Transmission networks of hepatitis C virus among HIV/HCV-coinfected patients in Guangdong, China

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Abstract

Background: Coinfection with hepatitis C virus (HCV) is common in human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) patients due to shared routes of transmission. We aimed to investigate the characteristics of HCV subgenotypes among HIV/HCV-coinfected patients in Guangdong and explore the molecular transmission networks and related risk factors for HCV strains.

Methods: Plasma samples were obtained from 356 HIV/HCV-coinfected patients for HCV NS5B region sequencing. A neighbor-joining phylogenetic tree was constructed to affirm HCV subgenotypes. The transmission networks based on maximum likelihood phylogenetic tree were determined by Cluster Picker, and visualized using Cytoscape 3.2.1.

Results: A total of 302 HCV NS5B sequences were successfully amplified and sequenced from the 356 plasma samples. A neighbor-joining phylogenetic tree based on the 302 NS5B sequences revealed the profile of HCV subgenotypes circulating among HIV/HCV coinfection patients in Guangdong. Two predominant strains were found to be 6a (58.28%, 176/302) and 1b (18.54%, 56/302), followed by 3a (10.93%, 33/302), 3b (6.95%, 21/302), 1a (3.64%, 11/302), 2a (0.99%, 3/302) and 6n (0.66%, 2/302). A molecular transmission network of five major HCV genotypes was constructed, with a clustering rate of 44.04%. The clustering rates of subgenotypes 1a, 3a, 3b, 1b, and 6a were 18.18% (2/11), 42.42%, 52.38%, 48.21%, and 44.89%, respectively. Multivariate logistic regression analysis showed no significant effects from sex, age, transmission route, geographical region, baseline CD4+ T cell count or subgenotype (P > 0.05), except marital status. Married or cohabiting people (compared with unmarried people) had more difficulty forming transmission networks.

Conclusions: In summary, this study, based on HCV NS5B subgenotypes, revealed the HCV subtype diversity and distribution among HIV/HCV-coinfected patients in Guangdong. Marital status inclined to be the factor influencing HCV transmission networks formation.

Keywords: Hepatitis C virus, Human immunodeficiency virus, Transmission network

Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver diseases worldwide, such as cirrhosis, steatosis, and hepatocellular carcinoma [1]. HCV displays high levels of genetic diversity and has been differentiated into seven major genotypes and approximately 100 subgenotypes [2]. Different genotypes and subgenotypes differ in clinical outcomes, responses to treatment and epidemiology. Coinfection with HCV and human
influencing factors of molecular transmission networks. In this study, we characterized the transmission patterns and features of HCV among HIV/HCV-coinfected patients in Guangdong, China.

Materials and methods

Study population

Plasma samples for NS5B sequencing were obtained from 356 HIV/HCV-coinfected patients recruited between January 2010 and September 2013 from Guangzhou Eighth People’s Hospital. The inclusion criteria were as follows: (1) older than 18 years of age at time of enrollment, (2) positive HIV-1 ELISA (Beijing Wantai, China) with a confirmatory Western blot (MP Biomedicals, Singapore), (3) positive IgG or IgM anti-HCV ELISA (Zhongshan Bioengineering, China) and detectable HCV RNA > 1000 IU/ml (Guangzhou DAAN Gene Limited Company, China). The exclusion criteria were as follows: (1) positivity HBV surface antigen (HBsAg) ELISA (Zhongshan Bioengineering, China), (2) evidence of liver disease due to other etiology, (3) excessive alcohol consumption or using liver-toxic drugs, (4) previously received antiviral (HIV or HCV) treatment, and (5) individuals with decompensated cirrhosis and hepatocellular carcinoma (HCC), severe cytopenias, pregnancy, breastfeeding status, renal failure, heart failure, or an AIDS-defining illness. Demographic information, including sex, age, transmission route, marital status, geographical region, and baseline CD4+ T cell count, was obtained at patient enrolment and extracted through chart review.

RNA extraction, amplification, and sequencing

Viral RNA was extracted from 140 µl of plasma using a QIAamp Viral RNA Mini Kit (Qiagen, Germany) following the manufacturer’s instructions. HCV NS5B (H77: 7996–8638 nt) fragments were amplified with a Prime-Script One-Step RT-PCR Kit and Premix Taq (Takara Bio, Dalian, China). The NS5B fragment was amplified with in-house degenerate primers (Table 1) under the following conditions: 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 40 s and 72 °C for 60 s for the first round and 95 °C for 2 min, followed by 35 cycles of 95 °C for 25 s, 55 °C for 40 s and 72 °C for 40 s for the second round. The PCR products were analysed using 1% agarose gel electrophoresis, and the positive products were sent for sequencing by a genomics company (Tianyi Huiyuan, China) with the primer R2.

Identification of HCV subgenotypes

The reverse complements of the obtained sequences were determined and aligned by using BioEdit 7.0. Then, sequence alignments were performed with HCV subtyping references from the Los Alamos HCV Sequence Database (https://hcv.lanl.gov/). All
sequences were manually edited. HCV subgenotypes were assigned based on phylogenetic analysis of NS5B region sequences. Neighbor-joining phylogenetic trees were constructed with the Kimura 2-parameter substitution model and evaluated by the bootstrap method with 1000 replicates by using MEGA 6.06.

### Analysis of HCV molecular transmission networks

The flow chart of transmission network analysis includes four steps [20]. First, PhyML 3.0 was used to construct a maximum likelihood phylogenetic tree (ML tree) using the GTR + G + I nucleotide substitution model. The phylogenetic tree's reliability

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**Table 1** HCV primers for the NS5B region by genotype

| Primers          | Primer Sequences (5’-3’) | H77 location (nt) | Amplified length (bp) |
|------------------|--------------------------|-------------------|-----------------------|
| **First round**  |                          |                   |                       |
| Forward (F1)     | CCACATCMRCCCTCGGTGTTG    | 7952–7970         | 696                   |
| Reverse (R1)     | GGRGCDGARTACCTGTCAT      | 8628–8647         |                       |
| **Second round** |                          |                   |                       |
| Forward (F2)     | ACMCCCAATWSMACBACCATCATG | 7996–8018         | 643                   |
| Reverse (R2)     | TACCTGGTCAATGCTCCCGTAA   | 8616–8638         |                       |

Fig. 1 Geographical region of Guangdong province. The geographical regions of Guangdong are represented by different colors on the map. Data is shown on the dataset tabulated in Table 2 and Table 4.
was determined with branch support based on the approximate likelihood ratio test (aLRT) with Shimodaira-Hasegawa (SH) supports of 1000 replicates [21]. Second, Cluster Picker [22] was used to determine extra transmission clusters with an intra-cluster maximum pairwise distance < 4.0% nucleotide substitutions per site [23] and bootstrap support value ≥ 0.9. Third, Mega 6.0.6 was used to calculate the Tamura-Nei 93 pairwise genetic distances to define the linkages within a cluster. Finally, the network data were visualized using Cytoscape 3.2.1 (http://cytoscape.org).

Statistical analysis
The database was established in Excel, and the statistical analyses were performed using IBM SPSS V25.0 (SPSS Inc. Chicago, IL). Categorical variables were compared using Fisher’s exact tests. Univariate and multivariate logistic regression models were used to estimate the potential factors associated with transmission within clusters. The variables considered were sex, age, transmission route, marital status, geographical region, baseline CD4+ T cell count, and HCV subgenotype. A multivariate logistic regression model was constructed in a forward manner to select variables independently associated with transmission within clusters. Odds ratios (ORs) and adjusted odds ratios (aORs) with 95% confidence intervals (95% CIs) were reported. For all statistical tests, the level of significance for the evaluation of two-sided P values was set at 0.05.

Results

Participant characteristics
For the 356 samples, 302 (84.8%) HCV NS5B sequences were successfully amplified, purified, and sequenced. The overall participant characteristics of those with NS5B sequences (n = 302) are shown in Table 2. Men constituted 79.47% of the study population. More than 80% of the patients were younger than 50 years when diagnosed. The most common transmission route was injectable drug use (65.89%), followed by heterosexual intercourse (31.46%). More than half of the patients were married or cohabiting (69.54%), and 22.52% were unmarried. The geographical regions of Guangdong mentioned in Table 2 are shown in Fig. 1, most patients originated from the Pearl River Delta region (56.62%). A total of 75.83% of the subjects exhibited a baseline CD4+ T cell count < 200 cells/mm³.

Table 3 Distribution of HCV subgenotypes in HIV/HCV coinfection patients in Guangdong, stratified by period, 2010–2013 (N = 302)

| year | Number (N = 302) | HCV subgenotypes (n/N, %) | P for fisher exact tests |
|------|-----------------|---------------------------|-------------------------|
|      |                 | 1a(n = 11) 1b(n = 56) 2a(n = 3) 3a(n = 33) 3b(n = 21) 6a(n = 176) 6n(n = 2) |                         |
| 2010 | 117             | 4 (3.42) 25 (21.37) 1 (0.85) 9 (7.69) 7 (5.98) 70 (59.83) 1 (0.85) | 0.951                   |
| 2011 | 72              | 3 (4.17) 12 (16.67) 0 (0.00) 8 (11.11) 7 (9.72) 42 (58.33) 0 (0.00) |                         |
| 2012 | 70              | 2 (2.86) 12 (17.14) 1 (1.43) 10 (14.29) 3 (4.29) 41 (58.57) 1 (1.43) |                         |
| 2013 | 43              | 2 (4.65) 7 (16.28) 1 (2.33) 6 (13.95) 4 (9.30) 23 (53.49) 0 (0.00) |                         |
HCV subgenotype determination
A neighbor-joining phylogenetic tree based on the 302 NS5B sequences revealed the profile of HCV subgenotypes circulating among HIV/HCV coinfection patients in Guangdong (Fig. 1). Two predominant strains were found to be 6a (58.28%, 176/302) and 1b (18.54%, 56/302), followed by 3a (10.93%, 33/302), 3b (6.95%, 21/302), 1a (3.64%, 11/302), 2a (0.99%, 3/302) and 6n (0.66%, 2/302). There was no significant difference in the distribution of HCV subgenotypes between 2010 and 2013 (Table 3).

Identification of transmission networks
A total of 11 subgenotype 1a, 56 subgenotype 1b, 33 subgenotype 3a, 21 subgenotype 3b, and 176 subgenotype 6a NS5B sequences were used for molecular transmission network analysis between 2010 and 2013. Forty-two transmission clusters containing 133 of the 302 HIV/HCV coinfection patients (total clustering rate: 44.04%) were identified. The average cluster size was 3.24, with a minimum of two (19 clusters) and a maximum of 11 (one cluster). The clustering rates of subgenotypes 1a, 3a, 3b, 1b, and 6a were 18.18% (2/11), 42.42% (14/33), 52.38% (11/21), 48.21% (27/56), and 44.89% (79/176), respectively (Fig. 2).

Among all 42 clusters, 88.10% (37/42) comprised at least one subject from the IDU group, 57.14% (24/42) comprised at least one subject from the HET group, and only 4.76% (2/42) comprised at least one subject from the MSM group or the blood transfusion group (Fig. 3). However, when we analysed the clustering rate of different risk groups, we found that the clustering rate of the MSM group was higher than that of the other groups (66.67% vs. approximately 40%) (Table 4). Of the 133 individuals in clusters, 57.89% (77/133) were linked to cases diagnosed in different regions. Individuals from eastern regions had a higher clustering rate than individuals from other regions (60.00% vs. approximately 40%) (Table 4).

Patients were divided according to whether they fell into the transmission networks, and sex, age, transmission route, marital status, geographical region, baseline CD4+ T cell count, and subgenotype were examined. The results of the multivariate logistic regression analysis
showed that no significant effects from these factors were observed (P > 0.05), except marital status. Married or cohabiting people (compared with unmarried people, aOR = 0.496, 95% CI: 0.285–0.863) had more difficulty forming transmission networks (Table 4).

**Discussion**

HCV subgenotypes 1b (62.78%) and 2a (17.39%) were the two predominant subgenotypes in China, according to data from epidemiological studies on hospitalized patients [24]. HCV subgenotypes exhibit significant divergence between regions. HCV subgenotypes 1b and 2a remain the two predominant subgenotypes in North China. While the prevalence of HCV subgenotype 3b in Southwest China is significantly higher than that in other regions [25], HCV 6a was the most frequently represented genotype in southern China [19, 26, 27].

This study revealed that the main circulating HCV subgenotypes among HIV/HCV-coinfected patients in Guangdong were 6a (58.28%, 176/302), followed by 1b (18.54%, 56/302), 3a (10.93%, 33/302), 3b (6.95%, 21/302), 1a (3.64%, 11/302), 2a (0.99%, 3/302), and 6n (0.66%, 2/302). The predominant HCV subgenotypes among HIV/HCV-coinfected individuals in Guangdong were similar to those in Guangxi (6a (46%), 3a (20%), 3b (16%)) [27] but distinct from those in Yunnan (3b (37.62%), 3a (23.76%), 1b (16.34%)) [28]. HCV genotypes vary in the Asia–Pacific region [29], HCV infections and HIV infections have the common transmission route of sharing contaminated injecting equipment, sexual transmission and blood related transmission [29]. The geographic proximity to Southeast Asia and the presence of drug trafficking and use likely explains the similarity of the HCV genotype distributions in HIV/HCV-coinfected individuals between Guangdong and Guangxi. Guangxi Province, which borders Vietnam, could have been the first region to contract 6a for circulation. Genotype 6a was introduced into Guangxi from Vietnam and then...
further spread to Guangdong through drug trafficking routes and IDU networks [28–30].

The main circulating HCV subgenotypes among HCV mono-infected individuals in Guangdong were 1b (67.7%), followed by 6a (17.2%), 3a (6.1%), 2a (5.0%), 3b (2.0%), 4a (1.0%) and 5a (1.0%) [31], which were quite distinct from that found in the HIV/HCV co-infected patients. The difference in HCV genotype distribution between mono- and co-infection is most likely due to the varied transmission routes, with blood transfusion being the more common route in monoinfection and injectable drug use being the more common route in coinfection [19, 31].

Real-world studies on the efficacy of direct-acting antiviral agents (DAAs) therapy for HCV mono-infected patients in China showed that the sustained virologic

| Table 4  Factors associated with transmission within clusters |
|-----------------------------------------------|
| Characteristics                        | Within transmission network, n = 133 (n/N, %) | Total sequences, N = 302 | P for fisher exact tests | OR (95% CI) | P–value | Adjusted OR (95% CI) | P–value |
|-----------------------------------------------|-----------------------------------------------|---------------------------|--------------------------|--------------|---------|---------------------|---------|
| Sex                                           | 103 (42.92)                                   | 240                       | 0.475                    | 1.000        |         |                     |         |
| Male                                          | 30 (48.39)                                    | 62                        | 1.247 (0.712–2.183)      | 0.440        |         |                     |         |
| Female                                        |                                               |                           |                          |              |         |                     |         |
| Age (years)                                    |                                               |                           |                          |              |         |                     |         |
| < 30 years                                     | 7 (58.33)                                     | 12                        | 0.889                    | 1.000        |         |                     |         |
| 30–39                                         | 55 (43.31)                                    | 127                       | 0.546 (0.164–1.812)      | 0.322        |         |                     |         |
| 40–49                                         | 57 (44.19)                                    | 129                       | 0.565 (0.170–1.876)      | 0.351        |         |                     |         |
| 50–59                                         | 13 (43.33)                                    | 30                        | 0.516 (0.134–1.993)      | 0.337        |         |                     |         |
| > =60                                         | 1 (33.33)                                     | 3                         | 0.357 (0.025–5.109)      | 0.448        |         |                     |         |
| Transmission routes                           |                                               |                           |                          |              |         |                     |         |
| Injecting drug use                            | 91 (45.73)                                    | 199                       | 0.681                    | 1.000        |         |                     |         |
| Heterosexual                                   | 38 (40.00)                                    | 95                        | 0.791 (0.482–1.300)      | 0.355        |         |                     |         |
| MSM                                           | 2 (66.67)                                     | 3                         | 2.374 (0.212–26.603)     | 0.483        |         |                     |         |
| Blood                                         | 2 (40.00)                                     | 5                         | 0.791 (0.129–4.839)      | 0.800        |         |                     |         |
| Marital status                                 |                                               |                           |                          |              |         |                     |         |
| Unmarried                                      | 39 (57.35)                                    | 68                        | 0.260                    | 1.000        |         |                     |         |
| Married or cohabiting                         | 84 (40.00)                                    | 210                       | 0.496 (0.285–0.863)      | 0.013        |         |                     |         |
| Divorced or separated                          | 5 (31.25)                                     | 16                        | 0.338 (0.106–1.080)      | 0.067        |         |                     |         |
| Widowed                                        | 5 (71.43)                                     | 7                         | 1.859 (0.337–10.266)     | 0.477        |         |                     |         |
| Unknown                                        | 0 (0.00)                                      | 1                         |                          | –            | –       |                     | –       |
| Geographical region                           |                                               |                           |                          |              |         |                     |         |
| Pearl River Delta                             | 69 (40.35)                                    | 171                       | 0.419                    | 1.000        |         |                     |         |
| Eastern                                        | 6 (60.00)                                     | 10                        | 2.217 (0.603–8.149)      | 0.230        |         |                     |         |
| Western                                        | 49 (48.51)                                    | 101                       | 1.393 (0.849–2.287)      | 0.190        |         |                     |         |
| Northern                                       | 9 (45.00)                                     | 20                        | 1.209 (0.476–3.073)      | 0.689        |         |                     |         |
| Baseline CD4 T cell count (cells/mm3)          |                                               |                           |                          |              |         |                     |         |
| < 200                                         | 104 (45.41)                                   | 229                       | 0.852                    | 1.000        |         |                     |         |
| 200–349                                       | 25 (39.68)                                    | 63                        | 0.791 (0.448–1.395)      | 0.418        |         |                     |         |
| 350–499                                       | 3 (37.50)                                     | 8                         | 0.721 (0.168–3.089)      | 0.660        |         |                     |         |
| > 500                                         | 1 (50.00)                                     | 2                         | 1.202 (0.074–19.451)     | 0.897        |         |                     |         |
| Subgenotypes                                   |                                               |                           |                          |              |         |                     |         |
| 1a                                            | 2 (18.18)                                     | 11                        | 0.282                    | 1.000        |         |                     |         |
| 1b                                            | 27 (48.21)                                    | 56                        | 4.190 (0.830–21.157)     | 0.083        |         |                     |         |
| 2a                                            | 0 (0.00)                                      | 3                         | –                        | –            | –       |                     | –       |
| 3a                                            | 14 (42.42)                                    | 33                        | 3.316 (0.618–17.800)     | 0.162        |         |                     |         |
| 3b                                            | 11 (52.38)                                    | 21                        | 4.950 (0.856–28.635)     | 0.074        |         |                     |         |
| 6a                                            | 79 (44.89)                                    | 176                       | 3.665 (0.770–17.453)     | 0.103        |         |                     |         |
| 6n                                            | 0 (0.00)                                      | 2                         | –                        | –            | –       |                     | –       |
response (SVR12) rate greater than 90% was achieved in most of the HCV genotypes[32, 33]. Subjects with compensated cirrhosis (92.73%) and prior treatment experience (77.78%) had significantly lower SVR rates when compared to chronic hepatitis C (98.15%) and treatment-naive (97.69%) groups[33]. The available DAA regimens were generally well-tolerated and with high efficiency in the treatment of HIV/HCV co-infected patients, with similar efficacy to those with mono HCV infection. There was no significant difference in adverse effects among patients with different baseline CD4+ T-cell count in those who received DAA regimens with or without Peg-IFN and RBV[34].

In this study, approximately 44% of the HIV/HCV coinfection patients were members of the HCV transmission networks, which was consistent with the clustering rate of HIV/HCV coinfection patients in Dehong, China[17] (39.1%, 95/243) but higher than the clustering rate of HCV infection patients in Australia (20.76%, 49/236) [9] and Vancouver, Canada (31.14%, 156/501) [35]. Subgenotype 3b and subgenotype 1b inclined to form transmission clusters easily, with comparatively higher clustering rates of 2.38% and 48.21%, respectively. It suggested that the two subgenotypes were transmitted persistently among certain population at high risks, compared to other subgenotypes. According to the results of multivariate logistic regression, sex, age, transmission route, geographical region, baseline CD4+ T cell count and subgenotype were not influencing factors for whether patients entered the transmission networks. Married or cohabiting people had more difficulty forming transmission networks than unmarried people (Table 4), which may be due to the relatively fixed sexual partners of married or cohabiting people, and their probability of high-risk behaviour is lower than that of unmarried people. More than 80% of clusters comprised at least one subject from the IDU group, and in the largest cluster, more than 60% of nodes were patients from the IDU group (Fig. 3). These results suggested that more attention should be given to IDUs in future prevention and control work.

There were several limitations in our study. First, our observations were obtained based on the individuals coinfected with HIV/HCV spanning January 2010 and September 2013 in Guangdong. The shorter terms of recruitment may affected the judgement of HCV prevalence in Guangdong. Second, we focus on the subjects of coinfection which mainly through IDU and heterosexual contact. These specific populations might bias the deduced factors facilitating HCV transmission clustering. Whatever, we indeed performed some work to explore the transmission network of HCV, which may be of help to block the transmission of HCV among HIV individuals and general population.

In conclusion, this study provides an overview of the HCV transmission network among HIV/HCV coinfection patients in Guangdong, China, by using the characteristics of phylogenetic analysis. The total clustering rate was 44.04%, with different subgenotypes varying from 18.18% to 52.38%. Sex, age, transmission route, geographical region, baseline CD4+ T cell count, and subgenotype were not influencing factors, but marital status was an influencing factor for whether subjects entered the transmission network. Additional attention should be given to coinfections among unmarried individuals or patients infected through drug injection in future prevention and control work.

Abbreviations
AIDS: Acquired immunodeficiency syndrome; aORs: Adjusted odds ratios; aRT: Approximate likelihood ratio test; DAA: Direct-acting antiviral agent; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HET: Heterosexual; IDU: Injection drug user; MSM: Men who have sex with men; ML tree: Maximum likelihood phylogenetic tree; NS5B: Nonstructural 5B; ORs: Odds ratios; SH: Shimodaira-Hasegawa; SVR: Sustained virologic response; 95% CIs: 95% Confidence intervals.

Acknowledgements
The authors would like to thank Prof. Ruolei Xin from the Beijing Center for Disease Prevention and Control and Prof. Xiang He from Guangdong Provincial Institute of Public Health, for their expert guidance on this paper. The authors also thank National Virus Resource Center (NVRC-PY-04).

Author contributions
YL, FH, XD, and ZL conceived the study and supervised all aspects of the study. XD and ZL participated in the experiment. JL and WC collected the data. YL, XD, ZL, and WC analysed the data and prepared the manuscript. YL, FH, XD, and FL. All authors read and approval the final manuscript.

Funding
This research was funded by Guangzhou Science and Technology Plan Project (202002030028).

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
The study was approved by the medical ethics committee of the Guangzhou Eighth People’s Hospital (No. 201816107). Written consent was obtained from all patients.

Consent for publication
Not Applicable.

Competing interests
The authors declare that they have no competing interests.

Received: 1 March 2022   Accepted: 1 July 2022
Published online: 14 July 2022

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