Expression of Cyclin-Dependent Kinase Inhibitor p27^{kip1} in Malignant Lymphomas

p27^{kip1} is a cyclin-dependent kinase inhibitor that regulates progression from G1 into S phase. Aberrations in cell cycle control are often observed in tumors and might even be necessary in tumor development. Recent reports showed that low p27^{kip1} expression is associated with poor prognosis in several tumors and leukemia. To investigate the expression of p27^{kip1} in malignant lymphomas and elucidate the role of p27^{kip1} as a possible prognostic indicator, the authors performed an immunohistochemical staining of p27^{kip1} correlated with Ki-67 labelling index and clinical parameters. p27^{kip1} expression was reduced variably in most malignant lymphomas and inversely correlated with Ki-67 labelling index (*p*=0.0151). Regarding chemotherapeutic response, p27^{kip1} expression in the complete remission group showed statistically significant difference in expression compared to the progressive disease group (*p*=0.0021). There were significant differences in survival between cases with low and high p27^{kip1} expression (*p*=0.0071). In a multivariate Cox analysis, p27^{kip1} expression was independent prognostic factors as well as other known prognostic factors including age, grade, stage and chemotherapeutic response. In conclusion, the study suggests that reduced expression of p27^{kip1} protein may play a role in the pathogenesis and biologically aggressive behavior of malignant lymphomas.

Key Words : *p27^{kip1}; Lymphoma; Prognostic Factor*

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INTRODUCTION

The cell cycle is regulated by a family of cyclin-dependent kinases (CDKs) and their regulatory subunit cyclins. These CDK/cyclin complexes are activated and inactivated at specific time points during the cell cycle in response to internal and external demands (1). The kinase activity of CDKs can be inhibited by a group of CDK-inhibitors that bind to cyclin-CDK complexes and block progression through the cell cycle (2). There are two families of CDK-inhibitors affecting the activity of kinase complexes contributing to the proper control of G1-S transition. The INK family of proteins (p15, p16, p18 and p19) consists of specific CDK-inhibitors which mainly affect cyclin-D-CDK4/CDK6 complexes (3). The other class of inhibitors, the CIP/KIP family (including p21, p27 and p57), has less selective inhibitory effect on many CDK-complexes that are mainly active during G1 (4). The activity of p27^{kip1} is upregulated in vitro by transforming growth factor-β (TGF-β) by contact inhibition, or by serum depletion (5, 6). Furthermore, p27^{kip1}

levels are increased during differentiation of cultured cells. p27^{kip1} might also be involved in terminal differentiation as observed for the promyelocytic cell line HL-60 (7,8). In lymphoid tissues, p27^{kip1} protein is expressed in nonproliferating lymphocytes whereas activated lymphocytes, e.g. in the germinal centers, are negative, suggesting an inverse relation between proliferation and p27^{kip1} expression in normal lymphocytes (9).

There is increasing evidence that cell cycle regulators are disrupted in various tumors (10). Similar findings have been observed for p27^{kip1} expression in several solid tumors (11-16) and leukemia (17). Recent reports showed that reduced p27^{kip1} protein expression correlates with poor survival in breast and colorectal carcinoma patients (13, 15, 18). However, there are a few reports regarding p27^{kip1} protein expression in malignant lymphomas (9, 19).

For this reason the authors investigated the expression of p27^{kip1} protein in malignant lymphomas and examined the correlations between this protein and other parameters such as grade, tumor cell kinetics, chemother-

apeutic response, clinical stage and survival rate.

MATERIALS AND METHODS

Patient population

The study group consisted of 69 patients with nodal non-Hodgkin's malignant lymphomas from 1985 to 1998 at Hanyang University Medical Center, and with available follow-up data. Lymphomas were initially classified by the Working formulation of NCI and reclassified according to the Revised European American Lymphoma classification (REAL) based on morphological examination of imprints and paraffin sections, and immunophenotyping (20). Small lymphocytic lymphoma (SLL, n=2), and follicular lymphoma (FL, n=5) were included in the indolent group and diffuse large B cell lymphoma (DLBL, n=35), medium to large T cell lymphoma (PTCL, n=11), Burkitt's lymphoma (n=6), lymphoblastic lymphoma (n=8), and anaplastic large cell lymphoma (ALCL, n=2) in the aggressive group. As a control group, six reactive hyperplasia of lymph nodes and tonsils were used.

Immunohistochemical staining

Four-micron sections were cut from formalin- or B5-fixed paraffin embedded tissue blocks. The sections were dehydrated and deparaffinized according to standard procedures. For antigen retrieval, the sections were heated in a microwave oven for a total of 30 min in 10 mM/L sodium citrate buffer at pH 6.0. Endogenous peroxidase activity was eliminated by preincubation in 2% H_2O_2 in methanol for 30 min followed by three washes in phosphate buffered saline (PBS). Sections were stained using standard streptavidin-biotin complex immunoperoxidase methods. Primary antibodies used were as follows: $p27^{kip1}$ (1:100, DAKO, Carpinteria, CA, U.S.A.) and MIB-1 antibody (1:20, Biogenex, San Ramon, CA, U.S.A.) produced by immunized mice against recombinant Ki-67 gene products. The slides were counterstained with hematoxylin.

Assessment of immunohistochemical staining

Expression of $p27^{kip1}$ protein and Ki-67 labelling index were assessed. For both $p27^{kip1}$ and Ki-67 immunostaining, only a distinct brown nuclear staining in tumor cells was determined as positive. The number of positively stained cells was counted using a 10×10 square grid fitted into the eyepiece of the microscope. Two to five high power fields were selected for counting positive

nuclei. The scoring system for $p27^{kip1}$ used was less than 10% as 0, between 10% to 30% as 1+, 30% to 60% as 2+, and more than 60% as 3+. Ki-67 labelling index was determined by counting at least 400 tumor cells between 2 to 5 high power fields.

Statistical analysis

Results from clinical investigations, staging, chemotherapeutic response and clinical outcome were studied retrospectively from the records. Kruskal-Wallis test was used for comparing variations between subgroups of clinical parameters and $p27^{kip1}$ expression, and χ^2 test for comparing proportions. Correlation between variables was tested according to Spearman's test. Kaplan-Meier was used to evaluate the relapse-free and overall survival data. A value of $p<0.05$ was considered significant.

RESULTS

Clinical data

The clinical data are summarized in Table 1. Sixty-nine cases included 43 males and 26 females with a mean age of 46.3 yr (range, 4-79 yr), and median follow- up was 24.3 months (range, 1-113 months). Chemotherapeutic response was evaluated and divided into four groups, including complete remission (n=23), partial remission (n=6), stable disease (n=3) and progressive disease (n=11). The majority of patients were clinical

Table 1. Clinical data of 69 cases with nodal non-Hodgkin's lymphomas

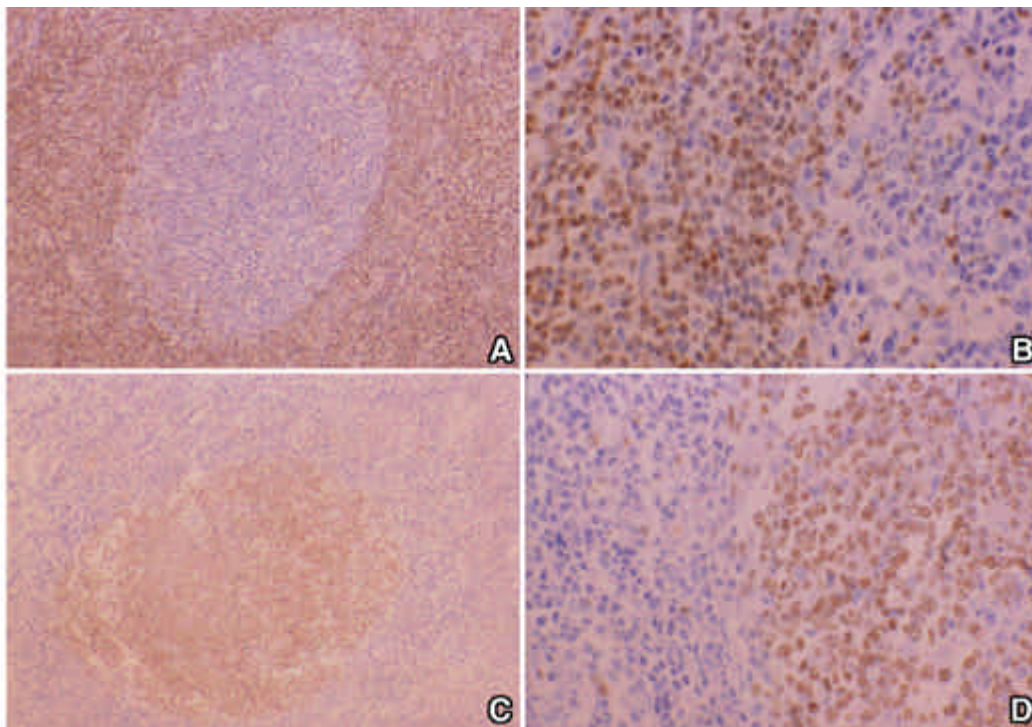|	
Sex	
Male	43
Female	26
Age (yr)	
Range (Mean)	4-70 (46.3)
Clinical follow up (months)	
Range (Median)	1-113 (24.3)
Chemotherapeutic response	
Complete remission	23
Partial remission	6
Stable disease	3
Progressive disease	11
Lost to follow-up / Undetermined	26
Clinical stage	
Stage I	5
Stage II	12
Stage III	12
Stage IV	25
Undetermined	15
Total	69

Fig. 1. Immunostaining for p27^{kip1} and Ki-67 in reactive lymphoid tissue. p27^{kip1} is strongly expressed in the nuclei of mantle cells and interfollicular small lymphocytes, whereas germinal center cells are negative (**A**, ×100; **B**, ×400). The pattern of Ki-67 expression is opposite to that seen with p27^{kip1} (**C**, ×100; **D**, ×400).

stage IV (n=25), and the remainder was stage I (n=5), stage II (n=12) and stage III (n=12). Thirteen patients were alive and twenty-eight were dead. Another twenty-one patients were lost during follow up.

p27^{kip1} and Ki-67 protein expression in reactive lymphoid tissue

A distinct nuclear staining was observed with very low background staining, allowing easy evaluation of the p27^{kip1} expression. In reactive tonsils, p27^{kip1} protein was strongly expressed in the nuclei of mantle cells and interfollicular small lymphocytes. In benign germinal centers, the centroblasts were typically negative whereas centrocytes were weakly to moderately positive forming the dark and light areas of each follicle in the immunostained slides. The pattern of expression was opposite to that seen with the proliferation-related antigen Ki-67. Ki-67 was strongly positive for activated germinal center cells and usually negative for parafollicular small lymphocytes (Fig. 1).

p27^{kip1} protein expression in relation to clinical parameters

The median value of p27^{kip1} positive cells in different lymphoma entities was about 33%. Results of p27^{kip1}

expression for different lymphoma entities were shown in Fig. 2. Two cases of small lymphocytic lymphoma revealed strong p27^{kip1} nuclear staining similar to the intensity seen in T lymphocytes. In follicular lymphomas, polarity of localization seen in reactive germinal center
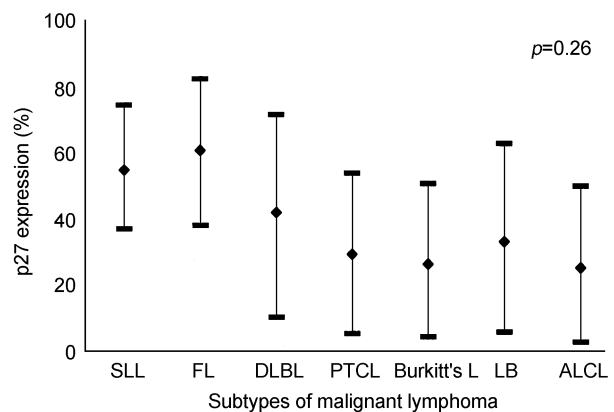

Fig. 2. Diagram shows p27^{kip1} expression for different lymphoma entities. Error bars represent mean values and 95% confidence intervals. There are no significant differences among different lymphoma entities (p=0.26). The histologic subgroups are: SLL (small lymphocytic lymphoma, n=2), FL (follicular lymphoma, n=5), DLBL (diffuse large B cell lymphoma, n=35), PTCL (peripheral T cell lymphoma, n=11), Burkitt's L (Burkitt's lymphoma, n=6), LB (lymphoblastic lymphoma, n=8), and ALCL (anaplastic large cell lymphoma, n=2)
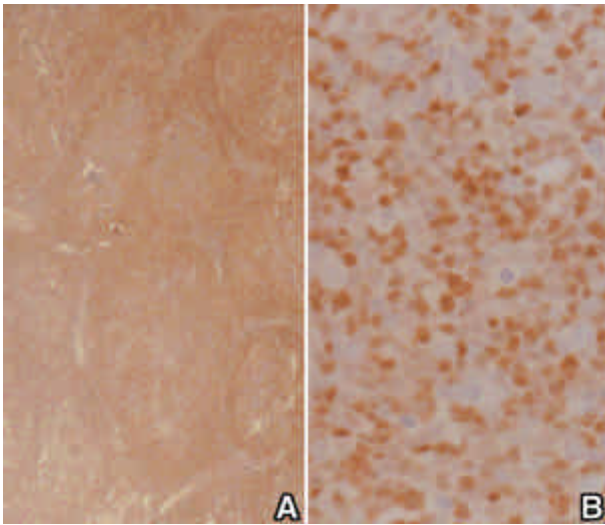
Fig. 3. p27[kip1] staining of a malignant follicles in an follicular lymphoma case. Polarity of localization seen in reactive germinal center disappears with a diffuse mixture of p27[kip1] positive and p27[kip1] negative cells (**A**, ×40; **B**, ×200).

Fig. 4. Expression of p27[kip1] in malignant lymphoma was inversely correlated with Ki-67 labelling indices (r= -.21, p= 0.0151).

disappeared, giving a positive appearance in the neoplastic follicles with a diffuse mixture of p27[kip1] positive and p27[kip1] negative cells (Fig. 3). In more aggressive lymphoma groups including diffuse large B cell lymphoma, peripheral T cell lymphoma, Burkitt's lymphoma, lymphoblastic lymphoma, and anaplastic large cell lymphoma, p27[kip1] expression was variable. Two cases of Burkitt's lymphoma and four cases of lymphoblastic lymphoma showed extremely low p27[kip1] expression. In contrast, the remaining cases of aggressive group showed wide range of p27[kip1] positive cells. There were no significant differences among different lymphoma entities.
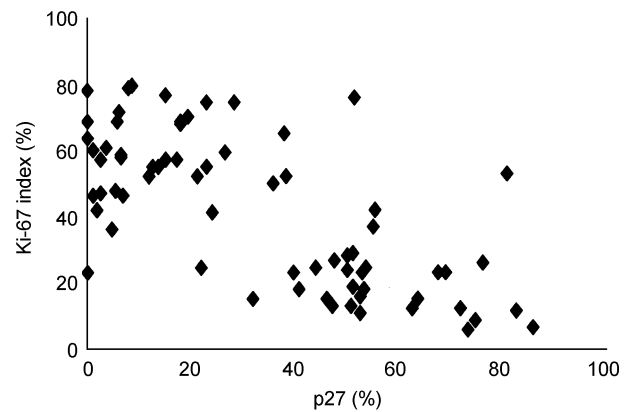
When the study cases were subdivided into indolent and aggressive group, p27[kip1] expression tended to be higher in the indolent group than in the aggressive group. But it was not statistically significant (p=0.26).

The expression of p27[kip1] was inversely correlated with Ki-67 labelling index as illustrated in Fig. 4 and it was statistically significant (r= -.21, p=0.0151). But, some cases demonstrated no linear link between p27[kip1] expression and Ki-67 labelling index. In a case of diffuse large B cell lymphoma consisting of large atypical tumor cells admixed with small reactive lymphocytes, reactive lymphocytes were positive for p27[kip1] and negative for Ki-67. In contrast, the atypical large tumor cells were, in general, positive for Ki-67 and negative for p27[kip1] but some of tumor cells were noted with distinct positive nuclear staining (Fig. 5).
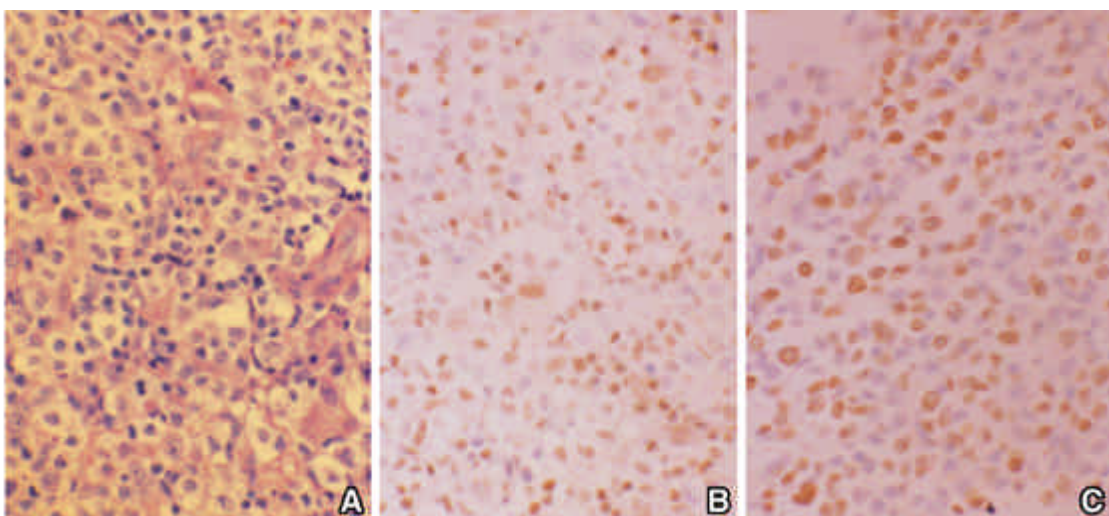

Fig. 5. Histologic findings of diffuse large B cell lymphoma (**A**, H&E, ×200); p27[kip1] is in general, negative for neoplastic lymphocytes but positive for reactive lymphocytes, but some of tumor cells are noted with distinct positive nuclear staining for p27[kip1] (**B**, ×400). In contrast, Ki-67 is positive for large tumor cells and negative for reactive lymphocytes (**C**, ×400).

Table 2. Relation between p27kip1 expression and clinical stages

p27kip1	Stage			
	I	II	III	IV
3+	2 (40%)	1 (8.3%)	1 (8.3%)	7 (28%)
2+	3 (60%)	3 (25%)	5 (41.7%)	6 (24%)
1+		6 (50%)	3 (25%)	3 (12%)
0		2 (16.7%)	3 (25%)	9 (36%)
Total	5 (100%)	12 (100%)	12 (100%)	25 (100%)

$p=0.23$
Scoring system for p27kip1: <10% (0), 10-30% (1+), 30-60% (2+), >60% (3+)

The relation between the p27kip1 expression and clinical stages were summarized in Table 2. Although cases with lower stage, especially stage I, tended to be associated with higher p27kip1 expression, there were no significant differences ($p=0.23$).

The result of p27kip1 expression according to chemotherapeutic response was shown in Fig. 6. Even if cases in the partial remission group and stable disease group were too small in number to compare statistically, there was a tendency for higher p27kip1 expression in the complete remission group that showed statistically significant difference in expression compared to the progressive disease group ($p=0.088$).

Survival analysis

Survival analysis was performed in 52 malignant lymphoma patients, with a median observation time of 46 months. When using 30% as cut off value, there was a significant difference in survival between cases with low and high p27kip1 expressions. Patients with low p27kip1
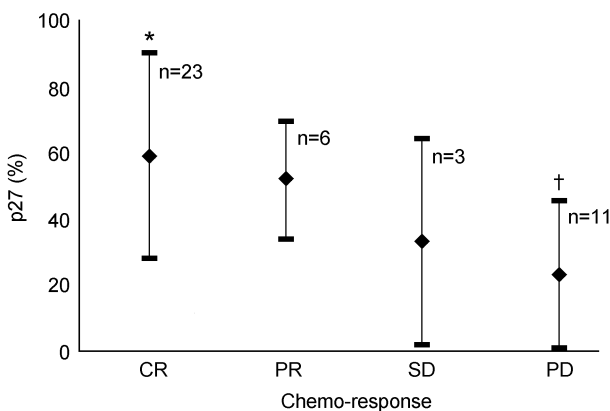

Fig. 6. Results of p27kip1 expression according to chemotherapeutic response. When comparing complete remission (*) and progressive disease group (†) with p27kip1 expression, it is statistically significant ($p=0.0021$) (CR, complete remission group; PR, partial remission group; SD, stable disease; PD, progressive disease group).

Table 3. Results of the mulitivariate Cox analysis for survival rate

Variable	Risk ratio (CI)*	p value[†]
Age[†]	1.05 (1.01-1.08)	0.027
Indolent vs aggressive	1.82 (1.17-3.39)	0.043
Stage[†]	2.11[§](1.20-3.46)	0.021
Complete remmission vs Progressive disease	2.27 (1.10-4.36)	0.012
p27 ≤30% vs >30%	0.60 (0.17-0.95)	0.037
Ki-67[†]		0.088

*CI, 95% confidence interval for the relative risk ratio; [†]From Wald chi-square; [†]Continuous variables; [§]Per step
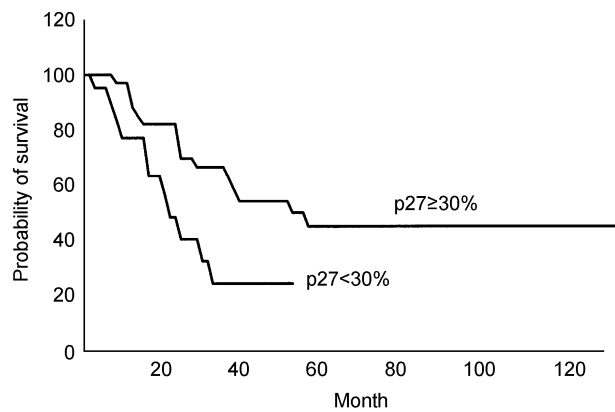

Fig. 7. Kaplan-Meier survival curve. When using 30% as cut off value, there is a significant difference in survival rate ($p=0.0071$).

expression (≤30% positive cells) had a significantly reduced survival rate and two times increased risk for death ($p=0.0071$) (Fig. 7).

Multivariate analysis by Cox was performed in 52 malignant lymphoma patients and presented in Table 3. Low p27kip1 expression (≤30% positive cells) was identified as a novel independent prognostic factor ($p=0.037$). Another independent prognostic factors includes age, grade, stage and chemotherapeutic response. Relative risk ratio for Ki-67 labelling indices was 1.27 but statistically insignificant ($p=0.037$).

DISCUSSION

p27kip1 is a protein of 198 amino acids, the function of which is crucial both for progression from G1 into S phase and for exit from the cell cycle (5). p27kip1 is present in large amounts in quiescent cells, and the level declines when cells proliferate in response to mitogenic signals (8). Recent studies suggest that p27kip1 mediates G1 arrest induced by transforming growth factor β, rapamycin, cAMP, contact inhibition and serum depri-

vation (5, 6, 8, 21, 22). The development of multiple organ hyperplasia and pituitary tumors in $p27^{kip1}$ knockout mice suggests that the loss of $p27^{kip1}$ disturbs the balance between cell cycle activators and inhibitors, leading to an alteration in the balance between proliferating and nonproliferating cells. This underscores the important role of $p27^{kip1}$ as a negative cell cycle regulator (22-25). $p27^{kip1}$ regulates progression from G1 into S phase by binding and inhibiting the cyclin E/CDK2 complex, which is required for entry into S phase (25, 26). Regulation of $p27^{kip1}$ protein occurs primarily through post-transcriptional mechanisms. In addition to ubiquitination, which leads to the degradation of $p27^{kip1}$ protein, $p27^{kip1}$ is regulated at the translational level and by noncovalent sequestration mediated by cyclin D1, which prevents inhibition of the cyclin E-CDK2 complex (5-7, 27, 28).

As a CDK inhibitor, $p27^{kip1}$ has been considered a potential candidate tumor suppressor gene. However, in contrast to p53 and $p16^{INK4a}$, no homozygous deletions and only rare mutations of the $p27^{kip1}$ gene have been found in cell lines or in human tumors (29-32). Although genetic abnormalities of $p27^{kip1}$ have not been detected, recent reports have shown that reduced expression of $p27^{kip1}$ protein correlates with poor survival in breast and colorectal carcinoma patients (13, 15, 18, 33).

In the present study, the authors examined the protein level of $p27^{kip1}$ in a panel of nodal non-Hodgkin's lymphoma specimens and evaluated whether deregulated expression of this cdk inhibitor correlated with clinical parameters. The $p27^{kip1}$ expression was easily evaluated as reported earlier, and even if occasional cells showed cytoplasmic staining, various fractions of clearly $p27^{kip1}$ positive nuclei were detected in the lymphomas (9,18). The authors found that $p27^{kip1}$ expression in normal lymphoid tissue and lymphoid neoplasias is inversely correlated to the proliferation index as measured by Ki-67. Small lymphocytes were $p27^{kip1}$ positive in general, whereas activated cells, such as centroblasts and immunoblasts, displayed weak or no $p27^{kip1}$ staining, suggesting an inverse association between proliferation and $p27^{kip1}$ expression. This association, verified statistically, was expected from the theoretical function of $p27^{kip1}$ as a CDK-inhibitor and cell cycle blocker. Normal lymphocytes also displayed an inverse correlation between $p27^{kip1}$ and proliferation. In the present study, there were inverse correlation between $p27^{kip1}$ expression and Ki-67 indices, which was statistically significant. On the other hand, other reports have observed conflicting results on various tumors (9, 13, 18). In the present study, the authors also observed no inverse relation between $p27^{kip1}$ expression and Ki-67 labelling index in several lymphoma cases. This result suggests that $p27^{kip1}$ may have additional functions that are not strictly related to cell cycle progression. Further studies are needed to clarify additional biologic functions of the $p27^{kip1}$.

In general, $p27^{kip1}$ expression was reduced variably in malignant lymphomas regardless of histologic subtypes. When the study cases were subdivided into indolent and aggressive group, there was a tendency for higher $p27^{kip1}$ expression in the indolent group than in the aggressive group. But it was not statistically significant (p=0.26). These results may be caused by a limited sample size of small lymphocytic lymphoma, follicular lymphoma and anaplastic large cell lymphoma in our studied cases. Additional prospective studies including larger numbers of lymphoma entities will be required.

The authors also found statistically significant correlation between $p27^{kip1}$ expression and chemotherapeutic response and survival rate. In the multivariate Cox analysis, $p27^{kip1}$ expression was highly significant as a independent prognostic factor. Other known risk factors including age, grade, stage and chemotherapeutic response also seemed to have an independent effect on prognosis. Low $p27^{kip1}$ expression has been found to be associated with a poor prognosis in tumors such as breast cancer, lung cancer, colorectal cancer and gastric cancer, suggesting that downregulation of $p27^{kip1}$ is a general phenomenon in malignancies associated with aggressive tumor growth (12, 13, 15, 16, 18).

The adverse effect of low $p27^{kip1}$ values in malignancies like lymphomas is not fully understood, but several contributing explanations are possible. Most obvious is an abrogated cell-cycle block due to downregulation of $p27^{kip1}$, leading to less strict G1/S checkpoint surveillance with facilitated transit into the S-phase. At low $p27^{kip1}$ levels, G0 cells are also recruited into the cell cycle. Therefore, the aberrant $p27^{kip1}$ expression might stimulate the recruitment of tumor cells into the cell cycle, affecting tumor growth fraction. Expression of $p27^{kip1}$ is influenced by cell-to-cell contact, and downregulation of $p27^{kip1}$ can inhibit cell adhesion. Tumors with low $p27^{kip1}$ expression may consequently have an impaired cell adhesion, promoting tumor dissemination.

Recent studies have shown the loss of detectable $p27^{kip1}$ protein expression in typical mantle cell lymphomas and hypothesized that this loss of expression may be related to the high level of cyclin D1 expression in these tumors rather than to the presence of structural abnormalities that occur in other genes with tumor suppressor activity (19). The authors also suggest that the loss of immunologically detectable levels of $p27^{kip1}$ has functional consequences that are likely to play an important role in mantle cell lymphoma tumorigenesis (19). Unfortunately, this study did not include mantle cell lymphoma cases, therefore the effect of $p27^{kip1}$ on this disease cannot be assessed.

In conclusion, our study suggests that reduced expression of p27^{kip1} may play a role in the pathogenesis and biologically aggressive behavior of malignant lymphomas.

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