

Poster # 650

Abstract

Treatment of HIV-1 infection with HAART can reduce viremia to below the limit of detection of “ultrasensitive” assays (50 copies of HIV-1 RNA/ml). However, a low level of virus production continues. In an initial cross-sectional study, we showed that this low-level viremia is comprised of drug-sensitive viruses lacking new resistance mutations to drugs in the HAART regimen. Here we describe longitudinal, clonal genotypic analysis of plasma virus from treated adults who had suppression of viremia to <50 copies/ml. We documented continuous production of virus in 8 HIV-1-infected adults who maintained suppression of viremia for up to 15 months. Using analytical approaches for distinguishing selected resistance mutations from non-selected mutations and PCR errors, we looked for evolution of resistance in the RT and protease genes. Despite continuous virus production, evolution was not observed. Sporadic resistance mutations were detected in some viral clones but were not selected for or fixed at subsequent times. Thus, in some patients HAART suppresses new cycles of replication to a level that does not allow the evolution of drug resistance over a time frame of years. These results provide insight into the nature of low-level viremia and illustrate complexities that will be inherent in genotypic analysis of low-level plasma virus.

Introduction

In HIV-1-infected patients who respond optimally to HAART, plasma virus levels fall below the limit of detection of “ultrasensitive” clinical assays (50 copies of HIV-1 RNA/ml) and remain there. However, even in such patients a low level of free virus can be detected in the plasma with more sensitive assays. Additionally, many patients on HAART have occasional positive plasma virus measurements, termed ‘blips’, following which virus levels fall back below 50 copies/ml. This and other evidence for continuing virus production in patients on HAART raises concerns regarding the eventual evolution of drug resistance despite apparently effective treatment. Testing for resistance mutations directly through genotyping is an obvious approach for detecting the evolution of drug-resistance. However, when viremia is below 50 copies/ml, the number of virions that can be analyzed is limited and the physiologic significance of resistance mutations found is unclear. In an initial cross-sectional study, we showed that this low-level viremia is comprised of drug-sensitive viruses lacking new resistance mutations to drugs in the HAART regimen. However, other studies provide evidence for the evolution of drug resistance on HAART. The present study was designed to determine whether drug resistance mutations could be detected in the plasma virus of patients whose HIV-1 RNA levels were below 50 copies/ml, and if so, to examine the significance of these mutations.

Methods

Patient Population. We studied asymptomatic adults with HIV-1 infection who had excellent responses to HAART with suppression of viremia to below 50 copies/ml. Patients were not excluded for isolated blips if subsequent measurements returned to below 50 copies/ml without adjustments to therapy.

HIV-1 plasma RNA quantification. Plasma HIV-1 RNA assays were performed by using the ultrasensitive Roche Amplicor Monitor System (Version 1.5, Roche Diagnostic Systems, Nutley, N.J.), which has a detection limit of 50 copies/ml.

Amplification and sequencing the pol gene from HIV+ plasma. Genotypic analysis of low levels of viremia was carried out by nested RT-PCR amplification of a segment of the *pol* gene from viral RNA isolated from pelleted plasma virions. PCR products were separated on agarose gels, purified, cloned, and sequenced.

Sequence analysis and quality control. Sequences were analyzed for known drug resistance mutations using the Los Alamos and Stanford HIV sequence databases. Initial phylogenetic analyses were performed using algorithms from PHYLIP 3.5 and implemented in NimbleTree version 2.0 (available from S.C.R. at <http://sray.med.som.jhmi.edu/>). Specifically, distance matrices were calculated using the F84 model (transition/transversion ratio 2.0), trees were inferred using the neighbor-joining algorithm, and support for clustering was assessed using bootstrap analysis.

Analysis of sporadic nonsynonymous substitutions. Sporadic substitutions (i.e. those observed in a single clone from a study subject) were classified as nonsynonymous or synonymous. The number of nonsynonymous sites in the region analyzed (protease residues 1-99 plus RT residues 1-229) was estimated from the number present in the HXB2. Nonsynonymous substitutions or sites were classified as resistance-associated.

Discussion

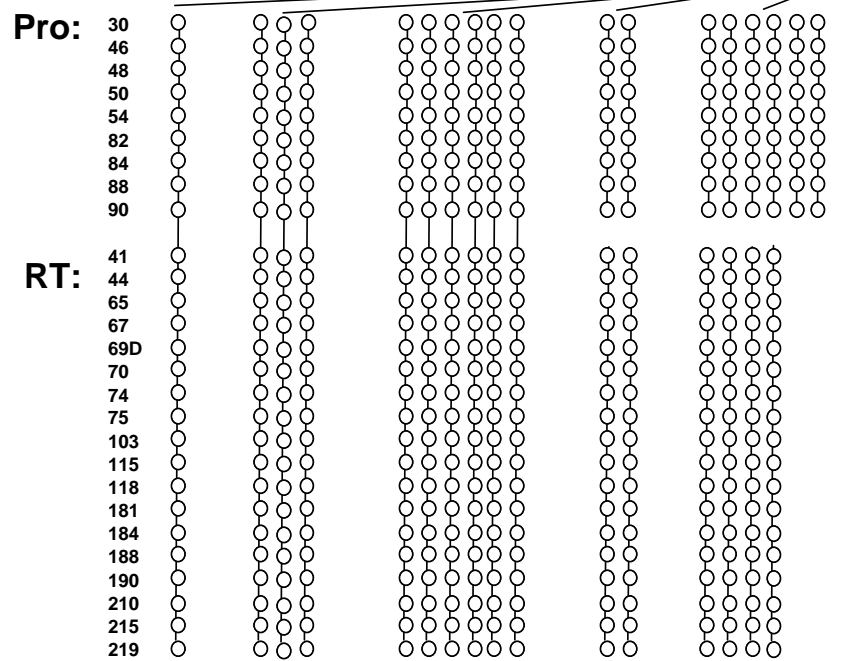
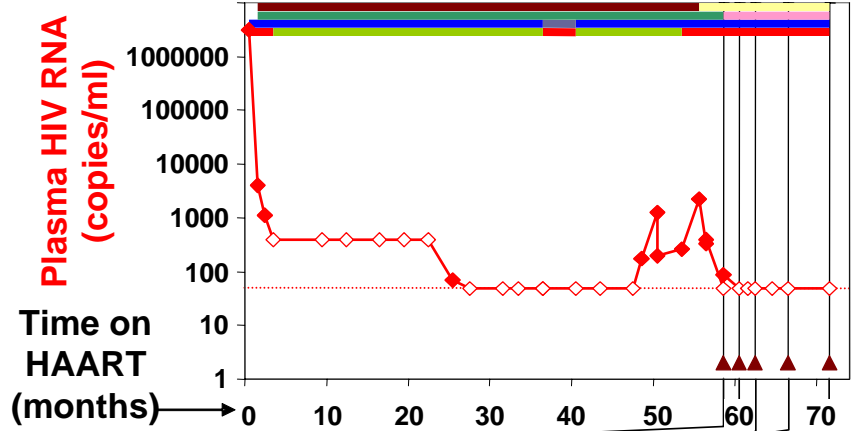
A central dilemma in the treatment of HIV-1 infection is that drug resistant variants will emerge if the drugs used do not adequately suppress viral replication. Therefore, it is important to know whether patients with plasma HIV-1 RNA levels below the limit of detection have suppressed replication sufficiently to preclude the emergence of drug resistance. As genotypic analysis is extended to lower and lower levels of viremia in an attempt to address this question, many potential concerns arise regarding the interpretation of the results. Current genotypic assays can work well only when viremia is >1000 copies/ml. We have developed a sensitive method of sequencing at the clonal level virus found in the plasma of patients on HAART with viral loads below 50 copies/ml. Our study evaluated analytical issues inherent in low level genotyping and provided evidence for adequate suppression to preclude the evolution of resistance at least in some patients.

With respect to analytical issues, the central problem is that only a small number of virions are present in each sample. We obtained multiple independent clones from each patient at many time points, generating a more comprehensive sampling of virions in the plasma. Another analytical problem is that the detection of a sporadic mutation may lead to the false assumption of emerging resistance. Clinical genotypes are not performed at the clonal level but can detect variant sequences present at frequencies above 10-20%. At low levels of viremia, sequences may be obtained from the amplification of 5 or fewer viral RNA molecules. If one of these is copied incorrectly in the early cycles of PCR or contains a new RT error that has not yet been selected for *in vivo*, the resulting mutation may be detected and incorrectly reported as evidence of resistance. Thus the sporadic drug resistance mutations observed may not have pathophysiologic significance. This conclusion is supported by longitudinal genotypic analysis which showed that sporadic mutations detected at <50 copies/ml did not become fixed. Therefore, overinterpretation of resistance mutations detected in genotypic analysis of low-level virus is a potential problem.

In standard evolutionary theory, selection drives changes in allele frequency. In this regard, our data show no evidence for evolution occurring in the low-level viremia that is found in patients suppressed below the limit of detection with HAART. Our results are also consistent with a study demonstrating that blips were not indicative of imminent treatment failure. It remains possible that evolution is occurring on a protracted time scale that would preclude the observation of drug resistance in these studies. Because stable reservoirs of HIV-1 exist in all patients, replication-competent virus can be continually released from these reservoirs into the plasma, providing an explanation for virus production without evolution. Whether originating from the small pool of latently infected memory CD4+ T cells or from cells harboring virus in drug sanctuary sites, it appears that plasma is never fully clear of HIV-1. Two of the possible sources of low level virus, stable long term reservoirs and ongoing replication, are not mutually exclusive; in fact both processes are most likely occurring and both probably contribute to differing degrees to the plasma viremia. What is important is that the antiretroviral drugs can, under optimal circumstances, reduce viral replication to a level that does not allow selection of drug resistant virus. Resistant viruses that do arise do not seem to be able to establish a foothold and expand within the population. This finding suggests that with appropriate monitoring of viremia, patients may be able to continue on therapy indefinitely without failure due to drug resistance and that treatment may be primarily limited by problems related to drug toxicity and adherence.

Figure 1

Pt 82



NRTI

AZT	Red
3TC	Blue
ddl	Yellow
ddC	Light Green
d4T	Green
ABC	Light Purple
TDF	Pink
Multi-NRTI	Yellow

NNRTI

EFV	Orange
DLV	Purple
NVP	Cyan

PI

IDN	Light Purple
RTV	Green
LPV	Yellow
APV	Grey
SQV	Brown
NFV	Light Purple
MULTI-PI	Red

Antiretroviral Drug Color Key

Pt 83

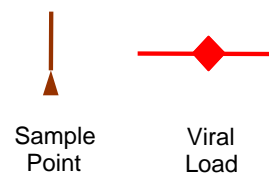
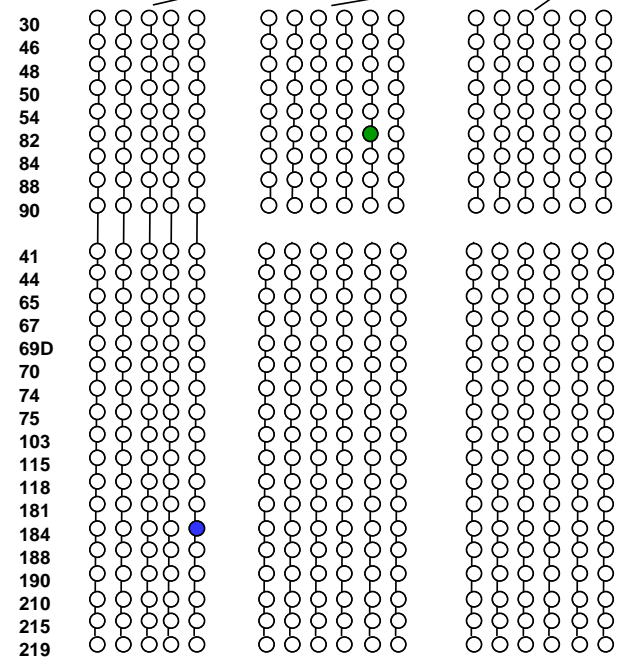
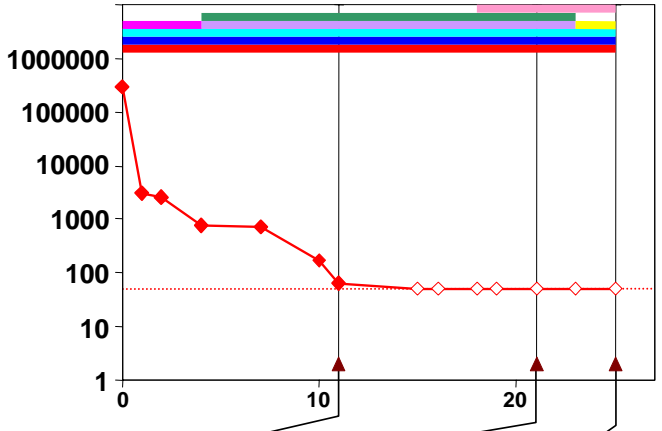


Figure 1: Genotypic analysis of patient HIV *pol* sequences of viruses isolated from the plasma of representative patients (82, 83) who had suppression of viremia to below the limit of detection. Treatment history is given by color coded bars found along the top of the graph showing plasma HIV-1 RNA levels over time. Open symbols represent a plasma HIV-1 RNA level less than 50 copies/ml. The dotted horizontal red line indicates the limit of detection of the assay (50 copies/ml). Vertical lines indicate sampling times. Genotypes are given for each time point with resistance mutations colored according to the drug to which they confer resistance. Each sequence is derived from an independent PCR reaction. Lines connecting the protease and RT sequences indicate linkage.

Figure 2: Genotypic analysis of HIV *pol* sequences of viruses isolated from the plasma of representative patients 79 (A) and 84 (B), both of whom had prior non-suppressive therapy. Treatment history is given by color coded bars found along the top of the graph showing plasma HIV-1 RNA levels over time. Open symbols represent a plasma HIV-1 RNA level less than 50 copies/ml. The dotted horizontal red line indicates the limit of detection of the assay (50 copies/ml). Vertical lines indicate sampling times. Genotypes are given for each time point with resistance mutations colored according to the drug to which they confer resistance. Each sequence is derived from an independent PCR reaction. Lines connecting the protease and RT sequences indicate linkage.

^a M, male; F, female; C, Caucasian; A, African-American.

^b Drugs taken as part of prior regimens that failed to suppress viremia or to maintain viremia below the limit of detection. Number of months on the regimen is indicated in parentheses.

^c Suppressive HAART regimen at time of sampling.

^d Time of sampling in months after the initiation of suppressive HAART regimen.

^e Mutations that could be attributed to selection by drugs in prior regimens that failed to suppress viremia.

^f Mutations that could not be attributed to selection by drugs in prior regimens that failed to suppress viremia.

^g Resistance mutations seen in more than one clone from a given patient.

^h Resistance mutations seen only in a single clone from a given patient

ⁱ Pt. 57 was started on d4T+3TC+IDV and sustained long term suppression with occasional blips. Treatment was interrupted, and viremia rebounded to 2×10^6 copies/ml. Initiation of treatment with ABC+EFV+3TC 2 months later was able to suppress viremia to undetectable levels once again.

^j Pt 79 started a HAART regimen consisting of 3TC+ddC+IDV/r and took this for 52 months. However, suppression of viremia was not maintained, there was early failure with detectable viremia in the range of 100-1000 copies/ml for the last 30 months. After a brief treatment interruption, suppression was achieved on a series of EFV-based HAART regimens.

Figure 2A

Pt 79

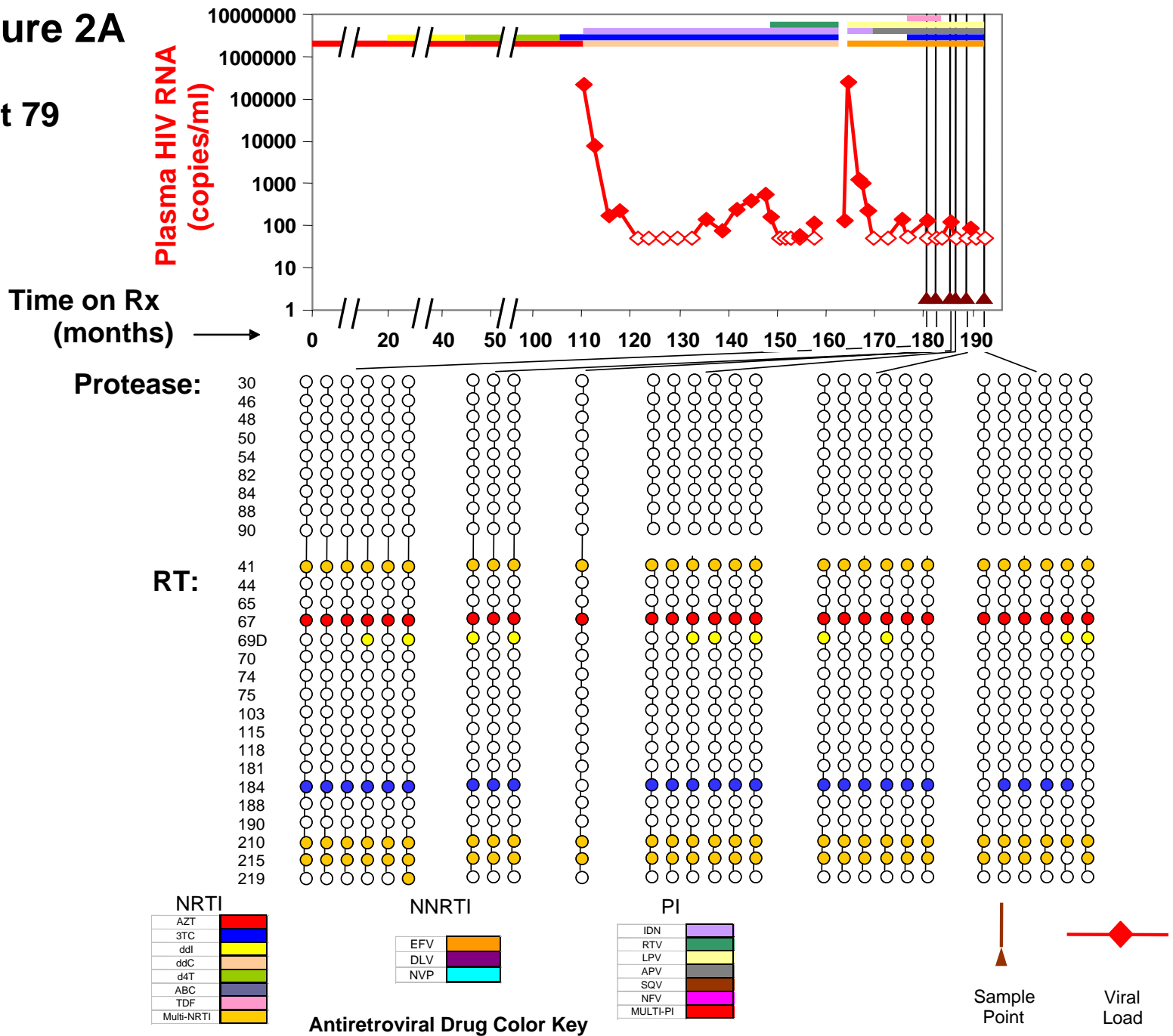
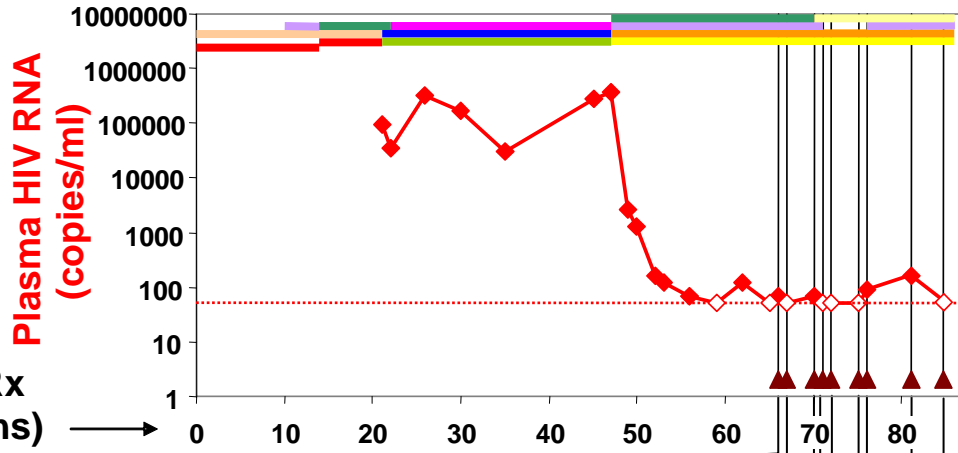
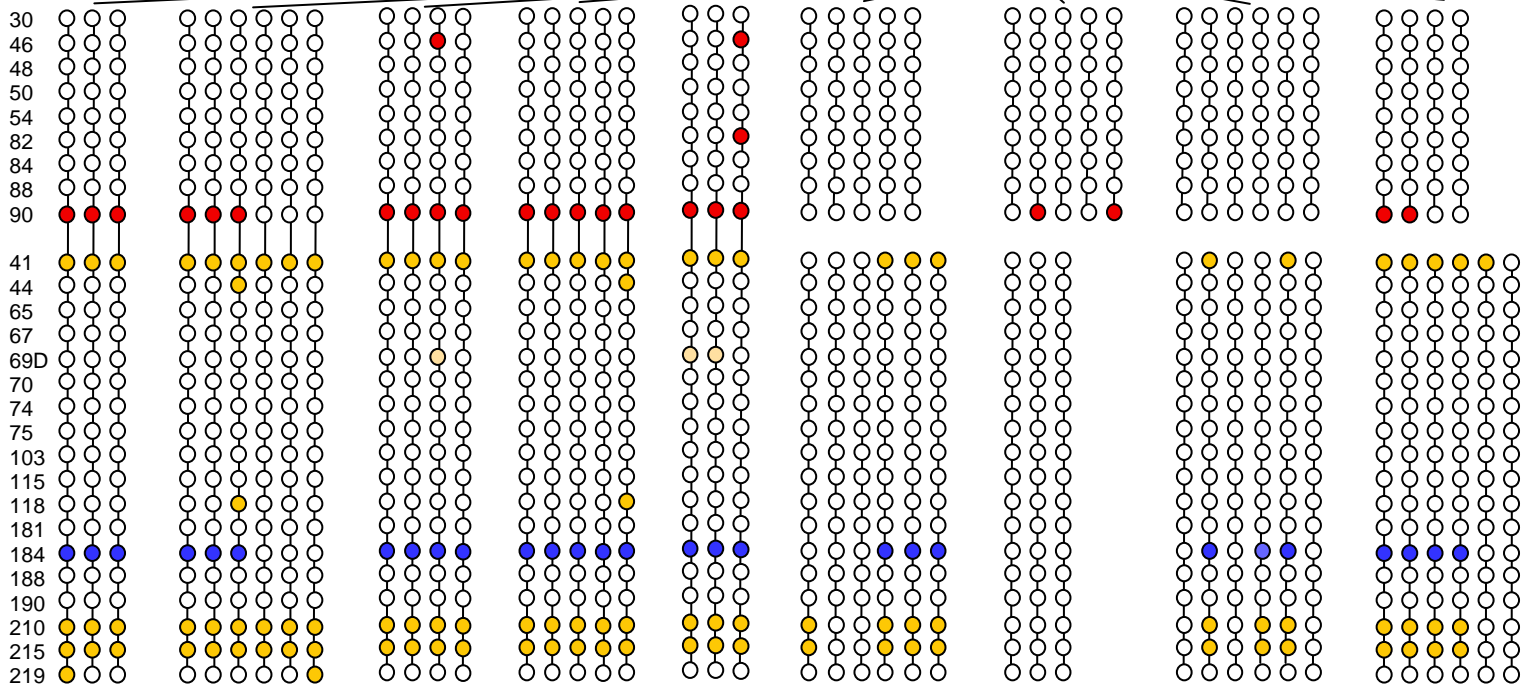


Figure 2B

Pt 84



Protease:



RT:

NRTI

AZT	Red
3TC	Blue
ddl	Yellow
ddC	Light Green
d4T	Light Yellow
ABC	Light Green
TDF	Pink
Multi-NRTI	Yellow

NNRTI

EFV	Orange
DLV	Purple
NVP	Cyan

PI

IDN	Purple
RTV	Green
LPV	Light Green
APV	Yellow
SQV	Grey
NFV	Brown
MULTI-PI	Red

Antiretroviral Drug Color Key

Sample Point

Viral Load

Figure 3

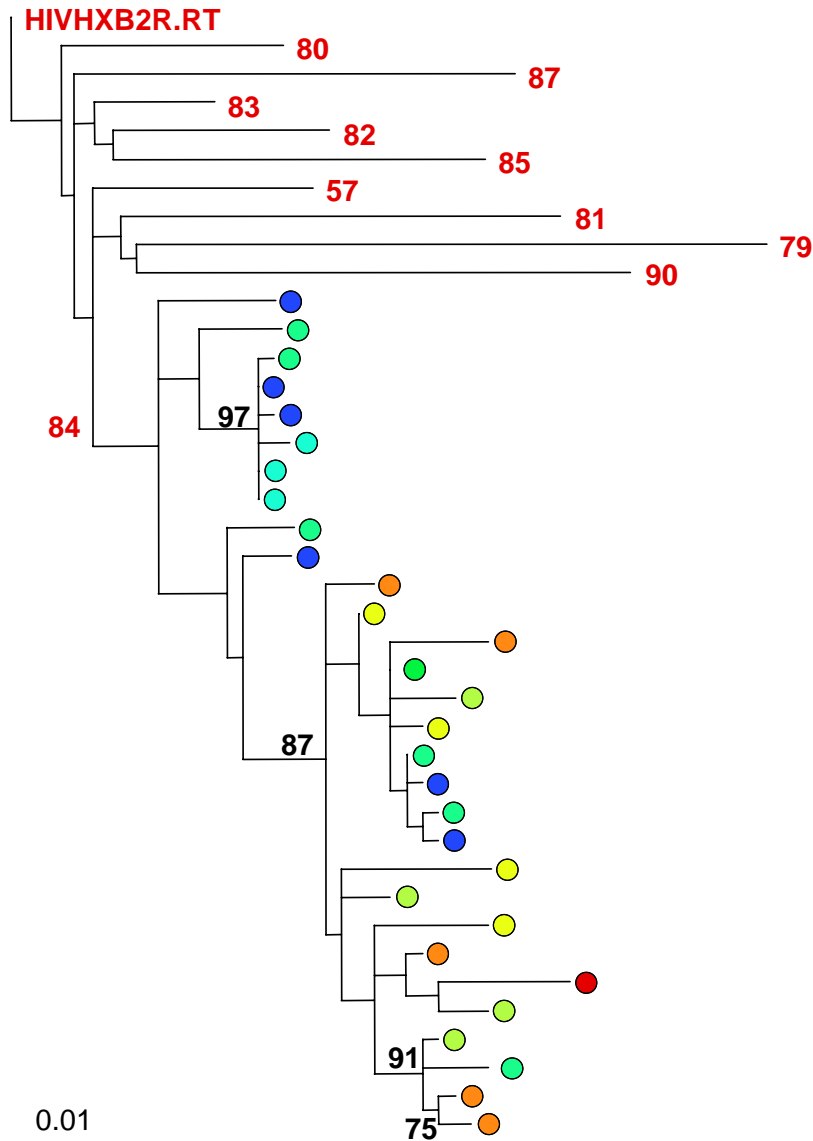


Figure 3: Maximum likelihood phylogenetic analysis of patient HIV RT sequences isolated from the plasma of patients with below detectable viral loads. Sequences are labeled in red with patient ID. Patient specific clustering is observed. The colored tree represents all the sequences obtained from one patient (84). Sampling time is indicated by coloring corresponding to the time scale key. Lack of temporal structure is demonstrated by the fact that genetic distances (horizontal scale) do not correlate with sampling time. The reference sequence HXB2 was used as an outgroup. Bootstrap values are given in black.

