Longer Failure-Free Survival Interval of Epstein-Barr Virus–Associated Classical Hodgkin's Lymphoma: A Single-Institution Study

Jens Krugmann, M.D., Alexandar Tzankov, M.D., Andreas Gschwendtner, M.D., Martina Fischhofer, Richard Greil, M.D., Falko Fend, M.D., Stephan Dirnhofer, M.D.

Institute of Pathology (JK, AT, AG) and Department of Internal Medicine, Division of Hematology and Oncology (MF, RG), University of Innsbruck, Austria; Institute of Pathology, Technical University of Munich, Munich, Germany (FF); and Institute of Pathology, University of Basel, Basel, Switzerland (SD)

We analyzed Epstein-Barr virus association in classical Hodgkin's lymphoma from a single center in Austria with special emphasis on the *latent membrane protein1* gene configuration and clinical outcome. All 119 (65 male, 54 female) patients were treated from 1974 to 1999 in the Division of Hematology and Oncology at the Department of Internal Medicine, University of Innsbruck, Austria. The mean follow-up time was 122 months (range, 3–333 mo). Epstein-Barr virus was examined by latent membrane protein1 immunohistochemistry and by *in situ* hybridization for Epstein-Barr virus–encoded early ribonuclein acid transcripts. For assessment of the Epstein-Barr virus subtype (A/B) and *latent membrane protein1* gene configuration, the polymerase chain reaction was employed. Fifty-four reactive tonsils were used as the control population. These results as well as clinical parameters such as age, gender, tumor stage, risk factors, and B symptoms were correlated with failure-free and overall survival. Latent membrane protein1 was detected in 31/119 (26%) classical Hodgkin's lymphoma, and Epstein-Barr virus subtyping was successful in 19 of the 31 virus-infected classical Hodgkin's lymphoma cases, as well as in 28 of 54 reactive tonsils. Subtype A was observed in all classical Hodgkin's lymphoma patients and in 26/28 (93%) tonsils. The 30–base pair *latent membrane protein1* gene deletion was found in only 4/31 (13%) Epstein-Barr virus-associated classical Hodgkin's lymphoma as well as in 20/54 (37%) reactive tonsils. Patients with Epstein-Barr virus–associated classical Hodgkin's lymphoma showed a significantly longer mean time to first relapse of 99 months, as compared with 49 months for the Epstein-Barr virus–negative cases (*P < .02*), and were more frequent in those aged >45 years (*P < .04*). Epstein-Barr virus–associated classical Hodgkin's lymphoma were predominantly of the mixed-cellularity subtype and occurred more frequently in male patients, in patients with Stage III and IV, and in patients with B symptoms as well as risk factors. However, overall survival did not correlate with Epstein-Barr virus association. The 30–base pair *latent membrane protein1* gene deletion had no influence on overall survival and failure-free survival time. Although the number of patients with this specific mutation was low, it further shows that an increased oncogenic potential of the *latent membrane protein1* deletion variant is unlikely. This large single-center study demonstrates a low prevalence of Epstein-Barr virus positivity in classical Hodgkin's lymphoma in western Europe. In accordance with results of similar studies, the presence of Epstein-Barr virus has a beneficial effect on the length of failure-free survival despite the higher frequency of risk factors such as higher tumor stage or advanced age.

KEY WORDS: Classical Hodgkin's lymphoma, EBV subtypes A and B, Epstein-Barr virus (EBV), *Latent membrane protein1* gene, Survival analysis.

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Epidemiological and molecular findings suggest that Epstein-Barr virus (EBV) directly contributes to the pathogenesis of a variable proportion of classical Hodgkin's lymphoma cases. The frequency of EBV association of classical Hodgkin's lymphoma depends on age, geographic location, socioeconomic status, and histological subtype (1–15). The prognostic relevance of EBV infection in classical

Hodgkin's lymphoma is still under investigation. EBV-associated classical Hodgkin's lymphoma are supposed to occur in advanced tumor stage (Stages III and IV) and in more aggressive histological subtypes (3, 4, 16, 17). Whereas earlier studies failed to demonstrate an effect of EBV association on outcome in classical Hodgkin's lymphoma (16, 18–24), more recent surveys involving larger number of patients demonstrated a beneficial effect on failure-free survival and relapse (13, 17, 18, 22).

Little is known about the influence of EBV strains and *latent membrane protein1* gene variants on the clinical behavior of classical Hodgkin's lymphoma. The two EBV subtypes A and B encode nine latently expressed genes, *i.e.*, six nuclear antigens (EBV nuclear antigen1, 2, 3A, 3B, 3C, and LP) and three membrane proteins (latent membrane protein1, latent membrane protein2a, and latent membrane protein2b) (25). Latent membrane protein1 is an established viral oncogene that is involved in the activation and transformation of human B-lymphocytes (26). Interestingly, the presence of a characteristic C-terminal 30–base pair in-frame deletion of the *latent membrane protein1* gene has been described in EBV-associated carcinomas and classical Hodgkin's lymphoma (27). Initially, a higher tumorigenic potential and an association with an aggressive histology was described for this variant (27). However, similar rates of the EBV *latent membrane protein1* deletion variant in both neoplastic and reactive lymphoid tissue from the same geographic area have been recently reported, thereby challenging the concept of an increased tumorigenicity (27–29). Nevertheless, clinical data about the potential prognostic impact of the *latent membrane protein1* gene deletion variant and EBV subtype are still largely lacking.

We previously reported a study of Mexican classical Hodgkin's lymphoma in which we found an unexpectedly high prevalence of the *latent membrane protein1* deletion in both reactive lymphoid tissues as well as classical Hodgkin's lymphoma (59% *versus* 80%, respectively). We concluded that this high percentage of the deletion variant simply reflects the distribution of EBV genotypes in the investigated population and not a tumor-specific gene variant (6).

On the background of these somewhat controversial findings, we aimed to examine the frequency and prognostic importance of EBV positivity as well as the impact of EBV variants in a large, well-documented series of classical Hodgkin's lymphoma patients treated at a single center in Austria.

MATERIALS AND METHODS

Cases

We analyzed 132 formalin-fixed, paraffin-embedded cases of classical Hodgkin's lymphoma from the files of the Institute of Pathology, University of Innsbruck, diagnosed between 1974 and 1999. All cases were reclassified according to the updated World Health Organization classification by two of the authors (JK, SD) by reviewing hematoxylin and eosin-stained slides and immunohistochemical stains (30). We excluded six cases of nodular lymphocyte-predominant Hodgkin's lymphoma, three cases of diffuse large B-cell lymphoma of the primary mediastinal subtype, and two cases of anaplastic large T-cell lymphoma. Two cases of classical Hodgkin's lymphoma were excluded because of insufficient paraffin material. In 8 of the remaining 119 cases, the histological subtype was changed. In addition, a total of 54 hyperplastic tonsils (33 male and 21 female patients; age range, 4 to 88 y; mean, 27 y) were analyzed for control purposes. Clinical data were obtained by reviewing the charts and contacting the treating physicians.

In Situ Hybridization

In situ hybridization was done on deparaffinized and dehydrated serial sections. Fluorescein-labeled oligonucleotides complementary to portions of the EBV-encoded early ribonuclein acid transcripts 1/2 were used for hybridization at 42° C, as described previously (31). After overnight incubation, the sections were washed with Tris/HCL-buffer, detected with the alkaline phosphatase/antialkaline phosphatase technique, and stained with Fast Red (Sigma, Vienna, Austria). A fluorescein-labeled poly-d (T) probe (Biogenex, San Ramon, CA) was used to assess mRNA integrity.

Immunohistochemistry

Immunohistochemical analysis was performed as described previously (6). Different pretreatment procedures used for antigen retrieval, as well as the primary antibodies employed, are listed in Table 1.

TABLE 1. Summary of Antibodies Used in this Study

Antibody	Clone	Source	Pretreatment	Dilution
CD15	Leu M1	Dakopatts, Copenhagen, DK	Wet autoclave	1:400
CD20	L26	Dakopatts	Microwave	1:200
CD30	Ber H2	Dakopatts	Wet autoclave	1:50
LMP1 of EBV	(CS 1–4)	Dakopatts	Pronase	1:1000

DNA Extraction, Polymerase Chain Reaction, and Southern Blot Hybridization for the *Latent Membrane Protein1* Gene Deletion

DNA extraction from paraffin-embedded material and polymerase chain reaction (PCR) analysis were performed as described previously (32). DNA integrity was assessed by amplification of a 268–base pair fragment of the β-globin gene (33). PCR analysis of the *latent membrane protein1* gene deletion was done using two oligonucleotide primers flanking the site of the characteristic 30–base pair, rendering a 161–base pair product for the wild-type sequence and a 131–base pair fragment for the deletion variant, respectively (32).

The results were confirmed by Southern blot hybridization using a digoxigenin-labeled internal probe specific for the deleted segment, thus hybridizing only against the wild-type *latent membrane protein1* gene and not the deleted *latent membrane protein1* gene, as well as an oligonucleotide specific for a sequence flanking the deleted segment, therefore binding to both *latent membrane protein1* gene variants.

EBV Genotyping

Identification of Type A and Type B strains of EBV was accomplished with oligonucleotide primers flanking a region of the *Epstein-Barr nuclear antigen2* gene, as reported previously (6, 34). The shorter 168–base pair fragment corresponded to Subtype A, and the longer 184–base pair fragment, to Subtype B.

Statistical Evaluation

Data registration and the statistical analysis were done using STATISTIKA software package (Microsoft Corp., Redmond, WA). The cumulative patient survival was estimated according to the Kaplan-Meier method (35). The survival curves were compared by the log-rank test. For correlation analysis, we used Spearman rank test and the Mann-Whitney U test (36). Statistical significance was defined by $P < .05$.

RESULTS

Clinical Data

The study group consisted of 65 (55%) male and 54 (45%) female patients. The mean age for men was 40.4 years (range, 14–83 y) and for women, 34.3 years (range, 16–77 y). Eighty-six of the 119 patients (72%) were younger than 45 years of age. The median follow-up time was 122 months (range, 3–333 mo). The clinical stage and the occurrence of B symptoms were known in 110 patients, and for 106 patients, we had information about the occurrence of risk factors. Data about the treatment were available for 108 patients. Fifty-four of the patients were treated with combined chemotherapy and radiotherapy; 30 patients, only by chemotherapy; and 24 patients, only with radiotherapy.

Histopathological and Immunohistochemical Findings

Nodular sclerosis was the most common subtype with 70 (59%) cases, followed by 39 (31%) cases of mixed-cellularity subtype, 7 (7%) cases of lymphocyte-predominant subtype, and 3 (3%) cases of lymphocyte-depleted subtype (Fig. 1A). All 119 classical Hodgkin's lymphoma expressed CD30, and 108/119 (91%) expressed CD15. EBV positivity was detected by latent membrane protein1 immunoreactivity in 31/119 (26%) cases (Fig. 1B). The four histological subtypes showed an increase in latent membrane protein1 positivity from 0/7 of the lymphocyte-predominant subtype, 15/70 (21%) of the nodular sclerosis subtype, 1/3 (33%) of the lymphocyte-depleted subtype, and 15/39 (38%) of the mixed-cellularity subtype.

In Situ Hybridization

Expression of EBV-encoded early RNA transcripts 1/2 was seen in Hodgkin and Reed-Sternberg cells of all latent membrane protein1–positive classical Hodgkin's lymphoma by *in situ* hybridization. The nuclei showed a strong positive signal in virtually all of the neoplastic cells. None of the latent membrane protein1–negative cases was positive by EBV-encoded early RNA transcripts in the neoplastic cells (Table 2).

EBV Genotyping

Latent membrane protein1 gene–derived PCR products were obtained in all latent membrane protein1–positive classical Hodgkin's lymphoma. Only 4 of 31 (13%) latent membrane protein1–positive cases contained the *latent membrane protein1* deletion variant (Fig. 2, Table 2). The remaining 27 (87%) cases showed the wild-type *latent membrane protein1* gene configuration. Interestingly, we also detected the 30–base pair gene deletion in 20 of 54 (37%) reactive hyperplastic tonsils.

PCR analysis of the *EBV nuclear antigen2* gene sequence to determine the subtype of EBV was done in cases of classical Hodgkin's lymphoma that were amplifiable for the *latent membrane protein1* gene and in the reactive tonsils. It was successful in 19/31 (61%) classical Hodgkin's lymphoma cases, all of which showed the specific 168–base pair fragment of Subtype A (100%). In the hyperplastic lymphoid tissue of 28 tonsils, Type A was detected in 26 (93%) cases, and Type B, in 2 (7%) cases.
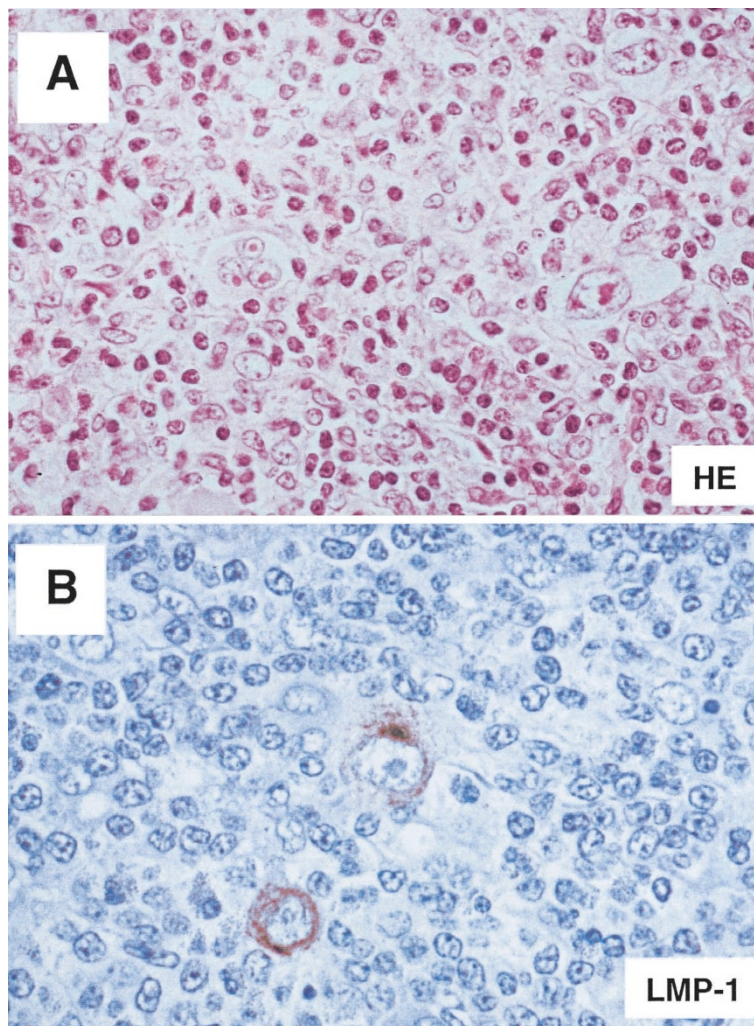
FIGURE 1. **A**, H&E stain of classical Hodgkin's lymphoma, mixed-cellularity type. Typical Hodgkin and Reed-Sternberg cells surrounded by reactive lymphoid cells (original magnification, 20×). **B**, latent membrane protein1 immunostain of classical Hodgkin's lymphoma. Hodgkin and Reed-Sternberg cells show the typical membranous staining pattern (original magnification, 40×).

TABLE 2. Immunohistochemical and Molecular Findings

Subtype	n	Sex		% Number				LMP1 genotype	
		M	F	CD15	CD30	LMP1	EBER-ISH	Wild Type	del
NS	62	31	31	85 (53/62)	100 (62/62)	23 (14/62)	24 (14/58)	13	1
NSII	8	5	3	100 (8/8)	100 (8/8)	12 (1/8)	17 (1/6)	0	1
MC	39	26	13	97 (38/39)	100 (39/39)	38 (15/39)	44 (15/34)	13	2
LP	7	2	5	100 (7/7)	100 (7/7)	0 (0/7)	0 (0/6)	0	0
LD	3	1	2	67 (2/3)	100 (3/3)	33 (1/3)	33 (1/3)	1	0
Total	119	65	54	91 (108/119)	100 (119/119)	26 (31/119)	30 (31/107)	27	4

EBER = Epstein-Barr encoded early RNA transcripts; ISH = *in situ* hybridization; LMP = latent membrane protein; IHC = immunohistochemistry; NS = nodular sclerosis; MC = mixed cellularity; LP = lymphocyte predominant; LD = lymphocyte depletion; del, deleted.

Clinical Data and Survival Analysis

In our study, we found an increased occurrence of EBV-positive cases in male patients, in advanced stage disease (Ann-Arbor III and IV), and in patients with B symptoms and risk factors (mediastinal bulk, extranodal involvement, increased erythrocyte sedimentation rate, involvement of three or more lymph node regions). None of these parameters reached statistical significance compared with the case of EBV-negative classical Hodgkin's lymphoma (Table 3). Patients older than 45 years showed a significant increase in EBV positivity, from 21 to 39% ($P < .04$; Table 3).

A recurrence of the disease was observed in 32/119 (27%, 20 male, 12 female) patients at approximately equal rates in EBV-positive and -negative
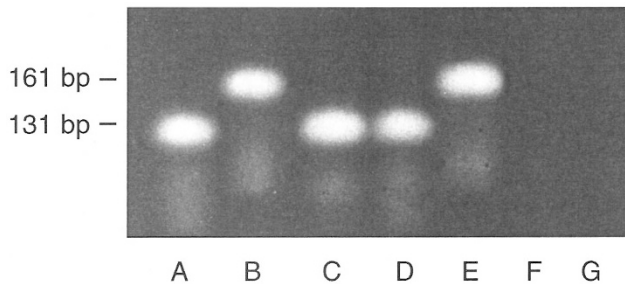
FIGURE 2. PCR analysis for the 30–base pair *latent membrane protein1* gene deletion in Hodgkin's lymphoma. In *Lanes B* and *E*, a 161–base pair fragment was detected, corresponding to the full-length (wild-type) *latent membrane protein1* gene, whereas in *Lanes A*, *C*, and *D*, a 131–base pair fragment was detected that corresponds to the 30–base pair *latent membrane protein1* gene deletion. *Lanes F* and *G* were negative controls (no DNA).

cases. Seven (23%) recurrences occurred in the latent membrane protein1–positive classical Hodgkin's lymphoma, and 25 (28%), in the latent membrane protein1–negative cases (*P* < .53). Twenty-three patients experienced a recurrence once; 5 patients, twice; 3 patients, three times; and 1 patient, five times. The mean time to the first recurrence was 49 months in latent membrane protein1–negative classical Hodgkin's lymphoma and was 99 months in latent membrane protein1–positive cases (*P* < .02; Fig. 3). In a multivariate analysis, EBV association was not recognized as an independent prognostic factor for failure-free survival.

Twenty-five (21%) of 119 patients died during the follow-up period. Seven patients died from tumor progression, and six died because of the development of a second malignancy (carcinoma of the lung in 2, oropharynx in 1, larynx in 1, pancreas in 1, and melanoma in 1). Nine patients died from cardiovascular disease, two from pneumonia, and one from trauma. Latent membrane protein1 expression was observed in 6 (24%) of these 25 patients, and only 1 of them died from the disease. None of the investigated clinical and pathological

TABLE 3. Correlation Analysis of Clinical Parameters of Patients with Known EBV Status

Parameter	EBV + n (%)	EBV− n (%)	P-value
Sex			
Male	20/65 (31%)	45/65 (69%)	.2
Female	11/54 (20%)	43/54 (80%)	
Age			
<45	18/86 (21%)	68/86 (79%)	.04
>45	13/33 (39%)	20/33 (61%)	
Stage			
I + II	15/68 (22%)	53/68 (78%)	.43
III + IV	14/42 (33%)	28/42 (67%)	
B symptoms			
Pos	16/51 (31%)	35/51 (69%)	.27
Neg	13/59 (22%)	46/59 (78%)	
Risk factors			
Pos	15/49 (31%)	34/49 (69%)	.26
Neg	12/57 (21%)	45/57 (79%)	

parameters, including EBV status, showed a significant influence on overall survival (Fig. 4; Table 3).

DISCUSSION

The present large single-center study from Austria demonstrates a low prevalence of EBV positivity of 26% in classical Hodgkin's lymphoma. EBV positivity is associated with a higher clinical stage and age as well as with mixed-cellularity subtype. Overall and failure-free survival did not differ significantly between EBV-positive and -negative cases, but a significantly shorter time to relapse was observed for EBV-negative cases (*P* < .02). This analysis of 119 classical Hodgkin's lymphoma cases confirms the data from eastern and western Europe, with EBV positivity in 26% of the Croatian and 27% of British patients and with increasing rates for patients older than 45 years of age (22, 24, 37, 38). The reasons for the significantly lower rate of EBV-associated classical Hodgkin's lymphoma in European and Western world populations, in contrast to the case of those in developing countries, are enigmatic and complex. One could speculate that the lower socioeconomic status in underdeveloped countries could be responsible for chronic immunosuppression attributable to malnutrition, a concept that could explain the marked increase of EBV infection in the pediatric population in these countries (4, 5). Chronic immunosuppression due to autoimmune disorders, human immunodeficiency virus infection, or posttransplantation status is also correlated with increased EBV infection and classical Hodgkin's lymphoma (39–41). However, various predisposing conditions and to-date-unknown possible interactions between virus and host seem to be responsible for the geographically different association rates of EBV and classical Hodgkin's lymphoma.

Irrespective of the underlying causes for the variability of EBV association, recent studies investigated the potential prognostic importance of EBV in classical Hodgkin's lymphoma. Whereas earlier, small studies failed to demonstrate any significant impact, more recent studies of larger series of patients provided evidence for a beneficial effect of EBV positivity. Montalban *et al.* (18) and Morente and coworkers (21) reported a better overall survival and a higher rate of complete remission and failure-free survival for EBV-associated classical Hodgkin's lymphoma in a Spanish population. In agreement with these results from Spain, three studies from Great Britain, Croatia, and India demonstrated a significantly longer failure-free survival in EBV-associated classical Hodgkin's lymphoma, but no correlation for overall survival was observed (17, 21–23). Our results, with an
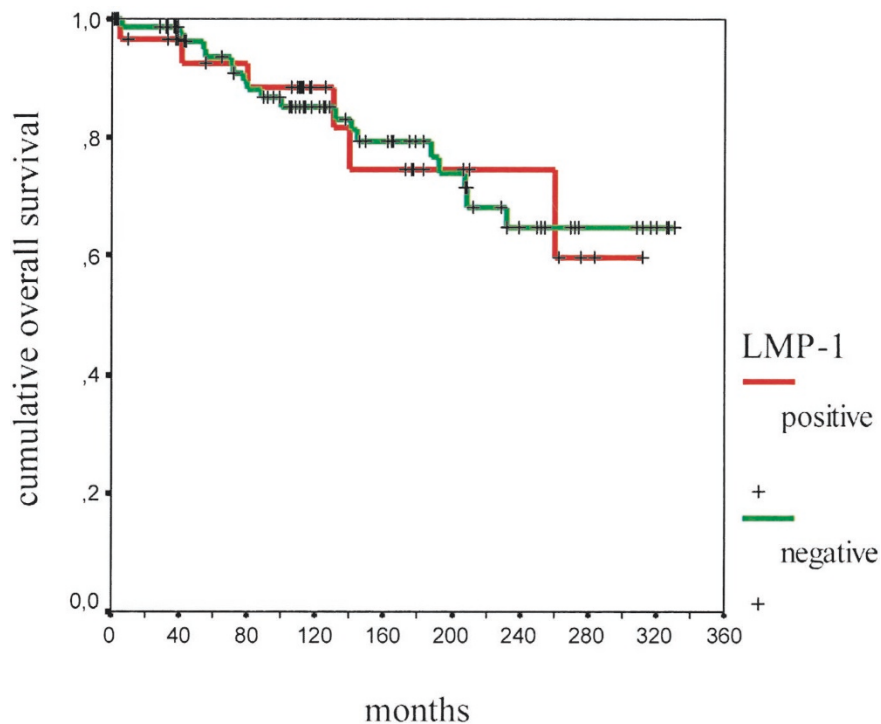
FIGURE 3. Overall survival and latent membrane protein1 expression in classical Hodgkin's lymphoma. No significant differences between the groups as compared by the log-rank test ($P < .92$).
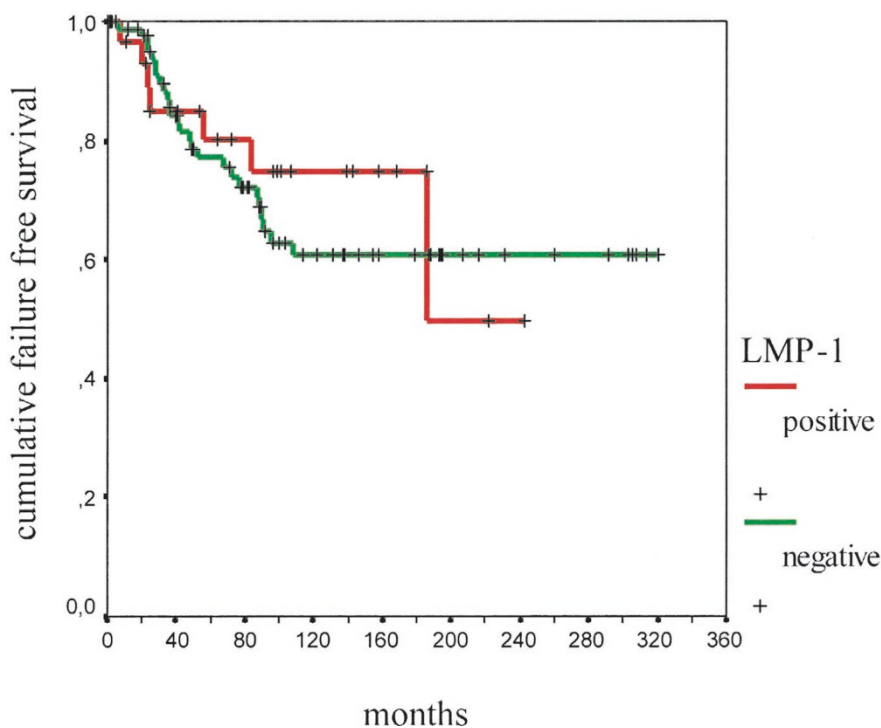

FIGURE 4. Failure-free survival and latent membrane protein1 expression in classical Hodgkin's lymphoma. No significant differences between the groups as compared by the log-rank test ($P < .53$). However, note significantly later first recurrences in the latent membrane protein1–expressing group ($P < .02$ as compared by the Spearman rank test).

increased failure-free survival interval of 99 months for EBV-positive patients, compared with 49 months for EBV-negative classical Hodgkin's lymphoma ($P < .02$), are in perfect agreement with those findings.

A positive interaction between EBV-infected Hodgkin and Reed-Sternberg cells and chemotherapeutic agents via apoptosis induced by BCL2 overexpression in EBV-negative, but lack of BCL2 expression in EBV-positive, Hodgkin and Reed-

Sternberg cells could explain the longer failure-free survival (42). Furthermore, residual EBV-positive tumor cells after cytoreduction by chemotherapy could be the target for immune cytolysis. This has implications for a potential development of specific immunotherapy. Some investigators obtained more activated cytotoxic T-lymphocytes and found increased levels of major histocompatibility complex Class I expression in Hodgkin and Reed-Sternberg cells in EBV-associated classical Hodgkin's lymphoma cases, compared with in EBV-negative cases (43–45).

Regarding the configuration of the *latent membrane protein1* gene, we found the characteristic 30–base pair in frame gene deletion in only 13% of the cases of classical Hodgkin's lymphoma in Austria. The low prevalence of this variant is in agreement with that found in other Western populations, apart from a childhood series from Spain (27, 28, 46–49). The even higher prevalence of the *latent membrane protein1* deletion in reactive lymphoid tissues from the same geographic region argues against a more potent tumor-promoting activity of this variant and confirms and extends our recent findings in a Mexican population. It is noteworthy that although the number of cases with *latent membrane protein1* gene deletion is too low for firm conclusions, we did not find a decreased failure-free or overall survival compared with the case of EBV-associated cases without the gene deletion.

The high prevalence of EBV Type A in this series, which is the common virus strain in European countries, both in classical Hodgkin's lymphoma (100%) and in reactive lymphoid tissue (93%), is in contrast with an increased occurrence of Type B in our previous Mexican series, thereby underlining the importance of geographical factors (47, 49).

In summary, our study of classical Hodgkin's lymphoma revealed a statistically significantly longer failure-free survival interval of EBV-positive classical Hodgkin's lymphoma. The low prevalence of EBV infection in classical Hodgkin's lymphoma from Austria (26%), the exclusive infection with Subtype A virus strain (100%), and the similar distribution of the *latent membrane protein1* deletion variant in neoplastic (13%) and reactive lymphoid cells (37%) highlights the paramount importance of geographical and/or ethnic factors in EBV-associated classical Hodgkin's lymphoma. Finally, increased oncogenic potential of the *latent membrane protein1* deletion variant is unlikely.

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