主　論　文

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

(高悪性度非ホジキンリンパ腫において Ezh2 は強発現し、ポリコーム群蛋白質複合体 PRC1.4 は PRC1.2 に対し優位に発現する)

[緒言]

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 multistep lymphomagenesis process. Aberrant epigenetic changes, including DNA methylation, histone modifications, chromatin remodeling, and noncoding RNAs, also contribute to the development and progression of malignancy.

Polycomb group (PcG) proteins are important regulators of lymphopoiesis. They form polycomb repressive complexes (PRCs), which control histone mod ifications. In mammals, there are two main PcG complexes: PRC1 and PRC2. PRC2 includes Ezh2, Eed, Suz12, RBBP4/7, and Jarid2. PRC1 six subgroups have been identified and numbered (PRC1.1–1.6). Bmi-1/PCGF4 is a component of the PRC1.4 complex and was described as a proto-oncogene that induced lymphomas in mice. Upregulation of Bmi-1 was reported in a variety of cancers. 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. .Ezh2 overexpression has been reported in solid organ cancers. It is associated with metastasis, poor prognosis, and treatment failure.

However, much remains unknown about the expression profiles of PcG proteins and their significance in terms of the pathology of 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.

[材料と方法]

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. Reactive lymphoid hyperplasia (RLH) and thymus 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.

Cell culture

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

The following mouse monoclonal antibodies were used: Ezh2 (clone M18) [26, 27], Bmi-1, (Abcam, MA, USA), CD3, CD20, CD56 (Novocastra, Newcastle upon Tyne, UK) and rabbit anti-Mel-18 (Santa Cruz Biotechnologies Inc., Texas, USA), and 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 ten high-power fields by three investigators. Positivity was defined as ≥25 % nuclear staining of tumor cells, in accordance with previous reports.

Double fluorescent immunocytochemical and immunohistochemical staining

A mixture of anti-mouse and anti-rabbit IgG for 2 to 3 h at room temperature was used. 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 examined with a conventional immunofluo- rescence microscope (IX71, Olympus, Tokyo, Japan).

Western blotting analysis

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

Statistical analyses were done with the SPSS version 11.5 software program. Pearson's chi-squared test and one-way analysis of variance (ANOVA) with the Bonferroni 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.

[結果]

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, nterfollicular CD3+ T cells, follicular CD4+ T helper cells and CD56+ NK/T cells were mostly Bmi-1+/Ezh2−. In 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-1+.

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

In B cell neoplasms, the overall expression of Bmi-1 was 84.4 %. Bmi-1 expression was found in all subtypes, ranging from 58.3 % in Burkitt's lymphoma (BL) to 100 % in four subtypes. No significant difference was found between the subtypes. The overall expression of Ezh2 in B cell neoplasms was 56.9 %. The highest percentage of positivity was found in BL, follicular lymphoma (FLG3), and diffuse large B-cell lymphoma DLBCL. FLG3 showed 87.5 % positivity versus 57.8 % in FLG1–2. Other B-cell neoplasms showed low expression of Ezh2.

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

The overall expression of Bmi-1 was 89.7% in T and T/NK cell lymphomas and 84.6 % for ezh2. Both were highly expressed in all subtypes.

Significant differences in Ezh2, but not Bmi-1, expression between high-grade 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 between them ($\chi 2$=37.561, P=0.000), and a one-way ANOVA with Bonferroni correction revealed that were significant differences between the aggressive subtypes (BL, DLBCL, and FLG3) and other indolent subtypes (P=0.000–0.030). One-way ANOVA with Bonferroni correction detected no significant differences in Bmi-1 between the subtypes.

Ezh2 expression correlated with the proliferative activity

The Ki67 labeling index showed a positive correlation with Ezh2 expression in B- and T/NK cell lymphomas. This correlation was not detected in the case of Bmi-1. Fluorescent immunohistochemical staining of Ezh2 and Ki67 in DLBCL showed that almost all cells strongly

coexpressed Ezh2 and Ki67. A low Ki67 proliferation index was observed in indolent lymphomas, which closely correlated with a low expression of Ezh2.

Expression of Mel-18 in RLH and malignant lymphomas

Immunohistochemical staining revealed low expression of Mel-18 in RLH. Mel-18 was weakly expressed in 14.7 % of B cell neoplasms and 20% in T/NK cell neoplasms.

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; stimulation of normal T cells with CD3/CD28 immunobeads induced a weak expression of all three proteins. Human mesenchymal stem cells and lymphoma cell lines showed a high expression of both Bmi-1 and Ezh2 and a weak expression of Mel-18. 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.

[考察]

Consistent with previous reports, the expression profiles of Bmi-1 and Ezh2 in normal lymphoid tissues were mutually exclusive. Bmi-1 was expressed in the resting cells of the mantle zone and interfollicular T zone while Ezh2 was expressed in the proliferating cells of the germinal center.

The present study showed that Bmi-1 is highly expressed in all subtypes of both B and T cell lymphomas, including the indolent types: No significant differences in the expression level of Bmi-1 were found between different subtypes. Enhanced expression of Bmi-1 compared to the normal counterpart samples was detected. 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 p16Ink4a, p19Arf, and p15Ink4b.

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 than the indolent subtypes. In T/NK cell lymphomas, Ezh2 showed high expression compared to the lack of expression observed in normal counterpart. This was supported by the positive correlation between Ki67 and the Ezh2 expression in B- and T/NK cell lymphoma. This is consistent with previous reports that Ezh2 overexpression is associated with increased proliferation, metastasis, and poor prognosis in breast, prostate, and stomach cancers..

Mel-18 was expressed weakly in B and T/NK cell neoplasms and in B and T/NK cell lymphoma cell lines. 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 normal lymphoid tissue is in favor of PRC1.4, rather than PRC1.2.

[結論]

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.