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Short Communication

CTLA-4 +6230G>A polymorphism and its impact on CTLA-4 level and risk of hepatocellular carcinoma: A case-control study in Batak patients with chronic hepatitis B

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Abstract

Genetic polymorphisms in cytotoxic T-lymphocyte-associated protein 4 gene (*CTLA-4*) vary by ethnic background, necessitating population-specific studies. The aim of this study was to assess the association between the *CTLA-4* +6230G>A polymorphism, serum CTLA-4 level, and hepatocellular carcinoma (HCC) in Batak patients with chronic hepatitis B, a group with high hepatitis B virus (HBV) endemicity. A case-control study was conducted among cases (Batak patients with chronic hepatitis B and HCC) and controls (chronic hepatitis B without HCC). Genotyping of the *CTLA-4 +6230G>A* polymorphism was performed using the TaqMan SNP Genotyping Assay. Serum CTLA-4 level was quantified using enzyme-linked immunosorbent assay (ELISA). Patient's demographic, clinical and laboratory data were recorded and assessed including age, sex, body mass index (BMI), smoking history, cirrhosis status, HBV DNA level, liver function markers (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)), hepatitis B e-antigen (HBeAg) status, smoking history, and alcohol consumption. This study found that G allele was significantly associated with an increased risk of HCC (OR: 2.69; 95%CI: 1.21–6.00; p=0.013). Individuals with GG/AG genotypes had a 2.89-fold higher risk of developing HCC compared to those with the AA genotype (p=0.032). Serum CTLA-4 level was significantly elevated in G allele carriers (GG: 159.9±57.1 pg/mL vs AA: 83.7±44.7 pg/mL; p<0.001). Multivariate analysis identified cirrhosis as the strongest predictor of HCC (OR: 7.60; p<0.001), followed by elevated ALT (OR: 3.42; p=0.018) and high HBV DNA levels (OR: 2.31; p=0.024). In conclusion, the *CTLA-4 +6230G>A* GG/AG genotype and elevated serum CTLA-4 level were significantly associated with an increased risk of HCC in Batak individuals with chronic HBV infection. Further research is needed to explore additional *CTLA-4* polymorphisms and immune regulatory mechanisms in HBV-related HCC to improve risk stratification and therapeutic strategies.

Keywords: Chronic liver disease, hepatocarcinogenesis, genetic polymorphism, *CTLA-4* gene, rs3087243

Introduction

Hepatitis B remains a major global health concern. According to a 2019 WHO report, the global chronic infection rate is estimated at 4%, with complications such as liver cirrhosis and hepatocellular carcinoma (HCC) contributing to a 0.3% mortality rate [1]. In 2020, HCC

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accounted for 900,000 new cases and 830,000 deaths worldwide, with the highest burden observed in East Asia, North Africa, and Southeast Asia. The incidence and mortality of HCC are projected to rise by 55% in 2040 [2]. Currently, HCC ranks among the three leading causes of cancer-related mortality in 46 countries and is among the top five in 90 countries [2]. Indonesia has one of the highest hepatitis B endemicity rate in Southeast Asia with hepatitis B surface antigen (HBsAg) prevalence of 7.1%, corresponding to approximately 18 million cases, with 20–30% of affected individuals at risk of progressing to liver cirrhosis or HCC [3]. HCC is the fourth most common cancer among men in Indonesia, with an incidence of 13.4 per 100,000 population [3]. Chronic hepatitis B remains the primary etiology, responsible for 67% of cases treated at Indonesian national referral hospitals [3]. However, survival outcomes remain poor due to late-stage diagnosis, with a median survival of 138 days and a one-year survival rate of 29.4% [4].

The development of HCC is influenced by viral, biochemical, and host-related factors, with hepatitis B virus (HBV) DNA levels and alanine aminotransferase (ALT) serving as key risk predictors [5,6]. Host factors, including age, sex, obesity, smoking, alcohol consumption, and cirrhosis, contribute to disease progression [7]. In males, androgen-mediated enhancement of viral replication and inflammation increases susceptibility to chronic hepatitis and cirrhosis [8]. Aging further promotes HCC development through the accumulation of oxidative stress over time [9]. Additionally, lifestyle factors such as obesity, alcohol consumption, and smoking exacerbate oxidative stress, further driving hepatocarcinogenesis [10-12].

Genetic factors are increasingly recognized as contributors to HCC risk. Polymorphisms in immune-regulating genes, particularly cytotoxic T-lymphocyte-associated protein 4 gene (*CTLA-4*), play a crucial role in tumor immunity. CTLA-4, an immune checkpoint molecule, functions as a negative regulator of T-cell activation [13]. Single nucleotide polymorphisms (SNPs) in *CTLA-4* can alter its expression and functionality, thereby influencing immune responses [14]. The *+6230G>A* (rs3087243) polymorphism in the 3′-untranslated region (3′-UTR) modulates mRNA stability and soluble CTLA-4 expression, impacting T-cell activity [15-18]. Furthermore, the +6230A allele has been associated with increased susceptibility to HBV progression and persistence, potentially due to its role in immune regulation [14].

CTLA-4 polymorphisms have been implicated in various immune-related conditions, including autoimmune diseases such as autoimmune hepatitis, type 1 diabetes, and systemic lupus erythematosus, as well as chronic viral infections, including HBV and hepatitis C virus (HCV) [14,19-21]. However, a previous meta-analysis found no statistically significant association between *CTLA-4* polymorphisms and HCC risk, suggesting a limited role in hepatocarcinogenesis [22]. Given that *CTLA-4* genetic polymorphisms may vary by ethnic background, population-specific studies are warranted to better understand their relevance in different genetic backgrounds. While the *+6230G>A* variant has been investigated in the context of HBV infection, previous studies have not accounted for ethnic variability, despite known regional differences in genetic associations [14,23]. This highlighted the need for population-specific research to elucidate the role of *+6230G>A* in disease progression. The aim of this study was to assess the association between the *CTLA-4* +6230G>A polymorphism, serum CTLA-4 levels, and HCC occurrence in Batak patients with chronic hepatitis B. As one of the largest ethnic groups in Medan, the Batak population provides a distinct genetic background for investigating HCC susceptibility. The findings from this study may contribute to improved early detection and risk stratification, ultimately enhancing clinical outcomes.

Methods

Study design and settings

A case-control study was conducted from in 2024 across six hospitals in Medan, Indonesia: Prof. Dr. Chairuddin P. Lubis Hospital, Mitra Medika Premiere Hospital, Mitra Medika Amplas Hospital, Mitra Medika Bandar Klippa Hospital, Mitra Medika Tanjung Mulia Hospital, and Wulan Windy Hospital. Eligible patients were identified from hepatology outpatient clinics and medical records based on a confirmed diagnosis of chronic hepatitis B. Patients were categorized into case and control groups based on predefined HCC diagnostic criteria. Clinical data, including

HCC status, laboratory findings, and patient characteristics, were recorded including age, sex, body mass index (BMI), smoking history, cirrhosis status, HBV DNA levels, liver function markers such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT), hepatitis B e-antigen (HBeAg) status, smoking history, and alcohol consumption. Blood samples were collected for *CTLA-4* +6230G>A genotyping and serum CTLA-4 measurement, with laboratory analyses conducted at Research Laboratory of Universitas Sumatera Utara, Medan, Indonesia. Genotyping of the *CTLA-4 +6230G>A* polymorphism was performed using the TaqMan SNP Genotyping Assay to determine the genotypes (GG, GA, and AA). Serum CTLA-4 levels were quantified via enzyme-linked immunosorbent assay (ELISA) to assess immunoregulatory differences.

Eligibility criteria

Inclusion criteria required patients to be at least 18 years old with a confirmed diagnosis of chronic hepatitis B, with or without HCC. Ethnicity was verified through documented lineage, requiring both paternal and maternal ancestry from the Batak ethnic group, specifically from one of six subgroups: Angkola, Karo, Mandailing, Pakpak-Dairi, Simalungun, or Toba. This verification was further supported by confirmation of the Batak clan name (*Marga*), a widely recognized marker of Batak descent. Exclusion criteria included recent use of immunomodulatory or immunosuppressive drugs within the past month and coexisting diagnoses of human immunodeficiency virus (HIV) infection, hepatitis C, chronic kidney disease, autoimmune disease, or other malignancies. Pregnant patients were also excluded.

Sample and sampling methods

Sample size calculation was performed using the Cochrane formula, referencing a prior study on *CTLA-4* polymorphisms in patients with chronic hepatitis B—indicated that 51% of patients with HCC carried the GG+GA genotype, compared to 17% of those without HCC [24]. To achieve 90% power with a 5% alpha error, a minimum of 39 patients per group was required, yielding a total sample size of at least 78 patients. A consecutive sampling technique was applied, in which all eligible participants who met the inclusion and exclusion criteria were recruited consecutively until the target sample size was obtained. Cases comprised Batak patients with chronic hepatitis B and HCC, while controls were Batak patients with chronic hepatitis B without HCC. Controls were selected from the same population and recruitment period as cases, ensuring comparability while allowing for an unbiased evaluation of the association between the *CTLA-4* +6230G>A polymorphism, serum CTLA-4 levels, and HCC.

Diagnosis of chronic hepatitis B and hepatocellular carcinoma

Patients were diagnosed with chronic hepatitis B and HCC based on clinical, serological, and imaging criteria in accordance with the Indonesian Ministry of Health's Guidelines for Adult Hepatocellular Carcinoma Management (HK.01.07/MENKES/1355/2022) [4] and international standards including the American Association for the Study of Liver Diseases (AASLD) [24] and European Association for the Study of the Liver (EASL) guidelines [25]. Chronic hepatitis B was confirmed by the persistence of HBsAg for ≥6 months. HCC diagnosis was established through triphasic liver computed tomography (CT) scan, characterized by arterial phase hyperenhancement with washout in the venous or delayed phase, supplemented by elevated serum alpha-fetoprotein (AFP) or protein induced by vitamin K absence-II (PIVKA-II) levels.

Demographic, clinical and laboratory assessments

Clinical assessments included structured interviews, physical examinations, and laboratory testing. Paper sheets were used to collect demographic and clinical data, including age, sex, BMI, smoking history, cirrhosis status, HBV DNA levels, liver function markers, specifically AST and ALT, HBeAg status, smoking history, and alcohol consumption. BMI was calculated using measured height and weight, with obesity defined as a BMI >25 kg/m² according to Asia-Pacific criteria [10]. Alcohol consumption was defined as any history of alcohol intake, with quantification based on daily alcohol consumption (grams) multiplied by the duration of alcohol use (years). Alcohol consumption was categorized as never, light (<24 grams/year), moderate, or heavy (>24 grams/year). Smoking history was assessed using the Brinkman Index, calculated as

the number of cigarettes smoked per day multiplied by the duration of smoking (years). Smoking status was classified as light (0–199), moderate (200–599), or heavy (≥600).

Abdominal ultrasonography was performed to evaluate liver cirrhosis status, with cirrhosis defined by the presence of ≥2 major imaging features (irregular liver surface, heterogeneous parenchyma, or reduced liver size) and ≥1 sign of portal hypertension (splenomegaly or ascites). Non-cirrhotic cases had either normal liver architecture or isolated abnormalities without concomitant signs of portal hypertension [9,11].

Liver function markers were evaluated using standard biochemical analyzers. AST, an enzyme predominantly found in the liver, heart, and muscles, was categorized as high (>98 IU/L) or low (<98 IU/L). ALT, a key enzyme for assessing hepatocellular injury, was classified as high (>90 IU/L) or low (<90 IU/L). HBV DNA levels were measured via real-time polymerase chain reaction (RT-PCR) and categorized as high (>6.4 $\log_{10}$ IU/mL) or low (<6.4 $\log_{10}$ IU/mL). HBeAg status, determined using ELISA, was classified as reactive or non-reactive.

Blood sample collection and processing

Venous blood samples (5 mL) were collected into EDTA vacutainer for genetic analysis and serum separation tube for *CTLA-4 +6230G>A* polymorphism and serum CTLA-4 level measurements. Samples were inverted 10 times for homogenization before centrifugation at 3000 rpm for 15 minutes. Plasma was stored at -70°C for DNA extraction, while serum was stored at -20°C for ELISA analysis. Upon completion of sample collection, specimens were transported on dry ice to the Research Laboratory of Universitas Sumatera Utara, Medan, Indonesia, for further analysis.

Genotyping of *CTLA-4* +6230G>A polymorphism

DNA extraction was conducted at the Research Laboratory of Universitas Sumatera Utara, using COBAS DNA Sample Preparation Kit (Roche Applied Science, Penzberg, Germany) following the manufacturer's protocol. Spectrophotometric quantification was performed using a Nanodrop 2000 at 260/280 nm (Thermo Fisher Scientific, Waltham, MA, USA) to assess DNA concentration and purity before proceeding with genotyping.

Genotyping of the *CTLA-4 +6230G>A* polymorphism was performed using the TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA). The PCR amplification was performed on a C1000 Thermal Cycler CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). The reaction mixture had a total volume of 25 µL, comprising 2X TaqMan GTXpress Master Mix (Applied Biosystems, Foster City, CA, USA), allele-specific TaqMan probes and primers [26], 50 ng of extracted genomic DNA, and nuclease-free water. The amplification process included an initial step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 92°C for 15 seconds and annealing/extension at 60°C for 1 minute. Allelic discrimination was based on real-time fluorescence data, which was analyzed using StepOnePlus Software (Applied Biosystems, Foster City, CA, USA). The fluorescence signals emitted by the TaqMan probes allowed for differentiation among the GG, GA, and AA genotypes. Each sample underwent genotyping once according to the standardized protocol, without duplicate testing.

Measurement of serum CTLA-4 levels

Serum CTLA-4 levels were quantified using the Quantikine ELISA Human CTLA-4 Immunoassay (R&D Systems, Minneapolis, MN, USA), a sandwich ELISA designed for high specificity and sensitivity. A total of 100 µL of serum samples and standards were tested using antibody-coated 96-well microplates. Absorbance was measured at 450 nm with a reference wavelength of 540 nm using a microplate reader (Multiskan Go, Thermo Fisher Scientific, Waltham, MA, USA). Serum CTLA-4 concentrations were calculated by interpolating absorbance values from a standard curve generated using known concentrations of recombinant CTLA-4. Serum CTLA-4 levels were categorized as low or high based on the median value.

Statistical analysis

The Kolmogorov-Smirnov test was used to assess data normality, while the Hardy-Weinberg equilibrium test evaluated the genotypic distribution of the *CTLA-4 +6230G>A* polymorphism in the study population. Differences in genotype and allele frequencies between case and control groups were analyzed using the Chi-squared test. Logistic regression was performed to determine

the association between the *CTLA-4 +6230G>A* polymorphism and HCC risk, adjusting for confounding variables such as age, cirrhosis status, HBV DNA level, and liver function markers. Results were presented as odds ratio (OR) and 95% confidence intervals (95%CI). Serum CTLA-4 levels were compared among genotypes using one-way ANOVA with least significant difference (LSD) post hoc analysis, while differences between HCC and non-HCC groups were assessed using independent Student t-tests. Multivariate logistic regression was conducted to evaluate the independent effects of the *CTLA-4 +6230G>A* polymorphism on HCC risk, incorporating significant covariates. Additionally, a two-way ANOVA was performed to examine interactions between the *CTLA-4 +6230G>A* polymorphism, serum CTLA-4 levels, and HCC status. Statistical analyses were conducted using SPSS v.26 (IBM, New York, USA), with $p<0.05$ considered statistically significant.

Results

Patient selections

Patient selection was conducted among individuals aged over 18 years with hepatitis B between 2020 and 2024 (**Figure 1**). Initially, 660 patients were identified, comprising 102 with HCC and 558 without HCC. Exclusion criteria were applied sequentially, resulting in the removal of patients with incomplete clinical data, leading to the exclusion of 14 patients with HCC and 37 without HCC. Further exclusions were made for patients diagnosed with other malignancies (6 with HCC, 25 without HCC), as well as those with HIV, hepatitis C, or chronic kidney disease (CKD) (3 with HCC, 36 without HCC). Additionally, 29 patients with HCC were excluded due to an inability to be contacted for blood collection for *CTLA-4* polymorphism testing or having passed away. Finally, the final study sample consisted of 100 patients, including 50 with HCC and the first 50 patients without HCC whose blood samples were collected consecutively (**Figure 1**).

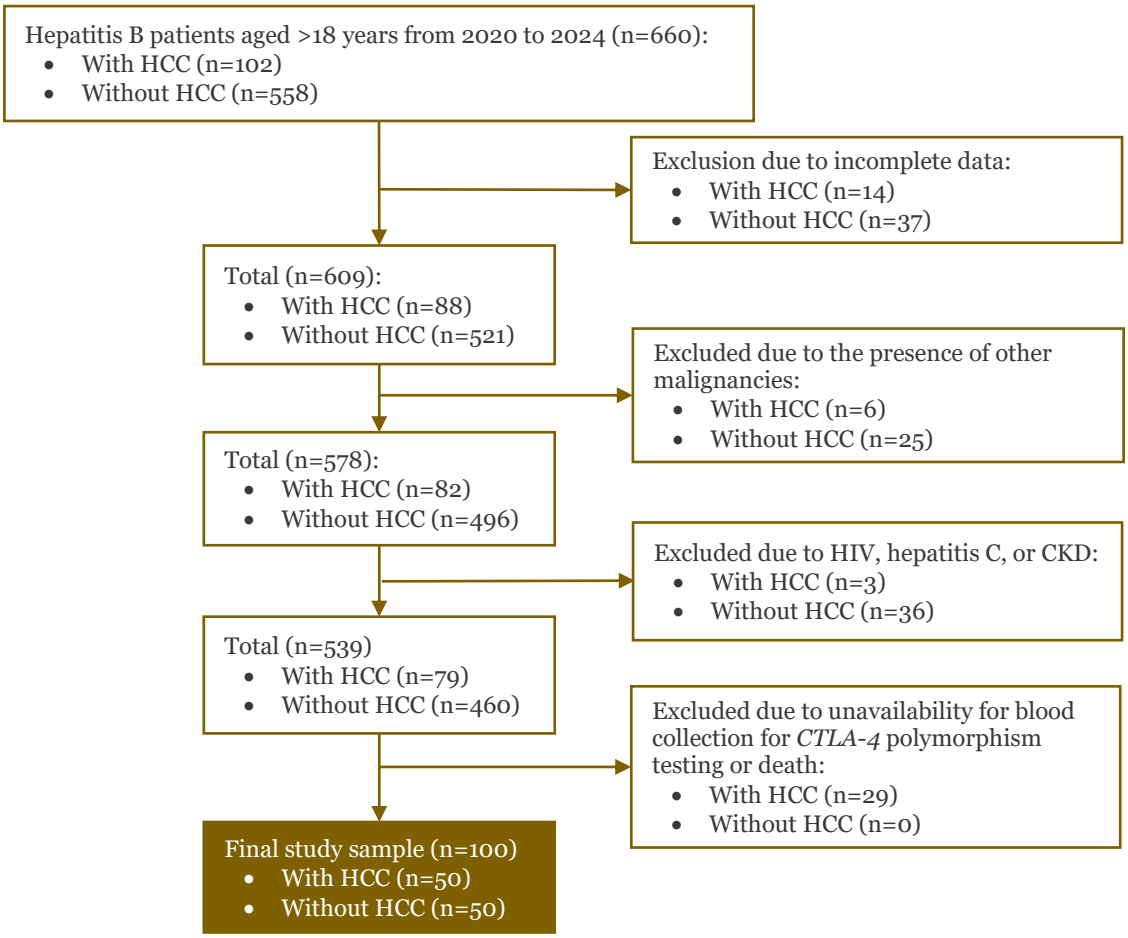

Figure 1. Flowchart of patient selection. CKD: chronic kidney disease; HIV: human immunodeficiency virus; HCC: hepatocellular carcinoma.

Characteristics of patients

The characteristics of patients within case and control groups are presented in **Table 1**. Among the 100 patients included in the study, 56% were male and 44% were female, with a higher proportion of males in the HCC group (66%) compared to the control group (46%) (**Table 1**). The mean age of the patients was 55±9.94 years, and a greater proportion of those aged >55 years was observed in the HCC group (62%) compared to controls (40%). Obesity was present in 36% of the total cohort, with a lower prevalence in the HCC group (28%) than in the control group (44%). Liver function markers were elevated in a greater proportion of HCC patients. The median AST level was higher in the HCC group (115 IU/L, range: 42–214) compared to controls (97.5 IU/L, range: 42–527), with elevated AST levels observed in 58% of HCC cases and 50% of controls. Similarly, the median ALT level was higher in HCC patients (113 IU/L, range: 31–230) than in controls (88 IU/L, range: 24–230), with high ALT levels found in 60% of HCC cases compared to 40% of controls. HBV DNA levels were also higher among HCC patients, with a median viral load of 6.60 $\log_{10}$ IU/mL (range: 4.40–8.10) compared to 6.00 $\log_{10}$ IU/mL (range: 1.60–7.90) in controls. High HBV DNA levels (>6.4 $\log_{10}$ IU/mL) were detected in 64% of HCC cases and 42% of controls. HBeAg reactivity was observed in 48% of HCC cases and 36% of controls. Cirrhosis was more prevalent among HCC patients, with 78% having liver cirrhosis compared to 32% in the control group. Serum CTLA-4 levels were also higher in the HCC group, with a median concentration of 105.90 pg/mL (range: 39.60–259.20) compared to 56 pg/mL (range: 36–264) in controls. High serum CTLA-4 levels were found in 62% of HCC cases compared to 40% of controls (**Table 1**).

Table 1. Characteristics of Batak patients with chronic hepatitis included in the study (n=100)

Variable	Total	Case (n=50)	Control (n=50)
	n (%)	n (%)	n (%)
Sex			
Male	56 (56)	33 (66)	23 (46)
Female	44 (44)	17 (34)	27 (54)
Age (years), mean±SD	55 ±9.94		
>55	51 (51)	31 (62)	20 (40)
≤55	49 (49)	19 (38)	30 (60)
Obesity status			
Yes	36 (36)	14 (28)	22 (44)
No	64 (64)	36 (72)	28 (56)
Alcohol consumption			
Moderate + heavy	20 (20)	13 (26)	7 (14)
Light/none	80 (80)	37 (74)	43 (86)
Smoking status			
Moderate + heavy	34 (34)	16 (32)	18 (36)
Light/none	66 (66)	34 (68)	32 (64)
AST levels (IU/L), median (min-max)	98 (42–527)	115 (42–214)	97.50 (42–527)
High	54 (54)	29 (58)	25 (50)
Normal	46 (46)	21 (42)	25 (50)
ALT levels (IU/L), median (min-max)	90 (24–230)	113 (31–230)	88 (24–230)
High	50 (50)	30 (60)	20 (40)
Normal	50 (50)	20 (40)	30 (60)
HBV DNA levels ($\log_{10}$ IU/mL), median (min-max)	6.40 (1.60–8.10)	6.60 (4.40–8.10)	6.00 (1.60–7.90)
High	53 (53)	32 (64)	21 (42)
Normal	47 (47)	18 (36)	29 (58)
HBeAg status			
Reactive	42 (42)	24 (48)	18 (36)
Non-reactive	58 (58)	26 (52)	32 (64)
Liver cirrhosis status			
Yes	55 (55)	39 (78)	16 (32)
No	45 (45)	11 (22)	34 (68)
Serum CTLA-4 levels (pg/mL), median (min-max)	90 (36–264)	105.90 (39.60–259.20)	56 (36–264)
High	51 (51)	31 (62)	20 (40)
Low	49 (49)	19 (38)	30 (60)

ALT: alanine transaminase; AST: aspartate aminotransferase; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; DNA: deoxyribonucleic acid; HBeAg: hepatitis B e-antigen; HBV: hepatitis B virus; HCC: hepatocellular carcinoma

CTLA-4 +6230G>A genotypic and allelic distribution in Batak patients with chronic hepatitis B

CTLA-4 +6230G>A polymorphism was analyzed in patients with chronic hepatitis B and the genotypic distribution showed AA as the predominant genotype (77%), followed by AG (13%) and GG (10%) (**Table 2**). Allele frequency analysis indicated that A allele was predominant, accounting for 83.5%, while the G allele was present in 16.5% of the included patients. In the dominant genetic model (GG + AG vs AA), 23% of patients carried at least one G allele (GG or AG), whereas 77% had the AA genotype. In the recessive model (GG vs AG + AA), only 10% of patients had the GG genotype, while 90% carried at least one A allele.

Table 2. Distribution of *CTLA-4* +6230G>A genotypic and allelic distribution in Batak patients with chronic hepatitis B

Polymorphism *CTLA-4* +6230G>A	Frequency (%)
Genotype	
Genotype GG	10 (10.0)
Genotype AG	13 (13.0)
Genotype AA	77 (77.0)
Allele	
Allele G	33 (16.5)
Allele A	167 (83.5)
Dominant model	
GG + AG	23 (23.0)
AA	77 (77.0)
Recessive model	
GG	10 (10.0)
AG + AA	90 (90.0)

CTLA-4: cytotoxic T-lymphocyte-associated protein 4

Hardy-Weinberg equilibrium for the *CTLA-4* +6230G>A polymorphism was assessed in both cases and controls. In the case group, the observed genotype frequencies did not deviate from the expected distribution (p=0.987), indicating equilibrium. Similarly, the control group remained in equilibrium (p=0.879) (**Table 3**). These results confirmed that the genotypic frequencies of *CTLA-4* +6230G>A polymorphism in both groups were in Hardy-Weinberg equilibrium (p>0.05).

Table 3. Hardy-Weinberg equilibrium analysis for *CTLA-4* +6230G>A polymorphism in case and control groups

CTLA-4 +6230G>A polymorphism	Observed (O)	Expected (E)	χ^2	p-value[a]
Case group (n=50)				
Genotype GG (n=7)	19	19.38	0.0263	0.987
Genotype AG (n=9)	46	45.23		
Genotype AA (n=34)	26	26.38		
Control group (n=50)				
Genotype GG (n=3)	12	10.90	0.2578	0.879
Genotype AG (n=4)	39	41.19		
Genotype AA (n=43)	40	38.90		

[a]Analyzed using Chi-squared test
CTLA-4: cytotoxic T-lymphocyte-associated protein 4

Association between demographic and laboratory factors with hepatocellular carcinoma in Batak patients with chronic hepatitis B

The analysis identified significant associations between sex, older age, high ALT levels, high HBV DNA levels, liver cirrhosis, and high CTLA-4 levels with HCC presence (p<0.05) (**Table 4**). Males had a 2.28-fold increased risk of HCC (p=0.044), while older age was associated with a 2.45-fold higher risk (p=0.028). High ALT levels associated with a 2.25-fold increased risk (p=0.046), whereas high HBV DNA levels were associated with a 2.46-fold greater likelihood of HCC (p=0.028). Liver cirrhosis represented the most significant risk factor, increasing the likelihood of HCC by 7.53 times (p<0.001). Moreover, high serum CTLA-4 levels were associated with a 2.45-fold elevated risk of HCC (p=0.028).

Table 4. Univariate analysis showing the associations between demographic and clinical characteristics with hepatocellular carcinoma in Batak patients with chronic hepatitis B

Variables	HCC, n (%)		OR (95%CI)	*p*-value[a]
	Case (n=50)	Control (n=50)		
Sex				
Male	33 (66)	23 (46)	2.28 (1.02–5.11)	0.044*
Female	17 (34)	27 (54)		
Age (years)				
>55	31 (62)	20 (40)	2.45 (1.10–5.47)	0.028*
≤55	19 (38)	30 (60)		
Obesity status				
Yes	14 (28)	22 (44)	0.50 (0.22–1.14)	0.096
No	36 (72)	28 (56)		
Alcohol consumption				
Moderate + heavy	13 (26)	7 (14)	2.16 (0.78–5.98)	0.134
Light/none	37 (74)	43 (86)		
Smoking status				
Moderate + heavy	16 (32)	18 (36)	0.84 (0.37–1.92)	0.673
Light/none	34 (68)	32 (64)		
AST levels (IU/L)				
High	29 (58)	25 (50)	1.38 (0.63–3.04)	0.422
Low	21 (42)	25 (50)		
ALT levels (IU/L)				
High	30 (60)	20 (40)	2.25 (1.01–5.00)	0.046*
Low	20 (40)	30 (60)		
HBV DNA levels				
High	32 (64)	21 (42)	2.46 (1.10–5.49)	0.028*
Low	18 (36)	29 (58)		
HBeAg status				
Reactive	24 (48)	18 (36)	1.64 (0.74–3.66)	0.224
Non-reactive	26 (52)	32 (64)		
Liver cirrhosis				
Yes	39 (78)	16 (32)	7.53 (3.08–18.44)	<0.001**
No	11 (22)	34 (68)		
Serum CTLA-4 levels				
High	31 (62)	20 (40)	2.45 (1.10–5.47)	0.028*
Low	19 (38)	30 (60)		

ALT: alanine transaminase; AST: aspartate aminotransferase; CI: confidence interval; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; DNA: deoxyribonucleic acid; HBeAg: hepatitis B e-antigen; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; OR: odds ratio

[a]Analyzed using logistic regression

*Statistically significant at $p<0.05$

**Statistically significant at $p<0.01$

Association between *CTLA-4* +6230G>A polymorphism and hepatocellular carcinoma in Batak patients with chronic hepatitis B

The association between the *CTLA-4* +6230G>A polymorphism and HCC was evaluated. A significant association was identified between the combined GG+AG genotypes and HCC, with these genotypes conferring a 2.89-fold increased risk compared to the AA genotype ($p=0.032$) (**Table 5**). Furthermore, the presence of the G allele was associated with a 2.69-fold higher risk of HCC compared to the A allele ($p=0.013$).

Multivariate logistic regression analysis of hepatocellular carcinoma risk factors in Batak patients with chronic hepatitis B

Seven variables identified in the univariate analysis—sex, age, ALT level, HBV DNA level, liver cirrhosis, serum CTLA-4 level, and *CTLA-4 +6230G>A* polymorphism—were included in a multivariate logistic regression model with HCC as the dependent variable. Liver cirrhosis emerged as the strongest predictor of HCC, increasing the risk by 7.6 times ($p<0.001$) (**Table 6**). High ALT level was associated with a 3.42-fold increased risk ($p=0.018$), while high HBV DNA level associated with a 2.31-fold higher likelihood of HCC ($p=0.024$). High CTLA-4 level and older age also demonstrated significant associations, with ORs of 2.16 ($p=0.040$) and 1.65 ($p=0.043$), respectively. The presence of the G allele in the *CTLA-4* +6230G>A polymorphism was associated with a 1.45-fold increased risk of HCC ($p=0.046$). Sex did not have a significant association with HCC in the multivariate analysis ($p>0.05$). The Nagelkerke R-squared value was 0.627, indicating

that the model explained 62.7% of the variability in HCC risk, with the remaining 37.3% attributable to other factors not included in the model.

Table 5. Association between *CTLA-4* +6230G>A polymorphism and hepatocellular carcinoma in Batak patients with chronic hepatitis B

CTLA-4 +6230G>A polymorphism	Case, n (%)	Control, n (%)	OR (95%CI)	*p*-value
Genotype				
GG	7 (14)	3 (6)	2.55 (0.59–10.86)	0.102[a]
AG	9 (18)	4 (8)	2.52 (0.81– 10.04)	0.094[a]
AA (Reference group)	34 (68)	43 (86)		
GG + AG	16 (32)	7 (14)	2.89 (1.07–7.82)	0.032[a*]
Allele				
G	23 (23)	10 (10)	2.69 (1.21–6.00)	0.013[b*]
A (Reference group)	77 (77)	90 (90)		

CI: confidence interval; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; OR: odds ratio
[a]Analyzed using logistic regression
[b]Analyzed using Chi-squared test
[*]Statistically significant at $p<0.05$

Table 6. Multivariate logistic regression analysis of factors associated with hepatocellular carcinoma in Batak patients with chronic hepatitis B

Variables	β	OR (95%CI)	*p*-value[a]
Liver cirrhosis	6.687	7.597 (1.53–8.66)	<0.001[**]
High ALT levels	3.310	3.420 (1.08–4.38)	0.018[*]
High HBV DNA levels	2.140	2.310 (1.05–3.62)	0.024[*]
High CTLA-4 levels	1.962	2.162 (1.04–3.56)	0.040[*]
Age >55 years	1.511	1.65 (1.04–3.20)	0.043[*]
Allele G *CTLA-4* +6230G>A polymorphism	1.206	1.45 (1.02–3.08)	0.046[*]
Male	1.180	1.24 (0.87–2.44)	0.108

ALT: alanine transaminase; CI: confidence interval; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; DNA: deoxyribonucleic acid; HBV: hepatitis B virus; OR: odds ratio
[a]Analyzed using multivariate logistic regression
[*]Statistically significant at $p<0.05$
[**]Statistically significant at $p<0.01$

Association between *CTLA-4* +6230G>A polymorphism and serum CTLA-4 levels in Batak patients with chronic hepatitis B

The association between the *CTLA-4* +6230G>A polymorphism and serum CTLA-4 levels was also analyzed. Significant differences in serum CTLA-4 level was observed among genotypes ($p<0.001$), with the GG and AG genotypes showing higher levels compared to the AA genotype (**Table 7**). Additionally, the presence of the G allele was associated with significantly high CTLA-4 level compared to the A allele ($p<0.001$).

Table 7. Association between serum CTLA-4 levels and *CTLA-4* +6230G>A genotypes and alleles in Batak patients with chronic hepatitis B

CTLA-4 +6230G>A polymorphism	Serum CTLA-4 levels (pg/mL), mean±SD	*p*-value
Genotype		
Genotype GG	159.86±57.10[#]	<0.001[* a]
Genotype AG	147.24±70.99[#]	
Genotype AA	83.66±44.72	
Allele		
Allele G	154.89±61.34	<0.001[* b]
Allele A	88.61±49.88	

CTLA-4: cytotoxic T-lymphocyte-associated protein 4; SD: standard deviation.
[a]Analyzed using two-way ANOVA
[b]Analyzed using independent Student t-test
[*]Statistically significant at $p<0.001$
[#]Significant compared to AA genotype

Discussion

Chronic hepatitis B posed a significant global health burden, particularly in Indonesia, due to its association with severe complications such as HCC. This study, which focused on the Batak ethnic

group, investigated the association between the *CTLA-4* +6230G>A polymorphism and its impact on serum CTLA-4 levels. The findings demonstrated that individuals carrying the G allele (GG and AG genotypes) had significantly higher serum CTLA-4 levels compared to those with the AA genotype. These results suggested that the G allele may have contributed to enhanced CTLA-4 expression, potentially influencing immune regulation in patients with chronic hepatitis B.

The *CTLA-4* +6230G>A polymorphism, located in the 3′-UTR of the *CTLA-4* gene, has been implicated in the regulation of mRNA stability and splicing efficiency [26,27]. Functional studies have demonstrated that variations in the 3′-UTR can influence the balance between membrane-bound and soluble forms of CTLA-4, thereby modulating its inhibitory effects on T-cell activation [27-29]. Specifically, the G allele has been associated with increased production of soluble CTLA-4, which enhances immune suppression by competitively binding to B7 ligands on antigen-presenting cells, thereby blocking the co-stimulatory signals required for full T-cell activation [28]. This competitive inhibition compromises T-cell activation, leading to immune exhaustion, impaired clearance of HBV-infected hepatocytes, and sustained viral replication [28,30,31]. Consequently, persistent HBV infection, coupled with chronic immune dysfunction, contributes to progressive hepatic injury, fibrosis, and hepatocarcinogenesis [32,33].

In the Batak population, high CTLA-4 expression level associated with the G allele was associated with an increased risk of HCC. This association highlights the pivotal role of CTLA-4 in regulating immune responses against HBV. Increased serum CTLA-4 levels have been shown to impair T-cell function, reduce cytokine production, and diminish cytotoxic activity—mechanisms essential for effective viral clearance and hepatocyte protection [30,31]. As a result, increased CTLA-4 expression may contribute to accelerated disease progression and an increased risk of HCC development in individuals with chronic hepatitis B.

A study conducted in Han Chinese individuals identified an association between the *CTLA-4* +6230G/A polymorphism and HBV persistence as well as HCC susceptibility, indicating that individuals carrying the A allele had a higher susceptibility to HBV-related liver cirrhosis and HCC, whereas the G allele was associated with HBV clearance [14]. These results contrasted with the present study, in which the G allele was associated with high serum CTLA-4 levels and an increased risk of HCC. This discrepancy may be attributed to genetic differences between the Han Chinese and Batak populations. The Batak ethnic group possesses a distinct ancestry and immune response profile, which may influence the functional impact of *CTLA-4* polymorphisms. Genetic studies have demonstrated that allele frequencies of immune-regulatory genes vary across ethnic groups, potentially contributing to differences in disease susceptibility and progression [34,35]. The mechanistic differences between these findings and those from the Chinese cohort may be explained by population-specific variations in linkage disequilibrium patterns and haplotype structures of the *CTLA-4* gene [36]. *CTLA-4* polymorphisms formed haplotypes that influenced immune responses, with the C-A-A and T-A-G haplotypes conferring an increased risk for HBV progression and persistence [14]. Although the present study did not conduct haplotype analysis, it remains possible that distinct *CTLA-4* haplotypes exist in the Batak population, resulting in different associations with chronic hepatitis B and HCC risk.

The Batak population may possess distinct genetic characteristics that contribute to the observed findings. Previous studies have indicated that Indonesian populations had a higher frequency of pro-inflammatory alleles in immune-related genes, including those involved in human leukocyte antigen (HLA) and cytokine signaling [37,38]. This predisposition to increased immune activation may lead to a more pronounced inflammatory response to HBV infection, promoting hepatic fibrosis and accelerating progression to HCC [6,8,31]. Such a mechanism may partially explain why individuals of Batak ethnicity with the GG genotype in *CTLA-4*-1661G>A demonstrated a stronger association with HCC risk compared to other populations. Additionally, the predominant HBV genotypes in Indonesia are B and C, with genotype B being the most prevalent [39,40]. Notably, HBV genotype C has been independently associated with an increased risk of HCC compared to genotype B [39]. The distribution of viral genotypes suggests that Batak individuals may experience a more aggressive course of chronic HBV infection, potentially amplifying the oncogenic impact of CTLA-4 polymorphisms.

Cirrhosis emerged as the strongest independent predictor of HCC, increasing risk more than sevenfold, likely due to its pro-inflammatory and fibrotic microenvironment, which promotes

oxidative stress, genomic instability, and aberrant cellular signaling [6,12,41]. High HBV DNA levels further exacerbated this risk by enhancing viral replication, facilitating immune evasion, and integrating viral DNA into the host genome—key processes in HBV-induced hepatocarcinogenesis [6,41]. Age also demonstrated a significant association, with individuals over 55 years exhibiting a higher risk, potentially due to cumulative inflammation, immune senescence, and prolonged HBV exposure [42]. Although male sex was associated with HCC in univariate analysis, it lost significance in multivariate analysis, possibly reflecting an indirect effect mediated through higher cirrhosis rates and other risk factors. The protective role of estrogen in modulating inflammation and enhancing viral clearance in women may further explain this difference [7]. Additionally, high ALT levels were identified as a significant predictor, reinforcing their specificity for liver injury and chronic inflammation, both of which are critical precursors to fibrosis and malignant transformation [43].

Established risk factors for liver disease, including obesity and alcohol consumption, did not show a significant association with HCC in Batak population, likely due to cultural and religious influences on dietary and lifestyle habits. Alcohol consumption remains relatively low in Indonesia, reducing its impact on liver disease at the population level [44]. Additionally, interview-based assessments may have been affected by social desirability bias, as discussing or admitting to alcohol consumption is often considered a social taboo, potentially leading to underreporting and obscuring any true associations [45].

Identifying individuals with high-risk *CTLA-4* genotypes could enable early intervention strategies, such as more frequent monitoring for liver fibrosis and HCC development. Additionally, therapies targeting CTLA-4 pathways, such as immune checkpoint inhibitors, may have potential applications in chronic hepatitis B patients with high CTLA-4 expression. However, caution is needed, as excessive immune activation could lead to liver inflammation and autoimmunity. Further studies are needed to explore the therapeutic modulation of CTLA-4 in chronic hepatitis B and its potential role in antiviral and anti-cancer strategies.

Despite these insights, certain limitations should be acknowledged. The focus on a single polymorphism does not fully capture the genetic complexity of immune regulation in chronic hepatitis B. The exclusion of other genetic markers, such as HBsAg quantification and covalently closed circular DNA (cccDNA) levels, may have restricted the ability to comprehensively assess HCC risk factors. Additionally, the absence of sequencing for other *CTLA-4* polymorphic sites limits the genetic scope of the study. Future research should incorporate haplotype analysis to elucidate how different *CTLA-4* variants interact to influence disease progression. Studies with larger sample sizes and multi-ethnic comparisons are warranted to validate these findings and assess their generalizability beyond the Batak population. Furthermore, functional studies investigating the impact of +6230G>A on *CTLA-4* mRNA stability and expression across different immune cell subsets would provide mechanistic insights into its role in HBV persistence and HCC development. A broader evaluation incorporating additional immune-related genetic markers and functional assays could enhance the understanding of the molecular mechanisms underlying HBV-induced hepatocarcinogenesis.

Conclusion

Batak patients with chronic hepatitis B carrying the *CTLA-4* +6230G>A GG+AG genotype had a significantly higher risk of HCC. Elevated CTLA-4 level was also associated with an increased risk of HCC, with significantly higher levels observed in patients with the GG and AG genotypes compared to those with the AA genotype. Liver cirrhosis, elevated ALT levels, high HBV DNA levels, and older age showed significant associated with HCC. Further investigations should examine additional *CTLA-4* polymorphisms, haplotype variations, HBsAg levels, and cccDNA to enhance understanding of immune regulation in HBV-induced HCC and its broader clinical implications.

Ethics approval

The study received ethical approval from the Health Research Ethics Committee of all relevant institutions in Indonesia prior to the commencement of any research activities (Approval

number: 791/KEPK/USU/2024). Patients meeting the inclusion criteria provided informed consent for inclusion in the study.

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

Declaration of artificial intelligence use

We hereby confirm that no artificial intelligence (AI) tools or methodologies were utilized at any stage of this study, including during data collection, analysis, visualization, or manuscript preparation. All work presented in this study was conducted manually by the authors without the assistance of AI-based tools or systems.

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