Journal of Medical Virology 81:545–551 (2009)

High-Risk HPV Types in Lesions of the Uterine Cervix of Female Commercial Sex Workers in the Philippines

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In order to prevent cervical cancer, vaccines against human papilloma virus types 16 (HPV-16) and 18 (HPV-18) have been implemented worldwide. However, the HPV types that cause cancer can differ according to geographical area and ethnicity. In this new era of the HPV vaccine, it is important to elucidate the prevalent HPV types in each area. Therefore, the prevalence of HPV infection and cervical abnormalities among 369 female commercial sex workers in the Philippines were examined. HPV *L1* gene was amplified by polymerase chain reaction (PCR) using modified GP5+/6+ primers, and genotyping was performed by sequencing cloned PCR products. HPV DNA was detected in 211 (57.2%) women, among whom 46 HPV types were identified. HPV-52 was most common and multiple-type infection was observed in 44.5%. Among 56 women with abnormal cervical cytology (low- and high-grade squamous intraepithelial lesions and adenocarcinoma in situ), HPV-52 was most common (23.2%), followed by HPV-16 (19.6%), -58 (10.7%), and -67 (10.7%). Only 27% of these women were positive for HPV-16 and -18. Multivariate analysis revealed that HPV-16, -39, -52, -67, and -82 were significantly associated with abnormal cytology. Repeated analysis of HPV-52 single-positive samples using the original GP5+/6+ PCR primers produced negative results in 57% of cases, suggesting that the prevalence of HPV-52 infection may have been underestimated in previous studies, and the current vaccines may not be sufficient for preventing infection and the development of premalignant lesions of the cervix in women in the Philippines. ***J. Med. Virol. 81:545–551, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: HPV high-risk type; cervical cytology; Philippines; female commercial sex workers

INTRODUCTION

Human papillomavirus (HPV) is the most important risk factor for cervical cancer [Muñoz et al., 2003], which is the second most common malignancy and the third most common cause of cancer-related death in women [Parkin et al., 2005]. The incidence and mortality of cervical cancer are very high in women of reproductive age, especially in developing countries [Parkin et al., 2005; Frain et al., 2006]. Cervical cancer screening using cytological testing and HPV vaccination are paramount for preventing cervical cancer in young women.

More than 40 HPV types have been identified in the mucosal epithelia of the human genital tract; these are classified into high-risk and low-risk types according to their ability to cause cancer [Muñoz et al., 2003]. HPV type 16 (HPV-16) is the most common high-risk type and is detected in 50–60% of high-grade squamous

Grant sponsor: Ministry of Health, Labor, and Welfare (International Health Cooperation Research); Grant number: 19C-4.

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Accepted 4 November 2008
DOI 10.1002/jmv.21416
Published online in Wiley InterScience (www.interscience.wiley.com)

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intraepithelial lesions and invasive cervical cancers [Muñoz et al., 2003; Wheeler, 2007]. HPV-18 is identified in 10–20% of cancers [Muñoz et al., 2003; Wheeler, 2007]. Thus, HPV-16 and -18 are thought to be responsible for about 70% of cervical cancer cases in many countries [Bosch et al., 1995; Brown et al., 1999; Muñoz et al., 2003].

To reduce the incidence of cervical cancer, vaccines against HPV-16 and -18 have been developed and have been found 100% effective in preventing infection by these HPV types [Harper et al., 2006; Wheeler, 2007]. However, the distribution of common HPV types may vary depending on the geographic area and ethnicity of the population. Thus, the impact of these HPV vaccines on the prevention of infection and cancer may differ in different areas. In Japan, the prevalence of HPV-16 and -18 in cancers and high-grade squamous intraepithelial lesions is approximately 50% and 33%, respectively [Sasagawa et al., 2001]. HPV-52 is more common than HPV-18 in Japan, Taiwan, and eastern Africa [de Sanjosé et al., 2007]. Thus, in order to estimate the effectiveness of the current HPV vaccines for preventing cervical cancer, it is essential to determine the predominant cancer-causing HPV type in each area.

Degenerate and/or consensus primers for polymerase chain reaction (PCR) have been used to amplify a variety of HPV types from clinical specimens. The GP5+/6+ primers that target the HPV *L1* gene have been considered one of the best primer sets for HPV PCR and have been used in many epidemiological studies. However, it has been reported that some HPV types, such as HPV-52, may not be amplified by GP5+/6+ PCR as effectively as HPV-16 and -18 because of sequence mismatches between the target gene and the primers [Matsukura and Sugase, 2004]. Therefore, the GP5+/6+ primers have been modified to broaden the spectrum of detectable HPV types [Yamada et al., 2008].

In a case–control study of the cause of cervical cancer in the Philippines [Ngelangel et al., 1998], the most common HPV type in women with squamous cell carcinomas was HPV-16 (42.9%), followed by HPV-18 (25.3%) and -45 (13.5%). HPV-45 (17.2%) was the most common type found in cytology-normal women (controls), followed by HPV-16 (14.3%) and -18 (14.3%). However, the original GP5+/6+ PCR primers were used in this study. Little other information about the prevalence of HPV types in premalignant lesions of the cervix in the Philippines is available.

In the current study, the prevalence of HPV types and their associations with abnormal cervical cytology among female commercial sex workers in the Philippines were examined. HPV types were detected by PCR using modified GP5+/6+ primers. The goal was to ascertain whether the current HPV vaccine is sufficient for preventing infection and the development of premalignant lesions of the cervix in the Philippines. The prevalence of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections and their association with HPV infection were also investigated.

SUBJECTS AND METHODS

Subjects and Sample Collection

Three hundred seventy female commercial sex workers who were attending the Makati Social Hygiene Clinic or its mobile clinic at a night bar in Manila, Philippines, for a regular check-up in January or July 2006 were enrolled in this study. Written informed consent was obtained from all participants.

Specially trained technicians used cervical brushes to collect two cervical specimens from each participant (Honest Uterine Cervical Brushes Type S, Honest Medical, Tokyo, Japan). The first sample was smeared onto a microscope slide, fixed with alcohol solution (Rapid Fix, Muto, Tokyo, Japan), and stained according to the Pap test. The second sample was suspended in 1 ml of cell lysis buffer (50 mM Tris–HCl, 5 mM EDTA, 2% SDS) and stored at −80°C for DNA extraction.

Classification of Cervical Cytology

Cervical cytology was diagnosed according to the Bethesda system [Solomon et al., 2002] and classified as normal (negative for intraepithelial lesion or malignancy), atypical glandular cells/atypical squamous cells of undetermined significance, low-grade squamous intraepithelial lesion, high-grade squamous intraepithelial lesion, or adenocarcinoma in situ.

Detection and Typing of HPV DNA

DNA was extracted from cervical cells using a DNA extraction kit (SMI test; Genome Science Laboratories, Fukushima, Japan) according to the manufacturer's instructions. The quality of the extracted DNA was evaluated by amplifying the glyceraldehyde-3-phosphate dehydrogenase gene (primers: 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′) [Fujimori et al., 2002]. All but one of the samples, (n = 369 of 370) were confirmed as adequate for HPV, *C. trachomatis*, and *N. gonorrhoeae* testing.

HPV DNA detection was carried out using three pairs of modified GP5+/6+ primers: GP5+M1-2 (5′-TTTRTTACTGTTGTWGATACTAC-3′); GP5+M2-2 (5′-TGTWACTGTTGTWGATACCAC-3′); GP5+M3-2 (5′-GTWACTGTTGTRGACACCAC-3′); GP6+M1-2 (5′-AATTGAAAWATAAACTGTAAWTCATATTC-3′); GP6+M2-2 (5′-GAAACATAAAYTGTAAATCAWATTC-3′); and GP6+M3 (5′-GAAAATYTGCAAATCAWACTC-3′).

These primers were designed to amplify a 140-bp fragment of the HPV *L1* gene. Amplification was performed as follows: one cycle at 95°C for 10 min followed by 45 cycles at 95°C for 30 sec, 45°C for 30 sec, and 74°C for 30 sec, with a final extension at 74°C for 10 min. The presence of HPV DNA was confirmed by ethidium bromide staining of the PCR products following agarose gel electrophoresis. HPV DNA-negative samples were retested using the original GP5+/6+ primers [de Roda Husman et al., 1995; van den Brule et al., 2002]. The PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and

sequenced as described previously [Ndembi et al., 2003]. The similarity between *L1* sequences obtained by PCR and those of various HPV genotypes in the GenBank database was determined by BLAST analysis (http:www.ncbi.nlm.nih.gov/BLAST/). Ten clones from each sample were analyzed. HPV types were classified as high-risk (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73, and -82), low-risk (HPV-6, -11, -40, -42, -43, -44, -54, -61, and -81), unknown risk (HPV-26, -30, -32, -34, -53, -55, -62, -66, -67, -70, -74, -83, -84, -86, -87, -90, -91, and -102), epidermodysplasia verruciformis (HPV-9 and -38), and unclassified types (JEB2 and unclassified), according to previous reports [Muñoz et al., 2003; Schiffman et al., 2005].

Detection of *C. trachomatis* and *N. gonorrhoeae*

C. trachomatis and *N. gonorrhoeae* were detected using the LAMP method as described elsewhere [Hong et al., 2004; Poon et al., 2005].

Statistical Analysis

Statistical analysis was performed using SPSS Version 15.0 J for Windows. Odds ratio (ORs) and 95% confidence intervals (CIs) were calculated as approximations of relative risks. Univariate analyses were performed to assess the association between HPV infection and demographic factors and between abnormal cervical cytology and HPV types. Any variables shown significant in univariate analysis were analyzed by a multivariate model. The level of statistical significance was set at $P < 0.05$.

Nucleotide Sequence Accession Numbers

GenBank accession numbers of the sequences reported in this study are EU911006–EU911930.

RESULTS

Risk Factors for Cervical HPV Infection

This study evaluated the presence of HPV, *C. trachomatis*, and *N. gonorrhoeae* DNA in cervical samples from 369 commercial sex workers (mean age ± SD: 24.5 ± 5.1 years; range: 18–40 years) working in Manila, Philippines. HPV DNA was detected in 198 of the 369 women by PCR using modified GP5+/6+ primers and in 13 of the remaining 171 women using the original GP5+/6+ primers. Ultimately, 211 (57.2%) women were positive for HPV DNA. *C. trachomatis* and *N. gonorrhoeae* DNA were detected in 84 (22.8%) and 24 (6.5%) women, respectively.

In order to determine the risk factors for cervical HPV infection, multivariate analysis using a logistic regression model was performed. Being 25 years of age and younger (OR: 2.5; 95% CI: 1.4–4.3) and having worked for at least 6 months to 2 years (OR: 3.3; 95% CI: 1.9–5.8) were significantly associated with HPV infection (Table I). Cervical *C. trachomatis* and *N. gonorrhoeae* infections,

TABLE I. Demographic Factors Associated With HPV Infection

Demographic factors	No. of subjects	No. of cases	%	Univariate analysis		Multivariate analysis		
				OR	95% CI	OR[a]	95% CI	*P*-value
Age (years)								
>25	127	59	47	1		1		
≤25	239	150	63	1.9	1.3–3.0	1.9	1.1–3.4	0.028
No answer	3	2	67					
Marital status								
Married	37	19	51	1		1		
Single	326	189	58	1.3	0.7–2.6	1.3	0.6–2.7	0.484
No answer	6	3	50					
Duration of sex work (months)								
≤6	120	58	48	1		1		
7–24	137	100	73	2.9	1.7–4.9	3.3	1.9–5.8	<0.0001
>24	97	45	46	0.9	0.5–1.6	1.5	0.8–2.8	0.212
No answer	15	8	53					
Age at first coitus (years)								
≥20	103	56	54	1		1		
<20	261	153	59	1.2	0.8–1.9	1.0	0.6–1.6	0.885
No answer	5	2	40					
C. trachomatis								
Negative	285	161	57	1		1		
Positive	84	50	60	1.1	0.7–1.9	1.2	0.7–2.1	0.507
N. gonorrhoeae								
Negative	345	195	57	1		1		
Positive	24	16	67	1.5	0.6–3.7	1.3	0.5–3.2	0.609

OR, odds ratio; CI, confidence interval.
[a]Adjusted for all other variables in the table.

marital status, and age at sexual debut were not significantly associated with HPV infection. Interestingly, having worked longer than 24 months did not increase the risk when compared with working at least 6 months (OR: 1.5; 95% CI: 0.8–2.8).

Profile of HPV Infection

Of the 211 women with HPV infection, 117 (55.5%) had a single-type infection and 94 (44.5%) had multiple-type infection. Among the infected women, 46 different HPV types were detected; HPV-52 (16.1%) was most prevalent, followed by HPV-66 (12.3%), -16 (11.8%), -45 (10.0%), and -67 (9.5%). One hundred eighty-two women (86.3%) were infected with high-risk types, 38 (18.0%) were infected with low-risk types, and 28 (13.3%) were infected with unknown-risk types. Among those with high-risk HPV infection, HPV-52 (18.7%) was the most common, followed by HPV-16 (13.7%) and -45 (11.5%). HPV-16 and -18 comprised only 20.3% of the high-risk HPV types (Fig. 1).

Risk Factors for Abnormal Cervical Cytology

Among the 369 women, 239 (64.8%) had normal cytology, 74 (20.1%) had atypical glandular cells/atypical squamous cells of undetermined significance, and the remaining 56 (15.2%) had abnormal cytology (low-grade squamous intraepithelial lesion, n = 42; high-grade squamous intraepithelial lesion, n = 12; adenocarcinoma *in situ*, n = 2). HPV DNA was detected in 91 (38.1%) of the 239 women with normal cytology, in 61 (82.4%) of the 74 women with atypical glandular cells/atypical squamous cells of undetermined significance, and in all (100%) of the 56 women with abnormal cytology. Stepwise regression analysis revealed that HPV infection was only the factor significantly associated with abnormal cytology ($P < 0.0001$; OR: 18; 95% CI: 7.6–52).

Twenty-five different HPV types were detected in the 56 women with abnormal cervical cytology. Of these types, HPV-52 was most prevalent (23.2%), followed by HPV-16 (19.6%), -58 (10.7%), -66 (10.7%), and -67 (10.7%). Multivariate analysis using a logistic regression model revealed that HPV-16, -39, -52, -67, and -82 were significantly associated with abnormal cytology (Table II).

PCR Using Modified Versus Original GP5+/6+ Primers

In order to evaluate the efficacy of the modified primers, HPV DNA samples from women with single-type infection according to PCR with modified GP5+/6+ primers were re-analyzed using the original GP5+/6+ primers (Table III). None (0%) of the seven women with HPV-16 infection, eight (57%) of 14 women with HPV-52 infection, and one (20%) of five women with HPV-67 infection tested negative for HPV DNA using the original GP5+/6+ PCR.

DISCUSSION

In this study, the prevalence of HPV infection among female commercial sex workers in the Philippines was 57.2%. The reported prevalences of HPV infection in this

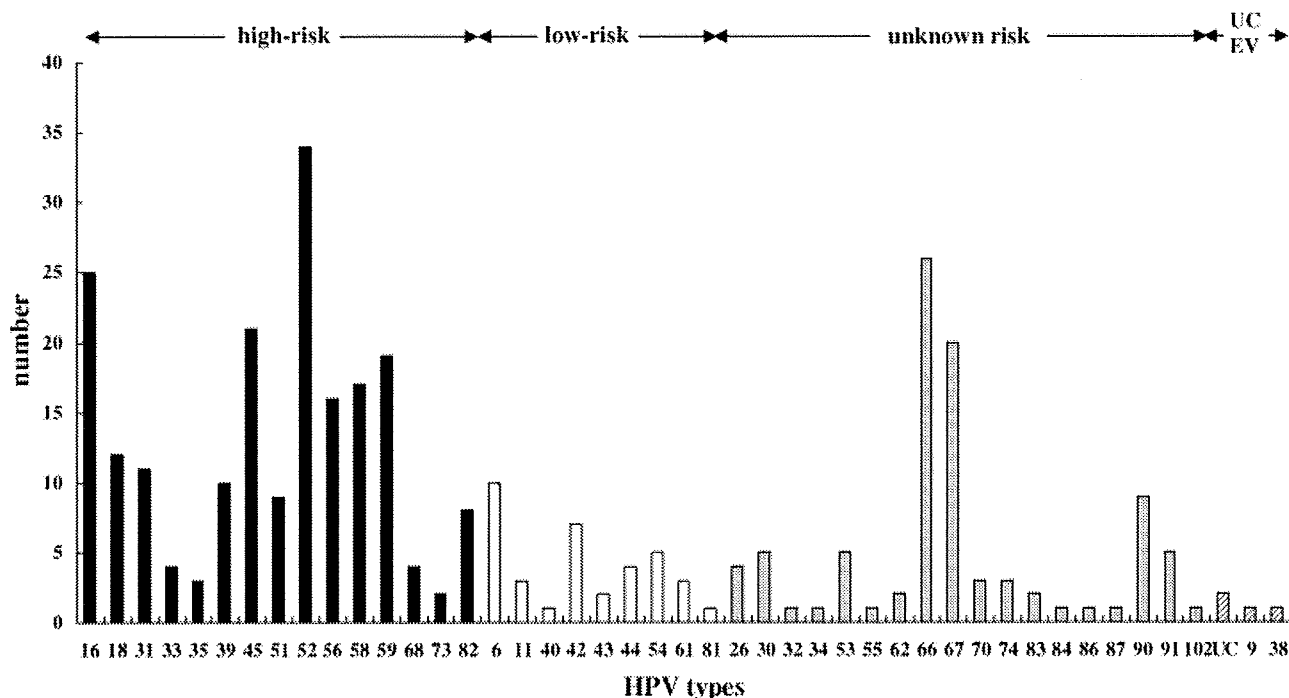

Fig. 1. The prevalence of HPV types among female commercial sex workers (n = 211) in the Philippines. ■, high-risk type; □, low-risk type; ▒, unknown-risk type; ▨, unclassified (UC) and epidermodysplasia verruciformis (EV) types.

TABLE II. HPV Types Associated With Abnormal Cervical Cytology*

HPV type	No. (%) of study participants positive for HPV DNA (n = 295): With abnormal cervical cytology (n = 56)	With normal cytology (n = 239)	Univariate analysis: OR (95% CI)	Multivariate analysis: OR[a] (95% CI)	P-value
16	11 (19.6)	10 (4.2)	5.6 (2.2–14)	7.3 (2.7–20)	<0.0001
18	4 (7.1)	6 (2.5)	3.0 (0.8–11)		
26	1 (1.8)	2 (0.8)	2.2 (0.2–24)		
30	2 (3.6)	2 (0.8)	4.4 (0.6–32)		
31	3 (5.4)	5 (2.1)	2.6 (0.6–11)		
34	1 (1.8)	0 (0.0)	—		
35	1 (1.8)	1 (0.4)	4.3 (0.3–70)		
39	5 (8.9)	2 (0.8)	12 (2.2–62)	14 (2.4–83)	0.004
40	1 (1.8)	0 (0.0)	—		
43	1 (1.8)	1 (0.4)	4.3 (0.3–70)		
44	1 (1.8)	2 (0.8)	2.2 (0.2–24)		
45	4 (7.1)	11 (4.6)	1.6 (0.5–5.2)		
51	3 (5.4)	3 (1.3)	4.5 (0.9–23)		
52	13 (23.2)	9 (3.8)	7.7 (3.1–19)	10 (3.9–28)	<0.0001
53	1 (1.8)	4 (1.7)	1.1 (0.1–9.7)		
54	2 (3.6)	0 (0.0)	—		
56	5 (8.9)	9 (3.8)	2.5 (0.8–7.8)		
58	6 (10.7)	8 (3.3)	3.5 (1.2–10)	3.4 (0.96–12)	0.057
59	2 (3.6)	11 (4.6)	0.8 (0.2–4.0)		
66	6 (10.7)	11 (4.6)	2.5 (0.9–7.0)		
67	6 (10.7)	7 (2.9)	4.0 (1.3–12)	4.3 (1.2–15)	0.024
82	3 (5.4)	2 (0.8)	6.7 (1.1–41)	7.6 (1.0–55)	0.046
86	1 (1.8)	0 (0.0)	—		
90	1 (1.8)	6 (2.5)	0.7 (0.1–6.0)		
91	1 (1.8)	3 (1.3)	1.4 (0.1–14)		

OR, odds ratio; CI, confidence interval.
*Abnormal cervical cytology: low-grade squamous intraepithelial lesions, high-grade squamous intraepithelial lesions, and adenocarcinoma in situ.
[a]Adjusted for HPV-16, -39, -52, -58, -67, and -82.

population in other countries are 28–39% in Spain [Cañadas et al., 2004; del Amo et al., 2005; Ortiz et al., 2006], 32% in Australia [Tideman et al., 2003], 47% in Korea [Choi et al., 2003], and 55% in Japan [Ishi et al., 2000]. Thus, the prevalence of HPV infection in this group of women in the Philippines is similar to that in Japan and Korea.

For the women in this group, being 25 years old or younger and having worked for a period of 6 months to 2 years were significantly associated with HPV infection, consistent with reports that the prevalence of HPV infection increases with decreasing age [Burk et al., 1996; Hassen et al., 2003; Matos et al., 2003; Baseman and Koutsky, 2005]. As expected, women who had worked longer than 6 months had a higher risk of being infected with HPV than did women who had worked <6 months. However, working longer than 2 years did not increase the risk of HPV infection, suggesting that these women may have acquired some immunity against common HPV types within 2 years of beginning this type of work. The role played by immunity against HPV in decreasing the risk of infection is supported by data from a recent report showing that age-dependent decrease in the prevalence of HPV is attenuated in HIV-infected women in Kenya [Yamada et al., 2008]. In the current study, no information was available for variables such as condom usage by sex partners, smoking, education, area of origin, and annual income,

TABLE III. Modified Versus Original GP5+/6+ PCR for Detecting HPV DNA

HPV infection	No. of positive samples: HPV-16 (n = 23) Modified[a]	HPV-16 (n = 23) Original[b]	HPV-52 (n = 34) Modified	HPV-52 (n = 34) Original	HPV-67 (n = 19) Modified	HPV-67 (n = 19) Original
PCR positive	23	22	34	23	19	17
Multiple infection	16	15	20	17	14	13
Single infection	7	7	14	6	5	4

[a]Modified: PCR using modified GP5+/6+ primers.
[b]Original: PCR using original GP5+/6+ primers.

which could be factors associated with HPV infection. The absence of these data could limit the types of conclusions that can be drawn from this study.

In the current study, HPV genotyping was performed by sequencing cloned PCR products. In previous studies, direct sequencing or hybridization with HPV type-specific oligo-probes has been used for HPV genotyping. Although these methods are easier and quicker than the method used in this study, their results can sometimes be difficult to interpret [Qu et al., 1997; Coutlée et al., 2002; Perrons et al., 2002; Asato et al., 2004; Gheit et al., 2006]. The direct sequencing method rarely detects multiple-type HPV infection, whereas the hybridization method can detect only HPV types for which probes are available and cross-hybridization of type-specific probe with untargeted HPV types can occur in the dot-blot hybridization method. In contrast, sequencing of cloned PCR products can detect multiple-type HPV infection and identify distinct HPV types. In fact, 46 different HPV types and many cases of multiple-type HPV infections (44.5%) were identified in this study. Therefore, sequencing of cloned PCR products should be considered a preferred method for assessing HPV infection, especially multiple-type infections. However, unless a sufficiently large number of clones are analyzed, a number of types might not be detected, especially in cases of multiple infection with three or more types.

HPV-52 was found to be the most prevalent infecting HPV type in the Philippines; this is not the case in western countries. This difference could be due in part to differences in the methods for detecting HPV DNA. In this study, 57% of the women with single-type HPV-52 infection detected by the modified GP5+/6+ PCR were missed by the original GP5+/6+ PCR, which has been used in previous studies. In contrast, there was no significant difference in the detection of HPV-16 and -67 DNA between modified and original GP5+/6+ PCR, suggesting that the prevalence of HPV-52 may have been underestimated in previous studies.

In the current study, high-risk HPV types were detected in 86% of female commercial sex workers with HPV infection; however, HPV-16 and -18 were detected in only 20% of these women and the most prevalent HPV type was HPV-52. Although HPV-16 is known to be the most prevalent type worldwide [Muñoz et al., 2003; Wheeler, 2007], it has been reported that HPV-52 and -58 are also prevalent in Japan and South Taiwan [Asato et al., 2004; Inoue et al., 2006; Lin et al., 2006]. These results suggest that in addition to HPV-16, HPV-52 may be common in Asian countries in general.

HPV-16, -39, -52, -67, and -82 were found to be significantly associated with abnormal cytology in this study group. HPV-16 and -18 were detected in only 27% of the women with abnormal cervical cytology. Furthermore, HPV-18, -34, -45, and -59 were identified in cases of adenocarcinoma in situ. These results suggest that many high-risk types of HPV other than HPV-16 and -18 might play important roles in cervical carcinogenesis in the Philippines. This is the first study to examine the prevalence of HPV types and their association with abnormal cervical cytology in the Philippines.

The results from clinical trials of first generation vaccines in humans look promising. The data show that an HPV vaccine can prevent HPV infection and precancerous lesions in vaccinated women [Koutsky et al., 2002]. Most HPV vaccines target both HPV-16 and -18 and have been shown highly effective for preventing type-specific HPV infections [Harper et al., 2006; Wheeler, 2007]. However, in the current study, high-risk types of HPV other than HPV-16 and -18, such as HPV-39, -52, -67, and -82, were significantly associated with abnormal cervical cytology in women in the Philippines. In addition, it has been reported that in Japan not only HPV-16 and -18 but also HPV-31, -33, and -58 are significantly associated with cervical cancer (OR > 100) [Asato et al., 2004]. Therefore, the current vaccines might not be sufficient for preventing premalignant and malignant lesions of the cervix in women in Asia, although some cross protection of the vaccine has been reported [Harper et al., 2006]. Area-specific vaccines might be needed. Alternatively, the development of type-common HPV vaccines might be more ideal, in light of data showing that a type-common neutralization epitope exists in minor capsid protein *L2* and that a vaccine using the *L2*-epitope is expected to be effective in preventing infection by all high-risk types of HPV [Kondo et al., 2007].

In conclusion, this study determined that HPV-52 is the most prevalent infecting HPV type among female commercial sex workers in the Philippines and that several high-risk HPV types other than HPV-16 and -18 are significantly associated with abnormal cytology. Therefore, the current vaccines may not be sufficient to prevent HPV infection and the subsequent development of pre-malignant lesions of the cervix in women in the Philippines.

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AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 25, Number 2, 2009
© Mary Ann Liebert, Inc.
DOI: 10.1089/aid.2008.0193

Profile of HIV Type 1 Infection and Genotypic Resistance Mutations to Antiretroviral Drugs in Treatment-Naive HIV Type 1-Infected Individuals in Hai Phong, Viet Nam

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Abstract

We evaluated the prevalence and profile of antiretroviral treatment (ART)-associated resistance mutations among HIV-1 strains in northern Vietnam by genotypically analyzing strains isolated from ART-naive individuals in Hai Phong, a city in which HIV-1 is highly prevalent. Plasma samples were collected from injecting drug users (IDU, $n = 760$), female sex workers (FSW, $n = 91$), seafarers ($n = 94$), pregnant women ($n = 200$), and blood donors ($n = 210$), and screened for HIV-1 antibodies. Plasma viral RNA was extracted from HIV-1-positive samples, amplified by reverse transcriptase (RT)-PCR of protease and RT genes, and analyzed for genotypes and ART-associated resistance mutations. HIV-1 prevalence among IDU, FSW, seafarers, pregnant women, and blood donors was 35.9%, 23.1%, 0%, 0.5%, and 2.9%, respectively. Phylogenetic analyses revealed that the most prevalent HIV-1 subtype was CRF01_AE (98.3%), similar to strains prevalent in southern China. Four (1.4%) subtype B strains and one (0.3%) unique recombinant between subtypes B and C were also identified. We found protease inhibitor-associated major resistance mutations in one of the 294 cases analyzed (0.3%; mutation M46I). We found RT inhibitor-associated major resistance mutations in 7/273 cases (2.6%; one occurrence each of L74I, M184I, and K219E; three cases of K103N; and two cases of G190E). One CRF01_AE strain harboring a protease codon 35 insertion was first identified in Vietnam. Thus, monitoring of drug-resistant HIV-1 and establishment of a database are required for the proper selection of ART in Vietnam.

Introduction

Combination antiretroviral drug therapy (ART) using reverse-transcriptase inhibitors (RTI) and protease inhibitors (PI) has been the gold standard for HIV/AIDS treatment since the late 1990s, and the prognosis for HIV/AIDS patients has correspondingly improved dramatically in developed countries. In contrast, many human immune deficiency virus type 1 (HIV-1)-infected individuals in developing countries were not able to access antiretrovirals (ARV) until early 2000, due mainly to the high price. However, the World Health Organization (WHO), the Joint United Nations Programme on HIV/AIDS (UNAIDS), and other international donors have been promoting the intensive introduction of ART to low- and middle-income countries through "3 by 5" initiatives and similar programs since 2003.

The number of people living with HIV-1 has risen steadily in Vietnam, from 122×10^3 in 2000 to 283×10^3 in 2006.[1] HIV-1 infection was first recognized in southern Vietnam in 1990 and had spread to all of the Vietnamese provinces by 2006 with variable epidemic status.[2] The majority of people infected with HIV-1 in Vietnam are intravenous drug users (IDU) and their sex partners.[1,3,4] A large number of governmental, civilian, and international programs have been implemented to reduce endemic HIV-1 infection in Vietnam, and the availability of treatment, care, and support programs for HIV-1-infected individuals has also increased in scale.[2] Beginning in 2003, ART has been intensively introduced to

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Vietnam by the Vietnamese government, WHO, and international donors, resulting in an increase in ART coverage of HIV-1-infected individuals from 1% in 2003 to 11% in 2005 and 28.4% in 2007.[2,5]

As ART is introduced into resource-limited countries, the appearance and spread of ART-resistant HIV-1 have become an emerging problem. In Ho Chi Minh City (HCM) in southern Vietnam, drug-resistant HIV-1 among ART-naive HIV-infected individuals was reported to be 6.5% in 2003, a time when ART was not yet common in Vietnam.[6] In Hanoi, the capital of Vietnam and located in the northern part of the country, the HIV drug-resistance threshold survey was conducted for the specimens collected in 2006 and showed low prevalence (<5%) of transmitted HIV-1 drug resistance to all drugs and drug classes evaluated.[7] However, little information is available regarding the current status of drug-resistant HIV-1 in Vietnam, where CRF01_AE is reported to be the predominant strain,[6,8–11] though further increases in drug-resistant HIV-1 are expected.

There are several well-established drug-resistance databases for subtype B HIV-1, which accounts for only 10% of the global HIV/AIDS pandemic.[12] However, databases for non-B subtypes, which are prevalent mainly in resource-limited countries, are far from comprehensive. It is important to investigate ARV resistance-associated mutations of non-B subtype HIV-1 strains, and to establish a database so that appropriate ARVs can be selected for individuals infected with ARV-resistant strains of HIV-1.

In the current study, we investigated the prevalence and profile of ARV resistance-associated mutations among ART-naive HIV-1-infected individuals in Hai Phong, a city in northern Vietnam in which HIV-1 is highly prevalent.[5]

Materials and Methods

Study population

Residents of Hai Phong, the largest port city in northern Vietnam, were invited to join this study in 2007. The participants had different risks of HIV infection and were categorized into five groups: (1) IDUs, who were concentrated in rehabilitation centers in Hai Phong ($n = 760$, all male, mean age: 34.1 years old, age range: 19–65); (2) female sex workers (FSW), who had previously been commercial sex workers and were concentrated in a rehabilitation center ($n = 91$, mean age: 24.8 years old, age range: 17–42); (3) seafarers, who worked for marine companies ($n = 94$, all male, mean age: 32.5 years old, age range: 20–56); (4) pregnant women, who attended antenatal clinics ($n = 200$, mean age: 30.8 years old, age range: 15–50); and (5) blood donors ($n = 210$; female/male: 69/140, one person whose sex was not known; mean age: 31.2 years old; age range: 16–58). None of the participants had any previous history of ART.

After thorough ethical clearance and informed consent, we collected blood samples from the participants from April to October in 2007. Plasma samples that were found to be reactive for HIV-1 antibody with an immunochromatography assay kit (Determine HIV ½; Abbott Japan, Tokyo, Japan) were confirmed with Western blotting (New Lab Blot 1, Bio-Rad Laboratories, Tokyo, Japan) and included in this study. The study protocol was reviewed and approved by the ethical committees of Hanoi Medical University in Vietnam and Kanazawa University in Japan.

Extraction and amplification of plasma HIV-1 viral RNA

HIV-1 RNA was extracted from 100 μl of HIV-1-positive plasma using SMITEST EX-R&D nucleotide extraction kit (Genome Science Laboratories, Fukushima, Japan) according to the manufacturer's instructions. Amplification of the HIV-1 *pol* gene, which encodes reverse transcriptase and protease, was performed by both one-step RT-PCR (SuperScript III One-step RT-PCR system with Platinum Taq DNA polymerase; Invitrogen, Carlsbad, CA) and nested PCR using AmpliTaq Gold (Applied Biosystems, Japan) and/or KOD FX (Toyobo, Osaka, Japan).

A region of the HIV-1 *pol* gene that includes the protease sequence (*pol-PR*, corresponding to nucleotides 2148–2611 in HIV-1_{HXB2}) was amplified by nested RT-PCR with primers DRPRO5 (5′-AGACAGGYTAATTTTTTAGGGA-3′) and DRPRO2L (5′-TATGGATTTTCAGGCCCAATTTTTGA-3′) in the first round and DRPRO1M (5′-AGAGCCAACAGCCCC ACCAG-3′) and DRPRO6 (5′-ACTTTTGGGCCATCCATT CC-3′) in the second round. A region of the HIV-1 *pol* gene that includes parts of the RT sequence (*pol-RT*, corresponding to nucleotides 2485–3372 in HIV-1_{HXB2}) was amplified by nested RT-PCR with primers DRRT1L (5′-ATGATAGGGGGAATTG GAGGTTT-3′) and RTout (5′-ATATACTCCATGCACAGG GGTTTT-3′) in the first round, and DRRT7L (5′-GACCTA CACCTGTCAACATAATTGG-3′) and DRRT6L (5′-TAATC CCTGCATAAATCTGACTTGC-3′) in the second round. For the amplification of HIV-1 *pol-RT*, the primer pairs RT18/K104 and K101/K102[13] were also used in the first and second rounds, respectively.

RT-PCR was performed with one cycle at 55°C for 30 min and one cycle at 94°C for 2 min, then 40 cycles at 94°C for 15 s, 55°C (for DRPRO5/DRPRO2L and RT18/K104 primer pairs) or 50°C (for the remaining primer sets) for 30 s, and 68°C for 1 min, with a final extension of 68°C for 5 min, using the One-step RT-PCR system (Invitrogen). Nested PCR for *pol-PR* was done with one cycle at 95°C for 10 min, followed by 40 cycle at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min, using AmpliTaq Gold. Nested PCR for *pol-RT* was done with one cycle at 94°C for 1 min, and 35 cycles at 98°C for 10 s, 55°C for 30 s, and 68°C for 1.5 min, using KOD FX (Toyobo, Japan). PCR amplification was confirmed by ethidium bromide staining of samples electrophoresed on an agarose gel. The amplified products were directly sequenced and analyzed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) with BigDye Terminator v1.1 (Applied Biosystems).

Genotype and drug-resistance determination

The sample nucleotide sequences were aligned with HIV-1 subtype/CRF reference sequences from the Los Alamos database and previously reported sequences of HIV-1 strains isolated from Southeast Asia and southern China using CLUSTAL W (version 1.83), with minor manual adjustments. Phylogenic trees were constructed and visualized as described previously.[13] Reference HIV-1 strains (accession number) used in this study were as follows: for subtype B, HXB2 (K03455) and China Yunnan RL42 (U71182); for subtype C, India (AF067155); for CRF01_AE, Thai CM240 (U54771), China Fujian (DQ859180), China Guangxi 2F (AY008714), China Guangxi 11F (AY008718), Vietnam HCM vr79 (AY238295 and AY238028), Vietnam HCM vr115 (AY238279

and AY238024), Vietnam HCM vr135 (AY238242 and AY238088), China Yunnan (AB213669), and China Liaoning (EF122521); for CRF15_01B, Thai (AF516184); for CRF 01B, Myanmar CSW (AB097866) and Myanmar IDU (AB097865); for CRF07_BC, China Xinjiang (AF286226) and China Yunnan (AB213675); for CRF08_BC, China Gansu (AF286229) and China Guangxi (AY008716); and as an outgroup, SIVcpz (X52154). To improve the accuracy of HIV-1 subtyping, we used the National Center for Biotechnology Information (NCBI) genotyping tool (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi) and the REGA subtyping tool (http://dbpartners.stanford.edu/RegaSubtyping/), as needed.

The *pol-PR* and a part of *pol-RT* nucleotide sequences (297 bps and 660 bps, respectively) were translated into the corresponding 99 and 220 amino acids, respectively. Using the Stanford University HIVdb sequence analysis program (http://hivdb.stanford.edu/pages/algs/HIVdb.html) and the International AIDS society-USA Spring 2008 list,[14] we analyzed the amino acid sequences for those ARV resistance-associated major and minor mutations that had been previously reported mainly in subtype B strains.

Results

HIV-1 prevalence

Of the 1355 individuals from five different groups in Hai Phong, 301 were positive for HIV-1 antibodies. The prevalence of HIV-1 among IDU, FSW, seafarers, pregnant women, and blood donors was 35.9% (273/760), 23.1% (21/91), 0% (0/94), 0.5% (1/200), and 2.9% (6/210), respectively.

Subtype distribution

Of the 301 HIV-1-positive samples, 272 could be analyzed in both the *pol-PR* and the *pol-RT* regions, 22 could be analyzed in the *pol-PR* region only, and one could be analyzed in the *pol-RT* region only. A total of 295 samples were successfully analyzed in the *pol-PR* and/or *pol-RT* region. The subtype or circulating recombinant form (CRF) of each sample was identified. Of the 295 HIV-1 strains, 290 (98.3%) were CRF01_AE, four (1.4%) were subtype B, and one (0.3%) was subtype B/C recombinant. Of these, 19 of the CRF01_AE strains and three of the subtype B strains were identified based on *pol-PR* sequences, one subtype B strain was identified based on the *pol-RT* sequence, and the remaining strains were identified based on both *pol-PR* and *pol-RT* sequences.

Phylogenetic analyses also revealed that most of the CRF01_AE strains from Hai Phong were similar to one another, and distinct from strains of HCM, Thai, and China Yunnan strains; however, a few of the Hai Phong strains were similar to the HCM strains. It is noteworthy that the CRF01_AE strains from Hai Phong were phylogenetically indistinguishable from the China Guangxi strains (Fig. 1).

The subtype-B/C recombinant strain found in this study was relatively similar to CRF08_BC strains from Guangxi province, in southern China (Fig. 1). However, further analysis with the Recombination Identification Program (RIP; Los Alamos National Laboratory, Los Alamos, NM) showed that a crossover event had taken place in the recombinant at a point in the *pol-RT* region different from that of the Guangxi CRF08_BC strain (data not shown).

PI resistance-associated mutations

Of the 294 cases that we analyzed, one (0.3%) had a strain with a major PI resistance-associated mutation, M46 I (a "flap" mutation); its determined subtype was CRF01_AE (Table 1A).

Minor PI resistance-associated mutations were also observed and are listed in Table 1A. M36I (99.0%) and H69K (99.3%), recently identified minor resistance mutations to the new PI tipranavir, were frequently observed in CRF01_AE strains and are considered to be natural polymorphisms. One strain (0.3%) harbored PR codon 35, a glutamic acid insertion (E35E_E). R41K (99.0%) and L89M (98.6%), which are not known to cause PI resistance, were also frequently observed in the *pol-PR* region of CRF01_AE strains (Table 2).

RTI resistance-associated mutations

Of the 273 cases analyzed, three (1.1%) had strains with major nucleoside reverse transcriptase inhibitor (NRTI)-resistance mutations: one case each with L74I, M184I, and K219E. Five (1.8%) cases had strains with major nonnucleoside reverse transcriptase inhibitor (NNRTI)-resistance mutations: three cases with K103N and two cases with G190E. One case (0.3%) had a strain that harbored both the M184I and the K103N mutation. Hence, the overall prevalence of RTI-resistance mutations was 2.6% (Table 1B and C).

We also observed the minor mutations V90I, V106I, and V179 D/F, which are related to the resistance of the new NNRTI etravirine. Other polymorphisms that are not associated with RTI resistance are summarized in Table 3. We did not identify any strains that harbored both PI-resistance and RTI-resistance mutations together.

All of the HIV-1 strains with major mutations were found to be CRF01_AE and only from the IDU group, which consisted of men.

Discussion

In the current study, we found ARV resistance-associated major mutations in 2.9% of our study population of ART-naive HIV-1-infected individuals in Hai Phong, Vietnam. To our knowledge, this is the first report on the current status of ARV-resistant HIV-1 strains in Hai Phong, northern Vietnam.

The prevalence of HIV-1 was first investigated among various risk groups in Hai Phong. Among IDU, it was found to be 35.9%, which is lower than the percentages reported by UNAIDS of 57.8% in 2005[2] and 65.8% in 2006.[5] This difference may be explained by the fact that the IDU in our study were recruited from rehabilitation centers and were not actively injecting drugs at the time of the study. Among FSW, the prevalence was 23.1%, which is far higher than what was reported by UNAIDS, which was 5.6% in 2005[2] and 7.2% in 2006.[5] These differences may be due to differences in the criteria used for FSW between our study and the study by UNAIDS. In our study, past and present FSW were recruited regardless of their history of injected drug use. It has been reported that around 30% of FSW inject drugs in Vietnam.[3,4,15–17] Therefore, it is very possible that several FSW in our study have had a history of injected drug use. The prevalence of HIV-1 in the pregnant women (0.45%), one of the representatives of the general population in our survey, was similar to that in UNAIDS reports in 2005.[2]

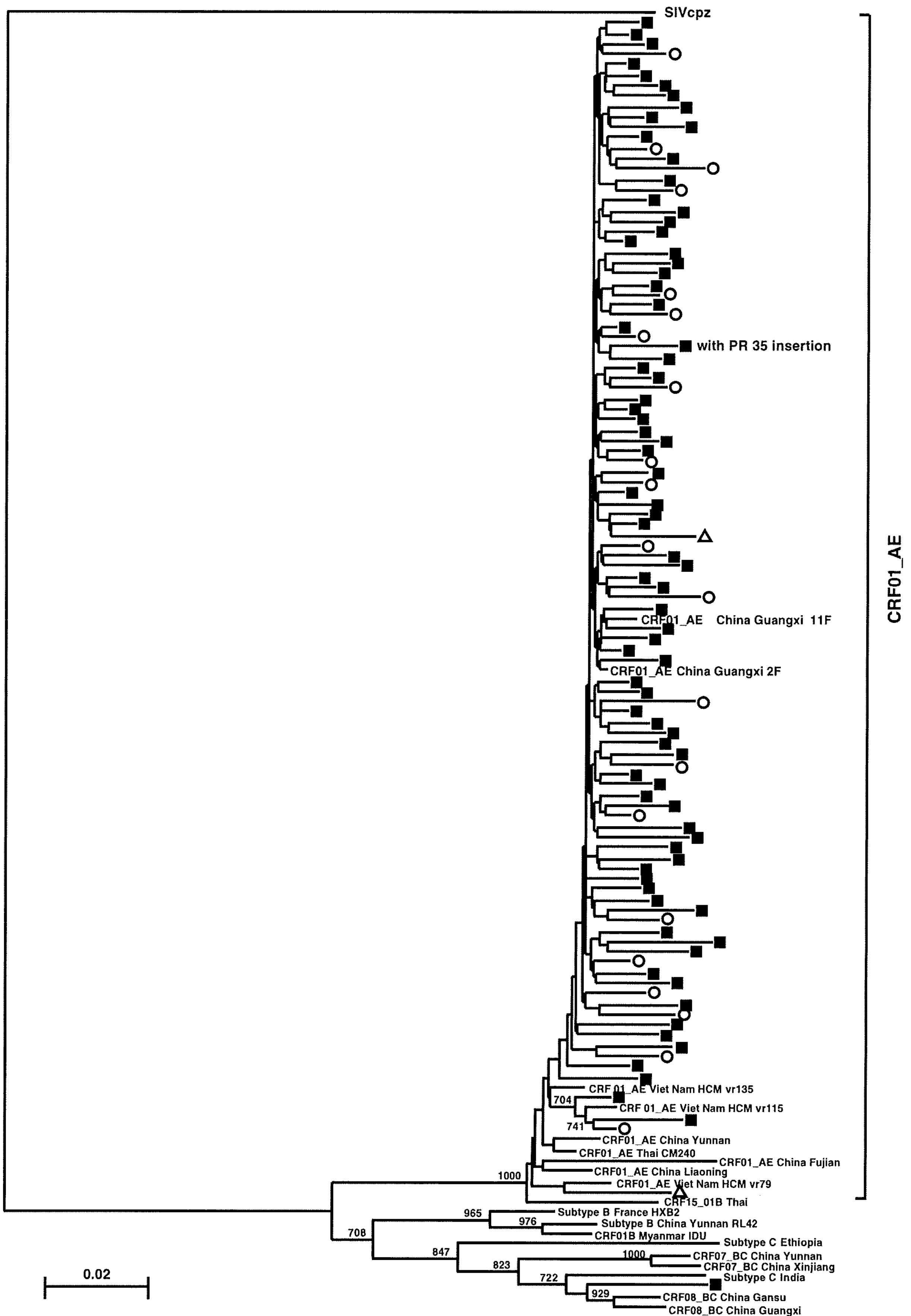

FIG. 1. Phylogenic tree of representative HIV-1 strains from IDU, all 21 strains from FSW, and two strains from blood donors, based on the *pol-PR* and *pol-RT* genes (approximately 957 bases). Filled squares, representative HIV-1 strains from IDU; open circles, HIV-1 strains from FSW; and open triangles, HIV-1 strains from blood donors. Boot strap values greater than 700 are shown.

TABLE 1. AMINO ACID SUBSTITUTIONS ASSOCIATED WITH RESISTANCE TO (A) PROTEASE INHIBITORS (PI), (B) NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NRTI), AND (C) NONNUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NNRTI)[a]

(A) Position		L10	V11	I13	G16	K20	*E35*	M36	**M46**	D60	I62	L63	I64	H69	A71	V77	V82	I93
01_AE	290	I (15) V (7)	I (1)	V (233)	E (68)	R (28) I (7) T (1)	*D (234)* *N (1)* *E insertion (1)*	I (287) V (1)	**I (1)**	E (6)	V (6)	P (25)	L (1) M (1)	K (288)	T (1) V (1)	I (1)	I (8)	L (45)
B+C	1							V (1)		E (1)				K (1)				L (1)
B	3																	

(B) Position		T69	L74	V75	V118	**M184**	*L210*	**K219**
01_AE	271	N (1) S (2)	I (1)	G (1)	I (1)[b]	**I (1)**[c]	*M (1)*	**E (1)**
B+C	1							
B	1							

(C) Position		V90	**K103**	V 106	*V 179*	**G190**	*P225*
01_AE	271	I (1)	**N (3)**	I (4)	*D (4)* *F (1)* *E (1)* *A (1)*	**E (2)**	*S (1)*
B+C	1	I (1)					
B	1						

[a]Text, minor mutations; boldface text, major amino acid mutations associated with drug resistance; italic text, PI- or NRTI-selected mutations, the significance of those substitutions is not known, or atypical substitutions.
[b]The significance of single V118I is unknown.
[c]With K103N.

HIV-1 CRF01_AE strains were found to predominate in Hai Phong in northern Vietnam, the same as was previously reported in both northern and southern Vietnam.[6–12,18–23] Phylogenetic analyses revealed that the majority of these strains were closely related to strains prevalent in Guangxi, southern China, and different from strains from HCM (southern Vietnam), Thailand, and Cambodia. This is consistent with the findings of previous studies, which reported that CRF01_AE strains in the northern provinces along the Vietnam–China border were introduced from Guangxi province and reached Hanoi through heroin-trafficking routes.[10,11,22,23] The CRF01_AE strains in southern Vietnam are believed to be derived from strains in Thailand and Cambodia.[11,18,22,23] In addition, the unique subtype-B/C recombinant strain that we found in our study was relatively similar to the CRF08_BC strain from Guangxi province (Fig. 1). However, it should be noted that we found strains in Hai Phong that were similar to those in HCM, showing that there may have been some mixing of strains from northern and southern Vietnam in this area.

Phylogenetic analysis also showed that the HIV-1 strains from the IDU group formed a cluster together with the strains from the FSW and blood donor groups (Fig. 1), suggesting that the HIV-1 epidemic in Hai Phong has already begun to spread from IDU into the general population through the FSW population, as has been observed in other Asian countries.[24]

We detected major mutations that cause PI and RTI resistance in ART-naive patients at rates of 0.3% and 2.6%, respectively, in Hai Phong as of October 2007. Our result is consistent with the previous findings in Hanoi in 2006,[7] though it is slightly lower than the findings in HCM in 2003.[6] These results are to be expected, because the current first line of ART in Vietnam is a combination of two NRTIs and one NNRTI, and PI use is still limited compared with developed countries. Further monitoring of changes in HIV-1

TABLE 2. POLYMORPHISM AT THE *Pol-PR* REGION NOT ASSOCIATED WITH RESISTANCE TO PROTEASE INHIBITORS

Position		*K14*	*I15*	*Q18*	*L19*	*N37*	*P39*	*R41*	*K43*	*K45*	*R57*	*Q61*	*K70*	*I72*	*L89*	*T91*	*Q92*
01_AE	290	R(15)	L (1) V (15)	E (1) M (2)	I (1) M (2) Q (4)	D (13) K (2) S (1) T (1)	Q (1) S (2)	K (287)	R (7)	R (8)	K (14)	E (3) H/P (1) Q (1)	Q (2) R (23)	T (1) V (4)	I (2) M (286)	A (2) I (1) S (2)	K (3)
B+C	1		V (1)		V (1)			K (1)					K (1)		M (1)		
B	3																

TABLE 3. POLYMORPHISM AT THE *Pol-RT* REGION AND NOT ASSOCIATED WITH RESISTANCE TO REVERSE TRANSCRIPTASE INHIBITORS

Position		*E6*	*K11*	*K20*	*V21*	*E29*	*V35*	*E36*	*T39*	*E40*	*K43*	*S48*	*V60*	*S61*	*K101*	*K102*	*T107*
01_AE	271	D (257) K (2) N (2)	A (2) Q (1) R (2) S (3) T (219)	R (6)	I (4)	A (1) K (3)	A (1) I (1) M (1) R (1) T (258) Y (1)		A (6) E (16) G (1) K (174) L (1) N (41) Q (3) R (1) S (2)	D (3)	E (6) Q (13) R (1)		I (3)		Q (2) R (1)	E (1) Q (5) R (4)	S (9)
B+C	1						T (1)	A (1)	D (1)			T (1)	I (1)	I (1)			
B	1																

Position		*V111*	*G112*	*D121*	*K122*	*K123*	*I132*	*I135*	*T139*	*I142*	*S162*	*Y173*	*Q174*	*D177*	*I178*	*V189*	*E194*
01_AE	271	I (10)	A (3)	A (1) E (1) H (6)	E (260)	E (1) G (4) K (7) N (18) S (237)	L (6)	R (1) T (28) V (3)	A (3)	V (12)	C (255) Y (5)	A (9) I (215) L (2) M (5) R (9) T (25) V (6)	K (262) L (2) N (3) R (3)	E (266) G (1) I (1)	M (136)	I (4)	D (2) G (1) K (1)
B+C	1			Y (1)				T (1)									
B	1																

Position		*G196*	*T200*	*K201*	*I202*	*E203*	*Q207*	*R211*	*E224*	*K238*	*V245*	*K249*	*S251*	*I257*	*Q258*	*L264*	*N265*	*W266*
01_AE	271	E (9) K (1) T (1)	A (58) E (1) I (19) Q (1) R (1) V (2)	R (3)	V (4)	D (3)	A (7) D (2) G (15) K (6) N (24) R (3) S (212)	G (1) H (1) K (1) N (4) S (262) T (1)	D (1) K (2)	R (212)	A (1) E (228) K (3) Q (4)	R (10)	H (1) I (1) N (2)	M (3) V (1)	E (2) R (4)	T (1) V (2)	I (9) K (1) S (4)	C (1) E (1) F (1)
B+C	1										Q (1)							
B	1																	

drug-resistance mutations in different areas of Vietnam is needed for the proper selection of ARV in this country.

Three cases who had HIV-1 with K103N mutation were found in the treatment-naive male IDU group in our study. K103N is selected by nevirapine (NVP) and is likely to be identified among pregnant women previously enrolled in the Prevention of Mother-to-Child Transmission (PMTCT) program.[25] In Vietnam, the PMTCT program using a single-dose NVP, two- or three-combination ARV regimen was first introduced in 2006, and those prophylaxis coverage rates of HIV-infected pregnant women were increased from 9.2% in 2006 to 13.9% in 2007.[5] It would be interesting to know whether those K103N mutations found among the IDU group in Hai Phong in 2007 were from those PMTCT program population or from outside of the country, such as southern China, where HIV-1 strains closely related to the CRF_01AE strains in IDU in Hai Phong were found.

In our study, we confirmed several minor drug-resistance mutations that are considered to be CRF01_AE-specific polymorphisms in the protease gene. We detected I13V (80.0%), G16E (23.4%), M36I (99.9%), and H69K (99.3%) at high frequencies. This profile is similar to those reported in previous studies from Vietnam and Southeast Asia.[6,26–31] Although no clinical survey has shown a significant correlation between natural polymorphisms and the development of ART failure,[13,32–38] the possibility should not be excluded that these polymorphisms might negatively affect the outcome of future ART.[39] Natural polymorphisms of CRF01_AE, which were recently identified as minor resistance mutations to the newly developed PIs tipranavir/ritonavir and darunavir/ritonavir[40,41] and the NNRTI etravirine,[42] highlight the importance of monitoring non-subtype B strains when planning new antiviral drug development.

A CRF01_AE strain harboring a PR codon 35 insertion, which is known to be related to PI treatment,[43–46] was first identified in Vietnam. In Asia, PR codon 35-inserted subtype B strains were reported to be circulating among ART-naive patients in Hong Kong,[47] but not in other countries. Careful monitoring of this mutation is needed to determine whether PR codon 35-inserted strains have begun to circulate more widely in Asian countries.

In conclusion, the most prevalent HIV-1 strains in Hai Phong, northern Vietnam, were CRF01_AE, and the majority were similar to those found in southern China. The prevalence of ARV-resistant HIV-1 among ART-naive individuals in Hai Phong was 2.9% in 2007, which is slightly lower than the

prevalence in HCM in 2003[6] and consistent with the threshold survey in Hanoi in 2006.[7] Further monitoring is necessary to establish a useful database of ARV-resistant HIV-1 in Vietnam. There is also a need for a consensus algorithm, based on what is known about drug-resistance mutations in subtype B strains, which can be used to predict the clinical outcomes of people who are infected with non-subtype B HIV-1 strains.[39]

Sequence Data

The GenBank accession numbers of the sequences reported in this study are as follows: from FJ006949 to FJ007345 for *pol-PR/RT*, FJ007346 to FJ007369 for *pol-PR*, and FJ007370 for *pol-RT*.

Acknowledgments

This work was supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) in Japan (the Program of Founding Research Centers for Emerging and Reemerging Infectious Disease). The authors are grateful to all the participants in this study; the staff (Ms. Thuy, Ms. Lan, Ms. Thanh, Ms. Xuan, Ms. Huong big, and M. Huong small) of Hai Phong Medical University, Hai Phong, Vietnam; Professor Nguyen Duc Hien (National Institute of Infectious and Tropical Disease, Hanoi, Vietnam); and the members (Ms. K. Matsushita, Ms. M. Miyashita, Dr. R. Lwembe, Mr. R. Lihana, Dr. S. Do, Mr. M. Saito, Professor H. Tanii, Dr. T. Higashi, Dr. F. Nishimura, and Dr. Y. Kimura) of the Graduate School of Medical Science, Kanazawa University, Japan.

Disclosure Statement

No competing financial interests exist.

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AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 25, Number 2, 2009
© Mary Ann Liebert, Inc.
DOI: 10.1089/aid.2008.0144

Changes in the HIV Type 1 Envelope Gene from Non-Subtype B HIV Type 1-Infected Children in Kenya

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Abstract

A switch of coreceptor usage from CCR5 to CXCR4 occurs in about half of HIV-1-infected individuals in the natural course of infection. To investigate whether antiretroviral therapy (ART) enhances the coreceptor switch of HIV-1, we genotypically analyzed the env-V3 amino acid sequences from 81 HIV-1-infected children in Kenya whose plasma samples were obtained between 2000 and 2007. Of 41 children on ART, 35 had HIV-1 using CCR5 as a coreceptor at baseline. In 7 (20%) of them HIV-1 switched the coreceptor usage during the follow-up period. The mean duration of ART to the time of coreceptor switch was 2.6 years (range: 0.5–5.2). Of the remaining 40 children without ART, 32 had HIV-1 using CCR5 as a coreceptor at baseline and in 3 (9.4%) HIV-1 switched the coreceptor usage. The mean age of the children with HIV-1 coreceptor switch with and without ART was 7.3 and 9.7 years, respectively. The difference in the rate and age of coreceptor switch between treated and untreated children was not significant ($p = 0.38$ and 0.31, respectively). Of the HIV-1-infected children, 10 started ART by the age of 5 years (rapid progressors) and 23 did not need ART by the age of 10 years (slow progressors). The rate of coreceptor switch was strongly higher in rapid progressors (40%) than slow progressors (8.7%) ($p = 0.053$). These results suggest that switching of coreceptor usage from CCR5 to CXCR4 among HIV-1-infected children is not influenced by ART, but by factors responsible for rapid disease progression.

Introduction

Human immunodeficiency virus type 1 (HIV-1) coreceptor usage plays a critical role in the virus tropism. HIV-1 infection requires interactions between the viral envelope (env) glycoprotein (gp120) and cellular receptors, CD4 as a major receptor and CCR5 or CXCR4 as a coreceptor.[1] Based on the coreceptor usage, HIV-1 variants are classified as CCR5-tropic (R5 variants), CXCR4-tropic (X4 variants), and dual tropic (R5/X4 variants).[2] R5 variants are responsible for the establishment of HIV-1 infection and predominate in the early stage of HIV-1 infection.[3–5] X4 variants emerge later as disease develops.[6–9] A switch in HIV-1 coreceptor usage from CCR5 to CXCR4, which correlates with the subsequent accelerated decrease in $CD4^+$ T cell count and disease progression, occurs in the late stage of HIV-1 infection in about half of HIV-infected individuals.[10–14]

It has been reported that in HIV-1 subtype C-vertically infected children, R5 variants are predominantly transmitted and the virus may evolve to use CXCR4 as a coreceptor in older children,[15] and that X4 variants emerge later as disease develops in HIV-1 subtype B-infected children.[6–9] However, the late appearance of X4 variants with relation to disease progression is less clear in children than in adults.[16] Especially in HIV-1-infected infants disease progression was not necessarily associated with the switch in HIV-1 coreceptor usage.[17,18] In one study the coreceptor switch of HIV-1 was found in two of six rapid-progressor infants after vertical infection.[19] In another study, 14 of 15 infants with rapid disease progression harbored viruses that used CCR5 as a coreceptor, and only the remaining one had a virus that used both CCR5 and CXCR4.

In developed countries the clinical profile of HIV-1 infection in children shows a bimodal distribution, with approximately one-quarter developing severe symptoms and dying within the first 24–36 months of life (rapid progressors). Most children, however, develop AIDS more slowly, with some surviving beyond 5 years (slow progressors).[20] A similar bimodal disease pattern is seen among children living in

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developing countries,[21] although considerably less is known about the underlying virological and immunological factors, which may differ from those in developed countries.

Recently several longitudinal studies regarding the effect of highly active antiretroviral therapy (HAART) in the dynamics of evolution of HIV tropism in patients under HAART were carried out and reported a higher prevalence of X4 variants in those HIV-1-infected individuals exposed to HAART than in drug-naive individuals.[22–29] In addition, effective HAART was reported to enhance CCR5 to CXCR4 coreceptor switch.[30] However, the dynamics of viral tropism during the course of HIV-1 infection in persons exposed to antiretroviral therapy (ART) still remain unclear. Moreover, there are indeed limited reports regarding the effect of ART on HIV-1 coreceptor usage among children.

The aim of this study was to investigate the dynamics of coreceptor usage and whether ART enhanced the coreceptor switch among non-B subtype HIV-1-vertically infected children in Kenya.

Materials and Methods

Study population

As of August 2007, 95 HIV-1-infected children resided in a children's home in Nairobi. These children were born to HIV-1-infected mothers who either died of, or were too debilitated by HIV/AIDS and could not offer basic care to the children. All children were admitted into the home by their first birthday, where their HIV-1 status was confirmed serologically at 18 months of age. None of these children had a history of previous exposure to ARVs by the time of admission. Of the 95 children 81 were followed up at least three times during the period between 2000 and 2007, and blood samples were obtained from them every 6 months since the year 2000. Of the 81 children, 41 received ART consisting of two nucleoside reverse transcriptase inhibitors (NRTIs) and one nonnucleoside reverse transcriptase inhibitor (NNRTI). The mean duration of ART varied among those children was 7.6 years with range of 1 to 15 years. The remaining 40 did not receive ART during the follow-up period.

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 antiretroviral, medical, and healthcare programs of the institution without additional demand for blood samples solely for research purposes.

$CD4^+$ T cell counts and plasma viral loads

$CD4^+$ T cell counts of peripheral blood were determined using the FACSCOUNT (Becton-Dickinson, Beiersdorf, Germany). Plasma HIV-1 RNA loads were determined by the Amplicor HIV-1 Monitor kit version 1.5 (Roche Diagnostics, Alameda, CA) using the standard procedure (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 & D (Medical & Biological Co. Ltd., Fukushima, Japan) according to the manufacturer's instructions. A part of the HIV-1 group M *env* gene covering the C2V3 region (corresponding to 6975–7520 nt in HIV-1 HXB2) was amplified by both one-step RT-PCR and nested polymerase chain reaction (PCR) with primers M5 (5′-CCAATTCCCATAC ATTATTGTGCCCCAGCTGG-3′ and M10 (5′-CCAATTGT CCCTCATATCTCCTCCTCCAGG-3′) in the first round and M3 (5′-GTCAGCACAGTACAATGCACACATGG-3′) and M8 (5′-TCCTTGGATGGGAGGGGCATACATTGC-3′) in the second round,[31] according to the manufacturer's instructions. Amplification was done with one cycle of 95°C for 10 min and 35 cycles of 95°C for 30s, 55°C for 30 s, and 72°C for 1 min with a final extension of 72°C for 10 min. PCR amplification was confirmed by visualization with ethidium bromide staining of the gel.

Sequencing, cloning, and subtyping of the env-C2V3 region

The amplified PCR products were cloned using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) and sequenced as described previously,[31] to take into account both the majority and minority virus populations.

The sample nucleotide sequences were aligned with HIV-1 subtype/circulating recombinant form (CRF) reference sequences from the Los Alamos database using CLUSTAL W (version 1.83), with minor manual adjustments. A phylogenetic tree was constructed by the neighbor-joining method, and its reliability was estimated by 1000 bootstrap replications. The profile of the tree was visualized with TreeViewPPC1.6.5.[31] To improve the accuracy of HIV-1 subtyping, we used the National Center for Biotechnology Information (NCBI) genotyping tool (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi) and the REGA subtyping tool (http://dbpartners.stanford.edu/RegaSubtyping/), as needed.

Determination of the predicted coreceptor usage of HIV-1

The predicted coreceptor usage of HIV-1 was determined based on the *env*-V3 loop amino acid sequences. R5 and X4 variants were identified according to (i) the 11/25 amino acid rule [uncharged residues at position 11 of V3 (mostly serine/glycine), negatively charged residues at position 25 (mostly glutamic (E)/aspartic (D) acid), and a net charge of the V3 loop less than +5 have been reported to predict CCR5 chemokine receptor usage. Conversely, positively charged residues at position 11 or 25 (mostly arginine (R)/lysine (K)) and a net charge of the V3 loop equal and more than ±5 have been reported to predict CXCR4 chemokine receptor usage], and (ii) the net charge of the V3 region, which was calculated by subtracting the number of acidic amino acids (aspartate and glutamate) from the number of basic amino acids [lysine, histidine (H), and arginine]. A net charge of equal and less than +5 in the V3 region has been shown to predict CCR5 chemokine receptor usage (R5 variants), whereas a net charge of more than +5 has been shown to predict CXCR4-chemokine receptor usage (X4 variants).[32–36] In our study, a net charge of +5 was considered to predict an R5 phenotype, unless this was accompanied by the appearance of either an arginine or lysine amino acid residue at positions 11 or 25.

Determination of the rate of disease progression

The children were categorized into three groups, rapid progressors, slow progressors, and the others, based on the rate of disease progression. *Slow progressors* were the children over 10 years of age when they received ART, and were classified in stage N1 or A1 according to the Centers for Disease Control and Prevention (CDC) classification for children. Children over 10 years of age who did not need ART were also included in this category. *Rapid progressors* were the children who received ART within the first 5 years of their lives either because they had an onset of severe clinical manifestations (CDC category C) and/or profound immune suppression (CDC category 3).

Results

Predicted HIV-1 coreceptor usage

Of the 41 children on ART, 35 had HIV-1 that used CCR5 as a coreceptor at baseline (the first time point at which sample analysis was done). The mean age, viral load,and $CD4^+$ T cell count of these 35 children at baseline were 5.5 years old (range: 1–12), 5.2 $\log_{10}$ copies/ml (range: 3.9–6.1), and 537 cells/μl (range: 93–1760), respectively. In 7 (20%) of them the virus switched coreceptor usage from CCR5 to CXCR4, and in 28 the virus used CCR5 as a coreceptor during the follow-up period. The duration from the start of ART to the time of HIV-1 coreceptor switch varied considerably (mean: 2.6 years, range: 0.5–5.2 years) (Table 1). The remaining six children on ART had HIV-1 that used CXCR4 as a coreceptor from baseline to the end of the study.

Of the 40 children without ART, 32 had HIV-1 that used CCR5 as a coreceptor at baseline. The mean age, viral load, and $CD4^+$ T cell count of these 32 children at baseline were 8.0 years old (range: 3–19), 4.8 $\log_{10}$ copies/ml (range: 2.3–6.0), and 684 cells/μl (range: 70–1335), respectively. In three (9.4%) of them the virus switched the coreceptor usage, and in 29 the virus used CCR5 as a coreceptor during the follow-up period. The remaining eight children without ART had HIV-1 that used CXCR4 as a coreceptor from baseline to the end of the study. Although more of the treated children had HIV-1 that switched coreceptor usage from CCR5 to CXCR4, the difference in the rate of the coreceptor switch between treated (7/35, 20%) and untreated (3/32, 9.4%) children ($p = 0.38$) was not statistically significant (Table 1).

The seven children whose viruses switched their coreceptor usage started ART at younger ages than the 28 children whose viruses used CCR5 as a coreceptor from baseline to the end of the study (mean 5.3 and 7.6 years, respectively). The mean age of the children with HIV-1 coreceptor switch with and without ART was 7.3 and 9.7 years, respectively. The difference in the age of the coreceptor switch between treated and untreated children was not significant ($p = 0.31$).

Chronological changes of the env-V3 amino acid sequences from the serial study points for the 10 children whose HIV-1 showed the coreceptor switch with and without ART are shown in Fig. 1. No significant association was observed between the changes in coreceptor usage and plasma viral load in the children.

HIV-1 coreceptor switch with different rates of disease progression

Of the HIV-1-infected children who had the virus that used CCR5 as a coreceptor at recruitment, 10 started ART by the age of 5 years (rapid progressors) and 23 did not need ART by the age of 10 years (slow progressors). The rate of coreceptor usage was strongly higher in rapid progressors (4/10, 40%) than slow progressors (2/23, 8.7%), though the association was not statistically significant ($p = 0.053$) (Table 2).

HIV-1 subtypes

Phylogenetic analysis based on the *env*-C2V3 region revealed that all the 81 children were infected with non-B subtype HIV-1: subtypes A1 ($n = 65$), A2 ($n = 4$), D ($n = 9$), C ($n = 2$), and CRF_02AG ($n = 1$) (data not shown). No significant relationship between HIV-1 subtype/CRF and

TABLE 1. CHARACTERISTICS OF THE STUDY CHILDREN AT BASELINE, ART START, AND CORECEPTOR SWITCH

		Baseline mean (range)			*ART start mean (range)*			*Coreceptor switch mean (range)*			
	Coreceptor usage	*Viral load*[a]	*CD4*[+b]	*Age*[c]	*Viral load*[a]	*CD4*[+b]	*Age*[c]	*Viral load*[a]	*CD4*[+b]	*Age*[c]	*Duration of ART (years)*
ART	R5▶R5 ($n = 28$)	5.3 (3.9–6.1)	472 (6–1566)	5.7 (1–12)	5.3 (3.9–6.1)	447 (93–1340)	6.6 (1–12)				
	R5▶X4 ($n = 7$)[d]	4.8 (3.6–5.4)	479 (178–1760)	5.0 (1–8)	4.7 (3.6–5.4)	357 (147–1442)	4.7 (2.5–7)	4.8 (4.2–5.4)	677 (157–1439)	7.3 (3–12)	2.6 (0.5.–5.2)
	X4▶X4 ($n = 6$)[e]	3.5 (2.3–5.0)	590 (17–1620)	11.2 (7–18)							
No ART	R5▶R5 ($n = 29$)	4.8 (2.3–6.0)	697 (70–1637)	8.1 (2–19)							
	R5▶X4 ($n = 3$)	4.4 (3.3–5.2)	573 (338–700)	7.7 (5–11)				4.8 (4.6–5.1)	462 (411–550)	9.7 (7–12)	
	X4▶X4 ($n = 8$)	4.4 (2.9–5.3)	716 (345–1570)	8.6 (6–13)							

[a]Log (copies/ml).
[b]$CD4^+$T cell count (cells/μl).
[c]Years old; R5, CCR5; X4, CXCR4.
[d]One of the seven children had already received ART at baseline.
[e]All the six children in this group had received ART at baseline.

Child ID	Date of sample collection	V3 amino acid sequence (11 … 25)	Viral load (log/ml)	net charge	11/25 amino acid	predicted phenotype
36m*	aug,02	CTRPGNNTRESVRIGPGQAFYATKDVIGDIRQAHC	4.9	+3	S/D	R5
	apr,03	.I..S.......................I.........	5.8	+3	S/D	R5
	feb,04	.........R..I.............IG........	5.6	+4	S/D	R5
	Oct,05	......K..R..I.....RV..T.NVIR........	5.1	+7	S/V	X4
38m*	mar,03	....ST...K..............GEIT........	4.6	+5	S/E	R5
	dec,03	....S....K..............GEIT........	4.8	+5	S/E	R5
	feb,04	....SSP..TR.A..R.......SAIT.T..K.Y.	4.6	+6	R/A	X4
	sep,05	....S.P..RR.A..........SAIX.T..T.Y.	4.9	+6	R/A	X4
51m*	apr,03	....N....KG.H.....S.FT.GNI.....K.Y.	5.3	+5	G/N	R5
	nov,04	....N....KG.H.....S.FT.GNI.....K.Y.	5.2	+5	G/N	R5
	apr,05	....N....KG.H.....SLFT.GNI.....K.Y.	5.5	+5	G/N	R5
	oct,05	....N....KG.H.....SLFT.GNI..N..K.Y.	5.4	+6	G/N	X4
69m*	mar,03	.I..N....QGIH.......WV.N....E....Y.	5.4	+4	G/D	R5
	may,04	.I..N....QGIH....R..WV..K.V.IK...Y.	5.1	+7	G/K	X4
85f*	feb,03	....N....K.I......T....G.IT........	4.5	+4	S/D	R5
	dec,03	....N....K.IH....RT....G.IT........	3.5	+5	S/D	R5
	apr,04	....N....K.IH....RT....G.IT........	4.9	+5	S/D	R5
	apr,05	....N....K.I......T....G.IT........	4.3	+5	S/D	R5
	sep,05	....N....K.IH....RT....G.I..N......	4.5	+6	S/D	X4
89f*	feb,04	..S.T...SRGIHM...RS....D.I..N......	5.2	+5	G/D	R5
	jul,04	..SNTSS.SRGIHM...RS....D.I..N......	5.0	+5	G/D	R5
	mar,05	..S.T...SRGIHM...RS....D.I..N......	5.5	+5	G/D	R5
	oct,05	..SRT...SRGIHM..LRS....DRI..N......	4.8	+6	G/R	X4
91f*	mar,05	....N....K.IHF.....L.T.DNI..N....Y.	4.9	+4	S/N	R5
	mar,06	....N....R.IH......L.T.NRI..N......	4.2	+6	S/R	X4
	aug,06	....N....KGIHF.....L.T.NRI....KK.Y.	4.0	+6	G/R	X4
21f**	jul,03	....S....K.IHL...R.....G.I.........	4.2	+5	S/D	R5
	dec,04	....N....K.IHL...R.....GRI..N......	4.7	+7	S/R	X4
	apr,06	....S....K.IHL.A.R.....GRI.........	4.2	+6	S/R	X4
49f**	aug,02	.S..S....K..............G.IV........	4.2	+5	S/D	R5
	jun,03	.S.......K..............G.IV........	4.5	+5	S/D	R5
	feb,04	.S.......K..........V...GAIV........	4.1	+5	S/A	R5
	mar,05	.S.......K..H...........GAIV....R...	4.6	+6	S/A	X4
72f**	Jul,02	.I.VN....Q.L..........MG.I..N..D...	5.2	+3	S/D	R5
	Jun,03	.I..Y.....GTHM...K.YFT...I.....D...	5.1	+4	G/D	R5
	feb,04	.I..N....Q..N.........MG.I.....D...	NT	+2	S/D	R5
	sep,04	....N....K.IHF.....L.TNNII..N..D...	5.1	+6	S/I	X4

FIG. 1. Changes in the HIV-1 V3 amino acid sequences during follow-up of the 10 children whose infected viruses switched from CCR5 to CXCR4 coreceptor usage. A net charge of less than and more than +5 in the V3 region was considered as CCR5-using (R5) and CXCR4-using (X4) variants, respectively, and a net charge of +5 was considered as R5 variants, unless this was accompanied by the appearance of either an arginine or lysine residue at position 11 or 25 of the V3 amino acid sequences. *, **Children whose HIV-1 showed a switch in coreceptor usage from CCR5 to CXCR4 with treatment (*) and without (**) treatment. NT, not tested.

TABLE 2. HIV-1 CORECEPTOR USAGE IN ASSOCIATION WITH THE RATE OF DISEASE PROGRESSION

	Change in coreceptor usage[a]	*Number of children (on ART)*	*Mean age (range) at ART start/recruitment*	*Mean age (range) at switch*	*Children with coreceptor switch*
Rapid progressor	R5→R5	6 (6)			
	R5→X4	4 (4)	3.0 (1–4)	4.8 (3–6)	40%[b]
Slow progressor	R5→R5	21 (6)			
	R5→X4	2 (0)	7.5 (6–9)	12.0 (10–14)	8.7%[b]

[a]R5, CCR5; X4; CXCR4.
[b]$p = 0.053$.

coreceptor usage was observed among the children (data not shown).

Discussion

In the current study we conducted a longitudinal study to investigate the evolution of the *env*-V3 region in terms of coreceptor usage among non-B subtype HIV-1-infected Kenyan children in relation to ART. These children were vertically infected with HIV-1 and have been virologically and immunologically followed up since the year 2000. Most studies on viral evolution and coreceptor usage from HIV-1-infected adults are often compromised by a lack of knowledge of the duration of infection. It makes this study particularly useful that the precise timing of HIV-1 infection is known in the children studied.

Recently it has been reported that the prevalence of X4 variants was higher in HIV-1-infected individuals exposed to ART than in drug-naive individuals,[22–25] and that effective HAART enhanced the coreceptor switch from CCR5 to CXCR4.[30] In our study, however, no significant difference in the rate of the coreceptor switch between the children with and without ART was observed ($p = 0.38$). In addition, the duration of time from the start of ART to the time of HIV-1 coreceptor switch varied considerably from 0.5 to 5.2 years (mean: 2.6 years) in our study, though it was expected to be synchronized if ART was directly associated with a switch in HIV-1 coreceptor usage. These results suggest that switching of coreceptor usage from CCR5 to CXCR4 among HIV-1-infected children is not directly influenced by ART.

We further analyzed the children who harbored HIV-1 who showed a switch in coreceptor usage according to the rate of disease progression. The rate of HIV-1 coreceptor switch was found to be strongly higher in the rapid progressors (40%, 4/10) than the slow progressors (8.7%, 2/23) ($p = 0.053$). The strong association between rapid disease progression and HIV-1 coreceptor switch in our study may suggest that those factors associated with rapid disease progression in children, such as high viral load at infection,[32] poor cell-mediated immune responses,[33] lack of neutralizing antibodies,[34] and the biological properties of the virus, would be more directly implicated. Studies using animal models also showed that reduced HIV-specific immunity may result in HIV-1 coreceptor switch.[35]

In the current study, the predicted coreceptor usages based on the env-V3 amino acid sequence according to previous reports[37,38] were not always correlated with those based on the amino acids residues at positions 11 and 25[39–41] (Fig. 1). Therefore, we mainly used the net charge of the env-V3 amino acid sequence to predict HIV-1 coreceptor usage, except when the net charge of the env-V3 amino acid sequence was +5, in which cases we used amino acid residues at positions 11 and 25 to predict the coreceptor usage.[39–41] Phenotypic assay for HIV-1 coreceptor usage might be needed to confirm our prediction of the coreceptor usage.

In conclusion, our data suggest that ART does not enhance the HIV-1 coreceptor switch from CCR5 to CXCR4. This switch in HIV-1 coreceptor usage was associated with rapid disease progression among non-B subtype HIV-1-infected children. We are currently carrying out more detailed analyses on the genetic host factors associated with delayed or rapid disease progression among HIV-1-infected children, hoping to focus more light on the possible factors that influence the HIV-1 coreceptor switch.

Sequence Data

GenBank accession numbers of the sequences reported in this study are EU602350 to EU603148 for env-C2V3.

Acknowledgments

This work was supported in part by the Ministry of Health, Labor and Welfare, Japan, and is published with the permission of the Director, KEMRI. 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. M.K. Wasunna, the Director of Kenya Medical Research Institute (KEMRI), whose facilitation ensured the successful completion of the study.

Disclosure Statement

No competing financial interests exist.

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