Follicular Origin of a Subset of CD5+ Diffuse Large B-Cell Lymphomas

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Abstract

Most follicular lymphomas (FLs) have a phenotype consistent with the origin from CD5–, CD10+, bcl-6+ follicular center cells and can progress to diffuse large B-cell lymphoma (DLBCL). CD5 is expressed in about 10% of DLBCLs, showing prognostic value, whereas expression is rare in FL. We present 6 cases with coexisting features of CD5+ FL and CD5+ DLBCL, supporting a follicular origin for some CD5+ DLBCLs. The follicular areas showed a meshwork of CD21+ follicular dendritic cells that were lacking in the DLBCL areas. All cases showed a clonal CD19+, CD20+, CD5+, and CD10+ population in both follicular and diffuse areas. Molecularly, 4 of 6 cases demonstrated immunoglobulin heavy chain rearrangements and 1 case, a bcl-2/immunoglobulin heavy chain gene rearrangement. Somatic hypermutations were high in all 4 cases, in keeping with their germinal center origin. Four of five patients died of disease within 42 months, consistent with the proposed prognostic value of CD5 expression in DLBCL. Our data describe an aggressive variant of CD5+ FL suggesting the follicular origin of some CD5+ DLBCLs.

CD5 is a 67-kd surface molecule expressed on T cells and on a subset of normal naive B cells.1,2 In the normal B-cell activation process, when entering the germinal center, CD5+ mantle cells down-regulate CD5 and acquire CD10 expression.3 Therefore, the vast majority of germinal center cells, ie, centrocytes and centroblasts, are negative for CD5 expression.4 Recent data suggest that the CD5 molecule can function as a negative regulator of B-cell receptor signaling that might help prevent inappropriate activation of autoreactive cells.5

In B-cell lymphomas, CD5 expression defines specific subsets of non-Hodgkin lymphoma (NHL), such as small lymphocytic lymphoma/chronic lymphocytic leukemia (SLL/CLL) and mantle cell lymphoma (MCL).6,7 However, a subset of marginal zone lymphomas (MZLs) and diffuse large B-cell lymphomas (DLBCLs) express CD5.8,9 By contrast, other NHLs are considered to be classically CD5–, such as follicular lymphomas (FLs) and Burkitt lymphomas. However, some reports describe the expression of CD5 in Burkitt lymphoma10 and in FL.11 In FL, CD5 expression is rare and described so far mainly in low-grade FL, possibly corresponding to the so-called floral variant.11-13

DLBCL is the most frequent subtype of aggressive lymphomas, arising de novo or originating from a transformation of a low-grade lymphoma.9 Recent gene expression profiling data suggest that more than 30% of DLBCLs derive from germinal center–like cells.14 The phenotype of DLBCL is heterogeneous, with variable expression of different markers. Approximately 10% of DLBCL cases express CD5 as a surface antigen,15,16 and, genetically, CD5+ DLBCLs are a heterogeneous and poorly described phenotypic variant that is distinct from the CD5+ cases of Richter transformation of SLL/CLL and from the aggressive variants of MCL, which carry the t(11;14) translocation leading to the overexpression...
of cyclin D1.\textsuperscript{17,18} It is interesting that CD5 expression in DLBCL has been regarded as a negative prognostic factor.\textsuperscript{16}

The histogenesis of CD5+ DLBCL is controversial, and few data are available on the cytogenetic alteration or the somatic hypermutation of the immunoglobulin heavy chain (\textit{IgH}) gene variable region (\textit{VH} gene).\textsuperscript{19,21} The lack of data regarding the histogenesis of CD5+ DLBCL and the description of a CD5+ variant of FL prompted us to search for the existence of DLBCL possibly derived from CD5+ FL. We present 6 cases of CD5+ DLBCL in which the derivation from germinal center cells or FL can be inferred based on morphologic aspects, immunophenotypic expression of follicle-associated antigens, and/or molecular biologic data.

\section*{Materials and Methods}

\subsection*{Case Selection}

We identified 41 consecutive cases of CD5+ aggressive lymphoma in the database of the Surgical Pathology Unit, S. Giovanni Battista Hospital, Turin, Italy, for the period January 1987 through December 2001. Among these, flow cytometric analysis identified a population of large cells expressing monotypic surface light chain and coexpressing CD5 and CD10 antigens in the majority of the neoplastic cells in 12 cases. Six cases were excluded from the study because they subsequently were classified as MCL or Richter syndrome in a previously diagnosed SLL/CLL or because they did not show morphologic or phenotypic signs suggestive of germinal center derivation. Formalin-fixed, paraffin-embedded tissue blocks were used for the study.

\subsection*{Immunohistochemical Analysis}

Immunohistochemical analysis was performed on deparaffinized and rehydrated sections, following an immunoperoxidase technique with an automatic staining device (Autostainer, DAKO, Glostrup, Denmark), according to the manufacturer’s instructions. Samples were analyzed with a monoclonal antibody panel comprising Ki-67 (DAKO), CD3 (MEDAC, Wedel, Germany), CD5 (Neomarkers, Fremont, CA), CD10 (DAKO), CD20 (DAKO), bcl-2 (DAKO), p53 (24/28, MEDAC), cyclin D1 (Neomarkers), CD21 (DAKO), CD23 (The Binding Site, Birmingham, England), bcl-6 (DAKO), and CD43 (Becton Dickinson, San Jose, CA). All cases were evaluated by at least 3 independent observers (R.C., D.N., and A.D.M.), and low (<25%), intermediate (25%-75%), and high (>75%) positivity scores were given to each sample.

\subsection*{Flow Cytometry}

Surface antigens were studied with a 3-color operating fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson) on mononuclear cells obtained from lymph nodes or biopsy specimens. Mononuclear cells were isolated after centrifugation on a density gradient; analysis gates included small and large lymphocytes; the following directly conjugated monoclonal antibodies were used in the study: CD3, CD5, CD10, CD11c, CD19, CD20, CD22, CD23, CD25, CD38, CD43, CD103, FMC7, anti–κ light chain, anti–λ light chain, anti-IgM, anti-IgD, anti-IgG, and anti-IgA. All were purchased from Becton Dickinson. Isotypic controls were defined before fluorescence-activated cell sorter acquisition: IgG1-fluorescein isothiocyanate and IgG2-phycoerythrin were used to set up the threshold for each analysis. Afterward, a gate was designed for CD19+ cells, and CD5+/CD10+ double-positive samples were studied.

\subsection*{Polymerase Chain Reaction Analysis of \textit{IgH} Rearrangements}

Formalin-fixed, paraffin-embedded samples were deparaffinized with a xylene-ethanol protocol. Tissue specimens were incubated overnight at 55°C in lysis buffer with Proteinase K (20 mg/mL), and DNA was obtained by phenol-isopropanol extraction. A seminested strategy was used for polymerase chain reaction (PCR) amplification of the \textit{IgH} rearrangements using an FR2A consensus primer complementary to the conserved framework-2 segment of the variable region (\textit{VH}) and, respectively, LJH and VLJH consensus primers for the joining region (\textit{JH}).\textsuperscript{22} The PCR mixture contained 1× Taq buffer (10 mmol/L of tris(hydroxymethyl)aminomethane hydrochloride, 50 mmol/L of potassium chloride, 4.5 mmol/L of magnesium chloride for the first step and 4 mmol/L for the second step, 0.8 mmol/L of deoxynucleoside triphosphates, 0.4 µmol/L of each primer, and 1.25 U of Taq polymerase. Amplification was carried out with 300 ng of genomic DNA in a final volume of 25 µL for 29 cycles at an annealing temperature of 55°C; then, 3 µL of the first-step product was reamplified for 20 cycles. Patient samples were evaluated together with monoclonal and polyclonal control samples and with a negative reagent control containing all PCR reagents without any template. Amplified DNA was analyzed by electrophoresis in a 2% agarose gel with ethidium bromide and visualized by UV light.

\subsection*{Heteroduplex Analysis}

PCR products were denatured for 5 minutes at 95°C and then cooled at 4°C for 15 minutes to induce duplex formation. The homoduplex and heteroduplex generated were loaded on 12% non-denaturing polyacrylamide gels in 1× tris borate EDTA buffer, run at room temperature, and stained with ethidium bromide. For sequencing, the homoduplex component was excised from the polyacrylamide gel to decrease the interference produced by the polyclonal component, resuspended in 20 µL of tris EDTA, pH 8.0, 0.1x, and treated for 45 minutes at 60°C; 5 µL of the sample then was
subjected to reamplification of the VH gene using FR2A and VLJH primers (20 cycles). PCR products were separated by size on a 2% agarose gel, specific bands were excised from the gel, and DNA was purified using the PCR clean-up gel extraction kit (Macherey-Nagel, Düren, Germany).

DNA Sequencing and Mutational Status Analysis

Sequencing was carried out using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), and the analysis was performed on an ABI 310 automated capillary system (Applied Biosystems), following the manufacturer’s instructions. All samples were sequenced in both directions. The sequences were compared with published germline sequences using the DNAplot on the Internet (http://imgt.cines.fr) to identify VH, D, and JH rearrangements and somatic mutations. The sequences with more than 2% deviation from a germline sequence in an expressed VH gene were considered mutated.

PCR Analysis of bcl-2/IgH Rearrangement

Formalin-fixed, paraffin-embedded tissue samples tested for IgH rearrangement were subjected to amplification to search for bcl-2/IgH rearrangement using a PCR technique aimed at the major breakpoint region (MBR) site on a standard thermal cycler (PCRExpress, Perkin Elmer, Boston, MA), according to published methods.23,24

Results

Clinical and Pathologic Findings

Six CD5+/CD10+ aggressive large cell lymphoma cases were selected from the period January 1987 through December 2001. All the cases were included based on the presence of following criteria: absence of a clinical history and immunophenotypic analysis compatible with SLL/CLL; absence of a t(11;14) translocation typical of MCL by cytogentic or cyclin D1 expression; presence of a diffuse component of large neoplastic B cells, consistent with the diagnosis of DLBCL; presence of a distinct follicular meshwork of follicular dendritic cells by immunohistochemical staining for the CD21 antigen in the same biopsy specimen or in previous or subsequent biopsy specimens from the same patient (cases 1 and 3); demonstration of the chromosomal translocation t(14;18) involving the bcl-2 gene, which is the genetic hallmark of FL; and expression of additional germinal center markers, such as the bcl-6 protein. The patients ranged in age from 50 to 74 years (median, 62.3 years); 4 were men, and 2 were women. Pathologic and clinical data are summarized in Table 1.

In all cases, histologic examination showed effacement of the nodal architecture by a neoplastic population composed of large B cells resembling centroblasts, with 1 or multiple nucleoli, dispersed nuclear chromatin, and mildly basophilic cytoplasm. In some cases (cases 3 and 5), the cells were medium-sized with round nuclei, resembling small noncleaved follicular center cells [Image 1]. The ratio between follicular and diffuse areas varied from 95% follicular and 5% diffuse in case 5 [Image 2] to 90% diffuse and 10% follicular in cases 2, 4, and 6 [Image 3]. Case 1 had a predominantly FL (>70%) in the first biopsy specimen, followed by a DLBCL relapse 3 years later. Conversely, case 3 had a DLBCL in 1990 followed by an FL in 1993. In these last 2 cases, samples in the first diagnosis and the relapse coexpressed CD5 and CD10. The mitotic rate was always very high, in the diffuse component and in the follicular areas. The follicular components could be classified as grade 3a or 3b according to the World Health Organization criteria.6

### Table 1

<table>
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<th>Case No./ Sex/Age(y)</th>
<th>Clinical Manifestations</th>
<th>Histologic Findings</th>
<th>Stage</th>
<th>Treatment</th>
<th>Outcome</th>
<th>Survival</th>
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<td>FL grade 3a (1987); DLBCL (1990)</td>
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<td>MACOP-B then HDS + ABMT</td>
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<td>70% FL grade 3b; 30% DLBCL</td>
<td>III</td>
<td>COP × 6 + LEM × 2 + CHOP × 1; bleomycin</td>
<td>DOD</td>
<td>22 mo</td>
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<td>3/M/69</td>
<td>Right tonsil and axillary lymphadenopathy</td>
<td>DLBCL (1990); FL grade 3b (1993)</td>
<td>IIE</td>
<td>Cyclophosphamide, then MINE</td>
<td>NED</td>
<td>14 y</td>
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<td>4/M/59</td>
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<td>NA</td>
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<td>NA</td>
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<td>IV</td>
<td>CHOP, rituximab, and ABMT</td>
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<td>24 mo</td>
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<tr>
<td>6/F/65</td>
<td>Cervical adenopathy</td>
<td>10% FL grade 3b; 90% DLBCL</td>
<td>IV</td>
<td>CHOP × 6 and rituximab × 4</td>
<td>DOD</td>
<td>42 mo</td>
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</table>

ABMT, allogeneic bone marrow transplantation; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone; COP, cyclophosphamide, vincristine, prednisone; DLBCL, diffuse large B-cell lymphoma; DOD, died of disease; FL, follicular lymphoma; HDS, high-dose sequential polychemotherapy; LEM, liposome-entrapped mitoxantrone; MACOP-B, methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone; MINE, methotrexate, ifosfamide, mitoxantrone, etoposide; NA, not available; NED, no evidence of disease.
Flow Cytometric Findings

Flow cytometric studies were performed on cell suspensions from freshly excised tissues. All 6 cases showed expression of CD19, IgM, and monotypic light chain, consistent with clonal B-cell processes. In all cases, neoplastic B cells expressed CD10, suggesting a follicle center origin for these lymphomas, as well as low to medium intensity levels of CD5 \[\text{Image 4}\]. Additional cell surface markers particularly useful for the subclassification of B-cell lymphomas included CD43, CD23, CD38, and FMC7. All cases were CD23− and CD38+; 4 of 6 were CD43+; and 2 cases expressed FMC7 at a high percentage. Flow cytometric results are summarized in Table 2.

To identify a possible normal counterpart of CD5+ FL, we studied reactive tonsils with florid follicular hyperplasia by triple staining for CD19, CD10, and CD5 surface antigens. A small but distinct population coexpressing CD19, CD10, and CD5 was evident in each tissue sample examined, indicating that in reactive germinal centers, there are cells normally expressing CD5 \[\text{Image 5}\]. Alternatively, these cells might represent a transitional population of mantle cells expressing CD10.

Immunohistochemical Results

Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded tissue samples, and results are summarized in Table 2. Neoplastic follicular and diffuse areas were composed of CD20+ B cells with admixed variable amounts of reactive CD3+ T cells. Similar to flow cytometric studies, CD10 expression was detected in the nodular and diffuse components. CD5 expression was detected in all cases with weak to medium intensity. All neoplastic B cells expressed bcl-2 and bcl-6 proteins in the follicular and diffuse components, in keeping with the germinal center origin of these tumors. Cyclin D1 expression was negative in all 6 cases.

The proliferative index was assessed by counting the percentages of the cell expressing the MIB-1 antigen. The proliferative rate was always high (range, 45%-90%), consistent with the diagnosis of high-grade FL or DLBCL. The diffuse areas showed the typical high proliferative rate of DLBCL, whereas the follicular areas had MIB-1 counts consistent with grade 3 in the World Health Organization classification.6

Molecular Biologic Findings

Molecular studies were performed on formalin-fixed, paraffin-embedded tissue samples to assess the clonality of the neoplastic B cells. Of 6 cases, 4 had amplifiable DNA, and all showed a clonal IgH rearrangement by FR2-JH PCR analysis. Rearrangement involving the bcl-2 gene (MBR site) was demonstrated by PCR in 1 of the 4 cases with amplifiable DNA (case 5). In case 1 with 2 biopsy specimens, the same clonal FR2-JH rearrangement was demonstrated.

More interesting, the clonal PCR products obtained from FR2-JH amplifications were isolated and sequenced to analyze the V\(_H\) family preferential use and the grade of somatic mutations of immunoglobulin genes from the germline sequences. In 3 (75%) of 4 cases, there was preferential use of the V\(_H\)4-34 gene. All 4 cases showed a very high degree of diversity from germline sequences (range, 7.6%-16.1%), indicating an active somatic hypermutation process in these lymphomas. Six control DLBCL cases were selected that did not show a follicular pattern of growth and lacked CD10 expression: control cases showed a variable percentage of somatic mutations (range, 2.7%-17.5%). The molecular results are summarized in Table 3.

Discussion

CD5+ de novo DLBCL cases have been identified as a distinct subset of DLBCL with particular clinical aspects, mainly the frequent association with poor prognostic components of the International Prognostic Index and with an aggressive clinical course.15,16,20 CD5+ DLBCLs have to be distinguished from aggressive CD5+ lymphomas that represent a transformed or a phenotypic variant of other NHLs. Richter syndrome-associated transformation of an SLL/CLL usually is composed of large B cells that can lose or retain CD5 expression and is genotypically distinct from de novo CD5+ DLBCL.17 MCL can manifest as aggressive variants, called pleomorphic or blastoid variants, with a very high fraction of growth and poor clinical outcome, but they are characterized as classical MCL by the t(11;14) translocation.
translocation, encoding for deregulated cyclin D1.\textsuperscript{18} Extranodal marginal zone B-cell (mucosa-associated lymphoid tissue) lymphomas or splenic MZLs express CD5+ in about 1% of the cases and can transform to CD5+ DLBCL.\textsuperscript{8} Moreover, rare cases of de novo CD5+ Burkitt lymphoma/leukemia have been described and can be recognized by the presence of the classic translocations involving the c-myc gene on chromosome 8, as well as other diagnostic surface markers.\textsuperscript{10}

Excluding these genetically distinct cases, the histogenesis of the majority of de novo CD5+ DLBCL remains unclear.

We report 6 cases of CD5+ DLBCL with morphologic, phenotypic, and genetic data in favor of an origin from follicle center cells. The cases were selected from a database of 41 CD5+ aggressive B-cell lymphomas collected during a 14-year period in our surgical pathology department. We excluded 35 cases because they could be demonstrated to be aggressive variants of MCL (23 cases) by the presence of overexpressed cyclin D1, transformation of CD5+ mucosa-associated lymphoid tissue lymphoma or splenic MZL (6 cases), Burkitt lymphoma by the cytogenetic demonstration of the t(8;14) (1 case), or CD5+ DLBCL with unclear origin, given the absence of diagnostic morphologic, phenotypic, or genetic data (5 cases).
**Image 3** (Case 2) Morphologic and immunophenotypic aspects. This case showed areas with a follicular growth pattern (A, H&E, ×100) adjacent to areas with diffuse growth (B, H&E, ×200) composed of large neoplastic cells mainly resembling centroblasts consistent with diffuse large B-cell lymphoma (inset, H&E, ×400). The neoplastic cells were bcl-2+ (C, ×100) and CD5+ (D, ×100). Immunostaining with CD21 highlighted a well-defined meshwork of follicular dendritic cells in the nodular areas (E, ×100) that was completely lacking in the diffuse areas (F, ×100). Inset, High-power magnification of a residual germinal center (CD21, ×400).
All 6 cases in our study showed monotypic surface light chain restriction on the neoplastic B cells and coexpressed CD5, as confirmed by flow cytometric and immunohistochemical studies. Large neoplastic B cells also expressed follicle center cell–associated antigens, CD10 and bcl-6 protein. Morphologically, all selected cases showed areas of nodular growth with a follicular nature demonstrated by the presence of CD21+ follicular dendritic cells and a surrounding area of IgD+ mantle cells. The proportions of the diffuse large cell components and the follicular areas varied from case to case and in 2 cases were evident in a previous (case 1) or subsequent (case 3) biopsy specimen.

The presence of the CD5 antigen in FLs was overlooked until recently. First, Tiesinga et al described 4 low-grade CD5+ FLs with features of the so-called floral variant of FL. In the 3 case series from Barry et al, cases 2 and 3 had a high proliferative index as evaluated with the MIB-1 antigen (with >50% of the cells positive), underlining the existence of high-grade CD5+ FL. However, the cases were described having a completely nodular pattern of growth, excluding the concomitant presence of a DLBCL. A large immunophenotypic study on CD5+/CD10+ lymphomas described 10 more cases of CD5+ FL. In this last series, however, no description was given of the grading of FL or of the presence of a high-grade diffuse component. Overall, CD5+ FLs are rare, probably representing less than 1% of the FLs in the different series.

Molecular analysis was performed on DNA extracted from formalin-fixed, paraffin-embedded tissue samples. In 2 cases, DNA was degraded and no amplification of the IgH genes was obtained. In the remaining 4 cases, IgH gene rearrangement confirmed clonality of the process. A t(14;18) involving the MBR site was demonstrated in 1 of 4 cases. However, PCR detection of the t(14;18) by means of MBR-specific primer sets have high false-negative rates on fixed material. Unfortunately, fluorescence in situ hybridization analysis for t(14;18) was unsuccessful owing to the presence
of acetic acid in our buffered formalin used to fix the samples. The VH family use and the percentage of somatic mutations was analyzed in the amplifiable cases. All 4 cases displayed a high rate of mutations compared with most control DLBCL cases (Table 3), indicating antigen selection in the somatic hypermutation process. The percentages and kind of somatic mutation found in our series are quite similar to those obtained in follicle center–derived lymphomas.26

The biologic and clinical relevance of CD5 expression in high-grade and transformed FL is unclear, given the small number of cases. Unusual CD5 expression has been associated with disseminated disease and aggressive behavior in low-grade lymphomas, such as MZL,8 and in high-grade lymphomas. CD5+ DLBCL cases have a more aggressive clinical course, with decreased failure-free survival and more frequent bone marrow involvement than CD5− DLBCL cases.16

In our series, all but 1 patient had peripheral adenopathy at diagnosis. The remaining case (case 1) had salivary gland involvement followed by a recurrence in the supraclavicular lymph node 3 years later. Two patients (cases 5 and 6) had stage IV disease, 1 had stage III, and 1 had stage IIIE. Of 6 patients, 4 died of disease (median survival, 28.5 months), 1 was lost to follow-up, and 1 (case 3), who had stage IIIE disease at diagnosis, was alive and free of disease 14 years after initial diagnosis. These clinical data are consistent with the aggressive clinical behavior of this phenotypic variant of lymphoma.

Our data indicate the existence of a small CD5+/CD10+ population in normal germinal centers, corresponding to about 1% of the reactive B cells. This population could represent CD10+ follicular center cells that express CD5. Alternatively, they could be CD5+ mantle cells that may be destined for the germinal center. It therefore is possible that a CD5+ FL can develop from this population directly as a high-grade lymphoma or as a low-grade lymphoma that can progress to higher grades and eventually to a CD5+ DLBCL. The frequency of CD5+ FL (around 1%) and the high rate of somatic hypermutation of the IgH variable regions is in agreement with this hypothesis.

The clinical and therapeutic relevance of CD5 expression in these lymphomas is still to be determined. In certain B cells27 or in B-cell tumors, such as CLL, CD5 stimulation can lead to apoptosis28; patients with CD5+ DLBCL then could be treated with chemotherapy and additional CD5-targeted antibodies or drugs.

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Table 3

Somatic Hypermutation Rate in Series and Control Cases

NA, not available.

Cases 7-12 are the control cases (CD5+/CD10− diffuse large B-cell lymphoma).
References


