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Envelope co-receptor tropism, drug resistance, and viral evolution among subtype C HIV-1 infected individuals receiving non-suppressive antiretroviral therapy

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Abstract

In resource-constrained settings, antiretroviral treatment (ART) is often continued based on clinical and CD4 responses, without virologic monitoring. ART with incomplete viral suppression was assessed in 27 subjects with subtype C HIV-1 by measuring plasma HIV-1 RNA, drug resistance, viral tropism, and evolution in polymerase (*pol*) and envelope (*env*) genes. The association between these viral parameters and CD4 cell change over time was analyzed using linear regression models. Increased area under the curve of HIV-1 RNA replication was a predictor of lower CD4 cell gains ($p < 0.007$), while less drug resistance measured as a genotypic susceptibility score (GSS) ($p=0.065$), and lower rates of evolution in *pol* and *env* genes ($p= 0.08$ and 0.097, respectively) measured as genetic distance were modestly associated with increasing CD4 cell counts. Evolution of *pol* and *env* were correlated ($R^2 = 0.48$, $p=0.005$), however, greater evolution was identified in *env* vs. *pol* ($p < 0.05$). CXCR4-usage (X4) was detected in 14/27 (52%) but no differences in CD4 cell change or plasma viremia were associated with X4-usage. Among subtype C HIV-1 infected patients in Zimbabwe receiving incompletely suppressive ART, higher virus replication and lower CD4 cell gains were associated with drug resistance and evolution of polymerase and envelope.

Keywords

Viral evolution; tropism; CD4 response; subtype C HIV; antiretroviral therapy

Introduction

In resource-constrained settings, clinical management of antiretroviral treatment (ART) is often based on drug tolerance, clinical stability and increasing CD4 counts, without virologic monitoring. However, continued ART with detectable viremia and active viral replication may lead to the selection of drug resistance and promote viral evolution, with opportunity for a switch in co-receptor usage independent of CD4 response.

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Viral evolution can be estimated by evaluating sequential viral sequences from the same individual. A change in viral tropism can also indicate ongoing viral evolution. R5 viruses are frequently identified in genital secretions and in recent infection [1, 2] while X4 viruses may appear later, associated with disease progression.[3] However, up to 17% of recently infected patients may have X4 virus.[4] Switching from R5 to X4 or D/M virus is observed in untreated individuals, associated with declining CD4 count, possibly as a result of diminished immune control or increased pathogenicity of X4 viruses.[5-9] Rapid progression to AIDS in cases of primary infection with X4 virus support the latter hypothesis.[2, 3, 10] Various techniques can be used to distinguish co-receptor usage. X4 viruses are associated with syncytia induction (SI) in culture, and may be estimated by the presence of basic amino acids at position 11 and 25 of the V3 loop,[11-13] although V-3 loop net charge, N-linked V1 and V2 glycosylation sites, and *env* length polymorphisms have also been implicated. [14, 15]

The relationship between a CD4 response to treatment, selection of drug resistance and changes in *env* tropism is not clear.[16] In the setting of ART, X4 virus is more prevalent among patients with lower CD4 cells and drug resistance.[17, 18] Drug resistance mutations may modulate HIV replication and the rate of CD4 decline. In primary infection, individuals with HIV RNA levels > 100,000 copies/ mL are more likely to have wild type viruses.[19] Treatment experienced individuals with drug resistance mutations have lower HIV RNA compared to antiretroviral naïve individuals.[20] This may be explained by decreased replication and fitness of viruses with drug resistance mutations.[21, 22]

Subtype C HIV-1 accounts for the majority of global infections.[23, 24] However, drug resistance, tropism, and ART have not been extensively studied in subtype C viruses. We previously described a high frequency of X4 viruses among a cohort of treatment experienced subtype C HIV-1 infected individuals in Zimbabwe, with multi-drug resistance and viremia despite ART.[25] Among these individuals followed for 1-3 years we now explored the relationships between persistent viremia, drug resistance, co-receptor usage, evolution of polymerase (*pol*) and envelope (*env*) genes, and the effect of these viral parameters on CD4 recovery and evolution of *env* and *pol*.

Methods

Study Population, CD4, and HIV-1 RNA testing

Study participants were recruited from The Centre, a community-based outpatient treatment program in Harare, Zimbabwe providing ART under the supervision of local physicians. Clinical and ART histories were obtained from 30 patients who provided blood samples for CD4, HIV-1 RNA, genotypic resistance testing, and culture in 2001, 2003, and 2004. Ethical approval for this study was obtained from the Human Subjects panel at Stanford University as an exempt protocol using de-identified samples obtained for clinical management. Results were returned to the patients and their physician.

CD4 testing was performed on fresh blood samples. Plasma was stored within 4 hours of collection, and maintained at -70°C. Plasma HIV-1 RNA was quantified using the Roche AMPLICOR™ Monitor test, version 1.5 (Roche Molecular Diagnostics, Branchberg NJ), with a lower limit of detection at 400 copies/ mL (2.6 $\log_{10}$ copies/ mL). An area under the curve (AUC) of plasma virus was calculated based on sequential HIV-1 RNA levels to provide a continuous representation of virus replication. The time points with HIV-1 RNA levels < 2.6 $\log_{10}$ copies/ mL were assigned a value of 2.59 $\log_{10}$ copies/ mL.

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Genotype Resistance Testing

HIV RNA was isolated from plasma using the Roche amplicor extraction. RNA was reverse transcribed to cDNA using random hexamer primers, and reverse transcriptase (Superscript III, Invitrogen). PCR amplification of cDNA generated an approximately 1,100 base pair amplicon of the *pol* gene for genotype resistance testing using primers MAW26 (forward-5′ TTGGAAATGTGGAAAGGAAGGAC) and RT21 (reverse-5′ CTGTATTTCTGCTATTAAGTCTTTTGATGGG) for first round amplification, and Pro1 (forward-5′CAGAGCCAACAGCCCCACCA) and RT20 (reverse-5′ CTTCGTCTCGATCTTGACCGTC) for second round amplification. RNA from the 2001 sample collection was extracted with the TRUPREP™ Viral RNA Kit (Visible Genetics Inc/ Bayer, Inc. Emeryville, CA), and reverse transcribed and amplified using enzymes and primers included in the TRUGENE™ Genotyping System kit, version 1.5 RT-PCR Research Use Only primers, optimized for non-subtype B HIV (Visible Genetics/Bayer, Inc.). Second round amplicon was purified using the QiaQuick PCR purification kit (Qiagen Inc.), and sequenced using Big Dye chemistry on an ABI 377 DNA sequencer. For population sequencing, chromatograms were manually inspected to verify mixtures at greater than 20% identified by the ABI or TRUGENE™ electropherogram interpretation algorithms. The presence of drug resistance and genotypic susceptibility score (GSS) was determined using the Stanford HIV Drug Resistance database, HIVDB.stanford.edu (accession date 12 December, 2006). The current antiretroviral drug regimen and resistance mutations for each individual were used to derive a GSS: a GSS of 3 indicates full susceptibility to 3 drugs, while a GSS of 0 indicates complete lack of activity of the treatment regimen against the patient's consensus virus. Genotypic susceptibility testing was not performed in real time, and treatment changes were undertaken by local physicians based on history of antiretroviral drug exposure and local availability of antiretroviral agents.

Tropism determination

Tropism was determined by passage of virus isolates in MT-2 cells and observation of SI/ NSI phenotype with *env* consensus sequence analysis. In some cases, a clonal *env* analysis was performed as previously described when accurate interpretation of the population sequence was not possible.[25] Among samples propagated in PBMC cultures, phenotype was determined in low passage virus isolates in GHOST cells, Human Osteosarcoma (HOS) cells expressing Green Fluorescent Protein (GFP) under the control of an HIV promoter. GHOST cells have been transfected with either CCR5 or CXCR4 to determine tropism. The web-based C-PSSM program (http://ubik.microbiol.washington.edu/computing/pssm/) for subtype C HIV-1 was used with V3 loop sequences to estimate tropism from plasma samples without a corresponding virus isolate.[26] MT-2 cells and GHOST cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

Phylogenetic Analysis

Sequences of nucleotides and amino acids were aligned using ClustalW, and manually edited in DAMBE. ModelTest and PAUP*4.0b10 were used to identify the optimal evolutionary model to estimate genetic distances in the C2-V5 of the *env* gene (approximately 650 base pairs), and *pol* gene sequences being analyzed.[27] The general time reversible model option in PAUP which accommodates six substitution types and selected gamma distribution was used to estimate genetic distance using the neighbor-joining method, and phylogenetic trees were generated in MEGA 3.1. 1000 replicate bootstrap values were calculated in PAUP to ascertain the relationship between viral sequences. Synonymous (dS) to nonsynonymous (dN) changes indicating positive or neutral selection was determined using SynScan (http://hivdb.stanford.edu/pages/synscan.html), with a dS/dN ratio greater than 1 indicating neutral selection, and less than 1 a change in the encoded amino acid interpreted as positive

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selection. The REGA Subtyping Tool (version 2.0) on HIVDB.stanford.edu was used to verify HIV-1 subtype of *pol* sequences. The Recombination Identification Program, RIP 3.0 (http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html) was used to assess recombination within sequences.

Statistical Analysis

Cross-tabulations were performed to describe the cohort. Differences between categorical variables were explored using the chi-square test or Fisher's Exact test where appropriate. Relationships between continuous non-parametric variables were examined using the Wilcoxon rank-sum test, and between paired non-parametric data using the Wilcoxon signed rank sum test and a two-sided p-value of < 0.05 considered statistically significant. Univariate linear regression models (PROC GLM; SAS, version 9.1) were used to model the rate of CD4 change over time. The relationship between genetic distance in sequential *pol* and *env* sequences, age, gender, CD4 change, viremia (AUC of HIV RNA), changing genotypic susceptibility to ART (GSS), and viral tropism was explored with univariate linear regression models. Multivariate analyses and mixed effects models were performed but are not reported due to indication of overfitting of variables in this small data set.

Sequence Accession Numbers

Nucleotide and amino acid sequences for the *pol* and *env* sequences have been deposited in Genbank. The *pol* sequences have the following accession numbers: AY090840-41, AY090843-49, AY090851, AY090853, AY090855-56, AY090859, XXXX-XXXX. The *env* sequences have the following accession numbers: AY265929, AY265934, AY265936-37, AY265941-43, AY265948-49, AY265951, AY265954, AY265957, XXXX-XXXX. Participants are identified as TC or ZI followed by a unique number in the submitted sequences.

Results

Baseline HIV RNA, CD4, and CD4 Response

Thirty individuals were repeatedly evaluated in 2001, 2003, and/or 2004, with two sequential CD4 tests performed on all participants, and three CD4 tests performed on 8 of the 30 participants. Patient characteristics in 2001 have been previously published,[28] and is updated in table 1 to include subjects recruited in 2003 with follow up in 2004. Among 12 men and 18 women, the median CD4 at enrollment was 122.5 per cu mm (range: 3-441) and HIV-1 RNA level was 4.46 (range: < 2.6 to 5.84 $\log_{10}$ copies/ mL). Median CD4+ T-lymphocyte count increased by 21.7/year (range:-111 to 357) and median percent CD4 by 20% (range: 95% to 5533%). Table 2 shows the CD4 and HIV RNA levels at initial enrolment and at the last study visit, and viral co-receptor usage.

Antiretroviral Regimens. HIV RNA Response, and Drug Resistance

Individuals had initiated ART between 1997 and 2001. Ten of the twenty-seven (37%) had been treated with mono or dual-therapy, and 2 were continuing mono or dual therapy in 2001. Most individuals had received multiple drug regimens; 5 were on their first treatment regimen, 8 individuals on their second, 8 on their third, 5 on their fourth, and one on their fifth regimen. Treatment interruptions were due to intermittent antiretroviral availability and toxicities. The last ART regimen included a protease inhibitor (PI) in 12 individuals, non-nucleoside reverse-transcriptase inhibitor (NNRTI) in 11 individuals, and 2 were receiving PI and NNRTI-containing regimens. One person was maintained on DDI and hydroxyurea, and subsequently on nevirapine monotherapy, with an HIV-1 RNA level <500 copies/ mL for three years. No treatment history was available for three subjects.

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Three individuals achieved virologic suppression to <2.6 $\log_{10}$ copies/ mL, while 27 individuals remained viremic. Among individuals with viremia, 16/27 (59%) had a CD4 increase over time, and 11/27 (41%) had a fall in CD4 count during the study period. The median genotypic sensitivity score (GSS) among 20 viremic individuals in whom *pol* sequence data and treatment history was available was 1.97 (predicted sensitivity to 2 of their drugs) compared with a median GSS of 2.89 among the three individuals who achieved virologic suppression. Treatment history was not available for three individuals, and *pol* sequence was not amplifiable in four individuals. Table 3 shows the treatment regimens at the time of blood sample collection for this study and genotypic drug resistance for the 23 individuals in whom *pol* sequence and treatment information was available. Among twenty individuals with viremia and sequential *pol* sequences, there was accumulation of drug resistance mutations in fourteen individuals. Figure 1a shows the phylogenetic tree of *pol* sequences, with clustering of sequences from the same individual.

Tropism and CD4 Response

Tropism was determined in 27 individuals, of whom 13 had exclusively R5 and 14 had X4 or D/M virus. Tropism determination was unsuccessful in 3 individuals, 1 of whom had achieved virologic suppression. Culture-based methods were unsuccessful for 9/30 samples, and tropism determination was performed using sequence only. Both culture-based assays and sequence analysis were performed on the remaining 21 samples, with agreement in tropism prediction in all but 1 subject. TC52 had two samples drawn in 2003: the first sample was NSI by culture but the PSSM interpretation of sequence was X4, while the second isolate was SI by culture with a PSSM sequence interpretation indicating R5 virus.

Paired *env* sequences are shown in the phylogenetic tree in figure 1b. Of the 27 individuals with detectable HIV-1 RNA levels despite ART, 12 had R5 virus, and 13 had X4 virus. Paired sequential env sequences were available from 14 individuals, and these are shown in the phylogenetic tree in figure 1b. 7 individuals had a tropism switch during the study period: 3 from R5 to X4, 2 from X4 to R5, and 1 each from R5 to D/M and D/M to R5; the five subjects in who tropism switch was detected, and for whom paired sequence data are available are depicted with arrows in figure 1b. Based on the tropism at the final sampling point, 8/16 women had X4 or D/M virus, and 3/11 men had X4 virus (p=0.43, Fisher's Exact Test). The median initial CD4 count per cu mm among individuals with exclusively R5 virus was 158 (range: 13-337; 25-75% IQR 75-272) compared to 76 (range: 3-441; 25-75% IQR 33-214) among individuals with evidence for X4 virus at some point during the study period (p=0.16, Wilcoxon rank-sum test). The median initial HIV RNA level among individuals with exclusive R5 virus was 4.48 (range: 2.88 – 5.84; 25-75% IQR 3.26 – 5.32) and 5.02 (range: 2.59 – 5.43; 25-75% IQR 2.61 – 5.07) among those with evidence for X4 virus (p=0.58, Wilcoxon rank-sum test).

Envelope and Polymerase Phylogenetic Analysis

Neighbor-joining phylogenetic trees for consensus *pol* and *env* sequences are shown in figure 1a and 1b, respectively. *Pol* sequences were subtype C HIV-1 with bootstrap values >97%. Sequential sequences from the same individuals were closely related to one another, with bootstrap values ≥ 88%. There was no evidence for viral recombination by sequence analysis. One consensus sequence from the plasma of TC28 in 2001 was not interpretable with respect to tropism, however, six molecular clones generated from second round amplicon from the 2001 sample showed two discrete viral populations by sequence, one X4, the other R5. The viral population consensus sequence in 2003 demonstrated only a pure R5 viral population phylogenetically closer to the R5 clonal population identified in the 2001 patient sample, suggesting a fitness advantage associated with R5 usage,.

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Sequential *pol* or *env* sequences were used to determine an intra-individual median genetic distance using the optimal evolutionary model identified by ModelTest. On treatment distribution of genetic distance between sequential time points in the *pol* gene among 21 individuals and the *env* gene among 14 individuals is shown in Figure 2. The genetic distance in *env* exceeded that in *pol* per unit time, with a median genetic distance in *env* of 0.013 compared with 0.0085 in *pol* (p=0.0494, Wilcoxon signed rank sum test). There was a significant correlation between genetic distance in *pol* and *env* ($R^2 = 0.48$, p=0.005). Genetic distance in *pol* was similar among individuals infected with R5 and X4 or D/M virus, with an arithmetic mean genetic distance of 0.014 among 10 individuals with R5 virus and 11 individuals with X4 virus. Positive selection was seen in the sequential consensus *env* sequence of 3/14 individuals and 1/19 consensus *pol* sequences.

Predictors of CD4 Response

Results of the linear regression models to evaluate predictors of CD4 cell numbers over time among individuals receiving non-suppressive ART are shown in table 4. Positive parameter estimates indicate an increase in CD4 cell numbers, while negative parameter estimates indicate a decline in CD4 in association with the variable being analyzed. By univariate analysis there was a significant negative correlation between rate of CD4 gain and increased viremia. Those with higher HIV RNA levels over time (higher AUC viremia) achieved less CD4 increase than those with lower levels of viremia (p=0.04). An increase in genotypic susceptibility score (GSS) to the drugs in a treatment regimen, indicating more potent drug activity, demonstrated a trend towards an increased CD4 gain, p=0.065. Greater viral evolution in *pol* and *env*, as measured by genetic distance, were associated with a trend towards lower rates of CD4 gain, p=0.08 and p=0.097 respectively (Figure 3). Age, gender, and tropism (measured as R5 only at all time points, or X4/ D/M on at least one time point) were not associated with the rate of CD4 change on treatment.

Predictors of Viral Evolution

The relationship between viral evolution measured as genetic distance between sequential plasma samples, sequential CD4 measurements, HIV-1 RNA levels, and viral tropism was explored using linear regression models, and the results shown in table 5. Evolution in *env,* represented by genetic distance, showed a trend towards association with level of HIV viremia (p=0.069), with increasing levels of viremia associated with greater evolution between sequential samples. CD4 change also showed a trend towards association with *env* evolution (p=0.097) with greater env evolution seen with among individuals with lower CD4 recovery. Evolution in *env* was not associated with age, gender, change in GSS, or tropism. There was a strong association between evolution in *pol* and level of viremia over time (p=0.007), with higher levels of viremia associated with increased *pol* evolution as measured by genetic distance between sequential samples. There was a trend towards an association between *pol* evolution and rate of CD4 change, p=0.08, with greater *pol* evolution seen among individuals with poor CD4 responses to treatment.

Discussion

Virologic predictors of clinical benefit, measured by changes in CD4 cell count, were explored among ambulatory subtype C HIV-1 infected individuals on non-suppressive ART being monitored in an outpatient community-based clinical setting. Linear regression modeling identified the AUC of HIV-1 RNA as the most significant predictor of CD4 cell change among ART-treated individuals with multi-drug resistance. Similarly, the area under the curve of viremia is the primary predictor of evolution in *env* and *pol*. Infrequent sampling for HIV RNA quantitation may affect the AUC of HIV RNA levels that was used in this analysis. However, individuals continued treatment on clinical grounds, without the provision of viral or

immunologic monitoring in real-time, and with very limited access to effective second-line or alternative drug regimens. While more frequent HIV RNA measurements, especially timed to reflect the impact of treatment changes, would have been ideal in the context of this study, we believe that the AUC HIV RNA levels that we used in this study reflect the virologic status of these participants, most of whose treatment changes provided less than one effective new antiretroviral agent. We hypothesize that reduced virus replication, and decreased area under the curve of viremia, may limit viral evolution. Less evolution is associated with higher CD4 gains as shown in Figure 3. In contrast, chemokine co-receptor use was not associated with area under the curve of viremia, nor evolution in *env* or *pol*. This may explain why X4-usage was not associated with decreased CD4 recovery. Despite the association of X4 virus with more rapid progression of disease in the absence of ART,[5-10] among these subtype C HIV-1 infected, ART treated individuals, there was no evidence of an association between X4 virus and either increasing HIV RNA or decreasing CD4 cell counts. X4 usage may not mark increased pathogenicity and effective modulation of viral immune escape in the presence of drug resistance associated mutations.

A high frequency of X4 or D/M virus has been identified in subtype B HIV, associated with failing therapy and nadir CD4 count.[18, 29] In ACTG 175, a pre-HAART era study of nucleoside RTI drugs among subtype B HIV-infected patients X4 virus (measured as SI) was independently associated with CD4 cell decline, AIDS and death [30, 31]. More recent studies of treatment and tropism in subtype B viruses suggest that X4 virus is associated with lower CD4 counts on ART,[17] and that X4 viruses have increased replicative capacity and reduced CD4 response to treatment associated with drug resistance and persistent viremia.[16] X4 viruses have been infrequently identified in prior studies of treatment naïve subtype C HIV-1 infected individuals in the absence of ART exposure.[23, 24] In the present study, about half of subtype C HIV-1 infected Zimbabweans receiving ART carried an X4 virus, providing additional evidence that incomplete viral suppression may select or allow X4 usage. Higher on-treatment HIV-1 RNA levels were associated with greater evolution in *env* than *pol*, and persistent viremia on ART was associated with accumulation of drug resistance associated mutations in *pol*. This is consistent with studies of subtype B HIV-1 in which infected individuals with viremia, despite ART develop new drug resistance mutations.[29, 32]

There are several limitations to this observational study. The patients who returned for repeated measures to this community clinic reflect a selected group with the potential for survival bias. Misclassification of reported drug regimens, treatment interruptions, poor adherence and limited access to medications may have contributed to continued viremia and drug resistance. Individuals continued treatment on clinical grounds, without viral or immunologic monitoring, and had very limited access to second-line or alternative drug regimens. Therapy with virologic non-suppressive (sub-optimal) regimens, as occurred here, is not recommended. However, in the absence of HIV RNA monitoring and with limited access to second line treatments, clinical monitoring alone and continued ART are the only option for many HIV infected individuals in resource-constrained settings.

Continued viremia with switches in tropism and the development of drug resistance on ART demonstrates that increasing ART access in resource-constrained settings may drive the evolution of HIV-1. The availability of a continuous supply of antiretroviral drugs is therefore critical to minimize treatment interruption, breakthrough viremia, and the potential for development of drug resistance and viral evolution. The virologic outcome of this treatment mono- and dual-therapy exposed cohort does not necessarily reflect the typical characteristics of current antiretroviral naïve populations receiving effective first-line combination ART. The relationship that we have explored in this treatment experienced group with multi-drug resistance should be further evaluated in a prospectively enrolled cohort more representative of the current recipients of combination ART globally. However, serial samples from these

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individuals in a community-based treatment program demonstrate an association between the level of viremia despite ART and increasing viral evolution. CD4 benefit was seen despite persistent viremia in the majority of patients, a finding which highlights the limitations of CD4 monitoring to gauge virologic response. A gain in CD4 was seen among 8/14 subjects with increasing drug resistance, suggesting that in some cases, the increase in drug resistance may be accompanied with a fitness cost to the viral population. In contrast, virologic outcomes, including greater HIV RNA levels, drug resistance and evolution are associated with lower CD4 cell responses. Where possible, access to virologic monitoring in resource-constrained settings will enhance timely access to effective second-line treatment regimens to reduce accumulation of drug resistance, diminish viremia and viral evolution, while sustaining clinical benefit.

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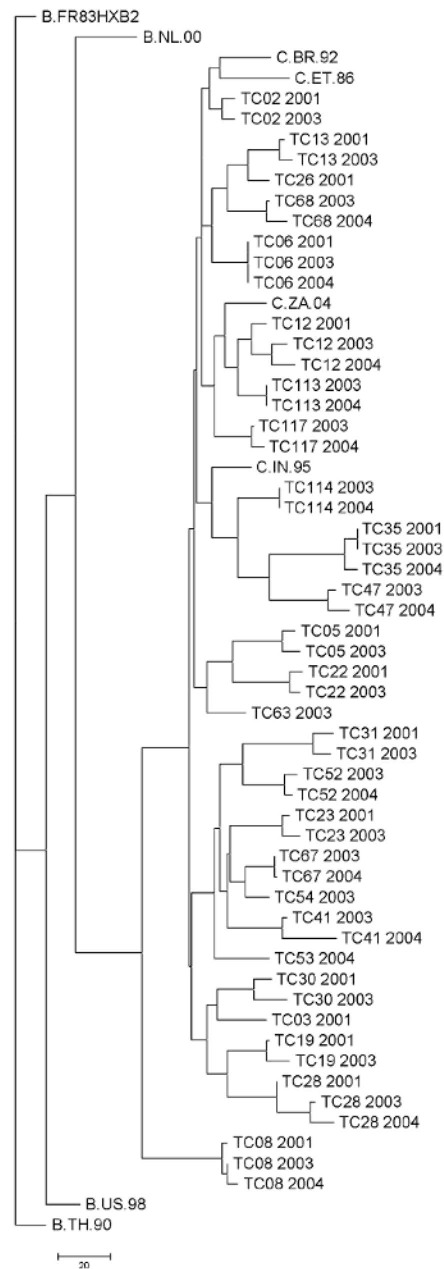
Figure 1a. Phylogenetic tree of *pol* sequences



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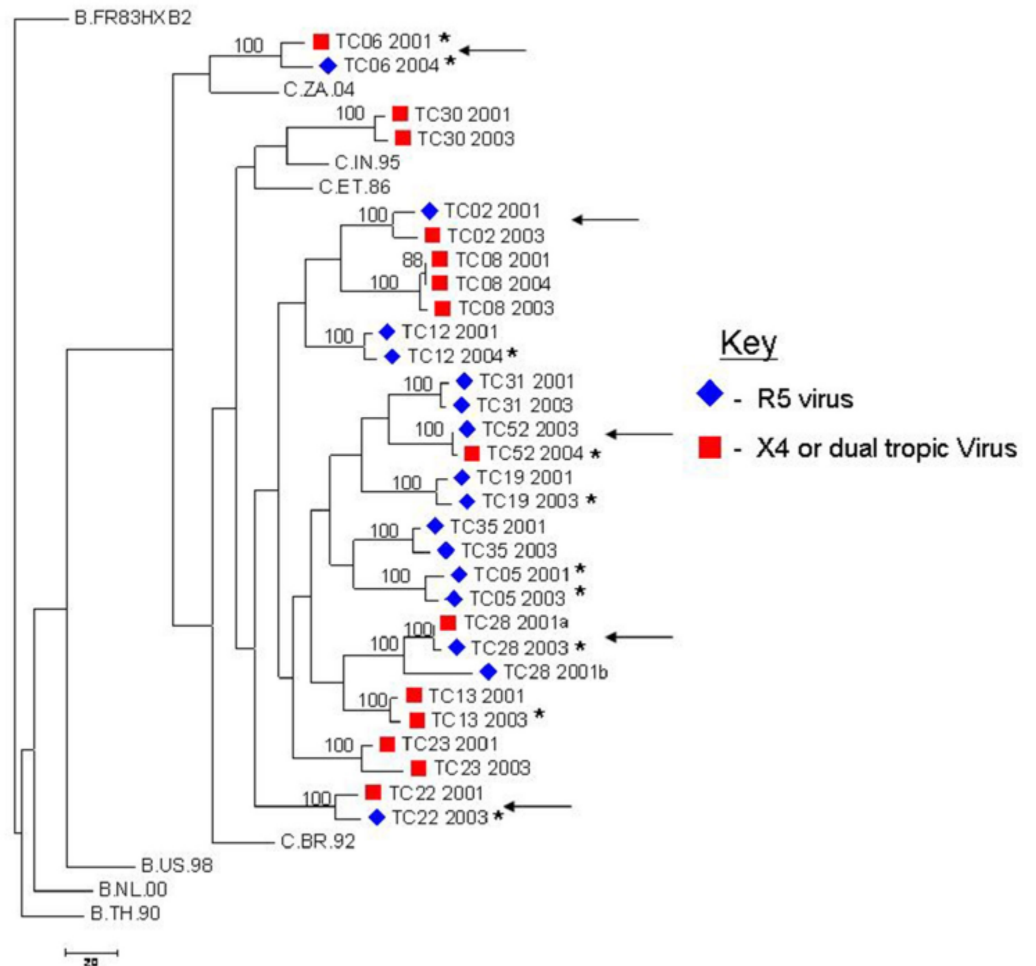

Figure 1.

Phylogenetic analysis of the *pol* (Figure 1a) and *env* genes (Figure 1b) of HIV among individuals with viremia while receiving combination antiretroviral therapy. Tree topology was inferred using the general time reversible evolutionary model based on parameters estimated using ModelTest. Optimal evolutionary model parameter estimates identified for *env* sequences based on the hierarchical likelihood ratio tests were as follows: Model selected: TVM+I+G; $f_A = 0.4309$, $f_C = 0.1744$, $f_G = 0.1885$, and $f_T = 0.2062$; R matrix values, RA->C = 1.5971, RA->G = 3.7064, RA->T = 0.8971, RC->G = 1.0596, RC->T = 3.7064, and RG->T = 1.0000; the proportion of invariable sites was 0.1994, and gamma distribution shape parameter=0.4728. Evolutionary model parameter estimates for *pol* sequences were as follows: Model selected: TVM+I+G $f_A = 0.3763$, $f_C = 0.1691$, $f_G = 0.2015$, and $f_T = 0.2531$; R matrix values, $R_{A\text{->}C} = 3.1596$, $R_{A\text{->}G} = 10.8420$, $R_{A\text{->}T} = 1.6226$, $R_{C\text{->}G} = 0.9179$, $R_{C\text{->}T} = 10.8420$, and $R_{G\text{->}T} = 1.0000$; the proportion of invariable sites was 0.4501, and gamma distribution shape parameter=0.7206. The subtype B HXB2 isolate was used as the outgroup to root the tree. Bootstrap values shown are based on 1000 replicates calculated in PAUP to ascertain the relationship between viral sequences. Samples from which tropism was determined by sequence only are indicated with an asterix, and the tropism determination of the *env* sequences

and/or isolates is indicated using solid blue diamonds for R5 virus, and solid red squares for X4 or dual/mixed virus. Sequential sequences from individuals in whom a tropism switch was detected are indicated with an arrow.

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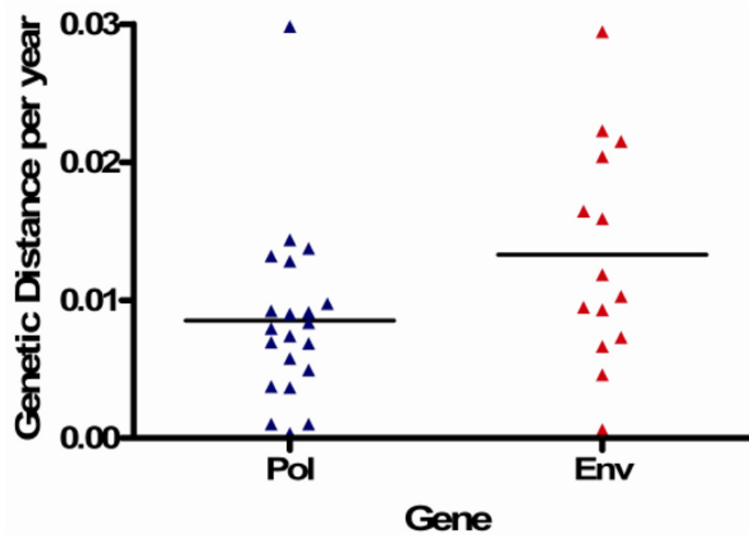

Figure 2.

Figure 2 shows the genetic distance per year in *pol* and *env* genes among individuals with sequential sequences available on treatment. The bold line represents the median genetic distance for the *pol* gene genetic distances (represented as blue triangles), and for *env* gene genetic distances (represented as red triangles). The Wilcoxon signed rank sum test was used to perform the equivalent of a paired t-test for non-parametric data, and showed genetic distance per year in *env* than in *pol*, p=0.0494.

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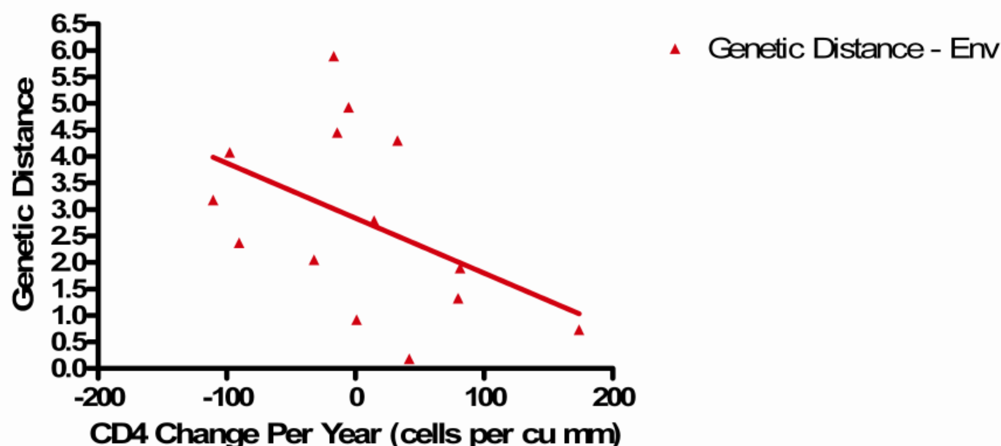

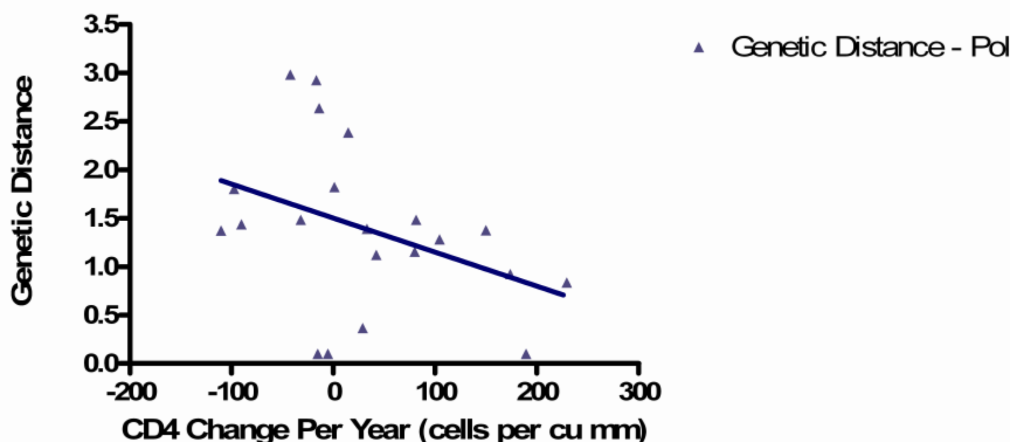

Figure 3.

Figure 3 shows the relationship between the rate of CD4 change and evolution in the envelope (figure 3a) and polymerase (figure 3b) genes as measured by genetic distance between sequential sequences of the respective gene. Each data point is indicated with a red triangle for envelope (*env*) and blue triangle for polymerase (*pol*), with the regression slope shown for each data set. An inverse relationship is seen between CD4 response and evolution, where greater evolution in the envelope and polymerase gene is associated with lower CD4 gain.

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Table 1

Characteristics at enrollment among subtype C HIV-1 infected Zimbabweans receiving antiretroviral therapy

Variable	Overall	Male (n=12)	Female (n=18)	* P-value
Age	42.5 (39-49)	48 (39.5-51.5)	40.5 (39-45)	0.06
Initial CD4 per cu mm	122.5 (42-264)	128.5 (48-260)	103 (42- 272)	0.93
Initial HIV-1 RNA $\log_{10}$ copies/ ml	4.46 (3.14-5.07)	3.87 (3.16-5.17)	4.46 (2.88- 5.07)	0.76
Change in CD4 per year	21.7 (-32 - 81)	14.9 (-39 – 81)	23.66 (-17 -104)	0.66
R5 virus at final determination – proportion (%)	16/27 (59)	8/11 (73)	8/16 (50)	0.43

Note: Data are median (interquartile range) unless otherwise indicated

* P-values were determined using the Wilcoxon rank-sum test for continuous, non-parametric data, and using the Fisher Exact test for categorical data

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Table 2

Cohort immunologic and viral characteristics at study enrolment and completion

Subject	Baseline CD4 (cells per cu mm)	Baseline HIV RNA (log_{10} copies/ mL)	Final CD4 (cells per cu mm)	Final HIV RNA (log_{10} copies/ mL)	Coreceptor Usage
TC23	255	5.02	25	5.47	X4
TC67	132	3.14	160	5.5	R5[a]
TC41	75	2.88	29	4.88	R5[a]
TC31	13	4.48	15	5.07	R5
TC52	33	5.43	222	4.61	R5 --> X4
TC28*	71	5.17	16	4.92	Dual/Mixed --> R5
TC05	321	3.26	133	4.7	R5[a]
TC06*	441	2.61	425	2.59	X4 [a] --> R5
TC68	86	2.59	249	5.35	R5 [a] --> X4 [a]
TC13	3	2.59	169	3.89	X4
TC19	41	5.53	149	4.92	R5
TC02	9	5.07	81	3.94	R5 --> Dual/Mixed
TC30	42	5.04	14	5.68	X4
TC08*	34	3.15	167	4.04	X4
TC22	214	4.23	11	5.29	X4 --> R5
TC35*	158	3.26	54	5.5	R5
TC47	337	4.95	459	4.45	R5 [a]
TC114	264	5.32	249	5.99	R5 [a]
TC12*	244	5.42	290	5.39	R5
TC113	323	3.12	507	4.23	R5 [a]
TC117	22	5.21	245	4.01	X4 [a]
TC01	272	4.46	46	2.69	R5 [a]
TC03*	81	5.02	476	3.04	X4
TC10*	286		113	3.98	X4

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Subject	Baseline CD4 (cells per cu mm)	Baseline HIV RNA (log_{10} copies/ mL)	Final CD4 (cells per cu mm)	Final HIV RNA (log_{10} copies/ mL)	Coreceptor Usage
TC26*	54	5.84	223	2.59	R5
TC53	120	2.59	196	4.9	R5 [a] --> X4
TC54	222	2.59	303		
TC63	81	4.99	86	5.79	R5 [a]
TC101	314	3.52	661	3.51	
TC107	125	3.18	81	4.4	

*Subjects in whom 3 measurements were available. Provided are the baseline and final CD4 and HIV RNA levels

**Coreceptor usage – R5 indicates CCR5-using virus; X4 indicates CXCR4-using virus; and Dual/mixed indicates the presence of both R5 and X4 using virus; arrows are used to indicate a change in coreceptor usage in sequential samples

[a]Coreceptor usage determined from sequence using C-PSSM program

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Table 3

Treatment history and drug resistance associated mutations among subtype C HIV-1 infected Zimbabweans[§]

Subject	Treatment Time 1*	Treatment Time 2 and 3*	Protease Mutations (Time 1)	Protease Mutations (Time2)	Protease Mutations (Time 3)	Reverse Transcriptase Mutations (Time 1)	Reverse Transcriptase Mutations (Time 2)	Reverse Transcriptase Mutations (Time 3)
TC23	AZT, DDC, SQV		L90M (2001)	L90M (2003)		D67G, K70R, K219Q (2001)	D67G, K70R, K103KR, V118I, K219Q (2003)	
TC67	AZT, 3TC, SQV		None (2003)	None (2004)		None (2003)	M184MV (2004)	
TC41	CBV, NVP	D4T,3TC,NVP	None (2003)	None (2004)		A62AV, K65R, K103R, Y181CY, K219KQ (2003)	A62V, K65R, K70R, V75I, F77L, Y115F, F116Y, V118I, Q151M, Y181C, M184I, K219E (2004)	
TC31	DDI, APV, NVP	EFV, LPV, IDV	I54FL (2001)	V32VG, M46MI, I47IM, L76V (2003)		M41L, D67N, L74V, Y181I, T215Y (2001)	D41L, D67N, L74V, K103N, Y181C, G190A, T215Y (2003)	
TC52	D4T, 3TC, NVP	D4T, DDI, IDV	None (2003)	None (2004)		M184V, G190A (2003)	D67N, K70R, M184V, K219E (2004)	
TC28	CBV, SQV	NVP, D4T, 3TC(2003) TDF,LPV,RTV (2004)	L90M (2001)	L90M (2003)	M46I, I84V, L90M (2004)	D67N, K70R, M184V, T215IT, K219Q (2001)	D67N, T69N, K70R, Y181C, M184V, T215V, K219Q (2003)	D67N, T69N, K70R, K101E, Y181C, G190A, T215F, K219Q (2004)
TC05	D4T, 3TC, SQV		L90M (2001)	L90M (2003)		M41L, M184V (2001)	M184V (2003)	
TC06	DDI, HYD	DDI, HYD (2003) NVP (2004)	None (2001)	None (2003)	None (2004)	None (2001)	None (2004)	None (2004)
TC68	D4T, 3TC, NVP	AZT, 3TC, NFV	None (2003)	I54L, L90LM (2004)		T69A, V106MV, K238KN (2003)	T69A, M184V (2004)	
TC13	AZT, 3TC, SQV	CBV, NFV	None (2001)	None (2003)		None (2001)	M184V (2003)	
TC19	CBV	DDI, D4T, EFV	None (2001)	None (2003)		M41LM, D67DN, K70KR, M184V, T215FIST (2001)	M41L, L74V, K103N, V106M (2003)	
TC02	3TC, D4T, SQV		None (2001)	L90M (2003)		None (2001)	M184V (2003)	
TC30	CBV, NFV	D4T, 3TC, NVP	None (2001)	I54V, I84V (2003)		M184V (2001)	M41L, D67N, K70R,Y181C, T215F, K219Q (2003)	
TC08	D4T, 3TC, SQV	3TC,D4T,SQV(2003) 3TC,D4T,EFV (2004)	None (2001)	None (2003)	None (2004)	M184V (2001)	L74LV, K103KR, M184V, M230LM, K238EGKR (2003)	K103R, V179D, M184V, T215F, M230L (2004)
TC22	IDV, NVP, SQV		None (2001)	L90M (2003)		M41L,K103N,M184V,T215Y (2001)	M41L, V75I, L100I, K103N, T215Y (2003)	
TC35	AZT, 3TC, NFV	D4T, 3TC, NVP	None (2001)	None (2003)	I54IF (2004)	M41L, D67N, K70R, Q151M, Y181C, M184V, G190A, T215Y, K219E (2001)	M41L, D67N, K70R, Q151M, Y181C, G190A, T215Y, K219E (2003)	M41L, A62V, K65R, K70R, V75T, Q151M,

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Subject	Treatment Time 1*	Treatment Time 2 and 3*	Protease Mutations (Time 1)	Protease Mutations (Time2)	Protease Mutations (Time 3)	Reverse Transcriptase Mutations (Time 1)	Reverse Transcriptase Mutations (Time 2)	Reverse Transcriptase Mutations (Time 3)
								Y181C, G190A, T215Y, K219E (2004)
TC47	D4T, DDI, NVP	D4T, 3TC, NVP	None (2003)	None (2004)		K103KN, V106MV, Q151KLMQ, Y181CY (2003)	K103N, F116Y, Q151M, Y181C, M184V (2004)	
TC12	3TC, DDI, HYD	D4T, 3TC, NVP	None (2001)	None (2003)	None (2004)	None (2001)	M41L, K103N, M184V (2003)	M41L, K103N, M184V, T215Y (2004)
TC03	DDI, HYD, AZT	IDV, D4T, 3TC	None (2001)			D67N,T69N,K70R,F77FL, Q151M,T215IT, K219EQ (2001)		
TC26	IDV, RTV, SQV	IDV, CBV	None (2001)			None (2001)		
TC 53	AZT, 3TC, NFV	D4T, 3TC, NVP	None (2004)			None (2004)		
TC54	AZT, 3TC, NVP	D4T, 3TC, NVP	None (2003)			V118I (2003)		
TC63	CBV, EFV		None (2003)			K103N (2003)		

§Individuals for whom treatment histories or *pol* sequence are unavailable are not included in the table.

Treatment histories indicate the antiretroviral regimen the individual was receiving prior to the collection of the blood sample.

Abbreviations: 3TC, lamivudine; D4T, stavudine; AZT, zidovudine; CBV, combivir; DDI, didanosine; EFV, efavirenz; HYD, hydroxyurea; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; NVP, nevirapine; RTV, ritonavir; SQV, saquinavir; TDF, tenofovir;

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Table 4

Predictors of rate of CD4 cell change per year among subtype C HIV-1 infected individuals receiving non-suppressive combination antiretroviral therapy, linear regression model (Proc GLM, SAS version 9.1).

	Univariate	
Variable	**Parameter Estimate**	**p-value**
Age	1.92	0.39
Gender	28.7	0.48
* AUC HIV RNA	-0.93	**0.04**
R5 Tropism	-28.6	0.43
Increase in GSS	65.63	0.065
Genetic Distance - env	-20.47	0.097
Genetic Distance – pol	-43.32	0.08

*AUC HIV-1 RNA level represents the area under the curve of viremia over time

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Table 5
Predictors of *Env* and *Pol* Gene Evolution by univariate linear regression analysis

Variable	*Env* Evolution Univariate		*Pol* Evolution Univariate	
	Parameter Estimate	p-value	Parameter Estimate	p-value
Age	-0.059	0.3687	-0.033	0.1178
Gender	1.462	0.1228	0.61	0.1053
CD4 Change	-0.01	0.097	-0.004	0.0805
*AUC HIV RNA	0.02079	0.0693	0.01	**0.0071**
Increase in GSS	-1.308	0.1876	-0.363	0.3148
R5 Tropism	-1.226	0.2187	-0.077	0.842