

[ATV, リトナビル(RTV), ネルフィナビル(NFV), EFV] を添加し, 脂肪細胞の分化, HMW-Ad 産生に与える影響を解析した。脂肪前駆細胞, 成熟脂肪細胞に EFV, NFV を添加すると, 脂肪滴は著明に減少した(図3)。成熟脂肪細胞において, HMW-Ad 産生は ATV, NFV, EFV の添加により著明に抑制されたが, ATV 添加の影響は軽度であった。また NFV, EFV によって著明に抑制された HMW-Ad は, 薬剤を ATV に変更することで回復した(図4)。

以上, 抗 HIV 薬の神経経路への影響は薬剤によって異なるが, ATV では影響が少なかった。また, 成熟脂肪細胞の減少に伴う HMW-Ad 低下が抗 HIV 薬における脂質代謝異常の一端と考えられた。

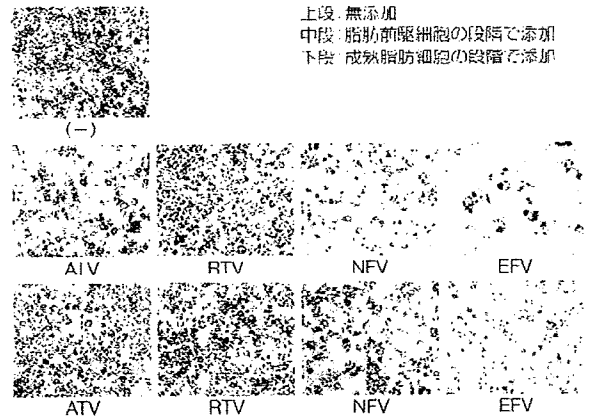


図3 3T3-L1の変化

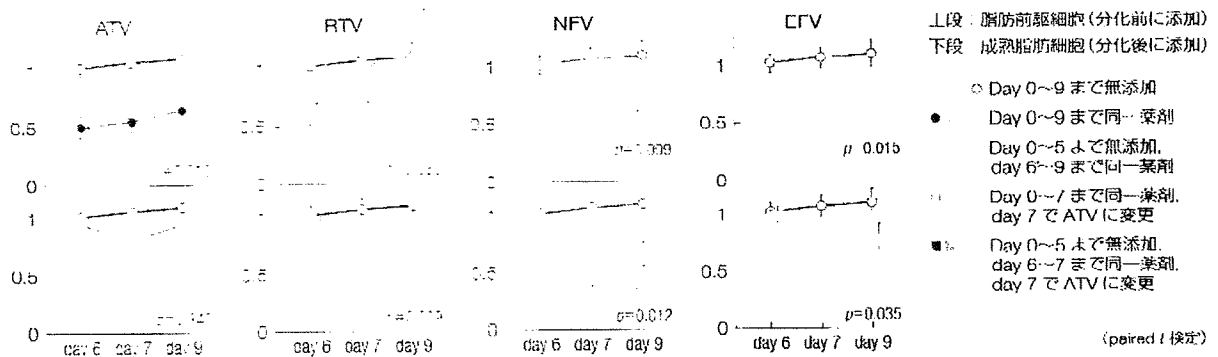


図4 アディポネクチンの産生(リアルタイム PCR)

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Trend of Drug-Resistant HIV Type 1 Emergence among Therapy-Naive Patients in Nagoya, Japan: An 8-Year Surveillance from 1999 to 2006

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ABSTRACT

We studied the emergence of drug-resistant human immunodeficiency virus type 1 (HIV-1) with major amino acid mutations in 402 therapy-naive patients at Nagoya Medical Center, Japan, between 1999 and 2006. The mean prevalence of drug-resistant HIV-1 was 6.7% (range, 2.3–10.0%; $n = 27$). HIV-1 variants with protease inhibitor (PI)-resistant mutations alone were most frequently found (3.5%, $n = 14$), followed by those with nonnucleoside reverse transcriptase inhibitor (NNRTI)-resistant mutations alone (1.7%, $n = 7$). Variants with nucleoside reverse transcriptase inhibitor (NRTI)-resistant mutations alone were sporadically found (1.0%, $n = 4$). A variant possessing both NRTI- and PI-resistant mutations was detected in one patient (0.2%) and a variant possessing both NNRTI- and PI-resistant mutations was identified in another patient (0.2%). In addition, another 17 variants (4.2%, $n = 17$) with only 215-revertant mutations (T215C/D/G/L/S) that can easily revert to the nucleoside analogue-associated mutation of T215Y/F were found. The 402 viruses were phylogenetically analyzed, revealing three independent clusters comprising PI-resistant variants with the M46I or L90M mutation, NNRTI-resistant variants with the K103N mutation, and 215-revertant variants. The PI-resistant and 215-revertant strains have been spreading since 2000, and the NNRTI-resistant strain has started spreading since 2003. The nature of the epidemic and information for successfully blocking the spread of drug-resistant HIV-1 were clarified in this study.

INTRODUCTION

COMBINATION THERAPY with three or more antiretroviral drugs (highly active antiretroviral therapy, HAART) can strongly suppress the replication of human immunodeficiency virus type 1 (HIV-1) and maintain the amount of HIV-1 RNA in plasma (viral load) under detectable levels in many cases.^{1–5} However, HIV-1 variants with decreased susceptibility to antiretroviral drugs are sometimes found under conditions in which the drug concentration is insufficient to suppress viral replication following poor adherence to treatment regimens.^{4–9} Such variants might become an origin for HIV-1 transmission, resulting in the finding of drug-resistant HIV-1 in therapy-naive individuals.

This represents a serious problem in therapy, as such variants hinder antiretroviral therapy from the first trial.^{10–16} Determining whether therapy-naive patients are infected by drug-resistant HIV-1 before starting HAART is thus important. The present study studied emergence trends for drug-resistant HIV-1 with major mutations among therapy-naive patients in the Nagoya Medical Center, Japan, between 1999 and 2006. We also studied the emergence of HIV-1 with 215-revertant amino acid mutations in the reverse transcriptase (RT), as 215-revertant variants can easily change to nucleoside RT inhibitor (NRTI)-resistant variants.^{17–20} The final aim of the study was to understand the epidemiological nature of drug-resistant variants and obtain information to successfully block their spread.

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MATERIALS AND METHODS

Patients

A total of 441 therapy-naïve HIV-1-infected patients underwent their initial consultation at Nagoya Medical Center in Nagoya, Japan, between January 1999 and December 2006. Genotypic drug-resistance testing for HIV-1 was performed on 402 of the 441 patients (91%) after obtaining patient consent. The characteristics of the 402 patients are shown in Table 1.

Genotypic drug-resistance testing for HIV-1

Genotypic drug-resistance testing for HIV-1 was performed as previously reported.^{21,22} HIV-1 RNA was purified from a plasma sample using a QIAamp viral RNA mini kit (QIAGEN, Tokyo, Japan). A single DNA fragment containing both protease (PR) and reverse transcriptase (RT) genes was amplified by reverse transcription-nested polymerase chain reaction (RT-nested PCR) using the Superscript one-step RT-PCR for long templates kit (Invitrogen, Tokyo, Japan) and LA Taq polymerase (Takara, Shiga, Japan). A labeling reaction for DNA sequencing was performed using the BigDye terminator cycle sequencing kit (Applied Biosystems, Tokyo, Japan), and DNA sequences were determined by the direct sequencing method using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). DNA sequences were converted to amino acid sequences, and then amino acid mutations were extracted through comparison with amino acid sequences of the HIV-1 HXB2 strain. Judgment of drug-resistant amino acid mutations was performed according to the latest version of the International AIDS Society USA panel, Fall 2006.²³

Phylogenetic analysis

Phylogenetic analysis was performed using the nucleotide sequences of HIV-1 obtained from all 402 therapy-naïve patients. Nucleotide sequences (1005 bases) containing both PR (codons 1–99) and RT (codons 1–236) genes were used. Multiple sequence alignment was performed using CLUSTAL W, and evolutionary distances were calculated using the Kimura two-parameter model. A phylogenetic tree was constructed by the neighbor-joining method with 1000 bootstrap replicates. These analyses were performed using MEGA software version 3.1.²⁴ Nucleotide sequences of 32 reference HIV-1 strains were obtained from the HIV sequence database in the Los Alamos National Laboratory. Subtyping of HIV-1 was also performed using the phylogenetic tree.

Measurement of viral load and CD4 cell count

Viral load was measured using an Amplicor HIV-1 monitor v1.5 system (Roche Diagnostics, Tokyo, Japan). CD4 cell counts were measured using a FACSCalibur flow cytometry system (Becton Dickinson, Tokyo, Japan).

Statistics

Multiple logistic regression analysis was performed to assess associations between patient characteristics and infection with drug-resistant or 215-revertant HIV-1 variants. Values of $p < 0.05$ were considered statistically significant. Analyses were performed using SYSTAT version 10.2 software (SYSTAT Software, California, USA).

RESULTS

Emergence trend of drug-resistant HIV-1 in therapy-naïve patients

The prevalence of drug-resistant HIV-1 fluctuated between 2.3% and 10.0% through the period from 1999 to 2006 (Fig. 1). The first wave was observed from 2001 to 2003, with prevalence increasing from a trough of 2.3% in 2001 and peaking at 10.0% in 2003. After that, the prevalence dropped to 4.2% in 2004, but increased again to reach 8.8% by 2006. The mean prevalence for the past 8 years was 6.7% (27/402).

Variants with NRTI-resistant mutations were sporadically found (Fig. 2A). Concerning nonnucleoside reverse transcriptase inhibitor (NNRTI)-resistant variants, none was found from 1999 to 2002 (Fig. 2B). However, two variants with the K103N mutation first emerged in 2003, and this type of variant was continuously detected thereafter. Variants with the V108I and P225H mutations first emerged in 2004 and 2006, respectively. Variants with protease inhibitor (PI)-resistant mutations appeared continuously from 2000 (Fig. 2C). The most abundant variant was that with the M46I mutation alone, found in a total of 12 cases (2000, $n = 1$; 2002, $n = 2$; 2003 and 2004, $n = 1$ each; 2005, $n = 2$; and 2006, $n = 5$). In contrast, variants with the L90M, L33F, or M46L mutation alone appeared once each in 2001, 2003, and 2006, respectively. A variant possessing

TABLE 1. CHARACTERISTICS OF 402 THERAPY-NAÏVE HIV-1-INFECTED PATIENTS

Age, years		
Median (IQR ^a)	33	(28–41)
Sex		
Male	362	90.0%
Female	40	10.0%
Nationality		
Japanese	335	83.3%
Foreign	67	16.7%
Risk factor for infection		
Homosexual	237	59.0%
Heterosexual	87	21.6%
Bisexual	32	8.0%
Unknown	46	11.4%
CD4 cell count, cells/ μ l		
Median (IQR ^a)	270	(94–400)
Viral load, log ₁₀ copies/ml		
Median (IQR ^a)	4.77	(4.26–5.26)
HIV-1 subtype		
B	346	86.1%
Non-B ^b	56	13.9%

^aIQR, interquartile range.

^bCRF01_AE, 30; A, 9; C, 8; D, 4; F, 2; G, 2; unclassified, 1.

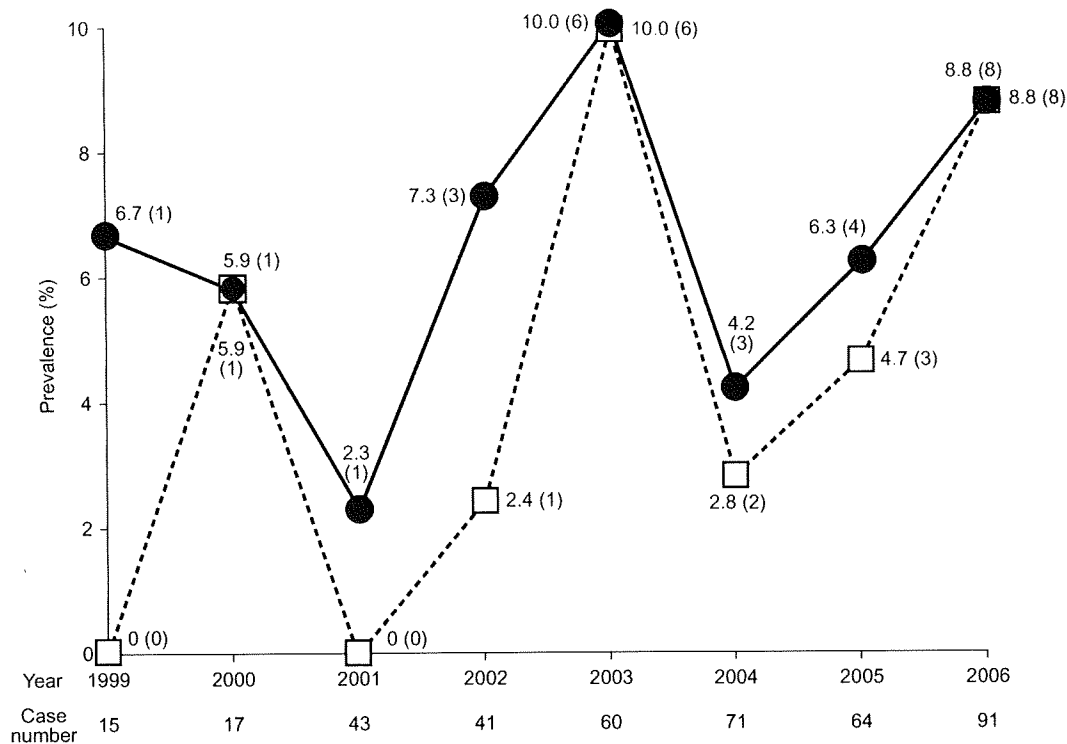


FIG. 1. Changes in prevalence of drug-resistant HIV-1 with major amino acid mutations (●—●) and 215-revertant variants (□—□) in therapy-naive patients. Genotypic drug-resistance testing was performed for 402 therapy-naive patients from 1999 to 2006. Detected numbers are shown in parentheses.

multiple mutations of V32I, M46I, I47V, and L90M was found very recently.

Characteristics of drug-resistant HIV-1

Characteristics of drug-resistant HIV-1 found in our surveillance are shown in Table 2. The most frequently found variant was a PI-resistant virus with the M46I mutation alone ($n = 12$), followed by an NNRTI-resistant virus with the K103N mutation alone ($n = 4$). Variants with two-class resistance were found in two cases, one possessing both PR- and NNRTI-resistant mutations, and the other with both PI- and NRTI-resistant mutations. Of note is the fact that no virus with resistance against all three classes was found in our surveillance.

Emergence trends for HIV-1 variants possessing the 215-revertant amino acid mutation in the reverse transcriptase

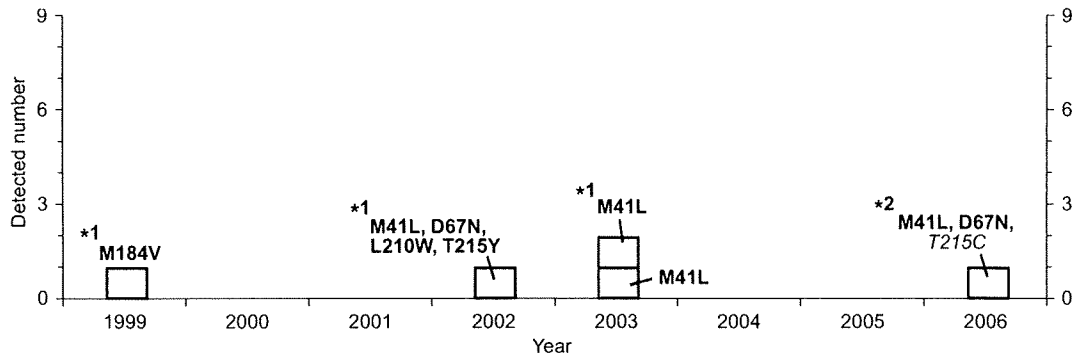
T215A/C/D/E/G/H/I/L/N/S/V amino acid substitutions in the RT represent revertant mutations of the T215Y/F NRTI-resistant mutation.^{2,3} The 215-revertant mutations do not exhibit NRTI resistance by themselves, but most can revert to the T215Y/F NRTI-resistant mutation by acquiring a single nucleotide mutation. In other word, most 215-revertant variants can much more easily change to NRTI-resistant variants under the

pressure of NRTIs than wild-type HIV-1.¹⁷⁻²⁰ We feel drug-resistant variants with the T215Y/F mutation are difficult to survive in the drug-free condition, as only one variant with the T215Y mutation has been found during an 8-year surveillance. The results of other researchers support our feelings.^{17,18} Examination of the emergence of the 215-revertant variant in addition to the T215Y/F possessing resistant variant is thus important. In our surveillance, variants possessing the T215A/C/D/E/G/L/S mutation were found in 21 cases; since T215G/D was found in 2000, such variants have been increasing (Fig. 2D). Among these, 17 cases (81%) can revert to the T215Y/F NRTI-resistant mutation by acquiring a single nucleotide mutation.

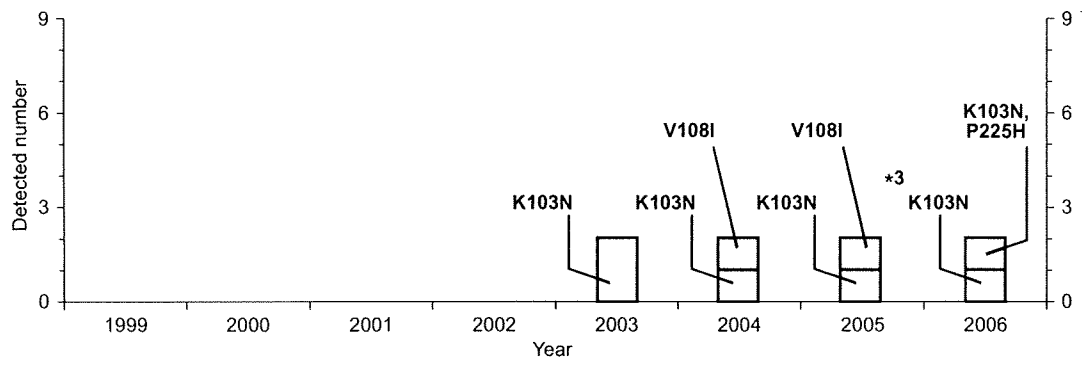
Phylogenetic analysis

This study identified 27 drug-resistant variants from 402 therapy-naive patients. We next performed phylogenetic analysis to clarify whether specific drug-resistant strains were spreading. Three different clusters were identified from 20 of 27 drug-resistant variants (#1-13, #14-18, and #19-20) on a phylogenetic tree (Fig. 3A). All the clusters were consisted of subtype B viruses. The remaining seven variants were dispersed over the tree (Fig. 3A, #21-27). Two out of the seven were non-B viruses, subtype D and CRF01_AE. Detailed divergence of

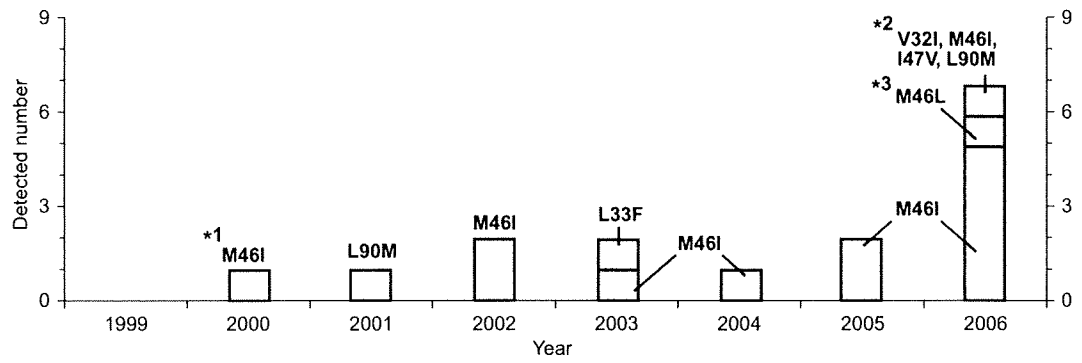
A



B



C



D

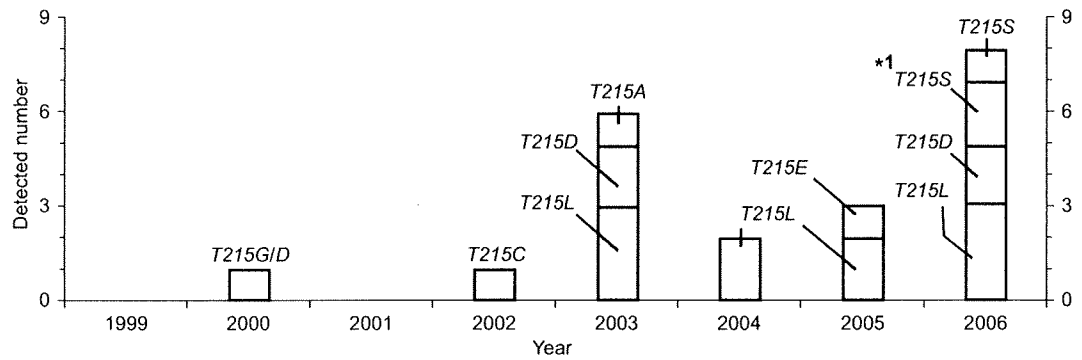


TABLE 2. CHARACTERISTICS OF DRUG-RESISTANT HIV-1

Type of drug resistance	n	Major drug-resistant amino acid mutations ^a
PI resistance alone	12	PR: M46I
14 (3.5%)	1	PR: L33F
1	1	PR: L90M
NNRTI resistance alone	4	RT: K103N
7 (1.7%)	2	RT: V108I
1	1	RT: K103N, P225H
NRTI resistance alone	2	RT: M41L
4 (1.0%)	1	RT: M41L, D67N, L210W, T215Y
1	1	RT: M184V
Two class resistance	1	PR: M46L
2 (0.5%)	1	RT: K103N
1	1	PR: V32I, M46I, I47V, L90M
		RT: M41L, D67N, T215C

^aMajor drug-resistant mutations and 215-revertant mutations are shown in bold and italics, respectively. PI, protease inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PR, protease; RT, reverse transcriptase.

the 13 PI-resistant variants (#1–13) with the M46I or L90M mutation is shown in Fig. 3B, indicating derivatives from a common ancestral strain. Four NNRTI-resistant variants and a two class-resistant variant possessing the K103N mutation formed another cluster (Fig. 3C, #14–18). Concerning the 215-revertant variants, 19 of 21 variants formed an independent cluster (Fig. 3A, #28–46; Fig. 3D). The clusters B and D were made by continual detection of the corresponding viruses from 2000 to 2006, but the cluster C from 2003 to 2006 (Fig. 3).

Statistical analysis

No significant differences in age, sex, nationality, risk factors for infection, CD4 cell count, viral load, or HIV-1 subtype were seen between patients with drug-resistant or 215-revertant variants and patients with wild-type viruses (data not shown).

DISCUSSION

The prevalence of drug-resistant HIV-1 among therapy-naive patients in Nagoya, Japan, was studied from 1999 to 2006. The mean prevalence was 6.7% (27/402), which is lower than that reported recently from European and North American countries (8.1–25.2%),^{25–37} but a tendency has recently been seen for increasing prevalence. Actually, prevalence has already exceeded the level at which the imple-

mentation of drug-resistance testing on therapy-naive patients is cost effective.^{38,39}

Over the past 8 years, the most abundant drug-resistant HIV-1 strains have been PI-resistant variants (3.5%, $n = 14$), and most have possessed the M46I mutation alone. The second most abundant variants were NNRTI-resistant HIV-1 (1.7%, $n = 7$), most of which possessed the K103N mutation. This type of variant with K103N was first found in therapy-naive patients in 2003. As the corresponding NNRTIs of nevirapine, efavirenz, and delavirdine were approved in Japan from 1998 to 2000, 3–5 years will be needed for the appearance of drug-resistant amino acid mutations in therapy-naive individuals after the start of drug usage. The sporadic finding of NRTI-resistant variants (1.0%, $n = 4$) in our surveillance seems curious, as NRTIs have been in use since 1987 in Japan. However, this may be explained by the finding that many HIV-1 variants with revertant mutations of the T215Y/F NRTI-resistant mutation have frequently been identified since 2000. Moreover, most (81%, 17/21) possessed 215-revertant mutations that could reconvert to the T215Y/F NRTI-resistant mutation through a single nucleotide change. Such highly resistant variants as three class-resistant variants have not yet been found, but two class-resistant variants were first identified in 2006.

Phylogenetic analysis yielded very important information, indicating that two independent major drug-resistant strains have been spreading in the Nagoya area, one possessing the M46I or L90M mutation and another possessing the K103N mutation. Furthermore, for 215-revertant variants, 19 of 21 variants were derivatives from the same strain, and have been independently spreading from 2000.

The present study succeeded in clarifying the epidemiological nature of drug-resistant variants and 215-revertant variants in Nagoya, Japan. Our data will provide information valuable for attempts to block the spread of these variants.

SEQUENCE DATA

The base sequences of drug-resistant HIV-1, 215-revertant HIV-1, and wild-type HIV-1 have been registered in the DNA databank of Japan (DDBJ) as #AB356098–AB356124, #AB356125–AB356145, and #AB356146–AB356499, respectively.

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FIG. 2. Emergence trends for drug-resistant HIV-1 and 215-revertant variants. The y-axis shows detected numbers of drug-resistant HIV-1 or 215-revertant variants: NRTI-resistant mutations (A), NNRTI-resistant mutations (B), PI-resistant mutations (C), and 215-revertant mutations (D). Major drug-resistant mutations and 215-revertant mutations are shown in bold and italic characters, respectively. *1, five variants detected in non-Japanese patients. *2, a variant simultaneously possessing M41L, D67N, and T215C mutations in the RT and V32I, M46I, I47V, and L90M mutations in the PR. *3, a variant possessing the K103N mutation in the RT and the M46L mutation in the PR.

FIG. 3. Phylogenetic analysis of HIV-1 strains from 402 therapy-naive patients. (A) A phylogenetic tree was constructed by the neighbor-joining method using nucleotide sequences (1005 bases) containing both the PR (codons 1–99) and RT (codons 1–236) genes. Bootstrap analysis was performed with 1000 replicates, and values greater than 70 were shown as orange dots at the nodes of the tree. The scale bars represent nucleotide substitutions per site. Green closed circles, NRTI-resistant variants; blue closed squares, NNRTI-resistant variants; red closed triangles, PI-resistant variants; brown closed diamonds, two-class-resistant variants. Green open symbols indicate HIV-1 variants with a 215-revertant mutation that can revert to the T215Y/F NRTI-resistant mutation by acquiring a single nucleotide mutation (green open circles) or more than two nucleotide mutations (green open triangles). Black open squares indicate reference HIV-1 strains. Group O_MVP5180 was used as the outgroup. Each cluster containing 13 variants with the M46I or L90M mutation in the PR (B), 5 variants with the K103N mutation in the RT (C), or 19 variants with the 215-revertant mutation in the RT (D) is shown as an enlarged figure. Major drug-resistant mutations and 215-revertant mutations are shown in bold and italics, respectively. PR, protease; RT, reverse transcriptase.

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Letter to the Editor

Pharmacokinetic Parameters of Lopinavir Determined by Moment Analysis in Japanese HIV Type 1-Infected Patients

EDITOR: We determined pharmacokinetic (PK) parameters of lopinavir (LPV) in Japanese HIV-1-infected patients, as PK data for LPV have not yet been reported from Japanese multiple-dose studies. Subjects comprised 65 HIV-1-infected patients (57 men, 8 women) with a mean age of 38 years (range: 22–62 years) and a mean weight of 63.8 kg (range: 50.0–80.8 kg), recruited at the Outpatient HIV Clinic of the National Hospital Organization Nagoya Medical Center, Japan, between March 2002 and January 2007. Patients were treated for >1 month with LPV/ritonavir (400/100 mg twice daily; Kaletra capsule) in combination with other antiretroviral agents. The combination of coadministered nucleoside reverse transcriptase inhibitors was ZDV-3TC in 37 patients, 3TC-TDF in 8 patients, 3TC-d4T in 8 patients, 3TC-ABC in 5 patients, ABC-TDF in 3 patients, TDF-FTC in 2 patients, ddI-TDF in 1 patient, and ddI-ABC in 1 patient. The drug adherence of each patient was confirmed by interview and viral load (<50 copies/ml). Also patients had not been administered any drugs metabolized through the CYP3A pathway. The hepatic and renal functions of all patients were normal.

Plasma sampling was performed in the afternoon at an outpatient HIV clinic. All samples were prospectively obtained for the purpose of routine therapeutic drug monitoring. LPV plasma concentrations were determined by high-performance liquid chromatography according to our previously described methods.¹ We measured LPV plasma concentrations six times on average (range: 1–32 times) for recruited patients. The distribution of LPV plasma concentrations ($n = 419$) is shown in Fig. 1. PK parameters (mean \pm SD) for LPV obtained using moment analysis were as follows: total area under the plasma concentration curve (AUC), $94.4 \pm 8.6 \mu\text{g} \cdot \text{h/ml}$; mean residence time (MRT), $10.0 \pm 1.2 \text{ h}$; elimination half-life ($t_{1/2}$), $5.1 \pm 0.9 \text{ h}$; appearance oral clearance (CL/F, $F =$ oral bioavailability), $4.23 \pm 0.3 \text{ liters/h}$; and volume of distribution (V/F), $42.6 \pm 1.6 \text{ l}$. AUC, $t_{1/2}$, CL/F, and V/F values obtained in this study were similar to those reported in American subjects.^{2–5} We have previously reported that the $t_{1/2}$ of LPV tended to be prolonged after food intake in a single-dose study of healthy Japanese individuals,⁶ but no such tendency was detected in this multidose study of Japanese HIV-1-infected patients. Our present data suggest that Japanese patients are sim-

ilar to American patients in terms of PK findings for LPV at steady state. The recommended dose of LPV in the guideline⁷ will thus also be suitable for Japanese HIV-1-infected patients.

The institutional review board of the National Hospital Organization Nagoya Medical Center approved this study and plasma samples were prepared from patients after obtaining written informed consent.

Moment analysis was performed using a PK analysis program developed by Tabata *et al.*⁸ Details of the method were previously reported by Yamaoka *et al.*⁹

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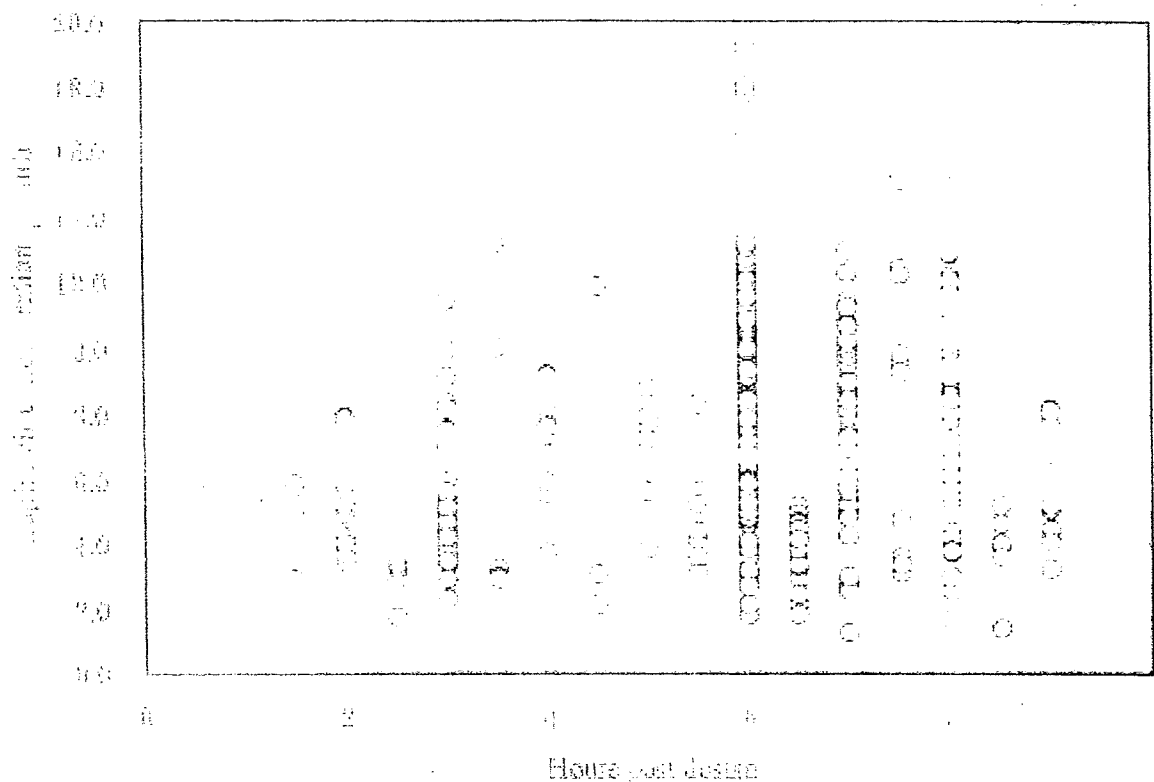


FIG. 1. Distribution of lopinavir plasma concentrations in 65 Japanese HIV-1-infected patients ($n = 419$). Measured concentrations are plotted versus hours postdosing.

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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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Naoto Mamiya, Motohiro Hamaguchi, and Tsuguhiro Kaneda

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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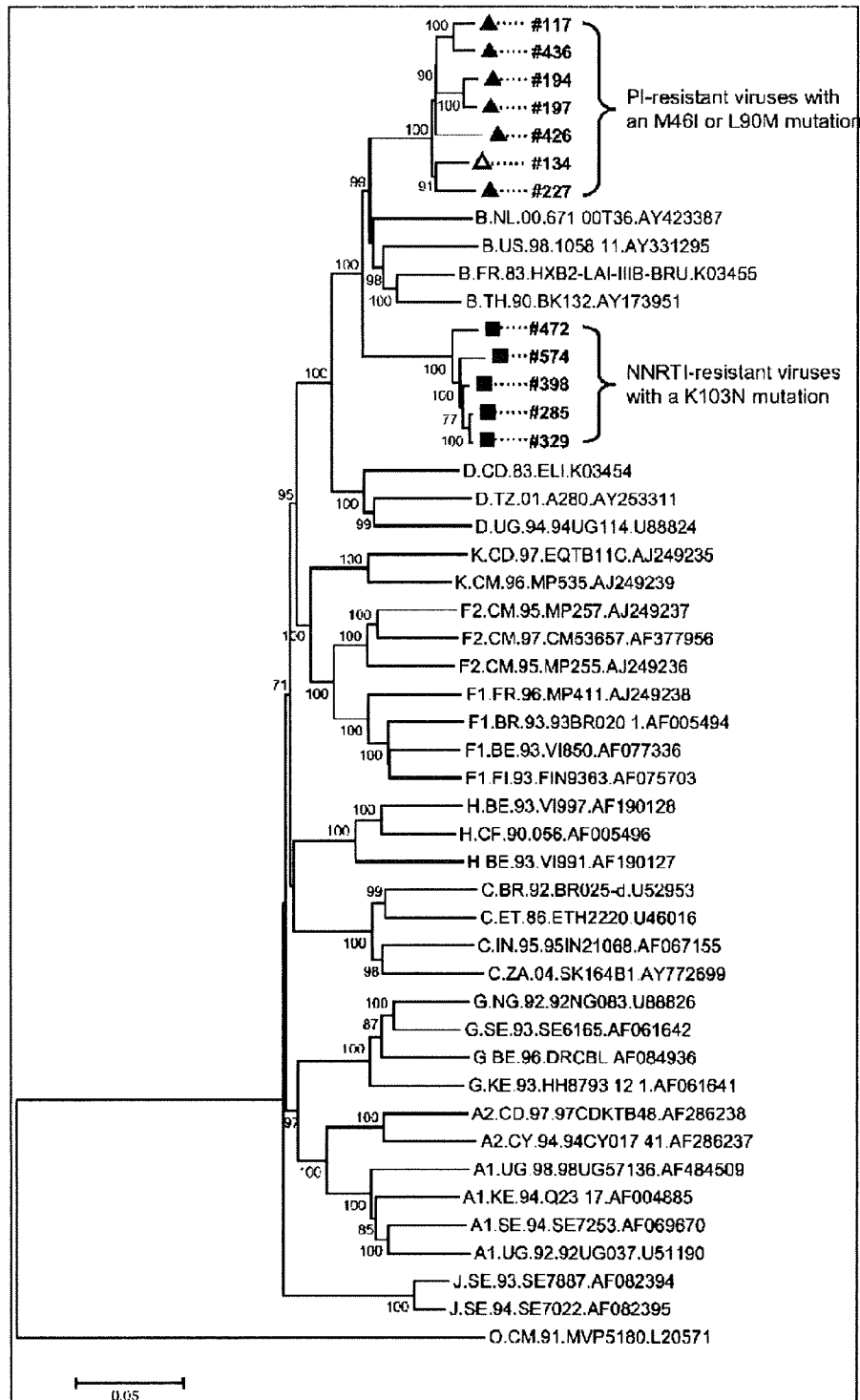


FIG. 1. Phylogenetic analysis of drug-resistant HIV-1. A phylogenetic tree was constructed using the neighbor-joining method with near full-length genomic sequences. Bootstrap values were calculated by 1,000 analyses and values greater than 70% were shown at the nodes of the tree. Scale bar represents 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			B Nef protein		
AA#	p6 ^{Pol}	Protease	AA#	Anchor domain	Core domain
	48	▼		57	▼
NL4-3	VSFSF	PQITL	NL4-3	ACAWL	EAQEE
HXB2	...N.	..V..	HXB2
117	I... L	117	DR.. V
134	..L NL	134	DR.. VD
194	I.. NL	194	D.. VD
197	I.. NL	197	D.. VD
227	..L. L	227	X ₂ X ₃ .. V
426	..L NL	426	.R.. V
436	I... L	436	DR.. V
285	285	D.V..	..H.D
329	329	D.V..	..H.D
398	398	D.V..	..H.D
472	472	D.V..D
574	574	D.V..	..H.D
C Gag p24 (capsid)			D Tat protein		
AA#	87		AA#	23	
NL4-3	PVHAGPIAP		NL4-3	CTNCYCKKCCFHCQVC	
HXB2		HXB2	
117	.. Q		117	.. NL.....	
134	.. AQ		134	.. N	
194	.. AQ		194	.. NY...A.	
197	.. AQ		197	.. NY.....	
227	.. Q		227	.. NL.....	
426	.. PQ		426	.. NS.....	
436	.. AQ ...X...		436	.. NW.....	
285	.. AQ ...HP.		285	.. N	
329	.. AQ ...HP.		329	.. N	
398	.. AQ ...HP.		398	.. N	
472	.. AQ ...HP.		472	.. N	
574	.. AQ ...HP.		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: X₁, D/A; X₂, R/C; and X₃, 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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A Conventional LC-MS Method Developed for the Determination of Plasma Raltegravir Concentrations

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Raltegravir belongs to a new class of antiretrovirals acting for a human immunodeficiency virus (HIV)-1 integrase inhibition. Clinical trials of this drug have demonstrated potent antiviral activity in both therapy naïve and experienced patients. Thus, raltegravir has become an important component of combination treatment regimens used to treat patients with multidrug-resistant HIV-1. The quantification of raltegravir in human plasma is important to support clinical studies and determine pharmacokinetic parameters of raltegravir in HIV-1 infected patients. Recently, the LC-MS/MS superfine system was developed to determine plasma concentration of raltegravir; however, the system needs to be delicately set and the equipment is very expensive. Therefore, we developed a conventional LC-MS method to overcome these difficulties. Subsequently the method was validated by estimating the precision and accuracy for inter- and intraday analysis in the concentration range of 0.010–7.680 µg/ml. The calibration curve was linear in this range. Average accuracy ranged from 97.2 to 103.4%. Relative standard deviations of both inter- and intraday assays were less than 10.4%. Recovery of raltegravir was more than 80.6%. This novel LC-MS method is accurate and precise enough to determine raltegravir levels in human plasma samples.

Key words human immunodeficiency virus-1; LC-MS; therapeutic drug monitoring; raltegravir

The clinical treatment of patients with human immunodeficiency virus (HIV)-1 infection has been advanced by the success of highly active antiretroviral therapy (HAART). However, it became clear that the long-term administration of HAART was limited by toxicity associated with many of these treatments^{1,2)} as well as by the development of resistance.^{3–6)} Therefore, new antiretroviral drugs, which act on different action points from DNA elongation and protein processing in HIV-1 life cycle, are required to continue effective HAART for the treatment of HIV-1.

Raltegravir is one of a new class of antiretroviral agents that work by inhibiting the insertion of viral DNA into the cellular genome, resulting in virus replication prevention.^{7–10)} Therefore, raltegravir is expected to treat therapy-experienced patients where protease inhibitor (PI) and/or reverse transcriptase inhibitor-resistant HIV-1 had developed.^{11–13)}

We have a routine system, by which all PI and efavirenz plasma concentrations are easily determined by HPLC,¹⁴⁾ and therapeutic drug monitoring was performed as needed.¹⁵⁾ In this study, we aimed to develop the determination method of plasma raltegravir.

Recently, a determination method using liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been reported.¹⁶⁾ However, the MS-MS detector needs to be delicately set and LC-MS/MS equipment is very expensive. In addition, isotope labeled raltegravir as an internal standard (IS) is needed. To bypass these difficulties, we aimed to develop more conventional procedures for determining raltegravir using liquid chromatography coupled with mass spectrometry (LC-MS).

MATERIALS AND METHODS

Chemicals and Reagents Raltegravir was supplied by Merck Research Laboratories (Rahway, NJ, U.S.A.) and the internal standard (IS), A-86093:(5*S*,8*S*,10*S*,11*S*)-9-hydroxy-2-cyclopropyl-5-[(1-methylethyl)-1-[(2-1-methylethyl)-4-thiazoly]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraaza-tridecan-13-oic acid, 5-thiazolylmethyl ester, was provided by Abbott Laboratories (Abbott Park, IL, U.S.A.). Their chemical structures are shown in Fig. 1. Methanol, hexane, methylene chloride, and acetonitrile (Kanto Chemical, Tokyo, Japan) were HPLC grade. Ammonium acetate, EDTA and acetic acid were purchased from Katayama Chemical (Osaka, Japan). Water was deionized and osmosed using a Milli-Q[®] system (Millipore Corp., Bedford, MA, U.S.A.). All other chemicals and solvents were of analytical grade.

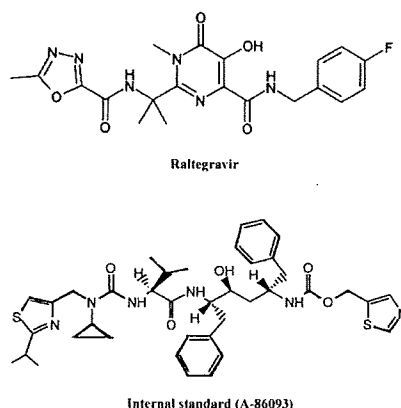


Fig. 1. Chemical Structures of Raltegravir and the Internal Standard A-86093

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Equipment A Waters Alliance 2695 HPLC and a Micromass ZQ-2000 MS (Waters Assoc., Milford, MA, U.S.A.), controlled with MassLynx version 4.0 software, were used for detection. The analytical column was a SunFire C₁₈ column (3.5 μ m, 2.1 \times 50 mm, Waters), protected by a SunFire C₁₈ Guard Column.

Chromatographic and Mass Spectrometric Conditions The mobile phase was a mixture of 0.1 mM EDTA in 0.1% acetic acid (A), 100% acetonitrile (B) and 100% methanol (C). An isocratic mobile phase consisting of A–B–C (65 : 15 : 20) was used during the first 2 min of the run, followed by a linear gradient elution consisting of A–B–C (10 : 70 : 20) for the next 8 min. The final conditions were maintained for the final 5 min. The system was then reequilibrated for an additional 13 min using the initial conditions. The flow rate of the mobile phase was 0.2 ml/min, the column temperature was 40 °C, and the amount of injected sample was 5 μ l.

The mass spectrometer was operated in positive ion electrospray mode. The capillary sprayer voltage was 3.5 kV and the sample cone voltage was 30 V for both raltegravir and A-86093. The source temperature was 120 °C and the desolvation temperature was 350 °C. The desolvation and cone gas flow-rates were set to 600 and 50 l/h, respectively. The acquisition mass range is m/z 200–800 at 0.5 s per scan with a 0.1 s interscan delay. All mass spectra are acquired in centroid mode.

Quantitative analysis, carried out in Selected-ion recording (SIR) mode, detected raltegravir at m/z 445, and the internal standard (IS), A-86093, at m/z 748, all in the form of ions. The quantitation calculations were performed using analytical software, MassLynx version 4.0 (Waters).

Standard Solutions Stock solutions of raltegravir and A-86093 were prepared by dissolving accurately weighed amounts of each reference compound in water/ethanol (50 : 50, v/v) to yield concentrations of 384.0 μ g/ml of raltegravir and 41.0 μ g/ml of A-86093. These stock solutions were stored at –80 °C and thawed on the day of analysis. The stock solution was diluted in drug-free plasma to yield ralte-

gravir concentrations of 0.010, 0.192, 1.920, 3.840 and 7.680 μ g/ml.

Sample Preparation Two milliliters of methylene chloride/hexane (50 : 50, v/v) containing the IS (0.328 μ g/ml) and 0.3 ml of 0.2 M ammonium acetate were added to a 500 μ l plasma sample prepared from peripheral blood anticoagulated with heparin. The mixture was vortexed for 5 min and then centrifuged at 3500 g for 5 min. The upper layer was separated and evaporated dry. The dried material was then dissolved in 50 μ l of a mobile phase solution. Lastly, 5 μ l of the upper solution was injected into the LC-MS system. The institutional review board of National Hospital Organization Nagoya Medical Center approved this study and each subject provided written informed consent.

Validation Inter- and intraday precision values using this method were estimated by assaying control plasma containing five different concentrations of raltegravir five times on the same day and on three separate days to obtain the relative standard deviation (RSD). Accuracy was determined as the percentage of the nominal concentration. To assess the absolute recoveries of raltegravir extracted from plasma, the peak area ratios of the analytes to the internal standard were compared with those obtained from the mobile phase having the same concentration. The mean recoveries were determined in triplicate.

RESULTS

LC-MS Chromatograms Figures 2A and B show selected-ion recording chromatograms obtained from a spiked plasma sample containing 0.192 μ g/ml of raltegravir and 0.328 μ g/ml of A-86093 (IS). Under the described chromatographic conditions, retention times were 8.2 min for raltegravir and 12.9 min for A-86093. Figures 2C and D show chromatograms obtained from a blank plasma sample. Assays performed on drug-free human plasma succeeded to show no interfering peaks during the interested intervals of the retention times. Figure 2D is the expanded figure of the

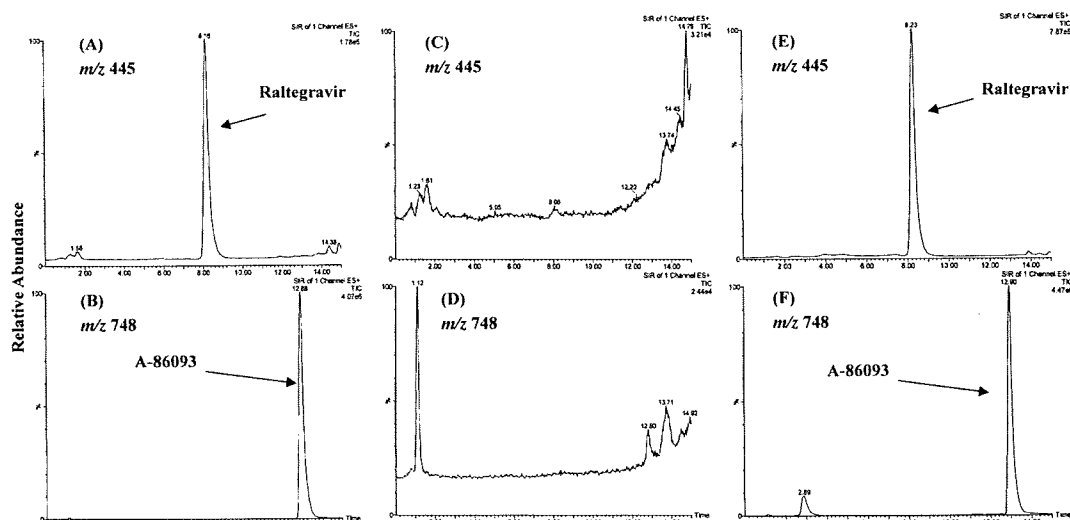


Fig. 2. Selected-ion Recording Chromatograms for Raltegravir and A-86093

(A) and (B) were obtained from a spiked plasma containing 0.192 μ g/ml of raltegravir and 0.328 μ g/ml of A-86093 (IS). (C) and (D) were obtained from a blank plasma sample. (E) and (F) were obtained from a plasma sample from an HIV-1-infected patient on raltegravir. (A), (C) and (E) were monitored with m/z 445. (B), (D) and (F) were monitored with m/z 748. (C) and (D) are the expanded figures of the baselines in (A) and (B), respectively.

Table 1. Intraday and Interday Precision and Accuracy for Raltegravir

Expected ($\mu\text{g/ml}$)	Intraday ($n=5$)		Interday ($n=5$)		Accuracy (%)	Recovery (%)
	Measured ($\mu\text{g/ml}$)	RSD (%)	Measured ($\mu\text{g/ml}$)	RSD (%)		
0.010	0.010 \pm 0.001	8.5	0.010 \pm 0.001	10.4	103.4 \pm 10.7	83.7 \pm 9.8
0.192	0.192 \pm 0.011	5.7	0.187 \pm 0.016	8.7	97.2 \pm 8.5	83.6 \pm 3.1
1.920	1.932 \pm 0.095	4.9	1.912 \pm 0.089	4.6	99.6 \pm 4.6	87.4 \pm 7.0
3.840	3.872 \pm 0.118	3.0	3.825 \pm 0.184	4.8	99.6 \pm 4.8	80.6 \pm 6.5
7.680	7.656 \pm 0.097	1.3	7.631 \pm 0.186	2.4	99.4 \pm 2.4	86.5 \pm 6.1

RSD, relative standard deviation. Means \pm S.D.

Table 2. Plasma Raltegravir Concentrations after the Oral Administration of 400 mg of Raltegravir to an HIV-1-Infected Patient

Time (h)	Raltegravir concentration ($\mu\text{g/ml}$)
0	1.24 \pm 0.03
0.5	1.78 \pm 0.02
1	5.21 \pm 0.17
2	3.14 \pm 0.05
3	2.67 \pm 0.04
6	1.46 \pm 0.02

Means \pm S.D.

baseline part of Fig. 2B. These peaks did not affect the quantification of IS. Figures 2E and F show chromatograms of a plasma sample from an HIV-1-infected patient treated with raltegravir. There were no interfering peaks affecting quantification of raltegravir in this chromatogram. Anticoagulants of heparin and EDTA did not hinder the selected-ion recording chromatograms for raltegravir and A-86093.

Validation: Linearity, Precision, Accuracy and Recovery Calibration curves of raltegravir appeared linear in the concentration range of 0.010 to 7.680 $\mu\text{g/ml}$ with a correlation of 1.000.

Precision, accuracy, and recovery of our LC-MS method are shown in Table 1. The selected concentration of raltegravir covers the expected plasma concentrations found in the patients. The RSDs calculated for raltegravir in the inter- and intraday assays ranged from 1.3 to 10.4%, which are similar to previously reported values.¹⁶⁾ Accuracies ranged from 97.2 to 103.4%. Recoveries from plasma ranged from 80.6 to 87.4%. Mean extraction recovery of the IS was 87.9%. These results indicate that this method achieves a high degree of reproducibility and accuracy.

Raltegravir Concentrations in Plasma Plasma raltegravir concentrations in an HIV-1-infected patient are shown in Table 2. The patient received oral administration of 400 mg raltegravir twice daily. The samples were collected on day 8 after the start of HAART. When raltegravir is administered by a single 400 mg dose, plasma concentrations are expected in the 0.01 to 4.71 $\mu\text{g/ml}$ range.^{16–18)} In this study, raltegravir concentrations at steady state following multiple-dose administration ranged from 1.2 to 5.2 $\mu\text{g/ml}$.

DISCUSSION

Clinical trials of raltegravir have demonstrated potent antiviral responses in both therapy naïve and experienced patients.^{11,19,20)} Moreover, raltegravir has demonstrated a clean safety profile in these studies and may not have the tox-

icity and tolerability issues as the current anti-HIV drugs. Thus, raltegravir has become an important component of combination treatment regimens and its use has been initiated for the treatment of heavily pretreated patients with a multidrug-resistant virus.

We first wanted to judge whether therapeutic drug monitoring of raltegravir is necessary. To achieve this, the development of determination method for raltegravir is essential. Until now there has been a methodological report for the determination of raltegravir using LC-MS/MS.¹⁶⁾ However, this method has several disadvantages in terms of cost performance and essential equipment; for example, the authors used isotoped labeled raltegravir and/or the setting of the LC-MS-MS equipment.

To avoid such disadvantages we decided to use an LC-MS method using an available IS (A-86093) for determining plasma protease inhibitor concentrations. The reason we chose ritonavir analogue A-86093 is the stability and better elution point of the compound on the HPLC as were reported previously.¹⁴⁾ Validation showed our method was successful in measuring plasma raltegravir with high precision and satisfactory RSD values. The raltegravir calibration curve was linear at the concentration range of 0.010 to 7.680 $\mu\text{g/ml}$, and the average accuracy ranged from 97.2 to 103.4%. Both inter- and intraday RSDs for raltegravir were less than 10.4%, which is similar to previously reported values.¹⁶⁾ Recovery of raltegravir was more than 80.6%. These results indicate our newly developed method achieves the same level of reproducibility and accuracy as the LC-MS/MS method. As plasma concentrations of raltegravir are expected in the 0.01 to 4.71 $\mu\text{g/ml}$ range when raltegravir is administered at single dose of 400 mg,^{16–18)} our method successfully covers this region with good precision and accuracy. Actually, the raltegravir concentration change was clearly demonstrated; it rose from 1.2 (0 h) to 5.2 $\mu\text{g/ml}$ (1 h), then decreased to 1.5 $\mu\text{g/ml}$ (6 h) when raltegravir was orally administered 400 mg twice daily in an HIV-1-infected patient.

Recently, Poirier *et al.* reported the HPLC method for determining plasma raltegravir concentration with fluorescence detection.²¹⁾ Our's and Poirier's methods can specifically determine the raltegravir concentration and the sensitivities seem almost equivalent. Therefore, an alternative use is possible according to the availability of the equipments.

This conventional LC-MS method can provide a routine clinical application, and permits management of drug interactions and toxicity.

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