

Protein Expression of Cell Cycle Regulator, p27^{Kip1}, Correlates with Histopathological Grade of Non-Hodgkin's Lymphoma

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The protein p27^{Kip1} is one of the cyclin-dependent kinase inhibitors that are known to play important roles in the regulation of cell-cycle progression. Low levels of p27 expression in malignant cells are associated with poor prognosis in patients with breast, lung, colorectal and gastric cancers. To determine the relation of cyclin-dependent kinase inhibitors to histopathological grades of B-cell non-Hodgkin's lymphomas, the expression of p27, cyclin D1 and cyclin E in lymph node tissues was investigated in 56 patients with B-cell non-Hodgkin's lymphomas by western blotting and immunohistochemical techniques. High levels of p27 expression were observed in most lymph node tissue samples (93%) obtained from patients with low grade B-cell non-Hodgkin's lymphomas, while expression was low in lymph node tissue taken from all patients with intermediate and high grade B-cell non-Hodgkin's lymphomas. The difference in p27 expression in lymphoma tissues was significant among the different histopathological grades of B-cell non-Hodgkin's lymphomas ($P < 0.01$). The analysis of the survival time of patients showed that the reduction of p27 expression correlated with poor prognosis. Cyclin D1, showed a high level of expression in mantle cell lymphomas and high grade B-cell non-Hodgkin's lymphomas. Cyclin E showed limited expression in 18 of 31 lymphoma tissues. Both cyclin D1 and E protein expression were not significantly different among the grades of B-cell non-Hodgkin's lymphomas. These results demonstrate that the level of p27 expression in lymphoma tissue is an important parameter in the classification of B-cell non-Hodgkin's lymphomas and in the prediction of prognosis.

Key words: p27^{Kip1} — Non-Hodgkin's lymphoma

Non-Hodgkin's lymphoma (NHL) consists of heterogeneous disorders of lymphoid cells derived from either B-cell or T-cell lineages. The precise molecular mechanisms leading to these lymphomas have not been clarified. Recently, several reports have demonstrated a relationship between NHL and the overexpression of proto-oncogenes such as *BCL-1/cyclin D1*,¹⁻⁶ *BCL-2*, *BCL-6*, *C-MYC*⁷ and *NPM/ALK*,^{8,9} the suppression of tumor suppressor gene *p53*¹⁰ and also viral infections such as Epstein-Barr virus and human herpes virus type-8.¹¹ Furthermore, recent molecular studies have suggested that abnormal regulation of the cell cycle is also involved in oncogenesis and the progression of malignant tumors through unrestrained cell growth and cell division.^{12,13}

Many cyclins and cyclin-dependent kinases (CDKs) have been identified as potentiators of the cell cycle¹⁴ and several CDK inhibitors (CDKIs) have also been identified as potential tumor suppressors.¹⁵⁻¹⁹ CDKIs have been

reported to bind to CDKs or the CDK-cyclin complex and thus to inhibit kinase activities.²⁰ Two classes of CDKIs have been postulated. The first class includes p14, p15, p16 and p18, which bind to CDK4 or CDK6 and prevent the formation of the CDK-cyclin D complex.²¹ The formation of CDK-cyclin D complex has been reported to be necessary for the phosphorylation of the retinoblastoma (*RB*) gene which initiates the release of E2F-related transcriptional activators and induces the cells to enter the S phase of the cell cycle.^{5,22,23} Thus, inactivation of the *RB* gene caused by the first class of CDKIs results in the induction of cells into the S phase of the cell cycle and appears to contribute to the formation of malignant phenotypes.

The second class of CDKIs includes p21 and p27, the genes of which are located on chromosome 6p and 12p, respectively.^{24,25} p21 has been reported to play an important role in the pathway related to the repair of cellular DNA damage.²⁶ p27 was first identified as a negative growth regulator present in cells that were rendered quiescent by contact inhibition,^{16,27} and was reported to be also

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present in cells that were treated with transforming growth factor β .^{16–18)} p27 binds to a wide variety of cyclin-CDK complexes containing CDK2 and CDK4, resulting in inhibition of kinase activities and in blockage of cell cycle progression.²⁸⁾ Therefore, p27 is considered to be a member of the cip/kip family of CDK inhibitors, and the gene encoding p27 is considered to be a candidate for a tumor suppressor gene.^{29–31)} However, mutations of the p27 gene have rarely been found in human cancer cells.^{32–34)} Striking findings regarding the significance of p27 in the progression of acute myeloid leukemia, breast, gastric and colorectal cancers have been published.^{32,35,36)} Reduced expression of p27 was correlated with poor prognosis in these patients.^{32,35,36)} However, the level of p27 mRNA in tumor cells was well maintained in several diseases.³⁶⁾ Decreased levels of p27 expression have been suggested to be due to proteasome-dependent degradation of protein. In these diseases, decreased levels of p27 expression may result in impairment of normal cell cycle regulation. This mechanism may be especially important in B-cell non-Hodgkin's lymphomas (B-NHLs), most of which show a sudden increase in tumor cell proliferation after long-term latency.

In the present study, we analyzed the expression of p27, as well as cyclins D1 and E, in different histopathological grades of B-NHLs by western blot and immunohistochemical techniques to determine whether these proteins have an effect on the aggressiveness of the disease.

MATERIALS AND METHODS

Patients and lymph node samples The expression of p27, cyclin D1 and E protein was investigated in the lymph nodes obtained from 56 patients with B-NHLs and eight patients with chronic nonspecific lymphadenitis. The level of p27 expression in cells from three lymphoma cell lines, DAUDI, NAMALWA and N-1, was also investigated. Sections of lymph node from patients with B-NHLs were stained with eosin-hematoxylin and were histopathologically diagnosed. Fifteen lymph nodes were diagnosed as low-grade B-NHLs according to histopathological classification,³⁷⁾ eighteen lymph nodes as intermediate grade (intermediate-grade B-NHLs) and the others as high grade (high-grade B-NHLs). Grading was based on the international working formulation that is strongly correlated with the clinical course of the disease.³⁷⁾ None of the patients had received chemotherapy prior to lymph node biopsy. A portion of the lymph node tissue was minced with scissors and the dispersed cells were utilized for cell culture and cytogenetic analysis.³⁸⁾ The remaining lymph node tissues were stored at -80°C and later used for the analysis of DNA/RNA and proteins. The lymph nodes obtained by our laboratory contained more than 80% tumor cells.

Western blotting The expression of p27 and cyclin D1

and E protein was examined by western blotting. Briefly, lymph node tissues stored at -80°C were washed twice with phosphate-buffered saline (PBS), homogenized and lysed in 500 μl of RIPA buffer (1% Nonidet-p40, 0.5% sodium deoxycholate, 0.1% SDS, 50 $\mu\text{g}/\text{ml}$ of PMSF, 50 $\mu\text{g}/\text{ml}$ of sodium orthovanadate, and 1.5 μl of aprotinin in PBS, pH 7.4). The lysates were centrifuged at 15,000 rpm for 20 min at 4°C . The concentration of protein in the cell lysate was determined using a protein assay kit (BioRad, Hercules, California). A 20 μg aliquot of the cell lysate was mixed with an equal volume of sample buffer (2% SDS, 10% glycerol, 60 mM Tris HCl [pH 6.7], 5% mercaptoethanol and 0.01% bromophenol blue) and heat-denatured at 100°C for 90 s. The denatured protein was applied to a 10% SDS-polyacrylamide gel (using a BioRad Transblot apparatus) and after electrophoresis, the separated proteins were transferred to a nitrocellulose membrane (Hybond ECL, Amersham, Buckinghamshire, England). The membrane was incubated in blocking buffer (4–10% bovine serum albumin [BSA] in PBS-Tween [PBS-T]) for 1 h and then was reacted with monoclonal antibodies against p27, cyclin D1 or cyclin E (1:3000 dilution in PBS-T) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 1 h at room temperature. After having been washed in PBS-T, the membrane was reacted with anti-goat IgG conjugated to horseradish peroxidase (1:3000 dilution in PBS-T; Santa Cruz Biotechnology Inc.) for 15 min at room temperature. Next, the membrane was washed again with PBS-T and the proteins on the membrane to which the horseradish peroxidase was bound were visualized by means of enhanced chemiluminescence (Amersham) according to the manufacturer's instructions.

The intensity of each band that appeared on the film was quantified by densitometry using MacBas-1500 (Fuji Films, Tokyo). The relative intensity of each band was graded, on an arbitrary scale from 0 to 200. The intensity of the bands was classified into three grades: an intensity between 0 to 9 was represented as "negative," a score between 10 to 99 was "low" and an intensity between 100 to 200 was classified as "high" expression. The χ^2 -test was used for comparing the three different histopathological grades and the levels of p27 expression.

Immunofluorescence staining The paraffin-embedded lymph nodes on slides were stained with hematoxylin-eosin to determine the grades and to establish the localization of lymphoma cells. Then the intensity of expression and the localization of p27 in low, intermediate and high-grade lymph node sections were analyzed by immunofluorescence microscopy. The paraffin sections were deparaffinized in xylene, dehydrated and microwaved for 10 min. The slides were washed with PBS and incubated with RNase (0.1 mg/ml) in a humidified incubator for 60 min at 37°C . Then, the sections were reacted with monoclonal antibodies against p27 for 1 h, washed with PBS, and

incubated with anti-goat IgG-FITC (10 mg/ml) (Santa Cruz Biotechnology Inc.) for 30 min at 37°C. They were mounted on slides with "Antifade" (Oncor, Gaithersburg, MD) after washing with PBS. Appropriate negative controls were prepared simultaneously.

Three different observers independently determined the intensity and localization of fluorescence, each observing more than 20 high power fields each. In lymphoma tissue, cells that showed the lowest level of intensity were considered as representing the "intensity" of the tumor tissue.

RESULTS

The expression of p27, cyclin D1 and cyclin E was examined by western blotting and immunohistochemical techniques in lymph nodes obtained from 56 patients with B-NHLs and eight patients with chronic nonspecific lymphadenitis and three lymphoma cell lines (DAUDI, NAMALWA and N-1).

Western blot analysis In low-grade B-NHLs, 93% of the lymph node lysates showed a high level of p27 expression. In the intermediate grade, 50% of the lymph node lysates showed high p27 expression and 50% showed low or no p27 expression. In high-grade B-NHLs, 13% of the lymph node lysates showed low p27 expression and 87% showed no p27 expression. The correlation between p27 expression and the grade of malignancy was statistically significant ($P < 0.01$). Survival analysis was performed at 70 months. As illustrated in Fig. 1, significant differences of survival were observed. The patients were divided into three groups; patients with high p27 expression ($n=6$), with low p27 expression ($n=15$) and with no p27 expression ($n=7$). The patients with low p27 expression were associated with shorter survival time ($P < 0.01$). All patients with high p27 expression survived more than 70 months.

The amount of p27 expressed in the lymph node tissue obtained from patients with low-grade B-NHLs was greater than that in the tissue obtained from patients with both intermediate and high-grade B-NHLs. Most lymph node tissue from patients with intermediate-grade B-NHLs exhibited only a small amount of p27 expression and two of the lymph node samples from high-grade B-NHLs expressed slight but detectable amounts of p27 (Fig. 2, Table I). Three out of eight samples from patients with lymphadenitis showed high expression of p27, while the other five showed low expression of p27. One low-grade follicular lymphoma cell line, N-1, showed high expression of p27, but two lymphoma cell lines, DAUDI and NAMALWA (high-grade lymphoma cell lines), showed low or barely detectable p27 expression. The cyclins D1 and E present in the lymph node tissues were also analyzed by the same methods using monoclonal antibodies against cyclins D1 and E. The results were divided into two groups, "positive" and "negative." Tables II and III

give the number of lymph nodes positive or negative for cyclins D1 and E expression in the different grades of B-NHLs. In low-grade B-NHLs, 62.5% of the lymph node lysates were positive for cyclin D1 expression. In intermediate-grade B-NHLs, 67% of the lymph node lysates were positive for cyclin D1 expression. In high-grade B-NHLs, 100% of the lymph node lysates were positive for cyclin D1 expression. All samples from mantle cell lymphomas were positive for cyclin D1 expression. The levels of cyclin D1 expression, particularly among the high-grade B-NHLs and mantle cell lymphomas, were much higher than levels in low- and intermediate-grade B-NHLs. Cyclin E expression in lymph node lysates was 50% positive in low grade, 29% positive in intermediate grade, 50% positive in high grade and 100% positive in mantle cell lymphomas. The level of cyclin E expression in tumor cells was fairly constant among "positive" lymph nodes.

Immunohistochemical analysis Immunohistochemical analysis was performed on lymph node sections obtained

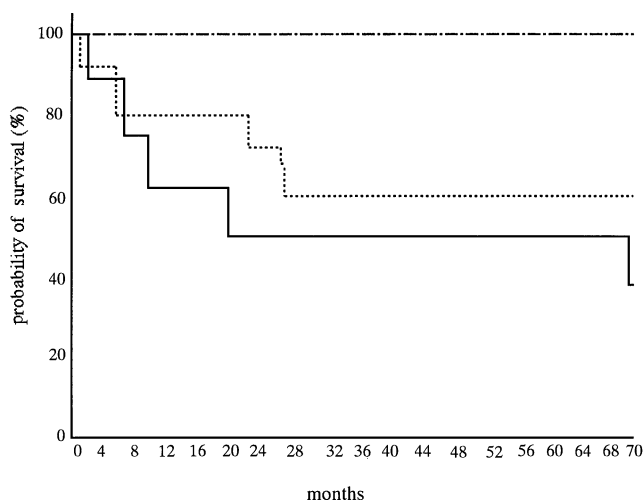


Fig. 1. Overall survival time in relation to p27 expression. p27 expression of the patients was divided into three levels; high ($n=6$, ---), low ($n=15$, ----) and undetectable ($n=7$, —).

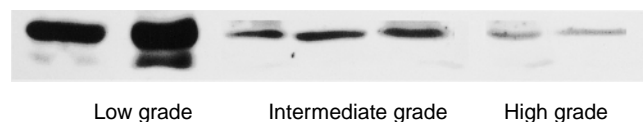


Fig. 2. Expression of protein p27^{Kip1} in B-cell non-Hodgkin's lymphoma. The p27 expression in lymph node tissues from two patients with low-grade B-NHLs was higher than that from three patients with intermediate-grade B-NHLs and two patients with high-grade B-NHLs.

Table I. p27 Expression in 56 Non-Hodgkin's Lymphoma Patients

Diagnosis	Level of p27 expression				
	High	Low	No	Total	
Low grade	14	1	0	15	
Small lymphocytic	3	0	0	3	
Follicular, predominantly small cleaved cell	6	0	0	6	
Follicular, mixed small cleaved and large cell	5	1	0	6	
Intermediate grade	0	9	9	18	
Follicular, predominantly large cell	0	4	4	8	
Diffuse, small cleaved cell	0	2	2	4	
Diffuse, mixed and large cell	0	1	2	3	
Diffuse, large cell	0	2	1	3	
High grade	0	2	13	15	
Large cell immunoblastic	0	0	3	3	
Lymphoblastic	0	0	2	2	
Small noncleaved cell (Burkitt's/non-Burkitt's)	0	2	8	10	
Miscellaneous	0	0	8	8	
Mantle cell lymphoma	0	0	8	8	
	Total	14	12	20	56
Control	4	2	5	11	
Lymphadenitis	3	1	4	8	
Lymphoma cell line	1	1	1	3	

Table II. Cyclin D1 Expression in 38 Non-Hodgkin's Lymphoma Patients

Diagnosis	Cyclin D1 expression			
	Positive	Negative	Total	
Low grade	5	3	8	
Small lymphocytic	3	0	3	
Follicular, predominantly small cleaved cell	0	3	3	
Follicular, mixed small cleaved and large cell	2	0	2	
Intermediate grade	10	5	15	
Follicular, predominantly large cell	1	1	2	
Diffuse, small and large cell	3	2	5	
Diffuse, mixed small and large cell	1	0	1	
Diffuse, large cell	5	2	7	
High grade	7	0	7	
Large cell immunoblastic	1	0	1	
Lymphoblastic	0	0	0	
Small noncleaved cell (Burkitt's/non-Burkitt's)	6	0	6	
Miscellaneous	8	0	8	
Mantle cell lymphoma	8	0	8	
	Total	30	8	38
Control	0	2	2	
Lymphadenitis	0	2	2	

from 22 patients with low, intermediate and high-grade B-NHLs, and from three patients with a non-malignant condition (chronic nonspecific lymphadenitis). The lymph nodes from patients with non-malignancy showed high

levels of p27 expression and more than 30% of the cells in the lymph nodes showed highly intense p27 staining. The cells in the lymph nodes that showed the highest intensity of fluorescence were endothelial cells, small lymphocytes

Table III. Cyclin E Expression in 31 Non-Hodgkin's Lymphoma Patients

Diagnosis	Cyclin E expression		
	Positive	Negative	Total
Low grade	4	4	8
Small lymphocytic	2	2	4
Follicular, predominantly small cleaved cell	1	1	2
Follicular, mixed small cleaved and large cell	1	1	2
Intermediate grade	2	5	7
Follicular, predominantly large cell	1	2	3
Diffuse, small and large cell	0	1	1
Diffuse, mixed small and large cell	0	1	1
Diffuse, large cell	1	1	2
High grade	4	4	8
Large cell immunoblastic	1	0	1
Lymphoblastic	1	0	1
Small noncleaved cell (Burkitt's/non-Burkitt's)	2	4	6
Miscellaneous	8	0	8
Mantle cell lymphoma	8	0	8
Total	18	13	38
Control	0	2	2
Lymphadenitis	0	2	2

and erythrocytes. The basement membrane of the lymph nodes also showed high intensity. These cells served as positive controls. Each lymphoid follicle in lymph nodes from patients with non-malignancy showed different levels of p27 expression although the patterns of p27 expression were heterogenous within a lymphoid follicle. The germinal centers showed the lowest level of p27 expression in every lymphoid follicle. Therefore, in the following experiments, the level of p27 expression was compared with both the intensity of germinal center cells, showing the lowest intensity, and the endothelial cells or small lymphocytes, showing the highest intensity, using confocal laser microscopy.

The analysis of lymph nodes from patients with low-grade B-NHLs revealed high p27 expression, although the pattern of the staining was heterogenous. The p27 expression in the dividing cells was lower than that of the non-dividing cells. In lymph nodes from patients with intermediate and high-grade B-NHLs, the level of p27 expression was low in tumor cells, while non-tumor cells, such as endothelial cells, showed high levels of p27 expression nearly identical to the levels seen in the lymph nodes from patients with non-malignancy. The p27 expression was the highest in the lymph node cell area of patients with non-malignancy, followed by tumor cells in lymph nodes from patients with low-grade B-NHLs. p27 expression was lowest in the tumor cell area of the lymph nodes from patients with intermediate and high-grade B-NHLs. The results of immunofluorescence correlated very well with the results

of western blotting. Representative photographs of p27 expression detected by immunofluorescence staining are shown in Fig. 3.

DISCUSSION

In the present study, we investigated the expression of p27, cyclin D1 and cyclin E in lymph node tissue of Japanese patients with B-NHLs of different histopathological grades. A significant difference in p27 expression was demonstrated in lymph node tissue of the patients with B-NHLs of different grades by both western blotting and immunofluorescence staining. However, the expression of cyclin D1 and E did not significantly differ between grades of B-NHLs.

Low p27 expression in tumor cells has been found to be associated with poor prognosis in patients with breast, lung, colorectal and gastric cancers, suggesting that down-regulation of p27 may be a general phenomenon associated with an aggressive tumor phenotype.^{32, 35, 36, 39, 40} It has been suggested that the downregulation of p27 expression may lead to less strict control of the checkpoint between the G1 and S phases, facilitating the entry of cells into the S phase.⁴¹⁻⁴³ The down regulation of p27 expression may also allow cells in the G0 phase to enter the cell cycle.^{12, 17} Both phenomena associated with the down regulation of p27 expression may also result in uncontrolled proliferation of cells with increased genetic instability. The down-regulation of p27 expression may also affect cell-to-cell

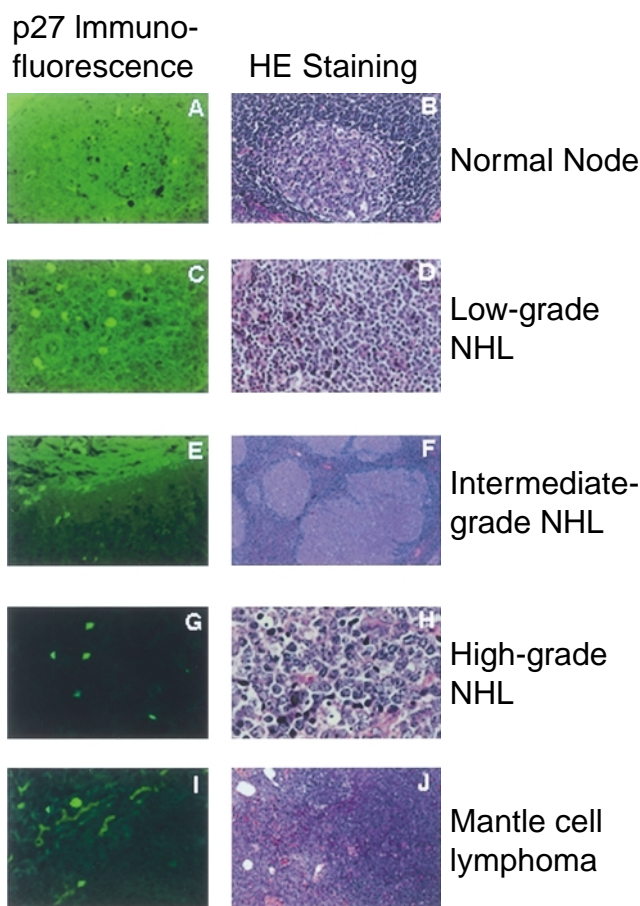


Fig. 3. H-E staining and immunofluorescence staining of p27 in lymph node tissue from patients with various grades of B-NHLs. Histopathological diagnosis was performed on H-E-stained sections (B, D, F, H, J). p27 expression was high in the lymph nodes from a patient with low-grade B-NHL (C) and low in lymph nodes from a patient with intermediate-grade B-NHL (E), high-grade B-NHL (G) and mantle cell lymphoma (I). A and B show sections of lymph node from a patient with non-malignancy (chronic nonspecific lymphadenitis).

contact inhibition, resulting in a decrease in cell adhesion and thus a promotion of tumor dissemination.^{16,44)}

The effect of p27 on the onset of leukemias and lymphomas is not fully understood. Erlanson *et al.* reported that the level of p27 expression may be an independent marker for prognosis of NHLs.⁴⁵⁾ In this study, we extended their work by demonstrating a close relationship between the level of p27 expression and clinical prognosis. Significant differences of survival times depending on the level of p27 expression were observed. A recent study also suggested that downregulation of p27 expression decreases with apoptosis, thereby giving lymphomas with

low p27 expression a growth advantage.⁴⁶⁾ Another report showed that rapamycin upregulates the expression of p27 and induces G1 arrest in exponentially growing T cells. The above observations suggest that p27 expression is an important parameter for diagnosis and also for the prediction of prognosis of B-NHLs. Interestingly, 25% of Burkitt's lymphoma cases, high-grade B-NHLs, showed a moderate level of p27 expression, while other high-grade B-NHLs did not show any p27 expression, suggesting that Burkitt's lymphoma may represent a different mechanism of tumor progression.

Cyclin D1 has been reported to be overexpressed in many malignant diseases and in transformed cells. Cyclin D1 is a putative proto-oncogene encoded by the *CCND1* gene on chromosome 11q13,⁴⁷⁾ and has been reported to be overexpressed through gene rearrangement or gene amplification mechanisms in several human tumors, including parathyroid adenoma, breast cancer, centrocytic lymphoma and other B-cell tumors with translocation t(11;14).^{1,2)} In this study, high-grade B-NHLs and mantle cell lymphomas showed a high level of cyclin D1 expression, while most of the B-NHLs showed various levels of cyclin D1 expression, as reported in other literature.⁴⁸⁾

In the present study, we could not find any relationship between cyclin E expression and the histopathological grade of B-NHLs. A small amount of cyclin E was expressed in relatively few cells and the variation in cyclin E expression in the different grades of B-NHLs was not significant. Other investigators have also failed to find any relationship between cyclin E expression and the proliferation rate of tumor cells.^{49,50)} On the contrary, Erlanson *et al.* reported a significant correlation between cyclin E expression and the grade of NHLs and also tumor aggressiveness.⁴⁵⁾ The expression of cyclin E, which is important in the cell cycle, may be aberrant in patients with B-NHLs and may be related to lymphomagenesis.

In the present study, it was demonstrated that p27 expression is an important parameter for the classification and prediction of prognosis of patients with B-NHLs. Aberrant expression of cyclins D1 and E shown in this study may indicate impaired checkpoints of the cell cycle in B-NHLs, that lead to malignant transformation.

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