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Minor drug-resistant HIV type-1 variants in breast milk and plasma of HIV type-1-infected Ugandan women after nevirapine single-dose prophylaxis

Daniel Pilger[1], Andrea Hauser[1,2], Claudia Kuecherer[2], Kizito Mugenyi[3], Rose Kabasinguzi[3], Sybille Somogyi[2], Gundel Harms[1,*], Andrea Kunz[1]

[1] Institute of Tropical Medicine and International Health, Charité - Universitâtsmedizin Berlin, Berlin, Germany
[2] Project HIV Variability and Molecular Epidemiology, Robert Koch Institute, Berlin, Germany
[3] MoH/GTZ PMTCT Project western Uganda, Fort Portal, Uganda

*Corresponding author e-mail: gundel.harms@charite.de

Abstract

Background: Nevirapine single-dose (NVP-SD) reduces mother-to-child transmission of HIV type-1 (HIV-1), but frequently induces resistance mutations in the HIV-1 genome. Little is known about drug-resistant HIV-1 variants in the breast milk of women who have taken NVP-SD.

Methods: Blood and breast milk samples of 39 HIV-1-infected Ugandan women were taken 6–12 weeks after NVP-SD intake. Samples were analysed by population sequencing and allele-specific real-time PCR (AS-PCR) with detection limits for NVP-resistant HIV-1 variants (K103N and Y181C) of <1% of the total viral population.

Results: AS-PCR results for both plasma and breast milk were obtained for 19 women who constituted the final study group (HIV-1 subtype frequencies were A1 n=11, D n=5, G n=2 and C n=1). A total of 7 (37%) and 10 (53%) women carried NVP-resistant virus in breast milk and plasma, respectively. Overall, 71% (5/7) women with NVP-resistant HIV-1 in breast milk displayed >1 drug-resistant variant. Resistance in breast milk was higher at week 6 (6/13 samples [46%]) compared with week 12 (1/6 samples [17%]). In total, 10 drug-resistant populations harbouring the K103N and/or Y181C mutation were detected in the 19 breast milk samples; 7 (70%) were caused by resistant minorities (<5% of the total HIV-1 population). In the four women with drug-resistant virus in both plasma and breast milk, the mutation patterns differed between the two compartments.

Conclusions: Minor populations of drug-resistant HIV-1 were frequently found in breast milk of Ugandan women after exposure to NVP-SD. Further studies need to explore the role of minor drug-resistant variants in the postnatal transmission of (resistant) HIV-1.

Introduction

Mother-to-child transmission of HIV is a major concern in sub-Saharan Africa, and postnatal HIV infection through breastfeeding is estimated to account for almost one-third to one-half of all HIV infections in infants and young children [1]. Nevirapine single-dose (NVP-SD) is still widely used to reduce the transmission of HIV type-1 (HIV-1) from the mother to the child in resource-limited settings, although it is associated with a high incidence of NVP-resistant HIV-1 [2–11]. To date, most studies have focused on the development of NVP-resistant virus in plasma, and it has been shown that 19–75% of women exposed to NVP-SD harbour NVP-resistant HIV-1 variants in their blood [3–11]. Although the proportion of resistant virus in plasma fades over time, minor drug-resistant populations can persist for long periods in this compartment [5,8,9]. However, little is known about the emergence and persistence of NVP-resistant HIV-1 variants in breast milk after NVP-SD and to what extent the mutation patterns differ from plasma. This is of great importance as breastfeeding is common in many developing countries where NVP-SD is used and the presence of resistant virus in breast milk might put children at risk of acquiring drug-resistant HIV-1 through breast milk [12].

So far, only three studies have determined the emergence of NVP-resistant virus in breast milk following intake of NVP-SD. Two studies analysed breast milk of women infected with HIV-1 subtype C and showed that HIV-1 variants in breast milk emerged frequently and often differed in sequences from resistant HIV-1 variants in plasma [13,14]. Recently, a third study analysed breast milk samples of women mainly infected with HIV-1 subtypes A and D using a population-based approach with a detection limit for drug-resistant HIV-1 variants of approximately 20% [15].

Here, we describe the emergence of NVP-resistant HIV-1 variants in breast milk and plasma of Ugandan women predominantly infected with HIV-1 subtypes A and D using highly sensitive allele-specific real-time PCR (AS-PCR), capable of detecting minor drug-resistant variants representing <1% of the total viral population.

Methods

We conducted an observational cohort study that was approved by the national ethical committee of Uganda (National Council of Science and Technology). HIV-1-positive pregnant women enrolled in the national prevention of mother-to-child transmission programme were recruited at Fort Portal Hospital (Fort Portal, western Uganda) after they had given informed written consent [16]. The women had taken NVP-SD (200 mg) at the onset of labour following the HIVNET 012 protocol [2]. None of the participating women received any other antiretroviral drugs aside from NVP-SD before or during the study period. Plasma and breast milk samples were collected at delivery (baseline) and at 1, 2, 6 and 12 weeks postpartum. Participants were included in the final analysis if plasma and breast milk samples from delivery were available and follow-up samples at week 6 or 12 were amplifiable. Whenever the baseline sample was amplifiable it was used as individual threshold for the detection of NVP-resistant variants as described by Hauser *et al.* [17]; otherwise a cutoff derived from plasmid mixtures was applied. HIV-1 variants with the K103N and/or Y181C mutation in the *pol* gene were detected and quantified by two-step AS-PCR assays, as previously described [17]; the detection limits for the three mutations as estimated from plasmid controls were 0.019% (K103N:AAC), 0.013% (K103N:AAT) and 0.29% (Y181C:TGT). To compensate for the expected lower viral loads in breast milk, the sample volume for RNA extraction was increased sixfold (3 ml) as compared with plasma (0.5 ml). Viral RNA was extracted (QIAamp Viral RNA Mini Kit; Qiagen GmbH, Hilden, Germany) and reverse transcribed (SuperScript II RT; Invitrogen, Karlsruhe, Germany) according to the manufacturer's recommendations. Viral loads were determined in the outer quantitative PCR (644 base pair amplimer with coordinates 2613–3256 of HXB2; GenBank accession number K03455) using a quantified virus stock (NL4.3) as standard. The lower limit of detection for viral load was 650 copies/ml; that is, the input of 3 ml breast milk allowed the quantification of viral loads down to 220 copies/ml. For population-based sequencing of the 644 base pair product (comprising the codons 21–236 of the reverse transcriptase), the automated sequencer 3130xl Genetic Analyzer (Applied Biosystems, Darmstadt, Germany) and the B-, D- and G-sequencing primers of the ViroSeq HIV-1 Genotyping System version 2.0 (Abbott GmbH & Co. KG, Wiesbaden, Germany) were used [17]. The sequences were subtyped with the REGA HIV-1 subtyping tool [18] or by the neighbour-joining method (PHYLIP package [19]). A neighbour-joining tree was constructed by aligning the *pol* sequences with 131 subtype reference sequences from the *pol* subtype reference alignment 2008 [20] using the CLUSTALW programme implemented in BioEdit version 7.0.5 [21]. All samples were measured at least in duplicates together with no template- and HIV-1-negative controls. A neighbour-joining phylogenetic tree was calculated to control for potential sample mix-up. Sequences derived from plasma and breast milk from one patient should colocalize in clades, whereas independent HIV-1 strains should not cluster together.

Results

Blood samples and breast milk samples of 39 women were available. For 20 (51%) women, the analyses of breast milk samples were PCR-negative because viral loads were below the detection level of the assays. Samples from these women were omitted from the final analysis, and the remaining 19 women constituted the final study group. The median age was 25 years (interquartile range [IQR] 19–29) and the median parity was 2 (IQR 1–5). A total of 11 (58%) women were infected with subtype A1, 5 (26%) with subtype D, 2 (11%) with subtype G and 1 (5%) with subtype C. The median viral load in breast milk was 1,100 copies/ml (IQR 500–2,800 copies/ml; Table 1).

Overall, 7 (37%) women harboured drug-resistant HIV-1 in breast milk and 10 (53%) women in plasma (Table 1). NVP-resistant virus in breast milk was detected in 5/7 subtype A1, 0/5 subtype D, 1/2 subtype G and 1/1 subtype C samples, respectively. A total of 13 breast milk samples were collected 6 weeks and 6 were collected 12 weeks after NVP-SD intake. At week 6, drug-resistant virus was detected in 6/13 (46%) breast milk samples, whereas at week 12, 1/6 (17%) breast milk samples exhibited NVP-resistant HIV-1. In total, 10 and 13 drug-resistant populations carrying the K103N or Y181C mutation were detected in breast milk and plasma samples, respectively. Among these, 7 (70%) in the breast milk and 5 (38%) in the plasma were present as resistant minor variants defined as <5% of the total viral population.

In 4 (21%) women, drug-resistant virus was identified in both breast milk and plasma. In these women (patient numbers 2, 4, 6 and 12; Table 1), the mutation patterns differed between the two compartments. In 5/7 (71%) women (patient numbers 2, 4, 6, 12 and 15; Table 1) with drug-resistant virus in breast milk, >1 NVP-resistant HIV-1 variants were found in the same breast milk sample.

All samples identified as wild-type HIV-1 (K103 and Y181) by AS-PCR yielded wild-type results by population-based sequencing. Whenever population-based sequencing detected HIV-1 variants carrying the K103N and/or Y181C mutation, the AS-PCR assays confirmed the presence of mutations. Population-based sequencing with its much lower sensitivity failed to detect 15 drug-resistant variants identified by the AS-PCR assays (Table 1). By contrast, population-based sequencing detected other NVP-associated resistance mutations, such as V106A and G190A in breast milk; however, it did no identify additional women with drug-resistant HIV-1 in breast milk, as all of these women carried the K103N and/or Y181C mutation, which had already been detected by the AS-PCR assays. The neighbour-joining tree topology did not reveal any indication of sample mix-up. Only HIV-1 sequences derived from plasma and breast milk from the same individual were closely related (supported by high bootstrap values) in contrast to sequences derived from independent infections (CK *et al.,* data not shown).

Discussion

Our findings confirm previous studies reporting high frequencies of drug-resistant virus in breast milk of NVP-SD-exposed pregnant women infected with HIV-1 [13–15]. Additionally, we found that most of the detected resistance mutations were present as minor drug-resistant variants accounting for <5% of the total viral population. The effect of such minor variants on the transmission of drug-resistant HIV-1 to the infant through breastfeeding is so far unknown, and needs further exploration.

Although it has been shown that minor drug-resistant variants can persist in plasma for prolonged periods [5,8,9], their persistence in breast milk is not well characterized. Knowledge of the persistence of drug-resistant variants is important to better estimate the risk of transmission of drug-resistant HIV-1 through breastfeeding. In this study, using highly-sensitive AS-PCR assays, we found that 46% and 17% of all women harboured drug-resistant HIV-1 variants in their breast milk at 6 and 12 weeks after NVP-SD intake, respectively. These findings suggest a fading of NVP-resistant virus in breast milk over time as it was shown for plasma [5,8,9]. Recently in Uganda, Hudelson *et al.* [15] detected NVP-resistant virus in samples of 40% of women 4 weeks after NVP-SD intake using population-based sequencing. It can be assumed that the frequency of drug-resistant variants would have been even higher if they had applied a more sensitive method, such as AS-PCR assays.

The percentage of women harbouring drug-resistant variants in our study was lower compared with studies analysing HIV-1 subtype-C-infected women [13,14]. Lee *et al.* [14] found drug-resistant virus in 65% of breast milk samples of women infected with HIV-1 subtype C 8 weeks after NVP-SD exposure using a population-based approach. Similarly, drug-resistant viruses in plasma have been shown to emerge at higher frequencies in subtype C as compared with subtype A and D [4,11], which also appears to be true for breast milk.

The persistence of drug-resistant variants in breast milk over months could have an effect on the transmission of drug-resistant HIV-1. As the risk of infection increases with the duration of breastfeeding [22], it is conceivable that prolonged breastfeeding increases the risk of acquiring drug-resistant HIV-1.

To estimate the risk of acquiring drug-resistant virus through breastfeeding, the emergence of drug-resistant virus in the breast milk compartment has to be understood. Our neighbour-joining analysis showed a close relationship between virus populations found in plasma and in breast milk of the same individual; however, viral strains differed with respect to resistance mutations in the two compartments. Different mutation patterns in breast milk and plasma is a consistent finding [13,14,23]. Furthermore, HIV-1 mutation patterns can also differ between samples from the right and left breast of the same women [14]. While it has been shown that a short-course of combivir (zidovudine and lamivudine) over 1 week can prevent the formation of NVP resistances in plasma after NVP-SD intake [24], it is not known whether this holds true for other compartments, that is, for breast milk.

Caution has to be taken when generalizing the results of this study. One-half of the initially enrolled women could not be included into the final resistance analysis because we did not obtain PCR amplificates. This constitutes a common problem in the analysis of breast milk because the viral load in breast milk is considerably lower than in plasma [25]. The real lower limits of detection for the K103N and Y181C variants using AS-PCR assays depend on the respective viral load. Therefore the sensitivity of the assays for breast milk samples with low viral load is reduced compared with the theoretical sensitivity as determined by plasmid controls. Thus, the presence of minor variants in breast milk is prone to be underestimated.

In conclusion, we have shown that minor drug-resistant variants frequently emerge in breast milk of Ugandan women infected with HIV-1 who took NVP-SD. Considering that breastfeeding is still the only option for many women in areas with a high HIV-1 prevalence, the exact role of minor drug-resistant variants in the postnatal transmission of HIV-1 should be subject to further investigations.

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Disclosure statement

The authors declare no competing interests.

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Table 1. Drug-resistant HIV-1 variants in plasma and breast milk detected by population-based sequencing and AS-PCR 6–12 weeks after NVP-SD intake

		Viral load, copies/ml		Sample collection, weeks after NVP-SD		NVP-associated resistant mutations by population sequencing		K103N (AAC) by AS-PCR, %		K103N (AAT) by AS-PCR, %		Y181C (TGT) by AS-PCR,%		NVP resistance	
Patient number	HIV-1subtype	Plasma	Breast milk	Plasma	Breast milk	Plasma	Breast milk	Plasma	Breast milk	Plasma	Breast milk	Plasma	Breast milk	Plasma	Breast milk
1	A1	96,000	420	6	6	wt	wt	wt	wt	wt	wt	wt	wt	No	No
2	A1	120,000	7,400	6	6	K103N	V106A	15.6	9.7	0.34	wt	wt	wt	Yes	Yes
3	D	91,000	3,400	6	6	wt	ND	6.8	wt	0.22	wt	0.62	wt	Yes	No
4	G	9,000	2,600	12	12	K103N	K103N, G190A	54.8	26.9	wt	0.03	wt	wt	Yes	Yes
5	D	95,000	14,000	6	6	wt	wt	wt	wt	wt	wt	wt	wt	No	No
6	C	21,000	2,800	12	6	Y181C	V106A	wt	0.67	wt	0.04	9.8	wt	Yes	Yes
7	G	22,000	360	12	12	wt	ND	wt	wt	wt	wt	0.5	wt	Yes	No
8	A1	19,000	1,160	6	12	wt	wt	wt	wt	wt	wt	wt	wt	No	No
9	A1	11,000	5,000	6	6	ND	wt	wt	wt	wt	wt	wt	2.6	No	Yes
10	A1	3,500	1,100	12	12	V106A	ND	wt	wt	wt	wt	wt	wt	Yes	No
11	D	2,000	500	6	6	wt	ND	wt	wt	wt	wt	wt	wt	No	No
12	A1	200,000	1,340	6	6	K103N[a], Y181C	K101E, K103N	26.1	100	35	0.04	100	wt	Yes	Yes
13	A1	4,700	340	12	12	wt	wt	wt	wt	wt	wt	wt	wt	No	No
14	D	4,600	660	6	6	Y188C	wt	19.2	wt	wt	wt	wt	wt	Yes	No
15	A1	12,000	1,000	12	6	ND	K101E	wt	wt	wt	0.02	wt	wt	No	Yes
16	A1	5,000	340	6	6	wt	wt	wt	wt	wt	wt	wt	wt	No	No
17	A1	5,400	1,400	12	12	wt	wt	1.2	wt	wt	wt	wt	wt	Yes	No
18	A1	8,100	600	6	6	wt	wt	wt	wt	wt	wt	wt	1.6	No	Yes
19	D	3,600	540	6	6	V106A	wt	wt	wt	wt	wt	wt	wt	Yes	No

[a]AAC+AAT. AS-PCR, allele-specific real-time PCR; HIV-1, HIV type-1; ND, not done; NVP, nevirapine; NVP-SD, nevirapine single-dose; wt, wild type.