Plasma levels of sPD-1 and PD-1 genetic variants are associated with hepatitis B infection and related liver disease progression

Pham Thi Minh Huyen  
108 Institute of Clinical Medical and Pharmaceutical Sciences

Dang Thi Ngoc Dung  
Hanoi Medical University

Peter Johann Weiß  
University of Tübingen

Dao Phuong Giang  
Vietnamese-German Center for Medical Research (VG-CARE)

Ngo Thi Uyen  
Hanoi Medical University

Ngo Tat Trung  
Vietnamese-German Center for Medical Research (VG-CARE)

Thirumalaisamy P Velavan  
University of Tübingen

Le Huu Song  
Vietnamese-German Center for Medical Research (VG-CARE)

Ngheim Xuan Hoan (ngheimxuanhoan@108-icid.com)  
108 Institute of Clinical Medical and Pharmaceutical Sciences

Research Article

Keywords: hepatitis B virus, chronic hepatitis B, liver cirrhosis, hepatocellular carcinoma, PD-1, sPD-1, PD-1.5, PD-1.9 polymorphism

DOI: https://doi.org/10.21203/rs.3.rs-853250/v1

License: © This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Background

Programmed cell death-1 (PD-1) variants and circulating levels of soluble PD-1 are associated with susceptibility to malignant and infectious disease. This study aimed to examine the association of PD-1.5 and PD-1.9 variants, and plasma sPD-1 levels with HBV infection and disease progression.

Methods

The study cohort consists of HBV-infected adults (n = 513) stratified by clinical course, including chronic hepatitis B (CHB, n = 173), liver cirrhosis (LC, n = 134), hepatocellular carcinoma (HCC, n = 206), and matched healthy controls (HC, n = 196). The PD-1.5 (rs2227981 C/T) and PD-1.9 (rs2227982 C/T) genetic variants were genotyped by Sanger sequencing, and then sPD-1 levels were quantified by enzyme immunoassay.

Results

The plasma sPD-1 levels were significantly high among HBV patients. The highest plasma sPD-1 levels were observed in CHB patients, followed by the LC and HCC groups. In addition, the plasma sPD-1 levels correlated positively with liver inflammation (aspartate transaminase, AST: rho = 0.57, P < 0.0001 and alanine aminotransferase, ALT: rho = 0.57, P < 0.0001) and were positively correlated with liver fibrosis (AST to Platelet Ratio Index, APRI score: rho = 0.53, P < 0.0001). The PD-1.9 TT genotype was less frequent in CHB patients compared to LC, HCC and HCC + LC patients in both codominant and recessive models (P < 0.01) and was found to be a risk factor for HCC predisposition [HCC vs. non-HCC: OR = 2.0 (95% CI: 1.13–3.7), P = 0.017]. The PD-1.5 CT genotype was associated with a reduced risk of acquiring HCC [OR = 0.6 (95%CI: 0.4–0.9), P = 0.031].

Conclusion

Our study concludes that sPD-1 levels are associated with liver inflammation and progression of liver fibrosis and the PD-1.5 and PD-1.9 variants are associated with HBV infection and progression of liver disease.

Introduction

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. HBV infection can cause a wide spectrum of liver diseases, including acute hepatitis B virus (AHB), chronic hepatitis B (CHB), liver cirrhosis (LC), and hepatocellular carcinoma (HCC). Notably, HBV is accountable for ∼53% of global HCC incidence.

The human cellular immune response (CD4+ and CD8+ T-cells) plays a vital role in HBV pathogenesis, in particular HBV-specific CD8+ T-cells. During chronic phase, HBV-specific CD8+ T cells responses are transient and relatively weak, probably interrelated to the immunosuppressive effects of the PD-1/PD-L1 (programmed cell death-1/programmed cell death ligand-1) signaling pathway. Several compelling evidences have shown that the activation of PD-1/PD-L1 pathway is crucial in modulation of T-cell dysfunction.

PD-1, encoded by the PDCD1 gene, is a type-I transmembrane immunoinhibitory receptor for PD-L1/2 ligands. 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. Recent findings have shown that, in addition to the membrane-bound form, the PD-1 protein also has a soluble form (sPD-1) that regulates immunological responses. Studies on the biological functions of sPD-1 in animal models showed that sPD-1 inhibited the growth of H22 hepatoma cells, enhanced the lysis of tumor cells and ultimately prolonged the overall survival of mice with tumors. Previous studies also found that sPD-1 levels are associated with the prognosis of cancer, including HCC. In addition, PD-1 overexpression is associated with T-cell dysfunction and exhaustion in chronic HBV infection and HCC development. Another study investigated the effects of sPD-1 levels on the long-term dynamics of HBV load and HCC risk showed that elevated sPD-1 levels correlated with a high viral load for greater than four and high sPD-1 levels were associated with an increased risk of HCC.

Genetic variations of the PDCD1 gene in the promoter and transcription binding sites that can alter gene function or expression have been associated with cancers, autoimmune diseases and infectious diseases. PD-1.5 (rs2227981) and PD-1.9 (rs2227982) were selected as candidates because they are located close to exon 5 of PDCD1, which encodes a cytoplasmic domain consisting of two tyrosine motifs associated with inhibitory activities. In addition, PD-1.9 is a non-synonymous substitution (valine to alanine) that modulates the structural and functional properties of PD-1.

There is very little data on the contribution of PD-1.5 and PD-1.9 polymorphisms with HBV infection, and there is only a case-control study that investigated the association between these two PD-1 polymorphisms and the progression of HBV-related liver disease, but not in particular on the circulating sPD-1 levels. In this line of research, we conducted a case-control study aiming to examine the association of PD-1.5 and PD-1.9 variants, but also plasma sPD-1 levels with HBV infection and disease progression.
Materials And Methods

Methods used in this study were in accordance with the relevant guidelines and regulations and were approved by the institutional review board and an independent Ethics Committee of the Institute of Clinical Medical and Pharmaceutical Sciences, Hanoi, Vietnam.

Ethics statement

This study was conducted in accordance with the Declaration of Helsinki. The study was approved by the institutional review board of the 108 Institute of Clinical Medical and Pharmaceutical Sciences, Hanoi, Vietnam. Informed written consent was obtained from all participants after explanation of the study at the time of sampling.

Study participants

This case-control study recruited 513 adult HBV-infected patients, who were referred for clinical management at the 108 Institute of Clinical Medical and Pharmaceutical Sciences, Hanoi, Vietnam, between March 2019 and December 2020. Chronic HBV infection is diagnosed by the presence of HBsAg (> 6 months) and being anti-HBc IgG positive. Other laboratory assessments, including hematological, biochemical, molecular, histological tests and imaging modalities were performed to establish the definitive diagnosis. HBV-infected patients were classified into three groups, including chronic hepatitis B (CHB; n = 173), liver cirrhosis (LC; n = 134), hepatocellular carcinoma (HCC; n = 206) based on clinical and subclinical manifestations. Briefly, individuals with clinical symptoms of hepatitis (intermittently or persistently elevated liver enzymes (ALT, AST)) were defined as patients with chronic hepatitis B (CHB). Patients with liver cirrhosis (LC) were diagnosed on the basis of the clinical features of liver cirrhosis by ultrasound or/and CT imaging or/and magnetic resonance imaging in combination with abnormal liver function tests, portal hypertension with esophageal varices, splenomegaly and ascites. The HCC cases were diagnosed according to the AASLD guidelines11. In case, if imaging modalities could not establish a definitive diagnosis, liver tumor biopsy was recommended to establish the exact diagnosis of tumor cells. Patients with HCC were further categorized as HCC stage-A, stage-B, stage-C and stage-D according to the Barcelona-Clinic Liver Cancer (BCLC) classification12. Patients with secondary HCC and HCC patients with metastases were excluded from this study.

None of HBV-infected patients had evidence of chronic comorbidities such as: autoimmune diseases, alcoholic liver disease, type 2 diabetes, addiction to smoking and alcohol or treatment with immune inhibitors. In addition, 196 individuals visited 108 hospital for routine medical check-up showed the normality of hematological and biochemical testing were collected as heathy control (HC) group. All patients and HCs were confirmed negative for anti-HCV and anti-HIV by ELISA assays. We collected 5 ml of peripheral blood from each participant. Plasma was immediately separated then frozen at -80°C until use.

Methods

PD1 genotyping

Genomic DNA was isolated from 200 µl of peripheral blood using a DNA isolation kit (Qiagen, Hilden, Germany), following manufacturer's instructions. The procedure of PD-1 genotyping was followed the study protocol as described 30. Briefly, the amplicon containing the variants PD-1.5 and PD-1.9 was amplified by PCR using the specific primer pairs PD-1.5/9_F: 5'-GCA AGA ATG CCA GGG ACA TTT CAG AG-3' and PD-1.5/9_R: 5'-TGC CTG GTG CAG GTG CAG-3'. PCR products were purified using the Exo-SAP-IT PCR product cleanup reagent (Afymetrix Santa Clara, USA) 5 µl of purified PCR products were used as sequencing templates. Direct sanger sequencing was performed using the BigDye terminator v.1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3130XL DNA sequencer according to the manufacturer's instructions.

Plasma sPD-1 measurement

Plasma sPD-1 levels were quantified in 191 patients (CHB = 96; LC = 42; HCC = 53) and in matched healthy controls (n = 73), using human PD-1 Simple Step ELISA Kit (ab214565, Abcam, Germany) according to the manufacturer's instructions. The limit of detection for sPD-1 is 9.6 pg/ml. All samples were measured in duplicates.

Statistical analysis

All statistical analyses were performed using R version 3.1.2 (http://www.r-project.org). Genotype and allele frequencies were determined by simple gene counting. Deviations from Hardy-Weinberg equilibrium were calculated for each group. Chi-square, Kruskal-Wallis and Mann-Whitney-Wilcoxon tests were used to compare differences between groups for qualitative or quantitative variables where appropriate. For genetic analyses, we used multivariate logistic regression models adjusted for age and gender to test for associations between variants and HBV-related liver diseases, applying different genetic models (codominant, dominant, recessive models) in which, adjusted odds ratios (OR) with 95% confidence intervals (CI) were calculated. Correlations between sPD-1 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.

Results

Baseline characteristics

Table 1 describes the baseline profiles of patient subgroups and 196 healthy controls. Most HBV patients were male (85%) compared to 49% in healthy group. Among the patient subgroups, the AST, ALT, total bilirubin and direct bilirubin levels were higher in CHB patients than in LC and HCC groups (P <
Albumin, prothrombin, and platelet counts were significantly lower in patients with LC compared to CHB and HCC group (P < 0.0001). AFP levels were significantly higher among the HCC compared to LC and CHB group (P < 0.0001).

Table 1: Clinical profiles of clinical subgroups of 513 HBV-infected patients

| Characteristics          | HBV patients (n = 513) | CHB (n = 173) | LC (n = 134) | HCC (n = 206) | HC (n = 196) | p$ | p$^a |
|--------------------------|------------------------|---------------|--------------|---------------|--------------|----|-----|
| Age (years)              | 54.1 ± 13.4            | 45.5 [19–77]  | 57 [21–81]   | 60 [32–90]    | 44.6 ± 8.6   | 2.5e-6$ | < 2.2e-16$ |
| Male (%)                 | 84.4                   | 81            | 77.8         | 91.8          | 49           | 0.0004$ | < 2.2e-16$ |
| HBsAg                    | Positive               | Positive      | Positive     | Positive      | Negative     | NA | NA  |
| Anti-HCV                 | Negative               | Negative      | Negative     | Negative      | Negative     | NA | NA  |
| Anti-HIV                 | Negative               | Negative      | Negative     | Negative      | Negative     | NA | NA  |
| AST (U/L)                | 90 [3-3935]            | 274 [4-3935]  | 71 [7-2938]  | 69 [3-1497]   | 21 [11–40]   | 5.8e-15$ | < 2.2e-16$ |
| ALT (U/L)                | 66 [11-4926]           | 425.5 [11-4926] | 51.5 [12-2456] | 50 [11-1119] | 18 [4–55] | < 2.2e-16$ | < 2.2e-16$ |
| Total bilirubin (µmol/l) | 21.6 [4.5–1504]        | 24.7 [5.3–802] | 26.6 [5.5-735.5] | 17.4 [4.5–1504] | 11.2 [1.5–20] | 1.1e-05$ | < 2.2e-16$ |
| Direct bilirubin (µmol/l)| 8.3 [0.5-542.7]        | 21.7 [0.7-410.3] | 13.27 [0.5-542.7] | 5.9 [0.7-236.2] | 2.2 [1.8-8] | 6.9e-09$ | 0.007$ |
| Albumin (g/L)            | 36.2 [17–49]           | 37.6 [20.3–48] | 31.5 [17-48.7] | 38.3 [22.3–49] | 43 [35.6–47.8] | 1.6e-07$ | 7.0e-15 |
| Prothrombin (% of standard) | 78.5 [14–126]      | 82 [14–126]  | 59 [18–116]  | 89 [34–123]   | ND           | 7.3e-16 $ | NA              |
| PLT (x10$^3$ cells/L)    | 184.2 [20–598]         | 195.5 [68–430] | 114.5 [20–464] | 188.5 [39–598] | 266.5 (156–320) | < 2.2e-16$ | < 2.2e-16$ |
| HBV-DNA (copies/ml)      | 7.1x10$^5$ [7-2.2x10$^{10}$] | 1.6x10$^6$ [1.2x10$^2$-2.3x10$^9$] | 6.7x10$^4$ [1.3x10$^2$.9x10$^9$] | 4.4x10$^4$ [10$^5$.5.04x10$^9$] | NA | 4.3e-05$ | NA |
| Alfa Feto Protein (IU/L) | 53.1 [1-52408]         | 8.8 [1-714.9] | 15.4 [1.4–1660] | 125.4 [1.2.52408] | ND | 4.8e-09$ | < 2.2e-16$ |

Abbreviation: CHB: chronic hepatitis B; LC: liver cirrhosis; HCC: hepatocellular carcinoma; HC: healthy control; PLT: platelets. AST and ALT: aspartate and alanine amino transferase; ND: not done; NA: not applicable. Values given are medians and range. $^a$ comparison between HBV subgroups (CHB, LC, HCC); $^b$ comparison between HBV patients and controls. P values were calculated by Kruskal-Wallis test (a) and Chi-square test (b).

Association of PD-1 variants with HBV infection and liver disease progression

The distribution of genotype and allelic frequencies of $PD-1.5$ and $PD-1.9$ variants and their association with hepatitis B and related liver diseases are summarized in the Table 2 and Table 3. The variants were in Hardy-Weinberg equilibrium for both cases and controls (P > 0.05). No significant association was found with susceptibility to HBV infection and the $PD-1.5$ variant, but only in chronically infected HBV-infected patients, where the frequency of the $PD-1.5$ CT genotype was significantly lower in HCC patients compared to non-HCC patients (CHB + LC). Among subgroups of HBV patients, the $PD-1.5$ CT genotype was found to be a protective factor for HCC (HCC vs. non-HCC: OR = 0.6, 95%CI: 0.4–0.9, $P_{adj} = 0.031$). For other $PD-1.5$ genotypes, no significant associations with HBV-related liver progression were found in various between-group comparisons (Table 3).
Table 2
Association of PD1 polymorphisms with susceptibility to HBV infection

| PD1 variants | HBV patients | Healthy controls | HBV patients vs. HC | p |
|--------------|--------------|------------------|---------------------|---|
|              | n = 513 (%)  | n = 196 (%)      | OR (95%CI)          |   |
| PD-1.5 (rs2227981) - synonymous codon, upstream variant | | | | |
| CC           | 287 (56.2)   | 95 (48.5)        | Reference           |   |
| CT           | 194 (38)     | 84 (42.9)        | 0.79 (0.5–1.2)      | NS |
| TT           | 30 (5.9)     | 17 (8.7)         | 0.58 (0.3–1.2)      | NS |
| Allele       |              |                  |                     |   |
| C            | 768 (75.1)   | 137 (69.9)       | Reference           |   |
| T            | 254 (24.9)   | 59 (30.1)        | 0.78 (0.58–1.05)    | NS |
| Dominant     |              |                  |                     |   |
| CC           | 287 (56.2)   | 95 (48.5)        | Reference           |   |
| CT&TT        | 224 (43.8)   | 101 (51.5)       | 0.76 (0.5–1.1)      | NS |
| Recessive    |              |                  |                     |   |
| CC&CT        | 481 (94.1)   | 179 (91.3)       | Reference           |   |
| TT           | 30 (5.9)     | 17 (8.7)         | 0.6 (0.3–1.3)       | NS |
| PD-1.9 (rs2227982) - missense, upstream variant | | | | |
| CC           | 173 (33.8)   | 72 (36.9)        | Reference           |   |
| CT           | 282 (55.1)   | 92 (47.2)        | 1.5 (0.9–2.3)       | NS |
| TT           | 56 (11.1)    | 31 (15.9)        | 0.9 (0.7–1.3)       | NS |
| Allele       |              |                  |                     |   |
| C            | 628 (61.3)   | 236 (61)         | Reference           |   |
| T            | 394 (38.7)   | 154 (39)         | 0.9 (0.73–1.3)      | NS |
| Dominant     |              |                  |                     |   |
| CC           | 173 (33.8)   | 72 (36.9)        | Reference           |   |
| CT&TT        | 338 (66.2)   | 123 (63.1)       | 1.3 (0.9–1.9)       | NS |
| Recessive    |              |                  |                     |   |
| CC&CT        | 455 (89.0)   | 164 (84.0)       | Reference           |   |
| TT           | 56 (11.0)    | 31 (16.0)        | 0.5 (0.3–0.9)       | 0.026 |

Abbreviation: OR: Odds Ratio; ORs and P-values were calculated by using binary logistic regression model adjusted for age and gender. n = number of chromosomes. NS: not significant. Missing data: Due to the quality of sequences, 2 sequences could not be visible for PD1.5 in two HBV patients. 1 sequence could not be visible for PD1.9 in a HBV patient and in a healthy individual.
### Table 3
Association of PD1 polymorphisms with liver disease progression

| PD1 variants | CHB | LC | HCC | LC vs. CHB | HCC vs. CHB | HCC vs. LC | HCC + LC vs. CHB | HCC vs. non-HCC |
|--------------|-----|----|-----|-----------|-------------|------------|-----------------|-----------------|
|              | n (%) | n (%) | n (%) | OR (95%CI) | P | OR (95%CI) | P | OR (95%CI) | P | OR (95%CI) | P |
| PD-1.5 (rs2227981) - synonymous codon, upstream variant |
| **CC**       | 95 (54.9) | 68 (51.5) | 124 (60.2) | Reference | Reference | Reference | Reference | Reference | Reference |
| **CT**       | 70 (40.5) | 57 (43.2) | 67 (32.5) | 1.1 (0.7–1.8) | NS | 0.7 (0.4–1.2) | NS | 0.6 (0.4–1.0) | NS | 0.9 (0.6–1.3) | NS | 0.6 (0.4–0.9) | 0.031 |
| **TT**       | 8 (4.6) | 7 (5.3) | 15 (7.3) | 0.9 (0.3–2.9) | NS | 1.1 (0.4–2.3) | NS | 1.2 (0.4–3.3) | NS | 0.9 (1.04–5.4) | NS | 1.1 (0.5–2.5) | NS |
| Allele       | C     | 260 (75.1) | 193 (73.1) | 315 (76.5) | Reference | Reference | Reference | Reference | Reference |
| **T**        | 86 (24.9) | 71 (26.9) | 97 (23.5) | 1.04 (0.7–1.53) | NS | 0.8 (0.8–1.3) | NS | 0.81 (0.6–1.2) | NS | 0.9 (0.7–1.3) | NS | 1.3 (0.9–1.7) | NS |
| Dominant     | **CC** | 95 (54.9) | 68 (51.5) | 124 (60.2) | Reference | Reference | Reference | Reference | Reference |
| **CT&TT**    | 78 (45.1) | 64 (48.5) | 82 (39.8) | 1.1 (0.7–1.7) | NS | 0.8 (0.6–1.3) | NS | 0.7 (0.4–1.1) | NS | 0.9 (0.6–1.3) | NS | 0.7 (0.5–1.03) | NS |
| Recessive    | **CC&CT** | 165 (95.4) | 125 (94.7) | 191 (92.7) | Reference | Reference | Reference | Reference | Reference |
| **TT**       | 8 (4.6) | 7 (5.3) | 15 (7.3) | 0.9 (0.3–2.75) | NS | 1.2 (0.5–3.2) | NS | 1.5 (0.5–3.9) | NS | 0.9 (0.4–2.4) | NS | 1.3 (0.6–2.9) | NS |
| PD-1.9 (rs2227982) - missense, upstream variant |
| **CC**       | 60 (34.7) | 46 (34.3) | 67 (32.7) | Reference | Reference | Reference | Reference | Reference |
| **CT**       | 106 (61.3) | 70 (52.2) | 106 (51.7) | 0.9 (0.6–1.6) | NS | 0.9 (0.53–1.5) | NS | 0.9 (0.6–1.5) | NS | 0.9 (0.6–1.4) | NS | 0.9 (0.6–1.4) | NS |
| **TT**       | 7 (4.0) | 18 (13.4) | 32 (15.6) | 4.2 (1.6–11.6) | 0.003 | 3.7 (1.4–9.5) | 0.0038 | 1.2 (0.6–2.4) | NS | 3.8 (1.6–9.3) | 0.0011 | 1.9 (1.02–3.4) | 0.042 |
| Allele       | C     | 226 (65.3) | 162 (60.4) | 240 (58.5) | Reference | Reference | Reference | Reference | Reference |
| **T**        | 120 (34.7) | 106 (39.6) | 170 (41.5) | 1.34 (0.9–1.9) | NS | 1.3 (0.9–1.8) | NS | 1.04 (0.7–1.4) | NS | 1.3 (0.97–1.8) | 0.07 | 1.2 (0.9–1.58) | NS |
| Dominant     | **CC** | 60 (34.7) | 46 (34.3) | 67 (32.7) | Reference | Reference | Reference | Reference | Reference |
| **CT&TT**    | 113 (65.3) | 88 (65.7) | 138 (67.3) | 1.14 (0.7–1.9) | NS | 1.1 (0.7–1.8) | NS | 0.9 (0.6–1.6) | NS | 1.1 (0.7–1.6) | NS | 1.1 (0.7–1.6) | NS |
| Recessive    | **CC&CT** | 166 (96.0) | 116 (86.6) | 173 (84.4) | Reference | Reference | Reference | Reference | Reference |
| **TT**       | 7 (4.0) | 18 (13.4) | 32 (15.6) | 4.3 (1.7–11.3) | 0.0012 | 4.1 (1.6–10.3) | 0.001 | 1.2 (0.6–2.4) | NS | 4.2 (1.8–9.9) | 0.00022 | 2.0 (1.1–3.7) | 0.017 |

**Abbreviation:** CHB: chronic hepatitis B (n = 193); LC: liver cirrhosis (n = 183); HCC: hepatocellular carcinoma (n = 306); non-HCC = CHB + LC; n = number of chromosomes; NS: not significant; OR: Odds Ratio; ORs and P-values were calculated by using binary logistic regression model adjusted for age and gender. Bold values present the statistical significance. Missing data: Due to the quality of sequences, 2 sequences could not be visible for PD1.5 in two LC patients. 1 sequence could not be visible for PD1.9 in a HCC patient.
For PD1.9 polymorphism, the frequency of the genotype TT was significantly lower in HBV-infected patients compared to healthy controls (HBV patients vs HC: OR = 0.53, 95%CI:0.31–0.9, \(P_{adj}=0.026\)). In addition, the TT genotype was more frequent in the LC, HCC and HCC + LC patient groups than in the CHB patient group (LC vs. CHB: OR = 4.3, 95%CI: 1.7–11.3, \(P_{adj}=0.0012\); HCC vs. CHB: OR = 4.1, 95%CI: 1.6–10.3, \(P_{adj}=0.0012\); HCC + LC vs. CHB: 4.2, 95%CI: 1.8–9.9, \(P_{adj}=0.00022\), respectively). The result also showed that PD-1.9 TT genotype was a risk factor for HCC (HCC vs. non-HCC: OR = 2.0, 95%CI: 1.1–3.7, \(P_{adj}=0.017\)).

No significant association was observed between PD-1.5 or PD-1.9 variants and any clinical laboratory parameters (data not presented).

**sPD-1 levels in HBV patients**

Plasma sPD-1 levels were significantly distributed between HBV patients and healthy controls (\(P < 0.0001\), Fig. 1A). Among HBV patients, the CHB group had high levels of sPD-1 (median: 230.2 pg/mL, range: 47.3–2171.4) followed by LC (median: 188.5 pg/mL, range: 92–1238) and HCC (median: 131.3 pg/mL, range: 37.9–449.6) groups (CHB vs. HCC: \(P < 0.0001\) and LC vs. HCC: \(P = 0.005\), Fig. 1B).

**Association of sPD-1 levels with PD-1 variants**

We analyzed whether PD-1.5 and PD-1.9 variants could influence the expression of sPD-1 in HBV infected patients. For SNP PD-1.5, patients carrying the CC and CT genotype had the higher sPD-1 levels than TT genotype (\(P = 0.0093\) and 0.036, respectively). Meanwhile, there were no significant differences in sPD-1 levels between PD-1.9 genotypes (Fig. 2).

**Correlation between sPD-1 and liver enzyme parameters**

The correlation between sPD-1 levels and biochemical parameters in 191 HBV infected patients is shown in Fig. 3. A positive correlation between sPD-1 levels and AST, ALT enzyme activity, total bilirubin, and direct bilirubin levels was observed (\(\rho = 0.57; 0.57; 0.27; 0.33\), respectively) (Fig. 3). We did not find the significant correlation between sPD-1 and other parameters including albumin, platelet counts, prothrombin and AFP levels, and HBV viral loads (data not presented).

**Association of plasma sPD-1 levels with fibrosis score**

Analysis of the association of plasma sPD-1 with HCC stages based on BCLC classification (BCLC-A/B/C/D) and liver fibrosis progression (FIB-4 and APRI scores) found no significant differences between plasma sPD-1 concentrations and HCC stages (data not shown).

The patients with an APRI score \(> 1\) had significantly higher plasma sPD-1 levels compared to those with APRI score \(\leq 1\) (\(P < 0.0001\)). Analysis of correlation between sPD-1 levels and APRI score also revealed a positive correlation (\(\rho = 0.53\), \(P < 0.0001\)). Nevertheless, the sPD-1 levels were not correlated with Fib-4 score (Fig. 4).

**Discussion**

Increased PD-1 expression on T lymphocytes, including CD4 and CD8 T lymphocytes, is believed to lead to T cell exhaustion and is an important mechanism for tumor cell immune defense. In this case-control study, we investigated PD-1.5 and PD-1.9 variants and soluble PD-1 levels in HBV-infected patients and controls. We observed that there is a potential clinical significance of \(PD-1.5/PD-1.9\) polymorphisms and plasma sPD-1 levels in HBV infection.

Several genetic association studies have investigated the association of polymorphisms of the PDCD1 gene with infectious diseases. In particular, five single nucleotide polymorphisms of \(PD1:\ PD-1.1\ (-538G/A), PD-1.3\ (+7146G/A), PD-1.5\ (+7785T/C), PD-1.6\ (+8669G/A) and \ PD-1.9\ (+7625C/T)\) have been associated with human malignancies and are highly expressed in several cancers\(^{22,33-36}\). Furthermore, few studies have investigated the association of these \(PD-1\) variants and susceptibility to HBV infection and liver disease progression\(^{28,37-40}\). In our current study, the \(PD-1.9\) variant but not \(PD-1.5\), is associated with risk of HBV infection and clinical outcome.

\(PD-1.9\) is a non-synonymous variant that results in the amino acid substitution from valine to alanine at codon 215, which may lead to a change in structure or function of PD-1. Our study has shown that \(PD-1.9\ TT\) genotype may be a risk factor for HBV infection as well as for disease progression, which is consistent with our previous findings\(^{30}\). Two of our earlier studies which utilized different HBV patient cohorts confirm the study conducted by Fang Li et al. showing that \(PD-1.9\) may be involved in hepatocarcinogenesis in HBV infection\(^{29}\). However, no significant association between \(PD-1.9\) variant and overall cancer susceptibility has been found in meta-analyses\(^{22,25,41}\).

The PD-1.5 SNP is located in the exon 5 region, the transition from C to T does not alter the amino acid sequence of PD-1. The significant associations between PD-1.5 and cancer are likely due to a linkage disequilibrium of the PD-1.5 variation with other PD-1 gene polymorphisms, which can lead to altered PD-1 expression levels\(^{21}\). Several meta-analyses have shown that the \(PD-1.5 TT\) genotype, as well as the \(T\) allele reduce the risk of cancers\(^{22,41,42}\). However, all studies did not include HCC patients. Although our current study did not show a significant association between this polymorphism and susceptibility to HBV infection, we reported for the first time that the \(PD-1.5 CT\) genotype may be a protective factor for HCC predisposition.

PD-1 has a soluble form (sPD-1) that can be detected in peripheral blood\(^{14,43}\). In our study, the circulating sPD-1 levels of HBV patients were significantly higher than those of the control group. The results were consistent with studies in HBV infections\(^{17,44,45}\), chronic HCV\(^{46}\) and in acute or chronic inflammatory conditions such as pancreatitis\(^{47}\), sepsis\(^{48}\), autoimmune hepatitis and inflammatory bowel disease\(^{49}\). These data 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. The above evidence could explain the significantly higher levels of plasma sPD-1 in CHB patients in this study cohort.

FIB-4 and APRI indices are considered good predictors of liver fibrosis in chronic hepatitis C and CHB. In the current study, we found for the first time a positive correlation between plasma sPD-1 levels and APRI scores, suggesting that sPD-1 could be a complementary marker for the diagnosis of fibrosis in patients with chronic hepatitis B infection. In addition, this marker has many advantages, such as being less invasive and monitoring the kinetics of fibrosis progression. This shall be a useful indicator in the diagnosis of HBV related diseases and in the treatment of patients. Therefore, sPD-1 could be a useful marker for monitoring the progression of HBV-related liver disease. However, studies in a larger number of patients are needed to confirm and extend the results of this study.

The current study has limitations as the results would have been more meaningful if we could have determined the expression of PD-1 membrane-bound proteins in PBMC (peripheral blood mononuclear cells) as well as protein expression in liver tissue by immunohistochemical assays. However, in this study, neither liver tissue samples nor blood samples were available for isolation of PBMC.

In summary, this study showed that plasma sPD-1 levels correlate with liver inflammation and are associated with liver fibrosis in chronically HBV-infected patients. The PD-1.5 and PD1.9 polymorphisms are associated with HBV infection and progression of HBV-related liver disease.

Declarations

Acknowledgements
We thank all study subjects for their participation. We acknowledge Bui Thuy Linh, Mai Thanh Hai Linh and Bui Dinh Tung for sample collection and support with experimental procedure.

Funding
Dr. Nghiem Xuan Hoan was financially supported by the Vietnam National Foundation for Science and Technology Development (NAFOSTED) under the Grant number: 108.02-2018.315 to conduct this study. The funder has no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

Author contributions
NXH, PTMH designed the study. PTMH, NTU, PJW performed the experiments. PTMH, NXH and DTND performed the statistical analyses and interpreted data. PTMH, NXH, NTT recruited patients. TPV and LHS contributed to the materials for experimental procedures. NTT, DPG contributed to data management. PTMH, NXH, DTND wrote the first draft. TPV, LHS revised the first and finalized the draft.

Competing interests: The authors declare no competing interests.

References
1. WHO, Hepatitis, B. & Epidemiology https://www.who.int/news-room/fact-sheets/detail/hepatitis-b. (2021).
2. Perz, J. F., Armstrong, G. L., Farrington, L. A., Hutin, Y. J. & Bell, B. P. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. J Hepatol, 45, 529–538 (2006). https://www.ncbi.nlm.nih.gov/pubmed/16879891
3. Rehermann, B. & Nascimbeni, M. Immunology of hepatitis B virus and hepatitis C virus infection. Nat.Rev.Immunol, 5, 215–229 (2005). http://www.ncbi.nlm.nih.gov/pubmed/15738952
4. Shuai, Z. et al. Adaptive immunity in the liver. Cell Mol.Immunol, 13, 354–368 (2016). http://www.ncbi.nlm.nih.gov/pubmed/26996069
5. Guidotti, L. G. et al. Viral clearance without destruction of infected cells during acute HBV infection. Science, 284, 825–829 (1999). http://www.ncbi.nlm.nih.gov/pubmed/10221919
6. Phillips, S. et al. CD8(+) T cell control of hepatitis B virus replication: direct comparison between cytolytic and noncytolytic functions. J Immunol, 184, 287–295 (2010). http://www.ncbi.nlm.nih.gov/pubmed/19949099
7. Yang, P. L. et al. Immune effectors required for hepatitis B virus clearance. Proc.Natl.Acad.Sci.U.S.A, 107, 798–802. doi: http://www.ncbi.nlm.nih.gov/pubmed/20080755. (2010).
8. Boni, C. et al. Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. J Virol, 81, 4215–4225 (2007). https://www.ncbi.nlm.nih.gov/pubmed/17287266
9. Bertoletti, A. & Ferrari, C. Adaptive immunity in HBV infection. J Hepatol, 64, S71–S83 (2016). https://www.ncbi.nlm.nih.gov/pubmed/27084039
10. Rehermann, B. Pathogenesis of chronic viral hepatitis: differential roles of T cells and NK cells. Nat Med, 19, 859–868 (2013). https://www.ncbi.nlm.nih.gov/pubmed/23836236
11. Fisicaro, P. et al. Combined blockade of programmed death-1 and activation of CD137 increase responses of human liver T cells against HBV, but not HCV. Gastroenterology: 143, 1576–1585 e1574. doi: https://www.ncbi.nlm.nih.gov/pubmed/22929808. (2012).
1. Schonrich, G. & Raftery, M. J. The PD-1/PD-L1 Axis and Virus Infections: A Delicate Balance. Front Cell Infect Microbiol, 9, 207 (2019). https://www.ncbi.nlm.nih.gov/pubmed/31263684

2. Sharpe, A. H. & Freeman, G. J. The B7-CD28 superfamily. Nat Rev Immunol, 2, 116–126 (2002). https://www.ncbi.nlm.nih.gov/pubmed/11910893

3. Khan, M. et al. PD-1: Predictive, Prognostic, and Therapeutic Value for Cancer Immunotherapy. Front Immunol, 11, 587460 (2020). https://www.ncbi.nlm.nih.gov/pubmed/33329567

4. He, L. et al. Blockade of B7-H1 with sPD-1 improves immunity against murine hepatocarcinoma. Anticancer Res, 25, 3309–3313 (2005). https://www.ncbi.nlm.nih.gov/pubmed/16101143

5. Elhag, O. A. et al. Reconstructed adeno-associated virus with the extracellular domain of murine PD-1 induces antitumor immunity. Asian Pac J Cancer Prev, 13, 4031–4036 (2012). https://www.ncbi.nlm.nih.gov/pubmed/23098512

6. Li, Z. (2010). Zhang, G. Observational Study. Ren, H. T. Chinese population. Endocrinol Invest, 8, 61–74 (2010). https://www.ncbi.nlm.nih.gov/pubmed/22892379

7. Haghshenas, M. R., Dabbaghmanesh, M. H., Miri, A., Ghaderi, A. & Erfani, N. Association of PDCD1 gene markers with susceptibility to thyroid cancer. Oncotarget, 8, 46020–46033 (2017). https://www.ncbi.nlm.nih.gov/pubmed/28545019

8. Isogawa, M., Chung, J., Murata, Y., Kakimi, K. & Chisari, F. V. CD40 activation rescues antiviral CD8+ T cells from PD-1-mediated exhaustion. PLoS Pathog, 9, e1003490 (2013). https://www.ncbi.nlm.nih.gov/pubmed/23853599

9. Shi, F. et al. PD-1 and PD-L1 upregulation promotes CD8+ T-cell apoptosis and postoperative recurrence in hepatocellular carcinoma patients. Int J Cancer, 128, 887–896 (2011). https://www.ncbi.nlm.nih.gov/pubmed/20473887

10. Cheng, H. Y. et al. Circulating programmed death-1 as a marker for sustained high hepatitis B viral load and risk of hepatocellular carcinoma. PLoS One, 9, e95870 (2014). https://www.ncbi.nlm.nih.gov/pubmed/25427199

11. Salmaninejad, A. et al. PD-1 and cancer: molecular mechanisms and polymorphisms. Immunogenetics, 70, 73–86 (2018). https://www.ncbi.nlm.nih.gov/pubmed/28642997

12. Dong, W. et al. Programmed Cell Death-1 Polymorphisms Decrease the Cancer Risk: A Meta-Analysis Involving Twelve Case-Control Studies. PLoS One, 11, e0152448 (2016). https://www.ncbi.nlm.nih.gov/pubmed/27031235

13. Lee, S. Y. et al. Functional polymorphisms in PD-L1 gene are associated with the prognosis of patients with early stage non-small cell lung cancer. Gene, 599, 28–35 (2017). https://www.ncbi.nlm.nih.gov/pubmed/27838455

14. Li, Y., Liu, Y., Liu, D., Zhang, Y. & Mu, K. Association of polymorphisms in the programmed cell death 1 (PD-1) and PD-1 ligand genes with ankylosing spondylitis in a Chinese population. Clin Exp Rheumatol, 29, 13–18 (2011). https://www.ncbi.nlm.nih.gov/pubmed/21269571

15. Zhang, J., Zhao, T., Xu, C., Huang, J. & Yu, H. The association between polymorphisms in the PDCD1 gene and the risk of cancer: A PRISMA-compliant meta-analysis. Med. (Baltim), 95, e4423 (2016). https://www.ncbi.nlm.nih.gov/pubmed/27749524

16. Gao, J. et al. Meta-analysis of programmed cell death 1 polymorphisms with systemic lupus erythematosus risk. Oncotarget, 8, 36885–36897 (2017). https://www.ncbi.nlm.nih.gov/pubmed/28415570

17. Mojtahedi, Z. et al. Programmed death-1 gene polymorphism (PD-1.5 C/T) is associated with colon cancer. Gene, 508, 229–232 (2012). https://www.ncbi.nlm.nih.gov/pubmed/22892379

18. Huang, C. et al. Association of rs10204525 genotype GG and rs2227982 CC combination in programmed cell death 1 with hepatitis B virus infection risk. Med. (Baltim), 98, e16972 (2019). https://www.ncbi.nlm.nih.gov/pubmed/31464942

19. Li, F. et al. Genetic association and interaction of PD1 and TIM3 polymorphisms in susceptibility of chronic hepatitis B virus infection and hepatocarcinogenesis. Discov Med, 27, 79–92 (2019). https://www.ncbi.nlm.nih.gov/pubmed/30939292

20. Hoan, N. X. et al. Genetic variants of programmed cell death 1 are associated with HBV infection and liver disease progression. Sci Rep, 11, 7772 (2021). https://www.ncbi.nlm.nih.gov/pubmed/33833369

21. Marrero, J. A. et al. Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. Hepatology, 68, 723–750 (2018). https://www.ncbi.nlm.nih.gov/pubmed/29624699

22. Forner, A., Reig, M. E., de Lope, C. R. & Bruix, J. Current strategy for staging and treatment: the BCLC update and future prospects. Semin Liver Dis, 30, 61–74 (2010). https://www.ncbi.nlm.nih.gov/pubmed/20175034

23. Yin, L., Guo, H., Zhao, L. & Wang, J. The programmed death-1 gene polymorphism (PD-1.5 C/T) is associated with non-small cell lung cancer risk in a Chinese Han population. Int J Clin Exp Med, 7, 5832–5836 (2014). https://www.ncbi.nlm.nih.gov/pubmed/25664115

24. Haghshenas, M. R., Dabbaghmanesh, M. H., Miri, A., Ghaderi, A. & Erfani, N. Association of PDCD1 gene markers with susceptibility to thyroid cancer. J Endocrinol Invest, 40, 481–486 (2017). https://www.ncbi.nlm.nih.gov/pubmed/27943063

25. Li, X. F., Jiang, X. Q., Zhang, J. W. & Jia, Y. J. Association of the programmed cell death-1 PD1.5 C>T polymorphism with cervical cancer risk in a Chinese population. Genet Mol Res, 15, doi: https://www.ncbi.nlm.nih.gov/pubmed/27050970. (2016).

26. Ren, H. T. et al. PD-1 rs2227982 Polymorphism Is Associated With the Decreased Risk of Breast Cancer in Northwest Chinese Women: A Hospital-Based Observational Study. Med. (Baltim), 95, e3760 (2016). https://www.ncbi.nlm.nih.gov/pubmed/27227944

27. Zhang, G. et al. Association of polymorphisms of programmed cell death-1 gene with chronic hepatitis B virus infection. Hum Immunol, 71, 1209–1213 (2010). https://www.ncbi.nlm.nih.gov/pubmed/20837075

28. Li, Z. et al. Immune checkpoint proteins PD-1 and TIM-3 are both highly expressed in liver tissues and correlate with their gene polymorphisms in patients with HBV-related hepatocellular carcinoma. Med. (Baltim), 95, e5749 (2016). https://www.ncbi.nlm.nih.gov/pubmed/28033288
39. Li, Z. et al. Genetic polymorphisms of immune checkpoint proteins PD-1 and TIM-3 are associated with survival of patients with hepatitis B virus-related hepatocellular carcinoma. Oncotarget, 7, 26168–26180 (2016). https://www.ncbi.nlm.nih.gov/pubmed/27034168
40. Bayram, S. et al. Lack of an association of programmed cell death-1 PD1.3 polymorphism with risk of hepatocellular carcinoma susceptibility in Turkish population: a case-control study. Gene, 511, 308–313 (2012). https://www.ncbi.nlm.nih.gov/pubmed/23041554
41. Hashemi, M. et al. Association between PD-1 and PD-L1 Polymorphisms and the Risk of Cancer: A Meta-Analysis of Case-Control Studies. Cancers (Basel), 11. doi: https://www.ncbi.nlm.nih.gov/pubmed/31405171. (2019).
42. Tang, W. et al. Programmed death-1 (PD1) rs2227981 C > T polymorphism is associated with cancer susceptibility: a meta-analysis. Int J Clin Exp Med, 8, 22278–22285 (2015). https://www.ncbi.nlm.nih.gov/pubmed/26885204
43. Zhu, X., Lang, J. & Soluble PD-1 and PD-L1: predictive and prognostic significance in cancer. Oncotarget, 8, 97671–97682 (2017). https://www.ncbi.nlm.nih.gov/pubmed/29228642
44. Bi, C. et al. Changes of serum sPD-1 levels in HBeAg-positive chronic hepatitis B patients with entecavir treatment and correlation with curative effect. Turk J Med Sci, 48, 286–292 (2018). https://www.ncbi.nlm.nih.gov/pubmed/29714442
45. Zhou, L. et al. Soluble programmed death-1 is a useful indicator for inflammatory and fibrosis severity in chronic hepatitis B. J Viral Hepat, 26, 795–802 (2019). https://www.ncbi.nlm.nih.gov/pubmed/30578715
46. Wang, D. et al. Aberrant production of soluble inducible T-cell co-stimulator (sICOS) and soluble programmed cell death protein 1 (sPD-1) in patients with chronic hepatitis C. Mol Med Rep, 7, 1197–1202 (2013). https://www.ncbi.nlm.nih.gov/pubmed/23426717
47. Yu, X. et al. Serum soluble PD-1 plays a role in predicting infection complications in patients with acute pancreatitis. Immun Inflamm Dis, 9, 310–318 (2021). https://www.ncbi.nlm.nih.gov/pubmed/33417300
48. Bakhshiani, Z., Fouladi, S., Mohammadzadeh, S. & Eskandari, N. Correlation of sPD1 with Procalcitonin and C-Reactive Protein Levels in Patients with Sepsis. Cell J, 23, 14–20 (2021). https://www.ncbi.nlm.nih.gov/pubmed/33650816
49. Hadley, T. et al. Soluble PD1 levels are increased with disease activity in paediatric onset autoimmune hepatitis and inflammatory bowel disease. Autoimmunity, 53, 253–260 (2020). https://www.ncbi.nlm.nih.gov/pubmed/32370568
50. Chang, B. et al. The correlation and prognostic value of serum levels of soluble programmed death protein 1 (sPD-1) and soluble programmed death-ligand 1 (sPD-L1) in patients with hepatocellular carcinoma. Cancer Immunol Immunother, 68, 353–363 (2019). https://www.ncbi.nlm.nih.gov/pubmed/30506460
51. Wai, C. T. et al. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatology, 38, 518–526 (2003). https://www.ncbi.nlm.nih.gov/pubmed/12883497
52. Teshale, E. et al. APRI and FIB-4 are good predictors of the stage of liver fibrosis in chronic hepatitis B: the Chronic Hepatitis Cohort Study (ChECS). J Viral Hepat, 21, 917–920 (2014). https://www.ncbi.nlm.nih.gov/pubmed/25131445

Figures
Figure 1

Distribution of sPD-1 levels in healthy controls and in patients (A). Distribution of sPD-1 levels in HBV infected patients and healthy controls. (B) in each patient subgroups. Box-plots illustrate median values with 25 and 75 percentiles with whiskers to 10 and 90 percentiles; p-values were calculated by Mann-Whitney test.
Figure 2

Association of sPD-L1 with PD-1 polymorphisms in HBV patients. (A) PD-1.5 (B) PD-1.9 polymorphism Box-plots illustrate median values with range (min-max) and outliers; NS: not significant, p-values were calculated by Wilcoxon tests (A) or Kruskal Wallis test (B).

![Graph A](image)

![Graph B](image)

Figure 3

Correlation between sPD-1 levels with laboratory parameters in HBV patients. Correlation between sPD-1 levels with AST (A); ALT (B); total bilirubin (C); direct bilirubin (D). The correlation coefficient between sPD-1 levels with laboratory parameters: was calculated by using Spearman's rank correlation coefficient. Spearman's rho (rho) and P value are given.

![Graph C](image)

![Graph D](image)
Figure 4

Association and correlation of sPD-1 and fibrosis scores in HBV patients. Box-plots illustrate median values with range (min-max) and outliers with P-values were calculated by Wilcoxon tests and Kruskal Wallis test. The correlation coefficients between sPD-1 levels with APRI (A) and FIB-4 (C) scores were calculated by using Spearman’s rank correlation coefficient. Spearman’s rho (rho) and P value are given.