Unique Polycomb Gene Expression Pattern in Hodgkin's Lymphoma and Hodgkin's Lymphoma-Derived Cell Lines

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Human Polycomb-group (PcG) genes play a crucial role in the regulation of embryonic development and regulation of the cell cycle and hematopoiesis. PcG genes encode proteins that form two distinct PcG complexes, involved in maintenance of cell identity and gene silencing patterns. We recently showed that expression of the *BMI-1* and *EZH2* PcG genes is separated during normal B-cell development in germinal centers, whereas Hodgkin/Reed-Sternberg (H/RS) cells co-express *BMI-1* and *EZH2*. In the current study, we used immunohistochemistry and immunofluorescence to determine whether the binding partners of these PcG proteins are also present in H/RS cells and H/RS-derived cell lines. PcG expression profiles were analyzed in combination with expression of the cell cycle inhibitor *p16^{INK4a}*, because experimental model systems indicate that *p16* is a downstream target of *Bmi-1*. We found that H/RS cells and HL-derived cell lines co-express all core proteins of the two known PcG complexes, including *BMI-1*, *MEL-18*, *RING1*, *HPH1*, *HPC1*, and -2, *EED*, *EZH2*, *YY1*, and the HPC2 binding partner, *CtBP*. Expression of *HPC1* has not been found in normal mature B cells and other malignant lymphomas of B-cell origin, suggesting that the PcG expression profile of H/RS is unique. In contrast to *Bmi-1* transgenic mice where *p16^{INK4a}* is down-regulated, 27 of 52 *BMI-1^{POS}* cases of HL revealed strong nuclear expression of *p16^{INK4a}*. We propose that abnormal expression of *BMI-1* and its binding partners in H/RS cells contributes to development of HL. However, abnormal expression of *BMI-1* in HL is not necessarily associated with down-regulation of *p16^{INK4a}*. (Am J Pathol 2004; 164:873–881)

Hodgkin Lymphoma (HL) is a malignant proliferation of mature lymphocytes, characterized by the presence of low numbers of neoplastic cells surrounded by high numbers of reactive T and B lymphocytes, histiocytes, and eosinophils.¹ The neoplastic cells in HL are the Reed-Sternberg cells (RS cells) and their mononuclear variants, the Hodgkin cells (H cells). The majority of classical H/RS cells are thought to be clonal expansions originating from germinal center B cells.^{2–4} The exact mechanism responsible for malignant transformation and subsequent development of HD is unclear. Various mechanisms might be involved, including aberrant expression of tumor necrosis factor family members,⁵ infection with Epstein-Barr virus,⁶ and deregulation of the apoptosis pathway.^{7,8} We recently proposed that altered expression of Polycomb-group (PcG) genes could also contribute to malignant transformation.⁹

PcG genes play a crucial role in embryonic development and have been associated with regulation of hematopoiesis and the cell cycle.^{10–17} PcG proteins form two evolutionary conserved multimeric complexes, that bind to DNA and maintain cell identity by gene suppression.^{18,19} The PRC1/HPC-HPH “maintenance” complex is the mammalian counterpart of the *Drosophila* Polycomb repressive complex 1 (PRC1) and contains the *BMI-1*, *RING1*, *HPH*, and *HPC* PcG proteins, and probably *MEL-18*.^{20–27} The PRC2/EED-EZH or *Esc-Ez* “initiation” complex, is much smaller and is composed of the *EED*, *EZH*, *SU(Z)12*, and *YY1* PcG proteins.^{28–33} PcG complexes can contain various transcription factors that may contribute to DNA binding.^{34,35} In addition, the presence of histone-methyltransferases and histone deacetylases strongly suggests that PcG complexes silence genes by histone modification.^{36–39}

The essential role of PcG genes in maintenance of cellular identity and normal cellular development is reflected by the relationship between abnormal PcG gene expression and malignant transformation. The best-known example is the *Bmi-1* transgenic mouse, where *Bmi-1* up-regulation induces down-regulation of *p16^{INK4a}* and *p19^{ARF}*, increased lymphoid proliferation, and devel-

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Table 1. Antibodies Used in this Study in Combination with Staining Conditions

Antigen	Antibody	Species	Subtype	Titer	Fluorochrome	Treatment	Control tissue
EED	M26	Mouse	IgG1	1:4000 o/n	strep ^{APC}	Citrate ABC/CARD	Tonsil
EZH2	K358	Rabbit		1:400 o/n	GaR ^{ALEXA 488}	EDTA ABC/CARD	Tonsil
HPC1	K350	Rabbit		1:100 o/n	GaR ^{ALEXA 488}	EDTA ABC/CARD	Kidney
HPC2	K326	Rabbit		1:100 o/n	GaR ^{ALEXA 488}	EDTA ABC/CARD	Tonsil
HPH1	K344	Rabbit		1:100 o/n	GaR ^{ALEXA 488}	EDTA ABC/CARD	Tonsil
RING1	K320	Rabbit		1:100 o/n	GaR ^{ALEXA 488}	Citrate ABC/CARD	Tonsil
BMI-1	K333	Rabbit		1:2000 o/n	GaR ^{ALEXA 488}	Citrate ABC/CARD	Tonsil
BMI-1	6C9	Mouse	IgG2b	undiluted	Rhodamine/tyramine	Citrate ABC/CARD	Tonsil
Ki67	Mib-1	Mouse	IgG1		strep ^{APC}	Citrate ABC/CARD	Tonsil
MEL-18		Rabbit		1:400 o/n	GaR ^{ALEXA 488}	Citrate ABC/CARD	Tonsil
YY1		Mouse	IgG1	1:250 o/n	strep ^{APC}	Citrate ABC/CARD	Testis
CtBP		Rabbit		1:4000 o/n	GaR ^{ALEXA 488}	Citrate ABC/CARD	Testis
P16		Mouse	IgG2a	1:500	Rhodamine/tyramine	Citrate ABC/CARD	Cervix carcinoma

Abbreviations: o/n, overnight at 4°C; citrate, antigen retrieval using 0.01M citrate; pH 6.0, 15 minute in the autoclave; EDTA, antigen retrieval using 10mM EDTA, pH 8.0, 15 minute in the autoclave; strepAPC, allophycocyanin-conjugated streptavidine (blue signal); GaR^{ALEXA}, ALEXA 488-conjugated goat-anti-rabbit (green signal); rhodamine/tyramine (red signal); ABC, horseradish-peroxidase-conjugated streptavidin-biotin-complex; CARD, catalyzed reporter deposition method. For details about isolation and characterization of PcG-specific antisera: see references in Materials and Methods section.

opment of lymphomas.^{40–42} Various BMI-1 binding partners have also been associated with malignant transformation, but appear to do so by mechanisms that do not involve the p16 pathway.^{22,43} In addition, overexpression of the *Eed* PcG gene results in diminished lymphoproliferation, suggesting that the two PcG complexes have opposing effects.⁴⁴ We recently discovered that predominance of PcG proteins associated with the PRC1/HPC-HPH complex is associated with resting cells in the germinal center, whereas predominance of PRC2/EED-EZH PcG complex proteins is related to proliferation.^{45,46} The opposing effects on lymphoproliferation in *Bmi-1*- and *Eed*-transgenic mice, and the PcG expression profiles in human germinal centers, suggest that a balance between the two PcG complexes is essential for proper regulation of lymphoproliferation.

Despite the correlation between malignant transformation and altered expression of PcG genes observed in experimental models, the role of human PcG genes in oncogenesis remains relatively unexplored. Previous data of our group showed that H/RS cells and neoplastic cells in high-grade large B-cell lymphomas and mantle-cell lymphomas are associated with nuclear co-expression of BMI-1 and EZH2.^{9,47,48} Co-expression of these two PcG genes is generally not observed in the normal counterparts of these cells,^{45,46} suggesting that aberrant PcG expression contributes to development of malignant lymphomas in man. It is unclear, however, whether BMI-1 expression in EZH2^{POS} malignant lymphomas has functional consequences for development of these tumors. One condition that should be met is that binding partners of BMI-1 and EZH2 are present in the tumor cells. In the current study, we investigated whether H/RS cells and HL-derived cell lines express other components of the PRC1/HPC-HPH- and PRC2/EED-EZH PcG complexes, and compared these patterns with the PcG expression profile of normal B cells. In addition, PcG expression was correlated with p16^{INK4a} expression.

Materials and Methods

Cell Lines

The characteristics of the HL-derived cell lines L428 and L1236 were described in detail previously.⁴⁹ Cell lines were maintained in RPMI 1640 (BioWhittaker, East Rutherford, NJ) medium supplemented with 25 mmol/L HEPES, 2 mmol/L L-glutamine, 10% fetal calf serum, 100 IU of streptomycin, and 100 µg of penicillin at 37°C in 5% CO₂.

Human Tissue

Formalin-fixed, paraffin-embedded tissue blocks of 54 primary biopsies of nodular sclerosing HL patients were retrieved from the Department of Pathology, LUMC, The Netherlands. Frozen tissue was retrieved from 24 cases as well. All cases were diagnosed between 1990 and 2001 and classified according to the World Health Organization new classification. The diagnostic immunohistochemical panel always included CD3, CD15, CD20, CD30, CD45, EMA, ALK1, LMP1, and EBER RNA *in situ* hybridization. This panel was extended if necessary for appropriate diagnosis. Only the first diagnostic biopsy specimen of the patients was investigated. As positive controls for immunohistochemistry, both paraffin-embedded and frozen material from tonsil, thymus, kidney, and testis was used (Table 1).

Immunohistochemistry

For immunohistochemistry, 3-µm sections of paraffin-embedded material were used for the detection of various PcG proteins and cell cycle-related proteins Ki67 and p16 (Table 1). After rehydration, endogenous peroxidase was quenched by incubation of the sections in 0.3% H₂O₂ diluted in methanol for 30 minutes. Thereafter, antigen retrieval was performed using either citrate (pH 6.0)

Table 2. Expression of PcG Genes in Hodgkin's Lymphoma and HL-Derived Cell Lines

PcG gene	Hodgkin lymphoma RS cells (n = 54)	Hodgkin lymphoma reactive lymphocytes (n = 54)	HL cell lines* (n = 2)
BMI-1	54/54	54/54 (30–80% positive cells)	Positive
HPH1	54/54	54/54 (30–80% positive cells)	Positive
HPC1	7/54**	0/54	Positive
HPC2	54/54	54/54 (30–80% positive cells)	Positive
RING1	54/54	54/54 (30–80% positive cells)	Positive
MEL-18	54/54	8/54 (~5% positive cells)	Positive
		46/54 (~90% positive cells)	
EZH2	54/54	54/54 (<5% positive cells)	Positive
EED	54/54	54/54 (<5% positive cells)	Positive
YY1	52/54†	54/54 (>95% positive cells)	Positive
CtBP	53/54‡	54/54 (>95% positive cells)	Positive

*Expression of PcG proteins was investigated in HL-derived cell lines L428 and L1236.

**HPC1 expression in neoplastic H/RS cells is only observed using immunofluorescence on frozen tissue sections (7 individual cases were studied). In paraffin-embedded HL tissue, HPC1 is not detected, most likely due to low expression in tumor cells.

†In 2 of 54 cases YY1 expression was absent from the neoplastic H/RS cells but present in the reactive lymphocytes.

‡In 1 of 54 cases CtBP expression was absent from the neoplastic H/RS cells but present in the reactive lymphocytes. CtBP/YY1- cases were not observed.

or EDTA (pH 8.0), depending on the antigen (Table 1), for 15 minutes in the autoclave. After cooling, the slides were rinsed with phosphate-buffered saline (PBS) containing 0.5% Triton X-100 (5 minutes), followed by PBS only (3 × 5 minutes). Subsequently, the slides were incubated with 0.1 mol/L glycine (10 minutes) and rinsed in PBS.

After pre-incubation with normal swine serum or normal rabbit serum, for polyclonal and monoclonal antibodies, respectively, the primary antibodies^{26,27,31,43,50,51} were applied at optimal dilution (Table 1). Secondary antibodies were biotinylated swine anti-rabbit or biotinylated rabbit-anti-mouse (Dako, Glostrup, Denmark). This was followed by application of horseradish peroxidase-conjugated streptavidine-biotin-complex (sABC-HRP) and further signal enhancement was achieved by the CARD-method (catalyzed reporter deposition method, DAKO). The signal was visualized by 3-amino-9-ethylcarbazole (AEC) and counterstained with hematoxylin. Photographs were taken with a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) and were digitized using an Agfa duoscan (Agfa, Morstel, Belgium).

Double and Triple Immunofluorescence

To confirm PcG protein staining patterns in neoplastic cells and the surrounding infiltrate, immunofluorescent double and triple staining was performed. This also allowed us to study PcG proteins in relation to other markers, such as p16^{INK4a}, CtBP, and Ki67. Frozen tissue sections were fixed in 1% formaldehyde, followed by quenching of endogenous peroxidase using 1% H₂O₂ diluted in PBS. After preincubation with 5% bovine serum albumin (BSA), a combination of two or three primary antibodies was applied overnight at 4°C. BMI-1 was detected by incubation with HRP-conjugated goat-anti-mouse IgG2b and subsequent rhodamine/tyramine intensification. Detection of HPC1, HPC2, HPH1, RING1, EZH2, YY1, p16, and CtBP was performed by incubating the slides with ALEXA 488-conjugated goat-anti-rabbit antiserum. The other markers were visualized by incubating the slides with biotinylated goat-anti-mouse IgG1 or

IgG2a, depending on Ig-subclass of the primary antibody, followed by allophycocyanin-coupled streptavidin. Cross-reactivity of the antisera was excluded by appropriate controls and for each double- or triple-immunofluorescence experiment; single-staining controls were included. In addition, positive and negative controls were routinely included. Sections were analyzed with a Leica DMR confocal laserscan microscope (Leica, Deerfield, IL). Images were stored digitally and processed using Adobe Photoshop 6.

Evaluation of Immunohistochemical Staining

Percentages of PcG expressing H/RS cells were determined visually and classified according to the percentage of positively staining tumor cells: 0, no staining in H/RS cells; 1, <25% of the H/RS cells positive; 2, 25% and <50% H/RS cells positive; 3, 50% and <75% H/RS cells positive; 4, 75% of the H/RS cells showed positive staining. In all cases, except for HPC1, small reactive lymphocytes served as an internal positive control.

Results

PcG Expression in Neoplastic H/RS Cells in Hodgkin's Lymphoma

We analyzed the expression of various components of the PcG PRC1/HPC-HPH complex (BMI-1, HPC1, HPC2, HPH1, RING1, MEL-18, and CtBP) and the PRC2/EED-EZH PcG complex (EED, EZH2, and YY1) by immunohistochemistry in 54 cases of HL (all cases classified as nodular sclerosing). For all stainings, appropriate controls were included (Table 1).

We initially focused on expression of PcG proteins belonging to the PRC1/HPC-HPH complex. Expression of the BMI-1 binding partners, RING1, HPH1, HPC2, and MEL-18, was found as strong nuclear staining in virtually all H/RS cells in HL (Table 2, Figure 1). The HPC1 PcG protein, however, was undetectable by immunohisto-

Figure 1. Immunohistochemical detection of PcG expression in H/RS cells. Neoplastic cells in HL express various members of the PRC1/HPC-HPH PcG complex (BMI-1, MEL-18, RING1, HPH1, HPC2, and CtBP) and the PRC2/EED-EZH complex (EED, EZH2, and YY1). HPC1, a member of the PRC1/HPH-HPC PcG complex, was undetectable in H/RS cells by immunohistochemistry.

chemistry in H/RS cells, although it can be part of the HPC-HPH complex and binds to BMI-1. Finally, the non-PcG transcriptional repressor CtBP that interacts with the HPC2 PcG protein, was detectable in the majority of H/RS cells examined in 53 of 54 cases of HL. These results collectively show that virtually all of the PRC1/HPC-HPH PcG complex core proteins are expressed by H/RS cells.

Our previous work on PcG expression in neoplastic cells of HL demonstrated that H/RS cells express BMI-1 in combination with EZH2, belonging to the PRC2/EED-EZH complex and normally present only in cycling B cells.⁹ We therefore examined whether other components of this complex are also present in H/RS cells. Immunohistochemical staining for EZH2, EED, and YY1 revealed strong nuclear expression of these proteins in the nuclei of virtually all H/RS cells (Table 2, Figure 1). In two instances, YY1 was undetectable in H/RS cells whereas lymphocytes in the surrounding infiltrate stained for this protein. The expression patterns of EZH2, EED, and YY1 confirm the presence of EZH2 in H/RS cells and show widespread expression of its binding partner EED in combination with YY1.

PcG Expression in Reactive Lymphocytes in Hodgkin's Disease

Variable numbers of reactive lymphocytes, but always more than 50%, expressed PcG proteins belonging to the PRC1/HPC-HPH complex. BMI-1 and its binding partners RING1, HPH1, and HPC2, displayed a similar staining pattern, both in numbers of positive cells as well as intensity of staining (Figure 1). Expression of HPC1, however, was never detected in reactive lymphocytes. Interestingly, MEL-18 expression in H/RS cells was always strongly positive, whereas in 8 of 54 cases (15%), only a small number of reactive lymphocytes stained positive (< 50% of the total number of lymphocytes). In the remaining cases, expression of MEL-18 in reactive lymphocytes was found in virtually every cell. Expression of EZH2 and

its binding partner EED on the other hand, was rarely detected in reactive infiltrating lymphocytes. The third PcG protein associated with the PRC2/EED-EZH complex, YY1, was found in virtually all of the reactive lymphocytes in all HL cases tested.

Co-Expression of PcG Complexes PRC2/EED-EZH and PRC1/HPC-HPH in Relation with Proliferation State of H/RS Cells

PcG expression profiles were further explored by double and triple immunofluorescence to gain insight in the (co-)expression of both PcG complexes in relation with cell cycle as determined by Ki67 expression in H/RS cells. Multiple combinations of primary antibodies were applied and co-expression was evaluated by confocal laser scan microscopy. The combinations of primary antibodies chosen included PcG proteins derived from both PRC1/HPC-HPH and the PRC2/EED-EZH complex in combination with Ki67 (Table 3).

H/RS cells are, in most instances, cycling cells expressing Ki67 and EZH2, and we therefore first performed triple immunofluorescent staining for Ki67 and EZH2 in combination with another PcG protein. We were unable to examine expression of the EZH2 binding partner EED in relation to Ki67, because antisera recognizing these proteins are both of IgG1 subtype. In agreement with our earlier findings, staining for BMI-1 and EZH2 clearly showed co-expression of these proteins in the nuclei of cycling neoplastic Ki67^{POS} H/RS cells (Figure 2A). Normal reactive lymphocytes in the surrounding infiltrate were primarily Ki67^{NEG} resting cells, expressing BMI-1 in the absence of EZH2 (Figure 2A). As previously shown,^{9,46} we occasionally observed dividing Ki67^{POS} reactive cells, which expressed EZH2 but lacked BMI-1.

Investigation of BMI-1 and EZH2 binding partners by triple immunofluorescence (Table 3) confirmed the staining patterns obtained by immunohistochemistry. Represen-

Table 3. Combinations of Antibodies Used for Double/Triple Immunofluorescence on Hodgkin's Lymphoma

Green fluorescence*	Red fluorescence†	Blue fluorescence‡	Coexpression in H/RS cells
HPC1	BMI-1	Ki67	Yes
HPC2	BMI-1	Ki67	Yes
HPH1	BMI-1	Ki67	Yes
RING1	BMI-1	Ki67	Yes
EZH2	BMI-1	Ki67	Yes
CtBP	BMI-1	Ki67	Yes
MEL-18	BMI-1		Yes
HPC1	BMI-1	EED	Yes
HPC2	BMI-1	EED	Yes
HPH1	BMI-1	EED	Yes
RING1	BMI-1	EED	Yes
EZH2	BMI-1	EED	Yes
CtBP	BMI-1	EED	Yes
MEL-18	Ki67		Yes

*Polyclonal rabbit-derived antibodies were visualized using an ALEXA 488-conjugated goat-anti-rabbit antiserum.

†Antibodies with an IgG2b subtype were visualized with an HRP-conjugated goat-anti-mouse IgG2b specific antibody followed by rhodamine/tyramine intensification.

‡Antibodies if IgG1 or IgG2a subtype were detected by a biotinylated goat-anti-mouse IgG1 or IgG2a followed by allophycocyanin-coupled streptavidine.

tative examples are shown in Figures 2 and 3. Staining for BMI-1 in combination with HPC2, HPH1, and RING1 revealed that these PcG proteins were co-expressed in the nuclei of large dividing Ki67^{POS} H/RS cells and in resting Ki67^{NEG} cells of the reactive infiltrate. A representative example of triple immunofluorescent staining for BMI-1 in combination with HPH1 and Ki67 is shown in Figure 2B. In contrast to the results obtained by immunohistochemistry, the HPC1 PcG protein was clearly detectable in neoplastic cells of HL by triple immunofluorescence (Table 3). This is illustrated by triple staining for BMI-1 in combination with HPC1 and EED in Figure 3B. Detection of HPC1 by immunofluorescence suggests low level HPC1 expression in the nuclei of malignant cells, because immunofluorescence on frozen sections is generally more sensitive than immunohistochemistry. We did not observe expression of HPC1 in normal reactive lymphocytes. Finally, a combination of EZH2, BMI-1, and EED showed that EZH2 and its binding partner EED are expressed in the nuclei of the malignant cells in combination with BMI-1, which does not bind EZH2 nor EED and is part of another PcG complex.

Figure 2. Immunofluorescent detection of BMI-1 in combination with EZH2 and HPH1 in Ki67^{POS} H/RS cells. **A:** Triple immunofluorescence staining for BMI-1 (red signal), EZH2 (green signal), and Ki67 (blue signal). Large Ki67^{POS} H/RS cells co-express BMI-1 and EZH2 (arrow) whereas BMI-1- and EZH2 expression are separated in healthy infiltrating cells (x denotes a resting (Ki67^{NEG}) BMI-1^{POS}/EZH2^{NEG} cell; * denotes a dividing (Ki67^{POS}) BMI-1^{NEG}/EZH2^{POS} cell). **Lower panel:** detail of a single H/RS cell, showing single fluorescence signals (upper left and right, and lower left) and the combination of these signals (lower right). **B:** Triple immunofluorescence staining for BMI-1 (red signal), HPH1 (green signal), and Ki67 (blue signal). Large Ki67^{POS} H/RS cells co-express BMI-1 and HPH1 (arrow) whereas dividing healthy infiltrating cells do not express BMI-1 and HPH1 (x). By contrast, BMI-1 and HPH1 are detectable in resting Ki67^{NEG} healthy infiltrating cells (*). **Lower panel:** as in A.

Figure 3. Immunofluorescent detection of BMI-1 and EED in combination with HPC1 and EZH2. BMI-1 was detected by a red signal, EED by a blue signal, and HPC1 and EZH2 by a green signal. **A:** Expression of the BMI-1 binding partner HPC1 in combination with EZH2 and EED. **B:** Large EZH2^{POS} tumor cells express the EZH2 binding partner EED in combination with BMI-1, which does not bind to EZH2 and is part of another PcG complex.

In summary, proliferating neoplastic H/RS cells co-express PcG core proteins derived from the PRC1/HPC-HPH and the PRC2/EED-EZH complex. The expression pattern of these proteins, BMI-1 in combination with RING1, HPH1, and HPC2, *versus* EZH2 in combination with EED, is always exclusive in reactive lymphocytes. In addition, H-RS cells express low levels of HPC1, which is undetectable in healthy reactive lymphocytes.

High Levels of BMI-1 Are Not Associated with p16 Down-Regulation

Mouse experimental model systems demonstrated that altered expression of PcG genes has strong effects on regulation of the cell cycle. For instance, overexpression of *Bmi-1* in mice leads to down-regulation of *p16^{INK4a}* and subsequent induction of lymphoproliferation and development of lymphomas.^{40–42} Therefore, the *p16* gene appears to be a downstream target of *Bmi-1*. Given the expression of BMI-1 in H/RS cells, we questioned whether the presence of BMI-1 in neoplastic cells is associated with absence of *p16^{INK4a}*. We found that *p16^{INK4a}* expression was detected as nuclear staining in the H/RS cells of 27 of 52 (52%) cases (Figure 4), which were all BMI-1^{POS}. As an internal positive

control, few scattered reactive lymphocytes stained. This demonstrates that expression of BMI-1 in H/RS cells is not necessarily associated with absence of *p16^{INK4a}* expression.

PcG Expression in HL-Derived Cell Lines L428 and L1236

Cytospin preparations were made from HL-derived cell lines L428 and L1236 and PcG expression was deter-

Figure 4. Immunohistochemical detection of *p16^{INK4a}* on two cases of nodular sclerosing HL. Two representative cases for *p16^{INK4a}* positive and negative cases of HL in **A** and **B**, respectively

mined using immunohistochemistry. The PcG staining pattern of these cell lines closely resembled the expression pattern of neoplastic H/RS cells in patient material. Both L428 and L1236 expressed all investigated proteins of the PRC1/HPC-HPH complex, including BMI-1, MEL-18, RING1, HPH1, and HPC1/2. In addition, these PRC1/HPC-HPH complex proteins were co-expressed with the EED, EZH2, and YY1 proteins of the PRC2/EED-EZH complex.

Discussion

Classical H/RS cells originate from follicular B cells in the germinal center.²⁻⁴ One group of genes that may contribute to malignant transformation and development of HL, belongs to the Polycomb-group (PcG).⁹ PcG proteins are responsible for preservation of cell identity, and contribute to regulation of lymphoid development at all stages of hematopoiesis.^{10,12,16,17} We recently showed that healthy follicular B cells in germinal centers display a mutually exclusive expression pattern of PcG genes encoding the PRC1/HPC-HPH- and PRC2/EED-EZH PcG complexes.^{45,46} Resting Ki67^{NEG} mantle cells and centrocytes preferentially expressed the PRC1/HPC-HPH *Bmi-1* and *RING1* PcG genes, whereas dividing Ki67^{POS} centroblasts were associated with detection of the PRC2/EED-EZH proteins EED and EZH2. Interestingly, from experiments in mutant mice it was concluded that core proteins of the two complexes have opposing functions. For instance, enhanced lymphoid proliferation was observed in transgenic mice overexpressing *Bmi-1*,⁴⁰⁻⁴² and in knockout mice with diminished expression of *Eed*.⁴⁴ These observations collectively suggest that a balance between the different PcG complexes is critical for normal division of hematopoietic cells.

In the current study and in our previous work,⁹ we demonstrated that this balance is lost in neoplastic H/RS cells and HL-derived cell lines. This is best illustrated by the expression pattern of BMI-1 and its binding partners. BMI-1 is not detected in healthy dividing EZH2^{POS} B cells, whereas H/RS cells and HL-derived cell lines co-express BMI-1 and EZH2 (this study, and^{9,45,46}). This expression pattern suggests abnormal expression of BMI-1 in transformed cells, and is in line with experimental model systems.⁴⁰ The fact that BMI-1 functions as part of a multiprotein complex, raises the question whether the presence of BMI-1 in neoplastic cells is functionally relevant. One condition that should be met is that the binding partners of BMI-1 are also present in the nucleus. In the current study we showed that H/RS cells and HL-derived cell lines display a highly conserved PcG expression profile of the core proteins that form the PRC1/HPC-HPH- and PRC2/EED-EZH PcG complexes. In all instances, BMI-1 was expressed in combination with its binding partners RING1, HPH1, HPC1/2, and MEL-18. In addition, various proteins constituting the PRC2/EED-EZH complex, including EZH2, EED, and YY1 were also expressed in the same nuclei. These data suggest that both PcG complexes can be assembled in neoplastic H-RS cells at the same time.

Three conclusions can be drawn from the available expression data: firstly, H/RS cells display abnormal expression of PRC1/HPC-HPH proteins. This is best illustrated by the expression of BMI-1 and its binding partners, because normal dividing B lymphocytes do not express BMI-1 in combination with MEL-18, RING1, HPH1, and HPC proteins (this study, and van Galen et al, manuscript submitted). Secondly, expression of this set of PRC1/HPC-HPH PcG genes in combination with the PRC2/EED-EZH complex indicates an additional pattern of abnormal PcG expression, because mature lymphocytes in germinal centers express core proteins of PRC1/HPC-HPH- and PRC2/EED-EZH complexes in a mutually exclusive pattern. Finally, if the PcG proteins that are present in H-RS cells associate to form PcG complexes, we suggest that this will result in formation of a different PRC1/HPC-HPH complex than the one seen in healthy resting B cells. The main difference in complex composition would most likely be related to low-level expression of HPC1 in the nuclei of H/RS cells, because this PcG protein is absent from healthy mature cells in the reactive infiltrate. Whether PRC1/HPC-HPH and PRC2/EED-EZH complexes actually bind to their target genes in tumor cells cannot be determined by this type of study, but we showed that various PcG proteins with DNA-binding activity, such as MEL-18⁵² and YY1,^{53,54} are present in the H/RS cells. We propose that abnormal PcG expression in H/RS cells results in altered composition of the PRC1/HPC-HPH PcG complex, and that this contributes to interference with normal gene silencing patterns in H-RS cells and loss of cell identity.

A causative relationship between abnormal expression of PcG genes, altered gene silencing patterns, and loss of cell identity is supported by various experimental model systems. The best-known example is the *Bmi-1* transgenic mouse, which identified *Bmi-1* as an oncogene capable of down-regulating the cell cycle-associated proteins p16^{INK4a} and p19^{ARF}.^{41,42} In non-proliferating cells, p16^{INK4a} and p19^{ARF} specifically bind to the cdk4/6 protein complex, thereby preventing cyclin D activation and subsequent transition toward the S-phase. *Bmi-1*-mediated down-regulation of p16^{INK4a} induces high levels of proliferating hematopoietic cells and formation of lymphomas. The inverse correlation between *Bmi-1* and p16^{INK4a} expression in mouse lymphocytes naturally leads to the question whether human p16^{INK4a} is also a downstream target of BMI-1. As reported in other studies,⁵⁵ we found that p16^{INK4a} is undetectable in a large number of HL cases. Although loss of p16^{INK4a} expression in malignant lymphomas is usually associated with altered methylation patterns or deletion of the gene,^{55,56} it is formally possible that BMI-1 overexpression is an additional contributing factor. However, our data show that expression of BMI-1 in H/RS tumor cells is not necessarily related to p16^{INK4a} down-regulation, because more than half of the HL cases expressed BMI-1 in combination with p16^{INK4a}. Although this suggests that human p16^{INK4a} may not be regulated by BMI-1, these results should be interpreted with caution because regulation of individual target genes of *Bmi-1* may be cell type-specific.⁵⁷ Indeed, recent studies of lung and penile

carcinomas^{58,59} reported an inverse correlation between the presence of BMI-1 and the absence of p16^{INK4a}. It is theoretically possible that an effect of BMI-1 on p16^{INK4a} in neoplastic cells of HL may be masked by the expression of other PcG genes. Several of these are associated with malignant transformation through mechanisms that do not involve p16^{INK4a}. Enhanced expression of RING-1, for instance, resulted in oncogenic transformation and up-regulation of proto-oncogenes *c-jun* and *c-fos*,²² but *Mel-18* seems to function as a tumor suppressor gene in the mouse.⁵² A further complication is that several PcG genes detected in H/RS cells, such as *HPC2*, are normally expressed by proliferating B cells (this study, and van Galen et al, submitted). However, it is unclear whether the level of *HPC2* expression is altered in tumor cells. This is an important issue to resolve because interference with the expression or function of *HPC2* is associated with abnormal cell cycle regulation.⁴³ In addition, enhanced expression of *EZH2* was recently associated with mantle-cell lymphomas⁴⁸ and progression of prostate and breast tumors,^{60–62} suggesting that this PcG gene may also contribute to development of neoplastic H/RS cells.

Concluding Remarks

The importance of PcG proteins in maintenance of cell identity is underscored by the relationship between abnormal expression of PcG genes and malignant transformation in experimental model systems. In the current study we demonstrated that neoplastic cells in HL abnormally express multiple PcG genes. Although this suggests that the individual regulation of several PcG genes is disturbed, it is also possible that a regulatory mechanism shared by multiple PcG genes is affected. A potential application of the observed PcG expression patterns in HL is improved diagnostics. For instance, expression of *HPC1* by H-RS cells may possibly be used to distinguish HL from CD30+ ALCL. Preliminary results of PcG expression patterns in T-NHL showed that CD30+ ALCL are *HPC1* negative (van Galen et al, in preparation), but this finding needs to be confirmed in a larger patient population. We propose that abnormal PcG expression in HL results in an altered composition of the PRC1/HPC-HPH PcG complex in H/RS cells, which probably affects expression of target genes involved in regulation of apoptosis and/or the cell cycle. Our observations add to the increasing evidence that PcG genes are important contributors to development of hematological malignancies.

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