Complex positive selection pressures drive the evolution of HIV-1 with different co-receptor tropisms

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HIV-1 co-receptor tropism is central for understanding the transmission and pathogenesis of HIV-1 infection. We performed a genome-wide comparison between the adaptive evolution of R5 and X4 variants from HIV-1 subtypes B and C. The results showed that R5 and X4 variants experienced differential evolutionary patterns and different HIV-1 genes encountered various positive selection pressures, suggesting that complex selection pressures are driving HIV-1 evolution. Compared with other hypervariable regions of Gp120, significantly more positively selected sites were detected in the V3 region of subtype B X4 variants, V2 region of subtype B R5 variants, and V1 and V4 regions of subtype C X4 variants, indicating an association of positive selection with co-receptor recognition/binding. Intriguingly, a significantly higher proportion (33.3% and 55.6%, P<0.05) of positively selected sites were identified in the C3 region than other conserved regions of Gp120 in all the analyzed HIV-1 variants, indicating that the C3 region might be more important to HIV-1 adaptation than previously thought. Approximately half of the positively selected sites identified in the env gene were identical between R5 and X4 variants. There were three common positively selected sites (96, 113 and 281) identified in Gp41 of all X4 and R5 variants from subtypes B and C. These sites might not only suggest a functional importance in viral survival and adaptation, but also imply a potential cross-immunogenicity between HIV-1 R5 and X4 variants, which has important implications for AIDS vaccine development.

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switch.

HIV-1 has high mutation rates and is often subject to strong selective pressures from human immune responses [7]. Positive (diversifying) selection has been widely detected in whole genomes, but especially in the env gene of HIV-1 group M, HIV-1 group O, HIV-2 and SIV [8–14]. Furthermore, an association between positive selection and AIDS disease progression was observed in pediatric and adult HIV-1 infections [15,16]. Although only the C2V3C3 region of the env gene of R5 variants was used in these studies, increasing evidence showed that positive selection was more prevalent in individuals with slow HIV-1 disease progression than those with rapid disease progression [7,17]. The reason for this was likely due, in part, to a stronger immune response in slow progressors and a destroyed immune system in rapid progressors. However, the reverse was observed in one study based on HIV-1 subtype B V3 sequences, in which syncytium-inducing (SI) variants appeared to evolve faster than the non-syncytium-inducing (NSI) variants [18], implying that SI variants were subject to stronger positive selection than NSI variants. The disagreement was likely due to the usage of only part of the env gene in these studies. To reveal the intricate nature of selection pressures driving the evolution of HIV-1 R5 and X4 variants, adaptive evolutionary analyses based on whole env genes, or even whole genomic sequences are required. Therefore, a genome-wide comparison between the adaptive evolution of R5 and X4 variants from HIV-1 subtypes B and C was performed in the present study. We found that R5 and X4 variants underwent obviously different evolutionary patterns and different HIV-1 genes were subject to various selection pressures. We found that a significantly higher proportion of positively selected sites were identified in the C3 region than in other conserved regions of Gp120 in all analyzed HIV-1 variants, indicating that the C3 region might be more important to HIV-1 adaptation than previously believed. In addition, approximately half of the positively selected sites identified in env genes were identical between R5 and X4 variants. These common positively selected sites might not only imply functional importance in viral survival and adaptation, but also have important implications for AIDS vaccine development.

1 Materials and methods

1.1 Sequences and alignment

All of the sequences used in this study were collected from the Los Alamos National Laboratory (LANL) HIV Sequence Database (http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html). Because the full-length HIV-1 sequences with known co-receptor tropism are very limited, we primarily downloaded all 624 subtype B and 466 subtype C full-length sequences from this database. To determine the co-receptor tropism of each full-length sequence, three robust online prediction tools WebPSSM (http://indra.mullins.microbiol.washington.edu/webpssm) [19], Geno2pheno (http://coreceptor.bioinf.mpi-inf.mpg.de/index.php) [20], and HIV-1PhenoPred (http://jxxy.ujs.edu.cn/R5-X4%20pred.rar) [21] were used based on hypervariable region 3 (V3) of Gp120. Only sequences that yielded consistent prediction results by the three tools were selected and divided into CCR5 and CXCR4 datasets. Because a very short evolutionary distance is able to reduce the discriminatory power [22], sequences with closer evolutionary distances were deleted. As a consequence, 37 R5 and 33 X4 sequences from subtype B, and 28 R5 and 13 X4 sequences from subtype C were kept. These sequences were divided into four HIV-1 sub-populations for adaptive evolutionary analyses and their GenBank accession numbers are provided in Appendix Table 1.

To compare the different HIV-1 genes that play various functions in the HIV-1 life cycle, each complete genome was divided into five genes: gag, reverse transcriptase (RT), integrase (IN), gpl20 and gpl41. For X4 variants of subtype C, only the C2V3C3 region was used to reconstruct phylogenetic trees. For R5 variants of subtype B, gpl20 was used in these studies. To reveal the intricate nature of selection pressures driving the evolution of HIV-1 R5 and X4 variants, adaptive evolutionary analyses based on whole env genes, or even whole genomic sequences are required. Therefore, a genome-wide comparison between the adaptive evolution of R5 and X4 variants from HIV-1 subtypes B and C was performed in the present study. We found that R5 and X4 variants underwent obviously different evolutionary patterns and different HIV-1 genes were subject to various selection pressures. We found that a significantly higher proportion of positively selected sites were identified in the C3 region than in other conserved regions of Gp120 in all analyzed HIV-1 variants, indicating that the C3 region might be more important to HIV-1 adaptation than previously believed. In addition, approximately half of the positively selected sites identified in env genes were identical between R5 and X4 variants. These common positively selected sites might not only imply functional importance in viral survival and adaptation, but also have important implications for AIDS vaccine development.

1.2 Analysis of positive selection and identification of positively selected sites

Positive selection is measured by comparing the rate of nonsynonymous nucleotide substitutions per nonsynonymous site (dN/s) with that of synonymous substitutions per synonymous site (dS). The dN/dS ratio (ω) is traditionally used as an index to assess positive selection. The ω greater than 1 is taken as evidence of positive selection, ω equal to 1 indicates neutral selection, and ω less than 1 reflects strong negative (purifying) selection. The analyses of adaptive molecular evolution on all datasets were performed using six codon substitution models: M0 (one-ratio), M1a (nearly neutral), M2a (positive selection), M3 (discrete), M7 (beta), and M8 (beta and ω), as implemented in PAML 4.0 [25]. The details of these models were described in a previous study [26]. The likelihood ratio test (LRT) comparing three pairs of nested codon evolutionary models (M0 vs. M3, M1a vs. M2a, and M7 vs. M8) was used to test against the null hypothesis of no positive selection [27]. The null hypothesis of three pairs of nested models were rejected and positive selection was inferred when the LRT statistic was significant for a χ^2 distribution with the degrees of freedom equivalent to the difference in the number of parameters between nested models. Then the datasets were subjected to the identification of positively selected sites using three
positive selection models (M2a, M3 and M8). The sites that were detected with high posterior probabilities ($P > 0.95$) within the class with $\omega$ greater than 1 by selection models were admitted as positively selected [26].

Because of the overestimate of the number of actual positively selected sites [27, 28], the results under model M3 were not used to identify positively selected sites. To reduce or avoid possible false-positive results, positively selected sites identified simultaneously by models M2a and M8 in the Codeml program were defined as positively selected [28]. To confirm the results obtained using the codon substitution models in PAML, similar analyses were also performed using online DataMonkey package [29].

2 Results

2.1 Positive selection on three major genes gag, pol and env

HIV-1 R5 and X4 variants exhibit different phenotypic characteristics in cellular tropism and replication ability [5]. Two viral proteins Gp120 and RT are closely associated with HIV-1 phenotypes since the former determines cellular tropism by specifically recognizing co-receptors and the latter determines viral replication ability. Here, gp120, gp41, RT, IN and gag of R5 and X4 variants from subtypes B and C were subjected to adaptive evolutionary analyses. The results of the codon-based maximum likelihood analyses are shown in Tables 1 and 2 for HIV-1 subtype B gp120 and gp41 genes, and in Tables 3 and 4 for subtype C gp120 and gp41 genes, respectively. The results of other genes are shown in Appendix Tables 2–7.

Positive selection was detected in all analyzed genes by three positive selection models (M2a, M3 and M8), except for the IN gene of R5 variants of the B subtype. Comparison between different genes showed that stronger positive selection acted on env and gag genes than on RT and IN genes. The env genes appeared to be under the strongest positive selection pressure [8–11]. The numbers of positively selected sites identified in different genes further supported stronger positive selection acting on env and gag genes. These results suggested that differential HIV-1 genes suffered differential positive selection patterns.

2.2 Identification of positively selected sites in env genes

In HIV-1 subtype B datasets, the numbers of positively selected sites in gp120 were 29 and 31 for R5 and X4
sub-populations, respectively. In gp120 of HIV-1 subtype C datasets, the numbers of sites were 16 and 24 for R5 and X4 sub-populations, respectively (Table 5). The number of positively selected sites identified in X4 sub-populations was greater than in the R5 sub-populations, suggesting that gp120 gene of X4 variants was subject to a stronger positive selection pressure. An opposite pattern was observed in gp41 genes, where 27 and 17 positively selected sites were identified in R5 sub-populations of B and C subtypes, respectively, which was greater than the 24 and 11 sites in the X4 sub-population of B and C subtypes, respectively (Table 5). This implied that gp41 gene of R5 variants underwent a stronger positive selection than that of X4 variants. Therefore, although as a whole there was no obvious difference in

**Table 2** Phylogenetic analysis by ML estimation for the gp11 gene of HIV-1 subtype B sequences with R5 and X4 tropismsa)

| Tropism | Model code | lnL | Estimates of parameters | ΔA | Positively selected sites |
|---------|------------|-----|-------------------------|-----|--------------------------|
| R5      | M0         | −10580.74 | α=0.57                 | 1518.12 | None |
|         | M3         | −9821.68 | p=0.6478, p=0.2360 (p=0.1162), α=0.08, α=0.86, α=3.74 | 871.72 | Not shown |
|         | M1a        | −10014.68 | p=0.7306 (p=0.2694) | 384.62 | Not allowed |
|         | M2a        | −9822.37 | p=0.6731, p=0.2198 (p=0.1072), α=3.96 | 418.48 | 7L 24M 2010 240L 2010 |
|         | M7         | −10032.47 | p=0.2010, q=0.3902 | 418.48 | 7L 24M 32Q |
|         | M8         | −9823.23 | p=0.8789 (p=0.1213), p=0.3113, q=0.8201 | 418.48 | 7L 24M 32Q |
| X4      | M0         | −8388.95 | α=0.63                 | 1183.34 | None |
|         | M3         | −7977.28 | p=0.6907, p=0.2243 (p=0.0497), α=0.11, α=1.29, α=5.08 | 327.88 | Not shown |
|         | M1a        | −7963.59 | p=0.7054 (p=0.2946) | 327.88 | Not allowed |
|         | M2a        | −7799.65 | p=0.6497, p=0.2545 (p=0.0959), α=4.57 | 327.88 | 24M 96A 108L 109E 110Q |
|         | M7         | −7980.27 | p=0.1591, q=0.2925 | 352.78 | 24M 32Q 96A 101A |
|         | M8         | −7803.88 | p=0.8908 (p=0.1092), p=0.2182, q=0.4835, | 352.78 | 24M 32Q 96A |

a) For details, see Table 1.

**Table 3** Phylogenetic analysis by ML estimation for the gp120 gene of HIV-1 subtype C sequences with R5 and X4 tropismsa)

| Tropism | Model code | lnL | Estimates of parameters | ΔA | Positively selected sites |
|---------|------------|-----|-------------------------|-----|--------------------------|
| R5      | M0         | −10763.38 | α=0.47                 | 1581.90 | None |
|         | M3         | −9972.43 | p=0.7142, p=0.2445 (p=0.0413), α=0.10, α=1.29, α=5.76 | 305.00 | Not shown |
|         | M1a        | −10127.16 | p=0.7172 (p=0.2828) | 305.00 | Not allowed |
|         | M2a        | −9974.66 | p=0.68058, p=0.2529 (p=0.0666), α=4.31 | 305.00 | 24M 101L 140T 141N |
|         | M7         | −10133.41 | p=0.1878, q=0.4031 | 312.76 | 7Y 10L 140T |
|         | M8         | −9977.03 | p=0.9223 (p=0.0777), p=0.2293, q=0.5610, | 312.76 | 7Y 10L 140T |
| X4      | M0         | −8030.39 | α=0.54                 | 993.48 | None |
|         | M3         | −7533.65 | p=0.6346, p=0.2910 (p=0.0744), α=0.10, α=1.30, α=6.87 | 224.46 | Not shown |
|         | M1a        | −7647.70 | p=0.6544 (p=0.3456) | 224.46 | Not allowed |
|         | M2a        | −7535.47 | p=0.5929, p=0.3111 (p=0.0959), α=5.31 | 224.46 | 7Y 137D |
|         | M7         | −7658.08 | p=0.1654, q=0.2725 | 242.32 | Not allowed |
|         | M8         | −7536.92 | p=0.8857 (p=0.1144), p=0.2070, q=0.3918, | 242.32 | 7Y 187G |

a) For details, see Table 1.
suggest that sub-populations of subtype C, respectively. These results was greater than the 17 and 11 sites in R5 and X4 sub-populations of subtype B, respectively, which between R5 and X4 sub-populations, the number of positively selected sites on env genes between R5 and X4 sub-populations, the gp120 and gp41 genes underwent different evolutionary pathways in R5 and X4 variants.

When taking subtypes into account, the number of positively selected sites in the env genes of subtype B was greater than in subtype C. For the subtype B gp120 genes, 29 and 31 sites were identified in R5 and X4 sub-populations, respectively. There were 16 and 24 sites in the R5 and X4 sub-populations of subtype C, respectively. For the gp41 genes, 27 and 24 sites were identified in R5 and X4 sub-populations of subtype B, respectively, which was greater than the 17 and 11 sites in R5 and X4 sub-populations of subtype C, respectively. These results suggest that env genes of subtype B underwent stronger positive selection than that of subtype C.

## 2.3 Identification of positively selected sites in gag and pol genes

The HIV-1 gag gene encodes four structural proteins. The gag products are crucial targets, recognized by the human immune system. In gag genes, 9 and 10 positively selected sites were detected in R5 sub-populations of subtypes B and C, respectively, obviously greater than the six sites in the X4 sub-population of subtype B (Appendix Tables 2 and 3), however, four of these sites (91, 138, 280 and 374) were all detected in both the R5 and X4 sub-populations of subtype B. Two (91 and 138) of the four common sites were also identified in the R5 sub-population of subtype C, possibly

### Table 4 Phylogenetic analysis by ML estimation for the gp120 gene of HIV-1 subtype C sequences with R5 and X4 tropisms

| Tropism | Model code | lnL | Estimates of parameters | 2Δl | Positively selected sites |
|---------|------------|-----|------------------------|-----|-------------------------|
| R5      | M0         | 764.17 | 0.53                  | 811.42 | None |
|         | M3         | 755.46 | 0.6953, p=0.02258 (p=0.0789), | 174.41 | Not allowed |
|         | M1a        | 762.79 | 0.01083, a=1.1385, a=3.39 | 141.38 | Not allowed |
|         | M2a        | 759.76 | 0.7019, p=0.02982 | 141.38 | Not allowed |
|         | M7         | 765.02 | 0.2013, q=0.3581 | 174.02 | Not allowed |
|         | M8         | 756.01 | 0.8674, p=0.1326, q=0.3539, q=0.9639, a=2.66 | 174.02 | Not allowed |
| X4      | M0         | 492.55 | 0.57                  | 430.82 | None |
|         | M3         | 471.34 | 0.6766, p=0.0427, a=0.09, a=1.46, a=5.67 | 174.38 | Not allowed |
|         | M1a        | 475.49 | 0.6561, p=0.03484 | 86.78 | Not allowed |
|         | M2a        | 471.35 | 0.6277, p=0.2940 (p=0.0783), a=3.97 | 86.78 | Not allowed |
|         | M7         | 476.49 | 0.0970, q=0.1519 | 98.00 | Not allowed |
|         | M8         | 471.49 | 0.9026, p=0.0975, q=0.1526, q=0.3012, a=3.64 | 98.00 | Not allowed |

### Table 5 Comparison of positively selected sites between different regions of Gp120 and Gp41

| Subtype | Co-receptor | Amino acid sites under Variable regions | Gp120 | Gp41 |
|---------|-------------|----------------------------------------|-------|------|
|         |             | V1 V2 V3 V4 V5 Total | C3 C1–C5 Total | HR1+HR2 Other regions Total |
| B       | R5          | Positive selection | 0 5 1 0 0 6 10 23 29 | 6 21 27 |
|         | Non-negative selection | 26 34 35 34 12 141 43 341 482 | 78 240 318 |
| X4      | Positive selection | 0 3 8 1 1 13 6 18 31 | 3 21 24 |
|         | Non-negative selection | 26 36 28 33 11 134 47 346 480 | 81 240 321 |
| C       | R5          | Positive selection | 0 1 3 0 5 5 11 16 | 2 15 17 |
|         | Non-negative selection | 26 38 35 31 12 143 48 353 485 | 82 246 328 |
| X4      | Positive selection | 4 2 2 6 1 15 5 9 24 | 1 10 11 |
|         | Non-negative selection | 22 37 34 28 11 132 48 355 487 | 83 251 334 |

### Notes

a) For details, see Table 1.

b) The numbers in boldface represent the statistically significant differences (P<0.05) between one variable region and all other variable regions or between variable (V1–V5) and conserved regions (C1–C5) using Fisher’s exact test. b) The numbers in boldface represent the statistically significant differences (P<0.05) between C3 and all other conserved regions (C1, C2, C4, and C5) using Fisher’s exact test.
implying importance of HIV-1 adaptation.

HIV-1 RT and IN are key enzymes in the HIV life cycle. A comparison of the selection pressures for the two genes in the R5 and X4 sub-populations could help to distinguish the difference in replication rates between R5 and X4 variants. In HIV-1 subtype B datasets (Appendix Table 4), two (162 and 376) sites in the R5 sub-population and one (211) site in the X4 sub-population were detected under positive selection in RT genes. Two sites (118 and 123) were detected under positive selection for the IN gene in the X4 sub-population, whereas no positive selection was detected in the R5 sub-population (Appendix Table 6). In the R5 sub-population of subtype C dataset, three sites (123, 344, and 377) in RT and three sites (50, 72, and 125) in IN were identified under positive selection (Appendix Tables 5 and 7).

2.4 Comparison of the locations of positively selected sites in HIV-1 Env

Gp120 is the HIV-1 surface glycoprotein that not only determines viral tropism, but also is the most important target for the host immune response. Gp120 contains five conserved (C1–C5) and five hypervariable regions (V1–V5). Comparing the location of positively selected sites in both conserved and hypervariable regions showed that significantly more positively selected sites occurred in hypervariable regions in the X4 sub-populations of subtypes B (41.9%, \( P = 0.041 \)) and C (62.5%, \( P = 0.0002 \)) than in conserved regions relative to the proportion (28.8%) of hypervariable regions in whole Gp120 (Table 5). This result implied that Gp120 hypervariable regions of X4 variants were subject to stronger positive selection pressures. We further analyzed the distribution of positively selected sites in five hypervariable regions. In X4 sub-population of subtype B, significantly more sites (61.5%, \( P = 0.0011 \)) appeared in the V3 region that was a critical determinant of co-receptor tropism and the main epitope for eliciting neutralizing antibody [30,31] compared with other hypervariable regions relative to the proportion (24.5%) of V3 in whole hypervariable regions. This implied a stronger positive selection pressure on V3, consistent with previous observations [7,18]. In X4 sub-population of subtype C, however, higher proportions (66.7%, \( P = 0.0316 \)) of positively selected sites were located in the V1 and V4 regions, both of which account for 34% of hypervariable regions (Table 5). Additionally, in R5 sub-population of subtype B, significantly higher proportions (83.3%, \( P = 0.0013 \)) of positively selected sites were located in the V2 region that accounts for only 26.5% of the hypervariable region (Table 5).

When taking conserved regions into account, all four sub-populations exhibited significantly higher proportions of positively selected sites in the C3 region than in other conserved regions (R5 sub-population of subtype B: 43.5%, \( P < 0.0001 \); X4 sub-population of subtype B: 33.3%, \( P = 0.0206 \); R5 sub-population of subtype C: 45.5%, \( P = 0.0032 \); X4 sub-population of subtype C: 55.6%, \( P = 0.0004 \); Table 5). These results suggested that C3 might be more important in Gp120 evolution than previously thought. With regard to Gp41, we found that relatively few positively selected sites (9.1%–22.2%) occurred in the two heptad repeat (HR) regions when compared with the proportion (24.3%) of HR1 and HR2 in whole Gp41 (Table 5 and Figure 1).

Figure 1 Mapping of positively selected sites across env for different HIV-1 subtypes with different co-receptor tropisms. Positively selected sites are detected with high posterior probabilities greater than 95% within the class with \( \omega \) greater than 1 by at least two of three selection models (M2, M3 and M8). The positions of the five variable regions (V1–V5) in Gp120 and the two HR regions in Gp41 are indicated. Positively selected sites identified in various HIV-1 sub-populations with different subtypes and different co-receptor tropisms are highlighted by different symbols. The solid and blank circles represent R5 and X4 variants of subtype B, respectively. The shaded and unshaded squares represent R5 and X4 variants of subtype C, respectively. The positively selected sites shared between R5 and X4 variants are highlighted with yellow and green for subtypes B and C, respectively. The positively selected sites shared by all four sub-populations are highlighted in pink.
We observed that approximately half of the positively selected sites identified in the \textit{env} genes were the same between R5 and X4 sub-populations regardless of whether they were subtypes B or C. As an example, in subtype B, 30 of 56 positively selected sites identified in the R5 sub-population were also detected in X4. In subtype C, 16 positively selected sites were common between the R5 and X4 sub-populations (Table 5 and Figure 1). Of additional note were three positively selected sites (96, 113 and 281) in \textit{gp41} genes that were common among all four sub-populations (Figure 1).

3 Discussion

By comparing selection pressures acting on several key genes of HIV-1 from subtypes B and C and from R5 and X4 sub-populations, we found that \textit{env} (\textit{gp120} and \textit{gp41}) and \textit{gag} genes underwent higher selection pressures than other genes. These results suggested that certain HIV-1 genes were subject to different selection pressures [8]. Comparison of positively selected sites identified in \textit{env} of R5 and X4 sub-populations showed that both variants experienced obviously different evolutionary patterns. For the \textit{gp120} genes, more positively selected sites were identified in the X4 than in the R5 sub-population (Table 5), similar to previous observations of highly positive selection in the V3 region of SI compared with NSI variants [18]. However, this pattern was reversed for Gp41, which underwent stronger positive selection in R5 compared with X4 variants (Table 5). These results suggested R5 and X4 had different evolutionary patterns.

At least two kinds of potentially positive selection pressures, the host immune system and the target cell range, can drive HIV-1 evolution in treatment-naïve HIV-1-infected individuals [2]. The host immune responses including humoral and cellular immune responses were generally assumed to be the most important evolutionary pressures for adaptive evolution observed in the HIV-1 genome [7]. The \textit{gag} gene encodes viral structural proteins that are less involved in viral replication and co-receptor recognition/binding. A total of 19 positively selected sites were identified in \textit{gag} genes of three analyzed HIV-1 sub-populations (R5 and X4 sub-populations from subtype B and R5 sub-population from subtype C) (Appendix Tables 2 and 3). All these sites were found to be associated with at least one of three kinds of epitopes (Ab, CTL and T-helper). In particular, 83.3% and 66.7% of these sites were associated with CTL and T-helper epitopes, respectively. These results indicated that the positive selection pressures on \textit{gag} genes were primarily imposed by the host immune response. Among these positively selected sites, two sites at 91 and 138 were detected in all three sub-populations, possibly implying an additional importance for HIV-1 adaptation. A previous study demonstrated that a residue change in site 30 of Gag was able to confer a species specific replication advantage in HIV or SIV to adapt to their hosts [32]. The potential roles of sites 91 and 138 in HIV-1 adaptation need to be assessed by site-directed mutagenesis analyses.

RT is a key enzyme responsible for HIV-1 replication. HIV-1 X4 viruses usually exhibit higher replication rates than R5 viruses [2]. A total of six positively selected sites were identified in RT of three sub-populations. All these sites were located in the DNA polymerase domain of RT [33], and the sites identified in R5 and X4 variants were different, implying that these positively selected sites might be associated with specific replication characteristics of R5 or X4 variants. All these sites were associated with CTL-specific epitopes. Therefore, cellular immune responses were also likely to drive the evolution of RT.

The envelope (Env) glycoprotein of HIV-1 is exposed on the surface of the virus particle and HIV-1-infected cells, playing an important role in viral survival. It not only determines the co-receptor tropism of HIV-1, but is also the major determinant of immunogenicity for humoral and cellular immune responses. Moreover, the highest mutation rate of the \textit{env} gene in HIV-1 genome confers a potential ability to escape host immune responses [34,35]. Therefore, the adaptive evolution of \textit{env} genes was thought to be complex and might involve multiple selection factors such as cell source, host immune responses and the virus itself [2,8].

The hypervariable rather than conserved regions were demonstrated to determine HIV-1 co-receptor tropism. The V3 region plays the most important role in the determination of HIV-1 co-receptor tropism [30,36,37] and other hypervariable regions, such as V1V2 and V4 regions, affect the co-receptor usage [38–44]. Comparing the location of positively selected sites in Gp120 showed that in X4 sub-populations significantly more sites were located in the hypervariable regions (\(P<0.05\)). However, a similar pattern was not observed in R5 sub-populations. Further analyses showed that significantly more positively selected sites were in the V3 region (61.5%, \(P=0.0011\)) of subtype B X4 variants, the V2 region (83.3%, \(P=0.0013\)) of subtype B R5 variants, and V1 and V4 regions (66.7%, \(P=0.0316\)) of subtype C X4 variants compared with other hypervariable regions of Gp120 (Table 5). These results distinctly indicated that positive selection acting on the Gp120 hypervariable regions was closely associated with the function of co-receptor recognition/binding. The V1V2, V4 and V5 regions have been demonstrated to contribute to autologous neutralization [45]. This implied that humoral immune response-imposed positive selection also contributed to the evolution of Gp120.

A higher proportion (33.3%–55.6%, \(P<0.05\)) of positively selected sites were located in the C3 region than in
other conserved regions of Gp120 in all four HIV-1 sub-populations (Table 5). This result indicated that the C3 region might be more important to the function of HIV-1 Gp120 than previously believed. Moreover, the C3 region of the subtype C virus was able to elicit early autologous neutralizing response to HIV-1 infection by forming an important structural motif together with the V4 region [45]. The results observed in the C3 region also supported that humoral immune response-imposed positive selection might play a role in the evolution of Gp120.

Like the S2 domain of SARS-CoV spike (S) protein, HIV-1 Gp41 contains two HR regions, which have been shown to be important in virus membrane fusion [46]. We found that low proportions (9.1%–22.2%) of positively selected sites occurred in two HR regions of Gp41 (Figure 1), possibly arguing against membrane fusion as a major selection factor for the evolution of HIV-1 Gp41 [47]. However, three sites (96, 113 and 281) were detected in Gp41 of all four HIV-1 sub-populations, and two of these sites (96 and 113) were located in the middle region between two HR regions. This finding suggested that the three mutual sites might play some role in the membrane fusion function of Gp41, supporting membrane fusion as a minor selection factor for the evolution of HIV-1 Gp41. Furthermore, we found that approximately half of the positively selected sites shared by all X4 and R5 variants might have important implications for AIDS vaccine development.

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Appendix Table 1 List of GenBank accession numbers for HIV-1 genomic sequences analyzed in the text

| Subtype | Co-receptor tropism | Sequence number | GenBank accession numbers |
|---------|-------------------|----------------|--------------------------|
| HIV-1 B | R5                | 37             | AB286956, AB253432, AF003888, AF042301, AF224507, AY173952, AY037282, EU576191, AY586543, AY713412, AY385748, AY713411, EU574998, AY561236, AY970946, AY839827, AY857022, D10112, DQ854714, DQ837381, DQ886031, FJ469746, EF363124, EF637046, EF514699, EF637049, EU786675, FJ460501, FJ469770, FJ495937, FJ469731, M93258, U23487, U63652, FJ496085, FJ496150, KO2007 |
| HIV-1 C | R5                | 28             | AB254141, DQ369991, AY734550, DQ275642, EU786673, AY788054, AF266227, AY945738, FJ496185, U46016, AY713414, AY110978, AY110981, AF266224, AF266231, AY444800, AY463217, AY563170, AF268233, AF286234, AF299007, AY403176, AY391231, AY118165, AY253503, AY28855, AY253521 |
|         | X4                | 13             | FJ846637, FJ846642, AY878064, DQ093600, AY529666, FJ846647, AY529678, DQ283262, DQ382372, DQ382378, AY529677, AY529673, AF411966 |
### Appendix Table 2

Phylogenetic analysis by ML estimation for gag gene of HIV-1 subtype B variants with different coreceptor tropisms

| Co-receptor tropism | Model code | lnL   | Estimates of parameters | 2Δl | Positively selected sites |
|---------------------|------------|-------|-------------------------|------|--------------------------|
| R5                  | M0(one-ratio) | -10957.60 | α=0.24 | 1329.12 | None                     |
|                     | M3(discrete)  | -10293.04 | | | 252N 280T 374A 375T 399I 425D 441Y 473P 478P 483L 487T |
|                     | M1a(Nearly neutral) | -10382.84 | p=0.8769 (p=0.2131) | 145.4 | Not allowed |
|                     | M2a(Positive selection) | -10310.14 | p=0.7719 (p=0.0381), α=3.82 | P=0.0000 | 847 91R 1381 2231 280T 374A 389I 473P 483L |
|                     | M7(beta)      | -10364.21 | p=0.1217, q=0.4289 | 157.08 | Not allowed |
|                     | M8(beta&α)   | -10285.67 | p=0.9509 (p=0.0491), p=0.1539, q=0.6937, α=3.06 | P=0.0000 | 67S 847 91R 1381 146A 2231 280T 374A 37ST 389I 425D 473P 478P 483L |
|                     | M0(one-ratio) | -8991.79 | α=0.25 | 850.6 | None                     |
|                     | M3(discrete)  | -8566.49 | p=0.6919, p=0.2246 (p=0.08350), α=0.02, q=0.49, α=2.06 | P=0.0000 | 12E 15R 30K 67S 79Y 84T 91R 93E 102D 119D |
| X4                  | M1a(Nearly neutral) | -8607.63 | p=0.7827 (p=0.2173) | 51.28 | Not allowed |
|                     | M2a(Positive selection) | -8581.99 | p=0.7777, p=0.0448 (p=0.1775), α=2.86 | P=0.0000 | 67S 91R 1381 280T 374A 478P |
|                     | M7(beta)      | -8603.18 | p=0.1191, q=0.4075 | 73.92 | Not allowed |
|                     | M8(beta&α)   | -8566.22 | p=0.9279 (p=0.0720), p=0.1844, q=0.9994, α=2.20 | P=0.0000 | 67S 847 91R 125S 1381 2231 280T 374A 478P 473L |

a) Positively selected sites were identified with posterior probability P>95%; in boldface, P>99%.

### Appendix Table 3

Phylogenetic analysis by ML estimation for gag gene of HIV-1 subtype C variants with R5 tropism

| Model code | lnL   | Estimates of parameters | 2Δl | Positively selected sites |
|------------|-------|-------------------------|------|--------------------------|
| M0 (one-ratio) | -9276.18 | α=0.27 | 1131.26 | None                     |
| M3 (discrete) | -8710.55 | p=0.7768, p=0.1893 (p=0.0338), α=0.05, α=0.93, α=3.99 | P=0.0000 | 28K 54S 79Y 90Q 91R 1381 146A 241S 371T 440S 458P |
| M1a (Nearly neutral) | -8778.63 | p=0.7926 (p=0.2074) | 135.54 | Not allowed |
| M2a (Positive selection) | -8710.86 | p=0.7825, p=0.1852 (p=0.0324), α=4.14 | P=0.0000 | 28K 79Y 90Q 91R 1381 146A 241S 374T 440S 458P |
| M7 (beta) | -8782.96 | p=0.1194, q=0.4021 | 147.9 | Not allowed |
| M8 (beta&α) | -8709.01 | p=0.9639 (p=0.0360), p=0.1426, q=0.5506, α=3.71 | P=0.0000 | 28K 54S 79Y 90Q 91R 1381 146A 223V 241S 371T 440S 458P |

a) Positively selected sites were identified with posterior probability P>95%; in boldface, P>99%.

### Appendix Table 4

Phylogenetic analysis by ML estimation for RT gag gene of HIV-1 subtype B variants with different coreceptor tropisms

| Co-receptor tropism | Model code | lnL   | Estimates of parameters | 2Δl | Positively selected sites |
|---------------------|------------|-------|-------------------------|------|--------------------------|
| R5                  | M0(one-ratio) | -7803.46 | α=0.14 | 602.52 | None                     |
|                     | M3(discrete)  | -7502.20 | p=0.7218, p=0.2236 (p=0.0546), α=0.02, α=0.27, α=1.46 | P=0.0000 | Not shown |
|                     | M1a(Nearly neutral) | -7534.98 | p=0.8974 (p=0.1026) | 22.66 | Not allowed |
|                     | M2a(Positive selection) | -7523.65 | p=0.8971, p=0.0897 (p=0.0132), α=3.05 | P=0.0000 | 162S 376T |
|                     | M7(beta)      | -7525.67 | p=0.1371, q=0.7393 | 49.5 | Not allowed |
|                     | M8(beta&α)   | -7500.92 | p=0.9604 (p=0.0396), p=0.2362, q=2.1903, α=1.25 | P=0.0000 | 162S 21R 245V 297E 332Q 360A 376T 386T |
|                     | M0(one-ratio) | -6749.04 | α=0.17 | 448.77 | None                     |
|                     | M3(discrete)  | -6504.69 | p=0.6640, p=0.2666 (p=0.0753), α=0.01, α=0.23, α=1.51 | P=0.0000 | Not shown |
| X4                  | M1a(Nearly neutral) | -6522.31 | p=0.8821 (p=0.1179) | 18.96 | Not allowed |
|                     | M2a(Positive selection) | -6512.83 | p=0.8834, p=0.0940 (p=0.0226), α=2.65 | P=0.0001 | 21R |
|                     | M7(beta)      | -6526.67 | p=0.1286, q=0.6409 | 46.92 | Not allowed |
|                     | M8(beta&α)   | -6503.21 | p=0.9479 (p=0.0521), p=0.2461, q=2.1554, α=1.86 | P=0.0000 | 207Q 21R 215T 245V 357M 376T |

a) Positively selected sites were identified with posterior probability P>95%; in boldface, P>99%.
### Appendix Table 5  Phylogenetic analysis by ML estimation for RT gene of HIV-1 subtype C variants with R5 tropism

| Co-receptor tropism | Model code     | lnL      | Estimates of parameters | 2Δl  | Positively selected sites |
|---------------------|----------------|----------|-------------------------|------|--------------------------|
|                     | M0(one-ratio)  | −7159.79 | α0=0.12, 100%           | 0.01 | None                     |
|                     | M3(discrete)   | −6873.07 | p0=0.8749, p1=0.1176, 100% | 573.44 | 123D 334Q 377T          |
|                     | M1a(Nearly neutral) | −6900.15 | p0=0.8841, p1=0.1159 | 0.02 | Not allowed              |
|                     | M2a(Positive selection) | −6873.63 | p0=0.8817, p1=0.1109, 100% | 53.04 | 123D 334Q 377T          |
|                     | M7(beta)       | −6909.81 | p=0.1378, q=0.6736 | 0.01 | Not allowed              |
|                     | M8(beta&ω)    | −6777.18 | p0=0.0019, (p1=0.0011, 100% | 65.26 | 123D 334Q 377T          |

a) Positively selected sites were identified with posterior probability \( P>95\% \) in boldface, \( P>99\% \).

### Appendix Table 6  Phylogenetic analysis by ML estimation for IN gene of HIV-1 subtype B variants with different coreceptor tropisms

| Co-receptor tropism | Model code     | lnL      | Estimates of parameters | 2Δl  | Positively selected sites |
|---------------------|----------------|----------|-------------------------|------|--------------------------|
| R5                  | M0(one-ratio)  | −4567.46 | α0=0.12, 100%           | 305.44 | 10E 16S 27L 38S 44L 71L 100L |
|                     | M3(discrete)   | −4415.29 | p0=0.82278, p1=0.1239, 100% | 0.00 | 111T 121T 124T 1991 |
|                     | M1a(Nearly neutral) | −4427.40 | p0=0.8994, p1=0.1006 | 6.9 | Not allowed       |
|                     | M2a(Positive selection) | −4423.95 | p0=0.9011, p1=0.0816, 100% | 0.03 | None               |
|                     | M7(beta)       | −4435.26 | p=0.1511, q=0.8502 | 0.01 | Not allowed       |
|                     | M8(beta&ω)    | −4416.53 | p0=0.9444, (p1=0.0556, 100% | 0.00 | 16S 71L 100L 124T |
| X4                  | M0(one-ratio)  | −3994.45 | α0=0.14, 100%           | 0.00 | 100L 118S 123A 124T |
|                     | M3(discrete)   | −3895.27 | p0=0.7992, p1=0.1833, 100% | 0.00 | None               |
|                     | M1a(Nearly neutral) | −3909.48 | p0=0.8738, p1=0.1262 | 0.12 | Not allowed       |
|                     | M2a(Positive selection) | −3903.47 | p0=0.8744, p1=0.1123, 100% | 0.00 | 118S 123A |
|                     | M7(beta)       | −3910.05 | p=0.1933, q=0.9867 | 0.00 | Not allowed       |
|                     | M8(beta&ω)    | −3896.57 | p0=0.9821, (p1=0.0179, 100% | 0.00 | 100L 118S 123A 124T |

a) Positively selected sites were identified with posterior probability \( P>95\% \) in boldface, \( P>99\% \).

### Appendix Table 7  Phylogenetic analysis by ML estimation for IN gene of HIV-1 subtype C variants with R5 tropism

| Model code     | lnL      | Estimates of parameters | 2Δl  | Positively selected sites |
|----------------|----------|-------------------------|------|--------------------------|
| M0(one-ratio)  | −4143.50 | α0=0.16, 100%           | 0.00 | None                     |
| M3(discrete)   | −3997.12 | p0=0.7776, p1=0.1910, 100% | 0.00 | 11E 50M 72V 125T 269R |
| M1a(Nearly neutral) | −4016.89 | p0=0.8952, p1=0.1048 | 0.00 | Not allowed       |
| M2a(Positive selection) | −4003.60 | p0=0.8940, p1=0.0851, 100% | 0.00 | 50M 72V 125T |
| M7(beta)       | −4021.05 | p=0.1546, q=0.7387 | 0.00 | Not allowed       |
| M8(beta&ω)    | −3996.88 | p0=0.9696, (p1=0.0304, 100% | 0.00 | 11E 50M 72V 125T |

a) Positively selected sites were identified with posterior probability \( P>95\% \) in boldface, \( P>99\% \).