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In aggressive variants of non-Hodgkin lymphomas, Ezh2 is strongly expressed and polycomb repressive complex PRC1.4 dominates over PRC1.2

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Running title: Ezh2 correlates with aggressiveness

Abstract

Polycomb group (PcG) proteins are important to the regulation of hematopoiesis by regulating chromatin compaction and silencing genes related to differentiation and cell cycle. The overexpression of Ezh2 and Bmi-1/PCGF4, has been implicated in solid organ cancers. Meanwhile, Mel-18/PCGF2 was reported as a tumor suppressor. The detailed expression profiles of PcG proteins and diagnostic significance in malignant lymphomas are still unknown. In this study, we analyzed the expression levels of Ezh2, Bmi-1, Mel-18 and Ki67 in 197 Hodgkin's and non-Hodgkin's lymphoma patient samples and in lymphoma cell lines using immunohistochemical, fluorescent immunocytochemical and Western blotting. Immunohistochemical staining showed that Ezh2 expression was significantly increased in aggressive compared to indolent subtypes of B-cell neoplasms (*P=0.000~0.030)* while no significant differences were found in Bmi-1 expression among these subtypes. Compared to the normal counterpart, T-cell lymphomas showed significant overexpression of Bmi-1 (*P=0.011)* and Ezh2 (*P=0.000)*. The Ki67 labeling index showed a positive correlation with Ezh2 in B-cell lymphomas (*correlation coefficient*: *Co*=0.983, *P*=0.000) and T/NK- cell lymphomas (*Co*=0.629, *P*=0.000). Fluorescent immunohistochemical staining showed co-expression of Ezh2 and Ki67 in the same tumor cells, indicating that Ezh2 expression correlates with cell proliferation. Both B-and T/NK cell neoplasms showed low expression of Mel-18 and high expression of both Bmi-1 and Ezh2. In conclusion, lymphomas show high expression of Ezh2 in the aggressive variants, and a dominance of polycomb repressive complex PRC1.4 over PRC1.2. Coexpression of Bmi-1 and Ezh2 is a characteristic of aggressive lymphomas**.** Ezh2 correlates with the proliferation and aggressive nature of non-Hodgkin's lymphomas.

Keywords: Ezh2, Bmi-1, Mel-18, malignant lymphoma, PRC1.2, PRC1.4

Introduction

Non-Hodgkin's lymphomas show a wide spectrum of morphology and variability in prognosis between different subtypes. Genetic abnormalities are not enough to explain the entire multi-step lymphomagenesis process. Aberrant epigenetic changes, including DNA methylation, histone modifications, chromatin remodeling and non-coding RNAs (ncRNAs) also contribute to the development and progression of malignancy [1-3].

Polycomb group (PcG) proteins are important regulators of lymphopoiesis that suppress gene expression through epigenetic modifications [4]. They form large multimeric complexes known as Polycomb repressive complexes (PRCs), which bind to chromatin and control histone modifications. In mammals, there are two main PcG complexes: Polycomb repressive complexes (PRC1) and 2 (PRC2). PRC2 includes Enhancer of zeste homologue 2 (Ezh2), Eed, Suz12, RBBP4/7 and Jarid2 [5]. PRC1 complexes include several complexes that differ in their localization and function. So far, six subgroups have been identified and numbered (PRC1.1~1.6). All of them contain RING1 A and RING1 B enzymes, which ubiquitinate histone H2A at the lysine 119 residue [6]. Ezh2 is a histone3 lysine27 methyltransferase that generates H3K27me3, which is reported to be the binding site of PRC1, resulting in the ubiquitination of histones [7, 8]. This association with H3K27me3 was recently proven to be specific to PRC1.2 and PRC1.4 [6]. The interaction between PRC1 and PRC2 facilitates chromatin compaction, silencing of important tumor suppressors and genes involved in development, differentiation, adhesion, cell cycle progression and proliferation. Both groups are important developmental regulators in both stem and cancer cells that help control the cell cycle, immortalization and self-renewal of normal stem/progenitor cells [4, 9-11].

Bmi-1/PCGF4 is a component of the PRC1.4 complex, and was described as a proto-oncogene that induced lymphomas in mice. The upregulation of Bmi-1 was reported in a variety of cancers, and sometimes correlated with a poor prognosis [6, 12-14]. Mel-18/PCGF2 is a component of the PRC1.2 complex, and was previously described as a tumor suppressor that is expressed in the normal state and suppressed in neoplastic conditions in gastric, prostate and breast cancers [15-17]. PRC1.2 and PRC1.4 complexes are formed, one with canonical PcG components (CBXs, PHCs and SCMs) and one with RYBP or YAF2, in addition to Bmi-1/PCGF4 or Mel-18/PCGF2 [6].

Ezh2 overexpression has been reported in solid organ cancers, including breast, prostate and stomach cancer. It is associated with metastasis, poor prognosis and treatment failure [18-20]. Somatic gain of function mutations in the SET domain of Ezh2, which result in increased histone methylation levels, were reported in follicular lymphomas (FL) and diffuse large B cell lymphoma (DLBCL). This is in contrast to the loss-of-function mutations reported in patients with myeloid malignancies, most commonly in those with myelodysplastic/myeloproliferative neoplasms and myelofibrosis [21-23]. However, a lot remains unknown about the expression profiles of PcG proteins and their significance in terms of the pathology of lymphoma, especially in T/NK-cell lymphomas. This study investigated the expression patterns of Bmi-1, Ezh2 and Mel-18 in different subtypes of B- and T/NK-cell neoplasms, and examined the possible correlation with Ki67 expression, as a marker of tumor proliferation.

Materials and Methods

Patient samples

Paraffin blocks of 197 lymphoma samples from the archives of the Department of Pathology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University were used for the present analyses. The demographic data of the patients and the locations of the neoplasms are summarized in Supplemental Table S1. Reactive lymphoid hyperplasia (RLH, 8 specimens), tonsil (5 specimens), inflammatory non-neoplastic skin lesions (4 specimens) and thymus (2 specimens) were used as normal controls. Informed consent was obtained from all patients for the analysis of their tissue samples. This study was approved by the Institutional Review Board of the Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University and related hospitals, in accordance with the Declaration of Helsinki. The classification of the neoplasms was done according to the REAL/WHO classification. DLBCL cases were further divided into two groups, germinal center type (DLBCL-GCB) and non-germinal center type (DLBCL non-GCB), according to the Hans algorithm [24, 25].

Cell culture

The cell lines and culture conditions are summarized in Supplemental Table S2. Peripheral blood mononuclear cells (PBMCs) and $CD4^+$ T-cells from healthy donors were used as normal controls. Immunomagnetic isolation was done using EasySep® Human $CD4^+$ T-Cell Enrichment Kit (#19052, Stemcell Technologies, Vancouver, BC, Canada). The Dynabeads® human T-cell activator CD3/CD28 was used to activate and expand human T-cells (Invitrogen, Oregon, USA).

Immunohistochemical staining

Paraffin blocks were retrieved, and new sections were used to ensure the quality of staining. Cases in which paraffin blocks were not available to make new sections were excluded. The following mouse monoclonal antibodies were used: Ezh2 (clone M18) [26, 27], Bmi-1 (Abcam, MA, USA), CD34 (Abcam, MA, USA), CD3 and CD20 (Novocastra, Newcastle upon Tyne, UK), CD56, MUM-1 (Novocastra, Newcastle upon Tyne, UK), CD4, CD8 (Nichirei Biosciences Inc., Tokyo Japan), CD10, CD68, CD138, (Dako, Glostrup, Denmark), BCL6 (Santa Cruz Biotechnologies Inc., Texas, USA), rabbit anti-Mel-18 (Santa Cruz Biotechnologies Inc., Texas, USA) and anti-Ki67 (Novocastra, Newcastle upon Tyne, UK). Staining was done with a Leica BOND-MAX™ autostainer (Leica Biosysytems, Melbourne, Vic., Australia). Both the intensity of staining and percentage of positive tumor cells were evaluated in 10 high-power fields by three investigators. A high Ki67 labeling index was defined as nuclear staining of $\geq$ 20% of tumor cells. Positivity was defined as $\geq$ 25% nuclear staining of tumor cells, in accordance with previous reports [14, 20]. Positive cases were further scored as (+), (++) and (+++) based on the intensity of the staining, which was judged by comparing it to internal control cells. Endothelial cells or adjacent normal/reactive lymphoid tissue served as an internal control for Bmi-1. For Ezh2, an adjacent germinal center in nodal and duodenal specimens, or the basal epithelial layer in the case of skin and mucous membrane specimens, was used as the internal control. For Mel-18, endothelial cells were used as the internal control. A control slide of either RLH or tonsil was also included in each staining run. For every case, the neoplastic nature of the stained cells was confirmed by its morphology and by using a selection from a panel of markers (including CD20, CD138, CD10, CD3, CD4, CD7, CD8, CD56, Cyclin-D1 and BCL2). A

combination of each marker (Ezh2, Bmi-1 or Ki67) with B- and T/NK cell markers (CD20, CD3, CD56, CD138) was done in normal lymphoid tissue and selected cases of lymphoma.

Double fluorescent immunocytochemical and immunohistochemical staining

The cells were washed with PBS then processed via cytospin (10^5 per slide) and fixed in cold acetone. Blocking was done by incubation in 5% skim milk for five hours at room temperature. After washing, the cells were treated with a mixture of two primary antibodies for three to five hours at room temperature. Washing was repeated, then samples were incubated with a mixture of anti-mouse and anti-rabbit-IgG for two to three hours at room temperature. The primary antibodies included rabbit monoclonal anti-Ezh2 (Cell Signaling, Beverly, MA, USA) and mouse monoclonal anti-Bmi-1 (Abcam, MA, USA). The secondary antibodies were anti-mouse IgG Alexa Flour 555 and anti-rabbit IgG Alexa Flour 488 (Invitrogen, Oregon, USA). Slides were mounted with SlowFade® Gold antifade reagent with DAPI (Invitrogen, Oregon, USA) and examined with a conventional immunofluorescence microscope (IX71, Olympus, Tokyo, Japan). The following combinations were used: Ezh2 with Bmi-1, Ezh2 with Ki67 and Bmi-1 with Ki67.

Western blotting analysis

The Western blot analyses were performed as described previously [28]. Rabbit anti-Ezh2, mouse anti-Bmi-1, rabbit anti-Mel-18 (Santa Cruz Biotechnologies Inc., Texas, USA) and mouse anti-beta-actin (Sigma Aldrich, St Louis, USA) antibodies and HRP-labeled anti-rabbit or anti-mouse antibodies (NA9340V GE and NA9310V, respectively, GE Healthcare, little Chalfont Buckinghamshire, UK) were used for the analyses.

Statistical analysis

All statistical analyses were done with the SPSS version 11.5 software program. Person`s Chi squared test and one-way ANOVA with the Bonferroni's correction were used to compare the different lymphoma subtypes. Spearman's and Pearson's coefficients were used for the correlation analyses of Bmi-1, Ezh2 and Ki67.

Results

Expression of Bmi-1 and Ezh2 in normal lymphoid tissues

In RLH, the expression of Bmi-1 and Ezh2 was mutually exclusive. Bmi-1 was mainly expressed in the mantle zone while Ezh2 was expressed in the germinal center; centroblasts showed stronger staining than centrocytes. $CD138^+$ plasma cells were Bmi-1+/- and Ezh2-. $CD68^+$ macrophages were Bmi-1-/Ezh2-. Interfollicular $CD3^+$ T, follicular CD4+ T-helper cells and $CD56^+$ NK/T cells were mostly $Bmi\text{-}1^+/Ezh2^-$ (Table 1 and Figure S1). The cutaneous CD3+ T-cells were Ezh2 negative. In the normal thymus, the cortical thymocytes were mostly Ezh2+/Bmi-1-. In the medulla, the pattern was reversed; the dominant population of $CD3^+$ cells was Ezh2 -/$Bmi\text{-}1^+$ (Table 1 and Figure S2). The staining intensity for Bmi-1 in both RLH and the thymus was generally weak to moderate (Table 1). The normal stratified squamous epithelium covering the tonsils showed nuclear staining for Ezh2, mostly in the basal layers while Bmi-1 staining generally showed low expression (Figure S3).

High expression of Bmi-1 and Ezh2 in Hodgkin's lymphoma and B-cell neoplasms

In Hodgkin's lymphomas, the coexpression of both Bmi-1 and Ezh2 was found in the tumor cells in 9/10 cases (90%), but was not found in the reactive background cells (Table 1). In B-cell neoplasms, the overall expression of Bmi-1 was 84.4%. The staining intensity was generally moderate (72 cases) to strong (nine cases). Bmi-1 expression was found in all subtypes, ranging from 58.3% in Burkitt's lymphoma (BL) to 100% in four subtypes: Mantle cell lymphoma (MCL), chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), mucosa associated lymphoid tissue (MALT) lymphoma and plasmacytome (PC) (Table 1). MCL showed a moderate staining in more than 90% of tumor cells in all cases. PC and plasma cell myeloma (PCM) showed a stronger staining intensity compared to the weak staining in 20% of normal CD138+ plasma cells ($X^2=8.718$, $P=0.033$). In both PCM and PC, the percentage of positive tumor cells was above 80% in 10 cases (Table 1, Figures 1 and S1). MALT lymphoma expressed Bmi-1 in 100% of cases. Strong staining was noted in 7/10 cases (70%), and the percentage of positive tumor cells was more than 90% in all cases.

Follicular lymphoma grade 1 and 2 (FLG1-2) expressed Bmi-1 in 73.7% of cases, compared to the negative germinal center light zone (Table 1, $X^2=14.246$, $P=0.003$). DLBCL-GCB showed moderate to strong staining in 85.7% of cases (Table 1 and Figure 1). The expression in BL, DLBCL-GCB and FLG3 showed significant differences compared to the germinal center dark zone (Table 1, $X^2=15.221$, $P=0.002$), and the percentage of positive tumor cells was above 70% in 10 cases. No significant difference was found between FLG1-2 and follicular lymphoma grade 3 (FLG3) in either the percentage or intensity of staining. In addition, no difference was noted between the DLBCL-GCB and DLBCL non-GCB subtypes.

The overall expression of Ezh2 in B-cell neoplasms was 56.9% (Table 1). The expression varied among the different subtypes. The highest percentage of positivity was found in BL, FLG3 and DLBCL (100%, 87.5% and 85.7%, respectively). The percentage of positive tumor cells was generally greater than 80% in the three entities. FLG3 showed 87.5% positivity versus 57.8% in FLG1-2. The strongest staining intensity was seen in DLBCL-GCB, in which 8/14 cases (57.1%) showed stronger staining intensity than the germinal center dark zone while the staining in FLG3 was comparable to the latter (Table 1 and Figure 1). The difference in the expression of Ezh2 in BL, DLBCL-GCB and FLG3 compared to the germinal center dark zone was not significant ($X^2=7.0279$, $P=0.064$). FLG1-2 expressed Ezh2 in 57.8% of cases, and the expression was comparable to that of the light zone ($X^2=5.836$, $P=0.054$). In MCL, an enhanced expression of Ezh2 was noted in 66.7% of cases, compared to 3.5% of normal mantle zone cells. CLL/SLL expressed Ezh2 in 20% of cases. Plasma cell myeloma (PCM) expressed Ezh2 in 14.3% of cases while PC, lymphoplasmacytic lymphoma and MALT lymphoma were negative for Ezh2 (Table 1). Plasma cell neoplasms did not show any noticeable difference in Ezh2 expression levels compared to normal CD138+ cells ($X^2=0.745$, $P=0.388$) (Table1, Figures 1 and S1).

High expression of Bmi-1 and Ezh2 in T- and NK/T-cell non-Hodgkin's lymphomas

The overall expression of Bmi-1 was 84.6% in T- and T/NK-cell lymphomas. Bmi-1 was expressed in all subtypes, ranging from 66.7% in adult T-cell leukemia/lymphoma (ATLL) and up to 100% in three subtypes: angioimmunoblastic T-cell lymphoma (AITL), cutaneous T-cell lymphoma

(CTCL) and anaplastic large cell lymphoma (Table 1). In all of the positive cases, Bmi-1 was expressed in more than 80% of the tumor cells. Moderate staining was noted in 37/78 cases (47.4%), and strong staining in 17/78 cases (21.8%), compared to the weaker staining in the normal CD3+, CD4+ and CD56+ cells in RLH and skin (Table1, Figures 2, S1 and S2).

Ezh2 expression was observed in all T- and T/NK-cell lymphoma subtypes, ranging from 66.7% in AITL to 100% in CTCL (Table 1). A moderate staining intensity in these neoplasms was noted, which was in contrast to almost a complete lack of expression of Ezh2 in normal CD3+, CD4+ and CD56+ cells in RLH and skin (Figures 2, S1 and S2). Among the positive cases, the highest percentage of positive tumor cells was in T-LBL (90%) and the lowest was in CTCL (40%). In mature T- and T/NK-cell lymphomas, a significant overexpression of both proteins compared to interfollicular, follicular and cutaneous CD3+ T-cells, as well as CD56+ NK- cells, was detected (*X^2=13.075, P=0.007 for Bmi-1 and X^2=38.942, P=0.000 for Ezh2).*

Significant differences in Ezh2, but not Bmi-1 expression between high- and low-grade B-cell neoplasms

High expression levels of Ezh2 were found mainly in the aggressive variants of B-cell neoplasms while Bmi-1 was highly expressed in all subtypes with no noticeable difference among them (Figure 3A). The Chi squared test for Ezh2 showed a significant difference among the subgroups of B-cell lymphomas *(X^2 = 37.561, P= 0.000)*, and a one-way ANOVA with the Bonferroni correction revealed that there were significant differences between the aggressive subtypes (BL, DLBCL-GCB, DLBCL non-GCB and FLG3) and the indolent subtypes (FLG1-2, PC, PCM, LPL, CLL/SLL and MALT lymphoma) (*mean difference = 1.06~2.31, P = 0.000~0.030)*. This difference corresponded to the differences in the Ki67 index *(mean difference = 0.56~1.00, P = 0.000~0.018)* among these subtypes (Table 2). The Chi squared test for Bmi-1 showed a difference in the expression among the subtypes of B-cell lymphomas *(X^2 = 99.257, P = 0.000)*. However, a one-way ANOVA with the Bonferroni correction detected a single statistically significant difference, between BL and MALT lymphoma (*mean difference = -1.38, P = 0.004)* (Table 2). No statistically significant

differences were detected among the subtypes of T/NK-cell lymphomas with regard to the expression of Bmi-1, Ezh2 and Ki67 (Figure 3B and Table S3).

Ezh2 expression correlated with the proliferative activity

The Ki67 labeling index showed a strong positive correlation with Ezh2 expression in B-cell neoplasms (*correlation coefficient* (*Co*) *= 0.983, P = 0.000*) and a considerable correlation in T- and T/NK-cell lymphomas (*Co = 0.629, P = 0.000*). This correlation was not detected in the case of Bmi-1 *(Co=-0.224, P=0.121)* in B-cell neoplasms, or *(Co=-0.251, P=0.146)* in T- and T/NK-cell lymphomas (Figure 4A). Fluorescent immunohistochemical staining of Ezh2 and Ki67 in DLBCL showed that almost all cells strongly co-expressed Ezh2 and Ki67. A low Ki67 proliferation index was observed in MALT lymphoma, which closely correlated with a low expression of Ezh2 (Figure 4B, white arrowhead). A few cells were stained weakly for Ki67 and Ezh2 (yellow arrowhead). Some cells showed intracellular co-localization of Ezh2 and Ki67 (red arrowhead). Fluorescent immunohistochemical staining revealed that the expression of Ezh2 closely correlated with that of Ki67 in almost all cells in B-cell lymphoma, in accordance with the correlation analysis (Figures 4A and B).

Expression of Mel-18 in RLH and malignant lymphomas

Immunohistochemical staining revealed low expression of Mel-18 in RLH. Double staining with CD20 revealed only a few double positive cells in the germinal center and mantle zones. The same pattern was detected in the interfollicular areas, and double staining with CD3 revealed a few double positive cells. CD138+ plasma cells and CD56+ NK/T cells were both generally negative for Mel-18 (Table 3 and Figure 5). The normal stratified squamous epithelium covering the tonsils showed nuclear and cytoplasmic staining in both the basal and superficial layers (Figure S3).

Mel-18 was expressed in 14.7% of B-cell neoplasms. DLBCL-GCB, DLBCL non-GCB, FLG1-2, PCM and PC all showed weak expression in 16.7% of cases. MALT lymphoma showed weak expression in 10% of cases. In T/NK-cell neoplasms, Mel-18 was expressed in 20% of cases. Both NK/T lymphoma and ALCL expressed Mel-18 in 33.4% of cases. T-LBL and ATLL showed

expression in 16.7% of cases while PTCL was negative in all cases. The staining intensity was typically weak, and the percentage of positive tumor cells was generally below 40% in the positive cases. Mel-18 was expressed weakly in 11/ 64 non-Hodgkin's lymphomas (Table 3 and Figure 5). Co-expression of Mel-18 and Bmi-1 was observed in 10 lymphoma cases. It is worth mentioning that one case of DLBCL-non GCB showed moderate staining for Mel-18 in 80% of tumor cells, and this case was Bmi-1 negative.

Expression of Ezh2, Bmi-1 and Mel-18 in hematopoietic cell lines

Normal resting PBMCs and $CD4^+$ cells were negative for Ezh2 and Mel-18 while the expression of Bmi-1 varied a little; a faint signal was observed in PBMC#1 and normal NK cells. The expression of the three proteins was not stable in PBMCs; stimulation of normal T-cells with CD3/CD28 immunobeads induced a weak expression of all three proteins (Figure 6C). Human mesenchymal stem cells (hMSCs) showed a strong expression of both Bmi-1 and Ezh2 and a weak expression of Mel-18. Lymphoma cell lines showed higher levels of both Bmi-1 and Ezh2 proteins compared to normal PBMCs. Mel-18 showed weak or no expression in B-cell lymphoma cell lines and T/NK lymphoma/leukemia cells compared to the high expression of Bmi-1. HTLV-1 immortalized cell lines (IWA1, MT4 and MT2) showed enhanced expression of Bmi-1, Mel-18 and Ezh2 proteins compared to PBMCs and normal CD4+ cells. The ratio of Bmi-1/Mel-18 was comparable in normal PBMCs and HTLV-I-immortalized cell lines; on the other hand, this ratio noticeably increased in malignant lymphoma cell lines (Figures 6A and B).

Immunofluorescent staining showed that normal resting PBMCs were negative for Ezh2, and a small population was weakly positive for Bmi-1. Immunofluorescent staining of several B and T/NK- lymphoma cell lines confirmed the higher expression levels of both proteins compared to those in normal PBMCs (Figure S4).

Discussion

The expression profile of Bmi-1 and Ezh2 in normal lymphoid tissues was mutually exclusive. Bmi-1 was expressed in the resting cells of the mantle zone, interfollicular T- zone and small

percentage of plasma cells while Ezh2 was expressed in the proliferating cells of the germinal center. In the thymus, Ezh2 expression predominated in the cortex while Bmi-1 predominated in the differentiated cells of the medulla. This is consistent with the findings of previous reports [29-32]. Mel-18 expression in RLH was generally low, which is also consistent with previous reports [33].

The present study showed that Bmi-1 is highly expressed in all subtypes of both B- and T-cell lymphomas, including the indolent types: MALT lymphoma, PC, PCM, LPL and CLL/SLL. Bmi-1 was previously reported to be preferentially expressed in aggressive B-cell lymphoma (DLBCL and BL) but not in indolent subtypes (FL and CLL/SLL) [34]. In the case of FL, the present study showed that Bmi-1 positivity is highly heterogeneous and dependent on each patient specimen; this might be due to differences in the patient populations (Table 1). No significant differences in the expression level of Bmi-1 were found in different grades of FL in the present study, suggesting that the expression of Bmi-1 does not contribute to the progression from low to high grade FL. Bmi-1 was reported to be one of the genes showing the signature of activated B-cell (ABC) type DLBCL, and could be used to distinguish it from the GCB type by a cDNA microarray analysis, and was therefore considered to be associated with a poor prognosis [35]. Later reports based on the immunohistochemical analyses showed that Bmi-1 expression was not restricted to the ABC type [14]. The present study showed that both GCB and non-GCB types expressed Bmi-1 with no significant difference noted between the subtypes (Tables 1 and 2), suggesting that the earlier differences might have been due to the differences between protein and mRNA expression since they are not always consistent. In this study, enhanced expression of Bmi-1 compared to the normal counterpart samples was found in some subtypes of lymphomas: BL, DLBCL-GCB, FLG1-3, PC, PCM and T-cell lymphomas (*P=0.002~0.033*). This is consistent with reports that Bmi-1 transgenic mice developed lymphoma, and that Bmi-1 is a negative regulator of the INK4a-ARF locus, which encodes tumor suppressor proteins $p16^{Ink4a}$, $p19^{Arf}$ and $p15^{Ink4b}$ [13, 36].

Meanwhile, Ezh2 showed a different expression profile in malignant lymphomas. The present study showed that Ezh2 expression was significantly stronger in the aggressive types of B-cell neoplasms (BL, DLBCL and FLG3) than the indolent subtypes (FLG1-2, SLL, LPL, PC, PCM and MALT lymphoma) *(P = 0.000~0.030)* (Table 2*)*. The Ezh2 positivity in FLG3 was higher than that in

FLG1-2. In T/NK-cell lymphomas, Ezh2 showed high expression compared to the negativity observed in normal counterpart samples (X^2=*38.942, P=0.000)*. However, no significant difference among subtypes was noted, which could be explained by the fact that most T/NK-cell lymphomas were aggressive with a high Ki67 proliferation index (Table 1). This was supported by the strong positive correlation between Ki67 and the Ezh2 expression in B-cell lymphoma (*Co* = 0.983, *P*=0.000) and the considerable correlation in T-cell lymphoma (*Co* = 0.629, *P* = 0.000) (Figure 4A). This is consistent with previous reports that Ezh2 overexpression is associated with increased proliferation, metastasis and poor prognosis in breast, prostate and stomach cancers [18-20]. These findings suggest that Ezh2 overexpression is also associated with the proliferation and aggressiveness in lymphoma.

It has been reported that Bmi-1 and other members of PRC1 function downstream of PRC2 [7, 8]. However, the present study showed that there are different expression patterns of Bmi-1 and Ezh2. These proteins do not always co-localize in RLH and malignant lymphomas indicating that, at least in some situations, they function independently. This is supported by evidence that there are genes targeted by PRC2 that lack the product of PRC1 catalysis (H2AK119ub), and genes targeted by PRC1 in the absence of PRC2 [37, 38].

McCabe et al reported that GSK126 is a potent and highly selective inhibitor of Ezh2 methyltransferase activity that decreased the global H3K27me3 level and induced pharmacological inhibition of the proliferation in Ezh2 mutant lymphoma. GSK126 also reduced the H3K27me3 levels in wild-type DLBCL cell lines [39]. In view of the low expression of Ezh2 in normal resting populations, selective pharmacological intervention to inhibit Ezh2 activity in both wild type and mutant lymphomas is a promising approach that warrants further research.

Mel-18 expression was previously reported in several normal adult tissues [15-17]. In RLH, we found that few cells expressed Mel-18 with moderate intensity while some populations expressed Bmi-1 with weak to moderate intensity, especially in the mantle zone. In B- and T/NK-cell neoplasms, Bmi-1 expression was found in 158/187 (84.5%) of cases, and the staining intensity was mostly moderate (109 cases) to strong (26 cases) while Mel-18 was expressed weakly in 11/64 cases (17.1 %) of B- and T/NK cell neoplasms (Tables 1 and 3). The Western blot analysis revealed that both B- and

T/NK-cell lymphoma cell lines showed weak or no expression of Mel-18, in contrast to their moderate/strong expression of Bmi-1 while normal PBMCs were negative for Mel-18 and negative/faintly positive for Bmi-1 (Figure 6). The ratio of Bmi-1/Mel-18 (PRC1.4/PRC1.2) was comparable in normal PBMCs and HTLV-I-immortalized cell lines, and increased noticeably in malignant lymphoma cell lines. Together, these data suggest that the regulation of PRC1 in the normal lymphoid tissue is in favor of PRC1.4, rather than PRC1.2, and that the difference increases with the development of malignancy. Only PRC1.2 and PRC1.4 complexes have been shown to accumulate in H3K27me3-rich regions; they induce chromatin compaction and gene silencing in different targets, and are suggested to play a central role in PRC1 function [6]. The altered expression state may induce changes in the downstream gene targeting and expression in lymphomas.

In conclusion, B-and T/NK- cell neoplasms show high expression of both Bmi-1 and Ezh2 while maintaining low expression of Mel-18. The regulation of PRC1.2 and PRC1.4 shifts more towards PRC1.4 dominance in the neoplastic state. The coexpression of Bmi-1 and Ezh2 is a characteristic of the aggressive variants of these lymphomas. Ezh2 correlates with tumor proliferation, and can be used to distinguish proliferative/aggressive lymphoma variants from indolent ones and normal resting cell populations. A detailed understanding of the precise role(s) of these proteins in lymphomagenesis is still lacking. However, correlation between the expression status of Ezh2 and the Bmi-1/Mel-18 ratio with the patients' prognosis may provide a useful method for the pathological evaluation of lymphomas in the future.

Disclosure/Conflict of Interest

The authors declare no conflict of interest.

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Titles and legends to figures

Fig. 1. Immunohistochemical staining of Bmi-1 and Ezh2 in reactive lymphoid hyperplasia and B-cell neoplasms

(a) Bmi-1 expression was detected mainly in the mantle zone (MZ) while the germinal center (GC) was negative. (**b**) Ezh2 expression was detected in the GC while the MZ was negative (Insets show a higher magnification of the GC). (**c**) and (**d**) DLBCL-GCB-type was strongly positive for Bmi-1 and Ezh2, respectively. (**e**) and (**f**) Follicular lymphoma grade 3 was strongly positive for Bmi-1 and Ezh2, respectively. (**g**) and (**h**) Plasma cell myeloma was strongly positive for Bmi-1 and negative for Ezh2, respectively (**a** and **b**: Magnification x 200, inset x 400 and **c** to **h**: Magnification x400).

Fig. 2. Immunohistochemical staining of Bmi-1 and Ezh2 in T- and NK-cell lymphomas

(**a**) and (**b**) ATLL was strongly positive for Bmi-1 and Ezh2, respectively. (**c**) and (**d**) NK/T lymphoma was strongly positive for Bmi-1 and Ezh2, respectively. (**e**) and (**f**) T-LBL was strongly positive for Bmi-1 and Ezh2, respectively (Magnification x400).

Fig. 3. Expression profiles of Bmi-1 and Ezh2 in malignant lymphoma subtypes and normal control cells

(a) B-cell neoplasms. (b) T/NK-cell lymphomas. MZ: Mantle zone, GC dark: Germinal center dark zone, GC light: Germinal center light zone, Norm PC: Normal plasma cells, BL: Burkitt's lymphoma, DLBCLGC: Diffuse large cell B-cell lymphoma germinal center type, DLBCLNG: Diffuse large B-cell lymphoma non-germinal center type, FLG3: Follicular lymphoma grade 3, FLG1-2: Follicular lymphoma grade 1 and 2, MCL: Mantle cell lymphoma, CLL/SLL: Chronic lymphocytic leukemia/small lymphocytic lymphoma, PCM: Plasma cell myeloma, PC: Plasmacytoma, LPL: Lymphoplasmacytic lymphoma, MALT: Mucosa-associated lymphoid tissue lymphoma, Norm NK/T: Normal natural killer T-cell, IF: Interfollicular, T-LBL: T-cell lymphoblastic lymphoma, ATLL: Adult T-cell leukemia/lymphoma, ALCL: Anaplastic large cell lymphoma, PTCL: Peripheral T-cell lymphoma, NK/T: Natural killer/T-cell lymphoma, CTCL: Cutaneous T-cell lymphoma and AITL: Angioimmunoblastic T-cell lymphoma.

Fig. 4. The correlation between Ezh2 and Ki67 expression, and between Bmi-1 and Ki67 expression based on the percentage of positive cells in both B- and T/NK-cell lymphomas

(A): (**a**) and (**c**) B-cell lymphomas. (**b**) and (**d**) T/NK-cell lymphomas. Co is the Pearson's correlation coefficient (The correlation was considered significant at the 0.01 level). **(a**) and (**b**) The correlation between Ki67 and Ezh2. (**c**) and (**d**) The correlation between Ki67 and Bmi-1. (**B)** Double fluorescent immunohistochemical staining of Ezh2 and Ki67 in DLBCL and MALT lymphoma patient specimens: (**a**) A high percentage of Ki67 (+) cells was found in DLBCL. (**b**) High expression of Ezh2 was found in the same case. (**c**) DAPI staining, (**d**) Merged Ezh2 and Ki67 staining showed coexpression in the same cells**. (e**) A low percentage of Ki67 (+) cells was found in MALT lymphoma. **(f**) A few cells expressed Ezh2 in the same case. (**g**) DAPI staining, (**h**) Merged Ezh2 and Ki67 staining. The yellow arrowheads show single positive for Ki67 and negative for Ezh2; many cells co-expressed Ki67 and Ezh2 (white arrowheads). The red

arrowheads show intracellular co-localization of Ezh2 and Ki67 (Magnification x400, Ezh2: red, Ki67: green).

Fig. 5. Expression of Mel-18 in normal tonsil and malignant lymphomas

(a) Few cells expressed Mel-18 in the germinal center and interfollicular zone of normal tonsils while the stratified squamous epithelial cells were positive (Inset from the germinal center showing one centroblast with moderate staining intensity for Mel-18) (b) Mucosa-associated lymphoid tissue (MALT) lymphoma showed a few cells with moderate staining intensity. (c) Diffuse large B-cell lymphoma showed a few cells weakly expressing Mel-18 (d) NK/T cell lymphoma showed a few cells weakly expressing Mel-18 (a: Magnification x100, inset: x400, b to d: Magnification x400).

Fig. 6. Results of the Western blot analyses of Bmi-1, Ezh2 and Mel-18 expression in malignant lymphoma cell lines and normal control cells

(**A**) Both Bmi-1 and Ezh2 were expressed in most B-cell lymphoma lines; however, Ezh2 showed a stronger signal in the more aggressive lymphoma lines. Mel-18 was negative in most B-cell lines **(B**) Both Bmi-1 and Ezh2 were expressed in most T-cell lymphoma lines. Mel-18 was weakly/faintly positive in ATLL leukemic cell lines (ED40515 and ATL55T). This was in contrast to HTLV-I immortalized cell lines (IWA1, MT4 and MT2), which showed an expression level comparable to that of Bmi-1. (**C**) Normal PBMCs#1 and normal NK cells showed a faint signal for Bmi-1 while they were negative for Ezh2 and Mel-18. Activated T-cells showed a faint expression of all three proteins. PBMCs#1 to #4: Peripheral blood mononuclear cells (PBMCs) from four healthy individuals, Activate T: T-cells activated with a CD3/CD28 cell expander and Norm NK: Normal natural killer (NK) cells.

Figure

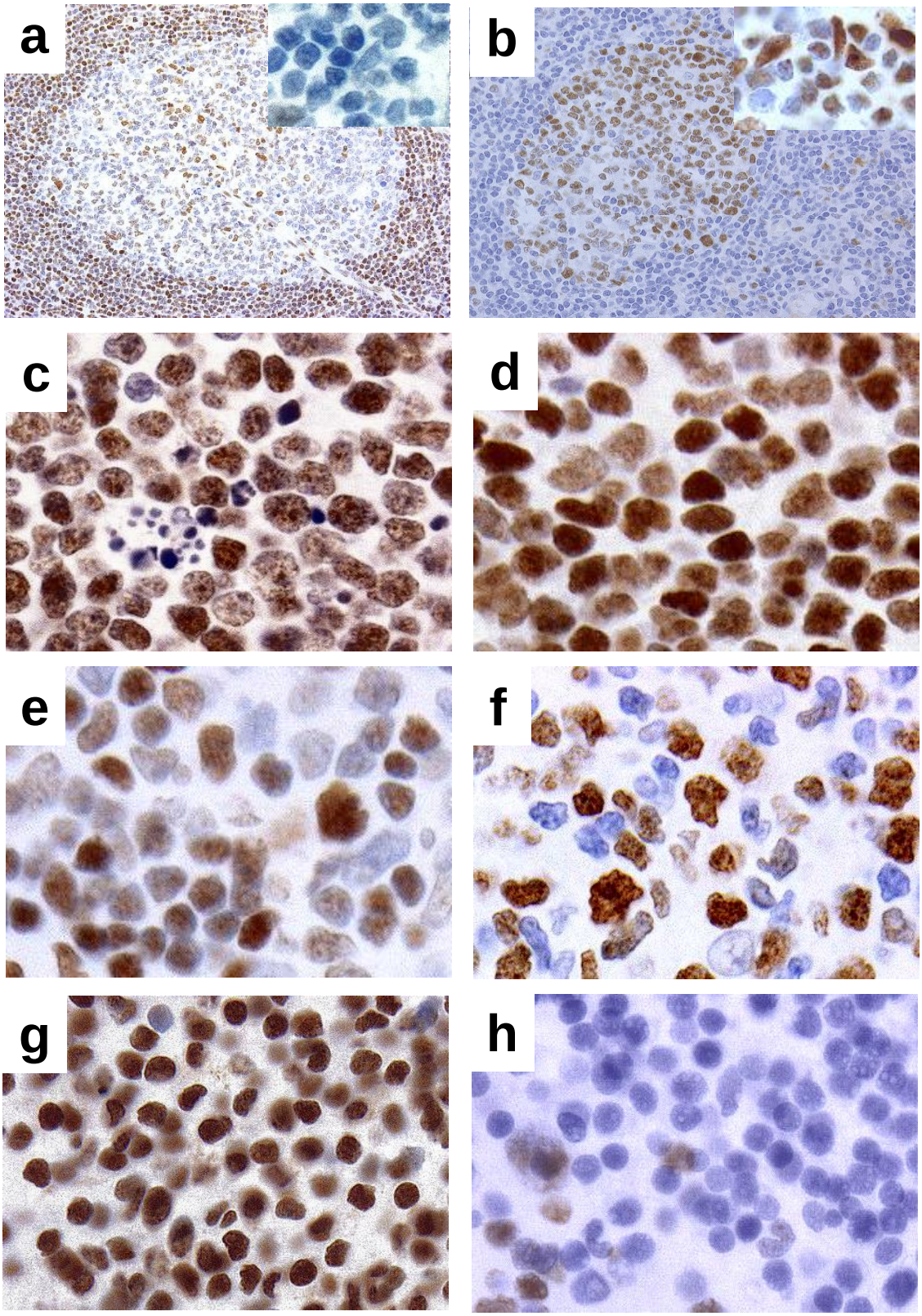


Fig.1

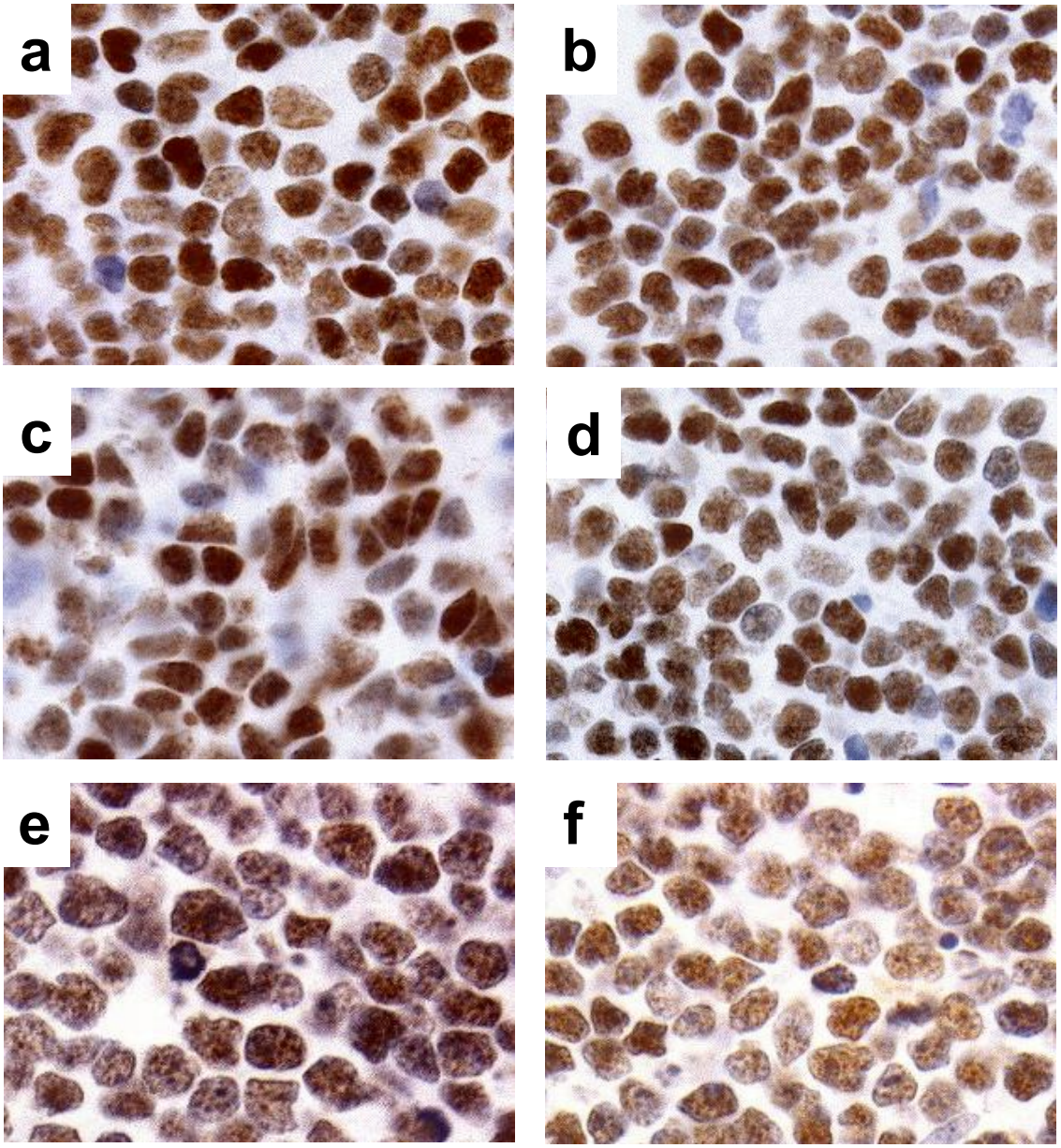
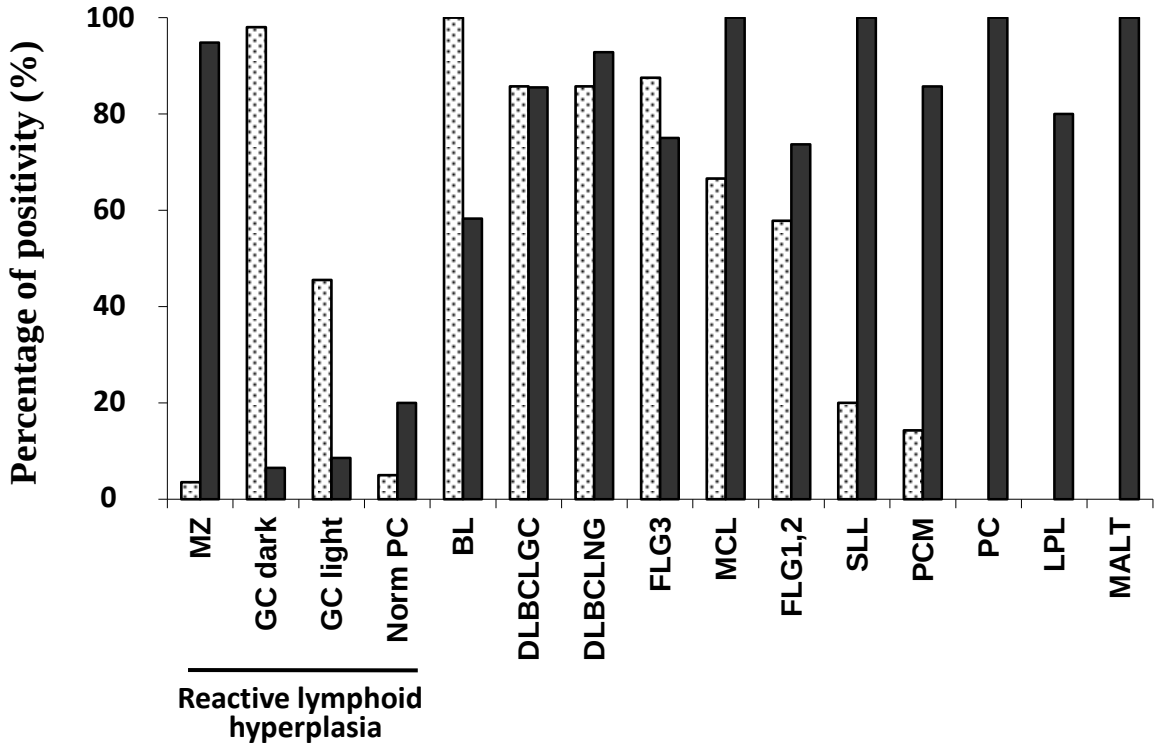


Fig. 2

A)


Percentage of positivity (%)
100
80
60
40
20
0
MZ
GC dark
GC light
Norm PC
BL
DLBCLGC
DLBCLNG
FLG3
MCL
FLG1,2
SLL
PCM
PC
LPL
MALT
Reactive lymphoid hyperplasia

B)

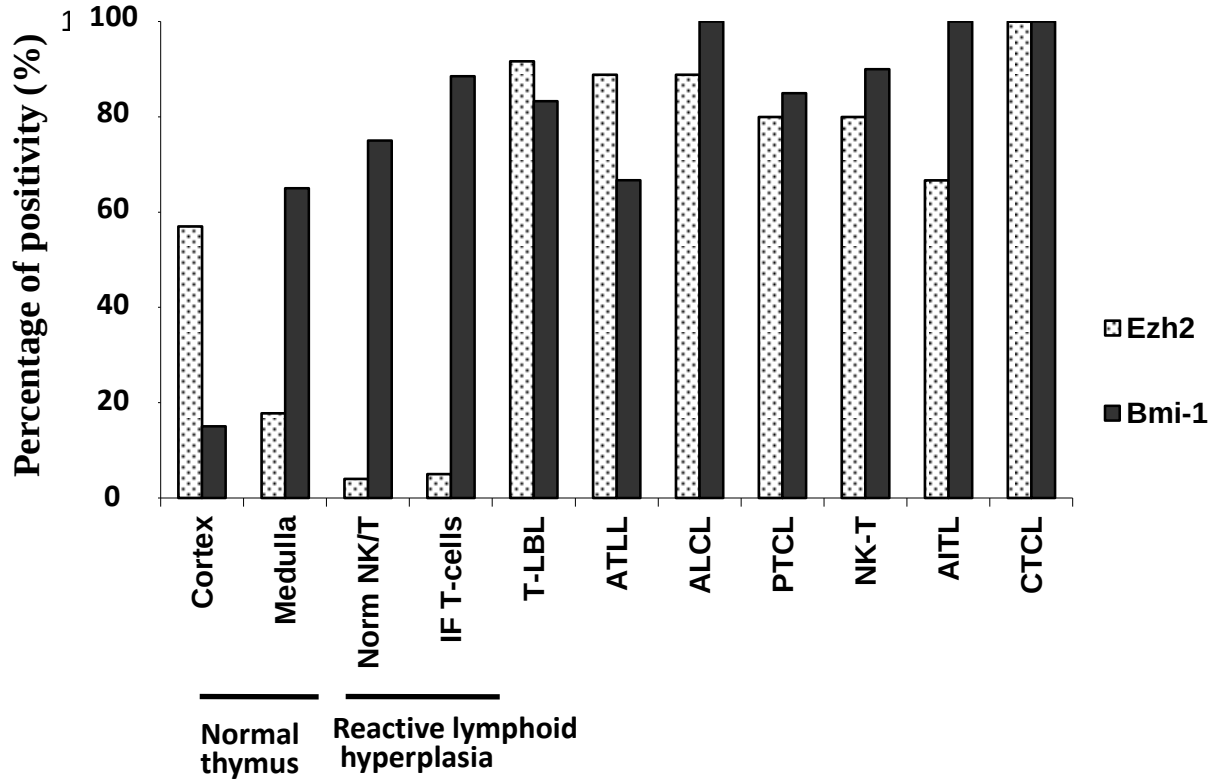
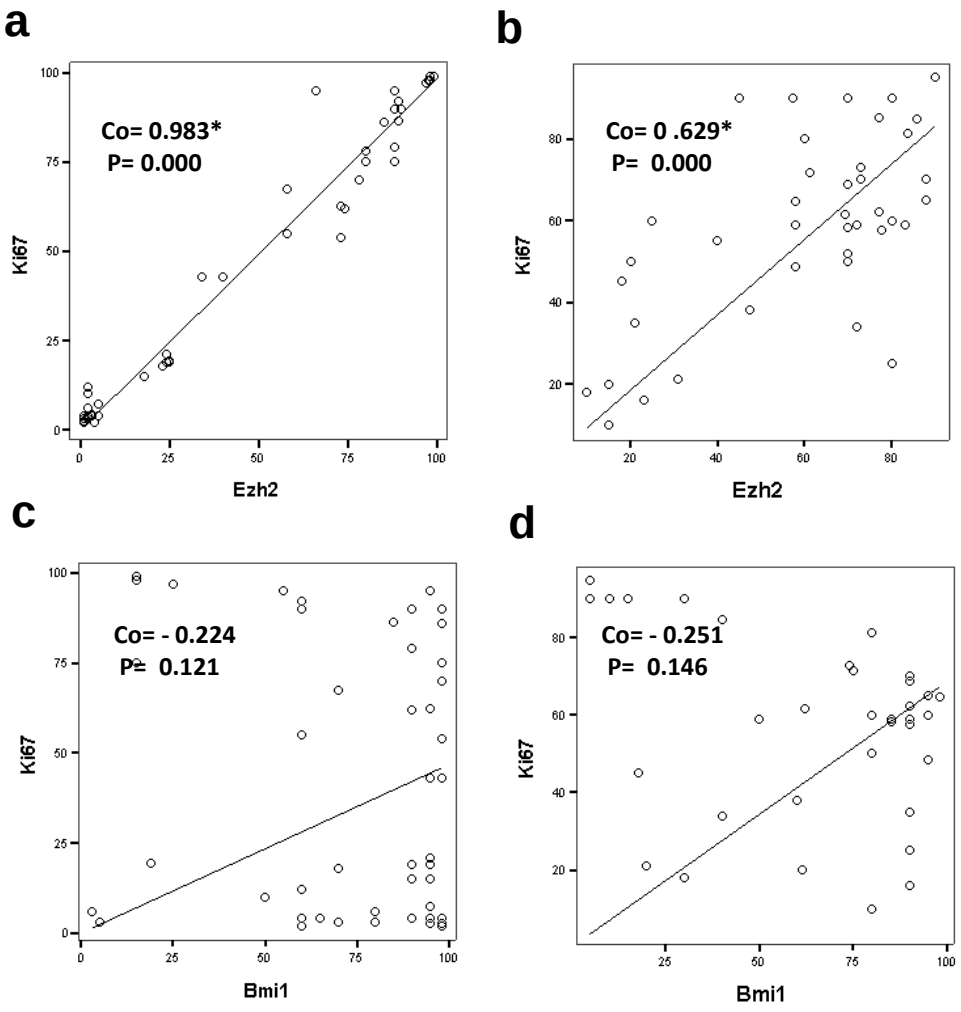

Percentage of positivity (%)
100
80
60
40
20
0
Cortex
Medulla
Norm NK/T
IF T-cells
T-LBL
ATLL
ALCL
PTCL
NK-T
AITL
CTCL
Ezh2
Bmi-1
Normal thymus
Reactive lymphoid hyperplasia

Fig. 3

A)


a
Co= 0.983*
P= 0.000
Ki67
Ezh2
b
Co= 0 .629*
P= 0.000
Ki67
Ezh2
c
Co= - 0.224
P= 0.121
Ki67
Bmi1
d
Co= - 0.251
P= 0.146
Ki67
Bmi1

B)

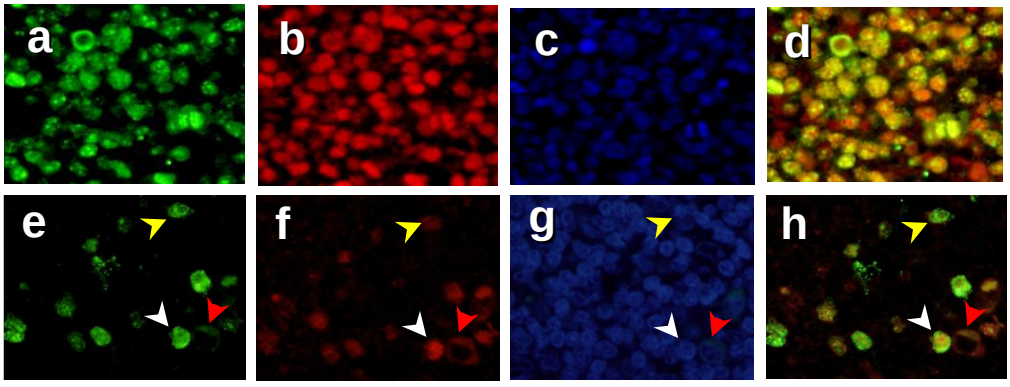

a
b
c
d
e
f
g
h

Fig. 4

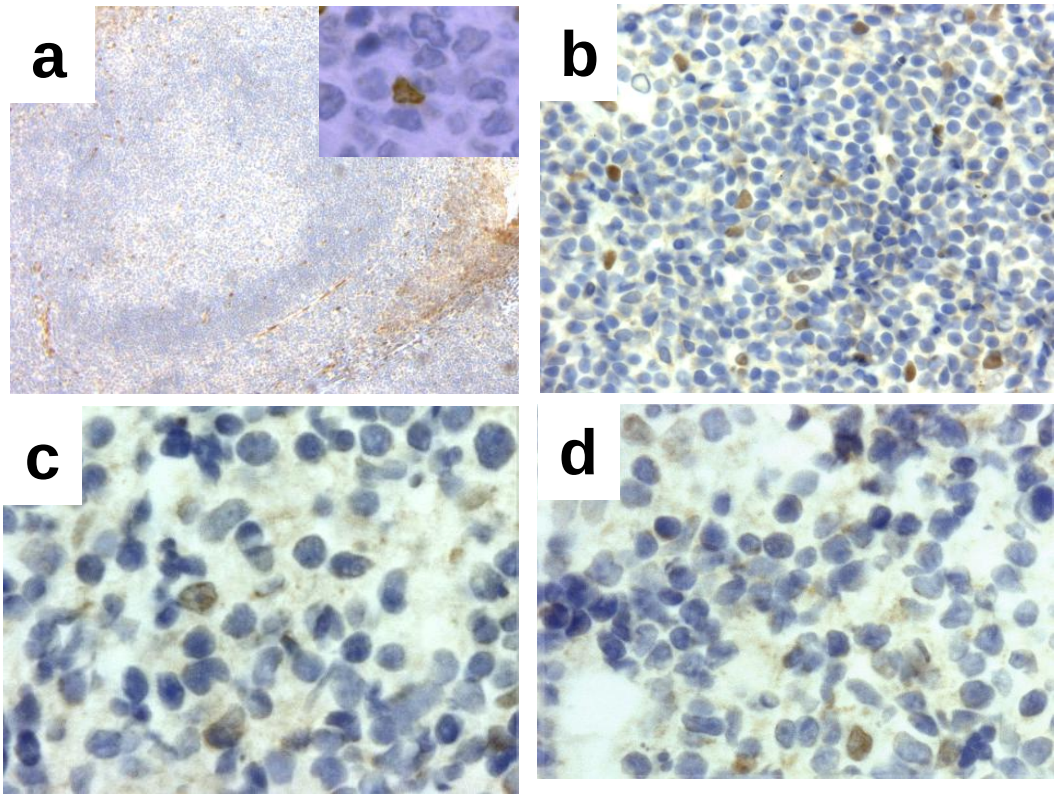


Fig. 5

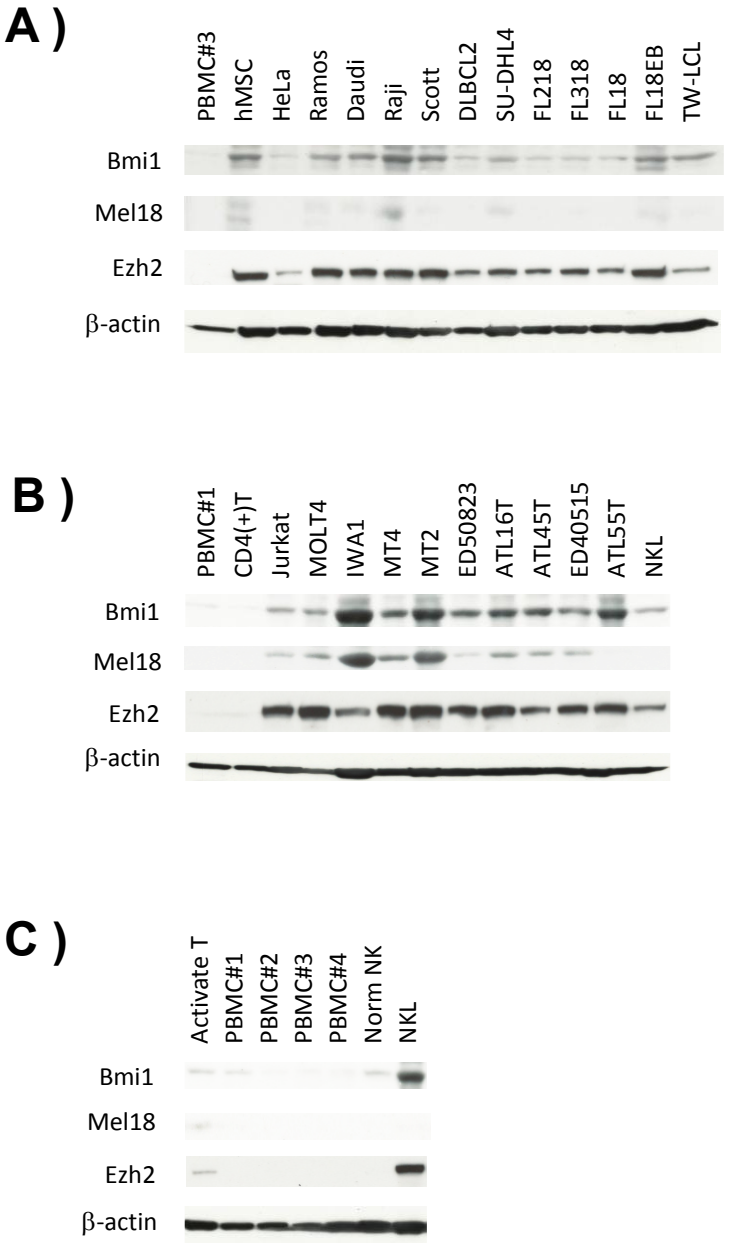

A)
PBMC#3
hMSC
HeLa
Ramos
Daudi
Raji
Scott
DLBCL2
SU-DHL4
FL218
FL318
FL18
FL18EB
TW-LCL
Bmi1
Mel18
Ezh2
β-actin
B)
PBMC#1
CD4(+)T
Jurkat
MOLT4
IWA1
MT4
MT2
ED50823
ATL16T
ATL45T
ED40515
ATL55T
NKL
Bmi1
Mel18
Ezh2
β-actin
C)
Activate T
PBMC#1
PBMC#2
PBMC#3
PBMC#4
Norm NK
NKL
Bmi1
Mel18
Ezh2
β-actin

Fig. 6

Table

Table 1. Ezh2 and Bmi-1 expression and Ki67 labeling index in different subtypes of lymphoma and normal control

Malignant lymphoma and normal control	Ezh2						Bmi-1						Ki67	
	-	+	++	+++	positive/ Total	Positivity %	-	+	++	+++	positive / Total	Positivity %	High/ Total	High %
HD	1	4	5	0	9/10	90%	1	2	7	0	9/10	90%	ND	ND
BL	0	0	10	2	12/12	100 %	5	3	4	0	7/12	58.3%	12/12	100%
DLBCL-GCB	2	2	2	8	12/14	85.7 %	2	1	9	2	12/14	85.7 %	13/14	92.8%
DLBCL non GCB	2	2	9	1	12/14	85.7%	1	3	10	0	13/14	92.9%	14/14	100%
FLG3	1	0	7	0	7/8	87.5%	2	2	4	0	6/8	75%	8/8	100%
MCL	3	4	2	0	6/9	66.7%	0	0	9	0	9/9	100%	4/9	44.4%
FLG1- 2	8	3	8	0	11/19	57.8 %	5	1	10	3	14/19	73.7 %	3/19	15.7%
CLL/SLL	4	0	1	0	1/5	20 %	0	0	5	0	5/5	100%	1/5	20%
PCM	6	0	1	0	1/7	14.3 %	1	1	5	0	6/7	85.7 %	2/7	28.6%
PC	6	0	0	0	0/6	0%	0	0	5	1	6/6	100%	1/6	16.7%
LPL	5	0	0	0	0/5	0%	1	0	4	0	4/5	80%	0/5	0%
MALT lymphoma	10	0	0	0	0/10	0 %	0	0	3	7	10/10	100 %	1/10	10%
B-cell neoplasms	47	11	40	11	62/109	56.9%	17	11	72	9	92/109	84.4%	59/109	54.1%
T-LBL	1	1	8	2	11/12	91.7 %	2	0	3	7	10/12	83.3 %	11/12	91.7%
ATLL	2	6	8	2	16/18	88.9%	6	2	7	3	12/18	66.7%	15/18	83.3%
ALCL	1	4	4	0	8/9	88.9%	0	0	9	0	9/9	100%	9/9	100%
PTCL	4	4	12	0	16/20	80 %	3	2	10	5	17/20	85 %	17/20	85%
NK/T	2	2	6	0	8/10	80%	1	5	2	2	9/10	90%	9/10	90%
AITL	2	0	4	0	4/6	66.7 %	0	1	3	2	6/6	100 %	6/6	100%
CTCL	0	0	3	0	3/3	100%	0	0	3	0	3/3	100%	3/3	100%
T- and NK-cell neoplasms	12	17	45	4	66/78	84.6%	12	10	37	17	66/78	84.6%	70/78	89.7%
RLH														
Mantle zone	386	10	4	0	14/400	3.5%	21	300	70	9	379/400	94.8%	10/400	2.5%
Germinal centre dark zone	4	110	80	6	196/200	98%	187	10	3	0	13/200	6.5%	192/200	96%
Germinal centre light zone	109	70	20	1	91/200	45.5%	183	12	5	0	17/200	8.5%	105/200	52.5%
CD 138 + plasma cells	190	7	3	0	10/200	5%	160	35	5	0	40/200	20%	0/200	0%
Interfollicular CD3+ cells	180	15	5	0	20/400	5%	56	250	80	14	354/400	88.5%	6/400	1.5%
CD56 + NK/T cells	192	8	0	0	8/200	4%	50	116	30	4	150/200	75%	7/200	3.5%
Follicular CD4+ T-helper cells	186	14	0	0	14/200	7%	31	140	25	4	169/200	84.5%	4/200	2%
Normal thymus:														
Cortical CD3 + T cells	172	60	140	28	228/400	57%	340	30	22	8	60/400	15%	200/400	50%
Medullary CD3 + T cells	329	30	30	11	71/400	17.8%	140	160	75	25	260/400	65%	16/400	4%

Positivity includes (+), (++) and (+++). HD: Hodgkin lymphoma, BL: Burkitt lymphoma, DLBCL-GCB: Diffuse large cell B cell lymphoma germinal centre type, DLBCL non GCB: Diffuse large B- cell lymphoma non germinal centre type, FLG3: Follicular lymphoma grade3, FLG1-2: Follicular lymphoma grade 1-2, MCL: Mantle cell lymphoma, CLL/SLL: Chronic lymphocytic leukemia/small lymphocytic lymphoma, PCM: Plasma cell myeloma, PC: Plasmacytoma, LPL: Lymphoplasmacytic lymphoma, MALT: Mucosa associated lymphoid tissue, T-LBL: T-cell lymphoblastic lymphoma, ATLL: Adult T-cell leukemia/lymphoma, ALCL: Anaplastic large cell lymphoma, , PTCL: Peripheral T-cell lymphoma, NK/T: Natural killer/T-cell lymphoma, AITL: Angioimmunoblastic T-cell lymphoma, CTCL: Cutaneous T-cell lymphoma, RLH : Reactive lymphoid hyperplasia. Data from RLH and thymus are based on cell count.

Table 2. Difference in expression of Bmi-1, Ezh2 and Ki67 among different subtypes of B-cell non Hodgkin lymphoma

Protein		BL	DLBCL-GCB	DLBCL Non-GCB	FL G3	FL G1-2
Bmi-1	**DLBCL-GCB**	-0.87 P=0.327				
	DLBCL non-GCB	- 0.73 P=1.000	0.14 P=1.000			
	FL G3	-0.33 P=1.000	0.54 P=1.000	0.39 P=1.000		
	FL G1-2	-0.66 P=1.000	0.21 P=1.000	0.06 P=1.000	-0.33 P=1.000	
	MCL	-1.08 P=0.128	-0.21 P=1.000	- 0.36 P=1.000	- 0.75 P=1.000	- 0.42 P=1.000
	PCM	-0.65 P=1.000	0.21 P=1.000	0.07 P=1.000	- 0.32 P=1.000	0.01 P=1.000
	PC	-1.25 P=0.108	- 0.38 P=1.000	- 0.52 P=1.000	- 0.92 P=1.000	- 0.59 P=1.000
	LPL	-0.68 P=1.000	0.19 P=1.000	0.04 P=1.000	- 0.35 P=1.000	- 0.02 P=1.000
	MALT	***-1.38**** ***P=0.004***	- 0.51 P=1.000	- 0.66 P=1.000	-1.05 P=1.000	- 0.72 P=1.000
	CLL/SLL	-1.08 P=0.607	- 0.21 P=1.000	-0.36 P=1.000	-0.75 P=1.000	-0.42 P=1.000
Ezh2	**DLBCL-GCB**	-0.14 P=1.000				
	DLBCL non-GCB	0.52 P=1.000	0.66 P=1.000			
	FL G3	0.42 P=1.000	0.56 P=1.000	-0.11 P=1.000		
	FL G1-2	***1.11**** ***P=0.007***	***1.25**** ***P=0.001***	0.59 P=1.000	0.69 P=1.000	
	MCL	***1.28**** ***P=0.011***	***1.42**** ***P=0.002***	0.75 P=1.000	0.86 P=1.000	0.17 P=1.000
	PCM	***1.88**** ***P=0.000***	***2.02**** ***P=0.000***	***1.36**** ***P=0.009***	***1.46**** ***P=0.015***	0.77 P=1.000
	PC	***2.17**** ***P=0.000***	***2.31**** ***P=0.000***	***1.64**** ***P=0.001***	***1.75**** ***P=0.002***	1.06 P=0.191
	LPL	***2.17**** ***P=0.000***	***2.31**** ***P=0.000***	***1.64**** ***P=0.003***	***1.75**** ***P=0.005***	1.06 P=0.346
	MALT	***2.17**** ***P=0.000***	***2.31**** ***P=0.000***	***1.64**** ***P=0.000***	***1.75**** ***P=0.000***	***1.06*** ***P=0.030***
	CLL/SLL	***1.77**** ***P=0.001***	***1.91**** ***P=0.000***	1.24 P=0.105	1.35 P=0.113	0.66 P=1.000
Ki67	**DLBCL-GCB**	0.00 P=1.000				
	DLBCL non-GCB	0.00 P=1.000	0.00 P=1.000			
	FL G3	0.00 P=1.000	0.00 P=1.000	0.00 P=1.000		
	FL G1-2	***0.80**** ***P=0.000***	***0.80**** ***P=0.000***	***0.80**** ***P=0.000***	***0.80**** ***P=0.000***	
	MCL	***0.56**** ***P=0.005***	***0.56**** ***P=0.004***	***0.56**** ***P=0.003***	***0.56**** ***P=0.018***	- 0.24 P=1.000
	PCM	***0.71**** ***P=0.000***	***0.71**** ***P=0.000***	***0.71**** ***P=0.000***	***0.71**** ***P=0.001***	-0.09 P=1.000
	PC	***0.80**** ***P=0.000***	***0.80**** ***P=0.000***	***0.80**** ***P=0.000***	***0.80**** ***P=0.001***	0.00 P=1.000
	LPL	***1.00**** ***P=0.000***	***1.00**** ***P=0.000***	***1.00**** ***P=0.000***	***1.00**** ***P=0.000***	0.20 P=1.000
	MALT	***0.90**** ***P=0.000***	***0.90**** ***P=0.000***	***0.90**** ***P=0.000***	***0.90**** ***P=0.000***	0.10 P=1.000
	CLL/SLL	***0.80**** ***P=0.000***	***0.80**** ***P=0.000***	***0.80**** ***P=0.000***	***0.80**** ***P=0.001***	0.00 P=1.000

One way ANOVA with Bonferroni correction, upper panel is the mean difference, * and bold font indicate significant values (P <0.05 level). BL: Burkitt lymphoma, DLBCL (GCB): Diffuse large cell B-cell lymphoma germinal centre type, DLBCL non GCB: Diffuse large B- cell lymphoma non germinal centre type, FLG3: Follicular lymphoma grade3, FLG1,2: Follicular lymphoma grade 1,2, MCL: Mantle cell lymphoma, CLL/SLL: Chronic lymphocytic leukaemia/small lymphocytic lymphoma, PCM: Plasma cell myeloma, PC: Plasmacytoma, LPL: Lymphoplasmacytic lymphoma, MALT: Mucosa associated lymphoid tissue

Table 3. Expression of Mel-18 in RLH and malignant lymphomas

Malignant lymphoma and normal control	Mel-18				Positive/ Total	Positivity %
	-	+	++	+++		
DLBCL-GCB	5	1	0	0	1/6	16.7%
DLBCL non-GCB	5	0	1	0	1/6	16.7%
FLG1-2	5	1	0	0	1/6	16.7%
PCM and PC	5	1	0	0	1/6	16.7%
MALT lymphoma	9	1	0	0	1/10	10%
B-cell neoplasms	29	4	1	0	5/34	14.7%
T-LBL	5	1	0	0	1/6	16.7%
ATLL	5	1	0	0	1/6	16.7%
PTCL	6	0	0	0	0/6	0%
NK/T lymphoma	4	2	0	0	2/6	33.4%
ALCL	4	2	0	0	2/6	33.4%
T/NK-cell neoplasms	19	5	0	0	6/30	20%
RLH						
Mantle zone	390	0	10	0	10/400	2.5%
Germinal centre dark zone	184	0	14	0	14/200	7%
Germinal centre light zone	195	0	5	0	2/200	2.5%
CD 138 + plasma cells	196	0	4	0	2/200	2%
Interfollicular CD3 + cells	192	0	8	0	8/200	4%
CD56 + NK/T cells	197	0	4	0	4/200	2%

Positivity includes (-), (+), (++) and (+++). DLBCL-GCB: Diffuse large cell B cell lymphoma germinal centre type, DLBCL non GCB: Diffuse large B- cell lymphoma non germinal centre type, FLG1-2: Follicular lymphoma grade 1-2, PCM: Plasma cell myeloma, PC: Plasmacytoma, MALT: Mucosa associated lymphoid tissue, T-LBL: T-cell lymphoblastic lymphoma, ATLL: Adult T-cell leukemia/ lymphoma, ALCL: Anaplastic large cell lymphoma, , PTCL: Peripheral T-cell lymphoma, NK/T: Natural killer/T-cell lymphoma, RLH : Reactive lymphoid hyperplasia. Data from RLH are based on cell count.

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