Identification of Cyclin D1 mRNA Overexpression in B-Cell Neoplasias by Real-Time Reverse Transcription-PCR of Microdissected Paraffin Sections[1]

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ABSTRACT

Purpose: Overexpression of cyclin D1 mRNA and protein as a result of the chromosomal translocation t(11; 14)(q13;q32) is a highly specific molecular marker of mantle cell lymphoma, but cyclin D1 dysregulation can also be found in other B-cell neoplasias. The aim of the study was to develop a precise and reliable tool for quantitation of cyclin D1 mRNA suitable for archival clinical specimens.

Experimental Design: A real-time reverse transcription-PCR (RT-PCR) assay was used to quantitate cyclin D1 mRNA copy numbers. Using 2000 microdissected cells as template, 104 formalin-fixed, paraffin-embedded lymph node, spleen, and decalcified bone marrow biopsies from a panel of 95 cases of B-cell non-Hodgkin's lymphomas (B-NHLs) were analyzed. In addition, cyclin D1 protein expression was assessed by immunohistochemistry.

Results: Strong cyclin D1 mRNA overexpression was detected in mantle cell lymphomas (23 of 23), hairy cell leukemias (5 of 19), and multiple myelomas (7 of 23) with particularly high levels in 2 of the latter cases. Intermediate transcript levels were found in 5 of 23 multiple myelomas and 7 of 19 hairy cell leukemias. B-cell chronic lymphocytic leukemias (10 of 10), follicular lymphomas (9 of 9), mucosa-associated lymphoid tissue lymphomas (5 of 5) and reactive lymphoid tissues with the exception of normal spleen had no or very low cyclin D1 expression. In comparison with real-time RT-PCR, immunohistochemistry showed a lower level of sensitivity, more variability, and did not allow accurate quantitation.

Conclusions: Real-time RT-PCR for cyclin D1 mRNA is an excellent tool for the differential diagnosis of B-NHLs and, in combination with microdissection, a powerful approach for retrospective trials using archival clinical specimens as tissue source. Furthermore, real-time RT-PCR may help to identify subgroups of B-NHLs according to cyclin D1 mRNA copy numbers and to investigate the possible influence of different chromosomal breakpoints on cyclin D1 expression.

INTRODUCTION

The cyclin D1 oncogene product derived from the *Prad-1/ bcl-1* locus on chromosome 11q13 is a major regulator of the G_1-S transition of the cell cycle (1). Aberrantly expressed cyclin D1 has been implicated in the pathogenesis of several types of human neoplasias (2). In lymphoid malignancies, cyclin D1 mRNA and protein overexpression is usually the consequence of the chromosomal translocation t(11;14)(q13;q32), which juxtaposes the immunoglobulin heavy chain gene complex on chromosome 14 with the cyclin D1/*BCL-1* region on chromosome 11q13 (3–6). The translocation t(11;14) is the molecular hallmark of MCL[4] and is found in up to 90% of cases (7). Cyclin D1 up-regulation resulting from this translocational activation has also been found occasionally in other B-NHLs such as MM (8) and cases classified as B-CLL (9) and prolymphocytic leukemia (10). In contrast to MCL, t(11;14) translocations in MM seem to involve an error in switch recombination rather than VDJ recombination, and breakpoints on chromosome 11q13 are widely scattered, without clustering in the MTC as observed in MCL (11). In HCL, as opposed to MCL and MM, the increased expression of cyclin D1 occurs independently of t(11;14) or other genetic abnormalities of the 11q13 region (12, 13).

Because cyclin D1 is normally not expressed in lymphoid cells, up-regulation of cyclin D1 in MCL is a highly specific and sensitive molecular marker and can serve as a diagnostic

[4] The abbreviations used are: MCL, mantle cell lymphoma; RT-PCR, reverse transcription-PCR; HCL, hairy cell leukemia; IHC, immunohistochemistry; MALT, mucosa-associated lymphoid tissue; B-NHL, B-cell non-Hodgkin's lymphoma; MM, multiple myeloma; B-CLL, B-cell chronic lymphocytic leukemia; FISH, fluorescence *in situ* hybridization; BM, bone marrow; MTC, major translocation cluster; FL, follicular lymphoma; LN, lymph node; NHL, non-Hodgkin's lymphoma; CV, coefficient of variation; CLL, chronic lymphocytic leukemia; Ct, cycle threshold.

tool for the differential diagnosis of this entity from other small B-NHLs with similar morphology, such as B-CLL with cleaved cells, MALT lymphomas, and splenic marginal zone lymphoma (9, 14).

In addition to its diagnostic utility in B-NHLs, cyclin D1 expression may also have prognostic relevance. In MM, the t(11;14), abnormalities of 11q13, and/or cyclin D1 overexpression have been shown to be associated with poor prognosis (15) and with an advanced clinical stage (16), although other studies have failed to confirm a prognostic significance (17, 18).

Methods for detection of t(11;14), including cytogenetics, Southern blot analysis, FISH, and PCR, are sometimes of limited practical use due to technical difficulties and availability, as well as the scattering of the 11q13 breakpoints. Currently, evaluation of cyclin D1 protein overexpression by paraffin section IHC is the most widely used diagnostic technique (14, 19–21). Whereas cyclin D1 immunostaining is well suited for routine purposes, it sometimes lacks sensitivity, does not allow quantitation of cyclin D1 expression levels, and is critically influenced by variations in tissue fixation and antigen retrieval technique (22, 23).

We recently reported that real-time RT-PCR in combination with laser microdissection allows reliable quantitation of gene expression in formalin-fixed, archival tumor tissues (24). Because cyclin D1 protein levels correlate well with mRNA levels (12, 19, 25), cyclin D1 is a suitable candidate for this approach. Given the practical importance of a reliable detection of cyclin D1 overexpression in hematological neoplasms and the limitations of currently used techniques, we decided to examine a large series of fixed, paraffin-embedded B-NHL samples including decalcified BM biopsies and normal lymphoid tissues, with real-time RT-PCR. Beyond its potential diagnostic value, exact quantification of cyclin D1 expression levels might provide an excellent tool to study causes and consequences of cyclin D1 deregulation.

MATERIALS AND METHODS

Tissue Samples. A total of 104 formalin-fixed, paraffin-embedded specimens of 95 patients with B-cell neoplasias diagnosed between 1993 and 2001 were selected from the files of the Institutes of Pathology, Technical University of Munich (Munich, Germany), the University of Innsbruck, and General Hospital Salzburg (Salzburg, Austria). All cases were classified according to the Revised European-American Lymphoma and the upcoming WHO classifications (26, 27). All cases were reviewed independently by three of the authors (M. K., L. Q.-M., and F. F.). The cases had been thoroughly characterized by paraffin section immunophenotyping. In addition, all cases of MCL had been examined by PCR for the presence of a t(11;14) translocation with primers directed against the MTC (28). Some of the cases have been reported previously as part of another study (29). In particular, 24 cases of MCL, 24 HCLs, 23 MMs, 10 B-CLLs, 9 FLs, and 5 MALT lymphomas were included in the study. In 3 cases of MCL, both LN and involved BM trephine biopsies were studied, whereas in the remaining 21 cases, only LN specimens (14 cases), BM biopsies (5 cases), or a gastrointestinal or a liver biopsy were available (1 case each). The 24 cases of HCL consisted of 2 splenectomy specimens, and

the remainder were BM biopsies. In all 23 cases of MM, undecalcified tissue obtained at surgery from large osteolytic bone lesions was available. A BM biopsy obtained in parallel was studied additionally in one case. All BM samples had been formalin (4%)-fixed (pH 7.4) for at least 24 h and decalcified with buffered EDTA (pH 7.0) for 48 h. As controls, formalin-fixed tissues of seven LNs, a normal spleen, and a BM biopsy with reactive changes were analyzed. Matching fresh frozen material of a LN with reactive changes, two cases of CLL and two MCL specimens were selected to assess the influence of fixation.

Cell Lines. In addition to the primary cases, a panel of three myeloid cell lines (HL-60, K562, and THP-1), six lymphoid cell lines (Jurkat, SUDHL-4, SUDHL-10, Ki-JK, NCEB, and Granta 519), and the A431 squamous carcinoma cell line was investigated. All cell lines, except A431 cells, which were grown as described previously (24), were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 10 units of penicillin, and 10 units/ml streptomycin (Life Technologies, Inc.). Formalin-fixed cell pellets from the Granta 519 cell line were prepared as follows: suspended cells were spun at $550 \times g$ for 5 min, and the supernatant was removed. The pellet was resuspended in PBS containing Ca^{2+}/Mg^{2+} and spun again for 5 min at $550 \times g$. After the supernatant was removed, three drops of human plasma were added directly into the pellet and mixed, thereafter, three drops of thrombin were added and left for 1 min. The resulting clot was fixed overnight in 4% formalin and embedded in paraffin.

IHC. Seventy-three of the 95 cases were stained for cyclin D1 (clone P2D11F11; Novocastra, Newcastle, United Kingdom; dilution, 1:10), CD20 (Dako, Copenhagen, Denmark; dilution, 1:500), and polyclonal CD3 (Dako; dilution, 1:200). Moreover, in selected cases, stains for CD10 (Novocastra; dilution, 1:10), CD5 (clone 4C7; Novocastra; dilution, 1:50), CD23 (Novocastra; dilution, 1:50), and DBA.44 (30) were accomplished. IHC was performed on an automated immunostainer (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's protocols, with minor modifications (31). After heat-induced antigen retrieval, incubations with the primary antibodies were performed overnight at room temperature. The rest of the procedure was completed on the Ventana immunostainer. To assure the staining quality of cyclin D1, a cyclin D1-positive MCL carrying a t(11;14) translocation was used as control in every experiment. Furthermore, scattered positive endothelial cells served as an internal control. All immunohistochemical stainings were reviewed by three pathologists (M. K., L. Q.-M., F. F.). Because cyclin D1 expression is undetectable in normal lymphatic cells, all unequivocal nuclear staining in tumor cells was classified as positive.

Microdissection and RNA Extraction. Laser microdissection and RNA isolation from formalin-fixed samples were performed on the 104 NHL specimens and 9 normal reactive tissues as described previously (24). The PALM Laser-Microbeam System (P.A.L.M., Wolfratshausen, Germany) was used to microdissect approximately 2000 cells from each sample, unless otherwise stated. Based on our previous experience, 2000 cells render highly reproducible quantitative RT-PCR results and can easily be obtained even from very small biopsies. RNA was isolated by proteinase K digestion followed by

phenol-chloroform extraction and dissolved in 20 μl of RNase-free water. Total RNA from cell lines and microdissected fresh frozen tissues was isolated using the acid-phenol guanidinium method (32).

Real-Time Quantitative RT-PCR. Real-time quantitative RT-PCR analyses were performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Intron-spanning primers and probes for *cyclin D1* and *TBP* as an endogenous reference (the *TBP* gene is coding for the TATA box-binding protein, a component of the DNA-binding protein complex TFIID) were designed using Primer Express software (Applied Biosystems) and synthesized by Applied Biosystems (Weiterstadt, Germany) as follows (designated by the nucleotide position relative to *cyclin D1* GenBank accession number X59798 and *TBP* GenBank accession number X54993): *cyclin D1*-307 sense, 5′-CCGTCCATGCGGAAGATC-3′; *cyclin D1*-376 antisense, 5′-CCTCCTCCTCGCACTTCTGT-3′; *TBP*-645 sense, 5′-GCCCGAAACGCCGAATAT-3′; and *TBP*-717 antisense, 5′-CCGTGGTTCGTGGCTCTCT-3′. The probes *cyclin D1*-331 (5′-CTCGCAGACCTCCAGCATCCAGGT-3′) and *TBP*-664 (5′-ATCCCAAGCGGTTTGCTGCGG-3′) were labeled with 6-carboxy-fluorescein as the reporter and 6-carboxy-tetramethyl-rhodamine as the quencher dye. Ten μl of RNA extracted from microdissected cells or 1 μg of cell line RNA was transcribed into cDNA using Superscript II-reverse transcriptase (Life Technologies, Inc.) and 250 ng of random hexamers (Roche, Penzberg, Germany) following the manufacturer's directions in a final volume of 20 μl. PCR was carried out with the TaqMan Universal PCR Master Mix (Applied Biosystems) using 3 μl of diluted cDNA, 200 nM probe, and 300 nM primers (except *cyclin D1*-307 sense, which was used at 900 nM) in a 30-μl final reaction mixture. For the construction of standard curves with serial dilutions of RNA from fresh as well as formalin-fixed cell pellets from the Granta 519 cell line, RNA was diluted in yeast-RNA (Ambion, Austin, TX) before reverse transcription. After a 2-min incubation at 50°C, AmpliTaq Gold was activated by a 10-min incubation at 95°C. Each of the 50 PCR cycles consisted of 15 s of denaturation at 95°C and hybridization of probe and primers for 1 min at 60°C. Amounts of *cyclin D1* and *TBP* mRNAs were calculated using linear regression analysis from an external standard curve. Final relative cyclin D1 levels of each unknown sample were obtained by division of *cyclin D1* copy numbers by *TBP* copy numbers. As cyclin D1 standard, an expressed sequence tag clone containing portions 27–1258 bp of cyclin D1 cDNA retrieved from GenBank accession number AA156380 was used. For construction of a TBP standard, a 565-bp fragment of TBP was amplified from A431 cDNA using *TBP*-391 sense (5′-CGGGATCCCGTTCAGCAGT-CAACGTCCCA-3′) and *TBP*-955 antisense (5′-GGAATTCCC-AGGAAATAACTCTGGCTCAT-3′) and cloned into the pCRII-Topo vector using the TA Cloning Kit (Invitrogen, Groningen, the Netherlands). After verification of both constructs by sequencing, molecule concentrations were calculated, and serial dilutions of standard DNA ranging from 2.2×10^7 to 220 molecules/reaction were prepared. Reactions were performed in at least two replicates, and all patient samples with SDs > 20% of the final cyclin D1/TBP transcript ratios were retested. To determine a cutoff value for altered cyclin D1 expression in neoplastic lymphoid tissues, the mean value of the cyclin D1:TBP ratio in reactive tissues from BM and LN was determined (mean, 0.84; range, 0.31–1.38). Cyclin

D1:TBP ratios below the value of 2.33 (mean + 5 SDs) were arbitrarily considered to represent low or negative cyclin D1 expression, ratios between 2.33 and 6.98 (3-fold the mean value in reactive LNs and BM) represent intermediate cyclin D1 overexpression, and ratios of 6.98 or more represent strong cyclin D1 overexpression.

Statistical Analysis. Differences in the mRNA levels between the various NHL groups were tested for significance using the Mann-Whitney *t* test with Bonferroni correction for multiple testing. Thus, to assure an overall significance level of $\alpha = 0.05$, between-group differences exhibiting $P < 0.001$ were reported as significant.

RESULTS

Assay Development. To determine cyclin D1 transcript levels in human formalin-fixed tissues, we used a real-time quantitative RT-PCR approach based on TaqMan methodology. TBP mRNA was measured as a reference to normalize cyclin D1 mRNA levels and to compensate for variations in the amount or quality of RNA and cDNA synthesis. Primers and probes were selected to avoid amplification from genomic DNA, and target sequences were kept small (70 and 73 bp) to ensure the detection of fragmented and partially degraded RNA. Standard curves of serially diluted and amplified cyclin D1 and TBP plasmids were linear over 5 orders of magnitude, with a R^2 value of 0.998 for cyclin D1 and 0.994 for TBP. As little as 220 copies of cyclin D1 and 200 copies of TBP could be reliably determined from the standard curve. Similarly, when using serially diluted RNA from the Granta 519 MCL cell line, either from fresh or formalin-fixed paraffin-embedded cell pellets, 2 pg of total RNA could be reliably determined, and the slope of the standard curves did not differ significantly between the two preparations, indicating that the formalin fixation procedure did not have an adverse effect on the assay (Fig. 1*A*). Control experiments confirmed that no amplification occurred when human genomic DNA or RNA prior to reverse transcription was used as a template (data not shown). We then tested the utility of the assay on routinely formalin-fixed, paraffin-embedded clinical samples. Fig. 1, *B* and *C,* shows representative amplification plots of cyclin D1 and TBP (each assayed in duplicate) generated from 2000 microdissected cells of a MCL specimen (Fig. 1*B*) and a normal LN (Fig. 1*C*). In this example, the MCL LN specimen yielded average Ct values of 20.13 and 26.36 for cyclin D1 and TBP, respectively, whereas the normal LN had average values of 27.89 and 26.33, respectively.

Sensitivity and Reproducibility. To determine the sensitivity of the method with regard to cell numbers from formalin-fixed tissue sections in more detail, we performed real-time RT-PCR after microdissection of defined numbers of cells. Twenty, 150, 1,000, and 10,000 cells were isolated from a MCL LN specimen and from a normal LN and subjected to real-time RT-PCR analysis. Cyclin D1 transcripts were detectable in 20 microdissected cells of the MCL specimen. Cyclin D1:TBP transcript ratios remained stable regardless of the number of microdissected cells (Fig. 2*A*). In the normal LN specimen, cyclin D1 could only be detected when using at least 150 microdissected cells as a template, and cyclin D1:TBP copy ratios were approximately 30 times lower than those in MCL.
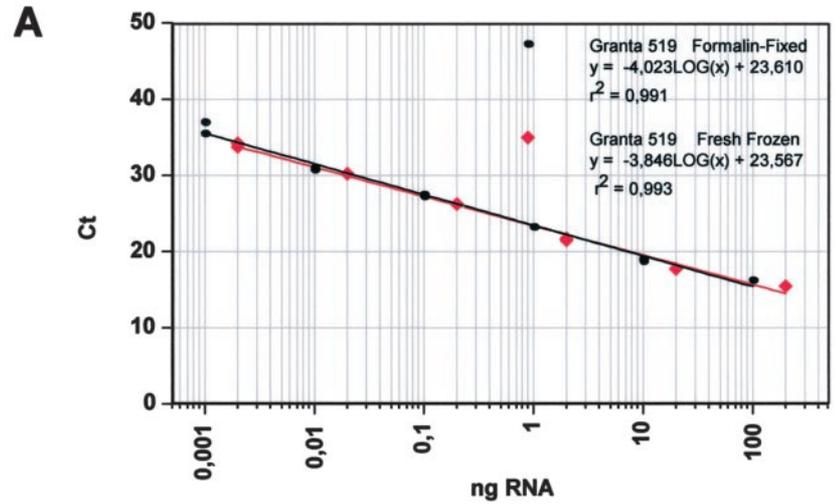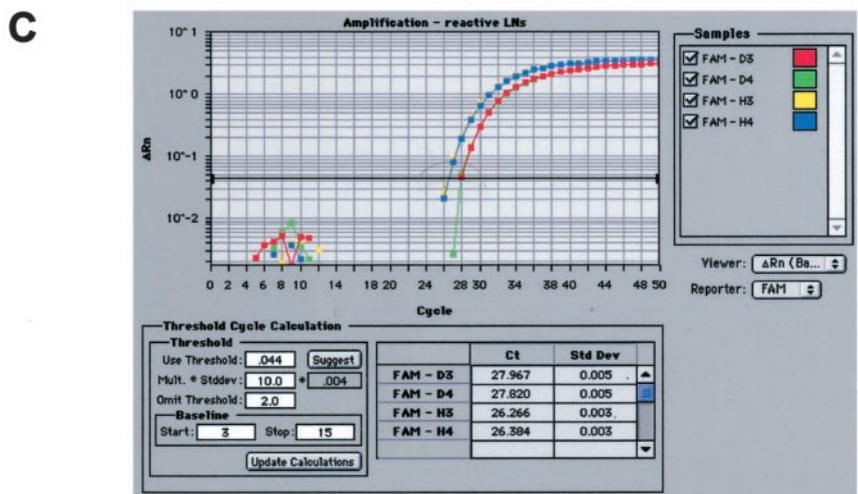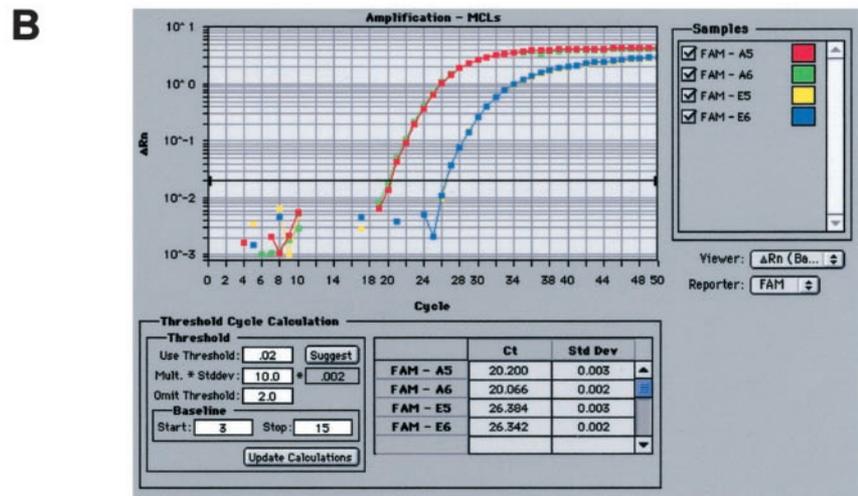
Fig. 1 Cyclin D1 mRNA quantitation by real-time RT-PCR. *A,* cyclin D1 standard curves generated by plotting the Ct values of serially diluted RNAs from fresh (*red*) and formalin-fixed, paraffin-embedded (*black*) cell pellets from the Granta 519 MCL cell line. The slopes of the standard curves do not differ significantly, indicating similar reaction efficiencies from both RNA preparations. *B* and *C,* duplicate cyclin D1 (*red* and *green signal*) and TBP (*yellow* and *blue signal*) amplification plots from a formalin-fixed MCL case (*B*) and a reactive LN (*C*). Note the low Ct value for cyclin D1 indicating strong overexpression in the MCL case as compared with the reactive LN.
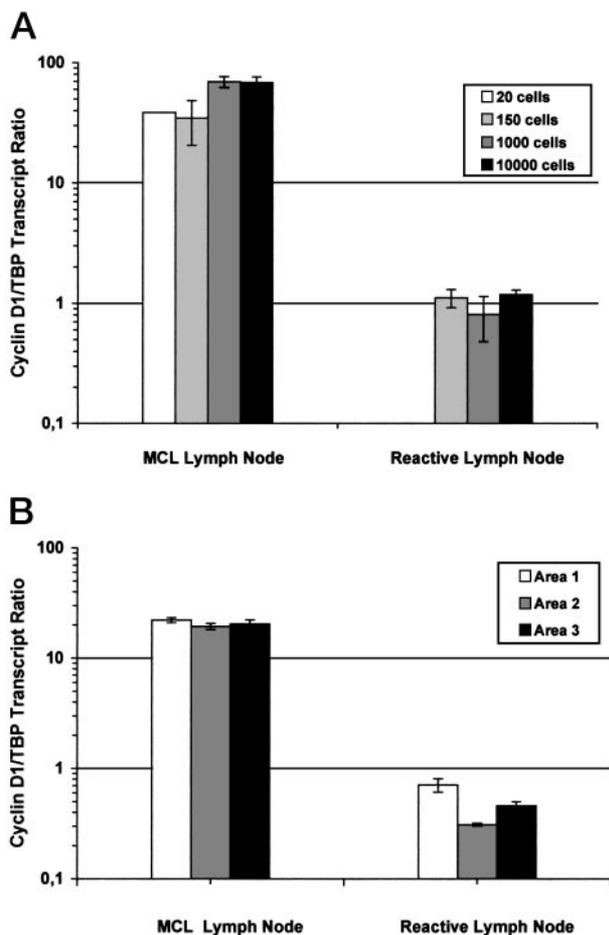
Fig. 2 Sensitivity and reproducibility of real-time RT-PCR for cyclin D1 expression in formalin-fixed tissues. *A,* cyclin D1:TBP transcript ratios in different amounts of microdissected cells from a 4-μm section remain stable. As few as 20 cells from a MCL can serve as template for the reaction, whereas in the reactive LN, at least 150 microdissected cells are necessary for the analysis. *B,* the reproducibilty of the assay was tested by microdissecting and analyzing in duplicate three different areas from a section of a MCL case and a reactive LN, respectively.

To determine the reproducibility of the whole assay, 2000 cells of a formalin-fixed normal LN and 2000 cells of a MCL LN specimen, respectively, were microdissected in triplicate and analyzed in duplicate. Intra-assay (within-run) precision was determined by calculating the mean, SD, and CV of the cyclin D1:TBP ratios and the Ct values for the six cDNA preparations. Mean cyclin D1:TBP ratios were 20.67 ± 1.15 for the MCL and 0.49 ± 0.16 for the reactive LN; thus, the cyclin D1:TBP copy ratios remained approximately constant in each of the three preparations (Fig. 2*B*). The CVs of the corresponding Ct values ranged between 0.4% and 2.9%. Inter-run variability analysis was performed with RNA isolated from 10 different cases of NHL. The CVs of the Ct values obtained on two different days varied between 3.5% for cyclin D1 and 2.8% for TBP.

Quantitation of Cyclin D1 mRNA in B-NHL in Normal Lymphoid Tissue. We next analyzed the cyclin D1 mRNA expression levels in 95 cases of B-cell lymphomas. Under the
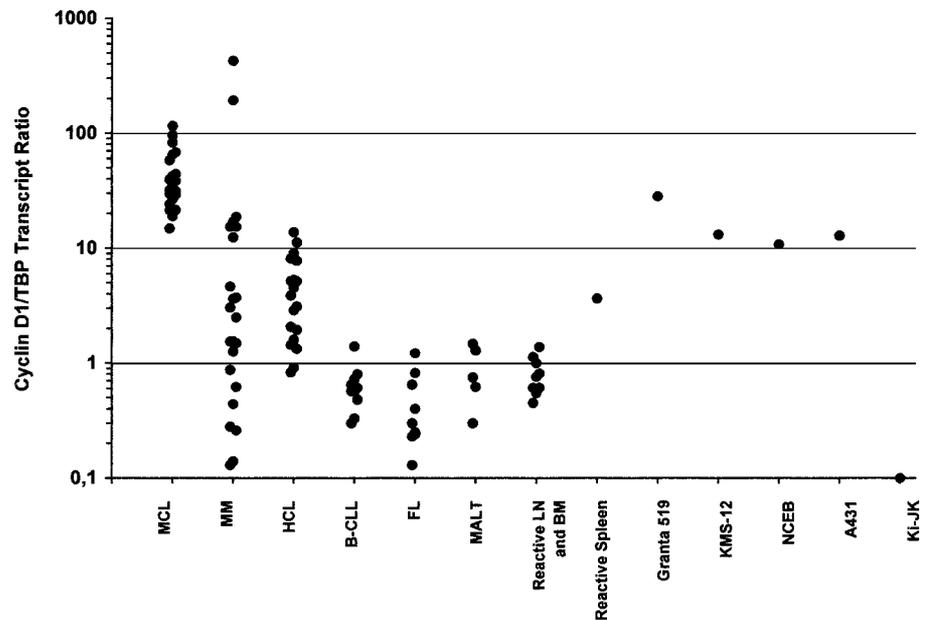
chosen experimental conditions, using 2000 microdissected cells as starting material, 90 of 95 (94.7%) cases of B-NHL rendered reproducible results. Six of 32 (18.8%) BM biopsies (5 HCLs and 1 MCL) failed to amplify. High cyclin D1 transcripts levels were observed in 23 of 23 (100%) MCLs (Fig. 3). No major differences in relative cyclin D1:TBP expression levels were observed in cases when different tissue samples (LN and BM) or multiple LN specimens from the same case were examined, although we noted that the Ct values for both cyclin D1 (Ct range, 25–29) and TBP (Ct range, 30–34) from BM biopsies were higher than those from LN specimens (cyclin D1 Ct range, 19–23; TBP Ct range, 26–28). Median Cyclin D1: TBP transcript ratios in MCLs (range, 14.8–129.6; median, 31.93) were significantly higher than those in all of the other NHLs and reactive tissues included in this study (Table 1). Median Cyclin D1:TBP transcript ratios were as follows: (*a*) MMs, range, 0.13–478; median, 2.5; (*b*) HCLs, range, 1.6–15.3; median, 3.87; (*c*) CLLs, range, 0.26–1.56; median, 0.59; (*d*) FLs, range, 0.12–1.22; median, 0.3; (*e*) MALT lymphoma, range, 0.3–1.48; median, 0.75; and (*f*) reactive LN and BM tissues, range, 0.31–1.38; median, 0.84 (Mann-Whitney nonparametric *U* test, $P < 0.001$). Whereas cyclin D1 transcript levels in MCLs were uniformly high, they showed very strong variations in MM. In two cases of MM, cyclin D1 mRNA levels were reproducibly approximately three times higher than the MCL case displaying the highest cyclin D1 level. In the 19 amplifiable cases of HCL, the median cyclin D1 level was at least 10-fold lower than that in MCL but was still significantly higher when compared with CLL, FL, and reactive tissues. Interestingly, cyclin D1 levels in normal spleen tissue were three times higher when compared with reactive LN and BM tissues, possibly due to the high number of sinusoidal endothelial cells that show immunohistochemically detectable expression of cyclin D1. To confirm our previous observations based on other mRNA species that expression level measurements by real-time RT-PCR in formalin-fixed tissues are comparable with those obtained in fresh frozen tissue (24), we also analyzed matching fresh frozen tissues in two MCLs, two CLLs, and a reactive LN and found no significant difference in cyclin D1:TBP transcript ratios, although the corresponding Ct values for both cyclin D1 and TBP were, on average, a value of 1–2 lower than the Ct values resulting from the formalin-fixed cases.

In comparison, cell lines with known t(11;14) (Granta 519, KMS12, and NCEB) or 11q amplification (A431) displayed levels of cyclin D1 that were similar to the formalin-fixed primary MCL cases. In contrast, the three myeloid cell lines (HL-60, K562, and THP-1) and the lymphoid cell lines Jurkat, SUDHL-4, SUDHL-10, and Ki-JK showed no or very low levels of cyclin D1 mRNA (Fig. 3).

Correlation between Real-time RT-PCR and IHC. Cyclin D1 protein expression was examined by immunohistochemical analysis in 73 of the 95 B-NHLs (23 MCLs, 23 MMs, 19 HCLs, and 8 CLLs). The results are summarized in Table 2 and Fig. 4. Twenty-two of 23 cases of MCL showed strong nuclear cyclin D1 staining as expected from this group of B-NHLs in complete concordance with the RT-PCR data. In one of three MCL cases, which was previously shown by genomic PCR to carry the t(11;14), the BM biopsy was repeatedly negative for cyclin D1 staining, whereas the LN showed a positive

Fig. 3 Relative cyclin D1 mRNA levels in formalin-fixed, microdissected specimens of 90 B-NHLs, reactive tissues, and various lymphoid and epithelial cell lines. Real-time RT-PCR analysis of cyclin D1 mRNA was performed relative to the *TBP* housekeeping gene, and results are depicted as the ratio of cyclin D1:TBP transcript numbers. A total of 90 of 95 (94.7%) B-NHLs and 9 of 9 (100%) reactive lymphoid tissues rendered reproducible results using approximately 2000 microdissected cells as template source. Note the high cyclin D1 levels in cell lines with known t(11;14) (Granta 519, KMS12, and NCEB) or 11q amplification (A431), whereas no cyclin D1 expression was found in the Ki-JK anaplastic large cell lymphoma cell line.

Table 1 Cyclin D1 mRNA expression levels in B-NHLs and reactive lymphoid tissues

| Histology | Cases | Ratio of cyclin D1:TBP mRNA expression | | |
		Mean	Range	Median
MCL	23	43.6 ± 25.58	14.84–129.57	31.93[a]
MM	23	31.46 ± 92.55	0.13–478.98	2.5
HCL	19	4.74 ± 3.61	1.6–15.3	3.87[b]
CLL	10	0.65 ± 0.29	0.26–1.56	0.59
FL	9	0.47 ± 0.34	0.13–1.22	0.3
MALT	5	0.89 ± 0.43	0.3–1.48	0.75
Reactive LN and BM	8	0.84 ± 0.3	0.31–1.38	0.79
Reactive spleen	1	3.66 ± 0.45		

[a] $P < 0.001$ when compared with reactive LN, MM, HCL, CLL, FL, and MALT.

[b] $P < 0.001$ when compared with reactive LN, CLL, and FL.

reaction. Nevertheless, real-time RT-PCR showed high cyclin D1 mRNA levels in both samples. The MMs showed intermediate to strong staining in 4 of 23 (17.4%) cases and weak staining in 7 of 23 (30.4%) cases, all of which were positive by RT-PCR, although the level of expression did not always correlate with staining intensity by IHC. In the group of HCLs, 2 of 18 (11%) showed intermediate to strong staining, and these 2 cases also had high mRNA levels. Another six cases of HCL showed weak staining. However, three of these had high cyclin D1 levels by real-time RT-PCR, and the remaining three showed intermediate levels. None of the CLL cases were positive for cyclin D1 by IHC, in agreement with the RT-PCR results.

DISCUSSION

In this study, we show that real-time RT-PCR and laser microdissection can be successfully combined for reliable quantitation of cyclin D1 mRNA levels in archival formalin-fixed, paraffin-embedded B-NHLs including EDTA-decalcified BM biopsies. Applying the TaqMan technology and using approximately 2000 cells as the standard template source, we found strong overexpression of cyclin D1 in all examined MCLs, 30% of MMs (with particularly high cyclin D1 transcript levels in two cases), and 26% of HCLs. Intermediate cyclin D1 mRNA overexpression was found in 22% of MMs, 37% of HCLs, and in one normal spleen. All other B-NHL subtypes and samples of normal LN and BM universally lacked cyclin D1 overexpression. The high reliability of the technique, the suitability of small amounts of routinely fixed tissue as template source, and the low dropout rate of 6% in our study (all of them decalcified BM biopsies) makes real-time RT-PCR an ideal tool for the detection and quantitation of cyclin D1 in hematological malignancies and potentially also in solid tumors.

D-type cyclins are expressed in a tissue-specific manner, and cyclin D1 is practically absent in lymphoid cells irrespective of the proliferation rate (33–35). Therefore, any significant expression of cyclin D1 mRNA or protein in lymphoid cells is abnormal and can virtually be equated with malignancy, irrespective of the underlying molecular mechanism (7). In MCL and, less frequently, other B-NHLs such as MM characterized by the t(11;14)(q13;q32) translocation, activation of the *cyclin D1* gene and subsequent overexpression of cyclin D1 mRNA and protein are due to the juxtaposition of the *cyclin D1* coding sequences with elements of the immunoglobulin heavy chain locus. Thus far, cyclin D1 status has mainly been examined by means of two methods, FISH and IHC. FISH studies have shown that the *cyclin D1 locus* is rearranged in virtually all MCLs (36) and in 12–18% of MMs (16, 17), but not in HCLs. However, overexpression of cyclin D1 mRNA and/or protein is detected in 50–90% of MCL patients (7), 25–34% of MM patients (16, 37), and 7–100% of HCL patients (12, 13, 20, 22, 38), depending on the methodology used.

In contrast to Northern blot or mRNA *in situ* hybridization

Table 2 Comparison of cyclin D1 mRNA and protein levels determined by real-time RT-PCR and immunohistochemistry in 73 cases of B-NHL

Histology[a]	Cases	Real-Time RT-PCR[b]			IHC[c]			
		−/+	++	+++	−	+	++	+++
MCL	23[d]	0	0	23	0	0	0	22
MM	23	11	5	7	12	7	3	1
HCL	19[d]	7	7	5	10	6	1	1
CLL	10	10	0	0	8	0	0	0

[a] Only the cases with reproducible RT-PCR results are shown.

[b] The scoring was performed as described in "Materials and Methods." +++, strong cyclin D1 overexpression; ++, intermediate cyclin D1 expression; −/+, low or negative cyclin D1 expression.

[c] The following semiquantitative scoring system was applied for assessment of cyclin D1 protein levels: +++ (strong), positive in ≥50% of tumor cells; ++ (intermediate), positive in 25–50% of tumor cells; + (weak), positive in ≤25% of tumor cells; −, negative.

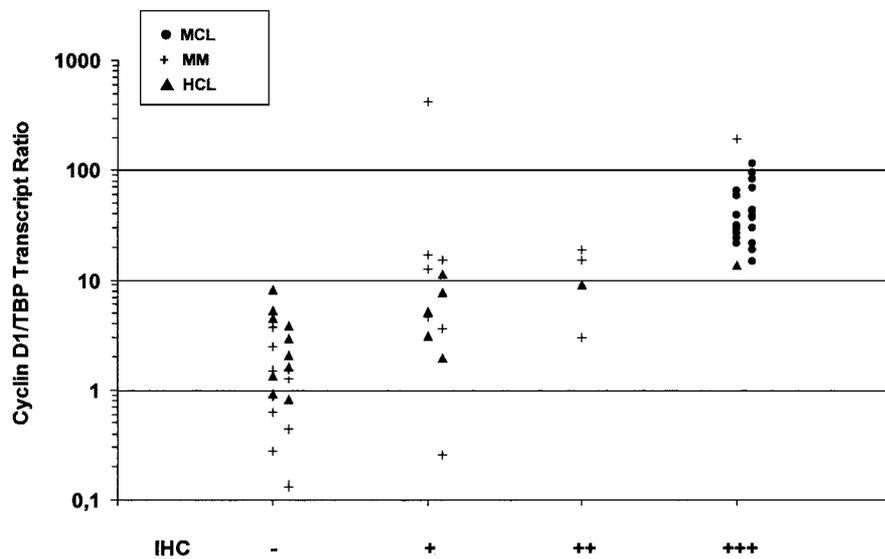[d] One case was not evaluable for cyclin D1 staining.

Fig. 4 Comparison of cyclin D1 mRNA and protein levels. Cyclin D1 levels were determined by real-time RT-PCR (*vertical axis*) and IHC (*horizontal axis*) in 73 cases of B-NHLs. All MCL cases uniformly show strong cyclin D1 mRNA and protein expression. Note that in MM, real-time RT-PCR and IHC give discordant results in two cases. This was even more pronounced in HCL, where IHC was negative in four cases, whereas cyclin D1 mRNA was intermediately overexpressed.

analysis, the recently introduced real-time TaqMan RT-PCR technology is particularly suitable for accurate quantitation of gene expression in minute amounts of tissue and is also applicable to the analysis of formalin-fixed specimens (24, 39). Real-time RT-PCR combines the speed and ease of a PCR-based system with an accurate and reproducible quantitation methodology and therefore has the potential to become a routine diagnostic tool. Importantly, it offers the possibility to screen and quantitate multiple mRNAs from the same cDNA preparation so that the expression level of multiple transcripts can be compared.

The universal overexpression of cyclin D1 mRNA in the MCL cases in our study is consistent with previous reports that used Northern blot analysis or competitive or real-time RT-PCR on frozen tissue for cyclin D1 quantitation (9, 35, 40). All our MCLs displayed cyclin D1 levels 15–120 times higher than reactive LN tissue, irrespective of whether frozen or fixed tissue was used. This is especially noteworthy considering the short half-life of cyclin D1 mRNA (approximately 30 min; Refs. 41–43) but is in accordance with our previous observations, based on other mRNAs, that fixation does not influence quantitative RT-PCR results as long as small amplicons are used

(24). Two recent studies also demonstrated the potential of formalin-fixed, paraffin-embedded tissues for cyclin D1 mRNA detection in MCL by either conventional, semiquantitative, or quantitative RT-PCR (44, 45). Aguilera *et al.* (44) used conventional RT-PCR and found high levels of cyclin D1 in most MCL cases, but they also observed positive results in a significant number of NHLs of other subtypes, indicating that RT-PCR without quantitation is not specific enough for discriminating MCL. However, by applying a semiquantitative RT-PCR assay, they could show that MCL could be distinguished from other NHL cases. Bijwaard *et al.* (45) used real-time RT-PCR for cyclin D1 quantitation and demonstrated that by using an optimal cutoff point for assessing overexpression, the assay could correctly separate 20 MCL cases from 21 non-MCLs. However, compared with our optimized RNA extraction and quantitative RT-PCR assay, the sensitivity of their assay, as judged by Ct values, was considerably lower than that of our approach, although they used serial whole sections. Furthermore, our study differs from these previous investigations in the routine employment of laser-assisted microdissection and the inclusion of MM and HCL, two other neoplasias known to overexpress cyclin D1.

Microdissection not only allows the examination of even

minute clinical samples but increases the purity of the examined sample and reduces the influence of tissue heterogeneity. Both dilution of tumor cells through admixed cyclin D1-negative cells (*e.g.,* in BM biopsies with small nodular NHL infiltrates) and "contamination" with normally cyclin D1-expressing non-lymphoid populations, such as epithelial cells in mucosal biopsies [as noted by Bijwaard *et al.* (45)], may render incorrect quantitative RT-PCR results. Given the excellent results of our study, quantitative RT-PCR compares favorably with IHC, the current gold standard for detection of cyclin D1 in archival tissues. Although the overall concordance with IHC was good, the sensitivity of quantitative RT-PCR was clearly higher. Whereas 12 of our 19 HCL cases showed elevated cyclin D1 levels by quantitative RT-PCR, only 6 of them showed weak immunostaining for cyclin D1 protein in a minority of cells. Furthermore, IHC cannot be quantified and is susceptible to subjective interpretation and variability in fixation and staining conditions.

Another B-cell neoplasia that sometimes shows a t(11;14) translocation is MM. In contrast to MCL, we found a broad range of cyclin D1 expression levels. This was reflected in part by the immunohistochemical results. The frequency of immunopositivity (47%) was slightly higher than the 25–34% reported by others (16, 37, 46). In line with our findings in the other entities, cyclin D1 overexpression detected by IHC was always accompanied by elevated mRNA levels, but the staining intensity did not correlate well with real-time RT-PCR. Two cases showed cyclin D1 transcript levels that were approximately three times higher than those seen in the MCL cases. It is tempting to speculate that this reflects the particular translocation breakpoints that occur in MM. Whereas in MCL, the 11q13 breakpoints are prevalently clustered in a 1-kb region, known as the MTC of the *bcl-1* locus, the breakpoints in MM are widely dispersed, and varying distances between the *cyclin D1* gene and juxtaposed promoter or enhancer elements may lead to different cyclin D1 levels. Only a careful mapping of the specific breakpoints in these cases could clarify this issue. Alternatively, additional mechanisms might contribute to the high cyclin D1 levels in MM, for example, deletions removing destabilizing sequences in the mRNA (47), alternative splicing (48, 49), or aberrant growth factor stimulation. Although the majority of MMs showing high levels of cyclin D1 likely carry a t(11;14), this is not a universal association, and the wide variations in cyclin D1 levels probably reflect an underlying biological heterogeneity. There are conflicting results regarding the frequency of the t(11;14) and cyclin D1 immunopositivity in MM, and there is no consensus on the phenotypic and prognostic consequences of cyclin D1 overexpression (15, 17, 18, 50). Interestingly, Pruneri *et al.* (16) observed that cyclin D1 protein overexpression does not always occur concomitant with t(11; 14). Similarly, Wilson *et al.* (51) speculated that cyclin D1 up-regulation in MM with low or intermediate proliferative activity occurs through t(11;14) but that the cyclin D1 overexpression in highly proliferative MMs occurs independently of t(11;14). The development of the real-time RT-PCR assay is therefore an important tool that in conjunction with strategies to detect and map 11q13 breakpoints might help to elucidate the diverse molecular mechanisms of cyclin D1 overexpression as well as the potential clinical implication.

Another B-cell neoplasia that has been shown to overexpress cyclin D1 is HCL. However, in contrast to MCL and MM, it lacks cytogenetically detectable molecular alterations of the *bcl-1/cyclin D1* locus (12, 13). We found strong cyclin D1 mRNA overexpression in 5 of 19 (26%) HCLs and intermediate cyclin D1 mRNA overexpression in 7 of 19 (37%) HCLs. This observation is in good agreement with other studies based on Northern blot analysis of frozen spleen specimens from HCL patients showing increased cyclin D1 expression in the majority of cases (12, 13). In these two studies as well as our own, median cyclin D1 expression levels were approximately 10 times lower than those observed in MCL.

In summary, quantitative RT-PCR in conjunction with microdissection is an excellent tool for the quantitation of cyclin D1 mRNA in archival tissue specimens. Ultimately, real-time RT-PCR may help to better define the importance of different cyclin D1 expression levels in MCL and MM and to investigate the possible influence of different breakpoints on cyclin D1 expression.

ACKNOWLEDGMENTS

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