The bcl-2 Oncogene in Hodgkin's Disease Arising in the Setting of Follicular Non-Hodgkin's Lymphoma

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Expression of the bcl-2 proto-oncogene on chromosome 18 is deregulated by the 14;18 chromosomal translocation, an abnormality that is consistently associated with follicular non- Hodgkin's lymphomas (NHL). Because bcl-2 is believed to function by prolonging cell survival rather than by increasing proliferation, the presence of t(14;18) in Hodgkin's disease (HD) would have profound implications for the pathogenesis of this neoplasm. We evaluated 32 cases of HD for t(14;18) by polymerase chain reaction (PCR). These results were correlated with expression of bcl-2 oncogenic protein by Hodgkin cells and with the presence of Epstein-Barr virus (EBV), as determined by immunohistochemistry or in situ hybridization. PCR provided evidence of t(14;18) in only 2 HD cases (6%), both of which were associated with a prior history of follicular lymphoma, and both of which were among the 7 cases (22%) with strong bcl-2 expression in Hodgkin cells. In at least 1 of the cases, the translocation involved identical chromosomal breakpoints in both types of lymphoma. Furthermore, 7 additional cases of combined follicular NHL and HD showed strong bcl-2 staining in Hodgkin cells. Although EBV was detected in 8 of 30 cases, it was not associated with t(14;18) and usually not with strong bcl-2 expression. These results suggest that a small proportion of HD cases might evolve from follicular NHL, possibly through molecular events superimposed on the t(14;18). High-level bcl-2 expression in Hodgkin cells is a potentially useful but not definitive marker for these cases.

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MATERIALS AND METHODS

Thirty-two cases of HD were selected from the files of the Laboratory of the Surgical Pathology at Stanford University (Stanford, CA) on the basis of availability of frozen tissue or DNA and the corresponding paraffin-tissue blocks or unstained paraffin sections. Paraffin sections stained with hematoxylin and eosin were reviewed from each case, and the cases were categorized according to the Rye classification.15 Of these cases, 4 were not classifiable; 2 met the criteria of interfollicular HD,16 and the remaining 2 cases had extranodal disease (gallbladder and lung) with pathologic findings diagnostic of HD but impossible to subclassify. Hospital charts were reviewed for the 3 cases with prior follicular lymphoma, whereas clinical information on the other cases was obtained from pathology reports. A group of 8 additional cases was assembled using the concurrent or sequential occurrence of HD and follicular lymphoma in the same patient as selection criteria.

For the PCR, high molecular weight DNA was purified, using...
techniques which have been previously described, 17 from snap-frozen biopsy specimens that had been stored at −70°C. Two micrograms of purified DNA was subjected to 30 cycles of amplification by PCR using the Perkin-Elmer DNA thermal cycler. Taq polymerase, synthetic oligonucleotide primers MC4, MC5, and MC8, and reagents obtained commercially (Perkin Elmer-Cetus, Emeryville, CA). Duplicate Southern blots were prepared, using one-fifth of the reaction products for each, and hybridized with 32P-labeled synthetic oligonucleotides MC6 or MC12 as probes. Oligonucleotide MC4, 5'-ACCTGAGGAGACGGTGACC-3', is complementary to a sequence common to the six JH regions of chromosome 14; MC5, 5'-TGCTGTGTTGATATTICGA-3', corresponds to a sequence within the bcl-2 gene on chromosome 18, immediately 5' to the major breakpoint region (MBR), and is complementary to the negative strand at that site; MC8, 5'-GACTCTTATAGGCTGGTACC-3', corresponds to a sequence immediately 5' to the minor cluster region (MCR) on chromosome 18; and MC6, 5'-GTATTTAGTTATGCTATACACTATTGGGAGCGA-3' and MC12, 5'-GATGGCCTTGGCTGAGAGGTAT-3', correspond to sequences within the anticipated PCR products from the MBR and MCR, respectively.18,19 DNA from 2 follicular lymphomas with known t(14;18)'s, one involving the MBR and the other the MCR, were run with each group of cases as positive controls. The minimum threshold of positivity was defined by control PCR samples containing 2 × 10−4 μg of positive control DNA diluted in 2 μg of DNA from a reactive tonsil. The method used for genomic Southern blot analysis for t(14;18) has been previously described.20 For case HH, PCR products of appropriate size were excised from an agarose gel and cloned into a plasmid vector (pCR 1000) using a commercially-obtained kit (TA Cloning Kit; Invitrogen, San Diego, CA) according to the instructions provided. Inserts were then sequenced using the Sequenase kit (US Biochemical, Cleveland, OH).

The monoclonal antibody (MoAb; clone no. 124) used for immunohistochemical detection of bcl-2 protein was provided by Dr. David Mason (Oxford University, Oxford, UK). The production and characterization of this reagent, as well as the avidin-biotin detection method used in immunohistochemical staining, have been previously described.21,22 Immunostained sections were examined under high magnification using a standard light microscope. Cytoplasmic staining of Reed-Sternberg cells and variants was considered strong if it was clearly more intense than in surrounding, non-neoplastic lymphocytes, weak if it was present but no more intense than in lymphocytes, or absent. In these latter cases, the staining of background lymphocytes served as an internal positive control. This precaution was necessary because processing of tissue for paraffin embedding can impair detectability of the bcl-2 epitope by MoAbs. In 25 cases in which additional snap-frozen tissue specimens were available, frozen section immunohistochemistry was performed for detection of the EBV-associated protein LMP-1 using a MoAb (CS1-4) provided by Drs. L. S. Young and A. B. Rickinson (University of Birmingham, Birmingham, UK). Production and characterization of this reagent have been previously described.23 The immunostaining method used was similar to that used to detect bcl-2, except that streptavidin-conjugated horseradish peroxidase and diaminobenzidine were replaced by alkaline phosphatase and Fast Red, respectively, to avoid difficulties in interpretation caused by endogenous peroxidase in eosinophils. In situ hybridization to detect EBV-specific EBER-1 region transcripts was performed on 27 cases from which a sufficient number of unstained paraffin sections were available. The technique used has been described in detail elsewhere.24

RESULTS

The results of PCR analysis of HD tissues for t(14;18) are shown in Table 1. Of 32 evaluated cases, 2 (6%) contained amplifiable t(14;18) products, both of which hybridized with a probe to the MBR. Both HH and HN, as well as a third case, VS, without a t(14;18), had a history of prior follicular lymphoma. Clinical details of these 3 cases are presented in Table 2. DNA from a snap-frozen biopsy specimen available from the follicular large-cell lymphoma of HH was examined by 14;18 PCR, which showed products of identical size to those from this patient's HD specimen (Fig 1). At least two bands were detected from these specimens because of priming by the consensus JH oligonucleotide at complimentary sites in contiguous JH genes. Genomic Southern blotting of the HH DNA specimens and hybridization with a DNA probe for the bcl-2 MBR (PFL-16) showed rearranged and germline bands in the follicular lymphoma and only a germline band in the HD specimen. The latter result was consistent with the presence of t(14;18) carrying cells in the HD specimen below the threshold of detection by Southern blot (1%). The presence of the same breakpoints in both lymphomas in case HH was confirmed by DNA sequencing (Fig 2). The relative abundance of the cells containing t(14;18) in the analyzed follicular lymphoma and HD tissue from HH was determined by dilution analysis of the DNA purified from these specimens (Fig 3). This indicated that fewer than 1% of the cells in the HD tissue from HH contained bcl-2–IgH fusion, a result consistent with the estimated abundance of Hodgkin cells as determined by morphologic and immunologic studies (see below) and by Southern blot analysis.

The immunohistochemical staining results are shown in Tables 3 and 4. Cytoplasmic bcl-2 staining was present in Hodgkin cells of 20 cases (63%). However, strong staining was present in only 7 cases (22%), including the 3 cases with previous follicular lymphoma (Fig 4). Thus, both cases in which a t(14;18) was detectable were among those with strong bcl-2 expression, an association of marginal statistical significance (P = .04). In the 3 cases with previous follicular lymphoma, enhanced bcl-2 expression was confined to the Hodgkin cells in contrast with the small lymphocytes that showed only background level staining. By these criteria, no NHL cells appeared to be present in the analyzed tissue sections. No t(14;18) was detected in any of the cases in which bcl-2 expression was weak or absent. LMP-1 was detected by immunostaining in 4 of 25 cases (16%), whereas in situ hybridization detected EBV-specific messenger RNA in 6 of 27 cases (22%; Table 4). Twenty-two cases were studied by both methods with complete concordance in the results (EBV detected in the same 4 cases by either method). Therefore, the results obtained by the two methods were consid-
Table 2. Cases With Follicular Lymphoma Preceding HD

<table>
<thead>
<tr>
<th>Patient</th>
<th>Lymphoma Type/Date</th>
<th>Treatment</th>
<th>HD Type/Date</th>
<th>t(14;18) in HD Tissue</th>
<th>Staining for BCL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH</td>
<td>FLC/1970</td>
<td>Lymphoid irradiation</td>
<td>NS/1985</td>
<td>Yes</td>
<td>Strong</td>
</tr>
<tr>
<td>HN</td>
<td>FSC/1976</td>
<td>Lymphoid irradiation</td>
<td>MC/1987</td>
<td>Yes</td>
<td>Strong</td>
</tr>
<tr>
<td>VS</td>
<td>FSC/1982</td>
<td>Chemotherapy</td>
<td>NS/1987</td>
<td>No</td>
<td>Strong</td>
</tr>
</tbody>
</table>

Abbreviations: FLC, follicular large cell; FSC, follicular small cleaved cell; NS, nodular sclerosing; MC, mixed cellularity.

The results prompted a retrospective immunohistochemical analysis of 8 additional cases in which HD and follicular NHL occurred in the same patient (Table 5). Strong staining of Hodgkin cells for BCL-2 was present in all but 1 of these cases (staining of small lymphocytes, used as an internal positive control, was also absent in this case making the result uninterpretable), and the association between strong BCL-2 expression in Hodgkin cells and prior or concurrent follicular lymphoma was statistically significant ($P < .001$). The results of diagnostic immunophenotyping supported the diagnosis of HD in each case. Molecular studies were not performed on these additional cases because of a lack of appropriately matched frozen tissues or the obvious coexistence of NHL and Hodgkin cells in the same specimens.

**DISCUSSION**

The Reed-Sternberg cell and its variants are recognized as the neoplastic component of the mixed population of hematolymphoid cells generally present in biopsy specimens of HD tissue. Although they have long been presumed to have arisen from a hematolymphoid precursor, study of Hodgkin cells at the molecular level has been hampered by their relative paucity in tissues involved by the disease. These cells generally comprise less than 1% of cells present in such tissues, whereas detection by traditional Southern blotting methods requires the DNA of interest to comprise at least 1% of the total DNA present in a sample. With its ability to amplify specific DNA sequences present in as few as 1 in $10^5$ cells, the PCR is potentially well suited to the study of HD. Furthermore, the tight clustering of breakpoints on chromosome 18 and in the JH genes on chromosome 14 have permitted us and others to design synthetic oligonucleotide primers for PCR complementary to sites that flank the chromosomal breakpoints involved in the vast majority of 14:18 translocations.

Our results from applying the PCR to 32 unselected cases of HD indicate that the 14:18 translocation occurs frequently in this neoplasm (6%). When t(14;18) does occur in HD, it appears to be restricted to cases associated with a previous follicular NHL. The translocation was detected in 2 of the 3 cases with such an association in our series. In 1 of these cases, the size and nucleotide sequence of PCR products from the HD tissue and the older follicular lymphoma tissue were identical, indicating involvement in the two neoplasms of the same breakpoints on chromosomes 14 and 18 and suggesting clonal evolution of HD from follicular lymphoma.

Alternatively, the presence of a minimal residual quantity of follicular lymphoma in the HD tissue could account for

**Fig 1.** PCR and Southern blot analysis of the t(14;18) chromosomal translocation in follicular lymphoma and HD biopsy specimens from patient HH are shown. Snap-frozen biopsy specimens of both follicular lymphoma and HD tissues were available from patient HH. DNA was purified from these and subjected to PCR and genomic Southern blot analysis. The identical size of PCR products from the two specimens strongly suggests involvement of the same chromosomal breakpoints in the two lymphoma types. The genomic Southern blot of the same DNA digested with the restriction enzyme BamHI and hybridized with the PFL-1 probe for the MBR on chromosome 18 showed an approximately 18-kb germline band visible in both samples (——) and an approximately 13-kb rearranged band that is visible in the follicular lymphoma specimen only (small arrow). The relative abundance of neoplastic cells in the HD specimen is probably too low for detection by Southern blotting without amplification.

**Fig 2.** DNA sequence analysis of t(14;18) breakpoint from case HH is shown. The same breakpoints on chromosomes 18 and 14 as well as an identical 11-nucleotide insertion (underlined) were identified in the PCR products from this patient’s follicular lymphoma and HD specimens.
Dilution of Sample DNA

Fig 3. Relative abundance of t(14;18)-containing cells by dilution analysis of DNA from follicular large-cell and HD specimens is shown. DNA from the follicular large-cell lymphoma and HD specimens from HH was serially diluted in nonneoplastic tonsillar DNA, and 2 μg of total DNA from each dilution was subjected to PCR amplification. Whereas the t(14;18) is detectable in the follicular lymphoma DNA after dilution to 1 in 10^4, as indicated by a faint hybridization signal, no hybridization signal is present from the HD DNA beyond a dilution of 1 in 10^6, indicating an approximately 100-fold lower abundance of t(14;18)-containing cells in the HD specimen relative to the follicular large-cell lymphoma. Because neoplastic cells constituted 50% to 75% of cells in the follicular lymphoma specimen as determined morphologically and immunologically, this result confirms an abundance of t(14;18)-containing cells in the HD tissue of less than 1%.

these results. Although we have not excluded this possibility rigorously, several findings make it unlikely. In our series, strong bcl-2 expression seems to be correlated with the presence of t(14;18) (2 of 2 v 5 of 30, P = .04) and is strongly correlated with the co-occurrence of follicular lymphoma (10 of 10 cases v 4 of 29, P < .001). Therefore, the clear localization to Hodgkin cells of abundant bcl-2 protein in both cases with detectable t(14;18) makes these cells the most likely site for the translocation. Furthermore, PCR amplification of serially diluted DNA specimens from one of the cases indicates the abundance of translocation-carrying cells to be approximately 0.5%, a quantity that is in agreement with morphologic and immunologic evaluation of the number of Reed-Sternberg cells and variants in the HD tissue from this case. Finally, no trace of follicular lymphoma was detectable morphologically or immunologically in the HD tissue from either of the t(14;18)-associated cases.

The term “composite lymphoma” refers to a tumor in which two distinct histologic subtypes of lymphoma are present in the same mass. Three large series of composite lymphomas include a total of 37 cases composed of HD and NHL. In 15 of these (41%) the NHL component was follicular, and the diagnosis of follicular lymphoma is noted to have antedated that of HD in 3 cases. These studies must be distinguished from studies of NHL that follow therapy for HD. In this setting, the most common secondary malignancy is acute myeloblastic leukemia. However NHL may also occur in this setting and are most often diffuse, histologically aggressive types.

It is also noteworthy that follicular lymphoma preceded HD in each of the 6 cases in our extended series in which the diseases were diagnosed on separate occasions. Of relevance to this observation is a recent study by Zarate-Osorno et al describing 9 cases of HD after NHL. The NHL was follicular in 7 cases (78%), although this prevalence decreased to 14 of 38 cases (37%) when additional cases from the literature were considered.

Our findings and those in the literature suggest that a small subset of lymphomas that meets the morphologic and immunologic criteria of HD has evolved from a preexisting follicular NHL. Although the low prevalence of t(14;18) in our initial group of HD cases indicates that such a progression does not account for the preponderance of HD, follicular lymphoma may represent merely one process among

Table 3. Immunohistochemical Detection of bcl-2 Expression in Hodgkin Cells

<table>
<thead>
<tr>
<th>HD Subtype</th>
<th>bcl-2 Staining</th>
<th>Strong</th>
<th>Weak</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte predominance</td>
<td></td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Mixed cellularity</td>
<td></td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Nodular sclerosis</td>
<td></td>
<td>5</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Unclassified</td>
<td></td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>7 (22%)</td>
<td>13 (41%)</td>
<td>12 (37%)</td>
</tr>
</tbody>
</table>

Table 4. Correlation of bcl-2 Expression With t(14;18) and EBV Status

<table>
<thead>
<tr>
<th>bcl-2 Staining</th>
<th>t(14;18) Present</th>
<th>EBV Detectable in Hodgkin Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong</td>
<td>2/7</td>
<td>1/6</td>
</tr>
<tr>
<td>Weak</td>
<td>0/13</td>
<td>4/12</td>
</tr>
<tr>
<td>Absent</td>
<td>0/12</td>
<td>1/12</td>
</tr>
<tr>
<td>Total</td>
<td>2/32 (6%)</td>
<td>6/30 (20%)</td>
</tr>
</tbody>
</table>

Values are the number of cases positive/number of cases evaluated.

* Refers to EBV detectability by either immunohistochemistry for LMP-1 or in situ hybridization for EBER transcripts.
bcl-2 AND HODGKIN'S DISEASE

Several processes that predispose to the later development of HD. A common feature of such processes may be the ability to produce a pool of inappropriately long-lived lymphoid cells. These cells may then constitute "fertile soil" for the occurrence of superimposed molecular events specific to HD. It is these latter molecular events, so far unidentified, that may enhance the proliferative activity of affected cells and bring about the emergence of a more aggressive neoplastic subclone. The same genetic alterations may also give rise to the constellation of features, such as polymorphous background of host cells and the acquisition by neoplastic cells of an immunophenotype similar to that of activated lymphocytes, which are currently used to distinguish HD from other hematolymphoid neoplasms. This process may be analogous to the superimposition of c-myc activation on a preexisting t(14;18) that has been documented to be associated clinically with progression from follicular to diffuse lymphoblastic lymphoma and experimentally with progression from polyclonal follicular hyperplasia to high-grade malignant lymphoma in t(14;18)-carrying transgenic mice. In addition, the association of HD with chronic lymphocytic leukemia and the rare association of HD with mycosis fungoides imply that similar mechanisms may be involved in the evolution of HD from several other indolent lymphoproliferative diseases.

B cells infected by EBV represent an attractive candidate for a Hodgkin-cell precursor. EBV infection is known to lead to a circulating population of long-lived B-lineage lymphocytes. Furthermore, EBV-specific nucleic acid sequences and proteins have been detected in HD cells in up to 48% of cases, with some studies indicating an especially high EBV prevalence in the mixed cellularity subtype. Recent evidence indicates that progression of B-cell survival in vitro by EBV is related to induction of bcl-2 expression by virus-encoded LMP-1. Interestingly, the EBV genome itself contains a gene that codes for BHRF1, a hypothetical protein of unknown function that has sequence homology to bcl-2.

In the current study, the possibility that induction by EBV may account for some cases in which bcl-2 expression was not associated with a detectable t(14;18) was explored by studying all except 2 cases in the series for the presence of either LMP-1 protein or EBER-1 transcript. EBV was detectable in 6 cases, only 1 of which showed strong expression of bcl-2. This case, in which there was no history of follicular lymphoma, was among the 4 cases with strong bcl-2 expression and no detectable t(14;18) or history of follicular lymphoma. Therefore, we were unable to find a plausible explanation for the enhanced bcl-2 expression in 3 of the cases in our primary series. Although we cannot rule out the possible occurrence of t(14;18) that involve breakpoints outside of the MBR or MCR, which our PCR primers would not have detected, it is also possible that elevated bcl-2 expression in HD cases may be brought about by other molecular mechanisms, including induction by viruses other than EBV.

Others have studied the t(14;18) in HD by PCR. Some investigators have found a low but significant prevalence, with the highest being 17 of 53 cases (32%). However, several groups have been unable to identify any cases with the translocation. Poppema et al detected the t(14;18) in 11 of 28 cases by PCR but in only 1 of these cases by cytogenetics and suggested that in some HD tissues the translocation might be located in "background" lymphocytes rather than Hodgkin cells. This would be consistent with the reported finding of t(14;18) in benign, hyperplastic lymphoid tissues. Reviews of cytogenetic studies also support a low incidence of t(14;18) in HD. Although abnormalities of band 14q32 have been reported in a number of fresh tissue specimens as well as in cell lines, the t(14;18)(q32;q21) translocation has been specifically recognized only rarely.

The discrepancies in PCR results are difficult to explain, but at least some may be related to technical differences. Unlike several other groups, we used control samples containing approximately 10^-4 μg of DNA (roughly the amount present in 10 human cells and far fewer than the anticipated number of Hodgkin cells in any of the samples) from lymphomas known to carry a 14;18 translocation to define the lower limit of hybridization signal intensity that we would consider as a "positive" result.

If follicular lymphoma does, in fact, occasionally progress to HD, it is difficult to estimate the relative contribution of such a process to cases of HD occurring in the general population. A prior history of follicular lymphoma is rare among patients with HD, although probably not so rare as...
in the general population. In a study published in 1948, Custer and Bernhard reviewed 700 cases of HD and identified 4 patients (0.57%) in whom follicular lymphoma had preceded HD. Interestingly, no cases were found in which HD had preceded follicular NHL. The relatively large proportion of HD patients in our initial series who had previously been treated for follicular lymphoma (almost 10%) almost certainly represents an overestimate and may be related to the large number of patients with follicular lymphoma who receive long-term follow-up at our institution. A high prevalence of these patients in our series relative to that in other studies might account for the failure of previous studies to detect an association between t(14;18) in HD and a prior history of follicular lymphoma.

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The bcl-2 oncogene in Hodgkin's disease arising in the setting of follicular non-Hodgkin's lymphoma

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