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

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

Conclusions

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

Introduction

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

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

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

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

Methods

Ethics Statement

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

Study Design and subjects

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

Measurements

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

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

Genetic polymorphisms

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

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

Pharmacogenetic analyses

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

Statistical analysis

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

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

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

Results

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

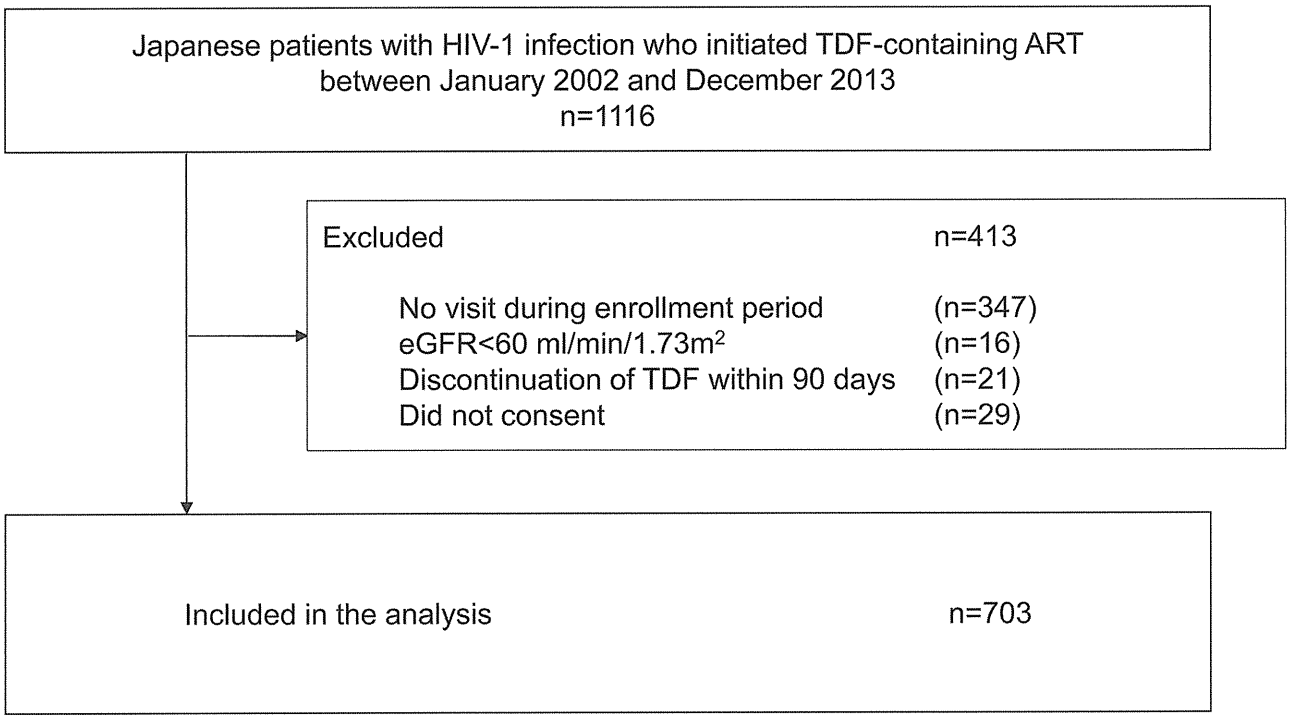

Fig 1. Flow diagram of the patient enrolment process.

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

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

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

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

Table 1. Baseline characteristics of the study patients.

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

†median (interquartile range)

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

doi:10.1371/journal.pone.0141931.t003

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

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

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

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

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

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

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

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

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

Supporting Information

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

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

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

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Author Contributions

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

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RESEARCH ARTICLE

What Triggers a Diagnosis of HIV Infection in the Tokyo Metropolitan Area? Implications for Preventing the Spread of HIV Infection in Japan

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Abstract

Background

Japan has not succeeded in reducing the annual number of new HIV-infected patients, although the prevalence of HIV infection is low (0.02%).

Methods

A single-center observational study was conducted at the largest HIV clinic in Tokyo, which treats 15% of the total patients in Japan, to determine the reasons for having diagnostic tests in newly infected individuals. HIV-infected patients who visited our clinic for the first time between 2011 and 2014 were analyzed.

Results

The 598 study patients comprised one-third of the total reported number of new patients in Tokyo during the study period. 76% were Japanese MSM. The reasons for being tested which led to the diagnosis was voluntary testing in 32%, existing diseases in 53% (AIDS-defining diseases in 22%, sexually transmitted infections (STI) in 8%, diseases other than AIDS or STIs in 23%) and routine pre-surgery or on admission screening in 15%. 52% and 74% of the study patients and patients presented with AIDS, respectively, had never been tested. The median CD4 count in patients with history of previous testing (315/μL) was significantly higher than that of patients who had never been tested (203/μL, $p<0.001$).

Conclusions

Only 32% of the newly HIV diagnosed patients were diagnosed because of voluntary testing, and 53% were diagnosed due to presence of other diseases. These results remain unchanged from our previous report 10 years earlier (2000–2004) on newly diagnosed

patients at the same clinic. HIV testing has not been widely used by newly diagnosed patients in the Tokyo metropolitan area.

Introduction

The advent and evolution of the combination antiretroviral therapy (cART) has substantially improved the prognosis of patients with HIV infection [1]. Furthermore, suppression of HIV viremia with cART does not only improve the prognosis of HIV-infected individuals regardless of their CD4 count [2,3], but also prevent the sexual transmission of HIV regardless of heterosexual or homosexual contact [4,5]. This "treatment as prevention" strategy is regarded as the main force in the attempt to prevent HIV transmission worldwide [6–8], since a preventive vaccine is currently unavailable. Based on this strategy, American Health and Human Services Guidelines recommend cART for all HIV-infected individuals and even the WHO guidelines were updated in 2015 to recommend that "ART should be initiated in all adults living with HIV at any CD4 cell count", with the hope of reducing the number of newly infected individuals [8,9]. At this stage, the importance of promoting HIV testing for individual at risk for HIV infection cannot be overemphasized, because diagnosis in the early stage of HIV infection and prompt introduction of cART will improve the prognosis of infected individuals [10,11] and at the same time prevent the transmission of HIV [4]. Together with other means, such as circumcision, condom usage, and needle and syringe program, efforts towards the prevention of HIV epidemic appear to be fruitful, with a reported decrease in the number of newly infected individual worldwide from 3.4 million in 2001 to 2.3 million in 2012 [12].

In Japan, however, efforts towards prevention of HIV epidemic have not been successful. Physicians are required to report a diagnosed HIV-infected patient by law, and since the first case was reported in the 1980s, the number of newly infected individuals continues to rise, and was 1,500 per year in 2007. Since 2007 approximately 1,500 new infections are being diagnosed every year for the last 8 years [13]. In Japan, majority of HIV-infected individuals are Japanese men who have sex with men (MSM) and only a few are injection drug users and females, and the majority reside in the metropolitan areas of Tokyo, Osaka, and Nagoya [13]. Especially the Tokyo metropolitan area, including Tokyo, Kanagawa, Saitama, and Chiba prefectures have the largest number of HIV infected individuals, as 46% of the total reported number of HIV-infected individuals in Japan have been reported in this area [13]. It is also noteworthy that approximately 30% of the patients were diagnosed in the advanced stage of HIV infection, following the development of AIDS-defining diseases [13].

Based on the abovementioned background, the present study was designed to understand the reasons for having diagnostic tests in newly infected individuals visiting the largest HIV clinic in Japan located in the Tokyo metropolitan area. Such understanding could help design effective intervention policies to prevent the spread of HIV infection in Japan.

Methods

Study design, setting, and participants

We conducted a single-center observational study to elucidate the reasons for having HIV diagnostic testing in newly infected individuals in the Tokyo metropolitan area. Our clinic, AIDS Clinical Center, National Center for Global Health and Medicine (NCGM), Tokyo, is the largest referral center for HIV infection in Japan [14] with approximately 4,000 registered patients. In this regard, the total reported number of patients with HIV infection in Japan at the end of

2014 was 26,000 [13]. Of these, 6,408 resided in Tokyo, and 12,032 in the Tokyo metropolitan area, including Tokyo, Kanagawa, Saitama, and Chiba prefectures. Thus, our clinic managed approximately 15% of the total HIV infected patients in Japan, and substantially higher percentage of HIV-infected patients in the Tokyo metropolitan area. The following inclusion criteria were applied for enrollment of patients in the study: 1) HIV-infected patients with over 19 years of age who visited our clinic for the first time between January 2011 and December 2014, 2) diagnosis of HIV infection was established preceding and within one year from the first visit to the clinic, thus including only recently diagnosed patients. All HIV-1-infected patients who visited our clinic for the first time were tested with the combined HIV-1 antigen and HIV-1/2 antibody fourth-generation assay, HIV-1 RNA PCR assay, and HIV-1 Western Blot, and only those who were confirmed to be HIV-1 positive based on the results of these assays were included as the study patients. Following exclusion criteria were applied: 1) patients who were vertically infected with HIV-1, 2) patients whose reason for undergoing the diagnostic tests for HIV infection was unknown, and 3) patients who did not undergo routine blood and urine tests in the first visit, such as those who visited the clinic for a second opinion.

The study protocol was approved by the Human Research Ethics Committee of National Center for Global Health and Medicine. Informed consent was waived because this study solely used the data gained from clinical practice. The clinical records were de-identified and analyzed anonymously. The study was conducted according to the principles expressed in the Declaration of Helsinki.

Definitions and measurements

The reasons for HIV diagnostic testing, the day (if not available, the month) of diagnosis of HIV infection, history of AIDS-defining diseases, perceived route of transmission, sexual orientation (men were asked whether they have sex with men), history of previous HIV testing, treatment status of HIV infection (treatment-naïve or experienced), and history of HBV vaccination, as well as the basic characteristics, such as age, sex, and ethnicity, were collected through a structured interview conducted at the first visit as part of routine clinical practice by the nurses specializing at the HIV outpatient care [15], and also through a structured interview by the treating physician. The patients who voluntarily tested for HIV infection were also asked whether they had tested because their sexual partners were diagnosed of HIV infection. Blood samples were also routinely collected at the first visit for CD4 count, HIV-1 RNA viral load, HIV-1 western blot testing, hepatitis B surface antigen (HBsAg), antibody to HBsAg (anti-HBs), antibody to hepatitis B core antigen (anti-HBc), hepatitis C antibody (anti-HCV), serum quantitative *Treponema pallidum* hemagglutination (TPHA) and rapid plasma reagin (RPR) test, and anti-Entamoeba histolytica antibody (anti-Eh).

The reasons for having HIV diagnostic tests were classified into 5 categories: (1) patients who were voluntarily tested (the voluntary group); voluntary testing was applied to those who visited public health center or other healthcare facilities for the purpose of receiving HIV diagnostic tests, or those who performed home-based self-test. This group also included subjects who were voluntary tested and later were diagnosed with AIDS-defining diseases. (2) occurrence of AIDS-defining diseases (23 diseases set by the Japanese Ministry of Health, Labour, and Welfare [16]) (the AIDS group), (3) diagnosis with sexually transmitted infections (STI) (the STI group), (4) occurrence of diseases other than AIDS-defining diseases and STIs (the non-AIDS disease group), (5) patients diagnosed incidentally after routine screening, such as before surgery, on admission to the hospital, or antenatal screening (the screening group). Acute HIV infection was defined as positive HIV nucleic acid testing and a non-reactive or indeterminate western blot [17]. Active syphilis infection which required treatment was

defined as patients with both serum RPR titer ≥8 and positive TPHA result [18]. History of syphilis was defined as patients with positive TPHA. Chronic HBV infection was defined as patients with positive HBsAg, whereas exposure to HBV was defined as those with either positive HBsAg, anti-HBsAg, or anti-HBc [19], because in Japan, universal HBV vaccination has not been introduced, except for health care professionals [20].

Statistical analysis

Baseline characteristics were described for the entire study patients. The median CD4 count of patients with history of previous HIV testing and no previous testing were compared with the Mann-Whitney U test. Statistical significance was defined as two-sided p values <0.05. All statistical analyses were performed with The Statistical Package for Social Sciences ver. 21.0 (SPSS, Chicago, IL).

Results

A total of 822 patients with HIV infection visited our clinic for the first time during the study period (Fig 1). Of them, 598 patients were analyzed as the study patients. The study patients comprised one-third of the total reported number of newly diagnosed HIV-infected individuals in Tokyo during the study period [13]. 95% of the study patients were males, and 88% were Japanese (Table 1). The median age was 37 [interquartile range (IQR) 30–44)], and 24% were in their 20's while 59% were <40 years old. Furthermore, 84% were MSM, and Japanese MSM comprised 76% of the study subjects. 96% were treatment-naïve, with a median CD4 count of 231 /μL (86–390), and HIV viral load of 4.92 $\log_{10}$copies/mL (4.38–5.44). 44 (7%) patients presented with acute HIV infection.

The reasons for undergoing the diagnostic tests for HIV infection was voluntary testing in 190 (31.8%), AIDS-defining diseases in 129 (21.6%), STIs in 50 (8.4%), diseases other than AIDS or STIs in 139 (23.2%), and before surgery, on admission, or antenatal routine screening in 90 (15%). Of the voluntary group, 47 (25%) individuals requested the tests because their partners had been diagnosed with HIV infection, and only 6 (3.1%) patients used a home-based self-test kit, including a mailing kit. The percentage of patients who were diagnosed of HIV infection because of voluntary testing among MSM was significantly higher than that among non-MSM [170 (34%) of 502 versus 20 (21%) of 96, p = 0.012)]. 52% of the study patients have never been tested previously for HIV infection, and among those with AIDS-defining diseases, 74% have never been tested previously. Furthermore, among patients who have never been tested previously for HIV infection, 71 (27%) had a history of STIs, including syphilis, hepatitis A, B, or C, gonorrhea, genital herpes, chlamydia, condyloma acuminatum, amoebiasis, and pubic lice. However, they were not screened for HIV infection at the time of STI presentation.

The median CD4 count at the first visit was 338 /μL (IQR 211–467) in the voluntary testing group, 292 /μL (IQR 147–408) in the screening group, 259 /μL (IQR 155–415) in the STI group, 234 /μL (IQR 122–392) in the non-AIDS disease group, and was the lowest in the AIDS group [54 /μL (IQR 23–98)]. Furthermore, the median CD4 count was significantly higher in patients with history of previous testing (315 /μL, IQR 175–450) than those without (203 /μL, IQR 81–354) (p<0.001, the Mann-Whitney U test).

Active syphilis infection that required treatment was diagnosed in 17% of the study patients, whereas history of syphilis defined by positive TPHA was observed in 34%. HBsAg was positive in 7% of the patients, and 51% were exposed to HBV. HCVAb was positive in 4% of the patients, and anti-Eh antibody was positive in 19%.

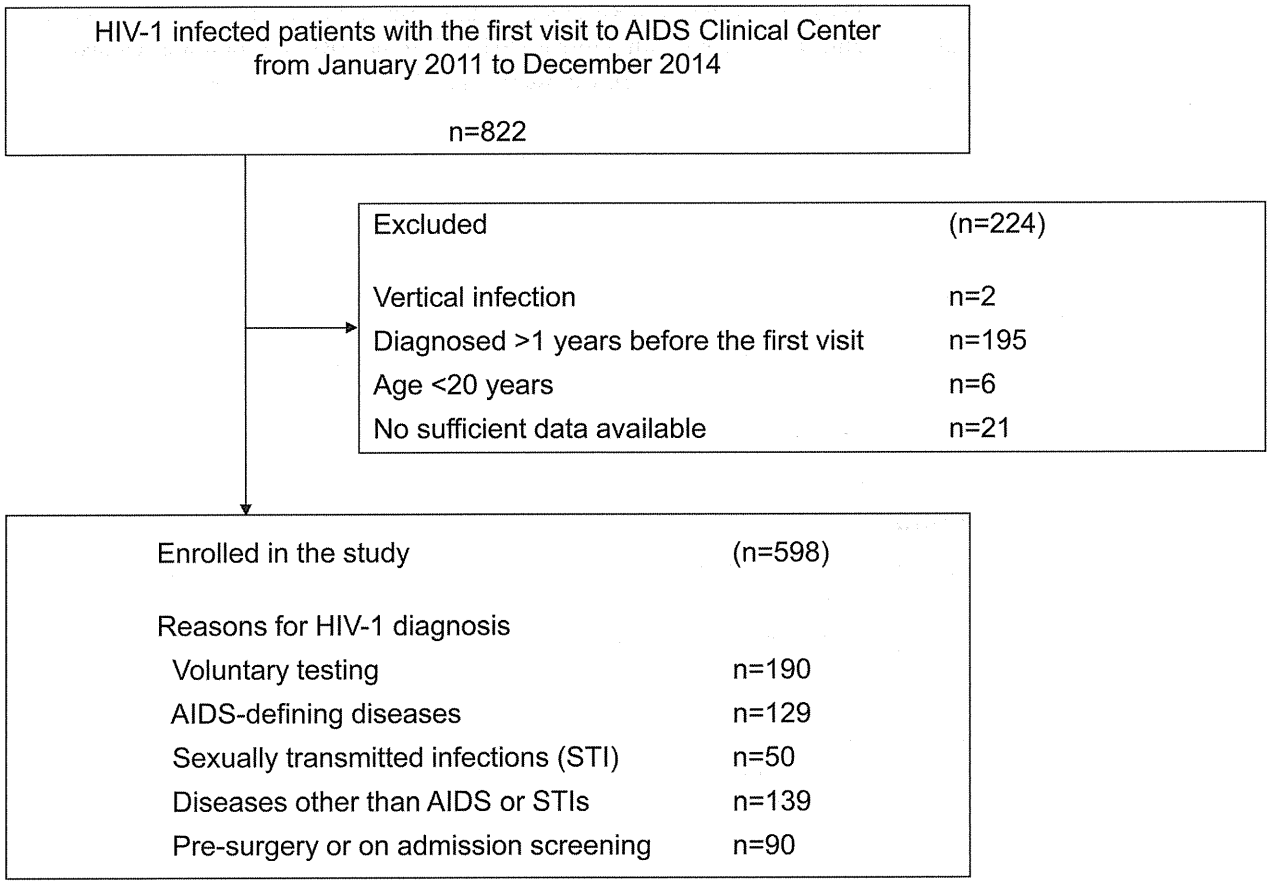

Fig 1. Patient enrollment process.

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Discussion

In this largest HIV clinic in Japan where approximately 15% of the total patients in Japan are treated, only 32% of the newly diagnosed patients between 2011 and 2014 were diagnosed with HIV infection because of voluntary testing. Alarmingly, 53% of the newly diagnosed patients underwent testing after the development of other diseases; which were either AIDS-defining diseases (22%), STIs (8%), or diseases other than AIDS and STIs (23%). Furthermore, 15% of the new diagnosis were incidentally made by the routine screening on admission to hospital, before surgery, or antenatal screening. More importantly, 52% of the newly diagnosed patients had never been tested for HIV infection, and this proportion was even higher (74%) among those who presented with AIDS-defining diseases. These results showed that HIV testing has not been widely utilized in newly diagnosed patients with HIV infection, who had been at high risk for HIV acquisition, in the largest HIV clinic in Japan located in the Tokyo metropolitan area.

Early establishment of the diagnosis of HIV infection and early initiation of treatment are both crucial for improvement of prognosis and lessening the spread of infection [10,11,21]. Our study also showed that the median CD4 count of the patients who were voluntarily tested and diagnosed [338 /μL (IQR 211–467)] was higher than that of patients with AIDS [54 /μL (IQR 23–98)] and non-AIDS diseases [234 /μL (IQR 122–392)]. In this regard, various efforts have been made to promote HIV testing in Japan, such as setting up free and anonymous

Table 1. Characteristics of the study patients (n = 598).

	n or median	% or interquartile range
Male sex, n (%)	565	94.5
Age (years)†		
20–29	144	24.1
30–39	210	35.1
40–49	175	29.3
>49	69	11.5
Ethnicity		
Japanese	523	87.5
Asians other than Japanese	41	6.9
Others	34	5.6
CD4 count (/μl)†¶	231	86–390
HIV RNA load (log_{10}/ml)†	4.92	4.38–5.44
Treatment-naive, n (%)	572	95.7
Men who have sex with men	502	83.9
No history of previous HIV testing¶	261	52
AIDS-defining illnesses	165	27.6
Acute HIV infection	44	7.4
Positive anti-Eh antibody¶	109	19
Rapid plasma reagin titer ≥8	103	17.2
Positive TPHA	204	34.1
Positive HCV antibody	22	3.7
Positive HBs antigen	43	7.2
HBV exposure*	306	51.3
Route of transmission		
Homosexual contact	491	82.1
Heterosexual contact	89	14.9
Injection drug or homosexual contact	11	1.8
Unknown	7	1.2

†Median (interquartile range). anti-Eh antibody, anti-entamoeba histolytica antibody; TPHA, *Treponema pallidum* hemagglutination; HCV, hepatitis C virus; HBs antigen, hepatitis B surface antigen

¶CD4 count is missing for one patient, history of previous HIV testing is missing for 96 patients, and anti-Eh antibody is missing for 25 patients.

*Two patients with history of HBV vaccination were excluded.

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testing sites at public health centers and other facilities across Japan, creating a website that provides information on HIV testing, publishing HIV testing guidelines for use of healthcare facilities, ensuring availability of rapid HIV tests at private clinics in the high-prevalence area, and outreaching events for sexual minorities to promote diagnostic testing. These efforts were spearheaded mainly by different study groups established by the Japanese Ministry of Health, Labour, and Welfare, in collaboration with various non-governmental organizations [22]. However, the annual reported number of newly infected cases reached its peak in 2007 with 1,500, and has stabilized with approximately 1,500 new patients every year in the last 8 years [13]. It is disappointing that the results of the present study are very similar to our previous report, which investigated the reasons for diagnostic testing in newly infected patients who visited our clinic for the first time between 2000 and 2005 [23]. In that study, the voluntary testing group comprised 35% of newly infected patients, while patients with diseases including AIDS

comprised 52%, and those who were incidentally diagnosed due to routine clinical testing formed 13% (Table 2). One major difference between the present and previous analyses is the percentage of patients without history of previous testing (decreased from 74% to 52%). These results highlight the difficulty in promoting HIV testing among high-risk population, such as MSM, in Japan, which has very low prevalence of HIV infection (prevalence: 0.02%, based on 26,000 reported patients by the end of 2014 [13] among a population of 127 million according to the census conducted in January 2015 [24]).

How can we reduce the number of newly HIV-infected individuals in Japan? Efforts to promote HIV diagnostic testing in high-risk population need to be continued and strengthened, however, it is probably not enough to reduce the number of new infections, as described above. Considering the difficulty of testing a high-risk population in a country with very low prevalence of HIV infection, the "treatment as prevention" strategy, which in principle encourages all HIV-infected individuals to start treatment, might be an efficient way to prevent transmission of HIV. Currently, the Japanese Guidelines for the treatment of HIV infection 2015 recommend initiation of cART in a treatment-naïve patient with CD4 count $\leq$350 /μL (strong recommendation), with CD4 351–500 /μL (strong/moderate recommendation), and also for those with CD4 count >500 /μL (moderate recommendation based on expert opinion) [25]. However, in Japan, most HIV-infected individuals obtain a certificate that allows them to receive financial assistance for out-of-pocket medical expenditure for cART, and for patients with CD4 count >500 /μL it is not always possible to obtain such assistance [26], and many such patients hesitate to start cART until CD4 count decreases to <500 /μL because of financial concern. It is desirable to remove this CD4 threshold from the requirement for obtaining financial assistance if "treatment as prevention" strategy is to be further promoted for the prevention of HIV epidemic in Japan, and to improve the prognosis of patients with CD4 count >500 /μL by early initiation of cART [2,3]. This "treatment as prevention" strategy is further backed up by a recently published article by Nosyk and colleagues which showed that treatment-for-all strategy in British Columbia, Canada, has been successful in not only reducing the number of new HIV-1 infected patients, but also being cost effective and furthermore, will be cost saving in the long-term [27].

Another important strategy for the diagnosis of HIV-infected patients in the early stage of infection is partner notification, counseling, and testing of sex partners of patients newly diagnosed with HIV infection, because such partners are at very high risk for HIV infection and diagnostic testing will benefit health of such partners [28]. Provider-assisted partner counseling and testing at our clinic has been very successful, as we had reported that 17 out of 86 (20%) of tested partners of patients with newly diagnosed infection were found to have HIV infection [29]. The present study also showed that among the patients who were voluntary tested for HIV, 25% had such test because of partner notification, suggesting the importance of such strategy.

Table 2. Comparison of reasons for HIV diagnostic testing in newly diagnosed patients between 2000–2005 and 2011–2014 time periods.

Reasons for HIV diagnostic testing	2000–2005 (n = 654)		2011–2014 (n = 598)	
	n	%	N	%
Voluntary testing	230	35	190	32
Presence of diseases (AIDS, non AIDS, or STIs)	338	52	318	53
Routine before-surgery or on admission screening	86	13	90	15

The data on newly diagnosed patients between 2000 and 2005 were cited from our previous study [23].

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Moreover, patients who present with STIs, especially syphilis and horizontally infected HBV, should be exhaustively tested for HIV infection, since such STIs are highly prevalent among patients with newly diagnosed HIV infection, as shown in the present study. The result that among the patients who had never been tested previously for HIV infection, 27% of such patients actually had a history of STIs but they were not tested for HIV, warrants the need for further raising awareness in healthcare personnel including primary care physicians.

In this study, only 3.2% of the patient who voluntarily tested for HIV infection used home-based HIV self-test. Consistent with this result, in Japan, home-based HIV self-test has a limited role in diagnosis of HIV infection, where the mainstream of the home-based test is "a mailing HIV self-check kit". The reported number of usage of such kit is increasing from 39,868 in 2006 to 73,863 in 2013, however, the reported number of positive result has been stable from 221 in 2006 to 192 in 2013 [30]. This mailing kit is a screening assay and cannot make definitive diagnosis of HIV infection. Also, they are expected to yield many false positive results due to low prevalence of HIV infection in Japan.

The strength of the present study include the uniqueness of detailed data on the reasons for diagnostic testing in newly infected individuals in Japan, a large-scale study which included one-third of the total reported number of newly diagnosed patients in Tokyo, and comparability of the present study with those of a previous study conducted by the same institution about 10 years earlier. Apart from the strengths of this study, few limitations need to be acknowledged. Being a single-center study, selection bias is not avoidable. However, as described above, our clinic treats approximately 15% of the patients in Japan, and the study patients covered one-third of the total newly diagnosed patients in Tokyo, where prevalence of HIV infection is highest [13]. Furthermore, the study population truly represents HIV-infected patients in Japan as a whole. For example, among the 1,546 newly reported HIV-infected individuals in 2014, 88.5% were Japanese males, 29.4% of the patients presented with AIDS-defining diseases, and 67.7% were infected through homosexual contact [13].

In conclusion, in the largest HIV clinic in Japan, only 32% of the newly diagnosed HIV-infected patients between 2011 and 2014 were diagnosed based on voluntary testing, and 53% were diagnosed because they had AIDS, non-AIDS diseases, or STIs. Furthermore, 52% of the newly diagnosed patients have never been tested for HIV infection. Importantly, these results largely remain unchanged from similar data analyzed 10 years ago by the same clinic [23]. While promoting diagnostic testing for the at-risk population for HIV infection remains important, the practice of "treatment as prevention" strategy needs to be encouraged in order to reduce the spread of HIV infection in Japan.

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Author Contributions

Conceived and designed the experiments: TN MT SO HG. Performed the experiments: MK YS MO KI. Analyzed the data: TN MT. Contributed reagents/materials/analysis tools: YK. Wrote the paper: TN MT SM SO HG.

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