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RANTES –28G Delays and *DC-SIGN* –139C Enhances AIDS Progression in HIV Type 1-Infected Japanese Hemophiliacs

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ABSTRACT

The relationships between host immune factors and HIV-1 disease progression are still in dispute. Unlike CCR5Δ32, which has been found to delay disease progression of HIV-1, there still remain several factors whose effect on the clinical course is unconfirmed. To clarify the relationships, we selected seven single-nucleotide polymorphisms (SNPs) out of the previously reported factors, namely, *RANTES* promoter −28G/−403A, *RANTES* In1.1C, *SDF-1* 3′A, *IL-4* promoter −589T, and *DC-SIGN* promoter −139C/−336C, and examined these in Japanese HIV-1-infected hemophiliacs ($n = 102$). The genotypes were examined by the direct sequencing method, and the distributions of genotype and allelic frequencies were compared between two groups, slow progressors ($n = 54$) who did not develop AIDS more than 10 years after intravenous infection and others (progressors) ($n = 48$). The allelic frequency of *RANTES* −28G was significantly higher in slow progressors (0.185) than in the progressor group (0.074) [$p = 0.023$, OR = 0.35, 95% CI (0.142, 0.880)]. *DC-SIGN* promoter −139C appeared in progressors with significantly higher allelic frequency (0.333) than slow progressors [0.204, $p = 0.040$, OR = 1.95, 95% CI (1.039, 3.677)]. With *RANTES* −403A, *RANTES* In1.1C, *SDF-1* 3′A, *IL-4* −589T, and *DC-SIGN* −336C, no significant difference was observed in allelic frequencies between the two groups. These results suggest that *RANTES* −28G was associated with delayed AIDS progression, while *DC-SIGN* −139C was associated with accelerated AIDS progression in HIV-1-infected Japanese hemophiliacs.

INTRODUCTION

THE INFLUENCE OF HOST IMMUNE FACTOR POLYMORPHISMS on AIDS progression has continuously been discussed. Previous studies, including multicenter meta-analyses, have almost concluded that CCR5Δ32[1–4] is related to delayed AIDS progression. These studies have mainly focused on sexually transmitted populations and intravenous drug users (IDUs) among whites, though some cohorts included hemophiliacs.

For years, factors other than CCR5, such as CCR2, SDF1, RANTES, and interleukin-4 (IL-4), have been studied. However, none of them has led to a definitive conclusion as to whether they delay AIDS progression in HIV-1-infected individuals or not. It is of note that most of the recent reports[4–8] have analyzed sexually transmitted individuals or IDUs, and, more importantly, most of the studied populations were whites.

In contrast to those subjects, HIV-1-infected hemophiliacs in Japan form a rather homogeneous population. Historically, Japanese hemophiliacs were thought to be infected with HIV-1 between 1982 and 1985 through contaminated blood coagulant, which means the time and mode of infection were virtually identical. We previously reported the relationships between CCR5 promoter polymorphism and the clinical courses in HIV-1-infected Japanese hemophiliacs.[9] In the current study, we tried to clarify further the impact of host immune factor single nucleotide polymorphisms (SNPs) on HIV-1 disease progression in the same population. The SNP sites analyzed were *RANTES* (*CCL5*) promoter −28/−403, *RANTES* intron 1.1

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(*RANTES* In1.1), SDF1 (*CXCL12*) 3′ untranslated region (UTR) position −801, *IL-4* promoter −589, and *DC-SIGN* (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin, also known as *CD209*) promoter −139/−336.

RANTES, the most potent ligand for CCR5, can compete the entry of and thus suppress replication of HIV-1 R5 (macrophage–tropic) strains, which use CCR5 as coreceptor.[10,11] The polymorphism in the promoter region, −28G, was reported to be associated with slower AIDS progression in Japanese HIV-1-infected cases,[12] though it was not supported by studies in other races.[13,14] *RANTES* −403A was initially reported to retard AIDS progression in HIV-1-infected European-Americans.[8] However, it was also reported to increase the rate of HIV-1 disease progression in cooperation with *RANTES* In1.1C, which is in strong linkage disequilibrium with *RANTES* −403A.[13]

SDF-1, the only natural ligand for CXCR4, can prevent T-lymphocyte infection with X4 (T cell tropic) strains of HIV-1, which use CXCR4 as coreceptor, through its direct blockade effect[15,16] and following CXCR4 downregulation.[17–19] The influence of *SDF1* 3′-UTR position −801A (*SDF1* 3′A) on AIDS progression is still in dispute.

IL-4 −589T was once reported to lower viral load and slow the rate of AIDS progression in whites,[7,20] however, this was not supported by other studies.[21,22] Further analyses are needed to confirm this issue.

The SNPs in the *DC-SIGN* promoter region have been reported to affect infectivity of HIV-1,[23] and recently that of *Mycobacterium tuberculosis*[24] and the severity of Dengue disease[25] as well. As for HIV-1 infection, a relationship between *DC-SIGN* −336C and acceleration of the primary parenteral infection has been reported.[23] Since dendritic cells play an important role not only at the initial phase of mucosal infection but in later expansion and reservoir function, we also evaluated whether *DC-SIGN* promoter −139C and −336C, two of the recently identified SNPs, could affect AIDS progression.

MATERIALS AND METHODS

Subjects

Cryopreserved peripheral blood mononuclear cells (PBMCs) collected from 104 HIV-1-positive Japanese hemophiliacs were used. These patients were presumed to be infected with HIV-1 virtually at the same period, between 1982 and 1985, through contaminated unheated blood products, and were enrolled in the study and followed up until 1996 by the Research Committee on Prevention of Developing Illness and Therapy for HIV-1-infected Patients in Japan.[9] The samples analyzed in this study did not overlap those in the previous report.[12]

All the patients were evaluated for their clinical stages according to 1987 CDC criteria.[26] They were divided into two groups: one consisted of 55 patients who did not proceed to AIDS without any treatment until the year 1994 (designated as "slow progressors" and the other included 49 patients who developed AIDS by 1994 ("progressors"). In this context, "slow progressors" were defined as those who did not progress to AIDS 10 years after HIV-1 infection, and "progressors" were defined as those who progressed AIDS within 10 years. None of the patients had been treated with antiretroviral drugs or other drugs, such as interferon-α, glycyrrhizin, or organic germanium compound.

Polymerase chain reaction and sequencing

In the current study, we examined seven SNPs, such as *RANTES* promoter −28G/−403A, *RANTES* In1.1C, *SDF-1* 3′A, *IL-4* promoter −589T, and *DC-SIGN* promoter −139C/ −336C. To genotype the SNPs, we extracted genomic DNA from the cryopreserved PBMC using a DNA extraction kit (Qiagen, Hilden, Germany), amplified the target DNA by polymerase chain reaction (PCR), and did direct sequencing.

For the analysis of *RANTES* promoters, we amplified the target DNA by PCR with primers RA1 (5′-AGAAGGCCTTACAGTGAGA-3′) and RA3 (5′-GCGCAGAGGGCAGTAGCAA-3′).[12] Amplification was done with one cycle of 94°C for 10 min and 35 cycles of 94°C for 30 sec, 49.2°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 10 min. For *RANTES* In1.1, we used 5′-CCTGGTCTTGACCACCACA-3′ and 5′-GCTGACAGGCATGAGTCAGA as forward and reverse primers, respectively.[13] Amplification was done with one cycle of 94°C for 10 min and 35 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 10 min. For *SDF1*, 5′-CAGTCAACCTGGGCAAAGCC-3′ was used as the forward primer and 5′-AGCTTTGGTCCTGAGAGTCC-3′ as the reverse primer.[5] Amplification was done with one cycle of 95°C for 10 min and 35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 10 min. For the *IL-4* promoter 4-1 (5′-GAATTCAATAAAAAACAA-3′) was used as the forward primer and 4-1190 (5′-GAAACAGAGGGGGAAGCA-3′) as the reverse primer.[27] Amplification was done with one cycle of 94°C for 10 min and 35 cycles of 94°C for 30 sec, 49.2°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 10 min. For *DC-SIGN* promoters, we designed a new primer set, PromF 5′-ACCTGACTACCCTAGGCATT-3′ (nt position −499 to −480) and PromR 5′-GGCCACAGCTTTTATTTCCC-3′ (nt position −38 to −57), and used them as forward and reverse primers, respectively. Amplification was done with one cycle of 94°C for 10 min and 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 10 min.

PCR was performed with an AmpliTaq Gold PCR kit (Applied Biosystems Japan, Tokyo, Japan) according to the manufacturer instruction. All the amplified products were purified with Montage PCR (Millipore Co., Bedford, MA) and then sequenced by the dye terminator method using BigDye v1.1 and ABI 310 (Applied Biosystems Japan, Tokyo, Japan) according to the manufacturer's instructions. All the sequence reactions except *RANTES* and *IL-4* were done with diluted PCR primers. For the sequencing of *RANTES* −403, RA2F (5′-ACTGATGAGCTCACTCTAGATG-3′)[12] was used as a primer. For the sequencing of the *IL-4* promoter, we designed another set of primers, 5′-GCCAAGGGCTTCCTTATGGGTAA-3′ (nt position −700 to −678) as forward primer and 5′-AATGCAGTCCTCCTGGGGAAAG-3′ (nt position −402 to −423) as reverse one.

Sample analysis

Genotypic distribution and allelic frequency of the SNPs were compared between the two groups, slow progressors and

progressors, with the c2 test or Fischer's exact test. A *p* value less than 0.05 was considered to be statistically significant. To confirm the associations between the SNPs and disease progression, odds ratios and 95% confidence intervals were further calculated by using unconditional logistic regression (SPSS 14.0J Regression Models).

RANTES haploypes were analyzed by an Expectation-Maximization algorithm utilizing Arlequin ver.3.01 (Genetica and Biometry Laboratory, Geneva, Switzerland).

RESULTS

The genotypic distribution and allelic frequency were analyzed for the SNPs, such as *RANTES* promoters −28G/−403A, *RANTES* Inl.1C, *SDF-1* 3′A, *IL-4* −589T, and *DC-SIGN* promoters −139C/−336C, and compared between slow progressors and progressors in Japanese HIV-1-infected hemophiliacs. These results are shown in Tables 1 and 2. As for the SNPs in the *RANTES* promoter and intron, haplotype analysis (−403/−28/In1.1) was also done (Table 3). All the genotypes were in Hardy–Weinberg equilibrium.

Genotype distribution analysis

In the genotypic analysis of *RANTES* −28, there was a weak tendency that C/G and G/G genotypes were more frequent in slow progressors ($p = 0.08$). Besides, the G/G genotype was detected only in slow progressors, though the number was small (three cases).

DC-SIGN −139 T/C and C/C genotypes appeared more frequently in progressors, though the difference was not significant ($p = 0.10$). As for *DC-SIGN* −336, the C/C genotype was not detected in the current study.

RANTES −403, *RANTES* In1.1, *SDF-1* 3′A −801, and *IL-4* −589 showed no significant difference in genotype distribution between the two groups.

Allelic frequency analysis

RANTES promoters. The allelic frequency of *RANTES* −28G was 0.185 in slow progressors. It was significantly higher compared with that of progressors (0.074) [$p = 0.023$, OR = 0.35, 95% CI (0.142, 0.880)]. The allelic frequency of *RANTES* −403A was also higher in slow progressors (0.343) than in progressors (0.271). There was, however, no statistically significant difference [$p = 0.29$, OR = 0.71, 95% CI (0.391, 1.230)].

RANTES In1.1. The allelic frequency of *RANTES* In1.1C was 0.330 in slow progressors. It was higher than that of progressors (0.245), though this difference was not significant [$p = 0.21$, OR = 0.66, 95% CI (0.353, 1.222)].

RANTES haplotype. Four haplotypes, I (ACC), II (ACT), III (AGC), and IV (GCT), were detected in Japanese hemophiliacs (Table 3), and their frequencies were compared between the progressor and slow progressor groups. Haplotype III (A/G/C at RANTES −403/−28/In1.1, respectively) was higher in slow progressors (0.186) than in progressors (0.065), though the difference was not statistically significant ($p = 0.052$).

SDF1 3′-UTR. As for the allelic frequency of *SDF-1* 3′A, there was no significant difference between slow progressors

TABLE 1. ASSOCIATION BETWEEN GENOTYPES OF HOST IMMUNE FACTORS AND CLINICAL OUTCOMES OF HIV-1-INFECTED JAPANESE HEMOPHILIACS

Polymorphism		*Genotype distribution (cases)*		p *value (Fisher's exact test)*
		Slow progressors	*Progressors*	
RANTES -28	C/C	37	40	
(n = 101)	C/G	14	7	0.08
	G/G	3	0	
RANTES -403	G/G	24	25	
(n = 101)	G/A	23	20	0.48
	A/A	7	3	
RANTES In1.1	T/T	24	25	
(n = 100)	T/C	23	21	0.21
	C/C	6	1	
SDF1 -801	G/G	24	21	
(n = 102)	G/A	24	23	0.96
	A/A	6	4	
IL-4 -589	C/C	5	5	
(n = 100)	C/T	23	22	0.84
	T/T	25	20	
DC-SIGN -139	C/C	3	5	
(n = 102)	C/T	16	22	0.10
	T/T	35	21	
DC-SIGN -336	C/C	0	0	
(n = 101)	C/T	4	4	1.00
	T/T	50	43	

TABLE 2. ASSOCIATION BETWEEN ALLELIC FREQUENCIES OF HOST IMMUNE FACTORS AND CLINICAL OUTCOMES OF HIV-1-INFECTED JAPANESE HEMOPHILIACS

Polymorphism		*Allelic frequency*		*p value*	*Odds ratio (95% CI*
		Slow progressors	*Progressors*		
RANTES -28	C	0.815	0.926		0.35
(n = 101)				0.023	
	G	0.185	0.074		(0.142, 0.88)
RANTES -403	G	0.657	0.729		0.71
(n = 102)				0.29	
	A	0.343	0.271		(0.391, 1.230)
RANTES In1.1	T	0.670	0.755		0.66
(n = 100)				0.21	
	C	0.330	0.245		(0.353, 1.222)
SDF1 -801	G	0.667	0.677		0.95
(n = 102)				0.88	
	A	0.333	0.323		(0.531, 1.713)
IL-4 -589	C	0.311	0.340		0.88
(n = 100)				0.76	
	T	0.689	0.660		(0.484, 1.584)
DC-SIGN -139	C	0.204	0.333		1.95
(n = 102)				0.40	
	T	0.796	0.667		(1.039, 3.677)
DC-SIGN -336	C	0.037	0.043		1.16
(n = 101)				1.00[a]	
	T	0.963	0.957		(0.281, 4.754)

[a]Fisher's exact test.

(0.333) and progressors (0.323) [$p = 0.88$, OR = 0.95, 95% CI (0.531, 1.713)].

IL-4 promoter. The allelic frequency of *IL-4* −589T was 0.689 in slow progressors and 0.660 in progressors. There was no significant difference between the two groups [$p = 0.76$, OR = 0.88, 95% CI (0.484, 1.584)].

DC-SIGN promoters. The allelic frequency of the *DC-SIGN* promoter −139C was 0.333 in progressors. It was significantly higher than that of slow progressors [0.204, $p = 0.040$, OR = 1.95, 95% CI (1.039, 3.677)]. The allelic frequency of the *DC-SIGN* promoter −336C was 0.037 in slow progressors and 0.043 in progressors, yielding no significant difference [$p = 1.00$, OR = 1.16, 95% CI (0.281, 4.754)].

To validate these univariate associations, we further analyzed all the SNPs chosen in a multivariate manner using an SPSS regression model. The same SNPs were found to be significantly and nonsignificantly associated with clinical outcomes of HIV-1-infected Japanese hemophiliacs (Tables 2 and 4).

DISCUSSION

In the current study, *RANTES* −28G was found to be associated with delayed disease progression in HIV-1-infected Japanese hemophiliacs. This result supports the previous report, in which the −28G mutation increased RANTES expression and secretion, and thus was concluded to retard AIDS progression in Japanese HIV-1-infected individuals including hemo-

TABLE 3. RANTES HAPLOTYPE AND THEIR ASSOCIATIONS WITH CLINICAL OUTCOMES OF HIV-1-INFECTED JAPANESE HEMOPHILIACS

	Slow progressors		*Progressors*		*Overall*	
Haplotype -403/-28/In1.1	*No. of alleles*	*Frequency (%)*	*No. of alleles*	*Frequency (%)*	*No. of alleles*	*Frequency (%)*
I ACC	13	(12.7)	16	(17.4)	29	(15.0)
II ACT	1	(1.0)	2	(2.2)	3	(1.5)
III AGC	19	(18.6)	6	(6.5)	25	(12.9)
IV GCT	69	(67.7)	68	(73.9)	137	(70.6)
	102		92		194	

TABLE 4. ASSOCIATION BETWEEN ALLELES OF HOST IMMUNE FACTORS AND CLINICAL OUTCOMES OF HIV-1-INFECTED JAPANESE HEMOPHILIACS (N = 102)

Allele	*Progressor (cases)*	*Slow progressor (cases)*	*Odds ratio*	*95% CIs*[a]
RANTES -28				
Non-G	41	37	1.000	
G	7	17	0.185	(0.051, 0.635)
RANTES -403				
Non-G	3	7	1.000	
G	45	47	2.371	(0.485, 11.595)
RANTES In1.1				
Non-C	26	25	1.000	
C	22	29	1.489	(0.544, 4.079)
SDF1 -801				
Non-G	4	6	1.000	
G	44	48	2.049	(0.484, 8.682)
IL-4 -589				
Non-C	21	26	1.000	
C	27	28	1.109	(0.468, 2.630)
DC-SIGN -139				
Non-C	21	25	1.000	
C	27	19	3.793	(1.451, 9.916)
DC-SIGN -336				
Non-C	44	50	1.000	
C	4	4	0.488	(0.093, 2.560)

[a]CIs, confidence intervals.

philiacs.[12] While its delaying effect was evaluated only by the decreased CD4 depletion rate in the previous study, a direct relationship between the allelic frequency and clinical outcomes could be observed in the present study. Moreover, *RANTES* −28 G/G homozygotes were found only in the slow progressor group.

RANTES In1.1C was reported to contribute to the rapid progression of AIDS in European-Americans and particularly in African-Americans.[13] In our study, however, the SNP was not found to influence disease progression in Japanese hemophiliacs. Our haplotype analysis showed that *RANTES* −28G, which was found to have AIDS-delaying effect, was always accompanied by *RANTES* In1.1C. The frequency of haplotype III, which contains both mutant alleles −28G and In1.1C, was significantly higher in Japanese hemophiliacs (0.129) than those reported in European-Americans (0.025) and in African-Americans (0.002).[13] In Japanese HIV-1-infected hemophiliacs, therefore, the protective effect of *RANTES* −28G might exceed the detrimental effect of *RANTES* In1.1C.

SDF-1 3′A was first reported to be associated with delayed onset of AIDS,[28] which was followed by conflicting reports,[6,29–31] concluding that *SDF-1* 3′A does not retard HIV-1 disease progression, either early or late in the course of infection. Our result was concordant with those of the reports denying the association, though Modi *et al.*[32] recently reported its protective effect as haplotype.

The polymorphism, *IL-4* promoter region −589T, was reported to be associated with delayed disease progression in HIV-1-infected nonhemophiliac whites.[7] In contrast, *IL-4* −589T was also reported to be associated with X4 strain acquisition,[27] which could lead to AIDS progression. In the current study, no significant difference was observed in allelic frequency of *IL-4* −589T between slow progressors and progressors. These results may be due to the bilateral functions of IL-4 in HIV-1 infection; it may suppress the primary infection of HIV-1 by downregulation of CCR5[33] and promote coreceptor switch by upregulation of CXCR4 as well.[34]

DC-SIGN is known to bind to HIV-1 gp120 and enhance *in trans* infection of HIV-1 from dendritic cells to T cells.[35] Recently, it was reported that an SNP in the promoter region of *DC-SIGN*, −336C, was associated with increased susceptibility to HIV-1 parenteral infection and not to mucosal infection among European-Americans.[23] In our study, however, the influence of *DC-SIGN* −336C on disease progression to AIDS was not observed. The allelic frequency of −336C was too low for statistical evaluation in Japanese hemophiliacs. Unexpectedly, *DC-SIGN* −139C was found to be associated with accelerated AIDS progression in HIV-1-infected Japanese hemophiliacs. These results may be explained by the report[36] that the SNP is located in the vicinity of a candidate binding site of transcription factor AP-1 (activator protein-1) in the *DC-SIGN* promoter region. The nucleotide substitution near the transcription factor-binding site in the promoter region may increase *DC-SIGN* expression level, resulting in the acceleration of AIDS progression.

In conclusion, our results suggest that *RANTES* promoter −28G is associated with delayed AIDS progression and *DC-SIGN* promoter −139C with accelerated AIDS progression in HIV-1-infected Japanese hemophiliacs, while *SDF-1* 3′-UTR, *RANTES* −403A, *IL-4* −589T, and *DC-SIGN* −336C do not

influence clinical courses. Further analysis is needed, particularly concerning the relationship among *DC-SIGN* promoter SNPs, modified *DC-SIGN* expression level, and the clinical course of HIV-1 disease.

SEQUENCE DATA

GenBank accession numbers of the sequences reported in this study are as follows: *RANTES* −28 (rs2280788), *RANTES* −403 (rs2107538), *RANTES* In1.1 (rs2280789), *SDF-1* −801 (rs1801157), *IL-4* −589 (rs2243250), *DC-SIGN* −139 (rs2287886), and *DC-SIGN* −336 (rs4804803).

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Anti-Retroviral Drug Resistance-Associated Mutations Among Non-subtype B HIV-1-Infected Kenyan Children With Treatment Failure

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Recently increased availability of anti-retroviral therapy (ART) has mitigated HIV-1/AIDS prognoses especially in resource poor settings. The emergence of ART resistance-associated mutations from non-suppressive ART has been implicated as a major cause of ART failure. Reverse transcriptase inhibitor (RTI)-resistance mutations among 12 non-subtype B HIV-1-infected children with treatment failure were evaluated by genotypically analyzing HIV-1 strains isolated from plasma obtained between 2001 and 2004. A region of *pol-RT* gene was amplified and at least five clones per sample were analyzed. Phylogenetic analysis revealed HIV-1 subtype A1 (n = 7), subtype C (n = 1), subtype D (n = 3), and CRF02_AG (n = 1). Before treatment, 4 of 12 (33.3%) children had primary RTI-resistance mutations, K103N (n = 3, ages 5–7 years) and Y181C (n = 1, age 1 year). In one child, K103N was found as a minor population (1/5 clones) before treatment and became major (7/7 clones) 8 months after RTI treatment. In 7 of 12 children, M184V appeared with one thymidine-analogue-associated mutation (TAM) as the first mutation, while the remaining 5 children had only TAMs appearing either individually (n = 2), or as TAMs 1 (M41L, L210W, and T215Y) and 2 (D67N, K70R, and K219Q/E/R) appearing together (n = 3). These results suggest that "vertically transmitted" primary RTI-resistance mutations, K103N and Y181C, can persist over the years even in the absence of drug pressure and impact RTI treatment negatively, and that appearing patterns of RTI-resistance mutations among non-subtype B HIV-1-infected children could possibly be different from those reported in subtype B-infected children. ***J. Med. Virol. 79:865–872, 2007.*** © 2007 Wiley-Liss, Inc.

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KEY WORDS: vertical transmission; anti-HIV resistance patterns; persistence of mutations; Kenya

INTRODUCTION

The emergence of anti-retroviral drug (ARV)-resistance mutations is a major cause of anti-retroviral treatment (ART) failure [D'Aquila et al., 1995; Lorenzi et al., 1999; Zolopa et al., 1999]. These drug-resistant HIV-1 strains can be transmitted through vertical, sexual, and parenteral routes [Erice et al., 1993; Conlon et al., 1994; Boden et al., 1999; Little et al., 1999; Brenner et al., 2000; Pillay et al., 2000; Salomon et al., 2000; Duwe et al., 2001]. Vertically transmitted multidrug resistant HIV-1 strain has been shown to persist for 9 months in an infant after postnatal therapy [Johnson et al., 2001]. Similarly, K103N-containing HIV-1 variants acquired after the administration of single dose-nevirapine, a non-nucleoside reverse-transcriptase inhibitor (NNRTI), have been reported to persist for more than 1 year in some women and infants after vertical transmission [Flys et al., 2005]. However, long-term persistence of vertically

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transmitted ARV-resistance mutations in the absence of drug pressure among infants and children is yet to be demonstrated.

Recently, the importance of ARV-resistant strains detected as minor populations has been reported. Minor drug-resistant HIV-1 populations have been detected both in the early phase of treatment failure [Coffin, 1995] and during successful structured treatment interruption [Metzner et al., 2003]. Minor drug-resistant populations undetectable by conventional assays can eventually overgrow and affect the clinical course [Dykes et al., 2004; Lecossier et al., 2005]. These minor drug-resistant populations have also been found to persist longer than expected previously in untreated patients, a favorable condition for wild-type virus to overgrow, which also indicates the risk of resistance transmission even from minor strains [Charpentier et al., 2004].

In patients experiencing treatment failure with nucleoside reverse-transcriptase inhibitors (NRTI), such as lamivudine plus either zidovudine or stavudine, the M184V mutation has been reported to always appear first, eventually followed by cumulative acquisition of thymidine-analogue-associated mutations (TAMs) if treatment with non-suppressive regimen is continued [Johnson et al., 2005]. Extensive studies on ARV-resistance suggest that HIV-1 may develop TAMs by either one of two distinct pathways; TAM 1 (M41L, L210W, and T215Y) or TAM 2 (D67N, K70R, and K219Q/E/N/R) [Flandre et al., 2003; Cozzi-Lepri et al., 2005]. However, most of these studies have focused on HIV-1 subtype B, which accounts for only 12% of the global HIV/AIDS pandemic, and data on non-subtype B HIV-1 is still limited. Furthermore, several differences in the development of ARV-resistance between subtype B and non-subtype B HIV-1 have been suggested [Apetrei et al., 1998; Quinones-Mateu et al., 1998; Pieniazek et al., 2000]. Most ARV-resistance studies have focused on adult populations [Yerly et al., 1998; de Ronde et al., 2001; Dykes et al., 2001; Brenner et al., 2002; Wainberg, 2003]. However, these findings may not be applicable directly to children, since several factors influencing selection of ARV-resistance such as pharmacokinetic properties; drug safety, tolerance, and antiviral activity of combination therapy, are usually different in the children [Kline et al., 1996].

The aim of this study was to investigate the patterns of emergence and the variable stability of ARV-resistance-associated mutations among non-subtype B HIV-1-vertically-infected children who developed eventually clinical failure with subsequent ART.

METHODS

Study Population

The subjects in this study resided in children's home in Nairobi, which housed 95 HIV-1-infected children. These children were born to HIV-1-infected mothers who either died of, or were too debilitated by HIV/AIDS hence could not offer basic care to the children. Of 95 children 55 were on ART as of August 2004. The duration of ART varied among children (mean: 23.3 months, range: 5–46 months). Of 55 children on ART 12 (8 males and 4 females, mean age: 7.4 years) experienced treatment failure, characterized by an initial decrease in plasma viral load (to undetectable level in one child) after treatment initiation and subsequent increase in the viral load as treatment continued. Seven of the 12 children received single ART regimen only during the study period: 5 received zidovudine/lamivudine/nevirapine, 1 zidovudine/didanosine/efavirenz, and 1 zidovudine/lamivudine/efavirenz (Table I). On the other hand, the remaining five children received multiple ART regimen during the study period: two received zidovudine/lamivudine/efavirenz followed by zidovudine/didanosine/efavirenz, two zidovudine/lamivudine/nevirapine followed by didanosine/lamivudine/efavirenz, and one didanosine/lamivudine/abacavir followed by zidovudine/didanosine/efavirenz and later didanosine/stavudine/efavirenz (Table I). These 12 children were admitted into the home by their first birthday and their HIV-1 status was confirmed serologically at 18 months of age. None of these children had history of previous exposure to any ARV.

This study was approved by the Kenya Medical Research Institute's National Ethical Review Committee on behalf of the Kenyan Government and conducted according to the national and international regulations governing the use of human subjects in biomedical research. The study was conducted within the continuing anti-retroviral, medical and healthcare programs of the institution without additional demand for blood samples solely for research purposes.

$CD4^+$ Cell Counts and Plasma Viral Loads

$CD4^+$ T cell counts of peripheral blood were determined using the FACSCOUNT (Becton-Dickinson, Beiersdorf, Germany) and plasma HIV-1 RNA loads using the Amplicor HIV-1 Monitor kit version 1.5 (Roche Diagnostics, Alameda, CA) with detection limit of 400 copies/ml according to the manufacturer's instructions.

Extraction and Amplification of Plasma HIV-1 Viral RNA

HIV-1 RNA was extracted from 100 µl of plasma using SMITEST EX-R and D (Sumitomo Metal Industries, Tokyo, Japan) according to the manufacturer's instructions. A region of the *pol-RT* gene (corresponding to nt 2480–3180 of HIV-1_{HXB2}) was amplified by both one-step RT-PCR (Invitrogen, Carlsbad, CA) and nested PCR with primer pairs, RT18 (5′-GGAAACCAAAAATGATAGGGGGAATTGGAGG-3′) and KS104 (5′-TGACTTGCCCAATTTAGTTTTCCCACTAA-3′) in the first round, and KS101 (5-GTAGGACCTACACCTGTTCAACATAATTGGAAG-3) and KS102 (5′-CCCATCCAAAGAAATGGAGGAGGTTCTTTCTGATG-3′) in the second round [Ndembi et al., 2004; Songok et al.,

TABLE I. General Characteristics of Non-B Subtype HIV-1-Infected Study Children

Sample ID	Age* (years)/sex	HIV-1 subtype/CRF	Study point (month, year)	ART[a] (initiation time)	$CD4^+$ T cell count (/μl)	Plasma viral load (copies/ml)	NRTI[b]-resistance mutations	NNRTI[c]-resistance mutations
NYU30	11/F	A1	Jul '02	ZDV, 3TC, EFV (Jun '01)	456	<400		
			Mar '03		475	24,857		
			Jan '04	ZDV,DDI,EFV (May 03)	267	89,063	*D67N + K70R + K219Q*	*L100I*
NYU33	11/F	A1	Jul '02	ZDV, 3TC, EFV (Jun '01)	549	3,449		
			Mar '03	ZDV,DDI,EFV (Oct 01)	556	122,419	*K219Q*	*K101Q*
			Feb '04		690	6,457	*K219Q + D218E*	*K101Q*
NYU36	11/M	D	Oct '01	ddI,3TC,ABC (Apr '01)	309	114,754	*M184V + T215F*	
			May '02			880,405	*M184V + T215F*	*I178M*
			Aug '02	ZDV,DDI,EFV (Oct 01)	321	81,870	*M184V + T215F*	*G190A*
			Apr '03		279	607,224	*T215F*	*G190A*
			Feb '04	D4T,DDI,EFV (Nov 02)	458	393,420	*T215F*	*G190A*
NYU38	10/M	C	Mar '03	ZDV,3TC,NVP (Sep '02)	388	38,459	*D67N*	
			Dec '03		188	60,695	*D67N + K70R + L210W + K219E*	
			Feb '04	DDI,3TC,EFV (Mar 04)	157	38,211	*D67N + K70R + L210W + K219E*	
			Aug '04		149		*D67N + K70R* + L210W *+ D218E + K219E*	
NYU44	9/M	A1	Feb '02		208	1,017,931		*K103N*
			Mar '03	ZDV, DDI, EFV (May '02)	370	71,895	*D67N + K70R + T215F + K219Q*	*K103N + G190A*
			Dec '03		474	150,549	*D67N + K70R + T215F + K219Q + M41L + V75M*	*K103N + G190A*
NYU62	8/M	A1	Dec '01		589	239,644		
			Sep '02	ZDV, 3TC, NVP (Sep '02)	828	2,838	*D67N + K70R*	*G190A*
			Mar '03		568		*D67N + K70R + T215F + K219E*	G190A
			May '04			6,901	*D67N + K70R + T215F + K219E*	*G190A + Y181C*
NYU69	6/M	A1	Mar '03		192	227,176		*K103N*
			May '04	ZDV, 3TC, NVP (Mar '03)	400	113,868	*M184V*	*K103N*
NYU70	7/M	D	Sep '02		718	700,563		*K103N*
			Jun '03	ZDV, 3TC, NVP (Jul '03)	169	1,323,431		*K103N*
			Dec '03		502	188,059	*K70R + M184V*	*K103N*
NYU79	6/M	A1	Feb '03	ZDV,3TC,NVP (Apr '03)	70	159,826		
			Feb '04		551	244,506	*V75M + M184V*	*K101E + G190A*
			Jun '04	DdI,3TC,EFV (Mar 04)	347	472,203	*V75M + M184V*	*K101E + G190A + Y181C*
NYU83	5/M	A1	May '01		876	634,644		
			Jul '02	ZDV, 3TC, EFV (May '04)	946	50,570	*M184V*	*K103N*
			Apr '03		1138	74,437	*M184V*	*K103N*
			Aug '04		1125	197,301	*M184V + T215Y*	*K103N*
NYU85	5/F	CRF02_AG	Feb '03		178	30,690		
			Dec '03	ZDV, 3TC, NVP (Apr '03)	1214	3,264	*D67N + M184V*	*K103N*
			Apr '04		1148	79,080	*D67N + M184V*	*K103N*
NYU90	2/F	D	Apr '03		6	523,950		*Y181C*
			Jan '04	ZDV, 3TC, NVP (Apr '03)	399	55,679	*M184V*	
			Mar '04		379	155,191		*K103N*

*As of August 2004.
[a]ART, anti-retroviral therapy; ZDV, zidovudine; ddI, didanosine; EFV, efivarenz; NVP, nevirapine; 3TC, lamivudine; d4T, Stavudine.
[b]NRTI, nucleoside analogue RTI.
[c]NNRTI, non-nucleoside RTI; blank, no mutation detected.

2004]. Amplification was done with 1 cycle of 95°C for 10 min and 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, with a final extension of 72°C for 10 min. PCR amplification was confirmed by ethidium bromide staining of samples electrophoresed on an agarose gel.

Cloning, Sequencing, and Subtyping

The amplified products were cloned using the TOPO TA Cloning kit (Invitrogen) and sequenced as described previously [Ndembi et al., 2004; Songok et al., 2004]. The sample nucleotide sequences were aligned with HIV-1 subtype reference sequences from the Los Alamos database by CLUSTALW (version 1.81) with minor manual adjustments. Phylogenetic trees were constructed and visualized as described previously [Ndembi et al., 2004; Songok et al., 2004]. To improve the accuracy of HIV-1 subtyping, we used the genotyping tool (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi), and the REGA subtyping tool (http://dbpartners.stanford.edu/RegaSubtyping/) as needed.

RTI Resistance-Associated Mutations

The RT nucleotide sequences (697 bps) were translated into the corresponding 232 amino acids and analyzed for previously reported drug resistance-associated mutations in subtype B strains using the Stanford university HIVdb sequence analysis program. For each sample, at least five clones were obtained and genotyped to detect the presence of minor populations.

RESULTS

General characteristics, treatment history, demographic, immunological, and virological data of the 12 HIV-1-infected children studied are summarized in Table I.

HIV-1 Subtypes

All children were infected with non-subtype B HIV-1: subtype A1 (n = 7), subtype C (n = 1), subtype D (n = 3), and circulating recombinant form (CRF)-02_AG (n = 1) (Table I).

RTI Resistance-Associated Mutations Before Treatment

Of the 12 children, 4 (33.3%) harbored NNRTI-resistance mutations before treatment. Three children, NYU44 (age, 7 years), NYU69 (5 years), and NYU70 (6 years), had K103N while NYU90 (1 year) had Y181C detected before treatment (Table I). All the mutations but one (one of seven clones in NYU69) were detected as full clones (Table IV). K103N detected in three children persisted, while Y181C detected in one child disappeared during treatment.

Emerging Pattern of NRTI Resistance-Associated Mutations

The patterns of NRTI-resistance mutations are summarized in Table II. M184V appeared as the first

TABLE II. Patterns of NRTI*-Resistance Mutations in Non-B Subtype HIV-1-Infected Children With Treatment

Child (ID)	Study point (mpti[a])					Treatment
	1st	2nd	3rd	4th	5th	
NYU69	*M184V* (10)					ZDV/3TC
NYU90	*M184V* (9)					ZDV/3TC
NYU83	*M184V* (13)	*M184V* (22)	*M184V* + 1TAM[b] (38)			ZDV/3TC
NYU70	*M184V* + 1TAM (6)					ZDV/3TC
NYU85	*M184V* + 1 TAM (9)	*M184V* + 1TAM (12)				ZDV/3TC
NYU36	*M184V* + 1 TAM (6)	*M184V* + 1 TAM (13)	*M184V* + 1TAM (18)	1 TAM (24)	1 TAM (34)	DDI/3TC/ABC, ZDV/DDI, D4T/DDI
NYU62	2 TAMs (6)	4 TAMs (12)	4 TAMs (22)			ZDV/3TC
NYU44	4 TAMs (11)	5 TAMs + *V75M* (19)				ZDV/DDI
NYU33		1 TAM (23)	2 TAMs (34)			ZDV/3TC, ZDV/DDI
NYU30			3 TAMs (31)			ZDV/DDI
NYU38	1 TAM (8)	4 TAMs (15)	4 TAMs (17)	5 TAMs (23)		ZDV/3TC, DDI/3TC
NYU79	*M184V* + *V75M* (10)	*M184V* + *V75M* (13)				ZDV/3TC, DDI/3TC

*NRTI, nucleoside analogue RTI.
[a]mpti, months post treatment initiation.
[b]TAM, thymidine analogue-associated resistance mutation; blank, no mutation detected.

primary NRTI-resistance mutation in 3 of 12 children (NYU69, NYU90, and NYU83), (later followed by the acquisition of one TAM in NYU83), while M184V appeared as first primary NRTI-resistance mutation with one TAM in three children (NYU36, NYU70, and NYU85) who received zidovudine/lamivudine, zidovudine/didanosine, or lamivudine/didanosine. The remaining five children (NYU30, NYU33, NYU38, NYU44, and NYU62) had a mixture of TAMs appearing as first mutations. Three of them (NYU44, NYU62, and NYU38) had both TAM 1 (M41L, L210W, and T215Y) and TAM 2 (D67N, K70R, and K219Q) profiles detected together. M184V appeared as the first primary NRTI-resistance mutation together with V75M in child NYU79. NYU33 developed K219Q only, a "secondary" NRTI-resistance mutation.

Emerging Pattern of NNRTI Resistance-Associated Mutations

In four of the five children who received nevirapine (NYU69, NYU70, NYU85, NYU90) K103N appeared as the first primary NNRTI-resistance mutation, while in one (NYU62) G190A appeared as the first mutation (Table III). In two of the five children who received efavirenz (NYU44 and NYU 83) K103N appeared as the first NNRTI-resistance mutation, while in two children (NYU30 and NYU33) L100I and K101Q, respectively, appeared as the first NNRTI-resistance mutation. One child (NYU36) who received didanosine/lamivudine/abacavir with subsequent change to an efavirenz-containing regimen developed I178M as the first NNRTI-resistance mutation, which was replaced later by appearance of G190A.

One child (NYU79) developed K101E and G190A as first NNRTI-resistance mutations with nevirapine therapy and developed additionally Y181C when ART was changed to efavirenz-containing regimen during the study period.

In the remaining one child (NYU38) no known NNRTI-resistance mutation was detected despite receiving nevirapine—and later efavirenz-containing regimen (Table III).

Growth of Minor Mutant Virus Population into Major One

Five of 12 children had RTI-resistance mutations detected as minor virus populations, which subsequently grew into full clones (Table IV). In the remaining seven children no RTI-resistant mutation was detected as a minor population (data not shown).

RTI-resistance mutations, such as T215F in child NYU36, T215F in NYU44, D67N/K70R/T215F in NYU62, and K101Q/K219Q in NYU33, appeared as minor populations after initiation of treatment, which overgrew subsequently to major populations.

In one child (NYU69), K103N was found as a minor population (1/5 clones) before initiation of treatment and became major population (7/7 clones) 8 months after treatment.

TABLE III. Patterns of NNRTI[*]-Resistance Mutations Among Non-B Subtype HIV-1-Infected Children With Treatment

Child (ID)	Study point (mpti[a])						Treatment
	Pre-treatment	1st	2nd	3rd	4th	5th	
NYU69	*K103N* (−4)	*K103N* (10)					
NYU70	*K103N* (−10, −1)	*K103N* (11)					
NYU85		*K103N* (9)	*K103N* (12)				NEVIRAPINE
NYU62		*G190A* (6)	*G190A* (12)	*G190A* + *Y181C* (26)			
NYU90	*Y181C* (−0.25)		*K103N* (11)				
NYU38							
NYU83		*K103N* (12)	*K103N* (22)	*K103N* (38)			
NYU30				*L100I* (31)			
NYU44	*K103N* (−3)	*K103N* + *G190A* (10)	*K103N*+*G190A* (18)				
NYU33			*K101Q* (11)	*K101Q* (34)			EFAVIRENZ
NYU36			*I178M* (13)	*G190A* (16)	*G190A* (24)	*G190A* (34)	
NYU79		*K101E* + *G190A* (10)	*K101E* + *G190A* + *Y181C* (14)				
NYU38							

NNRTI: non-nucleoside analogue RTI.
[a]mpti, months post treatment initiation; blank, no mutation detected.

TABLE IV. Evolution of Minor RTI-Resistance Mutant Populations Among Non-B HIV-1-Infected Children With Treatment

Child ID	Study point (months post treatment)	ART[a]	Plasma viral load (copies/ml)	NRTI[b]-resistance mutations	NNRTI[c]-resistance mutations
NYU36	1st (6)		114,754	**T215F (1/9)**[d] + **M184V (6/8)**	
	2nd (13)	DDI, 3TC, ABC	880,405	**T215F (1/8)** + **M184V (2/8)**	I178M (6/8)
	3rd (18)	ZDV, DDI, EFV	81,870	**T215F (9/9)** + **M184V (8/9)**	G190A (8/9)
	4th (24)		607,224	**T215F (5/5)**	G190A (5/5)
	5th (34)	D4T, DDI, EFV	393,420	**T215F (7/7)**	G190A (7/7)
NYU44	Pre-treatment		1,017,931		K103N (5/5)
	1st (10)	ZDV, DDI, EFV	71,895	D67N (5/5) + K70R (5/5) + **T215F (1/5)** + **K219Q (5/5)**	K103N (5/5) + G190A (5/5)
	2nd (17)		150,549	D67N (5/5) + K70R (5/5) + **T215F (5/5)** + **K219Q (5/5)** + **M41L (1/5)** + V75M (3/5)	K103N (5/5) + G190A (5/5)
NYU62	Pre-treatment		239,644		
	1st (6)	ZDV, 3TC, NVP	2,838	**D67N (1/5)** + **K70R (1/5)**	G190A(5/5)
	2nd (12)			**D67N (5/5)** + **K70R (5/5)** + **T215F (2/5)** + K219E (5/5)	G190A (5/5)
	3rd (26)		6,901	**D67N (5/5)** + **K70R (5/5)** + **T215F (2/5)** + K219E (5/5)	Y181C (4/5) + G190A (5/5)
NYU69	Pre-treatment		227,176		**K103N (1/5)**
	1st (10)	ZDV, 3TC, NVP	113,868	M184V (7/7)	**K103N (7/7)**
NYU33	1st (15)		3,449		
	2nd (23)	ZDV, 3TC, EFV	122,419	**K219Q (4/11)**	**K101Q (6/11)**
	3rd (34)	ZDV, DDI, EFV	6,457	**K219Q (14/14)** + D218E (14/14)	**K101Q (14/14)**

[a]ART, anti-retroviral therapy; ZDV, zidovudine; ddI, didanosine; EFV, efivarenz; NVP, nevirapine; 3TC, lamivudine; d4T, Stavudine.
[b]NRTI, nucleoside analogue RTI.
[c]NNRTI: non-nucleoside RTI, blank: no mutation detected.
[d]Number of clones with mutation/ total number of clones analysed; bold, minor RTI-resistant mutant populations that evolved.

DISCUSSION

In the current study, NNRTI resistance-associated primary mutations, K103N and Y181C, were found before ART in four (33.3%) of 12 HIV-1-vertically-infected Kenyan children with subsequent ART failure. Three children aged 5–7 years already had K103N mutation, while one child aged 1 year already had Y181C by the time ART was started. These children had no history of previous exposure to any ART or blood transfusion, suggesting that these drug-resistance mutations were transmitted vertically from their mothers. However, ART history of these children's mothers could not be confirmed, and the use of nevirapine to reduce transmission of HIV-1 from mother to child had not been started by the year 2002 in Kenya [NASCOP, 2002].

This is the first report on the long-term persistence of NNRTI-resistance mutation for upto 7 years in vertically HIV-1-infected children albeit in the absence of ART. The K103N mutation has been reported to have little impact on the replicative capacity of HIV-1, allowing K103N variants to persist as dominant species at the expense of the wild strains [Brenner et al., 2002]. Thus, these current findings emphasize the need for drug-resistance testing among HIV-1-infected children prior to starting any NNRTI-containing regimen to avoid earlier treatment failure.

The selection of some ARV-resistance mutations among minor HIV-1 populations after ART initiation has been reported previously [Coffin, 1995; Metzner et al., 2003; Charpentier et al., 2004; Dykes et al., 2004; Lecossier et al., 2005]. In this study, RTI-resistance mutations detected in five children as minor populations after ART initiation subsequently grew into major populations, resulting in ART failure. In addition, it is noted that a primary NNRTI-resistance mutation, K103N, was found in one of five HIV-1 clones from a drug-naïve Kenyan child (NYU69), and this minor drug-resistant virus became dominant (seven of seven clones) after 8-months ART, resulting in treatment failure. These findings indicate that minor ARV-resistant HIV-1 variants existing before therapy can also be an important cause of treatment failure, as suggested previously [Dykes et al., 2004; Lecossier et al., 2005; Johnson et al., 2006]. Standard genotyping methods can only detect more than 25% of the virus variants [Gunthard et al., 1998]. Therefore, in order to pick minor variant populations and pre-empt treatment failure, more sensitive detection methods for minor HIV-1 populations would be required [Edelstein et al., 1998; Gunthard et al., 1998; Grant et al., 2002; Schuurman et al., 2002; Malet et al., 2003; Shi et al., 2004; Palmer et al., 2005].

Results from this study suggest the possible existence of two different patterns of emergence or acquisition of the TAMs among children who receive thymidine-analogues such as zidovudine, lamivudine, and/or stavudine. Seven of the 12 children had an initial development of M184V mutation, followed by the cumulative acquisition of TAMs, consistent with previous studies of subtype

B HIV-1 [Johnson et al., 2005], which reported that TAMs always develop by either one of two distinct pathways, TAM1 (M41L, L210W, and T215Y) or TAM 2 (D67N, K70R, and K219Q/E/R), under the pressure of thymidine analogue-containing ARVs. The remaining five children, however, developed TAMs only without the initial appearance of M184V mutation. Additionally, three of these children developed both TAMs 1 and 2 members concurrently, discordant with previous reports [Flandre et al., 2003; Cozzi-Lepri et al., 2005]. One child (NYU33) developed K219Q and K101Q mutations only, after 2-year treatment with zidovudine, didanosine, and efavirenz. These two mutations have been previously grouped among the secondary RTI-resistance-associated mutations, unable to cause drug-resistance in the absence of other primary RTI-resistance-associated mutations such as K70R or T215F [Garcia-Lerma, 2005]. These findings therefore suggest the possible existence of different pathways for development of RTI-resistance in non-subtype B HIV-1-infected children, different from those reported in subtype B-infected individuals, and that secondary RTI-resistance-associated mutations namely K219Q and K101Q could independently cause ART resistance among non-subtype B HIV-1-infected children. Further studies are however needed in order to confirm these findings.

The K103N mutation has been reported as the most commonly selected NNRTI-resistance-associated mutation, usually appearing first [Johnson et al., 2005]. The results from the children who received nevirapine in this study agree with this observation. However, the children who received efavirenz developed a variety of NNRTI-resistance-associated mutations, such as L100I, K101Q, I178M, and G190A. This is the first report to show the possibility of the K101Q and I178M to appear as the first NNRTI-resistance mutations with efavirenz therapy. L100I, Y181C, and G190A have already been described [Johnson et al., 2005]. In addition, one child (NYU38) who received nevirapine and later efavirenz containing regimen did not have any NNRTI-resistance-associated mutation despite experiencing treatment failure, suggesting a possible difference in the initial selection of NNRTI-resistant mutations between non-subtype B and subtype B HIV-1-Infected children. However, considering recent reports on the association between a homozygous variant of multidrug-resistance transporter *C3435T* and good immune recovery [Saitoh et al., 2005], and the correlation of homozygous *CYP2B6* *6 with plasma efavirenz concentrations in HIV-1-infected individuals treated with efavirenz-containing regimen [Tsuchiya et al., 2004], further pharmacogenetic studies would also be needed to elucidate these phenomenon.

In conclusion, this study suggests a possible long-term persistence of "vertically transmitted" NNRTI-resistance mutations in the absence of drug pressure, that minor populations of RTI-resistant HIV-1 mutants may impact negatively on the outcome of ART, and that there is a possible difference in the pattern of appearance and profile of RTI-resistance mutations between non-subtype B and subtype B HIV-1-infected children. Further studies with large population size are needed to confirm these findings.

SEQUENCE DATA

GenBank accession numbers of the sequences reported in this study are DQ679541 to DQ679753 for *Pol-RT*.

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特集 感染症の新しい検査法と最近のトピックス

II．各論

HIV検査

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Key Words
HIV
母子感染
スクリーニング検査
確認検査

要旨

HIV感染診断のスクリーニング法として，HIV抗原抗体同時検出キットが認可され，感染初期の診断に役立っている．また，迅速検査試薬の開発により，即日検査が可能となってきた．確認検査として，ウエスタンブロット法だけでなくRT-PCR法の併用も保険適用となっている．HIV母子感染の診断には，生後18カ月未満の小児では，感染母体由来の移行抗体が存在するためRT-PCRによるHIV RNAの検出が必要である．

はじめに

HIV（human immunodeficiency virus）感染者の総数は，2006年末現在で3,950万人であり，そのうち15歳未満の子どもは230万人と推定されている[1]．また，2006年にHIVに新たに感染した人は，全世界で430万人であり，エイズによる死亡者は推定290万人に上る．そのうち15歳未満の子どもは，それぞれ53万人と38万人と推定されている．小児のHIV感染症は，その80～90％が母子感染とされているが，現在のわが国では，母子感染予防の充実により，小児のHIV感染症はきわめて少ない．現在までに41人の母子感染例が報告されている[2]が，その多くは，分娩時の検査あるいは分娩後の児の異常から母のHIV感染が判明し，予防対策がなされていなかった症例である．

しかし，年間1,000人以上（2006年1,358人）の新規HIV感染者がみられ[3]，その数が年々増加傾向にあるわが国においては，今後HIV感染妊娠の増加が予測され，感染母体からの出生児に対する対応と，感染診断・評価は感染症を専門としない小児科医にも求められている．また，近年の若年者におけるHIV感染者の増加を考慮すると，一般臨床の場でも急性HIV感染症を疑い，感染の診断あるいは除外をすることが，今後必要とされると考えられる．

本稿では，一般的なHIV感染とHIV母子感染の診断法について概説するともに，最新のHIV検査法を含めた話題を紹介する．

HIV感染症の診断[4]

HIV感染症の診断には，血清中の抗HIV抗体やウイルス学的（HIV抗原や遺伝子）検査が行われる．まず粒子凝集（PA）法，酵素抗体（ELISA）法などの高感度のスクリーニング検査

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を行う．スクリーニング検査には偽陽性が認められるため，陽性の場合にはウエスタンブロット（以下，WBと略す）法と，HIV遺伝子検査による確認検査を行い，診断を確定する（図1）．

1．スクリーニング検査

1）抗体検査

現在9種類のHIVスクリーニング抗体検査キットが市販されている（表1）．酵素抗体法が5種類，凝集法が3種類，イムノクロマト法を用いた迅速検査キットが1種類である．酵素抗体法，凝集法を用いたスクリーニング検査は，高感度であるが非特異的反応がみられ，偽陽性が0.3％ほど認められるため，陽性の場合には確認検査が必要となる．また，イムノクロマトグラフ法を用いた15分で結果が得られる，簡易迅速抗体検査キットによるスクリーニング検査が開発され，一部の保健所や民間のクリニックで即日検査が可能となってきている．ただし，この場合偽陽性率は約1％である．スクリーニング検査陽性検体について，異なるスクリーニング検査キットを用いて追加検査を行うと，偽陽性を大幅に減少させることができる（図1）．

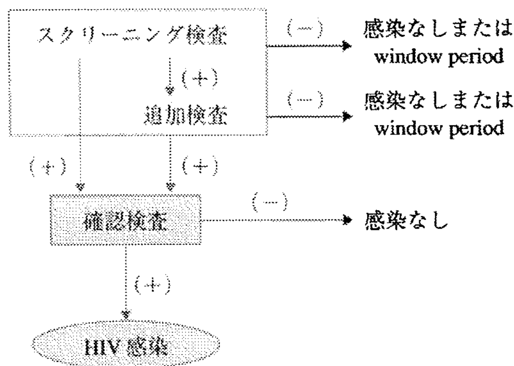

図1　HIV感染症診断の流れ

スクリーニング検査が陰性の場合，感染は否定できる．ただし，感染後，抗体が検出できない時期（window period）が数週間あること，また抗体産生が悪い個体が存在することも考慮する必要があり，検査の3カ月以内に感染リスク

表1　日本で使用されているHIV検査試薬

スクリーニング検査試薬

検　査	試薬名	販売会社	測定方法
抗体検査	ダイナスクリーン・HIV-1/2	アボットジャパン	イムノクロマト
	IMx HIV-1/HIV-2アッセイシステム	アボットジャパン	MEIA
	ジェンスクリーンHIV1/2	富士レビオ	ELISA
	ジェネディアHIV-1/2ミックスPA	富士レビオ	PA
	セロディア・HIV-1/2（HIV型別用）	富士レビオ	PA
	エンザイグノストanti-HIV1/2プラス	デイドベーリング	ELISA
	ルミパルスオーソHIV-1/2	オーソ・クリニカル・ダイアグノスティックス	CLEIA
	ビトロスHIV-1/2抗体	オーソ・クリニカル・ダイアグノスティックス	CLEIA
	ランリームHIV-1/2	シスメックス	ラテックス定量
抗原抗体同時検査	アキシムHIV Ag/Abコンボアッセイ・ダイナパック	アボットジャパン	MEIA
	ジェンスクリーンHIV Ag・Ab	富士レビオ	ELISA
	エンザイグノストHIVインテグラル	デイドベーリング	ELISA
	バイダスアッセイキットHIVデュオ	日本ビオメリュー	ELFA
抗原検査	ルミパルスI HIV-1p24（感染初期検出）	富士レビオ	CLEIA

確認検査試薬

検　査	試薬名	販売会社	測定方法
抗体検査	ラブブロット1	富士レビオ	WB
	ラブブロット2	富士レビオ	WB
	ペプチラブ1，2（HIV型別用）	富士レビオ	イムノブロット
遺伝子検査	アンプリコアHIV-1モニターVer. 1.5	ロシュ・ダイアグノスティックス	RT-PCR
	コバスアンプリコアHIV-1モニターVer. 1.5	ロシュ・ダイアグノスティックス	RT-PCR

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表2 HIV-1確認検査の判定と方針

WB法	RT-PCR法	判定
陽性	陽性	HIV-1感染者
	測定感度未満	HIV-1感染者 (高感度RT-PCR法による再検査 陰性であればHIV-2 WB法による確認検査)
保留	陽性	急性HIV-1感染者 (後日WB法による確認が必要)
	測定感度未満	2週間後に再検査
陰性	陽性	急性HIV-1感染者 (後日WB法による確認が必要)
	測定感度未満	2週間後に再検査

があった場合には，スクリーニング検査が陰性でも再検査をすすめることが基本である．

2）抗原抗体同時検査

抗HIV抗体と同時に血中のHIV抗原（HIV-1 p24抗原）を検出できる抗原抗体同時検出キットが認可され，window periodの短縮が可能となった．現在，酵素抗体法を用いた4種類のキットが市販されている（**表1**）．抗原抗体同時検査法では，抗体検出法に比べ5～7日ほど早期に陽性となるため，感染初期が強く疑われる場合には本検査法が推奨される．ただし，抗体を検出するWB法では，抗原のみ陽性例の確定診断が困難であり，確認検査には遺伝子検査（RT-PCR法など）が必要である．また，抗体についてはHIV-1/2に対応しているが，抗原は，HIV-1のみに対応しているので注意が必要である．

現在，抗原抗体同時検出が可能な迅速検査試薬（富士レビオ社）が日本で開発されている．本試薬は，HIV迅速検査試薬として高感度で，非常に特異性が高く（偽陽性率0.2％），感染早期のHIV検出が可能と報告[5]されており，早期の認可が期待される．

3）抗原検査

現在市販されているHIV p24抗原検査は他のウイルス学的検査法に比べて感度/特異度ともに劣るため，HIV感染診断には推奨されない．

2．確認検査

1）WBによる抗体確認試験

WB法は，HIV粒子を構成する全蛋白質がその分子量の順に並んでバンド上に結合されているニトロセルロース膜を用いて，各ウイルス構成蛋白質のおのおのに対する抗体の有無を調べる方法である．Env抗原（HIV-1:gp41，gp120，gp160，HIV-2:gp36/41，gp125，gp80，gp140）のバンドは，特異性が高く，診断的価値が高い．ただし，検出感度が低く，抗体検査のため感染初期には利用できない．WB法で陽性の場合には，HIV感染を確定できるが，判定保留の場合には，さらに遺伝子検査法などで感染初期か否かを確認する必要がある（**表2**）．また，国内のHIV-2感染者は非常にまれであるが，HIV-1 WB法で陰性/保留で疑わしい場合は，HIV-2 WB法の実施が必要となる．

2）遺伝子検査による確認検査

WB法の感度が低いこと，また陽性の場合治療方針の決定に有用であることから，確認検査にはWB法とRT-PCR法の併用が推奨されている（**表2**）．

①HIV-RNA検査（RT-PCR法）：現在市販されている遺伝子検査キットは，RT-PCR法と核酸ハイブリダイゼーション法を組み合せた方法により，HIV-1 RNAを高感度に検出することができる．血漿中のウイルス量定量によるHIV薬の効果・耐性の評価に有用で，患者の経過観察に用いられてきたが，抗原抗体同時検査法によ

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るスクリーニングが可能となり，確定診断用にも保険適用となった．測定感度は，高感度法で 50 copies/m*l*，標準法で 400 copies/m*l* である．ただし，これらの遺伝子検査キットの対象は HIV-1 のみで，HIV-2 は対象外である．

②HIV-DNA 検査（PCR 法）：PCR によるプロウイルス DNA を検出する方法で，HIV-RNA 検査より検出率が高いとされるが，検体としてリンパ球が必要となるため，日本ではほとんど普及していない．

③その他：われわれのグループは LAMP（Loop-Medicated Isothermal Amplification）法を用いた HIV-1 RNA の検出法を開発している．別項で述べられているように，LAMP 法は特異性が高く，一定温度（65℃付近）での反応で，短時間（15～45分）に，高度（10^9～10^{10}倍）の DNA の増幅が可能で，目視による判定も可能である．多様な HIV-1 が流行している中西部アフリカ（カメルーン，中央アフリカ）の HIV-1 感染者血漿を用いて検討したところ，グループ O を除くすべてのグループ M の検体で HIV-1 RNA の検出が可能であった（**図2**，論文投稿中）．検体からの HIV RNA 抽出法の更なる簡便化が必要であるが，感度（20 copies/反応，200 copies/m*l* に相当）もよく，一般検査室での HIV 感染確認検査試薬としての実用化をめざしている．

HIV 母子感染の診断

生後18カ月未満の小児では，感染母体由来の移行抗体が存在するため，HIV 感染の診断をするためには，直接的に HIV を検出するウイルス学的検査（RT-PCR による HIV RNA の検出，PCR によるプロウイルス DNA の検出，または培養によるウイルス分離）が必要である．生後18カ月以降の児では，HIV 感染の診断に抗体検査が使用でき，前述の基準に従い，感染の有無を診断できる．

1. 生後18カ月未満の小児の検査時期とその評価[2)6)]（図3）

HIV 感染母体から生れた児のウイルス学的検査は，生後48時間以内，14日，1～2カ月，3～6カ月の4時点で行うことがすすめられる．陽性であれば，ただちに新たな検体を用いて再検査し，陽性が確認されれば感染が確定する．陰性であれば，次の時点で再検査を行う．初回検査は生後48時間以内が望ましく，この時点で38%，生後14日で93%，1カ月までに96%以上で，さらに生後6カ月までに全例で感染の有無が確定できる．ウイルス量は生後2週間で急上昇することから，14日目の検査は早期診断に役立つ．早期診断により，早期の抗 HIV 薬併用療法やニューモシスティス肺炎予防などが可能

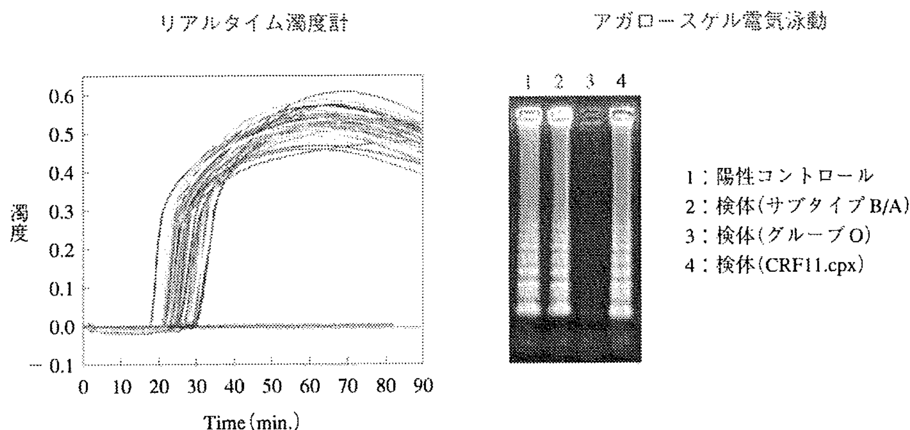

図2 LAMP 法による HIV-1 RNA の検出

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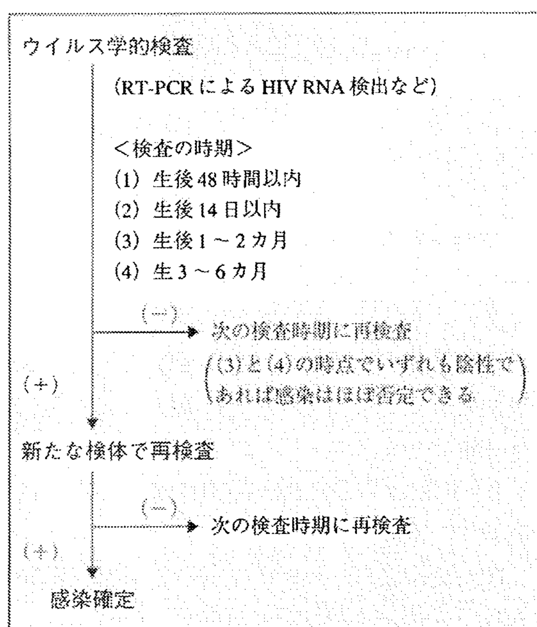

図3　HIV母子感染診断の流れ

となる．なお，生後48時間以内で陽性になった場合は，子宮内感染と考えられる．

2. 母子感染の否定

生後1カ月以降に行った2回以上のウイルス学的検査（RT-PCRによるHIV RNAの検出）が陰性であれば，感染はほぼ否定できる．なお，そのうち1回の検査は生後4カ月以降に行う必要がある．生後18カ月時に，低γ-グロブリン血漿がなく，HIV抗体陰性で，HIV感染の症候がなく，ウイルス学的検査も陰性であれば，感染は完全に否定できる．

その他の話題

1. HIV母子感染予防対策としての薬剤耐性検査

現在，わが国ではHIV母子感染予防対策として，母親への抗レトロウイルス薬（ARV）投与，予防帝王切開および断乳，児への予防的ARV投与が行われており，十分な予防効果をあげている（詳細は，「HIV母子感染予防対策マニュアル第4版（平成17年度，厚生労働省）」[2]を参照）．

しかしながら，世界的にARV抵抗性ウイルスの出現と蔓延が大きな問題となってきており，近い将来，日本でも薬剤耐性HIV感染妊娠の可能性が予想される．

また，ケニアでの自験例を含め[7]，薬剤耐性HIV-1株の母子感染例も報告されている．今後，児への予防的ARV投与を行う際に，分娩前の母親のHIV薬剤耐性関連遺伝子変異を調べ，有効なARVを選択することを考慮する必要があるかもしれない．

2. 郵送検査

現在インターネット上では，検査希望者が検査機関に行くことなしにHIV検査を受検することができる「HIV郵送検査」を取り扱うサイトが増えつつある．現在10社くらいが扱っているようである．須藤ら[8]の調査によると，2006年における年間郵送検査数は28,686件であり，スクリーニング検査陽性例も212例とされる．これは同年の保健所などHIV検査数（12万件）の約1/4に相当し，自発的にHIV検査を受けようとする人の中で，かなりの割合を占める．検査精度を含め，注意深く見守る必要がありそうである．

3. HIV-1/2関連検査の保険適用

HIV-1/2関連検査の診療報酬点数を表3に示

表3　HIV-1/2関連検査の保険適用

スクリーニング検査法	（診療報酬点数）
HIV-1抗体価	120点
HIV-1,2抗体価（抗原抗体同時検査を含む）	120点
確認検査法	
ウエスタンブロット法	
HIV-1抗体価精密測定	280点
HIV-2抗体価精密測定	370点
RT-PCR法	
HIV-1核酸増幅定量精密検査	510点
その他HIV関連検査法	
HIV-1核酸同定検査	430点
HIV-1抗原精密測定	600点

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