Circulating Biomarkers and their Possible Role in Pathogenesis of Chronic Hepatitis B and C Viral Infections

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Abstract The present study evaluated the plausible role of circulating biomarkers in immune pathogenesis of chronic hepatitis considered a priority in clinical hepatology. Total viral load of chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) patients was quantified and correlation studies were performed with circulating levels of Th1/Th2 cytokines; C reactive protein and circulating nucleosomes; glutathione reductase (GR) and superoxide dismutase. To our knowledge, the study is first among its kind that validates strong positive correlation of viral load with IL-4, IL-6, GR in HBV and IL-6, IL-10, GR in HCV infections. Although, multi-centric studies including large cohorts are required for translating our findings to clinical practice, however, role of these biomarkers with potential diagnostic or prognostic significance might be helpful in clinical assessment of high-risk individuals, thereby, designing interventional strategies, towards development of personalized medicare. The results of our study also offer valuable insights of immune signaling mediators engaged in development of hepatocellular carcinoma.

Keywords Hepatitis · Nucleosomes · Hepatocellular carcinoma · Translational research · Biomarkers

Introduction

Although enormous strides in prevention and control of hepatitis have been done in the past years, it still remains as a serious global problem due to its high morbidity and mortality rate worldwide. Among its viral constituents, hepatitis B virus (HBV) with two billion infected people and 500,000–700,000 deaths each year [1, 2] and hepatitis C virus (HCV) having an estimated 130 million infected persons which accounts for 23% cirrhosis and 25% of hepato-cellular carcinoma (HCC) worldwide [3], are the two main etiological agents. One of the foremost characteristic feature of these viral infections is that majority of the total affected population becomes chronically infected i.e., life long infections. The exact mechanism resulting in such a high chronic transformation still to be revealed, however, inability of host immune system to eliminate the organism is considered as an important cause of such persistent form of infection [4, 5].

The host response to hepatitis involves various components of the immune system, including T-lymphocyte immuno-regulatory cytokines which have distinct role in the outcome of the disease. While Th1 cytokine profile suggests a cell-mediated immunity and is associated with recovery [6], Th2 cytokine response takes place in development of persistent lifelong infection [7]. Thus, it can be stated that the imbalance of pro-inflammatory Th1 and anti-inflammatory Th2 cytokine production may play an important role in the pathogenesis of viral hepatic infections. Moreover, chronic inflammatory response involving inflammatory cytokines has been reported to be associated with liver injury. These cytokines recruit inflammatory cells, promote fibrogenesis and further activate oxidative burst [8]. Preliminary studies have also documented the role of pro-inflammatory cytokines and/or by a specific

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effect of HCV core protein in provoking oxidative DNA damage. This damage is further associated with an imbalance between apoptosis and cyto-proliferation and in the activation of the basic mechanisms of HCC [9–11]. Although, it is well known that reactive oxygen species (ROS) induction lies at the center of a complex network of tissue and inflammatory responses involving the expression of cytokines, growth factors and oncogenes, this network has not been thoroughly investigated in HBV/HCV-related liver diseases.

In addition, viral load in chronic hepatitis is considered as a strong predictor for the disease progression [12], yet, it is unclear whether there is any link between the increasing viral loads with the other circulating biomarkers. The aim of the work reported herein was to evaluate circulating biomarkers in patients with chronic HBV/HCV infections and their possible role in the pathogenesis of the disease. Initially, quantitative estimation of viral nucleic acid through COBAS Amplicor was done and later on, these results were correlated with the secreted levels of Th1/Th2 cytokines, IL-2, TNF- α , IFN- γ IL-4, IL-6 and IL-10; anti-oxidant defense system enzyme glutathione reductase (GR) and superoxide dismutase (SOD); C reactive protein (CRP) and levels of circulating nucleosomes.

Materials and Methods

Subject Selection

The study was approved by the Institutional Review Board, Bhopal Memorial Hospital and Research Centre, Bhopal (India). The study was classified into three groups i.e. group 1 comprised of patients with chronic HBV ($n = 60$); group 2 comprised of patients with chronic HCV ($n = 60$) infections and group 3 comprised of age and gender matched controls ($n = 60$). The mean age of the subjects included in the study was 35 years. The diagnosis of chronic HBV/HCV infections was based on the sero-positivity of HBV/HCV specific antibodies within the last

6 months. There was no serologic evidence of co-infection with other hepatotropic viruses (Table 1). Other possible causes of hepato-cellular injury, such as alcohol and drug-related injuries were also excluded. HBV/HCV samples were obtained from Medical and Surgical Gastroenterology Departments. The subjects were included in the study after informed consent was obtained and all clinical information pertaining to them was properly recorded. The controls incorporated in the study were without any clinical history of hepatitis and considered healthy through routine laboratory analysis. 10 ml of blood sample conjugated with EDTA were collected from each patient by routine venipuncture method. The blood obtained was centrifuged at 3000 rpm for 10 min for the separation of the plasma which was used for further investigations.

Reagents

Quantitative estimation of HBV DNA and HCV RNA was performed by using COBAS Amplicor HBV Monitor Test and COBAS Amplicor HCV Monitor Test from Roche Diagnostics, Mannheim, Germany. Activity of SOD and GR, enzymes of anti-oxidant defense system was quantified by using ELISA kits from Trevigen Inc., Gaithersburg, USA. Analysis of secreted levels of inflammatory cytokines was performed using BDTM Multiplex Cytometric Bead Array (CBA) and Human Th1/Th2 kit from BDTM Biosciences, San Diego, USA. Levels of CRP were determined by Quantikine Human CRP Immunoassay kit from R & D Systems., Minneapolis, USA. Cell death detection ELISA (CDDE) was performed by using kit from Roche Applied Science, Mannheim, Germany.

Quantitative Estimation of Viral Load

Quantitative estimation of HBV DNA/HCV RNA was performed as per supplier's instructions. Amplification and detection of the samples as well as the controls were done using COBAS Amplicor Analyzer (Roche Diagnostics, Mannheim, Germany) [13].

Table 1 Clinical characteristics of HBV, HCV and control groups

Characteristics	HBV (group 1)	HCV (group 2)	Control (group 3)
Duration of infection (years)	14.1 $\pm$ 8.7	13.4 $\pm$ 6.5	–
Co-infection	–	–	–
Serum ALT level (IU/l)	151.4 $\pm$ 34.1	163.5 $\pm$ 26.5	$\leq$ 40
Serum AST level (IU/l)	93.5 $\pm$ 28.6	112.6 $\pm$ 43.9	$\leq$ 40
Serum total bilirubin level (μ mol/ml)	19.8 $\pm$ 5.4	21.9 $\pm$ 10.1	$\leq$ 17.1

Data are expressed as mean $\pm$ SE

ALT Alanine Aminotransferase, AST Aspartate Aminotransferase

ELISA for Estimation of GR Activity

GR, a homodimeric flavoprotein disulfide oxidoreductase, plays an indirect but essential role in the prevention of oxidative damage within the cell by helping to maintain appropriate levels of intracellular reduced glutathione (GSH). The total GR activity was measured as per manufacturer's instruction. It is a spectrophotometric analysis in which the oxidation of NADPH to NADP⁺ is monitored by the decrease in absorbance at 340 nm. This rate of decrease in absorbance at 340 nm is directly proportional to the GR activity in the sample. The absorbance kinetics was measured at 340 nm by ELISA reader [14].

Estimation of SOD Activity by ELISA

Superoxide dismutase catalyzes the dismutation of the superoxide radical (O₂^{•−}) into H₂O₂ and elemental O₂, and as such, provides an important defense against the toxicity of superoxide radical. Activity of SOD was measured according to supplier's instructions. In this assay, O₂^{•−} ions that were generated from the conversion of xanthine to uric acid and H₂O₂ by xanthine oxidase (XOD) will convert WST-1 to WST-1 formazan. Superoxide dismutases in turn will reduce superoxide ion concentrations and thereby lowers the rate of WST-1 formazan formation. The extent of reduction in the appearance of WST-1 formazan is a measure of SOD activity present in the experimental sample. The optical density was measured at 450 nm on an ELISA reader [15].

ELISA for CRP

The assay was performed according to instructions supplied by the manufacturer. It employs a quantitative sandwich-enzyme immunoassay technique in which a monoclonal antibody specific for CRP has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any CRP present is bound by the immobilized antibody. The optical density was measured at 450 nm by ELISA reader [16].

Multiplex CBA Assay for Human Th1/Th2 Cytokines

Plasma from patients and controls was subjected for the analysis of Th1/Th2 cytokines by determining the levels of cytokines, IL-2, TNF- α , IFN- γ , IL-4, IL-6 and IL-10. The assay was performed according to the manufacturer's recommendation. Data acquisition and analysis were carried out on a flow cytometric platform [17].

Cell Death Detection ELISA

Measurement of circulating nucleosomes as the markers of apoptotic cell death was done through CDDE. The assay is based on the quantitative sandwich-enzyme-immunoassay-principle using mouse monoclonal anti-bodies directed against DNA and histones. The absorbance kinetics was measured at 405 nm by ELISA reader [18].

Statistical Analysis

Student's *t* test and Pearson's correlation coefficient (*r*) was performed for statistical analysis using SPSS software (SPSS Inc. Chicago, IL, USA). The data was of normal distribution with *P* value less than 0.05 and *r* value more than 0.5 being considered significant.

Results

Quantitative Estimation of Viral Load

Quantitative detection of HBV DNA and HCV RNA by COBAS Amplicor analyzer displayed viral load which ranged from 7.4×10^1 to 5.3×10^7 copies/ml in group 1 subjects (chronic HBV patients) and from 1620 to 6.1×10^6 copies/ml in group 2 subjects (chronic HCV patients). Neither HBV DNA nor HCV RNA was detected in the subjects of in group 3 (controls).

Correlation of HBV Viral Load with Circulating Biomarkers

Elevated mean level of circulating cytokines in group 1 (chronic HBV patients) in comparison to the controls were observed (Table 2). Furthermore, a positive correlation of viral load with Th2 cytokines IL-4 ($r = 0.589$, $P < 0.001$) and IL-6 ($r = 0.725$, $P < 0.001$) was also observed

Table 2 Comparative cytokine levels (pg/ml) in HBV, HCV and control groups

	HBV (group 1)	HCV (group 2)	Control (group 3)
IL-2	31.24 $\pm$ 2.407*	33.97 $\pm$ 2.63*	12.1 $\pm$ 0.81
IFN- γ	25.34 $\pm$ 1.74*	28.50 $\pm$ 2.16*	14.90 $\pm$ 1.06
TNF- α	23.92 $\pm$ 1.46*	24.88 $\pm$ 1.55*	13.05 $\pm$ 1.532
IL-4	55.71 $\pm$ 2.94*	59.42 $\pm$ 4.68*	14.67 $\pm$ 1.532
IL-6	37.17 $\pm$ 1.95*	51.68 $\pm$ 2.36*	13.69 $\pm$ 1.25
IL-10	35.9 $\pm$ 1.82*	48.72 $\pm$ 2.43*	12.88 $\pm$ 1.6

Data are expressed as mean $\pm$ SE

* $P \leq 0.05$

Fig. 1 Correlation studies performed between Hepatitis B viral load on y axis ($n = 60$) with circulating biomarkers on x axis. The results observed a strong correlation of IL-4, IL-6 and GR with increasing viral load in HBV group. **a** Correlation of viral load of HBV (copies/

ml) with IL-2 (pg/ml), IFN- γ (pg/ml), TNF- α (pg/ml), IL-4 (pg/ml), IL-6 (pg/ml), IL-10 (pg/ml). **b** Correlation of HBV viral load (copies/ml) with CDDE (AU/ml), CRP (ng/ml), GR (mU/ml), SOD (mU/ml)

Table 3 Comparative levels of circulating biomarkers in HBV, HCV and control groups

	HBV (group 1)	HCV (group 2)	Control (group 3)
CDDE (AU/ml)	0.36 ± 0.018*	0.71 ± 0.15*	0.17 ± 0.08
CRP (ng/ml)	0.64 ± 0.15*	0.96 ± 0.09*	0.32 ± 0.04
GR (mU/ml)	7.27 ± 0.67*	8.42 ± 0.79*	1.91 ± 0.76
SOD (mU/ml)	0.14 ± 0.02	0.18 ± 0.03*	0.08 ± 0.02

Data are expressed as mean ± SE

* $P \leq 0.05$

(Fig. 1a). The present investigation also observed a raise in the mean values of CDDE and CRP compared to their respective controls. The mean CDDE values were 0.35 ± 0.03 ($P < 0.001$) whereas in case of CRP the mean levels were 0.64 ± 0.09 ($P < 0.001$) (Table 3). Neither CDDE nor CRP values correlated with the viral loads of group 1 (Fig. 1b). Additionally, an increase in the levels of GR the enzyme of antioxidant defense system, was also observed in comparison to controls which strongly correlated with the increasing viral load ($r = 0.830$, $P < 0.001$). However, there was no such correlation observed with SOD level. The mean value of GR was 3.29 ± 0.50 while the SOD level was 0.04 ± 0.002 (Table 3; Fig. 1b).

Correlation Between HCV Viral Load and Circulating Biomarkers

In group 2 (chronic HCV patients) the mean level of circulating cytokines were observed to be elevated compared to the controls (Table 2). A positive correlation of viral load with Th2 cytokines IL-6 ($r = 0.799$, $P < 0.001$) and IL-10 ($r = 0.673$, $P < 0.001$) was observed in HCV group (Fig. 2a). In addition, the mean CDDE and CRP values were also observed to be raised in comparison to the control group. The mean CDDE values were 0.70 ± 0.05 ($P < 0.001$) and the CRP values were 0.97 ± 0.13 ($P < 0.001$) (Table 3). Both CDDE and CRP values did not show any correlation with the viral load in HCV group (Fig. 2b). Furthermore, in HCV group an increase in the levels of GR was also observed in HCV group comparison to controls which in turn was positively correlated with the increasing viral load ($r = 0.51$, $P < 0.001$). However, there was no such correlation observed in SOD level. The mean level of GR was 8.44 ± 0.47 ($P < 0.001$) and the mean level of SOD was 0.144 ± 0.006 ($P < 0.001$) in HCV group compared to their respective controls (Table 3; Fig. 2b).

Discussion

Chronic infection with hepatitis often results in cirrhosis and enhances the probability of developing HCC, with no

identified etiologic factors in majority of cases [19]. Since, viral load is considered as a strong predictor the disease progression in chronic hepatitis, identifying the status of circulating biomarkers in correlation to viral load might provide crucial clinical implications in disease management. The present study observed a strong positive correlation of increasing viral load with IL-4, IL-6 and GR in HBV and IL-4, IL-10 and GR in HCV.

Successful outcome of the disease requires a strong virus-specific cytotoxic response by removing infected hepatocytes and secreting cytokines that inhibit viral replication. While Th1 cytokines (IL-2, IFN- γ , TNF- α) are required for host antiviral immune response and are related with tissue injury, Th2 cytokines (IL-4, IL-6 and IL-10) on the other hand typically regulate humoral immune response and their rising levels are often associated with persistent infections [6]. Cytokines released by one type of Th lymphocyte population can down-regulate the functions of another Th population [20]. Earlier studies have reported a significant decreased Th1 and increased Th2 cytokines levels in patients with chronic HBV/HCV [21–24]. Further shift of Th1 to Th2 responses has been also implicated in the pathogenesis of some infectious diseases, such as HIV infection, mycobacterial and protozoal diseases [25]. Additionally, recent studies have indicated the involvement of Th2 cytokines in attributing towards the risk of developing HCC as serum levels of IL-6 and IL-10 were observed to be frequently elevated in patients with HCC, further serving as complementary tumor markers in these patients [26]. Interestingly, the present investigation observed an increase in the mean levels of circulating cytokines as compared with their controls (Table 2). A positive correlation between the viral load and Th2 cytokines in both group 1 (chronic HBV patients) and group 2 (chronic HCV patients) was also observed (Figs. 1a, 2a).

Another important mediator of host immune defense is CRP which is produced predominantly by the liver in pro-inflammatory conditions [27]. Normal baseline levels of circulating CRP are low, but may increase by 10,000-fold within hours of inflammation [28]. Although the physiological role of CRP is unclear, it is considered as a potential predictive biomarker for several pathological conditions [29–32]. Recently studies have attempted to correlate the serum levels of CRP with the prognosis of HCC patients and the usefulness of CRP as a tumor marker in HCC patients is debatable [33, 34]. In present study we observed the elevated level of CRP in the chronic patients in comparison to controls but without any correlation with viral loads of both HBV and HCV groups (Table 3; Figs. 1b, 2b).

Moreover, hepatic tissue homeostasis depends on maintaining the balance between cell proliferation and apoptosis. Disruption of this balance may lead to lethal

Fig. 2 Correlation studies conducted between Hepatitis C viral load ($n = 60$) on y axis and circulating biomarkers on x axis. A strong correlation of IL-6, IL-10 and GR with increasing viral load was observed in HCV group. **a** Correlation of HCV viral load (copies/ml)

with IL-2 (pg/ml), IFN- γ (pg/ml), TNF- α (pg/ml), IL-4 (pg/ml), IL-6 (pg/ml), IL-10 (pg/ml). **b** Correlation of HCV viral load (copies/ml) with CDDE (AU/ml), CRP (ng/ml), GR (mU/ml), SOD (mU/ml)

consequences including hepatic carcinogenesis [35]. Studies have also indicated that death of liver cells plays a crucial role in the pathogenesis of viral hepatitis as the expression levels of Fas antigen in hepatocytes of patients with HBV or HCV closely correlated with inflammatory activity and the increased cytotoxic response [36]. It is also evident from recent experimental data that increased levels of circulating nucleosomes in various disease conditions are associated with enhanced programmed cell death [37]. Therefore, our investigation focused on the possible relationship between increased levels of circulating nucleosomes as biomarker for apoptosis and pathogenicity of HBV and HCV and in this regard increasing levels of circulating nucleosomes in comparison with controls were observed (Table 3). However, no correlation with the increasing viral load in both group 1 (chronic HBV patients) and group 2 (chronic HCV patients) was observed (Figs. 1b, 2b).

Hepatocytes are continuously exposed to ROS and are protected from oxidative injury by a range of antioxidant pathways. Previous studies have suggested that oxidative stress induced by increased ROS and/or impairment of antioxidant mechanism is one of the reasons for hepatocellular damage in chronic infections [38–42] and might be associated with the development of HCC [9]. It has also been suggested that oxidative stress biomarkers could potentially be used as a useful clinical diagnostic tool to predict the duration of survival in patients with HCV-associated HCC [43]. Since patients with greater intra-hepatic oxidative stress are associated with increase risk of HCC, the present study looked at the potential role of antioxidant defense system enzymes as a useful clinical diagnostic tool in predicting the duration of disease in patients with chronic hepatitis-associated HCC. In the present investigation the increased levels of the antioxidant defense system enzyme GR was observed which positively correlated with increasing viral load of HBV and HCV. However no such correlation in the SOD activity was observed (Table 3; Figs. 1b, 2b).

The identification of biomarkers with potential diagnostic or prognostic significance for chronic hepatitis is considered a priority of clinical hepatology. To our knowledge, the study is first among its kind that validates a positive correlation of viral load with IL-4, IL-6, GR in HBV and IL-6, IL-10, GR in HCV infections. Although robust multi-centric studies including large cohorts are required for translating our findings to clinical practice, however, these might be helpful in clinical assessment of high-risk individuals for better disease prognosis and also for designing interventional strategies, towards development of personalized medicare. The results of our study also offer valuable insights of immune signaling mediators engaged in HCC development.

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References

1. Hepatitis B. Vaccines. *Wkly Epidemiol Rec.* 2004;9:255–63.
2. Shepard CW, Simard EP, Finelli L, Fiore AE, Bell BP. Hepatitis B virus infection: epidemiology and vaccination. *Epidemiol Rev.* 2006;28:112–25.
3. Alter MJ. Epidemiology of hepatitis C virus infection. *World J Gastroenterol.* 2007;13:2436–41.
4. Modlin RL, Nutman TB. Type 2 cytokines and negative immune regulation in human infections. *Curr Opin Immunol.* 1993;5: 511–4.
5. NIH Consensus Statement on Management of Hepatitis C. NIH Consensus State Sci Statements. 2002;19:1–46.
6. Hultgren C, Milich DR, Weiland O, Sallberg M. The antiviral compound ribavirin modulates the T helper (Th) 1/Th2 subset balance in hepatitis B and C virus-specific immune responses. *J Gen Virol.* 1998;10:2381–91.
7. Fan XG, Liu WE, Li CZ, Wang ZC, Luo LX, Tan DM, et al. Circulating Th1 and Th2 cytokines in patients with hepatitis C virus infection. *Mediators Inflamm.* 1998;7:295–7.
8. Ramadori G, Armbrust T. Cytokines in the liver. *Eur J Gastroenterol Hepatol.* 2001;13:777–84.
9. Moriya K, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, et al. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res.* 2001;61:4365–70.
10. Hoek JB, Pastorino JG. Ethanol, oxidative stress, and cytokine-induced liver cell injury. *Alcohol.* 2002;27:63–8.
11. Farinati F, Cardin R, Degan P, De Maria N, Floyd RA, Van Thiel DH, et al. Oxidative DNA damage in circulating leukocytes occurs as an early event in chronic HCV infection. *Free Radic Biol Med.* 1999;27:1284–91.
12. Chen CJ, Yang HI, Su J, Jen CL, You SL, Lu SN, et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA.* 2006;295:65–73.
13. Mishra PK, Bhargava A, Khan S, Pathak N, Punde RP, Varshney S. Prevalence of hepatitis C virus genotypes and impact of T helper cytokines in achieving sustained virological response during combination therapy: a study from Central India. *Indian J Med Microbiol.* 2010;28:358–62.
14. Mishra PK, Khan S, Bhargava A, Panwar H, Banerjee S, Jain SK, et al. Regulation of isocyanate-induced apoptosis, oxidative stress, and inflammation in cultured human neutrophils: isocyanate-induced neutrophils apoptosis. *Cell Biol Toxicol.* 2010;26: 279–91.
15. Mishra PK, Raghuram GV, Panwar H, Jain D, Pandey H, Maudar KK. Mitochondrial oxidative stress elicits chromosomal instability after exposure to isocyanates in human kidney epithelial cells. *Free Radic Res.* 2009;43:718–28.
16. Bhargava A, Punde RP, Pathak N, Dabadghao S, Desikan P, Jain A, et al. Status of inflammatory biomarkers in the population that survived the Bhopal gas tragedy: a study after two decades. *Ind Health.* 2010;48:204–8.
17. Bhargava A, Khan S, Panwar H, Pathak N, Punde RP, Varshney S, et al. Occult hepatitis B virus infection with low viremia induces DNA damage, apoptosis and oxidative stress in peripheral blood lymphocytes. *Virus Res.* 2010;153:143–50.
18. Mishra PK, Dabadghao S, Modi GK, Desikan P, Jain A, Mittra I, et al. In utero exposure to methyl isocyanate in the Bhopal gas disaster: evidence of persisting hyperactivation of immune system two decades later. *Occup Environ Med.* 2009;66:279.

19. Alacacioglu A, Somali I, Simsek I, Astarcioglu I, Ozkan M, Camci C, et al. Epidemiology and survival of hepatocellular carcinoma in Turkey: outcome of multicenter study. *Jpn J Clin Oncol.* 2008;38:683–8.
20. Fan XG, Yakkob J, Fan XJ, Keeling PW. Enhanced Th2 responses: immune mechanism of *H. pylori* infection. *Ir J Med Sci.* 1996;165:37–9.
21. Jirillo E, Greco B, Caradonna L, Satalino R, Pugliese V, Cozzolongo R, et al. Evaluation of cellular immune responses and soluble mediators in patients with chronic hepatitis C virus (CHCV) infection. *Immunopharmacol Immunotoxicol.* 1995;17:347–64.
22. Cacciarelli TV, Martinez OM, Gish RG, Villanueva JC, Krams SM. Immunoregulatory cytokines in chronic hepatitis C virus infection: pre-and post treatment with interferon alfa. *Hepatolgy.* 1996;24:6–9.
23. Piazzolla G, Tortorella C, Schiraldi O, Antonaci S. Relationship between interferon-gamma, interleukin-10, and interleukin-12 production in chronic hepatitis C and in vitro effects of interferon-alpha. *J Clin Immunol.* 2000;20:54–61.
24. Song LH, Binh VQ, Dinh ND, Kun JF, Bock TC, Kremsner PG, et al. Serum cytokine profiles associated with clinical presentation in Vietnamese infected with hepatitis B virus. *J Clin Virol.* 2003;28:93–103.
25. Clerici M, Shearer GM. TH1-TH2 switch is a critical step in the etiology of HIV infection. *Immunol Today.* 1993;14:107–10.
26. Hsia CY, Huo TI, Chiang SY, Lu MF, Sun CL, Wu JC, et al. Evaluation of interleukin-6, interleukin-10 and human hepatocyte growth factor as tumor markers for hepatocellular carcinoma. *Eur J Surg Oncol.* 2007;33:208–12.
27. Pepys MB, Hirschfield GM. C-reactive protein: a critical update. *J Clin Invest.* 2003;111:1805–12.
28. Shrive AK, Cheetham GM, Holden D, Myles DA, Turnell WG, Volanakis JE, et al. Three dimensional structure of human C-reactive protein. *Nat Struct Biol.* 1996;3:346–54.
29. Frank R, Hargreaves R. Clinical biomarkers in drug discovery and development. *Nat Rev Drug Discov.* 2003;2:566–80.
30. Black S, Kushner I, Samols D. C-reactive protein. *J Biol Chem.* 2004;279:48487–90.
31. Clearfield MB. C-reactive protein: a new risk assessment tool for cardiovascular disease. *J Am Osteopath Assoc.* 2005;105:409–16.
32. Di Napoli M, Papa F. C-reactive protein and cerebral small-vessel disease: an opportunity to reassess small-vessel disease pathophysiology? *Circulation.* 2005;112:781–5.
33. Lin ZY, Wang LY, Yu ML, Chen SC, Chuang WL, Hsieh MY, et al. Role of serum C-reactive protein as a marker of hepatocellular carcinoma in patients with cirrhosis. *J Gastroenterol Hepatol.* 2000;15:417–21.
34. Nagaoka S, Yoshida T, Akiyoshi J, Akiba J, Torimura T, Adachi H, et al. Serum C-reactive protein levels predict survival in hepatocellular carcinoma. *Liver Int.* 2007;27:1091–7.
35. Patel T. Apoptosis in hepatic pathophysiology. *Clin Liver Dis.* 2000;4:295–317.
36. Liu DX. A new hypothesis of pathogenetic mechanism of viral hepatitis B and C. *Med Hypotheses.* 2001;56:405–8.
37. Holdenrieder S, Eichhorn P, Beuers U, Samtleben W, Schoenermarck U, Zachoval R, et al. Nucleosomal DNA fragments in autoimmune diseases. *Ann N Y Acad Sci.* 2006;1075:318–27.
38. Swietek K, Juszczak JJ. Reduced glutathione concentration in erythrocytes of patients with acute and chronic viral hepatitis. *J Viral Hepat.* 1997;4:139–41.
39. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 3rd ed. Oxford: Science Publications; 1999.
40. Chrobot AM, Szaflarska-Szczepanik A, Drewa G. Antioxidant defense in children with chronic viral hepatitis B and C. *Med Sci Monit.* 2000;6:713–8.
41. Tanyalcin T, Taskiran D, Topalak O, Batur Y, Kutay F. The effects of chronic hepatitis C and B virus infections on liver reduced and oxidized glutathione concentrations. *Hepatol Res.* 2000;18:104–9.
42. Demirdag K, Yilmaz S, Ozdarendeli A, Ozden M, Kalkan A, Kilic SS. Levels of plasma malondialdehyde and erythrocyte antioxidant enzyme activities in patients with chronic hepatitis B. *Hepatogastroenterology.* 2003;50:766–70.
43. Maki A, Kono H, Gupta M, Asakawa M, Suzuki T, Matsuda M, et al. Predictive power of biomarkers of oxidative stress and inflammation in patients with hepatitis C virus-associated hepatocellular carcinoma. *Ann Surg Oncol.* 2007;14:1182–90.