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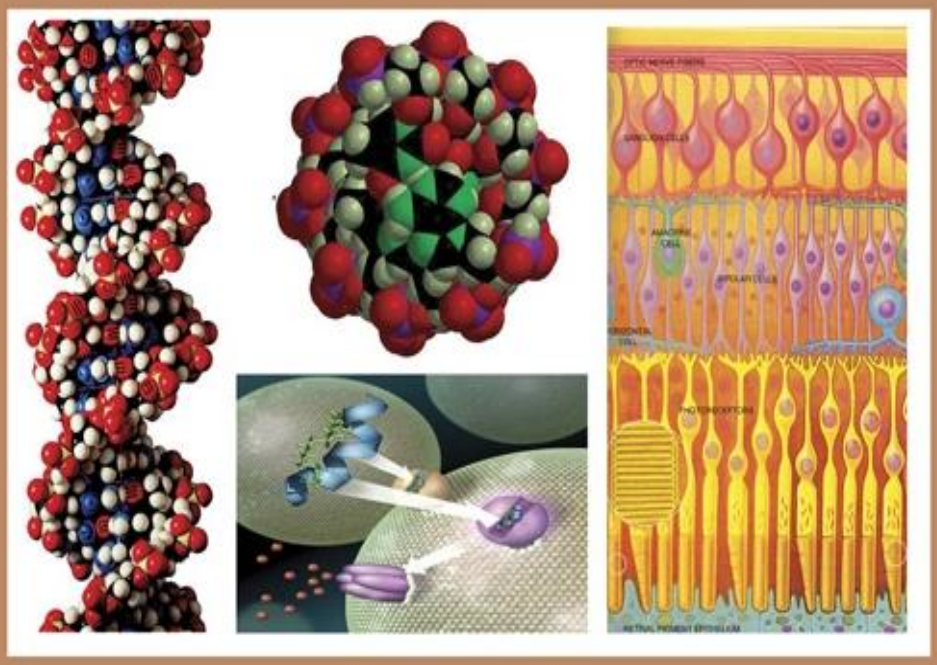

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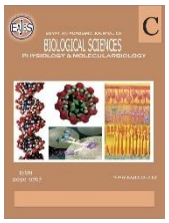

Association between Seroprevalence of Epstein–Barr Virus (EBV) and FIB4 Score-Based Liver Fibrosis Status in Chronic Hepatitis C patients

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ABSTRACT

BACKGROUNG: Epstein-Barr virus (EBV) infection represents ubiquitous etiology that can exert hepatic manifestations. The role of EBV in worsening liver fibrosis among chronic hepatitis C virus (HCV) infected patients is not well defined. The fibrosis index based on 4 factors (FIB-4) is used as a crucial non-invasive biomarker to diagnose liver fibrosis in chronic HCV infection. **OBJECTIVE:** This study aimed to investigate EBV seroprevalence in chronic HCV patients through the assessment of the corresponding antibodies, referring to their relationship with FIB4 score-based liver fibrosis status. **METHODS:** A total of 141 participants were involved in this study (81 chronic HCV patients and 60 controls). All participants were subjected to the measurements of baseline clinical parameters including HCV RNA. The FIB4 score was calculated to determine liver fibrosis. Serum samples were investigated for EBV-VCA IgG and EBV-VCA IgM antibodies by ELISA**. RESULTS:** EBV-VCA IgG antibodies exhibited 100% seropositivity in chronic HCV patients and controls, whereas the seropositivity of EBV-VCA IgM antibodies was detected in 6/81 (7.704%,) of chronic HCV patients (HCV/EBV co-infection) compared with 0/60 (0%) in controls. A significant increase in the seroprevalence of EBV-VCA IgM antibodies was observed in chronic HCV patients compared with controls (*P* 0.038). A significant increase in the total bilirubin level was reported among HCV/EBV co-infected patients compared with HCV mono-infected others ($P < 0.001$). At the FIB4 score high cutoff value of 3.25, a non-significant difference in EBV-VCA IgM antibody seropositivity between chronic HCV patients having significant (late) fibrosis (≥ F2, $n = 60$) and those having non-significant (early) fibrosis (< F2, $n = 21$) was reported, and no association between HCV/EBV co-infection and liver fibrosis was found. However, the incidence of increased EBV-VCA IgG antibody titre was found to be associated with having late fibrosis in chronic HCV patients (Odd's ratio 28.863, 95% C.I. 1.6691 to 499.1025, and *P* 0.020). Additionally, at the FIB4 score low cutoff value of 1.45, there was no significant association between seroprevalence of EBV-VCA IgM antibodies or incidence of increased EBV-VCA IgG antibody titre and a high probability of ruling out late fibrosis in chronic HCV patients. **CONCLUSION:** Chronic HCV patients predominantly have EBV-VCA IgG antibodies; the incidence of increased titre of these antibodies is associated with liver fibrosis progression. However, EBV reactivation is indicated by increased seroprevalence of EBV-VCA IgM antibodies, which does not show any association with liver fibrosis progression in the studied cohort of HCV patients.

INTRODUCTION

Epstein-Barr virus (EBV) represents a widespread etiology with double-stranded genomic DNA. EBV is a member of the herpes virus family (Roizman, 1982; Assaad *et al*., 2020), and is known as human herpes virus (HHV)-4 (Henry *et al*., 2013; Shoman *et al*., 2014). It is an enveloped virus with viral genome that is contained in a nucleocapsid (zur Husen *et al*., 1970; Issa *et al*., 2015). About 95% of the adult world-wide population is infected with EBV during life and becomes lifelong carriers (Baumforth *et al*., 1999; Assaad *et al*., 2020). Although EBV mainly infects B lymphocytes, it can also infect T cells and NK cells (Smatti *et al*., 2018). Prior to the entry of EBV into B cell, an envelope structure, major glycoprotein gp350, can bind to CD21 molecule on B cell surface which serves as a viral receptor. Moreover, the establishment of EBV infection is accompanied by involvement of the class II major histocompatibility complex (MHC) molecule, which serves as a cofactor for the infection of B cells (zur Hausen *et al*., 1970; Issa *et al*., 2015). EBV infection often becomes latent like other family members of herpes viruses (Petrova *et al*., 2010; Shoman *et al*., 2014). It persists for a life-long latency in B-lymphocytes (Gulley *et al*., 2001; Henry *et al*., 2013; Shoman *et al*., 2014) and expresses a limited number of genes (Gulley *et al*., 2001). As a response to EBV infection, immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies to viral capsid antigen (VCA) are expressed. These antibodies act as immunological markers for viral latency and reactivation (Smatti *et al*., 2018).

After EBV infection establishment ,a variety of diseases are caused, such as infectious mononucleosis and other EBV-associated complications (Yang 2017; Zhang *et al*., 2020). EBV infection manifestations include fever as well as enlargement of liver and lymph nodes (Han 2017; Zhang *et al*., 2020). This viral infection may cause disease deterioration in immunocompetent patients suffering from hepatitis C virus (HCV) infection (Yeung *et al*., 2007; Shoman *et al*., 2014). Indeed, the infection with EBV can promote HCV replication (Jang *et al*., 2018; Zhang *et al*., 2020). It has been revealed that Epstein-Barr nuclear antigen 1 (EBNA1) protein pears the responsibility for higher HCV replication (Sugawara *et al*. 1999; DaPalma *et al*. 2010; Shoman *et al*., 2014). It is suggested that the co-infections with EBV or CMV in patients infected with HCV have been proven to accelerate the chronic hepatitis C course of illness, thus leading to a more severe histological picture and facilitating the disease progression to fibrosis, cirrhosis, and hepatocellular carcinoma (Medina *et al*., 2007; Ghanem *et al*., 2014).

Liver fibrosis can be diagnosed by several methods, which include invasive and non-invasive approaches. Liver biopsy is an invasive approach that is very expensive and is not suitable for all patients due to the risk of bleeding (Sebastiani *et al.,* 2014; Gudowska *et al*., 2016; Catanzaro *et al*., 2020). Thus, alternative non-invasive approaches, including various imaging procedures and biomarkers, have been developed and validated to diagnose liver fibrosis accurately (Catanzaro *et al*., 2013a; Catanzaro *et al*., 2013b; and Catanzaro *et al*., 2020). In this regard, the fibrosis index based on 4 factors (FIB4) is a fibrosis scoring system based on four factors including the patient's age, alanine transferase (ALT), aspartate aminotransferase (AST), and platelet count. It is simple and easy to use as a biomarker to diagnose liver fibrosis (Sterling *et al*., 2006; Saviano *et al*., 2020). This serum biomarkers supposed to facilitate stratification during different disease stages and is used as crucial biomarkers to diagnose liver fibrosis (Kartoun, 2019; Eslam *et al*., 2015; Dawood *et al*., 2021). According to METAVIR scoring system, liver fibrosis score (F) is defined as F0 in absence of liver scaring, followed by four successive fibrosis stages: F1, mild fibrosis (portal fibrosis with no septa); F2, moderate fibrosis (portal fibrosis included rare septa); F3, severe liver fibrosis (numerous septa with no cirrhosis); and F4, cirrhosis or advanced liver scaring (Franciscus, 2010; Baranova *et al*., 2011;

Abdel-Rahman *et al*., 2013; Dawood *et al*., 2021). It is confirmed that FIB4 is able to predict severe fibrosis in chronic HCV infection (Catanzaro *et al*., 2021). The role of HCV/EBV co-infection and EBV reactivation in liver fibrosis progression among chronic HCV patients is not well known. The present study aimed to investigate EBV seroprevalence in chronic HCV patients through the assessment of the corresponding antibodies, referring to their relationship with FIB4 score-based liver fibrosis.

MATERIALS AND METHODS

Study Population:

A total of 141 Egyptian participants were included in this current study. The participants were divided into two groups: 60 healthy controls and 81 patients with chronic HCV infection, they were diagnosed at the Medical Center of Excellence, National Research Centre. Each participant provided written informed consent prior to blood sample collection. The study was conducted in accordance with the World Medical Association's Declaration of Helsinki guidelines published in 1964 and its later amendments.

Inclusion criteria: The study involved adult subjects of both genders. Chronic HCV patients were immunocompetent and characterized by the seropositivity of HCV antibodies and detected HCV RNA. On the other hand, healthy control participants were confirmed for seronegativity of HCV antibodies with no detectable HCV RNA or any other etiology.

Exclusion criteria: Children and immunocompromised patients excluded from sample recruitments. Participants who were positive for hepatitis B surface antigen (HBsAg) or human immunodeficiency virus (HIV) antibodies were excluded from the study.

All participants were subjected to clinical parameter investigations that involved biochemical assessment of liver functions, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, and total bilirubin (T Bil), in addition to platelet count (PLC) determination as a hematological parameter. HCV patients and controls were assessed for EBV-VCA IgG and EBV-VCA IgM antibodies serologically using the ELISA technique. The seropositivity of EBV-VCA IgM antibodies was considered in indicated to HCV/EBV coinfection in the studied cohort of HCV patients. Moreover, the liver fibrosis biomarker, FIB4 score, was calculated to evaluate liver fibrosis status.

Quantification of HCV RNA:

The extraction of HCV RNA was performed from sera specimens using QIAamp Viral RNA kit (Qiagen, Santa Clarita, CA) in accordance with standard manufacturer's instructions. HCV RNA detection was verified by one-step, real-time RT-PCR using the Artus HCV QS RGQ Kit (Qiagen, Santa Clarita, CA) in accordance with the manufacturer's instructions. The thermal profile of amplification was organized as follows: 51°C for initial incubation during 30 min, followed by 95°C for 10 min, 95°C for 50 cycles during 30s, 60°C during 1min, then followed by 95°C for 40 cycles during 15s, 60°C during 1 min, and 72°C during 30 s. The assessment of fluorescence signal was done at step of annealing/extension of each cycle. HCV RNA amplification was done by applying Rotor Gene real-time PCR (Qiagen, Santa Clarita, CA) (Dawood *et al*., 2022).

Serological Detection of EBV-VCA Antibodies:

Assessment of EBV-VCA IgM Antibodies:

The detection of EBV-VCA IgM antibodies was assessed in serum samples of recruited cases by a commercial ELISA kit, DRG EBV-VCA IgM (DRG International, Inc., USA), according to the manufacturer's instructions. In this procedure, solid-phase microtiter wells were coated with inactivated EBV-VCA gp 125. During incubation of patients' samples and controls, the positive sample of EBV-VCA antibodies was bound to the immobilized antigens. Horseradish peroxidase-conjugated anti-human IgM antibodies were added into the wells. During second incubation, the anti-IgM conjugate binds specifically to IgM antibodies, forming

enzyme-linked immune complexes, which were detected by incubation with TMB substrate and the development of a blue color that turned yellow by stopping an enzymatic reaction with sulfuric acid. The intensity of this color was directly proportional to the amount of EBV-IgM antibodies in the patient's sample. The color intensity was measured by a microtiter plate ELISA reader (TECAN; SUNRISE, Austria GmbH) at 450 nm. The results were expressed in DRG units [DU] according to the following equation: patients absorbance value ×10 / CO [CO referred to mean absorbance value of cut-off control] and classified according to their DU value as negative (< 9 DU), grey zone (9-11), and positive (> 11 DU).

Assessment of EBV-VCA IgG Antibodies:

The detection of EBV-VCA IgG antibodies was also assessed in serum samples of recruited cases by commercial ELISA kit, DRG EBV-VCA IgG (DRG International, Inc., USA), according to the manufacturer's instructions. In this procedure, solid-phase microtiter wells were coated with inactivated EBV-VCA p18 and p23. All the following steps were the same as those mentioned in the detection of EBV-VCA IgM except that horseradish peroxidase-conjugated anti-human IgG antibodies were added into the wells. During the second incubation, this anti-IgG conjugate binds specifically to IgG antibodies, forming enzyme-linked immune complexes revealed by enzymatic color reaction. Finally, the color intensity [directly proportional to the amount of EBV-IgG] was measured by microtiter plate ELISA reader (TECAN; SUNRISE, Austria GmbH) at 450 nm. The results were expressed in DU units with the same detection limit as of EBV-VCA IgM antibodies.

Calculation of FIB4 Score:

Noninvasive liver fibrosis estimation was carried out by calculating the FIB4 score according to Sterling's formula [age × AST (IU/L)]/[platelet count (10^9/L) × [$ALT^{1/2}$ (IU/L)] (Sterling *et al*., 2006; Dawood *et al*., 2021). Generally, liver fibrosis included four successive stages: F1, mild fibrosis; F2, moderate fibrosis; F3, severe fibrosis; and F4, cirrhosis (Dawood *et al*., 2021). Regarding liver fibrosis determination, there were two cutoff values of the FIB4 score: 3.25 and 1.45, referring to high and low World Health Organization (WHO) cutoff values respectively. The calculated FIB4 score value ≥ 3.25 was used to diagnose patients with significant (late) fibrosis ≥ F2, whereas the FIB4 score value < 3.25 was used to predict patients with non-significant (early) fibrosis < F2. On the other hand, the FIB4 score value < 1.45 referred to low risk and about 90% of the negative prediction value of having significant or late fibrosis. So, late fibrosis could be ruled out with high probability. In the determination of liver fibrosis, A high cutoff value provided the maximum specificity of more than 90 whereas a low cutoff value supplied the highest sensitivity, more than 82%. (Sterling et al., 2006; Sripongpun *et al*., 2019; Saviano *et al*., 2020).

Statistical Analysis:

Data analyses were done by application of the statistical program for social science (SPSS, Chicago, IL, USA) software version 20. The analyses included subject number, percentage, mean, and standard deviation. Chi-square was used to analyze qualitative data, whereas the t-test was used to analyze quantitative data. Additionally, a logistic regression test was applied to represent the association between the independent variable and a dependent variable (dichotomous) and assess an outcome. The significant results were indicated at p-value ≤ 0.05.

RESULTS

Demographic And Clinical Characteristics of The Studied Cohort:

The demographic and clinical features of the study population were summarized in Table 1. The cohort of study involved controls (n = 60) and HCV patients (n = 81). In chronic HCV patients, the results of the t-test revealed a significant increase in age, ALT, AST, ALP, GGT, T Bil, EBV-VCA IgG antibody titre, and FIB4 score, as well as a significant decrease in albumin and PLC (p < 0.05) compared with controls.

Table 1. Demographic data and baseline clinical features among subjects of study

Parameter	Subjects of study		*P*-value
	Group 1 (Controls, *n* = 60)	Group 2 (HCV patients, *n* = 81)	
Gender (M/F)	48(80%)/12(20%)	57(70.37%)/24(29.63%)	0.195
Age (years)	28.45±6.708	50.680±6.504	< 0.001*
ALT (U/L)	20.50±8.972	40.148±20.973	< 0.001*
AST (U/L)	21.80±8.283	60.518±37.568	< 0.001*
ALP (U/L)	75.70±18.364	136.630±58.190	< 0.001*
Albumin (g/dL)	4.64±0.324	2.870±0.474	< 0.001*
T Bil (mg/dL)	0.648±0.331	2.940±1.499	< 0.001*
PLC (10^3/cmm)	265.647±65.630	112.259±79.411	< 0.001*
HCV RNA (IU/ml)	-	116,192.167±118,435.739	-
EBV-VCA IgG (DU)	119.800±110.905	165.537±141.082	0.019*
FIB4 Score	0.552±0.199	6.076±3.604	< 0.001*

Where: M: male, F: female, ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, T Bil: total bilirubin, PLC: platelet count, FIB4: fibrosis 4 scores index, *n*: number of subjects, and *: significant value. Normal ranges were as follows: AST: from 0 to 40 U/L, ALT: from 0 to 40 U/L, GGT: from 10 to 55 U/L, ALP: from 30 to 120 U/L, albumin: from 3.5 to 5 g/dL, T.Bil: from 0 to 1.2 mg/dL, PLC: from 150 to 450 10^3/cmm, the detection limit of HCV RNA > 34 IU/ml, the positive EBV-VCA IgG > 11 DU, age ranges (20-41) for controls and (35-63) for HCV patients. For the t-test, data were expressed as mean and standard deviation values (M ± SD).

Seroprevalence of EBV Among Healthy Controls And Chronic HCV Patients:

The results of EBV-VCA IgG antibody seroprevalence were positive in 100% of the entire cohort of study **(**chronic HCV patients and controls**),** whereas the results of EBV-VCA IgM antibody seroprevalence were demonstrated with significant variation in this regard. The seroprevalence data of EBV-VCA IgG and EBV-VCA IgM antibodies among controls and chronic HCV patients were analyzed and recorded in Table 2. Chi-sqaure analysis revealed a non-significant variation in seroprevalence of EBV-VCA IgG antibodies between HCV patients and controls. Furthermore, a significant increase in seroprevalence of EBV-VCA IgM antibodies was described in 6/81 (7.407%) of HCV patients compared with 0/60 (0%) of controls (*P* 0.038).

Table 2. Comparison of seroprevalence of EBV-VCA IgG and IgM antibodies in HCV patients and controls.

EBV-VCA antibodies		Subjects of study				Chi-square	
		Group 1 (controls, *n*= 60)		Group 2 (HCV patients, *n* = 81)			
		n	%	*n*	%	X^2	*P*-value
IgG	Negative	0	0	0	0	0	1
	Positive	60	100	81	100		
IgM	Negative	60	100	75	92.593	4.314	0.038*
	Positive	0	0	6	7.407		

Where: *n*: number of patients.

Changes of Demographic And Clinical Features Among HCV Patients Based on The Seroprevalence of EBV-VCA IgM Antibodies:

Based on the seroprevalence of EBV-VCA IgM antibodies, HCV patients were divided into 2 groups: group 1 identified with negative EBV-VCA IgM antibodies (HCV mono-infection), $n = 75$, and group 2 identified with positive EBV-VCA IgM antibodies (HCV/EBV co-infection), $n = 6$. In **Table 3,** a significant increase in T Bil was reported in chronic HCV patients with positive EBV-VCA IgM antibodies compared with others with negative EBV-VCA IgM antibodies. Also, a tendency to increase in age, ALT, AST, ALP, and FIB4 score was demonstrated among HCV patients with seropositive EBV-VCA IgM antibodies compared with others with seronegative EBV-VCA IgM antibodies. Likewise, a tendency to a decrease in albumin was recoded in towards HCV patients with seropositive EBV-VCA IgM antibodies.

Table 3. Comparison between the groups of chronicHCV patients based on seroprevalence of EBV-VCA IgM antibodies.

Parameter	HCV Patients						*P*-value
	Group 1, seronegative EBV-VCA IgM antibodies (HCV monoinfection, $n = 75$)			Group 2, seropositive EBV-VCA IgM antibodies (HCV/EBV co-infection, $n = 6$)			
Gender (M/F)	54(72%)/21(28%)			3(50%)/3(50%)			0.620
Age (years)	50.680	±	6.504	54.167	±	0.983	0.393
HCV-RNA (IU/ml)	124,514	±	122,844.699	49,617.500	±	29,142.674	0.073
ALT (U/L)	39.760	±	21.742	44.833	±	3.125	0.286
AST (U/L)	60.240	±	38.944	64.000	±	10.954	0.394
ALP (U/L)	135.200	±	57.313	154.500	±	71.752	0.390
Albumin (g/dL)	2.892	±	0.486	2.600	±	0.109	0.354
T Bil (mg/dL)	2.823	±	1.442	5.530	±	0.318	< 0.001*
PLC (10^3/cmm)	111.560	±	87.898	121.000	±	93.113	0.391
EBV-VCA IgG (DU)	170.163	±	145.688	108.050	±	7.066	0.151
FIB4 Score	5.958	±	3.471	7.560	±	5.160	0.174

Where: M: male, F: female, ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, T Bil: total bilirubin, PLC: platelet count, FIB4: fibrosis 4 scores index, *n*: number of subjects, and *: significant value. Normal ranges were as follows: AST: from 0 to 40 U/L, ALT: from 0 to 40 U/L, GGT: from 10 to 55 U/L, ALP: from 30 to 120 U/L, albumin: from 3.5 to 5 g/dL, T.Bil: from 0 to 1.2 mg/dL, PLC: from 150 to 450 10^3/cmm, the detection limit of HCV RNA > 34 IU/ml, the positive EBV-VCA IgG > 11 DU. For the t-test, data were expressed as mean and standard deviation values (M ± SD).

Relationship Between Seroprevalence of EBV-VCA IgM Antibodies And FIB4 Score-Based Liver Fibrosis in HCV Patients:

Based on the FIB4 score higher cutoff value of 3.25, HCV patients were divided into two groups: a group with a FIB4 score ≥ 3.25 (patients with late fibrosis [≥ F2, $n = 60$]) and another group with a FIB4 score < 3.25 (patients with early fibrosis [< F2, $n = 21$]). In Table 4, the data on the relationship between seroprevalence EBV-VCA IgM antibodies and liver fibrosis status among HCV patients were demonstrated. In Table 4a, the results of the chi-square analysis revealed a non-significant change in the seroprevalence of EBV-VCA IgM antibodies between chronic HCV patients with early fibrosis and others with late fibrosis ($P > 0.05$). Moreover, the results of logistic regression analysis referred to a non-significant association between the seroprevalence of EBV-VCA IgM antibodies and liver fibrosis among chronic HCV patients. Furthermore, based on the FIB4 score low cutoff of 1.45, HCV patients were divided into two groups: one group with a FIB4 score < 1.45 (patients with a higher

probability of ruling out late fibrosis [≥ F2, *n* = 6]), and another group with a FIB4 score ≥ 1.45 (patients with a lower probability of ruling out late fibrosis [< F2, *n* = 75]). In Table 4b, the results of chi-square analysis revealed a non-significant change in the seroprevalence of EBV-VCA IgM antibodies between the two groups of HCV patients regarding the higher and lower probability of ruling out late fibrosis. In this regard, also, logistic regression analysis referred to a non-significant association ($P > 0.05$).

Table 4a. Seroprevalence of EBV-VCA IgM in relation to liver fibrosis among HCV patients according to the high cutoff value of the FIB4 score.

HCV patients	**EBV-VCA IgM antibodies**		**Chi-Square**		**Logistic Regression**		
	Negative, *n*=75	**Positive, *n*=6**					
	n (%)	*n (*%)	X^2	*P*- value	Odd's ratio	95% C.I.	*P* - value
FIB4 score < 3.25 (early fibrosis), *n=21*	18 (24%)	3 (50%)	1.956	0.162	0.316	0.0586 to 1.7042	0.180
FIB4 score ≥ 3.25 (late fibrosis), *n*= 60	57 (76%)	3 (50%)					
Total	75(100.00%)	6(100%)					

Where: *n*: number of patients, C.I.: confidence intervals (determined as 95% for Odd's ratio).

Table 4b. Seroprevalence of EBV-VCA IgM in relation to liver fibrosis among HCV patients according to the low cutoff value of the FIB4 score

HCV patients and probability of ruling out late fibrosis	**EBV-VCA IgM antibodies**		**Chi-Square**		**Logistic Regression**		
	Negative, *n*=75	**Positive, *n*=6**					
	n (%)	*n (*%)	X^2	*P*- value	Odd's ratio	95% C.I.	*P* - value
FIB4 score < 1.45 (high probability, *n=6*	6 (8%)	0 (0%)	0.518	0.472	0.8225	0.0415 to 16.3021	0.898
FIB4 score ≥ 1.45 (low probability), *n*= 75	69 (92%)	6 (100%)					
Total	75(100.00%)	6(100%)					

Where: *n*: number of patients, C.I.: confidence intervals (determined as 95% for Odd's ratio).

Relationship Between EBV-VCA IgG Antibody Titre And FIB4 Score-Based Liver Fibrosis in HCV Patients:

In **Table 5**, depending on the mean value (165.537) of EBV-VCA IgG antibody titre in HCV patients, the data on the relationship between the incidence of EBV-VCA IgG antibody titre and liver fibrosis

status among these patients were demonstrated. In Table 5a, at a FIB4 cutoff of 3.25, the results of the chi-square analysis revealed a significant elevation in the increased incidence of EBV-VCA IgG antibody titre (> 165.537) in chronic HCV patients with late fibrosis compared with those with early fibrosis (P > 0.05). Moreover, the results of logistic regression analysis referred to a significant association between the incidence of increased titre of EBV-VCA IgG antibodies and late fibrosis among chronic HCV patients. On the other hand, in Table 5b, at FIB4 cutoff of 1.45, a non-significant association between the incidence of the increased EBV-VCA IgG antibody titre and rulling out of late fibrosis was demonstrated.

Table 5a. EBV-VCA IgG antibody titre in relation to liver fibrosis in HCV patients based on FIB4 score high cutoff value.

HCV patients	**EBV-VCA IgGAntibody titre (mean value)**		**Chi-Square**		**Logistic Regression**		
	< 165.537, *n*=57	**> 165.537, *n*=24**					
	n (%)	*n* (%)	X^2	*P*-value	Odd's ratio	95% C.I.	*P* - value
FIB4 score < 3.25 (early fibrosis), *n=21*	21 (36.842%)	0 (0%)	11.937	0.005*	28.863	1.6691 to 499.1025	0.020*
FIB4 score ≥ 3.25 (late fibrosis), *n*=60	36 (63.158 %)	24 (100%)					
Total	57 (100.00%)	24 (100%)					

Where: *n*: number of patients, 165.537 is the mean value of EBV-VCA IgG antibody titre in HCV measured in Du, C.I.: confidence intervals (determined as 95% for Odd's ratio).

Table 5b. EBV-VCA IgG antibody titre in relation to liver fibrosis in HCV patients based on FIB4 score low cutoff value

HCV patients	**EBV-VCA IgGAntibody titre (mean value)**		**Chi-Square**		**Logistic Regression**		
	< 165.537, *n*=57	**> 165.537, *n*=24**					
	n (%)	*n* (%)	X^2	*P*-value	Odd's ratio	95% C.I.	*P* - value
FIB4 score < 1.45 (high probability, *n=6*	6 (10.526%)	0 (0%)	2.728	0.099	0.162	0.0088 to 2.9874	0.220
FIB4 score ≥ 1.45 (low probability), *n*= 75	51 (89.474%)	24 (100%)					
Total	57 (100.00%)	24 (100%)					

Where: *n*: number of patients, 165.537 is the mean value of EBV-VCA IgG antibody titre in HCV measured in Du, C.I.: confidence intervals (determined as 95% for Odd's ratio).

DISCUSSION

Beside the classic hepatotropic viruses, including hepatitis A through E viruses, EBV is also considered a hepatropic viral agent that has the ability to infect the liver, causing hepatitis (Gallegos-Orozco *et al.*, 2010; Shoman *et al*., 2014). The course of EBV infection has the property of latency and reactivation (Gandhi *et al*., 2004; Gredmark et al., 2007; Shoman *et al*., 2014). Among herpes viruses, the latency in EBV is the highest rate and may reach 90% of people approximately (Kang *et al*., 2017; Barakat *et al*., 2023). The latent phase of infection can last for the whole life (De Paschale *et al*., 2012; Chen *et al*., 2021). The virus reactivation is observed in different pathologic circumstances, including periods of downregulation of the immune system and disease-related stress, as well as co-infection with other pathogens (Gandhi et al., 2004; Gredmark *et al*., 2007; Ghanem *et al*., 2014; Shoman *et al*., 2014). After EBV infection, specific antibodies are induced, including EBV-VCA IgM and EBV-VCA IgG. Serum positivity for anti-VCA IgM indicates an acute infection or reactivated infection; the EBV-VCA IgG antibody appears at the primary infection stage, remaining positive for life during latency. Upon reactivation, EBV can express anti-VCA IgM (Berkun *et al*., 2009; Chen *et al*., 2021). The seroprevalence of EBV among HCV patients is a controversial issue, with a poor identified role in liver fibrosis progression.

Herein, we investigated the seroprevalence of EBV-VCA IgG and EBV-VCA IgM antibodies to identify the EBV infection among chronic HCV patients and healthy controls. Our results referred to 100% seroprevalence of EBV-VCA IgG antibodies in 60/60 of healthy controls and 81/81 of chronic HCV patients. Our findings were consistent with the previous literature, which revealed that EBV may infect more than 90% of adults over the world (Henry *et al*., 2013), as well as overall global populations (Oh and Weiderpass, 2014), and this infection may remain in a latent form. Furthermore, our findings agreed with Hu *et al*. (2019), who found 100% seroprevalence of EBV-VCA IgG antibodies in 68 cirrhotic patients of a study cohort consisting of 97 patients, with the availability of EBV-VCA IgG data for only 68 out of them (Hu *et al*., 2019). Contrary, our findings disagreed with those reported by Ghanem *et al*., (2014), who recorded the seroprevalence of EBV-IgG in 45/79 (56.9%) in HCV patients and 18/52 (34.6%) in healthy controls during their study for the prevalence of EBV among a cohort of Egyptian patients. The difference in seroprevalence might be due to differences in cohort characteristics as well as the stage of chronic liver disease. Also, our findings referred to a significant increase in EBV-VCA IgG antibody titre among chronic HCV patients than controls, which might refer to a state of EBV reactivation among chronic HCV patients. Indeed, it is stated that upon EBV reactivation, the level of EBV-VCA IgG antibodies could increase from the lower level that was maintained during latency (Gulley, 2001; Wood *et al*., 2021).

Our further investigations included the assessment of EBV-VCA IgM antibodies among chronic HCV patients and controls. In this regard, the incidence of EBV-VCA IgM antibodies was detected in 0/60 (0%) of controls compared to 6/81 (7.407%) among chronic HCV patients. Our results referred to a significant elevation in the seroprevalence of EBV-VCA IgM antibodies among HCV patients than controls. Indeed, our findings agreed with those reported by Ghanem *et al*. (2014), who recorded the seroprevalence of EBV IgM antibodies in 1/52 (1.9%) of controls and 3/79 (3.8%) in HCV patients (Ghanem *et al*., 2014). Indeed, our results regarding the serorevalence of EBV-VCA IgM highlighted the reactivation of EBV among chronic HCV patients. Our findings might be explained as follows: generally, in EBV infection, the EBV genome encodes several genes, resulting in the expression of lytic antigens, such as viral capsid antigen

(VCA) (Gulley, *2001*; Smatti *et al*., 2018). In the primary acute phase of EBV infection, the activated humoral response gives rise to IgM antibodies, followed by IgG antibodies to viral capsid antigen (VCA). After the acute phase of EBV infection and during the viral latency, EBV-VCA IgM declines and disappears (Smatti *et al*., 2018), while anti-VCA IgG remains at a lower level (Gulley, 2001). Upon EBV reactivation, EBV-VCA IgM antibodies were induced to be expressed and appear again (Berkun *et al*., 2009; Chen *et al*., 2021), and the level of EBV-VCA IgG antibodies could increase (Gulley *et al*., 2001; Wood *et al*., 2021).

The liver is one of the most important immune organs in the human body. The EBV virus can cause multiple diseases and multiple organ damage (Kobayashi, 2018; Zhang *et al*., 2020). Studies have shown that about 85% of patients with EBV infection have liver function impairment of varying degrees, and about 6% of hepatitis is caused by EBV infection. EBV infection increases the level of transaminase in the body, leading to liver function damage, which is usually characterized by swelling of liver cells (Mecadon *et al*., 2017; Zhang *et al*., 2020). Our findings referred to deterioration in liver function parameters among HCV patients with seropositive EBV-VCA IgM antibodies compared to others with seronegative EBV-VCA IgM antibodies. In this regard, our results revealed a significant increase in total bilirubin as well as a tendency to increase in liver enzymes including ALT, AST and ALP among HCV patients with seropositive EBV-VCA IgM antibodies. A tendency to decrease in albumin was also documented among these patients. Our findings agreed with Ghanem *et al*. (2014), who depended on the transaminases measurements in studding the role of EBV in liver pathogenicity among chronic HCV patients, they recorded an increased level of liver enzymes, including ALT and AST, in HCV patients co-infected with EBV (Ghanem *et al*. 2014). Indeed, CD8 + T cell lymphocytes are directly related to the liver's immunological activity. Furthermore, back in 1996, Russell *et al*. demonstrated that the number of CD8+ T cell lymphocytes in the liver would rapidly increase when the liver was wounded (Russell *et al*., 1998; Zhang *et al*., 2020). Larrubia *et al*., (2007) also revealed that CD8+ levels in peripheral blood of chronic HCV patients also increased (Larrubia *et al*., 2007; Zhang *et al*., 2020). It has the ability to attach to specific molecules expressed by particular cells and infiltrate into hepatic tissues (Cox *et al*., 1995; Zhang *et al*., 2020). EBV infection has the capability to cause liver injury. Therefore, the damage in liver function that caused by EBV infection may be related to immunological function of CD8 + T cell lymphocytes (Zhang *et al*., 2020).

Our further findings referred to a significant increase in total bilirubin among HCV patients with seropositive EBV-VCA IgM antibodies compared to others with seronegative EBV-VCA IgM antibodies, revealing an increase in hyperbilirubinaemia among HCV/EBV co-infected patients. Indeed, Herold and Grimaldo, (2019) demonstrated a marked elevation in bilirubin during a case report study as a major complication in EBV infection (Herold and Grimaldo, 2020). The previous study by Susan *et al*., (1987) revealed a marked increase in hyperbilirubinemia during EBV infection causing infectious mononucleosis (Fuhrman *et al*., 1987).

The previous studies suggested that EBV could promote the replication of hepatitis C virus (HCV) after infection (Jang *et al*., 2018; Zhang *et al*., 2020). Unfortunately, our results referred to a tendency toward a decrease in HCV RNA level among HCV patients with seropositive EBV-VCA IgM antibodies compared to other HCV patients with seronegative EBV-VCA IgM antibodies. This observation might be attributed to recent infection by EBV, and over time, the virus might promote the increase in HCV RNA level. Likewise, the tendency toward a decrease in EBV-VCA IgG antibody titre was observed in HCV patients with seropositive EBV-VCA IgM antibodies compared to those with seronegative EBV-VCA IgM antibodies. This observation might

be explained also due to the recent infection of EBV, and over time, the level of EBV-VCA IgM antibodies might decline and the EBV-VCA IgG antibody titre might increase during the course of EBV infection (Smatti *et al*., 2018; Wood *et al*., 2021).

The role of EBV in deteriorating liver fibrosis among HCV patients remains unclear due to the limited reports concerned with this issue. In the current study, our further investigations were directed to demonstrate the relationship between seroprevalence of EBV and liver fibrosis among chronic HCV patients using non-invasive biomarker assessment through the calculation of FIB4 score for recruited subjects. Indeed, the FIB4 score was applied with the high WHO cutoff value of 3.25. Consequently, FIB4 ≥ 3.25 was used to differentiate HCV patients into patients with late fibrosis (≥ F2) and others with early fibrosis (< F2) (Sripongpun *et al*., 2019). In our study, FIB4 score value ≥ 3.25 was detected in 60/81 of chronic HCV patients, identifying them with late fibrosis, whereas FIB4 score value < 3.25 was detected in 21/81 of chronic HCV patients, identifying them with early fibrosis. Our findings referred to a tendency toward an increase in FIB 4 score mean value in chronic HCV patients with seropositive EBV-VCA IgM antibodies compared with other`HCV patients who experienced seronegative EBV-VCA IgM antibodies. However, our results revealed a non-significant variation of seroprevalence of EBV-VCA IgM antibodies between the two groups of chronic HCV patients regarding having late fibrosis. Moreover, the logistic regression analysis revealed a non-significant association between the seroprevalence of EBV-VCA IgM antibodies or EBV serological reactivation and liver fibrosis among HCV patients (Odd's ratio 0.316, 95% C.I. from 0.0586 to 1.7042, and *P*-value 0.180). In addition, other findings in the current study referred to a non-significant change in the seroprevalence of EBV-VCA IgM antibodies between the two groups of HCV patients who were grouped based on the FIB4 score cutoff value of 1.45, which was considered a low WHO cutoff value. FIB4 score value < 1.45 was found in 6/81 of HCV patients identifying them with higher probability of ruling out late fibrosis (≥ F2). Moreover, the seroprevalence of EBV-VCA IgM antibodies among HCV patients was unassociated with the higher probability of ruling out late fibrosis.

Further findings in the current study revealed a significant association between the incidence of increased EBV-VCA IgG antibody titre (>165.537 as a mean value) and late fibrosis in HCV patients at the FIB4 cutoff of 3.25 (Odd's ratio 28.863, 95%C.I. from 1.6691 to 499.1025, and *P*-value 0.020), whereas, at FIB4 cutoff of 1.45, there was no association between the incidence of increased EBV-VCA IgG antibody titre and ruling out late fibrosis in HCV patients.

Indeed, there is deficient information in the literature regarding relation between EBV infection and liver fibrosis. However, Hu *et al.*, (2019) revealed that cirrhotic patients experienced a higher rate of EBV infection referring to viral reactivation, especially for patients older than 60 years in their studied cohort. They investigated 97 cirrhotic patients; 51.6% of them were defined with liver cirrhosis (F4 liver fibrosis) due to hepatitis B viral infection. In their study, data of EBV-VCA-IgG antibodies were available for only 68/97 with 100% seropositivity, and EBV-DNA was positive in 36/97 (37.1%), indicating EBV reactivation (Hu *et al.*, 2019).

In fact, few previous studies depended on liver fibrosis stage grouping to reveal the role of co-infection of HCV with other pathogens in increasing liver fibrosis progressions, the most important of which was a study conducted on cytomegalovirus (CMV), another herpes virus family member with a close similarity to EBV in latency and reactivation during the course of infection. Ibrahim *et al*. (2017) could observe and prove the effective role of HCV/CMV co-infection to increase liver fibrosis progression among chronic HCV patients (Ibrahim *et al*., 2017). Another previous study was conducted by Abdel-rahman *et al*., (2013), who could

not observe a significant role of HCV/schistosomiasis co-infection on liver fibrosis progression among HCV patients (Abdel-rahman *et al*., 2013). In addition, co-infections with other viruses such as HBV or HIV in patients diagnosed with HCV infection have been demonstrated to accelerate the HCV course of chronic pathogenicity, thus resulting in a worsening histological aspect and promoting the development of fibrosis and cirrhosis, as well as hepatocellular carcinoma (Park *et al*., 2005; Issa *et al*., 2015). The limitation of the current study was the unavailability to measure EBV-DNA beside EBV-VCA IgM and EBV-VCA IgG antibodies to highlight the role of EBV viremia in liver fibrosis progression during the viral reactivation that might provide additional valuable knowledge concerning EBV infection among HCV patients.

Conclusion

Chronic HCV patients predominantly have EBV-VCA IgG antibodies; the incidence of increased titre of these antibodies is associated with liver fibrosis progression. However, EBV reactivation is evidenced by increased seroprevalence of EBV-VCA IgM antibodies, which does not show any association with liver fibrosis progression. In seropositivity of EBV-VCA IgM antibodies, hyperbilirubinaemia is a remarkable observation among the studied cohort of HCV patients. Further studies are recommended to assess the role of HCV/EBV co-infection on liver fibrosis progression on the level of EBV-DNA measurements side by side with the serological assessments.

Abbreviations

HCV: Hepatitis C Virus
FIB4: Fibrosis Index Based on 4 Factors
EBV: Epstein–Barr virus
VCA: Viral Capsid Antigen.

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A. K.: Conceptualization, methodology, data collection and analysis, data interpretations, writing the manuscript, and revision of important contents. M. M.: Patient recruitment, resources and validation.

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