MAJOR ARTICLE

Clinical Significance of High Anti-*Entamoeba histolytica* Antibody Titer in Asymptomatic HIV-1-infected Individuals

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Background. Anti-*Entamoeba histolytica* antibody (anti- *E. histolytica*) is widely used in seroprevalence studies though its clinical significance has not been assessed previously.

Methods. Anti-*E. histolytica* titer was measured at first visit to our clinic (baseline) in 1303 patients infected with human immunodeficiency virus type 1 (HIV-1). The time to diagnosis of invasive amebiasis was assessed by Kaplan-Meier method and risk factors for the development of invasive amebiasis were assessed by Cox proportional-hazards regression analysis. For patients who developed invasive amebiasis, anti-*E. histolytica* titers at onset were compared with those at baseline and after treatment.

Results. The anti-*E. histolytica* seroprevalence in the study population was 21.3% (277/1303). Eighteen patients developed invasive amebiasis during the treatment-free period among 1207 patients who had no history of previous treatment with nitroimidazole. Patients with high anti-*E. histolytica* titer at baseline developed invasive amebiasis more frequently than those with low anti-*E. histolytica* titer. Most cases of invasive amebiasis who had high anti-*E. histolytica* titer at baseline developed within 1 year. High anti-*E. histolytica* titer was the only independent predictor of future invasive amebiasis. Anti-*E. histolytica* titer was elevated at the onset of invasive amebiasis in patients with low anti-*E. histolytica* titer at baseline.

Conclusions. Asymptomatic HIV-1-infected individuals with high anti-*E. histolytica* titer are at risk of invasive amebiasis probably due to exacerbation of subclinical amebiasis.

Keywords. seroprevalence; *Entamoeba histolytica*; HIV-1; anti-*E. histolytica* antibody; amebiasis.

Invasive amebiasis caused by *Entamoeba histolytica* is the second most common cause of parasite infection-related mortality worldwide, accounting for 40 000–100 000 deaths annually [1]. Recently, it was reported that invasive amebiasis is prevalent not only in developing countries where food or water is contaminated with stool, but also in East Asian developed countries (Korea, China, Taiwan and Japan) and Australia as a sexually transmitted infection (STI) [2–4]. On the other hand, the annual incidence of human immunodeficiency virus type 1 (HIV-1) infection is also on the rise among men who have sex with men (MSM) in these countries [5–8], with resultant growing concern regarding invasive amebiasis in HIV-1-infected MSM [9–14].

Serum anti-*E. histolytica* antibody (anti-*E. Histolytica*) is widely used as an index marker for the presence of amebiasis. It is used not only in developing countries [15–22] but also in developed countries where amebiasis is spreading as an STI [3, 9, 23–26]. Furthermore, the seroprevalence of anti-*E. histolytica* antibody in HIV-1-infected individuals is generally higher than in HIV-1 negative ones [3, 9, 15, 24]. However, only limited information is available on the seroprevalence of amebiasis in Japan [25, 26] despite the increasing number of invasive amebiasis among HIV-1-infected individuals reported lately [27, 28].

Received 18 September 2013; accepted 26 November 2013; electronically published 13 December 2013.

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The Journal of Infectious Diseases 2014;209:1801–7

DOI: 10.1093/infdis/jit815

Serum anti-*E. histolytica* antibody is also widely used for the diagnosis of invasive amebiasis based on the high sensitivity and good differentiation ability from other amoeba species, such as *Entamoeba dispar* and *Entamoeba moshkovskii* [29]. However, the primary disadvantage of this method is that it cannot distinguish current infection from past infection. Moreover, anti-*E. histolytica* antibody titer can be elevated even in asymptomatic infected individuals, and seroconversion of anti-*E. histolytica* was reported in the absence of any symptoms in longitudinal follow-up in endemic areas [14]. At present, the pathogenesis of amebiasis in asymptomatic anti-*E. histolytica* -positive individuals remains poorly understood.

In the present study, we found high seroprevalence of anti-*E. histolytica* antibody in HIV-1-infected adult Japanese. Retrospective analysis of these seropositive individuals indicated that those with high anti-*E. histolytica* titer are prone to future invasive amebiasis. These findings highlight the clinical significance of anti-*E. histolytica* positivity and enhance our understanding of the pathogenesis of invasive amebiasis.

MATERIALS AND 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. The study was conducted in accordance with the principles expressed in the Declaration of Helsinki.

Study Design and Population

The present study was a single-center retrospective cohort study. Our facility is one of the largest core hospitals for patients with HIV-1 infection in Japan, with >3000 registered patients. The study population was HIV-1-infected patients who were referred to our hospital for management of HIV-1 infection for the first time between January 2006 and April 2012.

Anti-*E. histolytica* Antibody Testing

Indirect fluorescent-antibody (IFA) assay was used for the detection of anti-*E. histolytica* antibody in serum by using a slide precoated with fixed *E. histolytica*. This method can distinguish amebiasis caused by *E. histolytica* from that caused by other amoeba species, such as *E. dispar* and *E. moshkovskii*. The sensitivity and specificity of this method for the detection of *E. histolytica* infection are comparable with other methods, such as counterimmunoelectrophoresis and indirect hemagglutination amebic serology [29, 30]. The commercial kit, Amoeba-Spot IF (bioMerieux SA), is currently approved for the diagnosis of *E. histolytica* infection in Japan. Based on the instructions enclosed with the kit, the biological samples were initially diluted at 1:100 with phosphate-buffered saline (PBS) and then incubated for 30 minutes at room temperature on slides precoated with fixed *E. histolytica*. Then, the slides were washed with PBS twice, treated with the fluorescent-labeled anti-human antibodies, and incubated for another 30 minutes at room temperature. The slides were washed again, and cover slips with buffered glycerol were placed over the slides. Fluorescence in each slide was examined with fluorescence microscope and compared with negative control slides. Seropositivity was defined as positive response in serum sample diluted at 1:100, and anti-*E. histolytica* titer was determined by the highest dilution for the positive response.

Development of Invasive Amebiasis in Patients Without History of Nitroimidazole Treatment

Newly registered HIV-1-infected individuals who underwent anti-*E. histolytica* testing at first visit were included in this analysis. Patients were excluded from the follow-up study (1) if they had been treated previously with nitroimidazole (metronidazole or tinidazole) or (2) if they were treated with nitroimidazole at first visit to the clinic. The clinical characteristics and results of serological tests for other STIs, such as syphilis and hepatitis B and C viruses (HBV and HCV), were collected from the medical records. The follow-up period spanned from the time of the first visit to May 2012, unless patients died from other causes during this period, dropped out, or were referred to other facilities.

The diagnosis of invasive amebiasis was based on the medical records of 3 different clinicians and satisfied one of the following 2 criteria, as described elsewhere [12–14] ; (1) identification of erythrophagocytic trophozoites in biological specimens (stool or biopsy sample) of HIV-1-infected patients with symptoms of invasive amebiasis, such as fever, tenesmus, and diarrhea, (2) identification of liver abscess by imaging studies in seropositive (titer $\geq\times100$) patients with symptoms related to invasive amebiasis who showed clinical improvement after nitroimidazole monotherapy. For patients who developed invasive amebiasis during follow-up, we compared anti-*E. histolytica* titer at the time of onset of invasive amebiasis with those at first visit (baseline) and after nitroimidazole therapy.

Statistical Analysis

The patients' characteristics and results of serological tests on STIs were compared using χ^2 test or Student t test for qualitative or quantitative variables, respectively. The time to the diagnosis of invasive amebiasis was calculated from the date of the first visit of our hospital to the date of diagnosis of invasive amebiasis. Censored cases represented those who died, dropped out, or were referred to other facilities during the follow-up. The time from first visit to the diagnosis of invasive amebiasis was calculated by the Kaplan-Meier method followed by log-rank test to determine the statistical significance. The Cox proportional-hazards regression analysis was used to estimate the impact of anti-*E. histolytica* titer at baseline on the incidence of invasive amebiasis. The impact of basic clinical characteristics,

Table 1. Characteristics of All Patients Who Underwent Anti-*E. histolytica* Testing (n = 1303)

	Anti-*E. histolytica* Negatives (n = 1026)	Anti-*E. histolytica* Positives (n = 277)	*P* Value
Age,years (range)	36 (18–77)	37 (19–74)	.06
Japanese nationality, no. (%)	921 (89.8%)	250 (90.3%)	.81
Male sex, no. (%)	960 (93.6%)	272 (98.2%)	.003
MSM, no. (%)	789 (76.9%)	245 (88.4%)	<.001
TPHA test positive, no. (%)	366/1012 (36.2%)	151/275 (54.9%)	<.001
HBV exposure,[a] no. (%)	524/1017 (51.5%)	187/272 (68.8%)	<.001
HCV Ab positive, no. (%)	40/1011 (4.0%)	5/273 (1.8%)	.09
Past history of IA, no. (%)	13 (1.3%)	60 (21.7%)	<.001
Diagnosis of IA at first visit, no. (%)	1 (0.1%)	7 (2.5%)	<.001

Abbreviations: Ab, antibody; Anti-*E. histolytica*, anti *Entamoeba histolytica* antibody; HBV, hepatitis B virus; HCV, hepatitis C virus; IA, invasive amebiasis; MSM, men who have sex with men; TPHA, *Treponema pallidum* hemagglutination.

[a] HBV exposure: HBsAg-positive or HBsAb-positive, and/or HBc-Ab positive.

such as sexuality and serology status of other STIs, was estimated with univariate Cox proportional hazards regression. We also conducted multivariate Cox hazards regression analysis using variables identified in univariate analysis with *P* values of < .20. In all analyses, statistical significance was defined as 2-sided *P* value of < .05. We used the hazard ratio (HR) and 95% confidence interval (95%CI) to estimate the impact of each variable on the development of invasive amebiasis. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL).

RESULTS

Clinical Characteristics of Asymptomatic Anti-*E. histolytica*-positive HIV-1-infected Patients

A total of 1519 patients were referred to our hospital during the study period. Anti-*E. histolytica* testing was conducted in 1303 patients at first visit, including 73 with history of invasive amebiasis, and anti-*E. histolytica* was positive in 277 of these (21.3%). Among the anti-*E. histolytica*-positive individuals, the rates of MSM (88.4%) and those with previous exposure to syphilis (TPHA test positive) (54.9%) and HBV (68.8%) were higher than those of anti-*E. histolytica*-negatives individuals, indicating that sexually active MSM are prone to *E. histolytica* infection among HIV-1-infected individuals in Japan (Table 1). Eight patients were diagnosed with invasive amebiasis at first visit, including 7 cases of amebic colitis and 1 case of amebic liver abscess, and they were treated immediately with metronidazole.

Incidence of Invasive Amebiasis During Follow-up of HIV-1 Infected Individuals

To assess the frequency of development of invasive amebiasis in patients free of symptomatic invasive amebiasis and who had not previously received nitroimidazole therapy, we excluded 96 patients from the analysis, including 73 patients because they had been treated previously for invasive amebiasis, and 23 patients (7 cases of amebic colitis, 1 case of amebic liver abscess, and 15 asymptomatic but anti-*E. histolytica*-positive cases treated preemptively) because they were treated with nitroimidazole at first visit (Figure 1). The remaining 1207 patients, including 195 anti-*E. histolytica*-positive patients (16.2%), were followed-up for median period of 25.3 months (interquartile range: 7.0–47.2). During the follow-up period, 18 patients developed invasive amebiasis (median time to onset: 9.1 months), including amebic appendicitis in 1 patient

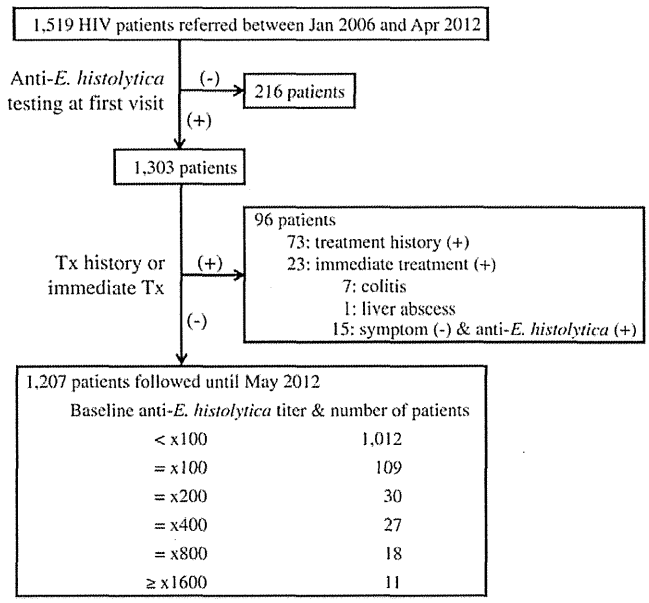

Figure 1. Flow diagram of patient recruitment process. Abbreviations: Anti-*E. histolytica*, anti-*Entamoeba histolytica* antibody; IA, invasive amebiasis; Tx, treatment.

Table 2. Comparison of Clinical Characteristics of Patients With and Without Invasive Amebiasis

	Amebic Colitis (n = 11)	Extraintestinal IA[a] (n = 7)	Non-IA (n = 1189)	*P* Value IA vs Non-IA
Age (years), average (SD)	35.9 (12.3)	38.2 (11.0)	37.5 (10.8)	.81
Japanese nationality, no. (%)	10 (90.9)	6 (85.7)	1068 (89.8)	.71
Male sex, no. (%)	11 (100)	7 (100)	1119 (94.1)	.62
MSM, no. (%)	11 (100)	6 (85.7)	929 (78.1)	.15
TPHA test-positive, no. (%)	5 (45.5)	2 (28.6)	451/1175 (38.4)	.91
HBV exposure,[a] no. (%)	6 (54.5)	5 (71.4)	630/1178 (53.5)	.15
HCV Ab-positive, no. (%)	0/11 (0)	0/7 (0)	42/1172 (3.6)	1.00
Anti-*E. histolytica* at baseline, median (IQR)	×100 (<×100–×800)	×400 (×100–×400)	<×100 (<×100–<×100)	<.001
Anti-*E. histolytica* at the onset of IA, median (IQR)	×800 (×200–×800)	×400 (×100–×800)	. . .	
Follow-up period, median months (IQR)	7.8 (3.3–25.1)	10.5 (4.9–17.9)	25.5 (7.0–47.3)	

Data were compared using χ^2 test, Student *t* test, or Mann–Whitney *U* test for qualitative or quantitative variables, respectively.

Abbreviations: Ab, antibody; Anti-*E. histolytica*, anti *Entamoeba histolytica* antibody; HBV, hepatitis B virus; HCV, hepatitis C virus; IA, invasive amebiasis; IA, invasive amebiasis; IQR, interquartile range; MSM, men who have sex with men; SD, standard deviation; TPHA, *Treponema pallidum* hemagglutination.[a]Extraintestinal cases include one case of appendicitis and 6 cases of liver abscess.

(confirmed by identification of erythrophagocytic trophozoites in surgically removed specimen), amebic liver abscess in 6, and amebic colitis in 11 (confirmed by identification of erythrophagocytic trophozoites in stool samples). The median anti-*E. histolytica* titer at baseline was significantly higher among patients who developed invasive amebiasis than that among those who did not, but the other clinical and laboratory parameters were not different between the 2 groups (Table 2). Although no significant differences in the frequency of invasive amebiasis were evident in patients with ×100 ($P = .77$) and ×200 ($P = .18$) anti-*E. histolytica* titers at baseline, compared with negative anti-*E. histolytica* patients (<×100), the frequency was higher in patients with ×400 ($P < .001$), ×800 ($P = .025$), and ≥×1600 ($P < .001$) anti-*E. histolytica* titers at baseline, compared with negative anti-*E. histolytica* patients. Univariate and multivariate analyses also showed that future development of invasive amebiasis correlated only with high titer of anti-*E. histolytica* antibody at baseline (≥×400: Univariate, HR: 20.985, 95% confidence interval [CI], 8.085–54.467; multivariate, HR: 22.079, 95% CI, 7.964–61.215) (Table 3). Furthermore, the risk of development of invasive amebiasis was significantly higher in the high anti-*E. histolytica* titer group (patients with anti-*E. histolytica* titer ≥×400 at baseline) than in the low anti-*E. histolytica* titer group (patients with anti-*E. histolytica* titer ≤×200 at baseline; log-rank test: $\chi^2 = 80.203$, $P < .001$, Kaplan-Meier estimate, Figure 2). Moreover, most patients of the high anti-*E. histolytica*

Table 3. Risk Analysis for Development of Invasive Amebiasis by Cox Proportional Hazard Regression Model

	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
older age (by 1 y)	0.989 (.947–1.033)	.624		
Japanese nationality	1.334 (.305–5.840)	.702		
Male sex	21.884 (.002–241297.39)	.516		
MSM	4.318 (.573–32.518)	.156	4.048 (.488–33.584)	.195
TPHA test-positive	0.901 (.348–2.335)	.831		
HBV exposure-positive	2.183 (.778–6.124)	.138	1.839 (.644–5.249)	.255
HCV Ab-positive	0.047 (.000–2697.344)	.584		
Anti-*E. histolytica* titer ≥×400	20.985 (8.085–54.467)	<.001	22.079 (7.964–61.215)	<.001

The Cox proportional-hazards regression analysis was used to estimate the impact of anti-*E. histolytica* titer at baseline on the incidence of invasive amebiasis. The impact of basic clinical characteristics, such as sexuality and serology status of other STIs, was estimated with univariate Cox proportional hazards regression. Multivariate Cox hazards regression analysis using variables identified in univariate analysis with *P* values of < .20. In all analyses, statistical significance was defined as *P* value of < .05.

Abbreviations: Ab, antibody; Anti-*E. histolytica*, anti *Entamoeba histolytica* antibody; CI, confidence interval; HBV, hepatitis B virus; HCV, hepatitis C virus; IA, invasive amebiasis; IA, invasive amebiasis; IQR, interquartile range; MSM, men who have sex with men; TPHA, *Treponema pallidum* hemagglutination.

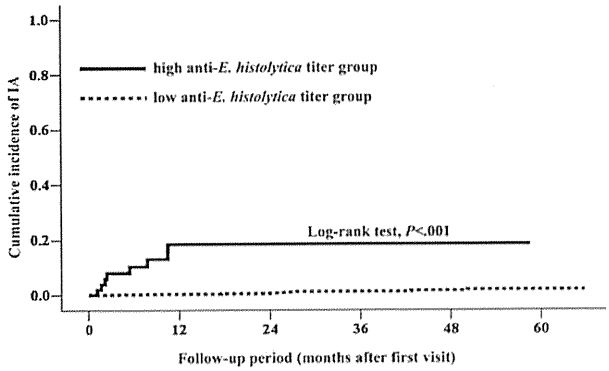

Figure 2. Incidence of invasive amebiasis in low and high anti-*E. histolytica* titer groups. Differences in the time from first visit to the diagnosis of invasive amebiasis (IA) between the low anti-*E. histolytica* titer group (≤×200 at baseline) and high anti-*E. histolytica* titer group (≥×400 at baseline) were analyzed by Kaplan-Meier method. Log-rank test was used to determine the statistical significance. Abbreviations: Anti-*E. histolytica*, anti-*Entamoeba histolytica* antibody; IA, invasive amebiasis.

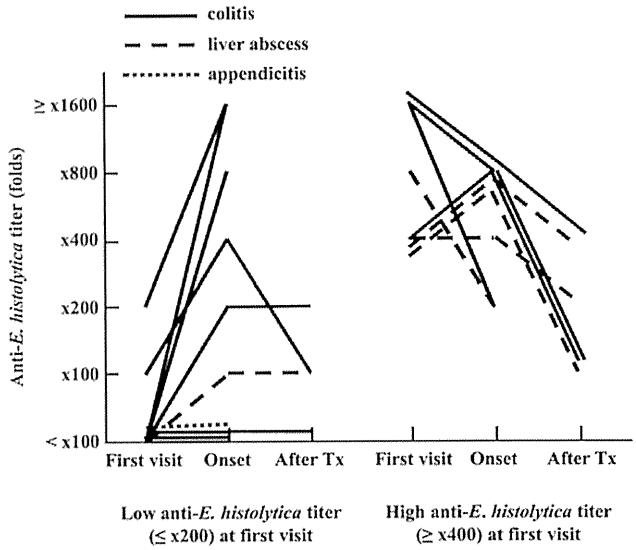

Figure 3. Anti-*E. histolytica* titer before and after diagnosis of invasive amebiasis. Anti-*E. histolytica* titer at the onset of IA was compared to that at baseline (first visit to the clinic) by Wilcoxon signed-rank test. Anti-*E. histolytica* titers after treatment were measured at 219 days [range: 174–252] and 367 days [272–841] after the completion of treatment of patients with low and high anti-*E. histolytica* titer at first visit, respectively. Abbreviations: Anti-*E. histolytica*, anti-*Entamoeba histolytica* antibody; IA, invasive amebiasis.

titer group developed invasive amebiasis during the first year of follow-up, whereas those of the low anti-E. histolytica titer group developed this complication more lately and new cases of invasive amebiasis were diagnosed throughout the follow-up period.

Transitional Changes in Anti-*E. histolytica* Titer Among Patients Who Developed Amebiasis

The median anti-*E. histolytica* titer was significantly higher at the onset of invasive amebiasis than that at first visit in patients with low baseline anti-*E. histolytica* titer (≤×200; $P = .028$, Wilcoxon signed-rank test) (Figure 3). In contrast, the median anti-*E. histolytica* titers at these 2 time points were not different in patients with high baseline anti-*E. histolytica* titer (≥×400; $P = .18$, Wilcoxon signed-rank test). Serum samples taken after nitroimidazole treatment (median time from the commencement of treatment 289 days [range 174–841]) were available in 10 patients. Anti-*E. histolytica* titers were lower after the treatment in 7 of the 10 patients, compared with the baseline values. To define the natural decay of anti-*E. histolytica*, we measured serum anti-*E. histolytica* titers at 9 months after study enrollment in 37 patients with high anti-*E. histolytica* titer at baseline but did not develop invasive amebiasis during the study period. The titers were lower, or similar to the baseline in 19 and 15 patients, respectively, whereas the remaining 3 patients showed 2-fold increase in the titer.

DISCUSSION

In the present study, the seroprevalence of anti-*E. histolytica* antibody among HIV-1-infected patients was 21.3%, which was much higher than those reported in other developed countries where amebiasis is considered as an STI [3, 9, 23, 24]. In addition, our results showed that sexually active MSM tend to be seropositive for *E. histolytica* infection, in agreement with previous studies from our group [27, 28].

The pathogenesis of amebiasis, such as incubation period after cyst ingestion and the mechanism of spontaneous remission, remains unclear. Although previous study showed anti-*E. histolytica*-positive children were more susceptible to *E. histolytica* infection than their seronegative counterparts [31], the clinical significance of anti-*E. histolytica* seropositivity and its titer in asymptomatic individuals had not been fully assessed. We measured serum anti-*E. histolytica* immunoglobulin M (IgM) levels in 18 patients at the onset of invasive amebiasis [32], but the level was detectable only in 3 patients with amebic colitis and 1 patient with liver abscess. The present study demonstrated that patients with high anti-*E. histolytica* titer (≥×400) at first visit developed invasive amebiasis much more frequently than those with low anti-*E. histolytica* titer (≤×200). The cumulative risk for invasive amebiasis among patients with high anti-*E. histolytica* titer at baseline rapidly increased during the first one year of follow-up but plateaued thereafter, suggesting that exacerbation of subclinical amebiasis occurs frequently within one year in these patients. On the other hand, the cumulative risk for invasive amebiasis among patients with low anti-*E. histolytica* titer at baseline increased more slowly and

developed at the same pace throughout the follow-up period, suggesting that the invasive amebiasis in these patients represented new infection rather than exacerbation of subclinical infection. The median anti-*E. histolytica* titer at the onset of invasive amebiasis in patients of high anti-*E. histolytica* titer group was not higher than that at first visit, whereas the titer increased at the onset compared with that at baseline in low anti-*E. histolytica* titer group. In addition, uni- and multivariate analyses identified high titer of anti-*E. histolytica* antibody at baseline as the only significant risk factor for future development of invasive amebiasis; seropositivity to other STIs was not a significant factor. These results add support to the aforementioned hypothesis regarding the difference in the pathology of invasive amebiasis between the high and low anti-*E. histolytica* groups. In this study, 15 asymptomatic but anti-*E. histolytica*-positive patients were treated with metronidazole at first visit (excluded from the follow-up analysis study), and none of them developed invasive amebiasis (median follow-up period, 11.7 months), suggesting the potential effectiveness of preemptive therapy for asymptomatic individuals with high anti-*E. histolytica* titer.

In conclusion, our results showed a relatively high prevalence of amebiasis in HIV-1-infected individuals in Japan, and that subclinical amebiasis is common among these individuals. The results emphasize the difficulty of disease control in not only individual patients with amebiasis but also in epidemiological control of this condition due to the long duration of subclinical infection of *E. histolytica*. Anti-*E. histolytica* testing for high-risk individuals could be helpful in early diagnosis of subclinical amebiasis, and early treatment of patients with such infection could prevent the development of invasive amebiasis and the transmission to others in the same community. Further studies to clarify the pathogenesis of invasive amebiasis are warranted.

Notes

Acknowledgments. We thank all clinical staff at the AIDS Clinical Center for their help in the completion of this study.

Financial support. This work was supported by a grant from the Ministry of Health, Labor, and Welfare of Japan (H25-promotion-general-014).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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J Antimicrob Chemother 2014; **69**: 1385–1389
doi:10.1093/jac/dkt514 Advance Access publication 29 December 2013

Journal of Antimicrobial Chemotherapy

Cumulative exposure to ritonavir-boosted atazanavir is associated with cholelithiasis in patients with HIV-1 infection

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Received 20 September 2013; returned 25 November 2013; revised 26 November 2013; accepted 7 December 2013

Objectives: This study aimed to examine the effect of long-term treatment with ritonavir-boosted atazanavir (atazanavir/ritonavir) on cholelithiasis.

Methods: A single-centre, cross-sectional study was conducted to elucidate the prevalence of cholelithiasis in patients with HIV-1 infection who underwent abdominal ultrasonography between January 2004 and March 2013. Univariate and multivariate logistic regression analyses were applied to estimate the effects of >2 years of atazanavir/ritonavir exposure on cholelithiasis as the primary exposure.

Results: Of the 890 study patients, 84 (9.4%) had >2 years of atazanavir/ritonavir exposure. Cholelithiasis was twice as frequent in those treated for >2 years with atazanavir/ritonavir [15 (18%) of 84 patients] compared with those treated for <2 years [72 (8.9%) of 806 patients] ($P=0.018$). Univariate analysis showed a significant association between >2 years of atazanavir/ritonavir exposure and cholelithiasis (OR=2.216; 95% CI=1.206–4.073; $P=0.010$) and the association almost persisted in multivariate analysis (adjusted OR=1.806; 95% CI=0.922–3.537; $P=0.085$). Long-term treatment (>2 years) with other commonly used protease inhibitors, such as ritonavir-boosted lopinavir and ritonavir-boosted darunavir, was not associated with cholelithiasis in univariate and multivariate analysis. Additional analysis showed that >1 year of exposure to atazanavir/ritonavir was significantly associated with cholelithiasis (OR=1.857; 95% CI=1.073–3.214; $P=0.027$), whereas >1 year of exposure to ritonavir-boosted lopinavir and ritonavir-boosted darunavir was not.

Conclusions: Long-term treatment of patients with HIV-1 infection for >2 years with atazanavir/ritonavir was associated with an increased risk of cholelithiasis compared with patients with shorter exposure. Long-term exposure to atazanavir/ritonavir appears to increase the risk of cholelithiasis in patients with HIV-1 infection.

Keywords: protease inhibitors, antiretroviral therapy, gallstones

Introduction

Ritonavir-boosted atazanavir (atazanavir/ritonavir) is a widely used protease inhibitor in the treatment of patients infected with HIV-1.[1–3] Cholelithiasis was not reported in atazanavir/ritonavir Phase 3 clinical trials;[4] however, recent post-marketing studies have suggested potential association between cumulative atazanavir/ritonavir exposure and cholelithiasis.[5–7] Only a couple of studies have so far reported the incidence of complicated cholelithiasis, such as cholecystitis, cholangitis and pancreatitis, in patients treated with atazanavir/ritonavir.[5,8] However, the effects of prolonged exposure to atazanavir/ritonavir on the incidence of cholelithiasis, including asymptomatic cholelithiasis, is unknown at this stage. This is of importance because ~20% of patients with cholelithiasis develop symptoms in the long term.[9]

The aim of this study was to elucidate the effects of atazanavir/ritonavir exposure on cholelithiasis, including asymptomatic cholelithiasis, in patients with HIV-1 infection.

Patients and methods

Study design

We performed a cross-sectional study of HIV-1-infected patients using the abdominal ultrasonography data and the medical records at the National Center for Global Health and Medicine, Tokyo, Japan.[10] The study

population was HIV-1-infected patients, aged >17 years, who underwent abdominal ultrasonography at the Physiological Examination Unit of the hospital between 1 January 2004 and 31 March 2013 as part of clinical practice. Atazanavir/ritonavir became available in Japan in January 2004. Exclusion criteria were: (i) patients with cholecystectomy performed before the study period; and (ii) patients with missing data on antiretroviral therapy (ART). At the Physiological Examination Unit, ultrasonography was conducted by certified medical technologists and the images and diagnosis were double-checked and confirmed by radiologists, hepatologists or gastroenterologists. If abdominal ultrasonography was conducted more than once during the study period, the latest ultrasonography data were used for the study. This study was approved by the Human Research Ethics Committee of the hospital. Each participant provided a written informed consent for the clinical and laboratory data to be used and published for research purposes.

Measurements

The primary exposure variable was a history of atazanavir/ritonavir use for >2 years, regardless of continuation of atazanavir/ritonavir at the time of abdominal ultrasonography. A 2 years threshold for atazanavir/ritonavir exposure was selected because cholelithiasis was not reported in atazanavir/ritonavir Phase 3 clinical trials with the primary endpoint set at week 48[4] and prolonged excretion of atazanavir in the bile appears necessary for gallstone formation.[5] The potential risk factors for cholelithiasis were collected from the medical records, together with the basic demographics.[9,11–13] They included age, sex, ethnicity, body mass index (BMI), cirrhosis, diabetes mellitus, CD4 count, HIV viral load, ART experienced or naive, duration of ART, length of exposure to atazanavir/ritonavir, ritonavir-boosted lopinavir (lopinavir/ritonavir) and ritonavir-boosted darunavir (darunavir/ritonavir), history of AIDS and hepatitis B or C coinfection. We used data collected within 3 months of the day ultrasonography was conducted.

Statistical analysis

Univariate and multivariate logistic regression analysis was used to estimate the effects of atazanavir/ritonavir exposure of >2 years, relative to <2 years or no atazanavir/ritonavir exposure, on cholelithiasis as the primary exposure. Basic demographics (age and sex), possible risk factors for cholelithiasis (BMI, cirrhosis and diabetes mellitus)[11–13] and variables with *P* values <0.05 in univariate analysis (HIV load and duration of ART) were added to the multivariate model. The variable 'treatment naive' was not added because of its multicollinearity with HIV load.

Statistical significance was defined as two-sided *P* values <0.05. We used ORs and 95% CIs to estimate the effects of each variable on cholelithiasis. All statistical analyses were performed with the Statistical Package for Social Sciences ver. 20.0 (SPSS, Chicago, IL, USA).

Results

Of the 890 study patients, cholelithiasis was diagnosed by abdominal ultrasonography in 87 patients, with a prevalence of 9.8% (see Figure S1, available as Supplementary data at *JAC* Online). Patients with cholelithiasis were significantly older, more likely to be females, have lower HIV-1 viral load, be diabetic, have cirrhosis and have longer exposure to ART (Table 1). On the other hand, patients without cholelithiasis were more likely to be treatment naive.

Of the 890 study patients, 186 (21%) were treated with atazanavir for a median duration of 1.79 years (IQR 0.68–3.78 years) and 84 (9.4%) patients were treated with atazanavir for >2 years. Of the 186 patients treated with atazanavir, 173 (93%) patients were on atazanavir/ritonavir, whereas only 13 (7%) were on non-boosted atazanavir. Cholelithiasis was twice as frequent in patients treated for >2 years with atazanavir [15 (18%) of

Table 1. Basic demographics of total study patients, patients with cholelithiasis and no cholelithiasis

	Total (*n*=890)	Cholelithiasis (*n*=87)	No cholelithiasis (*n*=803)	*P*[a]
Age, years[b]	41 (35–50)	45 (38–55)	40 (34–49)	<0.001
Female sex, *n* (%)	49 (5.5)	9 (10)	40 (5)	0.047
Race (Asian), *n* (%)	869 (98)	87 (100)	782 (97)	0.253
BMI, kg/m^2[b]	21.9 (20.1–24.6)	22.5 (20.1–25.7)	21.8 (20–24.4)	0.665
CD4 cell count, cells/μL[b]	365 (207–525)	370 (226–572)	365 (206–523)	0.206
HIV load, $\log_{10}$ copies/mL[b]	1.70 (1.07–4.04)	1.70 (1.70–1.90)	1.70 (1.70–4.20)	0.002
HIV load <50 copies/mL, *n* (%)	510 (57)	64 (74)	446 (56)	0.001
Diabetes mellitus, *n* (%)	53 (6)	10 (12)	43 (5)	0.030
Hepatitis B or C coinfection, *n* (%)	242 (27)	23 (26)	219 (27)	1.000
History of AIDS, *n* (%)	298 (34)	31 (36)	267 (33)	0.720
Cirrhosis, *n* (%)	14 (1.6)	6 (7)	8 (1)	0.001
Treatment naive, *n* (%)	267 (30)	14 (16)	253 (32)	0.003
History of atazanavir/ritonavir exposure, *n* (%)	186 (21)	25 (29)	161 (20)	0.070
History of lopinavir/ritonavir exposure, *n* (%)	294 (33)	32 (37)	262 (33)	0.472
History of darunavir/ritonavir exposure, *n* (%)	100 (11)	13 (15)	87 (11)	0.281
Duration of ART (years)[b]	2.7 (0–7.9)	4.8 (0.9–12)	2.2 (0–7.4)	<0.001

Cirrhosis was diagnosed by abdominal ultrasonography, diabetes mellitus was defined by use of antidiabetic agents or fasting plasma glucose >126 mg/dL or plasma glucose >200 mg/dL on two different days, hepatitis B infection was defined by positive hepatitis B surface antigen and hepatitis C infection was defined by positive hepatitis C virus viral load.
[a]The χ^2 test or Fisher's exact test was used for comparison of categorical data and Student's *t*-test was used for comparison of continuous variables.
[b]Median (IQR).

Table 2. Univariate and multivariate analysis to estimate the risk for cholelithiasis posed by long-term (>2 years) treatment with ritonavir-boosted atazanavir

	Model 1, crude (n=890)			Model 2, adjusted (n=890)			Model 3, adjusted (n=851)		
	OR	95% CI	P	OR	95% CI	P	OR	95% CI	P
>2 years of atazanavir/ritonavir exposure	2.216	1.206–4.073	0.010	2.096	1.131–3.883	0.019	1.806	0.922–3.537	0.085
Age per 1 year increment	1.034	1.016–1.053	<0.001	1.009	0.980–1.039	0.001	1.028	1.008–1.049	0.005
Female sex	2.201	1.030–4.705	0.042	2.005	0.921–4.368	0.080	2.183	0.986–4.834	0.054
BMI per 1 kg/m^2 increment	1.004	0.985–1.024	0.673				1.001	0.983–1.020	0.881
Cirrhosis	7.361	2.493–21.74	<0.001				6.947	2.133–22.63	0.001
Diabetes mellitus	2.295	1.110–4.748	0.025				1.017	0.417–2.481	0.971
CD4 count per 1 cell/μL increment	1.001	1.000–1.001	0.206						
HIV viral load per $\log_{10}$/mL increment	0.748	0.618–0.906	0.003				0.900	0.717–1.129	0.363
History of AIDS	1.111	0.700–1.765	0.655						
Treatment naive	0.417	0.231–0.753	0.004						
Hepatitis B or hepatitis C coinfection	0.958	0.581–1.582	0.868						
Duration of ART per 1 year increment	1.077	1.040–1.115	<0.001				1.030	0.983–1.080	0.216

Model 1 was the univariate analysis to estimate the risk of various factors for cholelithiasis for atazanavir/ritonavir exposure of >2 years, relative to <2 years or no atazanavir/ritonavir exposure. In Model 2, atazanavir/ritonavir exposure of >2 years, relative to <2 years or no atazanavir/ritonavir exposure, was adjusted by adding age and sex. In Model 3, possible risk factors for cholelithiasis (BMI, cirrhosis and diabetes mellitus) and variables with P values <0.05 in Model 1 (HIV load and duration of ART) were added. The variable 'treatment naive' was not added because of its multicollinearity with HIV load.

84 patients] compared with patients with no or <2 years of atazanavir [72 (8.9%) of 806 patients] (P=0.018).

Univariate analysis showed a significant association between >2 years of atazanavir/ritonavir exposure and cholelithiasis (OR=2.216; 95% CI=1.206–4.073; P=0.010) (Table 2, Model 1). Older age, female sex, cirrhosis, diabetes mellitus, low HIV viral load and duration of ART per 1 year increment were also significantly associated with cholelithiasis.

Multivariate analysis identified >2 years of atazanavir/ritonavir exposure as an independent risk factor for cholelithiasis after adjustment for age and female sex (adjusted OR=2.096; 95% CI=1.131–3.883; P=0.019) (Table 2, Model 2). The association was marginally significant after adjustment for other variables (adjusted OR=1.806; 95% CI=0.922–3.537; P=0.085) (Table 2, Model 3). Older age and cirrhosis also persisted in being significantly associated with cholelithiasis in multivariate analysis (age per 1 year increment, adjusted OR=1.028; 95% CI=1.008–1.049; P=0.005) (cirrhosis, adjusted OR=6.947; 95% CI=2.133–22.63; P=0.001).

Additional analyses focusing on the impact of other commonly used protease inhibitors demonstrated that 148 (16.6%) patients were treated with lopinavir/ritonavir for >2 years, while 29 (3.3%) were treated with darunavir/ritonavir for >2 years. Treatment for >2 years with lopinavir/ritonavir and darunavir/ritonavir was not associated with cholelithiasis in univariate and multivariate analysis adjusted with the same variables in Table 2, Model 3 (lopinavir/ritonavir: OR=1.246; 95% CI=0.710–2.185; P=0.443/adjusted OR=1.221; 95% CI=0.674–2.214; P=0.510) (darunavir/ritonavir: OR=1.067; 95% CI=0.316–3.601; P=0.916/adjusted OR=0.641; 95% CI=0.173–2.377; P=0.506). In univariate analysis, treatment for >1 year with atazanavir/ritonavir [n=124 (13.9%)] was also significantly associated with cholelithiasis (OR=1.857; 95% CI=1.073–3.214; P=0.027), whereas >1 year exposure to lopinavir/ritonavir [n=199 (22.4%)] and darunavir/ritonavir [n=53 (6%)] did not correlate with cholelithiasis (lopinavir/ritonavir: OR=1.367; 95% CI=0.830–2.252; P=0.220) (darunavir/ritonavir: OR=0.961; 95% CI=0.375–2.464; P=0.934).

Discussion

To our knowledge, this is the first study to investigate the effects of atazanavir/ritonavir exposure on cholelithiasis, including asymptomatic cholelithiasis. Patients treated for >2 years with atazanavir/ritonavir were twice as likely to develop cholelithiasis compared with patients with no or <2 years of atazanavir/ritonavir exposure. Univariate analysis demonstrated a significant association between >2 years of atazanavir/ritonavir exposure and cholelithiasis (OR=2.216; 95% CI=1.206–4.073; P=0.010) and the association almost persisted in multivariate analysis (adjusted OR=1.806; 95% CI=0.922–3.537; P=0.085) (Table 2). Thus, long-term treatment with atazanavir/ritonavir was associated with cholelithiasis in this cohort. On the other hand, exposure to lopinavir/ritonavir or darunavir/ritonavir, other widely prescribed protease inhibitors, was not associated with cholelithiasis.

Two mechanisms are suggested for the observed atazanavir-induced cholelithiasis. First, precipitation of atazanavir in the bile might enhance the formation of calculi composed of atazanavir and other biliary components. This hypothesis is supported by the documentation of atazanavir as a component of gallstones in several case reports.[5–7] Strong acidity (e.g. pH of 1.9) is required to achieve optimal dissolution of atazanavir, whereas biliary pH is usually >6.5.[4] This feature of atazanavir might result in precipitation of atazanavir and consequent cholelithiasis.[4] It is well known that atazanavir/ritonavir is a risk factor for nephrolithiasis[14,15] and, recently, a case of atazanavir-containing sialolithiasis in a patient treated with atazanavir/ritonavir was also reported.[16] These data further support the likelihood of atazanavir involvement in lithiasis. Second, because atazanavir is a competitive

inhibitor of uridine diphosphate glucuronyl transferase 1A1 (UGT1A1), a bilirubin-conjugating enzyme, atazanavir is known to cause hyperbilirubinaemia.[17] This might result in a rise in the bilirubin level in the bile, which could facilitate the formation of gallstones because bilirubin is also a component of such stones. This hypothesis is supported by a case report that showed the presence of indinavir, another protease inhibitor, in the gallstones of a patient on indinavir-containing ART.[18] Indinavir has similar characteristics to atazanavir: optimal solubility at low pH and being an inhibitor of UGT1A1.[18,19]

There are several limitations to our study. First, because stone composition analysis was not conducted in this study, one cannot rule out other causes of cholelithiasis in addition to atazanavir/ritonavir. Second, the prevalence of gallstones is generally lower in Asians than in Europeans and since most of the patients in this study were Asian, the effect of atazanavir/ritonavir might be different in other populations.[20] Third, because the study population included patients who had undergone abdominal ultrasonography in clinical practice with various indications, the prevalence of cholelithiasis might be overestimated.

In conclusion, the present study demonstrated that patients on long-term treatment (>2 years) with atazanavir/ritonavir were twice as likely to develop cholelithiasis compared with those treated for <2 years. A similar effect was not demonstrated in patients treated with lopinavir/ritonavir or darunavir/ritonavir. Long-term, large prospective studies are warranted to elucidate the incidence and risk factors for complicated cholelithiasis in patients exposed to atazanavir/ritonavir-containing ART.

Acknowledgements

We thank Motoshi Maejima, a senior staff member at the Physiological Examination Unit, and Mikiko Ogata and Michiyo Ishisaka for their invaluable contribution to the study. We also thank Akiko Nakano for supporting this study as a research coordinator and all the clinical staff at the AIDS Clinical Center for their help in the completion of this study.

Funding

This work was supported by Grants-in Aid for AIDS research from the Japanese Ministry of Health, Labour, and Welfare (H23-AIDS-001).

Transparency declarations

H. G. has received honoraria from MSD K.K., Abbott Japan, Co., Janssen Pharmaceutical K.K., Torii Pharmaceutical, Co. and ViiV Healthcare, Co. S. O. has received honoraria and research grants from MSD K.K., Abbott Japan, Co., Janssen Pharmaceutical K.K., Pfizer, Co. and Roche Diagnostics K.K., and has received honoraria from Astellas Pharmaceutical K.K., Bristol-Myers K.K., Daiichisankyo, Co., Dainippon Sumitomo Pharma, Co., GlaxoSmithKline K.K., Taisho Toyama Pharmaceutical, Co., Torii Pharmaceutical, Co. and ViiV Healthcare. All other authors: none to declare.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 30, Number 6, 2014

DOI: 10.1089/aid.2013.0252

Long-Term Use of Protease Inhibitors Is Associated with Bone Mineral Density Loss

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Abstract

HIV-infected patients are at high risk for bone mineral density (BMD) loss. The present study was designed to provide information on characteristics of BMD abnormalities in Japanese HIV-1-infected patients and risk factors involved in worsening of BMD. A total of 184 Japanese HIV-1-infected men were studied with a dual-energy X-ray absorptiometry scan (DXA) at the lumbar spine and femoral neck. Multivariate logistic regression models were used for comparison of the impact of risk factors on BMD loss. Osteopenia and osteoporosis were diagnosed in 46% and 10% of the patients at lumbar spine, and 54% and 12% at femoral neck, respectively. In logistic analysis, factors associated with low BMD at both lumbar spine and femoral neck were long-term treatment with a protease inhibitor (PI) [odds ratio (OR) 1.100 and 1.187 per 1 year increase of PI use; 95% confidence interval (CI) 1.003–1.207 and 1.043–1.351; $p=0.042$ and 0.009, respectively] and a low body mass index [OR: 0.938 and 0.852, CI 0.892–0.992 and 0.783–0.927; $p=0.024$ and <0.001, respectively]. Patients who discontinued PI had a significantly higher BMD than those who currently use PI at lumbar spine (t score -0.8 vs. -1.3, $p=0.04$) but not at femoral neck (-1.3 vs. -1.5, $p=0.38$). In HIV-infected Japanese patients, the duration of treatment with PI correlated significantly with BMD loss. Discontinuation of PI is a promising option in the treatment of BMD loss since it allows recovery of BMD, especially in the lumbar spine.

Introduction

FOR HIV-INFECTED PATIENTS, loss of bone mineral density (BMD) is an important age-related complication, in addition to chronic renal dysfunction, cardiovascular diseases, and metabolic disorders. A meta-analysis study reported that the prevalence of osteoporosis among HIV-infected patients was three times higher than in the HIV-negative population.[1] The etiology of low BMD in HIV-infected patients is multifactorial and is considered to include chronic HIV infection[2,3] and antiretroviral therapy, especially tenofovir disoproxil fumarate (TDF) and protease inhibitors (PI).[4–7] However, to our knowledge, information on the characteristics of BMD abnormalities in Asian HIV-infected patients is scarce and the exact risk factors involved in the worsening of BMD remain obscure. The present study was designed to provide new information on the above two aspects of Asian HIV-1 infection.

Materials and Methods

Setting and participants

We performed a cross-sectional study at the AIDS Clinical Center (ACC), National Center for Global Health and Medicine (NCGM) involving HIV-infected patients who were registered at the NCGM from February 2012 to June 2013. We excluded patients who had been on treatment for osteoporosis, current users of corticosteroids, and those with a history of bone fractures at the spine or bilateral femoral neck. A total of 184 Japanese HIV-infected men were enrolled in this study. This study was approved by the ethics committee of NCGM and a written informed consent was obtained from each patient.

Data collection

BMD was assessed using dual X-ray absorptiometry (DXA: QDR-4500W, Hologic Inc., Bedford, MA) at the lumbar spine

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and femoral neck. Osteopenia and osteoporosis were defined using the World Health Organization (WHO) criteria. Normal BMD was defined as a t score of -1 or higher, osteopenia as a t score between -1 and -2.5, and osteoporosis as a t score of -2.5 or lower.[8] Age, body mass index (BMI), smoking habit, hemophilia, history of an AIDS-defined illness, nadir CD4 cell count, time with low CD4 cell count (<200 cell/μl), time on antiretroviral therapy (ART), TDF, and PI, were obtained by interview or medical records. Estimated glomerular filtration rate (eGFR) was calculated using the modified Modification of Diet in Renal Disease (MDRD) equation for Japanese populations.[9]

Statistical analysis

t scores and BMD of the lumbar spine and femoral neck were compared using Student's paired t-test. To determine the impact of independent variables, multivariate logistic regression analysis was used. In logistic regression analysis, the dependent variable was set as low BMD (t score lower than -1.0) at both the lumbar spine and femoral neck. We used the odds ratio (ORs) and 95% confidence interval (95% CI) to estimate the impact of each variable on low BMD.

To assess the impact of PI discontinuation, we compared the t scores between PI-experienced patients and patients who discontinued such therapy, using the Student's unpaired t-test. For evaluation of the correlation between the t score at the lumbar spine and the time on PI, ritonavir (RTV) at different dosage (100 mg/day and 200 mg/day), and other types of PI, Pearson's correlation coefficient was used. For further evaluation of the relationship between the time on TDF and BMD, we compared the t scores between those who were treated with PI plus TDF and those treated with PI only and had never been treated with TDF, using the Student's unpaired t-test. All statistical analyses were performed with The Statistical Package for Social Sciences ver. 17.0 (SPSS, Chicago, IL).

Results

Patient characteristics

The characteristics of the 184 study patients are summarized in Table 1. All patients underwent DXA for the lumbar spine and 164 underwent DXA for the femoral neck. Hemophiliacs constituted 36% ($n=67$) of the study subjects. Seventy-one patients (39%) had a history of infection with hepatitis C virus (HCV), including all 67 hemophiliacs. Among them, 16 of the 71 (23%) patients showed spontaneous viral clearance, 23 (32%) achieved sustained virologic response after antiviral therapy, and 2 (3%) patients were still on treatment and had undetectable levels of HCV viral load. The remaining 30 (45%) patients with chronic hepatitis C were nonresponders or never users of antiviral therapy. Among them, 9 (14%) had liver cirrhosis diagnosed by radiological findings. Although 41 (21%) patients had a history of AIDS-defined illness, 172 (93%) patients had been treated with ART and 148 (80%) patients had an undetectable level of HIV viral load.

The median durations of ART, PI, and TDF of the total population were 88, 38, and 23 months, respectively. Among 139 TDF-treated patients, the median time on TDF was 38 months (IQR 14–68 months). One hundred and forty-four patients had previously received PI-based treatment, and the numbers of patients who had been treated with each type of PI were 30 patients with nelfinavir (NFV), 47 with lopinavir (LPV/r), 34 with atazanavir (ATV), 21 with fosamprenavir (FPV) or amprenavir (APV), 74 with darunavir (DRV), 4 with indinavir (IDV), and 1 with saquinavir (SQV). The total number of patients who had received RTV was 137, and of these, 102 and 63 patients had been treated with RTV at 100 and 200 mg/day, respectively.

TABLE 1. CLINICOPATHOLOGICAL CHARACTERISTICS OF THE 184 STUDY PATIENTS

Sex, (male/female)	184/0
Age: median (IQR)	43 (38–51)
Body mass index (kg/m^2)	22 (20–24)
Hypertension, n (%)	42 (23%)
Current smoking, n (%)	99 (54%)
Hemophilia, n (%)	67 (36%)
History of AIDS-defined illness, n (%)	40 (22%)
Positive HBsAg, n (%)	8 (4%)
Positive HCV-Ab, n (%)	71 (37%)
Liver cirrhosis, n (%)	10 (5%)
Diabetes mellitus, n (%)	7 (4%)
Current CD4$^+$ T cell count (cells/μl)	493 (322–623)
Nadir CD4$^+$ T cell count (cells/μl)	141 (54–218)
Low CD4$^+$ T cell count (<200 cells/μl) for>1 year, n (%)	52 (28%)
Current suppressed viral load (<20 copies/ml), n (%)	148 (80%)
Current use of ART, n (%)	172 (93%)
Time on ART (months)	88 (26–153)
Current use of protease inhibitors, n (%)	117 (64%)
Never use of protease inhibitors, n (%)	40 (22%)
Time on protease inhibitors (months)	38 (2–81)
Current use of tenofovir, n (%)	114 (62%)
Never use of tenofovir, n (%)	45 (24%)
Time on tenofovir (months)	22 (0–60)
Serum creatinine (mg/dl)	0.78 (0.68–0.89)
Estimated glomerular filtration rate (ml/min/1.73 m^2)	86.0 (74.7–100.3)

Values are median (IQR) or number (%) of patients.
HBsAg, hepatitis B virus surface antigen; HCV-Ab, hepatitis C virus antibody; ART, antiretroviral therapy; ART, antiretroviral therapy.

Prevalence of low bone mineral density

Based on the WHO criteria, osteopenia and osteoporosis were diagnosed in 46% and 10% of the patients at the lumbar spine and 53% and 12% at the femoral neck, respectively. The mean t scores were -1.1 [standard deviation (SD) 1.1] for the lumbar spine and -1.4 (SD: 1.1) for the femoral neck (Fig. 1A). The mean BMD scores were 0.914 g/cm^2 (SD: 0.199 g/cm^2) at the lumbar spine and 0.694 g/cm^2 (SD: 0.221 g/cm^2) at the femoral neck (Fig. 1B). Both the t score and BMD at the femoral neck were significantly lower than those at the lumbar spine ($p=0.008$ for t score and $p<0.001$ for BMD).

Impact of related risk factors

In multivariate logistic analysis, statistically significant regression models were built for low BMD (t score <-1) at the lumbar spine ($p=0.038$) and at the femoral neck

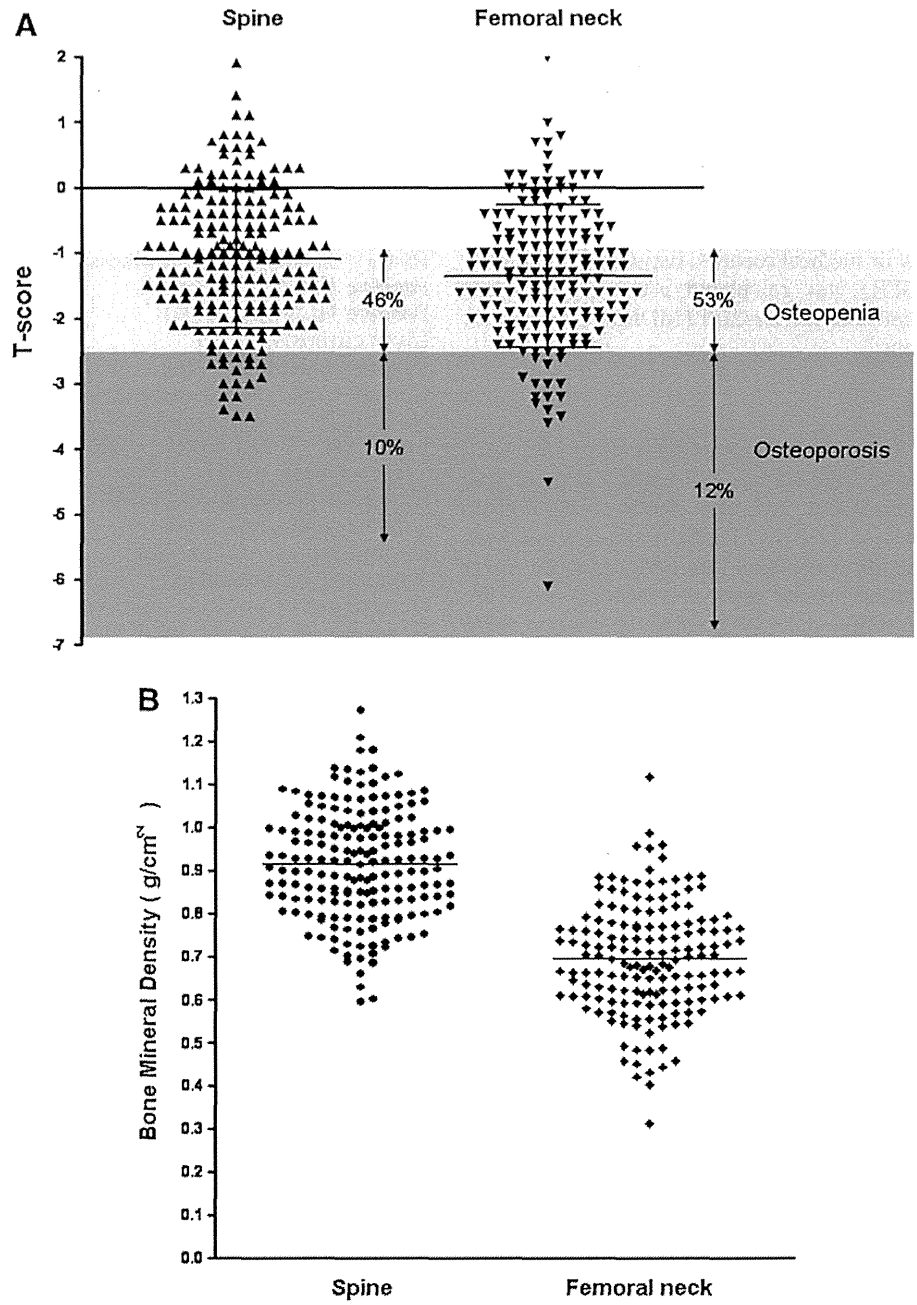

FIG. 1. **(A)** Distribution of *t* scores at lumbar spine and femoral neck. Light areas: osteopenia; dark gray areas: osteoporosis. **(B)** Distribution of bone mineral density (BMD) at lumbar spine and femoral neck. In both **(A)** and **(B)**, data are mean ± standard deviation. Differences in the mean scores of the spine and femoral neck were tested by the Student's paired *t*-test.

($p < 0.001$) (Table 2). In logistic analysis, the following factors were associated with low BMD at both the lumbar spine and femoral neck: longer duration of treatment with a PI [odds ratio (OR) 1.100 and 1.187 per 1 year increase of PI use and 95% confidence interval (CI) 1.003–1.207 and 1.043–1.351; $p = 0.042$ and 0.009, respectively] and lower body mass index [OR: 0.938 and 0.852, CI 0.892–0.992 and 0.783–0.927; $p = 0.024$ and < 0.001, respectively]. Low BMD at the femoral neck also correlated with age [OR: 1.071; CI 1.029–1.115; $p = 0.001$] and hemophilia [OR: 8.139; CI 2.594–25.337; $p < 0.001$].

Impact of PI use and discontinuation on bone mineral density

The *t* scores of both the spine and femoral neck were significantly lower in patients who received PI than in those who never used PI [−1.2 vs. −0.7 at the spine ($p = 0.02$) and −1.5 vs. −0.9 at the femoral neck ($p = 0.002$), respectively] (Fig. 2A). Moreover, patients who discontinued PI had a higher spine *t* score than those who currently used PI (−0.8 vs. −1.3, $p = 0.04$) and had a *t* score level comparable to those patients who never used PI (−0.8 in PI-discontinued patients

TABLE 2. RESULTS OF LOGISTIC ANALYSIS FOR BONE MINERAL ABNORMALITIES MEASURED FOR DIFFERENT JOINTS

	Univariate analysis			*Multivariate analysis*[a]		
	OR	*95% CI*	p *value*	*OR*	*95% CI*	p *value*
Low BMD at lumbar spine (t score < -1.0)						
Age (per 1 year increase)	1.015	0.986–1.045	0.309	1.016	0.989–1.042	0.249
Body mass index (per 1 increase)	0.924	0.845–1.011	0.086	0.938	0.892–0.992	0.024
Hemophilia	1.013	0.556–1.847	0.967			
Current smoking	1.690	0.942–3.302	0.078	1.651	0.903–2.971	0.104
History of AIDS-defined illness	1.630	0.800–3.323	0.176			
Nadir CD4 (per 1 increase of categories)						
≥350	1.000					
200–349	0.514	0.140–1.883	0.315			
≤199	0.799	0.241–2.653	0.714			
Time with CD4 <200/μl (per 1 year increase)	1.065	0.921–1.233	0.515			
Time on ART (per 1 year increase)	1.027	0.978–1.077	0.287	0.973	0.912–1.038	0.408
Time on TDF (per 1 year increase)	1.082	0.976–1.200	0.134	1.078	0.961–1.210	0.201
Time on PI (per 1 year increase)	1.081	1.009–1.159	0.026	1.100	1.003–1.207	0.042
Low BMD at femoral neck (t-score < -1.0)						
Age (per 1 year increase)	1.012	1.005–1.019	0.001	1.071	1.029–1.115	0.001
Body mass index (per 1 increase)	1.017	1.003–1.031	0.018	0.852	0.783–0.927	<0.001
Hemophilia	3.954	1.850–8.448	<0.001	8.139	2.594–25.337	<0.001
Current smoking	1.206	0.642–2.265	0.561	0.238	0.734–3.460	0.238
History of AIDS-defined illness	1.870	0.806–4.338	0.141	0.124	0.795–6.789	0.124
Nadir CD4 (per 1 increase of categories)						
≥350	1.000			1.000		
200–349	1.593	0.425–5.971	0.489	1.553	0.355–6.783	0.559
≤199	0.984	0.293–3.301	0.979	0.757	0.174–3.285	0.710
Time with CD4 <200/μl (per 1 increase of categories)	1.072	0.951–1.209	0.257	0.844	0.684–1.042	0.114
Time on ART (per 1 year increase)	1.070	1.034–1.117	<0.001	0.968	0.880–1.066	0.509
Time on TDF (per 1 year increase)	1.084	1.005–1.119	0.037	0.990	0.848–1.156	0.900
Time on PI (per 1 year increase)	1.151	1.079–1.225	<0.001	1.187	1.043–1.351	0.009

[a]In the analysis for lumbar spine, the final model obtained by backward stepwise elimination included the time on ART, TDF, and PI, current smoking, BMI, and age. OR, odds ratios; CI, confidence intervals; ART, antiretroviral therapy; TDF, tenofovir disoproxil fumarate; PI, protease inhibitors; BMD, bone mineral density.

vs. −0.7 in PI-never use patients, $p=0.97$) (Fig. 2B). In contrast, there was no significant difference in femoral neck t score between PI-discontinued patients and PI current-use patients (−1.3 vs. −1.5, $p=0.38$) or between PI-discontinued patients and PI-never use patients (−1.3 vs. −0.9, $p=0.24$) (Fig. 2C).

Impact of different types of PIs on bone mineral density

While the correlation between the duration of treatment of any PI and spine t score was significant ($r=-0.180$, $p=0.013$) (Fig. 3A), the duration of treatment with RTV showed a better correlation with spine t score (−0.207, $p=0.004$) (Fig. 3B). When both the time on RTV and the time on PI were entered as independent variables in logistic analysis for low BMD at the lumbar spine, a statistically significant model was built by elimination of the time on PI. In this model, the time on RTV was significantly associated with low BMD (OR: 1.146, 95% CI 1.032–1.273, $p=0.011$). At the femoral neck, RTV was associated with low BMD (OR: 1.267 per 1 year increase of RTV, 95% CI 1.010–1.589, $p=0.041$), whereas the time on PI was not (OR: 0.983 per 1 year increase of PI, 95% CI 0.803–1.202, $p=0.864$). There were no significant correlations between spine t score and the duration of treatment with RTV at either 100 mg/day ($r=-0.134$, $p=0.071$) (Fig. 3C) or 200 mg/day ($r=-0.133$, $p=0.073$) (Fig. 3D). No significant correlations were found between different types of PIs and spine t score (NFV: $r=-0.023$, $p=0.758$; LPV/r: $r=-0.080$, $p=0.239$; DRV: $r=-0.069$, $p=0.355$; ATV: $r=-1.123$, $p=0.097$; FPV or APV: $r=0.091$, $p=0.218$).

Comparison of BMD between PI- and PI-TDF-treated patients

For further confirmation of the poor association between TDF use and BMD loss, t scores were compared between patients who had been treated with both PI and TDF ($n=118$) and patients who received PI-based treatment and had never been treated with TDF ($n=26$). Neither spine nor femoral neck t scores were significantly different between the two groups (PI+TDF: −1.2, PI alone: −1.0, $p=0.414$ for spine t score, −1.5 vs. −1.5, $p=0.844$ for femoral neck, respectively).

Discussion

The present study showed that for Asian HIV-infected patients, PI use was the most significant determinant of low BMD at both the spine and femoral neck. Moreover, our logistic regression models strongly suggested that long-term use of PI has a gradual and cumulative effect on BMD.

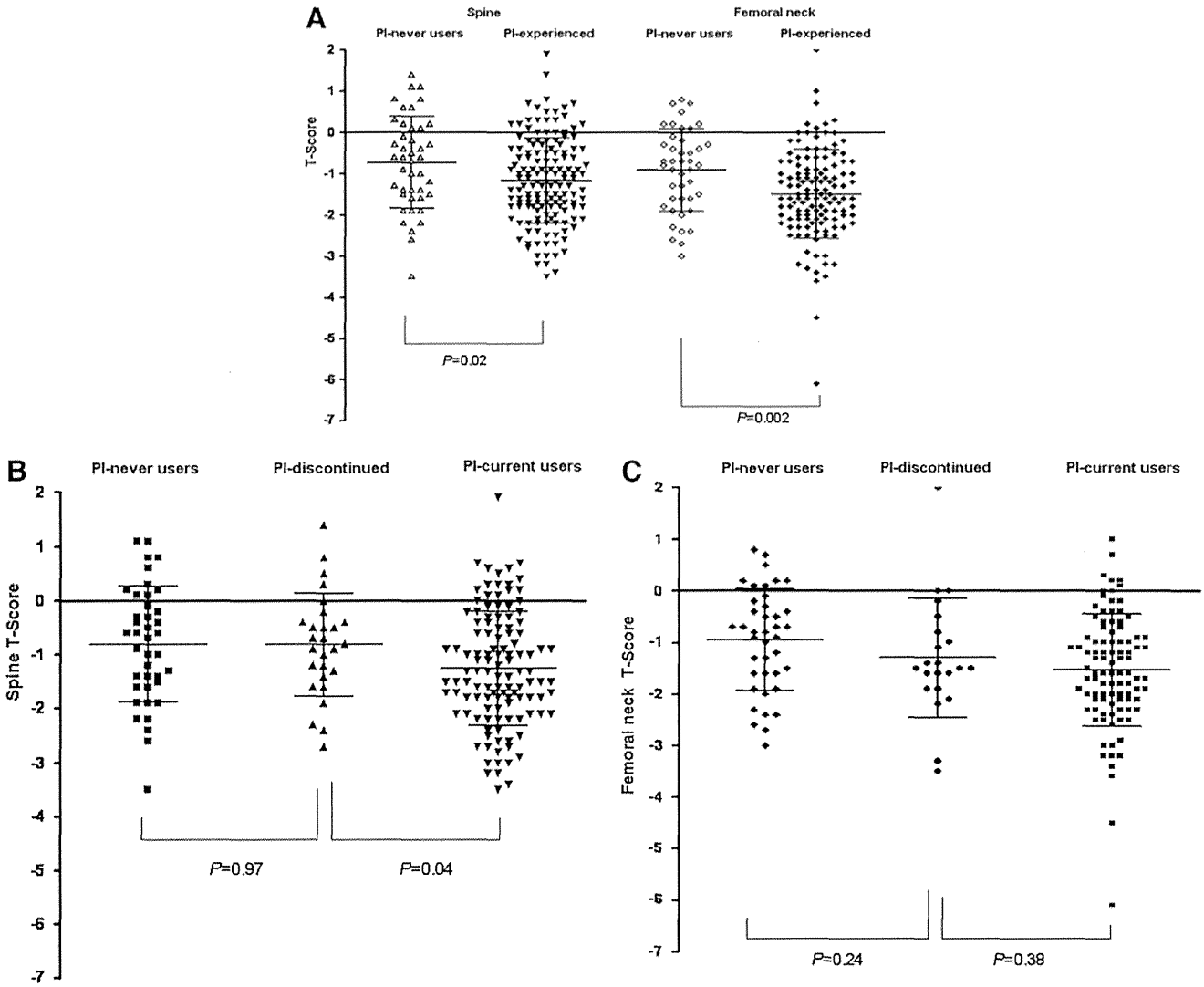

FIG. 2. (**A**) Comparison of *t* scores at lumbar spine and femoral neck between patients who were treated and never treated with a protease inhibitor (PI). Comparison of *t* score at lumbar spine (**B**) and at femoral neck (**C**) among patients who never used PI (left), discontinued PI (center), and are currently using PI (right). Data are mean ± standard deviation.

Although large cohort studies have already shown that PI use can cause BMD loss,[4–6] it still remains unclear which type of PI causes BMD loss. Our study found no significant association between the use of any particular type of PI and BMD loss, which is consistent with a previous *in vitro* study that evaluated the impact of different PIs on osteoblast activity using an osteoblast-like cell line.[10] Both *in vitro*[11,12] and *ex vivo* studies[13] reported that RTV promotes the proliferation/activation of osteoclasts, causing increased bone absorption. Our study added support to previous studies that RTV plays a major role in PI-associated BMD loss,[13] although there is insufficient data to conduct direct a comparison of BMD between patients treated with unboosted and boosted PI. The correlations between the two different dosages of RTV and BMD were almost comparable levels of strength, suggesting that RTV can cause BMD loss not dose dependently but time dependently irrespective of the dose. However, at this stage, we recommend further evaluation of the effect of each type of PI, since the subanalyses conducted in the present study have limited power for cause–effect evaluation due to the relatively small number of patients treated with certain types of PI.

Does discontinuation of PI lead to recovery of BMD? It seems there is no definitive answer to this question. A small cohort substudy showed possible BMD recovery after switching PI to raltegravir.[14] However, the change in BMD after switching was too small in that study to confirm the recovery effect of PI discontinuation. The present study provides additional data in support of a lower decrease in BMD by showing a large difference in BMD between PI-discontinued and -continued patients, although it is a cross-sectional study. A prospective longitudinal cohort study using a larger population on longer use of PI is necessary for a more precise evaluation of the reversibility of PI-associated BMD loss. It should be noted that the PI-discontinued patients showed a higher BMD level not in the femoral neck but in the lumbar spine, which is consistent with some large cohort study showing that PI causes greater BMD loss in the lumbar spine than the femoral neck.[4,5] This interesting discrepancy is well explained by the difference in bone tissue

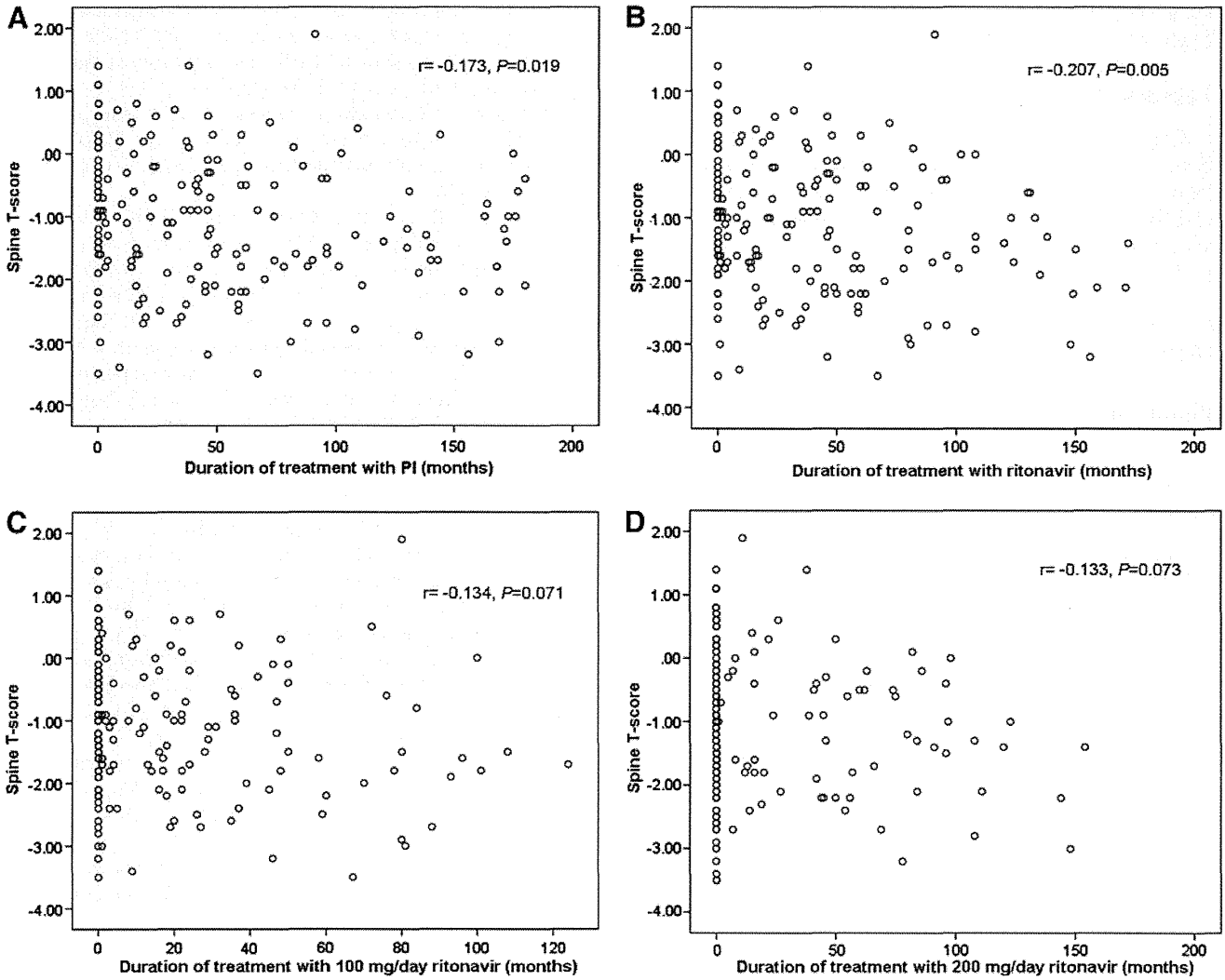

FIG. 3. Scattered dot plots of the correlation between *t* score at lumbar spine and duration of treatment with PI **(A)**, ritonavir (RTV) **(B)**, RTV at 100 mg/day **(C)**, and RTV at 200 mg/day **(D)**. Correlations were tested by Pearson's correlation coefficient.

type between the vertebrae and femur. While the femur contains abundant cortical substance with few osteoclasts, the vertebrae comprise osteoclast-rich trabecular substance. Therefore, discontinuation of osteoclast-activating agents, such as RTV, can cause a slower decrease of BMD in vertebrae compared with the femur.

TDF can cause BMD loss mainly through persistent urinary loss of phosphates.[4,7,15,16] However, our study did not show any significant association between TDF use and low BMD. While the exact reason for this finding is not clear, it could be related to the general clinical practice in Japan: TDF is often discontinued in Japan upon identification of modest proximal tubular dysfunction (a low level of percent tubular reabsorption of phosphates or a high level of urine-β_2-microglobulin) in HIV-infected patients.[16,17] This practice is an important limitation in the present study.

Hemophilia is a risk factor for BMD loss based on the associated hemophilic arthropathy and long-term disuse.[18,19] However, the present study demonstrated an almost equal prevalence of spine BMD abnormalities in hemophiliacs and HIV-infected patients [rate of osteoporosis, hemophiliacs: 5/67 (7%), other patients: 14/117 (12%); rate of osteopenia, hemophiliacs: 32/67 (48%), other patients: 52/117 (44%)]. Furthermore, the mean (standard deviation) *t* score of the lumbar spine was −1.1 (1.0) in hemophiliacs and −1.1 (1.1) in other patients. Thus, with regard to lumbar spine BMD, the present study well reflects the general Asian HIV-infected population. On the other hand, BMD abnormalities are common in hemophiliacs including abnormalities of the femoral neck [rate of osteoporosis, hemophiliacs: 15/57 (26%), other patients: 5/107 (5%); rate of osteopenia, hemophiliacs: 32/57 (56%), other patients: 56/107 (52%)]. The mean (standard deviation) *t* score of the femoral neck was −2.0 (1.1) in hemophiliacs and −1.0 (0.9) in other patients. Multivariate analysis identified age, BMI, and hemophilia as significant determinants of BMD at the femoral neck. Thus, BMD at the femoral neck is considered to be largely influenced by weight load and disuse.

In conclusion, long-term use of PI was identified as a significant risk factor for BMD loss in HIV-infected Asian patients. Furthermore, the results demonstrated that the negative effect of PI on BMD was time dependent. In particular, RTV plays a major role in PI-associated BMD loss irrespective of the dose. Discontinuation of PI seems to lessen the decrease in BMD, especially in the lumbar spine,

suggesting that withdrawal of PI is a promising option for treatment of BMD abnormalities.

Acknowledgments

The authors thank Hiroyuki Ito at the Clinical Research Center of NCGM and Yasuaki Yanagawa, Masahiro Ishikane, Takashi Matono, Kazuko Ikeda, Miwa Ogane, Michiyo Ishisaka, Akiko Nakano, Fumihide Kanaya, and all other staff at the AIDS Clinical Center for their help in the completion of this study.

This work was supported by a grant from the National Center for Global Health and Medicine, Japanese Ministry of Health, Labor, and Welfare.

Author Disclosure Statement

No competing financial interests exist.

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Low Prevalence of Transmitted Drug Resistance of HIV-1 During 2008–2012 Antiretroviral Therapy Scaling up in Southern Vietnam

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Background: The recent expansion of antiretroviral therapy (ART) program in resource-limited setting has raised concern about possible transmission of drug resistance (TDR). We assessed the prevalence of TDR over a 5-year period among treatment-naive individuals in Southern Vietnam during rapid ART scale-up.

Methods: Drug resistance mutations among antiretroviral-naive HIV-1–infected patients in Ho Chi Minh City were evaluated prospectively from 2008 to 2012 by HIV-1 pol gene sequencing. TDR was defined according to the World Health Organization list for surveillance of transmitted HIV-1 drug resistance in 2009.

Results: Pol sequence was obtained in 1389 individuals (median age: 30 years, males: 52.3%). Risks of HIV-1 infection included heterosexual contact in 60.7%, injection drug use in 22.4% and both 5.2%. The majority was infected with CRF01_AE (97%), whereas 19 were infected with subtype B. Over the 5-year study period, TDR was detected in 58 individuals (4.18%): 28 (2.02%) against nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), 19 (1.37%) against nonnucleoside reverse transcriptase inhibitors (NNRTIs), and 15 (1.08%) against protease inhibitors (PIs), including 4 (0.29%) against both NRTIs and NNRTIs. The most common TDR was K103N (0.5%) for NNRTI. The annual prevalence of TDR remained low to moderate (2008: 2.4%; 2009: 5.2%; 2010: 5.48%; 2011: 2.72%; 2012: 5.36%), and there was no clear trend over time.

Conclusions: There was no increase in TDR prevalence in Southern Vietnam during and after the 2008–2012 rapid scale up of ART.

Key Words: HIV, transmitted drug resistance, Vietnam

(*J Acquir Immune Defic Syndr* 2014;66:358–364)

INTRODUCTION

The recent roll-out campaigns in resource-limited settings to scale up antiretroviral therapy (ART) seem to have improved the morbidity and mortality of HIV-infected individuals. In Vietnam, where the HIV epidemic affected 249,660 individuals including 52,325 AIDS-related deaths up to the end of 2011, a national effort to facilitate ART supply has been implemented, and the ART coverage rate has rapidly increased from 18.1% in 2006 to 53% in 2011, saving 18,110 lives from AIDS-related deaths between 2000 and 2009.[1]

The expansion of ART program, however, has been accompanied by concerns on HIV drug resistance and risk of subsequent transmission of drug resistance (TDR) in new cases of HIV infection.[2] The WHO recommends surveillance of TDR where ART is being scaled up[3,4] and the Vietnam Authority of HIV/AIDS Control issued in 2008 a 5-year plan to assess and prevent HIV drug resistance. Because the large part of HIV epidemic in Vietnam has been driven by intravenous drug users (IDUs),[1,5] it is theoretically possible that the transmission of drug-resistant HIV spreads fast by sharing contaminated needles. The recent increase in HIV transmission by sexual intercourse in Vietnam also makes the TDR problem more difficult to control.[5] In addition, the pattern of antiretroviral drug use has been changing according to the global policy on ART recommendations or increased availability of second-line ART.[6–9] It is therefore important to monitor the prevalence of TDR and its pattern in Vietnam on a regular basis. Previous surveys and studies demonstrated low-to-moderate prevalence of TDR in Vietnam.[10–17] However, those studies were conducted using a cross-sectional setting or included monitoring for only a short period of time. To the best of our knowledge, there are no data on long-term monitoring of the prevalence of TDR in Vietnam.

This study was designed to assess the prevalence of TDR over a 5-year period in HIV-infected treatment-naive

Received for publication December 25, 2013; accepted March 27, 2014.

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Supported by the program of the Japan Initiative for Global Research Network on Infectious Diseases (10008050) from the Ministry of Education, Culture, Sports, Science and Technology, and the Grant for International Health Research (A22-2) from the Ministry of Health, Labour and Welfare, Government of Japan.

The authors have no conflicts of interest to disclose.

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