a family with partial androgen insensitivity syndrome can reduce the efficiency of protein translation (Choong *et al.*, 1996). Finally, it has been reported recently that a Graves'-disease–associated SNP that substitutes T for C at position –1 upstream from the open frame ATG codon of the CD40 gene enhances translation and could predispose to disease (Jacobson *et al.*, 2005). However, all these SNPs are located near the authentic translation initiation ATG codon of the proteins and directly affect the translation efficiency from the open frame ATG codon. In the case of the IL-7 gene, however, the mutation is located at –29 to –27 upstream from the open frame ATG codon, rather than near the authentic translation initiation codon. Nevertheless, it could up-regulate the IL-7 gene expression probably by changing the translation efficiency from the upstream, out-of-frame ATG codon. Our data showed that the consensus sequence for translation initiation is important, not only for the open-frame initiation ATG codon, but also for the upstream, out-of-frame ATG that is thought to reduce translation efficiency from the downstream initiation ATG. Similar to mutations within the consensus sequence of the open-frame initiation ATG codon, nucleotide substitution within the consensus sequence of the upstream, out-of-frame ATG can also modulate translation efficiency.

Our data also showed that the 1470-bp upstream non-coding region of the IL-7 gene exhibited extremely low levels of diversity in both Japanese and Thai populations. Also, no non-synonymous polymorphism has yet been identified in the IL-7 coding region. The reason for the low levels of diversity of the IL-7 gene is not clear at present, but is probably the result of its importance for the survival, development and proliferation of B and T cells. Experiments with IL-7 deficient mice proved that IL-7 is a non-redundant cytokine (von Freeden-Jeffry *et al.*, 1995). It is therefore reasonable to assume that low levels of diversity of the upstream non-coding region of the IL-7 gene that regulates the transcription of this gene are needed to provide a stable condition for IL-7 production.

In conclusion, we have identified a polymorphism in the upstream non-coding region of the IL-7 gene that could up-regulate gene expression. Although the frequency of this allele is very low in Japan and Thailand, it would be interesting to analyse this polymorphism in HIV-1-infected individuals with different rates of immune reconstitution after treatment with a highly active antiretroviral therapy. It would be important to analyse this polymorphism in other ethnic groups. On the other hand, an elevation of plasma IL-7 has also been reported in lymphopenia cases, including patients undergoing bone marrow transplantation or chemotherapy for cancer, or patients with idiopathic $CD4^+$ lymphopenia (Fry & Mackall, 2005; Bolotin *et al.*, 1999). It would also be of interest to analyse this polymorphism in those patients.

Acknowledgements

We thank J Sakuragi and S Sakuragi for their helpful discussions, S Bandou for technical assistance and N Teramoto for help. We also thank the Japanese and Thais who kindly donated blood for this study.

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AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 23, Number 5, 2007, pp. 686–692

DOI: 10.1089/aid.2006.0212

The Polymorphisms in *DC-SIGNR* Affect Susceptibility to HIV Type 1 Infection

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ABSTRACT

Dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3) grabbing nonintegrin (*DC-SIGN*) and its homologue *DC-SIGNR* (*DC-SIGN* related) have been thought to play an important role in establishing HIV infection by enhancing *trans*-infection of $CD4^+$ T cells in the regional lymph nodes. To identify polymorphisms associated with HIV-exposed seronegative (ESN) individuals in Thais, genomic DNA from 102 HIV-seronegative individuals of HIV-seropositive spouses, 305 HIV-seropositive individuals, and 290 HIV-seronegative blood donors was genotyped for two single nucleotide polymorphisms (SNPs) in *DC-SIGN* promoter (−139A/G and −336A/G), a repeat number of 69 bp in Exon 4 of *DC-SIGN* and *DC-SIGNR*, and one SNP in Exon 5 of *DC-SIGNR* (rs2277998A/G). We found that the proportion of individuals possessing a heterozygous 7/5 and 9/5 repeat and A allele at rs2277998 of *DC-SIGNR* in HIV-seronegative individuals of HIV-seropositive spouses was significantly higher than HIV-seropositive individuals [$p = 0.0373$, OR (95% CI) = 0.57 (0.32,1.01); $p = 0.0232$, OR (95% CI) = 0.38 (0.15,0.98); and $p = 0.0445$, OR (95% CI) = 0.61 (0.37,1.02), respectively]. Analysis after stratifying by gender showed that these associations were observed only in females but not in males. Moreover, HIV-seropositive females tend to have a homozygous 7/7 repeat more frequently than HIV-seronegative females with a marginal level of significance [$p = 0.0556$, OR (95% CI) = 1.79 (0.94,3.40)]. Haplotype analysis showed that the proportion of individuals possessing the 5A haplotype in HIV-seronegative females was significantly higher than HIV-seropositive females [$p = 0.0133$, OR = 0.50 (0.27,0.90)]. These associations suggest that *DC-SIGNR* may affect susceptibility to HIV infection by a mechanism that is different in females and males. Further studies are warranted to investigate the mechanisms of their function.

INTRODUCTION

SOME INDIVIDUALS, SUCH AS HIV-SERONEGATIVE COMMERCIAL SEX WORKERS and HIV-seronegative spouses of HIV-seropositive individuals, have been sexually exposed to HIV repeatedly, yet remain negative for anti-HIV antibody.[1,2] These individuals are recognized as HIV-exposed but seronegative persons (ESN). Our study in Thailand has identified a number of married couples in whom the serostatus of HIV infection was discordant between spouses and in the majority of cases, this discordance could not be explained by their sexual behavior, such as frequency of unprotected sexual contacts, or by viral load of their infected spouses (Rojanawiwat *et al.*, submitted). These observations made us speculate that these seronegative spouses were likely to have certain biological mechanisms that made them resistant to HIV infection.

A number of polymorphisms in host genes that encode products involved in HIV replication and/or immune regulation were reported to be associated with HIV infection and HIV disease progression.[3] Among these, only the homozygous *CCR5* 32-bp deletion (CCR5Δ32) was found to be consistently associated with resistance to HIV infection.[4–6] However, the CCR5Δ32 al-

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lele is very rare among Asians,[6–9] including Thais.[10] Thus other unidentified genes are likely to play a role in resistance to HIV infection among ESN in Thailand.

Dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3) grabbing nonintegrin (*DC-SIGN*, also called CD209) is a receptor on dendritic cells (DCs) that binds to ICAM-3 expressed on T cells to facilitate the initial interaction between DCs and T cells. *DC-SIGN* has been considered important in HIV research because it acts as an intermediate receptor for binding to HIV-1 at mucosal sites and then enhancing *trans*-infection of $CD4^+$ T cells in regional lymph nodes.[11] A homologue of *DC-SIGN* called *DC-SIGNR* (*DC-SIGN* related or CD209L or L-SIGN; the official name is C-type lectin domain family 4, member M, CLEC4M) shares 77% amino acid identity and exhibits a similar capacity of binding to HIV-1.[12,13] *DC-SIGN* is expressed at high levels on DCs and some types of macrophages,[14,15] whereas *DC-SIGNR* is expressed on endothelial cells in liver and lymph nodes.[12,13,16] Recently, the presence of *DC-SIGNR* mRNA was demonstrated in the human vaginal and rectal mucosa by nested reverse transcriptase polymerase chain reaction (RT-PCR).[17] Because *DC-SIGN* and *DC-SIGNR* have an apparent role in DC–T cell interaction and HIV infection, the polymorphisms associated with these genes may have an impact on the transmission of HIV as shown in several studies.[18–23] The objective of this study is, therefore, to identify those reported polymorphisms in *DC-SIGN* and *DC-SIGNR* that are associated with ESN individuals in Thais. Having investigated polymorphisms of these two genes among Thai couples, we found that polymorphisms in the *DC-SIGNR* but not in the *DC-SIGN* were significantly associated with ESN females but not with ESN males.

MATERIALS AND METHODS

Patients and samples

From 6 July 2000 to 15 October 2002 we conducted the Lampang HIV couple study at the HIV clinic in the Day Care Center of the Lampang Hospital, which is a referral hospital located about 600 km to the north of Bangkok (Rojanawiwat *et al.*, submitted). For this study of genetic polymorphisms, we recruited 188 couples, including 70 HIV-serodiscordant couples with 43 HIV-seronegative females and 27 HIV-seronegative males, and 118 HIV-seroconcordant couples. All 144 HIV-seropositive females and 155/161 (96.3%) HIV-seropositive males were heterosexually infected. There were two males with a history of injecting drug use, one male with blood transfusion, two males with more than one possible risk factor, and one male with no information. In terms of age, viral load, $CD4^+$ cell count, and clinical symptoms of HIV-infected spouses, there was no significant difference between discordant couples and concordant couples in both females and males, except that the proportion of HIV-infected males with HIV-seronegative wives was higher than that of HIV-infected males with HIV-infected wives (Table 1).

To increase the number of HIV seronegative spouses of HIV-infected individuals, we also included two HIV-seronegative individuals (one male, one female) who did not bring their HIV-seropositive spouses and 30 HIV-seronegative widows whose husband died of HIV/AIDS. These widows were included because they were significantly exposed to HIV: the median [interquartile range (IQR)] frequency of sexual contact was four (2, 6) times per month and 26/30 (86%) of them had never used a condom before they were aware of HIV. Furthermore, we believe that the level of HIV exposure was very high because their index case has already progressed and died: in 14/30 (46%) women, their husband died within 1 year of disclosure and in 21/30 women (70%) within 2 years of disclosure. There was one HIV-seropositive female who previously had an HIV-positive husband and remarried an HIV-negative husband after her first husband died. Therefore in total, 102 HIV-seronegative individuals (74 females and 28 males) at high risk for HIV infection and 305 HIV-seropositive individuals (144 females and 161 males) were enrolled.

The median age (IQR) of both HIV-seronegative and HIV-seropositive individuals was 32 (29, 36) years. Among 74 HIV-seronegative female spouses, one of them had known her HIV status before the marriage. In the other 73 females, the median (IQR) duration of marriage before they knew the HIV status of their husbands was estimated to be 5 (3, 8) years. The median (IQR) frequency of sexual contacts before the disclosure of the HIV status of their husband was five (3, 8) times per month. Of females, 64 (87.7%) reported that they had never used a condom during those sexual contacts and only four (5.5%) reported that they used a condom at every contact. Among 28 HIV-seronegative male spouses, nine of them had known the HIV status of their wives before the marriage. In the other 19 HIV-seronegative males, the median (IQR) duration of marriage before the disclosure of HIV status of their wife was estimated to be 1 (0, 2) year. The median (IQR) frequency of sexual contact before the disclosure of the HIV status of their wife was six (4, 24) times per month, and all of them reported that they had never used a condom during sexual contacts.

For a control HIV-seronegative group representing the Thai general population, 290 blood samples were collected from blood donors (171 males and 119 females) at the blood bank of the Lampang Hospital. All participants gave written informed consent. This study was approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand in January 2000.

EDTA-treated blood samples were separated for plasma and buffy coat, then stored at −80°C until used. Genomic DNA was extracted and purified from the frozen buffy coat using a kit (QIAamp mini blood kit; QIAGEN GmbH, Hilden, Germany).

HIV-1 serology was screened by an ELISA kit (Enzynost anti-HIV-1/2 plus; Dade Behring Marburg GmbH, Marburg, Germany). The positive samples were then confirmed by another ELISA kit (Genscreen HIV 1/2 Diagnostic Pasture Ltd., France) and a gel particle agglutination test (Serodia HIV-1; Fujirebio Inc., Tokyo Japan).

Polymorphism genotyping

DC-SIGN promoter: −139A/G (rs2287886) and −336A/G (rs4804803): *DC-SIGN −139A/G* was genotyped by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). Amplification of fragments containing this single nucleotide polymorphism (SNP) was performed using forward primer: 5′-GTCGGAATCCAAGGCCTCTC-3′ and

reverse primer: 5′-CAGGAAAGCCAGGAGGTCAC-3′. PCR was performed in a total of 25 μl containing 0.1 μM each primer, 100 μM dNTPs, 2.5 mM $MgCl_2$, and 0.5 unit of heat-activated DNA polymerase (IMMOLASE, Bioline USA Inc., MA) in 1× buffer supplied with the enzyme. The thermal profile was 7 min at 95°C, followed by 30 cycles of 30 sec at 95°C, 20 sec at 58°C, and 1 min at 72°C. The 574-bp PCR products were incubated with *Spe*I restriction enzyme. The product, which contained the −139A allele, was cut into 318-bp and 256-bp fragments, and determined by electrophoresis in 2% agarose gel.

For *DC-SIGN −336A/G* genotyping, we used an allelic discrimination (AD) assay by 7500 real-time PCR with SDS analysis software (Applied Biosystems, CA). Two primers (forward primer: 5′-TGTGTTACACCCCCTCCACTAG-3′; reverse primer: 5′-GGACAGTGCTTCCAGGAACT-3′) were used to amplify 68-bp products containing the *DC-366A/G* SNP site, which was detected by two probes labeled with different fluorescent dyes: 5′-VIC-TACCTGCCTACCCTTG-MGB-3′ to detect the "A" allele and 5-FAM-CTGCCCACCCTTG-MGB to detect the "G" allele. Real-time PCR was performed in a 10-μl reaction mixture containing 5 μl of 2 × TaqMan universal master mix, 1× primers and probes mix, and 10 ng of DNA sample. Thermocycling consisted of 10 min at 95°C, followed by 40 cycles of 15 sec at 92°C and 1 min at 60°C.

The 69-bp repeat number in Exon 4

The 69-bp repeat number was determined by the length of the PCR products. Primers and the thermal profile for *DC-SIGNR* 69-bp repeat number genotyping was performed as previously described.[13] For *DC-SIGN* 69-bp repeat number genotyping, forward primer: 5′-CCTTGGCTCTCACAAATGATGTCC-3′ and reverse primer: 5′-CACCCACTGCAGCCTTCAGCTG-3′ were used in the PCR condition as described above. The thermal profile was 7 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 15 sec at 64°C, and 1 min at 72°C. The PCR products were analyzed by electrophoresis in 2% agarose gel.

DC-SIGNR Exon 5 (rs2277998)

A/G SNP of rs2277998 was genotyped by PCR-RFLP as previously described.[18]

Data management and sample analysis

We conducted this study in a blinded manner between field investigators and laboratory investigators. The proportions of individuals possessing a certain allele/genotype among different groups were compared by a chi-square test or Fisher's exact test. Odds ratio (OR) and 95% confidence interval (95% CI) were used to measure the strength of the genetic influence associated with susceptibility to HIV infection. All statistical analyses were carried out using Epi Info version 3.01 (US-CDC). *DC-SIGNR* haplotypes were constructed and estimated for their frequencies by the expectation-maximization algorithm (Arlequin version 3.01, Genetica and Biometry Laboratory, Geneva, Switzerland).

RESULTS

Genotyping of polymorphisms in DC-SIGN and DC-SIGNR

We genotyped the five polymorphisms (three in *DC-SIGN* and two in *DC-SIGNR*) in 102 HIV-seronegative individuals, 305 HIV-seropositive individuals, and 290 blood donors (Table 2). We then compared the proportions of individuals possessing a specific genotype between HIV-seronegative and HIV-seropositive individuals (Table 3).

As for the two SNP sites in the *DC-SIGN* promoter, we did not find any significant differences in the proportion of individuals possessing the G allele (genotype G/G and A/G). The *DC-SIGN* 69-bp repeat number was highly conserved; the genotype 7/7 repeat was found almost exclusively at a frequency of 0.99. In contrast, we found some significant differences in genetic polymorphisms in *DC-SIGNR* associated with HIV infection.

TABLE 1. CHARACTERISTICS OF HIV-SEROPOSITIVE INDIVIDUALS

	Male		*Female*	
	With HIV −ve spouse (n = 43)	*With HIV +ve spouse (n = 118)*	*With HIV −ve spouse (n = 27[a])*	*With HIV +ve spouse (n = 118)*
Median age; years (IQR)[b]	34 (31, 38)	33 (30, 37)	31 (28, 35)	30 (27, 35)
Median viral load,[c] $\log_{10}$ copies/ml (IQR)	5.257 (4.915, 5.757)	5.305 (4.773, 5.756)	4.939 (4.358, 5.579)	4.993 (4.214, 5.477)
Median CD4 count,[c] cells/mm^3 (IQR)	28 (13, 72)	38 (11, 246)	321 (157, 524)	277 (148, 427)
HIV-1-related symptoms,[c] % (*n*)	79.1 (34)	59.5 (69)	33.3 (9)	27.4 (32)
Diagnosis of AIDS,[c] % (*n*)	65.1 (28)	38.8 (45)	11.1 (3)	12.8 (15)

[a]This includes a female who was counted as a concordant couple with her previous HIV +ve husband.
[b]IQR, interquartile-range.
[c]Data not available on two concordant couples.

TABLE 2. DISTRIBUTION OF GENOTYPES [NO. (%)] OF POLYMORPHISMS IN DC-SIGN AND DC-SIGNR AMONG HIV-SERONEGATIVE AND HIV-SEROPOSITIVE INDIVIDUALS

		HIV-seronegative			*HIV-seropositive*			*Blood donor*
		Male (n = 28)	*Female (n = 74)*	*Total (n = 102)*	*Male (n = 161)*	*Female (n = 144)*	*Total (n = 305)*	*Total (n = 209)*
DC-SIGN								
Pomoter region								
−139 (rs2287886)	A/A	16 (57.14)	31 (41.89)	47 (46.08)	64 (39.75)	74 (51.39)	138 (45.25)	146 (50.34)
	A/G	9 (32.14)	35 (47.30)	44 (43.14)	86 (53.42)	56 (38.89)	142 (46.56)	120 (41.38)
	G/G	3 (10.71)	8 (10.81)	11 (10.78)	11 (6.83)	14 (9.72)	25 (8.20)	24 (8.28)
−336 (rs4804803)	A/A	22 (78.57)	59 (79.73)	81 (79.41)	119 (73.91)	121 (84.03)	240 (78.69)	237 (81.72)
	A/G	6 (21.43)	13 (17.57)	19 (18.63)	40 (24.84)	21 (14.58)	61 (20.00)	51 (17.59)
	G/G	0 (0.00)	2 (2.70)	2 (1.96)	2 (1.24)	2 (1.39)	4 (1.31)	2 (0.69)
Exon 4	7/5	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.62)	0 (0.00)	1 (0.33)	0 (0.00)
69-bp repeat numbers	7/7	28 (100.00)	73 (98.65)	101 (99.02)	160 (99.38)	144 (100.00)	304 (99.67)	288 (99.31)
	8/7	0 (0.00)	1 (1.35)	1 (0.98)	0 (0.00)	0 (0.00)	0 (0.00)	2 (0.69)
DC-SIGNR								
Exon 5 (rs2277998)	G/G	19 (67.86)	46 (62.16)	65 (63.73)	111 (68.94)	115 (79.86)	226 (74.10)	215 (74.14)
	A/G	9 (32.14)	28 (37.84)	37 (36.27)	45 (27.95)	25 (17.36)	70 (22.95)	66 (22.76)
	A/A	0 (0.00)	0 (0.00)	0 (0.00)	5 (3.11)	4 (2.78)	9 (2.95)	9 (3.10)
Exon 4	5/5	0 (0.00)	0 (0.00)	0 (0.00)	4 (2.48)	4 (2.78)	8 (2.62)	11 (3.79)
69-bp repeat numbers	6/5	0 (0.00)	0 (0.00)	0 (0.00)	4 (2.48)	0 (0.00)	4 (1.31)	3 (1.03)
	6/6	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.34)
	7/5	6 (21.43)	21 (28.38)	27 (26.47)	33 (20.50)	19 (13.19)	52 (17.05)	47 (16.21)
	7/6	3 (10.71)	6 (8.11)	9 (8.82)	12 (7.45)	8 (5.56)	20 (6.56)	23 (7.93)
	7/7	9 (32.14)	22 (29.73)	31 (30.39)	52 (32.30)	62 (43.06)	114 (37.38)	133 (45.86)
	8/5	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.34)
	8/7	1 (3.57)	1 (1.35)	2 (1.96)	1 (0.62)	0 (0.00)	1 (0.33)	0 (0.00)
	9/5	3 (10.71)	7 (9.46)	10 (9.80)	8 (4.97)	4 (2.78)	12 (3.93)	8 (2.76)
	9/6	1 (3.57)	0 (0.00)	1 (0.98)	4 (2.48)	1 (0.69)	5 (1.64)	0 (0.00)
	9/7	5 (17.86)	14 (18.92)	19 (18.63)	39 (24.22)	40 (27.78)	79 (25.90)	56 (19.31)
	9/9	0 (0.00)	3 (4.05)	3 (2.94)	4 (2.48)	5 (3.47)	9 (2.95)	7 (2.41)
	10/7	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.69)	1 (0.33)	0 (0.00)

TABLE 3. COMPARISON OF PROPORTIONS [ODDS RATIO (95% CI) AND *p* VALUE] OF INDIVIDUALS POSSESSING CERTAIN GENOTYPE BETWEEN HIV-SERONEGATIVE AND HIV-SEROPOSITIVE INDIVIDUALS

		Total		*Male*		*Female*	
Polymorphism	*Genotype*	*OR (95% CI)*	p	*OR (95% CI)*	p	*OR (95% CI)*	p
DC-SIGN							
−139 (rs2287886)	G/G and A/G	1.03 (0.64, 1.66)	0.8838	2.02 (0.84, 4.91)	0.0856	0.68 (0.37, 1.25)	0.1839
−336 (rs4804803)	G/G and A/G	1.04 (0.58, 1.89)	0.8769	1.29 (0.47, 4.17)	0.6012	0.75 (0.34, 1.64)	0.4283
69-bp repeat numbers	7/7	0.33 (0.00, 26.34)	0.4389	Undefined	1.0000	0.00 (0.00, 20.04)	0.3394
DC-SIGNR							
Exon 5 (rs2277998)	A/A and G/G	0.61 (0.37, 1.02)	0.0445	0.95 (0.37, 2.46)	0.9088	0.41 (0.21, 0.81)	0.0049
69-bp repeat numbers	5/5	Undefined	0.2097	Undefined	1.0000	Undefined	0.3022
	6/5	Undefined	0.5760	Undefined	1.0000	—	—
	6/6	—	—	—	—	—	—
	7/5	0.57 (0.32, 1.01)	0.0373	0.95 (0.34, 3.9)	0.9105	0.38 (0.18, 0.82)	0.0061
	7/6	0.73 (0.30, 1.79)	0.4412	0.67 (0.16, 3.97)	0.4699	0.67 (0.19, 2.44)	0.5613
	7/7	1.37 (0.82, 2.28)	0.2022	1.01 (0.40, 2.60)	0.9870	1.79 (0.94, 3.40)	0.0556
	8/5	—	—	—	—	—	—
	8/7	0.16 (0.00, 3.21)	0.1562	0.17 (0.00, 13.73)	0.2750	0.00 (0.00, 20.04)	0.3394
	9/5	0.38 (0.15, 0.98)	0.0232	0.44 (0.10, 2.73)	0.2112	0.27 (0.06, 1.13)	0.0477
	9/6	1.68 (0.19, 80.39)	1.0000	0.69 (0.06, 35.12)	0.5556	Undefined	1.0000
	9/7	1.53 (0.84, 2.78)	0.1369	1.47 (0.50, 5.28)	0.4619	1.65 (0.79, 3.47)	0.1513
	9/9	1.00 (0.24, 5.88)	1.0000	Undefined	1.0000	0.85 (0.16, 5.64)	1.0000
	10/7	Undefined	1.0000	—	—	Undefined	1.0000

As for the *DC-SIGNR* 69-bp repeat number, we found a variation from 5 to10 repeats with 13 genotypes. The most common genotype in our study population was the 7/7 repeat, followed by the 9/7 and 7/5 repeats. We found that HIV-seronegative individuals had a significantly higher frequency of possessing the heterozygous 7/5 or 9/5 repeat than HIV-seropositive individuals ($p = 0.037$ and 0.023, respectively). Interestingly, we did not find any individuals with three or four repeats, which were found occasionally in whites. Instead we found one individual with 10 repeats that had not been reported elsewhere. For the SNP in *DC-SIGNR* Exon 5 (rs2277998), the proportion of individuals possessing the A allele (genotype A/A and A/G) was significantly higher in HIV-seronegative than that in HIV-seropositive individuals ($p = 0.0445$).

We further analyzed these associations after stratifying the study population into male and female groups (Table 3). Interestingly, we found that these associations remained significant in females ($p = 0.0061$ for the 7/5 repeat, $p = 0.0477$ for the 9/5 repeat, and $p = 0.0049$ for the A allele at the SNP rs2277998A) but not in males ($p = 0.9105$ for the 7/5 repeat, $p = 0,2112$ for the 9/5 repeat, and $p = 0.9088$ for the A allele at the SNP rs2277998A). Moreover, we found that HIV-seropositive females tend to have the homozygous 7/7 repeat more frequently than HIV-seronegative females with a marginal level of significance ($p = 0.0556$). Again, this association was not found in males.

TABLE 4. DISTRIBUTION OF DC-SIGNR HAPLOTYPE [NO. (%)] AMONG HIV-SERONEGATIVE AND HIV-SEROPOSITIVE INDIVIDUALS AND BLOOD DONORS

	HIV-seronegative			*HIV-seropositive*			*Blood donor*
DC-SIGNR haplotype[a]	*Male (n = 56)*	*Female (n = 148)*	*Total (n = 204)*	*Male (n = 322)*	*Female (n = 288)*	*Total (n = 610)*	*(n = 580)*
5A	9 (16.07)	28 (18.92)[b]	37 (18.14)	51 (15.84)	30 (10.42)[b]	81 (13.28)	77 (13.28)
6A	0 (0.00)	0 (0.00)	0 (0.00)	3 (0.93)	3 (1.04)	6 (0.98)	1 (0.17)
7A	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.31)	1 (0.35)	1 (0.16)	2 (0.34)
5G	0 (0.00)	0 (0.00)	0 (0.00)	2 (0.62)	1 (0.35)	3 (0.49)	5 (0.86)
6G	4 (7.14)	6 (4.05)	10 (4.90)	17 (5.28)	6 (2.08)	23 (3.77)	26 (4.48)
7G	33 (58.93)	86 (58.11)	119 (58.33)	188 (58.39)	190 (65.97)	379 (62.13)	390 (67.24)
8G	1 (1.79)	1 (0.68)	2 (0.98)	1 (0.31)	1 (0.35)	2 (0.33)	1 (0.17)
9G	9 (16.07)	27 (18.24)	36 (17.65)	59 (18.32)	55 (19.10)	114 (18.69)	78 (13.45)
10G	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.35)	1 (0.16)	0 (0.00)

[a]Haplotypes were constructed from repeat number of 69-bp (Exon 4) and A/G allele at rs227799 (Exon 5).
[b]HIV-seronegative female versus HIV-seropositive female; $p = 0.0133$, OR (95% CI) = 0.50 (0.27, 0.90).

We also analyzed frequencies of the *DC-SIGNR* haplotypes constructed from a 69-bp repeat number in Exon 4 and an allele at rs227799 in Exon 5 in relation to HIV infection as summarized in Table 4. We found nine haplotypes in our study population. The 7G was the most common haplotype in all groups. The 5A was the second most common haplotype in the HIV-seronegative group whereas the 9G was the second most common haplotype in the HIV-seropositive and blood donor groups. We found that the proportion of individuals possessing the 5A haplotype was significantly higher in HIV-seronegative females than HIV-seropositive females [$p = 0.0133$, OR (95% CI) = 0.50 (0.27,0.90)].

DISCUSSION

We identified the polymorphisms at the two loci in *DC-SIGNR* that showed a statistically significant association with Thai HIV-seronegative individuals of HIV-seropositive spouses, especially among females. Our data on their marital history and sexual behavior indicated that most of these seronegative females were exposed to HIV repeatedly. There were four HIV-seronegative females reporting condom use at every contact. However, three of the four women had had a child with their HIV-infected index case, indicating that there was a possibility of HIV exposure. Thus we included these women in our analysis. We have also done the analysis excluding this group, but the conclusions remain the same. Therefore we think that *DC-SIGNR* may play an important role in conferring resistance to HIV infection.

The previous study from the United States showed that the heterozygous 7/5 repeat in *DC-SIGNR* was associated with resistance to HIV infection, whereas the homozygous 7/7 repeat was associated with susceptibility to HIV infection.[19] Our study is the first conducted in Asia showing consistent results with the American study, although in our study the significance of the association between the homozygous 7/7 repeat and HIV infection was marginal and was found only in females. In another published study, however, such associations of repeated number with susceptibility to HIV infection were not shown.[20] We think this is because the comparison was made only with the HIV-negative general population but not with ESN. In our study population as well, we did not find a significance difference between HIV-seropositive individuals and HIV-negative blood donors. Gramberg *et al.*[21] investigated the effect of polymorphisms in the *DC-SIGNR* neck domain on the interaction with HIV the envelope protein in *in vitro* experiments, but they found that coexpression of seven repeats with five repeats did not decrease the interaction with HIV compared with seven repeats only. However, there remains a possibility that they did not show the inhibitory effect because their experiments were conducted under high level expression of the gene.

Our study is the first showing associations of the other two polymorphisms that are heterozygous 9/5 repeat and A allele at the SNP site rs2288997 in Exon 5 of *DC-SIGNR* with HIV-seronegative individuals. The A-to-G change at this SNP site is particularly interesting as it causes an aspatate-to-asparagine substitution in the carbohydrate recognition domain (CRD). This amino acid change may affect the binding affinity of CRDs to HIV-1 gp120 and/or ICAM-3. But the relevance of this genetic polymorphism to HIV infection has not yet been investigated in *in vitro* experiments. Since we found a significant linkage between the A allele in Exon 5 and five repeats in Exon 4, the association of the 7/5 and 9/5 repeat with HIV-seronegative individuals may merely be due to a confounding effect by the A allele in Exon 5 and it may be the polymorphism in the CRD coding region that truly affects susceptibility to HIV infection. It is also possible that these two polymorphisms reported here are in linkage disequilibrium with another variant elsewhere in this region that is actually responsible for the observed protective effect.

In our study population, we found that these associations with polymorphisms in *DC-SIGNR* were not observed when only males were included in the analysis. Instead, the stronger associations were observed when only females were analyzed than when males were combined. We have two possible reasons for this difference. First, there might be a different mechanism of HIV infection between female and male, and *DC-SIGNR* plays a role only in female HIV infection. Second, according to our information on marital history, the duration of marriage before the disclosure of HIV status was much longer in females than in males and one-third of HIV-negative males had known the HIV status of their wives before marriage. Therefore, a considerable proportion of HIV-seronegative males was unlikely to have been highly exposed to HIV; thus they may still be susceptible to HIV infection. In fact, our follow-up data of their serostatus showed a three times higher seroconversion rate in male seronegative individuals than female seronegative individuals (data not shown).

We found that the repeat number of *DC-SIGN* in Thais was highly conserved in the homozygous 7/7 repeat and was not associated with susceptibility to HIV infection as showed in the previous study.[22] We did not find any association between polymorphisms in the *DC-SIGN* promoter (−139A/G and −336A/G) and susceptibility to HIV infection in our study group, whose risk for acquiring HIV infection was heterosexual contact. This finding confirmed the previously reports, which showed an association of −336G with risk for parenteral risk, but not mucosal risk for HIV infection.[23]

Although *DC-SIGN* and *DC-SIGNR* are quite similar in amino acid sequences and both have a binding ability to carbohydrate ligands, there are differences in their characteristics, including expression distribution,[16,24] carbohydrate binding profiles,[25–29] alternatively splicing,[17,30] and level of polymorphism in repeat numbers.[13,19,21,29] Thus it is plausible that they may play a different role in HIV infection. *DC-SIGNR* expression at mucosal sites (vaginal and rectal) has been found to have an alternative splicing that produces predicted soluble isoforms of *DC-SIGNR* molecules.[17] This soluble isoform may modulate the efficiency of viral transmission and dissemination.[17] Our experiment in monocyte-derived DCs cultured *in vitro* revealed the expression of *DC-SIGNR* by nested RT-PCR (data not shown). The 375-bp nested PCR product had 100% identity to the *DC-SIGNR* mRNA isoform I [variant 1 (NM_04257) and variant 2 (NM_214675)] and isoform II [variant 3 (NM_214676)]. Further *in vivo* and *in vitro* studies are warranted to investigate the mechanisms of their functions.

ACKNOWLEDGMENTS

We are grateful to all the participants of the Lampang cohort and blood donors. We also thank Ms. Wimala Inunchot,

Ms. Suthira Kasemsuk, Ms. Sriprai Seneewong-na-ayuthaya, Ms. Anong-nard Suyasarojna, Ms. Nutira Boonna, and Mr. Prapan Wongnamnong for their technical assistance. This study was supported by The Ministry of Public Health Thailand, The Japan International Cooperation Agency, and The Japanese Foundation for AIDS Prevention.

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Journal of Medical Virology 79:865–872 (2007)

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.

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 multi-drug 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

Grant sponsor: Japan Society for the Promotion of Science; Grant number: 14256005; Grant sponsor: Ministry of Health, Labor and Welfare, Japan.

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Accepted 11 December 2006
DOI 10.1002/jmv.20912
Published online in Wiley InterScience (www.interscience.wiley.com)

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]) 1st	2nd	3rd	4th	5th	Treatment
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/3TC
NYU33		1 TAM (23)	2 TAMs (34)			ZDV/DDI
NYU30			3 TAMs (31)			ZDV/3TC, ZDV/DDI
NYU38	1 TAM (8)	4 TAMs (15)	4 TAMs (17)	5 TAMs (23)		ZDV/DDI
NYU79	*M184V* + *V75M* (10)	*M184V* + *V75M* (13)				ZDV/3TC, DDI/3TC
						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])						
	Pre-treatment	1st	2nd	3rd	4th	5th	Treatment
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)	*K101Q* (34)			EFAVIRENZ
NYU33			*K101Q* (11)		*G190A* (24)	*G190A* (34)	
NYU36			*I178M* (13)	*G190A* (16)			
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*.

ACKNOWLEDGMENTS

The authors are grateful to the children who joined this study. Without their contribution this work would not have been possible. The authors also wish to thank Dr. Davy Koech, the Director of Kenya Medical Research Institute (KEMRI), whose facilitation ensured the successful completion of the study. This work was supported in part by Japan Society for the Promotion of Science (Grant-in-Aid for Scientific Research, grants 14256005) and the Ministry of Health, Labor and Welfare, Japan. This work is published with the permission of the Director, KEMRI.

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Virology 362 (2007) 131 – 138
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Retro-transduction by virus pseudotyped with glycoprotein of vesicular stomatitis virus

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Received 28 September 2006; returned to author for revision 26 December 2006; accepted 27 December 2006
Available online 26 January 2007

Abstract

A virus pseudotyped with glycoprotein of vesicular stomatitis virus (VSV-G) can enter various cell types at a relatively high titer. We observed that the amount of viral antigen from VSV-G pseudotyped human immunodeficiency virus type 1 (HIV-1) producing cells was much higher than that from their non-pseudotyped counterparts. This enhanced viral antigen production was not observed when we used HIV-1 *pol* mutant, viral enzyme inhibitors, HIV Env protein, or VSV-G fusion defective mutants. The transfection experiment using GFP-expressing virus showed time-dependent expansion of GFP-positive cells and viral DNA integration. These results suggested that the increase in viral antigen yield was caused by the release of a progeny virus following retro-transduction by the pseudotyped virus of the cells within the transfected cell culture. The infectivity as well as the amount of VSV-G on virus particles per unit of viral antigen was significantly different before and after the onset of the yield enhancement. This suggests that results of infection assays of the virus pseudotyped with VSV-G may be affected by the occurrence of such enhancement. This means that, while pseudotyping with VSV-G is a simple and effective method, this procedure should be carefully considered when the virus is produced for infectivity assays.

Keywords: VSV-G; HIV-1; Pseudotyping; Viral vector; Retro-transduction

Introduction

Gene delivery using retroviral vectors has become a very popular and indispensable method. To ensure safety of the vectors and to increase their efficiency, envelope pseudotyping of viral vectors lacking their own envelope gene is used in almost all cases. Glycoprotein of vesicular stomatitis virus (VSV-G) in particular is one of the most frequently used envelope proteins for pseudotyping since it is very stable and promises highly effective and broad-spectrum gene delivery. However, there seems to be very little awareness of its adverse effect, that is, re-infection/retro-transduction. VSV-G interacts with a phospholipid component of the cell surface membrane and mediates viral entry by membrane fusion (Burns et al., 1993; Mastromarino et al., 1987). As viral entry does not seem to require specific protein receptors, VSV-G pseudotyped vectors could infect virtually all kinds of cells. Thus, the VSV-G pseudotyped viral vector has the potential to infect its producer cells to re-generate progenies, which may contain unexpected characteristics. In the study presented here, we examined retro-transduction of VSV-G pseudotyped HIV-1 vectors. Our findings suggest that this problem could confound the interpretation of the experimental results under certain conditions and must therefore be taken into careful consideration.

Results

Enhancement of virion production by VSV-G pseudotyping

We previously noticed that the viral antigen yield of VSV-G pseudotyped HIV-1 by transfection was always much higher than that of the non-pseudotyped virus. We first reconfirmed this phenomenon by using a simplified experimental design. For this purpose, 2.5 μg of HIV-1 proviral plasmid pNLNh, which lacks Env expression, was transfected into 3×10^6 293T cells

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0042-6822/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2006.12.030

along with 2.5 μg of either pCG-VSVG, which expresses VSV-G under the CAG hybrid promoter (Niwa et al., 1991), or pGEM-5Zf(+) (Promega, Madison, WI) as a control. The quantity of virus production was monitored by measuring CA-p24 antigen in the culture supernatant every 12 h. The amounts of CA-p24 detected in VSV-G pseudotyped virus and in control were almost equal up to 24 h post-transfection. However, CA-p24 production of the former became 10 times higher than that of control at 36 h and finally 20 times higher at 48 h (Fig. 1A). The actual amount of CA-p24 of virus from 3×10^6 of transfected cells with pseudotyping was typically 20–40 μg in total. This enhancement may have been caused by some transactivation effects of VSV-G, or by retro-transduction of the cells within the transfected cell culture, which then leads to production of the progeny virus. The absence of the enhancement at 12 or 24 h post-transfection suggests that transactivation was unlikely since the transactivation effect should become apparent from the onset of protein expression. In subsequent experiments, we therefore examined the possibility of retro-transduction of virus thus produced.

Retro-transduction of pseudotyped virus during transfection

The transfected cells were harvested 48 h post-transfection and viral antigens within the cells were detected by western blotting (Fig. 1B). The viral antigens within cells producing the *env*-lacking virus were mostly large, seemingly uncleaved, and with the precursor protein Gag-Pr55 dominant. On the other hand, the protein profile within the cells producing the VSV-G pseudotyped virus was strikingly different. The majority of viral antigens were mature proteins, such as CA-p24 and MA-p17, while the number of immature proteins was very limited. This observation was in agreement with a hypothesis that the viral antigen within cells producing the pseudotyped virus mainly came from mature virions, which would support the concept of retro-transduction of the released virus.

To examine this possibility, a *pol* mutant pNLN-RI was constructed from pNLNh. The mutant had a large deletion at the RT and IN genes in addition to an Env frameshift, while the expression of functional Env, RT, and IN was eliminated. The VSV-G pseudotyped NLN-RI was therefore not able to complete retro-transduction to produce its progeny. Next, 293T cells were co-transfected with pNLN-RI along with either pCG-VSVG or pGEM-5Zf(+), and the quantity of virus production was monitored by measuring CA-p24. No difference in virus production was observed between VSV-G pseudotyped and non-pseudotyped viruses during 48 h post-transfection (Fig. 1C). This indicates that the enhancement of virus production was impaired by the elimination of RT expression and subsequent inhibition of retro-transduction.

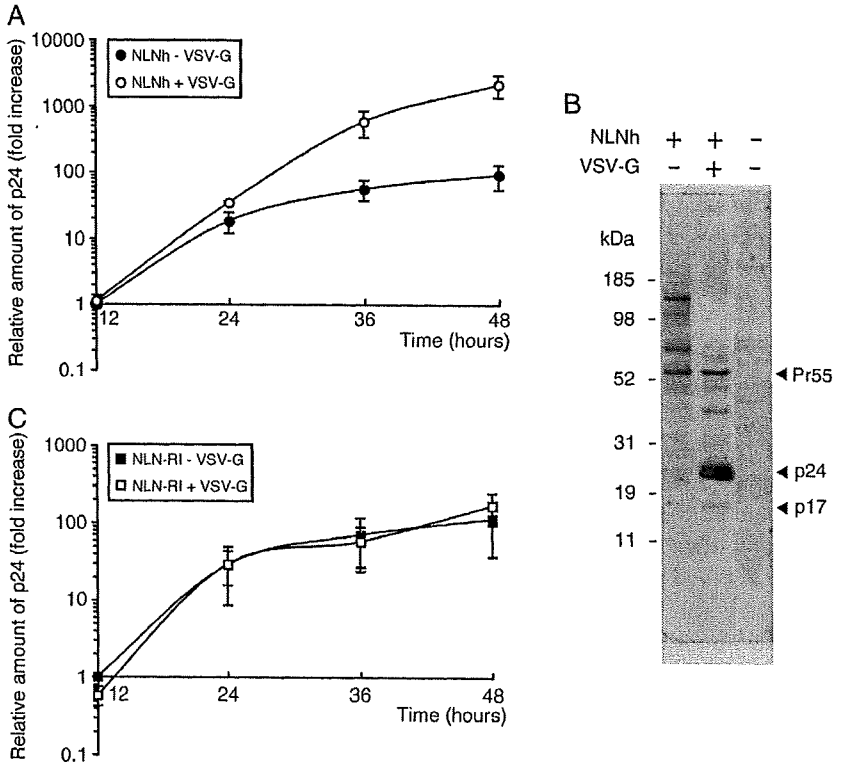

Fig. 1. Enhancement of virus production by pseudotyping. (A) Time course of HIV-1 production during transfection. The plasmid pNLNh was transfected into 293T cells along with either pCG-VSVG or pGEM-5Zf(+), and the quantity of virus production in the culture supernatant was monitored at 12, 24, 36, and 48 h post-transfection by measuring CA-p24 antigen. The value for NLNh without pseudotyping at 12 h was set at 1. Results show the averages of three separate experiments. Error bars represent the mean standard error for the difference between experiments. (B) Profiles of viral antigen in transfected cells. Cells were harvested at 48 h post-transfection and lysed. Viral antigens within cell lysates were detected by western blotting using serum from HIV-1 infected patients. (C) Similar experiment as in A except for the use of viral *pol* mutant pNLN-RI instead of the wild-type pNLNh.