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RESEARCH ARTICLE

Prevalence and patterns of drug-resistance mutations among HIV-1 patients infected with CRF07_BC strains in Sichuan province, China

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Little information is available on the prevalence of drug-resistance mutations in patients harboring the human immunodeficiency virus type 1 (HIV-1) circulating recombinant form (CRF)07_BC variant in Sichuan, China. This study examined 375 plasma samples from patients with HIV-1 who were infected with the CRF07_BC strain, including 104 drug-naive participants and 271 in whom antiretroviral therapy (ART) had failed. Only one participant in the drug-naive group had a drug-resistance mutation (M46L), compared with 31.73% of those in whom ART had failed. Further analysis showed that 19.56% of strains contained mutations conferring resistance to non-nucleoside reverse transcriptase inhibitors (NNRTIs) alone, 0.74% were resistant to nucleoside reverse transcriptase inhibitors (NRTIs) alone, and 11.44% were dual-resistant to both NRTIs and NNRTIs. The most common mutation in the ART-failure group was M184V (35.88%), K103N (45.01%), Y181C (17.33%), and G190S/A (15.88%). The percentages of HIV-1 strains resistant to lamivudine, emtricitabine, efavirenz, etravirine, and nevirapine were 10.70%, 10.70%, 28.04%, 7.75%, and 26.20%, respectively. To explore site variants possibly related to drug resistance, variations in the ancestor/consensus CRF07_BC sequences from the therapy-naive and ART-failure groups were compared, and seven mutations at six positions were identified as being significantly differently distributed between the two groups ($p<0.05$). Detailed sequence data will provide information on CRF07_BC genetic characterizations, and improve our understanding of antiretroviral susceptibility and the evolution of drug-resistance mutations. This will be valuable in developing and implementing local public-health approaches for HIV drug-resistance prevention and treatment.

KEYWORDS HIV-1; CRF07_BC; drug resistance; Sichuan; China

INTRODUCTION

Since the initiation of "Four Frees and One Care" policy by the Chinese government in 2003, 19,120 patients with AIDS in Sichuan, China, had undergone free highly active antiretroviral therapy (ART) treatment by the end of 2013. Highly active ART has remarkably reduced the morbidity and mortality caused by human immunodeficiency virus type 1 (HIV-1) infection (Pereira C F, et al., 2004). However, treatment efficiency is often limited by low drug potency, poor adherence to treatment regimens, and the appearance of HIV drug resistance, which is an important factor (Richman D D, et al., 2004). Thus, it is important to conduct HIV drug-resistance surveys in Sichuan in order to formulate effective ART regimens and develop a rational public-health strategy to control the local HIV epidemic.

HIV-1 circulating recombinant form (CRF)07_BC,

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which is descended from subtypes B and C, represents one of the most prevalent HIV-1 strains in China (Yu G, et al., 2009). CRF07_BC originated in intravenous drug users in Yunnan and rapidly spread to Sichuan (Tee K K, et al., 2008). HIV-1-prevalent strains with increasing complexity have been discovered in Sichuan. However, data from two national molecular epidemic surveys conducted from 1996 to 1998 and 2002 to 2004 have indicated that CRF01_AE and CRF07_BC are the absolute major forms in Sichuan. More and more evidence from Sichuan has shown that, in recent years, CRF07_BC infection is responsible for most cases of HIV in intravenous drug users and men who have sex with men (Yuan D F L, Xi J, 2011; Zeng P L Y, 2013). Most data on mutations conferring HIV drug resistance has been obtained with HIV-1 subtype B. The lack of such data for non-subtype B viruses limits the rational management of ART for the increasing number of patients infected with these subtypes (Ariyoshi K, et al., 2003; Li X P, et al.,2007). In this study, we aimed to summarize the prevalence of drug-resistance mutations in HIV-1 subtype CRF07_BC isolates prevailing in Sichuan, and to provide insights on subtype-specific variations and the drug-resistance spectrum of non-subtype B viruses.

MATERIALS AND METHODS

Participants and specimens

The present study has been ongoing in Sichuan province, China, since 2011. Our study cohort consisted of 375 randomized individuals infected with HIV-1 CRF07_BC, as verified by sequence analysis, including 104 treatment-naive patients and 271 patients in whom ART had failed. All of the treatment-naive and almost half of the ART-failure patients were recruited from Liangshan Yi Autonomous Prefecture, while the remaining ART-failure patients were recruited from another 16 cities in Sichuan. All patients provided written informed consent. Demographic data were obtained through individual interviews. Participants were considered treatment-naive if they had never been exposed to antiretroviral drugs. ART was considered to have failed if a patient had received ART for longer than 1 year but had a viral load of more than 1000 copies/mL.

HIV-1 RNA extraction, amplification, and sequencing

Viral RNA was extracted from 200 μL plasma using an automated extractor (MagNA Pure LC system, Roche, Branchburg, NJ, USA) according to the manufacturer's instructions. The viral protease gene and the first 255 codons of the reverse transcriptase (RT) gene were amplified by reverse transcriptional nested PCR, as described previously (Liao L, et al., 2007). Positive PCR products were purified and sequenced by the Nuosai Genomics Company (China) with a variety of internal specific primers (available on request).

Drug-resistance analyses

Sequence contig assembly was performed using the analysis software Sequencher 4.9 (Gene Codes, Ann Arbor, MI, USA). ClustalW multiple alignment and manual editing were performed using the BioEdit Sequence Alignment Editor (Ibis Biosciences, Carlsbad, CA, USA). Sequences from drug-naive individuals were analyzed for transmitted drug-resistance mutations using the Calibrated Population Resistance Tool (available at http://cpr.stanford.edu/cpr.cgi). Drug-resistance profiles of ART-experienced patients were analyzed based on genotypic and phenotypic interpretations defined by the Surveillance Drug Resistance Mutation list recommended by the World Health Organization, and the Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu).

Distinguishing mutations potentially conferring drug resistance

To identify ART-selected mutations, we compared the prevalence of mutations in ART-treated versus ART-naive individuals. Mutations with significantly higher frequency in the ART-failure group were identified. The chi-square test was used to calculate differences between mutations in the two groups. The potential impact of the screened mutations on antiviral drug response was assessed by submitting the sequences to the Stanford University HIV Drug Resistance Database.

Statistical analysis

Statistical analysis was conducted using SPSS 17.0 (SPSS, Chicago, IL, USA). Categorical variables were compared using chi-square analysis or Fisher's exact test. All tests were two-tailed, and *p*-values of less than 0.05 were considered significant.

RESULTS

Characterization of study participants

Plasma samples were collected from individuals diagnosed with HIV-1 infection in Sichuan province from 2011 to 2012. A total of 375 sequences of HIV-1 *pol* region that were verified as CRF07_BC by sequence analysis were successfully obtained, with 104 from therapy-naive patients and 271 from ART-failure patients. The demographics of these participants are summarized in Table 1. The median ages of the therapy-naïve and ART-failure patients were 32 years (interquartile range 14–50 years) and 36 years (interquartile range 7–72 years), respectively. Males predominated in the study population

(74.04% of therapy-naive and 71.23% of ART-failure individuals). Consistent with previously reported HIV-1 transmission routes in Sichuan, intravenous drug use and sexual conduct were the most common transmission routes in the current study. The average viral load was 4.59 lg copies/mL (interquartile range 2.89–6.74 lg copies/mL) in the ART-naive population and 4.51 lg copies/mL (interquartile range 1.70–6.27 lg copies/mL) in the ART-failure population.

ART was provided free of charge by the Chinese government. Lamivudine (3TC)/zidovudine (AZT)/nevirapine (NVP) was the most common regimen in ART-failure individuals (57.93%), as shown in Table 1. The proportion of individuals treated with 3TC/stavudine/NVP, 3TC/efavirenz/AZT and 3TC/tenofovir/efavirenz was 25.09%, 5.90%, and 5.54%, respectively. However, only one participant in the current study received protease inhibitor therapy with lopinavir and ritonavir.

Table 1. Demographic characteristics of study participants with HIV-1 CRF07_BC.

Characteristic	Patients	
	ART naive (n=104)	ART failure (n=271)
Age, years	32 (14–50)	36 (7–72)
Sex		
Male	77 (74.04)	193 (71.23)
Female	27 (25.96)	78 (28.78)
Viral load, RNA, lg copies/mL	4.59 (2.89–6.74)	4.51 (1.70–6.27)
Route of HIV infection		
Intravenous drug use	82 (78.85)	109 (40.22)
Homosexual sex	22 (21.15)	19 (7.01)
Heterosexual sex	–	137 (50.55)
MTCT	–	5 (3.68)
Other/unknown	–	1 (0.74)
Duration of ART, years	–	1.4 (0.3–6)
ART regimen (current used)		
3TC	–	1 (0.37)
AZT	–	1 (0.37)
d4T	–	1 (0.37)
3TC/AZT/NVP	–	157 (57.93)
3TC/d4T/NVP	–	68 (25.09)
3TC/EFV/AZT	–	16 (5.90)
3TC/EFV/d4T	–	8 (2.95)
3TC/AZT /LPV/r	–	1 (0.37)
3TC/TDF/ EFV	–	15 (5.54)
d4T/3TC/AZT/NVP	–	3 (1.11%)

Results are presented as n (%) or median (interquartile range). Abbreviations: 3TC, lamivudine; ART, antiretroviral therapy; AZT, zidovudine; d4T, stavudine; EFV, efavirenz; LPV/r, lopinavir with ritonavir; MTCT, mother-to-child transmission; NVP, nevirapine; TDF, tenofovir.

Prevalence of HIV drug resistance in ART-failure and ART-naive individuals

The presence of mutations in the HIV-1 *pol* gene was evaluated by comparing the sequences against the Stanford University HIV Drug Resistance Database to identify the known mutations related to drug resistance. In the ART-failure group, 31.73% of participants harbored at least one drug-resistance mutation, compared with only one participant (1%) in the ART-naive group. In this patient, the major mutation M46L was seen in the protease region, which reduced susceptibility to indinavir, nelfinavir, fosamprenavir, lopinavir, and atazanavir. All drug-resistance mutations identified in ART-failure individuals were in the RT region (Table 2). The most prevalent mutation was K103N (45.01%), followed by M184V/I (35.88%), Y181C (17.33%), G190S/A (15.88%), Y188C/L (9.20%), D67N/G (8.13%), V106M (9.13%) and K101Q/E (7.64%). For all strains carrying drug-resistance mutations, 19.56% were resistant to non-nucleoside RT inhibitors (NNRTIs) alone, 0.74% were resistant to nucleoside RT inhibitors (NRTIs) alone, and 11.44% were dual-resistant to both NRTIs and NNRTIs. Resistance to 3TC, emtricitabine, efavirenz,

Table 2. Drug-resistance mutations in the current populations among HIV variants epidemic in Sichuan province.

Resistant to nucleoside reverse transcriptase inhibitors			Resistant to non-nucleoside reverse transcriptase inhibitors		
	Frequency (%)			Frequency (%)	
Mutation	ART naive (n=104)	ART failure (n=271)	Mutation	ART naive (n=104)	ART failure (n=271)
M41L	0.00	2.45	V90I	0.00	1.96
D67N/G	0.00	8.13	K101Q/E	0.00	7.64
T69S/A	0.00	2.45	K103N	0.00	45.01
K70R	0.00	4.28	V106M	0.00	8.13
M184V/I	0.00	35.88	E138A	0.00	1.00
T215Y/I	0.00	4.28	V179D/E	0.00	6.80
K219E	0.00	4.28	Y181C	0.00	17.33
			Y188C/L	0.00	9.20
			G190S/A	0.00	15.88
			H221Y	0.00	1.44
			P225H	0.00	1.00
			F227L	0.00	1.44
			K238	0.00	2.84

Abbreviation: ART, antiretroviral therapy.

etravirine, and NVP was seen in 10.70%, 10.70%, 28.04%, 7.75%, and 26.20% of strains, respectively (Figure 1). Resistance to the other five drugs (abacavir, AZT, stavudine, didanosine, and rilpivirine) was present in less than 10% of strains for each.

Distinguishing mutations potentially conferring drug resistance

Since no transmission of drug resistance was found in ART-naive individuals in our study, we used the *pol* sequences identified from ART-naive individuals as a control background to explore potential drug-resistance–related mutations that are not included in the HIV Drug Resistance Database. Variations in the ancestor/consensus CRF07_BC sequences isolated from the therapy-naive and ART-failure groups were compared. No variations at the protease region were identified between the ART-naive and ART-failure groups. However, seven mutations at six RT amino acid sites (E28K, K32E, T35M, T200A, E248V, E248D, and K249Q) were found in the ART-failure group, with higher frequencies compared with the ART-naive group (Table 3).

DISCUSSION

By the end of 2013, the cumulative number of HIV/AIDS cases reported in Sichuan had reached 55,054. With the scaling up of ART in Sichuan, surveillance of the emergence and transmission of drug-resistant HIV-1 strains has became one of the most important tasks for HIV/AIDS prevention and control. The present study describes the prevalence of HIV drug-resistance mutation associated with CRF07_BC infections in Sichuan.

AIDS therapy in Sichuan uses the first-line regimens of the Chinese national free ART program, which contains

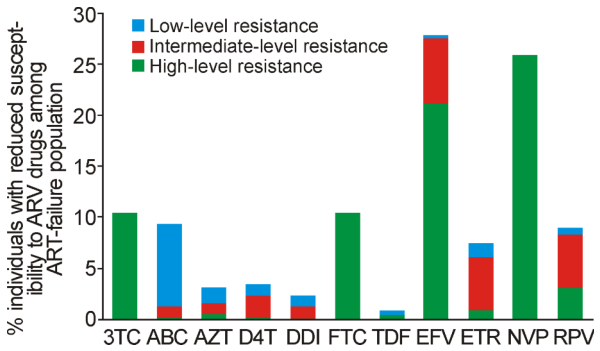

Figure 1. Antiretroviral drug-resistance levels among ART-failure individuals in Sichuan province. Abbreviations: 3TC, lamivudine; ABC, abacavir; AZT, zidovudine; d4T, stavudine; DDI, didanosine; EFV, efavirenz; ETR, etravirine; FTC, emtricitabine; NVP, nevirapine; RPV, rilpivirine; TDF, tenofovir.

Table 3. Mutations with significantly different distributions in the reverse transcriptase region between the therapy-naive (n=104) and ART-failure populations (n=271).

Position	Wild type (aa)	Mutation (aa)	Frequency (%)		X^2	*p*-value
			ART naive	ART failure		
28	E	K	0.00	14.02	–	–
32	K	E	1.90	15.50	13.37	<0.01
35	T	M	1.90	7.94	3.96	<0.05
200	T	A	4.76	12.92	5.18	<0.05
248	E	V	0.00	10.70	–	–
248	E	D	0.00	2.58	–	–
249	K	Q	0.00	13.28	–	–

Abbreviation: ART, antiretroviral therapy.

two NRTIs and one NNRTI. In our study, 3TC/AZT/NVP was the most common treatment strategy (57.93% of participants) followed by 3TC/stavudine/NVP (25.09%). Of all the ART-failure patients harboring HIV-1 CRF07_BC strains, only 0.74% were resistant to NRTIs alone, and 11.44% were dually resistant to NRTIs and NNRTIs. We can speculate that the use of NVP is responsible for most of the drug-resistance emergency. According to the Mutation ARV Evidence Listing (MARVEL), two mutations, V106M and Y181C, have been reported to be major drug-resistant mutations (Reuman E C, et al., 2010; Wu H, et al., 2012). Inconsistent with those data, the most common NNRTI mutation in the current study was K103N with a frequency of 45.01%. Y181C and G190S/A were present in 17.33% and 15.88% of strains, respectively. V106M exhibited a mutation rate of only 8.13%. These data suggest that, in Sichuan, HIV-1 CRF07_BC might exhibit a different evolutionary pattern under antiretroviral drug-selected pressure (Li Z, et al., 2013).

No protease-inhibitor–associated drug-resistance mutations were identified among the ART-failure individuals, probably as a result of the limited use of protease inhibitors by the participants in this study. However, one major protease-inhibitor–associated drug resistance mutation was found in an ART-naive patient. A possible explanation is that individuals administered self-procured protease inhibitors, or the mutation was introduced by transmission. Given a short period of access to ART in the sampling area, the prevalence of drug resistance was extremely low among the therapy-naive individuals. Further studies are required to elucidate the transmitted drug-resistance condition in Sichuan. In our study, more than 70% therapy-naive individuals were infected by intravenous drug use. The better adherence to therapy among intravenous drug users (Xiao L, et al., 2010) might result in a lower HIV drug-resistance prevalence in this population than in individuals infected through

sexual contact.

By comparing the 271 sequences obtained from ART patients with the 104 sequences from drug-naive individuals, this study identified seven novel mutations in six positions of RT (E28K, K32E, T35M, T200A, E248V, E248D, and K249Q). These mutations all occurred outside of the catalytic activity sites, which mainly locate in the 100–200 amino acids of RT. It is reasonable that the known resistance mutations related to NRTIs are mainly located in the palm domain and RT fingers domains, which help the RNA template bind to the catalytic sites. Resistance mutations related to NNRTIs are mainly located in the palm domain. However, interactions among mutations are complicated, and their effects on antiviral drug responses usually counteract each other (Li H, et al., 2012). It will therefore be worth conducting further investigations to verify the possible impacts of these mutations on the occurrence and development of drug resistance.

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COMPLIANCE WITH ETHICS GUIDELINES

All of the authors declare that they have no competing interests. Additional informed consent was obtained from all patients for whom identifying information is included in this article.

AUTHOR CONTRIBUTIONS

SL and LS designed the experiments. LS, DY, DBW, and HY carried out the experiments. LS, HY, and GMQ analyzed the data. LS and XZ wrote the paper. All authors read and approved the final manuscript.

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