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Original article

Analysis of HIV-1 sequences before and after co-infecting syphilis

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Abstract

Increasing syphilis incidence among men who have sex with men (MSM) has been reported. The index case was a human immunodeficiency virus type 1 (HIV-1)-positive MSM who presented coincidentally with the secondary syphilis and a rebound of plasma viral load after complete suppression of HIV-1 (below 50 copies/ml) for 13 months with potent antiretroviral therapy (PART), suggesting a possibility of HIV-1 superinfection. We analyzed HIV-1 sequences before and after syphilis in four HIV-1-positive patients including the index case to explore drug resistance mutations (DRMs) and a possibility of HIV-1 superinfection. There were patients who obtained DRMs around syphilis infection but no evidence of HIV-1 superinfection was obtained. Our results underline the importance of strict adherence to PART.
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Keywords: HIV-1 superinfection; Syphilis; Drug resistance mutations; STI; PART

1. Introduction

Syphilis has been noted as a reemerging infectious disease in large cities in the U.S. and Europe, where the incidence increased steeply among men who have sex with men (MSM) [1,2]. Sexually transmitted infections (STIs) such as syphilis not only imply practice of unsafe sex but actually contribute to the transmission of human immunodeficiency virus type 1 (HIV-1) [3,4]. Over 40% of Japanese HIV-1-positive patients who reported unprotected sex had a history of syphilis proven by *Tremponema pallidum* hemagglutination test (TPHA) [5]. Recently we have been experiencing increasing number of active syphilis cases in our HIV-1 outpatient clinic in Tokyo (Nakamura, H. unpublished).

Infection with drug-resistant HIV-1 is another problem in large cities in the world [6]. Although superinfection may be less frequent than initially concerned [7,8], superinfections with drug-resistant HIV-1 have actually been reported [9].

A patient with acquired immunodeficiency syndrome (AIDS)-lymphoma who had been treated successfully with potent antiretroviral therapy (PART) presented with syphilitic skin rash. Coincidentally his HIV-1 viral load (VL) exploded abruptly to 6.7×10^4/ml and his rebounded plasma HIV-1

Abbreviations for drugs: ddI, didanosine; d4T, stavudine; EFV, efavirenz; LPV, lopinavir; NFV, nelfinavir; RTV, ritonavir; ZDV, zidovudine; 3TC, lamivudine; CHOP, combination therapy with cyclophosphamide, vincristine, doxorubicin and prednisolone.

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was found to contain multiple drug-resistant mutations (DRMs). Therefore, a possibility of superinfection with drug resistant HIV-1 was considered in this case. Since it has been reported that syphilis may increase HIV-1 VL [10], it can be a driving force of new mutations for HIV-1. We analyzed HIV-1 sequences before and after syphilis in four HIV-1-positive patients including the index case to explore DRMs and a possibility of HIV-1 superinfection.

2. Patients, materials and methods

2.1. Patient 1

A 34 y/o MSM was referred to our hospital in April 2001. He had been diagnosed as AIDS with extranodular malignant lymphoma as an indicator disease. In parallel with CHOP (cyclophosphamide, vincristine, doxorubicin and prednisolone) therapy to lymphoma, he had been on PART with a combination of zidovudine (ZDV), lamivudine (3TC) and nelfinavir (NFV) since August 2000. As summarized in Fig. 1, his VL had been constantly less than 50 copies/ml for 8 consecutive time points in 13 months including time points 1−1 and 1−2.

He was asymptomatic in July 2002 when periodic blood samples were stored (Time point 1−3). Two months after 1−3 (time point 1−4), he developed skin rush and was examined. Serologic test for syphilis (STS, glass slide test) and TPHA at time point 1−4 was very high and the diagnosis of syphilis was confirmed by a skin biopsy. Retrospective examination using frozen plasma showed that the patient was TPHA-negative at time points 1−1 and 1−2 but turned positive at time point 1−3 indicating syphilis infection before that time point.

2.2. Patient 2

Patient 2 was treated with dual nucleosides (ZDV and 3TC) from 1996 to 1999. After a treatment interruption, he was reinitiated with a combination of stavudine (d4T), didanosine (ddI) and efavirenz (EFV) since July 2001. Although undetectable levels were not attained, his VLs were always under 1000 copies/ml for 20 months including August 2002 (time point 2−1) (Fig. 2). Eight months after time point 2−1 (time point 2−2), he presented with syphilitic skin rash and inguinal lymphadenopathy. At time point 2−2, his VL was unusually higher (4400 copies/ml) than his previous data.

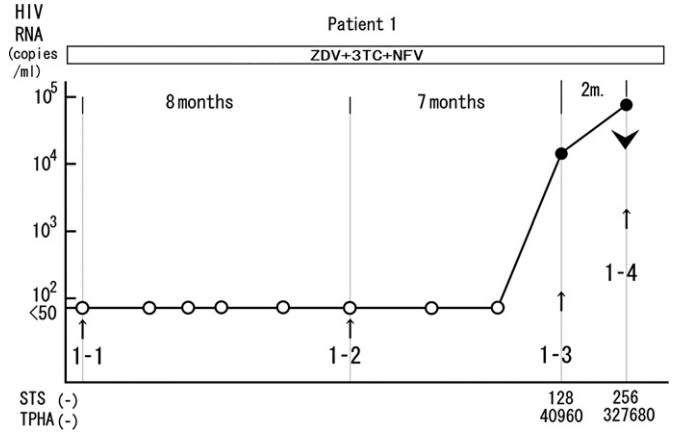

Fig. 1. Clinical course of patient 1. The graph shows VL along the clinical course. Open points indicate undetectable viral load (<50 copies/ml). Names of antiretroviral drugs are shown in the bar on the top. ZDV: zidovudine, 3TC: lamivudine, NFV: nelfinavir. Upward arrows indicate examined time points. A solid downward arrowhead indicates time points when the diagnosis of syphilis was made. STS: Serologic test for syphilis. TPHA: *Treponema pallidum* hemagglutionation test.

2.3. Patient 3

Patient 3 presented with syphilitic rash while he was treated with d4T, 3TC, lopinavir (LPV) and ritonavir (RTV, rtv). His VLs stayed between 100 and 1000 copies/ml three months before (time point 3−1) and at (time point 3−2) syphilis coinfection (Fig. 2).

2.4. Patient 4

Patient 4 was treated with d4T, 3TC and NFV since 1998. His VL became undetectable soon after the initiation of PART and kept undetectable for 41 months until October 2001 (time point 4−1). Although the diagnosis of syphilis was made based on a skin rash between time points 4−2 and 4−3, retrospective TPHA examination tested positive at time point 4−2 (Fig. 2). Except a couple of blips, his VL remained undetectable including time points 4−2, 4−3, and 4−4 after the infection of syphilis.

2.5. Extraction of provirus DNA

Aliquots of blood samples were collected periodically with informed consent. They were centrifuged in Ficoll density gradient and the isolated plasma and peripheral blood mononuclear cells (PBMCs) were preserved at −70 °C. DNA was extracted from frozen PBMC samples with QIAamp DNA mini kit (Qiagen). RNA was extracted from frozen plasma samples with Qiagen Viral RNA mini kit (Qiagen).

2.6. PCR amplification and sequencing

We amplified four regions of HIV-1, i.e., the coding regions of protease (PR), reverse transcriptase (RT), and *env*. For each region, outer polymerase chain reaction (PCR) and inner PCR were performed. Outer PCR, using 0.2−0.3 μg of genomic DNA, was performed in a volume of 50 μl with 1x ExTaq buffer, 0.2 mM of each deoxyribonucleoside mixture, 0.5 μM of each primer, and 1.25 U of Ex-Taq (TaKaRa). Hot start was employed for outer PCR by incubating the reaction mixtures at 94 °C for 1 min, and then the mixtures were subjected to 30 to 40 amplification (94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s) followed by a final incubation at 72 °C for 7 min. Five μl of outer PCR product was used for inner PCR. Inner PCR was performed in a volume of 50 μl under the same conditions as outer PCR. Exceptionally, for not being amplified sufficiently, the third PCR was performed for PR region of 1−2 in patient 1. And for not being amplified in one

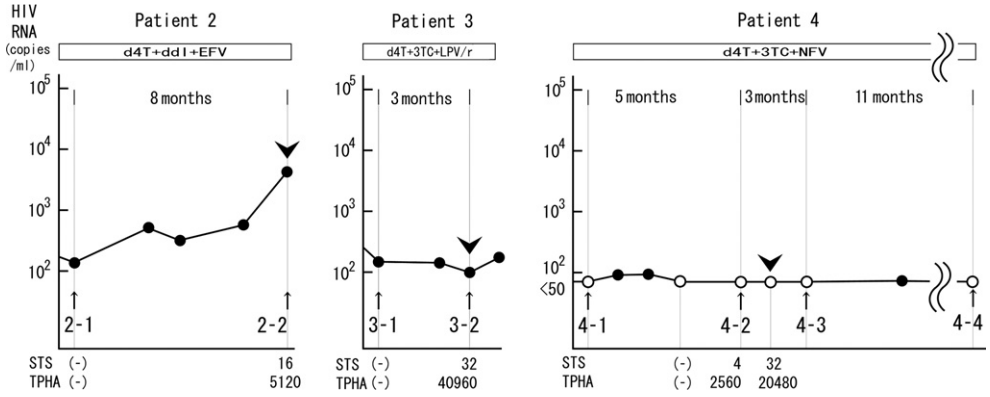

Fig. 2. Clinical course of patient 2, 3 and 4. The graph shows VL along the clinical course. Open points indicate undetectable viral load (<50 copies/ml). Names of antiretroviral drugs are shown in the bar on the top. d4T: stavudine, ddI: didanosine, EFV: efavirenz, 3TC: lamivudine, LPV/r: lopinavir/ritnavir, NFV: nelfinavir. Upward arrows indicate examined time points. A solid downward arrowhead indicates time points when the diagnosis of syphilis was made. STS: Serologic test for syphilis. TPHA: *Treponema pallidum* hemagglutionation test.

fragment, RT region of timepoints 1−2 and 1−4 in patient 1 were separately amplified in two fragments.

The primer sets used are as follows. Outer and inner primer pairs were used for the outer and inner PCR respectively.

2.7. The primer pairs for RT coding region

Outer primer pair: Forward (5′ATGATAGGGGGAAT TGGAGGTTT 3′; SF2 positions 2395−2417) Reverse (5′TACTTCTGTTAGTGCTTTGGTTCC 3′; SF2 positions 3432−3409).

Inner primer pair 1: Forward (5′GACCTACACCTGT CAACATAATTGG 3′; SF2 positions 2492−2516) Reverse (5′TAATCCCTGCATAAATCTGACTTGC 3′; SF2 positions 3379−3355).

Inner primer pair 2: Forward (5′GTACTTTAAATTT CCCCATTAGTCC 3′; SF2 positions 2543−2567) Reverse (5′CAGTCCAGCTGTCTTTTTCTGGC 3′; SF2 positions 3316−3294).

Inner primer pair 3: Forward (5′AACTCAAGACTTCTGG GAAGT 3′; SF2 positions 2805−2825) Reverse (5′TA ATCCCTGCATAAATCTGACTTGC 3′; SF2 positions 3379−3355).

Inner primer pair 4: Forward (5′GACCTACACCTGTCAA CATAATTGG 3′; SF2 positions 2492−2516) Reverse(5′TGG AATATTGCTGGTGATCC 3′; SF2 positions 3038−3019).

(Inner primer pair1 was used for 1−2 and 1−4 of patient 1, all time points of patients 3 and patient 4. Inner primer pairs 2 were used for patient 2. Internal primer pairs 3 and 4 were used for time points 1−2 and 1−4 of patient 1.)

2.8. Primer pairs for Protease coding region

Outer primer pair: Forward (5′AGACAGGYTAATTTTTT AGGGA 3′; SF2 positions 2081−2102) Reverse (5′TATG GATTTTCAGGCCCAATTTTTGA 3′; SF2 positions 2723−2698) (Y: T or C).

Inner primer pair: Forward (5′AGAGCCAACAGCCCC ACCAG 3′; SF2 positions 2155−2174) Reverse (5′ACTTTTG GGCCATCCATTCC 3′; SF2 positions 2618−2599).

3rd PCR primer pair: Forward (5′AGAGAGCTTC AGGTTTGGGG 3′; SF2 positions 2176−2195) Reverse (5′ACTTTTGGGCCATCCATTCC 3′; SF2 positions 2618−2599).

(3rd PCR was employed only for time point 1−2 of patient 1.)

2.9. Primer pairs for env

Outer primer pair: Forward (5′GAAAGAGCAGAAGA CAGTGG 3′; SF2 positions 6211−6230) Reverse (5′GCC CATAGTGCTTCCTGC 3′; SF2 positions 7822−7805).

Inner primer pair 1: Forward (5′GACCATGTACAAATG TCAGC 3′; SF2 positions 6951−6970) Reverse (5′TTCT CCAATTGTCCCTCATATCTCCTCCTCCA 3′; SF2 positions 7667−7636).

Inner primer pair 2: Forward (5′ACATGGAATTAGGCCA 3′; SF2 positions 6985−7000) Reverse (5′ATCTC TTGTTAATAGCAGCC 3′; SF2 positions 7594−7575).

Inner primer pair 3: Forward (5′ACATGGAATTAGGCCA 3′; SF2 positions 6985−7000) Reverse (5′TTCTCCAAT TGTCCCTCATATCTCCTCCTCCA 3′; SF2 positions 7667−7636).

2.10. Primer pairs for gag

Outer primer pair: Forward (5′CCAAATGAGAGAACC AAGG 3′; SF2 positions 1474−1492) Reverse (5′TCTTA CTTTGATAAAACCTCC 3′; SF2 positions 2430−2410).

inner primer pair 1: Forward (5′CCACCTATCCCAGT AGGAG 3′; SF2 positions 1556−1574) Reverse (5′GGTGG GGCTGTTGGCTC 3′; SF2 positions 2172−2156).

inner primer pair 2: Forward (5′CCACCTATCCCAGTA GGAG 3′; SF2 positions 1556−1574) Reverse (5′TTC CCTAAAAAATTAGCCTG 3′; SF2 positions 2103−2084).

PCR fragments were sequenced directly or sub-cloned into pGEM-T Easy vector (Promega) and sequenced bi-directionally. All the nucleotide sequences were determined by using Big dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) and an ABI-377 sequencer (Applied Biosystems).

The reaction solution consisted of 2 μl of Ready Reaction Premix, 1 μl of BigDye Sequencing Buffer, 0.5 μM of a primer, 2 to 4 μl of template DNA, filled with distilled water to 10 μl of final volume.

2.11. Phylogenetic analysis and evolutionary distance calculation

Alignments of the sequences and the phylogenetic reconstructions using the neighbor joining method with a maximum likelihood distance matrix were performed by CLUSTAL W program [11]. The final graphical output was created with the program TREEVIEW version 1.6.6 [12]. Consensus sequences for the analysis were extracted from Updated Proposal of Reference Sequences of HIV-1 Genetic Subtypes [13]. We also added some sequences determined as subtype B from previous study in our laboratory [14]. We used Stanford drug resostamce database (http://hivdb.stanford.edu/) for the definition of resistant mutations.

3. Results

3.1. Resistant mutations in patient 1

The initial genotypic assay of the plasma HIV-RNA was performed at time point 1−4, since a rebound of VL at the previous visit (time point 1−3) was recognized. The genotype revealed five nucleoside analogue reverse transcriptase inhibitor (NRTI)-associated mutations and four protease inhibitor (PI)-associated mutations.

Cloning and sequence analysis of proviral DNA at the baseline (time point 1−1) revealed a mixture of wild type and DRMs (Fig. 3). In RT region, 5 out of 16 clones (31%) at amino acid position 41 and all 16 clones at amino acid position 210 contained single point mutation, which confer DRMs known as M41L (31%) and L210W (100%), respectively. There were two nucleotide mutations at amino acid position 215 in all 16 clones, which would convert the amino acid at this position from the wild type Threonine (T) into Asparatic acid (D). This change (T215D) is known as ZDV sensitive but suggestive of back-mutation from T215Y [15,16]. In PR region, polymorphic change at amino acid position 63 (L63P) was detected [17]. At time point 1−2, when VL was still undetectable, populations of M41L elevated to 100% (20/20 clones). Third PCR was necessary to amplify the PR region at time point 1−2, presumably because the amount of proviral DNA in PBMC declined after one year of PART. However, a single point mutation which confers DRM at amino acid position 46 in PR region (M46I) were detected in 100% (13/13) of clones sequenced.

Proviral DNA contained nine DRMs at time point 1−3 when abrupt elevation of VL was first detected. Therefore, five DRMs (D67N, M184V, T215Y in RT and D30N, N88D in PR) had accumulated between time points 1−2 and 1−3. The great majority of proviral DNA sequences at time point 1−4 contained DRMs in accordance with the direct sequence data of plasma viruses described above. However, we could detect wild type sequences by cloning and sequencing at amino acid positions 67 (D), 184 (M) in RT and at amino acid positions 46 (M), 88 (N) in PR. Of note was the reemergence of T215D in 4 clones while ZDV-containing treatment was continued.

3.2. Analysis of env sequences in patient 1

To examine a possibility of superinfection by drug-resistant HIV-1 strain in patient 1, we compared nucleotide sequences of V3 region (105 bases) in *env* gene at four time points. 20 clones from time point 1−1, 15 clones from time point 1−2 and 17 clones from time point 1−3, and the direct sequence data from time point 1−4 were used. V3 sequences were most diverse at time point 1−1, in which we detected 8 different sequences among 20 clones. We detected 5 different sequences at time point 1−2. However, a clonal sequence (17/17) which was closely related to some sequences at time point 1−1 dominated at time point 1−3 (17/17) (Fig. 4).

3.3. Drug resistant mutations and env sequences in patient 2

Although PART with a combination of d4T/ddI/EFV was not completely suppressive in patient 2, his VL was always below before time-point 2−2. His VL rose to 4400 copies/ml at time point 2−2 coincidentally with syphilitic rash. We compared the nucleotide sequences of the proviral DNA looking for DRMs (Fig. 5). One non-nucleoside analogue reverse transcriptase inhibitor (NNRTI)-related mutation at amino acid 179 (V179D), was already observed at time point 2−1 when he was seronegative for syphilis. Although we could not detect any new DRMs at 2−2 by direct sequencing of the PCR product, cloning and sequencing revealed minor clones with DRMs at amino acid positions 106 (V106M) and 184 (M184V). It should be noted that wild type clones at amino acid 179 (V179) showed up at time point 2−2. Interestingly, V179D and M184V were not observed in the same clone.

Env V3 region sequences at time points 2−1 and 2−2 clustered together within a large cluster of many reference sequences of subtype B viruses (Fig. 4). There was no evidence of superinfection.

3.4. Drug resistant mutations and env sequences before and after syphilis co-infection in patient 3 and 4

VLs in patients 3 and 4 were well suppressed before and after syphilis co-infection (Fig. 2). We did not observe any drug-resistant mutations in RT at indicated time points (data not shown). Although we did not observe DRMs in PR in patient 4, amino acid substitution at position 36 (M36I) was observed in patient 3 both before and after syphilis co-infection (data not shown). V3 region sequences in patient 3 clustered together with reference sequences of AE recombinant viruses suggesting that the virus in this patient is AE recombinant in origin (3−1 and 3−2 in Fig. 4). Therefore, it is inferred that M36I in patient 3 was not a DRM but a polymorphism of

A. Nucleotide sequence of proviral DNA

		RT coding region							PR coding region				
Amino acid number		41	67		184	210	215		30	46	63	88	
Wt nucleotide		ATG	GAC		ATG	TTG	ACC		GAT	ATG	CTC	AAT	
Time point													
1-1	Nucleotide	...	...	11/16*	...	.G.	GA.	11/16*	...	...	.C.	...	16/16
	Nucleotide	C..	...	5/16*									
1-2	Nucleotide	C..	...	20/20	...	.G.	GA.	19/20	...	..A	.C.	...	13/13
	Nucleotide				.C.	.G.	GA.	1/20					
1-3	Nucleotide	C..	A..	20/20*	G..	.G.	TA.	20/20*	A..	..A	.C.	G..	20/20
1-4	Nucleotide	C..	A..	19/20	G..	.G.	TA.	15/20	A..	..A	.C.	G..	12/20
	Nucleotide	C..	...	1/20	...	.G.	GA.	4/20	A..	..A	.C.	...	2/20
	Nucleotide				...	.G.	TA.	1/20	...	..A	.C.	G..	1/20
	Nucleotide								A..	...	.C.	...	1/20
	Nucleotide								...	...	.C.	G..	1/20
	Nucleotide								...	...	.C.	...	3/20

B. Amino acid sequence of proviral DNA

		RT coding region							PR coding region				
Amino acid number		41	67		184	210	215		30	46	63	88	
Wt amino acid		M	D		M	L	T		D	M	L	N	
Time point													
1-1	Amino acid	-	-	11/16*	-	W	D	11/16*	-	-	P	-	16/16
	Amino acid	L	-	5/16*									
1-2	Amino acid	L	-	20/20	-	W	D	19/20	-	I	P	-	13/13
	Amino acid				T	W	D	1/20					
1-3	Amino acid	L	N	20/20*	V	W	Y	20/20*	N	I	P	D	20/20
1-4	Amino acid	L	N	19/20	V	W	Y	15/20	N	I	P	D	12/20
	Amino acid	L	-	1/20	-	W	D	4/20	N	I	P	-	2/20
	Amino acid				-	W	Y	1/20	-	I	P	D	1/20
	Amino acid								N	-	P	-	1/20
	Amino acid								-	-	P	D	1/20
	Amino acid								-	-	P	-	3/20

Fig. 3. Drug resistant mutations in patient 1. Wild type residues are shown on the top. Residues identical to the wild type are shown by dots (nucleotides) or dashes (amino acids). Squared capital letters indicate amino acid mutations related to the drug resistance. Fraction numbers are the number of subclones with the amino acid change indicated per total number analyzed subclones. Examined time points are shown on the left. RT at 1−2 and 1−4 were separately analyzed.

HIV-1 [18,19]. Since V3 sequences of patient 4 clustered together with reference sequences of subtype B viruses, it was likely that patient 4 was infected by a subtype B virus. From the serologic tests, it was likely that patient 4 had syphilis co-infection before time point 4−2 (Fig. 2). It is intriguing that a transient but substantial shift of V3 sequences was observed at time point 4−2 (Figs. 4 and 6). Since all V3 clones of patient 4 clustered compactly by themselves, however, it is unlikely that viruses at time point 4−2 were originated from superinfection.

4. Discussion

The coincidence of active syphilis and a break through of drug-resistant HIV-1 in a patient suggested us a possibility of superinfection at first. Therefore, we studied this case longitudinally using frozen samples. He was referred to our hospital after the initiation of PART. At time point 1−1, he was on his initial PART with ZDV, 3TC and NFV and VL was undetectable. However, the sequences of proviruses at time point 1−1 showed that a couple of thymidine analog-related mutations(TAMs) such as L210W and M41L had already been present in RT region. T215D was also present. T215D, referred to as a fossil mutation, is drug-sensitive but suggestive of a reversion from a drug resistant mutation T215Y [15,16]. L210W and M41L are TAMs that usually occur in the virus harboring T215Y [20]. Co-presence of L210W and M41L together with a fossil mutation T215D suggests that patient 1 had originally been infected by a ZDV-resistant virus, although we cannot rule out the possibility that these TAMs accumulated gradually while the patient had undetectable VL [21]. Proviruses at time point 1−2 contained increasing number of drug-resistant mutations while the patient's VL was still undetectable; all the 20 clones were now M41L in RT region, and M46I in PR region was also detected. The concomitant presence of M46I and L63P tends to lead the acquisition of D30N and N88D whose combination reduces susceptibility to NFV some 50 fold [22]. Actually both D30N and N88D were proven in patient 1 at time point 1−3 when the patient developed full-blown resistance. Analysis of serial V3 region sequences excluded the possibility of superinfection.

At time point 1−4 when VL went up higher, reversions towards the wild type residues were detected by cloning and sequencing. Reversions were observed in different classes of anti-retroviral drugs (ARVs) (thymidine and cytidine analogue-related mutations and protease inhibitor-related

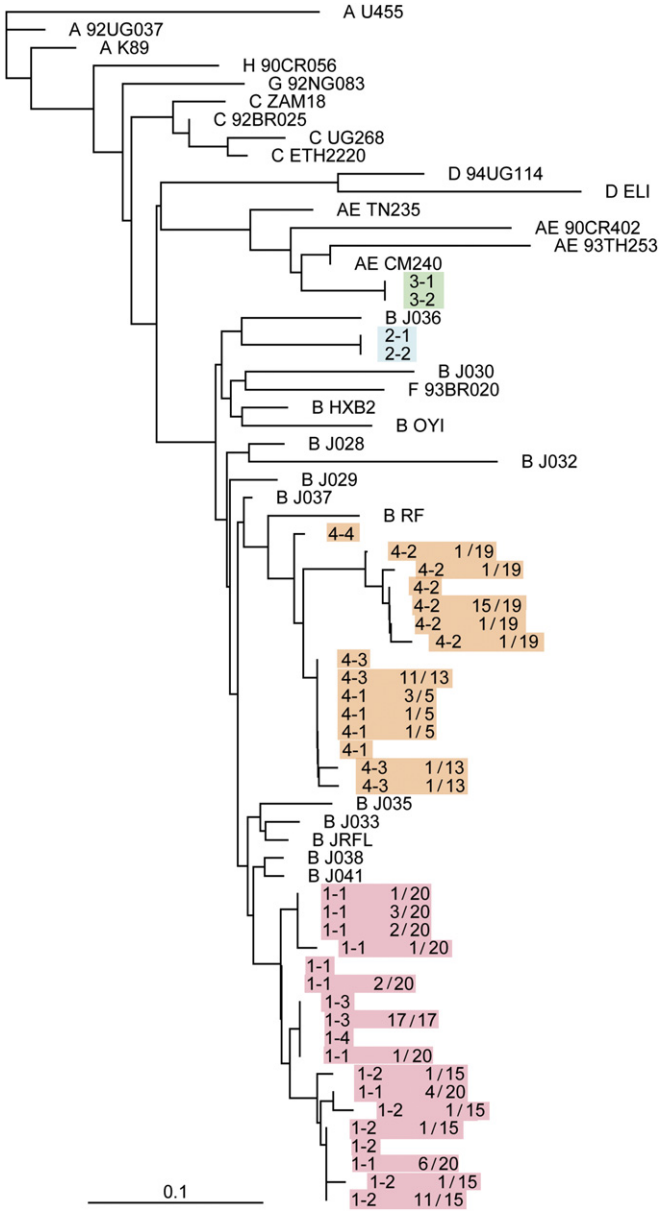

Fig. 4. Phylogenetic tree of V3 sequences in four patients. The phylogenetic tree was drawn according to the nucleotide sequences of *env* V3 regions (105 base). Time points with direct sequence indicate those derived from the direct sequencing. Time points with fraction numbers indicate the sequences from subclones per total numbers analyzed. The first or the first two capital letters of each consensus sequence indicates its subtype (e.g. B: subtype B, AE: AE recombinant).

mutations), which suggest general decrease of selective pressure by ARVs. Although he declared strict adherence to the treatment throughout the course, intended or unintended drug holidays were likely to be the cause of full-blown drug resistance mutations at time point 1−3 and subsequent appearance of revertants at time point 1−4. The importance of adherence to PART should not be underestimated.

The revertants at time point 1−4 could have occurred by simple back-mutation. However, it is noteworthy that the reversions were seen at only those mutations which occurred after time point 1−2 and not at mutations already present at time point 1−1. There could be a possibility that clones harboring wild type residues arose from latently infected reservoir cells. Although both simple back-mutation and reemergence of latent viruses could occur under the decreased selective pressure of ARVs, it was recently reported that back-mutation usually did not take place for more than a year among drug-resistant mutations transmitted at primary HIV-1 infection [23,24].

Patient 2 also obtained drug-resistant mutations around syphilis infection. One NNRTI- related mutation V179D was already present in 100% of the populations before syphilis infection, but another NNRTI-related mutation V106M emerged in 15% of the populations after syphilis infection. Similarly to time point 1−4 in patient 1, reversion of V179D to wild type residue was seen in about half of the populations after syphilis, suggesting a decreased selective pressure of ARVs in this case also.

Of interest was the emergence of M184V mutation, which is known to cause high level resistance to 3TC [25]. This patient was not taking 3TC during the examined period, although he had previously been treated with dual nucleoside therapy with ZDV and 3TC. Among the ARVs he took during the period, ddI may be a responsible drug for M184V mutation, although M184V's selective advantage over ddI is much lower

A. Nucleotide sequence of proviral DNA

Amino acid position	106	179	184	
Wt nucleotide	GTA	GTT	ATG	
Time point				
2-1				
Nucleotide	...	.A.	...	20/20
2-2				
Nucleotide	...	.A.	...	6/20
Nucleotide	A.G	.A.	...	2/20
Nucleotide	A.G	...	G..	1/20
Nucleotide	...	...	G..	4/20
Nucleotide	...	...	...	7/20

B. Amino acid sequence of proviral DNA

Amino acid position	106	179	184	
Wt amino acid	V	V	M	
Time point				
2-1				
Amino acid	-	[D]	-	20/20
2-2				
Amino acid	-	[D]	-	6/20
Amino acid	[M]	[D]	-	2/20
Amino acid	[M]	-	[V]	1/20
Amino acid	-	-	[V]	4/20
Amino acid	-	-	-	7/20

Fig. 5. Drug resistant mutations in patient 2. Wild type residues are shown on the top. Residues identical to the wild type are shown by dots (nucleotides) or dashes (amino acids). Squared capital letters indicate amino acid mutations related to the drug resistance. Fraction numbers are the number of subclones with the amino acid change indicated per total number analyzed subclones. Examined time points are shown on the left.

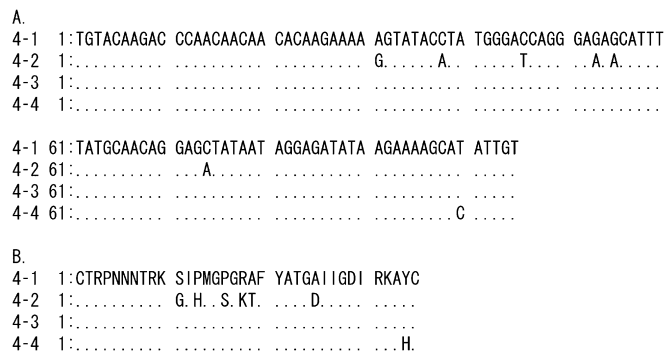

Fig. 6. Nucleotide (a); and amino acid (b) change in *env* V3 region in patient 4.

than over 3TC (1.4 fold versus more than 200 fold) [26]. Furthermore none of the clones containing M184V had V179D which was observed in all the clones at time point 2−1. Thus, it is likely that the mutants harboring M184V were derived from latently infected reservoir cells which had been established while the patient had taken 3TC without NNRTI. Syphilis infection may have predisposed the reactivation of these proviruses [10,27].

In both patients 1 and 2, decreased selective pressure appeared to be the principal driving factor for the emergence of drug-resistant mutants and the concomitant appearance of revertants and/or latently infected viruses. Our results may suggest that deteriorated adherence to ARV and committing unprotected sexual intercourse may be linked at least in some patients. Clinicians taking care of patients on ARV should pay special attention on ARV adherence when their patients get new STI.

It was recently reported that syphilis infection increased HIV-1 VL [10]. This increase was reported to be more prominent for patients without PART than patients under PART. Among the four cases under PART that we examined here, VL increased more than one log after syphilis co-infection in two patients. Both of them had already some DRMs before syphilis co-infection, and accumulated further DRMs after syphilis co-infection. The other two patients without any DRMs did not show significant changes in VL. It is possible that syphilis co-infection had some effect on the increase of VL for the former two cases that had some DRMs at baseline. However, as discussed above, a decrease of selective pressure was more probable cause of VL increase in these patients.

In patients 3 and 4, we could not detect any DRMs before and after syphilis co-infection, but longitudinal analysis of provirus sequences in patient 4 revealed a transient dynamic shift of env V3 region just after syphilis co-infection (time point 4−2). This transient shift of V3 sequences might be also attributable to the reactivation of latent proviruses in reservoir cells. However, the sequence shift was only seen in *env* V3 region, and no remarkable nucleotide changes were observed in *gag* (data not shown) or *pol* sequences. The reason for the dynamic change only in V3 region waits for further explanations.

In conclusion, we examined longitudinally the changes of provirus sequences of four patients under PART before and after syphilis co-infection. Two patients who had already some DRMs at baseline revealed VL increase and further accumulation of mutations around syphilis co-infection, whereas the other two that were devoid of any DRMs did not show VL increase or mutation accumulation. Reactivation of latent proviruses was suspected in some cases, but no evidence of superinfection was observed in any patient. HIV-1 superinfection appears to be less common than presumed before [7,8]. However, the prevalence of syphilis co-infection among HIV-1-infected patients indicates a spread of unprotected sexual behavior which may lead to HIV-1 superinfection as well as syphilis and other STIs. The importance of practicing safer sex should be underscored. Since it is most likely that the accumulation of DRMs in our cases was due to loosened adherence to ARVs, the importance of strict adherence should be emphasized.

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