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Immunohistochemical Expression of Epstein Barr Virus Antigen Latent Membrane Protein-1 and Bcl-2 in Classical Hodgkin Lymphoma

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Abstract

Background Different genetic and environmental factors appear to be involved in the pathogenesis of classical Hodgkin's lymphoma (HL) and among them is Epstein Barr virus.

Objective To evaluate the immunohistochemical expression of the Latent Membrane Protein-1 of Epstein-Barr virus and Bcl-2 in Classical Hodgkin Lymphoma and to correlates this expression with some clinicopathological parameters and finally to find if there is any relation between LMP-1 and Bcl-2 in classical Hodgkin's lymphoma.

Method Retrospective study of fifty paraffin-embedded blocks of lymph nodes biopsies from patients diagnosed as Classical Hodgkin's Lymphoma. Three representative sections were prepared for each case. The first stained with H&E and the other two sections stained immunohistochemically for LMP-1 and Bcl-2 .

Results Immunohistochemical expression of LMP-1 and Bcl-2 was detected in 90% and 66% of Hodgkin's lymphoma cases and in 60% and 80% of control subjects, respectively. The patients (9/50) who were less than 16 years were positive for LMP-1 antigen. 21/45 (46.6%) of positive cases for LMP-1 & 14/33 (42%) of positive cases for Bcl-2 were of mixed cellularity subtype. Intensity of LMP-1 but not Bcl-2 expression was significantly high in mixed cellularity compared to other subtypes. No statistically significant relation between LMP-1 and Bcl-2 expression in HRS cells of Hodgkin's lymphoma cases.

Conclusion The high prevalence and high expression of LMP-1 confirms the association of the virus with HL. Although Bcl-2 is highly expressed in HL, the two markers were not related to each other.

Keywords Classical Hodgkin's Lymphoma, Epstein Barr virus, Immunohistochemistry, Epstein Barr virus latent membrane protein-1, Bcl-2.

Introduction

Hodgkin's lymphoma (HL) is a lymphoproliferative malignancy of B-cell origin [(1)] According to the WHO classification, Hodgkin's lymphoma (HL) is divided into a classical variant and a nodular lymphocyte predominant variant which are characterized by the presence of Hodgkin's and Reed-Sternberg (H-RS) cells or lymphocytic and histiocytic (L&H) cells, respectively [(2)].

According to National Cancer institute in 2008, the worldwide incidence rate of HL is 3.1 per 100 000 population/year. Its incidence varies from 3.5 per 100,000 population/year [(3)] in Europe, 3.5 per 100,000 population/year in united state [(4)], >5.5 per 100,000/year in Yemen and Lebanon, to <1 per 100,000 population/year [(5)] in Bangladesh, Japan and China. In Iraq, the incidence of HL from

1995 to 2006 ranged between 1.49 and 1.01/100,000 population/year [6].

Viruses are etiologically associated with significant types of human leukaemia and lymphomas [7]. The Epstein-Barr virus (EBV) plays an important role and individuals with a history of infectious mononucleosis have an increased incidence of Hodgkin's lymphoma [8]. Approximately 30% to 40% of patients with Hodgkin lymphoma (HL) in the Western world and in some developing regions carry the EBV in the malignant Hodgkin Reed Sternberg (HRS) cells [9].

EBV infection is an early event in the development of HL as the viral genomes are found in a monoclonal form, indicating that infection of tumor cells has occurred before their clonal expansion [9]. Clonal viral genomes are found in the Hodgkin Reed-Sternberg cells (HRS). The latent infection results in expression of the viral oncogenes LMP-1 and LMP-2A. LMP-1 is the major transforming protein of EBV. It is a member of the tumor necrosis factor receptor (TNFR) superfamily and most closely resembles CD40. However, in contrast to CD40, LMP-1 signaling is constitutively active and requires no ligand. LMP-1 upregulates cellular Bcl-2 and other proteins that inhibit apoptosis and also stimulates cytokine production (interleukin IL-6 and IL-8) [10,11].

B cell lymphoma-2 (Bcl-2) family proteins are key regulators of the apoptotic process. Bcl-2 blocks the induction of apoptosis by inhibiting the activation of pro-apoptotic family members such as BAX and preventing mitochondrial membrane depolarization [12]. Dysregulation of Bcl-2 expression, which results in abnormal cell growth, certainly contributes to the development of some tumors [13]. Over expression of Bcl-2 may result in accumulation of cells in the G0 phase of cell cycle division [14]; causing resistance to chemotherapeutic drugs and radiation therapy, while decreasing Bcl-2 expression may promote apoptotic responses to anticancer drugs [12,15]. Consequently, Bcl-2 has become a very attractive target for the design of new anticancer drugs [16].

The study intended to evaluate the immunohistochemical expression of the Latent Membrane Protein-1 of Epstein - Barr virus and the anti-apoptotic protein Bcl-2 in Classical Hodgkin Lymphoma using a specified automated cellular image analysis system (Digimizer software analysis) then correlates their expression with clinicopathological parameters including: age of the patients and histological subtypes of the disease and to find if there is any relation between LMP-1 and Bcl-2 in classical Hodgkin's lymphoma.

Methods

This retrospective study was conducted on fifty paraffin-embedded blocks of lymph nodes biopsies from patients diagnosed as Classical Hodgkin's Lymphoma. The cases were selected from archive files of the Department of Pathology of the Teaching Laboratories, Specialized Surgical Hospital in Baghdad Medical City and Al-Kadhimiya Teaching Hospital from November 2010 to June 2011.The control group consist of 20 age matched subjects having reactive lymph nodes biopsies which were obtained from Al-Kadhimiya Teaching Hospital Laboratories. Clinicopathological parameters including the age and histological subtypes of the tumor were obtained from the available histopathological reports. Ethical approval for the use of all specimens was obtained.

For each case, three representative sections were prepared. One section stained with Hematoxylin and Eosin and the histopathological diagnosis was revised by a pathologist, while other two sections were stained immunohistochemically for LMP-1 and Bcl-2 with horseradish peroxidase (HRP)-labelled-streptavidin-biotin method. The work was done at Al-Kadhimiya Teaching Hospital Laboratories.

This technique basically uses an unlabeled primary antibody, which was mouse monoclonal antibody purchased from DAKO (code no. of the kits were IS 753 for LMP-1 and M 0887 for Bcl-2), it binds to its corresponding antigen, followed by a

biotinylated secondary antibody to which the avidin-biotin complex (one avidin molecule, three biotin-labeled peroxidase molecules) attaches. If the sought-after antigen is present in the section, there will be an antibody-antigen interaction and an enzymatic reaction that can be detected by the chromogen, diaminobenzidine (DAB), which can be visualized by light microscopy.

Negative technical controls were obtained by replacing the primary antibody with buffer saline and positive tissue controls for each antibody were included with the samples using follicular lymphoma for Bcl-2 and Nasopharyngeal carcinoma for LMP-1.

Using a Noval light microscope (Noval, 320M), representative areas of IHC spots were selected and captured through the 40×objective, with a Sony digital camera (digital still camera DSC-W210. Each picture was analyzed by Digimizer (Version 3.7.0) software image analysis. The analysis of micro images by Digimizer software will give rise to three main digital parameters which are the number of objects stained, the average intensity and the mean area stained. For purpose of statistical analysis we use the following variables:

A. **Color Intensity:** which means the average intensity of the brown color for the selected objects depending on the expression of antigens in the cells. Digimizer color scale range from 0.000 representing black intense color to 0.999 for white color, taking into consideration that the higher the digital number, the lesser the staining intensity is, (i.e. the digital number that represents color intensity is inversely proportional to the actual intensity).
B. **Fractional area stained:** which equals to *[(mean area* Number of objects)/area of a single image field]*100*

C. Digital Labeling Index: for better estimation of the immunohistochemical expression of the LMP-1 and Bcl-2 molecular markers we used an arithmetic tool named as Digital Labeling Index. This tool is calculated according to the following formula: (Fractional area * reverse Intensity).

The digital intensity obtained by digimizer image analysis software was converted into three categories (weak, moderate and strong) by referring to NordiQC laboratories participating in schemes, institute of pathology. The NordiQC classified the color intensity of DAB positive cells into three categories: strong +3(0.292-0.521) for dark brown immunostained slides, moderate +2(0.522-0.668) for brown immunostained slides, weak +1(0.669-0.72) for light brown to yellow color immunostained slides and negative for non-stained slides.

Data were analyzed using SPSS program (Statistical Package for Social Sciences) version 16 and Microsoft Office Excel 2007. A *p* value of less than 0.05 was considered a statistically significant.

Results

Most of HD cases were in childhood and early adult age, between 10-39 years with peak in 20-29 years and there was male predominance with M: F ratio of 1.27:1 (Table 1).

Table 1. Distribution of HD cases according to age groups

Age group (years)	Control	Hodgkin Lymphoma	Total
<16	6	9	15
16-34	10	22	32
35-49	3	11	13
≥50	1	8	9
Total	20	50	70

Regarding the histopatological subtype distribution of HD, the mixed cellularity was the commonest histological subtype (42%) followed by nodular sclerosis (22%) then lymphocyte depleted (24%) and only (12%) proved to be lymphocyte rich subtype. Moreover 74% of the cases presented with cervical lymphadenopathy.

Positive sections for both LMP-1 and Bcl-2 showed brown diffuse cytoplasmic stain of HRS cells of HD (Figure 1 & 2).

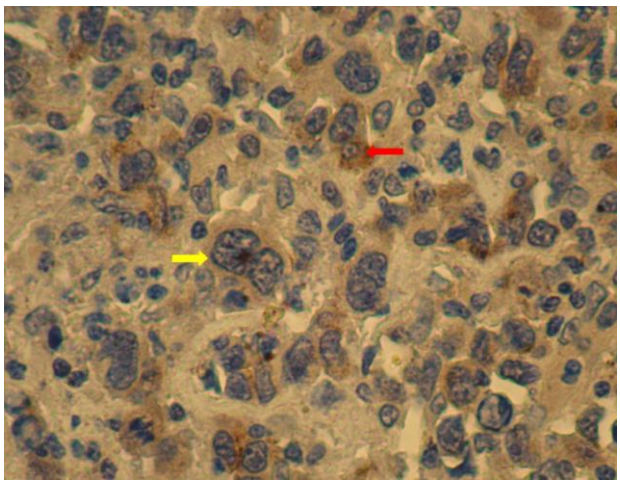

Figure 1. Moderate, brown, diffuse cytoplasmic LMP-1 expression in HRS cells in lymph node from patient with Hodgkin's lymphoma (40X)

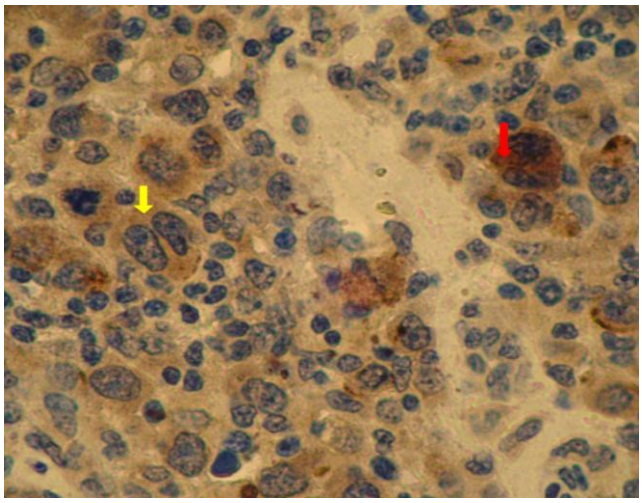

Figure 2. Moderate, brown, diffuse cytoplasmic Bcl-2 expression in HRS cells in lymph node from patient with Hodgkin's lymphoma (40X)

LMP-1 was detected in 90% of Hodgkin's lymphoma cases versus 60% of control, (*p* value =0.007). However when applying T-test for immunohistochemical expression in control and lymphoma cases, the three digital analysis parameters of the Digimizer were significantly higher in lymphoma than control group as shown in table 2.

Table 2. Comparison of the digital parameters of LMP-1 between control and Hodgkin's lymphoma cases

Parameter	Control	Lymphoma	P value
Intensity	0.91±0.14	0.32±0.23	<0.001
Fractional area	4.76±0.54	10.1±2.50	0.041
DLI	5.91±1.26	38.32±41.66	0.001

DLI = Digital labeling index

By using Digimizer software analysis for grading the intensity of the stained sections, all positive lymphoma cases showed high and moderate expression in contrast to control where all the positive cases showed weak expression as shown in table 3.

Table 3. Distriution of the control and Hodgkin's lymphoma cases into different grades of intensity of LMP-1 expression*

Intensity grade	Control	Lymphoma	Total
Negative	8(40%)	5(10%)	13(19%)
Weak	12(60%)	0	12(17%)
Moderate	0	9(18%)	9(13%)
Strong	0	36(72%)	36(51%)
Total	20(100%)	50(100%)	70(100%)

Chi square test was valid although the cases show different pattern of expression

By applying spearman rank linear correlation, there was significant inverse correlation between the age and LMP-1 in all three digital parameters of Digimizer. Figure 3 showed the positive correlation between the age and Digital labeling index of LMP-1 expression. Furthermore all children who were less than 16 years (9/50) were positive for LMP-1 expression as shown in table 4.

Table 4. Age dependent expression of LMP-1 in control and lymphoma cases

Age groups (years)	LMP-1	Control	Hodgkin Lymphoma	Total	*P*
<16	Negative	3 (50%)*	0	3	0.044
	Positive	3 (50%)	9 (100%)	12	
16-34	Negative	4 (40%)	2 (9.1%)	6	0.060
	Positive	6 (60%)	20 (90.9%)	26	
35-49	Negative	**1 (33.3%)**	**2 (18.2%)**	3	**1.000**
	Positive	2 (66.7%)	9 (81.8%)	11	
≥50	Negative	0	1 (12.5%)	1	**1.000**
	Positive	1(100%)	7 (87.5%)	8	

*The percentage showed in this table represented the percent of negative and positive cases in each age group.

Regarding the relation to histopathological subtype, all cases with mixed cellularity subtype express LMP-1 (21/50) and the digital labeling index was significantly high compared to other subtype (p=0.028), followed by nodular sclerosis subtype where 10/11 case were positive for LMP-1 (Table 5).

Table 5. Relation between LMP-1 and Histological subtypes of Hodgkin's Lymphoma

Marker	Parameter	Subtype	No.	Mean±SEM	*P* value
LMP-1	Intensity	Mixed Cellularity	21	0.24±0.07	0.028
		Nodular Sclerosis	11	0.25±0.06	
		Lymphocyte Rich	6	0.48±0.40	
		Lymphocyte Depleted	12	0.44±0.34	
		Total	50	0.32±0.23	
	Fractional area	Mixed Cellularity	21	12.36±15.34	0.503
		Nodular Sclerosis	11	10.12±10.88	
		Lymphocyte Rich	6	8.67±10.67	
		Lymphocyte Depleted	12	6.20±8.62	
		Total	50	10.10±12.50	
	Digital Labeling Index	Mixed Cellularity	21	52.62±47.58	0.307
		Nodular Sclerosis	11	40.48±39.27	
		Lymphocyte Rich	6	35.69±40.54	
		Lymphocyte Depleted	12	23.27±29.79	
		Total	50	38.32±41.66	

In the present study Bcl-2 showed positive staining in 66% of HL patients versus 80% of control (p=0.248). Table 6 showed the digital analysis parameters in control and HD.

Table 6. Comparison of the digital parameters of Bcl-2 in control and Hodgkin's lymphoma cases

Parameter	Control	Lymphoma	P value
Intensity	0.85±0.13	0.53±0.37	<0.001
Fractional area	4.56±1.02	19.00±6.44	0.330
DLI	5.40±1.27	52.45±10.37	0.047

DLI = digital labeling index

However, by using Digimizer analysis software for grading the intensity of the stained sections, 48% (24/50) of Hodgkin's cases showed strong expression and 16% (8/50) showed moderate expression and only one case showed weak expression, whereas 50% (10/20) of control subjects showed weak expression and 20% (4/20) showed negative expression as shown in table 7 .

Table 7. Distribution of lymphoma and control cases into different grades of intensity of Bcl-2 expression

Intensity grade	Control	Lymphoma	Total
Negative	4(20%)	17(34%)	21
Weak	10(50%)	1(2%)	11
Moderate	6(30%)	8(16%)	14
Strong	0	24(48%)	24
Total	20(100%)	50(100%)	70(100%)

In relation to the histopathological subtypes ,the Intensity, Fractional area and Digital Labeling Index of Bcl-2 immunohistochemical expressions did not significantly differed in relation to the histological subtypes of the tumor when applying ANOVA test. However Lymphocyte Depleted Hodgkin's lymphoma showed the highest Digital Labeling Index of Bcl-2 expression followed by Mixed Cellularity subtype.

By applying spearman rank linear correlation, there was significant positive correlation between the age of the patients and the three digital analysis parameters. Figure 4 showed the negative correlation between the age and Digital labeling index of Bcl-2 expression Furthermore Bcl-2 expression was high in patients older than 35 years old but this association was not statistically significant.

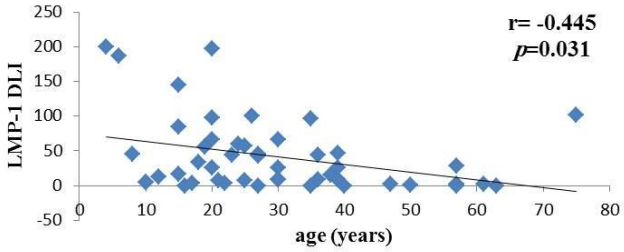

Figure 3. Correlation between LMP-1 DLI and age

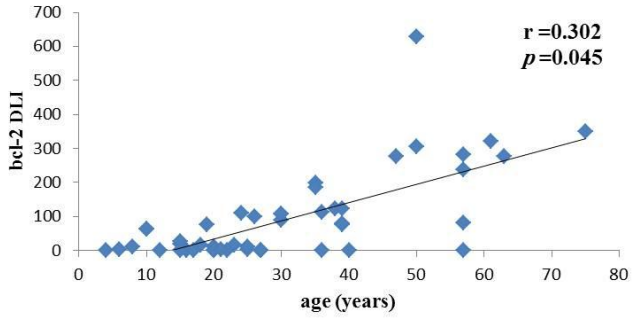

Figure 4. Correlation between Bcl-2 DLI and age

By applying spearman rank linear, there was no significant correlation between LMP-1 and Bcl-2 expression in Hodgkin's lymphoma cases (p=0.844).

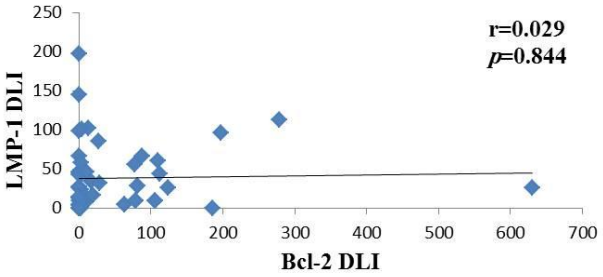

Figure 5. Correlation between LMP-1 DLI and Bcl-2 DLI

Discussion

In current study the age of most cases was between 10-39 years and more than half cases were below 27 years old. This was concordant with the results of Iraqi Cancer Registry in 2006 which showed that most of the Hodgkin lymphoma (HL) cases were between 15-35 years [6] and it was similar to other Iraqi studies done in 2007, 2005,2004 and 2001 [17-20] and to studies done in other Arab countries like Kuwait, Jordan and Egypt [21-23]. Thus we may conclude that in Iraq the age distribution of HL followed the pattern in developing countries in which the disease occurred earlier than in developed countries.

The slight male predominance observed in this study was in agreement with Iraqi Cancer Registry (ICR) results in 2006 [6] and in other Iraqi study in 2005 [20], but it differed from Al-Safi results in 2007 which stated that incidence of Hodgkin lymphoma was equal in both male and female [17]. This could explain by the small number of the samples.

Similar to this study, other studies done in other Arab countries like Kuwait in 2003 [21] and Egypt in 2010 [23] and worldwide [24-26] showed that there was male predominance with ratio of 1.2-2.4:1.

Similar to the results of ICR in 2006 [6] and other local studies in 2005 [18], 2003 [27], 200120 and a Kuwaiti study in 2003 [21], the mixed cellularity was the commonest histological subtype.

Cervical lymph nodes were the commonest site involved by tumour which was in agreement with the results of Iraqi study done in 2007 [19], Turkish study in 2005 [29] and a Kuwaiti study done in 2003 [21].

In the current study, LMP-1 expression was positive in 90% of HD versus 60% of control group. This high expression was comparable to an Iraqi study that was done by Al-Safi in 2007 [17] in which LMP -1 was found in 75% of Hodgkin lymphoma cases and also in line with other developing countries reaching 63% in Egypt [23], 60% in Nigeria [30], 82% in India [31], and 93% in Iran [32], whereas it was less common in developed countries, with percentages of 20-50% for North American [33] and European cases [34], and 39% in China [26].

Although LMP-1 EBV antigen was detected in HL and control subjects ,the expression was significantly higher in HL and since LMP-1 is the major EBV oncogene and is essential for B-cell immortalization; thus we may conclude that the presence of the virus have played an important role in the pathogenesis of the disease.

In the present study all patients below 16 years, were infected with EBV and showed high expression of LMP-1. These results were in agreement with Al-Safi study which revealed that EBV expression was highest in childhood [17]. This followed the pattern of EBV expression in developing countries such as Kuwait [21], Jordan [22] and Iran [35]. And this in contrast to the results found in USA [33], United Arab Emirates [36], and Netherlands [34], in which high frequency of LMP-1 expression was seen in young adult. In developing countries, infection usually occurs in early childhood and usually passed unnoticed and the vast majority will be persistently infected with a reservoir of infection in memory B-cells this may lead to Hodgkin's Lymphoma to develop in childhood group [21]. Whereas in industrialized countries primary infection is often delayed until adolescence and frequently results in infectious mononucleosis (IM) [7].

The high expression of LMP-1 in mixed cellularity HL that was seen in the present study was in concordance with several studies done in Jordan [22], China [26] and Rio de Janeiro [37].

Bcl-2 is an antiapoptotic protein, it was detected in 66% of HD cases, and this was in line with results of Rassidakis *et al* [38], Wang and Taylor [39], Kim *et al* [40] and Adelusola studies [30], which found that Bcl-2 expression was detected in HRS cells in 61%, 56.45%, 43.7%, 56% and 40% of the cases respectively. Moreover the highest expression was detected in the aggressive Lymphocyte depleted subtype followed by mixed cellularity. This result was concordant with results of Flangea *et al* [41] and it explain the role in the pathogenesis of the disease, since overexpression of Bcl-2 may result in accumulation of cells in the G0 phase of cell cycle [14]; causing resistance to chemotherapeutic drugs and radiation therapy thus Bcl-2 was considered as a

critical cellular factor contributing to the pathogenesis and progression of cancer [16].

Regarding the relation with age, the result of the present study was in agreement with Rassidakis *et al* study which had found that there was a significant association between Bcl-2 expression and patients older than 45 years old [38]. Thus we may propose that Bcl-2 expression is closely related to the age of the patients and is often associated with shorter survival time and generally poorer clinical outcomes [42].

Lastly, in current study there was no significant correlation between LMP-1 and Bcl-2 expression in classical Hodgkin's lymphoma, this result was in line with the result of Cickusic *et al* [43] study. Although escape from apoptosis represents the major oncogenic event in CHL pathogenesis [44], and LMP-1 upregulates cellular Bcl-2 and its homologues [45], therefore, interaction between LMP-1and Bcl-2 may be a critical factor contributing to the pathogenesis and progression of cancer. However both markers act independently on each other. So we may conclude that LMP-1 and Bcl-2 are independent biological markers in CHL.

The study conclude that the use of Automated Cellular Image Analysis System to quantitate immunohistochemical staining (Digmizer software) is easy to use, more objective, semiquantative flexible image analysis software and is simple to perform, The LMP-1 was detected in 90% of Hodgkin's lymphoma compared to 60% of control subjects however the expression of LMP-1 was strong and moderate in lymphoma cases, whereas it was weak in all control subjects. Thus we may suggest a role for the virus in the pathogenesis of Hodgkin's lymphoma. Bcl-2 was detected in control subjects as well as in Hodgkin lymphoma cases but the intensity of immunostaining of Bcl-2 was significantly stronger in lymphoma cases (p=0.001) which may suggest that escape from apoptosis represents the major oncogenic event in CHL pathogenesis. The EBV antigen LMP-1 and Bcl-2 were independent markers in HL.

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