Table 1. Prevalence of CKD and renal function at two time points in 771 HIV-infected Vietnamese on ART.

		CKD	Oct 2011	Apr 2012
Renal function	Ccl (ml/min)		n (%)	
Normal	90 or more	-	178 (23.0)	159 (20.6)
Mild reduction	60-89	-	519 (67.4)	501 (65.0)
Moderate reduction	30-59	54 (7.0)	72 (9.3)	108 (14.0)
Severe reduction	15-29	2 (0.3)	2 (0.3)	3 (0.4)
Renal failure	less than 15	0	0	0

Renal dysfunction was classified according to the guidelines of the National Kidney Foundation (18)

CKD was defined as Ccls of <60 ml/min at both time points (October 2011 and April 2012).

CKD; chronic kidney disease, ART; antiretroviral therapy

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Table 2. Baseline demographics and laboratory data of 771 patients measured at October 2011.

variables	Entire group	CKD (+)	CKD(-)	P value
Number of patients	771	56 (7.3%)	715 (92.7%)	
Age, years	36.4±7. 86	46.5±11.5	35.6±6.9	<0.001
Female, n (%)	296 (38.4%)	36 (64.3)	260 (36.4)	<0.001
Body weight, kg	55.0±8.4	47.1±6.3	55.6±8.2	<0.001
Diabetes mellitus, n (%)	32 (4.2%)	6 (10.7)	26 (3.6)	0.023
Serum creatinine, mg/dl	0.95±0. 15	1.11±0.22	0.94±0.13	<0.001
CD4+ count, /μl	349.0±202.8	337.0±215.2	349.9±201.9	0.648
HIV RNA, $\log_{10}$ c/ml	1.79±0.52	1.80±0.47	1.79±0.52	0.833
Duration of ART, years	1.34±1.54	1.69±1.96	1.32±1.51	0.083
Use of TDF, n (%)	171 (22.2%)	23 (41.1)	148 (20.7)	<0.001
Use of Lopinavir, n (%)	97 (12.6%)	13 (23.2)	43 (6.0)	0.013
Use of cotrimoxazole, n (%)	171 (22.2%)	18 (32.1)	153 (21.4)	0.062
AIDS defining disease, n (%)	69 (8.9%)	10 (17.9)	59 (8.3)	0.015

Data are mean±SD or n (%).

CKD; chronic kidney disease, ART; antiretroviral therapy, TDF; tenofovir

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Factors associated with CKD

Univariate analysis identified older age per year-increase, female sex, body weight per 1 kg-decrement, use of TDF, use of lopinavir boosted with ritonavir, diabetes mellitus, and AIDS-defining diseases as factors significantly associated with CKD. After adjustment by multivariate analysis, older age per year-increase (OR=1.229; 95%CI, 1.170-1.291; p<0.001), body weight per 1 kg-decrement (OR=1.286; 95%CI, 1.193-1.386; p<0.001), and use of TDF (OR=2.715; 95%CI, 1.028-7.168; p=0.044) were associated significantly with CKD (Table **3**).

Discussion

We documented in the present study the prevalence of CKD and the associated risk factors in our Vietnamese cohort. CKD was identified in 7.3% of the patients between October 2011 and April 2012. Although severe renal dysfunction was observed in only 2 cases, we consider this finding quite alarming in our study setting, since it is more than double that reported in a previous study (3.1%) on the prevalence of CKD among Vietnamese healthy volunteers aged more than 40 years [19]. Our cohort comprised relatively younger and stable patients on ART with a mean age of 36.4 years.

Table 3. Factors associated with CKD based on uni- and multivariate analyses (n=771).

	Univariate analysis		Multivariate analysis		
Variables	OR	95% CI	OR	95% CI	p value
Age per year-increase	1.135	1.102 - 1.168	1.229	1.170 - 1.291	<0.001
Female	3.150	1.786 - 5.556	2.124	0.892 - 5.056	0.089
Body weight per 1 kg-decrement	1.170	1.119 - 1.223	1.286	1.193 - 1.386	<0.001
Use of TDF	2.670	1.522 - 4.685	2.715	1.028 - 7.168	0.044
Use of Lopinavir	2.257	1.165 - 4.370	1.439	0.460 - 4.497	0.531
Diabetes mellitus	3.180	1.251 - 8.084	1.614	0.353 - 7.383	0.537
AIDS defining disease	2.417	1.160 - 5.035	2.042	0.628 - 6.643	0.236
CD4+ cell count per cell/μl	1.000	0.998 - 1.001			
HIV-RNA level per log 10 copies/ml	1.055	0.641 - 1.736			
Duration of ART per year	1.138	0.982 - 1.318			
Use of cotrimoxazole	1.740	0.966 - 3.134			

OR = Odds ratio; CI = confidence interval; CKD; chronic kidney disease, ART; antiretroviral therapy, TDF; tenofovir

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In addition to the high prevalence of CKD, a striking finding in this study was that TDF use has increased steeply since the 2010 WHO ART guidelines that recommended the use of TDF; TDF use was also an independent risk for CKD in Vietnamese, in addition to low body weight. We reported previously that Japanese patients with small body weight (<59 kg) treated with TDF were at high risk of renal dysfunction [16], whereas those with body weight of >67 kg had negligible risk, similar to the patients reported by Cooper et al [13]. One experimental study of rhesus macaques also reported that TDF-associated nephrotoxicity was dose-dependent [20]. The mean body weight of the patients enrolled in the present study was 55 kg, which is about 30 kg less than that of American males of similar age (88 kg) (URL:http://www.cdc.gov/nchs/data/nhsr/nhsr010.pdf). To prevent TDF-related CKD in patients with a small body weight, the efficacy and safety of low-dose TDF adjusted to low body weight should be evaluated in a clinical trial.

One study argued that the initial decline in eGFR following the commencement of TDF therapy stabilized later after the first 6 months [21]. However, whether or not the initial decline stabilizes later in patients with low body weight remains to be documented in a longitudinal study of our cohort. It is true that the future risk of TDF-related CKD is still uncertain. In this study, almost all patients who experienced renal dysfunction continued the same ART regimen because renal dysfunction was relatively moderate as shown in Table **1**. Although one severe case showed improvement of renal function after cessation of TDF, normalization of renal function after withdrawal of TDF was reported to be incomplete in some cases [22]. Previous studies recommended dose reduction of drugs that are cleared by the kidney, such as lamivudine and TDF, when Ccl falls below 50 ml/min [23], to avoid further worsening of renal dysfunction. Early detection of eGFR decline is important for switching from TDF to AZT or abacavir to preserve renal function. Despite those concerns, however, there is no doubt that TDF is still an important drug with enough anti-HIV potency and less mitochondrial toxicity among NRTIs. In this regard, serum creatinine should be monitored even in resource-limited situations.

Furthermore, another study that compared patients with or without TDF use depicted that TDF was more likely to be used in the salvage regimen so far; patients on TDF had the longer duration of ART and more positive viral load (Table **2**). Based on this analysis, patients on TDF were more likely to develop CKD, although the mean body weight was not significantly different between the two groups. In addition, in terms of another antiretroviral agent, protease inhibitor (PI), also known as a risk factor for CKD [11], 97 (12.6%) patients used PIs (all PIs were ritonavir boosted lopinavir). Of 97 patients, 83 (85.6%) were co-administered with TDF. Although univariate analysis suggested that the use of PIs was associated significantly with CKD, multivariate analysis did not (Table **3**). The reason of this result could be explained by the short duration of co-administration and its effect as a confounding factor for TDF use.

The present study has several limitations. Due to its cross-sectional nature, we can only draw association of events and not demonstrate causative relationship between TDF and renal dysfunction. Further longitudinal studies are required to determine the impact of the aforementioned factors on renal function. Second, co-infection with HCV, a known risk factor for CKD, was not included in this analysis due to lack of available data in our cohort. The prevalence of HCV in Vietnamese is relatively high because injecting drug use is one of the main routes of infection in Vietnam. We are adding data for a longitudinal study on TDF toxicity in our cohort. Lastly, the Modification of Diet in Renal Disease formula (MDRD) or Chronic Kidney Disease Epidemiology Collaboration (CKD-epi) is commonly used for evaluation of renal function at present [24-26], however, the racial coefficient for Vietnamese is currently not available. In addition, serum creatinine was measured by the Jaffe method in our study, which is difficult to apply to MDRD or CKD-EPI since those formulations are based on measurement of serum creatinine by the more widely used enzyme method. For this reason, our study utilized Ccl to assess renal function.

Despite these limitations, the results of the present study call for attention to active pharmacovigilance of TDF. The results identified TDF exposure as a significant and independent risk for CKD in Vietnam, although the duration of TDF use is still relatively short. Further longitudinal study is required to evaluate the impact of TDF on renal function in Vietnam and other countries with small-body weight patients.

Supporting Information

Table S1. Median and inter-quartile range of serum creatinine of 771 patients at October 2011 and April 2012.
(DOCX)

Table S2. Baseline (October 2011) demographics and laboratory data of 771 patients with or without TDF use in whom serum creatinine was measured at October 2011 and April 2012.
(DOC)

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Author Contributions

Conceived and designed the experiments: DM JT TN HG SO. Performed the experiments: NL ND NK YK. Analyzed the data: DM TN FK. Contributed reagents/materials/analysis tools: YK HG. Wrote the manuscript: DM TN HG SO.

References

1. Wyatt CM, Winston JA, Malvestutto CD, Fishbein DA, Barash I et al. (2007) Chronic kidney disease in HIV infection: an urban epidemic. AIDS 21: 2101-2103. doi:10.1097/QAD.0b013e3282ef1bb4. PubMed: 17885301.
2. Mocroft A, Kirk O, Gatell J, Reiss P, Gargalianos P et al. (2007) Chronic renal failure among HIV-1-infected patients. AIDS 21: 1119-1127. doi:10.1097/QAD.0b013e3280f774ee. PubMed: 17502722.
3. Palella FJ Jr., Baker RK, Moorman AC, Chmiel JS, Wood KC et al. (2006) Mortality in the highly active antiretroviral therapy era: changing causes of death and disease in the HIV outpatient study. J Acquir Immune Defic Syndr 43: 27-34. doi:10.1097/01.qai.0000233310.90484.16. PubMed: 16878047.
4. Michaels SH, Clark R, Kissinger P (1998) Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. N Engl J Med 339: 405-406. doi:10.1056/NEJM199808063390612. PubMed: 9696654.
5. Gardner LI, Klein RS, Szczech LA, Phelps RM, Tashima K et al. (2003) Rates and risk factors for condition-specific hospitalizations in HIV-infected and uninfected women. J Acquir Immune Defic Syndr 34: 320-330. doi:10.1097/00126334-200311010-00011. PubMed: 14600579.
6. Déti EK, Thiébaut R, Bonnet F, Lawson-Ayayi S, Dupon M et al. (2010) Prevalence and factors associated with renal impairment in HIV-infected patients, ANRS C03 Aquitaine Cohort, France. HIV Med 11: 308-317. doi:10.1111/j.1468-1293.2009.00780.x. PubMed: 20002500.
7. Overton ET, Nurutdinova D, Freeman J, Seyfried W, Mondy KE (2009) Factors associated with renal dysfunction within an urban HIV-infected cohort in the era of highly active antiretroviral therapy. HIV Med 10: 343-350. doi:10.1111/j.1468-1293.2009.00693.x. PubMed: 19490182.
8. Lucas GM, Lau B, Atta MG, Fine DM, Keruly J et al. (2008) Chronic kidney disease incidence, and progression to end-stage renal disease, in HIV-infected individuals: a tale of two races. J Infect Dis 197: 1548-1557. doi:10.1086/587994. PubMed: 18422458.
9. Menezes AM, Torelly J Jr., Real L, Bay M, Poeta J et al. (2011) Prevalence and risk factors associated to chronic kidney disease in HIV-infected patients on HAART and undetectable viral load in Brazil. PLOS ONE 6: e26042. doi:10.1371/journal.pone.0026042. PubMed: 22022501.

10. Crum-Cianflone N, Ganesan A, Teneza-Mora N, Riddle M, Medina S et al. (2010) Prevalence and factors associated with renal dysfunction among HIV-infected patients. AIDS Patient Care STDs 24: 353-360. doi:10.1089/apc.2009.0326. PubMed: 20515419.
11. Mocroft A, Kirk O, Reiss P, De Wit S, Sedlacek D et al. (2010) Estimated glomerular filtration rate, chronic kidney disease and antiretroviral drug use in HIV-positive patients. AIDS 24: 1667-1678. doi:10.1097/QAD.0b013e328339fe53. PubMed: 20523203.
12. Barrios A, Garcia-Benayas T, González-Lahoz J, Soriano V (2004) Tenofovir-related nephrotoxicity in HIV-infected patients. AIDS 18: 960-963. doi:10.1097/00002030-200404090-00019. PubMed: 15060449.
13. Cooper RD, Wiebe N, Smith N, Keiser P, Naicker S et al. (2010) Systematic review and meta-analysis: renal safety of tenofovir disoproxil fumarate in HIV-infected patients. Clin Infect Dis 51: 496-505. doi:10.1086/655681. PubMed: 20673002.
14. Nelson MR, Katlama C, Montaner JS, Cooper DA, Gazzard B et al. (2007) The safety of tenofovir disoproxil fumarate for the treatment of HIV infection in adults: the first 4 years. AIDS 21: 1273-1281. doi: 10.1097/QAD.0b013e3280b07b33. PubMed: 17545703.
15. Chaisiri K, Bowonwatanuwong C, Kasettratat N, Kiertiburanakul S (2010) Incidence and risk factors for tenofovir-associated renal function decline among Thai HIV-infected patients with low-body weight. Curr HIV Res 8: 504-509. doi:10.2174/157016210793499259. PubMed: 21073439.
16. Nishijima T, Komatsu H, Gatanaga H, Aoki T, Watanabe K et al. (2011) Impact of small body weight on tenofovir-associated renal dysfunction in HIV-infected patients: a retrospective cohort study of Japanese patients. PLOS ONE 6: e22661. doi:10.1371/journal.pone.0022661. PubMed: 21799928.
17. Nishijima T, Gatanaga H, Komatsu H, Tsukada K, Shimbo T et al. (2012) Renal function declines more in tenofovir- than abacavir-based antiretroviral therapy in low-body weight treatment-naive patients with HIV infection. PLOS ONE 7: e29977. doi:10.1371/journal.pone.0029977. PubMed: 22242194.
18. National Kidney Foundation (2002) K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis 39: S1-266. doi:10.1053/ajkd.2002.30571. PubMed: 11904577.
19. Ito J, Dung DT, Vuong MT, Tuyen do G, Vinh le D et al. (2008) Impact and perspective on chronic kidney disease in an Asian developing country: a large-scale survey in North Vietnam. Nephron Clin Pract 109: c25-c32. doi:10.1159/000134379. PubMed: 18497502.
20. Van Rompay KK, Durand-Gasselin L, Brignolo LL, Ray AS, Abel K et al. (2008) Chronic administration of tenofovir to rhesus macaques from infancy through adulthood and pregnancy: summary of pharmacokinetics and biological and virological effects. Antimicrob Agents Chemother 52: 3144-3160. doi:10.1128/AAC.00350-08. PubMed: 18573931.
21. Gallant JE, Moore RD (2009) Renal function with use of a tenofovir-containing initial antiretroviral regimen. AIDS 23: 1971-1975. doi: 10.1097/QAD.0b013e32832c96e9. PubMed: 19696652.
22. Wever K, van Agtmael MA, Carr A (2010) Incomplete reversibility of tenofovir-related renal toxicity in HIV-infected men. J Acquir Immune Defic Syndr 55: 78-81. doi:10.1097/QAI.0b013e3181d05579. PubMed: 20173649.
23. Dybul M, Fauci AS, Bartlett JG, Kaplan JE, Pau AK (2002) Guidelines for using antiretroviral agents among HIV-infected adults and adolescents. Recommendations Panel Clinical Practices Treat HIV MMWR Recomm Rep 51: 1-55. PubMed: 12617573.
24. Stevens LA, Levey AS (2005) Measurement of kidney function. Med Clin North Am 89: 457-473. doi:10.1016/j.mcna.2004.11.009. PubMed: 15755462.
25. Stöhr W, Walker AS, Munderi P, Tugume S, Gilks CF et al. (2008) Estimating glomerular filtration rate in HIV-infected adults in Africa: comparison of Cockcroft-Gault and Modification of Diet in Renal Disease formulae. Antivir Ther 13: 761-770. PubMed: 18839777.
26. Soares AA, Eyff TF, Campani RB, Ritter L, Weinert LS et al. (2010) Performance of the CKD Epidemiology Collaboration (CKD-EPI) and the Modification of Diet in Renal Disease (MDRD) Study equations in healthy South Brazilians. Am J Kidney Dis 55: 1162-1163. doi:10.1053/j.ajkd.2010.03.008. PubMed: 20497836.

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Ritonavir-Boosted Darunavir Is Rarely Associated with Nephrolithiasis Compared with Ritonavir-Boosted Atazanavir in HIV-Infected Patients

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Abstract

Background: Although ritonavir-boosted atazanavir (ATV/r) is known to be associated with nephrolithiasis, little is known about the incidence of nephrolithiasis in patients treated with ritonavir-boosted Darunavir (DRV/r), the other preferred protease inhibitor.

Methods: In a single-center cohort, the incidence of nephrolithiasis was compared between HIV-infected patients who commenced DRV/r-containing antiretroviral therapy and those on ATV/r. The effects of ATV/r use over DRV/r were estimated by univariate and multivariate Cox hazards models.

Results: Renal stones were diagnosed in only one patient (0.86 per 1000 person-years) of the DRV/r group (n=540) and 37 (20.2 per 1000 person-years) of the ATV/r group (n=517). The median [interquartile (IQR)] observation period in the DRV/r group was 27.1 months (IQR 18.1-38.4 months), and 40.6 months (IQR 17.5-42.7) for the ATV/r group. The total observation period was 1,163.6 person-years and 1,829.6 person-years for the DRV/r group and for the ATV/r group, respectively. In the 37 patients on ATV/r who developed nephrolithiasis, the median time from commencement of ATV/r to diagnosis was 28.1 months (IQR 18.4–42.7), whereas nephrolithiasis in the single patient of the DRV/r group occurred 11.2 month after the introduction of DRV/r. ATV/r use over DRV/r was significantly associated with nephrolithiasis by uni- and multivariate analyses (HR=26.01; 95% CI, 3.541–191.0; p=0.001) (adjusted HR=21.47; 95% CI, 2.879–160.2; p=0.003).

Conclusion: The incidence of nephrolithiasis was substantially lower in patients on DRV/r than those on ATV/r. The results suggest that DRV/r should be selected for treatment of HIV-infected patients at risk of chronic kidney disease.

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Introduction

Ritonavir-boosted darunavir (DRV/r) and ritonavir-boosted atazanavir (ATV/r) are the only two protease inhibitors (PI) selected as the preferred choices in the American Department of Health and Human Services (DHHS) guidelines for the initial treatment of patients infected with human immunodeficiency virus-1 (HIV-1) (http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf). Both drugs are widely used in combination with other antiretroviral drugs, based on their high efficacy, tolerability, favorable lipid profile, and once-daily dosing [1-4]. However, nephrolithiasis has been reported in patients receiving ATV/r-containing antiretroviral therapy (ART) [5,6]. Several case reports documented high concentrations of ATV in renal stones, suggesting the involvement of ATV in nephrolithiasis [5-8]. We recently reported in a single center cohort study that the incidence of renal stones is approximately 10 times higher among patients on ATV/r-containing

antiretroviral therapy (ART) than those on other PIs-containing ART [9].

Our study on the effects of ART on renal stone formation included only a small number of patients on DRV/r-containing ART [9,10], and no data are available at present on the incidence of nephrolithiasis in patients treated with DRV/r. Of note, de Lastours et al [11] recently reported higher ATV and DRV levels in urine samples than in plasma, whereas plasma and urinary levels of lopinavir, another commonly used PI, were comparable. They also reported the presence of PI-containing crystals in the urine of a small proportion of patients on ATV and on DRV, but not on lopinavir/ritonavir (LPV/r). The data presented by de Lastours et al suggest that DRV can crystallize in urine leading to nephrolithiasis.

The aim of the present study was to determine the incidence of DRV/r- and ATV/r-related nephrolithiasis. Such comparison is important for two reasons: 1) These two PIs are most frequently prescribed PIs in resource-rich settings, and 2) nephrolithiasis is a risk factor for chronic kidney diseases (CKD) and end-stage renal disease (ESRD), which are important comorbidities associated with AIDS and death [12-16].

Methods

Ethics statement

This study was approved by the Human Research Ethics Committee of the National Center for Global Health and Medicine, Tokyo. Each participant provided a written informed consent for the clinical and laboratory data to be used and published for research purposes. The study was conducted according to the principles expressed in the Declaration of Helsinki.

Study Subjects

We performed a retrospective, single-center cohort study of HIV-1-infected patients using the medical records kept at the National Center for Global Health and Medicine, Tokyo, Japan. Our facility is one of the largest clinics for patients with HIV infection in Japan with more than 2,700 registered patients. The study population was HIV-infected patients, aged >17 years, who commenced treatment with DRV/r or ATV/r-containing ART between January 1, 2004 and June 30, 2012. Both treatment-naïve and treatment-experienced patients were included. The follow-up period started at the time of commencement of ART containing the abovementioned drugs for the first time during the study period, and patients were followed until June 30, 2013. Patients were excluded if they had; 1) commenced the abovementioned ART during the study period at other facilities, 2) been prescribed unboosted ATV, or 3) been under treatment for nephrolithiasis at the time of commencement of the abovementioned ART. ATV/r became available in Japan in January 2004, and DRV/r in December 2007.

The attending physician selected either ATV/r or DRV/r at baseline. The use of these drugs was based on the Japanese guidelines, which placed ATV/r and DRV/r as the preferred choice, at least for 5 years during the study period (http://www.haart-support.jp/pdf/guideline2013.pdf. in Japanese). The attending physician also selected the concurrent drugs including nucleoside reverse transcriptase inhibitors (NRTI), non-NRTI, integrase inhibitors, and CCR5 inhibitors. None of the patients received two PIs during the study period.

Measurements

The main investigator reviewed the medical records of all study patients to identify those with renal stones. Then two other investigators reviewed the set of medical records of each patient with renal stones to determine whether the case fitted into the following pre-defined criteria for nephrolithiasis: cases with a clinical diagnosis by the attending physician based on new onset of acute flank pain plus one of the following: 1) new-onset hematuria confirmed by urine dipstick test, 2) documented presence of stones or radiological findings suggestive of renal stones, such as hydronephrosis or obstruction or dilatation of the ureter, by either abdominal ultrasonography or computed tomography, 3) stone passage confirmed by either the patient or attending physician [9]. Patients with acute flank pain due to etiologies other than nephrolithiasis were excluded. At the time of diagnosis of nephrolithiasis, the attending physician selected either discontinuation or modification of ART. In our clinic, it is customary for the patient to visit the clinic once a month before the initiation of ART and until the suppression of HIV-1 viral load, but the visit interval is extended up to every three months after viral load suppression.

In this study, the primary exposure variable was ATV/r use over DRV/r. The potential risk factors for nephrolithiasis were determined according to previous studies and collected from the medical records, together with the basic demographics [7,8,17]. They included age, sex, body weight, body mass index (BMI)={bodyweight (kg) / [(height (m)]^2}, baseline laboratory data [CD4 cell count, HIV viral load, estimated glomerular filtration rate (eGFR), serum uric acid], and presence or absence of other medical conditions [concurrent use of tenofovir (TDF), past history of nephrolithiasis, previous exposure to indinavir (IDV), co-infection with hepatitis B defined by positive hepatitis B surface antigen, and co-infection with hepatitis C defined by positive hepatitis C viral load]. eGFR was calculated using the equation of the 4-variable Modification of Diet in Renal Diseases (MDRD) study [18]. For patients on ATV/r-containing ART, the value of serum total bilirubin was collected in two ways: for stone cases, total bilirubin value on the day was collected, and for non-stone cases, the value of total bilirubin 2 years after initiation of ATV/r was collected. For patients who discontinued ATV/r within 2 years, the value closest to the day of discontinuation was used. At our clinic, weight was measured on every visit whereas other variables were measured in the first visit and at least once annually. We used the data on or closest to and preceding the day of starting ART by no more than 180 days, except for serum uric acid level, which were collected within 180 days from the day of starting ART.

Statistical analysis

Baseline characteristics were compared using the Student's *t*-test or χ^2 test (Fisher exact test) for continuous or categorical variables, respectively. The time to the diagnosis of nephrolithiasis was calculated from the date of commencement of DRV/r- or ATV/r-containing ART to the date of diagnosis of nephrolithiasis. Censored cases represented those who discontinued ATV/r or DRV/r, dropped out, were referred to other facilities, or at the end of follow-up period. The time from the start of ART to the diagnosis of nephrolithiasis was analyzed by the Kaplan Meier method for patients who started DRV/r (DRV/r group) and ATV/r (ATV/r group), 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 ATV/r use over DRV/r on the incidence of nephrolithiasis. The impact of each basic demographic parameter, 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 ATV/r use over DRV/r for nephrolithiasis, we conducted three models using multivariate Cox proportional hazards regression analysis. Model 1 was the aforementioned univariate analysis for ATV/r use over DRV/r. Model 2 included age, sex, and weight plus model 1 in order to adjust for basic characteristics. In model 3, we added variables with P values <0.05 in univariate analysis after adjustment (these included tenofovir use, serum uric acid per 1 mg/dl, and past history of renal stones). Possible risk factors for ATV/r-related nephrolithiasis identified in previous studies were also added to model 3 (these included prior exposure to IDV) [7,8].

In addition, to examine the impact of serum total bilirubin on ATV/r-containing ART and the incidence of nephrolithiasis, the median serum total bilirubin values were compared between the renal stone and non-renal stone groups using the Mann-Whitney U test.

Statistical significance was defined as 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 nephrolithiasis. All statistical analyses were performed with The Statistical Package for Social Sciences ver. 20.0 (SPSS, Chicago, IL).

Results

A total of 1,189 patients commenced either DRV/r- or ATV-containing ART between January 1, 2004 and June 30, 2012. Of the 1,057 patients who were included in the analysis, 540 (51%) started DRV/r-containing ART while 517 (48.9%) started ATV/r-containing ART (Figure 1). Table 1 shows the baseline characteristics of the study population. The ATV/r group included significantly younger (p=0.019), more patients of East Asian origin (p=0.009) with higher BMI (p=0.014), higher CD4 count (p=0.038), higher baseline serum uric acid (p=0.007), and a larger proportion of patients with past history of urinary stones (p=0.017) and previous exposure to IDV (p=0.036). In contrast, patients of the DRV/r group were significantly more likely to use tenofovir (p <0.001) and with higher viral load (p=0.002) (Table 1).

Thirty eight patients fulfilled the pre-defined criteria for nephrolithiasis. Nephrolithiasis was identified in 1 (0.2%) of the DRV/r group and 37 patients (7.1%) of the ATV/r group, with an estimated incidence of 0.86 and 20.2 per 1,000 person-years, respectively. The incidence of nephrolithiasis in the ATV/r group was approximately 20 times higher than that in the DRV/r group.

Of the patients with nephrolithiasis, 9 and 12 were diagnosed by hematuria and stone passage, respectively, as defined above. Furthermore, 17 were diagnosed by radiological studies, of which renal calcification was identified in 5 patients. Figure 2 shows the time from initiation or switching of DRV/r or ATV/r to the diagnosis of nephrolithiasis by the Kaplan Meier method. Patients of the ATV/r group were significantly more likely to develop renal stones, compared to those of the DRV/r group (p<0.001, Log-rank test).

The median time from the commencement of ART to the diagnosis of nephrolithiasis was 28.1 months [interquartile range (IQR), 18.4–42.7 months] for the ATV/r group and only one patient with nephrolithiasis in the DRV/r group was diagnosed 11.2 month after the introduction of DRV/r-containing ART. The total observation period was 1,163.6 patient-years [median, 27.1 months, IQR, 18.1–38.4 months] for the DRV/r group, and 1,829.6 patient-years [median, 40.6 months, IQR, 17.5–42.7 months] for the ATV/r group. Among the ATV/r group, the median total bilirubin value of the renal stone group was marginally higher than that of the non-renal stone group [2.7 (IQR 2-3.8) and 2.2 mg/dl (IQR 1.6-3.0), respectively, P=0.051].

Univariate analysis showed a significant relationship between ATV/r use and nephrolithiasis (HR=26.01; 95% CI, 3.541–191.0; p=0.001) (Table 2). Higher serum uric acid (HR=1.415; 95% CI, 1.173–1.705; p<0.001) and past history of nephrolithiasis (HR=2.658; 95% CI, 1.111–6.359; p=0.028) were also significantly associated with the nephrolithiasis. On the other hand, tenofovir use was negatively associated with nephrolithiasis (HR=0.435; 95% CI, 0.210–0.899; p=0.025) (Table 2). Multivariate analysis identified ATV/r use over DRV/r as an independent risk for nephrolithiasis after adjustment for age, male sex, and weight (adjusted HR=27.08 95% CI, 3.680–199.3; p=0.001) (Table 3, Model 2), and also after adjustment for other risk factors (adjusted HR= 21.47; 95% CI, 2.879–160.2; p=0.003) (Table 3, Model 3).

The chemical composition of the renal stones of the single case on DRV/r was analyzed with high performance liquid chromatography with ultraviolet detection (HPLC-UV) method as described elsewhere [19,20], but the analysis did not identify DRV. Renal stones of patients on ATV/r were not analyzed.

Discussion

To our knowledge, this is the first study that investigated the incidence of DRV/r-associated nephrolithiasis. Only a single case of nephrolithiasis was detected among 540 patients on DRV/r-containing ART with total observation period of 1,163.6 patient-years. The incidence of nephrolithiasis in the DRV/r group was only 0.86 per 1,000 person-years, comparable to that in the general population in Japan (1.14 per 1,000 person-

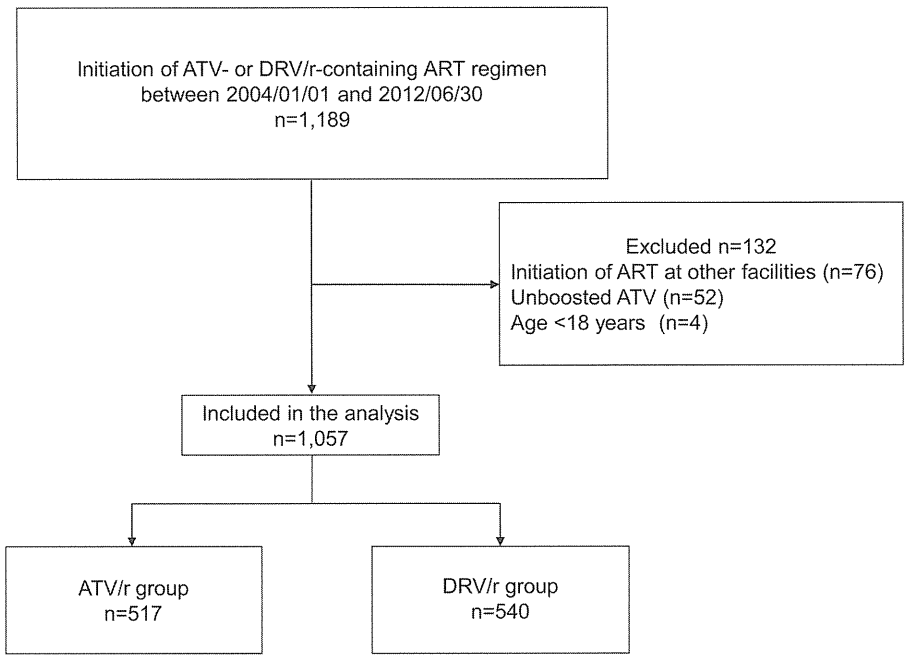

Figure 1. Flow diagram of patient selection. ART, antiretroviral therapy; ATV, atazanavir; DRV/r, ritonavir-boosted darunavir; ATV/r, ritonavir-boosted atazanavir.

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years) [21], whereas that in the ATV/r group was 20.2 per 1,000 person-years, approximately 20 times higher. Univariate and multivariate analyses identified ATV/r use over DRV/r as an independent risk factor for nephrolithiasis with a high hazard ratio. Furthermore, in the single patient with nephrolithiasis on DRV/r, DRV was not detected as a component of renal stones.

This study showed that the risk of nephrolithiasis is substantially lower among patients on DRV/r- than those on ATV/r-containing ART based on clinically feasible criteria. This finding is important considering DRV/r and ATV/r are the two PIs considered the preferred regimen for the treatment-naïve patients (http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf). Both PIs have similar characteristics; they are highly effective and tolerable with favorable lipid profile, and possess a high barrier to drug resistance [1-4]. One of the strengths of ATV/r is more abundant clinical evidence due to longer market availability than that of DRV/r. On the other hand, ATV/r often causes indirect hyperbilirubinemia, and requires acidic gastric environment for optimal absorption that requires some consideration on drug-drug interactions (http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf) (http://packageinserts.bms.com/pi/pi_reyataz.pdf). The substantially lower incidence of renal stones in patients on DRV/r than ATV/r adds another dimension to patient management in relation to the selection of a PI.

The development of renal stones, even a single episode, is a risk factor for CKD, doubling of serum creatinine level, and ESRD [12,13,16]. Many studies have also demonstrated that ATV/r use is a risk for renal dysfunction and CKD [22-25]. The high incidence of nephrolithiasis with ATV/r use identified in the present study may in part explain the risk of ATV/r for CKD. Thus, ATV/r should be introduced carefully in patients with concomitant predisposing factors for CKD. In this regard, there are no studies that show the association of DRV/r use with renal dysfunction or CKD, although this may in part be due to more recent introduction of DRV/r compared with ATV/r.

Why is nephrolithiasis less likely to occur with DRV/r compared to ATV/r? Although the mechanism of PI-induced nephrolithiasis is not fully understood, precipitation of pure PI is suggested as a possible etiology [8]. Up to 20% of IDV (an old PI well-known for its precipitation and renal stone formation) is

Table 1. Baseline demographics and laboratory data of patients who received ritonavir-boosted darunavir- or ritonavir-boosted atazanavir-containing antiretroviral therapy.

	DRV/r (n=540)	ATV/r (n=517)	P[a]
Age, years*	39 (33-46)	36 (31-44)	0.019
Male sex	498 (92.2)	480 (92.8)	0.727
Race (East Asian origin)	494 (91.5)	494 (95.6)	0.009
Body weight, kg*	62.1 (55.8-70)	64.0 (57.6-72)	0.074
body mass index, kg/m^2*	21.7 (19.8-24.1)	22.4 (20.4-24.6)	0.014
CD4 cell count, cells/μL*	251 (90-399)	260 (190-383)	0.038
HIV load, $\log_{10}$ copies/mL*	4.27 (1.70-5.17)	3.94 (1.70-4.66)	0.002
Treatment naïve	309 (57.2)	280 (54.2)	0.322
Tenofovir use	342 (63.3)	196 (37.9)	<0.001
eGFR, mL/min/1.73 m^2*	116 (102-131)	115 (103-130)	0.842
Serum uric acid, mg/dL*	5.7 (4.7-6.5)	5.9 (5.1-6.7)	0.007
HBV or HCV coinfection	78 (14.4)	64 (12.4)	0.367
Past history of nephrolithiasis	22 (4.1)	39 (7.5)	0.017
Previous exposure to IDV	25 (4.6)	41 (7.9)	0.030

Data are number (%) of patients or * median (interquartile range).

DRV/r, ritonavir-boosted darunavir; ATV/r, ritonavir-boosted atazanavir; eGFR, estimated glomerular filtration rate; HBV, hepatitis B virus, HCV, hepatitis C virus, HIV, human immunodeficiency virus; IDV, indinavir.

a. The χ^2 test or Fisher exact test was used for categorical data, and the Student *t* test was used for continuous variables.

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excreted unchanged in the urine, a property that contributes to the high incidence of nephrolithiasis in patients treated with IDV [26] (http://www.merck.com/product/usa/pi_circulars/c/crixivan/crixivan_pi.pdf). Unchanged DRV and ATV are reported to be excreted in urine at similar proportions of 7.7% and 7% of the administered dose, respectively (http://packageinserts.bms.com/pi/pi_reyataz.pdf) (http://www.merck.com/product/usa/pi_circulars/c/crixivan/crixivan_pi.pdf). However, strong acidity (e.g., pH of 1.9) is required to achieve optimal dissolution of ATV, and its solubility in urine is known to decrease with increase in pH (http://packageinserts.bms.com/pi/pi_reyataz.pdf). Because urine is usually mildly acidic [9], the difference in the solubility of DRV and ATV in urine might explain the different incidence of nephrolithiasis in patients using these two PIs. Although de Lastours et al [11] described the presence of DRV crystals in the urine of 4 (7.8%) out of 51 patients on DRV/r and suggested that DRV/r use might be a risk for renal stones, the number of enrolled patients in their study was relatively small to allow firm conclusions.

The present study has several limitations. First, due to the retrospective nature of the study, the baseline characteristics of the enrolled patients were not controlled. It is possible that more patients with potential risks for nephrolithiasis were included in the ATV/r group. In the ATV/r group, more patients were hyperuricemic, had history of renal stones, and previous exposure to IDV, which are known risk factors for nephrolithiasis. However, multivariate analysis clearly showed that ATV/r use is an independent risk factor with high hazard ratio even after adjustment for variables including the above three. Second, the median observation period was longer in the ATV/r group than in the DRV/r group (40.6 versus 27.1 months), suggesting that the risk of nephrolithiasis in the ATV/r group could be overestimated. Further studies are warranted to elucidate whether much longer use of DRV/r induces nephrolithiasis. However, it is noteworthy that in patients with nephrolithiasis, the median time from the commencement of ATV/r or DRV/r to the diagnosis of nephrolithiasis was 28.1 months (IQR: 18.4-42.7 months), which was similar to that of the DRV/r group [median 27.1 (IQR: 18.1-38.4)], backing up the result of the present study: the risk of nephrolithiasis is substantially lower among patients on DRV/r than those on ATV/r. Third, stone composition analysis was conducted in only one patient with renal stones (treated with DRV/r), therefore, it is possible that renal stones caused by other etiologies are included.

In conclusion, the present study demonstrated that the risk of nephrolithiasis, an important risk factor of CKD, is approximately 20 times lower among patients on DRV/r- than those on ATV/r-containing ART, providing DRV/r one advantage over ATV/r in the selection of PI. ATV/r use was identified as a significant and independent risk factor for nephrolithiasis in a robust statistical model that included ATV/r use over DRV/r as a primary exposure. ATV/r should be prescribed with caution in patients with predisposing factors for nephrolithiasis and those with CKD.

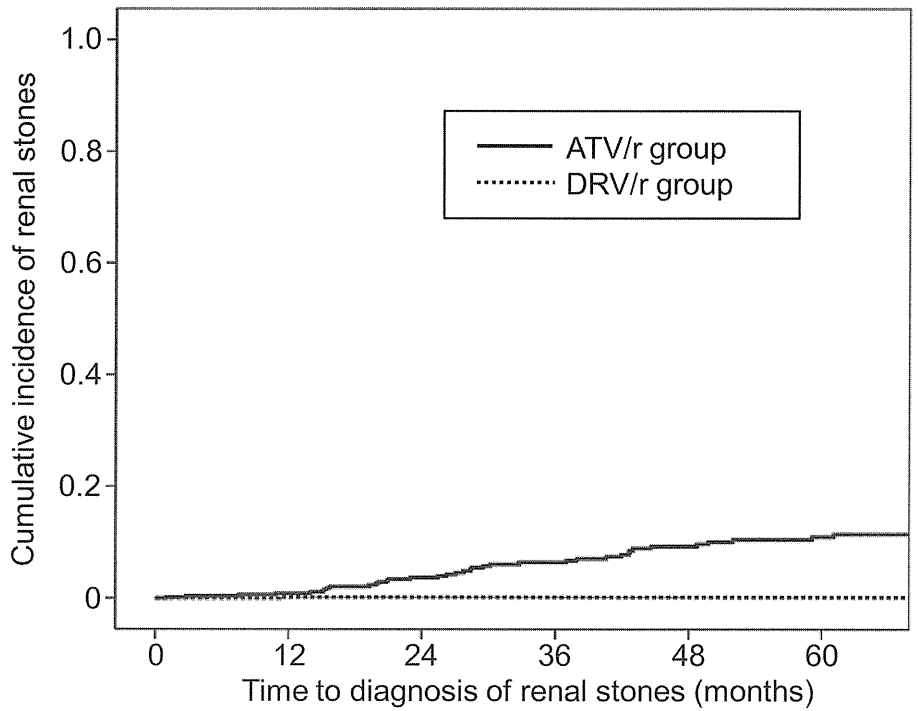

Figure 2. Kaplan-Meier curve showing time to the diagnosis of nephrolithiasis. ATV/r, ritonavir-boosted atazanavir; DRV/r, ritonavir-boosted darunavir.

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Table 2. Univariate analysis to estimate the risk of various factors for nephrolithiasis.

	Hazard ratio	95%CI	P value
ATV/r use over DRV/r	26.01	3.541-191.0	0.001
Age per 1 year	1.002	0.973-1.031	0.907
Male sex	1.665	0.401-6.919	0.483
Race (East Asian origin)	2.287	0.314-16.68	0.414
Weight per 1 kg increment	0.998	0.970-1.027	0.872
body mass index per 1 kg/m^2 increment	0.996	0.905-1.095	0.927
CD4 count per 10 /μl increment	0.999	0.983-1.016	0.901
HIV viral load per log10/ml	1.063	0.859-1.316	0.575
Treatment naïve	1.020	0.538-1.936	0.950
Tenofovir use	0.435	0.210-0.899	0.025
Baseline eGFR per 10 ml/min/1.73 m^2 decrement	1.103	0.980-1.242	0.105
Baseline serum uric acid per 1 mg/dl increment	1.415	1.173-1.705	<0.001
Hepatitis B or Hepatitis C	1.418	0.592-3.393	0.433
Past history of renal stone	2.658	1.111-6.359	0.028
Previous exposure to IDV	1.192	0.366-3.879	0.771

ATV/r, ritonavir-boosted atazanavir; DRV/r, ritonavir-boosted darunavir; HIV, human immunodeficiency virus; eGFR, estimated glomerular filtration rate; IDV, indinavir.

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Table 3. Multivariate analysis to estimate the risk of ATV/r- over DRV/r-containing antiretroviral therapies for nephrolithiasis.

	Model 1 crude (n=1,057)		Model 2 adjusted (n=1,056)		Model 3 adjusted (n=1,021)	
	HR	95%CI	HR	95%CI	HR	95%CI
ATV/r use over DRV/r	27.05	3.687-198.5	27.08	3.680-199.3	21.47	2.879-160.2
Age per 1 year			1.009	0.980-1.039	1.006	0.976-1.037
Male sex			1.939	0.441-8.528	1.202	0.262-5.512
Weight per 1 kg increment			0.988	0.956-1.021	0.979	0.947-1.012
Tenofovir use					0.678	0.313-1.470
Baseline serum uric acid per 1mg/dl increment					1.418	1.150-1.750
Past history of renal stone					1.661	0.520-5.307
Past exposure to IDV					0.491	0.100-2.403

HR, Hazard ratio; CI, confidence interval; ATV/r, ritonavir-boosted atazanavir; DRV/r, ritonavir-boosted darunavir; HIV, IDV, indinavir.

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Author Contributions

Conceived and designed the experiments: TN YH HG SO. Performed the experiments: TN YH KW K. Teruya. Analyzed the data: TN YH HK EK. Contributed reagents/materials/analysis tools: K. Tsukada YK. Wrote the manuscript: TN YH HG SO.

References

1. Squires K, Lazzarin A, Gatell JM, Powderly WG, Pokrovskiy V et al. (2004) Comparison of once-daily atazanavir with efavirenz, each in combination with fixed-dose zidovudine and lamivudine, as initial therapy for patients infected with HIV. J Acquir Immune Defic Syndr 36: 1011-1019. doi:10.1097/00126334-200408150-00003. PubMed: 15247553.
2. Molina JM, Andrade-Villanueva J, Echevarria J, Chetchotisakd P, Corral J et al. (2010) Once-daily atazanavir/ritonavir compared with twice-daily lopinavir/ritonavir, each in combination with tenofovir and emtricitabine, for management of antiretroviral-naive HIV-1-infected patients: 96-week efficacy and safety results of the CASTLE study. J Acquir Immune Defic Syndr 53: 323-332. doi:10.1097/QAI.0b013e3181c990bf. PubMed: 20032785.
3. Clotet B, Bellos N, Molina JM, Cooper D, Goffard JC et al. (2007) Efficacy and safety of darunavir-ritonavir at week 48 in treatment-experienced patients with HIV-1 infection in POWER 1 and 2: a pooled subgroup analysis of data from two randomised trials. Lancet 369: 1169-1178. doi:10.1016/S0140-6736(07)60497-8. PubMed: 17416261.
4. Mills AM, Nelson M, Jayaweera D, Ruxrungtham K, Cassetti I et al. (2009) Once-daily darunavir/ritonavir vs. lopinavir/ritonavir in treatment-naive, HIV-1-infected patients: 96-week analysis. AIDS 23: 1679-1688. doi:10.1097/QAD.0b013e32832d7350. PubMed: 19487905.
5. Chang HR, Pella PM (2006) Atazanavir urolithiasis. N Engl J Med 355: 2158-2159. doi:10.1056/NEJMc061892. PubMed: 17108352.
6. Anderson PL, Lichtenstein KA, Gerig NE, Kiser JJ, Bushman LR (2007) Atazanavir-containing renal calculi in an HIV-infected patient. AIDS 21: 1060-1062. doi:10.1097/QAD.0b013e3280c56ae1. PubMed: 17457108.
7. Chan-Tack KM, Truffa MM, Struble KA, Birnkrant DB (2007) Atazanavir-associated nephrolithiasis: cases from the US Food and Drug Administration's Adverse Event Reporting System. AIDS 21: 1215-1218. doi:10.1097/QAD.0b013e32813aee35. PubMed: 17502736.
8. Couzigou C, Daudon M, Meynard JL, Borsa-Lebas F, Higueret D et al. (2007) Urolithiasis in HIV-positive patients treated with atazanavir. Clin Infect Dis 45: e105-e108. doi:10.1086/521930. PubMed: 17879904.
9. Hamada Y, Nishijima T, Watanabe K, Komatsu H, Tsukada K et al. (2012) High incidence of renal stones among HIV-infected patients on ritonavir-boosted atazanavir than in those receiving other protease inhibitor-containing antiretroviral therapy. Clin Infect Dis 55: 1262-1269. doi:10.1093/cid/cis621. PubMed: 22820542.
10. Tattevin P, Revest M, Chapplain JM, Ratajczak-Enselme M, Arvieux C et al. (2013) Increased risk of renal stones in patients treated with atazanavir. Clin Infect Dis 56: 1186. doi:10.1093/cid/cis1213. PubMed: 23300244.
11. de Lastours V, Rafael De Silva Ferrari E; Daudon M, Porcher R, Loze B et al. (2013) High levels of atazanavir and darunavir in urine and crystalluria in asymptomatic patients. J Antimicrob Chemother, 68: 1850–6. PubMed: 23599359. PubMed: 23599359
12. Rule AD, Bergstralh EJ, Melton LJ 3rd, Li X, Weaver AL et al. (2009) Kidney stones and the risk for chronic kidney disease. Clin J Am Soc Nephrol 4: 804-811. doi:10.2215/CJN.05811108. PubMed: 19339425.
13. Jungers P, Joly D, Barbey F, Choukroun G, Daudon M (2004) ESRD caused by nephrolithiasis: prevalence, mechanisms, and prevention. Am J Kidney Dis 44: 799-805. doi:10.1016/S0272-6386(04)01131-X. PubMed: 15492945.
14. Gupta SK, Eustace JA, Winston JA, Boydstun II, Ahuja TS et al. (2005) Guidelines for the management of chronic kidney disease in HIV-infected patients: recommendations of the HIV Medicine Association of the Infectious Diseases Society of America. Clin Infect Dis 40: 1559-1585. doi:10.1086/430257. PubMed: 15889353.
15. Keddis MT, Rule AD (2013) Nephrolithiasis and loss of kidney function. Curr Opin Nephrol Hypertens 22: 390-396. doi:10.1097/MNH.0b013e32836214b9. PubMed: 23736840.
16. Alexander RT, Hemmelgarn BR, Wiebe N, Bello A, Morgan C et al. (2012) Kidney stones and kidney function loss: a cohort study. BMJ 345: e5287. doi:10.1136/bmj.e5287. PubMed: 22936784.
17. Parmar MS (2004) Kidney stones. BMJ 328: 1420-1424. doi:10.1136/bmj.328.7453.1420. PubMed: 15191979.
18. Levey AS, Coresh J, Greene T, Stevens LA, Zhang YL et al. (2006) Using standardized serum creatinine values in the modification of diet in renal disease study equation for estimating glomerular filtration rate. Ann Intern Med 145: 247-254. doi:10.7326/0003-4819-145-4-200608150-00004. PubMed: 16908915.
19. Hirano A, Takahashi M, Kinoshita E, Shibata M, Nomura T et al. (2010) High performance liquid chromatography using UV detection for the simultaneous quantification of the new non-nucleoside reverse transcriptase inhibitor etravirine (TMC-125), and 4 protease inhibitors in human plasma. Biol Pharm Bull 33: 1426-1429. doi:10.1248/bpb.33.1426. PubMed: 20686242.
20. Takahashi M, Yoshida M, Oki T, Okumura N, Suzuki T et al. (2005) Conventional HPLC method used for simultaneous determination of the seven HIV protease inhibitors and nonnucleoside reverse transcription inhibitor efavirenz in human plasma. Biol Pharm Bull 28: 1286-1290. doi:10.1248/bpb.28.1286. PubMed: 15997115.
21. Yasui T, Iguchi M, Suzuki S, Kohri K (2008) Prevalence and epidemiological characteristics of urolithiasis in Japan: national trends between 1965 and 2005. Urology 71: 209-213. doi:10.1016/j.urology.2007.09.034. PubMed: 18308085.
22. Mocroft A, Kirk O, Reiss P, De Wit S, Sedlacek D et al. (2010) Estimated glomerular filtration rate, chronic kidney disease and antiretroviral drug use in HIV-positive patients. AIDS 24: 1667-1678. doi:10.1097/QAD.0b013e328339fe53. PubMed: 20523203.
23. Albini L, Cesana BM, Motta D, Focà E, Gotti D et al. (2012) A randomized, pilot trial to evaluate glomerular filtration rate by creatinine or cystatin C in naive HIV-infected patients after tenofovir/emtricitabine in combination with atazanavir/ritonavir or efavirenz. J Acquir Immune Defic Syndr 59: 18-30. doi:10.1097/QAI.0b013e31823a6124. PubMed: 21992924.
24. Young J, Schäfer J, Fux CA, Furrer H, Bernasconi E et al. (2012) Renal function in patients with HIV starting therapy with tenofovir and either efavirenz, lopinavir or atazanavir. AIDS 26: 567-575. doi:10.1097/QAD.0b013e32834f337c. PubMed: 22398568.
25. Ryom L, Mocroft A, Kirk O, Worm SW, Kamara DA et al. (2013) Association between antiretroviral exposure and renal impairment among HIV-positive persons with normal baseline renal function: the D:A:D study. J Infect Dis 207: 1359-1369.
26. Deeks SG, Smith M, Holodniy M, Kahn JO (1997) HIV-1 protease inhibitors. A review for clinicians. JAMA 277: 145-153. doi:10.1001/jama.277.2.145. PubMed: 8990341.

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Original article

Identification of cross-clade CTL epitopes in HIV-1 clade A/E-infected individuals by using the clade B overlapping peptides

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Abstract

Identification of cross-clade T cell epitopes is one of key factors for the development of a widely applicable AIDS vaccine. We here investigated cross-clade $CD8^+$ T cell responses between clade B and A/E viruses in chronically HIV-1 clade A/E-infected Japanese individuals. $CD8^+$ T cell responses to 11-mer overlapping peptides derived from Nef, Gag, and Pol clade B consensus sequences were at a similar level to those to the same peptides found in clade B-infected individuals. Fifteen cross-clade CTL epitopes were identified from 13 regions where the frequency of responders was high in the clade A/E-infected individuals. The sequences of 6 epitopes were conserved between the clade B and clade A/E viruses whereas 9 epitopes had different amino acid sequences between the 2 viruses. $CD8^+$ T cells specific for the 6 conserved epitopes recognized cells infected with the clade A/E virus, whereas those for 8 diverse epitopes recognized both the clade A/E virus-infected and clade B-infected cells. All of the cross-clade $CD8^+$ T cells specific for conserved and diverse epitopes were detected in chronically HIV-1 clade A/E-infected individuals. These results show that in addition to conserved regions polymorphic ones across the clades can be targets for cross-clade CTLs.

Keywords: Cross-clade; CTL; HLA; Epitope; HIV

1. Introduction

The HIV-1 genome is characterized by genetic diversity wherein distinct HIV-1 clades are expanding not only in different geographical regions but also even in the same locality [1]. HIV-1 clade B is the most prevalent virus in Japan accounting for more than 80% of the patients in this country. CRF01_AE (clade A/E) is the second most prevalent virus, accounting for 6.1% (Sugiura W, unpublished report). In contrast, the clade A/E virus is mainly prevalent in south-east Asian countries including Thailand. An RV144 phase III vaccine trial, which was recently performed in Thailand, demonstrated a partial beneficial effect on HIV-1 infection [2]. In this trial, the recombinant canarypox virus-vectored HIV-1 *gag/pol/env* vaccine (ALVAC-HIV) and the recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E) were used for priming and boosting, respectively. These vaccines were generated by using genes from both the clade B and A/E viral strains [2] to cover a wider range of potential challenge strains in Thailand, where approximately 80% and 10% of HIV-1-infected individuals are infected with the clade A/E and clade B viruses, respectively [3]. Recent analyses confirmed $CD4^+$ cell-mediated and humoral immune responses in vaccines [3–6]. Thus, this clinical trial also highlighted the importance of the cross-clade immune responses to the clade B and A/E viruses. Although T cell functions in the acute and

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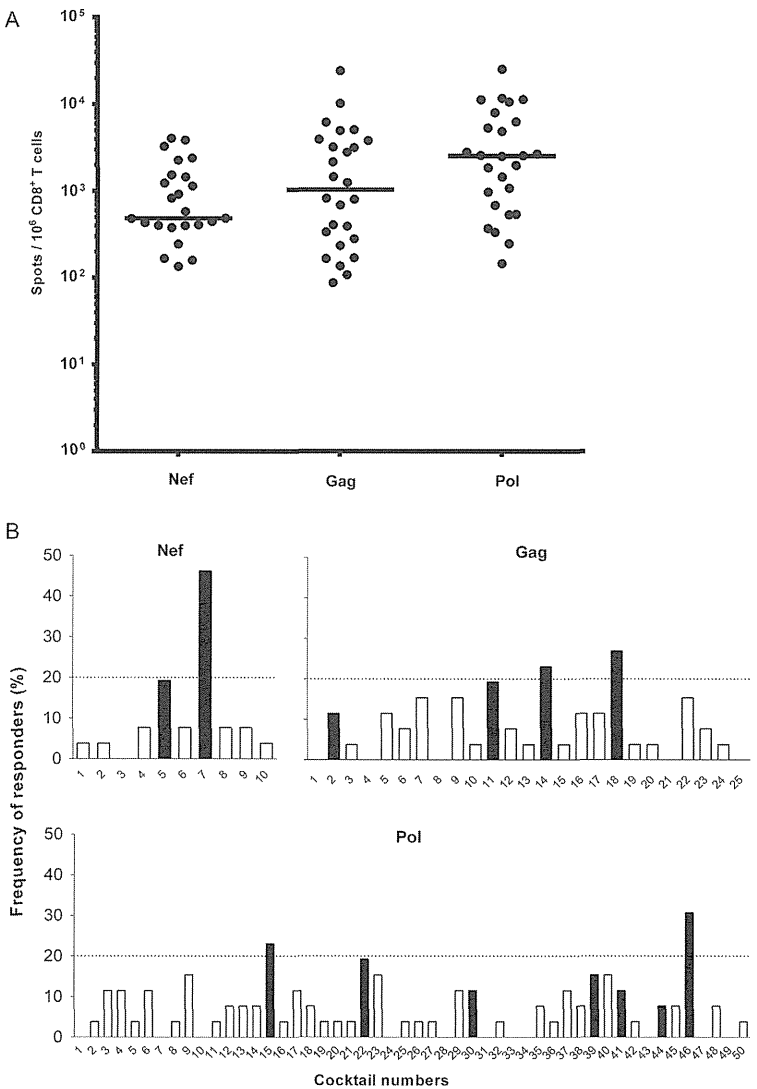
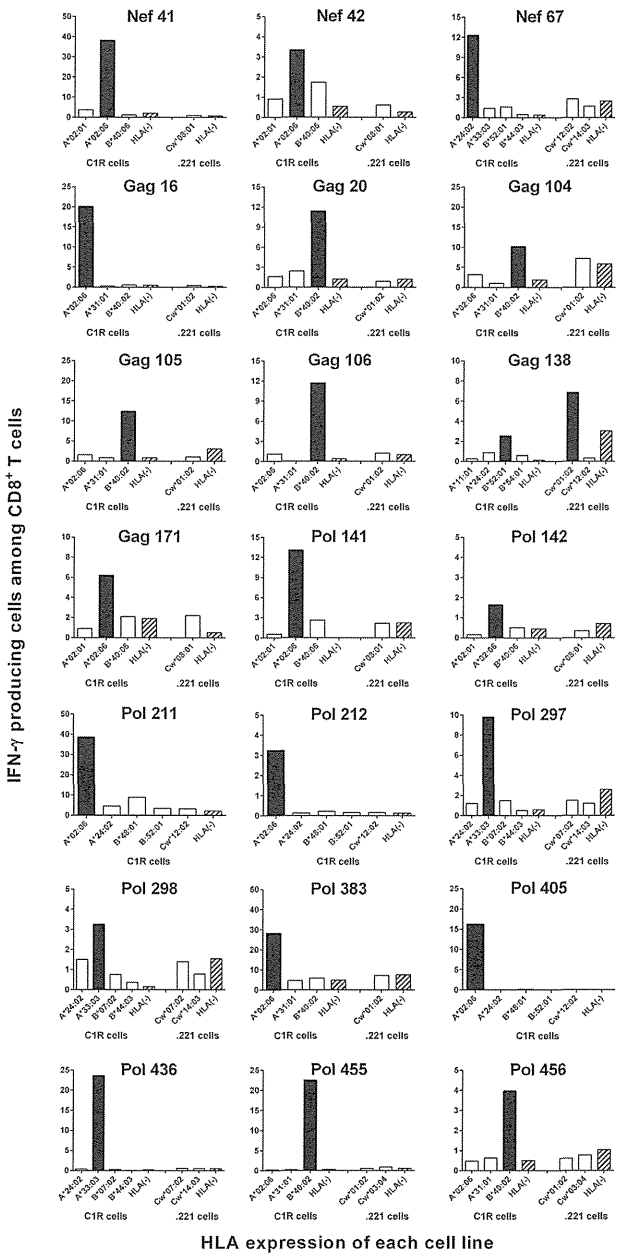


Fig. 1. $CD8^+$ T cell responses of clade A/E-infected individuals to HIV-1 clade B-derived overlapping peptides $CD8^+$ T cell responses to peptide cocktails containing clade B consensus overlapping 11-mer peptides were analyzed by performing the ELISPOT assay using $CD8^+$ T cells from 26 clade A/E-infected individuals. A. Total magnitude of $CD8^+$ T cell responses to the clade B consensus overlapping peptides spanning Nef, Gag and Pol regions. B. Frequency of the responders to each cocktail. Positive response is defined as more than 200 spots. The cocktails for which the frequency of responders was more than 20% or less than 20% are shown as solid bars. In the latter case, at least 1 patient showed a high response (>750 spots).



chronic phases of an HIV-1 infection were well studied in Caucasians infected with the clade B virus and in Africans infected with the clade C virus [7–12], there are only a limited number of studies about the cross-clade reactivity of CTLs [13–17]. However, even in such studies a comprehensive analysis of cross-clade reactivity of the CTLs was not performed.

In the context of HIV vaccine development, it is very important to choose vaccine immunogens capable of eliciting CTLs that can control the variable mutant viruses and exhibit cross-reactivity across the different clade viruses [18,19]. The conserved parts of HIV-1 are good candidates as vaccine antigens [11,12,16,20,21], since they include epitopes conserved among viruses not only in the same clade but also among those clades. Indeed, CTL vaccines containing conserved epitopes have been shown to elicit CTL responses to HIV-1 [22–25]. Although the safety of these consensus CTL vaccines was confirmed in humans [26–32], such vaccines were poorly immunogenic in previous phase I and II trials [27,28,30,31]. Thus more studies on cross-clade effective epitopes will be needed for the development of more potent vaccines.

In the present study, we analyzed cross-clade $CD8^+$ T cells between HIV-1 clade B and A/E viruses in chronically HIV-1 clade A/E-infected Japanese individuals. For this analysis, we sought to identify cross-clade CTL epitopes between the clade B and A/E viruses in the Japanese individuals by using 11-mer overlapping peptides derived from the clade B consensus sequence spanning Nef, Gag, and Pol regions. Thereafter, we analyzed cross-clade $CD8^+$ T cell recognition for epitope peptides between clade A/E and B as well as CTL recognition for cells infected with the clade B or the A/E virus. This is the first comprehensive study to identify cross-clade $CD8^+$ T cells by using overlapping HIV-1 peptides.

2. Materials and methods

2.1. Patient samples

This study was approved by the Ethical Committee of in National Center for Global Health and Medicine and Kumamoto University. Informed consent was obtained from all subjects, according to the Declaration of Helsinki. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from whole blood. HLA types of HIV-infected individuals were determined by standard sequence-based genotyping. HIV-1 subtypes were determined by the sequence results on Pol and Gag, and confirmed by Env sequencing. All samples were collected from the cohort in AIDS Clinical Center, National Center for Global Health and Medicine.

2.2. Sequence of autologous virus

Viral RNA was extracted from plasma samples from HIV-1-infected patients by the use of a QIAamp MinElute virus spin kit (Qiagen). cDNA was synthesized from the RNA by use of the SuperScript III First-Strand Synthesis System for RT-PCR and random hexamers (Invitrogen). Nef, Gag, and Pol regions were amplified by nested PCR using Taq DNA polymerase (Promega). The PCR products were purified by using ExoSAP-IT (GE). All DNA sequencing was performed with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and an ABI 3500 Genetic Analyzer.

2.3. Synthetic peptides

We previously designed overlapping peptides consisting of 11-mer amino acids and spanning Gag, Pol, and Nef of HIV-1 clade B consensus sequences. Each 11-mer peptide was overlapped by 9 amino acids [33]. These 11-mer peptides and truncated peptides were synthesized by utilizing an automated multiple peptide synthesizer and purified by high-performance liquid chromatography (HPLC). The purity was examined by HPLC and mass spectrometry. Peptides with more than 90% purity were used in the present study.

2.4. ELISPOT assay

$CD8^+$ T cells were sorted from cryopreserved PBMCs from 26 chronically HIV-1 clade A/E-infected Japanese individuals by using CD8 magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The sorted cells were plated in 96-well polyvinylidene plates (Millipore, Bedford, MA) that had been pre-coated with 5 mg/mL anti-IFN-γ mAb 1-D1K (Mabtech, Stockholm, Sweden). The appropriate amount of peptide cocktails including 10 overlapping 11-mer peptides were added in a volume of 50 μL, and then PBMCs were added at 1×10^5 cells/well in a volume of 100 μL. The plates were incubated for 16 h at 37 °C in 5% CO_2 and then washed with PBS before the addition of biotinylated anti-IFN-γ Mab (Mabtech) at 1 mg/mL. After the plates had been incubated at room temperature for 90 min and then washed with PBS, they were subsequently incubated with streptavidin-conjugated alkaline phosphatase (Mabtech) for 60 min at room temperature. Individual cytokine-producing cells were detected as dark spots after a 20-min. reaction with 5-bromo-4-chloro-3-idolyl phosphate and nitro blue tetrazolium by using an alkaline phosphatase-conjugate substrate (Bio-Rad, Richmond, CA, USA). The spot number was counted by using an Eliphoto-Counter (Minerva Teck, Tokyo,

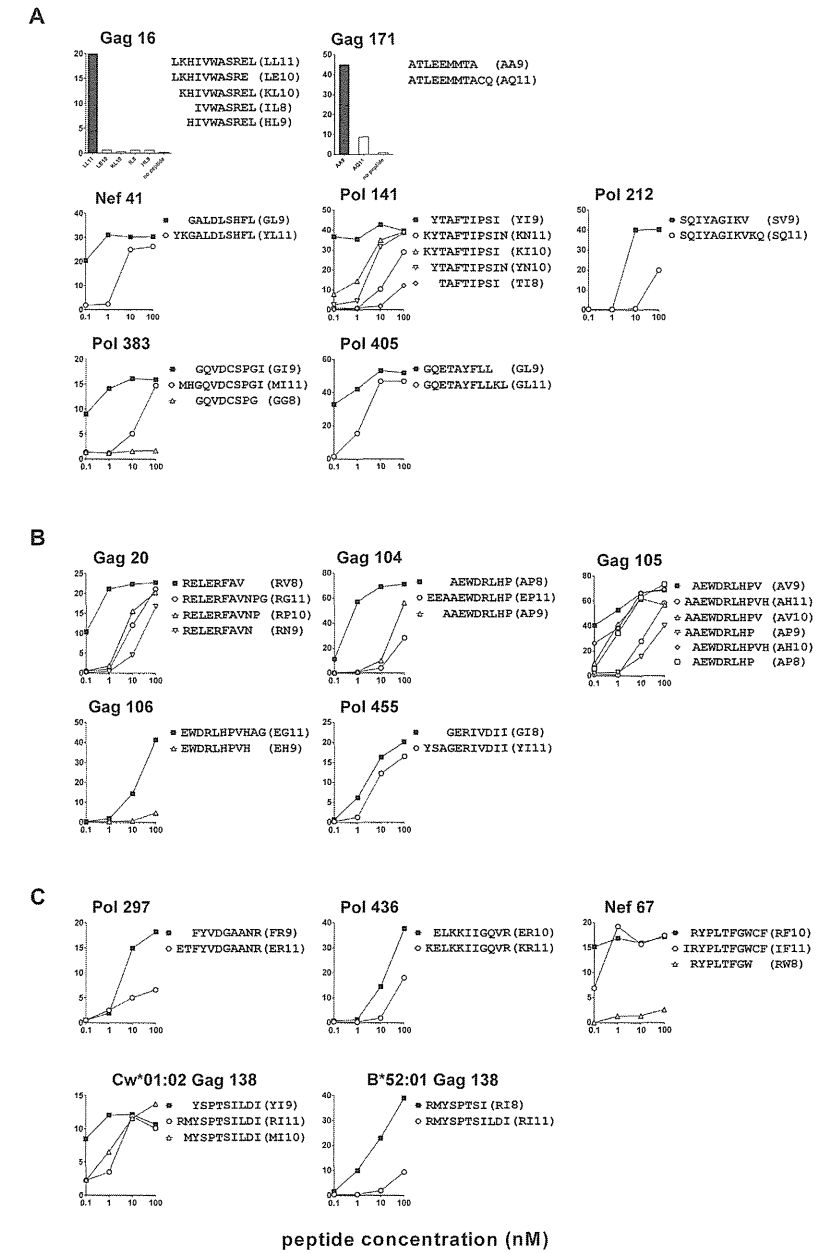


Fig. 2. Identification of HLA restriction of the responses to each 11-mer peptide Peptide-specific $CD8^+$ bulk T cells were induced from PBMCs of the following 6 responders by stimulating the cells with each single peptide. KI-648 for Nef 41, Nef 42, Gag 171, Pol 141, and Pol 142 peptides, KI-632 for Nef 67, Pol 297, Pol 298, and Pol 436 peptides, KI-388 for Gag 16, Gag 20, Gag 104, Gag 105, Gag 106, and Pol 383 peptides, KI-724 for Gag 138 peptide, KI-964 for Pol 211, Pol 212, and Pol 405 peptides, and KI-837 for Pol 455 and Pol 456 peptides. Induced $CD8^+$ bulk T cells were stimulated with the corresponding peptide-prepulsed C1R cells or 0.221 cells expressing each HLA-class I allele molecule. IFN-g production by $CD8^+$ T cells was detected by performing the intracellular cytokine staining (ICS) assay.

Japan). The $CD8^+$ T cells without peptide stimulation were used as a negative control. The number of spots for each peptide-specific T cell response was calculated by subtracting the number of negative-control spots (the number of spots in wells without peptides). Spots giving a mean of more than + 2 SD of the negative-control spots were defined as positive responses. In order to find cross-clade $CD8^+$ T cells in our cohort, we performed the ELISPOT assay by the same method with 11-mer single peptides that were the components of the cocktail peptides where 1) the frequency of responders was more than 20% or 2) the frequency was less than 20% but in which case at least 1 patient showed a high spot count (>750 spots).

2.5. Cells

721.221-CD4 cells expressing HLA-A*02:06, -A*33:03 or Cw*01:02 were generated by transfecting both human CD4 gene and one of these HLA-class I genes into 721.221 cells. These cells were maintained in RPMI medium containing 10% fetal calf serum (FCS) and 0.15 mg/mL hygromycin B. C1R cells expressing HLA-A*02:06 and those expressing HLA-A*33:03 were generated by transfecting C1R cells with HLA-A*02:06 and -A*33:03, respectively; and they were maintained in RPMI medium containing 10% FCS and 0.15 mg/mL hygromycin. C1R and 721.221 cells expressing other HLAs used in this study were previously generated and maintained in RPMI medium with 10% FCS and 0.15 mg/mL hygromycin B or 0.2 mg/mL neomycin [33–36].

2.6. Induction of peptide-specific CTLs from PBMCs

PBMCs from HIV-1-infected individuals who showed the responses to the cocktail peptides in the ELISPOT assay were stimulated with 11-mer single peptide or optimal peptide derived from consensus clade B HIV-1 (100 nM) and then cultured in culture medium (RPMI-1640 containing 10% FCS and 200 U/ml interleukin-2) for 2 weeks. These bulk cultured cells were used for intracellular IFN-γ staining assays.

2.7. HIV clones

The replication-competent molecular clones of p93JP-NH1 [37] and pNL-432 [38] reported previously were used in this study. Viral stocks were generated from plasmid DNA as described elsewhere [15,39].

2.8. HIV-1 infection of .221-CD4 cells expressing HLA molecules or not

.221-CD4 cells expressing HLA molecules or not were exposed to each virus for several days. These infected cells were used as stimulator cells for performing an intracellular cytokine staining assay (ICS) when approximately 30–60% of the cells had been infected, which infection was confirmed by intracellular staining for HIV-1 p24 antigen (KC-57-FITC; Beckman Coulter).

2.9. Intracellular cytokine staining assay (ICS)

After .221 cells or C1R cells had been incubated for 60 min with each peptide (0.01–100 nM), they were washed twice with RPMI-1640 containing 10% FCS. These peptide-pulsed or HIV-1–infected .221-CD4 cells (1×10^5 cells per well) and bulk cultured cells (2×10^4 cells per well) were added to wells of a 96-well round-bottomed plate, and then the cells were incubated for 2 h at 37 °C. Brefeldin A (10 μg/ml) was then added, after which the cells were incubated for a further 4 h. After having been stained with APC-labeled anti-CD8 mAb (DAKO, Glostrup, Denmark), the cells were fixed with 4% paraformaldehyde and then made permeable with the permeabilizing buffer (0.1% saponin and 5% FCS in PBS). Thereafter the cells were stained with FITC-labeled anti-IFN-γ mAb (BD Bioscience, CA). The percentage of $IFN\text{-}\gamma^+CD8^+$ cells was analyzed by flow cytometry.

3. Results

3.1. $CD8^+$ T cell responses to HIV-1 clade B-derived overlapping peptides by HIV-1 clade A/E-infected individuals

To clarify cross-clade responses of $CD8^+$ T cells between the clade B and A/E, we analyzed cross-clade responses of $CD8^+$ T cells from 26 clade A/E-infected Japanese individuals to 11-mer overlapping peptides derived from the consensus sequence of HIV-1 clade B Nef, Gag, and Pol regions. We measured the responses of $CD8^+$ T cells to cocktails including ten 11-mer overlapping peptides by performing the ELISPOT assay. The median of total magnitudes of the $CD8^+$ T cell responses to Nef, Gag, and Pol cocktails were 483, 1037, and 2538, respectively (Fig. 1A). There were no significant differences in total magnitude of the $CD8^+$ T cell responses between the clade A/E-infected and 401 clade B-infected Japanese individuals (the median of total magnitude against Nef, Gag and Pol in the clade B-infected individuals were 529, 1774, and 2300, respectively; H. Murakoshi et al. unpublished observation), although the identities of amino acid sequence in Nef, Gag, and Pol between clade the A/E and the clade B were 80.1, 84.3 and 92.3%, respectively. These results strongly suggest that cross-clade $CD8^+$ T cells were frequently elicited in the clade A/E-infected individuals.

Fig. 3. Identification of optimal epitopes Truncated peptides were designed based on HLA binding motif, and $CD8^+$ bulk T cells were induced from PBMCs of the following 6 responders. KI-648 for Nef 41, Gag 171, and Pol 141 peptides, KI-632 for Nef 67, Pol 297 and Pol 436 peptides, KI-388 for Gag 16, Gag 20, Gag 104, Gag 105, Gag 106, and Pol 383 peptides, KI-724 for Gag 138 peptide, KI-964 for Pol 212 and Pol 405 peptides, and KI-837 for Pol 455 peptide. IFN-g production by $CD8^+$ bulk T cells was measured by performing the ICS assay using the target C1R or .221 cells expressing HLA molecules prepulsed with truncated peptide or 11-mer peptide at a concentration of 100 nM. When the same level of response was seen at 100 nM, the ICS assay was performed again at concentrations from 0.1 to 100 nM. **A**. responses of HLA-A*02:06-restricted $CD8^+$ bulk T cells **B**. responses of HLA-B*40:02-restricted $CD8^+$ bulk T cells **C**. responses of HLA-A*33:03, A*24:02, Cw*01:02 or B*52:01-restricted $CD8^+$ bulk T cells.

3.2. Identification of cross-clade $CD8^+$ T cells elicited in HIV-1 clade A/E-infected individuals

To identify cross-clade $CD8^+$ T cells in the clade A/E-infected individuals, we focused on the $CD8^+$ T cell responses found to be strong or at a high frequency in these individuals (see Materials and methods). We selected the $CD8^+$ T cell responses to 13 cocktails including 2 Nef, 4 Gag, and 7 Pol cocktails (solid bars in Fig. 1B). First, to clarify which 11-mer peptides were recognized by the specific $CD8^+$ T cells, we selected the clade A/E-infected responders (KI-388, KI-632, KI-648, KI-659, KI-724, KI-837, and KI-964) and measured the $CD8^+$ T cell responses to ten 11-mer peptides in each cocktail by using the ELISPOT assay. We found positive responses to three 11-mer Nef peptides (Nef cocktail 5: Nef 41 and 42, Nef cocktail 7: Nef 67), to ten 11-mer Gag peptides (Gag cocktail 2: Gag 11, 16, 19, and 20, Gag cocktail 11: Gag 101, 104, 105, and 106, Gag cocktail 14: Gag 138, Gag cocktail 18: Gag 171), and to eleven 11-mer Pol peptides (Pol cocktail 15: Pol 141 and 142, Pol cocktail 22: Pol 211 and 212, Pol cocktail 30: Pol 297 and 298, Pol cocktail 39: Pol 383, Pol cocktail 41: Pol 405, Pol cocktail 44: Pol 436, Pol cocktail 46: Pol 455 and 456) (data not shown). We next sought to determine HLA restriction molecules in these responses. PBMCs from these responders were stimulated with the 11-mer peptides and then cultured for 14 days. In order to determine the HLA restriction molecules, responses of the cultured cells against the corresponding peptides were analyzed by performing the intracellular cytokine staining (ICS) assay using HLA class I gene-transfected C1R cells or 721.221 cells as stimulators. We found 10 HLA-A*02:06-restricted responses, 6 HLA-B*40:02-restricted responses, 3 A*33:03-restricted responses, 1 HLA-A*24:02-restricrted response, 1 HLA-B*52:01-restricted response, and 1 Cw*01:02-restricted response (Fig. 2).

We first analyzed the responses to the 10 HLA-A*02:06-restricted responses. Concerning the responses to overlapping peptides at 3 locations (Nef 41/42, Pol 141/142, and Pol211/212), we speculated that they would be the same epitope-specific $CD8^+$ T cell responses since the responses to these overlapping peptides were restricted by HLA-A*02:06. Therefore we focused on analyzing the response to Nef 41, Pol 141 or Pol 211, which showed higher responses than those to the other overlapping peptides (data not shown). We generated truncated peptides that were speculated based on HLA-A*02:06 binding motif (Ala, Thr or Gln at position 2) [40–43] and then analyzed these $CD8^+$ T cell responses to Nef 41, Pol 141, and Pol 212 by using them. As shown in Fig. 3A, we identified 3 optimal epitopes: Nef GL9 (GALDLSHFL), Pol YI9 (YTAFTIPSI), and Pol SV9 (SQIYAGIKV). $CD8^+$ T cell responses to Nef GL9 and Pol SV9 were detected among the responses to other overlapping peptides, Nef 42 and Pol 211, respectively (data not shown), indicating that the responses to Nef GL9 and Pol SV9 reflected those to Nef 42 and Pol 212, respectively. In contrast, Pol 142 did not contain Pol YI9. We analyzed the $CD8^+$ bulk T cells induced by Pol 142 by using the truncated peptides and identified Pol TI8 (TAFTIPSI) as an optimal epitope. However, the response to Pol TI8 in $CD8^+$ bulk T cells induced by Pol 142 (4.24% IFN-γ secretion at 100 nM peptide concentration) was much lower than that to Pol TI8 in $CD8^+$ bulk T cells induced by Pol 141 (12.07% at 100 nM; Fig. 3A), suggesting that Pol TI8 may have been a very weak epitope. Similarly, we analyzed the other 4 HLA-A*02:06-restricted responses (Gag16, Gag171, Pol383, and Pol405) by using truncated peptides and identified 4 optimal epitopes; Gag LL11 (LKHIVWASREL), Gag AA9 (ATLEEMMTA), Pol GI9 (GQVDCSPGI), and Pol GL9 (GQETAYFLL; Fig. 3A).

By using the same method identified the HLA-A*02:06-restricted epitopes, we attempted to identify other epitopes. We generated truncated peptides based on HLA binding motif [7,33,40,44–50] and then the responses to these truncated peptides were analyzed using the ICS assay. We finally identified 5 HLA-B*40:02-restricted epitopes (Gag RV8, Gag AP8, Gag AV9, Gag EG11, and Pol GI8; Fig. 3B), 2 HLA-A*33:03-restricted epitopes (Pol FR9 and Pol ER10; Fig. 3C), 1 HLA-A*24:02-restricted epitope (Nef RF10; Fig. 3C), 1 HLA-B*52:01-restricted epitope (Gag RI8; Fig. 3C) and 1 HLA-Cw*01:02-restricted epitope (Gag YI9; Fig. 3C).

Eleven of the above 17 peptides were reported as epitopes in previous studies [7,33,40–43,45,47–50], whereas the other 6 peptides (HLA-A*02:06-restricted Gag LL11, Pol SV9, and Pol GI9, as well as HLA-B*40:02-restricted Gag RV8, Gag AP8, and Gag EG11) had not been previously reported to be epitopes. Therefore, we examined whether the $CD8^+$ bulk T cells specific for these 6 epitopes could recognize HIV-1 clade B virus-infected cells. We measured the IFN-γ production from the $CD8^+$ bulk T cells for target cells infected with HIV-1 clade B clone, NL4-3. These $CD8^+$ bulk T cells effectively produced IFN-γ (data not shown), indicating that these 6 peptides had been naturally processed and presented in cells infected with HIV-1.

3.3. Cross-recognition between the clade B and A/E

We sequenced each epitope region in 26 HIV-1 clade A/E-infected Japanese individuals and then compared these sequences to those from the clade A/E and B viruses reported in the database of the Los Alamos National Library. The results showed that the consensus amino acid sequences of these epitopes in our cohort were the same as those in the database of Los Alamos National Library. The clade B consensus sequences of 6 epitopes (Gag AA9, Pol YI9, Pol SV9, Pol GI9, Pol GL9, and Pol ER10) were identical to the clade A/E consensus ones, whereas other 11 epitopes showed different consensus sequences between the clade B and A/E viruses (Table 1).

Table 1
Frequency of amino-acid sequence for each epitope region of clade A/E viruses from Los Alamos National Library database and our cohort patients.

Epitope	HXB2 region	Sequence	Frequency of amino acid sequence of each clade virus		
			Clade B viruses for Los Alamos database	Clade A/E viruses for Los Alamos database	Clade A/E viruses for our cohort patients
Nef GL9	Nef(83–91)	GALDLSHFL[a]	**529/1494**	0/76	1/26
		—F——F-	12/1494	**47/76**	**16/26**
		Others	953/1494	29/76	9/26
Nef RF10	Nef(134–143)	RYPLTFGWCF[a]	**800/1494**	0/76	2/25
		——C——	94/1494	**56/76**	**10/25**
		others	600/1494	20/76	13/25
Gag LL11	p17(31–41)	LKHIVWASREL[a]	**1182/1644**	18/315	2/26
		M—L———	0/1644	**147/315**	**14/26**
		others	462/1644	150/315	10/26
Gag RV8	p17(39–46)	RELERFAV[a]	**1299/1644**	20/315	4/26
		————L	111/1644	**217/315**	**17/26**
		others	234/1644	78/315	5/26
Gag AP8	p24(78–85)	AEWDRLHP[a]	**1263/1644**	53/315	1/26
		———V-	128/1644	**185/315**	**10/26**
		others	253/1644	77/315	15/26
Gag AV9	p24(78–86)	AEWDRLHPV[a]	**1135/1644**	47/315	1/26
		———V-	121/1644	**178/315**	**8/26**
		others	388/1644	90/315	17/26
Gag EG11	p24(79–89)	EWDRLHPVHAG[a]	**921/1644**	48/315	1/26
		——V———	107/1644	**149/315**	**5/26**
		others	616/1644	118/315	20/26
Gag RI8	p24(143–150)	RMYSPTSI[a]	**1033/1644**	30/315	2/26
		———V-	351/1644	**212/315**	**15/26**
		others	260/1644	73/315	9/26
Gag YI9	p24(145–153)	YSPTSILDI[a]	**1032/1644**	30/315	2/26
		——V———	345/1644	**212/315**	**15/26**
		others	267/1644	73/315	9/26
Gag AA9	p24(209–217)	ATLEEMMTA[a]	**1468/1644**	**285/315**	**25/26**
		others	176/1644	30/315	1/26
Pol YI9	RT(127–135)	YTAFTIPSI[a]	**574/1003**	**44/59**	**16/26**
		others	429/1003	15/59	10/26
Pol SV9	RT(268–276)	SQIYAGIKV[a]	**438/1003**	**38/59**	**18/26**
		others	565/1003	21/59	8/26
Pol FR9	RT(440–448)	FYVDGAANR[a]	**838/1003**	9/59	9/26
		————S-	47/1003	**45/59**	**15/26**
		others	118/1003	5/59	2/26
Pol GI9	Integrase(50–60)	GQVDCSPGI[a]	**915/1003**	**55/59**	**26/26**
		others	88/1003	4/59	0/26
Pol GL9	Integrase(94–102)	GQETAYFLL[a]	**519/1003**	**52/59**	**19/26**
		others	484/1003	7/59	7/26
Pol ER10	Integrase(157–166)	ELKKIIGQVR[a]	**770/1003**	**40/59**	**21/26**
		others	233/1003	19/59	5/26
Pol GI8	Integrase(197–204)	GERIVDII[a]	**535/1003**	0/57	0/26
		——I—	378/1003	**56/59**	**23/26**
		others	90/1003	3/59	3/26

[a] Amino acid sequence in clade B consensus used in this study.

We investigated the cross-recognition of these 11 epitope peptides by CD8$^+$ T cells that had been induced by stimulating PBMCs from clade A/E virus-infected individuals with clade B-derived epitope peptides. The CD8$^+$ bulk T cells induced by Nef RF10, Gag RV8, Gag AP8, Gag AV9, or Gag YI9 peptides recognized both clades B and A/E peptides evenly (Fig. 4A). The CD8$^+$ bulk T cells induced by Gag EG11, Gag RI8, or Pol FR9 more strongly recognized the clade B-derived peptide than the clade A/E-derived one, whereas those induced by Nef GL9 or Pol GI8 more strongly recognized the clade A/E-derived peptide than the clade B-derived one (Fig. 4A). Interestingly, the CD8$^+$ bulk T cells induced by Gag 16 LL11 failed to recognized the clade A/E-derived Gag LL11-1M-4L peptide (Fig. 4A).

Next, we investigated whether CD8$^+$ T cells recognizing these 10 clade A/E peptides could recognize clade A/E-infected cells. We measured IFN-γ production from the CD8$^+$ bulk T cells for target cells infected with HIV-1 clade A/E clone or for those infected with HIV-1 clade B clone. The CD8$^+$ bulk T cells induced by Nef RF10, Gag RV8, Gag AP8, Gag AV9, Gag EG11, Gag YI9, Gag RI8, or Pol GI8 recognized not only the clade B virus-infected cells but also the clade A/E virus-infected ones (Fig. 4B), indicating that these cross-clade epitopes had been naturally processed

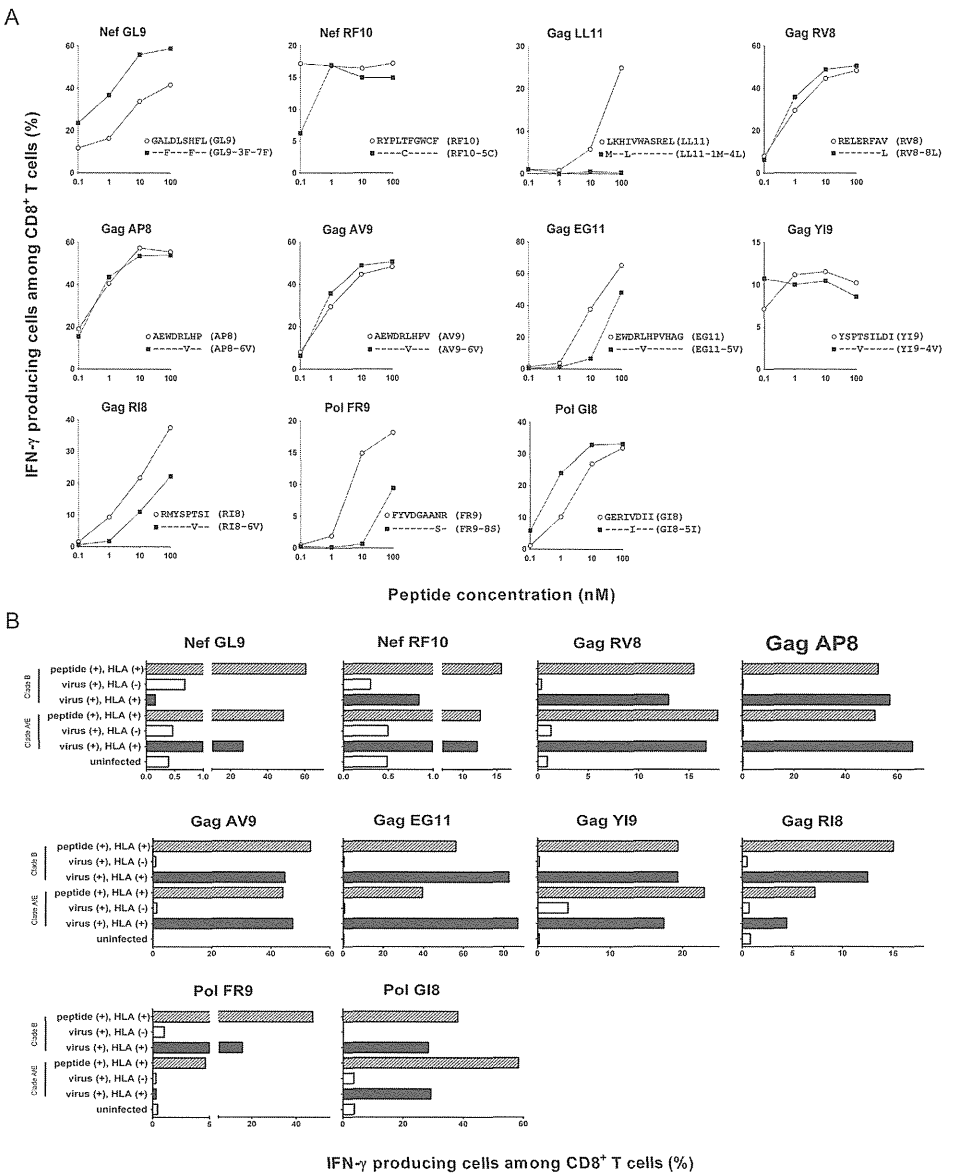


Fig. 4. Cross-recognition by CD8$^+$ T cells from HIV-1 clade A/E-infected individuals IFN-γ production by CD8$^+$ bulk T cells in response to stimulator cells with optimal epitope peptides and to cells infected with clade B or clade A/E viruses was measured by use of the ICS assay. Nef GL9 specific bulk T cells were induced from PBMCs of KI-648, Nef RF10 and Pol FR9 specific bulk T cells were induced from PBMCs of KI-632, Gag LL11, Gag RV8, Gag AP8, Gag AV9 and Gag EG11 specific bulk T cells were induced from PBMCs of KI-388, Gag RI8 and Gag YI9 specific bulk T cells were induced from KI-724, and Pol GI8 specific bulk

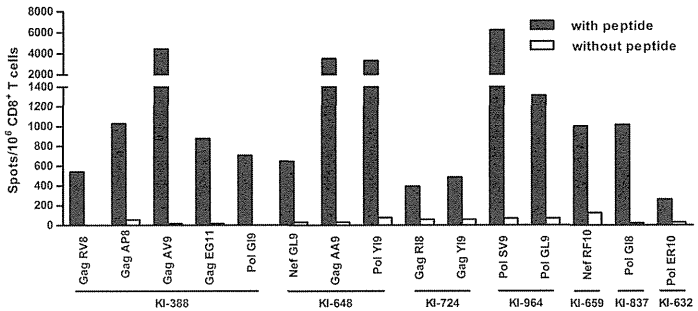


Fig. 5. CD8$^+$ T cell responses to clade B-drived epitope peptides in HIV-1 clade A/E-infected Japanese individuals CD8$^+$ T cell responses to 15 clade B-derived epitope peptides were analyzed by performing ELISPOT assay using CD8$^+$ T cells from seven clade A/E-infected individuals (KI-388, KI-632, KI-648, KI-659, KI-724, KI-837, and KI-964). >200 spots were evaluated as positive response.

and presented in cells infected with these viruses. On the other hand, CD8$^+$ bulk T cells induced by Pol FR9 recognized the clade B virus-infected cells but failed to recognize the clade A/E virus-infected cells (Fig. 4B). This finding is consistent with the low ability of these cells to recognize the clade A/E peptide (Fig. 4A). In contrast, CD8$^+$ T cells induced by Nef GL9 recognized the clade A/E virus-infected cells but failed to recognize the clade B virus-infected cells although these T cells could recognize GL9 peptide. This result may be explained by the fact that the amino acid sequence of the clade B consensus peptide is different than that of the clade B clone, NL4-3(Ala and Val at position 1 and 3, respectively, in Nef GL9 region). Thus, CD8$^+$ T cells induced by 8 out of 10 clade B-derived epitope peptides successfully recognized both the clade B virus-infected and clade A/E-infected cells.

3.4. Detection of cross-clade CD8$^+$ T cell responses in the clade A/E-infected Japanese individuals

To confirm CD8$^+$ T cell responses to the 15 epitopes including Nef GL9, we analyzed CD8$^+$ T cell responses to the clade B-derived epitope peptides in clade A/E-infected individuals who had HLA alleles restricting these epitopes. Positive CD8$^+$ T cell responses to these 15 clade B-derived epitope peptides were detected in PBMCs from chronically HIV-1 clade A/E-infected individuals (Fig. 5). These results indicate that these cross-clade CTLs are elicited in these individuals.

4. Discussion

Previous studies, which focused on known CTL epitopes for the clade B or C viruses, showed the existence of cross-clade CTLs in HIV-1-infected individuals by demonstrating that CTL clones established by using clade-matched peptides from the clade B-infected or the clade C-infected individuals recognize the cells infected with other clade viruses [13–17]. These studies also showed that conserved epitopes across the clades are more likely recognized by the T cell clones and suggested that conserved epitopes would be a more preferable target for a widely effective CTL vaccine than variable ones. In the present study, we for the first time performed a comprehensive analysis of cross-clade CD8$^+$ T cells by using 11-mer overlapping clade B-derived peptides to stimulate CD8$^+$ T cells from HIV-1 clade A/E-infected individuals. Interestingly, we found a similar level of CD8$^+$ T cell responses to clade B-derived Nef, Gag, and Pol peptides in the clade A/E virus-infected individuals as compared to those to the same peptides in clade B-infected individuals. These results strongly suggested the existence of a high number of cross-clade CTLs in the clade A/E virus-infected individuals. Indeed, we finally identified 15 cross-clade CTL epitopes from only 13 out of 85 overlapping peptide cocktails. These results strongly suggest that a large number of cross-clade CTLs were elicited in the clade A/E virus-infected individuals.

CD8$^+$ T cells induced by Pol FR9 recognized to a much lesser extent the clade A/E-derived peptide (FR9-8S) than the clade B-derived peptide and recognized cells infected with clade B virus but not those infected with A/E viruses, suggesting that PolFR9-8S was not an epitope. Indeed, the HLA-A*33:03$^+$ individuals were infected with the clade A/E virus carrying Pol FR9 sequence but not Pol FR9-8S one (data not shown). The CD8$^+$ T cells induced by Nef GL9 recognized the clade A/E virus-infected cells, whereas they failed to recognize the clade B-infected ones. These T cells could recognize GL9 peptide, though they recognized more effectively Nef GL9-3F-7F peptides than the Nef GL9 one. However, CD8$^+$ T cells specific for both Nef GL9 and Nef GL9-3F-7F were detected in 3 of 7 HLA-A*02:06$^+$ individuals (data not shown). These results suggest that Nef GL9-3F-7F had been presented in the clade A/E-infected individuals. Therefore, the failure of the T cells to recognize cells infected with NL4-3 virus may have resulted from a different amino acid sequence of this epitope between the clade B consensus peptide and NL4-3 (Ala and Val at position 1 and 3, respectively, in Nef GL9 region). CD8$^+$ bulk T cells induced by 8 other diverse epitopes effectively recognized both the clade B-infected and the clade A/E-infected cells, suggesting that these diverse epitopes could be cross-recognized by the T cells.

We previously reported that Phe at position 2 of Nef RF10 is an escape mutation in the clade B virus [48]. This escape mutation was frequently found in the clade A/E virus, though the consensus sequence was RF10-5C (RYPLCFGWCF; Table 1). Since RF10 and RF10-5C were cross-recognized by the CD8$^+$ T cells induced by the RF10 peptide, these T cells would be expected to select 2F mutants in the clade A/E-infected individuals. These results indicate that RF10-5C was an HLA-A*24:02-restricted epitope in the clade A/E-infected individuals and that RF10-5C-specific CD8$^+$ T cells could cross-recognize the RF10 epitope.

Since these epitopes were restricted by Asian HLA alleles, vaccine targeting these epitopes can cover Asian countries including south-east Asia and China where clade A/E and clade B viruses are prevalent. An HLA-B*40:02-restricted Nef epitope was known to be presented by world-wise HLA allele HLA-B*40:01 [33]. In addition, a previous study showed that Pol GL9-specific CD8$^+$ T cells were elicited in a vaccinated individual carrying world-wise HLA allele, HLA-A*02:01 [42]. These studies together suggest that some of the HLA-B*40:02-resticted and HLA-A*02:06-restricted epitopes identified in this study may be CTL epitopes presented by these world-wise HLA alleles. Thus, vaccine targeting the cross-clade epitopes identified in this study may cover countries in Europe, and northern and southern Americas in addition to Asian countries.

In conclusion, we here performed the first comprehensive study of cross-clade T cell responses and demonstrated that CD8$^+$ T cell responses to clade B-derived Nef, Gag, and Pol peptides were successfully induced in the clade A/E virus-infected individuals. We finally identified the 15 cross-clade epitopes which include not only conserved epitopes but also polymorphic epitopes across the different clades. These epitopes can thus be candidate targets of CTL-based vaccines.

T cells were induced from KI-837. **A**. Cross-recognition of HIV-1 clade B (open circle) and clade A/E (closed square) optimal epitope peptides of the consensus sequence. These analyses were performed at peptide concentrations from 0.1 to 100 nM. **B**. Cross-recognition of cells infected with clade B or clade A/E virus. IFN-γ production by CD8$^+$ bulk T cells in response to HLA-positive cells prepulsed with clade B or clade A/E consensus optimal peptide and that in response to HLA-negative cells infected with the virus and to uninfected HLA-positive cells were measured as positive and negative controls, respectively. All epitope sequences derived from the clade B or the clade A/E were identical to the sequences from clone virus (NL4-3 or 93JP-NH1) except for clade B Nef GL9 epitope (GALDLSHFL). NL4-3 has Ala and Val at positions 1 and 3 of this epitope, respectively.

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References

[1] B.D. Walker, B.T. Korber, Immune control of HIV: the obstacles of HLA and viral diversity, Nat. Immunol. 2 (2001) 473–475.
[2] S. Rerks-Ngarm, P. Pitisuttithum, S. Nitayaphan, J. Kaewkungwal, J. Chiu, R. Paris, N. Premsri, C. Namwat, M. de Souza, E. Adams, M. Benenson, S. Gurunathan, J. Tartaglia, J.G. McNeil, D.P. Francis, D. Stablein, D.L. Birx, S. Chunsuttiwat, C. Khamboonruang, P. Thongcharoen, M.L. Robb, N.L. Michael, P. Kunasol, J.H. Kim, MOPHTAVEG investigators, Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand, N. Engl. J. Med. 361 (2009) 2209–2220.
[3] J. Hemelaar, E. Gouws, P.D. Ghys, S. Osmanov, WHO-UNAIDS Network for HIV isolation and characterisation, global trends in molecular epidemiology of HIV-1 during 2000-2007, AIDS 25 (2011) 679–689.
[4] M.S. de Souza, S. Ratto-Kim, W. Chuenarom, A. Schuetz, S. Chantakulkij, B. Nuntapinit, A. Valencia-Micolta, D. Thelian, S. Nitayaphan, P. Pitisuttithum, R.M. Paris, J. Kaewkungwal, N.L. Michael, S. Rerks-Ngarm, B. Mathieson, M. Marovich, J.R. Currier, J.H. Kim, Ministry of Public Health-Thai AIDS Vaccine Evaluation Group Collaborators, The Thai phase III trial (RV144) vaccine regimen induces T cell responses that preferentially target epitopes within the V2 region of HIV-1 envelope, J. Immunol. 188 (2012) 5166–5176.
[5] M. Bonsignori, J. Pollara, M.A. Moody, M.D. Alpert, X. Chen, K.K. Hwang, P.B. Gilbert, Y. Huang, T.C. Gurley, D.M. Kozink, D.J. Marshall, J.F. Whitesides, C.Y. Tsao, J. Kaewkungwal, S. Nitayaphan, P. Pitisuttithum, S. Rerks-Ngarm, J.H. Kim, N.L. Michael, G.D. Tomaras, D.C. Montefiori, G.K. Lewis, A. DeVico, D.T. Evans, G. Ferrari, H.X. Liao, B.F. Haynes, Antibody-dependent cellular cytotoxicity-mediating antibodies from an HIV-1 vaccine efficacy trial target multiple epitopes and preferentially use the VH1 gene family, J. Virol. 86 (2012) 11521–11532.
[6] B.F. Haynes, P.B. Gilbert, M.J. McElrath, S. Zolla-Pazner, G.D. Tomaras, S.M. Alam, D.T. Evans, D.C. Montefiori, C. Karnasuta, R. Sutthent, H.X. Liao, A.L. DeVico, G.K. Lewis, C. Williams, A. Pinter, Y. Fong, H. Janes, A. DeCamp, Y. Huang, M. Rao, E. Billings, N. Karasavvas, M.L. Robb, V. Ngauy, M.S. de Souza, R. Paris, G. Ferrari, R.T. Bailer, K.A. Soderberg, C. Andrews, P.W. Berman, N. Frahm, S.C. De Rosa, M.D. Alpert, N.L. Yates, X. Shen, R.A. Koup, P. Pitisuttithum, J. Kaewkungwal, S. Nitayaphan, S. Rerks-Ngarm, N.L. Michael, J.H. Kim, Immune-correlates analysis of an HIV-1 vaccine efficacy trial, N. Engl. J. Med. 366 (2012) 1275–1286.
[7] P. Kiepiela, K. Ngumbela, C. Thobakgale, D. Ramduth, I. Honeyborne, E. Moodley, S. Reddy, C. de Pierres, Z. Mncube, N. Mkhwanazi, K. Bishop, M. van der Stok, K. Nair, N. Khan, H. Crawford, R. Payne, A. Leslie, J. Prado, A. Prendergast, J. Frater, N. McCarthy, C. Brander, G.H. Learn, D. Nickle, C. Rousseau, H. Coovadia, J.I. Mullins, D. Heckerman, B.D. Walker, P. Goulder, CD8+ T-cell responses to different HIV proteins have discordant associations with viral load, Nat. Med. 13 (2007) 46–53.
[8] R. Zuniga, A. Lucchetti, P. Galvan, S. Sanchez, C. Sanchez, A. Hernandez, H. Sanchez, N. Frahm, C.H. Linde, H.S. Hewitt,

W. Hildebrand, M. Altfeld, T.M. Allen, B.D. Walker, B.T. Korber, T. Leitner, J. Sanchez, C. Brander, Relative dominance of Gag p24-specific cytotoxic T lymphocytes is associated with human immunodeficiency virus control, J. Virol. 80 (2006) 3122–3125.

[9] S.A. Migueles, M.S. Sabbaghian, W.L. Shupert, M.P. Bettinotti, F.M. Marincola, L. Martino, C.W. Hallahan, S.M. Selig, D. Schwartz, J. Sullivan, M. Connors, HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors, Proc. Natl. Acad. Sci. USA 97 (2000) 2709–2714.

[10] E.L. Turnbull, A.R. Lopes, N.A. Jones, D. Cornforth, P. Newton, D. Aldam, P. Pellegrino, J. Turner, I. Williams, C.M. Wilson, P.A. Goepfert, M.K. Maini, P. Borrow, HIV-1 epitope-specific CD8+ T cell responses strongly associated with delayed disease progression cross-recognize epitope variants efficiently, J. Immunol. 176 (2006) 6130–6146.

[11] S.L. Rowland-Jones, T. Dong, K.R. Fowke, J. Kimani, P. Krausa, H. Newell, T. Blanchard, K. Ariyoshi, J. Oyugi, E. Ngugi, J. Bwayo, K.S. MacDonald, A.J. McMichael, F.A. Plummer, Cytotoxic T cell responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi, J. Clin. Invest. 102 (1998) 1758–1765.

[12] R.P. Johnson, A. Trocha, L. Yang, G.P. Mazzara, D.L. Panicali, T.M. Buchanan, B.D. Walker, HIV-1 gag-specific cytotoxic T lymphocytes recognize multiple highly conserved epitopes. Fine specificity of the gag-specific response defined by using unstimulated peripheral blood mononuclear cells and cloned effector cells, J. Immunol. 147 (1991) 1512–1521.

[13] D.M. McKinney, R. Skvoretz, B.D. Livingston, C.C. Wilson, M. Anders, R.W. Chesnut, A. Sette, M. Essex, V. Novitsky, M.J. Newman, Recognition of variant HIV-1 epitopes from diverse viral subtypes by vaccine-induced CTL, J. Immunol. 173 (2004) 1941–1950.

[14] M.S. Bennett, H.L. Ng, A. Ali, O.O. Yang, Cross-clade detection of HIV-1-specific cytotoxic T lymphocytes does not reflect cross-clade antiviral activity, J. Infect Dis. 197 (2008) 390–397.

[15] K. Fukada, H. Tomiyama, C. Wasi, T. Matsuda, S. Kusagawa, H. Sato, S. Oka, Y. Takebe, M. Takiguchi, Cytotoxic T-cell recognition of HIV-1 cross-clade and clade-specific epitopes in HIV-1-infected Thai and Japanese patients, AIDS 16 (2002) 701–711.

[16] H. Cao, P. Kanki, J.L. Sankale, A. Dieng-Sarr, G.P. Mazzara, S.A. Kalams, B. Korber, S. Mboup, B.D. Walker, Cytotoxic T-lymphocyte cross-reactivity among different human immunodeficiency virus type 1 clades: implications for vaccine development, J. Virol. 71 (1997) 8615–8623.

[17] D. Durali, J. Morvan, F. Letourneur, D. Schmitt, N. Guegan, M. Dalod, S. Saragosti, D. Sicard, J.P. Levy, E. Gomard, Cross-reactions between the cytotoxic T-lymphocyte responses of human immunodeficiency virus-infected African and European patients, J. Virol. 72 (1998) 3547–3553.

[18] H. Chen, Z.M. Ndhlovu, D. Liu, L.C. Porter, J.W. Fang, S. Darko, M.A. Brockman, T. Miura, Z.L. Brumme, A. Schneidewind, A. Piechocka-Trocha, K.T. Cesa, J. Sela, T.D. Cung, I. Toth, F. Pereyra, X.G. Yu, D.C. Douek, D.E. Kaufmann, T.M. Allen, B.D. Walker, TCR clonotypes modulate the protective effect of HLA class I molecules in HIV-1 infection, Nat. Immunol. 13 (2012) 691–700.

[19] M.P. Sanou, A.S. De Groot, M. Murphey-Corb, J.A. Levy, J.K. Yamamoto, HIV-1 vaccine trials: evolving concepts and designs, Open AIDS J. 6 (2012) 274–288.

[20] B.T. Korber, N.L. Letvin, B.F. Haynes, T-cell vaccine strategies for human immunodeficiency virus, the virus with a thousand faces, J. Virol. 83 (2009) 8300–8314.

[21] A.M. Masemola, T.N. Mashishi, G. Khoury, H. Bredell, M. Paximadis, T. Mathebula, D. Barkhan, A. Puren, E. Vardas, M. Colvin, L. Zijenah, D. Katzenstein, R. Musonda, S. Allen, N. Kumwenda, T. Taha, G. Gray, J. McIntyre, S.A. Karim, H.W. Sheppard, C.M. Gray, HIVNET 028 Study Team, Novel and promiscuous CTL epitopes in conserved regions of Gag targeted by individuals with early subtype C HIV type 1 infection from southern Africa, J. Immunol. 173 (2004) 4607–4617.

[22] Y. Roshorm, M.G. Cottingham, M.J. Potash, D.J. Volsky, T. Hanke, T cells induced by recombinant chimpanzee adenovirus alone and in prime-boost regimens decrease chimeric EcoHIV/NDK challenge virus load, Eur. J. Immunol. 42 (2012) 3243–3255.

[23] M.L. Knudsen, A. Mbewe-Mvula, M. Rosario, D.X. Johansson, M. Kakoulidou, A. Bridgeman, A. Reyes-Sandoval, A. Nicosia, K. Ljungberg, T. Hanke, P. Liljestrom, Superior induction of T cell responses to conserved HIV-1 regions by electroporated alphavirus replicon DNA compared to that with conventional plasmid DNA vaccine, J. Virol. 86 (2012) 4082–4090.

[24] M. Rosario, N. Borthwick, G.B. Stewart-Jones, A. Mbewe-Mvula, A. Bridgeman, S. Colloca, D. Montefiori, A.J. McMichael, A. Nicosia, E.D. Quakkelaar, J.W. Drijfhout, C.J. Melief, T. Hanke, Prime-boost regimens with adjuvanted synthetic long peptides elicit T cells and antibodies to conserved regions of HIV-1 in macaques, AIDS 26 (2012) 275–284.

[25] Y. Roshorm, J.P. Hong, N. Kobayashi, A.J. McMichael, D.J. Volsky, M.J. Potash, M. Takiguchi, T. Hanke, Novel HIV-1 clade B candidate vaccines designed for HLA-B*5101(+) patients protected mice against chimaeric ecotropic HIV-1 challenge, Eur. J. Immunol. 39 (2009) 1831–1840.

[26] I. Cebere, L. Dorrell, H. McShane, A. Simmons, S. McCormack, C. Schmidt, C. Smith, M. Brooks, J.E. Roberts, S.C. Darwin, P.E. Fast, C. Conlon, S. Rowland-Jones, A.J. McMichael, T. Hanke, Phase I clinical trial safety of DNA- and modified virus Ankara-vectored human immunodeficiency virus type 1 (HIV-1) vaccines administered alone and in a prime-boost regime to healthy HIV-1-uninfected volunteers, Vaccine 24 (2006) 417–425.

[27] N. Goonetilleke, S. Moore, L. Dally, N. Winstone, I. Cebere, A. Mahmoud, S. Pinheiro, G. Gillespie, D. Brown, V. Loach, J. Roberts, A. Guimaraes-Walker, P. Hayes, K. Loughran, C. Smith, J. De Bont, C. Verlinde, D. Vooijs, C. Schmidt, M. Boaz, J. Gilmour, P. Fast, L. Dorrell, T. Hanke, A.J. McMichael, Induction of multifunctional human immunodeficiency virus type 1 (HIV-1)-specific T cells capable of proliferation in healthy subjects by using a prime-boost regimen of DNA- and modified vaccinia virus Ankara-vectored vaccines expressing HIV-1 Gag coupled to CD8+ T-cell epitopes, J. Virol. 80 (2006) 4717–4728.

[28] M. Mwau, I. Cebere, J. Sutton, P. Chikoti, N. Winstone, E.G. Wee, T. Beattie, Y.H. Chen, L. Dorrell, H. McShane, C. Schmidt, M. Brooks, S. Patel, J. Roberts, C. Conlon, S.L. Rowland-Jones, J.J. Bwayo, A.J. McMichael, T. Hanke, A human immunodeficiency virus 1 (HIV-1) clade A vaccine in clinical trials: stimulation of HIV-specific T-cell responses by DNA and recombinant modified vaccinia virus Ankara (MVA) vaccines in humans, J. Gen. Virol. 85 (2004) 911–919.

[29] G.J. Gorse, L.R. Baden, M. Wecker, M.J. Newman, G. Ferrari, K.J. Weinhold, B.D. Livingston, T.L. Villafana, H. Li, E. Noonan, N.D. Russell, HIV Vaccine Trial Network, Safety and immunogenicity of cytotoxic T-lymphocyte poly-epitope, DNA plasmid (EP HIV-1090) vaccine in healthy, human immunodeficiency virus type 1 (HIV-1)-uninfected adults, Vaccine 26 (2008) 215–223.

[30] P. Spearman, S. Kalams, M. Elizaga, B. Metch, Y.L. Chiu, M. Allen, K.J. Weinhold, G. Ferrari, S.D. Parker, M.J. McElrath, S.E. Frey, J.D. Fuchs, M.C. Keefer, M.D. Lubeck, M. Egan, R. Braun, J.H. Eldridge, B.F. Haynes, L. Corey, NIAID HIV Vaccine Trials Network, Safety and immunogenicity of a CTL multiepitope peptide vaccine for HIV with or without GM-CSF in a phase I trial, Vaccine 27 (2009) 243–249.

[31] D. Salmon-Ceron, C. Durier, C. Desaint, L. Cuzin, M. Surenaud, N.B. Hamouda, J.D. Lelievre, B. Bonnet, G. Pialoux, I. Poizot-Martin, J.P. Aboulker, Y. Levy, O. Launay, ANRS VAC trial group, immunogenicity and safety of an HIV-1 lipopeptide vaccine in healthy adults: a phase 2 placebo-controlled ANRS trial, AIDS 24 (2010) 2211–2223.

[32] W. Jaoko, F.N. Nakwagala, O. Anzala, G.O. Manyonyi, J. Birungi, A. Nanvubya, F. Bashir, K. Bhatt, H. Ogutu, S. Wakasiaka, L. Matu, W. Waruingi, J. Odada, M. Oyaro, J. Indangasi, J. Ndinya-Achola, C. Konde, E. Mugisha, P. Fast, C. Schmidt, J. Gilmour, T. Tarragona, C. Smith, B. Barin, L. Dally, B. Johnson, A. Muluubya, L. Nielsen, P. Hayes, M. Boaz, P. Hughes, H. Hanke, A. McMichael, J. Bwayo, P. Kaleebu, Safety and immunogenicity of recombinant low-dosage HIV-1 A vaccine candidates vectored by plasmid pTHr DNA or modified vaccinia virus Ankara (MVA) in humans in East Africa, Vaccine 26 (2008) 2788–2795.

[33] T. Watanabe, H. Murakoshi, H. Gatanaga, M. Koyanagi, S. Oka, M. Takiguchi, Effective recognition of HIV-1-infected cells by HIV-1 integrase-specific HLA-B *4002-restricted T cells, Microbe. Infect. 13 (2011) 160–166.

[34] S. Karaki, A. Kariyone, N. Kato, K. Kano, Y. Iwakura, M. Takiguchi, HLA-B51 transgenic mice as recipients for production of polymorphic HLA-A, B-specific antibodies, Immunogenetics 37 (1993) 139–142.

[35] H. Koizumi, T. Iwatani, J. Tanuma, M. Fujiwara, T. Izumi, S. Oka, M. Takiguchi, Escape mutation selected by Gag28-36-specific cytotoxic T cells in HLA-A*2402-positive HIV-1-infected donors, Microbe. Infect. 11 (2009) 198–204.

[36] Y. Yagita, N. Kuse, K. Kuroki, H. Gatanaga, J.M. Carlson, T. Chikata, Z.L. Brumme, H. Murakoshi, T. Akahoshi, N. Pfeifer, S. Mallal, M. John, T. Ose, H. Matsubara, R. Kanda, Y. Fukunaga, K. Honda, Y. Kawashima, Y. Ariumi, S. Oka, K. Maenaka, M. Takiguchi, Distinct HIV-1 escape patterns selected by cytotoxic T cells with identical epitope specificity, J. Virol. 87 (2013) 2253–2263.

[37] H. Sato, Y. Tomita, K. Ebisawa, A. Hachiya, K. Shibamura, T. Shiino, R. Yang, M. Tatsumi, K. Gushi, H. Umeyama, S. Oka, Y. Takebe, Y. Nagai, Augmentation of human immunodeficiency virus type 1 subtype E (CRF01_AE) multiple-drug resistance by insertion of a foreign 11-amino-acid fragment into the reverse transcriptase, J. Virol. 75 (2001) 5604–5613.

[38] A. Adachi, H.E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, M.A. Martin, Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone, J. Virol. 59 (1986) 284–291.

[39] T. Akahoshi, T. Chikata, Y. Tamura, H. Gatanaga, S. Oka, M. Takiguchi, Selection and accumulation of an HIV-1 escape mutant by three types of HIV-1-specific cytotoxic T lymphocytes recognizing wild-type and/or escape mutant epitopes, J. Virol. 86 (2012) 1971–1981.

[40] A.C. Karlsson, A.K. Iversen, J.M. Chapman, T. de Oliviera, G. Spotts, A.J. McMichael, M.P. Davenport, F.M. Hecht, D.F. Nixon, Sequential broadening of CTL responses in early HIV-1 infection is associated with viral escape, PLoS One 2 (2007) e225.

[41] W. Kantakamalakul, M. de Souza, S. Bejrachandra, S. Ampol, J. Cox, R. Sutthent, Identification of a novel HIV type 1 CRF01_AE cytotoxic T lymphocyte (CTL) epitope restricted by an HLA-Cw0602 allele and a novel HLA-A0206/peptide restriction, AIDS Res. Hum. Retroviruses 22 (2006) 1271–1282.

[42] F. Li, A.C. Finnefrock, S.A. Dubey, B.T. Korber, J. Szinger, S. Cole, M.J. McElrath, J.W. Shiver, D.R. Casimiro, L. Corey, S.G. Self, Mapping HIV-1 vaccine induced T-cell responses: bias towards less-conserved regions and potential impact on vaccine efficacy in the Step study, PLoS One 6 (2011) e20479.

[43] S. Sabbaj, A. Bansal, G.D. Ritter, C. Perkins, B.H. Edwards, E. Gough, J. Tang, J.J. Szinger, B. Korber, C.M. Wilson, R.A. Kaslow, M.J. Mulligan, P.A. Goepfert, Cross-reactive CD8+ T cell epitopes identified in US adolescent minorities, J. Acquir. Immune Defic. Syndr. 33 (2003) 426–438.

[44] M. Altfeld, M.M. Addo, R.L. Eldridge, X.G. Yu, S. Thomas, A. Khatri, D. Strick, M.N. Phillips, G.B. Cohen, S.A. Islam, S.A. Kalams, C. Brander, P.J. Goulder, E.S. Rosenberg, B.D. Walker, HIV Study Collaboration, Vpr is preferentially targeted by CTL during HIV-1 infection, J. Immunol. 167 (2001) 2743–2752.

[45] M.S. Hossain, H. Tomiyama, T. Inagawa, S. Ida, S. Oka, M. Takiguchi, Identification and characterization of HLA-A*3303-restricted, HIV type 1 Pol- and Gag-derived cytotoxic T cell epitopes, AIDS Res. Hum. Retroviruses 19 (2003) 503–510.

[46] K. Falk, O. Rotzschke, M. Takiguchi, B. Grahovac, V. Gnau, S. Stevanovic, G. Jung, H.G. Rammensee, Peptide motifs of HLA-A1, -A11, -A31, and -A33 molecules, Immunogenetics 40 (1994) 238–241.

[47] Y. Ikeda-Moore, H. Tomiyama, K. Miwa, S. Oka, A. Iwamoto, Y. Kaneko, M. Takiguchi, Identification and characterization of multiple HLA-A24-restricted HIV-1 CTL epitopes: strong epitopes are derived from V regions of HIV-1, J. Immunol. 159 (1997) 6242–6252.

[48] M. Fujiwara, J. Tanuma, H. Koizumi, Y. Kawashima, K. Honda, S. Mastuoka-Aizawa, S. Dohki, S. Oka, M. Takiguchi, Different abilities of escape mutant-specific cytotoxic T cells to suppress replication of escape mutant and wild-type human immunodeficiency virus type 1 in new hosts, J. Virol. 82 (2008) 138–147.

[49] C. Brander, O.O. Yang, N.G. Jones, Y. Lee, P. Goulder, R.P. Johnson, A. Trocha, D. Colbert, C. Hay, S. Buchbinder, C.C. Bergmann, H.J. Zweerink, S. Wolinsky, W.A. Blattner, S.A. Kalams, B.D. Walker, Efficient processing of the immunodominant, HLA-A*0201-restricted human immunodeficiency virus type 1 cytotoxic T-lymphocyte epitope despite multiple variations in the epitope flanking sequences, J. Virol. 73 (1999) 10191–10198.

[50] S. Buranapraditkun, U. Hempel, P. Pitakpolrat, R.L. Allgaier, P. Thantivorasit, S.I. Lorenzen, S. Sirivichayakul, W.H. Hildebrand, M. Altfeld, C. Brander, B.D. Walker, P. Phanuphak, P. Hansasuta, S.L. Rowland-Jones, T.M. Allen, K. Ruxrungtham, A novel immunodominant CD8+ T cell response restricted by a common HLA-C allele targets a conserved region of Gag HIV-1 clade CRF01_AE infected Thais, PLoS One 6 (2011) e23603.

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Switching Tenofovir/Emtricitabine plus Lopinavir/r to Raltegravir plus Darunavir/r in Patients with Suppressed Viral Load Did Not Result in Improvement of Renal Function but Could Sustain Viral Suppression: A Randomized Multicenter Trial

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Abstract

Background: Whether tenofovir nephrotoxicity is reversible after its withdrawal is unknown. Furthermore, there are no data on the viral efficacy of raltegravir (RAL) plus ritonavir-boosted Darunavir (DRV/r) in patients with suppressed viral load.

Methods: This multicenter, randomized trial compared renal function and viral efficacy in patients with suppressed viral load treated with RAL+DRV/r and ritonavir-boosted lopinavir (LPV/r) plus tenofovir/emtricitabine (TVD), who had been previously on LPV/r+TVD. The primary endpoint was the proportion of patients with >10% improvement in estimated glomerular filtration rate (eGFR) at 48 weeks calculated with Cockcroft-Gault equation.

Results: 58 randomized and treatment-exposed patients were analyzed (28 on RAL+DRV/r and 30 on LPV/r+TVD). Greater than 10% improvement in eGFR was noted in 6 (25%) out of 24 with RAL+DRV/r and 3 (11%) of 28 with LPV/r+TVD, and the difference was not statistically significant (p=0.272, 95% CI -0.067 to 0.354). Sensitivity analyses using three other equations for eGFR showed the same results. Urinary β2 microglobulin, a sensitive marker of tenofovir tubulopathy, significantly improved with RAL+DRV/r than with LPV/r+TVD (-271 versus -64 μg/gCr, p=0.026). Per protocol analysis showed that the HIV-RNA was <50 copies/mL at week 48 in all patients of both arms (24 in RAL+DRV and 29 in LPV/r+TVD).

Conclusions: Switching LPV/r+TVD to RAL+DRV/r did not significantly increase the proportion of patients who showed >10% improvement in renal function among those with relatively preserved eGFR. However, the switch improved urinary β2 microglobulin, suggesting that discontinuation of TDF might be beneficial in the long-term. RAL +DRV/r showed favorable viral efficacy in patients with suppressed viral load.

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Introduction

Tenofovir disoproxil fumarate (TDF) is one of the most widely used nucleotide reverse transcriptase inhibitors (NRTI) for patients with HIV infection, with proven efficacy and safety [1-6]. However, tenofovir is excreted by both glomerular filtration and tubular secretion, and is known to cause renal proximal tubular dysfunction. Moreover, long-term TDF use reduces glomerular filtration rate more than other NRTIs [7-10]. Although the mechanism of tenofovir-induced kidney damage is not fully understood, mitochondria toxicity, a well-known adverse event of NRTIs [11,12], in the proximal renal tubular cells is considered to be the main mechanism [13,14]. In addition to renal dysfunction, TDF also reduces bone mineral density, and both complications might lead to serious outcomes with long-term use of TDF [9,15-19]. The concurrent use of ritonavir-boosted protease inhibitors (PI/r) is a risk factor for TDF-associated nephrotoxicity, since PI/r modifies tenofovir clearance and thus increases the severity of tenofovir nephrotoxicity [20,21].

Clinical manifestations such as lipoatrophy and neuropathy caused by NRTI-induced mitochondria toxicity are difficult to reverse [22,23], but whether TDF nephrotoxicity is reversible after discontinuation of TDF remains unknown at present. Unfortunately, the results of few small studies that have examined this issue are contradictory [24-26]. Of note, there is no randomized controlled study that has examined the reversibility of TDF-associated nephrotoxicity.

Recently, antiretroviral therapy (ART) not containing NRTIs (NRTI sparing regimens) has gained a wide attention, since these combinations can avoid NRTI toxicity. Despite high expectations, the results of studies on the efficacy and safety of NRTI sparing regimens for treatment-naïve patients showed dismal results. A small single arm study of CCR5 inhibitor maraviroc plus ritonavir-boosted Darunavir (DRV/r) showed a high rate of virologic failure, especially in patients with high baseline viral load of >100,000 copies/mL [27]. Raltegravir (RAL) plus unboosted atazanavir in a small randomized trial showed frequent grade 4 hyperbilirubinemia and emergence of raltegravir resistance [28]. Even the combination of RAL, a well-tolerated integrase inhibitor, and DRV/r, a protease inhibitor with high barrier to drug resistance and favorable lipid profile [29,30], showed a high prevalence of virological failure for patients with high baseline viral load in a single arm study [31].

At this stage, it is important to elucidate the effectiveness of NRTI sparing regimen for patients with suppressed HIV-1 viral load, because longer exposure with NRTIs tends to result in clinically overt NRTI-associated mitochondrial toxicity [22,32], and NRTI sparing regimens may avoid such long-term NRTI toxicity. Of note, the viral efficacy of NRTI-sparing regimen of RAL plus DRV/r has not been evaluated in patients with suppressed viral load [31].

Based on the above background, this multicenter randomized trial was conducted to elucidate 1) the reversibility of tenofovir nephrotoxicity, and 2) efficacy and safety of RAL +DRV/r for patients with suppressed viral load.

Methods

This clinical trial was designed and reported according to the recommendations of the Consolidated Standard of Reporting Trials (CONSORT) statement [33]. The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1 and protocol S1.

Ethics Statement

The Research Ethics Committees of Hokkaido University Hospital, Higashisaitama National Hospital, Niigata University Medical and Dental Hospital, the Institute of Medical Science, the University of Tokyo, Juntendo University School of Medicine, Shirakaba Clinic, Saku Central Hospital, Hiroshima University Hospital, Ehime University Hospital, National Hospital Organization Kyushu Medical Center, Kumamoto University Graduate School of Medical Sciences and National Center for Global Health and Medicine approved the study protocol. All patients enrolled in this study provided a written informed consent. The study was conducted according to the principles expressed in the Declaration of Helsinki.

Study Design

The SPARE trial is an on-going phase 3B, multi-center, randomized, open-label, parallel group study conducted in Japan to compare renal function and viral efficacy of NRTI-sparing regimen of RAL+DRV/r and a standard regimen of PI/r + 2NRTIs [(lopinavir/ritonavir (LPV/r) plus fixed dose of tenofovir/emtricitabine (TVD)] for 96 weeks, randomly allocated to patients on LPV/r+TVD with suppressed viral load. With one to one ratio, patients with suppressed viral load on LPV/r (800 mg/200 mg) plus fixed dose of TDF (300 mg)/emtricitabine (200 mg) were randomly assigned to either RAL (800 mg) plus DRV/r (800 mg/100 mg) or to continue LPV/r+TVD. Patient enrollment remained open between February 21, 2011 and December 2011, and the follow-up period is scheduled to end in December 2013. This report summarizes the findings after 48 weeks of treatment, including the primary endpoint.

Randomization was stratified based on baseline body weight of 60 kg because low body weight, especially body weight of <60 kg, is an important risk for tenofovir nephrotoxicity [4,18,34]. Randomization was conducted at the data center with independent data managers, using a computer-generated randomization list prepared by a statistician with no clinical involvement in the trial.

Study Patients

The study population included Japanese patients with HIV-1 infection, aged ≥20 years, who were on LPV/r plus TVD and with suppressed HIV-1 RNA viral load of <50 copies/ml over a period of more than 15 weeks. Patients were screened and excluded if found positive for hepatitis B surface antigen, or had history of virologic failure with regimens including protease inhibitor or integrase inhibitor, or if they were considered inappropriate for the study by the attending physicians. Candidates were also excluded if the level of alanine aminotransferase was 2.5 times the upper limit of normal, estimated glomerular filtration rate (eGFR) calculated by Cockcroft-Gault equation (CG equation) was <60 ml/min, {[(140 - age) × weight (kg)] / (serum creatinine × 72)] (× 0.85 for females)} [35], or on treatment for opportunistic infection. Actual body weight was used for the calculation of eGFR. Patients who provided written informed consent started the allocated regimens within 4 weeks of enrollment.

Study Procedure

Visits for clinical and laboratory assessments were required within 15 weeks before registration for screening, at registration, and every 12 weeks for the duration of the study. Patients of the RAL+DRV/r arm were required to visit within 4 weeks after commencement of the allocated regimen to screen for adverse events. Baseline evaluation and evaluations at each visit covered medical history, including history of AIDS-defining illness and other comorbidities, concurrent medications, concurrent smoking, physical examination, CD4 cell count, HIV-1 RNA viral load, complete blood cell count, blood chemistries (albumin, total bilirubin, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, alkaline phosphatase, creatine kinase, blood urea nitrogen, serum creatinine, sodium, potassium, calcium, phosphate, triglyceride, total cholesterol, low-density lipoprotein cholesterol, high density lipoprotein cholesterol, glucose), and urine examination (urine dipstick, phosphate, creatinine, β2 microglobulin, N-acetyl-β-D-glucosaminidase (NAG), and albumin). The values of urinary β2 microglobulin, NAG, and albumin were expressed relative to urinary creatinine of 1 g/L (/g Cr). Percent tubular resorption of phosphate was calculated by the following formula: {1 – [(urine phosphate × serum creatinine) / (urine creatinine × serum phosphate)]} × 100 [36]. All data, including HIV-1 RNA viral load, were collected at each participating site and then transferred to a central data center. Grade 3 or 4 serious adverse events were reported to the independent data and safety monitoring board and analyzed for their relation to the study drugs. The grade of adverse events was classified according to the Division of AIDS Table for grading the severity of adult and pediatric events, version 2004 (URL:http://www.mtnstopshiv.org/sites/default/files/attachments/Table_for_Grading_Severity_of_Adult_Pediatric_Adverse_Events.pdf). Independent monitors visited all facilities to conduct source document verification to ensure the accuracy of all submitted data by week 48 and compliance to the protocol. All authors participated in the trial design, data analysis, and preparation of the manuscript, and vouch for the completeness and accuracy of the presented data.

Statistical Analysis

The tested hypothesis was that more patients in the RAL+DRV/r arm will experience >10% improvement in eGFR from the baseline than patients in the LPV/r+TVD arm after switching from LPV/r+TVD to RAL+DRV/r. Sample size calculation was based on the assumption that 50% of the patients of the RAL+DRV/r arm and 10% of the patients of the LPV/r + TVD arm will experience >10% improvement in eGFR from the baseline to week 48. With a 2-sided alpha level of 0.05 and 80% power, the estimated population sample required in this study was 50 patients (25 per single arm). To account for dropouts, we planned to enroll 27 patients per one arm. The study was not fully powered for secondary analysis. Per protocol population while on the initial randomized regimen was used for the analysis of the primary endpoint.

The primary endpoint was the proportion of patients with >10% improvement in eGFR at 48 weeks from the baseline calculated with the CG equation [35]. The baseline eGFR was estimated from the average of serum creatinine measured at baseline and at screening for enrollment. eGFR at week 48 was estimated from the average of serum creatinine at weeks 36 and 48. The proportion of such patients was compared between the two arms by the Fisher exact test. The following three equations for eGFR were also used for sensitivity analysis: 1) A 3-variable equation for the Japanese set by the Japanese Society of Nephrology (JSN equation): [194 × (serum creatinine)$^{-1.094}$× (age)$^{-0.287}$× (0.739 for female patients)][37], 2) the Modification of Diet in Renal Disease (MDRD) equation adjusted with coefficient for the Japanese [0.808 × 175 × (serum creatinine)$^{-1.154}$ × (age)$^{-0.203}$× (0.742 for female patients)] [37], and 3) Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation adjusted for the Japanese [0.813 × 141 × min (serum creatinine/κ, 1)$^{\alpha}$× max (serum creatinine/κ, 1) $^{-1.209}$ × (0.993)^{age}× (1.018 for females)] (where κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, *min* represents the minimum of serum creatinine/κ or 1, and *max* is the maximum of serum creatinine/κ or 1) [38]. Furthermore, the percent improvement in eGFR from baseline to week 48, calculated with all four equations described above, was compared between the two arms by the Student's t-test. Because the percent improvement in eGFR may depend on the baseline value, a correlation between the percent improvement in eGFR and the baseline value was tested, and the results showed very weak correlation (0.001<r<0.2) for all four equations for eGFR. Accordingly, the comparison of the percent improvement was conducted by the t-test as described above.

The secondary renal endpoint was changes in per protocol renal tubular markers from the baseline to week 48, and the results were compared by the Mann-Whitney test. The secondary efficacy endpoint was the proportions of patients with HIV-1 RNA <50 copies/mL at weeks 24 and 48. Data of both per protocol population and the intent-to-treat (ITT) population, comprising all randomized treatment-exposed subjects were used for the assessment of efficacy. With regard to analysis on the viral efficacy in this study, per protocol analyses were more important than ITT analyses, because some patients enrolled in the RAL+DRV/r arm were expected to develop adverse events due to switching to the new medications and subsequent discontinuation of the allocated regimen, whereas new adverse events were not likely in patients of the LPV/r+TVD arm solely by continuing the same regimen as before. Baseline parameters were compared between the two arms by the Student's t-test for continuous variables and by either the χ^2 test or Fisher's exact test for categorical variables. Statistical significance was defined at two-sided p values <0.05. All statistical analyses were performed with The Statistical Package for Social Sciences ver. 21.0 (SPSS, Chicago, IL).

Results

Patient disposition and baseline characteristics

Between February and December of 2011, 59 patients from 11 centers were enrolled in the study and randomized. Of these, 29 and 30 patients were allocated to the RAL+DRV/r and the LPV/r+TVD arm, respectively (Figure 1). One patient in the RAL+DRV/r arm withdrew consent before starting the allocated regimen, thus was excluded from the analysis. The baseline demographics and characteristics of the participating patients are listed in Table 1. Most patients were men who have sex with men, with well-maintained CD4 count. Patients of the LPV/r+TVD arm were younger (p=0.040) and had lower CD4 count (p=0.029) than those of the RAL+DRVr arm. All other major variables were similar between the two arms.

Table 1. Baseline characteristics of the enrolled patients.

	RAL+DRV/r (n=28)	LPV/r+TVD (n=30)	P value
Sex (male), n (%)	28 (100)	29 (97)	1.000
Age (years)†	44 (37-51)	39 (34-45)	0.040
CD4 count (/μl)†	549 (384-710)	456 (330-592)	0.029
Route of transmission (homosexual contact), n (%)	27 (96)	24 (80)	0.151
History of AIDS, n (%)	10 (36)	11 (37)	1.000
Body weight (kg)†	66 (59-75)	66 (59-72)	0.502
Body mass index (kg/m^2)†	22 (21-25)	22.6 (19.9-24.6)	0.440
eGFR by JSN equation (ml/min/1.73 m^2)†	87 (76-103)	85 (70-90)	0.356
eGFR by CG equation (ml/min)†	119 (88-143)	108 (89-120)	0.456
Serum creatinine (mg/dl)†	0.78 (0.70-0.87)	0.76 (0.67-0.83)	0.184
Urinary albumin (mg/g Cre)†	8 (6-27)	7 (5-12)	0.075
Urinary β2 microglobulin (μg/g Cre)†	452 (178-1566)	424 (204-2275)	0.234
Tubular resorption of phosphate (%)†	92 (87-93)	90 (86-94)	0.886
NAG (U/g Cr)†	6.2 (3.7-11.6)	5.2 (3.7-8.3)	0.183
Hypertension, n (%)	2 (7)	1 (3)	0.605
Dyslipidemia, n (%)	17 (61)	8 (27)	0.016
Diabetes mellitus, n (%)	0 (0)	1 (3)	1.000
Current smoking, n (%)	13 (46)	13 (43)	1.000
Hepatitis C, n (%)	0 (0)	0 (0)	N/A
Duration of tenofovir use (weeks)	163 (109-224)	124 (85-212)	0.721

Hypertension was defined by current treatment with antihypertensive agents or systolic blood pressure >140 mmHg or diastolic blood pressure >90 mmHg. Dyslipidemia was defined by current treatment with lipid-lowering agents or low-density lipoprotein cholesterol >140 mg/dl, high-density lipoprotein cholesterol <40 mg/dl, total cholesterol >240 mg/dl, or triglyceride >500 mg/dl. IQR: interquartile range, AIDS: acquired immunodeficiency syndrome, eGFR: estimated glomerular filtration rate, LDL: low-density lipoprotein, JSN: the Japanese Society of Nephrology equation [37], CG: Cockcroft-Gault equation [35]

† median (interquartile range)

Primary endpoint

At week 48, six patients (25%) out of 24 in the RAL+DRV/r arm and 3 patients (11%) out of 28 in the LPV/r+TVD arm, experienced >10% improvement in eGFR from baseline, and the difference was not statistically significant (p=0.272, 95% CI -0.067 to 0.354). Sensitivity analysis with three other equations for eGFR (JSN, CKD-EPI, and MDRD) showed the same results; no difference in the proportion of patients with improvement of >10% in eGFR was noted between the two arms (JSN equation: 4/24 in RAL+DRV/r, 3/29 in LPV/r+TVD, p=0.688, 95% CI -0.126 to 0.267) (CKD-EPI equation: 2/24 in RAL+DRV/r, 2/29 in LPV/r+TVD, p=1.000, 95% CI -0.148 to 0.197) (MDRD equation: 5/24 in RAL+DRV/r, 3/29 in LPV/r +TVD, p=0.444, 95% CI -0.093 to 0.313) (Table 2).

Additional analysis showed that the percent improvement in eGFR from the baseline to week 48 calculated using all four equations was not significantly different between the two arms [CG equation: difference in mean % improvement (DRV/r+RAL versus LPV/r+TDF/FTC) -8.7%, 95% CI -18.2 to 0.8, p=0.071] (JSN equation: -1.1%, -6.9 to 4.8, p=0.720) (CKD-EPI equation: -1.6%, 95% CI -4.7 to 1.6, p=0.323) (MDRD equation: -1.1%, 95% CI -6.9 to 4.8, p=0.722) (Table 2). Thus, this study demonstrated that switching to NRTI-sparing regimen of RAL+DRV/r did not increase the proportion of patients who showed >10% improvement in eGFR, compared to continuation of LPV/r+TVD.

Secondary renal endpoints

Among the four renal tubular markers used in this study, the improvement in urinary β2 microglobulin from baseline to week 48 was significantly larger in the RAL+DRV/r arm (n=23) than

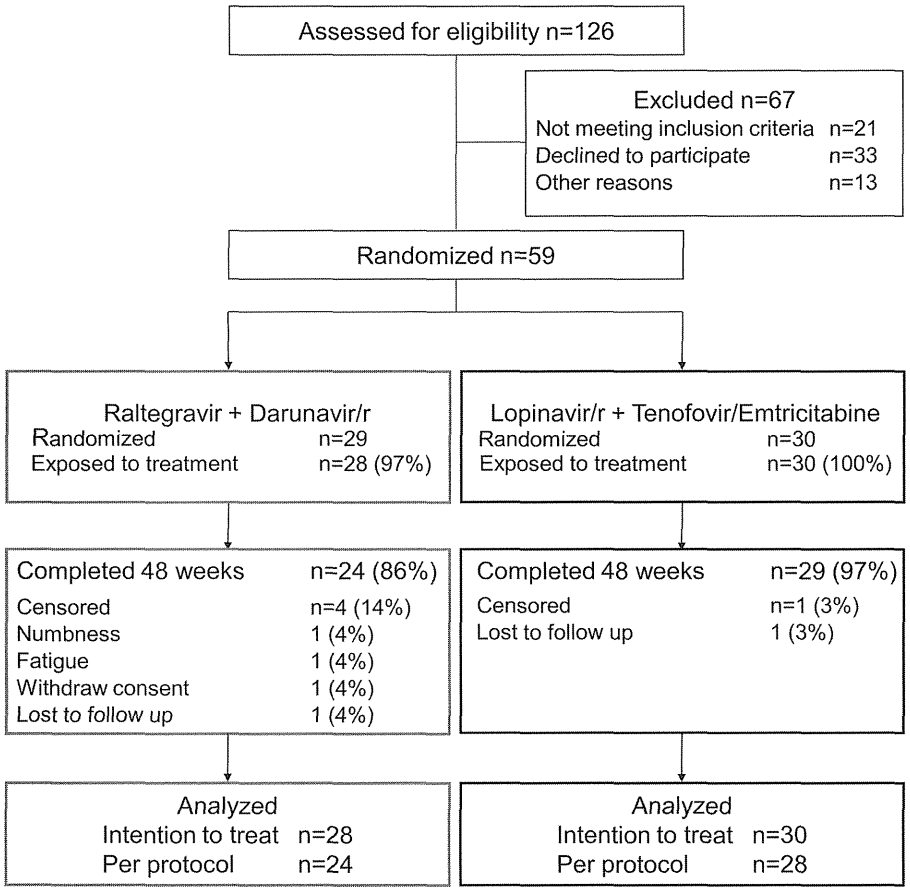

Figure 1. Enrollment, randomization, and disposition of patients. Darunavir/r, ritonavir-boosted darunavir; Lopinavir/r, ritonavir-boosted lopinavir.

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in the LPV/r+TVD arm (n=28) (-271 versus -64 μg/g Cr, p=0.026) (Figure 2A). However, urinary albumin, the percent tubular resorption of phosphate, and NAG showed little change from baseline, and the observed changes were not significantly different between the two arms (Figure 2B, C, D).

Secondary efficacy endpoints

Among the per protocol population, the proportion of patients with HIV RNA <50 copies/mL was 96.2% for the RAL+DRV/r arm and 96.7% for the LPV/r+TVD arm at week 24, with a difference of -0.5% (95% CI, -10% to 9%), and 100% for the both arms at week 48, with a difference of 0% (95% CI -0.1 to 0.1) (Figure 3A). ITT analysis showed that the proportion was 89.3% and 96.7% for the RAL+DRV/r and LPV/r+TVD arms, respectively, at week 24, with a difference of -7% (95% CI, -21% to 6%), and 85.7% and 96.7%, respectively, at week 48, with a difference of -11% (95% CI, -25% to 4%) (Figure 3B). There was no significant difference in viral efficacy between the

Table 2. Proportion of patients with >10% and mean percent improvement in eGFR at 48 weeks from the baseline calculated by the four equations.

	Cases with >10% increase from baseline	P value (95% CI)	Mean % improvement in eGFR from baseline	Difference in mean % improvement (95% CI) (DRV/r + RAL versus LPV/r + TDF/FTC)	P value
CG equation					
DRV/r + RAL	6/24	0.272 (-0.067 to 0.354)	5.4%	-8.7% (-18.2 to 0.8)	0.071
LPV/r + TDF/FTC	3/28		-3.3%		
JSN equation					
DRV/r + RAL	4/24	0.688 (-0.126 to 0.267)	2.5%	-1.1% (-6.9 to 4.8)	0.720
LPV/r + TDF/FTC	3/29		1.5%		
CKD-EPI equation					
DRV/r + RAL	2/24	1.000 (-0.148 to 0.197)	1.9%	-1.6% (-4.7 to 1.6)	0.323
LPV/r + TDF/FTC	2/29		1.7%		
MDRD equation					
DRV/r + RAL	5/24	0.444 (-0.093 to 0.313)	2.7%	-1.1% (-6.9 to 4.8)	0.722
LPV/r + TDF/FTC	3/29		1.7%		

DRV/r: ritonavir-boosted darunavir, RAL: raltegravir, LPV/r: ritonavir-boosted lopinavir, TDF: tenofovir, FTC: emtricitabine, CG: Cockcroft-Gault equation [35], JSN: the Japanese Society of Nephrology equation [37], CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation adjusted for the Japanese[38], MDRD: the Modification of Diet in Renal Disease equation adjusted with coefficient for the Japanese [37]

two arms at weeks 24 and 48. At week 48, all patients of the RAL+DRV/r arm on the allocated regimen (n=24) had a viral load of <50 copies/mL.

Safety and tolerability

One patient from each arm was lost to follow-up. Three patients of the RAL+DRV/r arm discontinued the allocated regimen by week 48 (one discontinued the regimen at week 4 due to weakness in the lower extremities and one at week 24 because of fatigue, which was later found to be related to acute hepatitis B infection). The other patient withdrew consent at week 24, because it was easier for him to maintain a good medication adherence with once-daily LPV/r+TVD (the regimen the patient used before enrollment). None of the patients of the LPV/r+TVD arm discontinued the allocated regimen by week 48. Thus, at week 48, 24 patients (86%) out of 28 in the RAL +DRV/r arm and 29 (97%) of 30 in the LPV/r+TVD arm, were on the allocated regimens.

The following grade 3 or 4 laboratory data or abnormal symptoms that were at least one grade higher than the baseline were encountered in this study: RAL+DRV/r arm: a rise in ALT (due to acute hepatitis B infection, n=1), and elevated LDL-cholesterol (n=3), LPV/r+TVD arm: elevated LDL-cholesterol (n=1), and hypophosphatemia (n=3). The above side effects did not lead to discontinuation of the study drugs.

Discussion

This randomized trial elucidated the recovery of TDF-associated nephropathy after discontinuation of TDF. The results demonstrated no significant increase in the proportion of patients who showed >10% improvement in eGFR after switching to NRTI sparing regimen of RAL+DRV/r, compared to continuation of LPV/r+TVD. This finding could be due to any of the following reasons; 1) Relatively preserved baseline renal function of the enrolled patients, with a median eGFR of 86 ml/min/1.73 m^2 (IQR 75-97, JSN equation), with only one patient with CKD stage 3 due to persistent +1 proteinuria, and no patients with stage 4 or more. Although the number of patients is relatively small, a previous pilot study of 21 patients reported improvement of eGFR (by CG equation) in most patients after switching from PI/r+TVD to PI/r+RAL in patients with proteinuria and suppressed HIV viral load [39]. Thus, improvement of eGFR after discontinuation of TDF might be more significant in patients with severe to moderately impaired renal function. Larger studies are needed to investigate this issue thoroughly. 2) Study patients had been on TDF for a long period of time at enrollment (median: 136 weeks, range 27-370 weeks, 72% were on TDF for more than 2 years), although shorter duration of TDF therapy is likely to be associated with greater eGFR improvement after discontinuation [26]. Furthermore, because TDF-induced renal dysfunction is mainly observed during the first 6 months after commencement of such therapy [18,19,40], it is possible that patients who developed severe renal dysfunction soon after starting TDF might have already discontinued TDF and therefore not included in the study.

Although the present study did not show an increase in eGFR after discontinuation of TDF, it is noteworthy that the value of urinary β2 microglobulin, a sensitive marker for TDF-induced tubulopathy [41,42], improved significantly in the RAL +DRV/r arm compared to LPV/r+TVD, even in patients with relatively preserved eGFR. It is of importance considering that

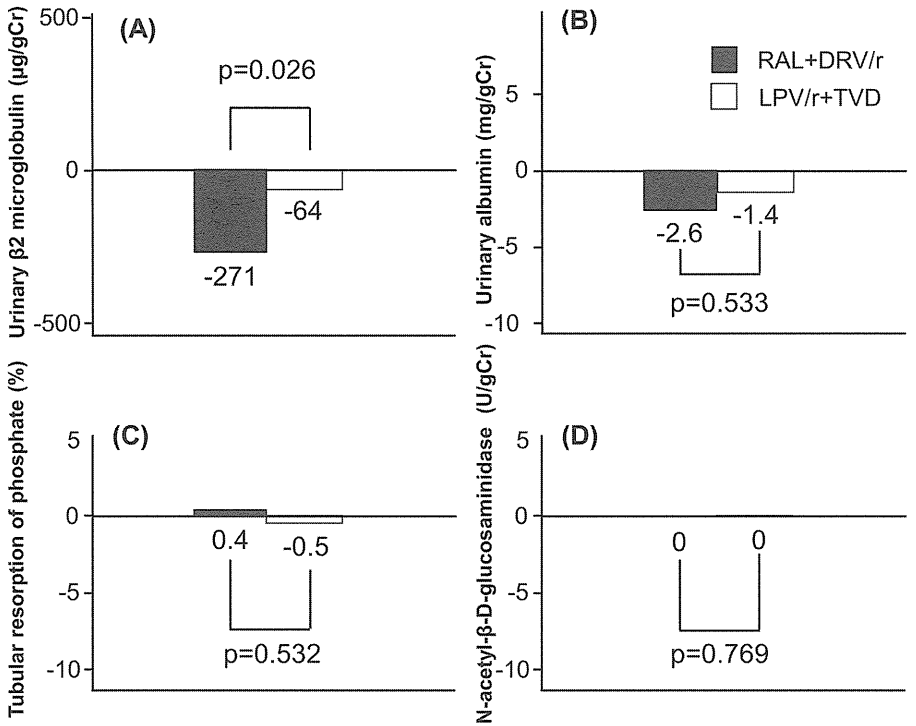

Figure 2. Median changes in markers of renal tubular function between baseline and 48 weeks. (A) Urinary β2 microglobulin, (B) Urinary albumin, (C) Percent tubular resorption of phosphate, (D) Urinary N-acetyl-β-D-glucosaminidase. RAL, raltegravir; DRV/r, ritonavir-boosted darunavir; LPV/r, ritonavir-boosted lopinavir; TVD, fixed dose of tenofovir/emtricitabine.

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proximal tubulopathy is associated with bone mineral density abnormality and possible long-term nephrotoxic effect [17,43-45]. Further large and long-term studies are needed to elucidate the long-term impact of TDF-induced tubulopathy on GFR.

With regard to the viral efficacy and safety of RAL+DRV/r, all patients in that arm who continued the allocated regimen accomplished viral suppression of <50 copies/ml at week 48 (n=24). Only one (3.6%) patient discontinued RAL+DRV/r due to a side effect possibly related to RAL+DRV/r (weakness of the lower extremities), confirming the safety of this combination. To our knowledge, this is the first study to examine the viral efficacy of RAL+DRV/r in patients with suppressed viral load. The KITE study, an industry-sponsored pilot study, examined the viral efficacy of RAL+LPV/r in patients with suppressed viral load [46]. However, LPV/r is placed as an alternative PI in the American Department of Health and Human Services Guidelines, mainly because of the higher rates of gastrointestinal side effects and hyperlipidemia compared with other PIs (URL: http://aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf). Because the number of enrolled patients is relatively small and this study does not have sufficient power to elucidate viral efficacy, further studies are needed to confirm the viral efficacy of RAL+DRV/r in patients with suppressed viral load. If the NRTI sparing regimen of RAL+DRV/r is proved to be efficacious in maintaining viral suppression in treatment-experienced patients, switching to this combination for patients with suppressed viral load should become an attractive treatment option for patients who cannot tolerate NRTI toxicity or to prevent further NRTI toxicity.

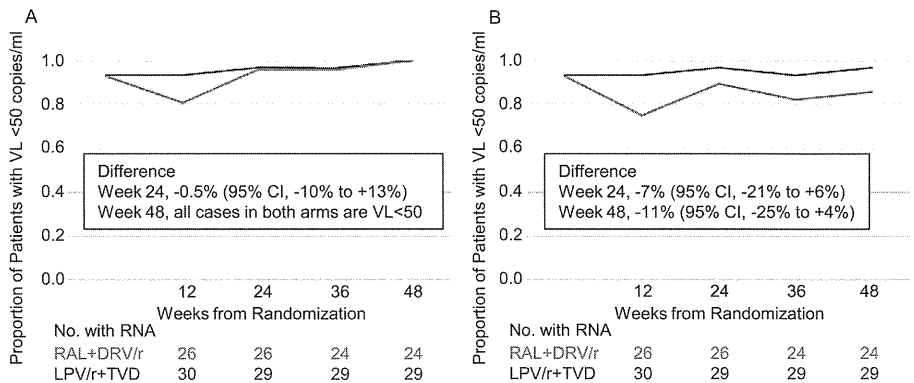

Figure 3. Proportion of patients with HIV RNA <50 copies/ml at 24 and 48 weeks. (A) Per protocol analysis. (B) Intention-to-treat analysis. VL, viral load; RAL, raltegravir; DRV/r, ritonavir-boosted darunavir; LPV/r, ritonavir-boosted lopinavir; TVD, fixed dose of tenofovir/emtricitabine.

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Several limitations must be acknowledged. First, as mentioned above, this trial has sufficient power for the primary endpoint only; other results should be interpreted with caution. Further larger studies are needed to confirm the improvement in urinary β2 microglobulin after switching ritonavir-boosted PI to NRTI sparing regimen of RAL+DRV/r and the viral efficacy of RAL+DRV/r in patients with suppressed viral load. Second, the enrolled patients had relatively preserved renal function. This was a study-design related issue; patients with severely impaired eGFR, the population in whom TDF nephrotoxicity can be reversible is clinically important, were excluded from the study. Based on the study design and need for randomization, patients of one arm needed to continue treatment with TDF, and it was considered ethically inappropriate to have patients with impaired renal function to continue TDF. Third, all study subjects were Japanese and almost exclusively men (mostly men who have sex with men). Further studies are needed to determine whether the findings of this study are also applicable to females, patients with different routes of transmission, and patients of different racial background.

In conclusion, this trial showed that discontinuation of LPV/r +TVD and switching to NRTI-sparing regimen of RAL+DRV/r did not result in improvement of renal function among patients with relatively preserved eGFR and suppressed HIV viral load. However, urinary β2 microglobulin, a sensitive marker of TDF-induced tubulopathy, improved after discontinuation of TDF plus ritonavir-boosted PI, suggesting switching TDF to NRTI sparing regimen might be beneficial in the long-term. RAL +DRV/r showed favorable viral efficacy and safety in patients with suppressed viral load, but further larger studies are needed to confirm the viral efficacy of this combination.

Supporting Information

Protocol S1. Trial protocol.
(DOCX)

Checklist S1. CONSORT checklist.
(DOC)

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References

1. Gallant JE, DeJesus E, Arribas JR, Pozniak AL, Gazzard B et al. (2006) Tenofovir DF, emtricitabine, and efavirenz vs. zidovudine, lamivudine, and efavirenz for HIV. N Engl J Med 354: 251-260. doi: 10.1056/NEJMoa051871. PubMed: 16421366.
2. Gallant JE, Staszewski S, Pozniak AL, DeJesus E, Suleiman JM et al. (2004) Efficacy and safety of tenofovir DF vs stavudine in combination therapy in antiretroviral-naive patients: a 3-year randomized trial. JAMA 292: 191-201. doi:10.1001/jama.292.2.191. PubMed: 15249568.
3. Izzedine H, Hulot JS, Vittecoq D, Gallant JE, Staszewski S et al. (2005) Long-term renal safety of tenofovir disoproxil fumarate in antiretroviral-naive HIV-1-infected patients. Data from a double-blind randomized active-controlled multicentre study. Nephrol Dial Transplant 20: 743-746. doi:10.1093/ndt/gfh658. PubMed: 15741212.
4. Nelson MR, Katlama C, Montaner JS, Cooper DA, Gazzard B et al. (2007) The safety of tenofovir disoproxil fumarate for the treatment of HIV infection in adults: the first 4 years. AIDS 21: 1273-1281. doi: 10.1097/QAD.0b013e3280b07b33. PubMed: 17545703.
5. Arribas JR, Pozniak AL, Gallant JE, Dejesus E, Gazzard B et al. (2008) Tenofovir disoproxil fumarate, emtricitabine, and efavirenz compared with zidovudine/lamivudine and efavirenz in treatment-naive patients: 144-week analysis. J Acquir Immune Defic Syndr 47: 74-78. doi: 10.1097/QAI.0b013e31815acab8. PubMed: 17971715.
6. Post FA, Moyle GJ, Stellbrink HJ, Domingo P, Podzamczer D et al. (2010) Randomized comparison of renal effects, efficacy, and safety with once-daily abacavir/lamivudine versus tenofovir/emtricitabine, administered with efavirenz, in antiretroviral-naive, HIV-1-infected adults: 48-week results from the ASSERT study. J Acquir Immune Defic Syndr 55: 49-57. doi:10.1097/QAI.0b013e3181f9c0f7. PubMed: 20431394.
7. Kinai E, Hanabusa H (2009) Progressive renal tubular dysfunction associated with long-term use of tenofovir DF. AIDS Res Hum Retroviruses 25: 387-394. doi:10.1089/aid.2008.0202. PubMed: 19361280.
8. Winston A, Amin J, Mallon P, Marriott D, Carr A et al. (2006) Minor changes in calculated creatinine clearance and anion-gap are associated with tenofovir disoproxil fumarate-containing highly active antiretroviral therapy. HIV Med 7: 105-111. doi:10.1111/j.1468-1293.2006.00349.x. PubMed: 16420255.
9. Gallant JE, Winston JA, DeJesus E, Pozniak AL, Chen SS et al. (2008) The 3-year renal safety of a tenofovir disoproxil fumarate vs. a thymidine analogue-containing regimen in antiretroviral-naive patients. AIDS 22: 2155-2163. doi:10.1097/QAD.0b013e3283112b8e. PubMed: 18832879.
10. Fux CA, Simcock M, Wolbers M, Bucher HC, Hirschel B et al. (2007) Tenofovir use is associated with a reduction in calculated glomerular filtration rates in the Swiss HIV Cohort Study. Antivir Ther 12: 1165-1173. PubMed: 18240857.
11. Nolan D, Mallal S (2004) Complications associated with NRTI therapy: update on clinical features and possible pathogenic mechanisms. Antivir Ther 9: 849-863. PubMed: 15651744.
12. Lewis W, Day BJ, Copeland WC (2003) Mitochondrial toxicity of NRTI antiviral drugs: an integrated cellular perspective. Nat Rev Drug Discov 2: 812-822. doi:10.1038/nrd1201. PubMed: 14526384.
13. Kohler JJ, Hosseini SH, Hoying-Brandt A, Green E, Johnson DM et al. (2009) Tenofovir renal toxicity targets mitochondria of renal proximal tubules. Lab Invest 89: 513-519. doi:10.1038/labinvest.2009.14. PubMed: 19274046.
14. Herlitz LC, Mohan S, Stokes MB, Radhakrishnan J, D'Agati VD et al. (2010) Tenofovir nephrotoxicity: acute tubular necrosis with distinctive clinical, pathological, and mitochondrial abnormalities. Kidney Int 78: 1171-1177. doi:10.1038/ki.2010.318. PubMed: 20811330.
15. Peyrière H, Reynes J, Rouanet I, Daniel N, de Boever CM et al. (2004) Renal tubular dysfunction associated with tenofovir therapy: report of 7 cases. J Acquir Immune Defic Syndr 35: 269-273. doi: 10.1097/00126334-200403010-00007. PubMed: 15076241.
16. McComsey GA, Kitch D, Daar ES, Tierney C, Jahed NC et al. (2011) Bone mineral density and fractures in antiretroviral-naive persons randomized to receive abacavir-lamivudine or tenofovir disoproxil fumarate-emtricitabine along with efavirenz or atazanavir-ritonavir: Aids Clinical Trials Group A5224s, a substudy of ACTG A5202. J Infect Dis 203: 1791-1801. doi:10.1093/infdis/jir188. PubMed: 21606537.
17. Fux CA, Rauch A, Simcock M, Bucher HC, Hirschel B et al. (2008) Tenofovir use is associated with an increase in serum alkaline phosphatase in the Swiss HIV Cohort Study. Antivir Ther 13: 1077-1082. PubMed: 19195333.
18. Nishijima T, Gatanaga H, Komatsu H, Tsukada K, Shimbo T et al. (2012) Renal function declines more in tenofovir- than abacavir-based antiretroviral therapy in low-body weight treatment-naïve patients with HIV infection. PLOS ONE 7: e29977. doi:10.1371/journal.pone.0029977. PubMed: 22242194.
19. Cooper RD, Wiebe N, Smith N, Keiser P, Naicker S et al. (2010) Systematic review and meta-analysis: renal safety of tenofovir disoproxil fumarate in HIV-infected patients. Clin Infect Dis 51: 496-505. doi:10.1086/655681. PubMed: 20673002.
20. Kiser JJ, Carten ML, Aquilante CL, Anderson PL, Wolfe P et al. (2008) The effect of lopinavir/ritonavir on the renal clearance of tenofovir in HIV-infected patients. Clin Pharmacol Ther 83: 265-272. doi:10.1038/sj.clpt.6100269. PubMed: 17597712.
21. Goicoechea M, Liu S, Best B, Sun S, Jain S et al. (2008) Greater tenofovir-associated renal function decline with protease inhibitor-based versus nonnucleoside reverse-transcriptase inhibitor-based therapy. J Infect Dis 197: 102-108. doi:10.1086/524061. PubMed: 18171292.
22. Tressler R, Godfrey C (2012) NRTI backbone in HIV treatment: will it remain relevant? Drugs 72: 2051-2062. doi: 10.2165/11640830-000000000-00000. PubMed: 23083109.
23. Moyle G (2000) Clinical manifestations and management of antiretroviral nucleoside analog-related mitochondrial toxicity. Clin Ther 22: 911-936; discussion 898 doi:10.1016/S0149-2918(00)80064-8. PubMed: 10972629.
24. Scherzer R, Estrella M, Li Y, Choi AI, Deeks SG et al. (2012) Association of tenofovir exposure with kidney disease risk in HIV infection. AIDS 26: 867-875. doi:10.1097/QAD.0b013e328351f68f. PubMed: 22313955.
25. Bonjoch A, Echeverría P, Perez-Alvarez N, Puig J, Estany C et al. (2012) High rate of reversibility of renal damage in a cohort of HIV-infected patients receiving tenofovir-containing antiretroviral therapy. Antiviral Res 96: 65-69. doi:10.1016/j.antiviral.2012.07.009. PubMed: 22892171.
26. Wever K, van Agtmael MA, Carr A (2010) Incomplete reversibility of tenofovir-related renal toxicity in HIV-infected men. J Acquir Immune Defic Syndr 55: 78-81. doi:10.1097/QAI.0b013e3181d05579. PubMed: 20173649.
27. Taiwo B, Swindells S, Berzins B et al. Week 48 results of the Maraviroc Plus Darunavir/ritonavir Study (MIDAS) for treatment-naive patients infected with R5-tropic HIV-1 [abstract TUPE099]. Presented at: 19th International AIDS Conference; 2012; Washington DC, USA.
28. Kozal MJ, Lupo S, DeJesus E, Molina JM, McDonald C et al. (2012) A nucleoside- and ritonavir-sparing regimen containing atazanavir plus raltegravir in antiretroviral treatment-naïve HIV-infected patients: SPARTAN study results. HIV Clin Trials 13: 119-130. doi:10.1310/hct1303-119. PubMed: 22592092.
29. Clotet B, Bellos N, Molina JM, Cooper D, Goffard JC et al. (2007) Efficacy and safety of darunavir-ritonavir at week 48 in treatment-experienced patients with HIV-1 infection in POWER 1 and 2: a pooled subgroup analysis of data from two randomised trials. Lancet 369: 1169-1178. doi:10.1016/S0140-6736(07)60497-8. PubMed: 17416261.
30. Madruga JV, Berger D, McMurchie M, Suter F, Banhegyi D et al. (2007) Efficacy and safety of darunavir-ritonavir compared with that of lopinavir-ritonavir at 48 weeks in treatment-experienced, HIV-infected patients in TITAN: a randomised controlled phase III trial. Lancet 370: 49-58. doi:10.1016/S0140-6736(07)61049-6. PubMed: 17617272.
31. Taiwo B, Zheng L, Gallien S, Matining RM, Kuritzkes DR et al. (2011) Efficacy of a nucleoside-sparing regimen of darunavir/ritonavir plus raltegravir in treatment-naive HIV-1-infected patients (ACTG A5262). AIDS 25: 2113-2122. doi:10.1097/QAD.0b013e32834bbaa9. PubMed: 21857490.
32. Dalakas MC, Illa I, Pezeshkpour GH, Laukaitis JP, Cohen B et al. (1990) Mitochondrial myopathy caused by long-term zidovudine therapy. N Engl J Med 322: 1098-1105. doi:10.1056/NEJM199004193221602. PubMed: 2320079.
33. Moher D, Hopewell S, Schulz KF, Montori V, Gøtzsche PC et al. (2010) CONSORT 2010 explanation and elaboration: updated guidelines for reporting parallel group randomised trials. BMJ 340: c869. doi:10.1136/bmj.c869. PubMed: 20332511.
34. Nishijima T, Komatsu H, Gatanaga H, Aoki T, Watanabe K et al. (2011) Impact of small body weight on tenofovir-associated renal dysfunction in HIV-infected patients: a retrospective cohort study of Japanese patients. PLOS ONE 6: e22661. doi:10.1371/journal.pone.0022661. PubMed: 21799928.
35. Cockcroft DW, Gault MH (1976) Prediction of creatinine clearance from serum creatinine. Nephron 16: 31-41. doi:10.1159/000180580. PubMed: 1244564.
36. Rodríguez-Nóvoa S, Labarga P, Soriano V, Egan D, Albalater M et al. (2009) Predictors of kidney tubular dysfunction in HIV-infected patients treated with tenofovir: a pharmacogenetic study. Clin Infect Dis 48: e108-e116. doi:10.1086/598507. PubMed: 19400747.
37. Matsuo S, Imai E, Horio M, Yasuda Y, Tomita K et al.. (2009) Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis 53: 982-992. doi:10.1053/j.ajkd.2008.12.034. PubMed: 19339088.
38. Horio M, Imai E, Yasuda Y, Watanabe T, Matsuo S (2010) Modification of the CKD epidemiology collaboration (CKD-EPI) equation for Japanese: accuracy and use for population estimates. Am J Kidney Dis 56: 32-38. doi:10.1053/S0272-6386(10)01197-2. PubMed: 20416999.
39. Bredeek UF, Guadron R, Yolo R et al. (2012) A Switch From TDF/FTC To Raltegravir In Patients On A Boosted Protease Inhibitor Is Effective In Reducing Proteinuria And Increasing GFR [abstract: H1-1399b]. Presented at: 52nd Interscience Conference on Antimicrobial Agents and Chemotherapy; September 9-12. USA: San Francisco.
40. Gallant JE, Moore RD (2009) Renal function with use of a tenofovir-containing initial antiretroviral regimen. AIDS 23: 1971-1975. doi: 10.1097/QAD.0b013e32832c96e9. PubMed: 19696652.
41. Gatanaga H, Tachikawa N, Kikuchi Y, Teruya K, Genka I et al. (2006) Urinary beta2-microglobulin as a possible sensitive marker for renal injury caused by tenofovir disoproxil fumarate. AIDS Res Hum Retroviruses 22: 744-748. doi:10.1089/aid.2006.22.744. PubMed: 16910829.
42. Nishijima T, Shimbo T, Komatsu H, Takano M, Tanuma J et al. (2013) Urinary beta-2 microglobulin and alpha-1 microglobulin are useful screening markers for tenofovir-induced kidney tubulopathy in patients with HIV-1 infection: a diagnostic accuracy study. J Infect Chemother. PubMed: 23467792.
43. Dauchy FA, Lawson-Ayayi S, de la Faille R, Bonnet F, Rigothier C et al. (2011) Increased risk of abnormal proximal renal tubular function with HIV infection and antiretroviral therapy. Kidney Int 80: 302-309. doi: 10.1038/ki.2011.124. PubMed: 21544066.
44. McComsey GA, Tebas P, Shane E, Yin MT, Overton ET et al. (2010) Bone Disease in HIV Infection: A Practical Review and Recommendations for HIV Care Providers. Clin Infect Dis 51: 937-946. doi:10.1086/656412. PubMed: 20839968.
45. Kudo K, Konta T, Mashima Y, Ichikawa K, Takasaki S et al. (2011) The association between renal tubular damage and rapid renal deterioration in the Japanese population: the Takahata study. Clin Exp Nephrol 15: 235-241. doi:10.1007/s10157-010-0392-y. PubMed: 21225309.
46. Ofotokun I, Sheth AN, Sanford SE, Easley KA, Shenvi N et al. (2012) A switch in therapy to a reverse transcriptase inhibitor sparing combination of lopinavir/ritonavir and raltegravir in virologically suppressed HIV-infected patients: a pilot randomized trial to assess efficacy and safety profile: the KITE study. AIDS Res Hum Retroviruses 28: 1196-1206. doi:10.1089/aid.2011.0336. PubMed: 22364141.

Illicit Drug Use Is a Significant Risk Factor for Loss to Follow Up in Patients with HIV-1 Infection at a Large Urban HIV Clinic in Tokyo

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Abstract

Background: Loss to follow up (LTFU) is an important prognostic factor in patients with HIV-1 infection. The impact of illicit drug use on LTFU of patients with HIV-1 infection is unknown in Japan.

Methods: A single center observational study was conducted to elucidate the impact of illicit drug use on LTFU at a large HIV clinic in Tokyo. LTFU was defined as those who discontinued their visits to the clinic for at least 12 months and were not known to be under the care of other facilities or have died within 12 months of their last visit. Patients who first visited the clinic between January 2005 and August 2010 were enrolled. Information on illicit drug use was collected in a structured interview and medical charts. Comparison of the effects of illicit drug use and no use on LTFU was conducted by uni- and multi-variate Cox hazards models as the primary exposure.

Results: The study subjects were 1,208 patients, mostly Japanese men, of relatively young age, and infected through homosexual contact. A total of 111 patients (9.2%) were LTFU (incidence: 24.9 per 1,000 person-years). Among illicit drug users and non users, 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. Uni- and multi-variate analyses showed that illicit drug use was a significant risk for LTFU (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). Multivariate analysis also identified young age, high CD4 count, no antiretroviral therapy, and no health insurance as risk factors for LTFU.

Conclusions: The incidence of LTFU among illicit drug users was almost twice higher than that among non users. Effective intervention for illicit drug use in this population is warranted to ensure proper treatment and prevent the spread of HIV.

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Introduction

The introduction of highly-active antiretroviral therapy (HAART) has markedly improved the prognosis of patients with HIV-1 infection [1,2]. Patients with HIV-1 infection need to maintain a good level of adherence to antiretroviral therapy (ART) and frequent visits to the health facilities for monitoring treatment efficacy and safety, with regard to the suppression of HIV-1 viral load, recovery of immune function, and improvement of prognosis and survival [3,4]. Those who discontinue medical follow up are likely to develop AIDS-defining illness and die, compared to those who continue their visits [5,6]. Thus, loss to follow up (LTFU) influences prognosis of patients with HIV-1 infection [7–11].

Among patients with HIV-1 infection, those who use illicit drugs are associated with lower ART uptake and inferior adherence to treatment [12–15], which lead to suboptimal treatment outcome, compared with patients with other risk categories [16–18]. However, illicit drug users are one of the "difficult to reach" populations and it is difficult to obtain accurate data on them [19]. It is especially difficult in Japan to collect data on illicit drug users, because of a strong 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

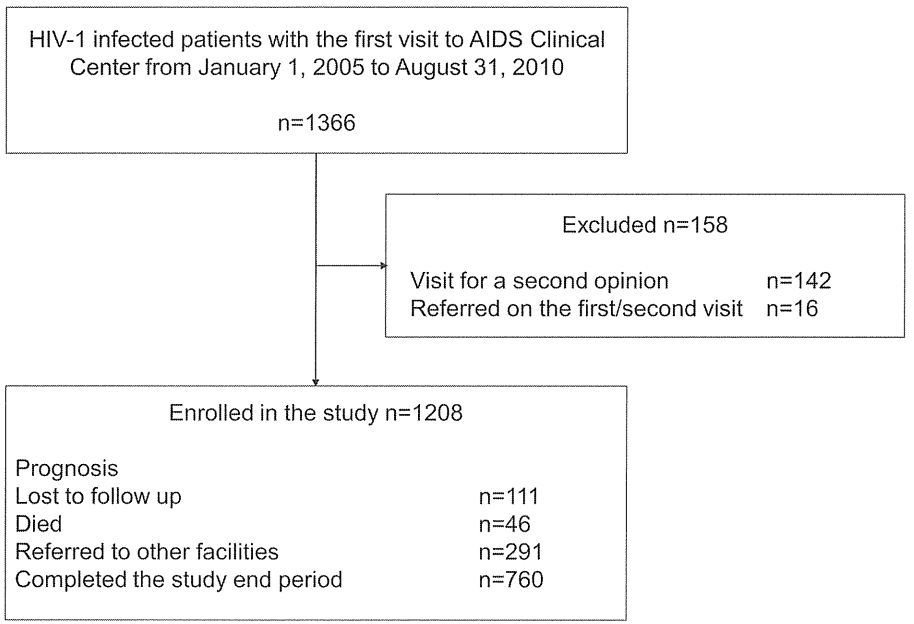

Figure 1. Patient enrollment process.

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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).

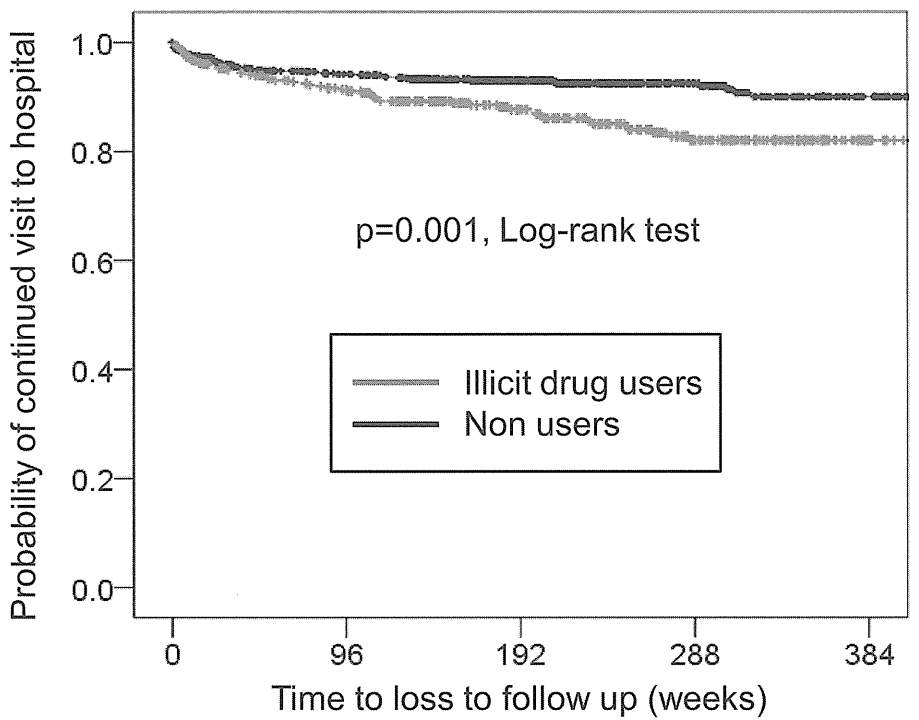

Figure 2. Kaplan-Meier curve showing time to loss to follow up for illicit drug users and non users. Compared to non drug users, illicit drug users were more likely to discontinue their visits to the hospital (p=0.001, Log-rank test).

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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

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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