

# Increased Expression of the *EZH2* Polycomb Group Gene in *BMI-1*-Positive Neoplastic Cells during Bronchial Carcinogenesis<sup>1</sup>

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## Abstract

**Polycomb group (PcG) genes are responsible for maintenance of cellular identity and contribute to regulation of the cell cycle. Recent studies have identified several PcG genes as oncogenes, and a role for PcG proteins in human oncogenesis is suspected. We investigated the expression of *BMI-1* and *EZH2* PcG oncogenes in human bronchial squamous cell carcinomas (SCCs) and bronchial premalignant precursor lesions (PLs). Whereas normal bronchial epithelium was associated with widespread expression of *BMI-1* in resting *EZH2*-negative cells, neoplastic cells in lung carcinomas displayed altered expression of both *BMI-1* and *EZH2*. Two patterns of abnormal PcG expression were observed: increased expression of *BMI-1* in dividing neoplastic cells of PLs and SCCs, and enhanced expression of *EZH2* and *Ki-67* in *BMI-1*-positive cells according to severity of the histopathologic stage. We propose that altered expression of *BMI-1* and *EZH2* is an early event that precedes high rates of proliferation in lung cancer. Because PcG complexes are normally involved in the maintenance of cell characteristics, abnormal PcG expression may contribute to loss of cell identity.**

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**Keywords:** Premalignant lesions, *BMI-1*, *EZH2*, polycomb genes, lung carcinoma.

## Introduction

Lung cancer is the leading cause of cancer-related deaths in the western world [1]. It is generally assumed that squamous cell carcinoma (SCC) in the central lung develops in a stepwise manner from normal bronchial epithelium to cancer through the accumulation of (epi)genetic alterations [2]. Our understanding of the carcinogenic process is continuously growing with new genes or classes of genes discovered that are involved in carcinogenesis. Polycomb group (PcG) genes form such a class of genes.

PcG genes have been identified in *Drosophila melanogaster* as repressors of homeobox genes throughout

development [3]. *Drosophila* PcG genes are evolutionarily conserved and several vertebrate homologues have been identified. PcG proteins form heterogeneous multimeric protein complexes [4]. Two distinct and evolutionary conserved PcG complexes have been identified, consisting of various PcG proteins and non-PcG proteins. The polycomb repressive complex 1 (PRC1) maintenance complex contains the *BMI-1*, *MEL-18*, *RING1*, *HPH*, and *HPC* PcG proteins, and the PRC2 initiation complex contains the *EZH2*, *EED*, *YY1*, and *SUZ* [12] PcG proteins [4–16]. PcG complexes most likely regulate gene activity by epigenetic mechanisms because they are associated with histone deacetylases and methyltransferases involved in chromatin modification [15,17–20]. Although PcG genes are essential for embryogenesis, they continue to contribute to the regulation of various processes in the adult, including cell cycle and hematopoiesis [21–25].

The pivotal role of PcG genes in normal development is underscored by studies of mice with altered PcG gene expression, which indicated that PcG genes are involved in carcinogenesis. For instance, overexpression of *Bmi-1* as a transgene results in a high predisposition for B-cell and T-cell lymphomas [26]. Similarly, binding partners of *BMI-1* in the PRC1 complex, including *RING1* and *HPC2*, have also been associated with malignant transformation [11,27]. PcG genes most likely contribute to oncogenesis by interfering with cell cycle control mechanisms. *Bmi-1* was shown to be an upstream regulator of the *INK4a-ARF* locus, and *Bmi-1* overexpression results in suppression of *p16<sup>INK4a</sup>* in various experimental settings [28–30]. Recent work on components of the PRC2 complex showed that *EZH2* is capable of providing a proliferative advantage to primary cells [31]. *EZH2* is regulated by the pRB–E2F pathway and appears essential for cellular proliferation [31]. Evidence is rapidly increasing that PcG genes are

Abbreviations: PcG, polycomb group; PRC, polycomb repressive complex

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also associated with carcinogenesis in humans. Several studies showed that *BMI-1* is overexpressed in malignant lymphomas, resulting in coexpression with *EZH2* in cycling neoplastic cells [32–36]. The observation that altered PcG gene expression also occurs in solid tumors, including carcinomas from the lung [37], breast [38–41], prostate [42,43], and colorectum [44], suggests that aberrant PcG expression may be a mechanism of transformation that is shared by various tumors. Given the role of PcG complexes in epigenetic modification of target gene expression, altered expression of PcG genes may contribute to the observed epigenetic modifications that occur in cancer cells (recently reviewed in Ref. [45]).

In the current study, we aimed to find out at what stage the expression patterns of *BMI-1* and *EZH2* are altered during bronchial carcinogenesis. To that end, expression of these PcG genes was analyzed in bronchial SCC and various stages of premalignant lesions (PLs).

## Materials and Methods

### Human Tissues

**Bronchial carcinomas and adjacent macroscopically normal tissues** Neoplastic tissues and adjacent macroscopically normal bronchial tissues were obtained from patients who underwent a surgical resection of a primary SCC of the bronchus at the VU Medical Center. Tissue parts of the tumor and tumor-distant, macroscopically normal bronchial tissues were collected and bisected, after which one part was formalin-fixed and paraffin-embedded, and the other part snap-frozen. We selected seven patients who were not pretreated by chemotherapy or radiotherapy prior to surgery. At the time of surgical resection, patients with bronchial SCC were aged 71 years (median; range 47–76 years). The male:female ratio was 5:2. Consecutive 4- $\mu$ m sections were cut from formalin-fixed and frozen tissues, for both histopathologic assessment and immunohistochemistry (IHC; formalin-fixed tissue) and immunofluorescence (IF; frozen tissue), respectively. After histologic examination, tumor samples were all classified as moderately or poorly differentiated SCC, and the normal samples as histomorphologically normal.

**Premalignant endobronchial lesions** Patients without clinically overt cancer who were at risk for developing bronchial carcinoma were investigated using autofluorescence bronchoscopy (AFB; Xillix Technologies Corporation, Richmond, BC, Canada) in combination with conventional white light bronchoscopy (WLB). Indications for performing AFB were suspicion of lung cancer or follow-up of treated cancer in the aerodigestive tract [46]. Paired biopsy materials were obtained from each area, revealing abnormal autofluorescence. Biopsies were formalin-fixed and used for routine histopathology, and snap-frozen in liquid nitrogen for IF. We randomly selected 29 samples of 19 patients, covering the spectrum of endobronchial PLs. At the time biopsies were obtained, patients were aged 63 years old (median;

range 40–73 years). The male:female ratio was 16:3. Consecutive sections were stained in hematoxylin and eosin (H&E) and a single pathologist (E. R.) examined the slides to confirm their histopathologic diagnosis. The slides were categorized as follows: normal (N;  $n = 3$ ), hyperplasia (H;  $n = 3$ ), squamous metaplasia (SM;  $n = 2$ ), mild dysplasia (MiD;  $n = 7$ ), severe dysplasia (SD;  $n = 13$ ), and carcinoma *in situ* (CIS;  $n = 1$ ).

### IHC

IHC was performed as described before [32,33,35,36, 41,47–49]. Briefly, sections were deparaffinized and rehydrated, and endogenous peroxidase was inhibited. For antigen retrieval, slides were boiled in 0.01 M sodium citrate buffer (pH 6.0) for 15 minutes. Primary antibodies were applied following preincubation with normal swine serum (1/:10 for *EZH2*), normal rabbit serum (1:50 for *BMI-1*), and MIB-1/Ki-67) for 10 minutes. For IHC detection of the PcG proteins BMI-1 and EZH2, slides were incubated with, respectively, the 6C9 mouse monoclonal antiserum for 60 minutes at room temperature and the K538 rabbit polyclonal antiserum (1: 800) overnight at 4°C. The primary antibody (Ab) (MIB-1; DAKO, Glostrup, Denmark) was used against MIB-1/Ki-67 (further notified as Ki-67). Secondary antisera used were a biotinylated rabbit antimouse (1:200) or a biotinylated swine antirabbit (1:300). Following incubation in streptavidin–biotin complex/horseradish peroxidase (sABC-HRP) (1:1000), slides were incubated in biotinylated tyramine for 10 minutes to enhance the signal. For the detection of Ki-67, no signal enhancer was used. Incubating the slides in sABC-HRP (1:100) completed the detection. 3,3'-Diaminobenzidine tetrahydrochloride (DAB) was used for light microscopic visualization of the signal. Slides were counterstained in hematoxylin for 40 seconds, dehydrated in methanol, and finally covered. All washing steps of the slides were performed using phosphate-buffered saline (PBS). Slides processed in the same manner, using PBS instead of the primary Ab, were considered as a negative control. A tonsil specimen, in which both BMI-1 and EZH2 are present [49,50], was included as a positive control for the IHC procedure during each experiment. Photographs were taken with a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) and digitized using an Agfa Duoscan scanner (Agfa, Mortsel, Belgium).

### IF

Immunofluorescent stainings were performed as described before [32,33,35,41,48,50]. Briefly, 4- $\mu$ m sections were cut from frozen tissues and mounted on poly-L-lysine-coated slides. Sections were fixed in 2% formaldehyde. Following inhibition of endogenous peroxidase, slides were preincubated with PBS containing 5% bovine serum albumin. For the detection of the PcG proteins, BMI-1, EZH2, and Ki-67 slides were incubated, respectively, with 6C9, a mouse IgG2b monoclonal Ab I K358 (1:100), a rabbit polyclonal Ab; and MiB-1 (IgG1) overnight at 4°C. The BMI-1 Ab was detected using biotinylated goat anti-mouse IgG2 antiserum (1:100) followed by rhodamine–thyramine (RT) in ethanol

(1:1000). The EZH2 Ab was detected using swine anti-rabbit Ig fluorescein isothiocyanate (FITC) as secondary step, followed by streptavidin Cy3 (1:300). Ki-67 Ab was detected using biotinylated anti-IgG1 (1:100) followed by streptavidin allophycocyanin (1:1000). All washing steps were performed using PBS containing 0.1% saponin. Slides processed in the same manner, using PBS instead of the primary Ab, were considered as negative controls. A tonsil was included as a positive control for the staining procedure during each experiment [50]. After slides were covered, sections were analyzed with a Leica DMR Confocal Laser-scan Microscope (Leica, Rijswijk, The Netherlands). Photographs were taken of representative areas of the slide; images were stored digitally at 1024 dpi and processed using Corel Photopaint 8.

## Results

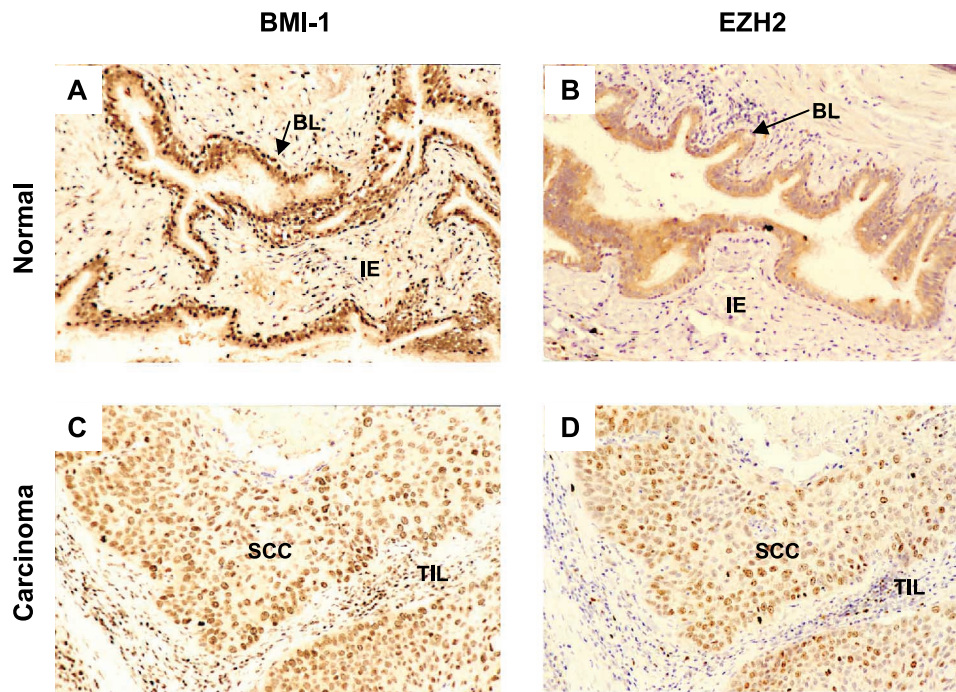
### IHC on Bronchial SCCs and Adjacent Normal Tissues

We started our analysis by IHC staining of tumor tissues and morphologically normal tissues of tumor distant sites, obtained from seven patients with bronchial SCC. BMI-1, EZH2, and Ki-67 were exclusively detected as nuclear staining of the epithelial cells. Representative examples of BMI-1-positive and EZH2-positive immunostainings are shown in Figures 1 and 2. Normal bronchial epithelia of all seven tumor distant samples showed abundant expression of BMI-1 (Figures 1A and 2B) and sporadic staining for Ki-67 (Figure 2C), suggesting that BMI-1 expression was primarily associated with resting Ki-67-negative cells. Normal epithe-

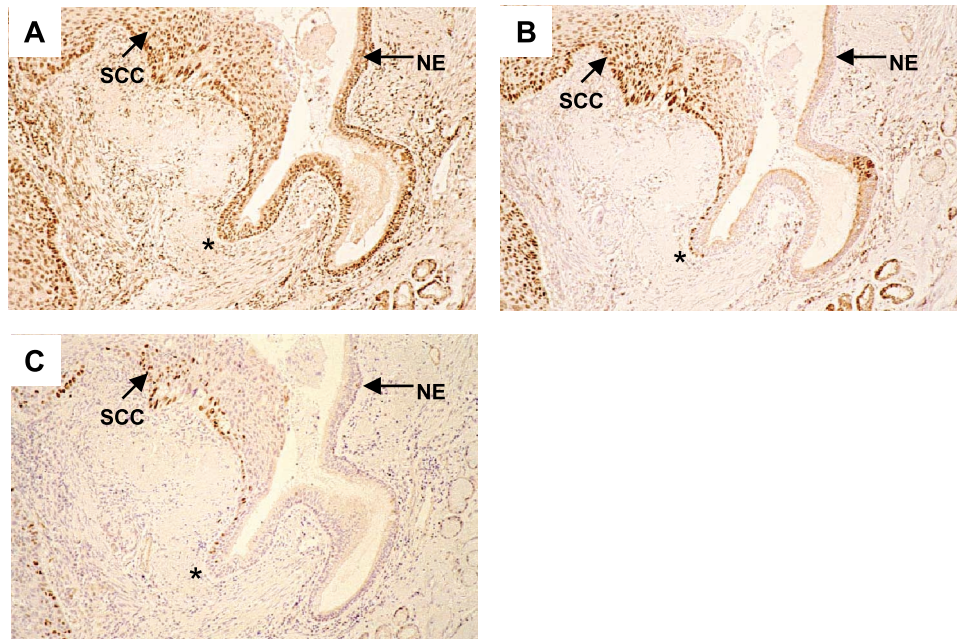
lium contained sporadic EZH2-positive cells, which were primarily associated with the basal layer (Figure 1B). The corresponding SCC samples revealed staining for Ki-67 in most of the nuclei of neoplastic cells (not shown). Six of the seven SCCs displayed diffuse nuclear staining for BMI-1 in a large proportion of the neoplastic cells. The remaining sample showed a weak nuclear staining for BMI-1 in the neoplastic cells. Furthermore, all except two SCCs showed diffuse clear nuclear staining for EZH2. The two exceptions included one sample with weak but diffuse nuclear EZH2 immunostaining and one sample that was EZH2-immunonegative. In one particular case (Figure 2), the transition from normal bronchial epithelium to invasive carcinoma could be observed. In this sample, expression of *BMI-1* was abundant in both normal epithelium and SCC. By contrast, EZH2 and Ki-67 were not detected in the normal bronchial epithelium, whereas EZH2/Ki-67 expression became evident in adjacent, noninvasive dysplastic nuclei, as well as in malignant cells. These staining patterns collectively show that neoplastic cells in SCC display increased expression of *EZH2*, and that this coincides with the presence of Ki-67. In addition, the immunohistochemical staining patterns suggest that proliferating Ki-67-positive neoplastic cells in SCC have retained expression of *BMI-1*, and that this PcG gene is coexpressed with *EZH2*.

### Immunofluorescent Staining on Bronchial SCCs and Adjacent Normal Tissues

To confirm the coexpression of *BMI-1* and *EZH2* in the nuclei of neoplastic cells of bronchial SCCs, IF double stainings for BMI-1 and EZH2 were performed on frozen



**Figure 1.** Expression of BMI-1 and EZH2 in normal bronchial epithelium and bronchial carcinoma. (A and B) Normal bronchial epithelium. (C and D) Bronchial carcinoma. Normal bronchial epithelium displays widespread expression of BMI-1 (A) and sporadic expression of EZH2 in the basal layer (BL). By contrast, both BMI-1 (C) and EZH2 (D) are expressed in the majority of neoplastic cells in bronchial carcinoma. Note that healthy intraepithelial (IE) cells in normal epithelium (A and B) and tumor-infiltrating lymphocytes (TILs) in bronchial carcinoma (C and D) are BMI-1-positive and EZH2-negative.



**Figure 2.** Expression of BMI-1, EZH2, and Ki-67 at the transition from normal bronchial epithelium to invasive cancer. BMI-1 expression (A) is detected throughout the normal epithelium (NE) and in SCC. By contrast, EZH2 is undetectable in NE but widespread in SCC (B). A minority of the EZH2-positive cells in SCC appear to express the proliferation marker Ki-67 (C). The transition of normal epithelium to SCC is indicated by (\*) and coincides with the appearance of EZH2 expression and the continued presence of BMI-1.

cryosections that were available in six of the abovementioned SCCs (including five cases that revealed both BMI-1 and EZH2 expression, and the case that was BMI-1-positive but EZH2-negative). The IF staining patterns confirmed the results obtained by IHC: normal bronchial epithelial samples showed widespread BMI-1 expression, whereas EZH2 positivity was rarely observed (Table 1) and not associated with the presence of BMI-1. The IF staining on frozen tumor samples showed colocalization of BMI-1 and EZH2 in the same nuclei in four of six cases that were BMI-1-positive and EZH2-positive by IHC. In one of these cases, EZH2 staining was less intense, which was in accordance with IHC findings of this case. In two of the cases without colocalization, only BMI-1 was detected by IF. In one of those cases, EZH2 was detected by IHC immunostaining but not by IF immunostaining. Representative IF staining patterns for tumor samples are shown in Figure 3. We conclude that

expression of BMI-1 and EZH2 are separated in normal bronchial epithelia (Table 1) and that BMI-1-positive neoplastic cells in SCC gain expression of EZH2.

*Immunofluorescent Staining on Premalignant Endobronchial Lesions*

To determine at what stage in bronchial tumorigenesis the expression of BMI-1 and EZH2 is altered, we performed IF triple stainings for BMI-1 and EZH2 in conjunction with Ki-67 in an additional group of patients at varying stages of the disease (Table 2). The proliferation marker was added to identify the proliferative cell fraction in PLs. The biopsies containing only normal bronchial epithelium (n = 3) showed a similar staining pattern as the tumor distant normal samples described above (i.e., positive staining for BMI-1 and infrequent detection of EZH2-positive cells) and occasional detection of Ki-67 in cells that were negative for BMI-1 and

**Table 1.** Results of IHC and IF Stainings on SCC and IHC Stainings on Adjacent Normal Tissues.

Age (years)	Histology	SCC (Ø; cm)	Sex	BMI-1/EZH2 Expression		
				IHC in SCC	IF in SCC	IHC and IF in Adjacent Normal Tissues
71	SCC	1	Male	±/+	+/-	+/-
67	SCC	4.5	Female	+/+	+/+	+/-
71	SCC	7	Male	+/+	+/+	+/-
73	SCC	4.5	Male	+/-	+/-	+/-
47	SCC	6	Male	+/+	+/+	+/-
48	SCC	4	Female	+/±	+/±	+/-
76	SCC	3	Male	+/+	ND	+/-

IHC, immunohistochemistry; IF, immunofluorescence; SCC, squamous cell carcinoma; (+) positive staining; (±) weak staining; (-) negative staining.

**Table 2.** Results of IF Stainings on Biopsies of Histomorphologically Normal Bronchial Epithelial Samples and Premalignant Endobronchial Lesions (Positive Lesions Per Histopathologic Diagnosis).

Histology	<i>BMI-1</i>	<i>EZH2</i>	<i>BMI-1</i> × <i>EZH2</i>	<i>EZH2</i> × Ki-67	<i>BMI-1</i> × MIB	<i>BMI-1</i> × <i>EZH2</i> × Ki-67
N ( <i>n</i> = 3)	3/3	0/3	0/3	0/3	0/3	0/3
H ( <i>n</i> = 3)	2/3	1/3	1/3	0/3	0/3	0/3
SM ( <i>n</i> = 2)	0/2	1/2	0/2	0/2	0/2	0/2
MiD ( <i>n</i> = 7)	5/7	7/7	4/7	7/7	3/7	3/7
SD/CIS ( <i>n</i> = 14)	12/14	14/14	12/14	14/14	10/14	10/14

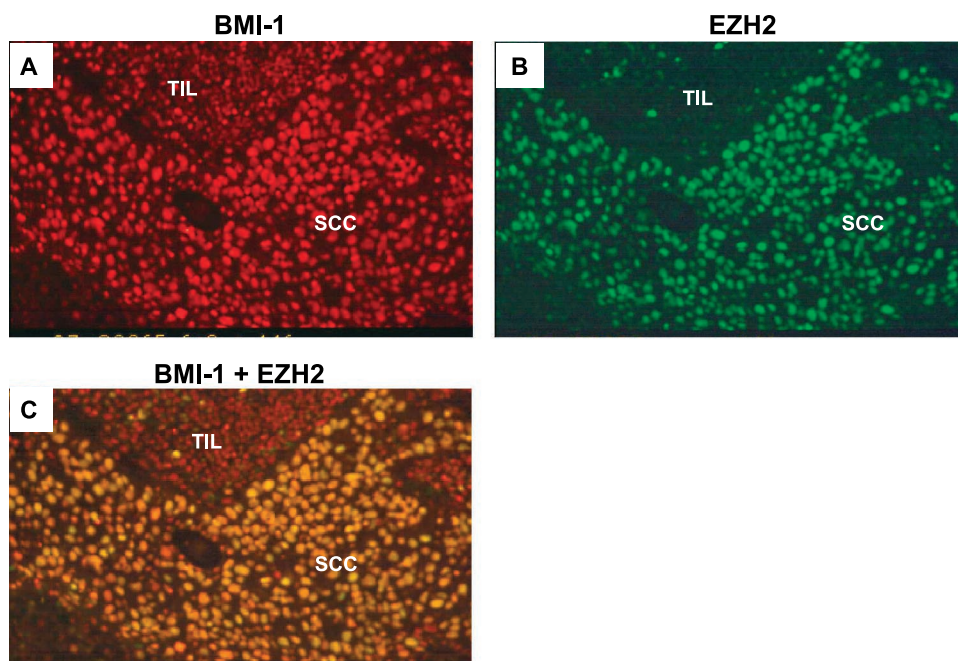
N, normal; H, hyperplasia; SM, squamous metaplasia; MiD, mild dysplasia; SD/CIS, severe dysplasia and/or carcinoma *in situ*.

*EZH2*. Lesions classified as hyperplasia (*n* = 3) showed positive staining for *BMI-1* in two of three cases. In one case, a coexpression with *EZH2* was evident, whereas the other two cases revealed absence of *EZH2* IF staining. Ki-67 staining was detected only occasionally, and in none of the lesions could colocalization of the PcG proteins with Ki-67 be found. Two lesions that were classified as squamous metaplasia revealed no staining for *BMI-1*, but one of them was *EZH2*-positive. Ki-67 staining was detected in the basal cell layer only and revealed no colocalization with *EZH2* in the single case that showed *EZH2* expression. Five of seven lesions that were classified as mild dysplasia revealed staining for *BMI-1*, whereas all seven lesions were *EZH2*-positive. Four of the lesions showed clear *BMI-1/EZH2* colocalization. Ki-67 was detected mainly in the lower third of the epithelial cell layer. All seven lesions showed colocalization of *EZH2* with Ki-67, whereas a very few cells showed colocalization of *BMI-1* and Ki-67 in only three lesions. These lesions revealed triple staining for *BMI-1*, *EZH2*, and Ki-67 in the same nuclei. All of the 14 lesions that were classified as severe dysplasia or carcinoma *in situ* had *EZH2* expression, 12 of which also revealed positivity for *BMI-1*. All these

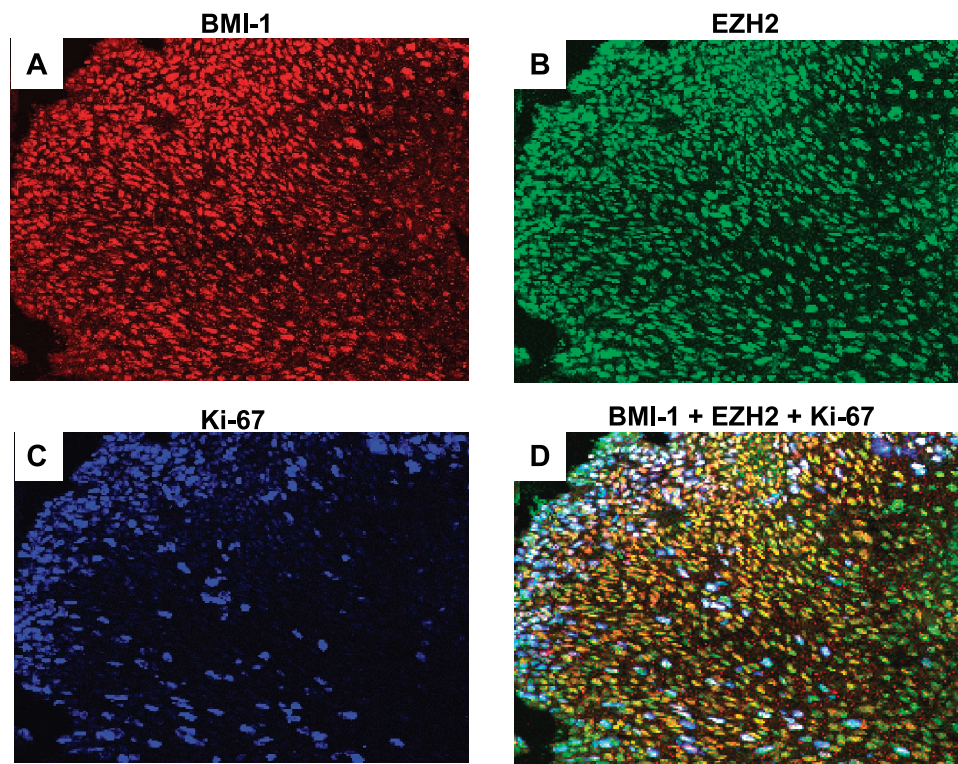
12 cases showed *BMI-1/EZH2* colocalization. Ki-67 could be found throughout the whole epithelial layer in all 14 lesions, although its IF staining was more pronounced in the lower segments of the epithelium. Colocalization of *EZH2* with Ki-67 was observed in all lesions, and coexpression of *BMI-1* and Ki-67 in the same nuclei was found in 10 of 14 lesions. If lesions showed colocalization of *BMI-1* with Ki-67, *EZH2* was also expressed in the same nuclei. A representative example is shown in Figure 4. We conclude that *EZH2* overexpression occurs at the level of PLs, which, in combination with disability to downregulate *BMI-1*, leads to coexpression. Furthermore, *EZH2* expression appears to precede increased proliferation in PLs.

### Discussion

PcG genes play a key role in maintenance of cell identity, and contribute to processes such as regulation of the cell cycle and hematopoiesis. Their essential function in normal cellular development is reflected by the fact that abnormal PcG gene expression is linked to carcinogenesis. In the current paper, we analyzed the expression of the *BMI-1* and *EZH2*



**Figure 3.** Coexpression of *BMI-1* and *EZH2* in bronchial carcinoma. Neoplastic cells in bronchial carcinoma (SCC) display widespread expression of *BMI-1* (A; detected by red immunofluorescence) and *EZH2* (B; detected by green immunofluorescence). Coexpression of *BMI-1* and *EZH2* in the same nucleus is indicated by yellow fluorescence (C), resulting from combination of the individual signals for *BMI-1* and *EZH2*. Note that TILs are *BMI-1*-positive and *EZH2*-negative.



**Figure 4.** Expression of BMI-1, EZH2, and Ki-67 in severe dysplasia. Neoplastic cells in severe dysplasia display widespread expression of BMI-1 (A; detected by red immunofluorescence), EZH2 (B; detected by green immunofluorescence), and Ki-67 (C; detected by blue fluorescence). Combination of signals for BMI-1, EZH2, and Ki-67 (D) displays a diverse population of neoplastic cells with extensive fluctuation in gene expression. Note that the majority of the cells coexpresses BMI-1 and EZH2, but that a subfraction of these appears to be in cycle (Ki-67–positive).

PcG oncogenes, representing the PRC1 PcG and PRC2 PcG complex, respectively. We discovered that bronchial SCCs and their PLs are associated with two altered patterns of PcG expression: the presence of BMI-1 protein in dividing neoplastic cells, and increased expression of *EZH2* resulting in coexpression with BMI-1.

*BMI-1* is abnormally expressed in neoplastic cells of bronchial SCCs and PLs. Analysis of histologically normal bronchial epithelium showed abundant expression of *BMI-1* in epithelial cells, and that *BMI-1* was primarily expressed in resting Ki-67–negative cells. This pattern is reminiscent of earlier studies of lymphocytes [48–50] and breast epithelia [41], where resting cells preferentially express *BMI-1*. By contrast, we found that cycling neoplastic cells in PL and SCCs display an increasing frequency of *BMI-1* expression: in 43% of the mild dysplasias and 71% of the severe dysplasias, BMI-1 was detected in proliferating cells. Given the association of BMI-1 with resting cells in the normal epithelium, we interpret this expression pattern as abnormal. Experimental model systems convincingly demonstrated that overexpression of *BMI-1* is strongly associated with increased cell proliferation and malignant transformation through suppression of *p16<sup>INK4a</sup>* and upregulation of telomerase [28,29,38]. Our study fits well with abnormal *BMI-1* expression patterns in lymphoid malignancies [32–36], breast carcinoma [38,41], and colorectal carcinoma [44], and supports the earlier observations that development of

lung carcinomas is associated with *BMI-1* overexpression and loss of *p16<sup>INK4a</sup>* expression [37]. The presence of BMI-1 in PLs suggests that *BMI-1* expression in proliferating neoplastic cells is an early event in lung carcinogenesis.

Development of lung cancer is associated with enhanced expression of the *EZH2* PcG gene. In addition to the abnormal expression pattern of *BMI-1* in cycling neoplastic cells, we also found an increased expression of the *EZH2* PcG gene in bronchial SCC and its PLs. Whereas *EZH2* expression was rare in normal bronchial epithelia, *EZH2* was present in nonproliferating and cycling cells of all mildly—and severely—dysplastic lesions. These findings are in line with a recent study by Bracken et al. [31], who demonstrated that *EZH2* expression is moderately to strongly enhanced in over 30% of primary human tumors. We found that the proportion of *EZH2*-positive cells in proliferating cell fraction increased with the severity of the histopathologic stage, and that the majority of bronchial SCCs showed expression of *EZH2*. Increased expression of *EZH2* appears to be an early event because *EZH2* was detected in one hyperplasia and one squamous metaplasia, and became predominantly manifest in dysplastic tissues. *EZH2* expression resulted in coexpression with the *BMI-1* PcG gene in neoplastic cells—a pattern that was not observed in normal epithelia. These observations collectively suggest that *EZH2* expression in BMI-1–positive neoplastic cells is associated with the development of lung cancer. Although *EZH2* expression is

linked to a variety of human epithelial tumors [31,40,42, 43,51], the mechanism responsible for enhanced expression of *EZH2* remains ill defined. *EZH2* appears to be controlled by the pRB pathway [31], which is frequently deregulated in human tumors [52]. An additional possible mechanism that may contribute to deregulation of *EZH2* expression is amplification of the *EZH2* gene, which was recently reported in up to 15% of human cancers [31].

The question remains as to whether induction of *EZH2* is responsible for cellular transformation, or whether it is mainly reflective of increased cell division. Current evidence supports both conclusions. Previous studies of lymphoid development demonstrated that *EZH2* expression is primarily associated with cycling normal lymphocytes and proliferating neoplastic cells [32–36,48–50]. A direct link between *EZH2* expression and proliferation is further supported by experimental suppression of *EZH2*, which resulted in diminished proliferation of cell lines [31], whereas overexpression of *EZH2* resulted in enhanced proliferation rates [34]. In the current work, we found that the *EZH2* expression pattern of histologically normal epithelia did not necessarily overlap with Ki-67, and that a considerable proportion of nuclei in PLs expressed *EZH2* in the absence of Ki-67. These observations suggest that *EZH2* expression in PLs precedes the induction of proliferation during bronchial carcinogenesis, as was recently observed in PLs of breast carcinomas [41]. To what extent *EZH2* expression is directly linked to cellular transformation cannot be determined by the current study, but the recent identification of *EZH2* as a bona fide oncogene suggests that *EZH2* may directly contribute to lung carcinogenesis and loss of cell identity [31]. Aside from being essential for proliferation, our current work also suggests that *EZH2* expression is related to differentiation because *EZH2* was detected at the transition from normal and hyperplastic epithelium (both consisting of cylindrical type of epithelium) to squamous metaplasia. Interestingly, enhanced expression of *EZH2* in breast carcinomas is primarily seen in ductal carcinoma *in situ* (DCIS) and carcinomas of poor differentiation [41]. In the present study, all bronchial SCCs were moderately or poorly differentiated, possibly explaining the high proportion of SCCs with frequent *EZH2* expression. Functional and follow-up studies are needed to elucidate the precise role of *EZH2* and other PcG genes in bronchial carcinogenesis.

In conclusion, we showed that neoplastic cells in lung carcinomas display altered expression of the *BMI-1* and *EZH2* PcG genes. We propose that altered expressions of *BMI-1* and *EZH2* are early events that precede high rates of proliferation. Because PcG complexes are normally involved in the maintenance of cell characteristics, abnormal expression of PcG genes may contribute to loss of cell identity. Several members of the PcG gene family have been identified as oncogenes, and recent studies in humans demonstrated that hematologic malignancies and solid tumors are all linked to altered PcG gene expression. Collectively, these studies strongly suggest that altered expression of PcG genes is an important contributing factor in the development of neoplasms in humans.

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