

THE EFFECT OF EPSTEIN–BARR VIRUS LATENT MEMBRANE PROTEIN-1 STATUS ON OUTCOME OF PATIENTS WITH CLASSIC HODGKIN'S LYMPHOMA

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ABSTRACT

Objective: This study determines the expression of latent membrane protein-1(LMP-1) in lymph nodes of patients with classic Hodgkin lymphoma (cHL) and its role in patients' survival.

Methodology: Specimen of 98 patients who had cHL was chosen and the samples were immunohistochemically stained with LMP-1 and Overall disease-free survival (DFS) was measured from the date of complete remission until the Relapse was confirmed by another lymph node biopsy.

We investigated the expression of LMP-1 in outcome of patients with cHL.

Results: LMP-1 was detected in RS cells of cHL in twenty five out of ninety eight (25.5%) patients. LMP-1 expression was significantly more frequent in mixed cellularity, 46.7%, than nodular sclerosis, 15.4% ($P= 0.005$). Patients with EBV-positive tumors had fewer DFS (22.6 vs. 25.9 mo), but the difference was not statistically significant ($p=0.06$).

Conclusion: Although in our study there is no relationship between age and LMP-1 expression. LMP-1 expression is associated with statistically different DFS in treated patients with cHL.

KEYWORDS: Classic Hodgkin Lymphoma, Latent membrane protein-1, LMP-1, Epstein-Barr virus, EBV, Survival.

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INTRODUCTION

This disease which was originally described by Thomas Hodgkin in 1832 and Samuel Wilks first proposed to be called Hodgkin's disease constitutes one of the richest chapters of history of oncologic pathology.¹ It is a malignant neoplasm of the hematopoietic-lymphoid system, meeting the usual criteria for malignancies, including the potential to spread to too many sites and the production of large tumoral masses containing neoplastic cells (Reed Sternberg cells). Clonality has been difficult to prove in HL, although chromosomal abnormalities² have been found in some cases and immunoglobulin gene rearrangements have been noted in others.³

The main candidate suggested as the infection causing Hodgkin's Lymphoma has been the Epstein-Barr virus (EBV). Several cohort studies following up individuals who have had infectious mononucleosis (IM) have shown a raised risk of Hodgkin's Lymphoma generally with a relative risk of about three. Although this might in itself be simply a function of the socio-economic circumstances conducive to late infection with EBV, this is rendered less likely by the results of a cohort study of relatives of 17,000 individuals with serologically confirmed IM, in whom no excess of Hodgkin's Lymphoma was found.⁴ It is surprising, therefore, that EBV genome has only been found within the tumor in 21–39% of cases of Hodgkin's Lymphoma with prior IM.⁵⁻⁷

The proportion of cases of Hodgkin's Lymphoma that are associated with EBV is greater for mixed cellularity and lymphocyte depleted than for nodular sclerosis and lymphocyte rich subtypes. It is also greater: (a) in children (but not adults) from less economically developed than from more developed areas; (b) in males than in females (except at ages over 50); (c) in Asians and Hispanics than in blacks and whites; (d) in children and older adults than in young adults.⁸

The aim of this study was to define the correlation between Expressions of Epstein - Barr virus Latent Membrane Protein-1 in Hodgkin and Reed-Sternberg Cells of Classical Hodgkin's Lymphoma and disease-free months of patients.

METHODOLOGY

Patients and specimens: This was a cross-sectional study with backward direction which included 98 patients with classic Hodgkin's lymphoma (cHL; 40 females, 58 males). Patients aged 14 to 53 years were eligible for enrollment in of this study if they were diagnosed with cHL between 2002 and 2005.

The sampling was performed by simple method and an inclusion criterion was:

* Patients with classic Hodgkin lymphoma at stage I or II with or without B symptoms whose diagnosis was based on Lymph node

biopsy and confirmed by immunohistochemical staining.

- * Success to terminate course of chemotherapy and/or radiotherapy and response to treatment (adriamycin, bleomycin, vinblastine and dacarbazine (ABVD) with or without low dose radiotherapy)
- * During 30 months after complete remission we had data from the course of the disease.

Clinical stage was defined as using the Ann Arbor staging system.⁹ Response to treatment was recorded as 'complete remission' if the disease completely disappeared for a minimum of one month. We wanted to investigate the expression of LMP-1 in outcome of patients with cHL.^{10,11} Choice of treatment was at the discretion of the supervising physician. The REAL/WHO scheme was used to categorize HL.

The histopathological diagnosis of HL and HL subtype, and Reed Sternberg (RS) cells were based on immunomorphological criteria using anti-CD15 (clone C3D-1), anti-CD30 (clone Ber-H2), anti-CD20 (clone L26), and anti-CD3 (clone T3) antibodies (Dako co, California).

Follow-up data regarding attainment of remission, date of birth, date of entry to trial, sex, histological subtype, Lymphoma stage, presence or absence of B symptoms and date of relapse were obtained either by statements from the physicians in charge of treatment or by review of the medical records by the study team.

After that we gathered paraffin blocks of patients and performed immunohistochemical staining for Latent Membrane Protein-1 of EBV (LMP-1) (Clone CS.1-4, Code M0897, Dako Co, California) to determine the reactivity. Overall disease-free months were measured from the date of complete remission until the Relapse was confirmed by another lymph node biopsy. All histopathological material was re-examined by two of the authors

Immunohistochemical staining procedures: Formalin-fixed, paraffin-embedded sections (5mm) were stained using the immunoperoxidase-streptavidin-biotin complex method. Monoclonal antibodies (MAbs)

against Latent Membrane Protein-1 (LMP-1) (Clone CS.1-4, Code M0897, Dako Co, California) were used.

Immunohistochemistry was performed using the streptavidin–biotin complex indirect immunoperoxidase method. Sections were dewaxed, rehydrated, and incubated with 3% hydrogen peroxide (H₂O₂) in water for five minutes to block endogenous peroxidase Activity. Antigen retrieval was performed by microwaving for 20 minutes in 0.01mol L-1 citrate buffer (pH 6) at 750 W. After rinsing in phosphate-buffered saline (PBS), the sections were incubated with the primary antibody (anti EBV antibody, used dilution of 1:100) for 30 minutes. After washing with PBS, the sections were incubated with biotinylated Rabbit antimouse immunoglobulin (Dako Ltd) for 30 minutes, washed with PBS, then incubated with streptavidin-peroxidase conjugate for 30 minutes. The sections were developed with three, 3'-diaminobenzidine tetrahydrochloride solution (Sigma-Aldrich, Poole, U.K.) and 0.1% H₂O₂ and counterstained with haematoxylin. The EBV-positive cell line B95-8 and K562 cells were used as external positive and negative controls, respectively.¹¹

Table-I: Patient, disease subtype, Stage and EBV positivity in all 98 patients.

	Number (%)
Male	58 (59.2%)
Female	40 (40.8%)
Age +/- SD	24 +/- 8.3 year
Max	14 year
Min	53 year
NS	65 (66.3%)
MC	30 (30.6%)
LL	3 (3.1%)
B-symptoms	38 (38.7%)
Stage-I	23 (23.4%)
Stage-II	75 (76.5%)
EBV ⁺	25 (25.5%)

NS= Nodular-Sclerosing;

Mc= Mixed-Cellularity;

LL= Lymphocyte-Rich

Statistical Analysis: Data were analyzed using SPSS version 11.5. In order to compare differences in proportions, x²-square analyses were carried out and an unpaired two tailed Student's t-test was undertaken to calculate differences in mean disease-free months (DFS). Facilities to perform EBER-in Situ hybridization are not available in Esfahan and it was not possible for the authors to do this procedure in other cities.

RESULTS

This study included 98 patients with classic Hodgkin's lymphoma (cHL; 40 females, 58 males). The mean age of the patient was 24 year old (14 – 53 year old). Sixty five (66%) of the patient had Nodular-Sclerosing; thirty (31%) Mixed-Cellularity and three (3%) Lymphocyte-Rich Hodgkin lymphoma (Table-I).

LMP-1 Expression: LMP-1 was detected in RS cells of cHL in 25 of 98 (25.5%) patients. LMP-1 expression was significantly more frequent in mixed cellularity, 46.7%, than nodular sclerosis, 15.4% ($P= 0.005$). LMP-1 was expressed in cHL in 28.6% of patients younger than 20 and in 25% of patients older than 40 compared with 24.2% in the patients aged between 20 and 40 ($P>0.05$). LMP-1 positivity was significantly more common in patients with B-symptoms.

Disease-free month's analyses: Patients with EBV-positive tumors had fewer disease-free months (22.6 vs. 25.9 mo), but the difference was not statistically significant ($p=0.06$) (Table-II).

When DFS was analyzed separately for patients age group (≤ 27 , >27) it showed that DFS difference was lower in the elder age group ($p=0.03$). An analysis of DFMs for all

Table-II: Disease-free survival (DFS) in both EBV positive and negative patients

	DFM			
	Mean	N	Std. Deviation	Minimum
EBV -	25.9452	73	6.90187	7.00
+	22.6000	25	9.34077	4.00
Total	25.0918	98	7.68730	4.00

patients was performed for the following factors: stage (I vs. II), histology (NS vs. MC) and gender but no significant relation was identified between them.

DISCUSSION

In this cross-sectional study patients with EBV-positive cHL had a poorer outcome than those with EBV-negative Lymphoma, with respect to DFS, but this did not remain significant following adjustment for the effects of sex, B symptoms, histology and stage except for those over twenty seven years. It was only in the elderly age group (>27 years) that EBV positivity was significantly associated with lower DFS.

There are conflicting reports in the literature regarding the clinical significance of latent EBV

infection in RS cells of cHL (Table-III).¹²⁻²⁰ These studies vary in methods of patient accrual, EBV detection, treatment regimens, and end point of analysis. Most studies included adults of both sexes, but one included only females. Treatment was uniform in only two studies, each of which analyzed few patients.^{13,14} The detection method of latent EBV infection also varied. In two reports of patients with uniform treatment, LMP-1 was detected immunohistochemically in one, and EBV small encoded RNA was detected by *in situ* hybridization in the other.^{13,14} End points also differed with overall survival (OS) being most common (Table-III). However, OS is affected not only by presenting patient characteristics and initial therapy but also by the post relapse salvage therapy and by the natural limitation

Table-III: Clinical significance of latent EBV infection in HRS cells of patients with HL

Author	Location	Patient age	No. of patients	Treatment	EBV detection	% EBV positive	Clinical end point	Prognostic Significance ^a
Morente <i>et al.</i> ¹²	Spain	All	140	Variable	LMP-1	51	OS	Favorable
Murray <i>et al.</i> ¹³	UK	Adult	190	ChIVPP/ PABLOE	EBER	27	OSFFS	None Favorable
Enblad <i>et al.</i> ¹⁵	Sweden	All	117	Variable	LMP- 1EBER ^b	27	DFS ^c Os	none
Naresh <i>et al.</i> ¹⁶	India	All	110	Variable	EBER	78	Os	Favorable
Glavina- Durdov <i>et al.</i> ¹⁴	Croatia	Adult	100	MOPP/ ABVD	LMP-1	26	DFSOs	Nonenone
Clarke <i>et al.</i> ¹⁸	USA	Adult	311	Variable	LMP-1 EBER ^d	17	Os	Adverse
Vassallo <i>et al.</i> ¹⁷	Brazil	Adult	78	Variable	LMP-1, EBER ^e	64	Os	Favorable
Herling <i>et al.</i> ¹⁹	USA, Europe	Adult	303	ABVD or equivalents	LMP-1	21	FFSOs	NoneNone
Kwon <i>et al.</i> ²⁰	Korea	All	56	Variable	LMP-1 EBER	26.841.1	Os	None Adverse

^a At the $P < 0.05$ level by log-rank test.

^b Tumors without LMP-1 expression were also tested for EBER and were considered positive if EBER was positive.

^c DFS, disease-free survival.

^d Tumors were considered EBV positive if HRS cells expressed either EBER or LMP-1.

^e EBER expression was not correlated with survival. However, expression of LMP-1 in $\approx 10\%$ of HRS cells was correlated with better OS.

of life expectancy. Because initial therapy was variable in most of these reports and salvage therapy was not even mentioned, OS is a problematic end point. Disease-free survival was used in two studies but does not account for primary treatment failures.^{14,15}

Three studies have demonstrated improved survival in relation to EBV and Chl.^{12,16,17} In the Indian study by Naresh et al, the patients were younger than in the present study, with 45% in the pediatric age group; overall, there was a high proportion of EBV-positive cases and these were younger than the EBV-negative cases.¹⁶ Thus the age distribution of the EBV-positive cases may explain their favorable outcome. In a study of adult patients from Brazil, Vassalo et al noted a favorable outcome in cases in which HRS cells expressed EBV latent membrane protein 1 (LMP1).¹⁷ EBV EBER expression, however, which is generally considered the most robust method to define EBV status, did not influence survival. Morente et al also observed a favorable impact of EBV tumor-cell positivity on OS in a study of 140 patients recruited from 11 Spanish centers. Only patients who received adriamycin, bleomycin, vinblastine, and dacarbazine (ABVD) or equivalent regimens were included in the analysis; this clearly represents a relatively selected population.¹² Selection criteria for cases could be the reason for the difference in results between that study and our own besides inclusion of pediatric cases.

In another study in South Korea Tissues from 56 patients were analyzed for the presence of EBV using the in situ hybridization (ISH) for EBV-encoded RNA (EBER) and immunohistochemistry for latent membrane protein (LMP-1). EBV infection was identified in 41.1% of cases by EBER ISH, 26.8% by LMP1 expression, and 26.8% by LMP1 and EBER ISH. EBER-positive HL were significantly more frequent in mixed cellularity (MC) subtype ($P=0.014$) and advanced stage ($P=0.034$). There was a trend toward shorter overall survival in EBER-positive patients without statistical significance ($P=0.238$). LMP1 expression also correlated with MC subtype ($P=0.006$) and

advanced stage ($P=0.007$), although it did not significantly influence the survival outcome. In subgroup analysis, both EBER and LMP1 positivities were associated with longer progression-free survival in patients with age <25 years old ($P=0.045$). Reverse trends were shown in patients ≥ 25 years of age. In this study, we demonstrated that the impact of tumor EBV status on prognosis may be age dependent and young patients with latent EBV infection have favorable prognosis.²⁰

In conclusion, our study showed that LMP-1 is detected in RS cells of 25.5% of patients with cHL and is not associated with statistically different DFS in treated patients with cHL.

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