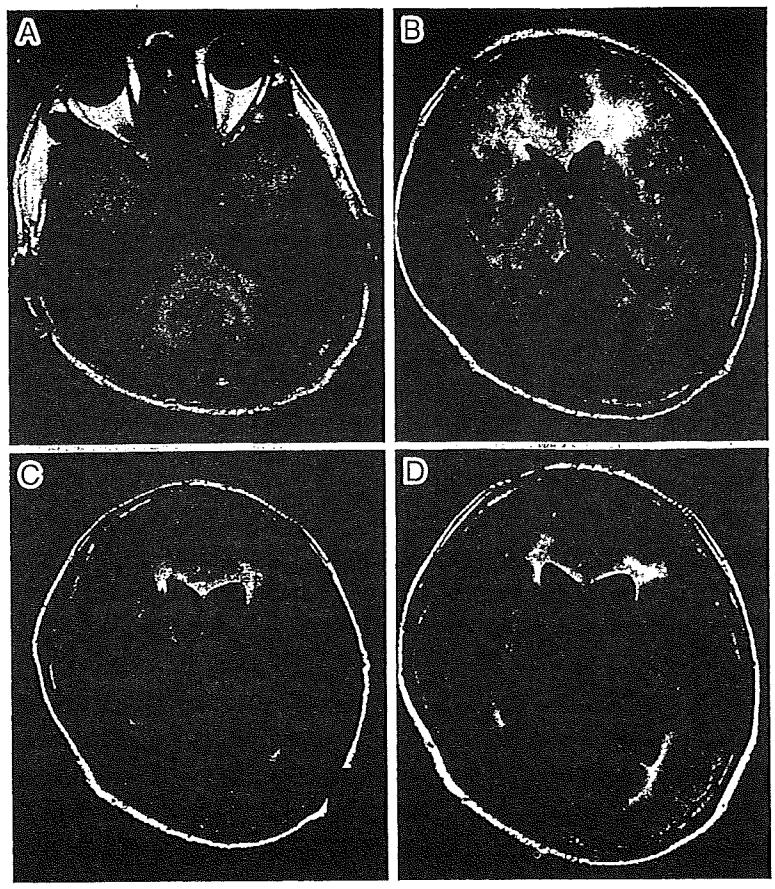

Fig. 1　Brain MRI (FLAIR) of case 1
A and B which were done at admission, revealed a diffuse lesion with high signal in the brain stem, cerebellar peduncles and cerebral white matter, atrophy of the basal ganglia and frontal lobes. C and D, which were done at 6 and 31 months respectively after HAART, showed that diffuse cerebral atrophy in the white matter had remarkably progressed.

時呼吸苦が出現．同年 8 月，発熱，呼吸苦にて前医入院した際に HIV 抗体陽性と判明し，カリニ肺炎と診断され治療を受けた．その頃からものわすれ，ふらつきを自覚．同年 9 月，当院に転院した．転院時，発動性の低下，動作緩慢，起居動作や歩行時にふらつきがあり，つぎ足歩行は不可能であり，その他の脳神経や筋力，感覚，腱反射には異常をみとめなかった．神経心理検査では，HDS-R 24 点，WAIS-R は言語性 IQ 86，動作性 IQ 65，全体 IQ 74 と認知機能障害をみとめた．また，50 音表を書くのに 80 秒かかり，立方体模写はできないなど，動作速度の低下や空間能力低下をみとめた．カリニ肺炎治療終了の 1 カ月後より HAART を開始したが，ふらつきの悪化，部屋をまちがえたり，前日のでき事を忘れたりといったことがしだいにめだつようになった．入院 1 カ月後に施行した頭部 MRI にて T_2 強調画像で両側前頭葉白質に左右対称性にびまん性に広がる高信号域をみとめ，HIV 脳症と診断した．HAART 開始 5 カ月後の神経学的診察では異常をみとめなかったが，MMSE は 28 点と認知機能障害の残存をみとめた．診断から約 1 年後，不安焦燥感を執拗に訴えて本人の強い希望で緊急入院したが，入院翌日に院内備品を持ち出したところを地下鉄職員に発見され，窃盗のうたがいで警察の事情聴取を受けた．その後，外来を不定期受診している．

症例 3　51 歳男性

職業は会社経営．2004 年 5 月に全身倦怠感と発熱が出現．同年 6 月に異常言動と失見当識が加わりしだいに傾眠となったため前医に入院し，HIV 抗体陽性と判明したため 7 月，当院に転院した．転院時 MRI では T_2 強調画像にて両側基底核に小病変をみとめた．当初，意識障害の原因としてクリプトコッカス髄膜炎，トキソプラズマ脳炎をうたがい治療を開始したが，血清および髄液中のクリプトコッカス抗原ならびに血清トキプラズマ抗体は陰性であった．血液培養から非定型抗酸菌が検出され，発熱は非定型抗酸菌敗血症によるものと診断し抗菌剤にて改善した．また，CD4 4μl と低値であったため HAART を施行した．その後，意識障害は改善し，尿失禁を残すも他の日常生活動作は自立となり約 4 カ月後に退院した．退院前後よりしだいに躁状態となり，入院中に高価な身の回りの品を換金して無断外出をしたり，退院後は妻が外出している隙に知人と旅行に行くといった行動障害が出現した．服薬アドヒアランスも不良となり，ふらつきが悪化し歩行困

難となるなどの運動機能障害が悪化したため再入院となった．再入院時，動作緩慢，失調性歩行で両側バビンスキー徴候陽性，尿失禁をみとめ，HDS-R 19点であった．血液検査はCD4 171/μl，HIVウイルス量は感度以下であった．髄液検査および頭部MRIでは日和見感染症を示唆する所見はみとめず，経過からHIV脳症を当初より合併していたと考えHAARTを継続した．深夜に家族と偽って知人を病室に招き入れるなど病棟のルールを守れずに強制退院となった．その後は精神科外来にて抗躁薬と抗精神病薬を投与し，徐々に落ち着きを取りもどしたが，HDS-Rは20点前後で推移している．

症例4　28歳男性

職業は代用教員．18歳時にバーキットリンパ腫に対し自己末梢血幹細胞移植を受け治癒している．2005年5月ころからものわすれを自覚した．同年6月，バーキットリンパ腫の経過観察のため施行した血液検査で汎血球減少を指摘され，前医に入院しHIV抗体を測定したところ，陽性と判明した．同年7月，職場で倒れているのを発見され救急車にて当院を受診した．体温37.7℃，朦朧状態で物品呼称および理解は比較的保たれているが復唱はできず，上肢の観念運動失行，右同名半盲，右注視麻痺，構音障害，右不全片麻痺，バビンスキー徴候右陽性をみとめた．入院時頭部MRIではT_2強調画像にて左右対称性びまん性の白質病変をみとめた(Fig. 2)．入院3日目より右片麻痺，失語は急速に改善し，入院1週間後の診察では失見当識をみとめるが失語や麻痺は消失していた．動作緩慢であり，50音表の書き取りに105秒かかった．MMSEは22点，立方体は模写できなかった．WAIS-Rは言語性IQ 84，動作性IQ 79，全体IQ 80と低下しており，空間能力低下，短期記憶障害などの認知機能障害をみとめた．SPECTでは両側前頭葉の血流低下に加え，左頭頂葉付近の血流増加をみとめ，脳波では左前頭部に棘波をみとめた．運動機能障害と認知機能障害をみとめ，頭部MRIでも白質病変をみとめることから，亜急性にHIV脳症を生じており，今回の入院契機であった一過性の大脳左半球症状はてんかん様発作であった可能性が考えられた．その後外来にてHAARTを施行した．発症より約6カ月後，見当識は良好だが時に単語がすっと出てこないことがある．運動障害はなく，HDS-Rは27点，MMSEは25点，50音表の書き取りは35秒で可能だがラ行が抜けていた．立方体模写は可能となった．HAART前後での頭部MRIを比較すると，わずかに病変は縮小しており，画像検査上もHIV脳症の改善をみとめた．転職し社会復帰を果たしている．

症例5　63歳男性

職業は会社員．2005年8月に微熱と全身倦怠感，体重減少を自覚した．10月初旬より上記に加えて湿性咳嗽，見当識障害，夜間せん妄が出現した．10月中旬に体重減少と呼吸苦の精査にて前医入院し，胸部CTにて間質性肺炎，胃内視鏡下生検にてサイトメガロウイルス胃炎と判明し，HIV抗体陽性であったため当院に転院した．転院時，呼吸不全をみとめ，神経学的診察では，軽度意識障害（Japan Coma Scale-2），自発性

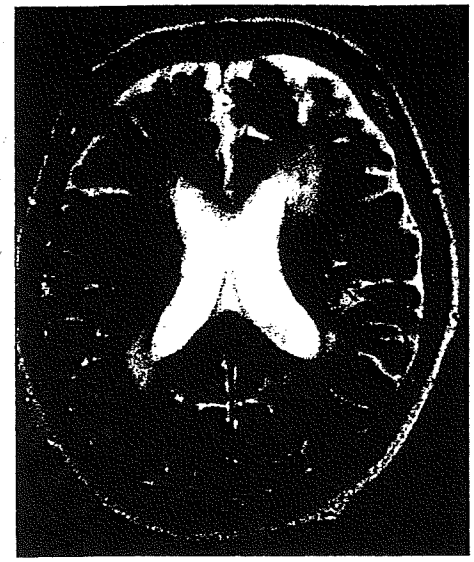

Fig. 2　Brain MRI (T2 weighted image) of case 4
It revealed that the diffuse high intensity area in cerebral white matter spared the subcortex.

低下，動作緩慢をみとめ，指鼻試験および膝踵試験は拙劣で，起居動作や歩行時にはふらつきがあり，つぎ足歩行はできなかった．立方体模写は不可能で，1から26の数唱に26秒，同じく書き取りに36秒かかった．50音表の書き取りは途中で止まってしまい遂行できなかった．全身状態が安定した後に施行したHDS-Rは12点，WAIS-Rは言語性IQ 62，動作性IQ 52，全体IQ 55と著明な低下をみとめた．頭部MRIにて，橋と両側大脳前頭葉白質から脳梁にT_2強調画像でびまん性の高信号域をみとめた．当院転院後，ST合剤およびステロイドによるカリニ肺炎の治療を施行し呼吸状態は改善したが，神経学的には変化がなかった．11月よりHAARTを施行したところ直後に一過性の譫妄をきたしたが，開始1週間後より病室で小説を読み，徐々に他の症状も改善していった．HAART開始1カ月後の診察では意識清明，歩行は自立しているがつぎ足歩行は不可能であった．HDS-R 29点，MMSE 30点と改善をみとめた．50音表書き取りは「な」行でとまってしまった．2カ月後に自宅退院され，現在も外来にてHAARTを施行している．

考　察

HIV感染症は近年，HAARTをはじめとする治療法の進歩によって当初恐れられていた日和見感染症や悪性腫瘍は減少傾向を辿っており[2)3)]，中枢神経合併症も同様の傾向を呈している[4)~7)]．しかし，HIV脳症はHAARTによっても発症頻度が減少しないとされ[5)8)]，その理由としてはHAARTによってHIV脳症をふくめたAIDS症例全体の生命予後が改善することが指摘されている[9)]．

今回のHIV脳症5例について，診断時のCD4とHIVウイルス量をTable 2にまとめた．いずれもCD4は200/μl以下

で平均 22.4/μl ときわめて低値であり，諸外国での HAART 導入以前と同様の傾向を示している[8]．他の日和見感染症を合併しており，高度の免疫不全状態であったと思われた．

次に，HIV 脳症診断時の髄液検査所見については，Table 3 に示すように症例 5 を除いていずれも細胞数は正常（症例 5 についても 1 週間後の再検査時には正常），蛋白は正常から微増であった．測定しえた症例では，髄液中 β-2 ミクログロブリンはいずれも 2μg/ml を超えていた．髄液中の HIV ウイルス量はばらつきが多いものの症例 1，3，4 では血液中のウイルス量と比較しても高値であった．髄液中の糖は全症例とも低値を示した．HIV 脳症において髄液中の細胞数増多や蛋白の上昇がときにみとめられることは知られているが，髄液中の糖についてはあまり検討がなされておらず，Navia らが HIV 脳症 41 症例中 1 例のみ糖が低値であったと報告している[1]．われわれが経験した 5 症例において，頭蓋内の細菌感染症は髄液培養検査が陰性であったことや経過から否定的であり，髄液中の糖が低値であった理由は不明であった．

HIV 脳症の症状は運動，認知，行動の 3 つに大別される[1]．今回の 5 症例において，運動障害と認知障害は程度の差異はあるものの全症例にみとめられたが，行動異常の有無については症例差がいちじるしかった．運動機能障害については動作緩慢は全症例とも改善をみとめた．失調は完全消失にはいたらないものの改善傾向であり，結果として関節炎による関節拘縮をきたした症例 1 以外を除いては日常生活動作が自立となっており，運動機能予後は良好と思われた．

認知機能について，経過中に適宜施行した HDS-R もしくは MMSE の結果からはいずれの症例も追跡しえた範囲では改善傾向にあり，症例 1 は 31 カ月を経過した時点でもなお改善傾向にあるが，依然障害は残存している．

認知障害とならんで，行動障害は服薬アドヒアランスを大きく低下させ療養を困難とする要因となった．症例 1，2，4 では経過中に顕著な行動異常が出現し，今回の症例で唯一症例 4 のみが就労を果たした．他の症例と比較すると認知機能障害の残存はみとめていたものの，診断当初より無気力をはじめとする行動障害をともなっていなかったことがその要因と思われた．このことからは，HIV 脳症に特徴とされる運動機能障害，認知機能障害，行動障害のうち，行動障害が強いばあいには就労は困難となりうることが示唆された．

HIV 脳症の治療として，全症例ともできるだけ早期に HAART を導入した[10][11]．今回追跡しえた期間内は死亡をみとめず，他の中枢神経疾患が多くのばあいに致死性である[7][12]ことを考えると，HIV 脳症の短期間の生命予後は良好であると思われた．その一方で機能予後は不良と考えられ，HAART のみでは治療効果は不十分であると思われた．今後，HAART に加えてあらたな治療の確立が望まれる[13][14][15]．

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Abstract

Clinical features and courses of 5 cases with HIV encephalopathy

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Clinical features and courses of 5 cases with HIV encephalopathy were reported. The subjects were among the patients registered as HIV patients at the Nagoya Medical Center, between 1996 and 2005. There were 458 patients with HIV infection including 127 cases of AIDS. All patients suffered from severe immunological deficiency when HIV encephalopathy developed. Other opportunistic infections had also occurred in three patients. HIV encephalopathy was one of the presenting manifestations of HIV infection in four patients, and no patients had received antiretroviral therapy. HAART improved motor disturbance and their ADL became independent except for one case. Improvements in neuropsychological examination scores were noted in all cases. Recovery from psychiatric symptoms, however, was incomplete. Four patients could not work, and 3 needed psychological treatment due to behavioral abnormalities. HIV encephalopathy is not a lethal disease but the functional prognosis was very poor. New therapy is needed for HIV encephalopathy.

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Key words: HIV, AIDS, cognitive impairment, behavioral change, prognosis

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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 reconvert 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-naive 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]

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/μl		
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%

[a]IQR, interquartile range.
[b]CRF01_AE, 30; A, 9; C, 8; D, 4; F, 2; G, 2; unclassified, 1.

Phylogenetic analysis

Phylogenetic analysis was performed using the nucleotide sequences of HIV-1 obtained from all 402 therapy-naive 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-naive 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

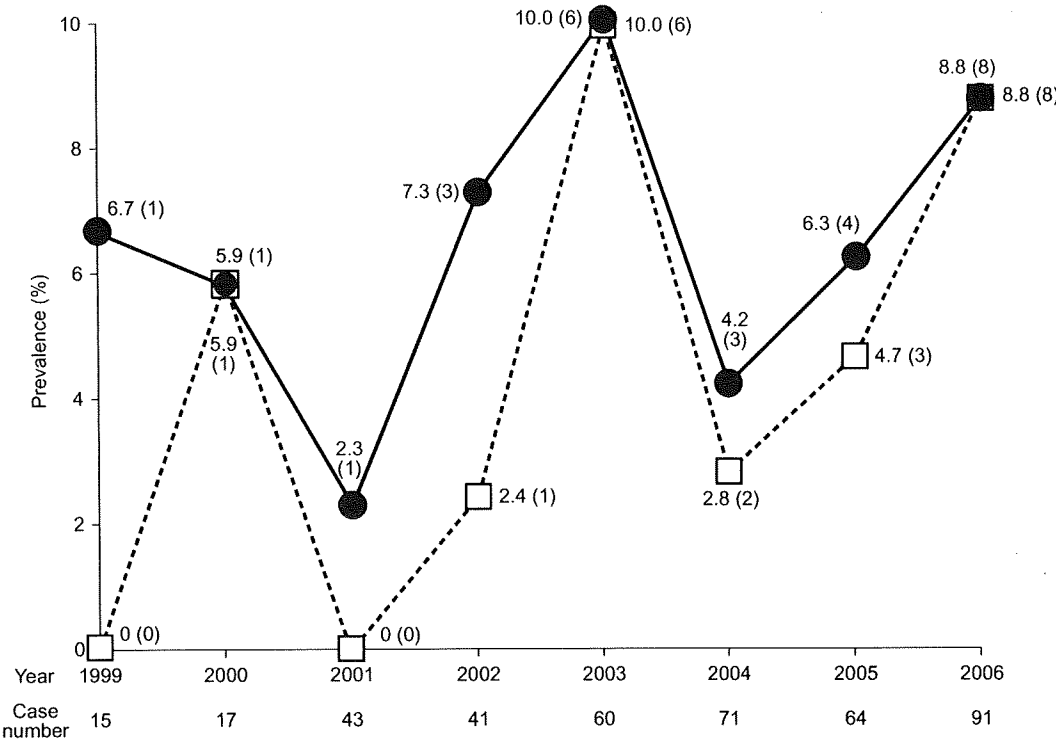

FIG. 1. Changes in prevalence of drug-resistant HIV-1 with major amino acid mutations (●—●) and 215-revertant variants (□—□) in therapy-naive patients. Genotypic drug-resistance testing was performed for 402 therapy-naive patients from 1999 to 2006. Detected numbers are shown in parentheses.

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.[23] The 215-revertant mutations do not exhibit NRTI resistance by themselves, but most can reconvert 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 reconvert 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

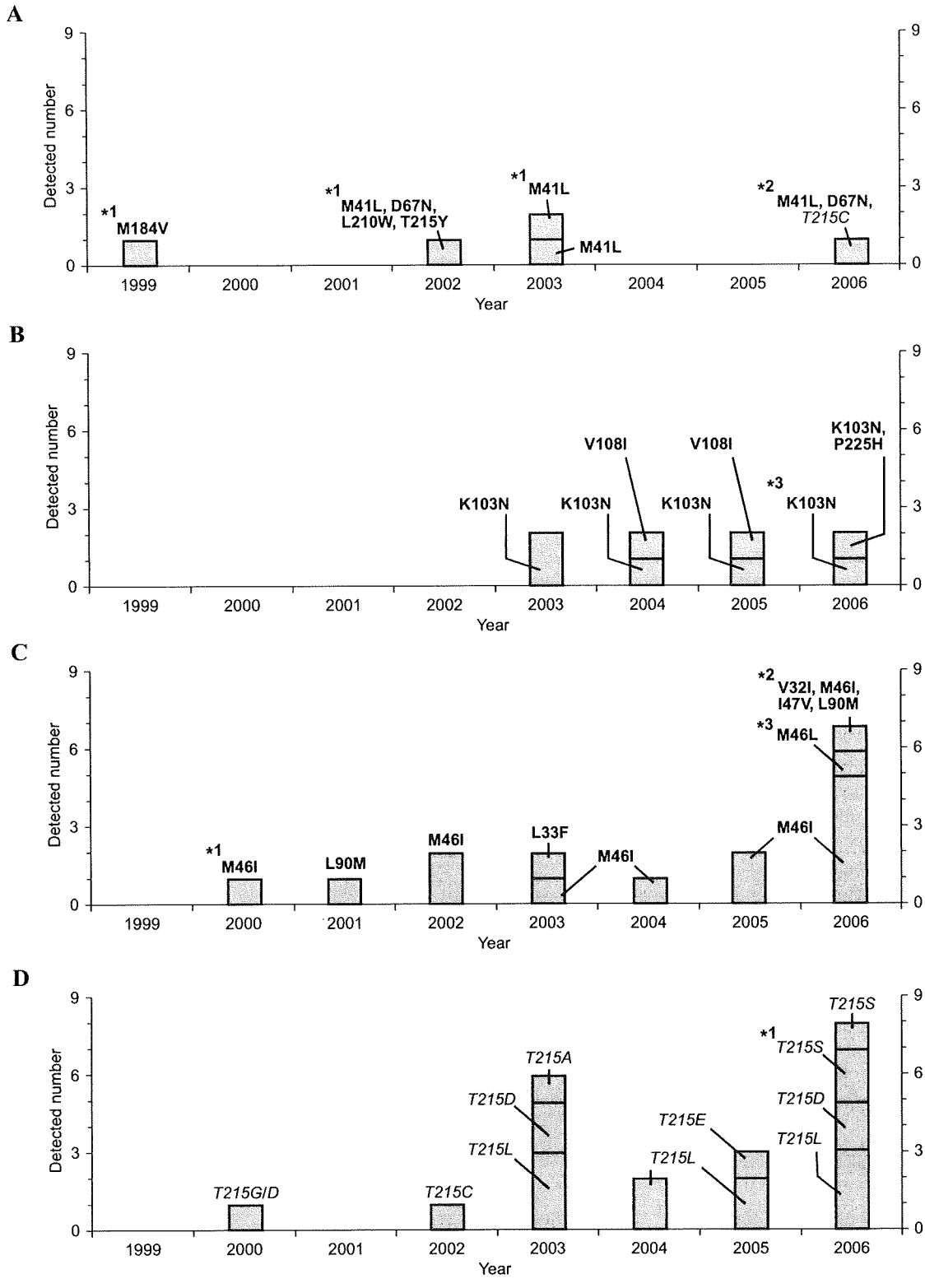
A
Detected number
Year
1999
2000
2001
2002
2003
2004
2005
2006
*1 M184V
*1 M41L, D67N, L210W, T215Y
*1 M41L
M41L
*2 M41L, D67N, T215C
B
K103N
K103N
V108I
K103N
V108I
*3 K103N
K103N, P225H
C
*1 M46I
L90M
M46I
L33F
M46I
*2 V32I, M46I, I47V, L90M
*3 M46L
M46I
D
T215G/D
T215C
T215A
T215D
T215L
T215L
T215E
*1 T215S
T215D
T215L
T215S

TABLE 2. CHARACTERISTICS OF DRUG-RESISTANT HIV-1

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

[a]Major 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 implementation 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-reveratant 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-reveratant 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.

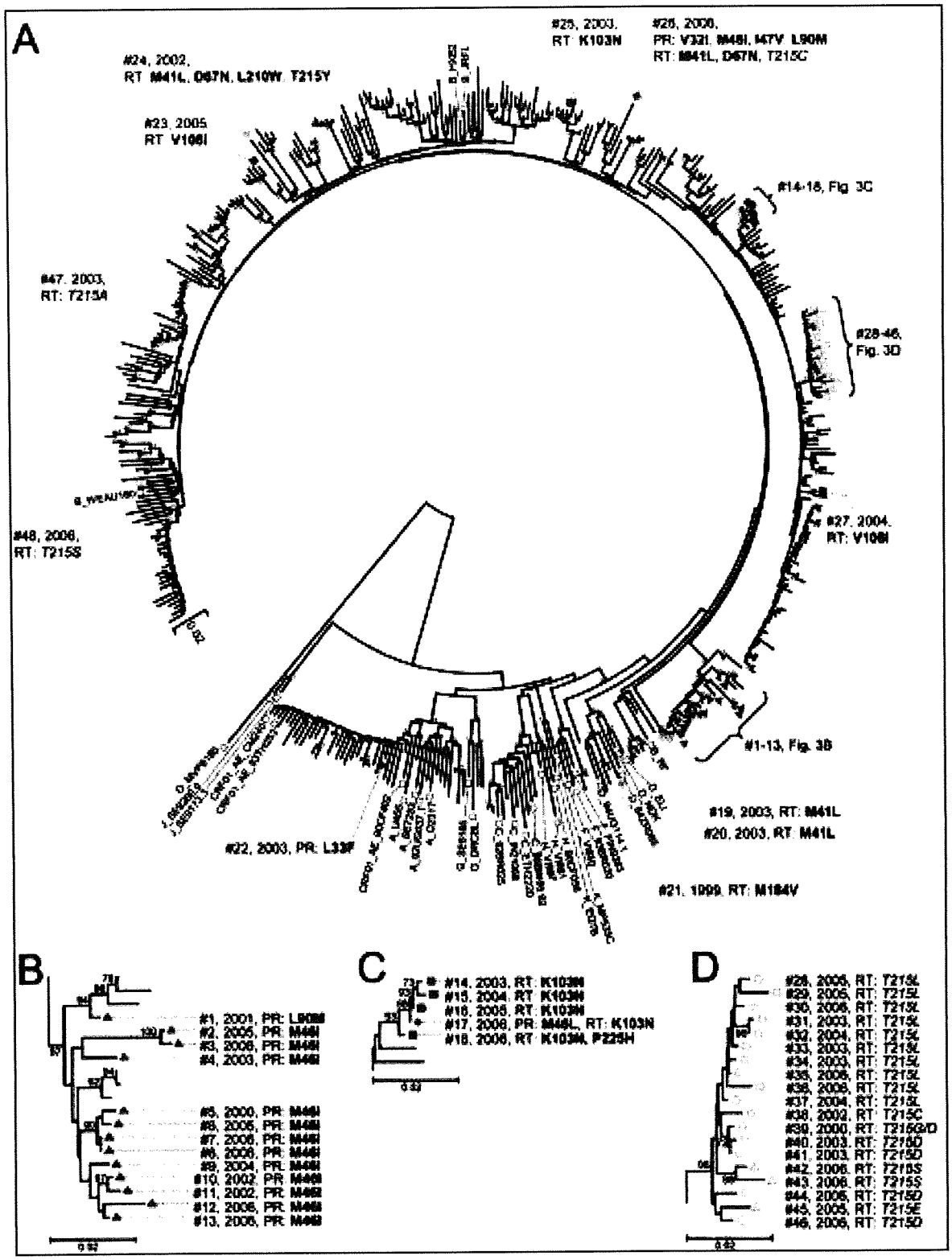
A
#24, 2002,
RT: M41L, D67N, L210W, T215Y
#23, 2005
RT: V106I
#25, 2000,
RT: K103N
#26, 2006,
PR: V32I, M46I, I47V, L90M
RT: M41L, D67N, T215C
#14-18, Fig. 3C
#47, 2003,
RT: T215A
#28-46,
Fig. 3D
#48, 2006,
RT: T215S
#27, 2004,
RT: V106I
#1-13, Fig. 3B
#19, 2003, RT: M41L
#20, 2003, RT: M41L
#22, 2003, PR: L33F
#21, 1999, RT: M184V
B
#1, 2001, PR: L90M
#2, 2005, PR: M46I
#3, 2006, PR: M46I
#4, 2003, PR: M46I
#5, 2000, PR: M46I
#6, 2005, PR: M46I
#7, 2006, PR: M46I
#8, 2006, PR: M46I
#9, 2004, PR: M46I
#10, 2002, PR: M46I
#11, 2002, PR: M46I
#12, 2006, PR: M46I
#13, 2006, PR: M46I
C
#14, 2003, RT: K103N
#15, 2004, RT: K103N
#16, 2005, RT: K103N
#17, 2006, PR: M46L, RT: K103N
#18, 2006, RT: K103N, P225H
D
#28, 2005, RT: T215L
#29, 2005, RT: T215L
#30, 2006, RT: T215L
#31, 2003, RT: T215L
#32, 2004, RT: T215L
#33, 2003, RT: T215L
#34, 2003, RT: T215L
#35, 2006, RT: T215L
#36, 2006, RT: T215L
#37, 2004, RT: T215L
#38, 2002, RT: T215C
#39, 2000, RT: T215G/D
#40, 2003, RT: T215D
#41, 2003, RT: T215D
#42, 2006, RT: T215S
#43, 2006, RT: T215S
#44, 2006, RT: T215D
#45, 2005, RT: T215E
#46, 2006, RT: T215D

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 reconvert 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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AIDS RESEARCH AND HUMAN RETROVIRUSES
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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 ± SD) for LPV obtained using moment analysis were as follows: total area under the plasma concentration curve (AUC), 94.4 ± 8.6 $\mu g \cdot h/ml$; mean residence time (MRT), 10.0 ± 1.2 h; elimination half-life ($t_{1/2}$), 5.1 ± 0.9 h; appearance oral clearance (CL/F, F = oral bioavailability), 4.23 ± 0.3 liters/h; and volume of distribution (V/F), 42.6 ± 1.6 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 similar to American patients in terms of PK findings for LPV at steady state. The recommended dose of LPV in the guideline[7] 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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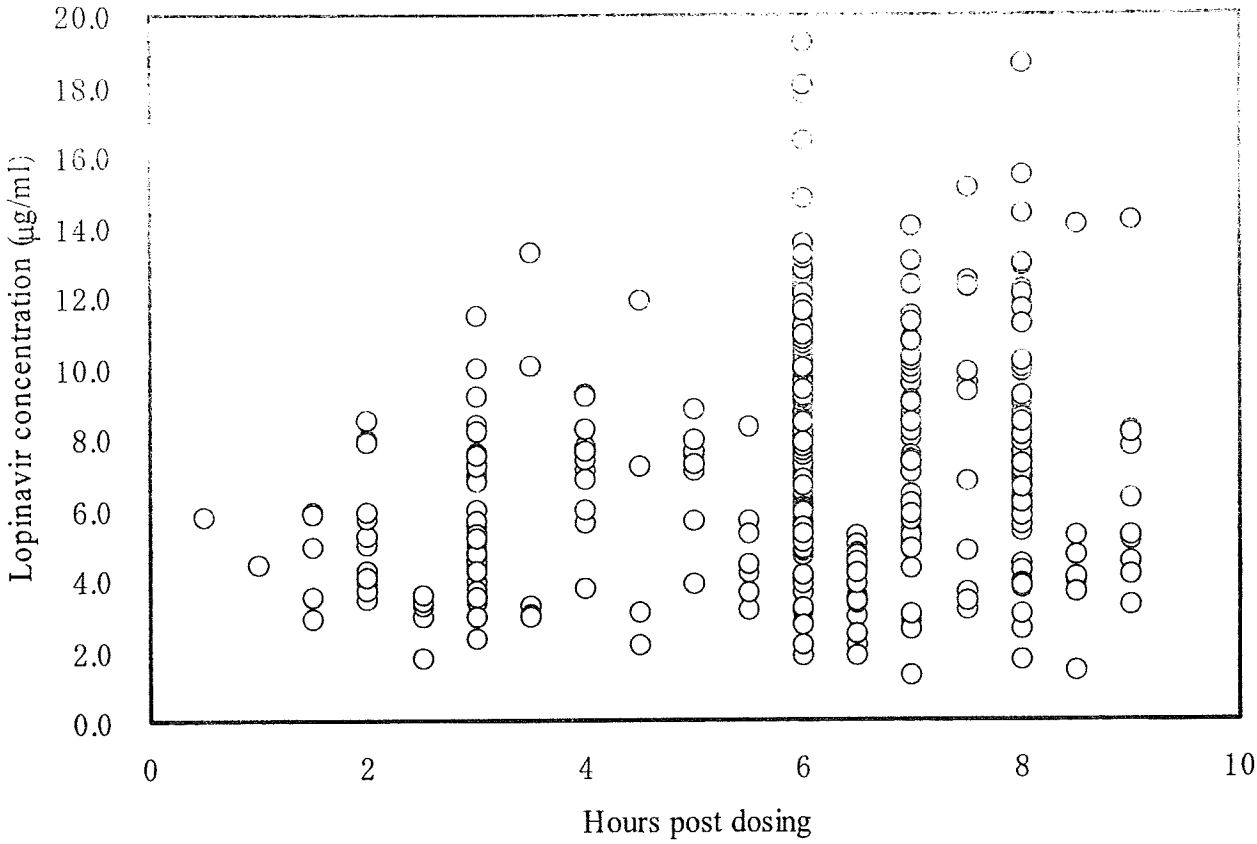

FIG. 1. Distribution of lopinavir plasma concentrations in 65 Japanese HIV-1-infected patients (n = 419). Measured concentrations are plotted versus hours postdosing.

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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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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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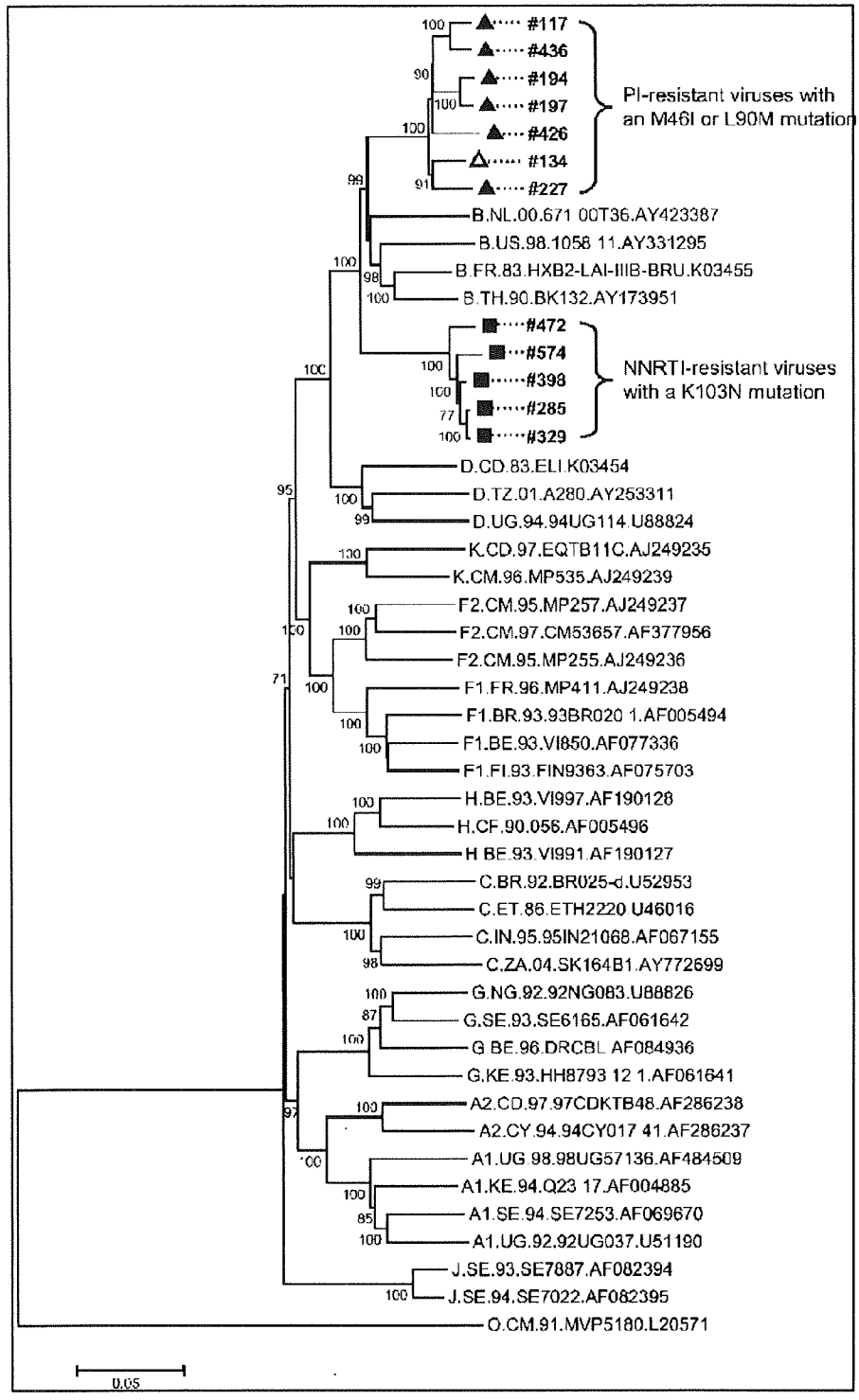

FIG. 1. Phylogenetic analysis of drug-resistant HIV-1. A phylogenetic tree was constructed using the neighbor-joining method with near full-length genomic sequences. Bootstrap values were calculated by 1,000 analyses and values greater than 70% were shown at the nodes of the tree. Scale bar represents 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-

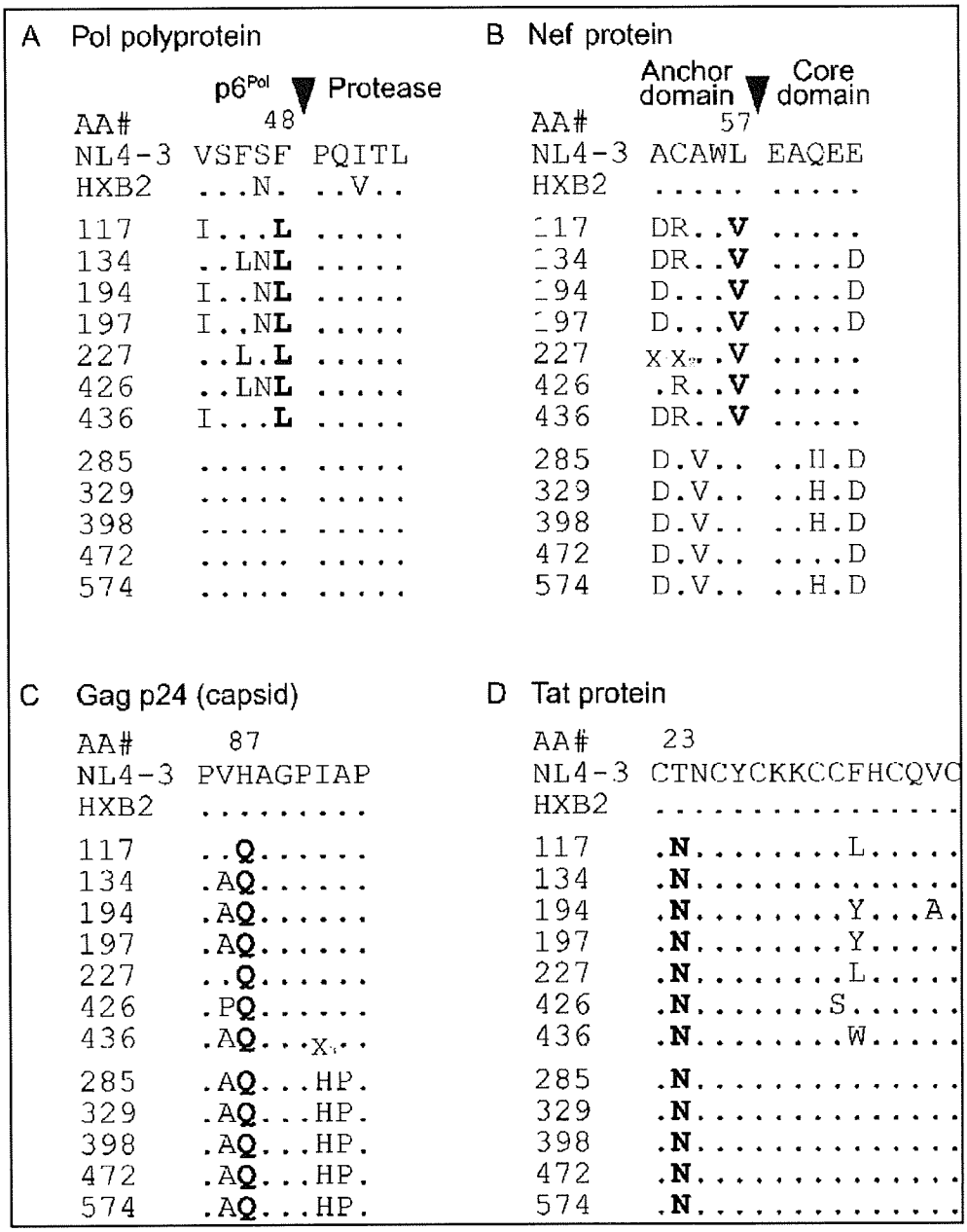

FIG. 2. Candidates for amino acid mutations that possibly enhance the replication activity of drug-resistant HIV-1. Protease inhibitor-resistant HIV-1 commonly possessed an F48L mutation in the carboxyl terminus of $p6^{Pol}$ (**A**) and an L57V mutation in the carboxyl terminus of Nef anchor domain (**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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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.

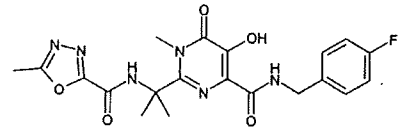
Raltegravir is one of a new class of antiretroviral agents that work by inhibiting the insertion of viral DNA into the cellular genome, resulting in virus replication prevention.[7—10] Therefore, raltegravir is expected to treat therapy-experienced patients where protease inhibitor (PI) and/or reverse transcriptase inhibitor-resitant 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.

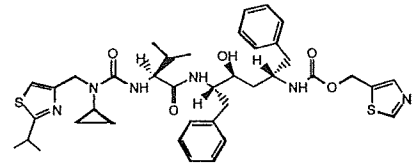
Recently, a determination method using liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been reported.[16] However, the MS-MS detector needs to be delicately set and LC-MS/MS equipment is very expensive. In addition, isotope labeled raltegravir as an internal standard (IS) is needed. To 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-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-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.



Raltegravir



Internal standard (A-86093)

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

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