High Prevalence of Human Parvovirus 4 Infection in HBV and HCV Infected Individuals in Shanghai

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Abstract

Human parvovirus 4 (PARV4) has been detected in blood and diverse tissues samples from HIV/AIDS patients who are injecting drug users. Although B19 virus, the best characterized human parvovirus, has been shown to co-infect patients with hepatitis B or hepatitis C virus (HBV, HCV) infection, the association of PARV4 with HBV or HCV infections is still unknown. The aim of this study was to characterise the association of viruses belonging to PARV4 genotype 1 and 2 with chronic HBV and HCV infection in Shanghai. Serum samples of healthy controls, HCV infected subjects and HBV infected subjects were retrieved from Shanghai Center for Disease Control and Prevention (SCDC) Sample Bank. Parvovirus-specific nested-PCR was performed and results confirmed by sequencing. Sequences were compared with reference sequences obtained from Genbank to derive phylogeny trees. The frequency of parvovirus molecular detection was 16–22%, 33% and 41% in healthy controls, HCV infected and HBV infected subjects respectively, with PARV4 being the only parvovirus detected. HCV infected and HBV infected subjects had a significantly higher PARV4 prevalence than the healthy population. No statistical difference was found in PARV4 prevalence between HBV or HCV infected subjects. PARV4 sequence divergence within study groups was similar in healthy subjects, HBV or HCV infected subjects. Our data clearly demonstrate that PARV4 infection is strongly associated with HCV and HBV infection in Shanghai but may not cause increased disease severity.

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Sample Bank. All human sera were de-identified before use in the study and all laboratory testing with samples was approved by SCDC Ethical Review Committee. All work involving human samples was approved by the Ethical Review Committee, Shanghai Municipal Center for Disease Control and Prevention. The samples were collected as part of routine surveillance activities undertaken by SCDC, and consent waived by the Ethical Review Committee.

Serological ELISA Assays

Anti-HCV IgM and HBsAg were assayed in all serum samples used. HBeAg was assayed in sera positive for HBsAg. Anti-HCV IgM, HBsAg and HBeAg were assayed using commercial ELISA kits (Diagnostic Kit for Anti-HCV IgM, HBsAg and HBeAg from Kehua Cooperation, Shanghai). Experiments were performed as per manufacturer’s recommendations. Positive and negative controls were included in each test. Any sample with OD of sample/average OD of negative control ≥2.1, was taken as positive.

PARV4 PCR

The total DNA of each sera sample (200 μl) was isolated on the MagNA Pure LC 2.0 (Roche, Switzerland) using MagNA Pure LC DNA Isolation Kit (Roche, Germany) following manufacturer’s recommendations; 60 μl of eluted DNA was used as template for PCR.

PARV4 nested PCR was performed in all samples using previously described primers PV4ORF1F and PV4ORF1R (outer primers) [3] and PV4NS1Fn2 and PV4NS1Rn2 (inner primers) [5], with GoTaq PCR Core System II (Promega, USA). PCR reaction conditions were as previously published [5].

B19 and Bocavirus Real-time PCR

Human Parvovirus (B19) Real Time PCR kit and HBoV Real Time PCR kit (Both from Shanghai ZJ Bio-Tech Co., Ltd., People’s Republic of China) were used to perform B19 and HBoV real-time PCR respectively, in all sera.

HBV DNA Test and ALT Assay

Serum specimens of chronic HBV patients were tested for presence of viral DNA with a Real Time PCR Quantitation kit (ZJ Bio-Tech, Shanghai). The PCR mix was amplified by Roche Light Cycler 480 (Roche, Switzerland). Alanine aminotransferase (ALT) was assayed in sera from chronic HBV patients using commercial kits (Kehua Cooperation, Shanghai) detected by the Synchron CX® 5 PRO Clinical Systems (Beckman, USA). Experiments were performed as per instructions; positive and negative controls provided by the company were included in each test. Serological positivity was accepted at titers ≥64 units per liter (U/L).

Sequencing and Phylogenetic Analysis

Identity of nested PCR products was confirmed by sequencing. PCR amplicons from 1564 to 1724 bp of ORF1 were sequenced by Biosune Company using the internal primers (BioSune, Shanghai). Sequences were analyzed by CLC Sequence Viewer 6 [9]. To facilitate phylogenetic analysis, PARV4 (PARV4 genotype 1; AY622943.1 and EU546211.1) and PARV5 (PARV4 genotype 2) sequences (DQ112361.1 and DQ873391.1) were downloaded from GenBank.

Data Analysis

Data were processed by SPSS for Windows version 13.0. Differences between nominal or ordinal variables were tested by the Pearson’s Chi-Square test. Continuous numeric data were compared by t test or nonparametric Kruskal-Wallis Chi-Square test. Significance was accepted at p≤0.05.

Results

Study subjects

The general information regarding study subjects is listed in Table 1. Groups were compared for gender and age distribution. 65.4% (100/153) of the HCV infected subjects and 67.6% (63/96) of matched controls were male (p = 0.703). Similarly, there was no difference in the gender distribution between HBV infected subjects and matched controls (p = 0.05). HCV infected subjects had the median age of 44.5 years, which was not different from that of matched controls (41 years; p = 0.227). The median age of HBV infected subjects was 41 years and did not differ from that of controls (36 years; p = 0.221).

B19 and Bocavirus Real-time PCR

Neither B19 nor HBoV were detected in serum samples from HBV, HCV infected subjects or their corresponding healthy controls.

HBV or HCV infected subjects had increased incidence of PARV4 DNA in sera compared to matched controls

Sera from 33.3% (51/153) of HCV infected subjects but only 16.7% (16/96) of matched controls were positive for PARV4 DNA (p = 0.004, Table 2). There was no difference in the gender distribution (p = 0.336) between HCV infected subjects who were positive for PARV4 (70.6%; 36/51 male) compared to those who were negative (62.7%; 64/102 male). Similarly, compared with the matched control group, PARV4 DNA detection was significantly higher in HBV infected subjects (p<0.0001), with no difference in the gender distribution (p = 0.122) between HBV infected subjects positive (60.3%; 47/78 male) or negative (50%; 55/110 male) for PARV4 (Table 2). Further analysis of PARV4 prevalence among different groups suggested that frequency of positive PARV4 DNA

| Gender       | Healthy Controls | Patients | p Value |
|--------------|------------------|----------|---------|
| **HBV group**|                  |          |         |
| Male         | 67.6% (65/96)    | 65.4% (100/153) | 0.703   |
| Female       | 32.3% (31/96)    | 34.6% (53/153) |         |
| **HCV group**|                  |          |         |
| Male         | 57.0% (110/193)  | 54.3% (102/188) | 0.590   |
| Female       | 43.0% (83/193)   | 45.7% (86/188) |         |

| Age (years) |          |         |         |
|            |          |         |         |
| **HBV group**|         |         |         |
| Median      | 41       | 44.5    | 0.227   |
| IQR         | 35–47    | 37–49   |         |
| Range       | 16–68    | 21–57   |         |
| **HCV group**|         |         |         |
| Median      | 36       | 41      | 0.221   |
| IQR         | 26–46    | 28–47.5 |         |
| Range       | 18–62    | 17–60   |         |

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Table 1. General information of study subjects.
PARV4 Infection is not associated with severity of HBV disease

Statistical analysis showed no difference in frequency of PARV4 DNA detection between the HBV infected (HBV carriers) and chronic HBV patients (p = 0.665), see Table 3. Further statistical analyses within the 60 chronic HBV patients showed similar frequency of PARV4 DNA detection between HBV DNA positive and negative subjects (p = 0.500). PARV4 positive subjects had an average ALT expression of 94.04 U/L, which was not significantly different from that of PARV4 negative subjects (107.3 U/L, p = 0.687; see Table 4).

High Sequence Similarity in PARV infecting Healthy, HBV, or HCV infected subjects

Ten sequences were selected from each study group and used to construct an evolutionary tree (Fig. 1). All 30 study subjects (Genbank accession number range from HQ267348 to HQ267375) were infected with PARV4, with nucleotide sequence homology ranging from 93.8% to 100%. The sequence homology between study samples and PARV4 genotype 1 reference strains ranged from 95.7 to 99.5%; between study samples and PARV4 genotype 2 reference strains from 90.1–95.6%. PARV4 detected in sample H122 showed the highest variability among the study samples (93.8-95.7%), with 95.7% homology with PARV4 genotype 1 reference sequences, and 90.1% with PARV4 genotype 2 reference sequences. The genetic distances between PARV4 and HBV59, HCV145 and H009 sequences were slightly larger than that of other study subjects. When an evolutionary tree was constructed on the basis of year of collection of samples no change in strains was observed in successive years.

Discussion

Previous studies have suggested a significant correlation between HBV and B19 co-infection [10,11]. PARV4 was originally detected in a HBV infected patient raising the possibility of an association with HBV infection as has been described for B19 [1,12]. To determine the association of PARV4 infection with HBV and HCV infection, we investigated the molecular prevalence of PARV4 in healthy individuals, HBV-infected subjects and HCV-infected subjects in Shanghai.

The frequency of PARV4 DNA detection was significantly higher among HBV infected and HCV infected subjects than matched controls. Our data demonstrate that PARV4 is probably able to co-infect with HBV and HCV. However, co-infection with PARV4 and HBV may not increase the severity of liver dysfunction in chronic HBV patients as no correlation between PARV4 prevalence and HBV DNA expression, or between PARV4 and blood ALT was observed. Our results show a similar trend to a study from Scotland which did not find any correlation between immunosuppression and PARV4 viral load or between PARV4 viral load and CD4 lymphocyte counts or HIV viral loads.

### Table 2. Detection Rate of PARV4 DNA.

| Characteristic | PARV4 Positive | PARV4 Negative | p Value |
|---------------|----------------|----------------|---------|
| Rate Between Patients and Healthy Controls | | | |
| HCV group | | | |
| Controls | 16.7% (16/96) | 83.3% (80/96) | 0.004 |
| Patients | 33.3% (51/153) | 66.7% (102/153) | |
| HBV group | | | |
| Controls | 22.2% (43/193) | 77.7% (150/193) | <0.0001 |
| Patients | 41.5% (78/188) | 58.5% (110/188) | |
| Rate Between Genders | | | |
| HCV group | | | |
| Male | 70.6% (36/51) | 62.7% (64/102) | 0.336 |
| Female | 29.4% (15/51) | 37.3% (38/102) | |
| HBV group | | | |
| Male | 60.3% (47/78) | 50.0% (55/110) | 0.122 |
| Female | 39.7% (31/78) | 50.0% (55/110) | |
| Rate Between HBV and HCV, as well as between Healthy Controls | | | |
| Hepatitis Patients | | | |
| HCV | 33.3% (51/153) | 66.7% (102/153) | 0.079 |
| HBV | 41.5% (78/188) | 58.5% (110/188) | |
| Healthy Controls | | | |
| 2008 | 16.7% (16/96) | 83.3% (80/96) | 0.283 |
| 2009 | 22.2% (43/193) | 77.7% (150/193) | |

### Table 3. General information & PARV4 DNA positive rate of HBV infected groups.

| Characteristic | HBV Infection (HBsAg+ HBeAg+) | Chronic HBV Infection (HBsAg+ HBeAg+) | p Value* |
|---------------|-----------------------------|---------------------------------|---------|
| Gender | | | |
| Male | 57.0% (107/188) | 56.7% (34/60) | 0.744 |
| Female | 43.0% (81/188) | 43.3% (26/60) | |
| Age | | | |
| Median | 41 | 42 | 0.245 |
| IQR | 28–47.5 | 36–48 | |
| PARV4 DNA | | | |
| Positive | 41.5% (78/188) | 38.3% (23/60) | 0.665 |
| Negative | 58.5% (110/188) | 61.7% (37/60) | |

*p value: data for 2 HBV patient groups was compared with Pearson’s χ²- test. doi:10.1371/journal.pone.0029474.t003

### Table 4. The PARV4 nucleic acid frequency in chronic HBV patients with/without HBV DNA, and with different ALT levels.

| Characteristic | PARV4 Positive | PARV4 Negative | P Value |
|---------------|----------------|----------------|---------|
| HBV DNA | | | |
| Positive | 60.9% (14/23) | 39.1% (9/23) | 0.597 |
| Negative | 67.6% (25/37) | 32.4% (12/37) | |
| ALT Quantitative | | | |
| Mean of ALT (U/L) | 94.04 | 107.30 | 0.687 |
| ALT Qualitative | | | |
| Positive (ALT≥64 U/L) | 52.2% (12/23) | 43.2% (16/37) | 0.500 |
| Negative (ALT<64 U/L) | 47.8% (11/23) | 56.8% (21/37) | |

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in HIV/AIDS patients [13]. 16.7–22.2% of healthy individuals in Shanghai had contracted PARV4 without any known acute viral infection symptoms, suggesting that PARV4 infection may often cause only subclinical infection.

The frequency of detection of PARV4 in sera from healthy subjects (16.7–22.2%) in our study is significantly higher than that reported in plasma from healthy blood donors from North America (5%, 7 of 137) [3] or Europe (none) [13]. Moreover, the PARV4 DNA prevalence in HCV and HBV infected individuals in Shanghai is markedly higher than that in highly exposed and symptomatic HIV patients from USA (33.3–41.5% vs. 6%) [12] or HCV-infected injecting drug users (IDUs) from London (30%; 3 of 10) [9] similar to that in plasma-derived coagulation factor concentrates from Germany (33%; 7/21) [14] and lower than that in the bone marrow and lymphoid organs of HIV-positive individuals (70%; 17 of 24) from Scotland [13]. Our data are in agreement with recent data from France where PARV4 DNA was detectable in 27.3% (6/22) and 38.7% (29/75) of plasma samples from haemodialysis patients positive for HCV and HBV, respectively [15]. The high prevalence of PARV4 DNA in HBV, HCV and HIV-infected subjects may simply reflect a generally higher exposure to infectious agents in these population groups. The variation in detection frequency in different countries may be multifactorial, such as the study sample size, sample types, the way and time of sample collection and storage, as well as detection protocols. In our study, the frequency of PARV4

Figure 1. Phylogenetic tree of PARV4 isolates infecting HBV, HCV patients and healthy controls. The nucleotide sequence from 1564 to 1724 bp of ORF1 gene of PARV4 were amplified, sequenced and analyzed. Bootstrap percentages are given at the node of each branch (1000 replicates). Sequences H009, H108, H110, H113, H121, H122, H142, H145 and H147 were from healthy controls. Sequences HBV02, HBV06, HBV09, HBV25, HBV42, HBV45, HBV50, HBV59, HBV63 and HBV73 were from HBV infected subjects with PARV4 infection. Sequences HCV037, HCV048, HCV052, HCV065, HCV074, HCV080, HCV112, HCV115, HCV130 and HCV145 were from HCV infected subjects with PARV4 infection. AY622943.1 and EU546211.1 represent 2 PARV4 genotype 1 reference strains, and DQ112361.11 and DQ873391.1 are sequences of 2 PARV4, genotype 2 reference strains. All reference strain sequences were downloaded from GenBank.

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PARV4 Co-Infection with Hepatitis B/C Virus

...clinical groups and that viral antigen persists to trigger and maintain T cell responses [17].

Phylogenetic analysis showed that all sequences of PARV4 ORF1 detected in this study clustered together with the two PARV4 genotype 1 reference strains and had a more distant phylogenetic relationship with PARV4 genotype 2 (PARV5). PARV4 genotype 1 may be the only PARV4 causing epidemics in Shanghai. Interestingly, the PARV4 genotype 1 variants circulating in Shanghai were substantially more divergent than those in Edinburgh [13].

Our study found that PARV4 has been circulating in Shanghai for the past 50 years with up to 22% of the population being affected. PARV4 had a significantly higher prevalence in HBV infected and HCV infected compared to healthy subjects. The pathogenic mechanisms of PARV4 co-infecting with other persistent pathogens remains to be elucidated.

Author Contributions
Conceived and designed the experiments: XY, JW, XZ, ZY. Performed the experiments: JZ, LH, JW. Analyzed the data: XY, XZ. Wrote the paper: XY, XZ, RG.

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