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# Prognostic Significance of Hypermethylation and Expression of *p16*, *p21*, and *p53* Genes in Diffuse Large B-cell Lymphoma

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**Abstract :** Diffuse large B-cell lymphoma (DLBCL) accounts for approximately 30—40% of non-Hodgkin's lymphomas. Significant molecular prognostic markers of DLBCL are not well known. In this study, hypermethylation of *p16*, *p21* and *p53* gene and protein expression of *p16*, *p21*, and *p53* in DLBCL were investigated with 91 paraffin blocks. Hypermethylation of *p16* gene was more common than hypermethylation of *p21* or *p53* in DLBCL. Hypermethylation of *p21* gene was highly related to recurrence and reduced expression of p21 protein showed a strong tendency of poor survival. For an individual patient with DLBCL, if the tumor shows hypermethylation of *p21* gene, it may make to predict the increased risk of recurrence. If the tumor shows reduced p16 expression, and/or methylation of *p53* gene, it is important to consider that this markers are related to higher international prognostic index, which help to predict outcome of patients.

**Key Words :** Large B-cell, Lymphoma, methylation, prognosis, *p16*, *p21*, *p53*

## Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common disease of aggressive lymphomas and accounts for approximately 30—40% of non-Hodgkin's lymphomas. Biologically and clinically, DLBCL shows considerable heterogeneity and usually occurred de novo, but it may arose from transformation of indolent lymphoma, such as follicular lymphoma, marginal zone B-cell lymphoma, or chronic lymphocytic leukemia /small lymphocytic lymphoma [1]. At the time of diagnosis it is important to identify patients

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who may benefit from more aggressive therapies, such as high dose chemotherapy [2].

Hypermethylation of cytosine residues of promoter CpG islands represses gene transcription, and is an alternative mechanism of gene inactivation [3-5]. In many malignant tumors, the CpG islands of tumor suppressor genes were aberrantly methylated. CpG hypermethylation of the *p16* promoters in DLBCL has been found in 27—46% of cases [6-8]. But hypermethylations of *p21* and *p53* were rarely studied in DLBCL. In this study, we investigated hypermethylations and immunohistochemical expressions of p16, p21 and p53 in DLBCL. We analyzed the correlation of these results with clinical parameters including recurrence, stage, IPI, and survival.

## Materials and Methods

### Tissue specimens and clinical study :

One hundred forty six cases of DLBCL were reviewed. The patients were diagnosed at Dongsan Medical Center from 1996 to 2005. We chose 91 cases of paraffin block in which remained enough tissue. Clinical parameters including age, sex, primary site, stage, recurrence, B symptoms, and survival status were reviewed retrospectively. The survival status of patients was evaluated until October 1st, 2006.

### DNA extraction :

Paraffin sections were deparaffinized and dehydrated. The tissues were digested by lysis buffer (10 mmol/L, Tris-HCl, pH 8.3, 1 mmol/L EDTA, 1% SDS, and 500 μg/mL proteinase K) at 48℃ for 48 hours. After inactivating the action of proteinase K at 99℃ for 10 minutes, the genomic DNA was available for polymerase chain reaction (PCR). All possible precautions were taken to avoid contamination.

### Methylation-specific PCR (MSP) :

Extracted DNA was subjected to a deamination reaction by incubation with sodium bisulfite, hydroquinone and sodium hydroxide at 50℃ for 16 hours. After removal of free bisulfite using the Wizard DNA purification resin (Promega, Madison, USA) according to the manufacturer's instruction, the modified DNAs were desulfonated with sodium hydroxide (final concentration, 0.3 M) for 5 minutes at room temperature. The DNAs were purified by ethanol precipitation [9]. For unmethylated control of MSP, DNA extracted from mononuclear cells of normal volunteer were used. For methylated control, methylated normal DNAs by SssI methylase (New England Biolabs, MA, USA) were used. For negative control, distilled water was used. The purified DNA was amplified by PCR using primers for specific modified methylated DNA or primers for specific modified unmethylated DNA (Table 1). The products were analyzed by electrophoresis on a 4% Metaphor agarose gel (FMC, Rockland, USA).

### Tissue microarray :

Tissue microarray was constructed from formalin-fixed paraffin-embedded specimens of 91 DLBCL. In each sample, the part

**Table 1.** Sequences of Primers Used in Methylation-specific PCR

| | Primer | Sequence | Size (bp) | AT (℃) |
|---|---|---|---|---|
| *p16* | M-sense | 5'TTATTAGAGGGTGGGGCGGATCGC3' | 150 | 65 |
| | antisense | 5'GACCCCGAACCGCGACCGTAA3' | | |
| | U-sense | 5'TTATTAGAGGGTGGGGTGGATTGT3' | 151 | 60 |
| | antisense | 5'CAACCCCAAACCACAACCATAA3' | | |
| *p21* | M-sense | 5'TTTCGGGGAGGGCGGTTTCGGGCGGCGCGG3' | 181 | 65 |
| | antisense | 5'CGATACCTCGACGAATCCGC3' | | |
| | U-sense | 5'GGTGGTGTGGTGGGTTGAGT3' | 132 | 62 |
| | antisense | 5'ACAAATCCACACCCAACTCC3' | | |
| p53 | M-sense | 5'GTAGTTTGAACGTTTTTATTTTGGC3' | 115 | 60 |
| | antisense | 5'CCTACTACGCCCTCTACAAACG3' | | |
| | U-sense | 5'GTAGTTTGAATGTTTTTATTTTGGT3' | 115 | 55 |
| | antisense | 5'CCTACTACACCCTCTACAAACA3' | | |

AT: Annealing temperature; M: methylated; U: unmethylated.

composed of tumor cells was selected by light microscopic examination and was used for tissue microarray. Cores measuring 0.5 cm in diameter were taken from the donor paraffin blocks of DLBCL, and rearranged in the recipient paraffin blocks using a manual tissue arrayer (MTA-1, Sun Prairie, WI, USA).

### Immunohistochemistry and interpretation :

Conventional 4 ㎛ sections were obtained from the tissue microarray blocks and incubated in an oven at 60℃, overnight. Sections were then dewaxed in xylene for 10 minutes and rehydrated through graded alcohol to distilled water. Activity of endogenous peroxidases was blocked with 3% hydrogen peroxide in methanol for 15 minutes. Subsequently, sections were subjected to antigen retrieval by microwaving in 10 mmol/L citrate buffer (pH 6.0) for 15 minutes at high power. Used primary antibodies were p16 (1:800, Neomarker, CA, USA), p21 (1:500, Lab vision, CA, USA), and p53 (1:1000, Novocastra, New Castle, UK). All of these slides were stained by BenchMark XT IHC/ISH Staining Module (Ventana medical systems, AZ, USA).

Immunohistochemical stain for p16 showed positive in nuclei or cytoplasms of the tumor cells. It was interpreted as score 0 (< 10%), score 1 (10—50%), score 2 (> 50%, weak), and score 3 (> 50%, strong). Immunohistochemical stains for p21, and p53 show positive in nuclei of the tumor cells. For p21, the results of immunostaining were interpreted as score 0 (< 10%) and score 1 (≥ 10%). For p53, the results of immuno-

staining were interpreted as score 0 (< 10%), score 1 (10—25%), score 2 (26—50% or > 50%, weak), and score 3 (> 50%, strong).

### Statistical analysis :

Pearson's $\chi^2$ test and Spearman's correlation analysis were used to determine the relationships between clinical features, hypermethylations of *p16*, *p21*, and *p53* genes, and immunohistochemical expression of p16, p21, and p53 proteins. Results were considered to be significant only when their *p*-value were less than 0.05.

Survival analysis was performed for 60 clinically available DLBCL patients. Overall survival was plotted using the Kaplan and Meier method and statistical significance was determined by the log rank test. Data were analysed with the SPSS system software (version 12.0, SPSS INC., Chicago, IL, USA).

## Results

### Patient characteristics :

The population of DLBCL patients consisted of 42 men and 49 women. Their age ranged from 9 to 90 years old. At the time of diagnosis, the mean age of the patients was 57.3 years old. There were 30 cases of nodal DLBCL and 61 cases of extranodal DLBCL. The major clinical features are shown in Table 2.

**Table 2.** Summary of Clinical Features of Patients

| Characteristics | No. of patients |
|---|---|
| Age (n = 91) | |
| ≥ 60 | 45 |
| < 60 | 6 |
| Sex (n = 91) | |
| Male | 42 |
| Female | 49 |
| B Symptoms (n = 58) | |
| A (absent) | 40 |
| B (present) | 18 |
| Recurrence (n = 57) | |
| Present | 12 |
| Absent | 45 |
| Primary site (n = 91) | |
| Lymph node | 30 |
| Extranodal | 61 |
| Ann Arbor stage (n = 60) | |
| 1 | 15 |
| 2 | 17 |
| 3 | 18 |
| 4 | 10 |
| IPI (score) (n = 53) | |
| Low (0,1) | 23 |
| Intermediate (2,3) | 27 |
| High (4,5) | 3 |

IPI: international prognostic index.

### MSP for *p16*, *p21*, and *p53* :

The purified DNAs were amplified by MSP in 87 cases for *p16* gene, 82 cases for *p21* gene, and 86 cases for *p53* gene out of 91 DLBCLs. Promotor hypermethylations were found in 31/87 cases (35.6%) for *p16*, 5/82 cases (6.1%) for *p21*, and 3/86 cases (3.5%)

for *p53* in MSP of DLBCL (Fig. 1). All three cases with *p53* hypermethylation revealed unmethylation of *p16* gene. Among of them, two cases revealed unmethylated *p21* gene (Table 3).

### Immunohistochemical Expression of *p16*, *p21*, and *p53* :

Immunohistochemical stain for p16 showed positivity in the nuclei and cytoplasms of tumor cells. Immunohistochemical stains for p21, p53 showed nuclear staining patterns (Fig. 2). Staining for p16 was positive in 67.1%, p21 was positive in 64.8%, and p53 was positive in 97.8% (Table 4).

### Comparision of MSP and immunohistochemical stains :

Twenty one cases with hypermethylation of *p16* gene were positive immunohisto-

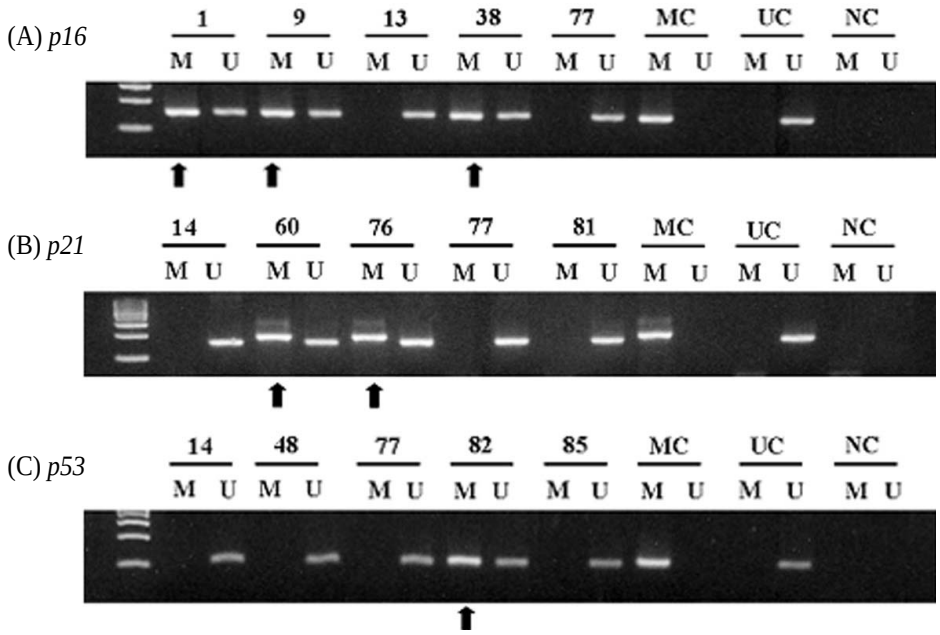


**Fig. 1.** Methylation-specific PCR analysis for *p16* (A), *p21* (B), and *p53* (C) genes in diffuse large B cell lymphomas. M: methylated; U: unmethylated; MC: methylated control; UC: unmethylated control; NC: negative control.

**Table 3.** Methylation Status of *p16* and *p21* according to *p53* in 91 Diffuse Large B-cell Lymphomas

| | | *p16* | | | *p21* | | |
|---|---|---|---|---|---|---|---|
| | | M | U | NA | M | U | NA |
| *p53* | M (n=3) | 0 | 3 | 0 | 0 | 2 | 1 |
| | U (n= 83) | 31 | 50 | 2 | 5 | 72 | 6 |
| | NA (n=5) | 0 | 3 | 2 | 0 | 3 | 2 |

M: methylated; U: unmethylated; NA: not amplified.

chemical stain for p16 protein. Five cases with hypermethylation of p21 gene showed positive immunohistochemical expression for p21 protein. Three cases with hypermethylation of *p53* gene showed positive expression for p53 protein (Table 5).

### Statistical analysis:

Results of immunohistochemical staining for p16 showed inversely related with stage ($r = -0.315$, $p = 0.019$) and IPI ($r = -0.291$, $p = 0.034$). Results of methylation-specific PCR for *p53* showed positive correlation with presence of B symptoms ($r = 0.291$, $p = 0.029$) (Table 6). Hypermethylation of *p21* gene was significantly related to recurrence ($p < 0.001$).

Patients with hypermethylation of *p16* gene were found a tendency of longer survival ($p = 0.258$). The hypermethylations of *p21* and *p53* showed no differences in overall survival between methylated and unmethylated groups. Patients with positive expression for p21 protein showed a strong tendency of longer survival ($p = 0.057$) (Fig. 3).

**Table 4.** Immunohistochemical Expressions for p16, p21 and p53 in 91 Diffuse Large B-cell Lymphomas

| Score | p16 | p21 | p53 |
|---|---|---|---|
| 0 | 30 (32.9%) | 32 (35.2%) | 2 (2.2%) |
| 1 | 36 (39.6%) | 59 (64.8%) | 38 (41.8%) |
| 2 | 12 (13.2%) | | 40 (43.9%) |
| 3 | 13 (14.3%) | | 11 (12.1%) |

**Table 5.** Comparison of the Immunohistochemical Expression and Methylation-specific PCR for *p16*, *p21*, and *p53*

| Score | *p16* MSP (n = 87) | | *p21* MSP (n = 82) | | *p53* MSP (n = 86) | |
|---|---|---|---|---|---|---|
| | M | U | M | U | M | U |
| 0 | 10 | 18 | 0 | 30 | 0 | 2 |
| | (32.3%) | (32.0%) | (0%) | (39.0%) | (0%) | (2.1%) |
| 1 | 14 | 21 | 5 | 47 | 1 | 35 |
| | (45.1%) | (37.5%) | (100%) | (61.0%) | (33.3%) | (45.8%) |
| 2 | 4 | 7 | | | 2 | 35 |
| | (12.9%) | (12.5%) | | | (66.7%) | (45.8%) |
| 3 | 3 | 10 | | | 0 | 11 |
| | (9.7%) | (18.0%) | | | (0%) | (13.3%) |

IHC: immunohistochemical stain; MSP: methylation-specific-PCR; M: methylated; U: unmethylated.

**Table 6.** Correlation between Expression or Methylation Status of Cell Cycle Regulator Molecules and Clinical Indicators

| Clinical indicators | p16-IHC score | | | | *p53*-MSP | | |
|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | M | U | NA |
| Stage (n=60) | | | | | | | |
| I | 3 | 7 | 3 | 2 | 0 | 15 | 0 |
| II | 4 | 7 | 3 | 3 | 1 | 16 | 0 |
| III | 8 | 6 | 1 | 3 | 0 | 17 | 1 |
| IV | 6 | 4 | 0 | 0 | 1 | 8 | 1 |
| | $r$ = -0.315, $p$ = 0.019 | | | | $r$ = 0.094, $p$ = 0.502 | | |
| IPI (n=53) | | | | | | | |
| 1 | 6 | 10 | 5 | 2 | 1 | 22 | 0 |
| 2 | 6 | 8 | 1 | 3 | 0 | 17 | 1 |
| 3 | 6 | 2 | 1 | 0 | 0 | 8 | 1 |
| 4 | 2 | 1 | 0 | 0 | 1 | 2 | 0 |
| | $r$ = -0.291, $p$ = 0.034 | | | | $r$ = 0.074, $p$ = 0.607 | | |
| B symptom (n=58) | | | | | | | |
| Absent | 13 | 18 | 4 | 5 | 2 | 39 | 1 |
| Present | 8 | 5 | 3 | 2 | 0 | 15 | 1 |
| | $r$ = -0.057, $p$ = 0.672 | | | | $r$ = 0.291, $p$ = 0.029 | | |

IHC: immunohistochemical stain; MSP: methylation-specific-PCR; IPI: international prognostic index; $r$: Spearman's correlation coefficient; $p$: $p$-value.

## Discussion

Overexpression of *p53* is an well known adverse prognostic indicator in many malignancies as well as DLBCL, but expression of other cell cycle regulators have not been extensively studied as prognostic indicators in DLBCL [10,11].

Hypermethylation of the *p16* promoters has been reported in colon, bladder, breast, and lung carcinomas, gliomas, leukemias, and lymphomas [12,13]. It is also one of common epigenetic alterations in DLBCL. Previous study reported that *p16* hypermethylation was found in 17 of 46 cases (36.9%) of large B cell lymphoma and overall survival is dependent on the accumulation of alterations in *p53*, *p16*, and *p27* [7]. In this study, 31 of 87 cases (35.6%) showed hypermethylation of *p16* gene. Among the 31 cases which revealed hypermethylation of *p16*, *21* cases showed immunohistochemical expression for

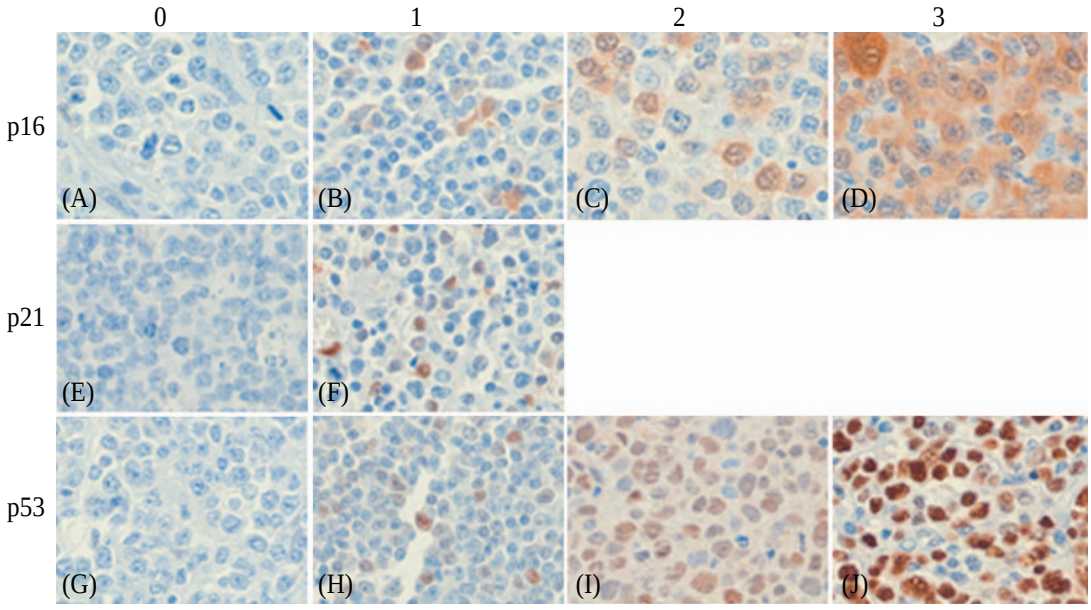


**Fig. 2.** Immunohistochemical stains for p16, p21, and p53 in diffuse large B-cell lymphomas. Immunohistochemical stains for p16 show positivity in nuclei or cytoplasms. Scores for p16 protein expression (a-d) range from 0 to 3. (a) 0: < 10% of the tumor cells. (b) 1: 10-50%. (c) 2: > 50%, weakly. (d) 3: > 50%, strongly. Immunohistochemical stains for p21 and p53 show positivity in the nuclei. Expressions of p21 protein (e and f) score 0 and 1. (e) 0 (negative): < 10% of the tumor cells. (f) 1 (positive): ≥ 10%. Scores for p53 protein expression (g-j) range from 0 to 3. (g) 0: < 10% of the tumor cells. (h) 1: 10-25%. (i) 2: 26-50% and/or > 50%, weakly. (j) 3: > 50%, strongly.

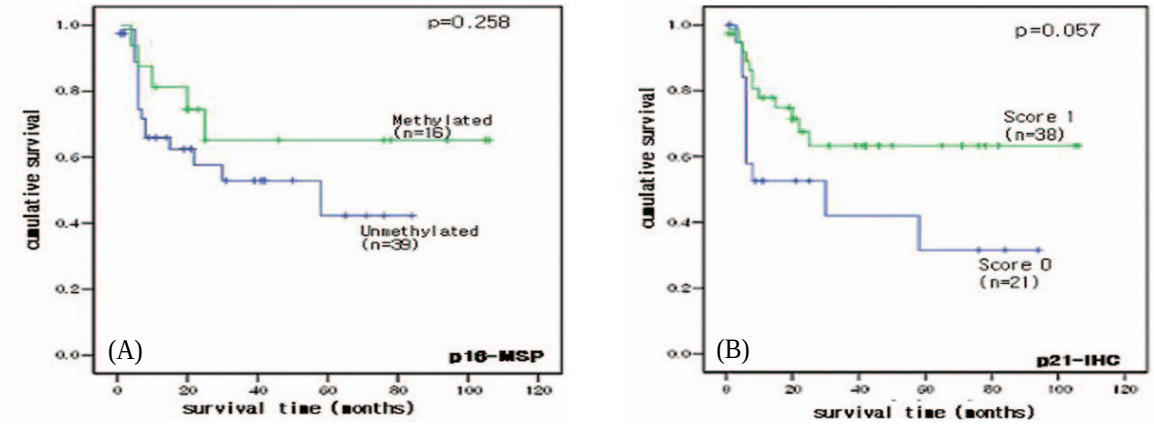


**Fig. 3.** Overall survival according to methylation status of *p16* and immunohistochemical expression of p21 in diffuse large B cell lymphoma. Hypermethylation of *p16* gene (A) and positive expression of p21 (B) show tendencies of longer survival.

p16 protein. Especially, 7 cases showed positive for p16 protein in more than 50% of tumor cells. These results represented that other abnormalities to make overexpression of p16 protein would be present in these cases. Appearing of this discordance in

DLBCLs is in contrast with colon cancers. In colon cancers, loss of p16 expression is correlated with DNA methylation of its promoter. It indicates that specific genes show hypermethylation in specific tumors [14,15]. In this study, tendency of longer survival was noted in patients with hypermethylation of *p16* gene. It is adverse to Shiozawa *et al*. [16] who reported that hypermethylation of *p16* is an useful indicator of poor survival in IPI high-risk patients.

Several studies in a wide variety of cancers have shown that alteration in p21 expression plays an important role in promoting the development or progression of human malignancies [17]. *p21* hypermethylation is rare in various lymphomas and carcinomas but demonstrated in acute lymphoblastic leukemia (ALL), natural killer cell (NK cell) disorders, rhabdomyosarcoma, and lung cancer[18-21]. Ying *et al*. [22] reported that only 3 out of 100 lymphomas exhibited p21 methylation and *p21* methylation was not found in DLBCL. In this study, hypermethylation of *p21* gene was 6.1% (5/82 cases) and it was very strongly correlated to recurrence. Other study about ALL reported that methylation of the *p21* gene was associated with poor prognosis, aggressiveness, and/or refractoriness [21]. However, Kawamata et al. [20] reported that methylation of the *p21* gene might not be associated with aggressiveness in NK cell disorders. In this study, immunohistochemistry for p21 protein was negative in 35% and showed a tendency of poor survival ($p$=0.057). Li *et al*. [17] reported that reduced p21 expression may provide prognostic information in gallbladder carcinomas. Also, Aoyagi *et al*. [23] reported that the prognosis of p53(+)/p21(-) cases in primary gastric lymphoma were poor.

The *p53* gene could be inactivated usually by genetic alterations of the gene by point mutation, deletion or less commonly by rearrangement. Mutation and rearrangement of *p53* gene were associated with 7.2—10.5% in non-Hodgkin lymphomas [24]. Also, it was reported that methylation of *p53* gene was important during hepatocarcinogenesis [25]. But studies for *p53* hypermethylations in DLBCL are rare. In this study, only three cases showed hypermethylation of *p53* gene. Two cases were negative in immunohistochemical study for p53 and they did not show hypermethylation of *p53* gene. So, reduced immunohistochemical expression of *p53* protein in DLBLC may not closely connected to hypermethylation of *p53* gene.

In conclusion, hypermethylation of *p16* gene is more common than hypermethylation of *p21* and *p53* in DLBCL. Hypermethylation of *p16* gene showed a tendency of longer survival. Reduced immunohistochemical expression of p16 protein was related to higher IPI and advanced stage. Hypermethylation of *p21* gene is highly related to recurrence. Reduced p21 expression showed a strong tendency of poor survival. If the tumor shows reduced p16 and p21 expression, and/or hypermethylation of *p16* or *p53* gene, it should be considered that these markers are related with clinical parameters to help for predicting outcome of patients such as IPI, stages, and presence or absence of B symptoms. Because the results of survival analysis failed to show significance and the correlation between biological markers and clinical parameters were relatively weak, so further studies including

more cases and other biological markers will be needed.

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