DOI: https://doi.org/10.53350/pjmhs22164464

ORIGINAL ARTICLE

The Role of Granzyme B Activity and Concentration, and Serpin B9 Concentration with Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Virus

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ABSTRACT

Backgrounds: Granzyme B (GzmB) is the main effective weapon of adaptive immune response that involved in killing of the infective as well as cancerous cells. Serum concentration and/or activity of GzmB may influence the course of chronic hepatitis B (CHB).

Aims of the study: To investigate the role of serum level of GzmB and its activity as well as the effect of serpin B9 inhibitor on serum level of GzmB in patients with HCC.

Patients and Methods: This is a cross-sectional study which included 85 patients with CHB. Those patients were divided into two groups: Group 1: includes 45 patients diagnosed with HCC, group 2: includes 40 Patients without HCC. Demographic data of the patients were collected from direct interview. Liver function test were measured spectrophotometrically. Granzyme B activity was estimated using spectrophotometry. Enzyme linked immunosorbent assay was used to measure Granzyme B concentration, serum level of and serpin B9.

Results: Patients without HCC had significantly higher activity (median: 58 pmol, range: 34.0-130.0 pmol) and concentration (median; 31 pg/ml, range: 0.0-383.0 pg/ml) than those without HCC (median activity: 26, range: 7.0-63.0, median concentration: 18.7 pg/ml, range: 0.0-52.0 pg/ml) with highly significant differences.

Conclusions: Granzyme B activity and concentration are significantly reduced in CHB patients with HCC compared with those without HCC. Thus, the reduction in GzmB activity cannot be attributed to increase Serpin B9, rather the GzmB polymorphism may be the main effector.

Keywords: Chronic hepatitis B, Hepatocellular carcinoma, Granzyme B activity and concentration, Serpin B9

INTRODUCTION

With an estimated 500,000–600,000 fatalities each year, hepatocellular carcinoma (HCC) is the second leading cause of cancer death globally (El-Serag and Rudolph; 2007; Venook et al., 2010). The interplay of genetic predisposition, environmental factors, and viruses (hepatitis B virus (HBV), hepatitis C virus (HCV)) drives the development of HCC. About 240 million people are chronically infected with HBV and remain at risk of developing liver cirrhosis and HCC despite the establishment of HBV vaccine programs since the early 1990s and the availability of potent antiviral treatments that lead to long-term inhibition of HBV replication [Levrero et al., 2016].

Several factors have been established as risk factor for development of HCC from chronic hepatitis B (CHB). Among these, patients' age and co-infection with human immunodeficiency virus (HIV) or other hepatitis viruses are well-documented (Campbell et al., 2021). Furthermore, other factors such as DM and hypertension were also suggested by some studies (Simon et al., 2018; Yang et al., 2011; Turati et al., 2013; Borena et al., 2012).

The main active immune arm against HBV is the cell-mediated immune response, in which Cytotoxic T lymphocytes (CTLs) eliminate virus-infected cells principally by releasing the contents of cytotoxic granules into the immune synapse formed with their target cell. Theses granules are serine proteases, known as granzymes (Gzms), which induce programmed cell death, after they are delivered into the target cell cytoplasm by the pore-forming protein perforin (Lieberman, 2010).

Granzyme B is the most important one that induces target cell apoptosis in a mitochondria-dependent manner, which is a highly regulated process involving the Bcl-2 family proteins that triggers early DNA damages (Safta et al. 2015). Gzms should work at maximum speed with lower Km and should be free of the inhibitors in order for the enzymes to successfully initiate apoptosis (Tan et al.,2015). In a previous study, Alshamary et al. (2019) demonstrated that GzmB concentration is reduced in treated and untreated CHB patients. However, the role of GzmB concentration and activity in the development of HCC in those patients was not fully illustrated. Therefore, the present study aimed to investigate the association between GzmB activity and concentration with HCC as well as to find out the effect of serpin B9 level on susceptibility of HCC.

MATERIALS AND METHODS

Study population: This is a cross-sectional study which was carried out in the Department of Chemistry and Biochemistry at the College of Medicine/Al-Nahrain university, in conjunction with the Department of Biochemistry Laboratory in Al Imamain Alkadhimain Teaching Hospital and Medical Research Unit/ College of Medicine/Al-Nahrain university during the period from 1st January 2021 to 31st December 2021. A total of 90 patients with CHB were included in the study. All adult patients diagnosed with chronic hepatitis B infection were included in this study. The diagnosis of CHB was primary based on positivity for HBsAg and anti-HBc-IgG antibodies accompanied with viral load beyond the reference range. The diagnosis of HCC was depended on ultrasound findings and Computed tomography (CT) scan. Patients with Renal failure, connective tissue disease, acute and Chronic infection, HCV infection or with other malignancy were excluded from the study. Eligible patients were divided into two groups:

Group 1: includes 45 patients diagnosed with hepatocellular carcinoma

Group 2: includes 40 patients without hepatocellular carcinoma.

Blood Samples: Five ml of venous blood was collected from each participant; 2 ml of which was kept in EDTA tube and the other 3ml in plan tube. The latter was undergone centrifugation where the serum was obtained and preserved at -20 ^{o}C until be used.

MATERIAL AND METHODS

Ready commercial ELISA kits (Biokit/Spain) were used to qualitatively measure each of HBsAg, anti-HBc IgG, HBeAg and anti-HBe. Liver function tests (total serum bilirubin (TSB), alanine aminotransferase determination (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were determination spectrophotometrically using ready kits (Agape/Switzerland). Serum level of alpha-fetoprotein, GzmB concentration and serpin

B9 concentrations were determined using ready commercial ELISA kits (Cell Sciences/USA). Finally, the human GzmB Activity was estimated using a ready kit (Ac-IEPD-pNA chromogenic granzyme B substrate by using Elisa commercial kit (Kemiya Biochemical/ USA).

Statistical Analysis: Statistical analyses were performed by using SPSS software version 25.0 (SPSS, Chicago). Continuous data were subjected to normality test (Shapiro Wilk test), Data with normally distribution were presented as mean and standard deviation, and analyzed with Student t-test (for two group comparison) or analysis of variance (ANOVA) (for three group comparison). Data with non-normal distribution were presented as median and range and analyzed Kruskal Wallis. Categorical variables were expressed as number and percentage and analyzed with Chi-square test. Receiver operating characteristic curve (ROC) was used to evaluate the diagnostic value of GzmB concentration and activity in the context of discrimination between CHB with and without HCC. Pearson's correlation test was used to explore the possible correlation of GzmB concentration and activity, AFT and serpin B9 with other numerical variables. A p-value less than 0.05 was considered to indicate a statistically significant difference.

RESULTS

Demographic and Laboratory Data of the Study Population: Although patients with HCC demonstrated higher mean age than those without HCC (42.7±14.18 years vs. 37.8±13.46 years), the difference was not significant. The two groups were comparable in terms of gender distribution and HBeAg positivity with no significant differences. However, patients without HCC had far more frequent positivity for HBeAb than those with HCC (46.67% vs. 7.5%) with a highly significant difference (Table 1). Interestingly, all patients in both groups were positive for HBsAg and HBcAb-IgG.

Table 1: Demographic and laboratory characteristics of the study

Variables	Without HCC (n= 45)	With HCC (n=40)	P- value
Age, years Mean±SD Range	37.8±13.46 22.0-71.0	42.7±14.18 25.0-75.0	0.116
Gender Male Female	26(57.78%) 19(42.22%)	29(72.5%) 11(27.5%)	0.156
HBeAg Positive Negative	27(60%) 18(40%)	29(72.5%) 11(27.5%)	0.225
HBeAb Positive Negative	21(46.67%) 24(53.33%)	3(7.5%) 37(92.5%)	<0.001

Table 2: Liver function tests

Variables	Without HCC (n= 45)	With HCC (n=40)	P- value
AST (IU/L) Mean±SD Range	30.18±9.67 14.0-51.0	61.57±8.93 45.0-86.0	<0.001
ALT (IU/L) Mean±SD Range	35.02±7.07 20.0-50.0	58.47±9.17 42.0-81.0	<0.001
ALP (U/L) Mean±SD Range	77.27±20.33 43.0-117.0	134.97±18.58 110.0-182.0	<0.001
TSB (mg/dL) Mean±SD Range	0.79±0.18 0.5-1.2	1.76±0.69 0.8-3.4	<0.001

Liver Function Test: Mean serum level of AST, ALT, ALP and TSB in patients with HCC was (61.57±8.93 IU/L, 58.47±9.17 IU/L, 134.97±18.58 IU/L and 1.76±0.69 mg/dl, respectively which was higher than those without HCC (30.18±9.67 IU/L, 35.02±7.07 IU/L, 77.27±20.33 IU/L and 0.79±0.18 mg/dl, respectively) with highly significant differences (Table 2).

Granzyme B Activity and Concentration: Data regarding GzmB activity and concentration and serpin B9 concentration were found to be non-normally distributed. Accordingly, these data were expressed and median and range and analyzed with non-parametric Mann Whitney U test). Patients without HCC had significantly higher activity (median: 58 pmol, range: 34.0-130.0 pmol) and concentration (median: 31 pg/ml, range: 0.0-383.0 pg/ml) than those without HCC (median activity: 26 pmol, range: 7.0-63.0 pmol, median concentration: 18.7 pg/ml, range: 0.0-52.0 pg/ml) with highly significant differences (figure 1).

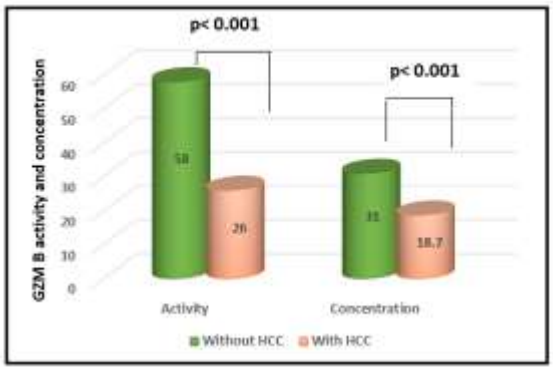

Figure 1: Median concentration and activity of granzyme B in hepatitis patients with and without HCC

Serpin B9: Although, serum level of serpine 9 in patients without HCC (median: 44.0 ng/ml, range: 11.2-79.2 ng/ml) was higher than that of patients with HCC (median: 28.1 ng/ml, range: 9.7-79.2 ng/ml), the difference was not significant (p= 0.216)

Diagnostic Value of Different Markers: Receiver operating characteristic (ROC) curve was used to evaluate the diagnostic value of GzmB activity and concentration in the context of discrimination between hepatitis patients with and without HCC. For GzmB activity, the AUC was 0.936, 95%CI=0.888-0.985, p< 0.001. The sensitivity and specificity of the test at cut off value of GzmB activity= 42.5 were 80% and 90% respectively. For GzmB concentration, the AUC was 0.735, 95%CI=0.627-0.843, p< 0.001. The sensitivity and specificity of the test at cut off value of GZB concentration = 21.5 were 71% and 62% respectively (Figure 2).

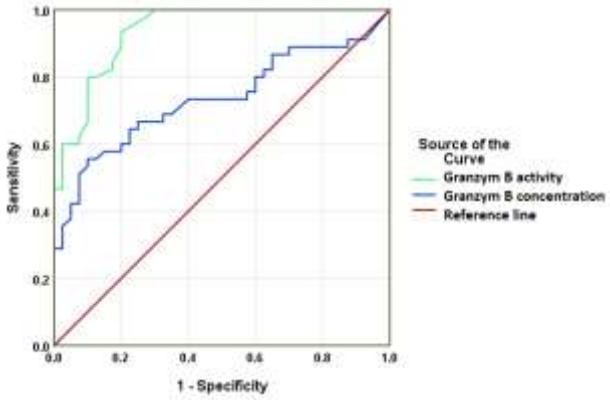

Figure 2: Receiver operating curve for granzyme B activity and concentration in the context of discrimination hepatitis patients with and without HCC

Correlation granzyme B concentration, and activity and serpin 9 with LFTs and age: Pearson's correlation and linear regression tests were used to find out the correlation of GzmB concentration, and activity, and serpin B9 with LFTs and age of the patients. In patients without HCC, none of these parameters had a significant association (Table 3).

Table 3: Pearson's correlation of granzyme B concentration and activity and serpin B9 with LFTs and age of hepatitis patients without HCC

Variables	Granzyme activity (pmol)		Granzyme conc. (pg/ml)		Serpin B9 (pg/ml)	
	r	p-value	r	p-value	r	p-value
Age	-0.197	0.194	-0.154	0.313	0.255	0.091
ALT	-0.218	0.151	0.181	0.234	-0.040	0.753
AST	-0.183	0.230	0.111	0.466	-0.083	0.586
ALP	-0.081	0.599	0.266	0.077	0.074	0.629
TSB	-0.213	0.160	0.269	0.074	-0.048	0.753
GzmB activity			-0.084	0.581	0.299	0.46
GZB conc.					-0.063	0.679

In patients with HCC, GzmB activity showed a positive significant correlation with TSB (r= 0.349, p= 0.027). Serpin B9 had a positive significant correlation with each of AST (r= 0.352, p= 0.026) and GzmB activity (r= 0.433, p= 0.005) as shown in table 4 and figure 6.

Table 4: Pearson's correlation of granzyme B concentration, and activity and serpin 9 with LFTs and age of hepatitis patients with HCC.

Variables	Granzyme activity (pmol)		Granzyme conc. (pg/ml)		Serpin B9 (pg/ml)	
	r	p-value	r	p-value	r	p-value
Age	-0.149	0.359	-0.172	0.289	-0.080	0.623
ALT	0.189	0.243	0.017	0.918	0.259	0.106
AST	0.291	0.068	0.170	0.293	0.352	0.026
ALP	0.240	0.136	-0.038	0.818	0.205	0.204
TSB	0.349	0.027	0.066	0.684	0.279	0.082
GZB activity			0.106	0.516	0.433	0.005
GZB conc.					-0.072	0.659

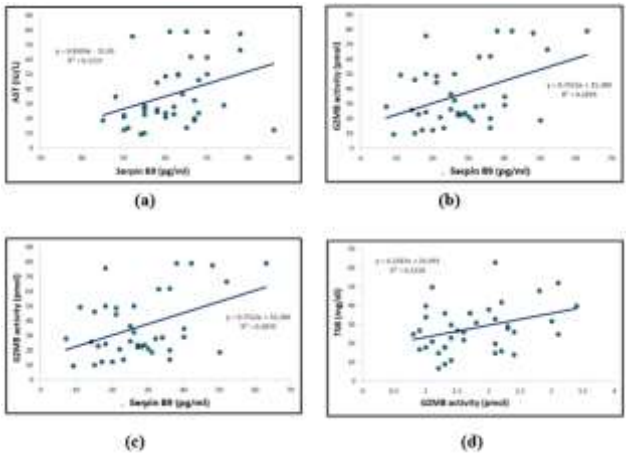

Figure 6: scatter plot and regression line between (a) serpin B9 and AST; (b) serpin B9 and GzmB activity; (c) GzmB activity and TSB; (d) in hepatitis patients with HCC

Table 5: Association of granzyme B concentration, and activity and serpin 9 with gender and hepatitis markers in hepatitis patients without HCC

Variables	Granzyme activity (pmol)	Granzyme conc. (pg/ml)	Serpin B9 (pg/ml)
Gender Male Female	 66(34-130) 49(36-118)	 27(0.0-383) 43(0.0-342)	 44.7(11.2-78.7) 37.7(11.7-79.5)
p-value	0.210	0.113	0.945
HBeAg Positive Negative	 64(34-130) 57(38-118)	 29.3(0.0-383) 36.5(0.0-326)	 37.7(11.2-79) 44.85(17.2-79.5)
p-value	0.899	0.626	0.494
HBeAb Positive Negative	 56(37.7-118) 65.5(34-130)	 36(0.0-326) 29.65(0.0-383)	 45.7(17.2-79.5) 32.7(11.2-79)
p-value	0.577	0.724	0.187

Association of granzyme B concentration, and activity and serpin B9 with Gender and Hepatitis Markers: In patients without HCC, there was no significant association of GzmB concentration, and activity and serpin B9 with gender and hepatitis markers. Although males had lower median GzmB concentration than females (27 pmol vs. 43pmol), the difference was not significant (Table 5).

In patients with HCC, females demonstrated higher median of serpin B9 than males (46.4 ng/ml vs. 24.6 ng/ml) with a significant difference. HBeAb negative patients showed lower median of GzmB activity than those with HBeAb positive patients (26 pmol vs 40 pmol) as demonstrated in table 6.

Table 6: Association of granzyme B concentration, and activity and serpin B9 with gender and hepatitis markers in hepatitis patients with HCC

Variables	Granzyme activity (pmol)	Granzyme conc. (pg/ml)	Serpin B9 (pg/ml)
Gender Male Female	 25(9.0-50) 32(7.0-63)	 20(0.0-40) 10.2(0.0-52)	 24.6(9.7-79.2) 46.4(14-79)
p-value	0.437	0.369	0.050
HBeAg Positive Negative	 26(9.0-50) 30(7.0-63)	 18.5(0.0-38.2) 22(0.0-52)	 28(9.7-79) 28.2(10.2-79.2)
p-value	0.511	0.254	0.835
HBeAb Positive Negative	 40(30-63) 26(7.0-52)	 7.9(0.0-40) 18.8(0.0-52)	 35(21.4-79) 28(9.7-79.2)
p-value	0.048	0.524	0.524

DISCUSSION

The current study aimed to investigate patients with CHB infection (with HCC and without HCC). Although patients with HCC demonstrated higher mean age than those without HCC (42.7±14.18 years vs. 37.8±13.46 years), the difference was not significant. It is reasonable to assume that patients with HCC are older than those without HCC. That is because the HCC development takes a considerable time of duration from hepatitis to fibrosis and then to liver cirrhosis until the carcinogenic process. However, it is well-known than only small percentage of HBV infection develop HCC, and most cases remain as CHB even with advances age.

In the present study, patients without HCC had far more frequent positivity for HBeAb than those with HCC (46.67% vs. 7.5%) with a highly significant difference. In fact, the presence of HBeAb indicating a non-replicating stage of the virus during which the HBeAg is no longer present in blood and HBeAb is generated. This HBeAg serconversion, or loss of HBeAg and the gaining of the antibody, HBeAb, can happen in many circumstances including response to treatment and spontaneous recovery due to immune response (Curry et al., 2010). Usually, these antibodies are decline after the acute stage which explains the low positivity in the majority of HCC patients. On the other hand, a relatively high proportion HBV patients without HCC were positive for these Abs indicating the persistence of replicating virus in those patients.

In the present study, all included LFTs (AST, ALT, ALP and TSB) increased in patients with HCC compared to without HCC despite the fact that almost all these levels in both groups were within normal limits. In accordance with these results are a Chinese study (Yang et al.; 2021) in which the median level of ALT, AST, ALP and TSB was 27.0 U/L, 33.0 U/L, 99.0 U/L and 20.41 µmol/L, respectively in patients with CHB without HCC compared with 32.0 U/L, 44.5 U/L, 134.5 U/L and 24.46 µmol/L, respectively in patients with HCC with highly significant differences. However, another study, Hann et al. (2012) did not observe a significant rise in ALT in patients with HCC compared with those without HCC. In contrast, AST and ALP exhibited a significant association with HCC risk. Ren et al. (2019) compared LFTs between patients with liver cirrhosis and HCC. Compared to the liver cirrhosis group, patients with HCC had higher albumin, g-glutamyltranspeptadase (GGT), and ALP, while TSB and total bile acid (TBA) were significantly reduced.

These variations between different studies are reasonable and can be attributed to several factors, the most important of which are the demographic characteristics of the study populations, disease duration, type and duration of received treatment, and the presence of comorbidities. Therefore, none of these tests could be used as reliable diagnostic or prognostic marker for the development of HCC in patients with HBV infection.

The increased serum level of all LFTs in the present study could be explained from two points of view: firstly, the increase destruction of hepatocytes by cancerous cells with a subsequent release of liver enzyme. Secondly, it has been confirmed that cancer cells can have certain properties of the source cells (Clark et al., 2007). For example, keratin can still be synthesized in epithelial cancers and the HCC cells can express hepatocyte markers in the embryonic stage such as epithelial cell adhesion molecule (Pineau et al., 1999).

The current study showed lower GzmB activity and concentration were significantly lower in patients with HCC compared with those without HCC. Very few studies addressed the association of GzmB with HCC. In a Chinese study including 547 HCC patients, 44 CHB patients, 86 liver cirrhosis patients, and 88 healthy individuals, Fu et al. (2013) compared the activity of CTL between those subjects. The most interesting finding in this study that there were intrinsic qualitative defects in CTLs in HCC patients associated with decreased production of GzmB. The authors further demonstrated that this defect is due to CD107a mobilization. CD107a is a lysosomal-associated membrane glycoprotein that surrounds the core of the GzmB and perforin granules in these cells. Upon T cell receptor (TCR) engagement, CD107a is exposed on the cell membrane of CTLs (Chen et al., 2006).

Several hypotheses explained reduction in GzmB in HCC. Among these, the presence of a defect in the intracellular signal cascades generated from the TCR and CD3 proteins specially defect in the proximal TCR signal transduction or distal signaling pathway lead to effects in the production of GzmB, or it may due to defect in the DNA transcription (Rousalova and Krepela, 2010). Another reason why GzmB level is reduced in HCC patients could be the defect in mRNA splicing or translation in the ribosomes as well as defect from the activation and proliferation of NK cells or cytotoxic cells in the immune system (Grossman et al., 2004).

Serum level of serpin B 9 in patients without HCC (median: 44.0 ng/ml, range: 11.2-79.2 ng/ml) was higher than that of patients with HCC (median: 28.1 ng/ml, range: 9.7-79.2 ng/ml); however the difference was not significant.

Unfortunately, there is no available previous study investigating serum level of serpin B9 in HCC. However, Rousalova et al. (2010) reported that non-small cell lung cancer (NSCLC) cells express serpin B9 and that there is a subset of NSCLC cells with upregulated mRNA of this protein. The authors suggested that the upregulation of serpin B9 expression in NSCLC cells may serve to protect them from apoptosis induced by GzmB.

As there were no significant differences in serpin B9 level between patients with and without HCC in the present study, the reduction in GzmB concentration in HCC patients could not be attributed to increase in serpin B9 in those patients.

CONCLUSIONS

Anti- Hepatitis B envelop antibodies are less common in CHB patients with HCC than those without HCC. Hepatocellular carcinoma is associated with an elevation of most liver enzymes and TSB compared with CHB patients without HCC, although these parameters still within normal limits. Granzyme B activity and concentration are significantly reduced in CHB patients with HCC compared with those without HCC. Thus, elevated liver enzymes and other LFTs could not be used for discrimination between CHB patients with and without HCC because they are usually within normal range. Alpha-fetoprotein could be efficiently used as additional diagnostic and even a predictor for the development of HCC in patients with CHB. Thus, regular measuring of this protein can help in early detection of HCC in CHB patients.

Acknowledgement: The authors highly appreciate the great cooperation of included patients to complete the study. Also, we acknowledge the great help form all staff at Medical Research Unit/ College of Medicine/ Al-Nahrain University for their effort in molecular assays.

Conflict of interest: The authors declare that they have no conflict of interest

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