

## Epstein-Barr virus in Hodgkin's lymphoma - immunohistochemical case series study

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### ABSTRACT

**Objectives:** 1- To determine the association between Hodgkin's lymphoma and Epstein-Barr virus. 2- To determine if it is related to certain age groups or specific histologic subtypes. 3- To compare the pattern with other developing or developed countries.

**Methods:** Biopsies of seventy cases with Hodgkin's lymphoma were collected from the pathology laboratories. The clinical data, including the patient's age, sex, site of lymph node affected and the histological classification according to the REAL classification, were retrieved from the pathologic reports. Immunoperoxidase stains for LMP-1 were performed on 40 cases.

**Results:** The mean age of all cases was 26.7 years, with a median of 25 years. There were 36 males and 34 females. The largest age group was seen in 15 - 40 years accounting for 68.5%. The third decade took the peak incidence (21/70, 30%). Nodular sclerosis Hodgkin's lymphoma was the most common subtype representing 58.5% of all the cases, followed by mixed cellularity (37.2% of cases). The lymphocyte depleted subtypes in 2.8%, whereas lymphocytic predominant seen in 1.4% of cases. Latent membrane protein-1 was observed in 37.5% of Hodgkin's lymphoma. The most frequent association was observed in lymphocytic depletion subtype (50%), followed by mixed cellularity subtype (45%). The least frequent association was in nodular sclerosis type (27.7%). The highest rate of EBV expression was seen in the pediatric age group (< 15 years; 66.6%) and the lowest rate was among young adults (15-40 years; 26.9%). Older age group (> 40 years) has a rate of EBV expression (50%) which is higher than the young adult patients, but lower than the pediatric age group. Epstein-Barr virus positive cases were mostly males (73.3%) compared to females (26.7%).

**Conclusion:** Hodgkin's lymphoma in our locality, in comparison with earlier studies, shows changing pattern with a gradual trend to those of developed countries including peak age group and subtypes. Moreover, Epstein-Barr virus is seen in slightly more than one third of cases and mostly seen in childhood, mixed cellularity and lymphocytic depletion subtypes with a male predominance.

**Keywords:** Hodgkin's lymphoma, Epstein-Barr virus, LMP1, developed and developing countries.

### الخلاصة

**الأهداف:** أولاً: لكشف العلاقة بين ورم الهوجكن اللمفاوي و فيروس ابشتاين-بار. ثانياً: تحديد علاقة الفيروس مع الفئات العمرية أو الأنواع المختلفة من أورام الهوجكن اللمفاوي. ثالثاً: مقارنة النتائج مع الدول المتطورة والنامية.  
**الطرق والأدوات:** هذه دراسة رجعية وسابقة. عينات سبعين حالة من أورام الهوجكن اللمفاوية تم جمعها من المختبرات المرضية النسيجية. البيانات السريرية والمتضمنة عمر المريض، الجنس، موقع العقد اللمفاوية المصابة والتصنيف النسيجي للحالة اخذ من تقارير المريض. صبغة المناعية النسيجية للبروتين الجداري المستتر-1 تم دراسته في أربعين حالة.

**النتائج:** معدل أعمار المرضى كان ٢٦,٧ سنة مع متوسط عمر ٢٥ سنة. تم جمع ٣٦ ذكر و ٣٤ أنثى مع نسبة ١:١.٠٦ أكبر فئة عمرية للورم كانت بين ١٥ إلى ٤٠ سنة وبنسبة ٦٨,٥%. العقد الثالث أخذ نسبة الذروة للإصابة وبمعدل ٣٠%, أتبع بالعقد الثاني وبنسبة ٢٤,٣%. نسبة المرضى الأقل من ١٥ سنة كانت ١٧,١%. بينما ١٤,٣% كانت بأعمار أكبر من ٤٠ سنة. التصلب العقدي للورم كان أكثر الأنواع شيوعا وبنسبة ٥٨,٥%. يليه مختلف الخلايا وبنسبة ٣٧,٢%. ثم مستنقذ الخلايا اللمفية وبنسبة ٢,٨%. وأخيرا سائد الخلايا اللمفية وبنسبة ١,٤%.

البروتين ألدجاري المستتر-١ لوحظ بنسبة ٣٧,٥% من أورام الهوجكن اللمفاوية. أكثر الأنواع ارتباطا مع الفيروس وجدت مع مستنقذ الخلايا اللمفية وبنسبة ٥٠%. يليه مختلف الخلايا وبنسبة ٤٥%. بينما أقل الأنواع ارتباطا مع الفيروس كان التصلب العقدي وبنسبة ٢٧,٧%. أعلى معدلات إصابات من الأورام الهوجكن للفيروس كانت لأعمار الأطفال أصغر من ١٥ سنة وبنسبة ٦٦,٦% بينما أدنى المعدلات كانت في الأعمار المتوسطة مابين ١٥ إلى ٤٠ سنة وبنسبة ٢٦,٩%. العمار أكبر من ٤٠ سنة كانت نسبة الارتباط ٥٠%. لوحظ تجلية الذكور على الإناث بالإصابة بفيروس ابشتاين-بار وبنسبة ١:١.٧.

**الاستنتاج:** أورام الهوجكن اللمفاوية في منطقتنا ومع مقارنتها بدراسات سابقة لوحظ تغير شكلي مع ميل تدريجي تجاه الدول المتطورة خاصة فيما يتعلق بذروة الفئة العمرية المصابة و بالأنواع الخاص للورم. وإضافة لذلك ظهور ارتباط الورم بالفيروس وبنسبة الثلث تقريبا خاصة في الأطفال و متعدد الخلايا و بغالبية ذكورية.

**H**odgkin's lymphoma (HL) is a heterogeneous condition that most probably comprises more than one etiological entity. As early as 1966 MacMahon proposed that Hodgkin's lymphoma might be caused by an infectious agent<sup>(1)</sup>. Epidemiological studies have indicated that many of the features of Hodgkin's lymphoma mimic those of an infectious process, including occurrence of disease in more than one member of the family of different ages, and clustering of cases in winter months<sup>(1)</sup>. EBV-associated disease is more common in low socio-economic class and in children with maternal deprivation<sup>(2-4)</sup>.

The first evidence that suggests the relationship between Hodgkin's lymphoma and Epstein-Barr virus (EBV) was provided by the detection of raised antibody titres to EBV antigens in patients with Hodgkin's lymphoma when compared with patients with other lymphomas and, further, that these raised values preceded the development of Hodgkin's disease by several years<sup>(1,5)</sup>. In addition, there is a specific association between EBV-associated HL in young adults and previous infectious mononucleosis with a relative risk of developing Hodgkin's lymphoma relative to those with no previous history, was shown to range between 2.0 to 5.0<sup>(1,6)</sup>.

Studies suggest that the EBV is associated with approximately one third of cases in developed countries, and this association is believed to be casual<sup>(7,8)</sup>. Whereas higher rates of association was reported in developing countries<sup>(9-14)</sup>.

EBV is more often associated with mixed cellularity HL than with nodular sclerosis type, and in children and older adults than in young adults<sup>(15-18)</sup>. Also it is more in males than females and more in Asians and Hispanics than whites or blacks<sup>(19)</sup>.

According to the age of the patients and EBV association: 4-disease models have been recognized, one EBV-negative group of cases, which accounts for the young adult peak in disease incidence as seen in developed countries, and three EBV-positive subgroups. The latter includes a childhood group, accounting for almost all cases of HL in early childhood; a young adult group, and an older adult group, which results from loss of the normal balance between latent EBV infection and host immunity (figure 1)<sup>(6, 20,21)</sup>.

EBV is an extremely efficient transforming agent infecting the Hodgkin/Reed Stenberg's (HRS) cells. The EBV genome is composed of linear-double-stranded DNA which code for different types of latent proteins. Six are nuclear antigens (EBNA1, 2, 3A, 3B, 3C, EBNA-LP), and three latent membrane

proteins (LMP1, LMP2A, LMP2B). LMP1 is the major transforming protein of EBV and is the only latent protein that can transform rodent fibroblast<sup>(22)</sup>. Expression of LMP1 appears to mimic a constitutively active CD40 receptor<sup>(23)</sup>. Both CD40 and LMP1 bind tumor necrosis factor (TNF) receptor-associated factors (TRAFs), initiating a signaling cascade that leads to activation of transcription factors NF-kappa B, AP-1 and STAT<sup>(22, 24-29)</sup>. By providing these CD40-like signals, it is conceivable that LMP1 allows EBV-infected HRS cells upregulate various antiapoptotic genes including bcl-2, mcl-1 & cytokine that bypass a germinal center checkpoint and escape apoptosis<sup>(27, 30-33)</sup>.

Older adults and children who are EBV positive have a poor prognosis possibly reflecting a poor immune status, which in turn means that those patients may tolerate disease and its treatment less well<sup>(3, 34)</sup>.

The aims of this study were first, to determine the association between Hodgkin's lymphoma and Epstein-Barr virus. Second, to determine if it is related to certain age groups or specific histologic subtypes. Third, to compare the pattern with other developing or developed countries.

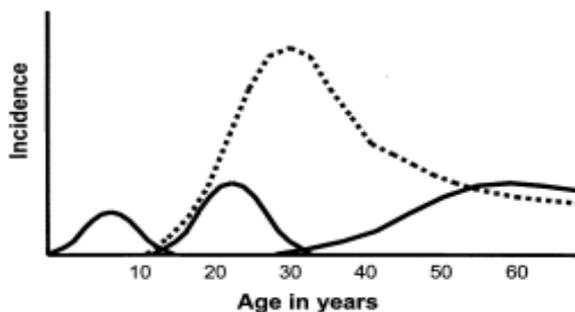


Figure (1): Four disease model of Hodgkin's lymphoma. Solid line is EBV-associated cases (three groups first below 10 years, second young adult between 14 and 34 years, the third are more than 55 years of age). Dashed line is non-EBV associated cases (one peak at young adults mainly seen in developed countries).

## Methods

This is a retro- and prospective study, conducted between 2007 - 2009. Seventy cases of Hodgkin's lymphoma were collected with their clinical data including age, sex, site of lymph node affected and the histological classification according to the REAL scheme). To some of the cases immunohistochemical stains including (CD15, CD30, CD43 and CD20) have been also applied as complementary tests.

Immunoperoxidase staining for LMP-1 was performed on 40 cases in this study. Three micron thick sections were cut from each selected paraffin block onto silane-coated slides; sections were then dewaxed, rehydrated to distilled water. Antigen retrieval was carried out by autoclaving at 95-99°C, for 20 minutes by a retrieval solution. The sections were then allowed to cool for at least 20 minutes, followed by washing 3 times each for 5-minutes in changes of phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked by placing sections in 3% hydrogen peroxide for 5 minutes and washed in 3 changes of distilled water. Sections were incubated with 1:100 diluted primary antibodies against LMP1 (Dako, Monoclonal Mouse Anti-Epstein-Barr virus, LMP1, Clone C4.1-4) for 30 minutes, followed by washing twice in 5-minutes changes of PBS. The sections were incubated for 30 minutes with secondary Dako envision antibody. Sections were washed twice by PBS and visualized using diaminobenzidine (DAB). Finally, the sections were lightly counterstained with hematoxylin, dehydrated and mounted. Negative control sections were treated in the same way except for the use of a negative control (Dako Mouse IgG1) instead of primary antibody. Positive control sections were taken from a positive case and were done in each run.

## Results

**Clinical findings:** From the 70 collected cases, the ages of the patients ranged from 2-80 years with a mean of 26.7 years, and a median of 25 years. There were 12 patients (17.1%) under 15 years of age. Ten cases

(14.3%) were above 40 years of age. Whereas the greatest group of Hodgkin's lymphoma was seen in young adult group (15 - 40 years) 48 patients (68.5%). The third decade took the peak incidence (21/70, 30%), followed by the second decade (17/70, 24.3%). There were 36 males and 34 females with male to female ratio of 1.06:1.

**Histopathologic findings:** Nodular sclerosis Hodgkin's lymphoma was the most common subtype in this series representing 58.5% (41/70) of all the cases; mixed cellularity represents 37.2% (26/70), lymphocyte depleted subtype represents 2.8% (2/70), and

lymphocytic predominant is reported in 1.4% (1/70), figure 2.

In patients <15 years old, the most frequent subtype was mixed cellularity (66.6%), followed by nodular sclerosis (25.0%). The most common subtype in patients aged 15-40 years was nodular sclerosis (70.8%), followed by mixed cellularity (27.1%), and then by lymphocytic predominant (2.1%). Patients >40 years, the most common type was mixed cellularity (50%), followed by nodular sclerosis (40%), then lymphocytic depletion subtypes (10%) (Table 1).

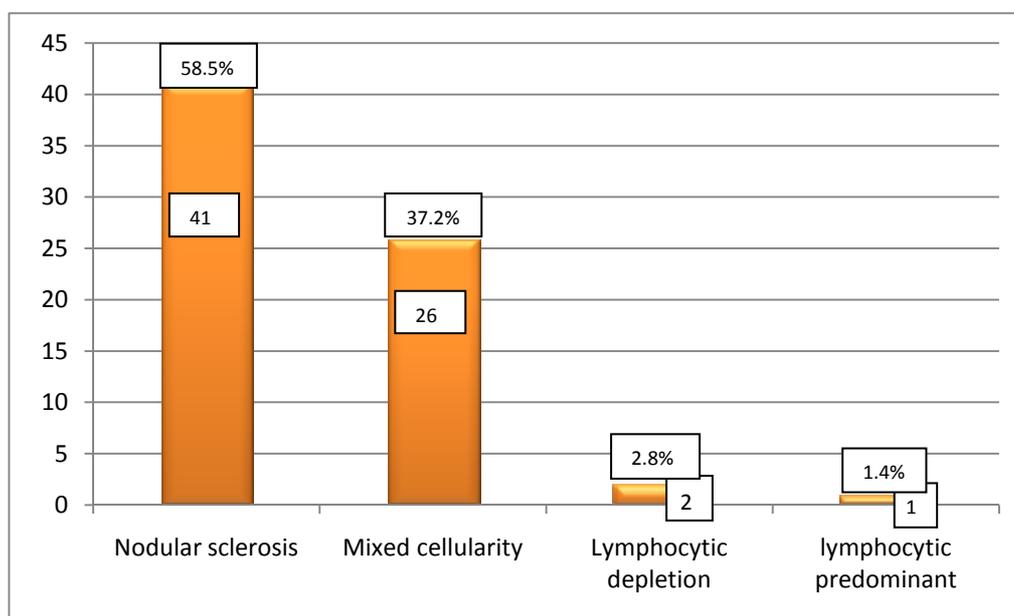


Figure (2): The percentage of various subtypes of Hodgkin's lymphoma.

Table (1): Distribution of Hodgkin's lymphoma subtypes according to the main age groups.

Age group (years)	Mixed cellularity		Nodular sclerosis		Lymphocytic depletion		Lymphocytic predominant		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
<15	8	(66.6)	3	(25.0)	1	(8.3)	0	(0.0)	12	17.1
15 - 40	13	(27.1)	34	(70.8)	0	(0.0)	1	(2.1)	48	68.5
>40	5	(50.0)	4	(40.0)	1	(10.0)	0	(0.0)	10	14.3
Total	26	(37.2)	41	(58.5)	2	(2.8)	1	(1.4)	70	100.0

**Epstein-Barr virus status:** Positive LMP-1 staining was seen in the cytoplasm and cell membranes of Hodgkin's/Reed-Sternberg cells. It was observed in 15 out of 40 cases (37.5%) of Hodgkin's lymphoma (figure 3).

The most frequent association was in cases of lymphocytic depletion subtype (1/2; 50%), followed by mixed cellularity subtype (9/20; 45%). The least frequent association was observed in nodular sclerosis type (5/18; 27.7%) as shown in table (2).

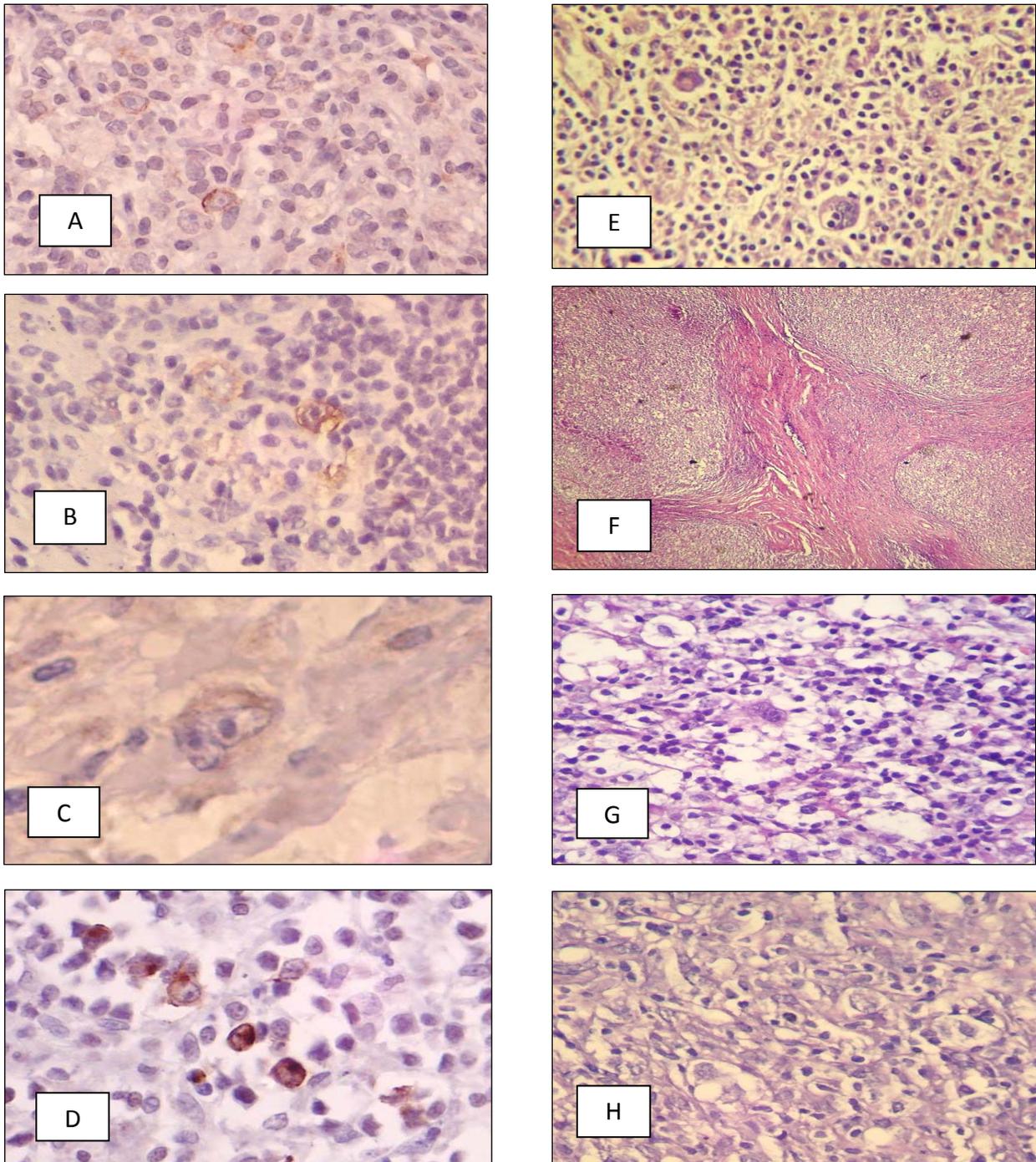


Figure (3): A, B, C, & D show positive LMP-1 staining seen in the cytoplasm and cell membranes of Hodgkin's/Reed-Sternberg cells. H & E stain of different subtypes of Hodgkin's lymphoma, E (MCHL), F (NSHL), G (Classical R-S cell), H (LDHL).

Table (2): Epstein-Barr virus association with various types of Hodgkin's lymphoma.

Histological Types	LMP-1 Positive	LMP-1 Negative	Total	
	No.	No.	Total No.	% of Positivity
Lymphocytic depletion	1	1	2	50
Mixed cellularity	9	11	20	45
Nodular sclerosis	5	13	18	27.7
Total	15	25	40	37.5

The frequency of EBV expression in relation to the age is shown in table (3). The highest rate of EBV expression was seen in the pediatric age group (< 15 years; 66.6%) and the lowest rate was among the young adults (15-40 years; 26.9%). The older adult (> 40 years) have a rate of EBV expression (50%) in between the 2 groups (higher than the young adult patients, but less than the pediatric age group).

Epstein-Barr virus positive cases were mostly in males (11/15; 73.3%) compared (4/15; 26.7%) females.

## Discussion

Most studies on Hodgkin's lymphoma have shown epidemiological differences between developed and developing countries. Correa and O'Connor introduced the concept of at least three epidemiological patterns of Hodgkin's lymphoma based upon country of residence. A type I pattern is characterized by relatively high incidence rates in male children, low incidence in the third decade, and a second peak of high incidence in older age groups. The histological subtypes are often those with a less favorable prognosis, usually either mixed cellularity or lymphocytic depletion. This pattern prevails in developing countries. Type III is the converse of the type I pattern, being characterized by low rates in children and a pronounced initial peak in young adults. The more favorable subtype of nodular sclerosis is common and

this pattern is typical of developed countries. Type II is an intermediate pattern, and reflects a transition between type I and type III. Correa and O'Connor interpreted these data as the result of the interplay of environmental and host factors influencing the natural history of a single disease<sup>(1, 35)</sup>.

In this study, young adult group (15-40 years) was the largest age group, and the third decade represented the peak incidence. There is predominance of nodular sclerosis subtype, which accounts for 58.5% of Hodgkin's lymphoma. There is relatively equal ratio between male and female (1.06:1). Al-Dewachi and Al-Irhayim<sup>(36)</sup> in a study done in Mosul in 1995 have observed the bimodal age distribution of the disease, with a peak age incidence coming one decade earlier than those reported in the Western countries and found the predominance of male sex in three histological types of mixed cellularity, lymphocytic depletion and lymphocytic predominance, while nodular sclerosis showed an almost equal gender distribution. In a more recent study done in Mosul in 2007, Abbas<sup>(37)</sup> found an increasing incidence of nodular sclerosis cases with the peak age incidence in the second decade, followed by the third decade. Comparing our findings with these previous studies shows the gradual changing trends of Hodgkin's lymphoma to that of developed countries as described previously by

Table (3): Distribution of Epstein-Barr virus in Hodgkin's lymphoma according to main age groups.

Age group (years)	LMP-1 Positive	LMP-1 Negative	Total	
	No.	No.	Total No.	% of Positivity
<15	4	2	6	66.6
15 - 40	7	19	26	26.9
>40	4	4	8	50
Total	15	25	40	37.5

Correa and O'Conor<sup>(1,35)</sup>. Similar findings have been documented from Kuwait, KSA & United Arab Emirates<sup>(38-40)</sup>.

Moreover EBV association studies have revealed differences between developed and developing countries with higher EBV related Hodgkin's lymphoma cases in developing countries. In an attempt to summarize the literature data (Table 4), we found that most North American and European countries have 20-40% EBV association, as opposed to much higher rates that may reach more than 90% in some of Far East and South American reports.

Results from this study confirmed the presence of EBV in the neoplastic cells of the Hodgkin's lymphoma cases. The proportion of HD cases associated with EBV was 37.5%. Such figures appear to be lower than those

reported in Far East, South America and some of Middle East Countries while similar to those reported in Jordan, Saudi Arabia, South Israel, North America and European Countries.

Takeuchi et al<sup>(58)</sup> have recorded a decreasing trend of EBV positive nodular sclerosis Hodgkin's disease during the last 4 decades, whereas Clarke et al<sup>(34)</sup> have observed that EBV positive Hodgkin's lymphoma patients are less likely to have nodular sclerosis histology. Flavell et al<sup>(59)</sup> have suggested that Hodgkin's lymphoma of childhood and elderly is commonly EBV associated, whereas the adult Hodgkin's disease is not significantly associated with EBV infection. All these explain the low percentage of EBV in our locality.

Table (4): Summary of literature on Epstein-Barr virus association with Hodgkin's lymphoma stained by Immunoperoxidase for LMP-1.

Study	Country	% of EBV	% of + in MC	% of + in NS
Alkuraya et al <sup>41</sup>	KSA	28.6	52.4	26.1
Yilmaz et al <sup>42</sup>	Turkey	61.5	91.3	NP
Almasri et al <sup>43</sup>	Jordan	39.1	52.9	25
Makar et al <sup>44</sup>	Kuwait	56	45.5	37.3
Benharroch et al <sup>45</sup>	South Israel	30	45	21
Chang et al <sup>46</sup>	USA	24	NP	NP
Krugmann et al <sup>47</sup>	Austria	26	NP	NP
Pinkus et al <sup>48</sup>	USA	26	69	14
Enblad et al <sup>49</sup>	Sweden	27	38	23
Jarrett et al <sup>7</sup>	U.K	33	60	23.8
Delsol et al <sup>50</sup>	France	34	58.2	10
Keresztes et al <sup>51</sup>	Hungary	43	50	35
Dirnhofer et al <sup>52</sup>	Mexico	61	75	56
Peh et al <sup>53</sup>	Malaysia	61	87	33.3
Zhou et al <sup>54</sup>	China	72	NP	NP
Belkaid et al <sup>11</sup>	Algeria	72	77	NP
Karnik et al <sup>55</sup>	South India	82	NP	NP
Dinand et al <sup>56</sup>	India	90.3	NP	NP
Araujo et al <sup>57</sup>	Brazil	About 100	NP	NP

EBV- Epstein-Barr virus. MC- Mixed cellularity. NS- Nodular sclerosis. NP- Not provided

Epstein-Barr virus positivity was related to the histologic subtypes of Hodgkin's lymphoma. The lymphocytic depletion and the mixed cellularity types were the most common subtypes associated with EBV. Only one fourth of the nodular sclerosis subtype had evidence of EBV association. These observations are in agreement with most data reported in the literature (Table 4).

Higher percentage of EBV positivity was observed in children below 15 years of age, followed by older adult group (> 40 years). Whereas the least association was seen in young adult age group. This is in agreement with many of the other studies (7, 11, 15, 19, 24, 43, 44, 46, 53, 54, 59,60)

EBV associated with Hodgkin's lymphoma was seen in males more than in females, this is in concordance with many other similar studies (7, 19, 44, 47, 60-62)

In conclusion, the data from this study and in comparison with earlier studies, show changing pattern of Hodgkin's lymphoma in our locality with a gradual trends to those of developed countries, including greatest age group, peak age incidence and subtypes. Moreover, Epstein-Barr virus is seen in slightly greater than one third of cases and mostly seen in childhood, mixed cellularity and lymphocytic depletion subtypes with a male predominance.

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