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Cyclin D1 (*Bcl*-1, PRAD1) Protein Expression in Low-Grade B-Cell Lymphomas and Reactive Hyperplasia

Woo-Ick Yang,* Lawrence R. Zukerberg,*† Toru Motokura,† Andrew Arnold,† and Nancy L. Harris*

From the Department of Pathology and Endocrine Oncology Unit,† Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts*

Mantle cell (centrocytic) lymphoma (MCL) and occasional cases of B-cell small lymphocytic lymphoma/chronic lymphocytic leukemia (B-SLL/CLL) show a characteristic translocation, t(11:14)(q13;q32) involving rearrangement of the Bcl-1 region. Recently it was shown that the key Bcl-1-region oncogene is cyclin D1/PRAD1; cyclin D1 mRNA was shown to be overexpressed in cases of MCL. We examined cyclin D1 protein expression in low-grade B-cell lymphomas and reactive lymphoid hyperplasias using polyclonal and monoclonal antibodies to cyclin D1 protein. Definite nuclear staining was seen in 15 of 15 MCLs, 1 of 7 B-SLL/CLLs, 0 of 7 reactive hyperplasias, 0 of 10 follicular lymphomas, and 0 of 4 lymphomas of mucosa-associated lymphoid tissue using immunoperoxidase stains on paraffin-embedded sections. Best results were obtained with the affinity-purified polyclonal antibody on microwave-treated, formalin-fixed, paraffin-embedded tissue. MCLs showed diffuse nuclear staining, whereas the one positive B-SLL/CLL showed dot-like or globular nuclear staining. Nuclear cyclin D1 protein can be detected in all cases of MCL and in rare cases of B-SLL/CLL using an immunohistochemical technique on formalin-fixed, paraffin-embedded tissue, and it does not appear to be detectable in reactive hyperplasias and other low-grade B-cell lymphomas. This protein may be useful in subclassification of low-grade B-cell lymphomas. (Am J Pathol 1994, 145:86–96)

Altered expression of proto-oncogenes by chromosomal translocations has been implicated in the pathogenesis of certain types of B cell non-Hodgkin's lymphomas and other tumors. C-*myc* overexpression by t(8;14)(q24;32) in Burkitt's lymphoma[1] and Bcl-2 overexpression by t(14;18)(q32;21) in follicular lymphomas[2] are common examples. Mantle cell lymphoma (MCL)[3], formerly known as centrocytic lymphoma[4,5]; intermediate lymphocytic lymphoma[6,7]; lymphocytic lymphoma of intermediate differentiation[8,9]; and mantle zone lymphoma[10] have a characteristic abnormality, t(11;14).[11–14] The sequences on 11q13 adjacent to the breakpoint of t(11;14) were designated *Bcl*-1,[15] and further studies demonstrated strong association of *Bcl*-1 rearrangement with MCL.[16–22] Rare cases of B-cell small lymphocytic lymphoma/chronic lymphocytic leukemia (B-SLL/CLL) also show this translocation and *Bcl*-1 rearrangement.[16,23,24] Although the *Bcl*-1 rearrangement was associated with lymphoma and was presumed to deregulate a nearby proto-oncogene similar to the c-*myc* and *Bcl*-2 rearrangements, transcribed sequences in the vicinity of the *Bcl*-1 locus were identified only recently.[25,26]

Cyclin D1/PRAD1 is a highly conserved oncogene on 11q13 which was found to be transcriptionally activated by clonal rearrangements in a subset of parathyroid adenomas.[25–27] In addition, cyclin D1/PRAD1 is amplified in 15 to 20% of breast and squamous cell carcinomas in which the *Bcl*-1-*int2*-*hst*1 region of 11q13 is amplified.[28] Cyclin D1/PRAD1 has recently been shown to be within 120 kb of the original *Bcl*-1 major translocation cluster region[29,30] with occasional breakpoints as close as 1 kb from PRAD1.[31] Furthermore, it was found that cyclin D1 mRNA transcripts were overexpressed in MCLs and in no control

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WI Yang and LR Zukerberg made equal contributions.

Address reprint requests to Dr. Nancy L. Harris, Pathology, Warren 2, Massachusetts General Hospital, Fruit Street, Boston, MA 02114.

lymphoma tissues.[18,29] Thus cyclin D1/PRAD1 has been implicated as the *Bcl*-1 oncogene and is involved in parathyroid adenomas, breast and squamous cell carcinomas, and MCLs.

Despite the importance of cyclin D1 as an oncogene, no studies have addressed cyclin D1 protein levels in tumors. In this study we describe an immunohistochemical staining method for routine detection of cyclin D1 protein using formalin-fixed paraffin-embedded tissue. We examined cyclin D1 protein levels in 36 cases of low-grade B-cell lymphomas and seven cases of reactive hyperplasia and correlated staining data with protein detection by immunoblots. We found cyclin D1 nuclear staining in MCLs and one case of B-SLL/CLL, but not in other types of low-grade B-cell lymphomas or reactive hyperplasias.

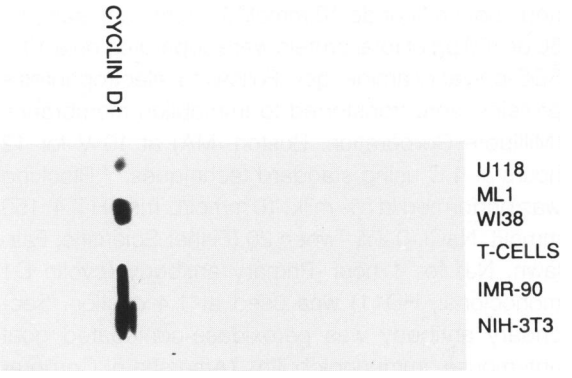

Figure 1. *Western blot of cell lines with affinity-purified polyclonal antisera no. 19 showing specificity of the antisera for cyclin D1. U118-glioblastoma cell line with overexpression of cyclin D1; ML1-myeloid leukemia cell line with cyclin D3 but not cyclin D1; WI38 and IMR-90-normal human fibroblast cell lines with high levels of cyclin D1; T cells PHA-stimulated primary buffy-coat T cells express cyclin D2 and D3 but not cyclin D1; NIH-3T3-transformed mouse fibroblast cell line with high levels of cyclin D1.*

Materials and Methods

Production of Anti-Cyclin D1 Antibodies

Full-length cyclin D1 protein overexpressed in bacteria was used to immunize female BALB/c mice and rabbits (Hazleton, Denver; PA).[26] After high titers of antibodies specific for cyclin D1 were detected, a representative mouse was sacrificed, and its splenocytes were fused with NS1 myeloma cells. Positive tissue culture supernatants were detected by enzyme-linked immunoadsorbent assay screening against cyclin D1. To characterize the cyclin D1 antibodies further, each was tested for the ability to immunoprecipitate *in vitro*-translated protein and native cyclin D1 from human cells and for the ability to recognize the protein on immunoblots. The correct cells were single-cell cloned twice by limiting dilution and single-cell picks. The antibodies used in this paper were specific for cyclin D1 and did not exhibit cross-reactivity for cyclins D2 or D3.

Rabbit antisera were affinity-purified by incubating overnight with cyclin D1 coupled to cyanogen bromide-activated Sepharose beads followed by extensive washing (250 mmol/L NaCl) and elution with 100 mmol/L glycine, pH 2.5 (neutralizing with TRIS, pH 8.0). The purified antiserum was tested by Western blot and immunoprecipitation against native cyclin D1. The specificity was shown by Western blot using extract from several cell lines with and without cyclin D1 overexpression (Figure 1). Antibodies were concentrated by ammonium sulfate precipitation and dialyzed against phosphate-buffered saline (PBS). Concentrated affinity-purified polyclonal antibody was stored with sodium azide.

Tissue Samples

Thirty-six cases of low-grade B-cell lymphoma were classified according to the morphological and immunophenotypical criteria of the updated Kiel classification.[5,32] Cases with typical histological and immunophenotypical features were included; equivocal or unclassifiable cases were not studied. Seven cases of reactive lymphoid hyperplasia were used as controls. Formalin- or B5-fixed tissue was embedded in paraffin and stained with hematoxylin and eosin and Giemsa stain. The frozen tissue was embedded in ornithine carbamoyltransferase compound (Miles Laboratories, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −70 C until use.

There were 15 cases of MCL (15/15 $CD20^+$, 14/15 $CD5^+$, 8/8 $CD23^-$), 7 cases of B-SLL/CLL (7/7 $CD20^+$, 7/7 $CD5^+$, 3/3 $CD23^+$), 10 cases of follicular (centroblastic/centrocytic) lymphoma, predominantly small cleaved-cell type (10/10 $CD20^+$, 0/6 $CD5^+$, 7/10 $CD10^+$), and 4 cases of lymphoma of MALT (4/4 $CD20^+$, 0/3 $CD5^+$, 0/4 $CD10^+$). Four of the cases of MCL (cases 2, 3, 4, 12) had been included in a previous study of PRAD1/cyclin D1 mRNA expression.[29] All showed cyclin D1 mRNA expression, and cases 3 and 4 had rearranged *Bcl*-1 genes using a single probe to the major translocation cluster.

Immunoblots

Tissue samples were teased and lysed in lysis buffer (50 mmol/L HEPES, pH 7.4, 0.1% NP40, and 250 mM NaCl) with proteases and phosphatase inhibitors (aprotinin, 5 µg/ml; phenylmethylsulfonyl fluoride, 100 µg/ml; leupeptin, 5 µg/ml; sodium vanidate, 5 µg/ml;

and sodium fluoride 10 mmol/L). From each sample, 50 or 100 μg of total protein were separated on a 10% SDS-polyacrylamide gel. Following electrophoresis proteins were transferred to Immobilon membranes (Millipore Corporation, Boston, MA) at 10 V for 12 hours at 4 C using standard techniques.[33] Blocking was performed in 5% milk, 10 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 0.2% Tween 20 (Fisher Scientific, Fairlawn, NJ) for 1 hour. Primary antibody (cyclin D1 monoclonal, HD11) was used at 1:4 dilution. Secondary antibody was peroxidase-conjugated goat anti-mouse immunoglobulin. (Amersham Corporation, Arlington Heights, IL; 1:5,000 dilution). The detection system used was chemiluminescence (ECL; Amersham).

Immunohistochemical Staining

Paraffin-Embedded Sections

Immunostaining was performed by the avidin-biotin peroxidase complex (ABC) method[34] using Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) as follows. Sections 4 μ thick were mounted on adhesive-coated slides, heated to 60 C for 1 hour, deparaffinized, and rehydrated through xylene and alcohol. Endogenous peroxidase was blocked with 3% H_2O_2 in methanol for 5 minutes. After rinsing in tap water and (PBS; 10 mmol/L phosphate-buffered saline, pH 7.4) slides were placed in plastic Coplin jars containing 10 mmol/L citrate buffer. Jars were covered with loose-fitting caps and heated in the microwave oven for 20 minutes at the medium-high setting (675 W) to unmask antigen. After being allowed to cool for 15 minutes, sections were incubated with blocking serum for 20 minutes. Slides were then incubated with the primary antibodies overnight at 4 C followed by PBS washes and incubation with the corresponding biotinylated goat anti-rabbit or horse anti-mouse immunoglobulin for 30 minutes at room temperature. Negative control sections were immunostained under the same conditions substituting murine monoclonal antibodies of irrelevant specificity (PAb419, antibody against SV40 large T antigen) and nonimmune rabbit serum for primary antibodies. Following PBS washes, sections were incubated with ABC Elite reagent for 60 minutes at room temperature and reaction products were developed using diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) as chromogen after PBS washes. Sections were washed in running tap water and lightly counterstained with hematoxylin followed by dehydration and coverslip mounting.

Frozen Sections and Cell Cultures

The basic staining procedures were the same as that for paraffin-embedded sections except that we used Vectastain. ABC kit (Vector Laboratories) and 3-amino-9-ethylcarbazole (AEC, Sigma) as the detection system. Briefly, frozen sections were cut at 5 μ and allowed to adhere to adhesive-coated slides. Adherent cells were cultured directly on glass slides before immunostaining. Slides of frozen sections or cell cultures were then air-dried for at least 30 minutes and fixed in acetone for 7 minutes. Other fixatives such as paraformaldehyde, formaldehyde, methanol, ethanol, or a combination of these were tested in an attempt to optimize staining. After brief air-drying, sections were incubated with blocking serum for 20 minutes followed by overnight incubation with primary antibodies at 4 C. After rinsing in PBS, slides were incubated with the corresponding biotinylated goat anti-rabbit or horse anti-mouse immunoglobulin for 30 minutes at room temperature. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxide for 20 minutes, and the signal was detected using AEC as chromogen after 60 minutes of incubation with ABC reagent.

Results

Immunohistochemical Study

Adherent cultured cell preparations from ZR-75-1 (breast cancer cell line with amplification of 11q13 and overexpression of cyclin D1) and WI38 (normal human fibroblast) showed scattered cells with nuclear staining with affinity-purified polyclonal antibody (no. 19) and 1 of 10 of the monoclonal antibodies tested (HD64). Frozen sections of MCL cases that showed cyclin D1 overexpression by Western blot analysis were tested with monoclonal and polyclonal antibodies, however, no definite staining could be detected with any of the antibodies irrespective of the fixative.

Monoclonal and polyclonal antibodies were tested using formalin-fixed paraffin-embedded cell blocks of ZR-75-1 and SAOS2 (osteosarcoma cell line with very low levels of cyclin D1 protein) as positive and negative controls, respectively, to find an antibody that stains paraffin sections. Selective nuclear staining of ZR-75-1 but not SAOS2 (Figure 2) was obtained only after microwave pretreatment with HD64 (among 10 monoclonal antibodies tested) and affinity-purified polyclonal antibody (no. 19).

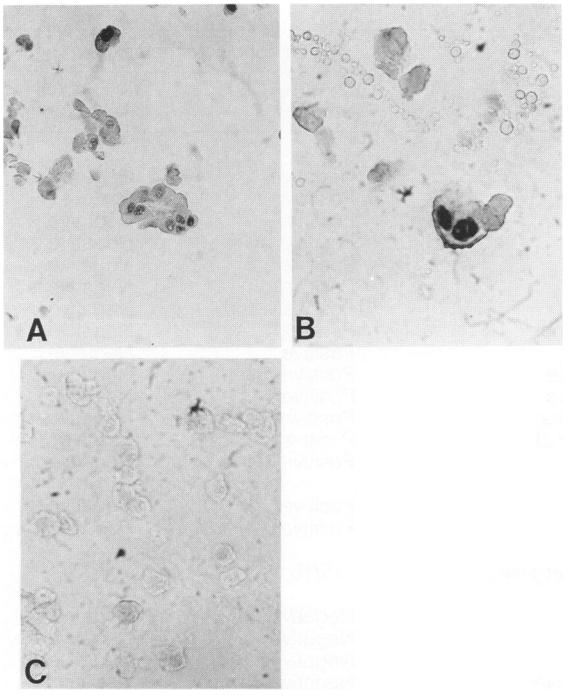

Figure 2. *Immunohistochemical staining of formalin-fixed, paraffin-embedded control cell lines: ZR-75-1 (positive control)* (**A** *and* **B**) *and SAOS2 (negative control)* (**C**). *Scattered ZR-75-1 cells show positive nuclear immunostaining with affinity-purified polyclonal antibody* (**A** *and* **B**). *SAOS2 cells are negative* (**C**) (*DAB with hematoxylin counterstain,* **A**: ×290, *B,C:* ×500).

Paraffin sections of lymphoid tissues were then stained with these antibodies. Using polyclonal antibody (no. 19), all 15 cases of MCL and one case of B-SLL/CLL showed nuclear staining (Table 1). Only formalin-fixed tissue sections showed nuclear staining; no definite staining was detected using B5-fixed tissue sections of positive cases. All other cases of low-grade B-cell lymphomas (6 B-SLL/CLL, 10 follicular lymphoma, 4 lymphoma of MALT) and reactive hyperplasia (7 cases) showed no nuclear staining. No case demonstrated positivity only with the monoclonal antibody, and in cases positive with both monoclonal and polyclonal antibodies the polyclonal antibody exhibited stronger staining. Positive cases of MCL showed diffuse nuclear staining of 20 to 90% of the lymphoma cells (Figure 3) but the one positive B-SLL/CLL case (case 21) demonstrated intranuclear globular or dot-like staining with both types of antibodies (Figure 4A). Cases of MCL with a mantle zone growth pattern revealed selective staining of tumor cells within widened mantle zones, leaving residual germinal center cells negative (Figure 5). One patient in this study (case 1) had two sequential biopsies, 1 year apart. The first biopsy (case 1a) showed a typical MCL, which had no cyclin D1 staining. The second biopsy (case 1b) showed typical MCL with focal areas of higher-grade lymphoma with slight increase in cell size, higher mitotic rate, and a starry sky pattern (Figure 6). Both areas were positive for cyclin D1, but the higher-grade area had stronger staining than the low-grade area (Figure 7).

Correlation of Immunoblotting and Immunohistochemical Staining

Eighteen cases in this study were included in a comparative mRNA/Western blot study (Zukerberg, unpublished data) allowing correlation of Western blots (Figures 8, 9) with immunostains (Table 1). Of five cases of MCL studied by both techniques, two (cases 9, 10) showed marked overexpression of cyclin D1 by Western blots, and three (cases 7, 11, 12) showed marginal overexpression; all 5 cases were immunohistochemically positive for cyclin D1. The remaining 13 cases of low-grade B-cell lymphoma and reactive hyperplasia were negative by both techniques, except for one case of B-SLL/CLL (case 21), which showed marked overexpression by Western blots and strong dot-like nuclear staining by immunohistochemistry.

Discussion

In this study, we were able to detect nuclear cyclin D1 protein in routinely processed, formalin-fixed, paraffin-embedded tissue sections using a microwave immunoperoxidase technique and affinity-purified polyclonal antibody. Monoclonal antibodies were less sensitive but helped to confirm the specificity of the nuclear staining. In addition, we found a strong correlation between cyclin D1 expression and MCL. Nuclear staining for cyclin D1 was detected in 15 of 15 MCLs and 1 of 7 B-SLL/CLLs. No cases of reactive hyperplasia, follicular lymphoma or lymphoma of MALT showed staining. Interestingly, MCLs as well as control cell lines (normal human fibroblast WI38s and breast cancer cell line ZR-75-1) showed diffuse nuclear staining, whereas the one positive B-SLL/CLL case showed a globular or dot-like nuclear staining. It is possible but unlikely that this is a fixation artifact, as diffuse nuclear staining was seen in this case with another nuclear protein, proliferating cell nuclear antigen (PCNA; data not shown). This difference may reflect biological differences in these lymphomas; however, further study is needed. In addition, one case of MCL showed focal areas of large-cell transformation with increased mitotic activity;

Table 1. *Results of Immunoblotting and Immunohistochemical Staining*

Condition and Case Number	Immunoblot	Immunohistochemistry	
		Polyclonal Antibody	Monoclonal Antibody
Mantle cell lymphoma			
1a		Negative	Negative
b		Positive	Positive
2		Positive	Negative
3		Positive	Negative
4		Positive	Negative
5		Positive	Negative
6		Positive	Negative
7	Marginal	Positive	Negative
8		Positive	Negative
9	Positive	Positive	Negative
10	Positive	Positive	Negative
11	Marginal	Positive	Negative
12	Marginal	Positive	Positive
13		Positive	Negative
Mantle cell lymphoma (*blastic variant*)			
14		Positive	Negative
15		Positive	Positive
Total	2/5 (3/5 marginal)	15/15	3/15
B-SLL/CLL			
16		Negative	Negative
17		Negative	Negative
18		Negative	Negative
19	Negative	Negative	Negative
20		Negative	Negative
21	Positive	Positive	Positive
22	Negative	Negative	Negative
Total	1/3	1/7	1/7
Follicular lymphoma			
23	Negative	Negative	Negative
24		Negative	Negative
25		Negative	Negative
26		Negative	Negative
27	Negative	Negative	Negative
28		Negative	Negative
29		Negative	Negative
30	Negative	Negative	Negative
31	Negative	Negative	Negative
32	Negative	Negative	Negative
Total	0/5	0/10	0/10
Lymphoma of MALT			
33		Negative	Negative
34	Negative	Negative	Negative
35	Negative	Negative	Negative
36		Negative	Negative
Total	0/2	0/4	0/4
Reactive hyperplasia			
37		Negative	Negative
38		Negative	Negative
39		Negative	Negative
40		Negative	Negative
41	Negative	Negative	Negative
42	Negative	Negative	Negative
43	Negative	Negative	Negative
Total	0/3	0/7	0/7

much greater intensity of cyclin D1 staining was seen in these areas. This suggests that high-grade transformation of MCLs might lead to a further heightened level of deregulation of cyclin D1 expression.

Recent evidence indicates that cyclin D1 has a role in regulation of the cell cycle. Cyclin D1 protein is expressed shortly after adding serum to quiescent mammalian cells and reaches a maximum in mid-G1 before S phase.[35–37] Microinjection studies with antibody against cyclin D1 or antisense plasmid prevented cells from entering S phase.[38] In addition, overexpression of cyclin D1 shortened the cell cycle

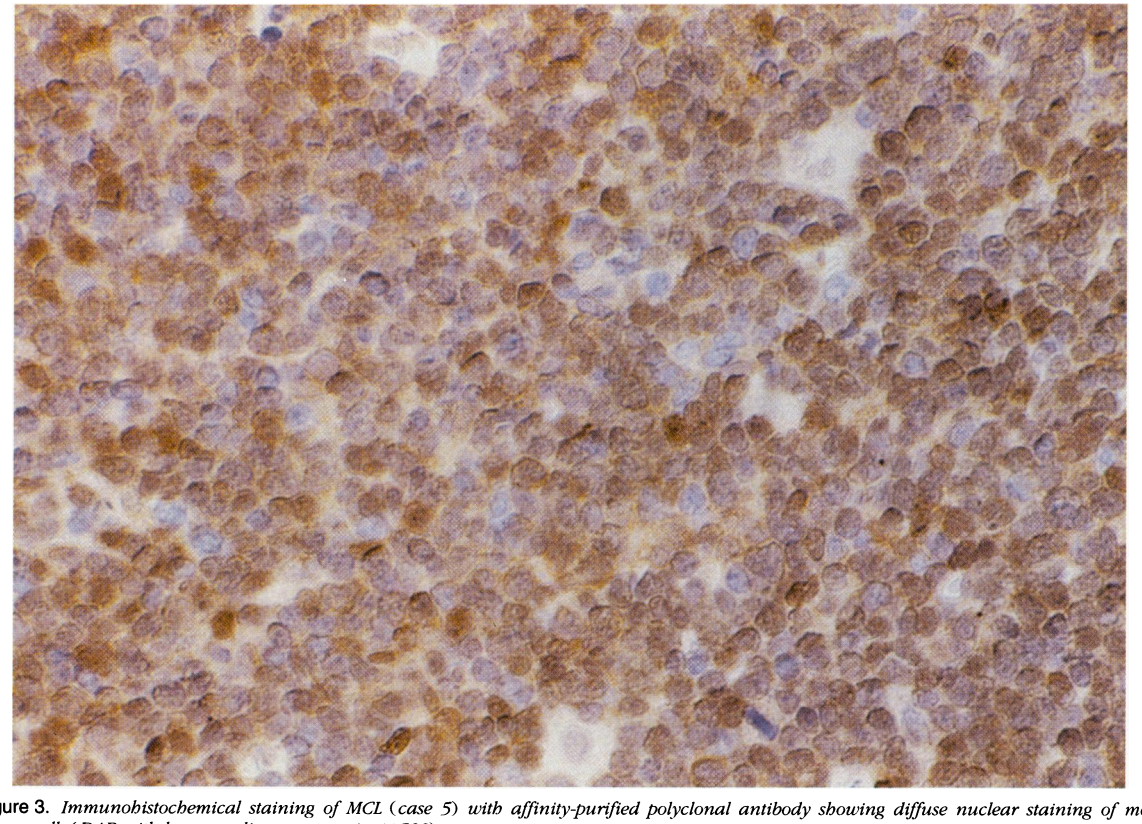

Figure 3. *Immunohistochemical staining of MCL (case 5) with affinity-purified polyclonal antibody showing diffuse nuclear staining of most tumor cells (DAB with hematoxylin counterstain, ×500).*

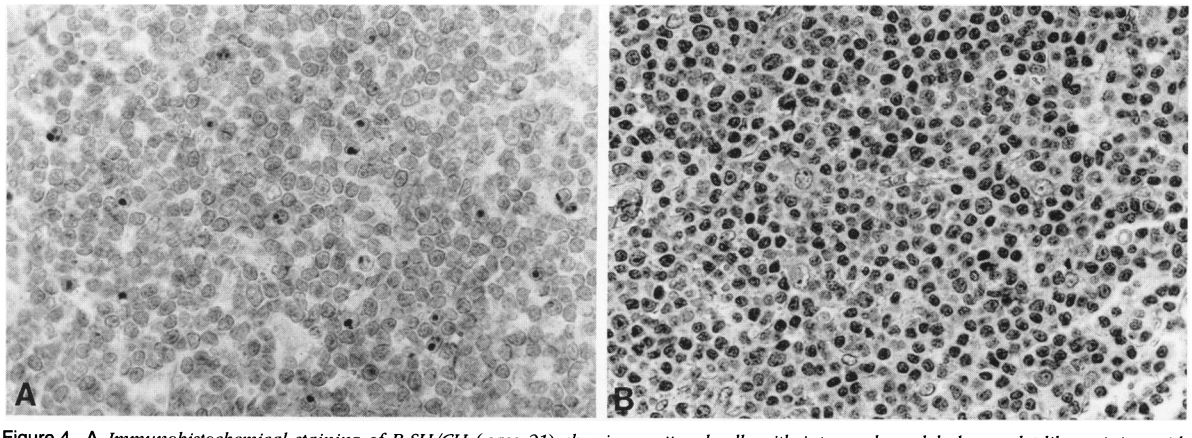

Figure 4. **A** *Immunohistochemical staining of B-SLL/CLL (case 21) showing scattered cells with intranuclear globular or dot-like staining with affinity-purified polyclonal antibody. (DAB with hematoxylin counterstain, ×500)* **B** *H&E section of the same case showing typical morphology of B-SLL/CLL: predominantly small round lymphoid cells with scattered prolymphocytes and paraimmunoblasts (×500).*

and increased the rate of G0 to S and G1 to S phase transit by several hours.[39] Cyclin D1 is associated with several proteins including numerous cyclin-dependent kinases (CDKs), PCNA, and a 21-kd inhibitor protein.[40] Association with the CDKs is a feature common to other cyclins, and by correlation one role of cyclin D1 should be to activate an associated kinase. Cyclin D1 is capable, *in vitro*, of phosphorylating the product of the retinoblastoma gene (pRb).[41]

Although the true substrate of cyclin D1 kinase activity is unknown, cyclin D1-associated kinase activity clearly prefers pRb over histone H1, casein, or other commonly used substrates. One study[42] has shown that cells arrested by transfection with pRb may be rescued by overexpression of cyclin D1, but rescue does not lead to pRb phosphorylation as is common with other cyclins. In light of this it has been proposed that cyclin D1 may bind to pRb directly through its

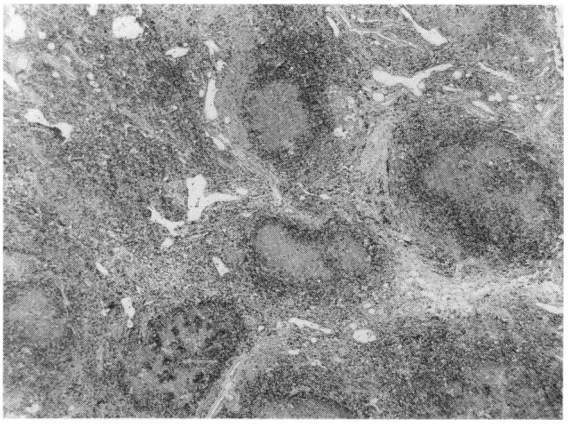

Figure 5. *Immunohistochemical staining of mantle cell lymphoma (case 9) showing selective nuclear staining of the widened mantle zone. Note that residual germinal center cells are negative (DAB with hematoxylin counterstain, ×71).*

Leu×Cys×Glu motif, which is required for pRb pocket binding.[42,43] One study[42] was able to demonstrate *in vivo* pRb-cyclin D1 association. Mutation of the Leu×Cys×Glu motif of cyclin D1 abolished pRb-cyclin D1 binding and led to increased rescue of pRb-arrested cells, suggesting that cyclin D1 is involved in a cell cycle event downstream of pRb and that pRb may keep D1 activity in check.[42]

Lymphoid cells and lymphomas in general do not express cyclin D1, but instead the closely related D-type cyclins D2 and D3.[36,44,45] In MCL and rare cases of B-SLL/CLL, the t(11;14) leads to increased transcription of the cyclin D1 as demonstrated in previous studies.[18,29] In this study, we show that this results in increased cyclin D1 protein, which can be detected by Western blot and immunohistochemistry. Detection of cyclin D1 expression by immunostaining can be a marker for the t(11;14) translocation, since lymphoid cells and lymphomas apparently do not express this protein in the absence of a translocation and may be useful in subclassifying low-grade B-cell lymphomas. If frozen tissue is available, CD5 is the most useful antigen in distinguishing B-SLL/CLL and MCL from follicular center cell lymphomas and MALT lymphomas. Additionally, CD23 distinguishes B-SLL/CLL from MCL.[32] However, this distinction is not absolute, and rare cases of MALT lymphoma may be CD5-positive. In ambiguous cases or when frozen tissue for evaluation of CD5 and CD23 is not available, cyclin D1 protein expression may therefore be diagnostically useful.

Our results indicate, however, that cyclin D1 protein overexpression, although characteristic of MCL, is not entirely specific, given that one of our cases of B-SLL/CLL showed marked overexpression. This case (case 21) contained prolymphocytes and paraimmunoblasts (Figure 4B), and was $CD23^+$, clearly distinguishing it from MCL.[32] The patient presented with bone marrow and peripheral blood involvement, typical for B-SLL/CLL. However, the disease was refractory to treatment and the patient died 9 months after the diagnosis. Thus, at least in this case, overexpression of cyclin D1 was associated with an unusually aggressive clinical course. Additional studies will be required to determine the clinical significance of *Bcl*-1 rearrangement and cyclin D1 overexpression in lymphomas other than MCL.

We found that, although two cases that showed definite cyclin D1 overexpression by immunoblot were also positive by immunohistochemistry, three additional cases that showed marginal overexpression slightly above background by immunoblot were clearly positive by immunoperoxidase. The increased sensitivity of immunohistochemical detection for cyclin D1 over Western blotting may be a reflection of

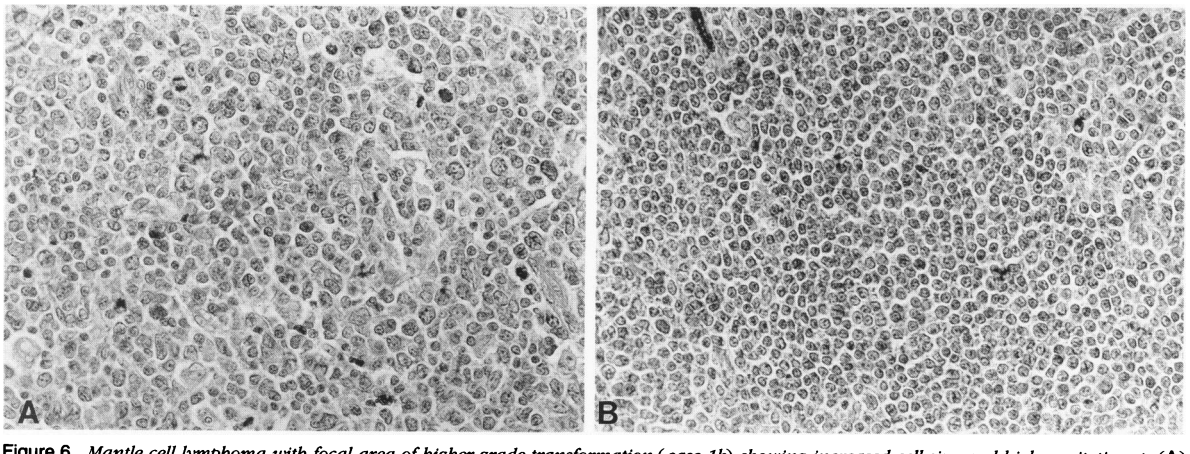

Figure 6. *Mantle cell lymphoma with focal area of higher grade transformation (case 1b) showing increased cell size and higher mitotic rate* (**A**) *compared to an area with typical morphology of mantle cell lymphoma* (**B**) *(Hematoxylin-Eosin,* **A,B:** *×500).*

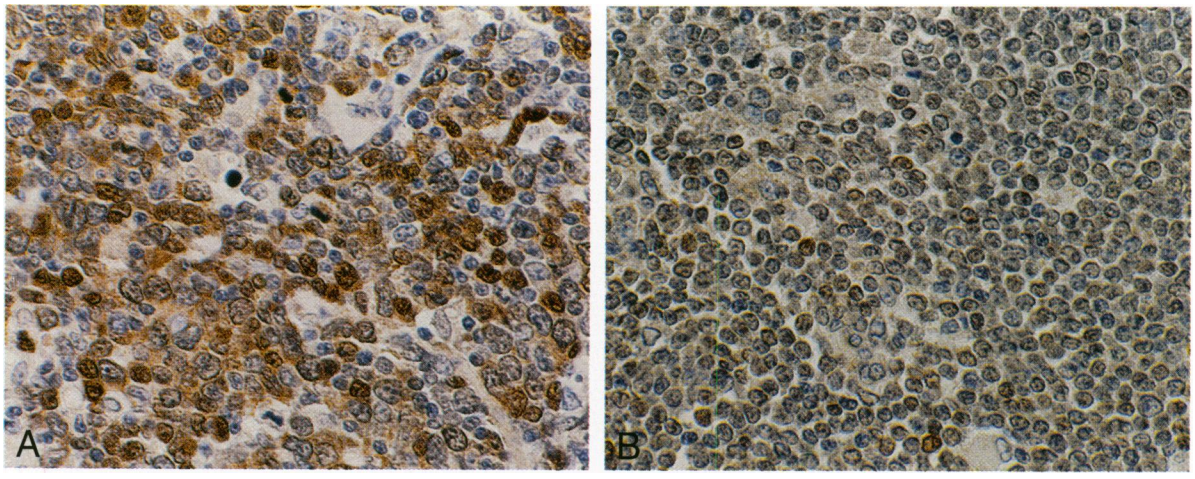

Figure 7. *Immunohistochemical staining of mantle cell lymphoma with focal higher grade transformation (case 1b) using affinity purified polyclonal antibody. There is increased immunostaining of the transformed foci (A) compared to the typical mantle cell lymphoma (B) (DAB with hematoxylin counterstain, A,B: ×500).*

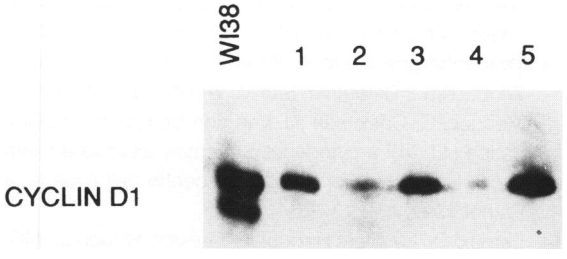

Figure 8. *Western blot of MCLs* (lanes 1–4), *positive B-SLL/CLL* (lane 5, *case 21*) *and normal human fibroblast cell line (WI38). Two MCLs* (lane 1, *case 10 and* lane 3, *case 9*) *and the B-SLL/CLL (case 21) show high levels of cyclin D1 while the other MCLs* (lane 2, *case 7 and* lane 4, *case 12*) *show marginal overexpression of cyclin D1 (only slightly greater than rare cases of reactive hyperplasia as seen in Figure 9).*

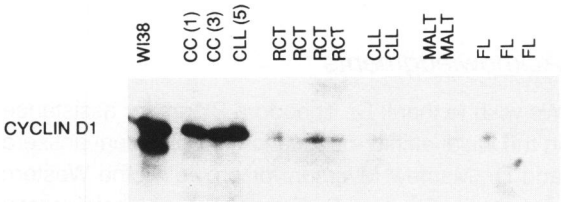

Figure 9. *Western blot of two MCLs (cases 9, 10) and positive B-SLL/CLL (case 21) with overexpression of cyclin D1 protein compared with reactive hyperplasia and representative other low-grade B-cell lymphomas. A low level of background cyclin D1, especially seen in reactive hyperplasia cases, may be caused by an admixture of fibroblasts and reticulum cells in the tissue; this lowers the sensitivity and specificity of Western blot analysis. CC, mantle cell (centrocytic) lymphoma; RCT, reactive hyperplasia; CLL, chronic lymphocytic leukemia; MALT, lymphomas of mucosa associated lymphoid tissue; FL, follicular lymphoma; WI38, normal human fibroblast cell line.*

both the short half-life of the protein[41] and the admixture of nonlymphomatous cells in most tissues. Since normal fibroblasts, macrophages, and dendritic cells express cyclin D1, it would be expected that rare cases of lymphoid lesions with a high percentage of these cells would show low levels of cyclin D1 protein by Western blots, because the protein extract used for Western blots is protein from all cell types in the tissue. Conversely, an admixture of benign T cells in an MCL may dilute the neoplastic B-cells so that overexpression of cyclin D1 may be obscured. These phenomena may lower both the sensitivity and specificity of the Western blot assay. For this reason, immunohistochemistry may well be the method of choice for cyclin D1 evaluation not only for lymphomas but in all tumors especially in cases where a reactive fibroblastic or desmoplastic response may make up a large percentage of the total protein.

It has become increasingly clear that cyclin D1 has a widely prevalent role as an oncogene. In addition to its clonal rearrangement in MCLs[18,29,31,46,47] and parathyroid adenomas,[25–27] the *Bcl*-1 region is amplified in 15 to 20% of breast carcinomas, a similar percentage of squamous cell carcinomas of the head, neck and lung,[28] and 32% of esophageal cancers.[48] 11q13 amplification has also been reported in 20% of transitional cell carcinomas of the bladder[49] and approximately 14% of epithelial ovarian carcinomas.[50] In addition, gene amplification and marked overexpression of cyclin D1 mRNA was found in advanced mouse skin carcinogenesis independent of the proliferation index indicating a role of cyclin D1 in the acquisition of autonomous growth.[51]

It is unclear how cyclin D1 causes transformation and is involved in carcinogenesis. No mutations have been detected in the cyclin D1 coding sequences from tumors with clonal rearrangement involving cyclin D1.[47] In MCLs cyclin D1 is associated with the CDKs, especially CDK4, but is not associated with

PCNA, in contrast to normal cells (WI38 fibroblasts) and most other transformed cell lines (Zukerberg, unpublished observation). Such associations indicate that its role as an oncogene is likely to be through its association with CDKs, whereas the lack of cyclin D1-PCNA association may or may not be important in tumorogenesis.

In conclusion, we have described an immunohistochemical method for detection of cyclin D1 protein in routinely processed formalin-fixed tissue and have shown that this staining technique is more sensitive and specific than Western blots for detection of cyclin D1 protein. In addition, we have shown that cyclin D1 protein can be detected in the majority of MCLs and in rare cases of B-SLL/CLL. It was not detected in reactive hyperplasias and other low-grade B-cell lymphomas, making it a potentially useful diagnostic tool in subclassification of low-grade B-cell lymphomas.

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