Published in final edited form as:

Biochem Biophys Res Commun. 2011 November 25; 415(3): 503–508. doi:10.1016/j.bbrc.2011.10.102.

Impact of host responses on control of hepatitis C virus infection in Chinese blood donors

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Abstract

A study was undertaken to explore the molecular mechanisms underlying control of HCV infection in blood donors in China. Factors including clinical information, anti-HCV reactivity (S/CO), IFN- α and IFN- γ , viral loads and genotypes were correlated with 160 index plasma samples at three statuses of 45 recovered, 76 chronic or 39 false positive anti-HCV reactive blood donors. The spontaneous recovery rate was 37.2%. Viral loads of 76 HCV plasmas ranged between 59.8 IU/ml and 2.43×10^6 IU/ml (median 3.67×10^4 IU/ml). Genotypes 1, 2, 3 and 6 of 63 HCV strains were identified phylogenetically. Recovered donors were significantly younger ($p=0.002$) and had lower level IFN- γ ($p=0.001$) than chronically HCV infected donors. Circulating levels of IFN- α and IFN- γ were higher in those with low viral load and were low in middle or high viral load samples. The ratio of IFN- α to IFN- γ (IFN- α/γ) was significantly positively correlated with viral load ($p=0.037$), and viral load was inversely correlated with IFN- γ in chronic HCV infection regardless of genotype. The study revealed clearly different relationships between IFN- α and IFN- γ in relation to viral load in HCV, which might provide a new approach to evaluate long term control of HCV infection.

Keywords

HCV; natural viral clearance; molecular factors; Chinese

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Conflict of interest: None of the authors has any potential financial conflict of interest related to this manuscript.

1 Introduction

More than 170 million people are infected with hepatitis C virus (HCV) worldwide. Approximately 30% of HCV infected individuals spontaneously clear the virus and 70% become chronically infected, the latter being at risk of developing chronic liver disease including liver failure, liver cirrhosis, hepatocellular carcinoma possibly requiring liver transplantation [1]. A systematic review of longitudinal studies reported rates of 0-80% spontaneous viral clearance following acute HCV infection [2], which varied according to ethnic groups [3,4]. However, to date, little is known about the rate of recovery of HCV infected population living in China.

HCV has been classified into six major genotypes 1 to 6, which have different geographical distributions, pathogenicity and genetic diversity around the world [5]. China has a high prevalence of HCV genotype 1b followed by 2a, 3 and 6 according to a previous report [6]. Genotypes may play a role in the severity and clinical outcome of HCV infection.

Specific antibodies and changes in levels of cytokines were detected in HCV infected individuals, which might be involved in determining spontaneous resolution or chronicity of HCV infection. Interferon- α (IFN- α) has been shown to inhibit HCV subgenomic RNA replication, and is used in combination therapy with ribavirin [7,8]. Levels of IFN- γ are reported to be significantly elevated in HCV infected patients compared to normal controls [9]. However, parameters such as antibody reactivity, cytokine level, viral load and genotype detected in HCV infected individuals, especially from Chinese populations have not been analytically correlated with clinical outcome of recovered or chronic HCV infection.

This study explored the mechanisms of HCV infections in Chinese blood donors at recovery or chronic status in relation with clinical information, viral features and host immune response.

2 Materials and methods

2.1 Blood donor screening and HCV antibody detection

Donor blood samples were collected from Shenzhen or Guangzhou Blood Centers in Guangdong or Blood Banks of Army Hospitals in Beijing. Donor plasmas were screened for HBsAg, anti-HCV and anti-HIV between September 2003 and January 2009. Anti-HCV reactive samples negative for both HBsAg and anti-HIV at the blood centers were re-tested by enzyme immunoassays (EIAs) (Murex anti-HCV v4.0, Murex Biotech SA Ltd, Abbott; Lizhu anti-HCV EIA, Zhuhai Lizhu Biotechnology Ltd, Zhuhai, China) according to the manufacturers' instructions. Anti-HCV positive plasma was confirmed by at least two reactive domestic and international commercial EIAs, and anti-HCV reactivity was reported as an average of ratios of signal to cut-off (S/CO).

2.2 Biochemical tests

The level of alanine aminotransferase (ALT) was measured in U/L for each anti-HCV reactive blood donor by ReflotronPlus kit (Roche Diagnostics, Mannheim, Germany).

2.3 HCV RNA detection and quantification

Viral RNA was extracted from 200 μ l of plasma using High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) as instructed by the manufacturer. The extracted RNA was eluted in 50 μ l elution buffer and stored at -80°C . Amplification of HCV genome was performed with two-step quantitative RT-PCR. Reverse transcription (RT) reaction mixture contained 1 $\times$ PrimeScript buffer, 0.5 μ l PrimeScript RT Enzyme Mix I, 5 μ M random 6 mers, and 7 μ l of template RNA preparation in 10 μ l of reaction volume (TaKaRa PrimeScript RT reagent Kit, TaKaRa Biotechnology Ltd., Dalian, China). The reaction was carried out at 37°C for 30 min, 85°C for 5 s. The quantitative PCR (qPCR) was performed as previously described [10]. QPCR mixture contained 1 $\times$ PCR reaction buffer (with 2 mM MgCl_2), 0.2 mM each dNTP, 0.4 μ M each primer, 0.2 μ M probe, 1 $\times$ Rox Dye II, 1 U of FastStart Taq DNA Polymerase, and 4 μ l of template cDNA in 25 μ l of reaction volume (Roche FastStart Taq DNA Polymerase kit). The amplification started with an incubation for 10 min at 95°C , then ran 45 cycles with two-step of 30 s at 95°C and 1 min at 55°C , and finished at 4°C .

The WHO International Standard for HCV RNA, which contained 3.0×10^5 IU/ml kindly provided by Mr. Qingtao Song (Xinchuang Scientific Ltd, Xiamen, China), was used for viral load quantification.

2.4 HCV RNA confirmatory testing and genotyping

The presence of HCV RNA in the anti-HCV reactive sample was confirmed by amplifying 5' un-translated region via RT nested PCR [11]. Amplicons (approximately 215 bp HCV cDNA fragment) were purified in 1.5% agarose gel using Axygen gel extraction kit and were sequenced for HCV genotyping. Nucleotide sequences were phylogenetically analyzed by MEGA version 4.1 in alignment with the 5'-NCR sequences of HCV references, which were retrieved from GenBank database with accession numbers as follows: HCV genotype 1: AF009606, AJ132996, D10749, D14853, D90208, M62321, M67463, U1214 and X61596; genotype 2: AB030907, AB031663, AF238484, AF238486, D00944, D10988 and D50409; genotype 3: AF046866, D17763, D28917, D49374, D63821 and X76918; genotype 4: Y11604; genotype 5: AF064490 and Y13184; genotype 6: D63822, D84262, D84263, D84264, D84265 and Y12083.

2.5 Cytokine assays

The cytokines IFN- α and IFN- γ were quantified in blood samples with a commercially available enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Inc. Minneapolis, MN, USA) according to the manufacturer's instructions.

2.6 Statistical analysis

Data analysis was performed using the SPSS software version 13.0. For descriptive analyses, the medians and interquartile ranges, or the means and standard deviations, were reported. Comparisons among continuous data were assessed by Median test with regard to median values or Independent-Samples T test regarding mean values, when appropriate. Pearson Chi-square test was used for comparisons of proportions between groups. Correlations

between continuous variables were assessed by Spearman's rho nonparametric correlation analysis. P value <0.05 was considered statistically significant.

3 Results

3.1 Identification of HCV infection status from blood donor samples

A total of 160 anti-HCV reactive blood donor samples were re-tested and stratified into 45 HCV antibody positive and RNA negative (Ab+/RNA-), 76 Ab+/RNA+ and 39 Ab-/RNA- corresponding to three statuses: recovered infection, chronic infection and false positive reactions, respectively. The rate of recovery or chronic HCV infection was 37.2% (45/121) and 62.8% (76/121) in the confirmed HCV antibody carrying blood donors, respectively. HCV RNA loads from 76 chronic infections ranged between 59.8 IU/ml and 2.43×10^6 IU/ml (median 3.67×10^4 IU/ml). Genotypes from 63 HCV strains were identified as 27 (42.9%) genotype 1, 9 (14.3%) genotype 2, 12 (19.0%) genotype 3 and 15 (23.8%) genotype 6. A high prevalence of HCV genotype 1 was detected in blood donors from Beijing and Guangdong, while genotype 6 (11/34, 32.4%) was mainly found in the Guangdong province.

3.2 Characterization of recovered, chronic and false positive HCV infections in Chinese blood donors

The parameters of clinical information, anti-HCV reactivity (S/CO) and IFN- α and IFN- γ productions were assessed in the statuses of recovery, chronic infection and false positive HCV reactions in blood donors (Table 1). Significant differences were observed in categories of age, S/CO and IFN- γ between the three groups ($p = 0.002$). The median age (26 years) of donors with recovered HCV infection was significantly younger than those (30 and 28 years) with chronic infection or false positive HCV reaction statuses ($p=0.002$). The median level of IFN- γ in donors with recovered status was similar to donors with a false positive status, but was significantly lower than those with chronic infection status ($p=0.001$). The calculated S/COs of anti-HCV reactivity in those with a recovered status were statistically lower than those with chronic infection ($p < 0.001$). Circulating levels of IFN- α from recovered HCV infected donors were higher than found in chronic infection and false positive HCV reaction populations (466.9 vs 364.3 or 370.6 pg/ml), but the difference was not statistically significant ($p=0.197$). Ratios of IFN- α to IFN- γ (IFN- α/γ) in recovered cases were similar to those with false positive status but was significantly higher in donors with chronic infection status ($p<0.001$).

3.3 Correlation of anti-HCV reactivity, IFN- α and IFN- γ in recovery and chronic HCV infections

To analyze the interactions of immune factors detected from recovered and chronic HCV infections, correlative analyses were performed for paired IFN- α and IFN- γ , IFN- α and S/CO, and IFN- γ and S/CO from 121 HCV infected blood samples, respectively (Fig. 1). A negative correlation between IFN- α and S/CO and a positive correlation between IFN- γ and S/CO were significant (Fig. 1B and 1C) ($p=0.005$ and 0.009 , respectively). IFN- α and IFN- γ appeared to be inversely related (Fig. 1A), but the correlation was not significant ($p=0.446$).

3.4 Relationship of IFN- α , IFN- γ and anti-HCV reactivity with HCV clearance

Considering the viral load of HCV in relation to the host immune response, the immunological parameters of anti-HCV reactive individuals were analyzed according to four levels of viral loads in three HCV infection groups. Viral loads in false positive and recovered groups were considered to be nil (0 IU/ml), and viral load in chronic infection was stratified into low, middle and high levels corresponding to 1 to $<10^3$, 10^3 to $<10^5$ and 10^5 to 10^7 IU/ml, respectively. Trends of IFN- α , IFN- γ , IFN- α/γ ratio and anti-HCV reactivity associated with viral loads are plotted in Figure 2. The levels of IFN- α , IFN- γ , S/CO and viral load in samples with false positive status were considered as baseline representative of healthy blood donor population. In chronic infection, levels of IFN- α and IFN- γ were highest in samples with low viral load (1 to $<10^3$ IU/ml), IFN- α was lower and stayed below the baseline in middle or high viral load (10^3 - 10^7 IU/ml), while IFN- γ decreased but maintained higher levels than in false positive controls. The level of anti-HCV reactivity (S/CO) was highest in middle viral load (10^3 – 10^5 IU/ml), while IFN- α level was the lowest.

Viral load was then correlated with each immunological factor IFN- α , IFN- γ , IFN- α/γ ratio or anti-HCV reactivity level in 74 individuals with chronic HCV infection (Fig. 3). IFN- α had no correlation with viral load (Fig. 3A) ($p=0.983$). IFN- γ and anti-HCV reactivity appeared to negatively and positively correlate with HCV viral load, respectively (Fig. 3B and 3D), but this was not statistically significant ($p=0.064$, 0.121). A significant positive correlation between IFN- α/γ ratios and viral loads was observed ($p=0.037$), which suggested that the balance between levels of these two cytokines was related to viral load control during chronic HCV infection.

The factors of clinical information, viral load, S/CO, IFN- α , IFN- γ and IFN- α/γ ratio were compared in relation to genotypes 1, 2, 3 and 6 of HCV detected in blood donors (data not shown). Only blood donors infected with genotype 2 had significantly higher anti-HCV reactivity than those infected with genotypes 1, 3 or 6 of HCV ($p=0.028$). Other factors showed no significant difference among HCV genotypes.

4 Discussion

In the study, we first determined the breakdown between spontaneous recovery and chronic HCV infection from blood donors in Beijing and Guangdong, China and systematically assessed the factors of clinical information, viral feature and host immune response in HCV infections associating with viral clearance or chronicity in a Chinese population.

HCV infection may resolve or evolve to chronic infection, reported as a variety of recovery rates in different reports. In West Africa, 45-53% of anti-HCV positive Ghanaian blood donors spontaneously recovered from HCV infection [3]. A rate of 29.9% spontaneous viral clearance was found in acute symptomatic hepatitis C in Poland [12], which was similar to a previous report from Japan [13]. A systematic review of longitudinal studies reported recovery rates ranging between 0-80% following acute HCV infection [2]. These widely variable rates might be related to the testing algorithm, the population and genetic variation [4], or the HCV genotype. To improve the precision of the estimated rate of spontaneous viral clearance in our study, the anti-HCV reactive samples from primary screening were

confirmed by at least two different EIAs. A 37% recovery ratio (45/121) was reported in this study in HCV infected Chinese blood donors. The samples with anti-HCV unconfirmed by alternative EIAs were finally classified as false positive status although some might represent recovered HCV infection with low or undetectable anti-HCV in plasma [14,15]. Thus 37% represents a lower limit and the true figure might potentially be even higher than this. This high value may be linked to the enrichment of favourable IL28B genotypes in this population [4].

Clinical information from individual donors including age, gender, race or ethnicity, education and body mass index were analyzed in correlation with the outcome of HCV infections, which suggested that race/ethnicity associated with the spontaneous clearance of HCV [2,16,17]. Whether age is associated with viral clearance remains controversial [18,19]. The median age (26 years) of recovered HCV infection status in our study was significantly lower than either chronic infection or false positive status (30 and 28 years, respectively) ($p=0.002$), suggesting that younger donors resolved infection more frequently than older donors. The odds of women presenting with recovered status were 8% higher than men, but this was not statistically significant ($p=0.597$).

The dynamic interaction between the virus and the host's immune response appears critical in determining the outcome of HCV infection. By comparing differences between recovery, chronic infection and false positive HCV status, the level of IFN- α was marginally higher (although not significantly so; $p=0.197$) while IFN- γ was significantly lower ($p=0.001$) in recovered donors compared to those with chronic HCV infection. Furthermore, the ratio of IFN- α/γ in recovered infection was significantly higher than in chronic infection ($p<0.001$). Although the causality of this result and its significance are not yet clear, it is consistent with the idea that IFN- α rather than IFN- γ played a dominant role in spontaneous recovery from HCV infection. Previous studies demonstrated that IFN- α production could be inhibited by HCV replication [20,21]. Consistent with this, in this study, the lowest levels of IFN- α were observed in samples with middle and high HCV viral loads and the highest level of IFN- α was found in donors with low viral load ($<10^3$ IU/ml). The levels of IL28B were not determined in this study, although it will be of interest in the future to address the impact of distinct IL28B polymorphisms on this balance of cytokine production *in vivo*.

Previous studies indicated that the level of IFN- γ increased in patients with HCV chronic infection and correlated with viral load and severity of liver disease [9]. IFN- γ can inhibit replication of subgenomic and genomic HCV RNAs *in vivo* [22]. In this area, we found that high levels of circulating IFN- γ were present in chronic infection but were lower in recovered HCV infection ($p=0.001$). Importantly, IFN- γ level inversely associated with HCV viral load in chronic infection ($p=0.064$). Interestingly, a significant positive correlation between IFN- α/γ ratio and HCV viral load was observed ($p=0.037$). Thus, amongst those with persistent infection, low viral loads are accompanied by relatively low levels of IFN- α and high levels of IFN- γ , while the converse is true in those with high viral loads. Prospective studies are required to assess whether of the value of IFN- α/γ ratio dynamics could be used to evaluate the outcome of HCV infection. IFN- γ production might play an important role in HCV viral load control during chronic infection. A similar

suggestion was made in a study where HCV RNA loads negatively correlated with IFN- γ produced by IL-12 stimulated CD56⁺ T cells [23].

Four genotypes 1, 2, 3, and 6 of HCV were detected in Beijing and Guangdong. Genotype 1 was widely distributed in south and north China, while genotype 6 was mainly identified in Guangdong, south China, closer to Hong Kong and Thailand where genotype 6 is predominant [6]. Whether the genotype of HCV, as a viral factor, played a role in determining the outcome of HCV infection remains controversial. Genotype 2 was associated with a high recovery rate (50%) of HCV infections in Ghanaian blood donors and was significantly associated with an efficient contribution of HLA-B57 [24]. Genotype 1b of HCV had been implicated in poor prognosis of the infection [25]. In our study, we found no statistical correlations between HCV genotype and factors such as gender, age, viral load and interferon levels.

The comparison of a range of parameters between spontaneous recovery, chronic and false positive HCV infections from blood donors in China suggested that the IFN- α response might play a major role in spontaneous recovery of HCV infection, while IFN- γ might play a role on viral load control in chronic HCV infection. A novel measure of IFN- α/γ ratio, which correlates with viral load in chronic disease, could be a new approach to evaluate long term outcome of HCV infection. Future studies to evaluate the role of antiviral cytokines in this population, which is highly enriched in protective IL28B genotypes, could provide new insights into the mechanisms of spontaneous and drug-induced control of HCV [26,27].

Acknowledgments

The authors thank the comments from Dr Daniel Candotti, National Health Service Blood & Transplant, Cambridge, England. This work was supported by the grants from National Basic Research Program of China (973 Program No: 2009CB522507 and No. 2010CB530204), S&T Grand Special Program (No: 2009ZX10004-305) and the Wellcome Trust, NIH U19 1U19AI082630-01, the James Martin School for the 21st Century Oxford and the NIHR Biomedical Research Centre Oxford.

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Fig. 1. Correlation between IFN- α , IFN- γ and S/CO in HCV infected individuals.

Paired IFN- α and IFN- γ , IFN- α and S/CO, IFN- γ and S/CO from 121 HCV infected blood samples were correlatively plotted. The power trends are indicated by the lines on Figure 1a to 1c. By Spearman's rho nonparametric correlation analysis, the 2-tailed p -values were 0.446, 0.005 and 0.009, respectively. The correlation coefficients were -0.072, -0.257 and 0.242, respectively.

Fig. 2. Variation of immune responses associating with viral load in three statuses of HCV reactivity.

Levels of HCV RNA loads were stratified into three statuses of HCV reactivity and in chronic infection in three grades of viral loads. The medians of IFN-α, IFN-γ, IFN-α/γ ratio and S/CO were plotted within each group.

Fig. 3. Correlation between HCV RNA load and immunological factors.

The power trend of viral load in association with each immune factor was plotted for 74 individuals with chronic HCV infection. By Spearman's rho nonparametric correlation analysis, the 2-tailed p -value from paired viral load and immune factor of IFN- α , IFN- γ , IFN- α/γ ratio or S/CO was 0.983, 0.064, 0.037 and 0.121, respectively.

Table 1

Characterization of recovery, chronic and false positive statuses of HCV infections from blood donors.

Variables		Recovery (N = 45)	Chronic (N = 76)	False positive (N = 39)	p-value ^a
Gender ^b	F/M	14/26	20/55	10/28	0.597
	F/M (%)	35/65	27/73	26/74	
	Nb Avail (n) ^d	40	75	38	
Age ^c	Median	26	30	28	0.002
	Interquartile ^e	23–30	26–36	25–32	
	Range	19–37	19–52	21–44	
	Nb Avail (n)	35	41	38	
ALT ^c (U/L)	Median	33	45	35	0.137
	Interquartile	23–53	25–66	27–49	
	Range	1–95	9–234	8–106	
	Nb Avail (n)	45	74	39	
S/CO ^c (Anti-HCV)	Median	2.31	4.85	0.49	<0.001
	Interquartile	1.78–4.14	3.35–15.64	0.40–0.66	
	Range	1.06–17.38	1.01–18.15	0.26–0.88	
	Nb Avail (n)	45	76	39	
IFN- α ^c (pg/ml)	Median	466.9	364.3	370.6	0.197
	Interquartile	207.9–568.4	164.6–722.7	344.8–457.4	
	Range	133.4–3191.3	95.0–2788.7	196.6–2613.6	
	Nb Avail (n)	44	75	39	
IFN- γ ^c (pg/ml)	Median	4.53	8.57	4.26	0.001
	Interquartile	3.32–7.25	4.44–20.02	2.06–8.20	
	Range	0.00–42.24	0.00–164.37	0.422–22.50	
	Nb Avail (n)	43	74	39	
IFN- α / γ ^c ratio	Median	91.43	33.20	106.14	<0.001
	Interquartile	36.16–165.45	13.33–75.33	40.59–233.71	
	Range	4.67–715.3	1.67–1237.6	19.10–864.7	
	Nb Avail (n)	41	67	39	

^a p-value indicated the difference among three groups of HCV infection statuses.

^b Comparison of proportions regarding gender was assessed by Pearson Chi-square.

^c Differences regarding other factors were calculated by Median test.

^d Nb Avail (n): Number available samples.

^e Interquartile: 75% of range.