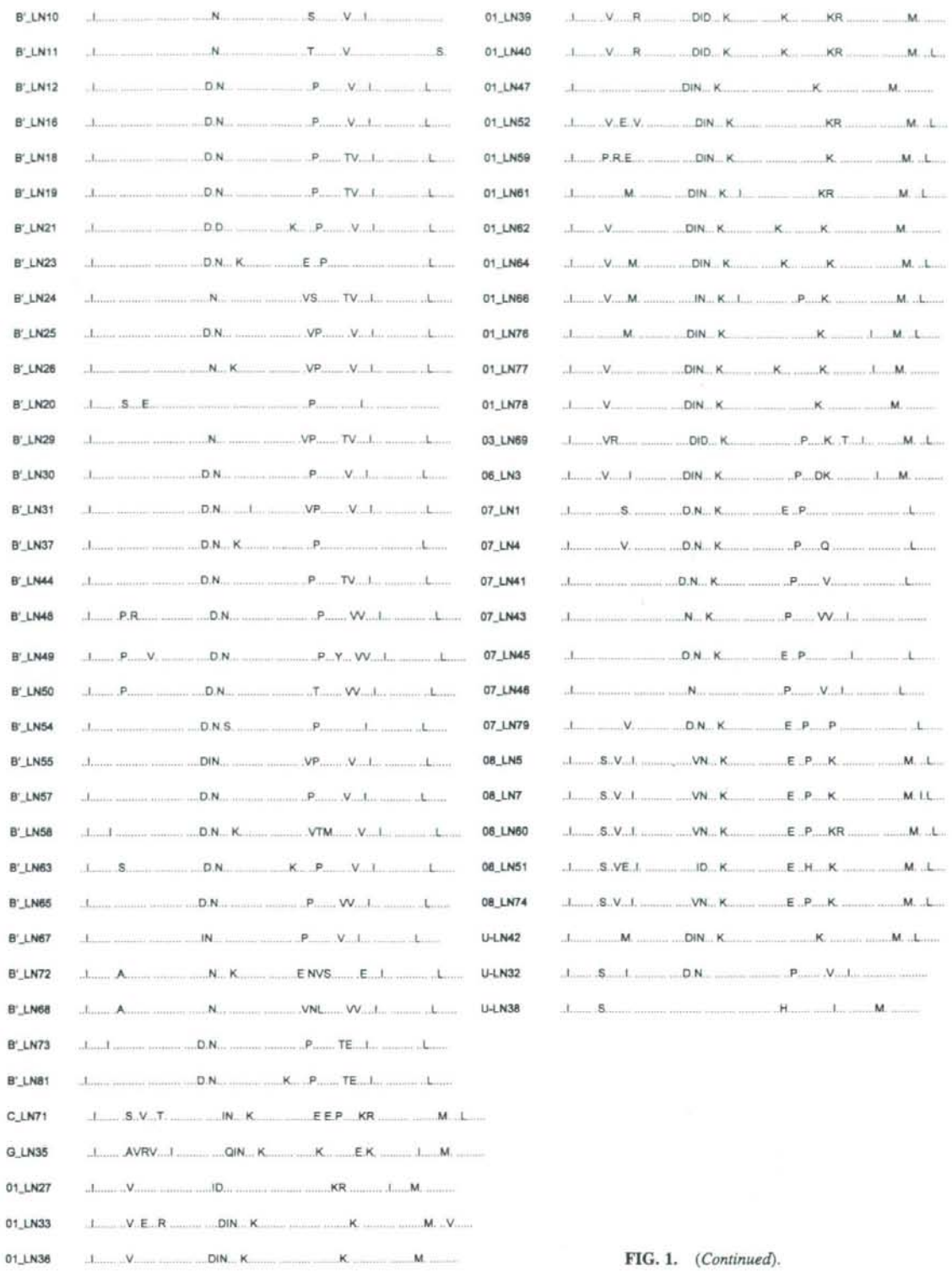

FIG. 1. (*Continued*).

(IDUs). And there were many HIV-1 subtypes in sexual infectors, in which both CRF01_AE and B′ were more popular; CRF07_BC, CRF08_BC, subtype A, subtype B, subtype C, subtype G, CRF03_AB, and CRF06_cpx can also be found. One mother-to-child transmission was subtype A (Table 1).

PI-related drug resistance mutations from treatment-naive HIV-1-infected patients

Major mutation M46I, which decreased the susceptibility to IDV RTV and NFV, was found in three patients (3 of 91). Various minor mutations were detected at the following positions in the PR gene: I93L (71.4%), L63P (62.6%), V77I (62.6%), M36I/V (33.0%), A71T/V (22.0%), K20R (6.6%), G16E (6.6%), and L10I (5.5%) (Table 2). Figure 1 shows the alignment of the amino acid sequences of the PR gene for each strain compared with an HXB_2.

RTI-related drug resistance mutations from treatment-naïve HIV-1-infected patients

One major mutation, M184I, associated with RT-related drug resistance, which confers high-level resistance to 3TC and FTC, was detected among 91 specimens (1.1%). Several other mutations including M41L (1 of 91), T69N (1 of 91), A62V (2 of 91), and K103R/Q (3 of 91) were detected (Table 3).

Subtype-specific differences in patterns of amino acid substitution

L63P, the second predominant minor mutation in this study, was observed in 36% of the non-B strains and in 77% of the B strains (chi-square 15.27, $p < 0.01$). V77I was observed in 13% of the non-B strains and in 96% of the B strains (chi-square 61.00, $p < 0.01$). A71V/T was observed in 5% of the non-B strains and in 34% of the B strains (chi-square 9.41, $p < 0.01$). Although M36I/V was not the predominant minor mutation, it was observed in 76% of the non-B strains and in 2% of the B strains (chi-square 52.54, $p < 0.01$); M36I/V was detected in all CRF08BC (5 of 5) strains but in none of the CRF07BC (0 of 7) strains. L10I was observed in 2% of the non-A strains and in 75% of the subtype A strains (Fisher exact test, $p < 0.01$). G16E and K20R/I were detected in 6 of 38 non-B strains, but in 0 of 53 B strains (Fisher exact test, $p < 0.01$). V82I was detected in 5 of 38 non-B strains, but in 0 of 53 B strains (Fisher exact test, $p < 0.05$). I93L was detected in 0 of 4 A strains, and in 64 of 87 non-A strains (Fisher exact test, $p < 0.01$). In the RT region, we found several drug resistance mutation; we cannot determine the subtype-specific mutation.

DISCUSSION

Our study aims to elucidate the emergence of drug resistance-associated mutations and the natural occurrence polymorphisms in both PR and RT genes of HIV-1-infected treatment-naive patients living in the northeast part of China.

This study showed that the prevalence of resistance mutations to PIs and RTIs was 3.3% and 1.1%, respectively, in treatment-naïve patients in Liaoning province, revealing that the primary resistance of HIV-1 circulating in this area is lower than in other previous reports.[15,16] In addition, we detected many minor mutations that were not always associated with a decrease in *in vitro* susceptibility and that can compensate for the reduced fitness of resistant mutants. This study demonstrated that the protease-encoding region of each HIV-1 strain had at least one secondary mutation associated with PI resistance. The clinical efficacy of a switch from one PI to another might therefore be compromised when the virus has had the opportunity to develop compensatory mutations.

Given the complexity of HIV-1 subtypes circulating in this area, we obtained information on different profiles of drug resistance-associated mutations from each subtype. Amino acid substitutions such as L63P, V77I, and A71V/T were frequently found in subtype B and B′, and were significantly higher in this study than in previous studies with subtype B isolates.[17,18] M36I was associated with HIV-1 non-B strains, especially with CRF01_AE and subtype A. L10I, G16E, K20R/I, and V82I were associated non-B, L10I was found in subtype A, and I93L was associated non-A. It is interesting that M36I/V was de-

TABLE 3. AMINO ACID SUBSTITUTIONS IN THE HIV-1 RT GENE (1–250 AMINO ACIDS) OF 91 TREATMENT-NAIVE PATIENTS AT POSITIONS ASSOCIATED WITH RESISTANCE TO NRTIS AND NNRTIS

Subtype	*Number of strains*	*M41L*	*A62V*	*T69N*	*K103R/Q*	*M184I*
A	4					
B	3					
B′	50					
C	1					
G	1					
CRF01-AE	15				2	
CRF03-AB	1		1			
CRF06-cpx	1					
CRF07-BC	7					1
CRF08-BC	5		1	1	1	
Unclassifiable	3	1				
Total	91	1 (1.1%)	2 (2.2%)	1 (1.1%)	3 (3.3%)	1 (1.1%)

tected in all CRF08BC strains but in none of the CRF07BC strains, reflecting the existence of a distinguished amino acid polymorphism in CRF07_BC and CRF08_BC strains. We compared the PRI-associated mutation patterns with the sequences from HIV-1 stains circulating in southern China, and found the CRF01_AE strains share a similar pattern of drug resistance mutations, except for I93L, for which nearly all sequences from Southern China keep 93I in the PR gene, whereas about 50% of the sequences in this study showed 93L. Our study detected both CRF07BC and CRF08 BC in Liaoning province, which by phylogenetic analysis were clustered together with CRF07_BC strains from Guangxi and Yunnan province, and CRF08_BC strains from Yunnan province, respectively, indicating the origins of HIV-1 transmission. However, the CRF08_BC strain found in Liaoning showed more complex polymorphism than that found in Yunnan.[19]

The presence of secondary mutations and/or the natural polymorphism of amino acids in the PR gene in subtype B strains have already been reported. However, information on the secondary mutation and/or polymorphism in the PR gene in non-B subtypes is still lacking, especially in China. Our study revealed that some secondary mutations, such as 93L, that are often present as a polymorphism in the PR gene were observed in non-B HIV-1 subtypes in the subjects studied. Furthermore, natural polymorphisms are often present in HIV-1 non-B subtypes at positions known to be associated with drug resistance in subtype B strains. These changes might influence the emergence of drug-resistant viruses, modifying drug susceptibility and/or virus replication capacity.[20] The improved viral replication of mutant 36I might favor a more rapid spreading of non-B subtypes of HIV-1; moreover, different pathways may lead to drug resistance accoring to HIV-1 subtype, based on a recent report.[21]

Only one patient, who was HCV coinfected, was found to harbor an M184I mutant that confers high-level resistance to 3TC and FTC. This patient has not received any ARV treatment for his HCV infection, indicating the M184I in RT in this patient might be primary resistance. In addition, several other mutations including M41L, T69N, A62V, and K103R/Q, were detected in this study. M41L usually occurs with T215Y, conferring an intermediate-to-high level resistance to AZT and d4T and a lower level of resistance to ddI, ABC, and TDF. However, we did not detect T215Y in any samples. The A62V amino acid substitution is known to be associated with multidrug resistance caused by Q151M; however, neither of these two patients had Q151M. These results revealed the presence of a particular pattern of the drug resistance-associated mutation occurred in Chinese patients. We also detected the K103R/Q (3 of 91) mutation, which confers resistance to NNRTIs. K103R occurs in about 1–2% of untreated persons and, by itself, has no effect on NNRTI susceptibility. The combination of K103R + V179D, however, reduces susceptibility to each of the NNRTIs about 15-fold. K103Q is a rare mutation at this position. Preliminary data suggest that K103Q causes minimal resistance to each NNRTI. The RTI-related mutation pattern and the low RTI-related mutation rate were quite different from the treatment-experienced patients.[22]

In conclusion, this study showed that 4.4% of patients have mutations conferring resistance to PIs and RTIs. The baseline prevalence of drug resistance mutations was rather low among antiretroviral-naive HIV-1 patients of Liaoning province. Most of patients will be sensitive to the free antiretroviral therapy provided by the Chinese government. However, many minor mutations reminded us to pay more attention to the observation of drug resistance mutations, preventing the occurrence of cross-drug resistance and multidrug resistance.

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HIV/AIDS in Asia: The Shape of Epidemics and Their Molecular Epidemiology*

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Abstract: The Asia-Pacific region is a home to 60% of the population in the world and to approximately one quarter of people with HIV/AIDS. Close to a million of people has been infected and a half million people died of AIDS annually in Asia, becoming the second largest epiçenter of global AIDS epidemic. Molecular epidemiology has been useful tool to track a course of HIV spread. In-depth knowledge from the studies on molecular epidemiology elucidates the dynamics of HIV spread and the interrelationship of epidemics in the different regions in Asia.

Key words: Molecular epidemiology; Genetic variability; Circulation recombinant form (CRF); Unique recombinant form (URF)

An estimated 8.6 (6.0-13.0) million people are living with HIV in Asia in 2006: 6.0 million men; 2.4 million women; ~200 000 children. In 1006 alone, there were 960 000 (640 000-900 000) new infections and 630 000 (430 000-900 000) AIDS death (29). The infection rates are still low in Asia, compared with some other continents, particularly Africa. However, since the populations of many Asian countries are so large, even low HIV prevalence rates means large numbers of people are living with HIV.

While HIV/AIDS epidemics come later in Asia, Asia is becoming an epicenter of 2nd largest epidemic after sub-Safaran Africa. In contrast to sub-Saharan Africa where the heterosexual transmission is a major driving force of the epidemic, the epidemic in Asia shows much complex structure. One third of HIV infections are acquired through injecting drug use in Asia. In addition to the epidemic through heterosexual route, the previously neglected epidemic among the men sex with men (MSM) has emerged in Asia (31). In the industrial countries in Asia, including Japan, Korea, Singapore, China Taiwan and Hongkong, MSM is the most important risk group and also significant numbers of HIV infection occurred among hemophiliacs by contaminated blood products in early 1980s. Molecular epidemiology has been a useful tool to analyze the origin of HIVs and to track a course of global HIV spread, providing the in-depth knowledge

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on AIDS epidemic: The study areas include the analyses of the distribution of HIV genotypes in different geographic areas, route of virus spread and specific association of genotypes with different epidemiologic features, such as risk behaviors. In this article, we overview the current status of HIV/AIDS epidemic and the recent advance in the study on the molecular epidemiology of HIV in Asia.

EPIDEMIOLOGY OF HIV IN ASIA

The Asia-Pacific region is vast and diverse and HIV epidemics in the region share that diversity. Table 1 summarizes the UNAIDS/WHO HIV estimates in different regions in Asia (29). We describe the outline of the epidemics in selected countries in Asia.

India

In India, the world's second most populous country, an estimated 5.7 million (The world's largest) were living with HIV in 2006. While the heterosexual transmission is the predominant mode (more than 80%) of HIV infections in most of regions in India, the injecting drug use is the main driver of the HIV epidemics in the northeast, especially in the state of Manipur and Nagaland, near the border with Myanmar. There is a substantial overlap between injecting drug use and paid sex in some areas of the country. In Tamil Nadu, HIV prevalence among sex workers is 50%.

China

In China, an estimated 650 000 were living with HIV/AIDS at the end of 2005. The highest numbers of HIV infection have been reported from Yunnan (southwest), Henan (East Central), Xinjiang (Northwest), Guangxi and Guangdong (Southeast) provinces. HIV epidemic began in rural areas before spreading to cities-unusual pattern of HIV spread. Injecting drug use accounts for nearly half (44%) of the people living with HIV. However, a half of the new HIV infection occurred during unprotected sex. As HIV is spreading gradually to the general population, the numbers of HIV infection among women are growing. The unique features of epidemic in China is that a substantial portion of infections were caused by unhygienic practice of commercial plasma collection in central China during the early 1990s. An estimated 250 000 people, mainly rural peasants, were infected in the provinces, including Henan, Anhui, Shanxi, Hubei and Shngdong. Recent study found the evidence of sexual transmission of HIV to non-donors. HIV epidemic starts to go beyond high risk groups into general population.

Thailand

In Thailand, an estimated 580 000 were living with HIV/AIDS with national adult HIV prevalence of 1.4% at the end of 2005. Because of intensive prevention measures, the new annual infection rate continues to drop: the estimated 18 000 new infections in 2005 from the peak incidence of 140 000 in early 1990s. A large proportion of new HIV infection is in general population. Recent studies show that more than one-third of HIV infections in 2005 were among women who had been infected by their long-term partners. Moreover, the previously neglected epidemic among MSMs has been recognized relatively recently. HIV prevalence among MSMs in Bangkok has increased steeply from 17% in 2003 to 28% in 2005.

Myanmar

An estimated 360 000 were living with HIV/AIDS at the end of 2005 with national prevalence of 1.3%. HIV prevalence among young people (15-24 years of age) was at concerned level of 2.2%. The high HIV

infection levels were found in the high risk groups: 43% in IDUs and 32% among sex workers. However, there are some indications that the epidemic might be slowing down: National prevalence among pregnant women has declined from 2.2% in 2000 to 1.3% in 2005.

Vietnam

An estimated 260 000 are living with HIV at the end of 2005, more than doubled since 2000. Approximately 40 000 people are newly infected with HIV each year. Injecting drug use and sex work are the main factors driving the epidemic. HIV prevalence among IDUs increased from 9% in 1996 to 32% in 2003. The levels of infection are reaching as high as 63% (in Hanoi) and 67% (in Hai Phong) in 2005. Up to 12% of sex workers and 18% of the sexual partners of IDUs were found to be infected with HIV, and the prevalence among pregnant women reached 1% mark in some regions.

Japan

An estimated 20 000 were living with HIV/AIDS in Japan at the end of 2005. More than 60% of new infections are among MSMs and 25% are acquired through heterosexual contacts. One out three new HIV/AIDS case reports is from AIDS patients, suggesting that a significant proportion of HIV-infected persons do not know their infections and visit clinics in the very late stage of diseases.

Table 1. HIV projection in various countries in Asia (end 2005) (UNAIDS/WHO)

Country	HIV Estimate[a]	Adult rate[b](%)
India	5 700 000	0.9
China	650 000	0.1
Thailand	580 000	1.1
Myanmar	360 000	1.3
Vietnam	260 000	0.5
Indonesia	170 000	0.1
Cambodia	130 000	1.6
Nepal	75 000	0.5
Malaysia	69 000	0.5
Papua New Guinea	60 000	1.8
Japan	17 000	<0.1
Korea	13 000	<0.1
Philippines	12 000	<0.1
Singapore	5 500	0.3
Sri Lanka	5 000	<0.1
Laos	3 700	0.1

[a] Adult (>15-yrs of age)+children (0-15 yrs)

[b] Adult prevalence rate exceeding 1.0% are bold-faced.

MOLECULAR EPIDEMIOLOGY OF HIV IN ASIA

GENOTYPE CLASSIFICATION OF HIVs

Human immunodeficiency virus (HIV) are divided into two related human retroviruses, HIV-1 (HIV type 1) and HIV-2 (HIV type 2) (Fig.1). HIV-1 is distributed worldwide, while HIV-2 is confined primarily to West Africa. Outside West Africa, a significant number of HIV-2 cases have been reported form southern/western India (6, 20) and Korea (9, 17).

HIV-1 strains were classified into three distinct groups of HIV-1 (M, N, and O) (Fig.1). The vast majority (more than 95%) of HIV-1 strains belong to group M (for Major or Main). Group O (for Outlier) infections are limited to people living in Central Africa (Cameroon, Gabon and Equatorial Guinea), but even in this area they represent a small minority of HIV-1 infections (3, 7, 13, 30). Only a few cases of group N (for New, or non-M/non-O) infections were identified in only limited numbers of patients from Cameroon (23). Groups O and N viruses were not detected in Asia.

Pandemic HIV-1 group M viruses are classified into at least 9 discrete genetic subtypes (A-D, F-H, J, K) based on the sequence of complete viral genomes (Fig. 1). In some subtypes, subclusters within subtypes were identified, leading to a classification into subsubtypes: subsubtypes A1 and A2 within the subtype F; subsubtypes F1 and F2 within subtype F.

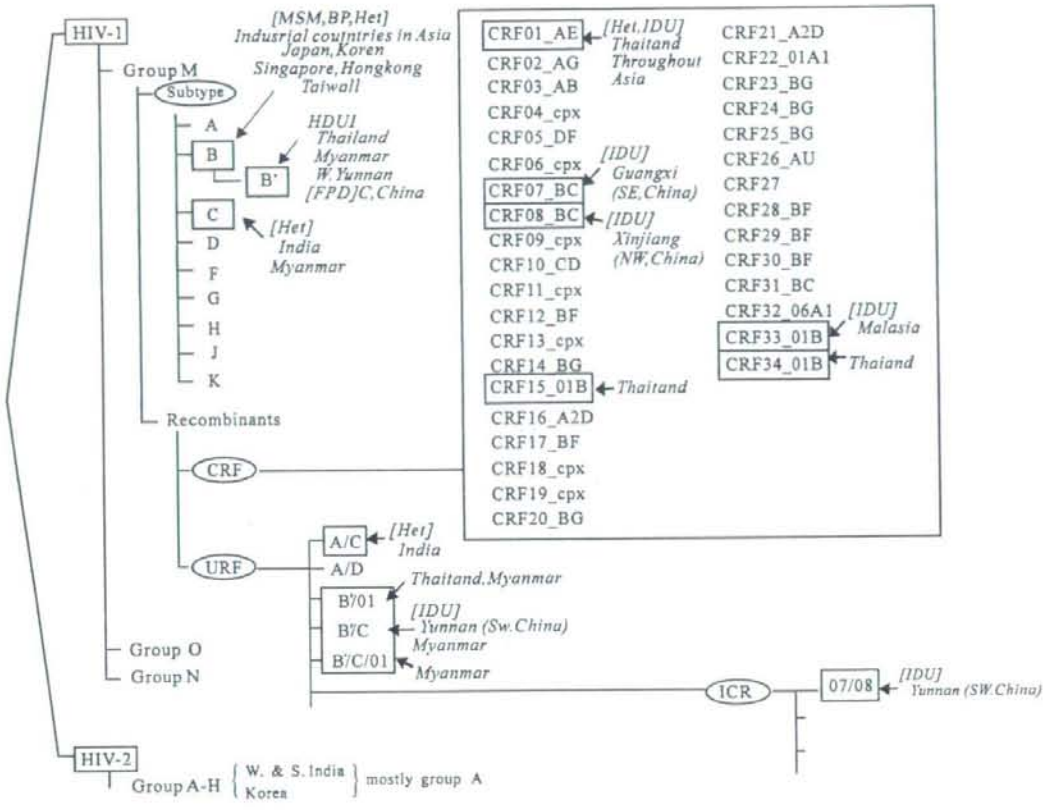

Fig. 1. Classification of HIV genotypes and their origins. HIVs are classified into types (types 1 and 2), groups (M, O and N for HIV-1; A through G for HIV-2), subtypes (A through K for HIV-1) and subsubtypes (A1 and A2, F1 and F2). HIV-1 recombinants are categorized into circulating recombinant forms (CRFs) and unique recombinant forms (URFs). HIV-1 subtype B' (B "prime") (Thailand variant of subtype B, also referred to as Thai-B), a unique regional subtype variant strongly associated with bloodborne transmission (prevalent among IDUs and former plasma donors/paid blood donors) in Asia (see text).

Circulating recombinant form (CRF)

It was realized that certain HIV-1 strains clustered with different subtypes in different regions of their genomes. Some of these mosaic HIV-1 genomes have been identified in several, apparently unlinked, individuals and play a major role in the global AIDS pandemic, designated as "circulating recombinant forms", or CRFs (2). A total of 34 CRFs are currently recognized (http://hiv-web.lanl.gov/CRFs/CRFs.html) (Fig.1). Six CRFs, CRF01_AE (former subtype E) (original site of isolation: Thailand), CRF07_BC (Xinjiang, China) and CRF08_BC (Guangxi, China), CRF15_01B (Thailand), CRF33_01B (Malaysia) and CRF34_01B (Thailand) have been identified so far in Asia (Fig. 1 and Fig. 2).

Unique recombinant form (URF)

In addition to CRFs, various types of 'unique' recombinant forms (URFs) have been reported, currently without evidence of epidemic spread (Fig. 1) (14). URFs are diverse forms of HIV-1 intergenotype recombinants with unique mosaic structures, seen only in a single person or in a few epidemiologically-

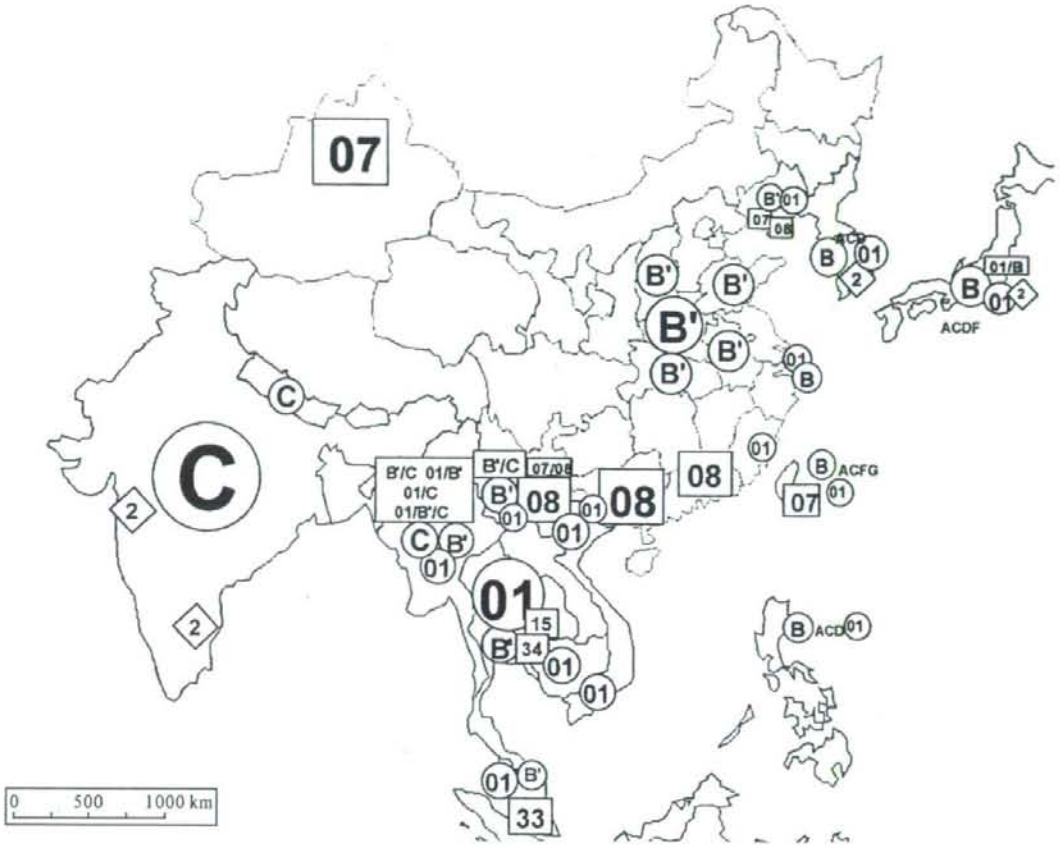

Fig. 2. Geographical distribution of HIV genotypes in Asia. The distributions of HIV genotypes (types, subtypes/subsubtypes and CRFs/URFs) in Asia are shown. '2' in rhombus, HIV-2; 01, CRF01_AE; 07, CRF07_BC, 08, CRF08_BC, 15, CRF15_01B; 33, CRF33_01B and 34, CRF34_01B _34. Various URFs comprised of the indicated genotype are shown in the shodowed box.

linked individuals. Most of URFs have been detected in the regions where multiple subtypes are co-circulating (Fig. 2). A various types of URFs were detected in several locales in Asia: the intersubtype A/C recombinants in India (12), CRF01_AE/B recombinants in Thailand (14); diverse recombinant forms comprised of various combination between subtypes B and C and CRF01_AE in Myanmar (15, 16, 26); the intersubtype B/C recombinants in Yunnan Province, China (34, 35) (Fig. 2). The detection of substantial numbers of different URFs worldwide suggests that dual/mutiple infections or super-infections with different lineages of HIV-1 strains might not be a rare event.

Distribution of HIV-1 genotypes in Asia

On a global scale, the most prevalent HIV-1 genotypes are subtypes C (56%), followed by A (23%), B (8%), D (5%) and CRF01_AE (5%) in 1999 (5). In Asia, HIV-1 subtypes B, US-European strain, is found among MSMs, hemophiliacs and some of hetero-sexual cases in the industrial countries in Asia, including Japan, Korea, Singapore, China Taiwan and Hongkong (Fig. 2 and Fig. 3). HIV-1 subtype C predominates in India (12). HIV-1 CRF01_AE is

widely circulating in Southeast Asia (18, 19, 32). CRF01_AE epidemic broke out in the late 1980s among female commercial workers and their clients in Thailand (19, 33) and became prevalent even among injecting drug users (IDUs) in this country. CRF01_AE quickly started disseminating throughout Southeast Asia, including Cambodia, Vietnam, Malyasia, China, China Taiwan, Korea and Japan (Fig. 3) (32, 33). Subtype B' (Thailand variant of subtype B; also referred to as Thai-B virus) (8, 18, 19) is a unique subtype B variant that spread primarily though IDU networks in southeast Asia (16, 33) and is the founder strain causing outbreaks among former plasma doors in Central China (24) (Fig. 2). Two closely-related CRFs, CRF07_BC and CRF08_BC are disseminating rapidly among IDUs in northwestern (Xinjiang Province) (25) and southeastern (Guangxi Province) China (21), respectively. A variety of novel CRFs comprised of CRF01_AE and subtype B (B') have been recently identified in Thailand (CRF15_01B (28) and CRF34_01B) and Malaysia (CRF33_01B) (27) (Fig. 1 and Fig. 2).

Geographical recombination hot spots in Asia

Recombinant viruses have already contributed substantially to the global pandemic as well as in Asia. We found that substantial proportions of HIV-1 strains were the URFs in Western Yunnan (65%) (34) and Central Myanmar (10%-30%) Myanmar (11, 15, 16, 26) (Fig. 2). Even recombinant viruses will recombine, leading to evolution of second-generation recombinants, inter-CRF recombinants (ICRs): ICR01_0708 identified among IDUs in southwesten China (Yunnan Province) is the first example of this category, which is comprised of two closely related CRFs in China, CRF07_BC and CRF08_BC (35).

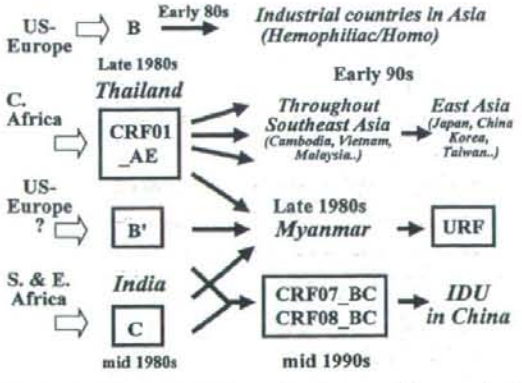

Fig. 3. The plausible origins and pathways of the spreads of Asian HIV-1 variants.

Genotype classification and geographic distribution of HIV-2

HIV-2s are known to be members of a broader HIV-2/Simian immunodeficiency virus (SIVsm) phylogenetic group. HIV/SIVsm phylogenetic groups are classified into eight genetic "groups" (A through H) (4). However, only HIV-2 groups A and B are disseminated into significant numbers of human populations (1, 22).

In Asia, HIV-2 infections were reported in west and south India, frequently associated with dual infections of subtype C strains (6, 20). Outside India, 10 cases of HIV-2 infections have been reported in Korea (17) (Fig. 1). While most of the HIV-2 infection cases in Asia were the infections with HIV-2 group A, one HIV-2 group B infection was identified in a person with Korean nationality living in Japan (10), who appears to be infected through heterosexual contacts in DR Congo (17).

CONCLUSION

Molecular epidemiology of HIV provides the basis of understanding the origins and genesis of the epidemics in different regions in Asia and their

relationship, particularly elucidating the pathway of viral spread over the regions and risk populations. Studies also provide information to delineate the mechanism of person-to-person transmission and viral evolution. However, the biological significance of the global diversity of HIV-1 strains remains to be defined. For instance, the relationship between HIV-1 genotype and biological phenotypes is not well established. In-depth knowledge obtained from the study on molecular epidemiology of HIV is critically important to elucidate the dynamics of HIV spread and to formulate future vaccine and other prevention strategies in Asia.

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Short report

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Optimal design and validation of antiviral siRNA for targeting HIV-1

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Abstract

We propose rational designing of antiviral short-interfering RNA (siRNA) targeting highly divergent HIV-1. In this study, conserved regions within HIV-1 genomes were identified through an exhaustive computational analysis, and the functionality of siRNAs targeting the highest possible conserved regions was validated. We present several promising antiviral siRNA candidates that effectively inhibited multiple subtypes of HIV-1 by targeting the best conserved regions in pandemic HIV-1 group M strains.

Findings

RNA interference (RNAi) is now widely used to knock-down gene expression in a sequence-specific manner, making it a powerful tool not only for studying gene function, but also for therapeutic applications including antiviral treatments [1,2]. The replication of a wide range of viruses can be successfully inhibited using RNAi with both short interfering RNA (siRNA) and siRNA expression vectors [3,4]. However, for RNA viruses such as HIV-1, designing functional siRNAs that target viral sequences is problematic because of their extraordinarily high genetic diversity. We analyzed 495 entries of near full-length HIV-1 group M sequences available in the Los Alamos HIV Sequence Database, and selected the highest-possible conserved target sites for designing optimal antiviral siRNAs. It is known that RNAi-resistant viral mutants emerge rapidly when targeting viral sequences due to their high mutation rate [5-7]. Since highly conserved sequences are likely to contain structurally or functionally constrained elements, our approach is anticipated to resist viral mutational escape.

First, we performed a detailed analysis on the HIV-1 genome to identify highly conserved targets by using 495 near full-length genome sequences of HIV-1 group M (listed in Additional file 1). Every possible 21-mer was generated from all of the HIV-1 group M sequences, and their conservations among the 495 HIV-1 sequences were exhaustively determined using siVirus engine [8]. We defined 'conservation' as the percentage of sequence entries out of the 495 HIV-1 sequences that showed perfect identity (*i.e.*, 21/21 matches) with the cognate 21-mer. Since many of the HIV-1 sequence entries lack 5' untranslated region (5' UTR), the 3' LTR sequence was used to compensate for the lack of 5' LTR sequences in order to avoid underestimating conservation in such regions. For the regions that cannot be compensated for in this way (depicted in Figure 1A and 1B left panel, colored

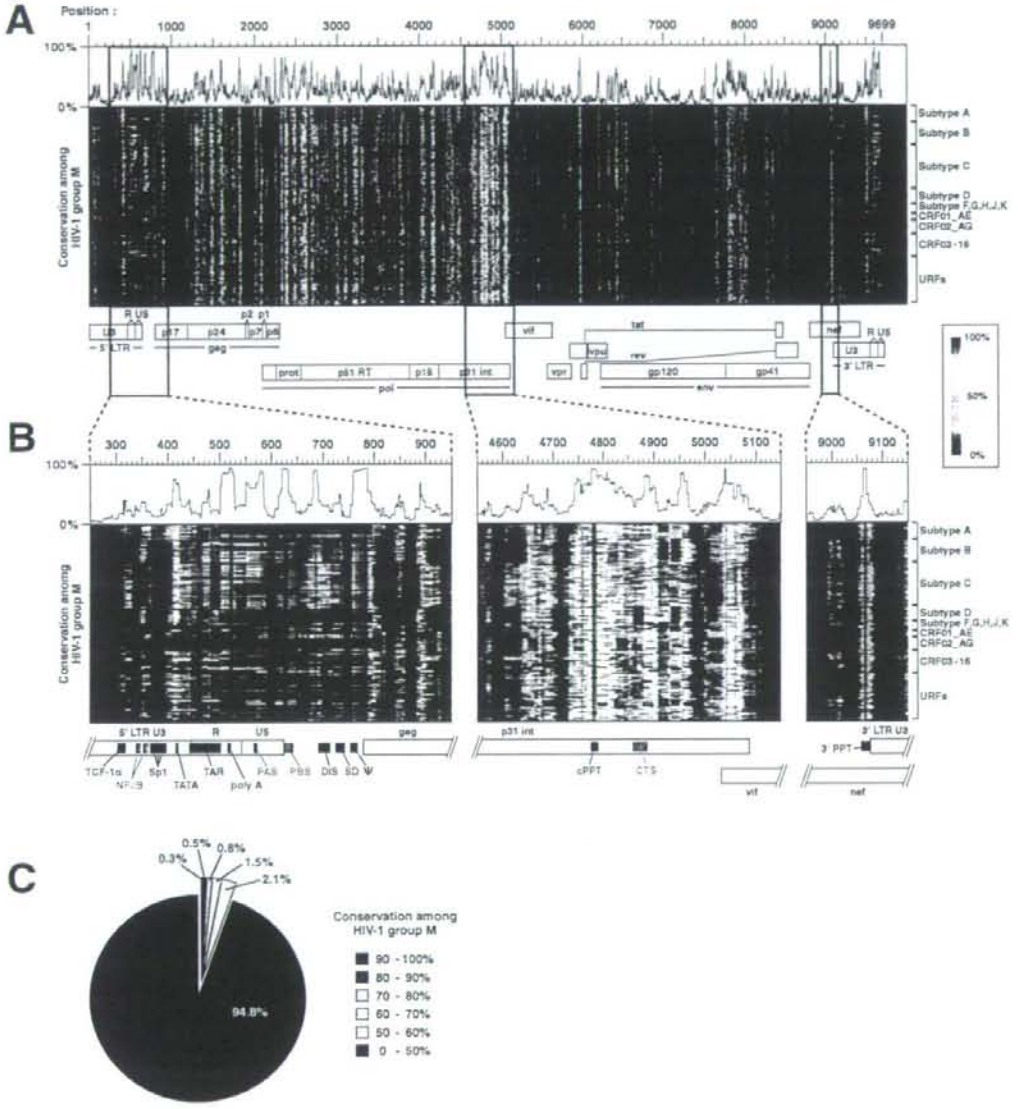

Figure 1

Conservations of siRNA target sequences among HIV-1 group M. (**A**) A total of 4,417,157 siRNA targets were generated from the 495 HIV-1 sequences, and their conservations within the HIV-1 genomes are represented using a color density plot. The line plot above the color chart represents the highest value in each position. (**B**) A detailed view of the three conserved regions; 5' LTR, the cPPT/CTS in the integrase gene, and 3' PPT. 'Position' indicates the 5'-most position of each 21-mer. The landmarks of the HIV-1 genome are adjusted to align at the center of the siRNAs by shifting 10 bp to the left. (**C**) Pie chart indicating the percentage of the 4,417,157 siRNA target sites at each conservation level.

black), conservation was calculated by considering only the HIV-1 sequences that contain the corresponding regions. The result revealed that HIV-1 genomes are not conserved for consecutive 21 bp for the most part, resulting in the poor conservation of many of the 21-mers over the HIV-1 sequences (Figure 1A, colored blue). As shown in Figure 1C, only 5.2% of the possible 21-mers are >50% conserved. Furthermore, highly (>70%) conserved 21-mers constitute only 1.6% of all 21-mers. It is of note that many of the published anti-HIV-1 siRNA sequences do not fall into this 'highly conserved' category (Additional file 2 and [9]). From these results, we anticipate that most of the possible siRNAs are not suitable for the efficient targeting of HIV-1.

However, our analysis has identified several distinct regions that are highly conserved in the HIV-1 genome (Figure 1B). Such regions include the regulatory domains responsible for the viral gene expression, such as the TATA sequence and polyadenylation signal (AAUAAA). In addition, several regions essential for the regulation of viral replication were also highly conserved, including the primer activation signal (PAS)[10], primer binding site (PBS), packaging signal (Ψ), central polypurine tract (cPPT), central termination sequence (CTS), and 3' polypurine tract (3' PPT). All of these highly conserved sequences are constrained at the nucleotide sequence level or by their RNA secondary structure in order to execute their functions. In contrast, regions constrained by amino acid sequences were not necessarily conserved at the nucleotide sequence level due to the wobbling of the third base in the codon (data not shown). siRNAs targeting the highly conserved regions are expected to overwhelm the high level of sequence diversity of the HIV-1 genome, and also to reduce the chances of viral mutational escapes.

Total of 216 highly conserved (>70%) siRNA targets identified in this study are listed in Additional file 3. In mammalian RNAi, the efficacy of each siRNA varies markedly depending on its sequence. According to our guidelines for the selection of effective siRNAs [11,12], 31 out of 216 siRNAs were predicted to be functional. Similarly, 30 and 44 siRNAs are functional according to the algorithms reported by Reynolds *et al.* [13], and Amarzguioui *et al.* [14], respectively (Additional file 3). This suggests that only a limited fraction of 21-mers is best suited for use as functional antiviral siRNAs.

For the functional validation, 23 siRNAs from Additional file 3, and 18 additional siRNAs targeting moderately-conserved regions were selected based on the following criteria: (I) predicted to be functional by the algorithm of Ui-Tei *et al.* [11,12], and (II) the sequence has perfect identity with pNL4-3 (GenBank M19921). The 41 siRNA sequences selected and their target sites are detailed in Additional file 4. We first tested the efficacy of each siRNA using target mRNA cleavage assay (Additional file 5 and [15]). Briefly, a vector expressing reporter mRNA that contains the siRNA target site was cotransfected into HeLa cells with the corresponding siRNA, and the mRNA cleavage activity of the siRNA was evaluated by measuring the quantity of surviving mRNA using real-time RT-PCR. This assay allows us to directly monitor the sequence-dependent potency of siRNA itself, without being affected by the differences in target gene expression level or target secondary structures. The result showed that 39 out of the 41 siRNAs gave >60% silencing at 5 nM (Figure 2, rightmost panel). si4794 and si4888 were not functional, probably due to the long consecutive Gs in si4794 and internal palindromes (AAAAUUUU) in si4888 [11,13].

Next, siRNAs were evaluated for their antiviral efficacy against three evolutionary-distant groups of HIV-1: subtypes B and B' (Thailand variant of subtype B [16]); subtype C; and CRF01_AE. Each siRNA was cotransfected into HeLa cells at 5 nM with one of the four infectious molecular clones: pNL4-3 (subtype B); 95MM-yIDU106 (subtype B'); 93IN101 (subtype C); or 93JP-NH1 (CRF01_AE). Culture supernatants were collected 48 h after transfection and the viral reverse transcriptase activity was measured (Additional file 5 and [17]). The results show that 26 of the 41 siRNAs effectively inhibited viral replication of all four strains by >80% (Figure 2, marked with red or orange circles). Of the remaining 15 siRNAs, 13 of them (except si4794/4888) were shown to be functional in the target mRNA cleavage assay, and 12 of them (except si690/4794/4888) inhibited the replication of at least one viral strain by >80%, indicating that the designed siRNAs have the potential to induce RNAi. In several viral strains, nucleotide substitutions in their target sites essentially abolished the inhibition of viral replication (Figure 2, blue bars with arrowheads). However, mismatches near the ends of the target sites (see Additional file 6) did not necessarily abolish the siRNA efficacy (Figure 2, blue bars with asterisks). si689 and si690 did not inhibit viral replication even though these siRNAs perfectly matched to their target sites (confirmed by DNA sequencing of the infectious molecular clones). This is probably due to the stable secondary structure at the si689-690 target sites in both BMH (branched multiple hairpin) conformation and LDI (long distance interaction) conformation of the HIV-1 leader RNA [18] (see Additional file 4). It should be noted that the efficacy of si575 differed when targeting pNL4-3 and 93IN101. One possible explanation for this is the secondary structure differences among HIV-1 subtypes, which may alter the accessibility of the si575 target site.

The approach described here enabled us to select highly effective siRNAs against divergent HIV-1 strains at a high

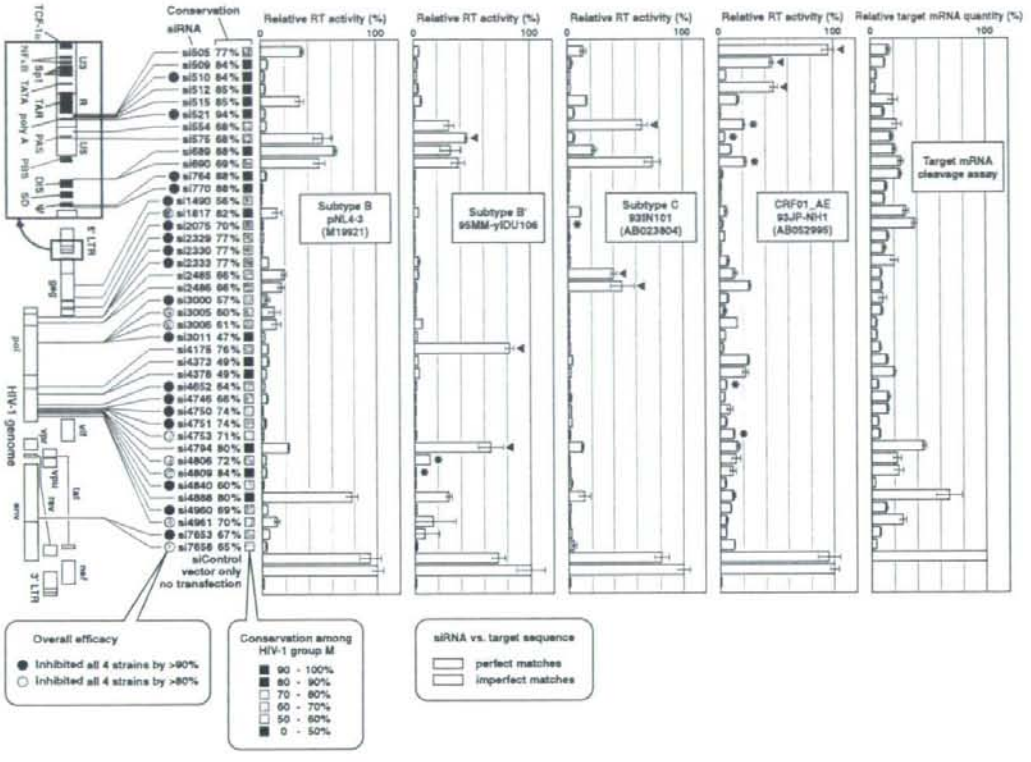

Figure 2
Validation of 41 siRNAs. The antiviral efficacy of each siRNA was tested against four HIV-1 infectious molecular clones: pNL4-2 (subtype B); 95MM-ylDU106 (subtype B'); 93IN101 (subtype C); or 93JP-NH1 (CRF01_AE). The potency of each siRNA was tested using the target mRNA cleavage assay (rightmost panel). The ability of each siRNA to cleave its target was evaluated by the target mRNA cleavage assay.

rate. The highly effective siRNAs (>90% inhibition) with maximal conservation (>70%) identified in our study include si521 (poly A site; 94% conservation), si764/770 (Ψ; 88%), si510 (TAR/poly A; 84%), si2075 (ribosomal slip site; 70%), si2329/2330/2333 (protease region; 77%), and si4750/4751/4753 (integrase region; 71–74%). These sites are found mostly in the 5' LTR, protease, and integrase regions (Figure 2). However, the extraordinarily high genetic diversity of HIV-1 obviously prevents us from designing a single siRNA that can nullify all HIV-1 strains currently circulating worldwide (Additional file 7). One possible approach is to combine multiple siRNAs targeting different conserved regions [19,20]. The siRNAs selected and validated in this study have the potential to target >99% of HIV-1 strains by combining only two siRNAs (Additional file 7), and also considered to resist viral mutational escape. Our approach is expected to be highly applicable to therapeutic intervention for other pathogens of public health importance, including HCV, influenza virus, and SARS coronavirus, that are known to show high genetic diversity.

Competing interests
The author(s) declare that they have no competing interests.

Authors' contributions
YN performed the computational analyses and the target mRNA cleavage assays, participated in the design of the study, and drafted the manuscript. KN and TO performed the viral replication assays. RU analyzed the data. KU-T participated in the target mRNA cleavage assays, and was

involved in critically revising the manuscript. KS and YT supervised the entire study and wrote the manuscript.

Additional material

Additional file 1

The list of 495 near full-length genome sequences of HIV-1 group M.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1742-4690-4-80-S1.pdf]

Additional file 2

The list of published siRNA/shRNAs targeting HIV-1.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1742-4690-4-80-S2.pdf]

Additional file 3

The list of highly conserved siRNA targets identified in this study.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1742-4690-4-80-S3.pdf]

Additional file 4

The siRNA sequences and their target sites. The sequences of 41 siRNAs and their target sites are shown. The siRNA numbers indicate the nucleotide position in HXB2 (GenBank K03455). The conservation level of each siRNA in HIV-1 group M sequence is depicted in color chart at the rightmost column. BMH (branched multiple hairpin) and LDI (long distance interaction) conformations of the HIV-1 leader RNA and siRNAs targeting them are shown.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1742-4690-4-80-S4.pdf]

Additional file 5

Supplementary materials and methods.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1742-4690-4-80-S5.pdf]

Additional file 6

Target sites of the 41 siRNAs used in this study. Sequence alignment of the target site from the four HIV-1 infectious molecular clones: pNL4-2 (subtype B); 95MM-yIDU106 (subtype B'); 93IN101 (subtype C); or 93JP-NH1 (CRF01_AE).
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[http://www.biomedcentral.com/content/supplementary/1742-4690-4-80-S6.pdf]

Additional file 7

Coverage of HIV-1 group M by single siRNA or two siRNAs. (A) Coverage of HIV-1 group M by 41 siRNAs used in this study. (B) Coverage of HIV-1 group M by combining two siRNAs from above. Coverage was calculated by considering only the HIV-1 sequences which contain the corresponding regions.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1742-4690-4-80-S7.pdf]

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Chronology of the HIV-1 CRF07_BC expansion in East Asia

(5)

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The HIV-1 epidemic among injecting drug users (IDU) in Taiwan is caused primarily by CRF07_BC infections. Evolutionary analyses, which utilize outgroup reference strains from northwestern China (Xinjiang), reveal that CRF07_BC was introduced into southern Taiwan in 1998–2001 and spread to central–northern Taiwan in 2001–2003, causing the largest HIV/AIDS epidemic in Taiwan. The separate introduction of CRF07_BC into Xinjiang occurred in 1992–1995. This study illustrates the temporal dynamics of CRF07_BC spread among IDU across east Asia.

The HIV-1 circulating recombinant form 07_BC (CRF07_BC) is a recombinant comprised of HIV-1 subtype B' (Thailand genotype of subtype B) and subtype C. This strain accounts for most infections among injecting drug users (IDU) in northwestern China (Xinjiang province), where an outbreak was detected in 1996 [1], although the most plausible origin of CRF07_BC as a whole is probably Yunnan province [2,3]. Outside mainland China, CRF07_BC is associated with a dramatic increase in HIV/AIDS cases in Taiwan; it was detected among IDU in prisons in southern Taiwan (Tainan) in 2002 and later detected in 2003–2004 in central (Nantou) and northern (Taipei) Taiwan [4]. By

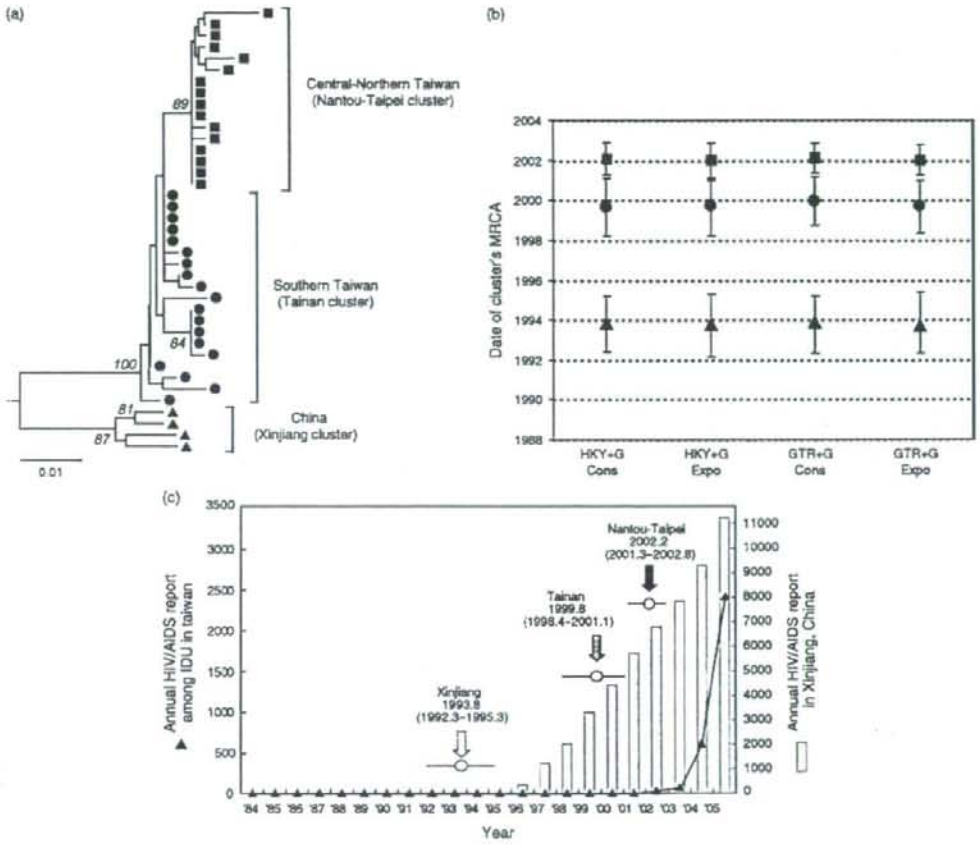

Fig. 1. Phylogenetic and evolutionary analysis of HIV-1 sequences from injection drug users in mainland China and Taiwan. (a) Estimated phylogeny for the *env* gene of HIV-1 CRF07_BC (HXB2 7077–7665 nt). For visual clarity, sequences are labeled with symbols according to location of isolation. (b) Estimated dates of the most recent common ancestors (MRCA) of CRF07_BC sequences from China (Xinjiang), southern Taiwan (Tainan), and central–northern Taiwan (Nantou–Taipei). Vertical lines denote the 95% highest posterior density credible intervals. Dates were estimated under various nucleotide substitution and evolutionary models: HKY, Hasegawa–Kishino–Yano model; GTR, general time reversible model; G, gamma distributed among-site rate heterogeneity; Cons, constant population size; Expo, exponential population growth. ■ Central–northern (Nantou–Taipei) cluster; ● southern Taiwan (Tainan) cluster; ▲ China (Xinjiang) cluster; (c) Change in annual HIV/AIDS cases in Xinjiang, China (□) and Taiwan (▲) between 1984 and 2005. The time to the MRCA of CRF07_BC clusters detected in the indicated locales are depicted with 95% highest posterior density credible intervals (in parentheses).

the end of September 2007, 15 183 HIV cases have been reported in Taiwan since 1984 and the majority of infections occurred relatively recently among IDU [5]. CRF07_BC accounts for 98% of the infections among IDU in Taiwan [4].

To estimate the timescale of CRF07_BC spread in mainland China (Xinjiang) and in Taiwan, we performed phylogenetic and Bayesian coalescent analyses on *env* sequences obtained from GenBank. The CRF07_BC *env* sequences used in this study are from Xinjiang province, China ($n=4$; sampled in 1997–1998), and from three cities in Taiwan; in particular, Tainan (southern; $n=19$), Nantou (central; $n=8$) and Taipei (northern; $n=8$), all sampled in 2004 (accession numbers EF078077–EF078079, EF078082–EF078105 and EF078107–EF078114). As shown in Fig. 1a, the CRF07_BC sequences group into three distinct phylogenetic clusters, denoted Xinjiang (China), Tainan (southern Taiwan) and Nantou-Taipei (central and northern Taiwan). Using the molecular clock approach implemented in BEAST v1.4 [6], we estimated the rate of evolution of the hypervariable region-stripped *env* gene (HXB2 7077–7665 nt) from an independent dataset of 41 HIV-1 subtype C strains with known sampling dates that ranged from 1989 to 2005. The

evolutionary rate estimate obtained ($4.7-5.0 \times 10^{-3}$ substitutions per site per year) was then incorporated as a prior probability distribution in the analysis of the CRF07_BC sequences [7]. A Bayesian Markov chain Monte Carlo method was used to estimate the dates of the most recent common ancestors (MRCA) of CRF07_BC in Xinjiang and Taiwan under various nucleotide substitution and evolutionary models. As illustrated in Fig. 1b, the likely year of origin of CRF07_BC from Xinjiang, China, was August 1993 [95% credible region (CR) March 1992–March 1995]. MRCA of CRF07_BC strains from southern and central-northern Taiwan were dated to August 1999 (95% CR April 1998–January 2001) and February 2002 (95% CR March 2001–August 2002), respectively. The evolutionary and statistical assumptions used have almost no effect on the estimated dates (Fig. 1b).

This study indicates that CRF07_BC was introduced into the IDU population in Xinjiang in the early to mid 1990s (1992–1995). The strain also spread into IDU in southern Taiwan in the late 1990s (1998–2001), and subsequently disseminated northward to IDU in central–northern Taiwan in the early 2000s (2001–2003), resulting in the largest ever HIV/AIDS epidemic in Taiwan. HIV/AIDS surveillance detected a dramatic upsurge of HIV cases in Xinjiang in the mid-1990s (1995–1996), and in 2003–2004 in Taiwan (Fig. 1c), suggesting that CRF07_BC may have been present among IDU for a year or two in each region before the epidemic spread and subsequent detection of the infection. It is most likely that both the Xinjiang and Taiwan outbreaks trace their origins back to Yunnan province, thought to be the geographical origin of CRF07_BC [2,3]. We note, however, that MRCA dates can also be more recent than the date of outbreak discovery, either because extant virus diversity has been incompletely sampled, or because the founding lineages of the outbreak have since gone extinct [8,9]. These results illustrate the history of the regional and international spread of HIV-1 CRF07_BC in east Asia.

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