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Evaluation of BTLA (CD272) Immune Checkpoint Levels Related to Hepatitis B Virus Infection in Diabetes Mellitus Patients

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ABSTRACT

Background: *Diabetes mellitus is a persistent disorder characterized by an imbalance in the regulation of glucose levels in the body. Hepatitis B is a hepatotropic virus that may trigger viral hepatitis, cirrhosis, and hepatocellular carcinoma. The B and T lymphocyte attenuator (BTLA/CD272) is a co-inhibitory receptor present in T lymphocytes and B lymphocytes. It plays a crucial role in controlling the activation of lymphocytes during periods of inflammation and infection. BTLA has high levels of expression in T-cells that are particular to the virus, resulting in a strong inhibitory impact on processes such as T-cell proliferation and cytokine release.* ***Objective****: The study aims to assess BTLA levels in relation to HBV infection in diabetes mellitus patients.* ***Subjects and methods****: A cross-sectional study was conducted from July to October 2023. The serum was obtained from 200 diabetics. All of the patients were tested using an ELISA technique for BTLA and HBc IgG. They were tested using a five-panel kit for HBsAb, HBsAg, HBcAb, HBeAg, and HBeAb. The statistical analysis approach was conducted using SPSS version 26.* ***Results****: Serum BTLA levels were correlated with serum HBc IgG positivity (P = 0.043) and total HBc Ab positivity (P = 0.017). There was no correlation between BTLA levels and serum HBs Ab (P = 0.151) or HBe Ab (p = 0.941). BTLA levels were not correlated with diabetes mellitus (P = 0.786).* ***Conclusions****: Elevated levels of BTlA in diabetic individuals who are exposed to the hepatitis B virus compared to those without this virus.*

Keywords: *Anti-HBc IgG, BTLA, Diabetes mellitus, Hepatitis B virus, Immune checkpoints.*

Article Information

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INTRODUCTION

Diabetes mellitus (DM) is a chronic disease characterized by an imbalance in glucose homeostasis, and it is becoming a worldwide epidemic (1). The prevalence of diabetes mellitus has increasingly become more predominant in recent decades, with an expected occurrence of DM reaching 592 million globally by the year 2035 (2). Diabetes mellitus arises from reduced insulin synthesis or insulin resistance, leading to elevated blood sugar levels (hyperglycemia) (3). The new coinhibitory protein BTLA potentially plays a regulatory function in retaining peripheral tolerance. Nevertheless, its involvement in autoimmune diabetes remains uncertain (4). However, there is a scarcity of information concerning the inhibitory pathway of BTLA-HVEM in the initiation and progression of T1D (5).

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The hepatitis B virus remains a persistent global health concern due to the ongoing complications associated with chronic infection, which continue to contribute significantly to morbidity and mortality (7). Hepatitis B virus produces a variety of antigens, such as hepatitis B core, surface, and envelope antigens. These antigens possess strong immunogenicity and can induce an immune reaction (8). A lot of IgM and then IgG anti-HBc are made because the hepatitis B core antigen is the part of HBV that makes people's immune systems react the strongest. Anti-HBc, typically assessed as a combination of IgG and IgM antibodies, is widely regarded as a highly sensitive and dependable marker for exposure to the hepatitis B virus. The levels of anti-HBc IgM rise fast after acute infection. Because of this, anti-HBc IgM levels are part of the first diagnostic tests (9). Patients who have recovered from the infection will have a lifelong presence of anti-HBc antibodies. IgM anti-HBc indicates acute infection, but IgG anti-HBc indicates prior infection (10, 11). This diminished immune response makes the host's immune system incapable of eradicating HBV, resulting in a persistent infection (12). After a long-term HBV infection, the host's immune system frequently exhibits deficiencies or lacks the reactivity of T-cells specific to the virus. T-cell exhaustion refers to a state in which T cells exhibit diminished immune responses characterized by cytotoxicity, reduced cytokine synthesis, and enhanced expression of several inhibitory molecules (13). B and T lymphocyte attenuator (BTLA), one of the immune checkpoints, is a crucial regulator of the immune system that moderates the immune response (14). Moreover, it is present on both T and B cells (15). BTLA, initially named CD272, was identified by a genetic analysis in 2003 for its potential to block Th1 cell expression (16). The BTLA gene is located on chromosome 3 at 3q13. It includes 5 exons and covers 870 base pairs long (17). The upregulation of BTLA by lymphocytes is associated with a decrease in T cell function during infectious viruses like HBV. BTLA facilitates T cell responses during chronic HBV infection. The restriction of T cell responses is worsened by high antigen levels, resulting in T cell exhaustion (18). BTLA is highly expressed in the peripheral blood of people who have HCC caused by HBV and is significantly related to the increase of CD4+CD25+Treg cells. BTLA may boost the efficacy of CD4+CD25+Treg cells, suppress the function of T cells and proliferation, and probably help the tumor immune escape (19). Now, there is a gap in research, particularly on the correlation between the amount of serum BTLA and the clinical parameters of HBV. This study is the first one that evaluates the BTLA levels and their relationship with clinical hepatitis B virus parameters, in individuals diagnosed with diabetes mellitus in Iraq. The purpose of this study is to examine BTLA levels in Iraqi patients with diagnosed DM and their link with clinical indicators of HBV infection.

SUBJECTS AND METHODS

The current research is a cross-sectional study carried out in Najaf province from July to October 2023. The study involved only Iraqi people who were the patients of the Endocrinology and Diabetes Department of Al-Sader Teaching Hospital in the city of Najaf. The study comprised a total of two hundred subjects. The age of the patients varied from 18 to 80 years. All patients were diagnosed with diabetes mellitus based on clinical diagnosis by specialists and serological tests. The specimens were obtained by extracting approximately 10 mL of venous blood from every participant. Blood samples were placed into a gel tube and allowed to coagulate at room temperature for thirty minutes. The serum was extracted using centrifugation and thereafter divided into 1.5-

ml Eppendorf tubes. A part of the serum was immediately utilized for a fasting blood glucose test. The other part was then preserved in a refrigerator at a temperature of -80°C for immunological investigation until further examination. Enzyme-linked immunosorbent assays (ELISA) and rapid diagnostic tests are the main methods used in clinical laboratories to find HBV serological markers. A fraction of the serum was used for a human hepatitis B virus panel test (five panel kit): HBc Ab, HBs Ag, HBs Ab, HBe Ag, HBe Ab (Eugene Biotech/China), anti-HCV rapid test, One Step Cassette Style HIV Rapid Test (Wondfo/China), After that, an ELISA method (Sun Long Biotech, China) was used to find a B and T lymphocyte attenuator (BTLA**)** and a qualitative HBc IgG. The measurement of fasting plasma glucose (FPG) was conducted using an enzymatic colorimetric method and a kit available commercially from Spinreact, a company in Spain. The procedures for all tests were conducted according to the instructions outlined in the kit's manual. The patients' data were obtained through the implementation of a questionnaire and the collection of a blood sample. Before enrolling in the research study, all patients received a comprehensive briefing on the study's aims and objectives and were then given their informed consent to participate.

Included criteria

All patients in the study were diagnosed with diabetes mellitus (only type 1 or type 2).

Exclusion criteria

Patients with a medical history of autoimmune disorders, cancer, and other viral infections such as the hepatitis C virus (HCV), COVID-19, HIV, and vaccinated individuals with the HBV vaccine.

Ethics Committee approval

The ethics council of the University of Kufa's Faculty of Medicine gave its approval before starting this research project. All participants gave their consent, and the Al-Sader Teaching Hospital in the province of Najaf gave its approval.

Statistical analysis

The data was inputted using Excel 2016 for Microsoft Windows, while the statistical analysis was performed using the Statistical Package for Social Sciences (SPSS-26). The distribution of the study group according to different parameters was assessed using frequency and percentage. The variables were reported as the mean and standard deviation for normally distributed (parametric) data and the median and interquartile range (IQR) for non-parametric data. The Shapiro test was used to evaluate the normality of the data distribution, whether parametric or non-parametric, when evaluating the lab. The non-parametric Mann-Whitney U test was employed to demonstrate the relationship between categorical and numerical data. With a p-value of less than 0.05, it was possible to determine the statistical significance of the results. To assess the difference in the medians, the Kruskal-Wallis test was utilized as an alternative to the one-way ANOVA. The chi-square test is utilized to determine the association between two category variables by analyzing the variables and parameters. The presentation of the results is done using MS Word and Excel 2016, with tables and figures along with an explanation paragraph.

RESULTS

The present study included a sample of 200 patients who were diagnosed with diabetes mellitus, specifically type 1 and type 2, while excluding other types. Based on the findings, it was observed that 56 patients (28%) were diagnosed with type 1 DM, whereas 144 patients (72%) were diagnosed with type 2 DM, ranging in age from 18 to 80 years old. The participants' median age was 50 years, and the mean age ± standard deviation was 49.71 ± 13.656.

Table 1 shows the serum BTLA values for the participants. No statistically significant (p-value > 0.05) but clinically significant increase in the mean serum BTLA levels was seen in patients with type 1 diabetes compared to patients with type 2 diabetes.

Females had a significantly higher mean level of BTLA [105.75 (ng/ml)] than males [89.34 (ng/ml)]. At the same time, the disparity did not exhibit statistical significance (P = 0.061). As seen in Tab 1.

Table 1: Comparative evaluation of BTLA levels between type 1, type 2 diabetes mellitus patients and sex.

	Variables		**N**	**Mean Rank**	**P value**
BTLA (ng/ml)	Types of DM	Type1 DM	56	102.29	P=0.786 Z=-0.272-
		Type 2 DM	144	99.81	
	Sex	Male	64	89.34	P=0.061 Z=-1.871-
		Female	136	105.75	
P values were calculated by the Mann-Whitney U test. * Significant difference (P < 0.05) between groups, DM (Diabetes mellitus).					

Table 2 shows the serum BTLA values for the participants. No statistically significant (p-value > 0.05) in the median serum BTLA levels was seen in patients within the different age groups. Table 2 shows the serum BTLA values for the participants .No statistically significant (p-value > 0.05) in the median serum BTLA levels was seen in patients with a variant duration of diabetes mellitus.

Table 2: The relation between BTLA and age group, duration of diabetes in diabetic patients.

	Variables		**N**	**Median (IQR)**	**P value**
BTlA (ng/ml)	Age group/ Year	< 20	4	4.3100 (3.96)	P= 0.462 df= 3
		20-39	31	6.5600 (5.09)	
		40-59	107	5.9900 (5.30)	
		60-79	58	5.6750 (4.81)	
	Duration of DM/ year	less than 5 years	51	5.1500 (5.17)	P= 0.366 df= 2
		From 5 to 15 years	116	6.3500 (4.89)	
		More than 15 year	33	5.3900 (5.45)	
P values were calculated by the Kruskal-Walli's test, * level of significance at < 0.05, IQR (interquartile range), DM (Diabetes mellitus).					

In this study, among the 200 diabetes mellitus patients, 151 (75.5%) of patients had HBc IgG +, 154 (77%) had HBc Ab +, 21 (10.5%) had HBs Ab +, and 33 (16.5%) had HBe Ab +. There was no positive result for HBs Ag, HBe Ag, HCV, or HIV. As shown in Fig. 1.

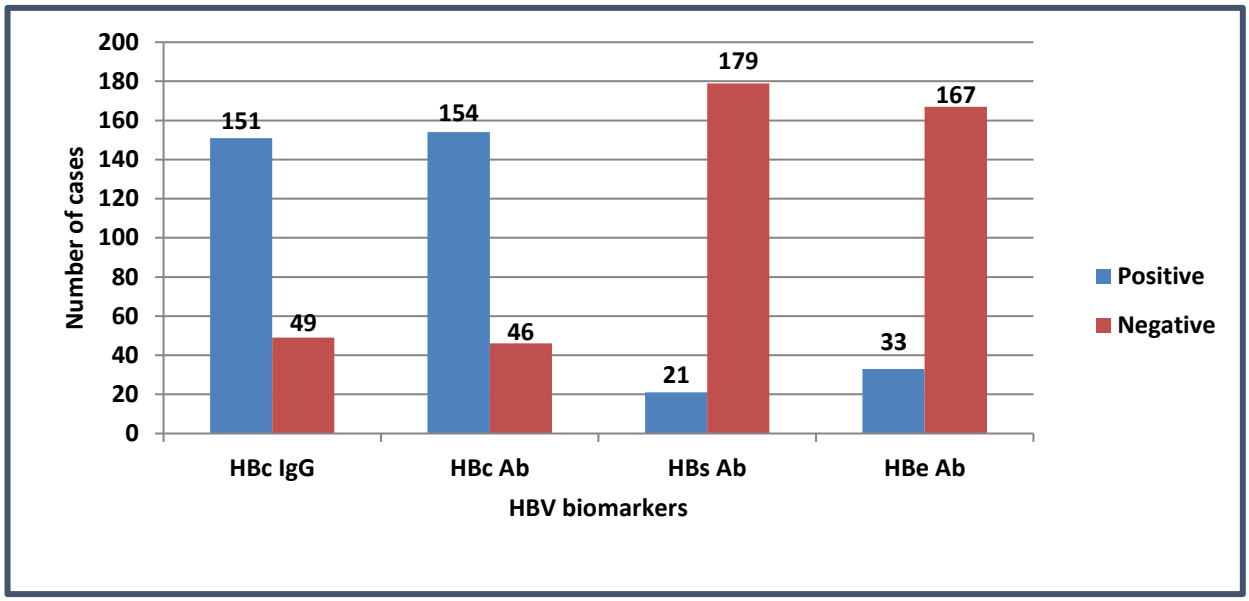

Figure 1: HBV biomarkers in diabetes mellitus patients.

Table 3 presents the serum BTL A measurements for the participants. The mean serum BTLA levels went up significantly (p-value < 0.05) in people who tested positive for HBc IgG and HBc AB. Additionally, the difference did not exhibit statistical significance (P >0.05) in the mean serum BTLA levels in patients who tested positive for HBs Ab and in patients who tested positive for HBe Ab.

Table 3: Assessment of BTLA levels across HBV markers HBc IgG, HBc Ab, HBs Ab, and HBe antibodies.

	HBV markers	positive		Negative		Test statistics
		N	Mean Rank	N	Mean Rank	
BTLA (ng/ml), Mean Rank	HBc IgG	151	105.23	49	85.93	P= 0.043* Z=-2.028-
	HBc Ab	154	105.84	46	82.63	P=0.017* Z=-2.386-
	HBs AB	21	117.67	179	98.49	P=0.151 Z=-1.437-
	HBe Ab	33	99.82	167	100.63	P=0.941 Z=-0.074-
P values were determined using the Mann-Whitney U test. * There is a statistically significant difference (P < 0.05) between the groups.						

As indicated in Tab 4, there was no statistically significant correlation between the BTLA value and FBG level (P = 0.110).

Table 4: Correlations between FBG and BTLA Level.

parameter	BTLA(ng/ml)	
	r	P-value
FBG(mg/dl)	-0.113-	0.055
At P-value < 0.01 (1-tailed) , FBG (Fasting blood glucose), r (Pearson correlation)		

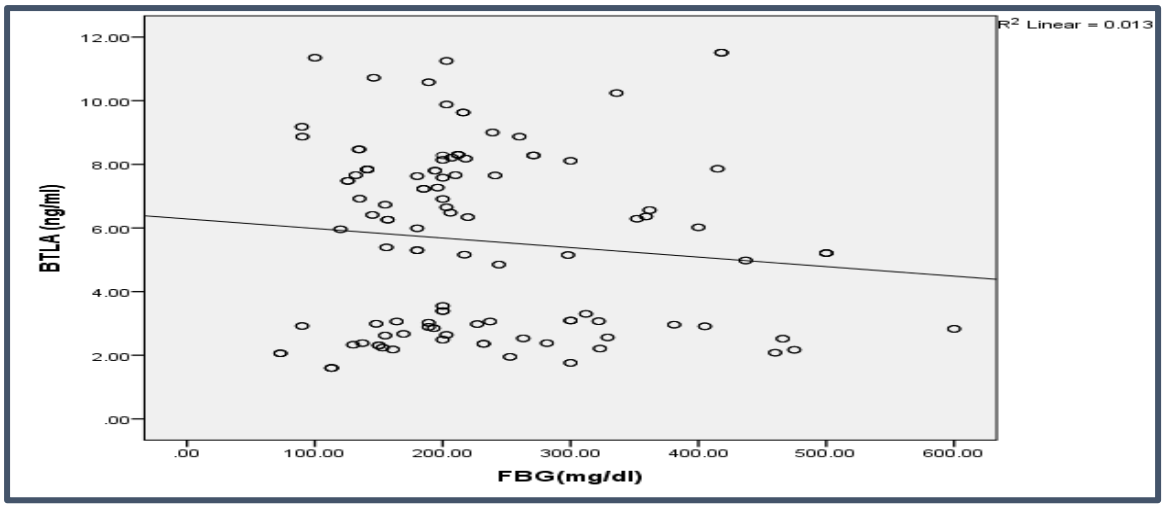

Figure 2: Serum BTLA and FBG levels demonstrated by Pearson correlation

DISCUSSION

The presence of co-stimulatory and co-inhibitory molecules is crucial in initiating and stopping the immune response. These molecules have been extensively researched as potential genes related to autoimmune illnesses (20, 21). However, so far, little is known about the BTLA-HVEM inhibition pathway in the initiation and growth of diabetes mellitus. The current investigation did not discover any statistically significant difference between serum BTLA amounts and type 1 or type 2 diabetes mellitus with a $P > 0.05$. Also, there is no substantial or significant correlation between the BTLA value and fasting blood glucose level ($P = 0.110$) or duration of diabetes ($P = 0.366$) in diabetic patients. This result is consistent with Inuo et al.'s (2009) study, which did not uncover any genetic influence of the BTLA gene on the origin of T1D among the Japanese population. Hence, the role of the BTLA gene in the progression of T1D is not yet established (22). Tsutsumi et al. (2006), a group in Japan, examined the role of the BTLA gene as a vulnerability factor in the development of type 1 DM. The research revealed no significant variations in terms of the genotypes, alleles, and haplotypes of the BTLA gene (rs2705534, rs2171513, and rs92889). The results provided from those investigations showed that the BTLA gene probably does not contribute to the emergence of T1D in the Japanese population (23).

In the current study, there were no statistically significant (p-value >0.05) serum BTLA levels and age group. The outcome is dissimilar with the one stated by Bian et al. study done in 2019. Kannan et al. (2015) study showed that levels of BTLA decrease with ageing, so in consequence, the immune response to the trivalent flu vaccine is reduced (24). The experimental group with an elevated amount of sBTLA had a shorter survival time than the group with lowered levels (25). An increased level of this protein increases the risk of mortality (26). This study found that the BTLA level in females is slightly higher than in males. However, this difference is not statistically significant ($P = 0.061$) and does not suggest any difference between the sexes. The reason for this may be due to the presence of the same immune response to the pathological conditions to which both sexes were exposed and the absence of clear differences in the levels of the immune factor, as they both suffer from the same diabetes condition, level of diseases, and state of the immune response, which may not differ between the sexes at that moment and in these particular disease groups.

Currently, there is a lack of research investigating the relationship between the amount of serum BTLA and the clinical parameters of the hepatitis B virus. The current study displays that BTLA was shown to have a higher significant correlation with the presence of HBc IgG (P = 0.043) and HBc Ab (P = 0.017) antibodies. The present outcome, similar with the one stated by Hua-Feng et al. in 2022, suggests that the elevated expression of BTLA and HVEM on CD4+ and CD8+ T cells' surfaces may have clinical relevance and serve a function in the progression of CHB after T cell exhaustion (27). As evidenced by Liao et al., there is a very notable increase in the levels of BTLA on HBV-related transformations of HCC from cirrhosis to liver cancer; therefore, this underscores the key role BTLA holds when it comes to the development of CHB infection (28).

In the year 2017, Shen et al. revealed that the CD8+ BTLA+ T lymphocytes, which can be extracted from the hepatic tissues of patients suffering from CHB, have an inhibitory function on regulatory T cells, thus helping HBV evade from the elimination by the immune system (29). Although the discovery implies that BTLA is not the only government factor, there is still a general divergence in the amount of BTLA in separate cytotoxic T lymphocyte (CTL) subtypes in both the hepatic cell and the peripheral blood cell of CHB people. Specifically, in the peripheral blood, BTLA is predominantly expressed in the Tcm subtype of T-lymphocytes; in reverse, within the hepatic cell, BTLA is mainly expressed in the Tem subtype. This discrepancy can be attributed to an increase in BTLA expression throughout the migration of peripheral CD8+ T-cells to the hepatocytes, thereby hindering the exaggerated transition of CD8+ T-cells from the central memory (CM) phase to the effector memory (EM) phase, which in turn aids in the evasion of immune clearance by HBV. Consequently, it is postulated that CD8+BTLA+T cells possess the capacity to exert an adverse modulatory impact on Treg cells (29). The BTLA expression in patients at different stages of HBV infection across various severities of CHB ought to be analyzed. More research is required to completely elucidate the connection between BTLA and HBV infection in people with diabetes.

Limitations of this research

Firstly, the study design (a cross-sectional study) cannot show cause and effect but can effectively validate or invalidate assumptions. Secondly, the study was conducted at a single center with a narrow sample size.

CONCLUSIONS

The study concluded that the relationship between the amount of serum BTLA and the clinical parameters of hepatitis B virus (HBV), such as anti-HBc IgG, anti-HBc Ab, and elevated levels of BTlA in diabetes mellitus individuals who are exposed to hepatitis B virus compared to those without this virus may be due to an excessive immunological response to the virus. This study has the potential to open up novel possibilities for future research on using the immune checkpoint BTLA as a treatment method for diabetic individuals with HBV infection.

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