

## Cyclin D1 Overexpression Detected by a Simple Competitive Reverse Transcription-polymerase Chain Reaction Assay for Lymphoid Malignancies

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**t(11;14)(q13;q32) observed in B-cell malignancies is associated with cyclin D1 (*bcl-1*, PRAD1, CCND1) overexpression. We devised a simple competitive reverse transcription-polymerase chain reaction (RT-PCR) assay for rapid detection of cyclin D1 overexpression. Sharing a single upstream primer derived from a homologous sequence in cyclins D1, D2 and D3, each PCR product serves as a competitor and cyclin D1 overexpression is determined by comparing the intensities of the three amplified products. We analyzed cyclin D1 in clinical specimens from 104 patients with lymphoid malignancies. Cyclin D1 overexpression was evident in 13 of 104 (7/72 non-Hodgkin's lymphomas, 0/6 adult T-cell lymphoma/leukemias, 0/4 Hodgkin's diseases, 0/11 acute lymphoblastic leukemias, 3/4 multiple myelomas, 1/2 Waldenström's macroglobulinemias, 1/2 prolymphocytic leukemias and 1/3 chronic lymphocytic leukemias). Among 72 patients for whom cytogenetic studies had been done, all 7 patients with t(11;14) were positive. The relative expression levels of D-type cyclins altered dramatically in the presence of t(11;14). Thus, this RT-PCR assay can identify tumors with cyclin D1 overexpression. Cyclin D1 overexpression was frequent in extranodal specimens (11 out of 32 vs. 2 of 72 lymph nodes) and was restricted to specific types of lymphoid malignancies, as observed using other methods. This reliable assay should be suitable to provide clinical guidance for the diagnosis and management of lymphoid malignancies, especially in the case of extranodal involvement.**

Key words: Cyclin D1 — Polymerase chain reaction — Lymphoma — Myeloma — Leukemia

The D-type cyclins (cyclins D1, D2 and D3) bind to and activate cyclin-dependent kinase 4 (or 6) and then the activated kinase phosphorylates the Rb protein and facilitates G1 phase progression toward the S phase.<sup>1, 2)</sup> All three human D-type cyclin genes encode small (33–34 kDa) proteins that share an average of 57% identity over the entire coding region and 78% in the cyclin box.<sup>3, 4)</sup> Among the D-type cyclins, the cyclin D1 gene was identified as a putative oncogene, designated PRAD1.<sup>5)</sup> *bcl-1*, an oncogene at the breakpoint on 11q13 of the t(11;14)(q13;q32) translocation frequently observed in B-cell malignancies, proved to be the cyclin D1/PRAD1 gene.<sup>6)</sup> t(11;14)(q13;q32) and its molecular counterpart, *bcl-1* rearrangement, leads to cyclin D1 overexpression, presumably because of the regulatory segment of the juxtaposed immunoglobulin gene.<sup>6, 7)</sup> These abnormalities were detected in 6% of B-cell lymphomas,<sup>8)</sup> 3% of plasma cell disorders,<sup>9, 10)</sup> 18% of prolymphocytic leukemia (PLL)<sup>11)</sup> and 6% of chronic lymphocytic leukemia (CLL).<sup>8, 12)</sup> Cyclin D1 overexpression was also

noted in many solid tumors with gene amplification on 11q13.<sup>13)</sup>

Mantle cell lymphoma (MCL) has been characterized by histopathology, immunophenotyping, t(11;14)(q13;q32) and cyclin D1 overexpression.<sup>8, 14, 15)</sup> Reliable methods for detection of t(11;14) and/or cyclin D1 overexpression are needed for clinical analysis and management of patients with MCL. Because of a low mitotic index in indolent lymphomas, which have to be distinguished from MCL, chromosome analysis is not always informative, and it is time-consuming. Detection of *bcl-1* rearrangement by classical Southern blot analysis or by polymerase chain reaction (PCR) methods is also hampered by the large area within the *bcl-1* locus in which rearrangement breakpoints can be located.<sup>6, 16–19)</sup> Fluorescence *in situ* hybridization techniques for detection of t(11;14)(q13;q32)<sup>20–23)</sup> and immunohistochemistry for cyclin D1 protein detection<sup>24–27)</sup> appear to be highly diagnostic procedures for MCL.

Recently, we developed a simple assay involving reverse transcription followed by competitive PCR (competitive RT-PCR). This approach allows the detection of cyclin D1 overexpression.<sup>28)</sup> In this assay, a single upstream primer is derived from a homologous region of the sequences in cyclin D1 and other D-type cyclins, cyclins D2 and D3, while

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three downstream primers are specific to their respective D-type cyclins. Because the upstream primer is shared in PCR amplification of the three sequences, each PCR product serves as a competitor and quantification of the target is made by comparing the intensities of the three products. This unique competitive RT-PCR is advantageous in its simplicity, as internal homologous sequences serve as competitors and there is no need for externally prepared competitors. This method is suitable for analysis of clinical specimens, the quality of which is difficult to control. In the present report, we demonstrate the clinical applicability of this competitive RT-PCR, using tissue specimens from patients with lymphoid malignancies.

MATERIALS AND METHODS

**Clinical specimens of lymphoid malignancies** A total of 104 tissue specimens from 104 Japanese patients with lymphoid malignancies was examined. Most of the specimens had been frozen with dimethyl sulfoxide in liquid nitrogen or in a deep-freeze unit at  $-80^{\circ}\text{C}$  until RNA preparation, and some were freshly prepared. These patients were selected from the files of the Fourth Department of Internal Medicine, University of Tokyo, School of Medicine (Tokyo) between December 1985 and July 1996, the Department of Hematology, Showa General Hospital (Tokyo) between March 1992 and February 1996, and the Cancer Institute Hospital (Tokyo) between November 1993 and February 1996 on the basis of the availability of frozen or fresh samples for molecular studies. Five patients had been referred from Nagano Red Cross Hospital (Nagano) and Kurobe City Hospital (Toyama).

Table I. Cyclin D1 Overexpression Detected by Competitive RT-PCR in Lymphoid Malignancies

Disease	No. of patients	Cyclin D1 overexpression
Non-Hodgkin's lymphoma		
B-lineage	55	7
T-lineage	5	0
Unclassified	12	0
Adult T-cell lymphoma/leukemia	6	0
Hodgkin's disease	4	0
Acute lymphoblastic leukemia		
B-lineage	6	0
T-lineage	3	0
Unclassified	2	0
Multiple myeloma	4	3
Waldenström's macroglobulinemia	2	1
Prolymphocytic leukemia	2	1
Chronic lymphocytic leukemia	3	1
Total	104	13

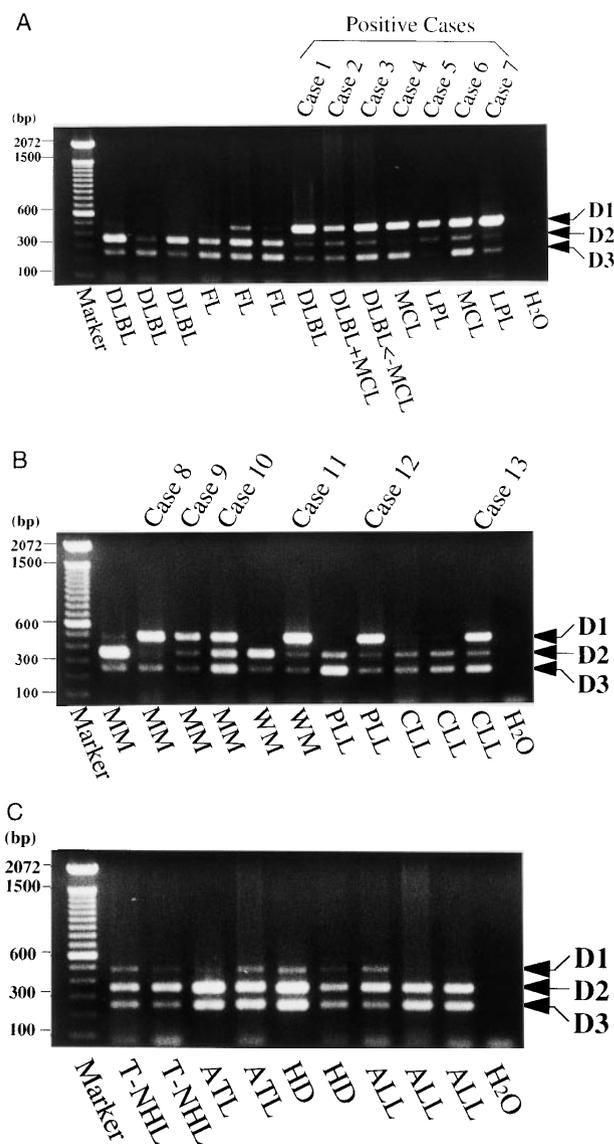


Fig. 1. Competitive RT-PCR analysis of clinical specimens for cyclin D1 overexpression. Representative results of the competitive RT-PCR analysis are shown, including all patients with cyclin D1 overexpression (positive cases 1–13). RNAs were prepared from patients with B-cell lymphoma (A), multiple myeloma (MM), Waldenström's macroglobulinemia (WM), B-cell prolymphocytic leukemia (PLL), B-cell chronic lymphocytic leukemia (CLL) (B), peripheral T-cell lymphoma (T-NHL), adult T-cell lymphoma/leukemia (ATL), Hodgkin's disease (HD), and acute lymphoblastic leukemia (ALL) (C). PCR products were analyzed on 1.2% agarose gels containing ethidium bromide and photographed. Arrows indicate PCR products corresponding to cyclins D1, D2, and D3. DLBL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; DLBL+MCL, diffuse large B-cell lymphoma with mantle cell lymphoma; DLBL<-MCL, diffuse large B-cell lymphoma transformed from mantle cell lymphoma; LPL, lymphoplasmacytoid lymphoma; H<sub>2</sub>O, negative control without cDNA. The sizes (bp) of 100 bp DNA ladders (Marker) are shown on the left.

The patients (59 men and 45 women) ranged in age from 14 to 89 years (median age: 58 years). Tissues consisted of 72 lymph nodes, 16 bone marrow (BM), 9 peripheral blood (PB), 4 pleural effusion, 2 extranodal tumors and 1 spleen. The lymphoid malignancies in these patients are summarized in Table I. Among them, 14 patients (10 non-Hodgkin's lymphoma (NHL) containing 2 MCL and 2 lymphoplasmacytoid lymphoma (LPL), 1 acute lymphoblastic leukemia (ALL), 1 multiple myeloma (MM), 1 CLL, and 1 PLL) were reported in our previous study.<sup>28)</sup> Data on 1 patient with MM<sup>29)</sup> and 1 patient with PLL<sup>30)</sup> were from the cited reports. Eighty-one tumors had been immunophenotyped using flow cytometry of cell suspensions. Cytogenetic analysis had been done for 72 patients, usually without specific mitogens. Results were recorded for 56 patients, while analyzable metaphases were not obtained for 16 patients. Survival was measured from the time of pathologic diagnosis of the disease.

**RNA preparation** Frozen cells were thawed rapidly in a bath prewarmed at 37°C, and RNAs of these cells were extracted by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method,<sup>31)</sup> boiled for 1 min, subjected to optical density measurement, and stored at -80°C. From freshly prepared peripheral blood or bone marrow, mono-

nuclear cells were separated by standard Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density-gradient centrifugation as recommended by the manufacturer. Fresh lymph node cells were prepared by sieving minced lymph nodes. RNAs of these cells were also extracted by the AGPC method.

**Competitive RT-PCR** Competitive RT-PCR was done as described<sup>28)</sup> but with minor modifications. Briefly, cDNA was synthesized with oligo(dT)<sub>15</sub> from total RNA and an aliquot (5 µl, 0.1 µg RNA equivalent) of cDNA was placed in 50 µl of 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, pH 8.3) with 200 µM each deoxyribonucleoside triphosphate, 0.2 µM each primer, and 2.5 U of recombinant Taq DNA polymerase (Takara, Kyoto). Reaction parameters were 30 rounds of 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min. The PCR products (10 µl) were electrophoresed on a 1.2% agarose gel containing ethidium bromide. A photograph of the gel was scanned with a ScanTouch (Nikon, Tokyo) and densitometric analysis was done using NIH Image software. The relative expression level of each D-type cyclin was calculated using the following formula: cyclin D1,  $a/(a+b+c)$ ; cyclin D2,  $b/(a+b+c)$ ; cyclin D3,  $c/(a+b+c)$ ; where  $a$  is a density of the cyclin D1 PCR product,  $b$  is that of the cyclin D2

Table II. Characteristics of the 13 Patients with Cyclin D1 Overexpression

Case No.	Age/ Sex	Disease	Stage	Tissue source	t(11;14)	Surface markers		M protein	RT-PCR relative expression level <sup>a)</sup> of			Survival (months)
						CD5	Immunoglobulins		cyclin D1	cyclin D2	cyclin D3	
1	77/M	NHL DLBL	III	BM <sup>d)</sup>	- <sup>e)</sup>	+	ND	-	0.64	0.20	0.16	40
2	60/F	NHL DLBL+MCL	IV	LN	+	+	IgM, λ	-	0.51	0.26	0.22	5
3	65/M	NHL DLBL<-MCL	IV	LN	+	+	IgM, λ	-	0.59	0.15	0.25	21
4	72/M	NHL MCL	IV	PB	- <sup>e)</sup>	+	ND	-	0.59	0.14	0.27	33
5	79/M	NHL LPL	IV	BM	- <sup>e)</sup>	+	IgM, IgD, λ	IgM-λ, BJP-λ	0.68	0.25	0.07	25+
6	62/M	NHL MCL	IV	PB	+	+	IgM, IgD, λ	-	0.57	0.16	0.27	35+
7	66/F	NHL LPL	IV	BM	- <sup>f)</sup>	-	-	IgG-λ, BJP-λ	0.71	0.16	0.13	18
8	60/M	MM	IIIB <sup>b)</sup>	PE	+	-	-	IgD-λ, BJP-λ	0.71	0.12	0.18	21
9	51/F	MM	IIIA <sup>b)</sup>	BM	+	-	-	IgG-κ, BJP-κ	0.60	0.23	0.17	14
10	79/F	MM	IIIB <sup>b)</sup>	BM	- <sup>e)</sup>	-	-	BJP-κ	0.37	0.30	0.33	8+
11	71/M	WM	-	BM	ND	ND	IgM, IgG, κ	IgM-κ, BJP-κ	0.63	0.21	0.16	3
12	72/M	PLL	-	PB	+	+	IgM, IgD, κ	-	0.73	0.14	0.13	34
13	69/F	CLL	II (A) <sup>c)</sup>	PB	+	+	IgM, IgD, λ	-	0.58	0.15	0.27	42+

Abbreviations: NHL, non-Hodgkin's lymphoma; MM, multiple myeloma; WM, Waldenström's macroglobulinemia; PLL, polyclonal lymphocytic leukemia; CLL, chronic lymphocytic leukemia; DLBL, diffuse large B-cell lymphoma; DLBL+MCL, diffuse large B-cell lymphoma and mantle cell lymphoma; DLBL<-MCL, diffuse large B-cell lymphoma transformed from mantle cell lymphoma; MCL, mantle cell lymphoma; LPL, lymphoplasmacytoid lymphoma; BM, bone marrow; LN, lymph node; PB, peripheral blood; PE, pleural effusion; ND, not done; BJP, Bence Jones protein.

a) The relative expression level of each D-type cyclin was calculated as described in "Materials and Methods."

b) Durie & Salmon.

c) Rai (Binet).

d) BM involvement was evident at recurrence.

e) Clonal cytogenetic abnormalities were not evident.

f) *bcl-1* rearrangement was evidenced by Southern blot analysis (data not shown).

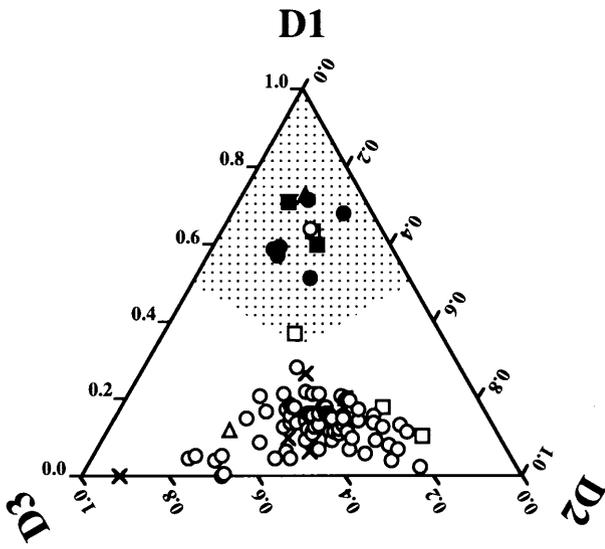


Fig. 2. Relative expression levels of D-type cyclins in clinical specimens from patients with lymphoid malignancies plotted in a diagram. Relative expression levels of D-type cyclins calculated using the formula described in "Materials and Methods" are plotted for each patient, as follows: 0–1.0 cyclin D1 from base to apex, 0–1.0 cyclin D2 from left side to right vertex, and 0–1.0 cyclin D3 from right side to left vertex. Each symbol denotes the following: open circle (○), NHL, ATL, and HD; closed circle (●), NHL with t(11;14) or *bcl-1* rearrangement and MCL; cross mark (×), ALL; open square (□), MM and WM; closed square (■), MM with t(11;14); open triangle (△), CLL and PLL without t(11;14); closed triangle (▲), CLL and PLL with t(11;14). Cyclin D1 overexpression is defined as the relative expression level of cyclin D1 exceeding those of cyclins D2 and D3, which is shown as a dotted area.

product and *c* is that of the cyclin D3 product. Cyclin D1 overexpression was considered positive when the relative expression level of cyclin D1 exceeded those of cyclins D2 and D3.

**Screening of peripheral blood and bone marrow aspirates** We analyzed 103 PB samples and 34 BM aspirates from consecutive patients in the outpatient clinic and in the Department of Hematology in the Branch Hospital, University of Tokyo, School of Medicine. The 103 patients for PB screening included 51 men and 52 women ranging in age from 20 to 84 years (median age: 60 years). There were 13 with active NHL, 2 with adult T-cell lymphoma/leukemia (ATL), 8 with myelodysplastic syndrome, 2 with primary amyloidosis, 8 with myeloproliferative disorders, 1 with CLL, 1 with carcinoid, 1 with signet ring cell carcinoma of the stomach, and 1 with colon cancer; the remaining 66 patients had no active neoplastic disease. The 34 patients for BM screening included 25 men and 9 women ranging in age from 20 to 82 years (median age: 63 years). In 22, there was no BM involvement of neoplastic disease.

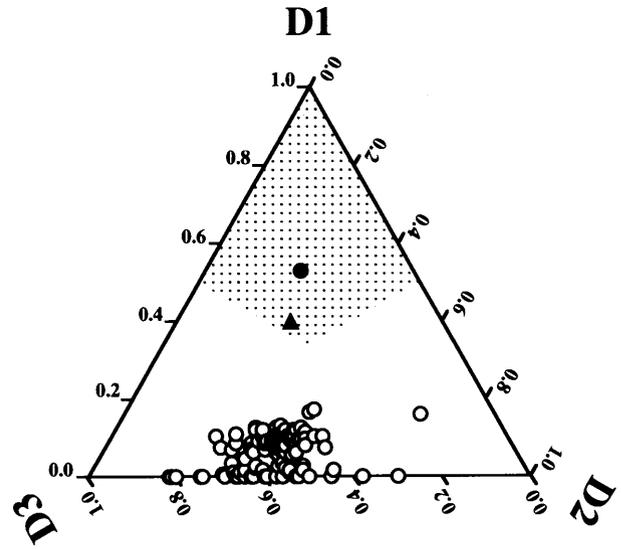


Fig. 3. Screening of peripheral blood from 103 patients for cyclin D1 overexpression. RNAs extracted from whole blood (100  $\mu$ l each) were subjected to the competitive RT-PCR assay and densitometric analysis, as described under "Materials and Methods." Relative expression levels of D-type cyclins are plotted for each patient, as described in the legend to Fig. 2. Each symbol denotes the following: closed circle (●), leukemic lymphoplasmacytoid lymphoma with *bcl-1* rearrangement (case 7); closed triangle (▲), leukemic mantle cell lymphoma with t(11;14) (case 6); open circle (○), other patients.

One-sixth of RNA derived from 100  $\mu$ l of peripheral blood or from 50  $\mu$ l of a bone marrow aspirate was subjected to RT in a volume of 20  $\mu$ l, and 5  $\mu$ l of the reaction products was subjected to PCR in a volume of 50  $\mu$ l. Clinical specimens used in this study were collected after written informed consent had been obtained. Analysis of 20 patients for PB screening and 4 patients for BM screening was reported in our previous paper.<sup>28)</sup>

RESULTS

**Competitive RT-PCR analysis of lymphoid malignancies** As shown in Table I, cyclin D1 overexpression was detected using the competitive RT-PCR assay in 13 of the 104 patients examined. Seven of the 72 NHLs (Fig. 1A), 3 of 4 MMs, 1 of 2 Waldenström's macroglobulinemias (WM), 1 of 2 PLLs and 1 of 3 CLLs (Fig. 1B) were clearly positive. None of the ATL, Hodgkin's disease (HD) or ALL specimens showed overexpression of cyclin D1 (Fig. 1C). All positive patients had B-cell malignancies (Table II). Interestingly, cyclin D1 overexpression was frequent in extranodal specimens, especially bone marrow and peripheral blood. Only two of 72 lymph nodes (2.8%) versus 11 out of 32 extranodal specimens (34.4%) were positive. The

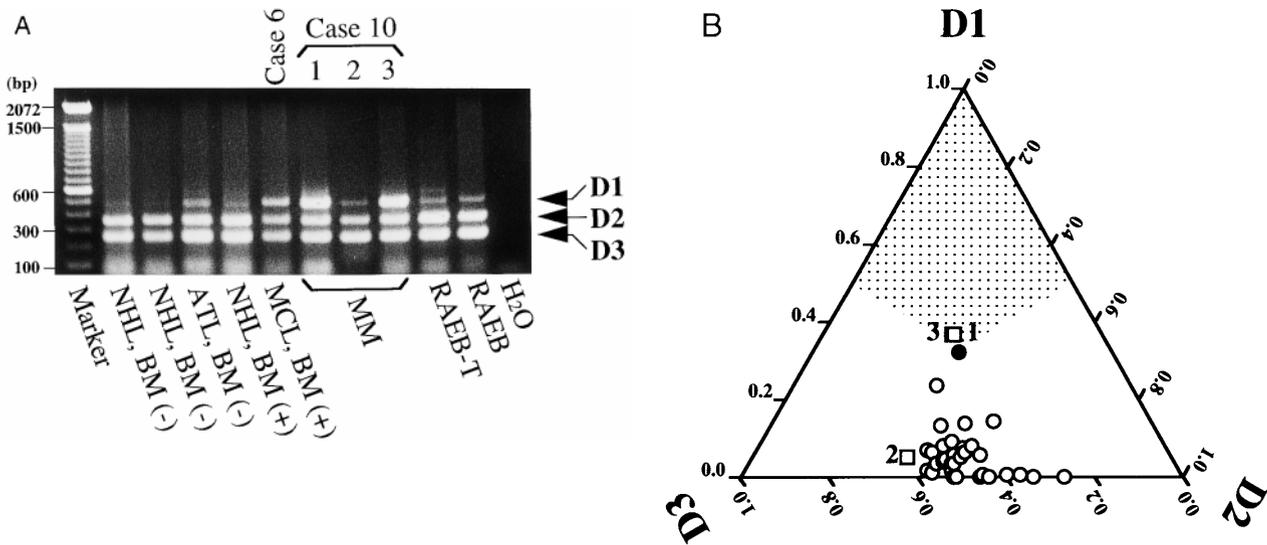


Fig. 4. Screening of bone marrow aspirates from 34 patients for cyclin D1 overexpression. A, RNAs extracted from aliquots (50  $\mu$ l each) of bone marrow aspirates were subjected to the competitive RT-PCR. Arrows indicate PCR products corresponding to cyclins D1, D2, and D3. Bone marrow aspirates from a myeloma patient (MM, case 10) were sequentially analyzed: Lane 1, at diagnosis; lane 2, after chemotherapy; lane 3, at the time when tumor regrowth was seen. Case 6 is a patient with mantle cell lymphoma (MCL). BM(+), bone marrow involvement; BM(-), no involvement of bone marrow; RAEB, refractory anemia with excess of blasts; RAEB-T, RAEB in transformation; H<sub>2</sub>O, negative control. The sizes (bp) of 100 bp DNA ladders (Marker) are shown on the left. B, Relative expression levels of D-type cyclins are plotted in a diagram. Relative expression levels of D-type cyclins calculated as described in "Materials and Methods" are plotted for each specimen as described in the legend to Fig. 2. Each symbol denotes the following: open square, case 10 at diagnosis ( $\square$  1), after chemotherapy ( $\square$  2), and at tumor regrowth ( $\square$  3); closed circle ( $\bullet$ ), case 6; open circle ( $\circ$ ), other patients.

result of cytogenetic analysis was definite for 56 patients, including 35 NHLs, 4 ATLs, 1 HD, 8 ALLs, 4 MMs, 2 PLLs, and 2 CLLs. t(11;14)(q13;q32) was detected in 7 patients (3 NHLs, 2 MMs, 1 PLL, and 1 CLL) while 6 patients with NHL had t(14;18)(q32;q21). Cyclin D1 overexpression was detected in all 7 patients with t(11;14) and in none of those with t(14;18)(q32;q21).

Among NHLs with cyclin D1 overexpression, 2 patients had typical MCL and 3 patients had a diffuse large B-cell lymphoma (DLBL) classified using the revised European-American classification of lymphoid neoplasms.<sup>32)</sup> Two were associated with MCL, sequentially or simultaneously. These DLBL cases were CD5-positive lymphomas and should be large-cell or blastic variants of MCL.<sup>15)</sup> LPL with M-protein was also a distinct type of lymphoma associated with cyclin D1 overexpression, as noted by other workers.<sup>33, 34)</sup> Almost all patients with cyclin D1 overexpression, including those with multiple myeloma, had advanced disease at the time of diagnosis. The median survival of all positive patients was 21 months from the time of diagnosis.

All patients are mapped according to the relative expression levels of D-type cyclins in the diagram shown in Fig. 2. Cyclin D1 expression is unequivocally predominant

in t(11;14)(q13;q32)-bearing B-cell malignancies and/or MCLs (closed symbols in Fig. 2). Major D-type cyclins expressed in other lymphoid malignancies are cyclins D2 and D3, with slightly higher expression levels of cyclin D2 in most of the patients.

**Screening of peripheral blood and bone marrow aspirates** To test the specificity of the assay, we examined PB and BM samples of consecutive patients in the Branch Hospital. In the screening of PB samples, two out of 103 patients were positive: one was a leukemic LPL with *bcl-1* rearrangement (the same patient as case 7 in Table II) and the other was a leukemic MCL with t(11;14) (case 6). Because of the low expression levels of cyclin D1 in all other PB samples, as shown in Fig. 3,<sup>28)</sup> these two patients were easily identified. One patient with MM (case 10 in Table II) was positive in the screening of BM aspirates (Fig. 4). Because her chromosome analysis was not informative and we could not exclude a possible false-positive result, we sequentially analyzed her BM. The first aspirate at diagnosis, in which myeloma cells accounted for 52.7% of all nucleated cells, was determined to be positive. The second one obtained after chemotherapy, in which the proportion of myeloma cells was 6%, was negative. The third aspirate taken at recurrence showed 70% myeloma cells and was

positive; thus cyclin D1 overexpression was attributed to myeloma cells. An MCL patient with BM involvement (case 6) could not be considered positive, according to our criteria, probably because the proportion of lymphoma cells in the analyzed BM specimen was small. Therefore, no false positivity was found in PB samples or in BM aspirates.

## DISCUSSION

We detected cyclin D1 overexpression in all the patients with t(11;14), using our competitive RT-PCR assay. Some patients without t(11;14) showed overexpression of cyclin D1, according to our criteria. These observations suggest that the potential for detection with this assay is better than with conventional chromosome analysis, because other mechanisms than t(11;14) could lead to cyclin D1 overexpression and/or because chromosome analyses were not informative, probably due to low proliferative rates in these patients or to contamination with normal cells. The assay can be done more rapidly and is less tedious than northern blot analysis and chromosome analysis. This method has higher rates of detection compared to classical Southern blot analysis and PCR methods for the detection of *bcl-1* rearrangement.<sup>6, 16-19</sup> Rapid and reliable detection of cyclin D1 overexpression with this assay would be a useful complement to fluorescence *in situ* hybridization techniques,<sup>20-23</sup> which are sensitive methods for detection of t(11;14)(q13;q32), and immunohistochemistry for cyclin D1 protein detection,<sup>24-27</sup> which appears to be highly diagnostic.

The competitive RT-PCR assay in the present study may show false-positives when tumor cells are mixed with normal cells that express cyclin D1 more than cyclins D2 and D3. However, as shown above, in both PB and BM, relative expression levels of cyclin D1 never exceeded those of cyclins D2 and D3 unless tumor cells overexpressing cyclin D1 were included. Therefore, a leukemic state or BM involvement in NHL is an appropriate situation for application of this assay with high specificity. In fact, MCL is frequently associated with extranodal involvement.<sup>15</sup> Furthermore, cyclin D1 overexpression was detected (11 out of 32) in extranodal specimens, especially PB and BM, more frequently than lymph nodes (2 of 72). Although there was some selection bias, our observations suggest direct or indirect association of cyclin D1 overexpression with extranodal distribution of tumor cells.

Theoretically speaking, with the expression of cyclins D2 and D3 genes used as internal controls, our assay quantifies the relative expression level of cyclin D1 not the absolute expression level. Therefore, false-positives may occur if cyclins D2 and D3 are downregulated. However, as shown above, in practice, no apparent false-positive was

found and the assay was useful for screening for cyclin D1 overexpression.

The sensitivity of this assay was previously determined so that 50% or more tumor cells are required for definite detection of cyclin D1 overexpression. In the present study, such sensitivity was again confirmed by sequential analysis of the patient with myeloma (case 10). Provided that tumor cells are a major component of a clinical specimen, a small aliquot of the sample is sufficient for detection of cyclin D1 overexpression, using this assay.

Dewald *et al.* reported that t(11;14)(q13;q32) was detected in 3% of plasma cell disorders (MM, plasma cell leukemia, and amyloidosis) and that it was the single most common abnormality.<sup>9</sup> In the current study, 3 of 4 myeloma patients had cyclin D1 overexpression and 2 had t(11;14). One patient was referred because of t(11;14) and two other positive patients might have been selected by chance. However, the reason for the high frequency might be that MM with t(11;14) tends to be aggressive<sup>9, 29</sup> and that the opportunity to collect and store such myeloma cells might have been greater. To our knowledge, no sizable studies on cyclin D1 overexpression in MM have been documented. With our assay, which is suitable for a large series of cases, the incidence of cyclin D1 overexpression could be easily clarified and might turn out to be much higher than the 3% determined by chromosome analysis, which could be hampered by low proliferation in the disease.

The types of lymphoid malignancies determined to overexpress cyclin D1 by our assay were identical with those previously reported for t(11;14)(q13;q32), *bcl-1* rearrangement and/or cyclin D1 overexpression with other methods.<sup>8-10, 12, 19, 34-36</sup> Heterogeneity of B-lymphoid malignancies associated with cyclin D1 overexpression was again confirmed. Thus, detection of cyclin D1 overexpression is not sufficient for the diagnosis of MCL. Relationships among these malignancies, i.e., MCL, LPL, MM, CLL and PLL, remain to be investigated.

Quantitative RT-PCR requires well-controlled conditions and good-quality RNA because of the exponential character of the reaction. Clinical specimens are sometimes difficult to preserve under good conditions and are not appropriate for conventional competitive RT-PCR. However, the present study shows that PCR can be used to detect oncogene overexpression in clinical specimens of variable quality. Competition with internally expressed homologous sequences would make quantitative PCR more reliable and simpler than conventional methods.

In conclusion, the competitive RT-PCR assay we have developed is reliable and provides clinical guidance in the diagnosis and management of lymphoid malignancies, especially in cases of extranodal involvement. It remained to be determined if this assay can detect cyclin D1 overexpression in solid tumors with gene amplification at 11q13.

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