Coexpression of BMI-1 and EZH2 polycomb-group proteins is associated with cycling cells and degree of malignancy in B-cell non-Hodgkin lymphoma

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Introduction

B-cell non-Hodgkin lymphomas (B-NHLs) are clonal disorders with a mature phenotype and rearranged immunoglobulin genes. These tumors show a wide spectrum of morphologic features that vary between a nearly intact preservation of nodal architecture in follicular lymphoma and a diffuse growth pattern in most large B-cell lymphomas and Burkitt lymphoma. The pathogenic mechanism leading to B-NHL is probably a multistep process related to the inherent genetic instability associated with immunoglobulin rearrangement, external factors such as impaired or suppressed immunity, and a variety of environmental factors.

Polycomb-group (PcG) proteins play a role in body plan formation (axial patterning through the repression of Hox genes), hematopoiesis, and checkpoints affecting cell cycle entry. Recent experiments also identified PcG proteins as a group of gene-regulatory factors that may contribute to oncogenesis and lymphomagenesis. PcG proteins form large, multimeric complexes that bind to chromatin and probably function by altering chromatin structure. Some PcG proteins may repress gene activity through histone deacetylation. So far, 2 PcG complexes have been identified: a complex containing the ENX/EZH2 and EED PcG proteins and another complex consisting of BMI-1, RING1, HPH1, HPH2, HPC1, HPC2, and HPC3. These complexes are hypothesized to have opposing roles: predominance of one complex may maintain cells in a proliferative state, whereas predominance of the other complex is seen in differentiated cells.

Previously, we demonstrated that the expression of PcG complexes during germinal center (GC) reaction is linked to the differentiation status of follicular B cells. We observed a mutually exclusive pattern of BMI-1/RING1 and EZH2/EED PcG proteins in reactive centroblasts and centrocytes. EZH2/EED expression was seen in dividing centroblasts of GC dark zones, and BMI-1/RING1 expression was dominant in resting B cells of the mantle zones and centrocytes in the light zones. These observations suggested that the expression of PcG complexes is highly regulated during GC reaction and that PcG proteins may contribute to antigen-specific B-cell maturation.

Deregulation of PcG gene expression in experimental model systems has clearly been linked to oncogenesis. For instance, the overexpression of Bmi-1 resulted in lymphomas in transgenic mice. In addition, the overexpression of RING1 caused anchorage-independent growth, cellular transformation, and metastatic activity in nude mice. Yet, little is known about a possible role for PcG genes in human lymphoma. We recently demonstrated that Mib-1/Ki-67+ Hodgkin–Reed-Sternberg (HRS) cells coexpress EZH2 and BMI-1. Because most HRS cells originate from B cells in reactive follicles, where the expression of BMI-1 and the expression of EZH2 are mutually exclusive, this pattern suggested that Hodgkin lymphoma is associated with deregulated expression of PcG complexes.
In the current study, we questioned whether B-NHL is also associated with BMI-1/EZH2 coexpression in Mib-1/Ki-67+ neoplastic B cells. Using unique antisera against BMI-1 and EZH2, we found BMI-1/EZH2 coexpression in Mib-1/Ki-67+ neoplastic large cells in intermediate- and high-grade B-NHL. Large Mib-1/Ki-67+ neoplastic cells in low-grade B-NHL showed weak coexpression of EZH2 and BMI-1. By contrast, small neoplastic cells in low-grade B-NHL showed reduced BMI-1 expression in the absence of EZH2 or Mib-1/Ki-67. We concluded that human B-NHL, such as Hodgkin lymphoma, is associated with irregular expression of BMI-1 and EZH2 PcG genes. In addition, the level of BMI-1/EZH2 coexpression correlated with clinical grade and the presence of Mib-1/Ki-67 expression. These findings suggest that the irregular expression of BMI-1 and EZH2 is an early event in the formation of B-NHL, and they point to a role for abnormal PcG expression in human lymphomagenesis.

Materials and methods

Patient material

Fifty-two lymph nodes from patients with B-NHL were obtained after surgery, immediately frozen or fixed in 10% buffered formalin, and embedded in paraffin (Table 1). Burkitt lymphoma (n = 5), mantle-cell lymphoma (MCL) (n = 6), follicular lymphoma (n = 30), diffuse large B-cell lymphoma (n = 6), and small lymphocytic lymphoma (n = 5) were retrieved by boiling for 10 minutes in citrate buffer (pH, 6), followed by successive rinses in phosphate-buffered saline (PBS) containing 0.5% Triton (1 × 5 minutes) and then in PBS only (3 × 3 minutes). Slides were incubated for 10 minutes in 0.1 M glycine (diluted in PBS) and rinsed in PBS only (3 × 5 minutes). Before application of the primary antisera or antibody, sections were incubated for 10 minutes in normal swine serum (diluted 1:10 in PBS + 1% BSA) or normal rabbit serum (diluted 1:50 in PBS + 1% BSA). Secondary antisera were biotinylated goat-antimouse or biotinylated swine-antirabbit (Dako, Glostrup, Denmark). Immunostaining was performed with 3-amin-9-ethylcarbazole using the streptavidin-biotin complex-horseradish peroxidase method (Dako) and tyramine intensification. Sections were counterstained with hematoxylin. Photographs were taken with a Zeiss Axioshot microscope and digitized using an Agfa duoscan scanner.

Tissue sections were fixed in 2% formaldehyde, and endogenous peroxidase was inhibited as above. After preincubation with 5% BSA, a combination of 2 primary antibodies was applied overnight at 4°C—either anti-BMI-1 (6C9; mouse IgG2b monoclonal antibody) and anti-EZH2 (K358; rabbit polyclonal antisera) or EZH2 with anti-Ki-67 (MIB1; mouse IgG1 monoclonal antibody; Immunotech). BMI-1 or Ki-67 was detected by biotinylated goat antimouse antisera followed by streptavidin-Cy3 (Immuno- research, Jackson, PA), whereas EZH2 was detected by swine-antirabbit Ig-fluorescin isothiocyanate (FITC; Dako). Alternatively, green fluorescence was performed using Alexa-linked goat-antirabbit immunoglobulin (Molecular Probes, Eugene, OR). For each double-immunofluorescence experiment, single-color controls were included.

Results

Coexpression of BMI-1, EZH2 in Mib-1/Ki-67+ neoplastic cells of large B-cell lymphoma

We started our study of PcG expression in human B-NHL with an analysis of large B-cell lymphoma (including follicular lymphoma grade III either with or without a residual follicular growth pattern) and diffuse large B-cell lymphoma. Neoplastic centroblasts in these lymphomas showed clear nuclear staining for BMI-1 (Figure 1A), to an extent almost similar to that of EZH2 (Figure 1B). Staining in these large B-cell lymphomas appeared comparable to the pattern obtained for Mib-1/Ki-67 (Figure 1C). Using double immunofluorescence, we confirmed that neoplastic centroblasts expressed BMI-1 (red signal in Figure 1D) and EZH2 (green staining in Figure 1E) in the same nucleus (producing a yellow signal in Figure 1F). Note that normal cells in the surrounding infiltrate are BMI-1+/EZH2− (see Figure 10). In addition, neoplastic cells expressed Mib-1/Ki-67 (red signal; Figure 1G) in combination with EZH2 (green signal; Figure 1H), resulting in yellow nuclear staining after combining the 2 signals (Figure 1I). From these patterns, we concluded that coexpression of BMI-1 and EZH2 coincided with cycling Mib-1/Ki-67+ neoplastic cells. We observed the same pattern in nodal and extranodal large B-cell lymphoma (not shown).

Table 1. Characteristics of 52 mature (peripheral) B-cell neoplasms tested

<table>
<thead>
<tr>
<th>Lymphoma Type</th>
<th>Number</th>
<th>Mean age, y (range)</th>
<th>Nodal/extranodal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small lymphocytic lymphoma</td>
<td>n = 5</td>
<td>55 (60-73)</td>
<td>2/3</td>
</tr>
<tr>
<td>Follicular lymphoma</td>
<td>n = 30</td>
<td>55 (12-91)</td>
<td>13/12</td>
</tr>
<tr>
<td>Mantle-cell lymphoma</td>
<td>n = 6</td>
<td>60 (31-48)</td>
<td>5/1</td>
</tr>
<tr>
<td>Diffuse large B-cell lymphoma</td>
<td>n = 6</td>
<td>52 (11-85)</td>
<td>3/3</td>
</tr>
<tr>
<td>Burkitt lymphoma</td>
<td>n = 5</td>
<td>5-21 (10)</td>
<td>−5</td>
</tr>
</tbody>
</table>
(single colors not shown), comparable to the pattern in large B-cell lymphoma. Note that large green EZH2$^+$ and Mib-1/Ki-67$^+$ cells are also present: these are probably pre-existing blasts.

The pattern of BMI-1 and EZH2 in virtually all Burkitt lymphoma cells showed overlap similar to that of large B-cell blasts in B-NHL. Burkitt blasts coexpressed BMI-1 (red signal) and EZH2 (green signal), producing a yellow signal in Figure 2C. Overlap between EZH2$^+$ neoplastic cells and Mib-1/Ki-67$^+$ cells was observed for almost all Burkitt blasts (Figure 2D).

**Figure 1.** Large B-cell lymphoma centroblasts are BMI-1$^+$/EZH2$^+$ that coexpress Mib1$^+$. (A-C, first row) Immunohistochemistry (IHC) of large B-cell lymphoma with numerous centroblasts shows clear expression of BMI-1 (A), EZH2 (B), and Mib-1/Ki67 (C). Large brown-staining cells are positive. Small lymphocytes also stain for BMI-1 (A), whereas these cells are negative for EZH2 and Mib-1. (D-F, second row) Immunofluorescence (IF) of large B-cell lymphoma shows red staining for BMI-1 (D), green staining for EZH2 (E), and overlay photographic exposure with yellow cells indicative of centroblasts expressing both BMI-1 and EZH2 (F). Note that bright BMI-1$^+$ (red) cells are probably macrophages (MØ), as indicated in panels D and F. Note that small (infiltrating) lymphocytes in panels D to F show expression of BMI-1 but not of EZH2, as expected. (G-I, third row) IF of large B-cell lymphoma shows expression of Mib/Ki67 in red (G) and EZH2 in green (H). Double fluorescence confirms the coexpression of these proteins in the nuclei of tumor cells (U, overlay photographic exposure with yellow nuclei). Same example as in panels D to F is shown in panels G to I. All IF pictures were taken with 63 x objective.

Decreased BMI-1 expression in neoplastic centrocytes of low-grade follicular lymphoma

Because the coexpression of BMI-1 and EZH2 correlated with cycling Mib-1/Ki-67$^+$ cells in intermediate- and high-grade lymphomas, we subsequently analyzed the expression profile of these PcG proteins in low-grade B-NHL. Figure 3 shows representative immunohistochemical staining patterns for BMI-1 and EZH2 in follicular lymphoma. In control tissue, expression of BMI-1 (Figure 3A) and EZH2 (Figure 3B) was as determined previously.20,21 In general, mantle cells, intrafollicular macrophages, and light zone centrocytes in reactive follicles were positive for BMI-1. These cells did not stain for EZH2, which was mainly detectable in dark-zone centroblasts. In low-grade follicular lymphoma with preserved follicular architecture, we observed reduced BMI-1 staining in neoplastic centrocytes (Figure 3C, see legend) compared to the more intense staining pattern in surrounding infiltrating cells and centrocytes of reactive follicles (Figure 3A). EZH2 expression in low-grade follicle center lymphoma was confined to reactive and neoplastic centroblasts (Figure 3D; overview). In diffuse areas, small numbers of neoplastic centroblasts were observed (Figure 3E; detail) comparable to the number of Mib-1/Ki-67$^+$-expressing cells (Figure 3F). The pattern in small lymphocytic lymphoma was similar to that in low-grade follicular lymphoma (not shown). We concluded that BMI-1 expression is decreased in neoplastic centrocytes in follicular lymphoma and neoplastic small cells in small lymphocytic lymphoma. EZH2 expression in neoplastic centroblasts appeared unchanged compared to that in reactive centroblasts.

Weak coexpression of BMI-1 and EZH2 in neoplastic centroblasts of low-grade follicular lymphoma

To determine whether EZH2$^+$ neoplastic centroblasts in low-grade follicular lymphoma coexpress BMI-1, we analyzed the expression of these proteins with double immunofluorescence. BMI-1 was detected in neoplastic centrocytes of low-grade follicular lymphoma and neoplastic small lymphocytes in small lymphocytic lymphoma. As can be seen in Figure 4A, BMI-1 expression was seen throughout, both in neoplastic centrocytes and in neoplastic...
Reactive centrocytes mainly on the left and neoplastic centrocytes mainly on the right (detail) showing Mib-1/Ki-67

Original magnification, 20× objective. (E-F, third row) IHC on low-grade follicular lymphoma

neoplastic (larger) centrocytes are BMI-1 and BMI-1 low/EZH2 exclusive (not shown, but discussed in detail elsewhere).20,21

Figure 3. Immunohistochemistry (IHC) and immunofluorescence (IF) of low-grade follicular lymphoma showing BMI-1low/EZH2+ neoplastic centrocytes and BMI-1high/EZH2− centroblasts. (A-B, first row) IHC on reactive follicle with BMI-1 (A) and EZH2 (B). Note that macrophages are BMI-1− (Mh) and EZH2−, centrocytes (cc) are BMI-1+ and EZH2+, whereas centroblasts (cb) are BMI-1 but EZH2−. M, mantle cells (BMI-1/EZH2+); DZ, dark zone; LZ, light zone. Original magnification, 20× objective. (C-D, second row) IHC on low-grade follicular lymphoma. (C) Reactive centrocytes mainly on the left and neoplastic centrocytes mainly on the right side of the image (arrows). Reactive centrocytes are clearly BMI-1+, whereas neoplastic (larger) centrocytes are weakly positive and negative. (D) Residual germinal center overrun by neoplastic centrocytes. Residual centroblasts are EZH2+. Original magnification, 20× objective. (E-F, third row) IHC on low-grade follicular lymphoma (detail) showing Mib-1/Ki-67+ neoplastic centroblasts (E) and, with similar magnification, EZH2+ centroblasts (F). Original magnification, 63× objective. All photographs are from a representative experiment with one low-grade follicular lymphoma (Berard grade I).

centroblasts. EZH2 expression was detected in a limited number of centroblasts (Figure 4B), and double immunofluorescence showed that large cells/blasts weakly expressed BMI-1 in the presence of strong EZH2 expression. This produced a yellowish hue in these centroblasts (Figure 4C). A similar pattern was observed in lymphoplasmacytoid lymphoma—weak but detectable BMI-1 expression (Figure 4D) in small numbers of EZH2-expressing neoplastic cells (Figure 4E), resulting in weak BMI-1/EZH2 coexpression (Figure 4F). Note that coexpression of BMI-1 and EZH2 rarely occurs in normal reactive follicles, whereas BMI-1 and EZH2 expression in centroblasts and centrocytes is mutually exclusive (not shown, but discussed in detail elsewhere).20,21

In follicular lymphoma Mib-1/Ki-67- and EZH2 expression overlapped (Figure 4G-I), similar in pattern to that in small lymphocytic lymphoma (not shown). We concluded from this pattern that Mib-1/Ki-67+ cells in low-grade B-NHL are cells that weakly coexpress BMI-1 and EZH2.

In summary, all B-NHL tested showed aberrant PcG expression compared to reactive follicular cells. In general, small centrocytes and lymphocytes in low-grade follicular lymphoma and small lymphocytic lymphoma expressed BMI-1 at reduced levels compared to their reactive counterparts. In these lymphomas, EZH2 expression was limited to large Mib-1/Ki-67+ cells that weakly coexpress BMI-1. In contrast, neoplastic mantle cells, Burkitt cells, and blast cells in large-cell B-NHL showed strong double expression of BMI-1/EZH2 that always overlapped with Mib-1/Ki-67+.

Discussion

PcG genes encode a new class of gene regulatory factors that contribute to normal lymphoid development and lymphomagenesis. They were originally discovered in Drosophila, where they regulate embryonic development as inhibitors of homeobox gene expression. Polycomb proteins function by forming multimeric protein complexes that bind chromatin. Two fundamental complexes have been identified, but their composition can differ in various cell types. This variation is most likely related to target gene specificity and the role of PcG complexes in the maintenance of cellular identity during cell division.

The 2 human PcG complexes are expressed at various stages of GC B-cell development.20,21 However, their expression depends on differentiation stage and stage in the cell cycle: dividing centroblasts express the complex identified by the EZH2 PcG protein, whereas resting mantle cells and centrocytes use the complex identified by BMI-1. BMI-1 and EZH2 are rarely detected in the same nucleus of follicular B cells, suggesting that expression of the 2 complexes is mutually exclusive and highly regulated.

There is increasing evidence that the deregulation of PcG expression is related to the formation of lymphomas. A well-studied example is the Bmi-1 transgenic mouse, which exhibits increased lymphoproliferation and induction of lymphomas.10,25,26 We recently demonstrated that one malignant counterpart of follicular B cells, the HRS cell in Hodgkin lymphoma, coexpresses BMI-1 and EZH2.21 This suggested that deregulated PcG expression may be related to human lymphomagenesis as well. In the current study, we analyzed BMI-1 and EZH2 expression in various classes of B-NHL and questioned whether neoplastic B cells coexpress BMI-1 and EZH2. We observed 2 aberrant expression patterns of these PcG proteins. Tumor cells in intermediate- and high-grade B-NHL (large B-cell NHL, Burkitt lymphoma, and mantle-cell lymphoma) expressed BMI-1 at high levels (BMI-1high), virtually always in the presence of EZH2. By contrast, tumor cells in low-grade B-NHL (follicular lymphoma, small lymphocytic lymphoma) expressed low levels of BMI-1 (BMI-1low), either in the presence (neoplastic centroblasts) or absence (neoplastic centrocytes) of EZH2. Furthermore, the detection of EZH2 in B-NHL neoplastic cells overlapped with expression of the Mib-1/Ki-67+ proliferation marker.

The irregular expression profile of BMI-1 and EZH2 in B-NHL suggests that the distinct balance between the BMI-1 and EZH2-containing PcG complex is disturbed in these lymphomas. Furthermore, the extent of irregular PcG expression correlated with the type of lymphoma (and, therefore, clinical behavior). Small neoplastic centrocytes in low-grade B-NHL exhibited decreased BMI-1 expression in the absence of EZH2, and larger neoplastic blasts in these low-grade B-NHL showed weak coexpression of BMI-1 and EZH2. By contrast, tumor cells in intermediate- and high-grade B-NHL were strongly positive for BMI-1 and EZH2. These results suggest that the balance between BMI-1 and EZH2 expression is progressively disturbed in dividing cells of intermediate- and high-grade B-NHL lymphomas. Because PcG complexes
are involved in the maintenance of the cellular differentiation program, altered PcG expression patterns could at least partially explain the different behavior of neoplastic cells.

Although our study does not resolve the mechanism that accounts for BMI-1/EZH2 coexpression in neoplastic cells, the expression profile in the normal counterparts of these cells allows us to speculate about a possible mechanism. Expression of EZH2 in Mib-1/Ki-67+ neoplastic B cells appears to be natural, because normal Mib-1/Ki-67+ follicular B cells express EZH2 as well. In addition, in vitro up-regulation of EZH2 transcription has been reported during the entry of lymphocytes into the cell cycle. We conclude that the aberrant PcG expression pattern in B-NHL is related to the presence of BMI-1 in dividing neoplastic cells, suggesting overexpression of this PcG gene in neoplastic cells. Normal follicular B cells do not express BMI-1 when they are dividing, and BMI-1 is only detected in EZH2+ resting centrocytes and mantle cells. Furthermore, the absence of BMI-1 expression in dividing healthy B cells is most likely related to the fact that expression of this PcG gene is cell cycle dependent; it dissociates from chromosomes during the late S-G2-M phase of the cell cycle. Consequently, the presence of BMI-1 in dividing neoplastic cells may reflect a failure to down-regulate BMI-1 expression. Theoretically, this occurs early during lymphomagenesis because the intensity of BMI-1/EZH2 coexpression, and the number of neoplastic cells in which this occurs, increases in B-NHL of higher grade. A recent study noted that a subset of mantle-cell lymphoma with blastoid transformation contained amplification of the Bmi-1 gene. Although the overexpression of BMI-1 in human neoplastic cells is in line with the induction of lymphomas in Bmi-1 transgenic mice, it is unclear whether BMI-1/EZH2 coexpression precedes cellular transformation or whether it is a consequence of this process.

One important aspect of PcG complex expression that should be addressed in future studies is the fine composition of the complexes expressed in normal and transformed cells. It is unknown whether PcG expression patterns, as determined in Hodgkin lymphoma and B-NHL, represent normally assembled PcG complexes. We recently found that BMI-1/RING1/EZH2/EED+ mantle cell lymphomas (MCL) up-regulate EZH2 when stimulated to proliferation. This resulted in the coexpression of BMI-1 and EZH2 in the presence of RING1. However, whereas the EED PcG protein is present in dividing normal EZH2+ centroblasts, EED remained unexpressed in the proliferating MCL cells. EED expression appears associated with the negative control of proliferation, and the imbalance between EZH2 and EED in MCL could be an alternative or additional contributing factor to MCL proliferation. In addition, we should mention at this point that the mutually exclusive expression of BMI-1/RING1 and EZH2/EED, as observed in follicular B cells, does not appear to be entirely universal for normal lymphoid cells. For instance, we recently found BMI-1/EZH2 coexpression in healthy thymocytes, whereas mature T cells expressed BMI-1 and EZH2 in a mutually exclusive pattern. This suggests that the fine composition of PcG complexes in normal and transformed cells could affect cellular behavior because a PcG complex may be composed of PcG proteins in different ratios. For instance, a BMI-1 PcG protein can interact with RING, HPC2, and HPC3, but also with other BMI-1 proteins. Therefore, the ratio in which PcG proteins are present in a complex may differ between cell types or between normal and diseased cells. Theoretically, a change in the relative ratio of PcG proteins could be an additional, or an alternative, explanation for altered cellular behavior. We are investigating whether the variation in PcG complex composition correlates with different clinical behaviors of lymphomas.

In conclusion, we demonstrated that low-, intermediate-, and high-grade B-NHL are associated with increased coexpression of the BMI-1 and EZH2 PcG proteins, whose normal expression pattern is mutually exclusive. The underlying mechanism of this expression pattern is most likely related to a failure to down-regulate BMI-1 in dividing neoplastic cells, which is in agreement with observations in Bmi-1 transgenic mice. The extent of BMI-1/EZH2 coexpression correlated with clinical grade and the presence of Mib-1/Ki-67 expression. This suggests that the irregular expression of BMI-1 and EZH2 is an early event in the formation of B-NHL and points to a role for abnormal PcG expression in human lymphomagenesis.
POLYCOMB GROUP PROTEIN EXPRESSION IN B-NHL

References


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