

Full Length Research Paper

Minor drug-resistant human immunodeficiency virus (HIV)-1 variants in the cellular DNA of Tanzanian women following triple antiretroviral regimen to prevent vertical transmission

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Antenatal zidovudine (AZT), intrapartum nevirapine (NVP) and postpartum lamivudine (3TC)/AZT to prevent mother-to-child transmission of human immunodeficiency virus (HIV)-1 as recommended in 2006 World Health Organization (WHO) guidelines has shown to select HIV-resistant plasma virus in Tanzanian women. During viral replication, HIV integrates into the cellular host genome where resistant strains may remain archived. This study analyzed the dimension of integration of drug-resistant HIV-strains into the host cells as provirus by analyzing corresponding peripheral blood mononuclear cells (PBMCs) for key resistance mutations selected by AZT/NVP/3TC, applying highly-sensitive allele-specific polymerase chain reaction (PCR). HIV-resistance was detected in PBMC-DNA of 10 (28%) and in plasma virus of 15 of 36 women (42%). Most resistance mutations were selected by AZT in comparable proportions in PBMCs (25%) and in plasma virus (22%). In conclusion, antenatal AZT may select for AZT-resistance potentially persisting as provirus, and therefore is likely to negatively impact future treatment options.

Key words: Tanzania, prevention of mother-to-child transmission of human immunodeficiency virus (HIV)-1, triple prophylaxis, HIV-1 drug resistance, peripheral blood mononuclear cells (PBMCs), proviral DNA, minor variants, allele-specific polymerase chain reaction (PCR).

INTRODUCTION

Antiretroviral regimens for the prevention of mother-to-child transmission (PMTCT) of human immunodeficiency

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virus (HIV)-1 have a proven efficacy in resource-limited countries. However, the temporary nature of such regimens poses the risk of developing resistance, extensively shown for nevirapine single-dose (NVP-SD) prophylaxis (Arrive et al., 2007). After discontinuation, the absence of selective drug pressure decreases the presence of resistant variants to undetectable levels in plasma (Eshleman et al., 2001). However, any viral variant, including drug resistant variants, that has been replicating for a sufficient length of time integrates into the genome of infected cells and may persist for the life span of the cell (Turriziani et al., 2010). Resistant HIV-strains are thus detectable in peripheral blood mononuclear cells (PBMC) for longer periods than in corresponding plasma samples, which has been shown for patients failing antiretroviral therapy (ART) who had a history of drug resistance (Turriziani et al., 2010; Ellis et al., 2004) as well as for drug naïve women after NVP-SD prophylaxis (Wagner et al., 2010). Previous research demonstrated that the presence of resistant proviruses negatively impacts future treatment options (Jourdain et al., 2010).

Applying a highly sensitive allele-specific real-time PCR (ASPCR), we recently reported the emergence of minor drug-resistant HIV-1 in the plasma of 40% (20/50) of Tanzanian women (Hauser et al., 2012), following the 2006 WHO recommended PMTCT regimen (WHO, 2006). The aim of this substudy was to assess the extent of drug resistance in provirus within the corresponding buffy coat samples and to compare the results to those with paired plasma samples.

MATERIALS AND METHODS

Clinical samples and study population

Of 1395 pregnant Tanzanian women recruited within an observational study at Kyela District Hospital (KDH), Mbeya Region, Tanzania, 202 were tested positive for HIV-1 (Kirsten et al., 2011). Of these, 87 treatment-naïve women initiated triple antiretroviral prophylaxis according to the national Tanzanian PMTCT guidelines of 2008 (Tanzania, 2008) and the WHO 2006 recommendations, consisting of antenatal zidovudine (AZT) starting at the 28th week of gestation, NVP-SD at labor onset and AZT/lamivudine (3TC) for one week postpartum (WHO, 2006). Maternal blood samples were collected before initiation of prophylaxis (baseline sample or "individual wildtype" sample) and during follow-up at delivery, 1-2, 4-6 and 12-16 weeks postpartum. EDTA stabilized blood was immediately processed into plasma and buffy coat/ peripheral blood mononuclear cells (PBMCs). The 4-6 and/or 12-16 weeks postpartum buffy coat-samples in addition to the corresponding baseline sample had to be available from those women which were previously analysed for drug-resistance in plasma virus. Informed written consent was obtained from all participants prior to enrolment. The study was approved by the local Mbeya Medical Research and Ethics Committee, the National Institute for Medical Research of Tanzania and the ethical committee of Charité, Universitätsmedizin Berlin, Germany.

Laboratory analysis

For plasma samples with expected low viral loads (<20,000 copies/

ml), we previously developed and evaluated seven highly sensitive allele-specific PCR (ASPCR) assays that allow the quantification of key resistance mutations in the viral reverse transcriptase (RT) with a detection limit of <1%. These include two AZT-selected mutations conferring high level resistance of the RT (T215Y, T215F); the low level AZT resistance mutation K70R, which occurs early and transient and thus is indicative for the emergence of AZT-resistance (Boucher et al., 1992); the three most common NVP-selected resistance mutations K103N (codon AAT and AAC) and Y181C; and the most frequently 3TC-selected mutation M184V (Johnson et al., 2013). To compensate for patient specific HIV-variability and thus the impact on real-time PCRs' measurements, baseline samples prior to drug initiation (assumed to be 100% wild-type in drug-naïve women) were used to calculate the true emergence of resistant variants.

Human genomic DNA was isolated from 200 µl buffy coat using the Qiagen QIAmp DNA Blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Cellular HIV-DNA copies (integrated and non-integrated in the human genomic DNA) were quantified by TaqMan real-time PCR of the HIV-1 LTR genomic region (Supplementary Text1). Only samples with at least 10 HIV-DNA copies/µl of the isolated total DNA were further applied to ASPCR assays to guarantee a minimal input of 100 copies/ASPCR and a detection limit of 1%. HIV-DNA amplification and ASPCR assays were conducted using published primers (Hauser et al., 2012) (Supplementary Text 2).

To rule out sample mix-up and to confirm the common origin of plasma virus and provirus, phylogenetic analysis of maternal sequences generated by population-based sequencing was performed (Supplementary Text 3). Statistical analyses were carried out using PASW Statistics 18 (SPSS Inc., Chicago, Illinois, USA). The non-parametric Mann-Whitney U test was used to assess significant differences between two independent samples. The Chi-square test or Fisher's exact test were used to analyze the independence of categorical variables and for descriptive analysis, median and interquartile ranges (IQR) were calculated. K103N (AAC) mutation and the K103N (AAT) mutation were summed to give the total proportion of viruses carrying the K103N mutation.

RESULTS

Study population

Corresponding baseline and at least one follow-up buffy coat samples with a minimum of 10 HIV-DNA copies/µl total DNA were available from 36 women out of 50 women previously tested for drug-resistance in plasma virus. The median baseline characteristics at initiation of prophylaxis were CD4-cell count of 395 cells/µl (IQR 260-454), a plasma viral load of 21,725 copies/ml (IQR 6,816-60,950) and a cellular HIV-1 load of 92 DNA copies/µg PBMC-DNA (IQR 53-161). Women took AZT during pregnancy for a median of 54 days (IQR 36-75). All women took intrapartum NVP-SD, while intra- and/or post-partal AZT/3TC intake was documented for 32/36 (89%) women. Plasma viral load was reduced to a median of 1,747 copies/ml (IQR 1,265-5,649) at week 1-2 and increased again to 15,123 copies/ml (IQR 8,830-63938) at week 4-6 (Fisher's exact test: both $p < 0.005$). 67% (24/36) of the women were infected with HIV-1 subtype C and 33% (12/36) with subtype A1.

HIV-resistance mutations detected in PBMC-DNA

Thirty-two buffy coat samples taken after 4 to 6 weeks,

Table 1. Drug resistant HIV-variants in plasma and PBMCs detected by ASPCR.

No	HIV-1 subtype	Antenatal AZT-intake (days)	Proportion of resistance mutation					
			Delivery-2weeks p.d.		4-6 weeks p.d.		12-16 weeks p.d.	
			Plasma		Plasma	PBMC	Plasma	PBMC
046	C	40	wt		wt	3.7% T215F	wt	-
081	C	57	-		wt	19% K103N*	wt	-
093	A1	105	wt		wt	-	wt	10% K70R
098	C	91	wt		wt	wt	wt	7.7% T215F
130	A1	33	12% K70R*		wt	1.5% K70R	-	-
02#	C	77	11% K70R* 0.7% M184V -		wt	-	-	18% K70R* 44% T215F* 6.7% M184V
11#	C	92	wt		2.7% K70R	1.2% K70R 13% K103N*	-	-
10#	A1	95	wt		4.9%K70R	5.5% K70R 1.2% T215F	-	-
16#	C	49	wt		36% K103N* 20% Y181C* 0.6% M184V	26% K103N*	4.0% K70R 12%K103N*	1.2% K70R 11% K103N* -
13#	C	32	wt		3.9% T215Y	wt	wt	1.3% T215Y

†Sample not available according to inclusion criteria; wt: No resistance mutation detected; *Mutation also detected by Sanger sequencing; #Patient numbers according to Hauser et al. (2012). p.d.: Post delivery.

and 11 buffy coat samples taken after 12 to 16 weeks were analysed for the emergence of HIV-resistance compared to the baseline sample (in total, 43 samples/36 women). Quantification of resistant HIV-strains in PBMCs was performed with a median HIV-DNA input of 123 copies/ASPCR (IQR 111-145).

HIV-resistance mutations were detected in 11 follow-up buffy coat samples of 10/36 (28%) women. AZT-selected mutations were identified in the cellular HIV-DNA of 9/36 (25%) women (four with the K70R mutation, five with T215Y/F +/- K70R). NVP-selected mutation (K103N) was found in the HIV-DNA of 3/36 (8%) women, and 3TC-selected mutation (M184V) was found in one woman (3%). Three women carried dual-resistant HIV-strains in their PBMCs: either selected by AZT (K70R) and NVP (K103N) or by AZT (T215Y) and 3TC (M184V) (Table 1). Women in whom AZT-resistance mutations were detected in HIV-DNA tended to have had a longer antenatal AZT intake, but this was not statistically significant (68 versus 49 days, Mann-Whitney U-test $p=0.11$).

Proportions of HIV-resistance mutations in PBMC compared to plasma

The previous ASPCR analysis of corresponding week 4-

16 plasma samples revealed resistant plasma virus in 17/54 samples (35 week 4-6 samples, 19 week 12-16 samples) constituting to 15/36 (42%) women. In 8/36 (22%) women, resistance was selected by AZT (five women with K70R and three with T215Y/F), in 5/36 (14%) women selected by NVP (K103N and/or Y181C) and in 2/36 (6%) women selected by 3TC (M184V) (Hauser et al., 2012).

The frequencies of resistant HIV-variants detected in PBMCs were not significantly different from those detected in plasma virus (11/43 versus 17/54; Fisher's exact test $p=0.67$). Additionally, the proportions of women in whom resistant HIV was selected by AZT/NVP/3TC were similar for both blood compartments (AZT (T215Y/F): $p=0.7$; AZT (K70R): $p=1.0$; NVP: $p=0.7$; 3TC: $p=1.0$ using Fisher's exact test; Figure 1). In detail, in six women, all but two plasma virus mutations were also identified in HIV-DNA from PBMCs collected at the same or later time points. In three of these six women, an additional resistance mutation was detected in the viral DNA genomes. In two of them this additional resistance mutation was selected by the same drug as the present one, thus not leading to an additional drug resistance. In four women, resistant HIV-variants were identified exclusively in the PBMCs, whereas in eleven women, resistant variants were detected in plasma samples only.

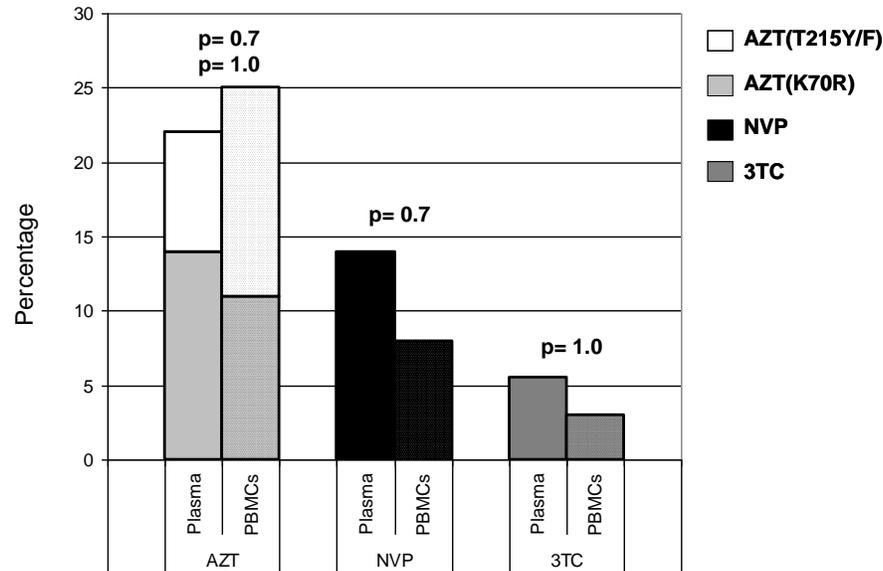


Figure 1. Drug-resistant HIV-1 detected in PBMCs and plasma. The relative proportions of women with AZT-, NVP- and 3TC- selected resistance mutations in RNA from plasma and HIV-DNA from buffy coat are shown (*p*-values of pairwise comparison are indicated for 3TC, NVP and both AZT mutations (not significant if >0.05); Fisher's exact test: 2-tailed).

HIV-resistance was found in neither the plasma viruses nor the PBMCs of fifteen women (Table 1).

DISCUSSION

In the present study, resistant HIV-1 strains in maternal PBMCs were identified in 28% of HIV-infected Tanzanian women 3-15 weeks after cessation of WHO 2006 transmission prophylaxis. NVP-resistance was detected in the HIV-DNA of 8% of the women, a considerably lower frequency than the 52.3% of women analysed 6 weeks after NVP-SD by Loubser et al. (2006). The efficacy of a seven day AZT/3TC-tail after NVP-SD exposure in order to reduce NVP-resistance has often been demonstrated for plasma virus (Farr et al., 2010) and also seems to apply to cellular HIV-DNA.

However, the antenatal mono-AZT administration resulted in the presence of proviral AZT-resistance mutations in one quarter of the women (25%), although the median antenatal AZT intake of 54 days (7.5 weeks) was shorter than recommended in the WHO 2006 guidelines (12 weeks). Prolongation of antenatal AZT intake to 26 weeks, as advocated by the WHO 2010 guidelines (WHO, 2010), may further increase the development of AZT-resistance mutations. Although the exact rate at which HIV-infected CD4+ cells return to a resting state as memory cells remains unclear, it is assumed that at least a proportion of the resistant proviruses will be archived in the host genome as a

“latent reservoir” (Lambotte et al., 2004; Turriziani et al., 2010) and persist for the life span of these cells (Alexaki and Wigdahl, 2008). Under the selective pressure of subsequent treatments, latently infected cells can re-activate the production of resistant virus to release resistant virus (Alexaki and Wigdahl, 2008; Turriziani et al., 2007, 2010). Wind-Rotolo et al. (2009) detected replication-competent proviruses carrying resistance mutations in re-activated PBMCs from 8% of women six months after NVP-SD treatment. Women with detectable NVP-resistance in their HIV-DNA were significantly more likely to experience virologic failure at initiation of a treatment involving NVP (Jourdain et al., 2010).

In the present study, the proportions of women with AZT, 3TC and/or NVP-resistance mutations in HIV from both blood compartments were comparable. This may reflect the ongoing process of viral replication after clearance of the drugs (Wind-Rotolo et al., 2009) and is supported by a 10-fold increased plasma viral load (viral rebound) one month after cessation of antiretroviral regimen and by the fact that HIV-resistance mutations in the plasma viruses were also found in HIV-DNA from PBMCs of six women. Viral rebound in the presence of resistant variants may enhance the integration of resistant genomes into the cellular reservoir.

Infection of cells by resistant plasma virus could not be confirmed for 11 women whose HIV-resistance mutations declined to minor (<5%) or undetectable levels in plasma. In these women, the mutant virus seemed to be rapidly replaced by wild-type variants, presumably through the

re-emergence of archived wild-type virus (Turriziani et al., 2010). Vice versa, HIV-resistance detected exclusively in HIV-DNA from buffy coat samples (four women) may be the result of viral infection and presumable integration at early stages of the prophylaxis regimen. This might explain the exclusive presence of the AZT resistance mutations in the HIV-DNA of three women. These women had taken antenatal AZT for 40, 91 and 105 days (Table 1), potentially allowing the selection and integration of AZT resistance mutations. The K103N mutation (detected in PBMCs only of one woman) has been reported to be detectable for longer periods and at higher frequencies in PBMC HIV than in plasma RNA (Saladini et al., 2010).

Such discrepancies in the occurrence and persistence of drug resistance mutations between plasma RNA and PBMC DNA have been reported in several studies, demonstrating that the PBMC compartment does not necessarily reflect the plasma compartment (Saladini et al., 2010; Turriziani et al., 2010; Wind-Rotolo et al., 2009). While plasma samples provide information on actively replicating viruses (Turriziani et al., 2010), cellular HIV-DNA reflects both actively and latently infected cells (Wirlden et al., 2011). Due to the low concordance of 46.7% between HIV-DNA of viral load suppressed patients and previous RNA RT-genotypes, Wirlden et al. (2011) concluded that archived mutated DNA is difficult to reach.

Contrary wise, it was concluded that using proviral DNA for HIV-genotyping is an adequate and even preferable approach to monitor resistance development following discontinuation of antiretroviral PMTCT interventions. The prolonged persistence of resistance mutations in the cellular DNA compared to plasma RNA makes PBMCs a useful resource to estimate the total spectrum and "resistance potential". This knowledge is of critical importance for the success of subsequent long-term therapy, especially in those countries that lack the resources to carry out genotypic resistance testing.

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Conflict of interest

Authors have none to declare

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