Gastroenterology and Hepatology From Bed to Bench.

ORIGINAL ARTICLE

A pilot study on some critical immune elements in HBV infection: evidence of Alpha-1 Antitrypsin as an immunological biomarker

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ABSTRACT

Aim: This study is an attempt to screen the key immune elements that participate during HBV infection and the related pathways that are modulated.

Background: The pathogenesis of Hepatitis B virus and the corresponding clinical manifestations in the host are primarily immune-mediated.

Methods: This study utilizes a PCR array to screen immune-related genes that are differentially expressed in the presence of the virus in HBV replicating HepG2.2.15 cells as compared to control HepG2 cells. The significantly up-regulated genes were subjected to bioinformatic analysis utilizing GSEA and STRING. Additionally, ELISA was used to corroborate the levels of Alpha 1 antitrypsin (AAT) from patients' sera.

Results: The expressions of 31% of the studied genes were significantly up-regulated (> 2-fold, $p<0.05$) in HepG2.2.15 cells compared to controls, and this included the SERPINA1, FN1, IL1R2, LBP, LY96, LYZ and PROC genes. When they were clustered based on biological processes, signaling pathways, and disease progression, the genes related to biotic stimulus, complement-coagulation cascades, and fibrosis, respectively, showed the highest ($p<0.05$) enrichment. Analysis of patients' sera by ELISA revealed that the serum AAT (SERPINA1) levels were significantly higher in asymptomatic carriers and in patients with chronic liver disease than in controls ($p<0.05$). Moreover, SERPINA1 levels were also positively correlated with the levels of serum ALT ($r=0.4495$, $p<0.05$) among HBV infected patients.

Conclusion: The current study highlights the key immune elements and pathways that are modulated during HBV infection and proposes the possible use of AAT as a non-invasive immunological biomarker to follow the progression of liver disease.

Keywords: HBV, Immune biomarker, Alpha 1 antitrypsin, Immune pathways.

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Introduction

Hepatitis B virus (HBV) infection is a global health problem, with an estimated 257 million chronic carriers. Studies indicate that of these, India alone harbors a predicted 17 million chronic HBV patients (1). Infection with HBV may lead to progressive liver disease, such as cirrhosis and hepatocellular carcinoma (HCC); HCC alone is the fifth most common type of cancer in the world (1, 2). The molecular determinants that could assist the prognosis of HBV infection are still to be acknowledged despite endless efforts to understand the molecular means.

Studies have documented that HBV itself is not cytolytic, and hepatic injuries are chiefly immune-mediated (3). This emphasizes the pivotal role of the immune system in the infectivity and propagation of the virus in a host. Adequate research has been done on the

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innate and adaptive immune responses against HBV infection, whereby several molecular determinants have come to light (4-9). On one hand, the Toll-like receptors (and downstream cytokines) belonging to the innate arm has a vital function during HBV infection (4-7), while on the other hand, CD8+ and CD4+ T lymphocytes (and associated cytokines) of the adaptive arm have a profound effect (8, 9). In order to decipher the key elements of the immune system that participate during HBV infection, this study utilized a PCR array to screen those sets of immune-related genes that are differentially expressed in the presence/absence of the virus. The data obtained was subjected to bioinformatics analyses to trace out the related pathways that are modified during HBV infection.

Owing to the substantial role of the immune system on HBV-mediated liver disease, it can be debated that there must be some immune molecules that could mark the progression of liver disease during the course of HBV infection. In light of the paucity of information relating to such immune markers, the current pilot study aimed to identify the key immune elements and pathways that are differentially modulated in hepatocytes during HBV infection with the intention of determining whether any of them can be established as a potential immune marker.

Patients and Methods

Study subjects

A total of 30 HBV infected patients were recruited for the current study from SCB Medical College, Cuttack, Orissa, and patients visiting our lab for HBV DNA testing. In addition, 14 healthy controls (C) comprising voluntary blood donors who tested negative for HBV, HIV, and HCV were also included. The HBV-infected patients were further distributed into 2 groups, namely asymptomatic carriers (ASC) (n=15, subjects who were positive for hepatitis B surface

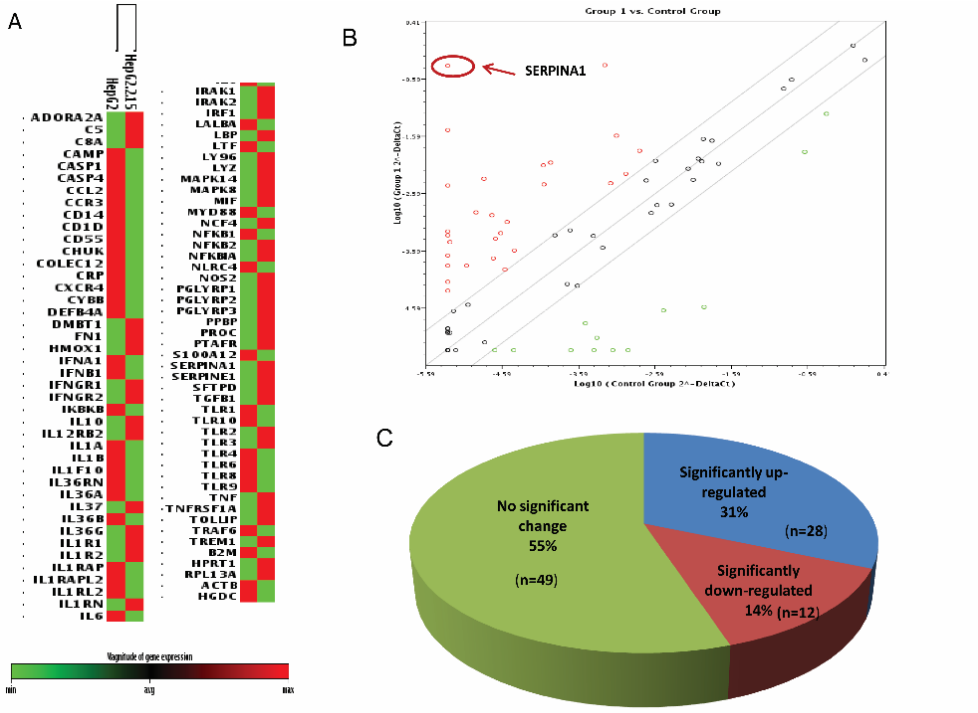

Figure 1. Innate and adaptive immune response gene expression in hepatoma cells, HepG2.2.15 (stable HBV replicating) and HepG2 (uninfected control); (A) Comparison of gene expression between HepG2 and HepG2.2.15 cells. Red color indicates genes expressed at least 2-fold higher in HepG2.2.15 or in HepG2 cells. Green color indicates genes expressed at least 2-fold lower in HepG2.2.15 or in HepG2 cells. (B) Scattered plot of 89 genes indicating $2^{-\Delta ct}$ numerical values in HepG2 cells (X axis) and HepG2.2.15 cells (Y axis). Red dots indicate genes expressed at least 2-fold higher in HepG2.2.15 than in HepG2. Green dots indicate genes expressed at least 2-fold lower in HepG2.2.15 than in HepG2 cells. Black dots indicate that the difference of gene expression between the two cells was within 2-fold. (C) A pie-chart representing the percentages and numbers of significantly up-regulated and significantly down-regulated genes as established by the PCR array. In the above case, a change of at least two-fold was used as the cut-off for both up- and down-regulation.

antigen (HBsAg) but had normal liver function tests, i.e. ALT≤45) and subjects with chronic liver disease (CLD) (n=15, subjects positive for HBsAg who had very high ALT levels with or without hepatomegaly).

Ethical code

Signed informed consent was obtained from all study subjects. The current study was part of a project entitled "Study of Toll-like Receptors and microRNAs Expression in Hepatitis B Virus Infection and Correlation with HBV Pathogenesis" and was approved by the Institute's Ethics Committee. None of the participating patients showed evidence of concomitant HCV, HDV, or HIV infection or metastatic or autoimmune liver disease.

Cell culture

The hepatoma cell lines HepG2 (uninfected) and HepG2.2.15 (stable HBV replicating), respectively, were maintained in DMEM and RPMI1640 medium with 10% fetal bovine serum (Sigma Aldrich, Munich, Germany) at 37 °C in a humidified atmosphere with 5% CO_2 (7). The supernatant was removed, and the cells were harvested 48 hours after seeding. These cells were then subjected to RNA isolation. HepG2.2.15 cells were very kindly gifted by Dr. T.K. Kanda, Ichikawa, Japan.

RNA isolation

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) from $5x10^5$ HepG2 and HepG2.2.15 cells. RNA quality and quantity was accessed by spectrophotometer and additional visualization in agarose gels.

PCR array

Gene expression profiling for innate and adaptive immune-related genes was performed using RT^2 Profiler assay (SAbiosciences, Frederick, USA) according to the manufacturer's instructions. In brief, 1

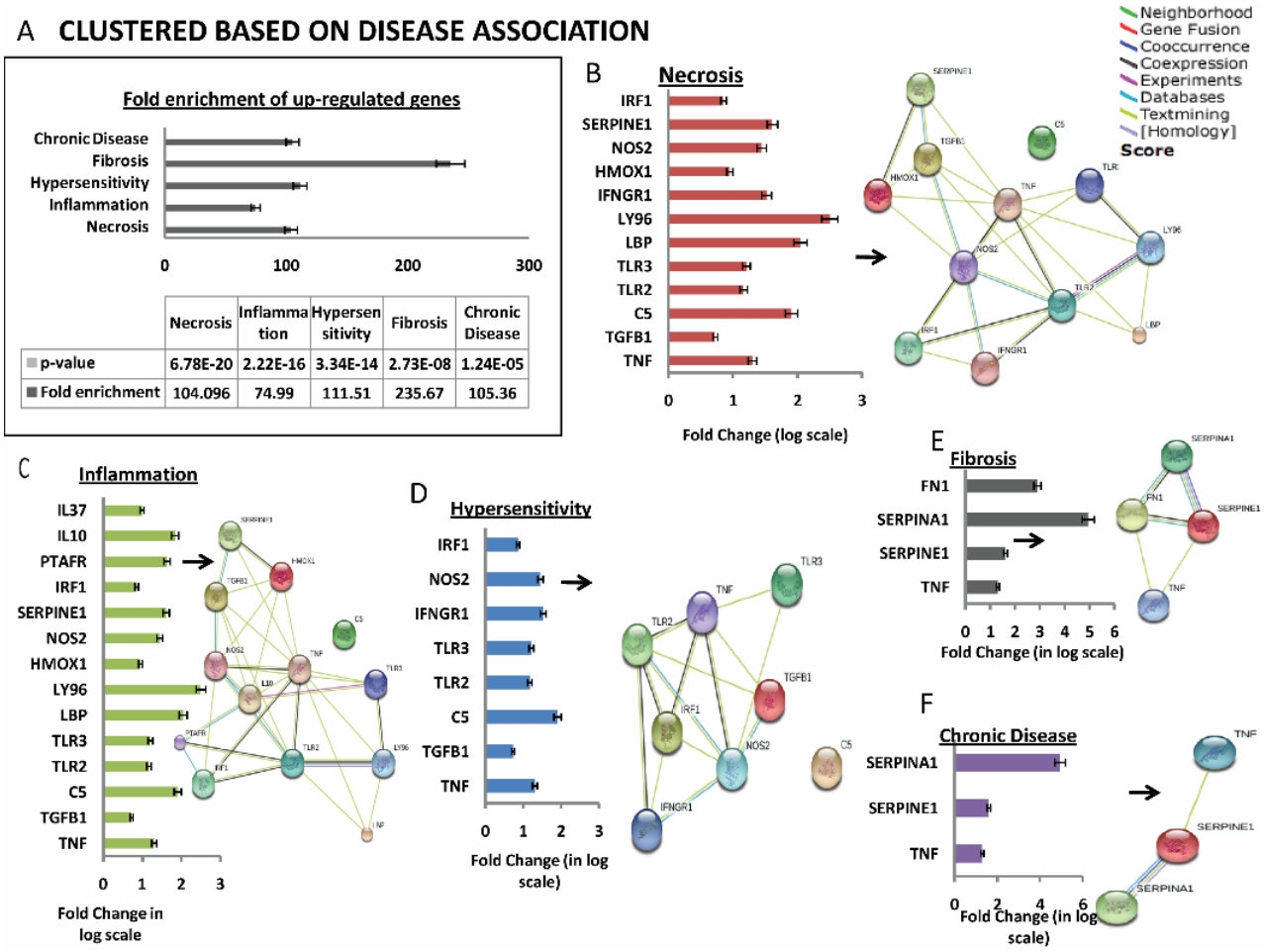

Figure 2. The enriched pathways when the up-regulated genes were clustered based on biological function. (A) Those genes responsible for "response to biotic stimuli" showed the highest enrichment ($p<0.05$). The interacting proteins in each of this cluster as evidenced by STRING viz. (B) Regulation of immune system. (C) Response to biotic stimulus. (D) Inflammatory response, respectively.

µg total RNA each from HepG2 and HepG2.2.15 cells were reverse transcribed with an RT^2 PCR Array First Strand Synthesis-Kit (SAbiosciences, Frederick, USA) followed by real-time-PCR. A change of at least two-fold was used as cut-off for both up- and down-regulation.

Pathway analysis

Various pathways were analyzed based on biological function, cell signaling network, and disease association using the web-based gene set Analysis Toolkit (GSEA) (Broad Institute, USA). The protein-protein interaction, if any, among the enriched pathways were retrieved by STRING, a database that predicts protein interactions.

ELISA

Alpha 1 Antitrypsin (SERPINA1) was quantified from the serum samples of patients by ELISA kit (Abcam) following the manufacturer's instructions.

Statistical analysis

Statistical analyses of the data generated by PCR array and patients' samples were executed using GraphPad Prism (GraphPad Software, USA, v 4.03). A t-test (unpaired, two-tailed) was used for comparison. Nonparametric statistical analysis was performed using the Mann–Whitney U test for unpaired observations. Correlations were determined using Spearman's test. Statistical analyses of the enriched pathways were given by the web-based gene set Analysis Toolkit (GSEA) (Broad Institute, USA). In all cases, a probability level of $p<0.05$ was set for statistical significance.

Results

Differential expression of innate and adaptive immune molecules in HepG2 Vs HepG2.2.15 cells

Hepatocytes are the primary site of HBV replication. The current study examined immune gene expression profiles in this system in the presence or absence of HBV using real-time PCR-based arrays. A comparison of innate and adaptive immune genes between HepG2.2.15 and HepG2 cells is shown in Figure 1A and 1B. Out of the 89 genes that were examined in the array, 28 (31%) were significantly up-

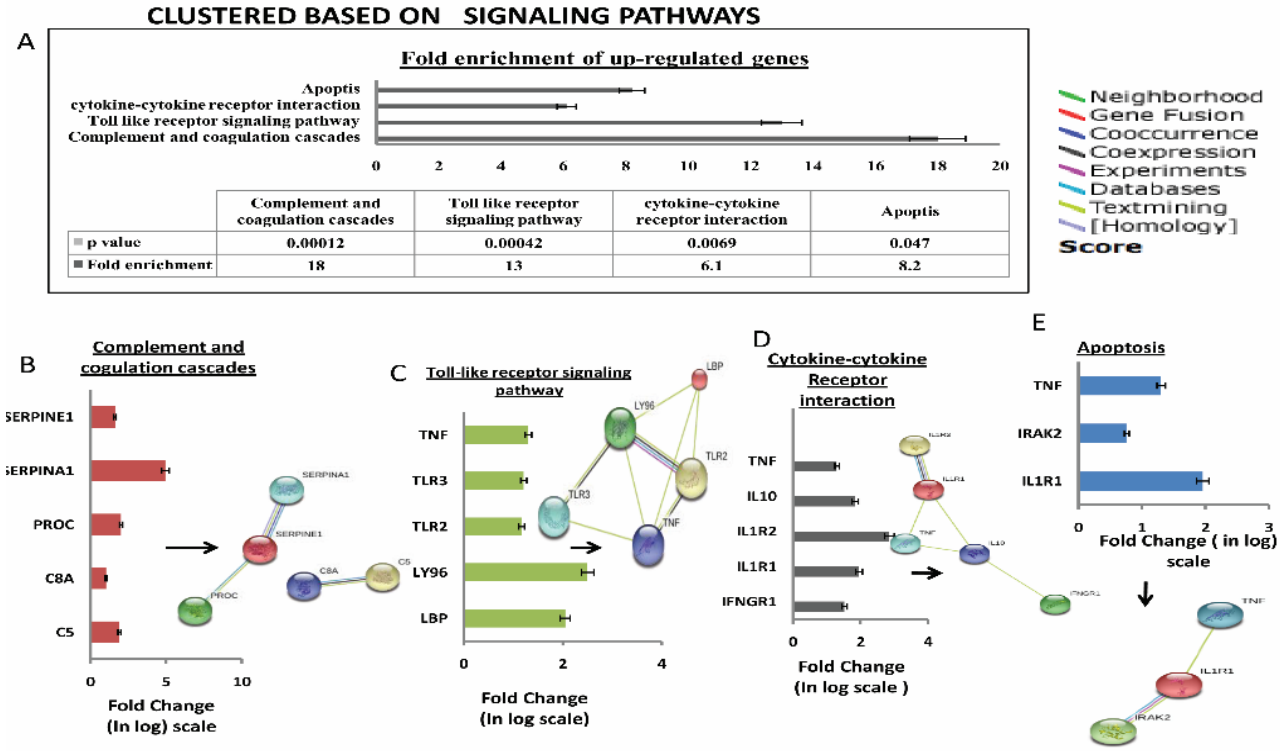

Figure 3. The enriched pathways when the up-regulated genes were clustered based on signaling pathways. (A) Those genes responsible for "complement and coagulation cascades" showed the highest enrichment ($p<0.05$). The interacting proteins in each of these clusters as evidenced by STRING viz. (B) Complement and coagulation cascades. (C) TLR signaling pathway. (D) Cytokine-cytokine receptor interaction. (E) Apoptosis, respectively.

regulated (>2-fold, p<0.05) in HepG2.2.15 cells when compared to HepG2 cells, while 12 genes (14%) were down-regulated (<2-fold, p<0.05). The remaining 49 genes (55%), however, showed no significant up- or down-regulation (Figure 1C). Among them, the genes that showed the highest levels of up-regulation included SERPINA1, FN1, IL1R2, LBP, LY96, LYZ, and PROC (ENTEREZ gene symbol), while genes that showed the highest levels of down-regulation included CASP1, CXCR4, CYBB, and NLRC4.

Pathway analysis of up-regulated genes

To interpret the pathways that were stimulated upon HBV infection, the genes that were up-regulated in HepG2.2.15 cells was subjected to gene ontology analysis, which was performed to identify the enriched genes. These were then clustered according to their biological processes, signaling pathways, and disease association by GSEA. Three enriched pathways were identified based on their biological functions (Figure 2A). This included inflammatory response (fold enrichment=18.35, p<0.05), response to biotic stimulus (fold enrichment=44.36, p<0.05), and regulation of immune response (fold enrichment=9.66, p<0.05). However, among them, response to biotic stimulus showed the highest enrichment. Those genes participating in this pathway were NOS2, TGFB1, TNF, TLR2, IRAK2, SERPINE1, IL10, LBP, and LY96. They also bore direct protein–protein interaction as per the protein interaction network depicted in Figure 2C. The genes that were associated in each of these clusters have been demonstrated in Figures 2B, 2C, and 2D. When the genes were clustered based on signaling pathways, four enriched pathways were identified (Figure 3A), i.e. complement and coagulation cascades (fold enrichment=18, p<0.05), Toll-like

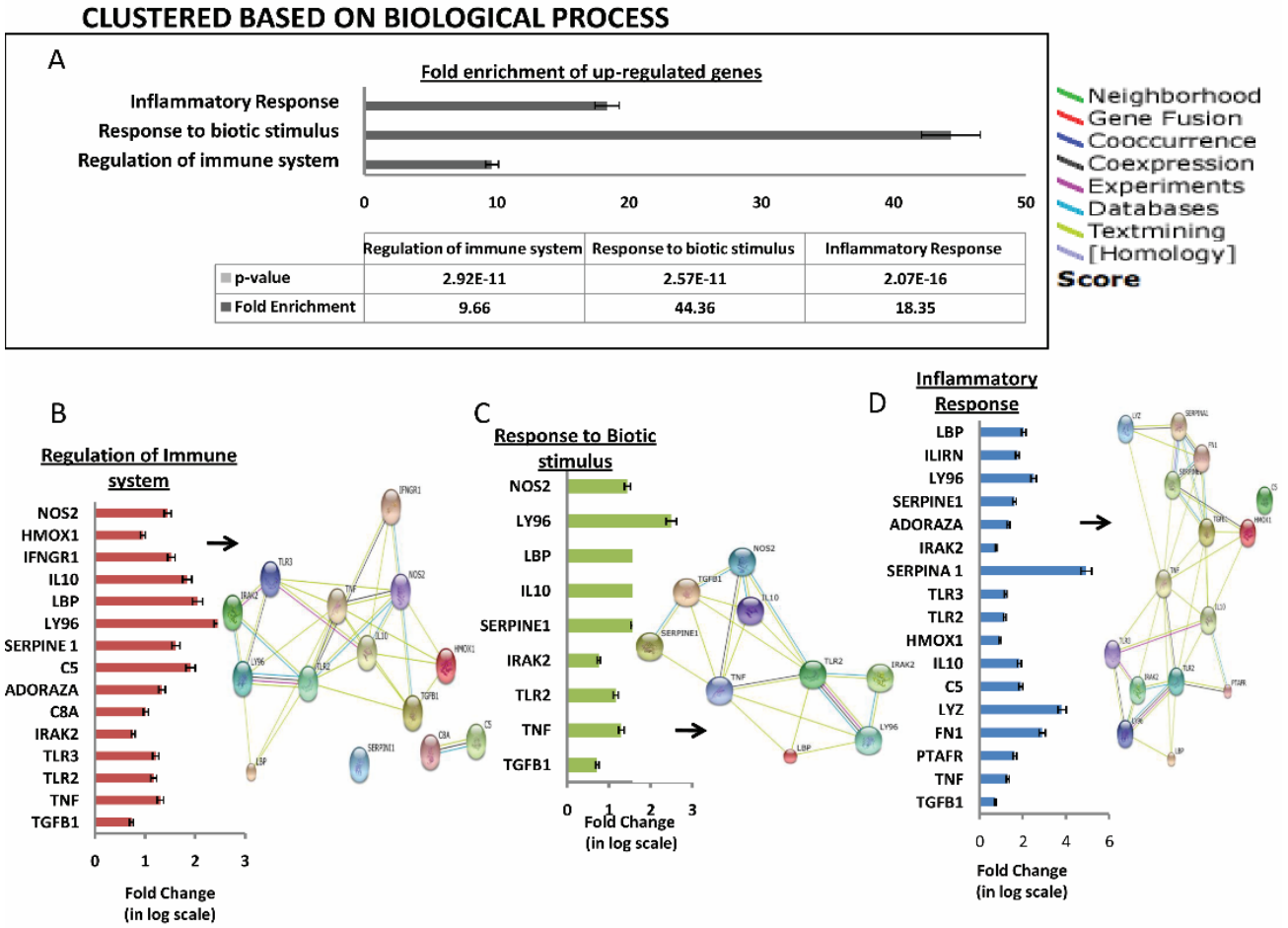

Figure 4. The enriched pathways when the up-regulated genes were clustered based on disease association. (A) Those genes responsible for "fibrosis" showed the highest enrichment (p<0.05). The interacting proteins in each of these clusters as evidenced by STRING viz. (B) Necrosis. (C) Inflammation. (D) Hypersensitivity. (E) Fibrosis. (F) Chronic disease, respectively.

receptor signaling pathway (fold enrichment=13, p<0.05), cytokine-cytokine receptor interaction (fold enrichment=6.1, p<0.05), and apoptosis (fold enrichment=8.2, p<0.05) (Figures 3B, 3C, 3D, and 3E). Among them, complement and coagulation cascades showed the highest enrichment. The key players in this pathway were the SERPINE1, SERPINA, PROC1, C8A, and C5 genes. Of them, only SERPINE1, SERPINA, and PROC1 bore direct protein-protein interaction as evidenced by STRING (Figure 3B). Based on disease association, five enriched categories were identified, namely necrosis (fold enrichment=104.096, p<0.05), inflammation (fold enrichment=74.99, p<0.05), hypersensitivity (fold enrichment=111.51, p<0.05), fibrosis (fold enrichment=235.67, p<0.05), and chronic disease (fold enrichment=105.36, p<0.05) (Figure 4). The highest enriched disease category was fibrosis. FN1, TNF, SERPINA1, and SERPINE1 clustered together in this disease category, and all these genes bore direct protein relationships as indicated by STRING (Figure 4E).

Assessment of SERPINA1 in patients

The results of PCR array showed that the SERPINA1 gene was most highly expressed in the HBV infected cell line model compared to the uninfected model, and pathway analysis further disclosed that SERPINA1 modulated the inflammation, fibrosis, and coagulation pathways during HBV infection (Figure 5A). In addition, early studies have shown that SERPINA1 is highly expressed in HBV-infected sera from severe chronic hepatitis and HBV-associated HCC patients (10). Therefore, this study

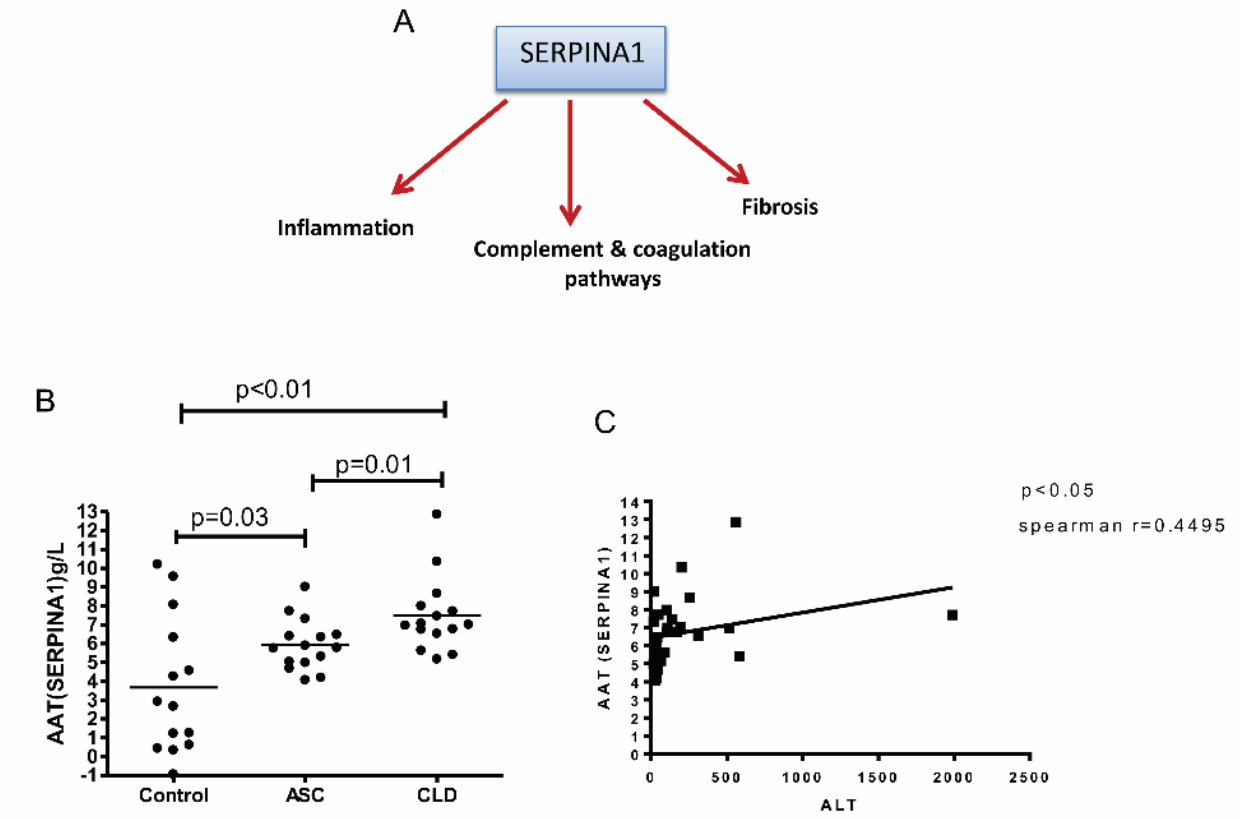

Figure 5. Serum levels of the protein alpha 1 antitrypsin (product of the gene SERPINA1) increases progressively with increased disease severity. (A) SERPINA 1, the most highly expressed gene in HBV infected cells compared to HBV uninfected cells participates in inflammatory, fibrosis, chronic disease, complement and coagulation pathways as evinced by prior pathway analyses (Figures 2D, 4E, F, 3B). (B) Alpha 1 antitrypsin (AAT) (protein product of SERPINA1 gene) levels were significantly high in asymptomatic patients compared to the controls, and AAT levels were significantly high in CLD patients compared to asymptomatic and control subjects, thus showing that AAT levels increase with disease severity during HBV infection. (C) AAT levels positively correlated (p<0.05) with the ALT levels among ASC and CLD patients. AAT levels were measured by ELISA from patients' sera, and statistical analyses were done by Graphpad PRISM 4.

attempted to determine whether Alpha 1 antitrypsin (AAT), the protein expressed from SERPINA1, could possibly serve as a biomarker of HBV-associated liver disease. To that end, AAT levels were measured with quantitative ELISA among healthy controls, HBV-infected asymptomatic carriers, and patients with HBV-associated chronic liver disease. The results revealed a significantly high expression of AAT in HBV-infected sera compared to the control. Moreover, it was determined that AAT levels increased significantly with the progression of liver disease; i.e. AAT levels were significantly high in ASC patients compared to controls, and again, there was a significantly high expression in CLD compared to ASC (Figure 5B). Furthermore, AAT levels positively correlated with the levels of ALT among ASC and CLD patients (Figure 5C).

Discussion

Despite the availability of numerous diagnostic as well as therapeutic options since the discovery of HBV, this disease remains unconquered. Several reports relating to its multiplication, propagation, growth, and elimination are still controversial. One most important aspect of the virus is its close association with the host immune response, compelling a school of thought to believe that the answer to most questions concerning the virus is the host immune system. In the current pilot study, therefore, we tried to analyze the major components of the immune system that can be linked to the viability or propagation of HBV. PCR array was conducted in stable HBV replicating HepG2.2.15 cells in comparison to its parental cells, HepG2, devoid of the virus, as it has been previously widely acknowledged (11, 12). The immune genes that showed the highest levels of mRNA expression in infected cells included SERPINA1, FN1, IL1R2, LBP, LY96, and PROC. Among them, SERPINA1, FN1, LY96, and PROC have been previously reported as being associated with HBV. Serine peptidase inhibitors (SERPINAs) are manufactured in the liver, and their levels are increased and secreted out of hepatocytes during HBV infection (10, 13). Fibronectin 1 (FN1), on the other hand, plays a vital role in the integration of the viral genome into the host genome during chronic infection (14, 15). The role of lymphocyte antigen 96 (LY96) during HBV infection is yet to be deciphered, though earlier was found to also be up-regulated in HepG2 cells transfected with the HBV core gene region (16). The role of protein C (PROC) has been investigated previously in children with chronic viral hepatitis, where abnormalities in blood coagulation are a major part of acute and chronic hepatitis; however, no significant correlation between PROC and hepatic histology activity or fibrosis was found (17).

The pathway analysis in this study was intended to identify the general immune-related pathways that are modulated upon HBV infection. The web-based gene set analysis toolkit was used for this purpose, as it has been extensively accredited in the past (18, 20). Analysis results revealed that some specific pathways were most enriched in each of the clustering criteria (biological function, signaling pathways, and disease association). The pathway that is responsible for response to biotic stimulus was most enriched under the biological function category. This included proteins nitric oxide synthase2 (NOS2), LY96 (lymphocyte antigen 96), lipopolysaccharide binding protein (LBP), interleukin 10 (IL10), serpin peptidase inhibitor, clade E (SERPINE1), interleukin-1 receptor-associated kinase 2 (IRAK2), Toll-like receptor 2 (TLR2), tumor necrosis factor (TNF), and transforming growth factor, beta 1(TGF-β). Among them, TLR2, LBP, and IRAK2 are responsible for pathogen recognition (21, 22, 23), whereas cytokines TNF and TGF-β perform downstream mechanisms to eliminate the virus (24, 25, 26). Both of these cytokines have been extensively studied in the context of HBV, whereby reports also indicate that even SNPs in the gene regions of these cytokines can affect viral persistence and disease propagation (27, 28). Nitric oxide (NO) production contributes to the pathological changes featured in some inflammatory diseases, and an early report has suggested that NOS expression participates in HBV clearance and the regulation of leukocyte infiltration in response to HBV antigens (29). The complement and coagulation cascades showed the highest enrichment under the category of signaling pathways. Proteins participating in this pathway are SERPINE1, SERPINA1, PROC, C5A, and C8. Assessment of protein interaction among them clearly disclosed two distinct networks, one being the coagulation cascade involving SERPINE1, SERPINA1, and PROC, and the other being the complement cascade involving C5A

and C8. Complement 5a (C5a) is a critical modulator of liver immunity. C5 and C5aR have been demonstrated to exhibit a significant role in liver fibrosis (30, 31). Studies have disclosed that plasma C5a concentration was significantly increased in patients with chronic hepatitis B, and its concentration was positively correlated with clinical parameters reflecting the severity of liver fibrosis, including type IV collagen and procollagen type III N-terminal peptide (30). Further studies have shown that increased C5a significantly activated hepatic stellate cells and up-regulated α-smooth muscle actin, hyaluronic acid and type IV collagen expression, leading to fibrosis (31). There is no direct evidence of the involvement of C8 with HBV; however, it should be worth analyzing its effect. Involvement of the complement cascades further emphasizes the imperative role of the innate immune system during HBV infection. When the genes were clustered based on their association with disease, those genes related to fibrosis were most enriched, including the SERPINA1, SERPINE1, FN1, and TNF genes. Studies in the past have proven through various experiments that the proteins generated from SERPINA1, SERPINE1, and FN1 are associated with increased disease progression (higher tendency towards chronicity), which might ultimately lead to liver fibrosis or cirrhosis (10-15). TNF, on the other hand, is increased in order to combat the virus. Sustained elevated levels of TNF, however, lead to a continuous inflammatory response which might be responsible for amplifying disease severity (32, 33).

The PCR array results in the current study suggest that SERPINA1 is the most highly expressed gene in the HBV-infected cell line model compared to the uninfected model, and pathway analysis further disclosed that SERPINA1 modulates the inflammation and coagulation pathways during HBV infection. It is also associated with fibrosis and chronic disease of the liver. SERPIN (SERine Proteinase INhibitor) is a super family of structurally-related proteins with notable structural homology. The SERPINA1 (serine proteinase inhibitor, clade A, member 1) gene encodes for the protein Alpha-1 antitrypsin (AAT), which is primarily synthesized in the hepatocytes (10, 13, 34). An early study established that the AAT protein synthesized in the liver is secreted out into the circulation. With the help of 2D gel electrophoresis, researchers have shown that the serum level of AAT is significantly high in patients with HBV-related HCC compared to normal HCC. Therefore, they hypothesized that elevated serum levels of AAT can be used as a biomarker (10). Studies have also revealed that α-1 Antitrypsin can be used as a potential biomarker in chronic heart failure (35). With the help of quantitative ELISA, we have established in the present study that serum AAT levels progressively increase with increasing disease severity. Its significant positive correlation with patients' ALT further proves this fact. Thus, we propose that AAT can be used as a biomarker to follow HBV-associated disease progression.

A limitation of this study is that the results need to be confirmed in higher study samples and a variety of different cell lines including hepatic stellate cells. Such studies in the future could open up novel diagnostic strategies to understand the immune status of an HBV-infected individual.

Conclusion

In conclusion, our study highlights the key immune elements and pathways that are modulated during HBV infection and proposes the possible use of AAT as a non-invasive biomarker to follow the progression of liver disease.

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Conflict of interests

The authors declare no conflict of interest.

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