Several studies suggest that the Epstein-Barr virus (EBV) is etiologically linked to Hodgkin's disease (HD). This study was undertaken to examine the role of EBV in familial HD (FHD). Among 60 FHD patients from 27 families with two or more cases per family, we tested available paraaffinized tumor tissues from 46 cases by in situ hybridization for EBV-encoded RNA (EBER1) expression. Thirteen of 46 FHD patients (28%) had EBER1 expressed in the Reed-Sternberg cells. Concordance rate of EBV positivity was evaluated among 34 first-degree related pairs from 17 families for which both cases had available paraaffinized tumor tissues. Only two of 17 pairs were concordant for EBER1 positivity. There was no excess of positive concordance (P = .38). Serologically, FHD patients had higher geometric mean antibody titers (GMTs) to the viral capsid antigen (VCA) and early antigen D (EA-D). There was no difference in seroprevalence between patients and control groups, nor was there concordance in elevated serology among 15 pairs of first-degree related FHD cases. Young adult unaffected family members (UFM) may not react to EBV in the same way as the general population as evidenced by the lower titer of VCA, although not statistically significant, and significantly lower titers of EA-D, compared with age-matched controls. While EBV might have some role in a subset of HD, lack of concordance of EBER1 expression and EBV serology among the FHD cases in the same family suggest that EBV does not play an important role in FHD.

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solution that consisted of nitroblue tetrazolium and X-phosphate. The reaction was stopped by briefly washing the slides in an appropriate buffer. The slides were counterstained with eosin. The integrity of the RNA in each tissue section was evaluated with a digoxigenin-labeled riboprobe (105 bp) directed at an abundant cellular mRNA transcript for a small nuclear ribonucleoprotein, snRNP U6.13 Sections that showed hybridization signal with the U6 probe were determined adequate for analysis with the EBER1 probe. A positive control slide, prepared from a paraffin-embedded tissue block that contained metastatic nasopharyngeal carcinoma to lymph node, was included in each hybridization run.

EBV serology. Immunoglobulin G (IgG) antiviral capsid antigen (anti-VCA), EBV early antigen D (EA-D), and EBV early antigen R (EA-R) were measured by indirect immunofluorescence assays according to Henle et al.14 Antibodies to EBV nuclear antigens (EBNA) were measured by anticomplement immunofluorescence using Raji cells based on the procedure by Reedman and Klein, but using modifications previously described.14 BJAB, an EBV-negative B-cell line, was used as a negative antigen control.

Statistical analysis. Familial pairs of HD were studied using two endpoints: (1) the presence of EBER1 in the tumors and (2) elevated EBV serology.

For the analysis of EBER1 results, families with pairs of cases tested for in situ hybridization were grouped by concordant positivity and discordant and concordant negativity. To test for excess concordance, we used a simple permutation test,15 ie, assuming the observed prevalence of positives, the number of pairs, and independence, we calculated the exact conditional probability of the observed number of concordant positives or more. This is the \( P \) value of the permutation test for excess concordance.

For the analysis of serologic data, titers from HD cases and UFM were compared with their respective age- and sex-matched controls. Fisher’s exact test and conditional logistic regression were used to compare rates of seroprevalence. To compare the geometric mean titer (GMT) of seropositive sera, we used standard unmatched \( t \) statistics for testing the difference between two means under the assumption of equal variance. The permutation test was again used to test for excess concordance. All tests were two-tailed.

RESULTS

Sixty FHD cases from 27 families were identified for this study (Tables 1 and 2). Most of the 27 kindreds had 2 cases of HD; however, 4 kindreds had 3 cases, and 1 kindred had 4 cases. Sixteen families presented as sibling pairs, 8 as parent-child pairs or parent-children combinations (trios), 1 as first cousin pairs, and 2 as siblings with a cousin or a niece. The male to female ratio was 1 (28 males and 32 females). Age at diagnosis (\( n = 60 \)) ranged from 11 to 57 years (mean, 28 years; median, 26 years) and 80% were \( \leq \) to 40 years of age (Fig 1); the mean age at examination and phlebotomy (\( n = 39 \)) was 32 (median, 31).

UFM were slightly older at the time of examination and phlebotomy, ranging from 10 to 69 years of age (mean, 40 years; median, 39 years). Half were siblings of FHD cases; the other half were their parents. The male to female ratio was approximately the same in both patient and control groups.

Histologic subtypes and positivity for EBER1. The majority, or 72% (43 of 60), of FHD cases were histologically classified as nodular sclerosis, and 18% (11 of 60) as mixed cellularity (Tables 2 and 3). Among 46 cases with available tissue blocks for EBV in situ hybridization testing, 83% were nodular-sclerosing, 11% were mixed-cellularity, 4% were lymphocyte-depleted, and 2% were lymphocyte-predominant HD. Among the 14 in which tissue blocks were no longer available, seven cases were mixed-cellularity or lymphocyte-predominant HD. Thirteen of 46 cases (28%) harbored EBER1 in the Reed-Stemberg cells. Within each histologic subtype, EBER1 was detected in 21% (8 of 38) of nodular-sclerosing, 60% (3 of 5) of mixed-cellularity, and 100% (2 of 2) of lymphocyte-depleted cases, but was not detected in the single lymphocyte-predominant case. Thirty-nine percent (5 of 13) of EBER1-positive cases and 6% (2 of 33) of EBER1-negative cases were classified as mixed cellularity or lymphocyte depletion.

When analyzed by age at diagnosis (Fig 1), 30% (3 of 10) of those who were 11 to 20 years old had EBV-positive tumors, compared with 20% (6 of 31) of those 21 to 30 years old. Ninety-one percent (32 of 46) of the FHD cases were younger than 40 years old at diagnosis, and most of them, or 76% (32 of 42), were EBV-genome-negative in the tumors. Although the numbers were small, the data suggest that those older than 40 years at diagnosis have a higher rate of EBV-positive tumors, or 75% (3 of 4) (\( P = .06 \)), compared with all other age subgroups.

Concordance for EBER1 positivity. Thirty-four of 46 cases occurred as pairs in 17 families (Table 2). Of those 17 pairs, only two pairs were concordant for EBER1 positivity, four were discordant, and 11 were concordant for EBER1 negativity. Given 17 pairs and eight positives, the conditional probability of two or more concordant positives at random is .18, ie, there was not a significant excess of concordant positivity.

EBV serology. As a group, FHD cases had a higher seroprevalence than their corresponding controls and UFM (Table 4). FHD cases had GMTs for VCA-G and EA-D that were significantly greater than their matched controls, which was not age-dependent. The GMTs of VCA-G and EA-D from FHD cases age 15 to 39 years were also significantly higher than their age-matched controls (\( P = .013 \) and .029, respectively). Although overall there was no difference in the GMT of EA-D for UFM in comparison to their controls, in the subgroup age 15 to 39 years, the EA-D was signifi-
Sibling pair only

1*  2  1. Brother  1964  1964 (21) II LD +
   2. Sister  1962  1969 (26) II NS +
   3. Sister  1951  1968 (16) II NS +
   5. Sister  1962  1991 (29) III NS +
   7. Brother  1966  1982 (14) IV NS −
   8. Brother  1969  1991 (21) II NS +
   10. Sister  1959  1982 (23) IV NS −

7*  2  1. Sister  1960  1971 (11) I NS −
   2. Brother  1962  1983 (21) III NS −
   4. Sister  1960  1985 (22) II NS −
   5. Brother  1967  1990 (21) III NS −
   7. Brother  1956  1977 (22) I NS −
   8. Sister  1953  1981 (27) II NS −
   10. Brother  1959  1968 (24) IV NS −

Family No.  No. of Cases per Family  Case No and Relationship  Year of Birth  Year (age) of Diagnosis  Clinical Stage  Histologic Subtype  EBV Genome by In Situ Hybridization

Parent-child(ren) pair

17*  3  1. Mother  1895  1950 (54) I MC +
   2. Daughter  1917  1969 (57) II LD +
   3. Daughter  1924  1981 (52) I MC N/A
   18*  2  1. Father  1937  1984 (47) I NS −
   2. Daughter  1972  1987 (14) II NS −
   19*  3  1. Mother  1952  1982 (31) II NS +
   2. Daughter  1954  1984 (29) III LP −
   3. Son  1956  1990 (33) IV NS −
   20*  2  1. Father  1942  1983 (40) III NS −
   2. Daughter  1977  1991 (14) II NS −
   21*  2  1. Mother  1948  1978 (29) III NS −
   2. Daughter  1970  1992 (22) I NS −
   22  2  1. Father  1936  1985 (49) IV NS N/A
   2. Son  1964  1985 (21) II NS −
   23  4  1. Son  1944  1964 (20) I MC +
   2. Daughter  1938  1966 (28) N/A NS N/A
   3. Father  1911  1967 (56) I MC N/A
   4. Daughter  1953  1982 (29) II NS N/A
   24  2  1. Mother  1934  1968 (34) N/A NS N/A

Cousin pair

25  2  1. Female  1939  1969 (30) I NS +
   2. Male cousin  1950  1975 (26) I MC N/A

Others

28  3  1. Brother  1955  1979 (23) II MC N/A
   2. Sister  1957  1991 (33) II NS −
   27  3  1. Brother  1920  1961 (40) I MC +
   2. Brother  1922  1963 (40) N/A NS N/A
   3. Niece  1939  1966 (49) III N/A N/A

Abbreviations: NS, nodular sclerosis; MC, mixed cellularity; LD, lymphocyte depletion; LP, lymphocyte predominance; N/A, not available; +, presence of EBER1 in Reed-Sternberg cells and variants; −, absence of EBER1 in Reed-Sternberg cells and variants.
* Seventeen families with 2 first-degree related cases tested for EBV in situ hybridization.
stantly lower than that from their similarly aged UFM (16 v 52; \( P = .023 \)). High GMT was not associated with EBERI positivity in the tumor. However, as expected, patients with mixed-cellularity subtype had high titers of VCA-G and EA-D.

**Concordance of high EBV serology titers.** Since most people are positive for EBV before the teens, EBNA and VCA-G titers were evaluated for high titer, defined as greater than 1:640 and 1:2,560, respectively (90th percentile level for population controls). There were 15 pairs of first-degree related FHD cases with available serology tests (Table 5). Two of those pairs demonstrated concordant high titer of EBNA (\( P = .06 \)) and two demonstrated high titer of VCA-G (\( P = .06 \)). One of these pairs from one family has elevated titers of both EBNA and VCA-G. There were two pairs that were concordant for positive titer of EA-D (\( P = .23 \)) and there were no concordant pairs for positive titer of EA-R.

**DISCUSSION**

These data provide three complementary aspects in the investigation of the relationship of EBV to FHD. Similar to previous studies, patients with FHD have higher seroprevalence and higher antibody titers to EBV compared with either UFM or general population controls. As in case series of HD, the familial patients with mixed-cellularity or lymphocyte-depleted HD are more likely to have EBV detected in the tumors by in situ hybridization than those with nodular-sclerosing HD. In addition, the age associations with EBV in situ positivity are similar to those described in other populations. However, the unique contribution of evaluating this group of patients is the examination of concordance within each family. If EBV were a shared environmental exposure important in the development of HD, one might expect to see high concordance rates, but we did not.

Previous seroepidemiologic studies have examined either cohorts \(^{16,17} \) or siblings \(^{18} \) as control groups for comparison of EBV titers. We used both first-degree relative controls and age-matched general population controls. Unaffected relatives of the HD patients had higher titers for EA-D than the population controls in the young adult age groups. The data suggest that family members from FHD may not be similar to the general population in exposure or reaction to EBV. The HD patients had higher titers for VCA-G and EA-D than either control group. These data are consistent with previously published findings, and indicate that other factors may be important in developing HD.

The in situ hybridization assay appears to be the most specific method to evaluate the association of EBV with HD. Overall, 28% (95% confidence interval, 0.15 to 0.41) of the evaluated cases were found to have the EBV genome in the tumor tissue. This is within previously reported ranges of EBV positivity. The relatively lower rate of positivity is not unexpected because of the histologic distribution of these cases, \(^{19} \) the young adult age of onset, \(^{20} \) and the high socioeconomic status of these families. \(^{21} \) It is noteworthy that the two families concordant for EBV positivity by in situ hybridization were not the same families that demonstrated concordance for high serologic titers. These and other data \(^{22} \) suggest that there is a discrepancy between EBV titers and the presence of EBV genome in the Reed-Sternberg cells. One possible explanation of the discrepancy is that, on average, these cases underwent phlebotomy about 4 years after diagnosis of HD (Table 1). The titers at the time of diagnosis of HD may have differed.

There are several sources of potential bias that may contribute to the lack of concordance in EBV by in situ hybridization or serology in this study. First, of 14 cases with no pathology blocks available, almost half were mixed-cellularity or lymphocyte-depleted HD, which are more likely to be EBV-positive. Of the 14, only five (two mixed cellularity, two nodular sclerosis, and one unknown type) occurred in families in which there was no EBV-positive HD. There are, therefore, missing data that could potentially affect the concordance rates found. To evaluate the effect of the missing data, we assumed that a missing case in each family was EBV positive to maximize the effect. Even in this extreme example, we would then have seven concordant positive pairs, eight discordant, and 11 concordant negative.

**Table 3. Distribution of Histologic Subtypes and EBV Genome Positivity**

<table>
<thead>
<tr>
<th>Subject</th>
<th>No.</th>
<th>Nodular Sclerosis</th>
<th>Lymphocyte Predominance</th>
<th>Lymphocyte Depletion</th>
<th>Mixed Cellularity</th>
<th>Unclassified</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHD case</td>
<td>60</td>
<td>43</td>
<td>2</td>
<td>2</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>In situ hybridization tested</td>
<td>46</td>
<td>38</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>EBER 1-positive</td>
<td>13</td>
<td>8</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>EBER 1-negative</td>
<td>33</td>
<td>30</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>-</td>
</tr>
</tbody>
</table>

**Fig 1. EBV genome positivity by age at diagnosis: EBV-positive (■), EBV-negative (■), and not tested □.**
EBV could be important in a subset of FHD with a specific genetic predisposition that is not yet identified. However, TAMM may be too crude a measure of gene-environment interaction. Data from a recent twin study, suggest that this may not account for the majority of FHD. Mack et al23 found that the data still would not be consistent with EBV playing a role in the etiology of familial HD. Further studies are required to define the role of EBV in EBV-positive cases, identify other factors in EBV-negative cases, and/or disentangle a gene-environmental interaction. Studies in high-risk cancer families, such as hereditary breast-ovarian cancer, or rare cancer family syndromes, such as Li-Fraumeni syndrome, which arise from inherited susceptibility to cancer, frequently result in identification of crucial gene(s). Given the rarity of FHD, continuing effort to collect more cases and perform a larger scale study, such as an international collaboration, could potentially lead to the understanding of its genetic basis.

ACKNOWLEDGMENT

The authors would like to thank the participating families for their generosity and cooperation that made this study possible; and Drs M.H. Greene, D.J. Tollerud, and J. Fraumeni for their contributions to the study.

REFERENCES


Table 4. GMTs (% Cases With Positive Titer) of Epstein-Barr Virus Serology Profile

<table>
<thead>
<tr>
<th>Subject</th>
<th>FHD cases</th>
<th>FHD controls</th>
<th>UFM</th>
<th>UFM controls</th>
<th>FHD cases with EBV in situ hybridization data available (n = 33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCA-G</td>
<td>39</td>
<td>8</td>
<td>63</td>
<td>126</td>
<td>EBER*</td>
</tr>
<tr>
<td>EA-D</td>
<td>39</td>
<td>8</td>
<td>63</td>
<td>126</td>
<td>EBER*</td>
</tr>
<tr>
<td>EA-R</td>
<td>39</td>
<td>8</td>
<td>63</td>
<td>126</td>
<td></td>
</tr>
<tr>
<td>EBNA1</td>
<td>39</td>
<td>8</td>
<td>63</td>
<td>126</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Distribution of EBV Serology by Number of Case Pairs From the Same Family (total no. of pairs = 16)

<table>
<thead>
<tr>
<th></th>
<th>Concordant</th>
<th>Disccordant</th>
<th>Concodant</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBNA</td>
<td>High Titer*</td>
<td>High Titer*</td>
<td>Low Titer*</td>
</tr>
<tr>
<td></td>
<td>or Positive*</td>
<td>or Positive*</td>
<td>or Negativity*</td>
</tr>
<tr>
<td>EA-D</td>
<td>2</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>EA-R</td>
<td>2</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>VCA-G</td>
<td>3</td>
<td>3</td>
<td>12</td>
</tr>
</tbody>
</table>

NOTE. IgG anti-VCA, EA-D, and EA-R were measured by indirect immunofluorescence. Antibodies to EBNA were measured by complement immunofluorescence using Raji cells.
* EBNA and VCA-G are compared as high v low titers.
† High titer for EBNA is defined as >1:540.
‡ High titer for VCA-G is defined as >1:2,560.
§ EA-D and EA-R are compared as presence (positive) v absence (negative) of immunofluorescence.
¶ P > .05.


Epstein-Barr virus and familial Hodgkin's disease

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