HIV-1 CRF01_AE and Subtype B Transmission Networks Crossover: A New AE/B Recombinant Identified in Japan

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Abstract

The major circulating HIV-1 strains in Japan have been subtype B (B) followed by CRF01_AE (AE) in newly diagnosed HIV/AIDS cases. These two subtypes have distinct epidemiological characteristics; B predominates in men who have sex with men, while AE is observed mostly in heterosexuals engaging in high-risk sex. However, transmission networks of these two high-risk populations appear to be crossing over and diffusing. Here we report the emergence of previously unidentified HIV-1 AE/B recombinants in Japan. We initially identified 13 cases with discordant subtyping results with AE (*gag* MA)/B (*pol* PR-RT)/AE (*env* C2V3) by molecular phylogenetic analysis of 1,070 cases who visited Nagoya Medical Center from 1997 to 2012. Genetic characterization of full-length sequences demonstrated that they shared an identical recombinant structure, and was designated as CRF69_01B by the Los Alamos HIV National Laboratory. By reviewing *gag*, *pol*, and *env* sequences collected in the Japanese Drug Resistance HIV-1 Surveillance Network, we found five other CRF69_01B probable cases from different areas in Japan, suggesting that the strain is transmitted widely throughout the country. The time of the most recent common ancestor analyses estimated that CRF69_01B emerged between 1991 and 1995, soon after AE was introduced from neighboring countries in the mid-1990s. Understanding the current epidemic strains is important for the diagnosis and treatment of HIV/AIDS, as well as for the development of globally effective HIV vaccines.

Introduction

HIV-1 CRF01_AE (AE) AND SUBTYPE B (B) are the two major strains circulating in Southeast and East Asian countries.^{1,2} This area has been recognized as a “recombination hotspot”¹ for AE and B. To date, 13 CRFs consistent with these two subtypes have been discovered, i.e., CRF15³

and 34⁴ in Thailand; CRF33,⁵ 48,⁶ 53,⁷ 54,⁸ and 58⁹ in Malaysia; CRF52¹⁰ in both Thailand and Malaysia; CRF51 in Singapore¹¹; and CRF55,¹² 59,¹³ 67, and 68¹⁴ in China.

In Japan, the number of HIV/AIDS patients at the end of 2013 was 23,015, including 7,203 AIDS and 15,812 non-AIDS patients.¹⁵ The major circulating HIV-1 subtype in Japan has been B, which is genetically related to the strain

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circulating in North America,^{16,17} followed by AE, with prevalences of 87.9% and 8.4%, respectively.¹⁸ These two strains have distinct epidemiological characteristics; B predominates in men who have sex with men (MSM), whereas AE is observed mostly in heterosexuals engaging in high-risk sex.¹⁸ However, the distinction between these two populations is becoming blurred as increasing numbers of AE cases have also been found in MSM.^{16,19} Therefore, the chance of recombination between the two strains is increasing in Japan. Indeed, among newly diagnosed cases in our study population, some cases had discordant subtyping results between *pol* and *env* sequences.

At Nagoya Medical Center (NMC), drug resistance genotypic testing and subtyping analyses have been performed since 1997 for all newly diagnosed patients to identify transmitted drug resistance and to monitor epidemiological trends of HIV-1. Of 1,070 cases tested between 1997 and 2012, our initial analyses of subtypes using the base sequences of *gag* matrix (MA), *pol* protease (PR) to the N-terminal region of reverse transcriptase (RT), and *env* C2V3 identified 13 probable cases of infection with AE/B recombinant strains. Here, we report the results of our thorough investigation on the genomic structure of these potential AE/B recombinants as well as our bioinformatics analyses of their origin and epidemic.

Materials and Methods

Sample

Thirteen probable cases infected with AE/B recombinant strains were selected by initial screening of 1,070 newly diagnosed patients admitted to NMC between June 1997 and April 2012 (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/aid). After drug resistance testing and subtyping of these 13 cases, the remaining peripheral blood mononuclear cells (PBMCs) or plasma specimens were stored at -80°C . These specimens were analyzed for near full-length sequences to determine the genetic structure of possible recombinant strains. In addition to the specimens collected at NMC, we reviewed *pol* PR-RT sequences collected in the Japanese Drug Resistance HIV-1 Surveillance Network.¹⁸ Among 3,899 cases classified as B in the region reviewed, five cases (three from Kanto and two from Hokuriku Areas, see Supplementary Fig. S1) were teased out as probable AE/B recombinant cases, and their sequences were analyzed along with the 13 NMC cases.

Ethics statement

This study was conducted according to principles in the Declaration of Helsinki. The study was approved by the ethical committee at NMC (#2010-310). All patients provided written informed consent for specimen collection and subsequent analyses.

Determination of HIV-1 subtypes

To determine subtypes of the specimens enrolled in this study, *gag* MA (396 bps; position 790–1,185 relative to HXB2), *pol* PR-RT (1,017 bps: 2,253–3,269), *pol* integrase (IN) (864 bps: 4,230–5,093), and *env* C2V5 (799 bps: 6,872–7,670) gene fragments were amplified and sequenced following a protocol reported elsewhere.^{20,21} Obtained sequence data

were subjected to phylogenetic analyses with reference sequences (Supplementary Table S2A) of HIV-1 subtypes A through K and CRF01_AE from the HIV sequence database at the Los Alamos National Laboratory (www.hiv.lanl.gov/). Multiple sequence alignment was performed using the MUSCLE program, and genetic distances were calculated based on the maximum composite likelihood model using MEGA software v.5.2.1.²² Phylogenetic trees were constructed using the neighbor-joining method (NJ) with 1,000 bootstrap replicates.

Analysis of near full-length sequences and determination of breakpoints

Cases with discordant subtype results among *gag* MA, *pol* PR-RT, *pol* IN, and *env* C2V5 were considered probable recombinant strains, and their near full-length sequences were determined. HIV-1 provirus DNA or viral RNA was extracted from patients' PBMCs or plasma, respectively, and four overlapping DNA fragments, 5' LTR-*gag* (position 48–2,272 relative to HXB2), *pol* (2,148–5,214), *pol* IN-*env* (4,175–6,841), and *env*-3' LTR (5,962–9,681), were amplified in the first-round PCR using LA Taq (Takara, Shiga, Japan) for DNA or Superscript III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen, Tokyo, Japan) for RNA, followed by nested PCR using LA Taq. Amplicons were sequenced using ABI 3500 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). Obtained sequences were edited by SeqScape software v.3.0 (Applied Biosystems) and assembled into near full-length sequences by GENETYX-WIN software v.5.0.4.

To clarify recombinant structures and breakpoints, we performed bootscanning and informative site analysis incorporated in the SimPlot software v.3.5.1.²³ Complete genomic sequences of HXB2 (subtype B, K03455), CM240 (CRF01_AE, U54771), and 04CMU11421 (subtype J as outgroup, GU237072) were used as reference sequences.

Fine analysis of the evolution of the recombinant strain

To investigate the evolutionary history of circulating recombinants between AE and B found in Japan, we reconstructed the phylogenetic relationships of *pol* PR-RT and *env* C2V5 genes of the recombinant strains with nonrecombinant strains collected in Japan and other East Asian countries by maximum clade credibility (MCC) trees of the Bayesian Coalescent Markov Chain Monte Carlo (MCMC) approach implemented in the BEAST v.1.8.1 package.²⁴ The sequence was partitioned into three-codon positions. The best-fit model for nucleotide substitution was evaluated by the hierarchical likelihood ratio test using PAUP v.4.0²⁵ with MrModeltest,²⁶ and the general time-reversible model was adopted with gamma-distributed site heterogeneity and invariant sites (GTR + G + I) with four rate categories.

To select a model for population growth and the molecular clock, we compared the marginal likelihood estimated by the stepping-stone sampling method^{27,28} using preliminary runs of BEAST with MCMC chains of 500 million iterations, and logistic growth (AICM = 58,506.49 and 86,555.36 at *pol* PR-RT and *env* C2V5 genes, respectively) and strict clock (AICM = 58,856.51 and 86,598.2 at *pol* PR-RT and *env* C2V5 genes, respectively) models were adopted as a best-fit model. The convergence of parameters was inspected using

Tracer v.1.5, with uncertainties depicted as 95% highest posterior density (HPD) intervals. The effective sample size of each parameter calculated in this inference was above 200. Tree samples in the MCMC were used to generate MCC trees using TreeAnnotator v.1.5.4 with a burn-in of 40,000 states. Resulting trees represent partial genealogies of the recombinant strains with some related viruses on B (*pol* PR-RT) and AE (*env* C2V5). References included in the trees are shown in Supplementary Table S2B.

Estimation of the time of the recombination event

The date of birth of CRF69_01B was estimated using the method of Tee *et al.*²⁹ In brief, it is assumed that a lineage “xy” is generated by a single recombination event between members of monophyletic groups *x* and *y*, and the times of the most recent common ancestor (tMRCA) of *x*, *y*, and *xy* are *tx*, *ty*, and *txy*, respectively. The time of the ancestral recombination event (ARE) originating the *xy* recombinant lineage (tARE) can be estimated as the interval between *txy* and the most recent of either *tx* or *ty*. We found the strain(s) most

closely related to the recombinant lineage (*xy*) in trees of *pol* PR-RT and *env* C2V5, which constituted the groups *x* and *y*, respectively, with *xy* members. We then inferred tMRCAs of groups *x* (*tx*), *y* (*ty*), and *xy* (*txy-pol* PR-RT and *txy-env* C2V5) used in an additional MCMC analysis consisting of 500 million iterations to estimate the evolutionary parameters using BEAST v.1.8.1.

To estimate the probable common ancestral sequence of CRF69_01B, we inferred the evolutionary history of the *env* gene from specimen sequences and AE references by the maximum likelihood method based on the GTR+G+I model³⁰ using MEGA6.³¹

Results

A new AE and B recombinant form, CRF69_01B, is identified in Japan

As shown in Fig. 1, 13 probable AE/B recombinant cases identified at NMC demonstrated identical discordance in subtyping of four regions: *gag* MA was identified as AE, *pol*

FIG. 1. Phylogenetic tree analyses of identified HIV-1 isolates. Neighbor-joining trees of different HIV-1 genes are shown. (A) HIV-1 *gag* MA (396 bps: positions 790 to 1185 coordinates of HXB2, K03455); (B) *pol* PR-RT (1,017 bps: 2,253 to 3,269); (C) *pol* IN (864 bps: 4,230 to 5,093); and (D) *env* C2V5 (799 bps: 6,872 to 7,670). Bootstrap values were calculated by 1,000 replicates; values above 90 are shown. Blue solid circles and letters show probable recombinant isolates identified at the Nagoya Medical Center (NMC) (K108, K132, K139, K214, K231, K255, K279, K320, K323, K372, K448, NMC172, and NMC200). Orange solid circles and letters show probable recombinants from the sequence collection of the Japanese Drug Resistance HIV-1 Surveillance Network (JP-B-29, -30, -31, -5, and -24). Reference sequences shown in black letters are summarized in Supplementary Table S2A. Color images available online at www.liebertpub.com/aid

PR-RT as B, *pol* IN as B, and *env* C2V5 as AE (Fig. 1A–D, blue circles and letters). In all four phylogenies, these 13 cases formed unique clusters with high bootstrap value (>90) within respective AE or B groups (Fig. 1A–D). Regarding the five additional probable AE/B recombinant cases found from the Surveillance Network, the same four regions were analyzed for three cases (JP-B-29, –30, and –31), and only *pol* PR-RT sequences were available from the other two cases (JP-B-5 and –24). As shown in Fig. 1, *pol* PR-RT for all five cases, and *gag* MA, *pol* IN, and *env* C2V5 sequences of JP-B-29, –30, and –31, clustered with 13 probable AE/B recombinant cases of NMC (Fig. 1A–D, orange circles and letters), suggesting their close genetic relationships.

From all 18 cases, we selected seven cases (K231, K279, K320, K323, K448, NMC172, and NMC200) for which we could obtain enough archive specimens of PBMCs and/or plasma to analyze their near full-length genomic sequences. Bootscanning analyses of these seven near full-length sequences revealed that all cases except K279 shared an identical recombinant structure (Fig. 2 and Supplementary Fig. S2), which differed from any previously reported AE/B recombinant forms.^{3–14} The requirement for declaring a new CRF, as proposed by the Los Alamos National Laboratory in 1999, is to have at least three cases with no direct epidemiological lineage, accompanied by near full-length sequences.^{32,33} From the medical records of the cases we confirmed that K231, K320,

FIG. 2. Determination of breakpoints for HIV-1 CRF01_AE/B recombinants. Bootscanning data (top) and schematic drawings (bottom) are shown for the genomic structures of (A) HIV-1 CRF69_01B and (B) K279. Bootscanning plots are shown for CM240 (CRF01_AE, U54771), HXB2 (subtype B, K03455), and 04CMU11421 (subtype J as outgroup, GU237072) in red, green, and gray, respectively. Analyses were performed with window size of 200 nucleotides and step size of 20 nucleotides, using the neighbor-joining algorithm with 500 replicates. Each breakpoint position is represented in aligned sequence data sets. In the schematic drawings, regions belonging to AE and B are shown in red and green, respectively. Numbering positions were adjusted to the HXB2 reference sequence. Each recombinant breakpoint is represented in Table 1 as the midpoint and range. Color images available online at www.liebertpub.com/aid

TABLE 1. EIGHT BREAKPOINT POSITIONS OF CRF69_01B

No.	Midpoint ^a	Range
1	1330	1306–1355
2	1482	1444–1520
3	1719	1669–1769
4	4320	4311–4330
5	4568	4527–4609
6	4961	4926–4996
7	5779	5749–5809
8	5911	5906–5916

^aNumbering positions were adjusted to the reference HXB2 sequence.

K323, K448, NMC172, and NMC200 were independently infected. Our data were carefully reviewed by editors of the Los Alamos HIV sequence database and this recombinant form was designated as CRF69_01B.

The genomic structure of CRF69_01B was identified as having eight breakpoints (Table 1 and Fig. 2A), where the N-terminal half of *gag* was identified as AE, the majority of *pol* as B, and the whole *env* as AE. The *vpr* and *tat* genes were composed of both B and AE. Regarding K279, the genomic structure was mostly identical to that of CRF69_01B, except that the AE fragment in the *pol* IN region (4,320–4,568) of CRF69_01B was not present (Fig. 2B and Supplementary Fig. S2B). This explains the observation that K279 in the *pol* IN tree lay outside the cluster containing CRF69_01B sequences with a bootstrap value of 97 (Fig. 1C). Therefore, K279 is classified as an independent recombinant strain and not CRF69_01B.

Regarding nine of the remaining cases (K108, K132, K139, K214, K255, K372, JP-B-29, –30, and –31), although near full-length sequences could not be analyzed due to the scarcity of PBMCs/plasma specimens, we were able to obtain sequences of the whole *pol* region. Bootscanning analyses of these sequences revealed that all nine cases contained an AE fragment insert in the IN region identical to that of CRF69_01B (Supplementary Fig. S3). From these results and the observation that all the probable recombinant cases were found within the clusters containing CRF69_01B for *gag* MA and *env* C2V5 (Fig. 1A and D), we concluded that these nine were also CRF69_01B cases.

For the remaining two cases, JP-B-5 and –24, because the nucleotide sequences of only the *pol* PR-RT region were available, we could not confirm the AE fragment in the *pol* IN region. Therefore, we decided to keep these two as “probable CRF69_01B cases.”

CRF69_01B emerged in the early 1990s as a result of recombination between subtypes B and AE that were cocirculating in Japan

To estimate the time of CRF69_01B emergence, tMRCAs of B and AE origins were calculated from the *pol* PR-RT and *env* C2V5 regions, respectively. For these analyses, 3,899 B *pol* PR-RT and 87 AE *env* C2V5 sequences collected in the Surveillance Network were used to construct NJ trees (Supplementary Fig. S4A and C). Clusters including CRF69_01B and genetically close sequences were trimmed out (Supple-

mentary Fig. S4B and D), and these sequences were reanalyzed by MCC trees of the Bayesian Coalescent MCMC method.

Figure 3 shows MCC trees of the *pol* PR-RT and *env* C2V5 regions with CRF69_01B cases making statistically significant clusters (0.94 and 1.0 posterior probability, respectively) in both regions. The B and AE sequences collected in the Surveillance Network are numbered in order of their collection years (Supplementary Table S3).

As summarized in Table 2, tMRCAs of the *pol* PR-RT and *env* C2V5 regions overlapped, sometime between 1986 and 1995 and between 1991 and 1996, respectively. Thus, the tARE of CRF69_01B were estimated to be sometime between 1991 and 1995.

Considering the origin of CRF69_01B, as shown in Fig. 3A, the MCC tree of the *pol* PR-RT region derived from CRF69_01B demonstrated a close genetic relationship with B strains collected in Japan (JP-B-1 to JP-B-42). Moreover, a *pol* PR-RT sequence from a full-length B case, JP-B-10, was found within a CRF69_01B cluster. This is clear evidence that CRF69_01B emerged from B strains circulating within Japanese high-risk populations.

Regarding the AE region of CRF69_01B, *env* C2V5 sequences of CRF69_01B cases were genetically close to AE strains collected in Thailand (Fig. 3B). Interestingly, JP-AE-2, –3, and –6 were found among Thai AE and CRF69_01B clusters, whereas other AE cases collected in Japan were located at genetically distant branches in the *env* C2V5 NJ tree (Supplementary Fig. S4C and D). These two findings indicate that the AE part of CRF69_01B was derived from AE strains circulating in Thailand. Furthermore, by searching the Los Alamos HIV sequence database, the strain most related to the common ancestral sequence of the CRF69_01B *env* region (Supplementary Table S4) was determined to be 93TH065 (AB220948) from Thailand, which had 91% identity with gaps of 33 bases.

Discussion

Since the late 1980s, subtype B' (Thai variant of subtype B)³⁴ has been the predominant HIV-1 subtype among injection drug users, while AE was found among commercial sex workers³⁴ in Southeast Asian countries. However, identification of multiple CRFs composed of AE and B', such as CRF15, 33, 48, 52, 53, 54, and 58, in these countries suggests that the two high-risk groups were not sequestered, but were crossing over each other. The discovery of CRF69_01B alerts clinicians that similar changes may be ongoing in interactions between high-risk groups in Japan and Thailand.

Although the first CRF69_01B was identified from a specimen collected in 2000, its tARE was estimated to be sometime between 1991 and 1995. The nearly 10-year gap between the time of its discovery and tARE suggests that the strain had initially been circulating within a limited area and/or community group before being widely transmitted throughout the country.

Where and how CRF69_01B emerged could have two scenarios. One is that the strain emerged within Japan by a crossover between two high-risk groups, MSM with B and heterosexual/MSM with AE. The other is that the strain was imported into Japan from neighboring countries where

FIG. 3. Subregion trees of CRF69_01B and its related viral variants. Partial maximum clade credibility trees are shown for (A) subtype B *pol* PR-RT sequences (1,017 bps) and (B) CRF01_AE *env* C2V5 sequences (799 bps) obtained by Bayesian MCMC analysis. The tips of the trees correspond to the sampling day, and the branch lengths reflect time. The dates of the times of the most recent common ancestor (tMRCA) means and 95% highest posterior density interval for the key nodes are displayed. Also shown are the CRF69_01B clade (red branches), members of CRF69_01B and probable cases (red letters), full-length B or AE strains that seemed to be the most closely related strains to the recombinant lineages (blue letters), strains isolated in Japan (green letters), and references registered in the Los Alamos HIV sequence database (black letters). Asterisks (JP-B-4, JP-B-31, JP-AE-1, and JP-AE-4) indicate the sequences obtained from non-Japanese patients. References and patient information are shown in Supplementary Tables S2B and S3, respectively. Color images available online at www.liebertpub.com/aid

the recombination event took place. The first scenario of CRF69_01B originating in Japan is supported by three of our findings: (1) the two full-length Japanese B strains with MSM as the risk behavior (Supplementary Table S3) were located upstream of the CRF69_01B cluster, (2) the *env* C2V5 region of this new recombinant form is unique as even the strain most closely related to the ancestral *env* C2V5 sequence (93TH065) was distant from the CRF69_01B lineage, and (3) CRF69_01B isolates were found only in Japan.

Our study serves to alert clinicians and policy makers that more attention must be paid to the international net-

work of high-risk populations, the inability of national boundaries to restrict sexual communications, and the chance that new recombinant AE/B strains may have emerged in Japan. It is not clear whether B and AE are virologically prone to recombination. Nonetheless, socially and/or virologically low barriers to recombination are suggested by the large number of reports in the past decade of more than 10 different types of CRFs between B and AE in Asian countries.^{3–14}

In conclusion, a new recombinant strain CRF69_01B was found in Japan. This discovery alerts stakeholders to the important need for continued surveillance of circulating HIV

TABLE 2. BAYESIAN MARKOV CHAIN MONTE CARLO EVOLUTIONARY PARAMETERS FOR NOVEL HIV-1 CIRCULATING RECOMBINANT FORMS IN JAPAN

Region (according to HXB2)	Substitution rate ^a	tMRCA of CRF (txy)	tMRCA of related groups (tx or ty)
<i>pol</i> PR-RT (2253–3269)	1.67 (1.36–2.01)	1995.6 (1991.4–1999.5)	1986.8 (1978.9–1993.5)
<i>env</i> C2V5 (6895–7653)	6.66 (5.91–7.45)	1996.1 (1994.4–1997.7)	1991.9 (1988.8–1994.4)

^aNucleotide substitution rates are expressed as 10^{-3} nucleotide substitutions per site per year, with the 95% highest posterior densities shown in parentheses.

tMRCA, time of the most recent ancestor; CRF, circulating recombinant form; PR, protease; RT, reverse transcriptase.

strains to understand changes in transmission networks of high-risk populations and to reconsider present intervention programs focusing mainly on Japanese MSM communities. Instead, such programs should widen their focus by collaborating with stakeholders in nearby countries to target high-risk populations internationally.

Sequence Data

Nucleotide sequences have been deposited in the DNA databank of Japan (DDBJ) as AB845344 through AB845349 for near full-length sequences of K231, K320, K323, K448, NMC172, and NMC200, respectively, and AB859012 for that of K279. Sequences of three regions for six cases, K108, K132, K139, K214, K255, and K372, have also been enrolled as AB845705 to AB845710 (*pol*), AB846829 to AB846834 (*env* C2V5), and AB442409, AB442426, AB442433, AB442491, AB442526, and AB442626 (*gag* MA), respectively.

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Author Disclosure Statement

No competing financial interests exist.

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