Dysregulated Expression of Stem Cell Factor Bmi1 in Precancerous Lesions of the Gastrointestinal Tract

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Abstract

Purpose: It is important to identify the definitive molecular switches involved in the malignant transformation of premalignant tissues. Cellular senescence is a specific characteristic of precancerous tissues, but not of cancers, which might reflect tumorigenesis-protecting mechanisms in premalignant lesions. Polycomb protein Bmi1, which is a potent negative regulator of the p16INK4 gene, suppresses senescence in primary cells and is overexpressed in various cancers. We hypothesized that Bmi1 expression would also be dysregulated in precancerous lesions in human digestive precancerous tissues.

Experimental Design: Bmi1 expression was investigated in cancerous and precancerous tissues of the digestive tract. The expression of p16, β-catenin, and Gli1 and the in vivo methylation status of the p16 gene were also analyzed in serial sections of colonic precancerous lesions.

Results: Bmi1 was clearly overexpressed across a broad spectrum of gastrointestinal cancers, and the expression of Bmi1 increased in a manner that reflected the pathologic malignant features of precancerous colonic tissues (low-grade dysplasia, 12.9 ± 2.0%; high-grade dysplasia, 82.9 ± 2.4%; cancer, 87.5 ± 2.4%). p16 was also strongly expressed in high-grade dysplasia, but not in cancers. p16 promoter methylation was detected only in some Bmi1-positive neoplastic cells.

Conclusions: Bmi1 overexpression was correlated with the malignant grades of human digestive precancerous tissues, which suggests that advanced Bmi1 dysregulation might predict malignant progression. The abnormal Bmi1 expression might link to malignant transformation via the disturbance of orderly histone modification.

In cancer research, the mechanism of switching from premalignancy to established malignancy is currently the focus of intensive investigation. Premalignant tumors often show oncogenic DNA changes that lead to abnormal proliferation, whereas a large proportion of the cells in tumors undergo oncogene-induced senescence. However, oncogenic cells bypass this process due to the lack of oncogene-induced senescence effectors, such as p16 or p53 (1), and establish malignant transformation. In other words, oncogene-induced senescence is a cellular response that might be crucial for protecting against cancer development (1, 2). Therefore, it is important to examine the mechanism of oncogene-induced senescence to better understand carcinogenic processes. p16 and p14 encoded by the Ink4a/Arf locus are important senescence biomarkers (1). Aberrant mitogen-activated protein kinase signaling in primary and immortalized fibroblasts induces the expression of both of these markers (3, 4), which is a potent fail-safe mechanism to prevent cells from engaging in uncontrolled proliferation. Indeed, in human malignancies, p16 is inactivated at high frequency (5) via aberrant epigenetic changes or genomic deletions. Importantly, gene-targeting analysis has shown that p16 suppression is indispensable for invasive cancer formation (6). Combined, these findings suggest that inactivation is the important step in oncogenesis and underline the significance of determining how expression inhibition is initiated in neoplastic lesions.

The epigenetic signatures of most cell types become fixed once the cells differentiate or exit the cell cycle. However, during normal development, or in disease, some cells undergo major epigenetic “reprogramming” (7). Epigenetic alterations are thought to be important factors for determining the characteristics of various cancer cells. For example, application to malignant cells of the “histone code” hypothesis, which posits that different combinations of histone modifications mediate unique cellular responses, suggests that abnormal histone modifications in cancers serve as one of the molecular switches for malignant transformation.
Recently, polycomb group genes, which play pivotal roles in gene expression through chromatin modifications, have been identified as oncogenes (8). Given the role of polycomb group proteins in epigenetic modification, it is possible that abnormal expression of polycomb group proteins contributes to the formation of cancer-specific cellular characteristics (8). The polycomb group gene Bmi1, which was originally identified as an oncogene that induces B-cell or T-cell leukemia (9, 10), is a potent negative regulator of the Ink4a/Arf locus (11–13). Bmi1 is overexpressed in several human cancers (14, 15). Interestingly, given that Bmi1 protein suppresses the senescence of primary cells (13) and is essential for maintaining stem cell fate (8, 16, 17), the overexpression of Bmi1 might play a role in the escape from senescence in premalignant cells.

This work explored whether Bmi1 plays a role in the malignant transformation of precancerous lesions of the human digestive tract.

Materials and Methods

Cell lines and human tissue samples. Cell lines from four human pancreatic carcinomas (SIU8686, AsPC1, BxPC3, and CFPAC1), two gastric cancers (AGS and MKN7), three hepatic carcinomas (Alexander, Hep3B, and HepG2), a cholangiocarcinoma (HuCCT), and embryonic kidney (HEK293T) were purchased from the American Type Culture Collection (Manassas, VA) or the Japanese Riken Cell Bank (Tsukuba, Japan). Normal hepatocytes were purchased from Dainippon Pharma (Osaka, Japan) and normal diploid colonocytes (CCD841) were purchased from American Type Culture Collection. Tissue specimens from each tissue examined by microscopy. Researchers analyzed the expression status in various human digestive tract tumors, including pancreas (Fig. 1A and B), bile duct (Fig. 1C and D), stomach (Fig. 1E and F), and colon (Fig. 1G and H) cancers. Bmi1 accumulation was detected in most cancerous cells, whereas tumor stromal tissues and normal epithelial cells did not express this protein (Fig. 1E and G), in contrast to bronchial epithelium, which shows widespread expression of Bmi1 (25). Similarly, in the cancer cell lines, Bmi1 was expressed in all 10 cell lines derived from stomach, liver, pancreas, and bile duct cancers, but weakly in normal cells (Fig. 1I). These results indicate the existence of cancer-specific induction mechanisms for Bmi1 in various cancers.

Bmi1 shows a gradient of expression in human colonic neoplasms. To evaluate Bmi1 expression in the precancerous lesions of digestive tissues, researchers examined the expression status in human colonic neoplastic lesions. Because colonic neoplasia is the most intensively studied of the multistep carcinogenesis models, it offers an excellent setting in which to examine the status of Bmi1 in human precancerous lesions. The tumors were pathologically classified into three groups depending on the malignant grade: low-grade intraepithelial dysplasia, high-grade intraepithelial dysplasia, and cancer. Interestingly, the precancerous lesions showed distinct Bmi1 expression patterns relative to the pathologic grade of neoplasia (Fig. 2A and B). As shown in Fig. 2C, even in a single gland, cells of different atypical grades showed distinct patterns. Researchers scored the percentages of Bmi1-positive cells by gland with different atypical grades (n = 50 glands per group). In low-grade

Results

Bmi1 is accumulated in the cancerous cells of a broad spectrum of gastrointestinal tumors. Bmi1 mRNA is detectable in the pancreas but not in the small or large intestines (24). We analyzed the expression in various human digestive tract tumor tissues, including pancreas, stomach, liver, pancreas, and bile duct cancers, but weakly in normal cells (Fig. 1I). These results indicate the existence of cancer-specific induction mechanisms for Bmi1 in various cancers.

Bmi1 Overexpression in Precancerous Cells

Validated siRNAs were purchased from Dharmacon and transfected into cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the protocol of the manufacturer. The knockdown efficiencies were examined by immunoblotting 48 hours after treatment. After siRNA treatment for 48 hours, the bromodeoxyuridine (BrdUrd) uptake of the cells over the following 24 hours was determined with BrdUrd ELISA (Roche, Basel, Switzerland). Three independent experiments were done.

Methylation-specific PCR. The methylation status of p16 was determined by bisulfite treatment of the DNA, followed by methylation-specific PCR amplification, as described previously (23). In brief, the microdissected genomic DNA was denatured in 2 N NaOH at 37°C for 10 minutes, and then incubated in 3 mol/L sodium bisulfite (pH 5.0) for 16 hours in the dark. The following primer sequences were used: methylated sense, 5′-TTATTAGGGTGGGGCCGG- GTCCG-3′; methylated antisense, 5′-GACCCCCAAACCGCCCGCCG-3′; unmethylated sense, 5′-TTATTAGGGTGGGGCGCCGATTGCT-3′; and unmethylated antisense, 5′-CAACCCCAACACACCAATGTA-3′.

Laser-capture microdissection. Microdissection of paraffin-embedded tissue was done on H&E-stained slides. Genomic DNA was extracted from microdissected tissues as previously described (23). Each specimen was treated with lysis buffer that contained proteinase K and incubated overnight at 56°C. The extracted DNA was purified with the QiAquick PCR Purification Kit (Qiagen, Valencia, CA).

Statistical analysis. The results are presented as the mean ± SE. Comparisons were made using Student’s two-tailed t test. P < 0.05 was considered statistically significant.
intraepithelial dysplasia, in which the nuclei were limited primarily to the lower halves of the atypical cells in the glands, the proportion of Bmi1-positive cells was 12.9 ± 2.0% (Fig. 2D). Conversely, for high-grade intraepithelial dysplasia, in which the nuclei were translocated to higher levels, Bmi1 was detected in 82.9 ± 1.6% of the cells, a frequency that is similar to that of cancerous cells (87.5 ± 2.4%; Fig. 2D). These findings indicate that Bmi1 expression is connected to the mechanism that determines malignant potential.

The p16 protein is expressed in precancerous colonic tissues, but not in the cancers. The Ink4a/Arf locus is a target of the polycomb complex, which includes Bmi1. Previous studies noted p16 deficiency in many cancers, and a distinct distribution of Bmi1 with p16 has been observed in colon cancers (26). The expression of p16 was not detected in either most of the colon cancer tissues or certain cancer cell lines in our experiments (Figs. 1I and 3A). Similarly, p16 was not detected in a large section of the normal colon or normal cell lines (ref. 27, and data not shown; Fig. 1I). However, p16 was clearly expressed in colonic precancerous lesions as previously reported (27), although the distribution was not uniform, even in identical tumors (Fig. 3B). We classified the distribution of p16-positive cells according to atypical grade, using the same tumors, with Bmi1 classification (Fig. 3C). Numerous p16-positive cells were noted in the high-grade intraepithelial dysplasia (14.3 ± 4.9%) compared with cancers (2.0 ± 1.4%; Fig. 3C). Given the

Fig. 1. Bmi1 expression in digestive tissue samples. Immunohistochemical Bmi1 staining of pancreas (A and B), bile duct (C and D), stomach (F), and colon (H) cancer tissues was done, as well as in normal stomach (E) and colon (G) tissues. All of the sections are counterstained with hematoxylin. Magnification, ×100 (A-D); ×200 (E-H). I, immunoblotting for Bmi1 and p16 in digestive tract cancer and normal cell lines.

Fig. 2. Distinct expression patterns of Bmi1 in precancerous lesions of the colon. H&E staining (A) and Bmi1 immunostaining (B and C) of precancerous colon specimens (magnification, ×200). D, quantitative analysis of Bmi1 expression in colon tumors. The results are shown as scatter plots of 50 glands derived from 12 specimens. Low, low-grade intraepithelial neoplasia; High, high-grade intraepithelial neoplasia. Columns, mean; bars, SE. * P < 0.05; n.s., not significant.
dissociation of the expression patterns in some cancer cells (26), it seems that a significant change in p16 expression is induced in Bmi1 and p16 double-positive cells with high-grade intraepithelial dysplasia, which ultimately leads to malignant transformation.

Methylation of the p16 gene promoter occurs in a part of Bmi1-positive cells of precancerous colonic tissues. Given the histone modification functions of polycomb protein Bmi1, aberrant epigenetic alterations were suspected in the cells overexpressing Bmi1. To determine whether dysregulated Bmi1 expression coexists with aberrant DNA methylation, the methylation status of the CpG island in the p16INK4a gene promoter was examined in the colonic tumors. The genomic DNA was selectively isolated from the neoplastic portions of four tumor tissues that had a distinct status for the Bmi1 and p16 proteins (Fig. 4A). In the Bmi1-positive glands, p16 methylation was detected in only some of the Bmi1 and p16 double-positive precancerous lesions (Fig. 4B), which indicates that p16 promoter methylation is not always induced by Bmi1 overexpression. In other words, Bmi1 might have a mechanism of p16 suppression that is independent of the promoter methylation during the transition from p16-positive precancerous tissues to p16-negative cancers.

The aberrant expression of Bmi1 is independent of Wnt or Hedgehog signaling in neoplastic gastrointestinal tissues. The induction mechanisms of Bmi1 in neoplastic cells have not been fully elucidated. The Wnt and Hedgehog (Hh) signaling pathways are important for the normal development of gastrointestinal tissues (20, 28–31), and it has been speculated that they are related to oncogenesis in gastrointestinal tumors (20, 29). To unravel the relationship between Wnt signaling and Bmi1 expression, the expression patterns of β-catenin and Bmi1 were compared in serial sections of precancerous colonic lesions (Fig. 5A and B). As shown in Fig. 5B, glands in which β-catenin was overexpressed in the cytoplasm (arrow) were Bmi1 positive, although the Bmi1-positive cells did not always show β-catenin accumulation (arrowhead). Because Bmi1 has been reported as being downstream of Hh signaling in cerebellar granule cells (32), we examined whether Hh signaling induces Bmi1 in cancer cells. As a result, neither Gli1 expression nor nuclear accumulation of Gli1 was significant, irrespective of the expression of Bmi1 in the colonic neoplasms (Fig. 5A and C). In addition, Hh signaling is activated in the upper gastrointestinal cancer cells (20, 29) and we reported that constitutive Hh expression activated the signaling in the human gastric cancer AGS cells (33). In the AGS cells, Gli1 overexpression did not induce Bmi1 up-regulation (Fig. 5D). Moreover, when the signaling cascade in the AGS cells was blocked by treatment with the Smo inhibitor cyclopamine, Bmi1 expression was not affected (Fig. 5D). Combined, these results suggest that Bmi1 expression is independent of Hh or Wnt signaling, and that different induction mechanisms exist in gastrointestinal neoplastic cells.

Bmi1 protein is not essential for DNA replication in cancer cells. Cyclin-dependent kinase inhibitor p16 leads to cell cycle arrest by affecting the retinoblastoma protein (34, 35). As Bmi1 is a potent negative regulator of p16 and has
an essential role in regulating the proliferative activities of normal and leukemic stem cells (16), we examined whether the Bmi1 protein is a positive cell cycle regulator in gastric cancer cells. Because Bmi1 expression was diminished 48 hours after siRNA treatment in AGS cells (Fig. 6A), the in vitro BrdUrd uptake assay was done after siRNA treatment for 24 hours. As a result, BrdUrd uptake was not affected by the knockdown of Bmi1 (Fig. 6B), which indicates that Bmi1 is not essential for the growth of AGS cells. Because Bmi1 is already overexpressed in precancerous lesions, the growth advantage with Bmi1 overexpression might be given full play in premalignant cells, rather than in established cancer cells. Previously, we reported that the knockdown of Gli1 leads to growth retardation of AGS cells (33). This result seems to be consistent with the finding that Bmi1 does not always mediate the Hh signaling activity in gastric cancer cells (Fig. 5).

Discussion

The global histone modification patterns have been described as being responsible for the heterogeneity of tumors and have been correlated with clinical behavior (36, 37). A recent report has documented that the Bmi1-associated gene signature is a powerful predictor of a short interval to distant metastasis and poor survival after therapy (36). Combined, the various aberrant histone modifications may contribute to the characteristics of cancer. Indeed, previous reports have documented that some histone modifiers are overexpressed in cancers. For example, high-level enhancer of zeste homologue 2 (EZH2) expression was localized to the more primitive malignant cell types, often in combination with high-level Bmi1 expression (38). The functional relationships between abnormal enzyme-based histone modification and oncogene-induced signaling in cancers need to be clarified.

Polycomb group protein Bmi1 can be recruited to histone H3 at Lys27 methylated by EZH2 (39–41). Both EZH2 and Bmi1 are up-regulated in many cancers (42–45). Altered epigenetic modifications caused by the dysregulated expression of EZH2 or Bmi1 might contribute to tumor progression from precancerous cells. A recent report has documented that EZH2 interacts with DNA methyltransferases and is associated with methyltransferase activities (46). Although our findings do not exclude situations in which Bmi1 overexpression cooperates with P16 promoter methylation, abnormal histone modifications could silence gene expression without the involvement of DNA methylation (47–49). Although it remains to be determined whether the overexpression of Bmi1 and EZH2 induces p16 gene suppression in precancerous cells, the mechanism linking the dysregulated histone modification to the epigenetic aspect of oncogenesis should be investigated further.
Given that our data do not show a perfect correlation between Bmi1 and p16 expression (Figs. 11 and 3), it is possible that Bmi1 functions through downstream molecules other than p16 in the tumors. In other words, the Bmi1-driven pathway might have various significant roles other than cell proliferation that benefit through p16 suppression in neoplastic cells (50). For example, Bmi1 might be linked to chemotherapy resistance (56) or to stem cell-like properties (17, 32). Recently, cancer stem cells were also found to reside within solid tumors, including several types of brain cancer (51, 52) and breast carcinomas (53). If stem cells are characterized by chemotherapy resistance (54), Bmi1 might play a role in the stem cell properties of cancer cells, as occurs in other stem cell lineages. Indeed, cancer stem cells cultured from a panel of pediatric brain tumors showed high-level expression of Bmi1 among various stem cell markers (51). It will be important to address whether Bmi1 determines the stem cell characteristics of cancer cells.

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