government policy against illicit drug use and extremely low lifetime prevalence of illicit drug use in the general population (2.9% in 2009 according to the Nationwide General Population Survey on Drug Use and Abuse) [20,21] (http://www.ncnp.go.jp/nimh/pdf/h21.pdf. in Japanese) (http://www.mhlw.go.jp/bunya/iyakuhin/yakubuturanyou/torikumi/dl/index-04.pdf. in Japanese). Thus, there are no data on illicit drug use among patients with HIV-1 infection, and the impact of such use on prognosis of HIV-1 infected patients in Japan [20,22].

Based on the abovementioned background, the aim of the present study was to elucidate the impact of illicit drug use on LTFU among patients with HIV-1 infection at a large urban HIV clinic in Tokyo, Japan.

Methods

Ethics Statement

This study was approved by the Human Research Ethics Committee of the National Center for Global Health and Medicine, Tokyo, Japan. The Committee waived a written informed consent, since this study only uses data of anonymized patients obtained from a routine practice. The study was conducted according to the principles expressed in the Declaration of Helsinki.

Study design

This study was designed and reported according to the recommendations of STROBE (Strengthening the Reporting of Observational studies in Epidemiology) statement [23]. We performed a single center observational study of patients with HIV-1 infection to elucidate whether illicit drug use is a risk factor for LTFU in a large urban HIV clinic in Tokyo. The AIDS Clinical Center is one of the largest clinics for HIV care in Japan with more than 3,300 registered patients. Considering that the total reported number of patients with HIV-1 infection is 21,415 by the end of 2011, this clinic treats approximately 15% of the HIV-1 infected patients in Japan (http://api-net.jfap.or.jp/status/2011/11nenpo/hyo_02.pdf. in Japanese).

Study subjects

The study population was patients with HIV-1 infection, aged >17 years, who visited our clinic for the first time from January 1, 2005 to August 31, 2010. The exclusion criteria were; 1) those who came for the second opinion and 2) those who were referred to other facilities on their first or second visit. They were excluded because the structured interview on social demographics was often not conducted for these patients. Patients who refused to have their data included in the study were also excluded. Patients were followed up until December 31, 2012.

Measurements

Variables were collected through a structured interview conducted at the first visit of each patient as part of routine clinical practice by the nurses specializing at the HIV outpatient care. The interview by these "coordinator nurses" included the following variables: history of illicit drug use and injection drug use (and type of illicit drugs if available), health insurance status, perceived route of transmission, sexuality, and whether living alone or with someone.

Because the interview could underestimate the prevalence of illicit drug use, we also searched the medical records for information on illicit drug use and related variables covering the period from the first visit to December 2012. Information on age, sex, ethnicity, treatment status for HIV infection, and history of AIDS [(defined as history of or concurrent 23 AIDS-defining diseases set by the Japanese Ministry of Health, Labour and Welfare) (http://www.haart-support.jp/pdf/guideline2012.pdf. in Japanese)] were extracted from the medical records. The laboratory data of CD4 cell count, HIV-1 viral load, and hepatitis C antibody on the first visit were also collected, and if these test results were not available on that day, the data within three months from the first visit were used.

Definition of loss to follow up

LTFU was defined according to the literature as follows: patients who discontinued their visits to the AIDS Clinical Center for at least 12 months after the last visit and who were not known to be under the care of other medical facilities or have died within 12 months of their last visit [24]. At our clinic, all patients provide their phone numbers at the first visit, and when they miss the scheduled visit, the abovementioned "coordinator nurse" calls the patient to make another appointment, or leave a message to visit if the patient does not answer the phone. If the patient does not visit the clinic after the first call, the nurses continue calling the patient every three months up to one year. For the majority of lost cases, we checked whether the patient went to seek care in another hospital, because in Japan only a few clinics provide HIV care, due to the low prevalence of HIV-1 infection (0.016%) (http://www.stat.go.jp/english/data/kokusei/pdf/20111026.pdf) (http://api-net.jfap.or.jp/status/2011/11nenpo/hyo_02.pdf. in Japanese). Thus, even if a patient stopped visiting our clinic and started seeking help at other facilities without informing the first health care provider, the new facility almost always contacts the original facility to obtain medical information.

Statistical analysis

Patients' characteristics and social demographics were compared between those who were LTFU and those who continued visiting the clinic by the Student's *t*-test for continuous variables and by either the χ^2 test or Fisher's exact test for categorical variables.

The time to LTFU as defined above was calculated from the date of the first visit to the date of LTFU. Censored cases represented those who were referred to other facilities, or who died within 12 months of their last visit, or at the end of follow-up period. The time from the first visit to LTFU was analyzed by the Kaplan Meier method for patients who experienced illicit drug use and those who did not, and the log-rank test was used to determine the statistical significance. The Cox proportional hazards regression analysis was used to estimate the impact of illicit drug use over non users on the incidence of LTFU as a primary exposure. The impact of each basic

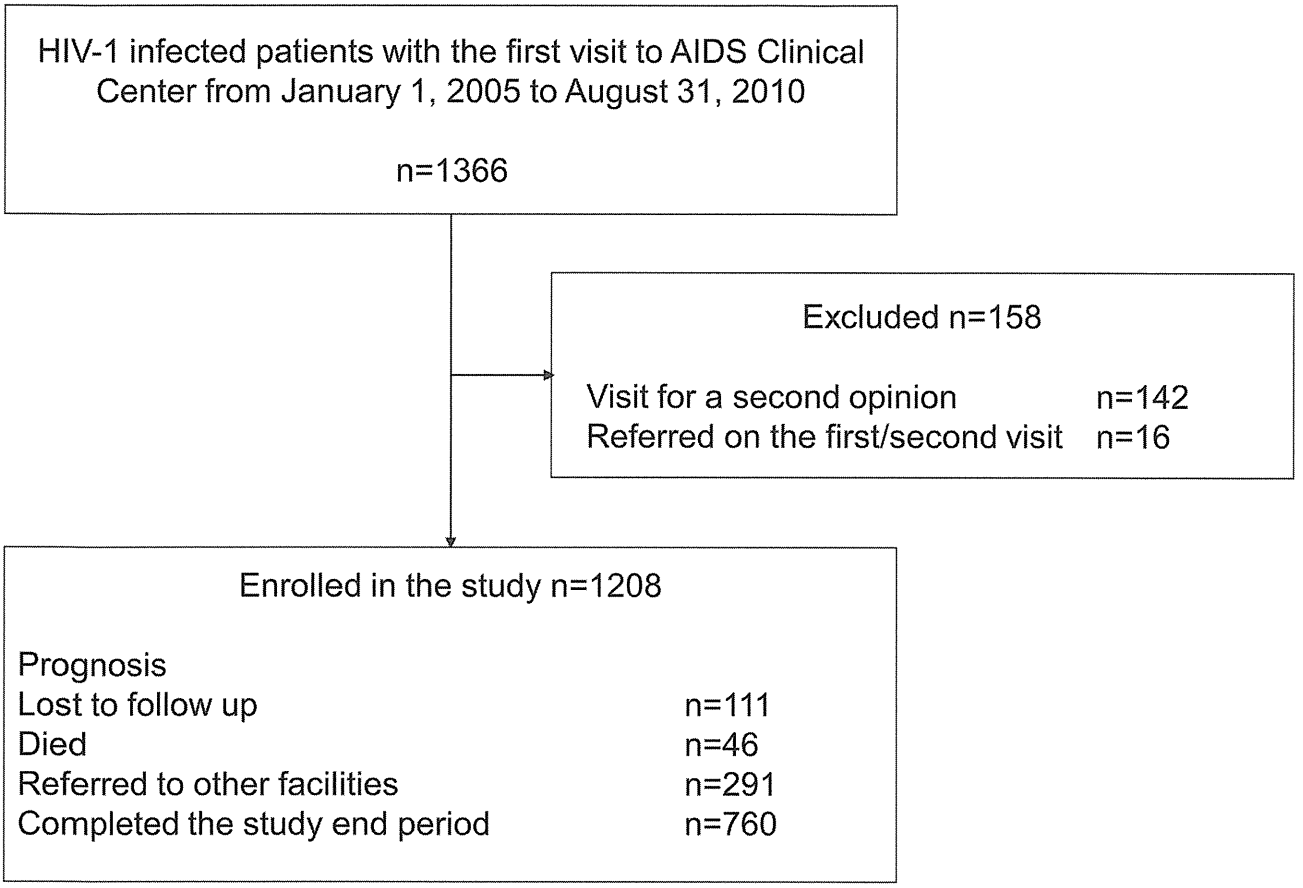

Figure 1. Patient enrollment process.

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demographics, baseline laboratory data, and other medical conditions listed above was also estimated with univariate Cox proportional hazards regression.

To estimate the unbiased prognostic impact of illicit drug use over non-users for LTFU, we conducted three models using multivariate Cox proportional hazards regression analysis. Model 1 was the aforementioned univariate analysis for illicit drug use over non users. Model 2 included basic demographics (age and Japanese) plus model 1. In model 3, we added CD4 count, ART, and health insurance status, because they showed significant relationship with LTFU in univariate analysis and the literatures showed a high CD4 count, without ART and without health insurance is a risk factor for LTFU [11,24,25]. History of AIDS and HIV-1 viral load were not added to the model, based on their multicollinearity with CD4 count and ART, respectively.

To elucidate whether the impact of illicit drug use on LTFU is affected by sexual behavior, we divided patients into MSM and non-MSM groups. Then, the abovementioned multivariate analysis was conducted for each group.

Statistical significance was defined at two-sided p values <0.05. We used hazard ratios (HRs) and 95% confidence intervals (95% CIs) to estimate the impact of each variable on LTFU. All statistical analyses were performed with The Statistical Package for Social Sciences ver. 20.0 (SPSS, Chicago, IL).

Results

A total of 1,366 patients with HIV-1 infection visited the AIDS Clinical Center for the first time during the study period. 142 patients visited for a second opinion and 16 patients were referred to other facilities on their first or second visit. Thus, 158 patients were excluded from the analysis (Figure 1). Table 1 summarizes characteristics of the 1,208 patients included in this study. The perceived route of transmission was homosexual contact in 948 (79%), heterosexual contact in 173 (14%), injection drug use in 22 (2%), contaminated blood product in 11 (1%), vertical transmission in 1 (0.1%), and unknown in 53 (4%). Further analysis indicated that 973 (81%) patients were MSM regardless of the perceived route of transmission (e.g., if a patient considered that they were infected with HIV-1 through injection drug use and they were MSM, they were classified to MSM in this study). The study patients were mostly Japanese men of relatively young age (mean: 36 years). Most patients were ART-naïve, with a median CD4 count of 245/μl.

Table 1. Baseline demographics and laboratory data for all study population, those who were lost to follow up and those who continued the visits.

	All (n=1,208)	Lost follow up (n=111)	Others (n=1,097)	P value
Sex (male), n (%)	1125 (93)	103 (93)	1022 (93)	0.84
Median (IQR) age	36 (29-43)	31 (25-39)	36 (30-43)	<0.01
Illicit drug use, n (%)	415 (34)	55 (50)	360 (33)	<0.01
Injection drug use, n (%)	53 (4)	8 (7)	45 (4)	0.14
Methamphetamine use, n (%)	63 (5)	10 (9)	53 (5)	0.07
Arrested due to illicit drug, n (%)	27 (2)	5 (5)	22 (2)	0.09
Median (IQR) CD4 count (/μl)[a]	245 (101-380)	391 (313-515)	231 (84-359)	<0.01
Median (IQR) HIV-1 viral load (log_{10}/ml)[b]	4.59 (3.89-5.18)	4.32 (3.80-4.75)	4.64 (3.91-5.20)	0.03
AIDS, n (%)	323 (27)	10 (9)	313 (29)	<0.01
On antiretroviral therapy, n (%)	131 (11)	5 (5)	126 (12)	0.02
Positive HCV antibody, n (%)	46 (4)	2 (2)	44 (4)	0.43
Men who have sex with men, n (%)	973 (81%)	89 (80)	884 (81)	0.90
Transmission category, n (%)				0.51
Homosexual contact	948 (79)	84 (76)	864 (79)	
Heterosexual contact	173 (14)	19 (17)	154 (14)	
Injection drug use	22 (2)	4 (4)	18 (2)	
Contaminated blood product	11 (1)	0	11 (1)	
Vertical transmission	1 (0.1)	0	1 (0.1)	
Unknown	53 (4)	4 (4)	49 (5)	
Ethnicity, n (%)[c]				0.02
Japanese	1070 (89)	92 (83)	978 (89)	
Asian	70 (6)	7 (6)	63 (6)	
White	27 (2)	2 (2)	25 (2)	
Black	26 (2)	7 (6)	19 (2)	
Latino	12 (1)	2 (2)	10 (0.9)	
Health insurance status, n (%)				<0.01
Without insurance	55 (5)	13 (12)	42 (4)	
With insurance/public assistance	1153 (95)	98 (88)	1055 (96)	
Working status, n (%)[d]				0.09
Unemployed	230 (19)	23 (21)	207 (19)	
With any job	909 (75)	77 (69)	832 (76)	
Student/housewife	68 (6)	11 (10)	57 (5)	
Living alone, n (%)[e]	532 (44)	46 (41)	486 (44)	0.62
Median (IQR) follow up days	1384.5 (732-1991)	266 (58-800)	1454 (914-2053)	<0.01

Data for [a] two, [b] four, [c] three, [d] one, and [e] fifteen cases, respectively, are missing

Based on the interview and medical records, 34% of the patients were illicit drug users (including injection drug users), 4% were injection drug users and 5% had used methamphetamine. Of the total, 2% were detained or arrested for possession or use of illicit drugs. Among illicit drugs, amyl nitrite and 5-methoxy-diisopropyltryptamine were the most commonly named by study patients (amyl nitrite and 5-methoxy-diisopropyltryptamine became prohibited substance by law in 2006 and 2005, respectively, in Japan) [26]. Methamphetamine, 3,4-methylenedioxymethamphetamine, cannabis, heroin, cocaine, and opium were also mentioned (numbers not counted except for methamphetamine).

LTFU patients were significantly more likely to be illicit drug users and tended to use methamphetamine and be arrested/detained due to illicit drug use than those who continued to visit the clinic. LTFU tended to be non-Japanese, younger age, had higher CD4 count, and less likely to have a history of AIDS, on ART, and covered by health insurance/public assistance, compared to the patients who continued to visit the clinic (Table 1).

Among the 1,208 patients included in the study, 111 (9.2%) were LTFU as defined above, with an incidence of 24.9 per 1,000 person-years. The median time from the first visit to LTFU was 266 days (IQR 58-800 days). Among illicit drug users (n=415) and non-users (n=793), 55 (13.3%) and 56 (7.1%) patients, respectively, were LTFU, with incidence of 35.7 and 19.2 per 1,000 person-years, respectively. Figure 2 shows the time from the first visit to LTFU by the Kaplan Meier method for the two groups. Illicit drug users were significantly more likely to stop visiting the clinic, compared to non-users (p=0.001, Log-rank test). The total observation period was 1,541.4 patient-years [median, 1,405 days, interquartile range (IQR), 674-2,029 days] for illicit drug users and 2,920.4 patient-years (median, 1,371 days, IQR, 759-1943 days) for non users.

Univariate analysis showed a significant relationship between illicit drug use and LTFU (HR=1.860; 95% CI, 1.282-2.699; p=0.001) (Table 2). Furthermore, young age, high baseline CD4 count, low HIV viral load, no history of AIDS, non Japanese, no ART, and no health insurance/public assistance were associated with LTFU. Injection drug use and methamphetamine use, respectively, were marginally associated with LTFU (injection drug use: HR=1.808; 95% CI, 0.880-3.713; p=0.107) (methamphetamine use: HR=1.684; 95% CI, 0.879-3.225; p=0.116).

Multivariate analysis identified illicit drug use as a significant risk for LTFU after adjustment for age and Japanese (adjusted HR=1.802; 95% CI, 1.209-2.686; p=0.004) (Table 3, Model 2), and also after adjustment for other risk factors (adjusted HR=1.544; 95% CI, 1.028-2.318; p=0.036) (Table 3, Model 3). Young age, high baseline CD4 count, no ART, and no health insurance/public assistance also persisted to be risk for LTFU in multivariate analysis.

Subgroup analysis of the patients stratified by sexual behavior showed that among MSM patients (n=973), the impact of illicit drug use on LTFU was slightly more evident (adjusted HR=1.641; 95% CI, 1.061-2.538; p=0.026) (Table 4) than in the total population (adjusted HR=1.544; 95% CI, 1.028-2.318; p=0.036) (Table 3, Model 3). On the other hand, illicit drug use had no significant impact in non-MSM patients (n=233) (adjusted HR=1.119; 95% CI, 0.248-5.053; p=0.883).

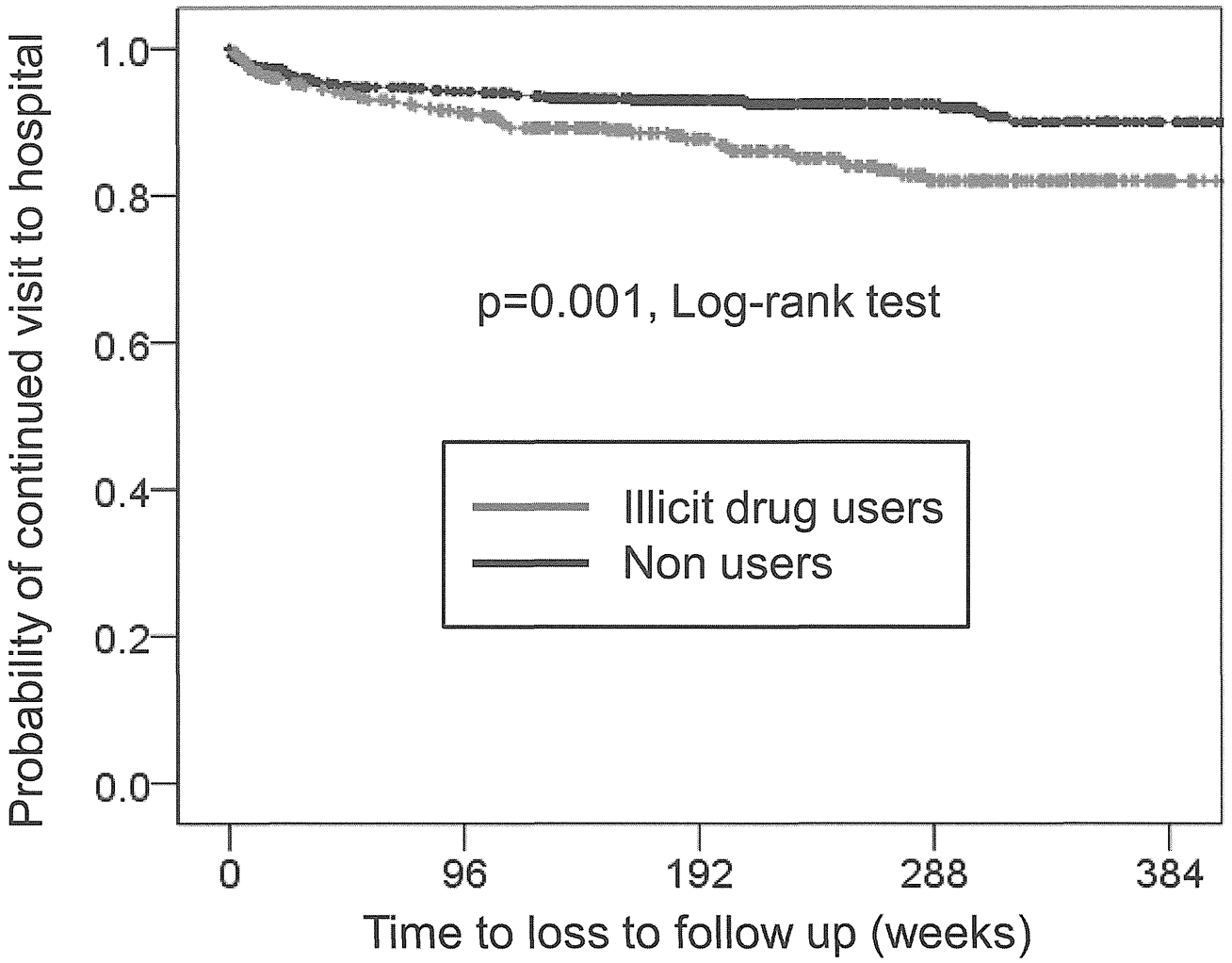

Figure 2. Kaplan-Meier curve showing time to loss to follow up for illicit drug users and non users. Compared to non drug users, illicit drug users were more likely to discontinue their visits to the hospital (p=0.001, Log-rank test).

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Table 3. Multivariate analysis to estimate the risk of illicit drug use for loss to follow up.

	Model 1 Crude (n=1,208)		Model 2 Adjusted (n=1,208)		Model 3 Adjusted (n=1,206)	
	HR	95% CI	Adjusted HR	95% CI	Adjusted HR	95% CI
Illicit drug use†	1.860	1.282-2.699	1.770	1.208-2.592	1.513	1.018-2.248
Age ≤30 years†			Reference		Reference	
30< Age ≤40 years†			0.462	0.304-0.703	0.467	0.303-0.720
Age >40 years†			0.360	0.212-0.609	0.442	0.259-0.752
Japanese			0.472	0.286-0.779	0.798	0.443-1.436
CD4 count ≤200/μl†					Reference	
200< CD4 count ≤350 /μl†					2.221	1.148-4.297
CD4 count >350/μl†					7.087	3.951-12.71
On antiretroviral therapy†					0.366	0.147-0.912
With health insurance/public assistance†					0.204	0.102-0.409

†
p<0.05 in Model 3

Table 2. Univariate analysis to estimate the risk of various factors for loss to follow up.

	Hazard ratio	95% CI	P value
Illicit drug use	1.860	1.282-2.699	0.001
Injection drug use	1.808	0.880-3.713	0.107
Methamphetamine use	1.684	0.879-3.225	0.116
Arrested/detained due to illicit drug	1.981	0.808-4.859	0.135
Male gender	0.961	0.468-1.974	0.961
Men who have sex with men	0.926	0.581-1.477	0.747
Age ≤30 years	Reference		
30 < Age ≤40 years	0.455	0.299-0.692	<0.001
Age >40 years	0.320	0.190-0.538	<0.001
CD4 count ≤200/μl	Reference		
200 < CD4 count ≤350/μl	2.536	1.318-4.878	0.005
CD4 count >350/μl	7.651	4.309-13.59	<0.001
HIV-1 viral load per $\log_{10}$/ml	0.846	0.730-0.981	0.027
History of AIDS	0.269	0.140-0.514	<0.001
Positive HCV antibody	0.466	0.115-1.888	0.285
Japanese	0.559	0.337-0.926	0.024
On antiretroviral therapy	0.402	0.164-0.986	0.046
With any job	0.870	0.549-1.376	0.551
On health insurance/public assistance	0.249	0.139-0.444	<0.001
Living alone	0.949	0.649-1.388	0.788

Table 4. Multivariate analysis to estimate the risk of illicit drug use for loss to follow up stratified by sexual behavior.

	Adjusted HR	95% CI	P value
MSM (n=973)	1.641	1.061-2.538	0.026
Non MSM (n=233)	1.119	0.248-5.053	0.883

Adjusted by variables in Table 3, Model 3 (age, Japanese, CD4 count, antiretroviral therapy, and health insurance)

MSM: men who have sex with men

Discussion

At this large urban HIV clinic in Tokyo, 9.2% of the patients were lost to follow up, with an incidence of 24.9 per 1,000 person-years. Furthermore, 34% of the study patients were illicit drug users and the incidence of LTFU for illicit drug users was almost twice higher than that for non users (35.7 and 19.2 per 1,000 person-years, respectively). Illicit drug use was identified as a significant risk for LTFU in uni- and multi-variate analyses (HR=1.860; 95%CI, 1.282-2.699; p=0.001) (adjusted HR=1.544; 95% CI, 1.028-2.318; p=0.036). The impact of illicit drug use on LTFU was slightly more evident among MSM than in the total study population.

To our knowledge, only a few studies have examined the impact of non-injection illicit drug use on LTFU [9,27], and this is the first such study conducted in Asia. The results showed that illicit drug use is a risk factor for LTFU, which is a marker for prognosis in patients with HIV-1 infection [7–11]. The result emphasizes the need for effective prevention and intervention strategies for illicit drug use in patients with HIV-1 infection in Japan. The finding of a more evident impact of illicit drug use in MSM patients also highlights the need for close monitoring of adherence to HIV care in this group of patients.

Among patients with HIV-1 infection, the prognosis of injection drug users is reported to be worse than that of non-injection drug users [28]. However, this study primarily focused on illicit drug use as a whole, rather than injection drug use for two main reasons; First, only a few studies focused on illicit drug use among HIV-1 infected patients, although a large number of studies focused on injection drugs [24,25,27,29,30]. Illicit drug use in patients with HIV-1 infection is an important issue, because not only illicit drug use lead to inferior treatment outcome compared with non users [16–18], but also non injection drug users are prone to practice high risk sexual behaviors, which might lead to transmission of HIV and other infectious diseases [14,31]. Furthermore, illicit drug use, especially opioid use, can be a trajectory into injection drug use [32,33]. Second, because only 0.5% of the patients were infected with HIV-1 through injection drug use by the end of 2011 in Japan (according to a nationwide surveillance conducted by the AIDS Surveillance Committee of the Ministry of Health, Labour and Welfare that covered all reported cases with HIV-1 infection), the anticipated prevalence of injection drug use was very low (http://api-net.jfap.or.jp/status/2011/11nenpo/hyo_02.pdf. in Japanese). Surprisingly, the prevalence of injection drug use was 4% in this study, the number is much higher than what the AIDS Surveillance Committee reported. This suggests a substantial underreporting for injection drug use as a route of transmission from the patients.

In the planning and design of effective prevention and intervention strategies for illicit drug users with HIV-1 infection in Japan, the unique circumstances related to this issue need to be taken into consideration. First, on one hand, the government maintains a strict punitive policy against illicit drug use and this policy has been one of the factors that helped maintain a relatively low prevalence of illicit drug use (lifetime prevalence 2.9%) [21] (http://www.ncnp.go.jp/nimh/pdf/h21.pdf. in Japanese). On the other hand, possibly due in part to severe criminalization of drug use, treatment and rehabilitation schemes for drug users remain poorly developed [20,34].

Second, most injected drugs in Japan are methamphetamine: In 2010, the number of arrested illicit drug users categorized by each drug was the largest for methamphetamine (12,200), while the numbers for other injectable drugs, such as heroin and cocaine were very small (22 and 112, respectively) (http://www.mhlw.go.jp/bunya/iyakuhin/yakubuturanyou/torikumi/dl/index-01.pdf. in Japanese). In the study patients, injection drug users and methamphetamine users also appeared to overlap considerably. Evidence from other countries shows that methamphetamine use has gained popularity among MSM, and methamphetamine use is strongly associated with high-risk sexual behavior [35–38]. Thus, any intervention for injection drug users with HIV-1 infection in Japan needs to take into consideration the frequent use of methamphetamines.

Several limitations need to be acknowledged. First, due to the nature of single-center study, the results of this study do

not necessarily represent all patients with HIV-1 infection in Japan. However, as abovementioned, our clinic treats approximately 15% of the total HIV patients in Japan, and furthermore, characteristics of the patients with HIV-1 infection newly diagnosed and reported to the Japanese National HIV Registry in 2011 (n=1529) is very similar to those of the study population: 94% male, 64% infected through homosexual contact, and 59% in their 20s and 30s of age (http://api-net.jfap.or.jp/status/2011/11nenpo/hyo_02.pdf. in Japanese). Most HIV-1 infected patients reside in urban areas such as Tokyo metropolitan area as well. Thus, the discrepancy between the study patients and all HIV patients in Japan should not be too large. Second, the structured interview designed for data collection does not prevent underreporting of illicit drug use. However, underreporting to a certain degree is unavoidable with regard to issues such as illicit drugs [19].

In conclusion, the incidence of LTFU in illicit drug users was almost twice higher than that in non users among patients with HIV-1 infection in Japan. Multivariate analysis identified illicit drug use as a significant risk factor for LTFU, which influences prognosis of patients with HIV-1 infection. Little data is available for illicit drug use in Japan, especially among patients with HIV-1 infection. However, all relevant parties in relation to this issue need to recognize that illicit drug use has spread among patients with HIV-1 infection, and that illicit drugs worsens adherence to HIV care in Japan. Appropriate measures for prevention and intervention of illicit drug use are urgently needed to ensure proper treatment and prevention of spread of HIV infection.

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Author Contributions

Conceived and designed the experiments: TN HG HK MT SO. Performed the experiments: MO KI. Analyzed the data: TN HK HG MT SO. Contributed reagents/materials/analysis tools: MO KI SO. Wrote the manuscript: TN HG MT SO.

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Pharmacokinetics of Rifabutin in Japanese HIV-Infected Patients with or without Antiretroviral Therapy

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Abstract

Objective: Based on drug-drug interaction, dose reduction of rifabutin is recommended when co-administered with HIV protease inhibitors for human immunodeficiency virus (HIV)-associated mycobacterial infection. The aim of this study was to compare the pharmacokinetics of rifabutin administered at 300 mg/day alone to that at 150 mg every other day combined with lopinavir-ritonavir in Japanese patients with HIV/mycobacterium co-infection.

Methods: Plasma concentrations of rifabutin and its biologically active metabolite, 25-*O*-desacetyl rifabutin were measured in 16 cases with HIV-mycobacterial coinfection. Nine were treated with 300 mg/day rifabutin and 7 with 150 mg rifabutin every other day combined with lopinavir-ritonavir antiretroviral therapy (ART). Samples were collected at a median of 15 days (range, 5–63) of rifabutin use.

Results: The mean C_{max} and AUC_{0-24} of rifabutin in patients on rifabutin 150 mg every other day were 36% and 26% lower than on 300 mg/day rifabutin, while the mean C_{max} and AUC_{0-24} of 25–*O*-desacetyl rifabutin were 186% and 152% higher, respectively. The plasma concentrations of rifabutin plus its metabolite were similar between the groups within the first 24 hours, but it remained low during subsequent 24 to 48 hours under rifabutin 150 mg alternate day dosing.

Conclusion: Rifabutin dose of 150 mg every other day combined with lopinavir-ritonavir seems to be associated with lower exposure to rifabutin and its metabolite compared with rifabutin 300 mg/day alone in Japanese patients. Further studies are needed to establish the optimal rifabutin dose during ART. The results highlight the importance of monitoring rifabutin plasma concentration during ART.

Trial registration: UMIN-CTR (http://upload.umin.ac.jp/cgi-open-bin/ctr/ctr.cgi?function=search&action=input&language=E) UMIN000001102

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Introduction

Rifabutin is commonly used for human immunodeficiency virus (HIV)-associated mycobacterial infections, especially during combination antiretroviral therapy (cART) containing HIV protease inhibitors (PIs), since it is less likely to induce hepatic microsomal enzymes than rifampicin [1–4]. Conversely, rifabutin and its active metabolite, 25–*O*-desacetyl rifabutin, are substrates of CYP 3A4 and concomitant use of PIs can elevate blood concentrations of rifabutin and 25–*O*-desacetyl rifabutin [3–8]. Such rise can increase the risk of side effects such as anterior uveitis [2,9–12]. Thus, a lower dose of rifabutin has been recommended in patients treated with PIs.

The previously recommended dose of rifabutin in combination with ritonavir-boosted PI (PI/r) [13] of 150 mg every other day, was associated with low rifabutin plasma concentrations and increases rate of acquired rifamycin resistance [14–17]. Furthermore, the Tuberculosis Trials Consortium (TBTC)/US Public Health Service Study 23 [14] suggested that AUC0–24 of 4.5 μg/mL is the cutoff value for risk of emergence of resistance to rifamycin. On the other hand, the combination of rifabutin at 150 mg thrice weekly with atazanavir-ritonavir provides exposure to rifabutin comparable to that of rifabutin 300 mg alone [11]. Thus, although 150 mg/day is the current recommended dose for rifabutin during PI/r-based cART [4], the optimal dose of rifabutin when used with a PI/r regimen remains to be established.

Ethnic differences, including body weight, renal clearance and various genetic factors like single nucleotide polymorphism (SNP), haplotype or DNA methylation [18,19], may alter the dose required to achieve a particular concentration of the drug in the circulation. Thus, pharmacokinetic studies involving different ethnic groups are needed to determine the recommended dose that take such factors into account. To our knowledge, there are no such pharmacokinetic studies for rifabutin use in Asians, who

are characterized by lower body weight compared with other ethnic groups. The present study was conducted to evaluate the pharmacokinetics of rifabutin in Japanese patients with HIV-1-related mycobacterial infection when used alone at 300 mg/day without cART and at 150 mg every other day when used in combination with lopinavir/ritonavir.

Methods

Ethics Statement

The study protocol was approved by the Ethics Committee of the National Center for Global Health and Medicine (NCGM-H20–580: approved on 7th February 2008). All participants provided their written informed consent before enrollment as indicated in the protocol.

The protocol for this study and supporting CONSORT checklist are available as supporting information; see File S1 for English translation of the protocol and File S2 for the Japanese original protocol and File S3 for CONSORT checklist.

Study design

Consecutive patients with HIV-1-related mycobacterial infection who received rifabutin-containing therapy at the National Center for Global Health and Medicine, Tokyo, Japan, between February 2008 and March 2009, were eligible for the study. After their written informed consent was provided, clinical history, physical examinations and laboratory tests (e.g., blood chemistry and complete blood cell count) were carried out within one week prior to the pharmacokinetic study. Patients were excluded if they were over 20 years of age or if they had abnormal liver function tests [aspartate aminotransferase (AST), alanine aminotransferase (ALT) or total bilirubin (>3 times the upper limit of normal: ULN)], or severe renal dysfunction (creatinine clearance <30 ml/min), and in the case of female patients if they were pregnant or breastfeeding. Rifabutin was administered while fasting at 300 mg/day and the dose was adjusted when used with cART as recommended by the treatment guideline at the time of the study [13]. Medications administered concomitantly or within 2 weeks before the first study day were recorded. To evaluate the impact of rifabutin plasma concentration on treatment efficacy and adverse events, participants were followed up for at least 2 years after stopping rifabutin. Any side effect noted during rifabutin use or within four weeks after stopping rifabutin, its association with rifabutin was assessed.

Pharmacokinetic assays

Pharmacokinetic sampling commenced after 5 days of rifabutin-containing anti-mycobacterial therapy without (Group I) or with (Group II) cART. Sequential enrollment of a patient into both groups was accepted. Blood samples were collected just before rifabutin administration and then 0.5, 1, 2, 4, 6, 8 and 24 hours afterward. Patients of Group II treated with 150 mg of rifabutin every other day underwent additional sampling at 48 hours. The plasma concentrations of rifabutin and its major metabolite, 25-O-desacetyl rifabutin [20–23] were determined simultaneously by validated high-pressure liquid chromatography (HPLC). Blood samples were taken in heparin-containing tubes, placed on ice and centrifuged at 3000×g for 10 min. Then, the obtained plasma was deproteinized by using three times volume of methanol and centrifuged 15,000×g for 5 min, and the supernatant was used for assay. The HPLC standard for rifabutin and 25-O-desacetyl rifabutin were kindly provided by Pfizer Co. (Pfizer, Inc., NY). The HPLC system consisted of Agilent 1100 series (Agilent Technologies, Santa Clara, CA). Isocratic elution was performed using the Inertsil ODS-3 column (5 μm, 4.6 mm I.D. ×150 mm; GL Sciences Inc, Tokyo, Japan) with a guard column (5 μm, 4.6 mm I.D. ×10 mm; GL Sciences Inc). The UV detection wavelength was 280 nm. The mobile phase consisted of 9 mM

Table 1. Characteristics of study subjects.

	All (n = 16)	Group I (without cART, n = 9)	Group II (with cART, n = 7)	*p* value [a]
Male sex, n	16	9	7	
Age, median years (range)	36 (23–60)	36 (23–55)	35 (23–60)	0.53
Body weight, median kg (range)	57.3 (44–66)	58.0 (46–64)	56.5 (44–66)	0.98
Mycobacterium, multiple choice, n				
M. tuberculosis	13	7	6	1.00
M. avium	4	3	1	0.94
M. kansasii	1	0	1	0.85
CD4 count, median cells/mm^3 (range)	63 (2–164)	63 (2–164)	63 (19–135)	0.84
Plasma viral load, median log copies/ml (range)	4.97 (3.43–6.62)	4.98 (4.18–6.62)	4.95 (3.43–5.18)	0.10
AST, median IU/L (range)	29 (16–70)	25 (16–59)	30 (17–51)	0.65
ALT, median IU/L (range)	27 (13–70)	26 (23–70)	29 (19–70)	0.31
Time on rifabutin, median days (range)	15 (5–63)	7 (5–20)	29 (10–63)	0.017
Time on cART, median days (range)	14 (10–29)	–	14 (10–29)	–
Concomitant medications, n				
lopinavir-ritonavir	7	–	7	–
clarithromycin	3	2	1	1.00
fluconazole	1	0	1	0.85

[a]By Fisher's exact test for categorical data and Mann Whitney's U test for continuous variables.
cART, combination antiretroviral therapy; AST, aspartate aminotransferase; ALT, alanine aminotransferase; IU, international unit.
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phosphate buffer (pH 6.8)-acetonitrile (30:70, v/v). The flow-rate was set at 1.0 ml/min and all separations were performed at 30°C in column oven.

Statistical and pharmacokinetic analyses

The area under the curve (AUC) was calculated using non-compartmental techniques (WinNonlin, ver. 5, Pharsight Corp., Mountain View, CA) based on the obtained values (AUC 0–24 h for all, AUC 0–48 h for Group II). The maximum plasma concentration (C_{max}) and time of C_{max} (T_{max}) were determined directly from the data.

Statistical analyses were performed using SPSS software package for Windows, version 17.0J (SPSS Japan Inc, Tokyo). Differences between groups were determined by using the Fisher's exact test for categorical data and the Mann Whitney's test for continuous variables. For all statistical analyses, differences were considered significant if the p value was less than 0.05.

Results

Patient characteristics

A total of 15 patients were enrolled in the study and 5 of 15 participated in both Group I and II. In total, twenty sampling was done for rifabutin pharmacokinetic analysis; 11 in Group I and 9 in Group II. Data from two sampling in Group I and 2 in Group II were excluded from the analysis because samples at 24-hour were unavailable or sampling was conducted earlier than 5 days of rifabutin use. As a result, data from 9 sampling in Group I and 7 sampling in Group II were used for analysis. The baseline characteristics of the 16 sampling cases are summarized in Table 1. All 7 patients of Group II were being treated with lopinavir/ritonavir as their cART, and thus rifabutin was administered at 150 mg every other day based on the guidelines at the time of the study [13]. Two cases of Group I and 1 of Group II were being treated with clarithromycin (CAM) [20] for systemic mycobacterial infection caused by *M. avium* or *M. intracullulare* (*M. avium* Complex: MAC). Five patients of Group I, in whom ART had been delayed several weeks after anti-mycobacterial therapy to prevent the immune reconstitution inflammatory syndrome (IRIS), were later enrolled in the study as patients of Group II (Figure 1). Accordingly, the median time of rifabutin use was longer in Group II than in Group I. There was no significant difference between the groups with regard to gender, age, body weight, CD4 counts, HIV-RNA load, type of mycobacteria and concomitant use of clarithromycin or fluconazole. All were Japanese and the median body weight was 57.3 kg. All patients completed their anti-

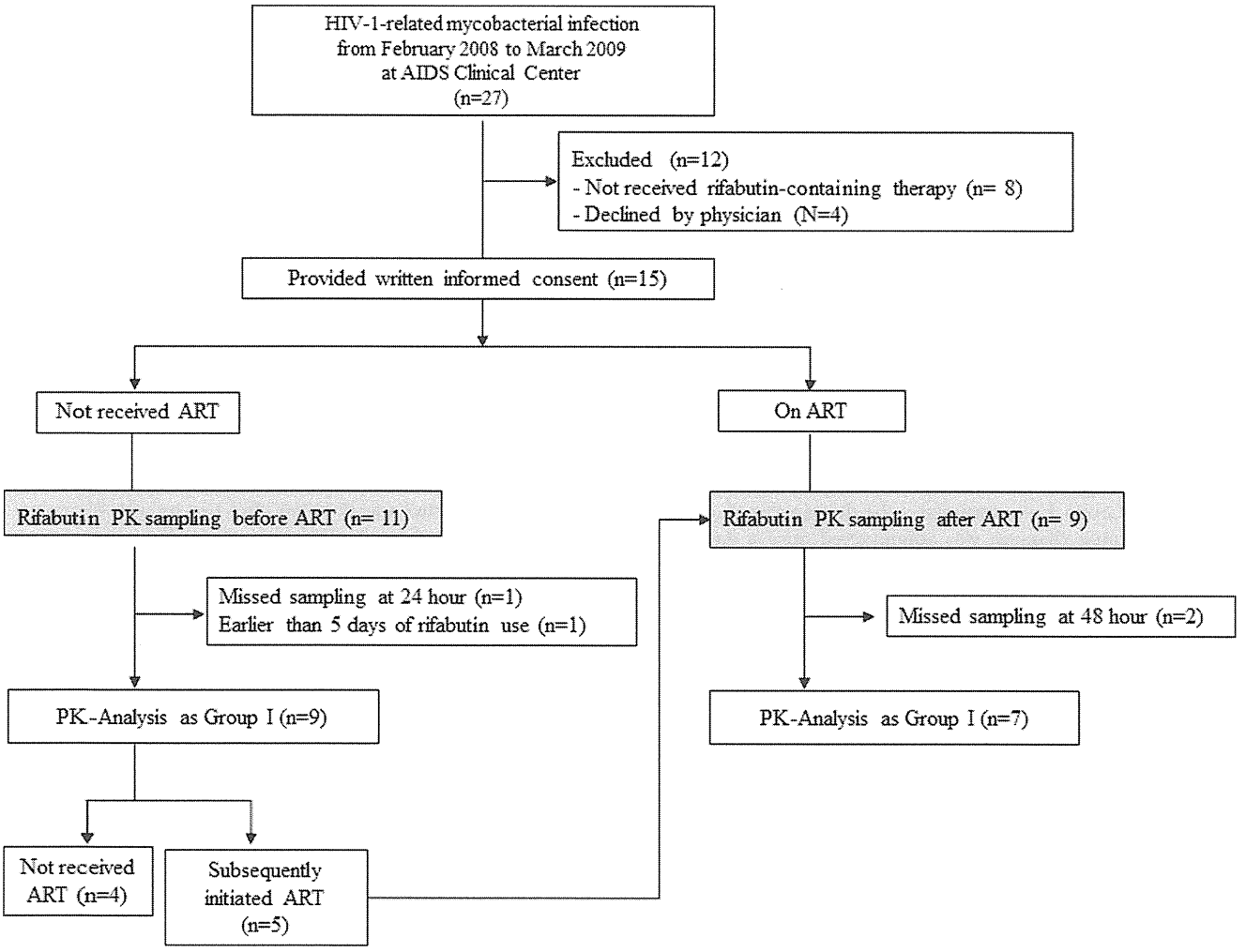

Figure 1. Flow chart of participants through the study. PK, pharmacokinetic; ART, antiretroviral therapy.
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Table 2. Pharmacokinetic parameters for rifabutin and 25-*O*-desacetyl rifabutin.

	Group I (without combination antiretroviral therapy, n = 9)				Group II (with combination antiretroviral therapy, n = 7)				*P* value[a]
	Median (range)		Mean (90% CI)		Median (range)		Mean (90% CI)		
Rifabutin									
C_{max} (μg/mL)	0.46	(0.15–0.86)	0.44	(0.39–0.49)	0.28	(0.10–0.44)	0.29	(0.25–0.33)	0.10
AUC_{0-24} (μg h/mL)	2.79	(1.32–15.7)	4.86	(3.83–5.90)	3.00	(1.13–5.43)	3.38	(2.92–3.84)	0.38
AUC_{0-48} (μg h/mL)[b]	5.59	(2.63–31.3)	9.71	(7.62–511.8)	4.21	(1.76–6.90)	4.58	(3.38–5.78)	0.32
T_{max} (h)	2.0	(2.0–4.0)	2.9	(2.6–3.1)	6.0	(2.0–12.0)	4.8	(4.1–5.1)	0.03
25-*O*-desacetyl rifabutin									
C_{max} (μg/mL)	0.00	(0.00–0.30)	0.05	(0.03–0.08)	0.13	(0.05–0.23)	0.14	(0.12–0.16)	0.05
AUC_{0-24} (μg h/mL)	0.00	(0.00–3.69)	0.82	(0.45–1.20)	1.52	(0.44–3.64)	2.07	(1.62–2.52)	0.12
AUC_{0-48} (μg h/mL)[b]	0.00	(0.00–7.38)	1.64	(0.89–2.39)	5.93	(0.44–7.21)	4.32	(3.27–5.38)	0.15
T_{max} (h)	6.0	(2.0–8.0)	5.3	(4.6–6.0)	6.0	(2.0–12.0)	5.7	(4.6–6.9)	0.87
Rifabutin plus 25-*O*-desacetyl rifabutin									
C_{max} (μg/mL)	0.47	(0.15–0.99)	0.49	(0.40–0.52)	0.42	(0.16–0.56)	0.39	(0.34–0.44)	0.54
AUC_{0-24} (μg h/mL)	3.36	(1.32–19.3)	5.49	(4.18–6.76)	6.23	(1.57–7.92)	5.27	(4.48–6.07)	0.93
AUC_{0-48} (μg h/mL)[b]	6.72	(2.63–38.7)	10.9	(8.35–13.5)	6.80	(2.20–14.1)	7.95	(6.40–9.49)	0.46

[a]By the Mann Whitney's *U* test.
[b]In Group I, AUC_{24-48} is assumed the same as AUC_{0-24} and AUC_{0-48} is calculated as double of AUC_{0-24} for comparison with Group II.
C_{max}, maximum plasma concentration; AUC, area under the curve; T_{max}, time of C_{max}; CI, confidence interval.
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mycobacterial treatment with clinical resolution of mycobacterial infections. None of the participants had treatment failure or relapse within more than 3 years of observation. Worsening of intra-abdominal lymphadenitis was observed in one patient with systemic M. avium infection at 8 months after stopping the 2-year rifabutin-containing anti-mycobacterial therapy, which excluded treatment failure or relapse. All patients confirmed complete adherence to anti-mycobacterial therapy and cART.

Pharmacokinetic parameters of rifabutin and its 25-*O*-desacetyl metabolite

The pharmacokinetic parameters of rifabutin and 25-*O*-desacetyl rifabutin are summarized in Table 2 and their mean plasma concentration-time data of 48 hours are illustrated in Figure 2A and 2B. For calculation of AUC_{0-48}, the data from 24 to 48 hours in Group I was assumed to be the same as that for 0–24 hours because rifabutin was administered once a day at the same dosage. As shown in Table 2, the mean values of C_{max} and AUC_{0-24} of rifabutin were 36% and 26% lower in Group II than in Group I, while the mean values of C_{max} and AUC_{0-24} of 25-*O*-desacetyl rifabutin were 186% and 152% higher in Group II than in Group I. However, the differences in the above values between the two groups were not statistically different. The low rifabutin concentration and high metabolite concentration in Group II may reflect the induction of rifabutin metabolism due to the longer duration of rifabutin use. Since 25-*O*-desacetyl rifabutin is microbiologically active against mycobacterium, total rifabutin activity might include rifabutin plus this metabolite. Figure 2C illustrates the mean plasma concentration of rifabutin plus the metabolite over time. Patients of Groups I and II had similar plasma concentrations of rifabutin plus the metabolite within the first 24 hours. However, the level of rifabutin plus the metabolite during the subsequent 24–48 hours was considerably lower in Group II than in Group I (dotted line in Figure 2C: Group I during 0–24 hours), whereas the AUC_{0-48} was not statistically different between the groups. Notably, 6 (67%) cases of Group I and 5 (71%) of Group II failed to achieve the AUC_{0-24} value suggested as risk for emergence of rifamycin-resistant *M. tuberculosis* [14] (4.5 μgh/mL). Neither C_{max} nor AUC_{0-24} of rifabutin and 25-*O*-desacetyl rifabutin were associated with age, body weight, body mass index, or CD4 count.

Rifabutin-associated side effects

Of the 15 participants, three patients developed side effects possibly related to rifabutin during the observational period; two of Group I developed skin rash and the other of Group II developed grade 2 rise in liver enzymes (ALT or AST 2.6–5.0 times of ULN). The skin rash appeared on day 11 of rifabutin-containing regimen in one patient and on day 28 in the other, and was resolved in both patients within several days after withdrawal of rifabutin. The rise in liver enzymes was detected after two months of rifabutin-containing regimen in combination with cART, and improved soon after discontinuation of rifabutin. Notably, the median CD4 counts in the three patients with rifabutin toxicity were significantly lower than in patients without rifabutin toxicity (12 *vs* 76, cells/mm^3, $p = 0.028$). However, rifabutin toxicity did not correlate with rifabutin AUC_{0-24}, C_{max}, or the concurrent use of cART (rifabutin AUC_{0-24}: $p = 0.37$, rifabutin C_{max}: $p = 0.86$, cART use: $p = 0.21$).

Discussion

In the present study, a low dose of rifabutin (150 mg every other day), in combination with lopinavir/ritonavir-containing cART, yielded comparable AUC_{0-24} of rifabutin and 25-*O*-desacetyl rifabutin to the commonly used dose of rifabutin of 300 mg/day. The advantage of the low-dose rifabutin included lower exposure to rifabutin and metabolite during the subsequent 24 to 48 hours in Japanese patients with HIV-mycobacteria co-infection. Since many participants started their cART after at least 1 month of

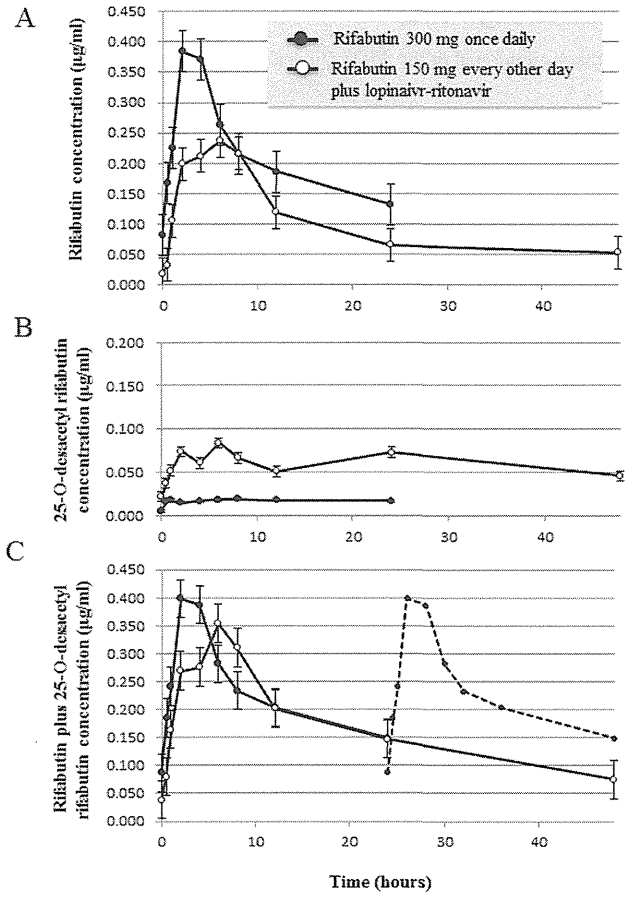

Figure 2. Mean plasma concentrations-versus-time plots of rifabutin (A), 25-*O*-desacetyl rifabutin (B), and rifabutin plus 25-*O*-desacetylrifabutin (C). Nine patients of Group I received 300 mg of rifabutin and 7 patients of Group II received 150 mg of rifabutin every other day with lopinavir/ritonavir-containing antiretroviral therapy. *Solid circles:* Group I, *open circles:* Group II. Data are mean ±1 standard errors. Dotted line in Figure C represents data of Group I during 0–24 hour for reference. RBT, rifabutin; PI/r, ritonavir-boosted protease inhibitor.
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anti-mycobacterial therapy in order to avoid deterioration by immune-reconstitution syndrome, the metabolism of rifabutin was induced upon the commencement of cART. This led to lower rifabutin concentration and higher 25-*O*-desacetyl rifabutin concentration in Group II but provided similar concentrations of rifabutin plus its active metabolite. However, on the day without medication, plasma concentrations of rifabutin and its active metabolite were lower in Group II, which were less than the susceptibility breakpoint level for *M. tuberculosis* proposed by others [20]. This suggests increased risk of emergence of rifamycin-resistant *M. tuberculosis* during the day without medication under low-dose rifabutin therapy, and that the currently recommended dosage 150 mg daily with PI/r is reasonable to this population as well. In this regard, Zhang et al. [11] reported that treatment with 150 mg/day rifabutin with atazanavir-ritonavir resulted in high risk of severe neutropenia. Furthermore, their post-hoc simulation showed that rifabutin 150 mg thrice weekly with atazanavir-ritonavir provided a comparable exposure to rifabutin compared with rifabutin 300 mg daily. Considering the risk of rifamycin-resistance and rifabutin toxicity, monitoring of rifabutin plasma concentration should be considered until the optimal rifabutin dosing during PI/r-based cART is fully established.

Although none of the patients showed treatment failure or relapse in this study, the rifabutin AUC0-24 observed in the study was in general close to the low end of the value reported in previous studies [7,14] and many participants [6 (67%) of Group I and 5 (71%) of Group II] failed to achieve AUC0-24 4.5 μgh/mL, the cutoff value suggested as risk for emergence of rifamycin-resistant M. tuberculosis [14]. One of the reasons for this discordant result might be the limitation of our study of small sample size involving several MAC and M. kansasii infections. Since acquisition of rifamycin-resistance M. tuberculosis was not frequent enough in this study group, it was difficult to evaluate the association with rifabutin pharmacokinetics and emergence of rifamycin-resistance. Other reasons may be the biological characteristics of rifabutin. Rifabutin has long postantibiotic effect against M. tuberculosis and MAC [20], shows extensive distribution in various tissues [21,22], and readily penetrates cell membranes of leucocytes [21,22]. These characteristics and their variations among patients can considerably influence the outcome of rifabutin-containing anti-mycobacterial therapy and therefore might be one of the explanations of favorable efficacy despite lower plasma concentrations of rifabutin in our study. Another limitation of this study is that plasma concentration of isoniazid was not measured, although low isoniazid plasma concentration is known to be independently related to treatment failure of HIV/TB co-infection [24]. Additionally, although there was no difference in rifabutin concentration among the patients with or without use of clarithromycin or fluconazole, those drugs can increase the rifabutin AUC and possibly affect the results. Since our study was enrolling patients with heterogeneous backgrounds in the real clinical setting, such as timing of sampling or different combination of anti-mycobacteial drugs, it was difficult to completely eliminate those impacts from the analysis. These conditions should be taken into account in the assessment of treatment outcome and associated factors in this study.

Among 15 study participants, 3 patients developed side effects related to rifabutin therapy, including skin rash and rise in liver enzymes. Notably, their CD4 counts were lower than those who did not show rifabutin toxicity, although rifabutin plasma concentrations and the concurrent use of cART were similar in the two groups. This is the first report implicating low CD4 count as a risk factor for rifabutin-related side effects. However, like other side effects of rifabutin, such as uveitis and leukocytopenia, which have been reported to be related to high-dose rifabutin or high rifabutin plasma concentrations [9–12], careful assessment involving larger population samples are needed to evaluate the association between high plasma concentrations of rifabutin and the related skin rash and hepatotoxicity.

In conclusion, in Japanese patients with HIV-mycobacteria co-infection, the plasma concentrations of rifabutin and active metabolite within the first 24 hours of treatment with low-dose rifabutin (150 mg every other day) combined with lopinavir-ritonavir, were similar to those encountered with 300 mg/day rifabutin alone. However, these concentrations decreased on the day without medication. Our findings could help determine the optimal dose of rifabutin during cART. Further studies are needed to establish the optimal dose of rifabutin during cART. Monitoring of rifabutin plasma concentration should be considered in patients with HIV-mycobacteria co-infection.

Supporting Information

Protocol S1 Summary in English. English translation of the protocol Summary.
(DOCX)

Protocol S1 Protocol and IC form in Japanese. The full version of the study protocol and the informed consent form in Japanese.
(PDF)

CONSORT Checklist S2.
(DOC)

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Author Contributions

Conceived and designed the experiments: JT. Performed the experiments: JT KS. Analyzed the data: JT. Contributed reagents/materials/analysis tools: JT KS. Wrote the paper: JT. Technical advice: YK. Patients' recruitment: KW TA HH HY K. Tsukada. Technical advice: K. Teruya HG SO.

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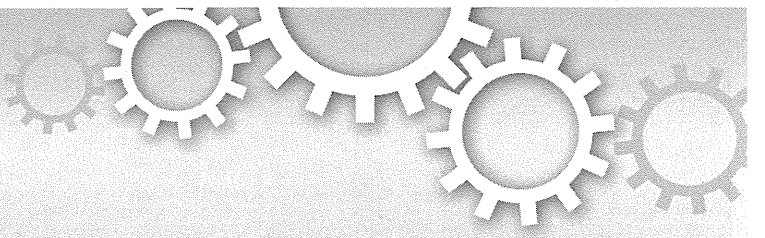

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Arginine insertion and loss of N-linked glycosylation site in HIV-1 envelope V3 region confer CXCR4-tropism

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The third variable region (V3) of HIV-1 envelope glycoprotein gp120 plays a key role in determination of viral coreceptor usage (tropism). However, which combinations of mutations in V3 confer a tropism shift is still unclear. A unique pattern of mutations in antiretroviral therapy-naive HIV-1 patient was observed associated with the HIV-1 tropism shift CCR5 to CXCR4. The insertion of arginine at position 11 and the loss of the N-linked glycosylation site were indispensable for acquiring pure CXCR4-tropism, which were confirmed by cell-cell fusion assay and phenotype analysis of recombinant HIV-1 variants. The same pattern of mutations in V3 and the associated tropism shift were identified in two of 53 other patients (3.8%) with CD4$^+$ cell count $<200/mm^3$. The combination of arginine insertion and loss of N-linked glycosylation site usually confers CXCR4-tropism. Awareness of this rule will help to confirm the tropism prediction from V3 sequences by conventional rules.

Since the introduction of maraviroc, a specific CCR5 antagonist, into clinical practice, scientific and clinical studies have focused on the coreceptor usage of human immunodeficiency virus type 1 (HIV-1)[1]. Evidence indicates the presence of a homogeneous population of predominantly CCR5-tropic variants[2,3] early in HIV-1 infection[4,5]. CXCR4-tropic variants[6,7], against which specific CCR5 antagonists are inefficient, can be distinguished from R5-tropic variants by their tendency for higher replication kinetics and a broader target cell range[8]. Their presence *in vivo* has been associated with accelerated fall in CD4$^+$ cell count and rapid disease progression[9,10]. CXCR4-tropic variants evolve from CCR5-tropic ones in the natural course of HIV-1 infection, though the exact mechanism of viral tropism evolution is not known yet. Long-term observation of natural course, which is indispensable for understanding the mechanism of tropism evolution, is usually difficult, because early use of antiretroviral therapy (ART) is highly recommended[11]. In this study, untreated natural course of one hemophiliac, who acquired HIV-1 infection through contaminated blood product before 1985 and exhibited slow disease progression, was followed until a rapid fall in CD4$^+$ cell count in 2007. The sequence change in the HIV-1 envelope (Env) glycoprotein gp120 V3 region (V3), the main determinant of HIV-1 tropism[12], was analyzed between 2003 and 2007. The results identified a unique change in 2007 associated with change in HIV-1 tropism. The same kind of sequence change in V3 was also identified in two other patients and in some of the registered sequences in the Los Alamos HIV sequence database.

Results

V3 sequence changes in Case 1. Case 1 was an ART-naive Japanese hemophiliac who acquired HIV-1 subtype B infection through contaminated blood product before 1985 and exhibited a slow disease progression. We reported previously the emergence of an escape mutation in HIV-1 Pol from cytotoxic T-lymphocyte (CTL) response in association with a mild increase in viral load in 1999 in this patient (KI-127)[13]. The HIV-1 viral load was steady around 10^4 copies/mL between 2002 and 2006 (Figure 1a). However, at the end of 2006, the viral load began to increase, coupled with a rapid fall in CD4$^+$ cell count. Since the emergence of CXCR4-tropic variants was suspected, changes in the V3 region were analyzed at five time points (June 2003, April 2006, and January, April, and November 2007) by sequencing 19–27 clones. Originally, most of the clones had identical or resembled V3

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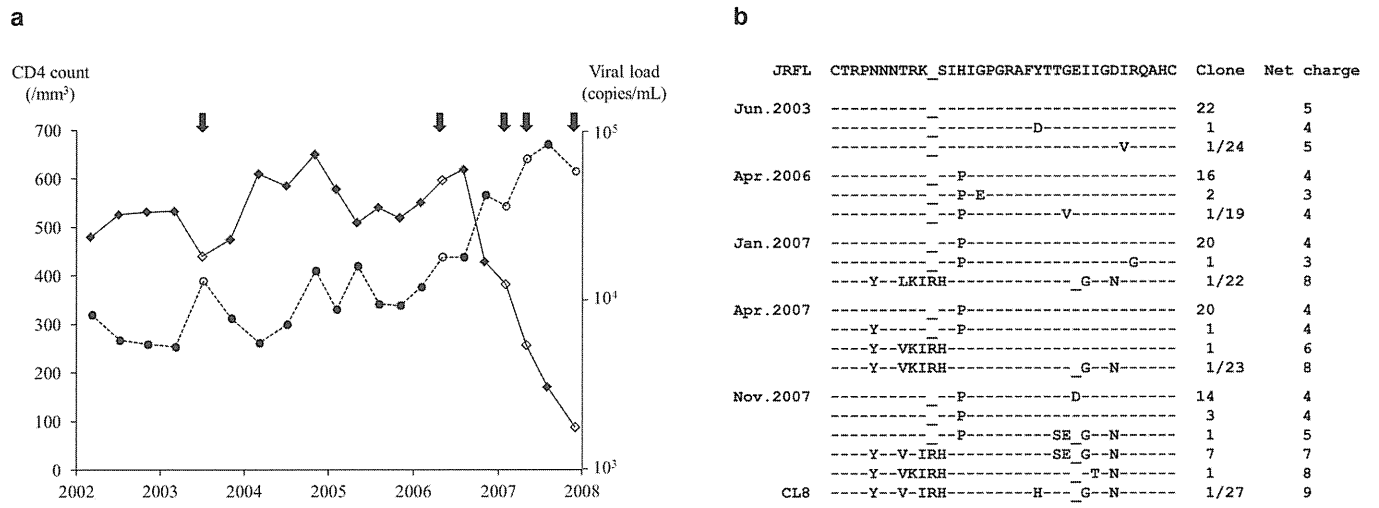

Figure 1 | (a). Clinical course of Case 1. The CD4$^+$ cell count (diamonds and solid lines) and HIV-1 load (circles and broken lines) from 2002 to 2008 are plotted. Arrows at the top indicate the five time points when V3 sequences were analyzed. Open diamonds and circles indicate the CD4$^+$ cell counts and HIV-1 loads at the same five time points. (b). V3 sequence changes in Case 1.Cloned sequences analyzed at the five time points are shown. The V3 sequence of HIV-1 JRFL is shown at the top column as a reference. Amino acids identical to those of HIV-1 JRFL are indicated as dashes. The numbers of clones harboring the corresponding V3 sequences are shown on the right.

sequences with CCR5-tropic HIV-1 JRFL (Figure 1b). Interestingly, a unique clone, harboring arginine insertion at position 11 of V3 (Ins11R), one amino acid deletion at position 25 (Del25), and other multiple amino acid substitutions, was identified at a frequency of 1/22 in January 2007, and the frequency of the same kind of the clones subsequently increased to 2/23 in April 2007, and 9/27 in November 2007, which was considered to be associated with the rapid fall in CD4$^+$ cell count.

Insertion and deletion confer CXCR4-tropism. In the next step, cell-cell fusion assay was performed to analyze the effect of the observed V3 changes on viral tropism, using Env-expressing 293 T cells and CD4$^+$ and CCR5$^+$/CXCR4$^+$ COS-7 cells. One V3 clone harboring Ins11R and Del25 identified in November 2007, named CL8-V3 (Figure 1b), was incorporated into JRFL Env-expressing plasmid. The cell-cell fusion assay demonstrated that CL8-V3 was purely CXCR4-tropic whereas JRFL was purely CCR5-tropic (Figure 2a). Ins11R occurred by the insertion of 'ACA' between 'G' and 'T' of 'AGT' at position 11 at nucleotide sequence level, and therefore, the substitution of serine (S) with histidine (H) at position 12 (S12H) was also associated with Ins11R in Case 1 ('AGT' → 'AGACAT' at nucleotide level; 'S' → 'RH' at amino acid level [Ins11R/S12H]). To identify the determinant of observed tropism change, the plasmids harboring Ins11R/S12H, Del25, and Ins11R/S12H/Del25 were also constructed using JRFL backbone. In the cell-cell fusion assay, Ins11R/S12H decreased CCR5-tropism of

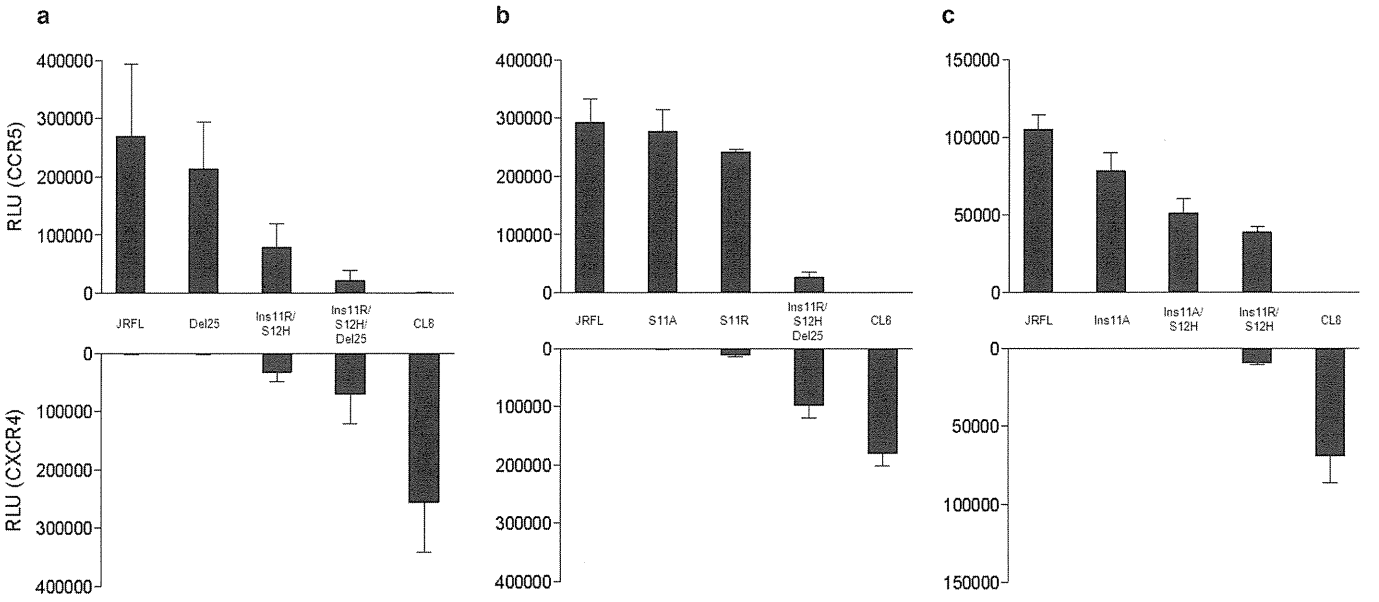

Figure 2 | Effect of Ins11R/S12H and Del25 in cell-cell fusion assay (a). Cell-cell fusion assay was performed using Env-expressing 293 T cells and CD4$^+$ and CCR5$^+$/CXCR4$^+$ COS-7 cells. Data are mean±SD values in relative luminescent unit (RLU) of six experiments (performed in duplicate and repeated three times). Analysis of two elements of Ins11R in cell-cell fusion assay (b and c). Effects of placing R at position 11 (b) and one amino acid elongation of V3 (c) were analyzed. Cell-cell fusion assay was performed using Env-expressing 293 T cells and CD4$^+$ and CCR5$^+$/CXCR4$^+$ COS-7 cells. Data are mean±SD values in relative luminescent unit (RLU) of six experiments (performed in duplicate and repeated three times).

JRFL-V3 and conferred CXCR4-tropism, resulting in dual-tropic (Ins11R/S12H vs. JRFL). Del25 further decreased the CCR5-tropism of Ins11R/S12H-V3 and increased CXCR4-tropism (Ins11R/S12H/Del25 vs Ins11R/S12H), though Del25 alone did not significantly change the JRFL-V3 tropism (Del25 vs JRFL). Considered together, the results suggest that Ins11R/S12H is indispensable for CXCR4-tropism of CL8-V3 and that Del25 strengthened the CXCR4-tropism in the presence of Ins11R/S12H. However, their combination was not enough to confer JRFL-V3 pure CXCR4-tropism (Ins11R/S12H/Del25-V3-expressing 293 T cells still fused with $CD4^+/CCR5^+$ COS-7 cells at low level), and some other mutations were necessary for pure CXCR4-tropism of CL8-V3.

Which is important, substitution or elongation? The above results suggested that Ins11R/S12H was indispensable for CXCR4-tropism of CL8-V3. According to the 11/25 rule, a basic amino acid residue (R or lysine [K]) at either position 11 or 25 of V3 is associated with CXCR4-tropism, whereas acidic or neutral amino acid residues at these positions are associated with CCR5-tropism[12,14,15]. Ins11R/S12H has two elements: one is to place R at position 11 and the other is one amino acid elongation of V3. To determine whether positioning R at 11 is sufficient for conferring CXCR4-tropism or whether V3 elongation is also necessary for this process, S at position 11 of JRFL-V3 was substituted with R (S11R) and alanine (A) (S11A) as reference. However, both substitutions did not alter the pure CCR5-tropism of JRFL (Figure 2b), indicating that not only R at position 11 but also one amino acid elongation was indispensable for dual tropism caused by Ins11R/S12H.

Is one amino acid elongation sufficient to induce CXCR4-tropism or is positioning R at 11 is also necessary? To answer this question, two V3-expressing plasmids were constructed; one harbored Ins11A only and the other harbored Ins11A and S12H (Ins11A/S12H). The cell-cell fusion assay indicated that Ins11A decreased and Ins11A/S12H further decreased infectivity with CCR5 though neither of them conferred CXCR4-tropism (Figure 2c). These results indicate that not only one amino acid elongation of V3 but also positioning R at 11 is indispensable for dual tropism caused by Ins11R/S12H.

Effect of net charge of V3. Ins11R/S12H conferred CXCR4-tropism and Del25 strengthened it. However, Ins11R/S12H/Del25-V3 was still dual-tropic, though CL8-V3 was purely CXCR-4 tropic. The next question was which mutation is necessary for Ins11R/S12H/Del25-V3 to become purely CXCR4-tropic, like CL8-V3 (to lose CCR5-tropism)? There are six amino acid substitutions in CL8-V3 (substitution of asparagine [N] with tyrosine [Y] at position 5 [N5Y], substitution of threonine [T] with valine [V] at position 8 [T8V], substitution of K with isoleucine [I] at position 10 [K10I], substitution of Y with H at position 22 [Y22H], substitution of V with glycine [G] at position 26 [V26G], and substitution of aspartic acid [D] with N at position 29 [D29N]), compared with Ins11R/S12H/Del25-V3. According to the net charge rule, the higher net charge of V3 is associated with CXCR4-tropism when calculated by subtracting the number of negatively charged amino acid residues (D and glutamic acid [E]) from the number of positively charged ones (K and R)[12,14]. D29N was the only amino acid substitution that increased the net charge of V3 among the six amino acid substitutions described above. Therefore, we analyzed the effect of D29N by adding D29N to Ins11R/S12H/Del25-V3 (Ins11R/S12H/Del25/D29N) and reverting it in CL8-V3 (CL8/N29D). In the cell-cell fusion assay, D29N reduced CCR5-tropism of Ins11R/S12H/Del25-V3 though it remained dual-tropic (Ins11R/S12H/Del25/D29N vs Ins11R/S12H/Del25), and the reversion of D29N did not change CL8-V3 tropism (CL8/N29D vs CL8) (see Supplementary Figure S1). These results indicate that D29N does not cause tropism difference between Ins11R/S12H/Del25-V3 and CL8-V3, indicating that the net charge rule did not work well.

***In silico* prediction of the effect of substitutions.** Adding D29N failed to alter the tropism of Ins11R/S12H/Del25-V3 from dual-tropic to purely CXCR4-tropic. There were five other amino acid substitutions between Ins11R/S12H/Del25-V3 and CL8-V3. Because the V3 conformation is important for coreceptor interactions[16] and because conformation of V3 loop is sensitive to V3 mutations[17,18], we examined how these V3 mutations could influence conformation of V3 in solution using molecular dynamics (MD) simulation[19]. In our MD simulation study, V3-loops of JRFL and Del25 (both CCR5-tropic) were placed in the opposite direction from the β20-β21 loop (Figure 3a), while CL8-V3 loop (CXCR4-tropic) was closed to and in the same direction with the β20-β21 loop (Figure 3c). The results were consistent with those obtained with $gp120_{LAI}$ recombinant outer domains containing CCR5-tropic and CXCR4-tropic V3 loop, respectively[17,18]. The loops of dual-tropic Ins11R/S12H-V3 and Ins11R/S12H/Del25 were located between Del25-V3 and CL8-V3 (Figure 3b). In fact, when the structural differences at the tip of the V3 tip region, i.e., the 'GPGR' amino acid residues were quantitatively measured with the root mean square deviation (RMSD) of the main chain[17], CL8-V3 was found to be located far from the loop of JRFL-V3 and Del25-V3, while those of Ins11R/S12H-V3 and Ins11R/S12H/Del25-V3 were between them (Table 1). These results suggest that our MD simulation could predict the V3 tropism based on the magnitude of the RMSD values of the V3 loop tip. In the next step, each of the six amino acid substitutions of CL8-V3 was incorporated into Ins11R/S12H/Del25-V3, and the location and conformation of the constructed loop was analyzed. When D29N was incorporated, the RMSD from JRFL-V3 decreased and that from CL8-V3 increased (Table 2), and the loop orientation was still similar to that of Ins11R/S12H/Del25 (Figure 3d), suggesting that D29N does not seem to change the tropism, compatible with the results of the cell-cell fusion assay (see Supplementary Figure S1). Among other single amino acid substitutions, only T8V was found to increase the RMSD from JRFL-V3 and decrease that from CL8-V3 (Table 2), and caused a positional shift of the V3 resembling that of the CL8-V3 (Figure 3e). N5Y did not change the orientation of the V3 loop (see Supplementary Figure S2a) though the RMSD from CL8-V3 increased and that from JRFL-V3 decreased (Table 2). K10I, Y22H, and V26G decreased the RMSD from JRFL-V3 and increased that from CL8-V3 (Table 2), and the V3 loop orientation was distinct from both Ins11R/S12H/Del25-V3 and CL8-V3 (see Supplementary Figure S2b, S2c, and S2d). These results suggest that among the six amino acid substitutions, T8V has the greatest impact on the tropism shift toward CXCR4-tropic.

Impact of T8V. Our *in silico* modeling predicted that T8V could alter the tropism of Ins11R/S12H/Del25-V3. In the next series of experiments, we incorporated T8V into JRFL-V3 (JRFL/T8V) and Ins11R/S12H/Del25-V3 (Ins11R/S12H/Del25/T8V), and analyzed the effect of such incorporation on their tropism using the cell-cell fusion assay. The incorporation of T8V into JRFL-V3 increased CCR5-tropism though it did not confer CXCR4-tropism (Figure 4a and 4b). However, T8V abrogated CCR5-tropism of Ins11R/S12H/Del25-V3 and converted it to purely CXCR4-tropic, although it did not increase CXCR4-tropism and Ins11R/S12H/Del25/T8V-V3 still had smaller CXCR4-tropism than CL8-V3 (Figure 4a). The combination of Ins11R/S12H/T8V was sufficient to confer CXCR4-tropism, although Del25/T8V did not (Figure 4b). T8V breaks the N-linked glycosylation motif 'NXT' at position 6–8 of V3, the loss of which was reported with tropism shift towards CXCR4-tropic[20,21]. Our results indicated that T8V was indispensable for pure CXCR4-tropism of CL8, which seemed to support the previous findings of the importance of the loss of N-linked glycosylation motif for CXCR4-tropism. The loss of the glycan moiety in V3 stem might lead to change gp120 interaction surface for coreceptor binding and influence coreceptor

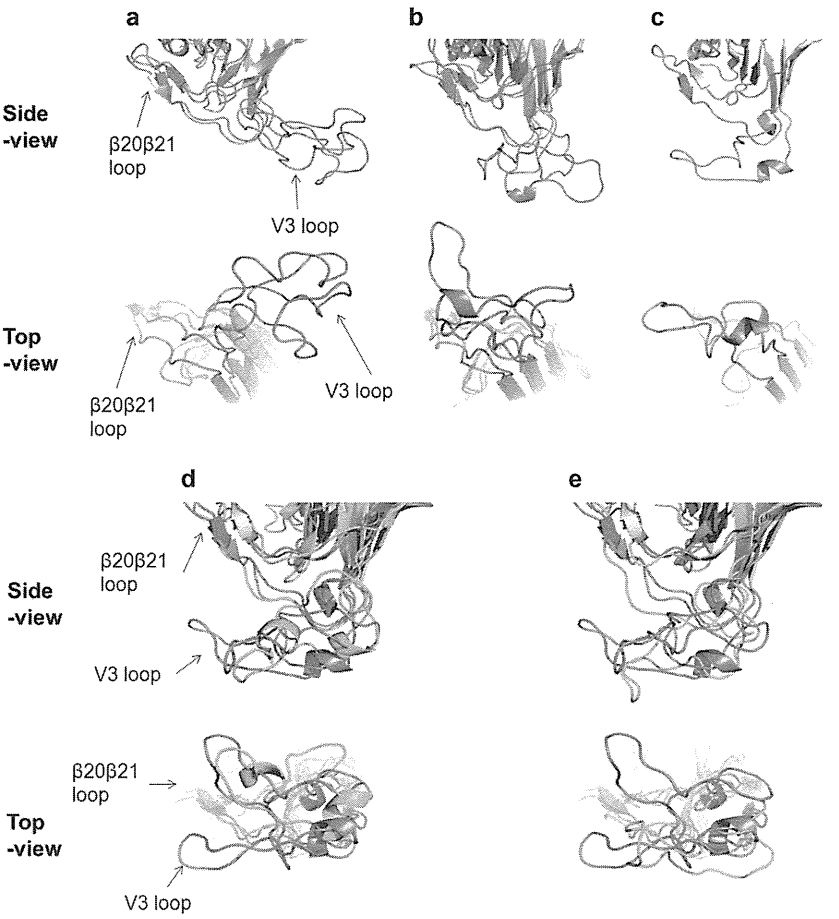

Figure 3 | Structural models of V3 loops on HIV-1 gp120 outer domains (a, b and c). MD simulations were performed for the HIV-1 JRFL gp120 outer domain with various V3 loops for CCR5 (a), dual (b), and CXCR4 (c) tropism. The most frequently appeared structures during 5–10 ns of MD simulations were extracted, and the top and side views of the structures around V3 loops are highlighted. (a) JRFL (gray) and Del25 (navy). (b) Ins11R/S12H/Del25 (gray) and Ins11R/S12H (navy). (c) CL8 (gray). Structural models of V3 loops of Ins11R/S12H/Del25-derived mutants (d and e). MD simulations were performed for the HIV-1 Ins11R/S12H/Del25 gp120 outer domain with D29N (d) or T8V (e) substitution in V3 loop. The most frequently appeared structures during 5–10 ns of MD simulations were extracted and superimposed with those of Ins11R/S12H/Del25 and CL8. (d) Superimposition of D29N (green), Ins11R/S12H/Del25 (gray), and CL8 (navy). (e) Superimposition of Ins11R/S12H/Del25/T8V (green), Ins11R/S12H/Del25 (gray), and CL8 (navy). Top and side views of the structures around V3 loops are shown.

tropism. However, available structural information was against this possibility, because the glycosylation site was exposed toward an opposite direction from the putative coreceptor binding site on V3[16,22,23]. Accordingly, presence or absence of the glycan moiety in V3 stem did not cause significant differences in V3 configuration in our MD simulation system[17,24]. Probably, amino acid substitution itself altered V3 configuration and coreceptor tropism.

Table 1 | Overall structural differences between the two V3 loop tips of various HIV-1 variants

	RMSD (Å)*			
ID of V3	JRFL	Del25	Ins11R/S12H	Ins11R/S12H/Del25
Del25	13.8	-	-	-
Ins11R/S12H	17.4	8.6	-	-
Ins11R/S12H/Del25	29.4	28.7	23.6	-
CL8	38.9	37.5	33.1	14.2

*RMSD values of the main chain atoms at V3 tips (GPGR) of two gp120 outer domain models from MD simulations. A smaller RMSD value means a closer conformation between two gp120s.

GHOST cell infection assay. Our cell-cell fusion assay indicated that Ins11R/S12H and T8V were indispensable for pure CXCR4-tropism of CL8. The next series of experiments were designed to confirm the findings using HIV-1 infection assay in GHOST cells[25,26]. HIV-1 JRFL and the recombinant HIV-1 variants harboring Del25-V3 and T8V-V3 had the same level of CCR5-tropism, although none could infect $CXCR4^+$ GHOST cells (Figure 4c). In comparison, Ins11R/S12H-V3- and Ins11R/S12H/Del25-V3-harboring variants had lower levels of CCR5-tropism. The latter variant, but not the former, infected $CXCR4^+$ GHOST cells though at low level. The Ins11R/S12H/Del25/T8V-V3-harboring variant lost the CCR5-tropism and acquired CXCR4-tropism, although the level of CXCR4-tropism was still lower than those of CL8-V3-harboring variant and HIV-1 NL4-3 (a CXCR4-tropic experimental strain). These results were compatible with the abovementioned results of the cell-cell fusion assay, though the CCR5-tropism of Ins11R/S12H/Del25-V3 seemed stronger in the cell infection assay. Dual-tropic Ins11R/S12H/Del25-V3 might have decreased susceptibility to AMD3100 used in the $CCR5^+$ GHOST Hi5 assay compared with pure CXCR4-tropic CL8-V3 and NL4-3-V3.

Same V3 pattern in two other cases. The analysis of V3 sequence changes in Case 1 demonstrated that Ins11R and the loss of N-linked

Table 2 | Effect of a single amino acid substitution on overall structure of the gp120 V3 tip

ID of V3	Added mutations*	RMSD (Å)¶ JRFL	RMSD (Å)¶ CL8
Ins11R/S12H/Del25	None	29.4	14.2
Ins11R/S12H/Del25/N5Y	N5Y	32.5	14.1
Ins11R/S12H/Del25/T8V	T8V	33.6	12.6
Ins11R/S12H/Del25/K10I	K10I	26.3	39.4
Ins11R/S12H/Del25/Y22H	Y22H	28.6	27.0
Ins11R/S12H/Del25/V26G	V26G	28.4	19.6
Ins11R/S12H/Del25/D29N	D29N	28.4	17.0

*Added amino acid substitution in the V3 loop of the Ins11R/S12H/Del25 gp120.
¶RMSD values of the main chain atoms at V3 tips (GPGR) of two gp120 outer domain models from MD simulations.

glycosylation site indispensably contribute to a shift toward CXCR4-tropism. To determine whether this finding was true only in Case 1 or could be generalized to other cases, HIV-1 subtype B V3 sequences were analyzed in 53 other treatment-naïve patients with $CD4^+$ cell count $< 200/mm^3$. The same pattern of mutations was identified in two cases (3.8%). In one case (Case 2), four of twenty analyzed sub-clones of V3 sequences harbored Ins11R associated with S12H, Del25, and N6A resulting in the loss of N-linked glycosylation site, compared with JRFL-V3 (Figure 5). In the other case (Case 3), three of twenty-two sub-clones harbored Ins11R associated with S11R, Del25, and T8V, resulting in the loss of N-linked glycosylation site. To delineate the tropism of the V3 abovementioned clones, two V3 clones in each case, one harboring Ins11R and the loss of N-linked glycosylation site (KF6 in Case 2, T16 in Case 3 [see Figure 5]) and the other harboring none of them (KF8 in Case 2, T02 in Case 3 [see Figure 5]), was incorporated into JRFL Env-expressing plasmid. As expected, cell-cell fusion assay indicated that the clones harboring Ins11R and the loss of N-linked glycosylation site (KF6 and T16) were purely CXCR4-tropic, although the clones harboring none of them (KF8 and T02) were purely CCR5-tropic (Figure 6a and 6b). The results of the GHOST cell infection assay using V3-incorporated HIV-1 JRFL (Figure 6c) were similar to those of the cell-cell fusion assay. Accordingly, it was concluded that the findings of the indispensability of Ins11R and the loss of N-linked glycosylation site for CXCR4-tropism were not only true in Case 1 but also in other cases.

Discussion

The phenotypic assay Trofile™ (Monogram Bioscience, South San Francisco, CA), which is based on recombinant virus technology, has been the most widely used diagnostic test for the detection of CXCR4-tropic HIV-1 variants[27]. However, this method has logistical and technical limitations that make it far from convenient as a diagnostic test in clinical practice. Genotypic methods based on V3 sequence represent a more feasible alternative[28] and are progressively replacing phenotypic assays, though their clinical use requires good genotypic-phenotypic correlations. The 11/25 rule and the net charge rule were proposed for the tropism prediction from V3 sequence[12,14,15], although they show only a moderate correlation with the results of phenotypic assays[12,15,28]. The results of specific genotypic tools, such as geno2pheno (Max-Planck Institute, Munich, Germany)[29,30] and position-specific scoring matrix (PSSM)[31,32] are comparable to those of phenotypic assays, suggesting that there should be some more genetic determinations for viral tropism. In this study, we successfully demonstrated two rules other than the 11/25 rule and the net charge rule on the association with CXCR4-tropic variants. One was that R insertion at position 11 of V3, not just placing R at position 11 but also one amino acid elongation, strongly shifted the HIV-1 tropism towards CXCR4-tropic. The other was that the loss of N-linked glycosylation site in V3 also shifted viral tropism towards CXCR4-tropic, which was previously described in some reports[20,21]. In the V3 analysis in our index case, R insertion at position 11 conferred dual-tropism to originally CCR5-tropic V3, and the loss of N-linked glycosylation site altered it totally CXCR4-tropic (see Supplementary Figure S3). We identified these mutation patterns not only in the index case but also in two other cases. When we surveyed V3 sequences with tropism confirmed by phenotypic assay registered at the Los Alamos HIV sequence database (Los Alamos National laboratory, Los Alamos, NM) (http://www.hiv.lanl.gov. as of September 25, 2012), 28 sequences had R insertion at position 11; 7 of 199 (3.5%) CXCR4-tropic, 14 of 513

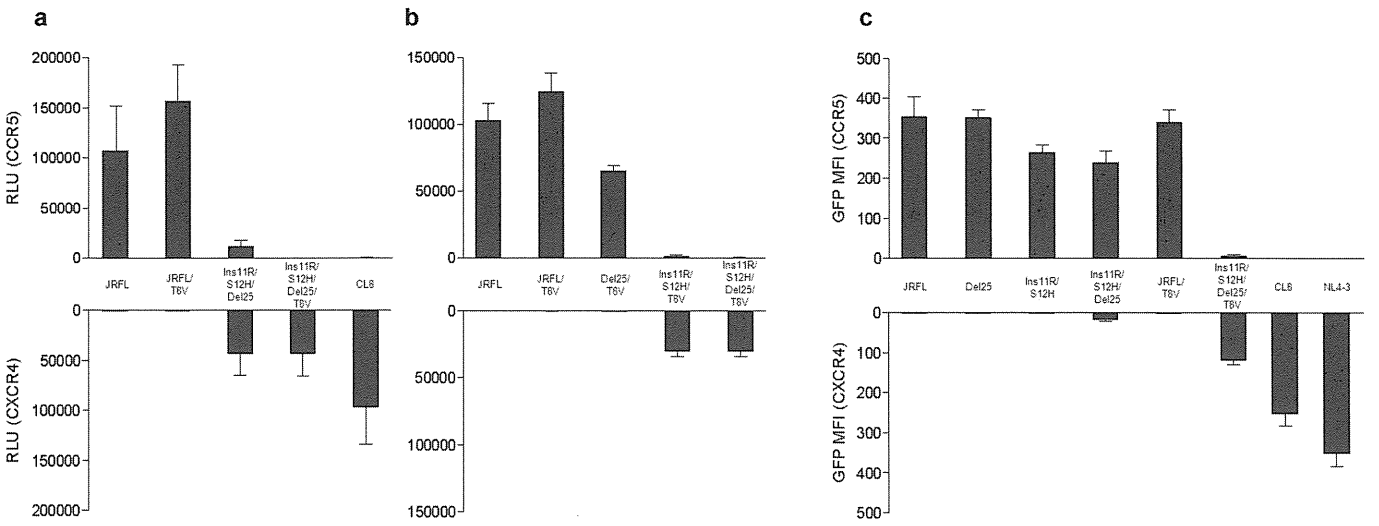

Figure 4 | Effects of T8V in cell-cell fusion assay (a and b). The effects of T8V were analyzed in combination with Ins11R/S12H/Del25 (a), and Del25 and Ins11R/S12H (b). Cell-cell fusion assay was performed using Env-expressing 293 T cells and $CD4^+$ and $CCR5^+/CXCR4^+$ COS-7 cells. Data are mean±SD values in relative luminescent unit (RLU) of six experiments (performed in duplicate and repeated three times). Tropism of recombinant HIV-1 variants harboring mutations identified in Case 1 (c). Tropism of HIV-1 variants was assessed in $CCR5^+$ GHOST Hi5 and $CXCR4^+$ GHOST CXCR4 cells. The mean fluorescent intensity (MFI) of infected cells expressing green fluorescent protein (GFP) was measured. Data are mean±SD values of six experiments (performed in duplicate and repeated three times).

		JRFL CTRPNNNTRK_SIHIGPGRAFYTTGEIIGDIRQAHC	Clone	Net charge
Case2	KF8	----------_G--M------F-DN_------K---	12	6
		---------R_G--M------F-DN_------K---	2	6
		----------_G--M---G--F-DN_------K---	1	5
		----------_G--M------F-DN_------K-Y-	1	5
	KF6	-----AI-K-RHF----------NN_KV----K---	2	10
		-----AI-KRRHF----------NK_-V----K---	1	9
		-----AI-K-RHF----------N_KV----K---	1/20	10
Case3	T02	----------_----------FA-D_---N--K-Y-	16	6
		----------_----------FA-D_------K-Y-	2	5
		----S-----_----------FA-D_------K-Y-	1	5
	T16	------KVIRRR---------VA-D_TT----K-Y-	3/22	7

Figure 5 | Cloned V3 sequences in Cases 2 and 3. The V3 sequence of HIV-1 JRFL is shown at the top column as a reference. Amino acids identical to those of HIV-1 JRFL are indicated as dashes. The numbers of clones harboring the corresponding V3 sequences are shown on the right.

(2.7%) dual-tropic, and 7 of 3301 (0.2%) CCR5-tropic sequences. Their frequency was significantly higher in CXCR4-tropic and dual-tropic sequences than CXCR5-tropic ones ($p < 0.0001$; Chi-square test). (All of the 7 CCR5-tropic sequences with R insertion at position 11 were sub-clones derived from one pair of a transmitter mother and her transmitted child[33], and the sequences were so unique that it was actually difficult to determine the exact site of one amino acid insertion). Interestingly, all of the 28 V3 sequences with R insertion at position 11, had lost the N-linked glycosylation site and had one amino acid deletion in the C-terminal half of V3 (one amino acid deletion at position 24 or 25 in 18 sequences [64.3%]), similar to our three cases. No other amino acid elongation patterns were found in the N-terminal half of V3 in the Los Alamos database. There were 3,301 CCR5-tropic V3 sequences registered in the database. Among them, 18 sequences had a basic amino acid residue at position 11 and therefore they were misjudged as CXCR4-tropic by the 11/25 rule. Only 7 of them had R insertion and the other 11 were recognized as CCR5-tropic by our rules. Therefore using our rules increased the specificity from 99.5% [(3,301–18)/3,301] to 99.8% [(3,301–7)/3,301] in identifying CXCR4- or dual-tropic V3 sequences in the Los Alamos database.

Considered together, amino acid elongation may be a rare event, but R insertion at position 11 sometimes occurs. The occurrence of such insertion seems to be always accompanied by loss of the N-linked glycosylation site and deletion of one amino acid in the C-terminal half of V3. The combination of these mutations usually confers CXCR4-tropism. Awareness of this rule will help to confirm the tropism prediction from V3 sequences by conventional rules.

Methods

Patients. Case 1 was an ART-naive Japanese hemophiliac who acquired HIV-1 subtype B infection through contaminated blood product before 1985 and exhibited slow disease progression, as described previously (KI-127)[13]. The study also included 53 other treatment-naïve HIV-1 subtype B-infected patients with $CD4^+$ cell count < 200/mm^3, who were newly diagnosed in 2008. The ethics committee of The National Center for Global Health and Medicine approved the study and all participants provided written informed consent.

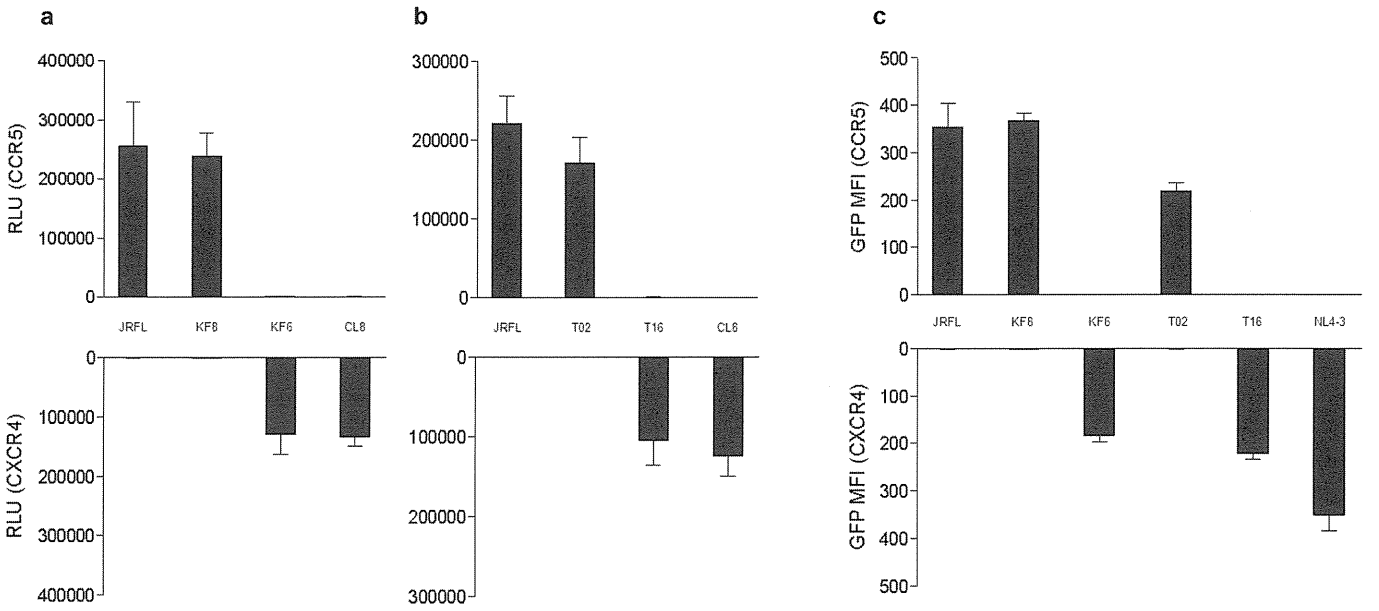

Figure 6 | Tropism of cloned V3 incorporated into JRFL gp120 backbone (a and b). Two distinct V3 clones from each of Case 2 (a) and Case 3 (b) were analyzed with the reference of JRFL-V3 and CL8-V3. Cell-cell fusion assay was performed using Env-expressing 293T cells and $CD4^+$ and $CCR5^+$/$CXCR4^+$ COS-7 cells. Data are mean±SD values in relative luminescent unit (RLU) of six experiments (performed in duplicate and repeated three times). Tropism of recombinant HIV-1 variants harboring V3 sequences derived from Cases 2 and 3 (c). Tropism of HIV-1 variants was assessed in $CCR5^+$ GHOST Hi5 and $CXCR4^+$ GHOST CXCR4 cells. The mean fluorescent intensity (MFI) of infected cells expressing green fluorescent protein (GFP) was measured. Data are mean±SD values of six experiments (performed in duplicate and repeated three times).

Cells. The 293 T and COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX). Parental GHOST cells[34] were cultured in DMEM supplemented with 10% FBS, 500 μg/ml G418 and 100 μg/ml hygromycin B. CCR5$^+$ GHOST Hi5 and CXCR4$^+$ GHOST CXCR4 cells[34] were cultured in DMEM supplemented with 10% FBS, 500 μg/ml G418, 100 μg/ml hygromycin B and 1 μg/ml puromycin.

Amplification, cloning and sequencing of Env V3 region. Total RNA was extracted from 200 μl of plasma using High Pure Viral RNA Kit (Roche, Indianapolis, IN) according to the instructions supplied by the manufacturer. HIV-1 cDNA was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) using the One Step RNA PCR kit (TaKaRa Bio, Kyoto, Japan). The DNA fragments were amplified by using the Ex Taq Hot Start Version (TaKaRa Bio) with the primer sets as follows. The Env fragment containing V3 region was amplified by RT-PCR with primers of C2 (5′ - AATGTCAGCACAGTACAATGTACAC - 3′) and C3 (5′ - ACAATTTCTGG GTCCCCTCCTGAGGA - 3′). S1 (5′ - ATGGAATTAGGCCAGTAGT - 3′) and A1 (5′ - CTCTTAATTTTATAACTATC - 3′) primer sets were used for nested PCR. The amplified PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and cloned by using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. At least 19 colonies were selected, inoculated into 4 ml of L broth, and incubated at 37°C overnight under vigorous agitation. In the next step, plasmids were isolated by using the QIAprep Spin Miniprep Kit (Qiagen). The purified plasmids were sequenced by using the ABI BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and processed with an automated ABI 3730 DNA Analyzer (Applied Biosystems).

Plasmid construction. The pcDNA6.2-CCR5 and pcDNA6.2-HIV-tat plasmids were constructed as described previously[35]. Briefly, the entire human CCR5 gene including a stop codon was amplified using pZeoSV-CCR5[36] as a template. The PCR product was ligated into pcDNA6.2/cLumio-DEST vector (Invitrogen), cloned using the method recommended by the manufacture, and termed as pcDNA6.2-CCR5. The CD4 expression vector (pcDNA6.2-CD4) and CXCR4 expression vector (pcDNA6.2-CXCR4) were generated using the same method. The CCR5-tropic HIV-1 JRFL[37] Env expression vector (pCXN-JRenv) and pLTR-LucE were used as described previously[35]. The full length Env and part of the Nef encoding regions of the HIV-1 genome was amplified using pHIV-1 JRFL. The PCR product was ligated into pGEM-T Easy Vector System (Promega, Madison, WI), cloned using the protocol supplied by the manufacturer, and termed as pGEM-T Easy-Env. Amino acid substitutions, insertion and deletion were introduced into the V3 region of pGEM-T Easy-Env using the Quikchange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) and applying the protocols supplied by the manufacturer. The V3 regions of pGEM-T Easy-Env containing mutations were digested with *Stu*I and *Xho*I, and the obtained fragments were introduced into pCXN-JRenv or pHIV-1 JRFL.

Cell-cell fusion assay. The assay was conducted as described in detail previously[35]. Briefly, the JRFL Env expression vector (WT or mutant) and Tat expression vector (0.5 μg each) were cotransfected into 293 T cells (2×10^5) using Lipofectamine 2000 (Invitrogen), while the CD4, CCR5 or CXCR4 expression vector and a reporter (luciferase) gene containing plasmid, pLTR-LucE (0.5 μg each) were cotransfected into COS-7 cells (2×10^5). On the next day, both cotransfected cells were harvested and mixed in a well of 96-well plates (2×10^4 cells each). The cotransfected cells were incubated further for 6 hrs and the luciferase activity in each well was detected using Bright-Glo Luciferase Assay System (Promega) and its luminescence level was measured using Wallac ARVO Sx 1420 multilabel counter (Perkin-Elmer, Waltham, MA).

MD simulation. MD simulation of gp120 outer domain containing V3 loop was performed as described previously[17,18] with some modifications. Initially, the gp120 outer domain structures with various V3 elements were constructed by homology modeling[38,39] using the Molecular Operating Environment (MOE) ver. 2008.10 (Chemical Computing Group Inc., Montreal, Quebec, Canada), as described previously[17,18]. As a modeling template, we used the crystal structure of HIV-1 gp120 containing the entire V3 element (PDB code: 2B4C)[19]. Subsequently, MD simulations were performed for individual models using the SANDER module in the AMBER 9 program package[40,41]. Heating calculations were achieved for 100 picoseconds until 310 K and MD simulations were subsequently executed at 310 K for 10 nanoseconds. The time step was set to 2.0 femtoseconds. The AMBER ff03ua force field[42] and the GB implicit solvent function by Hawkins, Cramer, and Truhlar[43,44] were applied. The "no cutoff" calculation was applied for the non-bonded energy calculation. In this study, we analyzed most frequently observed conformation among 5,000 snapshots obtained from 5.0–10.0 ns of MD simulation, which was selected by the Bayesian clustering algorithm[45].

Calculation of the RMSD. We compared the orientation of V3 loop between two gp120 outer domain models by the following procedure. We first superimposed two models by coordinating main chain atoms (N, Cα, and C) in amino acid residues other than those in the V3 loop using PyMOL ver. 0.99 rc6 (Schrödinger LLC, Portland, OR, http://www.pymol.org/). Subsequently, the RMSD values for the V3 loop tip (GPGR) between the two models were calculated using the coordinates of the main chain atoms using the in-house program.

Viral tropism assay. The wild type CCR5-tropic HIV-1 strain, pHIV-1 JRFL, CXCR4-tropic HIV-1 strain, pHIV-1 NL4-3[46], and each pHIV-1 JRFL Env derived from mutations containing the V3 region of pGEM-T Easy-Env were transfected into 293 T cells with Lipofectamine 2000 (Invitrogen), and the obtained infectious clonal viruses were harvested 48 hrs after transfection and stored at −80°C until use. The GHOST cell infection assay[25,47] was performed by incubating 1 ml containing 20 ng of p24 antigen of each virus with GHOST cells (2×10^4). Parental GHOST, CCR5$^+$ GHOST Hi5, and CXCR4$^+$ GHOST CXCR4 cells were infected for 72 hrs and then harvested. The mean fluorescent intensity (MFI) of infected cells expressing green fluorescent protein (GFP) was measured on a flow cytometer (FACSCalibur; BD Bioscience, San Jose, CA). GHOST cells express low levels of CXCR4 and therefore infection of GHOST Hi5 alone was performed in presence of the CXCR4 antagonist AMD3100 (Sigma-Aldrich, St. Louis, MO) at dose of 1 μM.

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