

Clinical Implications of HIV-1 Minority Variants

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Technologic advances in human immunodeficiency virus type 1 (HIV-1) sequencing have revolutionized the study of antiretroviral drug resistance and are increasingly moving from the laboratory to clinical practice. These techniques are able to detect HIV-1 drug resistance mutations present at low frequencies not detectable by current HIV-1 genotyping assays. For a number of commonly used antiretroviral medications, such as non-nucleoside reverse transcriptase inhibitors, the detection of these drug-resistant minority variants significantly increases the risk of treatment failure. The level of evidence, however, is insufficient to determine the impact of HIV-1 minority variants for several other classes of antiretroviral medications. Clinicians should be aware of the novel technologies that are moving into routine clinical use and the clinical implications of HIV-1 minority variants. Additional studies are needed to determine the optimal platform for clinical application of these new technologies and to provide guidance to clinicians on the type and frequency of clinically important HIV-1 minority variants.

Keywords. HIV-1 drug resistance; minority variants; treatment failure.

Genotypic tests for human immunodeficiency virus type 1 (HIV-1) drug resistance employ polymerase chain reaction (PCR) amplification and population sequencing techniques that detect resistance-associated mutations present at $\geq 15\%$ –25% of the viral population [1, 2]. These assays do not reliably detect the presence of low-frequency resistance mutations present as minority variants within the population of HIV-1 in an infected individual. A number of studies have now shown that such low-frequency mutations, also known as minority variants, can have significant clinical implications on the risk of combination antiretroviral treatment (cART) failure. However, there remains much confusion over which HIV-1 minority variants are clinically

significant and how their presence should affect clinical practice.

Minority HIV-1 drug resistance mutations found in treatment-naive patients originate from 1 of 2 sources: transmitted drug resistance or de novo generation as part of natural viral diversification. Compared to wild-type HIV-1, those viruses harboring resistance mutations generally have lower fitness. In the absence of drug-selective pressure, the frequency of such transmitted HIV-1 drug resistance mutations is likely to decay and at a certain time would no longer be detectable by current genotyping assays that rely on population sequencing [3, 4]. HIV-1 minority variants can also arise due to the underlying diversity of the viral population. This remarkable diversity stems from a high replication rate and the error-prone reverse transcriptase enzyme. It is estimated that up to 5 mutations may arise with each replication cycle [5]. The daily production of more than a billion new virions in a typical chronically infected patient implies that the virus undergoes 10–100 million rounds of replication daily, resulting in the rapid generation of viral progeny carrying every possible mutation throughout the viral genome [6, 7]. Because of this underlying diversity, it is estimated that

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drug resistance mutations are likely to be present during chronic infection even in the absence of drug exposure, with the frequency of the mutations dependent on their fitness costs [8]. This situation allows HIV-1 drug resistance to emerge rapidly in patients who are on antiretroviral therapy that is not adequately suppressive or during episodes of treatment interruption.

Minority HIV-1 drug resistance mutations can be detected by a number of ultrasensitive assays. The characteristics of the most commonly used assays are compared in Table 1. These assays can generally be categorized as point-mutation assays (eg, allele-specific PCR [ASPCR] and oligonucleotide ligation assay [OLA]) or deep-sequencing techniques. Although ASPCR is a highly sensitive assay with a limit of detection of much less than 1% of the viral population, this technique is limited by the select number of resistance mutations that can be interrogated concurrently, because the detection of each mutation requires a separate PCR reaction [9–11]. The OLA uses labeled probes that preferentially bind to either the wild-type sequence or a sequence with the mutation of interest [12]. This assay is relatively inexpensive and does not require costly equipment. However, like other point-mutation assays, the number of mutations it evaluates concurrently is limited and OLA is not quantitative. Recent advances in high-throughput sequencing have revolutionized HIV-1 sequencing and the study of HIV-1 minority variants. Unlike point-mutation assays, deep sequencing confers the benefit of evaluating an entire region of HIV-1 (eg, HIV-1 reverse transcriptase or the third variable

[V3] loop of HIV-1 envelope) and all mutations contained in that region. The most commonly used next-generation sequencing platforms are a pyrosequencing system developed by Roche/454 and a sequencing-by-synthesis system developed by Illumina. The general principle behind both of these technologies lies in the clonal amplification of millions of individual fragments of HIV-1 DNA that can then be sequenced in parallel. The Roche/454 system was the first to enter the market and has been the most popular platform for deep sequencing HIV-1 to date. As a whole, the deep sequencing field remains quite fluid due to the continual development and evolution of these and other next-generation sequencing platforms [13].

In this review, we explore the evidence behind 3 scenarios where HIV-1 minority variants have been shown to affect the risk of virologic failure and discuss the antiretroviral medications (ARVs) for which the impact of minority variants is still controversial.

NONNUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR RESISTANCE AFTER SINGLE-DOSE NEVIRAPINE EXPOSURE

The use of peripartum single-dose nevirapine (sdNVP) is an inexpensive and effective means of reducing the risk of mother-to-child transmission of HIV-1 [14]. This strategy was rapidly adopted in many developing countries, especially in Africa. However, the use of sdNVP has also been associated with the emergence of nonnucleoside reverse transcriptase

Table 1. Characteristics of Conventional Genotypic, Phenotypic, and Ultrasensitive Drug Resistance Assays

Assay	Advantages	Disadvantages	Currently in Clinical Use
Conventional genotypic (Sanger DNA sequencing)	<ul style="list-style-type: none"> Relatively inexpensive Rapid turnaround 	<ul style="list-style-type: none"> Difficult to interpret complex resistance patterns Misses minority variants 	Yes
Phenotypic	<ul style="list-style-type: none"> Avoids need to interpret complex mutational patterns Intuitive results 	<ul style="list-style-type: none"> Expensive Slow turnaround time Limited availability outside Europe or United States and Canada Less sensitive than standard genotype testing 	Yes
Ultrasensitive techniques			
Allele-specific polymerase chain reaction	<ul style="list-style-type: none"> Highly sensitive^a 	<ul style="list-style-type: none"> Detects only a few targeted resistance mutations 	No
Deep sequencing ^b	<ul style="list-style-type: none"> Moderately to highly sensitive^a Evaluates entire region of interest. 	<ul style="list-style-type: none"> Cost Need for computational support 	Yes ^c
Oligonucleotide ligation assay	<ul style="list-style-type: none"> Moderately sensitive^a Avoids expensive equipment or specialized technical expertise 	<ul style="list-style-type: none"> Only detects a few targeted resistance mutations Assay is not quantitative 	No

Adapted from [104].

^a Test sensitivity and limits of detection vary by testing site and patient-specific variables (eg, viral load, sample volume).

^b Refers to the Roche/454, Illumina, and other next-generation sequencing platforms.

^c The Roche/454 deep-sequencing system is currently used as part of a coreceptor tropism test developed by Quest Diagnostics (Madison, New Jersey).

inhibitor (NNRTI) resistance, detected by standard genotyping, in a significant subset of women and infants exposed to sdNVP [15–17]. Even more concerning was the finding that with the use of more sensitive assays, the majority of women and children who had received sdNVP harbored NNRTI resistance mutations, often at frequencies below the detection of standard genotyping [18–25]. The proportion of NNRTI-resistant minority variants decays over time, but they could still be detected in both women and infants a year or more after sdNVP exposure [23–27]. Three factors help explain these findings: (1) high-level resistance to the most commonly used NNRTIs can be conferred by a single mutation; (2) a long elimination half-life resulting in prolonged exposure to sub-therapeutic drug levels [28]; and (3) the relatively small negative impact on viral replication kinetics (fitness) conferred by these NNRTI resistance mutations.

But do these minority variants increase the risk of treatment failure? The OCTANE Trial 1 was performed to determine the efficacy of a nevirapine-based regimen compared to a regimen containing ritonavir-boosted lopinavir (LPV/r) for women previously exposed to sdNVP. The study found that women who had received sdNVP are more likely to reach the primary endpoint (virologic failure or death) when assigned to nevirapine-based antiretroviral therapy than when assigned to a regimen containing LPV/r (26% vs 8%) [29]. Interestingly, this outcome was observed even in participants in whom nevirapine resistance was not detectable in pretreatment samples by standard genotyping. A subsequent analysis of this trial revealed that women without NNRTI resistance by standard genotyping, but harboring K103N or Y181C detected by an ultrasensitive ASPCR assay, had >3 times the risk of virologic failure or death compared to women without these mutations [30]. This association between the presence of NNRTI-resistant minority variants and treatment failure has also been detected in several other studies of women and children exposed to sdNVP [31–34]. These results have led to several clinical trials evaluating the use of a nucleoside reverse transcriptase inhibitor (NRTI) regimen concurrent with sdNVP to prevent HIV-1 from developing resistance as the nevirapine is metabolized. These studies have shown that treatment with a short “tail” of NRTIs significantly decreases, but does not eliminate the emergence of NNRTI resistance mutations detectable by either conventional genotyping [35, 36] or by ultrasensitive techniques [37–42].

NNRTI RESISTANCE IN cART-NAIVE and -EXPERIENCED INDIVIDUALS

The effect of drug-resistant minority variants in cART-naive patients has been studied most rigorously for patients on an NNRTI-based first-line regimen. Interpretation of these studies has been challenging due to heterogeneity in the patient

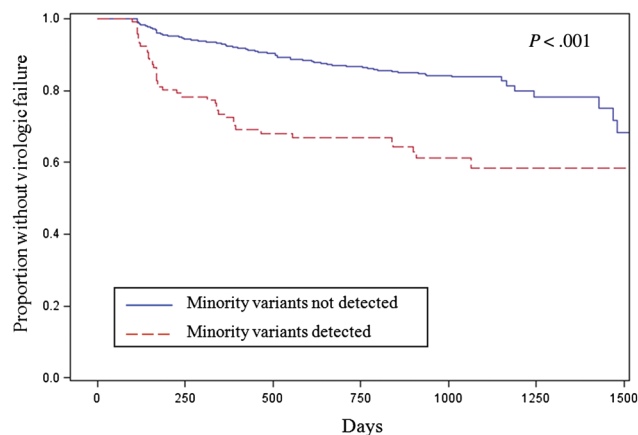


Figure 1. Kaplan-Meier curves for the proportion of patients without virologic failure on a first-line nonnucleoside reverse transcriptase inhibitor (NNRTI)-based combination antiretroviral therapy regimen by the presence of human immunodeficiency virus type 1 nucleoside reverse transcriptase inhibitor and NNRTI-resistant minority variants. Adapted from [55].

population and outcomes (ie, definition of virologic failure). To facilitate the interpretation of these studies, a pooled analysis was performed of 10 studies involving 985 participants [43–52]. This pooled analysis included only individuals with no detectable NNRTI and NRTI resistance by standard genotyping and standardized the definition of virologic failure across studies. Overall, 14% of participants were found to harbor either an NNRTI or NRTI minority variant, but this estimate varied depending on the sensitivity of the assay and the mutations tested. However, the finding was consistent with prior studies showing that ultrasensitive assays significantly increase the detection of HIV-1 drug resistance mutations in treatment-naive individuals [53, 54]. The pooled analysis also revealed that the presence of a minority HIV-1 drug resistance mutation at baseline is associated with more than twice the risk of virologic failure [55]. The increased risk of virologic failure was most evident early after therapy initiation (Figure 1) and was mediated primarily by NNRTI-resistant minority variants (hazard ratio, 2.6). To evaluate whether a threshold existed for the minority variant effect, analyses were performed categorizing participants by either the minority variant percentage or minority variant copy numbers, which takes into account both the minority variant percentage and viral load for each individual. The effect of the minority variants was dose-dependent and was detectable even after controlling for medication adherence and other potential confounding factors [55, 56]. In addition, an increased risk of treatment failure was detected even at very low minority variant frequencies (<0.5% and 10–99 mutant copies/mL).

Studies in treatment-naive African patients have also shown an association between NNRTI minority variant detection and

an increased risk of treatment failure [31, 57]. However, a recently reported analysis of OCTANE Trial 2 of treatment-naive African women did not detect a significant association between NNRTI minority variant detection and risk of treatment failure [58]. The reason behind this discrepant finding is unclear.

Ultrasensitive techniques may also be useful for detecting drug-resistant minority variants in NNRTI-experienced patients [59–62]. As in treatment-naive patients, presence of minority variants increases the risk of treatment failure [63, 64]. In addition, the use of ultrasensitive drug resistance assays in NNRTI-experienced patients frequently reveals the presence of minority variants associated with reduced efficacy of efavirenz [65, 66].

HIV-1 TROPISM

HIV-1 requires the use of either CCR5 or CXCR4 as a coreceptor for cellular entry. Maraviroc, a CCR5 antagonist that blocks HIV-1 entry, is approved by the US Food and Drug Administration for treatment of HIV-1 infection. Resistance to the CCR5 antagonists occurs either by adaptation of HIV-1 to use the drug-bound receptor or through the use of the CXCR4 coreceptor. Approximately 10%–15% of treatment-naive individuals and 50% of treatment-experienced individuals harbor virus that can use CXCR4 [67, 68]. To avoid prescribing maraviroc to patients harboring CXCR4-using variants, coreceptor usage of a patient's virus must first be determined by a genotypic or phenotypic tropism test. Historically, the phenotypic assay (Trofile, Monogram Biosciences) has been the preferred assay in the United States whereas the genotypic assay (by population sequencing of the V3 region of the HIV-1 envelope) has been used predominantly outside the United States. Despite tropism testing prior to the use of CCR5 antagonists, virologic failure is frequently accompanied by evidence of CXCR4-using virus. Using both cloning and deep sequencing, a number of studies have shown that virologic failure of a CCR5 antagonist can be caused by the outgrowth of a preexisting minority population of CXCR4-using HIV-1 not detected by current tropism assays [69–73]. In a retrospective analysis of 2 phase 3 clinic trials of maraviroc in treatment-experienced patients, the use of 454 deep sequencing was found to be a potentially better predictor of maraviroc response than the original Trofile phenotypic test [74]. Ultradeep sequencing also performed as well as an improved version of the Trofile assay in predicting the response to maraviroc [75]. A limitation of that analysis is that all the patients who received maraviroc were prescreened by the original Trofile assay. Thus, it was not possible to estimate the true sensitivity and specificity of deep sequencing for predicting treatment response to maraviroc.

A genotypic tropism assay that includes initial population sequencing with deep sequencing of samples that show only

R5-tropic virus by population sequencing is now available commercially in the United States (HIV-1 Coreceptor Tropism with Reflex to Ultradeep Sequencing, Quest Diagnostics). This Reflex assay was compared to both population sequencing alone and the enhanced sensitivity Trofile test in the retrospective analysis of 327 treatment-experienced patients who received maraviroc in the MOTIVATE and A4001029 studies [76]. The assay was found to have nearly identical ability to predict week 8 and 24 viral load declines and similar positive and negative predictive values when compared to the standard phenotypic assay. Population sequencing alone had significantly lower positive and negative predictive values compared to either the Reflex or phenotypic assay. These results represent the first commercial application of HIV-1 deep sequencing for the detection of HIV-1 minority variants. The use of deep sequencing as part of a genotypic tropism test has additional advantages as it is currently more cost-effective and faster to perform than phenotypic tropism testing.

CONTROVERSIES

Nucleoside Reverse Transcriptase Inhibitors

In the pooled analysis of treatment-naive patients initiating an NNRTI-based regimen, those with NRTI-resistant minority variants were found to have 1.6 times the risk of treatment failure compared to those without. However, only a subset of individuals was tested for NRTI minority variants, which were mainly limited to M184V and K65R [55]. Other studies evaluating the importance of NRTI-resistant minority variants have reported conflicting results. Several small studies have shown possible associations between NRTI-resistant minority variants and virologic failure [77–79], while others have detected no association [80–82]. There is evidence though, that low-frequency NRTI mutations can be detected after virologic rebound/failure [62, 83]. Additional studies evaluating the impact of NRTI-resistant minority variants would benefit from deep sequencing to assess the full spectrum of NRTI resistance mutations as there are currently ≥ 60 NRTI resistance mutations listed in the Stanford HIV Drug Resistance Database.

Integrase Inhibitors

Resistance to the integrase strand-transfer inhibitors (INSTI) raltegravir and elvitegravir share a number of characteristics with NNRTI resistance that may increase the likelihood that drug-resistant minority variants could contribute to the risk of virologic failure. First, these INSTIs have relatively low barriers to resistance and single amino acid changes are sufficient to confer a substantial decrease in antiretroviral activity. Integrase inhibitor resistance is usually selected more rapidly during *in vitro* passage experiments than is resistance to NRTIs or protease inhibitors (PIs). As with NNRTIs, clinical failure of

raltegravir and elvitegravir is frequently accompanied by genotypic evidence of INSTI resistance [84]. One report documents a case of virologic failure and emergence of raltegravir resistance in a patient with preexisting raltegravir-resistant minority variants [85]. The proportion of patients with low-frequency drug resistance mutations is also an important component in determining to what degree these minority variants contribute to the risk of treatment failure. In one study of 32 patients, 81% were found to have Q148R minority variants before exposure to integrase inhibitors [86]. In a retrospective case-control analysis of the BENCHMRK-2 study, 46% of those with treatment failure arm as compared to 31% with treatment success were found to harbor at least 1 primary or secondary minority raltegravir resistance mutation prior to raltegravir treatment [87]. However, neither study showed a significant association between the presence of raltegravir-resistant minority variants and an increased risk of treatment failure. In addition, a number of other studies have not detected either significant pretreatment raltegravir-resistant minority variants or an increased risk of virologic failure [88–91]. However, those studies have been relatively small and larger studies are needed. The investigational INSTI dolutegravir appears to have a higher genetic barrier to resistance than raltegravir and elvitegravir. Its activity is less likely to be affected by minority variants containing single INSTI resistance mutations.

Protease Inhibitors

The use of more sensitive genotyping methods has significantly increased the number of PI resistance mutations detectable in pre- [47, 52, 77, 92, 93] and posttreatment failure samples [62, 94–96]. However, evidence has not yet emerged for a significant association between low-frequency PI resistance mutations and a significantly increased risk of treatment failure [52, 77]. A number of factors may contribute to this lack of association. Unlike most NNRTIs and integrase inhibitors, the majority of ritonavir-boosted PIs have a high barrier to resistance as they require multiple mutations to confer significant resistance [97, 98]. The appropriate combination of mutations is unlikely to arise *de novo* in any significant proportion in the absence of drug selective pressure. Although a single resistance mutation (I50L) confers high-level resistance to atazanavir, the significant reduction in viral fitness associated with this mutation may minimize the frequency and hence the impact of minority variants carrying this mutation [99]. It is possible that drug-resistant minority variants could play a role in PI failure in patients with transmitted resistance or in those who have previously failed PI therapy with resistance mutations that have decayed in frequency over time. However, unless multiple linked mutations are detected, it can be difficult to distinguish between transmitted drug resistance and those generated *de novo*. Because of the number of PIs in clinical use and the

variety of mutations implicated in resistance to these drugs, it will be challenging to perform a pooled analysis (as was done for the NNRTIs) to assess the clinical significance of PI-resistant minority variants.

CONCLUSIONS

The detection of low-frequency variants of HIV-1 with altered drug susceptibility has been shown to be clinically significant in 3 settings: (1) detection of NNRTI-resistant minority variants after exposure to single-dose nevirapine; (2) detection of NNRTI-resistant minority variants prior to the initiation of a first-line NNRTI-based regimen; and (3) detection of CXCR4-using variants prior to treatment with CCR5 antagonists. In addition, there is evidence that ultrasensitive techniques could be useful after virologic failure of either NNRTI- or PI-based regimens to detect resistance mutations that emerged during treatment failure, but have decayed after drug discontinuation due to reduced fitness of the resistant virus in the absence of drug. Whether the efficacy of drugs in other classes are also affected by the presence of minority variants remains controversial. Uncertainty also exists as to the best method for detecting minority variants in a clinical setting, whether a threshold exists for what constitutes a significant frequency or number of resistant variants, and the importance of evaluating resistance mutations in the HIV-1 DNA reservoir.

At present, testing for HIV-1 minority variants is not available to clinicians for patient management with the exception of testing for coreceptor usage. However, efforts are ongoing worldwide to validate and implement deep sequencing for routine HIV-1 drug resistance genotyping [57, 100–103]. When compared to current HIV-1 genotyping assays, deep sequencing not only has improved sensitivity for detecting low-frequency resistance mutations, but will likely prove to be a lower-cost method of HIV-1 resistance testing for centers with a high demand for HIV-1 genotyping. However, the increased sensitivity of these novel assays for minority variant detection is also coupled with a heightened risk of detecting resistance artifacts generated in the laboratory during the amplification or sequencing steps. It is important to define conservative limits of detection for these ultrasensitive assays and to rigorously validate sequence analysis software that are to be used in clinical practice. In addition, the challenge for both clinicians and researchers alike will be in defining appropriate resistance interpretation algorithms for low-frequency variants detected by these ultrasensitive assays.

Additional research is also needed to provide clarity on the importance of minority variants outside of NNRTIs and CCR5 antagonists, and to define the frequency or copy number of minority variants that should prompt selection of an alternative regimen. For the time being, clinicians should be aware of these

novel technologies and the clinical implications of HIV-1 minority variants, especially for those who have received sdNVP or who are initiating a CCR5 antagonist.

Notes

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