

Original Article

Influence of Th17/Treg balance on progression of liver cirrhosis in patients with chronic hepatitis B

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Abstract: Objective: Our aim was to study the influence of T helper cell 17/regulatory T-cell (Th17/Treg) balance on progression of liver cirrhosis in patients with chronic hepatitis B (CHB). Methods: There were 86 patients diagnosed at our hospital with CHB and cirrhosis between January 2012 and March 2015. According to liver stiffness measurement (LSM), these patients were classified into low LSM group ($7 \text{ kPa} < \text{LSM} \leq 12 \text{ kPa}$, $n=42$) and high LSM group ($12 \text{ kPa} < \text{LSM} \leq 18 \text{ kPa}$, $n=44$). On the other hand, 30 healthy people examined at our medical center were taken as the control group (C group). In collecting general clinical data of the patients, enzyme linked immunosorbent assay (ELISA) and flow cytometry (FCM) were adopted to test serum levels of IL-10, TGF- β , IL-17, and expression rates of Th17 and Treg cells in peripheral blood (PB). We also calculated Th17/Treg ratio. Meanwhile, one-way analysis of variation (ANOVA) was adopted to analyze differences of the abovementioned indicators among patients with different degrees of liver cirrhosis. Pearson's correlation analysis and Cox regression analysis were employed to study relation between Th17/Treg ratio and degree of liver cirrhosis. Results: FCM results showed that expression rates of Th17 and Treg cells and Th17/Treg ratio of C group were (1.4 ± 0.8) , (1.8 ± 0.6) , and (0.82 ± 0.5) . Rates of the low LSM group were (3.6 ± 0.6) , (3.3 ± 0.3) , (1.7 ± 0.6) and rates of the high LSM group were (3.2 ± 0.5) , (1.4 ± 0.3) and (2.9 ± 0.7) . Differences among the three groups were of statistical significance ($P < 0.05$ or $P < 0.01$). ELISA results showed that serum levels of IL-10 of C group, the low LSM group, and high LSM group were $(47 \pm 9) \text{ pg/mL}$, $(78 \pm 17) \text{ pg/mL}$, and $(116 \pm 21) \text{ pg/mL}$, respectively. Serum levels of TGF- β of the three groups were $(23 \pm 7) \text{ pg/mL}$, $(69 \pm 10) \text{ pg/mL}$, and $(123 \pm 15) \text{ pg/mL}$, showing obvious differences ($P < 0.05$ or $P < 0.01$). IL-17 levels of the low LSM group and high LSM group were remarkably higher than that of C group ($(138 \pm 16) \text{ pg/mL}$ vs. $(118 \pm 11) \text{ pg/mL}$, $(145 \pm 20) \text{ pg/mL}$ vs. $(118 \pm 11) \text{ pg/mL}$) but differences between the two was not statistically significant. Pearson's correlation analysis showed that Treg, Th17/Treg ratio, and related cytokines like IL-10 and TGF- β were distinctly related to LSM ($P < 0.01$) while Cox regression analysis showed that Th17/Treg ratio, IL-10, and TGF- β were independent correlation factors for LSM. Conclusion: Th17/Treg balance is closely related to progression of liver cirrhosis in patients with CHB. It could be a critical indicator for clinical diagnosis of progression in patients with hepatitis B and liver cirrhosis and conditions of liver function.

Keywords: Hepatitis B virus, liver cirrhosis, helper T-cell, regulatory T-cell

Introduction

Hepatitis B virus (HBV) infection is one of the major diseases threatening human health. There are about 650,000 deaths of liver function failure, liver cirrhosis, and liver cancer caused by HBV infection [1]. It has been reported by studies that there is 8%-20% 5-year cumulative morbidity of liver cirrhosis among chronic hepatitis B (CHB) patients that did not undergo anti-viral treatment. For liver cirrhosis patients at compensated stage, morbidity is 20% and 5-year survival rate is 14%-35%. Annual incident rate of liver cancer among cirrhosis patients is about 2%-5% [2]. Immuno-

reaction is the key approach to learning what the host-mediated pathogen is. In patients under long-term HBV infection, immunoreaction is apparently inhibited, mainly reflected by the changing amount of T-cells and loss of T-cell function [3, 4]. In recent years, more and more researchers are starting to notice the influence of helper T17 lymphocyte/regulatory T-cell (Th17/Treg) balance on immune tolerance of HBV patients [5, 6]. However, the effects of Th17/Treg balance on progression of liver cirrhosis remain unclear.

In this study, by comparing expression rate of Th17 and Treg cells in peripheral blood (PB)

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Table 1. Patient general clinical data

Variable	Group			$\chi^2/F/T$	P
	Control group	Low LSM group	High LSM group		
Gender (male/female)	15/15	20/22	21/23	0.933	0.211
Age (years old)	49.3±8.9	51.6±10.2	50.8±9.4	1.071	0.144
BMI (kg/m ²)	22.3±4.1	21.7±3.8	23.5±4.5	0.802	0.311
ALT (IU/L)	21.2±5.7	38.3±8.2	43.2±9.5	3.422	0.013
AST (IU/L)	22.6±6.33	47.3±9.4	58.1±11.3	4.801	0.007
TBil (μmol/L)	10.5±3.3	14.7±4.2	16.4±4.8	2.095	0.031
Child-Pugh (A/B/C)	ND	21/12/9	13/16/15	1.861	0.037
HBV-DNA (copies/mL)	ND	6.3±1.2	7.4±0.9	6.578	0.000

Note: LSM, liver stiffness measurement; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBil, total bilirubin; HBV, Hepatitis B virus; ND, not determined.

and difference of Th17/Treg ratio in patients with different degrees of liver cirrhosis, we investigated influence of Th17/Treg balance on progression of liver cirrhosis in HBV patients.

Materials and methods

Objects of study

A total of 86 HBV patients that did not undergo anti-viral treatment were admitted to Yidu Central Hospital of Weifang, between January 2012 and March 2015. They were taken as the observation group. Diagnostic criteria were in compliance with Chronic Hepatitis B Guidelines [7]. According to liver stiffness measurement, these patients were classified into low a LSM group (7 kPa < LSM ≤ 12 kPa, n=42) and high LSM group (12 kPa < LSM ≤ 18 kPa, n=44).

Exclusion criteria: (1) Patients with other viral hepatitis; (2) Patients with other liver diseases like alcoholic liver disease, primary liver cancer, and autoimmune hepatitis; (3) Patients with other severe systemic diseases; (4) Patients with large seroperitoneum. On the other hand, 30 healthy people were examined at our medical center and were taken as our control group.

This study was approved by the Ethic Committee of Yidu Central Hospital of Weifang and all of the patients signed informed consent.

Methods

General clinical data: Basic data included patient age, gender, and body mass index (BMI). FibroScan-502 (Echosens, France) was used to detect patient LSM and an automatic biochemi-

cal analyzer (HITACHI, Ltd., Japan) was used to detect levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TBil) in patient serum.

Test expression rate of PB Th17 and Treg cells by FCM: First, we took 2 mL of fresh vein blood from patients, which had been treated with anticoagulant, and used density gradient centrifugation to collect peripheral blood monocyte (PBMC).

We then added 4 mL of RPMI-1640 medium (Thermo Fisher Scientific) to resuspend the cells. 6 μL of leukocyte irritant was added and cells were incubated with 5% CO₂ at 37°C for 5 hours. We then centrifuged at 1,500 rpm for 10 minutes. We discarded the supernatant and fully broke cell membrane by Ultrasonic Processor (Sonics & Materials, Inc.). We fixed and added fluorescent antibodies of CD8-FITC, CD3-FITC, Foxp3-PE, and IL-17-PE. We kept it away from light in ambient temperature for 30 minutes and then processed with intracellular staining. Finally, we added phosphate to rinse, slowly. We used Diva software for flow cytometry (FCM) testing and to analyze expression rates of Th17 and Treg and Th17/Treg ratio.

Test expression rate of serum IL-10, TGF-β, and IL-17: Double antibody sandwich ELISA method was adopted to test levels of IL-10, TGF-β, and IL-17 in patient serum. ELISA kit was purchased from Sino Biological Inc. Test operation was in compliance with instructions from the manufacturer. All samples used in the experiment were in triplicate. Absorbance of the samples in light of 450 nm wave length was tested. Cytokine concentration in these samples was calculated by standard curve method.

Statistical analysis

All data were analyzed by SPSS 19.0. Quantitative data are expressed as mean ± standard deviation ($\bar{x} \pm sd$). Comparison of indicators for patients of different groups was analyzed by one-way analysis of variance or t-test. Count data were expressed as case number and com-

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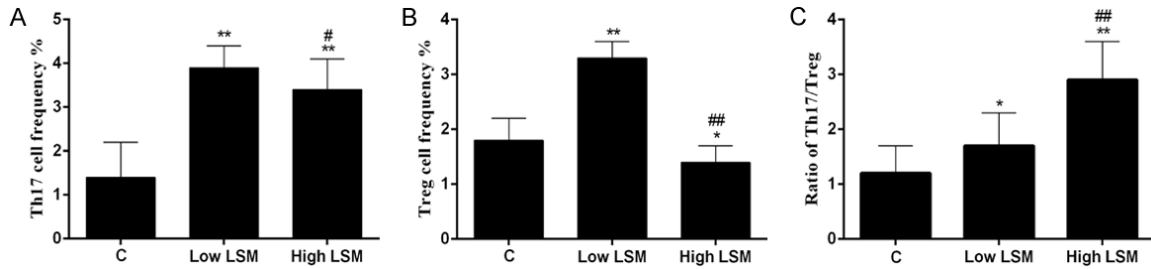


Figure 1. Comparable analysis of expression rate of Th17 and Treg cell and Th17/Treg ratio of patients of different groups. A. Comparable analysis of expression rate of patient Th17 cells; B. Comparable analysis of expression rate of patient Treg cells; C. Comparable analysis of patient Th17/Treg ratio. Compared with C group, * $P < 0.05$; compared with the C group, ** $P < 0.01$; compared with the low LSM group, # $P < 0.05$; compared with the low LSM group, ## $P < 0.01$. C group, control group; LSM, liver stiffness measurement.

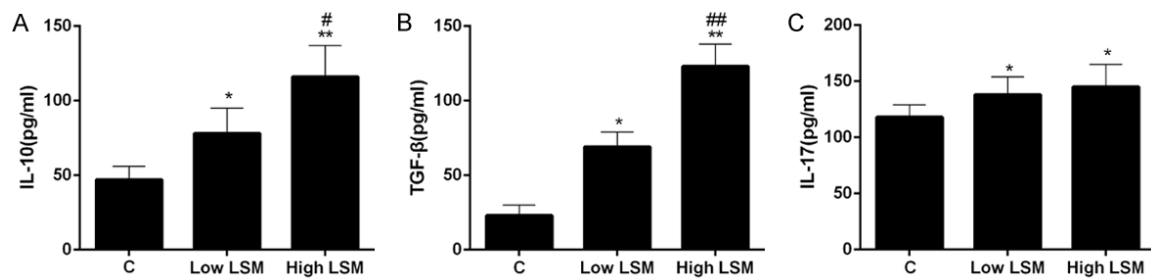


Figure 2. Comparable analysis of patient serum levels of IL-10, TGF- β , and IL-17. A. Comparable analysis of patient serum levels of IL-10; B. Comparable analysis of patient serum levels of TGF- β ; C. Comparable analysis of patient serum levels of IL-17. Compared with the C group, * $P < 0.05$; compared with the C group, ** $P < 0.01$; compared with the low LSM group, # $P < 0.05$; compared with the low LSM group, ## $P < 0.01$. C group, control group; LSM, liver stiffness measurement.

parison of indicators of patients for different groups was analyzed by Chi-square test. Correlation between LSM and influence factors was analyzed by Pearson's correlation. Related factors influencing incidence of liver cancer was analyzed by Cox regression analysis. $P < 0.05$ was considered statistically significant.

Results

General clinical data of patients

Differences of patient gender, age, and BMI were statistically insignificant ($P \geq 0.05$) while differences of ALT, AST, TBil, Child-Pugh, and HBV-DNA were noted ($P < 0.05$ or $P < 0.01$). See **Table 1**.

Analysis of expression rate of Th17 and Treg cell and Th17/Treg ratio of patients of different groups

FCM test results for expression rates of PBMC Th17 and Treg cell and Th17/Treg ratio showed that expression rates of Th17 and Treg cell and

Th17/Treg ratio of C group were respectively (1.4 ± 0.8) , (1.8 ± 0.6) , and (0.82 ± 0.5) . Rates of the low LSM group were (3.6 ± 0.6) , (3.3 ± 0.3) , and (1.7 ± 0.6) while those of high LSM group were (3.2 ± 0.5) , (1.4 ± 0.3) , and (2.9 ± 0.7) . Differences among the three groups were of statistical significance ($P < 0.05$ or $P < 0.01$). See **Figure 1**.

Analysis of serum levels of IL-10, TGF- β , and IL-17 of patients of different groups

Serum levels of IL-10 of patients of C group, the low LSM group, and high LSM group were respectively (47 ± 9) pg/mL, (78 ± 17) pg/mL, and (116 ± 21) pg/mL. Serum levels of TGF- β were respectively (23 ± 7) pg/mL, (69 ± 10) pg/mL, and (123 ± 15) pg/mL. IL-10 and TGF- β levels of the three groups showed distinct differences ($P < 0.05$ or $P < 0.01$). Though serum levels of IL-17 of low LSM group and high LSM groups were distinctly higher than the control group ((138 ± 16) pg/mL vs. (118 ± 11) pg/mL, (145 ± 20) pg/mL vs. (118 ± 11) pg/mL), differ-

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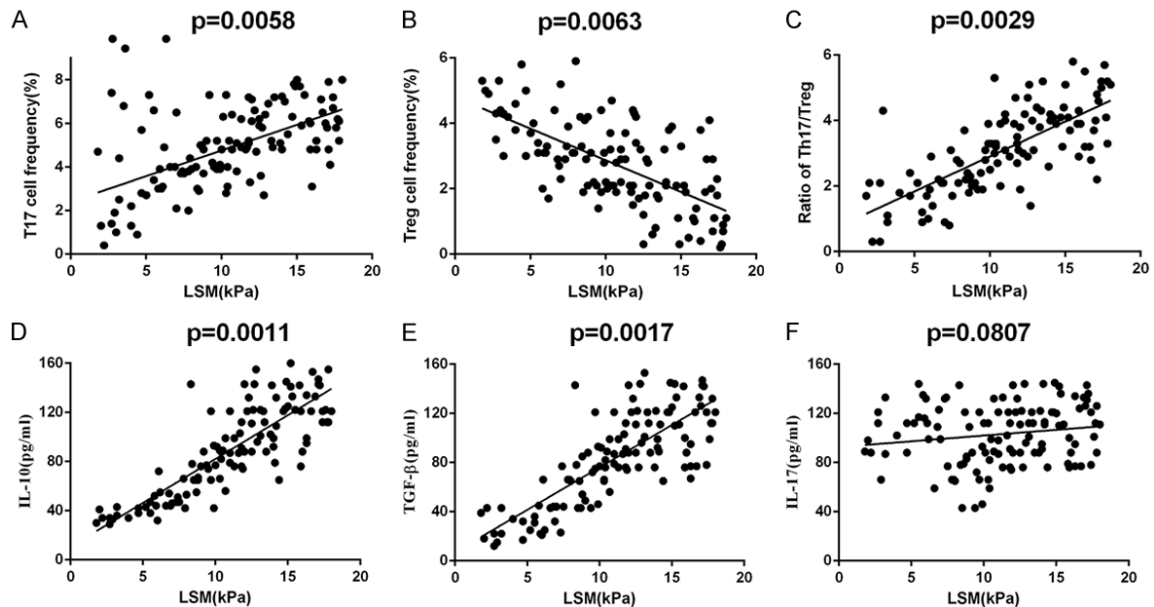


Figure 3. Analysis of correlation between influence factors and LSM. A. Correlation between Th17 expression rate and LSM; B. Correlation between Treg expression rate and LSM; C. Correlation between Th17/Treg ratio and LSM; D. Correlation between IL-10 and LSM; E. Correlation between TGF- β and LSM; F. Correlation between IL-17 and LSM. LSM, liver stiffness measurement.

Table 2. Cox regression analysis of related influence factors for progression of liver cirrhosis in patients with chronic hepatitis B

Variable	Hazard ratio	95% CI	P
Gender	0.184	0.025-0.377	0.077
BMI	0.678	0.230-0.859	0.458
AST	0.886	0.217-2.884	0.741
ALT	1.471	0.906-1.847	0.293
Child-Pugh	2.145	0.584-3.477	0.163
HBV-DNA	0.445	0.174-0.855	0.003
Th17/Treg	0.588	0.117-2.554	0.016
IL-10	1.056	0.451-1.583	0.032
TGF- β	0.581	0.151-1.274	0.019
IL-17	0.852	0.374-1.655	0.103

Note: CI, confidence interval; BMI, body mass index; AFP, alpha-fetoprotein; ALT, alanine aminotransferase; HBV, Hepatitis B virus.

ences between low LSM and high LSM groups were not distinct. See **Figure 2**.

Correlation between Th17, Treg cell, and Th17/Treg ratio and LSM

Pearson's correlation analysis showed that Treg expression rate was negatively correlated with LSM while Th17/Treg ratio was positively correlated with LSM, as were IL-10 and TGF- β with

LSM. Correlation between Th17 and LSM or IL-17 and LSM were not statistically significant. See **Figure 3**.

Analysis of related influence factors for LSM

Cox regression analysis on related influence factors for progression of liver cirrhosis in patients with CHB showed that HBV-DNA, Th17/Treg, IL-10, and TGF- β were main factors influencing progression of liver cirrhosis in patients with CHB. See **Table 2**.

Discussion

Liver injury and recovery imbalance are critical causes for progression of cirrhosis. To date, most studies have insisted that abnormal stacking of extracellular matrix (ECM) plays a key role in accelerating progression of liver cirrhosis. When the liver is infected by a virus and inflammation causes injury, hepatic stellate cell will be activated then extracellular matrix protein, glycoprotein, and collagen will continue to increase and cause ECM abnormal stacking, finally resulting in incidence and development of liver cirrhosis [8, 9]. In addition, inflammatory factor TGF- β generated after virus infections could activate Smad signal channel to activate hepatic stellate cell, accelerating incidence of

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ECM and inhibiting decomposition of collagenase [10]. Researches in recent years have shown that immunity-related cells could impact progression of liver cirrhosis. For instance, NK cell could slow down progression of liver cirrhosis by removing activated hepatic stellate cell, meaning that body immunity status plays an important role in incidence and progression of liver cirrhosis of HBV-infected patients [11]. Th17, a CD4⁺ T-cell different from Th1/Th2 cells, is a key player in mediating inflammation and body immunity. Treg cells, on the other hand, are immunosuppression cells. Th17 cells and Treg cells regulate activation and inhibition of inflammatory reaction. Th17/Treg imbalance also happens in various pathological processes like virus infections, chronic inflammation, and tumors [12, 13].

By testing expression rates of PB Th17 and Treg cells and Th17/Treg ratio of patients with CHB and liver cirrhosis and of the control group, we examined relation between expression rates of Th17 and Treg cell and Th17/Treg ratio and liver cirrhosis. We found that Th17 expression of low LSM group and high LSM group was distinctly higher than that of the control group while Treg expression was higher in low LSM group and lower in high LSM group. Research by Zhao et al. reported that Th17 cells increase prominently in HBV-infected cirrhosis patients [14]. Th17 cells release IL-22 to activate hepatic stellate cell to release chemotactic factor. Th17 cells, in turn, transfer to liver tissue and accelerate incidence and development of cirrhosis. This finding is similar to results of our study. Research by Paquissi et al. also found that Th17 can boost progression of liver cirrhosis in HBV patients [15]. Besides, a study by Katz et al. proved that though augment of Treg cells impacts function of lymphocytes in liver tissue, it helps slow down development of cirrhosis and fibrosis [16]. Our study also showed that expression rates of Treg cells of low LSM group was evidently higher than of the high LSM group, which might have something to do with Treg's inhibition of progression of cirrhosis. Thus, our results show that Th17 and Treg do not influence progression of liver cirrhosis of HBV patients, independently. Instead, Th17/Treg ratio is objective in regards to progression of liver cirrhosis in HBV patients. Research by Li et al. pointed out that Th17/Treg ratio can be a clinical predictor for progression of cirrhosis in HBV patients and also a diagnosis indicator

for liver cancer in HBV patients with cirrhosis [17].

Cytokine levels in HBV patient serum plays an important role in incidence and development of liver cirrhosis, fibrosis, and cancer [18, 19]. IL-17 is a cytokine secreted by Th17 cells. Virus infections and autoimmune-related inflammatory reactions mediated by Th17 cells mainly depend on secretion of IL-17. IL-10 and TGF- β , two major cytokines related to immunosuppression, are secreted by Treg cells. This study discussed serum levels of IL-17, IL-10, and TGF- β of patients at varied stages of cirrhosis. Although IL-17 is elevated in HBV-infected cirrhosis patients, its elevation is not evidently related with degree of cirrhosis, meaning that IL-17 probably plays an important role in HBV infection while it is irrelative to progression of liver cirrhosis. Our findings also show that IL-17 is not highly related to degree of patient cirrhosis ($P>0.05$). Research by Zhang et al., however, indicated that in the process of HBV infection, IL-17 could enhance capability of macrophages and monocytes releasing inflammatory factor, thereby aggravating liver injury, indirectly verifying IL-17's key role in HBV infection [20].

Our results show that IL-10 and TGF- β increase remarkably in HBV-infected cirrhosis patient serum and their elevation range is distinctly related to degree of cirrhosis. Studies in the past have found that TGF- β could be a clinical predictor and effective treatment for liver fibrosis and cirrhosis and that inhibiting TGF- β levels can slow down progression of cirrhosis [21]. Researches by Yu have pointed out that IL-10 and TGF- β tend to increase among HBV and HBV-infected cirrhosis patients and their elevation tendency appears more apparent among the latter, coinciding with our study [22]. Furthermore, Cox regression analysis shows that Th17/Treg, TGF- β , and IL-10 are independent related influence factors for progression of liver cirrhosis in HBV patients.

Overall, our study demonstrates that Th17 expression rate is not significantly correlated with progression of liver cirrhosis but Treg expression rate is negatively correlated. Moreover, Th17/Treg ratio is more objectively precise in regards to diagnosis of progression of liver cirrhosis in HBV patients. IL-17, a cytokine secreted by Th17 cells, is irrelative to progression of liver cirrhosis but IL-10 and TGF- β , two cyto-

kines secreted by Treg cells, are relative. Th17/Treg ratio and expression of IL-10 and TGF- β were independent risk factors for progression of liver cirrhosis in HBV patients.

This was a single-center study. Clinical samples were limited. Follow up period was just 12 months. Therefore, influence of Th17/Treg balance on incidence of liver cancer in HBV-infected cirrhosis patients requires further study. Although our conclusion indicates that Th17/Treg balance plays a key role in progression of liver cirrhosis in HBV patients, the exact mechanism of influence remains unknown. We will further examine this subject in the future.

Disclosure of conflict of interest

None.

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