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Detection of low-frequency K103N mutants after unstructured discontinuation of efavirenz in the presence of the *CYP2B6* 516 TT polymorphism

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***Objectives*: To measure antiretroviral drug plasma levels in newly diagnosed HIV-1 seropositive persons who presented with an undetectable plasma HIV-1 RNA load but gave no history of antiretroviral drug exposure and to determine the impact of interrupting undisclosed or unknown antiretroviral therapy on the emergence of drug resistance.**

***Patients and methods*: Five newly diagnosed, reportedly drug-naive HIV-1 seropositive persons were included in the study. Drug resistance was determined by population and clonal sequencing of reverse transcriptase and protease. *CYP2B6* polymorphisms were assayed by real-time PCR allelic discrimination on pre-amplified exons.**

***Results*: Efavirenz was detected in the plasma of one of the five persons coinciding with a viral load <40 copies/mL by two different assays. When efavirenz became undetectable, the viral load rebounded. The patient was *CYP2B6*-516T homozygous. Population sequencing showed wild-type subtype D virus, whereas clonal sequencing detected low-frequency (2%) K103N. The patient firmly denied antiretroviral exposure but described the use of Ugandan remedies.**

***Conclusions*: In migrating populations seeking HIV testing, careful and compassionate counselling is required to facilitate the disclosure of previous diagnosis and therapy. The use of remedies of dubious content should also be discussed and investigated.**

Keywords: remedies, low-frequency resistant mutants, efavirenz clearance

Introduction

The long plasma half-life of efavirenz is a desirable characteristic as it allows once-daily dosing, but caution is recommended when stopping therapy since withdrawing all drugs in efavirenz-containing regimens effectively results in efavirenz monotherapy, with the accompanying risk of resistance. A single mutation in the non-nucleoside reverse transcriptase inhibitor (NNRTI)-binding pocket or the surrounding domain can confer high-level resistance to the drug. Persons who undergo unstructured treatment interruption are likely to experience prolonged periods with plasma efavirenz concentrations in the optimal range for selective drug pressure, i.e. above those required to inhibit wild-type virus, but below those required to suppress efavirenz-resistant variants.[1] Efavirenz clearance is especially slow in persons carrying a G→T substitution at position 516 of the cytochrome P450 (CYP) *2B6* gene (rs3745274), a polymorphism over-represented in persons of black-African descent.[1]

The aim of this study was to measure antiretroviral drug plasma levels in newly diagnosed HIV-1 seropositive persons who presented with an undetectable plasma HIV-1 RNA load but gave no history of antiretroviral drug exposure and to determine the impact of interrupting undisclosed or unknown antiretroviral therapy on the emergence of drug resistance.

Patients, methods and results

Among five persons tested, one showed detectable efavirenz in plasma collected at the time of HIV diagnosis (Table 1).

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Table 1. Investigations and results

Time	Test	Result
July 2006	HIV-1 antibody	positive
	HIV-1 proviral DNA	positive
	plasma HIV-1 RNA load[a]	<40 copies/mL
	CD4 count	364 cells/mm^3
	plasma drug levels[b]	efavirenz 1683 ng/mL
August 2006	plasma HIV-1 RNA load[a]	<40 copies/mL
	plasma drug levels[b]	efavirenz 1500 ng/mL
October 2006	plasma HIV-1 RNA load	321 637 copies/mL
	CD4 count	158 cells/mm^3
	plasma drug levels[b]	no drug detected
	genotypic resistance test	wild-type virus, subtype D
	clonal sequencing	low-frequency resistant mutants

[a]Abbott Real-Time HIV-1 assay and Roche TaqMan HIV-1 test.
[b]Drugs tested: efavirenz, nevirapine, zidovudine, stavudine, lamivudine, amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir and tipranavir.

The other four patients did not show the presence of antiretroviral drugs in plasma. Their infection was confirmed by the detection of proviral DNA. Upon follow-up, three showed a persistently undetectable plasma viral load (<40 copies/mL) and preserved CD4 counts. The fourth patient was lost to follow-up.

The patient with detectable efavirenz was a middle-aged woman who migrated from Uganda to the UK in 2006 and sought voluntary HIV testing 6 months later. At diagnosis, the plasma viral load was <40 copies/mL by two different assays (Abbott Real-Time HIV-1 assay and Roche TaqMan HIV-1 test). Initiation of antiretroviral therapy was deferred in view of the relatively high CD4 count. After 3 months, the viral load became detectable and the CD4 count declined (Table 1).

With the patient's informed consent, reverse-phase high-performance liquid chromatography was used to measure antiretroviral drug levels retrospectively on plasma collected at the time of diagnosis, 2 weeks later and at the time of viral load rebound. The first two samples showed efavirenz concentrations of 1683 and 1500 ng/mL, respectively, whereas at the time of viral load rebound, there was no detectable efavirenz. Nevirapine, lamivudine, stavudine, zidovudine and protease inhibitors (PIs) were undetectable in all samples (Table 1). *CYP2B6* polymorphisms (516G>T and 983T>C) were genotyped by real-time PCR allelic discrimination on pre-amplified exons. The patient was homozygous for the T alleles at positions 516 (mutant allele) and 983 (wild-type allele).

Genotypic resistance testing by population sequencing (ViroSeq™ HIV-1 Genotyping System v2.0, Celera Diagnostics, USA) performed on plasma from the time of viral load rebound showed infection with subtype D, wild-type reverse transcriptase and protease. Clonal analysis was performed to detect low-frequency resistant mutants, using cDNA amplified in five PCR reactions and employing PfuUltra high-fidelity DNA polymerase (Stratagene, The Netherlands). The 1.2 kb amplicons (protease 1–99 and reverse transcriptase 1–250) were cloned in pCR2.1 by TOPO-TA technology (Invitrogen, UK). Of the 200 clones sequenced, 4 (2%) harboured the K103N resistance mutation, which confers high-level resistance to efavirenz and nevirapine. Additional mutations in the reverse transcriptase were detected at frequencies ranging from 0.5% to 1% (Table 2), a frequency above the expected error rate for the *Pfu* polymerase.[2] The mutations each occurred in independent clones.

Table 2. Mutations at recognized resistance sites in the reverse transcriptase detected by sequencing analysis of 200 clones

Mutation detected	Number of clones (%)	Drugs affected
A62V	1 (0.5)	possibly NRTIs
V75A	1 (0.5)	stavudine
K103N	4 (2.0)	efavirenz, nevirapine
V106A	1 (0.5)	efavirenz, nevirapine
Y115C	2 (1.0)	unknown
Q151R	1 (0.5)	unknown
V179G	1 (0.5)	possibly NNRTIs
M184T	1 (0.5)	lamivudine
Y188D	1 (0.5)	NNRTIs

Upon extensive counselling, the patient firmly denied exposure to antiretroviral drugs, but reported the previous use of Ugandan remedies, which had been prescribed regularly by a herbalist and traditional healer in her home village, when she consulted him for tiredness and diabetes. She described taking various remedies three times a day for approximately 4 years without significant side effects and running out sometime after the HIV diagnosis. Unfortunately, the preparations were not available for testing. Therapy was commenced with abacavir/lamivudine and ritonavir-boosted lopinavir. The patient currently maintains a viral load <40 copies/mL.

Discussion

In our pilot study, we investigated five HIV-1 seropositive persons who presented with an undetectable plasma viral load at the time of HIV diagnosis. In all five patients, the undetectable viral load was confirmed by two assays that target different regions (integrase and gag) of the HIV-1 genome. One of the patients showed the presence of efavirenz in plasma despite firmly denying a history of exposure to antiretroviral drugs. In the remaining four, the infection was confirmed by detection of proviral DNA. Among the latter four, three persons with available follow-up showed persistently undetectable viral load with preserved CD4 counts, suggesting an attenuated infection. Alternative explanations include the use of PI-based or nucleo-s(t)ide reverse transcriptase inhibitor (NRTI)-only regimens, which would have escaped detection due to the short half-lives of these drugs, or infection with an unusual HIV-1 strain that was not detected by PCR. Although the available viral load assays have shown good performance with prevalent non-B subtypes, it is possible that some assays underestimate viral load with some unusual virus sequences.[3] On this basis, it is

recommended that testing by two different methodologies is attempted in persons with confirmed HIV-1 seropositivity who present with an undetectable viral load at the time of diagnosis. Our findings suggest that measuring drug levels in plasma may be proposed as a further investigation.

One explanation for the presence of efavirenz in the plasma of one of the patients was that she had knowingly started highly active antiretroviral therapy (HAART) in her home country, achieving virological suppression and CD4 count gain, and subsequently depleted her supply, resulting in virological rebound and rapid immunological deterioration. As the patient strongly denied knowledge of her HIV-positive status and use of antiretrovirals, it can also be proposed that she was genuinely unaware of the nature of the remedies she had been prescribed. It can also be speculated that the patient obtained the drugs from sources within the UK, for example, through pill-sharing or attending another clinical centre. However, the timing of the viral rebound coincided with her stopping the remedy.

HAART first became available in Uganda at the end of 2002,[4] and drugs for first-line treatment include zidovudine, stavudine, lamivudine, efavirenz and nevirapine.[5] In addition to NNRTI resistance mutations, the patient showed V75A and M184T, which confer resistance to stavudine and lamivudine,[6] suggesting possible selective pressure with these drugs. While efavirenz monotherapy seems unlikely in view of the suppressed viraemia and good CD4 count, neither stavudine nor lamivudine were detected in plasma, probably as a result of their short plasma half-lives. Assays for assessing the plasma levels of PIs and NNRTIs are widely available in Western Europe. Testing for levels of NRTIs is less widespread. We only had access to testing for stavudine, zidovudine and lamivudine and were therefore unable to exclude the presence of other NRTIs. It can be proposed that the long half-life of efavirenz, prolonged further by the *CYP2B6* 516G→T polymorphism, led to extended efavirenz monotherapy after stopping treatment and the selection of resistant variants. After therapy discontinuation, plasma efavirenz concentrations with 516 GG, GT and TT genotypes are predicted to exceed the 95% inhibitory concentration for wild-type virus (46.7 ng/mL) for a median of 5.8 days (IQR 4.4–8.3 days), 7.0 days (IQR 5.0–8.0 days) and 14 days (IQR 11.1–21.2 days), respectively.[1] It is worth noting, however, that NNRTI resistance could have occurred regardless of the presence of the *CYP2B6* 516G→T polymorphism.

Rapid emergence of resistance during efavirenz therapy has been well documented, and K103N is the most frequent mutation selected.[7] While the case further underscores the limited sensitivity of standard genotypic resistance assays, the clinical significance of low-frequency mutants remains to be fully established.[8,9] Their detection was highly relevant in this case. According to the UK guidelines, the patient would have normally been offered an NNRTI-based regimen as her first-line HAART. Available evidence, however, indicates that low-frequency resistant mutants significantly increase the risk of virological failure among patients receiving NNRTI-containing regimens.[10]

In migrating populations seeking HIV testing, careful and compassionate counselling is required to facilitate the disclosure of previous diagnosis and therapy. This case illustrates the important point that the use of remedies of dubious content should also be discussed and investigated.

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Transparency declarations

M. J. has received reimbursement for either/or: attending a symposium; a fee for speaking; a fee for organizing education; funds for research; funds for a member of staff; fees for consulting from various pharmaceutical companies including Abbott, Boehringer-Ingelheim, Bristol-Myers Squibb, Gilead, GlaxoSmith Kline, Roche and Tibotec. A. M. G. has received consultancy fees, speaker honoraria, unrestricted educational and research grants and travel support from various pharmaceutical companies including Abbott Diagnostics, Abbott Pharmaceuticals, Boehringer-Ingelheim, Gilead Sciences, GlaxoSmithKline, MSD, Pfizer, Roche, Tibotec and Virco. Other authors: none to declare.

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