fractional excretion of uric acid (FE_{UA}) {[(urine uric acid × serum creatinine)/(serum uric acid × urine creatinine)] × 100)} > 15 %, β2-microglobulinuria (β2M > 1,000 μg/g Cr), α1-microglobulinuria (α1M > 16.6 mg/g Cr), and high NAG level in urine (NAG > 5.93 U/g Cr). The definition of KTD and the foregoing cutoff levels were determined based on the published reports [18, 25, 26].

The potential risk factors for KTD were determined according to previous studies and collected together with the basic demographics from the medical records [14, 27–30]: included were age, sex, body weight, and presence or absence of other medical conditions (concurrent use of other nephrotoxic drugs such as ganciclovir, sulfamethoxazole/trimethoprim, and nonsteroidal antiinflammatory agents; coinfection with hepatitis B, defined by positive hepatitis B surface antigen; coinfection with hepatitis C, defined by positive HCV viral load; hypertension, defined by current treatment with antihypertensive agents or two successive measurements of systolic blood pressure >140 mmHg or diastolic blood pressure >90 mmHg in the clinic; dyslipidemia, defined by current treatment with lipid-lowering agents or two successive measurements of either low density lipoprotein cholesterol >140 mg/dl, high density lipoprotein cholesterol <40 mg/dl, total cholesterol >240 mg/dl, triglyceride >500 mg/dl). At our clinic, blood pressure and body weight are measured every visit. We used the data on or closest to and preceding the day of blood/urine sample collection by no more than 180 days.

Statistical analysis

The baseline characteristics of patients with KTD and those without tubular dysfunction were compared by the Student's *t* test for continuous variables (e.g., kidney tubular markers), and by the χ^2 test or Fisher's exact test for categorical variables. Box plots were constructed for tubular markers of KTD and non-KTD. Diagnostic test comparison was performed using KTD as the dichotomous variable. Receiver operating characteristic (ROC) curves were constructed for individual markers, and the area under the curve (AUC) was estimated with 95 % confidence interval. The differences between the largest AUC and each of the other AUCs were tested using a nonparametric method [31], and multiple comparisons were adjusted with the Bonferroni correction. The cutoff value for each tubular marker was determined using raw data of 100 % sensitivity with maximal specificity because this point would diagnose all KTD cases with minimal false positives. For reference, two methods commonly applied for the identification of optimal cutoff points using the ROC curve were also applied: method 1 [the point on the curve closest to the point with sensitivity of one and one minus specificity of zero, calculated as the minimal value for $(1-\text{sensitivity})^2 + (1-\text{specificity})^2$]; and method 2 [the maximum vertical distance between the ROC curve and the diagonal line, calculated as the maximum value for (sensitivity + specificity − 1)] [32]. A p value <0.05 was considered statistically significant. Nonparametric methods to compare AUC of tubular makers were performed with Stata software SE ver. 12 (College Station, TX, USA). All other statistical analyses were performed with the Statistical Package for Social Sciences ver. 17.0 (SPSS, Chicago, IL, USA).

Results

A total of 190 patients were enrolled from whom blood and urine samples available for analysis (Fig. 1). Among them, 19 patients (10 %) fulfilled the criteria for KTD. The baseline characteristics and laboratory data for patients

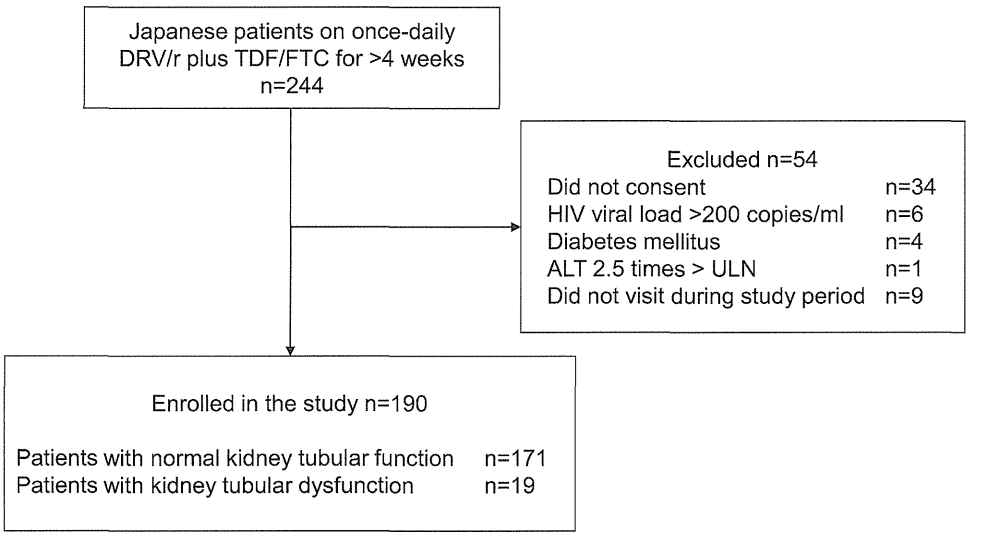


Fig. 1 Patient enrollment. *DRV/r* ritonavir-boosted darunavir; *TDF/FTC* tenofovir/emtricitabine; *ALT* alanine transaminase; *ULN* upper limit of normal

with KTD and patients without tubular dysfunction are listed in Table 1. Patients with KTD were older ($p < 0.001$), had a lower body weight ($p = 0.006$) and lower eGFR ($p = 0.003$), and were more likely to be hypertensive than patients with normal tubular function, although the difference was not significant ($p = 0.088$). The median duration of tenofovir therapy was 71.5 weeks [interquartile range (IQR), 36.8–109.2 weeks] for the entire study population and was not different between the two groups ($p = 0.888$).

Differences in tubular markers between patients with KTD and those without KTD are shown in Table 1 and box-and-whisker plots in Fig. 2. Patients with KTD had higher levels of all five tubular markers with p value <0.001 (Fig. 2). The performance of each tubular marker in differentiating patients with KTD from those with normal tubular function is illustrated by ROC curves (Fig. 3a). The AUCs and 95 % confidence intervals for the diagnosis of KTD by each tubular marker were β2M, 0.970 (0.947–0.992); α1M, 0.968 (0.944–0.992); NAG, 0.901 (0.828–0.974); FE_{IP}, 0.757 (0.607–0.907); and FE_{UA}, 0.762 (0.653–0.872). Results of comparisons of AUCs of β2M (with the largest AUC) and other markers are shown in Fig. 3b. The AUCs of β2M and α1M were not significantly different, whereas the AUCs of both FE_{IP} and FE_{UA} were significantly smaller than that of β2M. The AUC of NAG was marginally smaller than that of β2M with a single test, but the difference was no longer significant after adjustment of Bonferroni correction (Fig. 3b). Thus, urinary β2M and α1M had the best diagnostic performances for detecting KTD.

Identifying optimal cutoff point for tubular markers

The cutoff values for the different tubular markers with 100 % sensitivity and the maximal specificity were as follows: β2M, 1,123.2 μg/g Cr (specificity, 89 %); α1M, 15.4 mg/g Cr (specificity, 87 %); NAG, 3.58 U/g Cr (specificity, 46 %); FE_{IP}, 1.02 % (specificity, 0 %); and FE_{UA}, 3.92 % (specificity, 12 %) (Table 2). The cutoff values determined by the aforementioned two conventional methods are also listed in Table 2. The cutoff values of both β2M and α1M by method 1 yielded the high diagnostic accuracy (β2M, 1,612 μg/g Cr, sensitivity 95 %, specificity 93 %; α1M, 16.5 mg/g Cr, sensitivity 95 %, specificity 90 %), whereas the cutoff values for these two markers calculated with method 2 were the same as the aforementioned ones with 100 % sensitivity and maximal specificity. Methods 1 and 2 yielded the same cutoff value for NAG of 5.96 U/g Cr (sensitivity 90 %, specificity 86 %). For FE_{IP} and FE_{UA}, the sensitivity was relatively low with the cutoffs gained with method 1 and method 2, suggesting that FE_{IP} and FE_{UA} are not useful for screening KTD (Table 2).

Table 1 Characteristics of patients with and without kidney tubular dysfunction (KTD)

	KTD ($n = 19$)	Non-KTD ($n = 171$)	p value
Kidney tubular markers			
β2M > 1,000 μg/g Cr, *n* (%)	19 (100)	21 (12.3)	<0.001
α1M > 16.6 mg/g Cr, *n* (%)	18 (94.7)	17 (9.9)	<0.001
NAG > 5.93 U/g Cr, *n* (%)	17 (89.5)	23 (13.5)	<0.001
Fractional excretion of phosphate >18 %, *n* (%)	5 (26.3)	2 (1.2)	<0.001
Fractional excretion of uric acid >15 %, *n* (%)	2 (10.5)	4 (2.3)	0.112
Characteristics			
Sex (male), *n* (%)	18 (94.7)	166 (97.1)	0.473
Age (years)[a]	60 (41–62)	38 (32–42)	<0.001
Route of transmission (homosexual contact), *n* (%)	16 (84.2)	153 (89.5)	0.528
Weight (kg)[a]	56 (53.5–66.5)	67.2 (58.1–75)	0.006
eGFR (ml/min/1.73 m^2)[a]	75.5 (62.8–93.5)	87.7 (77.5–98)	0.003
Serum creatinine (mg/dl)[a]	0.85 (0.68–0.96)	0.80 (0.73–0.88)	0.168
CD4 cell count (/μl)[a]	380 (194–501)	379 (275–533)	0.261
Serum phosphate (mg/dl)[a]	3.4 (2.7–3.7)	3.2 (2.9–3.6)	0.815
Serum uric acid (mg/dl)[a]	4.7 (4.2–5.7)	5.6 (4.8–6.4)	0.080
Nephrotoxic drugs, *n* (%)	2 (10.5)	12 (7.0)	0.420
Hepatitis C, *n* (%)	0 (0)	3 (1.8)	0.728
Hepatitis B, *n* (%)	2 (10.5)	24 (14)	0.501
Dyslipidemia, *n* (%)	4 (21.1)	54 (31.6)	0.253
Hypertension, *n* (%)	8 (42.1)	42 (24.6)	0.088
C-reactive protein (mg/dl)[a]	0.07 (0.03–0.28)	0.07 (0.03–0.16)	0.277
TDF, weeks[a]	60.3 (17.7–115.4)	73.3 (37.7–109.1)	0.888

KTD kidney tubular dysfunction, *β2M* urinary β2-microglobulin, *α1M* urinary α1-microglobulin, *NAG* N-acetyl-β-D-glucosaminidase in urine, FE_{IP} fractional excretion of phosphate, FE_{UA} fractional excretion of uric acid, *eGFR* estimated glomerular filtration rate, *TDF* tenofovir disoproxil fumarate

[a] Median (interquartile range)

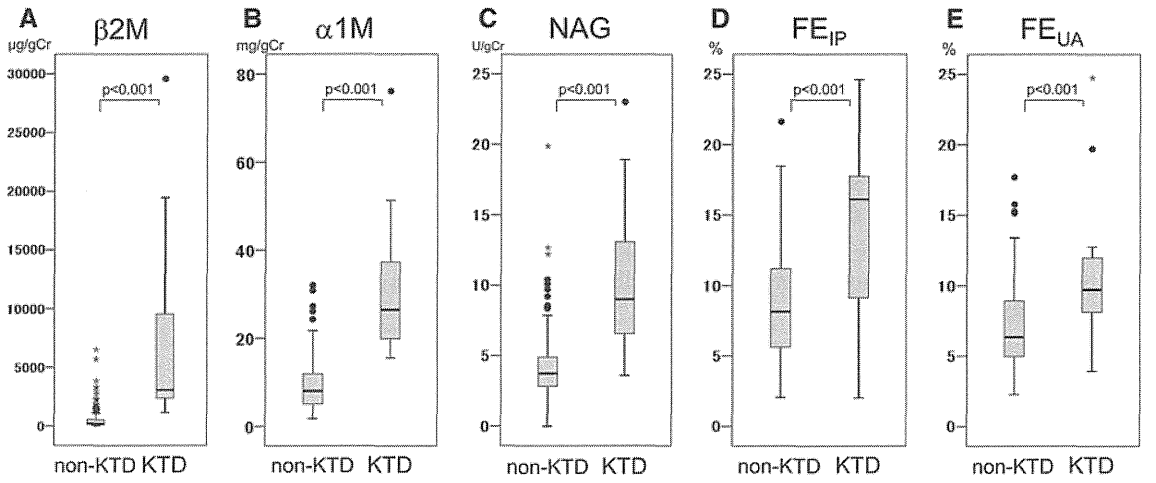
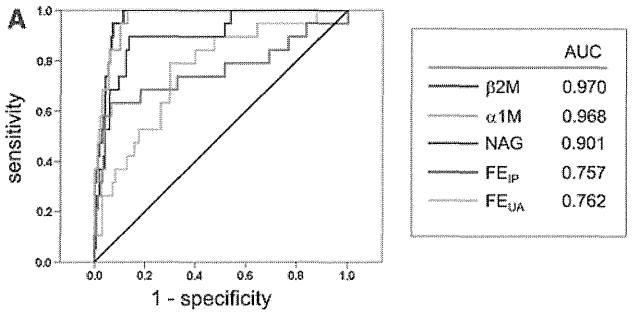


Fig. 2 Box-and-whisker plots of five tubular markers: urinary β2-microglobulin (*β2M*) (**a**), urinary α1-microglobulin (*α1M*) (**b**), *N*-acetyl-β-D-glucosaminidase in urine (*NAG*) (**c**), fractional excretion of phosphate (FE_{IP}) (**d**), and fractional excretion of uric acid (FE_{UA}) levels in patients with kidney tubular function (*KTD*) and those with normal tubular function (*non-KTD*) (**e**). In these plots, *lines within the boxes* represent median values; the *upper* and *lower lines of the boxes* represent the 25th and 75th percentiles, respectively; and the *upper and lower bars outside the boxes* represent the maximum and the minimum values or to the most extreme values within 1.5 interquartile ranges of the quartiles, respectively. *Closed circles* and *asterisks* in each graph represent outliers



B

	AUC	95% CI	Std. Err.	P value	P value†
β2M (standard)	0.970	0.947-0.992	0.011		
α1M	0.968	0.944-0.992	0.012	0.886	1
NAG	0.901	0.828-0.974	0.038	0.093	0.371
FE_{IP}	0.757	0.607-0.907	0.078	0.004	0.018
FE_{UA}	0.762	0.653-0.872	0.057	<0.001	<0.001

Fig. 3 The diagnostic accuracy of five tubular markers for tenofovir-induced tubulopathy. **a** Receiver operating characteristic (ROC) curves and areas under the curve (AUC) for five tubular markers. **b** The differences between the largest AUC (β2M) and each of the other AUCs were tested using a nonparametric method. *p* value†, value adjusted with Bonferroni correction

Discussion

To our knowledge, this is the first study to compare various kidney tubular markers for screening tenofovir-induced KTD in patients with HIV-1 infection. Both urinary β2M and α1M were identified as good screening markers with high diagnostic accuracy among the five tubular markers examined in this study. With a cutoff value of 1,123 μg/g Cr for β2M (sensitivity 100 %, specificity 89 %) and 15.4 mg/g Cr for α1M (sensitivity 100 %, specificity 87 %), these two markers are potentially suited for screening tenofovir-induced KTD. Although these low molecular weight proteins offered good screening ability, both FE_{IP} and FE_{UA}, which are traditional tubular function markers often used for the diagnosis of Fanconi syndrome, were not useful for screening KTD. NAG, a lysosomal enzyme of proximal tubular epithelial cells, had good diagnostic accuracy with a cutoff value of 5.96 U/g Cr (sensitivity 90 %, specificity 86 %). However, with cutoff of 3.58 U/g Cr, which yields 100 % sensitivity and maximal specificity, NAG had relatively low specificity of 46 %, and thus has a high false-positive rate.

TDF is one of the most important and widely used agents in the treatment of HIV-1 infection, as well as hepatitis B infection [4]. Fixed-dose tenofovir/emtricitabine is the only preferred NRTI in the American Department of Health and Human Services (DHHS) Guidelines and the revised British HIV Association Guidelines [33, 34]. TDF is also increasingly used in resource-limited settings, following the revised 2010 WHO guidelines that recommend TDF as one of the components of first-line therapies [35]. Although tenofovir nephrotoxicity is considered to be mild and tolerable [9], its long-term consequences are unknown. Thus, it is important to have a useful screening tool for tenofovir-induced nephrotoxicity.

Table 2 Cutoff values of five kidney tubular markers (calculated with 100 % sensitivity and maximal specificity) and two conventional methods

	Cutoff with 100 % sensitivity			Method 1			Method 2		
	Cutoff	Sensitivity (%)	Specificity (%)	Cutoff	Sensitivity (%)	Specificity (%)	Cutoff	Sensitivity (%)	Specificity (%)
β2M (μg/g Cr)	1,123	100	89	1,612	95	93	1,123	100	89
α1M (mg/g Cr)	15.4	100	87	16.5	95	90	15.4	100	87
NAG (U/g Cr)	3.58	100	46	5.96	90	86	5.96	90	86
FE_{IP} (%)	1.02	100	0	12.4	68	82	14.4	63	94
FE_{UA} (%)	3.92	100	12	8.1	79	70	8.1	79	70

β2M urinary β2-microglobulin, *α1M* urinary α1-microglobulin, *NAG* N-acetyl-β-D-glucosaminidase in urine, FE_{IP} fractional excretion of phosphate, FE_{UA} fractional excretion of uric acid

Previous studies identified old age, low body weight, preexisting renal impairment, concomitant use of nephrotoxic medications, concomitant use of ritonavir-boosted protease inhibitors, advanced HIV infection (low CD4 counts, AIDS), and other comorbidities (diabetes mellitus, hypertension, hepatitis C co-infection) as risk factors for tenofovir-induced reduction in renal function [14, 27–30]. The DHHS Guidelines recommend monitoring eGFR, urinalysis, and electrolytes in patients on TDF [33]. We suggest monitoring either urinary β2M or α1M in addition to the variables recommended by the DHHS guidelines every 6 months in patients under tenofovir use, especially in those with the aforementioned risk factors in resource-rich settings.

One of the strengths of the present study is the exclusion of factors that could otherwise predispose to KTD other than tenofovir, such as active infection, diabetes mellitus, preexisting renal impairment, and HIV-1 viremia, to make prevalence of KTD lower than that in the real-world settings [20]. The cutoff values of tubular markers for screening tenofovir-induced KTD with 100 % sensitivity were calculated in this setting. Thus, in applying these cutoff values in clinical practice with high prevalence rates of KTD, the false-positive rate will be lower than the one reported in the present study, making these cutoff values even more useful.

Another strength of the study is that the enrolled patients were on the same antiretroviral regimen (once-daily ritonavir-boosted darunavir plus fixed-dose tenofovir/emtricitabine). This practice helped proper evaluation of the diagnostic accuracy of the five tubular markers, because plasma concentrations of tenofovir and severity of tenofovir-induced KTD are influenced by concomitant use of antiretrovirals, and the delta change in plasma tenofovir concentration likely differs in the presence of each concomitant drug [36]. Thus, by enrolling patients on the same antiretroviral combination, this study excluded an important confounding factor for tenofovir-induced KTD.

Both β2M and α1M are low molecular weight proteins (<40 kDa) used as markers of kidney tubular function. β2M and the free unbound form of α1M are freely filtered by the glomerulus and reabsorbed almost completely in proximal tubular cells [37]. Serum β2M has been used as a surrogate marker of inflammation, because it is expressed on the surface of most nucleated cells, as part of class I major histocompatibility complex. On the other hand, α1M is mainly produced in the liver, and although its function is not fully understood, it has antioxidant properties and acts as a radical scavenger [38]. Of note, technical difficulty has been reported in the measurement of both markers; for β2M, acidic urine with pH <6.0 causes time- and temperature-dependent degradation of β2M [39]. Urinary α1M is more stable than β2M when stored in acidic urine; however, diurnal variation and gender differences have been reported [40–42].

There are several limitations in this study. First, there is no gold standard definition for KTD. The collection of abnormal tubular markers was used as a reference standard in this study, following their use in previous studies for the diagnosis of KTD [17, 43]. However, the criteria used for the diagnosis of KTD in each previous study included haphazard combination of tubular markers [16, 44]. Accordingly, this study selected five markers (β2M, α1M, NAG, FE_{IP}, and FE_{UA}) after taking into consideration their availability and cost. Thus, our study did not investigate other tubular markers, such as γ-glutamyl transpeptidase, retinol binding protein, and neutrophil gelatinase-associated lipocalin. Second, although this study evaluated the diagnostic values for five variables (β2M, α1M, NAG, FE_{IP}, and FE_{UA}), these variables were not fully independent from KTD, the reference standard, because KTD was defined as the collection of abnormal tubular markers [17, 43]. Third, the study subjects were mostly men, and thus the results of this study are not necessarily applicable to women, especially considering that gender variation should be taken into account in evaluation of α1M [40].

In conclusion, the present study identified urinary β2M and α1M as highly useful screening markers for tenofovir-induced KTD, in a setting designed to exclusively evaluate tenofovir-induced KTD. In the assessment of renal function

in patients under tenofovir therapy, regular monitoring of either urinary β2M or α1M, in addition to urinalysis, serum creatinine, and electrolytes, should be helpful in the diagnosis of early-stage tenofovir-induced KTD. Screening for tenofovir-induced KTD is especially important in patients with several risk factors for KTD, because undetected long-term tubular dysfunction might lead to premature osteopenia caused by phosphate wasting, and accelerated progression of renal dysfunction, both of which could result in a serious outcome.

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INTERNAL MEDICINE

□ CASE REPORT □

Idiopathic Oropharyngeal and Esophageal Ulcers Related to HIV Infection Successfully Treated with Antiretroviral Therapy Alone

Yohei Hamada[1], Naoyoshi Nagata[2], Haruhito Honda[1], Katsuji Teruya[1], Hiroyuki Gatanaga[1], Yoshimi Kikuchi[1] and Shinichi Oka[1]

Abstract

We herein report the case of an HIV-positive man who was diagnosed with idiopathic esophageal and oropharyngeal ulceration. The esophageal and oropharyngeal ulcers were considered to be idiopathic and related to HIV infection after excluding the possibility of infection with known pathogens. Both the esophageal and oropharyngeal ulcers showed significant improvements following antiretroviral therapy alone. Idiopathic esophageal ulcers are a well-known complication of late-stage HIV infection. However, involvement of both the esophagus and pharynx is rare. Furthermore, antiretroviral therapy without concomitant steroids is effective against idiopathic esophageal and oropharyngeal ulcers related to HIV infection.

Key words: HIV infection, idiopathic esophageal ulcer, pharyngeal ulcer, antiretroviral therapy, gastrointestinal diseases

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Introduction

Esophageal ulceration is a common complication in patients with human immunodeficiency virus-1 (HIV) infection, especially in the late stage. Although esophageal ulcerations can be caused by various infectious agents, such as *Candida* species, cytomegalovirus (CMV) and herpes simplex virus (HSV), a large proportion of patients are diagnosed with idiopathic esophageal ulcerations (1, 2) with no detectable etiology. Oropharyngeal ulcers are also an important comorbidity that can become progressive in HIV-infected patients (3, 4). The common infectious agents of esophageal ulcerations are known to also cause oropharyngeal ulcerations, although some cases are considered idiopathic with no identifiable etiology (5, 6). However, simultaneous involvement of the esophagus and oropharynx is uncommon outside of HSV esophagitis (5). We herein report a case of unusual discrete ulcers of the oropharynx and esophagus in a patient with HIV infection that showed a rapid improvement following treatment with antiretroviral therapy alone.

Case Report

A previously healthy 60-year-old Japanese homosexual man presented with severe odynophagia. He was diagnosed with oral candidiasis and HIV infection and therefore had been referred to our hospital (day-1). Laboratory tests showed a low CD4+ cell count (49/μL), a high HIV-RNA titer (1.0×10^6 copies/mL) and a low serum albumin level (Alb 2.9 g/dL). Whole-blood polymerase chain reaction (PCR) was negative for both CMV and HSV. The patient was treated with fluconazole for seven days for suspected esophageal candidiasis. Despite this treatment, the odynophagia did not improve. Since oral ulcers were noticed, treatment with oral valaciclovir at a dose of 1,000 mg/day was initiated based on a presumptive diagnosis of HSV infection. However, the odynophagia persisted, and the oral ulcers did not show any improvement despite a 3-week

[1]AIDS Clinical Center, National Center for Global Health and Medicine, Japan and [2]Department of Gastroenterology, National Center for Global Health and Medicine, Japan
Received for publication July 30, 2012; Accepted for publication October 30, 2012
Correspondence to Dr. Naoyoshi Nagata, nnagata_ncgm@yahoo.co.jp

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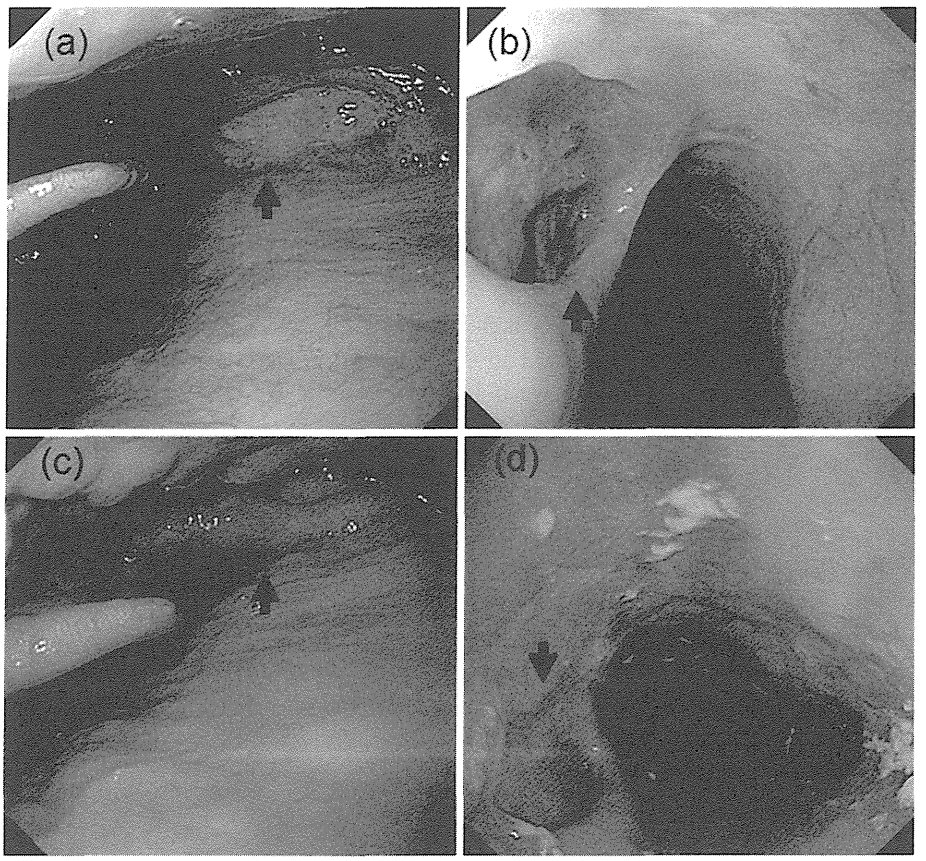

Figure. Endoscopic findings of the pharynx and esophagus. The pharyngeal (a) and esophageal (b) ulcers before the administration of antiretroviral therapy. The endoscopic appearance of the pharynx (c) and esophagus (d) on day 22 of antiretroviral therapy. Black arrows: ulcers.

course of anti-HSV therapy; thus, upper gastrointestinal endoscopy was performed. Endoscopy showed large, discrete and well-circumscribed esophageal and pharyngeal ulcers (Figure a, b). Because a diagnosis of CMV esophagitis was suspected based on the endoscopic appearance of the ulcers, treatment with intravenous gancicrovir at a dose of 5 mg/kg every 12 hours was initiated and the valaciclovir was discontinued. However, a histopathological examination of the biopsy specimen obtained from the base and edge of an ulcer before the initiation of gancicrovir therapy revealed lymphocytic infiltration without intranuclear or intracytoplasmic inclusion bodies. Immunohistochemical staining for CMV and HSV was negative. PCR assays of both pharyngeal and esophageal biopsies were negative for CMV-DNA and HSV-DNA (≤40 copies/μg DNA). Furthermore, repeat endoscopy performed after two weeks of gancicrovir therapy showed exacerbation of the ulcers. Based on these findings, we administered antiretroviral therapy consisting of ritonavir-boosted darunavir with abacavir/lamivudine. The gancicrovir therapy was discontinued after the completion of a 3-week course of treatment. The odynophagia gradually improved and ultimately disappeared two weeks later, while the CD4 count increased to 91/μL and the HIV-RNA titer decreased to 4×10^4 copies/mL. Endoscopy performed on day 22 of antiretroviral therapy demonstrated significant reductions in the size and depth of the pharyngeal and esophageal ulcers (Figure c, d). Additionally, resolution of the oral ulcers was noticed.

Discussion

To our knowledge, this is the first report of idiopathic esophageal and oropharyngeal ulcers successfully treated with antiretroviral therapy alone in a patient with late-stage HIV infection. Steroids are commonly used as the standard treatment for idiopathic esophageal ulcers (2, 7). However, steroids can lead to serious opportunistic infections due to their immunosuppressive effects. The efficacy of steroids is mostly based on reports from the pre-highly active antiretroviral therapy era, and the efficacy of antiretroviral therapy has not been examined. As described above, steroid therapy may not be necessary when a potent combination of antiretroviral therapy is administered. The etiology of idiopathic esophageal ulcers is still not fully understood. Although such ulcers are considered to be associated with HIV infection, they have been referred to as idiopathic when no identifiable etiologic agent other than HIV infection is present (8, 9). The potential pathogenesis of these ulcers includes apoptosis of the esophageal mucosa induced by HIV infection (10). Based on this probable pathogenesis, it is therefore considered to be rational to administer antiretroviral therapy to treat idiopathic esophageal ulcers.

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The diagnosis of idiopathic oropharyngeal and esophageal ulcers is established by excluding other infectious agents known to cause esophageal ulceration, including CMV, HSV and *Candida* sp, by performing histopathological and immunological examinations of biopsy specimens (1, 2, 5, 6). In our case, the histopathological findings showed no evidence of any infectious pathogens, and CMV and HSV infection were also excluded by PCR assays, which have a high sensitivity (11, 12). Furthermore, the oropharyngeal and esophageal ulcers were refractory to anti-CMV and anti-HSV therapy. In addition, the ulcers showed significant improvement following the administration of antiretroviral therapy alone. Therefore, the final diagnosis was idiopathic oropharyngeal and esophageal ulcers related to HIV infection.

Involvement of both the oropharynx and esophagus in HSV-related ulcers is not uncommon (5). However, in our patient, the esophageal and oropharyngeal ulcers were considered idiopathic, which is extremely rare. In this case, the ulcers in both regions were examined endoscopically. Therefore, performing careful endoscopic examinations of not only the esophagus, but also the pharynx, is considered to be important for establishing the cause of odynophagia in HIV-infected patients.

In conclusion, a pharyngeal and esophageal biopsy obtained using upper gastrointestinal endoscopy was useful for establishing the diagnosis in this case. Furthermore, antiretroviral therapy alone resulted in a significant improvement of the idiopathic ulcers in our HIV-infected patient. The initiation of antiretroviral therapy without steroids is therefore a reasonable option for treating idiopathic oropharyngeal and esophageal ulcers in HIV-infected patients.

The authors state that they have no Conflict of Interest (COI).

Acknowledgement

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PLOS | ONE

Highly-Sensitive Allele-Specific PCR Testing Identifies a Greater Prevalence of Transmitted HIV Drug Resistance in Japan

Masako Nishizawa, Junko Hattori, Teiichiro Shiino, Tetsuro Matano, Walid Heneine, Jeffrey A. Johnson, Wataru Sugiura

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Abstract

Background

The transmission of drug-resistant HIV in newly identified infected populations has become an underlying epidemic which can be better assessed with sensitive resistance testing. Since minority drug resistant variants cannot be detected by bulk sequencing, methods with improved sensitivity are required. Thus, the goal of this study was to evaluate if transmitted drug resistance mutations at minority levels in Japanese patients could be identified using highly sensitive allele-specific PCR (AS-PCR).

Materials and Methods

Samples were taken from newly diagnosed HIV/AIDS cases at the National Nagoya Hospital from January 2008 to December 2009. All samples were bulk sequenced for HIV protease and reverse transcriptase. To detect minority populations with drug resistance, we used AS-PCR with mutation-specific primers designed for seven reverse transcriptase inhibitor resistance mutations, M41L, K65R, K70R, K103N, Y181C, M184V, and T215F/Y, and for three protease inhibitor resistance mutations, M46I/L and L90M.

Results

We studied 149 newly identified HIV cases. Bulk sequencing detected 8 cases with NRTI resistance mutations (one with A62V, one D67E, one T215D, one T215E, two with T215L and two T215S) and 15 with PI resistance mutations (one with N88D and 14 with M46I). Results obtained by AS-PCR and bulk sequencing demonstrated good concordance but the AS-PCR enabled the detection of seven additional drug-resistant cases (one M41L, two with K65R, two with K70R, and one M184V) in the RT region. Additionally, AS-PCR assays identified 15 additional cases with M46I, five with M46L and four cases with L90M in the protease region.

Conclusions

Using AS-PCR substantially increased the detection of transmitted drug resistance in this population from 15.4% to 26.8%, further supporting the benefit of sensitive testing among drug-naïve populations. Since the clinical impact of minority drug-resistant populations is not fully comprehended for all mutations, follow-up studies are needed to understand their significance for treatment.

Figures

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Introduction

The use of combination antiretroviral therapy of (cART) has resulted in sustained reductions in morbidity and mortality from HIV infection [1,2]. Five classes of antiretrovirals (ARVs) are currently available in clinical use in Japan. However, selection of drug resistance mutations during cART is still a major issue affecting the clinical efficacy of ARVs and prognosis of HIV infected individuals [3,4].

A United States Department of Health and Human Services (DHHS) guideline recommends drug resistance testing for patients before they begin cART to guide their therapy[5]. Conventional bulk sequencing is used to detect drug resistance mutations in viral RNA from patient plasma, but the method generally does not detect mutants that comprise less than 20% of the viral population in individuals [4-7]. This detection limitation is a concern, both because transmitted minority variants might persist at low frequencies and most newly diagnosed HIV infections are in persons who have been infected for several months to years, providing time for drug resistant viruses with reduced viral fitness to decay to levels that conventional testing is not able to detect [8-10]. Therefore, the ability to detect low-frequency variants below 20% would improve identification of infections involving drug-resistant viruses and better inform decisions on the selection of active ARVs, especially for persons initiating treatment with NNRTI regimens. To detect low-frequency variants, several methods were developed and used to analyze drug-naïve persons and drug-experienced persons [11-14]. Several studies have shown the advantages of highly sensitive drug resistance assays with women who received intrapartum single-dose nevirapine (SD-NVP) for the prevention of mother-to-child HIV transmission. These reports on testing for NVP resistance have found that drug resistance emerges more frequently and persists for longer than previously demonstrated by bulk sequencing. Persisting minority NVP-resistant viruses may result in poor

emerges more frequently and persists for longer than previously demonstrated by bulk-sequencing. Persisting minority NVP-resistant viruses may result in poor virologic responses when subsequent regimens contain nevirapine-related drugs [15-19].. We previously reported that highly-sensitive drug resistance testing that is based on allele-specific real-time PCR can detect minority drug-resistant variants both in infections reported to be wildtype and infections involving other resistance mutations as determined by bulk sequencing. As with majority-level resistance, the amount of low-frequency resistance in new infections reflects both the prevalence of cART use in the region and behavior that is inconsistent with prevention practices for persons on therapy [20].

Recently, it has been reported that the prevalence of drug-resistant HIV transmission among newly diagnosed patients analyzed by bulk sequencing is increasing in Japan, rising from 5.9% in 2003 to 8.3% in 2008 [21]. As the study concluded, this observation was seen not only for recently infected persons, but also chronically infected but recently diagnosed cases, raising concern over the amount of resistance detection lost due to reversion. Therefore, by use of a highly sensitive method in the current study we attempted to examine for the possibility and prevalence of transmitted drug resistant mutations hidden as minority populations.

Materials and Methods

Ethics statement

Specimens were anonymous residual diagnostic material from subjects who provided written consent for HIV testing. The Ethical Committee for Biomedical Science of the National Institute of Infectious Diseases determined that this testing did not involve identifiable human subjects and has approved the study.

Samples

The 192 plasma samples were collected from HIV/AIDS cases for drug resistance analysis from January 2008 to December 2009 in National Nagoya Hospital (Table 1). Among these, 149 cases of newly diagnosed HIV-1 subtype B-infected ART-naïve individuals were selected and analyzed in this study (Figure 1). All samples were collected as part of HIV surveillance studies under Institutional Review Board of National Institute of Infectious Diseases, and written informed consent was obtained from each patient. These samples were directly sequenced for HIV protease (PR) positions 1-99 and reverse transcriptase (RT) positions 1-240. Drug resistance mutations were defined according to the mutation list proposed by Bennett et al. 2009[22]. All testing was performed by the NIID AIDS Research Center in Tokyo, Japan[21].

		2008	2009	Total
Total		75	74	149
Gender	male	74	73	147
	female	1	1	2
	unknown	0	0	
Age	median(Q1, Q3)	39	38	39
Risk behavior	MSM	52	49	101
	Sexual	9	11	20
	MSM/Sexual	8	13	21
	Hemophiliac	1	0	1
	Unknown	5	1	6
VL	Median	9.70E+04	7.00E+04	7.90.E+04
	mode	1.10E+05	2.70E+04	1.10.E+05
CD4	average	199.7	225.4	212.0

Table 1. Demographics of samples.

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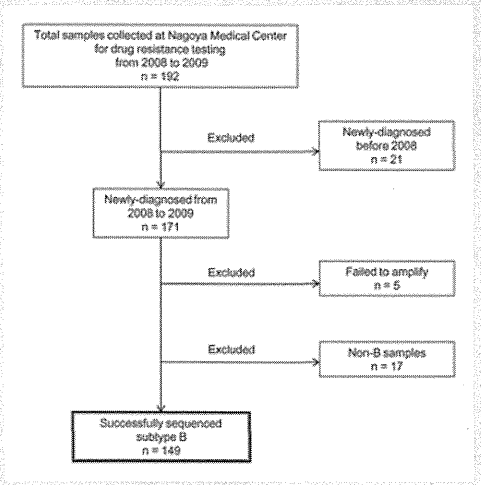

Figure 1. Flow Diagram of sample selection.
Flow diagram of sample selection for analysis of minority drug resistance mutations in the HIV-infected patient samples newly diagnosed at National Nagoya Hospital from 2008 to 2009.
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RNA extraction and virus template amplification

HIV RNA was extracted by Roche High Pure Viral RNA Kit from 200uL plasma samples. RNA was reconstituted in 100 uL of DEPC water and stored at -80°C until use. The HIV protease-reverse transcriptase (PR-RT) region was amplified by one-step RT-PCR (TAKARA One Step RNA PCR kit) with forward primer (DRPRO5 :

AGA CAG GYT AAT TTT TTA GGG A) and reverse primer (DRRT34 : GCT ATT AAG TCT TTT GAT GGG TCA TA). RT-PCR amplification conditions were 55°C for 40 minutes and 40 cycles of 95°C for 10 seconds, 52°C for 5 seconds, and 72°C for 90 seconds. In the case that the amplification of RT- PCR did not generate sufficient template, nested-PCR was performed using forward primer (PROFWD1F : CAG ATC ACT CTT TGG CAA CGA CC) and reverse primer (GEN4R : ATC CCT GGG TAA ATC TGA CTT GC)[23]. Nested-PCR amplification condition was 94°C 1 minute and 30 Cycles of 94°C for 10 seconds, 55°C for 4 seconds and 74°C for 15 seconds.

Real-time PCR (AS-PCR)

To detect minority populations with drug resistance, we used highly sensitive allele-specific PCR validated for subtype B HIV as described [17,23]. Briefly, mutation-specific primers were designed for seven reverse transcriptase inhibitor resistance mutations, M41L, K65R, K70R, K103N, Y181C, M184V, and T215F/Y. Results of highly sensitive allele-specific PCR and population sequencing data were compared for concordance and presence of additional mutations. The HIV-1 total copy primers, Com2F and Com4BR, span n.t. 258–420 in RT and were used with the common probes, Com1P and 2P (Table S1)[17,23]. For multiple mutation screening, several resistance mutation-specific reactions can be performed simultaneously. The cycle number at which the fluorescence emission exceeds the background fluorescence threshold is the threshold cycle (CT) and is the unit of measure for comparing the differences in amplification signals (ΔCT) between the total copy and mutation-specific reactions. All samples were tested in duplicate with the means of the total copy and mutation-specific CTs used for the determination of the ΔCT. Each ΔCT cutoff value for interpreting the presence of drug resistance mutations was determined previously [23] and were between 8.5 from 10.5 cycles, for validated assay cut-offs ranging from 0.03% to 2.0% mutant, depending on the assay.

Real-time PCRs were initiated with a hot-start incubation at 94°C for 11 minutes before proceeding to 45 cycles of melting at 94°C for 30 seconds, annealing at 50°C for 15 seconds and extension at 60°C for 30 seconds. All reactions were performed in a total volume of 50 uL/well in 96-well PCR plates using iQ5 real-time PCR thermocyclers with optical units (Bio-Rad) and AmpliTaq Gold polymerase (2.5 U/reaction; Applied Biosystems). Final reagent concentrations were 320 nM for the forward and reverse primers, 160 nM probe(s), and 400 mM dNTPs.

M46I/L primers for real-time-PCR

For this study, new primers for the detection of M46I and M46L protease inhibitor mutations were constructed as described before [17,23,24]. As with the RT primers, the protease mutation-specific primers (Table S2) were designed to preferentially anneal with the targeted mutation nucleotide(s), thus having reduced affinity for wild-type sequences. Specificity was enhanced by creating designed mismatches at the -2 nucleotide position relative to the primer 3'-end for each primer. Furthermore, to compensate for the spectrum of polymorphisms present, mixtures of three uniquely designed forward primers were required to detect M46L. Mutation-specific primer mixtures were experimentally evaluated and the ratios that best balanced differences in primer avidities and minimized cross-interference in primer annealing were selected.

Site-directed mutagenesis and cloning

M46I and M46L mutant clones for plasmid development were constructed by site-directed mutagenesis using HXB2 as a template. These constructs were used as positive control to verify the M46I/L primers and probes. To insert M46I or M46L mutations into HXB2, PCR with KODplus was performed using a pair of complementary primers (M46I-forward primer : GAA GAT GGA AAC CAA AAA TaA TAG GGG GAA TTc GAG G, M46L(ttg)-forward primer : GAA GAT GGA AAC CAA AAt TGA TAG GGG GAA TTG GAG G , M46L(ctg)-forward primer : GAA GAT GGA AAC CAA AAc TGA TAG GGG GAA TTG GAG G), and reverse primer : CTG GCA AAC TCA TTT CTT CTA ATA CTG TAT CAT CTG CTC C). PCR amplification conditions were 94°C for 2 minutes and 35 cycles of 98°C for 10 seconds, 68°C for 2 minutes and 30 seconds.

Evaluation of the new protease assays on plasmids and clinical samples

HXB2-M46I, HXB2-M46L and HXB2 (wild-type) plasmids were used in the preliminary selection of primer mixtures that provided the greatest sensitivity and specificity. The absolute mutation detection limits for the primer mixtures, that is, the greatest ΔCT that was able to distinguish mutant viruses from wild-type, were estimated from triplicate testing of mutant clone serial dilutions. The assays evaluated mutation-containing sequences at frequencies between 100%-0.0001% in a wild-type background, with each dilution having same total plasmid copies. The ΔCTs generated from the mutant dilutions were compared to the ΔCTs generated with the wild-type plasmids alone. Solely for the purpose of comparing relative assay detection limits with finite virus sequences, the ΔCT within the linear dilution range (R^2>0.995) that was equivalent to a frequency increase of 0.5 $\log_{10}$ above the wild-type mean ΔCT was chosen as the absolute assay detection limit. Selecting the detection limit in this manner provided an adequate buffer against variability in wild-type sequences and also took into account the PCR efficiency of the assay (slope of the dilution curve).

To evaluate cutoff values of M46I and M46L in patient samples, PR-RT sequences derived from ART-naïve patients were analyzed by real-time PCR. Forty-two PR-RT region sequences derived from 16 patients were cloned by TA-cloning to serve as heterogeneous wild-type sequences. Fifty-five samples with protease M46I were obtained from 20 individuals and 22 samples with M46L were obtained from 12 individuals. To increase the stringency of assay evaluations, specimens with substantial numbers of polymorphisms in primer binding sites were also included.

Assessing mutation associations in mutation-specific amplicons

To evaluate whether additional information on resistance mutations could be gained from the real-time PCR assays, we performed bulk sequencing (BigDye reagent, Prism 3130xl analyzer, Applied Biosystems) of the products from M46I/L or L90M-specific reactions to assess mutation linkage. Mutation-specific amplicon sequences were compared to their respective sample bulk sequence for evidence of nucleotide differences. Any other resistance mutation(s) found in the mutation-specific amplicons would indicate that they were on the same viral strand(s) as the mutation that was specifically targeted by the primers.

Phylogenetic analysis

Protease sequences were aligned by means of the clustal-W program with a set of reference sequences recommended by the Los Alamos sequence database (http://www.hiv.lanl.gov/content/index). The results of the alignment were then analyzed by the neighbor-joining method using MEGA5 [24,25]. In order to analyze the relationship between M46I/L-positive amplicon sequences and bulk sequences, we extended the M46I/L-positive amplicon by using the PRO2L reverse primer (Table S1B), which allowed sequencing from PR codon 47 to RT codon 36 of these M46I/L-positive amplicons (270 bp). In the case of L90M amplicon analysis, 209 bp DNA fragments extending from amino acid 20 in PR to amino acid 89 in PR were represented in the phylogenetic tree. The phylogenetic relatedness of these mutation-containing amplicons excluding the resistance codon position were represented in trees constructed using Kimura 2-parameter model with a discrete gamma distribution [1 +G] and 500 bootstrap replications in MEGA5.

Statistics

The Mann-Whitney U test was used to test for differences in CD4 counts and VL between the groups with minority drug resistance mutations and those without minority drug resistance mutations.

Results

Minority M46I could be detected as low as 0.04% and M46L could be detected as low as 0.03% in site-directed mutant clones

Relative limits of detection were compared in a simple laboratory setting using serial dilutions of HXB2-M46I or HXB2-M46L in backgrounds of HXB2 wildtype plasmid. The ΔCT that was equivalent to a 0.5 log greater reactivity than the wild-type mean ΔCT on the dilution curve (M46I : ΔCT=15 cycles, M46L : ΔCT=17 cycles) was used to compare assay sensitivities (Figure 2). This approach yielded detection limits of 0.04% and 0.03% for M46I and M46L, respectively. As this was derived from cloned sequences this is a theoretical detection limit against which clinical specimens are evaluated.

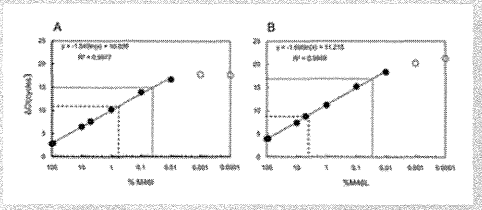

Figure 2. Mutation-specific assay reactivity on plasmids.
Cloned M46I (A.) and M46L (B.) mutant virus template was diluted 10-fold, from 100% to 0.0001%, in backgrounds of wild-type sequence to determine assay detection limits. Plotted are the mean ΔCT versus $\log_{10}$ of the mutant dilution series. The lower detection limit (lower dotted line) was placed at the ΔCT equivalent to 0.5 $\log_{10}$ below (0.5-log greater reactivity than) the wild-type ΔCT. For comparison, the mutant virus frequency equivalences for the established clinical cutoffs are also shown (dashed line).
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High sensitivity and specificity of M46I/L detection assays confirmed with clinical samples

Assay cutoff values intended for population-wide clinical screening were established using 42 cloned wild-type sequences derived from 14 patient-derived specimens collected by NIID from 2005-2007. The assay cutoffs selected based on plasmid sequences were evaluated against clinical specimens using a total of 55 samples with sequence-detectable M46I mutation and a total 22 samples with sequence-detectable M46L mutation. The resulting distribution of collated ΔCTs from the wild-type samples supported a ΔCT cutoff of 11 cycles for M46I clinical testing (ΔCTs ranged from 16.39–26.65 cycles) (Figure 3 and Table S3). Extrapolating from the dilution curve for cloned M46I sequences, this cycle difference corresponded to a frequency mean of 0.54% mutant virus (see Table S3). At this cutoff, all 55 genotyped M46I samples were positive (ΔCTs ranged from 1.39 to 10.1 cycles, Figure 3 and Table S3). For M46L assay, ΔCT cutoff was 9 cycles to avoid low-level amplification from spurious primer binding against clinical quasispecies specimens; this cutoff placement corresponded to a frequency mean of 4.01% mutant virus. All genotype M46L samples were positive (ΔCT ranged from 0.88 to 8.95 cycles) (Figure 3 and Table S3). Because of unusual polymorphisms, some samples comprised almost entirely of mutant virus produced ΔCTs near the cutoff. In these situations, elevated ΔCTs resulting from weak primer binding could be interpreted as mutant viruses present at low frequencies. Hence, this testing format is best-suited to provide highly specific population-level resistance screening and is not necessarily applicable to mutant virus quantitation.

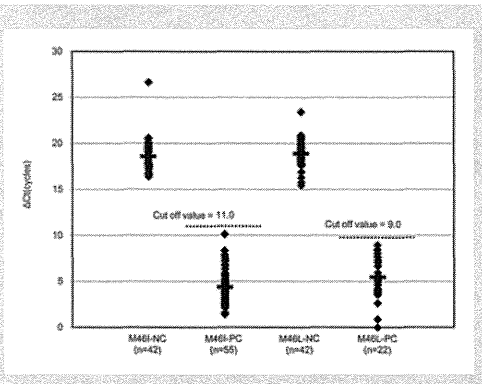

Figure 3. Assay reactivity with wild-type and M46I/L mutation clinical samples.
Dotted ΔCT values from clinical samples with sequence-detectable mutations and with wild-type sequences are shown. The range of reactivity for each assay is shown for wild-type and mutant samples. The mean of ΔCT (bar) for each group is indicated. Assay cutoffs (dotted horizontal line) were established to exclude all wild-type viruses from detection. PC; Positive clones with M46I/L, NC; Negative clone with no M46I/L(wild type).
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AS-PCR method identifies a greater prevalence of transmitted HIV drug resistance

Samples from a total of 149 subtype B cases were collected at Nagoya Medical Center for drug resistance testing. Drug resistance mutations were initially analyzed by bulk sequencing, and 23 cases were found to possess drug resistance mutations. As summarized in Table 2, all resistant mutations were found as sole mutation, one with A62V, one with D67E and six cases of intermediates at codon 215 (one with T215D, one with T215E, two cases of T215L and two cases of T215S), one with N88D and 14 cases of M46I mutation were detected by conventional bulk sequencing analysis, yielding a drug resistance mutation prevalence of 15.4% (23/149 cases) (Table 2). The sensitive screening detected an additional one case of M41L (0.67%), two cases of K65R (1.34%), two cases of K70R (1.34%), one case of M184V (0.67%), 15 cases of M46I (19.46%), 5 cases of M46L (3.36%) and 4 cases of L90M (2.68%) as minority-level drug resistance mutations (Table 2). The identified A62V, D67E, T215E, T215S and N88D mutations detected by bulk sequencing were not targeted by AS-PCR, and therefore were not included in determining changes in mutation frequency. All 17 mutations detected by bulk sequencing analysis that were also targeted by AS-PCR were likewise detected by the sensitive PCR method. The combined prevalence of drug resistance mutations in the total of 149 cases was 26.8% (40/149 cases) (Table 2). In one case, six mutations, M41L, K70R, M184V, M46I, M46L and L90M were detected as minority mutations by the highly sensitive assays (ID 29). These six mutations were undetectable by bulk sequencing analysis. In other cases, K70R and M46I were detected (ID 22), and M46I and M46L were detected in another case as minority drug resistance mutations (ID 5). Of those with minority drug resistance mutations, 11 cases were from 2008 and 12 cases were from 2009. The majority of patients with minority variants were MSM (90.9% in 2008 and 83.3% in 2009) and Japanese, and no significant differences were observed in viral load and CD4 counts by Mann-Whitney U test (p=0.17

and p=0.308, respectively) for persons with or without mutations. Though all of the samples from 2008 were Japanese patients, three cases from 2009 were non-Japanese patients (Table 2).

							Bulk-seq		AS-PCR	
ID	Gender	Risk behavior	Year	Nationality	VL	CD4	RT mutations	PR mutations	RT mutations	PR mutations
1	M	MSM	2008	Japan	2.0.E+04	402	A62A/V			
2	M	Heterosexual	2008	Japan	1.2.E+06	14		N88D/N		
3	M	Heterosexual	2008	Japan	3.1.E+05	38	T215L		T215F*	
4	M	MSM/Heterosexual	2008	Japan	5.8.E+05	222	D67D/E			M46I
5	M	MSM	2008	Japan	2.6.E+04	481				M46I, M46L
6	M	MSM	2008	Japan	1.7.E+06	10				M46I
7	M	MSM/Heterosexual	2008	Japan	4.1.E+05	39	T215S			
8	M	MSM	2008	Japan	2.2.E+05	14		M46I		M46I
9	M	MSM	2008	Japan	1.5.E+04	356				L90M
10	M	MSM/Heterosexual	2008	Japan	1.2.E+05	28	T215S			
11	M	MSM/Heterosexual	2008	Japan	1.3.E+05	348		M46I		M46I
12	M	MSM	2008	Japan	2.1.E+04	391			K65R	
13	M	MSM	2008	Japan	2.1.E+05	553		M46I		M46I, L90M
14	M	Heterosexual	2008	Japan	6.7.E+04	153		M46I		M46I
15	M	MSM	2008	Japan	2.2.E+05	10		M46I		M46I, M46L
16	M	MSM	2008	Japan	1.2.E+03	45	T215L		T215F*	M46I
17	M	MSM	2008	Japan	2.6.E+04	750		M46I		M46I
18	M	MSM	2008	Japan	1.1.E+05	146		M46I		M46I
19	M	MSM	2008	Japan	8.4.E+04	11				M46I
20	M	MSM	2008	Japan	1.4.E+05	86		M46I		M46I
21	M	MSM	2008	Japan	1.1.E+05	1050				M46I
22	M	MSM	2008	Japan	2.2.E+04	154			K70R	M46I
23	M	MSM	2009	Japan	7.2.E+03	319				M46L
24	M	MSM	2009	Japan	2.9.E+04	185				M46I

Table 2. Characteristics of HIV/AIDS patients with drug resistance mutations.

* T215F detection primers can detect T215L, T215I and T215V. **T215Y detection primers can detect T215D, T215H and T215N.

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Sequence analysis of M46I and M46L-specific amplicons showed that these mutations were not linked to L90M in the patient samples

To analyze the linkage between drug resistance mutations, we directly sequenced positive M46I/L or L90M-specific PCR products to ascertain whether additional genotypic information could be obtained from those amplicons. In ID 29, the I72V polymorphism observed in the bulk sequence was detected in M46L and L90M amplicons, but not in the M46I amplicon (Table 3). Additionally, M46I/L mutations were not detected in the minority L90M amplicon indicating these mutations were not linked. In ID 27, A71T was detected in the M46L amplicon, but this mutation was not found in the M46I amplicon or the bulk sequence (Table 3). In ID 22, the M46I amplicon matched the bulk sequence.

Sample	Sequences	Mutations
ID 29	Direct-sequencing	I62V, L63P, *I72V*, T74A, V77I, I93L
	M46I amplicon*	**M46I**, I62V, L63P, T74A, V77I
	M46L amplicon*	**M46L**, I62V, L63P, *I72V*, T74A, V77I
	L90M amplicon**	I62V, L63P, *I72V*, T74A, V77I, **L90M**
ID 27	Direct-sequencing	**M46I**, E21R, R41K, I62V, L63P, L89I, Q92K, I93L
	M46I amplicon*	**M46I**, I62V, L63P
	M46L amplicon*	**M46L**, I62V, L63P, *A71T*
ID 22	Direct-sequencing	E35D, M36I, L63P, H69K, V77I
	M46I amplicon*	**M46I**, L63P, H69K, V77I

Table 3. Genetic linkage of M46I/L or L90M and other mutations.

* M46I and M46L amplicons were spanned from M46 to N88.
** L90M amplicon was spanned from I15 to L90.

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Phylogenetic relatedness of minority and bulk sequence-detectable resistance mutations

Phylogenetic analysis conducted on all the protease sequences produced a pattern consistent with good separation of unrelated sequences even though they were not supported at the roots by significant bootstrap values due to somewhat short sequence lengths (Figure 4A). However, the branch tips show strong bootstrap support for the relatedness of minority variants to the patient bulk sequences from which they were derived. Moreover, some of the patients with bulk sequence-detectable M46I appeared to group together with relatively high bootstrap values (pairs X and Y, Figure 4A) and may represent infections linked within transmission clusters. The sequences within each X and Y pair were 100% identical in the 270 bp analyzed, with the exception of ≤3 mixed-base positions that included the nucleotide of the paired patient. In assessing the relatedness of the four detected minority L90M to infections that have the PR M46I mutation, three L90M were from patients that were wildtype at codon 46, the fourth was ID 13 which was also an M46I case (Figure 4B).

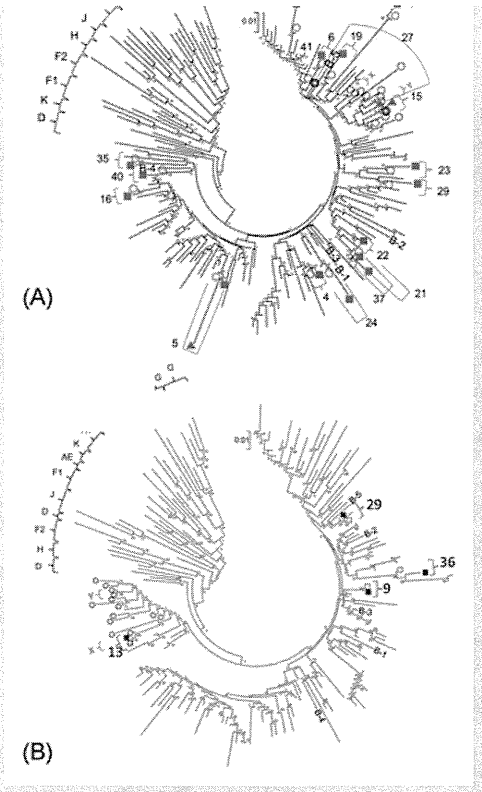

Figure 4. Phylogenetic tree of samples with or without minority variants of M46I/L or L90M drug resistance.
A neighbor-joining phylogenetic tree of all protease sequences using the Kimura 2-parameter model was generated in MEGA5. Numbers shown are IDs of patients with detectable minority drug resistance. Open circles (black color) are virus with M46I detected by bulk sequencing. A. Solid squares (red color) indicate sequences of M46I-specific amplicons and solid triangle (red color) indicate sequences of M46L amplicons; open circles (red color) indicate bulk sequences for persons with minority M46I/L mutations; X and Y are pairs of closely related transmitted M46I sequences. B. Solid squares indicate L90M-specific amplicon sequences; open squares indicate bulk sequences for persons with minority L90M mutations. Open circles (black color) are virus with M46I detected by bulk sequencing. Abbreviations of subtype B references: B-1; B.NL.00.671 00T36.AY423387, B-2; B.US.98.1058 11.AY331295, B-3; B.FR.83.HXB2 LAI IIIB BRU.K03455, B-4; B.TH.90.BK132.AY173951, B-5; B.US.98.15384 1.DQ853463.
doi:10.1371/journal.pone.0083150.g004

Discussion

In this study, we used a highly sensitive method to screen for minority drug-resistant populations in 149 cases of newly diagnosed HIV-infected patients. An additional six drug resistance mutations in the RT region and thirty drug resistance mutations in PR region were detected as minority-level drug resistance mutations. For the ten codons associated with resistance (RTI mutations: M41L, K65R, K70R, K103N, Y181C, M184V and T215F/Y, PI mutations: M46I/L and L90M) the prevalence of detectable drug resistance mutations increased from 15.4% to 26.8% using the highly sensitive assays. A previous surveillance study in Japan using bulk sequencing reported that in 2008 the prevalence of transmitted drug resistance was 8.3% [21]. Therefore, drug resistance mutation surveillance analyzed by bulk sequencing underestimates transmitted drug resistance, which potentially has both clinical and epidemiologic implications.

The epidemiologic implications of increased transmitted resistance may reflect prevention failures in persons who know they are infected and transmit HIV to their partners. The clinical implications of minority drug resistance center around the impact of these viruses on ART responses. Previous studies have reported that minority NNRTI-resistant variants are associated with increased risk of virologic failure in patients receiving first-line NNRTI-based ART regimens [20,26-32]. These findings are important because NNRTI resistance is the most commonly transmitted resistance in the US and Europe [33,34]. However, no evidence of either majority or minority NNRTI-resistance was found in this study, a unique finding that is explained by the historically infrequent use of NNRTIs in ART regimens in Japan. Instead, we note that the NRTI K65R and M184V mutations were both detected as minority populations. As major mutations K65R and M184V reduce the clinical efficacy of TDF and 3TC/FTC, respectively [35-37], however their clinical impact as minority mutations is not fully clear.

A previous study demonstrated no impact on therapy responses in patients who had minority-level K65R and M184V mutations when provided regimens that included protease inhibitors; however; because of the small number of patients representing different treatment regimens in that study, the bearing of these mutations could not be fully evaluated [29]. Additional studies are needed to assess clinically significant frequencies of different NRTI-resistant variants on various treatment regimens. In the present study, one of the 149 cases evaluated possessed six drug resistance mutations as minority variants (Table 2). Genotype interpretation by the Stanford HIV Drug Resistance Database showed that the patient possessed high-level resistance to NRTIs and some PIs. It was not possible to follow the clinical course of this patient to elucidate the significance of minority variants on subsequent cART.A major finding in this study was the high prevalence of transmitted PI resistance (20%) which accounted for about two-thirds the overall transmitted resistance. The high prevalence of transmitted PI resistance is supported both by detection at majority as well as minority variant levels, the latter comprising more than half of the transmitted PI cases. The high prevalence of PI resistance can be explained by the longstanding and predominant use of PI-based regimens in Japan, including darunavir and atazanavir in both first-line and second-line regimens

Genetic linkage analysis provided more insights into the composition of the viral population by showing that L90M, M46I and M46L in many patients existed on separate viral genomes. The capacity to identify linked mutations could be important for understanding the persistence [38] and clinical impact of mutant variants. A major factor that influences the persistence of drug-resistant mutants *in vivo* is their relative replicative capacity within the viral population. *In vitro* competition experiments conducted in the absence of drugs have shown that drug resistance mutations impair replicative fitness by different degrees. For instance, the M46I, the K70R, the 215 intermediate mutations have a lesser impact on fitness than L90M, K65R, and M184V [38-40], and, thus, such mutations are likely to persist longer *in vivo*. Moreover, accumulation of compensatory mutations such as L63P and A71V in protease have been demonstrated to increase or restore replicative fitness of PI resistant variants, and that once compensation has taken place reversion to wildtype is prohibited by a less fit intermediate [41]. This may explain, for example, the high prevalence of M46I we detected in this study as bulk and minority species.

Phylogenetic analysis showed the sequences of minority M46I/L and L90M-positive amplicons were closely related to their source bulk sequences, supporting that the

minority sequences detected were unique to the respective patients and were not the result of contamination. A few cases possessing the M46I mutation by bulk sequencing demonstrated a strongly supported identity which was not biased by including the resistance mutations in the analysis. However, with regard to minority M46I variants, we found they did not cluster closely with viruses from persons with majority-level M46I. This suggested that at least two pairs with majority-level M46I were phylogenetically related whereas those with minority M46I were scattered among the transmitted virus population, unrelated to any of the other cases in our analysis. While the sequence lengths used in the analysis might limit our ability to draw robust bootstrap values deeper in the tree nodes, the sequences for the pairs within the two clusters were identical with the exception of a few positions with mixed bases, and were further supported by high bootstraps.

The ability to conduct surveillance of minority-level drug resistance mutations is an important advancement to help understand transmission of HIV drug resistance in Japan. The finding from our select analysis of mutations of interest cannot be extrapolated to all codons associated with drug resistance; however, these results suggest that a substantial proportion of drug resistance-associated mutations are persist at low levels by the time HIV-infected persons are diagnosed and genotyped. Using an approach that can more broadly identify variants, such as next-generation sequencing [42-45], may identify other mutations that would further increase the prevalence of drug resistance. However, because the more commonly transmitted mutations are often targeted by AS-PCR, any additional increase in mutation prevalence identified by the more complex methods may be nominal. Hence, the lower cost and simplicity of AS-PCR offer advantages for routine surveillance, particularly when the sample burden may be high.

In conclusion, the relationship between minority drug resistance mutations and cART failure requires further exploration; nevertheless, the findings point to difficulties in getting infected persons diagnosed early and counseled to prevent forward spread of drug resistance.

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Author Contributions

Conceived and designed the experiments: WS JJ WH. Performed the experiments: MN JH. Analyzed the data: MN JH ST WS. Contributed reagents/materials/analysis tools: JJ WH WS TM. Wrote the manuscript: MN JJ WH WS.

Supporting Information

Table_S1.doc

Table S1. Oligonucleotide sequence Proportion(RTI mutations)

	Primer	Oligonucleotide sequence	Proportion
Total copy reaction	ComFWD	5'-CTT CTG GGA AGT TCA ATT AGG AAT ACC	
	ComREV	5'-TGG TGT CTC ATT GTT TRT ACT AGG TA	
	Com 1P	5'-*FAM*-TGG ATG TGG GTG A"T"G CAT ATT TYT CAR TTC CCT TA	60%
	Com 2P	5'-*FAM*-TAC TGG ATG "T"GGG TGA TGC ATA TTT TTC ART TCC CTT A	40%
Mutation			
Protease			
L90M	Rev1	5'-GAA AAT TTA AAG TGC AAC CAA KTT GAG TGA T	
	Fwd	5'-AGA TCA CTC TTT GGC AAC GAC C	
	P1	5'-*FAM*-TAG GGG GAA "T"TG GAG GTT TTR TCA AAG TAA GAC AGT AT	
Reverse transcriptase			
M41L	F1	5'-AAT AAA AGC ATT ART RGA AAT YTG TRC AGC AT	35%
	F2	5'-AAT WAA AGC ATT ART RGA AAT YTG TRC WGC AT	10%
	F3	5'-AAA AGC ATT ART RGA AAT YTG TRC AGG AC	32%
	F4	5'-TAA AAG CAT TAR TRG AAA TYT GTR CAK GTC	13%
	F5	5'-AAG CAT TAR TRG AAA TYT GTR CAK GGC	10%
	Rev	5'-CCT AAT TGA ACT TCC CAG AAG TCT TG	
	41-70p	5'-*FAM*-TTG GGC CTG AAA A"T"C CAT ACA ATA CTC CAG TAT TT	
K65R	F1	5'-ACA ATA CTC CAR TAT TTG CCA TAA RCA G	
	Rev	5'-CCT GGT GTC TCA TTG TTT ATA CTA GGT	
	K65R-P1	5'-*FAM*- TCA GAG AAC "T" TAA TAA RAG AAC TCA AGA CTT CTG GGA	80%
	K65R-P2	5'-*FAM*-TCA GAG AAC "T"CAA TAA GAG AAC TCA AGA CTT CTG GGA	20%
K70R	Rev1	5'- GTT CTC TRA AAT CTA YTA WTT TTC TCC CTC	70%
	Rev2	5'-TTC TCT RAA ATC TAY TAW TTT TCT CCC CC	30%
	Fwd	5'- AGA RAT TTG TAC AGA RAT GGA AAA GGA AG	
	41-70p	5'-*FAM*-TTG GGC CTG AAA A"T"C CAT ACA ATA CTC CAG TAT TT	

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Oligonucleotide sequence Proportion(RTI mutations).

Table S1.
Oligonucleotide sequence Proportion(RTI mutations).
doi:10.1371/journal.pone.0083150.s001
(DOC)

Table S2.
Oligonucleotide sequence Proportion(PI mutations).
doi:10.1371/journal.pone.0083150.s002
(DOC)

Table S3.
Assay ΔCt measures, cutoffs and sensitivities on clinical samples.
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(DOC)

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ORIGINAL

Development and application of a simple LC-MS method for the determination of plasma rilpivirine (TMC-278) concentrations

Masaaki Shibata[1], Masaaki Takahashi[1], Munehiro Yoshino[2], Takeshi Kuwahara[3], Toshiharu Nomura[1], Yoshiyuki Yokomaku[4], and Wataru Sugiura[4]

[1]*Department of Pharmacy, National Hospital Organization Nagoya Medical Center, Aichi, Japan.,*
[2]*Department of Pharmacy, National Hospital Organization Osaka Medical Center, Osaka, Japan.,*
[3]*Department of Pharmacy, National Cerebral and Cardiovascular Center Hospital, Osaka, Japan.,*
[4]*Department of Clinical Research Center, National Hospital Organization Nagoya Medical Center, Aichi, Japan*

Abstract : Rilpivirine (TMC-278) is a second-generation non-nucleoside reverse transcriptase inhibitor that is high potent against both wild-type and drug-resistant HIV-1 strains. Therefore, rilpivirine is expected to treat therapy-experienced patients who failed to use current drugs due to the emergence of drug-resistant HIV mutants. The quantification of rilpivirine in human plasma is important to support clinical studies and determine pharmacokinetic parameters of rilpivirine in HIV-1 infected patients. Consequently, simple and easy system to determine plasma rilpivirine concentrations has been required. In this study, we developed a conventional LC-MS method to quantify plasma rilpivirine. Subsequently the method was validated by estimating the precision and accuracy for inter- and intraday analysis in the concentration range of 18-715 ng/ml. The calibration curve was linear in this range. Average accuracy ranged from 100.0 to 100.6%. Relative standard deviations of both inter- and intraday assays were less than 3.3%. Recovery of rilpivirine was more than 82.0%. These results demonstrate that our LC-MS method provides a conventional, accurate and precise way to determine rilpivirine in human plasma. This method can be used in routine clinical application for HIV-1 infected patients, and permits management of drug interactions and toxicity for rilpivirine. J. Med. Invest. 60 : 35-40, February, 2013

Keywords **:** *rilpivirine, LC-MS, HIV, therapeutic drug monitoring*

INTRODUCTION

The clinical treatment of patients with human immunodeficiency virus (HIV)-1 infection has been advanced by the success of highly active antiretroviral therapy. The latest treatment guidelines recommend regimen including efavirenz, a first-generation non-nucleoside reverse transcriptase inhibitor (NNRTI), as one of the standard first-line regimen (1). However, efavirenz use is limited by low genetic barrier to resistance and central nervous system toxity (2, 3). Therefore, new antiretroviral drugs, which have long-term efficacy and good tolerability, are required to continue effective therapy for the treatment of HIV-1.

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Address correspondence and reprint requests to Masaaki Takahashi, Ph.D., Department of Pharmacy, National Hospital Organization Nagoya Medical Center, 4-1-1 Sannomaru, Naka-ku, Nagoya, Aichi 460-0001, Japan and Fax : +81-52-971-0776.