Andrea Freystetter / Christian Paar / Herbert Stekel / Jörg Berg

Analysis of HIV-1 Resistance Mutations from various Compartments of the Peripheral Blood in Patients with Low-Level Viremia

107 - Translationale Gesundheitsforschung-Brücken bauen von Grundlagenwissenschaft zu angewandter Forschung

Abstract

The aim of antiretroviral therapy (ART) is suppression of human immunodeficiency virus (HIV) RNA plasma loads (pVL) to levels below detection limits. Non-optimal suppressed HIV-RNA levels are associated with higher risks for accumulation of drug resistance mutations (DRM).These residual viremia may be observed as low-level viremia (LLV) episodes, i.e. HIV pVL between 20 and 500 copies/mL. DRM testing in LLV patients is thought to be essential for treatment optimization. However, genotyping assays usually fail in most LLV patients below 500 copies per mL due to technical limitations.

The aim of the study was to investigate in HIV-patients with LLV whether sequencing for DRMs from the Protease (Pr) and Reverse Transcriptase (RT) gene regions, from compartments of the peripheral blood other than plasma could serve as an alternative method to plasma-RNA analysis.

Thus, the following 6 different compartments of the peripheral blood were investigated: plasma RNA, proviral DNA from whole blood, proviral DNA from peripheral blood mononuclear cells (PBMCs) and $CD4^+$ cells, and cellular RNA from PBMCs and $CD4^+$ cells. Samples (n = 124) of 99 LLV patients with pVL between 20.5 and 480 copies per mL were analyzed by Sanger sequencing. Sequencing success rates, DRM profiles and whole sequence congruency were analyzed for sequence homologies and were compared across the different compartments by phylogenetic trees analysis.

Sequencing results were obtained in 119 of 124 samples. The sequencing success rate was 85.5% of proviral DNA from PBMCs, 84.7% of proviral DNA from whole blood and 61.3% of proviral DNA from $CD4^+$ cells. The sequencing success rate of cellular RNA analysis was only 18.5% and 19.4% respectively. Statistically, proviral DNA analysis had a 23-fold, 22-fold and 6-fold chance generating sequence results compared to plasma RNA. Comparison of DRM profiles of proviral DNA analysis from PBMCs and whole blood resulted in 67.7% of the samples to total matching DRMs and a comparison of proviral DNA analysis from PBMCs, whole blood and $CD4^+$ cells resulted in 52.2% of the samples to total matching DRMs. Whole sequence congruency resulted in a sequence homology in which 6 of 15 alignments were above 86% homology and 9 of 15 alignments above 95% homology. By the phylogenetic tree analysis all specimens of the same sample shared the same branch in the tree, i.e. they join the nearest neighborhoods, which is in line with the results of the sequence homologies.

In conclusion, sequencing analyses for DRMs in Pr and RT region of HIV using proviral DNA from whole blood or PBMCs are technically feasible. The compartments whole blood and PBMCs represent the most promising candidates to serve as an alternative to plasma RNA screening for DRM testing in LLV patients.

Keywords:

HIV-1, Low Level Viremia, Drug Resistance Mutations, Resistance Testing, Protease, Reverse Transcriptase, Proviral DNA, Cellular RNA

1. Introduction

Antiretroviral therapy (ART) is the current state-of-the-art treatment of infections with human immunodeficiency virus type 1 (HIV). The aim of ART is the suppression of the plasma viral load (pVL) under detectable limits (Maartens et al. 2014; Pasternak et al. 2013). Sometimes, even under compliant ART, patients develop episodes of detectable low pVL, i.e. low-level viremia (LLV), which means pVL between 20 and 500 copies/mL (Delaugerre et al. 2012; Wyl et al. 2012). Apart from an increased risk for virological failure, LLV patients are faced with higher immune activation and increased mortality (Delaugerre et al. 2012; McConnell et al. 2011). It is shown that treatment optimization in those patients according to their genotypic resistance profile may result in optimal treatment outcome within the next 12 months of optimized treatment (McConnell et al. 2011).

Even though genotypic resistance testing is essential for treatment optimization genotyping assays fail in most LLV patients below 500 copies/mL (Campbell et al. 2011; Delaugerre et al. 2012; McConnell et al. 2011). Therefore, attempts are being made to obtain DRM data either by increasing the sensitivity of sequencing methods or by sequencing from other viral sources e.g. proviral DNA and from compartments of the peripheral blood other than plasma (Campbell et al. 2011; Delaugerre et al. 2012; Armenia et al. 2015; Paar et al. 2011; Stelzl et al. 2010). Thus, whole blood, peripheral blood mononuclear cells (PBMCs) or $CD4^+$ selected cells can be envisioned as the source for HIV proviral DNA or HIV cellular RNA to attempts of sequencing.

The aim of the study was to investigate whether sequencing for DRM from the Protease (Pr) and Reverse Transcriptase (RT) gene region in patients with LLV; from other compartments of the peripheral blood could serve as an alternative method to plasma-RNA analysis. Thus, the following research questions are raised (1) Is it possible to assess the DRM-status of compartments of the peripheral blood as an alternative to plasma-RNA? (2) Is there a correlation between pVL and success rate of sequencing? (3) Are the profiles of DRMs the same in the various compartments within the same patients? (4) How comparable are the HIV-sequencing analysis from the various compartments? (5) Are there any differences in the nucleotide sequences to be observed between proviral DNA and cellular RNA analysis?

By the analysis of success rates and comparisons of DRMs and total sequences across the different compartments, the technical feasibility of this approach is investigated for LLV patients.

2. Material & Methods

Altogether, 133 back up blood samples of 104 patients were collected after a quantitative HIV PCR with COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 v2.0 (Roche) test system was performed. All patients were participants of the Austria HIV cohort study and approved by the Healthcare Ethics Committee Linz and Salzburg. Inclusion criteria for this study were pVL of 20 - 500 copies/mL.

Per sample two sample preparation methods were applied. First, sample preparation was applied within the quantitative HIV-PCR. For this, 3 mL plasma were lifted off into two sterile 2 mL tubes and stored at -70°C until the PCR analysis. For the second sample preparation, the back-up sample was refilled with 3 mL PBS Buffer to replace plasma. After re-suspension 500 µL for later whole-blood

analysis was collected. The remaining sample underwent a Ficoll® density centrifugation. After a washing step, 1mL of PBMCs was carried on a magnetic assisted cell sorting (MACS) separating for $CD4^+$ cell selection. The remaining PBMCs were collected in PBS and RLT in Eppendorf cups. $CD4^+$ cells were then collected in PBS and RLT. Next to these compartments the back-up plasma was collected.

All plasma samples for gaining plasma RNA were extracted on a MagNA Pure LV 2.0 (Roche) instrument with MagNA Pure LC Total Nucleic Acid Isolation Kit - Large Volume (Roche) test kit. Whole blood samples, PBMCs in PBS and $CD4^+$ selected cells in PBS for proviral DNA were extracted on a QIAcube (QIAGEN) instrument using the QIAamp DNA blood mini Kit (QIAGEN). Finally, PBMCs in RLT and CD4+ selected cells in RLT for gaining cellular RNA were extracted on a QIAcube instrument using QIAamp RNA blood mini Kit (QIAGEN).

After nucleic acid extraction, all samples were genotypically analyzed for their Pr and RT DRM status, using standard population based sequencing procedure. The sequence data was aligned and base-called by using SeqScape v2.9 software. For sequence interpretation, the data obtained in SeqScape software was submitted to the Stanford University HIV Drug Resistance Database.

Further sequence analysis included an alignment of all compartments within one sample, using the Multiple-Sequence-Alingment tool "Clustal omega" (EMBL-EBI), and sequence identities as percentage of total bases were recorded for further statistical analysis. Next to this, all results were separated into subtype B and non-subtype B, and collectively performed in phylogenetic tree analysis by using "Clustal X v2.0.12" software and "FigTree v1.4.0".

Statistical analysis included epidemiologic data analysis and descriptive statistical analysis of the sequencing success rates, the DRM profiles and of the whole sequence congruency based on the procentual identity analysis according to "Clustal omega". Further statistical analysis included a binary logistic regression analysis in which the compartments proviral DNA of whole blood, proviral DNA of PBMCs, proviral DNA of $CD4^+$ cells, cellular RNA of PBMCs and cellular RNA of cells were compared to the predefined "golden standard" plasma RNA.

Statistical analysis and corresponding graphical representations were performed with "IBM SPSS Statistics v22.0.0.0" statistic software and "Microsoft Office Excel 2007" software.

3. Results

From the obtained sequences, the HIV subtype distribution was determined in 119 samples. Sequencing analysis did not yield a result in 5 samples, and 9 samples were excluded as pVLs exceed 500 copies/mL. The most prevalent subtype was subtype B, remaining subtypes were identified as subtypes A, G, F, C, the circulating recombinant forms (CRF) CRF01_AE, CRF02_AG, CRF03_AB and CRF31_BC as well as the unique recombinant forms (URF) URF_CG and URF_BD.

Altogether, sequencing results were available in 106 samples (85.5%) from proviral DNA of PBMCs, 105 samples (84.7%) from proviral DNA of whole blood,76 samples (61.3%) from proviral DNA of $CD4^+$ cells, 28 samples (22.6%, n = 124) from plasma RNA, 24 samples (19.4%) from cellular RNA of $CD4^+$ cells and 23 samples (18.5%) from cellular RNA of PBMCs. A sequencing result from all 6

compartments was obtained in only 4 samples (3.2%, n = 124). About 9 samples (7.3%) had a sequencing result in 5 compartments, 22 samples (17.7%) had a sequencing result in 4 compartments, 49 samples (39.5%) had a sequencing result in three compartments, 23 samples (18.5%) had a sequencing result in two compartments, and 12 samples (9.7%) a sequencing result in one compartment. In 5 samples (4.0%) not a single compartment led to any sequencing analysis result at all. Sequencing results in 0, 1, 2, 3, 4 and 5 compartments are related to a 95%-CI pVL in between 20 - 200 copies/mL whereas sequencing results in 6 compartments are related to a 95%-CI pVL from 150 - 520 copies/mL. These results were not statistically significant but a tendency of a relation between successful sequencing from all 6 compartments and higher pVL was observed.

A distribution of the sequence results categorized into pVL groups, with 20.5 - 50 copies/mL, 51 - 150 copies/mL and 151 - 480 copies/mL showed differences between cellular RNA and proviral DNA analysis. Binary logistic regression showed a high probability ($p < 0.0001$) to obtain a positive sequencing result in all proviral DNA analyses compared to plasma RNA analysis. By contrast, when plasma RNA analysis was compared with cellular RNA analysis (Cellular RNA analysis from PBMCs $p = 0.423$, cellular RNA from $CD4^+$ cells $p = 0.524$), binary logistic regression showed no such probability. Next to the significance, compared to plasma RNA analysis, a 6-fold chance (95%-CI: 3.4 - 10.7) gaining a positive sequencing result from proviral DNA analysis from $CD4^+$ cells, a 22-fold chance (95%-CI: 11.5 - 43.8) from proviral DNA analysis from whole blood and a 23-fold chance (95%-CI: 12.2 - 47.2) from proviral DNA analysis from PBMCs is observed within this binary logistic regression.

In 73 specimens (61.4%, n = 119), protease inhibitor (PI) minor DRMs, in 49 specimens (61.2%) non-nucleoside reverse transcriptase inhibitor DRMs, in 35 specimens (29.4%) nucleoside / nucleotide reverse transcriptase inhibitor DRMs and in 11 specimens (9.2%) PI major DRMs were observed. According to the result of the binary logistic regression, DRM profiles of the proviral DNA compartments were compared. Identical DRM profiles were observed in 63 samples (67.7%, n=93) by comparing proviral DNA out of whole blood and from PBMCs. Identical DRM profiles were observed in 35 samples (52.2%, n=67) by comparing proviral DNA from whole blood, from PBMCs and from CD4+ cells.

A summary of sequence homology is shown in figure 1. Almost all alignments with their 95%-CI were at 95% homology and greater. Alignments with their 95%-CI of plasma RNA analysis and t of proviral DNA analysis from $CD4^+$ cells and cellular RNA analysis from PBMCs, were at 86% and greater.

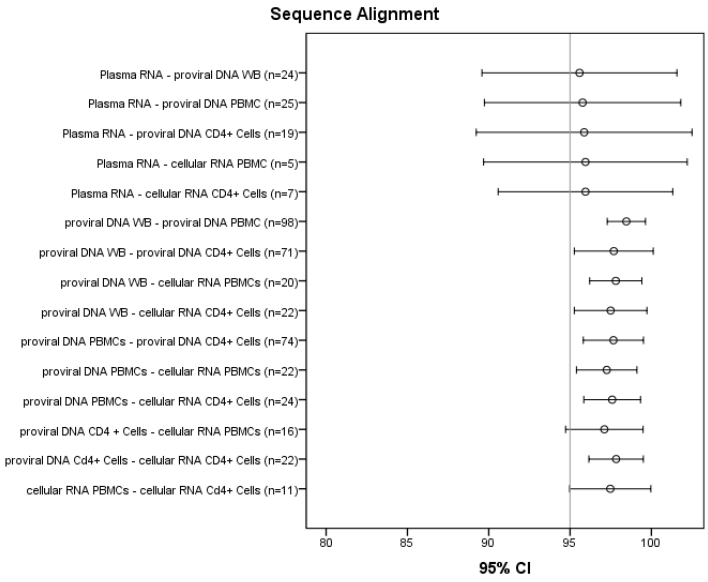

Figure 1. Summary of Sequence Alignment.For further information see text.

The phylogenetic tree analysis showed that most of the sequences from compartments of a given ID were nearest neighbors located on the same branch from the trunk of the tree. Replicate samples of patients collected during clinical course monitoring were omitted for the phylogenetic tree analysis.

4. Discussion

This study shows an investigation of genotypic DRM testing by population based sequencing of Pr and RT regions from compartments of the peripheral blood other than plasma RNA in HIV-patients with LLV.

Contamination of DNA in RNA samples and vice versa can be excluded, as RNA purification method comprised a DNA digestion step, and proviral DNA sequencing analysis were not preceded by an RT step in contrast to RNA analysis. The first and second round PCR products were analyzed by agarose gel electrophoresis prior to sequencing. In each PCR batch molecular grade water was used as a reagent control in order to exclude potential cross contamination. As the reagent control did not show any amplicons, contaminations were excluded.

Sequencing success rate results show that more than half of all potential sequencing results were not obtained across the various compartments. This fact can be explained by the sample selection criteria of the study, i.e. pVL between 20 - 500 copies/mL which is below recommendations for genotypic resistance testing (Campbell et al. 2011; Delaugerre et al. 2012; McConnell et al. 2011). Another explanation is the fact, that cellular RNA is only synthesized intra-cellularly in ongoing virus replication. Cellular RNA analysis from PBMCs led to sequencing results in 18.5% and cellular RNA analysis from $CD4^+$ cells led to sequencing results in 19.4%. Thus, ongoing virus replication may have accounted for this in these particular samples. Cell-associated HIV-RNA is discussed in various studies as biomarker for ongoing virus replication, and it is not clear whether sequence data obtained from this specimen is

predictive for drug response (Pasternak et al. 2013; Bruner et al. 2015; Pasternak et al. 2009; Hilldorfer et al. 2012; Cockerham et al. 2014).

According to the results of the binary logistic regression, DRM profiles were compared among proviral DNA analysis from whole blood and PBMCs, and, in a second step, among proviral DNA analysis from whole blood, PBMCs and $CD4^+$ cells. Identical DRM profiles were observed in 67.7% of 93 pairs and in 52.2% of 67 triplets. In addition, the concordance of DRM was described to be at 70% in the study of Blaimschein et al, but this study investigated not a LLV study population, which may be an explanation of different DRM concordance (Blaimschein 2014). The differing DRM profiles may reflect different cell pools which were present in the different compartments. Each sample preparation step of cell selection probably enriched compartments with different or varying cell population. Also, the variations of DRM profiles between two or three compartments could be due to the fact that a possibly considerable number of potential HIV-infected cells were lost in sample preparation, in particular, during the $CD4^+$ MACS procedure. Finally, differences between these DRM profiles among all proviral DNA compartments could be due to by-sequence-error-rates, which, applies to any population based sequencing analysis.

Entire sequence congruency was analyzed with different methods, i.e. sequence homologies and neighborhood joining analysis by phylogenetic trees. Thus, both methods were in line with each other.

Various studies investigated the concordance of plasma RNA sequencing and proviral DNA sequencing from PBMCs, especially for tropism testing of the V3-loop region of HIV gp120, one study also included Pr and RT testing. The concordance of sequence comparisons ranged from 84% to 98.4% dependent on the pVL of sample used (Paar et al. 2011; Fabeni et al. 2015; Blaimschein 2014; Svicher et al. 2010). Similar conditions with regard to the pVL were observed by Fabeni et al, who investigated samples of pVL between 50 and 500 copies/mL, and the concordance of sequence was at 84.5% (Fabeni et al. 2015). These results are in line with the sequence homology results of this study. Nevertheless, DRM analysis from proviral DNA may be a useful alternative to DRM analysis from plasma RNA and has already been suggested for the routine clinical laboratory work-up (Paar et al. 2011; Novitsky et al. 2015; Fabeni et al. 2015).

The study may be limited by the technical issue that back-up samples that were stored for several days at 4°C until sample preparation were used. It is conceivable that cell quality would have been better if the samples had been subjected to the compartment-specific preparations the same day that they were drafted from the patients. Despite this limitation, the sequencing results, when obtained were of an acceptable quality.

Treatment optimization according to the DRM profiles in LLV patients could not be observed within this study. Therefore, prospective studies based on the investigated methods in this study should be undertaken especially to answer the question of whether sequencing data obtained from proviral DNA of whole blood or from PBMCs are predictive for drug response.

5. Conclusion

Plasma RNA is the standard source for genotyping assays for the assessment of HIV drug resistance mutations. This approach, however, is limited to pVL greater than 500 copies/mL. In this study, different compartments of the peripheral blood apart from plasma RNA were investigated for use towards the analysis of HIV-1 drug resistance mutations in patients with LLV.

It is shown that in a majority of cases it is indeed possible to assess the DRM-status of the HIV-Pr and RT in patients with LLC from at least two other compartments of the peripheral blood. The most promising alternative compartments beside plasma RNA were both proviral DNA from whole blood and proviral DNA from isolated PBMCs. Interestingly, the data indicates that sequencing analysis of proviral DNA from $CD4^+$ cells and cellular RNA from both PBMCs and isolated $CD4^+$ cells may not be a suitable approach.

In conclusion, using proviral DNA for the analysis of Pr and RT DRMs by sequencing is technically feasible and may serve as an alternative approach in LLV HIV patients in whom sequencing results are not obtained by the examination of plasma RNA.

References:

Armenia, D. et al. (2015): HIV-1 integrase genotyping is reliable and reproducible for routine clinical detection of integrase resistance mutations even in patients with low-level viraemia. In: The Journal of antimicrobial chemotherapy 70, 1865–1873.

Blaimschein, Anna (2014) Bachelorarbeit. FH Gesundheitsberufe OÖ

Bruner, K. M./Hosmane, N. N./Siliciano, R. F. (2015): Towards an HIV-1 cure: measuring the latent reservoir. In: Trends in microbiology 23, 192–203.

Campbell, S./Caliendo, A. M./Griffith, B. P. (2011): Manual of Clinical Microbiology, 10th Edition, edited by J. Versalovic*, et al.* (American Society of Microbiology).

Cockerham, L. R. et al. (2014): CD4+ and CD8+ T cell activation are associated with HIV DNA in resting CD4+ T cells. In: PloS one 9, e110731.

Delaugerre, C. et al. (2012): Impact of low-level-viremia on HIV-1 drug-resistance evolution among antiretroviral treated-patients. In: PloS one 7, e36673.

Fabeni, L. et al. (2015): Genotypic Tropism Testing in HIV-1 Proviral DNA Can Provide Useful Information at Low-Level Viremia. In: Journal of clinical microbiology 53, 2935–2941.

Hilldorfer, B. B./Cillo, A. R./Besson, G. J./Bedison, M. A./Mellors, J. W. (2012): New tools for quantifying HIV-1 reservoirs: plasma RNA single copy assays and beyond. In: Current HIV/AIDS reports 9, 91–100.

Maartens, G./Celum, C./Lewin, S. R. (2014): HIV infection: epidemiology, pathogenesis, treatment, and prevention. In: The Lancet 384, 258–271.

McConnell, M. J. et al. (2011): Improved viral suppression after treatment optimization in HIV-infected patients with persistent low-level viremia. In: Journal of acquired immune deficiency syndromes (1999) 58, 446–449.

Novitsky, V. et al. (2015): Long-Range HIV Genotyping Using Viral RNA and Proviral DNA for Analysis of HIV Drug Resistance and HIV Clustering. In: Journal of clinical microbiology 53, 2581–2592.

Paar, C./Geit, M./Stekel, H./ Berg, J. (2011): Genotypic prediction of human immunodeficiency virus type 1 tropism by use of plasma and peripheral blood mononuclear cells in the routine clinical laboratory. In: Journal of clinical microbiology 49, 2697–2699.

Pasternak, A. O. et al. (2009): Cellular levels of HIV unspliced RNA from patients on combination antiretroviral therapy with undetectable plasma viremia predict the therapy outcome. In: PloS one 4, e8490.

Pasternak, A. O./Lukashov, V. V./Berkhout, B. (2013): Cell-associated HIV RNA: a dynamic biomarker of viral persistence. In: Retrovirology 10, 41.

Stelzl, E./Troppan, K. T./Winkler, M./Korn, K./Kessler, H. H. (2010): Optimized protocol for detection of HIV-1 drug mutations in patients with low viral load. In: Journal of virological methods 168, 152–154.

Svicher, V. et al. (2010): Performance of genotypic tropism testing in clinical practice using the enhanced sensitivity version of Trofile as reference assay: results from the OSCAR Study Group. In: The new microbiologica 33, 195–206.

Wyl, V. von et al. (2012): Incidence of HIV-1 drug resistance among antiretroviral treatment-naive individuals starting modern therapy combinations. In: Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 54, 131–140.