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EXPRESSION OF THE ALK PROTEIN BY ANAPLASTIC LARGE-CELL LYMPHOMAS CORRELATES WITH HIGH PROLIFERATIVE ACTIVITY

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A variable fraction of anaplastic large-cell lymphomas (ALCLs) exhibits a t(2;5)(p23;q35) translocation that results in expression of the chimeric hyperphosphorylated protein NPM-ALK (p80). Tumor cells expressing NPM-ALK exhibit markedly enhanced proliferative activity, but comparative cellular kinetic studies on ALK$^+$ (ALK lymphomas) and ALK$^-$ lymphomas are lacking. The present study showed that ALK$^+$ lymphomas, detected with the monoclonal antibody ALKc (n = 17), had significantly higher average values for the proliferation-associated parameters mitotic index, ana/telophase index, growth index (x × mitotic index – apoptotic index, assuming x = 3), percentages of Ki-67$^+$ cells and fraction of cells expressing cyclin A or B or the cell cycle–regulatory protein p34^{cdc2} than did ALK$^-$ ALCLs (n = 15). Whether this intense proliferative activity contributes to the good response to chemotherapy and favorable outcome of ALK$^+$ ALCLs remains to be assessed in a larger series of patients. Our findings support the notion that ALK$^+$ and ALK$^-$ ALCLs are 2 distinct disease entities. *Int. J. Cancer* 86: 777–781, 2000.
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Long after their first description in 1985 (Stein *et al.,* 1985), Ki-1 (CD30)$^+$ anaplastic large cell lymphomas (ALCLs) have remained a subject of controversy. This pertains not only to the origin of tumor cells, mostly T or null type, but also to ALCLs as a single entity and their subdivision into morphologically distinct subtypes (Kadin, 1997).

A major breakthrough occurred when it was recognized that ALCL tumor cells in 30% to 40% of cases exhibit a specific recurrent translocation, t(2;5)(p23; q35) (Le Beau *et al.,* 1988). This chromosomal aberration causes the novel tyrosine kinase ALK (anaplastic lymphoma kinase) gene (on chromosome 2) to fuse with the nucleophosmin (NPM) gene (on chromosome 5), which encodes a nuclear phosphoprotein (B23 or numatrin) shuttling between nucleus and cytoplasm. The resulting fusion gene encodes a chimeric protein, NPM–ALK (p80), consisting of the N-terminal portion of NPM fused to the entire cytoplasmic domain of ALK (Morris *et al.,* 1994).

The presence of NPM–ALK chimeric transcripts or proteins can be detected in ALCL samples by RT-PCR. Taking advantage of the fact that the ALK protein is not expressed in non-neoplastic lympho-hemopoietic tissues, immunostaining with polyclonal (Shiota *et al.,* 1994) and monoclonal (Pulford *et al.,* 1997; Falini *et al.,* 1998) antibodies (MAbs) specific for the cytoplasmic portion of ALK has been widely applied to provide evidence for the t(2;5) translocation (Falini *et al.,* 1998). MAbs directed against the ALK protein and the N-terminal portion of NPM (Cordell *et al.,* 1999) have been used in combination to distinguish between ALCLs bearing the NPM–ALK fusion protein (about 85% of cases) and those expressing variant ALK fusion proteins (about 15% of cases). The distinction between ALK$^+$ lymphomas (bearing NPM-ALK or ALK-variant fusion proteins) and ALK$^-$ ALCLs appears to be clinically important because of the apparently better prognosis of the former condition (Falini *et al.,* 1999).

It has long been understood and theoretically documented that the success of chemotherapy and radiotherapy in the treatment of malignant neoplasms depends to a great extent upon their cellular kinetic properties. To the best of our knowledge, differences in the growth rate of ALK$^+$ and ALK-negative ALCLs have so far not been reported. The present study was designed to provide some of this information. Since the combined use of several proliferation-associated parameters to separate groups may be superior to using any single variable (Leoncini *et al.,* 1999), we assessed not only the mitotic index (MI) and the percentage of Ki-67$^+$ cells but also the fraction of cells expressing cyclin A or B or the cell cycle–regulatory protein p34^{cdc2} (abbreviated as p34). The ana/telophase indices (ATI; Leoncini *et al.,* 1998) were registered because in Hodgkin's disease, another large group of CD30$^+$ malignant lymphomas, many mitoses are not successful in terms of cell production (Spina *et al.,* 1996). Since growth equals cell production minus cell deletion (Spina *et al.,* 1997), we also established, in each case, the apoptotic index (AI).

MATERIAL AND METHODS

Case selection and conventional histology/immuno-histochemistry

Paraffin blocks from a total of 32 ALCLs were collected from the files of our institutions in Sassari, Bologna and Siena. Biopsy specimens were sliced and fixed in a neutral, buffered, 4% aqueous formaldehyde solution and processed for conventional histology, as reported previously (Leoncini *et al.,* 1998). The various lymphomas were accepted based on the following criteria: no treatment prior to diagnosis, adequate fixation of biopsy material, sufficient amounts of tissue in paraffin blocks, absence of large areas of necrosis and satisfactory clinical data (response to therapy and status at the end of 1998). For the qualitative histologic evaluation, 4 μm-thick sections were stained with hemalum–eosin, Giemsa, periodic acid Schiff (PAS) reagent, Mallory trichrome and Gomori's silver impregnation. For enhanced antigen retrieval, sections were treated with microwaves (Cattoretti *et al.,* 1993) or boiled in a 10 mM citrate buffer, pH 6, for 10 min. Sections to be immunostained

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for ALK protein were subjected to microwave heating (750 W for 3 cycles of 5 min each) in 1 mM EDTA buffer, pH 8.0, as previously described (Falini *et al.,* 1998). Standard immunophenotyping was carried out using a large panel of antibodies, commonly applied in our institutions to the evaluation of malignant lymphomas, and the APAAP and/or the EnVision++ system (Sabattini *et al.,* 1998). Cases were separated into 2 groups, depending on the positive ($ALKc^+$) or negative ($ALKc^-$) immuno-histochemical reaction of neoplastic cells to the ALKc MAb (see below). Immunomorphologic criteria for ALCL were those originally established by Stein *et al.* (1985) and defined in subsequent reports on subtypes of this lymphoma (Falini *et al.,* 1997; Kadin, 1997). The first diagnosis was made independently by at least 2 experienced pathologists (LL, SP). After immunostaining with the ALKc antibody, a second, common review reached a consensus on 3 controversial cases. The morphologic, immunophenotypic and clinical features of the 32 cases of ALCL investigated in this study are summarized in Table I.

Antibodies used for quantitative immunohistology

The mouse MAb ALKc, which is directed against the cytoplasmic portion of the ALK protein (Falini *et al.,* 1998), was generated in the laboratory of one of the authors (BF). In our hands, this reagent gave stronger immunolabeling of tumor cells (especially nuclei) than the mouse MAb ALK1 (Dako, Milan, Italy) or the goat polyclonal anti-ALK anti-serum (Santa Cruz Biotechnology, Santa Cruz, CA), so we chose it for our immuno-histochemical studies. The mouse MAbs anti-$p34^{cdc2}$ serine/threonine kinase/cdk AB-1 (clone A17.1.1), for the detection of p34, and anti-cyclin B-1 AB-1 (clone V152), for that of cyclin B, were purchased from Neomarkers (Fremont, CA, U.S.A.). The anti-cyclin A MAb was generated by one of us (AG). We applied the mouse MAb MIB-1 (anti-Ki-67; Immunotech, Marseille, France) to detect the proliferation-associated antigen Ki-67. Immunostained cells were registered as positive, irrespective of the localization of the reaction product (nucleus-restricted, cytoplasm-restricted or nuclear and cytoplasmic).

TABLE I – ALCLS STUDIED[1]

	$ALKc^+$ ALCL	$ALKc^-$ ALCL
Number of cases	17	15
Age of patients [range and median (years)]	10–57 (21)	10–83 (60)
Female:male ratio	9:8	9:6
Cell line		
T	5	5
Null	12	10
Histologic subtype		
Common	14	12
Small-cell	2	—
Large-cell	1	2
Hodgkin's-like	—	1
Localization		
Lymph node	15	12
Tonsil	1	—
Breast	1	—
Skin	—	1[4]
Pharynx	—	1
Colon	—	1
Stage		
I	1	1
II	9	6
III	5	6
IV	2	2
Response to therapy[2]		
Complete remission	15	4
Relapse	2	11
Outcome[3]		
Alive	16	8
Dead	1	7

[1]If not stated otherwise, the values correspond to numbers of cases.–[2]Chemotherapy consisted of COP, 2 × COPADM and 4 × VEBBP/Sequence 1 (Brugières *et al.,* 1998).–[3]Outcome refers to 1.1.1999, *i.e.,* up to 9 years after diagnosis.–[4]This patient also had retroperitoneal lymph node involvement.

Cell counts and estimates of growth index

Cell counts were carried out as described before (Leoncini *et al.,* 1996, 1998). In a first step, the mean numbers per case of $CD30^+$ and $CD30^-$ lymphoid cells per high-power field [HPF; 56,000 μm^2, examined with an oil immersion objective (100×)] were registered in 20 HPFs chosen at random. In the entire section (>100 HPF), $CD30^+$ cells in individual phases (pro-, meta-, ana- and telophases) of mitosis were then assessed as percentages of the total number of $CD30^+$ cells and expressed as MI (all mitotic phases) or as ATI (only ana- and telophases). Similarly, we also established, in each case, the AI (Spina *et al.,* 1997). Neoplastic cells immunostained by any one of the above antibodies were counted and the numbers registered as percentages of $CD30^+$ cells. Intra- and inter-observer correlations, assessed by comparison of separate counts on the same section but different HPFs of a number of cases by 2 independent pathologists, were significant ($r \geq 0.95$, $p = 0.01$). Since mitotic and apoptotic times (duration) in each case are unknown but growth equals cell production minus cell deletion, we estimated the growth index (GI) by assuming the value 3 for x ($GI = x \times MI - AI$) (Spina *et al.,* 1997).

Statistical analysis

To evaluate univariately the significance level indicating that the 2 independent samples of patients with ALK^+ and ALK^- ALCLs were generated from different populations, the Wilcoxon rank sum test was used.

The statistical significance of the linear correlation coefficient between pairs of variables was tested and the corresponding regression analysis outlined. For the same variables, ALK^+ and ALK^- ALCL groups were compared both univariately and bivariately by computing the Hotelling T^2 and the related F-statistic values (Krzanowski, 1988).

A stepwise linear discriminant analysis was then applied to a wider set of measured variables, to seek the subset of variables that would allow the best multivariate, statistically significant distinction between the 2 groups. To select variables and stop the stepwise procedure, the Wilks λ criterion and the associated F statistic were used. Group classifications were evaluated with a jack-knifed classification matrix.

Data analysis was carried out by means of the BMDP statistical software and the statistical toolbox of the MATLAB package.

RESULTS

Qualitative histologic and immuno-histochemical findings

The MAb ALKc gave strong cytoplasmic and nuclear labeling of tumor cells, suggestive of the presence of NPM–ALK, in 17 of the 32 ALCL cases studied (Fig. 1). No labeling of obviously non-neoplastic elements was observed in any of the tissue samples investigated, thus confirming previous observations by Falini *et al.* (1998). The immunostaining pattern of the MAbs directed against p34, cyclins A and B and Ki-67 corresponded to that observed in other non-Hodgkin's lymphomas (Leoncini *et al.,* 1999). Identification of various mitotic phases and of apoptotic cells/bodies caused no difficulties (Spina *et al.,* 1996).

MI plotted against ATI

The scattergram shown in Figure 2 resulted from a plot of MI per case against ATI per case. It documents a highly significant positive linear correlation between these 2 variables ($r = 0.883$, $p < 0.0001$), so the regression line fits the data well. The values for MI and ATI, substantially higher in ALK^+ than in ALK^- ALCLs, are also clearly visible in Figure 2. On the basis of these variables,

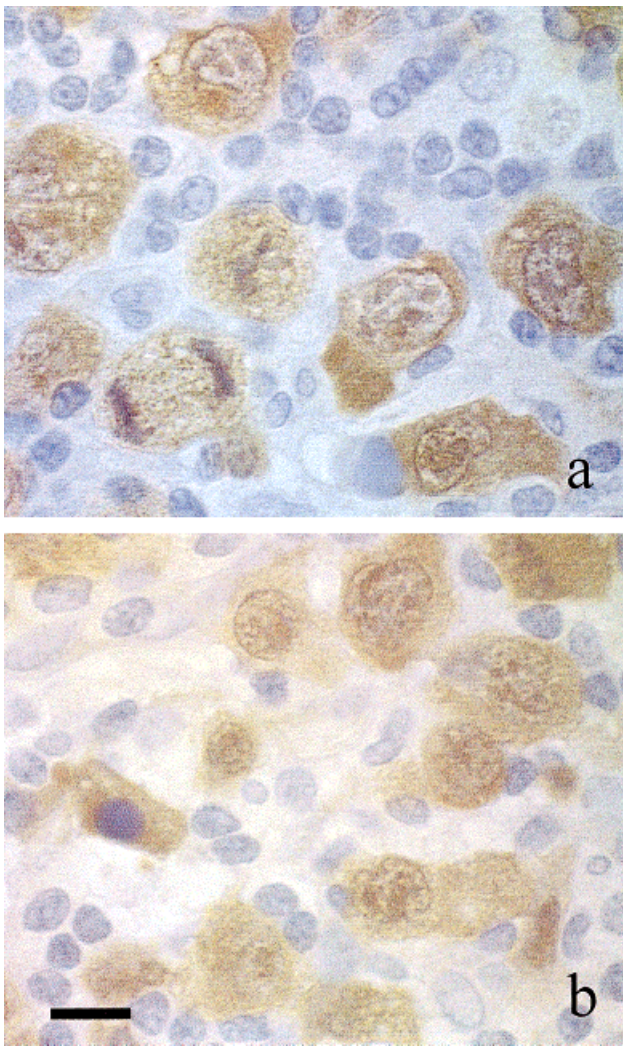

FIGURE 1 – Paraffin sections of ALCLs immunostained with the ALKc MAb, showing positively reacting large atypical cells, mitosis *(a)* and an apoptotic body *(b)* [EnVision++ technique (Sabattini *et al.,* 1998)]. Scale bar = 25 μm.

both univariate and bivariate tests pointed out a highly significant difference between the 2 groups of lymphomas ($p < 0.001$).

Comparison of ALK$^+$ and ALK$^-$ ALCLs with regard to the proliferation-associated variables GI, % p34$^+$ cells and % MIB-1$^+$ cells

All 3 proliferation-associated variables gave values that were markedly higher in ALK$^+$ than in ALK$^-$ ALCLs. The Wilcoxon rank sum test documented that the statistical significance of the difference between the 2 groups was greatest for GI ($p < 0.001$) but also proved to be very impressive for the percentages of MIB-1$^+$ cells ($p < 0.001$) and of p34$^+$ cells ($p < 0.001$). However, a considerable overlap can be seen in all 3 graphs of Figure 3, so simple evaluation of individual values for each of these parameters, taken alone, does not allow for a distinction between ALK$^+$ and ALK$^-$ ALCLs.

Correlations between expression of cyclins A and B and the percentages of MIB-1$^+$ cells and the MI, respectively

As visualized in Figure 4*a*, the percentages of MIB-1$^+$ cells correlated well with those of cyclin A$^+$ cells in a positive linear fashion ($r = 0.691$, $p < 0.001$). Almost equally good was the

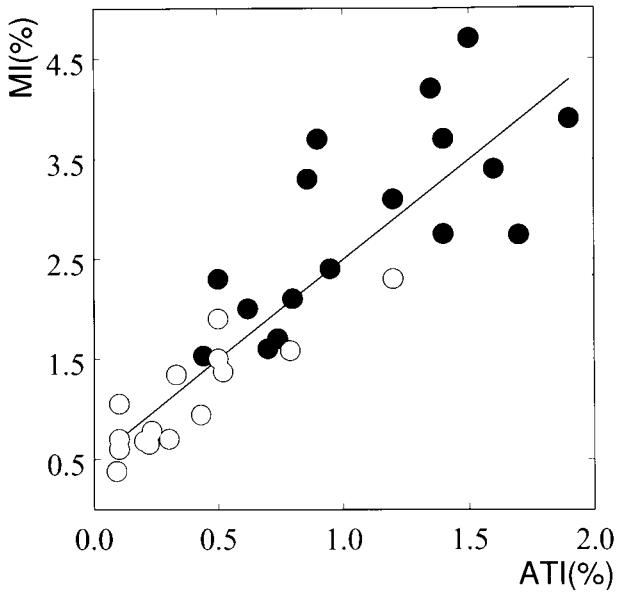

FIGURE 2 – Comparison of MI (%) and ATI (%) per case of ALKc$^-$ (open circles) and ALKc$^+$ (closed circles) ALCLs, illustrating the significant difference and the correlation between the 2 (line is a regression line).

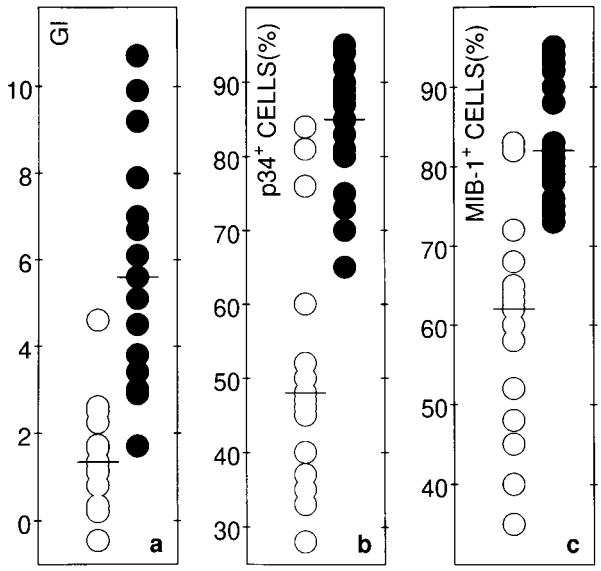

FIGURE 3 – Significantly higher values for the 3 proliferation-associated parameters GI (= 3 × MI – AI) *(a)*, % p34$^+$ cells *(b)* and % Ki-67 (MIB-1)$^+$ *(c)* cells per case in ALKc$^+$ than in ALKc$^-$ ALCLs (see legend to Fig. 2; medians are indicated by bars).

linear correlation between the MI and the fractions of cyclin B$^+$ cells ($r = 0.661$, $p < 0.001$; Fig. 4*b*). Similarly significant, though in part somewhat loose, correlations existed between the values for each of the other proliferation-associated variables (details not shown). Figure 4 further illustrates the markedly higher values for the percentages of cyclin A$^+$ and cyclin B$^+$ cells in ALKc$^+$ than in ALKc$^-$ ALCLs ($p < 0.001$). Hotelling's T^2 for both the percentages of cyclin A$^+$ cells against MIB-1$^+$ cells and the percentages of cyclin B$^+$ cells against MI supplied a high statistical significance, also in the bivariategroup comparison ($p < 0.001$).

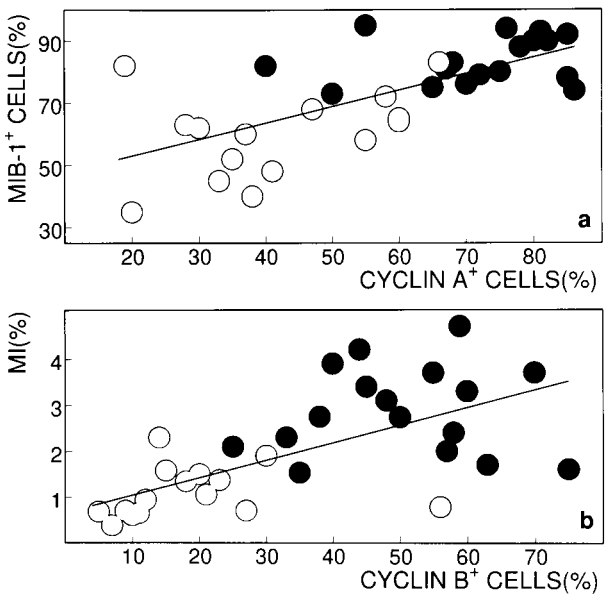

FIGURE 4 – Plots of % cyclin A^+ cells against % Ki-67 (MIB-1)$^+$ cells per case *(a)* and of % cyclin B^+ cells against MI per case *(b)* of ALCLs. Note the higher values for all above variables in $ALKc^+$ than in $ALKc^-$ ALCLs and the correlation between the values for the 2 pairs of variables (see legend to Fig. 2).

Discriminant analysis of proliferation-associated variables as to their power to distinguish ALK^+ and ALK^- ALCLs

Although we deal here with a limited number of cases and the results should, therefore, at best be taken as preliminary, it was of interest to carry out this analysis as it might suggest a certain order of correlations between individual proliferation-associated parameters and ALK positivity. It was not meant for diagnostic purposes.

All proliferation-associated variables, *i.e.,* GI, MI, ATI, AI, % cyclin A^+ cells, % cyclin B^+ cells, % $p34^+$ cells and % MIB-1^+ cells, were submitted to a stepwise linear discriminant analysis. After step 3, the entered variables were (values for Wilks' λ and associated F values in parentheses): GI ($\lambda = 0.336$, $F = 16.1$), % cyclin B^+ cells ($\lambda = 0.269$, $F = 6.50$) and % $p34^+$ cells ($\lambda = 0.243$, $F = 2.68$). However, since GI is based on an assumption ($x = 3$), this variable could not be used for factual calculations. Without using the GI as a parameter, the results of the stepwise discriminant analysis did not change significantly when MI was substituted for GI.

Classification results indicated that all ALK^- ALCLs (100%) were, based on the present variables, correctly classified, while only 1 (5.9%) of the 17 ALK^+ ALCLs was misclassified. This amounts to a total percent of cases correctly classified of 97.2%.

DISCUSSION

The main finding of the present study was the markedly higher values for proliferation-associated variables in ALK^+ than in ALK^- ALCLs. This pertains not only to MI and ATI (Fig. 2) but also to GI and the percentages of $p34^+$ and MIB-1^+ cells (Fig. 3) as well as the fractions of cyclin A^+ and cyclin B^+ cells (Fig. 4). While all ALCLs taken together already belong to the non-Hodgkin's lymphomas with the highest proliferative activity (Leoncini *et al.,* 1999), our study identified the ALK^+ subgroup of these neoplasms as even more intensely cycling. Also, our results give no indication that the high rate of cell production in ALK^+ ALCLs is counterbalanced by an excessive rate of apoptotic cell death. In a previous report (Spina *et al.,* 1997), growth was estimated by $GI = x \times MI - AI$ in 94 non-Hodgkin's lymphomas of various types, and the assumed value $x = 3$ reflects a situation where GI touches the zero line but does not become negative.

Another finding that we consider important lies in the rather close correlations between the values for distinct proliferation-associated variables. The strong positive linear relationship between MI and ATI (Fig. 2) favors the notion that in ALCLs most cell divisions are successful, in contrast to Hodgkin's disease, where many mitoses become arrested in metaphase and may end up in cell death (Leoncini *et al.,* 1996; Spina *et al.,* 1996). The correlations between % MIB-1^+ cells and % cyclin A^+ cells and between MI and the fraction of cyclin B^+ (the mitotic cyclin) cells (Fig. 4) support the view that most processes involved in the cell-cycle machinery are up-regulated in ALK^+ compared to ALK^- ALCLs. This is in line with experimental findings that expression of cDNAs encoding NPM–ALK (p80) in rat 1a fibroblasts induced accelerated cell-cycle entry into S phase, marked up-regulation of cyclin A and cyclin D1 expression and enhancement of AP-1–dependent transcriptional activation. In addition, markedly elevated expression of several immediate-early genes involved in cellular proliferation (*e.g., fos, jun* and *c-myc*) was noted (Wellmann *et al.,* 1997).

As alluded to under Results, ALK positivity may not correlate equally well with individual proliferation-associated parameters. More extended studies are needed to clarify this point.

Other observations made in our study confirm earlier reports of others on ALCLs expressing the NPM–ALK chimeric protein, *e.g.,* the younger age of patients, the frequency of the T- or null cell lines, the wide morphologic spectrum and the more favorable response to chemotherapy (Falini *et al.,* 1999) in ALK^+ *vs.* ALK^- lymphomas.

The molecular background of the intense proliferative activity in ALK^+ ALCLs has not been fully clarified. ALK, a novel receptor that exhibits a close relationship to leukocyte tyrosine kinases, is normally expressed by certain cells of the nervous system but not in the non-neoplastic lymphoid/hemopoietic tissue (Iwahara *et al.,* 1997; Morris *et al.,* 1997). The t(2;5) translocation brings the portion of the gene encoding the intracellular portion of ALK under the control of the strong NPM gene promoter, which leads to its ectopic expression in lymphoid cells and the resultant dysregulated growth (Morris *et al.,* 1994). The NPM portion of the fusion protein provides an oligomerization motif that promotes a self-association of NPM–ALK and results in its constitutive activation (Rodriguez and Park, 1994). The signaling pathway(s) leading to the mitogenic effect appears to involve the phosphorylation of target molecules. It has been shown, *e.g.,* that NPM–ALK forms a complex with phospholipase Cγ, which leads to tyrosine phosphorylation and activation of this enzyme (Bai *et al.,* 1998).

In conclusion, we have shown that the values for proliferation-associated parameters (MI, ATI, GI, % $p34^+$ cells, % cyclin B^+ cells and % MIB-1^+ cells) were markedly higher in ALK^+ than in ALK^- ALCLs. This finding suggests that these are 2 distinct disease entities. Whether the high mitotic activity of ALK^+ compared to ALK^- ALCLs correlates with a better outcome (Tilly *et al.,* 1997) remains to be assessed in larger clinical studies. The same pertains to the problem of multidrug resistance (Miller *et al.,* 1996).

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