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# Rapid Communication

## Correlation of the Expression of Epstein–Barr Virus Latent Membrane Protein and *In Situ* Hybridization with Biotinylated *Bam*HI-W Probes in Hodgkin's Disease

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***The detection of Epstein–Barr virus (EBV) nucleic acids by* in situ *hybridization (ISH) with biotinylated* Bam*HI-W probes was correlated with the expressions of EBV latent membrane protein (LMP) and EB nuclear antigen 2 (EBNA2), in 107 cases of Hodgkin's disease (HD) of different immunomorphologic subtypes. Epstein–Barr virus nucleic acids were present and restricted to the pathogenic cells in 4 of 40 (10%) cases of nodular sclerosis (NS) and 33 of 55 (60%) cases of mixed cellularity (MC), but were undetectable in other subtypes. Of the 37 cases positive for EBV nucleic acids, 35 (95%) showed the expression of LMP. Epstein–Barr virus nucleic acids and LMP were restricted to Reed–Sternberg cells and variants. Only 1 case (MC) showed LMP expression in the absence of EBV detection. The correlation was strengthened by the finding of LMP expression at first diagnosis in 6/7 EBV positive cases at relapse (14–126 months) (5/5 EBV negative cases at relapse were LMP negative at first diagnosis). EBNA2 was absent in all 13 (NS, 2; MC, 11) EBV+ and LMP+ cases tested. Both LMP and EBNA2 were expressed in control EBV-positive tissues and cell lines. EBV serology in MC HD was indicative of latent EBV infection, but neither serology nor clinical parameters correlated with the presence or the absence of EBV, over a short-term follow-up (median, 20 months). The findings, although not proving EBV as the etiologic agent of HD, suggest that: 1) LMP expression alone may be adequate for identifying EBV-associated HD, 2) the MC subtype has a stronger relation with EBV presence, and 3) the regulation of EBV genes in HD is different from other EBV-associated disorders. The clinical implications still remain to be discovered. (Am J Pathol 1992, 140:247–253)***

Hodgkin's disease has been on the list of Epstein–Barr virus (EBV)-related neoplasms by epidemiologic and serologic evidence.[1] Recent studies with Southern blot analysis,[2–6] DNA amplification,[5,7] and *in situ* hybridization,[2,4,8–10] have implicated EBV in the etiology of Hodgkin's disease (HD) in a proportion of cases. The detection rates depend on the technique employed, but a high frequency of EBV presence has been reported with polymerase chain reaction (PCR) analysis as compared with other studies.[5–7] *In situ* hybridization (ISH) has shown the presence of EBV genomic material exclusively in Reed–Sternberg (RS) and mononuclear variant (H) cells.[10] Further, the clonal presence of EBV in HD has been reported.[4,6,8] The concept of EBV as an etiologic agent in HD has been challenged by one group of authors because of similar findings in hyperplastic lymph nodes and the presence in both conditions of clonal, non-clonal episomal EBV, and linear replicating EBV genomes.[6] However, the finding of EBV latent membrane protein (LMP) in the majority of mixed cellularity (MC)

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subtype of HD by Pallesen et al,[11] has raised the possibility of this subtype being selectively an EBV-associated disorder.

Using ISH with biotinylated (nonisotopic) *Bam*HI-W probes, we had shown the presence of the EBV genomic material in H-RS cells of some cases of HD.[10] Most of the EBV-positive cases were of the MC subtype.[10] At the time of that report, anti-LMP and anti-EB nuclear antigen 2 (EBNA2) antibodies were not available for comparison with ISH. Our ISH method involved tissues fixed in acetone, for optimal preservation of both leukocyte differentiation antigens and morphology.[12] This particular method achieved localization of the ISH signals, to phenotypically (CD30+, CD15+) characteristic H-RS cells with greater precision than possible with isotopic probes and molecular methods. The current study correlates the presence of EBV nucleic acids with the expression of EBV-encoded proteins, for a more meaningful interpretation of the role of EBV in HD.

## Materials and Methods

### Tissue Source

The files of the Lymphoma Study Group, at CHU-Purpan in Toulouse, include lymph-node biopsies from 107 patients with Hodgkin's disease (HIV serology was negative). Portions of each tissue were processed by the modified AMeX method,[13] (ModAMeX)[12] since 1986. There were 16 cases in which the tissues were processed by this method at relapse but the biopsies at first diagnosis had been processed routinely in ethanol-based Bouin's fluid (anti-LMP antibody was tested in 12 of 16 cases). The diagnosis of the subtypes of HD was based on immunomorphologic criteria.[14,15] The distribution of cases according to subtype was as follows: lymphocyte predominance, nodular (LPn) = 10 cases (2 cases at relapse), with phenotype of lymphohistiocytic (L & H) cells as B-cell antigen +, epithelial membrane antigen (EMA)+, CD45+ and CD30−, CD15−; nodular sclerosis (NS) = 40 cases (4 cases at relapse), and mixed cellularity (MC) = 55 (10 cases at relapse). The morphologic diagnosis of NS was based on both the presence of collagen bands and lacunar variant of the R-S cells. However, the phenotype of Reed–Sternberg (RS) and mononuclear variants (H) in NS and MC was CD30+ and CD15+, EMA−, CD45−, in addition to the lack of definitive B or T-cell lineage on paraffin (or paraplast) embedded sections.[14] Two patients were unclassifiable according to the aforementioned scheme, but showed the phenotype common to NS and MC subtypes. One additional patient (MC) was associated with human immunodeficiency virus (HIV)+ serology. There were no patients who had the diagnosis of diffuse LP (LPd) or lymphocyte depletion (LD) types by immunomorphologic profile. The distribution of the patients (i.e., higher proportion of the MC subtype) is partly because of our practice of considering the so-called cellular phase of NS into the MC type. In addition, most patients of this study were referred to the Department of Hematology for chemotherapy, which often meant the presence of systemic symptoms and/or high tumor burden. The female predominance in the NS group (sex ratio M/F: 0.9 compared with 5 in MC) is similar to usual experience. In addition, patients in the NS group showed a tendency to present in lower stages (54% of stages I and II) than the clinical presentation of the MC subtype (38% in stages I and II). The patients were staged according to standard procedures that included computer-assisted scans of chest and abdomen, examination of bone marrow, and in most patients, a biopsy of liver.

### ModAMeX Method

The details and the possible advantages of this method are described elsewhere.[12] Briefly, the method consists of cutting the fresh tissue, in fragments of 2–3 mm thickness for fixation in cold acetone containing protease inhibitors (phenyl methyl sulfonyl fluoride and iodoacetamide), followed by clearance in methyl benzoate and xylene, and embedding in low melting point paraplast. Besides the original objective of preservation of membrane leukocyte differentiation antigens, the method was found suitable for ISH.[10]

### DNA Probes

Two probes, corresponding to the same *Bam*HI-W (3 kb and 3.1 kb respectively) internal repeat (IR1) of the EBV genome were used. One was obtained in the biotinylated form (dUTP-11-biotin) from a commercial source (Enzo Diagnostics Inc., NY). The other (kindly provided by Dr. G. W. Bornkamm) was subcloned in pBR 322, purified and labelled by nick translation using dATP-14-biotin (BRL, USA kit). The biotinylated probes recognizing the sequences of human papillomavirus 11 (HPV 11) and 18 (HPV 18) (Enzo Diagnostics Inc., NY) served as negative controls.

### In Situ *Hybridization*

The hybridizing conditions were described in detail in the previous report on 55 of these patients studied by ISH alone.[10] The signals obtained by this method were

strong, clear, and confined to the diagnostic cells by immunomorphology.[10] The effects of the ModAMeX procedure on nuclear components are not definitively known.[16] Initially, these signals were believed to be due to messenger RNA.[10] Further, investigations (using DNAse I and Ribonuclease A pretreatments) showed that with this protocol we detected mainly EBV DNA as well as some forms of EBV-related RNA.[17] However, in HD, the hybridization target seems to be EBV DNA since the transcription of EBV *Bam*HI-W sequence in HD has been questioned by several authors.[16] Positive and negative control tissues were the same as those used in our previous study.[10]

### *Immunohistochemistry with Anti-LMP and Anti-EBNA2 Antibodies*

Anti-LMP was available as a pool of four anti-LMP monoclonal antibodies (CS.1-4).[18] Anti-EBNA2 was available as a single reagent (PE2) reported to react with EBNA2A and EBNA2B antigens.[19] These antibodies were kindly provided by Dr. Alan B. Rickinson of the University of Birmingham, England, and Dakopatts, Copenhagen, Denmark. Double staining (i.e., immunostaining after ISH) was performed on randomly selected sections, from the batches of specimens of the ISH studies. Immunostaining was performed by the alkaline phosphatase–antialkaline phosphatase (APAAP) method.[20]

The anti-LMP antibodies were also tested on tissues at first diagnosis in 12/16 cases of relapse (NS, 3; MC, 9) because of the reactivity on our routinely fixed material.

### *Correlation with Clinical Data and Serology*

Previous analysis of clinical parameters and EBV serology was unselected for subtype.[10] Since the current patient population was larger, statistical methods were applied only to the MC subtype of HD, with respect to EBV positive and negative results. Details of EBV serology and clinical parameters were available in 53/55 patients of MC HD. Statistical analysis was performed with regard to the stage of the disease, response to treatment, and EBV serology by chi-2 test, with Yates correction for small samples. The median follow-up was 20 months (range, 14–126 months).

## *Results*

### In Situ *Hybridization*

The results of EBV nucleic acid detection on ModAMeX sections are shown in Table 1. Disregarding the subtype, a total of 37 of 107 (35%) tissues exhibited the presence of EBV nucleic acid material in the diagnostic cells of HD. The distribution of EBV nucleic acid positive cases according to subtype was as follows: LPn = 0/10 (0%); NS = 4/40 (10%); MC = 33/55 (60%); unclassified = 0/2. Thus, 33 of 37 (89%) EBV nucleic acid positive cases were of the MC subtype. In most EBV nucleic acid positive cases, virtually all H-RS cells were positive for ISH signals. The cells were easily visualized because of the complete absence of the background and the absence of signals in the surrounding small lymphocytes (Figure 1). Variability of ISH signals in H-RS cells was found in a small proportion (5/37) of cases. The signals ranged from few nuclear brown granules (Figure 1) to strong signals overlapping the nuclei (Figure 1). Nonrelevant probes (HPV 11 and HPV 18) excluded the possibility of a nonspecific signal.

### *Immunoreactivity with Anti-LMP Antibodies*

Anti-LMP antibody was found to be positive in 36 of 107 (33,6%) cases (Table 1). LMP-positive cells were detectable at low-power examination because of the strong cytoplasmic and membrane staining in most instances. Of the MC group, LMP was expressed by 31 of 33 (94%) cases which showed the presence of EBV nucleic acids (Table 1). Discordant results were obtained in three cases; two EBV nucleic acid positive cases were LMP negative and one EBV nucleic acid negative case was LMP positive. Anti-LMP reactivity was absent in the 36 of 40 cases of NS that were negative for EBV nucleic acids, but positive in all four EBV nucleic acid positive cases. The overall correlation of the presence of EBV nucleic acids and the expression of LMP was near unity although markedly skewed in favor of the MC subtype. Positive anti-LMP reactivity was restricted to H-RS cells (Figure 2). Significant numbers of large and atypical mononuclear

**Table 1.** *Hodgkin's Disease: Results of* In Situ *Hybridization by Nonisotopic EBV* Bam*HI-W Probes on ModAMeX Sections, and Immunostaining with Anti-LMP and Anti-EBNA2 antibodies*

| Types (Rye classification) | ISH +/tested | LMP +/tested | EBNA2 +/tested |
|---|---|---|---|
| Lymphocyte predominance | 0/10 | 0/10 | NT |
| Nodular sclerosis | 4/40 (10%) | 4/40 | 0/2 |
| Mixed cellularity | 33/55* (60%) | 32/55† | 0/11 |
| Unclassified | 0/2 | 0/2 | NT |
| Total | 37/107 | 36/107 | 0/13‡ |
| % | (34.6) | (33.5) | |

* Two cases positive for EBV nucleic acids were LMP negative.
† One case positive for LMP was EBV nucleic acids negative.
‡ All 13 EBV+/LMP+ cases.

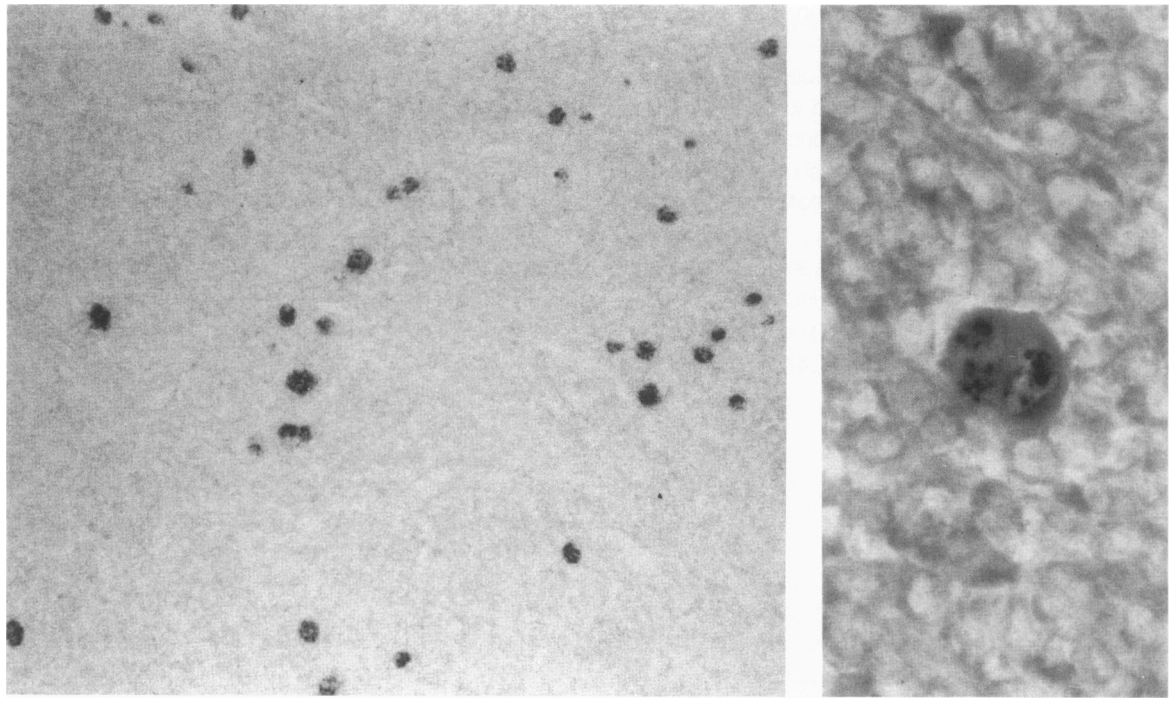

**Figure 1.** In situ *hybridization with EBV* Bam*HI-W probe on specially processed ModAMeX sections. At left, low-power magnification showing strong and clear signals, with complete absence of background staining, confined to the diagnostic cells as shown on the right (methyl green counterstain, ×200). At right, predominant nuclear localization of EBV nucleic acid hybridization signal (dark nuclear grains) in a diagnostic Reed–Sternberg cell. Note the absence of the signal in surrounding small lymphocytes (methyl green counterstaining, ×800).*

cells were labelled in addition to typical RS cells. Small lymphocytes in the background were negative.

Even though the anti-LMP antibodies have been studied on cryostat sections in the original report of Pallesen et al,[11] these antibodies were found to work on our routine fixative. The LMP positive reactions on routinely processed tissues were usually as strong as in ModAMeX processed material and interpretable without difficulties because of the clean background of the negative nonpathogenic cells. The correlation of EBV by ISH and LMP reactivity was further strengthened by findings in cases of relapse. In 12 of 16 cases of relapse (range 14–126 months), there were seven EBV nucleic acid positive (MC = 6, NS = 1) and five EBV nucleic acid negative (MC = 3, NS = 2) cases. Close agreement was found between LMP staining at the time of the first diagnosis and the presence of EBV in H-RS cells at relapse, in all cases tested. One EBV nucleic acid positive and LMP negative case at relapse showed strong positive staining for LMP in virtually all H-RS cells in the lymph node at first diagnosis, 10 years before relapse. In one other EBV nucleic acid positive case, only a few cells were positive for LMP at relapse, whereas virtually all H-RS cells were LMP positive in the original lymph-node biopsy.

An interesting observation was the tendency of EBV-positive cases to be richer in epithelioid cells and weaker in CD15 expression.

## *Immunoreactivity with Anti-EBNA2 Antibody*

The anti-EBNA2 antibody PE2 was tested in 13 (NS, 2; MC, 11) EBV nucleic acid positive cases. All were anti-EBNA2 negative. By contrast, 1 lymph node from a case of infectious mononucleosis showed scattered immunoblasts, the nuclei of which were strongly positive for EBNA2. EBNA2 was also detected in the two cases of non-HD lymphomas that were EBV nucleic acid positive and LMP positive.

## *Clinical and Serologic Correlations in the MC Subtype*

No differences were found as to the stage, response to chemotherapy, diseasefree survival, relapse, and death rate in the MC subtype, between EBV nucleic acid positive and EBV nucleic acid negative cases. The median follow-up (20 months) is short and longer follow-up is needed. Differences might emerge in the further short term in the nine MC cases of relapse. Six of these were EBV nucleic acid-positive and three were EBV nucleic acid negative by current ISH in the tissues at first diagnosis.

No significant differences between the two groups

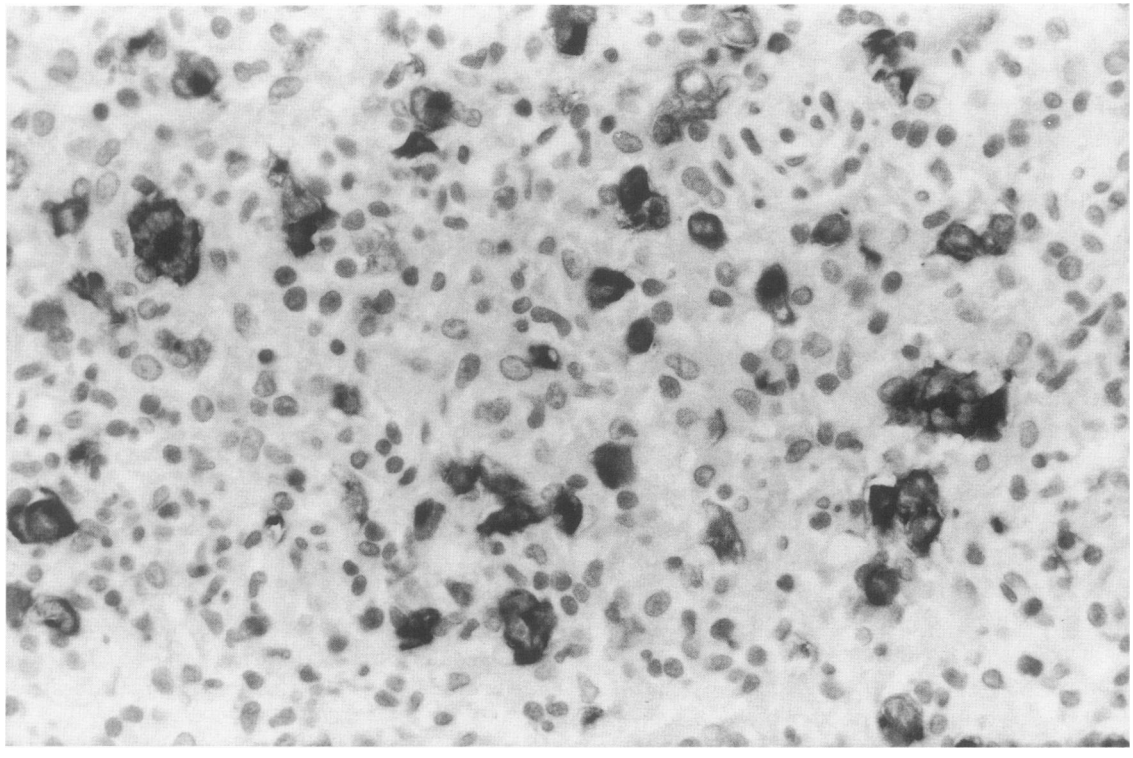

**Figure 2.** *Immunostaining with anti-LMP antibody of several Reed–Sternberg cells and mononuclear variants. In most cases the labelling was comparable to the number of positive cells observed by* in situ *hybridization with EBV* Bam*HI-W probes (APAAP with hematoxylin counterstaining, ×500).*

were observed in EBV serologic profiles. Majority of the patients presented with serologic profiles consistent with a past infection.

## *Discussion*

Several methods are described for the detection of the presence of EBV in HD: dot blot[6] and Southern blot analysis,[2–6] DNA amplification,[7] ISH with isotopic probes,[2,4,8,9] or nonisotopic probes,[10] and immunohistochemistry with anti-LMP antibodies.[7,11] In comparing the various detection rates by subtype, the results of dot blot and Southern blot analysis are similar.[6] However, the cold ISH method offers superiority for localization of EBV genomic material over isotopic probes because the latter often produces crude, poorly interpretable signals and high background.[6] The results by PCR analysis[5–7] can be criticized for overestimating the presence of EBV almost by a factor of two, without a clear difference in the various subtypes. The PCR technique may amplify DNA from the reservoir of nonpathogenic cells. The detection rate in LPn is 50% by PCR in two reports,[5,6] whereas the rate is closer to zero by ISH, Southern blot and LMP immunostaining in others.[10,11] The finding by Masih et al[6] of EBV nucleic acids in 17% and 34% in LP diffuse and 30% and 83% in LD by Southern blot and PCR, respectively, is similarly surprising since these subtypes can usually be reclassified as either LPn or MC, or NS by immunomorphologic criteria.[14,15,21] Considering LMP expression alone, the percentage of positive cases in the present report is lower than that of Pallesen et al[11] but similar to that of Herbst et al[7] (38% vs. 35%). In this and other studies, the highest frequency of EBV genomic material was found in cases of MC HD (60–96%)[7,10,11] whereas the association of EBV with NS HD,[3,10,11] (10–33%) is much weaker in other reports. It is uncertain if these variations are reflections of different criteria for the diagnosis of the subtypes.

The immunohistochemical assessment of LMP expression differs from ISH with *Bam*HI-W probes, in that LMP is encoded and transcribed from a different genomic sequence.[22] Nevertheless, a correlation of 91% between ISH with cold probes and the expression of LMP signifies parallel sensitivities of the two methods and the transcriptional activity of the EBV genome. This strong correlation was further borne out by the cases at relapse. Both ISH signals and LMP staining were restricted to the phenotypically characteristic H-RS cells. LMP may have been either below the detection threshold, or not synthesized, in the two cases that were EBV-positive by ISH.

Such discrepancies, between the presence of EBV DNA detected by PCR analysis and the lack of LMP immunostaining have been previously noted.[7] The cases of relapse also suggest that, in some, LMP expression may vary during the course of the disease. Because of the strong correlation of EBV presence and LMP expression, and the major restriction of EBV to a subtype such as MC, the view that the virus is nonspecifically derived from surrounding lymphoid cells[6] needs reevaluation.

The nonexpression EBNA2 in HD confirms and extends earlier reports.[7,11] By contrast, in one case of infectious mononucleosis and two cases EBV containing nonHodgkin's lymphoma, both LMP and EBNA2 were expressed. Thus, the LMP positive and EBNA2 negative phenotype of latent infection, appears to be the usual finding in H-RS cells.[7,11] We were also unable to find antigens expressed by EBV-producer cells, e.g., viral capsid antigen (EBV VCA), early antigen (EBV-EA-R) and late membrane antigen (MA) on H-RS cells, in a limited number of EBV positive cases tested by antibodies against these antigens (data not shown).

The detection of homogeneous episomal population of EBV by Southern blot using XhoI probe[4,8] suggests that there may be clonal expansion of a single EBV-infected H-RS precursor cell similar to the occurrence in Burkitt's lymphoma[23,24] and nasopharyngeal carcinoma.[23] Alternatively, as suggested by Raab-Traub and Flynn,[23] a single episome may be preferentially amplified in a progenitor of H-RS cells. The expression of LMP exclusively by EBV containing H-RS cells is evidence for functional activity of EBV in H-RS cells. LMP is reported to behave as a transforming oncogene for established rodent fibroblast cell lines[25] and may be similar to activated ras proteins.[26] LMP could inhibit terminal differentiation of lymphoid cells and promote their neoplastic state, similar to events in epithelial cells.[26] The proliferation of H-RS cells could be facilitated by the absence of EBNA2, which in addition to LMP, is of importance for T-cell immunosurveillance.[27] In the present study, no statistically significant correlation was found with EBV-positive and EBV-negative mixed cellularity Hodgkin's disease groups with regard to clinical presentation, stage, therapeutic response, relapse and EBV serology, thus confirming our previous findings.[10] It is, however, important to note that only cases with available ModAMeX-processed biopsy specimens were included in this study and that this procedure has been only recently developed (since 1987) thus precluding long-term follow-up at the present time. Since the anti-LMP antibodies were found to be suitable for use after routine fixatives, retrospective studies on a large population may better evaluate the clinical implications. Although the use of anti-LMP antibodies is technically simpler for identifying EBV-associated HD, both ISH and LMP staining should be used where possible. The present study and our previous results suggest that EBV plays a significant role in the development of a proportion of the cases of the MC subtype of HD but other etiologic agents (possibly viral) or mechanisms need to be considered, particularly for NS lesions.

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