ANAPLASTIC LARGE CELL LYMPHOMAS AND HODGKIN'S DISEASE: DISTINCTIVE GROWTH FEATURES AND EXPRESSION OF p34^{cdc2} / CYCLIN B-1

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ABSTRACT

The clinical aggressiveness of a neoplastic process is largely determined by its growth, which results from cell production minus cell loss. We evaluated the potential of growth-related parameters in the differential diagnosis of Hodgkin's disease (HD), anaplastic large cell lymphoma, common type (ALCL-C), and its Hodgkin's like variant (ALCL-HL). These three conditions share some common properties, e.g. CD30 positivity. Highly significant differences were found between HD and ALCL-C. In particular, CD30$^+$ cells in HD exhibited markedly higher mitotic indices (MI), multinucleation indices (MNI), DNA fragmentation indices (DFI, comparable to apoptotic indices) and percentages of mummified elements (MF), but distinctly lower ana-telophase indices (ATI, index of supposedly successful mitoses) than those in ALCL-C. Interestingly, the percentages of Ki-67 (MIB-1)$^+$ large atypical cells (LAC), often referred to as " growth fractions", did not differ significantly among the three lymphomas types tested. In view of the disturbed mitotic process of CD30$^+$ cells in HD, special attention was paid to the two major proteins regulating the G2-M phases of the cell cycle: distinctly lower percentages of LAC expressing cyclin B-1 (shortly: cyclin-B) in cytoplasm and nucleus (BCN) were registered in HD than in ALCL-C, while the reverse was true for the presence of p34^{cdc2} (shortly, p34). As regards the above parameters, ALCL-HL took a somewhat intermediate position between HD an ALCL-C, but was closer to the latter. A stepwise discriminant analysis revealed the following order of discriminant power of the parameters used to differentiate between the lymphomas: BCN > MNI > p34 > ATI > MF > MI. The other parameters tested, including the percentages of Ki 67$^+$ CD30$^+$ cells, were of little or no importance in this respect. Thus, anti-cyclin-B and anti-p34 antibodies may be useful tools in an immunohistochemical distinction of HD and ALCL.

Keywords: malignant lymphomas; growth; p34; cyclin B-1; Hodgkin's disease; anaplastic large cell limphoma.

Abbreviations used:
ATI, ana-telophase index; ALCL, anaplastic large cell (CD30$^+$) lymphoma; ALCL-C, anaplastic large cell lymphoma, common type; ALCL-HL, anaplastic large cell lymphoma, Hodgkin's like; BCN, C, cytoplasmic; C+N, cytoplasmic and nuclear; cdk, cyclin-dependent kinase; cyclin-B, cyclin B-1; DFI, DNA fragmentation index; H, Hodgkin; HD, Hodgkin's disease; HPF, high power field; ISEL, in situ end labeling of DNA; LAC, large atypical cells; MI, mitotic index; MNI, multinucleation index; N, nuclear; MPF, maturation/mitosis promoting factor; p34, p34^{cdc2} protein.

INTRODUCTION

The REAL classification of malignant lymphomas (Harris et. al., 1994) identifies disease entities, based on phenotypic and genotypic features, that may each have a range of morphologic grade and clinical aggressiveness. Thus, greater emphasis is now placed on parameters that reflect the state of growth of a given lymphoma, in addition to the histologic appearance, the immunophenotype, and, possibly, the genotype. Neoplastic growth results from cell production that exceeds cell loss (Steel, 1977), so that any attempt to estimate its magnitude must include the cell deletion factor (Del Vecchio et. al., 1991). However, apoptosis as a key physiological regulator of cell population size has, until recently, not received the attention it deserves (Hall and Coates, 1995).

The present study was undertaken to compare growth characteristics, including cell loss indices, of the two major CD30+ lymphomas, i.e. classical Hodgkin's Disease (HD) and anaplastic large cell lymphoma (ALCL), which occurs as a common type (ALCL-C) and as a provisional, Hodgkin's like entity (ALCL-HL, Harris et. al, 1994). Although HD and ALCL exhibit a number of quite distinctive, histologic, immunohistochemical, and cytogenetic features (Filippa et.al, 1996; Carbone et. al, 1996) they may in part show overlapping phenotypes (Leoncini et. al, 1990) and share, in rare cases of HD, identical genomic alterations, such as NPM-ALK gene rearrangements (Yee et. al, 1996). Since in HD many Hodgkin (H) cells exhibit abortive mitoses with an arrest at the metaphase-ana/ telophase transition and consecutive multinucleations (Reed Sternberg (RS) cells) and / or death (often in the form of so-called mummified cells) (Leoncini et.al, 1996; Spina et. al, 1996), it was necessary to introduce some additional indices that take these peculiar phenomena of disturbed proliferation into account (see under "Materials and Methods"). Since the progression of cells through the G2 and mitotic (M) phase of the cell cycle is largely dependent on the presence of the maturation/mitosis promoting factor (MPF) (Norbury and Nurse, 1992), which consists of cyclin B-1 complexed with the protein p34cdc2 (shortly, p34) (Reed, 1992; Grana and Reddy, 1995), we examined the presence of these two regulatory proteins by use of monoclonal antibodies that recognize epitopes of the respective molecules in formalin-fixed tissue (Kawamoto et. al., 1997).

MATERIALS AND METHODS

Selection of cases and conventional histology / immunophenotyping

A total of 71 cases of nodular sclerosis and mixed cellularity classical HD (nodular sclerosis n= 24, mixed cellularity n= 12, total n=36), ALCL-C (n=16), or ALCL-HL (n=19)

was retrieved from the files of the Unit of Hemolymphopathology, Institute of Hematology "L. & A. Seragnali", University of Bologna, and the Institute of Pathologic Anatomy and Histology, University of Siena. The various lymphomas were accepted to enter the study based on the following: no treatment prior to diagnosis, rapid post-surgery fixation of sliced specimens in a buffered 4% formaldehyde solution, pH = 7.4, adequate amounts of tissue in the paraffin blocks, and absence of large areas of necrosis. Details concerning age and sex of patients, and stage of the disease are summarized in Table I. For qualitative histologic evaluation, 4 µm thick sections were stained with hemalum-eosin, Giemsa, periodic acid Schiff reagent (PAS), Mallory trichrome, and Gomori's silver impregnation. For enhanced antigen retrieval, deparaffinized sections were subjected to microwaves (Cattoretti et. al, 1993) or boiling in a 10 mM citrate buffer, pH = 6.0, for 10 minutes. Standard immunophenotyping was carried out by use of a panel of antibodies, including the MIB-1 anti-Ki-67 antibody (immunotech, Marseille, France), and the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique as described previously (Leoncini et. al, 1997). Staining for CD30 proved to be useful because neoplastic cells, in particular H-RS cells, cannot always be recognized as such when they are in mitosis or when their nuclei are covered by other reaction products. We used normal human tonsils for positive control of standard immunophenotyping, including p34 and cyclin B-1, and ALCLs for the detection of CD30. Negative controls were obtained by replacing the primary antibody by normal mouse serum. Diagnoses were made with the knowledge of standard immunohistochemical reactions, first by 2 experienced pathologists (S.P. and L.L.) and then by consensus based on the REAL classification (Harris et. al, 1994).

Table 1. Summary of malignant lymphomas studied.

Lymphoma type	Number of cases	Age of patients(yrs); median (range)	Male/female ratio	Ann Arbor stage			
				I	II	III	IV
Hodkin's disease, nodular sclerosis (HD, NS)*	24	30 (15-57)	1.1. : 1	7	11	4	2
Hodkin's disease, mixed cellularity (HD, MC)*	12	44 (27-71)	1.8. : 1	3	4	2	3
Anaplastic large cell lymphoma, Hodkin's like (ALCL-HI)	19	35 (12-67)	1.4 : 1	5	4	6	4
Anaplastic large cell lymphoma, common type (ALCL-C)	16	29 (8.79)	1.9 : 1	3	4	5	4

* In this study, variants of classical Hodgkin's disease (HD = HD, NS+HD, MC) were treated as one entity.

Immunohistochemistry to detect p34 and cyclin B-1

Preparation of paraffin sections for these immunohistochemical reactions was the same as mentioned above, including the procedure to better unmask the antigens. We made use of two mouse monoclonal antibodies , purchased from Neomakers (Fremont, CA, USA), i.e., anti-p34[cdc2] serine/threonine kinase/cdk AB-1 (clone A 17.1.1) for p34, and anti-cyclin B-1

AB-1 (clone V152) for cyclin B-1. The staining intensity was not quantified but evaluated qualitatively. Because of overlap of reaction products over nuclei and/or cytoplasm, double staining for CD30 and p34 or cyclin B-1 was not carried out. As regards the immunohistochemical reaction to the anti-p34 antibody, we considered those cells as positive that showed cytoplasmic and nuclear staining. Cyclin B positivity was registered as either cytoplasmic (C) or cytoplasmic and nuclear (C+N).

Double staining for CD30 and DNA strand breaks

This was carried out by combining in situ end labeling (ISEL) of DNA (Ansari et al., 1993) with immunostaining for CD30. Since H-RS cells often disintegrate as so-called mummified cells (Leoncini et al., 1996) and not as apoptotic cells/bodies, we employed this methods rather than the assessment of an apoptotic index to register the relative magnitude of cell death (Spina et al., 1996).

Cells counts and indices related to growth, multinucleation and cell death.

In a first step, the mean number per case of CD30+ and CD30- lymphoid cells per high power field (HPF, 56000(m2, examined with an oil immersion objective (100x)), was assessed in 20 HPF chosen at random. In the entire section (>100 HPF) CD30+ cells in individual phases (pro-,meta-, ana- and telophase) of mitosis were then registered and expressed as percentages of all CD30+ cells, i.e., as mitotic index (MI) and as ana-telophase index (ATI). In the same way, the DNA fragmentation index (DFI), i.e., the fraction of CD30+ ISEL+ cells as percentage of all CD30+ cells, was calculated. We also assessed the relative number of mummified cells, expressed as fraction (%) of all CD30+ cells. Multinucleated cells were counted to obtain the multinucleation index(MNI (in %)= number of nuclei in multinucleated CD30+ cells/ total number of nuclei in CD30+ cells x 100). Neoplastic cells immunostained for p34 or cyclin B-1 were identified morphologically (large atypical cells, LAC) and their relative numbers were registered as percentages of all LAC. We did the same for KI-67+ cells.

Intra- and inter-observer correlations, assessed by comparisons of repeated counts on the same section, but different HPF, of a number of cases by two independent pathologists (SL and CB) were significant ($r \geq 0.95$; $p = 0.01$).

In situ hybridization to show EBV infection

The presence of EBV-encoded-RNAs (EBERs) was examined by use of an in situ hybridization technique, described in detail by Luzi et al.(1994).

Statistics

For each variable and group, we first computed mean, standard deviation, standard error, coefficient of variation, extreme values and frequencies. Then univariate analyses were carried out, using the one-way univariate analysis of variance (ANOVA), the Welch statistics (Brown and Forsythe, 1974), pair-wise t-tests of group means and the Bonferroni test. Finally, we performed a stepwise discriminant analysis based on Wilks' lambda (Jennrich, 1977) and the F-statistics (Rao, 1965). To obtain a reliable estimate of the misclassification rate when calculating the confusion matrix, the jackknife technique(leaving-one-out method, Artioli et al., 1991) was applied. We considered p values equal to or smaller than 0.05 as significant. Data were processed by use of the BMDP statistical software package and the MATLAB software.

RESULTS

Morphology and immunostaining

The histologic appearrance of ALCL-C and HD, immunostained for cyclin-B and p34, respectively, is shown in Fig.1.
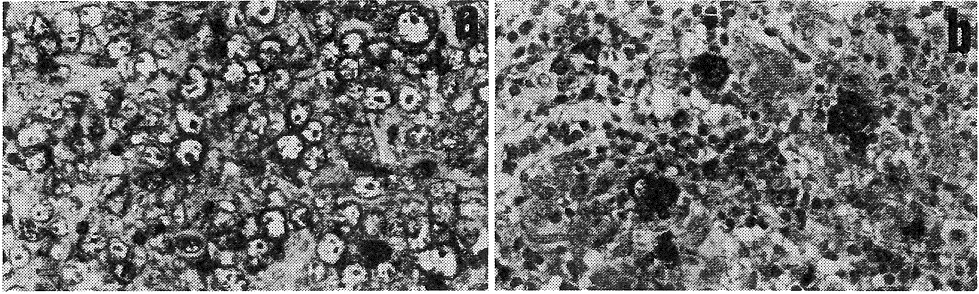

Fig. 1. *Immunostained paraffin sections. a) Anaplastic large cell lymphoma, common type,   with large atypical cells (LAC) showing predominantly cytoplasmic (C) cyclin-B positivity. b) Hodgkin's disease, nodular sclerosis type, with LAC exhibiting cytoplasmic and nuclear (C+N) immunoreactivity for p 34.*

Proliferative indices and multinucleation

As shown in Figure 2a, the MI of CD30^{+} cells was significantly ($p < 0.001$) higher in HD than in ALCL-C, while the latter did not differ in this respect from ALCL-HL. An inverse pattern was observed, when the ATI rather than the MI was chosen as parameter (Fig. 2b) since the values for this variable in ALCL-C were distinctly ($p < 0.001$) above those for HD.
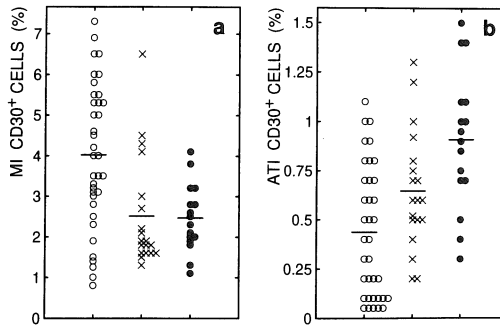

Fig. 2. *Mitotic indices (MI, a) and ana-telophase indices (ATI, b) per case of CD30+ cells in Hodgkin's disease (HD, open circles), anaplastic large cell lymphomas, Hodgkin's like (ALCL-HL, crosses), and anaplastic large cell lymphomas, common type (ALCL-C, closed circles). Means are indicated by bars.*

The cases of ALCL-HL took an intermediate position, without, however, being significantly different from either HD or ALCL-C in this respect. Interestingly and despite the differences noticed in Figure 1, the so-called growth fraction (%MIB-1% LAC) was about the same (60 - 70 %) in the three disorders tested (Fig. 3a, $p \gg 0.05$). As expected, the MNI was higher in HD than in ALCL ($p < 0.001$), and even the values for this parameter in ALCL.HL were significantly ($p = 0.01$) above those in ALCL-C (Fig. 3c).
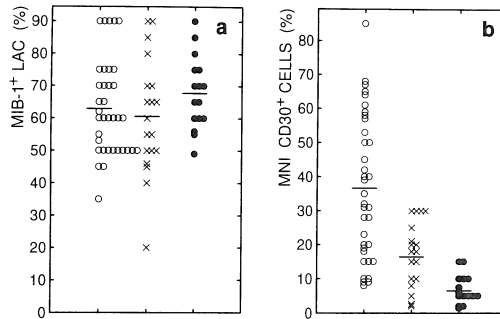

Fig. 3. Percentages of Ki-67 (MIB-1)+ LAC (a) and multinucleation indices (MNI) of CD30+ cells (b) in HD, ALCL-HL, and ALCL-C (see also legends to Figs 1 and 2).

Indices related to cell disintegration

Figure 4a documents that the DFI of CD30$^+$ cells was the highest in HD and the lowest in ALCL-C ($p < 0.01$), with the ALCL-HL taking an intermediate position, not significantly different from the other two groups. A similar but even more pronounced pattern is seen in Figure 4b, where the percentages of mummified cells in HD far exceeds th ose in ALCL-C ($p < 0.001$). Even the ALCL-HL exhibited distinctly ($p < 0.05$) less mummified cells than HD, while its difference in this respect from ALCL-C did not attain statistical significance.
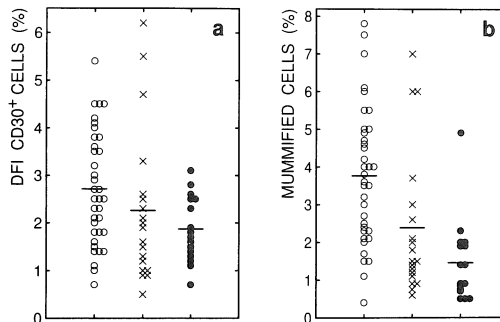

Fig. 4. DNA fragmentation indices (DFI, comparable to apoptotic indices, a) and percentages of CD30+ mummified cells (b) in HD, ALCL-HL, and ALCL-C (see also legends to Fig.2).

Expression of cyclin B-1 (C+N) and p34 by large atypical cells

As evidenced in Figure 5a, by far the highest mean percentage of LAC expressing cyclin B-1 (C+N) was found in ALCL-C, while the respective values in HD were rather low ($p < 0.001$). It may be noticed that cases of ALCL-HL took an intermediate position with regard to this variable (difference from ALCL, $p < 0.01$, from HD, $p < 0.05$). An opposite pattern emerged as regards the expression by LAC of p34. The largest mean fraction staining positively for this regulatory protein was observed in HD ($p < 0.001$), while the two other groups showed lower values and did not differ in this respect.
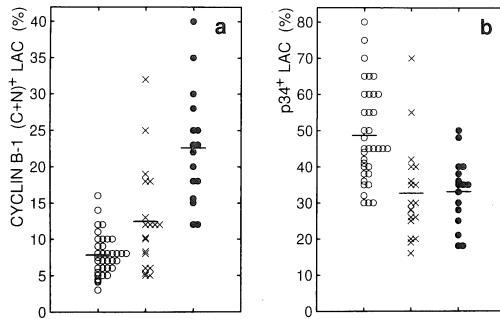

Fig. 5. *Percentages of cyclin-B(C+N)+ (a) and p34+ (b) LAC in HD, ALCL-HL, and ALCL-C (see also legends to Figs 1 and 2).*

EBV infection

EBV infection was registered in 12/24 (33.3%) of HD cases, in 2/14 ALCL-C (12,5%; chi-square test $= p < 0.01$) and in none of the ALCL-HL.

Stepwise discriminant analysis

Table 2. *Discriminant power of the various parametewrs in distinguishing between HD, ALCL-HL, and ALCL-C*.*

Parameter	F value**
% cyclin-B(C+N)$^+$ LAC (shortly, BCN$^+$ LAC)	38.21
Multinucleation index (MNI) of CD30+ cells	22.88
% p34$^+$ LAC	14.47
Ana-telophase index (ATI) of CD30$^+$ cells	11.65
% mummified CD30$^+$ cells	10.97
Mitotic index (MI) of CD30$^+$ cells	9.10
Infection by EBV	5.19
% cyclin-B(C)$^+$ LAC	2.68
% Ki-67(MIB-1)$^+$ LAC	1.04

*For abbreviation, see text.
**The F value (obtained at step 0 of the discriminant analysis) indicates the discriminant power of the respective parameter.

The F-statistics, computed from a one-way analysis of variance, of the various parameters at step O are summarized in Table II. These results suggest that, considering each variable alone, the most discriminant parameter was % cyclin B-1 $(C+N)^+$ LAC, followed by MNI of $CD30^+$ cells, % $p34^+$ LAC and the others, while the % $MIB-1^+$ LAC, the so-called growth fraction, was the least discriminant. The stepwise selection of variables, in which these were only entered and never removed from the analysis, converged after six steps. Figure 6 shows the territorial map for the three groups of lymphomas on the discriminant score plane, which had been obtained at the end of the stepwise analysis procedure. It illustrates that the boundary lines separate the three types of lymphomas reasonably well. In fact, there is no overlap between HD and ALCL-C. Only the ALCL-HL exhibits somewhat blurred contours. The confusion matrix obtained by the jackknife method gave a misclassification regarding ALCL-HL equal to 20%, but not betwen ALCL-C and HD.
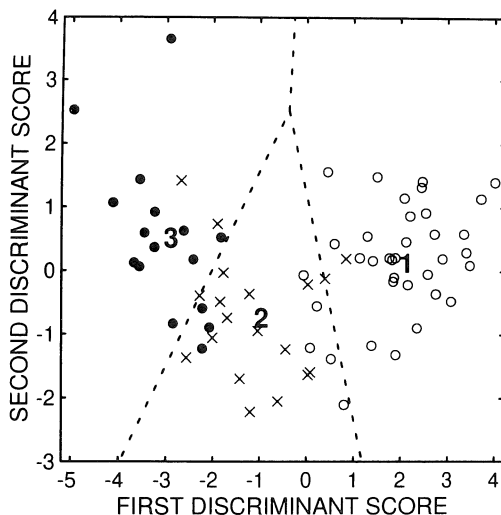

Fig. 6. *Territorial map for cases of HD, ALCL-HL, and ALCL-C on the discriminant score plane obtained at the end of the stepwise analysis procedure. There is an overlap between ALCL-HL and both HD and ALCL-C, but no between the latter two. Calculated boundaries are indicated by broken lines (HD: open circles; ALCL-HL: crosses; ALCL-C: closed circles).*

DISCUSSION

The results of the present study confirm and extend previous findings on the disturbed proliferation of H-RS cells in HD, in particular the frequent arrest of the metaphase-ana/telophase transition, the consecutive multinucleation and enhanced cell death (Leoncini et. al, 1996; Spina et. al, 1996). This was evident from a comparison of MI and ATI, the latter being a better index than MI for presumably successful cell division (Fig.2). The propensity of H-RS cells in HD for multinucleation (Fig. 3b) and mummification (Fig. 4b) could also clearly be documented. Since none of the cellular kinetic features of HD mentioned above was recognized as prominent in ALCL-C, we conclude that the growth characteristics of these two disorders differ profoundly. It was interesting to notice that ALCL-HL took an intermediate

position between the two other groups, so that the REAL classification's (Harris et. al, 1994) present contention to regard this disorder as a separate but provisional entity, finds some support also from a cytokinetic point of view.

   Some of our results raise the question what parameter(s) should be used to define growth. It is quite obvious from Figure 3a that the percentage of Ki-67^{+} (MIB-1^{+}) cells, which is often referred to as "growth fraction", was about the same in the three types of lymphomas studied. However, the DFI, the parameters chosen in our study to reflect cell loss, was significantly different in HD and ALCL-C, respectively. This exemplifies the necessity of considering cell deletion as well as cell proliferation when evaluating neoplastic growth (Steel, 1977; Del Vecchio et. al, 1991; Hall and Coates, 1995). Further, HD is a good example to question still another aspect of the term "growth fraction" as presently used by many authors. Cell proliferation implies the production of new cells. The fact that H-RS cells usually are Ki-67^{+} seemed to indicate that these are actively proliferating elements, like high grade lymphomas (Gerdes et. al, 1993). It became clear, however, that Ki-67 positivity in this disorder, because of abortive mitoses and cell deletion (Drexler et. al, 1989; Leoncini et. al, 1996), does not reflect the cell's capacity to give birth to daughter cells. ATI is probably the parameter closest to a successful cell division, because cells in ana-or telophase have overcome the metaphase/anaphase obstacle (Spina et. al, 1996). Figure 2, 3a and 4 are in full accord with these notions and strongly indicate that cell proliferation in terms of cell production is much more successful in ALCL than in HD. They also document that the term "growth fraction" in HD bears little meaning.

   Salient findings of this study were the markedly lower percentages of LAC expressing cyclin B-1 (C+N) in HD than in ALCL (Fig. 5a), while the reverse was true for the presence of p34 (Fig. 5b). In view of the multitude of factors regulating the eukaryotic cell cycle (Elledge, 1996) and the many mechanisms that may lead to uncontrolled cell proliferation in cancer (Sherr, 1996), it seems virtually impossible to offer a straightforward interpretation of this phenomenon. One possibility may be a relative lack of cyclin B-1 in HD. Alternatively, an unscheduled overexpression of this regulatory protein in ALCL, as was observed in several tumor cell lines (Gong et. al, 1994), or other disturbances could explain the differences. Further studies are warranted to clarify this problem.

   Irrespective of mechanisms involved, immunostaining for cyclin B-1 may prove valuable in the differential diagnosis of ALCL and HD, respectively, and for the evaluation of a characteristic that is somehow related to cell proliferation. The statistical analysis of our data revealed in fact that of the 10 variables studied, % cyclin B-1 (C+N)[+] LAC had the greatest power to discriminate between the lymphomas tested (Table 2), followed (in the order of decreasing discriminant power) by such growth-related parameters as MNI, % p34[+] LAC and ATI. Of interest, the often applied immunostaining for Ki-67 was the last in this list and thus offers little for the differentiation of HD and ALCL. Their territorial map for the three lymphomas tested on the discriminant score plan (Fig. 6) illustrates that it was possible, with the help of, somehow growth-related parameters to clearly distinguish between HD and ALCL-C. Overlaps were only seen between these two lymphomas and ALCL-HL, but not between the lymphomas themselves. This favors the view that ALCL-HL should be considered a separate disease entity, and stay as such until other - possibly cytogenetic - discriminators will show more uniform pattern.

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