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研究成果の刊行に関する一覧

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An 11-Year Surveillance of HIV Type 1 Subtypes in Nagoya, Japan

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Abstract

To monitor active HIV-1 transmission in Nagoya, Japan, we have been determining the subtypes of HIV-1 infecting therapy-naive individuals who have newly visited the Nagoya Medical Center since 1997. The subtypes were determined by phylogenetic analyses using the base sequences in three regions of the HIV-1 genes including *gag p17, pol protease* (*PR*) and *reverse transcriptase* (*RT*), and *env C2V3*. Almost all HIV-1 subtypes from 1997 to 2007 and 93% of all HIV-1 isolates in 2007 were subtype B. HIV-1 subtypes A, C, D, and F have been detected sporadically since 1997, almost all in Africans and South Americans. The first detected circulating recombinant form (CRF) was CRF01_AE (11-year average annual detection rate, 7.7%). Only two cases of CRF02_AG were detected in 2006. A unique recombinant form (URF) was first detected in 1998 and the total number of URFs reached 25 by year 2007 (average annual detection rate, 4.7%). Eleven of these 25 were detected from 2000 to 2005 and had subtypes AE/B/AE as determined by base sequencing of the *gag p17*, *pol PR* and *RT*, and *env C2V3* genes (average annual detection rate, 3.7%). Unique subtype B has been detected in six cases since 2006. All 17 of these patients were Japanese. Other recombinant HIV-1s have been detected intermittently in eight cases since 1998. During the 11-year surveillance, most HIV-1s in Nagoya, Japan were of subtype B. We expect that subtype B HIV-1 will continue to predominate for the next several years. Active recombination between subtype B and CRF01_AE HIV-1 and its transmission were also shown.

Introduction

The total number of HIV-1-infected individuals and the prevalence of HIV-1 in Japan were 13,842 and 0.01%, respectively, at the end of 2007, which is far less than the number and prevalence in other Asian countries.[1–6] However, the number of newly infected patients per year was still increasing in Japan in 2007, when it reached 1448. One-hundred and twenty-five (8.6%) new patients were identified in Aichi prefecture in the same year. The total number of HIV-1-infected individuals and the prevalence of HIV in Aichi prefecture in 2007 were 725 and 0.01%, respectively. The number of newly infected individuals is expected to increase further. Therefore, the rapid increase in the number of HIV-1-infected individuals and changes in HIV-1 subtype populations are matters of great concern.

The most prevalent HIV-1 subtype in the world is subtype C (50%) followed by A (12%), B (10%), G (6%), and D (3%).[7,8] In addition, there are circulating recombinant forms (CRFs), such as CRF01_AE, CRF02_AG, and other recombinant viruses. The prevalent HIV-1 subtypes in China, Japan's largest neighbor, are circulating B/C recombinant forms, CRF07_BC, and CRF08_BC, accounting for 50% of the HIV-infected population, and subtype B HIV-1 accounting for 32%.[9] In contrast, the most prevalent HIV-1 strains in Japan are subtype B (83.1%) and CRF01_AE (12.4%) according to the nationwide surveillance.[10] It is clear that the distribution of HIV-1 strains is very different in Japan than in the rest of the world. However, as subtypes were determined from the base sequence of the *pol protease* (*PR*) and *reverse transcriptase* (*RT*) genes, we could not identify recombinant HIV-1 subtypes.

To study the trend of HIV-1 subtypes and the emergence of recombinant HIV-1 subtypes in Nagoya, Japan, we selected the *gag p17*, *env C2V3*, and *PR* to *RT* gene. We determined the base sequences of these genes from 2003; the subtypes of HIV-1 samples from 1997 to 2002 were retrospectively determined by the same method. Finally, this study aimed to clarify the genetic changes in HIV-1 subtypes by monitoring the sequences of HIV-1 subtypes that infected therapy-naive patients between 1997 and 2007.

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Table 1. Subtype Distribution of Therapy-Naive HIV-1-Infected Patients

	Total	*A*	*B*	*C*	*D*	*F*	*CRF01_AE*	*CRF02_AG*	*Other recombinants*
Number of patients	534	10 (1.9%)	440 (82.4%)	11 (2.1%)	1 (0.2%)	4 (0.7%)	41 (7.7%)	2 (0.4%)	25 (4.7%)
Age									
Average	37	31	37	38	32	24	40	37	35
Range	17–73	20–40	17–73	22–66	—	21–28	20–69	33–41	20–53
$CD4^+$ T cells (cells/μl)									
Average	280	305	278	291	418	468	246	289	264
Range	0–1508	60–1003	0–1508	7–545	—	278–909	2–1200	210–367	1–716
Viral load (copies/ml)									
Average	3.1×10^5	1.8×10^5	3.3×10^5	8.6×10^4	2.5×10^4	2.2×10^4	2.7×10^5	5.1×10^4	2.9×10^5
Range	1.0×10^2–1.3×10^7	4.4×10^2–1.1×10^6	1.0×10^2–1.3×10^7	6.1×10^2–4.3×10^5	—	2.1×10^3–4.5×10^4	7.8×10^2–2.8×10^6	4.4×10^4–5.7×10^4	2.8×10^2–2.0×10^6
Clinical phase[a]									
AC	370	6	302	9	1	4	25	2	21
ARC	37	2	32	0	0	0	3	0	0
AIDS	127	2	106	2	0	0	13	0	4

[a]AC, asymptomatic carrier; ARC, AIDS-related complex.

Materials and Methods

Patients

We enrolled 534 therapy-naive HIV-1-infected patients who initially attended our HIV-1 clinic at Nagoya Medical Center in Nagoya, Japan, between June 1997 and December 2007 and gave their written, informed consent to participate in our study. Our research protocol was approved by the ethical committee in our hospital. The samples of this study were the same ones used for drug resistance testing, which was conducted in almost all therapy-naive individuals at their initial visit. The data, including age, nationality, sexual orientation, number of CD4-positive T cells, and viral load, were obtained from medical records.

Patient coverage by Nagoya Medical Center

Nagoya Medical Center is located in Nagoya City, the capital of Aichi prefecture, and is the central hospital for AIDS treatment and research in the Tokai area (which includes the Aichi, Mie, Gifu, and Shizuoka prefectures).

Amplification of HIV-1 DNA fragments and determinations of DNA sequences

HIV-1 RNA was extracted from plasma samples using a QIAamp viral RNA Mini Kit (QIAGEN, Tokyo, Japan). The DNA fragments were amplified by reverse transcription-nested polymerase chain reaction (RT-nested PCR) using the Superscript one-step RT-PCR system (Invitrogen, Tokyo, Japan) and LA Taq polymerase (Takara, Shiga, Japan). The primers used for DNA amplification were as follows. The *gag* fragment containing the region between *gag p17* and *p24* (codons 1–147 encoding *gag-pol polyprotein*) was amplified by RT-PCR with the primer set of 172A (5′-ATC TCT AGC AGT GGC GCC CGA ACA G-3′) and 173B (5′-CTG ATA ATG CTG AAA ACA TGG GTA T-3′).[11] 174A (5′-CTC TCG ACG CAG GAC TCG GCT TGC T-3′) and 175B(5′-CCC ATG CAT TCA AAG TTC TAG GTG A-3′) were used for nested PCR. The *pol* fragments containing the regions encoding *gag-pol polyprotein* (codons 425–500), *PR* (codons 1–99), and *RT* (codons 1–349) were amplified by RT-PCR with primers of K1 (5′-AAG GGC TGT TGG AAA TGT GG-3′) and U13 (5′-CCC ACT CAG GAA TCC AGG T-3′).[12] K4 (5′-GAA AGG AAG GAC ACC AAA TGA-3′) and U12 (5′-CTC ATT CTT GCA TAT TTT CCT GTT-3′) were used for nested PCR. The *env* fragment containing the region encoding *env C2V3* (codons 249–375) was amplified by RT-PCR with primers of 106A (5′-CAT ACA TTA TTG TGC CCC GGC TGG-3′) and 17B (5′-AGA AAA ATT CCC CTC TAC AAT TAA-3′).[11] 14A (5′-AAT GTC AGC TCA GTA CAA TGC ACA C-3′) and 10B (5′-ATT TCT GGG TCC CCT CCT GAG G-3′) were used for nested PCR. These are in-house primers capable of amplifying the target genes of different HIV-1 subtypes. The *gag*, *pol*, and *env* gene fragments were not amplified in RT-nested PCR using the above-mentioned primers in four, five, and nine cases, respectively. In such cases, different primer sets were used (not shown). Thus, amplified cDNAs were successfully obtained in all cases.

An HIV-1 DNA control, in which reverse transcriptase was omitted in the RT-PCR, was run in parallel with RT-PCR to control for the absence of genomic DNA. The PCR products were purified using the QIAGEN Gel Extraction Kit

(QIAGEN). A labeling reaction for DNA sequencing was performed using the same primers used for the PCR reactions as well as the BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems, Tokyo, Japan), and then labeled DNA fragments were purified on a AutoSeq G-50 column (GE Healthcare, Tokyo, Japan). The samples were analyzed by direct sequencing and electropherograms were obtained using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). DNA sequences were analyzed using Sequencing Analysis V3.7 (Applied Biosystems) and SeqScape V2.5 (Applied Biosystems).

Subtype determination

Sequences were analyzed and grouped into subtypes by phylogenetic tree construction. Phylogenetic analysis using nucleotide sequences of *gag* fragments from 534 therapy-naive patients was performed using the 62 reference HIV-1 sequences derived from the Los Alamos National Laboratory database.[13] The 62 sequences represent all subtypes of the major groups and 18 CRFs. Independent analysis was done using both *pol* fragments and *env* fragments. Sequences were aligned using CLUSTAL W software in MEGA software version 3.1. Evolutionary distances were calculated using the same software. Phylogenetic trees were constructed by the neighbor-joining method based on distances calculated with Kimura's two-parameter algorithm. The reliabilities of the branching patterns were tested by bootstrap analysis with 1000 replicates.

Results

HIV-1 subtypes in the last 11 years in Nagoya

Subtype B was the most prevalent subtype, identified in 440 out of 534 patients (82.4%, Table 1 and Fig. 1). The largest number, 388, was found in Japanese and the next largest, 45, in South Americans (Table 2). Of the total number, 319 were identified in Japanese men who have sex with men (MSM) and 14 in South American MSM. Of note, the 72.5% infected with subtype B HIV-1 were Japanese MSM. Infection with subtypes A, C, D, and F was detected in 10 individuals (seven Africans, one East European, and two Japanese), 11 individuals (two Africans, two South Americans, two Southwest

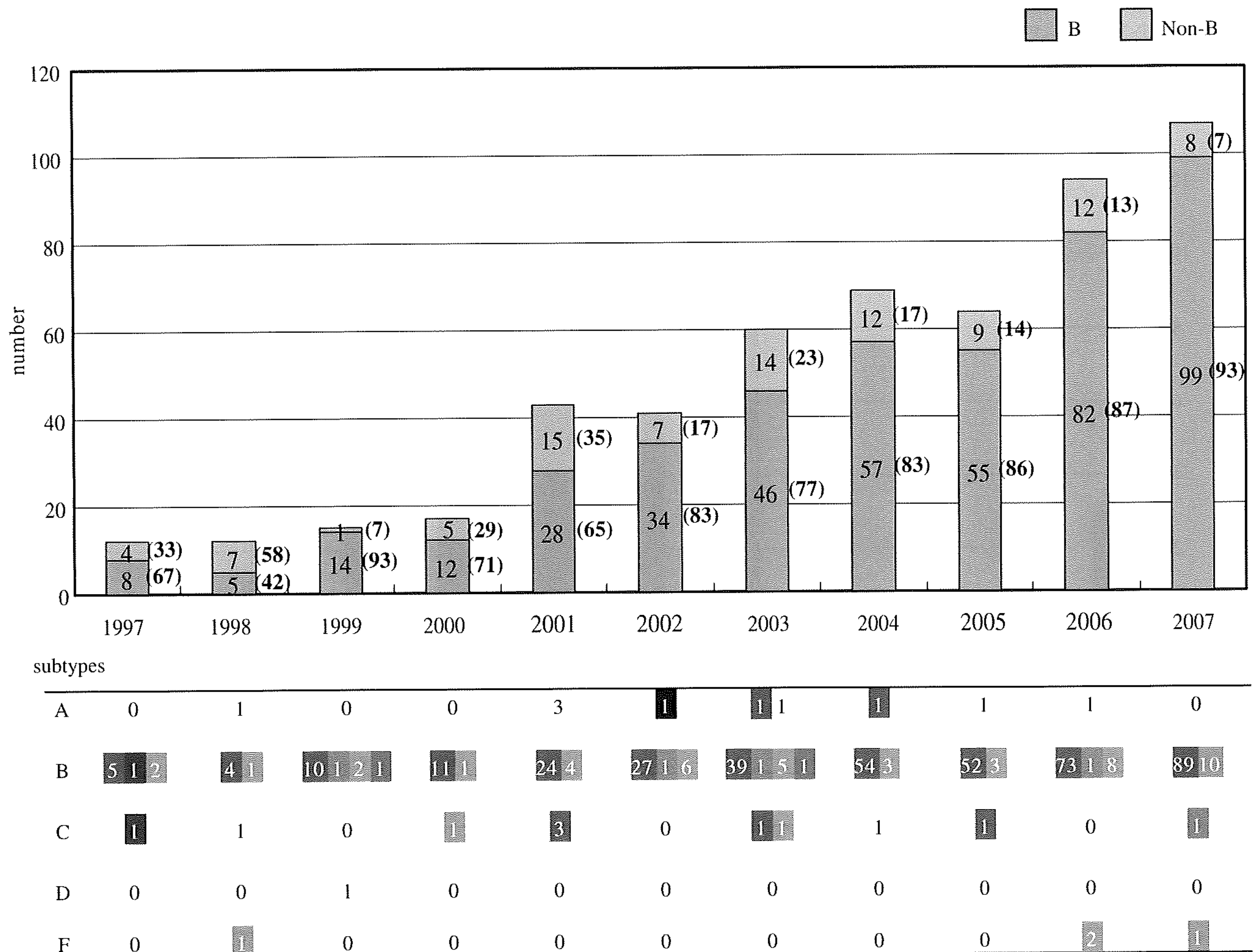


FIG. 1. Subtypes of HIV-1 found in therapy-naive infected patients in Nagoya Medical Center from 1997 to 2007 (upper panel). The numbers of patients (bars) and their proportions (%, in parentheses) are shown. The numbers of patients and their nationalities (indicated by a color: Japan, red; East Asia, green; Southwest Asia, blue; South America, orange; North America, pink; Eastern Europe, black; Africa, uncolored) are given in the lower panel.

TABLE 2. NATIONALITIES AND SEXUAL ORIENTATIONS OF THERAPY-NAIVE HIV-1-INFECTED PATIENTS BY SUBTYPES[a]

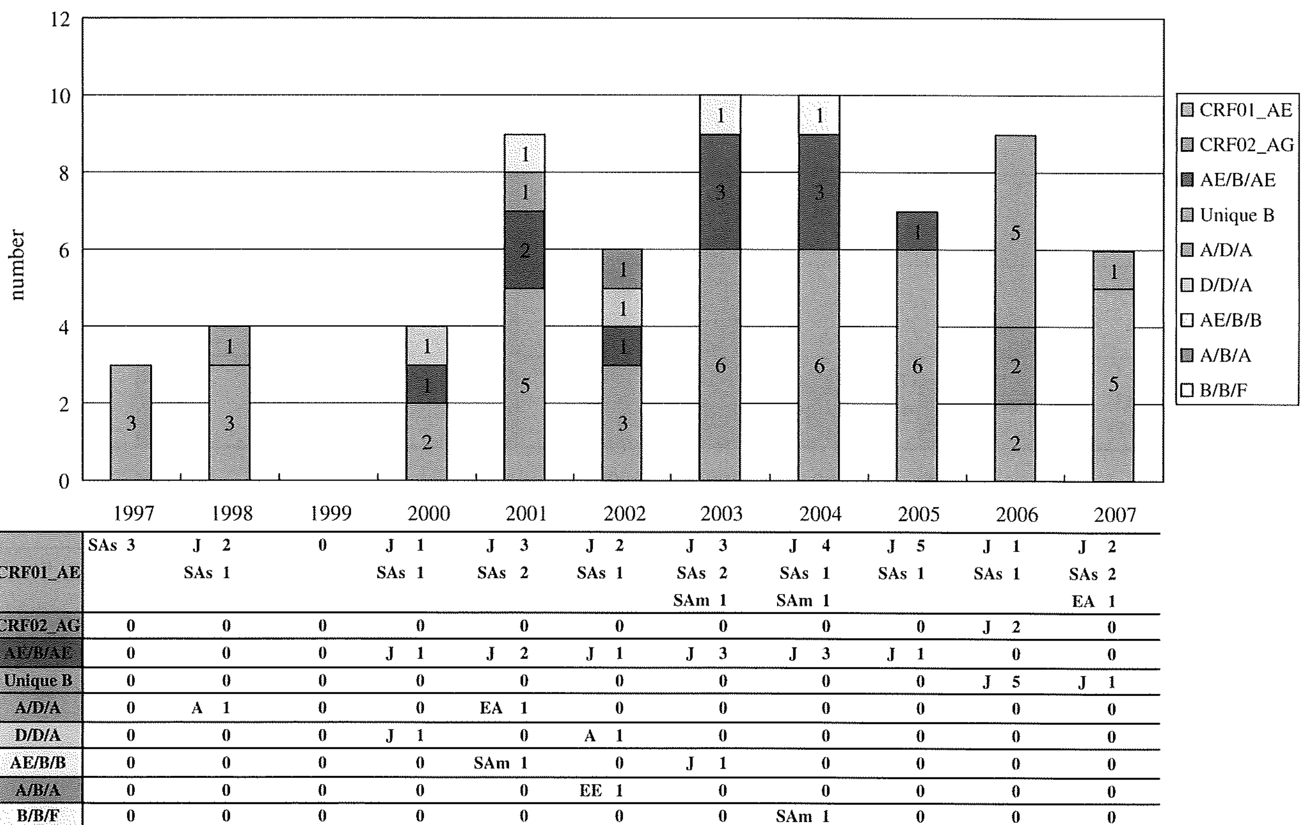
	Total	*A*	*B*	*C*	*D*	*F*	*CRF01_AE*	*CRF02_AG*	*Other recombinants*
Nationalities									
Japan	439 (420, 340)	2 (0, 0)	388 (381, 319)	5 (1, 1)	0	0	23 (20, 2)	2 (0, 0)	19 (18, 18)
East Asia	6 (4, 4)	0	4 (4, 4)	0	0	0	1 (0, 0)	0	1 (0, 0)
Southwest Asia	18 (9, 0)	0	1 (0, 0)	2 (1, 0)	0	0	15 (8, 0)	0	0
East Europe	2 (0, 0)	1 (0, 0)	0	0	0	0	0	0	1 (0, 0)
North America	2 (2, 2)	0	2 (2, 2)	0	0	0	0	0	0
South America	55 (36, 15)	0	45 (31, 14)	2 (1, 0)	0	4 (1, 0)	2 (1, 0)	0	2 (2, 1)
Africa	12 (9, 0)	7 (5,0)	0	2 (1, 0)	1 (1, 0)	0	0	0	2 (2, 0)

[a]The left and right numbers in parentheses are the numbers of males and MSMs, respectively.

Asians, and five Japanese), one African, and four South Americans, respectively. In foreigners, this distribution of HIV-1 subtypes represents the distribution of subtypes commonly found in the immigrants' region of origin.[14–20] However, subtypes A and C were detected mostly in Japanese females who seemed to have acquired the infection from foreign partners in Japan.

As for circulating recombinant forms (CRFs), CRF01_AE was detected in 41 individuals (7.7%), including 23 Japanese (18 non-MSM, two MSM, and three females) and 18 foreigners (nine non-MSM and nine females). Considering that only four individuals (two Japanese males, one Japanese female, and one South Asian female) were intravenous drug users, CRF01_AE has mainly spread through heterosexual contact in Nagoya. CRF02_AG was detected in two individuals (0.4%) in 2006 (Fig. 2).

Among other recombinant forms, the most frequent was AE/B/AE, found in 11 Japanese MSM. A unique subtype B HIV-1 strain that possessed subtype D fragments in the *gag* and *env* regions was also detected in six Japanese MSM. As for other recombinants, A/D/A, D/D/A, and AE/B/B were each identified in two individuals and A/B/A and B/B/F were each identified in one. Thirteen intravenous drug users (2.4%) were included: two male Japanese with subtype B, one female



	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007
CRF01_AE	SAs 3	J 2 SAs 1	0	J 1 SAs 1	J 3 SAs 2	J 2 SAs 1	J 3 SAs 2 SAm 1	J 4 SAs 1 SAm 1	J 5 SAs 1	J 1 SAs 1	J 2 SAs 2 EA 1
CRF02_AG	0	0	0	0	0	0	0	0	0	J 2	0
AE/B/AE	0	0	0	J 1	J 2	J 1	J 3	J 3	J 1	0	0
Unique B	0	0	0	0	0	0	0	0	0	J 5	J 1
A/D/A	0	A 1	0	0	EA 1	0	0	0	0	0	0
D/D/A	0	0	0	J 1	0	A 1	0	0	0	0	0
AE/B/B	0	0	0	0	SAm 1	0	J 1	0	0	0	0
A/B/A	0	0	0	0	0	EE 1	0	0	0	0	0
B/B/F	0	0	0	0	0	0	0	SAm 1	0	0	0

FIG. 2. Recombinant HIV-1s detected in therapy-naive patients in Nagoya Medical Center (upper panel). The bars indicate the number of recombinant HIV-1s detected from 1997 to 2007. The number of patients and their nationalities (indicated by J, Japan; EA, East Asia; SAs, Southwest Asia; SAm, South America; A, Africa; NA, North America; EE, Eastern Europe) are shown in the lower panel.

Japanese with CRF01_AE, four MSM with a unique subtype B, two male South Americans with subtype B, and two male and one female Southwest Asians with CRF01_AE.

The change of HIV-1 detection rates

The rate of subtype B detection, the most frequently detected HIV-1 subtype in the Nagoya Medical Center, increased to 93% in 2007 (Fig. 1). During this period, the absolute number of individuals with subtype B HIV-1 also increased. On the other hand, the number of individuals infected with non-B HIV-1 strains fluctuated between 7 and 15 after the year 2000. Major subtypes A, C, D, and F were sporadically detected (only two or three cases per year since 2004). The most frequently detected circulating recombinant HIV-1 was CRF01_AE, which was detected every year from 1997 to the present, except for 1999 (Fig. 2). About half of these infections were in Japanese and the other half were in Southwest Asians. No increase in the number of infections by this virus has been observed. The increase in the number of HIV-1-infected cases, especially of subtype B, during the past 11 years reflected the scaling up of clinical services as well as the increase in HIV-1 transmission.

Active recombination between subtype B and CRF01_AE HIV-1

The A/D/A recombinant was first detected in 1998 (Fig. 2), and unique recombinant forms (URFs) have been detected in all years except 1999. The AE/B/AE recombinant, the most frequently detected URF, had been detected between 2000 and 2005, and the accumulated number of cases with this virus was 11. A unique subtype B HIV-1 was detected in five patients and one patient in 2006 and 2007, respectively.

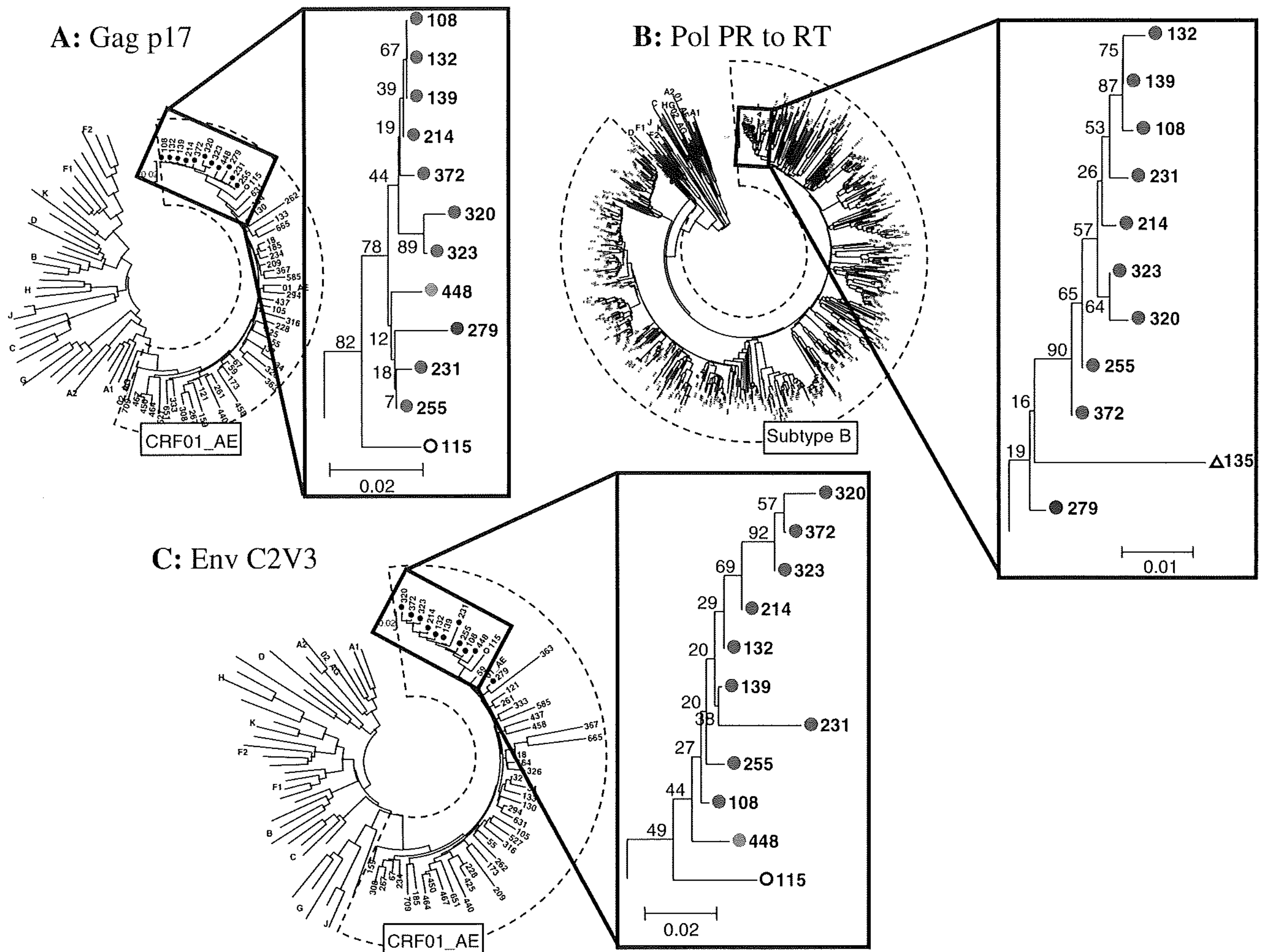


FIG. 3. Phylogenetic tree analyses of 11 recombinant AE/B/AE HIV-1s. Trees were constructed using nucleotide base sequences of *gag p17* (**A**), *pol PR* to *RT* (**B**), and *env C2V3* (**C**) gene regions. The base sequences of the *gag p17* and *env C2V3* genes of 41 CRF01_AE HIV-1s and those of the *pol PR* to *RT* gene of 440 subtype B HIV-1s in this study (Table 1) as well as 39 reference sequences obtained from the Los Alamos database were used for these analyses. Clustered AE/B/AE recombinant HIV-1s indicated by red, blue, and green circles are framed. Red circles represent HIV-1s that form a cluster in all trees. Green circles represent HIV-1s that form a cluster only in trees with *gag* and *env* genes. Blue circles represent HIV-1s that form a cluster only in trees with *gag* and *pol* genes. Candidate HIV-1s with subtype B or CRF01_AE accounting for AE/B/AE recombinant formation are depicted by open triangles (**B**) and open circles (**A** and **C**), respectively.

The detection of one A/D/A, one D/D/A, two AE/B/B, one A/B/A, and one B/B/F was sporadic from 2001 to 2004. The A/D/A, D/D/A, AE/B/B, A/B/A, B/B/F, and unique B recombinants may have been brought from foreign countries because no candidate sequences indicating the origin of A, D, F, and CRF01_AE have been found. Of course, patients infected with these recombinant viruses may have been partners in a sexual network with less access to medical care. Nine viruses of the AE/B/AE recombinant form (represented with red circles in Fig. 3) were clustered in three separate phylogenetic analyses of the *gag p17*, *env C2V3*, and *pol PR* to *RT* genes, indicating that they were closely related. In addition, CRF01_AE of patient 115 was positioned in the same cluster of *gag* and *env* sequences mentioned above, and subtype B of patient 135 in the same cluster of *pol* sequence, suggesting this AE/B/AE recombinant form may be originated from these two viruses.

Discussion

In this study, the emergence and transmission of recombinant HIV-1s were analyzed in detail by determining base sequences of major segments of three genes: *gag p17*, *pol PR* to *RT*, and *env C2V3*. The subtypes of HIV-1 strains prevalent in the Nagoya Medical Center for the past 11 years were B (82.4%), CRF01_AE (7.7%), C (2.1%), A (1.9%), F (0.7%), CRF02_AG (0.4%), D (0.2%), and other recombinant forms (4.7%). This result was similar to the result of a nationwide study (using only the *pol PR* to *RT* gene region to determine subtypes[10]), which found that only 5% of the total cases were due to recombinant forms. Worldwide, subtype C is the most prevalent HIV-1 subtype (50% of all cases).[8,9] This means the situation in Nagoya, Japan is quite different from that in other parts of the world, especially from that in Africa.

The recombinant HIV-1s, in addition to CRF01_AE and CRF02_AG, include 11 AE/B/AE, six unique subtype B recombinants possessing short D fragments in *gag* and *env* genes, two A/D/A, two D/D/A, two AE/B/B, one A/B/A, and one B/B/F. Among these seven, all except AE/B/AE appear to be immigrant types. Only the AE/B/AE type seems to be a newly emerged recombinant in our area. As subtype B was the predominant HIV-1 subtype and CRF01_AE was the second-most prevalent HIV-1 recombinant in Nagoya, we have speculated that a novel recombinant HIV-1 between subtype B and CRF01_AE will emerge in this area. The methodology for subtype determination using the base sequences of *gag p17*, *pol PR* to *RT*, and *env C2V3* genes is useful at the moment; however, to clarify the gene structure of these recombinant viruses, analysis of the full sequence and then computational analyses are required. Use of this approach revealed that the gene structure of AE/B/AE type recombinants found in this study is novel and differs from that of CRF15_01B, CRF33_01B, and CRF34_01B (i.e., CRFs resulting from recombination between subtype B and CRF01_AE HIV-1 found in Malaysia and Thailand).[21–23] The finding of two viruses with genes for both subtype B and CRF01_AE, which are the candidate origins of this AE/B/AE recombinant, supported our hypothesis of emerging recombinants. The number of HIV-1-infected individuals as well as the frequency of drug-resistant HIV-1 in therapy-naive individuals have increased, strongly implying that this increasing tendency will continue in Nagoya, Japan.[24,25] We will pursue this kind of surveillance to obtain information needed for suppressing the spread of HIV-1 infection.

Sequence Data

The base sequences of HIV-1 subtypes are registered in the DNA databank of Japan (DDBJ) as AB442228–AB443428 and AB356098–AB356499.

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Disclosure Statement

No competing financial interests exist.

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Analysis of Near Full-Length Genomic Sequences of Drug-Resistant HIV-1 Spreading among Therapy-Naïve Individuals in Nagoya, Japan: Amino Acid Mutations Associated with Viral Replication Activity

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Abstract

We analyzed a total of 12 near full-length genomes of drug-resistant HIV-1 spreading among therapy-naïve individuals in Nagoya, Japan. Genomes comprised seven protease inhibitor (PI)-resistant viruses possessing an M46I ($n = 6$) or L90M mutation ($n = 1$) and five non–nucleoside reverse transcriptase inhibitor–resistant viruses possessing a K103N mutation. All 12 viruses conserved both an H87Q mutation in the cyclophilin A–binding site of Gag p24 (capsid) and a T23N mutation in the cysteine-rich domain of Tat protein. PI-resistant viruses commonly possessed two cleavage site mutations in the $p6^{Pol}$/protease of Pol polyprotein (F48L in $p6^{Pol}$) and the anchor/core domains of Nef protein (L57V). These amino acid mutations represent candidates for enhancing replication activity of drug-resistant viruses and supporting expansion of such viruses in therapy-naïve individuals.

Transmission of drug-resistant HIV-1 in therapy-naïve individuals represents a serious problem in therapy, as such variants hinder antiretroviral therapy from the start.[1,2] Drug-resistant viruses were detected in 27 of 402 therapy-naïve patients (6.7%) in Nagoya, Japan, between 1999 and 2006.[3] Importantly, phylogenetic analysis has revealed that two main independent drug-resistant strains have been spreading in this area. One is a protease inhibitor (PI)-resistant strain possessing an M46I or L90M mutation in the protease. This strain started spreading in 2000 and was found in a total of 13 therapy-naïve patients. The other is a non–nucleoside reverse transcriptase inhibitor (NNRTI)-resistant strain possessing a K103N mutation in the reverse transcriptase. This strain started spreading in 2003 and was found in a total of five therapy-naïve patients.[3] Importantly, both strains are still growing. We recently started studying why or how these drug-resistant strains can spread in therapy-naïve individuals while maintaining drug-resistant amino acid mutations that generally confer replicative disadvantages. This study analyzed near full-length genomic sequences of drug-resistant viruses to identify clues to better understanding these epidemics.

Subjects comprised a total of 12 therapy-naïve patients. Among these, seven patients were identified with PI-resistant HIV-1 possessing an M46I mutation ($n = 6$) or L90M mutation ($n = 1$). The remaining five patients displayed NNRTI-resistant HIV-1 possessing a K103N mutation by routine genotypic drug-resistance testing from 2000 to 2006.[3] Genomic sequencing of HIV-1 was performed using plasma samples obtained at the first medical examination. HIV-1 RNA was purified from a plasma sample using a QIAamp viral RNA mini kit (QIAGEN, Tokyo, Japan). A single DNA fragment containing *gag* to *nef* genes was reverse transcribed and amplified by reverse transcription (RT)-nested polymerase chain reaction (PCR) using the Superscript III one-step RT-PCR system with platinum Taq high-fidelity kit (Invitrogen, Tokyo, Japan) and LA Taq polymerase (Takara, Shiga, Japan). Sense and antisense primers for RT-PCR were INF-13 and LTR-E, respectively. Sense and antisense primers for nested PCR were INF-12 and LTR-D, respectively. INF-19 or INF-11 primers were sometimes used instead of INF-13, and INF-20 or INF-10 primers were sometimes used instead of INF-12. Nucleotide sequences of primers were as follows: INF-13, 5′-GGT GAG TAC GCC ATT TAT TTG ACT

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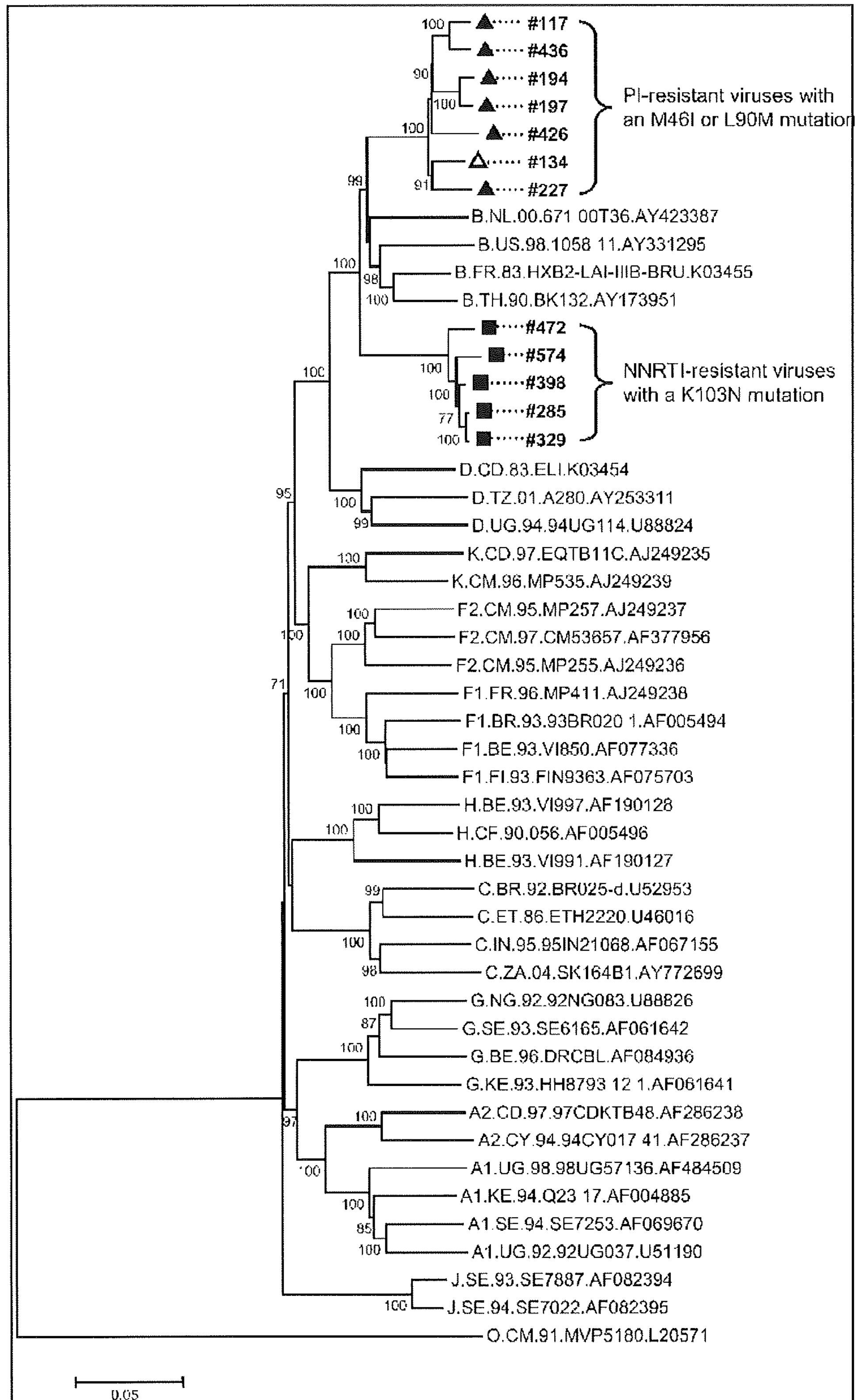

FIG. 1. Phylogenetic analysis of drug-resistant HIV-1. A phylogenetic tree was constructed using the neighbor-joining method with near full-length genomic sequences. Bootstrap values were calculated by 1,000 analyses and values greater than 70% were shown at the nodes of the tree. Scale bar represents nucleotide substitutions per site. Group O_MVP5180 was used as the outgroup. PI-resistant viruses possessing an M46I or L90M mutation are shown with closed or open triangles, respectively. NNRTI-resistant viruses possessing a K103N mutation are shown with closed squares. PI, protease inhibitor; NNRTI, non–nucleoside reverse transcriptase inhibitor.

AG-3′; LTR-E, 5′-CTT ATA TGC AGC TTC TGA GGG C-3′; INF-12, 5′-ATT TAT TTG GCG CGC GGA GGC TAG AA-3′; LTR-D, 5′-GCA TCA TTA ATT AAC CCT GGA AAG TCC CCA GCG GAA-3′; INF-19, 5′-GGT GAG TAC GCC AAA AAA CTT TTG ACT AG-3′; INF-20, 5′-AAA CTT TTG GCG CGC GGA GGC TAG AA-3′; INF-11, 5′-TCT CTC GAC GCA GGA CTC GGC TTG-3′; INF-10, 5′-GCT GAA GCG CGC ACA GCA AGA GGC GAG-3′. The RT-PCR program consisted of one cycle of RT reaction (60 min at 50°C), 1 cycle of pre-PCR (2 min at 94°C), and 40 cycles of PCR (15 s at 94°C, 30 s at 50°C, and 10 min at 68°C). The nested PCR program consisted of one cycle of pre-PCR (2 min at 94°C) and 40 cycles of PCR (15 s at 94°C, 30 s at 50°C, and 10 min at 70°C). A labeling reaction for DNA sequencing was per-

A Pol polyprotein

	p6[Pol] ▼	Protease
AA#	48	
NL4-3	VSFSF	PQITL
HXB2	...N.	..V..
117	I...**L**	.....
134	..LN**L**	.....
194	I..N**L**	.....
197	I..N**L**	.....
227	..L.**L**	.....
426	..LN**L**	.....
436	I...**L**	.....
285	.....	.....
329	.....	.....
398	.....	.....
472	.....	.....
574	.....	.....

B Nef protein

	Anchor domain ▼	Core domain
AA#	57	
NL4-3	ACAWL	EAQEE
HXB2	.....	.....
117	DR..**V**	.....
134	DR..**V**	....D
194	D...**V**	....D
197	D...**V**	....D
227	X_1X_2..**V**	.....
426	.R..**V**	.....
436	DR..**V**	.....
285	D.V..	..H.D
329	D.V..	..H.D
398	D.V..	..H.D
472	D.V..	....D
574	D.V..	..H.D

C Gag p24 (capsid)

AA#	87
NL4-3	PVHAGPIAP
HXB2	....
117	..**Q**......
134	.A**Q**......
194	.A**Q**......
197	.A**Q**......
227	..**Q**......
426	.P**Q**......
436	.A**Q**...X_3..
285	.A**Q**...HP.
329	.A**Q**...HP.
398	.A**Q**...HP.
472	.A**Q**...HP.
574	.A**Q**...HP.

D Tat protein

AA#	23
NL4-3	CTNCYCKKCCFHCQVC
HXB2	....
117	.**N**........L.....
134	.**N**..............
194	.**N**........Y...A.
197	.**N**........Y.....
227	.**N**........L.....
426	.**N**.......S......
436	.**N**........W.....
285	.**N**..............
329	.**N**..............
398	.**N**..............
472	.**N**..............
574	.**N**..............

FIG. 2. Candidates for amino acid mutations that possibly enhance the replication activity of drug-resistant HIV-1. Protease inhibitor-resistant HIV-1 commonly possessed an F48L mutation in the carboxyl terminus of p6[Pol] (**A**) and an L57V mutation in the carboxyl terminus of Nef anchor domain (**B**). All drug-resistant viruses displayed conservation of an H87Q mutation in the cyclophilin A–binding domain (Pro85 to Pro93) of Gag p24 (capsid) (**C**). All drug-resistant viruses also conserved a T23N mutation in the cysteine-rich domain (Cys22 to Cys37) of Tat protein (**D**). Candidate mutations are shown in bold. Cleavage points are represented as triangles in **A** and **B**. Mixed-type amino acids are represented as follows: X_1, D/A; X_2, R/C; and X_3, V/I. Amino acid sequences of NL4-3 and HXB2 were used as references. AA#, amino acid number.

formed using the BigDye terminator cycle sequencing kit (Applied Biosystems, Tokyo, Japan), and DNA sequences were determined using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). In phylogenetic analyses, multiple sequence alignment was performed using CLUSTAL W, and genetic distances were calculated based on the Kimura two-parameter model using MEGA software version 3.1.[4] Phylogenetic trees were constructed using the neighbor-joining method with 1,000 bootstrap analyses. Genomic sequences of reference HIV-1 strains were obtained from the HIV sequence database in the Los Alamos National Laboratory.[5] Recombinant formation was checked using the Recombinant Identification Program version 3.0 in the HIV sequence database.

A total of 12 near full-length genomic sequences of drug-resistant HIV-1 were successfully obtained from therapy-naïve patients. Seven PI-resistant viruses and five NNRTI-resistant viruses separately clustered together with reference subtype B viruses on a phylogenetic tree (Fig. 1). This is consistent with our previous result obtained by phylogenetic analysis using *pol* gene fragment alone.[3] We separately confirmed that they were subtype B and not recombinant forms using the Recombination Identification Program (data not shown).

We and others have previously reported that acquisition of an M46I, L90M, or K103N major mutation enables HIV-1 to survive under pharmacotherapeutic pressure but simultaneously sacrifices the replicative activity of such viruses in the absence of drug.[6,7] This fact forced us to hypothesize that our drug-resistant viruses restored reduced replication activity by acquiring some mutations in the genome, which thus consequently survive and expand under drug-free conditions such as in therapy-naïve patients. We therefore extensively searched for candidate amino acid mutations that might offer advantages in viral replication, revealing four interesting mutations.

The first was an F48L mutation located in the carboxyl terminus of $p6^{Pol}$, and the second one was an L57V mutation located in the carboxyl terminus of the Nef anchor domain (Figs. 2A, 2B). These were specified in the PI-resistant HIV-1 strain. Findings of A431V, L449F, and P453 mutations in the p7/p1 and $p1/p6^{Gag}$ cleavage sites of Gag polyprotein, and associated restorative activities on viral replication of PI-resistant HIV-1 have been reported,[8,9] but our viruses displayed no such mutations. The F48L mutation in $p6^{Pol}$ and/or the L57V mutation in the Nef protein might have restoration activity in our PI-resistant viruses.

The third was a non–cleavage site mutation found in Gag p24 (capsid). Several amino acid mutations in the non–cleavage site of Gag polyprotein have been reported to restore reduced replication activity of PI-resistant HIV-1.[10] One of these is an H219Q mutation also known as an H87Q mutation in the capsid. Amino acid 87H is located in the cyclophilin A–binding site, and the H87Q mutation reduces incorporation of cyclophilin A into HIV-1 virions, thus elevating HIV-1 replication.[11] Interestingly, all our drug-resistant viruses commonly possessed the H87Q mutation in the capsids, suggesting that replicative activities were enhanced by this mutation (Fig. 2C). Notably, in addition to the H87Q mutation, V86A/P, I91H/V, and A92P mutations were frequently found in the cyclophilin A–binding site. These additional mutations may also be associated with viral replication activity through binding modulation to cyclophilin A.

The fourth was again a non–cleavage site amino acid mutation found in the cysteine-rich domain of Tat protein. A previous study reported T23N as a polymorphic mutation that increased Tat transactivation activity on HIV-1 provirus gene expression.[12] Interestingly, all our drug-resistant viruses also commonly possessed this T23N mutation (Fig. 2D). Elevated Tat activity may plausibly support the replication of drug-resistant viruses.

As another interesting mutation, we found an insertion mutation of RPEP in the PTAPP motif of $p6^{Gag}$ in four cases of NNRTI-resistant HIV-1 (data not shown). At present, whether this insertion mutation confers any advantage for NNRTI-resistant viruses to survive under drug-free conditions is unclear.[13–15]

In conclusion, we successfully found primary candidates of amino acid mutations that might enhance the replicative activity of drug-resistant HIV-1 for surviving under drug-free conditions. Further investigations are required to elucidate whether these mutations substantially support the replication of drug-resistant viruses. Preliminary findings from our recent experiments have demonstrated positive roles of such mutations, particularly for H87Q mutation in the capsid.

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