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Research Article

Serological and molecular detection of Epstein –Barr virus (EBV) in patients with malignant lymphoid solids

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ABSTRACT

Background: Epstein-Barr virus (EBV) is a common herpesvirus can be associated with numerous infections and cancers. This study aimed to diagnosis of EBV among malignant lymphoid solids of patients in Basrah province/Iraq.

Patients and Methods: A 120 blood samples of both sexes with the age range 6 months to 93 years' old were collected from patients with malignant lymphoid solids. All serum samples were examined to detect EBV biomarkers including (anti-EBV-CA (IgG), anti-EBV-CA (IgM) and anti-EBV-NA-(IgG) antibodies. The nested PCR protocol was used to amplify two DNA fragment of EBV.

Results: The results of patients showed positive sera of 14/120(11.66%), 120/120(100%) and 111/120(92.5%) of anti-EBV capsid antigen IgM, anti-EBV capsid antigen IgG and anti-EBV nuclear antigen IgG, respectively. The present study was showed 4 statuses (Table, 1) among studied groups; a 99/120 (82.50%), 12/120 (10.0%), 7/120(5.83%) and 2/120(1.66%) patients as seropositive infection, reactivated infection, old infection and recent primary, respectively. EBV-DNA was detected in 44/120(36.7%) and 35/100(35%) in patients and control groups, respectively.

Conclusions: we concluded that highly seroprevalence of EBV among malignant lymphoid solids patients.

Keywords: Epstein-Barr virus, Acute lymphoblastic leukaemia, Hodgkin lymphoma.

INTRODUCTION

Leukemia is cancer of the blood or bone marrow that is characterized by an abnormal increase in blood cells, usually leukocytes (white blood cells). Leukemia is a wide term that covers a spectrum of diseases. In turn, it is part of the event of a larger group of diseases called hematological neoplasms.(Bacher et al., 2010).

The Epstein-Barr virus (EBV) is one of the most important viral causes of tumor development. It is a herpes virus that infects and establishes a persistent infection in humans. After primary infection in healthy individuals, EBV infects and immortalizes B lymphocytes, followed by lifelong viral latency.(Bacher et al.,2010). The viral particle is about 122-180 nm in diameter and consists of double-stranded DNA containing about 172 kb and 85 genes. The DNA is surrounded by protein. nucleocapsid which is surrounded by a tegument made of protein , which in turn is surrounded by a lipid envelope. Surface glycoproteins play an important role in the adsorption phase of the virus and then when the virus enters the cell. Epstein-Barr virus genes encode 100 to 200 polypeptides (Young and Rickinson , 2004). EBV is one of the eight viruses of the herpes family (Herpesviridae) and one of the most common viruses in humans. It belongs to the herpes virus subfamily γ Gammaherpesvirinae and is the prototype of the genus Lymphocryptovirus (Rickinson and Kieff, 2006). This virus is ubiquitous and infects over 90% of the human population worldwide with latent, asymptomatic and permanent infections (Saha and Robertso, 2011). The spectrum of disease associated with EBV has extended from infectious mononucleosis to overt leukemia / lymphoma. However, the definitions of the diseases are not yet clear. Some diseases are well defined and known, others are poorly defined and poorly understood (Cohen et al., 2009). Therefore, the present study was aimed at the serological and molecular detection of EBV in malignant lymphoid solids from patients in Basra Province / Iraq.

MATERIALS AND METHODS

Patients and samples

The blood samples were collected from Al-Sader teaching hospital and children hospital during the period December, 2018 into February 2019, comprising 120 individuals as patients group, 63 of them are males and 57 females with age range 6 months to 93 years, furthermore, 100 individuals as control group, 49 of them are males and 51 females with age range 6 months-

83 years, were selected randomly. A sample of 5 ml blood from each patient was collected by vein puncture; a 2ml of blood was put in EDTA tube used for extraction DNA. The remaining of blood was put in sterile plain tube left to clot at room temperature, and then centrifuged at 3000 rpm for 5 min. The serums were divided into several 0.25 µL aliquots and immediately stored at -40 until used in serological examination.

Serological tests

All serum samples were examined to detect EBV biomarkers including (anti-EBV-CA (IgG), anti-EBV-CA (IgM) and anti-EBV-NA-(IgG) antibodies. Determination was performed by using ELISA according to (EUROIMMUN kit, Germany).

Molecular detection

DNA extraction

DNA was extracted from blood using a DNA extraction Kit (Promega, USA) according to the manufacturers' instructions.

Nested PCR protocol

The nested PCR protocol was used to amplify a fragment of COOH-terminal part of the EBNA-1 gene (Wang et al., 2002). The purified EBV DNA was amplified over two rounds using outer and inner primers. The first round, involved usage of outer primers (5-GTAGAAGGCCATTTTTCCAC-3 (nt 109151–109170) and 5-CTCCATCGTCAAAGCTGCA-3 (nt 109741–109759) to amplify 609 bp of target region. The reaction mixture (25µl), was composed of 5 µl of DNA template, 2 pmol of primers, 12.5 µl of master mix (Biolab, UK) and the volume completed to 25µl with DD-Water. While in the second round, inner primers (5-AGATGACCCAGGAGAAGGCCCAAGC-3 (nt 109266–109290) and 5-CAAAGGGGAGACGACTCAATGGTGT-3 (nt 109549–109573) were used to amplify 309 bp of first product. The reaction mixture (25µl), was composed of 5 µl of the first PCR product as a template, 2 pmol of primers, 12.5 µl of master mix and the volume completed to 25µl with DD-Water. The reaction conditions were 94^0C for 30 min., 25 cycles of 94^0C for 30 sec. and 58^0C 30 sec., with a final extension at 72^0C for 40 sec. The Amplified products were visualized on 2 % agarose gel.

Statistical analysis

Analysis of the data obtained was made by using SSPS software version SPSS 24. P values <0.05 were considered statistically significant. Frequencies of each group were calculated by direct counting. Chi squares were performed to indicate the significance differences between groups.

RESULTS

Study populations

A total of 120 cancer individuals with the age range 6 months to 93 years' old were divided into 6 studied groups gives up the follows percentages (No,%): Acute Lymphoblastic Leukemia (ALL) [50 ,41.68%; 26(21.68%) females and 24(20%) mals], Non-Hodgkin Lymphoma (NHL) group [48, 40% ; 23(19.17%) females and 25(20.83%) males], Hodgkin lymphoma(HL) group [18, 15% ; 8(6.67%) females and 10(8.33%) males], Chronic Lymphocytic Leukemia (CLL) group [2, 1.66% ;1(0.83%) females and males], Burkitt's lymphoma (BL) group [1, 0.83% ; male only] and Nasopharyngeal carcinoma (NPC) group [1,0.83%; female only]. The ALL and NHL groups were the most groups diagnosed than other groups with significance differences at level of 0.05.

Serological tests

The present study was used Enzyme-linked immunosorbent assay (ELISA) test according to three types of antibodies. The results of patients showed positive sera of 14/120(11.66%), 120/120(100%) and 111/120(92.5%) of anti-EBV capsid antigen IgM, anti-EBV capsid antigen IgG and anti-EBV nuclear antigen IgG, respectively. While the results of healthy control showed positive sera of 5/100(5%), 97/100(97%) and 98/100(98%) of anti-EBV capsid antigen IgM, anti-EBV capsid antigen IgG and anti-EBV nuclear antigen IgG, respectively.

In EBV capsid antigen (IgM), the results were distributed among studied groups as follow: ALL group [7, 50%; 3(21.42%) females and 4(28.57%) males], NHL group [5, 35.71%; 2(14.2) females and 3(21.42%) males] and HL group [2, 14.28%; 1(7.14%) female and male]. In EBV capsid antigen (IgG), the results showed positive sera of 120(100%) and 97(97%) in the studied groups and control, respectively. Also in EBV nuclear antigen (IgG), the results were distributed among studied groups as follow: ALL group [44, 36.34%; 23(19.17%) females and 21(17.50%) males], NHL group [46, 38.34%; 22(18.34%) females and 24(20%) males], HL group [17, 14.17%; 7(5.83%) females and 10(8.34%) males] , CLL group [2, 1.66% ;1(0.83%) females and males], BL group [1, 0.83% ; male only] and NPC group [1,0.83%; female only].

Serological status of EBV infections

The current study was showed 4 statuses (Table, 1) among studied groups; a 99/120 (82.50%), 12/120 (10.0%), 7/120(5.83%) and 2/120(1.66%) patients as seropositive infection, reactivated infection, old infection and recent primary, respectively.

The seropositive or remote infection was distributed among studied groups as follow: 40(40.40%) for both NHL and ALL groups, 15(15.15%) for HL group, 2(2.02%) for CLL group and 1(1.01%) for both NFC and BL groups. Furthermore, reactivated infection was distributed among studied groups as follow: 5(41.66%) for both NHL and ALL groups and 2(16.66%) of HL group. Also, the Old infection was distributed among studied groups as follow: NHL 2(28.57%), ALL 4(57.15%) and 1(14.28%), While, recent primary was included (2, 100%) for ALL group (male only).

Table (1): statuses of EBV infections

No.	VCA Igm	VCA IgG	EBNA IgG	EBV status
99	_	+	+	Seropositive
12	+	+	+	Reactivated
7	_	+	_	Old infection
2	+	+	_	Recent primary
P <0.05				

Nested PCR

The extracting DNA was amplified by using PCR technique, the products of PCR was then subjected to gel electrophoresis. PCR products showed a sharp band on agarose gel (Figure, 1). Concerning to the results, the EBV DNA was registed in 44/120(36.7%) and 35/100(35%) in patients and control groups, respectively. The current results appeared that 25(56.8%) were females with significantly elevated (P<0.05) than 19(43.18%) males. Also, the positive results of EBV-DNA were distribution among the studied groups of patients as follow: NHL group (20, 45.45%; 11(55.0%) females and 9(45.0%) males), ALL group (18, 40.9%; 11(61.11%) females and 7(38.88%) males), HL group (3, 6.8%); 2(66.6%) females and 1(33.33%) males) and (1,2.27%) for NFC (female only), BL (male only) and CLL (male only) groups.

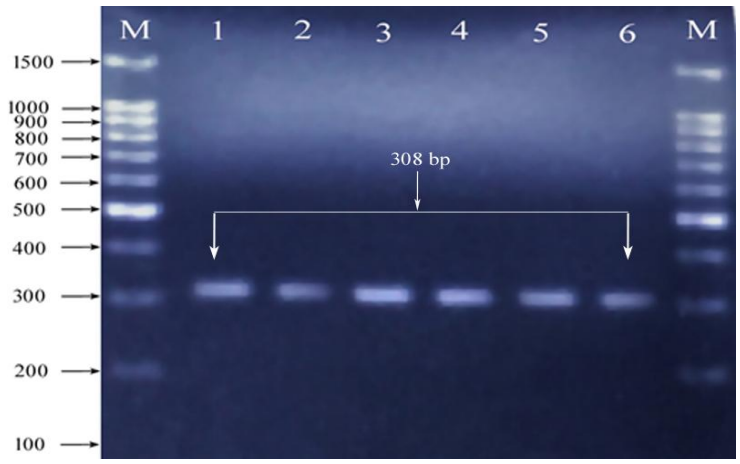

Fig.1:Gel electrophoresis of Nasted PCR products for Pateints

Lane M= DNA ladder (100-1500 bp DNA marker) , Lane 1-6 =positive results of EBV

DISCUSSION

The serological tests

The Epstein-Barr virus (EBV), the first human tumor virus to be isolated, was identified in 1964 by the Epstein group in a cell line derived from Burkitt's lymphoma (Epstein et al., 1964). In the present study, the enzyme immunosorbent assay (ELISA) was used according to three types of antibodies. Results showed positive sera of 14/120 (11.66%), 120/120 (100%) and 111

(92.5%) for anti-EBV IgM capsid antigen, anti-EBV capsid antigen IgG and anti- EBV IgG base antigen respectively.

There are several tests available that are commonly used to diagnose EBV infection that use different techniques but vary widely in performance(Gartner et al., 2001). VCA-IgM is transient and indicates a recent primary infection. VCA-IgM usually goes away after healing (a few weeks) and can persist for months (De Paschale & Clerici, 2012) and usually do not appear in life afterwards (Hess, 2004). Although VCA-IgM occurs early and helps diagnose acute EBV infection, there are some limitations to interpreting the VCA-IgM data as it has been found that some severely infected children and adults do not develop of VCA-IgM. (De Paschale and Clerici, 2012). The IgM VCA test has been widely used as an indicative test to diagnose a primary infection with EBV, because this antibody appears in a recurrent infection with this virus. detection of EBNA antibodies along with test for VCA IgG and/or IgM to diagnose primary EBV infection be used. A reliable EBNA antibody test could be used as a screening test, since the presence of EBNA antibodies precludes primary EBV infection. Antibodies to other EBV antigens should only be tested in the absence of EBNA antibodies (Svahn et al., 1997).

Regarding with results, the present study showed that seroprevalance of EBV infections were 100% and 97% in studied groups and healthy control, respectively, hence the increasing in the numbers of persons with malignancies in the future. In China, about 100% of children between the ages of 10 and 15 years have EBV antibodies, and it has been observed that in large region of China, nasopharyngeal carcinoma cancer is commen in the 20–40 age groups, possibly lead to its activation. primary infection with EBV (Xiong et al., 2004).

In present study, the results showed positive sera of 14(11.66%) to EBV capsid antigen (IgM), this was agreement with EBV IgM VCA in Croatia where IgM antibodies were detected in 9.0% (Nataša et al., 2018), also it was agreement with Kostadinova et al., 2018 that showed 18.2% IgM seropositivity of antibody against VCA. While the present study was disagreement with the study recorded in Qatar that showed only 1.8% IgM VCA.

The present results showed positive sera of 120(100%) to EBV capsid antigen (IgG), which was agreement with the prevalence rate in other developed countries (Pariente et al., 2007; Trottier et al., 2012; Henrik and Jeppe, 2007), and agreement with Nataša et al., 2018 that showed IgG seropositivity was 95.9%, and agreement with the seroprevalence of Epstein-Barr virus VCA IgG in Turkya which was found 96.4% seropositivity, also agreement with study of Qatar which appeared high rate of seropositivity (97.9%) was positive to VCA- IgG (Maria et al., 2017). While in USA they reported that 90% of healthy subjects pocessed IgG antibodies against EBV viral capsid (VCA) antigen (Elansary et al., 2016).

While the present study was disagreement with study that registed in Blood donors from the two major blood donation centers in Baghdad who found EBV seropositivity 79.8% among these blood donors (Amjad et al ., 2017), and higher than the seroprevalence was announced amongst blood donors in Iran that registed 85% (Chen et al .,2015), and in another study in Tehran, Iran that appear the total seroprevalence of IgG antibodies 81.4% (Sharifipour and Davoodi, 2020). furthermore, in Taiwan, Chen et al., 2015 reported that total seropositive ratio of EBV was 88.5% (Balfour et al., 2013).

The present study found that 97% of healthy control were EBV seropositivly, and this ratio agreement with the study that conducted in Qatar which found that 96% of blood donors under the age of 30 years old were EBV seropositive, and seropositivity ratio increased each to 100% in donors who are older than 40 years (Maria et al ., 2017). Similar results have been reported in several studies, including a new study from Thailand which found that the seroprevalence of EBV in people over 40 increased to 100% with age (Suntornlohanakul et al ., 2015).

Serological statuses of EBV infections

The present study was showed 4 status among studied groups including; seropositive infection (99, 82.50%), reactivated infection (12, 10.0%), old infection (7, 5.83%) and recent primary (2, 1.66%), this was agreement with study of Christina et al ., 2013 which recorded 89.6% demonstrated previous exposure, also agreement with Klutts et al., 2009 who found that 77.8% of patients who were proved to have had a past infection, and agreement with study in Bahrin that found 13.9% were negative for EBV, while 6.4% showed primary EBV infection, 9.4% showed reactivation of EBV infection, and 70.3% showed previous infection with EBV.(Eman and Mohammed ., 2016). While the present study was disagreement with Chan et al., 2003 which recorded 26 (83.4%) of 31 patients diagnosed with early primary infections, 11(41%) of 27 with recent primary infections, 1 (7%) of 14 with past infections and none (0%) of those with no infection.

The Molecular detection

We used polymerase chain reaction to detect EBV because the low copies of EBV DNA in Stiu hybridization are difficult to detect, but can be detected by PCR
(Hsieh et al., 1998). However, EBV DNA can also be detected from reactivated EBV infection (Dohno et al., 2010). Although most people would expect EBV DNA to be present in their lymphocytes, viral DNA is not normally found in plasma in the absence of active EBV disease because EBV is mainly associated with B cells and plasma viremia is rare(Gärtner and Preiksaitis, 2010 ; Qu et al., 2007).

The EBV-DNA was detected in 44/120(36.7%) and 35/100(35%) in patients and control groups, respectively. Also, the positive results of EBV-DNA were distribution among the studied groups of patients as follow: NHL group (45.45%), ALL group (40.9%), HL (6.8%) and (2.27%) for NFC, BL and CLL groups.

This present results agreement with the results that evidenced that 38 (76%) of patient subjects had EBV DNA in their serum (Shirin et al., 2015), also agreement with studies: chan et al., 2001; Okay et al., 2005 ; She et al., 2007; Kozic et al., 2006. Current results showed that 25(56.8%) were females with significantly elevated ($P<0.05$) than 19(43.18%) males. This difference is consistent with the concept that women generally develop stronger cell and antibody-mediated immune responses than men after infection or vaccination (Lazda, 2006; Beagley and Gockel, 2003). Therefore, these antibodies could be more easily detected in women than in men. In addition, the most common mode of transmission of EBV in adults is exposure to infected children (Xiong et al., 2014). Infected children actively excrete the virus in their saliva. As noted in this study, women in Iraq were at a higher risk of developing such infectious diseases in children due to their higher rate of contact with children compared to men, mainly mothers and teachers in schools. Kindergartens, daycares and elementary schools in these countries, most of them are teachers.

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