LETTER TO THE EDITOR

First description of two new HIV-1 recombinant forms CRF82_cpx and CRF83_cpx among drug users in Northern Myanmar

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Heroin trafficking routes have been integral to the human immunodeficiency virus (HIV) epidemic in Southeast Asia, and Myanmar has been central to all 4 known heroin trafficking routes associated with HIV-1 spread in this region.\textsuperscript{1} By the 1980s, 3 HIV-1 subtypes (B, C and circulating recombinant forms [CRF] 01\textunderscore AE) were being spread via these trafficking routes in Southeast Asia.\textsuperscript{2} The co-circulation of these 3 HIV-1 subtypes has resulted in the ongoing generation of inter-subtype recombinants among injection drug users (IDUs), particularly in the China-Myanmar border region where over 85\% of HIV-1 strains from IDUs are recombinants.\textsuperscript{3,4} Such generation of recombinant forms is likely because of dual infection within high risk individuals, like IDUs.\textsuperscript{5} When 3 or more recombinants are identified in at least 3 epidemiologically unlinked individuals and they share the same identical mosaic structures, these recombinants can be defined as a new CRF.\textsuperscript{6} Many CRFs have been identified in the world, and more than one third of them (28/79) have originated in Asia.\textsuperscript{7} However, no HIV-1 CRFs have been identified in Myanmar, in spite of high prevalence of HIV-1 unique recombinant forms (URF) in this country.\textsuperscript{5} On the other hand, few CRFs have caused epidemic in given regions. In China, 12 CRFs have been identified. Of them, CRF07\textunderscore BC, CRF08\textunderscore BC, and CRF55\textunderscore 01B led to a nation-wide prevalence.\textsuperscript{8-10}

Under the support of HIV/AIDS Asia Regional Program Yunnan Management Office and local government, 352 blood samples were collected from drug users who were at least 18 years old in detoxification centers in the Kokang Autonomous Region of Shan State (n = 252) and Mai Ja Yang region of Kachin State (n = 100) of Myanmar from November 2013 to November 2014 (Fig. 1A). Of these samples, 46 were HIV seropositive (Determine HIV-1/2, Alere Medical Co., Ltd, Chiba, Japan), Kokang (n = 31) and Kachin (n = 15). Near full-length HIV genomes (809–9124 base pairs, HXB2) were amplified and sequenced from 31 of 46 of these samples, Kokang (n = 26) and Kachin (n = 5) (Fig. 1B), according to methods described previously.\textsuperscript{11} To avoid the potential contamination, a negative control was included in each amplification experiment. The remaining 15 HIV-1 seropositive samples were unable to be amplified completely, probably due to the low level viremia and/or multiple freezing and thawing of these samples.

All the laboratory tests were performed in Key Laboratory of Animal Models and Human Disease Mechanisms of the Chinese Academy of Sciences & Yunnan Province, Kunming Institute of Zoology, Chinese Academy of Sciences. The obtained sequences were queried against all sequences obtained in this laboratory to check potential laboratory cross-contamination. These sequences have been submitted to the GenBank database under accession number KU820822-KU820852. The protocol of this study was approved by the Ethics Committee of the Kunming Institute of Zoology, Chinese Academy of Sciences (approval number: SWYX-2012017; date: October 10, 2012). All participants gave written informed consent.

To determine the genotypes of these 31 sequences, HIV-1 subtype reference alignment and all available near full-length HIV-1 sequences from Myanmar and the surrounding countries were downloaded from the Los Alamos HIV Sequence Database.\textsuperscript{7} Maximum likelihood (ML) tree was constructed using MEGA 7 with 1,000 bootstrap replications. To identify potential HIV-1
recombinants, bootscan analysis was performed using Simplot 3.5.1 software based on 100 replicates with a 200-bp sliding window moving in a step of 20 nt. Subtype B’ (AY173951), C (AF067155), F1 (AF077336), G (AF084936), and CRF01_AE (U54771) were used as subtype references in the bootscan analysis.

Phylogenetic analysis of these 31 sequences showed that only 4 clustered with known HIV-1 subtypes/CRFs: one CRF01_AE (fKSDU26), one CRF07_BC (mSSDU163) and 2 CRF08_BC (mSSDU91 and mSSDU220) (Fig. 2A). The other 27 sequences did not cluster with known HIV-1 subtypes/CRFs, suggesting that they may be new subtypes, unique recombinant forms or new CRFs. For six sequences (mSSDU12, mSSDU63, mSSDU75, mSSDU160, mSSDU191 and mSSDU195) from Kokang, phylogenetic analysis revealed that they formed an independent clade with high bootstrap value (clade I in Fig. 2A). Another 15 sequences (mSSDU21, mSSDU24, mSSDU28, mSSDU94, mSSDU109, mSSDU118, mSSDU137, mSSDU139, mSSDU144, mSSDU151, mSSDU153, mSSDU178, mSSDU180, mSSDU199 and mSSDU247) from Kokang formed another independent clade (clade II in Fig. 2A). Bootscan analyses revealed that clade I strains comprised recombinants of HIV-1 subtypes B, C and CRF01_AE with 7 identical breakpoints: 4 C, 3 B, and 1 CRF01_AE segments (Fig. 2B). Because of sharing same identical mosaic structures, they were designated as a new CRF (CRF82_cpx). Similar to clade I, clade II strains comprised recombinants of HIV-1 subtypes B, C and CRF01_AE, and 11 (mSSDU21, mSSDU24, mSSDU28, mSSDU94, mSSDU109, mSSDU118, mSSDU137, mSSDU144, mSSDU151, mSSDU178 and mSSDU180) of them shared 11 identical breakpoints: 5 C, 4 B, and 3 CRF01_AE segments and were designated another new CRF (CRF83_cpx) (Fig. 2C). To determine whether CRF82_cpx and CRF83_cpx existed previously, HIV BLAST (basic local alignment search tool) was performed using all CRF82_cpx and CRF83_cpx sequences obtained in this study as the query set. The top hit sequences generated by HIV BLAST were further subjected to bootscan analysis. Comparison of the recombination structures showed that none of the previously reported sequences shared same mosaic structure with the strains of CRF82_cpx and CRF83_cpx. These data strongly suggest that CRF82_cpx and CRF83_cpx have not been described previously.

Interestingly, in clade II, 4 strains (mSSDU139, mSSDU153, mSSDU199 and mSSDU247) were located outside the new CRF83_cpx cluster (Fig. 2A). Bootscan analyses found that these sequences (especially mSSDU199 and mSSDU247) shared some breakpoints with CRF83_cpx, suggesting that they might be an evolutionary intermediate of CRF83_cpx (Fig. 3A). To
evaluate this, we re-performed a bootscan analysis using subtype B, C, CRF01_AE, and mSSDU199 as the subtype references. If CRF83_cpx originated by recombination between mSSDU199 and subtype B, the % permuted trees line will break the genomes into fragments that resembled mSSDU199 and subtype B with high bootstrap values (rather than CRF01_AE, B and/or C) in most sequence regions. Except for a short fragment in vpr-vpu region that resembled subtype B, all other regions of CRF83_cpx resembled mSSDU199 with very high bootstrap values (more than 80% in most regions). This indicated that CRF83_cpx was most likely generated by the second generation recombination between mSSDU199 and subtype B (Fig. 3B and C). In fact, such second-generation recombination has been observed previously,10,13-16 and may serve as an evolutionary force to increase HIV diversity, and may explain why HIV-1 recombinants are becoming more complicated.3,4,17

In the Kokang region, the 2 newly identified CRFs_cpx accounted for 65.4% (17/26) of all strains sampled, being the predominant circulating strains. Overall, CRF01_AE/B/C recombinant forms (RF_01/B/C) represented 80.8% (21/26) of all strains sampled while RF_B/C accounted for 11.5% (3/26) (Fig. 1B). This is strikingly different from our previous study that found a high proportion of HIV-1 RF_B/C (37/79, 46.8%) and RF_01/B/C (29/79, 36.7%) among IDUs in Kachin state (RF_B/C 11.5% vs 46.8%, p = 0.01; RF_01/B/C 80.8% vs. 36.7%, p < 0.0001, Fisher Exact Test).3 It is unclear if the new predominance of these strains in the region represents a high transmissibility of these new CRFs_cpx or circulation within a susceptible high risk group. It is clear, however, that the generation of HIV-1 inter-subtype recombinants continues in Northern Myanmar and these recombinants are becoming more complex.3,4,17

This study has a number of limitations, mostly concerning the use of convenience sampling and the small sample size. Due to the unstable political environment in Myanmar, such as the armed conflict between Myanmar National Democratic Alliance Army and Tatmadaw in 2009 and 2015,18 only samples from Kokang and Kachin were included in this study. Because current HIV molecular epidemiology in Middle and Southern Myanmar is unavailable, we cannot be sure if there is an epidemiological link in HIV-1 migration between Kokang and other region of Myanmar. Although this study had a small sample size (5 from Kachin and 26 from Kokang) and likely sampling bias, the sequences from Kachin and Shan neither clustered together in the phylogenetic tree nor shared same recombination patterns (Fig. 2A). Also, HIV-1 strains isolated from Mandalay (Middle Myanmar) in 1999–2000 did not cluster with the strains in
Figure 3. Origin mechanism of CRF83_cpx via second-generation recombination between RF_01/B/C (mSSDU199) and HIV-1 subtype B. (A) Comparison of the bootscan plots between mSSDU199 and mSSDU94 (CRF83_cpx). (B) Bootscan plot of mSSDU94 (CRF83_cpx) using B', C, F1, G, CRF01_AE and mSSDU199 as subtype references. (C) Origin mechanism of CRF83_cpx via the 2nd generation recombination between RF_01/B/C (mSSDU199) and HIV-1 subtype B. Dotted line shows 2 distinct recombination breakpoints in the mosaic structures between mSSDU199 and mSSDU94 (CRF83_cpx), which just represent the recombination breakpoints between RF_01/B/C (mSSDU199) and HIV-1 subtype B.
Kachin and Shan (Figs. 1A and 2A). Altogether, these observations suggested that HIV-1 strains circulating in these areas were relatively separated from each other, and a nation-wide epidemiological investigation was needed to determine the prevalence status of these new CRFs_cpx in Myanmar.

It also remains unclear where these new CRFs_cpx arose. Although we identified the 2 new CRFs_cpx in Kokang, we cannot conclude that they originated in Kokang and spread to other places. As an important station for production and transport of illegal drugs, Northern Myanmar has the 4 heroin trafficking routes in Southeast Asia and plays a crucial role in the transmission of HIV-1 in Southeast Asia. Kokang Autonomous Region borders with Yunnan, China. Some drug users living in Kokang often cross the China-Myanmar border each month to inject drugs, and this population movement likely increases the risk of spread of new HIV CRFs_cpx on both sides of the border.

To better understand timing of the origins of the 2 CRFs_cpx, we performed Bayesian analysis to estimate the time to the most recent common ancestor (tMRCA) of CRF82_cpx and CRF83_cpx. Maximum clade credibility (MCC) trees were reconstructed based on the longest mutual region of subtype C origin of both CRFs (2695–3290 nt in HXB2) and unique CRF01_AE regions of each CRF (5758–6359 nt and 27006–7277 nt in HXB2). Subtype C and CRF01_AE sequences with known sampling years and location were downloaded from the Los Alamos HIV Sequence Database, and subjected to the phylogeographic analysis together with the sequences obtained in this study. The tMRCA of these CRFs were calculated, with 95% confidence interval. The results showed that CRF82_cpx originated around 2002–2006 and CRF83_cpx around 2007–2008 (Fig. 4). Phylogeographic analyses showed that the location states of tMRCA of CRF82_cpx and CRF83_cpx were Myanmar (Fig. 4).

Together with the fact that CRF82_cpx and CRF83_cpx were found in Northern Myanmar and that CRF82_cpx and CRF83_cpx strains have not previously been described in other countries/regions, these results suggest that both CRF82_cpx and CRF83_cpx most likely originated in Myanmar. A larger molecular investigation will be needed to determine how prevalent they are in Mandalay, Myanmar and Yunnan, China.

Myanmar has been a "hub" for the spread of HIV-1 in Asia. Thus, it is likely that once these 2 new CRFs_cpx spread to middle Myanmar and Yunnan, China, they have high possibility of spreading across Southeast Asia via drug-trafficking routes. Given previous epidemiologic patterns, it is likely that the outbreak of these 2 new CRFs_cpx, as well as the appearance of CRF07_BC and CRF08_BC in Myanmar, will change HIV-1 molecular epidemiology patterns in the region, and perhaps across Southeast Asia. We, therefore, propose international collaborative efforts to closely monitor the

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**Figure 4.** MCC trees of CRF82_cpx and CRF83_cpx. (A) The MCC tree based on the longest mutual region of subtype C origin of both CRF82_cpx and CRF83_cpx (2695–3290 nt in HXB2). (B) MCC tree based on the unique CRF01_AE region of CRF82_cpx (5758–6359 nt in HXB2). (C) MCC tree based on the unique CRF01_AE region of CRF83_cpx (7006–7277 nt in HXB2). Black dots indicate tMRCA of CRF82_cpx and CRF83_cpx. The time of tMRCA are shown with 95% confidence interval.
changing molecular epidemiology of HIV-1 in the region.

**Abbreviations**

- **BLAST** basic local alignment search tool
- **CRF** circulating recombinant form
- **HIV** human immunodeficiency virus
- **IDUs** injection drug users
- **MCC** maximum clade credibility
- **ML** maximum likelihood
- **RF** recombinant form
- **tMRCA** the most recent common ancestor
- **URF** unique recombinant form

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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