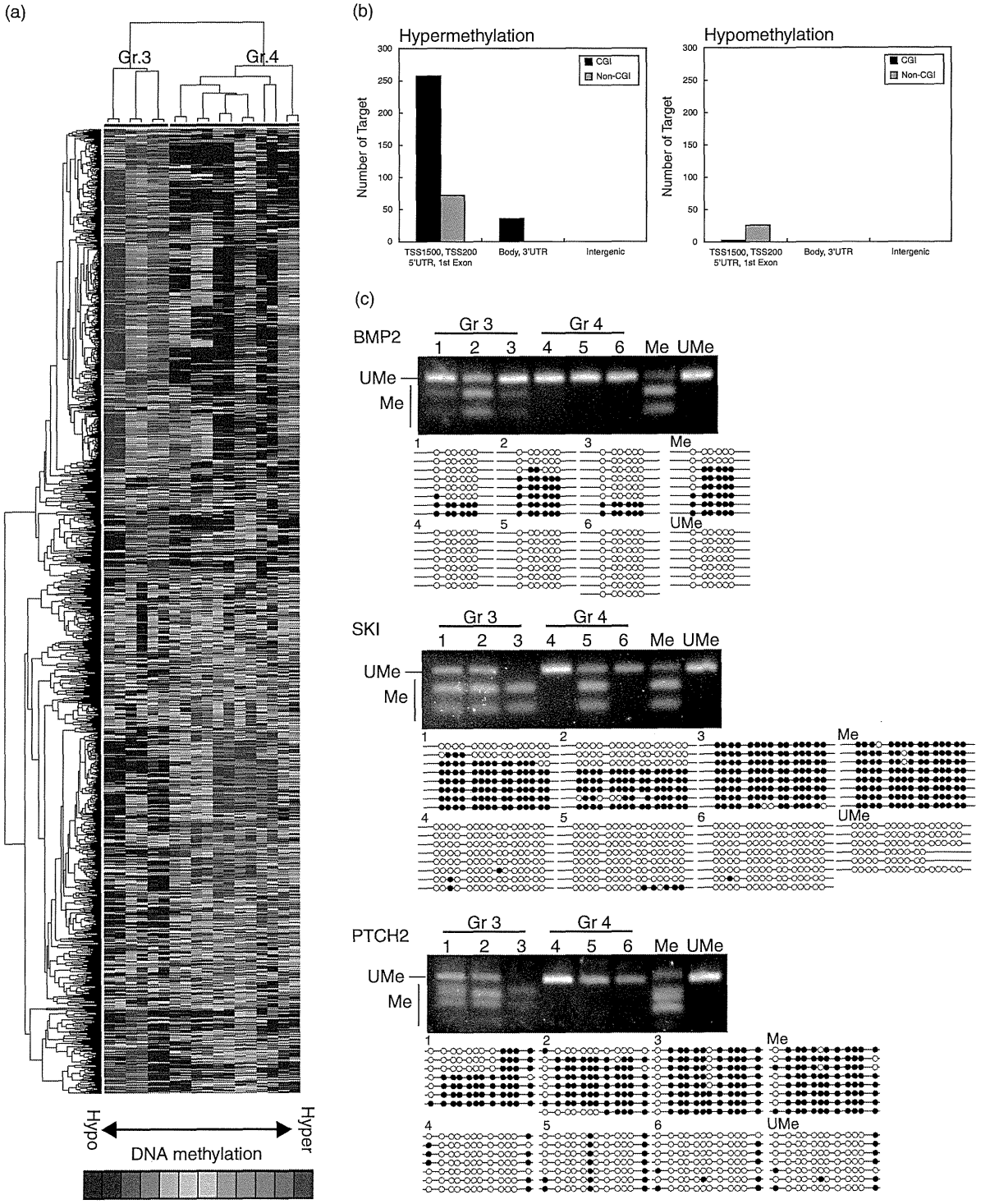

Fig. 2. Methylation profile clustering of HIV-associated lymphoma DNA in Cohort II, using Cancer Panel I. Cancer Panel I microarray analysis was performed for nine HIV-associated lymphomas in Cohort II. The color bar indicates hypermethylation and hypomethylation. Hierarchical clustering analysis of methylation gave two groups: Group 3 (Gr. 3) and Group 4 (Gr. 4). (b) Numbers of hypermethylation or hypomethylation targets in Group 3 compared with Group 4. (c) Validation by combined bisulfite restriction analysis (COBRA) and bisulfite DNA sequences. *BMP2* (bone morphogenetic protein 2), *SKI* (oncogene), and *PTCH2* (patched 2) are representative targets in the array analysis. Me, methylated allele or methylated control; UMe, unmethylated allele or unmethylated control; open circle, unmethylated CpG site; solid circle, methylated CpG site.

methylated, whereas fewer in Group 4 were methylated among those tested (Fig. 2c, upper). Bisulfite DNA sequencing clearly showed that Group 3 was highly methylated (Fig. 2c, lower), confirming the tendency toward hypermethylation in Group 3. Two cases in Group 3 subsequently showed recurrence, representing a significant patient characteristic ($P=0.083$), if 0.1 was considered a significant level (Table 2). In another case in Group 3, a tumor mass appeared in the cervical spinal cord about 17 months later, although recurrence was not confirmed pathologically. Notably, the methylation profile of nonrecurrent HIV-associated lymphomas (Group 4) did not differ significantly from that of non-HIV lymphomas (non-Group 3, Supplementary Fig. 5 and Supplementary Table 1, http://links.lww.com/QAD/A441). These data suggest that recurrent HIV-associated lymphomas have a specific methylation profile.

Discussion

The prognosis of HIV-associated lymphoma has improved with the development of HIV and cancer therapies [15]. Nevertheless, it is important to identify the mechanism responsible for the aggressiveness of HIV-associated lymphomas. Our data suggest that the DNA methylation profile is a molecular indicator of prognosis.

In the methylation analyses, we examined nine or 11 HIV-associated lymphomas. This number was relatively small because of the small HIV-positive population in Japan [13]. Even so, our data clearly suggest that DNA methylation profiles, especially CGI methylation in promoter regions, differ between HIV-associated and non-HIV lymphomas. As the tumor location varies in HIV-associated lymphoma [2], it is essential to know whether tumor location influenced our analyses. Lymph nodes were the most frequent tumor location and were broadly similar in Groups 1 and 2 ($P=0.45$; Supplementary Fig. 6a, http://links.lww.com/QAD/A441), although Group 1 had more extra-node variation, probably due to the high proportion of HIV-associated lymphoma. It is noteworthy that Group 1 had narrower correlation distances than Group 2, indicating that the DNA methylation profiles in Group 1 were quite similar, although Group 1 included various tumor locations (Supplementary Fig. 6b, http://links.lww.com/QAD/A441). Additionally, the lymph node cases in Group 1 were very dissimilar from the lymph node cases in Group 2. The data suggested that the clustered results were not due to tumor location. The differences between the profiles may not be related to antiretroviral therapy either, as only two HIV-positive lymphomas in Cohort I were treated with antiretroviral therapy. Coinfections such as EBV with HIV may influence DNA methylation profiles, but we found no significant difference between HIV-associated and non-HIV lymphomas in terms of EBV infection status in our study. However, we cannot exclude the influence of HIV infection on methylation profiles. One of our validation genes, *RARRES1*, is a cancer methylation target [16] that is differentially expressed in various tumors [17,18], although its clinical relevance to lymphomas remains unknown. *FGF5* is reported to be a bone metastasis-related gene related to angiogenesis [19]. As angiogenic growth factors have been implicated in a

Table 2. Patient characteristics of lymphoma samples in Cohort II for Cancer Panel I.

Items examined		HIV-associated lymphomas		*P* value (Group 3 vs. Group 4)
		Group 3	Group 4	
Sex	Female	1	0	
	Male	2	6	0.33
Age	Mean	36.66	35.00	
	SD	5.77	13.78	1.00
Histology	BL	2	3	
	DLBCL	1	1	
	HD	0	2	1.00
Bcl-2	+	0	1	
	−	3	5	1.00
Stage	I & II	0	2	
	III & IV	3	4	0.50
EBV	+	1	4	
	−	2	2	0.52
Recurrence	+	2	0	
	−	1[a]	6	0.083
IPI score[b]	0 or 1	0	1	
	2 or 3	1	1	
	4 or 5	2	2	1.00

The statistical significance of differences in the categorical variables was calculated by Fisher's exact test or Wilcoxon's rank-sum test. BL, Burkitt lymphoma; DLBCL, diffuse large B-cell lymphoma; EBV, Epstein–Barr virus; HD, Hodgkin's disease.
[a]A tumor mass appeared in the cervical spinal cord about 17 months later, although recurrence was not confirmed pathologically.
[b]IPI, International Prognostic Index for non-HD [stage, lactate dehydrogenase (LDH), performance status, age]. SD, standard deviation.

poor prognosis in non-Hodgkin lymphomas [20], hypomethylated *FGF5* may similarly influence the prognosis in HIV-associated lymphomas. Note that several significant pathways related to cell adhesion were found (Supplementary Table 2, http://links.lww.com/QAD/A441). Of these, those involving laminins, collagens, N-cadherin, and caveolin2 were significantly hypomethylated in HIV-associated lymphomas, suggesting that their increased expression initiates and promotes tumors and results in a poor prognosis [21–23]. These data partly support the poor prognosis seen in HIV-associated lymphomas.

Clustering analysis of the Cohort II data obtained using Cancer Panel I placed recurrent or suspicious and nonrecurrent HIV-associated lymphomas into separate groups, suggesting that recurrence of HIV-associated lymphomas is attributable to specific gene regulation involving DNA methylation. *PTCH2*, which was used for validation, was a significant component of the Hedgehog signaling pathway (Supplementary Table 3, http://links.lww.com/QAD/A441), which is related to relapse rate in carcinomas [24]. The data imply that the DNA methylation profile is a good indicator of prognosis. Recently, specific methylation targets have been reported as candidates for new biomarkers of prognosis or metastasis [25,26]. Careful determinations in more cases will identify biomarkers for recurrence in HIV-associated lymphomas.

To our knowledge, this is the first report using molecular technology to distinguish HIV-associated lymphomas from non-HIV lymphomas. Our findings contribute to the understanding of HIV-associated lymphomagenesis and suggest new prognostic biomarkers.

Acknowledgements

This study was supported by a Grant-in-Aid for Research on Advanced Medical Technology from the Ministry of Health, Labor, and Welfare of Japan (Grant number: H22-AIDS-I-002, and H25-AIDS-I-002), a Grant for International Health Research (104-A), Center of Excellence Research from the Ministry of Education, Sports, Culture, Science, and Technology of Japan, and Grants-in-Aid from the Japan Science and Technology Agency.

Author contributions: S.H. is responsible for the study design and the clinical data. S.H., M.S. and Y.I. planned the experiments; J.T. and S.O. analyzed the clinical information; T.H. and M.M. prepared the pathology samples; A.M. prepared gDNA from clinical samples; A.M. and M.S. analyzed the microarray data; N.T., M.Y., and L.Y. were in charge of the statistical analyses and bioinformatics; and M.S. and S.H. wrote the article. All authors discussed the results of the article.

Conflicts of interest

There are no conflicts of interest.

References

1. Cote TR, Biggar RJ, Rosenberg PS, Devesa SS, Percy C, Yellin FJ, *et al.* **Non-Hodgkin's lymphoma among people with AIDS: incidence, presentation and public health burden.** *Int J Cancer* 1997; **73**:645–650.
2. Raphael M, Said J, Borisch B, Cesarman E, Harris NL. **Lymphomas associated with HIV infection.** In: Swerdlow SH, Campo E, Harris NL, Jaffe E, Pillar SA, Stein H,*et al.* editors. *WHO Classification of tumours of haematopoietic and lymphoid tissues*. 4th ed . Lyon: IARC; 2008. pp. 340–342.
3. Simard EP, Engels EA. **Cancer as a cause of death among people with AIDS in the United States.** *Clin Infect Dis* 2010; **51**:957–962.
4. Borinstein SC, Conerly M, Dzieciatkowski S, Biswas S, Washington MK, Trobridge P, *et al.* **Aberrant DNA methylation occurs in colon neoplasms arising in the azoxymethane colon cancer model.** *Mol Carcinog* 2010; **49**:94–103.
5. Martin-Subero JI, Kreuz M, Bibikova M, Bentink S, Ammerpohl O, Wickham-Garcia E, *et al.* **New insights into the biology and origin of mature aggressive B-cell lymphomas by combined epigenomic, genomic, and transcriptional profiling.** *Blood* 2009; **113**:2488–2497.
6. Richter J, Ammerpohl O, Martin-Subero JI, Montesinos-Rongen M, Bibikova M, Wickham-Garcia E, *et al.* **Array-based DNA methylation profiling of primary lymphomas of the central nervous system.** *BMC Cancer* 2009; **9**:455.
7. Amara K, Trimeche M, Ziadi S, Laatiri A, Hachana M, Korbi S. **Prognostic significance of aberrant promoter hypermethylation of CpG islands in patients with diffuse large B-cell lymphomas.** *Ann Oncol* 2008; **19**:1774–1786.
8. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, Le JM, *et al.* **High density DNA methylation array with single CpG site resolution.** *Genomics* 2011; **98**:288–295.
9. Thirlwell C, Eymard M, Feber A, Teschendorff A, Pearce K, Lechner M, *et al.* **Genome-wide DNA methylation analysis of archival formalin-fixed paraffin-embedded tissue using the Illumina Infinium HumanMethylation27 BeadChip.** *Methods* 2010; **52**:248–254.
10. Wang D, Yan L, Hu Q, Sucheston LE, Higgins MJ, Ambrosone CB, *et al.* **IMA: an R package for high-throughput analysis of Illumina's 450K Infinium methylation data.** *Bioinformatics* 2012; **28**:729–730.
11. Davis S, Bilke S. methylumi: Handle Illumina methylation data. Bioconductor R package version 132 2010. http://www.bioconductor.org/packages/release/bioc/html/methylumi.html [Accessed 24 September 2012].
12. Bibikova M, Lin Z, Zhou L, Chudin E, Garcia EW, Wu B, *et al.* **High-throughput DNA methylation profiling using universal bead arrays.** *Genome Res* 2006; **16**:383–393.
13. Hagiwara S, Yotsumoto M, Odawara T, Ajisawa A, Uehira T, Nagai H, *et al.* **Non-AIDS-defining hematological malignancies in HIV-infected patients: an epidemiological study in Japan.** *AIDS* 2013; **27**:279–283.
14. Teschendorff AE, Menon U, Gentry-Maharaj A, Ramus SJ, Weisenberger DJ, Shen H, *et al.* **Age-dependent DNA methylation of genes that are suppressed in stem cells is a hallmark of cancer.** *Genome Res* 2010; **20**:440–446.
15. Shiels MS, Pfeiffer RM, Gail MH, Hall HI, Li J, Chaturvedi AK, *et al.* **Cancer burden in the HIV-infected population in the United States.** *J Natl Cancer Inst* 2011; **103**:753–762.
16. Youssef EM, Chen XQ, Higuchi E, Kondo Y, Garcia-Manero G, Lotan R, *et al.* **Hypermethylation and silencing of the putative tumor suppressor Tazarotene-induced gene 1 in human cancers.** *Cancer Res* 2004; **64**:2411–2417.
17. Davidson B, Stavnes HT, Risberg B, Nesland JM, Wohlschlaeger J, Yang Y, *et al.* **Gene expression signatures differentiate adenocarcinoma of lung and breast origin in effusions.** *Hum Pathol* 2012; **43**:684–694.
18. Kloth M, Goering W, Ribarska T, Arsov C, Sorensen KD, Schulz WA. **The SNP rs6441224 influences transcriptional activity and prognostically relevant hypermethylation of RARRES1 in prostate cancer.** *Int J Cancer* 2012; **131**:E897–E904.

19. Casimiro S, Luis I, Fernandes A, Pires R, Pinto A, Gouveia AG, *et al.* **Analysis of a bone metastasis gene expression signature in patients with bone metastasis from solid tumors.** *Clin Exp Metastasis* 2012; **29**:155–164.
20. Salven P, Orpana A, Teerenhovi L, Joensuu H. **Simultaneous elevation in the serum concentrations of the angiogenic growth factors VEGF and bFGF is an independent predictor of poor prognosis in non-Hodgkin lymphoma: a single-institution study of 200 patients.** *Blood* 2000; **96**:3712–3718.
21. Saha S, Lo PK, Duan X, Chen H, Wang Q. **Breast tumour initiating cell fate is regulated by microenvironmental cues from an extracellular matrix.** *Integr Biol (Camb)* 2012; **4**:897–904.
22. Montenegro RC, de Vasconcellos MC, Barbosa GD, Burbano RM, Souza LG, Lemos TL, *et al.* **A novel o-naphtoquinone inhibits N-cadherin expression and blocks melanoma cell invasion via AKT signaling.** *Toxicol In Vitro* 2013; **27**:2076–2083.
23. Ando T, Ishiguro H, Kimura M, Mitsui A, Mori Y, Sugito N, *et al.* **The overexpression of caveolin-1 and caveolin-2 correlates with a poor prognosis and tumor progression in esophageal squamous cell carcinoma.** *Oncol Rep* 2007; **18**:601–609.
24. Chaudary N, Pintilie M, Hedley D, Fyles AW, Milosevic M, Clarke B, *et al.* **Hedgehog pathway signaling in cervical carcinoma and outcome after chemoradiation.** *Cancer* 2012; **118**:3105–3115.
25. Bougel S, Lhermitte B, Gallagher G, de Flaugergues JC, Janzer RC, Benhattar J. **Methylation of the hTERT promoter: a novel cancer biomarker for leptomeningeal metastasis detection in cerebrospinal fluids.** *Clin Cancer Res* 2013; **19**:2216–2223.
26. Huang RL, Gu F, Kirma NB, Ruan J, Chen CL, Wang HC, *et al.* **Comprehensive methylome analysis of ovarian tumors reveals hedgehog signaling pathway regulators as prognostic DNA methylation biomarkers.** *Epigenetics* 2013; **8**:624–634.

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

Takeshi Nishijima[1,2], Takuro Shimbo[3], Hirokazu Komatsu[4], Yohei Hamada[1], Hiroyuki Gatanaga[1,2]*, Yoshimi Kikuchi[1] and Shinichi Oka[1,2]

[1]AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan; [2]Center for AIDS Research, Kumamoto University, Kumamoto, Japan; [3]Department of Clinical Study and Informatics, Center for Clinical Sciences, National Center for Global Health and Medicine, Tokyo, Japan; [4]Department of Community Care, Saku Central Hospital, Nagano, Japan

*Corresponding author. AIDS Clinical Center, National Center for Global Health and Medicine, 1-21-1, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan. Tel: +81-3-3202-7181; Fax: +81-3-3208-4244; E-mail: higatana@acc.ncgm.go.jp

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/).

References

1 Squires K, Lazzarin A, Gatell JM *et al.* Comparison of once-daily atazanavir with efavirenz, each in combination with fixed-dose zidovudine and lamivudine, as initial therapy for patients infected with HIV. *J Acquir Immune Defic Syndr* 2004; **36**: 1011–9.

2 Molina JM, Andrade-Villanueva J, Echevarria J *et al.* Once-daily atazanavir/ritonavir compared with twice-daily lopinavir/ritonavir, each in combination with tenofovir and emtricitabine, for management of antiretroviral-naive HIV-1-infected patients: 96-week efficacy and safety results of the CASTLE study. *J Acquir Immune Defic Syndr* 2010; **53**: 323–32.

3 Panel on Antiretroviral Guidelines for Adults and Adolescents. *Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents*. Department of Health and Human Services. http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf (25 November 2013, date last accessed).

4 *Reyataz (Atazanavir Sulfate): Full Prescription Information (Package Insert)*. Princeton: Bristol-Myers Squibb, 2012.

5 Rakotondravelo S, Poinsignon Y, Borsa-Lebas F *et al.* Complicated atazanavir-associated cholelithiasis: a report of 14 cases. *Clin Infect Dis* 2012; **55**: 1270–2.

6 Courbon E, Laylavoix F, Soulie C *et al.* Unexpected atazanavir-associated biliary lithiasis in an HIV-infected patient. *J Antimicrob Chemother* 2012; **67**: 250–1.

7 Jacques AC, Giguere P, Zhang G *et al.* Atazanavir-associated choledocholithiasis leading to acute hepatitis in an HIV-infected adult. *Ann Pharmacother* 2010; **44**: 202–6.

8 Hamada Y, Nishijima T, Komatsu H *et al.* Is ritonavir-boosted atazanavir a risk for cholelithiasis compared to other protease inhibitors? *PLoS One* 2013; **8**: e69845.

9 Barbara L, Sama C, Morselli Labate AM *et al.* A population study on the prevalence of gallstone disease: the Sirmione Study. *Hepatology* 1987; **7**: 913–7.

10 Nishijima T, Komatsu H, Higasa K *et al.* Single nucleotide polymorphisms in ABCC2 associate with tenofovir-induced kidney tubular dysfunction in Japanese patients with HIV-1 infection: a pharmacogenetic study. *Clin Infect Dis* 2012; **55**: 1558–67.

11 The epidemiology of gallstone disease in Rome, Italy. Part II. Factors associated with the disease. The Rome Group for Epidemiology and Prevention of Cholelithiasis (GREPCO). *Hepatology* 1988; **8**: 907–13.

12 Conte D, Fraquelli M, Fornari F *et al.* Close relation between cirrhosis and gallstones: cross-sectional and longitudinal survey. *Arch Intern Med* 1999; **159**: 49–52.

13 De Santis A, Attili AF, Ginanni Corradini S *et al.* Gallstones and diabetes: a case–control study in a free-living population sample. *Hepatology* 1997; **25**: 787–90.

14 Hamada Y, Nishijima T, Watanabe K *et al.* High incidence of renal stones among HIV-infected patients on ritonavir-boosted atazanavir than in those receiving other protease inhibitor-containing antiretroviral therapy. *Clin Infect Dis* 2012; **55**: 1262–9.

15 Rockwood N, Mandalia S, Bower M *et al.* Ritonavir-boosted atazanavir exposure is associated with an increased rate of renal stones compared with efavirenz, ritonavir-boosted lopinavir and ritonavir-boosted darunavir. *AIDS* 2011; **25**: 1671–3.

16 Le MP, Stitou H, Soulie C *et al.* Sialolithiasis in an HIV-1-infected patient treated with atazanavir/ritonavir monotherapy. *J Antimicrob Chemother* 2013; **68**: 727–9.

17 Zhang D, Chando TJ, Everett DW *et al.* In vitro inhibition of UDP glucuronosyltransferases by atazanavir and other HIV protease inhibitors

and the relationship of this property to in vivo bilirubin glucuronidation. *Drug Metab Dispos* 2005; **33**: 1729–39.

18 Verdon R, Daudon M, Albessard F *et al.* Indinavir-induced cholelithiasis in a patient infected with human immunodeficiency virus. *Clin Infect Dis* 2002; **35**: e57–9.

19 Siveke JT, Bogner JR. Cholelithiasis possibly induced by protease inhibitors in 3 patients. *Clin Infect Dis* 2003; **36**: 1498–500.

20 Shaffer EA. Epidemiology and risk factors for gallstone disease: has the paradigm changed in the 21st century? *Curr Gastroenterol Rep* 2005; **7**: 132–40.

AID-2013-0252-ver9-Kinai_1P
Type: research-article

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 30, Number 00, 2014

DOI: 10.1089/aid.2013.0252

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

Ei Kinai, Takeshi Nishijima, Daisuke Mizushima, Koji Watanabe, Takahiro Aoki, Haruhito Honda, Hirohisa Yazaki, Ikumi Genka, Junko Tanuma, Katsuji Teruya, Kunihisa Tsukada, Hiroyuki Gatanaga, Yoshimi Kikuchi, and Shinichi Oka

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

AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan.

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

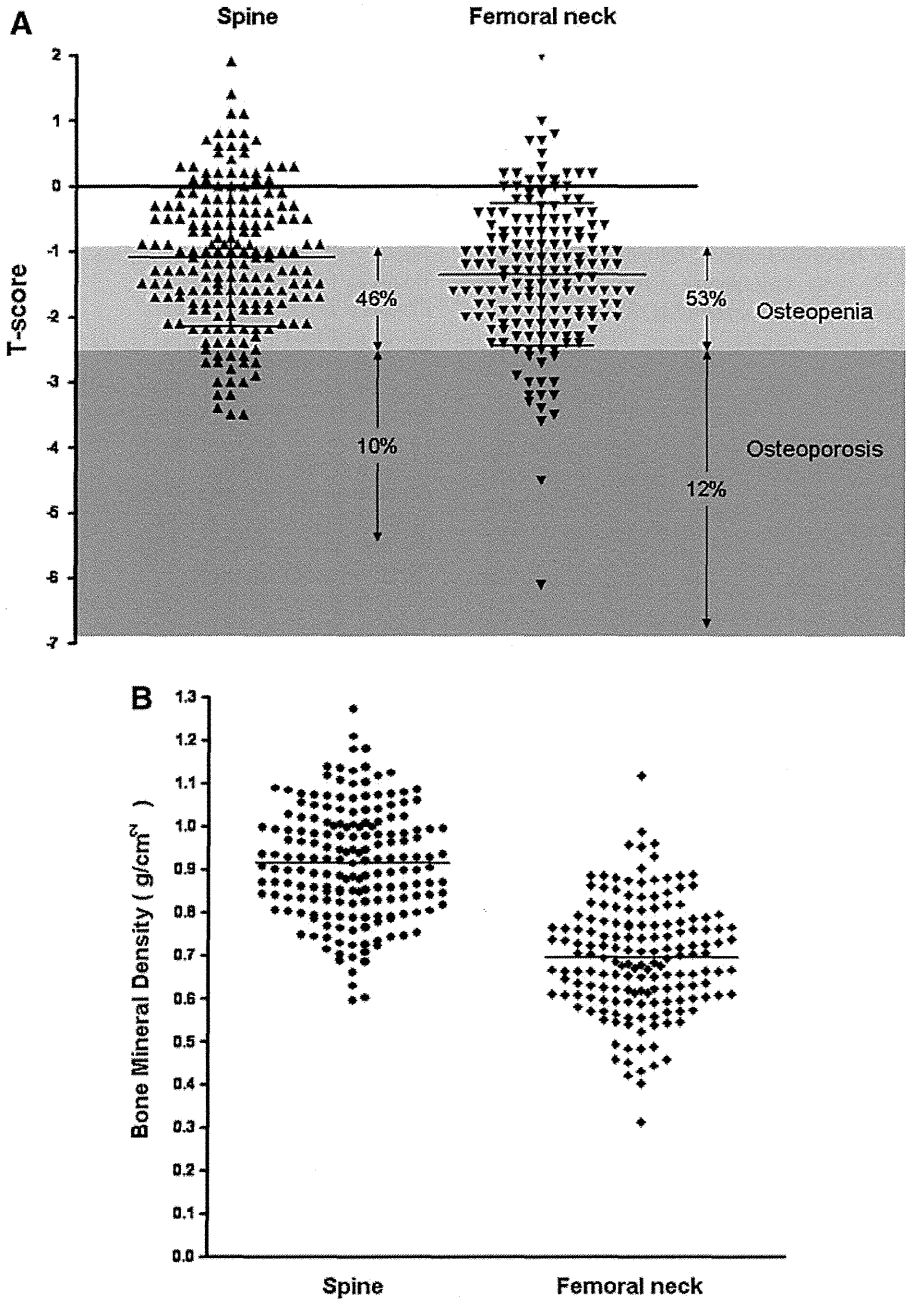

FIG. 1. **(A)** Distribution of *t* scores at lumbar spine and femoral neck. Light areas: osteopenia; dark gray areas: osteoporosis. **(B)** Distribution of bone mineral density (BMD) at lumbar spine and femoral neck. In both **(A)** and **(B)**, data are mean ± standard deviation. Differences in the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

T2 ▶ ($p < 0.001$) (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 ◀ F2 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: $r=0.091$, $p=0.218$).

F3 ▶

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.

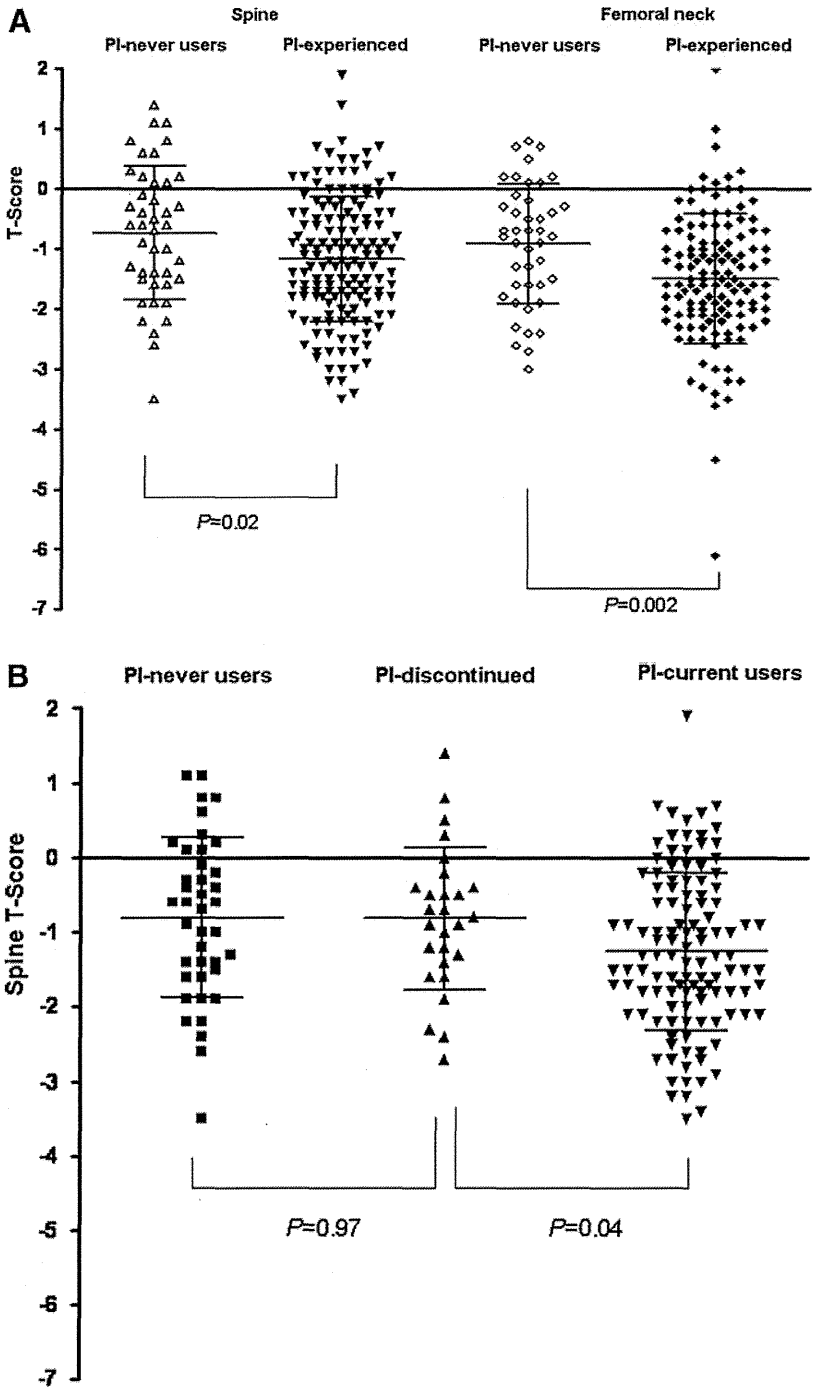

FIG. 2. (**A**) Comparison of *t* scores at lumbar spine and femoral neck between patients who were treated and never treated with a protease inhibitor (PI). Comparison of *t* score at lumbar spine (**B**) and at femoral neck (**C**) among patients who never used PI (left), discontinued PI (center),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

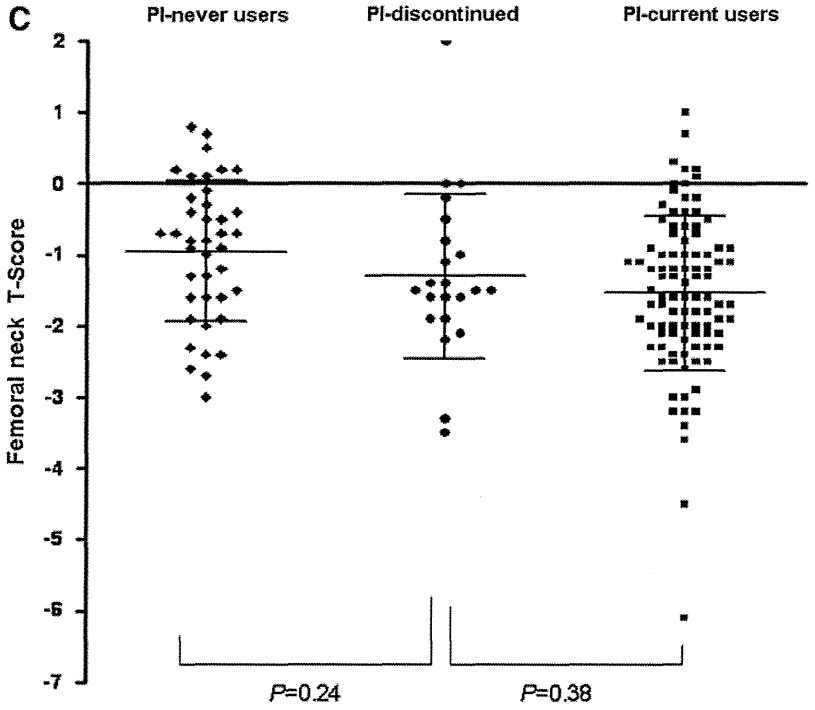

FIG. 2. (*Continued*)

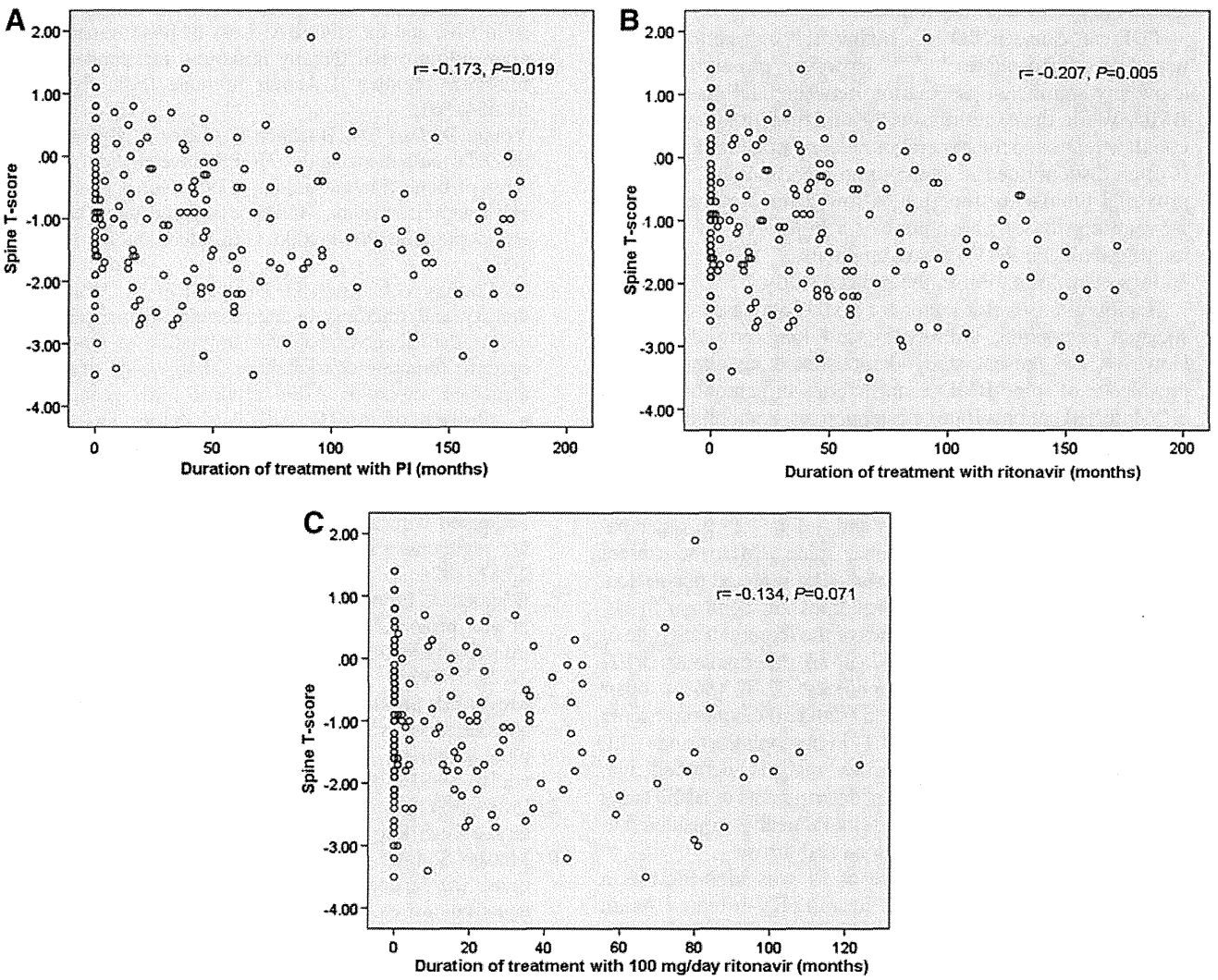

FIG. 3. Scattered dot plots of the correlation between *t* score at lumbar spine and duration of treatment with PI (**A**), ritonavir (RTV) (**B**), RTV at 100 mg/day (**C**), and RTV at 200 mg/day (**D**). Correlations were tested by Pearson's correlation coefficient.

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 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. ◀AU1

References

1. Brown TT and Qaqish RB: Antiretroviral therapy and the prevalence of osteopenia and osteoporosis; a meta-analytic review. AIDS 2006;20:2165–2174.
2. Brown TT, McComsey GA, King MS, Qaqish RB, Bernstein BM, and da Silva BA: Loss of bone mineral density after antiretroviral therapy initiation, independent of antiretroviral regimen. J Acquir Immune Defic Syndr 2009; 51:554–561.
3. Young B, Dao CN, Buchacz K, Baker R, Brooks JT, and the HIV outpatient study (HOPS) investigators: Increased rates of bone fracture among HIV-infected persons in the HIV outpatient study (HOPS) compared with the US general population, 2000–2006. Clin Infect Dis 2011;52:1061–1068.
4. McComsey GA, Kitch D, Daar ES, *et al.:* Bone mineral density and fractures in antiretroviral-naïve persons randomized to receive abacavir-lamivudine or tenofovir disoproxil fumarate-emtricitabine along with efavirenz or atazanavir-ritonavir: AIDS clinical trials group A5224s, a substudy of ACTG A5202. J Infect Dis 2011;203: 1791–1801.
5. Duvivier C, Kolta S, Assoumou L, *et al.:* Greater decrease in bone mineral density with protease inhibitor regimens compared with non-nucleoside reverse transcriptase inhibitor regimens in HIV-1 infected naive patients. AIDS 2009; 23:817–824.
6. Bonjoch A, Figueras M, Estany C, *et al.:* High prevalence of and progression to low bone mineral density in HIV-infected patients: A longitudinal cohort study. AIDS 2010; 24:2827–2833.
7. Moyle GJ, Stellbrink HJ, Compston J, *et al.:* 96-week results of abacavir/lamivudine versus tenofovir/emtricitabine, plus efavirenz, in antiretroviral-naïve, HIV-1-infected adults: ASSERT study. Antivir Ther 2013;18:905–913.
8. Prevention and management of osteoporosis. World Health Organ Tech Rep Ser 2003;921:1–164
9. Matsuo S, Imai E, Horio M, *et al.:* Collaborators developing the Japanese equation for estimated GFR: Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis 2009;53:982–992.
10. Gibellini D, Borderi M, de Crignis E, *et al.:* Analysis of the effects of specific protease inhibitors on OPG/RANKL regulation in an osteoblast-like cell line. New Microbiol 2010;33:109–115.

11. Modarresi R, Xiang Z, Yin M, and Laurence J: WNT/ β-Catenin signaling is involved in regulation of osteoclast differentiation by human immunodeficiency virus protease inhibitor ritonavir. Am J Pathol 2009;174:123–135.
12. Santiago F, Oguma J, Brown AMC, and Laurence J: Noncanonical Wnt signaling promotes osteoclast differentiation and is facilitated by the human immunodeficiency virus protease inhibitor ritonavir. Biochem Biophys Res Commun 2012;417:223–230.
13. Yin, MT, Modaressi R, Shane E, *et al.:* Effects of HIV infection and antiretroviral therapy with ritonavir on induction of osteoclast-like cells in postmenopausal women. Osteoporos Int 2011;22:1459–1468.
14. Curran A, Martinez E, Saumoy M, *et al.:* Body composition changes after switching from protease inhibitors to raltegravir: SPIRAL-LIP substudy. AIDS 2012;26:475–481.
15. Van Rompay KKA, Brignolo LL, Meyer DJ, *et al.:* Biological effects of short-term or prolonged administration of 9-[2-(phosphonomethoxy) propyl] adenine (tenofovir) to newborn and infant rhesus macaques. Antimicrob Agents Chemother 2004;48:1469–1487.
16. Kinai E and Hanabusa H: Progressive renal tubular dysfunction associated with long-term use of tenofovir DF. AIDS Res Hum Retroviruses 2009;25:387–394.
17. Gatanaga H, Tachikawa N, Kikuchi Y, *et al.:* Urinary beta2-microglobulin as a possible sensitive marker for renal injury caused by tenofovir disoproxil fumarate. AIDS Res Hum Retroviruses 2006;22:744–748.
18. Gerstner G, Damiano ML, Tom A, *et al.:* Prevalence and risk factors associated with decreased bone mineral density in patients with haemophilia. Haemophilia 2009;15:559–565.
19. Nair AP, Jjina F, Ghosh K, Madkaikar M, Shrikhande M, and Nema M: Osteoporosis in young haemophiliacs from western India. Am J Hematol 2007;82:453–457.

Address correspondence to:
Ei Kinai
AIDS Clinical Center
National Center for Global Health and Medicine, Tokyo
1-21-1, Toyama, Shinjuku-ku
Tokyo, 162-8655
Japan

E-mail: ekinai@acc.ncgm.go.jp

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

Koji Watanabe,[1,2] Takahiro Aoki,[1] Naoyoshi Nagata,[3] Junko Tanuma,[1] Yoshimi Kikuchi,[1] Shinichi Oka,[1,2] and Hiroyuki Gatanaga[1,2]

[1]AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan; [2]Center for AIDS Research, Kumamoto University, Kumamoto, Japan and [3]Department of Gastroenterology and Hepatology, National Center for Global Health and Medicine, Tokyo, Japan

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.

Correspondence: Dr Hiroyuki Gatanaga, AIDS Clinical Center, National Center for Global Health and Medicine, 1-21-1, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan (higatana@acc.ncgm.go.jp).

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

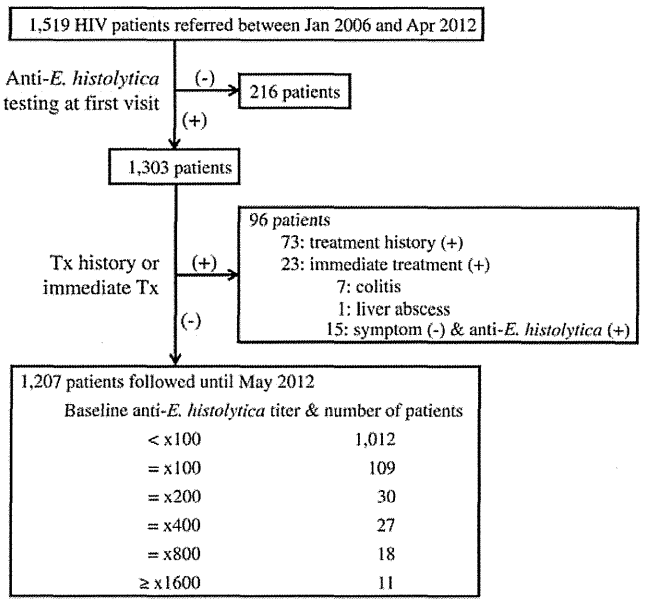

Figure 1. Flow diagram of patient recruitment process. Abbreviations: Anti-*E. histolytica*, anti-*Entamoeba histolytica* antibody; IA, invasive amebiasis; Tx, treatment.