Selection and fading of resistance mutations in women and infants receiving nevirapine to prevent HIV-1 vertical transmission (HIVNET 012)

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Objective: To examine the emergence and fading of NVP resistance (NVP^R) mutations in HIV-1-infected Ugandan women and infants who received single dose NVP to prevent HIV-1 vertical transmission.

Design: We examined NVP^R in women and infants who received NVP in the HIVNET 012 clinical trial, including 41 out of 48 women with infected infants, 70 randomly-selected women with uninfected infants, and 33 out of 49 infected infants.

Methods: Plasma HIV-1 was analyzed using the Applied Biosystems ViroSeq HIV-1 Genotyping System.

Results: NVP^R mutations were detected in 21 out of 111 (19%) women tested 6–8 weeks after delivery. The rate of NVP^R was similar among women whose infants were or were not HIV-1 infected. K103N was the most common mutation detected. NVP^R mutations faded from detection within 12–24 months in all 11 evaluable women. High baseline viral load and low baseline CD4 cell count were associated with development of NVP^R. NVP^R mutations were detected in 11 out of 24 (46%) evaluable infants who were infected by 6–8 weeks of age. The most common NVP^R mutation detected in infants was Y181C. Those mutations faded from detection by 12 months of age in all seven evaluable infants. Of nine evaluable infants with late HIV-1 infection, only one had evidence of NVP^R.

Conclusions: NVP^R was detected more frequently in infants than women following NVP prophylaxis, and different patterns of NVP^R mutations were detected in women versus infants. NVP^R was detected infrequently in infants with late HIV-1 infection. NVP-resistant HIV-1 faded from detection in women and infants over time.

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Introduction

Nevirapine (NVP) is a potent non-nucleoside inhibitor of HIV-1 reverse transcriptase (RT). In the HIVNET 012 clinical trial, administration of a single 200 mg dose of NVP to pregnant Ugandan women at the onset of labor and a single 2 mg/kg dose to their infants within 72 h of birth was shown to significantly reduce the rate of HIV-1 mother-to-child transmission (MTCT) [1,2]. That regimen was more effective than a short course of zidovudine prophylaxis starting in labor, and was simpler and much less expensive. The efficacy, simplicity and low cost of the HIVNET 012 NVP regimen make it attractive for use in developing countries. The World Health Organization recently recommended implementation of this regimen as one of several options for prevention of MTCT in resource-limited settings [3].

When NVP is administered as monotherapy for treatment of HIV-1 disease, rapid selection of HIV-1 variants with nevirapine resistance (NVP^R) mutations occurs [4]. We recently reported that NVP-resistant HIV-1 can also be selected in women who receive single dose NVP prophylaxis to prevent HIV-1 MTCT [5]. In that report, we analyzed HIV-1 from 15 women who were enrolled in the Ugandan Phase I/II trial, HIVNET 006 [6]. Women in HIVNET 006 received the same NVP prophylaxis regimen as women in HIVNET 012. In that cohort, the K103N NVP^R mutation was detected in HIV-1 from three out of 15 (20%) women 6–8 weeks after delivery. Our analysis of women in HIVNET 006 further suggested that a more prolonged exposure to NVP favors selection of NVP-resistant HIV-1 [5]. In this report, we extend our previous studies by examining the emergence and fading from detection of NVP-resistant HIV-1 in a large cohort of Ugandan women and infants who received NVP prophylaxis in the HIVNET 012 trial.

Materials and methods

HIV-1 Genotyping

HIV-1 genotyping was performed using the Applied Biosystems ViroSeq HIV-1 Genotyping System (Applied Biosystems, Foster City, California, USA). For some women, the amount of plasma available for analysis was limited and genotyping was performed with less than the recommended 0.5 ml plasma. Due to limitations in sample volumes, all infant samples were analyzed using 0.1 ml plasma. In this system, HIV-1 RNA is extracted from plasma samples and reverse transcribed with murine Moloney leukemia virus reverse transcriptase. A 1.8 kb DNA fragment is then amplified in a single 40-cycle PCR with AmpliTaq gold polymerase and uracil N-deglycosylase decontamination control. PCR products are purified using spin columns, analyzed by agarose gel electrophoresis, and sequenced with pre-mixed BigDye sequencing reagents in seven separate reactions. Sequencing products were analyzed using an ABI 377 automated sequencer. The resulting sequences were assembled and mutations associated with NVP^R (A98G, L100I, K103N, V106A, V108I, Y181C, Y188C, G190A) were identified using the HIV-1 Genotyping Software package v2.2 (Applied Biosystems). Bidirectional sequences were obtained in the region of interest (RT amino acids 98–190) for all samples analyzed in this report. For quality control, NVP^R mutations present as amino acid mixtures were identified only if the corresponding nucleotide mixture was present in the sequences of both DNA strands.

Study visits and results

The HIVNET 012 study protocol was reviewed and approved by institutional review boards in Uganda and the USA, and informed consent was obtained from all women prior to enrollment. Women had not received prior antiretroviral therapy and did not receive antiretroviral therapy after the single dose of NVP, consistent with the standard of care in Uganda. Women were evaluated at study entry, delivery, and 7 days and 6–8 weeks after delivery. Women gave separate informed consent for collection of follow-up blood samples at later time points. Infants were evaluated at birth, 7 days, 6, 10, and 14 weeks, and 6, 9, 12, and 18 months of age. Blood was obtained at birth, 6 and 14 weeks, and 12 and 18 months of age.

Detailed methods and results of HIVNET 012 have been presented elsewhere [1]. In the final analysis, 311 women receiving NVP had 320 live births (eight multiple births) [2]. HIV-1 infection was diagnosed in infants prior to 18 months of age using HIV-1 RNA PCR, confirmed by an additional HIV-1 RNA PCR or HIV-1 culture. At 18 months of age, HIV-1 infection was diagnosed by enzyme immunoassay (EIA), and if reactive, by confirmatory Western blot [1]. Forty-nine out of 320 infants were HIV-1 infected despite NVP prophylaxis, 37 (including one set of twins) by age 6–8 weeks and 12 after age 6–8 weeks (Table 1). Infected infants with negative virologic assays through age 6–8 weeks and who had their first positive virologic test after age 6–8 weeks were defined as having late infection.

NVP^R analysis study subjects

We analyzed plasma HIV-1 from samples collected 6–8 weeks after delivery from a subset of women enrolled in HIVNET 012. Forty-eight women had 49 infants (including one pair of twins) who became infected despite NVP prophylaxis. Plasma samples were available from 33 out of 36 (92%) women whose infants were infected by age 6–8 weeks; genotyping was successful for 32 of those samples. Of the remaining

Table 1. NVP^R analysis of study samples.

	Subjects in the HIVNET 012 NVP arm (n)	Subjects genotyped at 6–8 weeks (n)	Subjects with NVP^R at 6–8 weeks (n)	Subjects genotyped after 6–8 weeks (n)	Subjects with NVP^R after 6–8 weeks (n)
All women	311	111/311	21/111	11/21	0/11 (12–24 months)
Infant uninfected[a]	263	70/263	11/70	6/11 (24 months)	0/6 (24 months)
Infant positive by 6–8 weeks[b]	36	32/36	7/32	5/7 (12–18 months)	0/5 (12–18 months)
Infant positive after 6–8 weeks[b]	12	9/12	3/9	0/3	–
All infants[c]	320				
Uninfected	271	–	–	–	–
Infected[b]	49				
Positive by 6–8 weeks	37	24/37	11/24	9/11 (14–16 weeks) 3/11 (12 months)	5/9 (14–16 weeks) 0/3 (12 months)
Positive after 6–8 weeks	12	–	–	9/12	1/9[d]

[a]Testing was performed on a random set of 72 women (see Methods). Genotyping was successful for 70 out of 72 women. [b]Genotyping was performed on all available samples. Genotyping was unsuccessful for one of these women. [c]Including seven sets of twins and one set of triplets. [d]See Table 4 for the age at diagnosis and testing for each infant. Positive = diagnosed with HIV-1 infection.

263 women who had infants who were not HIV-1-infected, a random sample of 72 women was selected for evaluation. Those women had infants who were uninfected and alive at 6–8 weeks of age. Women were excluded if their viral load was < 2000 copies/ml at baseline or 6–8 weeks after delivery to provide sufficient HIV-1 RNA for genotyping. HIV-1 genotyping was successful for 70 of those 72 women. Additionally, samples collected 6–8 weeks after delivery were available from nine out of 12 (75%) of the mothers of the late-infected infants. Plasma samples were available and HIV-1 was genotyped from 24 out of 37 (65%) HIV-1 infected infants with infection diagnosed by age 6–8 weeks and nine out of 12 (75%) diagnosed after age 6–8 weeks (Table 1).

Statistics

Logistic regression was used to evaluate correlates of maternal NVP^R, including transmission status, baseline viral load, and CD4 cell counts. All statistical analyses were done with SAS (version 8.1).

Results

Quality control of sequence data

We analyzed 185 plasma samples from 144 individuals who received NVP in HIVNET 012, including 111 out of 311 (36%) women and 33 out of 49 (67%) infected infants. Samples collected at more than one time-point were genotyped for 23 out of 144 individuals. For each sample, a sequence corresponding to protease amino acids 1–99 and RT amino acids 1–324 was obtained. Phylogenetic reconstructions of the entire data set revealed the following: in cases where more than one sequence was analyzed from a given individual, all sequences from that individual clustered together; sequences from each infant clustered most closely with the sequence from the corresponding mother. Comparison of the genetic distances of all 185 sequences revealed only three cases where two sequences were identical: the sequence from one infant at 6–8 weeks of age was identical to the sequence from the same infant at 14–16 weeks of age; the sequence from one infant at birth was identical to the sequence from the same infant at 14–16 weeks of age; the sequence from one infant at 6–8 weeks of age was identical to the sequence from the corresponding mother at 6–8 weeks post-partum. This analysis provided evidence that the data set was valid, without evidence of sample cross-contamination or sample misidentification.

NVP^R mutations in women 6–8 weeks after delivery

We first analyzed samples from 102 women, including 70 whose infants were uninfected at 6–8 weeks, and 32 whose infants were diagnosed with HIV by 6–8 weeks. Analysis of women with late-infected infants is described later in this report. NVP^R mutations were detected in 18 out of 102 (18%) of women (Tables 1 and 2). The rates of resistance among women whose infants were and were not infected by 6–8 weeks were not significantly different. Baseline (pre-NVP) samples were available from all seven women whose infants were HIV-1 infected and who had NVP^R mutations detected 6–8 weeks after delivery. All seven samples lacked detectable NVP^R mutations. Those findings were consistent with our previous report that demonstrated an absence of NVP^R mutations in 27 antiretroviral drug-naive Ugandan adults [7]. Follow-up samples collected 12–24 months after delivery were available from 11 out of 18 women who had NVP^R detected 6–8 weeks after delivery. All 11 follow-up samples lacked detectable NVP^R mutations (Table 1).

Logistic regression analysis revealed an association be-

Table 2. NVP^R mutations detected in women and infants with HIV-1 infection diagnosed by age 6–8 weeks.

	Women			Infants
Mutations detected	Women whose infants were infected by age 6–8 weeks (n = 32)	Women with uninfected infants (n = 70)	All women (n = 102)	(n = 24)
K103N	5	6	11	1
K103N + Y181C	1	2	3	1
K103N + Y181C+V106A	1		1	
K103N + Y181C+G190A		1	1	
Y181C		1	1	6
Y181C + Y188C				2
Y181C + G190A				1
V108I		1	1	
Total	7	11	18	11

tween baseline viral load and development of NVP^R mutations and between baseline CD4 cell count and resistance. In a univariate model, women with high viral loads were more likely to develop resistance (per increase of one $\log_{10}$ HIV-1 RNA: odds ratio, 3.97; 95% confidence interval, 1.54–10.20; $P = 0.0042$). Similarly, women with low CD4 cell counts were more likely to develop resistance (per decrease of 100 cells: odds ratio, 1.63; 95% confidence interval, 1.20–2.21; $P = 0.0016$).

NVPR mutations in HIV-1 infected infants diagnosed by age 6–8 weeks

Plasma samples collected at 6–8 weeks of age were available for 24 out of 37 infants who were diagnosed with HIV-1 infection by 6–8 weeks of age. NVP^R mutations were detected in 11 out of 24 (46%) of those infants (Table 2). Birth samples (plasma from peripheral or cord blood) were available from 10 of those 11 infants. Analysis of NVP^R mutations was successful for nine of those samples, all of which lacked detectable NVP^R mutations. Follow-up samples collected at 14–16 weeks of age were available from nine out of 11 infants who had NVP^R mutations at 6–8 weeks of age. Of those, four out of nine lacked detectable NVP^R mutations. In contrast, five out of nine samples had the same NVP^R mutations detected in the 6–8 week sample from the same infant. The NVP^R mutations present in the 14–16 week samples from those five infants were: Y181C (three), K103N (one), and Y181C + G190A (one). Follow-up samples collected at 12 months were available for three out of those five infants, all of which lacked detectable NVP^R mutations.

Ten out of the 11 infants who had NVP^R mutations detected at 6–8 weeks of age were HIV-1 RNA positive at birth, compared to nine out of 13 infants who did not have NVP^R mutations. The death rate within the first year of life was similar among those with and without NVP^R mutations (3/11 versus 5/13, respectively). However, the numbers were too small for meaningful statistical analysis.

Comparison of NVP^R mutations in HIV-1 infected infants diagnosed by age 6-8 weeks and their mothers

Different patterns of NVP^R mutations were detected in women and infants. In women, the most common mutation was K103N, whereas in infants it was Y181C (Table 2). K103N was detected in 16 out of 18 (89%) women versus only two out of 11 (18%) infants. In contrast, Y181C was detected in six out of 18 (33%) women versus 10 out of 11 (91%) infants. NVP^R mutations were also compared in mother–infant pairs, in which both mother and infant had samples analyzed from 6–8 weeks after delivery. Twenty-two mother–infant pairs were available for analysis. In 12 pairs, samples from both the mother and infant lacked detectable NVP^R mutations. The NVP^R mutations detected in the other 10 mother–infant pairs are shown in Table 3. Six infants who had NVP^R mutations at 6–8 weeks of age had mothers who lacked detectable NVP^R mutations at that time; all of those infants had HIV-1 infection at the time of birth. In each case where both mother and infant had NVP^R, the pattern

Table 3 Comparison of NVP^R mutations in infants with HIV-1 infection diagnosed by age 6–8 weeks of age and their mothers.

Mother	Infant
WT	Y181C
WT	Y181C
WT	Y181C
WT	Y181C
WT	Y181C + G190A
WT	Y181C + Y188C
K103N	Y181C + Y188C
K103N	Y181C
K103N	Y181C + K103N
K103N + V106A + Y181C	K103N

WT, No NVP^R mutations detected.

of NVP^R mutations detected in the mother and infant was different.

NVP^R in late-infected infants and their mothers

In HIVNET 012, 98% of women breastfed their infants. Because of the potential for HIV-1 transmission by breastfeeding, infants who tested negative for HIV-1 infection at 6–8 weeks of age were followed for 18 months for evidence of late HIV-1 infection. Nine infants were diagnosed with HIV-1 at later time points. In addition, three infants who were not tested at 6–8 weeks of age, but who tested negative at birth, were diagnosed with HIV-1 at later times (Table 4). The median age of HIV-1 diagnosis in those 12 infants was 301 days (10 months; range, 77–550 days).

Samples collected 6–8 weeks after delivery were available from nine out of 12 of the mothers of the late-infected infants. NVP^R mutations were detected in three of those women (Table 4). Samples collected from those women at later time points were not available for analysis.

Samples collected after the diagnosis of HIV-1 infection were available from the corresponding nine late-infected infants. The age at diagnosis and at the time of subsequent resistance testing for each infant are shown in Table 4. Samples from eight out of nine infants lacked detectable NVP-resistance mutations. This included two out of three infants whose mothers had NVP^R mutations detected 6–8 weeks after delivery. Only one out of nine infants had NVP^R mutations detected following diagnosis of HIV-1 infection. That infant's mother had a mixture of codons AAA (K, lysine) and AAC (N, asparagine) at position 103, as well as the mutation Y181C detected 6–8 weeks postpartum. The infant tested negative for HIV-1 infection at 6–8 weeks, but tested positive for HIV-1 infection at day 367 (approximately 12 months of age). Samples from the infant at 15 months and 18 months of age both had the K103N mutation. Interestingly, both follow-up samples from the infant had an unusual mixture of codons at position 103: AAC and AGC (S, serine). The Y181C mutation was not detected in either of the infant's follow-up samples.

Discussion

We detected NVP-resistant HIV-1 6–8 weeks after delivery in 19% of 111 women tested who received single dose NVP in HIVNET 012. Several factors may have contributed to the selection of NVP-resistant HIV-1 in this setting. These include the high potency and long half-life of NVP in pregnant women during labor (median $t_{1/2} = 61.3$ h) [6,8], the ability of a single mutation (K103N or Y181C) to cause high level NVP^R, and the probability that minor HIV-1 variants with those mutations are likely to be present at low background levels in most infected women prior to NVP administration [4,9]. We found that the K103N mutation was selected more frequently than Y181C in women following single dose NVP. This is consistent with early detection of K103N in patients receiving chronic NVP therapy [10]. Early emergence of variants with K103N may reflect a fitness advantage of those variants compared to variants with Y181C. With increased NVP exposure, Y181C variants with higher levels of phenotypic NVP^R would be expected to emerge.

Frequent emergence of NVP^R among women in HIVNET 012 may in part reflect their advanced stage of HIV-1 disease. At study entry, women analyzed in this report had relatively high viral loads (median, 40 067 copies/ml) and relatively low CD4 cell counts (median, 412×10^6 cells/μl). Both of those factors were associated with development of NVP^R in this cohort. Therefore, the rate of NVP^R following single

Table 4 Analysis of NVP^R mutations in late-infected infants and their mothers.

Mutations detected in women 6–8 weeks after delivery	Age of infants at diagnosis of HIV-1 infection	Age of infants at time of sample collection	Mutations detected in infants
WT	7–8 months[a]	9 months	WT
WT	3–4 months[a]	8 months	WT
WT	2–3 months[a]	6 months	WT
WT	12–13 months	18 months	WT
WT	12 months	15 months	WT
WT	3–4 months	6 months	WT
K103N + G190A	3–4 months	12 months	WT
K103N	12–13 months	13 months	WT
K103N + Y181C	12–13 months	15 months	K103N
		18 months	K103N

[a]These three infants tested negative for HIV-1 infection at birth; one of these infants also tested negative for HIV-1 infection at day 7. Because of missed clinic visits, none of these infants was tested again until the age at diagnosis (shown). Infection of these three infants prior to 6–8 weeks of age cannot be excluded. WT, No NVP^R mutations detected.

dose NVP prophylaxis may be lower in cohorts with less advanced HIV-1 disease. In the USA, NVP prophylaxis is one of the recommended options for prevention of HIV-1 MTCT in women in labor who have not received antiretroviral therapy during pregnancy [11]. Initiation of fully suppressive, highly active antiretroviral therapy in such women during the immediate postnatal period would probably reduce the risk that NVP-resistant HIV-1 would emerge.

In the HIVNET 012 cohort, NVP-resistant HIV-1 faded from detection in women over time. HIV-1 variants with NVP^R mutations may continue to circulate in these women as minor variants, and be maintained as provirus in infected cells. However, replacement of the major HIV-1 population with NVP-sensitive HIV-1 makes it less likely that NVP-resistant HIV-1 would be transmitted from women to other adults. Furthermore, our inability to detect NVP-resistant HIV-1 12–24 months after delivery suggests that the single dose NVP prophylaxis regimen would remain effective for interruption of intrapartum transmission in subsequent pregnancies. This is because most of the HIV-1 population would be sensitive to NVP at the time of labor, when NVP is administered, and would be effectively inhibited during labor and delivery, when HIV-1 transmission is most likely to occur. This requires confirmation. In contrast, persistence of minor variants or proviruses with NVP^R mutations could potentially limit the use of NVP or other non-nucleoside RT inhibitors for subsequent treatment of HIV-1 infection. In developing countries, where the NVP prophylactic regimen is most likely to be implemented, current treatment options for HIV-1 infection are extremely limited. If treatment options in those countries were widely expanded in the future, women with NVP-resistant HIV-1 could be offered alternative treatment regimens with other antiretroviral drugs.

We found no association between post-partum selection of NVP-resistant HIV-1 in women and the risk of MTCT with the first use of NVP prophylaxis. We also found little evidence for transmission of NVP-resistant HIV-1 variants from women to infants who were infected by 6–8 weeks of age. The risk of transmission of NVP-resistant HIV-1 variants through breastfeeding requires further evaluation. The kinetics of the emergence and fading of NVP-resistant HIV-1 in women receiving single dose NVP has not been defined, and the duration NVP-resistant HIV-1 persists in the mother will affect the risk of late postnatal transmission of resistant virus. Of the nine late-infected infants in this study, only three had mothers with NVP^R detected at 6–8 weeks postpartum. Of those, two infants were infected with NVP-sensitive HIV-1 and one was infected with NVP-resistant HIV-1. Additionally, 11 women with NVP-resistant HIV-1 had uninfected infants and did not transmit HIV-1 despite breastfeeding. However, because evaluation of NVP^R in infants in HIVNET 012 was possible only using follow-up samples collected months after the diagnosis of HIV-1 infection for most infants, it is possible that additional NVP-resistant strains were transmitted to infants by breastfeeding, but that those variants faded from detection before follow-up samples were collected.

Our data suggest that NVP-resistant HIV-1 is selected independently in infants after NVP administration. The relatively high rate of NVP^R observed among infants in HIVNET 012 could reflect the high viral loads typically present in HIV-1 infected infants. Increased exposure to NVP in infants compared to their mothers may also favor emergence of NVP-resistant HIV-1. Infants in HIVNET 012 were essentially dosed twice: once by the placental transfer of maternally administered NVP, and once within 48–72 h of birth. The half-life of NVP in infants is long (median $t_{1/2} =$ 46.5 h) [6,8]. When pregnant women and infants received the same regimen used in HIVNET 012, the NVP concentration in infants was > 100 ng/ml (> 10 times the 50% inhibitory concentration of the drug) for 7 days [6,8]. The greater NVP exposure of infants versus women in HIVNET 012 may also explain the more frequent detection of the Y181C mutation (rather than K103N) in infants. The fading of NVP-resistant HIV-1 that we observed in infants paralleled that observed in women post-partum, and was consistent with reduced fitness of HIV-1 with NVP^R mutations in the absence of the drug.

The findings of this report emphasize the importance of evaluating the development of drug resistance among women receiving short-course antiretroviral prophylaxis regimens, particularly for prophylaxis regimens using antiretroviral drugs in which a single mutation can confer resistance, such as NVP or lamivudine. Such evaluations should include: (i) further characterization of the kinetics of the emergence and fading of NVP^R in women receiving single dose NVP prophylaxis; (ii) assessment of whether there is a risk for transmission of NVP-resistant virus to infants postnatally through breastfeeding or to sexual partners and the magnitude of that risk; and (iii) assessment of the association of NVP^R with disease progression. Such monitoring should be planned within future perinatal trials, as well as within the context of implementation efforts. Epidemiologic studies could also be considered to evaluate the effectiveness of the single dose NVP regimen in future pregnancies.

The potential for selection of NVP-resistant HIV-1 in women and infants receiving single dose NVP prophylaxis must be balanced against the documented efficacy, simplicity, and cost-effectiveness of the HIVNET 012 regimen. This regimen can significantly reduce HIV-1

MTCT in settings where other prophylactic regimens are impractical and treatment options are extremely limited. If implemented rapidly, this regimen can prevent HIV-1 infection in millions of HIV-1 exposed infants over the next decade.

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