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Hepatitis C virus genotype diversity and distribution among methadone maintenance treatment patients in Jiangsu, China

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ABSTRACT

Background: Heroin users are vulnerable and represent a highly-infected reservoir for hepatitis C virus (HCV) infection. This study investigated HCV prevalence and genotypes distribution among heroin users who received methadone maintenance treatment (MMT) in Jiangsu.

Methods: From June to December in 2016, a total of 534 patients among nine MMT clinics in six regions of Jiangsu were enrolled, with their demographic characteristics collected and serum samples tested for HCV antibody. 395 samples were seropositive and furthered to RNA extraction. HCV NS5B region fragments were amplified and subsequently sequenced.

Results: Among HCV seropositive samples, 240 were characterized by NS5B partial sequences and classified into four genotypes (GPs) and eight subtypes. HCV GP3 predominated and accounted for 66.3%, followed by GP1 (27.5%), GP6 (4.2%) and GP2 (2.1%). HCV subtypes 3b (41.7%) and 3a (24.6%) were the most common subtypes. None of the demographic characteristics showed a significant difference when comparing with HCV genotypes. The geographic feature shown GPs in six regions were the same, but the frequency of subtypes exhibited regional divergence. Phylogenetic analyses demonstrated that 3b had become a local endemic in Jiangsu.

Conclusion: The distribution of HCV subtypes among heroin users in Jiangsu province was complex. The data could provide more precise estimates for HCV prevalence and genotype distribution as well as heroin users of Jiangsu province.

1. Background

Hepatitis C virus (HCV) is the pathogen of Hepatitis C which causes chronic liver disease, cirrhosis, hepatocellular carcinoma, and liver-related mortality worldwide. Globally, the prevalence of viraemic HCV was estimated to be 1–1.1% which meant 71.1–80 million by 2015 (Polaris Observatory HCV Collaborators, 2017). In mainland China, the anti-HCV prevalence rate was 0.43–1.6% in the general population (Bennett et al., 2015; Cui and Jia, 2013), and the number would be much higher in high-risk groups, such as paid blood donors, patients on hemodialysis and heroin users. According to studies reported in China, the average seroprevalence of HCV infection among heroin abusers based on MMT clinics between 2004 and 2012 was 54.6%, furthermore the HCV antibody prevalence in heroin users was over 55% in some areas (Hser et al., 2012; Wang et al., 2016; Zhou et al., 2015), which made the great economic burden of society.

HCV is an enveloped, positive-sense, single-stranded RNA virus belongs to the genus Hepacivirus of the family Flaviviridae. In light of the updated criteria, the genetic heterogeneity of HCV had expanded to 7 genotypes and 67 subtypes due to the accumulation of nucleotide substitutions in the HCV genome (Smith et al., 2014). With the advent of new antiviral such as direct-acting antiviral drugs, HCV infection could be curable in nearly all patients, but HCV genotype might represent the essential index for disease progression and serve as a predictor for different responses to the same agent (European Association for the Study of the Liver, 2017; Gane et al., 2015, 2017; Jensen and Holle, 2016; Smith et al., 2015). In comparison with HCV GP2, 3, 5 and 6, patients with HCV GP1 and 4 were treated with a comparatively higher dosage of treatment taken for a longer course but were still less likely to bring about sustained virologic response (SVR) (Tao et al.,

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2015). Besides studies manifested that HCV GP1 might increase the risk of hepatocellular carcinoma (HCC), while GP3 was identified to be strongly associated with an increased risk of developing cirrhosis and HCC compared to HCV GP1 (Bennett et al., 2015; Kanwal et al., 2014).

HCV genotype prevalence exhibits high genetic diversity by regional variations. HCV subtypes 1b and 2a remain to be the two predominant in North, East and Central China, while HCV-3 stands to be a higher prevalence in the Southwest China (Li et al., 2017; Zhang et al., 2017). Furthermore, the distribution of HCV genotype in heroin users is more complicated than in general population (Qi et al., 2016). Jiangsu located in eastern of China and as a representative province for the societal characteristic, there is a paucity of large-scale data for HCV genotype distribution, and there is an urgent need to explore the prevalence and genotype in this high-risk population of Jiangsu province. The aim of this study was to determine the genotypes and subtypes distribution of HCV among heroin users recruited at National MMT program in Jiangsu province.

2. Materials and methods

2.1. Ethical statement

This study was done according to the Helsinki Declaration and was approved by the National Center for AIDS/STD Control and Prevention (NCAIDS), Chinese Center for Disease Control and Prevention (China CDC; # X120331210). All participants were voluntarily involved and signed informed consents before participating in this study.

2.2. Study participants

In order to better represent all heroin users in Jiangsu, we chose nine MMT clinics in total, and each clinic had over 50 outpatients per year. Participants were recruited in June to December 2016 from nine MMT clinics in Jiangsu province, including six regions of Nanjing, Suzhou, Wuxi, Zhenjiang, Changzhou and Lianyungang. All the participants were heroin dependent patients including injecting drug users and non-injecting drug users. A total of 534 patients were enrolled and consented for venous blood collection.

2.3. Serological assays

Six milliliters of venous blood were collected, and serum was stored at −20 °C after centrifugation. HCV antibody was tested with the enzyme immunoassay kit (Zhuhai Livzon Inc., Zhuhai, China), which was performed in accordance with the manufacturer's instructions. Anti-HCV positive samples were subjected to RNA extraction followed by genotyping.

2.4. Extraction and amplification of HCV RNA NS5B region

HCV RNA was extracted from 140 μL serum with QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany).HCV RNA positivity was identified by nested PCR based on NS5B fragment. For the amplification process, the first round was completed using SuperScript III Reverse Transcriptase kit (Invitrogen), and 2 × Taq PCR MasterMix (Tiangen) was used for the second round. The semi nested PCR was performed with primers as described previously (Cantaloube et al., 2005). The PCR products were sent to Nuosai company for sequencing and the resulting sequences were spliced together using the software SeqMan software (DNASTAR, Windows version 5.06)

2.5. HCV genotyping and phylogenetic analysis

The PCR product of fragment NS5B (8258 nt to 8643 nt according to the numbering of the H77 genome) was 386 bp. A variety of reference sequences were retrieved from the NCBI Nucleotide database for analysis, including 27 sequences of GP1, GP2, GP3 and GP6. The nucleotide sequences obtained were aligned with HCV reference sequences by the ClustalW method of MEGA software (Version 4.0). FastTree 2.3 was used to construct an approximately-maximum-likelihood phylogenetic tree (ML tree) using the GTR + G + I nucleotide substitution model (Price et al., 2010). The phylogenetic tree's reliability was determined with local support values based on the Shimodaira-Hasegawa (SH) test (Chan et al., 2011) and presented using FigTree v1.3.1 (http://beast.bio.ed.ac.uk).

2.6. Statistical analysis

Significant differences between the HCV genotype and subtype frequencies among different demographic characters were determined by a Fisher's test using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). A probability value < 0.05 was considered statistically significant.

3. Results

3.1. Demographic distribution based on HCV GP among heroin users in Jiangsu

In this study, we assessed the prevalence of HCV among 534 heroin users including six regions in Jiangsu province and 395 samples with positive of HCV antibody was subsequently diagnosed. After detecting HCV nucleic acid, 240 of them were successfully amplificated. The seroprevalence was 74.0% (395/534) while the viremia prevalence was 44.9% (240/534). Among the 240 heroin users with genotype determined, 81.3% were male, 75.0% were middle-aged adults (between the ages of 30–50 years old), 72.9% had middle school or less education, and 74.6% were unemployed. Detailed demographic characteristics are summarized in Table 1. HCV subtypes among different risk factors were checked, and no significant differences were identified.

Table 1
Subtype distribution of 240 drug users by demographic features.

Genotype		All	1a	1b	2a	3a	3b	6e	6n	6u	p-value
overall		240	9	58	5	59	100	1	5	3	
Sex											0.978
	Male	195	7	49	4	48	79	1	4	3	
	Female	45	2	9	1	11	21	0	1	0	
Age											0.858
	< 30	1	0	0	0	0	1	0	0	0	
	30–50	188	8	41	4	49	78	1	4	3	
	> 50	51	1	17	1	10	21	0	1	0	
Marriage											0.833
	Unmarried or Divorced	149	7	34	3	38	62	0	3	2	
	Married	84	2	21	2	18	37	1	2	1	
	other	7	0	3	0	3	1	0	0	0	
Ethnics											0.304
	Han	237	9	58	5	56	100	1	5	3	
	Tujia	1	0	0	0	1	0	0	0	0	
	Zhuang	2	0	0	0	2	0	0	0	0	
Education											0.755
	Elementary school	18	0	6	1	4	6	0	1	0	
	Middle school	157	8	36	2	40	66	1	2	2	
	High school or college	65	1	16	2	15	28	0	2	1	
Occupation											0.14
	Employed	61	4	10	0	21	24	0	2	0	
	Unemployed	179	5	48	5	38	76	1	3	3	
Drug use method											0.726
	Injection drug user	217	9	50	5	53	92	1	4	3	
	Non-injection drug user	23	0	8	0	6	8	0	1	0	

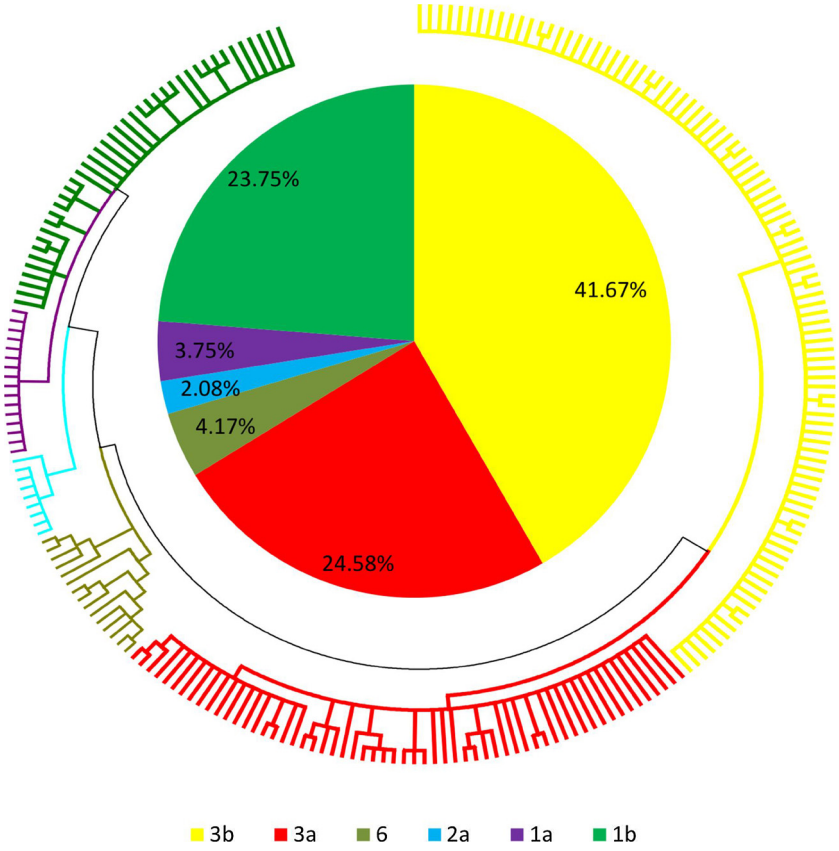

Fig. 1. A cicular form of phylogenetic tree based on NS5B sequences was generated. The pie chart inside the tree indicates the percentages of the different HCV subtypes of the 240 isolates obtained in this study.

3.2. Geographic distribution of HCV subtypes among heroin users in Jiangsu

Based on NS5B sequencing, we confirmed the circulation of four HCV GPs (GP1, 2, 3 and 6) and eight HCV subtypes (1a, 1b, 2a, 3a, 3b, 6e, 6n and 6u) in Jiangsu (Fig. 1). HCV GP3 predominated and accounted for 66.3%, followed by GP1 (27.5%), GP6 (4.2%) and GP2 (2.1%). Assessment of HCV subtypes revealed that the most common subtype was 3b, accounting for 41.7%, and followed by subtype 3a at 24.6%. For HCV genotype 1, two subtypes were included, 1a and 1b (3.8% and 23.8% percentage respectively). For HCV genotype 2, only 2a was identified. The most cataloged subtypes were found in genotype 6, which had three subtypes 6e, 6n and 6u (0.4%, 2.5% and 1.3% percentage respectively).

Fig. 2A showed the subtype distribution in various regions. From the diversity of distribution, subtypes 1b, 3a and 3b were detected in all six geographic regions. Subtypes 1a and 2a observed in two geographic regions, Changzhou and Wuxi. Other subtypes were encountered in Zhenjiang (subtype 6e), Nanjing (subtype 6n) and Lianyungang (subtype 6 u). Comparing the proportion of subtypes distribution, subtype 3a predominated in Zhenjiang and Suzhou, while 3b was the most prevalent in the other four regions.

From 2009 to 2012, three articles reported the distribution of HCV subtypes among DUs from Suzhou, Zhenjiang and other cities in Jiangsu respectively (Du et al., 2012; Qi et al., 2016; Zhang et al., 2011). Considering the selected regions were different, we summarized the data and analyzed them based on subtypes (Fig. 2B). Although the top three common subtypes among heroin users in current data were consistent with the data published before 2012, the constituent ratio of HCV distribution was significantly different ($P < 0.05$). In addition, subtype 6a is the fourth predominant subtype in this study, which is in contrast to the 2012data that showed no detection of this subtype.

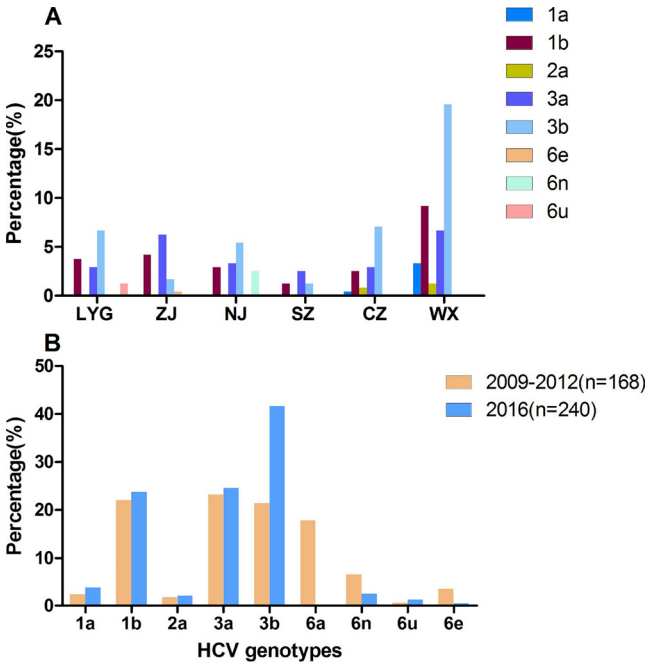

Fig. 2. HCV subtypes distribution in Jiangsu. A bar chart showing the HCV subtypes distribution by cities in Jiangsu (A). The abscissa was named by city abbreviation Where LYG, ZJ, NJ, SZ, CZ and WX, standing for six cities in Jiangsu, that is, Lianyungang, Zhenjiang, Nanjing, Suzhou, Changzhou and Wuxi respectively. Comparison of HCV subtypes characterization in Jiangsu at different times (B).

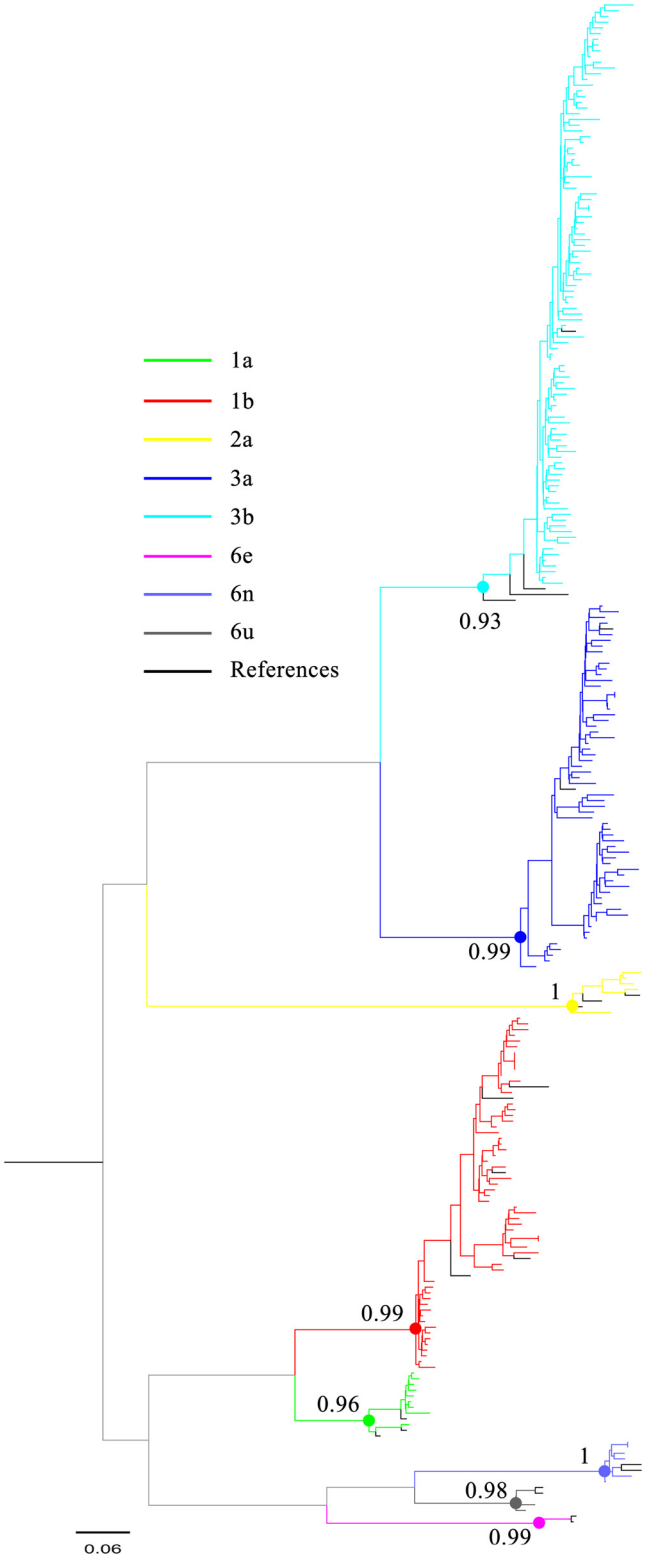

Fig. 3. Phylogenetic analysis of HCV genotype based on NS5B fragment sequences obtained from current study and reference sequences retrieved from NCBI. The bootstrap value ≥0.9 was identified as a subtype and the different subtypes were color-coded.

3.3. Phylogenetic analyses of HCV subtypes among heroin users in Jiangsu

Using these 240 sequences and 27 reference stains (Table S1) in NS5B region, a phylogenetic tree was reconstructed, and four groups were generated (Fig. 3). The same genotype was closely grouped, and each group was separated from the others with bootstrap support. HCV subtype1a, 2a and 6 showed obvious regional clustering. Subtypes in different regions were clustered separately in the phylogenetic tree and were not intermixed. Furthermore, sequences obtained from this study were closer to the reference strain from Chinese origin, while other reference sequences mainly from outside of China included other small clusters. The subtype 3a sequences obtained from this study were geographically mixed, showing a province-wide distribution. Compared to the tree described for subtype 3a, the subtype 3b sequences formed tree including more regions and appeared more orderly. The subtype 3b sequences obtained from this study were clustered together, similar to the reference strains from Chinese origin especially isolated from Shanghai and Jiangsu. The tree of HCV subtype 1b sequences could be clearly divided into two branches, but the sequences obtained from this study and references sequences were geographically mixed, showing simultaneous dissemination worldwide.

4. Discussion

In this study, we found a high level of HCV genetic diversity, with four major genotypes (1, 2, 3, and 6) and eight subtypes (1a, 1b, 2a, 3a, 3b, 6e, 6n, and 6u) circulating in Jiangsu province. We revealed that 3b was the dominant subtype. Previous studies reported that HCV subtype distribution was distinct between intravenous drug users (IDUs) and the general population. For example, GP1b and GP2 were observed more frequently among patients infected through blood transfusion than the other routes while GP3 and GP6 always predominated among IDUs internationally especially in China (Gong et al., 2016; Gower et al., 2014; Ju et al., 2015; Ruta and Cernescu, 2015; Yuan et al., 2017). According to the former studies in Jiangsu, GP3 was the prevalent genotype in IDUs (Du et al., 2012; Qi et al., 2016; Zhang et al., 2011); nevertheless, the HCV subtype distribution frequencies were different. The former studies found that subtype 3a as the dominant subtype and the proportion of subtypes 3b, 1b and 6a to be similar, while our study found that subtypes 3a and 1b's composition had similar proportion but lower than that of subtype 3b and no subtype 6a could be found. The divergence may be derived from the geographic difference, for the former samples of Jiangsu area is mainly from Zhenjiang city, but in our study, the majority of participants are from Wuxi. However, factors driving the decreasing of subtype 6a were not clear. According to Rong's report, HCV subtype 6a also increased in blood donors (Rong et al., 2014), so we speculate that subtype 6a may exist in the general population and not exceptionally prevalent among heroin users of Jiangsu province.

Rested on HCV NS5B sequencing, the phylogenetic analysis revealed different subtypes to show varied molecular evolutionary levels. Most HCV subtype 3b isolated in the current study clustered closer to local isolate indicates that the previous existence of HCV subtype 3b in Jiangsu might have spread and become prevalent province-wide. In contrast, some of the GP1 clusters close to the isolates from China and Russia, while some of theGP1 groups with isolates from China, Japan, Vietnam, and USA. Consistent with other studies both at home and abroad, this complex genetic connection showing HCV GP1 as the most prevalent genotype has been geographically integrated worldwide (Chen et al., 2017; Daw et al., 2016; Messina et al., 2015; Rao et al., 2014).

Illicit drug abuse especially drug injection is the main route of HCV transmission. The seroprevalence of HCV infection among MMT patients varied from 5.6 to 78.1% in China (Wang et al., 2016). In our study, the prevalence rate of anti-HCV was 73.97%, and the viremia prevalence was 44.94% among heroin users. Although needle exchange programs and methadone maintenance treatment significantly reduced HCV prevalence, the findings of this study appeared that relatively high HCV viremia remained prevalent in heroin users. One plausible explanation is that heroin users perform more high-risk behavior such as

abusive and unsafe sexual-behavior and refrainment of seeking medical attention. Some literature stated that the high prevalence of HCV infection was the result of low awareness of infection status and the reluctance in seeking medication among MMT patients. Some argued that people who inject drugs exhibit high response rates and low level of HCV reinfection after medication (Cheng et al., 2018; Ng et al., 2013; Ruta and Cernescu, 2015). Therefore, persistent screening and monitoring HCV prevalence in heroin users, encouraging medication of HCV infection may be a highly cost-effective way to reduce HCV incidence among heroin users and the general population.

Based on the latest HCV treatment program (European Association for the Study of the Liver, 2018), there are genotype-specific regimens and knowing the genotype will be useful to choose better therapeutic regimen. Our study is the first comprehensive report with respect to the HCV genotype diversity and subtypes distribution among MMT patients in Jiangsu province. Our target population better represented heroin users living in Jiangsu province in contrast to previous reports, which sample sizes were limited or performed in restricted areas. This study has some limitations. Selection bias might have occurred, because of convenient sampling. Moreover, by characterizing HCV genotype by partial NS5B region gene, the finding of recombinant subtype may have been concealed (Quer et al., 2015). To elucidate the epidemiology character of HCV genotype which influences the choice for the appropriate treatment regimen, it is urgent to analyze complete coding sequences or deep sequencing of HCV genome on a comparatively large scale.

5. Conclusion

Our study provided a more comprehensive scenario of HCV genotype distribution among heroin users in Jiangsu province. We investigated the prevalence of HCV and HCV genotype distribution among heroin users in Jiangsu, finding that the HCV seroprevalence remained high. With genotype analysis, four genotypes were detected and subtype 3b dominated which had been a local endemic in Jiangsu. Although MMT effectively controlled the use of illicit drugs and injections, most of the heroin users are still vulnerable and unaware of their HCV infection status, even less seeking of medication. We suggest that encourage testing and managing heroin users may play a crucial role in controlling the ongoing HCV epidemic.

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None declared.

Contributors

Jing Lu, Li Zhang, Gengfeng Fu and Guohong Chen conceived and designed the experiments. Jing Lu, Ying Zhou and Zhi Zhang conducted the experiments. Jing Lu, Xiaoqin Xu and Yuanfang Chen analyzed the data. Jing Lu and Li Zhang wrote the manuscript. All the authors listed have approved the manuscript that is enclosed.

Conflict of interest

The authors have declared that no competing interests exist.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.drugalcdep.2018.09.026.

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