CROSS-SECTIONAL STUDY

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Association Between Serum Cytotoxic T Lymphocyte Antigen (CTLA)-4 Level and Disease Progression in Patients With Chronic Hepatitis B

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ABSTRACT

Backgroud: Immune impairment, marked by increased expression of cytotoxic T lymphocyte antigen (CTLA)-4, promotes the disease progression of chronic hepatitis B. **Objective:** This study aimed to determine the association between serum CTLA-4 level and disease progression in patients with chronic hepatitis B. **Methods:** A cross-sectional study was conducted at Haji Adam Malik General Hospital Medan, Indonesia between October 2021 to September 2022. A total of 150 participants were enrolled. Patients aged 18 years or older with evidence of chronic hepatitis B, HBV-related liver cirrhosis, and HBV-related hepatocellular carcinoma (HCC) were enrolled. Exclusion criteria were history of chronic hepatotoxic drug consumption, underlying liver abnormalities other than HBV infection, and liver injury due to metastasized malignancy from other sites. Serum CTLA-4 level was determined from serum using human CTLA-4 enzyme linked immunosorbent assay kit. **Results:** Most participants were males and aged between 40 and 60 years. Serum CTLA-4 level was positively associated with chronic hepatitis B progression (P<0.001). Serum CTLA-4 level was negatively correlated with serum platelet (P<0.001) and albumin levels (P<0.001) but positively correlated with serum ALT (P=0.045) and total bilirubin levels (P<0.001). **Conclusions:** Serum CTLA-4 level is associated with disease progression in patients with chronic hepatitis B.

Keywords: chronic hepatitis B, CTLA-4, hepatocellular carcinoma, liver cirrhosis.

1. BACKGROUND

Hepatitis B virus (HBV) is a DNA virus from *Hepadnaviridae* family in which its infection may lead to chronic hepatitis and hepatocellular carcinoma (HCC) (1-5). HBV infection affects more than 400 million people and approximately 5% adults develop chronic infection (1). Around 75% of all chronic hepatitis B patients reside in Asia (6) with the highest being in China (7). Hepatocellular carcinoma itself is one of the most frequent malignancy worldwide (8) and contributes to global health burden (9). Globally, HCC is the sixth most frequent malignancy and the fourth most common cause of cancer-related mortality (3, 10). In Indonesia, HCC ranked as the 9th most common malignancy (4), and the 7th most common cause of death. The endemicity of hepatitis B surface antigen in Indonesia is intermediate to high. The male to female ratio for HCC was 4:1.4 (6). Its prevalence was highest between the 4th and 7th decade of life (4). The most common infecting HBV in Indonesia comes from genotype B (5, 6).

Several factors influencing disease progression from chronic hepatitis B to HCC including immune response (1, 2). Antigens produced by the virus in the chronic fashion impair immune response toward HBV (1). Anti-inflammatory cytokines is increased, immune cell differentiation is suppressed, and immune cell function is impaired following the chronic hepatitis B (11, 12). Immune impairment is marked by increased expression of co-inhibitory receptors such as cytotoxic T lymphocyte antigen (CTLA)-4 and programmed cell death protein (PD)-1 (3, 11, 13). Prolonged immune inhibition will give longer opportunity for HBV to reside in host's hepatocyte and induce disease

Variables	Chronic Hepatitis B	Liver Cirrhosis	Hepatocellular Carcinoma	P
Gender, n (%) Male Female	 26 (28.9) 24 (40.0)	 33 (36.7) 17 (28.3)	 31 (34.4) 19 (31.7)	 0.338
Mean age, years (SD)	46.7 (7.97)	57.7 (6.89)#	59.1 (7.89)#	<0.001*
Median serum platelet level, thousands/mm^3 (min-max)	265.5 (105-540)	83 (30 – 415) #	102 (42 – 677) #	<0.001*
Median serum AST level, IU/L (min-max)	44.46 (16-128)	56.34 (16 – 72)	62.12 (21 – 81)	0.324
Median serum ALT level, IU/L (min-max)	42 (18 – 130)	36 (20 – 61)	46 (24 – 98)	0.495
Median serum total bilirubin level, mg/dL (min-max)	0.8 (0.5 – 2.6)	2.4 (1.2 – 5.8) #	2.1 (0.91 – 5.6) #	<0.001*
Median serum albumin level, g/dL (min-max)	4.1 (2.8 – 4.9)	2.4 (1.2 – 3.8) #	2.6 (1.4 – 4) #	<0.001*
Median serum CTLA-4 level, pg/mL (min-max)	354.5 (93–780)	516 (253-1,212) #	1,018 (753-2,313)! #	<0.001*

Table 1. Associations between demographical and laboratory variables and chronic hepatitis B progression.*– P<0.05, # – significant compared to chronic hepatitis B group ! – significant compared to liver cirrhosis group

progression toward liver cirrhosis and hepatocellular carcinoma (13).

Cytotoxic T lymphocyte antigen (CTLA)-4 gene belongs to the immunoglobulin supergene family and located in chromosome 2q33 (8,12). CTLA-4 is a T cell co-stimulatory receptor which can attenuate immune response (2, 14). High concentration of soluble CTLA-4 is observed in patients with autoimmune diseases (14). Recently, serum CTLA-4 level is found to be positively associated with the risk of HCC in patients with chronic hepatitis B (2, 12).

2. OBJECTIVE

Our study aimed to determine the association between serum CTLA-4 level and disease progression in patients with chronic hepatitis B in Medan, Indonesia. The result of this study is expected to illuminate new therapeutic approach for patients with chronic hepatitis B, liver cirrhosis, and HCC.

3. MATERIAL AND METHODS

Study design and subjects

This cross-sectional study was conducted at Haji Adam Malik General Hospital Medan, Indonesia between October 2021 to September 2022. A total of 150 participants were enrolled in this study. Participants were obtained using consecutive sampling method. Patients aged 18 years or older displayed clinical biochemical, and virologic evidence of chronic hepatitis B, HBV-related liver cirrhosis, and HBV-related HCC were enrolled. The evidence of chronic hepatitis B consisted of positive plasma hepatitis B surface antigen (HBsAg) for more than 6 months with fluctuating serum alanine aminotransferase (ALT) and HBV deoxyribonucleic acid (DNA) levels. The diagnosis of HBV-related cirrhosis was confirmed by ultrasound and transient elastography while the diagnosis of HBV-related HCC was confirmed by triphasic liver computed tomography. The exclusion criteria were history of chronic hepatotoxic drug consumption, underlying liver abnormalities other than HBV infection, and liver injury due to metastasized malignancy from other sites. This study was approved by Institutional Review Board of Universitas Sumatera Utara with register number 1029/KEP/USU/2021.

Laboratory examinations

Data regarding serum platelet, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, and albumin levels were obtained from participants' medical record. Serum CTLA-4 level was determined from participant's serum using human CTLA-4 enzyme linked immunosorbent assay (ELISA) kit (Quantikine*(R)* ELISA, Human CTLA-4 Immunoassay R&D System Inc., Minneapolis, US) (15).

Statistical analysis

The association between categorical variable and chronic hepatitis B progression was evaluated using chi-square test. One-way ANOVA test was utilized to determine association between normally distributed numerical variables and chronic hepatitis B progression while Kruskal Wallis test was used for non-normally distributed numerical variables. The correlations between serum CTLA-4 level and serum platelet, AST, ALT, total bilirubin, and albumin levels were elevated with Spearman correlation test. Statistical analysis was conducted using Statistical Package for Social Science (SPSS) software version 24.0 at a confidence interval of 95%. P value of <0.05 was considered significant.

4. RESULTS

All of 150 participants enrolled in this study were divided into 3 groups equally: chronic hepatitis B, liver cirrhosis, and hepatocellular carcinoma groups. Participants were dominated by males and subjects aged 40-60 years. From statistical analysis, we found that serum CTLA-4 levels were associated with chronic hepatitis B progression (P<0.001). serum CTLA-4 levels were increased in patients from chronic hepatitis B, liver cirrhosis, and hepatocellular carcinoma groups. Additionally, several variables were negatively associated with chronic hepatitis B progression including serum platelet, total bilirubin, and albumin levels. In the other hand, participants' age was positively associated with the disease progression (Table 1).

Spearman correlation test revealed that serum CTLA-4 level was correlated with liver function laboratory parameters such as serum platelet, ALT, total bilirubin, and albumin levels. Serum CTLA-4 level was negatively associated with platelet and albumin levels while it was

Liver function laboratory parameters	P	r
Serum platelet level	<0.001*	-0.312
Serum AST level	0.224	0.100
Serum ALT level	0.045*	0.164
Serum total bilirubin level	<0.001*	0.319
Serum albumin level	<0.001*	-0.456

Table 2. Correlation between serum CTLA-4 level and serum platelet, AST, ALT, total bilirubin, and albumin levels *– P<0.05

positively associated with serum ALT and total bilirubin levels (Table 2).

5. DISCUSSION

Only 5% of the total HBV infection develops chronic infection and its complications but approximately 1 million people die every year due to HBV-related complications. Vertical transmission is the most common mode of transmission in chronic hepatitis B (11). Chronic hepatitis B is a risk factor for liver cirrhosis and HCC (9). Around 8-20% adults with chronic hepatitis B develops liver cirrhosis and 2-8% of those with cirrhosis suffer from HCC. The prevalence of HCC is increasing in developed countries (11). This malignancy is more prevalent in males compared to females (6) and peaked at the 4th and 7th decade of life (4). In accordance with the literatures, male participants dominate all groups in this study and participants' age was between the 4th and 6th decade of life.

HBV does not cause direct injury to hepatocyte (7, 11). It is the host's immune response that determines the fate of HBV infection in a person (7, 11, 13). HBV produces several antigens including hepatitis B core, envelope, and surface antigens. The antigens are immunogenic and may trigger immune reaction.[1] T cells play crucial role in eliminating HBV (10-12). CD4+ T cell activation is initiated by antigen presentation by antigen presenting cell. This process is followed by T cell proliferation and cytokine production which require positive co-stimulatory signal (3, 13, 14). CD4+ T cells induce cytotoxic T lymphocytes activation and neutralizing antibodies production, including TNF-α and IFN-γ. CD8+ T cells eliminate HBV infected hepatocytes and stimulate B cells to produce antibodies (3, 9, 11, 13).

Negative signal will lead to refractory or apoptosis of T cell (14). Negative signal is associated with prolonged exposure to hepatitis B surface antigen (HBsAg) (1). Hepatitis B surface antigen is believed to impair proper cellular and humoral along with innate immune response toward HBV (1, 11). Impairment of immune function, including B lymphocyte, cytotoxic T lymphocyte, T helper-1, and T regulatory cells, is observed in patients with chronic hepatitis B. The amount of hepatitis B surface antigen is related to the severity of HBV infection. In addition, there is an evidence of co-inhibitory receptor expression on T cells during chronic hepatitis B infection (1). CTLA-4 is one of co-inhibitory receptors that is expressed on T cells (2,8,12). CTLA-4 gene is located on chromosome 2q33 and codes the co-stimulatory molecule which is expressed on the surface of activated T cells (8, 14). CTLA-4 is homologue to CD28 in structure and functions as inhibitor of T cell activation and proliferation (2,3,8,9,13). After binding with its ligand, CTLA-4 will decrease T cell activation and enhance regulatory T cell activity (7, 13, 16). This effect is the result of competitive binding of CTLA-4 and CD28 receptor and direct inhibition of T cell receptor signalling by CTLA-4 (13). Furthermore, CTLA-4 is also associated with IL-10 mediated inhibition of T-cell proliferation and induction of T-cell apoptosis (10, 12). In addition, CTLA-4 decreases serum tumor necrosis factor-α and interferon-γ levels (2, 12). This decreased immune response causes host immune system unable to eliminate HBV and leads to chronic infection (2).

Prolonged HBV presence will activate hepatic stellate cells which will trans-differentiate into myofibroblast as the main source of extracellular matrix of liver and initiate fibrogenesis (11). Liver fibrosis is a reversible stage of chronic hepatitis B mediated by improper immune response (7, 9). Liver fibrosis should be controlled by IFN-γ which is suppressed in chronic hepatitis B (10, 11). Unstoppable fibrosis will end with liver cirrhosis and carcinogenesis (1-3, 11). Multiple factors including viral protein, chronic inflammation, and integration of HBV DNA to host's DNA affect the progression of chronic hepatitis B to liver cirrhosis and hepatocellular carcinoma (HCC). Mutation in viral HBx and PreS2 protein gene is associated with increased risk of liver damage including HCC (1, 5, 11). Several polymorphisms of CTLA-4 gene influence the expression of CTLA-4 (9). CTLA-4 gene rs231775 polymorphism with allele A is one of risk factors for HCC by its effect in increasing serum CTLA-4 level (8). Contrary, Wang et al found that CTLA-4 gene rs231775 polymorphism allele G increased serum CTLA-4 level and was a risk factor of hepatocellular carcinoma. The difference in the effect of gene polymorphism needs further evaluation (2).

Patients with chronic hepatitis B have upregulated CTLA-4 (16). Liu et al. reported that high expression of CTLA-4 was associated with higher risk for HCC (P=0.022) (8). Serum CTLA-4 levels were increased from asymptomatic HBV carrier to chronic hepatitis B, liver cirrhosis and hepatocellular carcinoma patients. Patients with high serum CTLA-4 levels had increased risk for developing HCC as high as 2.628 times compared to those with low serum CTLA-4 levels. Positive association between serum CTLA-4 level and poor outcome of patients with HCC was also observed (2). Another literature stated that patients with HCC have high expression of CTLA-4 and consequently decreased cytolytic granzyme B production by CD8+ T cells (10). In our study, there was an association between serum CTLA-4 level and chronic hepatitis B progression (P<0.001). Serum CTLA-4 level was increased from chronic hepatitis B to liver cirrhosis and hepatocellular carcinoma patients. Other factors which associated with disease progression were age, serum platelet count, serum total bilirubin and albumin levels. Serum albumin and platelet levels were negatively associated with disease progression in contrast with age and serum total.

Studies also stated that blockage of CTLA-4 would boost anti-HBV response and improve disease outcome. CTLA-4 blockage increased T cell activation and anti-tumor activity (2, 9, 13). Tremelimumab is a CTLA-4 inhibitor that being utilized in patients with HCC. Its administration gives favourable outcome of HCC (3, 10). Administration of 15 mg/kg tremelimumab intravenously every 90 days was proved effective in decreasing viral load in patients with HCC. Disease control rate was reported in 76.4% patients receiving the regimen (16). The effect of CTLA-4 blocker was also positive in regard of liver function laboratory parameters such as decreased serum ALT, AST, and total bilirubin levels along with increased serum albumin and platelet levels (10). This phenomenon was confirmed by another study which found that serum CTLA-4 level was positively associated with serum ALT, AST, and total bilirubin levels but negatively associated with serum albumin level (2). However, further study is mandatory to assess tremelimumab's efficacy and resistance (10). Similar with this study's findings, there was a positive correlation between serum CTLA-4 level and serum total bilirubin level while negative correlation was observed between serum CTLA-4 level and serum platelet and albumin levels.

Limitation of the study

This study had several limitations. We did not exclude confounding risk factors for HCC including alcohol consumption, tobacco use, diabetes, and obesity (3). We also did not include healthy participants as control group in this study. In conclusion, serum CTLA-4 level was associated with chronic hepatitis B progression and correlated with serum platelet, total bilirubin, and albumin levels in this study.

6. CONCLUSION

Serum CTLA-4 level is associated with disease progression in patients with chronic hepatitis B.

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- **Declaration of Patient Consent**: The authors certify that they have obtained all appropriate patient consent forms.
- **Authors Contribution**: D.D., D.L, J.S., T.W., M.R., M.M.A., F.Y., and P.C.E. gave substantial contributions to the conception or design of the work in acquisition, analysis. D.D., D.L, J.S., T.W., M.R., and M.M.A. gave contribution to acquistion of data. D.D., D.L., J.S., T.W., F.Y., and P.C.E. :gave contribution to data analysis and interpretation. D.D., M.L., and I.R. gave contribution in drafting article. D.D., D.L., J.S., T.W., M.L., I.R. gave final approval of the version to be published.
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