Assessment of Epstein-Barr (EBV) Virus Infection in Relation to the Response of Chronic Hepatitis C Virus Infected Patients to Interferon-Based Therapy

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Abstract

Background: **Epstein Barr (EBV) is a ubiquitous pathogen that infects the majority of humans. Co-infection of EBV and hepatitis C virus (HCV) may worsen the prognosis of HCV-infected patients.** ***Aim:*** **This study was conducted to examine the role of EBV reactivation in determining the response rate to treatment with interferon and ribavirin therapy in chronic HCV patients.** ***Patients and Methods:*** **One hundred patients with proven chronic hepatitis C who underwent PEG IFN + ribavirin therapy and twenty healthy controls (negative for both HCV and HBV antibodies) were enrolled in this study. HCV and EBV were assessed using both molecular (RT-PCR) and serological (enzyme-linked immunosorbent assay).** ***Results:*** **EBV was significantly undetectable in non-responders compared responders (96.2% *vs.* 77.0%, respectively, p<0.05). While there was a statistically significant higher percentage of EBV (>2000 copies/ml) among responders compared to non-responder patients (14.9% *vs.* 0%, respectively, p<0.05). Serologically, no statistically significant differences was found between both groups regarding EBV antibodies (p>0.05). However, a statistically significant higher percentage of past infection with EBV was detected among non-responder compared to responders (100% *vs.* 81.8%, respectively) (p<0.05).** ***Conclusions:*** **The results of this study suggest that there is a unidirectional relation between EBV and HCV in our study subjects.**

Key words: latent virus; co-infection; antiviral treatment

Introduction

Hepatitis C virus (HCV) infection is a major public health problem worldwide[(1-3)]. Egypt has the highest worldwide prevalence of HCV infection with an approximately 8 million infected inhabitants in 1999. In rural areas, HCV prevalence ranges from 10% in children to 45% in adults. The origins of this HCV epidemic have been attributed to the parenteral treatment of schistosomiasis by antimony salts between 1960 and 1982[(4)]. The estimated global prevalence of HCV infection is 2.2%, corresponding to about 130 000 000 HCV-positive persons worldwide. An estimated 27% of cirrhosis and 25% of HCC worldwide occur in HCV-infected people. Region-specific estimates range from <1.0% in Northern Europe to >2.9% in Northern Africa. The lowest prevalence

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(0.01%-0.1%) has been reported from countries in the United Kingdom and Scandinavia; the highest prevalence (15%-20%) has been reported from Egypt[5]. Hepatitis C virus is an enveloped, single stranded, positive sense RNA virus with a diameter of about 50 nm, classified as a separate genus (Hepacivirus) within the Flaviviridae family. The genomic organization and sequence of HCV resembles that of the pestiviruses and flaviviruses[6]. The HCV genome is ~9600 nt in length and carries a single, long open reading frame (ORF) flanked by 5' and 3' non-translated regions[7]. The main route of HCV transmission is parenteral[8]. Treatment of HCV is costly, beyond the reach of most patients in less-developed countries, requires 48 or more weeks to complete, and has serious adverse effects and low efficiency. HCV in a family member can be socially and economically detrimental. There is no vaccine for HCV[9]. HCV infection is infrequently diagnosed during the acute phase of infection. Clinical manifestations can occur, usually within 7 to 8 weeks (range, 2 to 26) after exposure to HCV, but the majority of persons have either no symptoms or only mild symptoms. Fulminant hepatitis has been described during this period, though it is very rare. In cases in which symptoms of acute hepatitis have been documented, they usually consisted of jaundice, malaise, and nausea. The infection becomes chronic in most cases, and chronic infection is typically characterized by a prolonged period in which there are no symptoms. An estimated 74 to 86 percent of persons will have persistent viremia[10]. HCV cirrhosis, fibrosis, and hepatocellular carcinoma are major sequelae of HCV chronic infection. The natural history of HCV infection remains surprisingly unclear. Large studies have suggested that up to 30% of infected individuals will develop cirrhosis, leading to end-stage liver failure and HCC[11]. Pegylated interferon (PEG IFN) and ribavirin combined therapy is the standard approved treatment for HCV and is only effective in around 50% of patients[12]. During the first three months of therapy, HCV viral loads usually fall to undetectable levels in response to IFN. However, in non-responder (NR) patients, HCV viral loads persist at pretreatment levels. Among those patients with an initial response to treatment, up to 50% will relapse after treatment is discontinued and are known as relapsed responders (RR), whereas the remainder will have sustained virological response (SVR) as determined by the absence of detectable viremia six months after the end of treatment[13]. IFN-α mediates its antiviral and pharmacological effects by binding to type I IFN receptors on the cell surface membrane, which leads to transcription of a large number of IFN-stimulated genes, presumably *via* the Janus-activated kinase 1-signaling transducers of activation and transcription (JAK1- STAT) signaling pathway. The transcription of IFN-inducible genes is usually activated through the IFN-stimulated response element (ISRE)[14]. Factors affecting viral morbidity, disease progression and response to therapy are diverse and can be classified into viral and host factors. Viral factors include viral genotype, pretreatment viral load, and pretreatment viral quasispecies diversity. Host factors include age, sex, co-infection with other pathogens, and the strength of the immune system response[15]. Virus–virus interactions have been demonstrated to modify the pathogenesis of human viral infections[16]. Several studies reported that HCV and HBV dual infection can coexist and suppress each other's replication, indicating a mutual interference[17]. Other studies indicated that EBV enhanced HCV replication and that EBV-encoded nuclear antigen 1 (EBNA1) was responsible for supporting HCV replication. EBV was the first human

virus directly implicated in carcinogenesis. Since its discovery it has been considered as a major player in the development of a wide range of cancers both in immune-competent and immunocompromised individuals[18]. Sugawara et al reported a higher amount of EBV DNA in HCV-positive HCC compared to HBV-associated HCC. EBV-infected cells support HCV replication better than uninfected cells, suggesting that EBV may act as a helper virus to promote HCV replication in the HCV-positive HCCs[19]. Epstein–Barr virus (EBV) was discovered by electron microscopy of cells cultured from Burkitt's lymphoma tissue by Epstein, Achong, and Barr in1964[20]. Four years later, in 1968, EBV was shown to be the etiologic agent of heterophile- positive infectious mononucleosis[21]. EBV DNA was detected in tissues from patients with nasopharyngeal carcinoma in 1970. EBV is a member of the herpesvirus family. The viral genome is encased within a nucleocapsid, which is, in turn, surrounded by the viral envelope. Before the virus enters the B cell, the major envelope glycoprotein, gp350, binds to the viral receptor, the CD21 molecule (the C3d complement receptor) on the surface of the B cell. Other factors in addition to CD21 are important for infection. The major histocompatibility complex (MHC) class II molecule serves as a cofactor for the infection of B cells[22]. Patients with X-linked agammaglobulinemia lack mature B cells, and their B cells cannot be infected with the virus either *in vitro* or *in vivo*[23]. The Epstein-Barr virus (EBV) is a member of the Gamma *herpesvirinae* subfamily of herpes viruses. It infects more than 90% of the adult population all over the world. EBV shares the tendency of establishing latency in the host with other herpes viruses[24]. Some reports have suggested EBV to be a trigger agent for an autoimmune hepatitis[25]. EBV infection is the main cause of post-transplant lymphoproliferative disease (PTLD). The incidence of PTLD ranges from 0.5% to 30% depending on the organ being transplanted, the EBV status of the transplant recipient and donor, and the therapies used to achieve immunosuppression[26]. In order to examine the possibility that infection with EBV virus affect response of HCV infected patients to interferon and ribavirin therapy, this study is conducted to examine the role of EBV reactivation in determining the response rate to treatment with interferon and ribavirin therapy in chronic HCV patients.

Patients and Methods

Patients

One hundred patients with proven chronic hepatitis C who underwent PEG IFN + ribavirin therapy and twenty healthy controls (negative for both HCV and HBV antibodies) were enrolled in this study. They were all Egyptians and unrelated to each other. Informed consent was obtained from each subject before collecting blood samples. All HCV patients were positive for both anti-HCV antibody and serum HCV RNA according to reverse transcription-polymerase chain reaction (RT–PCR). Viral RNA quantification was performed using HCV one step RT-PCR quantitative diagnostic kit (Hangzhou Bioer Technology, Hangzhou, China). IFN treatment and clinical follow up were performed at the treatment center of Viral hepatitis in Ismailia Hospital for tropical diseases, Ismailia. Prior to the start of the IFN therapy, none of patients had evidence of metabolic liver diseases, alcohol-induced, drug-induced or autoimmune hepatitis, diabetes, thyroid hypo or hyperfunction or had a history of schistosomiases. A pretreatment liver biopsy was carried out for histopathological examination, and histological staging. Patients were treated with IFN and ribavirin combination therapy. They received Peginterferon alfa-2b injections taken once weekly

at 1.5 micrograms/Kg, (Schering-Plough co. USA), in addition to oral ribavirin taken daily in a dose of 1000–1200 mg (according to bodyweight). Responders (*n*= 74) were defined as patients who tested negative for HCV RNA . All other patients, who did not respond to the therapy, were considered NR (*n* = 26).

HCV RNA PCR

Disappearance of viremia was confirmed by a variety of tests including RT–PCR using nested primers derived from the highly conserved HCV 5′UTR and real-time PCR. These tests were done after 12, 24 and 48 weeks from the start of IFN therapy. HCV RNA in plasma was detected using HCV RT-PCR Fluorescence Quantitative Diagnostic Kit (Hangzhou Bioer Technology, Hangzhou, China).

EBV DNA PCR

i) Extraction of DNA

Blood samples from all healthy controls and HCV-infected patients undergoing IFN-based therapy were collected. Total DNA was extracted from 200 µl whole blood using the commercially available DNA extraction kit QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) by the spin column procedure according to the manufacturer's instructions and eluted in 200 µl buffer AE then DNA eluate was stored in 3 aliquots for each sample (to prevent further thawing and freezing cycles with subsequent decrease in the viral load) at - 20°C until the real time PCR testing was performed. This method provides a high yield of a pure preparation of intact high molecular weight DNA.

ii) Detection of DNA

The EBV load was measured quantitatively using the commercially available Robogene EBV quantification kit (aj Roboscreen, GmbH, Germany) Using Rotor Gene 6000 analyzer (Corbett Research) according to the manufacturer's instructions. The RoboGene EBV Quantification Kit allows real-time in vitro quantification of EBV DNA isolated from clinical samples via amplification of the EBV nuclear antigen (EBNA-1).The increase in fluorescence is directly proportional to the specific target amplification during PCR. EBV control DNA provided as "intelligent tubes" were included in each experiment to obtain an immediate in vitro quantification of the EBV genomes in the tested samples. This control consists of 8 capillaries which are storage stably coated with different amounts of WHO reference calibrated synthetic virus control. The lower detection limit of the assay is 500 copies/ ml. EBV DNA peripheral blood load results were classified into 3 groups: undetectable, 500-2000 copies/ml and more than 2000 copies/ ml as 2000 copies/ ml whole blood was defined as cut-off for increased virus replication[27].

Serological Assay of EBV antibodies

Diagnosis of Epstein-Barr virus (EBV) infection is based on clinical symptoms and serological markers, including the following: immunoglobulin G (IgG) and IgM antibodies to the viral capsid antigen (VCA) , IgG and IgM antibodies to the EBV early antigen-diffuse (EA-D) and nuclear antigen (EBNA-1). The presence of these five markers in serum was determined using enzyme-linked immunosorbent assay kit (EUROIMMUN, Germany). Samples were processed according to the manufacturer's instructions.

Results

In order to explore whether any of the clinical, pathological, biochemical or virological parameters are associated with a specific response pattern, a comparison between R and NR was carried out and is outlined in Table 1. Among a total of 100 chronic HCV patients, the univariate analyses of all fac-

tors showed that there were no significant differences between the patient populations in gender, body mass index , Hemoglobin, PLT count, TLC, ALT, AST, Albumin, Total billirubin, Indirect bilirubin, Creatinine, fibrosis stage or activity grade. On the other hand results showed that there was significant higher mean age in non-responder than responder patients (p= 0.0015) while alpha-fetoprotein (AFP) levels were significantly higher in NR than R patients (*P*= 0.032). Also, the mean values of HCV RNA levels were significantly higher in non-responder than responder patients ($0.72x10^6$ *vs.* $0.34\ x10^6$) (p=0.049). The serological results of EBV as detected by ELISA among the studied control, responder and non-responder groups showed that there were not statistically significant differences between both groups regarding all EBV antibodies as detected by ELISA (p>0.05) as depicted in figure 1 .The interpretation of EBV serological results as detected by ELISA of the studied control, responder and non-responder groups showed that there was statistically significant higher percentage of past infection among non-responder than responder patients (100% *vs.* 81.8%, respectively) (p<0.05) as depicted in figure (2).

Table 1 Laboratory findings in 100 chronic HCV patients treated with pegylated interferon plus ribavirin therapy

Variables	Responder (n=74)	Non responder (n=26)	P-value
Demographic characteristics			
Age (years)	41.0±9.9	48.1±8.3	0.0015**
Gender (males/females)	52.7% / 47.3%	38.5% / 61.5%	0.31
BMI (kg/m^2)	26.0±2.3	25.1±3.1	0.12
Hematological parameters			
Hemoglobin (g/dl)	13.34±1.61	13.15±1.41	0.59
PLT count ($x10^3$)	233±97.2	217.9±72.1	0.47
TLC ($x10^3$)	6.97±1.99	6.23±2.10	0.11
Biochemical parameters			
ALT (IU/L)	65.6±32.2	66.8±31.9	0.87
AST (IU/L)	61.5±28.6	60.0±27.6	0.82
Albumin (g/dl)	4.3±0.49	4.2±0.42	0.73
Total billirubin (mg/dl)	0.67±0.22	0.65±0.22	0.69
Indirect billirubin (mg/dl)	0.36±0.18	0.39±0.16	0.45
Creatinine (mg/dl)	0.84±0.2	0.95±0.84	0.79
AFP (ng/mL)	3.5±2.8	5.2±4.0	0.032*
HCV RNA $x10^6$ (copies/ml)	0.34±0.56	0.72±1.4	0.049*
Histopathological parameters			
Low fibrosis (F0-F1)	27 (36.5%)	7 (26.9%)	0.52
High fibrosis (F2-F4)	47 (63.5%)	19 (73.1%)	
Low activity (A0-A1)	53 (71.6%)	20 (76.9%)	0.79
High activity (A2-A3)	21 (28.4%)	6 (23.1%)	

*Significant (p-value <0.05), **=highly significant (p-value <0.01), Mean ±SD; BMI= body mass index; PLT= platelets; TLC= total leucocytes count; ALT= alanine aminotransferase; AST= aspartate aminotransferase; AFP= alpha-fetoprotein; HCV= hepatitis C virus; RNA= ribonucleic acid

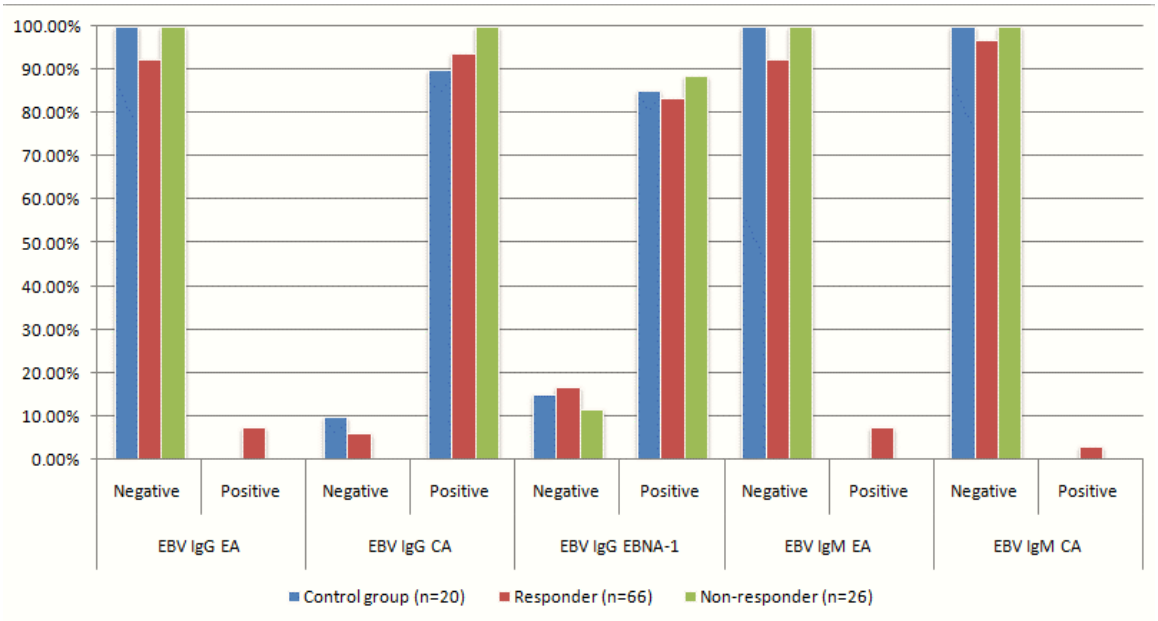

Figure 1: Serological results of EBV as detected by ELISA among the studied control, Responder, and non-responder groups

EBV viral load

The results showed that there was statistically significant higher percentage of EBV 500-2000 copies/ml in the control group than HCV patients group (35% *vs.* 7%, respectively) ($p<0.01$). Also, there was statistically significant higher percentage of undetectable EBV in HCV patients group than control group (82% *vs.* 60%, respectively) ($p<0.05$). The results also showed that there was statistically significant higher percentage of undetectable EBV in non-responder than responder patients (96.2% *vs.* 77%, respectively) ($p<0.01$), while there was statistically significant higher percentage of EBV >2000 copies/ml in responder than non-responder patients (14.9% *vs.* 0.0%, respectively, $p<0.05$) (Figure 3).

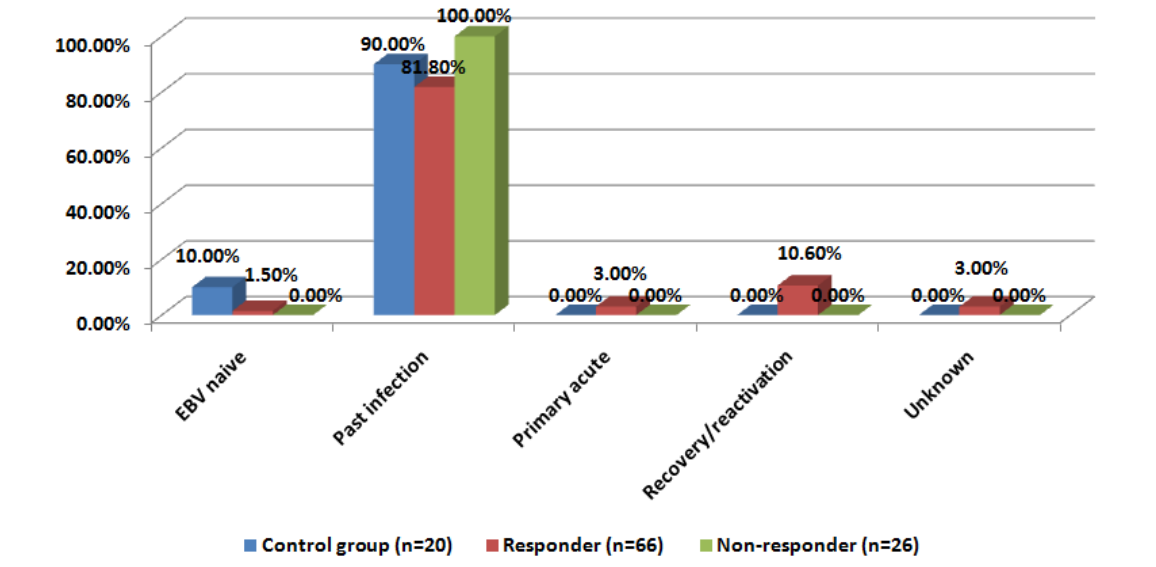

Figure 2: Interpretation of EBV results as detected by ELISA among the studied control, responder and non-responder groups

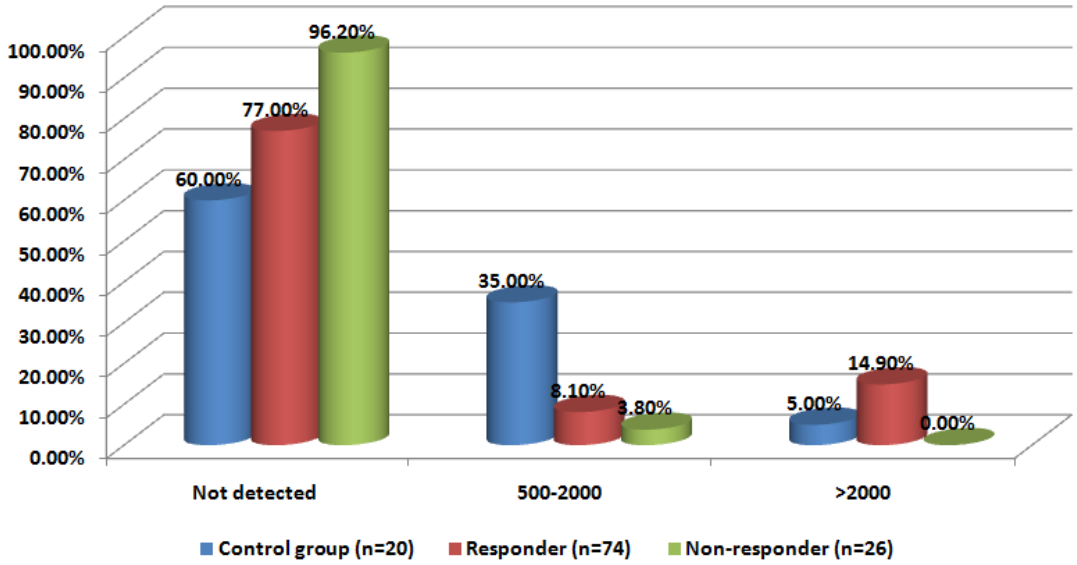

Figure 3: viral load of EBV as detected by PCR among the studied control, responder and non-responder groups

Discussion

Co-infections with HBV or HIV in patients infected with HCV have been proven to accelerate the course of chronic hepatitis C thus leading to a more severe histological picture and facilitating the disease progression to fibrosis, cirrhosis and hepatocellular carcinoma[28]. Specifically in HCV patients, co-infection with CMV can suppress the host immune system, affects the IFN-mediated antiviral pathway and thus becomes an additional threat to the host and may contribute to the complexity of disease outcome in HCV infection[12]. EBV is considered as the first virus associated with human malignancies. In Japan, Sugawara et al., found that EBV accelerates the onset of HCC and plays a role in oncogenesis of HCC. Probably by promoting HCV replication, exacerbating inflammatory processes in liver tissue. Promoting the proliferation of carcinoma cells or directly influencing tumorigenic potential[18]. The purpose of this study was to explore whether co-infection with EBV could significantly inhibit the response to IFN in chronic HCV patients and hence could be considered a significant risk factor for resistance to IFN therapy or not . Quantitative EBV DNA measurement is essential for differentiating the low-level infection of healthy carriers from the high levels characteristic of EBV-related disease. Patients with active infection or EBV-related cancer tend to have high levels of EBV DNA in the cell-free fraction of blood (plasma or serum), whereas in healthy carriers the virus is restricted to the intracellular compartment of the blood[29]. In the present study, EBV DNA load was measured in unfractionated whole blood samples not from peripheral blood mononuclear cells (PMNCs) nor from plasma samples because whole blood reflects the absolute viral burden in a defined unit of the circulation better than artificially enriched cell fractions[27], also this approach may increase the sensitivity by combining plasma and cellular blood compartments ,which may both harbor EBV DNA . The use of whole blood omits cell separation techniques and delayed preparation of plasma, factors that may possibly affect viral DNA characteristics by inducing uncontrollable cell lysis[30]. Plasma contains EBV DNA from free virus as well as any episomal DNA re-

sulting from the lysis of lymphocytes. Because the degree of lysis may vary depending on the EBV-specific cytotoxic T-cell response and the manipulation and storage of the sample, the testing of plasma can yield an underestimate of the total EBV DNA and may also lead to a higher degree of variability in DNA load measurement compared to the testing of whole blood[31]. EBV DNA peripheral blood load results were classified into 3 groups : undetectable, 500-2000 copies/ml and more than 2000 copies/ ml as 2000 copies/ ml whole blood was defined as cut-off for increased virus replication[27]. The results showed that there was statistically significant higher percentage of undetectable EBV in non-responder than responder patients (96.2% *vs.* 77%, respectively) ($p<0.01$), while there was statistically significant higher percentage of EBV >2000 copies/ml in responder than non-responder patients (14.9% versus 0.0%, respectively) ($p<0.05$) , the current study results disagree with those of Sugawara et al[18] who examined the effect EBV infection on HCV replication in vitro, indicated that HCV replication was promoted by EBV and that EBNA1 was responsible for supporting HCV replication. Yalcin et al[32] reported a reactivation of hepatitis B virus infection and a severe hepatitis flare in a patient with chronic hepatitis due to dual infection with hepatitis B and C viruses during combination therapy with alpha interferon and ribavirin. HBV reactivation following a decrease in HCV replication in an HBsAg-positive/anti-HCV positive patient on IFN therapy suggests that IFN therapy had decreased the suppressive effect of HCV on HBV to a level allowing HBV reactivation, as reported by Villa et al[33]. Therefore, there may be a potential risk of severe hepatitis if the clearance of one virus removes its suppressive effect on and facilitates the emergence of the other[34]. On the contrary other study investigated in vivo HCV and EBV in patients with AIDS[35], Their results showed that there was no difference between anti-HCV-positive and -negative patients or between HCV RNA-positive and -negative patients with regard to the prevalence of EBV markers, especially EBV replication markers. The presence of EBV replication markers was not related to HCV RNA seropositivity or to HCV viral load. This discrepancy could be explained by conditions differences between the two studies; at our study patients were underwent interferon based therapy where their patients did not have treatment; therefore suppressive effect of interferon on HCV was missing. Other study investigated correlation between HCV infection and reactivation of EBV in patients who succeeded in eradication of HCV after IFN therapy, their results suggested that the interferon therapy eradicated HCV but not EBV, the presence of HCV is required for reactivation of EBV in patients[36]. This discrepancy could be ascribed to the HCV genotype may be an interfering factor in the later study which performed in Japan with HCV genotype 1b being most predominant in Japan (63%) and genotype 4a in Egypt (93%)[37]. The diagnosis of EBV-related disease is based on clinical symptoms and laboratory testing[38]. The serological results of EBV as detected by ELISA among the studied groups shows that there were not statistically significant differences between both groups regarding all EBV antibodies as detected by ELISA ($p>0.05$). The interpretation of EBV results as detected by ELISA of the studied responder and non-responder groups showed that there was statistically significant higher percentage of past infection among non-responder than responder patients (100% *vs.* 81.8%, respectively) ($p<0.05$). These findings support those of kuniholm et al[39] who found that Among the 635 study women, 629 (99%) were EBV seropositive and among EBV-seropositive

women, HCV RNA status was not associated with level of EBV IgG. Also the current study results are contradictory also with those of Petrova et al[40] who investigated the possible role of the reactivated EBV infection in patients with chronic liver disease caused by HBV and HCV. Their patients with reactivated EBV infection had lower levels of HBV DNA and higher mean values of serum hepatitis C virus (HCV) RNA respectively, compared to EBV-seropositive patients without reactivation. This discrepancy between the present study results and those of Petrova et al. could be explained by conditions differences between two studies; at our study patients were underwent interferon based therapy where their patients did not have treatment ,therefore suppressive effect of interferon on HCV was missing.

Conclusion

In conclusion, these data exemplify the complexity of viral dominance in patients infected with multiple hepatitis viruses, and this has significant importance for treatment decisions. Also, we showed that high percentage of EBV viral load in responders and low EBV viral loads in non-responders. In conclusion, the results of this study suggest that there is a unidirectional relation between EBV and HCV in our study subjects.

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