Association of cytokine gene polymorphisms with chronic hepatitis C virus genotype 1b infection in Chinese Han population

An observational study

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Abstract
Cytokines are extensively involved in the process of hepatitis C virus (HCV) infection and take a crucial part in host immune regulation. We aimed to explore the potential correlation of cytokine single nucleotide polymorphisms (SNPs) with HCV susceptibility and response rate of interferon (IFN)-based antiviral therapy in Chinese Han population.

A case-control genetic association study was conducted between 198 patients with chronic HCV genotype 1b infection and 142 healthy controls. Genetic polymorphisms of TNF-\(\alpha\) (rs1800629), TGF-\(\beta\) (rs1800469), IL-10 (rs1800896, rs1800871, and rs1800872), IL-6 (rs1800795, rs1800796), IFN-\(\gamma\) (rs2430561), and IL-28B (rs12979860, rs12980275, and rs8099917) were analyzed by MassARRAY SNP technology. Patients were treated with IFN-\(\alpha\)-2b or pegylated-IFN-\(\alpha\)-2a plus ribavirin for 48 weeks. Sustained virological response (SVR) was assessed 6 months after the completion of the treatment.

The IL-28B rs12979860-C(CD) (odds ratio [OR] = 3.45, 95% confidence interval [CI]: 1.69–11.21, \(P=0.001\)), rs12980275-AA (OR = 3.41, 95% CI: 1.08–10.76, \(P=0.028\)), and rs8099917-TT (OR = 3.86, 95% CI: 1.49–10.12, \(P=0.004\)) were significantly associated with SVR, and IL-10 rs1800871-CT (OR = 0.25–1.00, \(P=0.049\)) and rs1800872-AA (OR = 0.50, 95% CI: 0.25–1.00, \(P=0.049\)) were also significant for SVR. No association was found between the cytokine SNPs and HCV susceptibility. Additionally, multivariate analysis showed that low baseline viral load (OR = 3.63, 95% CI: 1.01–13.02, \(P=0.048\)), pegylated-IFN (OR = 9.68, 95% CI: 1.14–82.13, \(P=0.037\)) and rs12979860-C(D) (OR = 6.08, 95% CI: 2.00–18.46, \(P=0.001\)) were independent factors for SVR.

IL-28B and IL-10 gene polymorphisms played an important role in predicting host response to IFN-based antiviral therapy in HCV genotype 1b infection.

Abbreviations: CHC = chronic HCV infection, CI = confidence interval, DAA = direct antiviral agents, HCV = hepatitis C virus, HIV = human immunodeficiency virus, IFN = interferon, IL = interleukin, OR = odds ratio, PCR = polymerase chain reaction, RBV = ribavirin, SNP = single nucleotide polymorphism, STAT1 = signal transducer and activator of transcription 1, SVR = sustained virological response, TGF = transforming growth factor, TNF = tumor necrosis factor.

Keywords: chronic hepatitis C, cytokine, single nucleotide polymorphism, sustained virological response

1. Introduction
Hepatitis C virus (HCV) infection poses a global healthcare burden. The estimated number of individuals with HCV infection is 160 million worldwide, with 3 to 4 million newly infected and 350,000 deaths each year.\[^1,2\]\(^{1}\) In China, the HCV prevalence rate is about 0.43%, and the number of infections is reported to be over 5.6 million.\[^3\]\(^{2}\] Chronic HCV infection (CHC) is the leading cause of end-stage liver disease, hepatocellular carcinoma and liver related death. Approximately 10% to 20% of patients will progress from persistent liver inflammation to cirrhosis in 20 to...
30 years after HCV infection. Once cirrhosis has developed, there is a 1% to 5% annual risk of HCC and a 3% to 6% annual risk of hepatic decompensation. Following an episode of decompensation the risk of death in the following year is between 15% and 20%.

Before the widespread use of direct antiviral agents (DAAs) in China, interferon (IFN) and ribavirin (RBV) combination is still the primary choice of therapy. Unfortunately, the rate of sustained virological response (SVR) is only around 50% in HCV genotype 1 infected patients. Genome-wide association studies indicated that single nucleotide polymorphisms (SNPs) near the interleukin (IL)-28B (IFN-λ) locus displayed association with treatment response. In addition, cytokines are extensively involved in the process of HCV infection and are taking crucial parts in immune regulation. Previous studies had examined the relationship between cytokine SNPs and the disease course in patients with HCV infection, which suggested that SNPs of tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), IL-10, IL-6, and IFN-γ may correlate with HCV susceptibility, natural clearance of HCV, response rate of antiviral therapy, and the incidence of liver cirrhosis or hepatocellular carcinoma. However, evidence still remain controversial because cytokine gene polymorphisms vary greatly among ethnicities. Further studies with subjects from different regions and different ethnic backgrounds would provide important information of interplays between host, HCV and the clinical course of infection. We aimed to explore the potential correlation of cytokine gene polymorphisms with HCV susceptibility and response rate of antiviral therapy in Chinese Han population.

2. Materials and methods

2.1. Study population

A total of 198 treatment-naive patients (68 males and 130 females) infected with HCV genotype 1b at Jurong People’s Hospital (Jiangsu, China) from January 2016 to December 2017 were consecutively enrolled. All patients were positive for HCV antibodies using a second-generation enzyme-linked immuno-sorbent assay and tested positive for HCV RNA with polymerase chain reaction (PCR) at least three times in a 6-month follow-up. Before being treated all patients had fulfilled the following investigation: blood routine tests, liver function tests, kidney function tests, and HCV RNA levels. Demographic information and clinical features were collected as well. Patients were excluded if they:

1. were co-infected with human immunodeficiency virus (HIV);
2. known to be chronic liver diseases of etiologies other than HCV infection;
3. suffered from other significant concurrent medical conditions.

In addition, 142 healthy volunteers (60 males and 82 females) were recruited from outpatient department of Jurong People’s Hospital (Jiangsu, China) and Huashan Hospital (Shanghai, China), and detailed history and regular laboratory tests’ data were collected. Volunteers who had disclosed predisposing conditions or apparent infectious diseases were excluded. All participants were of Chinese Han ethnicity and negative for HIV screening test. Ethical approval was obtained from the medical ethics committee of Jurong People’s Hospital and Huashan Hospital. All patients and volunteers gave their written informed consent before the study.

2.2. Treatment regimens and follow up

All patients were qualified for treatment with standard doses of IFNα-2b (Anterferon, Anke; 3–6 × 107 IU/day for 4 weeks and follow by 3–6 × 106 IU three times a week, n = 176) or PEG-IFN-α2a (PEGASYS, Roche; 180 µg or 135 µg/week; n = 22) combined with RBV (Ribavirin, Sinopharm; 1000mg/day if body weight was <75kg or 1200mg/day if body weight ≥75kg) for 48 weeks. A total of 176 CHC patients received IFN-based and 22 patients received PEG-IFN-based therapy.

All the patients attended to Jurong People’s Hospital for monitoring during treatment. Patients had been assessed before (on the day of treatment initiation) and at week 4, 12, 24, and 48 of treatment as well as 6 months after the end of treatment. At each review, laboratory tests were performed including blood routine tests, liver function tests, kidney function tests, and HCV RNA levels. Adverse events were also recorded, and appropriate dose modifications to IFN or RBV were made at physician discretion. Patients were classified as IFN “non-responders” if they had persistent viremia 6 months after the end of therapy and “responders” if they had loss of HCV RNA, which were known as SVR.

2.3. SNP selection

We selected cytokine SNPs that had been reported in previous studies to be associated with HCV infection with the selection criteria of r² > 0.8 and minor allele frequency >0.10 for Chinese Han population. Eleven SNPs were selected for genotyping: TNF-α rs1800629; TGF-β rs1800469; IL-10 rs12979860, rs12980275, and rs8099917; IL-10 rs1800871, and rs1800872; IL-6 rs1800795 and rs1800796; IFN-γ rs2430561.

2.4. PCR and SNP genotyping

Genomic DNA was extracted from the venous blood using the QIAamp DNA kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. Primers for PCR and single base extension were designed by using Assay Design Suite V2.0 online (Sequenom, California, USA) (Table 1). SNP genotyping was performed by using MassARRAY system (Sequenom) by means of matrix assisted laser desorption ionization-time of flight mass spectrometry method (MALDI-TOF) according to the manufacturer’s instructions. Briefly, the DNA sample to be queried was diluted to 5 to 10ng/µL, and 1µL of DNA was combined with 0.95µL of water, 0.625µL of PCR buffer containing 15mM MgCl2, 1 µL of 2.5mM dNTP, 0.325 µL of 25mM MgCl2, 1µL of PCR primers and 0.1 µL of 5units/µL HotStar Taq (Qiagen). The reaction was incubated at 94°C for 15 min followed by 45 cycles at 94°C for 20s, 56°C for 30s, and 72°C for 1min, and a final incubation at 72°C for 3 min. After PCR amplification, remaining dNTPs were dephosphorylated by adding 1.53 µL of water, 0.17 µL of SAP buffer, and 0.3 units of shrimp alkaline phosphatase (Sequenom). The reaction was placed at 37°C for 40 min, and the enzyme was deactivated by incubating at 85°C for 5 min. After shrimp alkaline phosphatase treatment, the single primer extension over the SNP was combined with 0.755 µL of water, 0.2 µL of 10 × iPLEX buffer, 0.2 µL of termination mix, 0.041 µL of iPLEX enzyme (Sequenom), and 0.804 µL of 10 µM extension primer. The single base extension reaction was carried out at 94°C for 30s, followed by 40 cycles at
94°C for 5s, 5 cycles of 52°C for 5s and 80°C for 5s, and a final incubation at 72°C for 3 min. The reaction mix was desalted by adding 6 mg of cation exchange resin (Sequenom), mixed and resuspended in 25 μL of water. The completed genotyping reactions were spotted onto a 384 well spectroCHIP using MassARRAY Nanodispenser (Sequenom) and determined by the matrix-assisted laser desorption-ionization time-of-flight mass spectrometer. Genotype calling was analyzed using the MassARRAY Typer software version 4.0 (Sequenom).

2.5. Statistical analysis

SNPStats (https://www.snpstats.net) was used for the genetic data analysis. The Hardy-Weinberg equilibrium was evaluated by chi-square test. The effect of genotypes was tested by multiple logistic regression models (dominant, recessive, and overdominant) for odds ratio (OR), 95% confidence interval (CI), and P-values. Data were expressed as median and range for quantitative variables and compared using Mann–Whitney tests. Proportions were compared with the Chi-squared test ($\chi^2$) or Fisher’s exact test, as appropriate. Logistic regression model was used for multivariate analysis. Data were analysed with the use of SPSS statistical package (version 17.0). All tests were two-sided, and a value of $P < .05$ denoted statistical significance.

3. Results

3.1. Demographic and clinical information

The detailed demographic and clinical information of CHC patients is shown in Table 2. Among these 198 patients, the median age was 52 years (range, 21–71 years), 68 patients were males and 130 patients were females. Most (77.8%, 154/198) patients got infected through the bloodstream, while others through the breaks of skin or mucous membrane barriers. One hundred fifty-four (77.8%) patients had high viral load (HCV RNA ≥ 4 × 10^5 IU/mL) before treatment. IFN-based therapy were stopped in 32 (16.2%) patients, of which 29 (14.6%) experienced severe IFN-related adverse events and 3 (1.5%) had no response to therapy. Of the 166 patients (55 males and 111 females) who finished IFN-based therapy, 120 (72.3%) had reached SVR.

3.2. Association of cytokine SNPs and HCV susceptibility

All samples were succeeded in genotyping. Allele distributions of the 11 tested SNPs were compared with the Chi-squared test ($\chi^2$) or Fisher’s exact test. Proportions of those SNPs were compared between CHC patients (n = 198) and control subjects (data not shown). The distribution of those SNPs was discovered. It was found that male and female (n, %) for OR, 95% confidence interval (CI), and P-values. Data were expressed as median and range for quantitative variables and compared using Mann–Whitney tests. Proportions were compared with the Chi-squared test ($\chi^2$) or Fisher’s exact test, as appropriate. Logistic regression model was used for multivariate analysis. Data were analysed with the use of SPSS statistical package (version 17.0). All tests were two-sided, and a value of $P < .05$ denoted statistical significance.

Table 1

| SNP ID | Primers of 11 tested cytokine SNPs designed for PCR. | Primers sequence (5’→3’) |
|-------|-----------------------------------------------------|--------------------------|
| rs1800629 | R ACGTTGGATGCAACACACTACTAAGGCTTC |  |
| rs180029 | F ACGTTGGATGGATAGTTCCAAACATGTGCG |  |
| rs1800796 | SBE SBE TTTCCTTTCTGTGAGCAAT |  |
| rs2430561 | R ACGTTGGATGCAACACACTACTAAGGCTTC |  |
| rs1800795 | F ACGTTGGATGGATAGTTCCAAACATGTGCG |  |
| rs1800469 | SBE SBE TTTCCTTTCTGTGAGCAAT |  |
| rs1800795 | F ACGTTGGATGGATAGTTCCAAACATGTGCG |  |

Table 2

| Demographic and clinical information of CHC patients. | Characteristics | CHC patients (n = 198) |
|-----------------------------------------------------|-----------------|------------------------|
| Age (year, median, range) | Male and female (n, %) for OR, 95% confidence interval (CI), and P-values. Data were expressed as median and range for quantitative variables and compared using Mann–Whitney tests. Proportions were compared with the Chi-squared test ($\chi^2$) or Fisher’s exact test. Proportions of those SNPs were compared between CHC patients (n = 198) and control subjects (data not shown). The distribution of those SNPs was discovered. It was found that male and female (n, %) for OR, 95% confidence interval (CI), and P-values. Data were expressed as median and range for quantitative variables and compared using Mann–Whitney tests. Proportions were compared with the Chi-squared test ($\chi^2$) or Fisher’s exact test, as appropriate. Logistic regression model was used for multivariate analysis. Data were analysed with the use of SPSS statistical package (version 17.0). All tests were two-sided, and a value of $P < .05$ denoted statistical significance. |  |

3.2. Association of cytokine SNPs and antiviral therapy outcome

Comparisons of genotype distribution of 17 tested SNPs as well as demographical and clinical features were made between responders (n = 120) and non-responders (n = 46). It was found that male patients accounted for a larger proportion in responder group (OR = 3.06, 95% CI: 1.31–7.13, P = .008). Proportions of patients with low baseline viral load (OR = 4.99, 95% CI: 1.45–
17.25, \( P = .006 \) and receiving PEG-IFN (OR = 9.55, 95% CI: 1.25–73.18, \( P = .01 \)) were significantly higher in responders than that of non-responders. In addition, rs12979860-CC (OR = 4.35, 95% CI: 1.69–11.21, \( P = .001 \)), rs12980275-AA (OR = 3.41, 95% CI: 1.08–10.76, \( P = .028 \)), and rs8099917-CC (OR = 3.86, 95% CI: 1.49–10.12, \( P = .004 \)) were more observed in responders, while rs1800871-CT (OR = .50, 95% CI: 0.25–1.00, \( P = .049 \)) and rs1800872-AA (OR = .50, 95% CI: 0.25–1.00, \( P = .049 \)) were less detected in responders (Table 3). Comparisons of other factors such as age, transmission route, and baseline laboratory routine tests found no significant differences between the two groups. We further developed a logistic regression model for SVR that included all variables with a value of \( P < .05 \) in the univariate analysis. Low baseline viral load (OR = 3.63, 95% CI: 1.01–13.02, \( P = .048 \)), PEG-IFN (OR = 9.68, 95% CI: 1.14–82.13, \( P = .037 \)) and rs12979860-CC (OR = 6.08, 95% CI: 2.00–18.46, \( P = .001 \)) were independently predictive of SVR (Table 3).

### 4. Discussion

IFN and RBV combination therapy is still the first choice for patients where DAAAs were not available. Even when combined with DAA, triple therapy of DAA, IFN, and RBV also showed better therapeutic effects than DAA alone.[15,16] Several viral and host related factors may influence the outcome of IFN and RBV combination therapy in CHC patients, of which cytokines played an important role in the initiation and regulation of antiviral immune responses.[17] Previous studies had observed differences in the expression profiles of cytokines among CHC individuals, which also displayed associations with treatment response and were considered to be related to their genetic polymorphisms. A case–control study of 440 CHC patients infected with HCV genotype 4 and 220 healthy controls in Egypt indicated that IL-28B rs12979860, TGF-\( \beta \) rs180469, and TNF-\( \alpha \) rs1800629 were significantly associated with susceptibility to HCV infection and response to antiviral therapy, while no association was found in IL-10 gene polymorphisms (rs1800896). Another study among HCV genotype 3 infected patients in Pakistan showed that cytokine gene polymorphisms were not correlated with SVR, whereas IL-10 (rs1800896) gene polymorphism was related to the grades of liver inflammation.[18] Pereira et al demonstrated a statistically significant difference in the frequency of TGF-\( \beta \) codon 25 polymorphism (rs1800471) among healthy subjects and CHC patients, while no associations were observed between polymorphisms of TNF-\( \alpha \), IFN-\( \gamma \), IL-10, TGF-\( \beta \) codon 10, or IL-6 and HCV infection.[19] Fabrício-Silva et al also carried out a study among 221 Brazilian HCV patients, including 184 patients infected with genotype 1 and 37 patients infected with genotype 2.[20] They concluded that IL-28B gene polymorphism (rs12979860 and rs12980275) can predict spontaneous HCV clearance rate and SVR rate, and IL-6 (rs1800795) G allele was involved in increased inflammation scores. Similarly, a meta-analysis indicated that patients carrying G allele of IL-6 (rs1800795) may be more likely to suffer from liver diseases, which was ethnic-dependent. Patients carrying IL-6 G allele were found to have high IL-6 expression in plasma and were more likely to cause persistent HCV infection.[21] In addition, other cytokines such as IL-10 and IFN-inducible protein-10 were also reported to have the potential to be the biomarkers for prognostic of HCV infection.[22,23]
We studied in Chinese Han population and enrolled 198 CHC patients and 142 controls to explore the correlation of 11 cytokine SNPs with susceptibility to infection and response to therapy. No associations were observed between cytokine gene polymorphisms and HCV susceptibility. With regard to the prediction of SVR, IL-28B, and IL-10 gene had shown significant importance. The SNPs (rs12979860, rs12980275, rs8099917) near the IL-28B gene on chromosome 19 coding for IFN-λ were previously reported to be associated with treatment response in HCV.[6] According to NCBI database, there are three genotypes in rs12979860, which are CC, CT, and TT. The genotype distribution varies among regions. The proportions of the three genotypes in African population are 0% to 10%, 0% to 42%, and 48% to 100%, respectively, while accounting for 80% to 93%, 0% to 20%, and 0% in Asia, respectively. Our study presents a proportion (87%, 12%, and 0.6%) consistent with that of Asian population. As for rs12980275, our data shows that the proportion of AA, AG, and GG genotypes are 92%, 7%, and 0.6%, respectively, which consists with the data from Asian that is 77% to 93%, 7% to 23%, and 0%. Three genotypes of rs8099917 are TT, TG, and GG. We present the corresponding proportions of 88%, 11%, and 0.6%, also in accordance with that of Asian population in database, which are 80% to 93%, 7% to 20%, and 0%. Moreover, genotype frequencies of TT, CT, and CC of rs1800871 (IL-10) and AA, AC, and CC of rs1800872 (IL-10) in our study are both 46%, 40%, and 13%, which are comparable with 44% to 56%, 36% to 51%, 5% to 9% and 37% to 60%, 32% to 52%, 4% to 10% of data from Asian population, respectively.

IL-10 belongs to Th2-type anti-inflammatory cytokine, acts in highly coordinated cytokine network and interferes with innate and adaptive protective immunity in infectious diseases.[25,26] IL-10 also interferes with the balance of Th1/Th2 cells, negatively regulates the response of Th1 lymphocytes, and prevents immunologic injury caused by exacerbated immune responses.[27] It is believed that ~50% to 70% of the observed variation in IL-10 production contributes to genetic factors, mainly the polymorphisms in the promoter region of IL-10 gene, those were rs1800896, rs1800871, and rs1800872.[22,28] In CHC patients receiving IFN-based therapy, IL-10 could attenuate IFN-α-activated signal transducer and activator of transcription 1 (STAT1) in the liver.[29] A meta-analysis of 14 studies involving 1687 CHC cases showed that the rs1800896 G allele was associated with decreased SVR rate, especially for the Egyptian and HCV genotype 4 infection, while the rs1800871 T allele was more likely to predict SVR in Caucasians.[30] Khan et al also revealed the significant increased infection risks for the high IL-10-producing rs1800896-GG and rs1800872-CC.[31] Furthermore, the determination of IL-10 serum level combining with IL-10-producing rs1800896-GG and rs1800872-CC genotypes was significantly associated with SVR, while no SNPs survived after adjusting for age, sex, baseline viral load, and types of IFN.

Strengths of this study include the prospective and complete follow-up of illness, and the combination of genetics and clinical susceptibility and prognosis. On the other hand, this study has noteworthy limitations. The number of cases recruited was limited. Some genotypes of SNPs have low frequencies, which may restrict statistical power. Therefore, our results should be interpreted with caution. Further studies comprise a large cohort, or conducted in other ethnic groups, or in patients infected with other HCV genotypes are necessary to reevaluate our findings.

In conclusion, our study suggested that IL-28 and IL-10 gene polymorphisms played an important role in predicting host response to IFN-based antiviral therapy in HCV genotype 1b infection. Further investigation of the causative mechanisms may be helpful to target cytokines on which to base host-directed immunotherapy.

Author contributions
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