Original Article

Serum Cytokine Analysis Reveals Predictors of Progression from Chronic Hepatitis B to Liver Cirrhosis

(chronic hepatitis B / liver cirrhosis / cytokine profiling / IL-9 / GM-CSF / Il-2Rα)

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Abstract: Hepatitis B virus (HBV) infection is more likely to develop into chronic and persistent infection in China, which is the main cause of chronic liver disease. We examined the cytokine profiles of chronic hepatitis B (CHB) and CHB-caused liver cirrhosis (LC) to look for the predictor of progression from CHB to LC. Serum samples of 15 healthy controls (HC), 15 CHB patients and 15 LC patients were collected to detect the profiles of 48 cytokines by multiplex biometric ELISA-based immunoassay. Partial least squares discriminant analysis (PLS-DA) and random forest were used to analyse significant cytokines, which were further validated by ELISA using an independent cohort of 60 CHB patients, 60 LC patients and 35 HC samples. There were 18 differentially expressed cytokines of CHB and LC. Three cytokines were identified by PLS-DA and random forest, including interleukin (IL)-9, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-2 receptor subunit α (IL-2Rα), which displayed significant changes in serum levels. Differentially expressed cytokine networks between HC, CHB and LC also indicated particular cytokine co-expression network patterns of CHB and LC. The receiver-operator characteristic (ROC) analysis demonstrated that IL-9, GM-CSF, IL-2Rα and their logistic regression panel are potential predictors that significantly differentiate CHB from LC ($P < 0.001$) and CHB from Child class A LC ($P < 0.001$). The three cytokines and the panel showed significant correlation with the Child-Pugh score. IL-9, GM-CSF, IL-2Rα and their logistic panel may be predictors for monitoring the progression of CHB to LC.

Received December 22, 2020. Accepted March 25, 2021.

This study was supported by the Shanghai Science and Technology Innovation Action Plan (20ZR1453700), the National Science and Technology Major Project of China (No. 2012ZX 10005001-004 and No. 2009ZX09311-003), the Programme of National Natural Science Foundation of China (No. 81330084 and No. 81503478), and the "Yang Fan" Programme of Shanghai Committee of Science and Technology Fund Annotation (No. 14YF1411400).

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Abbreviations: AFP – α-fetoprotein, ALB – albumin, ALT – alanine aminotransferase, AST – aspartate aminotransferase, AUC – area under the ROC curve, CHB – chronic hepatitis B, DBIL – direct bilirubin, ELISA – enzyme-linked immunosorbent assay, FDR – false discovery rate, GGT – γ-glutamyl transferase, GM-CSF – granulocyte-macrophage colony-stimulating factor, HBV – hepatitis B virus, HC – healthy controls, IL-2Rα – IL-2 receptor subunit α, LC – liver cirrhosis, PLS-DA – partial least squares discriminant analysis, ROC – receiver-operator characteristic, TBIL – total bilirubin, VIP – variable importance in projection.

Introduction

One of the main causes of hepatocellular carcinoma is chronic hepatitis B virus (HBV) infection (Sanyal et al., 2010). There are about 70 million cases of HBV infection in China (Liang et al., 2009; Lu and Zhuang, 2009), and 80 % of hepatocellular carcinoma cases are associated with liver cirrhosis (LC) after HBV infection (Tan, 2011).

The progression of CHB to LC is always asymptomatic; thus, the surveillance for LC of any aetiology is especially recommended for patients with CHB (Marshall et al., 2013). Early diagnosis of LC is expected to improve survival of HBV-infected patients. Liver biopsy is the gold standard to measure the degree of liver inflammation and fibrosis, but is not fully accepted by patients. The pathological changes in the liver are not evenly distributed, and only 80 % of the cases can be confirmed by liver biopsy.

Non-invasive tests for liver lesions initially consisted mainly of blood tests targeting cirrhosis, but their considerably low accuracy rendered them unsuitable for use in clinical practice (Calès et al., 2015). Most non-invasive tests are constructed and/or evaluated by a single

diagnostic target, such as serum hyaluronic acid (Halfon et al., 2005), thus providing a binary (yes or no) diagnosis. Aspartate aminotransferase (AST) to platelet count ratio index and FibroTest (Schmeltzer and Talwalkar, 2011) offer a fibrosis classification that reflects the histologic stage, but differ in methods and accuracies (Boursier et al., 2014). The ratio of AST to alanine aminotransferase (ALT) is also used to diagnose cirrhosis (Sheth et al., 1998; Lin et al., 2013).

Furthermore, most of the published studies were concerned with the use of serum markers for patients with chronic hepatitis C. In the case of patients with HBV infection, there were a few studies assessing non-invasive predictors (Yang et al., 2015). Currently, finding predictors for monitoring progression of CHB to LC would improve early diagnosis of LC. It remains a considerable challenge.

Cytokines are low-molecular-weight soluble proteins induced by immunogen, mitogen or other stimulants, which can participate in the immune response and immune regulation, regulate innate immunity and adaptive immune responses (Balkwill, 2004; Larrubia et al., 2008). It has been reported that cytokines have significance in CHB and LC (Costantini et al., 2009), so they have potential as diagnostic and therapeutic targets. With the tremendous developments seen in biotechnology and high-throughput analytical technologies, new non-invasive methods that are faster in diagnostic and therapeutic procedures challenge the longstanding "gold standard" (Martinez et al., 2011). In this study, we detected 48 cytokines in the serum of patients with CHB and LC by multiplex bead-based Luminex technology. Detection of cytokine expression patterns related to LC is not only essential for monitoring progression of CHB to LC, but also helpful in identifying LC early-stage biomarkers. We also developed a predictive cytokine-based prognostic classifier for HBV-associated CHB to LC based on these data.

Material and Methods

Objectives

Participants were recruited from Shuguang Hospital and Longhua Hospital affiliated with Shanghai University of Traditional Chinese Medicine (Shanghai, China) during 2012–2014. The study design is shown in Fig. 1. Venous blood samples from 15 healthy controls (HCs), 15 CHB patients and 15 LC patients were collected. The profiles of cytokines were detected by ELISA. In addition, serum samples from 60 CHB patients, 60 LC patients and 60 HCs were collected to verify the significant cytokines. The study was approved by the Ethics Committee of Shuguang Hospital (Approval number: 20122062202). The diagnostic criteria for CHB and LC were in accordance to "CHB prevention and treatment guidelines," (Zhang and Hai, 1994) while the diagnosis standard of cirrhosis followed "Chronic hepatitis B prevention and treatment guidelines" (Association, 2001). Patients who were pregnant or lactating, or had other infectious or inflammatory diseases, were excluded.

Sample preparation

Fasting venous blood samples were collected and placed at room temperature for 30 min and centrifuged at 4 °C and 5,700 × *g* for 10 min. The supernatant was packed in an EP tube, labelled and stored at −80 °C. Serum levels of total bilirubin (TBIL), direct bilirubin (DBIL), ALT, AST, γ-glutamyl transferase (GGT), albumin (ALB), and α-fetoprotein (AFP) were performed at Shuguang Hospital using an automatic biochemical analyser.

Measurements of serum cytokines

Cytokines were assessed using the Bio-Plex system (Luminex, Austin, TX). Two commercial kits were used for the multiplex biometric ELISA-based immunoassay

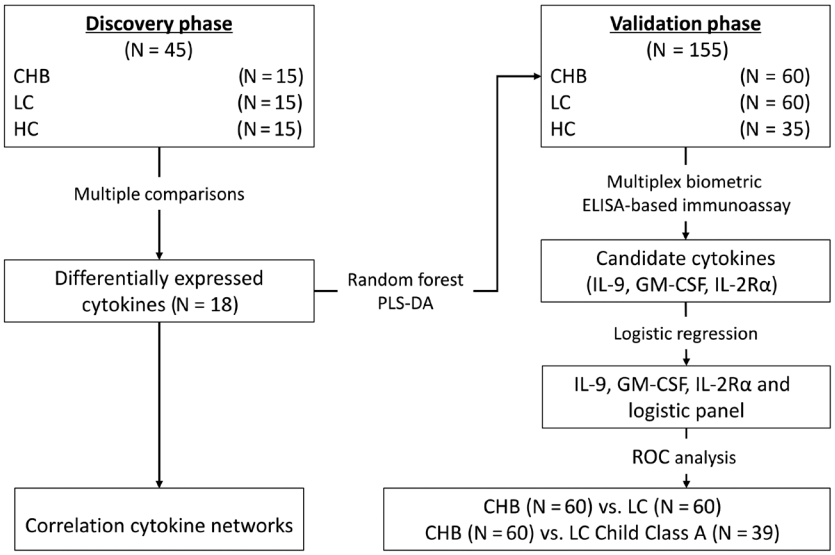

Fig. 1. Schematic overview of the study

according to the manufacturer's standard operating protocol (M50-0KCAF0Y, MF0-005KMII, Bio-Rad Laboratories Inc., CA). Based on previous reports on liver diseases, 48 specific cytokines were selected for detection in the present study (Steuerwald et al., 2013; Capone et al., 2015). A Millipore xMAP Kit (HCYTOMAG-60K-06; Merck Millipore, Billerica, MA) was used to verify the significant cytokines following the manufacturer's protocol (Codices et al., 2013).

Data analysis

Fisher's exact test was used for multiple comparison correction in statistical analysis of categorical variables. For continuous variables, Kruskal-Wallis test was used for nonparametric comparison. False discovery rate (FDR) was calculated to correct for multiple comparisons. All statistical tests were two-sided. The FDR q-value < 0.2 was defined as the significance threshold (Smith et al., 2007).

A "leave-one-out" cross-validation was performed using the supervised partial least squares discriminant analysis (PLS-DA) technology to identify LC-related proteins, which was conducted using the DiscriMiner package with R (Lebart et al., 1995). The discrimination ability of the experimental variables was selected according to the variable importance in projection (VIP) indices. Random forest used an ensemble of classification trees (Breiman et al., 1984), and used both bagging and random variable selection for the tree building (Hastie et al., 2009). The random forest analysis was performed using the randomForest package (Liaw and Wiener, 2002).

Logistic regression with stepwise backward selection was used to combine the diagnostic cytokines. When the P value was < 0.05, the differences detected were considered significant. Correlation networks were constructed by analysing the relationship among cytokine level datasets. Pair-wise Spearman correlation coefficients were calculated using Pearson correlation coefficients. The PCIT R package (Watsonhaigh et al., 2010) was used to identify and delete insignificant correlations.

Results

Clinical characteristics of participants

From 2012 to 2014, a total of 200 participants, including 75 patients with CHB, 75 patients with LC and 50 healthy controls, were screened in this study (Table 1). Thirty patients (15 CHB, 15 LC) and 15 HCs were enrolled in the discovery phase, while 120 patients (60 CHB, 60 LC) and 35 HCs were enrolled in the validation phase (Fig. 1).

Identification of differentially expressed cytokines between CHB and LC

In the cytokine analysis of 48 proteins (Table 2), the serum concentrations of PDGF-ββ, RANTES, IL-1α, IL-3, SDF-1α and TNF-β were beyond the detection range in both groups. Eighteen cytokines with significant differences in expression among groups were considered as differentially expressed cytokines ($P < 0.05$, $q < 0.2$).

LC and CHB discrimination and discovery of candidate cytokines

The results of PLS-DA showed that the LC group was well differentiated from CHB and HC groups (Fig. 2a). The VIP values of the variables that account for the difference in cytokine profiles among groups are shown in Fig. 2c. With 10-fold cross-validation of random forest, the most promising variable with the smallest cross-validation error was selected to optimize the model (Fig. 2b, 2d). Combining both the VIP of PLS-DA and the Gini score of random forest, IL-9, GM-CSF, and IL-2Rα were selected for subsequent experiments.

Table 1. Clinical characteristics of participants

Parameters	HC (N = 50)	CHB (N = 75)	LC (N = 75)		
			Child class A[a] (N = 44)	Child class B[b] (N = 23)	Child class C[c] (N = 8)
Age (years)	47.0 ± 13.8	41.1 ± 11.9	51.7 ± 8.5	53.7 ± 12.5	54.9 ± 8.6
Gender (M/F)	25 / 25	43/32	24/20	18/5	6/2
TBIL (μmol/l)	15.2 ± 3.6	20.7 ± 5.1	24.8 ± 11.6	17.1 ± 12.1	42.0 ± 9.1
DBIL (μmol/l)	4.3 ± 0.6	4.6 ± 1.2	4.5 ± 2.9	7.5 ± 5.6	14.4 ± 9.7
ALT (IU/l)	18.3 ± 6.9	47.9 ± 23.0	36.7 ± 17.9	36.2 ± 20.0	57.3 ± 26.7
AST (IU/l)	18.6 ± 6.0	39.7 ± 13.2	55.2 ± 29.1	36.7 ± 15.1	119.3 ± 88.2
GGT (IU/l)	17.4 ± 6.2	46.6 ± 44.4	45.7 ± 28.0	54.4 ± 48.4	38.0 ± 8.5
ALB (g/l)	43.3 ± 3.9	44.5 ± 1.9	31.5 ± 7.6	47.5 ± 3.2	24.3 ± 2.1
AFP (μg/l)	N/A	11.5 ± 18.0	12.8 ± 19.9	9.5 ± 24.5	51.3 ± 29.4

[a]Child class A = Child-Pugh score 5–6; [b]Child class B = Child-Pugh score 7–9; [c]Child class C = Child-Pugh score 10–15.

Table 2. Comparisons of cytokine levels (pg/ml) by the BioPlex assay

Cytokines	Means of concentration (pg/ml)			F test P value	Kruskal Wallis test P value	FDR *q*
	HC (N = 15)	CHB (N = 15)	LC (N = 15)			
IL-1β	3.68 ± 1.36	3.50 ± 1.66	7.01 ± 8.44	0.112	0.625	0.126
IL-1rα	229.53 ± 168.53	300.94 ± 196.11	227.91 ± 213.75	0.510	0.462	0.096
IL-2	1.70 ± 5.20	13.71 ± 33.18	8.92 ± 9.77	0.286	0.000	0.000
IL-4	4.29 ± 2.36	4.08 ± 2.30	4.42 ± 3.58	0.945	0.932	0.168
IL-5[a]	4.89 ± 2.00	5.23 ± 2.20	1.94 ± 1.05	0.004	0.002	0.001
IL-6	9.00 ± 4.99	25.84 ± 37.03	29.92 ± 25.44	0.087	0.002	0.000
IL-7	17.11 ± 7.73	19.09 ± 11.61	14.41 ± 11.54	0.499	0.140	0.035
IL-8[a]	39.48 ± 22.81	29.36 ± 14.91	144.93 ± 117.31	0.000	0.000	0.000
IL-9[a]	7.41 ± 4.95	51.32 ± 85.21	184.43 ± 281.93	0.019	0.000	0.000
IL-10	8.77 ± 3.31	11.79 ± 10.01	11.23 ± 15.77	0.716	0.333	0.074
IL-12p70	20.70 ± 9.33	37.34 ± 57.38	27.46 ± 44.07	0.557	0.473	0.096
IL-13	13.92 ± 5.24	16.65 ± 8.63	12.40 ± 5.01	0.231	0.319	0.074
IL-15	4.10 ± 3.52	15.08 ± 36.11	17.78 ± 14.16	0.246	0.039	0.013
IL-17	4.90 ± 4.22	8.38 ± 12.62	2.28 ± 5.00	0.163	0.006	0.005
Eotaxin[a]	7.34 ± 14.42	66.69 ± 95.18	99.33 ± 118.59	0.023	0.007	0.005
FGF basic	22.29 ± 8.49	28.84 ± 18.50	25.82 ± 20.01	0.550	0.716	0.139
G-CSF	3.26 ± 8.95	2.27 ± 4.51	26.82 ± 57.45	0.090	0.032	0.011
GM-CSF[a]	73.17 ± 35.35	40.57 ± 24.87	139.70 ± 107.61	0.001	0.008	0.005
IFN-γ	156.17 ± 115.06	164.46 ± 97.71	156.18 ± 70.76	0.965	0.734	0.139
IP-10[a]	462.86 ± 430.02	1,003.17 ± 596.42	1,803.40 ± 1,475.03	0.002	0.001	0.000
MCP-1[a]	74.78 ± 34.84	76.17 ± 33.07	116.10 ± 58.78	0.025	0.042	0.013
MIP-1α[a]	5.12 ± 2.35	5.32 ± 2.87	19.73 ± 22.38	0.005	0.024	0.008
MIP-1β	204.69 ± 166.44	191.46 ± 71.99	341.28 ± 195.98	0.025	0.068	0.021
PDGF-ββ	ADR	ADR	ADR	–	–	–
RANTES	ADR	ADR	ADR	–	–	–
TNF-α	24.43 ± 12.56	31.77 ± 15.31	29.79 ± 18.52	0.416	0.347	0.076
VEGF	173.94 ± 80.20	113.69 ± 51.69	164.76 ± 111.94	0.119	0.111	0.032
CTACK[a]	663.12 ± 275.33	1,470.67 ± 703.22	2,195.13 ± 920.32	0.000	0.000	0.000
GROα[a]	248.30 ± 109.04	246.98 ± 159.40	478.88 ± 260.23	0.002	0.016	0.008
HGF[a]	793.97 ± 222.09	1,167.47 ± 519.33	1,724.21 ± 891.72	0.001	0.001	0.000
IFN-α2[a]	194.73 ± 57.35	202.43 ± 69.63	128.11 ± 43.14	0.003	0.004	0.000
IL-1α	BDR	BDR	BDR	–	–	–
IL-2Rα[a]	386.20 ± 421.39	796.32 ± 334.93	503.26 ± 293.39	0.010	0.016	0.008
IL-3	BDR	BDR	BDR	–	–	–
IL-12p40	247.77 ± 246.19	1,285.45 ± 2,425.01	1,063.80 ± 1,036.49	0.171	0.129	0.035
IL-16	564.81 ± 351.69	1,310.01 ± 1,071.41	926.37 ± 504.52	0.026	0.062	0.019
IL-18[a]	179.15 ± 93.57	301.63 ± 162.18	236.84 ± 98.60	0.033	0.014	0.005
LIF	22.71 ± 12.66	32.63 ± 44.81	38.77 ± 24.87	0.383	0.145	0.035
MCP-3	38.18 ± 15.80	99.86 ± 216.02	55.17 ± 33.24	0.413	0.140	0.035
M-CSF	0.71 ± 1.70	8.77 ± 27.42	34.36 ± 108.81	0.341	0.200	0.047
MIF [a]	594.23 ± 336.77	887.26 ± 905.08	2,483.59 ± 1,781.91	0.000	0.000	0.000
MIG[a]	1,049.55 ± 867.37	1,472.80 ± 1,013.78	2,107.78 ± 1,370.05	0.047	0.021	0.008
β-NGF	4.26 ± 2.06	4.68 ± 4.33	4.36 ± 4.34	0.947	0.775	0.143
SCF[a]	146.81 ± 45.82	312.60 ± 191.90	488.80 ± 268.43	0.000	0.000	0.000
SCGF-β[a]	72,730.35 ± 21,1640.08	96,340.07 ± 26,2640.64	98,003.25 ± 40,673.86	0.049	0.044	0.013
SDF-1α	BDR	BDR	BDR	–	–	–
TNF-β	BDR	BDR	BDR	–	–	–
TRAIL	233.33 ± 88.45	469.65 ± 574.71	187.81 ± 127.60	0.080	0.198	0.047

[a]Cytokines selected into the PLS-DA and random forest analyses. Abbreviations: BDR – below the detection range, ADR – above the detection range.

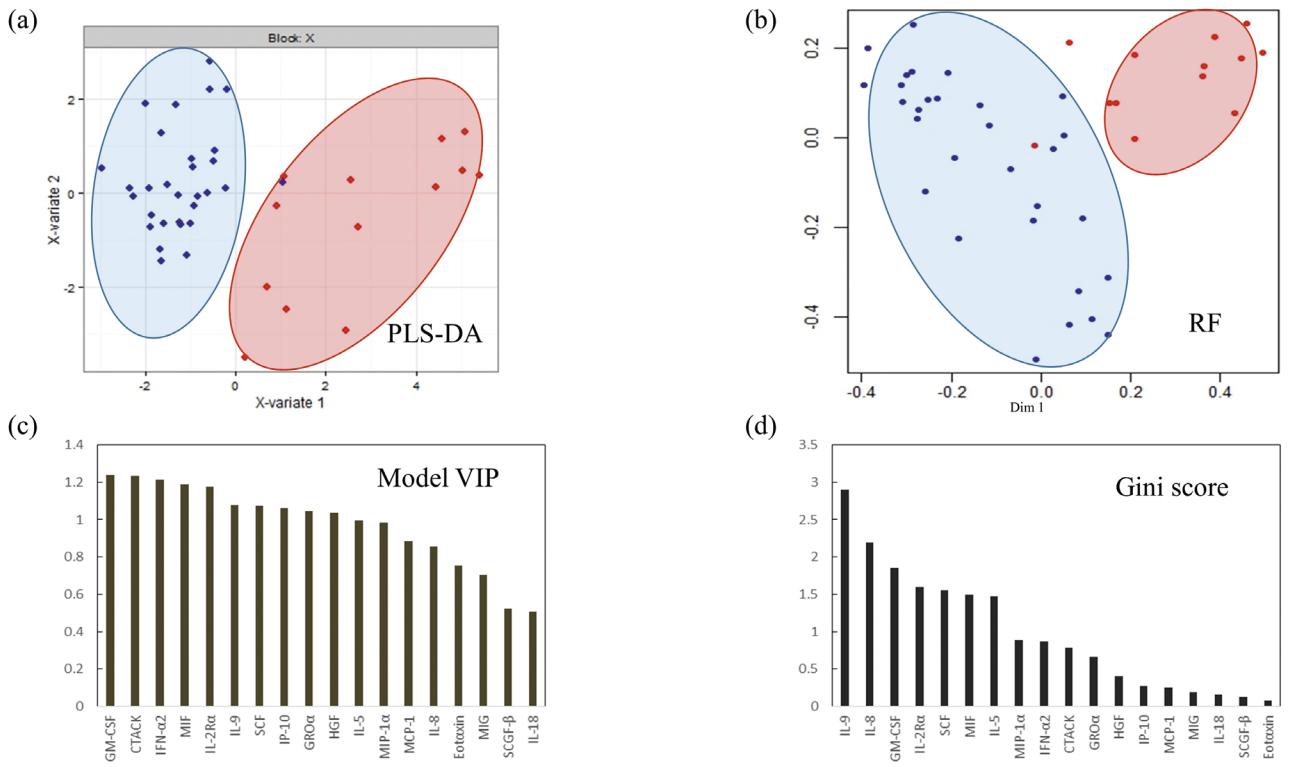

Fig. 2. Classification of CHB, LC and HC groups by cytokines
(**a**)-(**b**): PLS-DA and RF plots of the models discriminating the CHB, LC and HC groups. Red represents LC patients, blue represents CHB and HCs. (**c**)-(**d**): The relative importance of cytokines in the model was evaluated according to VIP and Gini score.

Differentially expressed cytokine networks between HC, CHB and LC

Several plasma cytokine mediators might respond as a complex interaction network during HBV infection progression from CHB to LC. These cytokines may bring changes in the correlation between CHB and LC stages, resulting in different correlation networks. Compared with HC, the significant correlation was increased in CHB and LC with the disease progression (Fig. 3). From CHB to LC, the significant positive correlations of 18 differentially expressed cytokines were lost, while negative correlations were gained. The three key cytokines, IL-9, GM-CSF and IL-2Rα, presented increasing interactions with other cytokines. There are particular cytokine co-expression network patterns of CHB and LC.

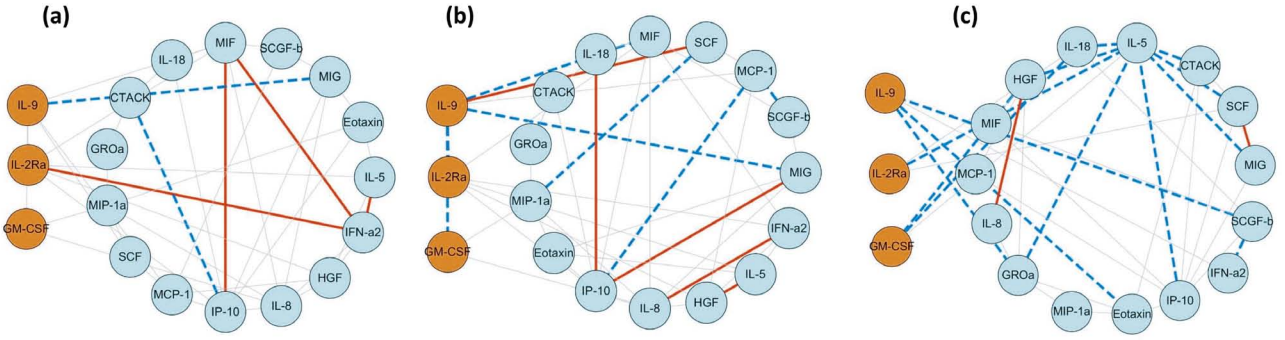

Fig. 3. Immune response networks of differentially expressed cytokines (DECs) in HC, CHB and LC
Correlations among 18 DECs under CHB and LC conditions were plotted in the network. Cytokines are represented by circles. Red solid lines: positive correlations ($0.7 \leq r \leq 1$); blue dotted lines: negative correlations.

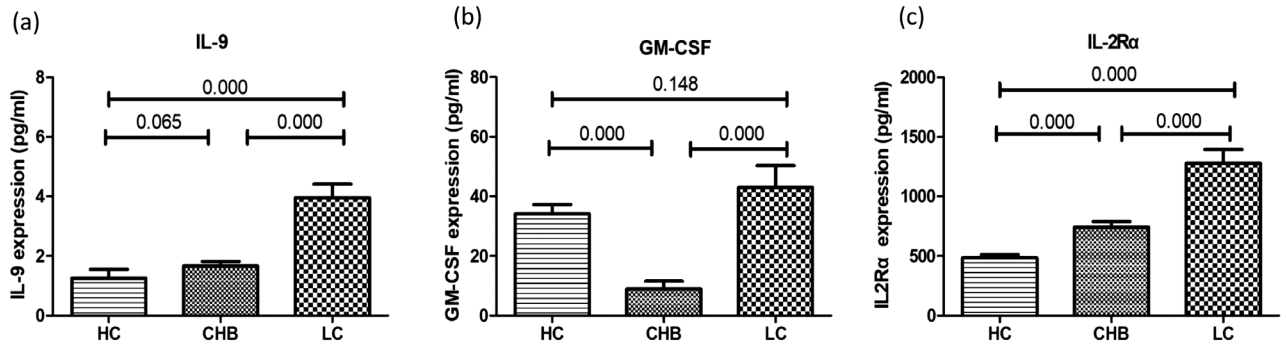

Fig. 4. Serum concentrations of candidate cytokines
Serum levels of IL-9 (**a**), GM-CSF (**b**) and IL-2Rα (**c**). HC: N = 35; CHB patients: N = 60; LC patients: N = 60.

Validation of IL-9, IL-2Rα and GM-CSF selected by random forest and PLS-DA

Referring to random forest calculation results, we used ELISA to detect samples from CHB patients (N = 60), LC patients (N = 60) and HCs (N = 35). Fig. 4a-c showed that the expression of IL-9, GM-CSF and IL-2R α was consistent with the results of multiple detection, with statistical significance. The IL-9 levels were elevated slightly in the CHB cases compared with HC, with no significance. Meanwhile, compared with the CHB group, the IL-9 levels were significantly increased ($P < 0.001$) in the LC group (Fig. 4a). The GM-CSF levels ($P < 0.001$) were also elevated in the LC group compared with the CHB group, while GM-CSF decreased dramatically during the progression from HC to CHB (Fig. 4b). The IL-2Rα expression levels of CHB were significantly different compared with the LC group ($P < 0.001$), while the CHB and LC groups both showed remarkably increased levels compared to the HC group (Fig. 4c). There was no significant difference between the CHB and HC groups in the case of IL-9 ($P = 0.065$) or the level of GM-CSF in the HC and LC groups ($P = 0.148$) (Fig. 4a-b).

Establishment and validation of the predictive cytokine panel

We used ROC analysis to evaluate the diagnostic ability of the three cytokines. The area under the ROC curve (AUC) for IL-9, GM-CSF and IL-2Rα was 0.729 ($P < 0.001$), 0.846 ($P < 0.001$) and 0.711 ($P < 0.001$), respectively (Fig. 5a). Logistic regression was also fit to find the diagnostic panel for discriminating between LC

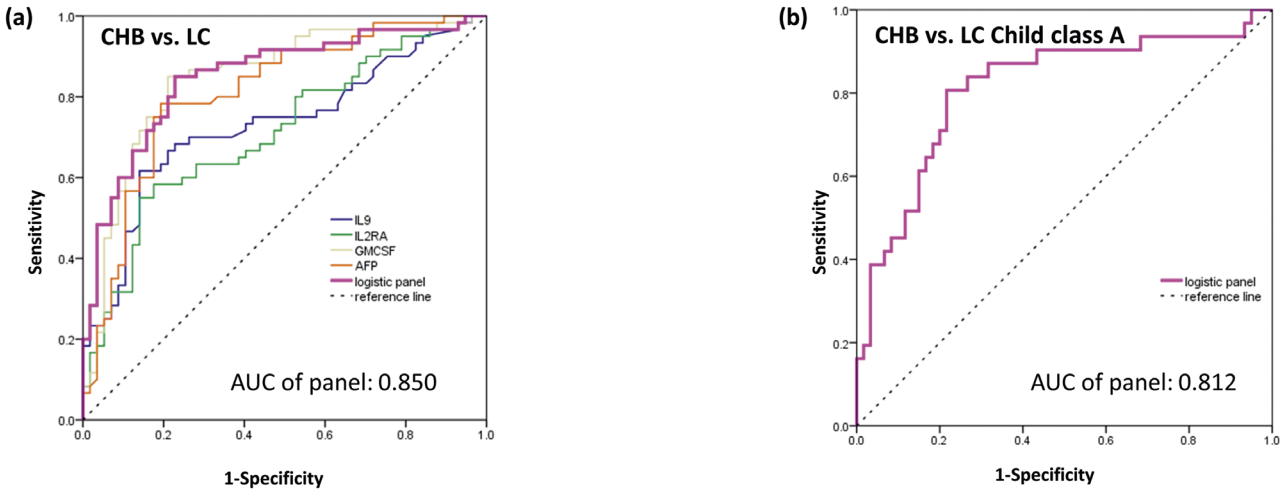

Fig. 5. ROC curves of cytokines for CHB and LC differentiation
(**a**) ROC curves were generated by cytokine expression data in CHB and LC patients. Logistic panel AUC = 0.850 (95% CI, 0.779 to 0.921); IL-9 AUC = 0.729 (95% CI, 0.636 to 0.822); IL-2Rα AUC = 0.711 (95% CI, 0.617 to 0.804); GM-CSF AUC = 0.846 (95% CI, 0.772 to 0.920); AFP AUC = 0.810 (95% CI, 0.730 to 0.890). (**b**) ROC curves were generated by cytokine expression data in CHB and LC Child class A patients. Logistic panel AUC = 0.812 (95% CI, 0.712 to 0.912).

and CHB. The formula for logistic model = −2.846 + 0.027 × GM-CSF + 0.34 × IL-9 + 0.001 × IL-2Rα + 0.082 × AFP was used to construct the ROC curve and evaluate the discriminatory power. The AUC for the panel was 0.850 ($P < 0.001$) and further demonstrated that the panel had high accuracy in discriminating LC from CHB. We next assessed the diagnostic capability of the panel for differentiating CHB from LC Child class A patients. The panel also showed high performance, with an AUC = 0.812 (Fig. 5b).

Significant positive correlation between serum levels of cytokines and Child-Pugh score

The cytokine levels of IL-9, GM-CSF and IL-2Rα were increased in the LC cohort (Fig. 4). Pearson's correlation analysis was used to estimate the significance between the Child-Pugh score and the three cytokines in the LC cohort. IL-9 ($r = 0.392$, $P = 0.002$), GM-CSF ($r = 0.399$, $P = 0.002$) and IL-2Rα ($r = 0.257$, $P = 0.047$) were positively correlated with the severity of liver disease. The logistic panel was also found significantly positively correlated with the Child-Pugh score ($r = 0.318$, $P = 0.013$), (Fig. 6).

Discussion

Fibrosis is a common pathological manifestation at the end stage of chronic liver disease. Surveillance for HBV-infected liver disease progression and accurate staging diagnosis of early LC are important for the treatment of chronic liver disease. At present, imaging and biomarker tests are mainly used to monitor LC. However, these diagnostic methods are unsatisfactory, particularly in the diagnosis of early-stage LC. Furthermore, there have not been many studies performed on predictors for LC (Yang et al., 2015). The occurrence of liver fibrosis is closely related to inflammation. Hepatic stellate cells are activated by pro-inflammatory cytokines released by macrophages (Karlmark et al., 2009).

In the present study, we used multiple comparisons and discovered that 18 cytokines were differentially expressed among the 48 cytokines simultaneously evaluated. We used the PLS-DA and random forest methods to predict which cytokines can discriminate LC from the sample population. Ten cytokines were selected from PLS-DA with VIP scores higher than 1. Additionally, large Gini scores for IL-9, IL-8, GM-CSF and IL-2Rα were found using random forest. Combining PLS-DA

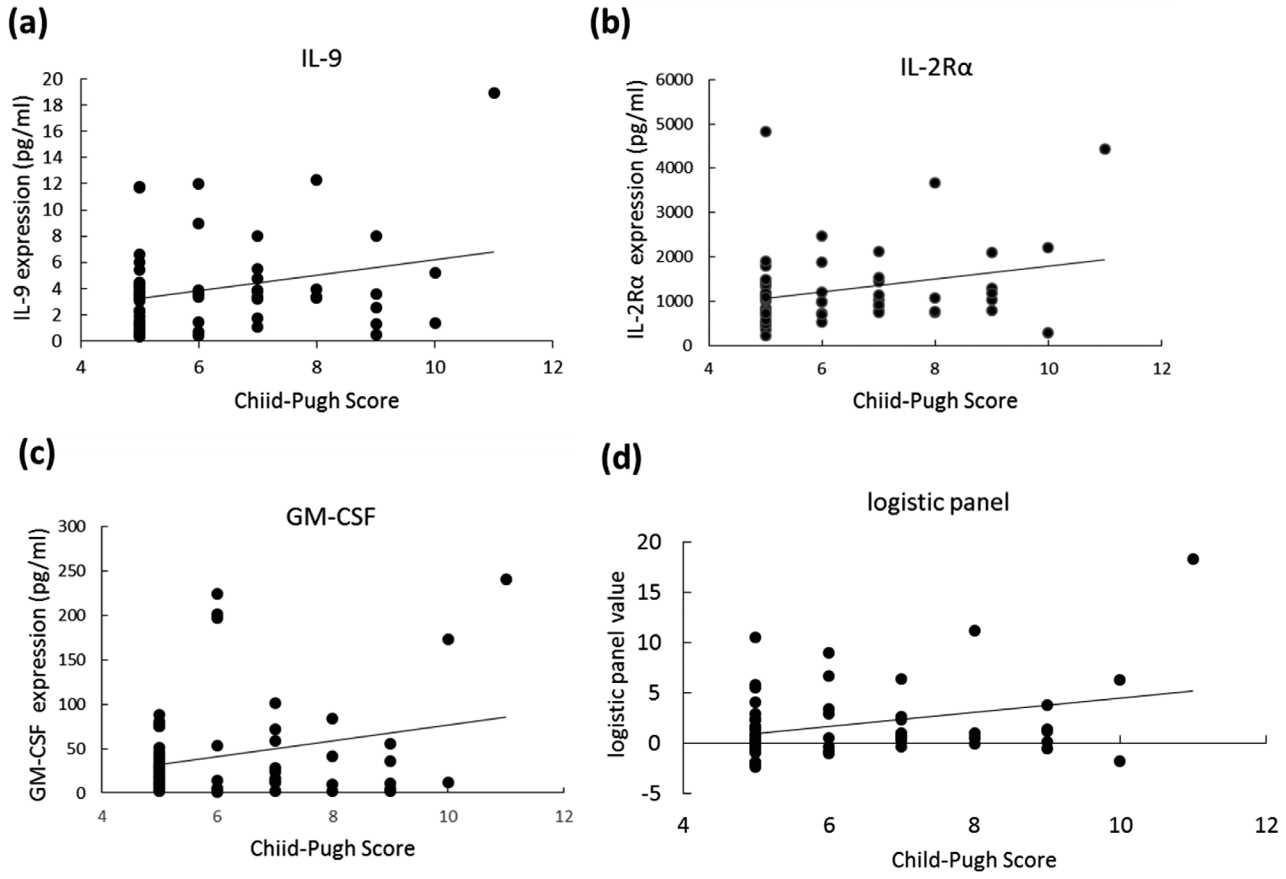

Fig. 6. Correlation between the Child Pugh score and cytokines and the logistic panel
IL-9, GM-CSF and IL-2Rα were positively correlated with the severity of liver disease. The cytokine name is on the top of each figure. The Spearman correlation (r) and probability (P) were used to evaluate the statistical significance.

and random forest, we chose the intersection of these two methods for use in validation. Thus, IL-9, GM-CSF and IL-2Rα were selected as potential candidates for predicting progression from CHB to LC.

Chronic damage resulted in progressive accumulation of scarring proteins (fibrosis). As the disease progressed, the tissue structure and function were altered, and cirrhosis or liver failure occurred (Wallace et al., 2008). IL-9 is a cytokine secreted by activated helper T cells, which can maintain long-term growth of antigen-free Th cells (Elyaman et al., 2009). Although described as a pleiotropic cytokine, IL-9 most importantly functions in mediating mast cell survival and function (Townsend et al., 2000; Kearley et al., 2011), which may participate in fibrotic processes. Mast cells are found in fibrogenic lesions in hepatic cirrhosis (Murata et al., 1972; Ambrust et al., 1997). Compared with CHB, the serum level of IL-9 significantly increased in LC patients in our study, which was consistent with the development from CHB to LC.

GM-CSF is an important haematopoietic growth factor and immune regulator, which can promote survival, proliferation and differentiation of precursor cells, and differentiation and colony formation of granulocytes and monocytes/macrophages (Burgess and Metcalf, 1980). GM-CSF appears to play an important role in the fibrotic tissue response (Xing et al., 1996). High levels of GM-CSF activate macrophages, causing the tissue injury syndrome (Lang et al., 1987). In our study, the GM-CSF levels decreased during the progression from HC to CHB, which was consistent with a previous study (Chen, 2003).

IL-2Rα, also known as CD25, is produced by activated T cells and is important for the formation of high-affinity receptors. Elevated serum IL-2Rα levels have been reported to be associated with reduced survival in patients with colorectal cancer (Huang et al., 2002). Moreover, circulating $CD4^+CD25^+$ Treg were correlated with the disease progression in acute hepatitis B patients and chronic severe hepatitis B patients (Xu et al., 2006), which was also consistent with the elevation of IL-2Rα in the LC patients compared with CHB patients in our study.

During HBV infection progression from CHB to LC, cytokine correlation changes reflect the progression of CHB to LC, resulting in different correlation networks. The logistic model of the cytokine panel of IL-9, GM-CSF and IL-2Rα was identified and validated using an independent cohort. The cytokine panel had higher accuracy in discriminating LC from CHB and CHB from Child class A. In the LC stage, a significant positive correlation was found between the serum level of cytokines and Child-Pugh score. These results indicated that the cytokine panels of IL-9, GM-CSF and IL-2Rα may be valuable predictors for the development of LC from CHB.

Altogether, CHB and LC showed particular cytokine co-expression network patterns. We were able to efficiently identify IL-9, GM-CSF, IL-2Rα and their combined panel with a higher accuracy for predicting the disease progression from CHB to LC.

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