Correlation between plasma levels of sPD-1 and sPD-L1 with hepatic inflammation in chronic HBV infected patients

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Summary

Objective: The immunosuppressive PD-1/PD-L1 (programmed cell death-1/ programmed cell death ligand-1) signaling pathway is considered to play an important role in the pathogenesis of HBV infection. We conducted a prospective cohort study to assess the relationship between circulating sPD-1 and sPD-L1 (soluble forms) levels in HBV infection and live disease outcome and progression. *Subject and method:* Plasma sPD-1 and sPD-L1 levels were quantified using commercial ELISA kits in a prospective cohort including asymptomatic carrier (ASY, n = 30), chronic hepatitis (CHB, n = 79), hepatocellular carcinoma (HCC, n = 47) and 73 healthy individuals as control group (HC). *Result:* The plasma sPD-1 and sPD-L1 levels were significantly higher in HBV infected patients or in each patient groups (ASY, CHB, HCC) compared to the controls ($p<0.0001$). Among HBV-infected patients, plasma sPD-1 and sPD-L1 levels were higher in the CHB followed by the HCC and ASY group. The plasma levels of sPD-1 and sPD-L1 were positively correlated with hepatic inflammation markers such as AST, ALT and GGT in HBV-infected patients. *Conclusion:* We could show that HBV infection can induce the expression of PD-1 and PD-L1 and significantly increased levels of plasma sPD-1 and sPD-L1 are correlated with the hepatic inflammation in chronic HBV-infected patients.

Keywords: HBV infection, chronic hepatitis B, hepatocellular carcinoma, sPD-1, sPD-L1

1. Background

Hepatitis B virus (HBV) infection is the most common chronic viral infection worldwide. The World Health Organization estimates that 296 million people are chronically infected with HBV in 2019 and 820.000 deaths mostly from cirrhosis and hepatocellular carcinoma [1]. HBV infection can cause a wide spectrum of clinical manifestations, including, asymptomatic carriers (ASY), acute hepatitis B virus (AHB), chronic hepatitis B (CHB), liver cirrhosis (LC), and hepatocellular carcinoma (HCC).

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During the course of chronic HBV infection, the mechanism of hepatocellular injury and liver disease progression is strongly dependent on the host immune responses and associated with persistently increased inflammatory cytokines released by T cells [2], [3] and depletion of HBV-specific $CD8^+$T cells, which plays a decisive role in pathogenesis of HBV infection [4]. Depletion of HBV-specific $CD8^+$T cells can occur through activation of suppressive immune signaling pathways, of which the PD-1/PD-L1 axis is the major signaling pathway [5].

PD-1, encoded by the *PDCD1* gene, is a type I transmembrane immunoinhibitory receptor for PD-L1 ligand. PD-1 is widely expressed on the surface of T cells, B cells, natural killer cells, natural T killer cells, activated monocytes and cancer cells [6]. On the other hand, PD-L1 is expressed in epithelial cells, especially in tumor cells. In vivo, both PD-1 and PD-

L1 exist in a membrane-bound and soluble form (sPD-1 and sPD-L1) [7]. Previous studies have shown that increased sPD-1 levels are associated with inflammation and higher inflammation may lead to higher plasma sPD-1 levels in HBV infections [8 - 10], chronic HCV [11] and in acute or chronic inflammatory conditions such as pancreatitis [12], sepsis [13], autoimmune hepatitis and inflammatory bowel disease [14].

In addition, PD-L1 mRNA, PD-L1 protein (mPD-L1 and sPD-L1) are upregulated by multiple inflammatory cytokines, such as IL-6, IL-10, IL-12, IL-17, IFN-γ, and TNF-α [15, 16]. The positive correlation between sPD-L1 and inflammatory cytokines has been also reported in HBV and HCV infected patients [17, 18]. In this line, we hypothesized that sPD-1 and sPD-L1 may involve in the immune-mediated liver damage in response to HBV infection. Therefore, we conducted this cross-sectional study to assess the association of circulating sPD-1 and sPD-L1 levels with live disease outcome and progression in chronic HBV-infected patients.

2. Subject and method

We conducted a cross-sectional study at the 108 Institute of Clinical Medical and Pharmaceutical Sciences, Hanoi, Vietnam.

Study subjects and blood sampling: Between March 2019 and January 2021, we collected chronic HBV infected patients, including asymptomatic carrier (ASY, n = 30), chronic hepatitis (CHB, n = 79), hepatocellular carcinoma (HCC, n = 47) and 73 healthy individuals as control group. Chronic ASY patients were defined as the presence of HBsAg (> 6 months) with anti-HBc IgG positive titters and having no episode of increased liver enzyme levels until sampling. Chronic HBV infection is diagnosed by the presence of HBsAg (> 6 months) with anti-HBc IgG positive titters with clinical symptoms of hepatitis (intermittently or persistently elevated liver enzymes (ALT, AST) due to chronic HBV infection. Patients with HCC were diagnosed based on either liver biopsy or imaging features of liver cirrhosis on ultrasound or/and computed tomography scan (CT scan) or/and magnetic resonance imaging (MRI) in combination with serological tests, according to the AASLD guideline for HCC. All participants were confirmed negative for anti-HCV and anti-HIV by ELISA assays.

Plasma sPD-L1 measurement: Plasma sPD-1 and sPD-L1 levels were quantified using enzyme-linked immunosorbent assay (human PD-1 ELISA kit-ab252360 and human PD-L1 ELISA kit-ab214565, Abcam, Germany) according to the manufacturer's instructions. The kit quantifies sPD-1 and sPD-L1 with 9.6 and 2.91pg/ml sensitivity respectively. The intra-and inter-assay variations for sPD-1 were 2.4% and 9.6% and for sPD-L1 were 5.4% and 4.1%, respectively.

Statistical analysis: All statistical analyses were performed using R version 4.1.1 (http://www.r-project.org). Chi-square, Kruskal-Wallis and Mann-Whitney-Wilcoxon tests were used to compare differences between groups for qualitative or quantitative variables where appropriate. Correlations between sPD-L1 and other laboratory parameters were assessed using the Spearman's rank correlation test. The level of significance was set at a two-sided p-value of <0.05.

3. Result

Demographic and clinical characteristics of studied groups

Table 1. Demographic and clinical characteristics of studied groups

Characteristics	HC (n = 73)	ASY (n = 30)	CHB (n = 79)	HCC (n = 47)	p values
Gender, male (%)	50%	89%	77%	91.5%	1.6e - 09[a,#]
Age	43 (19 - 69)	25 (19 - 44)	44 (20 - 74)	60 (34 - 85)	< 2.2e - 16[a,$]
WBC (x10^3/L)	5.1 (4.1 - 10.2)	4.9 (3.1 - 8.7)	6.5 (3.6 - 17.4)	6.0 (2.6 - 15.9)	NS[b,$]

RBC (x10^6/L)	4.7 (4.1 - 6.1)	4.2 (3.8 - 7.1)	4.9 (3.0 - 6.9)	4.6 (2.6 - 6.5)	0.01 [b,$]

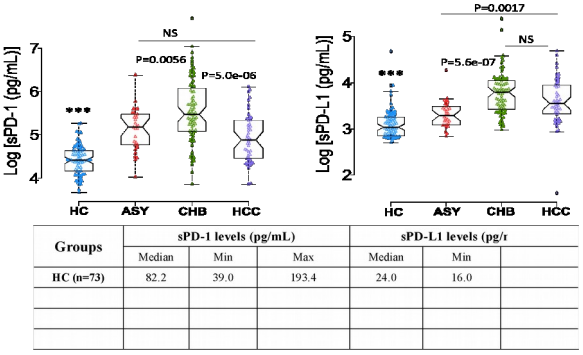
Table 1. Demographic and clinical characteristics of studied groups (Next)

Characteristics	HC (n = 73)	ASY (n = 30)	CHB (n = 79)	HCC (n = 47)	p values
PLT (x10^3/L)	258 (133 - 422)	203 (133 - 422)	206 (85 - 430)	149 (39 - 439)	0.0068 [b,$]
AST (U/L)	20 (9 - 36)	19 (7 - 38)	267 (4 - 3935)	72 (21 - 43868)	1.6e - 10 [b,$]
ALT (U/L)	17 (6 - 43)	16 (8 - 43)	425 (8 - 4926)	49 (11 - 916)	3.4e - 15 [b,$]
T-bilirubin µmol/l)	11.2 (5.1 - 22.4)	12.5 (5.1 - 18.4)	91 (7.3 - 492.8)	19 (5.2 - 1504)	0.0029 [b,$]
D-bilirubin (µmol/l)	6.7 (1.8 - 17)	5.9 (1.7 - 10.5)	37 (1.5 - 338)	5.6 (0.7 - 236.2)	0.0002 [b,$]
Albumin (g/L)	43 (41 - 46)	40 (37 - 46)	37 (20.3 - 45.6)	36 (25.3 - 49)	NS [b,$]
Prothrombin (%)	NA	NA	82 (14 - 120)	86 (46 - 117)	NS [b,$]
AFP (IU/L)	NR	2.6 (1.6 - 9.3)	7.0 (1.2 - 551)	89 (1.7 - 5248)	0.013 [b,$]
HBV DNA	NA	1.4e7 (1.0e2 - 2.4e9)	3.1e6 (1.0e2 - 9.8e8)	3.1e5 (6.4e3 - 5e8)	NS [b,$]

Abbreviations: ASY: Asymptomatic carrier; CHB: Chronic hepatitis B; HCC: Hepatocellular carcinoma; HC: Healthy control; PLT: Platelets. AST and ALT: Aspartate and alanine amino transferase; WBC, white blood cell; RBC, red blood cell; NA: Not applicable. Values given are medians and range, percentage. P-values were calculated by Chi-squared[#] test and Kruskal-Wallis test[$]; [a]comparisons between all groups and [b]comparisons between CHB and HCC.

Comments: Most of the HBV patients (84%) were male and the HCs were younger than CHB and HCC patients. The ALT, AST, bilirubin, and platelets were significantly higher in CHB patients than HCC group (p<0.01). Albumin and prothrombin levels were not significant difference between CHB and HCC groups (p>0.05). HCC patients had higher AFP levels than CHB patients (p=0.013).

sPD-1 and sPD-L1 levels in patient groups and healthy controls



Groups	sPD-1 levels (pg/mL)			sPD-L1 levels (pg/r	
	Median	Min	Max	Median	Min
HC (n=73)	82.2	39.0	193.4	24.0	16.0

Figure 1. Distribution of plasma sPD-1 and sPD-L1 levels in patient groups and healthy controls

Comments: The sPD-1 and sPD-L1 levels were significantly higher in HBV infected patients or in each patient groups (ASY, CHB, HCC) compared to the controls (p<0.0001). Among HBV-infected patients, plasma sPD-1 levels were higher in the CHB followed by the ASY and HCC group (CHB vs. HCC, p=5.0e-6; CHB vs. ASY, p=0.0056, HCC vs. ASY, p>0.05). Plasma sPD-L1 levels were also higher in the CHB patients followed by the HCC and ASY group (CHB vs. HCC, p>0.05; CHB vs. ASY, p=5.6e-7; HCC vs. ASY, p=0.0017).

Correlation between sPD-1 and sPD-L1 in HBV patients

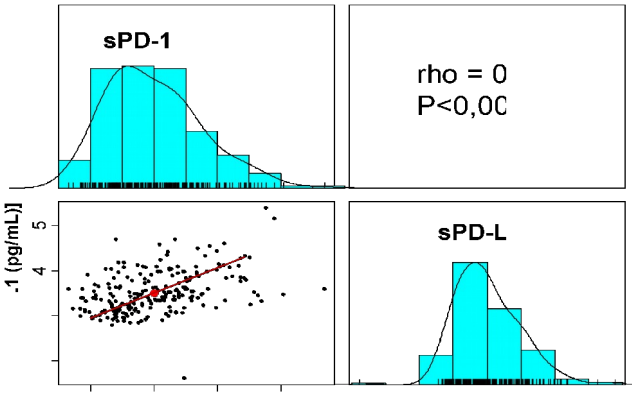


Figure 2. Correlation between plasma levles of sPD-1 and sPD-L1 in all HBV patients.

Comments: There was a positive correlation between the plasma levels of sPD-1 and sPD-L1 in HBV-infected patients with rho=0.51 (p<0.0001).

Correlation between plasma sPD-1 / sPD-L1 levels and liver inflammatory damage markers

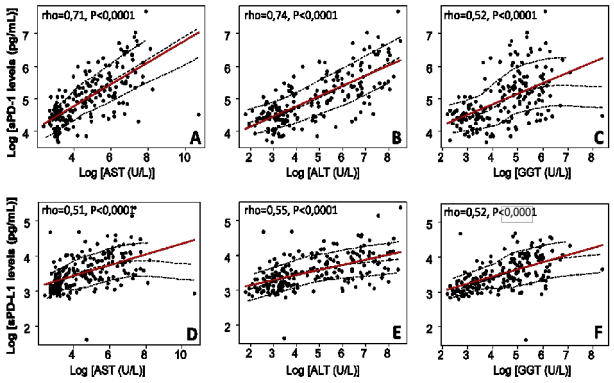


Figure 3. Correlation between plasma levles of sPD-1 / sPD-L1 and liver enzymes in all HBV patients.

Comments: There were positive correlations between the plasma levels of sPD-1 (upper panel) and sPD-L1 (lower panel) and levels of hepatocellular damage status indicated by AST, ALT and GGT (hepatocyte injury indicator) in HBV-infected patients. Briefly, the plasma sPD-1 levels positively correlated with AST (rho=0.71, p<0.0001), ALT (rho=0.74, p<0.0001) and GGT (rho=0.52, p<0.0001). The plasma sPD-L1 levels positively correlated with AST (rho=0.51, p<0.0001), ALT (rho=0.55, p<0.0001) and GGT (rho=0.52, p<0.0001). The correlation between plasma sPD-1 & sPD-L1 levels and liver enzymes in patients revealed a significant association of sPD-1 and sPD-L1 levels with liver disease severity.

Correlation between sPD-1 and sPD-L1 and other laboratory parameters

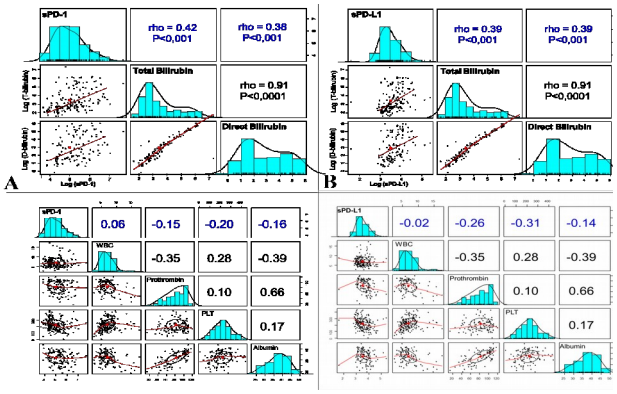


Figure 4. Correlation between plasma levles of sPD-1 and sPD-L1 and hematological and liver function parameters in all HBV patients

Comments: Significant correlations between plasma levels of sPD-1 & sPD-L1 and total-bilirubin and direct-bilirubin was observed (A: sPD-1, B: sPD-L1). Conversely, there was weak correlations or no correlations between plasma sPD-1 & sPD-L1 levels and other hematological and liver function parameters such as WBC, PLT, albumin, platelet counts, and prothrombin (|rho|<0.3; C: sPD-1, D: sPD-L1).

4. Discussion

The pathogenesis of liver disease outcomes and progression of chronic HBV infection is a multifactorial event. Of which, depletion of HBV-specific $CD8^+$T cells plays a decisive role [4]. Depletion of HBV-specific $CD8^+$T cells are mainly occurred through activation of suppressive immune signaling pathway PD-1/PD-L1. Hypothetically, sPD-1 and sPD-L1 may have a significant involvement in the immune-mediated liver damage in response to HBV infection. Thus, we conducted this study to assess the relationship between circulating sPD-1 and sPD-L1 levels in HBV infection and live disease outcome and progression. We have shown that HBV infection can induce the expression of sPD-1 and sPD-L1. In addition, plasma levels of sPD-1 and sPD-L1 were positively correlated with the liver injury condition.

The mechanism of hepatocellular injury and liver disease progression, during the course of HBV infection, is strongly dependent on the host immune responses and associated with persistently increased inflammatory cytokines released by T cells [2]. Previous studies have shown that upregulation

of the PD-L1 has been observed on Kupffer cells, liver sinusoidal endothelial cells, stellate cells, and infected hepatocytes and T cells expressing PD-1 are infiltrated in the liver microenvironment during chronic inflammation. Both PD-1 and PD-L1 have the soluble form (sPD-1 and sPD-L1) that can be detected in peripheral blood [19] indicating that these circulating proteins can be cleaved from the membrane-bound forms or by translation of alternative spliced mRNA.

Studies have found that sPD-1 and sPD-L1 can be upregulated in various human diseases, including cancers, autoimmune and infectious diseases. As found in our study, the circulating sPD-1 and sPD-L1 levels of HBV patients were significantly higher than those of the healthy control group. The results from our study were consistent with previous studies in HBV infections [8-10. Moreover, sPD-1 also increase in patients with chronic HCV infection [11], pancreatitis [12], sepsis [13], autoimmune hepatitis and inflammatory bowel disease [14]. These studies suggest that increased sPD-1 levels are associated with inflammation and higher inflammation tends to lead to higher plasma sPD-1 levels. Additional data from our current study was the positive correlation between plasma sPD-1 levels and indicators of liver damage such as AST and ALT. Furthermore, previous studies have shown that sPD-1 levels are elevated in HBV patients in a manner that corresponds with inflammatory factors such as AST, ALT, IL-10, IL-17, TNF-α and IFN-γ [8, 9, 17]

Regarding the expression of PD-L1, previous studies have shown that increased mPD-L1 expression in cancerous tumors correlates with worse overall survival in patients with solid tumors and the correlations differed according to tumor types suggesting that mPD-L1 plays as a marker for tumor prognosis. In addition to mPD-L1, soluble form of PD-L1 might be originated from the cleavage of mPD-L1 because of finding that sPD-L1 can be detectable in supernatants from cancer cell lines positive for mPD-L1 [20]. Upregulation of mPD-L1 in cancers in the context of persistent inflammation with the presence of the virus [21], corroborating our results where sPD-L1 levels were significantly elevated in HBV-infected patients than in healthy controls. Moreover, PD-L1 mRNA, PD-L1 protein (mPD-L1 and sPD-L1) are upregulated by multiple inflammatory cytokines, such as IL-6, IL-10, IL-12, IL-17, IFN-γ, and TNF-α [15, 16]. The positive correlation between sPD-L1 and inflammatory cytokines and negative effects on clinical outcomes of HCV [22] and HBV infection [23] have been reported. Our study is in accordance with previous study showing that sPD-L1 levels were positively correlated with ALT levels [23]. Juan Xia et al showed that the decreased sPD-L1 levels are associated with the improvement of liver inflammation in chronic HBV-infected patients who were under antiviral treatment. Another study has found the significantly positive correlations between the serum sPD-L1 and AST, ALT in chronic hepatitis C [23].

5. Conclusion

This study has shown that HBV infection can induce the expression of PD-1 and PD-L1 and significantly increased levels of plasma sPD-1 and sPD-L1 are possibly associated with the active or re-active phase of chronic HBV infected patients as indicated by elevated levels of hepatic inflammation.

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