adapted to the Japanese population by acquiring escape mutations from immune pressure restricted by human leukocyte antigens (HLAs) popular among the Japanese.[23,24] Based on this point of view, early diagnosis is even more important due to the shorter asymptomatic period before the development of AIDS.

The majority of our study participants were infected with HIV-1 subtype B, and HIV-1 subtype B infection correlated significantly with MSM (crude odds ratio 37.9, $p < 0.001$; Chi-square test). The non-AIDS patients were more likely to be infected with subtype B than AIDS patients (crude odds ratio 1.59, $p = 0.098$). The same was true for recent infection than chronic infection (crude odds ratio 2.81, $p = 0.009$). A previous Japan-wide survey also showed a close relationship between subtype B and MSM in Japan; all cases diagnosed with primary HIV-1 infection ($n = 45$) were caused by subtype B, and such primary infections were significantly frequent among MSM.[14] Considered together, the results indicate that subtype B is the major currently prevalent strain in Japan, especially among MSM, and such strains are probably adapting to the Japanese population by repeated exposure to immune pressure of the Japanese.

This study used case reporting-based surveillance to estimate the number of new HIV-1 infections in Tokyoites between 2002 and 2010. The data were collected at a single center and thus may have included some institutional bias. The study participants were statistically younger and were more likely to be MSM than those of the Tokyo registry. The BED assay was used in this study to determine the rate of recent infection in the selection study group and not to determine the national incidence rate. However, the data from this study suggest the following target-specific differential strategies for controlling the HIV epidemic and for AIDS prevention in Tokyo: campaigns aimed at promoting testing should be directed at older MSM for early diagnosis to prevent/halt the progression of AIDS; commencement of ART for HIV-infected younger MSM at early stages of the disease may effectively reduce the number of new cases based on the control of current hot-spots of HIV transmission among this group.

Acknowledgements

This work was supported in part by a Grant-in-Aid for AIDS research from the Ministry of Health, Labor, and Welfare, Japan (H20-AIDS-002), and the Global Center of Excellence Program from the Ministry of Education, Science, Sports and Culture of Japan.

Conflict of interest: The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2011.11.003.

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PLoS one

Renal Function Declines More in Tenofovir- than Abacavir-Based Antiretroviral Therapy in Low-Body Weight Treatment-Naïve Patients with HIV Infection

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Abstract

Objective: To compare the rate of decline of renal function in tenofovir- and abacavir-based antiretroviral therapy (ART) in low-body weight treatment-naïve patients with HIV infection.

Design: We conducted a single-center retrospective cohort study of 503 Japanese patients who commenced on either tenofovir- or abacavir-based initial ART.

Methods: The incidence of renal dysfunction, defined as more than 25% fall in estimated glomerular filtration rate (eGFR) from the baseline, was determined in each group. The effect of tenofovir on renal dysfunction was estimated by univariate and multivariate Cox hazards models as the primary exposure. Changes in eGFR until 96 weeks were estimated in both groups with a repeated measures mixed model.

Results: The median body weight of the cohort was 64 kg. The estimated incidence of renal dysfunction in the tenofovir and the abacavir arm was 9.84 per 100 and 4.55 per 100 person-years, respectively. Tenofovir was significantly associated with renal dysfunction by univariate and multivariate analysis (HR = 1.747; 95% CI, 1.152–2.648; p = 0.009) (adjusted HR = 2.080; 95% CI, 1.339–3.232; p<0.001). In subgroup analysis of the patients stratified by intertertile baseline body weight, the effect of tenofovir on renal dysfunction was more evident in patients with lower baseline body weight by multivariate analysis (≤60 kg: adjusted HR = 2.771; 95%CI, 1.494–5.139; p = 0.001) (61–68 kg: adjusted HR = 1.908; 95%CI, 0.764–4.768; p = 0.167) (>68 kg: adjusted HR = 0.997; 95%CI, 0.318–3.121; p = 0.995). The fall in eGFR was significantly greater in the tenofovir arm than the abacavir arm after starting ART (p = 0.003).

Conclusion: The incidence of renal dysfunction in low body weight patients treated with tenofovir was twice as high as those treated with abacavir. Close monitoring of renal function is recommended for patients with small body weight especially those with baseline body weight <60 kg treated with tenofovir.

Citation: 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(1): e29977. doi:10.1371/journal.pone.0029977

Editor: Claire Thorne, UCL Institute of Child Health, University College London, United States of America

Received September 21, 2011; **Accepted** December 7, 2011; **Published** January 5, 2012

Funding: This work was supported by a Grant-in Aid for AIDS research from the Japanese Ministry of Health, Labour, and Welfare (H20-AIDS-002), and the Global Center of Excellence Program (Global Education and Research Center Aiming at the Control of AIDS) from the Japanese Ministry of Education, Science, Sports and Culture. No additional external funding was received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Tenofovir disoproxil fumarate (TDF) and abacavir sulfate (ABC) are widely used nucleot(s)ide reverse transcriptase inhibitors (NRTIs) as part of the initial antiretroviral therapy for patients with HIV infection in the developed countries (URL:http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf) (URL: http://www.europeanaidsclinicalsociety.org/images/stories/EACS-Pdf/1_treatment_of_hiv_infected_adults.pdf). TDF is generally preferred to ABC, since ABC is reported to cause serious hypersensitivity reaction in 5–8% of the patients and its efficacy in viral suppression is reported to be inferior to TDF among patients with baseline HIV viral load of >100,000 copies/ml [1,2]. On the other hand, renal proximal tubular damage and renal dysfunction are well-known adverse effects of TDF [3–9]. A meta-analysis study that compared TDF and other NRTIs concluded that the decline in renal function with TDF use is significant but modest, and the ASSERT study conducted in Europe compared randomly-selected treatment naïve patients who commenced treatment with either TDF or ABC with efavirenz and showed no difference in estimated glomerular filtration

rate (eGFR) between the two groups at 48 weeks [9,10]. To date, the nephrotoxicity of TDF have been regarded as mild and tolerable [2,5–7,9–11].

However, the TDF-related nephrotoxicity has hardly been evaluated in patients with small body weight, who are potentially at higher risk for larger drug exposure and thus, more severe toxicity [12–15]. Indeed, some recent studies including ours reported a higher incidence of TDF-related renal dysfunction among Asian patients with low body weight compared with previous studies on mostly Whites and African Americans with larger body weight [13,16]. Thus, it is important to provide more evidence in support of TDF-associated nephrotoxicity in patients with low body weight since such data can elucidate whether TDF-related nephrotoxicity is as mild in low-body-weighted patients as previously reported in Europe and the USA. This is also important because there is increasing use of TDF in resource-limited settings, where patients are often of relatively small body weight, following the revised 2010 WHO guidelines that recommend TDF as one of the components of first line therapies (URL:http://whqlibdoc.who.int/publications/2010/9789241599764_eng.pdf)[13,16–19]. To our knowledge, there are no studies that compared renal function in treatment naïve Asian patients who commenced treatment with TDF or ABC.

Based on the above background, the present study was designed to compare the incidence of renal dysfunction and change in eGFR between treatment-naïve Japanese patients with low body weight who started either TDF or ABC as part of the antiretroviral regimen.

Methods

Ethics Statement

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

Study Subjects

We performed a retrospective, single-center cohort study of HIV-infected Japanese patients using the medical records 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 treatment-naïve patients with HIV infection, aged >17 years, who commenced treatment with either the recommended 300 mg/day dose of TDF or 600 mg/day dose of ABC-containing antiretroviral regimen at our clinic between January 1, 2004 and March 31, 2009. During this inclusion period, all except two patients at our clinic started ART with either ABC or TDF. Patients with an eGFR of >60 ml/min/1.73 m^2 were enrolled. Patients were followed up until March 31, 2011. They were excluded if they started ART with both TDF and ABC, their follow-up period at our facility was less than 24 weeks after commencement of ART, or if they had started ART at other facilities. Only Japanese patients were included in order to examine a population with comparatively homogenous basic demographics and background. The attending physician selected either TDF or ABC at baseline, and the use of these two drugs was based on the Japanese guidelines, which place both ABC and TDF as the preferred NRTIs (http://www.haart-support.jp/guideline2011.pdf. in Japanese). The attending physician also selected the key drug [non-nucleoside reverse transcriptase inhibitor (NNRTI), protease inhibitor (PI), or integrase inhibitor (INI)]. All patients received standard ART with two NRTIs combined with either PI, NNRTI, or INI.

Measurements

We defined renal dysfunction as more than 25% decrease in eGFR relative to the baseline [13,16,20,21]. The baseline eGFR was estimated for each patient from the average of two successive serum creatinine measurements made closest to and preceding the commencement of antiretroviral therapy by no more than 90 days. Changes in eGFR were plotted from the baseline measurement until the average value of two successive measurements diminished to less than 75% of the baseline, discontinuation of TDF or ABC, or at the end of the follow-up period. Discontinuation of TDF and ABC was the choice of the attending physician, and was based on virologic failure or ART-related side effects other than renal dysfunction. Before the initiation of ART and until suppression of HIV-1 viral load, patients visited our clinic every month. However, after viral load suppression, the visit interval was extended up to every three months. Serum creatinine and eGFR were measured in every visit, and the frequency of measurements was similar in patients on TDF and ABC. eGFR was calculated using the equation from the 4-variable Modification of Diet in Renal Disease (MDRD) study, $eGFR = 186 \times [\text{serum creatinine}]^{-1.154} \times [\text{age}]^{-0.203} \times [0.742 \text{ if patient is female}] \times [1.212 \text{ if patient is African American}]$ [22]. In this study, the primary exposure variable was TDF use over ABC as part of the initial ART.

The potential risk factors for renal dysfunction were determined according to previous studies and collected together with the basic demographics from the medical records [15,23–25]. They included age, sex, body weight, body mass index, (BMI) = {body weight (kg) / [(height (m)]^2}, baseline laboratory data (CD4 cell count, HIV viral load, and serum creatinine), and presence or absence of other medical conditions (concurrent use of ritonavir-boosted protease inhibitors, concurrent nephrotoxic drugs such as ganciclovir, sulfamethoxazole/trimethoprim, and non-steroidal anti-inflammatory agents, diabetes mellitus defined by using anti-diabetic agents or fasting plasma glucose >126 mg/dl or plasma glucose >200 mg/dl on two different days, co-infection with hepatitis B defined by positive hepatitis B surface antigen, co-infection with hepatitis C defined by positive HCV viral load, hypertension defined by current treatment with antihypertensive agents or two successive measurements of systolic blood pressure >140 mmHg or diastolic blood pressure >90 mmHg at the clinic, dyslipidemia defined by current treatment with lipid-lowering agents, and current smoking). At our clinic, weight and blood pressure were 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 90 days.

Statistical analysis

The time to 25% decline in eGFR from the baseline was calculated from the date of commencement of treatment to the date of diagnosis of the above-defined renal dysfunction. Censored cases represented those who discontinued ABC or TDF, dropped out, were referred to other facilities, or at the end of follow-up period. The time from the start of ART to >25% decrease in eGFR was analyzed by the Kaplan Meier method for patients who started TDF (TDF arm) and ABC (ABC arm), 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 TDF use over ABC on the incidence of more than 25% decrease in eGFR relative to the baseline. The impact of each basic 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 TDF use over ABC for renal dysfunction, we conducted three models using multivariate Cox proportional hazards regression analysis. Model 1 was the aforementioned univariate analysis for TDF use over ABC. Model 2 included age 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 for adjustment (these included age per 1 year, weight per 1 kg decrement, CD4 count per 1 /μl decrement, HIV viral load per log10/ml, serum creatinine per 1 mg/dl, concurrent use of nephrotoxic drug(s), hepatitis B infection, and diabetes mellitus). The eGFR and the BMI were excluded from multivariate analysis because of their multicollinearity with age and serum creatinine, and weight, respectively, since eGFR and BMI are gained by the equation of those variables [22,26]. We chose to add weight instead of BMI because our previous work showed that weight was more useful and handy information to estimate the risk for TDF-related nephrotoxicity than BMI [16].

As a sensitivity analysis, creatinine clearance was similarly calculated with Cockcroft-Gault equation for each patient, creatinine clearance = [(140 - age) × weight (kg)] / (serum creatinine×72)(×0.85 for females) [27]. Actual body weight was used for the calculation. The impact of TDF use over ABC for >25% decrement of creatinine clearance from the baseline was estimated in univariate analysis and multivariate analysis adjusted with the before mentioned variables with Cox proportional hazards model.

To estimate the impact of weight on TDF-related nephrotoxicity, we did subgroup analysis for intertertile baseline body weight categories: ≤60, 61–68, and >68 kg. Then, the abovementioned multivariate analysis with eGFR was conducted for each subgroup.

We also used a repeated measures mixed model to estimate and compare changes in eGFR between ABC and TDF from baseline to 2 years after initiation of ART by 6-month intervals adjusted for baseline eGFR and weight [10]. For each patient, the eGFR values at closest to and preceding 24, 48, 72 and 96 weeks after commencement of ART were collected. In this analysis, censoring occurred at discontinuation of TDF or ABC, leaving care, or reaching the end of the observation period before 96 weeks. Sensitivity analysis with creatinine clearance calculated by Cockcroft-Gault equation was similarly conducted.

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 renal dysfunction. All statistical analyses were performed with The Statistical Package for Social Sciences ver. 17.0 (SPSS, Chicago, IL).

Results

The study subjects were 199 patients in the TDF arm and 304 patients in the ABC arm who fulfilled the abovementioned criteria. Table 1 shows the demographics, laboratory data, and medical conditions of the study population at baseline. The majority of the study population was males, comparatively young and had a small stature (median weight, 64 kg, median BMI, 22.2 kg/m^2). More than 80% of the patients in the two arms had ritonavir-boosted PI. In the ABC arm, patients had significantly lower CD4 count (p = 0.006), were significantly more likely to have hypertension (p<0.001), and tended to use more nephrotoxic drugs (p = 0.109). On the other hand, in the TDF arm, patients had marginally higher baseline eGFR (p = 0.098) and were significantly more likely to have hepatitis B virus infection (P<0.001). However, all other major background parameters were similar in the two groups (Table 1).

More than 25% decrement in eGFR from baseline occurred in 44 patients (22.1%) in the TDF arm and 41 (13.5%) in the ABC arm, with an estimated incidence of 9.84 and 4.55 per 100 person-years, respectively. Figure 1 shows the time from ART initiation to >25% decrease in eGFR by the Kaplan Meier method in the two groups. Patients who started TDF-containing ART were significantly more likely to develop renal dysfunction, compared to the ABC group (p = 0.001, Log-rank test). The median time from commencement of ART to occurrence of >25% decrement in eGFR was 246 days (range, 1–1,339 days) for the TDF arm and 501 days (range, 7–2,022) for ABC arm. The total observation period was 447.2 patient-years [median, 839 days, interquartile range (IQR), 357–1137 days] for the TDF arm and 901.7 patient-years (median, 1,119 days, IQR, 660.5–1509 days) for the ABC arm.

Univariate analysis showed a significant relationship between TDF use and >25% decrement in eGFR (HR = 1.747; 95%CI, 1.152–2.648; p = 0.009) (Table 2). Furthermore, old age, small body weight, low baseline CD4 count, high HIV viral load, high eGFR, low serum creatinine, concurrent use of nephrotoxic drugs, hepatitis B infection, and diabetes mellitus were associated with renal dysfunction. On the other hand, concurrent use of ritonavir boosted PIs was not associated with renal dysfunction (HR = 1.220; 95%CI, 0.663–2.244; p = 0.523). Multivariate analysis identified TDF use as a significant risk for >25% decrement in eGFR after adjustment for age and weight (adjusted HR = 1.893; 95%CI, 1.243–2.881; p<0.003) (Table 3, Model 2), and also after adjustment for other risk factors (adjusted HR = 2.080; 95%CI, 1.339–3.232; p<0.001) (Table 3, Model 3). We also conducted a sensitivity analysis using BMI decrement instead of weight as a variable in Table 3, Model 3. The results were almost identical; TDF use over ABC use was a risk for renal dysfunction (adjusted HR 1.957, 95% CI 1.262–3.036, p = 0.003).

Sensitivity analysis with creatinine clearance confirmed the abovementioned findings: both univariate and multivariate analyses showed that TDF use was significantly associated with >25% decrement in eGFR (univariate analysis: HR = 2.212; 95%CI, 1.340–3.653; p = 0.002) (multivariate analysis: adjusted HR = 2.544; 95%CI, 1.493–4.335; p = 0.001).

Subgroup analysis of the patients stratified by intertertile baseline body weight showed that the lower the baseline body weight, the more evident the impact of TDF on renal dysfunction (≤60 kg: adjusted HR = 2.771; 95%CI, 1.494–5.139; p = 0.001) (61–68 kg: adjusted HR = 1.908; 95%CI, 0.764–4.768; p = 0.167) (>68 kg: adjusted HR = 0.997; 95%CI, 0.318–3.121; p = 0.995) (Table 4). These findings suggest that there is the effect modification by baseline body weight on TDF-associated renal dysfunction.

Data analysis by repeated measures mixed models showed a significant decrease in adjusted mean eGFR from the baseline to 96 weeks in both groups (TDF: -9.984 ml/min/1.73m^2, 95%CI -12.05 to -7.914 ml/min/1.73m^2, p<0.001; ABC: -5.393 ml/min/1.73m^2, 95%CI -7.087 to -3.699 ml/min/1.73m^2, p<0.001) (Figure 2). There was a statistically significant interaction between the two arms over time (p = 0.003), indicating that adjusted mean eGFR decreased more significantly in the TDF group than in the ABC group after initiation of ART. Analysis of eGFR in each group demonstrated a rapid decrease during the first 24 weeks,

Table 1. Baseline demographics and laboratory data of patients who received tenofovir- and abacavir-based antiretroviral therapy (n = 503).

	TDF (n = 199)	ABC (n = 304)	P value
Sex (male), n (%)	196 (98.5)	296 (97.4)	0.539
Median (IQR) age	36 (31–44)	37 (31–43)	0.436
Median (IQR) weight (kg)	64 (58–69)	64 (58.0–70.9)	0.426
Median (IQR) BMI (kg/m^2)	22.1 (20.4–23.9)	22.2 (20.3–24.6)	0.321
Median (IQR) eGFR (ml/min/1.73m^2)	119.4 (103.0–135.0)	115.6 (102.4–132.2)	0.098
Median (IQR) serum creatinine (mg/dl)	0.74 (0.67–0.84)	0.75 (0.68–0.83)	0.250
Median (IQR) CD4 count (/μl)	199 (109–272)	178.5 (75.3–234.8)	0.006
Median (IQR) HIV RNA viral load (log10/ml)	4.63 (4.20–5.20)	4.74 (4.23–5.20)	0.731
Ritonavir-boosted protease inhibitors, n (%)	173 (86.9)	256 (84.2)	0.441
Protease inhibitors (unboosted), n (%)	5 (2.5)	20 (6.6)	0.038
NNRTIs, n (%)	16 (8.0)	26 (8.6)	0.848
INIs, n (%)	5 (2.5)	2 (0.7)	0.119
Hypertension, n (%)	5 (2.5)	53 (17.4)	<0.001
Dyslipidemia, n (%)	4 (2.0)	4 (1.3)	0.718
Diabetes mellitus, n (%)	8 (4.0)	12 (3.9)	1.000
Concurrent use of nephrotoxic drugs, n (%)	65 (32.7)	121 (39.8)	0.109
Hepatitis B, n (%)	35 (17.6)	9 (3.0)	<0.001
Hepatitis C, n (%)	7 (3.5)	7 (2.3)	0.421
Current smoker, n (%)	93 (46.7)	149 (49.3)	0.585

TDF: tenofovir, ABC: abacavir, IQR: interquartile range, BMI: body mass index, eGFR: estimated glomerular filtration rate, NNRTI: non- nucleoside reverse transcriptase inhibitor, INI: integrase inhibitor.
doi:10.1371/journal.pone.0029977.t001

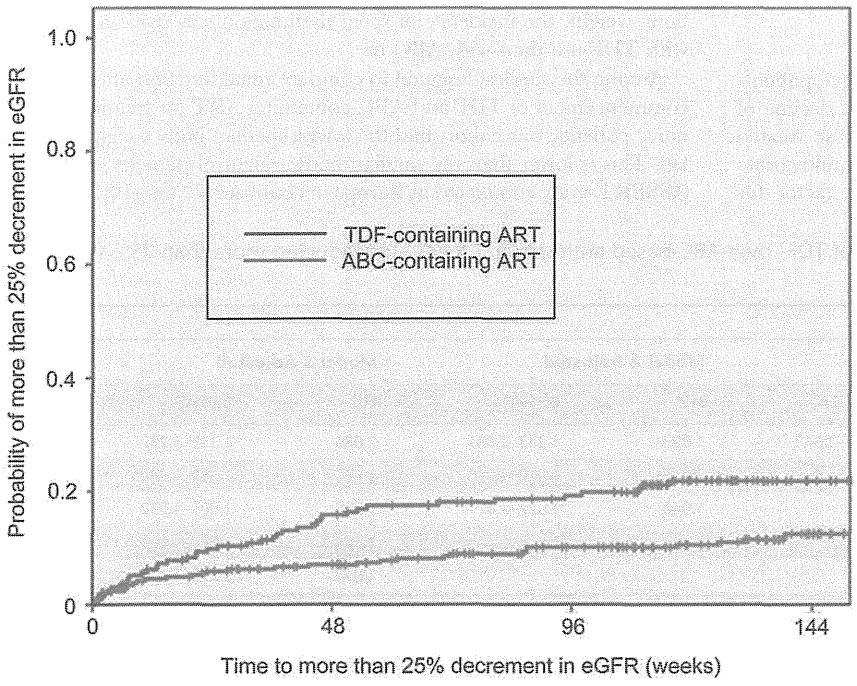

Figure 1. Kaplan-Meier curve showing the time to renal dysfunction in patients treated with TDF or ABC. Compared to treatment-naïve patients who commenced treatment with ABC, those on TDF were more likely to develop >25% fall in eGFR (p = 0.001, Log-rank test). TDF: tenofovir, ABC: abacavir, ART: antiretroviral therapy, eGFR: estimated glomerular filtration rate.
doi:10.1371/journal.pone.0029977.g001

Table 2. Univariate analysis to estimate the risk of various factors in inducing more than 25% fall in eGFR.

	Hazard ratio	95% CI	P value
TDF vs. ABC use	1.747	1.152–2.648	0.009
Female gender	0.048	0.000–16.93	0.310
Age per 1 year	1.031	1.011–1.051	0.002
Weight per 1 kg decrement	1.047	1.023–1.072	<0.001
BMI per 1 kg/m^2 decrement	1.152	1.066–1.244	<0.001
CD4 count per 1 /μl decrement	1.006	1.004–1.008	<0.001
HIV viral load per log10/ml	1.562	1.179–2.071	0.002
Ritonavir-boosted protease inhibitors	1.220	0.663–2.244	0.523
Baseline eGFR per 1 ml/min/1.73m^2	1.009	1.005–1.014	<0.001
Baseline serum creatinine per 1mg/dl	0.016	0.003–0.086	<0.001
Concurrent nephrotoxic drug	2.134	1.417–3.214	<0.001
Hepatitis B	1.866	1.038–3.356	0.037
Hepatitis C	1.721	0.631–4.695	0.289
Diabetes mellitus	2.558	1.181–5.540	0.017
Hypertension	0.865	0.448–1.669	0.664
Current smoking	0.989	0.657–1.489	0.958

eGFR: estimated glomerular filtration rate, CI: confidence interval, TDF: tenofovir, ABC: abacavir, BMI: body mass index.
doi:10.1371/journal.pone.0029977.t002

followed by a plateau until 96 weeks. In sensitivity analysis with creatinine clearance calculated by Cockcroft-Gault equation, the result was the same; a significant decrease from the baseline to 96 weeks in both groups (TDF: -10.62 ml/min, 95%CI -13.78 to -7.458 ml/min; ABC: -4.325 ml/min, 95%CI -6.893 to -1.756 ml/min) and significantly more eGFR decrement in the TDF group ($p = 0.019$).

Discussion

In this observational Japanese cohort, treatment-naïve patients who started TDF-containing ART experienced eGFR decline of >25% approximately twice as likely compared to those treated with ABC-containing regimen. Univariate and multivariate analyses identified TDF use as an independent risk factor for renal dysfunction. Subgroup analysis showed that the effect of TDF on renal dysfunction was more evident in patients with lower body weight. Furthermore, eGFR decrement was significantly larger in the TDF group than in ABC group over the 2-year observation period.

In our previous study, we demonstrated a high incidence of TDF-associated nephrotoxicity in patients with low body weight, and the use of a robust statistical model indicated a greater decline in renal function in patients of low body weight treated with TDF [16]. The results of the present study further emphasize the importance of low body weight as a risk factor for TDF-related nephrotoxicity by showing that in a cohort of patients with low body weight, the incidence of renal dysfunction was twice higher with TDF use than with ABC use.

Among the studies designed to compare renal function after the commencement of TDF and ABC-containing ART for treatment-naïve patients, our cohort had the lowest median body weight (64 kg). This is lower than the median body weight of patients of the ASSERT study conducted in European countries (72 kg) [10]. The

Table 4. Multivariate analysis to estimate the risk of TDF- over ABC-based antiretroviral therapy in the induction of more than 25% fall in eGFR according to baseline body weight.

	Adjusted HR	95% CI	P value
Baseline body weight ≤60 kg (n = 171)			
TDF vs. ABC use	2.771	1.494–5.139	0.001
Baseline body weight 61–68 kg (n = 167)			
TDF vs. ABC use	1.908	0.764–4.768	0.168
Baseline body weight >68 kg (n = 165)			
TDF vs. ABC use	0.997	0.318–3.121	0.995

TDF use was adjusted with the same variables indicated in Model 3, Table 3: age per 1 year, weight per 1 kg decrement, CD4 count per 1 /μl decrement, HIV viral load per log10/ml, serum creatinine per 1 mg/dl, concurrent use of nephrotoxic drugs, hepatitis B infection, and diabetes mellitus.
doi:10.1371/journal.pone.0029977.t004

Table 3. Multivariate analysis to estimate the risk of TDF- over ABC-based antiretroviral therapy in inducing more than 25% fall in eGFR.

	Model 1 Crude		Model 2 Adjusted		Model 3 Adjusted	
	HR	95% CI	HR	95%CI	HR	95%CI
TDF vs. ABC use†	1.747	1.152–2.648	1.893	1.243–2.881	2.080	1.339–3.232
Age per 1 year			1.029	1.010–1.048	1.020	1.000–1.040
Weight per 1 kg decrement†			1.046	1.022–1.071	1.028	1.005–1.052
CD4 count per 1 /μl decrement†					1.004	1.002–1.007
HIV viral load per log10/ml					1.048	0.749–1.466
Serum creatinine per 1 mg/dl†					0.053	0.009–0.304
Use of nephrotoxic drug					1.309	0.825–2.077
Hepatitis B					1.070	0.573–2.000
Diabetes mellitus					1.565	0.684–3.582

†P<0.05 in Model 3.
TDF: tenofovir, ABC: abacavir, eGFR: estimated glomerular filtration rate, HR: hazard ratio, CI: confidence interval.
doi:10.1371/journal.pone.0029977.t003

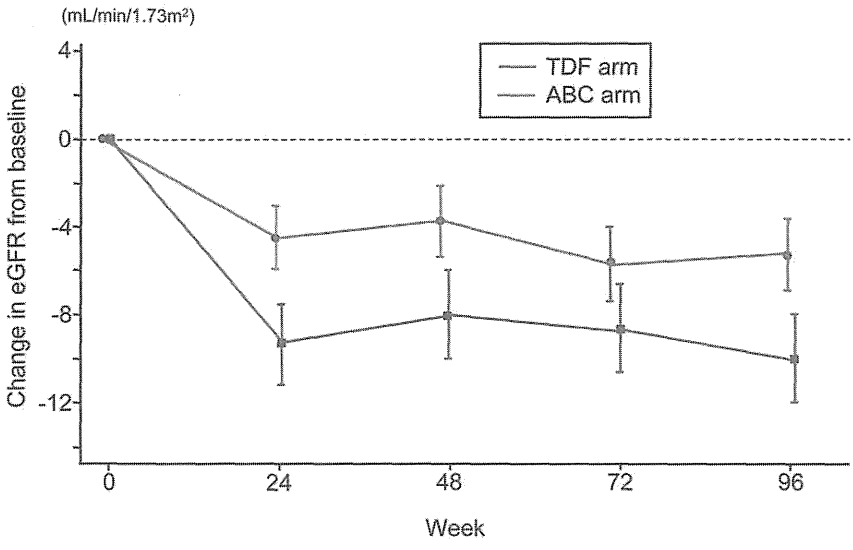

Figure 2. Changes in eGFR in patients treated with TDF or ABC between baseline and 96 weeks. The fall in eGFR was significantly greater in the TDF group than the ABC group ($p = 0.003$). Data are adjusted mean ±95% confidence interval. eGFR: estimated glomerular filtration rate, TDF: tenofovir, ABC: abacavir.
doi:10.1371/journal.pone.0029977.g002

results of the present study on TDF-related nephrotoxicity differ from the findings of randomized clinical trials that demonstrated no major change in renal function of TDF- and ABC-treated patients over 48–96 week follow-up [2,10,11]. The discrepant results might arise from differences between observational cohort and clinical trials, since observational studies tend to express the results in "real world setting" whereas clinical trials include patients who fulfill more strict criteria, therefore with better profile [9]. The discrepant results could be also due to the use of different definitions for renal dysfunction in these studies. However, the discrepant results could also reflect the difference in median body weight between the present study and these clinical trials. The results of our subgroup analysis support this hypothesis by showing that the effect of TDF on renal dysfunction was more evident in patients with low body weight. Apart from being low-body-weighted, the patients in this study did not appear to have many of other established risks for TDF-related nephrotoxicity; they were comparatively young, had relatively stable CD4 count, and had only a few co-morbidities (Table 1). Although the majority concurrently used ritonavir-boosted PIs, which are a probable risk for TDF-related nephrotoxicity, ritonavir-boosted PIs were not significantly associated with renal dysfunction in our cohort (Table 2) [24].

Changes in eGFR in those patients treated with TDF-containing ART were characterized by a rapid decline during the first 24 weeks of therapy, followed by a plateau until 96 weeks (Fig. 2). This finding is consistent with that reported from the Johns Hopkins group [9,28]. Together with the finding that the median time from commencement of ART to the >25% decline in eGFR in the TDF-treated patients was 246 days, these results suggest that careful monitoring of renal function is particularly warranted in the first year of TDF-based therapy. Thus, we suggest that renal function should be monitored by measurement of serum creatinine at least once annually in resource-limited settings and twice annually in resource-rich settings in patients starting TDF-containing ART, especially those with baseline body weight <60 kg.

The Department of Health and Human Services guideline for the treatment of HIV infection in the U.S. lists ABC as an alternative NRTI because it can potentially cause serious hypersensitivity reaction and cardiovascular diseases (URL:http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf). However, some international guidelines consider both TDF and ABC as the preferred NRTIs under the condition that ABC should be used with caution in patients with viral load >100,000 copies/mL, based on the low incidence of ABC-related hypersensitivity among HLA-B*5701-negative population and the controversial association between ABC and cardiovascular diseases [1,29–32] (URL: http://www.europeanaidsclinicalsociety.org/images/stories/EACS-Pdf/1_treatment_of_hiv_infected_adults.pdf) (http://www.haart-support.jp/guideline2011.pdf. in Japanese). The present study, together with our previous analysis that demonstrated preferential TDF-related nephrotoxicity in patients with low body weight, emphasize the advantage of ABC over TDF with regard to prognosis of renal function in low body weight patients [16].

TDF is the prodrug of acyclic nucleotide analog tenofovir, which is excreted by both glomerular filtration and active tubular secretion. Tenofovir is considered to cause mitochondrial damage in proximal renal tubular cells [33]. The concentration of tenofovir in the proximal renal tubules could be augmented with the complex interactions of pharmacological, environmental, and genetic factors, including small body weight, consequently resulting in renal tubular dysfunction [34]. Body weight has been identified as an important factor in TDF-related nephrotoxicity not only in clinical trials, but also in *in vitro* and pharmacokinetic studies [35–37].

The present study has several limitations. First, because of its retrospective nature, it was not possible to control the baseline characteristics of the enrolled patients. Thus, it is possible that patients with potential risk for TDF-related nephrotoxicity were not prescribed TDF. A proportion of patients treated with ABC had low CD4 count and others were hypertensive, both conditions are known risk factors for renal dysfunction [23,25]. However, for these reasons, the incidence of TDF-associated renal dysfunction might have been underestimated. Second, the definition of TDF-related nephrotoxicity, especially the criteria used to evaluate proximal renal tubular damage, is not uniformly established in the field and is different in the published studies. Accordingly, we

decided to adopt changes in eGFR, instead of parameters for proximal renal tubular damage. Using the eGFR as a marker for TDF-associated renal dysfunction, our results might have underestimated the incidence of TDF-related renal tubular dysfunction. However, the result of this study could be informative to resource-limited settings, where it is difficult to evaluate renal tubular markers. The rational and limitation of adopting more than 25% decrement in eGFR as the criterion for renal dysfunction were discussed in detail in our previous study [16]. Third, our cohort was characterized by the high prevalence of ritonavir-boosted PI use, which is considered by some groups a risk for TDF-related nephrotoxicity [24]. While it is difficult to completely exclude the impact of concurrent ritonavir-boosted PI in this study, it should be noted that the use of ritonavir-boosted PIs did not correlate with renal dysfunction in univariate analysis in this cohort (Table 2). Fourth, the study subjects were mainly men (mostly men who have sex with men and very few injection drug users). Further studies are needed to determine whether the findings of this study are also applicable to females, patients with different route of transmissions, and patients of different racial background.

In conclusion, the present study demonstrated a high incidence of renal dysfunction with TDF use, compared to ABC, among treatment-naïve patients with low body weight. TDF use was identified as an independent risk for renal dysfunction in a statistical model that included TDF as a primary exposure. At 96 weeks, patients with TDF showed greater eGFR decrement than patients treated with ABC. TDF is certainly a drug of choice in the treatment of HIV infection, but the importance of close monitoring of renal function in patients with small body weight, especially those with baseline body weight <60 kg should be emphasized for early detection of TDF-related nephrotoxicity. Further studies are warranted to elucidate the long-term prognosis of renal function with TDF use in patients with low body weight.

Acknowledgments

The authors thank Fumihiko Hinoshita, Ai Hori, Daisuke Tasato, Mahoko Kamimura, Kunio Yanagisawa, Daisuke Mizushima, Yohei Hamada, Aki Hashimoto, Akio Chiba, Yuko Yamauchi, Taiichiro Kobayashi, Kumi Tamura, and all other clinical staff at the AIDS Clinical Center for their help in completion of this study.

Author Contributions

Conceived and designed the experiments: TN HK HG TS EK JT SO. Performed the experiments: TN HK TS TA KW EK MH. Analyzed the data: TN HK HG TS HH HY K. Tsukada MH K. Teruya YK. Contributed reagents/materials/analysis tools: TA KW HH JT HY K. Tsukada MH K. Teruya YK. Wrote the paper: TN HK HG TS EK SO.

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Biochemical and Biophysical Research Communications 421 (2012) 291–295

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Minor contribution of HLA class I-associated selective pressure to the variability of HIV-1 accessory protein Vpu

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ARTICLE INFO

Article history:
Received 17 March 2012
Available online 7 April 2012

Keywords:
HIV/AIDS
Vpu
HLA class I
Viral evolution

ABSTRACT

Host HLA class I (HLA-I) allele-associated immune responses are major forces driving the evolution of HIV-1 proteins such as Gag and Nef. The viral protein U (Vpu) is an HIV-1 accessory protein responsible for CD4 degradation and enhancement of virion release by antagonizing tetherin/CD317. Although Vpu represents one of the most variable proteins in the HIV-1 proteome, it is still not clear to what extent HLA-I influence its evolution. To examine this issue, we enrolled 240 HLA-I-typed, treatment naïve, chronically HIV-infected subjects in Japan, and analyzed plasma HIV RNA nucleotide sequences of the vpu region. Using a phylogenetically-informed method incorporating corrections for HIV codon covariation and linkage disequilibrium among HLA alleles, we investigated HLA-associated amino acid mutations in the Vpu protein as well as in the translational products encoded by alternative reading frames. Despite substantial amino acid variability in Vpu, we identified only 4 HLA-associations in all possible translational products encoded in this region, suggesting that HLA-associated immune responses had minor effects on Vpu variability in this cohort. Rather, despite its size (81 amino acids), Vpu showed 103 codon–codon covariation associations, suggesting that Vpu conformation and function are preserved through many possible combinations of primary and secondary polymorphisms. Taken together, our study suggests that Vpu has been comparably less influenced by HLA-I-associated immune-driven evolution at the population level compared to other highly variable HIV-1 accessory proteins.

1. Introduction

Immune-mediated adaptation occurs during an HIV-1 infection. The HLA class I (HLA-I)-restricted $CD8^+$ cytotoxic T lymphocyte (CTL) response is one of the major forces driving HIV evolution, resulting in the selection of CTL escape mutants [1,2]. Despite the extensive genetic diversity of both HIV-1 and HLA-I alleles, escape pathways are reproducible and broadly predictable based on host HLA-I alleles [3–6]. Moreover, analysis of linked HLA-I and HIV datasets from large cohorts of HIV-infected subjects has facilitated our ability to map the landscape of immune escape mutations across HIV-1, identify immunogenic regions, and identify novel CTL epitopes [3,7].

Viral protein U (Vpu) is an accessory protein that is unique to HIV-1 and a subset of related simian immunodeficiency viruses. The HIV-1 Vpu protein has two major functions: degradation of newly synthesized CD4 molecules in the endoplasmic reticulum and enhancement of the release of progeny virions from infected cells by antagonizing tetherin/CD317, a host restriction factor that directly binds and retains viral particles on the surface of infected cells (reviewed in [8,9]). As such, Vpu is thought to play a role in virus spread and pathogenesis *in vivo*. Interestingly, Vpu is the most variable protein among all HIV proteins as evidenced by a cross-sectional comparison of HIV-1 sequences isolated from HIV-infected individuals [10], raising the possibility that Vpu undergoes adaptation in response to host immune responses. However, Vpu has been shown to be a minor target for CTLs as revealed by IFN-γ Elispot assays with overlapping peptides based on the subtype B consensus sequence [11]. Considering the highly variable nature of Vpu, it is possible to miss responses if the autologous virus sequence is markedly different from the peptide sequence when using this Elispot assay system [12].

In the present study, we sought to identify HLA-associated polymorphisms in Vpu and alternate reading frames and examine to

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.04.002

what extent they are involved in Vpu amino acid variability at the population level. We utilize a published phylogenetic dependency network model [13], a comprehensive evolutionary model that considers all important confounding effects such as HIV phylogeny, HIV codon covariation, and linkage disequilibrium of HLA alleles.

2. Materials and methods

2.1. Patient samples

A total of 240 chronically HIV-1-infected, treatment-naïve subjects (CD4, median 237; IQR, 160-397; viral load, median 33,200; IQR, 222,000-55,400) followed at the AIDS Clinical Center, International Medical Center of Japan were enrolled in this study. All participants provided written informed consent. HLA-I typing was performed as previously described [14]. The most frequently observed *HLA-A, B,* and *C* alleles in this cohort were *HLA-A*24:02*, *HLA-B*52:01*, and *HLA-C*01:02*, respectively, consistent with HLA class I allelic frequencies of the Japanese people [14].

2.2. Sequence analysis of vpu

HIV-1 particles were precipitated by ultracentrifugation (50,000 rpm, 15 min) of patients' plasma, after which the viral RNA was extracted using standard methods. Following reverse transcription, DNA fragments encoding Vpu proteins were amplified by nested PCR, and gel purified as previously described [15,16]. The primers used were as follows: the primers for the first round of amplification were VVVa-F (5′-TTAAAAGAAAAGGGGG GATTGGGGG-3′) and VVVb-R (5′-ATTCCATGTGTACATT GTACTGT-3′); and those for the second round, VVVc-F (5′-AGATAATAGTGAC ATAAAAGTAGTGCCAAGAAG-3′) and VVVd-R (5′-CCATAATAGACT GTGACCCACAA-3′). The *vpu* sequence was then directly analyzed with an automated sequencer (Applied Biosystems 3500xL) and aligned to the *vpu* sequence of the HIV-1 subtype B reference strain HXB2 (Accession No. K03455). More than 90% of the subjects were infected with subtype B, as determined by phylogenetic analysis of concatenated sequences of *vif*, *vpr*, and *vpu* reading frames.

2.3. Analysis of amino acid sequence variability

A Shannon entropy score for each position in the Vpu protein was calculated and used to analyze amino-acid sequence variability, as described previously [10]. Entropy is a measure of the amino acid variability at a given position that takes into account both the number of possible amino acid residues allowed and their frequency.

2.4. Analysis of association between Vpu sequence polymorphisms and host HLA class I alleles

To identify HIV-HLA polymorphism associations, we employed a phylogenetically dependency network model [13], which comprehensively includes all confounding effects of the analysis, such as HIV founder effects, HIV codon co-variation, and linkage disequilibrium of HLA-I alleles. Multiple comparisons are addressed using q-values (refer the detailed methods given in refs. [4,5,13]); in the present study, a cutoff of $q < 0.2$ was used to denote statistically significant associations. HLA-associated polymorphisms were classified into two categories. "Nonadapted" amino acids are enriched in the absence of the restricting HLA of interest. Usually, "nonadapted" forms represent the consensus amino acid at that position, and they can be thought of as the "wild-type" or "susceptible" form particular to that allele. Conversely, "adapted" amino acids are those enriched in the presence of the HLA allele; these can be thought of as the escape variant particular to that allele.

3. Results and discussion

3.1. Genetic variability of the vpu gene

We successfully amplified DNAs encompassing the *vpu* region from 216 of 240 samples (90%). Firstly, we analyzed the amino acid variability at each codon of Vpu by determining its Shannon entropy score. Two amino acid residues, Trp23 and Arg49, showed highly conserved (>98%) among individual sequences. Instead, most codons displayed substantial variability, with the average of the entropy score reaching 0.58 (Fig. 1A), confirming the findings by Yusim et al., which showed that Vpu is a highly variable protein [10]. Also, the amino acid variability of each codon in the present study correlated strongly with that of published subtype B sequence data from the Los Alamos database (Fig. 1B), suggesting that our observed pattern of amino acid variation in Vpu was generally representative of the variation observed in HIV-1 subtype B. In fact, the consensus amino acid sequences of subtype B and the present dataset were identical except for 3 amino-acid residues: positions 3, 5, and 24 (Fig. 1C). These amino acid residues were highly variable (Fig. 1A) and not directly associated with known Vpu functions (Fig. 1C).

3.2. HLA-associated polymorphisms in Vpu

As HLA-I-mediated selective contributes to HIV-1 sequence variability, especially the accessory protein Nef [4], we sought to examine whether HLA-I-mediated selective pressure substantially influenced the evolution of Vpu, another accessory protein. We applied a phylogenetic dependency network model [13], which adjusts for the confounding effects of HIV phylogeny, HIV codon covariation and linkage disequilibrium of HLA-I alleles.

In our dataset of 216 individuals, we identified only three HIV-HLA associations in Vpu: a nonadapted association between C*03 and Glu-5, a nonadapted association between A*33:03 and Arg-37, and an adapted association between A*33:03 and Lys-37. The presence of both nonadapted and adapted A*33:03-associated polymorphisms at Vpu codon 37 is consistent with an Arginine-to-Lysine escape mutation occurring at the C-terminus of the immunodominant HLA-A*33:03-restricted epitope in Vpu, ^{29}EYRKILRQR^{37} [11]. However, there was no HLA-restricted T cell epitopes around Vpu position 5 have been reported. Although we might have missed some polymorphisms due to the limited sample size in this study, these data suggest that HLA-I-mediated selective pressure toward Vpu does not substantially drive Vpu variability at the population level in this cohort.

3.3. HLA-associated polymorphisms in alternating reading frames

CTLs can recognize epitopes encoded by alternate reading frames including the antisense-strand sequences of HIV-1 *gag*, *pol*, and *nef* [17,18]. Therefore, we also investigated HIV-HLA polymorphism associations in peptide sequences encoded by alternative reading frames of the *vpu* gene. We observed no statistically significant HLA-associated polymorphisms in alternate or antisense reading frames, except for a single HLA-B*40:01 associated "adapted" lysine polymorphism at codon 2 of the overlapping Envelope reading frame which is initiated in the middle of the *vpu* gene (ORF + 2; Table 1, Fig. 2). Although this association was between Lys-2 of Env and *HLA-B*40:01*, no CTL epitopes have been reported in the context of HLA-B*40:01 in this region. Using bioinformatic prediction programs Epipred [19] and BIMAS [20] we

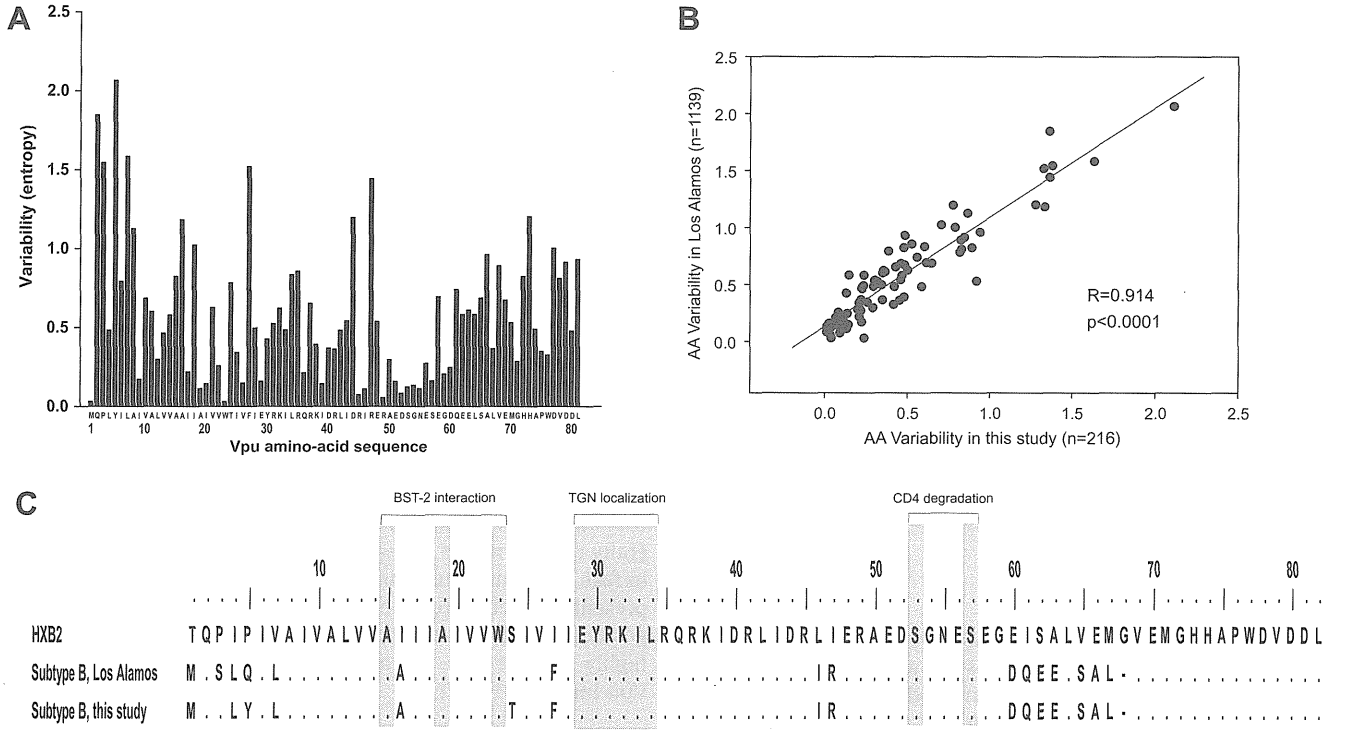

Fig. 1. Variability of the amino acid residues of HIV-1 Vpu. The amino acid sequence of Vpu was analyzed based on the cross-sectional studies on 216 HIV-infected subjects. The amino acid variability at each position of Vpu was analyzed by determining its Shannon entropy score (panel A). The Vpu sequence (subtype B, $n = 1139$) was retrieved from the Los Alamos HIV sequence database, analyzed for its amino acid variability, and compared with subtype B obtained from this study using Spearman Rank Order Correlation (panel B). The consensus sequences of Vpu obtained from Los Alamos database and this study were aligned with reference strain HXB2 and regions responsible for some key Vpu functions highlighted (panel C).

Table 1
Summary of HIV-HLA associations in the Vpu-encoded region.

RF	Protein	Pos HXB2	aa	HLA	Association	*p*-Value	*q*-Value	Known epitope	
								Sequence	Reference
+1	Vpu	5	E	C*03	Nonadapted	2.13×10^{-5}	1.52×10^{-1}	none	–
		37	R	A*22:03	Nonadapted	3.40×10^{-6}	5.50×10^{-2}	^{29}EYRKILRQR^{37}	[11]
		37	K	A*33:03	Adapted	2.80×10^{-5}	1.52×10^{-1}	^{29}EYRKILRQR^{37}	[11]
+2	Env	2	K	B*40:01	Adapted	1.63×10^{-5}	1.67×10^{-1}	none	–

RF, reading frame; Pos HXB2, amino acid position when aligned to HXB2 sequence.

Fig. 2. The Vpu and a part of Env proteins and their associations with host HLA class I alleles. The nucleotide sequence and its deduced amino acid sequence of Vpu and of an overlapping part of Env with reference to the subtype B consensus sequence of Los Alamos database is shown. The amino acid residues associated with the indicated HLA class I alleles ($p < 0.05$, $q < 0.2$) are shown with adapted (red) and nonadapted (blue) residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

attempted to predict B*40:01-restricted CTL epitopes, but found none (data not shown). This failure is most likely due to the presence of several basic amino acids, such as Arg and Lys, in this region of Env (Fig. 2), as it has been shown that HLA-B*40:01

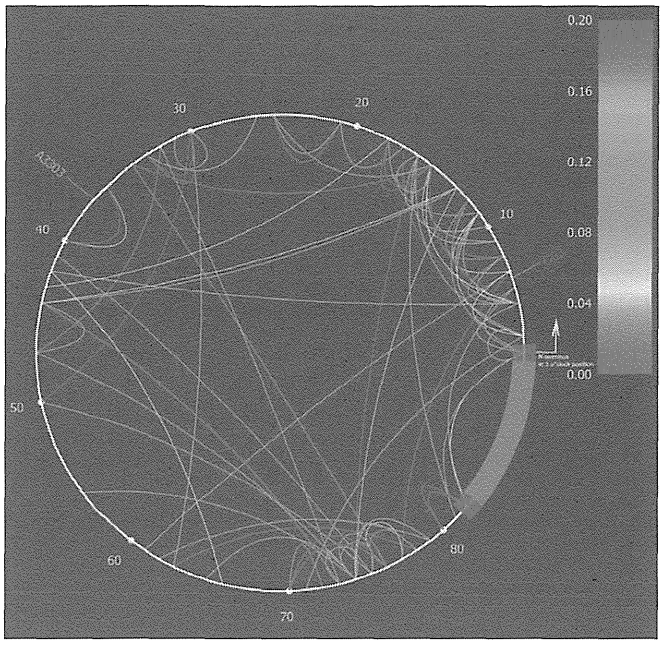

Fig. 3. Amino acid codon–codon covariation in Vpu. The circular map, generated by the PhyloDv software [13], shows Vpu codon–codon covariation associations as inner arcs connecting the association sites, with the HLA associations as tags pointing to their corresponding sites. Q values of individual codon pairs are represented as a heat map shown at the right.

preferentially binds peptides with acidic residues at their anchors [21]. This issue needs to be clarified in further studies using immunologic assays. Taken together, our results suggest that HLA-I-mediated selective pressure do not contribute to a large extent to population-level sequence variation in HIV-1 Vpu.

3.4. Codon–codon covariation of Vpu

Given that Vpu is functionally important in viral replication *in vivo*, the highly variable nature of Vpu amino acid sequences could be explained by complex networks of codon–codon covariation and/or secondary/compensatory mutation pathways. We therefore examined the codon–codon covariation of Vpu by using the phylogenetic dependency network model. Although Vpu consists of only 81 amino acids, we identified 103 covarying codon pairs in Vpu, displayed in Fig. 3. The covariation network in Vpu showed an uneven distribution, with a large number of codon–codon covariation networks at the N-terminal membrane-spanning region, a region responsible for BST-2 interaction [22]. Interestingly, the 3 HIV-HLA associations (Table 1, Fig. 2) were not significantly linked to any other amino acid residues. These data suggest that the conformation and function of Vpu may be preserved through many possible combinations of primary and secondary polymorphisms and that the HLA-I-associated immune-mediated selective pressure may have only a minor effect on such Vpu polymorphisms.

3.5. Association between Vpu polymorphisms and clinical parameters

Finally, we explored associations between Vpu polymorphisms and clinical parameters of HIV-infected patients (i.e., CD4 counts and plasma viral load). We observed no significant associations between Vpu polymorphisms and CD4 counts. However we identified a statistically significant association between amino acid residues at position 5 and viral load (Fig. 4). The patients harboring Val at Vpu-5 had significantly higher viral loads compared to those with amino acid residues other than Val at this position. Thus, amino acid residues at position 5 of Vpu showed several interesting features, i.e., the highest variability of all Vpu amino acids (Fig. 1A), nonadapted association of Glu-5 with *HLA-Cw*03*, and association of Val-5 with the increased viral load. Considering that the amino acid residue at this position is located in close proximity to the membrane-spanning region and that this region is functionally important for BST-2 binding, it would be interesting to examine functional effects of amino acid polymorphisms at position 5, whether they are mediated by host immune responses or otherwise.

In summary, we report here comparably fewer HLA-associated mutations in Vpu in this cohort although host HLA class I allele-associated immune responses are major forces driving the evolution of HIV-1 accessory proteins, such as Nef. Taken together, we conclude that the influence of immune selection on evolution of Vpu

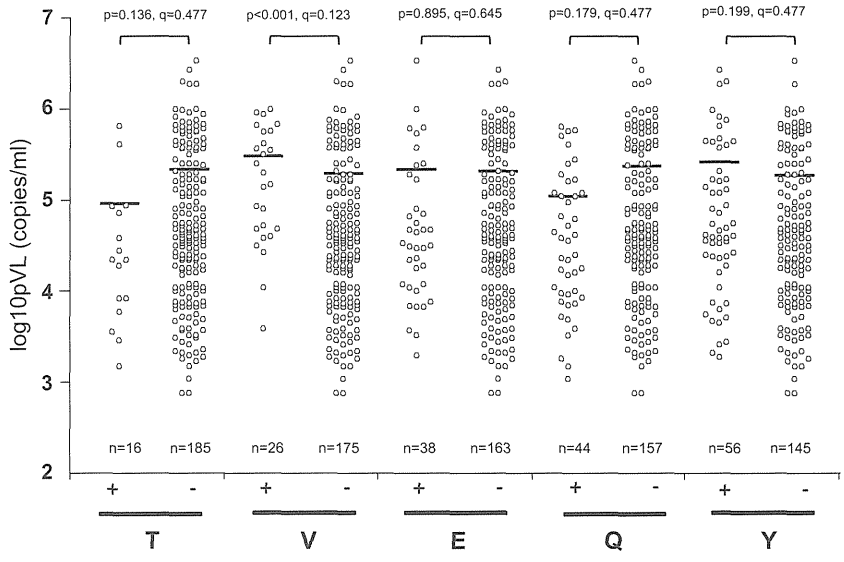

Fig. 4. Association between plasma viral load and amino acid polymorphism at position 5 of Vpu. HIV plasma viral loads, stratified by amino acid expression at Vpu codon 5, are shown. Vpu codon 5 exhibited 11 different amino acids positioning in our dataset; only those observed in >10 patients are shown here. Horizontal bars indicate medians. Statistical analysis was performed using the Mann-Whitney U-test.

at the population level may be reduced compared to other highly variable HIV-1 proteins, providing us with additional insight into differential evolutional pathways among viral accessory proteins.

Acknowledgments

This research was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture (MEXT) of Japan, by a Global COE Program (Global Education and Research Center Aiming at the control of AIDS), MEXT, Japan, and by a grant-in-aid for AIDS research from the Ministry of Health, Labor, and Welfare of Japan. Z.H. is supported by the scholarship for The International Priority Graduate Programs; Advanced Graduate Courses for International Students (Doctoral Course), MEXT, Japan. Z.L.B. is the recipient of a New Investigator Award from the Canadian Institutes of Health Research.

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HLA Class I-Mediated Control of HIV-1 in the Japanese Population, in Which the Protective HLA-B*57 and HLA-B*27 Alleles Are Absent

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We investigated the effect of HLA class I alleles on clinical parameters for HIV-1 disease progression in the Japanese population, where two strongly protective alleles, HLA-B*57 and HLA-B*27, are virtually nonexistent. HLA-B alleles showed a dominant role, primarily through HLA-B*67:01 and the HLA-B*52:01-C*12:02 haplotype. Neither a rare-allele nor a heterozygote advantage was found, suggesting that the effect of HLA alleles in the Japanese population is either different from those observed in Africans and Caucasians or undetectable due to limited power.

The presence of particular HLA class I alleles is associated with the rate of progression to AIDS and/or with clinical markers of disease progression, such as viral load (VL) and CD4 T cell count (2, 3, 7, 11, 13). HLA-B*57 and HLA-B*27 are well-known to associate with successful control of HIV-1 or slow progression to disease in Caucasians and Africans (1, 4, 5, 9–11) but not in Asians, since the frequencies of these alleles are very low (<1%) in this population. There is growing evidence that HIV-1-specific CTL responses restricted by HLA-B allotypes play a key role in determining disease outcomes relative to HLA-A and -C (7, 9), but it remains possible that the association between HLA-B and HIV-1 control is biased by the extremely strong effects of only a few HLA-B alleles, particularly the protective HLA-B*57 and -B*27 alleles and the detrimental HLA-B*58:02 and -B*35:02/B*35:03 alleles (6–8). The exceptional protection afforded by HLA-B*57 and -B*27 may mask weaker effects of other HLA alleles, in part due to the potential for inappropriate correction for the effects of these two alleles. Since both of these alleles are virtually nonexistent (i.e., present in 0% and 0.5% of the population, respectively) in Japan (12), studies of Japanese cohorts provide an unbiased means of determining the effects of other HLA alleles on HIV control. Therefore, we analyzed the association of HLA alleles with markers of disease progression, VL and CD4 count, in Japanese individuals chronically infected with HIV-1.

We recruited 504 chronically HIV-1-infected, antiretroviral therapy (ART)-naïve Japanese individuals (464 men and 40 women) who visited our hospital during 2000-2010 and who did not meet any criteria for clinical AIDS. The median (interquartile ranges) VL and CD4 count are 35,000 (9,175/100,000) and 288 (183/402), respectively. HLA alleles of these individuals were determined at the 4-digit level. Associations between HLA alleles and VL or CD4 count obtained at the first visit were determined among these individuals. We considered 48 HLA class I alleles occurring at a frequency of greater than 1% (11 HLA-A, 22 HLA-B, and 15 HLA-C), which excluded both B*57 (0 identified) and B*27:05 (1 identified) (see Table S1 in the supplemental material). The differential contribution of HLA-A, -B, and -C alleles on VL in the cohort as a whole was determined using the Kruskal-Wallis statistic H, which is a nonparametric measure of variation between data groups (i.e., among alleles at a given locus). The range of effects (protective to susceptible) across alleles at each given class I locus on VL was largest for HLA-B ($H = 81$, $P = 0.0005$) but was also significant for both HLA-A ($H = 37$, $P = 0.006$) and HLA-C ($H = 52$, $P = 0.0001$) (Table 1). HLA-B also showed the greatest range of effects on CD4 counts, and for this outcome, it was the only locus that showed significance ($H = 71$, $P = 0.004$ for HLA-B; $H = 18$, $P = 0.43$ for HLA-A; $H = 29$, $P = 0.084$ for HLA-C). These results indicate that the HLA-B locus has the greatest effect on HIV-1 control in Japanese and confirmed previous findings in Caucasians and Africans.

We found that 36% (4 of 11) of HLA-A alleles, 41% (9 of 22) of HLA-B alleles, and 27% (4 of 15) of HLA-C alleles were associated significantly with VL before correction for multiple tests (Fig. 1). Associations with CD4 count were observed for a more limited number of HLA class I alleles than those with VL: 18% (2 of 11) for HLA-A, 18% (4 of 22) for HLA-B, and 13% (2 of 15) for HLA-C (Fig. 1). Three alleles, HLA-B*67:01, -B*52:01, and -C*12:02, were significantly associated with both low VL and high CD4 count and two alleles, HLA-A*02:07 and -B*35:01, associated with both higher VL and lower CD4 count. After local false discovery rate estimation, the numbers of HLA alleles associated with VL and CD4 count were 14 and 5, respectively (see Table S2 in the supplemental material). HLA-B*52:01 and -C*12:02 form a known haplotype, which reaches a frequency of approximately 20% of the Japanese population (12), and given the protection associated with the individual alleles, it is possible that CTL or NK cell responses restricted by this haplotype play an important role in control of HIV-1 in Japanese. HLA-B*35:01 associated with high VL and low CD4 counts, which is consistent with data from Cauca-

Received 19 March 2012 Accepted 3 July 2012
Published ahead of print 18 July 2012
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Supplemental material for this article may be found at http://jvi.asm.org/.

doi:10.1128/JVI.00689-12

TABLE 1 Kruskal-Wallis test for associations of viral load and absolute CD4 count with alleles at three HLA class I loci in Japanese individuals chronically infected with HIV-1[a]

Allele (n)	VL		CD4 cell count	
	P	H	P	H
HLA-A (504)	**0.006**	37	0.43	18
HLA-B (504)	**0.0005**	**81**	**0.004**	**71**
HLA-C (503)	**0.0001**	**52**	0.084	29

[a] Values in bold are statistically significant.

sians, in which this allele was more commonly observed among HIV noncontrollers (5). On the other hand, HLA-B*35:02 and -B*35:03 associate with rapid disease progression in Caucasians, whereas HLA-B*35:01 does not (6), highlighting distinctions across allelic associations depending on the clinical outcome being considered. HLA-A*02:07 (frequency = 0.073), an allele that is

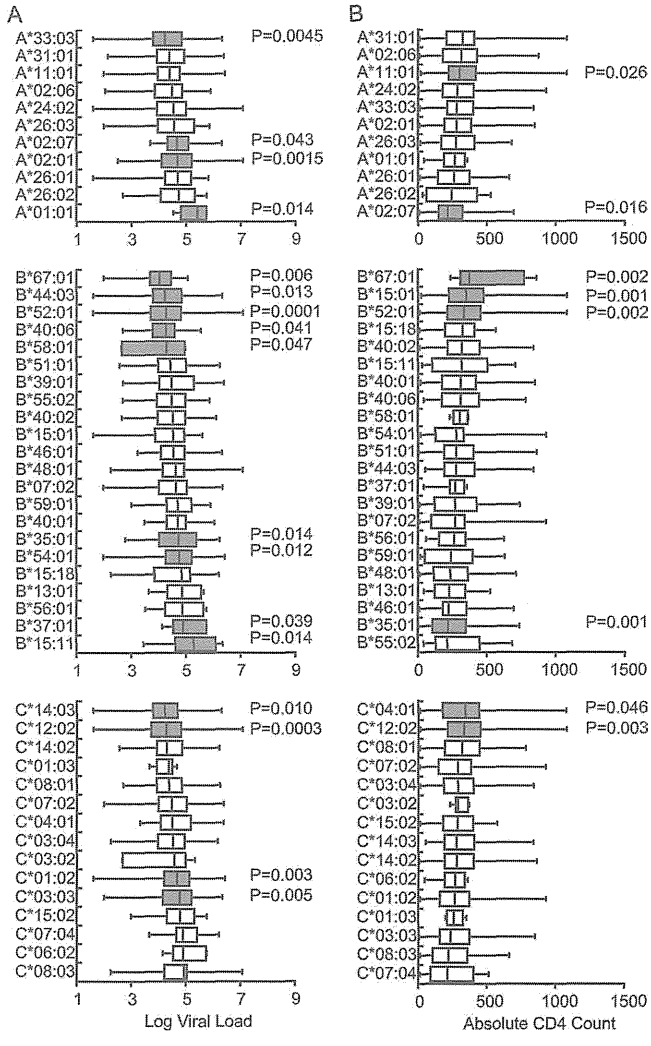

FIG 1 Association of HLA allele expression with viral load and absolute CD4 count in Japanese individuals chronically infected with HIV-1. All HLA alleles occurring at a phenotypic frequency greater than 1% were examined for their associations with viral load (A) and absolute CD4 count (B) in a cohort of 504 chronically HIV-1-infected Japanese individuals recruited from 2000 to 2010. Associations with a P value of <0.05 are highlighted in gray.

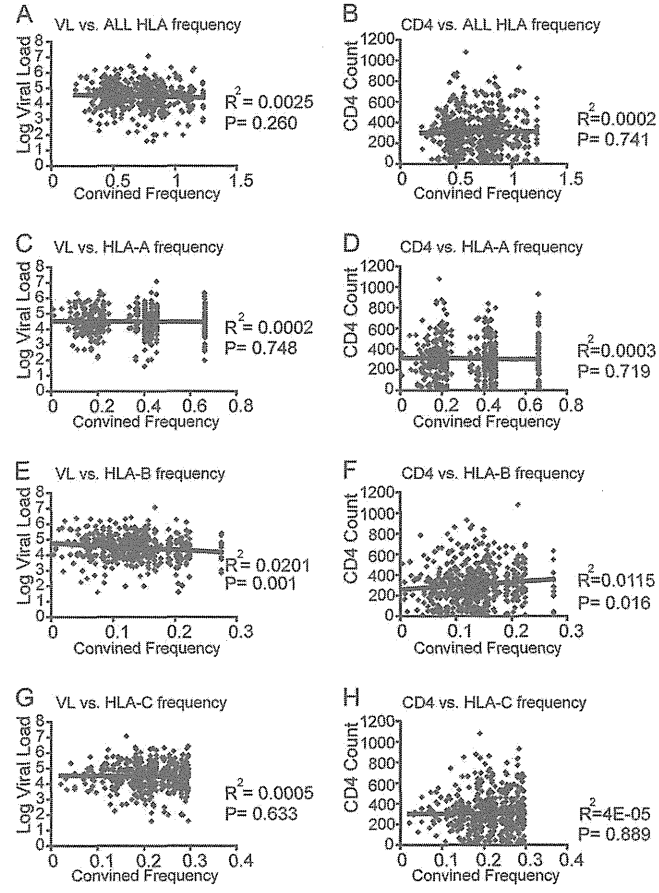

FIG 2 HLA frequency-dependent effects on viral load and CD4 counts. The correlations between VL or CD4 count with combined frequencies for all subjects (n = 504) are shown. HLA frequencies used to calculate the combined frequencies for each individual were derived from the overall allele frequencies in the entire cohort of 504 subjects. Linear regression lines are included in the plots. The distribution of HLA class I frequencies among 504 chronically HIV-1-infected Japanese individuals used in this analysis is shown in Table S4 in the supplemental material.

missing in Caucasians and Africans, also associated with susceptibility.

An advantage of rare HLA alleles in HIV-1 disease progression has been reported previously (13). Therefore, we investigated the effect of HLA frequency on VL or CD4 count in our cohort by comparing these clinical measurements first to the sum of the frequencies of the two alleles at each HLA locus individually (HLA-A, -B, and -C separately) and second to the sum of the frequencies of all HLA class I alleles (HLA-A, -B, and -C combined) for each subject. No significant correlation was observed between the overall HLA allele frequency and VL or CD4 count (Fig. 2A and B), nor was there a correlation between HLA-A allele frequencies or HLA-C allele frequencies with these clinical measurements (Fig. 2C, D, G, and H). In contrast to previous observations, the frequencies of HLA-B alleles were positively and negatively associated with CD4 count and VL, respectively (Fig. 2E and F). We also analyzed the effect of HLA supertype frequency on VL and found no effect (see Fig. S1 in the supplemental material). These results indicate no advantage of rare alleles on VL and CD4 count in the Japanese cohort. Further, we detected no significant

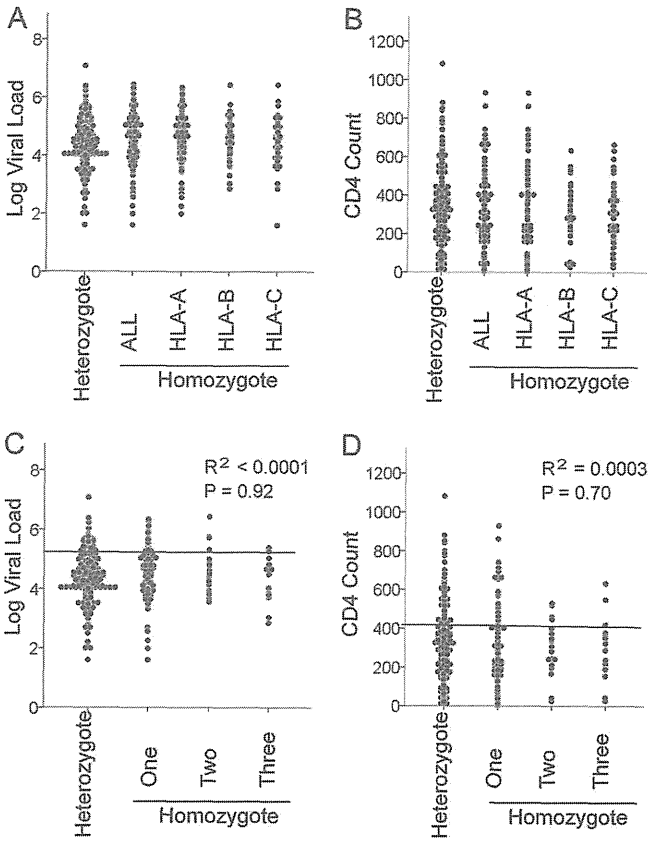

FIG 3 Effect of HLA class I homozygosity on VL and CD4. The comparison between heterozygotes at all three class I loci (n = 349) and homozygotes (n = 155) for at least one HLA allele is shown (A and B). (A and B) Homozygotes are grouped according to homozygosity for the HLA-A (n = 106), -B (n = 42), or -C (n = 64) locus. No significant difference was observed between these groups. Median VLs and CD4 counts, respectively, are 32,500 and 289 (fully heterozygous), 44,000 and 280 (HLA-A homozygous), 35,500 and 290 (HLA-B homozygous), 29,500 and 305 (HLA-C homozygous), and 42,000 and 288 (homozygous for at least one HLA locus). (C and D) Homozygotes were grouped according to homozygosity for one (n = 115), two (n = 23), or three (n = 17) HLA class I loci. Median VLs and CD4 counts, respectively, are 32,500 and 289 (fully heterozygous), 47,000 and 284 (homozygous at a single locus), 28,000 and 335 (homozygous at two loci), and 31,000 and 288 (homozygous at all three loci). No significant difference was observed between these groups. The lines in panels C and D are linear regression lines.

differences in either VL or CD4 count between heterozygotes and homozygotes at any individual HLA locus (Fig. 3A and B) or homozygosity at one, two, or all three class I loci (Fig. 3C and D). Thus, a heterozygote advantage of HLA class I was not observed in this cohort.

We also analyzed 147 ART-naïve Japanese individuals with clinical AIDS. There were no strong associations of HLA alleles with either VL or CD4 count (see Table S3 in the supplemental material). We excluded these individuals in the present study for the following reasons: (i) There were radical differences in VL and CD4 count between AIDS and non-AIDS groups, and (ii) since AIDS represents an effective breakdown of the immune response, a putative association observed in the non-AIDS group would not be comparable to that in the AIDS group even if HLA class I alleles had an effect on VL and CD4 count.

In summary, the HLA-B locus appears to have the strongest allelic effects on VL and CD4 counts in this Japanese cohort, with the HLA-B*52:01-C*12:02 haplotype showing the greatest significance. These findings in a Japanese cohort highlight the differences of the effects of HLA class I alleles on HIV-1 control between Japanese and Africans/Caucasians.

ACKNOWLEDGMENTS

This research was supported by the Global COE program "Global Education and Research Center Aiming at the Control of AIDS," launched as a project commissioned by the Ministry of Education, Science, Sports, and Culture, Japan, and by a grant-in-aid for scientific research from the Ministry of Health (no. H21-AIDS-010 and H24-AIDS-007), Japan. This project was funded in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract no. HHSN261200800001E. This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

We have no conflicting financial interests.

We thank Sachiko Sakai for her secretarial assistance.

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High Incidence of Renal Stones Among HIV-Infected Patients on Ritonavir-Boosted Atazanavir Than in Those Receiving Other Protease Inhibitor–Containing Antiretroviral Therapy

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(See the Brief Report by Rakotondravelo et al on pages 1270–2.)

Background. Little information is available on the incidence of renal stones with ritonavir-boosted atazanavir (ATV/r) use.

Methods. In a single-center study, the incidence of renal stones was compared between human immunodeficiency virus (HIV)–infected patients who commenced ritonavir-boosted atazanavir (ATV/r)–containing antiretroviral (ARV) therapy (the ATV/r group) and those who were receiving other protease inhibitors (the other PIs group). The effects of ATV/r were estimated by univariate and multivariate Cox proportional hazards regression models. Other possible risk factors were evaluated by univariate analysis, and those found to be significant were entered into multivariate analysis.

Results. Renal stones were diagnosed in 31 patients (23.7 cases per 1000 person-years) in the ATV/r group (n = 465) and 4 in patients (2.2 cases per 1000 person-years) in the other PIs group (n = 775). ATV/r use was significantly associated with renal stones, by univariate and multivariate analyses (adjusted hazard ratio, 10.44; 95% confidence interval [CI], 3.685–29.59; $P < .001$). ATV/r remained a significant risk factor for renal stones in all subgroups stratified by the median values of baseline variables. In the 31 patients receiving ATV/r who developed renal stones, the median time from commencement of ATV/r to diagnosis was 24.5 months (interquartile range, 14.7–34.6 months). Of the 18 patients who continued ATV/r despite the diagnosis of renal stones, 6 (33.3%) experienced recurrence. No patient who discontinued ATV/r experienced recurrence during the observation period (250.6 person-months).

Conclusions. The incidence of renal stones was substantially higher among patients in the ATV/r group, compared with patients in the other PIs group. Continuation of ATV/r after diagnosis of renal stones was associated with a high rate of recurrence. Switching ATV/r to other ARVs is warranted in patients who develop renal stones.

Ritonavir-boosted atazanavir (ATV/r) is a widely used protease inhibitor (PI) in combination with other antiretroviral drugs for patients infected with human immunodeficiency virus type 1 (HIV). According to the present guidelines, ATV/r is one of the key first-line drugs because of its high efficacy, tolerability, favorable lipid profile, and once-daily dosing [1–4]. However, renal stone formation has been reported in patients receiving ATV/r-containing antiretroviral therapy (ART) [5, 6].

Received 11 April 2012; accepted 19 June 2012; electronically published 19 July 2012.

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Clinical Infectious Diseases 2012;55(9):1262–9

DOI: 10.1093/cid/cis621

Urolithiasis is a well-known side effect of indinavir (IDV), and its etiology is considered to be drug crystallization in the urine [7]. Previous studies identified ATV-containing urolithiasis, suggesting a similar etiology [5, 6, 8, 9]. However, there is virtually no information on the incidence of ATV/r-induced renal stones, although ATV/r is one of the most frequently prescribed PIs. It is important to elucidate the incidence of ATV/r-associated renal stones, since renal stones are risk factors for chronic kidney diseases (CKD), an important comorbidity associated with AIDS and death [10–12].

On the basis of this background, we conducted a retrospective study to compare the incidence of renal stones among patients receiving an ATV/r-containing regimen with the incidence among patients receiving one of the following commonly used PIs: unboosted fosamprenavir (FPV), ritonavir-boosted fosamprenavir (FPV/r), lopinavir/ritonavir (LPV/r), and ritonavir-boosted darunavir (DRV/r).

METHODS

Ethics Statement

This study was approved by the Human Research Ethics Committee of our hospital, the National Center for Global Health and Medicine, Tokyo. Each participant provided a written informed consent. 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-infected patients using the medical records at our hospital. Our facility is one of the largest clinics for patients with HIV infection in Japan, with >2700 registered patients. The study population was HIV-infected patients aged >17 years who commenced treatment with ART containing ATV/r, FPV/r, FPV, LPV/r, or DRV/r between 1 January 2004 and 30 June 2010. Both treatment-naive and treatment-experienced patients were included. The follow-up period started at the time of commencement of ART that contained the above-mentioned drug for the first time during the study period, and patients were followed until 30 June 2011. Patients were excluded if (1) they had started the above-mentioned ART during the study period at other facilities, (2) they were prescribed unboosted ATV, or (3) they were receiving treatment for urolithiasis at the time they commenced the above-mentioned ART. Patients with previous exposure to one of the above-mentioned drugs before the present study and commenced the same drug in this study were also excluded from the analysis.

The attending physician selected ATV/r, FPV, FPV/r, LPV/r, or DRV/r at baseline. The use of these drugs was based on the Japanese guidelines, which placed all of the above-mentioned drugs as the preferred choice, at least for 3 years during the study period [13]. The attending physician also selected the concurrent drugs, including nucleoside reverse-transcriptase inhibitors (NRTIs), non-NRTIs (NNRTIs), integrase inhibitors, and CCR5 inhibitors. None of the patients received 2 PIs during the study period.

Measurements

The primary investigator (Y. H.) reviewed the medical records of all study patients who started new key drugs during the study period, to identify renal stone cases. Then 2 experienced HIV physicians (T. N. and K. W.) reviewed the set of medical records of each renal stone case to determine whether the cases fit into the following predefined criteria for renal stones: 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; or (3) stone passage confirmed by either the patient or the attending physician. Patients with acute flank pain due to etiologies other than renal stones were excluded. In case of disagreement between the 2 reviewers, a third independent reviewer (H. K.) evaluated the deidentified document set by the same criteria to make the final determination. At the time of diagnosis of renal stones, the attending physician selected either continuation or modification of ART. In our clinic, it is customary for the patient to visit the clinic every month before the initiation of ART and until suppression of HIV load, but the visit interval is extended up to every 3 months after viral load suppression.

In this study, the primary exposure variable was ATV/r use over other PIs (FPV, FPV/r, LPV/r, and DRV/r). The potential risk factors for renal stones were determined according to previous studies and were collected from the medical records, together with the basic demographic characteristics [8, 9, 14]. They included age, sex, body weight, body mass index (BMI; defined as the weight in kilograms divided by the square of the height in meters), baseline laboratory data (CD4 cell count, HIV load, estimated glomerular filtration rate [eGFR], serum uric acid, and urine pH), and presence or absence of other medical conditions (ie, concurrent use of tenofovir [TDF]; past history of renal stones; previous exposure to IDV; coinfection with hepatitis B virus [HBV], defined by detection of HBV surface antigen; and coinfection with hepatitis C virus [HCV], defined by detection of HCV load). eGFR was calculated using the equation from the 4-variable Modification of Diet in Renal Diseases study [15]. Among patients receiving ATV/r-containing ART, the total serum bilirubin level measured on the day of stone diagnosis (for patients with renal stones) or 2 years after ATV/r initiation (for patients without

renal stones) was used. 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 ≤180 days.

Statistical Analysis

Baseline characteristics were compared using the unpaired Student *t* test or the χ^2 test (ie, the Fisher exact test) for quantitative or qualitative variables, respectively. The time to the diagnosis of urolithiasis was calculated from the date of commencement of predefined PI-containing ART to the date of diagnosis for urolithiasis. Censored cases represented those who discontinued the PIs, dropped out, were referred to other facilities, or at the end of the follow-up period. The time from the start of ART to the diagnosis of renal stones was analyzed by the Kaplan-Meier method for patients who started ATV/r (the ATV/r group) and those who started other PIs (the other PIs 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, compared with other PIs, on the incidence of renal stones. The impact of basic demographic characteristics, 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 other PIs for renal stones, we conducted 3 models using multivariate Cox proportional hazards regression analysis. Model 1 was the aforementioned univariate analysis for ATV/r use over other PIs. 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 of < .05 in univariate analysis after adjustment (these included eGFR per 10 mL/min/1.73 m^2 and serum uric acid per 1 mg/dL). Possible risk factors for ATV/r-induced renal stones identified in previous studies were also added to model 3 (these included past history of renal stones and prior exposure to IDV) [8, 9].

To elucidate whether the impact of ATV/r on renal stones persist in subgroups, we divided patients into 2 groups on the basis of sex, age, baseline body weight, eGFR, and serum uric acid level, using the respective median value of each parameter. Then, the above-mentioned univariate analysis was conducted for each subgroup. In addition, to examine the association between total serum bilirubin level during ATV/r-containing ART and the incidence of renal stones, the median total serum bilirubin levels were compared between stone cases and nonstone cases, using the Mann-Whitney *U* test.

To explore the impact of urolithiasis on renal function, the change in eGFR was compared between stone cases (ie, the eGFR change between baseline and the diagnosis of renal stones) and nonstone cases (ie, the eGFR between baseline and 2 years after initiation of ATV/r) in patients receiving ATV/r, using the Student *t* test.

Statistical significance was defined as a 2-sided *P* value of < .05. We used hazard ratios (HRs) and 95% confidence intervals (CIs) to estimate the impact of each variable on renal stones. All statistical analyses were performed with SPSS, version 17.0 (SPSS, Chicago, IL).

RESULTS

A total of 1498 patients commenced or switched key drugs (PIs, NNRTIs, or an integrase inhibitor) between 1 January 2004 and 30 June 2010. Of the 1240 patients who were included in the analysis, 465 (37.5%) started ATV/r-containing ART, while 775 (62.5%) started other PI–containing ART (Figure 1). Table 1 shows the baseline characteristics of the study population. The majority of the study population was male, of East Asian origin, and comparatively young. The ATV/r group included significantly more patients of East Asian origin (*P* = .015) and had a significantly higher body weight (*P* < .001), higher CD4 cell count (*P* < .001), lower viral load (*P* < .001), higher baseline serum uric acid level (*P* = .034), and lower eGFR (*P* = .012). In contrast, patients in the other PIs group were significantly more likely to be treatment naive (*P* < .001) and significantly less likely to have had previous exposure to IDV (*P* = .036). However, all other major background parameters were similar in the 2 groups (Table 1).

The primary investigator (Y. H.) identified 37 renal stone cases, and 2 of these were excluded by the reviewers. Thirty-five patients fulfilled the predefined criteria for renal stones. Renal stones were identified in 31 patients (6.7%) from the ATV/r group and in 4 (0.52%) from the other PIs group, with an estimated incidence of 23.7 cases and 2.20 cases per 1000 person-years, respectively. The incidence of renal stones in the ATV/r group was approximately 10 times the incidence in the other PIs group. Of those renal stone cases, 4 and 14 patients were diagnosed on the basis of hematuria and stone passage, respectively, as defined above. Furthermore, 17 cases were diagnosed on the basis of radiological findings, of which renal calcification was identified in 4 cases. Figure 2 is a Kaplan-Meier curve of the time from initiation or switching of PIs defined above to the diagnosis of renal stones in the 2 groups. Patients in the ATV/r group were significantly more likely to develop renal stones, compared with those of the other PIs group (*P* < .001, by the log-rank test). The median time from the commencement of ART to the diagnosis of renal stones was 24.5 months (interquartile range [IQR], 14.7–34.6 months) for the ATV/r group and 21.9 months (IQR, 10.1–45.1 months) for the other PIs group. The total observation period was 1310.1 patient-years (median duration, 31.0 months; IQR, 15.0–48.7 months) for the