



LMP-1 IMMUNOHISTOCHEMICAL DETECTION OF EPSTEIN - BARR VIRUS IN LYMPHOMA AMONG SUDANESE PATIENTS

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ABSTRACT

Several studies have suggested a probable etiologic association between Epstein-Barr virus (EBV) and lymphoma; multiple EBV proteins can be expressed in infected lymphocytes, among which Latent membrane protein-1(LMP-1) is thought to be most important for malignant transformation, the aim of this study was to investigate the association of LMP-1 EBV in this tumors.

This study was carried at the Radio Isotope Center Khartoum (RICK)- Khartoum state –Sudan from January 2015 to July 2015, it was retrospective and prospective, the study include ninety (90) Formalin –fixed paraffin embedded biopsies from which seventy (70) of malignant lymphoma (study group) and twenty (20) lymph node hyperplasia (control group).

EBV was detected in (32%) (23/70), and (10%) (2/20) respectively, the (P.value =0.04), this result showed a significant difference between case and control groups, the, there is no statistically significant difference between the two lymphoma subtypes Hodgkin's and Non-Hodgkin's the (P.value= 0.333).

There is sufficient evidence for the carcinogenicity of EBV in the causation of lymphoma in Sudan. This requires further evaluation to find out whether this positivity is due to co-infection or has a role in pathogenesis.

Keywords:

Epstein-Barr virus, malignant lymphomas, lymph node hyperplasia, Hodgkin's disease, Immunohistochemistry, Sudan.

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1. INTRODUCTION

Malignant lymphomas make up an estimated 3–4% of all malignancies worldwide (Stewart and Kleihues. 2003). The lymphomas can be divided into two major groups, i.e. Hodgkin (20–30% of

all lymphomas) (Stewart and Kleihues. 2003; Stein. 2001) and non-Hodgkin lymphomas (HL and NHL respectively).

Infections and Hodgkin lymphoma:

The suspicion of an infectious etiology to HL is almost as old as the recognition of the disease as such (Mueller *et al.* 2007). Accordingly, both the disease's typical histological presentation dominated by inflammatory cells and its typical clinical presentation with sudden onset of fever, night sweats and Lymphadenopathy would be compatible with an infectious process. As mentioned, the clinical suspicion of an infectious etiology has been supported by epidemiological evidence. A hallmark of HL epidemiology is the bimodal age-specific incidence pattern that can be observed throughout the Western World (MacMahon, 1957). Different from the age-dependent monotonic incidence increase seen for NHL, this remarkable age distribution fostered the suggestion that HL in children, younger adults and older adults were etiologically distinct disease subtypes (MacMahon, 1957; MacMahon., 1966) More importantly, the typical histological presentation (nodular sclerosis HL), a more benign clinical behavior than HL in older adults, together with the impression of synchronicity of onset of familial cases, led to the proposition of an infectious etiology for HL in younger adults (MacMahon., 1966).

Non Hodgkin lymphoma (NHL) is a diverse group of neoplasms both in their natural history and in their response to treatment. Available epidemiological data from various parts of Asia indicate marked geographical variation in the incidence, Histopathologic and clinical behavior of NHL. Non Hodgkin lymphoma appears to be more common in developing countries, where a combination of environmental, infectious and genetic factors affect the development of these disorders. In the developed countries, the disease occurs more often in whites than in blacks, and it is about 50% more common among men than women (Mushtaq et al., 2008).

Epstein-Barr virus (EBV) is a widespread tumorigenic human herpes virus that establishes lifelong asymptomatic infection of B cells in the majority of humans, generally without causing disease (Williams et al., 2006; Mao et al., 2013). Epstein-Barr virus is associated with number of lymphoproliferative disorders including Burkitt's lymphoma (98%) (Nourse et al., 2012), post transplantation lymphoproliferative disease (PTLD), Angioimmunoblastic T-cell Lymphomas (AITL) (84.6%), natural killer (NK) cell lymphomas, lymphomatoid granulomatosis and Hodgkin's lymphoma (40%) (Nourse et al., 2012).

Substantial evidence implicates the Epstein-Barr virus (EBV) in the pathogenesis of Hodgkin's lymphoma (HL) (Flavell and Murray. 2000; Gulley *et al.*, 2002). EBV detection in HL may be used to risk-stratify patients and derive optimum treatment strategies. Investigation into the presence of EBV nucleic acids in affected tissues in EBV-associated diseases is performed by a variety of different techniques, including spot hybridization, in situ hybridization (ISH) and the polymerase chain reaction (PCR). (Ambinder, 2007; Gulley *et al.*, 2002) EBV-related proteins, including EBV nuclear antigen 1 (EBNA1) and the latent membrane proteins (LMP1, LMP2a and LMP2b) have also been examined by performing immunohistochemical assays. As previously stated, the percentage of EBV-positive cases of HL varied among studies, ranging between 20 and 70% (Flavell and Murray. 2000).

2. MATERIAL AND METHODS

This retrospective and prospective, was carried out in the Radio Isotope Center Khartoum (RICK)-Khartoum State –Sudan, in the period from January 2015 to July 2015,, the geographical and histoclinical data were achieved from the patients files , and Formalin –fixed paraffin embedded biopsies of seventy (70) of malignant lymphoma (study group) and twenty (20) lymph node hyperplasia (control group) , samples were used to investigate the positive rate of EBV, by anti (LMP-1) Immunostain according to standard Strepto Avididin Biotin (Thermo Fisher) protocol. The data was analyzed, using the statistical programs software Statistical Package for the Social Sciences (SPSS) version (11.5), Chi square test and different statistical measures were calculated.

The Immunohistochemical procedure was done as follows:

Three microns (3µm) sections from formalin-fixed, paraffin-embedded were cut and mounted onto salinized slides (Fisher brand). Monoclonal antibody (LMP- 1) was used as manufacture instructions to detect presence of (EBV) from both case and control samples. All sections were deparaffinized in two changes of xylene for 10 minutes in each change, then rehydrated in descending changes of ethanol as follows; sections were placed in two changes of absolute ethanol for 5 minutes in each change and then were placed in 90% ethanol for 3 minutes, and then were placed in 70% ethanol for 2 minutes, and then were washed in distilled water for 2 minutes. Sections were steamed for antigen retrieval for (EBV) using (PT link) in 10mM- citrate buffer Hydrochloric acid (HCl) (pH 7.6) for 20 minutes. Then slides were cooled in a sink containing cold tap water, the slides were removed once the buffer at room temperature, slides were rinsed in running tap water and placed in TBS at pH 7.4. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min, then ultra V block was applied and incubated for 8 min and then Slides were incubated with 100 µl of primary antibodies for 20 min at room temperature in a moisture chamber, and then were rinsed in Phosphate buffer saline. The primary antibody for EBV (Monoclonal antibody (LMP-1) was ready to use (Thermo Fisher). After washing with PBS for 3 minutes, binding of antibodies was detected by incubating with biotinylated goat anti polyvalent for 15 minutes, followed by incubating for 15 min with streptavidin peroxidase (Thermo Fisher kit). the slides then were be washed with two rinses of TBS pH 7.4 for 1-2 minutes followed, then the slides were be covered by 0.01% H₂O₂ and 0.05 3.3 diamino benzidine tetra hydro chloride (DAB) chromogen in Tris HCl pH 7.6 for 5 minutes at room temperature, (Thermo Fisher) as a chromogen to produce the characteristic brown stain for the visualization of the antibody/enzyme complex. the sections was counter stained with Mayer's Haematoxylin for one minute, washed in tap water for 2 minutes, dehydrated through 3 different baths of absolute alcohol, placed in xylene, mounted in (DPX) . For each run of staining, positive and negative control slides was also prepared. The positive control slides contain the antigen under investigation and the negative control slides prepared from the same tissue block, but were incubated with PBS instead of the primary antibody. Each slide was evaluated with investigator then the results were confirmed by consultant histopathologist.

3. RESULT

Males were the dominant gender, (73%) (51/70), and female represented (27%) of lymphoma case as illustrated in(Table.1).The frequency of malignant lymphomas according to the age groups, as the following (47%) (33/70), (30%) (21/70) and (23%) (16/70), for the age groups (less than 30

yrs), (above 50 yrs) and (30-50 yrs) respectively, as shown in (Figure .1). Considering the subtypes of malignant lymphoma, fifty nine (84%) (59/70) were Non Hodgkin` s lymphomas, and eleven (16%) (11/70) as Hodgkin` s lymphomas.

Comparing the association of EBV infection in malignant lymphomas and reactive lymph node hyperplasia, EBV were detected in (32%) (23/70), and (10%) (2/20) respectively, the (P.value =0.04), this result showed a significant difference between case and control groups, referring to (Table.2).

(Table. 3) illustrate that EBV positive Immunostain were reported in five (45%) (5/11) of Hodgkin` s lymphomas and (31%) (18/59) of Non Hodgkin` s lymphomas, the (P.value= 0.333), there is no statistically significant difference between the two lymphoma subtypes

Table 1: Distribution of study population according to the gender

Gender	Frequency	Present
Male	51	73
Female	19	27
Total	59	100

Table 2: EBV Immunostain, comparison between malignant lymphoma and the control group (normal and reactive lymph node)

Study group	IHC (EBV) result		Total	p. value
	Positive	Negative		
Malignant lymphoma	23	47	70	0.04
reactive lymph note	2	18	20	
Total	25	65	90	

Table 3: EBV Immunostain, comparison between Hodgkin` s lymphomas and Non-Hodgkin` s lymphomas

Type of Lymphoma	IHC (EBV) result		Total	p. value
	Positive	Negative		
H.L	5	6	11	0.333
N.H.L	18	41	59	
Total	23	47	70	

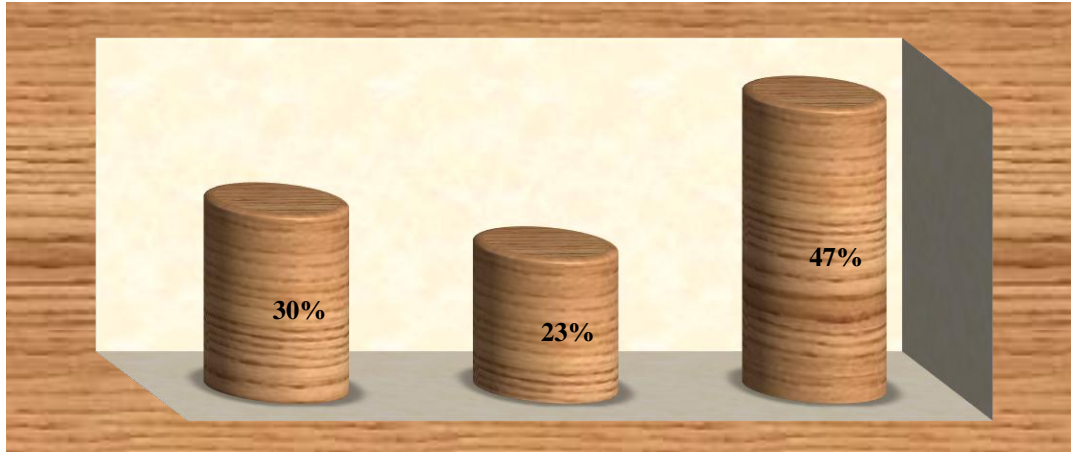


Figure 1: The frequency of age groups among malignant lymphomas
Discussion

The findings point out that, positive (LMP-1) Immunostain reaction for EBV, were reported in twenty three biopsies (32%) (23/70) among malignant tissues, compared with only two positive cases (10%) of lymph node hyperplasia, indicating a significant statistical difference, the (P.value =0.04), so there is sufficient evidence for the carcinogenicity of EBV in the causation of lymphomas, this finding is supported by (Orem, *et al.* 2007), their conclusion " Evidence for a causal relationship between EBV and Burkitt lymphoma in the endemic form is fairly strong. Frequency of association between EBV and Burkitt lymphoma varies between different patient groups and different parts of the world. EBV may play a role in the pathogenesis of Burkitt lymphoma by deregulation of the oncogenic c-MYC by chromosomal translocation". Also the strong evidence of EBV carcinogenicity came from (Gerald *et al.* 2001) they concluded "There is good evidence that EBV infection precedes expansion of the malignant cell populations in some virus-associated tumours. However, this is clearly not always the case and for some of these tumours there are indications that clonal genetic alterations may occur prior to EBV infection. Thus, whilst there is good evidence to suggest that EBV is a human carcinogen, its precise role in the development of virus-associated human tumours". Moreover the study showed that, the malignant lymphomas had higher frequency in male than female (73% versus 27%) the ratio: 2.7:1), similar findings were reported from Iran, by (Amir, *et al.* 2012), their results were "out of the 30 patients with primary GI lymphoma in the study, 12 were female (40%) and 18 were male (60%) (Male to female ratio: 3/2). The reason for this gender domination in lymphoma may be due to increase exposure to the environmental factors, this explanation, supported by Canadian study by (Chandima, *et al.* 2008) whom concluded that, an increased risk of developing NHL is associated with the following: long held occupations of faer and machinist; exposure to diesel fumes; and exposure to ionizing radiation (radium). The risk of NHL increased with the duration of employment as a farmer or machinist.).

The study revealed that, the most commonly affected age group in lymphoma, was children and young adults, this finding similar with that reported in first National Population-based Cancer Registry (2009), in Sudan (in children less than 15 years of age, leukemia was the most common cancer followed lymphoma) .

The most predominant subtypes was Non Hodgkin lymphoma (84%), while Hodgkin lymphoma was (16%). This finding was consistent with study done in China by (Wang, *et al.* 2006) as their results demonstrate that (86.1%) were confirmed to be non-Hodgkin lymphoma, while (13.9%) were Hodgkin lymphoma.

The present study clarified that there was no significant difference between HL and non HL existed as regard to association of EBV as the (P.value =0.333).

4. CONCLUSION

The study concluded that, there is sufficient evidence for the carcinogenicity of EBV in the causation of lymphoma. The study recommended to adopt EBV screening program, and to conduct further studies to find out whether the carcinogenicity of EBV need co-infection or not.

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