Determination of Cyclin D1 and CD20 mRNA Levels by Real-Time Quantitative RT-PCR from Archival Tissue Sections of Mantle Cell Lymphoma and Other Non-Hodgkin's Lymphomas

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Cyclin D1 overexpression is a valuable marker for the diagnosis of mantle cell lymphoma (MCL). We used a real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) method to quantify levels of cyclin D1, CD20, and cyclophilin A mRNA in manually microdissected, paraffin-embedded tissue sections using an ABI 7700 qRT-PCR system. The study group included 21 cases of MCL and 37 cases of other types of B-cell non-Hodgkin's lymphoma. Cyclin D1 mRNA copy number was normalized to CD20 and cyclophilin A mRNA and evaluated statistically by analysis of variance. The relative cyclin D1 levels were similar whether normalized to CD20 or cyclophilin A, indicating that CD20 levels are stable and can be used as a B-cell-specific normalizer. Statistically significant differences were found in the median levels of cyclin D1 mRNA (expressed as % CD20 mRNA) among cases of MCL (87.6), small lymphocytic lymphoma (9.9), follicular lymphoma (2.4), diffuse large B-cell lymphoma (5.9), marginal zone B-cell lymphoma (39.8), and Burkitt lymphoma (7.1) ($P < 0.05$). We conclude that qRT-PCR can be used to quantify cyclin D1 mRNA levels in archival tissue sections. Normalization of cyclin D1 to a B-cell-specific marker more accurately reflects overexpression by MCL than other methods that normalize using constitutively expressed mRNA species. (*J Mol Diagn* 2002, 4:201–208)

Mantle cell lymphoma (MCL) is a clinically and morphologically distinct type of non-Hodgkin's lymphoma (NHL) characterized by the t(11;14)(q13;q32).^{1,2} This translocation juxtaposes the *CCND1* gene (cyclin D1) at 11q13 with the immunoglobulin heavy chain gene, resulting in overexpression of cyclin D1.^{3,4} Mantle cell lymphoma has

an aggressive clinical course and is resistant to conventional therapies that have been used to treat other types of B-cell NHL.^{1,2} Consequently, the diagnosis of MCL has important prognostic implications.

Establishing the diagnosis of MCL is complicated by several factors. MCL exhibits a spectrum of morphological findings and distinguishing MCL from other types of NHL relies on the appreciation of subtle histological differences.^{1,2} The diagnosis of high-grade variants of MCL can be especially difficult. Immunophenotypic findings in MCL share many features with other low-grade B-cell NHLs, except for cyclin D1 overexpression, which is a characteristic feature of MCL. However, some cases of MCL have low level cyclin D1 overexpression that is difficult to detect by using immunohistochemical methods.^{5,6} Molecular methods to detect the t(11;14), thereby supporting the diagnosis of MCL, also have drawbacks. Polymerase chain reaction (PCR) methods, which target the major translocation cluster region, detect the t(11;14) in 40 to 50% of MCL.⁷ However, other chromosome 11 breakpoints are widely scattered over 120 kb and are not detected by PCR.^{8,9} Southern blot analysis using multiple probes can detect the t(11;14) in 70 to 80% of cases, but this method is time-consuming and requires high-quality DNA.^{8–10} Conventional cytogenetic analysis of MCL theoretically has the potential to identify the t(11;14) in all cases. However, for technical reasons, such as poor cell growth, the reported sensitivity using this method is approximately 60 to 80%.^{11,12} Fluorescence *in situ* hybridization (FISH) analysis of nuclei isolated from either fresh cells or paraffin-embedded tissue has been found to be highly sensitive for detecting the t(11;14),^{13–15} but this assay is not routinely performed in many laboratories.

An alternative approach to obtain support for the diagnosis of MCL is to determine expression levels of the *CCND1* gene, as all t(11;14) translocations result in over-

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expression. As lymphoid cells express very low levels of cyclin D1, not detectable by immunohistochemical methods,¹⁶ these methods are convenient and specific for distinguishing MCL from other NHL. However, a negative immunoreaction does not exclude the diagnosis of MCL, as some cases have relatively low levels of cyclin D1. Detection of cyclin D1 mRNA by RT-PCR, in contrast, is very sensitive. Unfortunately, this approach cannot distinguish signal contributed by stromal, epithelial, or lymphoid cells.^{6,16,17} Furthermore, conventional RT-PCR methods allow only semi-quantitative analysis of mRNA levels. Quantification of cyclin D1 mRNA from microdissected tissue sections would be an ideal solution coupling the requirements of high sensitivity and specificity. Numerical data, in addition, may permit more specific stratification of MCL cases for the purposes of classification and analysis of outcome.

In this study, we used qRT-PCR to quantify cyclin D1 mRNA levels in 21 MCL and 37 other B-cell NHLs to determine the value of this approach as an ancillary tool for the diagnosis of MCL. We extracted RNA from microdissected paraffin-embedded tissue sections as we have previously reported,¹⁸ and normalized cyclin D1 mRNA levels to two reference mRNA species, CD20 and cyclophilin A.

Materials and Methods

Tissue Samples

Paraffin blocks of 58 NHL (57 patients) and three reactive lymph nodes were retrieved from the files of the University of Texas M.D. Anderson Cancer Center and University of Texas-Houston/Memorial-Hermann Hospital. The diagnosis of MCL in 20 patients (21 specimens) was based on compatible morphological and immunophenotypic findings. Cyclin D1 overexpression was shown by immunohistochemical methods in all cases of MCL. The t(11;14) was proved to be present in nine of these neoplasms using various molecular methods. Four- μ m histological sections of a representative tissue block were placed on charged slides for immunohistochemical studies or uncharged slides for microdissection.

Cyclin D1 Immunohistochemical Methods

Deparaffinized tissue sections were subjected to microwave-enhanced antigen retrieval using 0.01 mol/L citrate buffer, pH 6.0, for 15 minutes.¹⁹ Sections were immunostained using a three-step avidin-biotin complex-peroxidase system. We used the DCS-6 monoclonal antibody (dilution 1:350, Dako, Carpinteria, CA) to assess for cyclin D1, followed by a biotinylated anti-mouse Ig, and avidin-biotin-peroxidase complex (LSAB2, Dako). Immunocomplexes were visualized by using 3,3'-diaminobenzidine tetrahydrochloride as the chromogen.

Microdissection and RNA Extraction

Formalin-fixed, paraffin-embedded tissue sections were dewaxed and rehydrated through two rinses each of

xylene and absolute ethanol and stained with 0.08% methylene blue. Non-Hodgkin's lymphoma tissue was separated from stroma and epithelial tissue using one to three tissue sections by manual microdissection under a dissecting microscope. The microdissected tissue was pooled and stored in absolute ethanol at room temperature until used. Microdissected tissue fragments were sedimented from ethanol by centrifugation at $10,000 \times g$ for 1 minute.

Total RNA was extracted from the tissue pellet using the Masterpure kit (Epicenter Technologies, Madison, WI) according to the manufacturer's instructions, with overnight proteinase K digestion. The extracted RNA was dissolved in 20 μ l of 1X PCR buffer supplemented with 5 mmol/L MgCl₂, 10 mmol/L dithiothreitol (DTT), 4 units of RNasin (Promega, Madison, WI), 3 units DNase I (Life Technologies, Gaithersburg, MD) and incubated for 30 minutes at 37°C to digest contaminating DNA. An additional 20 μ l of distilled water was added and the DNase I was inactivated at 75°C for 10 minutes. The RNA sample was stored at -70°C until analyzed.

Standards

To enable us to determine the number of molecules in the starting material, synthetic RNA (sRNA) (for cyclophilin A) or double-stranded DNA (for cyclin D1 and CD20) was used to construct standard curves. sRNA was transcribed from cyclophilin A cDNA that was tailed *via* PCR to introduce the promoter of the T7 RNA polymerase using the MEGAshortscript kit (Ambion, Austin, TX) as previously described.²⁰ Tenfold serial dilutions of the sRNA were used to generate the cyclophilin A standard curve.

CD20 and cyclin D1 were amplified from a fresh human tonsillectomy specimen by RT-PCR. The amplified material was purified by chromatography through a G50 spin column (Boehringer Mannheim, Indianapolis, IN). The DNA concentration was determined by optical density measurement and 10-fold serial dilutions were used as standards.

Quantitative RT-PCR

The TaqMan PCR technology^{21,22} makes use of an internally quenched, fluorescently labeled oligonucleotide (probe) that anneals to the denatured target sequence in the segment defined by the PCR primers. On primer extension, the probe is hydrolyzed by virtue of the 5' $\rightarrow$ 3' exonuclease activity of Taq polymerase, resulting in liberation and dequenching of a fluorescent nucleotide. The ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) detects in real-time the fluorescent signal of the hydrolyzed probe and determines the threshold cycle (C_T), the cycle number at which the signal exceeds background by $> = 10$ SD. The C_T value is a precise measure of the number of generated amplicons which, in turn, is a measure on a logarithmic scale of the amount of starting DNA target. This technique is sensitive, precise, and has a very wide dynamic range.²² The requirement

Table 1. Oligonucleotide Sequences and RT-PCR Reaction Conditions

Assay (amplicon length)	Oligonucleotide	5' nt position (orientation)	Conc (nM)	Mg2+ (mM)	Sequence	Accession no.	Reference
Cyclophilin A (66 bp)	Probe	69 (+)	100	4	CGCGTCTCCTTTGAGCTGTTTGCA	Y00052	24
	5' primer	52 (+)	300		ACGGCGAGCCCTTGG		
	3' primer	117 (-)	300		TTTCTGCTGTCTTTGGGACCT		
Cyclin D1 (75 bp)	Probe	358 (-)	100	3	TCTGTTCTCGCAGACCTCCAGCA	M64349	25
	5' primer	304 (+)	200		CCGTCCATGCGGAAGATC		
	3' primer	378 (-)	200		GAAGACCTCCTCTCGCACT		
CD20 (73 bp)	Probe	253 (+)	100	3	CACGCAAAGCTTCTTCATGAGGGAATCT	M27394	23
	5' primer	233 (+)	300		ATGTCTTCACTGGTGGGCC		
	3' primer	305 (-)	300		TAATCTGGACAGCCCCAA		

of probe hybridization makes this technique more specific than traditional PCR methods.

The RT and PCR reactions were run in 96-well plates. Each run consisted of a sRNA or DNA standard (typically 200 fg to 20 attog) in triplicate, 20 unknown samples in duplicate, 1 "no-RT" well for each duplicate unknown to control for tissue-derived DNA contamination, and a "no template" control well consisting of all of the components of the RT and PCR reaction mixtures except RNA to control for extraneous DNA contamination.

Reverse Transcription

The reaction mixture contained 2 μ l of extracted cellular RNA, 500 μ mol/L of each dNTP, 200 to 300 nmol/L of reverse primer, 1X first-strand buffer (Life Technologies), 10 mmol/L DTT (Boehringer Mannheim) and 10 units of Superscript II reverse transcriptase (Life Technologies) in a volume of 10 μ l. The reaction mixture was incubated at 50°C for 30 minutes followed by heat inactivation at 72°C for 10 minutes. RT reactions were run in triplicate for the standards and in duplicate for unknown samples. For each duplicate reaction, a "no-RT" control was run which included all components of the RT mixture except reverse transcriptase.

PCR

Following first-strand synthesis, all of the 10 μ l RT reaction mixture was used for subsequent PCR amplification by adding 40 μ l of PCR master mix to the same wells. The PCR reaction mixture included 1X PCR buffer, 200 μ mol/L of each dNTP, and 1.25 U *Taq* polymerase (Roche Molecular Biochemicals, Indianapolis, IN) in a final volume of 50 μ l. Depending on the target transcript, the optimal concentration of $MgCl_2$, primers, and the fluorogenic *Taqman* probe varied as tabulated in Table 1.

Amplification and real-time data acquisition were performed in an ABI Prism 7700 Sequence Detector using the following cycle conditions: initial denaturation at 95°C for 1 minute, followed by 40 cycles of denaturation at 95°C for 12 seconds, and annealing at 60°C for 1 minute.

Data Analysis and Statistical Evaluation

The ABI Prism 7700 instrument determines the C_T values of each well and the Sequence Detection Application converts these values to copy number if a calibrated

series of standards is included in the run. Only samples with C_T values at least two units less than that of the "no-RT" control were included in data analysis. To correct for variations of total RNA concentration, the copy number of cyclin D1 mRNA was expressed as a percentage of CD20 or cyclophilin A mRNA molecules. Cyclophilin A is a ubiquitous, constitutively expressed mRNA species.²⁶ These normalized cyclin D1 mRNA levels were used in data analysis.

We used Jandel's Sigmapstat software (SPSS Inc., Chicago, IL) for statistical analysis. To establish whether cyclin D1 mRNA level differences were statistically significant for any NHL group, one-way analysis of variance (ANOVA) was used. We used parametric analysis of variance for normally distributed values and the Kruskal-Wallis analysis of variance on ranks for values exhibiting asymmetrical distribution. If analysis of variance indicated a significant difference, we identified the specific diagnostic category that was significantly different from MCL by pair-wise comparison using either Dunn's test (for asymmetrical data) or Tukey's test (for normally distributed data). All procedures were performed at a significance level of $p = <0.05$. For correlation analysis, Pearson's correlation coefficient was calculated.

Results

The patients' age, biopsy sites, pathological diagnoses, and the relative cyclin D1 levels as determined by qRT-PCR are shown in Table 2. The MCL group included 14 men and 6 women. From one of the MCL patients, two lymph node biopsy specimens from different anatomical sites were analyzed (specimens 46 and 49).

The cyclin D1 mRNA level in any sample is a function of the abundance of message in individual cells and the concentration of total RNA in the sample. To correct for differences in RNA concentration and for uncontrolled variables during tissue processing, we expressed cyclin D1 levels as a percentage of reference message. One of the normalizers we selected was CD20 mRNA, a B cell-specific message. We also normalized to cyclophilin A mRNA. To determine whether normalizing to CD20 mRNA was valid, we plotted the CD20 mRNA copy number against that of cyclophilin A mRNA (Figure 1). This plot revealed a strong linear correlation of high statistical significance ($r = 0.85$, $P = 7.0 \times 10^{-18}$) between the two sets of logarithmic values, indicating that the level of one

Table 2. Patient Clinical Data, Biopsy Site, and Relative Cyclin D1 mRNA Levels Expressed as Percent of Either Cyclophilin A or CD20

	Diagnosis	Tissue, site	Sex	Age	Cyclin D1 mRNA (% CD20)	Cyclin D1 mRNA (% CyPA)
1	Reactive	LN, inguinal	M	55	7.0	3.2
2	Reactive	LN, inguinal	F	62	23.7	5.0
3	Reactive	LN, inguinal	F	39	53.1	10.3
4	BL	mass, nasal	M	17	17.8	1.6
5	BL	small intestine	F	13	1.6	0.4
6	BL	ileum	M	17	7.1	0.4
7	CLL/SLL	spleen	F	55	54.4	5.2
8	CLL/SLL	LN, inguinal	F	66	8.6	25.1
9	CLL/SLL	LN, axillary	F	62	12.9	3.7
10	CLL/SLL	LN, submandibular	M	58	10.4	0.6
11	CLL/SLL	LN, auricular	M	50	7.2	3.4
12	CLL/SLL	LN, inguinal	M	60	3.5	0.5
13	CLL/SLL	LN	M	67	17.4	7.2
14	CLL/SLL	LN, cervical	M	70	11.3	3.1
15	CLL/SLL	LN, supraclavicular	F	57	9.3	4.2
16	CLL/SLL	LN, axillary	F	79	1.3	0.8
17	FL, Gr1	LN, inguinal	F	46	4.4	1.9
18	FL, Gr1	LN, inguinal	M	48	1.3	1.8
19	FL, Gr1	LN, inguinal	M	83	2.0	0.9
20	FL, Gr1	LN, inguinal	M	68	3.1	1.2
21	FL, Gr1	LN, inguinal	F	46	4.9	2.5
22	FL, Gr2	LN, cervical	F	48	1.6	2.6
23	FL, Gr2	LN, inguinal	F	76	8.8	4.1
24	FL, Gr2	LN, inguinal	F	58	2.8	1.7
25	FL, Gr2	LN, axillary	M	52	2.0	1.8
26	FL, Gr3	LN, axillary	M	49	1.8	1.6
27	MZL	LN, cervical	F	67	18.7	8.5
28	MZL	parotid	F	56	3.6	1.1
29	MZL	skin	M	76	16.4	7.5
30	MZL	parotid	F	71	5.7	6.8
31	MZL	tongue	F	68	11.5	4.3
32	MZL	skin	F	75	5.4	7.8
33	MZL	LN, splenic	F	57	6.2	2.6
34	MZL	LN, paraaortic	F	65	3.4	7.5
35	MZL	spleen	F	79	14.5	8.6
36	DLBCL	mass, mediastinal	F	34	6.2	8.4
37	DLBCL	skin, breast	F	75	3.8	1.1
38	DLBCL	mass, mediastinal	F	49	11.7	3.3
39	DLBCL	mass, abdominal	M	58	5.9	1.8
40	DLBCL	nodule, chest	M	61	4.5	8.0
41	MCL	ileum	M	42	68.9	31.1
42	MCL	rectum	M	63	255.2	229.3
43	MCL	LN, cervical	F	78	106.5	52.4
44	MCL	LN, inguinal	M	62	90.4	27.1
45	MCL	colon	M	82	115.7	33.1
46	MCL	LN, hepatic	M	69	80.7	30.8
47	MCL	LN, axillary	M	56	131.2	181.6
48	MCL	LN, cervical	M	74	87.6	469.1
49	MCL	LN, omentum	M	69	192.9	101.8
50	MCL	LN, cervical	F	61	31.2	29.7
51	MCL	spleen	M	56	26.1	22.0
52	MCL	tonsil	F	68	36.1	26.4
53	MCL	LN, cervical	M	56	76.2	44.8
54	MCL	LN, inguinal	M	63	114.6	307.6
55	MCL	LN, inguinal	F	69	11.9	8.8
56	MCL	LN, splenic	M	69	31.6	25.2
57	MCL	LN	F	54	28.0	33.3
58	MCL	LN, axillary	M	59	88.8	30.2
59	MCL	LN, axillary	M	79	87.6	52.7
60	MCL	spleen	F	67	139.5	55.0
61	MCL	spleen	M	66	381.1	107.7

LN, lymph node, site not specified.

Cases 46, 49 represent different lymph nodes from same patient; #46 with blastic morphology.

Abbreviations: BL, Burkitt lymphoma; CLL/SLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma.

Figure 1. Scatter plot showing CD20 mRNA copy number as a function of cyclophilin A copy number (log-scale). The correlation coefficient (R) and P value were calculated using the logarithms of the absolute copy numbers.

normalizer was highly predictive of the other, ie, both were reliable measures of total cellular RNA.

The individual relative cyclin D1 mRNA values were very low and tightly clustered in each non-MCL group using CyPA as normalizer. In MCL, the CyPA-normalized values were much higher and showed a significant spread with extremes of several hundred percent of cyclophilin A. The CD20-normalized levels showed a similar distribution, but there were occasional outliers in the reactive and chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) groups.

Since the data points were not normally distributed, the medians were used to characterize the diagnostic groups. The median cyclin D1 mRNA levels expressed as a percentage of CyPA or CD20 for each diagnostic category are shown in Figure 2. As displayed, the CyPA-normalized cyclin D1 levels in all non-MCL cases were comparable, with a median value 4- to 70-fold lower than the MCL group. Statistical analysis by Dunn's pair-wise comparison for non-normally distributed data showed that these differences were significant between MCL versus all other groups ($P < 0.05$) except the reactive lymph node group (Table 3). When the distribution was normalized by logarithmic transformation and Tukey's test was used for pair-wise comparison, the difference in mean cyclin D1 mRNA levels between MCL and reactive lymphoid tissues also reached statistical significance ($P < 0.05$).

The results obtained by normalizing to CD20 mRNA were very similar, but not identical (Figure 2 and Table 3). Cyclin D1 levels in only follicular lymphoma (FL) and diffuse large B-cell lymphoma (DLBCL) were significantly different from MCL ($P < 0.05$) when we analyzed by a non-parametric statistical test. Cyclin D1 levels in marginal zone lymphoma (MZL), CLL/SLL, Burkitt lymphoma (BL), and reactive lymphoid tissues did not significantly differ from MCL due to the high variance of their expression levels. In a parametric test, however, cyclin D1 levels in all groups except the reactive lymph node group were found to be significantly different from MCL ($P < 0.05$).

To determine the possible cause of the higher variance of the CD20-normalized values, we calculated the levels of CD20 expression relative to cyclophilin A. As shown in

A. Relative Cyclin D1 mRNA Levels in non-Hodgkin's Lymphomas and Reactive Lymph Nodes (%CyPA)

B. Relative Cyclin D1mRNA Levels in non-Hodgkin's Lymphomas and Reactive Lymph Nodes (%CD20)

Figure 2. Relative cyclin D1 mRNA levels expressed as percentage of CyPA (A) or CD20 (B) mRNA copy number. The end points of the solid bars represent 25th and 75th percentile. Median is indicated by the star symbol. The inserted bar graphs show the median relative cyclin D1 mRNA levels for each normalizer (stippled bars).

Figure 3, the CD20 mRNA levels, when normalized to CyPA, were variable. They were very low in BL and reactive lymph nodes. The other non-MCL NHLs expressed comparable levels of CD20. MCL, as a group, expressed CD20 levels almost twice as high as CLL/SLL. Statistical analysis of log-transformed data showed that BL expressed significantly less CD20 mRNA than MCL, MZL, or FL ($P < 0.05$); all other groups were not significantly different from each other.

The mean relative cyclin D1 levels for male and female patients were 121.9% and 58.9% of CD20 and 112.9% and 32.3% of cyclophilin A, respectively. These differences reached only marginal statistical significance for the CD20-normalized values when the distribution was normalized by logarithmic transformation ($P < 0.05$). Similarly, the expression levels were not statistically different when we compared MCL cases with different architectural patterns (nod-

Table 3. Comparison of Relative Cyclin D1 mRNA Levels by Analysis of Variance

	Dunn's (medians)		Tukey's (means)	
	% CD20	% CyPA	log (% CD20)	log (% CyPA)
Reactive (<i>n</i> = 3)	NS	NS	NS	<i>P</i> < 0.05
BL (<i>n</i> = 3)	NS	<i>P</i> < 0.05	<i>P</i> < 0.05	<i>P</i> < 0.05
SLL (<i>n</i> = 10)	NS	<i>P</i> < 0.05	<i>P</i> < 0.05	<i>P</i> < 0.05
FL (<i>n</i> = 10)	<i>P</i> < 0.05	<i>P</i> < 0.05	<i>P</i> < 0.05	<i>P</i> < 0.05
MZL (<i>n</i> = 9)	NS	<i>P</i> < 0.05	<i>P</i> < 0.05	<i>P</i> < 0.05
DLBCL (<i>n</i> = 5)	<i>P</i> < 0.05	<i>P</i> < 0.05	<i>P</i> < 0.05	<i>P</i> < 0.05

Each diagnostic category was compared to MCL. To establish the existence of a statistically significant difference for any NHL category, one way ANOVA was used. To identify the specific group that is significantly different from MCL, pair-wise comparison was performed using Dunn's test for asymmetrical data (characterized by medians) or Tukey's test for normally distributed values (characterized by means).

ular or diffuse) or cytological features (classical or blastic). All patients were high clinical stage and thus stage could not be correlated with expression levels of cyclin D1. Our series included four blastoid MCL cases. In one patient, lymph nodes from two different anatomical sites harbored MCL with classical small cell and blastoid morphological features, respectively. In the classical small cell MCL sample (49) cyclin D1 was overexpressed at a higher level than in the sample of blastoid MCL (46). Anatomical site also did not influence the level of cyclin D1 overexpression (extranodal versus nodal, *P* = 0.12 normalized to CD20, *P* = 0.62 normalized to CyPA).

We also considered the possibility that a diagnostically useful cutoff level for cyclin D1 expression could be established. After examining the distribution of the data, we chose a cutoff value at 10 to 20% of CyPA or 20 to 25% of CD20, and obtained a sensitivity of 95.2% and specificity of 97.3% for both normalizers. (In these calculations samples 46 and 49 were included as independent data points).

Discussion

Our primary aim in this study was to develop a method for quantifying cyclin D1 mRNA levels that was applicable to routine archival material. We reasoned that this method may aid in the differential diagnosis of MCL with other types of NHL. Although immunohistochemical detection of cyclin D1 is commonly and successfully used in the diagnosis of MCL, this technique can be technically chal-

lenging and, due to occasional low level overexpression in MCL, requires subjective interpretation. More importantly, immunohistochemical detection relies on absolute quantity and preserved antigenicity of the target antigen and thus is sensitive to suboptimal tissue processing, an important consideration if the material is derived from disparate sources. By normalizing the level of cyclin D1 mRNA to a reference message, a quantitative procedure would obviate this dependence on optimal fixation and processing. As long as both messages are similarly affected, a valid relative level can be obtained even if the RNA yield is low due to sub-optimal recovery.

Previous attempts to apply qRT-PCR methods to the analysis of paraffin-embedded tissue sections have encountered limited sensitivity attributable to tissue fixation, particularly when cross-linking fixatives such as formalin are used, leading others to suggest the use of alternative fixatives.²⁷ We preferred to adapt this method to formalin-fixed and routinely processed tissue to enable us access to the vast tissue resources stored as archived paraffin blocks. The success of the method we have used may be attributed to the extensive proteolysis during RNA extraction and the design of assays with short target amplicons. In this study we were able to measure transcript levels from a single 4- μ m section of very small biopsy specimens, such as endoscopic biopsy specimens obtained from the gastrointestinal tract.

We detected cyclin D1 expression in all NHL and reactive lymph node specimens. Although some groups have reported that reactive lymphoid tissues and non-MCL (except for hairy cell leukemia) do not express cyclin D1,^{3,6,28} several groups have detected low levels of cyclin D1 mRNA or protein in non-neoplastic B lymphocytes and B-cell NHL other than MCL by Northern and Western blotting, respectively, or by RT-PCR.^{17,29} While the manual microdissection technique we used could not ensure the recovery of stromal cell-free lymphoid tissue, the fact that the cyclin D1 levels in various diagnostic categories changed in tandem whether normalized to CyPA or the B-cell-specific CD20 reference message suggests that these differences reflect truly variable expression by the B-cell compartment, rather than a predictably steady contribution by non-lymphoid cells. Furthermore, manual microdissection was adequate to obtain epithelium-free lymphoid tissue from gastrointestinal tract, skin, and salivary gland biopsy specimens. Since epithelial tissues are known to express

Figure 3. CD20 mRNA expression levels in various NHL (median of CD20/CyPA mRNA copy numbers).

cyclin D1, microdissection in these cases is necessary for accurate assessment of cyclin D1 levels from lymphoid tissue.

The reliability of our qRT-PCR method for the analysis of microdissected, paraffin-embedded tissue sections of lymphoid tissues is supported by several lines of evidence. The strong linear correlation between levels of two independent constitutive mRNA species confirmed that their recovery was proportional and apparently unaffected by mRNA species. There was a strong positive correlation between cyclin D1 levels and MCL, the diagnosis of which was based on unrelated methods. Other studies using qualitative or semi-quantitative RT-PCR on paraffin tissue or qRT-PCR on fresh-frozen material have reported results very similar to our own.^{16,30,31} The only studies using real-time qRT-PCR to measure cyclin D1 mRNA levels in paraffin sections of which we are aware are those of Bijwaard and colleagues and Suzuki and colleagues.^{30,31} These authors found significantly higher mean cyclin D1 mRNA levels in MCL compared with all other NHL types assessed. Bijwaard and colleagues³⁰ also observed large variability in the level of overexpression, approximately 16-fold differences, in MCL cases. Finally, the observed twofold difference in CD20 mRNA level between MCL and CLL/SLL is very similar to that reported for the protein as determined by quantitative flow cytometry by others.³²

The large differences in the level of cyclin D1 mRNA overexpression in MCL, resulting in a non-normal distribution of the mRNA copy number, suggest heterogeneity within the MCL group. This observation is in accordance with the results of cyclin D1 expression determined immunohistochemically. A review of the immunostained slides of the MCL cases in the study group showed that the source of this variability was attributable to both variable protein concentration in individual cells and a variable proportion of lymphoma cells overexpressing the protein. The observed variability of cyclin D1 overexpression in MCL cases possibly reflects the lack of regulatory constraint on the translocated cyclin D1 gene in a cell type that normally barely expresses it. This bias toward high level overexpression resulted in asymmetrical distribution of the cyclin D1 mRNA levels, making it necessary to compare the diagnostic categories by their median values. To maximize the resolving power of statistical calculations we also compared logarithmically transformed cyclin D1 mRNA levels. After such transformation, the data are amenable to analysis by the more powerful parametric statistical tests (parametric analysis of variance followed by Tukey's pair-wise comparison).

In an attempt to identify a factor that segregates the MCL cases into corresponding subgroups we correlated gender, architectural pattern, and cytological features with cyclin D1 mRNA copy number. We did not observe any difference in level of cyclin D1 overexpression that correlated with architecture (diffuse, nodular, or both), nor could we correlate cyclin D1 levels with cytologic features. Other authors also have failed to correlate morphological variants of MCL with levels of cyclin D1 mRNA or protein.³³

Cyclin D1 is up-regulated during estrogen-induced proliferation of estrogen target tissues,³⁴ yet MCL afflicts males

three to four times more frequently than females. To determine whether there were sex-related differences in cyclin D1 levels in MCL, we compared the values obtained in men and women. The mean cyclin D1 levels were higher in men, but these differences reached marginal statistical significance only for the CD20-normalized values.

Three of our MCL cases tested positive for the t(11;14) by PCR which targets the major translocation cluster (MTC). The MTC is approximately 120 kb distant from the *CCND1* gene, and PCR or Southern probes targeting this region demonstrate its involvement in approximately 40 to 50% of MCL cases.^{7,8,10} The other breakpoints are in closer proximity to the *CCND1* gene.^{8,9} Therefore, a possible positional effect of the IgH promoter on cyclin D1 transcription may be inferred by comparing cyclin D1 mRNA levels in PCR-positive and PCR-negative cases. This comparison showed no effect of proximity on cyclin D1 mRNA expression levels. Other authors similarly could not detect any effect of the proximity of the breakpoint site on the nuclear staining intensity by immunohistochemistry.³⁵ We found only a moderate level of cyclin D1 mRNA in one of our MCL cases (case 55) which, by our criteria, did not constitute overexpression. The lack of cyclin D1 mRNA or protein overexpression has been reported in 5 to 10% of MCL cases.^{29,33,36} Unlike the reported cases where cyclin D1 protein was undetectable or was not assayed for, our case did show cyclin D1 overexpression by immunohistochemical analysis. Altered post-translational regulation resulting in an enhanced translation of cyclin D1,³⁷ or a polymorphism or mutation of the affected allele preventing our assay from detecting mRNA overexpression, are possible explanations.

The use of a B-cell-specific normalizer is a novel feature of our study. By comparing the CyPA-normalized and CD20-normalized cyclin D1 mRNA levels, we were able to estimate the contribution of the B-cell fraction to the total cyclin D1 level. This can be especially useful if large numbers of reactive T cells or non-lymphoid cells are included in the specimen. We interpret the high CD20-normalized cyclin D1 values in reactive lymph nodes (23.7% of CD20) compared with B-cell NHLs other than MCL (2.4 to 9.9% of CD20) as indicative of a low overall CD20 level due to admixed non-B cells rather than true cyclin D1 overexpression, since the CyPA-normalized values were not elevated. In our study, the ratio of the CD20-normalized *versus* CyPA-normalized cyclin D1 levels ranged 0.09 to 0.59 for the NHL groups. In one case analyzed (and excluded from statistical analysis), the CD20-normalized value was 43 times higher than the CyPA-normalized value, suggestive of a very low level of CD20 expression. This patient had received rituximab (anti-CD20) therapy and the low level of CD20 expression might be the result of down-regulation of CD20 expression or selective proliferation of a CD20-negative B-cell clone.³⁸

In light of our finding that various NHLs express CD20 mRNA at slightly different levels, the use of another B-cell-specific message might be better suited as a normalizer. The concordance of the CD20 mRNA levels with the reported differences of membrane CD20 protein den-

sity measured by quantitative flow cytometry studies,³² however, is reassuring regarding the power of resolution of this paraffin section-based qRT-PCR procedure.

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