

The Effect of Epstein-Barr Virus Expression on the Clinical Outcome of Patients with Nodular Sclerosis Hodgkin's Disease

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ABSTRACT

Purpose: The aim of this work is to evaluate the effect of the immunological change associated with Epstein Barr Virus (EBV) on the clinical outcome of patients with nodular sclerosing Hodgkin's Disease.

Material & Methods: Forty-eight patients with the diagnosis of Hodgkin disease were studied retrospectively. The median age was 17 years (range 7-77), 31 males and 17 females, 5 patients had stage I disease, 17 stage II, 13 stage III and 13 stage IV.

B-symptoms were present in 24 patients, 27 patients were treated with chemotherapy alone, 2 patients with radiotherapy alone, 16 patients had combined modality treatment (chemotherapy & radiotherapy) and 3 patients received no treatment.

Results: Half of the patients had tumors positive for EBV, the mean helper to suppressor T lymphocytes (CD4/CD8) ratio was 5.27 (range 0.27-40), the mean T to B lymphocytes (CD3/CD19) ratio was 6.6 (range 0.6-32.5). The mean CD19/CD20 ratio was 23 (range 2-58).

Excluding the 3 patients who received no treatment, the 5 years overall survival was 82.7%, and the relapse free survival was 49%.

On univariate analysis patients who had a high CD4/CD8 ratio (≥ 1.5) had significantly better overall survival (OS) than those with lower CD4/CD8 ratio (88% vs. 58%, $p = 0.03$).

Patients who had a high CD4/CD8 ratio and were EBV negative had a significantly better OS compared to patients with low CD4/CD8 ratio and who were EBV positive (border-line immunity). Age was a determinant prognostic factor for relapse free survival (RFS), younger patients (40 years) did significantly better compared to older patients, (54% vs. 17%, $p = 0.007$), the effect of age on RFS was consistently seen on multivariate analysis with a p value of 0.03.

Key Words: Epstein barr virus - Hodgkin's disease.

INTRODUCTION

In our previous publication [10] we presented results indicating the role that Epstein-Barr virus (EBV) plays in the pathogenesis of Hodgkin's disease, also showed the profound immune compromise seen in patients with EBV infection.

In the present study we looked at the clinical outcome of patients with EBV infection, studying the pattern of disease relapse and survival in relation to the existing immune changes and also in relation to other known prognostic factors.

The current data in the literature looking into the effect of EBV on prognosis is anything but consistent mainly due to the small number of patients included in these studies. EBV was associated with Hodgkin's disease in patients presenting with advanced stage disease [2,6,20].

The ability to identify prognostic factors in Hodgkin's disease raises the possibility of optimizing treatment. Much is already known about the disease related factors (e.g. histology, tumor size and number of involved lymph nodes) and the patient related factors e.g. (age, sex and lymphocytopenia). More work needs to be done to identify immune related factors.

Recently, some biologic factors have been identified. The serum level of soluble CD30, the number of activated cytotoxic T cells and the presence or absence of CD15 in tumor biopsy have been correlated with outcome [13,14,15, 18,19].

MATERIAL AND METHODS

The charts of 48 patients with the histological diagnosis of Nodular Sclerosis Hodgkin's disease were retrospectively analyzed. All the data regarding the patients' characteristics including age, sex, clinical stage, histological subtype, laboratory and radiological work-up, treatment received, response to treatment, pattern and time of relapse, treatment of relapse, and status at last follow-up were collected.

All histological slides were reviewed by one pathologist. Fresh resected lymph nodes were submitted for flow-cytometric, surface marker analysis. The diagnosis was confirmed using standard histologic interpretation aided by immunohistochemical characterization using a panel of antibodies against CD3, CD15, CD20, CD30 and CD45. The typical phenotypic profile of Hodgkin and Reed-Sternberg cells was CD30+, CD15+, CD20-, CD45-. Sections from the formalin-fixed, paraffin-embedded sections of the lymph nodes were submitted for EBV mRNA in situ hybridization.

Cell suspensions were obtained from the lymph nodes either by scraping, *ex vivo* fine-needle aspiration, or by submitting fragments of the lymph nodes, or by a combination of these techniques. The tissue was submitted for flow-cytometric, surface-marker analysis. Representative portions of the lymph nodes obtained by evaluation of touch imprints of different portions of the node and by submission of portions of the most cellular areas. The following monoclonal antibodies were used:

CD45, CD13, CD3, CD4, CD8, CD19, CD20, Kappa and Lambda, CD15 and CD30 (Beckton Dickinson, San Jose, CA). For immunofluorescence staining, 0.1 ml. of adjusted cell count was incubated with FITC, PE, Percp, or PER-cy5 labeled monoclonal antibodies for 30 minutes at 37°C in the presence of 1% bovine serum albumin (BSA). Following incubation, the cells were washed once with 2 ml. of phosphate-buffered saline (PBS) plus 0.1 sodium azide plus 1% BSA.

Red cells were eliminated by lysing the specimens using Coulter IMMUNO-LYSE kit. The cells were then washed once with the above buffer, and the final cell concentration was adjusted by adding 0.4 ml. of 1% paraformaldehyde. The specimens were stored at 2-

8°C until they were ready for analysis. Analysis was performed on a FACS caliber flow cytometer (Coulter, Miami, FL) with Cellquest software (Beckton Dickinson, Brussels, Belgium). In a standard three-color immunofluorescence protocol, forward and side light-scatter were collected along with the three-color antibody combination to generate five characteristics per cellular event. Gating was concentrated on small- to medium-size cells for CD3, CD4, CD8, CD19 and CD20 large cells, which usually constituted < 5% of the events analyzed were excluded. When large cells constituted > 5% of the population, a separate gating was performed for this population for analysis of CD15, CD30 and CD45 expression. A profile of CD15+, CD30+ and CD45 cells was more evident of Hodgkin's disease.

The total percentage of CD3, CD4, CD8, and CD19 was estimated. Additionally, the ratios of CD4 to CD8 and CD3 to CD19 positive cells were estimated also. The reference range used in our laboratory for CD4 to CD8 ratio was 0.9-2.5. Ratios of < 1.5 were considered low. T-to B-lymphocyte ratios \leq 3.0 also were considered low. These reference ranges were determined in our laboratory after studying a group of healthy volunteers.

Histological grading was assessed to evaluate whether the case was Grade 1 and Grade 2 Hodgkin's disease. The grading was based on established criteria [11]. Cases were considered grade II if:

- 1- > 25% of the surface of the node was involved by anaplastic cells.
- 2- > 50% of the nodules showed involvement by Reed-Sternberg cells or
- 3- 30% of individual nodules showed REED-Sternberg cells.

Response evaluation:

All patients were evaluated for response clinically and by CT scan after primary therapy or therapy for relapse. Gallium scan results were not used in the definition of response. Complete response was defined as disappearance of all evidence of disease with patient being free from symptoms. Partial response was defined as \geq 50% reduction in the sum of the product of diameters of all measured lesions with no new lesions appearing and no lesions

increasing in size. Progressive disease was defined as any increase of more than 25% in the sum of the product of diameters of any measurable lesion or the unequivocal appearance of new lesions.

Statistical methods:

Survival was calculated from the day that treatment started to the date of death or last follow-up alive. Relapse-free survival applied only to patients achieving complete response. Analysis was done using univariate and multivariate techniques. The former was used primarily to screen the potential prognostic factors for those that were significantly related to survival. The log-rank test was used to assess their significance. The multivariate method used was the proportional hazards model, and was used to examine those variables found to be significant in the univariate analysis. The levels of significance in the multivariate analysis were calculated by the likelihood ratio test.

Comparison of lymphocyte subset ratios in relation to EBV expression, tumor grade, stage of the disease and age was done using the Kruskal-Wallis analysis of variance.

RESULTS

The median age was 17 years (range 7-77). There were 30 males and 18 female patients, 5 patients had stage I disease, 17 stage II, 13 stage III and 13 stage IV.

B-symptoms were present in 24 patients (50%), 11 patients (23%) had grade II Hodgkin disease.

Twenty-seven patients (56%) were treated with chemotherapy alone, 2 patients with radiotherapy alone, 16 patients (33%) had combined modality treatment (chemotherapy and radiotherapy) and 3 patients received no treatment.

Table (1) shows the relationship of clinical data in relation to EBV expression and T-lymphocyte subsets and T-to B-lymphocytes. EBV expression was seen in 24 patients (50%).

EBV expression was seen more often in Grade II disease than in Grade I. All grade II cases showed EBV expression whereas only 13(39%) cases of grade I disease were positive ($p = 0.03$).

Median CD4 to CD8 was 1.62 in EBV positive cases compared to a median of 3.86 in negative cases ($p=0.01$).

T to B ratios (CD3/CD19) were higher in EBV -negative and Grade I cases of Hodgkin disease. Children had a higher incidence of EBV expression than adults (58% vs. 29%). Low (< 1.5) CD4 to CD8 and low (< 3) T to B ratios were associated with higher stage disease.

Forty two (88%) patients achieved complete response after primary treatment, 3 patients achieved partial response and 3 patients received no treatment in view of their poor general condition.

Disease recurrence was observed in 17/42 patients (40%), 12 out of those 17(71%) recurrences were nodal only, 1(6%) was extranodal only and 4(23%) patients experienced nodal and extranodal recurrences.

Treatment of recurrence was by chemotherapy alone in 11(65%) patients, radiotherapy alone in 2(12%) patients and the remaining 4 (23%) patients received combined modality treatment.

With a median follow up of 3.4 years, the 5 year overall survival (OS) was 82.7% (Fig. 1) and the relapse free survival (RFS) was 49% (Fig. 2).

Tables (2,3) show the results of univariate analysis of the different prognostic factors in relation to OS and RFS respectively.

In terms of OS, patients who had a CD4/CD8 ratio ≥ 1.5 had significantly better OS compared to patients with low (< 1.5) CD4/CD8 ratio (88% vs. 58% respectively, $p = 0.03$) (Fig. 3), the same better OS was observed in patients with high CD4/CD8 ratio and who were EBV negative compared to patients with low CD4/CD8 ratio and who were EBV positive $p = 0.05$ (Fig. 4). No factor was significant for OS on multivariate analysis.

Patients who were below 40 years of age had a better 5 year RFS when compared to older patients (54% vs. 17%, $p = 0.007$) (Fig. 5). That age effect on RFS was also seen on multivariate analysis ($p = 0.03$).

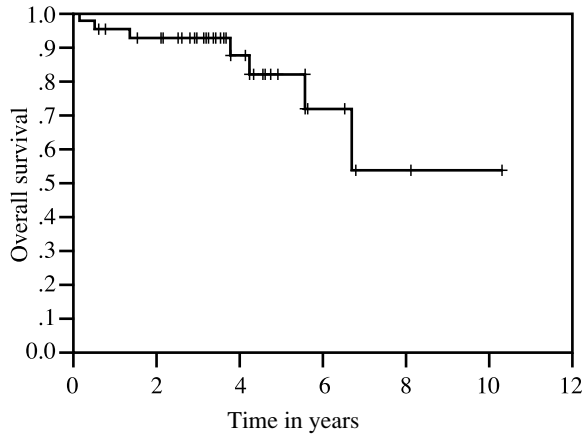


Fig. (1) Overall survival.

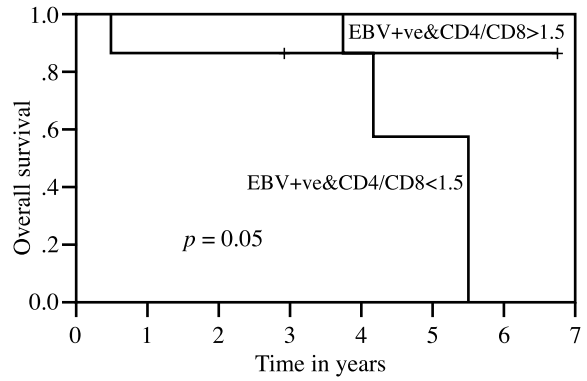


Fig. (4) Overall survival with CD/CD8 ratio and EBV expression.

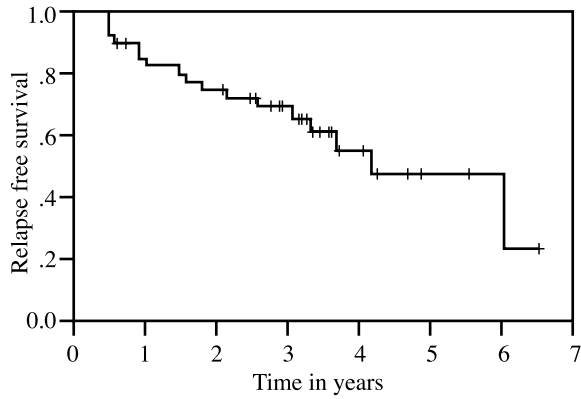


Fig. (2) Relapse free survival.

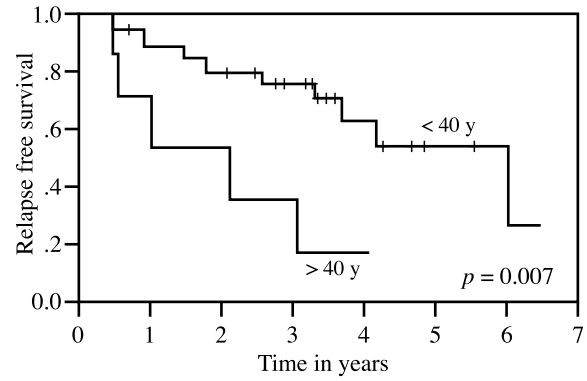


Fig. (5) Relapse free survival with age.

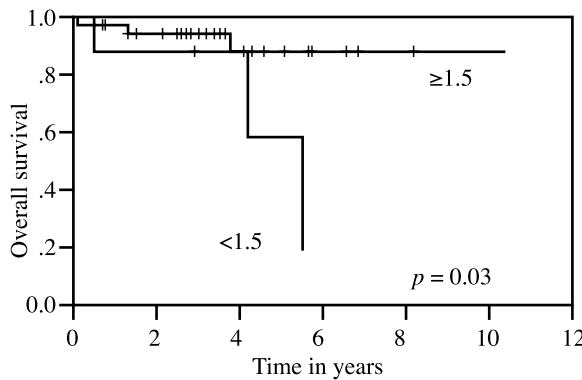


Fig. (3) Overall survival with CD4/CD8 ratio.

Table (1): Relation of age, stage and B symptoms with EBV expression and lymphocyte subset.

	EBV-% (No.)	EBV+% (No.)	P value
B symptoms	54 (13)	54 (13)	NS
High (III and IV)	54 (13)	58 (14)	NS
Low (≤ 1.5) CD4/CD8 ratio	51 (12)	81 (19)	NS
Low (≤ 3.0) T/B ratio	39 (9)	60 (14)	NS
Incidence in children (≤ 14 yrs)		59	0.08
Incidence in adults (> 14 yrs)		29	

Table (2): Overall survival with different prognostic factors.

Prognostic factor	% OS	P value	Significance
< 40 years	80.0	0.9	NS
≥ 40 years	58.3		
Male	89.6	0.1	NS
Female	65.0		
Stage I	100.0	0.7	NS
Stage II	90.0		
Stage III	83.3		
Stage IV	45.0		
Stage I & II	92.3	0.5	NS
Stage III & IV	71.9		
B symptoms	80.2	0.9	NS
No B symptoms	85.0		
Chemo. Only	74.0	0.5	NS
XRT only	100.0		
Combined	100.0		
EBV positive	78.5	0.2	NS
EBV negative	85.7		
CD4/CD8 < 1.5	58.3	0.03	Significant
CD4/CD8 ≥ 1.5	87.8		
CD4/CD8 < 1.5&EBV + ve	57.1	0.05	Significant
CD4/CD8 ≥ 1.5&EBV - ve	85.7		

*NS = not significant

Table (3): Relapse free survival with different prognostic factors.

Prognostic factor	% RFS	P value	Significance
< 40 years	54.0	0.0007	Significant
≥ 40 years	17.0		
Male	49.0	0.3	NS
Female	38.5		
Stage I	40.0	0.3	NS
Stage II	59.0		
Stage III	50.0		
Stage IV	44.0		
Stage I & II	92.3	0.5	NS
Stage III & IV	71.9		
B symptoms	30.0	0.3	NS
No B symptoms	34.0		
Chemo. Only	35.6	0.6	NS
XRT only	50.0		
Combined	73.0		
EBV positive	38.5	0.3	NS
EBV negative	56.3		
CD4/CD8 < 1.5	36.5	0.9	NS
CD4/CD8 ≥ 1.5	49.7		
CD4/CD8 < 1.5&EBV + ve	35.7	0.5	NS
CD4/CD8 ≥ 1.5&EBV - ve	55.6		

*NS = not significant

DISCUSSION

The bimodal age specific incidence pattern of Hodgkin disease is well known. Peak occurrence between ages 15 and 39 and after age 50 years in most populations, together with age specific differences in histologic and clinical features have lead to the assumption of infectious pathogenesis of the disease [12].

EBV, a B-lymphotropic herpesvirus [17], has long been implicated in the process, on the basis of epidemiologic and serologic findings [12].

The incidence of EBV association with Hodgkin disease is well known, in developing countries 70% - 100% are EBV associated compared to 30% - 50% in developed countries [7,8,9,21].

Half of our patients showed EBV infection. The true incidence of the EBV association with Hodgkin disease in this part of the world needs studies with larger number of patients.

Although the number of patients included in our study was small, the effect of EBV infection on the outcome of patients with Hodgkin disease was demonstrated. Patients with positive EBV infection had a border-line immune status as shown by a lower CD4/CD8 ratio that was 1.62 in EBV positive patients compared to 3.86 in EBV negative patients ($p = 0.01$). The overall survival of patients with poor immune status was lower than those patients with more competent immune system (58.3% vs. 87.8% respectively, $p = 0.03$).

Moreover, when the CD4/CD8 ratio was further correlated with EBV infection, the survival difference was consistently seen. Patients with low CD4/CD8 ratio and had EBV expression had a 5 year OS of 57% compared to 85.7% in patients with high CD4/CD8 ratio and who were EBV negative ($p = 0.05$).

Our results does not suggest that only infection with EBV will lead to a worse prognosis, as the difference in overall survival of EBV

positive and negative patients was not statistically significant (78.5% vs. 85.7%, respectively, $p = 0.2$). The same finding was reported by others [1,4,5,18].

Vestlev et al. [18] reported no statistical difference in progression free survival between EBV positive and negative cases (58.5% vs. 75.7% respectively). The only other factor that had significant impact in their study on the disease outcome was the histological subtype, as patients with lymphocyte predominant subtype did better than other histological subtypes, the number of cases analyzed in this study was not large enough (66 patients).

In a larger study of 130 patients, Fellbaum and colleagues [5] showed the same result for the influence of EBV infection on disease outcome with a p value of 0.64 (not significant). As the number of patients was large enough in the latter study, the effect of clinical stage was clearly observed, patients with earlier stages did significantly better in terms of survival ($p = 0.001$).

Enblad et al. [4] reported better overall survival and relapse free survival in EBV negative patients, however the differences were not statistically significant ($p = 0.17, 0.2$ respectively).

Our results also indicated a better RFS for patients who were younger than 40 years of age compared to older patients (54% vs. 17% respectively, $p = 0.007$). This finding was highly significant on univariate analysis and was consistently significant on multivariate analysis ($p = 0.03$). The effect of age on prognosis was not seen in the above mentioned studies [1,4,5,18].

However, recently a large study [3] by Clarke et al., age was found to be of important effect on the disease outcome. The study included 311 women in the Greater San Francisco area aged between 17-79 years. They observed no survival differences between EBV positive and negative women aged 19-44 years, but survival was significantly poorer in women aged 45-79 years with EBV positive Hodgkin disease. This finding was not related to stage or to treatment modality. This is in agreement with our finding, and it points to the importance of age as an independent prognostic factor. It is important that we only observed that in terms of RFS and not in terms of OS, the small number of patients included in our study might be the

reason for that. Also a major difference is that all patients in this study were women.

The effect of EBV infection on survival was different in the study of Naresh et al. [15], higher overall and relapse free survival were observed in EBV positive patients. They observed a 10 years OS of 85% and 64% ($p = 0.03$) in EBV positive compared to EBV negative patients respectively. The number of patients included in their study was 110. The authors are assuming a possible better response to chemotherapy in EBV positive patients. Murray et al. [13] reported a better response to chemotherapy in EBV positive compared to EBV negative patients, this may be because of higher proliferative rates.

Our study suggest that more work needs to be done in the area of biological markers especially those related to the immune system, as in the case of EBV infection and its possible effects on the immunity of patients with Hodgkin disease. Such understanding, in addition to the already known impact of other clinical prognostic factors may lead to better treatment design and more appropriate therapy. Larger studies with longer follow-up periods are needed for that.

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