Primary HIV-1 Drug-Resistant Minority Variants

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Abstract

Primary HIV drug-resistant mutations are mutations that occur in an HIV-infected individual prior to the initiation of antiretroviral therapy. These mutations may arise by de novo mutagenesis or result from transmission. Drug-resistant mutations may reduce the effectiveness of antiretroviral therapy, leading to inadequate virological outcomes. Currently, Sanger sequencing is the standard method for detection of drug-resistant mutations to inform treatment decisions, but it does not detect minor variant mutations. Drug-resistant minority variants can be detected by next generation sequencing. However, several challenges, including cost of infrastructure and the need for complex data analysis bioinformatics tools, remain major setbacks for next generation sequencing use. More importantly, the clinical impact of drug-resistant minority variants on antiretroviral therapy is not well understood, underscoring the importance for understanding whether the levels of primary drug-resistant minority variants for different mutations impact on the effectiveness of antiretroviral therapy and the rationale for inclusion in routine diagnostics. Understanding the impact of primary drug-resistant minority variants will help inform how next generation sequencing may be utilized in the future for pre-emptive clinical antiretroviral therapy decision making. (AIDS Rev. 2017;19:89-96)

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Key words

Primary drug resistance. Minority variant. Next generation sequencing. Antiretroviral therapy.

Introduction

Drug-resistant mutations (DRM) are a major obstacle to effective suppression of HIV by antiretroviral therapy (ART), which is the life-long use of at least three anti-retroviral (ARV) drugs from two drug classes in HIV treatment[1]. Drug resistance mutations in ART-naive patients, defined as primary drug resistance, can develop spontaneously by *de novo* mutagenesis or may result from transmitted resistance[2]. International guidelines recommend genotypic drug resistance testing before initiating ART to optimize treatment outcomes[3]. Genotyping relevant viral genes, such as the HIV pol gene or the HIV-1 envelope V3 loop, offers a cost-effective strategy[4] to customize effective, individualized drug regimens for use at ART initiation[5-7]. Curbing adverse consequences conferred by resistance, such as high programmatic costs, complex ART regimens, and multiple variable dosing strategies, will be major milestones toward achieving part of the 90-90-90 goals set

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by the World Health Organization (WHO), i.e. virological suppression in 90% of patients on ART[8].

Standard genotyping using Sanger sequencing does not reliably detect mutations at less than 15-25% of the viral quasispecies, and will therefore miss virus present at lower frequencies (i.e. < 20%)[9]. Recent technologies such as next generation sequencing (NGS) and point mutation assays such as allele-specific PCR (AS-PCR) are faster, cheaper, and can detect drug-resistant minority variants (DRMV). While point mutation assays detect specific targeted mutations at any given time, the inability of the assays to simultaneously detect multiple mutations limits their use in clinical practice.

On the other hand, NGS technologies, such as the 454-Pyrosequencing (GS FLX and GS Junior, Roche), MiSeq HiScan SQ system (Illumina), PacBio RS II (Pacific Biosciences), and Ion Torrent PGM (Life Technologies)[10], are effective in detecting multiple mutations in as low as 1% of the viral population[11]. The NGS also produces high-throughput data at relatively low costs per base[12]. However, the effect of primary HIV DRMVs in achieving virological suppression or informing patient clinical outcomes has not yet been fully elucidated. This review focuses on primary DRMVs, their clinical impact, challenges in their detection, and inclusion in routine diagnostics.

Drug-resistant minority variants in antiretroviral-naive patients

Primary DRMVs are mutations that occur as low-frequency viral variants in pre-ART patients that are not detected by Sanger sequencing[9]. In the absence of drug pressure, resistant viral variants are often outcompeted by wild-type viruses, which have better replicative capacity, making detection of resistant viral variants in treatment-naive patients less likely using Sanger sequencing[13]. However, NGS platforms are able to detect very low frequency viral variants, permitting the detection of these DRMVs in the absence of drug pressure and making these platforms an appealing tool for this application. Despite the enhanced sensitivity, the more important question pertains to the clinical impact of DRMVs and hence the clinical utility of their detection.

Clinical impact of primary drug-resistant minority variants

There is currently limited and varying evidence on the clinical impact of primary DRMVs on treatment outcomes. Much of what we understand about the clinical impact of DRMVs has come from nonnucleoside reverse transcriptase inhibitor (NNRTI)-based studies[7,14-22]. A pooled analysis by Li, et al. showed that there is more than double the risk of developing early virological failure on NNRTI-based regimens in patients with underlying NNRTI DRMVs. Additionally, a dose-dependent effect for the increased risk of treatment failure was also found in patients with higher DRMV copies per milliliter of plasma[22].

A cross-sectional study done in Spain amongst newly diagnosed HIV infected patients categorized DRMVs by frequencies at 1, 5, and 10% thresholds[23]. Using the GS-Junior (Roche-454), NGS showed a predictive increase in resistance to all drug classes (nucleoside reverse transcriptase inhibitors [NRTI], NNRTIs and protease inhibitors [PI]) when mutations were analyzed at 1% threshold[23]. In a case-control study by Johnson, et al., 2008, using AS-PCR to detect protease (PR) and reverse transcriptase (RT) mutations in treatment-naive patients, an increased risk of virological failure was seen when patients initiated efavirenz (EFV)-based treatment in the presence of primary DRMVs, compared to patients with no primary DRMVs (p = 0.0038)[17]. On the contrary, the STaR study did not show an association between mutations emerging during EFV and rilpivirine (RPV)-based treatment with DRMVs at baseline[24].

There is a paucity of data on rates of primary DRMVs in sub-Saharan Africa. In a clinical research cohort of treatment-naive patients in Ethiopia, NNRTI DRMVs were reported at 5.4% using AS-PCR that targeted K103N and Y181C mutations[25]. A cross-sectional study to determine DRMVs in Zambia reported a higher frequency of NRTI DRMVs (7/10) than NNRTI DRMVs (1/10) using 454 Pyrosequencing[26] in patients with no prior exposure to ART. However, the clinical impact of these DRMVs was not assessed.

A prospective study done in Cameroon in patients on first-line treatment showed incomplete adherence as a strong predictor of virological failure rather than primary DRMVs[27]. Interestingly, an ART cohort study in Malawi, investigating the effects of DRMVs in proviral DNA, reported 2/5 patients having DRMVs prior to treatment initiation[28]. However, the primary proviral DNA DRMVs were not associated with early emergence of DRMs on ART. Such conflicting findings warrant the need for continual research on the clinical impact of primary DRMVs in different settings.

Primary drug-resistant minority variants and prior antiretroviral drug exposure

Use of single and dual ARV drugs for prevention of mother-to-child transmission (PMTCT) of HIV poses a risk of ART drug resistance acquisition, thereby compromising future ART options[29,30]. The WHO PMTCT strategies of Option A, Option B, and Option B+ have been widely used for PMTCT in resource-limited settings over recent years[10]. Options A and B recommend the mother discontinue ARV drugs *post partum* until eligible to initiate ART, whilst Option B+ involves initiating life-long ART in recently diagnosed patients[10].

Exposure to ARV drugs for PMTCT (Option A and B) could select for mutations that persist for long periods of time[31]. This includes mutations such as K103N and Y181C, which are often associated with inadequate treatment outcomes and virological failure on first-line NNRTI-based regimens[14,32]. A study by Boltz, et al. following the OCTANE Trial 1 showed a higher risk of developing virological failure in women with baseline K103N and Y181C minority variants when initiated on a nevirapine (NVP)-based regimen with prior single dose (sd) NVP exposure[19]. High levels of primary NRTI and NNRTI DRMVs (70%) were also detected using AS-PCR in Tanzanian women who received a complex PMTCT prophylaxis[33].

A study conducted in Soweto, South Africa, using AS-PCR (K103N and Y181C) showed persistence of NVP resistance following exposure to sd NVP for PMTCT[31]. The study demonstrated presence of NNRTI DRMVs in 16 of 21 (76%) women, a year after sd NVP exposure. Similarly, a study in Johannesburg amongst women with and without prior exposure to sd NVP showed persistence of the K103N minority variant, and suggested the mutation could be a strong predictor of inadequate viral suppression and viral rebound[14]. With the maturing ARV program in sub-Saharan countries, there is need for more studies pertaining to the clinical relevance of these minority NNRTI-resistant variants, especially when patients initiate NNRTI-containing ART.

Post-exposure prophylaxis (PEP) involves use of ARV drugs following potential exposure to HIV to prevent infection. Pre-exposure prophylaxis (PrEP) involves use of ARV drugs before HIV exposure to lower the chances of infection. The WHO 2015 guidelines recommend offering PrEP not only to high-risk groups, but also to people at substantial risk of HIV infection (i.e. HIV incidence > 3/100 person-years) as part of HIV prevention strategies[34]. Both PrEP and PEP could result in the development of DRMs[35,36], but the evidence for the benefits of PrEP and PEP outweigh the risk of pre-ART resistance[36-38]. Mutations due to PrEP and PEP often decay rapidly[39] and could be missed by Sanger sequencing, underscoring the need for genotyping patients with a history of intermittent ARV exposure using NGS before initiating life-long ART.

Antiretroviral treatment and primary drug-resistant minority variants

The WHO recommends the use of two NRTIs and an NNRTI, as first-line regimens in adults[34]. Pre-ART DRMs can reduce the effectiveness of these ARVs in achieving viral suppression. However, there is little known about the frequencies of primary DRMVs in each ARV class.

Nucleoside reverse transcriptase inhibitors and primary drug-resistant minority variants

Nucleoside reverse transcriptase inhibitors inhibit viral replication by viral reverse transcription chain termination[40]. Most recommended first-line regimens and PrEP contain tenofovir (TDF)[34]. Minority TDF DRMs may negatively affect TDF-based ART. The most common mutation causing resistance to TDF is K65R[41]. Kozal, et al. in 2011 showed 4/411 (0.97%) treatment-naive patients had the K65R minority variants detected at > 1% by ultra-deep sequencing, with two of the four experiencing virological failure[42].

The prevalence of primary K65R minority variants in South Africa is 4%. A study amongst patients failing first-line TDF-based ART in South Africa found a high rate of TDF resistance (~ 60%)[43]. Thus, the potential for transmission of TDF resistance does exist, which may impact negatively on the effectiveness of PrEP. More studies that investigate the clinical impact of minority K65R variants are required.

In a German ART-naive cohort, the K65R minority variants occurred in 2.7% of chronically infected patients, but did not affect treatment outcome[44]. However, a case of an Eritrean immigrant showed the risk of early treatment failure associated with the K65R minority variants[45]. Determining prevalence of the K65R mutation in ART-naive patients may help inform the choice of first-line NRTI-based treatment[46]. This warrants the need for surveillance of TDF resistance in ART-naive patients, as TDF-associated mutations, most commonly the K65R mutation, could reduce the effectiveness of TDF in first-line ART and/or in its use for PrEP.

M184V is the main mutation that confers resistance to lamivudine and emtricitabine[47]. The mutation is known to reduce viral replicative capacity and increase viral susceptibility to stavudine, zidovudine, and TDF[48]. M184V is rarely detected in treatment-naive patients by Sanger sequencing, but is rapidly selected for under drug pressure[49], suggesting presence of M184V minority variants in ART-naive patients before initiating treatment[50]. The M184V minority variants are more common in recently and acutely infected patients than in chronically infected patients prior to ART initiation[2], suggesting rapid decay of the M184V minority variants due to the lower replicative capacity associated with the mutation[2]. There is, however, need for more evidence-based studies on the frequencies of such mutations in ART-naive patients and their implications on the currently recommended NRTIs.

Nonnucleoside reverse transcriptase inhibitors and primary drug-resistant minority variants

Nonnucleoside reverse transcriptase inhibitors inhibit viral replication by interacting with a non-active site of the viral reverse transcriptase[40]. The NNRTIs have a low genetic barrier to resistance[51]. This increases the chances of virological failure in patients with primary NNRTI DRMVs who initiate EFV- and NVP-based treatment[22]. The most common mutations associated with NNRTI resistance are K103N and Y181C[52].

High levels of NNRTI DRMVs in ART-naive patients have also been reported following use of sd NVP for PMTCT[14,31], with K103N and Y181C minority variants increasing the risk of virological failure on NNRTI-based treatment[16,20,21,53]. Coovadia, et al. in 2009 showed that the minority K103N mutation was associated with reduced virological response on NNRTI-based treatment[14]. The continued use of NNRTIs for PMTCT, the high level of transmitted NNRTI drug resistance[54], and the increasing evidence for the negative clinical impact of NNRTI DRMVs underscore the need for further studies describing primary NNRTI DRMVs.

Next generation sequencing platforms

Various NGS platforms are being used for detecting DRMVs. The NGS platforms vary in the sequencing coverage generated. Sequencing coverage is the average number of times a base is read from individual high-quality fragments across a genome during a sequencing run. It is calculated as $C = LN/G$ where C is the coverage, L is the length of the reads, N is the number of reads, and G is the length of the genome[55]. The more times a base is read from individual fragments, the higher the sensitivity and confidence in calling minor variant mutations. Illumina platforms generate relatively more sequence reads, which increases the sequencing depth obtained, with PacBio generating the least number of reads per unit[56]. Table 1 compares NGS platforms, showing the advantages and disadvantages of each. Instrument costs, data throughput, and run times have been shown as ranges to represent the different generations of equipment under each product line, which cater for different user preferences.

Challenges in next generation sequencing implementation

The NGS platforms have revolutionized genomic medicine by producing large amounts of data, with high depths of coverage, at lower sequencing costs, and in relatively shorter periods of time[57]. However, there are several challenges limiting use of NGS in routine clinical diagnostics. The analysis of NGS data is challenging and time consuming, partly due to the large amounts of data generated and high error rates, complicating its use in clinical decision-making compared to the current gold standard, i.e. Sanger sequencing (Table 2).

Automation of the complex steps is making NGS relatively simpler and faster to perform. However, such automated equipment is costly to acquire. Although batching and multiplexing of samples has helped reduce the cost of NGS, the technical expertise and bioinformatics tools required for data analysis, such as alignment and phylogenetics of multiple shorts reads, remain a challenge. To produce high-quality data there is need to separate actual sequence variants from background noise or contamination[58]. The ability to detect the low-level variants increases the chances of detecting carryover contamination, affecting data accuracy, and complicating the interpretation of data for patient management[12,59].

The large amounts of data ("data deluge") generated through NGS create challenges for data storage and ease of accessibility on public databases. There is need for uninterrupted high-performance computing systems that have the ability to store and process large data sets in a timely manner[60]. In resource-limited settings, NGS

Table 1. Comparison of next generation sequencing platforms

Platform (manufacturer)	454, GS FLX and GS Junior (Roche)	SOLID (LifeTechnologies)	MiSeq HiScan SQ system (Illumina)	Ion Torrent PGM (LifeTechnologies)	PacBio RS II (PacificBiosciences)
First introduced[64]	2005	2006	2007	2010	2011
Chemistry[64]	Pyrosequencing	Ligation-based sequencing	Sequencing by synthesis	Ion semiconductor sequencing	Real-time sequencing
Output/day[65]	0.04-0.7 Gb	8-24 Gb	5.5-600 Gb	0.2-64 Gb	2-20 Gb
Cost/Mb	$10[66]	$0.13[66]	$0.05-0.15[66]	$1.00[66]	$7[67]
Average accuracy	99.997%[68]	99.99%[68]	99.9%[67]	> 99.0%[68]	99.999%[65,69]
Quality score	> Q30[66]	> Q30[66]	> Q30[66,70]	> Q20[70]	> 50[69]
Most frequent error[71]	Deletions	A-T bias	Single nucleotide substitutions	Short deletions	CG deletions
Cost per instrument[65]	$125,000-500,000	$125,000-500,000	$125,000-1,000,000	$50,000-149,000	$350,000-700,000
Reads per unit[56]	20,000-700,000	81,500,000-266,666,667	1,000,000-400,000,000	400,000-60,000,000	22,000-47,000
Advantages	Long read length (400-700 bp)[64,68] Short run time[68]	Low error rate[68] Low reagent costs[68] High throughput[68] High sensitivity for detecting MVs	Low error rate[68] Low reagent costs[68] High throughput[68] High sensitivity for detecting MVs	Medium read lengths (200 bp) Short run time[68] Low instrument cost[66]	Long read lengths[68] Short run time[64] Simple sample preparation[68]
Disadvantages	Homopolymer errors[68] High costs of reagents[68] High cost per base and low throughput[66]	Short reads[68] Long run time[68] Palindromic sequence errors[66]	Short read lengths[68] Long run times	Longer hands-on time[68] High costs of reagents[68] Homopolymer errors[66,68]	High instrument cost[68] No paired end reads[68] Low sensitivity for detecting MVs

Gb: gigabases; Mb: megabases (equivalent to a million bases); MV: minority variants.

Table 2. Comparison of Sanger sequencing to next generation sequencing

	Advantages	Disadvantages
Sanger sequencing[63]	Higher accuracy (99.999%) Long read lengths (400-900 bp) Simpler data analysis Low instrument costs	High sequencing cost ($2,400/Mb) Not ideal for large genome sequencing Does not reliably detect minority variants Low throughput and low depth of coverage
Next generation sequencing	Lower sequencing costs (< $10/Mb) Ideal for whole genome sequencing[72] Detects minority variants High throughput and depth of coverage Multiplexing in a single run[73]	Higher error rates[70] Short read lengths Many complex procedures required Complex data analysis[74] High instrument costs

bp: base pairs; Mb: million bases.

is limited by instrument costs (Table 1), infrastructural requirements, expertise, and sophisticated data analysis tools required, resulting in most studies using AS-PCR to detect low-frequency mutations in ART-naive patients. Also, generation of high-quality genotypic results using a point-of-care approach remains a major challenge in HIV drug resistance testing (including Sanger sequencing). With the WHO recommendation on initiating ART in all recently diagnosed patients, irrespective of their CD4 counts[34], shorter NGS turn-around times are required if it is to become a useful tool for making decisions at ART initiation.

Current knowledge gaps in HIV resistance

A good starting point would be to adequately estimate the prevalence and patterns of primary resistance in resource-limited settings. Few prospective studies have determined the clinical impact of primary DRMVs. Moreover, use of point mutation assays, such as AS-PCR and oligonucleotide ligation assay, detects targeted mutations, thus missing several other DRMVs that could impact treatment. It would be important to determine the cumulative effect of the primary DRMVs detected by NGS and the clinical impact of these on ART rather than analyzing the mutations in isolation.

Sanger sequencing has been used for the past two decades to make clinical decisions on use of ART[12]. However, NGS is being used for routine clinical work in some settings[61,62], slowly replacing Sanger sequencing, which has higher sequencing costs (Table 2)[63]. Also, NGS has the advantage of whole genome

sequencing of HIV in single runs, allowing for more informed analyses of virus evolution. Unlike Sanger sequencing, NGS sequencing can detect HIV mutations at 1% and lower[12].

However, the effect of these low-level resistant mutations is still not well understood, compounded by the glaring gap that exists in the bioinformatics tools available to analyze such results. There is need to develop algorithms that are consistent and can be standardized in providing useful clinical information, such as the Stanford HIVdb mutation scoring algorithm (http://hivdb.stanford.edu) used in analyzing Sanger sequencing results. This entails determining cutoff values for each DRMV that might subsequently reduce the effectiveness of ART, as well as the cumulative effect of the mutations when they occur simultaneously. It is also important to understand whether the differences in the mutation frequencies, treatment adherence, as well as mutational loads result in different treatment outcomes.

Conclusion

Despite evidence for the presence of DRMVs detected in ART-naive patients for about a decade now, there still remains a knowledge gap on the clinical impact of DRMVs. It is plausible that primary DRMVs would outgrow wild-type virus under drug pressure to become the dominant viral population, leading to virological failure, as shown with NNRTIs[18]. Due to the complexities with DRMVs, viral evolution, the dynamic field of NGS, and the limited studies, there is a need for further in-depth research on primary DRMVs.

Acknowledgements

Declaration of interest

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