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Clinicopathologic significance of PELI1 expression and its correlation with MYC in malignant lymphoma

악성 림프종에서 PELI1 과발현의 임상 및 병리학적 중요성과 MYC 과의 관련성

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ABSTRACT

Background: Overexpression of Myc is known to guarantee a universally aggressive clinical course in B-cell lymphomas. The prototypic mechanism of *MYC* activation is *MYC/IGH* rearrangement, however, an alternative mechanism of Myc dysregulation is suspected in cases of Myc high tumors without structural alteration of *MYC* gene. This study aimed to explore possible link of Myc overexpression and E3 ubiquitin ligase PELI1 which regulates protein activity through post-translational modification.

Methods: Immunohistochemistry (IHC) for Myc and PELI1 was performed in a total of 495 cases including B, T or NK-cell and Hodgkin lymphomas, with additional IHC for BCL2, BCL6, CD10, IRF4/MUM1, CD5, CyclinD1, Ki-67 and Myc in diffuse large B-cell lymphoma (DLBCL) and mantle cell lymphoma (MCL). Flourescence in situ hybridization (FISH) for *MYC* rearrangemnt and *MYC* amplification was performed in DLBCL and MCL cases. Immunohistochemical expression of PELI1 was confirmed with western blot and real time RT-PCR in 14 malignant lymphoma cell lines.

Results: High expression of Myc or PELI1 was found in high grade B-cell lymphoma cases such as DLBCL, Burkitt lymphoma and

plasmablastic lymphoma, whereas low grade B-cell lymphoma, T / NK-cell lymphoma and Hodgkin lymphoma cases showed very low level of expression. The IHC results of PELI1 were concordant with in vitro cell line studies; RL7, Pfeiffer, SUDHL-2, DOHH2 and Ramos cell lines showed high levels of PELI1 protein and also mRNA. In DLBCL, PELI1 expression was positively correlated with expression of Myc, BCL6, BCL2 (all $p \leq 0.001$) and IRF4/MUM1 ($p=0.066$). In DLBCL, high expression of PELI1 was associated with frequent bone marrow involvement ($p=0.013$) and shorter disease-free survival ($p=0.002$). In MCL, high Myc expression was significantly frequent in blastoid/pleomorphic variants and was associated with shortened overall survival and disease-free survival by univariable and multivariable analyses (all $P < 0.05$).

Conclusions: These results suggest that PELI1 might participate in the pathogenesis of aggressive B-cell lymphomas, in collaboration with Myc. Overexpression of Myc or PELI1 can predict inferior outcome in patients with DLBCL or MCL.

Keywords: PELI1 protein; Lymphoma, B-cell; Proto-Oncogene Proteins c-myc; Proto-Oncogene Proteins c-bcl-6; Proto-Oncogene Proteins c-bcl-2

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INTRODUCTION

MYC, involved in the transcription of almost 15% of all genes regulating cell growth and cell cycle progression, is one of the most frequently deregulated oncogenes in human malignancies.(1) The rearrangement of *MYC* is the frequent and standard mechanism of Myc aberration, however, high expression of Myc can be also found in some cases of malignant lymphoma without demonstrable structural alteration of *MYC* gene.(2)

PELI is a small regulatory molecule which functions as an E3 ubiquitin ligase tagging proteins to be destroyed by the proteasome. Like other ubiquitin ligases, PELI takes part in intracellular enzymatic post-translational modification in animal cells. Among the three homolog forms of PELIs, particularly, PELI1 has been known to have a pivotal role in inflammatory and autoimmune processes mediating toll-like receptor signalling and regulating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) transcription activity. In addition, there is growing evidence that PELI is linked to proliferation and activation of B and T lymphocytes proven by in vitro experiments.(3, 4) However, the role of PELI1 in the oncogenesis has not been fully elucidated.

Recently, Park et al revealed PELI1 induced B-cell lymphomas through promotion of BCL6 stabilization in transgenic mice. It is very well known that lymphocytic development is highly dynamic, genetically unstable process, and also biology of lymphoma depends on the level of cell maturation at the corresponding stage of lymphocytic development.(5) Although little is known about PELI in the lymphomagenesis except BCL6, we suspected PELI1 plays a role in cooperation with other B-cell signaling molecules or Myc oncoprotein in a certain stage of lymphomagenesis.

Herein, we investigated PELI1 expression in 17 types of malignant lymphoma of B or T cell lineage, and analyzed its clinical significance and correlation with BCL2, BCL6, MUM1/IRF4 and Myc expression in diffuse large B-cell lymphoma (DLBCL) and mantle cell lymphoma (MCL).

MATERIALS AND METHODS

Patients

Samples were obtained from 495 patients diagnosed with malignant lymphoma at three university hospitals in Korea during 2005 to 2012. Diagnosis was confirmed by two experienced hematopathologists (JYC and JEK) according to the 2008 WHO classification.(6) Clinical data were obtained from electronic medical records. This study was approved by the Institutional Review Board of Seoul National University Boramae Hospital (IRB No. 16-2013-38) on April 2013.

Immunohistochemistry (IHC)

Tissue microarray blocks (TMAs) were constructed with a pair of 2-5mm wide core tissues from the most representative areas of paraffin-embedded blocks, and IHC was performed using TMAs. IHC for PELI1 (F-7, Santa Cruz Biotechnology, CA, USA) was conducted on all 495 cases. Additional IHC was done in diffuse large B-cell lymphoma (DLBCL) cases (N = 182) with following antibodies; Myc (Y69; Epitomics, CA, USA), BCL2 (M0887, Dako, CA, USA), BCL6 (LN22, Novocastra, Newcastle, UK), CD5 (M7194, Dako), CD10

(PA0270, Novocastra), IRF4/MUM1 (M7259, Dako) and Ki-67 (MIB1, Dako). Epstein-Barr virus (EBV) in situ hybridization (ISH) was performed in DLBCL, Hodgkin lymphoma (HL) and T or NK-cell lymphomas using an EBV-encoded RNA (EBER) probe (INFORM EBER Probe; Ventana Medical Systems). IHC and EBV ISH were performed using the Bench Mark automatic immunostainer (Ventana Medical Systems, Tucson, AZ, USA) with validated protocols.

Evaluation of IHC was quantitatively or semi-quantitatively graded for each antibody. Nuclear proteins (Myc, BCL6, IRF4/MUM1 and Ki-67) were measured by a percentage of positive tumor cells using automated image analysis software with slide scanner (Aperio, Vista, CA, USA). Cytoplasmic or membranous positivity of with/without nuclear staining (PELI1, BCL2 and CD10) was manually calculated using H score; 4 tiered intensity scores (0, absent; 0.1, faint; 1, weak; 2, moderate; 3, strong) multiplied by frequency scores (0, <1%; 0.1, 1-10%; 0.5, 10-50%; 1, 50-100%) of positive tumor cells.

Western blot analysis for PELI1

Expression of PELI1 protein was investigated by western blot with the same antibody as IHC in 14 malignant lymphoma cell lines. Cell lines consisted of Ramos, RL7 (lymphoblastic lymphoma, BL), Pfeiffer,

DOHH2 {DLBCL, germinal center B cell (GCB)}, SUDHL-2 (DLBCL, non-GCB), SP53, REC (mantle cell lymphoma, MCL), IM-9 (multiple myeloma), Jurkat (T-cell lymphoma), Karpas299, SUDHL-1 (anaplastic large cell lymphoma), NK-YS (NK/T-cell lymphoma), L428 and KMH2 (HL). Ramos, IM-9 and Jurkat were purchased from Korean cell line bank (Seoul, Korea). DOHH2, SP53, Karpas299, L428 and KMH2 cell lines were kindly provided by Dr. Francisco Vega-Vazquez (University of Miami, USA). REC and NK-YS were gifts from Dr. Yoon-kyung Jeon (Seoul National University Hospital, Seoul, Korea). Other cell lines obtained from American Type Culture Collection
(ATCC, VA, USA).

Western blot was performed as previously described.(7) Briefly, cells in log-phase growth were prepared, and 20 μg of protein resolved in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis was transferred to polyvinylidene difluoride membranes. The blot was incubated with primary antibodies against PELI1 (1:1000, Santa cruz) in TBST (TBS containing 0.05% Tween 20) with 5% skim milk at 4°C for overnight. The membranes were incubated for room temperature at 1 h with horseradish peroxidase conjugated anti-mouse IgG (Thermo Fisher Scientific, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) for PELI1

PELI1 mRNA levels were detected with RT-PCR in the same series of lymphoma cell lines. Total RNA was isolated using a TRIzol reagent (Thermo) and cDNAs were synthesized using the Maxima First Strand cDNA Synthesis Kit (Thermo). RT-PCR was performed with HotStarTaq Master Mix Kit (QIAGEN, CA, USA) using primers specific for PELI1(199 bp; forward, 5′ -TGTAGTAACTGACACGGTTCCT-3′ and reverse, 5′ -TCCATCTGATGTCTTCCATTTGG-3′) and GAPDH (452 bp; forward, 5′ -ACCACAGTCCATGCCATCAC-3′ and reverse, 5′ - TCCACCACCCTGTTGCTGTA-3′) genes.

Fluorescence in situ hybridization (FISH) for *MYC* rearrangement and *MYC* amplification

FISH was performed on TMA sections of DLBCL and MCL cases according to the manufacturer's protocols. A Vysis LSI MYC dual-color, break-apart rearrangement probe (Abbott Molecular, Abbott Park, IL, USA) was used to detect *MYC* translocation, and a Vysis MYC 8q24 probe mixed with a CEP8 probe (Abbott) was used for amplification. At least 100 cells from each case were assessed for split signals to identify *MYC* translocation and *MYC* copy numbers.

Statistical analysis

Spearman's rho was used to assess the correlation between protein expressions. Mann-Whitney U test and Fisher's exact test were performed to compare IHC results according to clinicopathologic variants. Overall survival (OS) was calculated from the date of diagnosis to the date of death, and disease-free survival (DFS) was calculated from the date of diagnosis to the date of disease progression or death. Univariable Kaplan-Meier survival functions with log rank tests were estimated to compare between binary groups based on protein expression. Multivariable survival analysis using the Cox multiple regression model was performed to identify independent prognostic markers. The results were considered statistically significant with a two-tailed p-value of < 0.05. All data were analysed with SPSS software, version 22.0 (SPSS Inc., IBM, NY, USA).

RESULTS

Expression of PELI1 in the normal lymphoid and malignant lymphoma cells

In the reactive lymph nodes, PELI1 was selectively positive in lymphocytes of the germinal centers (GCs) with both nuclear and cytoplasmic localization. Outside the GCs, PELI1 positive cells were absent or rarely identified. In mantle zone and marginal zone, PELI1 was negative. In paracortical area, PELI1 was almost negative in small lymphocytes but weakly positive in a few stromal cells such as endothelial cells (Figure 1).

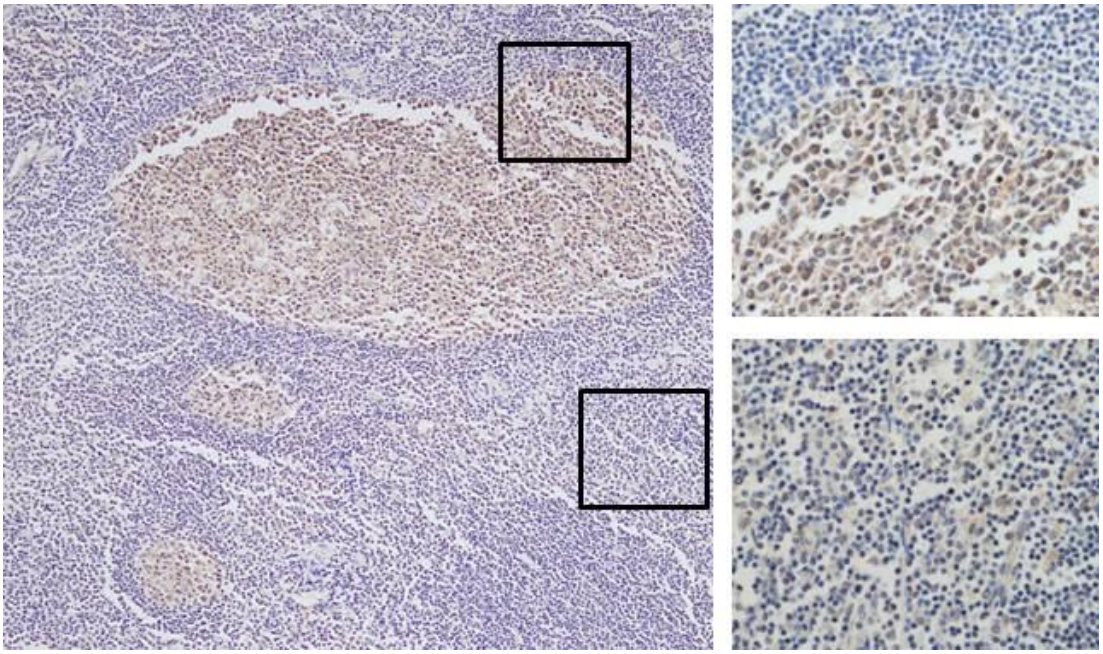


Figure 1. Immunohistochemical expression of PELI1 in normal lymphoid tissue. PELI1 expression was high in germinal center lymphocytes and absent or low in mantle zone and marginal zone (right upper). In paracortex and medulla, only a few stromal cells show PELI1 expression (right lower).

In malignant lymphoma tissues, PELI1 was variably positive according to the types of malignant lymphoma with both nuclear and cytoplasmic localization. Overall, PELI1 expression was higher in B-cell type lymphoma (mean ± SD, 0.92 ± 0.85) than that of T- or NK-cell lymphoma (0.25 ± 0.39) or HL (0.06 ± 0.06) ($p<0.001$). Among the all types of malignant lymphoma, DLBCL, BL and plasmablastic lymphoma (PBL) showed the highest levels of PELI1 expression. Other B-cell lymphomas including follicular lymphoma, mantle cell lymphoma, small lymphocytic lymphoma, lymphoplasmacytic lymphoma and nodal/extranodal marginal zone lymphoma showed low levels of PELI1 expression. Immature T- or B-cell lymphoma showed also low levels of PELI1 expression. T- or NK-cell lymphomas and HL showed very low levels of PELI1 protein (Figure 2, 3A, Table 1).

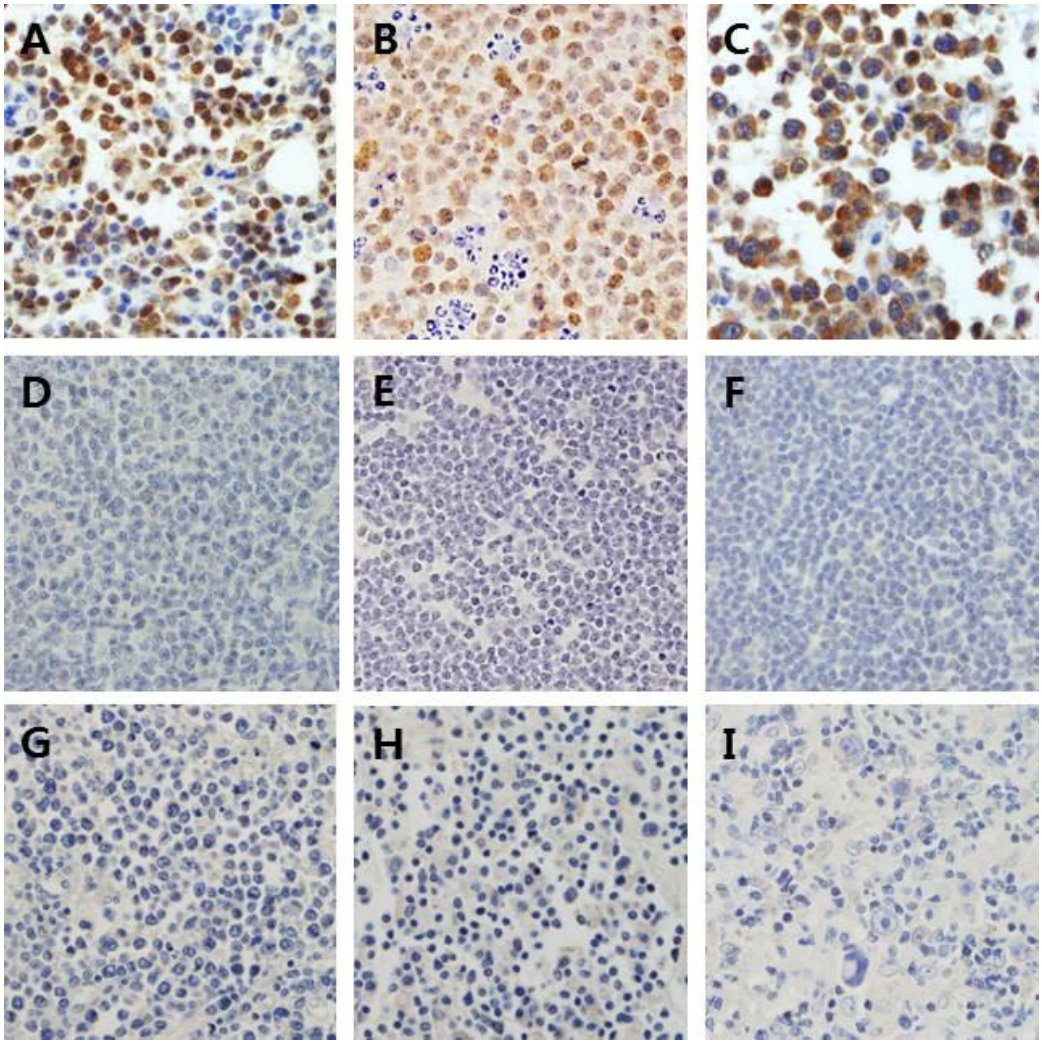


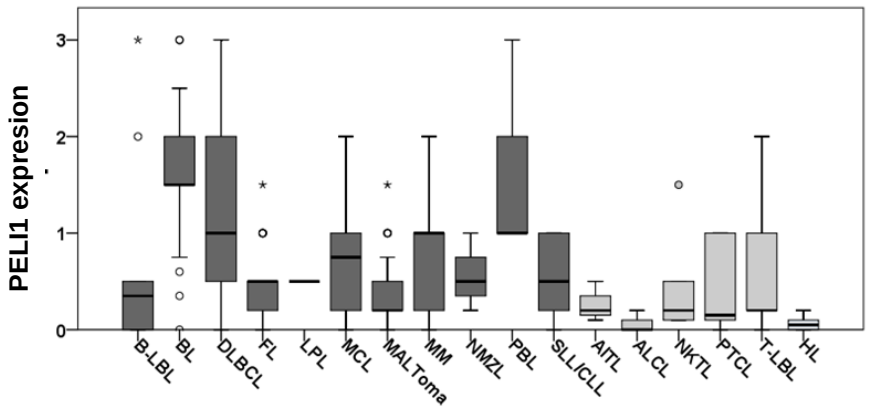
Figure 2. Immunohistochemical expression of PELI1 in various malignant lymphomas. Diffuse large B-cell lymphoma (A), Burkitt lymphoma (B) and plasmablastic lymphoma (C) showed the highest levels of PELI1 expression. Other low grade B-cell lymphoma including follicular lymphoma (D), mantle cell lymphoma (E) and small lymphocytic lymphoma (F) showed low level of PELI1 expression. T/NK-cell lymphomas such as exranodal NK/T-cell lymphoma (G) and angioimmunoblastic lymphoma (H) or Hodgkin lymphoma (I) show negative or very low level of PELI1 expression.

Table 1. PELI1 immunohistochemical expression in various type of lymphomas

Variables	No. of Cases	PELI1 expression (H score*)	
	(%)	Mean ± SD	Median (range)
Total	495 (100.0)	0.80 ± 0.83	0.50 (0.0-3.0)
B-cell lymphoma	406 (81.8)	0.92 ± 0.85	0.55 (0.0-3.0)
B-LBL	10 (2.0)	0.69 ± 1.01	0.35 (0.0-3.0)
BL	21 (4.2)	1.64 ± 0.81	1.50 (0.0-3.0)
DLBCL	187 (37.7)	1.26 ± 0.94	1.00 (0.0-3.0)
FL	33 (6.7)	0.44 ± 0.34	0.50 (0.0-1.5)
LPL	2 (0.4)	0.5 ± 0.00	0.50 (0.5-0.5)
MCL	22 (4.4)	0.65 ± 0.50	0.75 (0.0-2.0)
MALToma	70 (14.3)	0.33 ± 0.09	0.20 (0.0-1.5)
MM	21 (4.2)	0.93 ± 0.65	1.00 (0.0-2.0)
NMZL	4 (0.8)	0.55 ± 0.33	0.50 (0.2-1.0)
PBL	5 (1.0)	1.60 ± 0.89	1.00 (1.0-3.0)
SLL/CLL	30 (6.0)	0.51 ± 0.16	0.50 (0.0-1.0)
T-cell lymphoma	76 (15.4)	0.25 ± 0.39	0.1 (0.0-2.0)
AITL	8 (1.6)	0.25 ± 0.16	0.20 (0.1-0.5)
ALCL	36 (7.3)	0.05 ± 0.06	0.00 (0.0-0.2)
NKTL	6 (1.2)	0.43 ± 0.54	0.20 (0.1-1.5)
PTCL	8 (1.6)	0.44 ± 0.47	0.15 (0.0-1.0)
T-LBL	18 (3.6)	0.53 ± 0.27	0.20 (0.0-2.0)
Hodgkin lymphoma	14 (2.8)	0.06 ± 0.06	0.05 (0.0-0.2)

B-LBL, B-lymphoblastic lymphoma; BL, Burkitt lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; MALToma, extranodal marginal zone lymphoma of MALT; MM, multiple myeloma; NMZL, nodal marginal zone lymphoma; PBL, plasmablastic lymphoma; SLL/CLL, small lymphocytic lymphoma/chronic lymphocytic leukemia; AITL, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large cell lymphoma; NKTL, extranodal NK/T-cell lymphoma; PTCL, peripheral T-cell lymphoma; T-LBL, T-lymphoblastic lymphoma

*H-score was calculated by frequency of positive cells (0 (<1%), 0.1(<10%), 0.5(<50%), 1(≤100%)) x intensity of staining (0, 1, 2, 3).

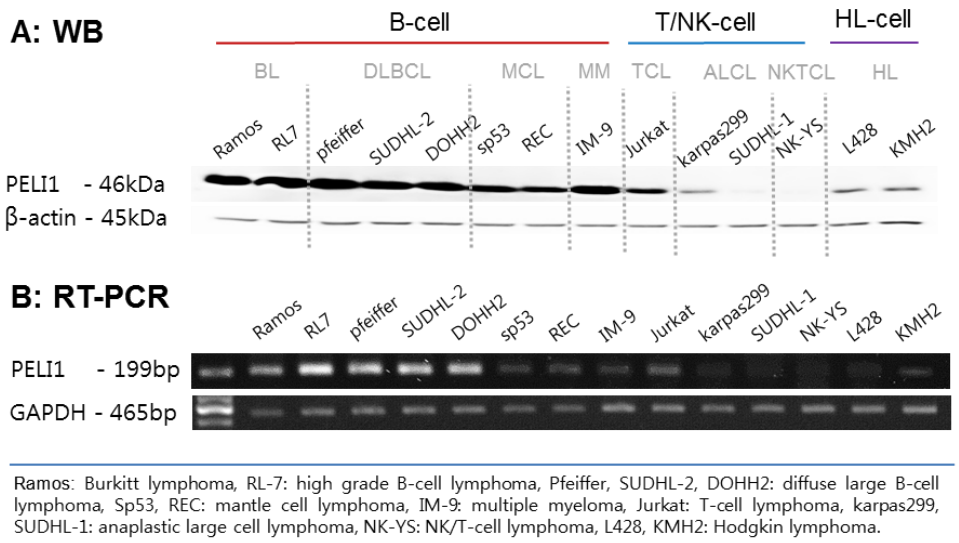


B-LBL, B-lymphoblastic lymphoma; BL, Burkitt lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; MALToma, extranodal marginal zone lymphoma of MALT; MM, multiple myeloma; NMZL, nodmal marginal zone lymphoma; PBL, plasmablastic lymphoma; SLL/CLL; small cell lymphoma/chronic lymphocytic leukemia; AITL, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large cell lymphoma; NKTL, extranodal NK/T-cell lymphoma; PTCL, peripheral T-cell lymphoma; T-LBL, T-lymphoblastic lymphoma; HL, Hodgkin lymphoma.

Figure 3. PELI1 expression levels in various malignant lymphomas.

Immunohistochemical (IHC) expression of PELI1 was variable according to types of malignant lymphoma. Some high-grade B-cell lymphomas showed high levels of PELI1 expression. However, low-grade B-cell lymphoma, T-cell lymphoma and Hodgkin lymphoma showed low level of PELI1 expression.

These IHC results were concordant to the results of western blot (Figure 4A) and also those of RT PCR (Figure 4B). DLBCL (Pfeiffer, SUDHL-2 and DOHH2) and BL cell lines (Ramos, RL-7) showed high levels of PELI1 protein and mRNA, whereas MCL cell lines (Sp53 and REC) showed lower levels of PELI1. Multiple myeloma (MM) cells (IM-9) displayed moderate density of PELI1. Lymphoma cells of T or NK lineage (Jurkat, Karpas299, SUDHL-1, NK-YS), and HL cell lines (L428 and KMH2) showed very low levels of PELI1 protein and mRNA.



Ramos: Burkitt lymphoma, RL-7: high grade B-cell lymphoma, Pfeiffer, SUDHL-2, DOHH2: diffuse large B-cell lymphoma, Sp53, REC: mantle cell lymphoma, IM-9: multiple myeloma, Jurkat: T-cell lymphoma, karpas299, SUDHL-1: anaplastic large cell lymphoma, NK-YS: NK/T-cell lymphoma, L428, KMH2: Hodgkin lymphoma.

Figure 4. PELI1 protein and mRNA levels in various malignant lymphoma cell lines. Western blot (A) results were concordant with immunohistochemistry in various lymphoma cell lines. PELI1 mRNA expression by RT-PCR (B) was similar with those of IHC and Western blot.

Clinicopathologic correlation of PELI1 in DLBCL patients

To find clinical significance of PELI1 in malignant lymphoma, we analysed clinicopathologic correlation in DLBCL cases, which showed distinctively high PELI1 levels among the various lymphoma types. Detailed profiles of182 DLBCL patients were presented in Table 2. The median follow-up of the patients was 42.6 months (range 0-261); remission, either complete or partial was achieved in 133/145 patients (91.7%) with 80 cases (55.2%) of deceased and 42 (31.8%) recurrent cases. Patients with higher PELI1 expression showed frequent bone marrow involvement (p=0.013) in DLBCL. Otherwise, there was no significant association between PELI1 expression and other clinical parameters including age, International Prognostic Index score, Ann Arbor stage and performance status. Also, PELI1 expression was not significantly associated with HANS classification, CD5 positivity or EBV positivity.

Table 2. Patient demographics of diffuse large B-cell lymphoma

Variables		**No of cases (%)**
Total		**182**
Age	Range (median)	8-87 (61)
Sex	Male	109 (40.1)
Primary site	Lymph node	117 (65.4)
IPI score	Low risk (0-1)	66 (41.0)
	Intermediate risk (2-3)	65 (40.4)
	High risk (4-5)	30 (18.6)
Ann Arbor stage	I-II	96 (55.2)
	III-IV	78 (44.8)
Extranodal site	≥2	35 (20.0)
LDH	Elevated	89 (56.0)
ECOG score	≥2	39 (22.5)
BM involvement	Yes	26 (15.8)
Bulky disease	Yes	24 (13.7)
B symptom	Yes	46 (26.4)
Hans classification	Germinal Center B-cell type	62 (34.3)
	Non-GCB type	119 (65.7)
Histologic subtypes	CD5-positive	15 (8.4)
	EBV-positive	12 (6.7)
Initial treatment	R-CHOP	86 (49.4)
	CHOP	55 (31.6)
	Other chemotherapy	23 (13.2)
	Supportive care only	10 (5.7)
Chemoresponse	Complete or partial response	133 (91.7)
	Stable or progressive disease	12 (8.3)
Follow-up (months)	Range (median)	0-261 (42.6)
	Dead/Alive/Missed	80/95/7
	Recurrence	42 (31.8)

IPI, international prognostic index; LDH, lactic dehydrogenase; ECOG, Eastern Cooperative Oncology Group; BM, bone marrow; EBV, Epstein Barr virus; R-CHOP, rituximab, cyclophosphamide, vincristine, adriamycin and prednisone

However, PELI1 expression was significantly correlated with expression of Myc, BCL6 and BCL2 (r=0.427, 0.507 and 0.246 respectively, all $p \leq 0.001$), and also marginally correlated with IRF4/MUM1 expression (r=0.137, p=0.066) (Figure 5, Table 3).

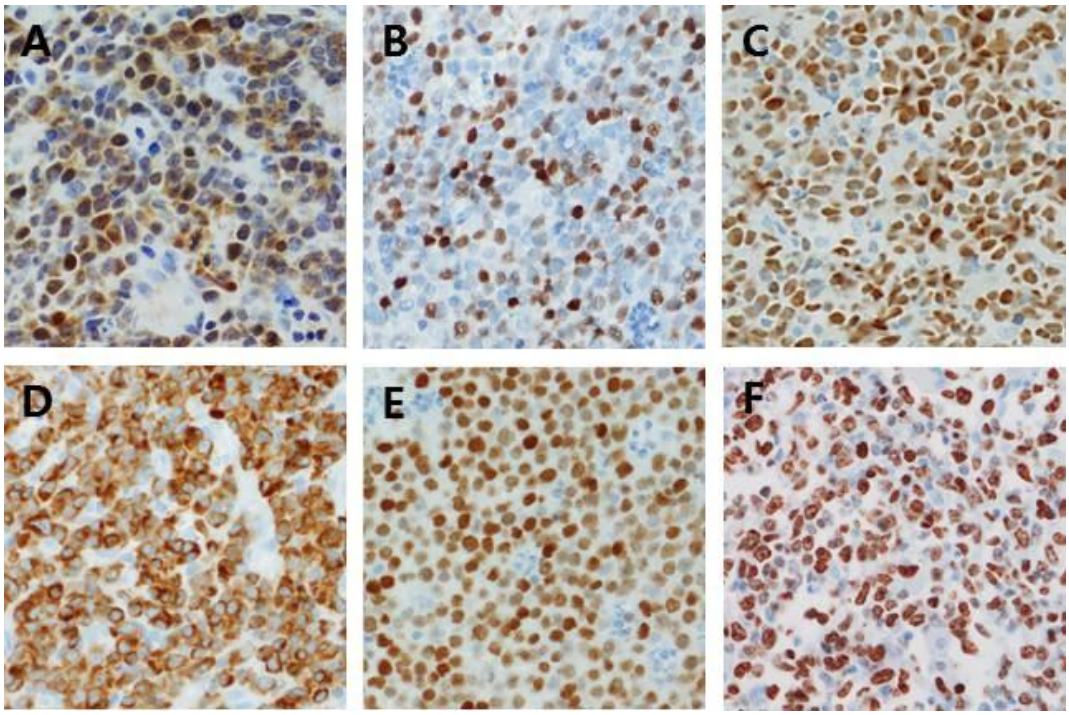


Figure 5. Immunohistochemical expression of PELI1 and correlated proteins in diffuse large B-cell lymphoma. PELI1 (A) was correlated with Myc (B), BCL6 (C) and BCL2 (D). PELI1 and IRF4/MUM1 (E) also had a tendency of correlation. Ki-67 proliferation index (F) was not correlated with PELI1.

Table 3. Correlations between PELI1 expression and other proteins in diffuse large B-cell lymphoma

Variables		**Ki-67**	**Myc**	**Bcl-6**	**Bcl-2**	**Mum1**
Peli1	r	0.108	0.427*	0.507*	0.246*	0.137
	p	0.156	<0.001	<0.001	0.001	0.066
	N	173	177	180	181	181
Ki-67	r		0.426*	0.204*	0.017	0.174*
	p		<0.001	0.007	0.820	0.022
	N		170	172	173	173
Myc	r			0.467*	0.157*	0.257*
	p			<0.001	0.037	0.001
	N			174	177	176
Bcl-6	r				0.031	0.051
	p				0.683	0.053
	N				180	178
Bcl-2	r					0.274*
	p					<0.001
	N					181

r, correlation coefficient of Spearman's rho; p, p-value

Fluorescence in situ hybridization (FISH) for MYC rearrangement and MYC amplification

FISH for *MYC* gene alteration was performed in 182 DLBCL, 6 PBL cases and in additional 53 cases of MCL. *MYC* rearrangement was detected in 3 of 89 DLBCL, 1 PBL and 2 MCL cases. *MYC* amplification (*MYC* gene copy number > 4) was found in 4 of 81 available DLBCL cases, and 2 of 53 MCL cases. Low polysomy or trisomy of *MYC* gene (*MYC* gene copy number < 4) was also found in 2 and 1 DLBCL cases, respectively (Figure 6). *IgH/MYC* translocation and *MYC* amplification was mutually exclusive. In DLBCL and PBL, expression of Myc is not different between the cases with structural alteration of *MYC* and the cases without it. Correlation between PELI1 and Myc expression was attenuated but sustained in the cases without structural alteration of *MYC* (r=0.268, p=0.052). In MCL cases, all cases harboring structural alteration of *MYC* showed high Myc IHC scores (range 61~ 85%, median 68.5%).

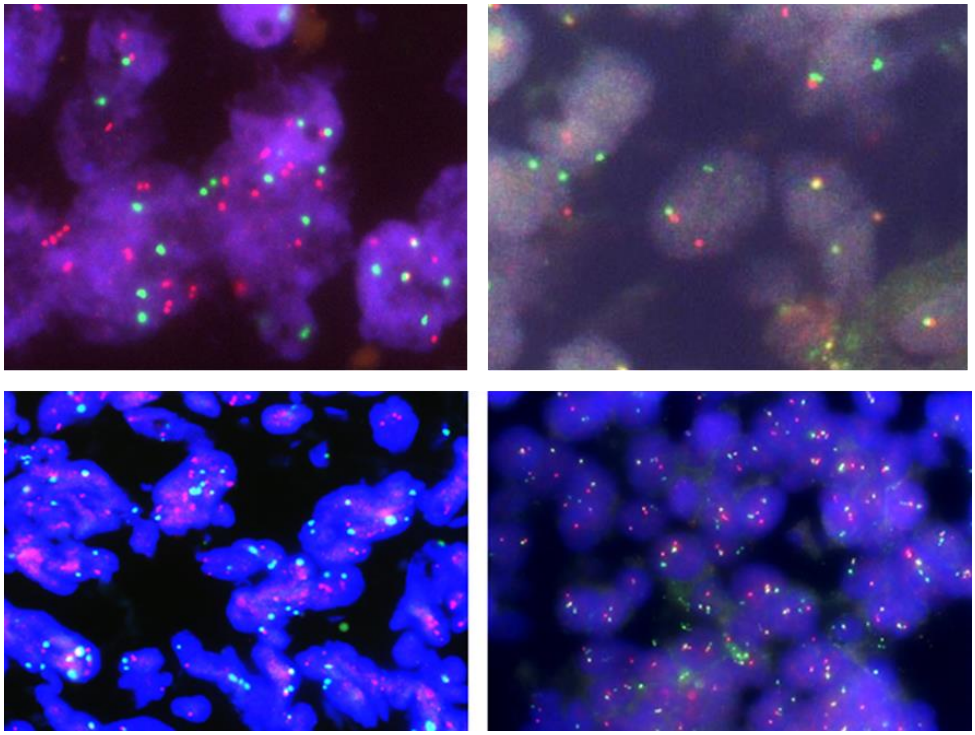


Figure 6. Representative features of diffuse large B-cell lymphoma (upper) and high grade mantle cell lymphoma (lower) showing *MYC* amplification (left) and translocation (right) by break-apart FISH.

Prognostic significance of Myc or PELI1 in DLBCL and MCL

A receiver operating characteristic (ROC) curve was drawn to identify the most sensitive and specific cut-off points for the expression of PELI1 and Myc regarding patient survival. Then, according to the ROC curves, cases were dichotomized into two groups based on the expression levels of each protein, as follows: "PELI1 high" (>2 by H score) and "PELI1 low (≤2 by H score); "Myc high" (>20%) and "Myc low" (≤20%). In DLBCL, patients with high PELI1 expression had shorter DFS but not OS than those with low PELI1 expression (p=0.002) by univariable analysis. Also, patients having high Myc expressing tumors had shorter OS and DFS than those with low Myc expressing tumors (p=0.003, p=0.036, respectively) (Figure 7). The cases with IgH/MYC translocation or MYC amplification showed no difference in OS or DFS.

In MCL, ROC curve for PELI1 was not generated because uniformly low expression. Patients harbouring high Myc IHC scores had shorter OS and DFS than those with low IHC scores ($P < 0.05$) (Figure 8).

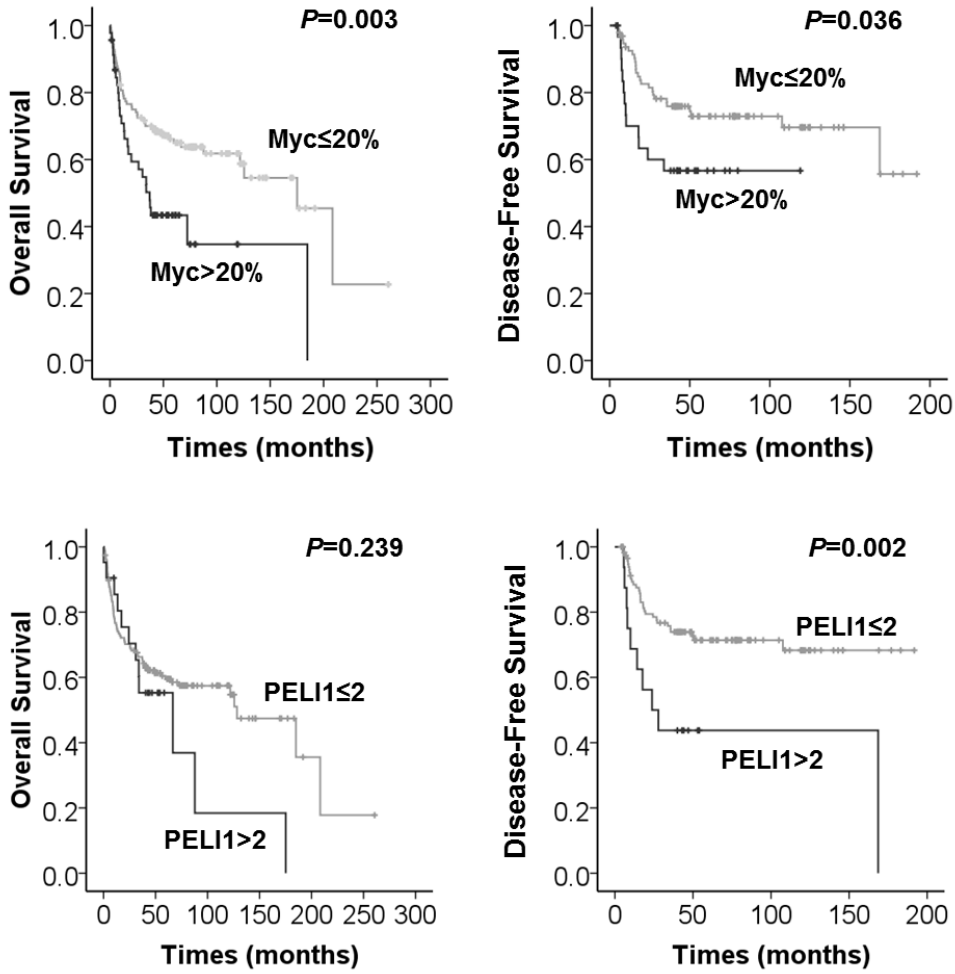


Figure 7. Survival analyses of diffuse large B-cell lymphoma patients with Myc and PELI1 expression levels. High PELI1 group (≥2, H score) showed significantly worse prognosis in disease-free survival (DFS), but not in overall survival (OS) in DLBCL in Kaplan-Meyer curve with log rank test. High Myc expression (≥20%) showed worse prognosis in both OS and DFS.

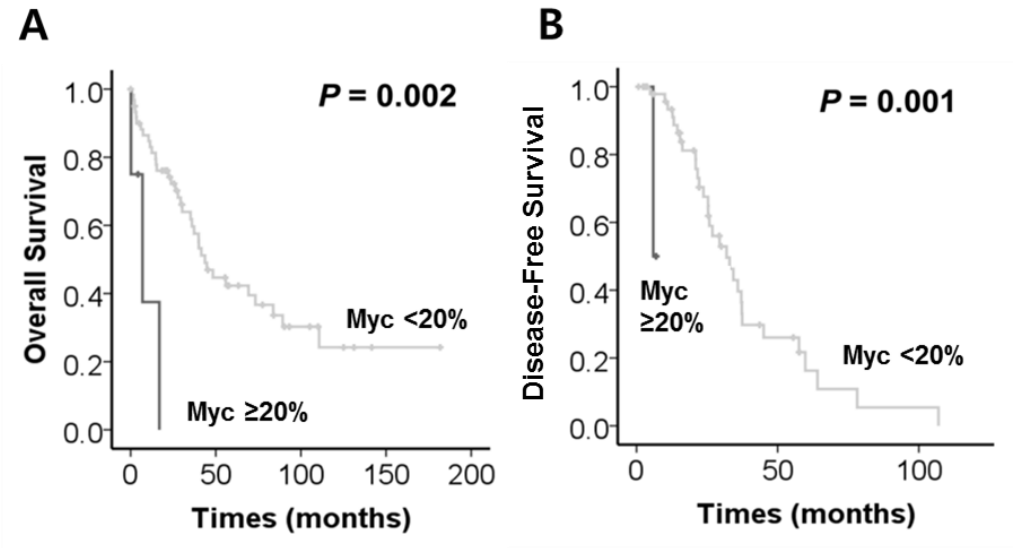


Figure 8. Survival analyses of mantle cell lymphoma patients with Myc expression level. Myc high group (≥ 20%) showed significantly worse overall survival and disease-free survival in mantle cell lymphoma.

Multivariable Cox regression analysis was performed with integration of several parameters including the IPI score, patients' age, Ann Arbor stage, performance status, B symptoms and therapeutic modalities which were significant prognostic indicators in univariable survival analysis, along with protein expression. Expression of PELI1 or Myc was analysed in a separated set because of the strong correlation between them. As a result, overexpression of PELI1 or Myc was not an independent predictor of patients' OS or

DFS (Table 4).

Table 4. Univariable and multivariable survival analyses in diffuse large B-cell lymphoma

Variables	**Total N**	**Univariable analysis**		**Multivariable analysis**	
		OS	DFS	OS (HR, 95%CI)	DFS (HR, 95%CI)
IPI score	167	<0.001	0.269		
Age (>60 years)*	175	0.005	0.197	0.003 (1.031, 1.010-1.051)	0.010 (1.034, 1.008-1.061)
Ann Arbor stage	170	0.001	0.235	0.003 (2.127, 1.284-3.522)	0.039 (1.961, 1.035-3.715)
ECOG score	169	<0.001	0.098	0.008 (2.089, 1.215-3.591)	0.220 (1.725, 0.722-4.122)
B symptom	170	<0.001	0.204		
Treatment	170	<0.001	0.003	<0.001 (0.104, 0.042-0.255)	<0.001 (0.229, 0.070-0.753)
Chemoresponse	142	<0.001	NA		
Recurrence	130	<0.001	NA		
PELI1* (>2)	175	0.239	0.002	0.521 (0.910, 0.681-1.215)	0,591 (1.121, 0.783-1.704)
Myc* (>20%)	173	0.003	0.036	0.385 (1.193, 0.801-1.776)	0.837 (1.054, 0.637-1.745)

Results of survival analyses are shown with p-value with univariable analyses by Kaplan-Meier with log rank test, and multivariable analysis by Cox proportional hazard model. *, continuous variables in multivariable analysis

DISCUSSION

This study revealed that PELI1 was frequently highly expressed in high grade B-cell lymphomas including DLBCL, and high levels of PELI1 was a negative predictor of patients' outcome along with Myc expression. Previous study suggested that PELI1 induces constitutive activation of B-cell signal transduction through upregulation of BCL6,(5) and the present study provides additional clue to the pivotal role of PELI1, especially in high grade B-cell lymphomas. It is possible that PELI1 works differently in the normal development, and in the neoplastic processes of B-cells. We found that expression of PELI1 was restricted in the GC B-cells in the reactive lymph nodes, reconfirming the close relationship of PELI1 with BCL6. However, in the B-cell lymphomas, the programmed tuning of PELI1 functions might be disrupted by oncogenic pathways, such as ones linked to Myc. In our series, BL revealed very high level of PELI1 expression whereas follicular lymphoma displayed relatively low PELI1, and also in DLBCL, PELI1 expression was not significantly different among immunohistochemical subgroups based on HANS classifier.(8) These findings indicate that not all lymphomas of GCB origin imposed PELI1 activation. B-cell lymphomas originated from pre-GC stages like small

lymphocytic lymphoma (SLL) and MCL showed extremely low PELI1. In post-GC stage tumors, marginal zone B-cell lymphoma and MM showed low expression of PELI1 in contrast PBL, in which Myc deregulation is frequently encountered showed very high levels of PELI1. Taken together these findings, we assume that activation of PELI1 starts from the GCs but can persist through post-GC stages with cooperation of Myc or B-cell signalling factors such as BCL6. However, expression of PELI1 protein or mRNA was uniformly low in T- or NK-cell lymphomas; even angioimmunoblastic T-cell lymphoma, a lymphoma originated from GC- T cells expressing constant BCL6 exhibited low PELI1. PELI1 has been shown to negatively regulate T-cell activation and maintain T-cell tolerance, which is the inverse of a function in B-cells.(3) Therefore, it is possible that PELI1 targets different signaling factors according to the types of immune cells and BCL6 or Myc is not the partner of PELI1 in T-cell lesions.

As is well known, PELI exerts its immunoregulatory functions via ubiquitination of some important transcription factors, and Myc gene has several binding sites for multiple ubiquitin ligases.(9) Since Myc is a short lived protein, the stability of Myc can be affected by ubiquitination.(9) In a development of BL, constant upregulation of Myc

is driven by the strong transcriptional control of immunoglobulin heavy chain, and additional mutation of the ubiquitin system binding sites contribute to the stability of Myc via decreased proteosomal degradation. (10, 11) Like the relationship of PELI1 to BCL6, deregulation of PELI1 might induce prolongation of Myc activity by preventing Myc degradation. However, the vice versa is also possible, that is, activation of Myc brings about PELI1 overexpression. In our study, DLBCL without t(8;14) or *MYC* amplificaiton, where the source of Myc overexpression is not obvious but more likely due to alternative or indirect pathogenetic mechanisms leading Myc deregulation, showed much less weaker correlation between PELI and Myc. This finding suggests that Myc is more likely to be a cause of PELI1 overexpression as it is known as a driver oncogene.

Stabilization of BCL6 by PELI1 was already demonstrated, however, relationship of PELI1 with Myc or Bcl2 has not been elucidated yet. Our study revealed correlation of PELI1 with Bcl2. Bcl2 works as anti-apoptotic protein and is thus considered as an oncogene in B-cell lymphoma. Recently, the importance of BCL2 overexpression with Myc was highlighted to contribute to the poor survival in DLBCL patients.(12, 13) Little is known about Bcl2 stabilization by ubiquitination system. Although nothing can be confirmed by this cross

sectional aspect of PELI1 and Bcl2 correlation, we may suggest that Myc alteration could be a causal factor of Bcl2 deregulation. One of the possible explanations is based on the Myc induced repression of some microRNAs targeting Bcl2 such as miR15a/16-1, which results in increased Bcl2 expression.(14)

Also, a modest association of PELI1 and MUM1/IRF4 was noted in this study. Previous study also confirmed MUM1/IRF4 expression in a small group of lymphomas arising in PELI1 transgenic mice but no further investigation was done. IRF4/MUM1 is a transcription factor which is necessary for development and activation of B lymphocytes. The expression of IRF4/MUM1 protein appears at the later stages of germinal center in B-cell differentiation after diminution of CD10 and Bcl-6 expression and at the post-germinal lymphocytes and plasma cells. The expression of IRF4/MUM1 and Bcl-6 in normal germinal center B cells appears to be mutually exclusive. In physiologic condition, IRF4/MUM1 with BLIMP1 represses Bcl6 and Myc and results in B-cell terminal differentiation into plasma cells.(15) However, in Myc-deregulated tumors such as plasmablastic lymphoma, Myc is thought to overcome the repressive activity of IRF4/MUM1. This phenomenon can partly elucidate the positive interaction of

PELI1-Myc- IRF4/MUM1.

The regulatory mechanism of PELI1 is currently not well known. PELI1 E3 ligase activity and stability can be modified by phosphorylation and auto-ubiquitination by IRAK or sumoylation.(4) In a previous study, miR21 was reported to target PELI1 and regulate cell proliferation in liver generation via NF-kB signaling.(14) In DLBCL cases, PELI1 amplification was reported in 6% (3/48) and PELI1 mRNA upregulation in 4% (2/48) of DLBCL cases according to genome-wide analysis of the Cancer Genome Atlas data available in the National Human Genome Research Institute. Further genetic or epigenetic alterations of PELI1 are remained unknown.

Although PELI1 deregulation in malignant lymphoma is an initiative event or just a concomitant incident is not clear, expression of PELI1 has a significant prognostic implication in DLBCL. Deregulation of PELI1 should be associated with pathogenesis of a subset of aggressive B-cell lymphomas. Correlation of PELI1 expression with other transcription factors such as Myc, Bcl-6, MUM1/IRF4 or BCL2 supports the notion that PELI1 is involved in the post-germinal center stages of B-cell maturation and lymphomagenesis.

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국문 초록

배경 : B-세포 림프종에서 Myc 단백의 과발현은 극히 불량한 예후를 표방하는 인자로 알려져 있다. MYC 유전자의 활성화 기전은 고전적으로 MYC과 면역글로블린 중쇄의 재조합에 의해 발생한다고 알려져 있으나, MYC 유전자의 구조적 이상이 없는 증례에서도 Myc 단백이 과발현되는 경우가 있기 때문에 추가적인 Myc 유전자의 이상 조절기전이 있을 것으로 의심이 된다. 본 연구는 Myc 과발현과 PELI1으로 대표되는 E3 유비퀴틴 연결효소와의 관계를 조사하기 위해 시행하였다.

방법 : 총 495 예의 B-세포, T-세포 및 NK-세포 림프종 및 호지킨 림프종에서 Myc 과 PELI1에 대한 면역조직화학염색 (immunohistochemistry, IHC)을 시행하였고, 그 중 미만성 대세포림프종 (diffuse large B-cell lymphoma, DLBCL) 및 외투세포림프종 (mantle cell lymphoma, MCL)에서는 Bcl-2, Bcl-6, CD10, Mum-1, CD5, CyclinD1, Ki-67 등에 대한 IHC를 추가하였다. MYC 유전자 재배열 및 증폭은 형광 제자리 교합법 (flourescence in situ hybridization, FISH)으로 확인하였다. PELI1 단백 및 mRNA 발현은 총 14 종의 악성 림프종 세포주에서 확인하였다.

결과 : Myc과 PELI1은 DLBCL, 버킷림프종, 형질모세포종 등의 고등급 B-세포 림프종에서 매우 높게 발현되었고, 저등급 B-세포 림프종, T/NK-세포 림프종 및 호지킨 림프종에서는 발현도가 매우 낮았다. 림프종 조직에서의 PELI1 IHC결과는 세포주에서의 western blot과 RT PCR 결과

와도 일치하였다. DLBCL에서 PELI1의 발현은 Myc, Bcl-6, Bcl2 등과 강한 연관성을 보였고 (모두 $p \leq 0.001$), IRF4/MUM1과는 상호 연관되는 경향을 보였다 ($p=0.066$). DLBCL에서 PELI1 과발현 증례는 그렇지 않은 증례에 비해 골수 침범이 잘 동반되는 특성과 무진행 생존기간이 짧은 특성을 보였다. MCL에서 Myc 의 과발현은 고등급 변종에서 자주 관찰되었으며 전체 생존률과 무진행 생존률에 모두 나쁜 영향을 주는 것으로 드러났다.

결론 : 본 연구 결과는 PELI1이 Myc과 상호 협조하여 고등급 B-세포 림프종의 발병 및 진행에 깊이 관여하고 있을 가능성을 제시한다. 또한 DLBCL과 MCL에서 Myc 또는 PELI1의 과발현은 나쁜 예후를 예측하는 인자로 사용할 수 있을 것이다.

주요어 : PELI1 protein; Lymphoma, B-cell; Proto-Oncogene Proteins c-myc; Proto-Oncogene Proteins c-bcl-6; Proto-Oncogene Proteins c-bcl-2

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