		JRFL CTRPNNNTRK_SIHIGPGRAFYTTGEIIGDIRQAHC	Clone	Net charge
Case2	KF8	----------_G--M------F-DN_------K---	12	6
		---------R_G--M------F-DN_------K---	2	6
		----------_G--M---G--F-DN_------K---	1	5
		----------_G--M------F-DN_------K-Y-	1	5
	KF6	-----AI-K-RHF----------NN_KV----K---	2	10
		-----AI-KRRHF----------NK_-V----K---	1	9
		-----AI-K-RHF----------N_KV----K---	1/20	10
Case3	T02	----------_----------FA-D_---N--K-Y-	16	6
		----------_----------FA-D_------K-Y-	2	5
		----S-----_----------FA-D_------K-Y-	1	5
	T16	------KVIRRR---------VA-D_TT----K-Y-	3/22	7

Figure 5 | Cloned V3 sequences in Cases 2 and 3. The V3 sequence of HIV-1 JRFL is shown at the top column as a reference. Amino acids identical to those of HIV-1 JRFL are indicated as dashes. The numbers of clones harboring the corresponding V3 sequences are shown on the right.

(2.7%) dual-tropic, and 7 of 3301 (0.2%) CCR5-tropic sequences. Their frequency was significantly higher in CXCR4-tropic and dual-tropic sequences than CXCR5-tropic ones ($p < 0.0001$; Chi-square test). (All of the 7 CCR5-tropic sequences with R insertion at position 11 were sub-clones derived from one pair of a transmitter mother and her transmitted child[33], and the sequences were so unique that it was actually difficult to determine the exact site of one amino acid insertion). Interestingly, all of the 28 V3 sequences with R insertion at position 11, had lost the N-linked glycosylation site and had one amino acid deletion in the C-terminal half of V3 (one amino acid deletion at position 24 or 25 in 18 sequences [64.3%]), similar to our three cases. No other amino acid elongation patterns were found in the N-terminal half of V3 in the Los Alamos database. There were 3,301 CCR5-tropic V3 sequences registered in the database. Among them, 18 sequences had a basic amino acid residue at position 11 and therefore they were misjudged as CXCR4-tropic by the 11/25 rule. Only 7 of them had R insertion and the other 11 were recognized as CCR5-tropic by our rules. Therefore using our rules increased the specificity from 99.5% [(3,301–18)/3,301] to 99.8% [(3,301–7)/3,301] in identifying CXCR4- or dual-tropic V3 sequences in the Los Alamos database.

Considered together, amino acid elongation may be a rare event, but R insertion at position 11 sometimes occurs. The occurrence of such insertion seems to be always accompanied by loss of the N-linked glycosylation site and deletion of one amino acid in the C-terminal half of V3. The combination of these mutations usually confers CXCR4-tropism. Awareness of this rule will help to confirm the tropism prediction from V3 sequences by conventional rules.

Methods

Patients. Case 1 was an ART-naive Japanese hemophiliac who acquired HIV-1 subtype B infection through contaminated blood product before 1985 and exhibited slow disease progression, as described previously (KI-127)[13]. The study also included 53 other treatment-naïve HIV-1 subtype B-infected patients with $CD4^+$ cell count < 200/mm^3, who were newly diagnosed in 2008. The ethics committee of The National Center for Global Health and Medicine approved the study and all participants provided written informed consent.

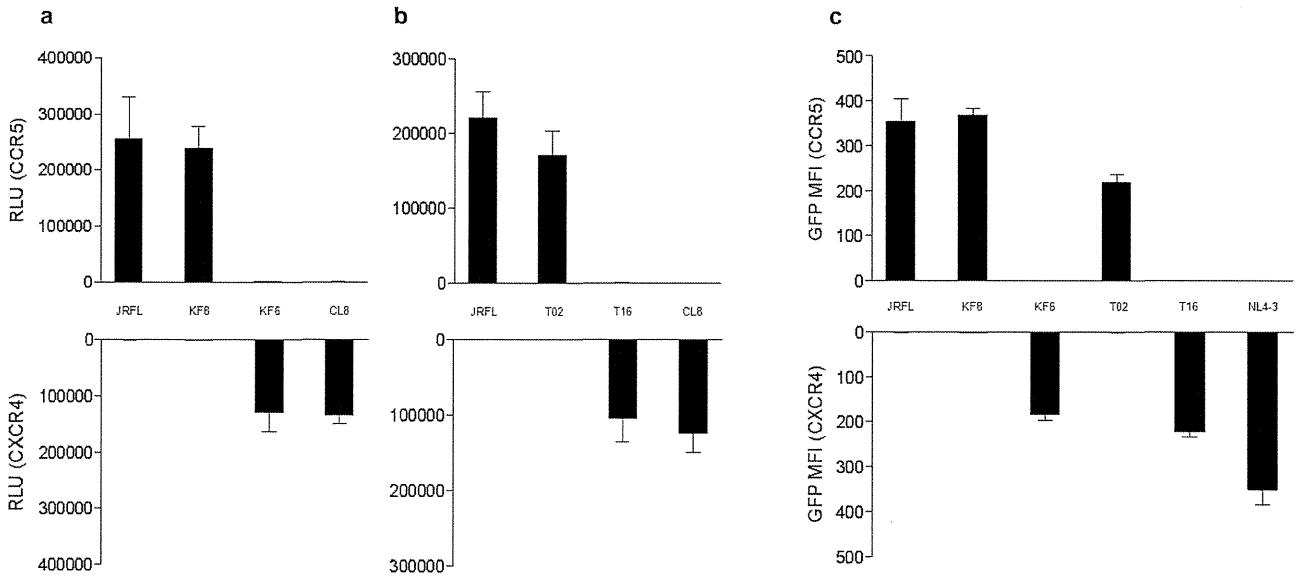

Figure 6 | Tropism of cloned V3 incorporated into JRFL gp120 backbone (a and b). Two distinct V3 clones from each of Case 2 (a) and Case 3 (b) were analyzed with the reference of JRFL-V3 and CL8-V3. Cell-cell fusion assay was performed using Env-expressing 293T cells and $CD4^+$ and $CCR5^+$/$CXCR4^+$ COS-7 cells. Data are mean±SD values in relative luminescent unit (RLU) of six experiments (performed in duplicate and repeated three times). Tropism of recombinant HIV-1 variants harboring V3 sequences derived from Cases 2 and 3 (c). Tropism of HIV-1 variants was assessed in $CCR5^+$ GHOST Hi5 and $CXCR4^+$ GHOST CXCR4 cells. The mean fluorescent intensity (MFI) of infected cells expressing green fluorescent protein (GFP) was measured. Data are mean±SD values of six experiments (performed in duplicate and repeated three times).

Cells. The 293 T and COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX). Parental GHOST cells[34] were cultured in DMEM supplemented with 10% FBS, 500 μg/ml G418 and 100 μg/ml hygromycin B. CCR5$^+$ GHOST Hi5 and CXCR4$^+$ GHOST CXCR4 cells[34] were cultured in DMEM supplemented with 10% FBS, 500 μg/ml G418, 100 μg/ml hygromycin B and 1 μg/ml puromycin.

Amplification, cloning and sequencing of Env V3 region. Total RNA was extracted from 200 μl of plasma using High Pure Viral RNA Kit (Roche, Indianapolis, IN) according to the instructions supplied by the manufacturer. HIV-1 cDNA was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) using the One Step RNA PCR kit (TaKaRa Bio, Kyoto, Japan). The DNA fragments were amplified by using the Ex Taq Hot Start Version (TaKaRa Bio) with the primer sets as follows. The Env fragment containing V3 region was amplified by RT-PCR with primers of C2 (5′ - AATGTCAGCACAGTACAATGTACAC - 3′) and C3 (5′ - ACAATTTCTGG GTCCCCTCCTGAGGA - 3′). S1 (5′ - ATGGAATTAGGCCAGTAGT - 3′) and A1 (5′ - CTCTTAATTTTATAACTATC - 3′) primer sets were used for nested PCR. The amplified PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and cloned by using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. At least 19 colonies were selected, inoculated into 4 ml of L broth, and incubated at 37°C overnight under vigorous agitation. In the next step, plasmids were isolated by using the QIAprep Spin Miniprep Kit (Qiagen). The purified plasmids were 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).

Plasmid construction. The pcDNA6.2-CCR5 and pcDNA6.2-HIV-tat plasmids were constructed as described previously[35]. Briefly, the entire human CCR5 gene including a stop codon was amplified using pZeoSV-CCR5[36] as a template. The PCR product was ligated into pcDNA6.2/cLumio-DEST vector (Invitrogen), cloned using the method recommended by the manufacture, and termed as pcDNA6.2-CCR5. The CD4 expression vector (pcDNA6.2-CD4) and CXCR4 expression vector (pcDNA6.2-CXCR4) were generated using the same method. The CCR5-tropic HIV-1 JRFL[37] Env expression vector (pCXN-JRenv) and pLTR-LucE were used as described previously[35]. The full length Env and part of the Nef encoding regions of the HIV-1 genome was amplified using pHIV-1 JRFL. The PCR product was ligated into pGEM-T Easy Vector System (Promega, Madison, WI), cloned using the protocol supplied by the manufacturer, and termed as pGEM-T Easy-Env. Amino acid substitutions, insertion and deletion were introduced into the V3 region of pGEM-T Easy-Env using the Quikchange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) and applying the protocols supplied by the manufacturer. The V3 regions of pGEM-T Easy-Env containing mutations were digested with *StuI* and *XhoI*, and the obtained fragments were introduced into pCXN-JRenv or pHIV-1 JRFL.

Cell-cell fusion assay. The assay was conducted as described in detail previously[35]. Briefly, the JRFL Env expression vector (WT or mutant) and Tat expression vector (0.5 μg each) were cotransfected into 293 T cells (2×10^5) using Lipofectamine 2000 (Invitrogen), while the CD4, CCR5 or CXCR4 expression vector and a reporter (luciferase) gene containing plasmid, pLTR-LucE (0.5 μg each) were cotransfected into COS-7 cells (2×10^5). On the next day, both cotransfected cells were harvested and mixed in a well of 96-well plates (2×10^4 cells each). The cotransfected cells were incubated further for 6 hrs and the luciferase activity in each well was detected using Bright-Glo Luciferase Assay System (Promega) and its luminescence level was measured using Wallac ARVO Sx 1420 multilabel counter (Perkin-Elmer, Waltham, MA).

MD simulation. MD simulation of gp120 outer domain containing V3 loop was performed as described previously[17,18] with some modifications. Initially, the gp120 outer domain structures with various V3 elements were constructed by homology modeling[38,39] using the Molecular Operating Environment (MOE) ver. 2008.10 (Chemical Computing Group Inc., Montreal, Quebec, Canada), as described previously[17,18]. As a modeling template, we used the crystal structure of HIV-1 gp120 containing the entire V3 element (PDB code: 2B4C)[19]. Subsequently, MD simulations were performed for individual models using the SANDER module in the AMBER 9 program package[40,41]. Heating calculations were achieved for 100 picoseconds until 310 K and MD simulations were subsequently executed at 310 K for 10 nanoseconds. The time step was set to 2.0 femtoseconds. The AMBER ff03ua force field[42] and the GB implicit solvent function by Hawkins, Cramer, and Truhlar[43,44] were applied. The "no cutoff" calculation was applied for the non-bonded energy calculation. In this study, we analyzed most frequently observed conformation among 5,000 snapshots obtained from 5.0–10.0 ns of MD simulation, which was selected by the Bayesian clustering algorithm[45].

Calculation of the RMSD. We compared the orientation of V3 loop between two gp120 outer domain models by the following procedure. We first superimposed two models by coordinating main chain atoms (N, Cα, and C) in amino acid residues other than those in the V3 loop using PyMOL ver. 0.99 rc6 (Schrödinger LLC, Portland, OR, http://www.pymol.org/). Subsequently, the RMSD values for the V3 loop tip (GPGR) between the two models were calculated using the coordinates of the main chain atoms using the in-house program.

Viral tropism assay. The wild type CCR5-tropic HIV-1 strain, pHIV-1 JRFL, CXCR4-tropic HIV-1 strain, pHIV-1 NL4-3[46], and each pHIV-1 JRFL Env derived from mutations containing the V3 region of pGEM-T Easy-Env were transfected into 293 T cells with Lipofectamine 2000 (Invitrogen), and the obtained infectious clonal viruses were harvested 48 hrs after transfection and stored at −80°C until use. The GHOST cell infection assay[25,47] was performed by incubating 1 ml containing 20 ng of p24 antigen of each virus with GHOST cells (2×10^4). Parental GHOST, CCR5$^+$ GHOST Hi5, and CXCR4$^+$ GHOST CXCR4 cells were infected for 72 hrs and then harvested. The mean fluorescent intensity (MFI) of infected cells expressing green fluorescent protein (GFP) was measured on a flow cytometer (FACSCalibur; BD Bioscience, San Jose, CA). GHOST cells express low levels of CXCR4 and therefore infection of GHOST Hi5 alone was performed in presence of the CXCR4 antagonist AMD3100 (Sigma-Aldrich, St. Louis, MO) at dose of 1 μM.

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Author contributions

K.T. designed and performed the research, analyzed the data and wrote the manuscript. H.O. and H.S. performed MD simulation and calculation of the RMSD, wrote the manuscript. T.H. and J.K. performed the cloning and sequencing. S.O. designed and supervised the study. H.G. designed the study, analyzed the data, wrote and edited the manuscript. All authors read and approved the final manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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Naturally Selected Rilpivirine-Resistant HIV-1 Variants by Host Cellular Immunity

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Background. Rilpivirine is listed as an alternative key drug in current antiretroviral therapy (ART) guidelines. E138G/A/K in human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) are rilpivirine resistance-associated mutations and can be identified in a few ART-naive patients, although at low frequency. The 138th position in HIV-1 RT is located in one of the putative epitopes of human leukocyte antigen (HLA)–B*18–restricted cytotoxic T lymphocytes (CTLs). CTL-mediated immune pressure selects escape mutations within the CTL epitope. Here we tested whether E138G/A/K could be selected by HLA-B*18-restricted CTLs.

Methods. The amino acid variation at the 138th position was compared between ART-naive HIV-1–infected patients with and without HLA-B*18. The optimal epitope containing the 138th position was determined and the impact of E138G/A/K on CTL response was analyzed by epitope-specific CTLs. The effect of E138G/A/K on drug susceptibility was determined by constructing recombinant HIV-1 variants.

Results. The prevalence of E138G/A/K was 21% and 0.37% in 19 and 1088 patients with and without HLA-B*18, respectively (odds ratio, 72.3; $P = 4.9 \times 10^{-25}$). The CTL response was completely abolished by the substitution of E138G/A/K in the epitope peptide. E138G/A/K conferred 5.1-, 7.1-, and 2.7-fold resistance to rilpivirine, respectively.

Conclusions. E138G/A/K can be selected by HLA-B*18-restricted CTLs and confer significant rilpivirine resistance. We recommend drug resistance testing before the introduction of rilpivirine-based ART in HLA-B*18-positive patients.

Keywords. rilpivirine; E138G/A/K; HLA-B*18; CTL.

Rilpivirine is a new-generation nonnucleoside reverse transcriptase inhibitor (NNRTI), with noninferior clinical efficacy demonstrated in large clinical trials, compared with efavirenz [1, 2], and is listed as an alternative key drug in current antiretroviral therapy (ART) guidelines [3, 4]. In those clinical trials, rilpivirine showed more-favorable safety and tolerance profiles compared with efavirenz, although it was also associated with a higher virological failure rate. The most commonly observed NNRTI resistance-associated mutation in rilpivirine-treated patients with virological failure has so far been E138 K [1, 2]. Not only E138 K, but also other substitutions at the 138th position in human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT), might confer significant rilpivirine resistance [5–7]. The glutamic acid at the 138th position (E138) is well conserved among HIV-1 strains and clinical isolates throughout clades [8]. However, some ART-naive patients are infected with HIV-1 variants harboring other amino acids at the 138th position (E138X), although the proportion of such patients is low [9]. The 138th position is located in one of the putative epitopes of human leukocyte antigen (HLA)–B*18-restricted cytotoxic T lymphocytes (CTLs) [10, 11]. Because CTL immune pressure often selects escape mutations within the epitope [11], E138X may be selected by HLA-B*18-restricted CTLs. In this study, we analyzed the frequency of amino acid variations at the 138th position in ART-naive patients with or without

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Table 1. Amino Acid Variations at the 138th Position of HIV-1 Reverse Transcriptase and Human Leukocyte Antigen–B*18

Amino Acid	HLA-B*18(+)	HLA-B*18(–)
E138 (wild-type)	15	1084
E138G	2	1
E138A	1	2
E138K	1	1

Abbreviation: HLA, human leukocyte antigen.

HLA-B*18, determined the impact of E138X on CTL response, and analyzed the drug susceptibility of recombinant HIV-1 variants harboring E138X.

METHODS

Sequences of HIV-1 Reverse Transcriptase

HIV-1 RT sequences were analyzed using viral RNA extracted from plasma samples [12], and HLA type was determined by standard sequence-based genotyping in 1107 ART-naive infected individuals who visited the Outpatient Clinic of the AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, between 2003 and 2012. The amino acid variation at the 138th position of HIV-1 RT was compared between individuals with and those without HLA-B*18, and the statistical significance of the difference was analyzed by Fisher exact test using the Statistical Package for Social Sciences, version 17.0 (SPSS, Chicago, Illinois). This study was approved by the institutional ethical committee of the National Center for Global Health and Medicine, and written informed consent was obtained from all the participants according to the Declaration of Helsinki.

Intracellular Cytokine Staining Assay

HIV-1-derived peptides and mutant peptides were synthesized using an automated multiple peptide synthesizer and purified by high-performance liquid chromatography. Peripheral blood mononuclear cells (PBMCs) from chronically HIV-1–infected HLA-B*18-positive patients were stimulated with the peptide (100 nM) in culture medium (RPMI 1640 medium supplemented with 10% fetal calf serum and 200 U/mL recombinant human interleukin 2). After 14 days in culture, the cells were assessed for interferon (IFN)-γ production activity using a FACSCanto II (BD Biosciences, San Jose, California) [13, 14].

Drug Susceptibility Assay

The desired mutations were introduced into the *XmaI-NheI* region of pTZNX, which encodes the 15th–267th positions of HIV-1 RT (strain BH10) [15, 16]. The *XmaI-NheI* fragment was inserted into pNL_{H219Q}, which was modified from pNL101 and encoded the full genome of HIV-1. Each molecular clone was transfected into COS-7 cells, and the obtained virions were harvested 48 hours after transfection and stored at −80°C until use. Efavirenz and nevirapine were generously provided by Merck Co, Inc (Rahway, New Jersey) and Boehringer Ingelheim Pharmaceutics Inc (Ridgefield, Connecticut), respectively. Etravirine and rilpivirine were purchased from Toronto Research Chemicals Inc (North York, Ontario, Canada). The susceptibility of recombinant HIV-1 variants to efavirenz, nevirapine, etravirine, and rilpivirine was determined in triplicate and repeated 3 times [16]. Fold resistance was calculated by comparing the viral 50% inhibitory concentration (IC_{50}) with that of monoclonal wild-type HIV-1.

Structural Modeling

We constructed structural models of the HIV-1 RT and rilpivirine complex by computational analysis, as described in our

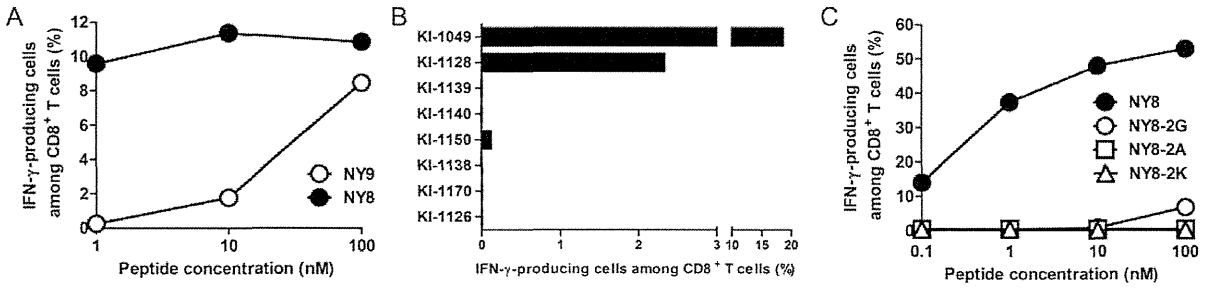

Figure 1. Recognition of human leukocyte antigen (HLA)–B*18-restricted $CD8^+$ T cells. *A*, Identification of the optimal epitope of HLA-B*18-restricted $CD8^+$ T cells. Peripheral blood mononuclear cells (PBMCs) from an HLA-B*18-positive individual chronically infected with human immunodeficiency virus type 1 (HIV-1) were stimulated with NY9 peptide and cultured for 2 weeks. Recognition of the bulk $CD8^+$ T cells toward each peptide was measured by the intracellular cytokine staining (ICS) assay. *B*, Induction of NY8-specific $CD8^+$ T cells in HLA-B*18-positive individuals chronically infected with HIV-1. PBMCs from 8 chronically HIV-1-infected HLA-B*18-positive individuals were stimulated with NY9 peptide and cultured for 2 weeks. Recognition of the bulk $CD8^+$ T cells toward NY8 peptide were measured by the ICS assay. *C*, Effects of E138G/A/K substitutions on the recognition of HLA-B*18-restricted $CD8^+$ T cells. Recognition of the bulk $CD8^+$ T cells toward each wild-type or mutant peptide was measured by the ICS assay. Abbreviations: IFN-γ, interferon gamma; NY8, NETPGIRY; NY8-2G, NGTPGIRY; NY8-2A, NATPGIRY; NY8-2 K, NKTPGIRY; NY9, NNETPGIRY.

Table 2. Susceptibility of Recombinant HIV-1 Variants to 4 Nonnucleoside Reverse Transcriptase Inhibitors

	IC_{50} (nM), Fold Resistance[a]			
Amino Acid	EFV	NVP	ETR	RPV
E138 (wild-type)	1.2 ± 0.2 (1)	31 ± 3 (1)	1.1 ± 0.1 (1)	0.16 ± 0.04 (1)
E138G	1.6 ± 0.2 (1.3)	30 ± 10 (0.97)	2.4 ± 0.3 (2.2)	0.82 ± 0.09 (5.1)
E138A	2.1 ± 0.3 (1.8)	30 ± 2 (0.97)	2.6 ± 0.2 (2.4)	1.13 ± 0.20 (7.1)
E138K	2.4 ± 0.4 (2.0)	50 ± 10 (1.6)	2.4 ± 0.1 (2.2)	0.43 ± 0.10 (2.7)

Data are presented as mean ± standard deviation.

Abbreviations: EFV, efavirenz; ETR, etravirine; IC_{50}, viral 50% inhibitory concentration; HIV-1, human immunodeficiency virus type 1; NVP, nevirapine; RPV, rilpivirine.

[a] Fold resistance was calculated by comparing viral IC_{50} with that of monoclonal wild-type HIV-1.

previous reports [15, 16]. In brief, the initial models of wild-type RT with rilpivirine were first constructed by homology modelling. The crystal structures of RT with NNRTI (PDB code: 2ZD1 [17]) was used for template structure. We also constructed the respective mutant RTs with rilpivirine by considering every possible conformer of the respective mutant models. The possible conformers were generated from the wild-type homology models using PyMOL software (http://www.pymol.org). Among the conformers, we selected those with the lowest energy as each mutant model.

RESULTS

First, we analyzed the frequency of amino acid variations at the 138th position of HIV-1 RT in 1107 ART-naive individuals. As expected, E138 was found in the majority (1099 cases [99%]) of the analyzed patients. However, 8 cases showed amino acid substitutions, including 3 cases of substitution with glycine (E138G), 3 cases with alanine (E138A), and 2 cases with lysine (E138 K). The frequency of E138G/A/K substitutions was 21% and 0.37% in 19 and 1088 individuals with and without HLA-B*18, respectively (Table 1). There was a significant difference in the frequency of the substitutions (odds ratio, 72.3; $P = 4.9 \times 10^{-25}$), suggesting that E138G/A/K could be selected by HLA-B*18-restricted CTLs.

Next, we delineated the impact of E138G/A/K on the response of HLA-B*18-restricted CTLs. The putative HLA-B*18-restricted CTL epitopes containing the 138th position of HIV-1 RT were NETPGIRYQY (NY10; position 137–146), NETPGIRYQ (NQ9; position 137–145), and NNETPGIRY (NY9; position 136–144) [10, 11]. These 3 peptides were used to stimulate PBMCs of 8 ART-treated HLA-B*18-positive patients chronically infected with HIV-1. IFN-γ production activity was detected in PBMCs from 1 of the 8 patients when stimulated with NY9. To determine the optimal epitope, the bulk $CD8^+$ T cells were further analyzed for NY9 and NETPGIRY (NY8; position 137–144). The bulk $CD8^+$ T cells more efficiently recognized NY8 than NY9 at 1-nM, 10-nM, and 100-nM concentrations (Figure 1*A*). These findings indicate that NY8 was the optimal epitope of HLA-B*18-restricted CTLs. Indeed, NY8-specific $CD8^+$ T cells were induced in 3 of the 8 patients (Figure 1*B*). A

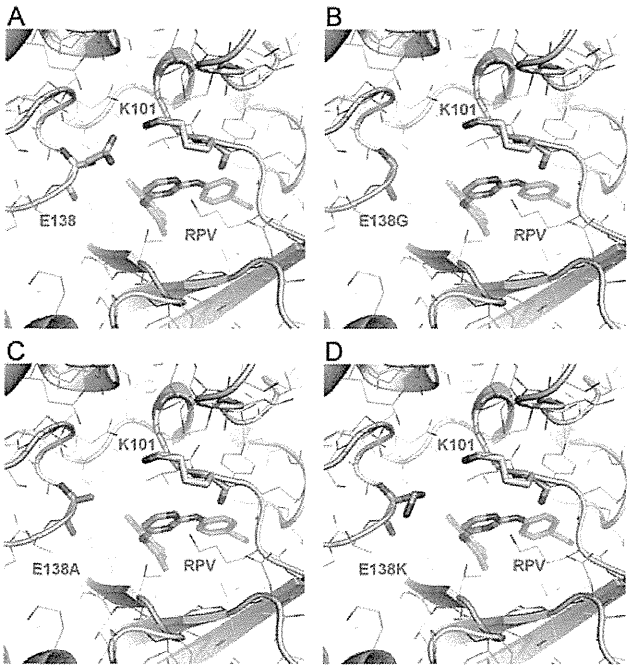

Figure 2. Structural models of human immunodeficiency virus type 1 reverse transcriptase (RT) and rilpivirine. The binding clefts of 4 complexes are shown: $RT_{E138(wild\text{-}type)}$ (*A*), RT_{E138G} (*B*), RT_{E138A} (*C*), and RT_{E138K} (*D*). Sticks indicate the amino acids at positions 101 and 138 of RT, and the atoms of rilpivirine. The mutated residues (E138G, E138A, and E138 K) and rilpivirine atoms are represented by orange and greenish-blue sticks, respectively. Abbreviation: RPV, rilpivirine.

previous study showed that HLA-B*18-binding peptides have 2 anchor residues, E at position 2 and Y/F at the C-terminus [18]. NY8 also had these 2 anchor residues, supporting that this peptide is a HLA-B*18-restricted CTL epitope. To analyze the effect of E138G/A/K on the CTL response, 3 mutant peptides, NGTPGIRY (NY8-2G), NATPGIRY (NY8-2A), and NKTPGIRY (NY8-2 K), were synthesized, and the recognition of the bulk CTLs for these mutant peptides was compared with that for NY8. The bulk CTLs failed to recognize these peptides at 0.1-nM, 1-nM, 10-nM, and 100-nM concentrations, although it effectively recognized NY8 (Figure 1*C*). These substitutions at the 138th position may affect peptide binding to the HLA-B*18 molecule because the second position of HLA-B*18-binding peptides is an anchor for HLA-B*18 [18]. These findings indicate that each of the E138G/A/K affected CTL recognition and allow escape from the HLA-B*18-restricted CTLs.

Finally, we analyzed the effect of E138G/A/K on viral susceptibility to NNRTIs by constructing recombinant HIV-1 variants. Each HIV-1 variant harboring one of E138G/A/K showed comparable replication fitness with wild-type HIV-1. Although the substitutions of E138G/A/K did not confer >2-fold resistance to efavirenz and nevirapine, they conferred mild resistance (2.2- to 2.4-fold) to etravirine. With regard to rilpivirine, E138 K, which was commonly observed in patients with virological failure under rilpivirine-based ART [1, 2], conferred mild resistance, whereas E138G and E138A conferred >5-fold resistance (Table 2). These findings indicate that in addition to E138 K, E138G and E138A can also reduce the clinical response to rilpivirine. The structural modeling suggests that substitution of E138 changes interactions around the rilpivirine-binding cleft (Figure 2). The side chain of E138 in the wild-type RT forms a salt bridge with the lysine at the 101th position (K101) at the edge of the cleft and establishes direct interactions with the pyrimidine moiety of rilpivirine, as seen in the crystal structure of RT with rilpivirine [17]. Meanwhile, mutant RTs with E138G/A/K substitutions could not create such a salt bridge, resulting in changes in the morphology of the binding cleft. In particular, RTs with E138G or E138A can reduce interactions with rilpivirine by creating large gaps between rilpivirine and the substituted 138th residues with small side chains, which seems to cause significant resistance to rilpivirine.

DISCUSSION

The major findings of the present study were as follows: (1) E138G/A/K substitutions were escape mutations of HLA-B*18-restricted CTLs and they were observed more frequently in HLA-B*18-positive patients than HLA-B*18-negative patients; and (2) we confirmed that these substitutions conferred significant resistance to rilpivirine, demonstrating that drug resistance–associated mutations can be selected naturally by CTL when its epitope is located in the viral protein of antiretroviral targets.

Studies of cellular immunology in HIV-1 have focused mainly on Gag [19, 20]. However, considering that many of the recently identified CTL epitopes are located in Pol [13, 14, 21], analysis of the interaction between CTL and drug susceptibility is warranted. Some escape mutations can persist after viral transmission to other hosts even if the new hosts do not have the corresponding HLAs [22]. Therefore, HIV-1 can adapt to HLA at a population level [23]. In fact, we identified E138G/A/K in ART-naive HLA-B*18-negative patients, although the frequency of such variations was extremely low. However, the same analysis performed in areas with higher prevalence of HLA-B*18, such as Eastern Europe [24], would probably detect higher frequency of E138G/A/K.

HIV drug resistance testing is recommended not only after treatment failure but also before the introduction of the initial treatment, considering the risk that the patient may have acquired drug-resistant viruses from those with treatment failure [3, 25]. The present study may add another reason for drug resistance testing of ART-naive patients: drug resistance–associated mutations may have evolved in the patients selected by their own immunity even if the original transmitted viruses were drug sensitive. At the very least, drug resistance testing should be performed before the introduction of rilpivirine-based ART in HLA-B*18-positive patients.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Is Ritonavir-Boosted Atazanavir a Risk for Cholelithiasis Compared to Other Protease Inhibitors?

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Abstract

Objective: To compare the incidence of complicated cholelithiasis in patients receiving ritonavir-boosted atazanavir (ATV/r)-containing antiretroviral therapy with those on other protease inhibitors (PIs).

Design: We conducted a single-center retrospective cohort study of patients who started either ritonavir-boosted ATV/r- or other PIs (ritonavir-boosted fosamprenavir, unboosted fosamprenavir, lopinavir/ritonavir, and ritonavir-boosted darunavir) -containing antiretroviral therapy.

Methods: The incidence of complicated cholelithiasis was determined in each group. Complicated cholelithiasis was defined as follows: 1) cholelithiasis complicated by cholecystitis, cholangitis, or pancreatitis or 2) symptomatic cholelithiasis or choledocholithiasis which required invasive procedures such as cholecystomy and endoscopic retrograde cholangiopancreatography. The effects of ATV/r were estimated by univariate and multivariate Cox hazards models as the primary exposure.

Results: Complicated cholelithiasis was diagnosed in 3 patients (2.23 per 1000 person-years) in the ATV/r group (n = 466), and 3 (1.64 per 1000 person-years) in the other PIs group (n = 776), respectively. The incidence was not statistically different in the two groups by log-rank test (P = 0.702). By univariate and multivariate analysis adjusted for age and body weight, ATV/r use was not associated with cholelithiasis. (HR = 1.365; 95% CI, 0.275–6.775; p = 0.704) (adjusted HR = 1.390; 95% CI, 0.276–7.017; p = 0.690). For the 3 patients who developed cholelithiasis in the ATV/r group, the time to the diagnosis of cholelithiasis was 18, 34, and 39 months, respectively.

Conclusion: In this study, the incidence of complicated cholelithiasis was low and was not different between patients on ATV/r and those on other PIs. On the contrary to ATV/r-associated nephrolithiasis, the possible risk of cholelithiasis should not preclude the use of ATV/r.

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Introduction

Ritonavir-boosted atazanavir (ATV/r) is a widely used protease inhibitor (PI) in combination with other antiretroviral drugs for patients with human immunodeficiency virus-1 (HIV) infection (URL: http://aidsinfo.nih.gov/contentfiles/lvguidelines/adultandadolescentgl.pdf) (URL: http://www.europeanaidsclinicalsociety.org/images/stories/EACS-Pdf/Eacs Guidelines-v6.1-2edition.pdf). ATV/r is one of the first-line antiretroviral drugs based on its high efficacy, tolerability, favorable lipid profile, and once-daily dosing [1,2]. However, recent studies suggested potential adverse effects associated with ATV/r, including nephrolithiasis and cholelithiasis [3,4].

Previous studies suggested a possible causal relation between protease inhibitors and cholelithiasis [4–8]. Of the 20 previously reported patients with PI-associated cholelithiasis, 16 (80%) were associated with the use of ATV [4–8]. In one of these studies, which reported 14 patients with ATV-associated cholelithiasis, the median duration of atazanavir exposure was 42 months, suggesting that prolonged exposure to ATV is a possible risk for cholelithiasis [4]. However, there is virtually no information on the incidence of ATV/r-related cholelithiasis compared to other PIs although ATV/r is one of the most frequently prescribed PIs.

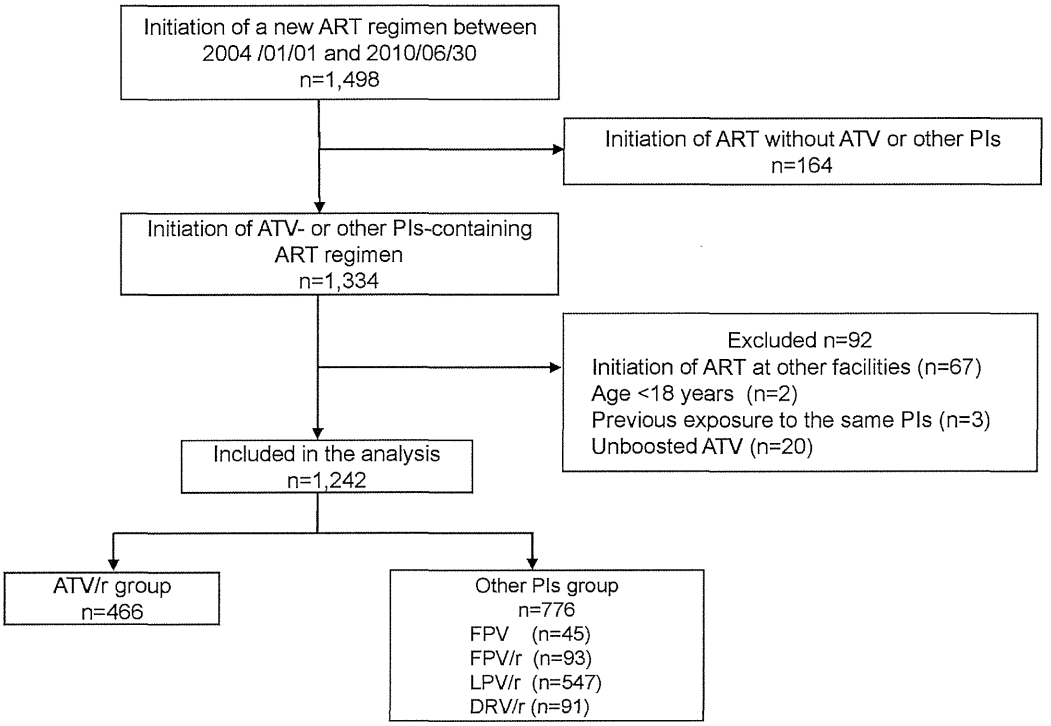

Figure 1. Flow diagram of patient selection. ART, antiretroviral treatment; ATV, atazanavir; PIs, protease inhibitors; LPV/r, lopinavir/ritonavir; ATV/r, ritonavir-boosted atazanavir; FPV, fosamprenavir; FPV/r, ritonavir-boosted fosamprenavir; DRV/r, ritonavir-boosted darunavir.
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Thus, we conducted a retrospective study to compare the incidence of complicated cholelithiasis in patients on ATV/r-containing antiretroviral treatment (ART) and those on other commonly used PIs [unboosted fosamprenavir (FPV), ritonavir-boosted fosamprenavir (FPV/r), lopinavir/ritonavir (LPV/r), and ritonavir-boosted darunavir (DRV/r)].

Table 1. Baseline demographics and laboratory data of patients who received ATV/r- and other-PIs-containing antiretroviral therapy (n = 1,242).

	ATV/r (n = 466)	Other PIs (n = 776)	P value
Age* [SD]	39.0 [10.6]	40.0 [11.5]	0.132
Male gender (%)	434 (93.1)	714 (91.9)	0.422
Race (East Asian origin) (%)	449 (96.4)	722 (93.0)	0.015
Body weight (kg)* [SD]	65.0 [10.5]	62.1 [10.7]	<0.001
BMI (kg/m^2)* [SD]	22.7 [3.14]	21.7 [3.25]	<0.001
CD4 count (/μl)* [SD]	304.0 [184.5]	176.2 [170.8]	<0.001
HIV viral load (log_{10}/ml)* [SD]	3.58 [1.38]	4.42 [1.40]	<0.001
Treatment naïve (%)	282 (60.5)	556 (71.6)	<0.001
TDF use (%)	177 (38.0)	326 (42.0)	0.162
eGFR (ml/min/1.73 m^2)* [SD]	117.4 [38.1]	121.7 [33.7]	0.012
Hepatitis B or C (%)	57 (12.2)	111 (14.3)	0.301

*Arithmetic mean.
ATV/r: ritonavir-boosted atazanavir, PI: protease inhibitor, SD: standard deviation, BMI: body mass index, TDF: tenofovir, eGFR: estimated glomerular filtration rate.
doi:10.1371/journal.pone.0069845.t001

Methods

Ethics statement

This study was approved by the Human Research Ethics Committee of National Center for Global Health and Medicine, Tokyo. All patients included in this study provided a written informed consent for their clinical and laboratory data to be used and published for research purposes. This study has been conducted according to the principles expressed in the Declaration of Helsinki (http://www.wma.net/en/30publications/10policies/b3/17c.pdf).

Study Subjects

This is a retrospective, single-center cohort study of patients with HIV-1 infection using the 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. The study population was HIV infected patients, aged >17 years, who commenced treatment with ATV/r, FPV/r, FPV, LPV/r, or DRV/r-containing ART between January 1, 2004 and June 30, 2010. Both treatment-naïve and treatment-experienced patients were included. The follow-up period started at the time of commencement of ART for the first time during the study period, and ended June 30, 2011. Patients were excluded; 1) if they had started the abovementioned ART during the study period at other facilities, 2) if they were prescribed unboosted ATV. Patients with previous exposure to one of the abovementioned drugs before the present study and commenced the same drug in this study were also excluded from the analysis.

The attending physician selected the PI drug at baseline, based on the Japanese guidelines, which placed all of the above-

Table 2. Uni-and multi-variate analyses to estimate the risk of ATV/r use over other PIs-containing antiretroviral therapies for cholelithiasis.

	Model 1 crude (n = 1,242)			Model 2 adjusted (n = 1,203)		
	HR	95%CI	P value	HR	95%CI	P value
ATV/r use	1.365	0.275–6.775	0.702	1.390	0.276–7.017	0.689
Age per 1 year	1.072	1.021–1.127	0.006			
Male gender	0.446	0.052–3.831	0.463			
Race (East Asian origin)	0.285	0.033–2.444	0.252			
Weight per 1 kg increment	0.990	0.914–1.073	0.807			
BMI per 1 kg/m^2 increment	0.997	0.780–1.274	0.980			
CD4 count per 10/μl increment	0.987	0.938–1.038	0.605			
HIV viral load per log10/ml increment	0.917	0.541–1.557	0.750			
Baseline eGFR 10 ml/min/1.73 m^2 decrement	1.140	0.842–1.557	0.394			
Hepatitis B or Hepatitis C	0.040	0.000–1138.5	0.538			

Model 2 was adjusted for age and body weight.
HR: hazard ratio, CI: confidential interval, ATV/r: ritonavir-boosted atazanavir, BMI: body mass index, eGFR: estimated glomerular filtration rate.
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mentioned drugs as the preferred choice, at least for 3 years during the study period (http://www.haart-support.jp/guideline2011.

pdf. in Japanese). The attending physician also selected the concurrent drugs, including nucleoside reverse transcriptase inhibitors (NRTI), non-NRTI, integrase inhibitors, and CCR5 inhibitors. None of the patients received two PIs during the study period.

Measurements

Complicated cholelithiasis was defined as follows: 1) cholelithiasis diagnosed by computed tomography or abdominal ultrasonography, together with cholecystitis, cholangitis, or pancreatitis, or 2) symptomatic cholelithiasis or choledocholithiasis requiring invasive procedures, such as cholecystomy or endoscopic retrograde cholangiopancreatography. Before the initiation of ART and until suppression of HIV-1 viral load, patients visited our clinic every month. However, after viral load suppression, the visit interval was extended up to every three months.

In this study, the primary exposure variable was ATV/r use over other PIs (FPV, FPV/r, LPV/r, and DRV/r). The potential risk factors for cholelithiasis were determined according to previous studies and collected from the medical records, together with the basic demographics [4,9,10]. They included age, sex, body weight, body mass index (BMI), baseline laboratory data [CD4 cell count, HIV viral load, estimated glomerular filtration rate (eGFR)], and presence or absence of other medical conditions [concurrent use of tenofovir (TDF), co-infection with hepatitis B, defined by positive hepatitis B surface antigen, and co-infection with hepatitis C, defined by positive hepatitis C viral load]. eGFR was calculated as described previously [11]. At our clinic, weight was measured on every visit whereas other variables were measured in the first visit and at least once annually. We used the data on or closest to and preceding the day of starting ART by no more than 180 days.

Statistical analysis

Baseline characteristics were compared using the unpaired Student's *t*-test or χ^2test (Fisher's exact test) for quantitative or qualitative variables, respectively. The time to the diagnosis of complicated cholelithiasis was calculated from the date of commencement of pre-defined PI-containing ART to the date of diagnosis of cholelithiasis. Censored cases represented those who discontinued the PIs, dropped out, were referred to other facilities, or at the end of follow-up period. The time from the start of ART to the diagnosis of cholelithiasis was analyzed by the Kaplan Meier method for patients who started ATV/r (ATV/r group) or other PIs (other PIs group), and the log-rank test was used to determine the statistical significance. The Cox proportional hazards regression analysis was used to estimate the impact of ATV/r use over other PIs on the incidence of cholelithiasis. The impact of each parameter listed above was also estimated by univariate Cox proportional hazards regression. We conducted multivariate analysis adjusted for age and body weight only, because of the small number of cases that were diagnosed with complicated cholelithiasis.

Statistical significance was defined as two-sided p value <0.05. We used the hazard ratio (HR) and 95% confidence interval (95%CI) to estimate the impact of each variable on cholelithiasis. All statistical analyses were performed with The Statistical Package for Social Sciences ver. 17.0 (SPSS, Chicago, IL).

Results

A total of 1,498 patients commenced or switched key drugs (PIs, non-NRTIs, or integrase inhibitor) between January 1, 2004 and June 30, 2010. Of the 1,242 patients who were included in the analysis, 466 (37.5%) started ATV/r-containing ART while 776 (62.5%) started other PIs-containing ART (Figure 1). Table 1 shows the demographics, laboratory data, and medical conditions of the study population at baseline. The majority of the study population was males, of East Asian origin, and comparatively young. The ATV/r group included significantly more patients of East Asian origin ($p = 0.015$) with significantly higher body weight ($P<0.001$), higher CD4 count ($p<0.001$), lower viral load ($p<0.001$), and lower eGFR ($P = 0.012$), compared with other PI groups. In contrast, patients of the other PIs group were significantly more likely to be treatment naïve ($p<0.001$). However, all other major background parameters were similar in the two groups.

Cholelithiasis was diagnosed in 3 patients (0.64%) of the ATV/r group and 3 (0.39%) in the other PIs group, with an estimated

Table 3. Clinical characteristics of patients who developed cholelithiasis.

n	Sex	Age (yrs)	BMI (kg/m^2)	Other conditions	Protease inhibitors	Other antiretroviral agents	Duration of PI therapy (months)	Diagnosis	Invasive procedures
1	F	63	20.0	Breast cancer, hypothyroidism, hypertriglyceridemia	ATV/r	ABC, 3TC	34	Choledocholithiasis	ERCP
2	M	59	25.8	hypertriglyceridemia	ATV/r	TDF, FTC	39	Cholecystitis	PTGBD
3	M	48	29.1	hypertriglyceridemia	ATV/r	ABC, 3TC	18	Gall stone pancreatitis	ERCP
4	M	56	22.7	hypertriglyceridemia	LPV/r	ABC, 3TC	39	Cholecystitis	PTGBD
5	M	37	16.6	hypertriglyceridemia	LPV/r	ABC, 3TC	1	Choledocholithiasis	ERCP
6	M	40	19.3	hypertriglyceridemia	LPV/r	ABC, 3TC	2	Cholelithiasis	Cholecystomy

BMI: body mass index, PI: protease inhibitor, ATV/r: ritonavir-boosted atazanavir, LPV/r: lopinavir/ritonavir, ABC: abacavir, 3TC: lamivudine, ERCP: endoscopic retrograde cholangiopancreatography, PTGBD: percutaneous transhepatic gall bladder drainage.

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incidence of cholelithiasis of 2.23 and 1.65 per 1000 person-years, respectively. The incidence was not statistically different in the two groups by log-rank test (P = 0.702). Univariate analysis showed that ATV/r use was not associated with the development of cholelithiasis (HR = 1.365; 95% CI, 0.275–6.775; p = 0.704) (Table 2). Furthermore, other variables, including gender, body weight, race, BMI, co-infection with hepatitis B or C, eGFR, CD4 count, and viral load were not associated with cholelithiasis. On the other hand, older age was associated with increased risk of cholelithiasis (per one year, HR = 1.072; 95% CI, 1.021–1.127; p = 0.006). Multivariate analysis adjusted for age and body weight indicated that ATV/r use was not associated with the development of cholelithiasis (HR = 1.390; 95% CI, 0.276–7.017; p = 0.690) (Table 2).

Table 3 shows the clinical characteristics of the patients diagnosed with cholelithiasis in the present study. For the three patients of the ATV/r group, the time to the diagnosis of cholelithiasis was 18, 34, and 39 months, respectively. They were diagnosed with gallstone pancreatitis, symptomatic choledocholelithiasis, and cholecystitis, respectively, and all patients required invasive therapies. The median observation period was 31.7 months (IQR 16.0–49.7 months) for the ATV/r group and 23.0 months (IQR 10.4–42.5 months) for the other-PIs group.

Discussion

To our knowledge, this is the first study that compared the incidence of complicated cholelithiasis between patients receiving ATV/r and those on other PIs. The incidence of cholelithiasis in the ATV/r group was low at 2.23 per 1000 person-years and was not statistically different from that in the other PIs groups based on uni- and multi-variate analyses.

Previous reports suggested the association between ATV/r use and cholelithiasis [4–6]. However, the association was not demonstrated in this cohort study of 1,242 patients. Rakotondravelo et al. reported 14 cases of PI-related cholelithiasis [4]. Although their study was not designed to calculate the incidence, the estimated incidence was 2.3 cases per 1000 person-years, which is similar to our result. This incidence is 10 times lower than that of ATV/r-associated renal stones reported in our previous study [3]. In fact, only 16 cases with ATV/r-induced cholelithiasis have been reported to date [4–6], compared with substantial number of ATV/r-associated renal stone reported by several groups [3,12–16]. Thus, the potential risk of cholelithiasis in patients on PIs seems low compared to urolithiasis and may not be a major factor in the selection of ART.

Siveke et al. suggested that all PIs could cause cholelithiasis based on 3 cases that developed cholelithiasis while on PIs-containing ART. It is possible that PIs other than ATV/r also contribute to the development of cholelithiasis [8]. However, this cannot be confirmed at this stage and further studies are needed to address this issue.

The exact mechanism of ATV/r-induced cholelithiasis is not fully understood, although several theories have been suggested. One such theory is the precipitation of ATV in the bile with associated ATV-induced hyperbilirubinemia [4]. Another proposed mechanism relates to end-stage liver disease, which results in increased plasma ATV concentration and subsequent ATV/r-induced cholelithiasis [4]. In this study, however, we could not identify any risk factor associated with cholelithiasis.

There are several limitations to our study. First, we could not investigate asymptomatic cholelithiasis and symptomatic gallstone without complications. Thus, the risk of developing cholelithiasis associated with ATV/r might have been underestimated in the

present study. Second, the prevalence of gallstones is generally lower in East Asians than in European descent and since most of the patients in this study were of East Asian origin, the effect of ATV/r might have been underestimated in our study [17]. Lastly, although prolonged exposure to ATV has been suggested as a possible etiology of ATV-induced cholelithiasis, the median observation period in our study (31.7 months) was shorter than the median latency between commencement of ATV-based therapy and the development of cholelithiasis reported in a previous study (42 months) [4]. Therefore, the short observation period in our study may have underestimated the risk of cholelithiasis. However, it remains to be determined whether ATV has a cumulative effect on the development of cholelithiasis due to the limited information available.

In conclusion, on the contrary to a substantially higher incidence of renal stones in the ATV/r group (23.7 cases per 1000 person-years) than in other PIs groups reported in the same cohort [3], the incidence of complicated cholelithiasis was low of 2.23 per 1000 person-years in the ATV/r group, and was not different between the two groups of PI-treated patients. Although the number of patients in our study might not have been large enough to show differences in the incidence of complicated cholelithiasis, the study at least suggested that the incidence of ATV/r-related cholelithiasis is low. Thus, on the contrary to ATV/r-associated nephrolithiasis, possible risk of cholelithiasis should not preclude the use of ATV/r.

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Author Contributions

Conceived and designed the experiments: YH. Analyzed the data: YH TN HK. Wrote the paper: YH TN HK KT HG YK SO.

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Illicit Drug Use Is a Significant Risk Factor for Loss to Follow Up in Patients with HIV-1 Infection at a Large Urban HIV Clinic in Tokyo

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Abstract

Background: Loss to follow up (LTFU) is an important prognostic factor in patients with HIV-1 infection. The impact of illicit drug use on LTFU of patients with HIV-1 infection is unknown in Japan.

Methods: A single center observational study was conducted to elucidate the impact of illicit drug use on LTFU at a large HIV clinic in Tokyo. LTFU was defined as those who discontinued their visits to the clinic for at least 12 months and were not known to be under the care of other facilities or have died within 12 months of their last visit. Patients who first visited the clinic between January 2005 and August 2010 were enrolled. Information on illicit drug use was collected in a structured interview and medical charts. Comparison of the effects of illicit drug use and no use on LTFU was conducted by uni- and multi-variate Cox hazards models as the primary exposure.

Results: The study subjects were 1,208 patients, mostly Japanese men, of relatively young age, and infected through homosexual contact. A total of 111 patients (9.2%) were LTFU (incidence: 24.9 per 1,000 person-years). Among illicit drug users and non users, 55 (13.3%) and 56 (7.1%) patients, respectively, were LTFU, with incidence of 35.7 and 19.2 per 1,000 person-years, respectively. Uni- and multi-variate analyses showed that illicit drug use was a significant risk for LTFU (HR=1.860; 95% CI, 1.282-2.699; p=0.001) (adjusted HR=1.544; 95% CI, 1.028-2.318; p=0.036). Multivariate analysis also identified young age, high CD4 count, no antiretroviral therapy, and no health insurance as risk factors for LTFU.

Conclusions: The incidence of LTFU among illicit drug users was almost twice higher than that among non users. Effective intervention for illicit drug use in this population is warranted to ensure proper treatment and prevent the spread of HIV.

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Introduction

The introduction of highly-active antiretroviral therapy (HAART) has markedly improved the prognosis of patients with HIV-1 infection [1,2]. Patients with HIV-1 infection need to maintain a good level of adherence to antiretroviral therapy (ART) and frequent visits to the health facilities for monitoring treatment efficacy and safety, with regard to the suppression of HIV-1 viral load, recovery of immune function, and improvement of prognosis and survival [3,4]. Those who discontinue medical follow up are likely to develop AIDS-defining illness and die, compared to those who continue their visits [5,6]. Thus, loss to follow up (LTFU) influences prognosis of patients with HIV-1 infection [7–11].

Among patients with HIV-1 infection, those who use illicit drugs are associated with lower ART uptake and inferior adherence to treatment [12–15], which lead to suboptimal treatment outcome, compared with patients with other risk categories [16–18]. However, illicit drug users are one of the "difficult to reach" populations and it is difficult to obtain accurate data on them [19]. It is especially difficult in Japan to collect data on illicit drug users, because of a strong

government policy against illicit drug use and extremely low lifetime prevalence of illicit drug use in the general population (2.9% in 2009 according to the Nationwide General Population Survey on Drug Use and Abuse) [20,21] (http://www.ncnp.go.jp/nimh/pdf/h21.pdf. in Japanese) (http://www.mhlw.go.jp/bunya/iyakuhin/yakubuturanyou/torikumi/dl/index-04.pdf. in Japanese). Thus, there are no data on illicit drug use among patients with HIV-1 infection, and the impact of such use on prognosis of HIV-1 infected patients in Japan [20,22].

Based on the abovementioned background, the aim of the present study was to elucidate the impact of illicit drug use on LTFU among patients with HIV-1 infection at a large urban HIV clinic in Tokyo, Japan.

Methods

Ethics Statement

This study was approved by the Human Research Ethics Committee of the National Center for Global Health and Medicine, Tokyo, Japan. The Committee waived a written informed consent, since this study only uses data of anonymized patients obtained from a routine practice. The study was conducted according to the principles expressed in the Declaration of Helsinki.

Study design

This study was designed and reported according to the recommendations of STROBE (Strengthening the Reporting of Observational studies in Epidemiology) statement [23]. We performed a single center observational study of patients with HIV-1 infection to elucidate whether illicit drug use is a risk factor for LTFU in a large urban HIV clinic in Tokyo. The AIDS Clinical Center is one of the largest clinics for HIV care in Japan with more than 3,300 registered patients. Considering that the total reported number of patients with HIV-1 infection is 21,415 by the end of 2011, this clinic treats approximately 15% of the HIV-1 infected patients in Japan (http://api-net.jfap.or.jp/status/2011/11nenpo/hyo_02.pdf. in Japanese).

Study subjects

The study population was patients with HIV-1 infection, aged >17 years, who visited our clinic for the first time from January 1, 2005 to August 31, 2010. The exclusion criteria were; 1) those who came for the second opinion and 2) those who were referred to other facilities on their first or second visit. They were excluded because the structured interview on social demographics was often not conducted for these patients. Patients who refused to have their data included in the study were also excluded. Patients were followed up until December 31, 2012.

Measurements

Variables were collected through a structured interview conducted at the first visit of each patient as part of routine clinical practice by the nurses specializing at the HIV outpatient care. The interview by these "coordinator nurses" included the following variables: history of illicit drug use and injection drug use (and type of illicit drugs if available), health insurance status, perceived route of transmission, sexuality, and whether living alone or with someone.

Because the interview could underestimate the prevalence of illicit drug use, we also searched the medical records for information on illicit drug use and related variables covering the period from the first visit to December 2012. Information on age, sex, ethnicity, treatment status for HIV infection, and history of AIDS [(defined as history of or concurrent 23 AIDS-defining diseases set by the Japanese Ministry of Health, Labour and Welfare) (http://www.haart-support.jp/pdf/guideline2012.pdf. in Japanese)] were extracted from the medical records. The laboratory data of CD4 cell count, HIV-1 viral load, and hepatitis C antibody on the first visit were also collected, and if these test results were not available on that day, the data within three months from the first visit were used.

Definition of loss to follow up

LTFU was defined according to the literature as follows: patients who discontinued their visits to the AIDS Clinical Center for at least 12 months after the last visit and who were not known to be under the care of other medical facilities or have died within 12 months of their last visit [24]. At our clinic, all patients provide their phone numbers at the first visit, and when they miss the scheduled visit, the abovementioned "coordinator nurse" calls the patient to make another appointment, or leave a message to visit if the patient does not answer the phone. If the patient does not visit the clinic after the first call, the nurses continue calling the patient every three months up to one year. For the majority of lost cases, we checked whether the patient went to seek care in another hospital, because in Japan only a few clinics provide HIV care, due to the low prevalence of HIV-1 infection (0.016%) (http://www.stat.go.jp/english/data/kokusei/pdf/20111026.pdf) (http://api-net.jfap.or.jp/status/2011/11nenpo/hyo_02.pdf. in Japanese). Thus, even if a patient stopped visiting our clinic and started seeking help at other facilities without informing the first health care provider, the new facility almost always contacts the original facility to obtain medical information.

Statistical analysis

Patients' characteristics and social demographics were compared between those who were LTFU and those who continued visiting the clinic by the Student's *t*-test for continuous variables and by either the χ^2 test or Fisher's exact test for categorical variables.

The time to LTFU as defined above was calculated from the date of the first visit to the date of LTFU. Censored cases represented those who were referred to other facilities, or who died within 12 months of their last visit, or at the end of follow-up period. The time from the first visit to LTFU was analyzed by the Kaplan Meier method for patients who experienced illicit drug use and those who did not, and the log-rank test was used to determine the statistical significance. The Cox proportional hazards regression analysis was used to estimate the impact of illicit drug use over non users on the incidence of LTFU as a primary exposure. The impact of each basic

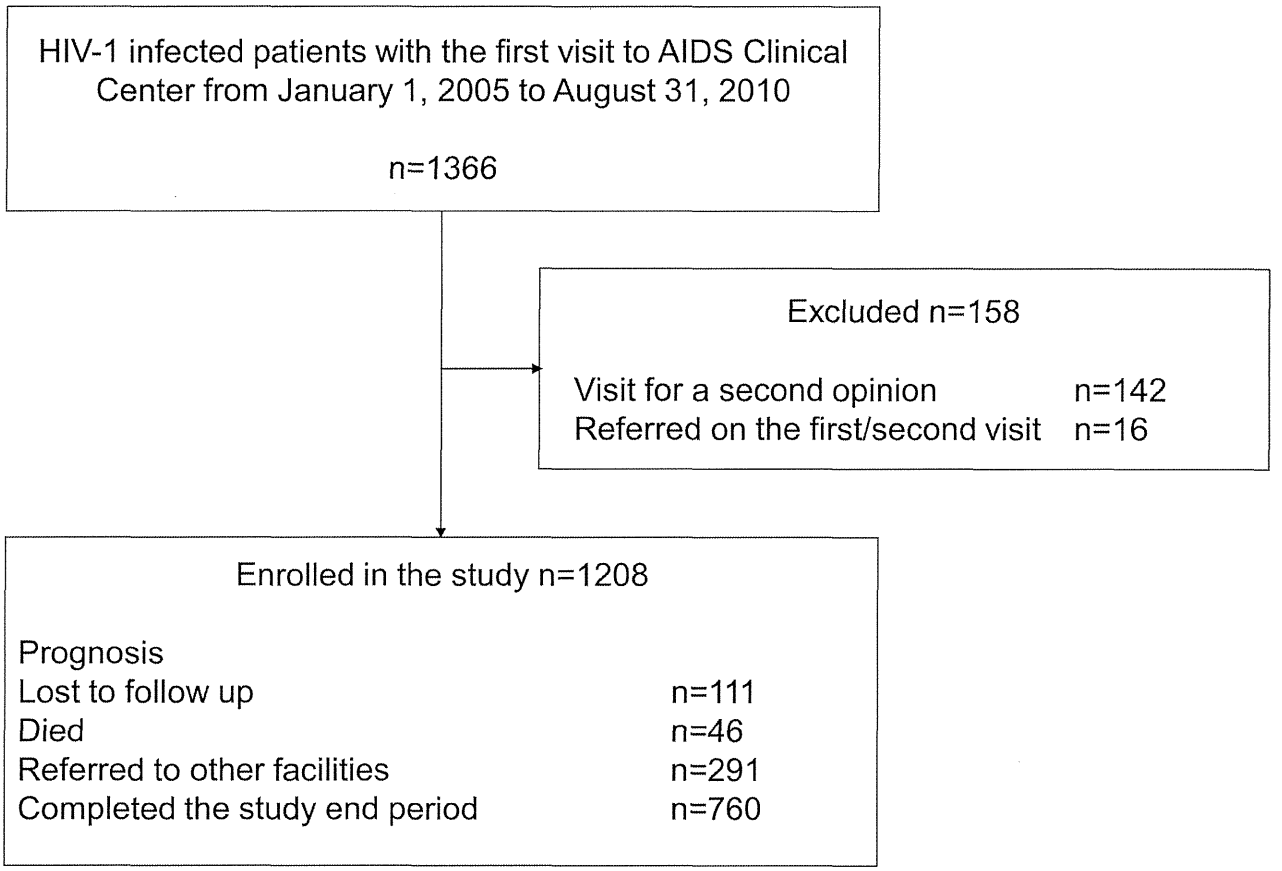

Figure 1. Patient enrollment process.
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demographics, baseline laboratory data, and other medical conditions listed above was also estimated with univariate Cox proportional hazards regression.

To estimate the unbiased prognostic impact of illicit drug use over non-users for LTFU, we conducted three models using multivariate Cox proportional hazards regression analysis. Model 1 was the aforementioned univariate analysis for illicit drug use over non users. Model 2 included basic demographics (age and Japanese) plus model 1. In model 3, we added CD4 count, ART, and health insurance status, because they showed significant relationship with LTFU in univariate analysis and the literatures showed a high CD4 count, without ART and without health insurance is a risk factor for LTFU [11,24,25]. History of AIDS and HIV-1 viral load were not added to the model, based on their multicollinearity with CD4 count and ART, respectively.

To elucidate whether the impact of illicit drug use on LTFU is affected by sexual behavior, we divided patients into MSM and non-MSM groups. Then, the abovementioned multivariate analysis was conducted for each group.

Statistical significance was defined at two-sided p values <0.05. We used hazard ratios (HRs) and 95% confidence intervals (95% CIs) to estimate the impact of each variable on LTFU. All statistical analyses were performed with The Statistical Package for Social Sciences ver. 20.0 (SPSS, Chicago, IL).

Results

A total of 1,366 patients with HIV-1 infection visited the AIDS Clinical Center for the first time during the study period. 142 patients visited for a second opinion and 16 patients were referred to other facilities on their first or second visit. Thus, 158 patients were excluded from the analysis (Figure 1). Table 1 summarizes characteristics of the 1,208 patients included in this study. The perceived route of transmission was homosexual contact in 948 (79%), heterosexual contact in 173 (14%), injection drug use in 22 (2%), contaminated blood product in 11 (1%), vertical transmission in 1 (0.1%), and unknown in 53 (4%). Further analysis indicated that 973 (81%) patients were MSM regardless of the perceived route of transmission (e.g., if a patient considered that they were infected with HIV-1 through injection drug use and they were MSM, they were classified to MSM in this study). The study patients were mostly Japanese men of relatively young age (mean: 36 years). Most patients were ART-naïve, with a median CD4 count of 245/μl.

Table 1. Baseline demographics and laboratory data for all study population, those who were lost to follow up and those who continued the visits.

	All (n=1,208)	Lost follow up (n=111)	Others (n=1,097)	P value
Sex (male), n (%)	1125 (93)	103 (93)	1022 (93)	0.84
Median (IQR) age	36 (29-43)	31 (25-39)	36 (30-43)	<0.01
Illicit drug use, n (%)	415 (34)	55 (50)	360 (33)	<0.01
Injection drug use, n (%)	53 (4)	8 (7)	45 (4)	0.14
Methamphetamine use, n (%)	63 (5)	10 (9)	53 (5)	0.07
Arrested due to illicit drug, n (%)	27 (2)	5 (5)	22 (2)	0.09
Median (IQR) CD4 count (/μl)[a]	245 (101-380)	391 (313-515)	231 (84-359)	<0.01
Median (IQR) HIV-1 viral load (log_{10}/ml)[b]	4.59 (3.89-5.18)	4.32 (3.80-4.75)	4.64 (3.91-5.20)	0.03
AIDS, n (%)	323 (27)	10 (9)	313 (29)	<0.01
On antiretroviral therapy, n (%)	131 (11)	5 (5)	126 (12)	0.02
Positive HCV antibody, n (%)	46 (4)	2 (2)	44 (4)	0.43
Men who have sex with men, n (%)	973 (81%)	89 (80)	884 (81)	0.90
Transmission category, n (%)				0.51
Homosexual contact	948 (79)	84 (76)	864 (79)	
Heterosexual contact	173 (14)	19 (17)	154 (14)	
Injection drug use	22 (2)	4 (4)	18 (2)	
Contaminated blood product	11 (1)	0	11 (1)	
Vertical transmission	1 (0.1)	0	1 (0.1)	
Unknown	53 (4)	4 (4)	49 (5)	
Ethnicity, n (%)[c]				0.02
Japanese	1070 (89)	92 (83)	978 (89)	
Asian	70 (6)	7 (6)	63 (6)	
White	27 (2)	2 (2)	25 (2)	
Black	26 (2)	7 (6)	19 (2)	
Latino	12 (1)	2 (2)	10 (0.9)	
Health insurance status, n (%)				<0.01
Without insurance	55 (5)	13 (12)	42 (4)	
With insurance/public assistance	1153 (95)	98 (88)	1055 (96)	
Working status, n (%)[d]				0.09
Unemployed	230 (19)	23 (21)	207 (19)	
With any job	909 (75)	77 (69)	832 (76)	
Student/housewife	68 (6)	11 (10)	57 (5)	
Living alone, n (%)[e]	532 (44)	46 (41)	486 (44)	0.62
Median (IQR) follow up days	1384.5 (732-1991)	266 (58-800)	1454 (914-2053)	<0.01

Data for [a] two, [b] four, [c] three, [d] one, and [e] fifteen cases, respectively, are missing

Based on the interview and medical records, 34% of the patients were illicit drug users (including injection drug users), 4% were injection drug users and 5% had used methamphetamine. Of the total, 2% were detained or arrested for possession or use of illicit drugs. Among illicit drugs, amyl nitrite and 5-methoxy-diisopropyltryptamine were the most commonly named by study patients (amyl nitrite and 5-methoxy-diisopropyltryptamine became prohibited substance by law in 2006 and 2005, respectively, in Japan) [26]. Methamphetamine, 3,4-methylenedioxymethamphetamine, cannabis, heroin, cocaine, and opium were also mentioned (numbers not counted except for methamphetamine).

LTFU patients were significantly more likely to be illicit drug users and tended to use methamphetamine and be arrested/detained due to illicit drug use than those who continued to visit the clinic. LTFU tended to be non-Japanese, younger age, had higher CD4 count, and less likely to have a history of AIDS, on ART, and covered by health insurance/public assistance, compared to the patients who continued to visit the clinic (Table 1).

Among the 1,208 patients included in the study, 111 (9.2%) were LTFU as defined above, with an incidence of 24.9 per 1,000 person-years. The median time from the first visit to LTFU was 266 days (IQR 58-800 days). Among illicit drug users (n=415) and non-users (n=793), 55 (13.3%) and 56 (7.1%) patients, respectively, were LTFU, with incidence of 35.7 and 19.2 per 1,000 person-years, respectively. Figure 2 shows the time from the first visit to LTFU by the Kaplan Meier method for the two groups. Illicit drug users were significantly more likely to stop visiting the clinic, compared to non-users (p=0.001, Log-rank test). The total observation period was 1,541.4 patient-years [median, 1,405 days, interquartile range (IQR), 674-2,029 days] for illicit drug users and 2,920.4 patient-years (median, 1,371 days, IQR, 759-1943 days) for non users.

Univariate analysis showed a significant relationship between illicit drug use and LTFU (HR=1.860; 95% CI, 1.282-2.699; p=0.001) (Table 2). Furthermore, young age, high baseline CD4 count, low HIV viral load, no history of AIDS, non Japanese, no ART, and no health insurance/public assistance were associated with LTFU. Injection drug use and methamphetamine use, respectively, were marginally associated with LTFU (injection drug use: HR=1.808; 95% CI, 0.880-3.713; p=0.107) (methamphetamine use: HR=1.684; 95% CI, 0.879-3.225; p=0.116).

Multivariate analysis identified illicit drug use as a significant risk for LTFU after adjustment for age and Japanese (adjusted HR=1.802; 95% CI, 1.209-2.686; p=0.004) (Table 3, Model 2), and also after adjustment for other risk factors (adjusted HR=1.544; 95% CI, 1.028-2.318; p=0.036) (Table 3, Model 3). Young age, high baseline CD4 count, no ART, and no health insurance/public assistance also persisted to be risk for LTFU in multivariate analysis.

Subgroup analysis of the patients stratified by sexual behavior showed that among MSM patients (n=973), the impact of illicit drug use on LTFU was slightly more evident (adjusted HR=1.641; 95% CI, 1.061-2.538; p=0.026) (Table 4) than in the total population (adjusted HR=1.544; 95% CI, 1.028-2.318; p=0.036) (Table 3, Model 3). On the other hand, illicit drug use had no significant impact in non-MSM patients (n=233) (adjusted HR=1.119; 95% CI, 0.248-5.053; p=0.883).

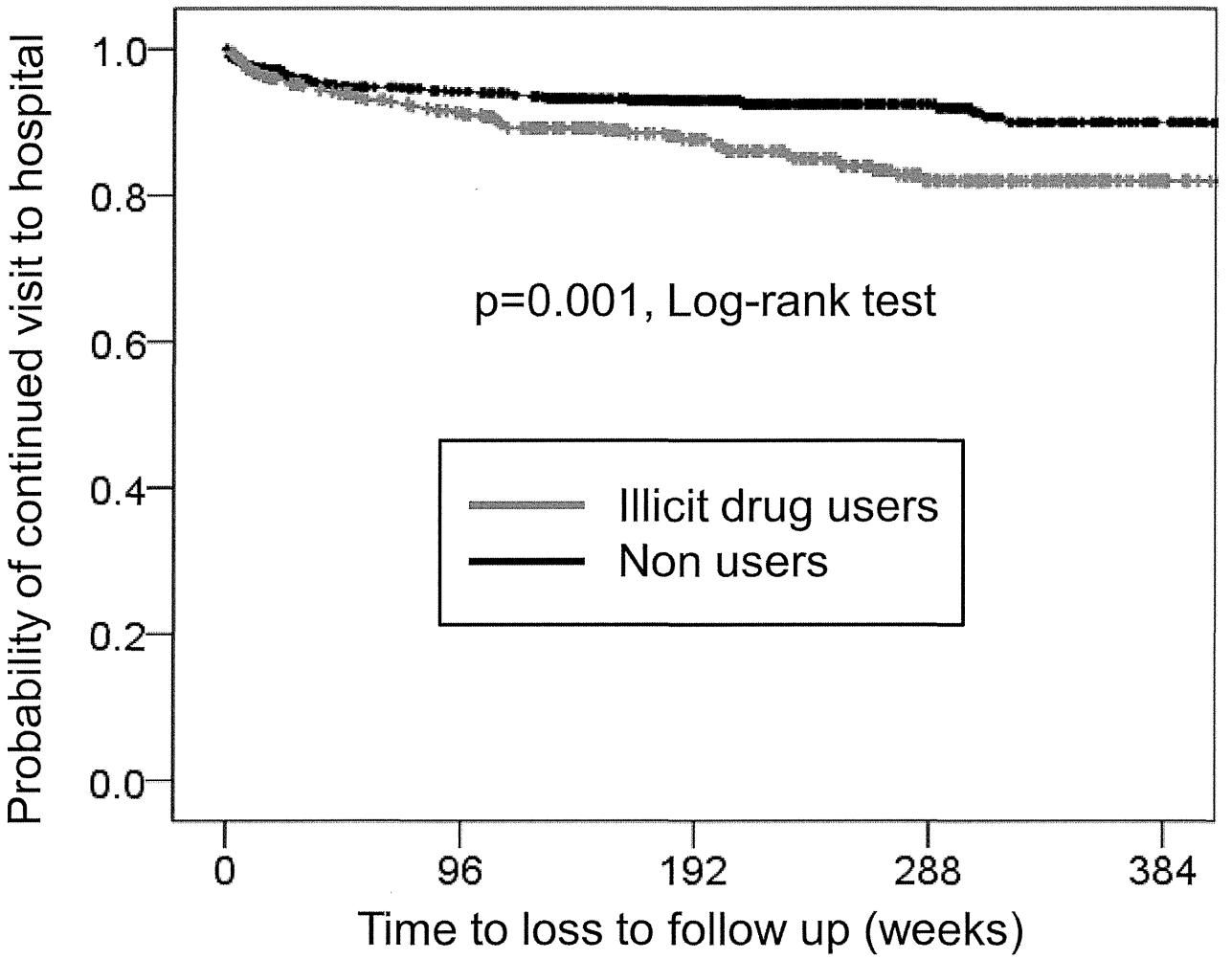

Figure 2. Kaplan-Meier curve showing time to loss to follow up for illicit drug users and non users. Compared to non drug users, illicit drug users were more likely to discontinue their visits to the hospital (p=0.001, Log-rank test).

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Table 3. Multivariate analysis to estimate the risk of illicit drug use for loss to follow up.

	Model 1 Crude (n=1,208)		Model 2 Adjusted (n=1,208)		Model 3 Adjusted (n=1,206)	
	HR	95% CI	Adjusted HR	95% CI	Adjusted HR	95% CI
Illicit drug use†	1.860	1.282-2.699	1.770	1.208-2.592	1.513	1.018-2.248
Age ≤30 years†			Reference		Reference	
30< Age ≤40 years†			0.462	0.304-0.703	0.467	0.303-0.720
Age >40 years†			0.360	0.212-0.609	0.442	0.259-0.752
Japanese			0.472	0.286-0.779	0.798	0.443-1.436
CD4 count ≤200/μl†					Reference	
200< CD4 count ≤350 /μl†					2.221	1.148-4.297
CD4 count >350/μl†					7.087	3.951-12.71
On antiretroviral therapy†					0.366	0.147-0.912
With health insurance/public assistance†					0.204	0.102-0.409

†
p<0.05 in Model 3

Table 2. Univariate analysis to estimate the risk of various factors for loss to follow up.

	Hazard ratio	95% CI	P value
Illicit drug use	1.860	1.282-2.699	0.001
Injection drug use	1.808	0.880-3.713	0.107
Methamphetamine use	1.684	0.879-3.225	0.116
Arrested/detained due to illicit drug	1.981	0.808-4.859	0.135
Male gender	0.961	0.468-1.974	0.961
Men who have sex with men	0.926	0.581-1.477	0.747
Age ≤30 years	Reference		
30 < Age ≤40 years	0.455	0.299-0.692	<0.001
Age >40 years	0.320	0.190-0.538	<0.001
CD4 count ≤200/μl	Reference		
200 < CD4 count ≤350/μl	2.536	1.318-4.878	0.005
CD4 count >350/μl	7.651	4.309-13.59	<0.001
HIV-1 viral load per $\log_{10}$/ml	0.846	0.730-0.981	0.027
History of AIDS	0.269	0.140-0.514	<0.001
Positive HCV antibody	0.466	0.115-1.888	0.285
Japanese	0.559	0.337-0.926	0.024
On antiretroviral therapy	0.402	0.164-0.986	0.046
With any job	0.870	0.549-1.376	0.551
On health insurance/public assistance	0.249	0.139-0.444	<0.001
Living alone	0.949	0.649-1.388	0.788

Table 4. Multivariate analysis to estimate the risk of illicit drug use for loss to follow up stratified by sexual behavior.

	Adjusted HR	95% CI	P value
MSM (n=973)	1.641	1.061-2.538	0.026
Non MSM (n=233)	1.119	0.248-5.053	0.883

Adjusted by variables in Table 3, Model 3 (age, Japanese, CD4 count, antiretroviral therapy, and health insurance)

MSM: men who have sex with men

Discussion

At this large urban HIV clinic in Tokyo, 9.2% of the patients were lost to follow up, with an incidence of 24.9 per 1,000 person-years. Furthermore, 34% of the study patients were illicit drug users and the incidence of LTFU for illicit drug users was almost twice higher than that for non users (35.7 and 19.2 per 1,000 person-years, respectively). Illicit drug use was identified as a significant risk for LTFU in uni- and multi-variate analyses (HR=1.860; 95%CI, 1.282-2.699; p=0.001) (adjusted HR=1.544; 95% CI, 1.028-2.318; p=0.036). The impact of illicit drug use on LTFU was slightly more evident among MSM than in the total study population.

To our knowledge, only a few studies have examined the impact of non-injection illicit drug use on LTFU [9,27], and this is the first such study conducted in Asia. The results showed that illicit drug use is a risk factor for LTFU, which is a marker for prognosis in patients with HIV-1 infection [7–11]. The result emphasizes the need for effective prevention and intervention strategies for illicit drug use in patients with HIV-1 infection in Japan. The finding of a more evident impact of illicit drug use in MSM patients also highlights the need for close monitoring of adherence to HIV care in this group of patients.

Among patients with HIV-1 infection, the prognosis of injection drug users is reported to be worse than that of non-injection drug users [28]. However, this study primarily focused on illicit drug use as a whole, rather than injection drug use for two main reasons; First, only a few studies focused on illicit drug use among HIV-1 infected patients, although a large number of studies focused on injection drugs [24,25,27,29,30]. Illicit drug use in patients with HIV-1 infection is an important issue, because not only illicit drug use lead to inferior treatment outcome compared with non users [16–18], but also non injection drug users are prone to practice high risk sexual behaviors, which might lead to transmission of HIV and other infectious diseases [14,31]. Furthermore, illicit drug use, especially opioid use, can be a trajectory into injection drug use [32,33]. Second, because only 0.5% of the patients were infected with HIV-1 through injection drug use by the end of 2011 in Japan (according to a nationwide surveillance conducted by the AIDS Surveillance Committee of the Ministry of Health, Labour and Welfare that covered all reported cases with HIV-1 infection), the anticipated prevalence of injection drug use was very low (http://api-net.jfap.or.jp/status/2011/11nenpo/hyo_02.pdf. in Japanese). Surprisingly, the prevalence of injection drug use was 4% in this study, the number is much higher than what the AIDS Surveillance Committee reported. This suggests a substantial underreporting for injection drug use as a route of transmission from the patients.

In the planning and design of effective prevention and intervention strategies for illicit drug users with HIV-1 infection in Japan, the unique circumstances related to this issue need to be taken into consideration. First, on one hand, the government maintains a strict punitive policy against illicit drug use and this policy has been one of the factors that helped maintain a relatively low prevalence of illicit drug use (lifetime prevalence 2.9%) [21] (http://www.ncnp.go.jp/nimh/pdf/h21.pdf. in Japanese). On the other hand, possibly due in part to severe criminalization of drug use, treatment and rehabilitation schemes for drug users remain poorly developed [20,34].

Second, most injected drugs in Japan are methamphetamine: In 2010, the number of arrested illicit drug users categorized by each drug was the largest for methamphetamine (12,200), while the numbers for other injectable drugs, such as heroin and cocaine were very small (22 and 112, respectively) (http://www.mhlw.go.jp/bunya/iyakuhin/yakubuturanyou/torikumi/dl/index-01.pdf. in Japanese). In the study patients, injection drug users and methamphetamine users also appeared to overlap considerably. Evidence from other countries shows that methamphetamine use has gained popularity among MSM, and methamphetamine use is strongly associated with high-risk sexual behavior [35–38]. Thus, any intervention for injection drug users with HIV-1 infection in Japan needs to take into consideration the frequent use of methamphetamines.

Several limitations need to be acknowledged. First, due to the nature of single-center study, the results of this study do

not necessarily represent all patients with HIV-1 infection in Japan. However, as abovementioned, our clinic treats approximately 15% of the total HIV patients in Japan, and furthermore, characteristics of the patients with HIV-1 infection newly diagnosed and reported to the Japanese National HIV Registry in 2011 (n=1529) is very similar to those of the study population: 94% male, 64% infected through homosexual contact, and 59% in their 20s and 30s of age (http://api-net.jfap.or.jp/status/2011/11nenpo/hyo_02.pdf. in Japanese). Most HIV-1 infected patients reside in urban areas such as Tokyo metropolitan area as well. Thus, the discrepancy between the study patients and all HIV patients in Japan should not be too large. Second, the structured interview designed for data collection does not prevent underreporting of illicit drug use. However, underreporting to a certain degree is unavoidable with regard to issues such as illicit drugs [19].

In conclusion, the incidence of LTFU in illicit drug users was almost twice higher than that in non users among patients with HIV-1 infection in Japan. Multivariate analysis identified illicit drug use as a significant risk factor for LTFU, which influences prognosis of patients with HIV-1 infection. Little data is available for illicit drug use in Japan, especially among patients with HIV-1 infection. However, all relevant parties in relation to this issue need to recognize that illicit drug use has spread among patients with HIV-1 infection, and that illicit drugs worsens adherence to HIV care in Japan. Appropriate measures for prevention and intervention of illicit drug use are urgently needed to ensure proper treatment and prevention of spread of HIV infection.

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Author Contributions

Conceived and designed the experiments: TN HG HK MT SO. Performed the experiments: MO KI. Analyzed the data: TN HK HG MT SO. Contributed reagents/materials/analysis tools: MO KI SO. Wrote the manuscript: TN HG MT SO.

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