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Monoclonal Antibody against PRAD1/Cyclin D1 Stains Nuclei of Tumor Cells with Translocation or Amplification at *BCL-1* Locus

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Mouse monoclonal antibodies were produced against the bacterial product encoded by human *PRAD1*/cyclin D1 gene, which is known to be involved in tumors with translocation or amplification at *BCL-1* locus of 11q13. The immunizing antigens used were GST-PRAD1 and T7 gene 10-PRAD1 fusion products. Four antibodies were reactive with both PRAD1 fusion products and cell lysates of B-cell tumor cell lines with t(11;14)(q13;q32) and a breast cancer cell line with 11q13 amplification, on immunoblotting. An immunofluorescence study showed that only one of them stained nuclei of cells with 11q13 abnormalities. Since this antibody proved applicable for conventional paraffin-embedded tissue sections, immunohistologic staining of various lymphoma tissues was performed. Eight of 11 mantle cell lymphomas showed intermediate to strong positivity and 6 of the positive cases demonstrated characteristic staining patterns that were either predominantly nuclear or both nuclear and cytoplasmic. The nuclear staining pattern was not observed with other types of lymphoma and thus may correlate with PRAD1 mRNA overexpression.

Key words: PRAD1 — Cyclin D1 — BCL-1 — Chromosomal translocation — Lymphoma

Chromosomal translocation and amplification are critical genetic alterations in neoplasia. The chromosome 11 band q13 region has been shown to be involved in a number of distinct malignancies including hematopoietic and non-hematopoietic tumors.[1–4] In B-cell malignancy, the *BCL-1* locus at 11q13 is one of the frequent breakpoint regions of chromosomal translocation with the immunoglobulin (Ig) heavy chain locus at 14q32.[5–8] Amplification at this locus has also been reported in 15–20% of primary breast cancers[3] and in an even higher proportion of squamous cell carcinomas of the head and neck.[9] Among the genes identified in the 11q13 amplification unit, U21B31 (*PRAD1*/cyclin D1) and *EMS1* were demonstrated to be overexpressed[10,11] in tumor cells with 11q13 amplification, whereas *INT2/FGF3* and *HSTF1/FGF4* genes were rarely expressed.[12–14]

The *PRAD1* gene was cloned from the breakpoint region of inv(11)(p15q13) found in parathyroid adenomas.[15,16] This inversion resulted in a juxtaposition of the parathyroid hormone gene regulatory region at 11p15 to the *PRAD1* gene at 11q13, leading to overexpression of *PRAD1*.[16] Gene walking for 110 kb from the *BCL-1* locus identified the *PRAD1* transcriptional unit[17] and the PRAD1 mRNA was shown to be overexpressed in mantle cell lymphomas associated with *BCL-1* translocation.[18] We have shown that PRAD1 mRNA is overexpressed in cells with a t(11;14)(q13;q32) translocation[19] and also in a lymphoma with t(11;22)(q13;q11), a variant translocation at the *BCL-1* locus.[20] The available evidence thus strongly suggests that *PRAD1* is a candidate *BCL-1* gene. It encodes a 295 amino acid polypeptide with sequence similarity to cyclins,[21] and subsequent study has identified this product as one of the G1 cyclins, D1, which plays a crucial role in regulating the cell cycle and proliferation.[22] Schuuring *et al.*[23] demonstrated that mRNAs of the *PRAD1* and *EMS1* genes were overexpressed in tumor cells with 11q13 amplification, the *EMS1* gene encoding a 550 amino acid polypeptide, a human homologue of chicken p80/p85, which is a substrate for the tyrosine kinase *src* oncogene product. The EMS1 protein was recently shown to be present at a level corresponding to the gene amplification and mRNA overexpression.[23] The question of whether the amount of PRAD1/cyclin D1 protein reflects the level of the mRNA expression, however, has remained unanswered. In the present report, we document the establishment of hybridomas producing monoclonal antibodies specific for the PRAD1 product and show that expres-

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sion of the protein corresponds to the mRNA level and that it is located predominantly in the nucleus of cell lines with 11q13 translocation or amplification. The results of an immunohistologic study of lymphomas, including mantle cell lymphomas, are also presented.

MATERIALS AND METHODS

Expression of PRAD1 fusion protein in *E. coli* Human PRAD1 cDNA clones were isolated from the SP-49 B-cell lymphoma cell line with a t(11;14)(q13;q32) translocation overexpressing a truncated form of PRAD1 mRNA.[19] One of the clones, SPO6, starting from nucleotide 43 according to the published PRAD1 cDNA sequence,[21] was used. An *Rsa*I fragment of 1174 bp (nucleotide 195 to 1368, encoding amino acids 18 to 295 of PRAD1) was isolated and the *Bam*HI linker (Pharmacia Biotech, #27-7725-01, Tokyo) was ligated. After *Bam*HI digestion, the fragment was cloned into the *Bam*HI site of pBluescriptII vector (Strategene, La Jolla, CA, USA) and the nucleotide sequence was confirmed using a Sequenase kit. Then, the *Bam*HI fragment was prepared and cloned into either pGEX-2T for the GST-fusion product (Pharmacia Biotech) or pET-3a for the T7 gene 10-fusion product (Promega, Madison, WI, USA). Sence or antisense constructs were then introduced into *E. coli* XL-1 cells for pGEX-2T-SPO6 or BL21 (DE3) cells for pET-3a-SPO6. Cells were cultured in LB medium containing 50 μg/ml ampicillin at 37°C overnight, then diluted 1:10 in 450 ml of fresh LB containing ampicillin and grown at 37°C. At an A_{600} of 0.4, expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 1 m*M*, and the cultures were further incubated for 2 h before harvesting. GST-RCK fusion product was used as a control. The *Pvu*II fragment of RCK cDNA[24] (nucleotide 438 to 1662) was prepared and the *Bam*HI linker was ligated. The fragment was cloned into pGEX-3X and subsequent procedures were conducted as described above.

Purification of PRAD1 fusion proteins After IPTG induction, the cells were pelleted and resuspended in 15 ml of 50 m*M* Tris pH 8.0, 0.25 *M* (w/v) sucrose. Lysozyme was added to 2 mg/ml and incubation on ice was performed for 1 h. Ethylenediaminetetraacetic acid (EDTA) was then added to 50 m*M*, and phenylmethylsulfonyl fluoride to 1 m*M*, and aprotinin and leupeptin to 10 μg/ml were also added. NP-40 was added to a final concentration of 0.25% and $MgCl_2$ to 10 m*M*, then the cells were lysed on ice by sonication and subjected to centrifugation at 10,000 rpm for 10 min at 4°C. Under the conditions employed, the PRAD1 fusion protein was recovered as inclusion bodies. They were washed with lysis buffer (100 m*M* NaCl, 1 m*M* EDTA, 50 m*M* Tris pH 8.0) containing 0.1% sodium deoxycholate. The gene 10-PRAD1 protein was partially soluble in lysis buffer containing 8 *M* urea at 4°C overnight, and the solubilized protein was dialyzed sequentially in Dulbecco's phosphate-buffered saline (−) (PBS) containing 4 *M*, 2 *M*, 1 *M*, 0.5 *M*, 0.25 *M* urea and finally without urea. In the GST-PRAD1 case, the inclusion bodies were not soluble even in 8 *M* urea, so they were washed once in 8 *M* urea solution and twice in PBS. The pellet was then resuspended in PBS and sonicated to disperse it. The fusion products thus processed were analyzed by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and visualized by Coomassie blue staining (Fig. 1a and 1b).

Production of hybridoma BALB/c mice were immunized intraperitoneally twice with 50 μg of GST-PRAD1 protein and twice with 50 μg of gene 10-PRAD1 protein with Freund's incomplete adjuvant every other week. Three days after the last immunization, spleen cells were harvested and fused with PAI myeloma cells derived from NS-1 as described previously.[25] Other procedures for hybridoma screening and immunoblot analysis were conducted as described below.

Enzyme-linked immunosorbent assay (ELISA) Immunoplates (Maxisorp F96; Nunc, Roskilde, Denmark) were coated overnight with 1 μg/ml of protein in 50 m*M* carbonate buffer, pH 9.6. After washing with PBS, nonspecific protein binding sites on Immuno-plates were blocked with PBS containing 3% dry milk and 5% normal goat serum at 37°C for 2h. After washing with PBS-0.05% Tween 20, 50 μl aliquots of hybridoma supernatants diluted 1:1 with PBS-0.05% Tween 20 were distributed into each well and incubated at 4°C overnight. After washing three times, 50 μl of 1/1000 diluted goat anti-mouse IgG+M (H+L, κ and λ) conjugated with horseradish peroxidase (HRP) was added, and incubated at room temperature for 1 h. After washings, HRP activity was visualized with 100 μl of OPD solution (1.7 mg/ml of *o*-phenylenediamine in 0.05 *M* citric acid-phosphate buffer containing 0.04% H_2O_2, pH 5.0). The reaction was stopped by adding 50 μl of 4 *N* H_2SO_4, and the absorbance at 490 nm was measured with an ELISA plate reader (Model 3550, Bio-Rad, Tokyo).

Cell lines Human hematopoietic cell lines were maintained in Iscove's culture medium containing 5% fetal calf serum (FCS). The cell lines with t(11;14)(q13;q32) translocation, SP-49 (B-cell lymphoma)[26] and KMS-12-PE (multiple myeloma)[27] were described previously.[19] The other cell lines without t(11;14)(q13;q32) were Jurkat and HPB-ALL (T-cell line), and Raji (Burkitt's lymphoma cell line). A breast cancer cell line with an 11q13 amplification, ZR-75-1, was obtained from the American Type Culture Collection (ATCC CRL 1500, Rockville, MD, USA).

Immunoblot analysis Whole cell lysates were prepared as described earlier[28] with some modifications. Briefly,

pellets of cultured cells were resuspended in the lysis buffer (0.1% sucrose monolaurate, 0.25 *M* sucrose, 0.01% EDTA, 1 m*M* phenylmethylsulfonyl fluoride) and sonicated on ice. Protein concentrations were measured with a DC protein assay kit (Bio-Rad). Fusion proteins or whole cell lysates were boiled in SDS-sample buffer (65 m*M* Tris pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue) for 5 min and 0.5 μg of the fusion protein or 50 μg of whole lysates was fractionated on 10% SDS-PAGE and electrophoretically transferred to PVDF membrane (Immobilon-P; Millipore Corp., Bedford, MA, USA) at 200 mA at 4°C overnight. The remaining protein binding sites on the membrane were blocked with 20% bovine serum albumin (BSA) at room temperature for 2 h. The membranes were cut into strips, incubated with hybridoma supernatants at 4°C overnight, washed with PBS-0.05% Tween 20, then incubated with biotinylated goat anti-mouse IgG (H+L, κ and λ) (diluted to 1/1000 in 3% dry milk, 1% normal goat serum in PBS-0.05% Tween 20) at room temperature for 1 h, and finally with avidin-biotinylated peroxidase complex (Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). The membranes were incubated in substrate solution (POD immunostain set, Wako Pure Chemical Industries, Ltd., Osaka) to develop color.

Immunofluorescence staining of cultured cells Cells were washed with PBS twice and loaded onto slide glasses with a cytospin apparatus (Shandon Southern Products Ltd., Cheshire, UK). After drying in a stream of cool air, cells were fixed with cold 70% methanol for 30 min and then air-dried. These slide glasses were stored at −80°C until use. The cells were first incubated with hybridoma supernatants, and then with fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulins (Dako, Glostrup, Denmark; diluted to 1/100 with 2% FCS-PBS) as described previously.[25]

Immunohistologic staining of lymphomas Immunostaining was conducted using both frozen and formalin-fixed paraffin-embedded tissues of 5 reactive lymph nodes and 24 lymphomas (11 mantle cell [diffuse centrocytic] lymphomas, 8 diffuse large cell [centroblastic] lymphomas and 5 follicular [centroblastic/centrocytic] lymphomas) using the 5D4 monoclonal antibody by the indirect immunoperoxidase technique. For formalin-fixed, paraffin-embedded sections, we applied the microwave oven heating technique, shown to be effective for the retrieval of masked epitopes of many antigens.[29, 30] Negative controls with normal mouse immunoglobulin were included in each staining run. Quantitative estimation of nuclear and cytoplasmic staining intensity (0 to 3+) was based on a comparison of the staining intensity of malignant cells with that of normal lymphoid organs, and was classified into four; negative (0), weak (1+), intermediate (2+) and strong (3+).

RESULTS

Expression and purification of PRAD1 fusion products in *E. coli* Bacterial colonies with sense or antisense constructs of pGEX-2T-SPO6 or pET-3a-SPO6 were cultured and induced for 2 h by IPTG. One ml of cultured medium was centrifuged and the pellet was resuspended in 100 μl of SDS-sample buffer and boiled. Ten μl of each sample was subjected to 10% SDS-PAGE and stained with Coomassie blue. As shown in Fig. 1, bands with the expected molecular masses, i.e., 58 kilodaltons (kDa) GST-PRAD1 fusion product (GST, 26 kDa; PRAD1, 32 kDa) and 33 kDa of T7 gene 10-PRAD1 fusion product (gene 10, 1 kDa; PRAD1, 32 kDa) were demonstrated with the sense construct, while these products were not present with the antisense constructs. Both GST-PRAD1 and gene 10-PRAD1 fusion products were found to be insoluble and were purified as inclusion bodies as described in "Materials and Methods."

Establishment of hybridomas producing specific monoclonal antibodies against PRAD1 products Hybridoma

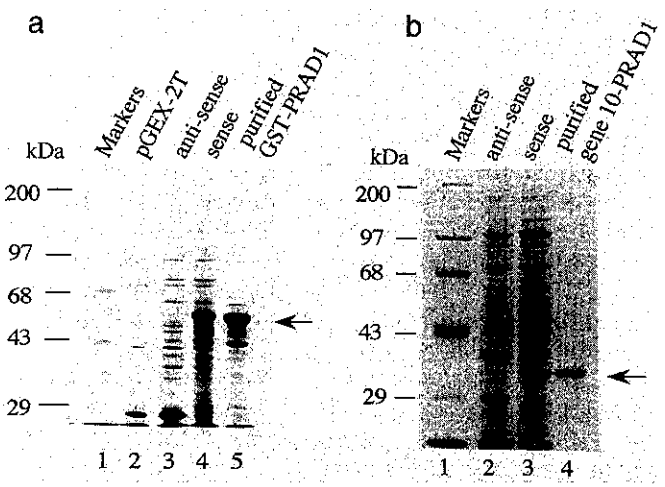

Fig. 1. Expression of GST-PRAD1 and gene 10-PRAD1 fusion products in *E. coli*. Cell lysates were fractionated by 10% SDS-PAGE and visualized by Coomassie blue staining. (a) GST-PRAD1 fusion products expressed by the pGEX-2T vector. Lane 1, molecular markers; lane 2, cell lysate with pGEX-2T vector without insert; lane 3, cell lysate with the SPO6 fragment in the anti-sense orientation; lane 4, cell lysate with the SPO6 fragment in the sense orientation; lane 5, purified GST-PRAD1 inclusion body. (b) Gene 10-PRAD1 fusion product expressed by the pET-3a vector. Lane 1, molecular markers; lane 2, cell lysate with the SPO6 fragment in the anti-sense orientation; lane 3, cell lysate with the SPO6 fragment in the sense orientation; purified gene 10-PRAD1 fusion product. The sizes of the GST-PRAD1 fusion product (58 kDa) (a) and the gene 10-PRAD1 fusion product (33 kDa) (b) are indicated by an arrow on each panel. The sizes of molecular markers (kDa) are indicated by dashes.

supernatants were screened against GST-PRAD1 and gene 10-PRAD1 fusion products and also against a control fusion product, GST-RCK by ELISA. Out of 190 clones, four clones, PRAD1-5D4 (IgG2a), -2D10 (IgG2b), -6H6 (IgG1), and -9E9 (IgG1) were shown to be positive for both GST-PRAD1 and gene 10-PRAD1, but negative for GST-RCK, suggesting that these clones produce antibodies specific for PRAD1 bacterial products (Fig. 2). The reactivity was further examined by immunoblot analysis. As shown in Fig. 3, these monoclonal antibodies demonstrated specific bands for GST-PRAD1 and gene 10-PRAD1, but not for GST-RCK. Smaller bands observed for GST-PRAD1 may be degradation products of the full length fusion product or prematurely terminated products. Immunoblot analysis of cultured cell lines with 11q13 abnormalities was conducted. Discrete 36 kDa signals were evident in both SP-49 and KMS-12-PE cell lines derived from B cell tumors with t(11;14)(q13;q32), and in a ZR-75-1 breast cancer cell line with an 11q13 amplification, but no such signals were recognized in Raji, Jurkat and HPB-ALL cell lines without such 11q13 abnormalities (Fig. 4a). Northern

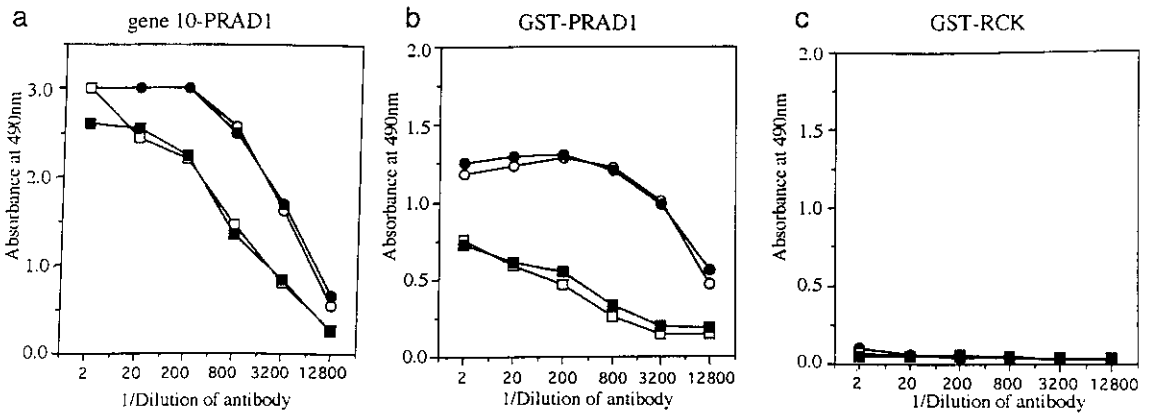

Fig. 2. Reactivity of anti-PRAD1 monoclonal antibodies tested against bacterial products by ELISA. Serially diluted culture supernatants of 5D4 (○), 2D10 (●), 6H6 (□) or 9E9 (■) in 5% FCS-Iscove's medium were mixed 1:1 with PBS-0.05% Tween 20, and the reactivity was assayed by ELISA against immuno-plates precoated with 1 μg/ml of products of either gene 10-PRAD1 (a), GST-PRAD1 (b), or a control product, GST-RCK (c). Abscissa, dilution of supernatants; ordinate, absorbance at 490 nm.

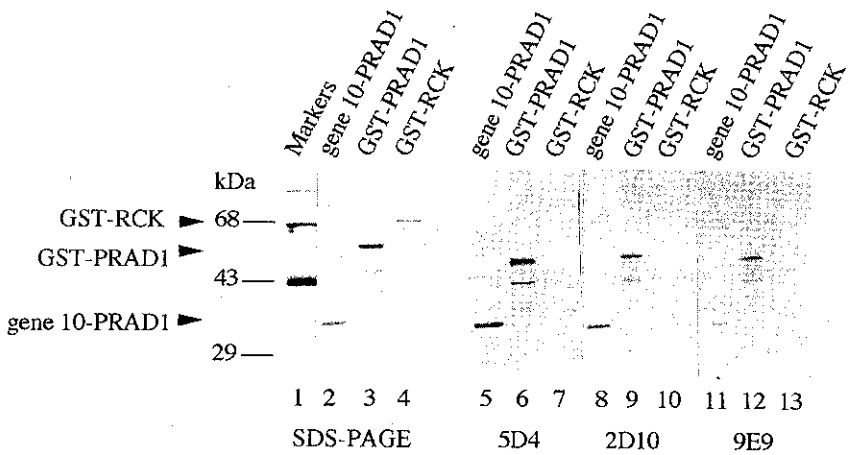

Fig. 3. Characterization of anti-PRAD1 monoclonal antibodies against bacterial fusion products by immunoblotting. Purified bacterial products of gene 10-PRAD1 (lanes 2, 5, 8, and 11), GST-PRAD1 (lanes 3, 6, 9, and 12) and GST-RCK (lanes 4, 7, 10, and 13) (ca. 0.5 μg in each lane) were fractionated by 10% SDS-PAGE, and then the blots were visualized by Coomassie blue staining (lanes 1 to 4), or stained with either 5D4 (lanes 5 to 7), 2D10 (lanes 8 to 10), or 9E9 (lanes 11 to 13) monoclonal antibodies to demonstrate immunoreactivity with PRAD1 fusion products. A similar reactivity was obtained with 6H6 (data not shown).

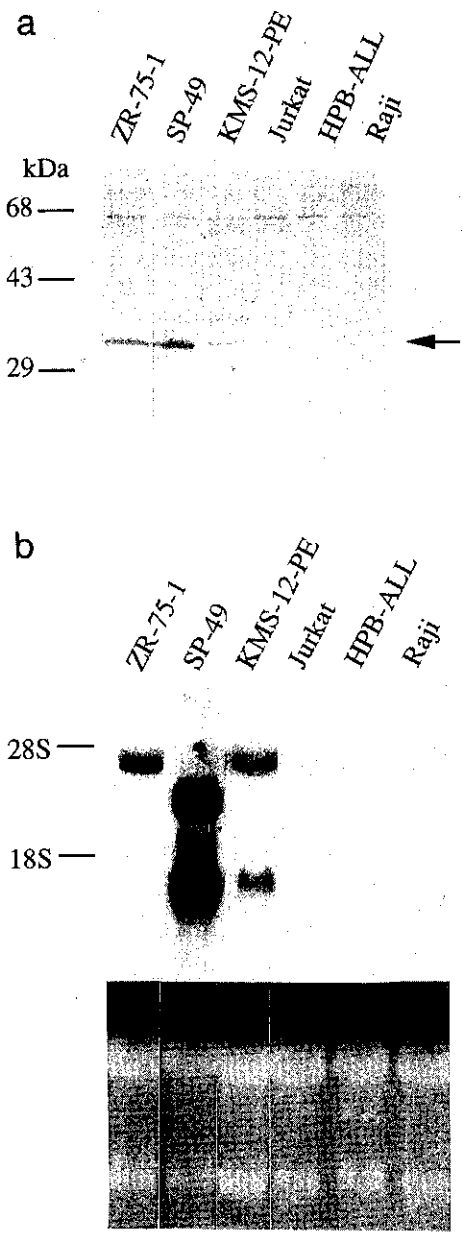

Fig. 4. Expression of PRAD1 products and mRNA in cell lines with t(11;14)(q13;q32) translocation or 11q13 amplification. (a) Immunoblot analysis with 5D4 anti-PRAD1 monoclonal antibody. Whole cell lysates separated by 10% SDS-PAGE were transferred to PVDF membrane and then the blots were stained with 5D4 antibody. (b) Northern analysis for PRAD1 mRNA expression. Northern blot analysis was performed as described previously[35] and 10 μg of total RNA were applied to each lane. Ethidium staining of the gel is shown below. ZR-75-1 is a breast cancer cell line with an 11q13 amplification; SP-49 cell line and KMS-12-PE are B-cell lymphoma and myeloma cell lines with t(11;14)(q13;q32), respectively; Jurkat and HPB-ALL are T-cell lines and Raji is a B cell line.

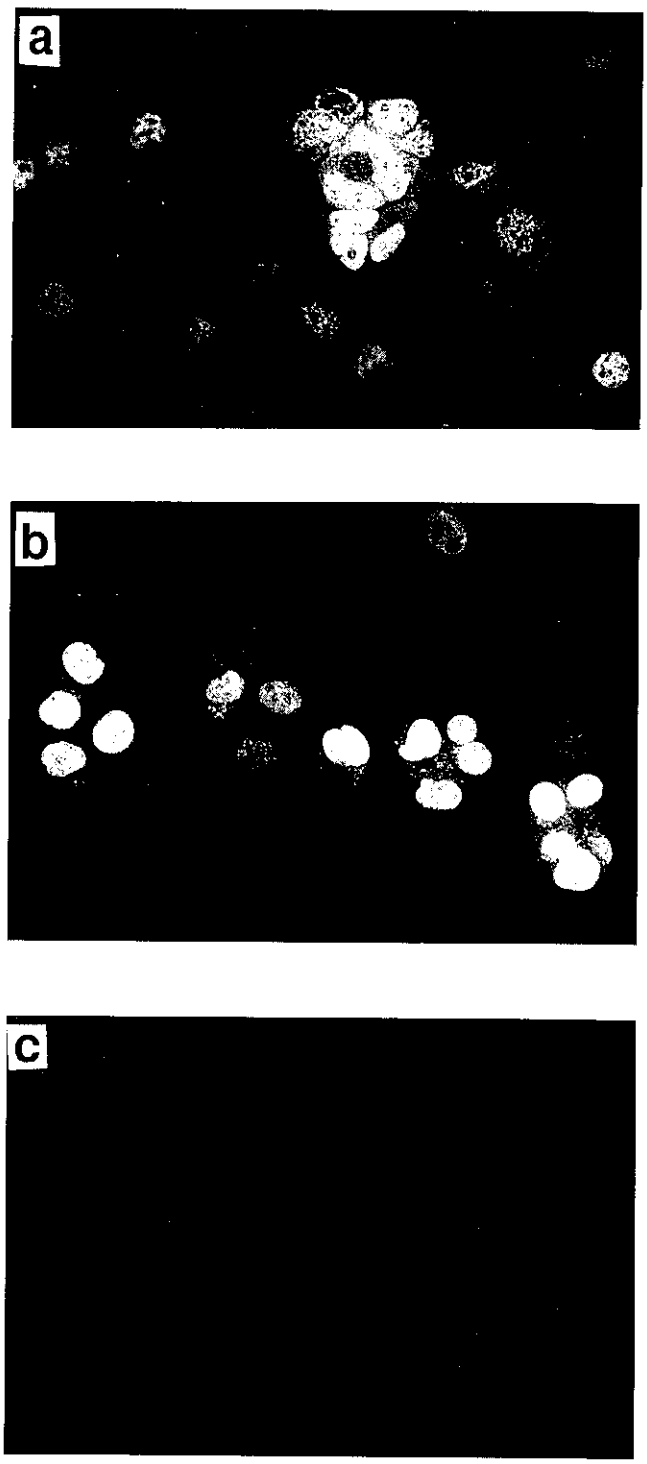

Fig. 5. Immunofluorescence staining of cell lines with 5D4 monoclonal antibody. Strong nuclear staining is seen in SP-49 cell line with a t(11;14)(q13;q32) translocation (a) and also in ZR-75-1 breast cancer cell line with an 11q13 amplification (b), but no such staining is observed in Jurkat cell line without any 11q13 abnormality (c). (magnification $\times$365).

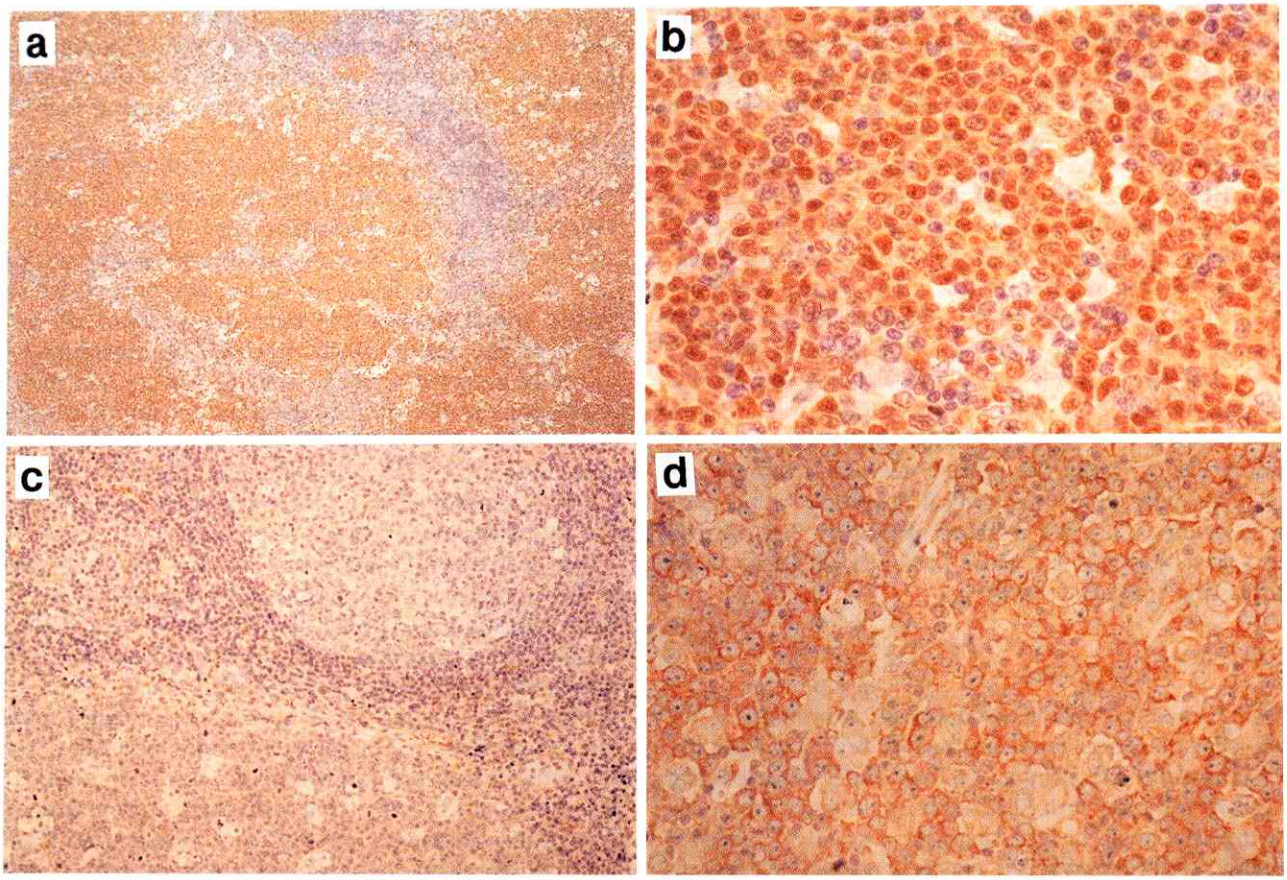

Fig. 6. Immunohistologic staining of formalin-fixed, paraffin-embedded sections of lymphomas and reactive lymph nodes with 5D4 monoclonal antibody. (a) Mantle cell lymphoma with a t(11;14)(q13;q32) showing strong staining (3+) for 5D4. Note the vague nodular pattern of positively stained tumor cells. (magnification ×40) (b) Higher magnification of Fig. 6a. The antibody staining appears to be predominantly nuclear, but cytoplasmic staining is also observed in a proportion of the tumor cells. (magnification ×400) (c) Reactive lymph node. The reactivity is negative (0). (magnification ×135) (d) Diffuse large cell lymphoma of B cell phenotype. The staining intensity is intermediate (2+) and the expression appears to be cytoplasmic without nuclear staining. (magnification ×270) See "Materials and Methods" for grading of the staining intensity. (immunoperoxidase, counterstained with hematoxylin)

analysis of these cell lines demonstrated that PRAD1 mRNA was expressed 10 to 50 times more abundantly in cell lines with 11q13 abnormalities than in cells without any 11q13 abnormalities (Fig. 4b), indicating that the expression of the PRAD1 protein detected by the monoclonal antibodies is well correlated with the level of mRNA expression.

Immunofluorescence staining with monoclonal antibodies of cell lines with 11q13 abnormalities Cell lines with 11q13 abnormalities were fixed with methanol and stained with each of the 4 monoclonal antibodies against PRAD1 products. One of them, antibody 5D4 showed nuclear staining in about 10 to 30% of the cells of SP-49, ZR-75-1 (Fig. 5a and 5b), and KMS-12-PE cell lines (data not shown). These staining signals were not recognized in cells without 11q13 abnormalities (Fig. 5c and data not shown). Three other antibodies 2D10, 6H6 and 9E9 did not exhibit any staining.

Immunohistologic staining of mantle cell lymphomas Since 11q13 translocation has been demonstrated to be associated with mantle cell lymphomas,[8] we conducted immunohistologic staining of various lymphomas and reactive lymph nodes. Preliminary study showed that not only frozen, but also formalin-fixed, paraffin-embedded sections were positively stained with the 5D4 antibody, so the latter were used for the immunohistologic staining study. Representative staining patterns are illustrated in Fig. 6. In five cases of reactive lymph nodes, the germinal centers of two showed barely detectable positive staining (1+), but nevertheless more intense than the mantle

zone, whereas the other three demonstrated no positivity (Fig. 6c) (see "Materials and Methods" for grading of the staining intensity). Eight of 11 mantle cell lymphomas (CD5+, CD10−) showed from 2 to 3+ positivity and 6 of the positive cases were characteristic in that their staining pattern appeared to be either predominantly nuclear or both nuclear and cytoplasmic (Fig. 6a and 6b). Two of these positive cases had the t(11;14)(q13;q32) chromosome abnormality but information was not available for the others. Four of 8 diffuse large cell lymphomas of B-cell phenotype (CD5−, CD10−) showed from 2 to 3+ positivity for cytoplasmic staining (Fig. 6d) but no nuclear staining was demonstrated. One of five follicular lymphomas (three CD5−, CD10− and two CD5−, CD10+) showed intermediate cytoplasmic staining (2+), while the other four were evaluated as weakly positive (1+). In several neoplastic follicles of the latter cases, a small number of positive centroblasts (2+) were admixed but their positivity appeared to be cytoplasmic (data not shown).

DISCUSSION

Using bacterial fusion products as immunogens, we established 4 monoclonal antibodies reactive with the PRAD1/cyclin D1 product. All of them detected p36 protein in cell lines with 11q13 abnormalities by immunoblotting, but only one of them gave positive immunostaining, reacting with the native product, suggesting differences in the epitopes detected by these antibodies. Recently, Baldin *et al.*[31] demonstrated that the cyclin D1 is a nuclear protein in human lung fibroblasts (IMR90) using an affinity-purified polyclonal antibody generated against recombinant cyclin D1. Jiang *et al.*[32] also reported a nuclear localized staining pattern in esophageal cancer cells with *PRAD1*/cyclin D1 gene amplification using an antibody raised against a C-terminal sequence. These results are in good agreement with our present finding that the PRAD1/cyclin D1 product is predominantly localized in the nucleus of tumor cell lines with 11q13 abnormalities.

A good correlation between PRAD1/cyclin D1 mRNA and the amount of PRAD1/cyclin D1 protein was demonstrated in the present study by immunostaining and immunoblotting. The fact that nuclear staining was observed in 10 to 30% of the cells of the tumor cell lines with mRNA overexpression, whereas no such staining was recognized in cells without such mRNA overexpression (Fig. 5), is good evidence for an identity between positivity for nuclear immunostaining and PRAD1/cyclin D1 mRNA overexpression. This finding is particularly important for clinical application, since immunostaining can be simply applied to far larger numbers of the cases than are accessible to mRNA detection. The polyclonal antibody raised against the C-terminal region has also been reported to stain formalin-fixed tissues as well,[32] but our monoclonal antibody should provide a more consistent reagent for clinical and basic studies.

The sequence similarity among cyclins D1, D2 and D3[33] raises the possibility that the monoclonal antibody, 5D4, might exhibit cross-reactivity against cyclin D2 and/or D3. Indeed, a cross-reaction between cyclin D1 and D2 products has been noted for polyclonal antibodies raised against the bacterial product of cyclin D1.[31] In the present study, however, immunoblot analysis only detected a single band of p36 protein and the expression of the protein detected by immunostaining correlated well with PRAD1 mRNA expression. Thus, it is likely that the 5D4 monoclonal antibody is specific for cyclin D1, although the possibility of cross-reaction can not be completely excluded at present.

The t(11;14)(q13;q32) translocation has been shown to be associated with mantle cell lymphomas[8] and we have demonstrated that it correlates with PRAD1 mRNA overexpression.[19] The immunohistologic results presented in this paper, with 8 of 11 mantle cell lymphoma cases showing 2 to 3+ positive staining, 6 involving the nucleus, are therefore of major interest. Two of the latter cases are known to have t(11;14)(q13;q32) translocations, in agreement with their nuclear immunofluorescence staining (Fig. 5). Since information regarding karyotype and gene rearrangement was not available for the other cases, it remains unclear whether all cases with t(11;14)(q13;q32) may show nuclear staining. Further study is needed to clarify this point. The present study demonstrated that some diffuse B-cell and follicular lymphomas showed cytoplasmic staining, although the intensity was weak as compared with that observed in mantle cell lymphomas. This cytoplasmic staining is also observed in samples with nuclear staining, and therefore it is difficult to speculate at this point whether the cytoplasmic staining signal may represent a different condition. It may represent a moderate level of PRAD1 overexpression, or alternatively, as demonstrated by Xiong *et al.*,[34] the interaction of PRAD1/cyclin D1 with other molecules such as PCNA, p21, or CDKs may determine the location of PRAD1/cyclin D1. To address this important question, the relation between the level of mRNA expression and the immunostaining pattern requires elucidation.

The immunostaining results suggest the possibility that overexpression of PRAD1/cyclin D1 mRNA plays an important role in the pathogenesis of mantle cell lymphomas. The major breakpoint cluster region of the *BCL-1* locus is about 120 kb away from the *PRAD1*/cyclin D1 gene,[17] but the translocated immunoglobulin gene has been speculated to exert effects over a range of

more than 250 kb.[35] Thus, it is possible that genes more 3′ to *PRAD1* are also influenced. One such candidate gene is *EMS1*, recently identified as a gene overexpressed in cells with 11q13 amplification.[11] It is not clear if either *PRAD1*/cyclin D1 or *EMS1*, or both, or other genes yet to be elucidated directly contribute to the growth advantage of tumors. It is, therefore, clearly of interest to determine whether *EMS1* is overexpressed in all lymphomas with either t(11;14)(q13;q32) or t(11;22)(q13;q11). Immunostaining studies with antibodies to both PRAD1/cyclin D1 and EMS1 products should provide an insight into tumorigenicity involving 11q13 abnormalities.

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