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, reserve transcriptase, protease

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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 (ddI), 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/mm^3 and 22 RTIs-treated patients with CD4 counts of less than 250 cells/mm^3

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 800*g* 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'-ACTGCACTGAGAGA CAGG-3' (nt 1622 to 1639) and PRRT-AS-First, 5'-CTACAGTCYACTTGTCCATG-3' (nt 3973 to 3954) were used for the first PCR, and PRRT-S-nested, 5'-AGACCAG AGCCAACAGCC-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

Table 1
CD4 count and viral load of patients enrolled in the study.

CD4 count (cells /mm3)

	Apr 2008	Jul 2008	Oct 2008	Jan 2009	Apr 2009
Drug-naïve patients					
CR2	1,116	1,129	1,113	1,057	1,109
CR3	685	735	680	595	470
CR4	587	510	543	590	437
CR5	572	509	526	490	411
CR6	566	405	359	422	368
CR7	529	574	564	456	533
CR8	506	603	440	391	328
CR9	432	471	444	879	654
CR11	328	321	546	369	496
CR12	321	351	306	727	398
CR14	310	582	487	506	511
CR15	281	472	536	456	565
CR16	266	259	257	256	135
CR17	239	160	316	507	477
CR18	716	444	572	672	791
RTIs-treated patients					
CR19	544	388	611	686	606
CR20	619	497	638	810	546
CR21	513	393	441	622	426
CR22	472	514	480	736	750
CR23	471	340	370	651	633
CR24	459	395	668	354	237
CR25	403	404	380	432	813
CR26	396	317	546	415	575
CR27	397	420	470	549	669
CR28	379	391	560	411	522
CR29	364	300	450	595	327
CR30	363	371	480	171	352
CR31	297	449	391	399	283
CR32	293	335	245	523	362
CR35	282	557	379	680	555
CR36	259	279	396	293	437
CR37	253	175	238	282	254
CR38	251	243	324	332	321
CR39	225	197	332	259	271
CR40	213	257	277	444	286

Viral load (RNA copy /ml)

	Oct 2008	Apr 2009
Drug-naïve patients		
CR2	2,410	10,700
CR3	2,410	3,630
CR4	84,900	6,300
CR5	531,000	1,760,000
CR6	26,500	11,800
CR7	2,980	950
CR8	273,000	255,000
CR9	1,540	1,711
CR11	336,000	78,600
CR12	19,800	125,000
CR14	2,600	2,520
CR15	682	838
CR16	172,000	472,000
CR17	130	<40
CR18	55,800	41,600
RTIs-treated patients		
CR19	<47	<40
CR20	<47	<40
CR21	<47	<40
CR22	<47	<40
CR23	<47	<40
CR24	<47	<40
CR25	<47	<40
CR26	<47	<40
CR27	<47	<40
CR28	<47	<40
CR29	<47	<40
CR30	<47	<40
CR31	<47	<40
CR32	<47	61.4
CR35	<47	<40
CR36	<47	<40
CR37	<47	<40
CR38	<47	<40
CR39	<47	<40
CR40	<47	<40

Drug treatment history

CR19	GPOvir (2007 - Present)
CR20	GPOvir (2004 - 2007), GPOvir-Z (2007 - Present)
CR21	GPOvir (2000 - 2000), AZT +3TC +EFV (2000 - Present)
CR22	GPOvir (2002 - 2007), GPOvir-Z (2007 - Present)
CR23	GPOvir (2003 - Present)
CR24	GPOvir (2004 - Present)
CR25	GPOvir (2004 - 2007), GPOvir-Z (2007 - Present)
CR26	GPOvir (2007 - 2007), GPOvir-Z (2007 - Present)
CR27	GPOvir (2004 - 2006), GPOvir-Z (2006 - Present)
CR28	GPOvir (2007 - 2007), d4T +3TC +EFV (2007 - Present)
CR29	GPOvir (2007 - Present)
CR30	GPOvir (2006 - Present)
CR31	GPOvir (2004 - Present)
CR32	GPOvir (2007 - Present)
CR35	GPOvir (2002 - 2003), AZT +3TC +EFV (2003 - Present)
CR36	GPOvir (2003 - Present)
CR37	GPOvir (2001 - Present)
CR38	GPOvir (2007 - Present)
CR39	GPOvir (2004 - Present)
CR40	GPOvir (2003 - Present)

CD4 count (left panel) and viral load (middle panel) of drug-naïve and RTIs-treated patients were monitored during the study period. 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. In addition, the history of drug treatment for RTIs-treated patients is shown on the right panel. GPOvir contains d4T, 3TC and NVP, while GPOvir-Z contains AZT, 3TC and NVP.

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-

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

	April 2008	July 2008	October 2008	January 2009	April 2009
Drug-naïve patients					
CR02		I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K
CR03	I13V, M36I, H69K	I13V, M36I, H69K		I13V, M36I, H69K	
CR04	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, **D60E**, H69K
CR05	I13V, M36I, H69K, **V82I**	I13V, M36I, H69K, **V82I**	I13V, M36I, H69K, **V82I**	I13V, M36I, H69K, **V82I**	I13V, M36I, H69K, **V82I**
CR06	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K		I13V, M36I, H69K
CR07		I13V, **G16E**, **K20I**, M36I, H69K	I13V, **G16E**, **K20I**, M36I, H69K	I13V, **G16E**, **K20I**, M36I, H69K	
CR09	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K
CR10	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K
CR11	I13V, M36I, **R41K**, H69K	I13V, M36I, **R41K**, H69K	I13V, M36I, H69K	I13V, M36I, **R41K**, H69K	
CR12	I13V, M36I, H69K		I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K
CR14		M36I, **I62V**, **I93L**	M36I, **I62V**, **I93L**	M36I, **I62V**, **I93L**	M36I, **I62V**, **I93L**
CR15	I13V, M36I, **L63P**, H69K	I13V, M36I, **L63P**, H69K	I13V, M36I, H69K	I13V, M36I, **L63P**, H69K	I13V, M36I, **L63P**, H69K
CR16	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K
CR17	I13V, M36I, **L63P**, H69K	I13V, M36I, **L63P**, H69K	I13V, M36I, **L63P**, H69K, **V82I**	I13V, M36I, H69K, **V82I**	I13V, M36I, H69K, **V82I**
CR18	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K
RTIs-treated patients					
CR19	**G16E**, M36I, **L63P**, H69K, **I93L**		**G16E**, M36I, **L63P**, H69K, **I93L**	**G16E**, M36I, **L63P**, H69K	**G16E**, M36I, **L63P**, H69K, **I93L**
CR20	**L10V**, I13V, **G16E**, M36I, H69K	**L10V**, I13V, **G16E**, M36I, H69K	**L10V**, I13V, **G16E**, M36I, H69K	**L10V**, I13V, **G16E**, M36I, H69K	**L10V**, I13V, **G16E**, M36I, H69K
CR21	M36I, H69K	**L10I**, M36I, H69K		**L10V**, M36I, H69K	
CR22	I13V, M36I, H69K	I13V, **K20R**, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	
CR23	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K
CR24	I13V, M36I, **I62V**, H69K	I13V, M36I, **I62V**, H69K	I13V, M36I, **I62V**, H69K	I13V, M36I, **I62V**, H69K	I13V, M36I, **I62V**, H69K
CR25	M36I, **L63P**, H69K, **I93L**	M36I, **L63P**, H69K, **I93L**	M36I, **L63P**, H69K, **I93L**	M36I, **L63P**, H69K, **I93L**	
CR26	**L10I**, I13V, **K20R**, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K
CR27	I13V, **K20R**, M36I, H69K		I13V, **K20R**, M36I, H69K	I13V, **K20R**, M36I, H69K	I13V, **K20R**, M36I, H69K
CR28	I13V, M36I, H69K	**L10I**, I13V, **K20R**, M36I, H69K	**L10I**, I13V, **K20R**, M36I, H69K	**L10I**, I13V, **K20R**, M36I, H69K	
CR29	I13V, **K20R**, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	**L10I**, I13V, **K20R**, M36I, H69K	I13V, **G16E**, **K20R**, M36I, H69K
CR30	**L10I**, I13V, M36I, H69K	**L10I**, I13V, **G16E**, M36I, H69K	**L10I**, I13V, M36I, H69K	**L10I**, I13V, M36I, H69K,	
CR31	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K		I13V, M36I, H69K
CR32	**K20R**, M36I, H69K,	**K20R**, M36I, H69K,	**K20R**, M36I, H69K,	**K20R**, M36I, H69K	**K20R**, M36I, H69K,
CR33	I13V, M36I, H69K	M36I, **I62V**, H69K	**L10V**, I13V, M36I, H69K	M36I, H69K	I13V, M36I, H69K
CR36	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	**L10I**, I13V, **G16E**, M36I, H69K
CR37	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	**L10I**, I13V, M36I, H69K,	
CR38	I13V, **K20I**, M36I, H69K	I13V, **K20I**, M36I, H69K	I13V, **K20I**, M36I, H69K	I13V, **K20I**, M36I, H69K	
CR39			I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K
CR40	I13V, M36I, H69K, **ATIT**	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K	I13V, M36I, H69K

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.

ance of drug resistance-associated mutation and viral load of the patient. The results showed that PR mutations, L10V, G16E, K20I/R, I62V, L63P and I93L, continuously appeared in samples derived from patients with a low viral load (Tables 1 and 2), while PR mutation, I64V, and RT mutations, V106I and V179D, continuously appeared in samples from patients with a relatively high viral load (Tables 1 and 3). In contrast, the continuous appearance of the PR mutation, V82I, was not correlated with the viral load of the patient (Tables 1 and 2).

DISCUSSION

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;

Table 3
Appearance of background mutations in CRF01_AE PR.

	April 2008	July 2008	October 2008	January 2009	April 2009
Drug-naïve patients					
CR2		I15V, E35D, R41K, L89M	I15V, E35D, R41K, L89M	I15V, E35D, R41K, L89M	I15V, E35D, R41K, L89M
CR3	K14R, E35D, R41K, L89M	K14R, E35D, R41K, L89M		E35D, R41K, L89M	
CR4	E35D, R41K, L89M	E35D, R41K, L89M	E35D, R41K, L89M	E35D, R41K, L89M	E35D, R41K, R57K, L89M
CR5	E35D, R41K, K45R, L89M	E35D, R41K, K45R, L89M	E35D, R41K, K45R, L89M	E35D, R41K, K45R, L89M	E35D, R41K, K45R, L89M, L97I
CR6	I15V, E35D, R41K, L89M	I15V, E35D, R41K, L89M	I15V, E35D, R41K, L89M		I15V, E35D, R41K, L89M
CR7		E35D, R41K, K43R, R57K, L89M	E35D, R41K, K43R, R57K, L89M	E35D, R41K, K43R, R57K, L89M	
CR8	E35D, R57K, L89M	E35D, R57K, L89M	E35D, R57K, L89M	E35D, R57K, L89M	E35D, R57K, L89M
CR9	E35D, R41K, L89M	E35D, L89M	E35D, R41K, L89M	E35D, R41K, L89M	E35D, R41K, L89M
CR11	E35D, R41K	E35D, R41K	E35D, R41K, R57K, L89M	E35D, R41K, R57K	
CR12	E35D, R41K, R57K, L89M		E35D, R41K, R57K, L89M	E35D, R41K, R57K, L89M	G16K, E35D, R41K, R57K, L89M
CR14		E35D, R41K, R57K, H69R, L89M	E35D, R41K, H69R, L89M	E35D, R41K, H69R, L89M	E35D, R41K, H69R, L89M
CR15	E35D, R41K, L89M	E35D, R41K, L89M	E35D, G40R, R41K, L63S, L89M	E35D, R41K, L89M	E35D, R41K, L89M
CR16	R41K, L63S, L89M	R41K, L63S, L89M	R41K, L63S, L89M	R41K, L63S, L89M	R41K, L63S, L89M
CR17	R41K, K43R, K70R, L89I	R41K, K43R, K70R, L89I	R41K, K43R, K70R, I72V, L89I	R41K, K43R, K70R, L89I	Q7H, R41K, K43R, K70R, L89I
CR18	E35D, R41K, R57K, L89M	E35D, R41K, R57K, L89M	E35D, R41K, R57K, L89M	E35D, R41K, R57K, L89M	E35D, R41K, R57K, L89M
RTIs-treated patients					
CR19	E35D, R41K, K45R, L89M		E35D, R41K, K45R, L89M	E35D, R41K, K45R, L89M	E35D, R41K, K45R, L89M
CR20	K14R, E35D, R41K, K70R, L89M	K14R, R41K, K70R, L89M	K14R, E35D, R41K, K70R, I72V, L89M	K14R, E35D, R41K, K70R, I72V, L89M	K14R, E35D, R41K, K70R, L89M
CR21	E35D, R41K, L89M	I15V, E35D, R41K, L89M		T12P, E35D, R41K, L63T, L89M	
CR22	I15V, E35D, R41K, L89M	I15V, E35D, R41K, L89M	I15V, E35D, R41K, L89M	I15V, E35D, R41K, L89M	
CR23	L10M, E35D, R41K, L89M	E35D, R41K, L89M	E35D, R41K, L89M	L10M, E35D, R41K, L89M	E35D, R41K, L89M
CR24	E35D, R41K, L89M	E35D, R57K, L89M	E35D, R57K, L89M	E35D, R57K, L89M	E35D, R57K, L89M
CR25	E35D, R41K, K43R, L89M	E35D, R41K, L89M	E35D, R41K, L89M	G17E, E35D, R41K, K45R, G49E, E65K, L89M	
CR26	K14R, E35D, R41K, D67G, L89M	T12S, E35D, R41K, R57K, L89M	E35D, R41K, R57K, L89M	E35D, R41K, R57K, L89M	T12S, E35D, R41K, R57K, L89M
CR27	E35D, N37D, R41K, R57K, L89M		E35D, N37D, R41K, R57K, L89M	E35D, N37D, R41K, R57K, L89M	E35D, N37D, R41K, R57K, L89M
CR28	E35D, R41K, L89M	K14R, E35D, R41K, D67G, L89M	K14R, E35D, R41K, D67G, L89M	K14R, E35D, R41K, D67G, L89M	
CR29	E35D, R41K, L89M	E35D, R41K, L89M	I15V, G16A, E35D, R41K, L89M	K14R, E35D, R41K, D67G, L89M	E35D, R41K, L89M
CR30	E35D, R41K, L89M	E35D, R41K, K70R, L89M	E35D, R41K, K70R, L89M	E35D, R41K, K70R, L89M	
CR31	K14R, E35D, R41K, R57K, L89M	K14R, E35D, R41K, R57K, L89M	K14R, E35D, R41K, R57K, L89M		K14R, E35D, R41K, R57K, L89M
CR32	E35D, R41K, I72V, L89M	E35D, R41K, I72V, L89M	E35D, R41K, I72V, L89M	E35D, R41K, I72V, L89M	E35D, R41K, I72V, L89M
CR35	L10M, E35D, R41K, L89M	E35D, R41K, D67Y, L89M	E35D, R41K, L89M	E35D, R41K, K45R, L89M	L10M, E35D, R41K, L89M
CR36	E35D, R41K, R57K, L89M	E35D, R41K, K43R, L89M	I15V, L19V, E35D, R41K, K43R, L89M	I15V, E35D, R41K, K43R, L89M	L19I, E35D, R41K, K43R, K70R, L89M
CR37	E35D, R41K, L89M	K14R, E35D, R41K, L89M	I15V, E35D, R41K, L89M	E35D, R41K, L89M	
CR38	G17E, E35D, R41K, K70R, L89M	K14R, G17E, E35D, R41K, L89M	G17E, E35D, R41K, K70R, L89M	G17E, E35D, R41K, L89M	
CR39			E35D, R41K, K70R, L89M	G16R, E35D, R41K, G68R, K70R, L89M	E35D, R41K, K70R, L89M
CR40	E35D, R41K, L63A, L89M	E35D, R41K, L89M	E35D, R41K, L63A, L89M	E35D, R41K, L89M	E35D, R41K, L63A, L89M

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

Table 4
Appearance of drug resistance-associated mutations in CRF01_AE RT.

NRTI resistance-associated mutations					
	Apr 2008	Jul 2008	Oct 2008	Jan 2009	Apr 2009
Drug-naive patients					
CR2		None	None	None	None
CR3	None	None		None	
CR4	None	None	None	None	None
CR5	None	None	None	None	None
CR6	None	None	None		None
CR7		**K70R**	None	None	
CR8	None	None	None	None	None
CR9	None	None	None	None	None
CR11	None	**K65R**	None	None	
CR12	None		None	None	None
CR14		None	None	None	None
CR15	None	None	None	None	None
CR16	None	None	None	None	None
CR17	None	None	None	None	None
CR18	None	None	None	None	None
RTIs-treated patients					
CR19	None		None	None	None
CR20	None	None	None	None	None
CR21	None	None		None	
CR22	None	None	None	None	
CR23	None	None	None	None	None
CR24	None	None	None	None	None
CR25	None	None	None	**M41I**	
CR26	None	None	**N348I**	None	None
CR27	None		None	None	None
CR28	None	**L210W**	None	None	None
CR29	**T69S**	None	None	None	None
CR30	None	**V75L**	None	None	
CR31	None	None	None	None	None
CR32	None	None	None	None	None
CR35	None	None	None	None	None
CR36	None	None	None	None	**T215S**
CR37	None	None	None	None	
CR38	None	None	None	None	
CR39			None	None	None
CR40	None	None	None	None	None

NNRTI resistance-associated mutations					
	Apr 2008	Jul 2008	Oct 2008	Jan 2009	Apr 2009
Drug-naive patients					
CR2		None	None	None	None
CR3	None	None		None	
CR4	**V106I**	**V106I**	None	None	**V106I**
CR5	None	None	None	None	None
CR6	None	None	None		None
CR7		None	None	None	
CR8	None	None	None	None	None
CR9	None	None	None	None	None
CR11	**V179D**	**V179D**	**V179D**	**V179D**	
CR12	None		None	None	None
CR14		None	None	None	None
CR15	None	None	None	None	**K238S**
CR16	None	None	None	None	None
CR17	None	None	None	None	None
CR18	None	None	None	None	None
RTIs-treated patients					
CR19	None		None	None	None
CR20	None	None	None	None	None
CR21	None	None		None	
CR22	None	None	None	None	
CR23	None	None	None	None	None
CR24	None	None	None	None	None
CR25	None	None	None	None	
CR26	None	None	None	None	None
CR27	None		None	None	None
CR28	None	None	None	None	None
CR29	None	None	None	None	None
CR30	None	None	None	None	
CR31	None	None	None		None
CR32	None	None	None	None	None
CR35	None	None	None	None	None
CR36	None	None	None	None	None
CR37	None	None	None	None	
CR38	None	None	None	None	
CR39			None	**G190E**	None
CR40	None	None	None	None	None

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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REFERENCES

Adachi A, Gendelman HE, Koenig S, *et al.* Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J Virol* 1986; 59: 284-91.

Auwanit W, Isarangkura-Na-Ayuthaya P, Kasornpikul D, Ikuta K, Sawanpanyalert P, Kameoka M. Detection of drug resistance-associated and background mutations in human immunodeficiency virus type 1 CRF01_AE protease and reverse transcriptase derived from drug treatment-naive patients residing in central Thailand. *AIDS Res Hum Retroviruses* 2009; 25: 625-31.

Birk M, Sonnerborg A. Variations in HIV-1 pol gene associated with reduced sensitivity to antiretroviral drugs in treatment-naive patients. *AIDS* 1998; 12: 2369-75.

Bon I, Gibellini D, Borderi M, *et al.* Genotypic

resistance in plasma and peripheral blood lymphocytes in a group of naive HIV-1 patients. *J Clin Virol* 2007; 38: 313-20.

Conway B, Wainberg MA, Hall D, *et al.* Development of drug resistance in patients receiving combinations of zidovudine, didanosine and nevirapine. *AIDS* 2001; 15: 1269-74.

DeGruttola V, Dix L, D'Aquila R, *et al.* The relation between baseline HIV drug resistance and response to antiretroviral therapy: re-analysis of retrospective and prospective studies using a standardized data analysis plan. *Antivir Ther* 2000; 5: 41-8.

Gulick RM, Mellors JW, Havlir D, *et al.* Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy. *N Engl J Med* 1997; 337: 734-9.

Hammer SM, Squires KE, Hughes MD, *et al.* A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team. *N Engl J Med* 1997; 337: 725-33.

Handema R, Terunuma H, Kasolo F, *et al.* Prevalence of drug-resistance-associated mutations in antiretroviral drug-naive Zambians infected with subtype C HIV-1. *AIDS Res Hum Retroviruses* 2003; 19: 151-60.

Hemelaar J, Gouws E, Ghys PD, Osmanov S. Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. *AIDS* 2006; 20: W13-23.

Holguin A, Paxinos E, Hertogs K, Womac C, Soriano V. Impact of frequent natural polymorphisms at the protease gene on the in vitro susceptibility to protease inhibitors in HIV-1 non-B subtypes. *J Clin Virol* 2004; 31: 215-20.

Johnson VA, Brun-Vezinet F, Clotet B, *et al.* Update of the drug resistance mutations in HIV-1. *Top HIV Med* 2008; 16: 138-45.

Lech WJ, Wang G, Yang YL, *et al.* In vivo sequence diversity of the protease of human immunodeficiency virus type 1: presence of protease inhibitor-resistant variants in untreated subjects. *J Virol* 1996; 70: 2038-43.

McCutchan FE. Global epidemiology of HIV. *J Med Virol* 2006; 78 (suppl 1): S7-12.

Nukoolkarn S, Pongthapisith V, Panyim S, Leelamanit W. Sequence variability of the HIV type 1 protease gene in thai patients experienced with antiretroviral therapy. *AIDS Res Hum Retroviruses* 2004; 20: 1368-72.

Pieniazek D, Rayfield M, Hu DJ, *et al.* Protease sequences from HIV-1 group M subtypes A-H reveal distinct amino acid mutation patterns associated with protease resistance in protease inhibitor-naive individuals worldwide. HIV Variant Working Group. *AIDS* 2000; 14: 1489-95.

Ross L, Johnson M, DeMasi R, *et al.* Viral genetic heterogeneity in HIV-1-infected individuals is associated with increasing use of HAART and higher viremia. *AIDS* 2000; 14: 813-9.

Shafer RW, Schapiro JM. HIV-1 drug resistance mutations: an updated framework for the second decade of HAART. *AIDS Rev* 2008; 10: 67-84.

Sukasem C, Churdboonchart V, Sirisidthi K, *et al.* Genotypic resistance mutations in treatment-naive and treatment-experienced patients under widespread use of antiretroviral drugs in Thailand: implications for further epidemiologic surveillance. *Jpn J Infect Dis* 2007; 60: 284-9.

Sukasem C, Churdboonchart V, Sukeepaisarncharoen W, *et al.* Genotypic resistance profiles in antiretroviral-naive HIV-1 infections before and after initiation of first-line HAART: impact of polymorphism on resistance to therapy. *Int J Antimicrob Agents* 2008; 31: 277-81.

Sutthent R, Arworn D, Kaoriangudom S, *et al.* HIV-1 drug resistance in Thailand: before

and after National Access to Antiretroviral Program. *J Clin Virol* 2005; 34: 272-6.

Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994; 22: 4673-80.

Vergne L, Peeters M, Mpoudi-Ngole E, *et al.* Genetic diversity of protease and reverse transcriptase sequences in non-subtype-B human immunodeficiency virus type 1 strains: evidence of many minor drug resistance mutations in treatment-naive patients. *J Clin Microbiol* 2000; 38: 3919-25.

Vergne L, Stuyver L, Van Houtte M, Butel C, Delaporte E, Peeters M. Natural polymorphism in protease and reverse transcriptase genes and in vitro antiretroviral drug susceptibilities of non-B HIV-1 strains from treatment-naive patients. *J Clin Virol* 2006; 36: 43-9.