Characterization of Nevirapine Resistance Mutations in Women With Subtype A Vs. D HIV-1 6–8 Weeks After Single-Dose Nevirapine (HIVNET 012)

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Objective: To compare the number and type of nevirapine (NVP) resistance mutations detected in Ugandan women with subtype A vs. D HIV-1 infection after single-dose NVP prophylaxis.

Design: In the HIVNET 012 trial, a higher rate of NVP resistance (NVPR) was seen in women with subtype D than A after single-dose NVP. In this study, the number and type of NVPR mutations detected 6–8 weeks after NVP were compared in women with subtypes A vs. D.

Methods: Plasma samples were available for 282 (92%) of 306 women who received NVP in HIVNET 012. Samples were analyzed with the ViroSeq HIV-1 Genotyping System (Applied Biosystems, Foster City, CA). Subtyping was performed by phylogenetic analysis of *pol* region sequences.

Results: Results were obtained for 279 women, including 147 with subtype A, 98 with subtype D, 6 with subtype C, and 28 with recombinant HIV-1. NVPR mutations were detected in 70 (25%) of 279 women. NVPR was more common in women with subtype D vs. A (35.7 vs. 19%, $P = 0.0035$). Complex patterns of NVPR mutations were detected in both subtypes. Among women with NVPR, 43% of women with subtype A and 46% of women with subtype D had ≥2 NVPR mutations. The mean number and pattern of NVPR mutations detected in women with subtypes A and D were similar.

Conclusions: This study confirms a higher rate of NVPR in women with subtype D than A and further defines the pattern of NVPR mutations that emerge 6–8 weeks after single-dose NVP prophylaxis in these subtypes.

Key Words: HIV-1, nevirapine, drug resistance, subtype, Uganda

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Nevirapine (NVP) is a potent inhibitor of HIV-1 reverse transcriptase (RT). The HIVNET 012 trial tested the efficacy of a single-dose regimen of NVP for prevention of HIV-1 mother-to-child transmission (MTCT). Pregnant women received a single 200-mg NVP dose at the onset of labor, and their infants received a single 2-mg/kg dose within 72 hours of birth. That regimen was shown to be more effective than a short course of zidovudine prophylaxis starting in labor and was simpler and much less expensive.[1–3] The HIVNET 012 regimen is now being implemented in resource-poor countries around the world.

In a previous study, we analyzed NVP resistance (NVPR) in a subset of the women in the NVP arm of HIVNET 012. HIV-1 variants with NVPR mutations were detected in 21 (19%) of 111 women by HIV-1 genotyping (population sequencing) 6–8 weeks after NVP administration.[4] NVPR mutations were no longer detectable in women 12–24 months after delivery. In an exploratory study that included 102 of the 111 women described here (excluding the 9 women whose in-

fants were infected after 6–8 weeks), we found a higher rate of NVPR among women with subtype D than subtype A.[5] The association between HIV-1 subtype and emergence of NVPR did not appear to reflect a difference in the stage of disease among women with subtype A vs. D, since the baseline viral loads and baseline CD4 cell counts were similar in the 2 groups.[5] The potential for HIV-1 subtype to influence the rate of NVPR after NVP prophylaxis suggests that the rate of NVPR in women receiving the HIVNET 012 regimen may vary from region to region depending on which subtypes are prevalent.

Recent studies have shown that HIV-1 subtype may influence the pattern of resistance mutations that emerge after exposure to antiretroviral drugs.[6,7] Subtype-specific differences in protease and RT sequences may influence the rate at which a specific mutation emerges[8] and the type of amino acid selected at a given position under drug pressure.[9,10] Differences in sequences of nonsubtype B viruses may also lead to emergence of novel subtype-specific drug resistance mutations at positions not associated with drug resistance in subtype B.[11] A detailed comparison of the number and type of NVPR mutations selected by the single-dose regimen in women with subtype A vs. D was not possible in the exploratory study described above, because the subset of 102 women included only 6 women with subtype A and 10 women with subtype D who had NVPR mutations detected at the 6- to 8-week time point.[5] By expanding analysis of NVPR in the HIVNET 012 cohort to include all available 6- to 8-week samples from women who received NVP prophylaxis (282 women), we are able to define further the relationship between subtype and emergence of NVPR and to compare the number and type of NVPR mutations that emerge in women with subtype A vs. D after NVP prophylaxis.

MATERIALS AND METHODS

Study Visits and Results From HIVNET 012

The HIVNET 012 study protocol was reviewed and approved by institutional review boards in Uganda and the United States, and informed consent was obtained from all women before enrollment. Women were antiretroviral drug naive before NVP administration and did not receive antiretroviral therapy after the single dose of NVP, consistent with the standard of care in Uganda. Detailed methods and results of HIVNET 012 are presented elsewhere,[1–3] including methods for determining baseline HIV-1 RNA levels and baseline CD4 cell counts. Plasma samples collected 6–8 weeks after delivery were used for analysis of NVPR mutations and for HIV-1 subtyping.

HIV-1 Genotyping

HIV-1 genotyping was performed using the Applied Biosystems ViroSeq HIV-1 Genotyping System (Applied Biosystems, Foster City, CA), as described previously.[4,5] HIV-1 genotyping was considered to be successful if a sequence corresponding to protease amino acids 1–99 and RT amino acids 1–324 (297 and 972 nucleotides, respectively) was obtained. Genotypes were analyzed only if bidirectional sequence data (sense and antisense) were obtained at all positions in RT associated with NVPR. HIV-1 sequences from each woman were examined for the presence of mutations associated with NVPR (A98G, L100I, K101E, K103N, V106A, V108I, V179D Y181C/I, Y188C/H/L, G190A/S, M230L), including accessory mutations (K101Q, V106I, P225H, Y318F) and mutations associated with hypersusceptibility to NVP (P236L) [IAS-USA Drug Resistance Mutations Group (2002), Stanford HIV RT and Protease Sequence Database (http://hivdb.stanford.edu/cgi-bin/NNRTIResiNote.cgi)].

Phylogenetic Analysis of HIV-1 Subtypes

Subtyping was performed using contiguous nucleotide sequences corresponding to protease amino acids 1–99 and RT amino acids 1–324. Sequences were examined for evidence of intersubtype recombination using the Recombinant Identification Program[12] (available at http://hivweb.lanl.gov/RIP/RIPsubmit.html) using a window size of 400 nucleotides. Reference sequences of known subtype were obtained from the HIV Sequence Database at the Los Alamos National Laboratory.[13] A detailed analysis of 13 of the 28 intersubtype recombinant sequences is presented elsewhere.[14]

Sequences without evidence of intersubtype recombination were aligned using the biosequence editor Seqapp v1.9a169 (http://iubio.bio.indiana.edu/soft/molbio/seqapp/). Alignments included reference sequences recommended by the Los Alamos National Laboratory for HIV-1 subtype analysis. Phylogenetic analysis of HIV-1 subtypes was performed using PHYLIP v. 3.572 (available at http://evolution.genetics.washington.edu/phylip.html). Alignments were performed manually and did not require gap stripping. Distances between the sequences were calculated with DNADist, utilizing the Kimura-2 parameter as an optimal substitution model and a transition-transversion ratio of 1.5. Neighbor-Joining and Consense were used to create phylogenetic trees with 500 bootstrap replications (SeqBoot). Consensus trees were displayed with TreeView.[15] Bootstrap values >80 were considered acceptable for subtype assignment.

Statistics

Logistic regression was used to evaluate correlates of maternal NVPR, including baseline viral load, baseline CD4 cell count, and HIV-1 subtype. All statistical analyses were done with SAS (version 8.1, SAS Institute, Cary, NC).

GenBank Accession Numbers

Sequences analyzed in this report included 110 sequences described previously[4,5] (GenBank accession num-

bers: AF388065–AF388098, AF388100–AF388166) and AY425349–AY425357, and 169 additional sequences AY435220–AY435388.

RESULTS

Genotyping Results

In HIVNET 012, 313 women were enrolled in the NVP arm and 306 received single-dose NVP at the onset of labor. Plasma samples collected 6–8 weeks after NVP (6–8 weeks after delivery) were available for 282 (92%) of those 306 women. All available plasma samples were analyzed with the Applied Biosystems ViroSeq HIV-1 Genotyping System. Genotyping analysis was successful for 279 (98.6%) of the 282 samples tested.

Analysis of HIV-1 Subtypes

HIV-1 subtyping of the *pol* (protease and RT) region was performed by phylogenetic methods. Intersubtype recombination in the *pol* region is common in Uganda.[14] Therefore, sequences were first analyzed for evidence of intersubtype recombination using the Recombinant Identification Program. HIV-1 sequences from 28 (10%) of the 279 women had evidence of intersubtype recombination. Unique patterns of recombination were observed in different women. The patterns of recombination observed in samples from 13 of those women are described in detail elsewhere.[14] There was clear evidence of intersubtype recombination within the RT region for 19 of the 28 women. RT sequences from the other 9 women appeared to be predominantly 1 subtype (7D, 1A, and 1C), with a second subtype in the protease/RT cleavage region and the N-terminus of the RT protein. The remaining 251 sequences, which showed no evidence of intersubtype recombination, were subtyped using phylogenetic methods described here. These methods identified 147 women with subtype A, 98 women with subtype D, and 6 women with subtype C infection.

Analysis of NVPR

NVPR mutations were detected in 70 (25%) of the 279 women (Table 1). Detection of NVPR mutations was associated with a higher baseline (pre-NVP) viral load and lower baseline CD4 cell count (per increase of 1 $\log_{10}$ HIV RNA: odds ratio [OR] = 3.1 [95% CI: 1.8–5.1, $P < 0.0001$], per decrease of 100 cells: OR = 1.3 [95% CI: 1.1–1.5, $P = 0.0055$]). The rate of NVPR was higher among women with subtype D than A. NVPR mutations were detected in 35 (35.7%) of the 98 women with subtype D, but in only 28 (19.1%) of the 147 women with subtype A (OR = 2.4 [95% CI: 1.3–4.2, univariate analysis]). In a logistic regression model controlling for baseline RNA and baseline CD4 cell count, all variables in the model were independent predictors of resistance. Controlling for baseline HIV-1 RNA and baseline CD4 cell count, the OR for subtype was 2.5 (95% CI: 1.3–4.9). This difference did not appear to reflect more advanced disease among women with subtype D vs. A, because baseline viral loads and baseline CD4 cell counts were similar for the 2 groups (median baseline viral load for A vs. D: 4.3 vs. 4.5 $\log_{10}$ HIV-1 RNA copies/mL, $P = 0.428$,Wilcoxon test; median baseline CD4 cell count for A vs. D: 495 vs. 405 cells/μL, $P = 0.099$, Wilcoxon test). These results are consistent with findings of our earlier exploratory studies.[4,5] Data from subtype C and recombinant HIV-1 are shown for comparison (Table 1) but were too limited for meaningful statistical analysis.

Analysis of NVPR Mutations

Seven different NVPR mutations were detected in this cohort: K101E, K103N, V106A, V108I, Y181C, Y188C, and G190A (Table 2). K103N and Y181C were the most common NVPR mutations detected (Table 2), representing 53.2% and 23.4%, respectively, of the 111 mutations detected among 70 women with NVPR. Both K103N and Y181C were detected at higher rates in women with subtype D than A. K103N was detected in 28 (28.6%) of 98 women with subtype D but in only 25 (17.0%) of 147 women with subtype A ($P = 0.0313$). Y181C was detected in 16 (16.3%) of 98 women with subtype D but in only 8 (5.4%) of 147 women with subtype A ($P = 0.005$). Deletion of codon 69, which has been associated with NVPR, was not observed. Accessory mutations were detected

TABLE 1. Median Baseline Viral Load, Median Baseline CD4 Cell Count, and HIV-1 Subtype in Women With and Without NVPR Mutations Detected 6–8 Weeks After Single-Dose NVP

	Viral Load $\log_{10}$ HIV-1 RNA	CD4 Cell Count Cells/μL	Subtype				
			A	D	C	R	Total
Resistant	4.9	300	28 (19%)	35 (35.7%)	2 (33.3%)	5 (17.9%)	70 (25%)
WT	4.3	519	119 (81%)	63 (64.2%)	4 (66.6%)	23 (82.1%)	209 (75%)
All women*	4.4	443	147	98	6	28	279

*The median baseline viral load ($\log_{10}$ HIV-1 RNA copies/mL) and median baseline CD4 cell count are shown for women with (resistant) and without (WT, wild type) NVPR mutations detected at 6–8 weeks. Data for median viral loads were not available for 6 women (1 resistant, 5 WT), and data for median CD4 cell counts were not available for 2 women (1 resistant, 1 WT). The numbers and percentages of women with and without NVPR mutations who had subtype A, D, C, and intersubtype recombinant (R) HIV-1 are shown.

TABLE 2. Detection of Individual NVPR Mutations in Women 6–8 Weeks After Single-Dose NVP*

	A	D	C	R	Total
K103N	25	28	2	4	59 (53.2%)
Y181C	8	16		2	26 (23.4%)
G190A	6	6			12 (10.8%)
K101E	1	4		1	6 (5.4%)
Y188C	2	3			5 (4.5%)
V106A	2				2 (1.8%)
V108I		1			1 (0.9%)
Total No. mutations	44	58	2	7	111

*A total of 111 NVPR mutations were detected among the 70 women with NVPR. The number of samples (number of women) with each mutation is shown for women with subtype A, D, C, and recombinant (R) HIV-1. Many of the women had >1 NVPR mutation detected (see text). The total number of NVPR mutations detected among women of each subtype is shown.

in 2 women: K101Q was detected in 1 woman with subtype C who also had the K103N mutation; V106I was detected in 1 woman with subtype D who did not have other NVPR mutations detected. Two of the NVPR mutations detected, K101E and V108I, are associated with low-level NVPR. The K101E mutation was detected in 5 women. All 5 women also had the K103N mutation detected, and 4 of those women had a 3rd mutation detected as well (2 with Y181C, 1 with Y188C, and 1 with G190A). The V108I mutation was detected in only 1 woman who had no other NVPR mutations detected. NVPR mutations detected in women with subtype C and recombinant HIV-1 are shown for comparison (Table 2) but were too limited for meaningful statistical analysis.

Complex patterns of NVPR mutations were detected. The type and number of NVPR mutations detected were similar among women with subtype A vs. D. Twenty-nine (41%) of the 70 women with NVPR had ≥2 NVPR mutations. This included 18 women with 2 mutations, 10 women with 3 mutations, and 1 woman with 4 mutations. More than 1 NVPR mutation was detected in 12 of 28 (42%) of women with subtype A and 16 of 35 (46%) of women with subtype D. Among the women with NVPR mutations, the mean number of NVPR mutations per woman was similar for women with subtype A vs. D (1.57 vs. 1.66, respectively).

DISCUSSION

This report reveals that complex patterns of NVPR mutations are detected 6–8 weeks after single-dose NVP in some women. It is likely that these mutations were selected as a result of the NVP exposure, because NVPR mutations were not detected in samples from >80 women enrolled in HIVNET 012 prior to antiretroviral drug administration (data not shown). Higher rates of both K103N and Y181C were detected in women with subtype D vs. A. However, the pattern of NVPR mutations, the percentage of women with ≥2 NVPR mutations, and the mean number of NVPR mutations per woman (among women with NVPR) were similar among women with these 2 subtypes. It is not known whether novel, subtype-specific NVPR mutations (other than those defined in subtype B) are selected in 1 or both of these subtypes. Because HIV-1 genotyping was performed by population sequencing, it was not possible to determine whether detection of multiple NVPR mutations in a sample represented selection of individual HIV-1 viruses with multiple, genetically linked mutations, or selection of different subpopulations of HIV-1 variants with different NVPR mutations. Further studies are needed to address that issue.

Data presented in this report further define the rate of NVPR following single-dose NVP prophylaxis. In a previous report, we found NVPR in 21 (19%) of 111 women who received NVP in HIVNET 012.[4] That study included a subset of the HIVNET 012 cohort, including 32 of 36 women whose infants were HIV-1 infected by 6–8 weeks of age (all available samples), 9 of 12 women whose infants were infected after 6–8 weeks (all available samples), and a randomly selected group of 70 women whose infants were not infected. That study, which was designed to include all women with infected infants, included a higher percentage of women with infected infants than the cohort of all women in the NVP arm of HIVNET 012 (28.8% vs. 11.5%, respectively). In HIVNET 012, women with infected infants tended to have higher baseline viral loads than women with uninfected infants. Because emergence of NVPR in HIVNET 012 was associated with higher baseline viral loads and lower baseline CD4 cell counts,[4] we speculated that the exploratory study may have overestimated the rate of NVPR across the entire cohort of women who received NVP in the HIVNET 012 trial. In contrast, the present expanded study (including all available samples) found a slightly higher rate of NVPR than the previous smaller study, with NVPR detected in 70 (25%) of 279 women. We do note, however, that the actual rate of NVPR in the entire cohort of 306 women who received NVP in HIVNET 012 may be slightly higher or lower, because genotyping results could only be obtained for 279 (91%) of those women.

Selection of NVP-resistant HIV-1 following single-dose prophylaxis is not unexpected, given the long-half life of NVP. HIV-1 variants with NVPR mutations are likely to be present as minority viral variants in HIV-1–infected patients before NVP exposure.[16,17] Rapid inhibition of the replication of NVP-sensitive virus would permit selection of NVPR variants. Indeed, NVPR variants were detectable in women in HIVNET 012 only 7 days after single-dose NVP,[18,19] and in women in HIVNET 023 only 2 weeks after administration of the same single-dose regimen.[20]

This expanded study confirms our earlier finding, that a high baseline viral load and low baseline CD4 cell count are

associated with emergence of NVPR in this setting. Because women in HIVNET 012 had relatively advanced HIV-1 disease, they may have been more likely to develop NVPR than women in other cohorts. In other settings, use of highly active antiretroviral therapy during pregnancy may reduce the rate of NVPR following single-dose NVP administration. However, results from PACTG 316 demonstrate that NVPR mutations can emerge after single-dose NVP in women receiving concurrent therapy with at least 1 other antiretroviral drug.[21]

This extended study confirms that the rate of NVPR following single-dose NVP is higher for women with subtype D compared with subtype A. This suggests that the rate of NVPR following administration of the HIVNET 012 NVP regimen may vary from region to region, depending on which subtypes are prevalent. Further studies are needed to compare resistance rates in women with different subtypes and to test whether the fading of NVPR variants over time is influenced by HIV-1 subtype. It is not known whether emergence of NVPR after single-dose NVP prophylaxis will affect the efficacy of NVP prophylaxis in subsequent pregnancies or limit use of NVP or other nonnucleoside RT inhibitors (NNRTIs) for subsequent treatment of HIV-1 infection. The HIVNET 012 regimen can significantly reduce HIV-1 transmission in settings where other regimens are impractical and where treatment options are limited. The emergence of drug resistance with simplified perinatal prophylaxis regimens must be balanced against the need for simple and affordable interventions to prevent HIV-1 transmission in resource-poor settings.

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