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ORIGINAL ARTICLE

Immunohistochemical Staining of Epstein - Barr Virus LMP-1 Expression in Patients with Lymphoma

Salwa S. Shihab [1], **Hayder Abdulhussein- Al-Hmudi** [2] and **Sadeq K. Ali. Al. Salait** [3]

[1] Basrah Youth and Sport Directorate, Basrah, Iraq
[2] College of Science, University of Basrah, Basrah, Iraq
[3] Hematologist, Oncology unit, Al-Sadr Hospital, Basra, Iraq

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Corresponding author:
Salwa S. Shihab
Email: salwaasab4@gmail.com
Basrah Youth and Sport Directorate
Basrah
Iraq

ABSTRACT

Background: Epstein-Barr virus (EBV) is a common herpes virus that can be linked to a variety of infections and cancers. Some kind of these diseases are good defined and known, others are poorly defined and weakly understood.

Objectives: The present study was aimed to study the expression of Epstein Barr virus (EBV) encoded oncoprotein LMP1 in the lymphoma patients.

Methods: A 67 blood samples of both sexes with the age range 3 to 93 years' old were collected from patients with malignant lymphoid solids. The nested PCR protocol it was used to amplify two DNA fragment of EBV. The immunohistochemical staining was used for detection of LMP1 proteins.

Results: EBV-DNA was detected in 24/67(35.82%) patients. The positive results of EBV-DNA were distribution among the studied groups of patients as follow: NHL group [20, 83.33%; 11(55%) females and 9(45 %) males], HL group [3, 12.5%; 2(66.66%) females and 1(33.34%) males] and [1, 4.17% (male only)] for BL. A 5/16 (31.25%) samples revealed positive staining of LMP1 protein.

Conclusion: we concluded that the positive EBV paraffin embedded samples was showing strong immunostaining for LMP1 protein.

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INTRODUCTION

Epstein-Barr virus (EBV) is one of the most important viral causes of tumor growth. It is a herpes virus that infects and creates a persistent infection in humans. Following primary infection in healthy individuals, EBV infects and immortalizes B lymphocytes, followed by lifelong viral latency [1]. The viral particle has a diameter of about 122-180 nm and consists of double-stranded DNA containing about 172 kb and 85 genes. Epstein-Barr virus genes encode 100-200 polypeptides [2]. 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 subfamily of the γ herpesvirus *Gammaherpesvirinae* and is the prototype of the genus *Lymphocryptovirus* [3]. This virus is ubiquitous, and infects more than 90% of the human population worldwide with a lifelong, asymptomatic, latent infection [4]. The virus persists in a

latent form for the rest of the person's life and is shed almost continuously. However, EBV infection during or after adolescence, can cause infectious mononucleosis [5]. The range 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 kind of these diseases are well defined and known, others are poorly defined and weakly understood [6]. Epstein-Barr virus (EBV) is known as the tumor virus, which expresses the viral cancer genes and immortalizes the infected lymphocytes. EBV infects not only B cells, but also T cells or natural killers (NKs). EBV causes benign lymphoproliferative diseases, infectious mononucleosis, and is link with several types of lymphoid neoplasms [3].

HL was originally described by Thomas Hodgkin in 1832. It is a unique type of lymphoma with a low number of neoplastic cells that comprise less than 1% of the total cell population. Neoplastic cells are found in a background of immune cells that consists of T-cells, eosinophils, neutrophils, plasma cells, fibroblasts and histiocytes. Despite their abundance, these immune cells are not able to induce an effective anti-tumor response. Instead the microenvironment protects neoplastic cells from anti-tumor .immune responses and provides survival signals for the neoplastic cells[7]. Spite of seriously chemotherapy result of EBV related T-cell non-Hodgkin's lymphomas (NHL) is always poor. Particularly, the fulminant T-cell NHL happening in the setting of a chronic active EBV infection has a dismal results [8]. Burkitt's lymphoma (BL) is a seldom but very vigorous non-Hodgkin's lymphoma (NHL) accounting for 0.8% of all B-cell lymphomas [9]. The present study was aimed to investigate the expression of EBV LMP1 in the lymphoma patients.

MATERIALS AND METHODS

Patients and samples: The blood samples were collected in EDTA tube from Al-Sader teaching hospital and children hospital during the period December, 2018 into February 2019, comprising 67 patients with lymphoma, 35 of them are males and 32 females with age range extend 3 to 93 years.

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 protocol of nested PCR was represented amplify a fragment of COOH-terminal part of the EBNA-1 gene [10]. 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 last extension at 72^0C for 40 sec. The Amplified products were visualized on 2 % agarose gel.

Immunohistochemical (IHC) staining: This study included 21 paraffin embedded tissue samples which include 16 paraffin tissue blocks of patients diagnosed positive for EBV and 5 paraffin tissue blocks which were diagnosed as negative for EBV. Blocks were collected from Al-Sader teaching hospital and children hospital. Sections were stained using hematoxyline and eosin stain following the procedure of Avwioro, 2011 [11]. Immunohistochemistry was procedure on the formalin-fixed paraffin-embedded tissues from previously diagnosed biopsies with monoclonal antibodies against the LMP-1.

RESULTS

A total of 67 lymphoma individuals with the age range 3 to 93 years' old were divided into 3 studied groups gives up the follows percentages (No,%): NHL group [48, 71.64%; 23(47.92%) females and 25(52.08%) males], HL group [18, 26.86% ; 8(44.45%) females and 10(55.55%) males], and BL group [1, 1.5%; male only].

Nested PCR: The extracting DNA was amplified by PCR technique, the PCR products was then subjected to gel electrophoresis. PCR products showed a sharp band on agarose gel (Figure 1). Regarding the results, EBV-DNA was detected in 24/67(35.82%) patients. Also, the positive results of EBV-DNA were distribution among the studied groups of patients as follow: NHL group [20, 83.33%; 11(55%) females and 9(45 %) males], HL group [3, 12.5%; 2(66.66%) females and 1(33.34%) males] and [1, 4.17% (male only)] for BL.

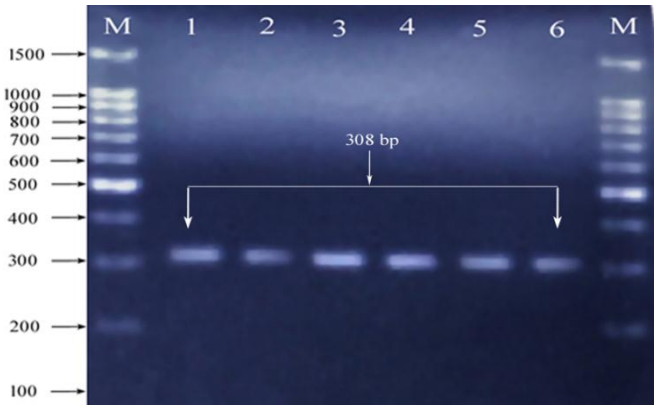

Figure 1. **Gel electrophoresis of Nasted PCR products for Pateints Lane M= DNA ladder (100-1500 bp DNA marker), Lane 1-6 =positive results of EBV**

LMP1 expression: The present study of IHC staining was included 16 pre-diagnosed EBV positive paraffin embedded samples, including; 12/16 (75%), 3/16(18.75%) and 1/16 (6.25%) samples of NHL, HL and BL groups, respectively, and 5 pre-diagnosed EBV negative paraffin embedded samples [3/5 (60%) and 2/5(40%) samples of NHL and HL groups, respectively]. The IHC staining was used for detection of LMP1 proteins. Of 16 paraffin embedded samples, LMP1 expression was detected in 5/16 (31.25%) samples including; 3/5 (60%) samples in HL group, 1/5 (20%) samples in NHL group, and 1/5 (20%) sample in BL group. The negative control did not show any immunohistochemical staining. In fact, LMP1 expression within each group was 3/3 (100%) samples in HL group, 1/1 (100%) sample in BL group and 1/12 (8.4%) sample in NHL group.

As shown in Figure 2, samples (a), (b), (c), showed positive staining for LMP1 protein. The negative sample (d) did not reveal any immunostaining. Sample (a) associated protein expression in cases of Hodgkin lymphoma, the protein expression usually strong among all tumor cells, changes with nucleus and cytoplasm, small lymphocytic cell obvious.

In sample (b); hyper proliferation of lymphocytes, large cells nuclear staining was positive, while sample (c); was showed a strong expression of LMP-1 associated with nucleus of lymphoma cells, dark stain nucleus, scanty cytoplasm, apoptotic cells and lymphocytic cells arrows showed positive staining in the diagnostic cells.

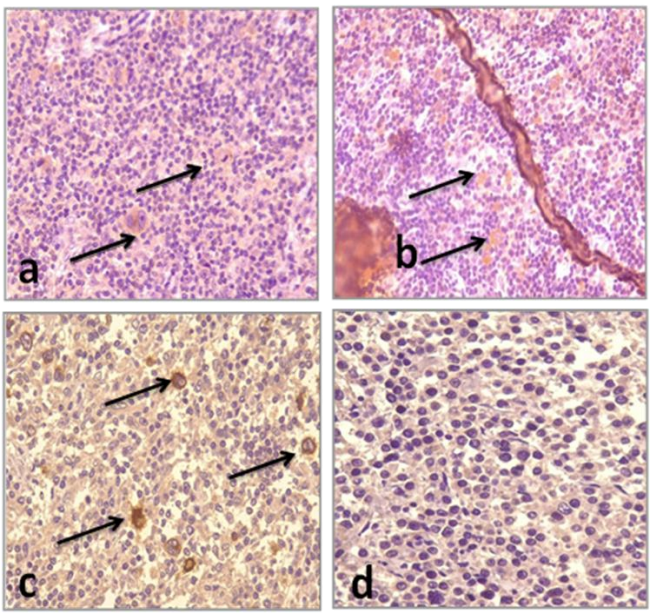

Figure 2. **Immunohistochemical findings**

DISCUSSION

Lymphomas originate from malignant transformation of lymphocytes, usually B-cells. T-cell and NK cell derived lymphomas are less common. The first lymphoma type that was recognized is Hodgkin lymphoma (HL). All the other lymphoma types have been collectively called non-Hodgkin lymphomas (NHL) and comprise various subtypes (like diffuse large B-cell lymphoma, follicular lymphoma and small lymphocytic lymphoma) as well as various T-cell lymphomas [9]. Over the last few years, LMP1 has attracted immense interest as it has been found to be associated with critical cellular signaling pathways. Latent membrane protein 1 (LMP1) is one of the major EBV encoded oncogenes associated with viral mediated transformation. It is known to encode an oncoprotein that functions as a constitutively active tumor necrosis factor receptor [12].

Our study reveals the association of EBV in lymphoma and demonstrates that immunohistochemical staining detects the presence of latent EBV in lymphoma tumors biopsy tissues. A 5/16 (31.25%) samples revealed positive staining of LMP1 proteins. LMP1 expression within each group was 3/3 (100%) samples in HL group, 1/1 (100%) sample in BL group and 1/21 (8.4%) sample in NHL group. In Iran, HL samples EBV LMP-1 was determined positive identicle to -35- percent [13].

The Molecular studies showed monoclonal EBV DNA in the Hodgkin tumor tissue, proposed that the virus was exiting prior expansion of the malignant clone [14]. Based on differences in histopathology, morphology, epidemiology and phenotype of the neoplastic cells, HL is divided into classical HL (cHL) and nodular lymphocyte predominant HL (NLPHL) [9]. These diagnosis is depend on the identification of multinucleated properties huge cells and an inflammatory environment. All These cells, called Reed Sternberg (RS) or diagnostic cells, form the body of the tumor; they measure 20-60 µm in diameter and appear a big border of cytoplasm within fully two nuclei with acidophilic or amphophilic nucleoli, coating more than 50% of the nuclear region [15].

In fact, correlation of EBV with HL cases is still argumentative [16]. various studies submited that EBV It is considered as a negative factor for advanced age and considered a positive factor for young [17,18,19]. In our study the age of patients was (7, 8 and 19) years old at HL, 7 years old at BL and 37 years old at NHL. The realization of prognostic importance and the role of EBV proto-oncogenes in tissues by immunohistochemical procedure can alter, therapy options [20].

There are several factors such as socioeconomic level, ethnical and geographical features have related with incidence of HL to EBV, and this varies between countries; a 90% of children less than six- years old undergo from virus in developing states, and -30% - -40% in developed countries [21]. The associated of HL cases with EBV have a ratio ranged from 30% - 50% in developed countries [22, 23] and 80% - 90% in developing countries [21, 24, 25]. Some findings suggesting that positive of EBV LMP-1 was not a prognosis factor for Hodgkin lymphoma [26, 27], while others results appeared that the virus was a negative parameter at the pathogenesis of Hodgkin lymphoma [28, 29].

In spite of EBV is assessed a B-lymphotropic virus, EBV can also infect other cells such as T-cells and

NK-cells. It has been assumed that EBV may move inside the T-cell through the processing that lead to killing B-cell by an stimulate activation cytotoxic T-cell [30]. It is the most prevalent, cancer in children in states where malaria is holo-endemic, such as Equatorial Africa, Brazil and Papua New Guinea [31]. The WHO classification of Burkitt's lymphoma describes three clinical variants: endemic, sporadic (the predominant type in areas unrelated to malaria) and linkage with immunodeficiency [32]. The endemic variant is related with endemicity of malaria and EBV is found in roughly all cases. The sporadic kind occurs fundamentally in the rest of the world (mainly North America and Europe) with no particular Climatic conditions or geographic association and is little linked with EBV infection. 1-2% of adult lymphomas and 30-40% of childhood non-Hodgkin's lymphomas in Europe and North America are sporadic Burkitt lymphomas [33, 34].

Because of all these circumstance, the negative ratio of EBV LMP-1 was 68.75% in the patients. This study has limitations due to the small number of studied cases, which might be viewed as a deceptive condition according to statistical significance. At the same time, the results were encouraging to LMP1 proteins staining of HL and BL cases that showed 100% positive results.

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