ABSTRACT

pRb/p105, p107, and pRb2/p130 compose the retinoblastoma (RB) family of proteins and regulate cellular growth and differentiation. Because recent functional studies have indicated that the expression of the RB-related proteins p107 and pRb2/p130 are tightly cell cycle regulated, we were interested in investigating their expression along with cellular kinetic characteristics and proliferative features of non-Hodgkin’s lymphomas (NHLs). p107 and pRb2/p130 expression was determined immunohistochemically in biopsy specimens from 83 untreated patients with NHLs of various histiotypes. The expression of these two RB-related proteins was correlated with the mitotic index, apoptotic index, and percentages of Ki-67(+) and cyclin A(+) cells. The overall survival rate was evaluated according to the Kaplan-Meier method and the log-rank test. We found a positive correlation between the percentages of cells positive for p107 and proliferative features such as mitotic index and percentage of Ki-67(+) and cyclin A(+) cells, whereas such correlation could not be demonstrated for the percentages of pRb2/p130 positive cells. Low immunohistochemical levels of pRb2/p130 detected in untreated patients with NHLs of various histiotypes inversely correlated with a large fraction of cells expressing high levels of p107 and proliferation-associated proteins. Such a pattern of protein expression is normally observed in continuously cycling cells. Interestingly, such cases showed the highest survival percentage (82.5%) after the observation period of 10 years. Thus, down-regulation of the RB-related pRb2/p130 protein could be one of the reasons why these cases display such a high rate of proliferation and why they respond so well to therapy.

INTRODUCTION

In recent years, the combined use of genetic and biochemical approaches has allowed us to identify regulatory mechanisms controlling cell cycle and cell growth (1, 2). A variety of experiments have documented the critical role of the RB3 protein family pRb/p105, pRb2/p130, and p107 in controlling the G1 checkpoint of the cell cycle (3). These proteins share considerable sequence homology and also the ability to interact with and regulate E2F transcriptional activity (4, 5).

Although pRb/p105, pRb2, and p107 interact with members of the E2F transcription factor family and have similar functional properties, each protein has a different temporal profile of interaction with different E2F members (6, 7). Whereas pRb (p105) is found in both quiescent and proliferating cells, the expressions of pRb2 and p107 are peculiar during the cell cycle (8). The binding of pRb2 to E2Fs is detected predominantly during G1, whereas that of p107 is detected during the G1 and S phases (9). Both depend on different upstream signals such as cyclin/CDK complexes or viral oncoproteins (10, 11). The flexibility of this pathway can explain the distinct activities of the three proteins in the regulation of cellular division and cellular differentiation (12). The three proteins exhibit different growth-suppressive properties in specific cell lines, suggesting that although the different members of the RB protein family may complement each other, they are not completely functionally redundant (13, 14). Although pRb (p105) has been extensively shown to play a key role in the negative regulation of
cellular proliferation (15, 16), little is known about pRb2 and p107 in human neoplasias.

Because of the importance of pRb2 and p107 in controlling cellular growth (8, 9), we investigated their expression in relation to the cellular kinetic characteristics and proliferative features of NHLs.

We used diffusely growing NHLs of different histopathological types as a model system because the NHLs exhibit a distribution of mitotic figures and apoptotic cells/bodies that approximates regularity better than other neoplasias (17) and are classified into disease entities that respect not only the phenotype but also cytogenetic alterations (18).

### MATERIALS AND METHODS

**Case Selection and Conventional Histopathology.** We retrospectively studied biopsy specimens from 83 untreated patients with various types of NHLs, collected from the Institutes of Pathology of the University of Siena and Bologna. Because comparison of histologically defined lymphomas was not the primary goal of this investigation, no attempt was made to select cases for comparable group size. Cases were chosen on the availability of sufficient amounts of diffuse or diffusely growing parts of NHL tissue in the paraffin blocks, absence of extended tissue necrosis, and adequate clinical information, especially regarding staging and survival. Follow-up data were available for all patients, with a median follow-up duration of 65 months (range, 24–120). Patients who died from causes other than lymphomas were considered as lost to follow-up evaluation, and therefore, their survival periods were censored at the date of death. All patients were treated with chemotherapy and/or radiotherapy according to established protocols (19–21). The clinical characteristics of lymphoma cases studied are summarized in Table 1. Biopsy materials from diseased tissues, obtained at different hours of the day, were immediately sliced and fixed in a neutral, buffered 4% formaldehyde solution (pH 7.4) for 24 h and embedded in paraplast at 56°C. For conventional histology, 4-μm sections were stained with H&E, Giemsa, and Gomori’s silver impregnation. Diagnoses were made independently by four experienced pathologists, based on the REAL classification (18). A final agreement, with the knowledge of immunohistochemical findings, was reached in every case.

**Immunohistochemical Procedures.** Immunophenotyping on paraffin sections was performed using a large panel of antibodies to detect formalin-resistant epitopes of malignant lymphoma cells and the EnVisionTM+/HRP method (Dako, Milan, Italy; Ref. 22). For antigen retrieval, deparaffinized sections of 3–4 μm were treated by microwaves or by pressure cooking in 1 mM EDTA (pH 8.0), for 5 min, followed by cooling at room temperature prior to incubation with the antibodies (23). Negative controls were obtained by replacing the primary antibodies with normal mouse serum. Normal human tonsils served as positive controls.

Immunostaining of the cell cycle regulator proteins examined in this study was carried out as described above. We used anti-p34cdc2 serine/threonine kinase/cdk AB-1 (clone A17.1.1; dilution, 1:100) for the detection of p34, anti-cyclin B-1 AB-1 (clone V152; dilution, 1:100) for the detection of cyclin B, and anti-cyclin A (clone 6E6; dilution, 1:100) for the detection of cyclin A. These mouse monoclonal antibodies were obtained from Neomarkers (Fremont, CA). The polyclonal antibodies anti-Rb2 and anti-p107 have been described previously (10, 12) and used at the working dilution of 1:1000. Immunostaining for Ki-67 with the MIB-1 antibody was performed as described previously (24).

**Cell Counts and Registration of Mitotic and Apoptotic Index.** In all sections, cells exhibiting a positive immune reaction to each antibody were counted in 30 representative fields (using a Leitz microscope with a Planapo 100/1 objective), and the results were expressed as percentages of all neoplastic cells in those areas. In selecting the fields for evaluation, the following criteria were applied: (a) their representativeness of the tumor’s histiotype; (b) the substantial homogeneity of the neoplastic population examined; and (c) the exact location and content of the reactive cells as revealed by previous phenotyping in paraffin sections (25). Intra- and interobserver reproducibility of counts was 95%. The intensity of immune reactions, i.e., the amounts of reaction product, was estimated qualitatively.

Because mitotic figures and apoptotic cells/bodies can be best recognized in semi-thin sections (26), we re-embedded part of the lymphoma tissue into Epon-Araldite resin (Monoej, Kidare, Ireland) and stained 1 μm section with Azur A for each case. MI and AI were registered in each case as described previously (24). Briefly, we used an oil immersion objective (×100) and first established the cellularity in 20 randomly chosen high-power fields, 56,000 μm²/field, and then assessed the MI and AI by registering the percentages of neoplastic cells in the mitosis or apoptosis of an entire section/case (100 high-power fields). With this approach, the relative number of apoptotic cells/bodies correlated with the percentages of cells showing in situ end-labeling of DNA strand breaks (27). Intra- and interobserver reproducibility of counts was 95%.

**Statistical Analysis.** A linear correlation analysis was performed to quantify the strength of the association among all of the parameters examined. For this purpose, the Pearson correlation coefficient r, was computed and evaluated together with its statistical significance in terms of error of probability P (28). Correlation coefficients were also examined, together with scatterplots and regression lines, for a graphical inspection of the effective linear association between variables at different ranges of values.

To investigate the existence of different populations on the basis of variables p107 and pRb2 and to locate cutoff values, the probability density functions were estimated by using the kernel method (29). This is a nonparametric method that does not impose any presupposed functional forms on the probabilities, for example the hypothesis of normality, and estimates proba-
probability density functions directly from the sample data. Given a sample of $N$ data points, the density function is estimated through the superimposition of $N$ identical kernel functions centered on each of the data points. We can smooth out the estimate by choosing different forms for the kernel function. For example, rectangular functions lead to the classical histogram representation. The major problem with the kernel-based methods is the choice of kernel width. When the width is too large, the estimated density is oversmoothed, and a possible multimodal nature of the distribution is lost. Conversely, a too small width gives a noisy estimation of the distribution. We chose a kernel of triangular form because it allows the width (basis of the triangle) of the kernel function to be estimated as a suitable compromise between noise and smoothing. To do that, a maximum likelihood technique was used. A great advantage of the kernel method is that, when a multimodal probability density function is found, cutoff values between different populations are unequivocally identified to correspond at the distribution minima. Cutoff values were used to form homogeneous subject groups.

Survival was evaluated according to the Kaplan-Meier method (30) and the log-rank test (31). Data analysis was performed with the SPSS statistical package, release 5.0.1 (SPSS, Inc., Chicago, IL).

RESULTS

**Qualitative Histological and Immunohistochemical Observations.** In Azur A-stained semithin sections, mitotic figures and apoptotic cells/bodies were easily identified (22). Signs of disturbed cell division, such as chromosome bridges, detached chromosomes, pyknomitoses, and asymmetrical mitoses, could be detected in only a very small fraction of mitotic figures. These were quite evenly distributed, although some focal accumulations did occur. More than one-half of the apoptotic cells exhibited a single, dark-stained, roundish mass with an effaced chromatin structure; the others appeared as apoptotic bodies with two or more nuclear fragments. Cells positively stained for p34 or cyclin B showed immune reaction over the cytoplasm and in part over the nucleus, whereas the localization of Ki 67, cyclin A, and p107 was essentially nuclear (Fig. 1, a–d). Cells stained for pRb2 showed a predominantly nuclear/or perinuclear positivity in the majority of the cases (Fig. 1e), but in a few cases, a diffuse cytoplasmic stain was also observed.
Correlation of p107 and pRb2 with Cellular Kinetic Features. The percentages of p107(+) cells were directly correlated with MI (r = 0.72, P < 0.001) and AI (r = 0.68, P < 0.001) as shown in Fig. 2, a and b.

On the other hand, a rather loose inverse correlation was found between the percentages of pRb2(+) cells and both MI (r = -0.35, P < 0.001) and AI (r = -0.35, P < 0.001) as shown in Fig. 2, c and d.
Correlation of p107 and pRb2 with Proliferative Features. The percentages of p107(+) cells were significantly correlated \( (P < 0.001) \) with the percentages of Ki67(+) \( (r = 0.81) \) and cyclin A(+) cells \( (r = 0.72) \). A similar correlation, although less significant, was also found between p107 staining and the percentages of p34(+) \( (r = 0.46) \) and cyclin B(+) cells \( (r = 0.44) \). The percentages of pRb2(+) cells in each case showed a weak inverse correlation with the percentages of Ki-67(+) \( (r = -0.41, P < 0.001) \), cyclin A(+) \( (r = -0.32, P < 0.01) \), and p34(+) \( (r = -0.29, P < 0.01) \) but not with the percentage of cyclin B(+) cells.

Figs. 3 and 4 show the scatterplots together with regression lines. It is evident from Figs. 3 and 4 that, although statistical correlation does exist, several cases are remarkably distant from the regression line.

Correlation between p107 and pRb2. A linear inverse correlation was found between the percentages of p107(+) and pRb2(+) cells \( (r = -0.45, P < 0.001) \), even if the scatterplot in Fig. 5 clearly shows that some points representing individual cases are again remarkably distant from the regression line. This finding prompted us to further investigate whether cases with distinct expression of p107/pRb2 could be identified. To test this hypothesis, the nonparametric kernel method (29) was then applied, and cutoff values, corresponding to 50 and 25% of cells positive for p107 and pRb2, respectively, were determined on the basis of the estimated probability density functions (Fig. 5). Four groups could then be identified in Fig. 5: values of p107 >50% and pRb2 >25% \( (p107+/pRb2+) \); values of p107 <50% and of pRb2 >25% \( (p107-/pRb2+) \); values of p107 <50% and pRb2 <25% \( (p107-/pRb2-) \); and values of p107 >50% and of pRb2 <25% \( (p107+/pRb2-) \).

Tables 2 and 3 illustrate the distribution of histiotypes and pathological stages among groups with distinct p107/pRb2 expression. The different histiotypes were represented in at least two or more groups, with the exception of B-CLL and MZL, which were present only in p107+/pRb2+ cases and BLL and ALCL, which were present only in p107+/pRb2- cases. Patients with different stages were included in each group.

Response to Therapy and Survival Analysis. Kaplan-Meier cumulative survival curves relative to the four groups are reported in Fig. 6. Although the variety of analyzed histological types of NHL, the diverging therapy, and the retrospective nature of the present study precluded an unequivocal assessment of actuarial survival and disease-free survival, it is of interest that the p107+/pRb2+ cases showed the highest survival rate after an observation period of 10 years.

**DISCUSSION**

Recent functional studies of p107 and pRb2 indicated that although the RB family members may be able to complement each other, these proteins are not completely functionally redundant (4). Although p107 specifically interacts with E2F4 and E2F5, as does pRb2, the constant presence of the E2F/p107/
cyclin A/CDK2 complex in proliferating cells (9) suggests that p107, rather than acting exclusively as a tumor suppressor protein (32), may also play a positive role in cellular proliferation (33). These findings confirm the presence of multifunctional domains on the nuclear RB products, which may allow complex interactions with cellular transcription machinery (34).

The expression of p107 and pRb2 in malignant lymphomas presented here are in accordance with these experimental data and support the concept of their different functions in cellular growth control. In fact, we found that the percentages of cells positive for p107 correlated in a linear fashion with proliferative features such as MI and the percentages of Ki-67(+) and cyclin A(+) cells, whereas such correlation could not be demonstrated for the percentages of pRb2(+) cells.

On the basis of the experimental data, a model for pRb2 and p107 control during cell proliferation has been proposed previously (33). In quiescent G0 cells, the E2F-pRb2 complex is responsible for the active repression of a number of cellular promoters, including those of E2F1, E2F2, and p107 (33–35). After the release into the cell cycle, pRb2 is phosphorylated by G1 CDKs and subsequently degraded through a proteasome-dependent mechanism (36). pRb2 protein level thus drops dramatically to almost undetectable levels, resulting in the derepression of a variety of genes, including p107 (32, 34, 37). The accumulated p107 is then able to interact with E2F4 and E2F5 that have been released from the pRb2 complex and associate with cyclin A/CDK2 (38). Thus, in an ideally controlled proliferating cell population with identical cell cycle and cycle phase times, during which a certain protein can be detected by the correspondent antibody, the percentages of cells expressing p107 and pRb2 should be inversely related. Our finding of an inverse correlation between the percentages of positive cells for p107 and pRb2 supports these observations. However the scatter plot in Fig. 5 clearly shows that some points representing individual cases are remarkably distant from the regression line, indicating that we were dealing with considerable variation. There are many tentative explanations for the deviation of individual cases from the ideal line. Many factors may be altered in neoplastic cell populations; the interaction between individual proteins and the E2F family members and the timing of formation of particular E2F complexes during the cell cycle can be deregulated (39), or a functional interaction with cyclins may be disturbed because of an overexpression and/or an unscheduled expression of these latter molecules (40). Yet, proteins with lost or diminished function(s) may still be detectable by immunostaining (41), and circadian variation in the nuclear expression of cyclins has been reported (42). Genetic alterations disrupting the nuclear localization of RB2 in human cell lines and primary tumors have been documented recently.4 Such mutations may reduce growth inhibition, because RB2 has been shown to have antiproliferative and tumor-suppressive potentials in vivo and in vitro using a tetracycline-dependent overexpression system (43, 44). Alterations in the p107 gene have also been identified in a limited number of human hematological malignancies (45). Furthermore, it should be considered that checkpoint control is usually performed by more than one system. These and other influences make it virtually impossible to interpret the present results in terms of molecular events.

Nevertheless, previous reports have shown a tight inverse correlation between tumor malignancy and pRb2 expression in several human neoplasia, suggesting a direct involvement of pRb2 in the course of those diseases (46–49). In this study, we present data showing a distinct expression of p107 and pRb2 among histological types of malignant lymphomas. B-CLL and MZL are represented only in the p107−/pRb2+ group of cases, whereas BLL and ALCL are represented only in the p107+/pRb2− cases. Conversely, the other histiotypes are represented in at least two or more groups with different p107 and pRb2 expression levels. In particular, this pattern pertains to follicular cell lymphoma and diffuse large B-cell lymphoma and suggests the possibility of different pathogenetic mechanisms within these types of lymphoma. Our results emphasize the REAL Classification recommendation to examine each disease entity with respect to possibly divergent molecular features, growth patterns, and clinical aggressiveness (18). Interestingly, the p107+/pRb2− cases showed the highest survival rate (82.5%) after the observation period. These cases demonstrated low values of pRb2 that inversely correlated with a large fraction of cells expressing high levels of p107 and proliferation-associated proteins. Such a pattern of protein expression is observed when cells enter G1 after mitosis in continuously cycling cells (50). Thus, down-regulation of the RB-related pRb2 protein in malignant lymphomas can be in part responsible for an enhanced cellular proliferation (51) and a better response to therapy (52, 53). It would be extremely interesting to look at the pRb2 expression in a uniform group of patients to determine whether this can be an additional marker for prognosis.

Unfortunately, few data are known about the transcriptional, posttranslational, and regulatory events that involve the RB-related proteins and E2Fs, so that little more than speculation can be made at this time. Although complex analysis of important genetic pathways are technically challenging, they are now possible and can provide powerful genetic, cellular, and biochemical approaches to determine the basic mechanism of cellular growth control.

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Retinoblastoma-related p107 and pRb2/p130 Proteins in Malignant Lymphomas: Distinct Mechanisms of Cell Growth Control

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