Impact of triplicate testing on HIV genotypic tropism prediction in routine clinical practice

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Abstract

Guidelines state that the CCR5-inhibitor Maraviroc should be prescribed to patients infected with R5-tropic HIV-1 only. Therefore, viral tropism needs to be assessed phenotypically or genotypically. Preliminary clinical trial data suggest that genotypic analysis in triplicate is associated with improved prediction of virological response by increasing the detection of X4-tropic variants. Our objective was to evaluate the impact of triplicate genotypic analysis on prediction of co-receptor usage in routine clinical practice. Samples from therapy-naive and therapy-experienced patients were collected for routine tropism testing at three European clinical centres. Viral RNA was isolated from plasma and proviral DNA from peripheral blood mononuclear cells. Gp120-V3 was amplified in a triplicate nested RT-PCR procedure and sequenced. Co-receptor usage was predicted using the Geno2Pheno$_{[coreceptor]}$ algorithm and analysed with a false-positive rate (FPR) of 5.75%, 10%, or an FPR of 20% and according to the current European guidelines on the clinical management of HIV-1 tropism testing. A total of 266 sequences were obtained from 101 patient samples. Discordance in tropism prediction for the triplicates was observed in ten samples using an FPR of 10%. Triplicate testing resulted in a 16.7% increase in X4-predicted samples and to reclassification from R5 to X4 tropism for four cases rendering these patients ineligible for Maraviroc treatment. In conclusion, triplicate genotypic tropism testing increases X4 tropism detection in individual cases, which may prove to be pivotal when CCR5-inhibitor therapy is applied.

Keywords: CCR5, CXCR4, genotypic, HIV-1, maraviroc, R5-tropic, tropism, X4-tropic
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Introduction

Maraviroc (MVC) is the first available antiretroviral drug targeting a human receptor. It binds to the CCR5 co-receptor thereby inhibiting replication of CCR5 using (R5-tropic) HIV-1 [1,2]. MVC has been approved for HIV-1-infected patients that exclusively harbour R5-tropic viruses and is licensed in Europe for therapy-experienced patients and in the USA for both therapy-experienced and therapy-naive patients. As MVC has no antiretroviral effect on strains using the CXCR4 co-receptor (X4-tropic), determination of co-receptor usage (viral tropism testing) is needed to exclude the presence of X4-tropic HIV-1 strains. For determination of viral tropism several phenotypic and genotypic assays have been developed. Among phenotypic tropism tests, the 'enhanced sensitivity Trofile™ assay' (ESTA; Monogram Biosciences, San Francisco, CA) is most often used [3,4]. However, for clinical centres, ESTA has several limitations: testing is only performed in California (USA), resulting in logistical problems, long turnaround time and high costs. Furthermore, the assay

is only available in Europe for samples with HIV RNA ≥1000 copies/mL. For these reasons tropism testing is increasingly performed using genotypic assays.

Genotypic tropism tests analyse the sequence of the HIV-1 envelope gp120 variable 3 (V3) loop, the main determinant for co-receptor usage. To predict viral tropism the generated V3 sequences are interpreted using publicly available algorithms, such as Geno2Pheno$_{[coreceptor]}$ (G2P) and position-specific scoring matrices (PSSM$_{X4\text{-}R5}$) [5,6]. Genotypic tropism testing can be applied on population sequences obtained from either HIV RNA or HIV proviral DNA. The latter is recommended if HIV RNA levels are below the level of reliable amplification [7]. Population sequencing, the most frequently used method of genotypic tropism testing, is hampered by limited sensitivity for detecting minority X4-tropic strains in the quasi-species. As such, minority X4-tropic variants may remain undetected when they represent <10–25% of the total population [8–10].

Despite limitations in sensitivity compared with ESTA, population genotypic tropism testing demonstrated equal predictive value for virological outcome of MVC-containing therapy in antiretroviral naive individuals [11]. In this particular retrospective analysis a genotypic testing procedure was performed in triplicate to increase detection of minority X4 populations.

The rationale for performing genotypic tropism testing in triplicate, instead of using a single procedure as usually performed for resistance testing on *pol*, is based on differences in selective pressure on the viral envelope protein compared with *pol*, which are reflected by the nine-fold higher nucleotide substitutions/site/year in *env* [12]. The relatively high levels of variation in *env* may be better captured in a triplicate procedure.

In therapy-experienced patients, re-analysis of three clinical trials demonstrated that triplicate genotypic tropism testing increased the number of X4-predicted samples [13]. Preliminary data suggest that testing in triplicate has a beneficial effect on predicting clinical outcome of MVC-containing regimens [13].

However, in clinical cohort studies triplicate genotypic tropism testing is not performed routinely. Still a good correlation between genotypic tropism testing and ESTA in predicting virological outcome to MCV-containing therapy has been observed [14–17]. As such, the added value of triplicate testing in routine care is still under debate.

In the absence of a direct comparison of single and triplicate test procedures in clinical practice, the recently formulated European guidelines advise triplicate testing with a false-positive rate (FPR) of 10%. If single testing is performed then a more conservative FPR of 20% for RNA samples with a viral load <1000 copies/mL and for proviral DNA samples is recommended [7].

We investigated the influence of triplicate testing on tropism prediction during routine clinical practice in three European clinical centres.

Materials and Methods

Patient samples on which routine tropism testing was performed in clinical practice were randomly selected from three European centres. HIV-1 plasma RNA levels and counts of $CD4^+$ cells/mm^3 at nadir and at time of sampling were collected, HIV proviral DNA was not measured. HIV-1 pol subtyping was based on IDNS (Smartgene, Lausanne, Switzerland) or the Rega HIV-1 subtyping tool [18].

Viral RNA, DNA isolation

Viral RNA was isolated from 200–500 μL EDTA-plasma with the Viroseq HIV-1 sample preparation module (Abbott, Hoofdorp, the Netherlands) or a high pure viral RNA kit (Roche, Vilvoorde, Belgium). If no plasma was available or the HIV RNA level was below the level of amplification, proviral DNA was extracted from $1.0E^7$ peripheral blood mononuclear cells with QIAamp DNA Blood Mini Kit (QIAGEN, Madrid, Spain). For each sample, one isolation was performed. Subsequent processing of the samples, amplification and sequencing, were performed in triplicate. In each isolation and amplification round two or three negative controls were included, depending on the number of isolations and amplifications.

Viral RNA amplification

For amplification of the V3-loop, two in-house protocols were used. Protocol one; 10 μL of RNA, with primers 6206V3F 5′-AGAGCAGAAGACAGTGGCAATGAGAGT GA-3′, 7785R 5′-AGTGCTTCCTGCTGCTCCYAAGAA CCC-'3 (Titan One Tube RT-PCR kit, Roche, Woerden, the Netherlands) for RT-PCR. Subsequently a nested-PCR was performed using primers 6658F 5′-TGGGATCAAAGCCT AAAGCCATGTG-'3, 7371R 5′-GAAAATTCCCCTCCACAA TT-'3 (Expand High-Fidelity PCR-System, Roche, Woerden, the Netherlands). Sequencing was performed with primers 6957F 5′-GTACAATGTACACATGGAAT-'3 and 7371R or V3-4 5′-ACAGTACAATGTACACATGGAATTA-3′ and V3-3 5′-AATTCCCCTCCACAATTAAAASTGTG-3′ (Big dye Terminator Cycle seq kit v3,1, Applied Biosystems, Nieuwekerk ad IJsel, the Netherlands). Protocol two; for the RT-PCR 10 μL RNA and a mixture of the primers sense ENV_11 5′-GGATATAATCAGYYTATGGGA-3′, antisense ENV_22 5′-GGTGGGTGCTAYTCCYAITG-3′, sense-ENV1 5′-GAG-