

## D Cyclins in CD5+ B-Cell Lymphoproliferative Disorders

### Cyclin D1 and Cyclin D2 Identify Diagnostic Groups and Cyclin D1 Correlates With ZAP-70 Expression in Chronic Lymphocytic Leukemia

Howard J. Meyerson, MD,<sup>1</sup> Grayden MacLennan,<sup>1</sup> William Husel,<sup>1</sup> William Tse, MD,<sup>2</sup> Hillard M. Lazarus, MD,<sup>2</sup> and David Kaplan, MD, PhD<sup>1</sup>

**Key Words:** Chronic lymphocytic leukemia; Flow cytometry; Cyclin D1; Cyclin D2; Cyclin D3

DOI: 10.1309/7C2VV961P60RMLHD

#### Abstract

*We analyzed protein expression of cyclin D1, cyclin D2, and cyclin D3 using high-resolution enzymatic amplification staining and flow cytometry in the neoplastic cells from 80 patients with CD5+ B-cell lymphoproliferative disorders. The D cyclins were expressed differentially in chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), and mantle cell lymphoma (MCL) with strong staining of cyclin D1 and D2 in MCL, strong staining of cyclin D1 but weak staining of cyclin D2 in 4 of 5 PLLs, and low-level staining for both cyclins in most CLLs. No correlation between cyclin D1 and D2 and growth rates or CD38 expression was observed. However, cyclin D1 levels were significantly higher in ZAP-70+ CLL cases, although no association between ZAP-70 and cyclin D2 was detected. The results indicate that flow cytometric analysis of D cyclins may help in classification of CD5+ B-cell lymphoproliferative disorders.*

Chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), and mantle cell lymphoma (MCL) are related clonal CD5+ B-cell lymphoproliferative disorders with overlapping clinical, morphologic, and phenotypic features.<sup>1-17</sup> Although criteria for differentiating these tumors and identifying prognostic features exist,<sup>1-8</sup> classification by current methods is difficult owing to subjectivity in the morphologic evaluation, variability within the diagnostic categories, inconsistencies in the expected antigen expression profile, and insensitivity of the analytic methods.<sup>5,8-12</sup>

The most reliable method to distinguish between PLL and CLL remains morphologic evaluation of peripheral blood smears.<sup>1</sup> However, the morphologic definition of a prolymphocyte is subjective and dependent on the quality of the stained material, limiting diagnostic precision.<sup>1</sup> The detection of cyclin D1 protein in immunohistochemically stained tissue sections has been suggested to be a sensitive and specific method to discriminate MCL from PLL and CLL because most MCL cases will have detectable nuclear expression of the protein.<sup>18-21</sup> Nevertheless, in some MCL cases, the protein is not identified and occasional CLL and PLL tumors show detectable protein by immunohistochemical staining.<sup>22-26</sup> Furthermore, the cyclin D1 immunostain is technically difficult to perform and often weak with only a subpopulation of cells positive, and the staining is assessed subjectively.<sup>18-21,24,27</sup>

Recently, Kaplan et al<sup>28</sup> adapted a highly sensitive signal-amplifying staining method, enzymatic amplification staining (EAS), to detect cyclin D1, cyclin D2, and cyclin D3 in lymphocytes by flow cytometry. All 3 D cyclins are key cell cycle regulatory molecules involved in oncogenesis. Their importance in the pathogenesis of lymphoproliferative disorders is highlighted by the occurrence of translocations targeting these

proteins for overexpression in B-cell lymphomas.<sup>29-31</sup> Previous analyses of cyclin D1 expression in low-grade B-cell lymphoproliferative disorders by flow cytometry have utilized used an antibody that cross-reacts with cyclin D2.<sup>32-34</sup> Moreover, a comprehensive analysis of the expression of all 3 D cyclins in the malignant cells from patients diagnosed with CLL, PLL, and MCL has not been performed. Therefore, we evaluated the expression of cyclin D1, cyclin D2, and cyclin D3 using EAS and flow cytometry in 80 CD5+ B-cell lymphoproliferative disorders. The results indicate that high-resolution flow cytometric evaluation of the D cyclins discriminates between the CD5+ B-cell lymphoproliferative disorders and might impart prognostic information in CLL. The findings also might have significant biologic implications.

## Materials and Methods

### Cases

The flow cytometry laboratory, University Hospitals of Cleveland, Cleveland, OH, identified 80 cases of CD5+ B-cell lymphoproliferative disorders. For diagnosis, all 5 MCL cases had tissue biopsy specimens with the characteristic phenotype and showed typical cyclin D1 nuclear immunoreactivity. Specimens included 2 lymph node biopsies, 1 tonsillectomy specimen, 1 gastrointestinal biopsy specimen, and 1 bone marrow biopsy specimen. For CLL, the diagnosis was based on peripheral blood morphologic features and phenotype according to the Matutes criteria.<sup>1,35,36</sup> The diagnosis of PLL was established by the presence of a predominant population of cells with prolymphocytic morphologic features in the peripheral blood.<sup>1</sup> Two patients also had lymph node biopsies, and 1 had a bone marrow biopsy supportive of the diagnosis. Of the 80 cases, 66 were classified as CLL, 5 as PLL, and 5 as MCL; 3 were deemed atypical CLL based on the atypical morphologic features and/or phenotype; and 1 transformed from CLL to PLL.

### Tumor Cell Growth

To establish the rate of tumor cell growth in the subset of cases (15 CLL and 3 PLL) with available clinical data, we used a best-fit exponential growth curve (Excel software, Microsoft, Redmond, WA) based on the change in lymphocyte count over time, assuming early-phase gompertzian growth.<sup>37</sup> Growth rates were determined only if a minimum of 3 separate lymphocyte counts were obtained during a period of at least 2 months and the cyclin D flow cytometric analysis was performed during the same period. The relative growth rate was estimated by using the exponential growth rate constant ( $r$ ) from the following equation:

$$P_t = P_o e^{rt}$$

where  $P_o$  is the initial lymphocyte count,  $P_t$  the final lymphocyte count,  $t$  the time (in months), and  $r$  the relative rate of tumor growth (per month).

### Cells

Peripheral blood samples were obtained from healthy volunteers, and peripheral blood, bone marrow, and lymph node samples from patient's with CD5+ low-grade B-cell malignant neoplasms were obtained from the clinical flow cytometry laboratory, University Hospitals of Cleveland, with institutional review board approval. Mononuclear cells were isolated in all cases by Ficoll-Hypaque discontinuous gradient centrifugation.

### Antibodies

A fluorescein-conjugated murine monoclonal antibody specific for cyclin D1, clone DCS-6, was obtained from BD Bioscience, San Diego, CA, along with fluorescein-conjugated isotype-matched control immunoglobulin. A murine monoclonal antibody to cyclin D2, clone DCS-3.1, was obtained from Abcam, Cambridge, MA. A fluorescein-conjugated murine monoclonal antibody to cyclin D3, clone G107-565, was obtained from BD Bioscience, along with a fluorescein-conjugated isotype-matched control immunoglobulin. The antibodies to cyclin D1, cyclin D2, and cyclin D3 are specific and do not bind other D cyclins.

### Flow Cytometry

We analyzed 84 specimens from 80 patients, including 72 peripheral blood specimens, 9 bone marrow specimens, 2 lymph nodes, and 1 tonsillectomy specimen. All tissue specimens were from patients with MCL. Three patients had specimens evaluated from multiple sources. All cells were analyzed within 48 hours of venipuncture or surgical removal.

Cell staining by EAS was performed as described previously.<sup>28</sup> Briefly, cells were fixed and permeabilized and stained with 20  $\mu$ L of the fluorescein-conjugated anti-cyclin D1 or isotype control as recommended by the manufacturer, 15  $\mu$ g/mL of anti-cyclin D2 or isotype control, or 20  $\mu$ L of fluorescein-conjugated anti-cyclin D3 or isotype control. Cells were incubated in a reaction volume of 50  $\mu$ L for 10 minutes at room temperature.

EAS was performed subsequently using FlowAmp Systems kits (Cleveland, OH) as follows: After washing, a secondary horseradish peroxidase-conjugated mouse monoclonal antibody to fluorescein isothiocyanate was added for cyclin D1 and D3, and a secondary polyclonal horseradish peroxidase-conjugated goat antimouse immunoglobulin was added for the cyclin D2-stained cases per manufacturer's recommendations. After incubation, cells were washed and amplification was performed with the addition of 50  $\mu$ g/ $\mu$ L of fluorescein isothiocyanate-tyramide in amplification medium

(FlowAmp Systems) and 0.01% peroxide (Sigma, St Louis, MO). Cells were washed in diluent, and fluorescence data were acquired on a FACScan (BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

The mean fluorescence index (MFI) was obtained for each D cyclin on the tumor cells. The *MFI* is defined as the ratio of the mean fluorescence intensity for the specific monoclonal antibody compared with the mean fluorescence intensity for the isotype-matched control that was performed in all cases. The MFI for cyclin D1, D2, and D3 on unstimulated, quiescent lymphocytes was determined using 8 normal control samples. Linearity was established previously for EAS using antibody-coated beads.<sup>38</sup> We assessed reproducibility of the staining by analyzing multiple frozen samples from the same case.

Flow cytometric data on the specimens also were obtained in the clinical flow cytometry laboratory using directly labeled antibodies as detailed elsewhere.<sup>39</sup> Analysis was performed on a BD FACSCalibur flow cytometer (BD Biosciences) using CellQuest software. Quantitation of CD38 on the CLL cells was performed based on the percentage of positive cells over background fluorescence using an allophycocyanin-conjugated antibody to CD38. Previous data from our laboratory established a cutoff of 24.5% for CD38+ CLL cases.<sup>39</sup> All CLL cases were analyzed for CD38 expression.

ZAP-70 staining (21 CLL cases) was performed using an Alexa-488 directly conjugated murine monoclonal antibody to ZAP-70 (Caltag Laboratories, Burlingame, CA). For ZAP-70 staining, we added 50  $\mu$ L of whole blood to 2 tubes containing CD5-phycoerythrin, CD45–peridinin chlorophyll protein, and CD19-allophycocyanin and incubated the tubes for 15 minutes at room temperature. Cells were permeabilized using Fix and Perm (Caltag). The ZAP-70–specific Alexa-488 conjugated antibody and an Alexa-488 conjugated isotype control were added along with Caltag “Reagent B” during the permeabilization step and incubated for 20 minutes. Cells were washed and resuspended in 200  $\mu$ L of phosphate-buffered saline with 1% paraformaldehyde before analysis. A normal peripheral blood sample was processed in parallel for each sample analyzed. The staining characteristics were confirmed using internal T cells as control samples and verified using the normal peripheral blood sample. CLL cells were identified by gating on the CD5+ and CD19+ cells, and the specific reactivity for ZAP-70 over the isotype control was determined. Cases were considered ZAP-70+ if the staining was greater than 20% over control.

## Statistics

Comparison of D cyclin levels between disease categories and normal lymphocytes and comparison between D cyclin levels and growth rate, CD38, and ZAP-70 expression was performed using the Student *t* test (Excel software).

## Results

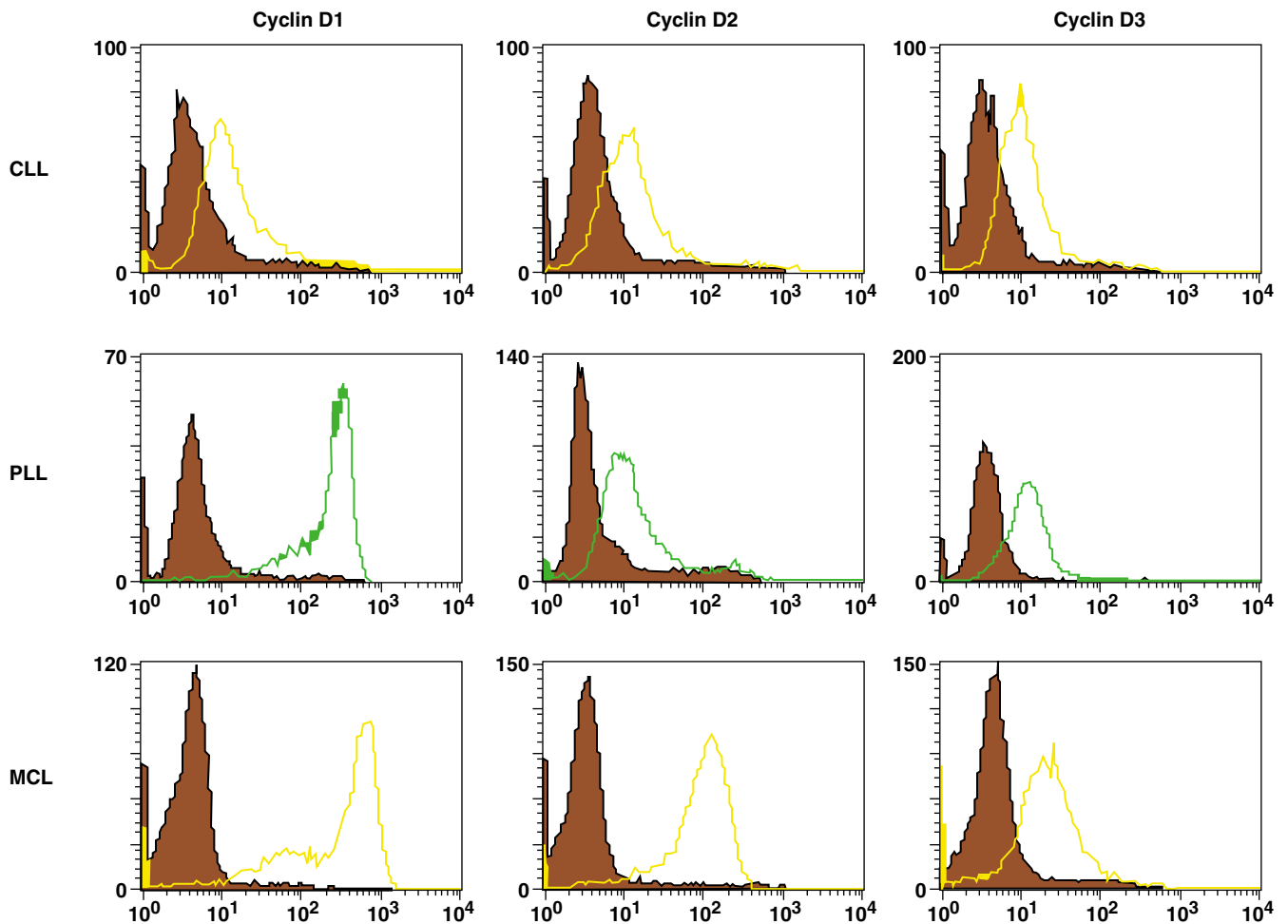
### Patterns of D Cyclin Expression in Low-Grade B-Cell Lymphoproliferative Disorders

We analyzed CD5+ B-cell lymphoproliferative disorders for the expression of cyclin D1, cyclin D2, and cyclin D3 using EAS and flow cytometry. Our study included 66 cases of CLL, 5 cases of PLL, and 5 cases of MCL. Typical staining patterns are shown in **Figure 1**. In most cases, MCL, PLL, and CLL could be distinguished based on their relative expression of cyclin D1 and D2 with MCL characterized by strong staining for cyclin D1 and D2, PLL characterized by strong staining for cyclin D1 and weak staining for cyclin D2, and CLL characterized by weak staining for cyclin D1 and D2. Cyclin D3 staining was weak in all 3 neoplasms.

Because cyclin D1 and D2 seemed to give the best discrimination between cases, we plotted the MFI of cyclin D1 vs cyclin D2 for the majority of our cases **Figure 2**. An MFI of 15 for cyclin D1 and cyclin D2 was used to separate the cases into 4 groups. The resulting graph allowed us, in a general way, to categorize these 3 disorders with some exceptions. For example, PLL cases could be distinguished readily from MCL by their lower cyclin D2 expression and most CLL cases based on the strong expression of cyclin D1 (lower right quadrant). One PLL case had a cyclin D2–predominant pattern.

The majority of CLL cases (73.5%) showed low cyclin D2 and D1 expression (lower left quadrant). However, CLL cells from some cases (13.2%) approximated the D cyclin pattern observed in the PLL cases. All 5 MCL cases showed strong expression of cyclin D1 and D2. MCL could be discriminated from CLL in most cases, although overlap occurred such that 4 cases (5.9%) classified as CLL by standard methods, showed cyclin D1 and D2 reactivity similar to MCL (upper right quadrant). Five CLL cases (7.4%) and 1 PLL case showed strong expression of cyclin D2 with low levels of cyclin D1 (upper left quadrant). These cases were not otherwise distinguishable from their counterparts. It is interesting that 1 CLL case analyzed 16 months before PLL transformation showed a MCL-like cyclin D1 and D2 pattern. This case was not analyzed after transformation.

We compared the expression of the D cyclins in the lymphoproliferative disorders with that observed in normal peripheral blood lymphocytes **Figure 3**. Previously, we had not observed differences in the level of the D cyclins expressed in peripheral blood B and T cells.<sup>28</sup> There is variability in the expression of cyclin D1 in normal lymphoid cells. Nevertheless, for CLL, the expression of cyclin D1 was significantly weaker (MFI  $\pm$  SD, 10.4  $\pm$  8.5 vs 44.3  $\pm$  21.4; *P* < .0001) and the expression of cyclin D2 somewhat stronger (MFI  $\pm$  SD, 9.1  $\pm$  6.4 vs 3.1  $\pm$  1.2; *P* = .01) than that in normal lymphoid cells. In MCL, cyclin D2 protein, as detected by



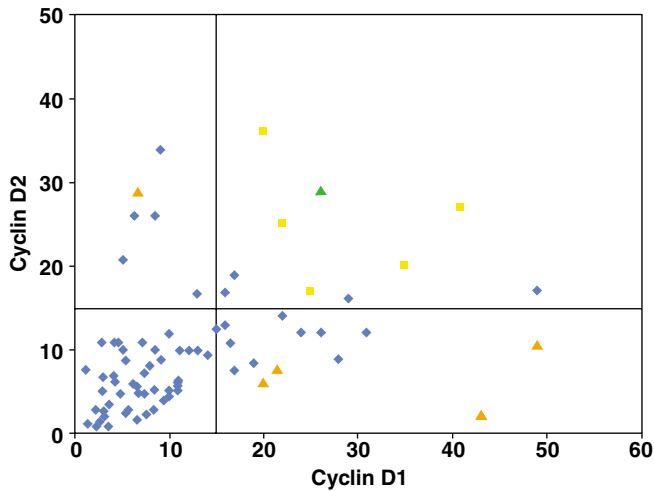
**Figure 1** Flow cytometric histograms of D cyclins in CD5+ B-cell lymphoproliferative disorders. Representative flow cytometric histograms of chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), and mantle cell lymphoma (MCL) cells stained with cyclin D1, cyclin D2, and cyclin D3 using enzymatic amplification and flow cytometry. CLL cells typically showed low-level staining for cyclin D1 and D2, PLL cells showed strong staining for cyclin D1 and weak staining for cyclin D2, and MCL cells showed strong staining for cyclin D1 and D2. Cyclin D3 staining was weak in all 3 disorders.

EAS and flow cytometry, was strikingly overexpressed compared with normal peripheral blood lymphocytes (MFI  $\pm$  SD,  $25 \pm 7.3$  vs  $3.1 \pm 1.2$ ;  $P < .0001$ ). However, cyclin D1 expression was not significantly different from nonmalignant lymphoid cells (MFI  $\pm$  SD,  $28.6 \pm 9.0$  vs  $44.3 \pm 21.4$ ;  $P = .68$ ). The PLL cases exhibited expression of cyclin D1 (MFI  $\pm$  SD,  $33 \pm 14.8$  vs  $44.3 \pm 21.4$ ;  $P = .18$ ) and D2 (MFI  $\pm$  SD,  $6.6 \pm 3.5$  vs  $3.1 \pm 1.2$ ;  $P = .05$ ) similar to normal peripheral blood lymphocytes, although cyclin D2 was at the level of statistical significance. No significant difference in expression of cyclin D3 was noted between any of the neoplasms and benign lymphocytes.

#### Correlation of Cyclin D1 and D2 With Cell Proliferation

We wondered whether the rate of cell proliferation influenced the expression level of the D cyclins and affected our ability to categorize the neoplasms based on their relative D

cyclin expression patterns. Therefore, we examined the in vivo growth rate of the lymphoproliferative disorders by review of the clinical laboratory data. We were able to establish the growth rate in 15 cases of CLL and 3 of PLL. Growth rate analysis of the CLL and PLL cases is illustrated in **Figure 4A**. The exponential growth rate of the tumors segregated into 2 groups based on a rate of growth greater or less than 0.1. All PLL cases showed a fast growth rate, as expected. However, roughly 40% of the CLL cases also showed a rate of growth greater than 0.1. We next compared the growth rate of the disorders with their relative expression of cyclin D1 and D2 **Figure 4B**, **Figure 4C**, and **Figure 4D**. However, neither the levels of expression of cyclin D1 ( $P = .09$ ) and cyclin D2 ( $P = .63$ ) nor combined cyclin D1 and D2 levels ( $P = .13$ ) correlated with the in vivo growth rate, regardless of whether CLL and PLL cases or only the CLL cases were analyzed.



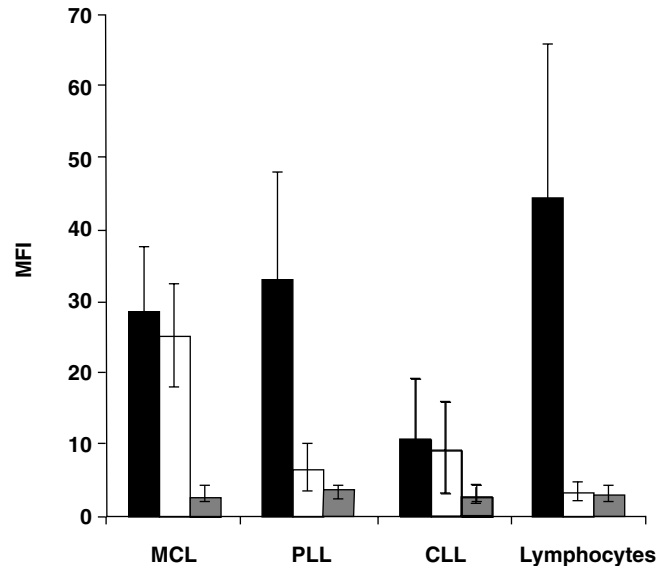
**Figure 2** Cyclin D1 and D2 distinguish between CD5+ B-cell lymphoproliferative disorders. The mean fluorescence index (MFI) of cyclin D1 vs cyclin D2 for 79 CD5+ B-cell lymphoproliferative disorders analyzed by enzymatic amplification staining and flow cytometry: 5 mantle cell lymphomas (MCLs; yellow squares), 5 prolymphocytic leukemias (PLLs; red triangles), 68 chronic lymphocytic leukemias (CLLs; blue diamonds), and 1 CLL that transformed to PLL (green triangle) were analyzed. Three atypical CLLs were included with the standard CLL cases. One CLL case was omitted because only cyclin D1 was analyzed. Quadrants are based on an MFI cutoff of 15. Note that the CLL cases cluster in the lower left quadrant, MCL cases in the upper right quadrant and PLL cases in the lower right quadrant.

### Correlation Between Cyclin D1 and D2 and CD38 and ZAP-70 in CLL Cells

We next evaluated whether the expression levels of cyclin D1 and D2 were related to the expression of CD38 and ZAP-70 on CLL cells because their expression identifies patients with a more aggressive course of disease. We found no association between the expression of cyclin D1 ( $P = .81$ ) and D2 ( $P = .57$ ) with CD38 (data not shown). However, in a subset of 21 cases for which ZAP-70 data were available, the mean  $\pm$  SD level of cyclin D1 expression was significantly greater in the ZAP-70+ ( $14.3 \pm 5.8$ ) than in the ZAP-70- cases ( $6.9 \pm 3.5$ );  $P = .002$  **Figure 5**. No association was observed with ZAP-70 expression and cyclin D2 expression: ZAP-70+, MFI  $\pm$  SD,  $13.9 \pm 9.1$  vs  $11.4 \pm 8.0$  in the ZAP-70- cases;  $P = .45$ .

### Discussion

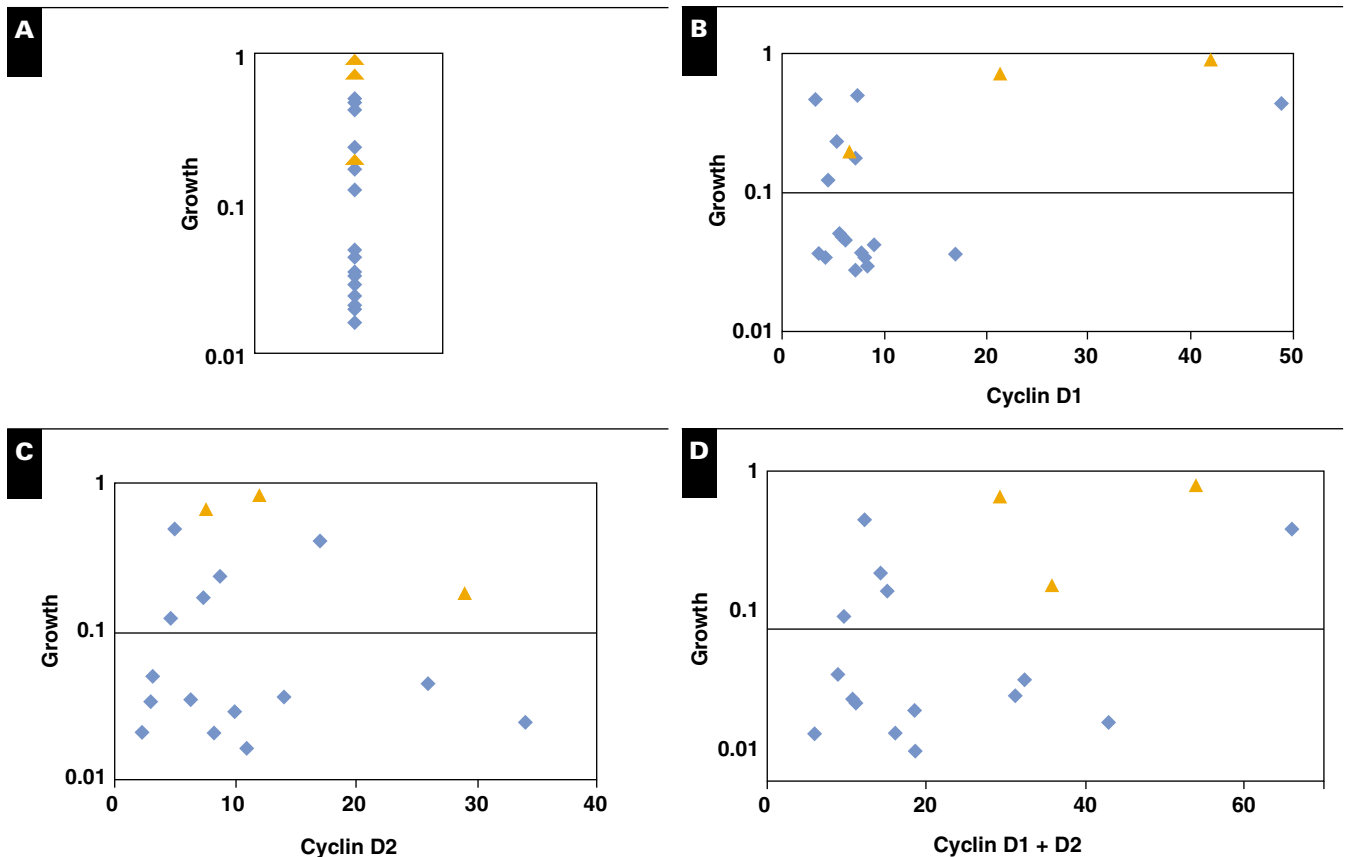
Our findings indicate that MCL, PLL, and CLL can be distinguished by their D cyclin expression patterns as detected by flow cytometry. Strong staining for cyclin D1



**Figure 3** D cyclin levels in the CD5+ lymphoproliferative disorders in comparison with normal lymphocytes. Comparison of cyclin D1 (black bar), cyclin D2 (white bar), and cyclin D3 (gray bar) levels in chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), and prolymphocytic leukemia (PLL) with that observed in normal peripheral blood lymphocytes. Compared with normal lymphocytes (mean fluorescence index [MFI]  $\pm$  SD,  $44.3 \pm 21.4$ ), cyclin D1 levels were reduced in CLL cells (MFI  $\pm$  SD,  $10.4 \pm 8.5$ ;  $P < .0001$ ) but not significantly different in MCL (MFI  $\pm$  SD,  $28.6 \pm 9.0$ ;  $P = .68$ ) and PLL (MFI  $\pm$  SD,  $33 \pm 14.8$ ;  $P = .18$ ) cells. Cyclin D2 levels were increased modestly in CLL cells (MFI  $\pm$  SD,  $9.1 \pm 6.4$ ;  $P = .01$ ), significantly increased in MCL cells (MFI  $\pm$  SD,  $25 \pm 7.3$ ;  $P < .0001$ ), and not appreciably different in PLL cells (MFI  $\pm$  SD,  $6.6 \pm 3.5$ ;  $P = .05$ ) compared with normal lymphocytes (MFI  $\pm$  SD,  $3.1 \pm 1.2$ ). Cyclin D3 levels were not significantly different from resting lymphocytes in all 3 disorders. Previously we have shown that cyclin levels are comparable in T- and B-lymphoid cells.

and D2 was observed in all 5 MCL cases examined, strong staining for cyclin D1 but weak staining of cyclin D2 was present in the cells from 4 of 5 cases of PLL, and low-level staining for both cyclins was noted in most cases (73.5%) of CLL. The distinction between these neoplasms is sometimes difficult owing to overlapping clinical and phenotypic features and the reliance on subjective staining criteria.<sup>1</sup> Although the study is somewhat limited by the small number of PLL and MCL cases evaluated, an objective flow cytometric analysis of cyclin D1 and D2, as shown here, may help distinguish these neoplasms. Analysis of additional cases could help further clarify the significance of the D cyclin expression patterns observed.

It should be noted that not all cases fell neatly into the diagnostic groups based on the level of cyclin D1 and D2



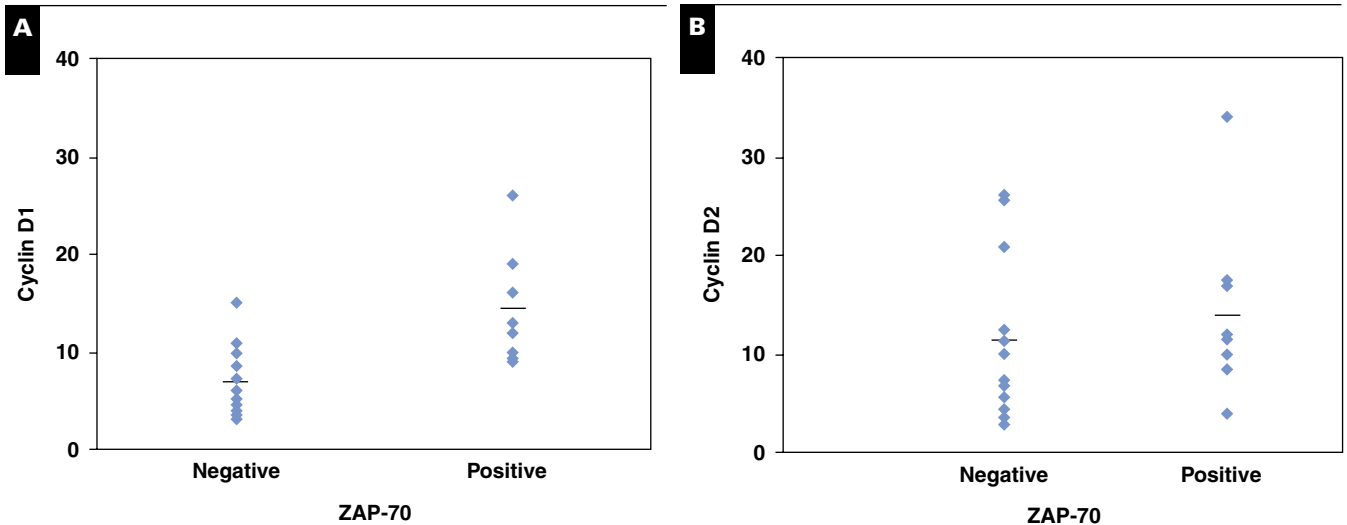
**Figure 4** Cyclin D1 and cyclin D2 levels do not correlate with in vivo growth rate. The growth rate of 15 chronic lymphocytic leukemia (CLL) cases (blue diamonds) and 3 prolymphocytic leukemia (PLL) cases (orange triangles) using best-fit exponential growth curves as described in the “Materials and Methods” section was determined. **A**, The cases segregated roughly into 2 groups based on an exponential growth rate greater or less than 0.1 with means of 0.24 and 0.03, respectively. Note that 40% of the CLL cases exhibited a fast growth rate. Growth rates were compared with the level of expression of cyclin D1 (**B**), cyclin D2 (**C**), and combined levels of cyclin D1 and D2 (**D**). No correlation of the cyclin D1 ( $P = .09$ ), D2 ( $P = .63$ ), or combined ( $P = .13$ ) levels with in vitro growth rate was observed, regardless whether all cases or only the CLL cases were examined.

expression. Most notably, variation was present within the CLL cases, particularly with regard to cyclin D1 levels. Some cases demonstrated a D cyclin staining pattern that approximated that observed in the PLL cases (13.2%) with strong cyclin D1 staining and weak cyclin D2 staining. Fewer CLL cases (5.9%) demonstrated the strong cyclin D1 and D2 staining pattern observed in MCL cases. The variation in cyclin levels did not seem to be accounted for by differences in the in vivo cell growth based on an analysis of a subset of tumors. We had limited clinical data on the patients because the specimens were accrued from multiple outpatient sources and private clinicians offices. Consequently, the clinical outcome of the patients diagnosed with CLL with cyclin D1 and cyclin D2 levels in the MCL and PLL ranges could not be ascertained. It is not known whether these patients diagnosed with CLL will demonstrate a more aggressive clinical course. It is interesting that Ravandi-Kashani et al<sup>40</sup> noted that patients with CLL

cells with increased cyclin D1 expression, as determined by radioimmunoassay analysis, had a worse outcome.

We also noted that the cells from 5 CLL cases (7.4%) and 1 of PLL displayed a cyclin D2–predominant staining pattern. In addition, we identified 3 cases in which cyclin D3 staining was increased. In all 3 cases, only a subpopulation of cells showed cyclin D3 expression. These cases were thought to be CLLs, although all 3 had atypical phenotypic features. It is reasonable to speculate on the significance of the samples that did not conform to the pattern observed in the other cases. One plausible explanation for the observed differences in the samples is that the D cyclin expression profile identifies distinct pathogenetic subgroups of tumors.

No correlation was noted between the expression of cyclin D1 and D2 and CD38 expression, and although an association between cyclin D2 and ZAP-70 was not observed, the expression of cyclin D1 was significantly



**Figure 5** Cyclin D1 levels correlate with ZAP-70 expression in chronic lymphocytic leukemia (CLL). ZAP-70 expression was determined in 21 cases and compared with cyclin D1 (**A**) and D2 (**B**) levels. Cyclin D2 levels were similar in the ZAP-70+ (mean fluorescence index [MFI]  $\pm$  SD,  $13.9 \pm 9.1$ ) and ZAP-70- (MFI  $\pm$  SD,  $11.4 \pm 8.0$ ) cases;  $P = .45$ . However, cyclin D1 levels were significantly higher in the ZAP-70+ (MFI  $\pm$  SD,  $14.3 \pm 5.8$ ) than the ZAP-70- (MFI  $\pm$  SD,  $6.9 \pm 3.5$ ) cases;  $P = .002$ .

stronger in the ZAP-70+ cases. This latter finding is intriguing and raises the possibility of a link between cell cycling and enhanced signaling through the B-cell receptor complex by ZAP-70 in CLL cells.<sup>41,42</sup> In B cells, cross-linking of the B-cell receptor results in activation of SYK, leading to the formation of a membrane signaling complex incorporating phosphatidylinositol-3 (PI3) kinase.<sup>43</sup> This pathway is known to up-regulate cyclin D2 in murine B cells.<sup>44</sup> It is unclear why cyclin D2 levels in CLL cells do not significantly differ from those of benign lymphocytes.

PI3-kinase also initiates a cascade of events, including activation of Akt-1.<sup>45</sup> Akt-1 has been identified as an upstream modulator of cyclin D1 by decreasing cyclin D1 proteolytic degradation via phosphorylation and inactivation of glycogen synthase kinase-3 (GSK-3).<sup>46,47</sup> Chen et al<sup>41,42</sup> have shown that ZAP-70 augments the activation of SYK in CLL cells. Therefore, it is conceivable that in CLL cells overexpressing ZAP-70, amplified PI3-kinase signaling ensues, leading to a relative increase in cyclin D1 compared with the ZAP-70- cases. It is interesting that Akt-1 is dysregulated in CLL cells.<sup>48</sup> Nevertheless, in most CLL cases, we noticed reduced expression of cyclin D1 compared with that observed in normal lymphocytes, suggesting additional mechanisms must be active in controlling cyclin D1 protein levels. Indeed, cyclin D1 also is regulated by the Wnt-1 signaling cascade, a pathway operative in CLL cells.<sup>49,50</sup> Control of cyclin D1 expression also might occur by other means in nonneoplastic T cells and B cells.

Previous studies have indicated that cyclin D1 is overexpressed in MCL. Although the qualitative expression of cyclin

D1 has been examined extensively using immunohistochemical analysis, most quantitative studies of cyclin D1 expression in MCL, however, have been based on RNA levels with fewer analyses of the amount of protein produced. Sola et al<sup>51</sup> noted that cyclin D1 protein expression was not correlated quantitatively with cyclin D1 messenger RNA levels in MCL and CLL cells. Two studies used flow cytometry to determine the expression of cyclin D1 protein in malignant lymphocytes and identified overexpression of cyclin D1 protein in MCL lymphoma cells.<sup>32,33</sup> However, the analysis was carried out using the monoclonal antibody, 5D4, which is known to cross-react with cyclin D2.<sup>34</sup> We identified significant overexpression of cyclin D2 rather cyclin D1 in our cases, which may explain their findings.

Several analyses of cyclin D1 protein expression by Western blot, immunoblots, and radioimmunoassays also have identified overexpression of the cyclin D1 protein in MCL compared with that in normal lymphocytes.<sup>19,40,52</sup> The reason for the discrepancy between these results and ours is unclear but could relate to a number of factors. Our flow cytometric assay involves fixation of the cells before cellular permeabilization and staining, whereas the other methods require cell lysis, which may lead to enhanced proteolytic degradation of cyclin D1. The detection methods may be affected differentially by cyclin D1's subcellular localization or complexed proteins. Finally, there are differences in the sensitivity of the analyses. Of note, we previously validated the specificity of our staining method for cyclin D1.<sup>28</sup> Moreover, we have shown that the amplified fluorescence signal produced with EAS maintains linearity.<sup>38</sup>

We did not identify a relationship between D cyclin expression and cell proliferation similar to that observed in studies involving other tumors.<sup>53,54</sup> The failure to identify a link between cell proliferation and cyclin D1 or cyclin D2 levels may be explained by their intracellular distribution because nuclear expression of the protein is necessary for cell cycle control. We have shown that cyclin D1 shuttles from the cytoplasm to the nucleus in peripheral blood lymphocytes without a noticeable change in protein level on stimulation and proliferation.<sup>28</sup> The subcellular localization of the protein is controlled by a number of factors, including its interaction with p21 and p27 and phosphorylation of the molecule via the GSK-3 $\beta$ .<sup>47,55-57</sup> It has been suggested that the consequence of the molecular translocation (t(11;14)) in MCL is accumulation of the protein in the nucleus rather than true cellular overexpression of the protein, perhaps due to some of these molecular interactions.<sup>51</sup> Our results would be consistent with that premise.

There was significant overexpression of cyclin D2 protein in MCL cells in our cases. Before this analysis, only a limited set of studies have examined cyclin D2 expression, via message levels, in primary MCL tumors.<sup>58,59</sup> We found cyclin D2 protein also was overexpressed in CLL cases, although only to a relatively small amount compared with MCL. PLL cases did not show overexpression of cyclin D2. The role of cyclin D2 in distinguishing the unique biology of MCL from CLL and PLL is not known. Cyclin D2 has importance in B-cell lymphoma behavior because elevated cyclin D2 message levels are a risk factor for patients with diffuse large B-cell lymphoma and cyclin D2 messenger RNA is up-regulated on B-lymphocyte transformation by the Epstein-Barr virus.<sup>60,61</sup>

We evaluated the protein expression of the 3 D cyclins in CLL and the related CD5+ B-cell lymphoproliferative disorders, PLL and MCL, by flow cytometry. Previous studies have left an incomplete picture of D cyclin expression in these disorders, likely owing to the difficulty in analyzing these proteins and the inconsistent correlation between protein and message levels. We could distinguish the 3 disorders, with some exceptions, by their relative expression of cyclin D1 and D2. In addition, cyclin D1 levels were significantly higher in ZAP-70+ CLL cases. Therefore, the analysis of the D cyclins by flow cytometry might help in the classification of these tumors and could have the added benefit of identifying high-risk subgroups. These studies underscore the power and usefulness of high-sensitivity flow cytometric analysis in lymphoproliferative disorders.

*From the Departments of <sup>1</sup>Pathology and <sup>2</sup>Medicine, Case Western Reserve University, and the Ireland Cancer Center, University Hospitals of Cleveland/Case Western Reserve University, Cleveland, OH.*

*Supported by in part by grant CA77428 from the National Institutes of Health, Bethesda, MD.*

*Address reprint requests to Dr Meyerson: Dept of Pathology, Case Western Reserve University, 11100 Euclid Ave, Cleveland, OH 44106.*

*Acknowledgments: We thank Kristine Lewandowska for technical help and the members of the University Hospitals of Cleveland Flow Cytometry Laboratory, Dawn Thut, Jennifer Powers, Alison Edinger, and Ebenezer Osei, for assistance.*

## References

- Jaffe ES, Harris NL, Stein H, et al, eds. *Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: IARC Press; 2001:127-132, 168-170. *World Health Organization Classification of Tumours*.
- Schroers R, Griesinger F, Trumper L, et al. Combined analysis of ZAP-70 and CD38 expression as a predictor of disease progression in B-cell chronic lymphocytic leukemia. *Leukemia*. 2005;19:750-758.
- Damle RN, Wasil T, Fais F, et al. IgV gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood*. 1999;94:1840-1847.
- Mainou-Fowler T, Dignum HM, Proctor SJ, et al. The prognostic value of CD38 expression and its quantification in B cell chronic lymphocytic leukemia (B-CLL). *Leuk Lymphoma*. 2004;45:455-462.
- Crespo M, Bosch F, Villamor N, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. *N Engl J Med*. 2003;348:1764-1775.
- Wiestner A, Rosenwald A, Barry TS, et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. *Blood*. 2003;101:4944-4951.
- Dorfman DM, Pinkus GS. Distinction between small lymphocytic and mantle cell lymphoma by immunoreactivity for CD23. *Mod Pathol*. 1994;7:326-331.
- Kilo MN, Dorfman DM. The utility of flow cytometric immunophenotypic analysis in the distinction of small lymphocytic lymphoma/chronic lymphocytic leukemia from mantle cell lymphoma. *Am J Clin Pathol*. 1996;105:451-457.
- Kumar S, Green GA, Teruya-Feldstein J, et al. Use of CD23 (BU38) on paraffin sections in the diagnosis of small lymphocytic lymphoma and mantle cell lymphoma. *Mod Pathol*. 1996;9:925-929.
- Huh YO, Pugh WC, Kantarjian HM, et al. Detection of subgroups of chronic B-cell leukemias by FMC7 monoclonal antibody. *Am J Clin Pathol*. 1994;101:283-289.
- Catovsky D, Cherchi M, Brookss D, et al. Heterogeneity of B-cell leukemias demonstrated by the monoclonal antibody FMC7. *Blood*. 1981;58:406-408.
- Ginaldi L, De Martinis M, Matutes E, et al. Levels of expression of CD19 and CD20 in chronic B cell leukaemias. *J Clin Pathol*. 1998;51:364-369.
- Tefferi A, Bartholmai BJ, Witzig TE, et al. Heterogeneity and clinical relevance of the intensity of CD20 and immunoglobulin light-chain expression in B-cell chronic lymphocytic leukemia. *Am J Clin Pathol*. 1996;106:457-461.
- DiRaimondo F, Albitar M, Huh Y, et al. The clinical and diagnostic relevance of CD23 expression in the chronic lymphoproliferative disease. *Cancer*. 2002;94:1721-1730.

15. Wohlschlaeger CH, Lange K, Merz H, et al. Aberrant immunophenotypes of mantle cell lymphomas. *Leuk Lymphoma*. 2003;44:269-273.
16. Hulkkonen J, Vilpo L, Hurme M, et al. Surface antigen expression in chronic lymphocytic leukemia: clustering analysis, interrelationships and effects of chromosomal abnormalities. *Leukemia*. 2002;16:178-185.
17. Kampalath B, Barcos MP, Stewart C. Phenotypic heterogeneity of B cells in patients with chronic lymphocytic leukemia/small lymphocytic lymphoma. *Am J Clin Pathol*. 2003;119:824-832.
18. Yang WI, Zukerberg LR, Motokura T, et al. Cyclin D1 (bcl-1, PRAD1) protein expression in low-grade B-cell lymphomas and reactive hyperplasia. *Am J Pathol*. 1994;145:86-96.
19. de Boer CJ, Schuurin E, Dreef E, et al. Cyclin D1 protein analysis in the diagnosis of mantle cell lymphoma. *Blood*. 1995;86:2715-2723.
20. Zukerberg LR, Yang WI, Arnold A, et al. Cyclin D1 expression in non-Hodgkin's lymphomas: detection by immunohistochemistry. *Am J Clin Pathol*. 1995;103:756-760.
21. Swerdlow SH, Yang WI, Zukerberg LR, et al. Expression of cyclin D1 protein in centrocytic/mantle cell lymphomas with and without rearrangement of the bcl1/cyclin D1 gene. *Hum Pathol*. 1995;26:999-1004.
22. Yatabe Y, Suzuki R, Tobinai K, et al. Significance of cyclin D1 overexpression for the diagnosis of mantle cell lymphoma: a clinicopathologic comparison of cyclin D1-positive MCL and cyclin D1-negative MCL-like B-cell lymphoma. *Blood*. 2000;95:2253-2261.
23. O'Malley DP, Vance GH, Orazi A. Chronic lymphocytic leukemia/small lymphocytic lymphoma with trisomy 12 and focal cyclin D1 expression: a potential diagnostic pitfall. *Arch Pathol Lab Med*. 2005;129:92-95.
24. Cheuk W, Wong KO, Wong CS, et al. Consistent immunostaining for cyclin D1 can be achieved on a routine basis using a newly available rabbit monoclonal antibody. *Am J Surg Pathol*. 2004;28:801-807.
25. Wong KF, So CC, Chan JK. Nucleolated variant of mantle cell lymphoma with leukemic manifestations mimicking prolymphocytic leukemia. *Am J Clin Pathol*. 2002;117:246-251.
26. Schlette E, Bueso-Ramos C, Giles F, et al. Mature B-cell leukemias with more than 55% prolymphocytes: a heterogeneous group that includes an unusual variant of mantle cell lymphoma. *Am J Clin Pathol*. 2001;115:571-581.
27. Korin HW, Schwartz MR, Chirala M, et al. Optimized cyclin D1 immunoperoxidase staining in mantle cell lymphoma. *Appl Immunohistochem Mol Morphol*. 2000;8:57-60.
28. Kaplan D, Meyerson H, Husel W, et al. D cyclins in lymphocytes. *Cytometry*. 2005;63:1-9.
29. Raffeld M, Jaffe ES. bcl-1, t(11;14), and mantle cell-derived lymphomas. *Blood*. 1991;78:259-263.
30. Hoglund M, Johansson B, Pedersen-Bjergaard J, et al. Molecular characterization of 12p abnormalities in hematologic malignancies: deletion of KIP1, rearrangement of TEL, and amplification of CCND2. *Blood*. 1996;87:324-330.
31. Sonoki T, Harder L, Horsman DE, et al. Cyclin D3 is a target gene of t(6;14)(p21.1;q32.3) of mature B-cell malignancies. *Blood*. 2001;98:2837-2844.
32. Elnenaï MO, Jadayel DM, Matutes E, et al. Cyclin D1 by flow cytometry as a useful tool in the diagnosis of B-cell malignancies. *Leuk Res*. 2001;25:115-123.
33. Jain P, Giustolisi GM, Atkinson S, et al. Detection of cyclin D1 in B cell lymphoproliferative disorders by flow cytometry. *J Clin Pathol*. 2002;55:940-945.
34. Kuroda H, Komatsu H, Nakamura S, et al. The positive nuclear staining observed with monoclonal antibody against PRAD1/cyclin D1 correlates with mRNA expression in mantle cell lymphoma. *Jpn J Cancer Res*. 1995;86:890-898.
35. Matutes E, Owusu-Ankomah A, Morilla R, et al. The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. *Leukemia*. 1994;8:1640-1645.
36. Moreau EJ, Matutes E, A'Hern RP, et al. Improvement of the chronic lymphocytic leukemia scoring system with the monoclonal antibody SN8 (CD79b). *Am J Clin Pathol*. 1997;108:378-382.
37. Laird AK. Dynamics of tumor growth. *Br J Cancer*. 1964;18:490-502.
38. Kaplan D. Enzymatic amplification staining for cell surface antigens. In: Darzynkiewicz Z, Dean P, Orfao A, et al, eds. *Current Protocols in Cytometry*. New York, NY: Wiley; 2003:6.14.1-6.14.11.
39. Cocco A, Edinger A, Thut D, et al. Bimodal cell populations are common in chronic lymphocytic leukemia but do not impact prognosis. *Am J Clin Pathol*. 2005;123:818-825.
40. Ravandi-Kashani F, O'Brien S, Manshouri T, et al. Variations in the low levels of cyclin D1/bcl1 have prognostic value in chronic lymphocytic leukemia. *Leuk Res*. 2000;24:469-474.
41. Chen L, Widhopf G, Huynh L, et al. Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. *Blood*. 2002;100:4609-4614.
42. Chen L, Apgar J, Huynh L, et al. ZAP-70 directly enhances IgM signaling in chronic lymphocytic leukemia. *Blood*. 2005;105:2036-2041.
43. Tsubata T, Wienands J. B cell signaling. Introduction. *Int Rev Immunol*. 2001;20:675-678.
44. Glassford J, Soeiro I, Skarell SM, et al. BCR targets cyclin D2 via Btk and the p85alpha subunit of PI3-K to induce cell cycle progression in primary mouse B cells. *Oncogene*. 2003;22:2248-2259.
45. Nicholson KM, Anderson NG. The protein kinase B/Akt signaling pathway in human malignancy. *Cell Signal*. 2002;14:381-395.
46. Liang J, Slingerland JM. Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. *Cell Cycle*. 2003;2:339-345.
47. Diehl JA, Cheng M, Roussel MF, et al. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev*. 1998;12:3499-3511.
48. Plate JM. PI3-kinase regulates survival of chronic lymphocytic leukemia B-cells by preventing caspase 8 activation. *Leuk Lymphoma*. 2004;45:1519-1529.
49. Karim R, Tse G, Putti T, et al. The significance of the Wnt pathway in the pathology of human cancers. *Pathology*. 2004;36:120-128.
50. Lu D, Zhao Y, Tawatao R, et al. Activation of the Wnt signaling pathway in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A*. 2004;101:3118-3123.
51. Sola B, Salaun V, Ballet JJ, et al. Transcriptional and post-transcriptional mechanisms induce cyclin-D1 over-expression in B-chronic lymphoproliferative disorders. *Int J Cancer*. 1999;83:230-234.
52. Alkan S, Schnitzer B, Thompson JL, et al. Cyclin D1 protein expression in mantle cell lymphoma. *Ann Oncol*. 1995;6:567-570.
53. Wong SC, Chan JK, Lee KC, et al. Differential expression of p16/p21/p27 and cyclin D1/D3, and their relationships to cell proliferation, apoptosis, and tumour progression in invasive ductal carcinoma of the breast. *J Pathol*. 2001;194:35-42.

54. Tut VM, Braithwaite KL, Angus B, et al. Cyclin D1 expression in transitional cell carcinoma of the bladder: correlation with p53, waf1, pRb and Ki67. *Br J Cancer*. 2001;84:270-275.
55. Diehl JA, Zindy F, Sherr CJ. Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev*. 1997;11:957-972.
56. Alt JR, Gladden AB, Diehl JA. p21(Cip1) Promotes cyclin D1 nuclear accumulation via direct inhibition of nuclear export. *J Biol Chem*. 2002;277:8517-8523.
57. LaBaer J, Garrett MD, Stevenson LF, et al. New functional activities for the p21 family of CDK inhibitors. *Genes Dev*. 1997;11:847-862.
58. Delmer A, Ajchenbaum-Cymbalista F, Tang R, et al. Overexpression of cyclin D2 in chronic B-cell malignancies. *Blood*. 1995;85:2870-2876.
59. Rosenwald A, Wright G, Wiestner A, et al. The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma. *Cancer Cell*. 2003;3:185-197.
60. Hans CP, Weisenburger DD, Greiner TC, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. *Blood*. 2004;103:275-282.
61. Arvanitakis L, Yaseen N, Sharma S. Latent membrane protein-1 induces cyclin D2 expression, pRb hyperphosphorylation, and loss of TGF-beta 1-mediated growth inhibition in EBV-positive B cells. *J Immunol*. 1995;155:1047-1056.