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Selection of escape mutation by Pol154-162-specific cytotoxic T cells among chronically HIV-1-infected HLA-B*5401-positive individuals

Masao Hashimoto [a], Mitsutaka Kitano [a], Kazutaka Honda [a], Hirokazu Koizumi [a], Sachi Dohki [a], Shinichi Oka [b,c], Masafumi Takiguchi [a,*]

[a] Division of Viral Immunology, Centers for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan
[b] Division of Infectious Disease, Centers for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan
[c] AIDS Clinical Center, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan

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ABSTRACT

Most escape mutations have been identified on cytotoxic T lymphocyte (CTL) epitopes presented by Caucasian or African human leukocyte antigen (HLA) class I alleles, whereas a limited number of studies have identified the escape mutations on epitopes presented by Asian alleles. HLA-B54 is a common HLA allele in Asian countries. We recently identified five HLA-B*5401-restricted HIV-1-specific CTL epitopes. We here investigated escape mutations in these CTL epitopes in Japanese HIV-1-infected individuals. The frequency of substitution from Glu (E) to Asp (D) at position 7 (FV9-7D) in the Pol 154-162 (FV9) epitope was significantly higher in HLA-B*5401$^+$ HIV-infected individuals than in HLA-B*5401$^-$ individuals, whereas substitutions that were significantly higher in HLA-B*5401$^+$ individuals than in HLA-B*5401$^-$ individuals were not found in the other four epitopes. FV9-specific CTLs showed reduced killing activity against target cells pulsed with the FV9-7D mutant peptide and failed to kill those infected with the FV9-7D mutant virus, strongly suggesting that FV9-7D is an escape mutant. Furthermore, longitudinal sequence analysis of the FV9 epitope in two HLA-B*5401$^+$ individuals revealed that the sequence had changed from the wild type to the FV9-7D during the clinical course. Taken together, these results indicate that the FV9-7D escape mutant had been selected by FV9-specific CTLs among chronically HIV-1-infected HLA-B*5401$^+$ individuals.

1. Introduction

Cytotoxic T lymphocytes (CTLs) play an important role in controlling HIV-1 replication during acute and chronic phases of an HIV-1 infection [1,2]. However, HIV-1 escapes from the host immune system by various mechanisms, including mutations of immunodominant CTL epitopes [3–7]. The escape of HV-1 from CTLs has been proposed to be a major obstacle for HIV-1 vaccine development [8–10].

A number of studies have demonstrated that CTL-mediated immune pressure selects escape mutants during acute and chronic phases of HIV-1 infections [3–5,11]. Several escape mechanisms have been proposed when mutations occur within CTL epitopes: a substitution of an amino acid abrogates peptide binding to HLA molecules, reduces the recognition of T-cell receptor, and/or interferes with efficient antigen processing [8]. Any of these mechanisms results in impaired CTL activities against target cells infected with HIV-1 mutants, which contribute to the selection of HIV-1 escape mutants. The appearance of escape mutants can lead to the loss of immune control and eventually accelerate the disease progression [2,3,6,12].

Many studies have focused on HIV-1 escape mutants associated with Caucasian or African human leukocyte antigen (HLA) alleles [6,8,13–18]. In contrast, a paucity of data is available on HIV-1 escape mutants selected in Asian populations [7]. HLA-B54 is a common HLA allele in Asia, including Japan. HLA-B*5401, which is the only genotype of HLA-B54 in the Japanese population, is reported in approximately 13% of Japanese people [19]. Previously, we identified five HLA-B*5401-restricted HIV-1-specific CTL epitopes [20]: Pol154-162 (FPISPIETV, FV9), Pol303-312 (LPQGWKGSPA, LA10), Pol792-800 (HVASGYIEA, HA9), Nef125-133 (FPDWQNYTP, FP9), and Nef150-160 (VPVEPEKVEEA, VA11). However, it remains unknown whether escape mutants among these epitopes are selected by HIV-1-specific CTLs in the Japanese population.

The objective of the present study is to determine whether some escape mutants are selected among those five HLA-B*5401-restricted HIV-1-specific CTL epitopes. Here, we first analyzed the sequences of those five HLA-B*5401-restricted HIV-1-specific CTL epitopes in chronically HIV-1-infected Japanese individuals. When the frequency of some amino acid substitutions was significantly higher in HLA-B*5401$^+$ than in HLA-B*5401$^-$ individuals, we ex-

* Corresponding author.
E-mail address: masafumi@kumamoto-u.ac.jp (M. Takiguchi).

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amined whether such substitutions indeed contributed to a failure of recognition by CTLs. In addition, we performed longitudinal analyses of sequences of those epitopes to confirm that those substitutions had actually been selected in chronically HIV-1-infected HLA-B*5401$^+$ individuals.

2. Subjects and methods

2.1. Samples of HIV-1-infected individuals

This study was approved by the International Medical Center of Japan and the Kumamoto University Ethical Committee. Informed consent was obtained from all subjects according to the Declaration of Helsinki. For sequence analyses, blood specimens were collected in EDTA. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood. The patients' HLA types were determined by standard sequence-based genotyping.

2.2. Sequence of autologous virus

Viral RNA was extracted from samples of plasma from HIV-1-infected individuals using a QIAamp MinElute virus spin kit (Qiagen GmbH, Germany), and cDNA was synthesized from RNA with SuperScript RNase H-reverse transcriptase and random primers (Invitrogen, Carlsbad, CA). Proviral DNA was extracted from PBMCs of HIV-1-infected individuals using a QIAamp DNA blood mini kit (Qiagen). Corresponding Pol or Nef regions were amplified by nested PCR using Taq DNA polymerase (Promega, Madison, WI). The PCR products were then purified with agarose gel and sequenced directly. DNA sequencing was performed using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 310 genetic analyzer (Applied Biosystems).

2.3. Cells

C1R cells expressing HLA-B*5401 (C1R-B*5401) were previously generated [20] and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.2 mg/ml neomycin. 721.221-CD4-B*5401 cells were generated by transfecting the CD4 and HLA-B*5401 genes into 721.221 cells and maintained in RPMI 1640 medium supplemented with 10% FCS and 0.15 mg/ml hygromycin B. MT2 and H9 cells were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.1 mg/ml kanamycin. MAGIC-5 cells (CCR-transduced HeLa-CD4/LTR-β-gal cells) were cultured and used as described previously [21].

2.4. Generation of CTL clones

Peptide-specific CTL clones were generated from established peptide-specific bulk CTLs by seeding 0.8 cells/well into U-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μl of cloning mixture (RPMI 1640 medium containing 10% FCS and 200 U/ml human recombinant interleukin-2), 5×10^5 irradiated allogeneic PBMCs from a healthy donor, and 1×10^5 irradiated C1R-B*5401 pulsed with 1 μM concentration of the appropriate HIV-1-derived peptides. Wells positive for the growth after about 2 weeks were examined for CTL activity by the standard ^{51}Cr-release assay. All CTL clones were cultured in RPMI 1640 containing 10% FCS and 200 U/ml recombinant human interleukin-2. CTL clones were stimulated biweekly with irradiated target cells pulsed with the corresponding peptides.

2.5. HIV-1 clones

An HIV-1 mutant was generated by introducing the FV9-7D mutation into NL432 (NL432-FV9-7D) using site-directed mutagenesis (Invitrogen) based on overlap extension.

2.6. CTL assay for target cells pulsed with HIV-1 peptide

Cytotoxic activity was measured by the standard ^{51}Cr-release assay, as previously described [7]. Target cells (2×10^5) were incubated for 1 hour with 100 mCi $Na_2{}^{51}CrO_4$ in saline and then washed three times with RPMI 1640 medium containing 10% newborn calf serum. Labeled target cells (2×10^3/well) were added to 96-well round-bottom microtiter plates (Nunc) along with the appropriate amount of the corresponding peptide. After 1 hour of incubation, effector cells were added and the mixtures were then incubated for 4 hours at 37°C. The supernatants were collected and analyzed with a gamma counter. Spontaneous ^{51}Cr release was determined by measuring the counts per minute (cpm) in supernatants from wells containing only target cells (cpm spn). Maximum ^{51}Cr release was determined by measuring the cpm in supernatants from wells containing target cells in the presence of 2.5% Triton X-100 (cpm max). Specific lysis was defined as (cpm exp − cpm spn)/(cpm max − cpm spn) × 100, where "cpm exp" is the counts per minute in the supernatant in the wells containing both target and effector cells.

2.7. CTL assay for target cells infected with HIV-1

721.221-CD4$^-$B*5401 cells were exposed to NL432 or NL432-FV9-7D for several days. The cells were used as target cells for CTL assays when approximately 60% of cells were infected, which was confirmed by intracellular staining for HIV-1 p24 antigen. Infected cells were labeled with ^{51}Cr as described above. Labeled target cells were added along with effector cells into round-bottom microtiter plates (Nunc), and the mixtures were incubated for 6 hours at 37°C.

2.8. Replication kinetics assay

MT-2 cells (1×10^5) were exposed to each infectious virus preparation (500 blue cell-forming units in MAGIC-5 cells) for 2 hours, washed twice with phosphate-buffered saline (PBS), and

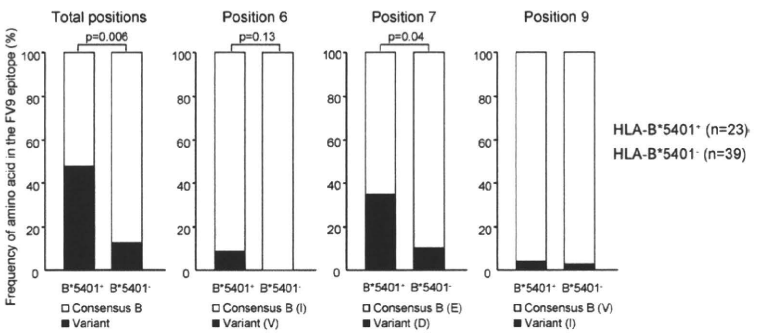

Fig. 1. Frequency of mutations in the FV9 epitope among HLA-B*5401$^+$ and HLA-B*5401$^-$ HIV-1-infected individuals. The consensus sequence of this epitope in clade B is FPISPIETV. The frequency of mutations in the total sequence and at a given position of the epitope is illustrated for both HLA-B*5401$^+$ and HLA-B*5401$^-$ HIV-1-infected individuals. p values were determined using Fisher's exact test.

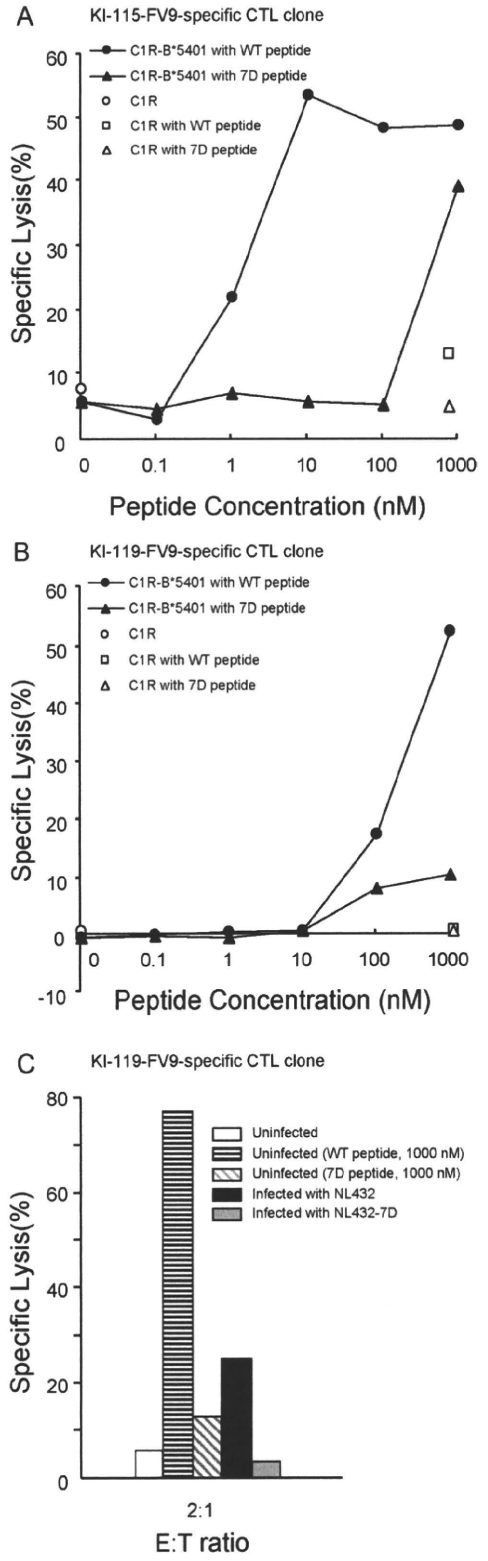

Fig. 2. Cytolytic activity of FV9-specific CTLs against target cells pulsed with the mutant (FV9-7D) peptide or those infected with the mutant virus (NL432-FV9-7D). A. Cytolytic activity of KI-115-FV9-specific CTL clone to kill C1R-B*5401 cells pulsed with the wild-type (FV9) or FV9-7D peptide. C1R-B*5401 cells were pulsed with various concentrations of the FV9 or FV9-7D peptide. B. Cytolytic activity of KI-119-FV9-specific CTL clone to kill C1R-B*5401 cells pulsed with the FV9 or FV9-7D peptide. C1R-B*5401 cells were pulsed with various concentrations of the FV9 or FV9-7D peptide. C. Cytolytic activity of KI-119-FV9-specific CTL clones against 721.221-CD4-B*5401 cells infected with the NL432 or NL432-FV9-7D virus. 721.221-CD4-B*5401 cells were used as target cells at an E:T ratio of 2:1. The percentages of p24 antigen-positive cells among target cells infected with NL432 and NL432-FV9-7D were 53.4 and 56.4%, respectively.

cultured in 1 ml of complete medium [21]. Aliquots (0.1 ml) of the culture were harvested every other day, and the volume removed was replaced with fresh medium. The concentration of p24 antigen was measured using an enzyme immunoassay (HIV-1 p24 antigen enzyme-linked immunosorbent assay kit; ZeptoMetrix, Buffalo, NY). Replication kinetics assays were performed in duplicate.

2.9. Competitive HIV-1 replication assay

Freshly prepared H9 cells (3×10^5) were exposed for 2 hours to mixtures of paired virus preparations (300 blue cell-forming units each; NL432 vs NL432-FV9-7D) for examination of their replication ability, washed twice with PBS, and cultured as described previously [21]. On day 1, one third of the infected H9 cells were harvested and washed twice with PBS, and proviral DNAs were sequenced (0 passage). Every 7 days, the supernatant of the virus culture was transmitted to new uninfected H9 cells. The cells harvested at the end of each passage were then subjected to direct DNA sequencing of the HIV-1 RT gene. The change in viral population was determined from the relative peak height on sequencing electrograms. The persistence of the original amino acid substitution was confirmed for each infectious clone used in this assay.

3. Results

3.1. Association of an HLA-B*5401 allele with mutations among five HLA-B*5401-restricted HIV-1-specific CTL epitopes

To clarify whether HLA-B*5401-restricted HIV-1-specific CTLs select the escape mutant at the population level, we analyzed the sequences of 5 HLA-B*5401-restricted CTL epitopes (FPISPIETV; FV9, LPQGWKGSPA; LA10, HVASGYIEA; HA9, FPDWQNYTP; FP9, and VPVEPEKVEEA; VA11) in HIV-1 infected individuals. Among these 5 CTL epitopes, HLA-B*5401-associated mutations were reported only in the FV9 epitope. There were no HLA-B*5401-associated mutations within or at the flanking regions of the other 4 HLA-B*5401-restricted CTL epitopes (data not shown). The sequence analysis of the FV9 epitope and its flanking regions of HIV-1 from 23 HLA-B*5401$^+$ and 39 HLA-B*5,401$^-$ HIV-1-infected individuals exhibited several mutations at positions 6, 7, and 9 of the epitope (Fig. 1). The frequency of the substitution of Glu (E) to Asp (D) mutation at position 7 (FV9-7D) was significantly higher in HLA-B*5401$^+$ than in HLA-B*5401$^-$ HIV-1-infected individuals, whereas no significant differences were observed at other positions between HLA-B*5401$^+$ and HLA-B*5401$^-$ donors (Fig. 1). In the flanking regions of the FV9 epitope, there was also no significant difference among these donors (data not shown). These results suggest that the FV9-7D mutant was selected by FV9-specific CTLs.

3.2. FV9-7D is an escape mutant from FV9-specific CTLs

To confirm that FV9-7D was an escape mutant for FV9-specific CTLs, we investigated whether FV9-specific CTLs could recognize the FV9-7D mutant. First, we tested the activity of FV9-specific CTL clones to kill target cells pulsed with the FV9-7D mutant peptide. Two FV9-specific CTL clones, which were generated from two HLA-B*5401$^+$ HIV-1-infected individuals (KI-115 and KI-119), effectively killed target cells pulsed with the FV9 wild-type peptide. In contrast, both CTL clones showed a reduced ability to kill target cells pulsed with the FV9-7D mutant peptide (Fig. 2A, 2B).

To clarify whether FV9-specific CTLs failed to recognize target cells infected with HIV-1 mutant virus containing FV9-7D muta-

tion, we generated the HIV-1 mutant by introducing the FV9-7D mutation into NL432 (NL432-FV9-7D) and examined whether FV9-specific CTLs could kill target cells infected with the FV9-7D mutant virus. The FV9-specific CTL clone killed target cells infected with NL432 effectively but failed to kill those infected with NL432-FV9-7D (Fig. 2C). These results indicate that FV9-7D was indeed a mutant that had escaped from FV9-specific CTLs.

We performed longitudinal analysis of a sequence of the FV9 epitope in two HIV-1-infected HLA-B*5401$^+$ individuals, KI-091 and KI-160 (Table 1). In KI-091, the wild-type sequence was detected 11 months after the first visit in the early phase of the infection (September 10, 2001). Four months later, a FV9-7D mutation appeared and then remained stable over 3 years. In KI-160, the sequence of the FV9 epitope was the wild type at the first visit in the early phase of the infection (July 25, 2002) and then had changed to FV9-7D within the next 3 years. These results support the idea that FV9-7D was the escape mutant selected by FV9-specific CTLs.

3.3. FV9-7D mutation does not impact viral replication in vitro or in vivo

To examine the impact of the FV9-7D mutation in the FV9 epitope on viral replication, we investigated the replication kinetics for NL432 and NL432-FV9-7D. NL432-FV9-7D exhibited replication equivalent to that of NL432 (Fig. 3A). Moreover, NL432 and NL432-FV9-7D exhibited comparable replication efficiency in a competitive HIV-1 replication assay using H9 cells (Fig. 3B).

Finally, to examine whether the FV9-7D mutation was stable or reverted to the wild-type sequence in HLA-B*5401$^-$ HIV-1-infected individuals, we performed longitudinal analysis of the sequence of the FV9 epitope in HLA-B*5401$^-$ HIV-1-infected individuals, those who had the FV9-7D mutation (Table 2). Among four HLA-B*5401$^-$ HIV-1-infected individuals with the FV9-7D mutation, plasma samples from three of them (KI-060, KI-068, and KI-107) were available for this analysis. In all samples, the FV9-7D mutation had remained stable during the period tested (range: 17–71 months, median period: 41 months).

4. Discussion

In the present study we identified FV9-7D as an escape mutation among the five HLA-B*5401-restricted CTL epitopes. Although it is known that a restricted number of HIV-1-specific T cells select escape mutants, the factors selecting escape mutants remain unclear. Given that HLA-associated polymorphisms are predominantly driven by immunodominant CTL responses [22], the FV9 epitope is the immunodominant one among chronically HIV-1-infected HLA-B*5401$^+$ individuals. In fact, our previous study showed that FV9-specific memory CTLs could be detected more frequently in chronically HLA-B*5401$^+$ HIV-1-infected individuals than in the other four epitope-specific ones [20].

The FV9-7D mutation may be assumed to have little impact on viral fitness and not to revert *in vivo* for the following reasons: first, the FV9-7D mutant virus exhibited a replication capacity equivalent to that of the wild-type virus *in vitro*. Second, the FV9-7D mutation remained stable in HLA-B*5401$^-$ hosts in the present study. Reversion to wild-type sequence would likely occur if the escape mutant is transmitted to a non-HLA-matched recipient in the absence of CTL selective pressures and if the escape mutation is located in a region within the viral genome where an escape mutation is accompanied with a high replication fitness cost for the virus [23,24]. Consequently, the FV9-7D mutation is speculated to accumulate in our cohorts. However, compared with other escape mutations previously reported in Japanese cohorts [7,25], the accumulation rate of the FV9-7D mutation was not as high, with the mutant being reported in only about 10% of HLA-B*5401$^-$ HIV-1-infected individuals in the present study. Gag28-3R and Nef138-2F are escape mutants selected by HLA-A*2402-restricted HIV-1-specific CTLs, and those mutants were shown to accumulate among HLA-A*2402$^-$ hosts with an approximate frequency of 30–50% [7,25,26]. One explanation for this discrepancy in accumulation rates of the escape mutants is that the prevalence of the population with the relevant HLA-allele by which HIV-1-specific CTLs responses are restricted is different. HLA-B*5401 is reported in approximately 13% of the Japanese population, whereas HLA-A*2402 is expressed

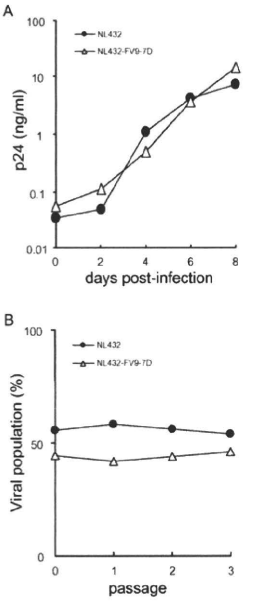

Fig. 3. Replication kinetics of HIV-1 clones. A. HIV-1 clones were propagated in MT-2 cells. The concentration of p24 in the culture medium was measured every other day. The assay was performed in duplicate, and the data represent the logarithmic mean values of p24 concentrations. B. Two infectious HIV-1 clones to be compared for their fitness (L432 vs NL432-FV9-7D) were mixed and used to infect H9 cells. The cell-free supernatant was transferred to fresh H9 cells every 7 days. High-molecular-weight DNAs extracted from infected cells on day of the culture (0 passage) and at the end of each passage were subjected to nucleotide sequencing, and the proportion of Glu and Asp at position 7 of the FV9 epitope was determined. The black circle and white triangle indicate the population of Glu and Asp at position 7 of the FV9 epitope, respectively.

Table 1
Longitudinal analysis of the FV9 epitope sequence of HLA-B*5401$^+$ HIV-1-infected individuals

ID	Sample date (month/day/year)	Sample	Sequence[a]
KI-091	09/10/2001	Proviral DNA	---------
	01/09/2002	Proviral DNA	------D--
	08/04/2005	RNA	------D--
KI-160	07/25/2002	RNA	---------
	12/27/2005	RNA	------D--

[a]The consensus sequence of the FV9 epitope in clade B is FPISPIETV.

Table 2
Longitudinal analysis of the FV9 epitope sequence of HLA-B*5401$^-$ HIV-1-infected individuals

ID	Sample date (month/day/year)	Sample	Sequence[a]
KI-060	08/18/2001	RNA	------D--
	07/03/2007	RNA	------D--
KI-068	09/27/2001	RNA	------D--
	03/06/2003	RNA	------D--
KI-107	06/12/2001	RNA	------D--
	11/29/2004	RNA	------D--

[a]The consensus sequence of the FV9 epitope in clade B is FPISPIETV.

in about 70%. Other possible explanations are variability in the frequency of CTL responses induced among the populations with relevant HLA-alleles and the ability of those epitope-specific CTLs to select escape mutants. An FV9-specific CTL response was detected in 50% of chronically HIV-1-infected HLA-B*5401$^+$ individuals [20], whereas Gag28- and Nef138-specific CTL responses were detected in 67 and 58% of chronically HIV-1-infected HLA-A*2402$^+$ individuals, respectively [7,25]. Furthermore, the FV9-7D mutation was selected by FV9-specific CTLs in 35% of HLA-B*5401$^+$ HIV-1-infected individuals, whereas both Gag28-3R and Nef138-2F mutations were selected by those epitope-specific CTLs in approximately 70% of HLA-A*2402$^+$ individuals. Together, these differences might influence the pace of accumulation of the HIV-1 mutants, including the FV9-7D mutation at the population level.

The appearance of the FV9-7D mutation in HIV-1-infected HLA-B*5401$^+$ individuals would not lead to a preferable outcome of the disease because escape mutations often undermine immune control. Taking into account that FV9-specific CTLs failed to recognize cells infected with FV9-7D mutant virus *in vitro*, those CTLs may fail to respond to the mutant virus in HLA-B*5401$^+$ HIV-1-infected hosts. By contrast, because it is well known that some escape mutants are associated with reduced viral replication capacity [18], they are sometimes advantageous for HIV-1-infected individuals [27]. Such a situation, however, might not be applicable to the FV9-7D mutation for HLA-B*5401$^+$ hosts because the FV9-7D mutation is not accompanied by low fitness. Taken together, the data indicate that this mutation ultimately might be disadvantageous for HLA-B*5401$^+$ HIV-1-infected individuals.

The present study demonstrated that the FV9-7D mutation had been selected as an escape mutation by FV9-specific CTLs among HIV-1-infected HLA-B*5401$^+$ individuals. Further studies of CTL escape mutations selected in Japanese and other Asian populations are necessary to understand the interaction between the hosts and HIV-1 in those populations.

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Trends in transmitted drug-resistant HIV-1 and demographic characteristics of newly diagnosed patients: Nationwide surveillance from 2003 to 2008 in Japan

Junko Hattori[a], Teiichiro Shiino[b], Hiroyuki Gatanaga[c], Shigeru Yoshida[d], Dai Watanabe[e], Rumi Minami[f], Kenji Sadamasu[g], Makiko Kondo[h], Haruyo Mori[i], Mikio Ueda[j], Masao Tateyama[k], Atsuhisa Ueda[l], Shingo Kato[m], Toshihiro Ito[n], Masayasu Oie[o], Noboru Takata[p], Tsunefusa Hayashida[c], Mami Nagashima[g], Masakazu Matsuda[q], Shiro Ibe[a], Yasuo Ota[r], Satoru Sasaki[n], Yoshiaki Ishigatsubo[l], Yoshinari Tanabe[o], Ichiro Koga[r], Yoko Kojima[i], Masahiro Yamamoto[f], Jiro Fujita[k], Yoshiyuki Yokomaku[a], Takao Koike[s], Takuma Shirasaka[e], Shinichi Oka[c], Wataru Sugiura[a,b,t,*]

[a] National Hospital Organization, Nagoya Medical Center, Clinical Research Center, 4-1-1 Sannomaru, Naka-ku, Nagoya 4600001, Japan
[b] National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 1628640, Japan
[c] AIDS Clinical Center, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku-ku, Tokyo 1628655, Japan
[d] Faculty of Health Sciences, Hokkaido University, N12-W5, Kita-ku, Sapporo 0600812, Japan
[e] National Hospital Organization, Osaka Medical Center, 2-1-14 Houenzaka, Chuo-ku, Osaka 5400006, Japan
[f] National Hospital Organization, Kyushu Medical Center, 1-8-1 Jigyohama, Chuo-ku, Fukuoka 8108563, Japan
[g] Tokyo Metropolitan Institute of Public Health, 3-24-1 Hyakunin-cho, Shinjuku-ku, Tokyo 1690073, Japan
[h] Kanagawa Prefectural Institute of Public Health, 1-3-1 Shimomachiya, Chigasaki, Kanagawa 2530087, Japan
[i] Osaka Prefectural Institute of Public Health, 1-3-69 Nakamichi, Higashinari-ku, Osaka 5370025, Japan
[j] Ishikawa Prefectural Central Hospital, 2-1 Anngetsu-higashi, Kanazawa 9208530, Japan
[k] University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 9030125, Japan
[l] Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 2360004, Japan
[m] Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 1608582, Japan
[n] National Hospital Organization, Sendai Medical Center, 2-8-8 Miyagino, Miyagino-ku, Sendai 9830045, Japan
[o] Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Chuo-ku, Niigata 9518510, Japan
[p] Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 7348551, Japan
[q] Mitsubishi Chemical Medience Corporation, 3-30-1 Shimura, Itabashi-ku, Tokyo 1748555, Japan
[r] Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 1738605, Japan
[s] Hokkaido University Graduate School of Medicine, N15-W7, Kita-ku, Sapporo 0608638, Japan
[t] Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 4668550, Japan

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ABSTRACT

The emergence and transmission of drug-resistant human immunodeficiency virus-1 (HIV-1) compromises antiretroviral treatment for HIV-1. Thus, testing for drug resistance is recommended at diagnosis and before initiating highly active antiretroviral treatment. We conducted an epidemiological study enrolling newly diagnosed patients between 2003 and 2008 in our nationwide surveillance network. In the 6-year study period, the prevalence of drug-resistant HIV-1 among 2573 patients, consisting mainly of Japanese men in their late-30s and infected through male-to-male sexual contacts, followed an increasing trend from 5.9% (16/273) in 2003 to 8.3% (50/605) in 2008. Nucleoside reverse transcriptase inhibitor-associated mutations predominated in each year, with T215 revertants being the most abundant. The predictive factor for drug-resistant HIV-1 transmission was subtype B (OR = 2.36; $p = 0.004$), and those for recent HIV-1 infection were male gender (OR = 3.79; $p = 0.009$), MSM behavior (OR = 1.67; $p = 0.01$), Japanese nationality (OR = 2.31; $p = 0.008$), and subtype B (OR = 5.64; $p < 0.05$). Continued activities are needed to raise awareness of the risks of HIV-1 infection and complications of drug-resistant strains. Continued surveillance is also needed to understand trends in the HIV-1 epidemic.

Abbreviations: HIV-1, human immunodeficiency virus type 1; HAART, highly active antiretroviral therapy; PI, protease inhibitor; HBV, hepatitis B virus; HCV, hepatitis C virus; PR, protease; RT, reverse transcriptase; RT-PCR, reverse transcription polymerase chain reaction; CRF, circulating recombinant form; NRTI, nucleoside RT inhibitor; NNRTI, non-nucleoside RT inhibitor; OR, odds ratio; CI, confidence interval; MSM, men who have sex with men; IDU, intravenous drug user.

* Corresponding author at: Clinical Research Center, National Nagoya Medical Center, 4-1-1 Sannomaru Naka-ku, Nagoya, Aichi 4600001, Japan.
Tel.: +81 52 951 1111; fax: +81 52 963 3970.
E-mail address: wsugiura@nih.go.jp (W. Sugiura).

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1. Introduction

The emergence of drug-resistant human immunodeficiency virus type 1 (HIV-1) among patients under highly active antiretroviral therapy (HAART) limits the successful suppression of HIV-1 replication. Several years after the introduction of HAART, drug-resistant strains are being detected among newly diagnosed HAART-naïve patients, suggesting the transmission of drug-resistant HIV-1 from the treatment-exposed population. Thus, treatment-naïve patients have been recommended by the US Department of Health and Human Services, International AIDS Society-USA, and other drug-resistance testing guidelines to undergo drug resistance testing at diagnosis and before initiation of HAART (DHHS, 2009; Hirsch et al., 2000, 2008). Indeed, choosing effective antiretrovirals according to the results obtained from this testing has led to successful control of HIV-1 infection. Furthermore, the drug resistance testing at diagnosis helps to understand transmission of drug-resistant HIV-1 in HAART-naïve individuals which in turn may help prevent transmission events.

The prevalence of drug-resistant HIV-1 among treatment-naïve patients has been closely monitored and reported from many countries. Before and early in the HAART era, when only mono or dual therapy was available, the prevalence was as high as 10–20% (Boden et al., 1999; Gómez-Cano et al., 1998; Tambussi et al., 1998). However, after the introduction of antiretrovirals with better pharmacokinetics, such as ritonavir-boosted protease inhibitor (PI), the emergence of drug-resistant viruses seemed to decrease (Gallego et al., 2001; Maia Teixeira et al., 2006).

Furthermore, despite the great number of HIV-1-infected patients, the prevalence tended to be low in developing countries where patients had limited or no access to antiretroviral drugs, e.g., 0–4.2% in Africa (Bártolo et al., 2009; Mintsa-Ndong et al., 2009; Ndembi et al., 2008; Pillay et al., 2008), 1.5% in Cambodia (Nouhin et al., 2009), and 2.6% in Vietnam (Ishizaki et al., 2009). In contrast, in countries where antiretroviral drugs are more accessible, the prevalence has been higher, e.g., 5.2% in Thailand (Apisarnthanarak et al., 2008), 9.4% in Taiwan (Chang et al., 2008), 10.0% in India (Lall et al., 2008), 7.8% in Portugal (Palma et al., 2007), 9.0% in Germany (Sagir et al., 2007), 9.5% in Belgium (Vercauteren et al., 2008), 10.9% in France (Chaix et al., 2009), and 15.9% in the US (Eshleman et al., 2007).

In Japan, since the first HIV-1-infected case was identified in 1985, the annual number of reported cases has been increasing every year, reaching 15 451 by the end of 2008. With more people getting infected, larger numbers of patients are starting anti-HIV-1 treatment and the risk of emerging drug-resistant HIV-1 is increasing. To understand the trends in drug-resistant HIV-1 in Japan, a nationwide surveillance project has been in effect since 2003. In our previous report of surveillance results from 2003 to 2004, the prevalence of drug-resistant HIV-1 in newly diagnosed patients was 4.0% (Gatanaga et al., 2007). We have continued collecting and analyzing data from newly diagnosed HIV-1-infected patients at participating clinical and research facilities in Japan. We report here the prevalence of drug-resistant HIV-1 among newly diagnosed therapy-naïve patients between 2003 and 2008.

2. Materials and methods

2.1. Sample

The study population included all the HIV-1-infected patients newly diagnosed between January 2003 and December 2008 at any of the participating HIV/AIDS clinics. Drug resistance genotypic tests were performed at 12 laboratories including 8 clinical laboratories at HIV/AIDS clinics, 3 public health laboratories, and the National Institute of Infectious Diseases. After patients agreed to participate in our surveillance project and gave informed consent, peripheral blood was drawn with EDTA added, and their demographic and clinical information were collected. Demographic information included age, gender, nationality, and risk behavior. Clinical data included HIV-1 viral loads, $CD4^+$ T cell counts, status of hepatitis B and C virus (HBV, HCV) co-infection, baseline sequence data, and drug-resistant amino acid mutations.

This study was conducted according to the principles in the Declaration of Helsinki, and was approved by the ethical committee of the National Institute of Infectious Diseases, Japan. By Japanese law, HIV-1-infected patients must be reported to the Japanese Ministry of Health, Labour, and Welfare upon diagnosis. The numbers reported to the Ministry are considered the "official numbers" of newly diagnosed HIV/AIDS cases, and were used as comparison controls to evaluate our study population.

2.2. Drug resistance genotypic testing

Drug resistance genotypic testing was performed using in-house protocols. Briefly, viral RNA was extracted from patient plasma samples. HIV-1 protease (PR, 1–99 amino acids) and the N-terminal region of reverse transcriptase (RT, 1–240 amino acids) were amplified in reverse transcription polymerase chain reaction (RT-PCR) followed by nested PCR using in-house primer sets. Subsequently, the amplified PCR products were purified and their sequences were analyzed by direct sequencing method using an automated sequencer. The resulting electropherograms were analyzed using commercially available software. The quality of testing methods used at each participating facility was assessed and confirmed for detection of drug-resistant mutations (Fujisaki et al., 2007). Thus, detection of drug-resistant mutations was consistent among facilities.

2.3. Determination of HIV-1 subtypes and drug-resistant HIV-1

HIV-1 subtypes were determined using the sequences of HIV-1 PR and RT genes obtained in the drug resistance genotypic testing explained above. Each sequence was aligned with the reference sequences of HIV-1 subtypes A through K, and circulating recombinant forms (CRFs), all of which were obtained from the Los Alamos HIV Databases (Los Alamos, 2010), using ClustalW, and phylogenetic trees were constructed using the neighbor-joining method with bootstrap value of 1000.

The resulting sequences were compared to that of HXB2 to judge the presence of amino acid mutations. The drug-resistant mutations were determined according to criteria of the HIV Drug Resistance Database of Stanford University (Bennett et al., 2009). Thus, a sample was considered to harbor drug-resistant HIV-1 if it possessed any of the following mutations: in the PR gene, L23I, L24I, D30N, V32I, M46I/L, I47V/A, G48V/M, I50V/L, F53L/Y, I54V/L/M/A/T/S, G73S/T/C/A, L76V, V82A/T/F/S/C/M/L, N83D, I84V/A/C, I85V, N88D/S, and L90M (indicating PI resistance); in the RT gene, M41L, K65R, D67N/G/E, T69D/insertion, K70R/E, L74V/I, V75M/T/A/S, F77L, Y115F, F116Y, Q151M, M184V/I, L210W, T215Y/F/I/S/C/D/V/E, K219Q/E/N/R (indicating nucleoside RT inhibitor [NRTI] resistance), and L100I, K101E/P, K103N/S, V106M/A, V179F, Y181C/I/V, Y188L/H/C, G190A/S/E, P225H, M230L (indicating non-nucleoside RT inhibitor [NNRTI] resistance).

2.4. BED assay

The time of HIV-1 seroconversion was estimated in randomly selected samples as recent (within 155 days) or not recent using the BED assay (Calypte HIV-1 BED Incidence EIA, BioRad) according to the Manufacturer's instruction. Briefly, 5 μL of plasma was diluted

with 500 μL of sample diluent in the kit, and the proportion of anti-HIV-1 IgG to a total IgG in the sample was measured by optical density.

2.5. Statistical analysis

Statistical analyses were performed using R software (SAS Institute). Chi-square or Fisher's exact probability tests were used to determine associations among patients' demographic characteristics, nationality, BED assay results, and transmission of drug resistance. The odds ratio (OR) and 95% confidence intervals (CI) were calculated for all the variables. Recent and not-recent seroconversion groups were examined for differences in HIV-1 viral loads by analysis of covariance (ANCOVA), with $CD4^+$ T cell count as the covariate.

3. Results

3.1. Majority of treatment-naïve patients are Japanese men who have sex with men (MSM) in mid-30s

The demographics of the 2573 newly diagnosed HIV-1-infected patients enrolled between 2003 and 2008 are summarized in Table 1. Male ($n = 2397$, 93.2%), Japanese (90.1%), and those infected through male-to-male sexual contact (68.9%) predominated, and the median age was 35. For the female cases ($n = 170$), high-risk heterosexual contact was the major risk factor ($n = 152$, 89.4%), and approximately half were non-Japanese ($n = 63$, 41.4%). Further analysis showed a significant association between the transmission route and nationality, i.e., most Japanese patients were infected through male-to-male sexual contact, while non-Japanese patients were infected by other routes (OR = 5.60; 95% CI 4.14–7.63; $p < 0.01$) (Table 2). It should be noted that sexual contacts (92.1%) are the major risk factor for HIV-1 infection in Japan. On the other hand, injecting drug usage, one of the high risk factors in other countries, accounts for only 0.4%.

HBV and/or HCV co-infection, an important clinical factor affecting prognosis and treatment of HIV infection (Ockenga et al., 1997; Piroth et al., 2000), was found to have a prevalence of 8.4% of 2101 patients, and 4.7% of 2071, respectively (Table 1). These prevalence rates did not change significantly throughout the study period (supplementary Table 1). HBV co-infection was found to be significantly associated with subtype B (OR = 2.04; $p < 0.05$) or infection through male-to-male sexual contact (OR = 1.66; $p < 0.05$).

3.2. Subtype B HIV-1 predominates in Japan

Of 2573 plasma samples collected during the study period, the sequences of PR and RT genes were successfully amplified and analyzed in 2536 (98.6%) and 2534 (98.5%) samples, respectively. Of these, we examined sequences of the PR-RT region from 2496 cases by phylogenetic tree analysis to determine the distribution of HIV-1 subtypes in Japan. Subtype B HIV-1 was found to predominate among the study population ($n = 2194$, 87.9%). The remaining non-B subtypes included 210 (8.4%) CRF01_AE, 30 (1.2%) C, 19 (0.8%) CRF02_AG, 18 (0.7%) A, 9 (0.4%) G, 7 (0.3%) F, 5 (0.2%) D, and 1 (0.04%) CRF08_BC (Table 1). In addition, 1 recombinant case of K/C, A/K, and D/B was detected in 2005, 2006, and 2007, respectively. These non-B subtype viruses were found mostly among the heterosexually infected population (223/302, 73.8%). In contrast, subtype B HIV-1 was found in the vast majority of MSM (1700/1773, 95.9%). In terms of nationality, Japanese patients, most of whom were MSM, were infected with subtype B HIV-1. On the other hand, only about a half of non-Japanese patients harbored subtype B HIV-1, and the remaining half were infected with non-B HIV-1, such as CRF01_AE (OR = 8.85; 95% CI 6.46–12.1; $p < 0.01$) (Table 2). This result is reasonable considering that the predominant HIV-1 subtype differs by country, and our study population included many Thais and Malaysians. In addition, this result suggests that subtype B HIV-1 is transmitted in a closed community of MSM, while non-B subtype strains are spread in wider areas among those infected through high-risk heterosexual contacts.

Table 1
Demographic characteristics of newly diagnosed HIV/AIDS patients.

	6-Year total (2573)	
Age		
Average	37.4	
Median	35	
Mode	35	
Quartile (Q1, Q3)	29, 43	
Nationality	*n*	(%)
Japanese	2319	(90.1)
Non-Japanese	225	(8.7)
Asian	83	(3.2)
Oceanian	4	(0.2)
North American	17	(0.7)
South American	58	(2.3)
European	10	(0.4)
African	26	(1.0)
Unspecified[a]	27	(1.0)
Unknown	29	(1.1)
Transmission category		
Male	2397	(93.2)
Male-to-male sexual contact	1773	(68.9)
High-risk heterosexual contact	369	(14.3)
Sexual contact	75	(2.9)
IDU	8	(0.3)
Other[b]	26	(1.0)
Unidentified	146	(5.7)
Female	170	(6.6)
High-risk heterosexual contact	152	(5.9)
IDU	3	(0.1)
Other[b]	5	(0.2)
Unidentified	11	(0.4)
Unknown	6	(0.2)
Unidentified	6	(0.2)
Hepatitis co-infection[c]		
HBV		
(+)	176	(8.4)
(−)	1925	(91.6)
Unknown	472	
HCV		
(+)	98	(4.7)
(−)	1973	(95.3)
Unknown	502	
HIV-1 subtype[c]		
B	2194	(87.9)
non-B	302	(12.1)
AE	210	(8.4)
C	30	(1.2)
AG	19	(0.8)
A	18	(0.7)
G	9	(0.4)
F	7	(0.3)
D	5	(0.2)
Other	4	(0.2)
Unidentified	77	

[a] Unspecified individuals in the nationality category were identified only as of non-Japanese origin.
[b] Other transmission categories include mother-to-child, blood products, transfusion, and needle stick.
[c] Prevalence of subtypes, HBV, and HCV was calculated after omitting the unidentified or unknown data. DU, intravenous drug user; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1.

3.3. Prevalence of drug-resistant HIV-1 is increasing in Japan

A total of 194 cases (7.7%) in the 6-year study period were found to harbor HIV-1 strains with at least one major drug-resistant muta-

Table 2
Characteristics of newly diagnosed Japanese and non-Japanese HIV/AIDS patients.

	Nationality (*n*)			Odds ratio
	Japanese	Non-Japanese	Unknown	
Gender				
Male	2224	151	22	11.45*
Female	95	74	1	
Unknown[b]			6	
Transmission category				
Male-to-male sexual contact	1691	73	9	5.60[a,*]
High-risk heterosexual contact	399	114	7	
Sexual contact	72	4	0	
Other	29	10	2	
Unidentified[b]	128	24	11	
Subtype				
B	2051	118	25	8.85*
Non-B	198	101	3	
Unidentified[b]	70	6	1	
BED assay (*n* = 640)				
Recent	220	13	0	2.31*
Not recent	351	48	8	
Drug-resistant HIV-1				
Detected	173	16	5	1.05
Not detected	2146	209	24	

[a] Odds ratios for the transmission category were calculated between male-to-male sexual contact and other categories which include high-risk heterosexual contact, sexual contact, and other.
[b] Unknown and Unidentified cases were omitted in calculation of odds ratio.
* $p < 0.01$.

tion conferred by PIs, NRTIs, or NNRTIs. The annual prevalence of drug-resistant mutations shown in Fig. 1 had an overall tendency to increase from 5.9% (16/273) in 2003 to 8.3% (50/605) in 2008. The most prevalent mutation in each year was NRTI-associated resistance, with 11 (4.0%), 12 (4.0%), 21 (5.0%), 23 (5.2%), 28 (5.9%), and 23 (3.7%) cases, followed by PI- and NNRTI-associated mutations. PI-resistant major mutations were detected in 63 cases (2.5%), and NNRTI-associated mutations were detected only in 20 cases (0.8%). These data reflect the type of antiretrovirals being prescribed in treated population. In other words, NRTIs have a long history of being prescribed including the period of mono and dual therapy; thus, NRTIs have been more frequently used. As a consequence, NRTI-resistant HIV-1 has emerged and been transmitted more frequently to treatment-naïve patients. Regarding the drug-resistant mutations shown in Table 3, T215 revertants (T215X) (3.2%), M184I/V (0.5%), K103N (0.6%), and M46I/L (1.7%) accounted for the majority of detected mutations in contrast to other muta-

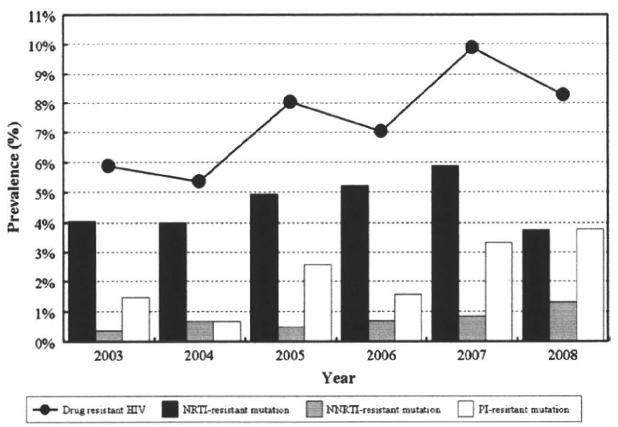

Fig. 1. Annual overall prevalence of drug-resistant HIV-1 (solid circles) in Japan increased in treatment-naïve patients in Japan from 2003 to 2008. The most prevalent mutation in each year was associated with resistance to nucleoside reverse transcriptase inhibitor (NRTI) treatment. Annual prevalence of drug-resistance mutations was categorized by antiretroviral drug class (NRTIs, solid black bars; non-nucleoside reverse transcriptase inhibitors [NNRTIs], horizontally striped bars; protease inhibitors [PIs], solid white bars). Drug-resistant HIV-1 was counted once even when the strain contained multiple drug-resistant mutations. Each drug-resistant mutation was counted even when multiple mutations were detected in one patient.

Table 3
Drug-resistant mutations in newly diagnosed HIV/AIDS patients, by class of antiretroviral drugs.

	6-Year total (2573)	
	n	(%)
NRTI[a]		
M41L	11	(0.4)
K65R	1	(0.0)
D67N/G/E	7	(0.3)
T69D	8	(0.3)
69INS	1	(0.0)
K70R/E	2	(0.1)
L74V/I	3	(0.1)
V75A/M	2	(0.1)
Y115F	3	(0.1)
M184V/I	12	(0.5)
L210W	5	(0.2)
T215X	81	(3.2)
K219Q/E/N/R	4	(0.2)
NNRTI[a]		
L100I	1	(0.0)
K101E	2	(0.1)
K103N	14	(0.6)
V106A/M	1	(0.0)
Y181C/I/V	3	(0.1)
P225H	1	(0.0)
P236L	1	(0.0)
PI[a]		
L24I	1	(0.0)
D30N	5	(0.2)
V32I	3	(0.1)
M46I/L	44	(1.7)
I47V/A	2	(0.1)
V82A/L	2	(0.1)
I85V	5	(0.2)
N88D/S	7	(0.3)
L90M	4	(0.2)

[a] Numbers of cases and the proportions in parentheses are listed.

Table 4
Predictive factors for transmission of drug-resistant HIV-1.

	Drug-resistant HIV-1 (*n*)		Odds ratio
	(+)	(−)	
Gender			
Male	183	2214	1.92
Female	7	163	
Nationality			
Japanese	173	2146	1.05
Non-Japanese	16	209	
Transmission category			
Male-to-male sexual contact	130	1643	0.91
High-risk heterosexual contact	37	484	
Sexual contact	15	60	
Other	1	40	
Unidentified[a]	11	152	
Subtype			
B	180	2014	2.36**
Non-B	11	291	
Unidentified	3	77	

[a] For calculation of odds ratio, unidentified cases were omitted.
** $p < 0.01$.

tions that were detected only sporadically throughout the study period (supplementary Table 2).

Analysis of possible predictive factors for transmission of drug-resistant HIV-1 showed that individuals infected with subtype B HIV-1 had a significantly higher tendency to harbor drug-resistant HIV-1 than non-B subtypes (OR = 2.36; 95% CI = 1.27–4.88; $p < 0.01$) (Table 4). Other possible predictive factors, including male gender (OR = 1.92; 95% CI = 0.89–4.93; $p = 0.1$), Japanese nationality (OR = 1.05; 95% CI = 0.62–1.92; $p = 1$), and MSM behavior (OR = 0.91; 95% CI 0.66–1.26; $p = 0.57$), were not significant predictive factors in our study population. These results indicate that the chance of getting infected with drug-resistant HIV-1 was the same for anyone regardless of gender, nationality, or risk behavior.

3.4. MSM are diagnosed earlier than heterosexually infected individuals

To examine awareness of HIV infection, especially of risk behavior, and to characterize HIV-testing patterns among the HIV-infected population, we estimated the time of seroconversion by quantifying the amount of anti-HIV antibody in plasma samples. Of 640 randomly selected samples in 2007 and 2008, 233 (36.4%) were classified by BED assay with a cut-off value of 0.8 as recently infected (<155-day seroconversion), while the remaining 407 (63.4%) were classified as not recently infected (Table 5). For the recently and not recently infected groups, the average $CD4^+$ T cell count and HIV-1 viral load were 285 and 215 cells/μL and 5.1×10^5 and 1.4×10^5 copies/mL, respectively. Recently infected individuals were shown by ANCOVA with $CD4^+$ T cell counts as the covariate, to have significantly higher HIV-1 viral loads than not recently infected cases (Fig. 2). These data support that the BED assay had precisely determined early infected cases.

With respect to risk behavior, the highest rate of recent infection was in MSM (39.2%), followed by either homo- or heterosexual contacts (38.9%), and heterosexual contacts (25.0%). No patients infected through a risk behavior other than sexual contacts were categorized as recently infected. Whereas 37.8% of male patients were determined to be recently infected, only 13.8% of female patients were categorized as recently infected. These findings were reinforced by statistical analysis. Recent HIV-1 infection was significantly predicted by male gender (OR = 3.79; 95% CI 1.29–15.17; $p < 0.01$), MSM behavior (OR = 1.67; 95% CI = 1.11–2.54; $p = 0.01$), Japanese nationality (OR = 2.31; 95% CI 1.20–4.76; $p < 0.01$), and infection with subtype B HIV-1 (OR = 5.64; 95% CI = 2.37–16.33; $p < 0.01$) (Table 5). In other words, Japanese males, especially those who were MSM, were more aware of being at high risk of HIV-1 infection and got tested more often than non-Japanese. In contrast, females, individuals of non-Japanese origin, heterosexuals, and non-subtype-B-infected persons, had low awareness of the risks of HIV-1 infection.

Table 5
Predictive factors for recent or not-recent seroconversion determined by BED assay, $n = 640$.

	Seroconversion (*n*)		Odds ratio
	Recent (*n* = 233)	Not recent (*n* = 407)	
Gender			
Male	229	377	3.79**
Female	4	25	
Unknown[b]	0	5	
Nationality			
Japanese	220	351	2.31**
Non-Japanese	13	48	
Unknown[b]	0	8	
Transmission category			
Male-to-male sexual contact	189	293	1.67[a,*]
High-risk heterosexual contact	24	70	
Sexual contact	7	11	
Other	0	4	
Unidentified[b]	13	29	
Subtype			
B	224	350	5.64**
Non-B	6	53	
Unidentified[b]	3	4	
Drug-resistant HIV			
Detected	14	37	0.64
Not detected	219	370	

[a] Odds ratio for the transmission category was calculated between male-to-male sexual contact and other categories which include high-risk heterosexual contact, sexual contact, and other.
[b] Unknown or unidentified cases were omitted in calculation of odds ratio.
* $p < 0.05$.
** $p < 0.01$.

Regarding associations between the time of diagnosis and drug-resistant HIV transmission event, time of diagnosis did not differ significantly between those harboring and those not harboring drug-resistant HIV-1 (OR = 0.64; 95% CI = 0.31–1.24; $p = 0.18$) (Table 5), suggesting that transmission of drug-resistant HIV-1 is not a recent trend, but has been ongoing since the first antiretroviral, AZT, was introduced in 1986.

4. Discussion

Our study results show that the proportion of drug-resistant HIV-1 among newly diagnosed cases in Japan increased slightly (by 2.4%) from 2003 to 2008, with fluctuations from year to year. Drug-resistant HIV-1 in HAART-naïve patients are transmitted from HAART-experienced patients with inadequate adherence or from other treatment-naïve individuals with drug-resistant strains, but not yet diagnosed or tested for drug-resistant HIV-1 (de Mendoza et al., 2005). Hence, drug-resistant mutations detected in the naïve population should be tightly related to trends in antiretroviral use in the treated population. Antiretrovirals available in the early days of the HAART era, especially, had short half-lives and low genetic barriers for drug resistance acquisition, making the viruses easily resistance prone. On the other hand, new antiretroviral drugs, such as lopinavir, atazanavir, amprenavir and darunavir, have been developed so that they have improved pharmacokinetics and higher genetic barriers, thus the viruses have less chance of developing drug resistance (Dunn et al., 2008; Lima et al., 2008; Zajdenverg et al., 2009). In the present study, we found that drug-resistant mutations detected among treatment-naïve patients were

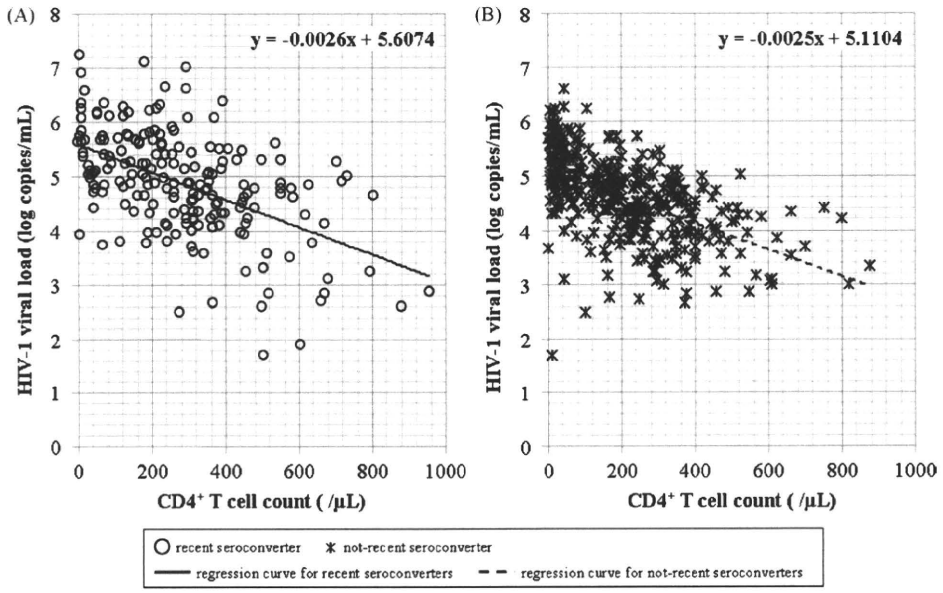

Fig. 2. Scatter plots of viral load and CD4+ T cell counts for (A) recently seroconverted patients (○), and (B) not recently seroconverted patients (*) determined by BED assay. Regression curves and their equations are shown for each group.

associated especially with antiretrovirals used prior to and early in the HAART era. It should be noted that contrary to the reports from the United States and many of European countries (Audelin et al., 2009; Vercauteren et al., 2009; Wheeler et al., 2010), the prevalence of NNRTI-resistant variants have been determined to be low in Japan, less than 1% in the study period 2003–2007 and 1.3% in 2008 being the highest. This difference is due to the situation in Japan that delavirdine had never been used and even nevirapine is only rarely prescribed. Nonetheless, strains with T215X, M46I/L, K103N, and M184V/I mutations were detected every year, suggesting that these strains are stably maintained in individuals and in high-risk populations even under antiretroviral drug-free environments. This finding is supported by the insignificant difference in prevalence of drug-resistant HIV-1 between recently and not recently infected groups. These results raise the concern that such drug-resistant strains may have become some epidemic strains actively transmitted among newly diagnosed HIV/AIDS patients. Furthermore, considering the presence of low frequent variants, the prevalence of drug-resistant mutations in this report may be higher if more sensitive techniques, such as allele-specific PCR and ultra-deep sequencing, are applied to test the samples (Halvas et al., 2010; Varghese et al., 2009). Further studies employing such techniques are needed to understand the detailed epidemic in Japan.

In investigating predictive factors for transmission of drug-resistant strains, we found that the only predictive factor was subtype B HIV-1 (OR = 2.36, $p < 0.01$). The lower transmission risk of drug-resistant strains in non-B HIV-1 can be explained by patients' countries of origin. We observed a significant relationship between non-B subtype HIV-1 and non-Japanese patients, most of whom were from developing countries with limited access to antiretrovirals. Thus, our finding agrees with reports of low prevalence drug-resistant HIV-1 transmission in developing countries (Bártolo et al., 2009; Ishizaki et al., 2009; Mintsa-Ndong et al., 2009; Ndembi et al., 2008; Nouhin et al., 2009; Pillay et al., 2008).

Interestingly, a high proportion of Japanese MSM was diagnosed as recently infected compared to patients of non-Japanese origin, and females determined by BED assay. This result may be due to successful prevention programs targeting the MSM community, so that they have become more aware of their risks of HIV-1 infection. On the other hand, many of non-Japanese patients are seen at hospitals long after HIV infection is established. In addition, women tend to be ignorant of the risks of HIV infection, thus they are often diagnosed upon a prenatal HIV screening test.

Although MSM was not a predictive factor for transmission, this group included 130 cases with drug-resistant HIV-1, the highest prevalence among all the transmission categories. Therefore, those who are involved in prevention programs should take one step further to remind the MSM community about drug-resistant HIV-1 and the limited choice of effective antiretrovirals. HIV-1 transmission has been reported to be prevented in models that assessed the effect of HIV-1 testing for wider populations and immediate initiation of antiretroviral therapy (Granich et al., 2009). Although this model seems very appealing, our results suggest the importance of not forgetting the emergence and transmission of drug-resistant HIV-1 and the limited selection of antiretroviral drugs. It is important to continue surveying newly diagnosed HIV/AIDS patients to keep track of trends in drug-resistant HIV-1 transmission, to reveal high-risk populations with low awareness of HIV infection, to propose effective programs to prevent transmission of drug-resistant HIV-1, and to develop antiretroviral drugs with improved pharmacokinetics/pharmacodynamics. All these efforts may bring us one step closer to eradicating HIV-1.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2010.07.008.

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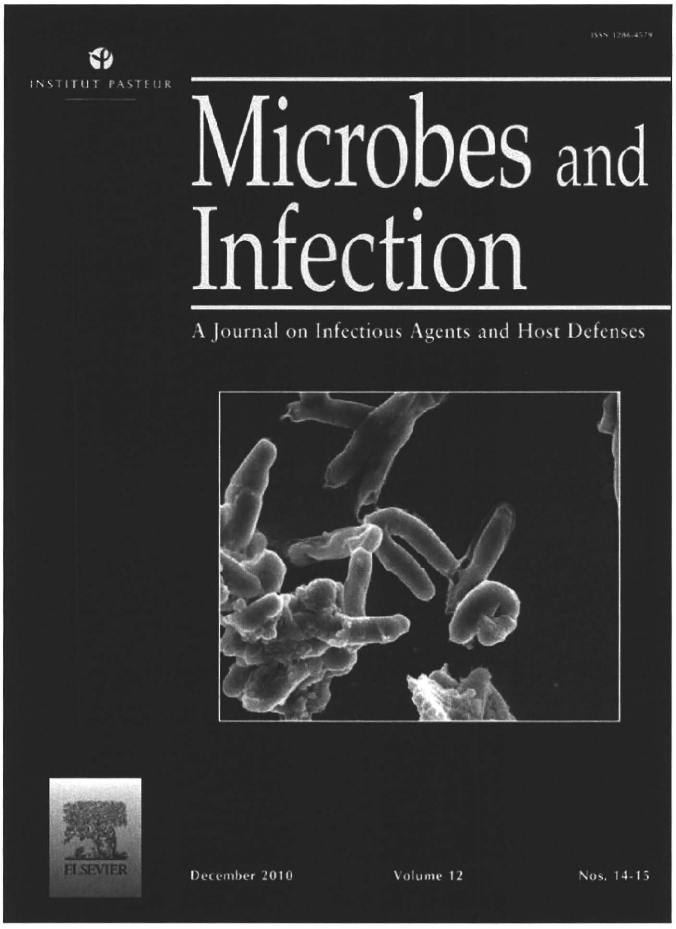

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Original article

Impact of CRF01_AE-specific polymorphic mutations G335D and A371V in the connection subdomain of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) on susceptibility to nucleoside RT inhibitors

Junko Tanuma [a,c,*], Atsuko Hachiya [a], Kyoko Ishigaki [a], Hiroyuki Gatanaga [a], Trinh Thi Minh Lien [b], Nguyen Duc Hien [b], Nguyen Van Kinh [b], Mitsuo Kaku [c], Shinichi Oka [a]

[a] *AIDS Clinical Center, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan*
[b] *National Hospital for Tropical Diseases., 78 Giai Phong Street, Dong Da District, Hanoi, Vietnam*
[c] *Department of Infection Control and Laboratory Diagnostics, Internal Medicine, Tohoku University Graduate School of Medicine. 1-1 Seiryo-cho, Aoba-ku, Sendai, Japan*

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Abstract

Certain mutations in the connection subdomain and RNase H domain of reverse transcriptase (RT) of subtype B HIV-1 contribute to resistance to nucleoside reverse transcriptase inhibitors (NRTIs). However, the impact of non-B subtype polymorphisms in this region on drug resistance remains unclear. In this study, we determined the frequencies of drug resistance mutations of the entire RT in patients with treatment failure from a cohort of Circulating recombinant form (CRF) 01_AE HIV-1-infected patients in Hanoi, Viet Nam. Subsequently, we assessed the impact of CRF01_AE polymorphisms G335D and A371V with or without thymidine analogue mutations (TAMs) on susceptibility to NRTI with recombinant viruses. In 49 patients with treatment failure, resistance mutations to NRTIs in the N-terminal half of RT were observed in 89.8%. In the C-terminal half, G335D (100%), N348I (36.8%), A371V (100%), A376S (5.3%) and A400T (97.4%) were detected, although G335D, A371V and A400T were considered polymorphisms of CRF01_AE. Drug susceptibility showed G335D, A371V, or both did not confer resistance by themselves but conferred significant resistance to NRTIs with TAMs, especially in mutants containing G335D, A371V and TAM type 2. Our results suggest the important role of CRF01_AE polymorphisms in the C-terminal half of RT in drug resistance.

Keywords: Drug resistance; Reverse transcriptase; G335D; A371V; CRF01_AE

1. Introduction

In Viet Nam, where the epidemic of human immune deficiency virus type 1 (HIV-1) has been in a rapid growth phase with an estimated number of HIV-1-infected individuals rising from 122×10^3 in 2000 to 283×10^3 in 2006, the intensive introduction of antiretroviral therapy (ART) has been implemented with two nucleoside reverse transcriptase inhibitors (NRTI) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) [1,2] and ART coverage of HIV-1-infected individuals has increased from 1% in 2003 to 28.4% in 2007 [3–5]. At the same time, concern regarding drug resistance has emerged [6].

HIV-1 reverse transcriptase (RT) is a heterodimer of two subunits: a 66-kDa subunit (p66) and a 51-kDa subunit and the p66 contains the N-terminal polymerase (codons 1–321), the connection subdomain (codons 322–440) and RNase H (codons 441–560). Although the majority of commercially available genotypic and phenotypic assays have not targeted

* Corresponding author. AIDS Clinical Center, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan. Tel.: +81 3 3202 7181x5642; fax: +81 3 3202 7198.
E-mail address: jtanuma@acc.ncgm.go.jp (J. Tanuma).

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the C-terminal half of RT: the connection subdomain and RNase H domains, certain mutations in this region have been recently found to be associated with resistance to NRTIs and NNRTIs [7–20]. Despite the accumulation of data on the prevalence and mechanisms of mutations in the C-terminal half of RT, most data are from subtype B viruses and little information is available on those of non-B subtype. Since amino acid sequence diversity in the *pol* gene is 10–15% among subtypes [21–23] and the subtype have an impact on drug resistance mutations [19–31], there is a need to determine whether inter-subtype diversity influences the spectrum of drug resistance mutations in the C-terminal half of RT as well.

Circulating recombinant form (CRF) 01_AE is the most predominant subtype in Viet Nam [32–34] and accounting for 83% of all HIV-1 infections in Southeast Asia [28–34]. Recently, Delviks-Frankenberry demonstrated that the substitution A400T, a common polymorphism in RNase H of CRF01_AE, is responsible for the high AZT resistance [16], although A400T usually emerged after AZT exposure in subtype B [19]. As well as A400T, we found a high frequency of G335D and A371V in treatment-naïve CRF01_AE patients [20]. Although G335D and A371V are assumed as common polymorphisms in CRF01_AE in the Stanford HIV Drug Resistance Database [http://hivdb.stanford.edu/index.html, accessed as late as July 20th 2010], they are thought to be associated with AZT resistance in subtype B [7,11]. However, the role of substitutions G335D and A371V in drug resistance to NRTIs has not been well characterized.

In the present study, we first investigated drug resistance mutations of CRF01_AE HIV-1 including the connection subdomain and RNase H domain of RT from HIV-1-infected patients failing ART. In addition, since we again found high frequencies of the double mutation of G335D and A371V in this population, we examined phenotypic resistance levels of these mutations by using mutant recombinant viruses containing G335D, A371V or both with or without TAMs, to determine the impacts of these mutations on drug resistance.

2. Materials and methods

2.1. Study population

HIV-1-infected patients who had taken antiretroviral therapy for more than 6 months at the National Hospital for Tropical Diseases (NHTD) in Hanoi between October 1, 2007 and June 30, 2008, were enrolled in this study. Each participant provided a written informed consent. Plasma viral load (pVL) was measured by the Cobas AmpliPrep-Cobas TaqMan system (Roche Diagnostics, Tokyo, Japan) and plasma samples were stored at −80 °C for genotypic resistance testing. When pVL was >1000 copies/ml, the patient was defined as treatment failure and the frozen plasma was shipped to the National Center for Global Health and Medicine (NCGM) in Tokyo for genotypic resistance testing.

The study protocol was approved by the institutional ethical review boards of NTHD and NCGM (IMCJ-H18-360) and by the ethics committee of the Vietnamese Ministry of Health (#1468,1469/QD-BYT dated April 19, 2007).

2.2. Reagents and cells

AZT, stavudine (d4T) and didanosine (ddI) were purchased from Sigma (St. Louis, MO). Lamivudine (3 TC) and tenofovir (TDF) were purchased from Moravek Biochemicals, Inc. (Brea, CA). Abacavir (ABC) was generously provided by GlaxoSmithKline (Philadelphia, PA). Cos-7 and MAGIC-5 cells (CCR5-transduced HeLa-CD4/LTR-β-Gal cells) were cultured and used as described previously [35].

2.3. Genotypic resistance and subtype analysis

Drug resistance genotyping was carried out by in-house protocols in NCGM. In brief, total RNA was extracted from plasma with a High Pure Viral RNA kit (Boehringer Mannheim, Mannheim, Germany), followed by reverse transcription-polymerase chain reaction (PCR) with a One Step RNA PCR kit (TaKaRa Shuzo, Otsu, Japan). Nested PCR was subsequently conducted with a Prime STAR Max Premix kit (TaKaRa Shuzo, Otsu, Japan) to amplify nearly the entire RT region (codons 1–560) and protease region. The primer sets for amplification of the N-terminal half of RT (codons 1–318) were T1-AE [5′-AGGGGGAATTGGAGGTTT; nucleotides (nt) 2393–2410] and T4-AE (5′-TTCTGTTAGTGCTTTGGTT; nt 3422–3404) for the first PCR, and T12-AE (5′-CCAGTAAAATTAAAGCCAG; nt 2574–2592) and T15-AE (5′-TCCCACTAACTTCTGTATGTC; nt 3335–3315) for the second PCR. The primer sets for amplification of the C-terminal half of RT (codons 319–560) were 3120F-AE (5′-TCTGATTTAGAAATAGGGCAG; nt 3120–3140) and 4428R-AE (5′-GTGTGC AATCTAATTGCCATAT; nt 4428–4407) for the first PCR, and 3240F-AE (5′-GGATATGAACTCCATCCTGA; nt 3240–3259) and 4316R-AE (5′-GTGGCAAATTAAAATCACTAGCC; nt 4316–4295) for the second PCR. Primer sets for amplification of protease were PR01-AE (5′-CCAACAGCCCCACCAGC; nt 2152–2168) and PR02AE (5′-ATTTTCAGGCCCAATT TTTGA; nt 2711–2691) for the first PCR, and PR03-AE (5′-AGCAGGAGCAGAAAGACAAGG; nt 2213 to) and PR04-AE (5′-CTGGCTTTAATKTTACTGGTA; nt 2592–2572) for the second PCR. The PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and subjected to direct sequencing with an ABI PRISM 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA). Amino acid sequences were deduced with the Genetyx-Win program version 8.0 (Software Development, Tokyo).

Resistance-associated mutations in the N-terminal half of RT were identified according to the International AIDS Society Resistance-USA Panel revised in December 2009 [36] and subtypes of HIV-1 in RT gene were determined by software "Genotyping/NCBI" using BLAST algorithm [http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi]. Resistant mutations in the connection subdomain and the RNase H domain of RT in the previous reports were determined if greater than three-fold increase of EC_{50} compared to

that of NL4-3 was noted in the reports. Since all sequences of the study participants belonged to CRF01_AE subtype, data on frequencies of each mutation in the C-terminal half of RT in CRF01_AE and subtype B in treatment-naïve patients was obtained from the Stanford HIV Drug Resistance Database [http://hicdb.stanford.edu/index.html, accessed as late as July 20th, 2010] for reference. Nucleotide sequences of the C-terminal half of CRF01_AE RT from 38 patients have been deposited in the DDBJ database (accession numbers AB545813–AB545850).

2.4. Construction of recombinant HIV-1 harboring G335D and/or A371V with or without TAMs

To examine the influence of G335D and A371V on drug susceptibility to NRTIs, we constructed mutant HIV-1 recombinants that included G335D, A371V or both with or without TAMs. TAM-1 virus was constructed as combination of M41L, L210W and T215Y and TAM-2 as combination of D67N, K70R and T215F. Mutant recombinant plasmid clones of the virus were generated by oligonucleotide site-directed mutagenesis as described previously [10], using pBS-RT_{WT}, which contains the entire RT coding sequences (amino acid position 14–560) and three silent restriction sites (XmaI, NheI and XbaI from the 5′ to 3′ end of RT at codons 15, 267, and 560). After site-directed mutagenesis, the mutated RT was ligated into pNL4-3, which contains the entire genome of HIV-1 and the same silent restriction sites as pBS-RT_{WT}. The infectious virus was generated by transfection of each molecular clone into Cos-7 cells, harvested and stored at −80 °C until use. Infectivity was measured as blue cell-forming units (BFU) of MAGIC-5 cells. All mutations in recombinant viruses were confirmed by full-length sequencing of the entire RT coding region.

2.5. Drug susceptibility assay

Susceptibility to NRTIs was determined by using MAGIC-5 cells as described previously [35] in more than three experiments. MAGIC-5 cells were infected with diluted virus stock (100 BFU) in the presence of increasing concentrations of RTIs, cultured for 48 h, fixed and stained with X-Gal (5-bromo-4chloro-3-indolyl-βD-galactopyranoside). The stained cells were counted under a light microscope. Drug concentrations that reduced the cell count to 50% of that of the drug-free control (EC_{50}) were determined by referring to the dose–response curve.

2.6. Statistical analysis

Data are expressed as mean ± SD. The Student's t-test was used to compare two groups of continuous variables and a p-value less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSSII software package for Windows, version 11.0J (SPSS Japan Inc, Tokyo, Japan).

3. Results

3.1. Characteristics of patients failing antiretroviral therapy

A total of 416 individuals on ART were consecutively enrolled in the present study and their pVLs were assayed between October 1, 2007 and June 30, 2008 at the NTHD in Hanoi. Among them, 49 individuals were confirmed as treatment failure by the definition described above and assigned for genotypic resistance analysis. The characteristics of the 49 individuals are listed in Table 1. All patients had received AZT or d4T plus 3TC combined with NVP, EFV or lopinavir/ritonavir (LPV/r) at the time of enrollment. The most frequently used combination was AZT, 3TC, and NVP, followed by d4T, 3TC and NVP. Protease inhibitors (PIs) were used by 17 (34.7%) patients, while the Vietnamese national ART guideline recommends d4T, 3TC plus 1 NNRTI for the first line regimen [2]. This was probably due to the inclusion of patients who had started ART when the guideline had not been issued yet. The median duration of ART exposure was 2.98 years (IQR 2.17–4.00).

3.2. Genotypic resistance patterns including C-terminal domain of RT

We successfully amplified the N-terminal half of RT and protease of all the 49 patients and C-terminal region of RT of 38 patients. The proportion of patients with at least one NRTI resistance mutation in the N-terminal half of RT was 89.8%.

Table 1
Characteristics of patients failing antiretroviral therapy.

		$n = 49$	(%)
Sex, n (%)			
males		33	(67.3)
females		16	(32.7)
Median Age, years (range)		31	(21–50)
Risk of HIV-1 infection (multiple choice), n (%)			
sexual contact		46	(93.9)
intravascular drug use		20	(40.8)
CD4 count, median cells/mm^3 (IQR)		145	(84–195)
Plasma viral load, median log copies/ml (IQR),		4.23	(3.59–4.94)
Duration of prior ART, median years (IQR)		2.98	(2.17–4.00)
Experienced ART, n (%)			
NRTI	AZT	39	(79.6)
	d4T	24	(49.0)
	3TC	49	(100)
	ddI	7	(14.3)
	ABC	2	(4.1)
	TDF	2	(4.1)
NNRTI	NVP	43	(87.8)
	EFV	15	(30.6)
PI	IDV	12	(24.5)
	SQV	6	(12.2)
	LPVr	3	(6.1)

IQR: interquartile range. ART: antiretroviral therapy. NRTI: nucleoside reverse transcriptase inhibitor. NNRTI: non-nucleoside reverse transcriptase inhibitor. PI: protease inhibitor.

Of those, M184V was the most common (81.6%) and TAMs were also observed frequently in 71.4%: M41L (22.4%), D67N (24.5%), K70R (18.4%), L210W (14.3%), T215F (16.3%), T215Y (28.6%), K219E (12.2%) and K219Q (6.1%), whereas K65R (6.1%), L74V (4.1%), Y115F (2.0%) and mutations driven by Q151M complex (4.1%) were relatively rare. Similar to previous reports on drug resistance in CRF01_AE [28–30], mutations classified into TAM type 2 (TAM-2): D67N, K70R, T215F and K219E/Q, were more frequently observed than those of TAM type 1 (TAM-1): M41L, L210W and T215Y/F (30.6% v.s. 26.5%), except for a patient having only T215F. With regard to codon 215, T215F were more frequently seen with other TAM-2 mutations (six out of eight sequences that contain T215F), concurring with the previous reports showing the introduction of T215F into TAM-2 backbone increase relative fitness in the presence of AZT but resulted in decreased viral fitness in TAM-1 backbone [37]. The resistance mutations of NNRTIs in the N-terminal half of RT were detected in 79.6%. The most frequent NNRTI-resistance mutations were Y181C/I/V (32.7%), K103N (26.5%) and G190A (26.5%). In 17 PI experienced patients, no major mutations were found, but 9 minor mutations were detected: L10I/V (11.8%), I13V (88.2%), G16E (11.8%), K20R (17.6%), M36I (100%), L63P (29.4%), H69K (100%), V82I (11.8%) and I93L (8.2%). However, the mutations in protease are considered as consensus amino acids in most non-B subtype HIV-1 (I13V, M36I and H69K) or common polymorphic mutations (L10V, G16E, K20R, L63P, V82I and I93L) and could not be determined as mutations that emerged after treatment.

The frequencies of mutations in the C-terminal half of the RT reported previously as NRTI or NNRTI resistance [7–20] are described in Table 2. As shown, G335D (100%), N348I (36.8%), A371V (100%), A376S (5.3%), E399D (28.9%) and A400T (97.4%) were detected in the patients failing ART. However, as we reported previously [20], G335D and A371V were also commonly observed in untreated patients infected with non-B subtype HIV-1 and the frequencies of G335D and A371V in CRF01_AE subtype shown in the Stanford HIV Drug Resistance Database are 95.2% and 97.1%, respectively, while those are rare in subtype B (G335D: 1.3%, A371V: 3.2%). A400T is also one of the known polymorphisms in CRF01_AE [16]. Therefore, it is unlikely that G335D, A371V and A400T in this population were selected by ART exposure or involved in the resistance mutations.

Table 2
Frequencies of mutations associated with RTI-resistance in the connection and RNase H domain of reverse transcriptase of HIV-1.

Mutations[b]	Study participants (Treatment failure)		Stanford database[a] (RTI-naïve)	
	CRF01_AE		CRF01_AE	Subtype B
	$n = 38$			
	%	(n)	%	%
G333	100	(38)		
D	0	(0)	0	0.7
E	0	(0)	0	7.5
G335	0	(0)		
C	0	(0)	0	0.5
D	100	(38)	92.0	1.3
N348	57.9	(22)		
I	36.8	(14)	0	0.5
T	5.3	(2)	0	0
A360	97.4	(37)		
I	0	(0)	0	0
V	0	(0)	0	0.7
S	2.6	(1)	1.1	0
V365	100	(38)		
I	0	(0)	0	3.2
T369	94.7	(36)		
I	0	(0)	0	0
A	2.6	(1)	19.3	3.3
V	2.6	(1)	2.8	1.2
A371	0	(0)		
V	100	(38)	97.1	3.2
A376	94.7	(36)		
S	5.3	(2)	1.7	5.8
E399	*68.4*	*(26)*		
D	*28.9*	*(11)*	*2.6*	*14*
K	*2.6*	*(1)*	*0*	*0.1*
A400	0	(0)		
T	97.4	(37)	89.2	25.3
L	2.6	(1)	0	1
Q475	100	(38)		
A	0	(0)	0	0
Q509	97.4	(37)		
L	0	(0)	0	0
R	2.6	(1)	0	0

[a] Available from http://hicdb.stanford.edu/index.html.
[b] Resistance mutations reported previously [8–21] are indicated in bold. Resistance was defined as greater than three fold increase of EC_{50} compared to that of NL4-3.

3.3. Drug susceptibility assay for mutant recombinant HIV-1

To address whether G335D or A371V have an impact on NRTI susceptibility depending on the pattern of TAMs, we constructed recombinant viruses containing G335D and/or A371V in the background of TAM-1 or TAM-2 by site-directed mutagenesis. As shown in Table 3, G335D, A371V or their double mutant did not increase the resistance levels to all NRTIs by themselves. In contrast, as shown in Table 4, variants with G335D, A371V or both exhibited higher resistance to AZT in the background of TAM-1 (8.2- to 23.2-fold) and the increased resistance level was the greatest in the double mutant G335D/A371V. Although G335D/A371V showed statistical increase in resistance to all the other NRTIs except 3TC, the fold increase from TAM-1 mutant was the greatest in AZT (Table 4). Similar to TAM-1 background, G335D, A371V or G335D/A371V with TAM-2 exhibited considerable increase in susceptibility to AZT (52.7-, 21.1-, 52.6-fold, respectively). In addition, there were marginal changes in d4T susceptibility (Table 5) in the three patterns of the mutants, G335D, A371V or G335D/A371V. In TAM-2 background, we also found G335D alone increased susceptibility to ABC (4.2-fold) and to TDF (2.4-fold), and that G335D/A371V increased susceptibility to ddI (7.2-fold), ABC (3.1-fold) and

Table 3
Drug susceptibilities of HIV-1 variants with G335D or A371V.

Mutation[a]	EC_{50} (μM)[b] (fold increase)											
	AZT		d4T		ddI		3TC		ABC		TDF	
Wild Type	0.050 ± 0.002		2.55 ± 0.07		1.90 ± 0.17		0.45 ± 0.035		2.48 ± 0.21		0.020 ± 0.0023	
335D	0.052 ± 0.004	(1)	3.19 ± 0.14	(1.3)	4.56 ± 0.20	(2.4)	0.45 ± 0.022	(1)	2.71 ± 0.17	(1.1)	0.018 ± 0.0019	(0.9)
371V	0.047 ± 0.003	(0.9)	3.26 ± 0.17	(1.3)	5.30 ± 0.02	(2.8)	0.55 ± 0.027	(1.2)	2.32 ± 0.09	(0.9)	0.027 ± 0.0014	(1.3)
335D/371V	0.052 ± 0.010	(1)	3.52 ± 0.06	(1.4)	3.38 ± 0.21	(1.8)	0.65 ± 0.023	(1.5)	2.39 ± 0.12	(1)	0.025 ± 0.0031	(1.2)

AZT, zidovudine; d4T, stavudine; ddI, didanosine; 3TC, lamivudine; ABC, abacavir; TDF, tenofovir.
[b] Data are mean ± SD from at least three independent experiments. Fold increase was the relative change in EC_{50} value compared with that of HIV-1 WT.
[a] See Materials and Methods for the construction of clones.

TDF (5.2-fold). Of note, the increased resistance levels to AZT, d4T, ddI and TDF were greater in G335D/A371V in TAM-2 background than that in TAM-1 background. Our data suggest double mutant G335D/A371V in TAM-2 background would have the most impact on NRTI susceptibility.

4. Discussion

In the present study, we described the drug resistance mutations in the entire RT of CRF01_AE HIV-1-infected Vietnamese patients who had high pVL levels despite 6-month ART. According to the criteria used for evaluation of drug resistance proposed by Shafer et al. [38,39], correlations between mutations and treatment should be confirmed by extensive resistance surveillance. However, limited sequences of CRF01_AE in the connection subdomain and RNase H domain of the RT have been available so far especially from treatment-experienced patients [40]. Santos et al. [19] previously compared amino acid variations between treatment-naïve and treatment-experienced patients in connection subdomain (280 naïve vs. 230 treated) and RNase H domain (334 naïve vs. 234 treated). Although their study included substantial number of patients, larger number of cases belonged to subtype B (80–82% of treatment-experienced patients) and the unique characteristics of CRF01_AE, accounting for only 10% of their study, could not be fully assessed. Since our present study focused on CRF01_AE sequence alone, the data provide direct information on the evaluation of drug resistance mutations in CRF01_AE, although sequences before ART initiation were not available. The largest study to date exploring treatment-related mutation in RT C-terminal site in CRF01_AE infection is the report from Thailand by Saeng-aroon et al. [40], in which significantly higher frequencies of N348I, E399D, P537S and I542M in treatment-exposed patients than treatment-naïve patients (76 naïve vs. 49 treated) was noted. Although the former two mutations have already known to be associated with exposure to NRTI or NNRTI and were detected in our treatment-experienced patients, the results of P537S and I542M were different from us: no patients in our study had P537S and I542M. Further studies are required to determine the prevalence of drug resistance mutations in the C-terminal half of RT in CRF01_AE.

Among the mutations previously reported as drug resistance in the connection subdomain and RNase H domain of RT, we found no mutations except G335D, N348I, A371V, A376S, E399D and A400T in treatment-experienced individuals with CRF01_AE infection. Of these mutations, N348I is one of the most extensively assessed mutations in the RT connection domain and has been established as multiclass resistance to both NRTIs and NNRTIs by being identified in clinical isolates in treatment-experienced individuals in subtype B and by *in vitro* drug susceptibility assay [9,10,12,13]. Since N348I is rare in treatment-naïve of both subtype B and CRF01_AE, N348I observed in 35.8% of CRF01_AE sequences in our study was considered to be treatment-related. The wide use of NVP in Viet Nam might be one of the causes of the higher prevalence of N348I in this population than in subtype B. In addition to N348I, E399D has been thought to be associated with resistance to AZT and to EFV when combined with K103R and 179D [41,42]. Although our results of E399D prevalence of in treatment-exposed patients (28.9%) was relatively higher than those in the Stanford database (9%), it was similar to the previous study by Saeng-aroon et al. of treatment-exposed patients with CRF01_AE infection (32.7%) and considered to be selected after treatment. In contrast, A376S detected in this study was not clearly identified as a treatment-related mutation because the frequency (5.3%) was similar to those of treatment-naïve

Table 4
Drug susceptibilities of HIV-1 variants with G335D or A371V in the TAM-1 background.

Mutation	EC_{50} (μM) (fold change)											
	AZT		d4T		ddI		3TC		ABC		TDF	
Wild Type	0.050 ± 0.002		2.55 ± 0.07		1.90 ± 0.17		0.45 ± 0.035		2.48 ± 0.21		0.020 ± 0.0023	
TAM-1	0.200 ± 0.016	**(4)**	4.78 ± 0.30	(1.9)	5.35 ± 0.79	(2.8)	2.37 ± 0.017	**(5.3)**	4.20 ± 0.25	(1.7)	0.043 ± 0.0030	(2.2)
TAM-1/335D	0.411 ± 0.028	**(8.2)**[a]	6.63 ± 0.05	(2.6)	5.71 ± 0.57	(3.0)	2.14 ± 0.099	**(4.8)**	3.17 ± 0.23	(1.3)	0.024 ± 0.0026	(1.2)
TAM-1/371V	0.473 ± 0.052	**(9.4)**[a]	6.07 ± 0.12	(2.4)	6.30 ± 0.48	**(3.3)**	2.45 ± 0.110	**(5.5)**	3.88 ± 0.32	(1.6)	0.046 ± 0.0018	(2.3)
TAM-1/335D/371V	1.160 ± 0.078	**(23.2)**[a]	9.01 ± 0.20	**(3.5)**[a]	7.87 ± 0.35	**(4.1)**[a]	2.40 ± 0.016	**(5.4)**	7.57 ± 0.57	**(3.1)**[a]	0.056 ± 0.0004	(2.8)

Boldface indicates an increase greater than threefold.
[a] Increases in fold change were significant compared to TAM-1 without G335D or A371V.

Table 5
Drug susceptibilities of HIV-1 variants with G335D or A371V in the TAM-2 background.

Mutation	EC_{50} (μM) (fold increase)											
	AZT		d4T		ddI		3TC		ABC		TDF	
Wild Type	0.050 ± 0.002		2.55 ± 0.07		1.90 ± 0.17		0.45 ± 0.035		2.48 ± 0.21		0.020 ± 0.0023	
TAM-2	0.3960 ± 0.076	**(7.9)**	6.18 ± 0.11	(2.4)	6.71 ± 0.57	**(3.5)**	2.57 ± 0.089	**(5.7)**	2.97 ± 0.29	(1.2)	0.033 ± 0.0026	(1.7)
TAM-2/335D	2.6390 ± 0.396	**(52.7)**[a]	7.97 ± 0.47	**(3.1)**[a]	5.74 ± 0.63	**(3)**	2.37 ± 0.082	**(5.3)**	10.43 ± 0.41	**(4.2)**[a]	0.049 ± 0.0014	(2.4)[a]
TAM-2/371V	1.0600 ± 0.131	**(21.1)**[a]	8.29 ± 0.23	**(3.3)**[a]	6.00 ± 0.64	**(3.2)**	2.58 ± 0.072	**(5.8)**	3.43 ± 0.21	(1.4)	0.036 ± 0.0012	(1.8)
TAM-2/335D/371V	2.6340 ± 0.132	**(52.6)**[a]	13.71 ± 0.76	**(5.4)**[a]	13.76 ± 0.51	**(7.2)**[a]	2.45 ± 0.062	**(5.5)**	7.57 ± 0.21	**(3.1)**[a]	0.105 ± 0.0030	**(5.2)**[a]

Boldface indicates an increase greater than threefold.
[a] Increases in fold change were significant compared to TAM-2 without G335D or A371V.

subtype B (5.8%) and CRF01_AE (1.7%) infected individuals in the Stanford database. On the other hand, G335D, A371Vand A400T were found in almost all the patients in our study. Although these three mutations are thought to be related to NRTI resistance in subtype B [7,11,16], they are common polymorphisms of wild-type CRF01_AE HIV-1 with prevalence of more than 90% in our previous study [20] and in the Stanford database. Therefore, we conclude that G335D, A371V and A400T detected in the present study were not selected after treatment but had existed before the introduction of treatment. Consequently, N348I was the only drug resistance mutation in the C-terminal half of RT observed in our cohort of treatment-experienced Vietnamese infected with CRF01_AE HIV-1.

Our results demonstrated that common CRF01_AE polymorphisms G335D and A371V play considerable role in drug resistance to NRTIs. Recent studies suggested that each of G335D or A371V is associated with drug resistance; G335D emerged after AZT exposure exhibits greater AZT resistance (8 to 53-fold over WT) when combined with TAM [11] and A371V selected in the background of D67N and K70R by high concentrations of AZT *in vitro* shows strong resistance to AZT in the presence of TAMs [7]. In agreement with those reports, our results showed that mutant containing G335D or A371V did not increase the resistance levels to NRTIs by themselves but they conferred higher resistance when combined with TAMs, especially to AZT (8.2–52.7 fold increase). Furthermore, we found that the dual mutation G335D/A371V had the greater impact than each single mutation on resistance in the presence of TAM. As G335D and A371V always appear together in treatment-naïve CRF01_AE, this finding is more critical for CRF01_AE HIV-1 infection than for subtype B infection. In addition, the fold change increased by G335D and A371V was greater with TAM-2 than that with TAM-1. Since TAM-2 is more frequent in CRF01_AE than in subtype B [28–30], this data is important for CRF01_AE HIV-1. Although the impact of G335D and A371V was the greatest in AZT resistance and seemed to be minor in other NRTIs' resistance, the fold-increase in TDF of G335D/A371V plus TAM-2 variant were above the clinical cut-off values [43], which can cause treatment failure. As TDF is often used in second line ART [2], this data is crucial for decisions on the next therapeutic strategies for CRF01_AE HIV-1-infected patients failing first line ART. Since our recombinant viruses were created with pBS-RT_{WT}, which was derived from subtype B RT but not from CRF01_AE RT, our results cannot be applied directly to CRF01_AE infection. CRF01_AE/B recombinants have been emerged and highly prevalent in Southeast Asian countries [32,44,45] and the breakpoint analysis showed some CRF01_AE/B recombinants consisted of subtype B N-terminal site and CRF01_AE C-terminal sites [45]. Therefore, our data suggests the potential influence of those CRF01_AE/B recombinants as well as CRF01_AE strain on the selection of second line therapy in Southeast Asia.

In summary, we reported the frequencies of drug resistance mutations in the connection subdomain and RNase H domain of RT in CRF01_AE HIV-1-infected Vietnamese who experienced ART. Then we demonstrated that the combination of G335D and A371V, a common pattern of polymorphisms in wild-type CRF01_AE, confer significant resistance to various NRTIs in the presence of TAMs. Our findings emphasize the important role of polymorphisms in C-terminal half of RT in CRF01_AE HIV-1 on drug resistance, especially in consideration of the second line therapy. Further investigation is needed on drug resistance mutations in widely prevailing non-subtype B HIV-1.

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