HIV-1 RESISTANCE PROFILE IN PLASMA AND PERIPHERAL BLOOD LYMPHOCYTES IN A GROUP OF NAIVE PATIENTS

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Abstract - Transmitted HIV-1 drug resistance (TDR) is a persisting problem, even though the prevalence of primary resistance may remain stable or start to decline. Proviral DNA detectable in peripheral blood mononuclear cells (PBMCs) is a reservoir of drug resistant viral variants and could be an alternative marker to viral RNA for the detection of drug resistance mutations. The aim of this study was to compare the HIV-1 resistance profile between plasma viral RNA and proviral DNA in a group of untreated patients. Thirty-one HIV-1 seropositive patients without prior ARV treatment were included in the study. The presence of non-polymorphic drug resistance mutations was identified in 10 cases in proviral DNA and in 11 cases in plasma according to different scoring systems. Our results show a similar resistance profile between plasma RNA and proviral DNA, but with some discordances present. The sequencing of proviral DNA could provide useful additional information with regard to primary resistance.

Key words: HIV, resistance profile, proviral DNA

INTRODUCTION

The primary goals of antiretroviral therapy (ART) are to restore and preserve the immunologic function of an HIV-infected person, reduce morbidity, prolong survival, improve quality of life, and prevent HIV transmission (Hammer et al., 2008; Paredes et al., 2010). All drug classes currently available for first-line ART are efficacious and have good tolerability (Camacho and Teófilo, 2011). The differences between individual drugs must be carefully considered when deciding a first-line regimen. Antiretroviral agents are divided into 6 classes according to their mechanism of action: nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), entry inhibitors, coreceptor CCR5 inhibitors and integrase inhibitors (Ribera et al., 2011). However, the emergence of drug-resistant HIV and the long-term toxicity of antiretrovirals is still of great concern. Importantly, an immediate consequence of ARV resistance is the loss of treatment efficacy and the increased probability of the transmission of drug-resistant viruses from person-to-person as primary infection or superinfection. The possibility that resistant strains in drug-naive individuals may also arise from viral genetic diversity cannot be excluded, although it is rare to find major drug resistance mutations in a virus never exposed to drugs using routine genotypic resistance tests (Brenner et al., 2008).

The presence of transmitted drug resistance mutations (TDR) in patients not exposed to highly active antiretroviral treatment (HAART) is a major problem in the management of HIV-1 infection. Drug resistance mutations reduce the effectiveness of initial
therapy for HIV and have the disturbing potential of limiting future therapy options. Also, the widespread dissemination of drug-resistant HIV variants could have negative impact on the potency of post-exposure prophylaxis (Oette et al., 2006; Bannister et al., 2008; Wittkop et al., 2011). Transmitted resistance to PR and RT inhibitors has been observed in most European countries, with a prevalence of around 10% (Frentz et al., 2012). At this point, the prevalence of primary resistance may remain stable or start to decline (Frentz et al., 2012).

Current international guidelines recommend resistance testing in cases of primary or recent HIV infection before commencement of the first treatment regimen for HIV-infected patients. This strategy attempts to maximize the chances of detecting transmitted drug resistance. The cost-effectiveness of genotypic resistance before initiation of therapy does not only depend on the cutoff of acceptable cost but also on the prevalence of TDR. It is considered cost-effective for both recent and chronic infection when the prevalence level of TDR is >1.5% (Weinstein et al., 2001; Sax et al., 2005; Vandamme et al., 2011).

Genotypic resistance using direct sequencing of plasma-derived viral RNA has been used to detect resistant viruses, because plasma viruses have a rapid turnover and are considered to represent actively replicating HIV. However, current genotypic resistance assays are unlikely to identify a resistant virus if present in less than 15-25% of the total viral population (Brun-Vézinet et al., 2004; Messiaen et al., 2012). More sensitive approaches are being evaluated in research settings. (Palmer et al., 2005; Bon et al., 2007; Wang et al., 2007; Vandenbroucke et al., 2011; Messiaen et al., 2012). One of them is based on the detection of minority variants by standard genotyping resistance of proviral DNA in peripheral blood mononuclear cells (PBMCs). The cell compartment harbors archival proviral DNA, either wild type or drug resistant, that arise during infection. The durable persistence of archived drug-resistant DNA may significantly reduce the effectiveness of targeted drugs, and represents the ‘resistance potential’ profile of a patient (Siliciano and Siliciano, 2006).

Recent reports present different results concerning the concordance between the HIV strains in plasma and PBMCs in therapy-naive patients. Some authors recommend simultaneous genotypic determination in both compartments for a more complete determination of drug resistance mutations (Parisi et al., 2007). However, contradictory results have been reported on the usefulness of each compartment, depending, for example, on whether naive or treated patients are studied. In this study, we tested whether the sequencing of proviral DNA increases the sensitivity of detection of primary resistance.

**MATERIALS AND METHODS**

Thirty-one HIV-1 seropositive patients older than 18 years, without prior exposure to antiretroviral treatment were included in the study, having given informed consent.

Plasma and PBMC samples were obtained from a 3 mL EDTA-treated blood sample. Whole-blood EDTA samples for resistance genotyping were first routinely centrifuged to separate plasma and blood cells. Each fraction was kept at -80°C. Total RNA was extracted from 280 µL of each plasma sample using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), and then subjected to reverse transcription using the One Step RNA PCR Kit (Qiagen, Hilden, Germany) with outer primer pair 01 and 02. The procedure employed a nested PCR protocol using Taq PCR Core Kit (Qiagen, Hilden, Germany) and inner primer pairs 03 and 04. The final concentration of primers used in the reaction of reverse transcription was 0.6µM, while in nested PCR it was 0.3 µM. DNA samples were extracted from 200 µl of whole blood using a QIAamp DNA kit (Qiagen Inc., CA, U.S.A.) according to the manufacturer’s protocol. Partial pol regions were PCR-amplified using nested primers as previously described. Each PCR procedure consisted of 35 cycles of denaturation (94°C), annealing (50°C) and elongation (72°C). Primer sequences were as follows: sense primer 01: 5’-GGTATTAAATAAAATTGAAG-3’ (HIV-1 HXB2 positions 1138 to 1155); 03: 5’-AATTGTTTTACATCATTTAAGTGTG-3’(HIV-1 HXB2 positions 1355 to 1383); antisense
primers, 02: 5’-GCTACACTAGAAGAAATGATGAC-3’ (HIV-1 HXB2 positions 3199 to 3176), 04: 5’-CTTGATAAATTGATATGTCCATTG-3’ (HIV-1 HXB2 positions 3126 to 3101) (Snoeck et al., 2005). Products were purified using the Qiagen PCR purification kit (Qiagen Hilden, Germany) according to the manufacturer’s protocol. Purified DNA was sequenced using the ABI BigDye Terminator v.3.1 cycle sequencing ready reaction kit (Applied Biosystem, Foster City, CA, USA), and processed with an automated ABI 310 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). Sequencing reactions were performed with the described inner primer pair, as well as additional sequencing primers: 5’-TGTACTGAGAGACAGGCTAATTTTTAGGG-3’, 5’-GGCAAATACTGGAGTATTGTATG-3’, 5’-CACCTGTCAACATAAT-3’, 5’-TACTAGGTATGGTAAATGCAGT-3’, 5’-CAGTACTGGATGTGGGTGATG-3’ (Snoeck et al., 2005). Analysis of viral sequences was performed using the BLAST tool from the National Center for Biotechnology Information (NCBI) and the Los Alamos HIV database (accessible at www.ncbi.nlm.nih.gov/BLAST/ and www.hiv.lanl.gov). Resistance mutations were determined and interpreted with the HIVdb Genotypic Resistance Interpretation Algorithm available at the Stanford University HIV Drug Resistance Database (www.hivdb.stanford.edu). The World Health Organization (WHO) list of Surveillance Drug Resistance Mutations (SDRM) was used to determine the presence of TDR mutations. HIV-1 subtypes were determined with the Rega subtyping tool (available at www.jose.med.kuleuven.be/subtypetool/html).

Besides genotypic testing, clinical data were collected regarding the number of CD4+ T lymphocytes per mm³ and disease stage according to the Center for Disease Control (CDC) criteria (Centers for Disease Control and Prevention, 1992). All results were processed using standard statistical methods (chi-square test).

RESULTS

Rega subtyping tool analysis revealed that most [90% (28/31)] of the patients were infected with subtype B virus. Among the non-B subtypes, two isolates were classified as subtype G and one patient as CRF01_AE, as shown in Table 1. The clinical presentation of patients included in the study was as follows: 35% of patients in CDC stages A and C each, while 30% of the patients presented CDC stage B. The average value of the CD4 cell count was 280 cells/mm³; range (11-1320), SD 304.

The presence of SDRM was identified in 4 cases in both PBMCs and plasma: patient no. 14 had a major protease mutation V82L correlated to decreasing tipranavir (TPV) susceptibility; patient no. 17 had an amino acid change at codon 215 (T215I) that reduces NRTI susceptibility; patient no. 18 had K65KR, Y181C plus an amino acid change at codon 151 (Q151M) belonging to a cluster of mutations conferring MDR (Multi Drug Resistance), and patient no. 31 had an amino acid change at codon 103 (K103N) preventing the clinical utility of all NNRTIs.

Besides SDRM mutations, additional resistance-related mutations from the International Antiviral Society-USA (IAS-USA) list were identified. Five patients carried amino acid changes at position 33, 74, 101 and 118 in both compartments (Table 1) However, in two patients reverse transcriptase mutations was identified only in the RNA viral sequence but not in a proviral compartment: patient no.6 had a NRTI-correlated mutation T69I and patient no.27 had a NNRTI correlated mutation H221NY. In one patient (no. 24) we disclosed PR minor non-polymorphic mutation L10F only in proviral DNA but not in a plasma viral RNA. Polymorphic mutations
Table 1. Resistance profile of plasma derived RNA sequences and PBMCs derived DNA sequence
CD4 cell count – (range 11-1320 per mm3); disease stage according to Center for Disease Control (CDC) criteria; Major mutations conferring resistance to multiple drugs are shown in bold; Drug resistance mutations found in only one compartment (either plasma pr PBMCs) are italicized and underlined.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Subtype</th>
<th>CD4 count Cells per mm³</th>
<th>CDC stage</th>
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<th>Other resistance correlated mutations</th>
<th>SDRM</th>
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correlated to resistance were identified in 13 cases in PBMCs and in 14 cases in the plasma, wherein patients nos. 15 and 21 carried an amino acid change only in proviral DNA and patients nos. 13, 23 and 26 carried the mutation only in the plasma. The overall frequency of these mutations in both compartments was significantly greater in the PR gene than in the RT gene.

The chi-square test did not reveal significant difference between plasma-derived RNA sequences and PBMC-derived DNA sequences, taking into account SDRMs and other resistance-related mutations.

As shown in Fig. 1, the HIV-1 RNA and DNA sequences derived from PBMCs and plasma were closely related, and the phylogenetic tree revealed a tight cluster of both viral sequences. Nucleotide divergence among all DNA and RNA B clade sequences; only the DNA sequences and only the RNA sequences were: 3.3%, range (0-8.9%), SD 2; 3.8%, range (0.3-8.5%), SD 1.4; 2.9%, range (0-7.7%), SD 1.4, respectively. The average nucleotide divergence between RNA and DNA sequences isolated from the same samples was 0.7% (range 0-2.7%), SD 0.7%. The chi-square test showed a statistically significant connection between the greater nucleotide divergence of RNA vs. DNA sequences from the same sample and a more advanced disease stage (CDC stage B and C) ($\chi^2 = 4.5; 3.84-6.64; p < 0.05; H_1$).

**DISCUSSION**

Previous reports have given evidence of the genetic heterogeneity between plasma viral RNA and proviral DNA. However, most of these works are focused on patients treated with suppressive antiretroviral therapy (ART) (Chew et al., 2005; Turriziani et al., 2007; Diaz et al., 2008; Quan et al., 2008). Current resistance testing guidelines do not provide clear indications whether primary resistance genotyping of HIV proviral DNA would provide additional information compared to plasma RNA testing. Reports on the resistance patterns in DNA and RNA suggest different degrees of discordance between two compartments in therapy naive patients. However, there are still limited data concerning the investigation of genetic heterogeneity in drug-naive HIV-infected subjects.

Several recent studies have shown that resistance genotyping of proviral DNA may disclose major mutations conferring resistance to reverse transcriptase (RT) inhibitors more frequently than data obtained from sequencing of plasma viral RNA. Also, in these reports the authors have found major mutations in the protease (PR) region only in peripheral blood mononuclear cells (PBMCs) but not in plasma-derived samples (Bon et al., 2007; Rangel et al., 2009). However, other authors found a high correlation between RNA- and DNA-derived sequences of the pol region, suggesting that proviral DNA could be used as an alternative template for genotyping resistance (Vicenti et al., 2007; Demetriou et al., 2010). These results, limited by the small number of studies, may be due to the applied methodology, disease stage and virus dynamics in the infected subjects. Different results might also depend on the duration of infection and on the viral load level at the time of sampling, as the main reasons for discrepancy between plasma RNA and proviral DNA heterogeneity. In addition, other reports have revealed that different observations in the plasma and PBMC compartment are likely attributed to stochastic effects, given that the detection of minority variants is at the limit of detection sensitivity (Jakobsen et al., 2010). In one of the latest findings, it was shown that in cases of suppressed viremia historical HIV-RNA resistance test results are more informative than proviral DNA genotyping (Wirden et al., 2011). Furthermore, comparison of episomal cDNA and integrated HIV-1 proviral DNA genomes has suggested that the majority of proviral DNA has a limited ability to reveal the dynamics of the viral population that persists in patients on suppressive ART. In contrast to proviral DNA, extrachromosomal (episomal) viral DNA is labile and could give better information about recent infection events (Sharkey et al., 2011).

Our results, albeit limited to a restricted number of cases, show similar resistance profiles between plasma RNA and proviral DNA, with discrepancies
Fig. 1. Phylogenetic tree denoting relationships between plasma derived RNA sequences and PBMCs derived DNA sequences of 27/31 subjects included in the study. Sequences were analyzed along with hiv-1 subtype B, G, CRF01_AE and HIV-2 as an out-group member (accession numbers: K03455; AF084936; GQ477441; NC_001722) by using the Kimura's formula distance matrix fed into the neighbor-joining tree construction algorithm. Branches are shown with patient number, extraction template used for genotypic resistance testing and disease stage.
present. Overall, our findings show that the analysis of PBMC DNA could be as useful as the sequencing of plasma viral RNA and in some cases could give additional information, as correlated in some previous data (Vicenti et al., 2007; Parisi et al., 2007; Jakobsen et al., 2010; Demetriou et al., 2010).

According to the WHO list of mutations for surveillance of transmitted drug resistance, we identified four cases with SDRMs in both cell and cell-free compartments (Table 1). In all four mentioned cases the CD4 count was greater than average (average CD4 = 280, patients with SDRM CD4 count >400). This is in correlation with the recent results indicating that patients with TDR have a higher initial CD4 count than persons with wild-type virus, which is due to the reduced fitness and pathogenicity of drug-resistant variants (Bhaskaran et al., 2004; Booth et al., 2007). However, evidence of these is still contradictory.

Mutations from the WHO SDRM list are considered as markers of transmitted resistance. However, other changes in the viral genome may also be present. We found other resistance-related mutations at several positions, mostly in the PR gene in both compartments (Table 1). These mutations are associated with resistance when present with other mutations and may compensate for the reduced activity of viral enzymes caused by primary mutations improving the fitness of viruses. In addition, these mutations may suggest a previous contact of the virus with ARV drugs (Nijhuis et al., 1999; Johnson et al., 2011) and thus a possible simultaneous presence of hidden TDR mutations, underscoring the need for caution in the choice and follow-up of first-line treatment.

In one patient, we observed the minor mutation L10F which confers decreased susceptibility to nelfinavir (NFV) and fosamprenavir (FPV), both belonging to the protease inhibitor class, only in proviral DNA but not in the plasma RNA. This patient was in an advanced disease stage with a very low CD4 count, which may be due to the long period of infection before HIV diagnosis. This finding suggests that a reverse mutation could have occurred in the course of chronic infection.

In two patients we found resistance-related mutations at RT positions 69 and 221 only in plasma viral RNA but not in the proviral DNA. This difference may be due to spontaneous mutations that occurred during the replication course of the virus. In addition, the detection of resistance mutations is dependent on the sensitivity of the genotyping technique used. Routine genotyping resistance does not detect a resistance virus representing less than 15% of the total viral population (Brun-Vézinet et al., 2004; Messiären et al., 2012). These minority genotypes of the virus may exist in a very small percent of PBMCs and thus not be detectable by standard resistance testing. Some authors suggest that a highly sensitive method such as ultra-deep sequencing might give better results, but its cost and implementation are not yet compatible with routine use (Wang et al., 2007; Messiären et al., 2012). However, during the course of infection these minority genotypes may become dominant in a plasma viral population. Furthermore, some studies state that resistant variants observed in the plasma compartment may originate from sources other than proviral DNA (Potter et al., 2003; Sharkey et al., 2011).

In our work, we compared the higher nucleotide divergence of RNA vs DNA sequences from the same sample at more advanced disease stages (CDC stages B and C) and found a statistically significant connection. Low nucleotide similarity (defined as a more than average nucleotide divergence, 0.7%) between proviral DNA and plasma RNA in the same sample may be due to a longer period of infection and increased virus diversity. In addition, one explanation for these findings could be the different dynamics of the divergent evolution of HIV in infected patients.

The greater nucleotide divergence among DNA sequences in total, observed in our study, probably reflects a chronic duration of infection in the majority of naïve patients tested. In fact, epidemiological data indicate that at present in Serbia, a significant number of patients present at an advanced disease...
stage, although the trend is towards earlier diagnosis – from a majority of 56% CDC stage C patients in 2002 to 25% in 2010, among newly diagnosed patients (Simic, 2010).

On the basis of our findings and previous data, we conclude that cellular DNA sequencing and plasma RNA sequencing are both useful for the detection of drug resistance mutations in antiretroviral-drug-naive patients. Furthermore, the sequencing of proviral DNA could give additional information, especially in cases of chronically infected patients. In some circumstances, HIV DNA can be conveniently used as an alternative source for the detection of transmitted resistance, such as in cases of low viremia and when plasma viral RNA cannot be isolated. For a better understanding of the viral evolution and compartmentalization between residual plasma viremia and proviruses in PBMCs, further investigation could include a larger group of patients and patients receiving HAART therapy.

Acknowledgements - This study was supported in part by the sixth framework European Commission grant LSHP-CT-2006-518211 and by the Republic of Serbia Ministry of Education and Science, Project No 175024.

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http://hiv.lanl.gov


