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Resistance detected by pyrosequencing following zidovudine-monotherapy for prevention of HIV-1 mother-to-child-transmission

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Abstract

To prevent mother-to-child-transmission-of-HIV-1, the 2010 WHO guidelines recommended prenatal zidovudine monotherapy (Option A). To determine if ZDV-monotherapy selects for HIV-resistance in antiretroviral-naïve women during pregnancy, specimens from 50 were examined using pyrosequencing. ZDV-resistance mutations were detected at delivery in 7 (14%, 95% confidence interval 6.6-26.5%). These data raise the question whether women administered zidovudine monotherapy for PMTCT could have higher risk of virologic failure when later started on combination ARV therapy, as has been demonstrated following single-dose-nevirapine prophylaxis.

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Authors' contributions:

Ingrid Beck developed the 454-pyrosequencing protocol in our laboratory and assisted in processing subject samples, interpreting test findings, and managing data.

Paula Britto and David E. Shapiro performed statistical analysis of subject characteristics included in study, including comparison of those subjects with and without detected resistance mutations, and contributed to the manuscript.

Roger E. Bumgarner directs the 454-pyrosequencing facility that sequenced subject samples and assisted in interpretation of data, and contributed to the manuscript.

Wenjie Deng and James I. Mullins performed data analysis of 454-pyrosequencing reads with assistance in interpretation of resistance data, and contributed to the manuscript.

Lisa M. Frenkel conceived and obtained funding for the current study, she mentored Dr. Olson in this project and assisted with interpretation of data, and crafting of final manuscript.

Gonzague Jourdain managed the original P1032 cohort in Chiang Mai, Thailand. He helped conceive and obtain funding for the current study, and contributed to the manuscript.

Nicole Ngo-Giang-Huong managed the original P1032 cohort in Chiang Mai, Thailand. She helped conceive and obtain funding for the current study, help select the study subjects and specimens.

Scott Olson processed subject samples and served as primary author of manuscript.

Russell B. Van Dyke conceived of the original P1032 study. He helped conceive and obtain funding for the current study.

Keywords

HIV-1; zidovudine; resistance; prophylaxis; mother-to-child-transmission; pyrosequencing

Introduction

Antiretroviral drugs (ARV) for prevention of mother-to-child transmission (PMTCT) of HIV-1, as recommended by the World Health Organization (WHO), have led to decreased rates of MTCT in resource-limited regions of the world. Single-dose nevirapine (sdNVP) administered to mothers during labor and infants at delivery was recommended due to its moderate efficacy, ease of administration, and affordability. However, due to strong evidence that resistance mutations selected following sdNVP negatively impact future combination antiretroviral therapy (cART) in both mothers and infected infants, in 2010 the WHO recommended that mothers with CD4 T-lymphocyte counts >350/μ[illegible]pactE[illegible] [illegible]ovirE[illegible]therE[illegible]010 E[illegible] **1**]. The higher cost of cART compared to ZDV and resource restrictions led many nations that previously relied on sdNVP to adopt Option A, despite the fact that in most women ZDV monotherapy does not completely suppress HIV replication. Implementation studies suggest that cART for all HIV-infected pregnant women increases cost effectiveness and associated health benefits [**2**], leading the WHO to suggest cART for all pregnancies in July 2013 [**3**].

ZDV monotherapy was used for PMTCT early in the HIV pandemic, and studies from this period demonstrated that resistance mutations were detected infrequently [**4**]. In these studies, however, ZDV treatment averaged only ten weeks, and resistance detection relied on consensus sequencing, which has poor sensitivity when mutations are present at less than 20-50% of the viral population [**5,6**]. Studies evaluating longer periods of ZDV monotherapy or using more sensitive assays (techniques generating multiple-parallel sequences or oligonucleotide ligation assay) have reported a wide range of detected resistance (0-30%) [**7,8**].

If longer periods of ZDV-monotherapy for PMTCT selects resistance mutations at higher rates, and these persist in the viral population, ZDV-resistance could negatively impact future treatment, particularly as ZDV mutations impart cross-resistance to other nucleoside reverse transcription inhibitors (NRTIs).

To better assess the selection of resistance following ZDV-monotherapy for PMTCT, we performed 454-pyrosequencing, defining the prevalence of resistance mutations across a cohort of women and the proportion of those mutations in each woman’s viral population at the time of delivery and at discontinuation of ZDV. For those with resistance mutations detected, samples at 24 weeks postpartum were evaluated for evidence of persistence or decay of resistance.

Methods

Study population

Participants in the IMPAACT P1032 study, conducted from June 2006 through June 2008 in Thailand, were pregnant, ARV-naïve, HIV-1 CRF01_AE infected, receiving ZDV-monotherapy plus sdNVP at delivery [**9**]. They were randomized to one of three postnatal ARV-tails (Arm A: ZDV/didanosine (ddI)/lopinavir/ritonavir (LPV/r) for 7 days; Arm B: ZDV/ddI for 30 days; or Arm C: ZDV/ddI/LPV/r for 30 days). The objective of the P1032 study was to compare various postpartum ARVs for the reduction of incident NVP-resistance mutations versus no postpartum ARV. Women with plasma HIV-1 RNA greater than 500 copies/mL at the time of delivery and adequate available specimens for 454-pyrosequencing were selected for this study.

Plasma viral load was quantified by the Amplicor HIV-1 Monitor test, v1.5 (Roche Molecular Systems, Branchburg NJ, USA), with a lower limit of quantification of 50 copies/mL. Plasma samples from delivery were analyzed, as well as plasma from the date of ZDV discontinuation in those women from Arm B (prolonged postnatal ZDV exposure), and PMBC DNA from 24-weeks postpartum for those women who were found to have resistance mutations, due to longer persistence in DNA compared to RNA once selected [**10,11**]. Clinical data collected included age, plasma viral load and CD4 count at enrollment, duration of ZDV monotherapy, and plasma viral load at delivery (**Table 1**).

454-Pyrosequencing

RNA was extracted from 1000 μL of plasma using NucliSENSE miniMAG kit (Biomerieux, Durham NC, USA), and reverse transcribed using random hexamers with the Blue Print 1st Strand cDNA synthesis kit (Takara Bio Inc., Shiga, Japan). DNA was extracted from whole blood collected at 24-weeks postpartum using silica extraction [**12**]. Real time PCR of the HIV-1 LTR-region was performed on each sample in duplicate to quantify the amplifiable viral cDNA or DNA templates [**13**] (**supplemental table**).

Amplification of HIV-1 *pol* region that encodes reverse transcriptase (RT) was performed using FastStart High Fidelity polymerase (Roche Diagnostics, Mannheim, Germany), with an input of 1000 amplifiable viral cDNA/DNA templates per subject. Nested PCR amplified two regions encoding RT using CRF_AE specific first- and second-round primers. Each 2nd-round primer included pyrosequencing adapters for later emulsion PCR, and a multiplex identifier (MID) allowing 14 samples to be sequenced in the same pool (**supplemental table**).

Following PCR amplification, each sample was purified using the High Pure PCR purification kit (Roche Applied Science, Mannheim, Germany), quantified with the Quant-iT PicoGreen dsDNA Reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA), then diluted to 1×10^9 molecules/μL. Equal volumes of amplicons sequenced together were pooled, and each pool diluted to 1×10^7molecules/μL.

Clonal amplification on beads (emulsion PCR) of each pool used reagents that allowed bidirectional sequencing (454 Life Sciences, Roche Diagnostics Corporation, Branford, CT,

USA). Two-million enriched beads were loaded per region of a PicoTiter plate divided by a gasket for sequencing by GS FLX Titanium System following the manufacturer's instructions (454 Life Sciences). An HIV-1 plasmid was amplified and sequenced with each plate as control for PCR and pyrosequencing error rates at codons of interest.

Data analysis

454-sequencing reads containing ambiguous bases, <100 bp, or with average quality score <25 were not analyzed. Remaining reads were mapped to a reference HIV-1 sequence using the BLAST algorithm, aligned using the Needleman-Wunsch algorithm, and manually refined [**14**].

454-pyrosequences from each subject were evaluated for ZDV-resistance mutations encoding M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E. If resistance was detected, the proportion of mutant variant was determined based on forward, reverse, and overall nucleotide frequencies at each site across the refined alignments.

P-values were calculated using the two-sample Wilcoxon test, with two-sided p-values <0.05 considered statistically significant.

Results

Subject characteristics

The 50 subjects in this study had a median age of 27.5 years, CD4 T-lymphocytes of 424/μL, and plasma HIV-1 RNA of 12,464 c/mL at study entry and 7,250 c/mL at delivery. The median duration of prepartum ZDV therapy was 10.7 weeks (**Table 1**). The distribution of subjects across study arms was relatively balanced, with 15 subjects from Arm A, 17 from Arm B, and 18 from Arm C.

HIV-1-ZDV-resistance by 454-pyrosequencing

Analysis of the plasmid control demonstrated an error rate <0.5% at each codon of interest. Combined with analysis of ~1000 viral templates from each subject, a conservative limit of 1% mutant was used for analyses of ZDV-resistance mutations.

Plasma samples from the day of delivery revealed ZDV-resistance mutations at levels ≥1% in 7 subjects (14%, 95% CI 6.6-26.5%). Five subjects had the K70R mutation, three had the D67N mutation, and one had the M41L mutation. No L210W, T215Y/F, K219Q/E, or intermediate T215 mutations were detected. One subject had three resistance mutations (M41L, D67N, K70R), while all others had one mutation each (**Table 2**). At delivery, the proportion of mutant in the viral population ranged from 1.1 to 24.5%, with only one codon near the sensitivity of consensus sequencing (K70R at 24.5%). Among 17 subjects who received 30 days of ZDV/ddI as a postpartum-tail, three had mutations detected at discontinuation of ZDV: Two had new resistance mutations detected (Subjects 6 and 8; the latter with no mutants detected at delivery), and the third (Subject 2) had increases in the concentration of mutants (**Table 2**). Testing at 24-weeks postpartum, detected ZDV-resistance in the PBMC of only one of the eight subjects with mutations: K70R in Subject 2 at 1.6% (**Table 2**).

A comparison of subjects with and those without mutations at delivery revealed no statistically significant differences in age (p=0.5), plasma HIV-1 RNA load or CD4 T-lymphocyte counts at entry (p=0.72 and 0.31, respectively), plasma HIV-1 RNA load at delivery (p=0.16), or duration of ZDV-monotherapy before delivery (p=0.51) (**Table 1**).

Discussion

In our cohort of women receiving a median of 10.7 weeks of ZDV-monotherapy for PMTCT, 14% of subjects had ZDV-resistance detectable by pyrosequencing at delivery. Among the subset of 17 who received 30 days of ZDV/ddI postpartum, one additional subject had selection of a ZDV-mutation and the mutant population increased in size in two others. While the duration of ZDV varied widely, it was not statistically different between those with and without resistance at delivery, possibly due to the small sample size. ZDV-resistance detected following PMTCT was mostly at levels below the threshold detectable by genotypic consensus sequencing. The impact of these minority ZDV-resistant variants on the efficacy of 1st-line-cART is still uncertain. Given that PMTCT programs in some countries have relied on Option A (ZDV-monotherapy), it is important to assess the selection of ZDV-associated mutations and the risk of both majority and minority ZDV-resistant populations on virologic failure of future cART, as has been described with NNRTI-mutations following use of sdNVP [**15,16**].

Most detected ZDV-mutations faded below the limit of detection in PBMCs by 24-weeks postpartum, likely due to decay of recently infected cells and poor replication capacity compared to wild-type variants [**17**]. The decrease to levels below detection in the peripheral blood does not guarantee that the HIV-1 reservoir is free of replication-competent mutants. Rather, women may be at increased risk of virologic failure for a long time as observed with prolonged selection during virologic failure [**18**], or for a limited time as with sdNVP [**16**].

A limitation of our study is the absence of genotypes before ZDV to compare to resistance profiles after ZDV. Given that a single HIV variant has been observed to found most infections and that the subjects were ARV-naïve, it is unlikely that the ZDV-resistance detected represents transmitted drug resistance. Another limitation is that despite an additional 30-days of ZDV in 17 subjects, its administration in combination with sdNVP at delivery and ddI adds to the genetic barrier for selection of resistance and precludes interpreting the postpartum data as reflective of Option A. Furthermore, enrollment in our cohort occurred when cART was recommended for women with CD4 <250 cells/μL. Three women with mutations in our study would qualify for cART under the 2010 guidelines, which was recommended for women with CD4 counts ≤350 cells/μL.

In our study, 14% of women who received ZDV-monotherapy for a median of 10.7 weeks had resistance mutations detected at delivery, albeit at a low proportion of the HIV population in most subjects. Although the significance of these resistance mutations on later cART is uncertain, recent findings that combination cART is superior to ZDV in PMTCT makes rapid implementation of cART in all programs the most circumspect approach **[19]**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Characteristics of HIV-1 infected pregnant women and comparison of those with and without ZDV-resistance mutations at delivery.

		Total (n=50)	Subjects with ZDV Mutations at Delivery (n=7)	Subjects without ZDV Mutations (n=43)	p-value
Age (years)	Median	27.5	30	27.3	0.5
	IQR	24 – 30.3	26.3-31.3	23.9-30.3	
Plasma HIV RNA at study entry (c/mL)	Median	12,464	14,366	11,500	0.72
	IQR	7,635 – 30,150	9,760-34,000	7,670-25,550	
Plasma HIV RNA at delivery (c/mL)	Median	7,250	15,586	7,100	0.16
	IQR	4,500 – 14,771	8,704-35,400	4,610-13,702	
$CD4^+$ T-cells at study entry (cells/μL)	Median	424	365	430	0.31
	IQR	334 – 528	304-457	344-538	
ZDV duration (weeks)	Median	10.7	11.85	10.57	0.51
	IQR	10 – 12.28	9.64-13.78	10-12	

Table 2

Zidovudine (ZDV)-resistant mutations by time, plasma HIV RNA and CD4 T-cell counts. Resistance detected by pyrosequencing of plasma (HIV-1 RNA) at delivery and after 30 days of postpartum tail, and PBMC (HIV-1 DNA) at 24 weeks postpartum.

Subject	ZDV-resistance mutations detected	ZDV duration at delivery (weeks)	ZDV-resistance mutation representation in HIV population by duration of ZDV			Plasma HIV RNA (copies/mL)		CD4 T-cells at study entry
			Delivery	After 30 day ZDV+ddI tail†	24 weeks postpartum	Study entry	Delivery	
1	K70R	10.0	2%	^	0%	28,200	24,000	431
2	M41L	9.0	1.1%	0%	0%	51,300	55,000	252
	D67N		11%	13.8%	0%			
	K70R		24.5%	87.0%	1.6%			
3	D67N	7.5	3.2%	^	0%	39,800	46,800	317
4	K70R	14.4	5%	^	0%	6,900	6,200	586
5	K70R	31.0	2.5%	^	0%	5,200	3,500	484
6	D67N	12.0	1.5%	2.0%	0%	14,366	15,586	365
	K70R		0%	2.0%	0%			
7	K70R	13.0	2%	^	0%	12,621	11,208	291
8	D67N	11.9	0%	1.6%	0%	5,800	7100	368

^ not tested

† subjects in Arm B tested at completion of a 30-day ZDV+ddI tail