Expression of Human Cytokine Genes Associated with Chronic Hepatitis B Disease Progression

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ABSTRACT

Background: Hepatitis viruses are non-cytopathic viruses that lead to the infection and pathogenesis of liver diseases as a result of immunologically mediated event. **Objective:** To investigate the expression of human inflammatory cytokines in chronic hepatitis B patients according to the severity of the infection. **Methods:** We recruited a total of 120 patients, 40 of whom from cirrhotic, 40 non-cirrhotic, and 40 acute flare chronic hepatitis B and 40 healthy controls. For all groups total cellular RNA was extracted from whole blood samples, genomic DNA was eliminated, and cDNA was synthesized using the RT2 first strand kit, as instructed by the manufacturer. The real-time profiler PCR array was performed on an a master cycler ep realplex and the data were analyzed using an online data analysis software. **Results:** Non-cirrhotic chronic hepatitis B patients were found to significantly upregulate interleukin 10 receptors that regulate the balance between T helpers 1 and 2. On the other hand, patients with cirrhosis were found to have significant upregulated interleukin 3 gene expression. **Conclusion:** Our finding suggests that upregulation of anti-inflammatory and downregulation of pro-inflammatory cytokines may play a roles in the progression of non-cirrhotic chronic hepatitis B patients to cirrhotic and acute flare. However, a multi-center study with a larger sample size is needed to confirm our findings.

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Keywords: Acute Flare, Hepatitis B, Inflammatory Cytokines, Interleukins, Liver Cirrhosis

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INTRODUCTION

Acute and chronic liver infections are mostly characterized by the expression of pro- and anti-inflammatory cytokines, which lead to many inflammatory diseases of the liver, fibrosis, and subsequent cirrhosis of the liver. These cytokines are regulatory peptides that are pleiotropic in nature and can be produced by nearly every nucleated cell in the body, including hepatocytes (1,2). Cytokine family is composed of several subfamilies, such as the interleukins (ILs), tumor necrotic factors (TNFs), interferons (IFNs), chemokines, and interleukin 6-type cytokines. Recently, there have been increasing lines of evidence supporting the major role of several inflammatory cytokines in liver disease progression and tissue repair (3,4), including the current study. The cytokine families that are considered as the key factors in various stages of liver diseases include the pro-inflammatory molecule TNF-α, the anti-inflammatory cytokine IL-10, and the adipokine adiponectin; these cytokines also correlates with biomarkers of autoimmunity (5).

Hepatitis B virus (HBV) and hepatitis C virus are among numerous viruses affecting the human liver and are distinct because of their phenomenal capacity to cause persistent infection, cirrhosis, and hepatocellular carcinoma (HCC). Hepatitis B, C, and E are non-cytopathic viruses as such the outcome of infection and the pathogenesis of liver diseases are the result of immunologically mediated events. Adaptive immune response mediates almost all of the liver pathology associated with viral hepatitis, as evidenced by the fact that the hepatic immunopathology is induced by antigen-nonspecific inflammatory cells exacerbating cytotoxic T lymphocytes (CTLs) and the accumulation of CTLs in the liver. This study aimed to study the expression of human inflammatory cytokine genes in chronic hepatitis B patients related to the severity of the infection.

MATERIALS AND METHODS

Patients. This study is an analytical cross-section trial involving three disease groups (cirrhotic, non-cirrhotic, and acute flare chronic hepatitis B) and one healthy control group. Patients were recruited consecutively from the Hepatology Department, Selayang Hospital, Selangor, which serves as a tertiary referral center for hepatology cases in Malaysia. The control group included healthy volunteers of similar ages and racial origin to the disease groups. In this work, 10 mL of blood was collected from each patient using an EDTA blood tube and kept at -80℃ prior to experiments. Next, 20 samples were collected from each group of chronic non-cirrhotic, cirrhotic, and acute flare chronic hepatitis B patients and healthy controls using computer-generated random number online software (https://www.randomizer.org/). The severity of a disease is defined as the extent of organ or system damage or its physiological decompensation of the patient's condition.

Total RNA Extraction. Total cellular RNA was extracted from the patient's whole blood using the QIAamp RNA blood mini kit, as described by the manufacturer (Qiagen, Hilden, Germany). Concisely, 20 µl of the protease was added to the bottom of a 1.5 ml tube, followed by the addition of 200 µl plasma. Buffer AL was added to all samples and was mixed thoroughly for 15 sec and incubated for 10 min at 56℃. Ethanol (200 µl) was added to the sample and vortexed for 15 sec. The mixture was centrifuged at 10000×g for 60 min, followed by washing it using AW1 and AW2 buffers. The RNA

was then eluted by adding 50 µl of elution buffer and stored at -70℃. The extracted RNA was subjected to purity and concentration checks.

cDNA Synthesis Using an RT2 First Strand Kit. The RT2 first strand kit was used for cDNA synthesis according to manufacturer's guidelines (Qiagen, Hilden, Germany). Briefly, a reverse transcription mixture was prepared by adding 4 µl of Buffer BC3 x 5, 1 µl of Control P2, 3 µl of RNase free water, and 2 µl of reverse transcriptase RE3 mix. This reverse transcription mixture (10 µl) was then added to the genomic elimination mixture (10 µl) and mixed by pipetting, and then was incubated for 15 min at a temperature of 42℃; the reaction was immediately stopped by incubating at 95℃ for 5 min. To each reaction mixtures, 91 µl of RNase-free water was added and mixed by pipetting it for several times; before placing the mixture on ice and prior to real-time PCR, it was stored at -30℃.

Real-time Profiler PCR Array. The RT2 SYBR green master mix was briefly centrifuged. This master mix contained hot-start polymerase. The PCR component for real-time PCR for 96-wells was prepared by mixing 1350 µl of RT2 SYBR green x 2 master mix, 1248 µl RNase-free water, and 10^2 µl cDNA synthesis reagent in a 14 ml tube. The RT2 profiler PCR array was carefully removed from its sealed packaging and 25 µl of the PCR component mixture was added to each of the wells by changing tips to avoid cross-contamination. An optical thin wall cap was then used to seal the microplate, followed by centrifuging it briefly for 1 min at 1000 x g to remove bubbles. The plate was placed on an Eppendorf master cycler ep realplex (Eppendorf, Canada) and the cycling conditions were set to 95℃ for 10 min, 95℃ for 15 sec, and 60℃ for 1 min for 40 cycles. The threshold cycle (CT) value for each of the samples was then calculated using the real-time PCR cycler software. The threshold was set manually at 140 using the log view of the amplification plot.

Statistical Analysis. The Ct values were exported to an excel spreadsheet and the data were analyzed using SABioscience online data analysis software. A Multi-Gene qPCR Assay was also chosen. The MS Excel file containing the PCR data was then uploaded. In the "Basic Setup" section, samples were assigned to different groups including Group 1 (non-cirrhotic chronic hepatitis B patients), Group 2 (cirrhotic CHB patients), Group 3 (CHB patient with acute flare), and a control group (healthy individuals). The "Data QC" section was reviewed to assess each groups' PCR reproducibility, reverse transcription efficiency, and the presence of genomic DNA contamination. Housekeeping Genes were selected for data normalization by clicking the appropriate checkboxes. The data were then analyzed by clicking on the "analyze" button. The "Average Ct", "2^{-Ct}", "Fold Change", "p-value", and "Fold Regulation" sections for the results were processed by the software using the imported data.

RESULTS

The results from the clinically diagnosed chronic hepatitis B patients who have never undergone HBV treatment (Group 1), cirrhotic patients with liver stiffness score of more than 11 KPa (Group 2), and acute flare chronic hepatitis B patients with elevated serum ALT more than 5 times higher than the upper normal limit (Group 3) were compared with healthy normal control group that is HBsAg negative. Cytokine gene expression results showed that, out of the 84 genes (Supplementary) associated with inflammatory pathways that were tested, only 7 (8.3%) were expressed in non-cirrhotic

chronic hepatitis B patients compared to the healthy hepatitis B negative control group. On the other hand, gene expression was high among the cirrhotic and acute flare chronic hepatitis B groups in which 75 (89.3%) and 68 (81%) genes were expressed, respectively.

Patients with non-cirrhotic chronic hepatitis B infection were found to have significant upregulation of the interleukin 10 receptors (IL-10R) 1 and 2 ($p<0.05$), while acute flare and cirrhotic chronic hepatitis B infection were found to have the highest number of upregulated genes (Table 1).

This observation is explained by the fact that the upregulated genes, mostly pro-inflammatory chemokines such as Chemokine (C-C motif) ligands (CCL) and also interleukin receptor one (IL-1R1), might play a significant role in acute flaring in chronic hepatitis B patients. Besides, non-cirrhotic chronic hepatitis B infected patient showed significant downregulation of only CCR6 ($p<0.05$), whereas cirrhotic and acute flare chronic hepatitis B infected patients showed downregulation of several anti-inflammatory cytokine genes such as interleukin 13 and 17, interleukin 1 receptor antagonist (IL-1RN), and gamma interferon (Table 1).

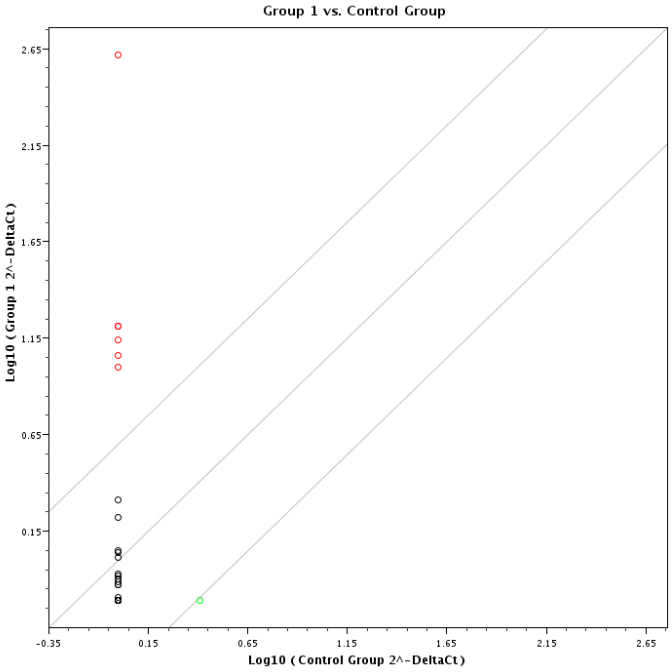

Figure 1. Up-regulated genes (red cycles) are in the non-cirrhotic (group 1) patients as well as a significantly down-regulated gene (green cycle). The other genes (black cycles) were also up- and down-regulated but their expression is not significant when compared with the normal control group. The central lines indicate unchanged gene expression, while genes that are over-expressed are above the line and under-expressed genes are below the line.

The delta Ct values for each gene in the control and disease groups with p-values less than 0.05 are indicated in red in a scattered plot for non-cirrhotic (Fig. 1), cirrhotic (Fig. 2), and acute flare chronic hepatitis B (Fig. 3) patients. The scattered plot compares the normalized expression of every gene on the array between the two groups plotted together in other to quickly visualize large changes in gene expression. A fold-change ($2^{-\Delta Ct}$) was obtained by dividing the normalized gene expression in the disease sample

with the normalized gene expression of the control sample. Fold change values greater than unity represent an up-regulation.

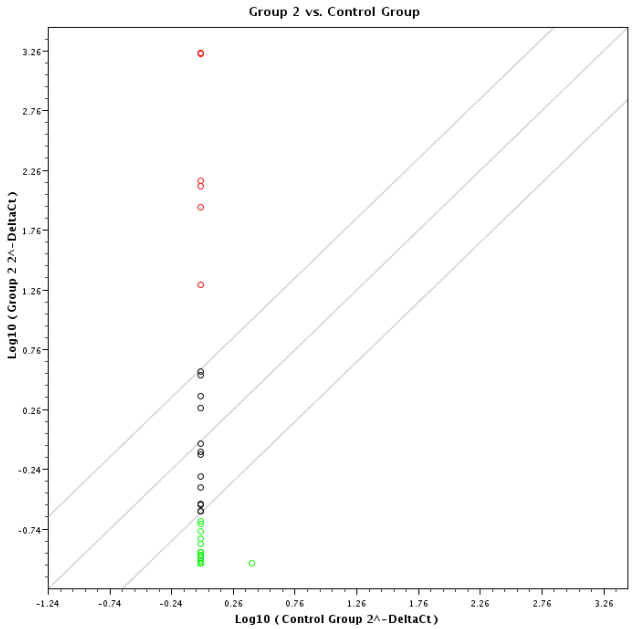

Figure 2. Up-regulated (red cycles) and down-regulated genes (green cycle) in the cirrhotic (group 2) patients. The central lines indicate unchanged gene expression, while genes that are over-expressed are above the line and under-expressed genes are below the line.

This change is equal to the fold regulation in a biologically meaningful way. Similarly, fold change values less than the unity indicate downregulation, for which the fold regulation is the negative inverse of the fold-change.

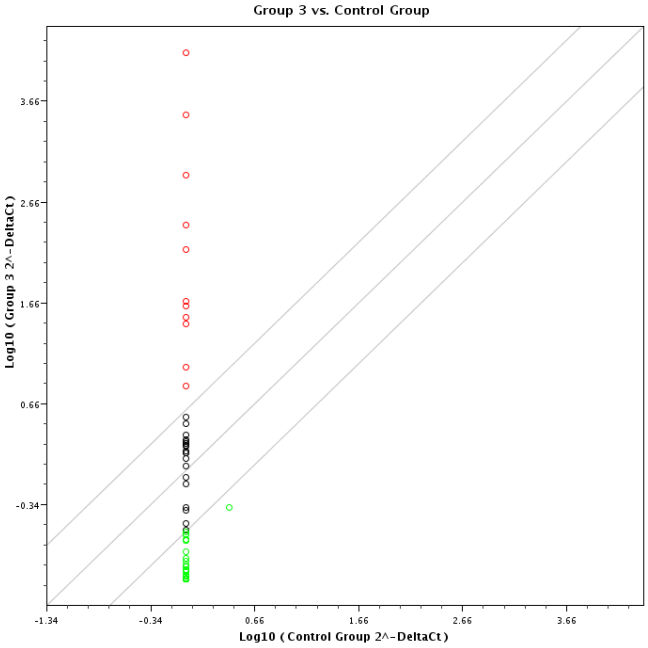

Figure 3. Scattered plot of the expressed genes in the acute flare (group 3) patients. Significantly up-regulated (red cycles) and down-regulated genes (green cycle), while the remaining (black cycles) show no significant difference with the control group. The central lines indicate unchanged gene expression, while genes that are over-expressed are above the line and under-expressed genes are below the lines.

Table 1. Up and Down-regulated cytokines expression as compared to normal control group

Upregulated Cytokines			Down regulated Cytokines		
Non-cirrhotic CHB	**Cirrhotic CHB**	**Acute flare**	**Non-cirrhotic CHB**	**Cirrhotic CHB**	**Acute flare**
CD40LG	CCL26	CCL1	CCR6	BMP2	C5
CSF3	CCL4	CCL16		C5	CCL11
CX3CL1	CCR2	CCL17		CCL1	CCL13
IL-10RA	CXCL9	CCL26		CCL11	CCL15
IL-10RB	IL-3	CCL3		CCL13	CCL2
	TNFSF13	CCL4		CCL15	CCL20
		CCL7		CCL16	CCL22
		CCR2		CCL17	CCL23
		CCR4		CCL2	CCL24
		CCR5		CXCL12	IFNA2
		IL-1R1		CXCL13	IFNG
				CXCL2	IL15
				CXCL3	IL17A
				CXCL5	IL17C
				CXCL6	IL17F
				CXCR1	IL1A
				CXCR2	IL1B
				FASLG	IL1RN
				IFNA2	IL21
				IL10RB	IL27
				IL15	IL3
				IL16	IL33
				IL17A	IL5
				IL17C	IL5RA
				IL17F	IL7
				IL1A	IL9
				IL1B	IL9R
				IL1R1	TNF
				IL1RN	TNFRSF11B
				IL21	TNFSF10
				IL27	TNFSF11
				IL33	TNFSF13
				IL5	TNFSF13B
				IL5RA	TNFSF4
				IL7	VEGFA
				CXCL8	
				IL9	

CCR6: Chemokine (c-c motif) receptor 6; CD40LG: Cluster of differentiation 40 ligand; CSF3: Colony stimulating factor 3; CX3CL1: Chemokine C-X3-C motif ligand 1; IL-10RA: Interleukin 10 receptor alpha; IL-10RB: Interleukin 10 receptor beta; CCL26: Chemokine (C-C motif) ligand 26; CCL4: Chemokine (C-C motif) ligand 4; CCR2: Chemokine (C-C motif) receptor 2; CXCL9: Chemokine (C-X-C motif) ligand 9; IL-3: Interleukin 3; TNFSF13: Tumour necrosis factor (ligand) superfamily, member 13; CCL1: Chemokine (C-C motif) ligand 1; CCL16: Chemokine (C-C motif) ligand 16; CCL17: Chemokine (C-C motif) ligand 17; CCL3: Chemokine (C-C motif) ligand 3; CCL7: Chemokine (C-C motif) ligand 7; CCR4: Chemokine (C-C motif) receptor 4; CCR5: Chemokine (C-C motif) receptor 5; IL-1R1: Interleukin 1 receptor typeI

The fold-change was found to be higher in the liver cirrhotic group in relation to the expressed genes ($p<0.05$). The results of the average Ct values show that more inflammatory cytokine genes were expressed as the diseases increase in severity from non-cirrhotic ($p<0.05$) to cirrhotic ($p<0.05$) and even higher in the acute flare group ($p<0.05$). This finding substantiates the role of the immune system in the progression of hepatitis B infection; the intensity of inflammatory response is concomitant with the physiological and pathological presentation of chronic hepatitis B patients.

DISCUSSION

In the present study, chronic hepatitis B patients were classified into three groups: non-cirrhotic, cirrhotic, and acute flare. The non-cirrhotic chronic hepatitis B patients were found to have significant upregulation of the interleukin 10 receptors (IL-10 R) 1 and 2, which provide binding sites for interleukin 10 (IL-10) to mediate its anti-inflammatory and hepatoprotective functions, in agreement with the finding of Zhang *et al* (6). IL-10 is a promoter of Th2 response; its upregulation in patients with chronic hepatitis B infection is of significant benefit as it is an anti-inflammatory cytokine that mediates its effects via B cells. Thus, it mediates viral clearance without having destructive effects on hepatocytes (7), which is supported by the findings of this study. Other upregulated genes associated with non-cirrhotic chronic hepatitis are colony stimulating factor 3 (CSF3) as reported previously in HCV infected patients (8), chemokine C-X3-C motif ligand 1 (CX3CL1) that was reported to enhance progressive liver fibrosis (9), and the cluster of differentiation 40 ligand (CD40LG) that is consistent with previous findings (10,11). G-CSF3 encodes a glycoprotein that supports the formation of haematopoietic colonies, thereby influencing the proliferation, survival, maturation of haematopoietic progenitors, and regulating the function of matured effector cells. Hence, G-CSF3 upregulation as also reported in this study is significant in preventing chronic non-cirrhotic patients from developing hepatocellular carcinoma this. However, similar studies have established the apoptotic effects of hepatocytes (12,13). The upregulation of the chemokine (C-C motif) ligand 1(CXCL1) gene, which is the only member of the CX3C chemokine family commonly known as fractalkine in humans, as found in this study to be associated with hepatic inflammation, is in agreement with similar previous findings (14,15). However, its function depends on its form; in the soluble form, it functions as a chemoattractant, while in the membrane form, it mediates the adhesion of leukocytes such as integrins (16,17). On the other hand, only chemokine (C-C motif) receptor 6 (CCR6) gene was found to be downregulated among the non-cirrhotic chronic hepatitis B patients; this gene encodes the CCR chemokine protein also refrrered to as cluster of differentiation 196 (CD196) (18) and has been reported to be associated with different diseases stages (19). It was also found to be associated with hepatic cirrhosis (20), as such its down-regulation may favor non-progression of chronic hepatitis B patients to cirrhosis and HCC, as revealed in this study and other related studies (21,22). In chronic hepatitis B patients with cirrhosis, upregulation of interleukin 3 (IL-3), a biological signalling cytokine that is capable of improving the body's natural response to diseases by binding to the IL-3 receptor and a member of the hematopoietic cytokines play significant role in the pathogenesis of malignancies as previously reported (23-25). Accordingly, upregulation of the IL-3 gene in liver cirrhotic patients indicates poor prognosis and may be associated with the development of HCC (26,27),

tumour necrosis factor (ligand) superfamily member 13 (TNFSF-13) in agreement with finding of Wu, Chen (28), chemokines such as the chemokine (C-C motif) ligand 4, and 26 (CCL4, CCL26) as reported to enhance the progression of liver cirrhosis to HCC in previous studies (29,30) and serve as a potential therapeutic target (31,32).

Similarly, chemokine (C-C motif) receptor 2 (CCR2) mediates the function of CC chemokines in regulating myeloid cell recruitment to the liver. The upregulation of chemokine suggests that myeloid cells recruited to the liver might lead to liver cirrhosis, as previously reported in non-alcoholic fatty liver diseases (33) and other related animal studies (34-36). Chemokine (C-X-C motif) ligand 9 (CXCL9) is a strong chemoattractant that plays a significant role in recruiting Th1 in chronic hepatitis C infection; this result is in agreement with the current study, wherein CXCL-9 was found to be significantly upregulated in hepatitis B liver cirrhosis (37,38).

A significant number of cytokine genes were also found to be downregulated in this study. Most important of them are IL-10Rb, Interferon alpha, TNF, CC chemokines, CXC chemokines family, and complement 5 (C5); others include IL-1, -5, -7, -8, -9, -15, -16, -17, -21, -27, and -33 (39,40). Among these cytokines, IL-10Rb which is an anti-inflammatory cytokine that mediates its effects via B cells, thereby mediating viral clearance without having destructive effects on the hepatocytes. Thus, it has been found significant in the clinical state of infection (41,42). Moreover, IL-10Rb downregulation in cirrhotic patients as revealed in this study is an indication of the significant role of this cytokine in preventing liver damage and the effect of its polymorphism (43) as well as its role in sustained virological response to PEGylated Interferon (44). Similarly, interferon alpha has been also considered as a cytokine with anti-viral, immunomodulatory, anti-fibrogenic, and anti-inflammatory activity, most especially in HCV infection (45,46). Therefore, its downregulation favors the cirrhotic state of the patients (47,48). The significant role it plays in the prevention of progression of chronic hepatitis B and C infection to cirrhosis makes it an important therapeutic agent.

The primary role of tumor necrosis factor (TNF) is to regulate immune cells; it is capable of inducing inflammation and inhibits tumorigenesis and viral replication (49). TNF and its related pro-inflammatory cytokines have been reported to play key roles in chronic infection (50,51), which concur with the findings in this study, according which modulating the TNF inflammatory pathway may have potential effects against pro-inflammatory diseases like viral hepatitis. Furthermore, the effects of CC chemokine are mediated by specific surface cell receptors (CCR1 to CCR10). The deletion of a CCR-2 receptor for CCL-2 in liver cells was found to be sufficient to inhibit a hepatic fibrogenic response (36) and has been associated with polarization of lymphocytes towards Th1 or Th2 (52). Similarly, CXC chemokine family includes CXCL-1 to CXCL-17; ligands of this receptor are highly expressed on organs that have a predilection for tumor metastasis (53). However, in this study, these genes were downregulated, which indicate poor patient condition as supported by previous studies (54,55). Complement 5 (C5) is the fifth component of the complement system, which plays a significant role in inflammation and has been reported to play a critical role in liver fibrosis (56). A similar study also reported an increased risk of fibrosis in hepatitis C patients (56-58). Tumour necrosis factor superfamily member 13 (TNFSF-13) on the other hand, was reported in a recent study to be associated with solid tumor pathogenesis (59), which is further supported by the findings of this study where significant upregulation was observed in cirrhotic patients but not in normal controls and non-cirrhotic patients.

Chronic hepatitis B acute flare has been reported to associate with pro-inflammatory cytokines as a result of systemic inflammatory response (60,61); the elevation of helper-2 (Th2)-associated cytokines like interleukin 4, 5, 10, and 13 has been also reported (62-64). However, in this study, only IL-1 receptor and some CC chemokines were found to be upregulated in acute flare patients, while other interleukins such as interferon-alpha, interferon-gamma, and TNFs were downregulated in line with the findings of other researchers (65,66). Spontaneous acute flare in chronic hepatitis B is a dynamic process of immune response involving the HBV, liver cells, and host immune cells. When the immune response to viral antigens is vigorous, it increases the prospect of inflammatory changes, leading to acute flare, and damage to the liver cells, leading to fibrosis (67).
In conclusion, acute flare chronic hepatitis B patients were found to have the highest number of upregulated genes, most of which being chemokines. Chemokines associate with enhancing the inflammatory process, which might play a significant role in acute flaring of chronic hepatitis B patient. Non-cirrhotic chronic hepatitis B patients were found to significantly upregulate interleukin 10 receptor, which is a promoter of Th2 response. Upregulation of this receptor in patients with chronic hepatitis B infection is of significant benefit since it mediates its effects via B cells immune response thereby mediating viral clearance without having destructive effects on the hepatocytes. In cirrhotic chronic hepatitis B patients, the upregulated genes found in this study were interleukin 3 (IL-3), which is a biological signal cytokine capable of improving body's natural response to diseases by binding to the IL-3 receptor and is a member of hematopoietic cytokines.

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