Research on the Pathogenesis of Chronic Hepatitis B: From T lymphocyte to HMGB1-PTEN Pathway

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Abstract

Background: Viral hepatitis is still a major health problem in the world, such as chronic hepatitis B(CHB). Hepatitis B virus (HBV) easily causes liver cirrhosis and hepatocellular carcinoma that seriously endanger human health. However, the cellar and molecular pathology of hepatitis B virus is still unclear. Currently, the level of immunity and the inflammatory response are recognized as close relationship with the prognosis of CHB.

Methods: Peripheral blood samples from 36 CHB patients and 14 healthy volunteers as control subjects in this study. We studied the change of T lymphocytes and the proteins levels of HMGB1-PTEN pathway in the CHB patients and the healthy controls by Flow cytometry and enzyme-linked immunosorbent assay (Elisa), and then further analyzed the correlation between T lymphocytes and HMGB1-PTEN pathway by constructing multiple regression equation and Pearson test.

Results: The percentages of $CD4^+T$, $CD8^+T$ cells in CHB patients were lower than those of the healthy controls, and the percentages of Th17, Treg, ratio of Th17/Treg and Tfh cells in CHB patients were higher than those of the healthy controls ($P<0.01$). HMGB1, PI3K, PDK1, Akt proteins in CHB patients were higher than healthy people ($P<0.01$). Furthermore, HMGB1-PTEN pathway proteins were negatively correlated with $CD4^+T$, $CD8^+T$ cells, and positively correlated with Th17, Treg cells.

Conclusions: Long term HBV infection could lead to decrease of immune function and activation of inflammatory response, which makes it difficult to cure chronic hepatitis B.

Background

At present, Hepatitis B Virus (HBV) infection cause many attentions because of high morbidity, and the prevalence of the disease greatly varied in different regions. In 2017, the number of HBV infections worldwide reached about 2 billion, of which 257 million were chronic infection [1]. Every year, about 1 million people were died of chronic liver failure, cirrhosis, or primary hepatocellular carcinoma in which HBV infection is main pathogenesis [2]. Before the wide use of hepatitis B vaccine, the rate of hepatitis B virus surface antigen (HBsAg) positivity was close to 10% in China [3]. Recently, the HBsAg positive rate has been declining, that is about 5–6%, however, there were still approximate 70 million people with chronic HBV infection, in which 20–30 million are CHB patients[4]. Therefore, the current epidemic situation of HBV infection is severe, which has seriously threatened human life and health.

It is well-known that HBV does not directly cause liver cell injury in CHB but the immune response caused by pathogens that promotes the development of the disease. The removal of HBV in the body is primarily completed by the specific immune response that is mainly induced by cellular immunity [5, 6]. The immune response happened at the begin of the infection, and the number and ratio of lymphocytes changed which resulted in disorder of immune status [7, 8]. $CD4^+T$ and $CD8^+T$ cells, as the two main subtypes of T lymphocytes, involves into the process of the disease, which reveal the immune status and serve as a direct indicator [9]. Some studies have shown that Th17 cells and Treg cells play very important role in chronic and severe HBV infection [10–15]. Treg cells control immune responses, including maintaining immune tolerance,

regulating lymphocyte proliferation, and antagonize the Th17 cells pro-inflammatory effect. The complementation of the two functions constitutes the balance axis of the body's immune regulation [16]. Follicular helper T cells (Tfh) are a new subset of CD4+Th cells. They mainly locate in lymphoid follicles which work as mediators of connecting T and B cells, promoting the activation of B lymphocytes, enhancing the differentiation and proliferation of plasma cells [17]. Recently, some reports uncovered that the number of Tfh cells and its cytokine IL-21 increased in CHB patients, indicating that Tfh cells involved into the process of disease[18–20].

There is a strong relationship between CHB and proinflammatory factors. High mobility group box-1 protein (HMGB1) is a ubiquitous DNA binding protein secreted by immune cells. Endotoxin and various inflammatory factors induce the secretion of HMGB1 and promote the inflammatory response [21]. HMGB1 is speculated to be a potential inflammatory mediator and a "risk signal" of tissue damage because of early increases and longer duration [22, 23]. The result of a Meta-Analysis showed HMGB1 serum levels were higher in severe hepatitis B or acute-on-chronic liver failure patients [24]. The phosphatase and tension homology deleted on chromosome ten (PTEN) is a gene on chromosome 10 which regulate some cellular processes, including proliferation, survival, energy metabolism, cellular architecture, and motility[25]. The expression of PTEN is affected by the HMGB1, and the HMGB1-PTEN pathway plays an important role in tissue injury, inflammation, cell necrosis[26]. Recruiting 3-phosphoinositide dependent kinase-1(PDK1) to cell membrane is regulated by PI3K promoting the activation of protein kinase B(Akt), and forms PI3K/PDK1/Akt signaling pathway [27, 28]. The activation of PI3K/Akt pathway also increases IL-12 production, which is a typical proinflammatory cytokine of CHB [29]. Research indicated that the lack or decrease of PTEN caused increase of PI3K [30], thus, elevated the level of PDK1 and Akt in the downstream[31, 32]. PTEN negatively regulates PI3K/Akt pathway, inhibits cell growth, and accelerates cell apoptosis[33]. Since the PTEN is another gene closely related to tumorigenesis p53 gene, PTEN/PI3K/Akt pathway seems to be related to some cancers, such as liver cancer, lung cancer, gastric cancer, lymphatic cancer[34–37]. Nevertheless, such pathway has been recognized as an inflammatory regulator, which has been confirmed to regulate the proliferation of mouse mesangial cells[38].

Methods

Study subjects

The ethics committee of the Affiliated Hospital of Chengdu University of Traditional Chinese Medicine has approved the study and all subjects gave informed consent. The two groups of subjects required for this study, CHB patients and healthy controls, were recruited from August to October 2020 in the Affiliated Hospital of Chengdu University of Traditional Chinese Medicine.

CHB group: The diagnostic criteria of CHB was referred to the diagnostic criteria of CHB was referred to the Guidelines for the Prevention and Treatment of Chronic Hepatitis B (2019) [39]. A total of 36 CHB patients including 20 males (55.6%) and 16 females (44.4%) comprised the CHB group. Inclusion criteria were Age 18 to 65 years, positive HBsAg at least half a year and/or HBV-DNA positive, ALT levels continue or repeatedly increase within 1 year; The exclusion criteria included the following: Acute, subacute or chronic severe hepatitis B; Complications such as cirrhosis and hepatocellular carcinoma; Other infectious diseases such as HAV, HCV, and HIV infection; Other types of liver diseases such as drug-induced liver damage, autoimmune

hepatitis; Other serious diseases of the heart, lung, kidney, endocrine, blood and other systems; Immune diseases or using immunomodulators within the past six months; Alcohol abuse (female>20g/d or male>30g/d) or taking drugs etc. within half a year; Pregnant and lactating women; Taking immune-regulating drugs or health products in the past three months.

Control group: Fourteen healthy individuals from the Physical Examination Center at the Affiliated Hospital of Chengdu University of Traditional Chinese Medicine comprised the control group, including 6 males (42.86%) and 8 females (57.14%). Inclusion criteria included the following: Age 18 to 65 years; Healthy, without any diseases; No use of immune-regulating drugs or health products in the past six months.

Sample collection

The anticoagulant blood was collected from these participants by using EDTA tube, 2 copies per person. One specimen was sent to flow cytometry within 6 hours. The another was centrifuged, then the supernatant was taken and stored in a sterile dry cryotube, and placed at -80°C.

T lymphocyte assay

Flow cytometry was used to detect the percentages of $CD4^+T$ cells, $CD8^+T$ cells, Th17, Treg and Tfh cells. The fluorescent antibodies and reagents required for flow cytometry testing are listed in Supplementary Table. The antibodies were added to 50 uL whole blood. After staining for 30min, add 600µL erythrocyte lysate for 10-15min, centrifuge at 1000r for 4min, then add 200µL lysate for 3min, after centrifugation at 1000r for 4min, add 300µL of PBS and test $CD4^+T$, $CD8^+T$, Treg and Tfh cells by flow cytometer (CytoFLEX, Beckman Coulter, Inc. USA). Peripheral blood mononuclear cells (PBMC) extraction is required in the detection of Th17 cells. Add 1µL PMA, 1µL Ionomycin and 2µL Brefeldin A to stimulate and incubate for 4h, and measure the levels of $CD4^+T$ and IL-17 in the cytoplasm for detection Th17 cells level.

HMGB1-PTEN proteins assay

Elisa was used to detect the expression of signal proteins (HMGB1, PTEN, PI3K, PDK1 and Akt). These proteins were detected by using human protein ELISA kits (Jianglai Biological, China). The operation protocols were according to manufacturer's instructions.

Construction of association network

In order to better understand the interaction between proteins in HMGB1-PTEN pathway, a protein association map was built. The STRING database(https://string-db.org/), and the GeneMANIA database(http://genemania.org/) were used to analyze and construct the functional expression correlation between HMGB1, PTEN, PI3K, PDK1, and Akt.

Statistical Analysis

Statistical analysis was performed using SPSS22.0 software (IBM, Chicago IL, USA). Since this experiment was a small sample study, all data were tested for normal distribution by Shapiro-Wilk test. Then select the appropriate test method (Student's T test or Mann Whitney U test) depending on the result. Multiple stepwise

regression analysis and Pearson or Spearman test were used to explore the correlation between the HMGB1-PTEN pathway axis and immune cells. For all tests, $P<0.05$ was considered statistically significant.

Results

T lymphocyte alteration in CHB patients

Compared with the healthy controls, the percentages of CD4$^+$T and CD8$^+$T cells in CHB patients were lower ($P<0.01$). The percentages of Th17, Treg, ratio of Th17/Treg and Tfh cells in CHB patients were significantly higher than the healthy controls ($P<0.01$). But there was no statistically significant difference in ratio of CD4$^+$T/CD8$^+$T between the two groups ($P>0.05$) (Table1 and Fig. 1).

Table 1 Difference statistics of T lymphocyte subsets (n=50, Mean ±SD)

T lymphocytes	CHB (%)	Health (%)	Value P
CD4$^+$T	31.17±6.59	36.58±4.76	0.007
CD8$^+$T	24.18±7.54	32.07±6.27	0.001
CD4$^+$T /CD8$^+$T	1.37±0.35	1.18±0.30	0.092
Th17	11.07±4.83	0.49±0.24	0.000
Treg	9.76±2.07	4.67±3.08	0.000
Th17/Treg	1.18±0.57	0.20±0.22	0.000
Tfh	11.22±3.18	8.67±1.82	0.001

The pathway proteins alteration in CHB patients

Compared with the healthy controls, the serum levels of HMGB1, PI3K, PDK1, and Akt in CHB patients were significantly higher ($P<0.01$). No difference in PTEN was found in CHB patients and healthy controls ($P>0.05$) (Table 2 and Fig. 2).

Table 2 Statistic of HMGB1-PTEN pathway protein level (n=50, Mean ±SD)

HMGB1-PTEN pathway	CHB	Health	value P
HMGB1	0.7053±0.0040	0.6699±0.0106	0.000
PTEN	0.2348± 0.0823	0.2759±0.0022	0.376
PI3K	0.3287±0.0024	0.2795±0.0054	0.000
PDK1	0.5058±0.0003	0.4535±0.0016	0.000
Akt	1.3899±0.0043	0.7762±0.0049	0.000

HMGB1-PTEN pathway protein network

The protein interaction network diagram was constructed through the STRING database, and then the relationship between HMGB1-PTEN pathway was analyzed. As shown in Fig. 3, we found that Akt was the core factor, which connect the HMGB1 with other proteins (PTEN, PI3K, PDK1). The connection involved varied aspects such as literature mining, experiments, databases, co-expression, gene neighborhood, gene fusion, co-occurrence, etc. The data showed that the combined score of evidence suggesting a functional link among PI3K, PDK1 and Akt were above 0.8. The combined scores among PTEN, PI3K and Akt were above 0.9. The results suggested that HMGB1, PTEN, PI3K, PDK1 and Akt had close relationship.

The GeneMANIA database was used to further analyze the functional expression correlation between HMGB1, PTEN, PI3K, PDK1, and Akt. As shown in Fig. 4, HMGB1, PTEN, PI3K, PDK1, and Akt mainly interacted through physical interactions (67.64%), co-expression (13.50%), co-localization (6.17%), pathway-mediated (4.35%) and other aspects. Depending on Akt, these proteins (HMGB1, PTEN, PI3K, and PDK1) were linked. The activated relationship among PI3K, PDK1and Akt, and the negative relationship among PTEN, PI3K and Akt have been found. HMGB1 and PDK1are co-expressed, suggesting that there may be a positive regulatory relationship between HMGB1 and PI3K, PDK1 and Akt

The correlation analysis between HMGB1-PTEN pathway and immune cell

In order to analyze the relationship between HMGB1-PTEN pathway and immune cell, we performed Pearson/Spearman test (Table 3). HMGB1 was negatively correlated with CD8$^+$T cells(P<0.05) and positively correlated with Th17, Treg and Tfh cells(P<0.01). PTEN was negatively correlated with Treg cells(P<0.05), while no significant correlations were found with CD4$^+$T, CD8$^+$T, Th17, Tfh cells. PI3K, PDK1 and Akt were all negatively correlated with CD4$^+$T and CD8$^+$T cells, and positively correlated with Th17, Treg and Tfh cells (P<0.01).

Table 3 Correlation analysis of HMGB1-PTEN pathway and T lymphocytes

Pathway	CD4$^+$T	CD8$^+$T	Th17	Treg	Tfh
HMGB1	-0.276/0.055	-0.364/0.010	0.736/0.00	0.611/0.00	0.393/0.005
PTEN	0.261/0.071	0.052/0.721	0.105/0.471	-0.283/0.049	0.125/0.391
PI3K	-0.365/0.010	-0.444/0.001	0.766/0.00	0.688/0.00	0.377/0.008
PDK1	-0.379/0.007	-0.451/0.001	0.759/0.00	0.701/0.00	0.378/0.007
Akt	-0.380/0.007	-0.455/0.001	0.759/0.00	0.698/0.00	0.385/0.006

The numbers to the left and right sides of each slash line represent the correlation coefficient and P value, respectively.

To further identify the important parameters that HMGB1-PTEN pathway axis can affect, stepwise regression analysis was conducted. The multiple regression equation of HMGB1-PTEN pathway and T lymphocytes was

constructed in CHB. The adjusted R^2 of all proteins were good. The results showed that it was highly consistent in five proteins, mainly linearly correlated with CD4$^+$T, CD8$^+$T, and ratio of CD4$^+$T/CD8$^+$T (Table 4). Similarly, in the Healthy controls, the regression results showed that the HMGB1-PTEN pathway axis could significantly affect the T lymphocyte and was highly consistent, which were significantly correlated with CD4$^+$T, CD8$^+$T, and ratio of CD4$^+$T/CD8$^+$T. There was a linear correlation between HMGB1-PTEN pathway protein and the number of immune cells, and did not change with pathophysiological status.

Table 4 Multiple stepwise regression analysis of HMGB1-PTEN pathway and T lymphocytes in CHB

Pathway	CD4$^+$T	CD8$^+$T	CD4$^+$T/CD8$^+$T	Th17	Treg	Th17/Treg	Tfh
HMGB1	-7.973/0.997**	16.105/0.997**	22.261/0.997**	—	—	—	—
PTEN	6.006/0.926**	—	—	—	—	3.597/0.926**	—
PI3K	-8.233/0.997**	16.469/0.997**	22.814/0.997**	—	—	—	—
PDK1	-8.215/0.997**	16.371/0.997**	22.624/0.997**	—	—	—	—
Akt	-8.153/0.997**	16.254/0.997**	22.437/0.997**	—	—	—	—

The numbers to the left and right sides of each slash line represent the regression coefficient and coefficient of determination, respectively. *, **: p=0.05,0.01, respectively.

Discussion

Chronic hepatitis B is a major health problem that needs to be solved urgently, so that the pathological mechanism should be clarified. The process of chronic HBV infection is closely related to the immune system. Previous studies have proved that the main pathogeny of chronic hepatitis B is not HBV infection, but the replication and proliferation promotes immune response finally leads to liver injury [40, 41]. At one side, the immune response brings protective function to remove the virus. At the other side, the immune activities lead to liver cell damage, and even induce virus mutation. Unlike acute HBV infection, the cellular immune response decreased in chronic infection. And the chronic cellular immune response caused the persistence of HBV and finally developed the disease [42]. As the key members of cellular immunity, CD4$^+$T and CD8$^+$T are of great significance in the clearance of hepatitis B virus. It was reported that the number of CD4$^+$T, CD8$^+$T and ratio of CD4$^+$T/CD8$^+$T in patients of CHB significantly declined, which indicated that the cellular immunity was disordered in chronic HBV infection [43]. Furthermore, the liver cells immunity gradually decreased with the development of CHB [44]. As a helper, both CD4$^+$T cells and CD8$^+$T can directly kill viruses, which provide an important defense line to fight against viruses. However, these T cells significantly decreased in CHB, suggesting that the immune system was seriously damaged. After the antigen activating initial CD4$^+$T, they differentiate into different subtypes, such as Th17 and Treg, and perform different functions. Th17 and Treg keep a relatively stable balance, and the disorder of the balance take part into the persistence and severity of CHB. Th17 cells mainly participate in the pathological process of various inflammatory reactions by secreting a variety of cytokines. Studies have found that the number of Th17 cells in peripheral blood of CHB patients significantly increased [45], which suggests that Th17 cells play an immune activation role in chronic HBV

infection. Th17 cells abnormally differentiated, and secreted inflammatory factors, such as IL-17, which aggravated the inflammation of the body. However, Treg cells are mainly immunosuppressive. In chronic HBV infection, Treg cells inhibit the differentiation and activation of CD4$^+$T, CD8$^+$T and other effector T lymphocytes. Treg cells decrease the secretion of some cytokines, such as IL-10, TGF - β, which weakens the ability to clear target cells, immune response, and liver inflammation. It also reduces the ability to clear the pathogen, which leads to the chronic progress of HBV infection [46]. Th17 and Treg cells can restrict each other [47], and cooperate with each other [48]. Under certain conditions, they transform each other. Excessive Treg cells reduction induces Th17 cell proliferation [49]. Th17/Treg cell balance is an important component of maintaining the normal cellular immune function, and it has been recognized as main cause of the development of CHB. The number of Th17 and Treg and the ratio of Th17/Treg altered in CHB, which indicated that the balance of Th17/Treg cells were broken. The imbalance was the risk factor for the development of CHB into cirrhosis and HCC [50].

Humoral immunity is also vital in virus clearance. Antibodies control or eliminate the virus but also improve the long-term prognosis of CHB [51]. Tfh promotes the lymphocyte differentiation, B lymphocyte activation, and antibody production [52]. Tfh in CHB patients significantly increased, which involved into the immune inflammatory response of hepatitis B virus infection[19]. Tfh originated IL-21 promote HBeAg seroconversion in patients with chronic HBV infection [53, 54].

HMGB1 levels increased in CHB that accompanied with the severity of liver inflammation, and closely related to the progress of liver fibrosis after hepatitis [55, 56]. During HBV infection, the PI3K/Akt pathway was activated [57]. IL-12, as one of the proinflammatory factors, significantly increase in CHB patients and HBV-induced IL-12 expression involved into the activation of the PI3K-Akt pathway [29]. In addition, the evidence has demonstrated that the change of PTEN activity leads to development of CHB by deregulating the PI3K/Akt pathway [58]. Therefore, the main role of the HMGB1-PTEN mediated PI3K/PDK1/Akt pathway is to promote progress of inflammation in CHB patients. In contrast, the levels of some HMGB1-PTEN pathways proteins (HMGB1, PI3K, PDK1, and Akt) of CHB patients were higher, revealing that the inflammatory state were activated.

Excessive immune response cause inflammation but also affects the expression of T lymphocytes. Studies have shown that HMGB1 levels increased in CHB patients, which in turn inhibit the activation of Treg cells through TLR-IL-6 receptor signal transduction, and further promote the activities of Th17 cells [59, 60]. PTEN regulates the stability of Treg cells through Akt, and the lack of PTEN activates Tfh cells and leads to spontaneous inflammation [61]. HMGB1 can also regulate the differentiation of Treg cells by deregulating PTEN [62]. Moreover, in vivo and vitro, knocking out the PDK1gene of T cells decreased the number of Th17 and Treg cells [63]. Otherwise, PDK1 gene could mediate Akt level to and elevate Treg cells [64]. The level of Treg cells in the peripheral blood of lung cancer patients significantly increased, which might be related to the PTEN weak expression and PI3K-Akt signaling pathway activation [65]. So, PI3K/PDK1/Akt signaling pathway is regulated by HMGB1-PTEN signaling axis that takes effect on the differentiation of Treg cells (Fig. 5). We hypothesized that HMGB1-PTEN pathway affects the balance of immune cell subtypes by regulating the activation of T lymphocytes and inducing the differentiation of cell subtypes.

As a result, we studied the relationship between pathway and immune cells. From the correlation analysis, we can see that HMGB1-PTEN negatively directly affect CD4$^+$T and CD8$^+$T cells. In other words, the higher the levels of HMGB1, PI3K, PDK1 and Akt, the lower the levels of CD4$^+$T and CD8$^+$T cells, which means the lower the immune function in chronic hepatitis B. Th17, Treg, Tfh cells increased with the increase of HMGB1, PI3K, PDK1, Akt levels, which participate into the development of inflammation. PTEN is negatively correlated with Treg, which is different from other proteins in the pathway. All indicated the close relationship between HMGB1-PTEN pathway and T lymphocytes. The HMGB1-PTEN pathway might reflect the changes of T lymphocyte levels. But this is only a first step, and further experiments need to clarify the regulatory mechanism of HMGB1-PTEN pathway.

Conclusions

Chronic hepatitis B decreased immune function but activated inflammatory response, which directly affect the prognosis. There is a close relationship between HMGB1-PTEN pathway and T lymphocytes.

Abbreviations

Akt: Protein Kinase B; CHB: Chronic Hepatitis B; Elisa: Enzyme linked immunosorbent assay; HBV: Hepatitis B Virus; HMGB1: High mobility group box-1 protein; PDK1: Pyruvate Dehydrogenase Kinase Isozyme 1; PI3K: Phosphatidylinositol 3-kinase; PTEN: Phosphatase and tension homology deleted on chromosome ten; Tfh: Follicular Helper T cells.

Declarations

Statement

The study we performed is in compliance with institutional, national, or international guidelines.

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Authors' contributions

XL was involved in drafting the manuscript and acquisition of data; CL completed analysis and interpretation of data; GYL helped the acquisition and analysis of data; JM and LSX contributed to the statistical analysis and correction of the manuscript. QSF and CJ designed this study and gave final approval of the version to be published. All authors have read and approved the final version of the manuscript.

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Ethics declarations

Ethics approval and consent to participate

The ethics committee of the Affiliated Hospital of Chengdu University of Traditional Chinese Medicine has approved the study and all subjects gave informed consent. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Figures
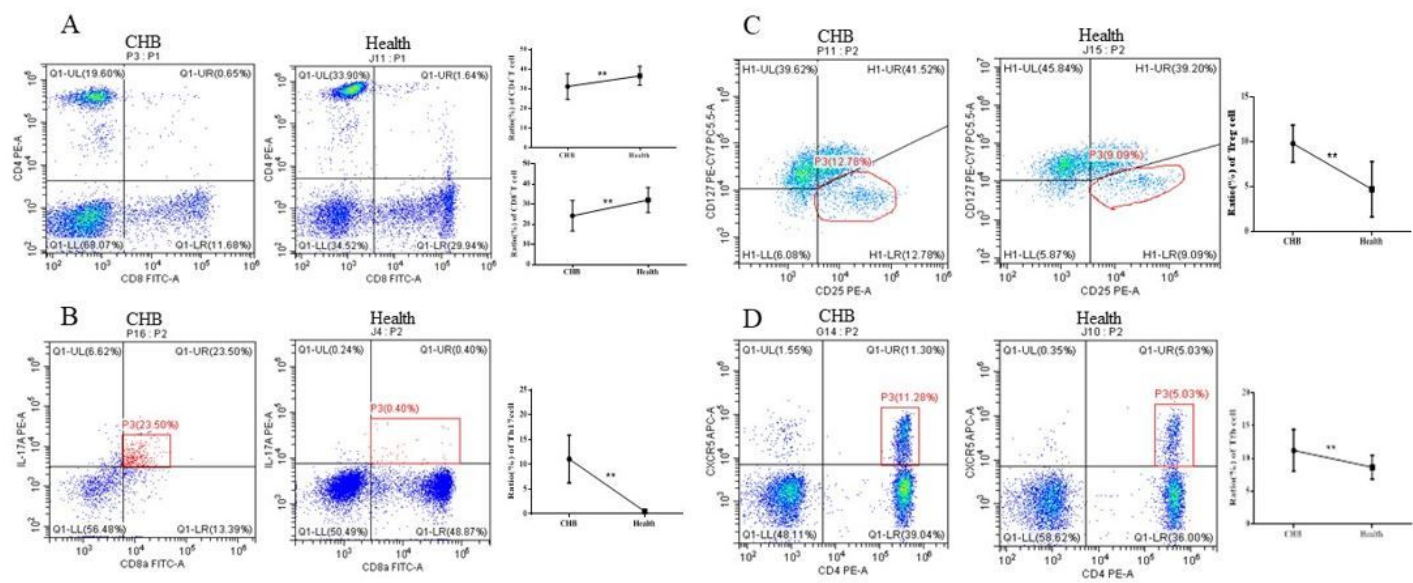
Figure 1

T lymphocyte alteration in CHB patients (A-D). Blood cells were stained with BD MultiTEST kit and the corresponding percentages of T cells were summarized and counted. Patients in the CHB group had significantly reduced percentages of CD4+T and CD8+T cells(A), while increased percentages of Th17(B), Treg(C) and Tfh(D) cells. **P<0.01.
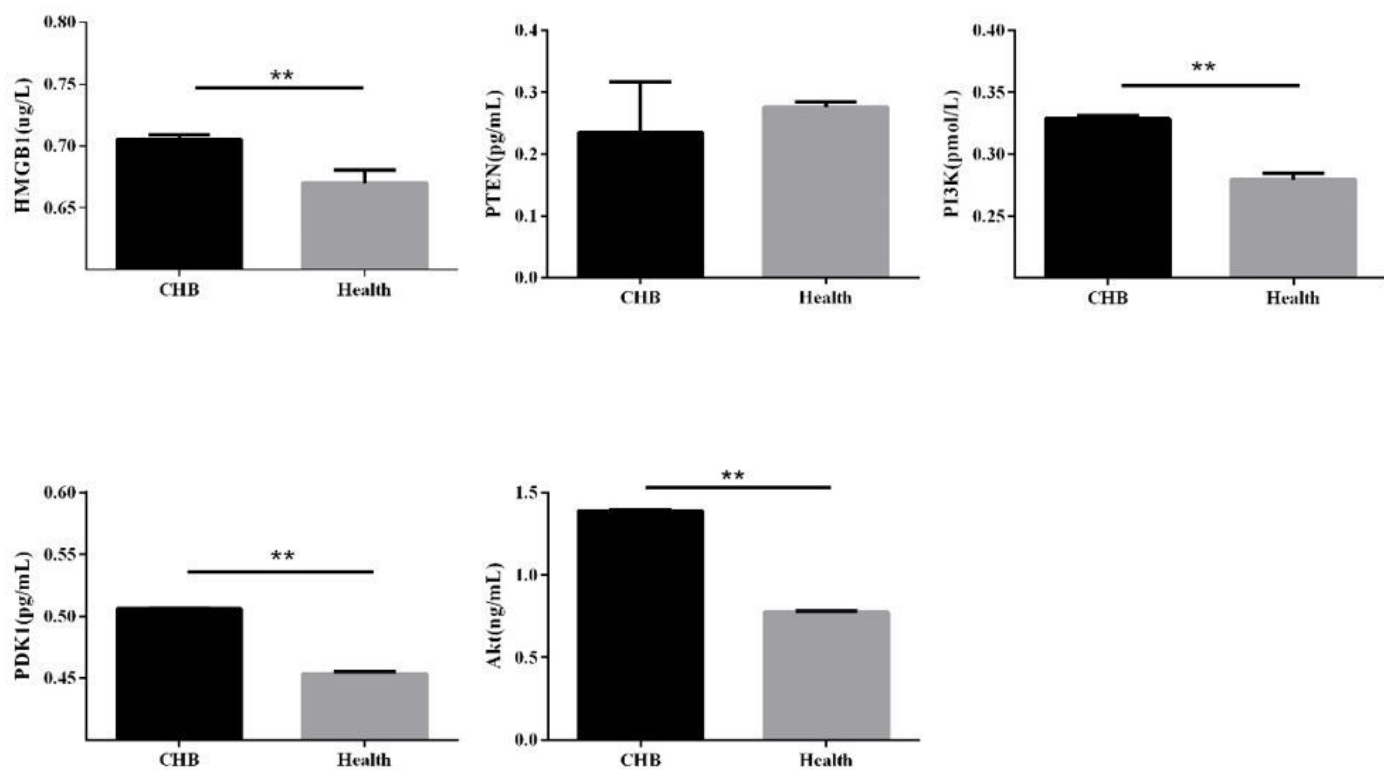
Figure 2

The HMGB1-PTEN pathway proteins alteration in CHB patients. **P<0.01.
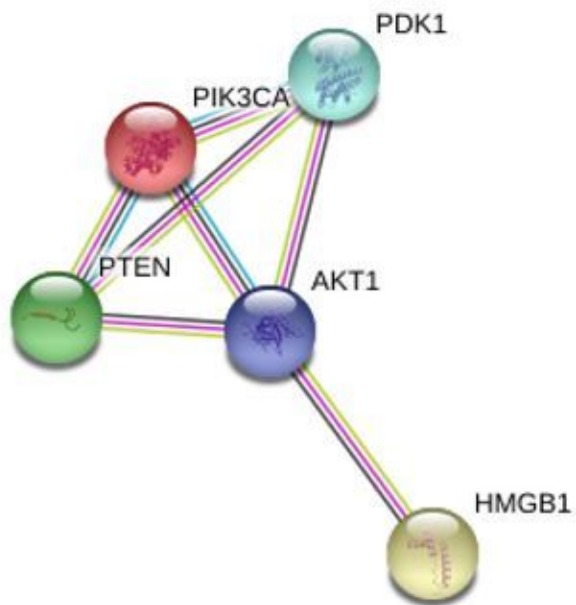
Figure 3

Conducting a HMGB1-PTEN pathway protein interaction network diagram, different connection colors represent different interaction types. The thickness of the line represents the strength of the association. The thicker the line, the stronger the association, and the thinner the line, the weaker the association.
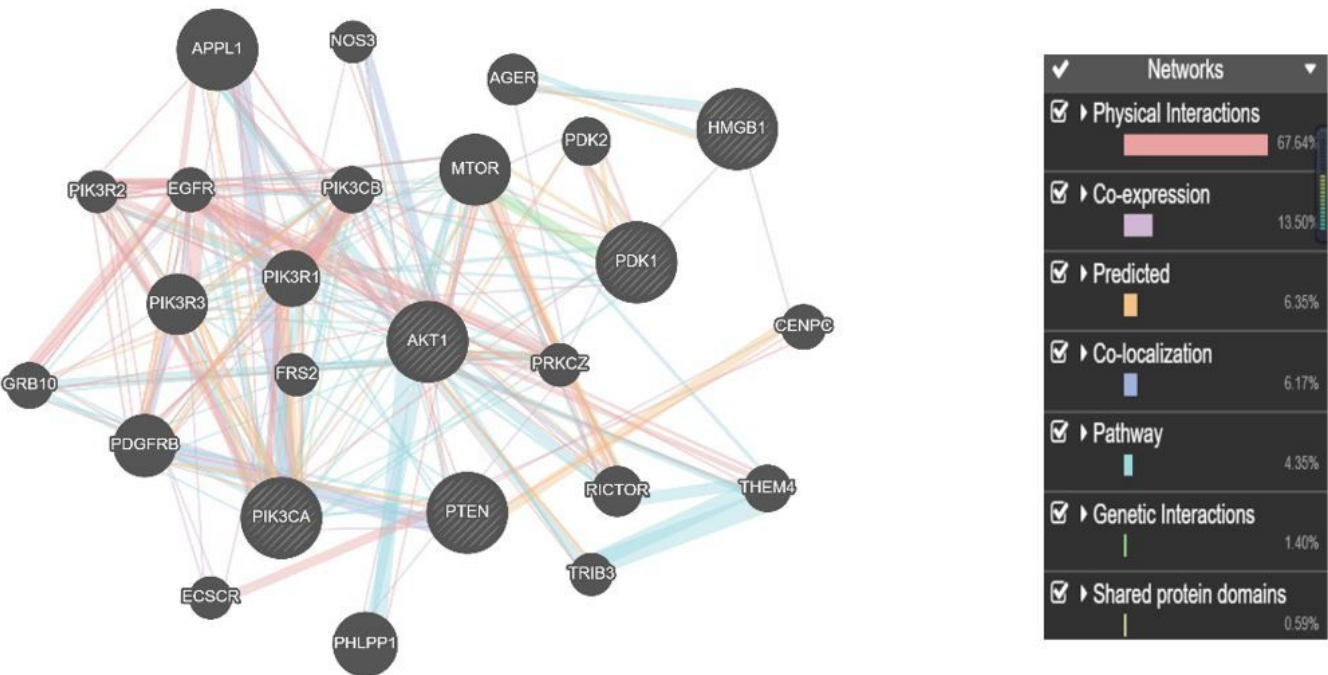
Figure 4

HMGB1-PTEN pathway protein function correlation diagram
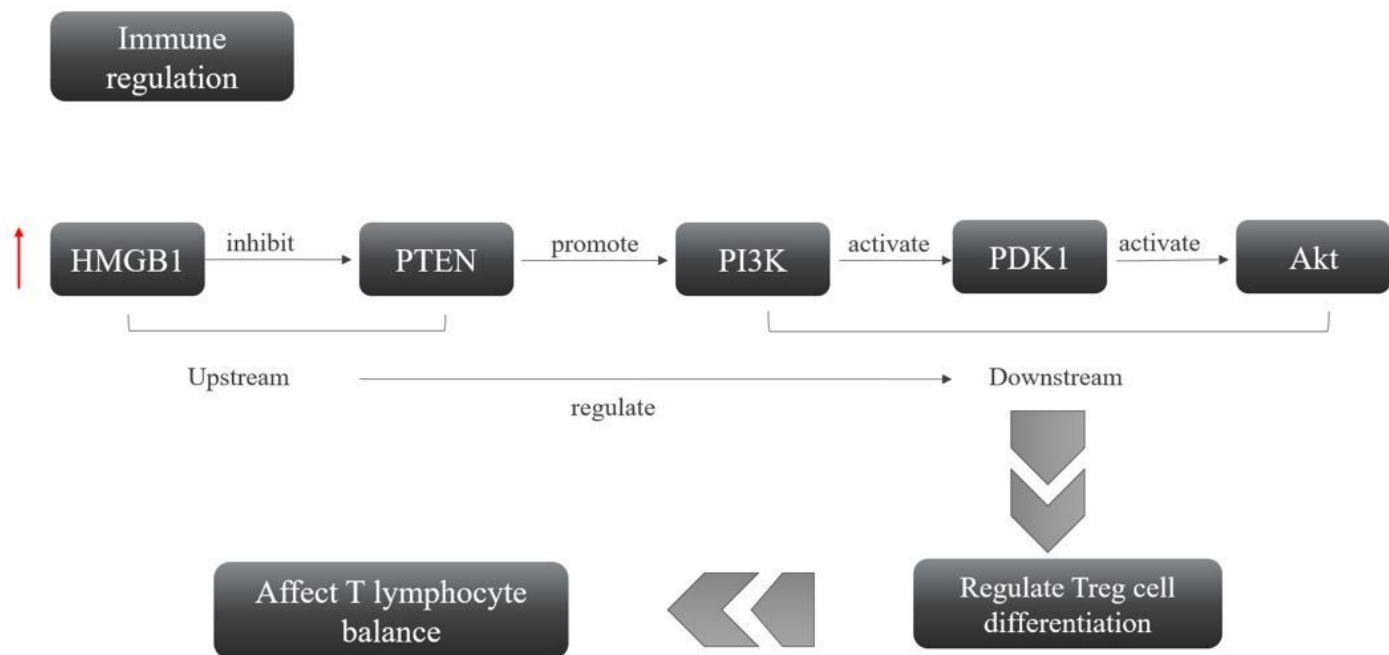
Figure 5

HMGB1-PTEN pathway regulate T lymphocytes