Bmi-1 Is a Novel Molecular Marker of Nasopharyngeal Carcinoma Progression and Immortalizes Primary Human Nasopharyngeal Epithelial Cells

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Abstract

The Bmi-1 oncoprotein regulates proliferation and oncogenesis in human cells. Its overexpression leads to senescence bypass in human fibroblasts and immortalization of human mammary epithelial cells. In this study, we report that compared with normal nasopharyngeal epithelial cells (NPEC), Bmi-1 is overexpressed in nasopharyngeal carcinoma cell lines. Importantly, Bmi-1 was also found to be overexpressed in 29 of 75 nasopharyngeal carcinoma tumors (38.7%) by immunohistochemical analysis. In contrast to nasopharyngeal carcinoma, there was no detectable expression of Bmi-1 in noncancerous nasopharyngeal epithelium. Moreover, high Bmi-1 expression positively correlated with poor prognosis of nasopharyngeal carcinoma patients. We also report that the overexpression of Bmi-1 leads to bypass of senescence and immortalization of NPECs, which normally express p16INK4a and exhibit finite replicative life span. Overexpression of Bmi-1 in NPECs led to the induction of human telomerase reverse transcriptase activity and reduction of p16INK4a expression. Mutational analysis of Bmi-1 showed that both RING finger and helix-turn-helix domains of it are required for immortalization of NPECs. Our findings suggest that Bmi-1 plays an important role in the development and progression of nasopharyngeal carcinoma, and that Bmi-1 is a valuable marker for assessing the prognosis of nasopharyngeal carcinoma patients. Furthermore, this study provides the first cellular proto-oncogene immortalized nasopharyngeal epithelial cell line, which may serve as a cell model system for studying the mechanisms involved in the tumorigenesis of nasopharyngeal carcinoma. (Cancer Res 2006; 66(12): 6225-32)

Introduction

Nasopharyngeal carcinoma is a common cancer in southern China (1–3). The etiologic factors associated with nasopharyngeal carcinoma are believed to be genetic susceptibility, EBV infection, and other environmental factors (1–3). However, the precise genetic changes that are responsible for nasopharyngeal carcinoma progression are largely unknown. The development and progression of nasopharyngeal carcinoma may involve accumulation of multiple genetic alterations over a long period of time (1). Various nasopharyngeal carcinoma–derived cell lines and viral oncogene (human papillomavirus E6/E7 or SV40T antigen)–immortalized nasopharyngeal epithelial cell (NPEC) lines have been established (2–4), which can be used to study the mechanisms of nasopharyngeal carcinoma development. However, such cell lines do not offer an ideal system, as tumor-derived cell lines and cells immortalized with viral oncogenes cultured over long period may contain genomic abnormalities unrelated to nasopharyngeal carcinoma development. On the other hand, an NPEC strain immortalized with a cellular oncogene, which also overexpressed in nasopharyngeal carcinoma, would provide an ideal cellular system to precisely delineate the steps involved in nasopharyngeal carcinoma development. Apart from the human telomerase reverse transcriptase (hTERT), two cellular oncogenes (c-Myc and Bmi-1) have been described in the literature, which can immortalize certain cell types (5, 6). These oncogenes are also overexpressed in a variety of cancers (7, 8). In the present study, we focused on the expression of Bmi-1 oncogene in nasopharyngeal carcinoma and examined its potential as an immortalizing oncogene in NPECs.

Bmi-1 was first isolated as an oncogene that cooperates with c-Myc in generating lymphomas in a murine model (9, 10). It is a transcriptional repressor belonging to the Polycomb-group (PcG) family of proteins involved in axial patterning, hematopoiesis, regulation of proliferation, and senescence (11, 12). Bmi-1-deficient mouse embryonic fibroblasts (MEF) overexpress INK4a/ARF locus-encoded genes p16INK4a and p19ARF (mouse homologue of human p14ARF) and undergo premature senescence in culture (13). Conversely, overexpression of Bmi-1 reduces expression of p16INK4a and p19ARF and immortalizes MEFs (13). Recently, it has been found that Bmi-1 is overexpressed in a variety of human cancers, such as mantle cell lymphomas (14), non–small cell lung cancer (15), B-cell non-Hodgkin's lymphoma (16), breast cancer (17), colorectal cancer (18), and prostate cancer (19). At present, it is not known if Bmi-1 is overexpressed in human head and neck cancers, such as nasopharyngeal carcinoma.

Recently, we have shown that Bmi-1 overexpression alone was able to immortalize post-selection p16INK4a-deficient human mammary epithelial cells (HMEC) and induced telomerase activity in these cells (5). In human fibroblasts, which expresses p16INK4a, Bmi-1 overexpression results in extension of replicative life span but no immortalization (20). Bmi-1 was also reported to immortalize bone marrow stromal cells and cementoblast progenitor cells, albeit in combination with other oncogenes (21, 22). However, there is no report of Bmi-1 overexpression leading to immortalization of p16INK4a-expressing primary human cells.

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Here, we report that Bmi-1 is overexpressed in nasopharyngeal carcinoma cell lines at both mRNA and protein levels. Importantly, overexpression of Bmi-1 was also observed in a significant number of nasopharyngeal carcinoma tumors, which correlated with advanced invasive stage of the tumor progression and poor prognosis. We also report that overexpression of Bmi-1 lead to the induction of telomerase activity, reduction of p16INK4a expression, and immortalization of NPECs.

Materials and Methods

Cell culture. One well-differentiated nasopharyngeal carcinoma cell line (CNE-1), three poorly differentiated nasopharyngeal carcinoma cell lines (CNE-2, C666, and SUNE-1), and two SUNE-1 subclones (6-10B and 5-8F) were maintained in our laboratory. These four nasopharyngeal carcinoma cell lines and two subclones of SUNE-1 were cultured in DMEM supplemented with 10% fetal bovine serum. Primary cultures of NPECs were established as described previously (23). Briefly, fresh biopsies of the nasopharynx, which were pathologically exclusive of nasopharyngeal carcinoma, were collected from the Department of Nasopharyngeal Carcinoma, Cancer Center, Sun Yat-sen University, Guangdong, China, and each sample was divided into two parts. One part was directly cultured for the primary epithelial cells. The other part was fixed in formaldehyde and embedded in paraffin. Prior consent of the patients and approval from the research ethics committee were obtained for the use of these surgical materials. To initiate primary culture of nasopharyngeal epithelial cells, the biopsies were cut into ~1 mm² size explants and placed onto T-25 culture flask (Falcon) containing 2 mL of keratinocyte-serum-free medium (Invitrogen, Grand Island, NY). The cells grown out from the biopsies were characterized with an anti-cytokeratin antibody (pan ZM-0069; ref. 23).

Clinical samples and clinical staging system. A total of 75 nasopharyngeal carcinoma tissue samples and 11 non-nasopharyngeal carcinoma tissue samples were collected from the archives of the Department of Pathology of the Cancer Center, Sun Yat-sen University. All nasopharyngeal carcinoma samples were classified as nonkeratinizing carcinomas (WHO II). Fifty-seven patients (76%) received radiotherapy only, whereas the other 18 patients (24%) received concomitant and/or adjuvant chemotherapy combined with radiotherapy. For the use of these clinical materials for research purposes, prior patient’s consent and approval from the Institute Research Ethics Committee was obtained. The disease stages of all the patients were classified or reclassified according to the 1992 nasopharyngeal carcinoma staging system of China (24).

Reverse transcription-PCR analyses. Total RNA from fresh tissues, tumor cell lines, Bmi-1-overexpressing cells, and pMSCV vector control cells were extracted using a Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. The RNA was treated with DNase, and 2.5 μg of total RNA was used for cDNA synthesis using random hexamers. Full-length open reading frame of Bmi-1 was amplified by PCR from cDNA samples of non-nasopharyngeal carcinoma and nasopharyngeal nasopharyngeal carcinoma cell lines. The following primers were used for amplification of Bmi-1: sense primer, 5'-ATGGCATGGAACAGGAGAATCGACTGACT-3'; antisense primer, 5'-TCAACAGAAGATGTGCTGAT-GACC-3'. The primers used for p16INK4a amplification were sense primer, 5'-AGCTTGCTGCTGGCTTG-3' and antisense primer, 5'-CTGGCATCATGACCTTGGA-3'. The primers used for detection of the genes in the Bmi-1-driven pathway were as described by Ginsky et al. (19). The primers used for glyceraldehyde-3-phosphate dehydrogenase (internal control) were 5'-AAATCCATACCCATCTCTC-3' and 5'-CTGGCTCTACACCTCTTGTG-3'. The PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide and/or ethidium bromide.

Immunohistochemical analysis. Immunohistochemistry was done to examine Bmi-1 expression in 75 human nasopharyngeal carcinoma tissue specimens and 11 non-nasopharyngeal carcinoma tissue specimens. Bmi-1 was detected using a mouse monoclonal antibody against Bmi-1 (Upstate Biotechnology, Lake Placid, NY). Briefly, a paraffin section of the nasopharyngeal carcinoma tissue from the patient was deparaffinized with xylene and rehydrated. Antigenic retrieval was processed by submerging the sample in citrate buffer (pH 6) and microwaving. The sections were then treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity followed by incubation with 1% bovine serum albumin to block the nonspecific binding. The sections were then stained with anti-Bmi-1 antibody (1:150) for 90 minutes at room temperature. After washing, the tissue sections were then incubated with the biotinylated anti-mouse secondary antibody followed by further incubation with streptavidin-horseradish peroxidase complex. The tissue section was immersed in 3-amino-9-ethyl carbazole and counterstained with 10% Mayer's hematoxylin, dehydrated, and mounted in crystal mount. In the negative control, primary antibody was replaced by the non-immune mouse IgG of the same isotype. The degree of immunostaining of formalin-fixed, paraffin-embedded sections was evaluated by two independent observers, and moderate to strong nuclear staining was scored as a positive reaction. The distribution of Bmi-1 was scored as follows: negative (<10% of the cells being positive) and positive (where ≥10% of the cells were positive).

Western blot and immunofluorescence analysis. Western blot analysis was done as described (25). Where relevant, the blots were probed with anti-Bmi-1 and anti p53 (DO-1), anti p21 (F-5), or anti p16INK4a (50.1) antibodies (Santa Cruz, CA, USA), and signal was detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were stripped and probed with an anti-α-tubulin mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to confirm equal loading of the samples. Immunostaining of Bmi-1 in cultured cells was done as described (26).

Immortalization studies. Full-length Bmi-1 was amplified from NPEC cDNA and cloned into pMSCV vector. pBabe-Bmi-1 and pBabe-Bmi-1 deletion mutants have been described previously (5). The RING finger (ΔRF) and helix-turn-helix (ΔHT) mutants of Bmi-1 contain deletion of the NH2-terminal RING finger domain and the centrally located H-T-H-T region, respectively (5). Retroviruses were generated by transient transfection as described (25). The Bmi-1 gene was introduced into NPEC1 cells by infecting cells with a retrovector pMSCV-Bmi-1. Control cells were infected with the empty retrovector pMSCV. For mutational analysis, pBabe-Bmi-1 and pBabe-Bmi-1ΔRF and ΔHT as well as the vector control viruses were used to infect a different normal cell line (NPEC2). Retrovirus-infected cells were selected and maintained in 0.5 μg/mL puromycin for 3 to 5 days. Western blot analysis was done to confirm the expression of Bmi-1 and Bmi-1 mutants in the NPEC cells using the specific antibody against Bmi-1. Cells were serially passaged to determine the replicative life span as described (5, 20).

Telomerase assay. The presence of telomerase in immortalized NPEC was examined using the PCR-based telomeric repeats amplification protocol assay according to the previously published protocol (27).

DNA damage checkpoint analysis. DNA damage checkpoint analysis was done as previously described (5). The cells were treated with 0.5 μg/mL Adriamycin or vehicle (DMSO) alone for 24 hours, and the total cell lysates were prepared for p53 and p21 expression by Western blot analysis. To determine the % labeled nuclei, cells were pulsed with [3H]thymidine for 6 hours, fixed, and processed for autoradiography as described (28).

Statistical analysis. The Pearson χ^2 and Kruskal-Wallis test were used to analyze the relationship between Bmi-1 expression and clinicopathological characteristics by using the SPSS 10.0 software package, and test for the trend of odds was used to analyze correlation between Bmi-1 expression and T classification by using STATA (8.0) software package. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. Multivariate analysis was done by Cox’s proportional hazards regression model. P < 0.05 was considered statistically significant.

Results

Establishment of a primary human NPEC cell line. A primary cell line (NPEC1) grew from the biopsy of a patient diagnosed with nonmalignant nasal disease. The cells had a cobblestone-like morphology, which is a characteristic of epithelial cells grown in culture (Fig. 1A). These cells proliferated in culture for 8 to 10
passages before undergoing senescence. To confirm the epithelial origin of these cells, expression of cytokeratin (pan) was determined. The results showed positive staining for cytokeratin in the cytoplasm of every individual cells, indicating that they were of epithelial origin (Fig. 1B). Similarly, another NPEC cell line (NPEC2) was established and characterized.

**Bmi-1 is overexpressed in nasopharyngeal carcinoma cell lines.** As a first step to assess the potential role of Bmi-1 in NPEC transformation, we asked whether Bmi-1 is overexpressed in nasopharyngeal carcinoma cell lines and nasopharyngeal carcinoma biopsies. For this purpose, we analyzed the expression of Bmi-1 in several human nasopharyngeal carcinoma cell lines using reverse transcription-PCR (RT-PCR) and Western blot analyses. A weak expression of Bmi-1 at the mRNA and protein level was detected in normal NPEC1 (Fig. 1C and D). However, all of the nasopharyngeal carcinoma cell lines showed overexpression of Bmi-1 at both mRNA (Fig. 1C) and protein levels compared with NPEC1 (Fig. 1D). C666 cell line, which contains EBV, showed the highest level of expression of Bmi-1. Another cell line (CNE2) also showed very high expression of Bmi-1 (Fig. 1C and D).

**Bmi-1 is overexpressed in nasopharyngeal carcinoma tissues and correlates with clinical outcome.** As we detected Bmi-1 overexpression in nasopharyngeal carcinoma cell lines, we were interested in investigating the status of Bmi-1 expression in nasopharyngeal carcinoma biopsies. By immunohistochemical analysis, 29 of 75 (38.7%) paraffin-embedded archival nasopharyngeal carcinoma biopsies showed moderate to strong nuclear staining of Bmi-1 in most of the tumor cells and in some scattered infiltrated lymphocytes. No positive nuclear staining of Bmi-1 was detected in the adjacent noncancerous epithelial cells and 11 noncancerous tissue biopsies (Fig. 2A-D).

We further analyzed the relationship between the expression of Bmi-1 and clinical characteristics of the patients (Table 1). There was no significant correlation between the expression of Bmi-1 and age, gender, N classification, M classification, or clinical staging of nasopharyngeal carcinoma patients. However, there was a significant difference of Bmi-1 expression in patients categorized according to T classification ($P = 0.024$). Test for the trend of odds showed that the expression of Bmi-1 positively correlates with T classification: higher T classification showed high Bmi-1 expression ($P = 0.0024$; Table 1). The 5-year survival rate, as assessed by the Kaplan-Meier method, was 84.2% in the Bmi-1-negative group, whereas it was only 47.6% in the Bmi-1-positive group (Fig. 2). There was a significantly higher 5-year survival rate in the Bmi-1-negative group than in the Bmi-1-positive group by log-rank test ($P = 0.019$) as well. The clinical stage, a comprehensive index reflecting T, N, and M classifications, is the most commonly used prognostic factor in the clinic. Therefore, the multivariate analysis, which included Bmi-1 expression, clinical stage, and the treatment, was done to determine if Bmi-1 is an independent prognostic factor of outcomes. The results showed that Bmi-1 and the clinical stage but not the treatment were independent prognostic indicators of survival (for Bmi-1: $P = 0.014$, for clinical stage: $P = 0.015$, for treatment: $P = 0.63$). Thus, our finding indicates that Bmi-1 protein expression has a significant correlation with prognosis of nasopharyngeal carcinoma (Fig. 2).

**Overexpression of Bmi-1 bypasses senescence and results in immortalization of NPECs.** The overexpression of Bmi-1 in

![Figure 1. Establishment of a NPEC cell line and the overexpression of Bmi-1 in nasopharyngeal carcinoma cell lines. NPEC, primary NPEC1; NPEC-Bmi-1, Bmi-1-overexpressing NPECs used as a positive control. SUNE-1, 5-8F, 6-10B, CNE-1, CNE-2, and C666 are nasopharyngeal carcinoma cell lines. A, primary culture of NPEC1 (the arrow shows a piece of biopsy). B, positive staining of cytokeratin in the NPEC1 cytoplasm. Magnification, ×400. C, RT-PCR analysis of Bmi-1 mRNA in NPEC1 and various nasopharyngeal carcinoma cell lines. β-Actin was used as an internal control. D, Western blot analysis of Bmi-1 in NPEC1 and various nasopharyngeal carcinoma cell lines. α-Tubulin was used as a loading control.
nasopharyngeal carcinoma cell lines and tumors suggests a possible role of it in the development of nasopharyngeal carcinoma. Bypass of senescence and immortalization are considered early steps in the tumor development. Moreover, Bmi-1 can bypass senescence and immortalize HMECs. Similar to HMECs, NPECs has a finite life span and do not spontaneously undergo immortalization. To test the possibility that Bmi-1 overexpression may also bypass senescence and cause immortalization of NPECs, we overexpressed Bmi-1 in these cells. The NPECs were infected at 10 population doublings with a retroviral vector expressing Bmi-1 or pMSCV control vector. After puromycin selection, overexpression of Bmi-1 was confirmed by Western blot analysis and immunostaining (Fig. 3A and B). As expected, NPECs infected with the control retrovirus proliferated for a limited number of passages (12 population doublings after selection) and then underwent senescence. In contrast, Bmi-1-overexpressing cells were capable of continuous proliferation over 150 population doublings (Fig. 3C) and thus seem to be immortal. Similar to HMECs overexpressing Bmi-1 (5), NPEC showed no extensive crisis period during Bmi-1-induced immortalization. Compared with NPEC, which showed typical epithelial cells of different sizes and shapes, the Bmi-1-immortalized culture at late passages mostly had uniform colonies with small cell size. Late-passage cells also grew in tight colonies (Fig. 3D). Similar results were obtained using a different NPEC cell line derived from an independent donor.

**Bmi-1 induces telomerase activity in NPECs.** As Bmi-1 induces telomerase activity in HMECs, next, we asked whether Bmi-1 overexpression leads to induction of telomerase activity in NPECs. We assessed telomerase activity in control and Bmi-1-overexpressing NPECs after puromycin selection (passages 3 and 20). Indeed, there was significant up-regulation of telomerase activity in Bmi-1-overexpressing cells. In contrast, control cells were devoid of any detectable telomerase activity under identical conditions (Fig. 4). We used extract from NP69, a SV40T-immortalized NPEC cell line, as a positive control. Because early-passage culture of Bmi-1-expressing cells exhibited significant telomerase activity, the ability of Bmi-1 to induce telomerase activity seems to be an early event.

**Bmi-1 down-regulates p16INK4a in NPECs.** Down-regulation of p16INK4a by overexpression of Bmi-1 is associated with the extension of replicative life span of human fibroblasts (20). Down-regulation of p16INK4a promotes hyperphosphorylation of pRb and thus suppresses its growth inhibitory function. Inactivation of pRb

<table>
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<th>Bmi-1+</th>
<th>Bmi-1−</th>
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<td>Age (y)</td>
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<td></td>
<td></td>
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<tr>
<td>≤45</td>
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<td>17 (36.2%)</td>
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<tr>
<td>&gt;45</td>
<td>16 (57.1%)</td>
<td>12 (42.9%)</td>
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<tr>
<td>Gender</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>22 (53.7%)</td>
<td>19 (46.3%)</td>
<td>0.714</td>
</tr>
<tr>
<td>Female</td>
<td>14 (58.3%)</td>
<td>10 (41.7%)</td>
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<tr>
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<tr>
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<td>16 (61.5%)</td>
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<tr>
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function by Bmi-1 results in extension of replicative life span in human fibroblasts (20). We therefore assessed expression of p16INK4a and the phosphorylation status of pRb in control and Bmi-1-overexpressing cells by Western blot analysis. The representative result of three independent experiments is shown in Fig. 4, which indicates that compared with control cells, p16INK4a is down-regulated in Bmi-1-overexpressing cells. As a consequence of p16INK4a down-regulation, increased hyperphosphorylation of pRb was observed in Bmi-1-overexpressing cells at passage 3 and passage 42 (Fig. 5A). In contrast to p16INK4a, the level of p53 and its target p21 were comparable in control and Bmi-1-overexpressing cells (Fig. 5A). Thus, in agreement with previous results, Bmi-1 seems to specifically target pRb pathway by down-regulating p16INK4a expression in NPECs. Bmi-1 is known to regulate p16INK4a through transcriptional repression in MEFs. Our RT-PCR data suggest that similar to MEFs, p16INK4a is transcriptionally down-regulated in Bmi-1-overexpressing NPECs (Fig. 5B).

**Bmi-1-immortalized NPECs maintain a normal DNA damage response.** Bmi-1-immortalized HMECs maintain a normal p53 checkpoint and undergo growth arrest in response to treatment with genotoxic agent, such as Adriamycin (5). We therefore determined if p53 is maintained in Bmi-1-immortalized NPECs. The control and Bmi-1-overexpressing NPECs were treated with Adriamycin for 24 hours. As expected, p53 and p21 were induced by Adriamycin treatment in both control and Bmi-1-overexpressing NPECs (Fig. 5D). In contrast, SV40T-immortalized NP69 cells did not show any p53 or p21 induction in response to Adriamycin treatment (data not shown). Cell cycle arrest was also observed in control as well as Bmi-1-overexpressing NPECs as determined by incorporation of [3H]thymidine after Adriamycin treatment.
Thus, DNA damage checkpoint remains intact in Bmi-1-immortalized NPECs.

**Analysis of Bmi-1-driven pathway in Bmi-1-immortalized NPECs.**

Recently, an 11-gene signature was described as a conserved Bmi-1-driven pathway, which defines stem cell-ness of highly invasive tumors of multiple tissue origin and correlates with therapy failure (19). We therefore asked whether the expression of these 11 genes was similarly regulated in Bmi-1-immortalized cells. We carried out RT PCR analysis to determine the expression pattern of these 11 genes in control and Bmi-1-immortalized cells. In agreement with the published report (19), BUB1, HEC1, and KI67 were found to be consistently up-regulated, whereas CES and ANK3 were consistently down-regulated in Bmi-1-immortalized cells (Fig. 6). However, there was no significant difference in the expression level of other six target genes (Gbx2, cyclin B1, KIAA1063, RNF2, HCFC1, and FGFR2) in Bmi-1-immortalized and control NPECs.

**Both RF and HT motifs of Bmi-1 are required for immortalization of NPECs.** To determine the specificity of immortalization function of Bmi-1 in NPECs, we overexpressed full-length Bmi-1 (WT), RF deletion, and ΔHT deletion mutants of Bmi-1 in another primary normal NPEC cell line NPEC2. Western blot analysis showed that the full-length and mutant Bmi-1 proteins were expressed as expected. Consistent with earlier results (5), p16INK4a down-regulation was only observed in cells expressing wild-type Bmi-1 (Fig. 7B). Also consistent with our previous results in human fibroblasts (20), ΔRF mutant expressing cells showed up-regulation of p16INK4a (Fig. 7B). The replicative life span of the control and Bmi-1 (wild type and mutants) overexpressing cells was determined. The result showed that only WT Bmi-1 was able to induce significant extension of the replicative life span and cause immortalization (Fig. 7A). Control as well as ΔRF and ΔHT mutants expressing NPECs underwent senescence and completely ceased growth in about 10 population doublings after selection. Thus, both RF and HT motifs of Bmi-1 are required for the immortalization of NPECs.

**Discussion**

Although PcG proteins function as the epigenetic gene silencers, increasing numbers of PcG proteins seem to play a key role in oncogenesis and regulation of the cell cycle (29). Recently, it has been shown that abnormal expression of PcG genes is associated with different type of human cancers (8, 30–33). For example, Bmi-1 is overexpressed in lymphomas (14), non-small cell lung cancer (15), B-cell non-Hodgkin’s lymphoma (16), breast cancer (17), and colorectal cancer (18). Most recently, Bmi-1 was showed to be a useful molecular marker for predicting occurrence of myelodysplastic syndrome and prognosis of the patients (34). The EZH2 polycomb transcriptional repressor also has been reported to be up-regulated in bladder carcinomas (35), prostate cancer (36), hepatocellular carcinoma (37), colorectal cancer (38), and breast cancer (39, 40). The products of the newly identified PcG gene products of the newly identified PcG gene...
(hPCL3) and its short form (hPCL3S) are overexpressed in colon, skin, lung, rectal, cervical, uterus, and liver cancers, and its expression is correlated with tumor progression (41). SU(Z)12, PcG member, is up-regulated in a number of different human tumors, including tumors of the colon, breast, and liver (42).

Our study suggests an important role for PcG proteins in the development of head and neck cancers. Here, we showed that Bmi-1 is overexpressed at both transcriptional and translational level in nasopharyngeal carcinoma cell lines. It is interesting to note that C666, the only well-known nasopharyngeal carcinoma cell line consistently carrying EBV has the highest Bmi-1 expression. Our primary data showed that LMP1, an EBV oncoprotein, induces Bmi-1 expression in nasopharyngeal carcinoma cells (data not shown). However, whether the EBV induces expression of Bmi-1, or whether high Bmi-1 level helps to maintain EBV in nasopharyngeal carcinoma cells needs to be further investigated. Importantly, overexpression of Bmi-1 protein was observed in 38.7% of nasopharyngeal carcinoma specimens, and the expression of Bmi-1 protein was found to correlate with the invasion of nasopharyngeal carcinoma primary tumor and the prognosis of nasopharyngeal carcinoma patients. Our results suggest that Bmi-1 is a valuable molecular marker of nasopharyngeal carcinoma progression. The pathologic diagnosis together with detection of Bmi-1 in tumor tissue could aid in evaluating new cases of nasopharyngeal carcinoma and designing optimal individualized treatment modalities.

Nasopharyngeal carcinoma is a disease with remarkable racial and geographic distribution. Over the years, numerous studies have revealed that multiple factors, such as virological, genetic, and environmental factors, are involved in the etiology of nasopharyngeal carcinoma. However, the molecular basis of nasopharyngeal carcinoma is far from being fully understood. The development and progression of nasopharyngeal carcinoma may involve the accumulation of multiple genetic alterations over a long period of time (1). To test this hypothesis and study the mechanism of nasopharyngeal carcinoma development, for the first time, we have established a NPEC cell line immortalized with a single cellular oncogene Bmi-1, which is overexpressed in nasopharyngeal carcinoma tumors and nasopharyngeal carcinoma cell lines, as our study suggest. This immortal NPEC cell line maintains a normal p53 checkpoint and is unlikely to have other undefined genetic lesions, which is always the case with tumor-derived cell lines and cells immortalized with viral oncopgenes. Bmi-1-immortalized NPEC cell line, thus, can be used to further study the mechanism of nasopharyngeal carcinoma development using defined genetic elements.

In NPECs, we have shown that Bmi-1 induces telomerase activity and represses p16^{INK4a} expression. To our knowledge, this is the first report where Bmi-1 has been shown to immortalize p16^{INK4a}-expressing cells. We further confirmed that the repression of p16^{INK4a} expression by Bmi-1 is at the transcriptional level. Thus, Bmi-1 is unlikely to target p16^{INK4a} degradation through ubiquitination pathway, although it contains a RF domain, which is commonly present in a subgroup of E3 ligases (43). Structural analysis of Bmi-1 shows that the NH2-terminal RF domain and the central HT domain of it are required for the immortalization of NPECs, which is consistent with our previous report that HT and RF domains of Bmi-1 are essential for telomerase induction and immortalization of HMECs (5). It remains possible that these two domains regulate different set of Bmi-1 target genes.

Apart from regulating p16^{INK4a} and telomerase, Bmi-1 may also promote immortalization by modulating the expression of other genes. Recently, by applying a mouse/human comparative translational genomics approach, a Bmi-1-driven 11-gene signature was identified. This cohort of 11 genes was suggested to be a magic
marker of stem-cell ness and therapy failure in patients with a variety of aggressive tumors. Five of the 11 genes from this cohort were significantly deregulated in Bmi-1-immortalized NPEC when compared with control cells. Among them, Bub1 is similar to the Aurora B/Plk1 kinase in having roles in both the checkpoint control and microtubule binding (44). HEC1 plays a important role in spindle checkpoint signaling and kinetochore recruitment of Mad1/Mad2 (45). It has been shown recently that RNA interference against HEC1 inhibits tumor growth in vivo (46), suggesting that HECH1 plays a role in maintaining malignant phenotype of cancer cells. Ki67 is a well-known molecular marker of the proliferation index of tumors (47, 48), and may not be involved in immortalization per se. The exact role of these Bmi-1 target genes in immortalization and nasopharyngeal carcinoma development remains to be investigated. Regardless of the role of the additional Bmi-1 target genes in NPEC immortalization, our results suggest that Bmi-1 plays an important role in the development and progression of nasopharyngeal carcinoma.

Acknowledgments

References

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