The Positive Nuclear Staining Observed with Monoclonal Antibody against PRAD1/Cyclin D1 Correlates with mRNA Expression in Mantle Cell Lymphoma

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Recently, we produced a monoclonal antibody, 5D4, against the PRAD1/cyclin D1 product and suggested positive nuclear staining to be associated with mantle cell lymphoma (MCL). Now we have further characterized the specificity of this antibody and studied the relation of immunohistochemical detection to PRAD1/cyclin D1 mRNA expression and DNA rearrangement. Immunofluorescence and immunoblotting studies demonstrated the 5D4 antibody to be crossreactive with cyclin D2, but not cyclin D3. On immunostaining, 15 of 19 MCL cases (79%) presented the nuclear staining pattern and PRAD1/cyclin D1 mRNA expression was detected by Northern blot analysis in 12 of 15 MCL cases studied (80%): all cases with the mRNA expression showed the nuclear staining pattern. Southern blot analysis with 11q13 BCL-1 probes detected DNA rearrangements in 8 of 19 MCL cases (42%), all 8 exhibiting PRAD1/cyclin D1 mRNA expression. In 21 lymphoma cases of types other than MCL, neither the mRNA expression nor the nuclear staining were observed, although cytoplasmic staining was often apparent. These results indicated that positive nuclear staining of lymphoma cells by 5D4 antibody reflects PRAD1/cyclin D1 mRNA expression, and showed that this monoclonal antibody has diagnostic value for differentiating MCL from other types of lymphomas.

Key words: PRAD1 — Cyclin D1 — BCL-1 — Malignant lymphoma

The *BCL-1* (B cell leukemia/lymphoma-1) locus at chromosome 11 band q13 region (11q13) is a major translocation cluster site with the immunoglobulin heavy chain gene (*IgH*) at 14q32 in B-cell malignancies.[1] The *PRAD1* which was isolated by Arnold and his colleagues as a proto-oncogene for parathyroid adenomas[2,3] was shown to be the first transcriptional unit at the telomere side of the *BCL-1* locus by gene walking,[4] and we reported that PRAD1 mRNA overexpression in hematopoietic cell lines is concordant with the presence of t(11;14)(q13;q32),[5] shown to be associated with the mantle cell lymphoma (MCL).[6] We also demonstrated that the putative *BCL-1* gene is *PRAD1* by studying an MCL case with the variant translocation t(11;22) (q13;q11) showing overexpression of PRAD1 mRNA and juxtaposition of the *Igλ* gene at exon 5 of the *PRAD1* gene.[7,8] These results demonstrated that the *PRAD1* gene is confined between the translocation breakpoints with the *IgH* and *Igλ* genes, indicating identity with the *BCL-1* gene, because *PRAD1* mRNA is the sole transcriptional unit between these breakpoints.

The *PRAD1* gene was found to encode a 295-amino-acid polypeptide with sequence similarity to cyclins,[9] which was identified as cyclin D1, a member of the G1 cyclin group, playing a key role in cell cycle regulation with cyclin-dependent kinases (CDKs).[10,11] Recently, the gene for the CDK4 inhibitor (*p16/MTS1*), which forms a complex with cyclin D1, has been reported to be frequently mutated or deleted in various human cultured tumor cell lines and also in certain types of patients' tumors,[12,13] suggesting that deregulation of cyclin D1 and related genes plays an important role in the development of malignancies.

MCL is considered to be derived from follicular mantle cells, possessing pan-B-cell markers and CD5, but not CD10,[6] and is essentially identical to the lymphocytic lymphoma of intermediate differentiation of Berard[14] and also to the centrocytic lymphoma in the Kiel classification.[15] PRAD1/cyclin D1 mRNA overexpression was reported to be shown in many MCL,[16–19] whereas it was rarely detected in B-cell lymphoma cases other than MCL.[17,18] Recently, we produced monoclonal antibodies against PRAD1/cyclin D1[20] and suggested positive nuclear staining to be associated with MCL.[21]

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Yang *et al.* also reported a monoclonal antibody against cyclin D1, HD64, and found that only 3 cases out of 15 MCL showed positive nuclear staining, although all MCL demonstrated a positive nuclear signal with polyclonal antibody.[22]

In order to study the discrepancy between HD64 and our monoclonal antibody, 5D4, in the immunohistochemical studies, we carried out a further specificity analysis of 5D4 antibody, and also examined the relation of immunohistochemical staining to PRAD1/cyclin D1 mRNA expression and DNA rearrangement in various types of lymphomas.

MATERIALS AND METHODS

Tumor samples Nineteen cases of MCLs were analyzed. Each case was classified without knowledge of the molecular results based on the histologic criteria previously described by Lennert and Feller.[15] Other types of malignant lymphomas including 6 follicular lymphomas (FL), 7 diffuse lymphomas (DL), 5 Hodgkin's lymphomas (HD), 2 T cell lymphomas and one large cell anaplastic lymphoma case were also selected and studied. Immunophenotyping was performed either on cell suspensions prepared at the time of biopsy by flow cytometry or on frozen sections by immunohistochemistry.[23] All samples of MCL were positive for CD5 and negative for CD10.
Northern and Southern blot analyses These analyses were performed as described previously.[5, 7] In brief, 5 μg of total RNA for Northern analysis or 10 μg of DNA digested with *Bam*HI, *Pst*I or *Hin*dIII endonuclease for Southern analysis was applied in each lane. For Northern hybridization analysis, sheared human placenta DNA was included in the hybridization solution at the concentration of 100 μg/ml to eliminate crossreactivity with 28 S ribosomal RNA. Probes used for Southern blot analysis are as follows: PRAD1 cDNA probes (a mixture of fragments from overlapping cDNA clones, λP1-4, λP1-5 and λP2-3), kindly provided by Dr. A. Arnold (Massachusetts General Hospital, Boston, MA),[9] BCL-1 MTC probe (*Sac*II/*Sac*II 2.1 kb), a gift from Dr. Y. Tsujimoto (Osaka University School of Medicine)[1] and p94PS (*Pvu*II/*Sma*I 460 bp) that lies approximately 23.4 kb 5′ of the BCL-1 MTC, kindly provided by Dr. T. Meeker (University of California, San Francisco, CA).[24] DNA rearrangements at the 11q13 region were studied with PRAD1 cDNA, BCL-1 MTC, and p94PS probes. PRAD1/cyclin D1 mRNA expression was studied with a PRAD1 cDNA probe, a 0.9 kb *Pst*I/*Hin*dIII fragment of λP1-4 containing a part of the open reading frame. Cyclin D2 and D3 expression was also studied using respective cDNA probes. For comparison, 24-h exposure films were used for densitometric analysis. Densitometric scanning of autoradiographic signals was performed

using a Shimadzu CS-930. The level of PRAD1 mRNA expression was evaluated based on comparison with PRAD1 expression in KMS-12-BM (+++) and Raji (−). The PRAD1 mRNA signal of patient 6 was scored as (+). Based on the PRAD1 signal intensity, signals for cyclin D2 and cyclin D3 were also scored.
Serological analysis The antibody used for immunostaining was the IgG2a monoclonal antibody, 5D4, prepared against recombinant human PRAD1/cyclin D1.[20] To analyze further the specificity of 5D4 antibody, we used a COS7 cell transient transfection system.

Human and mouse PRAD1/cyclin D1, D2, and D3 were expressed using a pCXN2 vector containing the SV40 ori and β-actin promoter[25] (kindly provided by Dr. J.-I. Miyazaki, University of Tokyo). COS7 cells grown on glass coverslips in 6 cm dishes (Iwaki, Tokyo) were transfected and used for immunofluorescence staining. For immunoblot analysis, 10 cm dishes were used for growing COS7 cells. Twenty-four hours after plating at 4×10^5 per 6 cm dish or 2×10^6 per 10 cm dish, cells were transfected with 5 to 12 μg plasmid using Lipofectamine (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's protocol. Forty-eight hours after the transfection, cells were processed for immunofluorescence analysis or immunoblot analysis as described previously.[20]

Human and mouse cyclin D cDNAs used in this study were as follows: human cyclin D2, kindly provided by Dr. G. Peters (Imperial Cancer Research Fund Laboratories, London), human cyclin D3, kindly provided by Dr. A. Arnold (Massachusetts General Hospital, Boston, MA), and mouse cyclins D1, D2, and D3, kindly provided by Dr. H. Matsushime (Institute of Medical Science, University of Tokyo).
Immunohistochemical staining Immunostaining was conducted on formalin-fixed paraffin-embedded lymphomas using 5D4 by the indirect immunoperoxidase technique as described previously.[21] Tissue sections were subjected to microwave oven heating,[26] shown to be effective for retrieval of masked epitopes of antigens. Peroxidase activity was detected by incubation with a solution of diaminobenzidine (DAB) and counterstained with hematoxylin. Semi-quantitative estimation of the intensity of nuclear and cytoplasmic staining was based on comparison of the staining intensity of lymphoma cells with that of normal lymphoid organs. Hence, four categories of staining intensity were established; negative (−), weak (+), intermediate (++), and strong (+++). "Nuclear" staining pattern indicates nuclear staining with or without cytoplasmic positivity, while "cytoplasmic" staining corresponds to cytoplasmic localization without nuclear staining. Three pathologists independently evaluated and then agreed on the immunostaining pattern and intensity.
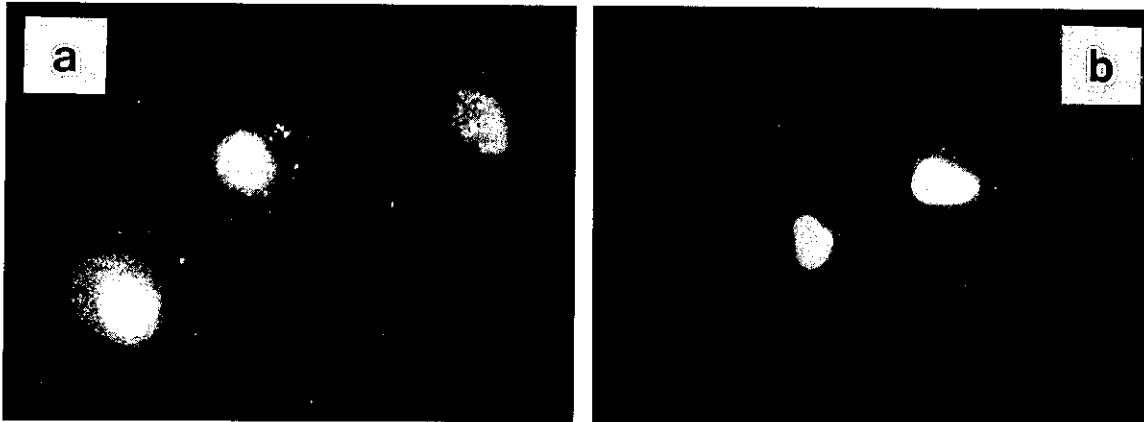
Fig. 1.   Immunofluorescence study of cyclin D1, D2, and D3 transfectants with 5D4 monoclonal antibody. COS7 cells were transfected with cDNAs encoding human or mouse cyclin D and stained with 5D4 after fixation in 70% cold methanol. The nuclear staining pattern was observed for (a) human PRAD1/cyclin D1 and (b) human cyclin D2 transfectants. An untransfected COS7 cell exists in (b), upper-left region. Mouse cyclin D1 and cyclin D2 transfectants also showed the nuclear staining pattern, but human and mouse cyclin D3 transfectants did not show any staining (data not shown). Therefore, 5D4 crossreacts with cyclin D2, but not with cyclin D3. (×330)

RESULTS

Serological specificity of 5D4 monoclonal antibody produced against recombinant human PRAD1/cyclin D1 product   Our previous immunoblotting study showed that 5D4 antibody is reactive with bacterial fusion products of the T7 gene 10-PRAD1/cyclin D1 and GST-PRAD1/cyclin D1.[29] In the present study, not only human cyclin D1, D2, and cyclin D3 products, but also mouse cyclin D products were expressed in a COS7 transient transfection system, and used for specificity analysis. As shown in Fig. 1, positive staining with 5D4 was observed in both human cyclin D1 and human cyclin D2 transfectants, but not in the human cyclin D3 transfectant (data not shown). Mouse cyclin D1 and D2 transfectants also showed positive staining but the D3 transfectant did not (data not shown). The specificity was further confirmed by immunoblot analysis. As shown in Fig. 2, a band with a size of 36 kd was observed in both human and mouse cyclin D1 lanes. In the lanes of human and mouse cyclin D2, a 35 kd band was observed, showing crossreactivity. The antibody, however, was not reactive with cyclin D3 of either human or mouse origin. **Immunohistochemical analysis with 5D4 monoclonal antibody**   Sections of formalin-fixed, paraffin-embedded lymphoma tissues were stained with 5D4 antibody by the indirect immunoperoxidase technique and representative staining patterns are shown in Fig. 3. Seventeen of 19 MCL cases (89%) showed positive staining with intermediate or strong intensity. Fifteen of the 19 (79%) showed the nuclear staining, while the other 4 cases (patients 16, 17, 18, and 19) showed only cytoplasmic
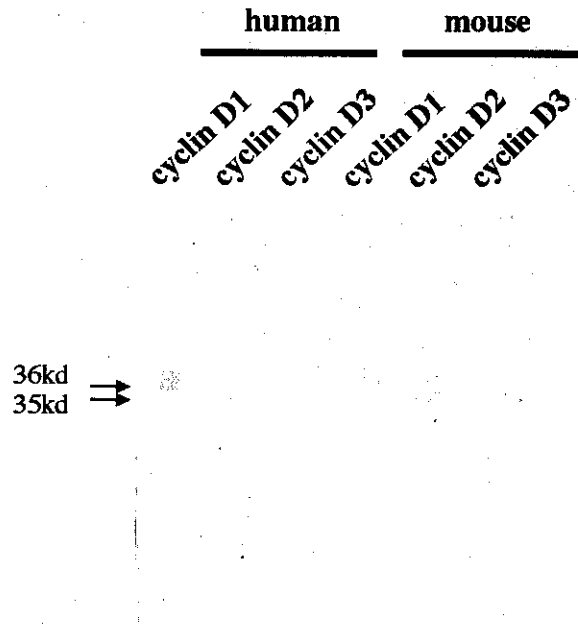

Fig. 2.   Western blot analysis of cyclin D1, D2, and D3 transfectants with 5D4 monoclonal antibody. Human PRAD1/cyclin D1 (36 kd) and cyclin D2 (35 kd) as well as mouse cyclin D1 and D2 were detectable with 5D4, whereas human and mouse cyclin D3 (34 kd) were not.

staining (Table I). In lymphomas of types other than MCL, 9 of 21 cases (43%) showed positive staining with intensities ranging from intermediate to strong (4 of 6 FL cases, 4 of 7 DL cases, 1 of 5 HD, 0 of 3 others),
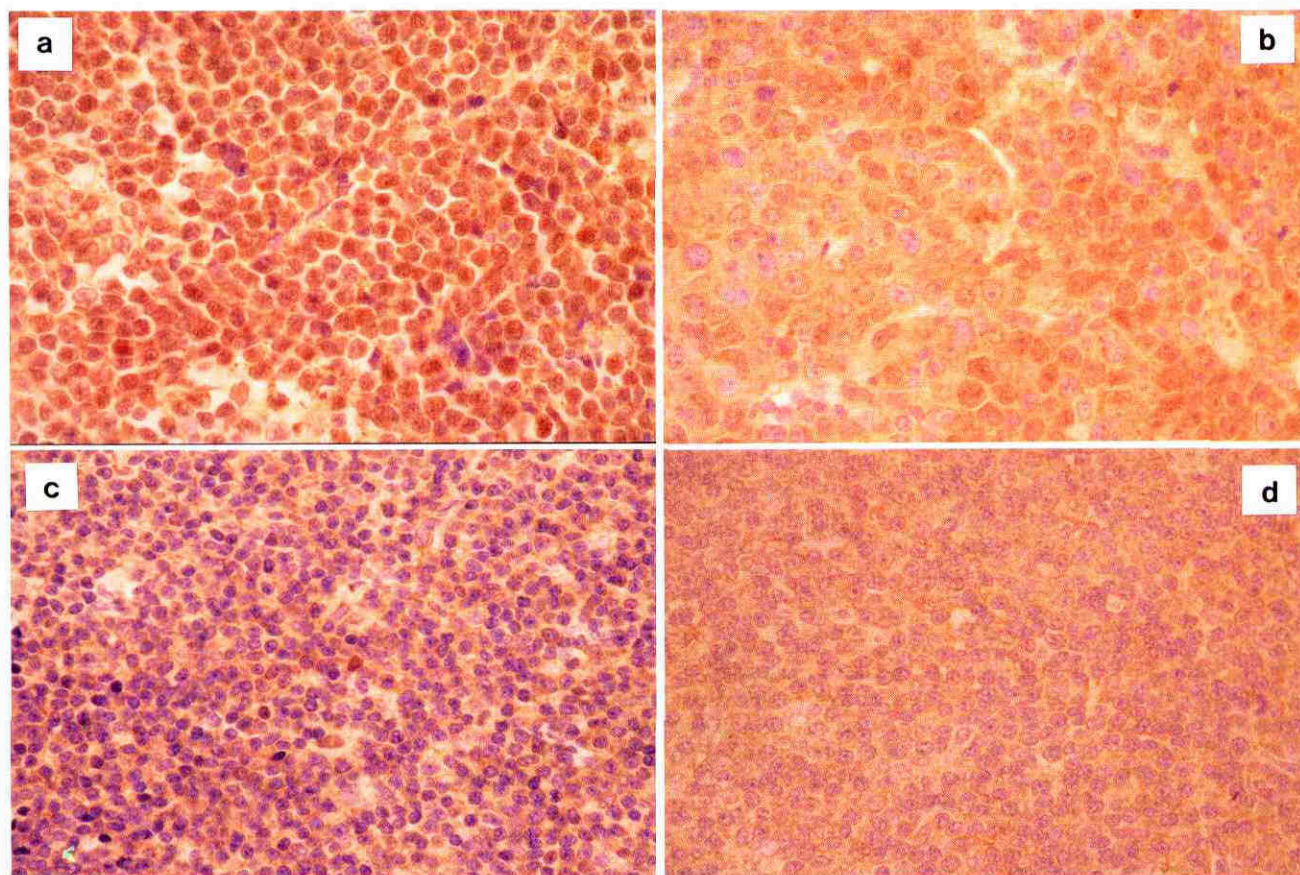
Fig. 3. Immunohistochemical staining of formalin-fixed, paraffin-embedded sections of MCL with 5D4 monoclonal antibody. (a) Patient 3; the majority of the lymphoma cells show strong staining ($+++$) for 5D4, which appears to be predominantly nuclear. (b) Patient 13; the staining intensity is intermediate ($++$) and the expression appears to be both cytoplasmic and nuclear. (c) Patient 14; only a few cells show nuclear staining ($++$). (d) Patient 19; the reactivity appears to be weakly cytoplasmic ($+$) without nuclear staining. (DAB with hematoxylin counterstain, $\times 560$)

strictly limited to the cytoplasm (Table II). Thus, in the tests so far, the nuclear pattern was observed only in MCL cases.

Correlation of immunohistochemical staining to mRNA expression All the cases studied for immunostaining except for 4 MCL cases were available for Northern blot analysis. Twelve of 15 MCL cases (80%) demonstrated PRAD1/cyclin D1 mRNA expression (Fig. 4) and all 12 showed the nuclear staining pattern, ranging in intensity from intermediate to strong (Table I). Two cases without any detectable cyclin D1 mRNA signal (patients 16 and 17) showed no nuclear staining. None of the lymphoma cases other than MCL showed PRAD1/cyclin D1 mRNA expression (Table II). Thus, the correlation between cyclin D1 mRNA expression and the nuclear staining pattern was very good, with only a single exceptional case: patient 14 showed nuclear staining with no detectable mRNA signal. This patient demonstrated in-

termediate intensity with N/C pattern (Table I), but there are only two cells at most with positive nuclear staining as shown in Fig. 3c (less than 0.2%), indicating that the nuclear positivity of this patient is significantly different from that of the other nuclear-positive patients who showed 30 to 70% positivity.

With regard to cyclin D2, none of the lymphoma cases with cyclin D2 mRNA expression (patients 29, 36, 37, and 39) showed nuclear staining; two of the cases (patients 29 and 37) did not show any immunohistological staining and the other 2 patients showed only a weak cytoplasmic pattern (Table II). No correlation was seen between cyclin D3 mRNA expression and immunohistochemical staining (Table II). These results again consistently supported a good correlation between PRAD1/cyclin D1 mRNA expression and nuclear staining. There are patients with cytoplasmic staining but without any detectable cyclin D1, D2, and D3 mRNA, such as pa-

Table I. PRAD1/Cyclin D1 Staining, mRNA Expression and DNA Rearrangement in MCL Cases

Patient No.	Immunostaining		Northern analysis[c]			Southern analysis with probes[d]		
	Intensity[a]	Staining pattern[b]	PRAD1 /cyclin D1	cyclin D2	cyclin D3	MTC	p94PS	PRAD1/cyclin D1 probe mixture
1	+++	N	+++	—	—	—	—	R
2	+++	N	+++	—	—	R	—	—
3	+++	N	+++	—	—	—	R	—
4	++	N	+++	—	—	—	—	—
5	++	N	++	—	—	—	—	—
6	+++	N	+	—	—	R	—	—
7	++	N	+	—	—	R	R	R
8	+++	N	n.d.	n.d.	n.d.	—	—	R
9	+++	N/C	+++	—	—	R	—	—
10	++	N/C	++	—	—	—	—	—
11	++	N/C	++	—	—	—	—	—
12	++	N/C	+	—	—	—	—	R
13	++	N/C	+	—	—	—	—	—
14	++	N/C	—	—	—	—	—	—
15	+++	N/C	n.d.	n.d.	n.d.	—	—	—
16	+	C	—	—	—	—	—	—
17	++	C	—	—	—	—	—	—
18	++	C	n.d.	n.d.	n.d.	—	—	—
19	+	C	n.d.	n.d.	n.d.	—	—	—

a) +++, strong; ++, intermediate; +, weak; —, negative.
b) N, nuclear staining pattern; N/C, nuclear and cytoplasmic staining pattern; C, cytoplasmic staining pattern.
c) mRNA expression after 24-h exposure measured by densitometry (A_{590nm}): +++, 160000–490000; ++, 90000–160000; +, 30000–90000; —, <30000. n.d., not done due to the poor quality of mRNA.
d) Southern analysis was conducted with various probes for the BCL-1 breakpoint region after *Bam*HI, *Pst*I or *Hin*dIII digestion: —, germline: R, rearrangement.

Table II. PRAD1/Cyclin D1 Staining and mRNA Expression in Lymphomas Other than MCL

Patient No.	Diagnosis[d]	Immunostaining		Northern analysis[c]		
		Intensity[a]	Staining pattern[b]	PRAD1 /cyclin D1	cyclin D2	cyclin D3
20	FL	+	C	—	—	+
21	FL	+	C	—	—	+
22	FL	+++	C	—	—	+
23	FL	+++	C	—	—	+
24	FL	++	C	—	n.d.	n.d.
25	FL	+++	C	—	—	++
26	DL	+	C	—	—	—
27	DL	+++	C	—	—	—
28	DL	++	C	—	—	—
29	DL	—		—	+++	—
30	DL	—		—	—	—
31	DL	++	C	—	—	—
32	DL	+++	C	—	—	—
33	HD	++	C	—	—	—
34	HD	+	C	—	—	—
35	HD	—		—	—	—
36	HD	+	C	—	++	+
37	HD	—		—	+	+
38	IBL	—		—	—	—
39	IBL	+	C	—	++	—
40	ALCL	+	C	—	—	—

a), b), c), see footnotes to Table I.
d) FL, follicular lymphoma; DL, diffuse lymphoma; HD, Hodgkin's lymphoma; IBL, large cell immunoblastic lymphoma; ALCL, anaplastic large cell lymphoma.
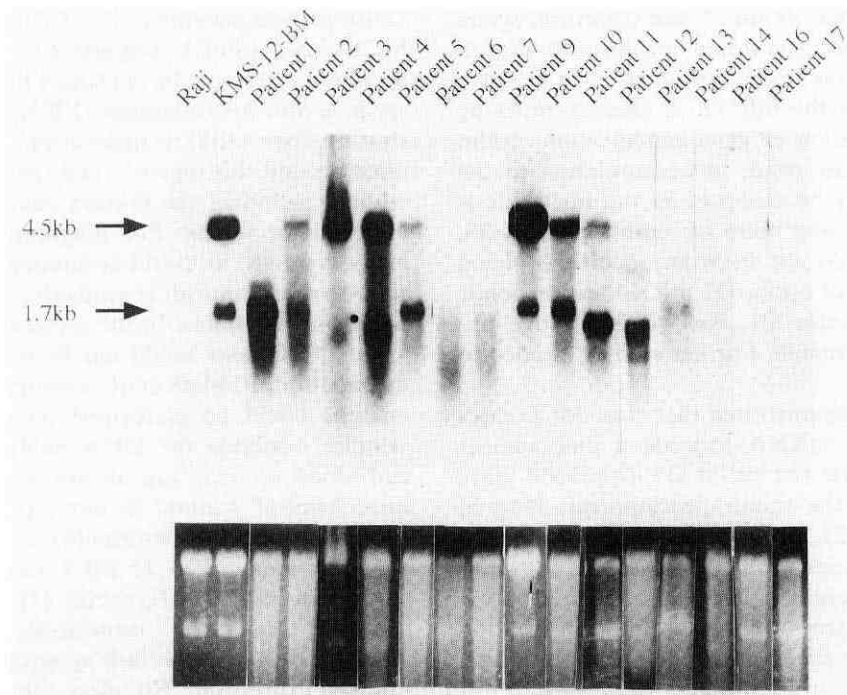
Fig. 4. PRAD1/cyclin D1 mRNA expression in MCL cases. Raji, a Burkitt's lymphoma cell line (as a negative control); KMS-12-BM, a multiple myeloma cell line with t(11;14)(q13;q32) (as a positive control). Two bands of 4.5 kb and 1.7 kb are normal PRAD1/cyclin D1 mRNAs. In 12 of 15 MCL cases, PRAD1/cyclin D1 mRNA expression was detected. In patients 1 and 12, truncated mRNAs are shown (2.5 kb and 1.7 kb for patient 1; 3.4 kb and 1.2 kb for patient 12), possibly caused by gene alterations as described previously.[5, 8] The sizes of mRNA signals of patients 6, 7, and 13 are not clear because of RNA degradation as shown in the ethidium staining of the gel below. Under the conditions employed (see "Materials and Methods"), PRAD1/cyclin D1 mRNA was not detectable in patients 14, 16, and 17.

tients 27 and 32. There is no direct evidence at present, however, to show whether the antibody is detecting cross-reactive molecule(s) in the cytoplasm.

Correlation of PRAD1/cyclin D1 staining pattern and mRNA expression to DNA rearrangement at 11q13 All the MCL cases were also studied by Southern blot analysis using probes for the 11q13 locus; PRAD1/cyclin D1 cDNA probe mixture, BCL-1 MTC, and p94PS probe. DNA rearrangements were detected in 8 of 19 MCL cases (42%) (Table I). All 8 showed the nuclear staining pattern, and 7 of these expressed the PRAD1/cyclin D1 mRNA. RNA was not available from the remaining one case with gene rearrangement (patient 8). Five cases with PRAD1 mRNA expression did not show any gene rearrangement with the probes described above.

DISCUSSION

The present study, involving simultaneous analysis of each lymphoma case for immunostaining, mRNA expression, and DNA rearrangements, demonstrated that nu-

clear staining of lymphoma cells with our monoclonal antibody, 5D4, is a useful marker for differentiating MCL from other types of lymphomas. Yang *et al.* reported that with their polyclonal antibody against PRAD1/cyclin D1, nuclear staining was detected in all of 15 MCL and only one of 28 cases of other lymphoproliferative disorders.[22] They also used PRAD1/cyclin D1 monoclonal antibody, HD64, and found that only 3 of the 15 MCL cases showed nuclear staining, suggesting that their monoclonal antibody has a narrower specificity than ours. Since their HD64 antibody was not crossreactive with cyclin D2, in contrast to the 5D4 antibody, it is possible that some of the positive 5D4 antibody staining was due to cyclin D2. However, this is unlikely because cyclin D2 mRNA expression was not evident in our MCL cases.

Why is this specific nuclear staining pattern observed in MCL cells expressing PRAD1/cyclin D1 mRNA? PRAD1/cyclin D1 protein is known to localize in the nucleus of the cells during G1 phase and translocates to the cytoplasm at the beginning of S phase.[27] It has a very

short half-life of less than 30 min,[28] and therefore, cyclin D1 in normal lymphoid tissue would be difficult to detect. Indeed, we observed no immunostaining in 5 reactive lymph nodes.[20] When the mRNA is overexpressed by chromosome translocation or gene amplification, cyclin D1 overproduction may result in accumulation of the protein so that it may be observed in the nuclei. It is not known at present why there are cases (patients 29, 36, 37, and 39) who do not show any nuclear staining even though the level of cyclin D2 mRNA expression is similar to that for cyclin D1, for which positive nuclear staining was detectable. Further study is needed to clarify this point.

The present study demonstrated that most MCL cases have both cyclin D1 mRNA expression and nuclear staining, suggesting that the cyclin D1 expression plays an important role in the tumor development. Normal counterpart cells of MCL are thought to be located in the follicular mantle zone, and such cells have been shown to be inactive in the cell cycle.[29] The cyclin D1 mRNA expressed under the control of chromosome translocation may thus lead mantle cells to enter the cell cycle, which would bring about cell proliferation.

Another interesting point observed in this study is that the staining intensity was not always directly related to the mRNA expression. In a report concerning another anti-cyclin D1 monoclonal antibody, DCS-6, which is not crossreactive with cyclin D2 or cyclin D3,[27] a clear correlation between the intensity of staining and DNA amplification was found in breast cancers.[30] A preliminary study by our group, however, revealed that DCS-6 antibody (kindly provided by Dr. J. Bartek, Copenhagen) showed nuclear staining in only one case out of 10 MCL found to be nuclear signal-positive with 5D4 antibody, indicating that nuclear positivity with 5D4 antibody correlates well with the mRNA expression in lymphomas. Further comparative specificity analysis of the three monoclonal antibodies so far reported, 5D4, HD64, and DCS-6, is needed to explain the variation in incidences of positive staining and staining pattern in MCL cases. Our cytoplasmic staining detected by 5D4 antibody was not always due to the presence of cyclin D1 or D2 mRNA (for example, patients 27 and 32), suggesting that the antibody is also detecting crossreacting molecule(s) in the cytoplasm other than cyclin D1 and D2. This cytoplasmic staining without mRNA expression remains to be elucidated. Thus, the fact that a correlation does exist between the 5D4 nuclear staining and mRNA expression is particularly significant.

Southern analysis detected DNA rearrangement in 42% (8/19) of the MCL cases, which is in general agreement with the previous reports.[17, 19, 31] For example, Rimkoh et al. described rearrangements in 19 of 33 MCL cases (58%).[17] Boer et al.[31] and Bosch et al.[19] detected DNA rearrangements in 10 of 20 (50%) and in 6 of 12 MCL cases (50%), respectively. These relatively low detection rates may be explained by the result of fluorescence in situ hybridization (FISH) experiments demonstrating that 11q13 translocation breakpoints are scattered around this region[32] and the probes used for these studies, including the present one, cover only a limited albeit major region. For diagnosis of MCL, Northern analysis proved as useful as immunohistochemical analysis with our monoclonal antibody, 5D4, but mRNAs are not always available. In the present study, mRNAs from 4 out of 40 cases could not be investigated because of degradation. Rimkoh et al. similarly reported that RNA analysis could be performed on only 15 of 33 MCL samples available for DNA analysis.[17] Since paraffin-embedded sections are always available, the immunohistochemical method is more practical for detecting PRAD1/cyclin D1 abnormality in the clinical situation.

In our study, 3 of 15 MCL cases did not show any expression of PRAD1/cyclin D1 mRNA. Rimkoh et al.[17] and Oka et al.,[18] respectively, also reported one of 15 and 2 of 8 MCL to lack apparent PRAD1/cyclin D1 mRNA expression. No clear clinical differences were recognized between MCL cases with or without mRNA expression in our study or theirs. Further analysis is thus necessary to elucidate why the same MCL histology can arise without involvement of PRAD1/cyclin D1 in those cases lacking mRNA expression. One possibility is that the cell cycle deregulation involving cyclin D1 may take place without cyclin D1 overexpression because of alterations in other targets. One such candidate is the p16 which inhibits CDK4 by binding in competition with cyclin D1.[33] If p16 were lost, cyclin D1-CDK4 complex formation would not be inhibited, leading to a situation similar to overexpression of cyclin D1. In this regard, it would be of interest to study other cell cycle-regulatory molecules linked to cyclin D1 in mantle cell lymphomas.

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