Association between Detection of HIV-1 DNA Resistance Mutations by a Sensitive Assay at Initiation of Antiretroviral Therapy and Virologic Failure

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Background. Antiretroviral therapy (ART) has become more available throughout the developing world during the past 5 years. The World Health Organization recommends nonnucleoside reverse-transcriptase inhibitor–based regimens as initial ART. However, their efficacy may be compromised by resistance mutations selected by single-dose nevirapine (sdNVP) used to prevent mother-to-child transmission of human immunodeficiency virus (HIV)–1. There is no simple and efficient method to detect such mutations at the initiation of ART.

Methods. One hundred eighty-one women who were participating in a clinical trial to prevent mother-to-child transmission and who started NVP-ART after they had received sdNVP or a placebo were included in the study. One hundred copies of each patient’s HIV-1 DNA were tested for NVP-resistance point-mutations (K103N, Y181C, and G190A) with a sensitive oligonucleotide ligation assay that was able to detect mutants even at low concentrations (≥5% of the viral population). Virologic failure was defined as confirmed plasma HIV-1 RNA ≥50 copies/mL after 6 to 18 months of NVP-ART.

Results. At initiation of NVP-ART, resistance mutations were identified in 38 (26%) of 148 participants given sdNVP (K103N in 19 [13%], Y181C in 8 [5%], G190A in 28 [19%], and ≥2 mutations in 15 [10%]), at a median 9.3 months after receipt of sdNVP. The risk of virologic failure was 0.62 (95% confidence interval [CI], 0.46–0.77) in women with ≥1 resistance mutation, compared with a risk of 0.25 (95% CI, 0.17–0.35) in those without detectable resistance mutations (P < .001). Failure was independently associated with resistance, an interval of <6 months between sdNVP and NVP-ART initiation, and a viral load higher than the median at NVP-ART initiation.

Conclusions. Access to simple and inexpensive assays to detect low concentrations of NVP-resistant HIV-1 DNA before the initiation of ART could help improve the outcome of first-line ART.
tations and a higher risk of NNRTI-based ART failure [1, 2]. This creates a diagnostic challenge, because NVP-resistant mutants decay to concentrations not detected by consensus sequencing within 6 to 18 months after sdNVP [3]. Studies have found that the longer the period between sdNVP and the initiation of ART, the more effective NNRTI-based ART is at achieving suppression of HIV-1 replication [2, 4]. Other studies have found that the selection and decay rate of NVP-resistant viruses appears to vary by subtype [5], by codon [6], between individuals [3, 7–9], and by methods used to detect mutations [7, 10, 11]. Recent studies suggest that detection of persistent resistance mutations would help identify individuals with a higher risk of virologic failure [12–16]. Because NVP-resistance mutations may persist longer in cellular DNA than in plasma RNA [17, 18], we hypothesized that, in women who have previously received sdNVP or a placebo, resistance that was detected using a sensitive assay (ie, capable of detecting point-mutations at concentrations as low as 5% of the viral population) that was applied to HIV-1 DNA at the time ART is initiated would be associated with virologic failure of NVP-ART.

METHODS

Study population and design. We studied women who were participating in the Program for HIV Prevention and Treatment (PHPT)–2 (ClinicalTrials.gov Identifier: NCT00398684), a randomized, placebo-controlled trial of adding sdNVP in labor to ongoing stavudine and lamivudine (NVP-ART) [19]. We determined whether detection of NVP resistance in the pre-ART specimen was associated with the virologic response to NVP-ART.

Banked plasma-poor whole blood specimens that were obtained from women immediately before initiating NVP-ART were retrospectively assessed for 3 HIV-1 pol mutations that confer high-level resistance to NVP (K103N, Y181C, and G190A). The personnel who tested the specimens did not have high-level resistance to NVP (K103N, Y181C, and G190A). The personnel who tested the specimens did not have previously received sdNVP or a placebo, resistance that was detected using a sensitive assay (ie, capable of detecting point-mutations at concentrations as low as 5% of the viral population) that was applied to HIV-1 DNA at the time ART is initiated would be associated with virologic failure of NVP-ART.

DNA extraction and quantification. DNA was extracted from plasma-depleted whole blood by means of QiAmp DNA Midi kits (Qiagen). DNA was quantified using a Hoefer DyNA Quant 200 Fluorometer (Amersham Pharmacia Biotech).

HIV-1 quantification and amplification. The HIV-1 DNA concentration in each participant’s genomic DNA was determined using real-time polymerase chain reaction (PCR) amplification of HIV-1 long terminal repeat [20]. DNA that contained a total of 100 copies of HIV-1 was split between 2 separate nested PCRs and then was tested by oligonucleotide ligation assay (OLA) [21].

OLA. The OLA that we developed uses an enzyme-linked immunosassay to detect HIV-1 point-mutations that are associated with resistance to antiretroviral agents [21]. Two PCR amplicons from each participant were evaluated in duplicate by OLA for mutations in 3 codons (K103N, Y181C, and G190A) that are associated with resistance to NVP, with reagents that are adapted to HIV-1 CRF01_AE viruses. OLA results were interpreted by comparison to subtype- and codon-specific controls. The assay was repeated in cases of discordance between duplicates, and resistance was determined by majority. Samples that tested positive for a mutant codon in either or both of the 2 PCRs performed for each woman were classified as NVP resistant, on the assumption that rare drug-resistance mutants would be distributed randomly across independent PCRs. Samples in which neither the wild-type nor the mutant oligonucleotides produced a positive signal were considered to be indeterminate.

Statistical analysis. The primary analysis was performed using the cases previously reported [1] for which there were OLA data from specimens obtained at ART initiation. The primary end point was a plasma HIV-1 RNA >50 copies/mL within 6 to 18 months after initiation of NVP-ART, confirmed as sustained viremia by a second viral load >50 copies/mL in the next blood specimen obtained from the participant. Observations were censored at time of switch to a protease inhibitor–based regimen or at the last visit, in cases of discontinuous follow-up. A secondary analysis was performed using the plasma HIV-1 RNA threshold of 400 copies/mL, and sensitivity analyses evaluated the outcome when death was classified as failure and when specimens that yielded insufficient HIV-1 DNA to perform OLA were assumed to have no NVP resistance.

To compare distributions of categorical data and continuous variables, we used the Fisher exact test and the Wilcoxon rank-sum test, respectively. For multivariate analyses, variables were selected on the basis of the results of univariate analyses (P < .20). A multivariate logistic regression model was used to study the association between the detection of resistance mutations by OLA at ART initiation and the presence of NNRTI resistance mutations by consensus sequencing at 10 days postpartum, the interval between sdNVP and ART initiation, and the HIV RNA load and the CD4 cell count at time of ART initiation. Kaplan-Meier survival estimates of the 18-month virologic failure rate were calculated. Cox proportional hazards models were used to assess the respective effect on virologic failure of the detection of NNRTI resistance mutations by consensus sequencing at 10 days postpartum; the interval between sdNVP and ART initiation; and the plasma HIV RNA load, the CD4 cell count, and the detection of resistance mutations by OLA at ART initiation. Statistical analyses were performed using Stata, version 10.1 (StataCorp).

Ethics. The PHPT-2 and the PHPT Cohort Study protocols and their amendments were approved by the ethics committees of the Thai Ministry of Public Health and the Chiang Mai University Faculty of Associated Medical Sciences.
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Figure 1. Flow chart showing specimens analyzed by oligonucleotide ligation assay (OLA) at initiation of nevirapine (NVP)-based antiretroviral therapy (ART). HIV, human immunodeficiency virus; PCR, polymerase chain reaction.

RESULTS

Study population. OLA results were obtained from 181 of the 269 women in the PHPT-2 trial who started NVP-ART at a median 9.3 months postpartum (148 of the 221 women who had been exposed to sdNVP and 33 of the 48 unexposed women) (P = .87). Eighty-eight women were not analyzed by OLA, primarily because the available plasma-poor whole blood specimens yielded insufficient HIV-1 DNA (Figure 1). The characteristics of the 181 women tested by OLA were compared with those of the 88 women who were not tested by OLA (73 who had been exposed to sdNVP and 15 unexposed) (Table 1). The median age, the CD4 cell count during pregnancy and at ART initiation, the interval between sdNVP and NVP-ART, the proportion exposed to sdNVP, and the risk of virologic failure within 6 to 18 months of therapy were similar between the 2 groups. However, median plasma HIV-1 RNA during pregnancy and at the initiation of NVP-ART was ~0.5 log10 copies/mL greater in those assessed by OLA. The risk of virologic failure was 0.32 in the group of women with OLA results and 0.33 in those without (P = .90). The CRF 01_AE HIV-1 subtype was identified in 243 (96%) of the 253 subjects, with no differences between the 2 groups.

NVP resistance mutations detected by OLA. At least 1 of the 3 resistance mutations was detected at NVP-ART initiation in 38 (26%) of the 148 women who were exposed to sdNVP and who had OLA results: 19 (13%) with the K103N mutation, 7 (5%) with the Y181C, and 28 (19%) with the G190A. At least 2 mutations were detected in 15 women (10%). None of the 33 women unexposed to sdNVP had resistance mutations at NVP-ART initiation. The OLA result was indeterminate for all 3 codons in 1 woman, who was excluded from analysis (Figure 1), and for a single codon in 4 other women (one at position 103, one at 181, and two at 190, including one with an identified K103N mutation). OLA results for duplicate PCRs from the same DNA sample were discordant in 38 (7%) of 543 samples, suggesting low concentrations of mutant. Repeat OLA results from individual PCRs were discordant in 69 (6%) of 1086 samples, mostly with readings near the 5% limit of detection and across plates, suggesting interassay variability.

Factors associated with the detection of mutations by OLA. Of the 148 women with available OLA results, 145 had been

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Women tested by OLA (n = 181)</th>
<th>Women not tested by OLA (n = 88)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median years (IQR)</td>
<td>28.8 (25.4–32.5)</td>
<td>28.2 (24.9–33.1)</td>
<td>.94</td>
</tr>
<tr>
<td>CD4 cell count during pregnancy, median cells/µL (IQR)</td>
<td>186 (146–242)</td>
<td>187 (125–236)</td>
<td>.46</td>
</tr>
<tr>
<td>Plasma HIV-1 RNA during pregnancy, median log10 copies/mL (IQR)</td>
<td>4.63 (4.08–4.96)</td>
<td>4.25 (3.64–4.88)</td>
<td>.003</td>
</tr>
<tr>
<td>Received intrapartum sdNVP</td>
<td>148 (82)</td>
<td>73 (83)</td>
<td>.87</td>
</tr>
<tr>
<td>HIV-1 subtype CRF01_AE (253 sequences available)</td>
<td>169 (96)</td>
<td>74 (96)</td>
<td>1.0</td>
</tr>
<tr>
<td>Interval from sdNVP to initiation of NVP-ART, median months (IQR)</td>
<td>9.3 (3.8–15.9)</td>
<td>6.2 (2.7–14.0)</td>
<td>.10</td>
</tr>
<tr>
<td>Started HAART &lt;6 months after delivery</td>
<td>74 (41)</td>
<td>43 (49)</td>
<td>.24</td>
</tr>
<tr>
<td>CD4 cell count at initiation of NVP-ART, median cells/µL (IQR)</td>
<td>166 (83–217)</td>
<td>184 (99–231)</td>
<td>.47</td>
</tr>
<tr>
<td>Plasma HIV-1 RNA at initiation of NVP-ART, median log10 copies/mL (IQR)</td>
<td>4.81 (4.27–5.17)</td>
<td>4.27 (3.46–4.84)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Risk of virologic failure after 6 to 18 months of therapy, median (95% CI)</td>
<td>0.32 (0.26–0.40)</td>
<td>0.33 (0.23–0.45)</td>
<td>.90</td>
</tr>
</tbody>
</table>

NOTE. Data are no. (%) of patients unless otherwise specified. All 269 women initiated NVP-based ART after they had participated in a randomized double-blind trial of adding single-dose NVP (sdNVP) or placebo to zidovudine starting in the third trimester of pregnancy [1]. CI, confidence interval; HAART, highly active ART; HIV, human immunodeficiency virus; IQR, interquartile range.

* By Wilcoxon rank-sum test for continuous variables, Fisher exact test for categorical data, and log rank for risk of failure.
tested for NNRTI resistance by consensus sequencing 10 days after sdNVP exposure. Women with NVP-resistance mutations detected by consensus sequencing were more likely to have resistance mutations detected by OLA at the initiation of ART. Specifically, 24 (50%) of the 48 women with NNRTI resistance detected by consensus sequencing 10 days after sdNVP had ≥1 mutation by OLA, compared with 14 (14%) of 97 women who had no mutations by consensus sequencing (P < .001). A higher percentage of women who initiated ART within 6 months of sdNVP had at ≥1 NVP mutation detected by OLA, compared with the corresponding percentage of women who initiated ART >6 months after sdNVP (26 [37%] of 70 women versus 12 [15%] of 78 women; P = .004). NVP mutations by OLA were not significantly associated with a higher viral load (P = .09) or a lower CD4 cell count at time of ART start (P = .71).

Upon multivariate logistic regression, only NVP mutations at 10 days postpartum (adjusted odds ratio [aOR], 5.7; 95% CI, 2.5–13.0; P < .001) and initiation of ART within 6 months of delivery (aOR, 3.0; 95% CI, 1.3–7.1; P = .01) remained independently associated with the detection of mutations by OLA, and no significant interaction was detected between these 2 factors.

**NVP-ART outcome according to resistance mutations detected by OLA.** In the group of 148 women who were exposed to sdNVP and who had available OLA results, 3 women (2%) died (at 7, 7, and 10 months after NVP-ART initiation, respectively), 4 (3%) withdrew from the study because they moved to another province, and 16 (11%) were lost to follow-up. In the group of 48 women unexposed to sdNVP, 2 (4%) died, 1 (2%) withdrew because of moving, and 1 (2%) was lost to follow-up. There were no cases of switching to a protease inhibitor regimen before failure.

Among the 148 women who were exposed to sdNVP and who had available OLA results, the risk of virologic failure was 0.62 (95% CI, 0.46–0.77) in women with at ≥1 resistance mutation detected by OLA at the time of NVP-ART initiation, compared with 0.25 (95% CI, 0.17–0.35) in those with no detectable resistance mutations (P < .001) (Figure 2). The detection of ≥2 mutant codons was associated with a higher rate of virologic failure (hazard ratio [HR], 5.6; 95% CI, 1.3–22.1; P < .001) (Figure 2). Among the 33 women unexposed to sdNVP who had available OLA results, the risk of failure was 0.13 (95% CI, 0.05–0.32) (Figure 2).

**Other risk factors associated with virologic failure.** Virologic failure was also increased (Table 2) with the detection by OLA of each of the 3 mutations (K103N and Y181C, P < .001; G190A, P = .001); a viral load higher than the median at enrollment during pregnancy (4.58 log_{10} copies/mL) (P = .004) or higher than the median at initiation of NVP-ART (4.77 log_{10} copies/mL) (P = .01); a CD4 cell count below the median during pregnancy (183 cells/μL) (P = .02) (but virologic failure was not increased with a CD4 cell count below the median at initiation of NVP-ART [153 cells/μL] [P = .61]); and with an interval of <6 months between delivery and NVP-ART initiation (P < .001). Notably, women with ≥1 resistance mutation detected by consensus sequencing at 10 days postpartum did not have a higher risk of virologic failure, compared with the risk of women with no mutations (P = .28).

In the multivariate analysis, the risk factors that remained independently associated with an increased rate of failure were an interval of <6 months between delivery and NVP-ART initiation (adjusted HR, 3.2; 95% CI, 1.7–6.2; P = .001), the detection of any NVP resistance by OLA at the time of NVP-ART initiation (adjusted HR, 2.5; 95% CI, 1.3–4.5; P = .004), and a viral load higher than the median at initiation of NVP-ART (adjusted HR, 2.4; 95% CI, 1.3–4.5; P = .007).

**Secondary and sensitivity analyses.** A secondary analysis used a confirmed viral load >400 copies/mL as the threshold for virologic failure. In this model, the HRs for failure and the levels of statistical significance were very similar to those reported in the primary analysis, except that a CD4 cell count higher than the median during pregnancy (183 cells/μL) was not significantly associated with virologic failure (P = .1). A sensitivity analysis that classified deaths with virologic failures also gave very similar results.

**DISCUSSION**

In this study, genotypic NVP resistance in proviral HIV-1 DNA obtained at the initiation of NVP-ART was strongly associated with virologic outcome. Specifically, women with a proviral population that contained >5% resistant mutants (K103N, Y181C, or G190A) were significantly more likely to experience virologic failure. In contrast, the risk of virologic failure was not significantly associated with resistance detected by consensus sequencing in the period after exposure to sdNVP. The marked association between NVP resistance detected by OLA and virologic failure provides a rationale for testing patients with previous exposure to NNRTI at the initiation of ART to guide selection of antiretrovirals for treatment. From a public health perspective, the selective use of an alternative regimen, such as a protease inhibitor–based regimen, would improve the chance of successful suppression of HIV-1 replication in women with NVP resistance. Importantly, women without mutations had a rate of virologic failure similar to that of women who had not been exposed to sdNVP, suggesting that it may be reasonable for these women to start NVP-based ART, which is a simpler, better tolerated, and less expensive regimen. Although there are many communities where testing for HIV-1 drug resistance is not feasible today, other communities may realize a cost benefit with resistance testing by OLA. OLA costs US$20–$40 (depending on whether multiplexed) for the 3 co-
dons that correspond to the most prevalent NNRTI resistance mutations, whereas use of a protease inhibitor–based ART cost US$300–$400 per year more than an NNRTI-based ART in June 2009 [24].

Multiple studies [2, 4], including this study, suggest that sdNVP selects NVP-resistant viruses that then decay over the subsequent months, decreasing to clinically insignificant levels in many women. The detection of HIV-1 drug-resistance mutations by more-sensitive assays is associated with virologic failure of ART in case reports and in a few larger studies [12–16], although 1 study found no association between low-frequency mutations and ART outcome [22]. In our study, women with ≥5% NVP-resistant mutants in cellular DNA had a high risk (0.62) of virologic failure. Yet, some women with detected resistance experienced virologic success. This may be because resistance mutations can be detected as a part of an integrated yet nonfunctional proviral genome [23], falsely indicating a risk of virologic failure.

The persistence of NVP-resistant mutants for >6 months after sdNVP in association with virologic failure suggests that the effects of sdNVP can linger for >6 months. The women with NVP resistance that persisted until the initiation of NVP-
ART did not have lower levels of CD4 lymphocytes or higher concentrations of plasma HIV-1 RNA at the initiation of NVP-ART; however, these women had a shorter interval between sdNVP and treatment. A threshold for the effect of time between sdNVP and initiation of NVP-ART could not be identified, which was also true for the analysis that combined cohorts from Zambia, Kenya, and Thailand (not inclusive of our subjects) [4]. Both our cohort and the combined cohort [4] detected an effect of sdNVP beyond the 6-month interval, which had previously been suggested as the outer limit for the effect of sdNVP on the virologic outcome of NVP-ART [2].

Several novel and important observations come from our study. First, drug-resistant HIV-1 variants appear to decay at different rates across individuals; therefore, mutations that present immediately before ART appear more relevant to treatment outcome than those detected postpartum. Second, we assayed peripheral blood mononuclear cell–derived HIV-1 DNA rather than plasma-derived HIV-1 RNA. Comparative studies have shown that HIV-1 resistance mutations are detectable for a longer period in peripheral blood cellular DNA, compared with the corresponding period in plasma RNA, both in women after sdNVP [18] and in individuals for whom ART fails [17]. These studies have also shown that HIV-1 resistance mutations are detectable for a longer period by means of OLA, compared with the corresponding period by consensus sequencing, both in women after sdNVP [18] and in individuals for whom ART fails [17]. Although other studies have observed the persistence of resistance for longer periods in HIV-1 RNA, compared with the periods in DNA [9, 14], these findings may have been affected by sampling too few copies of HIV-1 DNA. Third, OLA that detected mutants present at ≥5% of the total viral population, which is considerably more sensitive than consensus sequencing, captured a large part of the risk for virologic failure. More sensitive methods, such as allele-specific PCR, may be able to detect more resistance, but the concentration of clinically relevant mutant is uncertain, and in the case of our study, it is not likely that a more sensitive assay would have improved the predictive power.

A limitation of our study is that a significant number of samples could not be analyzed by OLA, mostly because of insufficient available DNA. Although the exclusion of these subjects could have the potential to bias our findings, the only difference between these subjects and the group analyzed by OLA was slightly higher plasma HIV-1 RNA among the latter. Nevertheless, the rate of virologic failure among the 88 excluded women was similar to that of the women included in the study, suggesting that the study was not biased toward women for whom therapy was more likely to fail.

In summary, testing women who had been exposed to sdNVP just before the initiation of NVP-ART identified NVP-resistant HIV-1, and the women with detected resistance were at increased risk of virologic failure. Identification of ≥2 NVP-resistance mutations conferred an extremely high likelihood of virologic failure. Also, these results may be of interest for patients who previously discontinued an NVP-based regimen for toxicity and who are later candidates for an efavirenz-based ART, or even for antiretroviral-naive patients who are starting an NNRTI-based regimen, because NVP-resistant viruses have also been detected in patients who have not been treated with sdNVP [14, 16], presumably because of the transmission of drug-resistant viruses. These observations call for further investigations to determine the thresholds above which drug-resistant viruses are clinically relevant and warrant access to simple and inexpensive assays that evaluate low concentrations of drug-resistant HIV-1.

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Potential conflicts of interest.

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