EUROPEAN JOURNAL OF CANCER 43 (2007) 161–168

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Hodgkin's lymphoma in Indian children: Prevalence and significance of Epstein–Barr virus detection in Hodgkin's and Reed–Sternberg cells

Veronique Dinand[a,*], *Ramesh Dawar*[b], *Laxman S. Arya*[c], *Rajani Unni*[b], *Binimaya Mohanty*[b], *Rajvir Singh*[d]

[a]*Department of Paediatrics, Division of Paediatric Oncology, All India Institute of Medical Sciences, New Delhi 110 029, India*
[b]*Department of Pathology, All India Institute of Medical Sciences, New Delhi, India*
[c]*Cancer Institute, Indraprastha Apollo Hospital, New Delhi, India*
[d]*Department of Biostatistics, All India Institute of Medical Sciences, New Delhi, India*

ARTICLE INFO

Article history:
Received 6 August 2006
Accepted 21 August 2006
Available online 20 November 2006

Keywords:
Hodgkin's lymphoma
Children
Epstein–Barr virus
Treatment outcome
Immunohistocytochemistry
Hybridisation *in situ*
Prevalence
Socioeconomic status

ABSTRACT

Aim: This study was done to document the prevalence of Epstein–Barr virus (EBV) in Hodgkin's lymphoma (HL) in children of North India.
Methods: 145 previously untreated children diagnosed with HL from 1991 to 2003 were included. Lymph node (LN) biopsies were studied and classified using World Health Organisation (WHO) classification. EBV detection was done by immunohistochemistry (IHC) and *in situ* hybridisation (ISH) in 145 cases and 25 age- and sex-matched controls. Patients were treated with chemotherapy alone.
Results: EBV was detected by IHC in 131 (90.3%) cases and by ISH in 126 (93.3%) out of 135 cases, and in none of the controls examined. With IHC and ISH combined, EBV positivity was seen in 96.6% and was significantly associated with younger age ($p = 0.012$) and lower socioeconomic level ($p = 0.007$). EBV status had no implication on treatment response and survival.
Conclusion: EBV detection in 96.6% of childhood HL in a population with almost universal EBV seroconversion, and in none of the control lymph nodes, suggests a causative role of EBV in most cases of Indian childhood HL.

1. Introduction

Epstein–Barr virus (EBV) has been detected in a large subset of Hodgkin's lymphoma (HL) cases all over the world, especially in countries with poor socioeconomic conditions and among children.[1] Four publications from South and West India are available,[2–5] and report 31% to 82% of EBV detection in all cases including adults, 28% to 98% in children with HL. However, there is no data available from North India. We conducted a study to assess the frequency and significance of EBV detection by immunohistochemistry (IHC) and in situ hybridisation (ISH) in lymph node (LN) biopsies of children with HL in North India.

2. Materials and methods

2.1. Patients inclusion

A retrospective and prospective study of children under 15 years of age diagnosed with HL from 1991 to 2003, was con-

* *Corresponding author:* Tel.: +91 11 26594376; fax: +91 11 26588663.
E-mail address: verodinand@fastem.com (V. Dinand).
0959-8049/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2006.08.036

ducted in the Division of Oncology, Department of Paediatrics, All India Institute of Medical Sciences (AIIMS), New Delhi. During that period, 274 children with HL were registered at the Paediatric Oncology Clinic, AIIMS, for evaluation and treatment. Of those, only 236 had analysable data. Patients who had previously received treatment for HL or any other malignancy were excluded. All available LN biopsies were reviewed to confirm the diagnosis, and all cases with paraffin-embedded LN or mass biopsies available from the archives of the Department of Pathology, AIIMS, were included in the study. Ethical committee approval was granted and informed consent was obtained from the parents or guardian of the patients included prospectively on a consent form provided either in English or in Hindi.

2.2. Controls selection

Twenty-five sex- and age-matched control children with significant lymphadenopathy leading to a clinical suspicion of lymphoma were randomly selected from the list of LN biopsies reported as follicular hyperplasia of the LN between January 2001 and April 2003 in the Department of Pathology. Out of 411 such cases, 55 were children. The controls were randomly selected among those whose paraffin blocks were available in the Department of Pathology, following the sex and age-group distribution of the cases included in the study.

2.3. Assessment of socioeconomic status

Kuppuswamy's socioeconomic scale, based on education of the household head, occupation of the household head, and family income was used for patients from urban areas.[6] A modification of Pareek's socioeconomic status scale,[7] based on the occupation and education of the head of the family, land holding and family type, was used for patients from rural areas. Socioeconomic classes were further categorised into high socioeconomic group, including the upper and upper-middle classes I and II of Kuppuswamy's and Pareek's scales, and middle-low socioeconomic group, including the middle and lower classes III, IV and V of the same.

2.4. Staging

Patients' initial work-up consisted of a history relative to constitutional symptoms and demographic data, complete clinical examination, complete blood counts with Westergren erythrocyte sedimentation rate (ESR), chest X-ray, contrast enhanced computerised tomography of chest, abdomen, and pelvis, and unilateral iliac crest bone marrow (BM) biopsy. Clinical staging was performed according to the Ann Arbor staging system.[8]

2.5. Treatment protocol

Most patients were treated with chemotherapy alone. Four cycles of COPP (cyclophosphamide, vincristine, procarbazine and prednisolone) alternating with four cycles of ABVD (doxorubicin, bleomycin, vinblastine and dacarbazine) as described earlier[9] were given on an outpatient basis over a period of 8 months. Eight patients treated in 1990 received six to eight COPP cycles (cyclophosphamide, vincristine, procarbazine and prednisolone). Patients were assessed for remission status 4 to 8 weeks after completion of chemotherapy. Follow-up was performed every 3 months during the first year, every 6 months until the fifth year, then once a year.

2.6. Immunohistochemistry

Formalin-fixed paraffin-embedded LN specimens were stained using the avidin–biotine technique with primary monoclonal antibodies against CD45, CD20, CD45RO, CD15 and CD30 antigens (Dako, Denmark) for immunological classification, and with a pool of anti-EBV latent membrane protein-1 (LMP1) antibodies (clones CS1-CS4, Dako, Denmark). Paraffin-embedded BM trephine specimens involved with HL were stained with CD30, CD15 and EBV-LMP1 to confirm involvement and to assess concordance of EBV status between LN and BM at the time of initial diagnosis. Twenty-five LN biopsies of sex- and age-matched controls diagnosed morphologically as follicular hyperplasia were stained for EBV-LMP1. Controls used for standardisation and with each batch of tissue sections were sections of a reactive LN for CD45, CD20 and CD45RO; sections of a LN biopsy from known CD15 positive and CD30 positive HL cases were used for CD15 and CD30 respectively and a LN biopsy from a mixed cellularity (MC) HL in an 8 year-old boy for EBV-LMP1.

Paraffin sections on coated slides were processed for IHC. They were deparaffinised in xylene and rehydrated before microwave oven processing for antigen retrieval. Microwave antigen retrieval was done for 30 min at 700 W power in citrate buffer (pH 6.00) for antibodies to CD45, CD20, CD45RO, CD15, and EBV-LMP1, and in Tris-EDTA buffer (pH 9.00) for antibodies to CD30. Endogenous peroxidase activity was blocked with 1.2% methanol – hydrogen peroxide. Primary antibody, raised in mouse (Dako, Denmark) was diluted in Tris buffer (pH 7.50) at the optimal concentration previously standardised and applied to the sections. Overnight incubation of the primary antibody in a moist chamber was followed by 30 min incubation with biotinylated anti-mouse antibody (Dako) and subsequent 30 min incubation with horseradish peroxidase-labelled streptavidine (Dako). Freshly prepared 3,3 diaminobenzidine substrate (DAB, Dako) was applied to the sections. DAB reaction was controlled at low power microscope and stopped in water. Light haematoxylin counterstaining was done and slides were air dried and mounted with DPX mountant. All HL cases were classified according to the World Health Organisation (WHO) classification.[10]

2.7. In situ hybridisation

Five μm-sections of formalin-fixed paraffin-embedded HL LN biopsies were taken on Teflon coated glass slides. After initial standardisation, a known EBV-LMP1 positive HL case was chosen as positive control for EBER ISH. Pre-baked sections were deparaffinised and air-dried. Proteolytic treatment and hybridisation procedure were done with RNAse free measures. Pepsin digestion (2.5 mg/ml) was done at 37 °C in a moist chamber for 30 min. Subsequently, the slides were dehydrated in graded ethanol series (70%, 95% and 100%) and air-dried before starting the hybridisation procedure.

Twenty μl of digoxigenin-labelled EBER probe (PanPath, The Netherlands) was applied to each section, covered with a glass coverslip and incubated overnight at 37 °C in a moist chamber. After TBS washes, alkaline phosphatase-conjugated anti-digoxigenin antibody (PanPath) was applied and incubated at 37 °C for 30 min. NTB/BCIP was applied to the sections and incubated in the dark for about 5 min. Colour development was examined with a light microscope. Substrate reaction was stopped by immersing the slides in deionised water. A light methylgreen counterstain was given, and sections were mounted in DPX and examined under light microscope.

2.8. Statistical methods

Statistical analysis was done using SAS 8.0 software. EBV status was correlated with various epidemiological, clinical, biological and pathological parameters by chi-square test. Univariate and multivariate binary logistic regression was applied to see the strength of association between EBV status and various parameters. Overall survival (OS) was calculated from diagnosis to death or last visit (censored). Event-free survival (EFS) was calculated from diagnosis to death, progression of the disease, relapse, second malignancy, whichever came first, or last visit (censored). OS and EFS were estimated by Kaplan–Meier actuarial survival method. Patients who were lost to follow-up early were excluded from survival analysis. For all candidate prognostic factors, whether clinical or pathological, Cox regression univariate as well as multivariate analysis were performed to find independent as well as combined risk factors for poor EFS. The p value of <0.05 was considered as statistical significance level.

3. Results

3.1. Clinical analysis

One hundred and forty five untreated Hodgkin's lymphoma patients aged less than 15 years were included in the study, with a median age of 8 years (range 2–14 years). Fifty seven percent of the patients presented with stage III or IV disease, and 62% had constitutional symptoms. BM was involved in 14 (10.3%) cases. A summary of clinical data is given in Table 1. Geographical distribution of the patients included Delhi in 22% of cases, nearby states (Haryana, Rajasthan, Uttar Pradesh, Uttaranchal) in 59% and more remote states (Kashmir, Bihar, Jharkhand, Meghalaya and Madya Pradesh) in 19%. One hundred and nineteen patients were categorised into high (29.4%) and middle-low (70.6%) socioeconomic groups.

The 25 controls selected, respecting the sex and age distribution of the 145 HL cases (Table 1), included 22 males (88%) and 3 females (12%) aged 3 to 15 years old; 3 (12%) controls were less than 5 years old, 15 (60%) were 5 to 9 years old and seven (28%) were 10 to 15 years old.

3.2. Pathological classification

Review of HL and its subtyping was done with diagnostic antibodies (LCA, CD20, CD45RO, CD15 and CD30). All but two cases were classical HL; that is, Reed–Sternberg (RS) cells had the following immuno-phenotype: LCA (CD45) negative, B cell (CD20) negative, T cell (CD45RO) negative, CD30 positive. In all but 13 cases of classical HL, Reed–Sternberg (RS) cells were CD15 positive. In two cases of mixed cellularity HL, a proportion of RS cells expressed the B cell marker CD20, as well as CD15 and CD30 markers. Two cases were classified as nodular lymphocyte predominant HL (NLPHL), with the following immuno-phenotype for the Hodgkin's cells: CD20 and LCA positive; CD45RO, CD15 and CD30 negative. Both NLPHL cases presented with stage I disease.

Table 1 – Patients' characteristics (n = 145)

Characteristics	n	%
Sex		
Male	130	89.7
Female	15	10.3
Age		
<5 years	17	11.7
5–9 years	93	64.1
10–14 years	35	24.2
Stage		
I	22	15.2
II	41	28.3
III	64	44.1
IV B	18	12.4
B symptoms present (n = 143)	88	61.5
Extra-lymphatic organs or tissues involved		
Bone Marrow (n = 136)	14	10.3
Lung (n = 144)	7	4.9
Pleura (n = 144)	2	1.4
Liver (n = 144)	2	1.4
Other characteristics		
Hb <10.5 g/dL (n = 139)	86	61.9
Leucocytosis ⩾12,000/cmm (n = 139)	20	14.4
Lymphopenia <1000/cmm (n = 96)	8	8.3
ESR 1st hour ⩾40 mm (n = 98)	59	60.2

Notes: LN: lymph node; Hb: haemoglobin; ESR: erythrocyte sedimentation rate at 1st hour.

Final subtyping in 145 cases of HL was MC in 105 (72.4%) cases, nodular sclerosis (NS) in 33 (22.8 %) cases, NLPHL in two (1.4%) cases, lymphocyte depletion (LD) and lymphocyte rich classical HL (LRCHL) in one (0.7%) case each. Three (2.5%) cases of classical HL could not be subtyped because of poor tissue preservation and/or processing. Using the Ann Arbor staging, it was seen that HLNS was significantly associated with advanced stage disease (stage III and IV, p = 0.039), bulky mediastinal disease (p = 0.018) and the presence of para-aortic lymph nodes (p = 0.040) as compared with HLMC (Table 2).

3.3. EBV-LMP1 detection

EBV-LMP1 was expressed in 131 out of 145 cases (90.3%). Staining was restricted exclusively to Hodgkin's and Reed–Sternberg (H-RS) cells and was both membrane and cytoplasmic in most cases (Fig. 1). Table 3 shows the percentage of EBV positivity according to histological subtype. Nine BM biopsies involved with HL were stained for CD30, CD15 and EBV-LMP1. BM involvement was confirmed and all cases showed concordant EBV status between the LN and the BM at the time of ini-

Table 2 – Mixed cellularity and nodular sclerosis subtypes of HL (*n* = 138)

Features	MC		NS		*p*	OR	95% CI
	n	%	*n*	%			
Stage of the disease							
I–II	50	47.6	9	27.3	0.039	2.4	1.03–5.7
III–IV	55	52.4	24	72.7			
B symptoms (*n* = 136)							
A	42	40.4	9	28.1	0.21		
B	62	59.6	23	71.9			
Bulky mediastinal (*n* = 137)							
Yes	10	9.6	9	27.3	0.011	3.5	1.3–9.6
No	94	90.4	24	72.7			
Para-aortic LNs (*n* = 135)							
Yes	43	41.7	20	62.5	0.040	2.3	1.03–5.3
No	60	58.3	12	37.5			
Hb (*n* = 132)							
<10.5 g/dl	59	59.0	24	75.0	0.103		
⩾10.5 g/dl	41	41.0	8	25.0			
TLC (*n* = 132)							
<12,000/cmm	91	90.1	22	71.0	0.008	3.7	1.4–10.3
⩾12,000/cmm	10	9.9	9	29.0			

Notes: OR: unadjusted odds ratio (univariate binary logistic regression); CI: confidence interval; LNs: lymph nodes; Hb: haemoglobin; TLC: total leucocyte count.

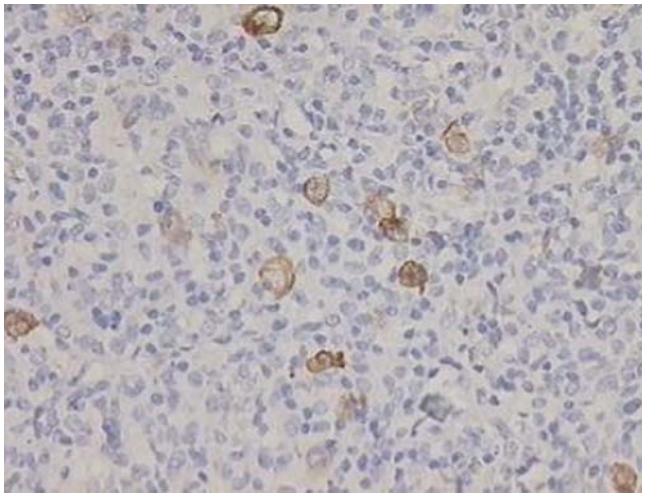

Fig. 1 – Membrane and cytoplasmic immunohistochemical labelling of Epstein–Barr virus latent-membrane protein-1 restricted to Hodgkin's and Reed–Sternberg cells in a case of mixed cellularity Hodgkin's lymphoma.

Table 3 – EBV detection in H-RS cells by combined IHC and ISH in various subtypes of HL (*n* = 145)

WHO classification	Total	EBV positive		EBV negative	
	n	*n*	%	*n*	%
Classical HL MC	105	102	97.1	3	2.9
NS	33	32	97.0	1	3.0
LD	1	1	100.0	0	0.0
LRCHL	1	1	100.0	0	0.0
Unclassified	3	3	100.0	0	0.0
NLPHL	2	1	50.0	1	50.0
Total	145	140	96.6	5	3.4

Notes: IHC: immunohistochemistry; ISH: *in situ* hybridisation; MC: mixed cellularity; NS: nodular sclerosis; LD: lymphocyte depletion; LRCHL: lymphocyte-rich classical Hodgkin's lymphoma.

tial diagnosis. Twenty five lymph nodes diagnosed as follicular hyperplasia from sex- and age-matched controls were stained for EBV-LMP1. None of the controls was EBV-LMP1 positive.

There was a significant statistical association between EBV-LMP1 detection and younger age, as well as between EBV-LMP1 detection and middle-low socioeconomic status as compared with high socioeconomic status (Table 4). EBV-LMP1 positive cases presented were earlier stage disease (stage I–II disease in 45% of EBV-LMP1 positive cases *versus* 31% of EBV-LMP1 negative cases, $p = 0.33$) and a lower frequency of bulky disease (41% *versus* 67%, $p = 0.075$), ESR ⩾ 40/mm (58% *versus* 78%, $p = 0.26$) and leucocytosis ⩾12,000/cmm (13% *versus* 23.1%, $p = 0.35$) than EBV-LMP1 negative cases, but statistical significance was not reached. There was no significant difference between sexes, nutritional status, number of siblings, household size or size of community (village, small town or large town).

3.4. *EBER-1 and EBER-2 detection*

EBV detection by ISH was done on 135 HL cases and 25 controls. EBV small encoded RNAs EBER-1 and EBER-2 were detected by the presence of a nuclear dark blue colour precipitate in H-RS cells and occasional lymphocytes (Fig. 2)

Table 4 – EBV-LMP1 detection by immunohistochemistry in HL (n = 145)

Factor	EBV-LMP1 positive	%	p	OR	95% CI
Sex					
Male	117/130	90.0	0.20		
Female	15/15	100			
Age					
<5 years	17/17	100	$p < 0.001$	9.2[a]	2.6–32.1[a]
5–9 years	89/93	95.7			
⩾10 years	26/35	74.3			
Socioeconomic group (n = 119)					
Middle-low	81/84	96.4	$p = 0.003$[b]	9.7[b]	2.1–44.6[b]
High	28/35	80.8			

Notes: OR: odds ratio; CI: confidence interval.
a Odds ratio and 95% CI are obtained by comparing the groups <10 years and ⩾10 years.
b After adjustment for age by multivariate binary logistic regression.

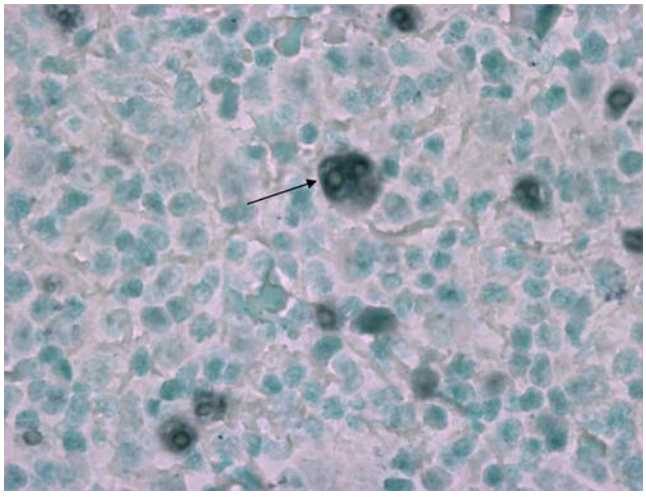

Fig. 2 – *In situ* hybridisation detection of Epstein–Barr virus encoded RNAs in Hodgkin's and Reed–Sternberg cells (arrow) and reactive lymphocytes in a case of mixed cellularity Hodgkin's lymphoma.

in 126 out of 135 cases (93.3%) and in none of the 25 controls examined. IHC and ISH were concordant for EBV positivity in 95% of the cases. With combined IHC and ISH, EBV was detected in 140 out of 145 cases (96.6%) and was significantly associated with younger age ($p = 0.012$) and middle-low socioeconomic status ($p = 0.007$).

3.5. *Response to chemotherapy and survival*

Response to chemotherapy was assessed in 125 patients who received alternating COPP and ABVD and in eight patients treated with six to eight cycles of COPP. One hundred and fourteen (92.7%) patients achieved complete remission (Table 5).

Table 5 – Treatment results

Response to treatment (n = 123)	n	%
Complete remission	114	92.7
Partial remission	3	2.4
Progression	2	1.6
Early death	4	3.3
Status at last follow-up (n = 118)	n	%
Alive in 1stcomplete remission	97	82.2
Alive after relapse	12	10.2
Dead	9	7.6
Survival (n = 118)	5-year	10-year
OS	92.4% (SE 2.6%)	88.4% (SE 4.7%)
EFS	85.3% (SE 3.6%)	65.6% (SE 7.7%)

Notes: Early death: death on therapy; OS: overall survival; SE: standard error; EFS: event-free survival.

After exclusion of 12 cases for protocol violation and further exclusion of 15 cases lost to follow-up less than a year after completion of chemotherapy, 118 patients were analysed for OS and EFS. With a median follow-up duration of 3.7 years, 97 patients are in first complete remission, 12 are alive after having relapsed and nine have died. A total of 14 out of 112 patients (12.3%) who achieved complete remission and were not lost to follow-up have relapsed 4 months to 7.9 years after completing chemotherapy. Six cases were salvaged with additional chemotherapy and low-dose involved field radiotherapy, five have progressive disease and three have died. The projected OS at 5 years is 92.4% (standard error (SE) 2.6%) and the EFS 85.3% (SE 5.6%).

Kaplan–Meier univariate survival analysis showed significantly lower OS in case of advanced disease (stage III and IV as compared with stage I and II, log rank $p = 0.002$), presence of B symptoms (log rank $p = 0.007$), hepatomegaly at the time of presentation (log rank $p = 0.020$), more than two LN areas involved by HL (log rank $p = 0.009$), haemoglobin (Hb) <10.5 g/dL (log rank $p = 0.016$) and CD15 negative H-RS cells in classical HL (log rank $p = 0.0002$). EFS was significantly lower in cases with stage IV disease, presence of B symptoms, hepatomegaly, more than five LN areas involved by HL, belonging to the middle-lower socioeconomic group as compared with the high socioeconomic group, and Hb <10.5 g/dL (Table 6). Multivariate Cox regression analysis identified belonging to the lower socioeconomic group as a single independent risk factor for poor EFS (adjusted hazard ratio 4.8, 95% confidence interval 1.1–21.8). EBV status did not have any prognostic implications in this study.

4. Discussion

Published reports show that epidemiological characteristics of HL have geographical variations and show marked differences between the poorer developing countries and the affluent societies of North America and Europe.[11,12] The high male to female ratio (8.7:1) and the young age at presentation of our report confirm previous data from countries with limited

Table 6 – Five-year event-free survival (Kaplan–Meier) and univariate Cox regression analysis (*n* = 118)

Factors	*n*	5-yr EFS	SE	*p*	HR	95% CI
Sex						
Male	107	86.8	3.4	0.72		
Female	11	60.0	21.9			
Socioeconomic group (*n* = 105)						
High	34	93.2	4.7	0.012*	5.4	1.2–23.6
Middle-low	71	81.5	5.2			
Stage of the disease						
I–III	102	89.9	3.2	0.0001	5.5	2.2–13.8
IV	16	49.4	17.1			
B symptoms (*n* = 116)						
A	45	97.3	2.7	0.005	4.3	1.4–13.0
B	71	76.8	5.6			
LN areas involved						
1–4	94	89.0	3.5	0.010	3.2	1.3–8.3
⩾5	24	69.1	11.3			
Hepatomegaly (*n* = 115)						
Yes	37	76.2	7.5	0.003	3.6	1.5–8.7
No	78	90.9	3.6			
Hb (*n* = 116)						
<10.5 g/dl	73	82.8	4.9	0.003	5.4	1.6–19.1
⩾10.5 g/dl	43	92.0	4.5			
CD15 (CHL only, *n* = 116)						
Positive	105	87.8	3.5	0.011	3.4	1.2–9.3
Negative	11	60.6	15.7			
EBV-LMP1						
Positive	109	86.0	3.7	0.67		
Negative	9	77.8	13.9			

Notes: EFS: event-free survival; SE: standard error; HR: unadjusted hazard ratio; CI: confidence interval; Hb: haemoglobin; CHL: classical Hodgkin's lymphoma.
* Independent factor for poor EFS in multivariate Cox regression analysis.

Table 7 – EBV association in Indian Hodgkin's lymphoma

Author	Study site	*n*	EBV detection	Overall EBV positivity	EBV positivity in children
Radha et al.,[2] 1997	Chennai	45, all ages	IHC	31%	9/32 (28%)
Naresh et al.,[3] 2000	Mumbai	110, all ages	IHC + ISH	78%	49/50 (98%)
Karnik et al.,[4] 2003	Vellore	100, all ages	IHC	82%	24/25 (96%)
Rajalakshmi et al.,[5] 2006	Bangalore	40, all ages	IHC	55%	4/9 (56%)
Present study	Delhi	145 children	IHC + ISH	-	140/145 (97%)

Notes: IHC: immunohistochemistry; ISH: *in situ* hybridisation.

resources and from India.[13–15] Male predominance in HL is more pronounced in developing countries and among children, a feature also present in Indian HL.[16]

EBV association with HL is particularly frequent in children below 10 years of age, in males and in patients with lower socioeconomic status.[1] Industrialised countries report EBV detection in 30 to 60% of childhood HL, whereas developing countries of Asia, Africa and Latin America report 60 to 100% of EBV positive childhood HL. In India, discordant results have been reported in childhood HL, with an association with EBV varying from 28%[2] to 98%.[3] The number of childhood cases in our study is the largest as compared with other Indian series. Our results are similar to the two Indian series that include 100 or more patients of all ages[3,4] (Table 7). Thus over 95% of Indian paediatric HL cases are EBV-associated. Our results highlight the significant association of EBV positivity with younger age and lower socioeconomic status respectively. We could not identify any clinical or biological factor significantly associated with EBV positivity.

More than 90% of adults are infected by EBV worldwide, mostly in early childhood in developing countries like India, where seroconversion is seen in 90% of children by the age of 4 years.[17] Only a few will develop EBV-associated diseases. The detection of EBV-LMP1 protein and EBV encoded RNAs in H-RS cells does not necessarily imply EBV's direct involvement in the pathogenesis of HL, as EBV may be a secondary bystander in HL. The presence of EBV in a monoclonal episomal form[18,19] however, indicates that EBV infection precedes the expansion of the neoplastic clone, thus strengthening its causal role. The total absence of EBV detec-

tion by either IHC or ISH in reactive LNs in sex- and age-matched controls suggests a strong association between HL and EBV, although establishing a causal relationship is beyond the scope of this study. Further studies might assess reactive LNs from immunodeficiency states for the presence of EBV.

The presence of EBV negative HL cases suggests that other possible pathogenic pathways have a role in the development of these tumours. Search for other virus associated with HL, such as cytomegalovirus, human herpes virus 6 (HHV-6), HHV-7, HHV-8, has not yielded any positive results.[20–22] Garbuglia et al. detected the torquetenovirus (TT virus) recently in more than 30% of NSHL tumour cells, and there was often co-infection with EBV in these cases.[23]

Various studies have shown an improved prognosis of EBV-associated HL[3,24,25] while others have shown a poorer prognosis, particularly in patients older than 50 years.[26,27] This conflicting data might be due to the heterogeneous nature of the disease, the treatment protocol used and the age distribution of the patients. We found that several poor prognostic factors, such as advanced stage, bulky disease, raised ESR and leucocytosis, were more frequently seen in EBV negative cases, though statistical significance was not reached. Weinreb et al. have shown that stage IV HL is independently associated with EBV in children[28] In our series, stage IV disease was more frequently associated with EBV positivity by combined IHC and ISH as compared with stage I to III disease (100% *versus* 96.1%, $p = 0.39$). We failed to identify the potential effect of EBV association on survival, which may be due to the small number of EBV negative cases in our study.

The association between lower socioeconomic status and EBV association, which our results highlight, might also be found in affluent European and North American countries. Further studies are necessary to better assess the causative role and prognostic implications of EBV detection in childhood HL, if any, particularly in Western countries where the proportion of EBV negative cases is higher.

5. Conclusion

Almost all childhood cases of HL in India are EBV-associated, while none of the sex- and age-matched controls are positive for EBV detection. The causative role of EBV in the etiopathogenesis of this disease remains to be proven, but larger scale studies might bring oncologists to a better understanding of its clinical implications, and eventually to better therapeutic strategies leading to an improved long-term survival.

Conflict of interest statement

None declared.

Acknowledgement

This study was supported by a grant from the Indian Council of Medical Research, New Delhi, India.

REFERENCES

1. Glaser SL, Lin RJ, Stewart SL, et al. Epstein-Barr virus-associated Hodgkin's disease: epidemiologic characteristics in international data. *Int J Cancer* 1997;**70**:375–82.
2. Radha K, Shanthi P, Madhavan M, Senthamarai A. Study of association of Epstein-Barr virus with Hodgkin's disease. *Indian J Pathol Microbiol* 1997;**40**(3):351–4.
3. Naresh KN, Johnson J, Srinivas V, et al. Epstein-Barr virus association in classical Hodgkin's disease provides survival advantage to patients and correlates with higher expression of proliferation markers in Reed-Sternberg cells. *Ann Oncol* 2000;**11**:91–6.
4. Karnik S, Srinivasan B, Nair S. Hodgkin's lymphoma: immunohistochemical features and its association with EBV LMP-1. Experience from a South Indian hospital. *Pathology* 2003;**35**(3):207–11.
5. Rajalakshmi T, Payal K, Purnima Makhija, Karuna V. Epstein-Barr virus in Hodgkin's lymphoma – Incidence and prognostic implications. *Indian J Med Paed Oncol* 2006;**27**(1):23–6.
6. Kuppuswamy B. *Manual of socioeconomic status (urban)*. Delhi: Manasayan; 1981.
7. Pareek U, Trivedi. *Manual of socioeconomic status (rural)*. Delhi: Manasayan; 1985.
8. Carbone PP, Kaplan HS, Musshoff K, et al. Report of the committee on Hodgkin's disease staging. *Cancer Res* 1971;**31**:1860–1.
9. Arya LS, Dinand V, Thavaraj V, et al. Hodgkin's disease in Indian children: Outcome with chemotherapy alone. *Pediatr Blood Cancer* 2006;**46**(1):26–34.
10. Harris NL, Jaffe ES, Diebold J, et al. The World Health Organisation classification of neoplasms of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting—Airlie House, Virginia, November, 1997. Hematol J 2000;1 (1):53-66.
11. Correa P, O'Conor GT. Epidemiologic patterns of Hodgkin's disease. *Int J Cancer* 1971;**8**:192–201.
12. Mac Mahon B. Epidemiology of Hodgkin's disease. *Cancer Res* 1966;**26**:1189–200.
13. Kapoor G, Advani SH, Dinshaw KA, et al. Treatment results of Hodgkin's disease in Indian children. *Pediatr Hematol Oncol* 1995;**12**(6):559–69.
14. Sripada PV, Tenali SG, Vasudevan M, et al. Hybrid (COPP/ABV) therapy in childhood Hodgkin's disease: a study of 53 cases during 1988-1993 at the Cancer Institute, Madras. *Pediatr Hemat Oncol* 1995;**12**(4):333–41.
15. Nandakumar A, Anantha N, Appaji L, et al. Descriptive epidemiology of childhood cancers in Bangalore, India. *Cancer Causes Control* 1996;**7**(4):405–10.
16. Dawar R, Mangalik A. Hodgkin's disease, an analysis of 128 cases. *Am J Hematol* 1978;**4**(3):209–15.
17. Venkitaraman AR, Seigneurin JM, Lenoir G, John TJ. Infections due to the human Herpesviruses in Southern India: a seroepidemiologic survey. *Int J Epidemiol* 1986;**15**(4):561–6.
18. Weiss LM, Strickler JG, Warnke RA, et al. Epstein-Barr viral DNA in tissues of Hodgkin's disease. *Am J Pathol* 1987;**129**:86–91.
19. Anagnostopoulos I, Herbst H, Niedobitek G, Stein H. Demonstration of monoclonal EBV genome in Hodgkin's disease and Ki67 positive anaplastic large cell lymphomas by combined Southern and in situ hybridisation. *Blood* 1989;**74**:810–6.
20. Berneman ZN, Torelli G, Luppi M, Jarrett RF. Absence of a causative role for human herpesvirus 7 in human lymphoma and a review of herpesvirus-6 in human malignancy. *Ann Hematol* 1998;**77**(6):258–75.

21. Armstrong AA, Shield L, Gallagher A, Jarrett RF. Lack of involvement of known oncogenic DNA viruses in Epstein-Barr virus-negative Hodgkin's disease. *Br J Cancer* 1998;**77**(7):1045–7.
22. Caussinus E, Meggetti F, Delsol G, Brousset P. Simultaneous occurrence of Epstein-Barr virus associated Hodgkin's disease and HHV-8 related multicentric Castelman's disease: a fortuitous event? *J Clin Pathol* 2001;**54**(10):790–1.
23. Garbuglia AR, Iezzi T, Capobianchi MR, et al. Detection of TT virus in lymph node biopsies of B-cell lymphoma and Hodgkin's disease, and its association with EBV infection. *Int J Immunopathol Pharmacol* 2003;**16**(2):109–18.
24. Murray PG, Billingham LJ, Hassan HT, et al. Effects of Epstein-Barr virus infection on response to chemotherapy and survival in Hodgkin's disease. *Blood* 1999;**94**(2):442–7.
25. Montalban C, Abraira V, Morente M, et al. Epstein-Barr virus-latent membrane protein 1 expression has a favorable influence in the outcome of patients with Hodgkin's disease treated with chemotherapy. *Leukemia Lymphoma* 2000;**39**(5–6): 563–72.
26. Stark GL, Wood KM, Jack F, Angus B, Proctor SJ, Taylor PR. Northern Region Lymphoma Group. Hodgkin's disease in the elderly: a population-based study. *Br J Haematol*. 2002;**119**(2):432–40.
27. Jarrett RF, Stark GL, White J, et al. Scotland and Newcastle Epidemiology of Hodgkin's Disease Study Group. Impact of tumour Epstein-Barr virus status on presenting features and outcome in age-defined subgroups of patients with classic Hodgkin lymphoma: a population-based study. *Blood* 2005;**106**(7):2444–51.
28. Weinreb M, Day PJ, Murray P, et al. Epstein-Barr virus (EBV) and Hodgkin's disease in children: incidence of EBV latent membrane protein in malignant cells. *J Pathol* 1992;**168**(4):365–9.