On-going generation of multiple forms of HIV-1 intersubtype recombinants in the Yunnan Province of China

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**Objectives:** To investigate the molecular epidemiology of HIV in China's Yunnan Province, where the initial HIV-1 outbreak among injecting drug users (IDU) occurred in 1989, and to analyse the genesis and interrelationship of the epidemic with that in surrounding areas.

**Design:** A molecular epidemiological investigation was conducted among IDU in three prefectures in Yunnan Province, including Wenshan (east), Honghe (southeast) and Dehong (west).

**Methods:** Thirty-nine specimens were collected from consenting IDU in 2000–2001. The nucleotide sequences of 2.6 kb gag–RT and 340 base pair (bp) env (C2/V3) regions were determined. Phylogenetic tree and recombination breakpoint analyses were performed.

**Results:** The circulating recombinant form (CRF), CRF08\_BC, predominated in east Yunnan near Guangxi Province (89% in Wenshan and 81% in Honghe), whereas it was not detected in Dehong (0/14) in the west. In contrast, 71\%(10/14) of the Dehong isolates were unique recombinant forms (URF), mostly between subtypes B' (Thailand variant of subtype B) and C, with distinct profiles of recombination breakpoints. The subtype B' accounts for the remaining 29\%(4/14) of Dehong isolates. Interestingly, two Honghe isolates (2/16) shared some of the precise B'/C recombination breakpoints with CRF07\_BC.

**Conclusion:** New recombinant strains are arising continually in west Yunnan near the Myanmar border. Some appeared to be secondary recombinants derived from CRF07\_BC that had further recombined with other strains. The uneven distribution of subtypes, CRF and URF, suggests the presence of independent transmission networks and clusters among IDU in Yunnan.

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Introduction

HIV-1 infection continues to spread in China, primarily through injecting drug user (IDU) networks [1,2]. A total of 22,517 HIV infections were reported in China to September 2000; IDU accounted for 71% of infections (http://www.aids.net.cn). Unlike other HIV-1 epidemics, which have spread from urban to rural areas, the first HIV epidemic in China began in a rural area (Dehong Prefecture) in western Yunnan Province in 1989, and then spread to urban areas along the routes of drug trafficking [3–6]. Yunnan Province borders the heroin-producing areas (‘Golden Triangle’) of southeast Asia, consisting of northern and eastern Myanmar, northern Thailand and western Laos, and is thought to be a crucial intersection of the drug trafficking routes to other areas in China. The largest number of HIV infections (8,317 cases, 40% of the total cases in China) were reported in Yunnan Province. Injecting drug use is the major risk factor in Yunnan, accounting for approximately 80% of HIV infections in this province. The highest HIV prevalence among IDU (over 70% since 1995) was found in Dehong Prefecture in western Yunnan near the border with Myanmar [7]. Average HIV prevalence among IDU in Yunnan rose from 6.8% in 1995 to 27.8% in 1999 [7]. During the period 1996–1997, HIV outbreaks occurred among Chinese IDU outside Yunnan, including Xinjiang Province in northwestern China and Guangxi Province in southeastern China [1]. Overland heroin trafficking routes through Yunnan Province apparently played a key role in the spread of HIV in China [5–9].

Genetic subtyping has been a useful tool to track HIV spread and to study the genesis of the epidemic in specific areas. The HIV-1 epidemic among IDU in Yunnan was initiated with both HIV-1 subtype B (same lineage of subtype B strains isolated in the United States and Europe) and subtype B’ (Thailand variant of subtype B, also referred to as Thai-B) strains [10–12]. The subtype B’ strain appeared to replace subtype B of US–European lineage, increasing from 20% of all subtype B in 1990 to 90% in 1996 [10–12]. HIV-1 subtype C strains were identified among IDU in China in 1992 [13]. CRF01_AE strains were found among women who had returned from Thailand to Yunnan after working as commercial sex workers in Thailand [14].

More recently, it has been reported that two circulating recombinant forms (CRF), CRF07_BC and CRF08_BC, arose and began to circulate widely among IDU in China [5,6]. These two CRF are composed mostly of subtype C and contain a few small segments of subtype B’. Their structures are closely related, but have distinct differences (Fig. 1a). It has been proposed that each CRF was associated with a different overland heroin trafficking route: CRF07_BC has been spreading northwestward to Xinjiang Province, whereas CRF08_BC spread eastward to Guangxi Province from their common origin presumably in Yunnan, where subtypes B’ and C are co-circulating [5,6].

Despite the importance of Yunnan Province in the genesis and spread of HIV-1 strains in China, the genetic structure of HIV-1 strains circulating in Yunnan has not been extensively investigated. In the present study, we explored the molecular epidemiology and genetic structures of HIV-1 strains in three sites in Yunnan Province, and found evidence of the ongoing generation of multiple forms of intersubtype recombinants in western Yunnan. On the basis of our findings, we discuss the genesis of the HIV epidemic in this particular area of China, and the implications for prevention strategies.

Materials and methods

Study subjects and specimens

Blood specimens were collected from 39 HIV-positive consenting IDU from three prefectures in Yunnan Province, including Wenshan (east), Honghe (southeast) and Dehong (west), during the period from August 2000 to March 2001. Subjects included 35 men and four women with the age range of 17–43 years (mean 29.4 years). The mean duration of drug use was 5 years (range 2–11 years). Peripheral blood mononuclear cells (PBMC) were separated on Ficoll gradients. Viruses were isolated by co-cultivation of PBMC from seropositive IDU with CD8-depleted phytohemagglutinin-stimulated donor PBMC. Positive virus cultures were detected from cell culture supernatants by using virion-associated reverse transcriptase (RT) assays. Plasma samples were saved for nucleotide sequence determination of virion HIV-1 RNA. Seven additional serum specimens from sexually transmitted-disease (STD) patients collected in Kunming between 1994 and 1997 were subjected to analysis for comparison.

Nucleotide sequence determination and data analyses

The nucleotide sequences of 2.6 kb gag-RT and 340 base pair (bp) env (C2/V3) regions [15–17] were determined from viral RNA in the plasma or virus culture supernatants on an automated ABI PRISM310 DNA sequencer (Applied Biosystems, Inc., USA) by using cycle-sequencing and dye terminator methods. The details of procedures are available upon request. All the nucleotide sequences obtained in the present study were screened using the BLAST 2.0 program (National Center For Biotechnology Information, USA) to search for sequence similarities to previously reported sequences in the databases, and
to rule out potential laboratory errors. Nucleotide sequences were aligned with the reference strains [18] and HIV-1 subtype B' (RL42) [10], using CLUSTAL W version 1.4 [19], and corrected manually to ensure that gaps did not alter the reading frame. Frequencies of nucleotide substitutions were estimated by Kimura’s two-parameter method [20]. Phylogenetic trees were constructed by the neighbour-joining method [21] with 100 bootstrap replicates [22]. Analyses were implemented by PHYLIP, version 3.573 [23]. Simplot 2.5 software was used to identify the recombination breakpoints [24]. Informative site analysis was performed as described [25,26] to confirm the precise boundaries of intersubtype recombinations.

Nucleotide sequence accession numbers
The Genbank accession numbers of nucleotide sequences reported in this article are AB078639–AB078716.

Results
Experimental design for characterization of HIV-1 strains circulating in Yunnan Province
As the large-scale determination of full-length HIV-1 nucleotide sequences is not practical for molecular epidemiological investigation, we developed a convenient and less labour-intensive protocol optimized for the characterization of genetic structures of HIV-1 strains circulating in Yunnan Province. In addition to the 340 bp env (C2/V3) regions, we determined the nucleotide sequences of the 2.6 kb gag-RT regions of the HIV-1 genome, in which the two B/C recombinants, CRF07_BC and CRF08_BC, circulating widely among IDU in China, exhibit the distinct profiles of recombination breakpoints [5,6] (Fig. 1a and b). Thirty-nine specimens from IDU at three sites in Yunnan (Wenshan, Honghe, and Dehong Prefectures) (Fig. 2) were subjected to the analyses. For comparison, seven archival specimens from HIV-positive STD patients in Kunming were also analysed (Fig. 2). We classified HIV-1 strains in Yunnan on the basis of: (i) the phylogenetic relationship of the nucleotide sequences in the 2.6 kb gag-RT and env (C2/V3) regions (data not shown); and (ii) the profile of recombination breakpoints in gag-RT segments. The recombination breakpoints were screened and identified by bootscanning plots (Fig. 1) and informative site analyses (data not shown). Corroborating the data obtained by these recombination identification program, the unique recombinant forms (URF) are identified as ‘outliers’ outside the clusters of known subtypes and CRF on phylogenetic trees (data not shown).

Overall trends in the distribution of HIV-1 subtypes and circulating recombinant forms
The distinct pattern of the distribution of subtypes CRF and URF has emerged (Fig. 2). The majority of specimens from eastern Yunnan near Guangxi Province showed a structural profile identical to CRF08_BC in the gag-RT regions (Fig. 1a). They belonged to subtype C in the env (C2/V3) region, as expected for CRF08_BC (Fig. 1a). CRF08_BC accounted for 89% (8/9) and 81% (13/16) of IDU specimens from Wenshan and Honghe, respectively, whereas it was not detected in Dehong Prefecture in western Yunnan Province (Fig. 2). In contrast, HIV-1 subtype B’ was found only in Dehong Prefecture (4/14, 29%). HIV-1 subtype C and CRF01_AE were not found among IDU in the present study. Only one CRF01_AE was detected among seven archival specimens from STD patients in Kunming (14%, 1/7) (Fig. 2).

Evidence for the formation of secondary recombinants in Honghe Prefecture
Although CRF07_BC was found in low prevalence in Yunnan (2/39, 5.1%) (Fig. 2), the bootscanning plots and informative site analyses (data not shown) revealed that two Honghe isolates, HH069 and HH086, are closely related to CRF07_BC (Fig. 1c). These two strains shared three or four of the precise subtype B/C recombination breakpoints with CRF07_BC in the gag-RT region (Fig. 1c). Compared with CRF07_BC, the B’ segment in the middle of the gag-RT region were missing in HH086, whereas the B’ segment in the middle of the gag-RT region was shortened, and instead a short CRF01_AE segment was inserted in the 3’-RT region of HH069 (Fig. 1c). We designated them ‘CRF07_BC-related unique recombinant form’ (07-URF) (Fig. 1 and Fig. 2). The env (C2/V3) subtypes of HH069 and HH086 were B’ and C, respectively (Fig. 1c). HH069 appeared to be a much complicated recombinant, composed of CRF07_BC and CRF01_AE with subtype B’.

Majority of HIV-1 strains circulating in Dehong Prefecture are unique recombinant forms
In contrast to the eastern prefectures, Dehong showed a distinct feature in the distribution of HIV-1 subtypes and CRF. HIV-1 subtype B’ strains were found only in Dehong Prefecture (4/14, 29%), whereas no CRF08_BC was detected (0/14) in this area (Fig. 2). Interestingly, 71% of Dehong isolates (10/14) were various types of URF (Fig. 2) harbouring different recombination breakpoints (Fig. 1d). Most Dehong URF (9/10) were chimeras between subtypes B’ and C, whereas only one isolate (DH006) had a recombination breakpoint between B’ and CRF01_AE in the gag-RT region (Fig. 1d). The env (C2/V3) regions of most Dehong URF (7/10) belonged to subtype C, whereas three URF (DH006, 017 and 019) that were composed mostly of HIV-1 subtype B’ in the gag-RT
Fig. 1. Schematic representation of subtype structures of HIV-1 strains from Yunnan Province based on the phylogenetic and recombination breakpoint analyses of the 2.6 kb gag-RT and 340-bp env (C2/V3) regions. The regions of subtypes B' and C, and CRF01_AE are depicted in different colours as indicated in the inset. Regions in white indicate the areas that were not analysed or could not be decisively assigned to either subtype B' or C. The bootscanning plots for the 2.6 kb gag-RT regions of the selected strains, depicting the relationship to the indicated reference strains (inset in the right), are shown at the right. The bootstrap values were plotted for a window of 200 bp moving in increments of 50 bp along the alignment. As for circulating recombinant forms (CRF) and HIV-1 subtype B', the bootscanning plot for only one representative strain from Yunnan is shown. The vertical lines show the B'/C recombination breakpoints that appear to be shared among isolates. (a) CRF08_BC; (b) CRF07_BC; (c) 07-URF (CRF07_BC-related unique recombinant form); (d) Dehong URF; (e) subtype B'.
regions belonged to subtype B' in the env (C2/V3) region (Fig. 1d). Among Dehong URF, DH009 was subtype B′ throughout the 2.6 kb gag-RT region, but was subtype C in the env (C2/V3) region (Fig. 1d).

Recombination breakpoints in Dehong URF differed from isolate to isolate, and were not identical to any known CRF, including CRF07_BC and CRF08_BC. Interestingly, however, bootscanning plot and informative site analyses (data not shown) revealed that some of the B′/C recombination breakpoints in the gag-RT regions were shared between different sets of Dehong URF (Fig. 1d). The two B′/C recombination breakpoints in DH003, DH012 and DH015 and the single B′/C breakpoint in DH008 and 016 appeared to be identical, respectively.

Discussion

Yunnan Province, where the initial HIV outbreak among IDU occurred in 1989, is thought to be the epicentre of the HIV epidemic among IDU in China [3–6]. In the present study, we demonstrated that HIV-1 subtypes, CRF and URF showed a unique...
geographical distribution in Yunnan Province (Fig. 2). We found that CRF08_BC predominated in the eastern part of Yunnan. The prevalence of CRF08_BC was highest in Wenshan in the east (89%, 8/9), followed by Honghe Prefecture (81%, 13/16), whereas it was not detected in Dehong Prefecture (0/14) in the west, showing the gradient towards the east (Fig. 2). This may be related to the CRF08_BC epidemic in neighbouring Guangxi Province [5,9]. Although the exact origin of CRF08_BC is not known, it is plausible that CRF08_BC might have arisen in Yunnan Province and spread along the southeastern drug trafficking route to Guangxi Province [8], where CRF08_BC is reported to be the principal circulating strain among IDU [5,9].

In contrast, CRF07_BC was detected in low prevalence (2/39, 5.1%) among IDU in Yunnan (Fig. 2). This might suggest that CRF07_BC had emerged sometime in the past in Yunnan, but subsequently migrated far along the northwestern drug trafficking route to Xinjiang Province [8]. Alternatively, CRF07_BC might have originated outside Yunnan somewhere along the northwestern drug trafficking route. To address these questions, it would be interesting to analyse the archival specimens that were sampled at the earlier timepoints in Yunnan and the surrounding areas.

The two Honghe URF (HH069 and HH086) showed the possible linkage to CRF07_BC, because both shared some of the precise B'/C recombination breakpoints with that of CRF07_BC (Fig. 1c). This implies that these URF are ‘secondary’ recombinants derived from CRF07_BC that have further recombined with other HIV-1 strains. This could be the first evidence that CRF can recombine with other strains, leading to the emergence of ‘secondary’ recombinants. Conversely, however, as HH086 exhibits a much simpler recombinant structure than that of CRF07_BC, it would be possible that the HH086-like strain may reflect the structure of the ‘parental’ form of CRF07_BC rather than that of the ‘secondary’ recombinant generated by the ‘back-cross’ of CRF07_BC with other subtype C strains.

Multiple forms of URF harbouring a variety of unique recombination breakpoints were found in Dehong Prefecture (Fig. 1d) near the Myanmar border (Fig. 2). To our surprise, 71% of the strains circulating among IDU in Dehong (10/14) were URF (Fig. 2). The structures of URF identified in Dehong differed from each other, and did not show any similarity to known CRF or other recombinants (Fig. 1d). This strongly suggests that new recombinants are arising continually in Dehong Prefecture. The highest HIV prevalence among IDU has been over 70% since 1995 in Dehong Prefecture [7], suggesting that the IDU in this region are highly and continuously exposed to new virus strains. The mixing of the subtypes in such a highly exposed population may quickly lead to the generation of new recombinant viruses. It has been reported previously that HIV-1 subtype B' and subtype C were co-circulating among IDU in the western part of Yunnan Province, on the basis of a study carried out at two sites in Dehong in 1992 [13]. These two strains may represent a potential reservoir for the intersubtype recombination.

Interestingly, the generation of Dehong URF appears to be not completely independent. The sets of Dehong URF (DH003, 012 and 015, and DH008 and 016) shared the precise B'/C recombination breakpoints in gag-RT regions (Fig. 1d), suggesting the presence of intricate networks of HIV-1 transmission among IDU in the western part of Yunnan Province.

It is also noted that the pure (non-recombinant) form of HIV-1 subtype C was not detected (0/39) in Yunnan Province in the present study. In contrast, HIV-1 subtype B' was detected in the Dehong area and apparently remains intact, despite such high-risk settings where the frequent mixings between different lineages of HIV-1 strains are expected. This may suggest the presence of independent transmission clusters and networks among IDU in western Yunnan. Similar observations showing evidence for independent transmission networks among IDU were reported in studies in the Netherlands [27] and Russia [28]. Alternatively, it may imply that there are continual inflows of HIV-1 subtype B' strains into the Dehong area from surrounding regions, most likely from neighbouring Myanmar, where subtype B' is one of the most prevalent strains [17,29]. A molecular epidemiological study with a large sample size, coupled with extensive epidemiological investigation, is necessary to elucidate the detailed mechanisms of the uneven distribution of subtypes and of the formation of new URF in Yunnan Province.

The emergence of a new generation of recombinants may further complicate the development of effective vaccines to limit the HIV-1 epidemic. HIV vaccine efficacy, once established, may be challenged by novel recombinant strains. Finally, it remains to be investigated whether recombination may confer selective advantages over parental viruses. Studies are now underway to elucidate the biological consequence of the intersubtype recombination observed in this area.

**Conclusion**

We demonstrated that new forms of recombinants have been generated continually among highly exposed IDU
in western Yunnan, and provide insights into the genesis and spread of HIV-1 strains in China.

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