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Full Length Research Paper

Dynamics of drug resistance development in HIV-positive Ugandan mother-child pairs during 18 months after nevirapine single-dose exposure for PMTCT

Andrea Hauser[1,2], Andrea Kunz[1], Kizito Mugenyi[3], Rose Kabasinguzi[3], Stefanie Theuring[1*], Claudia Kuecherer[2] and Gundel Harms[1]

[1]Institute of Tropical Medicine and International Health, Charité – Universitätsmedizin Berlin, Germany.
[2]Center for HIV and Retrovirology, Robert Koch Institute, Berlin, Germany.
[3]Ministry of Health/German Agency for International Cooperation - PMTCT Project Western Uganda, Fort Portal, Uganda.

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Single-dosed nevirapine (NVP), which frequently selects for resistant virus, is still often applied in resource-limited settings to prevent vertical human immunodeficiency virus (HIV) transmission. We followed-up 83 NVP-exposed HIV-positive mothers and newborns between delivery and 18 months postpartum, testing for vertical transmission and for common NVP-selected resistance mutations through highly sensitive allele-specific polymerase chain reaction (PCR). Ten infants turned seropositive within 18 months; 9 mother-child-pairs were available for resistance testing. Mutations were detected in plasma virus of 7/9 (78%) mothers and 4/9 (44%) infants. Resistant virus predominantly emerged at 2 to 8 weeks after NVP-exposure. NVP resistant HIV-1 variants did not persist longer in infants than in their mothers; however, the success of non-nucleoside reverse transcriptase inhibitors (NNRTI)-containing treatment might be limited for HIV-infected infants if initiated within 6 months after NVP exposure.

Key words: Human immunodeficiency virus (HIV), preventing mother-to-child transmission (PMTCT), nevirapine (NVP), resistance, antiretroviral treatment (ART) initiation, paediatric ART.

INTRODUCTION

Single-dosed nevirapine (sdNVP) for human immunodeficiency virus (HIV)-infected women and their newborns has been a standard regimen for prevention of mother-to-child transmission of HIV (PMTCT) in endemic Sub-Saharan Africa since 2001 (Guay et al., 1999). Although the current World Health Organization (WHO) PMTCT guidelines recommend a triple antiretroviral regimen (WHO, 2006, 2010), sdNVP is still offered as a minimal intervention in many resource-limited countries. In Uganda, 58% of all PMTCT clients still received sdNVP in 2010 (UNAIDS, 2010). However, exposure to NVP seems to have a negative impact on the virologic response to a subsequent NVP-containing antiretroviral treatment (ART) if started within 6 months of exposure (Lockman et al., 2007; Stringer et al., 2010). SdNVP frequently selects for resistance mutations, conferring cross resistance to non-nucleoside reverse transcriptase inhibitors (NNRTI), as verified for mothers (Arrive et al.,

*Corresponding author. E-mail: Stefanie.theuring@charite.de. Tel: +49-30-30116 835. Fax: +49-30-30116 710.

2007) and infants (Eshleman et al., 2001, 2005; Martinson et al., 2007).

Even the presence of minor drug resistant variants was reported to reduce the efficacy of subsequent treatment in adults (Lecossier et al., 2005; Coovadia et al., 2009; Rowley et al., 2010; Li et al., 2011) and children (MacLeod et al., 2010; Sigaloff et al., 2011).

In the absence of drug-selective pressure, resistant virus populations fade over time (Eshleman et al., 2001, 2005; Loubser et al., 2006). However, previous studies showed persistence of minor resistant variants for a period of more than a year in NVP-exposed women (Flys et al., 2005, 2007), and a 12-months NVP-free interval after sdNVP exposure has been recommended to ensure unrestricted treatment response to non-nucleoside reverse transcriptase inhibitors (NNRTI)-containing ART (Stringer et al., 2010). At the same time, it has not been clearly answered yet whether such a time interval is also required for children. Most data regarding the presence of NVP-resistant virus populations in infants is based on results of the less sensitive Sanger sequencing method (Martinson et al., 2007) or on samples taken 4 to 12 weeks after birth or after treatment failure (Eshleman et al., 2001; MacLeod et al., 2010; Sigaloff et al., 2011). So far, the emergence and persistence of minor resistant HIV-variants in infants following sdNVP has not been assessed in tight time intervals during the first year of life or beyond.

In the present study, we monitored the presence of NVP resistance mutations for a period of 18 months in HIV of Ugandan mothers and their vertically infected infants after sdNVP exposure. Therefore, a highly sensitive allele-specific real-time PCR (ASPCR) assay with detection limits of <1% was applied to detect the NVP-selected resistance mutations K103N and Y181C in the reverse transcriptase (Hauser et al., 2012). The aim of the study was to determine the time of emergence and persistence of resistance mutations in plasma virus of infants compared to their mothers within 18 months after birth.

METHODOLOGY

Study population

During 2003 to 2005, we observed 90 HIV-1-infected, treatment-naïve mothers and their newborns who participated in a PMTCT program in Fort Portal District Hospital, Western-Uganda. In accordance with national guidelines, mothers received 200 mg NVP at the onset of labour; newborns received 2 mg/kg NVP syrup within the first 72 h after birth (HIVNET012 protocol). Blood samples of mothers and babies were taken at birth, week 2 to 4, week 6 to 8, and months 3, 6, 12 and 18. All women had given informed consent. The presence of NVP was confirmed in 83/90 maternal delivery blood samples. These 83 samples were the basis for the present investigation, as well as for other studies (Kunz et al., 2009; Hauser et al., 2011; Pilger et al., 2011). The 83 HIV-positive women gave birth to 86 newborns (80 single and 3 twin births); 74 (86%) infants were exclusively breastfed and 12 (14%) received replacement feeding. The study was approved by the National Council of Science and Technology of Uganda and by the Ethical Committee of Charite-Universitätsmedizin Berlin, Germany.

Tests applied to determine HIV status and resistance mutations

Since maternal HIV-1 antibodies are transferred to the fetus through the placenta during pregnancy, HIV-PCR has to be performed for diagnosis of HIV-1 infections in early paediatric blood samples. The maternal antibodies in infants usually disappear 18 months after birth, and uninfected infants revert to HIV-seronegative status (Gulia et al., 2007). In the present study, only those paediatric specimens taken 18 months after birth were tested in two commercial HIV-1 antibody tests (Murex HIV-1.2.0, Abbott GmbH & Co. KG, Wiesbaden, Germany and Gene Screen HIV1/2 version 2, BioRad Laboratories, Munich, Germany). For reactive and indifferent HIV-1 antibody test results, as well as for samples available only before the age of 18 months (in case of death or loss to follow-up of the child), HIV-PCR was performed using the “outer” PCR of ASPCR (Hauser et al., 2012). In case of a positive PCR-result, all samples of the infected child were tested by PCR to determine the time of vertical transmission. Per our definition, positive PCR-results for samples taken at birth indicated “*in-utero* transmission” (Bryson et al., 1992), a negative PCR-result for a sample taken at birth followed by positive PCR-results for samples taken at week 2 to 4 indicated “intrapartum transmission” (via late *in utero* transmission or early breastfeeding), and a positive PCR-result in the sample taken at week 6 (or later), but negative for samples taken earlier, indicated “postpartum transmission” (via late breastfeeding).

Since subtypes A and D are the predominant HIV-1 subtypes circulating in Uganda (Gale et al., 2006), plasma samples of HIV-positive infants and their mothers were investigated for the most common NVP-selected resistance mutations K103N (AAC and AAT codons) and Y181C (TGT codon), using the subtype A and D-specific ASPCR with detection limits < 1% (Hauser et al., 2012). If maternal delivery sample (baseline sample; assumed to contain HIV-1 wild-type only; Church et al., 2007) was not available, a wild-type DNA-standard was used to determine the proportion of resistant variants in the total viral population. In case of HIV-negative or lacking newborn birth sample, the corresponding maternal sample was used. Proportions of K103N mutants encoded either by codon AAC or AAT were summarized. Sensitivity of ASPCR assays for detection of drug-resistant HIV-1 depends also on the input viral load. In order to avoid false-positive results, we established a threshold considering the respective viral load of any given sample (Hauser et al., 2012). If the calculated proportion of drug-resistant HIV-1 was below the calculated theoretical threshold, it was considered to be false-positive and presence of HIV-1 wild type was assumed.

Population-based sequencing was conducted using the Viroseq HIV-1 Genotyping System version 2.0 (Abbott, Wiesbaden, Germany) and the automated sequencer 3130xl Genetic Analyzer (Applied Biosystems, Darmstadt, Germany). HIV-1 subtyping was performed using the REGA HIV subtyping tool.

RESULTS

Eight out of 86 infants were tested HIV-positive at 6 to 8 weeks after delivery or earlier (transmission rate 9.3%). In total, ten children were infected within the study period of

18 months (transmission rate 11.6%). One vertically infected newborn was lost to follow-up, so nine HIV-positive mother-child pairs were included into the resistance analysis. Delivery samples were available from seven mother-child pairs. From all mother-child-pairs, we obtained 3 to 6 follow-up samples from the time span between 2 to 4 weeks and 18 months postpartum. According to our definition, three babies were infected *in utero*, one intrapartum and three postpartum. For two infants, time of infection could not be identified, since samples of birth and/or 2 to 4 weeks after were lacking. Mother-child pairs were infected with HIV-1 subtypes A1 (n = 4), D (n = 3), G (n = 1) and K (n = 1). One child each had died by month 6, month 12, and month 18.

For resistance testing by ASPCR, a mean of 4 maternal and 3 infant follow-up samples were investigated for K103N and Y181C mutations. The dynamics of resistance development in all nine mother-child-pairs are shown in detail in Table 1. In 7/9 mothers and 4/9 infants, NVP-resistant HIV-1 variants could be identified during the study period. In six out of these seven mothers, HIV-resistance emerged 2 to 8 weeks after delivery and persisted for at least six months in all but one woman. In two of these women, resistant HIV-1 was still detectable in the 12 months samples in low (0.05%) and high (100%) proportions. For 4/7 (57%) mothers, resistant variants were identified as minority (< 5%) only. NVP-resistant HIV-variants in infants were also present in the 2 to 8 week postpartum samples of 3/4 newborns. In one infant, viral resistance emerged later (3 months postpartum) and in very low proportions (0.06%) only. For 2/4 infants (50%) resistant HIV-1 were detected in low proportions (< 5%). High proportions of resistant variants (18.7 and 24.0%, respectively) in early follow up samples (week 2 to 4) were identified in two out of three *in utero* infected newborns.

In contrast, in the three postpartum-infected infants, no resistant virus was identified, although their mothers carried resistant HIV-1 during the observation period: In one of these mothers, resistant variants were present in high proportions during the first twelve months postpartum, whereas in two mothers, resistant virus variants were reduced to low or undetectable proportions. Persistence of resistant variants in infants was observed for a maximum of six months (n = 1). NVP-resistance mutations were observed in 3 of 6 subtype D, and in 5 of 8 subtype A infected mothers and infants, while drug-resistant variants in proportions above 5% of the total viral population were identified in 3 of 3 subtype D and in 1 of 5 subtype A infected mothers and infants.

DISCUSSION

In the present study, the 6 to 8 week postnatal HIV-1 transmission rate after sdNVP intervention for mother and newborn was 9.3%, hence comparable to published data (15%; Guay et al., 1999). Investigations on the emergence of NVP-resistant HIV-1 in this mother-child-cohort revealed the presence of at least one drug-resistant variant (K103N and/or Y181C) in 44% of infants and 78% of women during the observation period. These frequencies are consistent with previously published data, showing NVP-resistant HIV-variants after sdNVP exposure in 46% of infants at week 4 to 12 (Eshleman et al., 2001; Martinson et al., 2007) and 87% of women at week 6 to 8 (Loubser et al., 2006; Flys et al., 2007).

Due to the long half-life of NVP, HIV is exposed to slowly decreasing NVP levels over weeks providing resistance-selective conditions (Kunz et al., 2009). For mothers with established HIV-1 infection, emergence of NVP-resistance mutations 1 to 6 weeks after exposure is well documented (Hauser et al., 2011) and seems to occur in infected newborns as well: presence of drug resistant variants 2 to 8 weeks postnatally could be detected in three out of four infants. The highest proportions of drug resistant variants 2 to 8 weeks after birth were identified in infants infected during pregnancy. Since vertical HIV-transmission in those cases took place prior to intrapartum NVP-exposure, high proportions of resistant variants were likely to be selected in the infant itself. Viremia in infants infected *in utero* under NVP-selective pressure increases the development of resistant HIV-variants, fosters proviral integration and thus the archiving and persistence of NVP-resistance mutations (Ghosn et al., 2006).

In the postpartum-infected infants of the present study (n = 3), no resistant virus was identified, although NVP-resistance was detected in plasma virus of their mothers (Table 1). While in two mothers, resistant viral variants had faded to low proportions (no.9: 0.05% K103N) or wild-type virus only (no.8: K103K and Y181Y) at the estimated time of transmission (assumed between last HIV negative and first positive sample of the child), the third mother (no.7) displayed high proportions of resistant plasma virus (< 100% Y181C) in all follow-up samples. Nevertheless, transmission of resistant virus from this mother to her child could not be documented. Hence, the breast milk of this mother is assumed to have carried wild-type virus (K103K and Y181Y) only. Studies on distribution of resistant HIV-variants in plasma and breast milk have shown that different resistance patterns and proportions are present in respective body compartments (Pilger et al., 2011; Raisler et al., 2005).

Half of the mothers and infants displayed resistant HIV-variants in low proportions (< 5% of the total HIV-population). The presence of minor NVP-resistant variants is reported to be correlated with virologic failure for mothers (Lecossier et al., 2005; Coovadia et al., 2009; Rowley et al., 2010; Li et al., 2011) and for NVP-exposed infants (MacLeod et al., 2010). According to this so-called “Mashi” study, which observed 26 NVP-exposed infants,

Table 1. Drug resistant HIV-variants in plasma of mother-child pairs detected by ASPCR.

No.	Transmission	Sub-type		Delivery		Week 2-4		Week 6-8		Month 3		Month 6		Month 12		Month 18	
				K103N	Y181C	K103N	Y181C	K103N	Y181C	K103N	Y181C	K103N	Y181C	K103N	Y181C	K103N	Y181C
1	IU	K	Child	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	Child died	
		K	Mother	<ld	<ld	2.2	1.1	1.7	<ld	<ld	<ld	0.12	<ld	<ld	<ld	–	
2	IU	A1	Child	<ld	<ld	17.8	18.7	3.6	1.3	–		–		–		<ld	<ld
		A1	Mother	<ld	<ld	–		<ld	1.1	<ld	<ld	<ld	3.2	–		<ld	<ld
3	IU	D	Child	<ld	<ld	24.0	4.8	0.8	<ld	10.7	<ld	47.6	<ld	<ld	<ld	<ld	<ld
		D	Mother	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld
4	IP	A1	Child	O		<ld	<ld	<ld	<ld	0.06	<ld	<ld	<ld	Child died		n.a.	
		A1	Mother	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	–		–		–	
5	§	G	Child	–		–		0.02	<ld	0.03	<ld	Child died		n.a.		n.a.	
		G	Mother	–		–		9.3	<ld	54.8	<ld	7.3	<ld	–		–	
6	§	A1	Child	–		–		<ld	<ld	<ld	<ld	<ld	<ld	–		<ld	<ld
		A1	Mother	–		–		–		1.2	<ld	0.4	<ld	–		<ld	<ld
7	PP	D	Child	O		O		<ld	<ld	<ld	<ld	<ld	<ld	–		–	
		D	Mother	<ld	<ld	3.3	100	60.9	100	1.4	100	<ld	100	<ld	100	–	
8	PP	D	Child	O		O		O		O		O		–		<ld	<ld
		D	Mother	<ld	<ld	0.9	8.4	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld
9	PP	A1	Child	O		O		O		O		O		O		<ld	<ld
		A1	Mother	<ld	<ld	0.05	<ld	<ld	<ld	-		<ld	<ld	0.05	<ld	<ld	<ld

<ld = <limit of detection; – = no sample available; O = HIV PCR negativ; IU = in-utero transmission; IP = intrapartal transmission; PP = postpartal transmission; § = time of transmission unknown.

virologic failure is also correlated with an early initiation of therapy of less than seven months after NVP-exposure. Higher rates of NVP-associated resistance mutations in HIV-1 subtype D strains as compared to subtype A strains have been observed in other studies (Eshleman et al., 2005). While in our study the emergence of NVP-resistance was observed slightly more frequently in NVP-SD exposed mothers and infants infected with HIV-1 subtype A during the observation period, the presence of drug-resistant variants reaching higher proportions (above 5%) in the total viral population was more frequent in HIV-1 subtype D infections as compared to subtype A. However, statistical significance could not be calculated due to the small sample size.

In our study, HIV resistance mutations persisted longer in mothers (12 months) than in infants (six months). Since the presence of resistant variants may expand to become the predominant population under drug selection pressure (Lee et al., 2005),

a minimum of a 12-months interval between sdNVP and NNRTI-containing treatment is recommended for the mothers (Stringer et al., 2010). However, applying a time interval to sdNVP-exposed children is much more challenging: our data (despite the very small sample size) and data of Persaud et al. (2011) (26% of children with NVP-resistant HIV six months after sdNVP exposure) suggest that the start of therapy within seven months cannot be recommended. Rather, the fact that resistant HIV-1 variants could be archived in the cellular reservoir of antenatally infected infants (Ghosn et al., 2006; Persaud et al., 2007, 2011) indicates the need of extended intervals in children. On the other hand, HIV-disease progression in children is much faster than in adults, and consequently, 85% of infants are in need of ART within the first six months of life (Mphatswe et al., 2007; Violari et al., 2007). Therefore, recommendations for an interval longer than 7 months before ART initiation in sdNVP exposed infants are not realistic.

The small sample size of seropositive NVP-exposed mother-infant-pairs is a limitation of the study. In rural settings like our study area in Uganda, loss to follow-up of pregnant, HIV-positive women is a common problem, especially over long time periods (Lubega et al., 2013). At the same time, due to transmission rates at 10 to 15% for sdNVP, even with a reasonable number of HIV-positive pregnant women, the number of infected infants will always be limited. Accordingly, most comparable studies focussing on HIV-infected mother-infant-pairs are also based on small sample sizes (Kiptoo et al., 2008; Delaugerre et al., 2009; Permar et al., 2013). On the other hand, our study is the first one to follow up HIV-infected NVP-exposed mother-infant-pairs until 18 months postpartum to analyse the dynamics of resistance development. Thus, regardless of the small cohort, our findings are of high interest especially when considering that even today, many PMTCT clients in Uganda and also in other countries still receive only sdNVP for prophylaxis, despite current recommendations for combination regimens.

Conclusion

Due to the limited study panel, any conclusion can only be drawn with great caution. Bearing this in mind, the results from this study show that the use of sdNVP and extended NVP for newborns in PMTCT interventions in Sub-Saharan Africa could implicate a large risk of resistance development in case the infant became vertically infected. Since resistance testing prior to the start of ART can not be performed in many resource-limited settings, NNRTIs should be replaced by other antiretroviral drugs if ART is initiated in women within the first twelve months after sdNVP intake. At the same time, a seven months-interval between NVP exposure and ART initiation seems to be sufficient to prevent a reduced NNRTI-containing treatment response in infants.

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