ɔse, M184V was the most common (81.6%) and TAMs also observed frequently in 71.4%: M41L (22.4%), (24.5%), K70R (18.4%), L210W (14.3%), T215F %), T215Y (28.6%), K219E (12.2%) and K219Q (6.1%), as K65R (6.1%), L74V (4.1%), Y115F (2.0%) and ions driven by Q151M complex (4.1%) were relatively Similar to previous reports on drug resistance in 1_AE [28–30], mutations classified into TAM type 2 -2): D67N, K70R, T215F and K219E/Q, were more ɛntly observed than those of TAM type 1 (TAM-1): ., L210W and T215Y/F (30.6% v.s. 26.5%), except for ɛnt having only T215F. With regard to codon 215, T215F more frequently seen with other TAM-2 mutations (six ' eight sequences that contain T215F), concurring with evious reports showing the introduction of T215F into 2 backbone increase relative fitness in the presence of but resulted in decreased viral fitness in TAM-1 back- [37]. The resistance mutations of NNRTIs in the N- ıal half of RT were detected in 79.6%. The most frequent TI-resistance mutations were Y181C/I/V (32.7%), N (26.5%) and G190A (26.5%). In 17 PI experienced ts, no major mutations were found, but 9 minor muta- were detected: L10I/V (11.8%), I13V (88.2%), G16E %), K20R (17.6%), M36I (100%), L63P (29.4%), H69K ɔ), V82I (11.8%) and I93L (8.2%). However, the muta- in protease are considered as consensus amino acids in non-B subtype HIV-1 (I13V, M36I and H69K) or ıon polymorphic mutations (L10V, G16E, K20R, L63P, and I93L) and could not be determined as mutations that ɛd after treatment.

ɛ frequencies of mutations in the C-terminal half of the ported previously as NRTI or NNRTI resistance [7–20] ɛscribed in Table 2. As shown, G335D (100%), N348I %), A371V (100%), A376S (5.3%), E399D (28.9%) and Γ (97.4%) were detected in the patients failing ART. ver, as we reported previously [20], G335D and A371V also commonly observed in untreated patients infected ıon-B subtype HIV-1 and the frequencies of G335D and V in CRF01_AE subtype shown in the Stanford HIV Resistance Database are 95.2% and 97.1%, respectively, those are rare in subtype B (G335D: 1.3%, A371V: . A400T is also one of the known polymorphisms in 1_AE [16]. Therefore, it is unlikely that G335D, A371V 400T in this population were selected by ART exposure olved in the resistance mutations.

ɔrug susceptibility assay for mutant recombinant

address whether G335D or A371V have an impact on susceptibility depending on the pattern of TAMs, we ucted recombinant viruses containing G335D and/or V in the background of TAM-1 or TAM-2 by site- ɛd mutagenesis. As shown in Table 3, G335D, A371V or louble mutant did not increase the resistance levels to all ɜ by themselves. In contrast, as shown in Table 4, vari- ɔith G335D, A371V or both exhibited higher resistance to

Table 2
Frequencies of mutations associated with RTI-resistance in the connection and RNase H domain of reverse transcriptase of HIV-1.

Mutations[b]	Study participants (Treatment failure)		Stanford database[a] (RTI-naïve)	
	CRF01_AE		CRF01_AE	Subtype B
	$n = 38$			
	%	(*n*)	%	%
G333	100	(38)		
D	0	(0)	0	0.7
E	0	(0)	0	7.5
G335	0	(0)		
C	0	(0)	0	0.5
D	100	(38)	92.0	1.3
N348	57.9	(22)		
I	36.8	(14)	0	0.5
T	5.3	(2)	0	0
A360	97.4	(37)		
I	0	(0)	0	0
V	0	(0)	0	0.7
S	2.6	(1)	1.1	0
V365	100	(38)		
I	0	(0)	0	3.2
T369	94.7	(36)		
I	0	(0)	0	0
A	2.6	(1)	19.3	3.3
V	2.6	(1)	2.8	1.2
A371	0	(0)		
V	100	(38)	97.1	3.2
A376	94.7	(36)		
S	5.3	(2)	1.7	5.8
E399	*68.4*	*(26)*		
D	*28.9*	*(11)*	*2.6*	*14*
K	*2.6*	*(1)*	*0*	*0.1*
A400	0	(0)		
T	97.4	(37)	89.2	25.3
L	2.6	(1)	0	1
Q475	100	(38)		
A	0	(0)	0	0
Q509	97.4	(37)		
L	0	(0)	0	0
R	2.6	(1)	0	0

[a] Available from http://hicdb.stanford.edu/index.html.
[b] Resistance mutations reported previously [8–21] are indicated in bold. Resistance was defined as greater than three fold increase of EC_{50} compared to that of NL4-3.

AZT in the background of TAM-1 (8.2- to 23.2-fold) and the increased resistance level was the greatest in the double mutant G335D/A371V. Although G335D/A371V showed statistical increase in resistance to all the other NRTIs except 3TC, the fold increase from TAM-1 mutant was the greatest in AZT (Table 4). Similar to TAM-1 background, G335D, A371V or G335D/A371V with TAM-2 exhibited considerable increase in susceptibility to AZT (52.7-, 21.1-, 52.6-fold, respectively). In addition, there were marginal changes in d4T susceptibility (Table 5) in the three patterns of the mutants, G335D, A371V or G335D/A371V. In TAM-2 background, we also found G335D alone increased susceptibility to ABC (4.2-fold) and to TDF (2.4-fold), and that G335D/A371V increased susceptibility to ddI (7.2-fold), ABC (3.1-fold) and

e 3
; susceptibilities of HIV-1 variants with G335D or A371V.

ation[a]	EC_{50} (μM)[b] (fold increase)											
	AZT		d4T		ddI		3TC		ABC		TDF	
l Type	0.050 ± 0.002		2.55 ± 0.07		1.90 ± 0.17		0.45 ± 0.035		2.48 ± 0.21		0.020 ± 0.0023	
)	0.052 ± 0.004	(1)	3.19 ± 0.14	(1.3)	4.56 ± 0.20	(2.4)	0.45 ± 0.022	(1)	2.71 ± 0.17	(1.1)	0.018 ± 0.0019	(0.9)
/	0.047 ± 0.003	(0.9)	3.26 ± 0.17	(1.3)	5.30 ± 0.02	(2.8)	0.55 ± 0.027	(1.2)	2.32 ± 0.09	(0.9)	0.027 ± 0.0014	(1.3)
)/371V	0.052 ± 0.010	(1)	3.52 ± 0.06	(1.4)	3.38 ± 0.21	(1.8)	0.65 ± 0.023	(1.5)	2.39 ± 0.12	(1)	0.025 ± 0.0031	(1.2)

', zidovudine; d4T, stavudine; ddI, didanosine; 3TC, lamivudine; ABC, abacavir; TDF, tenofovir.
)ata are mean ± SD from at least three independent experiments. Fold increase was the relative change in EC_{50} value compared with that of HIV-1 WT.
)ee Materials and Methods for the construction of clones.

F (5.2-fold). Of note, the increased resistance levels to T, d4T, ddI and TDF were greater in G335D/A371V in M-2 background than that in TAM-1 background. Our data gest double mutant G335D/A371V in TAM-2 background ıld have the most impact on NRTI susceptibility.

)iscussion

n the present study, we described the drug resistance :ations in the entire RT of CRF01_AE HIV-1-infected tnamese patients who had high pVL levels despite 6-month T. According to the criteria used for evaluation of drug stance proposed by Shafer et al. [38,39], correlations ween mutations and treatment should be confirmed by :nsive resistance surveillance. However, limited sequences CRF01_AE in the connection subdomain and RNase H ıain of the RT have been available so far especially from tment-experienced patients [40]. Santos et al. [19] previ-ly compared amino acid variations between treatment-ve and treatment-experienced patients in connection domain (280 naïve vs. 230 treated) and RNase H domain 4 naïve vs. 234 treated). Although their study included stantial number of patients, larger number of cases)nged to subtype B (80–82% of treatment-experienced ents) and the unique characteristics of CRF01_AE,)unting for only 10% of their study, could not be fully :ssed. Since our present study focused on CRF01_AE uence alone, the data provide direct information on the luation of drug resistance mutations in CRF01_AE, ough sequences before ART initiation were not available. largest study to date exploring treatment-related mutation RT C-terminal site in CRF01_AE infection is the report n Thailand by Saeng-aroon et al. [40], in which signi-ntly higher frequencies of N348I, E399D, P537S and I542M in treatment-exposed patients than treatment-naïve patients (76 naïve vs. 49 treated) was noted. Although the former two mutations have already known to be associated with exposure to NRTI or NNRTI and were detected in our treatment-experienced patients, the results of P537S and I542M were different from us: no patients in our study had P537S and I542M. Further studies are required to determine the prevalence of drug resistance mutations in the C-terminal half of RT in CRF01_AE.

Among the mutations previously reported as drug resistance in the connection subdomain and RNase H domain of RT, we found no mutations except G335D, N348I, A371V, A376S, E399D and A400T in treatment-experienced individuals with CRF01_AE infection. Of these mutations, N348I is one of the most extensively assessed mutations in the RT connection domain and has been established as multiclass resistance to both NRTIs and NNRTIs by being identified in clinical isolates in treatment-experienced individuals in subtype B and by *in vitro* drug susceptibility assay [9,10,12,13]. Since N348I is rare in treatment-naïve of both subtype B and CRF01_AE, N348I observed in 35.8% of CRF01_AE sequences in our study was considered to be treatment-related. The wide use of NVP in Viet Nam might be one of the causes of the higher prevalence of N348I in this population than in subtype B. In addition to N348I, E399D has been thought to be associated with resistance to AZT and to EFV when combined with K103R and 179D [41,42]. Although our results of E399D prevalence of in treatment-exposed patients (28.9%) was relatively higher than those in the Stanford database (9%), it was similar to the previous study by Saeng-aroon et al. of treatment-exposed patients with CRF01_AE infection (32.7%) and considered to be selected after treatment. In contrast, A376S detected in this study was not clearly identified as a treatment-related mutation because the frequency (5.3%) was similar to those of treatment-naïve

e 4
; susceptibilities of HIV-1 variants with G335D or A371V in the TAM-1 background.

ıtion	EC_{50} (μM) (fold change)											
	AZT		d4T		ddI		3TC		ABC		TDF	
l Type	0.050 ± 0.002		2.55 ± 0.07		1.90 ± 0.17		0.45 ± 0.035		2.48 ± 0.21		0.020 ± 0.0023	
1-1	0.200 ± 0.016	**(4)**	4.78 ± 0.30	(1.9)	5.35 ± 0.79	(2.8)	2.37 ± 0.017	**(5.3)**	4.20 ± 0.25	(1.7)	0.043 ± 0.0030	(2.2)
1-1/335D	0.411 ± 0.028	**(8.2)**[a]	6.63 ± 0.05	(2.6)	5.71 ± 0.57	(3.0)	2.14 ± 0.099	**(4.8)**	3.17 ± 0.23	(1.3)	0.024 ± 0.0026	(1.2)
1-1/371V	0.473 ± 0.052	**(9.4)**[a]	6.07 ± 0.12	(2.4)	6.30 ± 0.48	**(3.3)**	2.45 ± 0.110	**(5.5)**	3.88 ± 0.32	(1.6)	0.046 ± 0.0018	(2.3)
1-1/335D/371V	1.160 ± 0.078	**(23.2)**[a]	9.01 ± 0.20	**(3.5)**[a]	7.87 ± 0.35	**(4.1)**[a]	2.40 ± 0.016	**(5.4)**	7.57 ± 0.57	**(3.1)**[a]	0.056 ± 0.0004	(2.8)

lface indicates an increase greater than threefold.
ncreases in fold change were significant compared to TAM-1 without G335D or A371V.

ısceptibilities of HIV-1 variants with G335D or A371V in the TAM-2 background.

›n	EC_{50} (μM) (fold increase)											
	AZT		d4T		ddI		3TC		ABC		TDF	
ype	0.050 ± 0.002		2.55 ± 0.07		1.90 ± 0.17		0.45 ± 0.035		2.48 ± 0.21		0.020 ± 0.0023	
	0.3960 ± 0.076	**(7.9)**	6.18 ± 0.11	(2.4)	6.71 ± 0.57	**(3.5)**	2.57 ± 0.089	**(5.7)**	2.97 ± 0.29	(1.2)	0.033 ± 0.0026	(1.7)
/335D	2.6390 ± 0.396	**(52.7)**[a]	7.97 ± 0.47	**(3.1)**[a]	5.74 ± 0.63	**(3)**	2.37 ± 0.082	**(5.3)**	10.43 ± 0.41	**(4.2)**[a]	0.049 ± 0.0014	(2.4)[a]
/371V	1.0600 ± 0.131	**(21.1)**[a]	8.29 ± 0.23	**(3.3)**[a]	6.00 ± 0.64	**(3.2)**	2.58 ± 0.072	**(5.8)**	3.43 ± 0.21	(1.4)	0.036 ± 0.0012	(1.8)
/335D/371V	2.6340 ± 0.132	**(52.6)**[a]	13.71 ± 0.76	**(5.4)**[a]	13.76 ± 0.51	**(7.2)**[a]	2.45 ± 0.062	**(5.5)**	7.57 ± 0.21	**(3.1)**[a]	0.105 ± 0.0030	**(5.2)**[a]

:e indicates an increase greater than threefold.
·eases in fold change were significant compared to TAM-2 without G335D or A371V.

›e B (5.8%) and CRF01_AE (1.7%) infected individuals Stanford database. On the other hand, G335D, A371V and Γ were found in almost all the patients in our study. ugh these three mutations are thought to be related to resistance in subtype B [7,11,16], they are common ıorphisms of wild-type CRF01_AE HIV-1 with preva- of more than 90% in our previous study [20] and in the ›rd database. Therefore, we conclude that G335D, A371V 400T detected in the present study were not selected after ıent but had existed before the introduction of treatment. ·quently, N348I was the only drug resistance mutation in ·terminal half of RT observed in our cohort of treatment- ıenced Vietnamese infected with CRF01_AE HIV-1.

r results demonstrated that common CRF01_AE poly- ıisms G335D and A371V play considerable role in drug ınce to NRTIs. Recent studies suggested that each of D or A371V is associated with drug resistance; G335D ;ed after AZT exposure exhibits greater AZT resistance (8 fold over WT) when combined with TAM [11] and A371V ɛd in the background of D67N and K70R by high ntrations of AZT *in vitro* shows strong resistance to AZT in esence of TAMs [7]. In agreement with those reports, our ; showed that mutant containing G335D or A371V did not se the resistance levels to NRTIs by themselves but they ·red higher resistance when combined with TAMs, espe- to AZT (8.2–52.7 fold increase). Furthermore, we found ıe dual mutation G335D/A371V had the greater impact ach single mutation on resistance in the presence of TAM. 335D and A371V always appear together in treatment- CRF01_AE, this finding is more critical for CRF01_AE infection than for subtype B infection. In addition, the hange increased by G335D and A371V was greater with 2 than that with TAM-1. Since TAM-2 is more frequent in 1_AE than in subtype B [28–30], this data is important for 1_AE HIV-1. Although the impact of G335D and A371V ıe greatest in AZT resistance and seemed to be minor ıer NRTIs' resistance, the fold-increase in TDF of D/A371V plus TAM-2 variant were above the clinical f values [43], which can cause treatment failure. As TDF ɛn used in second line ART [2], this data is crucial cisions on the next therapeutic strategies for CRF01_AE -infected patients failing first line ART. Since our ıbinant viruses were created with pBS-RT_{WT}, which was ·d from subtype B RT but not from CRF01_AE RT, our ; cannot be applied directly to CRF01_AE infection. CRF01_AE/B recombinants have been emerged and highly prevalent in Southeast Asian countries [32,44,45] and the breakpoint analysis showed some CRF01_AE/B recombinants consisted of subtype B N-terminal site and CRF01_AE C-terminal sites [45]. Therefore, our data suggests the potential influence of those CRF01_AE/B recombinants as well as CRF01_AE strain on the selection of second line therapy in Southeast Asia.

In summary, we reported the frequencies of drug resistance mutations in the connection subdomain and RNase H domain of RT in CRF01_AE HIV-1-infected Vietnamese who experienced ART. Then we demonstrated that the combination of G335D and A371V, a common pattern of polymorphisms in wild-type CRF01_AE, confer significant resistance to various NRTIs in the presence of TAMs. Our findings emphasize the important role of polymorphisms in C-terminal half of RT in CRF01_AE HIV-1 on drug resistance, especially in consideration of the second line therapy. Further investigation is needed on drug resistance mutations in widely prevailing non-subtype B HIV-1.

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References

[1] C.F. Gilks, S. Crowley, R. Ekpini, S. Gove, J. Perriens, Y. Souteyrand, D. Sutherland, M. Vitoria, T. Guerma, K. De Cock, The WHO public-health approach to antiretroviral treatment against HIV in resource-limited settings, Lancet 368 (2006) 505–510.

[2] Ministry of Health of Viet Nam, Decision by the Minister of Health on the Issuance of the Guidelines for HIV/AIDS Diagnosis and Treatment: Guidelines for HIV/AIDS Diagnosis and Treatment. Health Publishing House, 2005, No: 06/2005QD-BYT.

NAIDS/WHO, AIDS epidemic update ASIA regional summary 2007 vailable from: http://data.unaids.org/pub/Report/2008/jc1527_epibriefs_ia_en.pdf (2008).
NAIDS/WHO, Epidemiological fact sheets on HIV/AIDS and sexually nsmitted infections Available from: http://apps.who.int/GlobalAtlas/edefinedReports/EFS2008/index.asp (2008).
inistry of Health of Viet Nam, The third country report on following up e implementation to the declaration of commitment on HIV and AIDS vailable from: http://data.unaids.org/pub/Report/2008/viet_nam_2008_untry_progress_report_en.pdf (2008).
E. Bennett, M. Myatt, S. Bertagnolio, D. Sutherland, C.F. Gilks, ecommendations for surveillance of transmitted HIV drug resistance in untries scaling up antiretroviral treatment, Antivir. Ther. 13 (2008) 25–36.
H. Brehm, D. Koontz, J.D. Meteer, V. Pathak, N. Sluis-Cremer, W. Mellors, Selection of mutations in the connection and RNase H mains of human immunodeficiency virus type 1 reverse transcriptase at increase resistance to 3′-azido-3′-dideoxythymidine, J. Virol. 81 007) 7852–7859.
A. Delviks-Frankenberry, G.N. Nikolenko, R. Barr, V.K. Pathak, utations in human immunodeficiency virus type 1 RNase H primer grip hance 3′-azido-3′-deoxythymidine resistance, J. Virol. 81 (2007) 37–6845.
A. Delviks-Frankenberry, G.N. Nikolenko, P.L. Boyer, S.H. Huges, J. Coffin, A. Jere, V.K. Pathak, HIV-1 reverse transcriptase connection bdomain mutations reduce template RNA degradation and enhance ZT excision, Proc. Natl. Acad. Sci. USA 105 (2008) 10943–10948.
Hachiya, E.N. Kodama, S.G. Sarafianos, M.M. Schuckmann, Y. kagami, M. Matsuoka, M. Takiguchi, H. Gatanaga, S. Oka, Amino id mutation N348I in the connection subdomain of human immuno-ficiency virus type 1 reverse transcriptase confers multiclass resistance nucleoside and nonnucleoside reverse transcriptase inhibitors, J. Virol. (2008) 3261–3270.
N. Nikolenko, K.A. Delviks-Frankenberry, S. Palmer, F. Maldarelli, J. Fivash Jr., J.M. Coffin, V.K. Pathak, Mutations in the connection domain HIV-1 reverse transcriptase increase 3′-azido-3′-deoxythymidine resis-nce, Proc. Natl. Acad. Sci. USA 104 (2007) 317–322.
H. Yap, C.W. Sheen, J. Fahey, M. Zanin, D. Tyssen, V.D. Lima, B. ynhoven, M. Kuiper, N. Sluis-Cremer, P.R. Harrigan, G. Tachedjian, 348I in the connection domain of HIV-1 reverse transcriptase confers dovudine and nevirapine resistance, PLoS Med. 4 (2007) e335.
Ehteshami, G.L. Beilhartz, B.J. Scarth, E.P. Tchesnokov, S. McCor-ick, B. Wynhoven, P.R. Harrigan, M. Götte, Connection domain utations N348I and A360V in HIV-1 reverse transcriptase enhance sistance to 3′-azido-3′-deoxythymidine through both RNase H-depen-nt and -independent mechanisms, J. Biol. Chem. 283 (2008) 222–22232.
Zelina, C.W. Sheen, J. Radzio, J.W. Mellors, N. Sluis-Cremer, echanisms by which the G333D mutation in human immunodeficiency rus type 1 reverse transcriptase facilitates dual resistance to zidovudine d lamivudine, Antimicrob. Agents Chemother. 52 (2008) 157–163.
H. Brehm, J.W. Mellors, N. Sluis-Cremer, Mechanism by which glutamine to leucine substitution at residue 509 in the ribonuclease H main of HIV-1 reverse transcriptase confers zidovudine resistance, ochemistry 47 (2008) 14020–14027.
A. Delviks-Frankenberry, G.N. Nikolenko, F. Maldarelli, S. Hase, Y. kebe, V.K. Pathak, Subtype-specific differences in the human immu-deficiency virus type 1 reverse transcriptase connection subdomain CRF01_AE are associated with higher levels of resistance to 3′-azido--deoxythymidine, J. Virol. 83 (2009) 8502–8513.
D. Kemp, C. Shi, S. Bloor, P.R. Harrigan, J.W. Mellors, B.A. Larder, A vel polymorphism at codon 333 of human immunodeficiency virus pe 1 reverse transcriptase can facilitate dual resistance to zidovudine d L-2′,3′-dideoxy-3′-thiacytidine, J. Virol. 72 (1998) 5093–5098.
Ntemgwa, M.A. Wainberg, M. Oliveira, D. Moisi, R. Lalonde, V. icheli, B.G. Brenner, Variations in reverse transcriptase and RNase H main mutations in human immunodeficiency virus type 1 clinical olates are associated with divergent phenotypic resistance to zidovu-ne, Antimicrob. Agents Chemother. 51 (2007) 3861–3869.
[19] A.F. Santos, R.B. Lengruber, E.A. Soares, A. Jere, E. Sprinz, A.M. Martinez, J. Silveira, F.S. Sion, V.K. Pathak, M.A. Soares, Conservation patterns of HIV-1 RT connection and RNase H domains: identification of new mutations in NRTI-treated patients, PLoS ONE 3 (2008) e1781.
[20] A. Hachiya, K. Shimane, S.G. Sarafianose, E.N. Kodama, Y. Sakagami, F. Negishi, H. Koizumi, H. Gatanaga, M. Matsuoka, M. Takiguchi, S. Oka, Clinical relevance of substitutions in the connection subdomain and RNase H domain of HIV-1 reverse transcriptase from a cohort of antiretroviral treatment-naïve patients, Antiviral. Res. 82 (2009) 115–121.
[21] L. Buonaguro, M.L. Tornesello, F.M. Buonaguro, Human immunodeficiency virus type 1 subtype distribution in the worldwide epidemic: pathogenetic and therapeutic implications, J. Virol. 81 (2007) 10209–10219.
[22] R. Kantor, Impact of HIV-1 pol diversity on drug resistance and its clinical implications, Curr. Opin. Infect. Dis. 19 (2006) 594–606.
[23] R. Kantor, D.A. Katzenstein, B. Efron, A.P. Carvalho, B. Wynhoven, P. Cane, J. Clarke, S. Sirivichayakul, M.A. Soares, J. Snoeck, C. Pillay, H. Rudich, R. Rodrigues, A. Holguin, K. Ariyoshi, M.B. Bouzas, P. Cahn, W. Sugiura, V. Soriano, L.F. Brigido, Z. Grossman, L. Morris, A.M. Vandamme, A. Tanuri, P. Phanuphak, J.N. Weber, D. Pillay, P.R. Harrigan, R. Camacho, J.M. Schapiro, R.W. Shafer, Impact of HIV-1 subtype and antiretroviral therapy on protease and reverse transcriptase genotype: results of a global collaboration, PLoS Med. 2 (2005) e112.
[24] J. Snoeck, R. Kantor, R.W. Shafer, K. Van Laethem, K. Deforche, A.P. Carvalho, B. Wynhoven, M.A. Soares, P. Cane, J. Clarke, C. Pillay, S. Sirivichayakul, K. Ariyoshi, A. Holguin, H. Rudich, R. Rodrigues, M.B. Bouzas, F. Brun-Vézinet, C. Reid, P. Cahn, L.F. Brigido, Z. Grossman, V. Soriano, W. Sugiura, P. Phanuphak, L. Morris, J. Weber, D. Pillay, A. Tanuri, R.P. Harrigan, R. Camacho, J.M. Schapiro, D. Katzenstein, A.M. Vandamme, Discordances between interpretation algorithms for genotypic resistance to protease and reverse transcriptase inhibitors of human immunodeficiency virus are subtype dependent, Antimicrob. Agents Chemother. 50 (2006) 694–701.
[25] E. Caride, R. Brindeiro, K. Hertogs, B. Larder, P. Dehertogh, E. Machado, C.A. de Sá, W.A. Eyer-Silva, F.S. Sion, L.F. Passioni, J.A. Menezes, A.R. Calazans, A. Tanuri, Drug-resistant reverse transcriptase genotyping and phenotyping of B and Non-B subtypes (F and A) of human immunodeficiency virus type I found in Brazilian patients failing HAART, Virology 275 (2000) 107–115.
[26] B. Montes, L. Vergne, M. Peeters, J. Reynes, E. Delaporte, M. Segondy, Comparison of drug resistance mutations and their interpretation in patients infected with non-B HIV-1 variants and matched patients infected with HIV-1 subtype B, J. Acquir. Immune. Defic. Syndr. 35 (2004) 329–336.
[27] V. Novitsky, C.W. Wester, V. DeGruttola, H. Bussmann, S. Gaseitsiwe, A. Thomas, S. Moyo, R. Musonda, E. Van Widenfelt, R.G. Marlink, M. Essex, The reverse transcriptase 67N 70R 215Y genotype is the predominant TAM pathway associated with virologic failure among HIV Type 1C-infected adults treated with ZDV/ddI-Containing HAART in Southern Africa, AIDS Res. Hum. Retrovir. 23 (2007) 868–878.
[28] K. Ariyoshi, M. Matsuda, H. Miura, S. Tateishi, K. Yamada, W. Sugiura, Patterns of point mutations associated with antiretroviral drug treatment failure in CRF01_AE (subtype E) infection differ from subtype B infection, J. Acquir. Immune. Defic. Syndr. 33 (2003) 336–342.
[29] L.Y. Hsu, R. Subramaniam, L. Bacheler, N.I. Paton, Characterization of mutations in CRF01_AE virus isolates from antiretroviral treatment-naive and -experienced patients in Singapore, J. Acquir. Immune. Defic. Syndr. 38 (2005) 5–13.
[30] W.C. Yam, J.H. Chen, K.H. Wong, K. Chan, V.C. Cheng, H.Y. Lam, S.S. Lee, B.J. Zheng, K.Y. Yuen, Clinical utility of genotyping resistance test on determining the mutation patterns in HIV-1 CRF01_AE and subtype B patients receiving antiretroviral therapy in Hong Kong, J. Clin. Virol. 35 (2006) 454–457.
[31] C. Sukasem, V. Churdboonchart, W. Sukeepaisarncharoen, W. Piroj, T. Inwisai, M. Tiensuwan, W. Chantratita, Genotypic resistance profiles in antiretroviral-naive HIV-1 infections before and after initiation of first-line HAART: impact of polymorphism on resistance to therapy, Int. J. Antimicrob. Agents 31 (2008) 277–281.

H.L. Nguyen, P. Recordon-Pinson, V.H. Pham, N.T. Uyen, T.T. Lien, .T. Tien, I. Garrigue, M.H. Schrive, I. Pellegrin, M.E. Lafon, J.P. oulker, F. Barré-Sinoussi, H.J. Fleury, HIV type 1 isolates from 200 ıtreated individuals in Ho Chi Minh City (Vietnam): ANRS 1257 study. ırge predominance of CRF01_AE and presence of major resistance utations to antiretroviral drugs, AIDS Res. Hum. Retrovir. 19 (2003) 5–928.

T.H. Tran, I. Maljkovic, S. Swartling, D.C. Phung, F. Chiodi, T. Leit- r, HIV-1 CRF01_AE in intravenous drug users in Hanoi, Vietnam, IDS Res. Hum. Retrovir. 20 (2004) 341–345.

. Ishizaki, H.C. Nguyen, P.V. Thuc, V.T. Nguyen, K. Saijoh, S. ageyama, K. Ishigaki, J. Tanuma, S. Oka, H. Ichimura, Profile of HIV pe 1 infection and genotypic resistance mutations to antiretroviral ugs in treatment-naive HIV type 1-infected individuals in Hai Phong, et Nam, AIDS Res. Hum. Retrovir. 25 (2009) 175–182.

. Hachiya, S. Aizawa-Matsuoka, M. Tanaka, Y. Takahashi, S. Ida, H. atanaga, Y. Hirabayashi, A. Kojima, M. Tatsumi, S. Oka, Rapid and mple phenotypic assay for drug susceptibility of human immunodefi- ency virus type 1 using CCR5-expressing HeLa/CD4$^+$ cell clone 1–10 MAGIC-5), Antimicrob. Agents Chemother. 45 (2001) 495–501.

A. Johnson, F. Brun-Vézinet, B. Clotet, H.F. Gunthard, D.R. Kuritzkes, . Pillay, J.M. Schapiro, D.D. Richman, Update of the drug resistance utations in HIV-1: December 2009, Top. HIV Med. 17 (2009) 8–145.

Hu, F. Giguel, H. Hatano, P. Reid, J. Lu, D.R. Kuritzkes, Fitness mparison of thymidine analog resistance pathways in human immu- odeficiency virus type 1, J. Virol. 80 (2006) 7020–7027.

W. Shafer, S.Y. Rhee, D. Pillay, V. Miller, P. Sandstrom, J.M. Schapiro, .R. Kuritzkes, D. Bennett, HIV-1 protease and reverse transcriptase utations for drug resistance surveillance, AIDS 21 (2007) 215–223.

[39] D.E. Bennett, R.J. Camacho, D. Otelea, D.R. Kuritzkes, H. Fleury, M. Kiuchi, W. Heneine, R. Kantor, M.R. Jordan, J.M. Schapiro, A.M. Vandamme, P. Sandstrom, C.A. Boucher, D. van de Vijver, S.Y. Rhee, T.F. Liu, D. Pillay, R.W. Shafer, Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update, PLoS ONE 4 (2009) e4724.

[40] S. Saeng-aroon, N. Tsuchiya, W. Auwanit, P.I. Ayuthaya, P. Pathipvanich, P. Sawanpanyalert, A. Rojanawiwat, M. Kannagi, K. Ariyoshi, W. Sugiura, Drug-resistant mutation patterns in CRF01_AE cases that failed d4T+3TC+nevirapine fixed-dosed, combination treatment: follow-up study from the Lampang cohort, Antivir. Res. 87 (2010) 22–29.

[41] S. Gupta, S. Fransen, E. Paxinos, W. Huang, R. Dua, E. Stawiski, C. Petropoulos, N. Parkin, Infrequent occurrence of mutations in the C-terminal region of reverse transcriptase modulates susceptibility to RT inhibitors, Antivir. Ther. 11 (2006) S143.

[42] E. Poveda, C. de Mendoza, T. Pattery, M. González, J. Villacian, V. Soriano, Phenotypic impact of resistance mutations on etravirine susceptibility in HIV patients with prior failure to nonnucleoside analogues, AIDS 22 (2008) 2395–2398.

[43] B. Winters, E. Van Craenenbroeck, K. Van der Borght, P. Lecocq, J. Villacian, L. Bacheler, Clinical cut-offs for HIV-1 phenotypic resistance estimates: update based on recent pivotal clinical trial data and a revised approach to viral mixtures, J. Virol. Methods 162 (2009) 101–108.

[44] B. Wang, K.A. Lau, L.Y. Ong, M. Shah, M.C. Steain, B. Foley, D.E. Dwyer, C.B. Chew, A. Kamarulzaman, K.P. Ng, N.K. Saksena, Complex patterns of the HIV-1 epidemic in Kuala Lumpur, Malaysia: evidence for expansion of circulating recombinant form CRF33_01B and detection of multiple other recombinants, Virology 367 (2007) 288–297.

[45] K.K. Tee, C.K. Pon, A. Kamarulzaman, K.P. Ng, Emergence of HIV-1 CRF01_AE/B unique recombinant forms in Kuala Lumpur, Malaysia, AIDS 19 (2005) 119–126.

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Trends in transmitted drug-resistant HIV-1 and demographic characteristics of newly diagnosed patients: Nationwide surveillance from 2003 to 2008 in Japan

Junko Hattori[a], Teiichiro Shiino[b], Hiroyuki Gatanaga[c], Shigeru Yoshida[d], Dai Watanabe[e], Rumi Minami[f], Kenji Sadamasu[g], Makiko Kondo[h], Haruyo Mori[i], Mikio Ueda[j], Masao Tateyama[k], Atsuhisa Ueda[l], Shingo Kato[m], Toshihiro Ito[n], Masayasu Oie[o], Noboru Takata[p], Tsunefusa Hayashida[c], Mami Nagashima[g], Masakazu Matsuda[q], Shiro Ibe[a], Yasuo Ota[r], Satoru Sasaki[n], Yoshiaki Ishigatsubo[l], Yoshinari Tanabe[o], Ichiro Koga[r], Yoko Kojima[i], Masahiro Yamamoto[f], Jiro Fujita[k], Yoshiyuki Yokomaku[a], Takao Koike[s], Takuma Shirasaka[e], Shinichi Oka[c], Wataru Sugiura[a,b,t,*]

[a] National Hospital Organization, Nagoya Medical Center, Clinical Research Center, 4-1-1 Sannomaru, Naka-ku, Nagoya 4600001, Japan
[b] National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 1628640, Japan
[c] AIDS Clinical Center, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku-ku, Tokyo 1628655, Japan
[d] Faculty of Health Sciences, Hokkaido University, N12-W5, Kita-ku, Sapporo 0600812, Japan
[e] National Hospital Organization, Osaka Medical Center, 2-1-14 Houenzaka, Chuo-ku, Osaka 5400006, Japan
[f] National Hospital Organization, Kyushu Medical Center, 1-8-1 Jigyohama, Chuo-ku, Fukuoka 8108563, Japan
[g] Tokyo Metropolitan Institute of Public Health, 3-24-1 Hyakunin-cho, Shinjuku-ku, Tokyo 1690073, Japan
[h] Kanagawa Prefectural Institute of Public Health, 1-3-1 Shimomachiya, Chigasaki, Kanagawa 2530087, Japan
[i] Osaka Prefectural Institute of Public Health, 1-3-69 Nakamichi, Higashinari-ku, Osaka 5370025, Japan
[j] Ishikawa Prefectural Central Hospital, 2-1 Anngetsu-higashi, Kanazawa 9208530, Japan
[k] University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 9030125, Japan
[l] Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 2360004, Japan
[m] Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 1608582, Japan
[n] National Hospital Organization, Sendai Medical Center, 2-8-8 Miyagino, Miyagino-ku, Sendai 9830045, Japan
[o] Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Chuo-ku, Niigata 9518510, Japan
[p] Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 7348551, Japan
[q] Mitsubishi Chemical Medience Corporation, 3-30-1 Shimura, Itabashi-ku, Tokyo 1748555, Japan
[r] Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 1738605, Japan
[s] Hokkaido University Graduate School of Medicine, N15-W7, Kita-ku, Sapporo 0608638, Japan
[t] Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 4668550, Japan

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ABSTRACT

The emergence and transmission of drug-resistant human immunodeficiency virus-1 (HIV-1) compromises antiretroviral treatment for HIV-1. Thus, testing for drug resistance is recommended at diagnosis and before initiating highly active antiretroviral treatment. We conducted an epidemiological study enrolling newly diagnosed patients between 2003 and 2008 in our nationwide surveillance network. In the 6-year study period, the prevalence of drug-resistant HIV-1 among 2573 patients, consisting mainly of Japanese men in their late-30s and infected through male-to-male sexual contacts, followed an increasing trend from 5.9% (16/273) in 2003 to 8.3% (50/605) in 2008. Nucleoside reverse transcriptase inhibitor-associated mutations predominated in each year, with T215 revertants being the most abundant. The predictive factor for drug-resistant HIV-1 transmission was subtype B (OR = 2.36; $p = 0.004$), and those for recent HIV-1 infection were male gender (OR = 3.79; $p = 0.009$), MSM behavior (OR = 1.67; $p = 0.01$), Japanese nationality (OR = 2.31; $p = 0.008$), and subtype B (OR = 5.64; $p < 0.05$). Continued activities are needed to raise awareness of the risks of HIV-1 infection and complications of drug-resistant strains. Continued surveillance is also needed to understand trends in the HIV-1 epidemic.

Abbreviations: HIV-1, human immunodeficiency virus type 1; HAART, highly active antiretroviral therapy; PI, protease inhibitor; HBV, hepatitis B virus; HCV, hepatitis C virus; PR, protease; RT, reverse transcriptase; RT-PCR, reverse transcription polymerase chain reaction; CRF, circulating recombinant form; NRTI, nucleoside RT inhibitor; NNRTI, non-nucleoside RT inhibitor; OR, odds ratio; CI, confidence interval; MSM, men who have sex with men; IDU, intravenous drug user.

* Corresponding author at: Clinical Research Center, National Nagoya Medical Center, 4-1-1 Sannomaru Naka-ku, Nagoya, Aichi 4600001, Japan. Tel.: +81 52 951 1111; fax: +81 52 963 3970.
E-mail address: wsugiura@nih.go.jp (W. Sugiura).

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1. Introduction

The emergence of drug-resistant human immunodeficiency virus type 1 (HIV-1) among patients under highly active antiretroviral therapy (HAART) limits the successful suppression of HIV-1 replication. Several years after the introduction of HAART, drug-resistant strains are being detected among newly diagnosed HAART-naïve patients, suggesting the transmission of drug-resistant HIV-1 from the treatment-exposed population. Thus, treatment-naïve patients have been recommended by the US Department of Health and Human Services, International AIDS Society-USA, and other drug-resistance testing guidelines to undergo drug resistance testing at diagnosis and before initiation of HAART (DHHS, 2009; Hirsch et al., 2000, 2008). Indeed, choosing effective antiretrovirals according to the results obtained from this testing has led to successful control of HIV-1 infection. Furthermore, the drug resistance testing at diagnosis helps to understand transmission of drug-resistant HIV-1 in HAART-naïve individuals which in turn may help prevent transmission events.

The prevalence of drug-resistant HIV-1 among treatment-naïve patients has been closely monitored and reported from many countries. Before and early in the HAART era, when only mono or dual therapy was available, the prevalence was as high as 10–20% (Boden et al., 1999; Gómez-Cano et al., 1998; Tambussi et al., 1998). However, after the introduction of antiretrovirals with better pharmacokinetics, such as ritonavir-boosted protease inhibitor (PI), the emergence of drug-resistant viruses seemed to decrease (Gallego et al., 2001; Maia Teixeira et al., 2006).

Furthermore, despite the great number of HIV-1-infected patients, the prevalence tended to be low in developing countries where patients had limited or no access to antiretroviral drugs, e.g., 0–4.2% in Africa (Bártolo et al., 2009; Mintsa-Ndong et al., 2009; Ndembi et al., 2008; Pillay et al., 2008), 1.5% in Cambodia (Nouhin et al., 2009), and 2.6% in Vietnam (Ishizaki et al., 2009). In contrast, in countries where antiretroviral drugs are more accessible, the prevalence has been higher, e.g., 5.2% in Thailand (Apisarnthanarak et al., 2008), 9.4% in Taiwan (Chang et al., 2008), 10.0% in India (Lall et al., 2008), 7.8% in Portugal (Palma et al., 2007), 9.0% in Germany (Sagir et al., 2007), 9.5% in Belgium (Vercauteren et al., 2008), 10.9% in France (Chaix et al., 2009), and 15.9% in the US (Eshleman et al., 2007).

In Japan, since the first HIV-1-infected case was identified in 1985, the annual number of reported cases has been increasing every year, reaching 15 451 by the end of 2008. With more people getting infected, larger numbers of patients are starting anti-HIV-1 treatment and the risk of emerging drug-resistant HIV-1 is increasing. To understand the trends in drug-resistant HIV-1 in Japan, a nationwide surveillance project has been in effect since 2003. In our previous report of surveillance results from 2003 to 2004, the prevalence of drug-resistant HIV-1 in newly diagnosed patients was 4.0% (Gatanaga et al., 2007). We have continued collecting and analyzing data from newly diagnosed HIV-1-infected patients at participating clinical and research facilities in Japan. We report here the prevalence of drug-resistant HIV-1 among newly diagnosed therapy-naïve patients between 2003 and 2008.

2. Materials and methods

2.1. Sample

The study population included all the HIV-1-infected patients newly diagnosed between January 2003 and December 2008 at any of the participating HIV/AIDS clinics. Drug resistance genotypic tests were performed at 12 laboratories including 8 clinical laboratories at HIV/AIDS clinics, 3 public health laboratories, and the National Institute of Infectious Diseases. After patients agreed to participate in our surveillance project and gave informed consent, peripheral blood was drawn with EDTA added, and their demographic and clinical information were collected. Demographic information included age, gender, nationality, and risk behavior. Clinical data included HIV-1 viral loads, $CD4^+$ T cell counts, status of hepatitis B and C virus (HBV, HCV) co-infection, baseline sequence data, and drug-resistant amino acid mutations.

This study was conducted according to the principles in the Declaration of Helsinki, and was approved by the ethical committee of the National Institute of Infectious Diseases, Japan. By Japanese law, HIV-1-infected patients must be reported to the Japanese Ministry of Health, Labour, and Welfare upon diagnosis. The numbers reported to the Ministry are considered the "official numbers" of newly diagnosed HIV/AIDS cases, and were used as comparison controls to evaluate our study population.

2.2. Drug resistance genotypic testing

Drug resistance genotypic testing was performed using in-house protocols. Briefly, viral RNA was extracted from patient plasma samples. HIV-1 protease (PR, 1–99 amino acids) and the N-terminal region of reverse transcriptase (RT, 1–240 amino acids) were amplified in reverse transcription polymerase chain reaction (RT-PCR) followed by nested PCR using in-house primer sets. Subsequently, the amplified PCR products were purified and their sequences were analyzed by direct sequencing method using an automated sequencer. The resulting electropherograms were analyzed using commercially available software. The quality of testing methods used at each participating facility was assessed and confirmed for detection of drug-resistant mutations (Fujisaki et al., 2007). Thus, detection of drug-resistant mutations was consistent among facilities.

2.3. Determination of HIV-1 subtypes and drug-resistant HIV-1

HIV-1 subtypes were determined using the sequences of HIV-1 PR and RT genes obtained in the drug resistance genotypic testing explained above. Each sequence was aligned with the reference sequences of HIV-1 subtypes A through K, and circulating recombinant forms (CRFs), all of which were obtained from the Los Alamos HIV Databases (Los Alamos, 2010), using ClustalW, and phylogenetic trees were constructed using the neighbor-joining method with bootstrap value of 1000.

The resulting sequences were compared to that of HXB2 to judge the presence of amino acid mutations. The drug-resistant mutations were determined according to criteria of the HIV Drug Resistance Database of Stanford University (Bennett et al., 2009). Thus, a sample was considered to harbor drug-resistant HIV-1 if it possessed any of the following mutations: in the PR gene, L23I, L24I, D30N, V32I, M46I/L, I47V/A, G48V/M, I50V/L, F53L/Y, I54V/L/M/A/T/S, G73S/T/C/A, L76V, V82A/T/F/S/C/M/L, N83D, I84V/A/C, I85V, N88D/S, and L90M (indicating PI resistance); in the RT gene, M41L, K65R, D67N/G/E, T69D/insertion, K70R/E, L74V/I, V75M/T/A/S, F77L, Y115F, F116Y, Q151M, M184V/I, L210W, T215Y/F/I/S/C/D/V/E, K219Q/E/N/R (indicating nucleoside RT inhibitor [NRTI] resistance), and L100I, K101E/P, K103N/S, V106M/A, V179F, Y181C/I/V, Y188L/H/C, G190A/S/E, P225H, M230L (indicating non-nucleoside RT inhibitor [NNRTI] resistance).

2.4. BED assay

The time of HIV-1 seroconversion was estimated in randomly selected samples as recent (within 155 days) or not recent using the BED assay (Calypte HIV-1 BED Incidence EIA, BioRad) according to the Manufacturer's instruction. Briefly, 5 μL of plasma was diluted

with 500 μL of sample diluent in the kit, and the proportion of anti-HIV-1 IgG to a total IgG in the sample was measured by optical density.

2.5. Statistical analysis

Statistical analyses were performed using R software (SAS Institute). Chi-square or Fisher's exact probability tests were used to determine associations among patients' demographic characteristics, nationality, BED assay results, and transmission of drug resistance. The odds ratio (OR) and 95% confidence intervals (CI) were calculated for all the variables. Recent and not-recent seroconversion groups were examined for differences in HIV-1 viral loads by analysis of covariance (ANCOVA), with $CD4^+$ T cell count as the covariate.

3. Results

3.1. Majority of treatment-naïve patients are Japanese men who have sex with men (MSM) in mid-30s

The demographics of the 2573 newly diagnosed HIV-1-infected patients enrolled between 2003 and 2008 are summarized in Table 1. Male ($n = 2397$, 93.2%), Japanese (90.1%), and those infected through male-to-male sexual contact (68.9%) predominated, and the median age was 35. For the female cases ($n = 170$), high-risk heterosexual contact was the major risk factor ($n = 152$, 89.4%), and approximately half were non-Japanese ($n = 63$, 41.4%). Further analysis showed a significant association between the transmission route and nationality, i.e., most Japanese patients were infected through male-to-male sexual contact, while non-Japanese patients were infected by other routes (OR = 5.60; 95% CI 4.14–7.63; $p < 0.01$) (Table 2). It should be noted that sexual contacts (92.1%) are the major risk factor for HIV-1 infection in Japan. On the other hand, injecting drug usage, one of the high risk factors in other countries, accounts for only 0.4%.

HBV and/or HCV co-infection, an important clinical factor affecting prognosis and treatment of HIV infection (Ockenga et al., 1997; Piroth et al., 2000), was found to have a prevalence of 8.4% of 2101 patients, and 4.7% of 2071, respectively (Table 1). These prevalence rates did not change significantly throughout the study period (supplementary Table 1). HBV co-infection was found to be significantly associated with subtype B (OR = 2.04; $p < 0.05$) or infection through male-to-male sexual contact (OR = 1.66; $p < 0.05$).

3.2. Subtype B HIV-1 predominates in Japan

Of 2573 plasma samples collected during the study period, the sequences of PR and RT genes were successfully amplified and analyzed in 2536 (98.6%) and 2534 (98.5%) samples, respectively. Of these, we examined sequences of the PR-RT region from 2496 cases by phylogenetic tree analysis to determine the distribution of HIV-1 subtypes in Japan. Subtype B HIV-1 was found to predominate among the study population ($n = 2194$, 87.9%). The remaining non-B subtypes included 210 (8.4%) CRF01_AE, 30 (1.2%) C, 19 (0.8%) CRF02_AG, 18 (0.7%) A, 9 (0.4%) G, 7 (0.3%) F, 5 (0.2%) D, and 1 (0.04%) CRF08_BC (Table 1). In addition, 1 recombinant case of K/C, A/K, and D/B was detected in 2005, 2006, and 2007, respectively. These non-B subtype viruses were found mostly among the heterosexually infected population (223/302, 73.8%). In contrast, subtype B HIV-1 was found in the vast majority of MSM (1700/1773, 95.9%). In terms of nationality, Japanese patients, most of whom were MSM, were infected with subtype B HIV-1. On the other hand, only about a half of non-Japanese patients harbored subtype B HIV-1, and the remaining half were infected with non-B HIV-1, such as CRF01_AE (OR = 8.85; 95% CI 6.46–12.1; $p < 0.01$) (Table 2). This result is reasonable considering that the predominant HIV-1 subtype differs by country, and our study population included many Thais and Malaysians. In addition, this result suggests that subtype B HIV-1 is transmitted in a closed community of MSM, while non-B subtype strains are spread in wider areas among those infected through high-risk heterosexual contacts.

Table 1
Demographic characteristics of newly diagnosed HIV/AIDS patients.

	6-Year total (2573)	
Age		
Average	37.4	
Median	35	
Mode	35	
Quartile (Q1, Q3)	29, 43	
Nationality	*n*	(%)
Japanese	2319	(90.1)
Non-Japanese	225	(8.7)
Asian	83	(3.2)
Oceanian	4	(0.2)
North American	17	(0.7)
South American	58	(2.3)
European	10	(0.4)
African	26	(1.0)
Unspecified[a]	27	(1.0)
Unknown	29	(1.1)
Transmission category		
Male	2397	(93.2)
Male-to-male sexual contact	1773	(68.9)
High-risk heterosexual contact	369	(14.3)
Sexual contact	75	(2.9)
IDU	8	(0.3)
Other[b]	26	(1.0)
Unidentified	146	(5.7)
Female	170	(6.6)
High-risk heterosexual contact	152	(5.9)
IDU	3	(0.1)
Other[b]	5	(0.2)
Unidentified	11	(0.4)
Unknown	6	(0.2)
Unidentified	6	(0.2)
Hepatitis co-infection[c]		
HBV		
(+)	176	(8.4)
(−)	1925	(91.6)
Unknown	472	
HCV		
(+)	98	(4.7)
(−)	1973	(95.3)
Unknown	502	
HIV-1 subtype[c]		
B	2194	(87.9)
non-B	302	(12.1)
AE	210	(8.4)
C	30	(1.2)
AG	19	(0.8)
A	18	(0.7)
G	9	(0.4)
F	7	(0.3)
D	5	(0.2)
Other	4	(0.2)
Unidentified	77	

[a] Unspecified individuals in the nationality category were identified only as of non-Japanese origin.
[b] Other transmission categories include mother-to-child, blood products, transfusion, and needle stick.
[c] Prevalence of subtypes, HBV, and HCV was calculated after omitting the unidentified or unknown data. DU, intravenous drug user; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1.

3.3. Prevalence of drug-resistant HIV-1 is increasing in Japan

A total of 194 cases (7.7%) in the 6-year study period were found to harbor HIV-1 strains with at least one major drug-resistant muta-

Table 2
Characteristics of newly diagnosed Japanese and non-Japanese HIV/AIDS patients.

	Nationality (*n*)			Odds ratio
	Japanese	Non-Japanese	Unknown	
Gender				
Male	2224	151	22	11.45*
Female	95	74	1	
Unknown[b]			6	
Transmission category				
Male-to-male sexual contact	1691	73	9	5.60[a,*]
High-risk heterosexual contact	399	114	7	
Sexual contact	72	4	0	
Other	29	10	2	
Unidentified[b]	128	24	11	
Subtype				
B	2051	118	25	8.85*
Non-B	198	101	3	
Unidentified[b]	70	6	1	
BED assay (*n* = 640)				
Recent	220	13	0	2.31*
Not recent	351	48	8	
Drug-resistant HIV-1				
Detected	173	16	5	1.05
Not detected	2146	209	24	

[a] Odds ratios for the transmission category were calculated between male-to-male sexual contact and other categories which include high-risk heterosexual contact, sexual contact, and other.
[b] Unknown and Unidentified cases were omitted in calculation of odds ratio.
* $p < 0.01$.

tion conferred by PIs, NRTIs, or NNRTIs. The annual prevalence of drug-resistant mutations shown in Fig. 1 had an overall tendency to increase from 5.9% (16/273) in 2003 to 8.3% (50/605) in 2008. The most prevalent mutation in each year was NRTI-associated resistance, with 11 (4.0%), 12 (4.0%), 21 (5.0%), 23 (5.2%), 28 (5.9%), and 23 (3.7%) cases, followed by PI- and NNRTI-associated mutations. PI-resistant major mutations were detected in 63 cases (2.5%), and NNRTI-associated mutations were detected only in 20 cases (0.8%). These data reflect the type of antiretrovirals being prescribed in treated population. In other words, NRTIs have a long history of being prescribed including the period of mono and dual therapy; thus, NRTIs have been more frequently used. As a consequence, NRTI-resistant HIV-1 has emerged and been transmitted more frequently to treatment-naïve patients. Regarding the drug-resistant mutations shown in Table 3, T215 revertants (T215X) (3.2%), M184I/V (0.5%), K103N (0.6%), and M46I/L (1.7%) accounted for the majority of detected mutations in contrast to other muta-

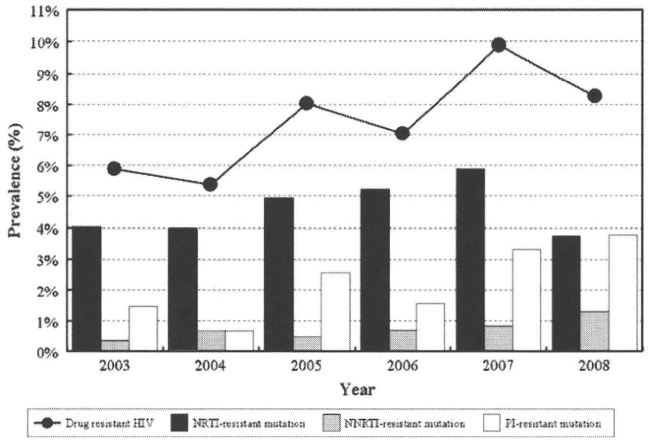

Fig. 1. Annual overall prevalence of drug-resistant HIV-1 (solid circles) in Japan increased in treatment-naïve patients in Japan from 2003 to 2008. The most prevalent mutation in each year was associated with resistance to nucleoside reverse transcriptase inhibitor (NRTI) treatment. Annual prevalence of drug-resistance mutations was categorized by antiretroviral drug class (NRTIs, solid black bars; non-nucleoside reverse transcriptase inhibitors [NNRTIs], horizontally striped bars; protease inhibitors [PIs], solid white bars). Drug-resistant HIV-1 was counted once even when the strain contained multiple drug-resistant mutations. Each drug-resistant mutation was counted even when multiple mutations were detected in one patient.

Table 3
Drug-resistant mutations in newly diagnosed HIV/AIDS patients, by class of antiretroviral drugs.

	6-Year total (2573)	
	n	(%)
NRTI[a]		
M41L	11	(0.4)
K65R	1	(0.0)
D67N/G/E	7	(0.3)
T69D	8	(0.3)
69INS	1	(0.0)
K70R/E	2	(0.1)
L74V/I	3	(0.1)
V75A/M	2	(0.1)
Y115F	3	(0.1)
M184V/I	12	(0.5)
L210W	5	(0.2)
T215X	81	(3.2)
K219Q/E/N/R	4	(0.2)
NNRTI[a]		
L100I	1	(0.0)
K101E	2	(0.1)
K103N	14	(0.6)
V106A/M	1	(0.0)
Y181C/I/V	3	(0.1)
P225H	1	(0.0)
P236L	1	(0.0)
PI[a]		
L24I	1	(0.0)
D30N	5	(0.2)
V32I	3	(0.1)
M46I/L	44	(1.7)
I47V/A	2	(0.1)
V82A/L	2	(0.1)
I85V	5	(0.2)
N88D/S	7	(0.3)
L90M	4	(0.2)

[a] Numbers of cases and the proportions in parentheses are listed.

Table 4
Predictive factors for transmission of drug-resistant HIV-1.

	Drug-resistant HIV-1 (*n*)		Odds ratio
	(+)	(−)	
Gender			
Male	183	2214	1.92
Female	7	163	
Nationality			
Japanese	173	2146	1.05
Non-Japanese	16	209	
Transmission category			
Male-to-male sexual contact	130	1643	0.91
High-risk heterosexual contact	37	484	
Sexual contact	15	60	
Other	1	40	
Unidentified[a]	11	152	
Subtype			
B	180	2014	2.36**
Non-B	11	291	
Unidentified	3	77	

[a] For calculation of odds ratio, unidentified cases were omitted.
** $p < 0.01$.

tions that were detected only sporadically throughout the study period (supplementary Table 2).

Analysis of possible predictive factors for transmission of drug-resistant HIV-1 showed that individuals infected with subtype B HIV-1 had a significantly higher tendency to harbor drug-resistant HIV-1 than non-B subtypes (OR = 2.36; 95% CI = 1.27–4.88; $p < 0.01$) (Table 4). Other possible predictive factors, including male gender (OR = 1.92; 95% CI = 0.89–4.93; $p = 0.1$), Japanese nationality (OR = 1.05; 95% CI = 0.62–1.92; $p = 1$), and MSM behavior (OR = 0.91; 95% CI 0.66–1.26; $p = 0.57$), were not significant predictive factors in our study population. These results indicate that the chance of getting infected with drug-resistant HIV-1 was the same for anyone regardless of gender, nationality, or risk behavior.

3.4. MSM are diagnosed earlier than heterosexually infected individuals

To examine awareness of HIV infection, especially of risk behavior, and to characterize HIV-testing patterns among the HIV-infected population, we estimated the time of seroconversion by quantifying the amount of anti-HIV antibody in plasma samples. Of 640 randomly selected samples in 2007 and 2008, 233 (36.4%) were classified by BED assay with a cut-off value of 0.8 as recently infected (<155-day seroconversion), while the remaining 407 (63.4%) were classified as not recently infected (Table 5). For the recently and not recently infected groups, the average $CD4^+$ T cell count and HIV-1 viral load were 285 and 215 cells/μL and 5.1×10^5 and 1.4×10^5 copies/mL, respectively. Recently infected individuals were shown by ANCOVA with $CD4^+$ T cell counts as the covariate, to have significantly higher HIV-1 viral loads than not recently infected cases (Fig. 2). These data support that the BED assay had precisely determined early infected cases.

With respect to risk behavior, the highest rate of recent infection was in MSM (39.2%), followed by either homo- or heterosexual contacts (38.9%), and heterosexual contacts (25.0%). No patients infected through a risk behavior other than sexual contacts were categorized as recently infected. Whereas 37.8% of male patients were determined to be recently infected, only 13.8% of female patients were categorized as recently infected. These findings were reinforced by statistical analysis. Recent HIV-1 infection was significantly predicted by male gender (OR = 3.79; 95% CI 1.29–15.17; $p < 0.01$), MSM behavior (OR = 1.67; 95% CI = 1.11–2.54; $p = 0.01$), Japanese nationality (OR = 2.31; 95% CI 1.20–4.76; $p < 0.01$), and infection with subtype B HIV-1 (OR = 5.64; 95% CI = 2.37–16.33; $p < 0.01$) (Table 5). In other words, Japanese males, especially those who were MSM, were more aware of being at high risk of HIV-1 infection and got tested more often than non-Japanese. In contrast, females, individuals of non-Japanese origin, heterosexuals, and non-subtype-B-infected persons, had low awareness of the risks of HIV-1 infection.

Table 5
Predictive factors for recent or not-recent seroconversion determined by BED assay, $n = 640$.

	Seroconversion (*n*)		Odds ratio
	Recent (*n* = 233)	Not recent (*n* = 407)	
Gender			
Male	229	377	3.79**
Female	4	25	
Unknown[b]	0	5	
Nationality			
Japanese	220	351	2.31**
Non-Japanese	13	48	
Unknown[b]	0	8	
Transmission category			
Male-to-male sexual contact	189	293	1.67[a,*]
High-risk heterosexual contact	24	70	
Sexual contact	7	11	
Other	0	4	
Unidentified[b]	13	29	
Subtype			
B	224	350	5.64**
Non-B	6	53	
Unidentified[b]	3	4	
Drug-resistant HIV			
Detected	14	37	0.64
Not detected	219	370	

[a] Odds ratio for the transmission category was calculated between male-to-male sexual contact and other categories which include high-risk heterosexual contact, sexual contact, and other.
[b] Unknown or unidentified cases were omitted in calculation of odds ratio.
* $p < 0.05$.
** $p < 0.01$.

Regarding associations between the time of diagnosis and drug-resistant HIV transmission event, time of diagnosis did not differ significantly between those harboring and those not harboring drug-resistant HIV-1 (OR = 0.64; 95% CI = 0.31–1.24; $p = 0.18$) (Table 5), suggesting that transmission of drug-resistant HIV-1 is not a recent trend, but has been ongoing since the first antiretroviral, AZT, was introduced in 1986.

4. Discussion

Our study results show that the proportion of drug-resistant HIV-1 among newly diagnosed cases in Japan increased slightly (by 2.4%) from 2003 to 2008, with fluctuations from year to year. Drug-resistant HIV-1 in HAART-naïve patients are transmitted from HAART-experienced patients with inadequate adherence or from other treatment-naïve individuals with drug-resistant strains, but not yet diagnosed or tested for drug-resistant HIV-1 (de Mendoza et al., 2005). Hence, drug-resistant mutations detected in the naïve population should be tightly related to trends in antiretroviral use in the treated population. Antiretrovirals available in the early days of the HAART era, especially, had short half-lives and low genetic barriers for drug resistance acquisition, making the viruses easily resistance prone. On the other hand, new antiretroviral drugs, such as lopinavir, atazanavir, amprenavir and darunavir, have been developed so that they have improved pharmacokinetics and higher genetic barriers, thus the viruses have less chance of developing drug resistance (Dunn et al., 2008; Lima et al., 2008; Zajdenverg et al., 2009). In the present study, we found that drug-resistant mutations detected among treatment-naïve patients were

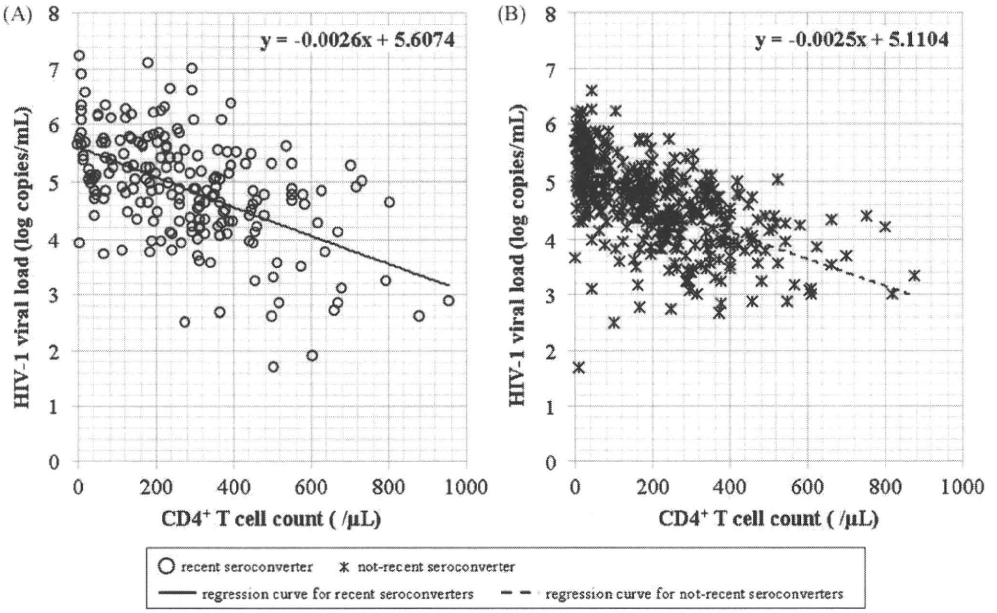

Fig. 2. Scatter plots of viral load and CD4+ T cell counts for (A) recently seroconverted patients (○), and (B) not recently seroconverted patients (*) determined by BED assay. Regression curves and their equations are shown for each group.

associated especially with antiretrovirals used prior to and early in the HAART era. It should be noted that contrary to the reports from the United States and many of European countries (Audelin et al., 2009; Vercauteren et al., 2009; Wheeler et al., 2010), the prevalence of NNRTI-resistant variants have been determined to be low in Japan, less than 1% in the study period 2003–2007 and 1.3% in 2008 being the highest. This difference is due to the situation in Japan that delavirdine had never been used and even nevirapine is only rarely prescribed. Nonetheless, strains with T215X, M46I/L, K103N, and M184V/I mutations were detected every year, suggesting that these strains are stably maintained in individuals and in high-risk populations even under antiretroviral drug-free environments. This finding is supported by the insignificant difference in prevalence of drug-resistant HIV-1 between recently and not recently infected groups. These results raise the concern that such drug-resistant strains may have become some epidemic strains actively transmitted among newly diagnosed HIV/AIDS patients. Furthermore, considering the presence of low frequent variants, the prevalence of drug-resistant mutations in this report may be higher if more sensitive techniques, such as allele-specific PCR and ultra-deep sequencing, are applied to test the samples (Halvas et al., 2010; Varghese et al., 2009). Further studies employing such techniques are needed to understand the detailed epidemic in Japan.

In investigating predictive factors for transmission of drug-resistant strains, we found that the only predictive factor was subtype B HIV-1 (OR = 2.36, $p < 0.01$). The lower transmission risk of drug-resistant strains in non-B HIV-1 can be explained by patients' countries of origin. We observed a significant relationship between non-B subtype HIV-1 and non-Japanese patients, most of whom were from developing countries with limited access to antiretrovirals. Thus, our finding agrees with reports of low prevalence drug-resistant HIV-1 transmission in developing countries (Bártolo et al., 2009; Ishizaki et al., 2009; Mintsa-Ndong et al., 2009; Ndembi et al., 2008; Nouhin et al., 2009; Pillay et al., 2008).

Interestingly, a high proportion of Japanese MSM was diagnosed as recently infected compared to patients of non-Japanese origin, and females determined by BED assay. This result may be due to successful prevention programs targeting the MSM community, so that they have become more aware of their risks of HIV-1 infection. On the other hand, many of non-Japanese patients are seen at hospitals long after HIV infection is established. In addition, women tend to be ignorant of the risks of HIV infection, thus they are often diagnosed upon a prenatal HIV screening test.

Although MSM was not a predictive factor for transmission, this group included 130 cases with drug-resistant HIV-1, the highest prevalence among all the transmission categories. Therefore, those who are involved in prevention programs should take one step further to remind the MSM community about drug-resistant HIV-1 and the limited choice of effective antiretrovirals. HIV-1 transmission has been reported to be prevented in models that assessed the effect of HIV-1 testing for wider populations and immediate initiation of antiretroviral therapy (Granich et al., 2009). Although this model seems very appealing, our results suggest the importance of not forgetting the emergence and transmission of drug-resistant HIV-1 and the limited selection of antiretroviral drugs. It is important to continue surveying newly diagnosed HIV/AIDS patients to keep track of trends in drug-resistant HIV-1 transmission, to reveal high-risk populations with low awareness of HIV infection, to propose effective programs to prevent transmission of drug-resistant HIV-1, and to develop antiretroviral drugs with improved pharmacokinetics/pharmacodynamics. All these efforts may bring us one step closer to eradicating HIV-1.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2010.07.008.

References

Apisarnthanarak, A., Jirayasethpong, T., Sa-nguansilp, C., Thongprapai, H., Kittihanukul, C., Kamudamas, A., Tungsathapornpong, A., Mundy, L.M., 2008. Antiretroviral drug resistance among antiretroviral-naive persons with recent HIV infection in Thailand. HIV Med. 9, 322–325.

Audelin, A.M., Lohse, N., Obel, N., Gerstoft, J., Jørgensen, L.B., 2009. The incidence rate of HIV type-1 drug resistance in patients on antiretroviral therapy: a nationwide population-based Danish cohort study 1999–2005. Antivir. Ther. 14, 995–1000.

Bártolo, I., Rocha, C., Bartolomeu, J., Gama, A., Fonseca, M., Mendes, A., Cristina, F., Thamm, S., Epalanga, M., Silva, P.C., Taveira, N., 2009. Antiretroviral drug resistance surveillance among treatment-naive human immunodeficiency virus type 1-infected individuals in Angola: evidence for low level of transmitted drug resistance. Antimicrob. Agents Chemother. 53, 3156–3158.

Bennett, D.E., Camacho, R.J., Otelea, D., Kuritzkes, D.R., Fleury, H., Kiuchi, M., Heneine, W., Kantor, R., Jordan, M.R., Schapiro, J.M., Vandamme, A.M., Sandstrom, P., Boucher, C.A., van de Vijver, D., Rhee, S.Y., Liu, T.F., Pillay, D., Shafer, R.W., 2009. Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. PLoS One 4, e4724.

Boden, D., Hurley, A., Zhang, L., Cao, Y., Guo, Y., Jones, E., Tsay, J., Ip, J., Farthing, C., Limoli, K., Parkin, N., Markowitz, M., 1999. HIV-1 drug resistance in newly infected individuals. JAMA 282, 1135–1141.

Chaix, M.L., Descamps, D., Wirden, M., Bocket, L., Delaugerre, C., Tamalet, C., Schneider, V., Izopet, J., Masquelier, B., Rouzioux, C., Meyer, L., Costagliola, D., 2009. Stable frequency of HIV-1 transmitted drug resistance in patients at the time of primary infection over 1996–2006 in France. AIDS 23, 717–724.

Chang, S.Y., Chen, M.Y., Lee, C.N., Sun, H.Y., Ko, W., Chang, S.F., Chang, K.L., Hsieh, S.M., Sheng, W.H., Liu, W.C., Wu, C.H., Kao, C.L., Hung, C.C., Chang, S.C., 2008. Trends of antiretroviral drug resistance in treatment-naive patients with human immunodeficiency virus type 1 infection in Taiwan. J. Antimicrob. Chemother. 61, 689–693.

de Mendoza, C., Rodriguez, C., Eiros, J.M., Colomina, J., Garcia, F., Leiva, P., Torre-Cisneros, J., Aguero, J., Pedreira, J., Viciana, I., Corral, A., del Romero, J., Ortiz de Lejarazu, R., Soriano, V., 2005. Antiretroviral recommendations may influence the rate of transmission of drug-resistant HIV type 1. Clin. Infect. Dis. 41, 227–232.

DHHS, 2009. Panel on antiretroviral guidelines for adults and adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents.

Dunn, D., Geretti, A.M., Green, H., Fearnhill, E., Pozniak, A., Churchill, D., Pillay, D., Sabin, C., Phillips, A., 2008. Population trends in the prevalence and patterns of protease resistance related to exposure to unboosted and boosted protease inhibitors. Antivir. Ther. 13, 771–777.

Eshleman, S.H., Husnik, M., Hudelson, S., Donnell, D., Huang, Y., Huang, W., Hart, S., Jackson, B., Coates, T., Chesney, M., Koblin, B., 2007. Antiretroviral drug resistance, HIV-1 tropism, and HIV-1 subtype among men who have sex with men with recent HIV-1 infection. AIDS 21, 1165–1174.

Fujisaki, S., Fujisaki, S., Ibe, S., Asagi, T., Itoh, T., Yoshida, S., Koike, T., Oie, M., Konda, M., Sadamasu, K., Nagashima, M., Gatanaga, H., Matsuda, M., Ueda, M., Masakane, A., Hata, M., Mizogami, Y., Mori, H., Minami, R., Okada, K., Watanabe, K., Shirasaka, T., Oka, S., Sugiura, W., Kaneda, T., 2007. Performance and quality assurance of genotypic drug-resistance testing for human immunodeficiency virus type 1 in Japan. Jpn. J. Infect. Dis. 60, 113–117.

Gallego, O., Ruiz, L., Vallejo, A., Ferrer, E., Rubio, A., Clotet, B., Leal, M., Soriano, V., 2001. Changes in the rate of genotypic resistance to antiretroviral drugs in Spain. AIDS 15, 1894–1896.

Gatanaga, H., Ibe, S., Matsuda, M., Yoshida, S., Asagi, T., Kondo, M., Sadamasu, K., Tsukada, H., Masakane, A., Mori, H., Takata, N., Minami, R., Tateyama, M., Koike, T., Itoh, T., Imai, M., Nagashima, M., Gejyo, F., Ueda, M., Hamaguchi, M., Kojima, Y., Shirasaka, T., Kimura, A., Yamamoto, M., Fujita, J., Oka, S., Sugiura, W., 2007. Drug-resistant HIV-1 prevalence in patients newly diagnosed with HIV/AIDS in Japan. Antiviral Res. 75, 75–82.

Gómez-Cano, M., Rubio, A., Puig, T., Pérez-Olmeda, M., Ruiz, L., Soriano, V., Pineda, J.A., Zamora, L., Xaus, N., Clotet, B., Leal, M., 1998. Prevalence of genotypic resistance to nucleoside analogues in antiretroviral-naive and antiretroviral-experienced HIV-infected patients in Spain. AIDS 12, 1015–1020.

Granich, R.M., Gilks, C.F., Dye, C., De Cock, K.M., Williams, B.G., 2009. Universal voluntary HIV testing with immediate antiretroviral therapy as a strategy for elimination of HIV transmission: a mathematical model. Lancet 373, 48–57.

Halvas, E.K., Wiegand, A., Boltz, V.F., Kearney, M., Nissley, D., Wantman, M., Hammer, S.M., Palmer, S., Vaida, F., Coffin, J.M., Mellors, J.W., 2010. Low frequency nonnucleoside reverse-transcriptase inhibitor-resistant variants contribute to failure of efavirenz-containing regimens in treatment-experienced patients. J. Infect. Dis. 201, 672–680.

Hirsch, M.S., Brun-Vézinet, F., D'Aquila, R.T., Hammer, S.M., Johnson, V.A., Kuritzkes, D.R., Loveday, C., Mellors, J.W., Clotet, B., Conway, B., Demeter, L.M., Vella, S., Jacobsen, D.M., Richman, D.D., 2000. Antiretroviral drug resistance testing in adult HIV-1 infection: recommendations of an International AIDS Society-USA Panel. JAMA 283, 2417–2426.

Hirsch, M.S., Günthard, H.F., Schapiro, J.M., Brun-Vézinet, F., Clotet, B., Hammer, S.M., Johnson, V.A., Kuritzkes, D.R., Mellors, J.W., Pillay, D., Yeni, P.G., Jacobsen, D.M., Richman, D.D., 2008. Antiretroviral drug resistance testing in adult HIV-1 infection: 2008 recommendations of an International AIDS Society-USA panel. Clin. Infect. Dis. 47, 266–285.

Ishizaki, A., Cuong, N.H., Thuc, P.V., Trung, N.V., Saijoh, K., Kageyama, S., Ishigaki, K., Tanuma, J., Oka, S., Ichimura, H., 2009. Profile of HIV type 1 infection and genotypic resistance mutations to antiretroviral drugs in treatment-naive HIV type 1-infected individuals in Hai Phong, Viet Nam. AIDS Res. Hum. Retroviruses 25, 175–182.

Lall, M., Gupta, R.M., Sen, S., Kapila, K., Tripathy, S.P., Paranjape, R.S., 2008. Profile of primary resistance in HIV-1-infected treatment-naive individuals from Western India. AIDS Res. Hum. Retroviruses 24, 987–990.

Lima, V.D., Gill, V.S., Yip, B., Hogg, R.S., Montaner, J.S., Harrigan, P.R., 2008. Increased resilience to the development of drug resistance with modern boosted protease inhibitor-based highly active antiretroviral therapy. J. Infect. Dis. 198, 51–58.

Los-Alamos, 2010. HIV Databases, http://www.hiv.lanl.gov/content/index.

Maia Teixeira, S.L., Bastos, F.I., Hacker, M.A., Guimarães, M.L., Morgado, M.G., 2006. Trends in drug resistance mutations in antiretroviral-naïve intravenous drug users of Rio de Janeiro. J. Med. Virol. 78, 764–769.

Mintsa-Ndong, A., Caron, M., Plantier, J.C., Makuwa, M., Le Hello, S., Courgnaud, V., Roques, P., Kazanji, M., 2009. High HIV Type 1 prevalence and wide genetic diversity with dominance of recombinant strains but low level of antiretroviral drug-resistance mutations in untreated patients in northeast Gabon, Central Africa. AIDS Res. Hum. Retroviruses 25, 411–418.

Ndembi, N., Lyagoba, F., Nanteza, B., Kushemererwa, G., Serwanga, J., Katongole-Mbidde, E., Grosskurth, H., Kaleebu, P., 2008. Transmitted antiretroviral drug resistance surveillance among newly HIV type 1-diagnosed women attending an antenatal clinic in Entebbe Uganda. AIDS Res. Hum. Retroviruses 24, 889–895.

Nouhin, J., Ngin, S., Martin, P.R., Marcy, O., Kruy, L., Ariey, F., Peeters, M., Chaix, M.L., Ayouba, A., Nerrienet, E., 2009. Low prevalence of drug resistance transmitted virus in HIV Type 1-infected ARV-naive patients in Cambodia. AIDS Res. Hum. Retroviruses 25, 543–545.

Ockenga, J., Tillmann, H.L., Trautwein, C., Stoll, M., Manns, M.P., Schmidt, R.E., 1997. Hepatitis B and C in HIV-infected patients. Prevalence and prognostic value. J. Hepatol. 27, 18–24.

Palma, A.C., Araújo, F., Duque, V., Borges, F., Paixão, M.T., Camacho, R., 2007. Molecular epidemiology and prevalence of drug resistance-associated mutations in newly diagnosed HIV-1 patients in Portugal. Infect. Genet. Evol. 7, 391–398.

Pillay, V., Ledwaba, J., Hunt, G., Rakgotho, M., Singh, B., Makubalo, L., Bennett, D.E., Puren, A., Morris, L., 2008. Antiretroviral drug resistance surveillance among drug-naive HIV-1-infected individuals in Gauteng Province, South Africa in 2002 and 2004. Antivir. Ther. 13 (Suppl. (2)), 101–107.

Piroth, L., Grappin, M., Cuzin, L., Mouton, Y., Bouchard, O., Raffi, F., Rey, D., Peyramond, D., Gourdon, F., Drobacheff, C., Lombart, M.L., Lucht, F., Besnier, J.M., Bernard, L., Chavanet, P., Portier, H., 2000. Hepatitis C virus co-infection is a negative prognostic factor for clinical evolution in human immunodeficiency virus-positive patients. J. Viral Hepat. 7, 302–308.

Sagir, A., Oette, M., Kaiser, R., Däumer, M., Fätkenheuer, G., Rockstroh, J.K., Knechten, H., Schmutz, G., Hower, M., Emmelkamp, J., Pfister, H., Haussinger, D., 2007. Trends of prevalence of primary HIV drug resistance in Germany. J. Antimicrob. Chemother. 60, 843–848.

Tambussi, G., Boeri, E., Carrera, P., Gianotti, N., Lazzarin, A., 1998. Prevalence of mutation associated to resistance with nucleoside analogues in a cohort of naïve HIV-1 positive subjects during the period 1984–1997. J. Biol. Regul. Homeost. Agents 12, 32–34.

Varghese, V., Shahriar, R., Rhee, S.Y., Liu, T., Simen, B.B., Egholm, M., Hanczaruk, B., Blake, L.A., Gharizadeh, B., Babrzadeh, F., Bachmann, M.H., Fessel, W.J., Shafer, R.W., 2009. Minority variants associated with transmitted and acquired HIV-1 nonnucleoside reverse transcriptase inhibitor resistance: implications for the use of second-generation nonnucleoside reverse transcriptase inhibitors. J. Acquir. Immune Defic. Syndr. 52, 309–315.

Vercauteren, J., Derdelinckx, I., Sasse, A., Bogaert, M., Ceunen, H., De Roo, A., De Wit, S., Deforche, K., Echahidi, F., Fransen, K., Goffard, J.C., Goubau, P., Goudeseune, E., Yombi, J.C., Lacor, P., Liesnard, C., Moutschen, M., Pierard, D., Rens, R., Schrooten, Y., Vaira, D., Van den Heuvel, A., Van Der Gucht, B., Van Ranst, M., Van Wijngaerden, E., Vandercam, B., Vekemans, M., Verhofstede, C., Clumeck, N., Vandamme, A.M., Van Laethem, K., 2008. Prevalence and epidemiology of HIV type 1 drug resistance among newly diagnosed therapy-naive patients in Belgium from 2003 to 2006. AIDS Res. Hum. Retroviruses 24, 355–362.

Vercauteren, J., Wensing, A.M., van de Vijver, D.A., Albert, J., Balotta, C., Hamouda, O., Kücherer, C., Struck, D., Schmit, J.C., Asjö, B., Bruckova, M., Camacho, R.J., Clotet, B., Coughlan, S., Grossman, Z., Horban, A., Korn, K., Kostrikis, L., Nielsen, C., Paraskevis, D., Poljak, M., Puchhammer-Stockl, E., Riva, C., Ruiz, L., Salminen, M., Schuurman, R., Sonnerborg, A., Stanekova, D., Stanojevic, M., Vandamme,

A.M., Boucher, C.A., 2009. Transmission of drug-resistant HIV-1 is stabilizing in Europe. J. Infect. Dis. 200, 1503–1508.
Wheeler, W.H., Ziebell, R.A., Zabina, H., Pieniazek, D., Prejean, J., Bodnar, U.R., Mahle, K.C., Heneine, W., Johnson, J.A., Hall, H.I., 2010. Prevalence of transmitted drug resistance associated mutations and HIV-1 subtypes in new HIV-1 diagnoses U.S.-2006. AIDS 24, 1203–1212.
Zajdenverg, R., Badal-Faesen, S., Andrade-Villanueva, J., 2009. Lopinavir/ritonavir (LPV/r) tablets administered once- (QD) or twice-daily (BID) with NRTIs in antiretroviral-experienced HIV-1 infected subjects: results of a 48-week randomized trial (study M06-802). In: 5th IAS Conference on HIV Pathogenesis, Treatment and Prevention, Cape Town, South Africa.

Emergence of raltegravir-resistant HIV-1 in the central nervous system

K Watanabe MD, **M Honda** MD, **T Watanabe** MD, **K Tsukada** MD PhD, **K Teruya** MD PhD, **Y Kikuchi** MD PhD, **S Oka** MD PhD **and H Gatanaga** MD PhD

AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan

Summary: Integrase inhibitor-resistant HIV-1 was detected in the cerebrospinal fluid, but not in the plasma of a 42-year-old man with HIV encephalopathy treated with a raltegravir (RAL)-containing regimen. Raltegravir resistance may develop in the central nervous system when the virus is already multi-drug resistant because of different penetration into cerebrospinal fluid of individual antiretroviral agents.

Keywords: integrase, resistant, cerebrospinal fluid, Q148R, HIV, raltegravir

INTRODUCTION

Raltegravir (RAL) is the first approved HIV-1 integrase inhibitor that has demonstrated potent antiretroviral activity in combination with an optimized background regimen in treatment-experienced patients.[1] However, RAL resistance develops relatively easily after virologic failure.[2,3] We describe here a case of HIV encephalopathy, in whom a major resistance mutation of integrase inhibitors was detected in the cerebrospinal fluid (CSF) but not in the plasma during RAL-containing antiretroviral treatment.

CASE REPORT

A 42-year-old man infected with HIV-1 presented with asthenia, skin hyperesthesia, loss of memory and psychomotor slowing. Magnetic resonance imaging (MRI) of the brain revealed diffuse bilateral and symmetrical increase in T2-weighted signal in the periventricular matter of the frontal and parieto-occipital regions. He was diagnosed with HIV encephalopathy based on the MRI findings and negative test results for other viral and bacterial infections, supported by a higher HIV-1 load in the CSF than in the plasma. During eight years of combination antiretroviral treatment (cART) (including zidovudine, didanosine, abacavir, efavirenz (EFV), indinavir and nelfinavir), multiple drug-resistance mutations were detected in plasma HIV-1, including A62V, V75I, F77L, Y115F, F116Y, Q151M, M184V and Y188L in reverse-transcriptase, and L10I, K20R, E35D, M36I, M46I, H69K, V82F and I93L in protease (at day −505).[4] The CD4 count was 246 cells/μL and the HIV-1 load was incompletely suppressed in the plasma (70–1200 copies/mL) over the preceding 15 months with cART of tenofovir (TDF), stavudine (d4T), lamivudine (3TC) and ritonavir-boosted lopinavir (LPVr), whereas active HIV-1 replication seemed to continue in the central nervous system (CNS) (120,000 copies/mL in CSF; Figure 1). In order to control HIV-1 replication, the cART regimen was modified to the combination of TDF, emtricitabine (FTC), EFV, darunavir (DRV), ritonavir (RTV), RAL and enfuvirtide (ENF) (day 0), resulting in successful HIV-1 suppression to below the level of detection (50 copies/mL) in both plasma and CSF on day 33, and the patient's CNS symptoms improved. ENF injection was stopped on day 58 though the other antiretroviral agents were continued. The CNS symptoms gradually deteriorated, although the plasma HIV-1 load was persistently suppressed below the level of detection (50 or 40 copies/mL), but the CSF HIV-1 load was found to have rebounded to a level of 440 copies/mL on day 224. ENF treatment was re-introduced, which resulted in the suppression of the CSF viral load to below the level of detection on day 253, and improvement of CNS symptoms; however, the patient showed gradual mood changes and developed psychiatric symptoms. ENF injection became difficult and it was stopped on day 358. By day 371, the HIV-1 load in the CSF was 4300 copies/mL but still undetectable in the plasma. Direct sequencing of the HIV-1 integrase gene identified a major mutation of RAL resistance (Q148R) in CSF, which was not detected by sequencing of earlier plasma (day −8) and CSF (day 224) samples. The above resistance mutations in reverse transcriptase and protease genes were detected in all successfully polymerase chain reaction-amplified samples.[4] The antiretroviral treatment was stopped on day 371 because of potential choking by the patient during swallowing. The HIV-1 load rebounded to 18,000 copies/mL in the plasma and 28,000 copies/mL in the CSF by day 405, in both of which no major RAL-associated mutation was detected.

Correspondence to: H Gatanaga, AIDS Clinical Center, National Center for Global Health and Medicine, 1-21-1, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan
Email: higatana@acc.ncgm.go.jp

DISCUSSION

In the present case of HIV encephalopathy HIV-1 load was persistently higher in CSF than in plasma, suggesting active viral

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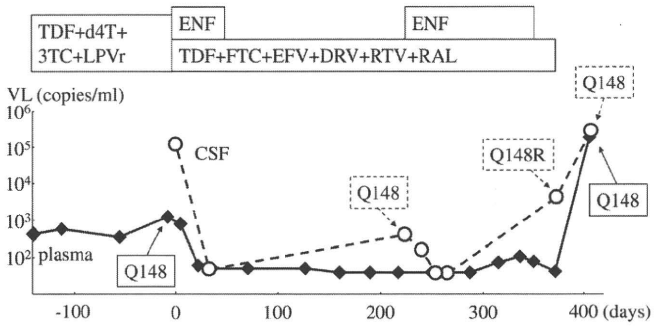

Figure 1 **HIV-1 resistance mutations in the integrase gene and changes in viral loads (VL) in plasma and cerebrospinal fluid (CSF). HIV-1 integrase sequence was successfully analysed in two plasma samples (days −8, and 405) and three CSF samples (days 224, 371 and 405). A major mutation of raltegravir resistance (Q148R) was detected in only one CSF sample (day 371) which reverted to wild-type (Q148) after cessation of RAL-containing regimen (day 405). HIV-1 integrase gene could not be amplified from the plasma sample taken at day 371. No other integrase mutations listed in mutations figures or described in user notes of the International AIDS Society-USA Drug Resistant Mutation Groups were detected.[4] Multiple drug resistance mutations in reverse transcriptase (A62V, V75I, F77L, Y115F, F116Y, Q151M, M184V and Y188L) and protease genes (L10I, K20R, E35D, M36I, M46I, H69K, V82F and I93L) were persistently detected in all successfully amplified samples**

replication in the CNS, which continued under RAL-containing regimen. RAL has a lower genetic barrier to resistance compared with protease inhibitors (PIs) to which multiple mutations are necessary for HIV-1 to attain significant resistance.[2,3] Recently, RAL was reported to penetrate well into CSF, whereas the penetration of DRV and EFV are extremely limited.[5–7] In the present case, the altered cART seemed to suppress multidrug resistant HIV-1 replication successfully in the systemic circulation and lymph nodes in the presence of effective concentrations of RAL, DRV, and EFV. However, in the CNS, active replication was suppressed only imperfectly, most likely due to lower concentrations of DRV and EFV in the CSF, which induced the emergence of HIV-1 variants resistant to RAL, the only drug fully active against the previous HIV-1 variants. This case study illustrates the potential for RAL resistance developing in the CNS when the virus is already multi-drug resistant.

REFERENCES

1 Steigbigel RT, Cooper DA, Kumar PN, *et al.* Raltegravir with optimized background therapy for resistant HIV-1 infection. *N Engl J Med* 2008;**359**:339–54

2 Lennox J, DeJesus E, Lazzarin A, *et al.* STARTMRK, a phase III study of the safety & efficacy of raltegravir (RAL)-based vs efavirenz (EFV)-based combination therapy in treatment-naïve HIV-infected patients [abstract H-896a]. In: *Program and Abstracts of the 48th Interscience Conference on Antimicrobial Agents and Chemotherapy (Washington, DC).* Washington, DC: American Society for Microbiology, 2008

3 Cooper DA, Steigbigel RT, Gatell JM, *et al.* Subgroup and resistance analyses of raltegravir for resistant HIV-1 infection. *N Engl J Med* 2008;**359**:355–65

4 Johnson VA, Brun-Vezinet F, Clotet B, *et al.* Update of the drug resistance mutations in HIV-1. *Top HIV Med* 2008;**16**:138–45

5 Calcagno A, Banora S, Bertucci R, *et al.* Raltegravir penetration in the cerebrospinal fluid of HIV-positive patients. *AIDS* 2010;**24**:931–32

6 Iwamoto M, Wenning LA, Petry AS, *et al.* Safety, tolerability, and pharmacokinetics of raltegravir after single and multiple doses in healthy subjects. *Clin Pharmacol Ther* 2008;**83**:293–9

7 Antinori A, Perno CF, Giancola ML, *et al.* Efficacy of cerebrospinal fluid (CSF) – penetrating antiretroviral drugs against HIV in the neurological compartment: different patterns of phenotypic resistance in CSF and plasma. *Clin Infect Dis* 2005;**41**:1787–93

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Autoimmune Diabetes in HIV-Infected Patients on Highly Active Antiretroviral Therapy

Daisuke Takarabe,* Yuka Rokukawa,* Yoshihiko Takahashi, Atsushi Goto, Maki Takaichi, Masahide Okamoto, Tetsuro Tsujimoto, Hiroshi Noto, Miyako Kishimoto, Yasushi Kaburagi, Kazuki Yasuda, Ritsuko Yamamoto-Honda, Kunihisa Tsukada, Miwako Honda, Katsuji Teruya, Hiroshi Kajio, Yoshimi Kikuchi, Shinichi Oka, and Mitsuhiko Noda

Department of Diabetes and Metabolic Medicine (D.T., Y.R., Y.T., A.G., M.T., M.O., T.T., H.N., M.K., Y.Ka., K.Y., R.Y.-H., H.K., M.N.), and AIDS Clinical Center (K.Ts., M.H., K.Te., Y.Ki., S.O.), National Center for Global Health and Medicine, Tokyo 162-8655, Japan

Context: Various autoimmune diseases, especially autoimmune thyroid disease, are known to occur in HIV-infected patients on highly active antiretroviral therapy (HAART). However, no reports have described the development of autoimmune diabetes during HAART.

Objective: Our objective was to investigate the clinical course of the development of autoantibodies and diabetes during HAART.

Patients and Methods: Based on their high antiislet autoantibody titers and requirement for insulin therapy, we diagnosed three HIV-infected patients with autoimmune diabetes. To clarify the relationship between the development of an autoimmune reaction against pancreatic β-cells and recovery of $CD4^+$ T lymphocyte (CD4) counts, we retrospectively assayed stored samples of the patients' plasma for antiglutamic acid decarboxylase antibody (GAD-Ab).

Results: No GAD-Ab was detected in the plasma samples of any of the three patients prior to HAART, and their CD4 counts were below 20 cells/μl at their nadir. The GAD-Ab tests became positive from 6 to 38 months after the start of HAART, and their conversion to positive followed a dramatic increase in the patients' CD4 count. Two patients developed diabetes after testing positive for GAD-Ab. Although one patient had mild diabetes prior to testing positive for GAD-Ab, the rapid worsening of glycemic control and introduction of insulin therapy almost coincided with the detection of GAD-Ab. The high magnitude of the CD4 increase during HAART and the timing of the detection of autoantibody were similar to the magnitude and timing reported in HAART-associated autoimmune thyroid disease.

Conclusions: Autoimmune diabetes develops in some HIV-infected patients after immune restoration during HAART. (***J Clin Endocrinol Metab*** **95: 0000–0000, 2010)**

Autoimmune diabetes is characterized by the presence of antiislet autoantibodies and is caused by autoimmune-mediated destruction of pancreatic β-cells (1). Although a high prevalence of diabetes has been reported in HIV-infected patients, most cases are considered attributable to insulin resistance induced by antiretroviral drugs (2, 3). The immunodeficiency of HIV-infected patients is characterized by a low $CD4^+$ T-lymphocyte (CD4) count, but highly active antiretroviral therapy (HAART) can reduce the HIV plasma viral load (pVL), and the CD4 count sometimes increases dramatically (immune restoration). As a result, some patients experience clinical deterioration

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* D.T. and Y.R. contributed equally to this work.

Abbreviations: AITD, Autoimmune thyroid disease; CD4, $CD4^+$ T lymphocyte; GAD-Ab, glutamic acid decarboxylase antibody; HAART, highly active antiretroviral therapy; HbA_{1c}, glycosylated hemoglobin; HLA, human leukocyte antigen; IA2-Ab, insulinoma-associated antigen-2 antibody; pVL, plasma viral load; T1D, type 1 diabetes; T2D, type 2 diabetes.

due to restoration of an inflammatory immune response against both infectious and noninfectious antigens (4). Various autoimmune diseases have been reported after immune restoration (5, 6), especially autoimmune thyroid disease (AITD) (7–11), but none have described the development of autoimmune diabetes during HAART. We report the cases of three HIV-infected patients who developed autoimmune diabetes after immune restoration during HAART.

Ethics

This report was approved by the local ethics committee of the National Center for Global Health and Medicine. We obtained written informed consent from all three patients.

Case reports

The characteristics and laboratory findings of the three patients are indicated in Table 1. All of the three patients are currently on intensive insulin therapy, and recent doses of insulin are shown. The diagnosis of diabetes was made on the basis of repeated measurements of fasting and/or casual plasma glucose levels. To investigate the clinical course of immune restoration and development of autoimmunity, we retrospectively measured the titers of several autoantibodies sequentially by RIA of plasma samples that had been stored frozen.

Patient 1 was a 30-yr-old Japanese man who had been diagnosed with HIV infection and hepatitis C virus infection at 19 yr of age. Although HAART had been started then, the HAART regimen had often been suspended and switched to other regimens because of adverse effects, and his CD4 count and pVL had been uncontrolled for years. Although the patient was overweight and had a family history of type 2 diabetes (T2D), he had never been diagnosed with diabetes until he was 29 yr old. At 29 yr of age, HAART was resumed with a new regimen, which the pa-

TABLE 1. Characteristics and laboratory findings at the diagnosis of autoimmune diabetes

	Patient 1	Patient 2	Patient 3
Age (yr)	30	31	68
Sex	Male	Male	Female
Body mass index (kg/m^2)	24.2	20.0	19.1
Weight loss within 3–6 months (%)	20	11	27
Family history of diabetes	+	–	–
Regimen of the tolerated HAART	3TC TDF LPVr	3TC d4T LPVr	3TC, ETR RTV, DRV RAL
Duration of the tolerated HAART (months)[a]	18	10	55
Duration of HIV infection (yr)[a]	11	17	5
Recent dose of insulin injection (U/kg)	0.9	0.7	0.9
Data related to HIV infection			
pVL	Undetectable	Undetectable	Undetectable
CD4 count (cells/μl)	311	172	316
CD4 count at nadir (cells/μl)	12	14	19
Data related to diabetes			
Casual plasma glucose (mmol/liter)	26.6	9.2	8.1
HbA_{1c} (%)	10.8	10.9	12.2
HbA_{1c} increment within 3–6 months (%)	5.3	5.5	5.8
Fasting serum insulin (pmol/liter)	20.1	15.3	16.0
Fasting serum C-peptide (nmol/liter)	0.33	0.22	0.13
Urinary C-peptide (nmol per 24 h)	40.5	3.8	9.3
Urine ketone body	+	–	–
Autoantibody tests [positive test after the start of HAART regimen (months)][b]			
GAD-Ab (U/ml)	606 (6)	26000 (7)	1023 (38)
IA2-Ab (U/ml)	22.5 (9)	<0.4	5.9 (38)
TSHR-Ab (IU/liter)	12.6 (13)	<1.0	<1.0 (64)[c]
TPO-Ab (U/ml)	33.4 (6)	29.3 (4)	>60.0 (26)
Tg-Ab (U/ml)	1.6 (6)	<0.3	13.2 (38)
Antiadrenal cortex antibody (fold)	<10	<10	<10

3TC, Lamivudine; TDF, tenofovir; LPVr, lopinavir/ritonavir; d4T, sanilvudine; ETR, etravirine; RTV, ritonavir; DRV, darunavir; RAL, raltegravir; TSHR-Ab, anti-TSH receptor antibody; TPO-Ab, antithyroid peroxydase antibody; Tg-Ab, antithyroglobulin antibody.

[a] Interval before the diagnosis of autoimmune diabetes.

[b] Normal ranges include the following: GAD-Ab, less than 1.4 U/ml; IA2-Ab, less than 0.4 U/ml; TSHR-Ab, less than 1.0 IU/liter; TPO-Ab, less than 0.3 U/ml; Tg-Ab, less than 0.3 U/ml; antiadrenal cortex antibody, less than 10-fold.

[c] TSHR-Ab test became positive after the diagnosis of autoimmune diabetes.

tient tolerated, and his CD4 count gradually rose, but about 9 months later he was diagnosed with diabetes. At first, he was thought to have antiretroviral drug-induced diabetes, and his glycosylated hemoglobin (HbA_{1c}) levels (standardized by the Japan Diabetes Society) remained less than 5.5% in the absence of treatment with any antidiabetic agents. However, 18 months after resuming HAART, the patient's HbA_{1c} level began to increase, sometimes reaching as high as 10.8%. Insulin secretion gradually decreased, and the patient required intensive insulin therapy. He also had a high antiglutamic acid decarboxylase antibody (GAD-Ab) titer and a high insulinoma-associated antigen-2 antibody (IA2-Ab) titer (Table 1). A retrospective GAD-Ab test revealed that the patient had become GAD-Ab-positive in the period between the recovery of his CD4 count and the diagnosis of diabetes (Fig. 1A). Thus, the autoimmune response against β-cells actually began before the diagnosis of antiretroviral drug-induced diabetes, and we concluded that the patient's diabetes was caused by autoimmune mechanism. At 34 yr of age, the patient was admitted to our hospital because of ketoacidosis after omitting insulin on a sick day.

Patient 2 was a 31-yr-old Japanese man who had been diagnosed with HIV infection and hepatitis C virus infection at 13 yr of age. HAART was instituted then, but the HAART regimen had often been suspended and switched to other regimens because of the emergence of drug-resistant HIV and the occurrence of adverse effects, and his CD4 count and pVL had been uncontrolled for years. At 19 yr of age, the patient was diagnosed with diabetes, which was thought to be antiretroviral drug-induced diabetes or T2D. Good glycemic control had been maintained ($HbA_{1c} < 5.5\%$) for more than 10 yr with an α-glucosidase inhibitor. At 30 yr of age, HAART was resumed with a regimen that was tolerated, and his CD4 count gradually rose. Nine months later, however, his glycemic control rapidly deteriorated (HbA_{1c} 10.9%), and the GAD-Ab test became positive at that time (Table 1). The diabetes was not insulin dependent, but an iv glucagon challenge test demonstrated severely impaired insulin secretion. The patient required intensive insulin therapy, and the insulin dose was gradually increased. A retrospective GAD-Ab test revealed that the patient had become GAD-Ab positive in the period between the recovery of his CD4 count and the rapid deterioration of his glycemic control (Fig. 1B). We therefore concluded that his diabetes had worsened because of autoimmune destruction of β-cells, although he basically had antiretroviral drug-induced diabetes or T2D.

Patient 3 was a 68-yr-old Japanese woman who had been diagnosed with HIV infection at 63 yr of age, and HAART had been started then. She had tolerated the regimen from the beginning, and her CD4 count and pVL remained well controlled. About 36 months after the start

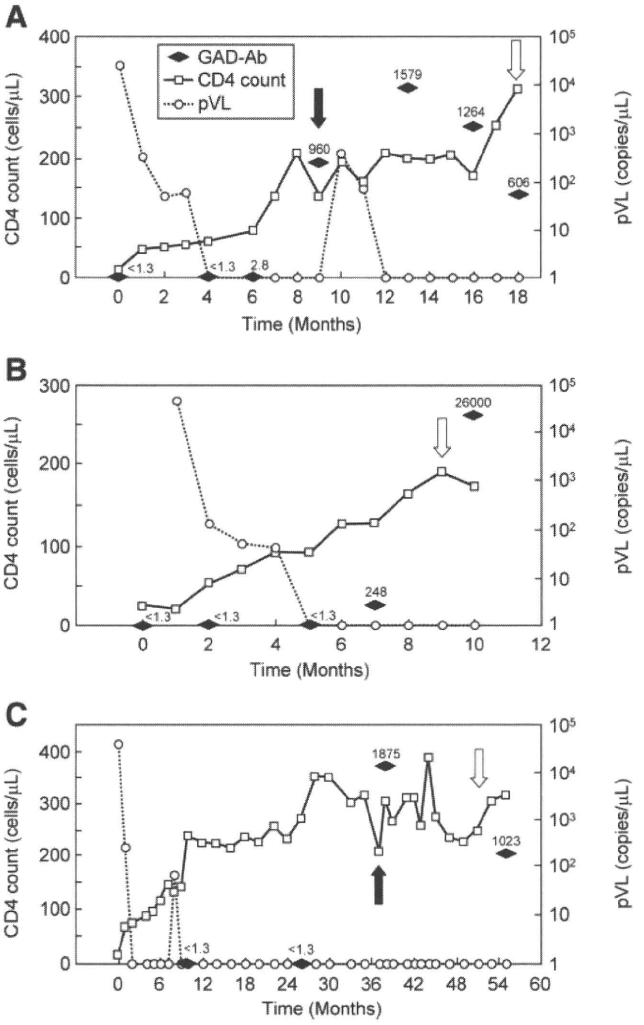

FIG. 1. Clinical courses of three patients before autoimmune diabetes diagnosis. Course of CD4 counts, pVL values, and GAD-Ab titers (units per milliliter) in patient 1 (A), patient 2 (B), and patient 3 (C) after the introduction of HAART. *Black arrows* indicate the time of the initial diagnosis of diabetes. *White arrows* indicate the beginning of progressive worsening of glycemic control. Because the initial diagnosis of diabetes in patient 2 was made several years before the introduction of HAART, the time of the diagnosis is not indicated.

of HAART, however, the patient was diagnosed with diabetes, which was thought to be antiretroviral drug-induced diabetes or T2D. She was treated with a sulfonylurea, and her glycemic control improved for approximately 5 months (representative HbA_{1c} value 6.1%). However, 55 months after the start of HAART, her HbA_{1c} level rapidly increased to as high as 12.2% and was accompanied by a marked decrease in insulin secretion, and she required intensive insulin therapy. Both GAD-Ab test and IA2-Ab test were positive (Table 1), and retrospective examination showed that GAD-Ab had appeared between the recovery of her CD4 count and the diagnosis of diabetes (Fig. 1C). Based on these findings, the diagnosis was changed to autoimmune diabetes.

Follow-up examination revealed a fasting plasma glucagon level in patients 1, 2, and 3 of 57, 82, and 61 pg/ml,

respectively, and the corresponding plasma glucose level was 12.3, 6.3, and 13.6 mmol/liter. Although data on glucagon response to hypoglycemia were unavailable, these values suggested that glucagon secretion did not seem to be severely impaired.

Other autoimmune endocrine diseases

The three patients had from one to three types of antithyroid antibodies (Table 1). In fact, patient 1 had thyrotoxicosis that showed spontaneous remission, and patient 3 was diagnosed with Graves' disease after the development of autoimmune diabetes (Rokukawa Y., Y. Takahashi, and M. Noda, *et al.*, manuscript in preparation).

The patients' serum cortisol levels indicated normal adrenal function. We measured the titer of antiadrenal cortex antibody, but none of the patients were positive or had any evidence of other endocrinological disorders. Thus, none of the three patients met the diagnostic criteria for autoimmune polyendocrine syndrome (12).

Human leukocyte antigen (HLA) genotyping

The HLA genotyping of these patients showed that patient 1 was heterozygous for DRB1*0405-DQB1*0401$^{\#}$ and DRB1*0901-DQB1*0303$^{\#\#}$, patient 2 was heterozygous for DRB1*0803-DQB1*0601 and DRB1*0901-DQB1*0303$^{\#\#}$, and patient 3 was heterozygous for DRB1*0403-DQB1*0302 and DRB1*0406-DQB1*0302 ($^{\#}$ and $^{\#\#}$ are haplotypes for increased susceptibility to type 1 diabetes (T1D) among Japanese (13)].

Discussion

We diagnosed the three patients with autoimmune diabetes because they had high GAD-Ab titers, which had never been observed in T2D with β-cell failure and because they required intensive insulin therapy for glycemic control. Antiislet antibodies are not always specific for T1D, and previous reports have stated that combined assays for multiple antiislet autoantibodies are more sensitive for the diagnosis of T1D (14, 15). In fact, patients 1 and 3 were positive for both GAD-Ab and IA2-Ab. The limitation of our study is that direct evidence of autoimmune destruction of β-cells such as histological findings was not available in our cases. Classic T1D, which is commonly observed in children, progress rapidly, whereas the slowly progressive form generally occurs in adults and is sometimes referred to as latent autoimmune diabetes in adults (16) or slowly progressive insulin-dependent diabetes mellitus (17). Insulin secretion decreased slowly and progressively in our patients, suggesting that their course of diabetes was close to the latent autoimmune diabetes in adults/slowly progressive insulin-dependent diabetes mellitus type. Hampe *et al.* (18) have shown that the characteristics of amino-terminal-specific GAD65 autoantibodies can be used to differentiate between severe and milder phenotypes of patients with GAD-Ab and ketosis-prone diabetes. Because we were not able to explore the biochemical nature of the patients' GAD-Ab, we cannot exclude the possibility that the characteristics of the GAD-Ab are different in the present cases and in classic T1D. Although we cannot draw conclusions as to whether insulin resistance caused by the antiretroviral drugs or obesity really existed in our patients, we have to consider a possibility that insulin resistance may have affected their clinical course because the acceleration mechanism of the autoimmunity against β-cells via insulin resistance-associated β-cell stress is proposed (19).

Zandman-Goddard and Shoenfeld (5) suggested that autoimmune diseases in HIV-infected patients may develop as a result of molecular mimicry in the acute phase of HIV infection as well as due to altered autoimmune regulation in the phase when the CD4 count has been restored by HAART. Autoimmune diabetes did not develop in the acute phase of the HIV infection in our patients, and pVL was undetectable for more than 2 months before the first positive test for GAD-Ab. Moreover, examinations did not detect evidence of other viral infections that are thought to be associated with T1D (20). Thus, molecular mimicry by HIV itself or other viruses was unlikely to have caused the autoimmune diabetes in our patients.

In most of the cases of HAART-associated AITD, there was a severe decline in the CD4 count at the nadir that was followed by a marked increase in the CD4 count before the development of AITD (7–11), and the large magnitude of the increase may be a risk factor for AITD (8). The changes in the CD4 counts of our patients seemed consistent with those in HAART-associated AITD (Fig. 1). Chen *et al.* (8) suggested that the T-cell repopulation induced by HAART is biphasic and that AITD develops in the second phase, when a substantial increase in naive CD4 occurs (~6 months after the first phase). Although we did not analyze the T-cell population in detail, the emergence of GAD-Ab and onset of the autoimmune diabetes in our patients were likely to have occurred in the second phase.

Patients 1 and 2 both had HLA haplotypes that has been reported to increase susceptibility to T1D in Japanese. Patient 3, however, did not have any of the known HLA haplotypes associated with increased susceptibility to diabetes in Japanese. No role of HLA in the development of HAART-associated autoimmune diseases has yet been established (6), and further investigation is warranted at this point.

In conclusion, autoimmune diabetes develops in some HIV-infected patients after immune restoration induced

by HAART, and some of the patients require insulin therapy. Testing for GAD-Ab is important in HIV-infected patients whose glycemic level deteriorates after immune restoration.

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Address all correspondence and requests for reprints to: Yoshihiko Takahashi, Chief, Department of Diabetes and Metabolic Medicine, National Center for Global Health and Medicine, Toyama 1-21-1, Shinjuku-ku, Tokyo 162-8655, Japan. E-mail: yotakaha@hosp.ncgm.go.jp.

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References

1. **Alberti KG, Zimmet PZ** 1998 Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 15:539–553
2. **Florescu D, Kotler DP** 2007 Insulin resistance, glucose intolerance and diabetes mellitus in HIV-infected patients. Antivir Ther 12:149–162
3. **Aboud M, Elgalib A, Kulasegaram R, Peters B** 2007 Insulin resistance and HIV infection: a review. Int J Clin Pract 61:463–472
4. **Murdoch DM, Venter WD, Van Rie A, Feldman C** 2007 Immune reconstitution inflammatory syndrome (IRIS): review of common infectious manifestations and treatment options. AIDS Res Ther 4:9
5. **Zandman-Goddard G, Shoenfeld Y** 2002 HIV and autoimmunity. Autoimmun Rev 1:329–337
6. **Calabrese LH, Kirchner E, Shrestha R** 2005 Rheumatic complications of human immunodeficiency virus infection in the era of highly active antiretroviral therapy: emergence of a new syndrome of immune reconstitution and changing patterns of disease. Semin Arthritis Rheum 35:166–174
7. **Knysz B, Bolanowski M, Klimczak M, Gladysz A, Zwolinska K** 2006 Graves' disease as an immune reconstitution syndrome in an HIV-1-positive patient commencing effective antiretroviral therapy: case report and literature review. Viral Immunol 19:102–107
8. **Chen F, Day SL, Metcalfe RA, Sethi G, Kapembwa MS, Brook MG, Churchill D, de Ruiter A, Robinson S, Lacey CJ, Weetman AP** 2005 Characteristics of autoimmune thyroid disease occurring as a late complication of immune reconstitution in patients with advanced human immunodeficiency virus (HIV) disease. Medicine (Baltimore) 84:98–106
9. **Jubault V, Penfornis A, Schillo F, Hoen B, Izembart M, Timsit J, Kazatchkine MD, Gilquin J, Viard JP** 2000 Sequential occurrence of thyroid autoantibodies and Graves' disease after immune restoration in severely immunocompromised human immunodeficiency virus-1-infected patients. J Clin Endocrinol Metab 85:4254–4257
10. **French MA, Lewin SR, Dykstra C, Krueger R, Price P, Leedman PJ** 2004 Graves' disease during immune reconstitution after highly active antiretroviral therapy for HIV infection: evidence of thymic dysfunction. AIDS Res Hum Retroviruses 20:157–162
11. **Vos F, Pieters G, Keuter M, van der Ven A** 2006 Graves' disease during immune reconstitution in HIV-infected patients treated with HAART. Scand J Infect Dis 38:124–126
12. **Betterle C, Dal Pra C, Mantero F, Zanchetta R** 2002 Autoimmune adrenal insufficiency and autoimmune polyendocrine syndromes: autoantibodies, autoantigens, and their applicability in diagnosis and disease prediction. Endocr Rev 23:327–364
13. **Kawabata Y, Ikegami H, Kawaguchi Y, Fujisawa T, Shintani M, Ono M, Nishino M, Uchigata Y, Lee I, Ogata T** 2002 Asian-specific HLA haplotypes reveal heterogeneity of the contribution of HLA-DR and -DQ haplotypes to susceptibility to type 1 diabetes. Diabetes 51:545–551
14. **Verge CF, Stenger D, Bonifacio E, Colman PG, Pilcher C, Bingley PJ, Eisenbarth GS** 1998 Combined use of autoantibodies (IA-2 autoantibody, GAD autoantibody, insulin autoantibody, cytoplasmic islet cell antibodies) in type 1 diabetes: Combinatorial Islet Autoantibody Workshop. Diabetes 47:1857–1866
15. **Sera Y, Kawasaki E, Abiru N, Ozaki M, Abe T, Takino H, Kondo H, Yamasaki H, Yamaguchi Y, Akazawa S, Nagataki S, Uchigata Y, Matsuura N, Eguchi K** 1999 Autoantibodies to multiple islet autoantigens in patients with abrupt onset type 1 diabetes and diabetes diagnosed with urinary glucose screening. J Autoimmun 13:257–265
16. **Zimmet PZ, Tuomi T, Mackay IR, Rowley MJ, Knowles W, Cohen M, Lang DA** 1994 Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency. Diabet Med 11: 299–303
17. **Kobayashi T, Tamemoto K, Nakanishi K, Kato N, Okubo M, Kajio H, Sugimoto T, Murase T, Koska K** 1993 Immunogenetic and clinical characterization of slowly progressive IDDM. Diabetes Care 16:780–788
18. **Hampe CS, Nalini R, Maldonado MR, Hall TR, Garza G, Iyer D, Balasubramanyam A** 2007 Association of amino-terminal-specific antiglutamate decarboxylase (GAD65) autoantibodies with β-cell functional reserve and a milder clinical phenotype in patients with GAD65 antibodies and ketosis-prone diabetes mellitus. J Clin Endocrinol Metab 92:462–467
19. **Hyppönen E, Virtanen SM, Kenward MG, Knip M, Akerblom HK** 2000 Obesity, increased linear growth, and risk of type 1 diabetes in children. Diabetes Care 23:1755–1760
20. **Jun HS, Yoon JW** 2003 A new look at viruses in type 1 diabetes. Diabetes Metab Res Rev 19:8–31