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Cyclin D2 is overexpressed in proliferation centers of chronic lymphocytic leukemia/small lymphocytic lymphoma

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The D cyclins are important cell cycle regulatory proteins involved in the pathogenesis of some lymphomas. Cyclin D1 overexpression is a hallmark of mantle cell lymphoma, whereas cyclins D2 and D3 have not been shown to be closely associated with any particular subtype of lymphoma. In the present study, we found that cyclin D2 was specifically overexpressed in the proliferation centers (PC) of all cases of chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) examined (19/19). To examine the molecular mechanisms underlying this overexpression, we immunohistochemically examined the expression of nuclear factor (NF)-κB, p15, p16, p18, and p27 in the PC of six patients. Five cases showed upregulation of NF-κB expression, which is known to directly induce cyclin D2 by binding to the promoter region of *CCND2*. All six PC examined demonstrated downregulation of p27 expression. In contrast, upregulation of p15 expression was detected in five of six PC examined. This discrepancy suggests that unknown cell cycle regulatory mechanisms involving NF-κB-related pathways are also involved, because NF-κB upregulates cyclin D2 not only directly, but also indirectly through c-Myc, which is believed to downregulate both p27 and p15. In conclusion, cyclin D2 is overexpressed in the PC of CLL/SLL and this overexpression is due, in part, to the upregulation of NF-κB-related pathways. (*Cancer Sci* 2011; 102: 2103–2107)

The D-type cyclins (D1, D2, and D3) play key roles in cell cycle machinery and the biochemical functions of these cyclins partially overlap.[1,2] The D cyclins positively regulate cell proliferation by binding to cyclin-dependent kinase (CDK) 4 and CDK6, resulting in the phosphorylation of the retinoblastoma protein and the G_1/S transition of the cell.[2,3]

Dysregulation of D cyclins has been implicated in the pathogenesis of lymphoid malignancies.[4] Among the three D cyclins, the expression pattern of cyclin D1 has been well documented;[5] indeed, overexpression of cyclin D1 induced by translocation of t(11;14)(q13;q32) serves as a hallmark for the diagnosis of mantle cell lymphoma.[6] Recent studies have identified a rare type of cyclin D1-negative mantle cell lymphoma that overexpresses cyclin D2 or D3.[7,8] Cyclins D2 and D3 are also detected in various lymphomas,[9,10] but these two proteins are not as specific to certain lymphomas as is cyclin D1.[10]

Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) is an indolent lymphoma/leukemia.[11] At least two major types of CLL/SLL are known to exist, one with mutated immunoglobulin heavy-chain variable region (*IGHV*) genes, which is negative for zeta-chain-associated protein kinase 70 (ZAP-70), and the other with unmutated *IGHV* genes, which is positive for ZAP-70.[11–14]

Previous reports have described that peripheral blood neoplastic cells of patients with CLL/SLL are closely related to cyclin D2.[15–17] In these studies, the vast majority of circulating tumor cells were arrested in the G_0/early G_1 phase and were not the proliferative component.[11] The proliferating cells are located in the proliferation centers (PC) of lymph nodes, which show a pseudofollicular pattern of pale areas on a dark background of small cells.[18,19] In the PC, the growth of tumor cells is believed to be favored by advantageous T-cell help, and the circulating tumor cells are the offspring of the proliferative compartment.[19] Although several previous studies have revealed that the PC play an important role in the pathogenesis of CLL/SLL,[11,19,20] the cell cycle in the PC microenvironment has not been well analyzed.[21]

In the present study, we sought to clarify the cell cycle regulation of CLL/SLL, with special reference to the PC, in terms of the expression of cyclins D2 and D3, and the molecular mechanisms involved.

Materials and Methods

Patients and materials. Samples from 19 Japanese patients with CLL/SLL, diagnosed between 1999 and 2010, as recorded in our surgical pathology files, were examined. All samples were collected with patients' informed consent.

All 19 tissue biopsies (17 lymph nodes and two tonsils) expressed the typical morphology and immunophenotype of CLL/SLL. All samples were confirmed immunohistochemically to be sox11 negative (to exclude cyclin D1-negative mantle cell lymphoma) by using an anti-sox11 antibody (polyclonal; 1:100; Sigma-Aldrich, St Louis, MO, USA).[8]

The clinical characteristics of the patients at the time of the biopsy are summarized in Table 1. The patients included 12 men and seven women between 34 and 84 years of age (median age 65 years). Forty-seven per cent of patients (9/19) had increased white blood cell (WBC) counts and 31% (4/13) had hepatosplenomegaly. Seven and 12 patients were SLL and CLL, respectively.[22] Of the 12 patients with CLL, three had incomplete clinical data. Seven of the other nine patients were in Binet Stage B or C and four were in Rai Stage III–IV.

Histological examination and immunohistochemistry. Tissue samples were fixed in 10% formalin and embedded in paraffin. Sections (4 μm) were stained with H&E.

Immunohistochemistry was performed on paraffin-embedded sections using automated Bond-max stainer (Leica Biosystems, Melbourne, Vic., Australia) and primary antibodies for the

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Table 1. Characteristics of 19 patients with chronic lymphocytic leukemia/small lymphocytic lymphoma

Parameter	No. patients (%)
Median age (range), years	65 (34–84)
Gender (M/F)	12/7
Increased WBC count	9 (47)
Presence of hepatosplenomegaly†	4 (31)
Small lymphocytic lymphoma	7 (37)
Chronic lymphocytic leukemia	12 (63)
Binet stage	
A	2
B	3
C	4
NA	3
Rai stage	
0	0
I–II	5
III–IV	4
NA	3

†Data available for 13 patients. M, male; F, female; NA, not available; WBC, white blood cell.

following antigens: cyclin D1 (SP4; 1:40; Nichirei Biosciences Inc., Tokyo, Japan), cyclin D2 (polyclonal; 1:150; Proteintech Group Inc., Chicago, IL, USA), cyclin D3 (DCS-22; 1:10; Progen Biotechnik GmbH, Heidelberg, Germany), nuclear factor (NF)-κB p65 (polyclonal; 1:1500; Abcam Inc., Tokyo, Japan), p15 (15p06; 1:25; Lifespan Biosciences Inc., Seattle, WA, USA), p16 (JC8; 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p18 (118.2; 1:100; Santa Cruz), and p27 (1B4; 1:150; Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK).

The PC were defined as pale areas containing large cells, prolymphocytes and paraimmunoblasts, surrounded by a dark background of small lymphocytes.

In every immunostained slide, 10 high-power fields were recorded in the PC and surrounding areas, quantified, and averaged for the estimated percentage of positively immunostained cells. Based on previous studies, a sample was considered positive if ≥20% tumor cells were stained.[16]

Double immunofluorescence. For indirect double immunofluorescence, paraffin-embedded sections of the tonsil biopsy specimens were stained for cyclin D2 and Ki-67. The primary antibodies used were anti-cyclin D2 (rabbit; polyclonal; 1:150; Proteintech Group) and anti-Ki-67 (mouse; MIB-1; 1:100; Santa Cruz). Alexa fluor anti-mouse 555 (Invitrogen, Carlsbad, CA, USA) and Alexa fluor anti-rabbit 488 (Invitrogen) were used as secondary antibodies at a dilution of 1:400. Specimens were examined under a conventional immunofluorescence microscope (IX71; Olympus, Tokyo, Japan).

Assessment of ZAP-70 and CD38 expression. The expression of ZAP-70 was determined by immunostaining with an anti-ZAP-70 antibody (9E11; 1:100; Leica Biosystems Newcastle Ltd), as described previously.[23] Expression of CD38 in the tissues was evaluated using flow cytometry and was considered to be increased when it was ≥30%.[24]

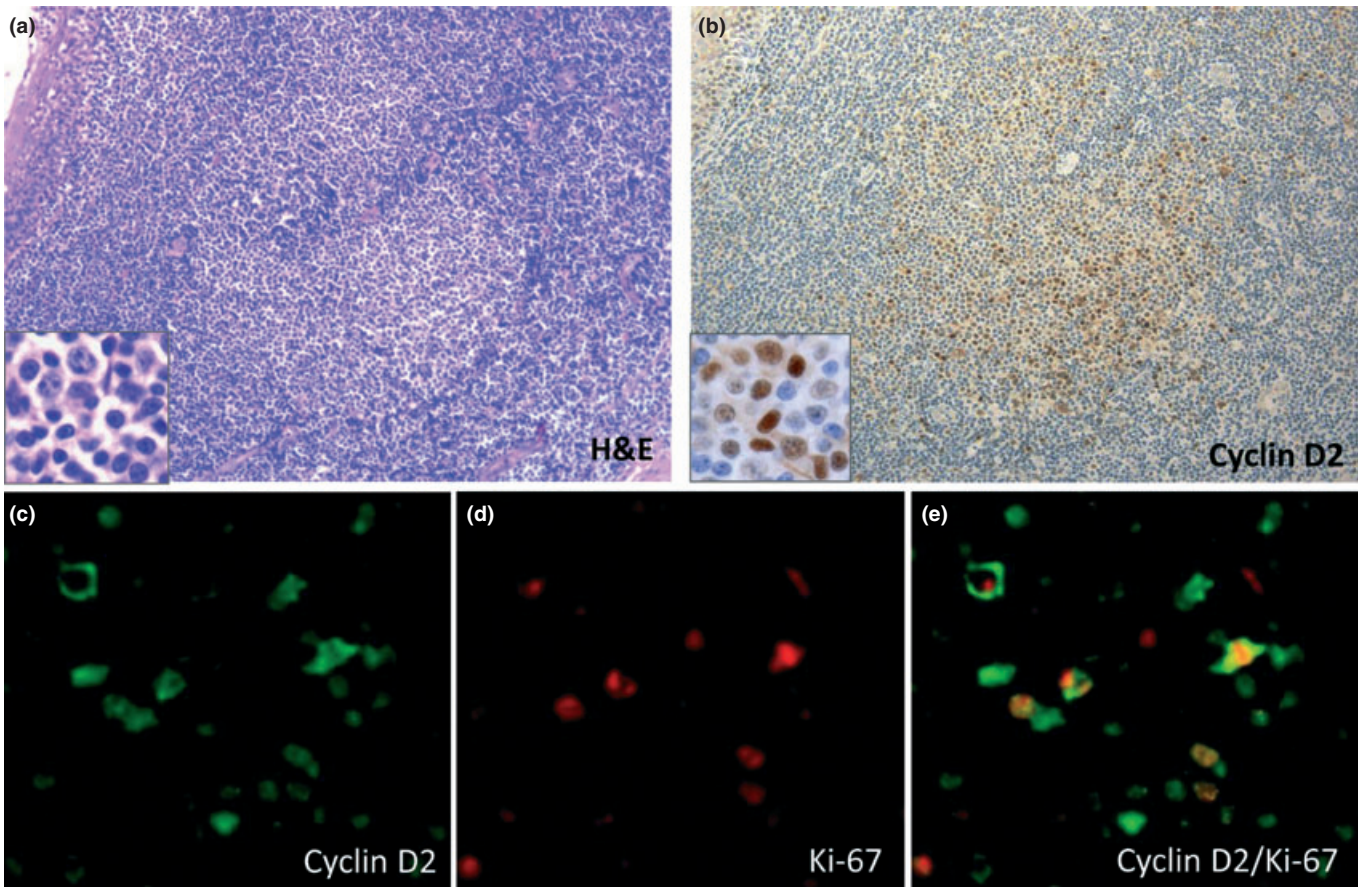

Fig. 1. Cyclin D2 expression in the proliferation center of chronic lymphocytic leukemia/small lymphocytic lymphoma: (a) H&E staining and (b) cyclin D2 staining. (Original magnification ×100; inset ×400.) Fluorescent double staining for (c) cyclin D2, (d) Ki-67, and (e) cyclin D2/Ki-67. (Original magnification ×400).

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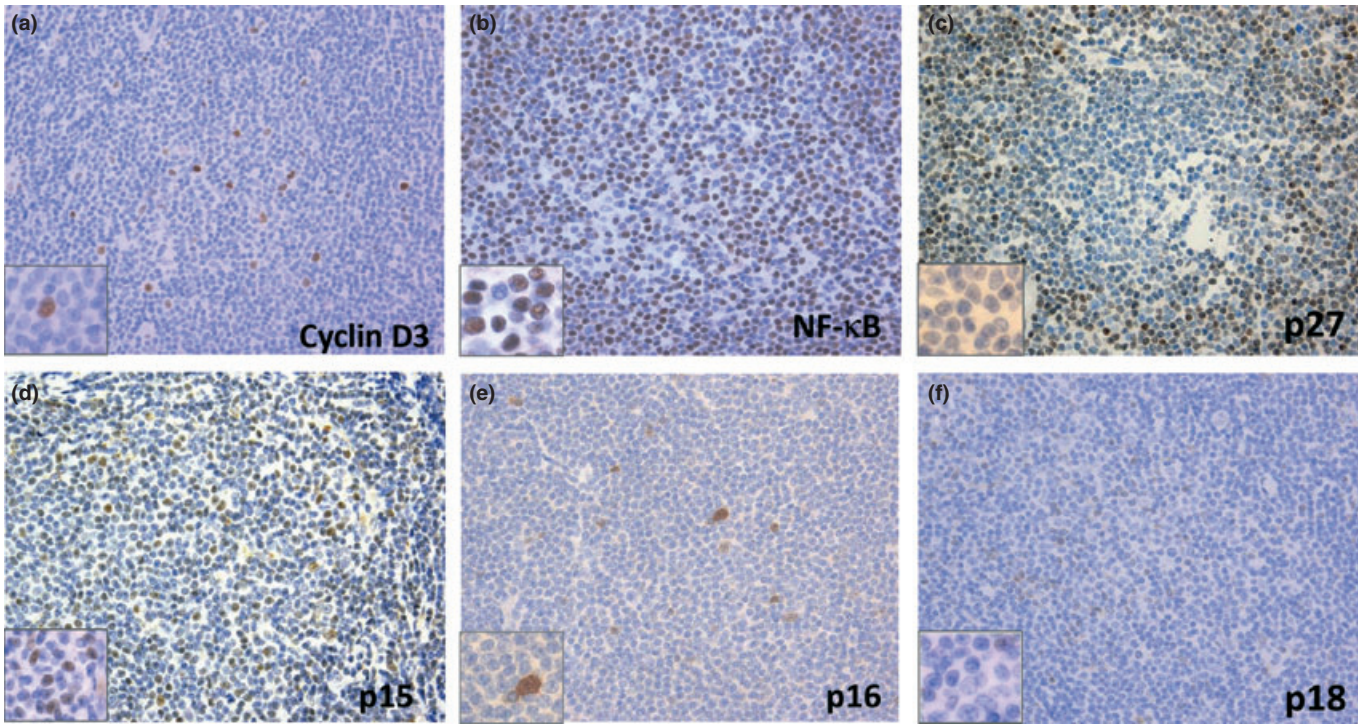

Fig. 2. Expression of cyclin D3, nuclear factor (NF)-κB, and cyclin-dependent kinase inhibitors (p27, p15, p16, and p18) in the proliferation center of chronic lymphocytic leukemia/small lymphocytic lymphoma. (Original magnification ×200; inset ×400).

Results

Cyclin D2 and D3 expression. Immunostaining for cyclins D2 and D3 was performed in 19 samples from CLL/SLL patients. Cyclin D2 was clearly detected in all PC examined, but not in the surrounding areas (Fig. 1). Cyclin D2 was expressed mainly in the nuclei of prolymphocytes and paraimmunoblasts. In all cases examined, cyclin D3 expression was negative in both the PC and surrounding areas, except for some large cells in the PC that showed a nuclear staining pattern (Fig. 2).

To examine the proliferative activity of cyclin D2-positive cells in the PC, we performed fluorescent double staining for cyclin D2 and Ki-67. This revealed that most of the cyclin D2-positive cells coexpressed Ki-67, indicating that the coexpressing cells were in the cell cycle (Fig. 1).

Analysis of the NF-κB/cyclin D2 pathway. To examine the molecular mechanisms underlying cyclin D2 overexpression in the PC of CLL/SLL patients, immunostaining for NF-κB was performed in six samples (Table 2; Fig. 2). Five samples were positive for NF-κB in the PC and three of these were also positive for NF-κB in areas surrounding the PC.

Cyclin-dependent kinase inhibitor analysis. To examine the correlation between CDK inhibitors (CDKI) and cyclin D2, immunostaining for p15, p16, p18, and p27 was performed in six samples from CLL/SLL patients (Table 2; Fig. 2). Five of six cases were positive for p15 in the PC. The surrounding areas were negative for p15 in all samples examined. Five of six cases were negative for p16 in both the PC and surrounding areas, but large cells were sporadically positive for p16; one case was completely negative for p16 in both the PC and surrounding areas. All cases examined were p18 negative in both the PC and surrounding areas. In all cases, the PC were negative for p27; however, p27 expression was upregulated in the surrounding areas.

Table 2. Immunohistochemical findings and ZAP-70 and CD38 status in chronic lymphocytic leukemia/small lymphocytic lymphoma

Patient no.	Tissue	ZAP-70	CD38	Location	D1	D2	D3	NF-κB	p15	p16	p18	p27
1	LN	–	NA	PC	–	+	–†	+	+	–†	–	–
				Non-PC	–	–	–	+	–	–†	–	+
2	LN	–	NA	PC	–	+	–†	+	+	–†	–	–
				Non-PC	–	–	–	+	–	–†	–	+
3	Tonsil	–	NA	PC	–	+	–†	+	+	–†	–	–
				Non-PC	–	–	–	+	–	–†	–	+
4	LN	–	NA	PC	–	+	–†	–	+	–	–	–
				Non-PC	–	–	–	–	–	–	–	+
5	LN	–	–	PC	–	+	–†	+	+	–†	–	–
				Non-PC	–	–	–	–	–	–†	–	+
6	LN	–	NA	PC	–	+	–†	+	–	–†	–	–
				Non-PC	–	–	–	–	–	–†	–	+

†Some large cells were positive. LN, lymph node; PC, proliferation center; NA, not available; D1, D2, D3, cyclin D1, cyclin D2, and cyclin D3, respectively; NF-κB, nuclear factor-κB.

Cyclin D1 expression. Immunostaining for cyclin D1 was performed in six samples from CLL/SLL patients (Table 2). All cases examined were cyclin D1 negative in both the PC and surrounding areas.

ZAP-70 and CD38 expression. The proportion of patients with increased ZAP-70 and CD38 expression was 11.8% (2/17) and 60% (3/5), respectively.

Discussion

Although cyclins D2 and D3 reportedly lack a specific relationship with certain subtypes of lymphoma,[10] we found that cyclin D2 was specifically overexpressed in the PC of all CLL/SLL cases examined (19 of 19 cases). To the best of our knowledge, this is the first report that has revealed the specific overexpression of cyclin D2 using immunohistochemistry. Cyclin D3 was expressed only in a minority of tumor cells in the PC, and CLL/SLL is typically immunohistochemically negative for cyclin D1,[25] except for a few rare cases.[26] These data indicate that the cell cycle of CLL/SLL is mainly controlled by cyclin D2, supporting the hypothesis that CLL/SLL is derived from CD5-positive B1 cells,[11] because cyclin D2 plays a crucial role in mediating proliferative signaling and is essential for CD5-positive B cell development.[27]

The *CCND2* gene is located on chromosome 12p13, and 14% of CLL/SLL patients have a trisomy of chromosome 12.[28] However, only a single case report has shown a translocation involving *CCND2*,[29] and the amplification of this gene has not been detected.

Recent studies have demonstrated that cyclin D2 expression is controlled by multiple signaling pathways, such as the NF-κB-related pathways (Fig. 3).[30] High NF-κB activity is a major finding of CLL/SLL, as well as in other B cell malignancies.[31,32] In the present study, we also identified activation of NF-κB in the PC of CLL/SLL (five of six cases). Nuclear factor (NK)-κB upregulates cyclin D2 not only by binding the promoter region of *CCND2*,[33] but also through c-Myc,[34,35] which also upregulates cyclin D2 expression.[36,37] Although we were not able to demonstrate the upregulation of c-Myc in the PC in the present study, it has been reported that c-Myc is upregulated in peripheral blood CLL cells activated by dipeptidyl peptidase 2 inhibition.[38]

The Ink4 family (p15, p16, p18, and p19) proteins bind to CDK4 or CDK6 and prevent interactions with their cognate D cyclins;[39] p27 (Kip1) facilitates the assembly of D cyclins and CDK4,[40] which is the primary CDK in the cell cycle of CLL/SLL (Fig. 3).[16] Although there were no significant findings for p16 and p18 in the present study, p15 was rather overexpressed in the PC examined (five of six cases); in contrast, p27 was downregulated in the PC of all CLL/SLL cases (six of six cases), in agreement with previous studies.[21] This discrepancy suggests that unknown cell cycle regulatory mechanisms involving NF-κB-related pathways exist, because c-Myc is believed to downregulate both p15 and p27.[36,41]

The proportion of mutated variants in CLL/SLL is higher in Japan than in Western countries.[14,42] In the present study, most cases (88.2%) were negative for ZAP-70. Cyclin D2 overexpression occurred in the PC of CLL/SLL samples, irrespective of ZAP-70 expression; this is in agreement with a previous study that used peripheral blood and reported that cyclin D2 can be induced in circulating CLL cells by B cell antigen receptor (BCR) stimulation, irrespective of ZAP-70 expression.[43]

It is known that ZAP-70 and CD38 are the most reliable negative prognostic markers for CLL/SLL.[44,45] Several lines of evidence imply that ZAP-70 and CD38 are not only prognostic markers, but also key elements in the pathogenesis of CLL/SLL.[46] For example, ZAP-70 can enhance and prolong activation of Syk kinase and modulate BCR signaling[47,48] and CD38 ligation induces tyrosine phosphorylation of ZAP-70, further sustaining the signal (Fig. 3).[49] These findings imply that signaling pathways regulating cyclin D2 expression can differ depending on ZAP-70 and CD38 expression. Unfortunately, in the present study the six cases whose cell cycle regulation was further analyzed were ZAP-70 negative and data for CD38 expression were only available for one of the six cases. Further investigations are required.

In conclusion, cyclin D2 is overexpressed in the PC of CLL/SLL and this overexpression is due, in part, to the upregulation of NF-κB-related pathways. These findings may play an

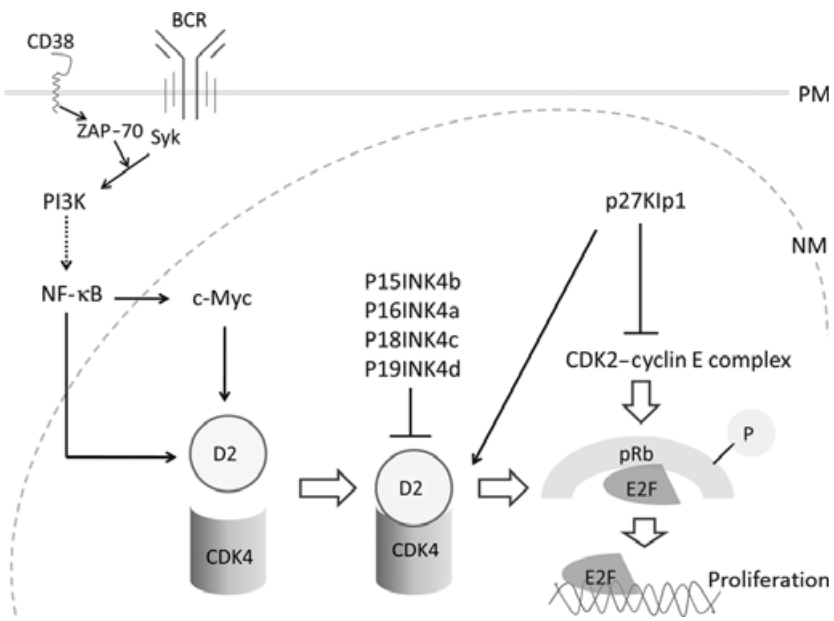

Fig. 3. The nuclear factor (NF)-κB/cyclin D2 pathway and cyclin D2 cell cycle regulation in chronic lymphocytic leukemia/small lymphocytic lymphoma. PM, plasma membrane; NM, nuclear membrane; BCR, B cell antigen receptor; PI3K, phosphatidylinositol 3-kinase; CDK, cyclin-dependent kinase; pRb, retinoblastoma protein; E2F, E2F transcription factor; P, phosphate group.

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important role in understanding cell cycle regulation in CLL/SLL because the importance of the PC is being recognized now more than before.[11,19,20]

Disclosure Statement

The authors have no conflict of interest.

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