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

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Recently increased availability of anti-retroviral therapy (ART) has mitigated HIV-1/AIDS prognoses especially in resource poor settings. The emergence of ART resistance-associated mutations from non-suppressive ART has been implicated as a major cause of ART failure. Reverse transcriptase inhibitor (RTI)-resistance mutations among 12 non-subtype B HIV-1-infected children with treatment failure were evaluated by genotypically analyzing HIV-1 strains isolated from plasma obtained between 2001 and 2004. A region of *pol-RT* gene was amplified and at least five clones per sample were analyzed. Phylogenetic analysis revealed HIV-1 subtype A1 ( $n = 7$ ), subtype C ( $n = 1$ ), subtype D ( $n = 3$ ), and CRF02\_AG ( $n = 1$ ). Before treatment, 4 of 12 (33.3%) children had primary RTI-resistance mutations, K103N ( $n = 3$ , ages 5–7 years) and Y181C ( $n = 1$ , age 1 year). In one child, K103N was found as a minor population (1/5 clones) before treatment and became major (7/7 clones) 8 months after RTI treatment. In 7 of 12 children, M184V appeared with one thymidine-analogue-associated mutation (TAM) as the first mutation, while the remaining 5 children had only TAMs appearing either individually ( $n = 2$ ), or as TAMs 1 (M41L, L210W, and T215Y) and 2 (D67N, K70R, and K219Q/E/R) appearing together ( $n = 3$ ). These results suggest that “vertically transmitted” primary RTI-resistance mutations, K103N and Y181C, can persist over the years even in the absence of drug pressure and impact RTI treatment negatively, and that appearing patterns of RTI-resistance mutations among non-subtype B HIV-1-infected children could possibly be different from those reported in subtype B-infected children. **J. Med. Virol.** 79:865–872, 2007. © 2007 Wiley-Liss, Inc.

**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

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

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

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

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

## METHODS

### Study Population

The subjects in this study resided in children's home in Nairobi, which housed 95 HIV-1-infected children. These children were born to HIV-1-infected mothers who either died of, or were too debilitated by HIV/AIDS hence could not offer basic care to the children. Of 95

children 55 were on ART as of August 2004. The duration of ART varied among children (mean: 23.3 months, range: 5–46 months). Of 55 children on ART 12 (8 males and 4 females, mean age: 7.4 years) experienced treatment failure, characterized by an initial decrease in plasma viral load (to undetectable level in one child) after treatment initiation and subsequent increase in the viral load as treatment continued. Seven of the 12 children received single ART regimen only during the study period: 5 received zidovudine/lamivudine/nevirapine, 1 zidovudine/didanosine/efavirenz, and 1 zidovudine/lamivudine/efavirenz (Table I). On the other hand, the remaining five children received multiple ART regimen during the study period: two received zidovudine/lamivudine/efavirenz followed by zidovudine/didanosine/efavirenz, two zidovudine/lamivudine/nevirapine followed by didanosine/lamivudine/efavirenz, and one didanosine/lamivudine/abacavir followed by zidovudine/didanosine/efavirenz and later didanosine/stavudine/efavirenz (Table I). These 12 children were admitted into the home by their first birthday and their HIV-1 status was confirmed serologically at 18 months of age. None of these children had history of previous exposure to any ARV.

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

### CD4<sup>+</sup> Cell Counts and Plasma Viral Loads

CD4<sup>+</sup> 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<sub>HXB2</sub>) was amplified by both one-step RT-PCR (Invitrogen, Carlsbad, CA) and nested PCR with primer pairs, RT18 (5'-GGAAACCAAAAATGATAGGGGGAATTGGAGG-3') and KS104 (5'-TGAC-TTGCCCAATTTAGTTTTCCCACTAA-3') in the first round, and KS101 (5'-GTAGGACCTACACCTGTTC-AACATAATTGGAAG-3) and KS102 (5'-CCCAT-CCAAAGAAATGGAGGAGGTTCTTTCTGATG-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*<br>(years)/sex | HIV-1<br>subtype/CRF | Study point<br>(month, year)                        | ART <sup>a</sup> (initiation time)                 | CD4 <sup>+</sup> T cell<br>count (/μl) | Plasma viral load<br>(copies/ml)                   | NRTI <sup>b</sup> -resistance mutations                                     | NNRTI <sup>c</sup> -resistance<br>mutations |
|-----------|---------------------|----------------------|---|--|--|--|---|---|
| NYU30     | 11/F                | A1                   | Jul '02<br>Mar '03<br>Jan '04                       | ZDV, 3TC, EFV (Jun '01)<br>ZDV, DDI, EFV (May '03) | 456<br>24,857<br>267                   | <400<br>89,063                                     | D67N + K70R + K219Q   | L100I                                       |
| NYU33     | 11/F                | A1                   | Jul '02<br>Mar '03<br>Feb '04                       | ZDV, 3TC, EFV (Jun '01)<br>ZDV, DDI, EFV (Oct 01)  | 549<br>556<br>690                      | 3,449<br>122,419<br>6,457                          | K219Q<br>K219Q + D218E  | K101Q<br>K101Q                              |
| NYU36     | 11/M                | D                    | Oct '01<br>May '02<br>Aug '02<br>Apr '03<br>Feb '04 | ddI, 3TC, ABC (Apr '01)<br>ZDV, DDI, EFV (Oct 01)  | 309<br>321<br>279<br>458               | 114,754<br>880,405<br>81,870<br>607,224<br>393,420 | M184V + T215F<br>M184V + T215F<br>M184V + T215F<br>T215F<br>T215F           | I178M<br>G190A<br>G190A<br>G190A            |
| NYU38     | 10/M                | C                    | Mar '03<br>Dec '03                                  | ZDV, 3TC, NVP (Sep '02)                            | 388<br>188                             | 38,459<br>60,695                                   | D67N + K70R + L210W<br>+ K219E  |   |
|           |                     |                      | Feb '04<br>Aug '04                                  | DDI, 3TC, EFV (Mar 04)                             | 157<br>149                             | 38,211   | D67N + K70R + L210W<br>+ K219E<br>D67N + K70R + L210W<br>+ D218E + K219E    |   |
| NYU44     | 9/M                 | A1                   | Feb '02<br>Mar '03<br>Dec '03                       | ZDV, DDI, EFV (May '02)                            | 208<br>370<br>474                      | 1,017,931<br>71,895<br>150,549                     | D67N + K70R + T215F + K219Q<br>D67N + K70R + T215F + K219Q<br>+ M41L + V75M | K103N<br>K103N + G190A<br>K103N + G190A     |
| NYU62     | 8/M                 | A1                   | Dec '01<br>Sep '02<br>Mar '03<br>May '04            | ZDV, 3TC, NVP (Sep '02)                            | 589<br>828<br>568                      | 239,644<br>2,838                                   | D67N + K70R<br>D67N + K70R + T215F + K219E<br>D67N + K70R + T215F + K219E   | G190A<br>G190A<br>G190A + Y181C             |
| NYU69     | 6/M                 | A1                   | Mar '03<br>May '04                                  | ZDV, 3TC, NVP (Mar '03)                            | 192<br>400                             | 227,176<br>113,868                                 | M184V   | K103N<br>K103N                              |
| NYU70     | 7/M                 | D                    | Sep '02<br>Jun '03<br>Dec '03                       | ZDV, 3TC, NVP (Jul '03)                            | 718<br>169<br>502                      | 700,563<br>1,323,431<br>188,059                    | K70R + M184V  | K103N<br>K103N<br>K103N                     |
| NYU79     | 6/M                 | A1                   | Feb '03<br>Feb '04<br>Jun '04                       | ZDV, 3TC, NVP (Apr '03)<br>DdI, 3TC, EFV (Mar 04)  | 70<br>551<br>347                       | 159,826<br>244,506<br>472,203                      | V75M + M184V<br>V75M + M184V  | K101E + G190A<br>K101E + G190A<br>+ Y181C   |
| NYU83     | 5/M                 | A1                   | May '01<br>Jul '02<br>Apr '03<br>Aug '04            | ZDV, 3TC, EFV (May '04)                            | 876<br>946<br>1138<br>1125             | 634,644<br>50,570<br>74,437<br>197,301             | M184V<br>M184V<br>M184V + T215Y   | K103N<br>K103N<br>K103N                     |
| NYU85     | 5/F                 | CRF02_AG             | Feb '03<br>Dec '03<br>Apr '04                       | ZDV, 3TC, NVP (Apr '03)                            | 178<br>1214<br>1148                    | 30,690<br>3,264<br>79,080                          | D67N + M184V<br>D67N + M184V  | K103N<br>K103N                              |
| NYU90     | 2/F                 | D                    | Apr '03<br>Jan '04<br>Mar '04                       | ZDV, 3TC, NVP (Apr '03)                            | 6<br>399<br>379                        | 523,950<br>55,679<br>155,191                       | M184V   | Y181C<br>K103N                              |

\*As of August 2004.  
<sup>a</sup>ART, anti-retroviral therapy; ZDV, zidovudine; ddI, didanosine; EFV, efavirenz; NVP, nevirapine; 3TC, lamivudine; d4T, Stavudine.  
<sup>b</sup>NRTI, nucleoside analogue RTI.  
<sup>c</sup>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 <sup>a</sup> ) |                    |                                |             |            | Treatment                     |
|------------|----------------------------------|--------------------|--------------------------------|-------------|------------|-------------------------------|
|            | 1st                              | 2nd                | 3rd                            | 4th         | 5th        |                               |
| NYU69      | M184V (10)                       |                    |                                |             |            | ZDV/3TC                       |
| NYU90      | M184V (9)                        |                    |                                |             |            | ZDV/3TC                       |
| NYU83      | M184V (13)                       |                    |                                |             |            | ZDV/3TC                       |
| NYU70      | M184V + 1TAM (6)                 | M184V (22)         | M184V + 1TAM <sup>b</sup> (38) |             |            | ZDV/3TC                       |
| NYU85      | M184V + 1TAM (9)                 | M184V + 1TAM (12)  |                                |             |            | ZDV/3TC                       |
| NYU36      | M184V + 1TAM (6)                 | M184V + 1TAM (13)  |                                | 1 TAM (24)  | 1 TAM (34) | DDI/3TC/ABC, ZDV/DDI, D4T/DDI |
| NYU62      | 2 TAMs (6)                       | 4 TAMs (12)        |                                |             |            | ZDV/3TC                       |
| NYU44      | 4 TAMs (11)                      | 5 TAMs + V75M (19) |                                |             |            | ZDV/3TC                       |
| NYU33      |                                  | 1 TAM (23)         |                                |             |            | ZDV/DDI                       |
| NYU30      |                                  | 4 TAMs (15)        |                                |             |            | ZDV/3TC, ZDV/DDI              |
| NYU38      | 1 TAM (8)                        | M184V + V75M (13)  |                                |             |            | ZDV/DDI                       |
| NYU79      | M184V + V75M (10)                |                    |                                | 5 TAMs (23) |            | ZDV/3TC, DDI/3TC              |

\*NRTI, nucleoside analogue RTI.

<sup>a</sup>mpti, months post treatment initiation.

<sup>b</sup>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<sup>a</sup>-Resistance Mutations Among Non-B Subtype HIV-1-Infected Children With Treatment

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

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

<sup>a</sup>ART, anti-retroviral therapy; ZDV, zidovudine; ddI, didanosine; EFV, efavirenz; NVP, nevirapine; 3TC, lamivudine; d4T, Stavudine.

<sup>b</sup>NRTI, nucleoside analogue RTI.

<sup>c</sup>NNRTI, non-nucleoside RTI, blank: no mutation detected.

<sup>d</sup>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 [NASCO, 2002].

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

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

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

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

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

In conclusion, this study suggests a possible long-term persistence of "vertically transmitted" NNRTI-resistance mutations in the absence of drug pressure, that minor populations of RTI-resistant HIV-1 mutants may impact negatively on the outcome of ART, and that there is a possible difference in the pattern of appearance and profile of RTI-resistance mutations between non-

subtype B and subtype B HIV-1-infected children. Further studies with large population size are needed to confirm these findings.

### SEQUENCE DATA

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

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