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Detection of Epstein Barr Virus in Hepatocellular Carcinoma Patients in Suez Canal Region: A Case-Control Study

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ABSTRACT
Background: Epstein-Barr virus (EBV), a gamma-1 herpes virus, is a lifelong asymptomatic infection that is carried by the vast majority of human populations. However, it appears etiologically related to several pre-malignant lymphoproliferative diseases. It often accompanies chronic liver disease and cirrhosis. EBV acts as a catalyst for virus C, demonstrating a connection between Hepatocellular carcinoma (HCC) and EBV virus. **Objective:** The aim of the current study is to evaluate the EBV prevalence in HCC patients, and whether it is contributed to the development of HCC by working synergistically with hepatitis C virus. **Patients and methods:** The study was conducted and included 90 HCC patients, 45 chronic liver disease patients, and 45 controls. Full history was taken, and clinical data of the patients were noted. Blood samples were drawn, and the following lab tests were performed: CBC, prothrombin time, alpha-fetoprotein, and liver function tests (AST, ALT, albumin, bilirubin). EBV IgM and IgG were done by ELISA technique. DNA extraction and EBV detection by PCR were performed for those who had positive EBV IgM. **Results:** EBV IgM antibodies was higher among HCC group compared to CLD group, while there was no difference in EBV IgG antibodies between the two groups. According to EBV detection by PCR, no difference was observed between the HCC and CLD groups in samples that tested positive for EBV IgM. **Conclusion:** EBV may have a role in development of HCC.
Keywords: EBV, HCC, CLD, Case control study, Suez Canal University.

INTRODUCTION
Epstein-Barr virus (EBV) spreads through body fluids, saliva, blood, and semen during sexual contact [(1)] and causes infectious mononucleosis known as kissing disease [(2)]. Most people get EBV during childhood and do not experience any symptoms. But in some cases, the virus switches off the normal process that controls cell growth and leads it to divide out of control and they can develop cancer after years of being infected with EBV [(3)]. EBV was the first virus to be identified as causing cancer in humans. It is linked to a number of human cancers that originate from lymphocytes, mesenchymal cells, and epithelial cells. Both immune-competent hosts and immune-compromised patients are susceptible to EBV-associated neoplasia. Examples of epithelial cancers associated to EBV include gastric adenocarcinoma and nasopharyngeal carcinoma [(4)].

Hepatocellular carcinoma (HCC) is a primary liver malignancy. It arises in those who suffer from chronic liver disease and cirrhosis [(5)]. HCC is regarded the sixth most common cancer worldwide [(6)], and the fourth most common cancer in Egypt [(7)]. It comes in third most prevalent cause of cancer-related mortality worldwide [(8)]. Because of the high endemic prevalence of both Hepatitis viruses B and C, Asia and Africa have the highest incidence of HCC. Both viruses increase the risk of developing chronic liver disease (CLD) and ultimately HCC [(9)]. Additionally, HCC has been observed in patients without liver cirrhosis, confirming virus-driven oncogene events. With hepatitis C virus, such kind of dual aetiology has been observed. Although these viruses seem to be crucial contributing factors to HCC, they are not enough for the hepato-carcinogenesis process, as it is a multistep process [(6)]. Since the last two decades, concern on EBV-related epithelial cancers, which account 80% of all EBV-related cancers, has been grown. It is well established that EBV is related with about 10% of gastric carcinoma (GC) and nasopharyngeal carcinoma (NPC) [(10)]. Some studies suggested that EBV has been associated to HCC, which is an epithelial cell malignancy, during induced infectious mononucleosis [(11)].

The aim of this study was to evaluate the prevalence of EBV in HCC patients, and whether it is contributed to the development of HCC by acting synergistically with hepatitis C virus.

PATIENTS AND METHODS
A case-control study was carried out and included 90 HCC patients, 45 CLD patients, and 45 healthy controls. Blood samples were taken from healthy adult blood donor volunteers for control group, HCC patients and chronic liver disease (CLD) patients who visited Suez Canal University hospital from January 2021 to July 2021.
Lab work was carried out in Clinical Pathology Department at Faculty of Medicine, and Botany Department at Faculty of Science, Suez Canal University.

Inclusion criteria:
1- HCC patients.
2- Chronic liver disease patients.
3- Both genders.
4- Age 18-60 years

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Exclusion criteria:
-Refusal
-Children

Study Tools:
1. Full history was taken including age, sex, education, material status, employment, duration, course, onset, and associated chronic disease.
2. The clinical data (cachexia, jaundice, splenomegaly, and ascites) of HCC patients was noted and ultrasound was used to detect the focal lesions.
3. Blood samples were drawn without anticoagulant, held at room temperature for 20 min, centrifuged at 3000 rpm for 15 minutes; the resulting supernatant was used to estimate liver function, alpha-fetoprotein (AFP) test, and EBV IgM and IgG antibodies by enzyme-linked immunosorbent assay (ELISA) (DIAsource Immunoassay human EBV IgM and IgG Antibodies ELISA kits, Belgium). Other blood samples were drawn in citrate tubes for estimation of the prothrombin time (PT). Another blood samples were drawn in EDTA tubes for estimation of complete blood picture (CBC) and for detection of viremic status of EBV by Polymerase chain reaction (PCR). DNA was extracted from blood samples (QIAamp® DNA Mini kit), then DNA concentration was quantified to be sure that the concentrations are pure enough to make RT-PCR by NanoDrop® ND–1000 Spectrophotometer (NanoDrop Technologies; Wilmington, Delaware, United States) and real-time RT-PCR was performed in a Mx3005P Real-Time PCR System (Agilent Stratagene, USA) using FAM primer and probe mix TaqMan® principle.

Ethical approval:

The study was conducted with approval from a Scientific Research Ethical Committee of Suez Canal University. A written consent was obtained from all individuals to take part in the current research. They were informed about research, aim, benefits of this study. Consent for using the data of the patients' files in the study was taken from Suez Canal University Hospital and Patients. To ensure data confidentiality a code number for linking the data from each subject was used. All of the data collected from everyone were strictly confidential and were not used outside this study. This research was conducted in agreement with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for human studies.

Statistical analysis

The gathered data were coded, processed, and interpreted using the SPSS (Statistical Package for Social Sciences) version 25.0 for Windows® (IBM SPSS Inc, Chicago, IL, USA). Data were tested for normal distribution using the Shapiro Walk test. Qualitative data were expressed as frequencies and relative percentages. Chi square test ($\chi 2$) and Fisher's exact test to determine the difference between two or more groups of qualitative variables. Quantitative data were expressed as mean and standard deviation (SD). Independent samples t-test or Mann-Whitney U test was used for comparison between two independent groups. The data were examined using ANOVA to test the statistical significance of differences between groups. P-value at ≤ 0.05 was significant.

RESULTS

Table 1 summarizes the numbers of focal lesions in HCC group.

Table 1: Focal lesion number among HCC patients.

HCC Patient number	Focal lesion number	Patient percent
52	1	57.8 %
24	2	26.6%
4	3	4.4%
10	4	11.1%

EBV IgM antibodies were positive in 29% of HCC patients 27% of CLD patients. EBV IgM antibodies were higher among HCC group compared to CLD group, while no difference in EBV IgG antibodies was observed between the two groups (Table 2).

Although according to EBV detection by PCR, no difference was observed between the HCC and CLD groups in samples that tested positive for EBV IgM (Table 3).

According to Child-Pugh score, there were more CLD patients in class A than HCC patients, while there were no patients with CLD in classes B or C (Table 4).
There was no correlation between EBV IgM antibodies and clinical manifestations of HCC patients (Table 5).

Table 2: EBV IgM and IgG antibodies in HCC versus CLD groups:

Variable	HCC N(%)	CLD N(%)	P value
Positive IgM	26(29)	12(27)	0.016*
Negative IgM	64(71)	33(73)	
Positive IgG	36(40)	20(44)	0.814
Negative IgG	54(60)	25(56)	

Means with different superscript are significantly different by Duncan's multiple range test. P ≤ 0.05.

Table 3: EBV PCR Results in HCC and CLD groups positive for EBV IgM antibodies:

EBV	HCC N (%)	CLD N (%)
Positive	0 (0)	0 (0)
Negative	26 (100)	12 (100)

Means with different superscript are significantly different by Duncan's multiple range test. P ≤ 0.05.

Table 4: Comparison between HCC and CLD groups regarding Child-Pugh score classification.

Child-Pugh class	HCC N (%)	CLD N (%)	P value
A	18 (20)	45 (100)	<0.001**
B	38 (42.2)	0	
C	34 (37.8)	0	

Means with different superscript are significantly different by Duncan's multiple range test. P ≤0.05.

Table 5: Relation between EBV IgM antibodies and the clinical data of HCC group.

Variable		Positive n (%)	Negative n (%)	P value
Cachexia	No	7(30.4)	16(69.6)	0.815
	Yes	6(27.3)	16(72.7)	
Jaundice	No	9(32.1)	19(67.9)	0.537
	Yes	4(23.5)	13(76.5)	
Splenomegaly	No	1(11.1)	8(88.9)	0.188
	Yes	12(33.3)	24(66.7)	
Ascites	No	7(28)	18(72)	0.883
	Yes	6(30)	14(70)	

Means with different superscript are significantly different by Duncan's multiple range test. P ≤0.05.

Hb concentration, TLC and platelet count did not differ among the studied groups. Prothrombin time and INR values were higher in HCC and CLD groups than control one, while there was no difference between HCC group and CLD one (Tables 6 and 7).

Table 6: Hematological parameters and prothrombin time in Control, HCC and CLD groups.

Variable	Control	HCC	CLD	P value
Hemoglobin Concentration	12.98 ± 1.48	10.13 ± 1.1	10.42 ± 1.76	0.074
TLC	7.8 ± 1.2	5.71 ± 1.4	6.85 ± 1.5	0.601
Platelet count	170 ± 2.3	104.9 ± 2.7	150.5 ± 1.8	0.6
Prothrombin time (sec)	12.4 ± 0.34	15.4 ± 0.58	16.3 ± 0.38	< 0.001**
INR	1.14 ± 0.25	1.4 ± 0.39	1.55 ± 0.36	< 0.001**

Means with different superscript are significantly different by Duncan's multiple range test. P ≤0.05.

Table 7: Post Hoc test between each two groups of study regarding to prothrombin time and INR results.

Test	Groups	Mean Difference	P value
Prothrombin time (sec)	HCC & CLD	0.83±0.79	0.294
	Control & HCC	12.96±1.01	< 0.001**
	Control & CLD	12.13±1.01	< 0.001**
INR	HCC & CLD	0.141±0.09	0.074
	Control & HCC	0.56±0.12	0.002**
	Control & CLD	0.51±0.11	< 0.001**

Means with different superscript are significantly different by Duncan's multiple range test. P ≤0.05.

AST, ALT, total and direct bilirubin were higher in the HCC and CLD groups than control group, whereas they were higher in CLD group than HCC group. On the other hand, albumin level was declined in the HCC and CLD groups compared to controls, and no difference between HCC and CLD groups was observed. Meanwhile, when AFP was compared to the control group, it was considerably increased in both the HCC and the CLD groups, it was statistically higher in HCC group than CLD group (Tables 8 and 9).

Table 8: Liver function and AFP tests in control, HCC and CLD groups.

Variable	Control	HCC	CLD	P value
AST	**32.4 ± 0.25**	**64.7 ± 0.19**	**101.6 ± 0.34**	**0.0028***
ALT	**32.7 ± 0.38**	**76.3 ± 0.43**	**85.3 ± 0.29**	**0.004****
T. bilirubin	**0.73 ± 0.1**	**3.11 ± 0.2**	**4.19 ± 0.3**	**0.0139**
D. bilirubin	**0.43 ± 0.05**	**1.68 ± 0.04**	**3.19 ± 0.01**	**0.0144**
Albumin	**4.26 ± 0.45**	**2.9 ± 0.17**	**2.7 ± 0.32**	**< 0.001****
AFP	**3.61 ± 0.44**	**787.5 ± 2.36**	**17.3 ± 1.33**	**0.001****

Means with different superscript are significantly different by Duncan's multiple range test. P ≤0.05.

Table 9: Post Hoc test between each two groups of study regarding to the significant laboratory Liver function and AFP results.

Test	Groups	Mean Difference	P value
AST	**HCC & CLD**	**17.5 ± 2.6**	**0.493**
	Control & HCC	**62.2 ± 2.2**	**0.036***
	Control & CLD	**79.08 ± 1.8**	**0.008****
ALT	**HCC & CLD**	**22.49 ± 2.4**	**0.117**
	Control & HCC	**62.18 ± 1.8**	**0.001****
	Control & CLD	**42.78 ± 1.2**	**0.17***
Albumin	**HCC & CLD**	**0.08 ± 0.005**	**0.559**
	Control & HCC	**1.08 ± 0.21**	**0.001****
	Control & CLD	**1.38 ± 0.2**	**0.001****
AFP	**HCC & CLD**	**793.2 ± 2.0**	**0.001****
	Control & HCC	**662.8 ± 1.8**	**0.006***
	Control & CLD	**9.74 ± 2.4**	**0.982**

Means with different superscript are significantly different by Duncan's multiple range test. P ≤0.05.

DISCUSSION

HCC represents 80% of liver cancer cases. Liver cirrhosis, the most common precipitating factor for HCC, is caused by chronic liver diseases like chronic hepatitis, autoimmune hepatitis, HBV, and HCV infection [10]. EBV infection affects approximately 90% of people worldwide. EBV is responsible for 5.6% of infection-associated cancer and 1% of the world's cancer burden [12]. A study by **Sharaf *et al.*** [13] found genome of EBV in Egyptian breast cancer patients and showed that virus was linked to unfavorable prognostic factors suggesting that it may contribute to the aggression of the tumor.

In our study, we found that EBV IgM antibodies were positive in 29% of HCC patients 27% of CLD patients. While EBV IgG antibodies did not differ between HCC and CLD groups. This may be because EBV serves as a supporting virus. It invades the cell and aids HCV replication, aggravating liver tissue inflammation, encouraging the growth of carcinoma cells, or affecting tumorigenic potential [14]. A study by **Sugawara *et al.*** [15] found that EBV was positive among 33% of HCC patients, 40% of anti HCV positive patients, and 14% of HBsAg positive patients. It had been established that HBV and HCV had a part in the development of HCC. Thus, HCV and EBV work together synergistically, although HBV and EBV do not [15]. In a study made in China, **Cheng *et al.*** [16] were able to convert HCV from HCV positive patients to long-lasting lymphoblastoid cell lines using EBV. They discovered that, with the aid of EBV, HCV might persist and function for a very long time in a cultured cell line. Moreover, **Wei *et al.*** [17] found EBV DNA in 28.2% of the HCC tissues by PCR. In contrast, a study in the United States carried by **Chu *et al.*** [18] examined 41 cases of HCC for any EBV infection evidence. The authors came to the conclusion that there is no correlation between EBV and the development of HCC. Furthermore, later experiments conducted in Germany, the United Kingdom, and the Netherlands by **Akhter *et al.*** [19] and **Herrmann *et al.*** [20] also found very low rates of EBV detection in HCC cases, indicating that EBV has not much to do with the development of hepatocellular carcinogenesis. Thus, the relation between EBV and HCC is controversial. We assume that these differences may be due ethnic and regional variations.

We found that EBV IgM antibodies did not correlate with clinical manifestations in HCC patients as cachexia, jaundice, ascites, or splenomegaly. Cachexia is

a complex syndrome. It may be because of the effect of cancer or anti-neoplastic treatments and may be due to worsening of cancer. In HCC patients, the prevalence of muscle loss can reach 40.3% [21]. Jaundice can result from a tumor compressing the major bile duct or from metastatic lymphadenopathy at the porta hepatis. 40% of patients with HCC have jaundice, which is often secreted in late stages of the disease. Jaundice caused by EBV has been associated in roughly 6.6% of cases [22]. One of the complications of cirrhosis is ascites, which is a main feature of portal hypertension. It is related to a high tumor burden, vascular invasion of HCC, and liver function impairment. Half of the patients infected with EBV have splenomegaly, which begin to decrease in the third week of infection with splenic rupture but is uncommon [23].

In the current study, we observed that Hb concentration, TLC and platelet count did not differ among the studied groups. In chronic liver disease patients, Hb concentration is a poor predictor of prognosis. Anemia may worsen with advanced stages of HCC, due to metastasis, chemotherapy, or nutritional problems. The abnormal TLC number of individuals with liver diseases may result from the underlying condition or its treatment [24]. CLD patients may have both abnormal Platelet counts and functions. The degree of liver disease is not clearly correlated with poor platelet kinetics. In patients with normal liver functions, low platelet count is mostly related with portal hypertension and splenomegaly [25].

This study revealed that according to Child-Pugh score, there were more CLD patients in class A than HCC patients, while there were no patients with CLD in classes B or C. Unlike other solid malignancies, prognosis of HCC depends not only on the cancer itself but also on the severity of the underlying liver cirrhosis [13].

Moreover, we found that prothrombin time and INR values were higher in HCC and CLD groups than controls, while no difference between HCC and CLD groups was noticed. When predicting the prognosis of cancers, coagulation parameter may be used, as solid tumors such as HCC are affected by the coagulation system [26]. A study by **Goa *et al.*** [26] found that prothrombin time was higher in HCC patients than non-HCC patients**.** A prolonged prothrombin time indicates the liver's potential for biosynthesis. Hepatic insufficiency causes a decrease in plasma coagulation factors, an increase in inflammation, and increased neuro-hormonal activity in the microenvironment of tumor. INR is considered as an indicator of liver disease and the level of liver synthetic function impairment rather than to assess the risk of a liver hemorrhage episode or probability for bleeding. In chronic liver disease, there is an impairment in protein synthesis, which leads to poor hemostasis and increased INR [27].

Our study found that AST, ALT, total and direct bilirubin were more in the HCC and CLD groups than control group, whereas they were higher in CLD group. AST is detected more in the heart than in other tissues such as liver, kidney, or skeletal muscle. Its elevation is primarily related to liver disease, which often raises ALT [28]. The liver has higher ALT concentrations than any other tissue (kidney, heart, and muscle). It is very specific to hepatocellular diseases and does not correlate with the degree of damage to the liver cells. The primary cause of AST elevation in chronic liver disease is hepatic tissue necrosis and degradation. Elevated AST is used by clinicians to detect HCC presence. ALT elevation in patients with HCC may be due to hepatocytes damage which is resulting from tumor growth as well as damage to more distant liver cells with concurrent chronic active hepatitis [27]. Bilirubin is produced from the breakdown of old red blood cells in cases with hereditary or acquired hepatic excretion abnormalities [29].

In this study, Albumin level was declined in the HCC and CLD groups compared to controls, and no difference was found between HCC and CLD groups. In CLD, albumin concentration is decreased because of its defective synthesis. Albumin is a crucial prognostic indicator for advanced cancer survival [30]. It is produced by the liver and could be a synthetic marker of inflammation and liver function. Some studies used serum albumin level for prediction of HCC and revealed that its decreased level might lead to the aggressiveness of HCC [31]. **Okonkow *et al.*** [32] studied 120 patients without HCC and 64 HCC patients. He found that among HCC patients, 30(46.9%) had increased AST, while 31(48.4%) had raised ALT, 34 (53%) of the patients had hyperbilirubinemia, while 54(84.3%) had hypoalbuminemia. Liver function tests were more often abnormal in HCC cases than in non-HCC cases, apart from bilirubin.

We observed that when AFP was compared to the control group, it was considerably increased in both the HCC and the CLD groups, although it was significantly more in the HCC group than the CLD group. Hepatocyte regeneration, hepato-carcinogenesis, and embryonic carcinomas all showed pathological increase of AFP. High level of AFP in chronic liver disease is considered a risk factor for HCC development. It has been shown that it has sensitivity for HCC detection in 39- 65% of patients. Its persistent elevation together with cirrhosis aids in early HCC detection 6 months before its detection by ultrasonography in 85% of patients [33].

CONCLUSION

EBV may have a role in development of HCC. More research with larger sample sizes is needed. It is recommended that patients with CLD undergo routine checks for EBV infection. We also recommend screening

of donated blood for EBV as blood transfusion is regarded as a substantial risk factor for acquiring and transmitting viruses that cause HCC.

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