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Detection of Epstein-Barr Virus in Multiple Sites Involved by Hodgkin's Disease

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Tissues obtained from 14 patients with multiple anatomic sites involved by Hodgkin's disease were studied for Epstein-Barr virus (EBV) using **in situ** ***hybridization for EBV-encoded RNA (EBER) 1 and immunohistochemical methods for EBV latent membrane protein (LMP) expression. Each patient in this study had two to five separately involved anatomic sites, and all biopsy sites, a total of 43 specimens, were analyzed for EBV. EBV was detected in 6 of 14 (42.8%) patients with Hodgkin's disease, including 5 of 11 (45.4%) with nodular sclerosis and 1 of 3 (33%) with mixed cellularity. In these six patients, all biopsy sites were positive for both EBER1 and LMP. In the EBV-positive cases we analyzed the 3′-end of the EBV LMP1 gene in all sites of disease using polymerase chain reaction. In three patients all sites of disease had a 30-base pair deletion. In two patients, there was discordance between sites of disease, with LMP1 gene deletions in some sites and other sites with the LMP1 gene in the germline configuration. The results of this study demonstrate that EBV, when found in Hodgkin's disease, is detectable in all anatomic sites involved. The presence of the same 30-base pair deletion in the EBV LMP1 gene in all sites of disease in three patients suggests that the deletion occurred before dissemination and that all sites are clonally related. However, the discordance between anatomic sites in two patients suggests that LMP1 gene deletion may also occur as a later event, after dissemination. These results lend further support to the hypothesis that EBV plays a role in the pathogenesis of a subset of cases of Hodgkin's disease. (Am J Pathol 1995, 147:1408–1415)***

For many years an association between Epstein-Barr virus (EBV) and Hodgkin's disease has been known. For example, EBV is known to cause infectious mononucleosis and, after this illness, patients have an increased risk of Hodgkin's disease.[1] Patients with Hodgkin's disease also have high antibody titers specific for EBV antigens,[2] and elevated antibody titers have been shown in patients preceding the diagnosis of Hodgkin's disease.[3] More direct evidence demonstrating EBV in Hodgkin's disease has been established using molecular techniques,[4] such as Southern blot hybridization, polymerase chain reaction (PCR), and *in situ* hybridization.[5–9] Southern blotting, in addition to demonstrating the presence of EBV, has shown that the virus is monoclonal, indicating that EBV is likely to be present before neoplastic transformation. *In situ* hybridization has localized EBV DNA and RNA within the Reed-Sternberg (R-S) cells and mononuclear variants (Hodgkin cells) of Hodgkin's disease. In summary, these studies suggest that EBV plays a role in the pathogenesis of Hodgkin's disease. The expression of EBV-latent membrane protein by R-S cells and Hodgkin cells, as demonstrated immunohistochemically,[10] is additional evidence suggesting that EBV is involved in the pathogenesis of a subset of cases of Hodgkin's disease.

Presently, most studies of EBV in Hodgkin's disease have analyzed one biopsy specimen per patient. To further substantiate the role of EBV in Hodgkin's disease, we reasoned that the study of multiple anatomic sites of disease in a given patient might be revealing. If the virus were present in all biopsy sites, its role in pathogenesis of Hodgkin's disease would be further supported. Therefore, for this study we collected the cases of 14 patients with Hodgkin's disease who had multiple anatomic sites

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of disease and assessed these sites for EBV. Our results indicate that EBV, when found in Hodgkin's disease, is present in all sites of disease, further supporting the hypothesis that EBV is involved in the pathogenesis of this subset of cases of Hodgkin's disease.

Materials and Methods

Fourteen patients with Hodgkin's disease, each with two to five separately involved anatomic sites, were evaluated for EBV. A total of 43 biopsy specimens were studied. All cases had been diagnosed as Hodgkin's disease, using standard histological criteria,[11] at Stanford University Medical Center between the years 1983 and 1990.

Immunohistochemical Studies

Immunohistochemical studies were performed on all biopsy specimens using formalin-fixed, paraffin-embedded tissue sections and previously published methods.[12] The antibodies used included: Leu M1 (CD15) (Becton-Dickinson, San Jose, CA), Ber H2 (CD30) (Dako, Carpinteria, CA), and CS1–4 reactive with EBV-latent membrane protein (LMP) (Dako). The CD15 and CD30 antibodies were used at a dilution of 1:10 to 1:15 and 1:20 to 1:30, respectively. After heat-induced epitope retrieval and enzymatic digestion, the CS1–4 antibody was used at a dilution of 1:25 to 1:50. Reactivity was detected using avidin-biotin technique and 3′,3′ diaminobenzidine-tetrahydrochloride dihydrate as the chromogen (Biotek, Santa Barbara, CA).

In Situ *Hybridization*

The EBV RNA *in situ* hybridization studies were performed using a 30-base oligonucleotide complementary to a portion of the EBV-encoded RNA1 (*EBER1*) gene, a region of the EBV genome that is actively transcribed (up to 10^7 copies per cell) in latently infected cells.[8,13] The oligonucleotide was biotinylated using methods previously described.[14] The procedure used for these studies has been described elsewhere.[14] Briefly, the slides were deparaffinized, dehydrated, predigested with pronase, prehybridized, and hybridized overnight at a concentration of 0.25 ng/μl of probe. After washing, detection was accomplished using avidin-alkaline phosphatase conjugate followed by development of the signal with McGadey's substrate. A brown or blue-brown color in the nucleus over background levels was considered a positive reaction. This methodology detected EBV RNA from the EBV-infected Raji cell line, but not from the non-EBV infected T-cell line Molt 3. In addition, lymphoid tissue from an EBV-seronegative patient and tissues infected with herpesvirus I, papillomavirus 16, and adenovirus showed no cross-reactivity. A known EBV-positive neoplasm served as a positive control and EBV-negative lymphoid tissue served as a negative control in each run. Any slide negative for EBV RNA was tested for preservation of total cellular RNA using a poly d(T) probe as described elsewhere by us.[15]

PCR Studies

Genomic DNA was extracted from 5 μm sections cut from formalin-fixed, paraffin-embedded tissue blocks of the EBV-positive neoplasms (cases 1 to 6) using standard methods. The PCR was then used to amplify the 3′ end of the *EBV-LMP1* gene, the region of the gene that has been reported to be deleted in a subset of cases of Hodgkin's disease.[16] Two 20-base oligonucleotide primers flanking the site of the characteristic 30-bp deletion were used: 5′-CGG-AAG-AGG-TGG-AAA-ACA-AA-3′ and 5′-GTG-GGG-GTC-GTC-ATC-ATC-TC-3′. Each reaction was performed with 2 μl of extracted DNA in a 50 μl mixture containing 10 pmol of each primer, 0.2 mmol/L each deoxynucleotide triphosphate; 1.5 mmol/L Mg^{2+}, and 2.5 units of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). After an initial denaturation for 5 minutes at 94°C, 35 cycles were performed as follows: 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute. A final extension at 72°C for 7 minutes completed the PCR amplification. The amplified products were electrophoresed in 7% polyacrylamide gels with $\phi\chi$ 174-puc 19/Hae III size markers and visualized with ethidium bromide. Using this method, the amplified germline product of the 3′ end of the *EBV-LMP* gene is 161 bp, while a product containing the characteristic deletion is 131 bp. The results were confirmed by slot-blot hybridization using a P^{32}-labeled internal probe that is specific for the deleted segment (and thus hybridizes against the germline but not deleted *LMP* gene) (5′-GCC-GTC-ATG-GCC-GGA-ATC-AT-3′), as well as a probe that is specific for a region flanking the deleted segment (and thus hybridizes against both the germline and deleted *LMP* genes) (5′-GGC-GGG-CCC-TGG-TCA-CCT-CC-3′).[16]

Table 1. *Summary of Results*

Case	Age/Sex	Type	Sites	EBER 1	LMP	3′ LMP1 gene segment size
1	30/F	NS	Axillary LN*	Positive	Positive	131 bp
			Celiac LN	Positive	Positive	131 bp
			Para-aortic LN	Positive	Positive	131 bp
			Splenic LN	Positive	Positive	131 bp
			Spleen	Positive	Positive	131 bp
2	22/F	NS	Splenic LN	Positive	Positive	131 bp
			Spleen	Positive	Positive	131 bp
3	49/M	MC	Celiac LN	Positive	Positive	161 bp
			Splenic LN	Positive	Positive	161 bp
			Spleen	Positive	Positive	161 bp
			Liver	Positive	Positive	131 bp
4	39/F	NS	Portal LN	Positive	Positive	131 bp
			Splenic LN	Positive	Positive	131 bp
			Spleen	Positive	Positive	131 bp
5	27/M	NS	Splenic LN	Positive	Positive	131 bp
			Spleen	Positive	Positive	161 bp
6	32/F	NS	Splenic LN	Positive	Positive	Not amplified
			Spleen	Positive	Positive	Not amplified
7	23/M	MC	Iliac LN	Negative	Negative	ND
			Spleen	Negative	Negative	ND
8	34/F	NS	Cervical LN*	Negative	Negative	ND
			Para-aortic LN	Negative	Negative	ND
			Porta hepatic LN	Negative	Negative	ND
			Splenic LN	Negative	Negative	ND
			Spleen	Negative	Negative	ND
9	43/M	NS	Lung†	Negative	Negative	ND
			Cervical LN†	Negative	Negative	ND
10	26/M	NS	Splenic LN	Negative	Negative	ND
			Spleen	Negative	Negative	ND
			Liver	Negative	Negative	ND
11	8/M	MC	Cervical LN*	Negative	Negative	ND
			Porta hepatic LN	Negative	Negative	ND
			Spleen	Negative	Negative	ND
12	28/M	NS	Cervical LN*	Negative	Negative	ND
			Spleen	Negative	Negative	ND
			Liver	Negative	Negative	ND
13	32/M	NS	Splenic LN	Negative	Negative	ND
			Spleen	Negative	Negative	ND
14	34/M	NS	Splenic LN	Negative	Negative	ND
			Celiac LN	Negative	Negative	ND
			Spleen	Negative	Negative	ND
			Accessory spleen	Negative	Negative	ND
			Liver	Negative	Negative	ND

F, female; M, male; *initial biopsy site; †recurrent site. All other biopsy sites obtained at the time of staging laparatomy. LN, lymph node; NS, nodular sclerosis; MC, mixed cellularity; bp, base pairs; ND, not done.

Results

The patient age, sites of disease studied, and histological subtype of Hodgkin's disease are summarized in Table 1.

The age of the patients ranged from 8 to 49 years with a median age of 31 years. Most patients presented with lymphadenopathy, most commonly of the cervical or supraclavicular regions. Two patients had palpable splenomegaly. In nine patients all specimens were obtained at staging laparatomy, in four patients the initial biopsy site and staging specimens were assessed, and in one patient two sites of recurrent disease were analyzed. The sites biopsied included 24 lymph nodes (perisplenic 10, cervical 4, porta hepatis 3, celiac 3, para-aortic 2, axillary 1, and iliac 1), 13 spleen, 1 accessory spleen, 4 liver, and 1 lung.

The Hodgkin's disease was classified as nodular sclerosis in 11 patients or mixed cellularity in three patients. At all sites of disease in each patient the histological findings were similar. In all biopsy specimens, the R-S and Hodgkin (H) cells were positive for Ber H2 (CD30); in 12 of 14 tumors the RS and H cells were also positive for Leu M1 (CD15). These results support the diagnosis of Hodgkin's disease.[17] All R-S and H cells stained by Leu M1 and Ber H2 showed a membranous and perinuclear ("Golgi") pattern of staining.

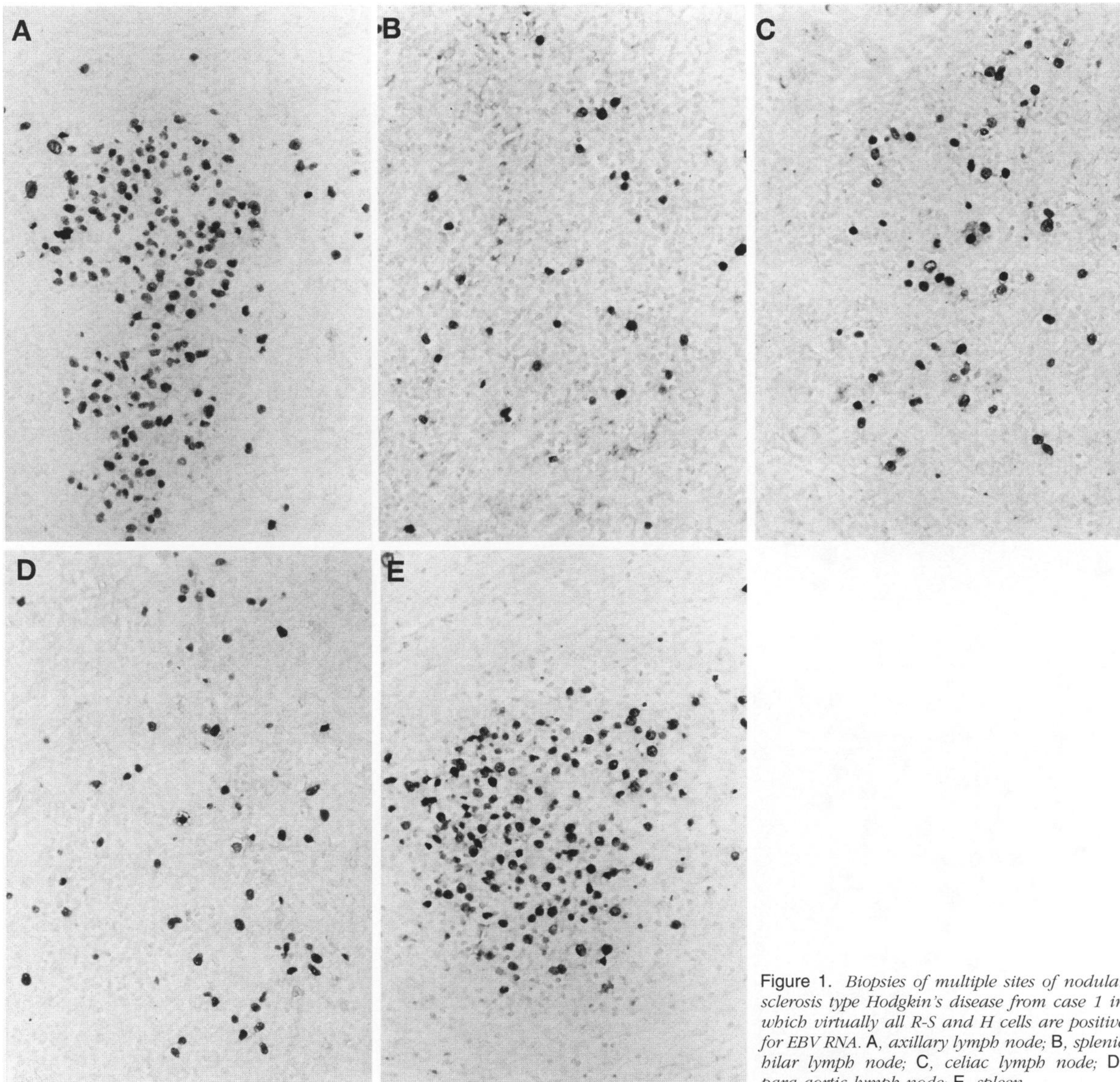

Figure 1. *Biopsies of multiple sites of nodular sclerosis type Hodgkin's disease from case 1 in which virtually all R-S and H cells are positive for EBV RNA.* A, *axillary lymph node;* B, *splenic hilar lymph node;* C, *celiac lymph node;* D, *para-aortic lymph node;* E, *spleen.*

EBV was detected in 6 of 14 (42.8%) cases studied; 1 of 3 (33%) cases of mixed cellularity and 5 of 11 (45.4%) cases of nodular sclerosis were positive (Figure 1, A–E). All EBV-positive cases showed abundant hybridization of EBER1 in virtually all R-S and H cells. All cases positive by *in situ* hybridization also expressed EBV LMP with a membranous and paranuclear pattern of staining (Figure 2, A–E). More than 75% of the R-S and H cells in each biopsy specimen were positive for EBV LMP. Whenever EBV was detected in a case of Hodgkin's disease, the virus was detected in all anatomic sites involved by disease, both in tissues obtained at the time of the staging laparatomy and in the initial biopsy site (when available). In the patient with recurrent Hodgkin's disease, involving two different anatomic sites separated by a two year interval, both biopsy specimens were negative for EBV.

In five of six EBV-positive cases, DNA was amplified by PCR (Figures 3 and 4). In cases 1, 2, and 4, all sites of disease had an identical 30-bp deletion in the 3′ end of the *EBV-LMP1* gene. In contrast, in cases 3 and 5 there were discordant amplified products. In case 3, the *EBV-LMP1* gene was in the germline configuration in the celiac and splenic lymph nodes, and the spleen but the EBV-LMP1 gene in the liver had a 30-bp deletion. In case 4, in the spleen the EBV-LMP1 gene was germline but the EBV-LMP1 gene in the splenic lymph node exhibited a 30-bp deletion. The DNA in case 6 was degraded and could not be analyzed. In all specimens, the 161-bp fragment hybridized with both the probe spe-

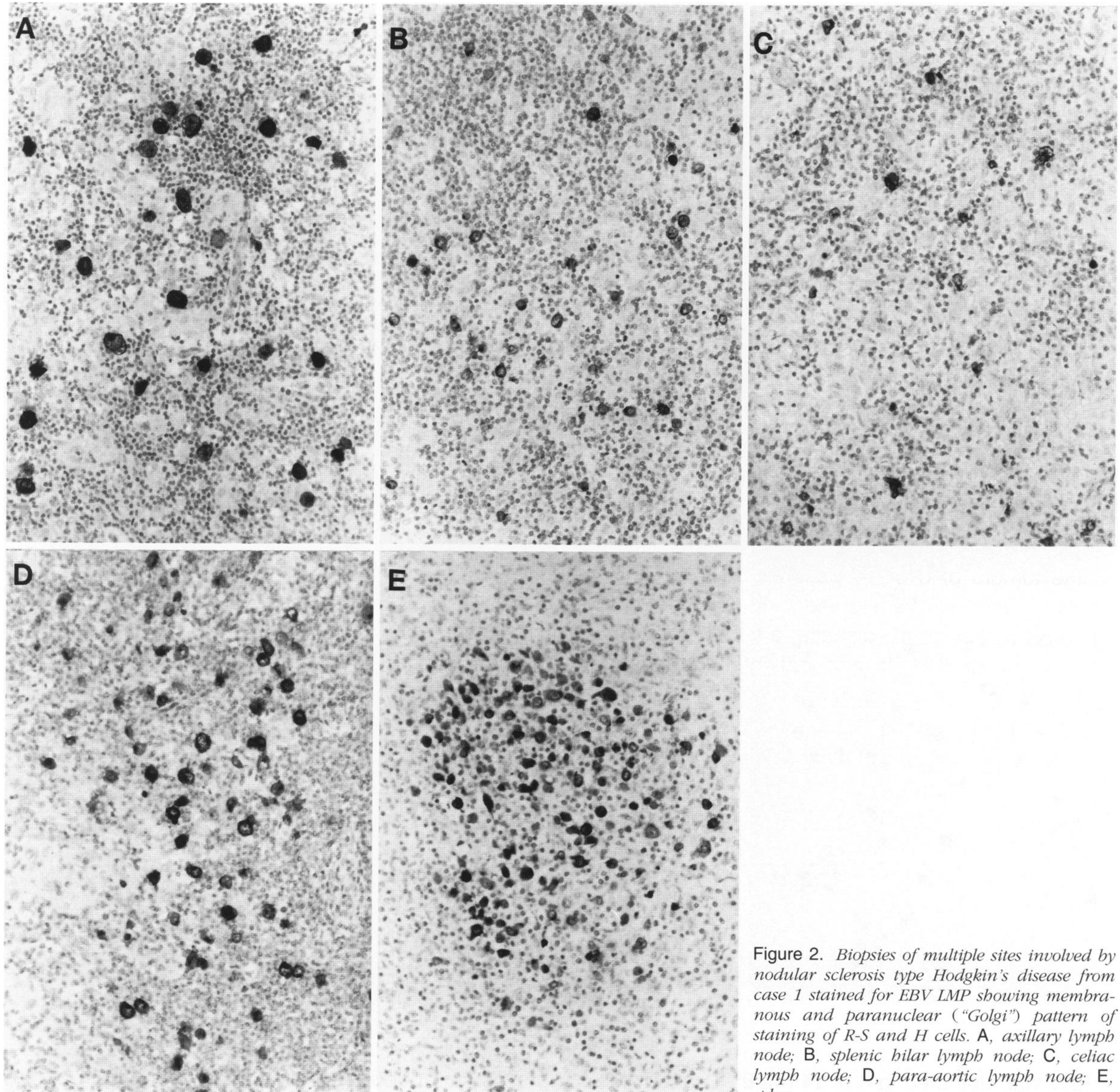

Figure 2. *Biopsies of multiple sites involved by nodular sclerosis type Hodgkin's disease from case 1 stained for EBV LMP showing membranous and paranuclear ("Golgi") pattern of staining of R-S and H cells.* A, *axillary lymph node;* B, *splenic hilar lymph node;* C, *celiac lymph node;* D, *para-aortic lymph node;* E, *spleen.*

cific for the deletion segment and the probe from the flanking segment in slot-blot studies (data not shown). In contrast, the 131-bp fragment only hybridized with the flanking segment probe and did not hybridize with the probe specific for the deletion segment, confirming the absence of the deletion segment as the reason for the lower molecular weight product.

Discussion

EBV is known to be present in a significant proportion of cases of Hodgkin's disease. Direct demonstration of EBV DNA in biopsy specimens involved by Hodgkin's disease was first reported in 1987.[4] *In situ* hybridization and immunohistochemical studies subsequently demonstrated that EBV DNA, RNA, and proteins are localized in R-S and H cells.[5–8,9,18–21] These observations suggest that EBV infection plays a role in the pathogenesis of a subset of cases of Hodgkin's disease.

Most studies that have analyzed EBV in Hodgkin's disease have studied one involved anatomic site per patient. Few studies have assessed patients with multiple sites involved by Hodgkin's disease.[22–24] In this study we analyzed 14 patients with multiple an-

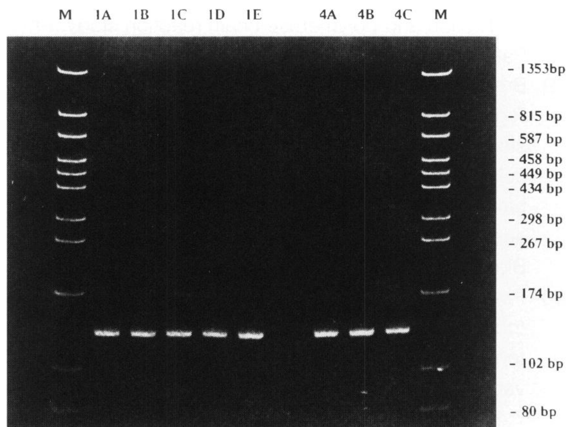

Figure 3. *PCR amplification of the 3′ end of the* EBV-LMP1 *gene. In cases 1 and 4, all anatomic sites involved by Hodgkin's disease contain the characteristic 30-bp deletion in the* EBV-LMP1 *gene.* **M**, *size markers* **1A**, *axillary lymph node;* **1B**, *splenic hilar lymph node;* **1C**, *spleen;* **1D**, *para-aortic lymph node;* **1E**, *celiac lymph node;* **4A**, *spleen;* **4B**, *splenic hilar lymph node;* **4C**, *portal lymph node.*

atomic sites, a total of 43, involved by Hodgkin's disease for EBER1 and EBV LMP. EBV was identified in the tumors of 6 of 14 patients. In the six EBV-positive cases, EBV was present in all anatomic sites involved by Hodgkin's disease, a total of 18 biopsy specimens studied. In one patient, EBV was detected in the initial biopsy site and in multiple involved sites obtained at the time of staging laparatomy. In five cases, EBV was detected in all sites of disease sampled at the time of staging laparatomy.

In the six EBV-positive cases of Hodgkin's disease, we analyzed the 3′ end of the *EBV-LMP1* gene for deletions using a PCR method. This region of the gene has been reported to contain deletions in a subset of cases of Hodgkin's disease.[16] In three

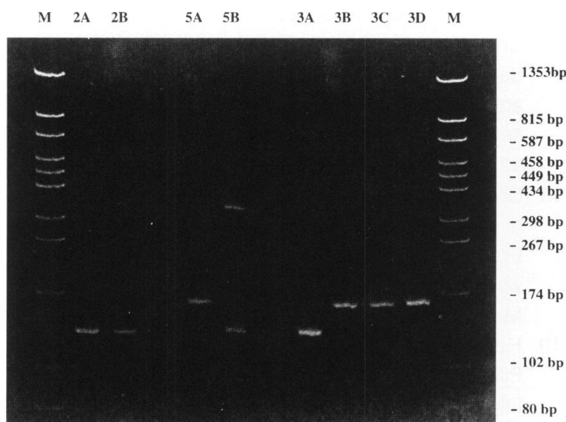

Figure 4. *PCR amplification of the 3′ end of the* EBV-LMP1 *gene. In case 2, both anatomic sites involved by Hodgkin's disease contain the characteristic 30-bp deletion in the* EBV-LMP1 *gene. In cases 3 and 5, anatomic sites of Hodgkin's disease contain either deleted or germline EBV-LMP1 genes.* **M**, *size markers;* **2A**, *splenic hilar lymph node;* **2B**, *spleen;* **5A**, *splenic hilar lymph node;* **5B**, *spleen;* **3A**, *liver;* **3B**, *celiac lymph node;* **3C**, *splenic hilar lymph node;* **3D**, *spleen.*

patients, the *EBV-LMP1* gene had identical 30-bp deletions in all sites of disease. These results suggest that in these patients, all anatomic sites involved by Hodgkin's disease contained the same *EBV-LMP1* gene, supporting the hypothesis that all sites were involved by the same neoplastic clone. These results also suggest that the *EBV-LMP1* gene deletion preceded dissemination. In two cases, however, there were discordant EBV findings. In cases 3 and 5, one or more anatomic sites contained the germline *EBV-LMP1* gene, whereas one site had a 30-bp deletion in the *EBV-LMP1* gene. These results suggest that deletions in the *EBV-LMP1* gene also may occur as a later event in the pathogenesis of Hodgkin's disease, after dissemination of disease. Knecht et al[25] found an incidence of *LMP1* deletions of 10%, with a suggestion that the *LMP1* deletions were preferentially found in cases with more aggressive histological features. The higher incidence of *LMP1* gene deletions in our study than in the study of Knecht et al[25] may be due to the selection of higher stage cases in our series.

Our results support and expand the observation of others. Coates et al[22] studied 55 cases of Hodgkin's disease using *in situ* hybridization and an EBV DNA probe. EBV was localized in the R-S and H cells of 9 of 55 (16.3%) cases. Furthermore, six of the EBV-positive tumors had more than one site of disease; all sites were positive. Similarly, Boiocchi et al[23] described two human immunodeficiency virus (HIV)-positive men with Hodgkin's disease, each with metachronous involvement of multiple anatomic sites. In both patients, EBV was present in all sites analyzed. Furthermore, Southern blot hybridization using EBV terminal repeat region probes showed that the EBV episomal DNA was monoclonal, and the same-sized episome was present in all sites in each patient. Brousset et al[24] also studied cases of EBV-positive Hodgkin's disease that had relapsed. In one case studied by Southern blotting with EBV terminal repeat region probes, both the original diagnostic biopsy specimen and two subsequent relapse specimens contained the same size EBV episome. In addition, in two tumors, analysis of *EBV-LMP1* gene revealed identical deletions in both the diagnostic and relapse specimens of Hodgkin's disease.[24]

The consistent detection of EBV in multiple anatomic sites of disease in an individual patient with EBV-positive Hodgkin's disease, as well as the demonstration of identical deletions in the EBV-LMP1 gene in multiple sites in a subset of patients, strongly supports the hypothesis that EBV is involved in the pathogenesis of this subset of cases of Hodgkin's disease. Had EBV been identified in R-S and H cells

in one or more but not all sites, it would have implied that infection occurred after or at the time of dissemination. The presence of EBV at all sites of involvement in EBV-positive cases implies that infection occurred at an early stage of lymphomagenesis, before spread from one site to another. The consistent positivity of virtually all R-S and H cells in each positive site is consistent with the hypothesis that EBV is present before clonal expansion. The finding of the same 30-bp deletion in the *EBV-LMP1* gene at all sites of disease in three cases further suggests that these sites are clonally related and the *LMP1* gene deletion preceded dissemination. However, the absence of *EBV-LMP1* gene deletions in some anatomic sites in two of the patients in this study suggests that *EBV-LMP1* gene deletions may also occur as a later event, after dissemination.

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