Viral RNA was extracted from a plasma sample using the High Pure System Viral Nucleic Acid (Roche, Basel, Switzerland). The viral load then was measured using the Cobas AmpliPrep/Cobas TaqMan HIV-1 version 5.1 Assay (Roche).

**Amplification of HIV-1 genomic fragment encoding viral PR and RT**

Plasma was isolated from peripheral blood by centrifugation at 800g for 10 minutes. In addition, peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Buckinghamshire, UK). RNA and DNA were extracted from plasma and PBMC using the QIAamp viral RNA mini-kit and the QIAamp DNA blood mini-kit (Qiagen, Hilden, Germany), respectively. Viral RNA was reverse transcribed to cDNA using the SuperScript III First-Stand Synthesis kit (Invitrogen, Carlsbad, California, USA) with the reverse primer, K-env-R1, 5’-CCAATCAGG GAAGAAGCCTTG-3’ [corresponding to nucleotide (nt) 8736 to 8716 of CRF01_AE reference strain, CM240 (GenBank accession no. U54771)]. To amplify the HIV-1 genomic fragment encoding PR and RT, generated cDNA, as well as DNA extracted from PBMC, was serially diluted and then subjected to nested polymerase chain reaction (PCR) using BIO-X-ACT DNA polymerase (Bioline, Luckenwalde, Germany) and the following primers: PRRT-S-First, 5’-ACTGCAGTGAAGAAGCCTTG-3’ (nt 1622 to 1639) and PRRT-AS-First, 5’-CTACAGTGYACTTGTCCATG-3’ (nt 3973 to 3954) were used for the first PCR, and PRRT-S-nested, 5’-AGACCAG AGCCACAGCC-3’ (nt 1702 to 1719) and PRRT-AS-nested, 5’-ATCACTAGCCATT GTTCTCCAATTGC-3’ (nt 3878 to 3853) were used for the nested PCR. In order to examine the genomic fragment of the major viral population in the samples, PCR products amplified at the end-point dilution of cDNA or DNA templates were subjected to sequence analysis.

**Sequence analysis**

Sequence analysis of the HIV-1 genomic fragment encoding PR and RT was carried out using the BigDye Terminator v3.1 Cycle Sequencing kit with an ABI PRISM 3130XL genetic analyzer (Applied Biosystems, Foster City, California, USA), and data were assembled using SeqScape v2.5 software (Applied Biosystems). The deduced amino acid sequences were then aligned with the sequence of the subtype B reference strain, pNL4-3 (Adachi et al, 1986), using the ClustalW algorithm (Thompson et al, 1994) with slight manual adjustment. Subtype classifications of the newly cloned HIV-1 PR and RT genes were carried out using the Recombinant Identification Program: RIP 3.0 (www.hiv.lanl.gov).

**Nucleotide sequence accession numbers**

The nucleotide sequence of the viral gene encoding PR and RT has been deposited in the GenBank database under acces-
sion numbers GQ857285-GQ857437 and GQ857132-GQ857284, respectively.

RESULTS

Amplification and sequence analysis of HIV-1 genomic fragment encoding PR and RT derived from samples continuously collected from HIV-1-infected Thai patients

We collected peripheral blood samples every 3 months for a year from 17 drug-naïve and 22 RTIs-treated, HIV-1-infected patients. HIV-1 genomic fragments encoding PR and RT were amplified from plasma or PBMC samples derived from these patients by RT-PCR or PCR, respectively, and subjected to sequence analysis. We first tried to amplify the genomic fragment of plasma virus by RT-PCR. However, for some samples, we failed after multiple attempts and instead amplified proviral DNA from PBMC by PCR. The viral loads of RTIs-treated patients were quite low (< 70 RNA copies/ml) (Table 1); thus, we mainly analyzed proviral DNA for samples derived from these patients after failing to amplify the viral genome from plasma samples. We collected peripheral blood samples 5 times from April 2008 to April 2009, and successfully amplified viral genomic fragments at least 3 out of 5 times from samples derived from 35 patients, including 15 drug-naïve and 20 RTIs-treated patients. Therefore, we examined the deduced amino acid sequences of HIV-1 PR and RT derived from these samples in this study. The CD4 count and viral load of 35 patients are shown in Table 1. CD4 counts of all patients were not drastically altered during the study period. In addition, the viral loads of drug-naïve patients varied, whereas these RTIs-treated patients remained less than 70 copies per milliliter during the study period. The history of drug treatment for 20 RTIs-treated patients is also shown in Table 1.

Drug resistance-associated mutations detected in CRF01_AE PR and RT

The deduced amino acid sequences of HIV-1 PR were compared with that of the subtype B reference strain, pNL4-3. Drug resistance-associated mutations were identified according to the classification described previously (Johnson et al, 2008). We detected CRF01_AE genomic fragments from all samples studied (data not shown). In addition, I13V (amino acid substitution from isoleucine (I) to valine (V) at position 13), M36I and H69K, which are known to appear as a natural polymorphism in CRF01_AE PR (Nukoolkarn et al, 2004), were continuously detected in most samples (Table 2). Furthermore, other PI resistance-associated mutations were detected, as follows. L10I (continuously detected in samples derived from the patient, CR30), L10V (CR20), G16E (CR7, 19 and 20), K20I (CR7 and 38), K20R (CR27, 28, 29 and 32), I62V (CR14 and 24), L63P (CR15, 17, 19 and 25), I64V (CR11), V82I (CR5 and 17) and I93L (CR14, 19 and 25) were frequently detected in at least 3 of 5 continuously collected samples (Table 2). Next, we examined the appearance of background mutations in the samples, and found that E35D, R41K and L89M were detected in most samples (Table 3). In addition, K14R (CR20, CR28 and CR31), I15V (CR2, CR6 and CR22), G17E (CR38), K43R (CR7, CR17 and CR36), K45R (CR5 and CR19), R57K (CR18, CR27 and CR31), L63S (CR16), K70R (CR17, CR20, CR30 and CR39) and I72V (CR32) were continuously detected in several samples.

We next studied RTI resistance-associated mutations in CRF01_AE RT derived from drug-naïve and RTIs-treated patients. The results showed that NRTI resistance-associated mutations, M41I (sporadically
detected in samples derived from the patient, CR25, K65R (CR11), T69S (CR29), K70R (CR7), V75L (CR30), L210W (CR28), T215S (CR36) and N348I (CR26), as well as NNRTI resistance-associated mutations, G190E (CR39) and K238S (CR15), were only sporadically detected in samples derived from drug-naïve and RTIs-treated patients (Table 4). In contrast, NNRTI resistance-associated mutation V106I and V179D was continuously detected in samples derived from two drug-naïve patients, CR4 and CR11, respectively (Table 4).

Taken together, these results demonstrated that many drug resistance-associated mutations continuously appeared in CRF01_AE PR derived from drug-naïve as well as RTIs-treated patients (Table 2), whereas such mutations appeared rarely in CRF01_AE RT derived from these patients (Table 4). In addition, drug resistance-associated mutations did not continuously appear in CRF01_AE RT derived from RTIs-treated patients within the study period (Table 4).

Correlation between the continuous appearance of drug resistance-associated mutation and viral load of the patient

Finally, we examined the possible correlation between the continuous appear-
HIV-1 genomic fragment encoding CRF01_AE PR was amplified from plasma (highlighted with gray background) or PBMC samples (no background color) by RT-PCR or PCR respectively, and the nucleotide sequence of the PCR product was determined by cycle sequencing. Patient IDs are shown on the left side of the panel, while the dates of sample collection are shown on the top of the panel. Deduced amino acid sequence was compared with that of the subtype B reference strain, pNL4-3. Information regarding mutations associated with drug resistance was obtained from the literature (Johnson et al, 2008). Mutations other than I13V, M36I and H69K are shown in bold. Empty column represents a sample that failed to amplify the HIV-1 gene fragment.

Table 2
Appearance of drug resistance-associated mutations in CRF01_AE PR.

<table>
<thead>
<tr>
<th>Date</th>
<th>Drug naive patients</th>
<th>Drug RTI patients</th>
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<tr>
<td>April 2000</td>
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<td>July 2000</td>
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In this report, we examined the sustained appearance of drug resistance-associated mutations in HIV-1 CRF01_AE PR and RT, using peripheral blood samples continuously collected for a year from 15 drug-naïve and 20 RTIs-treated Thai patients. Drug resistance-associated mutations have been detected in HIV-1 PR derived from drug-naïve patients in many countries (Birk and Sonnerborg, 1998; Pieniazek et al, 2000; Vergne et al, 2000; Handema et al, 2003; Holguin et al, 2004;
Background mutations in CRF01_AE PR were examined as described in the Table legend for Table 2. Patient IDs are shown on the left side of the panel, while the dates of sample collection are shown on the top of the panel. Empty column represents a sample that failed to amplify the HIV-1 gene fragment.

Vergne et al, 2006; Bon et al, 2007; Sukasem et al, 2007, 2008; Auwanit et al, 2009); however, it was still unclear whether viruses in drug-naïve patients stably harbored such mutations in the absence of drug pressure. Our results showed that several drug resistance-associated mutations continuously appeared in CRF01_AE PR derived from PI-naïve Thai patients.

We detected not only mutations which appeared as a natural polymorphism in CRF01_AE viruses, I13V, M36I and H69K, but also several other mutations, L10I/V, G16E, K20I/R, I62V, L63P, I64V, V82I and I93L, in CRF01_AE PR. In addition, our results suggested that the appearance of a particular mutation was probably correlated with that of another drug resistance-associated or background mutation. It has been reported that the appearance of the mutation, V82I, is correlated with that of M46F and L63P in subtype A and
HIV-1 genomic fragment encoding CRF01_AE RT was amplified from plasma (highlighted with gray background) or PBMC samples (no background color) by RT-PCR or PCR respectively, and the nucleotide sequence of the PCR product was determined by cycle sequencing. Patient IDs are shown on the left side of the panels, while the dates of sample collection are shown on the top of the panels. Deduced amino acid sequence was compared with that of the subtype B reference strain, pNL4-3. Information regarding mutations associated with drug resistance to NRTI (left panel) and NNRTI (right panel) was obtained from the literature (Johnson et al., 2008). None denotes no detection of drug resistance-associated mutations, while empty column represents a sample that failed to amplify the HIV-1 gene fragment.

CRF01_AE (subtype E in the literature) PR (Lech et al., 1996). In our study, no mutation was detected at amino acid position 46 of CRF01_AE PR (data not shown); however, the samples derived from patient CR5 and 17 that continuously contained V82I harbored a background mutation K45R and K43R respectively (Table 3), sug-
gesting a correlation in the appearance of V82I with these mutations. In addition, the early samples contained L63P, and then V82I subsequently appeared in the late samples derived from CR17 (Table 2), suggesting a correlation in the appearance between V82I and L63P, as reported previously (Lech et al, 1996). Moreover, I93L appeared simultaneously with I62V or L63P in samples derived from CR14, 19 and 25 (Table 2), suggesting correlations in the appearance of these mutations. However, we could not statistically analyze these correlations because of the limited number of samples. Thus, further studies will be required to confirm our observations.

A previous report showed that PI resistance-associated major mutations were detected in proviral DNA, but not in genomic RNA of plasma viruses derived from drug-naïve patients (Bon et al, 2007). In contrast to the previous report, no drug resistance-associated major mutations were detected in CRF01_AE PR derived from either the viral RNA genome of plasma virus or proviral DNA in this study (Table 2). In other words, all mutations continuously detected in CRF01_AE PR were drug resistance-associated minor mutations, according to the criteria described previously (Johnson et al, 2008). Thus, these mutations might not play a major role in reducing viral drug susceptibility to PIs. However, we cannot reject the possibility that mutations present before drug treatment may play a role in worsening the prognosis of ART as a combination with the mutations eventually appeared after long-term drug treatment. Therefore, further surveillance studies are necessary to reveal the existence of drug resistance-associated mutations in drug-naïve, non-B subtype HIV-1-infected patients in Asian and African countries, as well as to establish a drug-resistance database, including the potential role of such mutations in the prognosis of ART for non-B subtype viruses.

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