Epstein–Barr virus expression in Hodgkin's disease in Jordan

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ABSTRACT

Objective: Epstein-Barr virus (EBV) has been associated with many hematopoietic malignancies including Hodgkin's disease (HD). The association of HD correlates with the histologic subtype, age of presentation and geographic location. Our aims were to find out if EBV is associated with Jordanian HD; and if EBV association exists, to determine its relationship to certain age groups or specific histologic subtypes of HD; and finally to establish whether such association follows patterns seen in developing or developed countries.

Methods: We have examined 64 cases of HD diagnosed in 2 major medical centers in Jordan for evidence of EBV association. We used immunohistochemistry and in-situ hybridization techniques to detect latent membrane protein (LMP-1) and Epstein-Barr virus encoded RNA (EBER) in the Reed-Sternberg cells. The study was conducted at the Department of Pathology, Jordan University of Science and Technology, Irbid, Jordan in the years 2000 and 2001.

Results: Epstein Barr virus was seen in 47% of our cases: 22 (65%) of the 34 mixed cellularity and 8 (29%) of 28 nodular sclerosis HD. None of our 2 lymphocyte predominant HD cases showed evidence of EBV. Epstein-Barr virus was seen in 73% of HD cases in children below 15 years of age as opposed to 34% of the young adult group.

Conclusion: Our results confirm the presence of EBV in Jordanian HD in approximately half of the cases, a figure close to those reported in the West. Epstein-Barr virus association with HD in Jordan is seen mostly in the mixed cellularity subtype and childhood HD.

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Perhaps no other malignant tumor has been more historically linked to an infectious etiology than Hodgkin's disease (HD) since its recognition. Indeed both Reed and Sternberg believed that HD represent an infectious disease.[1] Epidemiological studies have indicated that many of the features of HD mimic those of an infectious process. These features include bimodal age distribution in developed countries, case clustering, and high economic standards association with the young adult peak.[2-6] Early epidemiological data suggested a relationship between HD and Epstein-Barr virus (EBV);[7] however, the presence of EBV genomic DNA in HD was first reported in 1987.[8] Several studies using different methods have confirmed EBV association with certain subtypes of HD. In summary, results of such studies have indicated that the frequency of EBV association with HD is related to the subtype of HD, the age of patients, and the geographic location i.e. developed versus developing countries. Mixed cellularity HD has the highest rate of EBV association,[9-15] whereas the lymphocyte predominance type has the lowest rate of EBV association.[16,17] As for the age, most studies have found higher rates of EBV presence among childhood HD as opposed to adult onset HD.[11,14,18,19] Also most studies with a few exceptions have found higher rates of EBV among HD patients in

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developing countries.[20-25] In Jordan, little work has been carried out to evaluate the association of EBV with HD. In this study, we have used immunohistochemistry and in situ hybridization to detect the presence of EBV in Jordanian HD. Our objectives were first to determine if EBV is associated with Jordanian HD. Second, if EBV association exists, to determine if it is related to certain age groups or specific histologic subtypes of HD. Third to determine whether such association follows patterns seen in developing or developed countries.

Methods. This is a retrospective study, conducted in the years 2000 and 2001, dealing with cases of HD, from 2 large medical centers, diagnosed at the Department of Pathology, Faculty of Medicine, Jordan University of Science and Technology (JUST) between 1996-1999, and cases from Al-Basheer Hospital diagnosed between 1996-1999. All cases were reviewed and sub-classified by one of us (NMA), according to the World Health Organization (WHO) classification of hematologic malignancies,[26] after performing immunohistochemical stains of at least CD45, CD15 and CD30, and in some cases CD20 and CD45RO. Sixty-four cases of confirmed HD cases were included in this study.

Immunoperoxidase staining. Immunoperoxidase stains for LMP-1 were performed on all the 64 cases in this study. Three micron thick sections were cut from each selected paraffin block onto saline-coated slides; sections were then dewaxed, rehydrated to distilled water and placed in 0.01 M sodium citrate solution (pH 6.0). Antigen retrieval was carried out by autoclaving at 121^{0}C, 15 PSI for 20 minutes. The sections were then allowed to cool in the sodium citrate solution for at least 30 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. To minimize non-specific background staining, sections were blocked for 30 minutes with normal rabbit or goat serum diluted 1:1 in PBS. After removing excess serum, sections were incubated with antibodies against LMP1 (Dako, Carpinteria, CA, USA), followed by washing twice in 5-minutes changes of PBS. The sections were incubated for 30 minutes with biotinylated rabbit anti-mouse antibody, washed twice again, followed by 30 minutes incubation with a streptavidin-biotin-horseradish peroxidase complex. Sections were washed in 45-minutes changes of PBS and visualized using diaminobenzidine (DAB). Finally, the sections were lightly counterstained with hematoxylin, dehydrated and mounted. Control sections were treated in the same way except for omitting the application of the primary antibody.

In situ hybridization staining. In situ hybridization was performed on 59 case; 5 cases were not evaluated because of unavailability of adequate tissue in the blocks. Detection of EBV genome was carried out with DAKO EBV [EBER] probe (mixture of fluorescein-conjugated peptide nucleic acid [PNA] probes) intended for the detection of latent EBV infection by binding to the 2 nuclear EBER RNAs encoded by EBV. Briefly, formalin-fixed, paraffin-embedded tissue sections were mounted on glass slides pretreated with 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO, USA) and deparaffinize with xylene and rehydrated in alcohol. The sections were digested with proteinase K (Gibco, BRL, USA) at a concentration of 15 ug/ml for 30 minutes at 37^{o}C, washed in water and dehydrated in 95% alcohol. They were incubated for 2 hours at 37^{o}C with 20 mL of hybridization mixture containing EBER PNA probe. After washing twice in Tris-buffer saline (TBS) containing 0.1% Triton X-100, the slide were further incubated for 30 minutes at 37^{o}C with rabbit anti-fluorescein isothiocyanate antibody conjugated with alkaline phosphatase (1:50, Dakopatts). Slides were washed in TBS, followed by application of nitro blue tetrazolium as a chromogen. Sections were finally counterstained with hematoxylin.

Results. ***Clinical findings.*** The ages of the patients ranged were from 4-70 years with a mean of 28 years, and a median of 24 years. There were 11 patients (17.3%) below 15 years of age (childhood group). Fifty-three patients (82.7%) presented at age ≥15 years, 59.6% of these cases were ≤40 years (young adult group). There were 4 patients (6.2%) between 55 and 74 years of age. The age distribution showed 2 peaks, the first in the second decade of life, and the second in the fifth decade of life. There were 35 males and 29 females with a male to female ratio of 1.2:1. Cervical lymph nodes were the most commonly affected site representing 56.3% of all cases.

Histopathologic findings. Mixed cellularity HD was the most common subtype in this series representing 53.1% of all the cases; nodular sclerosis represent 43.8% and lymphocyte predominance subtypes represent 3.1% of the cases. In patients <15 years old, the most frequent subtype was mixed cellularity (63.6%), followed by nodular sclerosis (27.3%). The most common subtype in patients aged 15-40 years was nodular sclerosis (57.9%), followed by mixed cellularity (39.5%).

Epstein-Barr virus status. Positive LMP-1 staining was seen in the cytoplasm and cell membranes of Reed-Sternberg cells, whereas positive EBER expression was localized in the nuclei of these cells (**Figure 1**). The distribution of LMP-1 and EBER expression in Reed-Sternberg cells or variants is summarized in **Table 1**. Latent

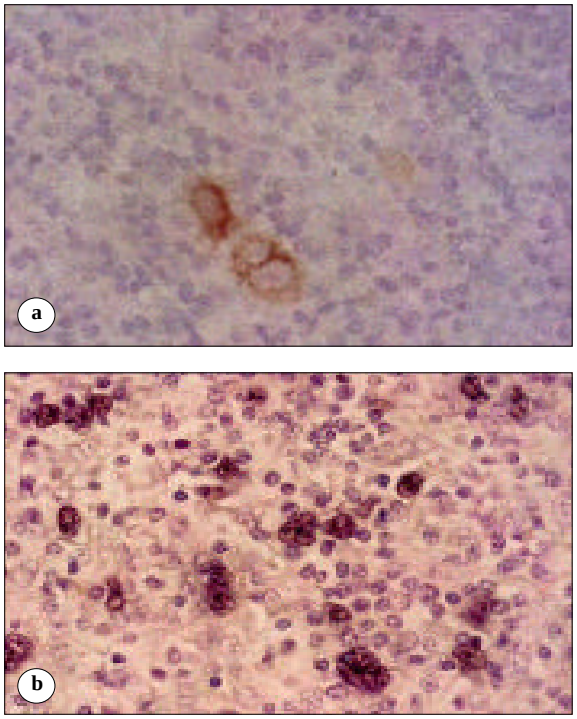

Figure 1 - Photograph showing **a)** Positive latent membrane protein-1 immunohistochemical stain showing brown staining of the cytoplasm of Reed-Sternberg cells. **b)** In situ hybridization for Epstein-Barr virus encoded RNA showing purple staining of the nuclei of Reed Sternberg cells.

membrane protein-1 was observed in 25 out of 64 cases (39.1%) of HD in our study, with the most frequent association in cases of mixed cellularity subtype (18/34; 52.9%). Only 7 out of 28 (25%) nodular sclerosis type were LMP-1 positive. Epstein-Barr virus encoded RNA expression was detected in 40.6% of HD cases with the highest frequency seen in the mixed cellularity subtype (58.1%). Cases with expression of LMP-1 or EBER were considered to be EBV-associated HD cases. Four cases were negative for LMP-1, but positive for EBER; 2 additional cases were LMP-1 positive, but they were EBER negative. In the final analysis, these 6 cases were considered positive for EBV expression. In all the remaining cases, no discrepancy between LMP-1 and EBER expression was noted. None of the 2 lymphocyte predominance HD cases were positive for either LMP-1 or EBER. The difference in rates of expression EBV in the mixed cellularity versus the nodular sclerosis types was statistically significant (p=0.0046). The frequency of EBV expression in relation to the age of the patients is shown in **Table 2**. The highest rate of EBV expression was seen in the pediatric age group, and the lowest rate was among the young adult age group. The difference in EBV expression among these 2 groups was statistically significant (p=0.02). The elderly age group has a rate of EBV expression higher than the young adult patients, but less than the pediatric age group.

Table 1 - Epstein-Barr virus association with Hodgkin's disease according to histologic subtype.

HD subtypes	LMP-1 positive	EBER positive	EBV expression (LMP-1 or EBER positive)
	n (%)	n (%)	n (%)
MC	18/34 (52.9)	18/31 (58.1)	22/34 (64.7)
NS	7/28 (25)	6/26 (23.1)	8/28 (28.6)
LP	0/2	0/2	0/2
Total	**25/64 (39.1)**	**24/59 (40.6)**	**30/64 (46.8)**

HD - Hodgkin's disease, EBV - Epstein-Barr virus, NS - nodular sclerosis, MC - mixed cellularity, LP - lymphocyte predominant, LMP-1 - latent membrane protein 1, EBER - Epstein-Barr virus encoded ribonucleic acid

Table 2 - Epstein-Barr virus association with Hodgkin's disease according to age groups.

Age group (years)	LMP-1 positive	EBER positive	EBV expression (LMP-1 or EBER positive)
	n (%)	n (%)	n (%)
<15	6/11 (54.5)	6/9 (66.6)	8/11 (72.7)
15-40	13/38 (34.2)	11/35(31.4)	13/38 (34.2)
>40	6/15 (40)	7/15(46.6)	9/15 (60)
Total	**25/64(39.1)**	**24/59 (40.6)**	**30/64 (46.8)**

EBV - Epstein-Barr virus, LMP-1 - latent membrane protein 1, EBER - Epstein-Barr virus encoded ribonucleic acid

Discussion. Most studies on HD have shown epidemiological differences between developed and developing countries. Hodgkin's disease in the United States of America and Europe countries tend to have a bimodal age distribution with a young adult peak occurring between 20 and 34 years of age and a second peak occurring in the elderly patients between 50 and 74 years of age.[3,4] On the other hand, HD in developing countries lack the young adult peak; but there is an increase in the incidence of HD in children <15 years of age.[4-6,48] It has also been shown that the nodular sclerosis subtype predominates in developed countries, whereas the mixed cellularity subtype is the most common type in developing countries.[4] Moreover EBV association studies have revealed differences between developed and developing countries with higher EBV related HD cases in developing countries. In an attempt to summarize the literature data (**Table 3**), we found that most developed

Table 3 - Summary of literature on Epstein-Barr virus association with Hodgkin's disease including studies using immunoperoxidase or in situ hybridization techniques on tissue sections.

Study	Country	EBV expression in histologic subtypes of HD								EBV expression in all HD cases	
		MC		NS		LP		LD		Unclassified	
		n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Pallesen et al[9]	UK	23/24	(95.8)	16/50	(32)	1/10	(10)	0/0	-	0/0	-	40/84	(48)
Weiss et al[10]	USA	6/8	(75)	4/12	(33.3)	0/14	-	1/2	(50)	0/0	-	11/37	(30)
Khan et al[11]	UK	15/22	(68)	9/38	(24)	0/10	-	1/7	(14)	0/0	-	25/77	(32)
Peh et al[14]	Malaysia	27/31	(87)	12/33	(33.3)	0/14	-	1/2	(50)	1/1	(100)	41/67	(61)
Enblad et al[15]	Sweden	8/21	(38)	20/67	(23)	1/5	(20)	3/3	-	0/1	-	32/97	(27)
Brousset et al[16]	France	12/26	(46.1)	4/22	(18.2)	0/5	-	0/0	-	0/1	-	16/54	(30)
Armstrong et al[19]	UK	33/60	(55)	25/111	(23)	6/45	(13)	NP		NP	NP	66/206	(32)
Monterroso et al[24]	Costa Rica	12/14	(86)	3/20	(15)	0/5	-	1/1	-	-	-	16/40	(40)
Herbst et al[27]	Italy & Germany	9/18	(50)	9/27	(33.3)	0/1	-	0/1	-	0/0	-	18/47	(38)
Herbst et al[28]	Germany	7/18	(38.9)	8/24	(33.3)	2/2	(100)	1/2	(50)	0/0	-	18/46	(39)
Delsol et al[29]	France	32/55	(58.2)	4/40	(10)	0/10	-	0/0	-	0/2	-	36/107	(34)
Murray et al[30]	UK	6/7	(85.7)	12/24	(50)	1/12	(8.3)	3/3	(100)	0/0	-	22/46	(48)
Weinreb et al[31]	UK	17/20	(85)	14/36	(38.8)	4/13	(30.8)	1/2	(50)	1/3	(33.3)	37/74	(50)
Bellas et al[32]	Spain	1/3	(33)	5/10	(50)	1/2	(50)	0/1	-	0/0	-	7/16	(44)
Hamilton-Dutoit and Pallesen[33]	Denmark	NP	-	NP	-	NP	-	NP	-	NP	-	46/102	(45)
Hamilton-Dutoit and Pallesen[33]	China	NP	-	NP	-	NP	-	NP	-	NP	-	17/28	(61)
Pinkus et al[34]	USA	27/39	(69)	18/127	(14)	0/14	-	2/3	(66.7)	1/3	(33.3)	48/186	(26)
Zarate-Osorno et al[35]	Mexico	18/27	(67)	7/7	(100)	6/13	(46)	0/1	-	5/6	(83)	36/54	(67)
Quintanilla-Martinez et al[36]	Mexico	18/22	(81)	10/20	(50)	1/1	(100)	6/7	(86)	-	-	35/50	(70)
Weinreb et al[37]	Kenya	18/18	(100)	51/64	(80)	4/5	(80)	7/9	(78)	5/5	(100)	85/101	(85)
Paulino et al[38]	Philippines	6/9	(66.7)	3/10	(30)	0/2	-	0/0	-	-	-	9/21	(42)
Huh et al[39]	Korea	38/51	(75)	10/17	(59)	5/8	(63)	7/11	(64)	-	-	60/87	(69)
Hayashi et al[40]	USA	4/6	(66.7)	7/17	(41.2)	0/2	-	-		1/1	(100)	12/26	(46)
Hayashi et al[40]	Brazil	23/23	(100)	2/3	(66.7)	0/0	-	1/1	-	-	-	26/27	(96)
Andriko et al[41]	USA	3/4	(75)	5/13	(39)	1/16	(6)	-	-	8/11	(73)	17/44	(39)
Mourad et al[42]	KSA	8/9	(89)	21/45	(47)	0/8	-	-	-	-	-	29/62	(47)
Preciado et al[43]	Argentina	NP	(72)	0	-	NP	(33)	NP		NP	NP	21/37	(57)
Murray et al[44]	UK	10/27	(37)	30/116	(26)	2/13	(15)	1/6	(17)	8/25	(32)	51/190	(27)
Bosch Princep et al[45]	Spain	7/12	(58)	11/29	(38)	0/5	-	1/1	(100)	1/2	(50)	20/49	(41)
Vassallo et al[46]	Brazil	14/15	(93)	36/61	(59)	0/0	-	0/1	-	0/1	-	50/78	(64)
Elgui de Oliveira et al[47]	Brazil	47/56	(84)	8/27	(30)	0/4	-	1/4	(25)	5/5	(100)	61/96	(64)
Present study	Jordan	22/34	(64.7)	8/28	(28.6)	0/2	-	-	-	-	-	30/64	(46.8)

EBV - Epstein-Barr virus, MC - mixed cellularity, NS - nodular sclerosis, LP - lymphocyte predominant, LD - lymphocyte depleted, NP - not provided, USA - United States of America, UK - United Kingdom, HD - Hodgkin's disease

countries have 30-40% EBV association with HD as opposed to much higher rates that may reach the 90% level in some developing countries. Results from this study have shown that epidemiologically HD in Jordan shares some of the features seen in developing countries. Specifically, mixed cellularity subtype accounted for more than half of the cases in this series. The peak incidence in our series was between 10 and 20 years of age, which is a decade earlier than the peak seen in developed countries. The association of EBV with HD has not been evaluated in Jordan except for one study, which included only pediatric patients.[49] In that study, 50% of the pediatric HD cases were EBV positive. In this current series, a much larger group of patients have been studied including all age groups. Results from this study confirmed the presence of EBV in the neoplastic cells of Jordanian HD cases. The proportion of HD cases associated with EBV was 46.6%. Such figures appear to be lower than those reported in most developing countries and closer to those reported in the Western world. It is not clear why EBV association with HD in Jordan is following the trend seen in developed countries when the epidemiology of HD in Jordan is different from that seen in such countries. One may speculate that the relatively low proportion of EBV related HD cases seen in Jordan is a reflection of the high standards of living. Monterroso et al used this argument to explain the low rates seen in Costa Rica.[24] Similarly, identical low rates of EBV associated HD were seen in a study from Saudi Arabia,[42] a developing country with a high living standards. Epstein-Barr virus association was related to the histologic subtypes of HD. The mixed cellularity type was the most common type associated with HD, and none of the 2 lymphocyte predominant cases had evidence of EBV infection. Only one fourth of the nodular sclerosis subtype had evidence of EBV association, this is less than half the rate seen in the mixed cellularity subtype. These observations are in agreement with most data reported in the literature (**Table 3**) with the exception of those reported from Mexico,[35] Kenya[37] and Brazil[46] where EBV was found in most cases of HD regardless of the histologic subtype. We were able to detect EBV in 73% of our pediatric HD group as opposed to 34% in the young adult group. Such difference between the 2 groups is statistically significant and may indicate a more important role for EBV in the childhood HD. Our data in this regard are consistent with most of the studies carried out in the past, which showed higher frequencies of EBV in children with HD.[20,31,50] The results from this study are supportive of Armstrong's argument that pediatric HD is an EBV associated disease.[19] Indeed, all of the tissues from children <10 years of age harbored EBV lending more support to the concept that HD in different age groups have different etiologies.

In summary, the data from this study have shown that HD in Jordan share some of the epidemiologic features of HD in developing countries. However, the rates of EBV association are similar to those seen in developed countries. Epstein-Barr virus association with HD is mostly seen in childhood and the mixed cellularity subtype.

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