Japanese, on ART, and history of AIDS were associated with illicit drug use. On the other hand, without a job, living alone, and positive HCV antibody were not associated with illicit drug use. Multivariate analysis identified MSM to be significantly associated with illicit drug use after adjustment for age and Japanese (adjusted OR = 4.60; 95% CI, 2.88–7.36; p<0.01) (Table 2, Model 2).

Subgroup analysis of the patients stratified by three age groups (≤30, 31 to 40, and >40) showed that the odds of association of MSM with illicit drug use was the strongest in the youngest age group (≤30 years: adjusted OR = 7.56; 95% CI, 2.86–20.0; p<0.01), followed by the oldest (>40 years: adjusted OR = 6.15; 95% CI, 2.40–15.8; p<0.01), and the weakest in the group aged 31 to 40 (adjusted OR = 3.39; 95% CI, 1.73–6.63; p<0.01) (Table 3).

Table 3. Results of multivariate analysis of the association of MSM over non-MSM for illicit drug use according to age.

	Adjusted OR	95% CI	P value
Age ≤30 years (n = 369)			
MSM vs. non-MSM	7.56	2.86–20.0	<0.01
Age 31 to 40 years (n = 473)			
MSM vs. non-MSM	3.39	1.73–6.63 85	<0.01
Age >40 years (n = 354)			
MSM vs. non-MSM	6.15	2.40–15.8	<0.01

MSM was adjusted with the same variables as Model 2, Table 2.
MSM: men who have sex with men.
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Discussion

The prevalence of illicit drug use among patients with HIV-1 infection in this large urban HIV clinic in Tokyo, which treats approximately 15% of patients with HIV-1 infection in Japan, was high at 35%. The prevalence was higher among HIV-1 infected MSM (40%), especially among young MSM aged ≤30 years (52%). Furthermore, HIV-1 infected MSM were more likely to use methamphetamine and to be arrested due to illicit drugs, compared with non-MSM. It should be emphasized that these numbers are likely to be underreported, since some patients would not admit illicit drug use to the interviewers on their first visit.

To our knowledge, this is the first study on the prevalence of illicit drug use among patients with HIV-1 infection in Japan. Although the prevalence of illicit drug use is considered extremely low among the general population in Japan with lifetime prevalence of 2.9% in 2009, high prevalence of illicit drug use in patients with HIV-1 infection, especially among HIV-1 infected MSM, was demonstrated [4,5] (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). The prevalence of methamphetamine use and incarceration due to illicit drug was also high, suggesting a substantial impact of illicit drugs, not only on the well-being of this population in terms of both medical and social perspectives, but also on public health perspectives [11,12].

In Japan, the number of illicit drug users arrested in 2010 was 14,965. Among these, 12,200 used methamphetamine, followed by cannabis (2,367), while only several hundred at most used other drugs (http://www.mhlw.go.jp/bunya/iyakuhin/yakubuturanyou/torikumi/dl/index-01.pdf in Japanese). Of note, the number of arrestees due to other injectable drugs, such as heroin and cocaine, was small (22 and 112, respectively). Thus, most injection drug users in Japan are methamphetamine users. Majority of the patients identified as IDU in this study were considered to be methamphetamine users as well.

By the end of 2011, of 19,976 patients (excluding those infected with contaminated blood products) reported to be infected with HIV-1, 108 (0.5%) were reported to be infected through injection drug use according to the surveillance conducted by the AIDS Surveillance Committee of the Japanese Ministry of Health, Labour and Welfare (http://api-net.jfap.or.jp/status/2011/11nenpo/hyo_02.pdf in Japanese). The prevalence of IDUs in this study is substantially higher; 53 (4%) of the 1,196 were IDUs, suggesting a considerable underreporting of IDU in the surveillance data. It is well known that for IDUs, prognosis is much worse than non-injecting drug users, as one multicenter study conducted in Europe and North America reported that IDUs experienced approximately five times higher mortality rates than patients infected through sexual intercourse [18]. Although the prevalence of IDUs among patients with HIV-1 infection in Japan is still much lower than that in neighboring countries, such as Taiwan (27.6%) and China (24.3%), there is an urgent need to develop effective prevention programs for HIV-1 infected illicit drug users [19] (http://www.unaids.org.cn/download/2009%20China%20Estimation%20Report-En.pdf) (http://www.cdc.gov.tw/english/list.aspx?treeid = 00ED75D6C887BB27&nowtreeid = 334C2073091C8677).

Although the prognosis of injection drug users is reported to be worse than that of non-injection drug users [20], this study primarily focused on illicit drug use as a whole, rather than injection drug use. This is because only a few studies focused on illicit drug use among HIV-1 infected patients, although a large number of studies focused on injection drugs [21–25]. 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 [10–12], 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 [8,26]. Studies from the US reported that especially MSM who use illicit

Table 2. Results of multivariate analysis of the association of MSM over non-MSM for illicit drug use.

	Model 1 Crude n = 1,196		Model 2 Adjusted n = 1,196	
	OR	95% CI	OR	95% CI
Men who have sex with men†	5.87	3.74–9.20	4.60	2.88–7.36
Age per 1 year†			0.95	0.94–0.97
Japanese†			1.74	1.07–2.82

†p<0.05.
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drugs are at high risk for HIV and sexual transmitted infections due to close associations between risky sexual behaviors and illicit drug use [27,28] Furthermore, illicit drug use, especially opioid use, can be a trajectory into injection drug use [29,30].

Several limitations need to be acknowledged. First, due to the nature of single-center study, this is a convenience sample and the results of this study do not necessarily represent the prevalence of illicit drug use in all patients with HIV-1 infection in Japan. However, as mentioned above, our clinic treats approximately 15% of the total HIV patients in Japan, and furthermore, most HIV-1 infected patients reside in urban areas such as Tokyo metropolitan area (http://api-net.jfap.or.jp/status/2011/11nenpo/hyo_02.pdf in Japanese). Thus, the discrepancy in the prevalence of illicit drug use between the study patients and all HIV patients in Japan should not be too large. Second, the structured interview method to collect data cannot avoid underreporting of illicit drug usage. Thus, the prevalence of illicit drug use in this population is very likely to be higher than what is reported here. However, underreporting to a certain degree is unavoidable with regard to issues such as illicit drug use [3].

In conclusion, the prevalence of illicit drug use in patients with HIV-1 infection in this large HIV clinic in Tokyo was high at 35%, and was higher in HIV-1 infected MSM (40%). Despite the low prevalence of IDUs (0.5%) among HIV-infected patients reported by the AIDS Surveillance Committee, 5% of patients in this study were IDUs. All relevant parties to the issue of illicit drug use in patients with HIV-1 infection need to recognize that illicit drug use is a huge burden in care and well-being of this population even in Japan, a country with very low prevalence of illicit drug use in the general population. Appropriate measures for prevention and intervention of illicit drug use are urgently needed to ensure proper treatment and prevention of spread of HIV infection.

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Author Contributions

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

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WHO Antiretroviral Therapy Guidelines 2010 and Impact of Tenofovir on Chronic Kidney Disease in Vietnamese HIV-Infected Patients

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Abstract

Objective: The 2010 WHO antiretroviral therapy (ART) guidelines have resulted in increased tenofovir use. Little is known about tenofovir-induced chronic kidney disease (CKD) in HIV-infected Vietnamese with mean body weight of 55 kg. We evaluated the prevalence and risk factors of CKD in this country.

Design: Cross-sectional study was performed.

Methods: Clinical data on HIV-infected Vietnamese cohort were collected twice a year. To evaluate the prevalence of CKD, serum creatinine was measured in 771 patients in October 2011 and April 2012. CKD was defined as creatinine clearance less than 60 ml/min at both time points. Multivariate logistic regression was used to determine the factors associated with CKD

Results: Tenofovir use increased in Vietnam from 11.9% in April 2011 to 40.3% in April 2012. CKD was diagnosed in 7.3%, of which 7% was considered moderate and 0.3% was severe. Multivariate analysis of October-2011 data identified age per year-increase (OR: 1.229, 95%CI, 1.170-1.291), body weight per 1 kg-decrement (1.286, 1.193-1.386), and tenofovir use (2.715, 1.028-7.168) as risk factors for CKD.

Conclusions: Older age, low body weight and tenofovir use were independent risk factors for CKD in Vietnam. Further longitudinal study is required to evaluate the impact of TDF on renal function in Vietnam and other countries with small-body weight patients.

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Introduction

Advances in antiretroviral therapy (ART) had turned HIV/AIDS into a chronic□disease [1-5]. As a consequence of living longer, chronic kidney disease (CKD) has become an important cause of morbidity and mortality in HIV-infected patients [1,3-5]. Several studies have reported increased prevalence of CKD, ranging from 4.9% to 8.4% in such patients [6-9]. In addition to the established risk factors, such as aging, diabetes mellitus (DM) and hypertension [2,10], other factors related to the virus itself and to the treatment [e.g., exposure to tenofovir (TDF), a commonly used antiretroviral (ARV)], are thought to be related to nephrotoxicity in HIV-infected patients[2,11,12].

To date, the benefit of TDF first line treatment is considered to outweigh the risk of TDF-induced nephrotoxicity. A recent meta-analysis study has reported that the use of TDF is associated with a statistically significant though only modest renal dysfunction, and recommended no restriction of TDF use when regular monitoring of renal function and serum phosphate levels is impractical [13]. Furthermore, the 2010 WHO guidelines for ART in adults and adolescents recommended TDF as part of the first line regimens (URL: http://whqlibdoc.who.int/publications/2010/9789241599764_eng.pdf).

However, several studies have reported that low body weight is an independent risk factor for TDF-associated nephrotoxicity and might lead to potentially higher risk for larger drug exposure and thus, more severe toxicity [14-17]. Under such

scenario, regional prevalence of CKD may influence the approach to screening and monitoring of HIV-infected patients initiated on ART. In particular, most nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), such as TDF and 3TC/FTC, are excreted by the kidney and may require dose adjustment in individuals with reduced glomerular filtration rate (GFR), and may require more intensive monitoring in patients with pre-existing CKD.

Following the 2010 WHO guidelines, the use of TDF has been increasing rapidly in Vietnam, where patients are more likely to have smaller body weight compared to Caucasians. At this stage, little is known about CKD among Vietnamese HIV-infected patients. In this context, it is important to determine the prevalence of CKD and its risk factors including TDF exposure and low body weight in this region. The present study was conducted to evaluate the above factors in Vietnamese HIV-infected patients.

Methods

Study design

We performed a cross-sectional study with an observational single-center cohort of Vietnamese HIV-infected patients on ART. This cohort was established since 2007 at the National Hospital of Tropical Disease in Hanoi, one of the largest outpatient clinics for HIV infected-patients in Vietnam. Clinical data are collected twice a year (in April and October) in this cohort. The population of this cohort comprised HIV-infected patients on ART aged more than 17 years. To evaluate CKD in this group, serum creatinine had been examined since October 2011. Serum creatinine was measured in October 2011 and April 2012. Patients whose creatinine was not obtained at both time points were excluded from the study. Other clinical data were collected twice a year (in April and October) as well. The study was approved by the Human Research Ethics Committee of National Hospital of Tropical Disease and Hanoi city. Each patient included in this study provided a written informed consent for the clinical and laboratory data to be used for publication. The study was conducted according to the principles expressed in the Declaration of Helsinki.

Measurements

Data included demographic variables (height, weight, sex and age); a complete history of ART; use of cotrimoxazole; CD4 cell count (cell/mm^3, measured by flow cytometry); plasma HIV-RNA (copies/ml, measured by the Roche COBAS TaqMan HIV monitor assay); serum creatinine (mg/dl, measured by Jaffe method); date of HIV diagnosis and other comorbidities. CKD was defined as creatinine clearance (Ccl) estimated by the Cockcroft-Gault formula of <60 ml/min at October 2011 and April 2012 (6 months apart). Renal dysfunction at each time point was also classified into five stages according to the guidelines of the National Kidney Foundation [18]: normal renal function: Ccl ≥90 ml/min; mild renal dysfunction, Ccl between 60-89 ml/min; moderate, Ccl 30-59 ml/min; severe renal dysfunction, Ccl 15-29 ml/min; and renal failure or dialysis, with Ccl of <15 ml/min.

Statistical analysis

Statistical analysis included descriptive (mean and standard deviation), univariate and multivariate analyses. Absolute and relative frequencies were utilized for continuous and categorical variables, respectively. To evaluate the association between CKD and categorical variables, the chi-square test or Fisher exact test was applied as required. Independent T test or one-way analysis of variance (ANOVA) was used to compare mean values of normally distributed data and the Mann Whitney test or Kruskal-Wallis test for parameters with skewed data distribution. Variables significantly associated with renal dysfunction in univariate analysis ($p<0.05$) were entered into multivariate analysis. Logistic regression was used to determine the factors associated with CKD in univariate and multivariate analyses. Statistical significance was defined at two-sided p value <0.05. We used the odds ratio (OR) and 95% confidence interval (95% CI) to estimate the association of each variable with renal dysfunction. All statistical analyses were performed with The Statistical Package for Social Sciences ver. 17.0 (SPSS, Chicago, IL).

Results

Patients on TDF

The percentage of TDF use in our cohort increased from 11.9% in April 2011 to 40.3% in April 2012. In contrast, stavudine (d4T) use decreased from 37.8% in April 2011 to 14.6% in April 2012. The patterns of use of TDF and d4T well reflected the recommendation of the 2010 WHO ART guidelines; recommendation for the use of TDF or zidovudine (AZT) and phasing out of d4T.

Prevalence of CKD and renal dysfunction at each time point

To determine the prevalence of CKD, serum creatinine was measured in 771 patients in October 2011 and April 2012. CKD was diagnosed in 56 (7.3 %) patients and classified as moderate in 54 and severe in 2 (Table **1**). The number of patients with moderate and severe renal dysfunction increased from 74 (9.6%) in October 2011 to 111 (14.4%) in April 2012. The data of serum creatinine by CKD stage are shown in Table **1**.

Baseline demographics and laboratory data

Table **2** compares the baseline demographics and clinical variables of patients with or without CKD for the data of October 2011. Patients with CKD were significantly older, more likely to be diabetic females treated with TDF and lopinavir boosted with ritonavir, and of significantly lower body weight with higher serum creatinine, and with history of AIDS-defining disease, compared to those without CKD. CD4 count, HIV RNA viral load, and duration of ART were not significantly different between the two groups. The mean CD4 count was >300/mm^3 and the mean HIV RNA load was <100 copies/ml in both groups.

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.

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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-

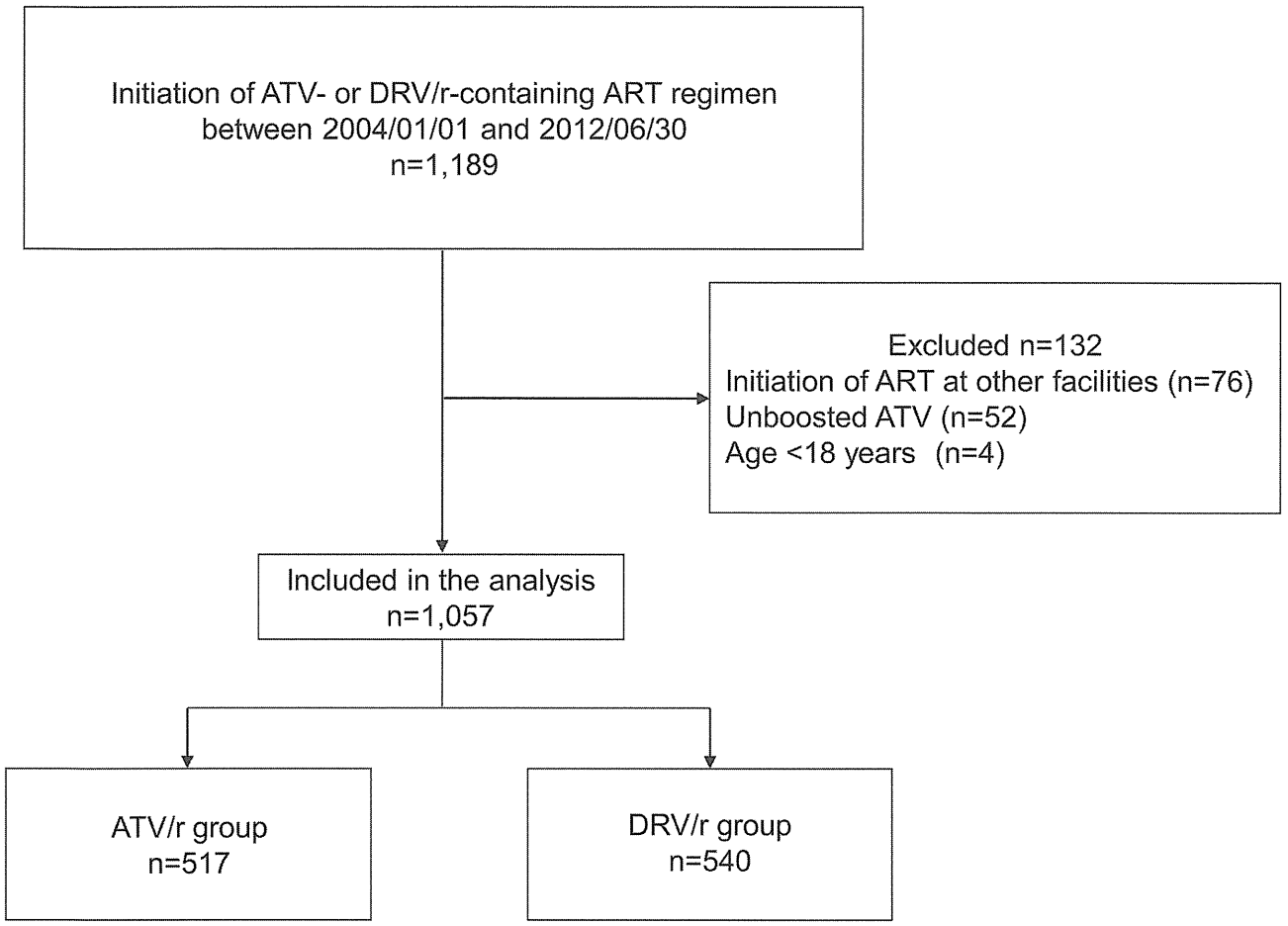

Figure 1. Flow diagram of patient selection. ART, antiretroviral therapy; ATV, atazanavir; DRV/r, ritonavir-boosted darunavir; ATV/r, ritonavir-boosted atazanavir.

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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.

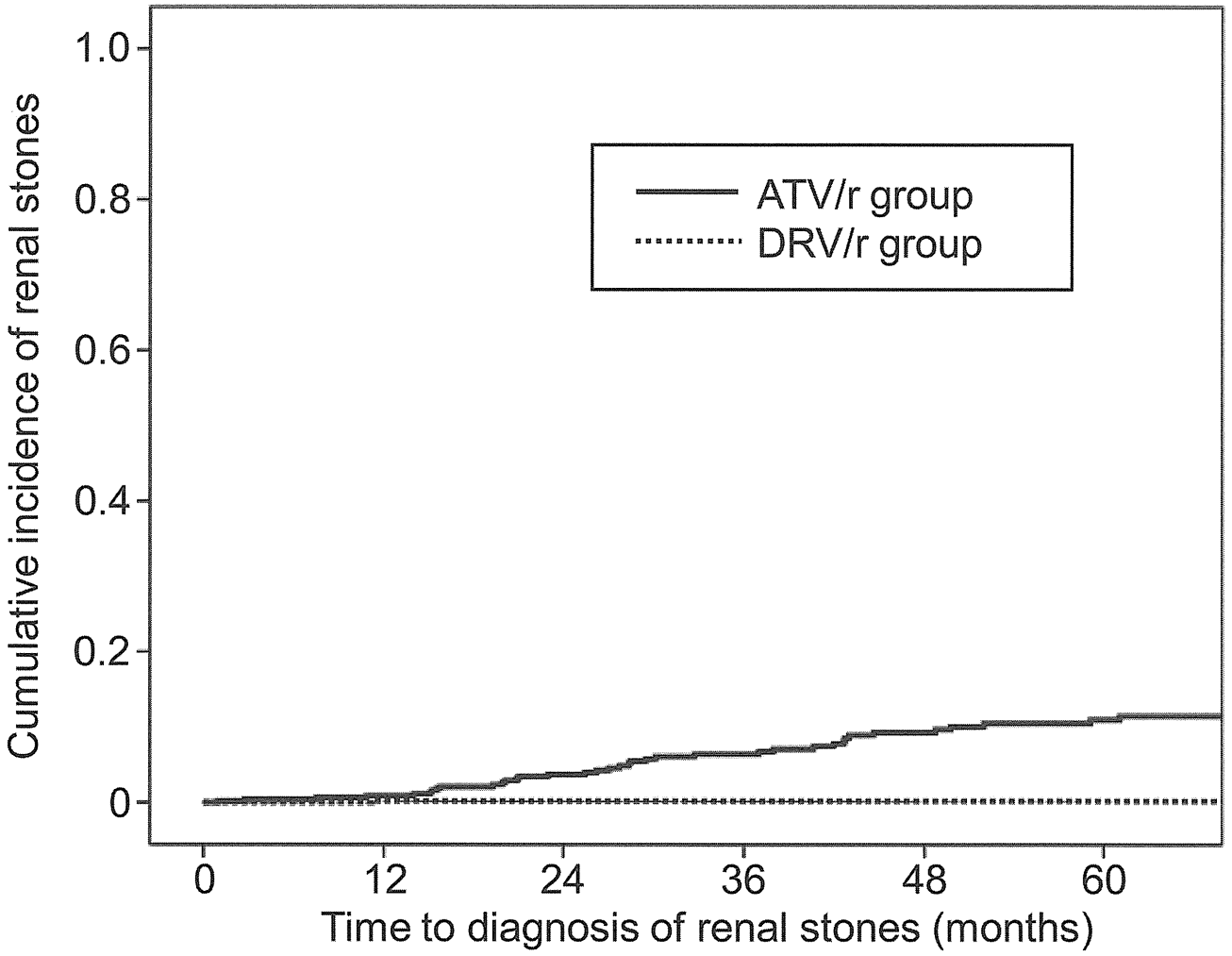

Figure 2. Kaplan-Meier curve showing time to the diagnosis of nephrolithiasis. ATV/r, ritonavir-boosted atazanavir; DRV/r, ritonavir-boosted darunavir.

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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.

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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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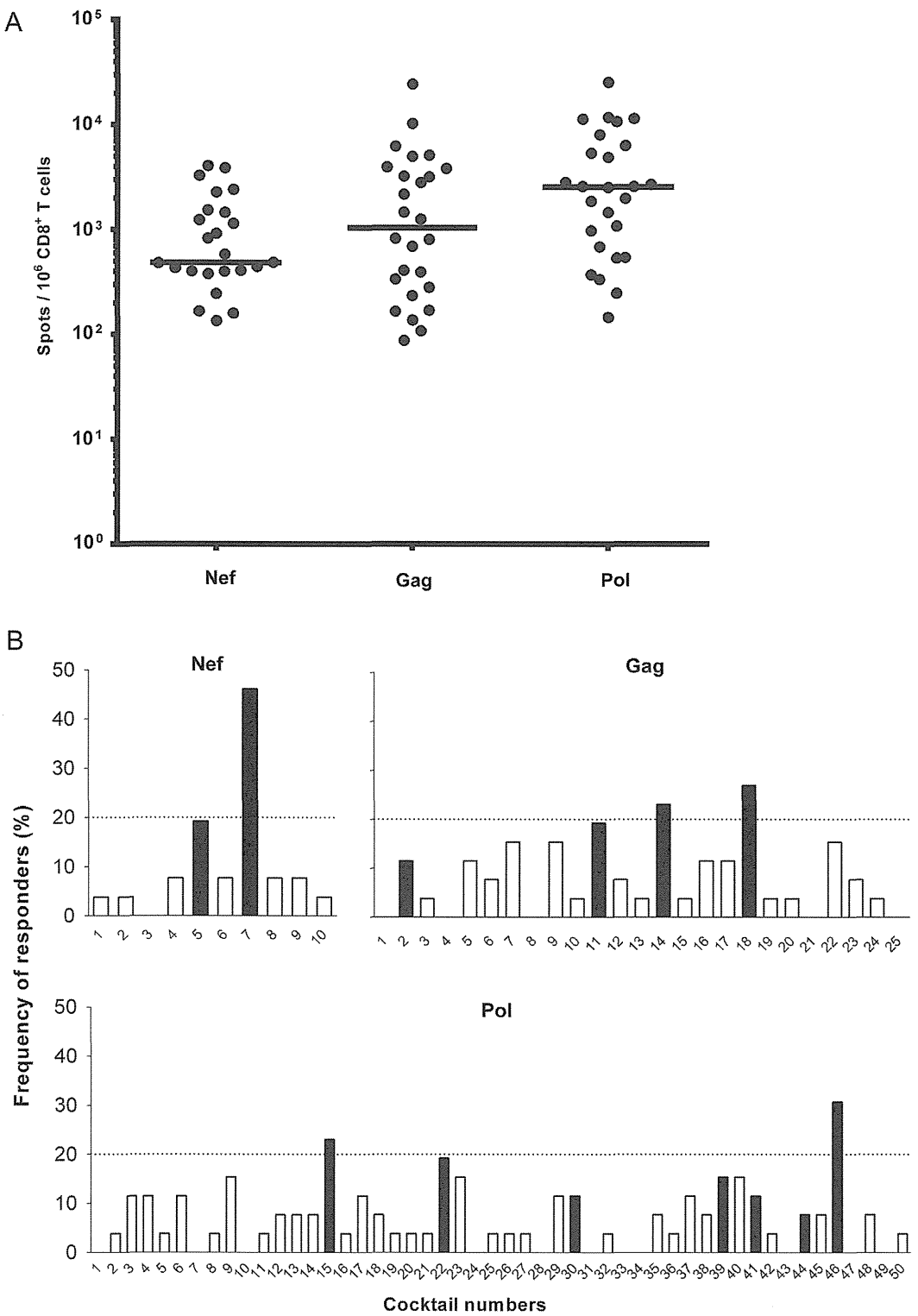
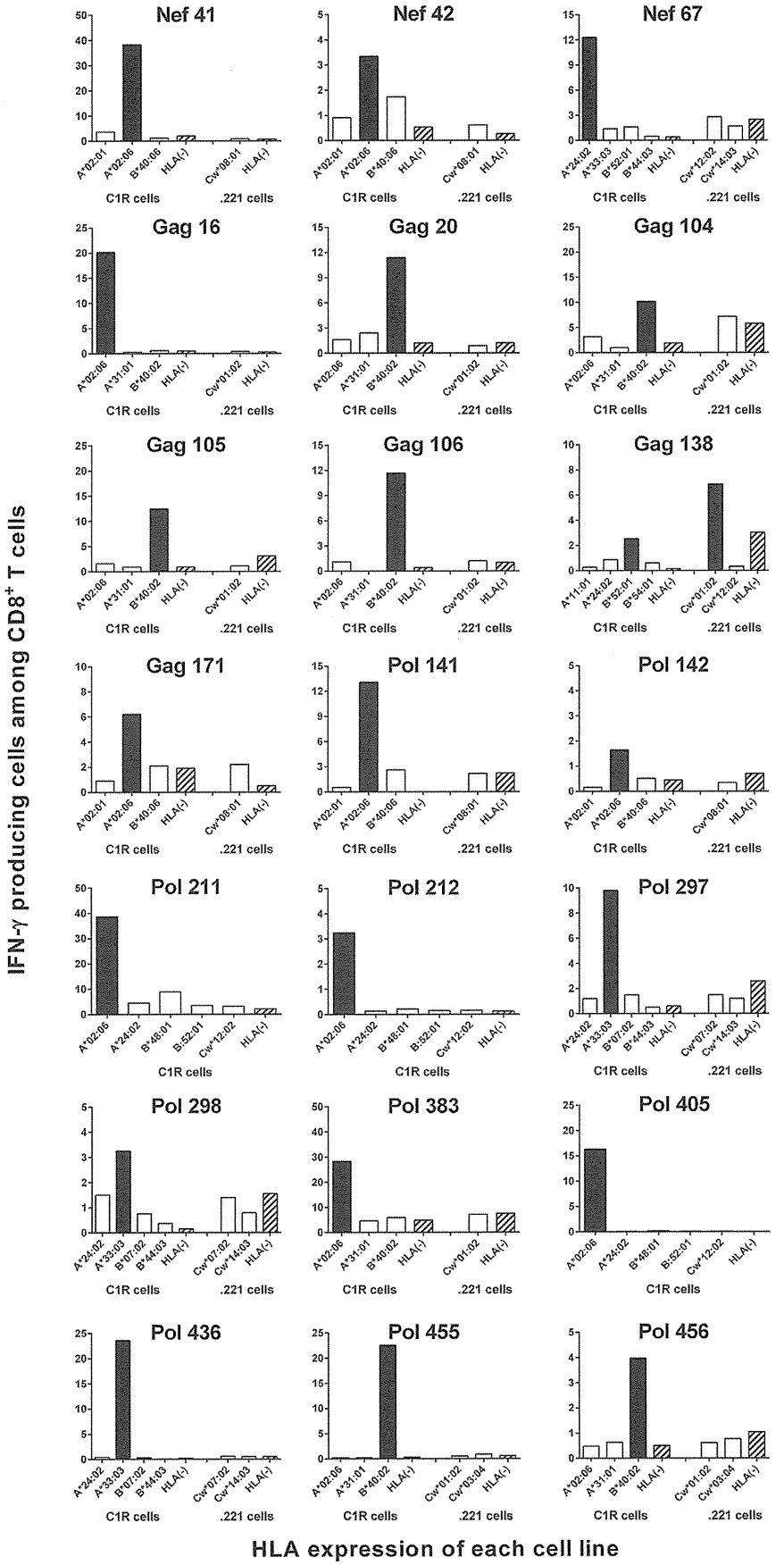


Fig. 1. $CD8^+$ T cell responses of clade A/E-infected individuals to HIV-1 clade B-derived overlapping peptides $CD8^+$ T cell responses to peptide cocktails containing clade B consensus overlapping 11-mer peptides were analyzed by performing the ELISPOT assay using $CD8^+$ T cells from 26 clade A/E-infected 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).


Nef 41
Nef 42
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Gag 16
Gag 20
Gag 104
Gag 105
Gag 106
Gag 138
Gag 171
Pol 141
Pol 142
Pol 211
Pol 212
Pol 297
Pol 298
Pol 383
Pol 405
Pol 436
Pol 455
Pol 456
C1R cells
.221 cells
IFN-γ producing cells among CD8+ T cells
HLA expression of each cell line

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.