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Brief Article

Peptide HIV-1 Integrase Inhibitors from HIV-1 Gene Products

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Anti-HIV peptides with inhibitory activity against HIV-1 integrase (IN) have been found in overlapping peptide libraries derived from HIV-1 gene products. In a strand transfer assay using IN, inhibitory active peptides with certain sequential motifs related to Vpr- and Env-derived peptides were found. The addition of an octa-arginyl group to the inhibitory peptides caused a remarkable inhibition of the strand transfer and 3′-end-processing reactions catalyzed by IN and significant inhibition against HIV replication.

Introduction

Many antiretroviral drugs are currently available to treat human immunodeficiency virus type 1 (HIV-1) infection. Viral enzymes such as reverse transcriptase (RT[a]), protease and integrase (IN), gp41, and coreceptors are the main targets for antiretroviral drugs that are under development. Because of the emergence of viral strains with multidrug resistance (MDR), however, new anti-HIV-1 drugs operating with different inhibitory mechanisms are required. Following the success of raltegravir, IN has emerged as a prime target. IN is an essential enzyme for the stable infection of host cells because it catalyzes the insertion of viral DNA inside the preintegration complex (PIC) into the genome of host cells in two successive reactions, designated as strand transfer and 3′-end-processing. It is assumed that the enzymatic activities of IN have to be negatively regulated in the PIC during its transfer from the cytoplasm to the nucleus. Otherwise, premature activation of IN can lead to the autointegration into the viral DNA itself, resulting in an aborted infection. We speculate that the virus, rather than the host cells, must encode a mechanism to prevent autointegration. The PIC contains in association with the viral nucleic acid, viral proteins such as RT, IN, capsids ($p24^{CA}$ and $p7^{NC}$), matrix ($p17^{MA}$), p6 and Vpr, cellular proteins HMG I (Y), and the barrier to autointegration factor (BAF).[1−4] It is likely that, due to their spatial proximity in the PIC, these proteins physically and functionally interact with each other. For instance, it is already known that RT activity inhibited by Vpr,[5] and that RT and IN inhibit each other.[5−9] Vpr also inhibits IN through its C-terminal domain.[5,10] Because these studies suggest that PIC components regulate each other's function, we have attempted to obtain potent inhibitory lead compounds from a peptide fragment library derived from HIV-1 gene products, an approach which has been successful in finding a peptide IN inhibitor from LEDGF, a cellular IN binding protein.[11]

In this paper, we describe the screening of an overlapping peptide library derived from HIV-1 proteins, the identification of certain peptide motifs with inhibitory activity against HIV-1 IN, and the evaluation of effective inhibition of HIV-1 replication in cells using the identified peptide inhibitors possessing cell membrane permeability.

Results and Discussion

An overlapping peptide library spanning HIV-1 SF2 *Gag*, *Pol*, *Vpr*, *Tat*, *Rev*, *Vpu*, *Env*, and *Nef*, provided by Dr. Iwamoto of the Institute of Medical Science at the University of Tokyo (Supporting Information, SI, Figure 2A), was screened with a strand transfer assay[12] in search of peptide pools with inhibitory activity against HIV-1 IN. The library consists of 658 peptide fragments derived from the HIV-1 gene products. Each peptide is composed of 10−17 amino acid residues with overlapping regions of 1−7 amino acid residues. Sixteen peptide pools containing between 16 and 65 peptides were used for the first screening at the final concentration of 5.0 μM for each peptide (SI Figure 2B). This initial screening gave the results shown in Figure 1. Both Vpr and Env4 pools showed remarkable inhibition of IN strand transfer activity, and consequently a second screening was performed using the individual peptides contained in the Vpr and Env4 pools. A group of consecutive overlapping peptides in the Vpr pool (groups 13−15) and groups 4−6 and 20−21 in the Env4 pool were found to possess IN inhibitory activity (Figure 2). We focused on Vpr15 and Env4-4 peptides because they showed inhibitory activity against IN strand transfer reaction in a dose-dependent manner (Figure 3). The IC_{50} values of Vpr15

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[a]Abbreviations: HIV, human immunodeficiency virus; IN, integrase; RT, reverse transcriptase; MDR, multidrug resistance; PIC, preintegration complex; BAF, barrier to autointegration factor; R_8, octa-arginyl.

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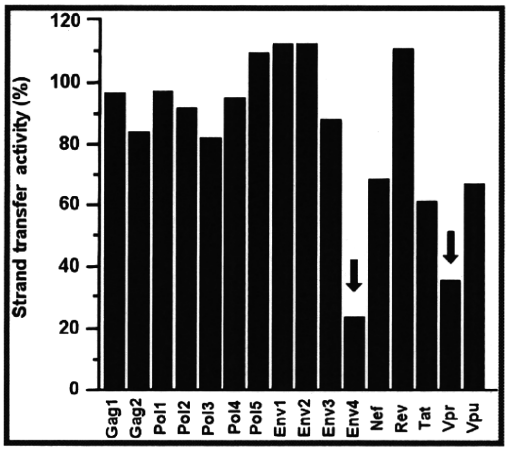

Figure 1. Inhibition of the IN strand transfer activity by peptide pools. Inhibition of the IN strand transfer activity was strongly inhibited by Env4 and Vpr pools (arrows). The *y*-axis represents the IN strand transfer activity relative to the solvent control (DMSO).

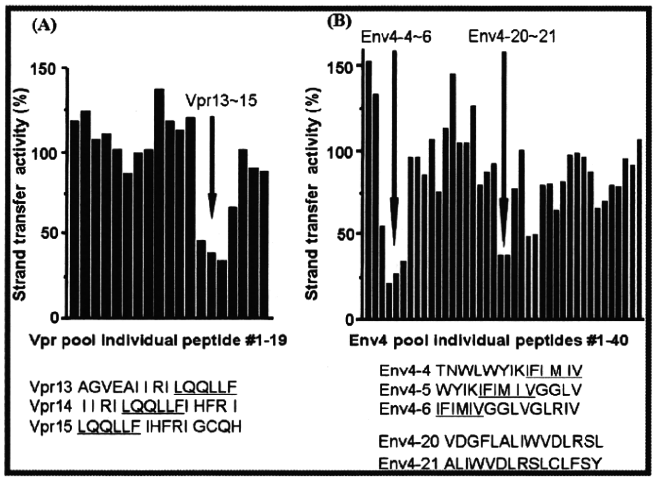

Figure 2. Identification of IN inhibitory peptides in the Vpr (A) and Env4 (B) pools based on the strand transfer activity of IN. The consecutive overlapping peptides display the inhibition of the strand transfer activity of IN (arrows). The *y*-axis represents the IN strand transfer activity relative to the solvent control (DMSO). The concentration of each peptide was 5 μM. The common sequences of individual peptides derived from Vpr and Env4 pools with anti-IN activity are underlined.

and Env4-4 were estimated at 5.5 and 1.9 μM, respectively. These peptides did not show any significant inhibitory activity against HIV-1 RT, suggesting that they might inhibit IN strand transfer reaction selectively.

The overlapping peptides of Vpr13-15 and Env4-4-6 have the vommon hexapeptide sequences LQQLLF and IFIMIV, respectively. The LQQLLF sequence covers positions 64−69 of Vpr, which is a part of the second helix of Vpr. The IFIMIV sequence corresponds to positions 684−689 of gp160, which is a part of the transmembrane domain of TM/gp41. These hexapeptides are thought to be critical to inhibition of IN activity. It was recently reported[5] that similar peptides derived from Vpr inhibit IN with IC_{50} values of 1−16 μM, which is consistent with our data. In this report,[5] the peptide motif was found to be 15 amino acid residues spanning LQQLLF from the overlapping Vpr peptide library. In our study, more precise mapping of inhibitory motif in Vpr peptides was achieved by identifying the shorter effective peptide motif. We focused on the Vpr-derived peptide, LQQLLF (Vpr-1) to develop potent inhibitory peptides. However, the expression of inhibitory activity against IN

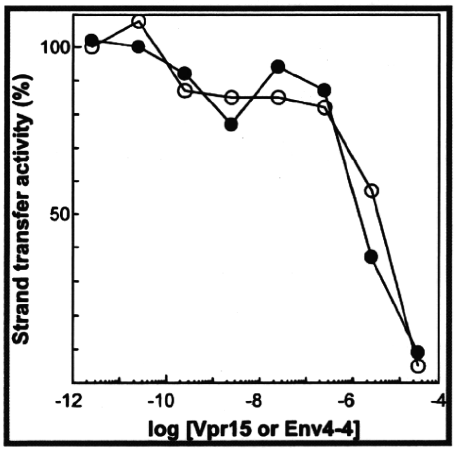

Figure 3. Concentration-dependent inhibition of IN strand transfer activities by Vpr15 (○) and Env4−4 (●) peptides. The *y*-axis represents the IN strand transfer activity relative to the solvent control (DMSO).

in vivo by only hexapeptides might be difficult because these hexapeptides penetrate the plasma membrane very poorly and to achieve antiviral activity, it is essential that they penetrate the cell membrane. To that effect, an octa-arginyl (R_8) group[13] was fused to the Vpr-derived peptides (Table 1). R_8 is a cell membrane permeable motif and its fusion with parent peptides successfully generates bioactive peptides without significant adverse effects or cytotoxicity.[14−18] In addition, the R_8-fusion could increase the solubility of Vpr-derived peptides which have a relatively hydrophobic character.

The inhibitory activity of Vpr-1 and Vpr-1-4 R_8 peptides against IN was evaluated based on the strand transfer and 3′-end-processing reactions in vitro (Table 1).[19,20] Vpr-1 did not show strong inhibition of either IN activity, but the IC_{50} of Vpr-1 R_8 toward the strand transfer reaction of IN was 10-fold lower than that of Vpr-1 lacking the R_8 group. This indicates that the positive charges derived from the R_8 group might enhance the inhibitory activity of the Vpr-1 peptide. Because we were concerned that the strong positive charges close to the LQQLLF motif might interfere with the inhibitory activity, the 6 amino acid sequence (−IHFRIG−) was inserted as a spacer between LQQLLF and R_8 (Vpr-3 R_8). The IHFRIG sequence was used to reconstitute the natural Vpr. The IC_{50} values of Vpr-2 R_8 for the strand transfer and 3′-end-processing activities of IN were 0.70 and 0.83 μM, respectively, while Vpr-3 R_8 showed potent IN inhibitory activities of 4.0 and 8.0 nM against the strand transfer and 3′-end- processing activities, respectively. This result indicates the additional importance of the IHFRIG sequence for inhibitory activities against IN. The increased IN inhibitory activities might be achieved presumably by the synergistic effect of the LQQLLF motif, the IHFRIG sequence, and the R_8 group. Vpr-4 R_8, in which the EAIIRI sequence was attached to further reconstitute the Vpr helix 2, showed inhibitory activities similar to those of Vpr-3 R_8, suggesting that reconstitution of helix 2 of Vpr is not necessary for efficient IN inhibition. Vpr-3 R_8 and Vpr-4 R_8, with $IC_{50} > 0.5\,\mu$M,[21] were less potent inhibitors of RT-associated RNase H activity, indicating that these peptides can selectively inhibit IN. These results suggest that Vpr-derived peptides are novel and distinct from any other IN inhibitors reported to date.

For rapid assessment of the antiviral effect of Vpr-derived peptides, we established an MT-4 Luc system in which MT-4 cells were stably transduced with the firefly luciferase expression cassette by a murine leukemia viral vector (SI Figure 3).

Table 1. Sequences of Vpr-Derived Peptides and Their IC_{50} Values toward the Strand Transfer and 3′-End Processing Reactions of IN

	sequence	IC_{50} (μM) strand transfer	IC_{50} (μM) 3′-end processing
Vpr-1	LQQLLF	68 ± 1.0	> 100
Vpr-1 R_8	Ac-LQQLLF -RRRRRRRR-NH_2	6.1 ± 1.1	> 11
Vpr-2 R_8	Ac-IHFRIG-RRRRRRRR-NH_2	0.70 ± 0.06	0.83 ± 0.07
Vpr-3 R_8	Ac-LQQLLF IHFRIG-RRRRRRRR-NH_2	0.004 ± 0.0001	0.008 ± 0.001
Vpr-4 R_8	Ac-EAIIRI LQQLLF IHFRIG-RRRRRRRR-NH_2	0.005 ± 0.002	0.006 ± 0.006

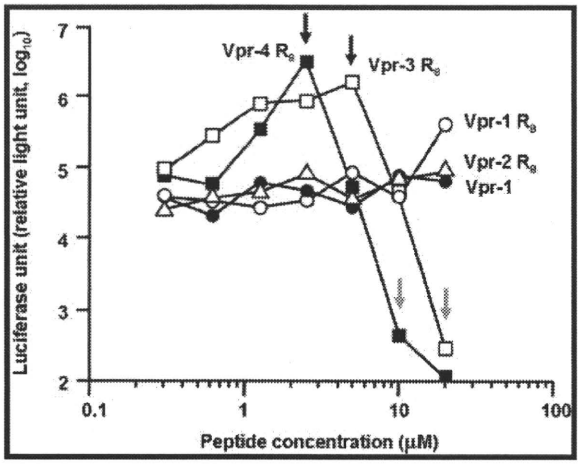

Figure 4. Luciferase signals in MT-4 Luc cells infected with HIV-1 in the presence of various concentrations of Vpr-derived peptides: Vpr-1 (●), Vpr-1 R_8 (○), Vpr-2 R_8 (△), Vpr-3 R_8 (□), Vpr-4 R_8 (■).

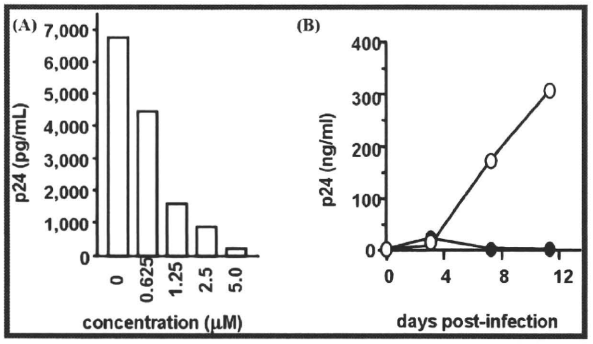

Figure 5. (A) The inhibition of HIV-1_{JR-CSF} replication in NP2-CD4-CCR5 cells in the presence of various concentrations of Vpr-3 R_8. (B) The replication kinetics of HIV-1_{HXB2} in MT-4 cells in the presence of Vpr-3 R_8 (●). The concentration of Vpr-3 R_8 was fixed at 0.5 μM. Absence of Vpr-3 R_8 (○).

MT-4 Luc cells constitutively express high levels of luciferase which are significantly reduced by HIV-1 infection due to their high susceptibility to cell death upon HIV-1 infection. Protection of MT-4 Luc cells from HIV-1-induced cell death maintains the luciferase signals at high levels. In addition, the cytotoxicity of Vpr-derived peptides can be evaluated by a decrease of luciferase signals in these MT-4 Luc systems. Vpr-2 R_8, which is a weak IN inhibitor, showed no significant anti-HIV-1 activity below concentrations of 20 μM, suggesting that its moderate IC_{50} level in vitro is not sufficient to suppress HIV-1 replication in tissue culture and that the R_8 group is not significantly cytotoxic (Figure 4). Vpr-1 did not show any inhibitory effects against HIV-1 replication; however, Vpr-1 R_8 displayed a weak antiviral effect at a concentration of 20 μM and both Vpr-3 R_8 and Vpr-4 R_8 showed significant inhibitory effects against HIV-1 replication. The R_8 peptide did not show significant anti-HIV activity (IC_{50} > 50 μM, data not shown). These results suggest that the addition of the R_8 group enables Vpr-derived peptides to enter the cytoplasm and access IN, with the result that HIV-1 replication could be effectively inhibited.

Because Vpr-3 R_8 was less cytotoxic than Vpr-4 R_8, the inhibitory activities of Vpr-3 R_8 were further investigated. Two replication assay systems, R5-tropic HIV-1_{JR-CSF} on NP2-CD4-CCR5 cells and X4-tropic HIV-1_{HXB2} on MT-4 cells, were utilized. NP2-CD4-CCR5 cells were infected with HIV-1_{JR-CSF} in the presence of various concentrations of Vpr-3 R_8. On day 4 postinfection, the culture supernatant was collected and the concentration of viral p24 antigen was measured by an ELISA assay. The p24 levels decreased in a dose-dependent manner with increasing the concentration of Vpr-3 R_8; 50% inhibition of p24 expression was obtained with approximately 0.8 μM of Vpr-3 R_8 (Figure 5A). This concentration was approximately 10-fold lower than the concentration of Vpr-3 R_8 known to be cytotoxic (Figure 4). Second, MT-4 cells were infected with HIV-1_{HXB2} and the replication kinetics was monitored in the presence of 0.5 μM Vpr-3 R_8. The degree of replication of HIV-1_{HXB2} was quite low in the presence of Vpr-3 R_8, while replication of HIV-1_{HXB2} was robust in the absence of Vpr-3 R_8 (Figure 5B), suggesting that Vpr-3 R_8 strongly suppresses the replication of HIV-1 in cells. To examine whether the HIV-1 replication was blocked through the inhibition of IN activity, quantitative real-time PCR was performed. If IN is inhibited, the efficiency of viral genome integration should be decreased while the reverse transcription of viral genome should not be affected. Accordingly, NP2-CD4-CXCR4 cells were infected with HIV-1_{HXB2} in the presence or absence of 0.5 μM Vpr-3 R_8. Genomic DNA was extracted on day 2 postinfection, and the viral DNA was quantified at the various steps of viral entry phase. The level of "strong stop DNA", representing the total genome of infected virus in Vpr-3 R_8-treated cells, was similar (139.7%) to that in DMSO-treated control cells and the level of viral DNA generated at the late stage of reverse transcription in Vpr-3 R_8-treated cells was slightly decreased (84.4%) compared to control cells. This small decline can probably be attributed to the weak anti-RNase H activity of Vpr-3 R_8. On the other hand, a drastic decrease of Alu-LTR products was observed in Vpr-3 R_8-treated cells (15.8%), indicating an inhibition of integrated viral genome. Concomitantly, the double LTR products, representing the end-joined viral genome catalyzed by host cellular enzymes, were increased by a factor of 8 (779.8%). These results strongly suggest that Vpr-3 R_8 blocks viral infection by inhibiting IN activity in cells, consistent with our in vitro observations. Judging by these results, Vpr-derived peptides with the R_8 group are potent IN inhibitors that suppress HIV-1 replication in vivo.

Finally, in silico molecular docking simulations of Vpr-derived peptides and HIV-1 IN were performed. The Vpr-derived peptides are located in the second helix of Vpr and were thus considered to have an α-helical conformation.[22] Docking simulations of three peptides (Vpr13, Vpr14, and Vpr15), using the predicted structure of the HIV-1 IN dimer as a template,[23] were performed by GOLD software to investigate the binding mode of the peptides, the binding affinity of

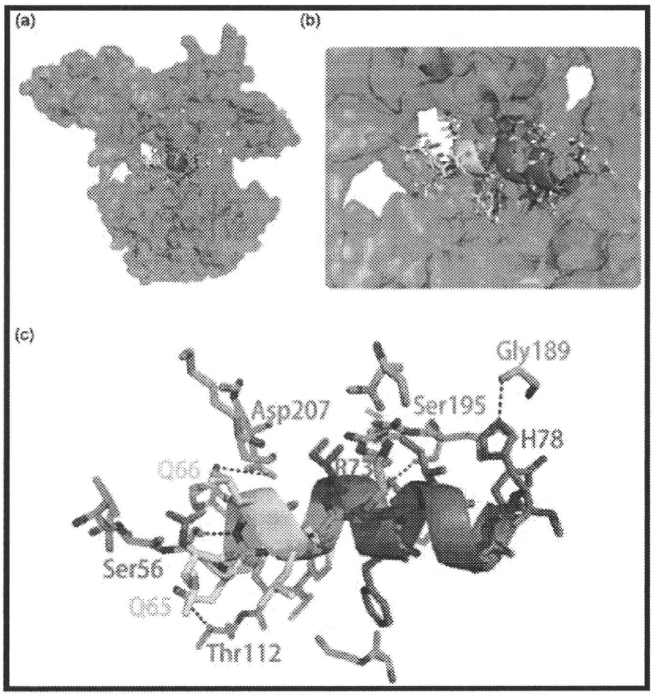

Figure 6. Predicted binding mode of Vpr15 to HIV-1 IN by GOLD. An overall view of (a) the complex obtained by docking Vpr15 with the HIV-1 IN dimer and (b) the closer view of the complex. The predicted structure of full-length HIV-1 IN was used as a template. Each HIV-1 IN monomer was shown as green or cyan surface. The docked Vpr15 is shown as a cartoon. The yellow-colored region is the LQQLLF motif. The GOLD score representing the docking complementarity is 69.83, indicating the high binding affinity between Vpr15 and IN. The hydrogen-bond interactions between HIV-1 IN and Vpr15 were presented by LIGPLOT software shown as blue dotted line (c).

the peptides being evaluated by GOLD Fitness score. The predicted binding mode of Vpr15 to IN is shown in Figure 6. Our results predict that the three Vpr-derived peptides interact with the cleft between the amino-terminal domain and the core domain of HIV-1 IN. This region is distinct from the nucleic acid interacting surfaces, indicating that the Vpr-derived peptides inhibit IN function in an allosteric manner. A previous report provided a model in which a Vpr peptide was bound to IN in a manner similar with our model[5] and, interestingly, the peptides were bound to IN with an exterior surface of Vpr. This earlier report that the full-length Vpr inhibits IN[10] strongly supports the predicted binding mode of Vpr15. Five hydrogen-bond interactions between HIV-1 IN and Vpr15 were identified by LIGPLOT analysis,[24] which invoked the following IN-Vpr amino acids: IN Thr112-Vpr Gln65, IN Ser56-Vpr Gln66, IN Asp207-Vpr Gln66, IN Ser195-Vpr Arg73, and IN Gly189-Vpr His78. The numbering of Vpr amino acids is based on the Vpr full-length coordinate, Figure 6. Additional hydrophobic contacts between IN and Vpr15 were found in which the following IN-Vpr amino acid pairs are involved: IN Lys211-Vpr Gln66, IN Pro109-Vpr Phe69, IN Arg262-Vpr His71, and IN Arg187-Vpr Gln77. These data indicate that the Gln65, Gln66, and Phe69 residues in Vpr-derived peptides play a major role in the interaction between IN and Vpr-derived peptides.

Conclusions

In summary, two peptide motifs, LQQLLF from Vpr and IFIMIV from Env4, possessing inhibitory activity against HIV-1 IN, were identified through the screening of overlapping peptide library derived from HIV-1 gene products. We initially speculate that HIV encodes a mechanism to prevent autointegration in the PIC because integration activity must be regulated until the virus infects cells. This speculation is supported by the finding that IN inhibitors exist in the viral PIC components. Vpr-derived peptides with the R_8 group showed remarkable inhibitory activities against the strand transfer and 3′-end-processing reactions catalyzed by HIV-1 IN in vitro. In addition, Vpr-3 R_8 and Vpr-4 R_8 were shown to inhibit HIV-1 replication with submicromolar IC_{50} values in cells using the MT-4 Luc cell system. In the quantitative analysis of p24 antigen, 50% inhibition of HIV-$1_{JR\text{-}CSF}$ replication was caused by approximately 0.8 μM of Vpr-3 R_8, and the replication of HIV-1_{HXB2} was extensively suppressed in the long term by Vpr-3 R_8 at 0.5 μM concentrations. Our finding suggest that these peptides could serve as lead compounds for novel IN inhibitors. Amino acid residues critical to the interaction of Vpr-derived peptides with IN were identified by our in silico molecular docking simulations, and suggests that more potent peptides[25] or peptidomimetic IN inhibitors represent a novel avenue for future small molecule inhibitors of IN and HIV integration.

Experimental Section

Peptide Synthesis. Vpr-derived peptides containing the R_8 group were synthesized by stepwise elongation techniques of Fmoc-protected amino acids on NovaSyn TGR resin. Coupling reactions were performed using 5.0 equiv of Fmoc-protected amino acid, 5.0 equiv of diisopropylcarbodiimide, and 5.0 equiv of 1-hydroxybenzotriazole monohydrate. Cleavage of peptides from resin and side chain deprotection were carried out with 10 mL of TFA in the presence of 0.25 mL of *m*-cresol, 0.75 mL of thioanisole, 0.75 mL of 1,2-ethanedithiol, and 0.1 mL of water as scavenger by stirring for 1.5 h. After filtration of the deprotected peptides, the filtrate was concentrated under reduced pressure, and crude peptides were precipitated in cooled diethylether. All crude peptides were purified by RP-HPLC and identified by MALDI-TOFMS. Purities of all final compounds were confirmed (>95% purity) by analytical HPLC. Detailed data are provided in SI.

Enzyme Assays. The strand transfer assay for the first screening was performed as described previously.[12] The IN strand transfer and 3′-end-processing assays for peptide motif characterizations were performed as described previously.[19,20] RNase H activity was measured as described by Beutler et al.[21]

Replication Assays. For HIV-1 replication assays, 1×10^5 cells were incubated at room temperature for 30 min with an HIV-1 containing culture supernatant (ca. 0.2−50 ng p24) and then washed and incubated. Culture supernatants were collected at different time points, and then the cells were passaged if necessary. Levels of p24 antigen were measured using a Retro TEK p24 antigen ELISA kit, according to the manufacture's protocol. Signals were detected using an ELx808 microplate photometer.

For MT-4 Luc assays, MT-4 Luc cells (1×10^3 cells) grown in 96-well plates were infected with HIV-1_{XHB2} (ca. 0.2−10 ng p24) in the presence of varying concentrations of Vpr-3 R_8. At 6−7 d postinfection, cells were lysed and luciferase activity was measured using the Steady-Glo assay kits according to the manufacture's protocol. Chemiluminescence was detected with a Veritas luminometer.

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Supporting Information Available: Additional experimental procedures including MS data and figures; HPLC charts of final compounds, explanation for HIV-1 genes and the peptide pools, and illustration of MT-4 Luc system. This material is available free of charge via the Internet at http://pubs.acs.org.

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HIV-2 CRF01_AB: First Circulating Recombinant Form of HIV-2

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Background: Five HIV-2–seropositive cases were recently identified in Japan, outside the HIV-2 endemic area of West Africa. To clarify the molecular epidemiology of HIV-2 in Japan, we analyzed sequences of these cases in detail.

Methods: HIV-2 genetic groups were determined by *gag* and *env* sequences. For suspected recombinant isolates, the genetic structure was determined by full-length genomic analyses. To understand the history and evolution of HIV-2 recombinant isolates, we estimated the time of most recent common ancestor by Bayesian Markov chain Monte Carlo method.

Results: Three isolates were determined as recombinants of groups A and B, and their mosaic genome structures were identical with that of 7312A, a recombinant isolate reported in 1990 from Côte d'Ivoire. Our 3 isolates and 7312A fulfilled the criteria for determining a circulating recombinant form (CRF). These isolates were verified by the Los Alamos HIV sequence database as the first CRF of HIV-2, HIV-2 CRF01_AB. The mean time of most recent common ancestor of CRF01_AB was estimated as between 1964 and 1973, several decades after the estimated emergence of HIV-2.

Conclusions: We recently identified HIV-2 CRF01_AB cases in Japan. This ectopic observation of the virus outside its original endemic area suggests an ongoing global spread of HIV-2 CRF01_AB.

Key Words: circulating recombinant form, CRF01_AB, HIV-2, molecular epidemiology

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INTRODUCTION

One million people worldwide are infected with HIV-2. The distribution of HIV-2, unlike the global epidemic of HIV-1, is still mainly restricted to West Africa and several European and Asian countries.[1–4] HIV-2 has been characterized as less pathogenic than HIV-1,[5–11] with more than 75% of HIV-2–infected cases remaining asymptomatic throughout their clinical course.[4] HIV-2 can be genetically classified into 8 groups, A to H, which have equivalent genetic distances to those of HIV-1 groups but not subtypes, with groups A and B circulating in the human population.[12–16] In addition, 2 different AB recombinants (7312A and 510-03) have been identified in West Africa,[12,13,17–19] but their circulation has not been identified to date.

In Japan, only 2 HIV-2–infected cases have been reported, but both were infected abroad.[20,21] Inside the country, there has been no evidence of HIV-2 transmission and circulation. Here we report 5 HIV-2–infected cases recently identified in Japan. Of these 5 cases, 3 were shown by full-length genomic analysis to be infected with the same type of recombinant virus determined to be the first circulating recombinant form (CRF) of HIV-2.

METHODS

HIV-2 Samples and Quantification of HIV Plasma Viral Loads

Among 843 HIV/AIDS cases registered at the Nagoya Medical Center (NMC), Japan from 1994 to 2008 (for demographic characteristics, see **Table, Supplemental Digital Content 1**, http://links.lww.com/QAI/A49), 5 cases (3 males and 2 females) were diagnosed serologically as HIV-2 infected. To better understand the molecular epidemiology of HIV-2 infection in Japan, we analyzed the HIV-2 genetic groups of the 5 cases.

Plasma HIV-1 viral loads were measured by the Cobas Amplicor HIV-1 monitor test v1.5 (Roche Diagnostics, Tokyo, Japan) or the Cobas TaqMan HIV-1 test (Roche Diagnostics),

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whereas plasma HIV-2 viral loads were measured by an in-house quantification assay, the Poisson quantification method described elsewhere.[22,23] In brief, total RNA was extracted from 500 μL of plasma sample using the QIAamp UltraSens Virus Kit (QIAGEN, Tokyo, Japan). Reverse transcription (RT) and nested polymerase chain reaction (PCR) (RT-nested PCR) were performed using serially diluted RNA samples, and HIV-2 viral loads were statistically calculated using results from samples diluted to near the endpoint. (For details of RT-nested PCR reaction mixtures and thermal programs, see **Table, Supplemental Digital Content 2**, http://links.lww.com/QAI/A50).

Genomic DNA Sequencing

HIV-2 proviral DNAs were purified from peripheral blood mononuclear cells using the DNA blood mini kit (QIAGEN). To determine HIV-2 genetic groups, *gag* (777 bps: 1163 to 1939 according to SIVmac239) and *env* (454 bps: 7300 to 7753) gene fragments were amplified by nested PCR using LA Taq polymerase (Takara Bio, Shiga, Japan) and previously reported[13,24] primers: gagA, gagB, gagC, and gagF for *gag*, and PFD1, LTR9574, EB2, and EB5 for *env*. To determine full-length genomic sequences, 4 DNA fragments containing (1) 5′ long terminal repeat (LTR) (915 bps: 31 to 945), (2) *gag* to *nef* genes (9122 bps: 899 to 10020), (3) 3′ LTR (791 bps: 9463 to 10252), and (4) the joining point of the circular 2 LTR form (597 bps: 10085 to 10279 and 1 to 402) were amplified by nested PCR using 8 primer pairs (see **Table, Supplemental Digital Content 3**, http://links.lww.com/QAI/A51). The following PCR program was used: denaturation (2 minutes at 94°C) followed by 40 cycles of PCR (94°C: 15 seconds, 60°C: 30 seconds, and 70°C: 1 minute/1000 bps). Sequencing was performed using a 3730 DNA Analyzer (Applied Biosystems, Tokyo, Japan).

Phylogenetic Tree Analysis and Determination of Recombinant Genome Structures

Multiple sequence alignment was performed using CLUSTAL W, and genetic distances were calculated based on the maximum composite likelihood model using MEGA software v4.[25] Phylogenetic trees were constructed using the neighbor-joining method.

Complete full-length genomic sequences of 4 HIV-2 group A strains (ALI, BEN, CAM2CG, and UC2), 3 HIV-2 group B strains (D205, EHO, and UC1), and SIVmac239, (a rhesus macaque-adapted simian immunodeficiency viral isolate) were used as reference sequences. After realigning the sequence set, recombinant breakpoints were determined by similarity plotting, bootscanning, and informative site analysis using SimPlot software, v3.5.1.[26]

Estimated Times of the Most Recent Common Ancestors

Evolutionary rates, chronological phylogenies, and other evolutionary parameters were estimated from 17 full-length or near full-length HIV-2/SIV genomic sequences (see **Table, Supplemental Digital Content 4**, http://links.lww.com/QAI/A52) using the Bayesian Markov chain Monte Carlo (MCMC) method implemented in BEAST v1.4.8.[27] The alignment data for the full-genome sequences were processed into 2 subsets consisting of sequences corresponding to the group A or B region of HIV-2 AB-recombinant virus. Bayesian MCMC analyses were performed using a relaxed molecular clock model.[28] The nucleotide substitution model was evaluated by the hierarchical likelihood ratio test using PAUP v4.0 beta[29] with MrModeltest (Nylander JAA. 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University), and the general time-reversible model[30] was adopted with both invariant sites and gamma-distributed site heterogeneity for 4 rate categories. The coalescent model used in the analyses was a logistically growing population because the population size of HIV-2 seemed constant in the early phase followed by exponential growth in the recent period.[31] Each Bayesian MCMC analysis was run for 40 million states and sampled every 10,000 states. Posterior probabilities were calculated with a burn-in of 4 million states and checked for convergence using Tracer v1.4. The posterior distribution of the substitution rate obtained from the heterochronous sequences was subsequently incorporated as a prior distribution for the evolutionary rate of HIV-2 genome regions A and B, thereby adding a timescale to the phylogenetic histories of the HIV-2 strains and enabling the times of most recent common ancestor (tMRCAs) to be estimated.[32]

Accession Numbers

Nucleotide sequences have been registered as #AB499685 to AB499695 in the DNA databank of Japan.

TABLE 1. Demographic and Clinical Characteristics of Patients Diagnosed as HIV-2 Infected

Patient #	Year	Sex	Age (Yrs)	Nationality	Risk Factor for Infection	$CD4^+$ Cell Count (Cells/μL)	HIV-1 Viral Load (Copies/mL)*	HIV-2 Viral Load (Copies/mL)	Western Blot†		Opportunistic Infections
									HIV-1	HIV-2	
NMC307	2004	M	28	Nigerian	Hetero	241	<50	350,000	I	P	Tuberculosis
NMC678	2007	F	28	Japanese	Hetero	883	<50	ND	I	P	—
NMC716	2007	M	36	Nigerian	Hetero	4	<50	680,000	I	P	Candidiasis
NMC786	2008	M	38	Ghanaian	Hetero	1	<40	60,000	N	I	Candidiasis, CMV infection
NMC842	2008	F	34	Japanese	Hetero	110	<40	25,000	N	P	—

*Detection limits of Cobas Amplicor HIV-1 monitor v1.5 and Cobas TaqMan HIV-1 tests were 50 and 40 copies/ml, respectively.
†New LAV Blot I and II kits (Bio-Rad Laboratories, Tokyo, Japan) were used.
CMV, cytomegalovirus; F, female; Hetero, heterosexual contact; I, intermediate; M, male; ND, not detected; N, negative; P, positive.

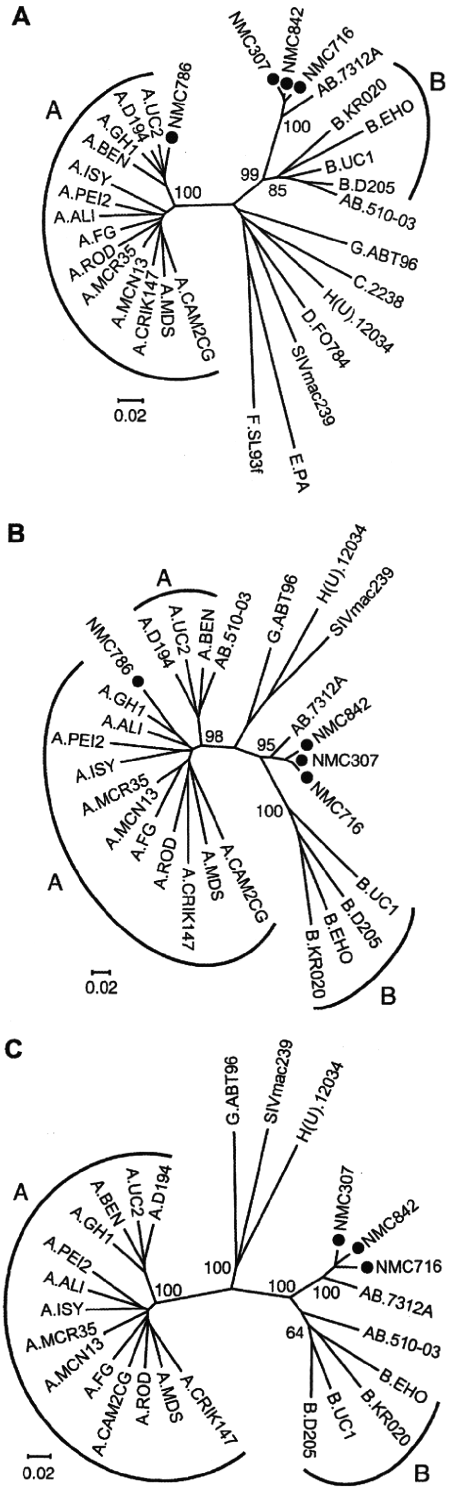

FIGURE 1. Phylogenetic tree analyses of HIV-2 isolates identified in this study. Phylogenetic tree analyses are shown using the following: A, HIV-2 *gag* gene sequences (bps: 1163 to 1939 in the reference SIVmac239 sequence); B, *env* gene sequences (bps: 7300 to 7753); and C, full-length or near full-length genomic sequences. Phylogenetic trees were constructed by the neighbor-joining method. Bootstrap values were calculated by 1000 analyses and are shown at the major tree nodes. Scale bar represents 0.02 nucleotide substitutions per site. Each reference HIV-2 strain is represented by its genetic group and name. HIV-2 isolates identified in this study (NMC307, NMC716, NMC786, and NMC842) are shown by filled circles.

RESULTS

HIV-2 Infection Confirmed by Nucleotide Amplification in Four AIDS Cases

Profiles of 5 HIV-2–seropositive cases are summarized in Table 1. The 3 males were from West African countries, a major endemic area for HIV-2, and suspected as seropositive before arriving in Japan. However, 2 females, both Japanese, were suspected to be recently infected within Japan based on their interviews. All their risk factors were heterosexual contacts, and no personal connection was confirmed among any of these cases. Thus, these 5 cases were independently infected with HIV-2 on different occasions. Notably, 4 cases (NMC307, NMC716, NMC786, and NMC842) were found at advanced stage AIDS with low $CD4^+$ cell counts and high HIV-2 viral loads, accompanied by opportunistic infections (Table 1). One case (NMC678) was found at an asymptomatic stage with high $CD4^+$ cell count and undetectable viremia. HIV-1 RNAs were undetectable in all 5 cases, indicating that they were infected by HIV-2 alone.

The First Circulating Recombinant Form Discovered in HIV-2: HIV-2 CRF01_AB

HIV-2 genetic groups were determined by both *gag* and *env* sequences. We were successful in analyzing 4 AIDS cases, however, we failed to amplify these 2 genes and analyze in asymptomatic case NMC678. One isolate (NMC786) was clearly classified into group A in phylogenetic tree analysis (Fig. 1A, B). On the other hand, isolates NMC307, NMC716, and NMC842 formed an independent cluster with a reference AB recombinant isolate 7312A (Fig. 1A, B). To better understand the detailed genomic structures of the 3 suspected AB recombinants, full-length genomic sequences of the 3 cases were analyzed. In the phylogenetic tree with full-length or near full-length reference sequences (Fig. 1C), NMC307, NMC716, NMC842, and 7312A formed an independent cluster with a high bootstrap value of 100%, suggesting these 4 isolates are the same type of AB-recombinant virus.

We next compared their genomic structures. As shown in Fig. 2A, similarity plotting and bootscanning analyses revealed that the recombinant breakpoints of our 3 isolates perfectly matched those of 7312A. This finding was supported by subregion phylogenetic analyses (Fig. 2B). In conclusion, NMC307, NMC716, and NMC842 are AB-recombinant forms with a mosaic genome structure identical to that of 7312A, demonstrating that they are the same type of HIV-2 AB-recombinant form.

The minimum requirement for declaring a new CRF, as proposed by the Los Alamos HIV sequence database in 1999, is at least 3 cases with no direct linkage, accompanied with near full-length sequences.[33,34] These CRF nomenclature

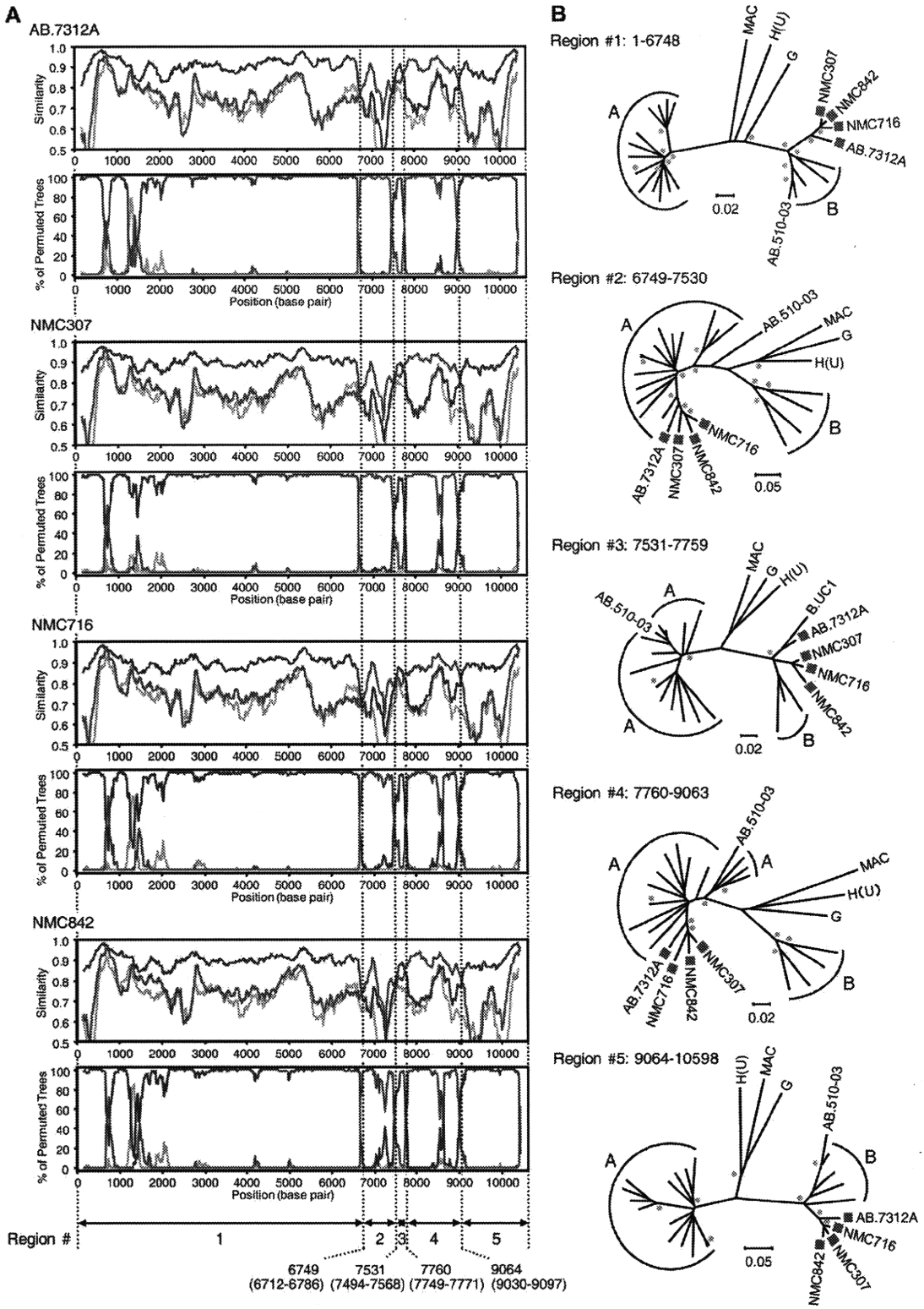

FIGURE 2. Determination of mosaic genome structures of HIV-2 AB recombinants. A, Similarity plotting (top) and bootscanning (bottom) data for each case of AB.7312A, NMC307, NMC716, and NMC842. Plots for consensus group A, consensus group B, and SIVmac239 are shown in red, blue, and gray, respectively. Both similarity plotting and bootscanning were performed with window and step sizes of 300 and 20 nucleotides, respectively. Bootscanning was performed using the neighbor-joining algorithm with 500 replicates. Each position of the 4 recombinant breakpoints is represented in the aligned sequence data set as the midpoint and

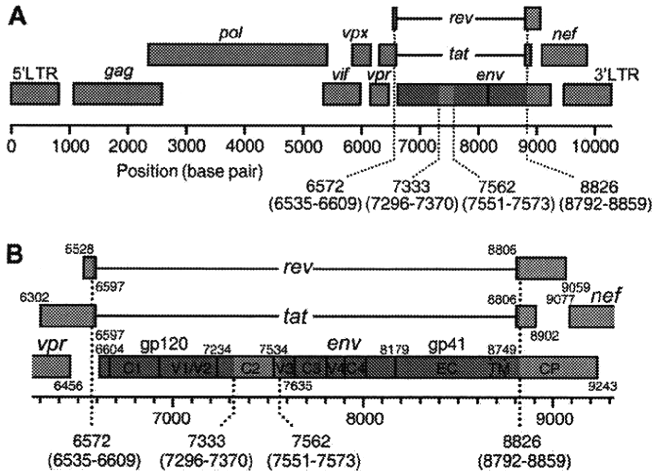

FIGURE 3. Schematic drawings for the genomic structure of HIV-2 CRF01_AB. A, Whole genomic structure; and B, Details around the *env* gene are represented. Regions belonging to group A and B are shown in red and blue, respectively. Numbering positions were adjusted to the reference SIVmac239 sequence.[35,36] Each position of 4 recombinant breakpoints is represented as the midpoint and range. C, constant region; CP, cytoplasmic domain; EC, extracellular domain; gp, glycoprotein; TM, transmembrane domain; V, variable region.

requirements are perfectly fulfilled with full-length genomic sequence information for 4 cases independently infected on different occasions with the AB recombinant identified by us and others.[12,13,19] Our data were carefully reviewed by editors of the Los Alamos HIV sequence database and confirmed as the first CRF discovered in HIV-2. They decided that the least confusing and most consistent way to name this new strain was to call it HIV-2 CRF01_AB.

The genomic structure of CRF01_AB is shown in Fig. 3. Interestingly, all 4 recombinant breakpoints of the CRF were located near or within the *env* gene (Fig. 3A). Further detailed analysis revealed that CRF01_AB possessed a chimeric gp120 containing a backbone of group A and a partial C2V3 fragment of group B and a chimeric gp41 containing extracellular and transmembrane domains of group A and a cytoplasmic domain of group B (Fig. 3B).

CRF01_AB Emerged Approximately in the Mid 20th Century

To estimate the time of CRF01_AB emergence, the time of the most recent common ancestor (tMRCA) of the recombinant was calculated by the Bayesian MCMC method. The mean substitution rates per year for the group A and B regions were estimated as 2.22×10^{-3} and 1.64×10^{-3}, respectively (Table 2), and the mean tMRCAs for groups A and B were estimated from 1921 to 1929, and from 1909 to 1948, respectively (Table 3). Similar results[31,37] validate our estimations. Finally, the mean tMRCA of CRF01_AB was estimated from 1964 to 1973. As the emergent times for groups A and B were estimated in the early 20th century, several decades seem to have been required for CRF01_AB to emerge. Concerning the geographical origin of the recombinant form, 3 of 4 isolates (7312A, NMC307, and NMC716) were identified in West Africans from Côte d'Ivoire and Nigeria. As these 2 countries were reported as sites of an epidemic in HIV-2 group A and B strains,[38,39] the most likely geographical origin of CRF01_AB is the south coastal area of West Africa.

DISCUSSION

In this study, we identified 3 HIV-2 AB recombinants with the same recombination pattern as 7312A, an isolate reported in Côte d'Ivoire in 1990.[12,13,19] These 4 isolates are determined as the first CRF of HIV-2, named CRF01_AB. It is noteworthy that all 3 of our cases infected with CRF01_AB were found at the AIDS stage. Considering that more than 75% of HIV-2–infected cases have a prognosis of remaining asymptomatic throughout their lifetimes[4] and that few HIV-2–seropositive cases were reported in Japan in the last 2 decades, 3 HIV-2 cases in the AIDS stage infected with the same CRF and identified in the past 5 years is highly unusual. Regarding the incubation periods for AIDS development in the 3 cases, not much information was available except for NMC842. This case was found to be seronegative for HIV-1/2 when tested in 2000. Thus, this case seems to have developed AIDS at most within 8 years, same as the median incubation period for AIDS development in HIV-1 infections (7.7–12.3 years).[40–45] As for the other 2 cases (NMC307 and NMC716), they developed AIDS at 28 and 36 years old (Table 1), which is significantly younger than age 65, reported as the peak of death by HIV-2 infections.[46,47] Though the number of cases identified is still small, we are concerned that the CRF01_AB might have acquired higher pathogenicity through recombination and adaptation to humans. As shown in Figure 3B, CRF01_AB has a recombination in the C2V3 region, the site of the major determinant for anti-envelope host immune responses and a functional domain for the chemokine receptor–binding site. The chimeric structure in the C2V3 region may confer advantages in host immune escape and viral replication capacity.

According to tMRCA analysis of the 4 isolates, CRF01_AB is estimated to have emerged sometime between 1964 and 1973. Interestingly, the mean tMRCA of the 3 isolates collected at NMC was estimated from 1982 to 1995 (Table 3), a later estimate than that of the 4 isolates, suggesting ongoing selection and evolution of CRF01_AB through transmission which has been taking place from the era of the 7312A isolate to the NMC isolates.

In conclusion, we report here the first CRF of HIV-2, CRF01_AB. Although national borders worldwide have

range (bottom). B, Subregion phylogenetic tree analyses. Phylogenetic trees were individually constructed by the neighbor-joining method using 5 subregion sequences. The HIV-2 isolates identified in this study (NMC307, NMC716, and NMC842) and AB.7312A are shown by green filled squares. Bootstrap values were calculated from 1000 analyses, and values greater than 95% are shown as orange dots at tree nodes. Scale bar represents 0.02 or 0.05 nucleotide substitutions per site. MAC, SIVmac239.

TABLE 2. Parameters in Bayesian MCMC Analysis for HIV-2/SIV Phylogenetic Inferences

	Substitution Rate Per Year		Coefficient of Variation		Population Size	
Data Set	Mean	95% HPD	Mean	95% HPD	Mean	95% HPD
Group A Region	2.22×10^{-3}	6.86×10^{-4}–3.68×10^{-3}	0.173	0.076–0.293	405.2	98.3–830.2
Group B Region	1.64×10^{-3}	5.99×10^{-4}–2.87×10^{-3}	0.269	0.170–0.395	341.2	93.3–668.9
Combined*	1.87×10^{-3}	6.39×10^{-4}–3.32×10^{-3}	0.235	0.088–0.382	357.9	93.3–709.2

*Combined data were produced from the 2 subsets, "group A region" and "group B region," using a LogCombiner program.
HPD, highest posterior density.

TABLE 3. Estimated TMRCAs of Monophyletic Clades in the HIV-2/SIV Lineage

	Group A region		Group B region		Combined	
Data set	Mean	95% HPD	Mean	95% HPD	Mean	95% HPD
Clade						
NMC isolates*	1982	1960–1996	1995	1987–2002	1990	1974–2002
CRF01_AB†	1964	1933–1985	1973	1956–1986	1971	1949–1986
Group A	1921	1864–1963	1929	1882–1964	1927	1879–1964
Group B	1909	1837–1962	1948	1915–1973	1934	1879–1973
HIV-2/SIV	1818	1670–1923	1821	1697–1930	1822	1693–1926

*This clade consisted of our 3 CRF01_AB isolates: NMC307, NMC716, and NMC842.
†This clade consisted of all 4 CRF01_AB isolates: 7312A, NMC307, NMC716, and NMC842.
HPD, highest posterior density;
SIV, simian immunodeficiency virus.

become more porous than ever, it is still surprising that the same recombinant strain was harvested in Japan, an island nation remote from the original endemic area, West Africa. This ectopic observation of the virus outside its endemic area suggests an ongoing global spread of HIV-2 CRF01_AB.

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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

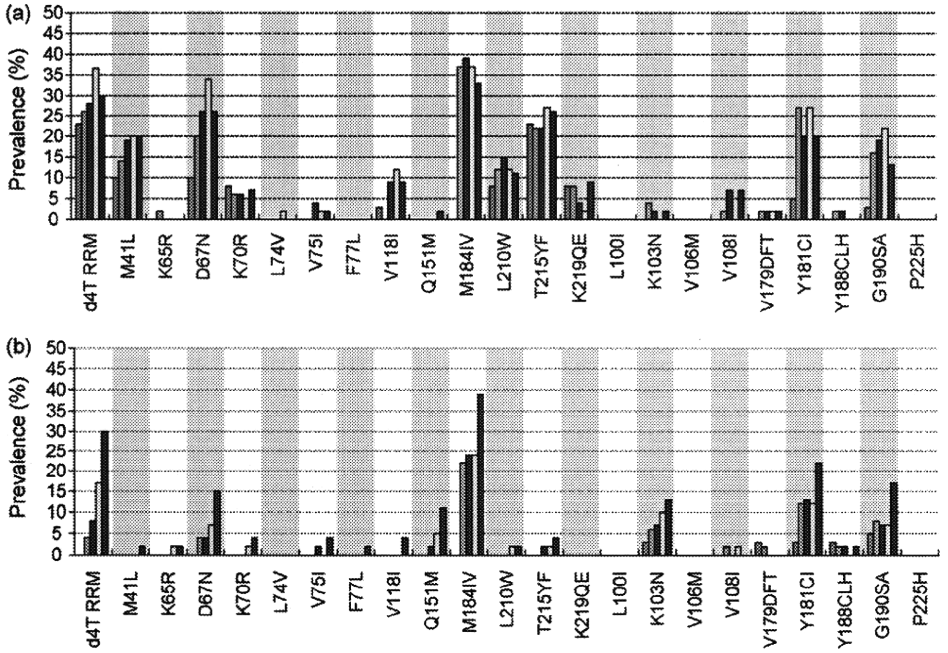

Fig. 1. Summary of drug resistant mutations detected in (a) antiretroviral exposed group, and (b) naive group. Light blue, gray, green, yellow and red bars indicate prevalence of mutation detected at baseline, 6, 12, 18 and 24 months after initiation of GPOvir treatment, respectively. d4T related resistance 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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