PLOS | ONE

RESEARCH ARTICLE

Drug Transporter Genetic Variants Are Not Associated with TDF-Related Renal Dysfunction in Patients with HIV-1 Infection: A Pharmacogenetic Study

Takeshi Nishijima[1,2], Tsunefusa Hayashida[1], Takuma Kurosawa[3,4], Noriko Tanaka[4], Shinichi Oka[1,2], 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, **3** Department of Mathematical Science for Information Sciences, Graduate School of Science, Tokyo University of Science, Tokyo, Japan, **4** Biostatistics Section, Department of Clinical Research and Informatics, Clinical Science Center, National Center for Global Health and Medicine, Tokyo, Japan

* higatana@acc.ncgm.go.jp

Citation: Nishijima T, Hayashida T, Kurosawa T, Tanaka N, Oka S, Gatanaga H (2015) Drug Transporter Genetic Variants Are Not Associated with TDF-Related Renal Dysfunction in Patients with HIV-1 Infection: A Pharmacogenetic Study. PLoS ONE 10(11): e0141931. doi:10.1371/journal.pone.0141931

Editor: Roger Le Grand, Commissariat a l'Energie Atomique(cea), FRANCE

Received: July 29, 2015

Accepted: October 14, 2015

Published: November 4, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by a Grant-in Aid for AIDS research from the Japanese Ministry of Health, Labour, and Welfare (H23-AIDS-001), The Grant for National Center for Global Health and Medicine (25-106 and 27-6001), and a grants-in-aid of The IMAI MEMORIAL TRUST FOR AIDS RESEARCH. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Objective

To investigate whether single nucleotide polymorphisms (SNP) of drug transporter proteins for TDF is a risk factor for TDF-related renal function decrement.

Methods

This study investigated the association between 3 SNPs (*ABCC2*–24, 1249, and *ABCB1* 2677), which are shown to be associated with TDF-induced tubulopathy, and clinically important renal outcomes (>10ml/min/1.73m^2 decrement in eGFR relative to baseline, >25% decrement in eGFR, and eGFR <60ml/min/1.73m^2) in 703 HIV-1-infected Japanese patients who initiated TDF-containing antiretroviral therapy (ART). Genotyping was performed by allelic discrimination using TaqMan 5'-nuclease assays.

Results

95% of the study patients were males and 66% were treatment-naïve, with median CD4 count of 249/μl, median baseline eGFR of 96ml/min/1.73m^2 (IQR 84.6–109.2), and median exposure to TDF of 3.66 years (IQR 1.93–5.59). The frequencies of genotypes at -24, 1249 of *ABCC2*, and 2677 of *ABCB1* were neither different between patients with decrement in eGFR of >10ml/min/1.73m^2 and those without such decrement (*ABCC2*: -24, p = 0.53, 1249, p = 0.68; *ABCB1*: 2677, p = 0.74), nor between those without and with the other two renal outcomes (>25% decrement: *ABCC2*: -24, p = 0.83, 1249, p = 0.97, *ABCB1*: 2677, p = 0.40; eGFR <60ml/min/1.73m^2: *ABCC2*: -24, p = 0.51, 1249, p = 0.81, *ABCB1*: 2677, p = 0.94). Logistic regression analysis showed that the risk genotype of the three SNPs were

Competing Interests: S.O. has received honoraria and research grants from MSD K.K., has received honoraria from Torii Pharmaceutical, Co., Abbie, and ViiV Healthcare. H.G. has received honoraria from MSD K.K., Abbott Japan, Co., Janssen Pharmaceutical K.K., Torii Pharmaceutical, Co., Roche Diagnostics K.K., and ViiV Healthcare, Co. The remaining authors declare no conflict of interest. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

not associated with any of the three renal outcomes, respectively. Logistic regression model that applied either dominant, recessive, or additive model yielded the same results.

Conclusions

SNPs of the drug transporters for TDF are not associated with clinically important renal outcomes in patients who initiated TDF-containing ART.

Introduction

Tenofovir disoproxil fumarate (TDF), a prodrug of tenofovir, is one of the most widely used nucleotide reverse transcriptase inhibitors (NRTI) for the treatment of HIV-1 infection in both resource-rich and resource-limited settings [1,2], and also for the treatment of hepatitis B infection [3]. Furthermore, the use of TDF, either as a fixed dose with emtricitabine (FTC) or alone, has been recently recommended by the WHO and American CDC guidelines, as pre-exposure prophylaxis for prevention of transmission of HIV-1 in high-risk uninfected adults [4,5].

Tenofovir is predominantly excreted by the kidney through the combination of glomerular filtration and active tubular secretion [6]. TDF is known to cause renal proximal tubular dysfunction, such as Fanconi's syndrome [7], and also reduces the estimated glomerular filtration rate (eGFR) more than other NRTIs [8,9]. Although the exact mechanism of tenofovir-induced nephrotoxicity is not fully understood, mitochondria toxicity in proximal renal tubular cells has been suspected as the main cause of TDF-related renal function decrement [10].

Because the severity of tenofovir nephrotoxicity varies widely among individuals, the role of host genetics has drawn a particular attention. Many single nucleotide polymorphisms (SNPs) of the genes encoding transporter proteins in renal tubular cells, such as organic anion transporter (OAT) 1 and 3, multidrug resistance protein (MRP) 2, 4, and 7, and P-glycoprotein, have been investigated to elucidate their roles in tenofovir-induced tubulopathy [11–15]. As a result, genotype C/C at -24 (rs717620) and genotype A/A at 1249 (rs2273697) on the *ABCC2* gene, which encode MRP2, consistently shown the association with tenofovir-induced tubulopathy [11,13,16]. However, whether individuals with such SNPs are more susceptible to TDF-related renal function decrement than those without such genetic variants remains to be elucidated. This issue is important because HIV-1 infection requires life-long antiretroviral therapy (ART) and renal dysfunction and chronic kidney diseases are important comorbidities that can influence mortality [17,18].

Based on the above background, the present study was designed to elucidate the association between polymorphisms in genes encoding drug transporters in renal tubular cells and tenofovir-related renal function decrement among HIV-1-infected patients who initiated TDF-containing ART.

Methods

Ethics Statement

This study was approved by the Human Genetics Research Ethics Committee of the National Center for Global Health and Medicine, Tokyo, Japan. Each patient included in this study provided a written informed consent for genetic testing and publication of clinical data for research purposes. The study was also conducted according to the principles expressed in the Declaration of Helsinki.

Study Design and subjects

We performed a single-center cohort study to investigate the association between TDF-related renal function decrement and SNPs in genes encoding renal tubular transporters in Japanese HIV-1-infected patients who initiated TDF-containing ART. The inclusion criteria for the study patients were: 1) Japanese patients with HIV infection who initiated TDF-containing ART at our clinic between January 2002 and December 2013, 2) patients who continued TDF for ≥90 days, and 3) patients who provided a written informed consent for the study. Patients with eGFR <60 ml/min/1.73m^2 at initiation of TDF were excluded. The written informed consent was obtained from the candidate patients between June 2014 and October 2014.

Measurements

The eGFR was calculated using the equation developed by the Japanese Society of Nephrology (JSN): eGFR = 194 × [serum creatinine]$^{-1.094}$ × [age]$^{-0.287}$ × [0.739 if female] [19]. This equation is used because this is more suitable for patients with small body stature, such as Japanese, than The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [20,21]. The 2013 practice guidelines for patients with CKD published by JSN also recommend the use of this equation for the Japanese, rather than the CKD-EPI equation [21]. The baseline eGFR was estimated for each patient from age, sex, and serum creatinine measurements made closest to and preceding the initiation of ART by no more than 90 days. Patients visited our clinic at least every three months for monitoring CD4 cell count, HIV-1 viral load, and eGFR, since the prescription period under the Japanese health care system is limited to three months [22]. Thus, for calculation of follow-up eGFR values, we collected serum creatinine values measured closest to every 90 day within a range of 45 days from initiation of ART. Follow up eGFR data were collected from the baseline measurement until discontinuation of TDF or at the end of the follow-up period (August 2014).

The potential risk factors for renal dysfunction were determined according to previous studies and collected together with the basic demographics from the medical records [23–26]. They included age, sex, body weight, body mass index (BMI) = {body weight (kg) / [(height (m)]^2}, history of AIDS, route of HIV-1 transmission, HIV-1 treatment status (either treatment-naïve or experienced), combination of ART, baseline laboratory data (CD4 cell count, HIV viral load, and serum creatinine), and presence or absence of other medical conditions [diabetes mellitus defined by using anti-diabetic agents or fasting plasma glucose >126 mg/dl or plasma glucose >200 mg/dl on two different days, hypertension defined by current treatment with antihypertensive agents or two successive measurements of systolic blood pressure >140 mmHg or diastolic blood pressure >90 mmHg at the clinic, dyslipidemia defined by current treatment with lipid-lowering agents, co-infection with hepatitis B defined by positive hepatitis B surface antigen, co-infection with hepatitis C defined by positive HCV viral load, and current smoking] [22], concurrent use of ritonavir-boosted PIs (PI/r), concurrent use of nephrotoxic drugs, such as ganciclovir and sulfamethoxazole/trimethoprim. At our clinic, body weight and blood pressure were measured on every visit whereas other variables were measured in the first visit and at least once annually [22]. We used the data on or closest to and preceding the day of starting ART by no more than 180 days.

Genetic polymorphisms

The selected SNPs were *1)* -24C→T (in the promoter; rs717620) and *2)* 1249G→A (Val417Ile; rs2273697) of *ABCC2* gene, because they are the only SNPs that have consistently shown close association with tenofovir-induced tubulopathy in previous studies [11,13,16]. In addition, 2677T→A/G (A:Ser893Thr, G:Ser893Ala; rs2032582) of *ABCB1* gene, which encodes P-

glycoprotein, was also selected, because this triallelic SNP is functionally significant and appears to influence the absorption of TDF at the intestine and affect exposure of tenofovir [6,27,28].

Pharmacogenetic analyses

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Mini-Kit and the protocol provided by the manufacturer (Qiagen, Valencia, CA). All genotyping was performed by allelic discrimination using TaqMan 5'-nuclease assays with standard protocols (TaqMan SNP Genotyping Assays; Applied Biosystems, Foster City, CA). The primer and probe sequences are available on request.

Statistical analysis

Three renal endpoints were applied in this study; we focused primarily on 1) decrement in eGFR of >10 ml/min/1.73 m^2 relative to the baseline, because this endpoint is considered appropriate for patients with well maintained renal function [22,29], such as the study population. We also set two commonly used renal endpoints; 2) >25% decrement in eGFR relative to the baseline [30,31], and 3) eGFR <60 ml/min/1.73 m^2 [32].

The baseline characteristics were compared between patients with decrement in eGFR of >10 ml/min/1.73 m^2 and those without such decrement by the Student's t-test or the Wilcoxon signed-rank test for continuous variables and by either the χ^2 test or Fisher's exact test for categorical variables. The χ^2 test was used to test for deviation of allele frequency from the Hardy-Weinberg equilibrium. Statistical comparisons for genotype frequencies between the two groups were tested by the Fisher's exact test or the χ^2 test where appropriate. The logistic regression model was used to estimate the association of risk genotype/allele of each SNP with the occurrence of these renal endpoints. We applied the following four genetic models for the analysis: genotype model (a model that postulates no mode of inheritance), dominant model, recessive model, and additive model. Each genetic effect in logistic regression models was estimated with the adjustment for the variables which were determined a priori; they included baseline eGFR, age, baseline body weight, nephrotoxic drug use, PI/r use, CD4 count, hypertension, and dyslipidemia, which are established risk factors for TDF nephrotoxicity [9,23,24,26]. Sex and diabetes mellitus were not added to the models due to their low frequency. The above statistical analyses were repeated using eGFR calculated by the CKD-EPI equation adjusted with the Japanese coefficient [33].

Statistical significance was defined at two-sided p values <0.05. We used the odds ratio (OR) with 95% confidence intervals (95% CI) as a measure of the effect of risk allele/genotype on each renal endpoint. All statistical analyses were performed with SAS software, version 9.3 (SAS Institute, Cary, NC).

Results

A total of 703 patients who satisfied the inclusion and exclusion criteria and provided a written informed consent during the inclusion period were enrolled in the study (Fig 1). The study patients were mostly homosexual male with a median age of 38 (IQR 33–46) (Table 1). The median CD4 count at baseline was 249 /μL (IQR 127–385), and 66% of the study patients were treatment-naïve for HIV-1 infection. With regard to ART, 75% of the patients started TDF with PI/r. The median baseline eGFR was 96 ml/min/1.73 m^2 (IQR 84.6–109.2) [by CKD-EPI equation: 94.2 ml/min/1.73 m^2 (88.3–100.3)]. The median duration of TDF use was 3.66 years (IQR 1.93–5.59).

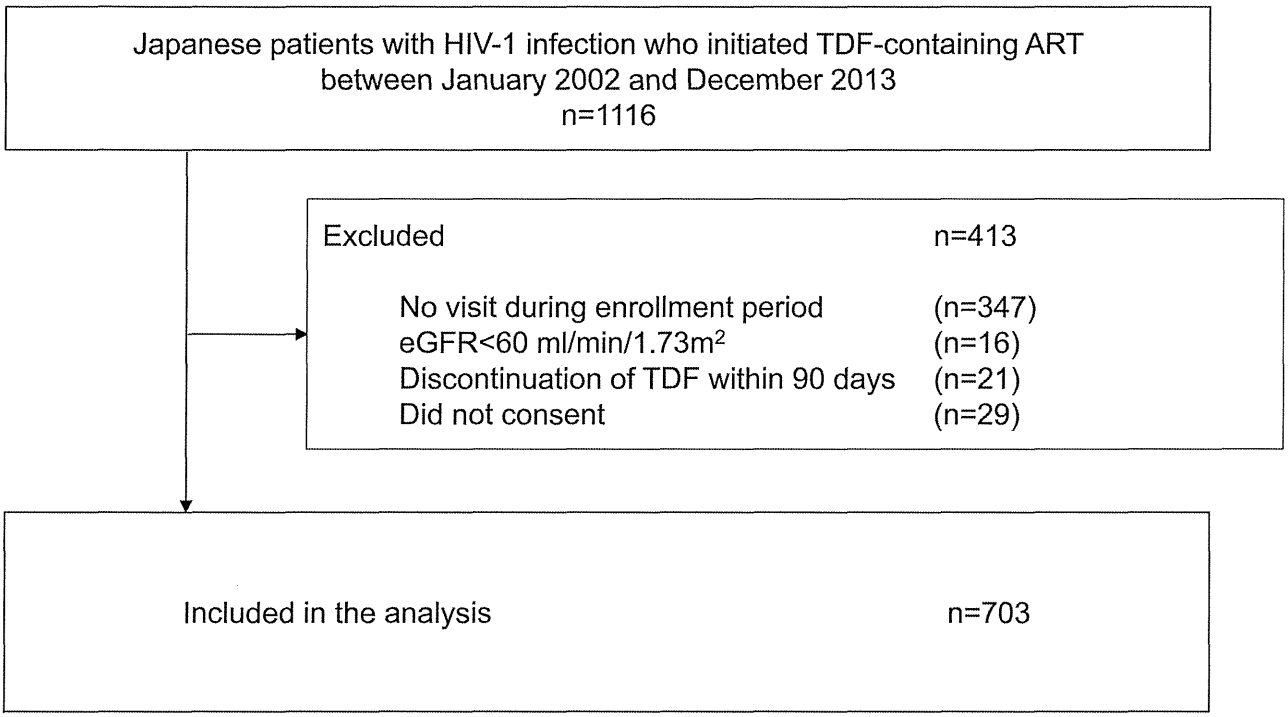

Fig 1. Flow diagram of the patient enrolment process.

doi:10.1371/journal.pone.0141931.g001

Of the 703 study patients, >10 ml/min/1.73 m^2 decrement in eGFR relative to the baseline occurred in 624 (89%), >25% decrement in 119 (17%), and eGFR <60 ml/min/1.73 m^2 in 126 (18%). Patients with >10 ml/min/1.73 m^2 decrement in eGFR had higher baseline eGFR ($p<0.0001$), lower CD4 count ($p = 0.0058$), had more frequent HBV co-infection ($p = 0.0070$), and had longer exposure to TDF ($p<0.0001$), compared to those without decrement in eGFR (Table 1).

Table 2 summarizes the distribution of genotypes at -24 and 1249 of *ABCC2* gene and at 2677 of *ABCB1* gene, for patients with each renal endpoint and those free of decrement in eGFR. All polymorphisms were in Hardy-Weinberg equilibrium. The frequencies of genotypes at -24, 1249 of *ABCC2* gene and at 2677 of *ABCB1* gene were not different between patients with >10 ml/min/1.73 m^2 decrement in eGFR and those without decrement in eGFR (-24 of *ABCC2*, $p = 0.53$, 1249 of *ABCC2*, $p = 0.68$, 2677 of *ABCB1*, $p = 0.74$), between patients with >25% decrement in eGFR and those without (-24 of *ABCC2*, $p = 0.83$, 1249 of *ABCC2*, $p = 0.97$, 2677 of *ABCB1*, $p = 0.40$), and between patients with decrement in eGFR to <60 ml/min/1.73 m^2 and those without (-24 of *ABCC2*, $p = 0.51$, 1249 of *ABCC2*, $p = 0.81$, 2677 of *ABCB1*, $p = 0.94$).

The results of additional analyses using eGFR calculated by the CKD-EPI equation are shown in Table 3. Similarly, the frequencies of genotypes at -24, 1249 of *ABCC2* gene and at 2677 of *ABCB1* gene were not different between patients with each renal endpoint and those who reached no such endpoint (>10 ml/min/1.73 m^2 decrement in eGFR: -24 of *ABCC2*, $p = 0.59$, 1249 of *ABCC2*, $p = 0.20$, 2677 of *ABCB1*, $p = 0.95$) (>25% decrement in eGFR: -24 of *ABCC2*, $p = 0.62$, 1249 of *ABCC2*, $p = 0.86$, 2677 of *ABCB1*, $p = 0.22$) (eGFR <60 ml/min/1.73 m^2: -24 of *ABCC2*, $p = 0.91$, 1249 of *ABCC2*, $p = 1.00$, 2677 of *ABCB1*, $p = 0.76$).

The logistic regression model which evaluated genotypic effect of the *ABCC2* gene showed that the risk genotype (i.e., genotype CC at -24) was not associated with any of the three renal

Table 1. Baseline characteristics of the study patients.

	Study patients (n = 703)	>10 ml/min/1.73 m^2 decrement (n = 624)	No decrement in eGFR (n = 79)	P value
Sex (male), n (%)	669 (95)	592 (95)	77 (97)	0.41
Age†	38 (33–46)	38 (33–46)	39 (34–47)	0.18
Weight (kg)†	63.3 (57.3–70)	63 (57–70)	66 (60–73)	0.077
BMI (kg/m^2)†	22 (20.2–24.1)	21.9 (20–24.1)	22.8 (20.9–24.2)	0.26
eGFR: JCKD-EPI ($ml/min/1.73m^2$)†	94.2 (88.3–100.3)	95.4 (89–101.1)	88.4 (76.8–92.4)	<0.0001
eGFR: JeGFR ($ml/min/1.73m^2$)†	96 (84.6–109.2)	98.2 (86.7–112.2)	82.1 (68.3–91.6)	<0.0001
Serum creatinine (mg/dl)†	0.74 (0.65–0.82)	0.72 (0.65–0.80)	0.84 (0.79–0.96)	<0.0001
CD4 count (/μl)†	249 (127–385)	244.5 (110–377.5)	304 (188–436)	0.0058
HIV RNA viral load (log_{10}/ml)†	4.51 (2.66–5.11)	4.5 (2.6–5.1)	4.4 (2.8–4.9)	0.52
Treatment naive, n (%)	467 (66)	416 (67)	51 (64)	0.71
Ritonavir-boosted protease inhibitors, n (%)	529 (75)	470 (75)	59 (75)	0.89
Protease inhibitors (unboosted), n (%)	22 (3)			
NNRTIs, n (%)	71 (10)			
INSTIs, n (%)	89 (13)			
Hypertension, n (%)	110 (16)	93 (14)	17 (22)	0.14
Dyslipidemia, n (%)	311 (44)	274 (44)	37 (47)	0.63
Diabetes mellitus, n (%)	19 (3)	18 (3)	1 (1)	0.71
Concurrent use of nephrotoxic drugs, n (%)	110 (16)	99 (16)	11 (14)	0.74
Hepatitis B, n (%)	79 (11)	77 (12)	2 (3)	0.0070
Hepatitis C, n (%)	35 (5)	34 (5)	1 (1)	0.16
History of AIDS, n (%)	207 (29)	188 (30)	19 (24)	0.30
Homosexual contact, n (%)	565 (80)			
Injection drug user	7 (1)			
Current smoker, n (%)	307 (44)	275 (44)	32 (41)	0.63
TDF duration (years)†	3.66 (1.93–5.59)	3.89 (2.14–5.67)	1.52 (0.96–3.15)	<0.0001

†median (interquartile range)

Nine patients were taking both PI/r and NNRTI, 1 patient with NNRIT and INSTI. 1 patient was treated with 2 NRTIs and 1 with 3 NRTIs. Other patients were treated with 2 NRTIs together with either PI, NNRTI, or INSTI.

Differences between the two groups were compared by the Student's t-test for continuous variables and by Fisher's exact test for categorical variables, except for CD4 count, HIV RNA viral load, and TDF duration, which were compared by the Wilcoxon signed-rank test.

BMI: body mass index, TDF: tenofovir disoproxil fumarate, eGFR: estimated glomerular filtration rate, NNRTI: non- nucleoside reverse transcriptase inhibitor, INSTI: integrase strand transfer inhibitor.

doi:10.1371/journal.pone.0141931.t001

outcomes (Table 4) (>10 ml/min/1.73 m^2 decrement in eGFR: genotype C/C versus T/T, adjusted OR 0.5, 95%CI 0.06–3.91, p = 0.62; genotype C/T versus T/T, adjusted OR 0.4, 95%CI 0.05–3.33, p = 0.34) (>25% decrement in eGFR: genotype C/C versus T/T, adjusted OR 1.2, 95%CI 0.42–3.20, p = 0.62; genotype C/T versus T/T, adjusted OR 1.0, 95%CI 0.35–2.83, p = 0.81) (eGFR <60 ml/min/1.73 m^2: genotype C/C versus T/T, adjusted OR 0.6, 95%CI 0.20–2.08, p = 0.61; genotype C/T versus T/T, adjusted OR 0.6, 95%CI 0.17–1.93, p = 0.36). Similarly, the risk genotype (genotype A/A) at 1249 of *ABCC2* or a genotype at 2677 of *ABCB1* was not associated with either of the three renal outcomes (S1 and S2 Tables). Furthermore, logistic regression analysis, which applied the dominant model, recessive model, and additive model, showed no association between each allele/genotype at the SNPs and any of the three renal endpoints (S3 Table). Logistic regression analysis using eGFR calculated by the CKD-EPI

Table 2. Genotype frequencies at three SNPs of *ABCC2* and *ABCB1* in patients with and without three renal outcomes.

		>10 ml/min/1.73 m^2 decrement in eGFR from baseline			>25% decrement in eGFR from baseline			eGFR <60 ml/min/1.73 m^2		
	Amino acid	>10 ml/min/1.73 m^2 decrement (n = 624)	No decrement (n = 79)	P value*	>25% decrement (n = 119)	No decrement (n = 584)	P value*	<60 ml/min/1.73 m^2 (n = 126)	No decrement (n = 577)	P value*
ABCC2 (MRP2)										
-24 C→T, rs717620										
C/C		382 (61)	51 (65)		76 (64)	357 (61)		83 (66)	350 (61)	
C/T		215 (35)	27 (34)	0.53	38 (32)	204 (35)	0.83	38 (30)	204 (35)	0.51
T/T		27 (4)	1 (1)		5 (4)	23 (4)		5 (4)	23 (4)	
1249 G→A, rs2273697	Val417Ile									
G/G		483 (78)	61 (77)		93 (78)	451 (77)		100 (79)	444 (77)	
A/G		132 (21)	16 (20)	0.68	24 (20)	124 (21)	0.97	24 (19)	124 (21)	0.81
A/A		9 (1)	2 (3)		2 (2)	9 (2)		2 (2)	9 (2)	
ABCB1 (P-glycoprotein)										
2677T→A/G, rs2032582	A: Ser893Thr G: Ser893Ala									
T/T		112 (18)	13 (16)		19 (16)	106 (18)		21 (17)	104 (18)	
T/A		77 (12)	13 (16)		22 (18)	68 (11)		18 (14)	72 (12)	
G/G		122 (20)	13 (16)	0.74	20 (17)	115 (20)	0.40	21 (17)	114 (20)	0.94
G/T		195 (31)	29 (37)		39 (33)	185 (32)		41 (32)	183 (32)	
G/A		96 (15)	9 (12)		17 (14)	88 (15)		20 (16)	85 (15)	
A/A		22 (4)	2 (3)		2 (2)	22 (4)		5 (4)	19 (3)	

*By use of Fisher's exact test for 2×3 table (2×6 table for rs2032582).

doi:10.1371/journal.pone.0141931.t002

equation yielded the same results. Post-hoc analysis with the logistic models after further adjustment for duration of TDF therapy also yielded the same results.

Discussion

This pharmacogenetics study investigated the association between drug transporter genetic variants and TDF-related renal function decrement in Japanese HIV-1-infected patients who initiated TDF-containing ART. The results showed that none of the three examined SNPs was associated with any of the three selected renal outcomes: >10 ml/min/1.73 m^2 decrement in eGFR relative to the baseline, >25% decrement in eGFR, and eGFR <60 ml/min/1.73 m^2. The results were reproduced using the dominant, recessive, and additive models, in addition to the genotype model for the estimation of association between genetic variants and renal outcomes.

Two main aspects of our study are important. First, this study showed that genetic factors do not need to be taken into account as predisposing factors for TDF–related renal dysfunction, using three clinically important renal outcomes (>10 ml/min/1.73 m^2 decrement in eGFR relative to the baseline [22,29], >25% decrement [30,31], and eGFR <60 ml/min/1.73 m^2 [32], which are known to be associated with morbidity and mortality in HIV-1-infected patients

Table 3. Genotype frequencies of three SNPs of *ABCC2* and *ABCB1* in patients with and without three renal outcomes calculated by the CKD-EPI equation.

		>10 ml/min/1.73 m2 decrement in eGFR from baseline			>25% decrement in eGFR from baseline			eGFR <60 ml/min/1.73 m2		
	Amino acid	>10 ml/min/ 1.73 m^2 decrement (n = 624)	No decrement (n = 79)	P value*	>25% decrement (n = 119)	No decrement (n = 584)	P value*	<60 ml/ min/1.73 m^2 (n = 126)	No decrement (n = 577)	P value*
ABCC2 (MRP2)										
-24 C→T, rs717620										
C/C		302 (62)	131 (61)		79 (64)	354 (61)		38 (66)	395 (61)	
C/T		166 (34)	76 (36)	0.59	39 (31)	203 (35)	0.62	18 (31)	224 (35)	0.91
T/T		22 (4)	6 (3)		6 (5)	22 (4)		2 (3)	26 (4)	
1249 G→A, rs2273697	Val417Ile									
G/G		386 (79)	158 (74)		98 (79)	446 (77)		45 (78)	499 (77)	
A/G		95 (19)	53 (25)	0.20	24 (19)	124 (21)	0.86	12 (21)	136 (21)	1.00
A/A		9 (2)	2 (1)		2 (2)	9 (2)		1 (1)	10 (2)	
ABCB1 (P-glycoprotein)										
2677T→A/G, rs2032582	A: Ser893Thr G: Ser893Ala									
T/T		83 (17)	42 (20)		19 (15)	106 (18)		9 (15)	116 (18)	
T/A		62 (13)	28 (13)		24 (19)	66 (11)		8 (14)	82 (13)	
G/G		95 (19)	40 (19)	0.95	21 (17)	114 (20)	0.22	12 (21)	123 (19)	0.76
G/T		157 (32)	67 (31)		41 (33)	183 (32)		15 (26)	209 (32)	
G/A		75 (15)	30 (14)		17 (14)	88 (15)		12 (21)	93 (14)	
A/A		18 (4)	6 (3)		2 (2)	22 (4)		2 (3)	22 (4)	

*By use of Fisher's exact test for 2×3 table (2×6 table for rs2032582).

doi:10.1371/journal.pone.0141931.t003

[17,18]. In this regard, one study from Thailand reported the association between -24 C/C genotype of *ABCC2* gene and lower eGFR in treatment-naïve patients who initiated TDF-containing non-NRTI based regimen [34]. However, the relatively small sample size of 117 patients and, more importantly, the cross-sectional analysis used for the assessment of the

Table 4. Effects of SNP at -24 of *ABCC2* on three renal outcomes in patients who initiated TDF-containing antiretroviral therapy: Multivariate logistic regression with genotype model.

	>10 ml/min/1.73 m^2 decrement in eGFR			>25% decrement in eGFR			eGFR<60 ml/min/1.73 m^2		
	OR	95%CI	P value	OR	95%CI	P value	OR	95%CI	P value
Genotype C/C versus T/T	0.5	0.06–3.91	0.62	1.2	0.42–3.20	0.62	0.6	0.20–2.08	0.61
Genotype C/T versus T/T	0.4	0.05–3.33	0.34	1.0	0.35–2.83	0.81	0.6	0.17–1.93	0.36

Odds ratios for each genotype were adjusted for baseline eGFR, age, CD4 count, body weight, nephrotoxic drug use, hypertension, dyslipidemia, and use of PI/r. OR: odds ratio, CI: confidence interval, eGFR: estimated glomerular filtration rate, PI/r: ritonavir-boosted protease inhibitor.

doi:10.1371/journal.pone.0141931.t004

association between eGFR and genotype at 48 and 96 weeks undermine the reliability of their findings, because in using such design, the value of eGFR at 48 or 96 weeks is inevitably affected by the baseline eGFR. Furthermore, another recent Thai study of 238 patients showed that SNPs of drug transporters, including -24 and 1249 of *ABCC2* gene, were not associated with a change in creatinine clearance from the baseline to 1 and 3 years of TDF exposure [35]. Nevertheless, our sample size is the largest (n = 703) among the studies investigating the effect of genetic variants of drug transporters on TDF-related renal dysfunction, and by using clinically relevant renal outcomes, our study showed that SNPs were not associated with TDF nephrotoxicity.

Second, to our knowledge, this is the first study to apply not only genotype model (a model that postulates no mode of inheritance), but also dominant, recessive, and additive models, to investigate the association between genetic variants and TDF nephrotoxicity [16,34,35]. It is noteworthy that none of the four genetic models showed any association between genetic variants of transporter proteins and TDF-associated renal dysfunction, especially considering that it is unknown which genetic model is appropriate for the evaluation of the effect of SNPs on TDF nephrotoxicity. Another strength of this study is that the results were reproduced with eGFR calculated by the CKD-EPI equation, in addition to the results based on eGFR calculated by the JSN equation.

It is noteworthy that although the association between the SNPs of *ABCC2* investigated in this study and TDF-induced tubulopathy has been well established [11,13,16], the exact mechanism by which these SNPs pose a risk for TDF tubulopathy remains unknown [13,16]. In this regard, MRP2 encoded by *ABCC2* is not likely to take part in the transportation of TDF at the luminal membrane of kidney tubular cells [16,36]. At this point, because the results of this study showed that genetic variants of the drug transporters for TDF are not associated with clinically important renal outcomes, we think that these SNPs do not count as a risk factor for TDF-related renal dysfunction, at least in the clinical setting, and efforts should rather be focused on the management of traditional risk factors for renal dysfunction, such as diabetes mellitus and hypertension [37], as well as the management of PI/r, antiretroviral agents that are reported to increase TDF exposure [38] and thus are a risk factor for TDF-related renal dysfunction [23]. It is also notable that among PI/r, ritonavir-boosted atazanavir and lopinavir/ritonavir are reported to be associated with CKD [39].

Several limitations need to be acknowledged. First, the patients who discontinued TDF within 90 days from the initiation of this therapy were excluded from the study. It is difficult to completely exclude the possibility that inclusion of such patients would have resulted in misleading results, because the subjects would have included some who experienced substantial decrement in renal function due to causes other than TDF, such as death shortly after initiation of ART or immune reconstitution inflammatory syndrome of opportunistic infections, considering that two-third of the study patients were treatment-naive. Second, although we selected the target SNPs that have been identified to associate with TDF-induced tubulopathy reported in previous studies, there might be other unknown transporter proteins for tenofovir excretion or transportation that contribute to susceptibility to tenofovir nephrotoxicity. Third, this study did not measure TDF plasma concentration, which could correlate with TDF-induced renal dysfunction [40]. Fourth, our cohort was characterized by the high prevalence of PI/r use, which can affect plasma concentration of TDF [41], and it is difficult to completely exclude the impact of concurrent PI/r in this study.

In conclusion, the present study demonstrated that genetic variants of the drug transporters for TDF do not associate with clinically important renal outcomes in patients who started TDF-containing ART. Such SNPs are not considered to be a risk factor for clinically relevant TDF-related renal dysfunction.

Supporting Information

S1 Table. Effects of SNP at 1249 of *ABCC2* on three renal outcomes in patients who initiated TDF-containing antiretroviral therapy: Multivariate logistic regression with genotype model.
(DOCX)

S2 Table. Effects of SNP at 2677 of ABCB1 on three renal outcomes in patients who initiated TDF-containing antiretroviral therapy: Multivariate logistic regression with genotype model.
(DOCX)

S3 Table. Effects of SNPs on three renal endpoints using dominant, recessive, and additive genetic models for multivariate logistic analysis.
(DOCX)

Acknowledgments

The authors thank the study patients. The authors also thank Yoshimi Kikuchi, Katsuji Teruya, Kunihisa Tsukada, Junko Tanuma, Haruhito Honda, Ei Kinai, Koji Watanabe, Takahiro Aoki, Kiyoto Tsuchiya, Daisuke Mizushima, Yasuaki Yanagawa, and all other clinical staff at the AIDS Clinical Center for their help in completion of this study.

Author Contributions

Conceived and designed the experiments: TN SO HG. Performed the experiments: TN TH TK NT. Analyzed the data: TN TH TK NT. Contributed reagents/materials/analysis tools: NT SO HG. Wrote the paper: TN NT SO HG.

References

1. 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 (18 December 2013, date last accessed).
2. World Health Organization. Consolicated Guidelines on the Use of Antiretroviral Drugs for Treating and Preventing HIV Invection: Recommendations for a Public Health Approach. http://apps.who.int/iris/bitstream/10665/85321/1/9789241505727_eng.pdf. (19 January 2014, date last accessed).
3. Fung S, Kwan P, Fabri M, Horban A, Pelemis M, Hann HW, et al. (2013) Randomized Comparison of Tenofovir Disoproxil Fumarate vs Emtricitabine and Tenofovir Disoproxil Fumarate in Patients with Lamivudine-resistant Chronic Hepatitis B. Gastroenterology.
4. Consolidated guidelines on HIV prevention, diagnosis, treatment and care for key populations. World Health Organization. July 2014 Available at http://www.who.int/hiv/pub/guidelines/keypopulations/en/ Accessed 19 February 2015.
5. Preexposure Prophylaxis for the Prevention of HIV Infection in the United States 2014 Clinical Practice Guideline. American Center for Disease Prevention and Control. Available at:http://www.cdc.gov/hiv/pdf/PrEPguidelines2014.pdf. Accessed 19 February 2015.
6. Moss DM, Neary M, Owen A (2014) The role of drug transporters in the kidney: lessons from tenofovir. Front Pharmacol 5: 248. doi: 10.3389/fphar.2014.00248 PMID: 25426075
7. Verhelst D, Monge M, Meynard JL, Fouqueray B, Mougenot B, Girard PM, et al. (2002) Fanconi syndrome and renal failure induced by tenofovir: a first case report. Am J Kidney Dis 40: 1331–1333. PMID: 12460055
8. Gallant JE, Winston JA, DeJesus E, Pozniak AL, Chen SS, Cheng AK, et al. (2008) The 3-year renal safety of a tenofovir disoproxil fumarate vs. a thymidine analogue-containing regimen in antiretroviral-naive patients. AIDS 22: 2155–2163. PMID: 18832879
9. Nishijima T, Gatanaga H, Komatsu H, Tsukada K, Shimbo T, Aoki T, et al. (2012) Renal function declines more in tenofovir- than abacavir-based antiretroviral therapy in low-body weight treatment-

naive patients with HIV infection. PLoS One 7: e29977. doi: 10.1371/journal.pone.0029977 PMID: 22242194

10. Kohler JJ, Hosseini SH, Hoying-Brandt A, Green E, Johnson DM, Russ R, et al. (2009) Tenofovir renal toxicity targets mitochondria of renal proximal tubules. Lab Invest 89: 513–519. doi: 10.1038/labinvest.2009.14 PMID: 19274046
11. Izzedine H, Hulot JS, Villard E, Goyenvalle C, Dominguez S, Ghosn J, et al. (2006) Association between ABCC2 gene haplotypes and tenofovir-induced proximal tubulopathy. J Infect Dis 194: 1481–1491. PMID: 17083032
12. Kiser JJ, Aquilante CL, Anderson PL, King TM, Carten ML, Fletcher CV (2008) Clinical and genetic determinants of intracellular tenofovir diphosphate concentrations in HIV-infected patients. J Acquir Immune Defic Syndr 47: 298–303. PMID: 18398970
13. Rodriguez-Novoa S, Labarga P, Soriano V, Egan D, Albalater M, Morello J, et al. (2009) Predictors of kidney tubular dysfunction in HIV-infected patients treated with tenofovir: a pharmacogenetic study. Clin Infect Dis 48: e108–116. doi: 10.1086/598507 PMID: 19400747
14. Pushpakom SP, Liptrott NJ, Rodriguez-Novoa S, Labarga P, Soriano V, Albalater M, et al. (2011) Genetic variants of ABCC10, a novel tenofovir transporter, are associated with kidney tubular dysfunction. J Infect Dis 204: 145–153. doi: 10.1093/infdis/jir215 PMID: 21628669
15. Rodriguez-Novoa S, Labarga P, Soriano V (2009) Pharmacogenetics of tenofovir treatment. Pharmacogenomics 10: 1675–1685. doi: 10.2217/pgs.09.115 PMID: 19842939
16. Nishijima T, Komatsu H, Higasa K, Takano M, Tsuchiya K, Hayashida T, et al. (2012) 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 55: 1558–1567. doi: 10.1093/cid/cis772 PMID: 22955427
17. Choi AI, Rodriguez RA, Bacchetti P, Bertenthal D, Volberding PA, O'Hare AM (2007) The impact of HIV on chronic kidney disease outcomes. Kidney Int 72: 1380–1387. PMID: 17805235
18. Brennan A, Evans D, Maskew M, Naicker S, Ive P, Sanne I, et al. (2011) Relationship between renal dysfunction, nephrotoxicity and death among HIV adults on tenofovir. AIDS 25: 1603–1609. PMID: 21646902
19. Matsuo S, Imai E, Horio M, Yasuda Y, Tomita K, Nitta K, et al. (2009) Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis 53: 982–992. doi: 10.1053/j.ajkd.2008.12.034 PMID: 19339088
20. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF 3rd, Feldman HI, et al. (2009) A new equation to estimate glomerular filtration rate. Ann Intern Med 150: 604–612. PMID: 19414839
21. Horio M, Imai E, Yasuda Y, Watanabe T, Matsuo S (2013) Performance of GFR equations in Japanese subjects. Clin Exp Nephrol 17: 352–358. doi: 10.1007/s10157-012-0704-5 PMID: 23080048
22. Nishijima T, Kawasaki Y, Tanaka N, Mizushima D, Aoki T, Watanabe K, et al. (2014) Long-term exposure to tenofovir continuously decrease renal function in HIV-1-infected patients with low body weight. AIDS 28: 1903–1910. PMID: 25259702
23. Goicoechea M, Liu S, Best B, Sun S, Jain S, Kemper C, et al. (2008) Greater tenofovir-associated renal function decline with protease inhibitor-based versus nonnucleoside reverse-transcriptase inhibitor-based therapy. J Infect Dis 197: 102–108. doi: 10.1086/524061 PMID: 18171292
24. Nelson MR, Katlama C, Montaner JS, Cooper DA, Gazzard B, Clotet B, et al. (2007) The safety of tenofovir disoproxil fumarate for the treatment of HIV infection in adults: the first 4 years. AIDS 21: 1273–1281. PMID: 17545703
25. Gatanaga H, Tachikawa N, Kikuchi Y, Teruya K, Genka I, Honda M, et al. (2006) Urinary beta2-microglobulin as a possible sensitive marker for renal injury caused by tenofovir disoproxil fumarate. AIDS Res Hum Retroviruses 22: 744–748. PMID: 16910829
26. Lucas GM, Ross MJ, Stock PG, Shlipak MG, Wyatt CM, Gupta SK, et al. (2014) Clinical practice guideline for the management of chronic kidney disease in patients infected with HIV: 2014 update by the HIV Medicine Association of the Infectious Diseases Society of America. Clin Infect Dis 59: e96–138. doi: 10.1093/cid/ciu617 PMID: 25234519
27. Kurata Y, Ieiri I, Kimura M, Morita T, Irie S, Urae A, et al. (2002) Role of human MDR1 gene polymorphism in bioavailability and interaction of digoxin, a substrate of P-glycoprotein. Clin Pharmacol Ther 72: 209–219. PMID: 12189368
28. Neumanova Z, Cerveny L, Ceckova M, Staud F (2014) Interactions of tenofovir and tenofovir disoproxil fumarate with drug efflux transporters ABCB1, ABCG2, and ABCC2; role in transport across the placenta. AIDS 28: 9–17. PMID: 24413260
29. Roldan V, Marin F, Fernandez H, Manzano-Fernandez S, Gallego P, Valdes M, et al. (2013) Renal impairment in a "real-life" cohort of anticoagulated patients with atrial fibrillation (implications for

thromboembolism and bleeding). Am J Cardiol 111: 1159–1164. doi: 10.1016/j.amjcard.2012.12.045 PMID: 23337836

30. Chaisiri K, Bowonwatanuwong C, Kasettratat N, Kiertiburanakul S (2010) Incidence and risk factors for tenofovir-associated renal function decline among Thai HIV-infected patients with low-body weight. Curr HIV Res 8: 504–509. PMID: 21073439

31. Nishijima T, Komatsu H, Gatanaga H, Aoki T, Watanabe K, Kinai E, et al. (2011) Impact of small body weight on tenofovir-associated renal dysfunction in HIV-infected patients: a retrospective cohort study of Japanese patients. PLoS One 6: e22661. doi: 10.1371/journal.pone.0022661 PMID: 21799928

32. Mocroft A, Kirk O, Reiss P, De Wit S, Sedlacek D, Beniowski M, et al. (2010) Estimated glomerular filtration rate, chronic kidney disease and antiretroviral drug use in HIV-positive patients. AIDS 24: 1667–1678. PMID: 20523203

33. Horio M, Imai E, Yasuda Y, Watanabe T, Matsuo S (2010) Modification of the CKD epidemiology collaboration (CKD-EPI) equation for Japanese: accuracy and use for population estimates. Am J Kidney Dis 56: 32–38. doi: 10.1053/j.ajkd.2010.02.344 PMID: 20416999

34. Manosuthi W, Sukasem C, Thongyen S, Nilkamhang S, Sungkanuparph S (2014) ABCC2*1C and plasma tenofovir concentration are correlated to decreased glomerular filtration rate in patients receiving a tenofovir-containing antiretroviral regimen. J Antimicrob Chemother 69: 2195–2201. doi: 10.1093/jac/dku129 PMID: 24788661

35. Sirirungsi W, Urien S, Harrison L, Kamkon J, Tawon Y, Luekamlung N, et al. (2015) No Relationship Between Drug Transporter Genetic Variants and Tenofovir Plasma Concentrations or Changes in Glomerular Filtration Rate in HIV-Infected Adults. J Acquir Immune Defic Syndr 68: e56–59. PMID: 25559596

36. Ray AS, Cihlar T, Robinson KL, Tong L, Vela JE, Fuller MD, et al. (2006) Mechanism of active renal tubular efflux of tenofovir. Antimicrob Agents Chemother 50: 3297–3304. PMID: 17005808

37. Lucas GM, Ross MJ, Stock PG, Shlipak MG, Wyatt CM, Gupta SK, et al. (2014) Clinical Practice Guideline for the Management of Chronic Kidney Disease in Patients Infected With HIV: 2014 Update by the HIV Medicine Association of the Infectious Diseases Society of America. Clinical Infectious Diseases.

38. Kearney BP, Mathias A, Mittan A, Sayre J, Ebrahimi R, Cheng AK (2006) Pharmacokinetics and safety of tenofovir disoproxil fumarate on coadministration with lopinavir/ritonavir. J Acquir Immune Defic Syndr 43: 278–283. PMID: 17079992

39. Ryom L, Mocroft A, Kirk O, Worm SW, Kamara DA, Reiss P, et al. (2013) Association between antiretroviral exposure and renal impairment among HIV-positive persons with normal baseline renal function: the D:A:D study. J Infect Dis 207: 1359–1369. doi: 10.1093/infdis/jit043 PMID: 23382571

40. Poizot-Martin I, Solas C, Allemand J, Obry-Roguet V, Pradel V, Bregigeon S, et al. (2013) Renal impairment in patients receiving a tenofovir-cART regimen: impact of tenofovir trough concentration. J Acquir Immune Defic Syndr 62: 375–380. PMID: 23196828

41. Kiser JJ, Carten ML, Aquilante CL, Anderson PL, Wolfe P, King TM, et al. (2008) The effect of lopinavir/ritonavir on the renal clearance of tenofovir in HIV-infected patients. Clin Pharmacol Ther 83: 265–272. PMID: 17597712

A strong association of human leukocyte antigen-associated Pol and Gag mutations with clinical parameters in HIV-1 subtype A/E infection

Giang Van Tran[a,*], Takayuki Chikata[a,*], Jonathan M. Carlson[b], Hayato Murakoshi[a], Dung Hoai Nguyen[c], Yoshiko Tamura[a], Tomohiro Akahoshi[a], Nozomi Kuse[a], Keiko Sakai[a], Sachiko Sakai[a], Kyle Cobarrubias[d], Shinichi Oka[a,e], Zabrina L. Brumme[d,f], Kinh Van Nguyen[c], Masafumi Takiguchi[a,g,h], for the NHTD Treatment-Naive Cohort Study Group

Objectives: Identification of human leukocyte antigen-associated HIV-1 polymorphisms (HLA-APs) in different global populations furthers our understanding of HIV-1 pathogenesis and may help identify candidate immunogens for HIV vaccines targeted to these populations. Although numerous population-based studies identifying HLA-APs have been conducted in HIV-1 subtype B- and subtype C-infected cohorts, few have focused on subtype A/E.

Design: We investigated HLA-APs in a cohort of chronically HIV-1 subtype A/E-infected Vietnamese individuals.

Methods: HLA-APs in HIV-1 Gag, Pol, and Nef regions from 388 treatment-naive individuals chronically infected with HIV-1 subtype A/E were analyzed using phylogenetically informed approaches.

Results: A total of 303 HLA-APs were identified. HLA-APs occurring at six positions in Gag and six positions in Pol were significantly associated with higher plasma viral load (pVL), whereas HLA-APs occurring at two positions in Gag and 13 positions in Pol were significantly associated with lower $CD4^+$ T-cell counts. Furthermore, the proportion of Pol codons harboring an HLA-AP specific to the host's HLA correlated positively with HIV-1 pVL ($R=0.22$; $P<0.0001$) and inversely with $CD4^+$ T-cell counts ($R=-0.32$; $P<0.0001$). Similarly, the proportion of HLA-associated Gag codons harboring host-specific HLA-AP correlated inversely with $CD4^+$ T-cell counts ($R=-0.13$; $P=0.01$).

Conclusion: These significant associations between HIV-1 amino acids adapted to Vietnamese HLA alleles and higher pVL and lower $CD4^+$ T-cell counts suggests that accumulation of cytotoxic T cells escape mutations may influence clinical outcomes in HIV-1 subtype A/E-infected Vietnamese individuals.

AIDS 2016, **30**:681–689

Keywords: cytotoxic T cells, escape mutations, human leukocyte antigen-associated polymorphism, human leukocyte antigen

[a]Center for AIDS Research, Kumamoto University, Kumamoto, Japan, [b]eScience Group, Microsoft Research, Los Angeles, California, USA, [c]National Hospital of Tropical Diseases, Dong Da District, Hanoi, Vietnam, [d]Faculty of Health Sciences, Simon Fraser University, Burnaby, BC, USA, [e]AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan, [f]British Columbia Centre for Excellence in HIV/AIDS, Vancouver, Canada, [g]International Research Center for Medical Sciences, Kumamoto University, Kumamoto, Japan, and [h]Nuffield Department of Medicine, University of Oxford, Oxford, UK.

Correspondence to Masafumi Takiguchi, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan.

Tel: +81 96 373 6529; fax: +81 96 373 6532; e-mail: masafumi@kumamoto-u.ac.jp

* Giang Van Tran and Takayuki Chikata contributed equally to this study.

Received: 9 July 2015; revised: 19 October 2015; accepted: 5 November 2015.

DOI:10.1097/QAD.0000000000000969

Introduction

HIV-1-specific cytotoxic T cells (CTLs) play an important role in the control of HIV-1 [1–9]. They eliminate HIV-1-infected cells via their recognition of virally derived peptide epitopes, which are presented by human leukocyte antigen (HLA) class I molecules on the surface of HIV-1-infected cells. However, CTL escape mutations occur in HIV-1 sequences that allow the virus to evade CTL recognition; indeed the adaptation of HIV-1 to HLA-associated immune pressures has been demonstrated at both the individual and the population levels [10–12]. Numerous population-based studies identifying HLA-associated polymorphisms (HLA-APs) in HIV-1 subtypes B and C have been conducted in North American, European, Australian, and African cohorts [13–18]. However, fewer studies have been undertaken in Asian cohorts [19,20] where HIV-1 prevalence is also substantial and other HIV-1 subtypes predominate in certain populations. Specifically, HIV-1 subtypes B, C and A/E predominate in East, South, and South-east Asia, respectively. A few large-scale studies of HLA-APs have been undertaken in China and Japan, but all in HIV-1 subtype B [19,21]. Only one study of HLA-APs in subtype A/E has been performed in Southeast Asia [20]. Undertaken in a cohort of 116 Thai individuals and focused solely on Gag, the study identified 56 HLA-AP at P less than 0.05.

As Asian populations differ in both host and viral genetics (in terms of HLA allele distributions and circulating HIV-1 sequences) from white and African cohorts, the analysis of HLA-APs helps further our understanding of HIV-1 pathogenesis in Asia and may help inform the design of HIV vaccine targeted to these populations. In particular, the analysis of HLA-APs in subtype A/E is relevant to the design of HIV-1 immunogens relevant to Southeast Asian countries [22–24].

In the present study, we identified HLA-APs in HIV-1 subtype A/E Gag, Pol, and Nef proteins in a cohort of 388 treatment-naïve Vietnamese individuals chronically infected with HIV-1 subtype A/E using phylogenetically informed approaches. In addition, we investigated their associations with clinical parameters such as $CD4^+$ T-cell count and plasma viral load (pVL). Importantly, HLA genotyping was undertaken at the subtype level, allowing us to map HLA-AP at high resolution. This is the first larger-scale study of HLA-APs in HIV-1 subtype A/E infection.

Materials and methods

Ethical statement

The study protocol was approved by the institutional ethical review boards of National Hospital of Tropical Diseases (18/HĐĐĐ-NĐTU) and Kumamoto University (Rinri-540) and by the Ethics Committee of the Vietnamese Ministry of Health (1666/QĐ-BYT). All study participants provided their written informed consent for the collection of the samples and their subsequent analysis.

Study population

This cross-sectional study included 388 antiretroviral-naive individuals chronically infected with HIV-1, enrolled at their first visit to the National Hospital of Tropical Diseases (NHTD) in Hanoi between October, 2012 and November, 2014. All participants were adults with HIV-1 infection confirmed by ELISA at recruitment. Pregnant women, individuals with AIDS-related symptoms, and those with previous antiretroviral exposure were excluded.

Plasma samples and human leukocyte antigen-typing

Participant $CD4^+$ T-cell count (cells/μl) and plasma HIV-RNA (copies/ml, measured by the Roche COBAS TaqMan HIV monitor assay) were measured at enrollment. HLA class I typing at subtype-level (four-digit) resolution was performed using a probe-based sequence-specific oligonucleotide typing method (HLA Laboratory, Kyoto, Japan).

RT-PCR and sequencing of plasma HIV RNA

HIV-1 RNA was extracted from plasma samples using either a QIAamp MinElute virus spin kit (Qiagen, Valencia, California, USA) or an EZ1 Virus Mini Kit v2.0 (Qiagen). Reverse transcription was undertaken using random hexamers with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, California, USA). Nested PCR of HIV-1 Gag, Pol, and Nef genes was subsequently conducted with Taq DNA polymerase (Promega, Fitchburg, Wisconsin, USA) using 14 HIV-1 subtype A/E sequence-specific primer pairs. The second-round PCR product was analyzed by gel electrophoresis, excised and purified with EXOSAP-IT (GE Healthcare, Buckinghamshire, UK). Gag, Pol, and Nef sequences were bi-directionally sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, California, USA), on an ABI 3500 genetic analyzer (Applied Biosystems). Sequences were analyzed and aligned to the subtype A/E reference sequence using SeqScape software (Applied Biosystems). Sequences have been deposited in the DDBJ/EMBL/GenBank under accession numbers LC100161 to LC100529 (Gag), LC100902 to LC101260 (Pol), and LC100530 to LC100901 (Nef).

Identification of human leukocyte antigen-associated polymorphisms

Associations between HLA class I alleles and HIV-1 amino acid polymorphisms (termed HLA-associated HIV-1 polymorphisms or 'HLA-APs') in the Vietnamese cohort were identified using a previously published statistical-association approach that accounts for the

confounding effects of HLA linkage disequilibrium, HIV phylogeny, and HIV codon covariation using a phylogenetically corrected logistical regression model [13,17,19,25–27]. HLA-APs that are overrepresented in individuals harboring the relevant HLA allele are termed 'adapted' forms, whereas those underrepresented in individuals harboring the relevant HLA are termed 'nonadapted' forms. All HLA alleles and HIV-1 polymorphisms observed in a minimum of 10 individuals were examined for potential associations. In addition, analysis of codon covariation within HIV Gag, Pol, and Nef proteins was performed using the same phylogenetically corrected logistical regression approach [13,26,27]. Multiple testing was addressed using *q* values, the *P*-value analog of the false discovery rate (FDR). The FDR is the expected proportion of false positive results deemed significant at a given *P*-value threshold [28]. For example, at *q* less than 0.2, we expect 20% of identified associations to represent false positives. The present study employed a *q*-value cutoff of *q* less than 0.2 to define statistical significance, as was done in previous studies identifying HLA associated polymorphisms in HIV-1 [13,15,17,18,29–32].

Statistical analysis

Spearman's correlation was used to evaluate the relationship between the total number (or overall proportion) of HLA-associated substitutions specific to each individual's HLA profile and clinical parameters ($CD4^+$ T-cell counts and pVL). In these analyses, HLA-associated substitutions were defined as HLA-associated HIV-1 codons expressing the specific 'adapted' residue (and/or not expressing the specific 'nonadapted' residue) at a given HLA-associated site. The Mann–Whitney test was used to evaluate differences in the distribution of pVL or $CD4^+$ T-cell counts in the presence/absence of HLA-associated substitutions in Gag and Pol. Multiple testing was addressed using *q* values, with *q* less than 0.2 defined as statistically significant.

ELISPOT assay

HLA-C*01 : 02-restricted YI9 epitope (YSPVSILDI, Gag277–285) or mutant epitope (YSPTSILDI) [26] and PBMCs from HIV-1-infected individuals were added to a 96-well polyvinylidene plate precoated with 5 mg/ml of anti-interferon (IFN)-γ mAb 1-D1K (Mabtech, Stockholm, Sweden). After incubation for 16 h at 37°C, biotinylated anti-IFN-γ Mab 7-B6–1 (Mabtech) at 1 mg/ml was added. Individual cytokine-producing cells were detected as previously described [9]. PBMCs without peptide stimulation were used as a negative control. The number of spots for each peptide-specific T-cell response was calculated by subtracting the number of negative-control spots.

Intracellular cytokine staining assay

HLA-C*01:02-expressing 721.221 cells prepulsed with the peptide for 1 h at 37°C were co-cultured with T cells in the presence of Brefeldin A (10 μg/ml) at 37°C for 4 h. Subsequently, the cells were stained with PE-labeled anti-CD8 monoclonal antibody (mAb) at 4°C for 30 min, fixed with paraformaldehyde solution at 4°C for 20 min, and then permeabilized with buffer containing 0.1% saponin and 10% fetal bovine serum in phosphate-buffered saline at 4°C for 10 min. Thereafter, the cells were stained with fluorescein isothiocyanate-conjugated anti-IFN-γ mAb at room temperature for 20 min and then washed twice with the permeabilization buffer. The percentage of $CD8^+$ cells producing IFN-γ was analyzed by flow cytometry (FACSCanto II).

Results

Identification of HLA-associated polymorphisms in chronically HIV-1 subtype A/E infected Vietnamese individuals

HLA-APs in Vietnamese individuals were identified in a cohort of 388 antiretroviral therapy-naïve individuals chronically infected with HIV-1 subtype A/E enrolled from 2012 to 2014. All of recruited individuals were successfully HLA class I typed. Gag, Pol, and Nef genotyping was successful for 369 (95%), 359 (93%), and 372 (96%) individuals, respectively. A total of 97 HLA class I alleles (25 HLA-A, 52 HLA-B and 20 HLA-C), identified at subtype-level resolution, were observed in this cohort. The distributions and frequencies of these HLA alleles were consistent with the database of Vietnamese HLA class I alleles (Supplemental figure 1, http://links.lww.com/QAD/A841), with HLA-A*11 : 01 (28.4%), HLA-B*15 : 02 (13.1%), and HLA-C*07 : 02 (17.0%) representing the highest-frequency HLA-A, B, and C alleles observed.

Of these 97 HLA class I alleles, 43 (13 HLA-A, 15 HLA-B, and 15 HLA-C alleles) were observed in more than 10 individuals and therefore were included in the statistical analysis of HLA-APs. HLA-APs were identified using a phylogenetically corrected logistic regression model with multiple tests addressed using *q* values. A total of 303 HLA-associated polymorphisms were identified in Gag (79 associations), Pol (114 associations), and Nef (110 associations) at *q* less than 0.2 (Supplemental Tables 1 and 2, http://links.lww.com/QAD/A841). These HLA-APs were observed at 45 of 498 Gag codons (9.0%), 59 of 1003 Pol codons (5.9%), and 47 of 206 Nef codons (22.8%) (Fig. 1). The higher frequency of HLA-APs in Nef compared with Gag and Pol is consistent with previous studies of HIV-1 subtype B and the subtype C-infected cohorts [13,15,19,25,30,33]. The number of HLA-A-, HLA-B-, and HLA-C-APs were 87, 123, and 93, respectively (Supplemental Tables 1 and 2, http://links.lww.com/QAD/A841). The predominance of HLA-B associated polymorphisms is also consistent with studies in other cohorts [15,19,30,34,35].

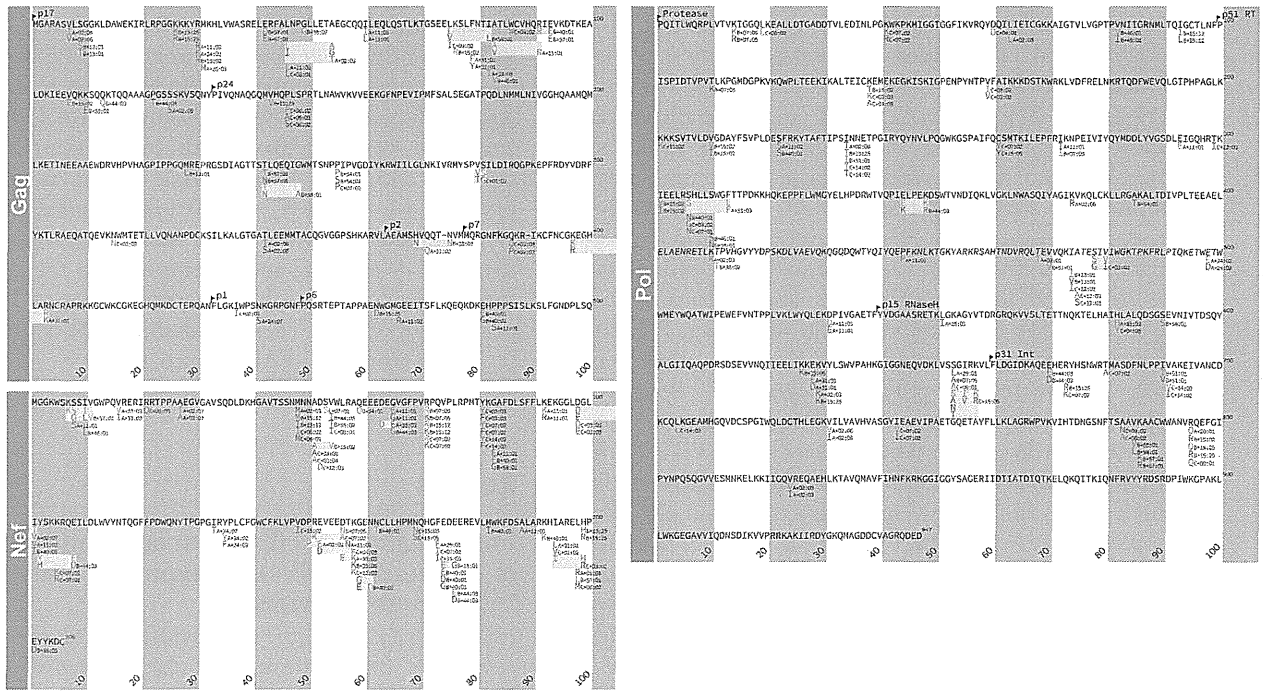

Fig. 1. Map of HLA-associated polymorphisms in Gag, Pol, and Nef. Escape maps indicate the locations, amino acids substitutions and HLA restrictions of HLA-associated polymorphisms (all $q < 0.2$). The HIV-1 subtype A/E consensus amino acid sequence, constructed from cohort sequences, is used as a reference. 'Nonadapted' (susceptible form) and 'adapted' (escape form) associations are shown in blue and red, respectively. Polymorphisms restricted by the same HLA allele that occur in proximity to one another are grouped together in yellow. A list of all HLA-associated polymorphisms is provided in Supplemental Table 1.

Association of the number and proportion of human leukocyte antigen-associated polymorphisms with clinical parameters

We next investigated the association between the proportion of sites exhibiting an HLA-associated substitution in HIV-1 sequences and pVL or $CD4^+$ T-cell counts in our cohort. The proportion of sites was calculated as the number of HLA-associated substitutions divided by the total number of HIV-1 codons associated with the host's HLA profile. Briefly, HIV-1 codons associated with each individual's HLA profile were counted as exhibiting an HLA-associated substitution if the codon harbored the specific 'adapted' residue (and/or did not harbor the specific 'nonadapted' residue) associated with that HLA allele at that site (see methods) [17,25,26,28,36]. A significant positive correlation was observed between the proportion of sites exhibiting an HLA-associated substitution in Pol and pVL ($R = 0.22$; $P = < 0.0001$). Similarly, a significant inverse relationship was observed between the proportion of sites exhibiting an HLA-associated substitution in Pol and $CD4^+$ T-cell counts ($R = -0.32$; $P = < 0.0001$) (Fig. 2). A significant inverse correlation was also observed between the proportion of sites exhibiting an HLA-associated substitution in Gag and $CD4^+$ T-cell counts ($R = -0.13$; $P = 0.01$), though no significant correlation was observed with pVL. No significant correlations were observed in Nef.

We also analyzed correlations between the 'number' of HLA-associated amino acid substitutions in HIV-1 sequences and clinical markers. Consistent with the previous analysis, a significant positive correlation was observed between the total number of HLA-associated substitutions in Pol and pVL ($R = 0.22$; $P = < 0.0001$), whereas a significant inverse correlation was observed with $CD4^+$ T-cell counts ($R = -0.3$; $P = < 0.0001$) (Supplemental figure 2, http://links.lww.com/QAD/A841). A significant positive correlation was also observed between the total number of HLA-associated substitutions in Gag and pVL ($R = 0.14$; $P = 0.007$). Again, no significant correlations were observed in Nef. Taken together, these results suggest that escape mutations adapted to Vietnamese HLA alleles, particularly those in Pol, may increase in-vivo viral fitness, presumably via their influence on evasion of HIV-1 control by specific CTLs.

To identify specific HLA-associated substitutions associated with clinical markers in our cohort, we stratified HIV-1 sequences based on the presence/absence of HLA-associated substitutions in Gag and Pol, and analyzed differences in pVL and $CD4^+$ T-cell counts by Mann–Whitney test. HLA-associated substitutions at Gag codons 54, 79, 107, 122, 280 and 369 and Pol codons 63, 138, 306, 410, 657 and 824 were associated with significantly higher pVL ($P < 0.05$, $q < 0.2$) (Table 1),

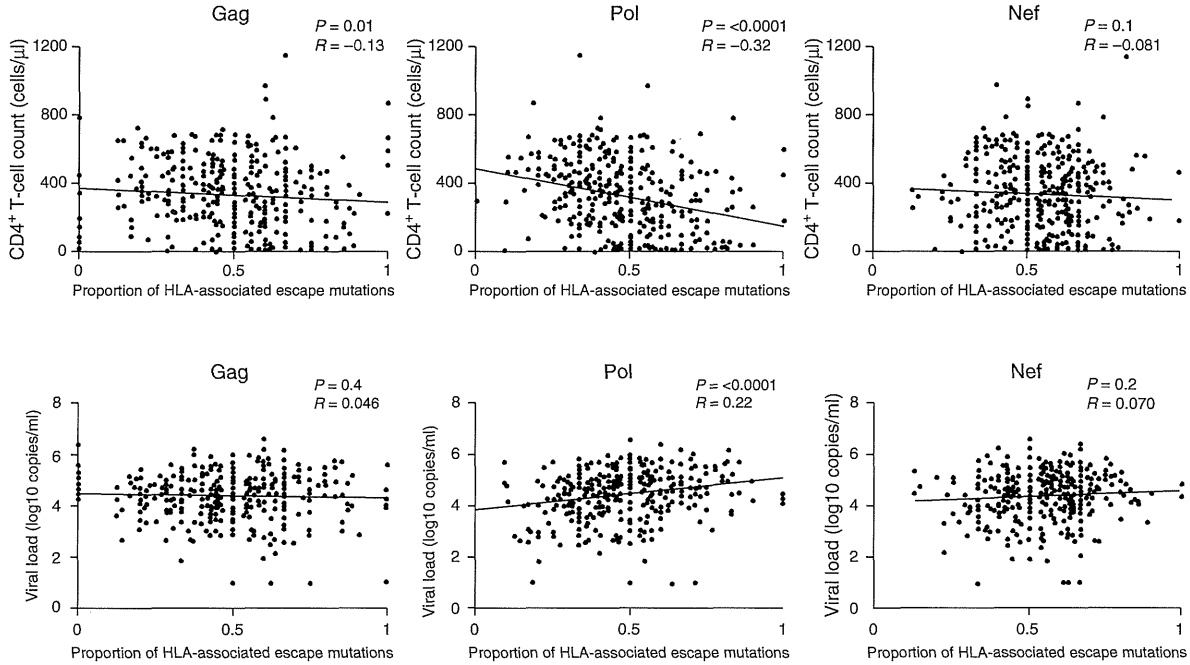

Fig. 2. Correlation between the proportion of HLA-associated escape mutations and plasma viral load or CD4$^+$ T-cell count. The proportion of HLA-associated codons exhibiting the 'escape' form specific for the host's HLA profile was calculated for each individual's Gag, Pol, and Nef amino acid sequence (where 'escape' was defined as the specific adapted form and/or any amino acid other than the 'nonadapted' form). Correlations between the proportion of HLA-associated escape mutations in Gag, Pol or Nef and pVL or CD4$^+$ T-cell count were analyzed using Spearman correlation. Trend lines are included to help visualize the relationship.

whereas HLA-associated substitutions at Gag codons 122 and 280 and Pol codons 19, 41, 63, 138, 385, 410, 411, 478, 657, 680, 731, 785, and 824 were associated with significantly lower CD4$^+$ T-cell counts ($P < 0.05$, $q < 0.2$) (Table 2). Gag codons 122 and 280 and Pol codons 63, 138, 410, 657, and 824 were significantly associated with both higher pVL and lower CD4$^+$ T-cell counts, suggesting these as possible markers of disease progression. In contrast, Gag codon 110 and Pol codon 673 were significantly associated with lower pVL, whereas Pol codon 222 was significantly associated with higher CD4$^+$ T-cell counts. To investigate the possibility that some of these HIV amino acids covary with one another, we cross-referenced them against the full list of covarying HIV codons from our intra-protein analysis (see methods). However, none of the HIV amino acids listed in Tables 1 and 2 exhibited strong covariation (defined as $q < 0.2$, data not shown), suggesting that the

Table 1. Association between HLA-associated substitutions and plasma viral load.

Protein	Codon	HLA allele	Amino acid	Direction	Number of patients with mutation	Median viral load (log_{10}, copies/ml) Without mutation	Median viral load (log_{10}, copies/ml) With mutation	*P* value	*q* value
Gag	54	A0203	A	Nonadapted	61	4.5	4.8	0.005	0.09
Gag	79	A3101	F	Nonadapted	111	4.5	4.8	0.03	0.17
Gag	107	B1502	E	Nonadapted	352	3.9	4.6	0.03	0.17
Gag	110	B5801	E	Adapted	9	4.6	3.7	0.007	0.09
Gag	122	B4403	T	Adapted	79	4.6	4.8	0.02	0.15
Gag	280	C0102	V	Nonadapted	42	4.5	4.8	0.005	0.09
Gag	369	B3802	N	Adapted	64	4.5	4.7	0.02	0.15
Pol	63	A0203	L	Nonadapted	123	4.5	4.8	0.0001	0.006
Pol	138	C0303	K	Nonadapted	188	4.5	4.7	0.005	0.06
Pol	306	A3303	S	Nonadapted	166	4.5	4.7	0.01	0.07
Pol	410	A0203	K	Nonadapted	41	4.5	4.9	0.006	0.06
Pol	657	C1505	K	Nonadapted	54	4.5	4.9	0.007	0.06
Pol	673	B1525, C0702[a]	R, K	Adapted	356	5.8	4.6	0.007	0.06
Pol	824	A0203	V	Nonadapted	75	4.5	4.9	0.001	0.04

[a]HLA-B*15:25 and C*07:02 were associated with R and K, respectively.

Table 2. Association between HLA-associated substitutions and CD4$^+$ T-cell counts.

Protein	Codon	HLA allele	Amino acid	Direction	Number of patients with mutation	Median of CD4$^+$ T-cell count (cells/μl)		*P* value	*q* value
						Without mutation	With mutation		
Gag	122	B4403	T	Adapted	79	341	237	0.01	0.19
Gag	280	C0102	V	Nonadapted	42	339	224	0.01	0.19
Pol	19	C0602	L	Nonadapted	23	339	207	0.01	0.1
Pol	41	C0702	K	Nonadapted	16	335	173	0.03	0.13
Pol	63	A0203	L	Nonadapted	123	344	274	0.04	0.16
Pol	138	C0303	K	Nonadapted	188	365	270	<0.0001	0.002
Pol	222	A1102, B4001[a]	S	Adapted	273	260	350	0.001	0.03
Pol	385	B5401	T	Adapted	119	351	293	0.02	0.11
Pol	410	A0203	K	Nonadapted	41	340	223	0.003	0.03
Pol	411	B3802	T	Nonadapted	76	357	251	0.001	0.03
Pol	478	C1203	S	Nonadapted	15	334	108	0.04	0.16
Pol	657	C1505	K	Nonadapted	54	340	218	0.001	0.03
Pol	680	C0702	A	Adapted	60	333	301	0.04	0.16
Pol	731	A0206	V	Nonadapted	45	339	252	0.02	0.1
Pol	785	B5801	V	Nonadapted	44	340	209	0.007	0.06
Pol	824	A0203	V	Nonadapted	75	340	244	0.01	0.1

[a]Both HLA-A*11:02 and B*40:01 were associated with S.

associations shown in Tables 1 and 2 are independently associated with pVL or CD4$^+$ T-cell counts.

Selection of escape mutation at Gag280 by YI9-specific cytotoxic T cells

Of the HLA-associated substitutions that correlated with higher pVL and/or lower CD4$^+$ T-cell counts (Tables 1 and 2), only Gag280 was located within a published CTL epitope restricted by the same HLA allele associated with the substitution: this was the HLA-C*01 : 02-restricted YI9 (YSPVSILDI) epitope spanning Gag codons 277–285 [37]. At codon 4 of this epitope, the subtype A/E consensus 'V' and the variant 'T' represent nonadapted (susceptible) and adapted (escaped) forms, respectively, associated with HLA-C*01 : 02 (Fig. 1 and Table S1, http://links.lww.com/QAD/A841). Therefore, we analyzed the response to YI9–4 V and YI9–4T peptides in two HIV-1 subtype A/E-infected HLA-C*01 : 02$^+$ individuals (KI-974 and KI-1071) by ELISPOT assay. Responses to YI9–4 V were detected in both individuals at a peptide concentration of 100 nmol/l, but responses to the 4T mutant peptide were negligible at this concentration (Fig. 3a). We further analyzed the recognition of the YI9–4T mutant peptide by YI9-specific T cells by intracellular cytokine staining (ICS). YI9-specific T cells were induced by simulating PBMCs from KI-974 and KI-1071 with YI9 peptide. YI9-specific T cells exhibited significantly poorer recognition of YI9–4T mutant

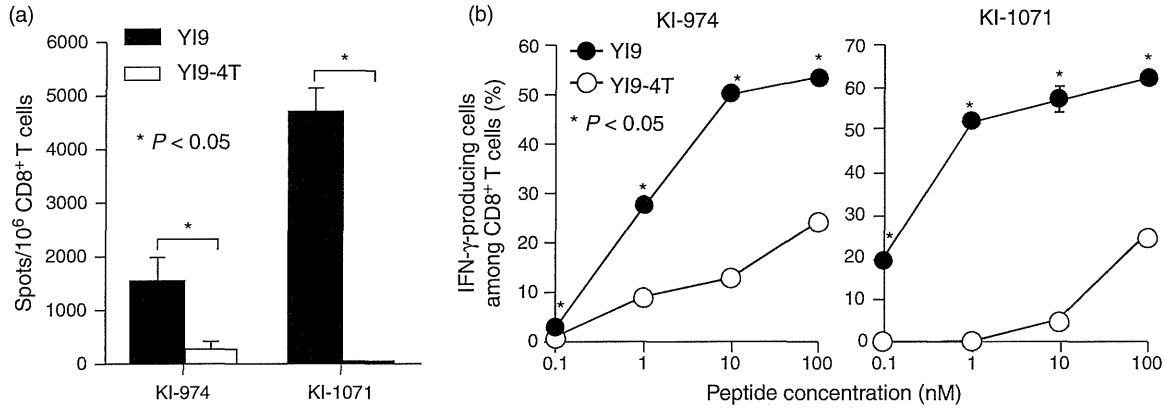

Fig. 3. Recognition of YI9–4T mutation by YI9-specific CTL. (a) Recognition of Gag YI9 wild-type or YI9–4T mutant peptides by specific T cells in 2 HIV-1 cladeA/E-infected individuals carrying HLA-C*01 : 02. Peptide-specific CD8$^+$ T cell responses at a peptide concentration of 100 nM were analyzed by ELISPOT. The results are shown as the mean and SD of triplicate experiments. (b) Recognition of YI9 and YI9–4T peptides by YI9-specific CD8$^+$ T cells. YI9-specific bulk T cells were stimulated with HLA-C*01 : 02-expressing 721.221 cells prepulsed with YI9 or YI9–4T peptide, after which IFN-γ production by bulk T cells was detected by ICS. The results are shown as the mean and SD of triplicate experiments. Statistical analyses were performed using the paired *t* test.

peptide at all concentrations tested (Fig. 3b). Taken together, these results support GagV280T as an escape mutation selected by YI9-specific CTLs in HLA-C*01 : 02$^+$ individuals infected with HIV-1 subtype A/E.

Discussion

This study is the first to identify associations between HLA alleles and amino acid substitutions in multiple HIV-1 proteins in the context of subtype A/E infection. We identified a total of 303 HLA-APs using phylogenetically informed methods: 79 in Gag, 114 in Pol, and 110 in Nef (all $q < 0.2$). Consistent with previous studies in subtypes B and C [13,15], Nef harbored the largest frequency of HLA-APs (these occurred at 22.8% of Nef codons), followed by Gag (9.0%) and Pol (5.9%). A previous study of HLA-AP in HIV-1 subtype A/E, undertaken in a Thai cohort, identified 56 HLA-A- and HLA-B-associated polymorphisms at 45 codons in HIV-1 Gag [20]. Of these, only 11 Gag codons (9, 30, 54, 75, 82, 83, 93, 228, 242, 248 and 403) overlapped with those identified in the present study; differences may be explained by the smaller size of the Thai cohort, different methods used to identify HLA-APs (including correction for multiple comparisons), and different HLA distributions between the cohorts.

Certain CTL escape mutations appear to be accumulating at the population level in HIV-1 sequences [10,11,38]. Moreover, certain CTL escape mutations affect viral replication, to an extent that may influence HIV clinical markers [30,39,40]. Previous population studies have also reported associations between HLA-APs and clinical markers. An HIV-1 subtype B cohort study reported significantly higher viral loads in the presence of HIV-1 polymorphisms S162X and K166X in reverse transcriptase among persons expressing the restricting HLA allele [11]. The proportion of escaped sites in Gag has also been reported to correlate positively with pVL in subtype B [41]. Furthermore, HLA-associated polymorphisms in protease/RT as well as Nef have been reported to correlate inversely with CD4$^+$ T-cell counts in subtype B [30]. HLA-associated polymorphisms associated with significantly lower pVL have also been reported in HIV subtype C [15]. In our Vietnamese cohort with subtype A/E infection, we observed significant positive correlations between the total number of HLA-associated substitutions (as well as the total proportion of sites harboring HLA-AP) in Pol and pVL, and inverse correlations with CD4$^+$ T-cell counts. These results suggest that HLA-associated substitutions in HIV-1 subtype A/E Pol may enhance in-vivo viral replication fitness, presumably via escape from HIV-1 specific CTL responses.

Although 20 HLA-APs in Gag and Pol were significantly associated with higher pVL and/or lower CD4$^+$ T-cell counts, the only one located within a known HIV-1 CTL epitope was the HLA-C*01 : 02-associated V-to-T substitution at Gag280, located at position 4 of the HLA-C*01 : 02-restricted YI9 epitope (Gag277–285; YSPVSILDI). Responses to wild-type YI9 were detected in 2 HLA-C*01 : 02$^+$ individuals infected with HIV-1 subtype A/E virus. However, responses to YI9 containing the 4T mutation were negligible in these individuals, strongly supporting Gag V280T as a bona-fide HLA-C*01 : 02 escape mutation in HIV-1 subtype A/E. In general, the observation that a majority of identified HLA-APs did not lie within known epitopes underscores the ongoing need to identify novel HLA-restricted CTL epitopes relevant to Asian populations.

We identified 14 and 12 HLA-APs associated with protective HLA alleles HLA-B*57 : 01 and B*58 : 01, respectively. However, the majority of these have not previously been reported in studies of subtype B or C infection. For example, within the well known HLA-B*57 : 01-restricted IW9 epitope (Gag147–155; ISPRTLNAW), GagI147L has been identified as an HLA-B*57 : 01-associated polymorphism in subtype B [13,25], that (along with I147 M) confers escape from IW9-specific CTLs [42]. However, we did not observe these HLA-APs in our subtype A/E cohort. Similarly, GagT242N confers escape from HLA-B*57 : 01-restricted CTL specific for the TW10 (Gag240–249; TSTLQEQIAW) epitope in subtype B infection [43–45]. Although GagT242N reduces viral fitness and thus could contribute to B*57 : 01-associated protective effects in HIV-1 subtype B infection [46–49], we did not observe any correlation between Gag 242 substitutions and HIV-1 clinical markers in our cohort. Of the HLA-B*58 : 01-restricted HLA-APs, NefA83G has previously been identified as a HLA-B*58 : 01-restricted polymorphism in subtypes B and C [13,17,25] that confers escape from KF9 (Nef82–90; KAAVDLSHF)-specific CTLs [38]. This escape mutation does not generally revert to wild type after transmission to individuals lacking HLA-B*58 : 01 [38], which may explain the observation that G represents the consensus amino acid in HIV-1 subtypes B and C. We observed no correlations between Nef 83 substitutions and HIV-1 clinical markers in our cohort; to our knowledge, there are no reports that NefA83G reduces viral fitness in subtypes B or C. Taken together, results suggest that HLA-B*57 : 01 and HLA-B*58 : 01-restricted immune responses may be different in subtype A/E than in subtypes B and C.

We previously identified 284 HLA-APs in treatment-naive Japanese individuals with chronic HIV-1 clade B infection and compared these with HLA-APs identified in a cohort of treatment naive individuals with chronic clade B infection in North America and Australia [19]. Of 238 Japanese HLA-APs associated with HLA alleles commonly observed in both cohorts, only 96 (40%) were also identified in the North American/Australian cohort,

supporting differential immune escape pathways across host populations (even those infected with the same HIV-1 subtype). Similarly, we compared HLA-APs in Vietnamese and Japanese cohorts to identify differences between them. Twenty-three HLA alleles (53%) were commonly observed in both cohorts and 156 HLA-APs identified in Vietnam were associated with these HLA alleles. Of these, only 21 (13%) were identified in the Japanese cohort (data not shown). These differences may be attributable to the circulating viral subtype and/or different immune responses between these two Asian countries. The location of HLA-APs could be used to guide the discovery of novel CTL epitopes in order to further elucidate the role of T-cell immune responses in driving these inter-cohort differences.

The present study is the first to identify HLA-APs in HIV-1 subtype A/E infection on a large scale. HLA-associated substitutions at 6 Gag and 6 Pol codons were significantly associated with higher pVL, whereas HLA-associated substitutions at two Gag positions and 13 Pol positions were significantly associated with lower $CD4^+$ T-cell counts. In particular, the number (and proportion) of HLA-associated substitutions in Pol correlated positively and significantly with pVL and negatively with $CD4^+$ T-cell counts. This suggests that Pol mutations adapted to Vietnamese HLA may modulate clinical markers of HIV-1 infection, an observation that contrasts somewhat with previous subtype B and C cohort studies that have identified Gag as a major target of CTL responses where immune escape may herald clinical consequences.

Acknowledgements

The authors thank Rie Maruyama for technical assistance.

NHTD Treatment-Naïve Cohort Study Group. Additional authors: Dung Thi NGUYEN, Bich Thi DANG, Ha Thi Nhu NGUYEN, Trung Vu NGUYEN- National Hospital of Tropical Diseases, Dong Da District, Hanoi, Vietnam, and Madoka KOYANAGI- Center for AIDS Research, Kumamoto University, Kumamoto, Japan.

Author contributions: G.V.T., T.C., M.T. conceived and designed the experiments. G.V.T., T.C., H.M., Y.T. performed the experiments. G.V.T., T.C., J.M.C., Z.L.B. analyzed the data.

K.C. supported to make figures. G.V.T., T.C., H.M., D.H.N., Y.T., T.A., N.K., K.S., M.K., S.S., D.T.N., B.T.D., H.T.N.N., T.V.N., S.O., K.V.N., M.T. contributed collection of samples. J.M.C., Z.L.B., T.M. participated in discussion on the data, commented on the manuscript and revised the manuscript. G.V.T., T.C., Z.L.B., M.T. wrote the article.

S.S. assisted in manuscript submission. All authors critically reviewed and gave final approval of the article. Justification of the number of contributors greater than 10. All authors contributed to establishment and maintenance of this cohort study and satisfy the conditions of authorship.

Financial support: This research was supported by a JSPS KAKENHI grant-in-aid for scientific research (A) (grant No. 15H02658), Japan; and by a Joint Research Grant with the Institute of Tropical Medicine, Nagasaki University. ZLB was supported by a New Investigator Award from the Canadian Institutes of Health Research and a Scholar Award from the Michael Smith Foundation for Health Research.

Conflicts of interest

The authors have no financial conflicts of interest.

References

1. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. **Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection.** *J Virol* 1994; **68**:6103–6110.
2. Matano T, Shibata R, Siemon C, Connors M, Lane HC, Martin MA. **Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques.** *J Virol* 1998; **72**:164–169.
3. Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S, *et al.* **Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA.** *Science* 1998; **279**:2103–2106.
4. Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, *et al.* **Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes.** *Science* 1999; **283**:857–860.
5. Carrington M, O'Brien SJ. **The influence of HLA genotype on AIDS.** *Annu Rev Med* 2003; **54**:535–551.
6. Goulder PJ, Watkins DI. **Impact of MHC class I diversity on immune control of immunodeficiency virus replication.** *Nat Rev Immunol* 2008; **8**:619–630.
7. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, *et al.* **Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome.** *J Virol* 1994; **68**:4650–4655.
8. Kawashima Y, Kuse N, Gatanaga H, Naruto T, Fujiwara M, Dohki S, *et al.* **Long-term control of HIV-1 in hemophiliacs carrying slow-progressing allele HLA-B*5101.** *J Virol* 2010; **84**:7151–7160.
9. Murakoshi H, Akahoshi T, Koyanagi M, Chikata T, Naruto T, Maruyama R, *et al.* **Clinical control of HIV-1 by cytotoxic t cells specific for multiple conserved epitopes.** *J Virol* 2015; **89**:5330–5339.
10. Kawashima Y, Pfafferott K, Frater J, Matthews P, Payne R, Addo M, *et al.* **Adaptation of HIV-1 to human leukocyte antigen class I.** *Nature* 2009; **458**:641–645.
11. Moore CB, John M, James IR, Christiansen FT, Witt CS, Mallal SA. **Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level.** *Science* 2002; **296**:1439–1443.
12. Goulder PJ, Phillips RE, Colbert RA, McAdam S, Ogg G, Nowak MA, *et al.* **Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS.** *Nat Med* 1997; **3**:212–217.
13. Brumme ZL, John M, Carlson JM, Brumme CJ, Chan D, Brockman MA, *et al.* **HLA-associated immune escape pathways in HIV-1 subtype B Gag, Pol and Nef proteins.** *PLoS One* 2009; **4**:e6687.