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Low-Abundance Drug-Resistant HIV-1 Variants: Finding Significance in an Era of Abundant Diagnostic and Therapeutic Options

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Several prospective and retrospective studies have shown that genotypic testing for drug-resistant HIV-1 variants has helped health-care providers better understand patient responses to antiretroviral therapy (ART) and make wiser choices for both initial and salvage therapy. The standard approach to genotypic resistance testing begins with plasma HIV-1 RNA extraction, reverse transcription, and polymerase chain reaction (PCR) amplification, and it ends with the direct sequencing of PCR products by use of the standard dideoxynucleotide terminator sequencing method developed by Sanger 30 years ago. The high mutation rate of HIV-1 and the complex population genetics of HIV-1 in infected patients complicate the interpretation of standard genotypic tests for resistant variants because these tests are generally unable to detect minority or low-abundance drug-resistant mutations (DRMs) that are present at levels <20% of the virus quasispecies in a clinical sample.

Until recently, 2 main approaches have been used to detect low-abundance drug-resistant HIV-1 variants: point mutation assays and clonal sequencing. Point mutation assays depend on the differential hybridization of oligonucleotide probes to the wild type and mutant variants at a drug-resistance mutation position. Point mutation assays may depend entirely on differential hybridization, or they may be followed by a ligation step to improve specificity and/or by PCR to improve sensitivity [1–5]. Molecular and limiting-dilution clonal sequencing processes use the standard Sanger sequencing method to sequence multiple virus variants from a plasma sample [6].

During the past 4 years, several new high-throughput sequencing technologies have been developed. The first one to become commercially available, developed by 454 Life Sciences (now owned by Roche Diagnostics), is based on the massive parallelization of pyrosequencing in picoliter-sized wells [7]. One application of this technology is the sequencing of many individual DNA molecules in a complex mixture of genetic populations, a process called ultra-deep pyrosequencing (UDPS).

In patients who have received ART, the presence of low-abundance DRMs before a change in therapy often results in virologic failure for regimens that do not contain antiretroviral

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drugs to which the low-abundance DRMs are susceptible. For example, women who have received single-dose nevirapine to prevent mother-to-child HIV-1 transmission are at increased risk of virologic failure as a result of the replication of low-abundance nevirapine-resistant variants when treated with a subsequent nevirapine-containing regimen [2, 8]. Similarly, there is a higher than expected level of clinical cross-resistance when patients change from one antiretroviral drug to another of the same drug class, as a result of low-abundance DRMs present at the time of the first virologic failure causing cross-resistance to the antiretroviral drugs used in the second regimen [9–11].

Fewer studies have examined the prevalence and clinical significance of low-abundance DRMs in ART-naive patients. Prior to the study by Simen et al. in this issue of the *Journal* [12], 4 research groups had reported the frequent detection of low-abundance DRMs in ART-naive patients [5, 13–15]. Two of these groups also reported statistically significant associations between the low-abundance DRMs and subsequent virologic failure [5, 15]. However, only 1 study found, albeit in a small number of subjects, that the low-abundance DRMs present before the initiation of ART had emerged into majority variants at the time of virologic failure (in 4 of the 5 subjects for whom plasma samples were available) [5].

Simen et al. [12] used UDPS to assess the prevalence and clinical significance of minority variants in plasma samples from a random subset (264 subjects) of the Terry Beirn Community Programs for Clinical Research on AIDS 058 Flexible Initial Retrovirus Suppressive Therapies (FIRST) Study. The FIRST study compared the following 3 treatment strategies in 1397 previously untreated US patients who began ART in the years 1999–2002: a combination of nonnucleoside reverse-transcriptase inhibitors (NNRTI) and nucleoside reverse-transcriptase inhibitors (NRTI), a combination of protease inhibitors (PI) and NRTI, and a combination of NNRTI, PI, and NRTI. [16]. UDPS was performed on samples with plasma HIV-1 RNA levels >170 IU/μL (~100,000 RNA copies/mL) to ensure that a the 140 μL of available plasma would yield a sufficient number of viral genomes to allow the detection of low-abundance DRMs, which were present at levels as low as 1%–3% of the viral population.

Simen et al. [12] reported that standard genotypic testing for resistant variants and UDPS detected DRMs in samples from 14% and 28% of subjects, respectively. Of the 84 subjects in the subset who were randomized to the NNRTI and NRTI regimen, all 11 subjects whose plasma sample contained an NNRTI-resistance mutation (including 7 for whom the mutation was detectable only by UDPS) experienced virologic failure. The rate of virologic failure was higher for the 11 subjects who had an NNRTI-resistance mutation identified (91.6 episodes per 100 person-years), compared with subjects who did not have an NNRTI-resistance mutation (28.8 episodes per 100 person-years), with a hazard ratio of 2.73 (P = .007) adjusted for plasma HIV-1 RNA level, CD4 cell count, and a history of an AIDS-defining event. Compared to those who had no NNRTI-resistance mutation identified, the 7 patients who had mutations detectable only by UDPS had an unadjusted hazard ratio of 2.41 (P = .03).

Simen et al. [12] do not indicate whether the low-abundance DRMs present before the start of therapy became dominant at the time of virologic failure. The presence of this follow-up

information would have provided evidence that the low-abundance DRMs were directly responsible for virologic failure. The absence of such evidence, however, does not eliminate the possibility that the low-abundance variants created a replication foothold from which more highly resistant variants eventually emerged.

Simen et al. [12] hypothesize that the low-abundance DRMs may be naturally occurring, low-level quasispecies background mutations [17] or transmitted resistant variants that receded to low levels in the absence of selective drug pressure [18]. The mutations they found indicate that both possibilities are plausible. The 3 most common low-abundance NNRTI-resistance mutations were K103R, V108I, and V179D. These mutations are polymorphisms that occur as dominant variants in about 1%–2% of untreated infected individuals, which is most consistent with their occurring as low-level quasispecies background mutations. These 3 mutations cause only low levels of NNRTI resistance, making it likely that if they contributed to virologic failure, they did so by way of providing a replication foothold. In contrast, several of the other mutations detected only by UDPS, such as K103N, Y181C, and G190A/E, are nonpolymorphic mutations that cause high levels of NNRTI resistance. These mutations may have been more likely to be the result of transmitted resistance and to have directly contributed to virologic failure.

What are the implications of this study for HIV-1 drug resistance research? UDPS is performed by using a standardized, all-purpose sequencing platform that is being increasingly adopted for HIV research [19–23]. It is an exciting new method for obtaining a comprehensive picture of evolving HIV-1 drug resistance in patients and is well suited for testing large numbers of samples in a clinical trial or well-characterized patient cohort. For these purposes, it has a number of advantages when compared with the alternative approaches of point mutation assays and clonal sequencing.

The sensitivity of UDPS for detecting minority HIV-1 variants is not limited by the 454 Life Sciences sequencing technology, but rather by the number of virus templates that can be successfully extracted and amplified from a plasma sample. This limitation—which exists for all methods of detecting minor HIV-1 variants—explains why the authors confined their use of UDPS to those samples with the highest plasma HIV-1 RNA levels.

The specificity of UDPS depends on the number of mismatch errors generated during the processes of PCR amplification and pyrosequencing. The reported mismatch error rate of 0.1% and the approximately random distribution of errors mean that its positive predictive value is high for minority variants present at a level of ≥1% [19, 21]. The use of PCR enzymes with increased fidelity can improve sensitivity by improving the reliability of variants detected at levels <1%, but this comes at the cost of decreased amplification efficiency.

In contrast to UDPS, point mutation assays must be individually optimized for each mutation they are designed to detect and are at risk for false-negative and false-positive results caused by primer binding site variability. Their low cost provides a rationale for their use in epidemiological studies in which information about only the most common DRMs may be required. Their platform independence also provides an advantage in smaller

research laboratories. However, the large number of different point mutation assays may make it difficult to compare the results obtained in different laboratories.

The main disadvantage of clonal sequencing is that it is labor-intensive, because 50–100 clones must be sequenced to obtain a sensitivity of 1%–2%. Although molecular clonal sequencing is less labor-intensive than limiting-dilution clonal sequencing, molecular clonal sequencing is at a higher risk for erroneous results due to PCR errors and for biased estimates of the proportions of low-abundance DRMs.

What are the implications of this study for HIV-1 drug-resistance testing in clinical settings? An assessment of new technologies for detecting low-abundance DRMs should begin with retrospective studies of samples obtained prior to initiation of therapy from ART-experienced and ART-naive patients who have well-defined virologic outcomes. These studies should assess the prevalence of low-abundance DRMs in a wider variety of clinical situations and validate their clinical significance in patients who are receiving the highly efficacious treatment regimens currently being used [24].

Improvements in ART have gone hand-in-hand with improvements in diagnostic testing. From the initial use of virus load and genotypic resistance testing to the development of phenotypic resistance testing and virus tropism assays, HIV care providers have a sophisticated array of tools to help them use antiretroviral drugs optimally. The recent improvements in therapy brought about by existing diagnostic tests and by the introduction of new antiretroviral drugs inevitably make it challenging to show that a new technology is beneficial, let alone cost-effective. Nonetheless, we should greet this challenge with enthusiasm because with it will lead to insights into HIV-1 infection therapy that were not previously possible.

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