Long non-coding RNA expression in T and NK cell lymphomas : in relation to polycomb repressive complex pathway

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Directed by Professor Sun Och Yoon

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ABSTRACT

Long non-coding RNA expression in T and NK cell lymphomas
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Long non-coding RNA (lncRNA) is a non-protein-coding RNA molecule longer than 200 nucleotides functioning in target gene regulation at transcriptional or post-transcriptional level. Recently, tumor type specific expression patterns of lncRNAs have been reported in several types of tumors and thus draw attention to lncRNA as a prognostic marker as well as a therapeutic target. However, the clinicopathologic studies on the expression pattern of lncRNAs in T and NK cell lymphoma have not yet been reported.

Thus, the expression pattern of seven lncRNAs (ANRIL, H19, HEIH, HOTAIR, KCNQ1, MALAT1, and TUG1) and their relationship with polycomb repressive complex (PRC) pathway markers (BMI1, EZH2, H3K27me3, and SUZ12) were investigated using 167 clinical samples and 6 lymphoma cell lines. Their role as a prognostic marker was also analyzed.

Real-time polymerase chain reaction using RNAs prepared from formalin-fixed paraffin-embedded tissue samples and T and NK cell lymphoma cell lines demonstrated statistically significant high expression of lncRNA MALAT1 in both clinical samples and cell lines. Immunohistochemistry using clinical samples revealed correlation of BMI1 with all other PRC-pathway markers. Multiple linear regression analysis demonstrated independent correlation of BMI1 with H3K27me3 expression and tendency of correlation between lncRNA MALAT1 and BMI expression.

RNA immunoprecipitation assay in a HH cell line demonstrated direct binding of lncRNA MALAT1 with EZH2, SUZ12 and H3K27me3. Transfection of si-MALAT1 induced no significant changes of all four PRC pathway markers and significantly decreased biding of lncRNA MALAT1 with EZH2 and SUZ12. Although there was no distinct correlation between lncRNA MALAT1 expression status and clinicopathologic variables, high lncRNA MALAT1 expression demonstrated statistically significant inferior overall survival in cases of peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS) and T-lymphoblastic lymphoma. High BMI1 expression was also associated with statistically significant inferior overall survival in mature T cell lymphoma and PTCL-NOS, additionally, poor overall survival of marginal significance in extranodal NK/T-cell lymphoma.

So lncRNA MALAT1 upregulated in T and NK cell lymphomas and associated with inferior overall survival interacts with PRC2 proteins by direct binding and induce BMI1 activation possibly through histone modification of H3K26. LncRNA MALAT1, like BMI1, could serve as a prognostic marker as well as a therapeutic target in T and NK cell lymphoma.

Key words: long noncoding RNA, MALAT1, polycomb repressive complex pathway, T and NK cell lymphoma

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I. INTRODUCTION

T and NK cell lymphoma preferentially occurs in East Asians but therapeutic options are limited to conventional chemotherapy and radiotherapy with no available target agents.[1] Long non-coding RNAs (lncRNAs) are known as a non-protein-coding RNA molecule longer than 200nucleotieds regulating gene expression in chromosome remodeling, transcription and post-transcriptional process.[2] The lncRNAs are located in the cytoplasm, although there are some found in both cytoplasm and nucleus.[3] LncRNAs are mostly transcribed by RNA polymerase II, and follow co-transcriptional modifications such as splicing and polyadenylation. The biological functions of lncRNAs are results of the basic structural and biochemical properties of RNA molecules.[4] Various lncRNAs are encoded in regions proximal to the promoters of known coding genes, or as antisense transcripts to coding genes. LncRNAs are regulated independently of adjoining genes and have their own specific histone modifications and splicing signals; in some cases, lncRNAs are located in genomic regions distant from

known protein coding genes.[5] Recently, aberrant expression of lncRNAs are investigated in many human diseases including various malignant tumors.[2,6-10] LncRNAs are deregulated in a number of cancers, demonstrating both oncogenic and tumor suppressive roles, thus suggesting their aberrant expression may be a substantial contributor in cancer development. Moreover, many studies have been reported that its clinicopathologic meaning in aspects of prognosis or therapeutic benefit, as a possible therapeutic target or an anticipated biomarker.[11-13] Some hematologic malignancy also have been studied focused on B cell lymphoma/leukemia[14-19], but not on T and NK cell lymphoma.

Some lncRNAs is known to work on the regulation of gene expression via a mechanism involving interaction with the polycomb repressive complex (PRC) pathway, e.g., HOTAIR by PRC2 and ANRIL by PRC1 and PRC2.[20] The PRC proteins consist of two main families of complex, PRC1 and PRC2. The PRC2 complex contains the three core proteins of enhancer of zeste homolog 2 (EZH2), suppressor of zeste 12 homolog (SUZ12), and embryonic ectoderm development (EED). The other complex, PRC1, is more complicated but the main of the complex formed by RING finger protein 1 (RING1), RING finger protein 2 (RNF2), B lymphoma Moloney murine leukemia virus integration site 1 (BMI1), melanoma nuclear protein 18/polycomb group ring finger2 (MEL18/PCGF2), polyhomeotic homolog 1 (PH), nervous system polycomb1 (NSPC1), MEL18- and BMI1-like ring-finger protein (MBLR), and chromobox homolog (CBX)proteins.[21]Generally, PRC2 is related to trimethylation of histone H3 at

lysine 27 (H3K27), whereas PRC1 interacts the genome regions via H3K27me3. According to current concept, PRC2 initiates transcriptional repression by inhibiting transcription initiation, whereas the PRC1 maintains the repressive conditions. PRC1 and PRC2 have intrinsic histone modifying activities specific for ubiquitination of lysine 119 of histoneH2A (H2AK119ub) and trimethylation of lysine residue 27 of histone H3 (H3K27me3), respectively.[22] In malignant tumor, the abnormal polycomb group (PcG) activity induces a repression of differentiation promoting genes as well as tumor suppressor genes.[23] Increased expression of lncRNA MALAT1 in renal cell carcinoma was reported to promote aggressive biologic behavior through EZH2 and interacts with microRNA.[24] Also other study about bladder cancer showed that the expression of lncRNA HOTAIR may be regulated by EZH2 and it has prognostic value.[25]

Overexpression of BMI1 was correlated with tumor progression and chemoresistance in B cell leukemia/lymphoma.[26] In breast cancer and myeloid leukemias, BMI-1 expression was associated with aggressive behavior.[27,28] Various regulations of long non-coding RNAs and PRC-related markers has been reported in different type of malignant tumors although their functional mechanisms remains unclear in T and NK cell lymphomas.

Thus, the present study aims to compare and analyze 1) the patterns of long non-coding RNA expression between various T and NK cell lymphomas, 2) relation between long non-coding RNA and polycomb repressive complex pathway and 3) its clinicopathologic implication focused of prognostic value in

T and NK cell lymphomas.

II. MATERIALS AND METHODS

1. Patients and samples

A total of 167 T and NK cell lymphoma clinical samples diagnosed in the Department of Pathology at Severance Hospital from 1999 to 2013 were included for study by the following criteria; 1) available paraffin blocks 2) confirmed diagnosis by two pathologists (S.H.K and S.O.Y) according to current World Health Organization criteria.[1]Extranodal natural killer/T-cell lymphoma (ENKTL; n = 56); peripheral T cell lymphoma, not otherwise specified (PTCL-NOS; n = 44); angioimmunoblastic T cell lymphoma (AITL; n = 16); anaplastic large cell lymphoma (ALCL; ALK-positive, n=16; ALK-negative, n = 16); T lymphoblastic leukemia/lymphoma (T-LBL; n = 19) were selected for this study. Clinical information was obtained from medical record review, and prognostic implications were analyzed in 135 patients with available clinical data. The clinicopathologic characteristics of patients are summarized in Table 1. This study was approved by the Institutional Review Board of Severance Hospital.

Table 1. Characteristics of patients

Characteristic	n (%)
All patients	135
Sex	
Male	91(67.4%)
Female	44(32.6%)
Age (years)	
<60	86(63.7%)
≥60	49(36.3%)
Primary site of tumor	
Head and neck	36(26.7%)
Lymph node	72(53.3%)
Gastrointestinal tract	9(6.7%)
Soft tissue and bone	12(8.9%)
Others (solid organs)	6(4.4%)
Ann Arbor stage	
Stage I & II	28(20.7%)
Stage III&IV	75(55.6%)
Not evaluable	32(23.7%)
LDH level	
Normal	35(25.9%)
Elevated	60(44.4%)
Not evaluable	40(29.6%)
Bone marrow involvement	
Absent	72(53.3%)
Present	33(24.4%)
Not evaluable	30(22.2%)
IPI score	
0-2	61(45.2%)
3-5	43(31.9%)
Not evaluable	31(23.0%)

Abbreviations: LDH, lactate dehydrogenase; IPI, International Prognostic Index

2. Selection of long non-coding RNA

Among numerous lncRNAs, HOTAIR, MALAT1, KCNQ1, TUG1, H19, ANRIL, HEIH were selected based on the previous study results which demonstrated relationship with PRC pathway.[5,20,25,29-31]

3. Analysis for long non coding RNA expression in clinical samples

Paraffin-embedded and formalin-fixed (FFPE) tissue sections were prepared and stained with hematoxylin and eosin, and then tumor areas were confirmed and marked under the microscope. The marked FFPE areas were dissected at 10-mm thickness using a microtome. Generally, four slices of tissue section per case were used for RNA extraction. Total RNA was isolated using an RNeasy FFPE Kit (Qiagen, Hilden, Germany) according to the supplier's instructions. Extracts of RNA were verified by measuring the ratios of A260/A280 and A260/A230 with a ND-1000 NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, USA). One step of reverse transcription and real-time PCR was performed using a one-step SYBR Prime Script RT-PCR kit (Takara, Shiga, Japan). Primers for seven types of long non-coding RNA (lncRNA), HOTAIR, MALAT1, KCNQ1, TUG1, H19, ANRIL, and HEIH and the reference gene *GAPDH* were summarized in Table 2, and these primers were designed using the Primer3-web interface (http://frodo.wi.mit.edu/primer3/input.htm).

Table 2. Primers for seven types of long non coding RNA (lncRNA), and the reference gene GAPDH

gene	primer-F
HOTAIR	AGCCAGAGGAGGGAAGAGAG
MALAT1	GCA GGG AGA ATT GCG TCA TT
KCNQ1	TCT CCC CAT GAT TTG CTG GT
TUG1	GTA CCT CCA CTC AGC ACA GT
H19	GAG GTT TAG GGG ATC GAG GG
ANRIL	TTT ATT CCT GGC TCC CCT CG
HEIH	ACA TAC CAG TGG CCA GAA GT
GAPDH	CAAATTCCATGGCACCGTCA

gene	primer-R
HOTAIR	TCCCGTTCCCTAGATTTTCC
MALAT1	TTC TTC GCC TTC CGA TAC TT
KCNQ1	TGA GAA AGG AAG GGG CAG AG
TUG1	CTG GAG TGG AGG GCT GTT AA
H19	TCT TGC TCT TTC TGC CTG GA
ANRIL	CGG AGC GGC TTT TAG TTC AA
HEIH	GTA GTC AGC CTC CCC TTC TG
GAPDH	ATCGCCCCACTTGATTTTGG

Amplification was performed using an ABI StepOnePlus™ (Applied Biosystems, Foster City, California, USA). Relative lncRNA expression levels were normalized to *GAPDH* by the comparative method (2^{-ddCt}). The experiment was performed three times. After stepwise quality assessment, appropriate cases were selected for statistical analyses for lncRNA expression in tissue samples (Figure 1).

< Flow of case selection for lncRNA expression analysis>

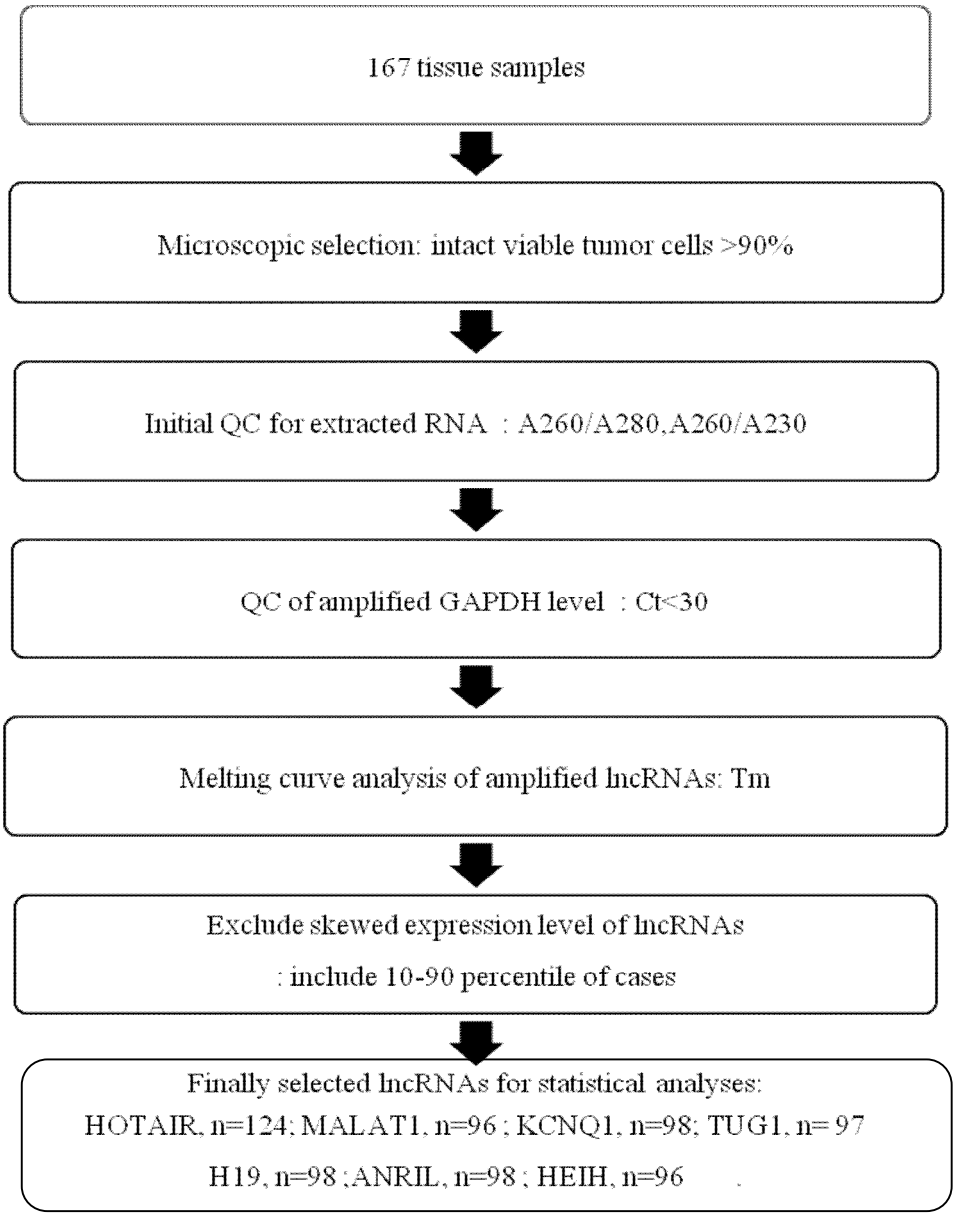


Figure 1. Case selection for lncRNA expression analysis

4. Tissue microarray construction

TMA blocks were manufactured as previously studied.[32]

The hematoxylin–eosin slides were reviewed and representative formalin-fixed, paraffin-embedded (FFPE) archival blocks were selected in each case. Each of blocks was included nonneoplastic tonsil tissues. Core tissue biopsies (3 mm in diameter) were taken from the individual FFPE blocks (donor blocks) and arranged in recipient paraffin blocks (tissue array blocks) using a trephine apparatus.[33] All TMA blocks were confirmed to contain suitable lymphoma lesions and nonneoplastic tonsils after hematoxylin and eosin staining.

5. Immunohistochemistry of polycomb repressive complex markers

Immunohistochemical staining for EZH2, SUZ12, BMI1 and Tri-Methyl-Histone H3(H3K27me3) was performed on the TMA blocks following a standard protocol using a Ventana automatic immunostainer (Ventana, Benchmark, Tuscan, AZ). The primary antibodies used in this study were summarized in Table 3. After deparaffinization, heat-induced antigen retrieval was performed using citrate buffer (CC1 protocol; Ventana) of pH 6.0. Reactivity was detected using the Ultra-View detection kit (Ventana). Positive rate for each marker was determined and scored independently by two pathologists (S.H.K and S.O.Y). EZH2, SUZ12, BMI1 and H3K27me3 showed nuclear expression. The percentage and intensity of positive tumor cells were recorded by manual counting of at least 500 tumor cells from representative

fields in each case. The cut-off value for high expression of EZH2, SUZ12, H3K27me3, and BMI1 was 75% of tumor cells with moderate to strong intensity as our previous study.[32] Representative cases showing high expressions of EZH2, SUZ12, BMI1 and H3K27me3 are presented in Figure 2A,B,C and D.

Table 3. The list of antibodies used for immunohistochemistry

Antibody	Clone	Manufacturer	Dilution
EZH2	ZMD309	Invitrogen, Carlsbad, CA, USA	1:100
SUZ12	SUZ220A	Abcam, Cambridge, MA, USA	1:50
Tri-Methyl-HistoneH3 (Lys27)	C36B11	Cell Signaling Technology, Beverly, MA, USA	1:100
BMI1	1.T.21	Abcam, Cambridge, MA, USA	1:50

6. In situ hybridization (ISH) of EBV-encoded RNA (EBERs)

In situ hybridization (ISH) of EBV-encoded RNA (EBERs) was performed using digoxigenin-labeled probes (Novocastra, Newcastle, UK) and an automated staining system (Ventana XT, AZ). EBV-positive gastric carcinoma tissues were used as positive controls. Positivity was visualized as strong blue-black signals in the tumor cell nuclei. Positivity of EBERs ISH was regarded as more than 10% of tumor cells. Representative cases showing high expressions of EBER is presented in Figure 2E.

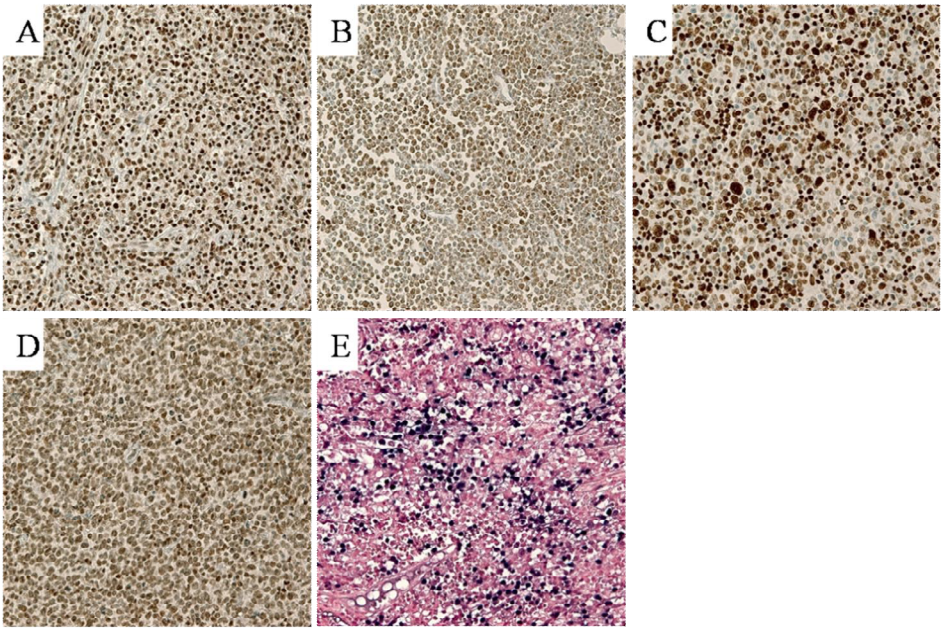


Figure2. A representative case showing high grade expression of polycomb proteins and EBER in T cell lymphoma (x400). (A) EZH2, (B)SUZ12, (C) BMI1,(D) H3K27me3, (E) EBER

7. Cell culture

Five cell lines derived from T or NK cell lymphomas, and one cell line derived from B cell lymphoma were used in the present experiment; YT, established from an EBV-positive human NK cell line, was cultured in IMDM media supplemented with 20% heat-activated FBS. SNK-6, established from EBV-positive NKTL, was cultured in RPMI-1640 media supplemented with 10% heat-inactivated human plasma, Jurkat, established from human T

lymphoblastoid cell line, was cultured in PMI-1640 media supplemented with 20% heat-activated FBS. HH, established from cutaneous T cell lymphoma cell line and Mac-1 , established from CD30 positive lymphoma cell line, were cultured in RPMI-1640 media supplemented with 20% heat-activated FBS.

All the cell lines derived from T or NK cell lymphomas were kindly provided by Prof. Y.K.J (Seoul National University College of Medicine, Seoul, Korea). Toledo, established from diffuse large B-cell lymphoma, purchased from American Type Culture Collection, was also used in the analysis.

8. Western blot analysis

The antibodies used were specific for Ezh2 (#5246; Cell Signaling Technology), SUZ12 (#3737; Cell Signaling Technology), Tri-Methyl-Histone H3(Lys27) (#9733; Cell Signaling Technology) and Bmi1 (#6964; Cell Signaling Technology). For western blot analysis, cells were harvested after 48 hours of trasfection and were lysed with lysis buffer (5mM/l ethylenediaminetetraacetic acid; 300mM/l NaCl; 0.1% NP-40; 0.5mM/l NAF; 0.5mM/l Na3VO4; 0.5mM/l phenylmethylsulfonyl fluoride; and 10μg/ml each of aprotinin, pepstatin and leupeptin; Sigma-Aldrich, St Louis, MO, USA). After centrifugation at 15,000 g for 30 minutes, the concentrations of supernatant proteins were analyzed by Bradford reagent (Bio-Rad, Hercules, CA, USA). For the analysis of protein contents, 50μg total proteins was electophoresed in 10% SDS PAGE gel, trasferred to polyvinylidenedifluoride

membranes (Millipore, Bedford, MA, USA) and were then incubated with the respective antibodies indicated above. For visualization, proteins were enhanced using Amersham ECL Plus Western Blotting Detection System (GE Healthcare Life Sciences, UK). The protein expression level was expressed relative to GAPDH level. The used antibodies and their dilutions were summarized in Table 4.

Table 4. The list of antibodies used for western blot analysis

Antibody or IgG	Clone or Cat No.	Dilution	Manufacturer
EZH2	Rabbit monoclonal antibody D2C9 XP®	1:1000	Cell Signaling Technology, MA, USA
SUZ12	Rabbit monoclonal antibody D39F6 XP®	1:1000	Cell Signaling Technology
Tri-Methyl-Histone H3 (Lys27)	Rabbit monoclonal antibody C36B11	1:1000	Cell Signaling Technology
BMI1	Rabbit monoclonal antibody D20B7XP®	1:1000	Cell Signaling Technology
GAPDH	Rabbit monoclonal antibody 14C10	1:1000	Cell Signaling Technology
Anti-rabbit IgG (whole molecule) antibody	R5506		Sigma-Aldrich

9. Transfection, RNA isolation, and real-time PCR

Knockdown of lncRNAMALAT1 was performed using three types of si-MALAT1 duplex, si-MALAT1-a, -b, and –c (Bioneeroligosynthesis,Daejeon, South Korea) and scramble negative control, si-NC (SN-1002, Bioneeroligo synthesis). The siRNAs were transfected using 4D-Nucleofector™ X Unit

(V4XC-1024, Lonza, 50829 Cologne, Germany) and Lonza 4D Nucleofector™ system (Lonza) according to the manufacturer's instruction. In brief, cells were grown in 6-well plates and transfected with cocktails of three si-MALAT1 (a, b, and c) at a concentration of 200pmol per well. After checking transfection efficiency, each si-MALAT1 of a 200pmol concentration was individually transfected. Sequences of si-MALAT1 are summarized in Table 5.

Table 5. Sequences of si-MALAT1

si-MALAT1 type	Sequences
si-MALAT1-a	5'-CACAGAUGCUAUAGUACUA(dTdT)-3', 5'-UAGUACUAUAGCAUCUGUG(dTdT)-3'
si-MALAT1-b	5'-GUGGUAAACUAUACCUACU(dTdT)-3', 5'-AGUAGGUAUAGUUUACCAC(dTdT)-3'
si-MALAT1-c	5'-CAGAAGUGGAUUCAGUGAA(dTdT)-3', 5'-UUCACUGAAUCCACUUCUG(dTdT)-3'

After 48hours, cells were harvested. Experiments were performed independently three or more times. After initial screening of transfection efficiency in the studied cell lines of T and NK cell lymphoma lines, a HH cell line was finally selected in further study.

RNA was isolated with RNeasy plus mini kit (Qiagen), and cDNA was constructed using a cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). The TaqMan probes were as follows: GAPDH (Hs99999905-m1, ABI; Life Technologies), MALAT1 (Hs00273907-s1, ABI). Quantitative PCR was

performed using ABI StepOnePlus™ (Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 60°C for 1min. Relative expression levels of lncRNAMALAT1 was determined by the comparative method (2^{-ddCt}) against the reference GAPDH.

10. RNA immunoprecipitation assay (RIP)

To investigate potential binding of lncRNA MALAT1 to the polycomb repressive complex-related markers (EZH2, SUZ12, H3K27me3, and BMI1), RNA immunoprecipitation was performed using Imprint® RNA Immunoprecipitation Kit (RIP, Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's protocols. From the RNA immunoprecipitation fraction, RNA was purified with TRI Reagent (T9424, Sigma-Aldrich), and then DNase I (AMPD1, Sigma-Aldrich) was treated. cDNA was synthesized using the cDNA Synthesis Kit (Invitrogen), and fold enrichment of lncRNA MALAT1 was finally generated through quantitative real time PCR. LncRNA MALAT1 fold enrichment of RNA immunoprecipitation fraction per each target antibody was normalized to RNA immunoprecipitation fraction of control antibody (IgG).

11. Statistical analysis

High or low expression of lncRNA was determined using Youden's index for survival. Pearson's χ^2 test or Fisher's exact test was used to compare differences between variables and the Spearman coefficient was also used for correlation

analysis. Overall survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. Multiple linear regression analysis was used for probing interactions of markers. A P value <0.05 was considered statistically significant. Statistical analyses were performed using IBM SPSS 22 software for Windows (IBM Corp, Somers, NY, USA).

III. RESULTS

1. Expression of seven long non-coding RNAs in clinical samples of T and NK cell lymphomas and cell lines

Among tested 7 types of lncRNAs, MALAT1 revealed distinctively high expression when compared to other types of lncRNAs in both clinical tumor samples and cell line. Expression of HOTAIR was also slightly higher in clinical tumor samples. Other lncRNAs demonstrated distinctively lower expression in clinical tumor samples and cell lines (Table 6, Figure 3 and 4).

To compare the expression patterns of lncRNA between T cell lymphoma and B cell lymphoma, B cell lymphoma -derived cell line, Toledo, is included in this study. LncRNA MALAT1 is highly expressed in B cell lymphoma cell line. Though, the expression of other lncRNAs is rarely detected (Figure 4).

Table 6. Results of quantitative analysis of 7 long non-coding RNAs in clinical samples of T and NK cell lymphomas

	N	Median	Mean	SD
HOTAIR ratio	124	0.1545	0.654716	1.14159
MALAT1ratio	96	3.678171	4.647954	3.734142
KCNQ1ratio	98	0.018664	0.161392	0.314458
TUG1ratio	98	0.019104	0.046071	0.055809
H19ratio	97	0.091559	0.338243	0.651711
ANRIL ratio	98	0.007295	0.020028	0.027378
HEIH ratio	96	0.02978	0.21036	0.423051

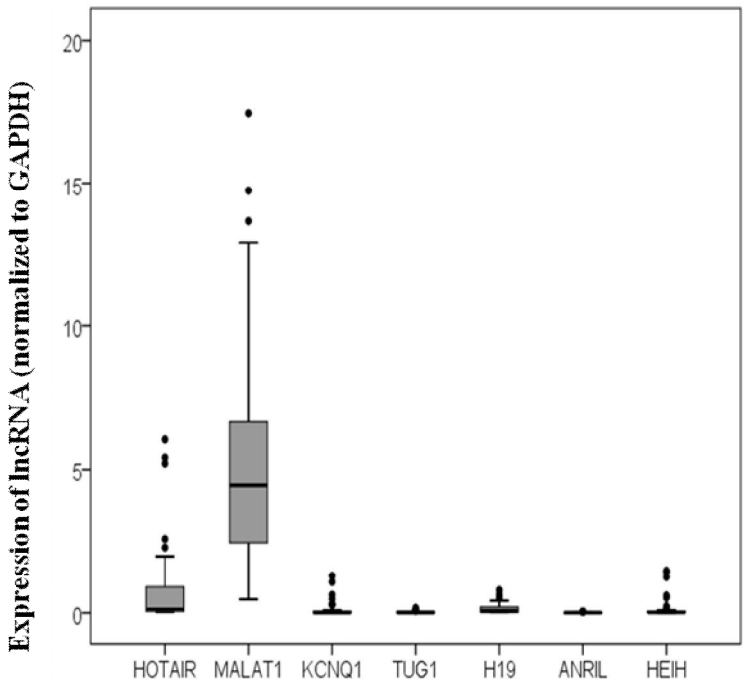


Figure 3. Results of quantitative expression analysis of 7 long non-coding RNAs normalized to GAPDH in clinical samples of T and NK cell lymphomas

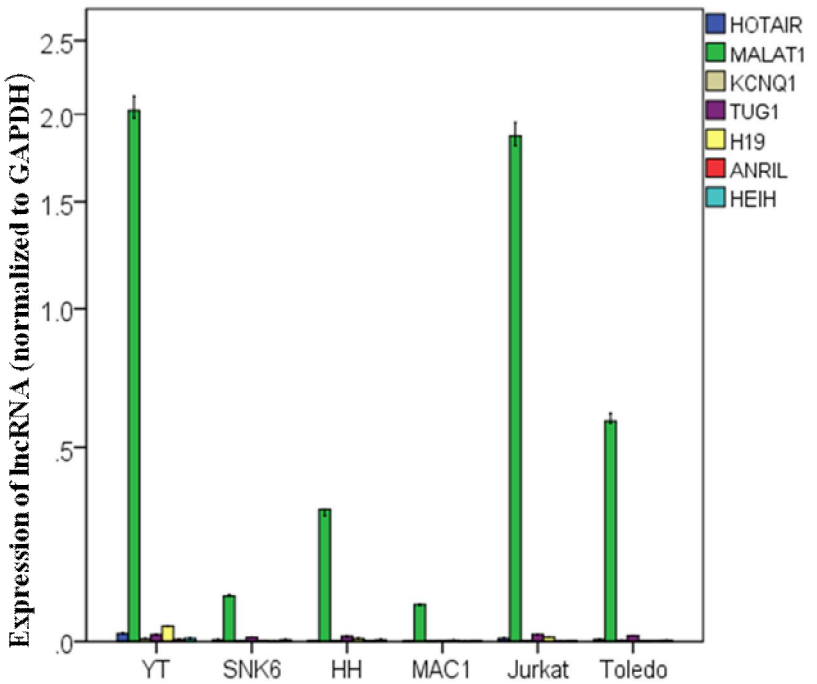


Figure 4. Results of quantitative expression analysis of 7 long non-coding RNAs normalized to GAPDH in six cell lines tested

2. Expression of lncRNA MALAT1 according to subtypes of T and NK cell lymphomas in clinical samples

There was no significant difference of lncRNA MALAT1 expression among extranodal NK/T cell lymphoma of nasal type (ENKTL), peripheral mature T cell lymphoma (PTCL), angioimmunoblastic T cell lymphoma (AITL), anaplastic large cell lymphoma (ALCL) and precursor T lymphoblastic lymphoma/leukemia (T-LBL) (Figure 5).

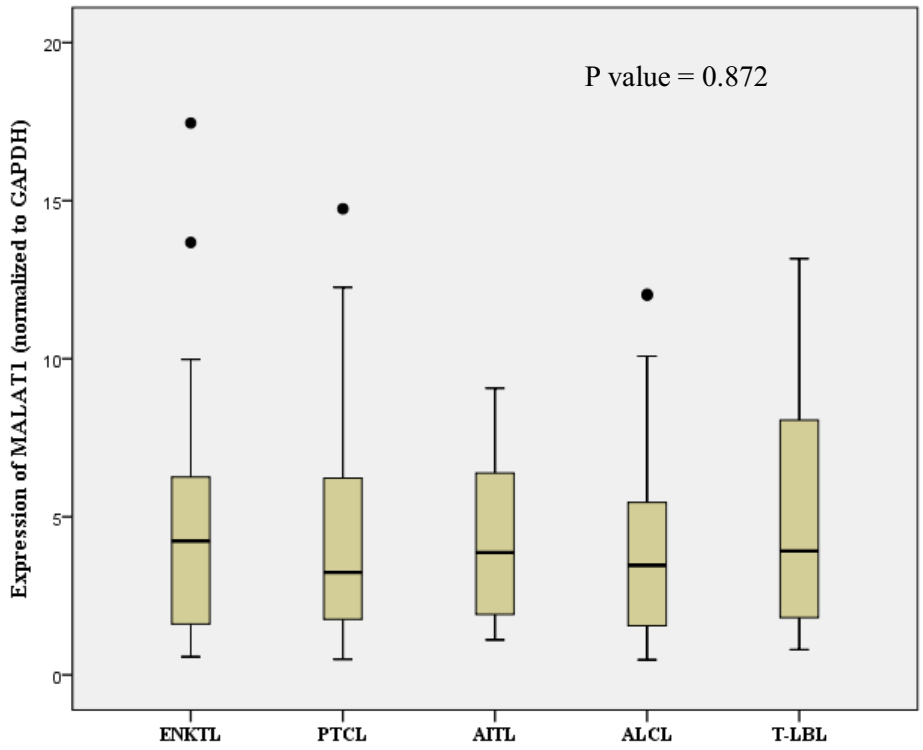


Figure 5. Results of quantitative expression analysis of lncRNA MALAT1 normalized to GAPDH in various subtypes of T and NK cell lymphomas

3. Correlation between expression of PRC pathway markers and lncRNA MALAT1 in clinical samples of T and NK cell lymphomas

BMI1 was positively correlated with all other PRC pathway markers as well as lncRNA MALAT1, while lncRNA MALAT1 was positively correlated with BMI1 but not with EZH2, SUZ12, or H3K27me3 (Figure 6).

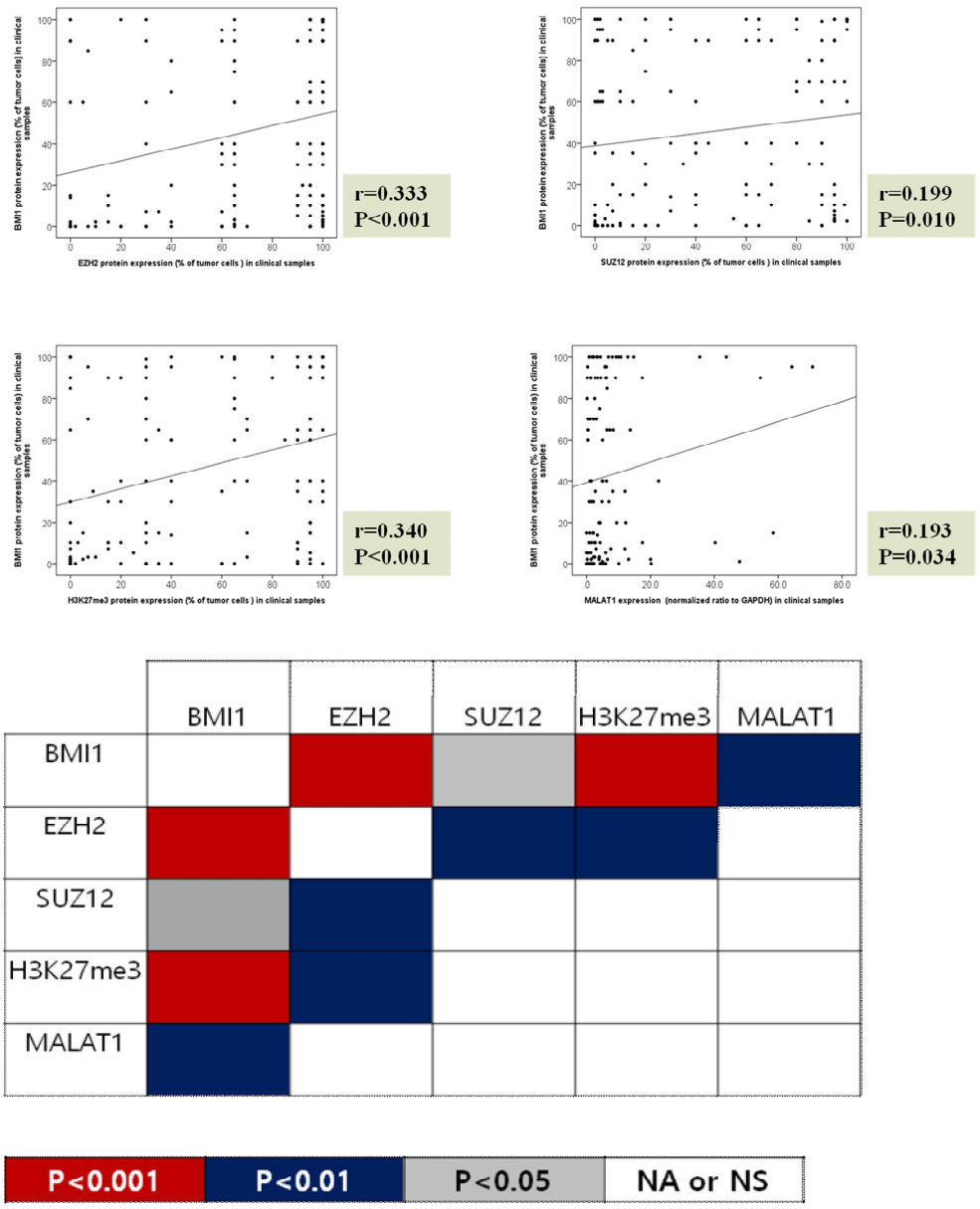


Figure 6. BMI1 expression and association with EZH2, SUZ12, H3K27me3 and lncRNA MALAT1 of T and NK cell lymphoma by correlation analysis in clinical samples

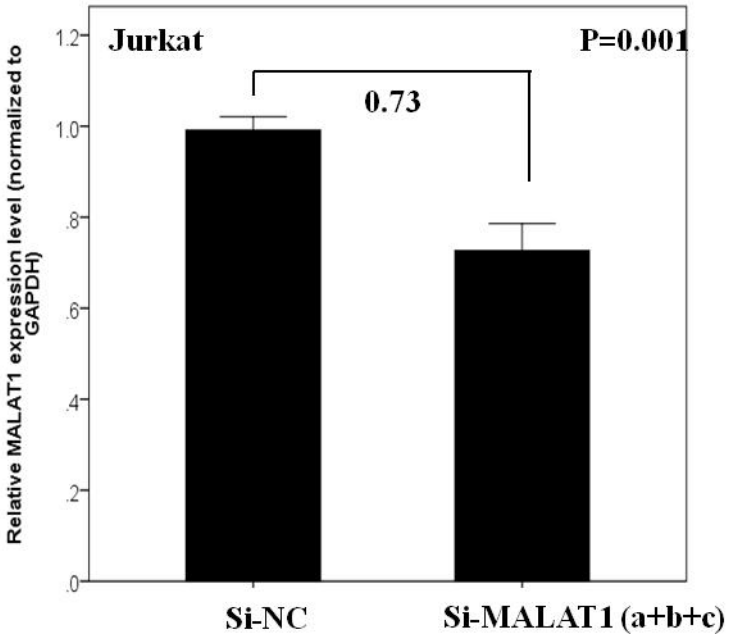
In multiple linear regression analysis, H3K27me3 was independently associated with BMI1 expression, and lncRNA MALAT1 also revealed a tendency to be associated with BMI1 expression (Table 7).

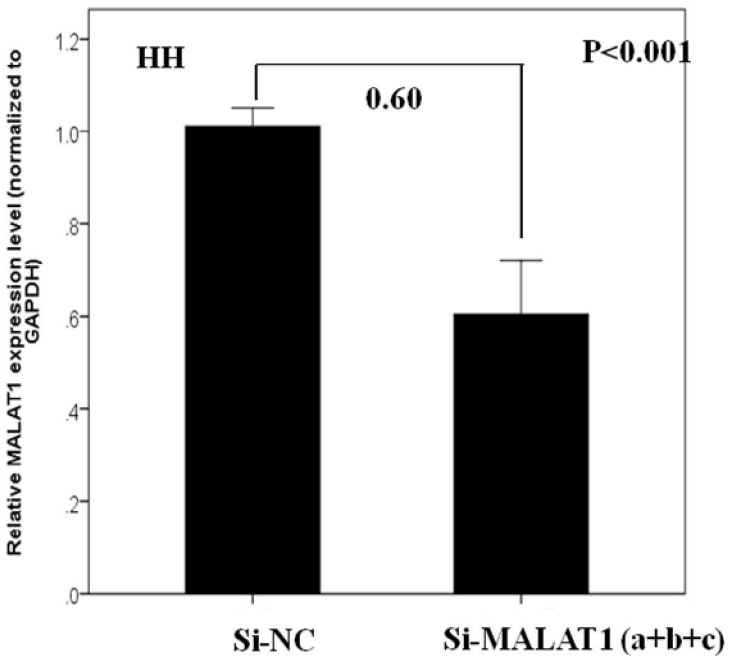
Table 7. Results of multiple linear regression analysis of BMI1 with other PRC pathway markers and lncRNA MALAT1 in clinical samples of T and NK cell lymphomas

Variables	ß (95% CI)	t	P value
EZH2	0.012 (-018 to 0.21)	0.136	0.892
SUZ12	0.155 (-0.02 to 0.34)	1.816	0.072
H3K27me3	0.383 (0.21 to 0.56)	4.332	<0.001
MALAT1	0.165 (-0.02 to1.07)	1.913	0.058

4. LncRNA MALAT1 mRNA expressions lncRNA MALAT1 knockdown in two T cell lymphoma cell lines

As shown in figure 8, the knockdown effect was more prominent in the HH cell line compared with the Jurkat cell line. The transfected oligomers of si-MALAT1, especially, si-MALT1-a, efficiently inhibited lncRNA MALAT1 expression (Figure 8). So the HH cell line showing high transfection efficiency was selected for further experiments.





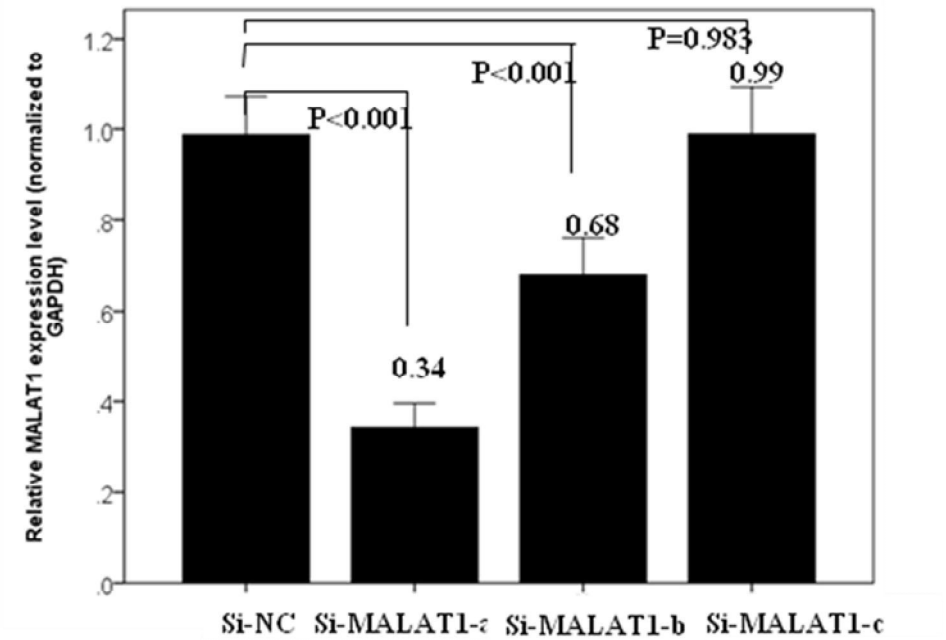


Figure 8. LncRNA MALAT1 expression after lncRNA MALAT1 knockdown using 3 oligomers in the HH and Jurkat cell lines

5. Baseline expression of PRC pathway proteins in the HH cell line

Western blot analysis of PRC pathway proteins, EZH2, SUZ12, and BMI1 proteins and H3K27trimethylation, demonstrated strong baseline expression of all proteins in the HH cell line (Figure 9).

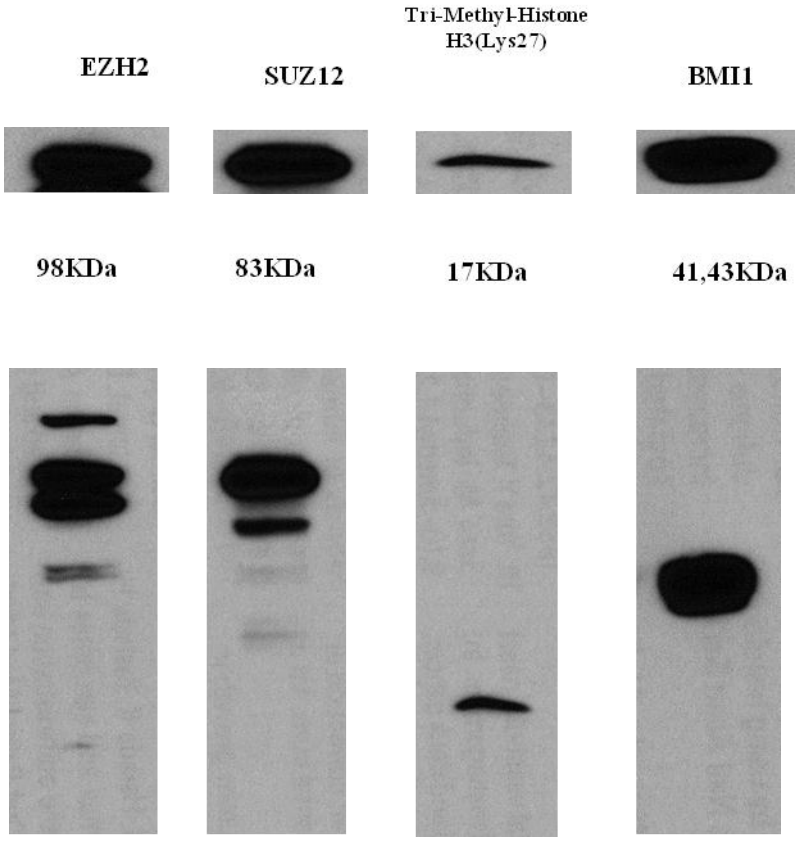


Figure 9. Results of Western blot analysis of EZH2, SUZ12, H3K27me3 and BMI-1 proteins in the HH cell line. All PRC pathway markers showed strong baseline expression in the HH cell line.

6. Interaction between lncRNA MALAT1 and PRC pathway proteins in the HH cell line

To investigate the potential interaction of lncRNA MALAT1 with PRC pathway proteins, RNA immunoprecipitation was performed. In the non-treated

HH cell lines, lncRNA MALAT1 was significantly enriched with the antibodies of EZH2, SUZ12, and H3K27me3 when compared to the control antibody IgG. However, significant interaction of lncRNA MALAT1 with BMI1 antibody was not observed (Figure 10).

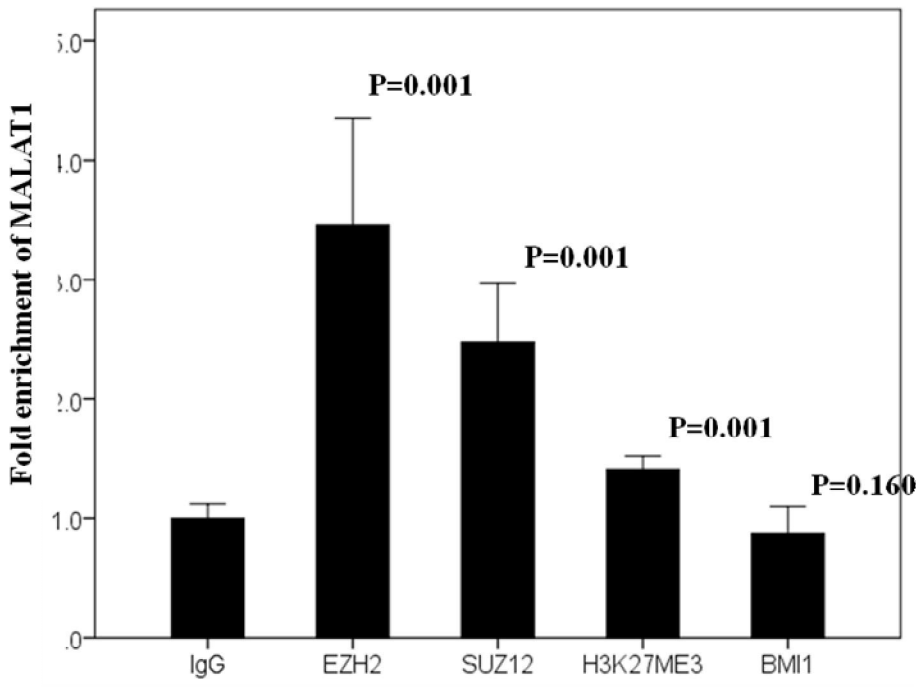


Figure 10. Results of RNA immunoprecipitation in the HH cell line

: LncRNA MALAT1 directly binds with EZH2, SUZ12 and H3K27me3 but not with BMI1.

7. Changes PRC pathway proteins after lncRNAMALAT1 inhibition in the HH cell line

In the Western blot analysis after 48hours transfection of si-MALAT1, expression of EZH2, SUZ12, H3K27me3, and BMI were not significantly decreased in the HH cell line (Figure 11).

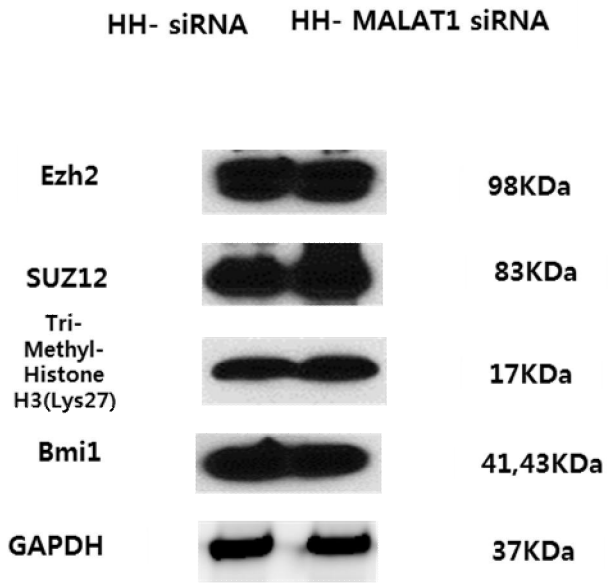


Figure 11. Results of Western blot analysis of 4 PRC pathway proteins after 48hours transfection of si-MALAT1 in the HH cell line

8. Interaction between lncRNA MALAT1 and PRC pathway proteins after transfection of si-MALAT1 in the HH cell line

In the RNA immunoprecipitation analysis after transfection of si-MALAT1, fold enrichment of lncRNA MALAT1 interacting with SUZ12 was most significantly changed in the HH cell line. In detail, the level of lncRNA MALAT1 binding to EZH2 and SUZ12 was significantly decreased after lncRNA MALAT1 inhibition, but the level of lncRNA MALAT1 binding to IgG was not changed even after lncRNA MALAT1 inhibition (Figure 12). However, fold enrichment of lncRNA MALAT1 interacted with the antibody of H3K27me3 and BMI1 was not significantly changed even after lncRNA MALAT1 inhibition.

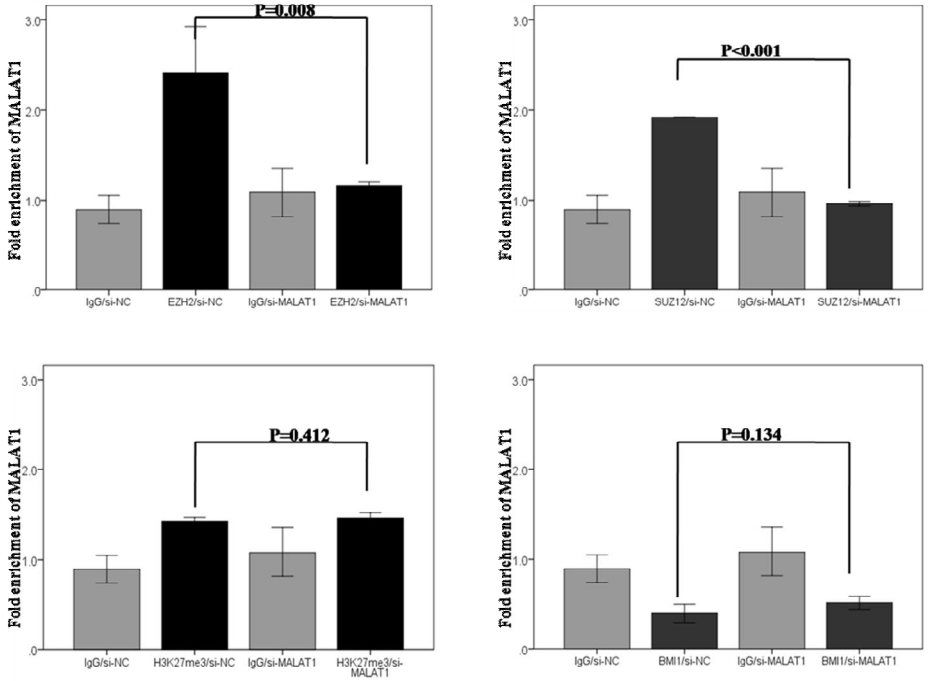


Figure 12. Results of Western blot analysis of 4 PRC pathway markers after lncRNA MALAT1 knockdowns in HH cell lines

9. Clinical significance of lncRNA MALAT1 and BMI1 expression

A total of 96 clinical samples were used for lncRNA MALAT1 analysis as shown in figure 1.No distinct correlation between lncRNA MALAT1 expression status and clinicopathologic variables, such as age, pretreatment LDH level, Ann-Arbor stage, IPI score, anatomical sites of tumor, bone marrow involvement, was demonstrated EBER mRNA expression status also didn't reveal significant association with lncRNAMALAT1 expression (Table 8).

Table 8. Correlation between lncRNAMALAT1 expression status and clinicopathologic variables

	Low MALAT1 expression (%)	High MALAT1 expression (%)	P value
Subtype			0.872
ENKTL	11(52.4)	10(47.6)	
PTCL, NOS	15(60.0)	10(40.0)	
AITL	7(53.8)	6(46.2)	
ALCL	16(66.7	8(33.8)	
T-LBL	7(53.8)	6(46.2)	
Sex			0.250
Male	31(62.0)	19(38.0)	
Female	14(48.3)	15(51.7)	
Age (years)			1.000
<60	30(56.6)	23(43.4)	
≥60	15(57.7)	11(42.3)	
Primary site of tumor			0.394
Head and neck	7(53.8)	6(46.2)	
Lymph node	27(55.1)	22(44.9)	
Gastrointestinal tract	5(71.4)	2(28.6)	
Soft tissue and bone	5(83.3)	1(16.7)	
Others (solid organs)	1(25.0)	3(75.0)	
Ann Arbor stage			0.219
Stage I & II	11(78.6)	3(21.4)	
Stage III&IV	27(58.7)	19(41.3)	
LDH level			1.000
Normal	12(66.7)	6(33.3)	
Elevated	25(69.4)	11(30.6)	
Bone marrow involvement			0.397
Absent	28(63.6)	16(36.4)	
Present	9(50.0)	9(50.0)	
IPI score			0.165

0-2	26(72.2)	10(27.8)	
3-5	12(52.2)	11(47.8)	
EBER1 mRNA positivity			1.000
Low	42(57.5)	31(42.5)	
High	12(57.1)	9(42.9)	

Abbreviations: LDH, lactate dehydrogenase; IPI, International Prognostic Index; ENKTL, Extranodal natural killer/T-cell lymphoma; PTCL, NOS, peripheral T cell lymphoma, not otherwise specified; AITL, Angioimmunoblastic T cell lymphoma; T-LBL, T lymphoblastic leukemia/lymphoma
Information was not available in some cases. Valid number of case (percentage) is presented in the parenthesis.

High lncRNA MALAT1 expression showed a tendency of inferior overall survival in T and NK cell lymphomas (Figure 13) and there was a statistically significant correlation between high lncRNA MALAT1 expression and inferior overall survival in cases of mature T cell lymphomas including PTCL-NOS, AILT and ALCL. In subtype analysis of T and NK cell lymphoma, relationship between lncRNA MALAT1 expression and overall survival was not statistically significant.

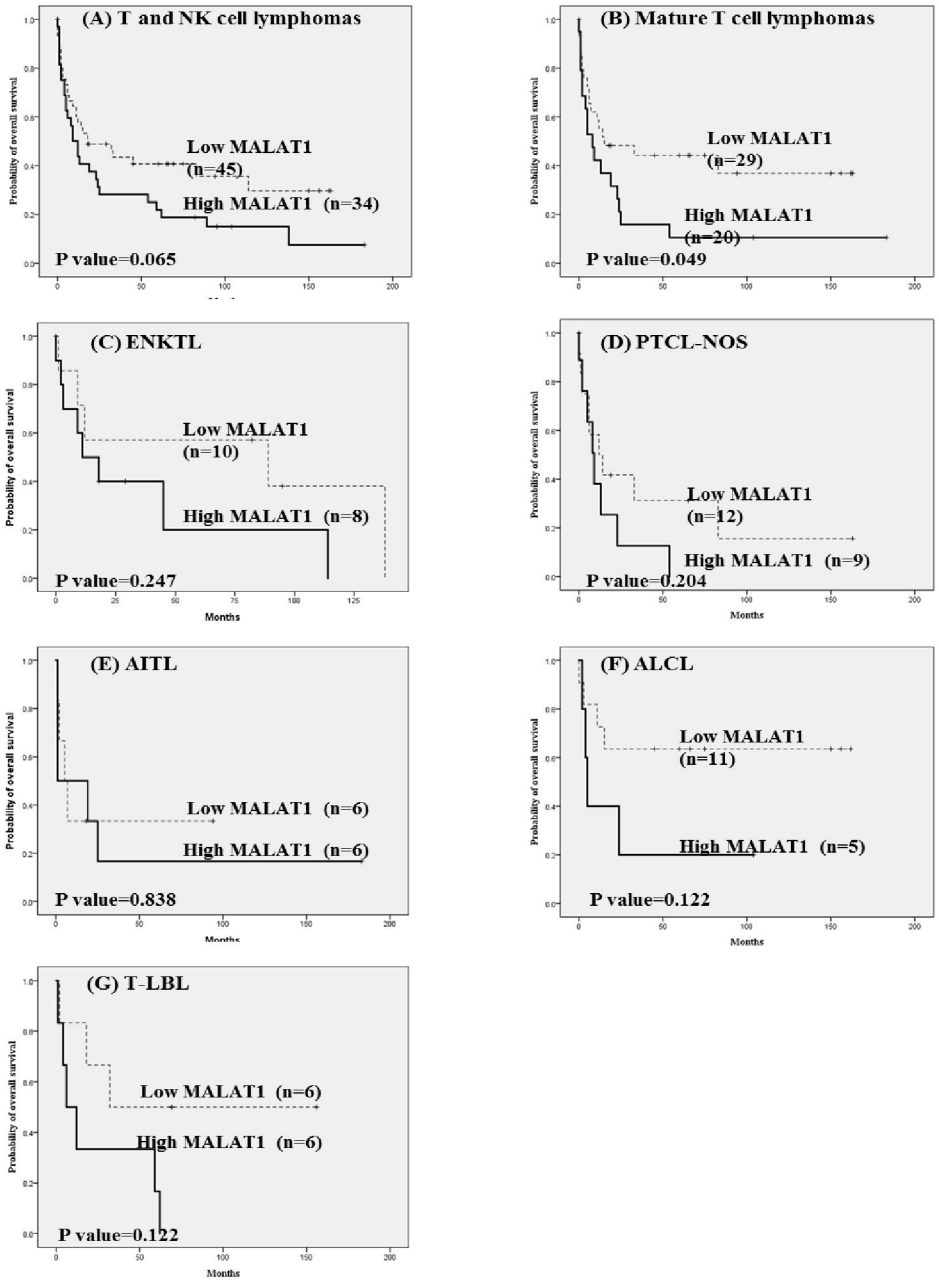


Figure 13. Results of overall survival analysis according tolncRNAMALAT1 expression in various subtypes of T and NK cell lymphomas

Kaplan Meier survival analysis for evaluating prognostic implication of BMI1 expression showed that high BMI expression was significantly associated in poor prognosis (P<0.001) in all T and NK cell lymphomas as noted in the previous study.[32] Lymphoma subtype analysis demonstrated that high BMI1 expression was associated with statistically significant inferior overall survival rate in mature T cell lymphomas and PTCL-NOS (p<0.001 and =0.001, respectively) and poor overall survival with marginal significance in ENKTL (p=0.053). But there was no statistically significant correlation between BMI1 expression and survival in AITL and T-LBL cases (p=0.284 and 0.237, respectively) (Figure 14). ALCL cases could not be analyzed because it is not included a valid value.

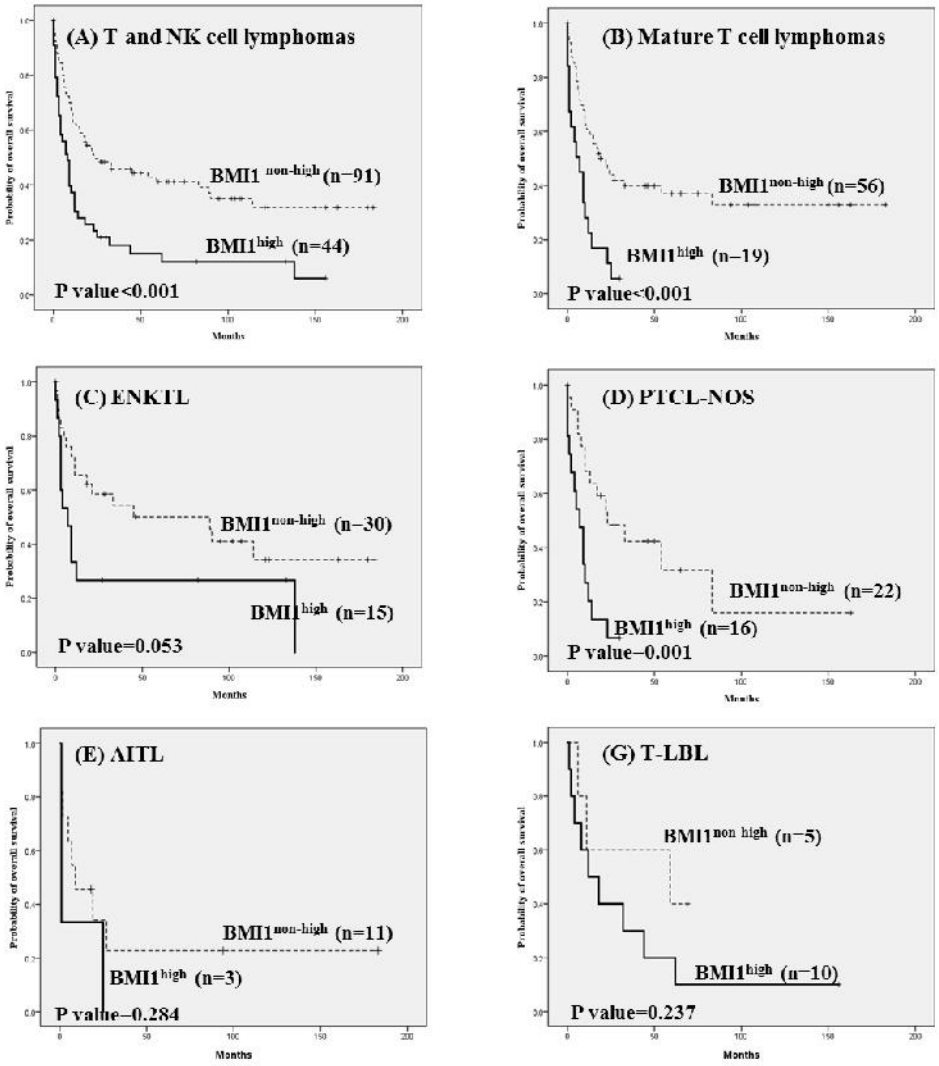


Figure 14. Results of overall survival analysis according to BMI expression in various subtypes of T and NK cell lymphomas

IV. DISCUSSION

T and NK cell lymphoma is highly aggressive, and current treatment options are clearly sub-optimal and many patients are resistant in conventional chemotherapy. Accordingly, advanced understanding of the tumor development will be imperative and precious. However, their oncogenic molecular mechanism is not fully understood.[1] In the present study, the expression of seven lncRNAs(ANRIL, H19, HEIH, HOTAIR, KCNQ1, MALAT1, and TUG1) in clinical samples of T and NK cell lymphomas was investigated and increased expression of lncRNA MALAT1 compared to other lncRNAs was identified. The lncRNA MALAT1 (Metastasis-Associated Lung Adenocarcinoma Transcript 1) locus at 11q13.1 has been identified to harbor chromosomal translocation break points associated with cancer.[2,10,34] LncRNA MALAT1, which is also known as nuclear-enriched abundant transcript2 (NEAT2), is firstly identified as a novel non coding RNA in non-small cell lung cancer.[35] Recently, the function and role of lncRNA MALAT1 in tumor development has been extensively reported.[24,30,34,36-42] In osteosarcoma, lncRNA MALAT1 has been observed to promote malignancy as its knockdown inhibits cell proliferation and invasion and suppresses metastasis.[43-45] A study of renal cell carcinoma reported that silencing of lncRNA MALAT1 reduces cell proliferation and invasion and increases apoptosis.[24]In addition, recent reports indicate that the lncRNA MALAT1 expression is significantly associated with the poor prognosis in various malignant tumors.[36,38,40,46,47] The results of the present study also demonstrate poor prognosis of a high lncRNA MALAT1 expression

group among T and NK cell lymphoma patients, especially mature T cell lymphoma.

A recent study showed that the lncRNA MALAT1 level is upregulated and promotes the activity of the PRC2 complex by interacting SUZ12 protein in bladder cancer.[48] In chemotherapy resistant prostate cancer, lncRNA MALAT1 directly interacted with EZH2 protein and their data also showed lncRNA MALAT1 may be a key role RNA cofactor of EZH2 and that the EZH2-lncRNA MALAT1 association may be a new therapeutic target.[37]

In my previous study, T and NK cell lymphomas revealed frequent activation of PRC pathway-related markers (EZH2, SUZ12, H3K27me3, and BMI1) and the expression of each marker was closely correlated with others. Moreover, overexpression of BMI1 was related to poor prognosis of T and NK cell lymphoma patients. Previous study implied that activation of PRC pathway, especially BMI1 activation, which is induced by PRC2-H3K27me3 activation, may be related to clinically aggressive behaviors of T and NK cell lymphomas.

As expected and also studied in our previous study, the lncRNAMALAT1 as well as polycomb repressive complex (PRC)-related markers, EZH2, SUZ12, H3K27me3, and BMI1 revealed positive correlation with each other. The lncRNA MALAT1 closely related with polycomb repressive complex pathway. Notably, H3K27me3 was the independently related factor for BMI1 activation. This finding could confirm the sequential interaction of lncRNA MALAT1 and PRC2 (EZH2 and SUZ12) and, in addition, subsequent trimethylation of H3K27me3 for BMI1

activation in development of T and NK cell lymphoma. To assess direct interaction between lncRNA MALAT1 and PRC pathway markers, the RNA immunoprecipitation study was performed using a cell line which showed high endogenous expression of PRC-related markers. In the present study using a HH cell line derived from cutaneous T cell lymphoma, the direct binding of lncRNA MALAT1 to EZH2 and SUZ12 was noted. Bound lncRNA MALAT1 level was decreasing according to following orders, EZH2, SUZ12, and H3H27me3. LncRNA MALAT1 binding to BMI1 was not significant. To exclude the possibility that the interaction of lncRNA MALAT1 and PRC-related markers may be HH cell line-dependent effect, lncRNA MALAT1 level was modified using a si-RNA transfection method. The lncRNA MALAT1 bound to control (IgG) didn't change even after knockdown of lncRNA MALAT1. When knocking down lncRNA MALAT1, it bound to EZH2 and SUZ12 was significantly decreased. LncRNA MALAT1 bound to trimethylated H3K27 (H3K27me3) was slightly higher compared with that bound to control (IgG) after regardless of lncRNA MALAT1 modification. LncRNA MALAT1 bound to H3K27me3 didn't change even after lncRNA MALAT1 knockdown. The lncRNA MALAT1 bound to BMI1 was consistently low regardless of lncRNA MALAT1 modification. All these findings suggest that lncRNA MALAT1 directly and specifically binds to PRC2 subunits (EZH2 and SUZ12) when tumor cells express high level of lncRNA MALAT1. The direct binding of lncRNA MALAT1 to PRC2 proteins, EZH2, H3K27me3and SUZ12, was previously described by other authors. [24,37,49] This

results suggest that direct interaction of lncRNA MALAT1 with BMI1 might not occur regardless of lncRNA MALAT1 expression status within T and NK cell lineage tumor cells. Considering the expression pattern of PRC pathway markers by immunohistochemical staining in clinical samples and results of RNA immunoprecipitation in the HH cell line, PRC1 seems to be recruited in response to H3K27me3 after lncRNA MALAT1 and PRC2 interaction. The results about direct binding of lncRNA MALAT1 to PRC2 were known, especially lncRNA MALAT1 binding to EZH2, the H3K27 methyltransferase.[24,49] Even if rarely reported, direct binding of lncRNA MALAT1 to SUZ12 also has been noted.[48] The present study confirmed that the direct interaction between lncRNA MALAT1 and PRC2 subunits of EZH2 and SUZ12 in T cell lineage lymphoma. However, direct interaction between lncRNAMALAT1 and BMI1, the subunit of PRC1, was not identified in the present study. In terms of PRC pathway activation and chromatin remodeling, the effect of upregulated lncRNAMALAT1 seems to be sustained till the trimethylation of H3K27. However, BMI1 might be recruited in response to H3K27me3 mark, and lncRNA MALAT1 does not directly recruit BMI1.

Although lncRNA MALAT1 doses not directly interact to BMI1, lncRNA MALAT1 may be one of important molecules to sustain H3K27trimethylation and then subsequent BMI1 activation in T and NK cell lymphomas. The observed correlation of lncRNA MALAT1 expression with BMI1, not with EZH2 or SUZ12, within tissue samples might imply that the final outcome of upregulated lncRNA MALAT1 is BMI1 activation in tumor tissues of T and NK cell lymphoma patients.

Activated BMI1 might involve in the progression to a high risk phenotypes of T cell lymphomas, when considering the poor prognosis of cases with high BMI1 expression in this study.

LncRNA MALAT1 which is highly expressed in T and NK cell lymphomas may play a role in the development of T and NK cell lymphomas. LncRNA MALAT1 directly binds to PRC pathway proteins, particularly PRC2, along with trimethylation of histone H3 at lysine 27 (H3K27). BMI1, a member of PRC1, does not directly interact with lncRNA MALAT1 (Figure 15). Though, the results of this study suggest upregulated lncRNA MALAT1 may induce BMI1 activation through histone modification of H3K27 and recruitment of PRC2 subunits.

Figure 15. Hypothetical illustration about interaction of lncRNA MALAT1 and polycomb repressive pathway markers

In the present study, lncRNA MALAT1 knock down could not suppress the protein expression of EZH2, SUZ12,BMI1, and the H3K27me3 in vitro analysis. Because the endogenous expression level of these molecules was very high, the 48-hour transfection time using siRNA oligonucleotides was not enough to decrease these molecules, although functional interaction of lncRNA MALAT1 and those PRC-related proteins could be changed during the 48-hour transfection time. The short duration of observation time and no quantitative changes of PRC pathway markers might be also the cause why lncRNA MALAT1 knock down didn't effect on cell survival or death in the present in vitro study. Further study with longer observation time should be followed using shRNA transfection method or vector-based system.

V. CONCLUSION

In T and NK cell lymphomas, lncRNA MALAT1 was upregulated and high MALAT1 expression was associated with inferior overall survival, especially mature T cell lymphoma subtypes. Direct binding of lncRNA MALAT1 with EZH2, SUZ12and H3K27me3, PRC pathway markers was verified through the RNA immunoprecipitation method and upregulated lncRNA MALAT1 may induce BMI1 activation which is related to poor patient prognosis through histone modification of H3K27. LncRNA MALAT1, like BMI1, could serve as a prognostic marker as well as a therapeutic target in T and NK cell lymphomas.

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ABSTRACT(IN KOREAN)

T and NK 세포 림프종에서 long non-coding RNA 발현과 polycomb repressive complex pathway와의 관계

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Long non-coding RNA (lncRNA)는 200 뉴클레오티드 이상 길이의 단백질을 코딩하지 않는 RNA로서, 목표 유전자를 전사 또는 전사 후에 조절하는 기능을 한다. 최근에 다양한 종양에서 lncRNA의 특이적 발현이 연구 되고 있으며, 치료의 새로운 목표 물질로서 또는 예후와 연관된 요소로서 보고 되고 있다. 하지만, T/ NK 세포 림프종에서 lncRNA의 임상병리학적 의의에 관한 연구는 아직 보고되지 않았다. 따라서, 167례의 환자조직과 6개의 림프종 세포주를 이용하여 7종류(ANRIL, H19, HEIH, HOTAIR, KCNQ1, MALAT1, and TUG1)의 lncRNA와 polycomb repressive complex (PRC) pathway관련 물질 (EZH2, SUZ12, H3K27me3 and BMI1)의 발현 양상을 조사하여 그들의 예후적 의미를 분석하였다.

T/NK 세포 림프종 환자의 포르말린에 고정된 파라핀 포매 조직과 림프종 세포주에서 RNA를 각각 분리하여 실시간 중합효소 연쇄반응기법을 이용해 lncRNA발현 양상을 분석하였다. 대상이 된 7종류의lncRNA중, lncRNA MALAT1은 환자 조직과 세포주에서 모두 통계학적으로 의미 있게 높은 발현이 관찰되었다. 환자 조직을 이용한 면역염색에서 BMI1은 다른 PRC 관련 물질들과 양의 상관관계가 관찰되었으며, 다중회귀분석에서는 BMI1은 H3K27me3와 독립적으로 연관되어 있으며, lncRNA MALAT1은 BMI1발현과 연관된 경향성이 있음이 관찰되었다.

MALAT1과 polycomb repressive pathway의 연관성을 알아 보기 위해 HH세포주를 이용하여 RNA면역침강법과 western blot을 시행하였으며,

lncRNA MALAT1과 polycomb repressive pathway 관련 물질인 EZH2, SUZ12 그리고 H3K27me3는 직접적으로 결합을 하지만, BMI1은 직접적으로 결합하지 않는 것을 확인하였다.
LncRNA MALAT1 발현과 임상병리학적 변수들과의 사이에 의미 있는 상관 관계는 발견되지 않았지만, lncRNA MALAT1의 높은 발현은 mature T 세포 림프종과 T 세포 림프모구 림프종 환자에서 나쁜 예후와 관련이 있었다. BMI1의 높은 발현은 T/NK 세포 림프종 환자의 생존율과 연관되어 있었으며, T /NK 세포 림프종을 아형별로 구분했을 때는 mature T세포 림프종과 말초성T세포 림프종에서 나쁜 예후와 관련되어 있음이 밝혀졌으며, NK 세포 림프종에서는 나쁜 예후와 관련된 경향성이 확인 되었다.
요약하면, T/NK 세포 림프종에서lncRNA MALAT1의 발현은 특이적으로 높게 관찰되며, 이러한 발현은 환자의 예후와 관련되어있다. 아울러, BMI1의 과발현은 T/NK 세포 림프종 환자의 예후를 결정지을 수 있는 중요한 요소이다. 과발현된 lncRNA MALAT1은 polycomb repressive protein2 (EZH2, SUZ12)와 직접적으로 상호작용하여 H3K27 tri-메틸레이션을유도하며, BMI1은 H3K27 tri-메틸레이션에 반응하는 단백질로 생각된다. 이러한 기전을 통해, lncRNA MALAT1은 직접적으로 BMI1과 연결되지는 않으나, H3K27 tri-메틸레이션을 유지하고, 결과적으로 BMI1을 활성화시키는 중요한 요소 중 하나일 것이다. BMI1과 lncRNA MALAT1은 T/NK 세포 림프종에서 예후 지표 및 치료제의 목표 물질이 될 수 있을 것으로 생각된다.

핵심되는말: long noncoding RNA, MALAT1, polycomb repressive complex pathway, T/NK 세포림프