

Clonal Analysis of Week 12 Virologic Non-responders Receiving Tenofovir/Abacavir/Lamivudine in ESS30009

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Introduction

- Early termination of the ESS30009 tenofovir, abacavir and lamivudine treatment arm (TDF/ABC/3TC) occurred after a high incidence of virologic non-response was observed within the first 12 weeks of treatment (50/102; 49%)¹.
- By conventional population genotyping, virologic non-response at week 12 was associated with treatment-emergent resistance mutations. M184V was detected in all but one of the virologic non-responders (VNR, 40/41; 98%) however, K65R was only found in 22 of 41 (54%).
- In this study, clonal genotyping was conducted to determine the incidence of underlying treatment-emergent mutations that were not detected by population genotyping at week 12.

Methods

- Viral load determinations were by Quest. Drug susceptibility and population genotyping (GT) were by ViroLogic.
- Four subjects that did not have detectable K65R at week 12 were selected for clonal GT. We selected samples from subjects with a range of week 12 plasma HIV-1 RNA levels (6,500 - 320,000 copies/mL). The clonal GT methodologies implemented by our lab (GlaxoSmithKline Clinical Virology) were similar to those recently reported by both the Demeter and Siliciano labs^{2,3}.
- Briefly, viral RNA was extracted from 1 mL of patient plasma using a magnetic silica particle method (miniMag™, bioMérieux; Durham, NC).
- RT-PCR amplification of the viral *pol* gene and confirmatory population GT were performed using the TruGene™ HIV-1 Genotyping kit and OpenGene™ System (Bayer HealthCare; Tarrytown, NY).
- Amplicons were directly cloned from the same RT-PCR using the Zero-Blunt® TOPO PCR-cloning system (Invitrogen; Carlsbad, CA). Individual colonies grown on selective media were separately isolated, followed by PCR amplification with HIV-1 specific primers. The colony-PCR product was purified and sequenced with HIV specific primers.
- To verify the absence of PCR cross-contamination, all clonal sequences underwent phylogenetic analysis (PHYLIP; neighbor-joining method) illustrated in figure 2. Individual clones are listed with a letter corresponding to the four patients shown in figure 1 (patients A, B, C and D), followed by the clone number. Coding changes that were distinctive for individual clones are shown. Treatment emergent mutations are in bold and were defined as coding changes observed at week 16 by population GT, but not at week 12 or baseline (figure 2; patients A and B).

References

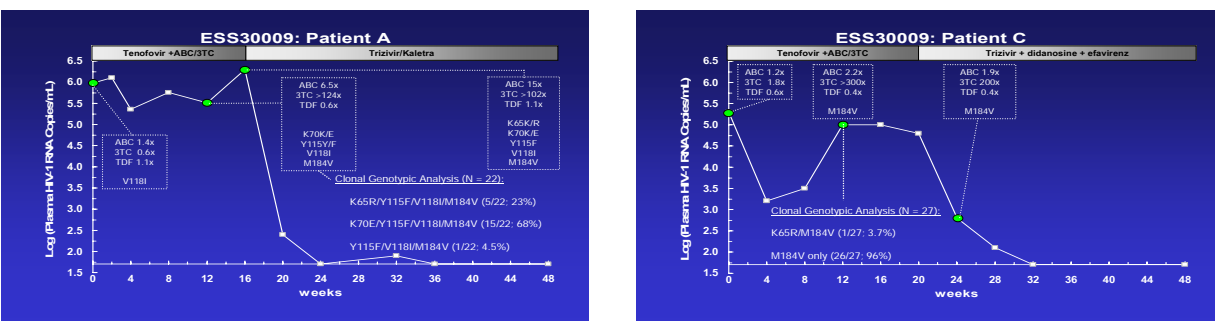
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Results

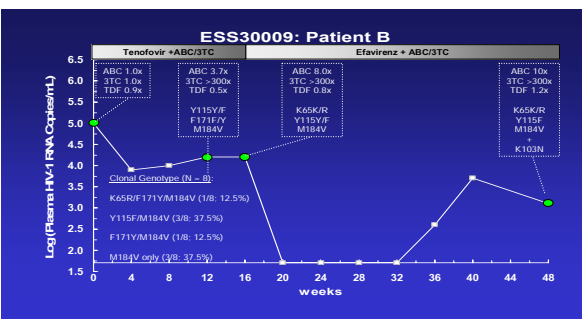
Figure 1. Longitudinal Patient Profiles of VNRs with Detectable M184V but not K65R at Week 12 by Population Genotyping
Note: Treatment Regimen (grey bars, top), population phenotype and genotype (boxed) and clonal genotypic analysis are shown.



Clonal genotyping (GT) at week 12:

- Consistent with the population GT at week 12, Y115F/V118I/M184V was detected in almost all clones (21/22; 95%). In addition, the K70E mutation was observed in 15 of 22 clones (68%).
- However, clonal GT also resulted in the detection of the K65R mutation in 5 of 22 clones (23%).
- Interestingly, K65R and K70E were not observed in the same clone and as such, no evidence of linkage between these two mutations was observed (figure 2).
- Other mutations detected by clonal but not by population GT were as follows: N54S, K66R, S68N, R72K, R72G, K103R, A114V, D123N, K154E, E169K, I195M and H198R.

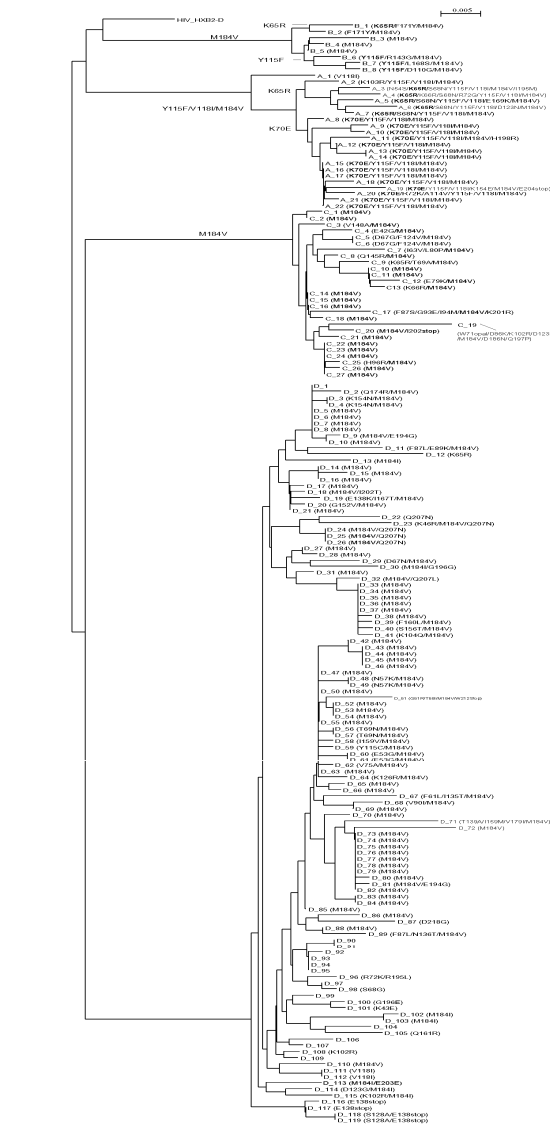
- Therapy switch to Trizivir/Kaletra was efficacious.



Clonal genotyping (GT) at week 12:

- Consistent with the population GT at week 12, all clones (8/8) had M184V. Only 3/8 had Y115F (37.5%) and 2/8 had the F171Y (25%) mutation.
- Clonal GT also resulted in the detection of the K65R mutation in 1 of 8 clones (12.5%).
- Linkage was observed between F171Y and K65R, but not with Y115F (see fig. 2).
- Other mutations detected by clonal but not by population GT were: D110G, R143G and L168S.
- Substitution of efavirenz for tenofovir resulted in the selection for K103N.

Figure 2. Phylogenetic comparison of all clones from each patient and wt HIV-(HXB2-D)



- Phylogenetic analysis did not reveal evidence for the occurrence of PCR cross-contamination.

Discussion

- Limitations in the sensitivity of standard population genotyping may result in the failure to detect underlying treatment emergent resistance mutations. In addition to the intrinsic limits of detection of these techniques, resistance testing is often conducted at points when resistant variants are just emerging.
- The purpose of our study was to determine if underlying resistance (i.e. below the sensitivity of standard population genotyping and phenotyping methods) could help explain the poor efficacy observed with TDF/ABC/3TC despite initial selection for a major population resistant to only one of three drugs.
- Two IAS-defined NRTI-resistance mutations, K65R (Figure 1; patients A-D) and V118I (patient D), were detected at the week 12 time point by clonal but not by population genotyping. In contrast to the week 12 population genotype, K65R was detected by population genotyping at week 16 (Patients A and B). Consistent with the increase in sensitivity for the genotypic detection of K65R observed between weeks 12 and 16, an increase in tenofovir and abacavir phenotypic fold-change was also detected (patients A and B). Together, these data suggest that the underlying K65R detected by clonal genotyping at week 12 was indeed treatment emergent.
- Clonal genotyping also detected atypical coding changes at codons associated with NRTI-resistance (D67G, K70E, V75A and Y151C), in addition to a treatment emergent mutation at a novel codon (F171Y).
 - D67G (patient C) has been previously associated with resistance to dOTFC⁵ *in vitro*. D67G was observed in 2 of 27 clones (7.4%) and found to be linked with F124V and M184V.
 - K70E (patient A) has been previously associated with *in vitro* and clinical resistance to adefovir^{6,7}. K70E was strongly selected for by week 12 (15/22 clones; 68%) and was almost 3-fold more prevalent than the underlying K65R observed in this patient. K70E was also detected at week 16 along with K65R by standard population genotyping. However, by clonal genotyping at week 12 virus was present with either K70E+Y115F/V118I/M184V or K65R + Y115F/V118I/M184V suggesting that both patterns conferred a selection advantage in this regimen. This may suggest that like K65R, K70E may confer a selection advantage and possibly resistance to tenofovir. It is interesting to note that at least 6 other patients have been identified from the tenofovir treatment arm with the K70E mutation (data not shown). Further work is needed to understand the impact of this mutation on tenofovir resistance.
 - V75A (patient D) and F171Y (patient B) have been detected in isolates from NRTI-experienced patients⁸.
 - Y115C (patient D) has not been previously reported⁹.

Conclusions

- Population genotyping/phenotyping may miss underlying resistance. This may be especially likely if the testing is conducted at time of virologic rebound, prior to the establishment of viral quasiespecies equilibrium.
- Previously unreported coding changes were found in one or more clones at week 12. These may be associated with non-response to this regimen and include K66R, D67G, S68N, S68G, T69A, K70E, V75A, K103R, Y115C, F171Y and V179I.
- The incidence of K65R in virologic non-responders to TDF + ABC/3TC is likely higher than the 54% originally reported. Underlying K65R was observed in 4 out of 4 subjects with no detectable K65R at week 12 by population genotyping.
- These results suggest that resistance for some agents may be present following virologic failure even in the absence of detection by standard genotyping and phenotyping.