

Short communication

Evaluation of minority populations of HIV type-1 with K103N and M184V drug resistance mutations among children in Argentina

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Background: The aim of this study was to describe the frequency of minority populations of viruses carrying mutations K103N and M184V in drug-naïve HIV type-1 (HIV-1)-infected children, and to further evaluate their effect on the selection of drug-resistant viruses within highly active antiretroviral therapy (HAART).

Methods: Newly diagnosed vertically HIV-1-infected children were evaluated. The HIV-1 *pol* gene was sequenced for subtyping and antiretroviral drug resistance analysis. Standard genotypic sequencing and sequence-selective real-time PCR (SPCR) to quantify minority viral populations were used.

Results: From December 2004 to July 2006, we included 35 children who were studied at baseline and during their first HAART regimen (follow-up median time 29.4 months). Of them, 82.9% were infected with intersubtype

B/F recombinant variants. At baseline, all children had a drug-susceptible viral population that was studied by bulk sequencing. SPCR showed that 4 children had between 2–10% of M184V, 11 had <0.7%, 18 had no detectable mutation and 2 could not be amplified. No K103N minority populations were found. Once under HAART, children who had 2–10% of M184V at baseline further selected it in percentages >20% in less time than those with 0.1–0.6% or without minority populations ($P=0.01$).

Conclusions: It was shown that having 2–10% of M184V at baseline enhanced its selection in high percentages in a short time after HAART initiation. Further research regarding the presence of minority quasispecies before initiation of HAART in large paediatric populations should be undertaken to evaluate their clinical effect during HAART.

Introduction

The use of antiretroviral therapy (ART) has been shown to be effective in reducing morbidity and mortality in HIV type-1 (HIV-1)-infected individuals [1]. As seen in adults, significant decreases in mortality and in HIV-1-related morbidity and hospitalizations have been reported among HIV-1-infected children, concomitant with increased use of highly active antiretroviral therapy (HAART) [2]. It has been well described that the emergence of antiretroviral (ARV) drug resistance compromises HAART efficacy and reduces the range of choices of active ARV drugs [3]. This is particularly important in the paediatric population. Because of the greater overall duration of ARV exposure among children, there is a more significant need to prevent the emergence of resistance in order to preserve future ARV

options. It is known that ARV drug-resistant viruses can develop in drug-experienced children, but can also be found in ARV-naïve children who have become infected with HIV despite maternal/infant ARV prophylaxis [4].

Studies evaluating the presence of drug-resistant viral strains that constitute <20% of the total viral population, which cannot be detected by standard genotypic assays, have been extensively performed during the past few years [5,6]. The effect of these minority populations on clinical progression while receiving HAART has been described for drug-naïve adults [7–10], but has not yet been described in the paediatric population.

The aim of this study was to describe the frequency of minority populations of viruses carrying one primary

mutation associated with nevirapine (NVP) resistance (K103N) and one mutation associated with lamivudine (3TC) resistance (M184V) in drug-naive HIV-1-infected children, and to further evaluate their effect on the selection of drug-resistant viruses during HAART.

Methods

Patients

This study was performed in collaboration with two public hospitals treating children living in the suburbs of Buenos Aires, Argentina, where low socio-economic and educational conditions predominate. This study was approved by the School of Medicine Ethics Committee of the University of Buenos Aires (Buenos Aires, Argentina). The eligibility criteria were newly diagnosed HIV-1-infected children (born to an HIV-1-infected mother), aged ≤ 14 years, who were treated at these hospitals and whose parents provided informed consent for participation. The study was carried out as a convenience observational cohort study. Diagnosis of children was performed through virological tests in children aged < 18 months and with HIV antibody assays in older children according to international recommendations [11]. ARV drug history of the children and their mothers was obtained. A blood sample was obtained from each child at the time of enrolment (baseline) and, when possible, every 3 months thereafter. Biological samples from the mothers were not available.

Laboratory assays

Plasma HIV-1 RNA was extracted, amplified to obtain all of the protease (PR) and codons 1–400 of the reverse transcriptase (RT) of the HIV-1 *pol* gene, and further bulk-sequenced using an in-house assay as previously described [12]. The Stanford University genotypic resistance interpretation algorithm [13] was used to search for mutations, and their association to drug resistance was determined following the International AIDS Society–USA recommendations [14]. For subtyping, all sequences were analysed individually for their similarity to reference consensus sequences [15] using Simplot 2.5 [16]. Sequence-selective real-time PCR (SPCR) was performed to search for minority populations of viral quasispecies with resistance mutations. The lower detection limit of SPCR for the mutations studied was 0.1%. Mutations K103N and M184V were evaluated as previously described [17]. The primers and probes used for detection of the K103N and M184V mutations were those published by Lecossier *et al.* [5] and Bergroth *et al.* [18], respectively, with modifications described elsewhere [17]. These modifications were introduced to improve their affinity with the sequences that circulate in Argentina

(predominantly HIV-1 subtype B and intersubtype B/F recombinants) [19]. Amplification, data acquisition and analyses were performed using the 7500 Real-Time PCR System and SDS software version 1.3 (Applied Biosystems, Singapore). Plasma HIV-1 RNA quantification was performed using the Versant HIV-1 RNA 3.0 Assay (Siemens, Tarrytown, NY, USA) with a detection limit of < 50 copies/ml.

Adherence to HAART was evaluated on the basis of caregiver self-reports, on-time clinic visits and/or on-time pharmacy refill visits. The dates on which the medication was obtained at the hospital pharmacy were documented in the clinical records. With the knowledge of how long the prescribed medication would last, it was possible to calculate, together with the clinic visits, if > 3 doses were missed per month. If so, adherence was estimated as being $< 95\%$.

Statistical analyses

Virological failure was defined as a $< 1.0 \log_{10}$ copies/ml decrease in HIV-1 RNA concentration from baseline after 8–12 weeks of therapy or repeated HIV-1 RNA $> 2.6 \log_{10}$ copies/ml after 6 months of therapy [11]. The time to selection of mutation M184V after HAART initiation was analysed by Kaplan–Meier and Cox regression methods. All statistical analyses were performed using SPSS for Windows version 13.0 (SPSS Inc, Chicago, IL, USA). A two-sided $P < 0.05$ value was considered as statistically significant.

Results

From December 2004 to July 2006, 35 children who had initiated their first HAART regimen and who were followed-up for a median time of 29.4 months (interquartile range [IQR] 22.9–38.3) were studied. Of these 35 children, 29 (82.9%) were infected with HIV-1 intersubtype B/F recombinant variants (in the *pol* region). The remaining 6 (17.1%) were infected with subtype B strains. In terms of ARV resistance, all of them had a drug-susceptible viral population according to standard bulk sequencing, on the basis of the absence of primary mutations. By means of the SPCR, 35 and 33 samples were evaluated to identify minority populations of viruses carrying mutations K103N and M184V, respectively. We found that 4 (12.1%) children had between 2% to 10% of minority populations with the M184V mutation at baseline, 11 (33.3%) children had the mutation in $< 0.7\%$ and 18 (54.6%) children had no detectable mutation (below the detection limit [BDL]). When K103N was evaluated, no minority populations were found. Details of the 35 children studied, regarding age, gender, ART history, drug resistance, HAART regimens prescribed, viral load suppression and adherence to therapy are shown in Table 1.

Table 1. Characteristics of the study population at baseline and during HAART

Patient identification	Age at entry, months	Gender	ART		Baseline		HAART						
			ART in mother	ART prophylaxis in child	HIV-1 subtype	Drug resistance (standard genotyping)	Minority K103N, %	Minority M184V, %	HAART regimen	Time to selection of M184V (>20%), months ^a	Suppressed VL	Optimal adherence ^b	Drug resistance (standard genotyping)
1	47	M	No	No	B/F	No	BDL	10.0	ddl, 3TC, NVP	1.9	No	Yes	Yes
2	12	M	No	No	B/F	No	BDL	7.4	3TC, d4T, NVP/3TC, d4T, NVP	6.1	No	No	Yes
3	2	F	No	No	B	No	BDL	3.5	3TC, d4T, NVP	1.1	No	Yes	Yes
4	1	M	No	No	B/F	No	BDL	3.0	3TC, d4T, NVP	0.2	No	No	Yes
5	105	M	No	No	B/F	No	BDL	0.6	AZI, 3TC, EFV	6.0	No	No	Yes
6	42	M	No	No	B/F	No	BDL	0.4	AZI, 3TC, NVP/AZI, ddl, NVP	5.8	Yes	Yes	Yes
7	108	F	No	No	B	No	BDL	0.3	AZI, 3TC, NVP	No	Yes	Yes	No
8	104	F	No	No	B/F	No	BDL	0.2	3TC, d4T, NVP	10.4	No	No	Yes
9	26	M	No	No	B/F	No	BDL	0.2	3TC, d4T, NVP	3.2	No	No	Yes
10	3	F	No	No	B/F	No	BDL	0.2	3TC, d4T, NVP	No	No	No	No
11	4	F	No	No	B/F	No	BDL	0.2	3TC, d4T, NVP	0.9	No	No	Yes
12	168	F	No	No	B/F	No	BDL	0.1	AZI, 3TC, NVP	13.8	Yes	No	Yes
13	6	M	No	No	B/F	No	BDL	0.1	3TC, d4T, NVP/AZI, ddl, NVP	5.4	No	No	Yes
14	55	F	No	No	B	No	BDL	0.1	3TC, d4T, NVP	No	Yes	Yes	Yes
15	32	M	No	No	B/F	No	BDL	0.1	AZI, 3TC, NVP	2.0	No	Yes	Yes
16	6	M	No	No	B/F	No	BDL	BDL	3TC, d4T, NVP	No	No	No	Yes
17	2	M	No	AZI	B/F	No	BDL	BDL	3TC, D4T, NVP	No	Yes	Yes	No
18	12	F	No	No	B/F	No	BDL	BDL	3TC, d4T, NVP/3TC, d4T, NVP	No	No	No	Yes
19	5	M	No	No	B/F	No	BDL	BDL	AZI, 3TC, NVP	6.1	No	No	Yes

^aNo^c implies that the mutation was not selected up to the last sample collected. ^bEvaluated on the basis of caregiver self-reports; on-time clinic visits and/or on-time pharmacy refill visits. ^cSingle dose nevirapine (NVP), ART, antiretroviral therapy; AZI, zidovudine; BDL, below detection limit; ddl, didanosine; d4T, didanosine; EFV, efavirenz; F, female; HAART, highly active antiretroviral therapy; HIV-1, HIV type-1; LPV/r, ritonavir-boosted lopinavir; M, male; NA, not available; NVP, nevirapine; VL, viral load; 3TC, lamivudine.

Table 1. Continued

Patient identification	Age at entry, months	Gender	ART in mother	ART in child	HIV-1 subtype	Baseline			HAART				
						Drug resistance (standard genotyping)	Minority K103N, %	Minority M184V, %	HAART regimen	Time to selection of M184V (>20%), months ^c	Suppressed VL	Optimal adherence ^d	Drug resistance (standard genotyping)
20	17	M	No	No	B/F	No	BDL	BDL	AZI, 3TC, NFV	No	No	No	No
21	10	F	AZI	AZI	B/F	No	BDL	BDL	3TC, ddI, NFV	5.7	No	No	Yes
22	61	F	No	No	B/F	No	BDL	BDL	3TC, ddI, NFV/AZI, 3TC, NVP	11.9	No	No	Yes
23	148	M	No	No	B/F	No	BDL	BDL	AZI, 3TC, NFV	2.9	No	No	Yes
24	93	F	No	No	B/F	No	BDL	BDL	AZI, 3TC, NFV	1.8	Yes	Yes	Yes
25	52	M	No	No	B	No	BDL	BDL	AZI, 3TC, NFV	No	Yes	Yes	No
26	144	F	No	No	B/F	No	BDL	BDL	AZI, 3TC, NFV	No	Yes	Yes	No
27	10	F	No	No	B/F	No	BDL	BDL	AZI, 3TC, NVP	8.2	No	No	Yes
28	78	M	No	No	B/F	No	BDL	BDL	AZI, 3TC, NFV (or NVP or LPV/r)/AZI, ddI, NFV	7.3	No	No	Yes
29	72	M	No	No	B/F	No	BDL	BDL	AZI, 3TC, NFV	No	Yes	Yes	No
30	0.5	M	No	No	B/F	No	BDL	BDL	3TC, ddI, NFV	2.6	No	No	Yes
31	153	F	No	No	B/F	No	BDL	BDL	AZI, 3TC, NPV	No	Yes	Yes	No
32	7	M	No	AZI, NPV ^e	B/F	No	BDL	BDL	AZI, 3TC, NFV	5.2	No	No	Yes
33	166	F	No	No	B/F	No	BDL	BDL	AZI, 3TC, NFV	6.5	No	No	Yes
34	4	F	No	AZI	B	No	BDL	NA	3TC, ddI, NVP/AZI, 3TC, NPV	31.0	Yes	Yes	Yes
35	36	M	No	No	B/F	No	BDL	NA	AZI, 3TC, LPV/r	No	Yes	Yes	No

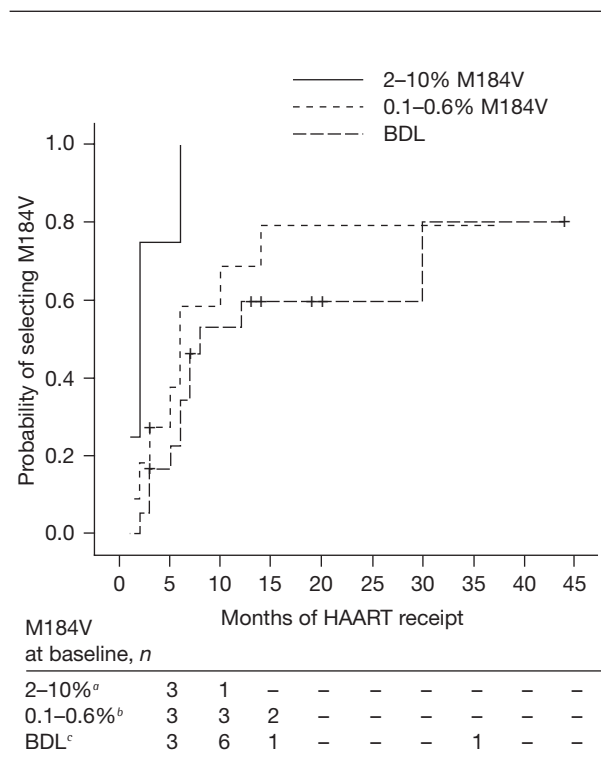
Cox regression showed that the percentage of M184V at baseline was a statistically significant predictor of the time of its selection once under HAART (hazard ratio [HR] 1.21, 95% confidence interval [CI] 1.03–1.42; $P=0.02$). The baseline variable (M184V percentage) was further categorized into three groups: >2%, 0.1–0.6% and BDL. The difference in time of M184V selection during HAART between these three groups was statistically significant (Mantel–Cox test $P=0.01$; Figure 1). The Kaplan–Meier estimator of the probability of selecting the mutation during the first 6 months of HAART was 35% for children with no detectable mutation, 58% for those with 0.1–0.6% and 100% for those with >2% of the mutation at baseline (Mantel–Cox test $P=0.01$). Cox regression, considering the grouped variable as the predictor, showed that there was no significant difference between children with 0.1–0.6% of M184V at baseline and those with BDL percentages (HR 1.42, 95% CI 0.57–3.55; $P=0.45$). By contrast, there was a significant difference between children with percentages >2% and those with BDL percentages (HR 5.71, 95% CI 1.69–19.3; $P=0.005$). In addition, the probability of selecting M184V at a given time was higher among children with virological failure than among those with suppressed viral loads (Mantel–Cox test $P=0.016$).

The frequency of suboptimal adherence to therapy was not significantly different when comparing children who had 2–10% of mutation M184V at baseline with those with 0.1–0.6% and with those with BDL percentages (50%, 64% and 67%, respectively; Fisher’s exact test $P=0.88$). The frequency of virological failure among these three groups of children (100%, 64% and 67%, respectively; Fisher’s exact test $P=0.58$) was also not significantly different. However, the occurrence of poor adherence to HAART was associated with a higher frequency of virological failure and population ARV drug resistance (Fisher’s exact test $P<0.001$ and $P=0.006$, respectively).

Discussion

In this study, we analysed the presence of viral strains with two primary mutations associated with ARV drug resistance (K103N and M184V) in percentages <20% before HAART initiation in a group of 35 children who then initiated their first HAART regimen. We found that almost half of the children studied carried viral quasispecies with the mutation M184V in different percentages (Table 1) at baseline, but mutation K103N was not detected among any of them. Although the cohort was largely ARV-naive prior to starting HAART (one mother had received ARV drugs during pregnancy and four children had received prophylactic therapy after birth; Table 1) and all had

Figure 1. Kaplan–Meier survival analysis of the time to selection of M184V after HAART initiation



Kaplan–Meier survival analysis of the time to selection of M184V (>20%) after highly active antiretroviral therapy (HAART) initiation in children who, at baseline, had 2–10% of the mutation versus those who had 0.1–0.6% and versus those with below detection limit (BDL) percentages. The number of patients in each group (related to the total number [*n*]), who had already selected the M184V mutation at each corresponding time point is also shown below the graph. ^aTotal $n=4$. ^bTotal $n=11$. ^cTotal $n=18$.

a drug-susceptible viral populations at baseline as determined by bulk-sequencing. It is noteworthy that 45.4% of the children had between >0.1% and 10% of the total viral population strains with the M184V mutation. Minor species in this population could have been transmitted from parent to child (implying either resistance selection in the parent or transmission from a donor to parents) or could be low-level circulating virus species containing these mutations in the absence of drug exposure. Transmission of drug-resistant variants might suggest high levels of exposure to HAART in the studied community and a considerable level of primary drug resistance in the local community, likely as a result of widespread use of non-suppressive 3TC-containing HAART regimens over many years. This might not be the case in the population studied here, where most of the parents did not know about their HIV infection (the children were frequently the index cases of their families) and were consequently ARV-naive. Besides, previous surveillance studies of primary resistance in the adult HIV-infected population

of Buenos Aires did not show a high prevalence of mutation M184V [20].

We further explored the consequences of having minority populations of viruses with M184V at baseline once the children began HAART, which always included 3TC plus two other drugs. We found that 63.6% of the total population eventually selected mutation M184V (detectable by bulk sequence analysis). We found that having 2–10% of M184V at baseline increased the likelihood of selecting it in high percentages in a short time after HAART initiation. Although no significant differences in the frequencies of virological failure and optimal adherence according to the baseline M184V percentages were found, a trend can be observed of a higher frequency of virological failure in children with higher percentages of baseline M184V than in the rest. Nonetheless, these results should be analysed with caution considering the small number of patients evaluated in each group.

It was also shown that patients with virological failure had a greater probability of selecting M184V during therapy compared with those who suppressed their viral load. As expected, poor adherence to therapy will cause suboptimal drug concentrations and consequently result in incomplete viral load suppression. In this scenario, the ongoing viral replication under suboptimal drug pressure can allow for the selection of resistance mutations. In our study, we found that once under HAART, poor adherence was related to a higher frequency of virological failure and ARV drug resistance. It should be noted that 80% of the patients studied here were treated with NVP-based regimens, in which cases of virological suppression might have also been related to the inferior antiviral activity described for this drug compared with ritonavir-boosted protease inhibitors [21]. In addition, the presence of resistant minority variants might have a greater effect when suboptimal treatment regimens are used.

These results shed light on the prevalence of two minority drug resistance mutations in a group of recently diagnosed children and on the effect of mutation M184V on the selection of 3TC-, abacavir- and/or emtricitabine-resistant viruses within HAART. However, because of the low number of patients studied here, these results should be considered cautiously; therefore, we highlight the importance of more research regarding the presence of minority quasispecies before initiation of HAART in large paediatric populations to evaluate their clinical effect during HAART. This should be of great importance considering that children will need therapy for a life time. In addition, further research studies in paediatric populations, particularly addressing adherence issues, are needed to accomplish long-term and successful treatment of HIV infection in children.

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Disclosure statement

The authors declare no competing interests.

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