Anaplastic Large Cell Lymphomas Expressing the Novel Chimeric Protein p80<sup>NPM/ALK</sup>: A Distinct Clinicopathologic Entity


Anaplastic large cell lymphoma (ALCL) is a subtype of non-Hodgkin's lymphoma characterized by the CD30<sup>+</sup> large neoplastic cells and sometimes carries a t(2;5)(p23;q35). Recently, we found a novel hyperphosphorylated 80-kD protein tyrosine kinase, p80, in ALCLs with t(2;5). Subsequent cDNA cloning showed p80 to be a fusion protein of two genes, the novel tyrosine kinase gene and the nucleophosmin gene, in accordance with the sequence of the NPM/ALK gene (Morris et al, Science 263:1281, 1994). Meanwhile, the clinicopathologic features of p80-carrying ALCLs have remained unclear. Paraffin sections of 105 cases of ALCL were immunostained using anti-p80 antibody, and 30 of them were shown to express p80. Clinicopathologic comparison between p80-positive and -negative ALCLs showed that p80-positive cases occurred in a far younger patient age group (16.2 ± 12.9 years; p80-negative cases, 51.0 ± 22.3 years; P < .0001) and the patients showed a far better 5-year survival rate (79.8%; p80-negative group, 32.9%; P < .01). These data showed that p80-positive ALCL is a distinct entity both clinically and pathogenetically and should be differentiated from p80-negative ALCL.

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CLINICOPATHOLOGIC STUDY OF ALCL WITH t(12;5)

MATERIALS AND METHODS

Selection of cases. Cases that satisfied the morphologic criteria of ALCL and also expressed CD30 were selected. Cases that had been diagnosed as ALCL were collected from the investigators' case records. Microscopic slides of these cases were reviewed by two hematopathologists (S.M. and M.S.) and those that satisfied the morphologic criteria of ALCL were selected. The criteria were based on the recently published REAL classification (Revised European-American Classification of Lymphoid Neoplasms), as follows:

"Neoplastic cells composed of large blastic cells with often horse-shoe-shaped or multiple nuclei with multiple or single prominent nucleoli. Occasional presence of multinucleated forms resembling Reed-Sternberg cells. Tumor cells much larger in size than the cells of usual large cell lymphomas, with more abundant cytoplasm. Cells growing in a cohesive pattern, often preferentially involving the lymph node sinuses."

Subsequently, cases for which sufficient clinical data and good paraffin sections were available were selected. Cases whose clinical courses remained unclear were excluded. Cases lacking information for CD30 were immunostained with anti-CD30; if unstained, the cases were excluded. To avoid any misjudgement of specimens whose antigenicity was lost during fixation and paraffin-embedding, all of the specimens were immunostained with antibodies that have been applied for staining of routine paraffin sections, including L26 (CD20, pan-B-cell antibody; Dakopatts, Copenhagen, Denmark), UCHL1 (CD45RO, pan-T-cell antibody; Dakopatts), or proliferating cell nuclear antigen (PCNA; Dakopatts); if unstained, these cases were excluded. As controls, 20 NHLs other than ALCL and 20 cases of Hodgkin's disease were also studied. The NHLs consisted of 2 small lymphocytic lymphomas; 2 mantle cell lymphomas; 7 follicular center cell lymphomas, follicular; 3 extranodal marginal zone lymphomas; 21 diffuse large lymphomas; 1 Burkitt's lymphoma; 10 peripheral T-cell lymphomas, unspecified; 2 angioimmunoblastic lymphomas; and 2 adult T-cell lymphomas. The 20 Hodgkin's disease cases consisted of 1 lymphocytic predominance, 10 mixed cellular, 5 nodular sclerosis, and 4 lymphocytic depletion subtypes.

Immunostaining. Anti-p80 antibody as well as BerH2 (CD30; Dakopatts), L26, UCHL1, and PCNA were used as the first reagents for immunostaining. The labeled avidin-biotin-peroxidase method was used as the staining procedure. Antibodies other than anti-p80 were used for two purposes: (1) to clarify the immunophenotypes of the neoplastic cells and (2) to evaluate the quality of paraffin-embedded tissue sections, as described above. The immunologic specificities of anti-p80 antibody have been reported previously. For the second- and third-phase immunostaining reagents, biotinylated swine antirabbit or rabbit antimouse IgG (Dakopatts, Denmark) and peroxidase-conjugated streptavidin (Dakopatts) were used.

To expose antigens from fixed tissues, the paraffin sections were first treated with microwave radiation. Deparaffinized slides were placed in a plastic Colpin jar (Fischer, California) containing 5% urea in dH2O, heated twice with microwave radiation at 950 W (Hitachi MRO 4500, Tokyo, Japan) for 5 minutes each, and washed with phosphate-buffered saline (PBS), pH 7.4. To remove endogenous peroxidase, the slides were then rinsed in 80% methanol containing 0.8% H2O2 for 20 minutes. After washing with PBS and blocking of nonspecific antibody binding with 20% swine serum, the slides were incubated with appropriately diluted first antibodies for 1 hour at 37°C, washed with PBS, incubated with the second- and third-phase reagents for 30 minutes each, and washed again. They were then colored with 0.6 mg/mL 3-3' diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO) in PBS containing 0.01% H2O2 for 5 minutes, dehydrated, and mounted.

To substantiate the validity of immunostaining with anti-p80 antibody on paraffin sections, 10 ALCLs for which unfixed frozen tissues were available were processed into unfixed-frozen and paraffin sections and immunostained and the results were compared. As another control study, paraffin sections of 50 NHLs other than ALCLs and 20 cases of Hodgkin's disease were immunostained with anti-p80 antibody. As a control for the anti-p80 antibody, rabbit preimmune serum that had been taken in advance from the rabbit in which the anti-p80 antibody was raised was used.

Statistical analysis. Clinical data, including age at onset, sex, primary site, symptoms, vital signs, laboratory data, response to treatment, and clinical endpoint, were compiled from the patients' clinical charts. Immunologic data for neoplastic cells were also compiled from the charts, and in cases in which the data were insufficient, the results of the present immunostaining were added. The mean age at onset was tested using the Mann-Whitney U test, and the 5-year survival curves were compared using the Cox-Mantel test. Correlations between p80 expression and various clinical, pathologic, or immunologic features were determined by chi-square test. Multivariate analysis was performed to clarify the degree of variable factors in p80-positive ALCLs with the introduction of multiple regression analysis.

RESULTS

One hundred forty cases were collected as the initial case sets. Thirty-five cases were excluded during case selection. The remaining 105 cases were finally used for further analysis. The excluded cases comprised 4 with insufficient clinical data, 20 that were histopathologically different from the diagnostic criteria of ALCL, and 11 that showed a poor immunoreaction to the control antibodies. Among the 105 selected cases, 30 reacted immunohistologically with anti-p80 antibody. In a large proportion (25/30), the staining was heavy and clear in whole-tissue sections. However, in 5 of 30 cases, good staining was observed only in the marginal portions, leaving the central portion unstained or very faint, probably because of a difference in fixation efficiency. The sites mainly stained were the nuclei in some cases (7/30) and the cytoplasm in others (16/30), whereas in the remaining cases both the nuclei and cytoplasm were stained evenly (7/30; Fig 1). In controls, the preimmune serum did not react with any of the 105 ALCLs. Also, paraffin sections of the 50 NHLs other than ALCLs and the 20 cases of Hodgkin's disease remained unstained. For 10 ALCLs whose unfixed-frozen and paraffin-embedded sections were studied, both sections in each case showed identical results, ie, both positive or both negative. Thus, these intensive control studies substantiated the validity of the present immune reaction on paraffin sections of 105 cases selected.

Clinical aspects of the p80-positive and -negative ALCLs were then analyzed (Table 1). As to the age at onset, the p80-positive ALCLs were found to occur mostly in childhood or adolescence. The mean age of the patients was 16.2 ± 12.9 years, in sharp contrast to patients with p80-negative ALCLs, whose mean age was 51.0 ± 22.3 years (P < .0001, Mann-Whitney U test; Fig 2). However, it must be remembered that the vast majority of young ALCL patients did not show the p80 protein, because 14 of the 40 ALCLs who were 30 years of age or younger were negative for this protein. In the study of clinical outcome, the p80-positive group was shown to have a far better course, with a 5-year survival rate of 79.8%, again contrasting sharply to the p80-negative cases, whose 5-year survival rate was only 32.9%. The sur-
vival curves of these groups were shown in Fig 3 ($P < .01$, Cox-Mantel test$^{27}$). In addition, the survival curve and rate of the patients less than 30 years of age were compared. It was found that even in this age group, p80-positive cases showed a far better survival curve ($P < .05$ by both Cox-Mantel test and generalized Wilcoxon test) and 5-year sur-

Fig 1. Representative morphology and immunohistology of p80-positive ALCLs. (A) Immunostaining with anti-p80; (B) immunostaining with anti-CD30 (BerH2; Dakopatts); (C) hematoxylin-eosin staining. p80-positive ALCL showing monomorphic histology.

Fig 4. Representative morphology and immunohistology of p80-negative ALCLs showing, in part, the pleomorphic morphology. (A) Immunostaining with anti-p80; (B) immunostaining with anti-CD30; (C and D) hematoxylin-eosin staining. A part of p80-negative ALCL shows pleomorphic histology (C), whereas, in the remaining part, typical morphology of ALCL is noted (D).
Table 1. Clinical, Pathologic, and Immunophenotypic Differences Between p80-Positive and -Negative ALCLs

<table>
<thead>
<tr>
<th></th>
<th>p80 Positive</th>
<th>p80 Negative</th>
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<tbody>
<tr>
<td>Total no.</td>
<td>30</td>
<td>75</td>
</tr>
<tr>
<td>Average age (yr)*</td>
<td>16.2 ± 12.9</td>
<td>51 ± 22.3</td>
</tr>
<tr>
<td>5-yr survival rate (%)†</td>
<td>79.8</td>
<td>52.9</td>
</tr>
<tr>
<td>Primary site (LN/others)‡</td>
<td>23/7</td>
<td>40/35</td>
</tr>
<tr>
<td>Immunophenotype (T/B/N)</td>
<td>14/0/16</td>
<td>36/11/28</td>
</tr>
</tbody>
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Abbreviations: LN, lymph node; T, T-cell type; B, B-cell type; N, null cell type.

P < .0001, Mann-Whitney U test.
† P < .01, Cox-Mantel test.
‡ P < .02, Chi² test.

The primary sites of the p80-positive ALCLs were lymph nodes in 23 cases and extranodal sites in 7 (skin in 4, mediastinum in 1, soft tissue in 1, and bone in 1). In the p80-negative group, the primary sites consisted of lymph nodes in 40 cases and extranodal sites in 35 (skin in 16, mediastinum in 8, liver in 2, soft tissue in 2, bone in 2, salivary gland in 2, stomach in 1, intestine in 1, and chest wall in 1). Upon statistical analysis of these data, although extranodal occurrence was observed in both groups, p80-positive cases were found to occur more frequently in lymph nodes (P < .01, Chi² test). Meanwhile, our clinical data were insufficient to make a generalization of the difference between the two groups in their responses to various therapeutic protocols. Also, no differences were noted in sex (p80-positive group, M:F = 21:9; p80-negative group, M:F = 52:23), clinical symptoms, vital signs, or laboratory data between the two groups.

After immunophenotyping, about half of the p80-positive cases showed the CD45RO+ CD20- phenotype (14/30), whereas the remainder was CD45RO+ CD20+ (16/30). One noteworthy point was the absence of p80-positive ALCLs expressing CD20. In contrast, 11 of 75 p80-negative cases were of the CD20+ CD45RO+ immunophenotype, 36 of 75 were of the CD45RO+ CD20+ phenotype, and 28 of 75 were of CD45RO+ CD20- phenotype (Table 1). These findings suggest that B-cell NHLs can express CD30 and a large anaplastic cell morphology but are apparently different from p80-positive ALCLs.

Histopathology showed that a large proportion of p80-positive cases (26/30) had the typical morphology of ALCL; the neoplastic cells were rather uniform large anaplastic cells growing in a cohesive pattern with a poor stromal reaction (Fig 1). In the remaining 4 cases, proliferation of small vessels was observed in the stroma, whereas the neoplastic cells themselves showed morphology identical to that of these 26 cases. Cytomorphology showed that p80-positive ALCLs were basically uniform in size, although huge and did not show the pleomorphic morphology that is a common finding in ordinal peripheral T-cell NHLs. Such pleomorphic morphology was found somewhat frequently in the p80-negative group (23/75; Fig 4C and D). In these cases, the pleomorphic morphology was noted in a part of the slides, whereas the typical morphology of ALCL, which is composed of uniform large cells growing in cohesive pattern, was observed in other fields of the same slides (Fig 4C and D). These findings suggest that the ordinal peripheral T-cell lymphomas can sometimes show a morphology closely similar to ALCL but are different from p80-expressing ALCLs.

To clarify the degree of variability in the clinicopathologic factors that characterize the p80-positive group, a multivariate analysis was performed (Table 2). This analysis showed that, among five variables (age, sex, primary site, prognosis, and immunophenotype), age was the most powerful predictor of p80 positivity.

DISCUSSION

The classification of NHLs has long been based on morphology. The recent introduction of immunophenotyping
has led to drastic remodeling of this classification, and new NHL subtypes based on this new method have been proposed. Immunology is a very powerful tool for the clarification of phenotypes, including lineages, differentiation levels, clonality, and ontogeny of new and old NHL subtypes. However, it cannot elucidate the pathogenetic mechanisms of each subtype alone, and such mechanisms have been clarified only through the introduction of molecular biology. As the pathogenesis has become clearer, some subtypes have been shown to contain pathogenetically heterogeneous cases. ALCL is an example of the latter. The heterogeneity of ALCLs has been suggested recently by marker studies (existence of T-cell– and B-cell–derived ALCLs), by a bimodal distribution of age at onset, and especially by the presence of the chromosomal translocation t(2;5) in some but not all ALCLs. However, because of the limited number of cases analyzed in detail, various aspects of this heterogeneity have remained unclear.

The association of various chromosomal translocations with specific NHL subtypes has been well studied. For each of these translocations, some cell multiplication-associated proteins, such as c-myc in Burkitt’s lymphomas, bcl-2 in follicular lymphomas, the PRAD gene in mantle cell lymphoma, and bcl-6 gene in diffuse large B-cell lymphoma, have been shown to play important roles in tumorigenesis. With regard to ALCLs with t(2;5), recent molecular studies by our group and Morris et al have shown that the novel chimeric protein p80NPM/ALK may be the primary causative agent. Indeed, p80 is greatly suspected to cause ALCL, primarily because transfection of p80 cDNA into NIH3T3 cells induces their transformation very easily, and hence this protein is assumed to be a new oncogenic protein (manuscript submitted). Thus, the classical ALCLs are now divided into p80-positive and -negative groups. Here, p80-positive ALCLs are considered to be pathogenetically identical, whereas p80-negative ALCLs are composed of still heterogeneous cases.

Immunohistology is an easy and convincing method for differentiating these two ALCL groups. However, in performing such an analysis, intensive control studies must be performed in advance. Control studies would include checking the specificity of the antibody and the reliability of the immune reaction on paraffin sections. The specificity of anti-p80 antibody for immunohistology on unfixed-frozen sections has been studied intensively in a previous investigation. We concluded that the antibody reacted specifically with t(2;5)-carrying ALCLs among various NHL tissues. Through this study, we also confirmed that the antibody reacted with formalin-fixed and paraffin-embedded pathology specimens. This was a valuable finding because it proved the applicability of the antibody to the study of many ALCLs stored in pathology files. However, for immunostaining of paraffin sections, other intensive control studies were required because many pathologists have found that formalin-fixed, paraffin-embedded specimens sometimes lose their antigenicity through improper fixation or embedding. Therefore, we first compared the results of immunostaining on paired unfixed-frozen sections and paraffin sections from 10 ALCLs. Because all of the results from these paired samples matched perfectly, we performed another control study that involved the staining of paraffin sections from all ALCLs with antibodies that are used routinely on paraffin sections (L26, UCHL1, and PCNA). In this control study, 11 cases were found to be unstained with some of them and were therefore excluded. We believe that the results of our control studies sufficiently justify the reliability of the present findings, although it is still theoretically possible that some p80-positive but immunohistologically unstained cases may be present, because the preservation of CD antigens or PCNA in certain tissues does not necessarily imply preservation of p80 antigenicity.

Thus, 30 cases were selected as p80-positive ALCLs. It may be interesting to know if all ALCLs with t(2;5) express p80. However, cytogenetic studies have not been performed in most of the present cases and, thus, we could not show the incidence directly. The introduction of reverse transcription-polymerase chain reaction (RT-PCR) on p80 mRNA seemed invalid on the present specimens that were formalin-fixed and paraffin-embedded. Few reports have ever referred to the incidence of t(2;5) in ALCLs. Three t(2;5)-carrying cases among 9 ALCLs, 2 t(2;5) among 5 ALCLs, and 3 t(2;5) among 10 ALCLs are the only reliable data reported so far. Furthermore, the latter study (our previous work) was the only one that referred directly to the incidence of p80-positive cases so far. In that report, we had shown that all 3 of the t(2;5)-carrying ALCLs but none of the 7 t(2;5)-negative ALCLs expressed p80.

It might be worth adding a comment on Hodgkin’s disease. Recently, Orscheshek et al and Bullrich et al identified t(2;5)-associated PCR products in considerable number of cases of Hodgkin’s disease. Meanwhile, our present study as well as our previous study could not detect p80 or t(2;5)-associated PCR products in more than 100 cases of Hodgkin’s disease. At present, we cannot clarify why such a gap appeared among these groups. Some cooperative studies among these groups will be required to solve this mystery.

The differences in clinical features between p80-positive and -negative ALCLs were striking. p80-positive cases were shown to occur in far younger patients, with a mean age at onset of 16.2 years. In previous reports, a bimodal distribution or occurrence in a younger age group have been described for ALCLs in general. The present study showed very clearly that p80-positive cases account for the majority of cases.
of ALCLs occurring in younger patients, limiting the view of Nakamura et al only to p80-positive cases. The difference in the 5-year survival rate was also remarkable. The rate for p80-positive ALCLs was around 80%, placing these cases in the low-grade malignancy group. Some previous reports have also indicated a favorable outcome of ALCLs, whereas the present study clearly showed that such a favorable course was true only for p80-positive cases and not for the p80-negative group, because the 5-year survival rate of the latter group was only 33%. These clinical data suggest that p80-positive and -negative ALCLs will behave differently in response to various therapeutic procedures. The assumption that the better natural course of p80-positive cases may be a reflection of the better course in the younger age group seemed rather unlikely because the difference in survival between these two groups was again evident among patients less than 30 years of age. New clinical studies of p80-positive ALCLs now seem warranted.

Histopathologically, the difference between p80-positive and -negative ALCLs was not as striking as the clinical features. Routine histopathology studies and immunophenotyping cannot differentiate between them, and the use of immunostaining with anti-p80 is essential in this respect. A noteworthy morphologic feature was that cellular and nuclear pleomorphism, which is commonly observed in ordinal peripheral T-cell lymphomas, was never found in p80-positive cases. This finding suggests that the ordinary peripheral T-cell lymphomas bearing CD30+ large cells, which are categorized in the high-grade malignancy group, are entirely different from t(2;5)-associated ALCLs and thus can and have to be differentiated from p80-positive ALCLs. Also, it may be worth stressing that not a single case of p80-positive ALCL showed a B-cell phenotype. This finding again suggests that B-cell NHLs can at times express CD30 and a large, anaplastic cell morphology, but are apparently different from t(2;5)-associated ALCLs. The new REAL-classification as well as the updated Ki-1 classification seems intent on excluding cases with B-cell markers from ALCLs. The present results support this idea on p80-positive ALCLs but not on p80-negative ALCLs.

Finally, the present study clearly showed that p80-positive ALCL is a distinct clinicopathologic entity. Considering the importance of clinical and pathogenetic differences, all of the ALCLs should thereafter be separated into p80-positive and -negative groups.

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