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

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Abstract

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

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

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

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

Trial Registration: ClinicalTrials.gov NCT01294761 http://clinicaltrials.gov/ct2/show/NCT01294761?term=SPARE&rank=2, Umin Clinical Trials Registry UMIN000005116 http://upload.umin.ac.jp/cgi-open-bin/ctr/ctr.cgi?function=brows&action=brows&type=summary&recptno=R000006083&language=J)

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Introduction

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

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

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

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

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

Methods

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

Ethics Statement

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

Study Design

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

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

Study Patients

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

Study Procedure

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

Statistical Analysis

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

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

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

Results

Patient disposition and baseline characteristics

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

Primary endpoint

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

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

Table 1. Baseline characteristics of the enrolled patients.

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

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

† median (interquartile range)

Secondary renal endpoints

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

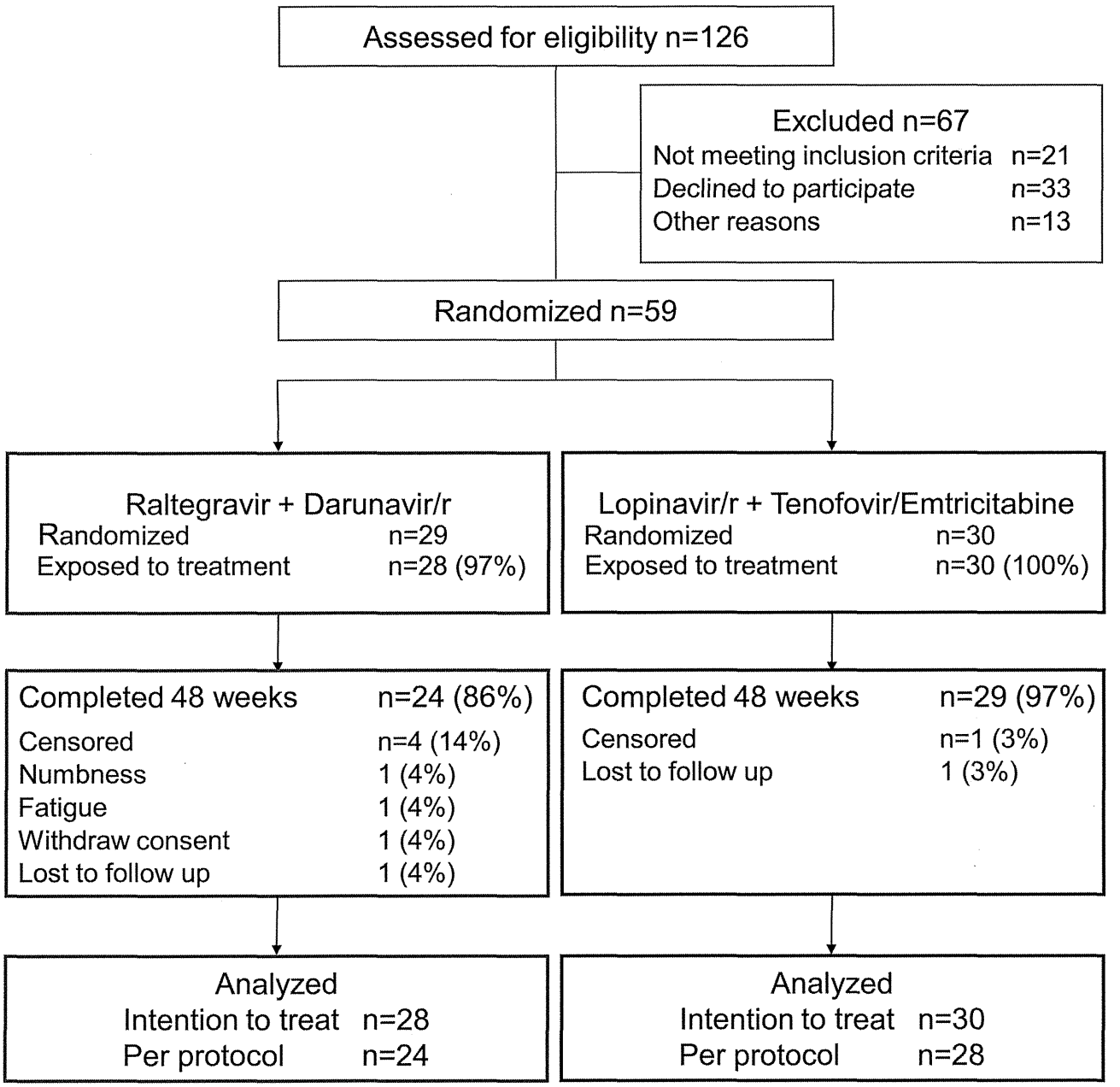

Figure 1. Enrollment, randomization, and disposition of patients. Darunavir/r, ritonavir-boosted darunavir; Lopinavir/r, ritonavir-boosted lopinavir.

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

Secondary efficacy endpoints

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

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

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

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

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

Safety and tolerability

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

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

Discussion

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

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

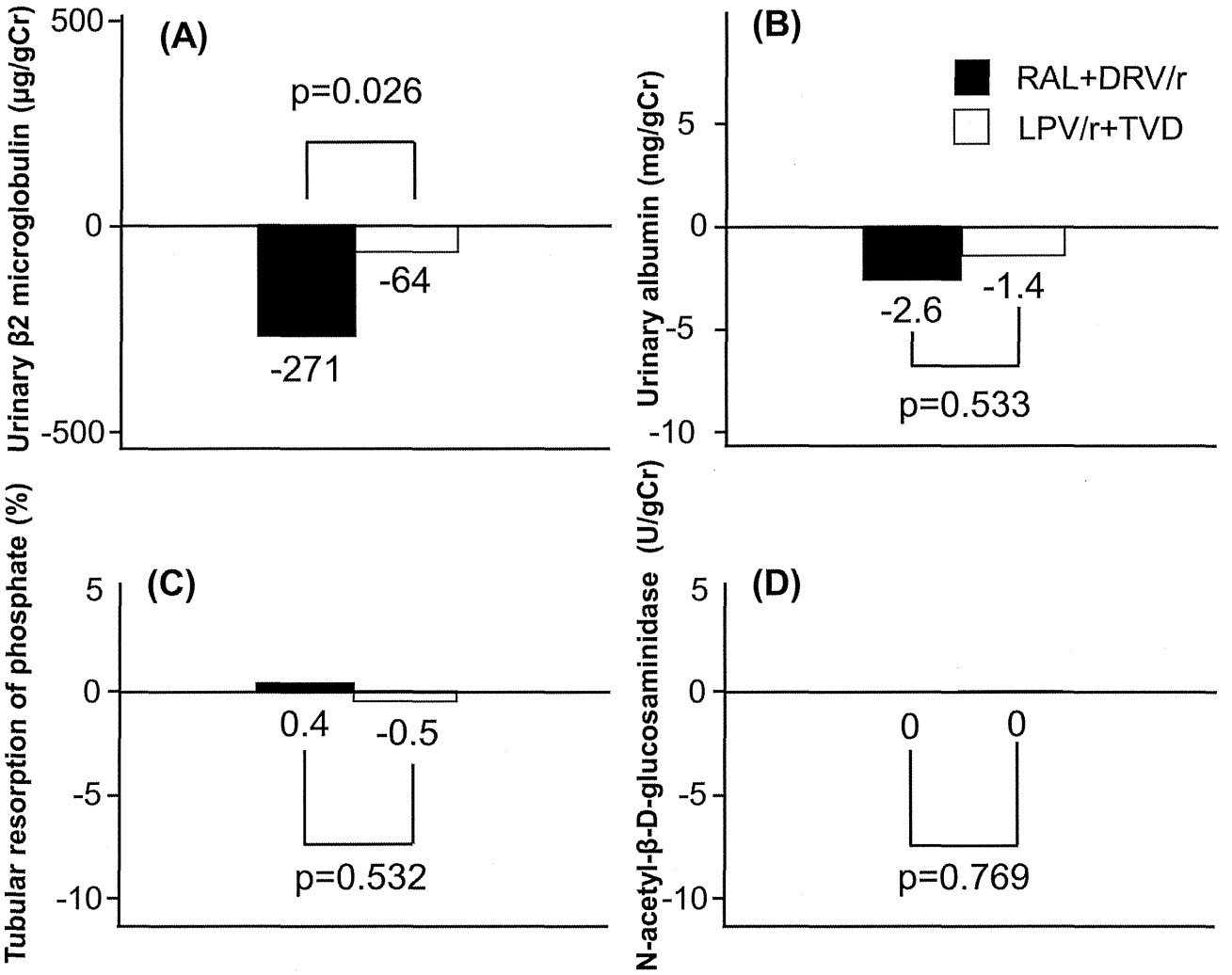

Figure 2. Median changes in markers of renal tubular function between baseline and 48 weeks. (A) Urinary β2 microglobulin, (B) Urinary albumin, (C) Percent tubular resorption of phosphate, (D) Urinary N-acetyl-β-D-glucosaminidase. RAL, raltegravir; DRV/r, ritonavir-boosted darunavir; LPV/r, ritonavir-boosted lopinavir; TVD, fixed dose of tenofovir/emtricitabine.
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proximal tubulopathy is associated with bone mineral density abnormality and possible long-term nephrotoxic effect [17,43-45]. Further large and long-term studies are needed to elucidate the long-term impact of TDF-induced tubulopathy on GFR.

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

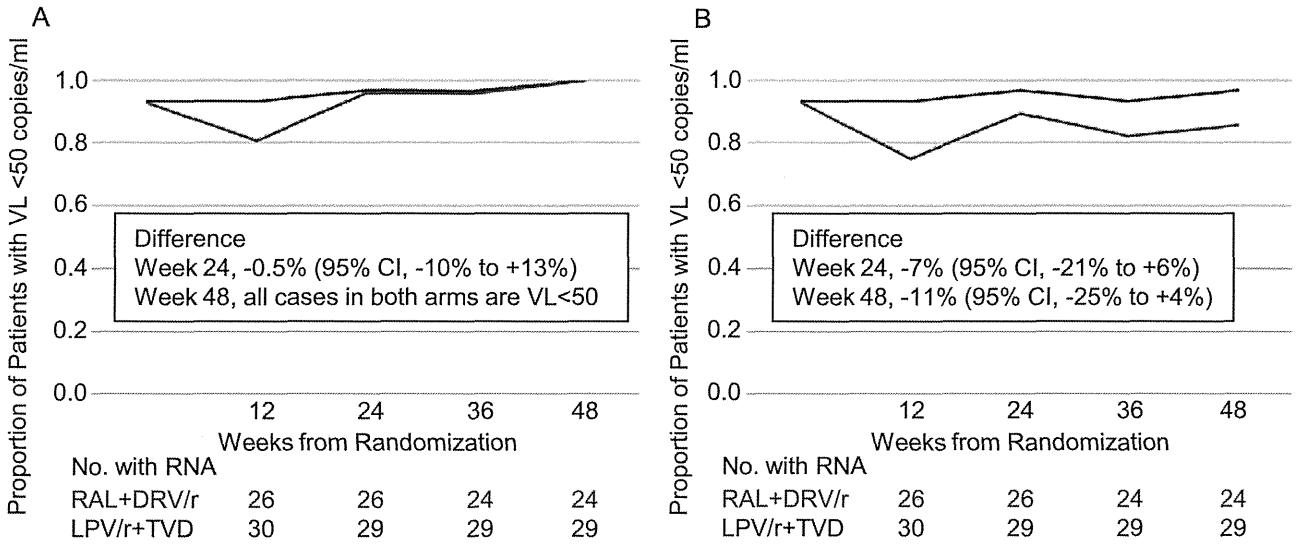

Figure 3. Proportion of patients with HIV RNA <50 copies/ml at 24 and 48 weeks. (A) Per protocol analysis. (B) Intention-to-treat analysis. VL, viral load; RAL, raltegravir; DRV/r, ritonavir-boosted darunavir; LPV/r, ritonavir-boosted lopinavir; TVD, fixed dose of tenofovir/emtricitabine.

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

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

Supporting Information

Protocol S1. Trial protocol.
(DOCX)

Checklist S1. CONSORT checklist.
(DOC)

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Conceived and designed the experiments: T. Nishijima HG TS TE MH T. Naito TF KT MY TM YT HM SO. Performed the experiments: T. Nishijima HG TE MH MK T. Naito II MT TF KT MY TM YT SO. Analyzed the data: T. Nishijima HG TS HK SO. Wrote the manuscript: T. Nishijima HG TS HK TE MH MK T. Naito II MT TF KT MY TM YT HM SO.

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PLOS ONE

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

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Abstract

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

Design: Cross-sectional study was performed.

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

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

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

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Introduction

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

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

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

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

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

Methods

Study design

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

Measurements

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

Statistical analysis

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

Results

Patients on TDF

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

Prevalence of CKD and renal dysfunction at each time point

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

Baseline demographics and laboratory data

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

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

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

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

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

CKD; chronic kidney disease, ART; antiretroviral therapy

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

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

Data are mean±SD or n (%).

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

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Factors associated with CKD

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

Discussion

We documented in the present study the prevalence of CKD and the associated risk factors in our Vietnamese cohort. CKD was identified in 7.3% of the patients between October 2011 and April 2012. Although severe renal dysfunction was

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

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

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

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observed in only 2 cases, we consider this finding quite alarming in our study setting, since it is more than double that reported in a previous study (3.1%) on the prevalence of CKD among Vietnamese healthy volunteers aged more than 40 years [19]. Our cohort comprised relatively younger and stable patients on ART with a mean age of 36.4 years.

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

One study argued that the initial decline in eGFR following the commencement of TDF therapy stabilized later after the first 6 months [21]. However, whether or not the initial decline stabilizes later in patients with low body weight remains to be documented in a longitudinal study of our cohort. It is true that the future risk of TDF-related CKD is still uncertain. In this study, almost all patients who experienced renal dysfunction continued the same ART regimen because renal dysfunction was relatively moderate as shown in Table **1**. Although one severe case showed improvement of renal function after cessation of TDF, normalization of renal function after

withdrawal of TDF was reported to be incomplete in some cases [22]. Previous studies recommended dose reduction of drugs that are cleared by the kidney, such as lamivudine and TDF, when Ccl falls below 50 ml/min [23], to avoid further worsening of renal dysfunction. Early detection of eGFR decline is important for switching from TDF to AZT or abacavir to preserve renal function. Despite those concerns, however, there is no doubt that TDF is still an important drug with enough anti-HIV potency and less mitochondrial toxicity among NRTIs. In this regard, serum creatinine should be monitored even in resource-limited situations.

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

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

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

Supporting Information

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

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

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Author Contributions

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

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DNA methylation profiling can classify HIV-associated lymphomas

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Background: HIV-positive patients have a 60-fold to 200-fold increased incidence of non-Hodgkin lymphomas, including Burkitt lymphoma, diffuse large B-cell lymphoma, and primary central nervous system lymphoma. HIV-associated lymphomas frequently have features such as extranodal involvement, decreased responses to standard chemotherapy, and high relapse rates, which indicate a poor prognosis. General pathological features do not clearly differentiate HIV-associated lymphomas from non-HIV lymphomas.

Methods: To investigate the features of HIV-associated lymphomas, we performed genome-wide DNA methylation profiling of HIV and non-HIV lymphomas using Illumina GoldenGate Methylation Cancer Panel I and Illumina Infinium HumanMethylation450 BeadChip microarrays. DNA methylation profiles in HIV-associated and non-HIV lymphomas were characterized using unsupervised hierarchical clustering analyses.

Results: The analyses of promoter regions revealed unique DNA methylation profiles in HIV-associated lymphomas, suggesting profile differences compared with non-HIV lymphomas, which implies specific gene regulation in HIV-associated lymphoma involving DNA methylation. Based on HumanMethylation450 BeadChip data, 2541 target sites were selected as differing significantly in comparisons between HIV-associated and non-HIV-associated lymphomas using Wilcoxon's rank-sum test ($P<0.05$) and $\Delta\beta$ values more than 0.30. Recurrent cases of HIV-associated lymphoma had different profiles compared with nonrecurrent HIV lymphomas.

Conclusion: DNA methylation profiling indicated that 2541 target sites differed significantly in HIV-associated lymphoma, which may partly explain the poor prognosis. Our data indicate that the methylation profiles of target genes have potential in elucidating HIV-associated lymphomagenesis and can serve as new prognostic markers.

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Keywords: CpG islands, DNA methylation microarray, HIV, HIV-associated lymphomas, poor prognosticators

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Introduction

The incidence of non-Hodgkin's lymphoma is 60-fold to 200-fold higher in patients with HIV infection [1,2]. Most HIV-associated lymphomas are high-grade B-cell lymphomas such as diffuse large B-cell lymphoma, Burkitt lymphoma, and primary central nervous system lymphoma. The clinical course is often aggressive, with a poor prognosis [2]. Since the introduction of highly active antiretroviral therapy, the risk for opportunistic infections and the incidence of AIDS-defining malignancies, including HIV-associated lymphomas, have declined, and prognoses have improved. Nevertheless, lymphomas remain a major cause of death for HIV-infected patients [3]. It is important to identify differences between HIV-associated lymphomas and non-HIV lymphomas, as their clinical and general pathological features do not clearly distinguish them [2]. Recent studies have revealed that the DNA methylation patterns can differentiate among disease subtypes, suggesting that epigenetic DNA alterations are related to carcinogenesis [4,5]. Epigenetic silencing of functionally important genes may contribute to the development of lymphomas [5,6], and promoter hypermethylation of CpG islands (CGIs) in some genes has been reported in aggressive-phenotype lymphoma with a poor prognosis [7]. In this study, we examined DNA methylation of CGIs in a promoter region clustered with HIV-associated lymphomas and non-HIV lymphomas, and investigated the prognostic significance of DNA methylation. Our findings contribute to an understanding of the lymphomagenesis of HIV-associated lymphomas and suggest specific DNA methylation as a useful prognostic biomarker.

Methods

Patients

HIV-associated lymphoma is a pathologically diagnosed malignant lymphoma in HIV patients. Two cohorts were studied. Cohort I consisted of 11 HIV-associated and 18 non-HIV lymphoma patients who visited Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital (CICK), and two non-HIV lymphoma patients who visited the National Center for Global Health and Medicine Hospital (NCGM). Cohort II included nine HIV-associated and 12 non-HIV lymphoma patients who visited NCGM. Formalin-fixed, paraffin-embedded tissues and fresh-frozen tissues were collected from NCGM and CICK, following approval by the ethics committees of both hospitals and in accordance with the Declaration of Helsinki. All patients gave written informed consent for their tissue to be used and for review of their clinical records. Diagnosis was made using the 2008 WHO classification [2]. Hematologists reviewed the tumor specimens and classified them histologically as diffuse large B-cell lymphoma, Burkitt lymphoma, primary central nervous system lymphoma, follicular lymphoma, or Hodgkin's lymphoma. Non-HIV lymphoma samples were randomly selected from among the Burkitt lymphomas, diffuse large B-cell lymphomas, follicular lymphoma, and Hodgkin's lymphoma. Epstein–Barr virus (EBV) status was determined by Epstein–Barr encoded RNA (EBER) *in situ* hybridization and Southern blotting. BCL2 expression was examined by immunostaining.

HumanMethylation450 microarray analysis

Cohort I was analyzed using an Infinium HumanMethylation450 BeadChip microarray [8], which covered 485 577 methylation sites. Genomic DNA was isolated using a DNeasy mini kit (QIAGEN, Valencia, California, USA) according to the manufacturer's protocol. After 1 μg of DNA was ligated at 24°C for 30 min, the reaction was stopped by 5 min at 95°C (REPLI-g FFPE kit; QIAGEN) [9]. The DNA was subjected to genome-wide DNA methylation profiling using an Infinium HumanMethylation450 BeadChip (Illumina, San Diego, California, USA) [8], according to the manufacturer's instructions. The methylation status of specific cytosines is indicated by the β value, with 1 indicating complete methylation and 0 indicating no methylation. We first filtered the probes and samples using the Bioconductor IMA package to load files created by Illumina GenomeStudio software, using the IMA.methy450R function. With this package, we performed filtering steps using the IMA.methy450PP function. The inclusion criteria were as follows: sample call rate, more than 99.5%; detection P value, <0.05; site call rate, more than 90%; probes with no SNPs based on snpsite.txt provided in the IMA package [10]; and probes outside the XY chromosomes. We converted the initial file created by Illumina GenomeStudio to a new file to reflect the filtering results. The data were normalized by entering the filtered data into the Bioconductor lumi package [11]. Using the lumi package, methylation data were first analyzed by the color balance check and then scaled based on the mean of all probes, using methylation simple scaling normalization (SSN) implemented in the lumi package. The Infinium array methylation data are available in the Gene Expression Omnibus database under the accession number GSE42372.

Cancer Panel I microarray analysis

Cohort II was analyzed using the Illumina GoldenGate Methylation Cancer Panel I microarray, a cancer-focused methylation analysis covering 1505 CpG loci from 807 genes (Illumina) [12]. Genomic DNA was isolated (Agencourt FormaPure kit; Beckman Coulter, Brea, California, USA), subjected to sodium bisulfite conversion, labeled with fluorescent dyes, and hybridized to the microarrays according to the manufacturer's protocol. The methylation status of specific cytosines was indicated by the β value (1, complete methylation; 0, no methylation). Only probes with detection P value at

<0.01 were used for the analyses. The X chromosome loci were removed from the analysis, leaving 1421 CpG loci. Raw average β values were not normalized and were used for analyses as per the manufacturer's recommendations. The GoldenGate array methylation data are available in the Gene Expression Omnibus database under the accession number GSE42626.

For the statistical analysis, enrichment analysis of target genes, validation by combined bisulfite restriction analysis (COBRA), and bisulfite DNA sequences, see the Supplementary Methods, http://links.lww.com/QAD/A441.

Results

To identify differences between HIV-associated and non-HIV lymphomas, genome-wide DNA methylation array analyses were performed using Infinium HumanMethylation450 BeadChip technology. DNA from formalin-fixed and paraffin-embedded or fresh-frozen lymphoma tissues collected from the 11 HIV-positive and 20 HIV-negative Asian patients in Cohort I was analyzed (Table 1). DNA methylation throughout the genome was examined using probes targeting six gene regions (Fig. 1a): within 1500 bps of a transcription start site (TSS1500), within 200 bps of a transcription start site (TSS200), and the 5′ untranslated region (5′UTR), first exon (1stExon), body, and 3′ untranslated region (3′UTR) and intergenic regions. Three HIV-negative lymphomas were excluded from the analyses in the filtering steps (see Methods for details). The differences in methylation status between HIV-associated and non-HIV lymphomas were significantly greater for CGIs in the various target regions, compared with non-CGI methylation (Supplementary Fig. 1, http://links.lww.com/QAD/A441). Hierarchical clustering analysis of CGI methylation markers of TSS1500, TSS200, 5′UTR, and 1stExon (Fig. 1b) produced roughly two groups that distinguished HIV-associated lymphomas from non-HIV lymphomas (Groups 1 and 2; Fig. 1b, upper left), with a few exceptions. By contrast, the analysis of non-CGI methylation and CGI methylation in the body and 3′UTR and intergenic gene targets did not give clear groupings (Fig. 1b, upper right and lower images, Supplementary Fig. 2, http://links.lww.com/QAD/A441). As all HIV patients in this study were men (Table 1), we next analyzed male patients only. The CGI results for TSS1500, TSS200, 5′UTR, and 1stExon again clustered into two groups (Supplementary Fig. 3, http://links.lww.com/QAD/A441), suggesting that gender does not affect the results. Generally, patients with HIV-associated lymphomas were younger than patients with non-HIV lymphomas (Table 1) [13]. When we excluded age-related target sites, as previously suggested [14], the analysis of CGI methylation in TSS1500, TSS200, 5′UTR, and 1stExon again produced two groups that distinguished between HIV-associated and non-HIV lymphomas (Supplementary Fig. 4, http://links.lww.com/QAD/A441). These results suggest that DNA methylation of CGIs in promoter regions (TSS1500, TSS200, 5′UTR, and 1stExon) probably distinguishes HIV-associated from non-HIV lymphomas. Among the targets measured, those with a significant absolute difference between HIV-associated and non-HIV lymphomas were used for further analyses (Supplementary Methods, http://links.lww.com/QAD/A441). Compared with non-HIV lymphoma DNA, HIV-associated lymphoma DNA tended to be hypomethylated (Fig. 1c). Representative genes were used to validate the array analyses. Using COBRA, three of the five non-HIV lymphomas cases were methylated as positive controls, whereas none of the HIV-associated lymphomas was detected as methylated at either *RARRES1* or *FGF5* (Fig. 1d, upper). Bisulfite DNA sequencing gave consistent results (Fig. 1d, lower), confirming this tendency toward hypomethylation in Group 1 (Fig. 1d). These findings encouraged us to examine previously analyzed cases in Cohort II.

Table 1. Patient characteristics of lymphoma samples for Human Methylation450 (450K) microarray analysis in Cohort I.

Items examined		HIV	Non-HIV	*P* value (HIV vs. non-HIV)
Sex	Female	0	10	
	Male	11	10	0.0049*
Age	Mean	45.27	64.35	
	SD	16.92	10.60	0.018*
Histology	BL	2	3	
	DLBCL	8	17	
	HD	1	0	0.57
Stage	I & II	3	5	
	III & IV	8	12	
	ND	0	3	0.63
EBV	+	3	7	
	−	8	9	
	ND	0	4	0.22

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; ND, not determined; SD, standard deviation.
*P <0.05

Data from nine HIV-associated lymphoma samples derived from the first visit of Cohort II, which had been previously analyzed using Illumina GoldenGate Methylation Cancer Panel I (see Methods), were used for hierarchical clustering analyses. The results showed two apparent methylation profiles for HIV-associated lymphomas (Groups 3 and 4, Fig. 2a). The genes with a significant absolute difference between two clusters were used for further analyses (Supplementary Method, http://links.lww.com/QAD/A441). Group 3 tended to be hypermethylated compared with Group 4 (Fig. 2b). COBRA indicated that all of the Group 3 cases were

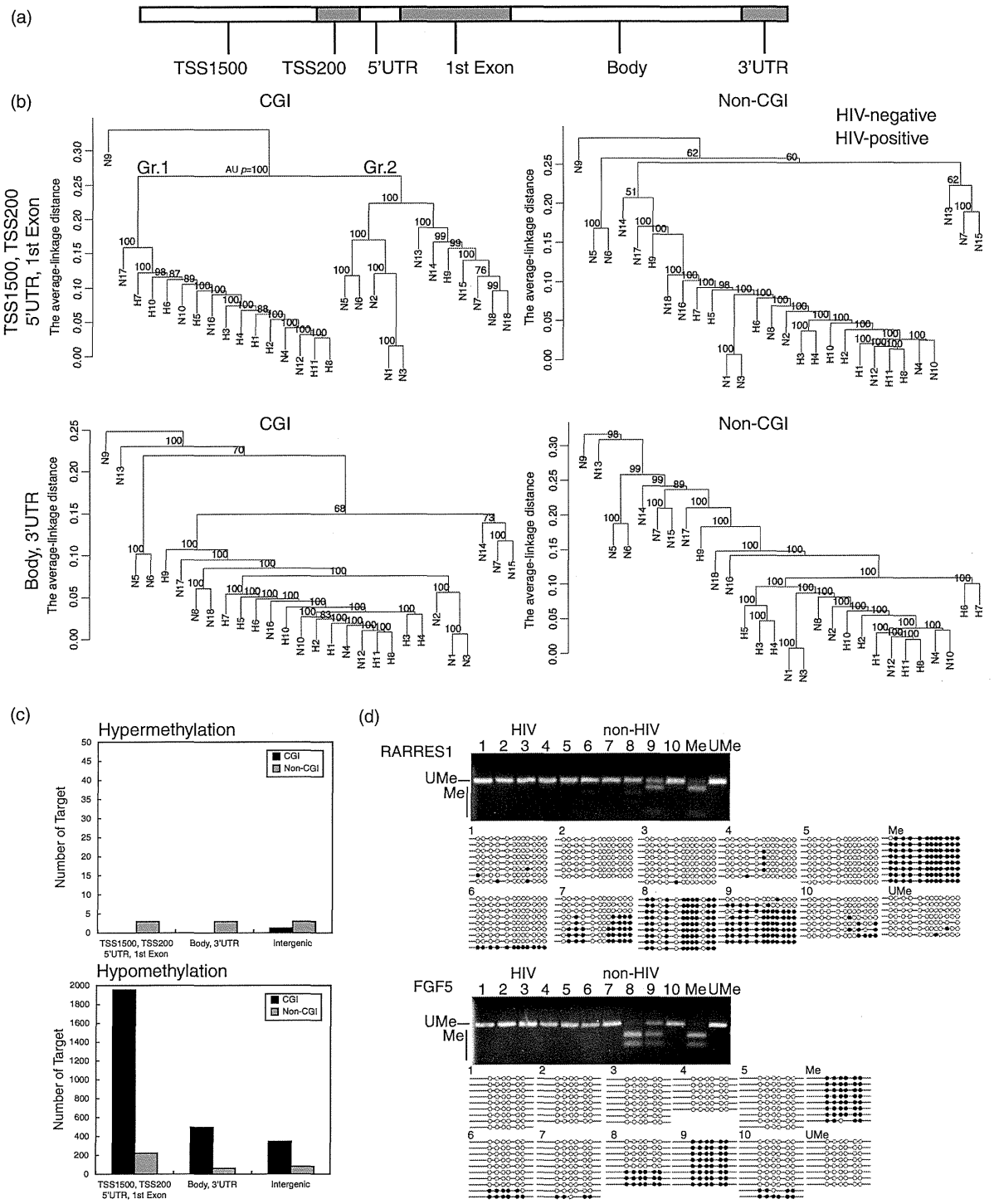

Fig. 1. Methylation profile analysis of HIV-associated and non-HIV lymphoma DNA in Cohort I, using Infinium HumanMethylation450 BeadChip technology. (a) Schematic of the gene regions examined for methylation. (b) Hierarchical clustering analysis of CpG island (CGI) and non-CGI methylation of lymphoma DNA in Cohort I. The analysis of CGI methylation in the promoter regions (TSS1500, TSS200, 5'UTR, and 1stExon) produced two groups that distinguished between HIV-associated lymphomas (Group 1, Gr. 1) and non-HIV lymphomas (Group 2, Gr. 2). TSS, transcription start site; AU *p* value, approximately unbiased *P* value computed using multiscale bootstrap resampling. (c) Numbers of hypermethylation or hypomethylation targets in HIV-associated lymphomas compared with non-HIV-lymphomas. (d) Validation by combined bisulfite restriction analysis (COBRA) and bisulfite DNA sequences. Retinoic acid receptor responder 1 (*RARRES1*) and fibroblast growth factor 5 (*FGF5*) 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; HIV, HIV-associated lymphoma; non-HIV, non-HIV lymphoma.