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Differential Expression of Cyclin D1 in Mantle Cell Lymphoma and Other Non-Hodgkin's Lymphomas

Nadine S. Ives Aguilera,* Karen E. Bijwaard,† Beverly Duncan,† Amy E. Krafft,† Wei-Sing Chu,* Susan L. Abbondanzo,* Jack H. Lichy,† and Jeffery K. Taubenberger†

From the Department of Hematologic and Lymphatic Pathology and Division of Molecular Pathology,† Department of Cellular Pathology, Armed Forces Institute of Pathology, Washington DC*

Mantle-cell lymphomas are associated with a characteristic chromosomal translocation, t(11;14)(q13;q32). This translocation involves rearrangement of the bcl-1 proto-oncogene from chromosome 11 to the immunoglobulin heavy chain gene on chromosome 14, resulting in an overexpression of cyclin D1 mRNA (also known as bcl-1 and PRAD1). In the current study performed on paraffin-embedded tissue, cyclin D1 mRNA could be detected in 23 of 24 mantle-cell lymphomas by reverse transcription polymerase chain reaction (RT-PCR) whereas only 9 of 24 demonstrated a t(11;14) by PCR. However, we also found that cyclin D1 mRNA could be detected in the majority (11 of 17, 65%) of non-mantle-cell lymphomas and in a minority of atypical lymphoid hyperplasias (3 of 7, 43%). Cyclin D1 mRNA expression was not observed in floridly reactive lymph nodes (0 of 9) or in unstimulated lymph nodes (0 of 20), suggesting that it is a sensitive adjunct marker for malignant lymphoproliferative processes, but not specific for mantle-cell lymphoma. A semiquantitative RT-PCR assay was developed that compared the ratio of cyclin D1 to the constitutively expressed gene β_2-microglobulin. Using this assay on a limited number of our specimens, cyclin D1 overexpression in mantle-cell lymphoma could be reliably distinguished from its expression in other non-Hodgkin's lymphomas. This assay for cyclin D1 expression, designed for formalin-fixed, paraffin-embedded tissue, was a very sensitive and specific marker for mantle-cell lymphoma. *(Am J Pathol 1998, 153:1969–1976)*

Mantle-cell lymphoma (MCL) is a non-Hodgkin's B-cell lymphoma classically described as showing either a mantle zone, nodular, or diffuse pattern and being composed of a monotonous population of small to intermediately sized slightly cleaved B cells. Sometimes, however, there are overlapping features between mantle-cell lymphoma and other small-B-cell lymphomas, making histological distinction problematic. For example, these features may include cytology, which shows predominantly round cells or extreme nodularity of the architecture. Clinically, mantle-cell lymphoma is more aggressive than many other so-called small-B-cell lymphomas, necessitating the importance of an accurate diagnosis.[1–4] Recently developed antibodies for paraffin-embedded tissue, including cyclin D1 and CD5, are not always helpful in differentiating histologically low-grade B-cell lymphomas (because of varying staining with different fixation), and other modalities are sometimes necessary for definitive diagnosis. Specific genetic markers for various types of lymphomas and non-lymphoid neoplasms have proven useful for diagnosis and prognosis. The most recent classification scheme for lymphoid neoplasms, the revised European-American Classification of Lymphoid Neoplasms (REAL),[5] includes morphology, immunophenotype, and genetic features for defining neoplasms. Although immunophenotype of lymphomas has always been important, with the advent of the REAL classification, immunophenotype is no longer optional in diagnosing lymphoid neoplasms, and genetic studies are often necessary. The t(11;14)(q13;q32) translocation with its molecular counterpart, bcl-1 rearrangement, is seen in 50% to 80% of MCLs, using Southern blot or cytogenetics,[1–3,6–18] with even greater incidence (up to 95% of MCLs) using DNA fiber fluorescence *in situ* hybridization (FISH).[19] This rearrangement appears to be very specific, although occasionally other lymphoproliferative disorders have been reported to show t(11;14).[13,17,20–22] The expression of cyclin D1 mRNA has been examined with Northern blot[9–12,20,22] and *in situ* hybridization[23,24] and has been found to be present in MCL. Competitive reverse transcription polymerase chain reaction (RT-PCR) in fresh tissue has also been used to demonstrate the overexpression of cyclin D1 in MCL compared with expression patterns of cyclin D2 and cyclin D3.[25]

In this paper we compared three different methods for detecting markers associated with MCL in formalin-fixed, paraffin-embedded (FFPE) tissue: immunohistochemical profile including immunoreactivity for cyclin D1 and CD5, PCR for bcl-1 rearrangement corresponding to t(11;14),

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Address reprint requests to Dr. Jeffery K. Taubenberger, Armed Forces Institute of Pathology, Division of Molecular Pathology, Department of Cellular Pathology, Room G-137, 14th Street and Alaska Avenue, NW, Washington DC, 20306-6000. E-mail: taubenbe@afip.osd.mil.

Table 1. Antibodies

Antibody	Source	Dilution
CD45RB(LCA)*	DAKO, Carpinteria, CA	1:200
CD20(L26)*	DAKO	1:200
CD45RO(UCHL-1)*	DAKO	1:200
CD3†‡	DAKO	1:500
CD43(MT-1)*	Biotest, Denville, NJ	1:50
Kappa†‡	DAKO	1:25,000
Lambda†‡	DAKO	1:50,000
Bcl-2	DAKO	1:100
CD23(BU38)*‡	Binding Site Unlimited, Birmingham, UK	1:200
CD5 (NCL-CD5-4C7)*§	Novocastra, Newcastle-upon-Tyne, UK	1:100
Cyclin D1/Bcl-1(5D4)*	Immunotech, Westbrook, ME	1:3000

LCA, leukocyte common antigen.
*Mouse monoclonal.
†Rabbit polyclonal.
‡Required 20-minute predigestion with 0.4% pepsin.
§Microwave antigen retrieval.

and RT-PCR for cyclin D1 mRNA expression. We found that, although the RT-PCR assay for cyclin D1 was very sensitive for MCL, it was not specific in that cyclin D1 expression was observed in the majority of non-MCLs examined. To further characterize expression of cyclin D1 in MCL from its expression in other lymphomas, a semiquantitative RT-PCR assay was developed. This assay reliably distinguished high levels of cyclin D1 mRNA in MCL from the low levels found in other malignant lymphoproliferative processes.

Materials and Methods

Histological Classification

Cases diagnosed as either MCL, intermediately differentiated lymphocytic lymphoma, or diffuse small cleaved lymphoma from 1980 to 1997 were retrieved from the files of the Armed Forces Institute of Pathology Hematopathology Registry and classified according to the REAL classification. Lymphomas with classic, unequivocal histological features of MCL were used.[5,26] These showed either a mantle zone, nodular, or diffuse pattern with small to intermediately sized lymphocytes with slightly cleaved nuclei. No transformed cells or proliferation centers were seen. The blastic variant, which made up a subset of these lymphomas, occasionally showed a starry-sky appearance with diffuse effacement of the lymph node with medium-sized lymphocytes with finely dispersed chromatin. So-called variant forms (large-cell or anaplastic) were also accepted for this study.[14,27,28]

Non-MCLs were selected, as available, and included examples of follicle center lymphoma, diffuse large-B-cell lymphoma, peripheral T-cell lymphoma, or anaplastic large-cell lymphoma. Atypical cases were selected, as available, and were defined as suspicious for malignant lymphoma, but they lacked definitive histological features of lymphoma (see Table 4). Nonstimulated lymph nodes from node-negative mastectomy specimens and florid reactive lymph nodes without suspicion of malignancy were used as controls.

Immunohistochemical Studies

Five-micron sections from paraffin-embedded tissue blocks were prepared for immunophenotypic analysis according to the standard avidin-biotin complex method of Hsu.[29] The antibody panel for each case included CD45RB, CD20, CD45RO, CD3, CD43, bcl-2, CD5, kappa (Ig light chain), lambda (Ig light chain), CD23 (BU38), and cyclin D1 (Table 1). Of the above antibodies, kappa, lambda, CD3, and CD23 required predigestion for 20 minutes with 0.4% pepsin (P-7000, Sigma Chemical Co., St. Louis, MO) in 0.1 mol/L HCl buffer solution at pH 2.0 at 40°C to 42°C. Microwave antigen retrieval was necessary for CD5.[30]

Positive immunoreactivity for CD45RB and the B-cell marker CD20 (or kappa or lambda restriction) with negative CD45RO and/or CD3 was used to determined B-cell immunophenotype. Co-expression of CD43 in B-cell neoplasms was considered consistent with a B-cell malignancy.[31,32] CD23 was considered positive if individual cells showed cytoplasmic staining but negative if only reticular staining was seen.[33] Cyclin D1, a marker considered highly specific and sensitive for MCL,[8,13,34] was used to help confirm the diagnosis of MCL. We considered cyclin D1 to be positive if over 10% of the cells showed nuclear positivity. Cytoplasmic staining was not considered positive.

Molecular Diagnostic Studies

PCR for t(11;14) and Immunoglobulin Heavy Chain (IgH)

Molecular diagnostic studies for t(11;14) and IgH were performed by PCR. DNA was extracted from the FFPE sections and amplified as previously described.[35] A PCR master mix containing PCR buffer II, pH 8.3 (Perkin-Elmer, Norwalk, CT), $MgCl_2$ (2.5 mmol/L final concentration), 1.25 U of Ampli*Taq* polymerase (Perkin-Elmer), and 10 pmol of appropriate primer, listed in Table 2, was added for a final volume of 50 μl. The reaction mixture

Table 2. Primers and Probe Sequences

Name	Sequence
t(11;14)	
bcl-1 primer (22-mer)	5′-ATTCGGTTAGACTGTGATTAGC-3′
CFW-1 primer (24-mer)	5′-ACCTGAGGAGACGGTGACCAGGG-3′
bcl-1 probe	5′-AAGTGGTTTTGTTAGATGTA-3′
PRAD-1	
PRAD 5′	5′-ATGCTGAAGGCGGAGGAGACC-3′
PRAD 3′ (RT-primer)	5′-TCCTCGCACTTCTGTTCCTCGC-3′
Probe	5′-CCTCGGTGTCCTACTTCAAA-3′
IgH	
FR3A, V region primer	5′-ACACGGC(C/T)TGTATTACTGT-3′
CFW1, J region primer	5′-ACCTGAGGAGGTGACCAGGGT-3′

was amplified in a 9600 Gene Amp PCR system (Perkin-Elmer) programmed for a 5-minute denaturation at 94°C, followed by 40 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, with a final 7-minute extension at 72°C. The t(11;14) PCR products (18 μl) were separated by electrophoresis through a 2.5% agarose gel in Tris/borate/EDTA buffer, pH 8.4, and visualized by ethidium bromide staining. Specific products were detected by Southern blotting with end-labeled oligonucleotide probes,[36,37] as listed in Table 2. End-labeling was performed with T4 polynucleotide kinase (Life Technologies) and [γ-^{32}P]ATP (10 mCi/ml; Amersham, Arlington Hts, IL) or terminal transferase and digoxigenin-ddUTP (Boehringer Mannheim, Indianapolis, IN). A positive result for a major translocation cluster of t(11;14) produced a band of 180 to 280 bp. The positive control for the assay was the M02058 cell line.[38] PCR for IgH was performed according to previously described methods.[39] A monoclonal IgH rearrangement appeared as a dominant band of 105 to 120 bp.

RT-PCR for Cyclin D1 and β_2-Microglobulin

All RT-PCR studies were performed as previously described[37] and according to the guidelines of the College of American Pathologists checklist for molecular pathology with appropriate positive and negative controls.

RNA was extracted from the formalin-fixed, paraffin-embedded tissue for RT-PCR as previously described.[37] RT-PCR for the ubiquitously expressed β_2-microglobulin (β_2M) gene was used as a control for the presence of amplifiable RNA. Reverse transcription was performed with a specific antisense primer, listed in Table 2. The RT step was performed on 1- and 5-μl templates at 37°C for 60 minutes in 20-μl mixtures containing First Stand Buffer (Life Technologies, Gaithersburg, MD), 10 mmol/L dithiothreitol, 60 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies), 0.2 mmol/L each deoxynucleotide triphosphate, and 15 pmol of antisense primer. A PCR master mix containing PCR buffer II, pH 8.3 (Perkin-Elmer), $MgCl_2$ (2.5 mmol/L final concentration), 1.25 U of Ampli*Taq* polymerase (Perkin-Elmer), and 15 pmol of sense primer (Table 2) was added for a final volume of 50 μl. The reaction mixture was amplified in a 9600 Gene Amp PCR System (Perkin-Elmer) programmed for a 5-minute denaturation at 94°C, followed by 40 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, with a final 7-minute extension at 72°C. Cyclin D1 RT-PCR products (18 μl) were separated by electrophoresis through a 2.5% agarose gel in Tris/borate/EDTA buffer, pH 8.4, and visualized by ethidium bromide staining. Specific products were detected by Southern blotting with an end-labeled oligonucleotide probe as listed in Table 2. A positive result for β_2M produced a band of 158 bp. A positive result for cyclin D1 produced a band of 134 bp. The positive control for the cyclin D1 assay was RNA extracted from the t(11;14)-bearing cell line M02058.[38]

For semiquantitative RT-PCR, 5 μl of lysate was reverse transcribed as described above for cyclin D1 and β_2M. As absolute RNA amounts in FFPE lysates cannot be easily quantitated, a semiquantitative assay was designed in which the relative expression of cyclin D1 was compared with the expression of β_2M. To determine the linear range of amplification, control lysates were amplified by RT-PCR for β_2M with 20, 25, 30, and 40 cycles. It was determined that 30 cycles of PCR was still in the linear range and was optimal for this assay. For quantification, the amplified samples were subjected to gel electrophoresis and Southern blotting as above. Southern blots were visualized and bands were quantitated using a Storm 860 PhosphorImager equipped with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). For cyclin D1 quantitation, the ratio of cyclin D1/β_2M was calculated for each case. The resultant ratios were normalized to the cyclin D1/β_2M ratio of the positive control, cell line M02058, arbitrarily set as 1.0.

Statistics

The Statistics Program for Social Sciences (STSS 7.5) for windows (STSS, Chicago, IL) was used for determining statistical significance between the cyclin D1/β_2M ratios of MCLs and non-MCLs with a two-tailed *t*-test for equality of means.

Results

Twenty-four cases of lymphoma were collected prospectively and retrieved from the files at the Armed Forces Institute of Pathology that fit the criteria for MCL (Table 3).

Table 3. Mantle Cell Lymphoma

ID	Diagnosis	Age (years)/sex	Site	t(11;14)	PRAD1	CCND1	IgH	CD5	CD43	Bcl-2
1	MCL	74/M	LN, Rt axillary	Pos	Pos	Pos	Mo	Pos	Pos	Pos
2	MCL	78/M	LN, Rt groin; Lt/Rt tonsils	Neg	Pos	Neg	Mo	Pos	Pos	Pos
3	MCL-B	50/M	Nasopharynx	Pos	Pos	Pos	Mo	Pos	Pos	Pos
4	MCL	76/F	LN, Rt inguinal	Neg	Pos	Pos	Mo	Pos	Pos	Pos
5	MCL	82/M	Skin and LN, Rt inguinal	Pos	Pos	Neg	Mo	ND	ND	ND
6	MCL	76/M	LN, submental	Neg	Pos	Neg	Mo	Neg	Pos	Pos
7	MCL	67/F	LN, cervical	Neg	Pos	Neg	Mo	ND	Pos	ND
8	MCL	53/F	Mass, parotid	Pos	Pos	Pos	Mo	Pos	ND	Pos
9	MCL-B	57/M	LN, Rt inguinal	Neg	Pos	Pos	Mo	Pos	Pos	Pos
10	MCL-B	77/M	LN, Rt axillary	Pos	Pos	Pos	Mo	Pos	Pos	Pos
11	MCL	63/M	LN, Lt neck	Neg	Pos	Neg	Mo	Pos	Pos	Pos
12	MCL-MZ	67/M	LN, para-aortic	Pos	Pos	Pos	Mo	Neg	Pos	Pos
13	MCL-B	70/M	Lt tonsil	Neg	Pos	Pos	Mo	Neg	Neg	Pos
14	MCL	47/M	LN, Rt post cervical	Neg	Pos	Pos	Mo	Pos	Neg	Pos
15	MCL-B	41/M	LN, Lt axillary	Neg	Neg	Neg	Mo	Pos	Pos	Pos
16	MCL	66/M	LN, Rt femoral	Neg	Pos	Pos	Mo	Pos	Pos	Pos
17	MCL	74/F	LN, Rt neck	Neg	Pos	Pos	Mo	Pos	Pos	Pos
18	MCL	57/M	Mass, Lt parotid	Pos	Pos	Pos	Mo	Pos	Pos	Pos
19	MCL	55/M	Mass, Lt hard palate	Neg	Pos	Pos	Mo	Pos	Pos	Pos
20	MCL	34/M	LN, Lt inguinal	Neg	Pos	Neg	Mo	Pos	Pos	Pos
21	MCL	84/M	Mass, right neck	Pos	Pos	Pos	Mo	Pos	Pos	Pos
22	MCL-V	73/M	LN, cervical	Pos	Pos	Pos	Mo	Neg	Pos	Pos
23	MCL	57/M	LN, submandibular	Neg	Pos	Pos	Mo	ND	Pos	Pos
24	MCL	70/M	LN, axillary	Neg	Pos	Pos	Mo	ND	Pos	Pos

MCL, mantle cell lymphoma; B, blastic; V, variant; M, male; F, female; LN, lymph node; Rt, right; Lt, left; post, posterior; Pos, positive; Neg, negative; Mo, monoclonal; ND, not done; CCND1, cyclin D1; IgH, immunoglobulin heavy chain.

Other cases examined included 17 non-mantle-cell lymphomas (9 follicle center lymphomas, 3 peripheral T-cell lymphomas, 3 diffuse large-B-cell lymphomas, and 2 anaplastic large-cell lymphomas) and 7 atypical lymphoid proliferations (Table 4). Twenty axillary lymph nodes from node-negative breast carcinoma cases and nine floridly reactive lymph nodes were also examined for cyclin D1 expression and t(11;14).

Immunohistochemistry

The immunohistochemistry is summarized in Table 3. All MCLs were B-cell immunophenotype with CD20 immunoreactivity and CD3 and/or CD45RO negativity. Cyclin D1 was immunoreactive in 16 of 23 cases (70%). Results of other immunohistochemical markers included the following: 20 of 22 showed CD43 coexpression (91%); 21 of

Table 4. Non-Mantle Cell Processes

ID	Diagnosis	t(14;18)	t(11;14)	PRAD1	IgH
1	Atypical	Neg	ND	Neg	Polyclonal
2	Atypical	Neg	ND	Neg	Polyclonal
3	Atypical	ND	ND	Neg	ND
4	Atypical	ND	Neg	Pos	Polyclonal
5	Atypical	ND	Neg	Pos	Polyclonal
6	Atypical	ND	Neg	Pos	Indeterminate*
7	Atypical	ND	Neg	Neg	Polyclonal
8	FCL	Pos	Neg	Pos	Monoclonal
9	FCL	Pos	Neg	Pos	Monoclonal
10	FCL	Neg	ND	Neg	Polyclonal
11	FCL	Neg	ND	Neg	ND
12	FCL	Pos	Neg	Pos	Polyclonal
13	FCL	Neg	ND	Neg	Polyclonal
14	FCL	Pos	Neg	Pos	Polyclonal
15	FCL	Pos	Neg	Pos	Polyclonal
16	FCL	Pos	Neg	Pos	Monoclonal
17	DLBCL	Neg	ND	Pos	Monoclonal
18	DLBCL	ND	ND	Neg	Non-Amplifiable
19	DLBCL	Neg	Neg	Pos	ND
20	PTCL	ND	Neg	Pos	ND
21	PTCL	ND	Neg	Pos	Polyclonal
22	PTCL	ND	ND	Neg	ND
23	ALCL	ND	ND	Neg	ND
24	ALCL	ND	Neg	Pos	ND

FCL, follicle center lymphoma; DLBCL, diffuse large-B-cell lymphoma; PTCL, peripheral T-cell lymphoma; ALCL, anaplastic large-cell lymphoma; Neg, negative; ND, not done; Pos, positive immunoreaction.
*Amplifiable but not clearly monoclonal.

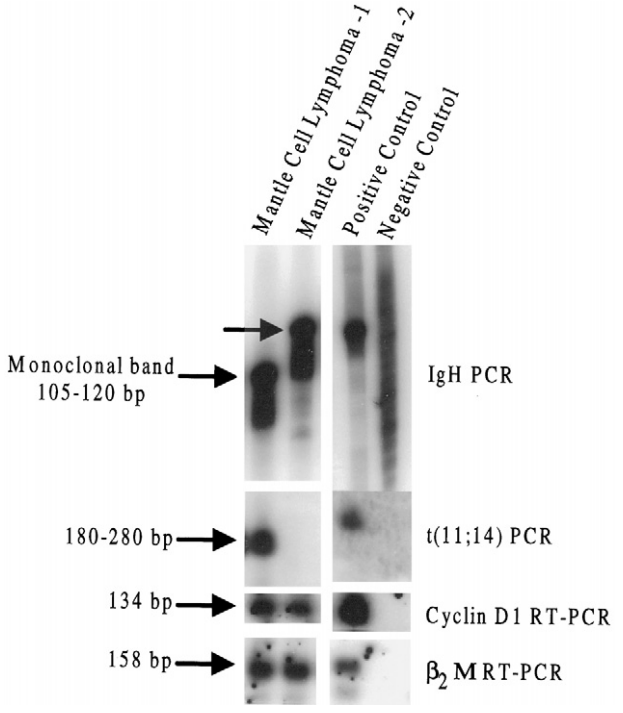

Figure 1. Examples of two mantle cell lymphomas both are cyclin D1 positive, but only one shows the t(11;14) by PCR (lane 1). Positive and negative controls for cyclin D1 are seen in the last two lanes. The negative control for IgH (immunoglobulin heavy chain) is reactive lymph node. For all other assays, the negative control is molecular grade water.

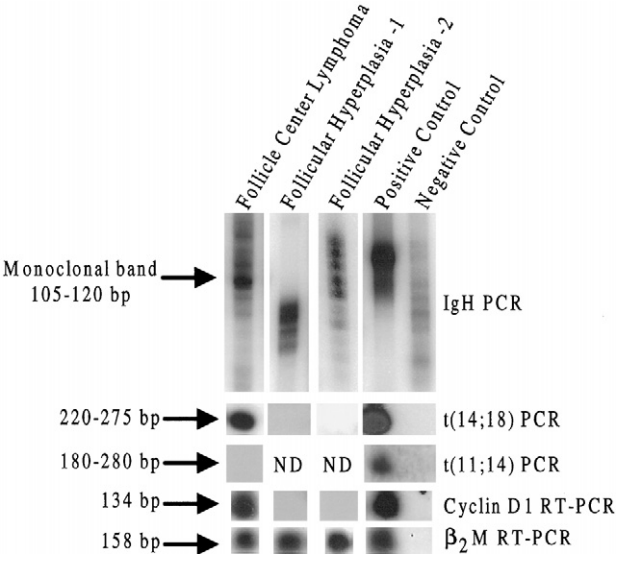

Figure 2. A follicle center lymphoma (FCL) expresses cyclin D1 (lane 1) whereas two follicular hyperplasias do not express cyclin D1 (lanes 2 and 3). The FCL also shows t(14;18) and is negative for t(11;14). Controls are seen in the last two lanes. The negative control for IgH is reactive lymph node. For all other assays, the negative control is molecular grade water.

21 MCLs were bcl-2 immunoreactive (100%); and 15 of 19 MCL were CD5 positive (79%). All cases tested were CD23 negative. All cases of non-MCLs studied were negative with cyclin D1.

Molecular Diagnostic Studies

All cases of MCL showed a monoclonal IgH rearrangement by PCR (24 of 24, 100%). PCR for t(11;14) translocation was performed on all MCLs and showed a distinct band in 9 of 24 cases (38%); no band was seen in 15 of 24 cases. Cyclin D1 mRNA as detected by RT-PCR was expressed in 23 of 24 cases (96%) of MCL (Table 3). All nine t(11;14)-positive MCL cases were cyclin D1 positive. Of the 15 MCL cases negative for t(11;14) by PCR, 14 (93%) were cyclin D1 positive. One case with a negative result for t(11;14), cyclin D1 mRNA and protein, had results showing $CD20^+$, $CD43^+$, $CD5^+$, $CD23^-$, and bcl-2 immunoreactivity and was considered MCL by morphology and immunohistochemistry. Results for two MCL cases are shown in Figure 1.

The majority (11 of 17, 65%) of non-MCLs also showed cyclin-D1 expression by RT-PCR. Cyclin-D1-positive cases by type included six of nine follicle center lymphomas (67%), two of three peripheral T-cell lymphomas (67%), two of three diffuse large-cell lymphomas (67%), and one of two anaplastic large-cell lymphomas (50%). Of the atypical lymphoid proliferations, three of seven (43%) were cyclin D1 positive. The floridly reactive lymph node cases were all negative for cyclin D1 mRNA (0 of 9). The cases of nonstimulated axillary lymph nodes from node-negative mastectomy specimens were also all cyclin D1 negative (0/20). Twelve cases of cyclin-D1-positive non-mantle-cell lymphomas were tested for t(11;14), and all were negative (Table 4). Representative cases are also shown in Figure 2.

A semiquantitative RT-PCR assay was developed for use with FFPE tissues to establish whether cyclin D1 expression in MCLs and non-MCLs could be distinguished. The relative expression of cyclin D1 was compared with the expression of the constitutively expressed gene β_2M. Relative cyclin D1 expression in 10 MCL cases (six t(11;14) positive and four t(11;14) negative) was compared with expression in 8 other cyclin-D1-positive non-MCLs and atypical hyperplasias, including 3 follicular center lymphomas, 2 peripheral T-cell lymphomas, 1 anaplastic large-cell lymphoma, and 2 atypical follicular hyperplasias. The calculated case ratios were normalized to the cyclin D1/β_2M ratio of the positive control cell line, M02058, which was arbitrarily set at 1.0 (see Materials and Methods). As shown in Figure 3, the MCL cases showed significantly higher expression of cyclin D1 than did other non-MCLs. There was no statistically significant difference between the cyclin D1/β_2M ratio of MCLs that were t(11;14) positive and those that were t(11;14) negative. Mean relative cyclin D1/β_2M ratio of MCL $\pm$ SEM was 1.44 $\pm$ 0.25. Mean relative cyclin D1/β_2M ratio for non-MCLs was 0.17 $\pm$ 0.05 ($P < 0.001$, two-tailed *t*-test for equality of means).

Discussion

Mantle-cell lymphoma was first recognized in the 1970s as a lymphoma with intermediate differentiation that could not be readily classified as either follicle center lymphoma (poorly differentiated lymphoma) or small lym-

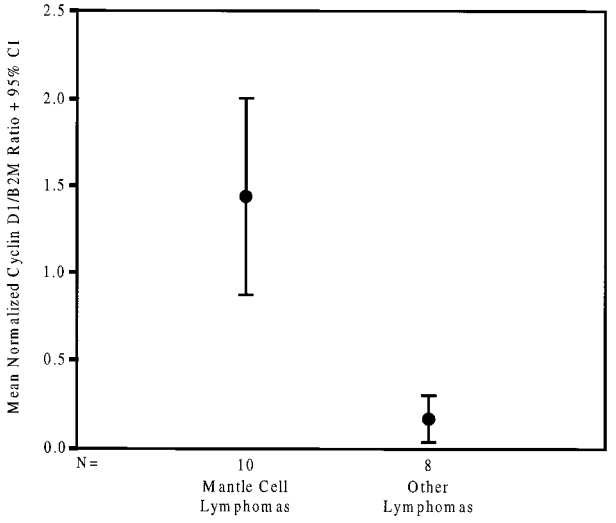

Figure 3. Mean normalized cyclin D1/β_2M ratio. This represents a 95% confidence interval when comparing mantle cell lymphoma and non-mantle cell lymphoma. Positive control line, M02058, was arbitrarily set at 1.0.

phocytic lymphoma (well differentiated lymphoma).[40–42] Lennert and Feller later included this entity in the Kiel classification as centrocytic lymphoma,[43] and in the United States the terms lymphocytic lymphoma of intermediate differentiation and intermediate lymphocytic lymphoma were used for what we now refer to as mantle-cell lymphoma.[40] Recent immunohistochemical, genetic, and clinical studies have confirmed the previously held views that MCL is a distinct clinicopathological entity.[5]

With the advent of the REAL classification, there has been an augmentation of knowledge and treatment of lymphomas. With this expanding knowledge, there is increasing necessity to subclassify small-B-cell lymphomas. In the case of MCL, this is especially justified because the natural history of MCL, which has low-grade histological features, has proven to be worse than its more indolent simulators, and its clinical behavior is more similar to intermediate histological grade.[1–4] The recent development of immunohistochemical markers, such as CD5,[44] cyclin D1,[5,13,14] and CD23,[33] has made the classification of small-B-cell lymphomas considerably easier, although there are still cases that are difficult to classify unequivocally by immunohistochemistry alone.

MCL has been associated with the cytogenetic abnormality t(11;14)(q13;q32). This translocation, which involves the bcl-1 breakpoint and cyclin D1, appears to be restricted to MCLs with occasional exceptions.[13,17,20–22,45] This translocation leads to deregulation of the bcl-1 gene and plays a role in the overexpression of cyclin D1 mRNA and subsequent overexpression of the cyclin D1 protein.[1,8–10,12,13] Cyclin D1 mRNA overexpression in MCL has been demonstrated by *in situ* hybridization, by Northern blot,[11,12,23,24] and more recently by competitive RT-PCR, with cyclin D2 and D3, in fresh tissue.[25] The cyclin D1 protein overexpression has been demonstrated with both Western blot and immunohistochemical studies in fresh and fixed tissue.[13,14,17,34]

The bcl-1 locus is rearranged in 50% to 80% of MCLs when using Southern blot or 75% to 80% using cytogenetics,[13,18] but using a direct visualization method with DNA fiber fluorescence *in situ* hybridization, this translocation can be seen in as many as 95% of MCLs.[19] As a result of the characteristic t(11;14), the bcl-1 locus, on chromosome 11, is juxtaposed to the immunoglobulin heavy chain (IgH) gene locus located on chromosome 14. The breakpoints are widely scattered on chromosome 11q13, but it has been shown that 70% to 80% of the breakpoints are localized to a 1-kb DNA segment known as the major translocation cluster (MTC).[18,24] Within the MTC, the breakpoints occur in a relatively small region of approximately 80 bp on chromosome 11 and the 5′ area of one of the IgH joining (J_H) regions on chromosome 14.[46] Because of the localization of these breakpoints, the translocation is amenable to PCR techniques for detection, although some of the breakpoints falling outside the MTC cannot be identified with PCR. This technique has been demonstrated to be sensitive when the MTC is present,[46] although because of the low percentage of MCLs exhibiting the MTC, the technique has a low sensitivity for MCL overall. It is estimated that only in 33% to 50% of patients with MCL can the breakpoints be detected by PCR using primers in the region of the MTC.[18,47,48] Our findings in this study fall within this expected range of detection.

Although there is low sensitivity for the t(11;14) molecular counterpart of the bcl-1 rearrangement by PCR,[47] the expression of the cyclin D1 protein in MCL is similar to that of the presence of t(11;14) itself, approximately 50% to 90%.[13,16,17] This can be demonstrated by immunohistochemical studies.[13,49] In the current study, cyclin D1 protein expression was observed in 70% of MCLs, which may reflect the variability of fixation and processing of cases submitted to the Armed Forces Institute of Pathology. Cyclin D1 is associated with progression of the cell cycle through G1 but is generally not found in normal lymphoid tissue or B-cell lines without t(11;14).[50] As with the translocation, the presence of this protein is highly characteristic of MCL, although it has been demonstrated occasionally in other hematopoietic malignancies, including hairy cell leukemia, splenic marginal zone lymphoma, multiple myeloma, and plasmacytoma.[20–22,45] Nonhematopoietic neoplasms, such as parathyroid adenoma,[51] breast carcinoma, and squamous cell carcinomas[52] have also demonstrated cyclin D1. Although the weak nuclear expression of this protein, detected by the anti-cyclin-D1 antibody, and high background in some cases can hinder interpretation, we feel that in conjunction with other immunohistochemical stains and diagnostic modalities, it is extremely helpful in definitive classification of MCL.

Because cyclin D1 protein appears helpful in diagnosis of MCL, we speculated that cyclin D1 mRNA would also be useful, even at low levels. We investigated the use of RT-PCR in FFPE tissue to demonstrate the presence of cyclin D1 mRNA and create a specific and sensitive test for MCL. The use of RT-PCR is often helpful in demonstrating the presence of low-level target mRNA in tissue, often lower than can be detected with Northern blot or *in situ* analysis. This was recently demonstrated in a study by Uchimaru et al in which competitive RT-PCR

was used to detect cyclin D1 in MCL and lymphoid cell lines.[25] The finding of cyclin D1 in non-MCL cell lines was also demonstrated in the study by Uchimaru et al but was seen rarely and in conjunction with the presence of t(11;14).[25] Our study shows that cyclin D1 can be demonstrated in MCL in a high percentage of cases by RT-PCR from paraffin-embedded tissue. The presence of cyclin D1 mRNA by RT-PCR was highly sensitive, but the extensive presence of cyclin D1 in non-MCL was an unexpected finding. In contrast to our study of cyclin D1 expression by RT-PCR, other studies, in which cyclin D1 expression was examined, used Northern blot and demonstrated only rare non-MCLs to be positive: 3/122 (2.5%),[10] 0/10 (0%),[53] and 1/50 (2%).[11] Only one study by De Boer et al also found low levels of expression in 36/56 (64%), a high percentage of non-MCLs.[9] In our current study and that of de Boer and colleagues, cyclin D1 expression (without quantitation) did appear to be a sensitive marker for lymphoid malignancy but not specific for MCL as has been previously reported.[25,11] Quantitation of the cyclin D1 expression was essential in differentiating MCL from non-MCL. Although Uchimaru used comparative quantitation,[25] to our knowledge, the present study is the first to use a semiquantitative, comparative method in paraffin-embedded tissue.

Cyclin D1 mRNA has also rarely been demonstrated in non-neoplastic lymphoid tissues by Northern blot.[11,17,22,50,53] Only a single study by de Boer et al[9] demonstrated low levels of cyclin D1 expression in widespread non-neoplastic lymphoid tissue, including normal lymph node, spleen, and tonsil. This finding is unique to the study of de Boer et al and has not been demonstrated by other authors, nor was it seen in our study. In our study, all of the non-neoplastic lymph nodes we examined, both nonstimulated and floridly reactive, were negative for cyclin D1 mRNA. The differences in sensitivity between the Northern blot assays developed by these different groups and our RT-PCR assay are difficult to assess.

Our study showed that the majority of non-MCLs expressed cyclin D1 mRNA, albeit at low levels. The presence of cyclin D1 expression in our study appears to be a sensitive adjunctive marker for malignancy in lymphoproliferative processes, but it is not specific for MCL. Because cyclin D1 expression is not seen in normal hematopoietic cells, the demonstrated expression of cyclin D1 in lymphomas other than MCL may indicate that cyclin D1 is a ubiquitous oncogene, however, at significantly lower levels than is seen in MCL. As the vast majority of these lymphomas do not possess the t(11;14) responsible for cyclin D1 overexpression in MCL, the mechanism for cyclin D1 expression/overexpression in these cases likely is different from that of MCL. However, using the semiquantitative assay described herein, cyclin D1 overexpression in MCL can be readily distinguished in paraffin-embedded tissues from its low expression/overexpression in non-MCLs. This finding may be helpful in diagnosis of morphologically and immunophenotypically ambiguous cases of suspected MCL. This method of examining differential expression of mRNA, although somewhat arduous, may also be helpful in other studies using paraffin-embedded fixed tissue where there is a necessity to quantitate mRNA expression.

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