Association of the Subtype 2 of the Epstein-Barr Virus With T-Cell Non-Hodgkin’s Lymphoma of the Midline Granuloma Type

By Bettina Borisch, Ivo Hennig, R. Hubert Laeng, Ernst R. Waelti, Rainer Kraft, and Jean Laissue

Lethal midline granuloma (LMG) is associated with Epstein-Barr virus (EBV). The latter has at least two subtypes with different biological properties. The subtypes can be identified by their genomic configuration. Using EBV-RNA (EBER) in situ hybridization and EBV polymerase chain reaction (PCR), we have looked for the presence of EBV in six LMGs and six non-Hodgkin’s lymphomas (NHLs) located in the nasopharyngeal region, and determined the subtype of EBV. Six of six LMGs were positive by PCR and EBER in situ hybridization, whereas NHLs were either negative or in three of six cases, showed few EBER-positive cells considered to be nonneoplastic lymphocytes. The subtype 2 was found in LMG lesions of three of six patients; the remaining three of six patients with LMG had the generally occurring subtype 1. The results indicate that the association of EBV with NHL may depend more on tumor type than on its localization. The occurrence of the rare subtype 2 in LMG may relate to a covert immune defect.

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MATERIALS AND METHODS

Case Selection

Twelve lymphomas of the oropharynx, hypopharynx, or epipharynx and upper respiratory tract were selected from the files of the Institute of Pathology, Bern (Table 1). Six T-cell lymphomas fulfilled the following morphological and clinical criteria of LMG: (1) nasal and midfacial involvement by pleomorphic lymphoma, displaying angiocentricity, angioinvasion, large areas of necrosis, and severe inflammatory reaction in many cases (Fig 1A and B); (2) a clinically and histologically documented initial phase characterized by an inflammatory pattern (the follow-up time was between 1 and 9 years); (3) a T-cell phenotype, but sometimes with partial loss of some T-cell antigens (Figs 2 and 3).

The other six NHLs, high-grade B- or T-cell lymphomas, were classified according to the updated Kiel classification and the International Working Formulation.

Immunophenotypic Analysis

Each of the 12 lymphomas was immunophenotyped using paraffin-embedded sections of formalin-fixed tissues and the following antisera: L26 (CD20), UCHL1 (CD45RO), CD3 (Dako A452), ODP4 (CD45RO), and antibody Cs 1-4 directed against the latent membrane protein (LMP) of EBV. All antibodies were obtained from Dako (Copenhagen, Denmark). An avidin-biotin method was used with peroxidase and dianaminobenzidine (DAB). Fresh-frozen material was not available. Therefore, more detailed immunophen-
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Table 1. Summary of Clinical and Histologic Features: Results of Immunophenotyping, EBER In Situ Hybridization, and PCR Studies

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age/Sex</th>
<th>Site</th>
<th>Histology</th>
<th>Phenotype</th>
<th>EBER</th>
<th>EBV-PCR</th>
<th>EBV Subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25/M</td>
<td>Endonasal, LN, cervical</td>
<td>LMG</td>
<td>T</td>
<td>+</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>10/M</td>
<td>Endonasal</td>
<td>LMG</td>
<td>T</td>
<td>+</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>41/M</td>
<td>Tonsil, nose, sinus</td>
<td>LMG</td>
<td>T</td>
<td>+</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>19/M</td>
<td>Nose</td>
<td>LMG</td>
<td>T</td>
<td>+</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>30/M</td>
<td>Hypopharynx</td>
<td>LMG</td>
<td>T</td>
<td>+</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>39/M</td>
<td>Epipharynx</td>
<td>IWF: H (Kiel: ib)</td>
<td>T</td>
<td>+t</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>69/M</td>
<td>Pharynx, upper respiratory tract</td>
<td>IWF: H (Kiel: ib)</td>
<td>Non-B, non-T</td>
<td>+t</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>69/M</td>
<td>Epipharynx</td>
<td>IWF: H (Kiel: ib)</td>
<td>Non-B, non-T</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>75/M</td>
<td>Oropharynx</td>
<td>IWF: I(Kiel: ib)</td>
<td>T</td>
<td>+t</td>
<td>-*</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>33/M</td>
<td>Nose, mediastinum</td>
<td>IWF: I (Kiel: ib)</td>
<td>T</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>70/F</td>
<td>Tonsil</td>
<td>IWF: G (Kiel: cb polym.)</td>
<td>B</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: LN, lymph node; ib, immunoblastic non-Hodgkin's lymphoma; lb, lymphoblastic non-Hodgkin's lymphoma; cb, polym., centroblastic non-Hodgkin's lymphoma, polymorphic subtype; IWF, International Working Formulation (G, diffuse, large noncleaved cell; H, large-cell, immunoblastic, including clear cell; I, lymphoblastic, convoluted cell/nonconvoluted cell), Kiel, Kiel classification; -, negative; +, positive; 0, could not be typed.

* House-keeping gene (β-globulin)-negative in PCR.
† EBER positivity is confined to few small lymphocytes.

nototypic studies and/or demonstration of clonal gene rearrangements, especially of the TCR genes could not be performed. Thus, a NK phenotype (CD56+, CD2-) could not be established, since all available antibodies are not reactive in paraffin-embedded, formalin-fixed tissues, even after microwave treatment.

**In Situ Hybridization**

The methods have been described in detail in a previous study.23-25 Plasmid and probes. Genes for RNAs to be analyzed were cloned into SP6 vectors. The pJJ1 vector, used for EBER1, was digested with MstII/PvuII, and the fragment containing 89% of the EBER1 gene was treated with Klenow fragment and ligated into pSP65 digested with Smal. After determining the orientation, the pSP65/EBER1 plasmids (both orientations, sense and antisense) were digested with BamHI and transcribed to obtain RNAs of 175 nt (83% EBER1-specific). The RNA was labeled with digoxigenin-UTP using the RNA Labeling Kit (SP6/T7) from Boehringer Mannheim (Mannheim, Germany). Previously prepared probes and labeled probes were obtained from Professor H. Wolf, Regensburg, Germany.

Technique. In situ hybridization was performed on formalin-fixed, paraffin-embedded tissues according to the method of Macmahon et al.24 Hybrids were detected by use of the Boehringer Mannheim digoxigenin system. The sections were mounted without counterstaining.

Controls, interpretation. Sections of formalin-fixed, paraffin-embedded EBV-positive Raji and B95.8 cells and of EBV-negative cells were used as positive and negative controls, respectively. Sections of EBV-positive lymphomas (as shown by previous Southern blot hybridization, PCR, and in situ hybridization25-28) were included in each batch of experiments. Hybridization with the digoxigenin system (Boehringer Mannheim) and nitroblue tetrazolium (NBT)-X-phosphate produces dark bluish-brown positive signals. Positive controls displayed a predominantly nuclear label, sometimes with visible exclusion of the nucleolus. All specimens that contained cells with a comparable hybridization signal were scored positive for EBER if the hybridization result with control probes (ie, EBER1-sense) was negative.

**PCR**

The paraffin block sections were processed and PCR was performed as described previously.23 Primers glycoprotein (gp)220 1 and gp220 2 were used together with an appropriate probe; the primers are specific for the gp220 region of the EBV genome. To define the subtypes of EBV, primers EBNAl2 gen 1 and gen 2 were used as the outer primers in a seminested reaction with subtype-specific primers as the next step; each time, probes were applied to confirm the results. Primers, probes, and their sequences are listed in Table 2. As control for the PCR, the β-globulin gene was amplified in each case.25 Only cases with positive β-globulin sequences were used for further EBV-PCR. Negative controls (water substituted for template DNA) were performed with each experiment. After Southern blot and hybridization with the appropriate end-labeled probes, the detection of positive bands was performed using the digoxigenin system. Samples of two cases of undifferentiated nasopharyngeal carcinomas and tonsilar tissue (two cases with infectious mononucleosis, three hyperplastic tonsils) were used as controls.

**RESULTS**

The results are summarized in Tables 1 and 3. EBV-DNA was amplified by PCR with gp220 primers from six of 12 NHLs investigated. EBV-RNA (EBER) were detected by in situ hybridization in 9 of 12 lymphomas. All cases positive for both EBER in situ hybridization and by PCR (6 of 12) were T-cell lymphomas of the LMG type. EBER-positive elements in LMG, ie, large polymorphic tumor cells, were often found in the wall of vessels or in their vicinity (Fig 1A). Few EBER-positive cells, all considered to be nonneoplastic small lymphocytes, diffusely distributed over neoplastic tissue, were found in three NHLs studied for comparison (cases no. 7, 8, and 10). Hybridization with EBER1-sense probe always showed lack of annealing (Fig 1B).

The T-cell phenotype of the LMG lymphomas established by use of monoclonal antibodies and paraffin-embedded tissues is outlined in Table 3. The tumor cells in all LMGs reacted with the antibody UCHL1. In addition, four LMGs showed positive tumor cells after use of CD3 (Fig 2), or of antibodies directed against OPD4 (Fig 3), or of both compounds. In two cases (no. 2 and 3), the reactions (CD3, OPD4) were negative, possibly due to loss of antigens. Nevertheless, these cases fulfilled the morphological and clinical criteria of LMGs. In case no. 2, over the course of 6 years, a
Fig 1. (A) EBER-positive cells in LMG are found close to vessels and in the wall of a vessel. (EBER-in situ hybridization; original magnification × 480.). (B) Same case as in (A) hybridized with control-probe (EBER-sense) demonstrates lack of annealing. (EBER in situ hybridization; original magnification × 360.)

Fig 2. LMG (case no. 6) stained with CD3-antibody shows numerous positive tumor cells. (Immunohistochemistry; original magnification × 360.)
pleomorphic medium-to-large-cell lymphoma with extensive invasion of and damage to blood vessels destroyed extensively midline facial structures. In case no. 3, a LMG with a comparable morphology spread systematically. The patient died from generalized lymphoma, as shown at the autopsy. Clinical course is listed in Table 4.

The subtype of EBV was determined in all LMG cases. Three cases had EBV type 2, and three cases had subtype 1 (Table 1 and Fig 4). No double infections with both subtypes were found. All PCR results, particularly all subtypes, were then checked by hybridizing the amplified DNA with the corresponding digoxigenin labeled probes. The results were identical to those obtained by PCR alone: three of six EBV-positive LMGs had subtype 2 EBV.

Using a chi-square test for two independent samples and EBER-positivity alone as a criterion we found that the prevalence for EBV subtype 2 in LMG was significantly different from that in the other non-LMG lymphomas tested ($P = .05$). The application of two criteria, ie, positivity by EBER and PCR, yielded a probability of .01.

The nasopharyngeal carcinomas and the tonsils with/without infectious mononucleosis carried the subtype 1 of EBV. Carcinoma cells, as well as some cells in the tonsils with infectious mononucleosis, were LMP-positive. In LMGs, only one case showed a few scattered LMP-positive tumor cells.

DISCUSSION

In Western populations, T-cell NHLs are associated with EBV in a higher percentage than B-cell NHLs. In vitro, partly explained by the fact that EBV-specific receptor CD21 is found on human B lymphocytes, but not on mature peripheral T cells. Some subtypes of T-cell NHLs, more prone to EBV-association than others, primarily present with a granulomatous or inflammatory aspect, such as T-cell NHL of the angioimmunoblastic lymphadenopathy with dysproteinemia (AILD) type, and T-cell NHLs of the LMG type. Both lesions were erroneously considered to be reactive diseases of the lymphatic system. Both the absence and the presence of TCR rearrangement have been reported in LMGs. In our series, clinical follow-up showed the malignant nature of these lesions. It is conceivable that the end stage of initially polyclonal LMGs might be clonal. However, there are no longitudinal data available to support this hypothesis. The T-cell nature of the cases in our series has been determined by immunohistochemistry on paraffin sections. The drawbacks of this technique are known. The density of available epitopes may be altered and thus differs from case to case, eg, because of differences in fixation. Furthermore, immunohistochemical methods and criteria for evaluation may differ. These observations relate especially to differences seen with UCHL1 and OPD4 in our series. Generally, OPD4 is thought to give a more reliable staining on paraffin sections than UCHL1. However, our cases had been collected from the files of the insti-

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**Table 2. List of Probes and Primers Used in This Study**

<table>
<thead>
<tr>
<th>Probe/Primer</th>
<th>5'-Sequence-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Globin</td>
<td>5'ACACAAGCTGTCTACTAGC3'</td>
</tr>
<tr>
<td>PC03: gp220 of EBV</td>
<td>5'ACACTTCATCCAGTTCAC3'</td>
</tr>
<tr>
<td>gp220 1</td>
<td>5'GGCTGTGTCACCTGTGTTA3'</td>
</tr>
<tr>
<td>gp220 2</td>
<td>5'CCTTAGAGGAAGAACAGTCC3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'AGGAGGCTGACTTGAAGTTA3'</td>
</tr>
<tr>
<td>EBNA2</td>
<td>5'GTGCTGTGACTGCTGTTG3'</td>
</tr>
<tr>
<td>EBNA2 subtype 1</td>
<td>5'ACGGTGGCTGACTGCTGTTG3'</td>
</tr>
<tr>
<td>EBNA2 A1</td>
<td>5'TCTGTCACCAAGCGCCCTGCTG3'</td>
</tr>
<tr>
<td>EBNA2 A2</td>
<td>5'AGGCCTACTCTCTCAACCAG3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'CAGTTTACACTGCCCAG3'</td>
</tr>
</tbody>
</table>

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tute over a 30-year period. Changes in technical procedures for tissue processing have occurred during this period.

The LMG type of T-cell lymphoma is regularly associated with EBV. It has been suggested that EBV replicates and resides in cells of the oropharynx. However, the issue of EBV-latency in oropharynx is still controversial. Therefore, it was tempting to suggest that oropharyngeal localization predisposes to the development of EBV-associated NHLs. However, in our series, EBV was confined to LMG; only a few EBV-positive cells, considered to be nonneoplastic small lymphocytes, were detectable in three of six NHLs from the same localization. Thus, in the nasopharyngeal region, the type of lymphoma, ie, LMG, disposes to an asso-
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Association of the subtype 2 of the Epstein-Barr virus with T-cell non-Hodgkin’s lymphoma of the midline granuloma type [see comments]

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