However, their mitochondrial toxicity made long-term use difficult (4-7). Due to HLA-B*5701-related hypersensitivity, abacavir (ABC) is listed as the second line drug under the United States Department of Health and Human Services (DHHS) guidelines. However, HLA-B*5701 is quite rare among Japanese, and thus the incidence of hypersensitivity to ABC in Japanese patients is lower than that of Caucasians (8-10). Although tenofovir (TDF) is widely used as the first line drug, the dose-dependent nephrotoxicity is a major concern in Japanese because Japanese body weight is lighter than that of Caucasians (11, 12).

The present study was designed in 2006, when the combination of TDF, lamivudine (3TC) or entiricitabine (FTC), and EFV was the first line regimen of antiretroviral treatment (13). To explore the optimal antiretroviral combination for the best clinical outcome among Japanese HIV-1 patients (14), a selection study was designed to compare the efficacy and safety of once daily treatment with EFV or ATV/r combined with a fixed-dose ABC and 3TC (ABC/3 TC).

Objective

The objective of this study was to select a once daily regimen for further non-inferior study comparing the virological efficacy and safety of the first line once daily antiretroviral treatment regimens in the current HIV/AIDS guideline.

Subjects and Methods

Study design

The study was designed as a randomized, open label, multicenter selection study, which means the superior regimen at the end point is to be selected as alternate arm to compare with the current first line regimen in the next step. Therefore, this study was not to compare superiority or non-inferiority of both arms. As the selection study, the main objective is to select a treatment regimen for further pivotal study and the secondary objective is safety. The primary endpoint was the proportion of patients in each arm who achieved virologic success (HIV-1 RNA less than 50 copies/mL in plasma) at week 48. The secondary endpoints were death, AIDS and serious non-AIDS events, non-AIDS defining cancer, treatment-related serious or grade 3 to 4 adverse events, and discontinuation of antiretroviral treatment before week 96.

The inclusion criteria of this study were those who were treatment-naïve, HIV-1 positive Japanese men with a CD4+ count ranging from 100 to 300 cell/mm^3. The exclusion criteria included current active AIDS, acute retroviral syndrome and persistent active hepatitis B infection (HBs-Ag positive). Patients with a history of 3TC treatment for hepatitis B infection were also excluded. After obtaining informed consent, eligible participants were randomized into once daily 600 mg EFV or 100 mg RTV and 300 mg ATV (EFV arm vs ATV/r arm). All participants received a fixed dose of 600 mg of ABC and 300 mg 3TC (ABC/3TC).

At baseline, the demographic characteristics and a complete medical history were recorded, physical examination was performed, and various laboratory tests were obtained (CD4+ count, HIV-1 RNA, complete blood count, biochemistry, liver and renal function tests, and total cholesterol). Participants were examined at baseline, then every 4 weeks until week 96. Careful clinical examination was provided at each visit, including history taking of any adverse event, adherence to treatment, and physical examination. Furthermore, blood tests were obtained including complete blood count, biochemistry, liver and renal function tests, CD4+ count and HIV-1 RNA. When HIV-1 RNA became less than 50 copies/mL, participants were rescheduled to be seen every 4 to 12 weeks. All participants underwent clinical examination at week 48 as the primary endpoint, then every 12 weeks until week 96 as the secondary follow-up period for evaluation of safety.

The study recruitment period was started on September 1st of 2005 for 2 years. The study protocol was originally designed to follow patients for 48 weeks, however, during the study period, cardiovascular adverse events of ABC-containing regimen were reported (15, 16). Considering the importance of adherence to safety, the follow-up period was extended to 96 weeks.

Independent data and safety monitoring board reviewed virology and safety data by treatment allocation were obtained when all participants had completed 24 weeks of the study. A total of 18 academic medical institutions in Japan participated in this study. The study protocol was approved by the ethics committee of each site and was registered at Clinicaltrials. Gov (NCT00280969) and the University Hospital Medical Information Network (UMIN000000243).

Statistical analyses

The estimated proportion of virologic failure, representing HIV-1 RNA of more than 50 copies/mL at 48 weeks of treatment, was 30% over one year. To choose one treatment group with a probability of 0.90, if it is superior to another treatment by >10%, if any, a sample size of 40 participants per group was necessary according to the selection design (17).

To assess differences in proportions, we used Fisher's exact test and calculated exact confidence intervals (CIs). We conducted intent-to-treat analysis and used the T test to compare the efavirenz arm and the ritonavir boosted atazanavir arm, unless the data showed skewed distribution, in which case the Wilcoxon's test was used. All analyses used a two-sided alfa of 0.05. No adjustment for each test was made for multiple comparisons due to the fact that we have several tests to compare the efficacies and safeties of two groups. All analyses, unless otherwise specified, were determined a priori and were hypothesis driven. Statistical analyses were performed using SAS version 9.1.

Table 1. **Baseline Characteristics of Participants**

Variable	efavirenz	atazanavir/r	p
Number of patients	36	35	NS
Age (yrs) median	35	36	NS
HIV-RNA (log_{10} copies/mL)			
median	4.6	4.4	NS
range	2.8–5.4	3.0–5.3	
CD4 count (cells/mm^3)			
median	220	226	NS
range	121–323	103–324	
Total Cholesterol (mg/dL)			
median	155.5	159.5	NS
range	122–208	112–215	
Total bilirubin (mg/dL)			
median	0.6	0.5	NS
range	0.3–1.7	0.3–1.5	
ALT (IU/L)			
median	24	20	NS
range	8–71	8–78	
Creatinine (mg/dL)			
median	0.80	0.75	NS
range	0.6–1.03	0.6–1.02	

Results

Participants

In the study recruitment period, 71 participants were randomly assigned to two groups (36 in EFV arm and 35 in ATV/r arm). The baseline characteristics of the subjects are listed in Table 1. Among the 71 participants, 62 (87.3%) for the primary endpoint and 58 (80.6%) for the secondary endpoint completed the study protocol. By week 96, 9 participants had withdrawn due to clinical events, 2 declined to continue the study for personal reasons, one died by accident and 3 were transferred to other non-participating institutions.

Primary endpoint

At week 48, by intent-to-treat, missing-equals-failure analysis, 28 of 36 participants (77.8%, 95% CI: 60.9-89.9) in the EFV arm and 27 of 35 (77.1%, 95% CI: 59.9-89.9) in the ATV/r arm achieved the goal of HIV-1 RNA less than 50 copies/mL. There was no significant difference between the two arms (p=0.95).

Virologic success over time

Figure 1 shows the intent-to-treat analysis of participants who reached virologic success. At week 96, the rates of virologic success in the EFV arm were 55.6% (20 of 36) and 68.6% (24 of 35) in the ATV/r arm (p=0.33). The number of participants with a baseline HIV-1 RNA level of more than 100,000 copies/mL was 5 in the EFV arm and 2 in the ATV/r arm. One participant in each arm withdrew from the study at week 4 due to skin rash. The rest of the participants achieved virologic success in the EFV arm (4 out of 4) and in ATV/r arm (1 out of 1).

Secondary endpoints

In the EFV arm, 7 of 36 participants did not complete the study; 5 of the 7 developed psychiatric symptoms, including suicidal idealization, insomnia and irritation, 2 developed skin rashes and the remaining 2 were lost to follow-up because they were transferred to non-affiliated hospitals. In the ATV/r arm, 6 of 35 patients could not complete the study; one died by accident for unknown reason (the cause of death according to the coroner's report was not related to the cardiovascular system), 2 participants required treatment change (this was due to suicidal idealization in one and to skin rash in the other), one participant withdrew by own wish, one enrolled into another study, and one was transferred to another non-affiliated medical care facility.

Figure 2 shows the change of total cholesterol, liver function and total bilirubin from the baseline. At enrollment in the study, the median total cholesterol in the EFV arm was 155.5 mg/dL (range: 122-208) and in the ATV/r arm was 159.5 mg/dL (range: 112-215). The total cholesterol was not more than 220 mg/dL in any of the participants of both arms at baseline, and there was no significant difference between the two arms. During the study period, the total cholesterol increased to more than 220 mg/dL and required treatment with hypolipidemic agents in 52.8% of the EFV arm and 34.3% of the ATV/r arm. There was a significant increase in total cholesterol from the baseline in both arms ($p < 0.05$). There was no significant change in liver function tests during the study. New onset grade 3 hyperbilirubinemia was noted in 27 of 35 (77.1%) of the ATV/r arm but in none of the EFV arm. None of the hyperbilirubinemia in the ATV/r arm was associated with altered liver function, altered renal function, nephrolithiasis, or cholelithiasis.

Discussion

This study was designed as selection study, which means the superior regimen at the endpoint is to be selected as an alternate arm to compare with the current first line treatment in the next step. By definition of the selection study, the superior arm does not require statistical significance (17). At week 48, 77.8% of ATV/r arm and 77.1% of EFV arm reached HIV-VL of less than 50 copies/mL. Based on the definition of the selection study, the combination ABC/3TC/EFV was selected to compare the current first line treatment while the efficacy of each arm was almost even in this study.

In this clinical trial of 71 participants over a period of 96 weeks, no cardiovascular events or severe hypersensitivity reaction against ABC was observed. In this study, the efficacy of EFV combined with ABC/3TC and ATV/r combined with ABC/3TC was similar. Therefore, ABC based regimen can be selected as a safe combination to compare the efficacy of the first line combinations, such as EFV plus TDF/FTC or ATV/r plus TDF/FTC (18-20), in the next step for the best clinical benefits in Japanese patients.

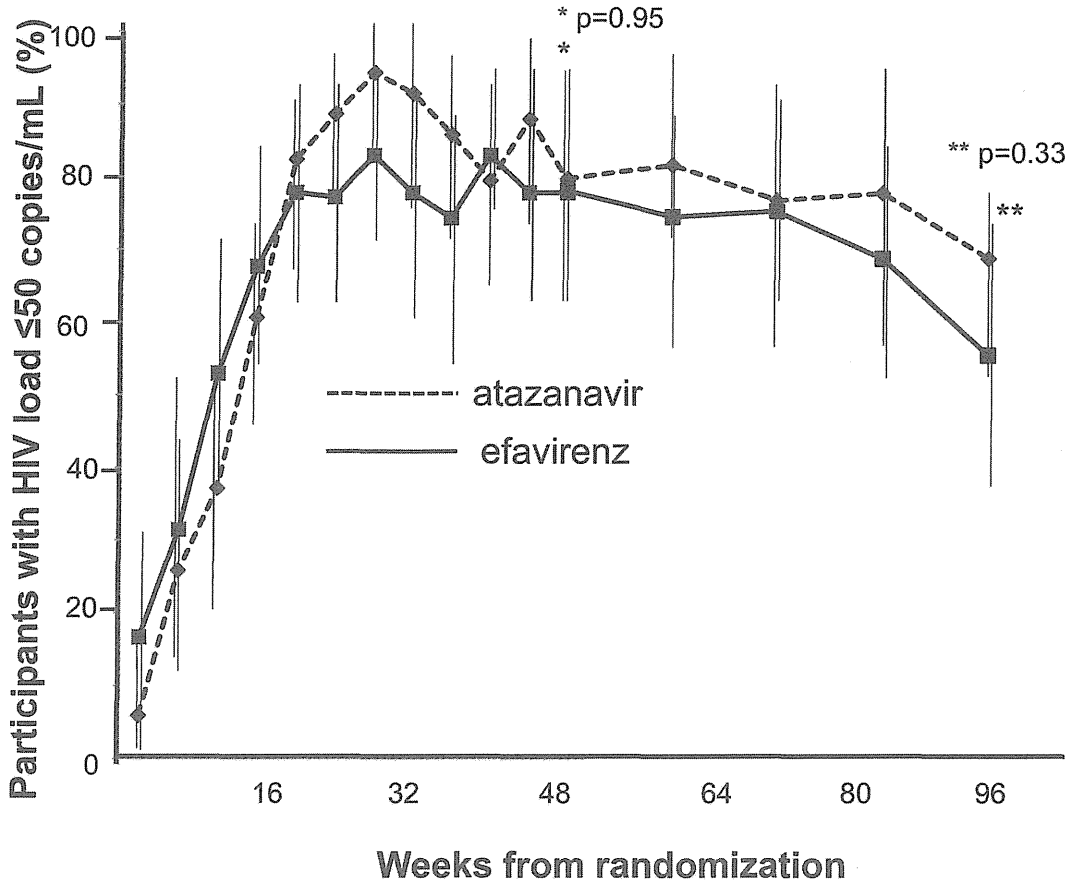

Figure 1. Proportions of participants with HIV-RNA less than 50 copies/mL.
The efficacies of the efavirenz arm and ritonavir-boosted atazanavir arm were compared with intent-to-treat analysis. There were no significant difference between arms at both week 48 (p=0.95) and week 96 (p=0.33).

In February 2008, the United States National Institution of Allergy and Infectious Disease announced that the data and safety monitoring board of ACTG 5202 recommended a modification of the study design because they found that among participants with high viral loads (100,000 or more copies/mL) at the time of screening, treatment combinations that included ABC/3TC were not as effective in controlling the virus as those of regimens containing TDF/FTC (19, 21). At that point, all of the present 71 participants were already enrolled in the study and the baseline HIV-1 RNA of 7 participants was more than 100,000 copies/mL. Of these 7 participants, 2 had already withdrawn from the study by week 4, and the rest of participants had reached HIV-1 RNA of less than 50 copies/mL. The safety monitor board made no recommendation to amend the protocol.

As a primary endpoint, 77.8% of the EFV arm and 77.1% of the ATV/r had reached virological success, however, total cholesterol in 58.1% of the EFV arm and 46.9% of the ATV/r arm increased to more than 220 mg/dL, which required treatment. Thus, the overall proportion of participants with good viral suppression and without severe adverse events or treatment modification was 39.6% for the EFV arm and 62.3% for the ATV/r arm. Considering the reasons for treatment modification, the neuro-psychiatric side effects required a regimen change in the EFV arm. Although several studies concluded that the neuro-psychiatric side effects are transient in nature, one study reported that treatment had to be changed in 16% of patients on EFV due to neuro-psychiatric side effects (22-24). Although there was no significant difference even with the small sample size, 5 out of 36 (13.9%) participants on EFV in our study required treatment change, compared with only 1 out of 35 (2.9%) of the ATV/r arm. This aspect of our study was similar to that reported in the Euro SIDA study (24). In the Swiss Cohort study, the treatment-limiting CNS adverse events was 3.8 (95% CI 2.7-5.2) per 100 person-years and it was clearly related to EFV (25). Considered together, these results emphasize the need for close observation of patients treated with EFV.

The incidence of hyperbilirubinemia in the present study was 77.1% in the ATV/r arm but none of these patients was above grade 4. Furthermore, none of the patients in this study developed liver function abnormality, altered renal function, renal stones, or cholelithiasis. As reported by Torti et al and Josephson et al, such clinical outcome can be used as a marker of adherence to ATV therapy (26, 27).

Limitations of this study include a small sample size.

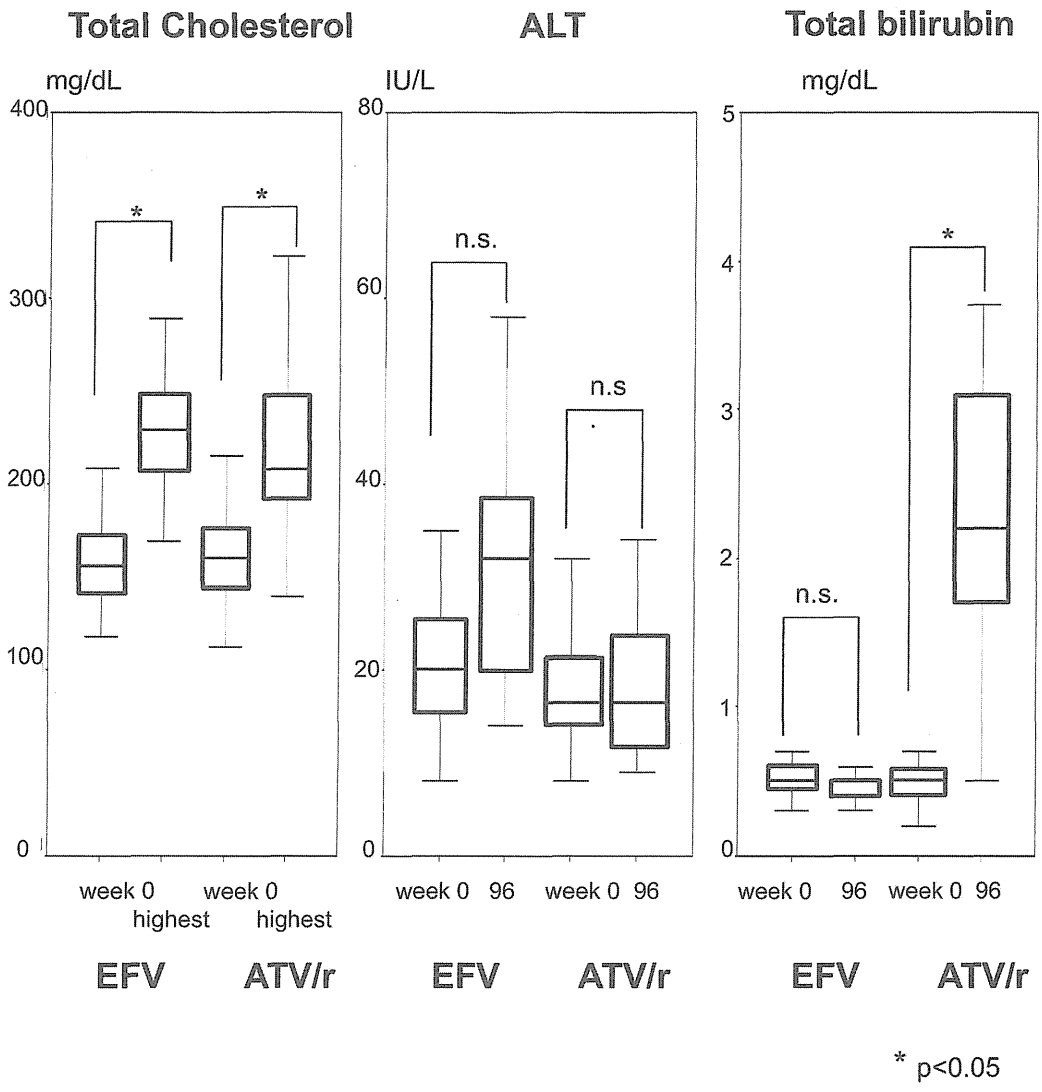

Figure 2. Changes from baseline in total cholesterol, ALT and total bilirubin.
ALT and total cholesterol at week 96 were compared with the baseline values. Since participants who developed hyperlipidemia were treated with lipid-lowering agents during the study period, the highest levels registered in each participant during the follow-up were collected for analysis. There were no significant differences in total cholesterol and ALT between the two arms, while hyperbilirubinemia was significantly higher in the ATV/r arm. Modification of treatment due to hyperbilirubinemia was not required in any of the patients of the ATV/r arm. In these box-and-whisker plots, the 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 90th and 10th percentiles, respectively.

Considering many studies on HIV treatment held in western countries that enrolled few Asian HIV-1 patients, it is important to collect data from Asian population. The current United States Department of Health and Human Services guidelines recommend TDF/FTC as the first line regimen, while the European AIDS Clinical Society recommends 3TC and ABC addition to TDF and FTC alone (28, 29). TDF/FTC is a known potent antiretroviral agent, however, its long-term efficacy and safety remain unclear (11, 12). Considering that the combinations of NRTI are limited, the efficacy and safety of ABC in the low HLA-B*5701 population need to be evaluated for wider treatment options for HIV-1 patients (9, 10).

Conclusion

This study was designed as a selection study to compare the virologic efficacy and treatment safety of EFV and ATV/r, both with ABC/3TC, in Japanese patients. The results showed no significant differences in efficacy between the two regimens at week 48. The evaluation of safety was extended to 96 weeks, which also showed no significant difference in both arms. The results of the present study have already been applied as the basis of a follow-up study that is

currently being conducted in Japan to compare NRTI combinations of ABC/3TC and TDF/FTC with ATV/r as key drugs.

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

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Identification of a Current Hot Spot of HIV Type 1 Transmission in Mongolia by Molecular Epidemiological Analysis

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Abstract

We investigated the current molecular epidemiological status of HIV-1 in Mongolia, a country with very low incidence of HIV-1 though with rapid expansion in recent years. HIV-1 *pol* (1065 nt) and *env* (447 nt) genes were sequenced to construct phylogenetic trees. The evolutionary rates, molecular clock phylogenies, and other evolutionary parameters were estimated from heterochronous genomic sequences of HIV-1 subtype B by the Bayesian Markov chain Monte Carlo method. We obtained 41 sera from 56 reported HIV-1-positive cases as of May 2009. The main route of infection was men who have sex with men (MSM). Dominant subtypes were subtype B in 32 cases (78%) followed by subtype CRF02_AG (9.8%). The phylogenetic analysis of the *pol* gene identified two clusters in subtype B sequences. Cluster 1 consisted of 21 cases including MSM and other routes of infection, and cluster 2 consisted of eight MSM cases. The tree analyses demonstrated very short branch lengths in cluster 1, suggesting a surprisingly active expansion of HIV-1 transmission during a short period with the same ancestor virus. Evolutionary analysis indicated that the outbreak started around the early 2000s. This study identified a current hot spot of HIV-1 transmission and potential seed of the epidemic in Mongolia. Comprehensive preventive measures targeting this group are urgently needed.

Introduction

MONGOLIA HAS A LOW PREVALENCE of HIV with estimated infected individuals comprising less than 0.01% of the general population. However, this number is increasing rapidly and recent statistical data estimated a 10-fold increase in HIV/AIDS incidence during the past 5 years. Since 1992, when data on HIV/AIDS began to be compiled in Mongolia, there had been only five cases reported as of December 2004. In 2005, the number of infected cases increased sharply and 11 cases were registered in that year. According to an unpublished report from the Ministry of Health Mongolia, the total number of HIV infected cases was 56 as of May 2009. The infected individuals were men who have sex with men (MSM) 64.3%, heterosexual males (HSM) 14.3%, and females (HSF) 21.4%, of whom 50% were female sex workers (FSW).

The Second Generation HIV/Sexually Transmitted Infections (STI) surveillance program (SGS) for HIV/STI serological studies in various behaviors was initiated in Mongolia in 2002. According to the latest results of the SGS conducted in 2007, the HIV prevalence among blood donors and pregnant women was zero and among 1350 tuberculosis patients was 0.15% (95% CI 0.00–0.35).[1] The results of our 2007 prevalence survey of 2465 individuals (1415 high-risk and 1050 healthy control populations) in Mongolia demonstrated that the current HIV prevalence is low, but according to the high prevalence of syphilis (anti-TP 23.1%) and HCV (anti-HCV 8%) in high-risk populations, the risk status for HIV-1 infection is estimated to be high.[2] The high-risk populations included FSW, MSM, mobile men, tuberculosis (TB) patients, and male STI clinic clients; healthy control populations were youth and blood donors.

Knowledge about current patterns and trends of HIV infections is essential for planning and evaluating prevention programs and for resource allocation. In the past, epidemiological data on newly diagnosed HIV/AIDS and results of a

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surveillance study of HIV/STI have been used for planning and targeting HIV prevention programs in Mongolia. However, these data were not sufficient to implement a comprehensive, effective, targeted strategy for prevention of HIV infection in Mongolia. To gain a better understanding of the current HIV status, a more detailed molecular epidemiological study using phylogenetic analyses is needed. Molecular epidemiological analyses are useful tools to gain information about the origin of HIV epidemics and transmission patterns.[3,4] Moreover, information regarding the genetic diversity of HIV strains, and the geographic prevalence of genotypes, is important for the evaluation of diagnostic tests and vaccines and for their effective application.[5,6]

In the present study, we used phylogenetic analyses to determine the HIV-1 subtypes circulating in Mongolia, especially the dominant subtypes responsible for the outbreak in the infected population. Then, we performed a Bayesian coalescent-based framework to investigate the origin and estimate the onset year of the dominant HIV-1 subtypes in Mongolia.

Materials and Methods

Study population

After obtaining informed consent, blood samples were collected anonymously from 39 Mongolian HIV-1-positive patients attending the National Center for Communicable Diseases of Mongolia (NCCD) in November 2007 and May 2009. For all of them, HIV infection was diagnosed from 1997 to 2009, and all participants were infected through sexual contact. In this study, we also examined two stored serum samples at the NCCD obtained from a Russian married couple who were diagnosed with HIV-1 in Mongolia in 2007. Therefore, a total of 39 (69.6%) samples out of all 56 reported cases in Mongolia as of May 2009 was examined in this study. The study subjects were 32 men [mean age (±SD): 31.3±7.8 years, range: 17–52 years] and 9 females (25.9±5.1, 18–33 years) and 63.4% of them had a high level (college/university) of education (Table 1). The *StatView* software version 5.0 (SAS Institute, Cary, NC) was used for statistical analyses. The collected serum samples were sent to the AIDS Clinical Center, National Center for Global Health and Medicine (NCGM), Tokyo, Japan, for further analysis. The study protocol was approved by the ethics committees of NCGM (H19-448 and H20-545) and the Ministry of Health, Mongolia (2007-#7 and 2008-#3).

Amplification and sequencing of HIV-1

Total RNA was extracted from 140 μl of serum, using the QIAamp RNA Mini Kit (Qiagen, Valencia, CA) according to the instructions supplied by the manufacturer. HIV-1 cDNA was obtained by reverse transcriptase polymerase chain reaction (RT-PCR) using the TaKaRa One Step RNA PCR kit (AMV, TaKaRa Bio Inc., Japan) and then DNA fragments were amplified using the TaKaRa Ex Taq Hot Start Version (TaKaRa Bio Inc, Japan) with the following primer sets. A total of 1065 bp of the polymerase (*pol*) fragment (HXB2: 2243–3308) containing the regions encoding the full protease and first 560 nucleotides of RT was amplified by RT-PCR with primers of F-1849 (5′-GAT GAC AGC ATG TCA GGG AG-3′) and R-3500 (5′-CTA TTA AGT CTT TTG ATG GGT CAT AA-3′). DRPRO5 (5′-AGA CAG GYT AAT TTT TTA GGG A-3′), DRPRO2L (5′-TAT GGA TTT TCA GGC CCA ATT TTT GA-3′), DRRT1L (5′-ATG ATA GGG GGA ATT GGA GGT TT-3′), and DRRT4L (5′-TAC TTC TGT TAG TGC TTT GGT TCC-3′) primer sets were used for nested PCR. A total of 447 bp of the envelope (*env*) fragment (HXB2: 6834–7281) containing the region C2V3 was amplified by RT-PCR with primers of V1V2-1 (5′-TGT GTA CCC ACA GAC CCC AAC CC-3′) and IC462M (5′-GCC CAT AGT GCT TCC TGC TGC T-3′). ENV02 (5′-ATG GTA GAA CAG ATG CAT GA-3′) and E115 (5′-AGA AAA ATT CCC CTC CAC AAT TAA-3′) primer sets were used for nested PCR. The amplified DNA was purified using the QIAquick PCR purification kit (Qiagen Inc.) according to the protocol provided by the manufacturer. Purified DNA was sequenced by using the ABI BigDye Terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) and processed with an automated ABI 3730 DNA Analyzer (Applied Biosystems).

Table 1. Epidemiological Characteristics of the 41 HIV-1 Infected Patients

Characteristics	*Male (%)*	*Female (%)*
Gender	32 (78)	9 (22)
Age groups (based on age at first diagnosis)		
<20	1 (2.4)	1 (2.4)
20–29	15 (36.6)	5 (12.2)
30–39	10 (24.4)	2 (4.9)
40–49	4 (9.8)	0 (0)
>50	1 (2.4)	0 (0)
Unknown	1 (2.4)	1 (2.4)
Ethnicity		
Mongolian	31 (75.6)	8 (19.5)
Russian	1 (2.4)	1 (2.4)
Transmission category		
Homosexual	27 (65.8)	0 (0)
Heterosexual	4 (9.8)	9 (22)[a]
Unknown	1 (2.4)	0 (0)
Education		
Uneducated	1 (2.4)	0 (0)
Primary	0 (0)	0 (0)
Incomplete secondary	2 (4.9)	3 (7.3)
Complete secondary	5 (12.2)	2 (4.9)
College/university	23 (56.1)	3 (7.3)
Unknown	1 (2.4)	1 (2.4)
HIV-1 subtype		
B	30 (73.1)	2 (4.9)
C	0 (0)	2 (4.9)
G	2 (4.9)	0 (0)
CRF01_AE	0 (0)	1 (2.4)
CRF02_AG	0 (0)	4 (9.8)

[a]Five (55.6%) of the female cases were female sex workers.

HIV-1 subtype determination and distance-based phylogenetic inference

Analyses of HIV-1 subtype and circulating recombinant form were performed using the REGA HIV-1 Subtyping Tool.[7] The basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST) was used to search and select the HIV-1 reference sequences with the highest similarity score (>95%) to the Mongolian strains. Additional reference

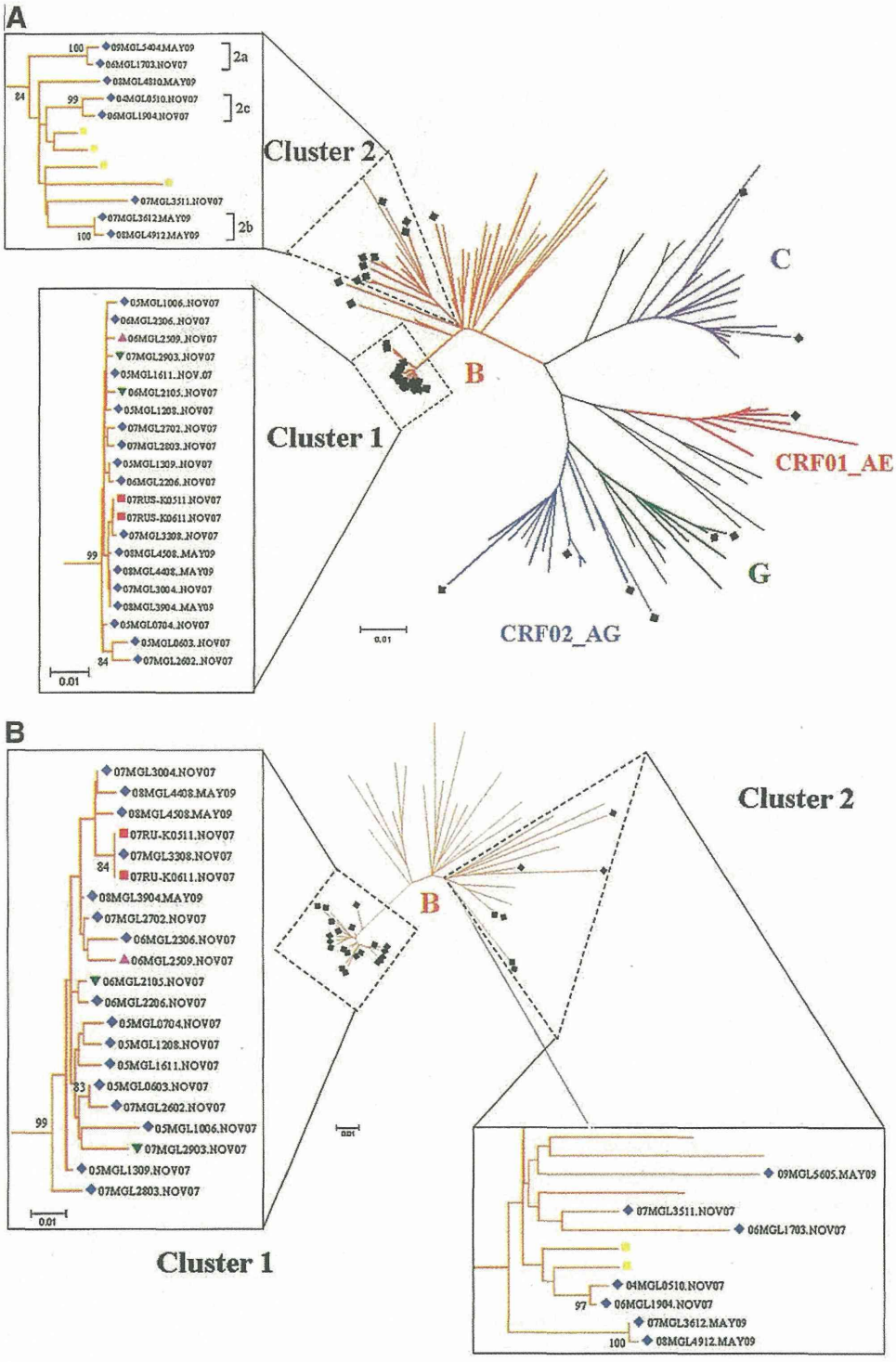
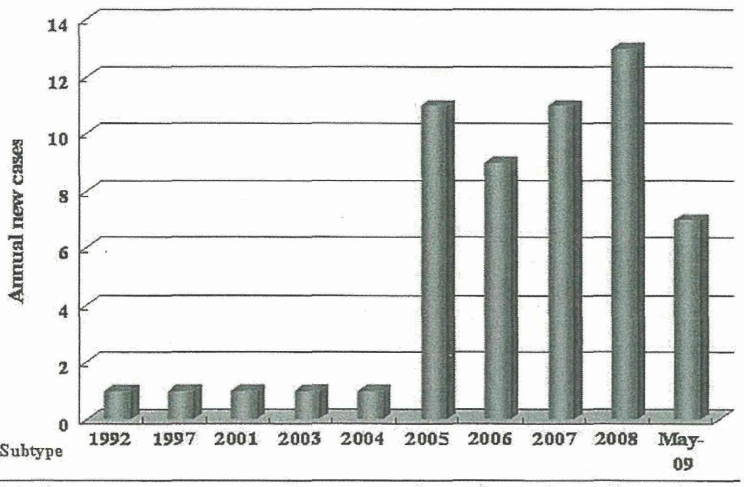


FIG. 1. *Distance-based phylogenetic tree.* Unrooted radial phylogeny of **(A)** 41 tested sequences and 67 reference HIV-1 *pol* (PR to RT; 1065 nt) sequences with different subtypes; **(B)** 28 tested sequences and 21 reference HIV-1 subtype B *env* (C2V3; 447 nt) sequences. The color of the branches represents the different subtypes and recombinant forms. *Solid diamonds:* sequences from Mongolians. The clustered sequences are framed and subtrees of the clusters are represented in the rectangle panels on the left. Numbers on the branch in the subtrees represent bootstrap probabilities. The different symbols and different colors in the subtrees indicate risk factors and/or countries of origin of sequences [*blue diamonds:* MSM (men who have sex with men); *green triangles:* HSM (heterosexual male); *pink triangle:* HSF (heterosexual female) for Mongolians only; *red squares:* sequences isolated from Russian patients; *yellow squares:* reference sequences of Korean origin].

sequences of different subtypes and recombinant forms were retrieved from the Los Alamos National Laboratory (www.hiv.lanl.gov). Sequences were aligned using the CLUSTAL-W software in the MEGA (molecular evolutionary genetics analysis) version 4.1.[8] Phylogenetic trees were constructed by the neighbor-joining (NJ) method based on the Maximum Composite Likelihood distance matrix listed in the MEGA software. The reliabilities of the branching patterns were tested by bootstrap analysis with 1000 replicates.

Bayesian coalescent inference using the subtype B sequences

Evolutionary rates, molecular clock phylogenies, and other evolutionary parameters were estimated from heterochronous genomic sequences of subtype B in Mongolian, non-Mongolian, and some reference sequences by using the Bayesian Markov chain Monte Carlo (MCMC) method. The reference sequences were obtained from the HIV sequence



HIV-1 Subtype	1992	1997	2001	2003	2004	2005	2006	2007	2008	May-09
B (Cluster 1)						6	4	6	3	
B (Cluster 2)					1		2	2	2	1
B									2	1
C						1	1			
G									1	1
CRF01_AE								1		
CRF02_AG		1				1		2		
Unavailable	1		1	1		3	2		5	4
Total	1	1	1	1	1	11	9	11	13	7

FIG. 2. Year-diagnosis rates of HIV-1 infection in Mongolia between 1992 and May 2009 (upper panel). The number of patients and their identified HIV-1 subtypes are listed in the lower panel.

database in the Los Alamos National Laboratory with their sampling time. The reference alignment set for the *pol* region consists of 22 sequences of subtype B viruses covering Korea, China, Japan, Russia, Europe, and North America, with the sequence of D.KE.97.ML415_2 as the subtype's outgroup. The set for the *env* region consists of 20 sequences of subtype B viruses covering Korea, China, Japan, Russia, Europe, and North America, with the sequence of C.US.98US_MSC5016 as the subtype's outgroup. Each reference set was piled up with the Mongolian sequence analyzed in the present study and realigned using CLUSTAL-W. The following analyses were performed in each region of the sequence alignment. The nucleotide substitution model used in the analyses was evaluated by the hierarchical likelihood ratio test using PAUP v4.0 beta[9] with MrModel test,[10] and the general time-reversible (GTR) model[11] with both invariant sites (I) and gamma-distributed site heterogeneity (G) with four rate categories had maximum likelihood. Bayesian MCMC analyses were performed by BEAST v1.4.8[12] using the GTR+I+G and a relaxed molecular clock model (the uncorrelated lognormal-distributed model).[13] Three different population dynamic models, Exponential growth, Logistic growth, and Bayesian Skyline Plot (BSP), were tested in the analyses, and the exponential model was adopted as the most likely phylogeny according to the BSP property. Each Bayesian MCMC analysis was run for 30 million states and sampled every 10,000 states. Posterior probabilities were calculated with a burn-in of 4 million states and checked for convergence using Tracer v1.4. The maximum clade credibility tree for the analyzed set of the MCMC data was annotated by TreeAnotator in the BEAST package. The posterior distribution of the substitution rate obtained from the heterochronous sequences was subsequently incorporated as a prior distribution for evolutionary rate of the *pol* region as well as the *env* region, thereby adding a timescale to the phylogenetic histories of the given viruses and enabling the estimation of the times of the most recent common ancestors (tMRCA).[14]

Results

The *pol* region (1065 bp) was successfully amplified and sequenced in 41 sera. According to the REGA HIV-1 Subtyping Tool, the distribution of HIV-1 genotypes in the study population was as follows: 32 cases (78%) of subtype B, two (4.9%) subtype C, two (4.9%) subtype G, four (9.8%) CRF02_AG, and one case (2.4%) of CRF01_AE.

To investigate the geographic origin of the Mongolian strains, the 41 *pol* sequences were compared against all HIV-1 sequences in the NCBI database using a BLAST similarity search. A distance-based phylogenetic tree (NJ tree) was constructed with 67 *pol* reference sequences (Fig. 1A). As shown in the phylogenetic tree of the *pol* gene region, the majority (78%) of the sequences belonged to subtype B, in which two distinct clusters, named cluster 1 and cluster 2, were identified. Twenty-one (65.6%) of the total 32 subtype B sequences, which included 16 MSM, two HSM, and one HSF Mongolians, were grouped into "cluster 1." This cluster showed a remarkable monophyly with a long branch against the other sequences and high bootstrap value (<98%). The mean nucleotide diversity within the cluster was low (<0.01), indicating that members of this cluster were closely related. Russian B strains (07RUS-K0511NOV07 and 07RUS-K0611NOV07) were included in cluster 1, suggesting a Russian carrier was responsible for the Mongolian HIV-1 epidemic. Cluster 2 consisted of sequences from eight (25%) MSM Mongolian patients and four reference strains from Korean subtype B origin. Cluster 2 was more divergent than cluster 1, and had a relatively low bootstrap value (<90%). We also identified three small groups, group 2a, 2b, and 2c, in cluster 2. They had high clade credibility (bootstrap value >98%) and low genetic divergences (<0.01).

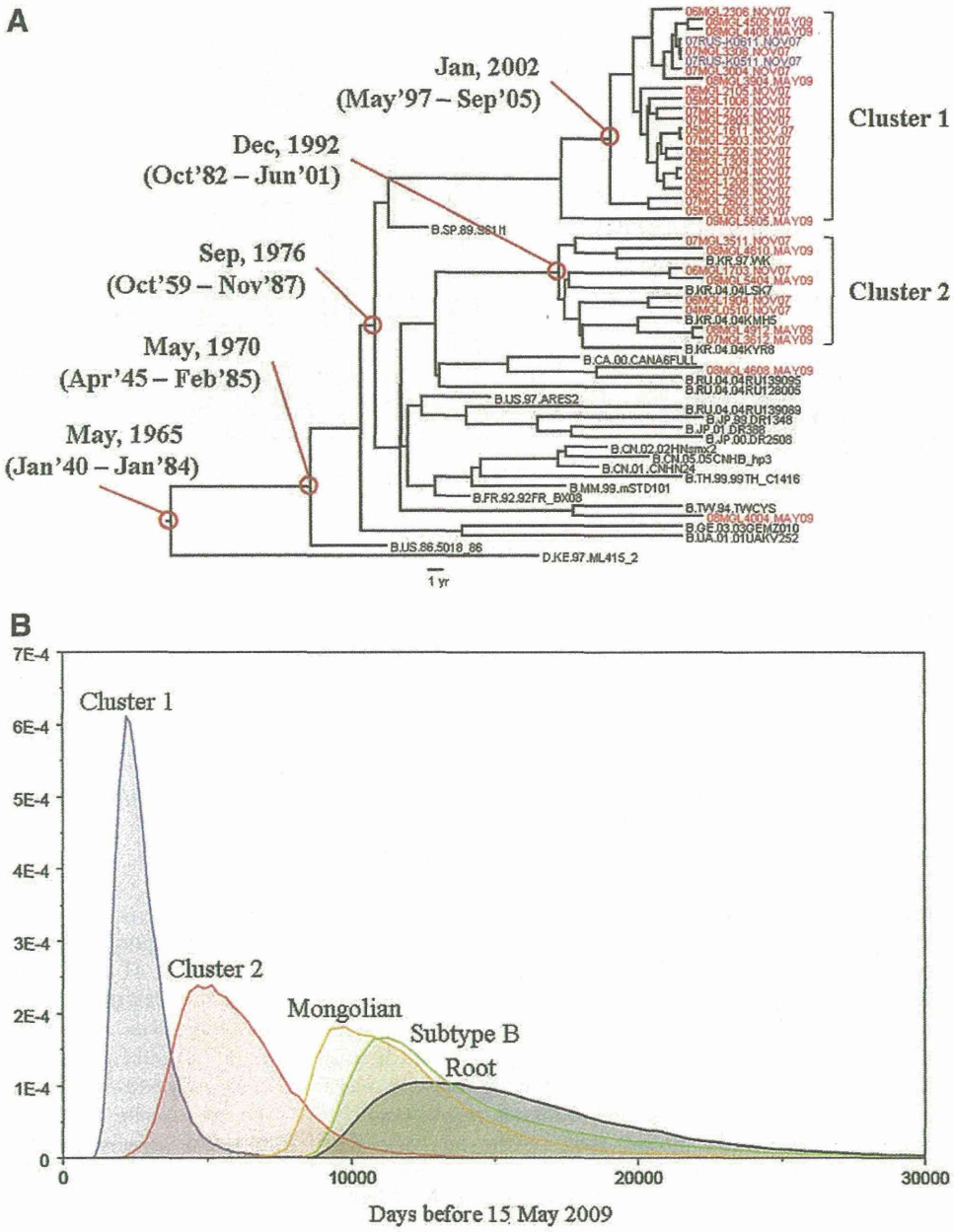


FIG. 3. Bayesian coalescence analysis of *pol* gene HIV-1 subtype B. **(A)** Maximum clade credibility tree of the *pol* gene by Bayesian Markov chain Monte Carlo (MCMC) analysis. The rooted tree illustrates the chronological phylogenetic relationship of 30 Mongolians and 22 reference HIV-1 subtype B *pol* (PR to RT; 1065 nt) sequences. The branch length of phylogeny is in units of time, and a yearly scale is shown under the tree. Red-purple sequence names represent the sequences in Mongolian and Russian patients, respectively. The clusters detected in the neighbor-joining (NJ) trees are annotated by brackets on the right of the tree. Month and year labels with red lines and circles indicate times of the most recent common ancestors (tMRCAs) of corresponding monophyletic groups, and the labels in parentheses indicate the 95% highest posterior density (HPD) interval of the tMRCA. **(B)** Marginal densities of tMRCAs on the Bayesian MCMC analysis. Blue, red, orange, green, and black represent the distribution of tMRCA estimates of cluster 1, 2, whole Mongolian subtype B, subtype B, and root height, respectively.

Two sequences isolated from two Mongolian heterosexual men belonged to subtype G. They were close relatives and seemed to have diverged from the reference sequences of subtype G of Central African (Nigeria) origin. All other remaining Mongolian sequences were from female patients. Two sequences (one was FSW) were close to the reference sequences subtype C of Asian (India) and Northeast African (Ethiopia and Burundi) origins, respectively. The sequences of four sex workers represented CRF02_AG. These sequences were close to reference strains isolated from Uzbekistan, Cameroon, and Ghana. The remaining isolated sequence was CRF01_AE, which was close to reference strains from Vietnam.

Twenty-eight out of the 32 subtype B samples based on the *pol* gene sequences were successfully amplified and the *env* (C2V3) gene region was sequenced and a phylogenetic tree was constructed (Fig. 1B). All samples were also classified as subtype B, indicating that they were not intersubtype recombinant forms if judged from the *pol* and *env* genes. Since the *env* gene region of HIV-1 has high sequence diversity, it is suitable for gene evolution analysis. Nevertheless, the intragroup nucleotide diversity of all 21 sequences on the *env* gene region in cluster 1 was very low, reflecting the rapid expansion of transmission of this lineage of HIV-1 in Mongolia. In contrast, seven other sequences on the *env* gene region belonging to cluster 2 were considerably divergent, suggesting a multiple origin of cluster 2.

Newly diagnosed cases of HIV-1 infection in Mongolia markedly increased in 2005 (Fig. 2, upper panel). Since that year, 7–13 new cases of HIV-1 infection had been diagnosed annually. Although the dominant subtype in Mongolia is currently subtype B, the first subtype-confirmed case in our analysis was CRF02_AG and was detected in 1997 (Fig. 2, lower panel). In 2004, one virus was classified into subtype B.

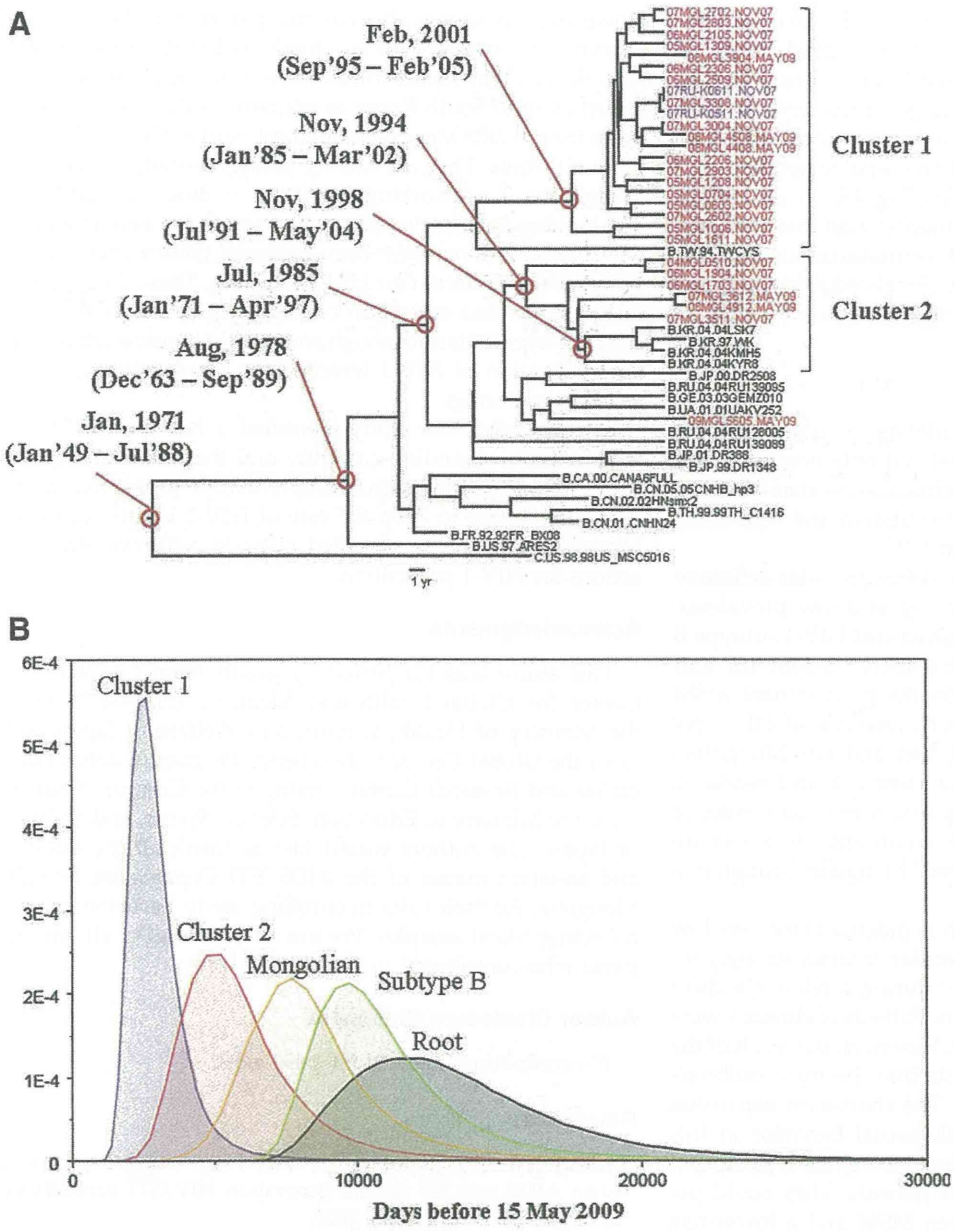


FIG. 4. Bayesian coalescence analysis of the *env* gene of subtype B. **(A)** Maximum clade credibility tree of the *env* gene by Bayesian MCMC analysis. The rooted tree illustrates the chronological phylogenetic relationship of 26 Mongolians and 23 reference HIV-1 subtype B *env* (C2V3; 447 nt) sequences. The branch length of the phylogeny is in units of time, and a yearly scale is shown under the tree. Red-purple sequence names represent the sequences of Mongolian and Russian patients, respectively. The clusters detected in the NJ trees are annotated by brackets on the right of the tree. Month and year labels with red lines and circles indicate tMRCAs of the corresponding monophyletic groups, and the labels in parentheses indicate 95% HPD confidence interval of the tMRCA. **(B)** Marginal densities of tMRCAs on the Bayesian MCMC analysis. Blue, red, orange, green, and black represent the distribution of tMRCA estimates of cluster 1, 2, whole Mongolian subtype B, subtype B, and root height, respectively.

This virus belonged to cluster 2, and was assumed to be of Korean origin. In 2005, viruses from six patients were classified as subtype B while one was subtype C and CRF02_AG, respectively. All subtype B viruses belonged to cluster 1 in our phylogenetic analysis. In 2006, viruses from six patients were also classified as subtype B, and two of them belonged to cluster 2. One of these cluster 2 viruses was also assumed to be of Korean origin (i.e., group 2c). From 2006, cluster 1 viruses had been twice as dominant as cluster 2 viruses. Other subtypes and CRFs were rarely collected in Mongolia.

To assess the result of the distance-based analysis, and to estimate tMRCAs of the Mongolian clusters, we performed a Bayesian coalescent-based phylogenetic inference. Appling the Bayesian relaxed molecular clock method to the phylogeny, the estimated mean evolutionary rates per year per site were 1.90×10^{-3} and 6.66×10^{-3} for *pol* and *env*, respectively (Supplementary Table S1; Supplementary Data are available online at www.liebertonline.com/aid). The estimated mean coefficients of variation were 0.72 and 0.48 for *pol* and *env*, indicating substantial heterogeneity in the evolutionary rate among viral lineage (Supplementary Table S1). The topology of the maximum clade credibility trees of both regions by the Bayesian MCMC analysis was similar to the NJ trees (Figs. 3 and 4), although some differences were observed between trees on the *pol* and *env* regions. One of these differences was found in Korean subtype B sequences in cluster 2. Thus, the Korean sequences formed a monophyletic group in the *pol* tree, but not in the *env* tree. Another notable difference was 09MGL5605.MSM.MAY09, which was close to cluster 1 in the *pol* tree but was related to cluster 2 in the *env* tree. The remaining differences were caused by differences in the samples used for analysis; while we were able to sequence 26 samples for the *env* region, 30 sequences were available for the *pol* region. The Bayesian chronological phylogenies showed that cluster 2 had older ancestors than cluster 1. Based on the estimates of the evolutionary rate, the mean tMRCA of cluster 1

was dated January 2002 on the *pol* region (Fig. 3A) and February 2001 on the *env* region (Fig. 4A). The marginal density of the posterior probabilities of tMRCA in both regions indicated that tMRCA of cluster 1 viruses ranged from 1995 to 2005 (Figs. 3B and 4B). On the other hand, the mean tMRCA of cluster 2 was dated December 1992 on the *pol* region (Fig. 3A) and November 1994 on the *env* region (Fig. 4A). The marginal density of tMRCA of cluster 2 was blunter than that of cluster 1, showing less reliability of tMRCA estimates of cluster 2. The root height of all Mongolian and U.S.-related subtype B reference sequences dated from the late 1960s to early 1970s.

Discussion

It is important to monitor circulating subtypes and the emerging genetic diversity of HIV-1 not only because it has implications for global surveillance but also because it should facilitate risk analysis of HIV-1 transmission and help effective strategies for HIV-1 prevention.[15–19]

The present study is the first report that provides definitive evidence of HIV-1 infection occurring in a low prevalence country, Mongolia. Our results showed that HIV-1 subtype B is responsible for nearly 78% of the analyzed samples, and possibly by sexual network within the predominant MSM (84.8%) risk group. The phylogenetic analyses of HIV-1 *pol* subtype B sequences from Mongolian and non-Mongolian origins showed that sequences of cluster 1 and cluster 2 formed monophyletic groups compared with other viruses of the same and different subtypes from around the world, indicating that HIV-1 subtype B entered Mongolia through two distinct origins.

The most intriguing feature of this epidemic is the very low genetic diversity of cluster 1. Molecular analysis strongly indicated that HIV-1 spread rapidly during a relatively short period with the same ancestor virus. Patients of cluster 1 were diagnosed between 2005 and 2008. However, the result of the Bayesian MCMC analyses suggests that the main outbreak occurred around the early 2000s. The short-term expansion also strongly suggests a high-risk sexual behavior in this population.[20] Although most patients were MSM, the group also included bisexual and female patients. They could potentially serve as a bridge between MSM and a lower-risk population, such as heterosexually active adults. Based on the extremely high prevalence of syphilis in FSW as determined in our nationwide surveillance of HIV/STI in 2007,[2] Mongolia is at high risk of an expansion of HIV-1 infection. In Russia, subtype B is not a major subtype in the total HIV epidemic but is predominant among MSM.[21] However, it is possible that the Russian subtype B strain may become the major strain in the Mongolian population in the future. Based on this, comprehensive preventive measures are urgently needed for this group and our team has already started taking action.

The median evolution rates estimated for Mongolian subtype B in the *pol* (1.9×10^{-3} substitution site per year) and *env* region (6.66×10^{-3} substitution site per year) were comparable with the rates reported previously for these genomic regions for subtype B in other countries (*pol* 2.5×10^{-3} substitution site per year,[22] *env* $5–7\times10^{-3}$ substitution site per year[23,24]). Considering these evolutionary rates, the older origin was probably from Korean HIV-1 subtype B, and first emerged in Mongolia around the early 1990s, almost a decade before the first detection of HIV-1 subtype B in Mongolia. However, this group also has the potential to be a major cluster in the future. This conclusion is based on the demographic data that documented that most of the patients in this group lived in South Korea as migrant workers. At present, more than 10,000 Mongolian migrant workers live and work in South Korea. They are usually young, sexually active, and living alone. Their working and living conditions are unstable and looking for friends and sex partners is not easy in a new environment. Given these circumstances, these workers are a vulnerable population for HIV-1 infection. These data clearly demonstrate that education on HIV-1 infection among the migrant workers is not enough and comprehensive actions for the prevention of HIV-1 infection are needed before these workers go abroad.

In conclusion, our study identified a hot spot of HIV-1 transmission expanding currently and the potential seed of the epidemic in Mongolia. Comprehensive preventive measures are crucial to keep the rate of HIV-1 infection low in Mongolia. Our study provided clues for effective strategic actions for HIV-1 prevention.

Acknowledgments

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Author Disclosure Statement

No competing financial interests exist.

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Impact of Small Body Weight on Tenofovir-Associated Renal Dysfunction in HIV-Infected Patients: A Retrospective Cohort Study of Japanese Patients

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Abstract

Background: Treatment with tenofovir is sometimes associated with renal dysfunction. Limited information is available on this side effect in patients with small body weight, although the use of tenofovir will spread rapidly in Asia and Africa, where patients are likely to be of smaller body weight.

Methods: In a single-center cohort, Japanese patients with HIV infection who started tenofovir-containing antiretroviral therapy were retrospectively analyzed. The incidence of tenofovir-associated renal dysfunction, defined as more than 25% decrement of estimated glomerular filtration rate (eGFR) from the baseline, was determined. The effects of small body weight and body mass index (BMI) on tenofovir-associated renal dysfunction, respectively, were estimated in univariate and multivariate Cox hazards models as the primary exposure. Other possible risk factors were evaluated by univariate analysis and those found significant were entered into the multivariate analysis.

Results: The median weight of 495 patients was 63 kg. Tenofovir-related renal dysfunction occurred in 97 (19.6%) patients (incidence: 10.5 per 100 person-years). Univariate analysis showed that the incidence of tenofovir-related renal dysfunction was significantly associated with smaller body weight and BMI, respectively (per 5 kg decrement, HR = 1.23; 95% CI, 1.10–1.37; $p<0.001$)(per 1 kg/m^2 decrement, HR = 1.14; 95% CI, 1.05–1.23; $p = 0.001$). Old age, high baseline eGFR, low serum creatinine, low CD4 count, high HIV viral load, concurrent nephrotoxic drugs, hepatitis C infection, and current smoking were also associated with tenofovir-related renal dysfunction. Multivariate analysis identified small body weight as a significant risk (adjusted HR = 1.13; 95% CI, 1.01–1.27; $p = 0.039$), while small BMI had marginal significance (adjusted HR = 1.07; 95% CI 1.00–1.16; $p = 0.058$).

Conclusion: The incidence of tenofovir-associated renal dysfunction in Japanese patients was high. Small body weight was identified as an independent risk factor for tenofovir-associated renal dysfunction. Close monitoring of renal function is advocated for patients with small body weight treated with tenofovir.

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Introduction

Tenofovir disoproxil fumarate (TDF) is one of the most widely used nucleotide reverse transcriptase inhibitors (NRTI) for patients with HIV infection, with proven efficacy and safety [1–6]. However, TDF is known to cause renal proximal tubular dysfunction, and several case reports have been published with TDF-related Fanconi syndrome, diabetes insipidus, and acute tubular necrosis, which sometimes lead to acute renal failure [7–10]. Long-term TDF use also reduces glomerular filtration rate more than other NRTIs [11–14]. To date, the nephrotoxic effect of TDF is regarded as mild and tolerable. A recently published meta-analysis has reported that the use of TDF is associated with a statistically significant but only modest renal dysfunction, and recommended that TDF use should not be restricted even when regular monitoring of renal function and serum phosphate levels is impractical [15]. However, the TDF-related renal dysfunction has hardly been evaluated in patients with small body weight, who are potentially at higher risk for larger drug exposure and thus, more severe toxicity [16–19].

The 2010 WHO guideline on antiretroviral therapy for HIV infection in adults and adolescents, usually applied to resource-constrained settings, recommends TDF as one of the components of first line therapies (URL:http://whqlibdoc.who.int/publications/

2010/9789241599764_eng.pdf). It is expected that the use of TDF will spread rapidly in Asia and Africa in the near future, where patients are more likely to be of small body weight. Thus, at this stage, it is important to establish the relationship between TDF-associated renal dysfunction and body weight. A small body weight is considered a risk factor for TDF-associated renal dysfunction, in addition to old age, high baseline serum creatinine level, low CD4 count, concurrent use of ritonavir-boosted protease inhibitor, and concurrent use of nephrotoxic drugs [4,17,19–21]. To our knowledge, there is almost no report that primarily analyzed the influence of body weight on TDF-associated renal dysfunction. Since Japanese are generally of smaller stature and have a lower median body weight than Whites and African Americans, who mostly comprise the cohorts of studies published to date, it is important to investigate the impact of TDF-associated renal dysfunction in Japanese patients.

Based on the above background, the present study was designed to determine the incidence of TDF-associated renal dysfunction in Japanese patients and analyze the impact of small body weight on TDF-associated renal dysfunction.

Methods

Ethics Statement

This study was approved by the Human Research Ethics Committee of National Center for Global Health and Medicine (Text S1). All patients included in this study provided a written informed consent for their clinical and laboratory data to be used and be published for research purposes. This study has been conducted according to the principles expressed in the Declaration of Helsinki.

Study Design and Settings

We performed a single-center, retrospective cohort study of HIV-infected Japanese patients using medical records at the National Center for Global Health and Medicine, Tokyo, Japan. Our facility is one of the largest clinics for patients with HIV infection in Japan with more than 2,700 registered patients.

Study Subjects

The study population were patients >17 years of age who commenced treatment with standard 300 mg/day of TDF-containing antiretroviral regimen at our clinic between January 1, 2002 to March 31, 2009. Both treatment-naive and patients with experience in antiretroviral treatment but not TDF, with an estimated glomerular filtration rate (eGFR) of >60 ml/min/1.73 m^2 were enrolled. Patients were followed up until September 31, 2009. Patients were excluded if their follow-up period at our facility was less than 24 weeks after commencement of TDF-based therapy, if they had started TDF at other facilities, or if there was evidence of prior TDF use. We only included Japanese patients in order to examine a population with comparatively homogenous basic demographics and background.

Measurements

Outcome measure: TDF-associated renal dysfunction. We defined TDF-associated renal dysfunction as more than 25% decrease in eGFR relative to the baseline [17,22,23]. Baseline eGFR was estimated for each patient from the average of two successive serum creatinine measurements made closest to and preceding the commencement of TDF by no more than 90 days. Changes in eGFR were plotted from the baseline measurement until the value diminished to less than 75% of the baseline or at the end of the follow-up period. The eGFR values at occurrence of TDF-associated renal dysfunction, at censoring, and closest to and preceding 24, 48, and 96 weeks to the diagnosis were collected. Patients generally visited our clinic between every month to every 3 months, and measurement of eGFR was usually conducted on every visit. eGFR was calculated using the equation from the 4-variable Modification of Diet in Renal Disease (MDRD) study [24].

Primary exposure variable. Our primary exposure variables were body weight and body mass index (BMI) at the time of commencement of TDF-containing antiretroviral therapy (ART). BMI was calculated by the equation: BMI = [body weight (kg)/height $(m)^2$].

Other variables: potential risk factors. Potential risk factors for TDF-associated renal dysfunction were determined according to previous studies and collected together with the basic demographics from the medical charts [4,19,20,25]. They included sex, age, baseline laboratory data: CD4 cell count, HIV viral load, and serum creatinine, and other medical conditions (antiretroviral treatment-naïve or experienced, concurrent ritonavir-boosted protease inhibitors, concurrent nephrotoxic drugs such as ganciclovir, sulfamethoxazole/trimethoprim, ciprofloxacin, and NSAIDs, diabetes mellitus, co-infection with hepatitis B defined by positive hepatitis B surface antigen, co-infection with hepatitis C defined by positive HCV viral load, hypertension defined by current treatment with antihypertensive agents, dyslipidemia defined by current treatment with lipid-lowering agents, and current smoking) [26]. We used the data on or closest to and preceding the day of starting TDF-containing ART by no more than 90 days. The data on weight change from the baseline to the end of follow-up period and the frequency of eGFR monitoring for each patient were collected.

Statistical analysis

The time to 25% decline in eGFR from the baseline was calculated from the date of treatment initiation to the date of occurrence of TDF-associated renal dysfunction. Censored cases represented those who discontinued TDF, dropped out, referred to other facilities, or at the end of follow-up period. The time from TDF initiation to 25% decrease in eGFR was analyzed by the Kaplan Meier method for the whole cohort. To estimate the impact of body weight on the incidence of TDF-associated renal dysfunction, we calculated the impact of every 5 kg decrement from the median weight using Cox proportional hazards regression analysis. The impact of every 1 kg/m^2 decrement in BMI on the incidence of TDF-associated renal dysfunction was estimated by the same method. The impact of each basic demographics, baseline laboratory data, and other medical conditions listed above was also estimated with univariate Cox proportional hazards regression.

To estimate the unbiased prognostic impact of weight on TDF-associated renal dysfunction, we conducted three models using multivariate Cox proportional hazards regression analysis. Model 1 was the aforementioned univariate analysis for every 5 kg decrement. Model 2 included sex, age plus model 1 in order to adjust for basic characteristics. In model 3, we added variables with P values<0.05 in univariate analysis for adjustment (these included age per 10 years, serum creatinine >0.8 mg/dl, CD4 count <200/μl, HIV viral load per log10/ml, concurrent nephrotoxic drugs, co-infection with hepatitis C, and current smoking). Concurrent ritonavir-boosted protease inhibitors were also added in Model 3 although their p value was 0.116 in the univariate analysis. This was based on the results of several studies suggesting that concurrent use of ritonavir-boosted protease inhibitors is a risk factor for TDF-associated renal dysfunction

[19,20]. The eGFR was excluded from multivariate analysis because of its multicollinearity with sex, age, and serum creatinine, since eGFR was gained by the equation of those variables. The impact of every 1 kg/m^2 decrement in BMI on the incidence of TDF-associated renal dysfunction was estimated by the same method with Model 1 to Model 3.

Four other analyses were conducted to further examine the relationship between low body weight and TDF-associated renal dysfunction. First, the time from initiation of TDF therapy to 25% decrease in eGFR was analyzed by the Kaplan Meier method for intertertile baseline body weight categories: <59, 59–67, and >67 kg. The log-rank test was used to determine statistical significance. Second, to investigate the impact of changes in muscle mass on changes in the eGFR as calculated by MDRD, we compared weight changes with one-way ANOVA among intertertile baseline weight categories. We also conducted the sensitivity analysis by adding the variable "weight change" in multivariate analysis. Third, the median and interquartile value for the actual fall in eGFR from the baseline to 24, 48, and 96 weeks for the whole cohort and three baseline weight categories, respectively, were calculated. The eGFR value at 24, 48, and 96 weeks included those that were censored before reaching 24, 48, and 96 weeks, respectively, so that we could interpret the data for actual fall in eGFR, including not only survived cases but also censored cases. Fourth, we counted the number of patients whose eGFR decreased to <60 and <10 ml/min/1.73 m^2, and who discontinued TDF with the clinical diagnosis of renal dysfunction due to TDF. Chi-square test was used to determine whether the difference among the weight categories was statistically significant.

Statistical significance was defined at two-sided p values< 0.05. We used hazard ratios (HRs) and 95% confidence intervals (CIs) to estimate the impact of each variable on TDF-associated renal dysfunction. All statistical analyses were performed with The Statistical Package for Social Sciences ver. 17.0 (SPSS, Chicago, IL).

Results

Between January 1, 2002 to March 31, 2009, 599 patients started TDF-containing ART (Figure 1). Of these, 104 patients were excluded based on the abovementioned criteria. Thus 495 patients were included in the present study (Dataset S1). Table 1 shows the demographics, laboratory data, and medical conditions of the study population at baseline. Two patients received ART with 3 NRTIs, 3 patients received ART with one protease inhibitor (PI), one non-NRTI (NNRTI), and tenofovir/emtricitabine, and the remaining patients had a standard ART with 2 NRTIs and either PI, NNRTI, or integrase inhibitor (INI). The median body weight and BMI were 63 kg and 21.9 kg/m^2, respectively. The median age of the patients was 38 years and 95.2% were males. The eGFR was well maintained (median: 120.9 ml/min/1.73 m^2), and the median baseline CD4 count was 247/μl. Of the total, 208 patients (42%) were antiretroviral treatment naïve, while 287 were treatment-experienced patients. Viral load was suppressed to <50 copies/ml in 162 (32.7%) patients. 403 (81.4%) were on concurrent PIs as the key drug, 367 (74.1%) were on ritonavir-boosted PIs, and only 83 (16.8%) had NNRTIs as the key drug. Smoking was prevalent among the study population, as 240 (48.5%) were identified as a current smoker.

TDF-associated renal dysfunction defined by more than 25% decrease of eGFR from baseline occurred in 97 patients (19.6%), with an estimated incidence of 10.5 per 100 person-years. The median time from commencement of TDF to occurrence of TDF-associated renal dysfunction was 39 weeks (IQR 13.5–99.4 weeks) (range: 1–1,841 days). The total observation period was 924.7 patient-years (median 72 weeks, IQR 38.6–139.3 weeks). Figure 2 shows the Kaplan-Meier survival curve for the occurrence of TDF-associated renal dysfunction for the whole cohort.

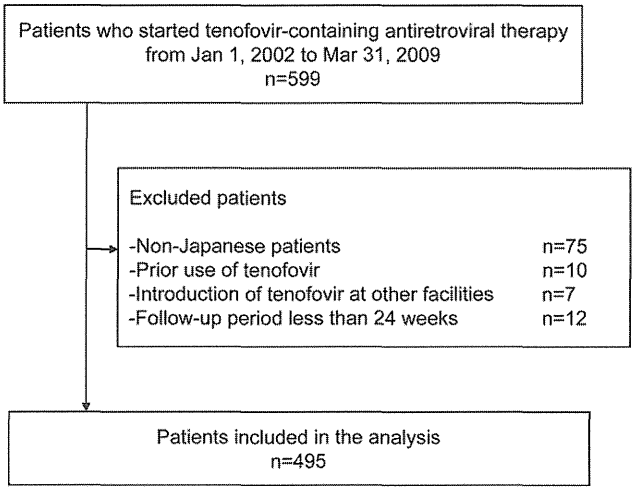

Figure 1. Flow diagram of patient selection.
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Univariate analysis showed a significant relationship between TDF-associated renal dysfunction and every 5 kg less than the median body weight (HR = 1.23; 95% CI, 1.10–1.37; p<0.001), and 1 kg/m^2 less BMI than the median BMI (HR = 1.14; 95% CI, 1.05–1.23; p = 0.001) (Table 2). Furthermore, old age, high eGFR, low serum creatinine, low CD4 counts, high HIV viral load, concurrent use of nephrotoxic drugs, presence of chronic hepatitis C, and smoking were associated with TDF-related renal dysfunction. On the other hand, concurrent use of PIs, ritonavir boosted PIs, and LPV/r tended to be associated with TDF-related renal dysfunction, albeit statistically insignificant. Treatment-naïve or Treatment-experienced was not associated with TDF-related renal dysfunction.

Multivariate analysis showed that every 5 kg less than the median body weight was a significant risk for TDF-associated renal dysfunction after adjustment for sex and age (adjusted HR = 1.21; 95% CI, 1.07–1.36; p = 0.002) (Table 3, Model 2), and also after adjustment for other risk factors (adjusted HR = 1.13; 95% CI, 1.01–1.27; p = 0.039) (Table 3, Model 3). Similarly, every 1 kg/m^2 less than the median BMI was also a significant risk factor for TDF-associated renal dysfunction even after adjustment for sex and age (adjusted HR = 1.13; 95% CI 1.05–1.22; p = 0.002) (Table 4, Model 2), and tended to be a significant factor after adjustment for other variables (adjusted HR = 1.07; 95% CI 1.00–1.16; p = 0.058) (Table 4, Model 3). Old age and current smoking were also independent risk factors in both multivariate analysis for body weight and BMI (Table 3, Model 3 and Table 4, Model 3).

In complementary analyses, First, Figure 3 shows the relation between probability of TDF-associated nephrotoxicity and time from initiation of TDF therapy to 25% decrease in eGFR analyzed by the Kaplan Meier method for intertertile baseline weight categories. Compared to patients with baseline body weight >67 kg, patients with baseline weight <59 kg were significantly more likely to develop >25% decline in eGFR (p = 0.002). On the other hand, the difference in this probability between patients with baseline weight 59–67 kg and those >67 kg was only marginally significant (p = 0.073, log-rank test). Secondly, one-way ANOVA

Table 1. Baseline demographics and laboratory data.

Characteristics		
Median (IQR) weight (kg)	63	(57–69)
Median (IQR) BMI (kg/m^2)	21.9	(20.3–23.8)
Male, n (%)	471	(95.2)
Median (IQR) age	38	(33–46)
Median (IQR) eGFR (ml/min/1.73 m^2)	120.9	(104.8–138.2)
Median (IQR) serum creatinine (mg/dl)	0.72	(0.64–0.81)
Median (IQR) CD4 count (/μl)	247	(159–371)
Median (IQR) HIV viral load (log10/ml)	3.73	(1.60–4.81)
HIV viral load <50 copies/ml, n (%)	162	(32.7)
Antiretroviral therapy naïve, n (%)	208	(42.0)
Key drugs, n (%)*		
PIs	403	(81.4)
Ritonavir-boosted PIs	367	(74.1)
LPV/r	175	(35.4)
ATV/r	131	(26.5)
FPV/r	52	(10.5)
DRV/r	9	(1.8)
FPV	14	(2.8)
ATV	4	(0.8)
NFV	15	(3)
SQV	2	(0.4)
IDV	1	(0.2)
NNRTIs	83	(16.8)
EFV	65	(13.1)
NVP	17	(3.4)
ETR	1	(0.2)
INI		
RAL	10	(2.0)
Concurrent use of nephrotoxic drug, n (%)	131	(26.5)
Diabetes mellitus, n (%)	30	(6.1)
Hepatitis B, n (%)	75	(15.2)
Hepatitis C, n (%)	52	(10.5)
Hypertension, n (%)	28	(5.7)
Dyslipidemia, n (%)	40	(8.1)
Smoking, n (%)	240	(48.5)
Median (IQR) weight change (kg)	0.0	(−2.0–2.25)
Median (IQR) frequency of eGFR monitoring	16	(9.0–27)

(n = 495).
*Two patients did not take any key drugs. Three patients took both PI and NNRTI.
IQR: interquartile range, BMI: body mass index, eGFR: estimated glomerular filtration rate, PI: protease inhibitor, LPV/r: lopinavir/ritonavir, ATV: atazanavir, FPV: fosamprenavir, DRV: darunavir, NFV: nelfinavir, SQV: saquinavir, IDV: indinavir, NNRTI: non-nucleos(t)ide reverse transcriptase inhibitor, EFV: efavirenz, NVP: nevirapine, ETR: etravirine, INI: integrase inhibitor, RAL: raltegravir.
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showed that weight changes among the three baseline weight categories were not significantly different (p = 0.206). Sensitivity analysis after adding the variable "weight change" in Model 3 multivariate analysis (Table 3) showed that adjusted hazard ratio for weight per 5 kg decrement hardly changed (adjusted HR 1.131; 95% CI, 1.007–1.271; p = 0.038). Thirdly, Table 5 shows

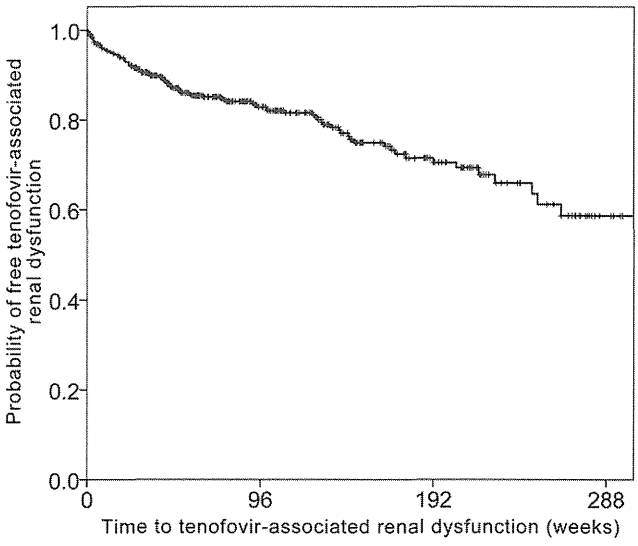

Figure 2. Kaplan-Meier curve showing the time to 25% reduction in eGFR for the whole cohort. eGFR: estimated glomerular filtration rate.
doi:10.1371/journal.pone.0022661.g002

the median and interquartile values for the actual falls in eGFR from the baseline to 24, 48, and 96 weeks. The eGFR decreased gradually in all categories, except for patients with baseline weight

Table 2. Univariate analysis for TDF-associated renal dysfunction.

	HR	95%CI	P value
Weight per 5 kg decrement	1.23	1.10–1.37	<0.001
BMI per 1 kg/m^2 decrement	1.14	1.05–1.23	0.001
Male gender	0.54	0.26–1.11	0.094
Age per 10 years	1.22	1.02–1.45	0.027
eGFR per 10 ml/min/1.73 m^2	1.10	1.05–1.15	<0.001
Serum creatinine >0.8 mg/dl	0.51	0.30–0.88	0.014
CD4 count <200/μl	1.97	1.32–2.93	0.001
HIV viral load per log10/ml	1.15	1.01–1.30	0.037
Antiretroviral therapy naïve	0.98	0.63–1.52	0.927
Concurrent key drugs			
Any PIs	1.52	0.89–2.59	0.124
Ritonavir boosted PIs	1.46	0.91–2.33	0.116
LPV/r	1.45	0.97–2.17	0.072
ATV/r	1.05	0.66–1.68	0.826
Concurrent nephrotoxic drug	1.59	1.04–2.42	0.031
Diabetes mellitus	1.57	0.76–3.24	0.220
Hepatitis B	1.36	0.82–2.24	0.231
Hepatitis C	1.80	1.07–3.04	0.028
Hypertension	1.18	0.51–2.69	0.702
Dyslipidemia	0.97	0.47–2.00	0.932
Smoking	1.57	1.05–2.36	0.028

TDF: tenofovir, HR: hazard ratio, CI: confidence interval, BMI: body mass index, eGFR: estimated glomerular filtration rate, PI: protease inhibitor, LPV/r: lopinavir/ritonavir, ATV: atazanavir.
doi:10.1371/journal.pone.0022661.t002

Table 3. Multivariate analysis to estimate the effect of lower body weight on TDF-associated renal dysfunction.

	Model 1 Crude		Model 2 Adjusted		Model 3 Adjusted	
	HR	95%CI	HR	95%CI	HR	95%CI
Weight per 5 kg decrement¶	1.23	1.10–1.37	1.21	1.07–1.36	1.13	1.01–1.27
Male gender			0.88	0.41–1.89	0.57	0.26–1.26
Age per 10 years¶			1.16	0.98–1.38	1.24	1.04–1.49
Serum creatinine >0.8 mg/dl					0.62	0.35–1.07
CD4 count <200/μl					1.65	0.97–2.79
HIV viral load per log10/ml					1.05	0.90–1.23
Boosted PIs					1.54	0.93–2.54
Concurrent use of nephrotoxic drug					1.23	0.77–1.97
Hepatitis C					1.57	0.92–2.69
Smoking¶					1.65	1.09–2.48

¶P<0.05 in Model 3.
TDF: tenofovir, HR: hazard ratio, CI: confidence interval, PI: protease inhibitor.
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>67 kg. Fourthly, the number (percentage) of patients whose eGFR decreased to <60 ml/min/1.73 m^2 was not different among the baseline weight categories (p = 0.229), whereas the number of patients who discontinued TDF with a clinical diagnosis of renal dysfunction due to TDF varied significantly according to body weight (p = 0.001, chi-square test, Table 6). None of the patients showed reduction of eGFR to <10 ml/min/1.73 m^2.

Discussion

In this Japanese cohort, 19.6% of the patients experienced eGFR decline of more than 25% from the baseline after commencement of TDF. The incidence of TDF-associated renal dysfunction was 10.5 per 100 person-years. Multivariate analysis identified smaller body weight and smaller body mass index as significant and almost significant factors, respectively, for TDF-associated renal dysfunction.

The incidence of TDF-associated renal dysfunction in patients with small body weight might be higher than previously reported in studies of patients with larger statures. Studies from North America, Europe, and Australia reported an incidence of <1% to 4.3% for TDF-related renal dysfunction, although the definition used for the diagnosis of renal impairment was different among the studies and varied from an increase in serum creatinine from >0.5 to >2 mg/dL from baseline [1–3,5,13]. Several studies conducted in these regions indicated that the range of patients' mean body weight was 69–74 kg, indicating that their patients were heavier than those of the present study with a median weight of 63 kg [2,6,12,14]. The impact of the comparatively lower body weight seems stronger in our patients probably because they do not appear to have many of the other established risk factors for TDF-associated renal dysfunction despite the high incidence of 10.5 per 100 person-years. For example, they were comparatively young with a median age of 38 years, CD4 count was relatively maintained, and approximately 30% had suppressed HIV viral load at baseline (Table 1). Furthermore, they were less likely to have hypertension, dyslipidemia, and diabetes mellitus.

Table 4. Multivariate analysis to estimate the impact of BMI decrement on TDF-associated renal dysfunction.

	Model 1 Crude		Model 2 Adjusted		Model 3 Adjusted	
	HR	95%CI	HR	95%CI	HR	95%CI
BMI per 1 kg/m^2 decrement	1.14	1.05–1.23	1.13	1.05–1.22	1.07	1.00–1.16
Male gender			0.67	0.32–1.38	0.48	0.23–1.03
Age per 10 years¶			1.20	1.01–1.43	1.27	1.06–1.52
Serum creatinine >0.8 mg/dl					0.60	0.35–1.04
CD4 count <200/μl					1.64	0.97–2.79
HIV viral load per log10/ml					1.05	0.90–1.23
Boosted PIs					1.49	0.90–2.45
Concurrent use of nephrotoxic drugs					1.22	0.76–1.94
Hepatitis C					1.62	0.94–2.76
Smoking¶					1.63	1.08–2.46

¶P<0.05 in Model 3.
BMI: body mass index, TDF: tenofovir, HR: hazard ratio, CI: confidence interval, PI: protease inhibitor.
doi:10.1371/journal.pone.0022661.t004

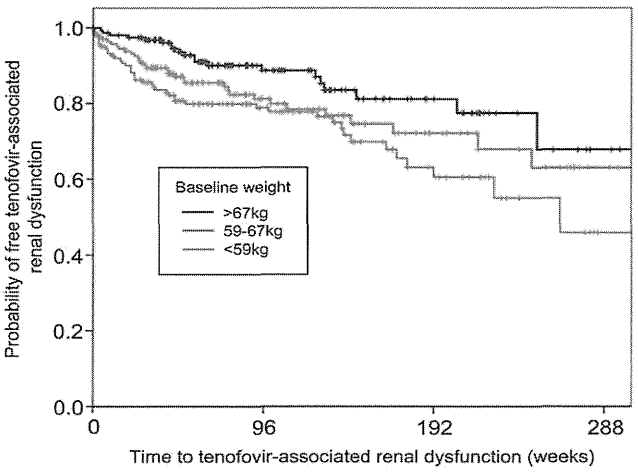

Figure 3. Kaplan-Meier curve showing the time to 25% reduction in eGFR according to baseline weight categories. Compared to patients with body weight >67 kg, those with weight <59 kg were more likely to develop >25% decline in eGFR (P = 0.002), whereas those with weight 59–67 kg showed only a marginal significance (P = 0.073, log-rank test). eGFR: estimated glomerular filtration rate.
doi:10.1371/journal.pone.0022661.g003

The results of multivariate analysis that each 5 kg decrement in body weight was significantly associated with TDF-associated renal dysfunction but not each 1 kg/m^2 decrement in BMI suggests that weight might be more useful and handy information to estimate the risk for TDF-associated renal dysfunction than BMI. Thus, patient's body weight is an important risk factor to consider at the time of TDF prescription.

Our study is one of a few that have examined the impact of TDF-associated renal dysfunction in patients with small body weight, but is the first to examine the impact of small body weight as a primary exposure by creating the model used for multivariate analysis [16–18]. One study from Thailand that included patients with a median weight of 56.5 kg reported a similar incidence of 16.2 per 100 person-years for developing TDF-associated renal dysfunction [17]. They concluded that the small body weight of their patients was probably associated with the high incidence of TDF-associated renal dysfunction. Our study confirmed that conclusion and provided statistically-backed evidence that small body weight is a significant risk factor of TDF-associated renal dysfunction by using a multivariate model with least multi-collinearity to evaluate the impact of small body weight. The results of the present study could be applied to many countries in Asia and Africa, where stature and body weight of the population are comparatively smaller.

This study adopted a decrease in eGFR of >25% as a definition for TDF-associated renal dysfunction. This criterion is one of common methods in evaluating renal function [22,23]. Using this definition, however, does not mean that all patients with >25% fall in eGFR have severe renal dysfunction. However, the definition of renal dysfunction based on a fall in eGFR of >25% is probably more sensitive than that based on eGFR <60 ml/min/1.73 m^2, in patients with comparatively good baseline renal function, such as patients of our study. Adopting this definition could be useful in detecting early renal dysfunction and in the clinical decision making regarding the need for certain interventions, for example, discontinuation of TDF. Early detection of renal dysfunction is particularly important in patients with HIV infection, because kidney disease may be associated with AIDS and death, and TDF-associated renal dysfunction might be irreversible [27,28].

Since the calculation of eGFR using the MDRD formula is based on serum creatinine, age, race and gender, any fall in eGFR is influenced by hypercreatininemia caused by increased muscle mass [29]. It is possible that the muscle mass increases in patients on ART, especially those with low weight at baseline compared to those with higher weight, reflecting reversal of wasting in those patients who were most malnourished. Such increase in muscle mass could then result in a fall in eGFR despite no change in actual renal elimination of creatinine. However, complementary analysis showed that weight change throughout the follow-up period was not significantly different among patients with different baseline weight, and the sensitivity analysis demonstrated that weight change did not alter the significance of every 5 kg decrement.

In the present study, high eGFR and low serum creatinine levels at baseline were identified as risk factors for falls in eGFR of more than 25%, in contrast to several previous studies that showed high serum creatinine and low eGFR were risk factors [4,10,25]. While the exact reason for this discrepancy is unknown at present, it could be related to differences in the definition of TDF-associated renal dysfunction. The aforementioned Thai study used the same definition applied in the present study and a Canadian study that used the definition of 1.5-fold increase in serum creatinine from baseline also reported high eGFR and low serum creatinine level at baseline as risk factors [17,30]. Thus, it is plausible to observe a fall in eGFR when the baseline value is high, since Horberg et al. reported that patients with baseline eGFR of >80 ml/min/1.73 m^2 were likely to show a pronounced fall in eGFR with TDF use [31].

Multivariate analysis also suggested that old age and current smoking are significant risks for TDF-associated renal dysfunction

Table 5. Median and interquartile range of the actual fall in eGFR from the baseline to 24, 48, and 96 weeks, according to body weight.

	Total (n = 495)		<59 kg (n = 167)		59–67 kg (n = 168)		>67 kg (n = 160)	
	fall in eGFR(ml/min/1.73 m^2)		fall in eGFR		fall in eGFR		fall in eGFR	
	median	IQR	median	IQR	median	IQR	median	IQR
to 24 weeks	7.8	(−1.7–18.1)	9.8	(−3.6–22.6)	6.8	(−1.5–17.3)	7.3	(−1.8–15.4)
to 48 weeks	9.0	(−0.7–21.9)	13.0	(−0.2–29.3)	7.2	(−1.2–20.0)	8.1	(−0.6–18.6)
to 96 weeks	9.3	(−0.5–23.1)	13.4	(1.2–33.2)	8.6	(−0.2–21.7)	7.5	(−2.4–19.8)

eGFR: estimated glomerular filtration rate, IQR: interquartile range.
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