# Implication of Polycomb Members Bmi-1, Mel-18, and Hpc-2 in the Regulation of p16INK4a, p14ARF, h-TERT, and c-Myc Expression in Primary Breast Carcinomas

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**Abstract**

**Purpose:** Deregulation of mammalian Polycomb group (PcG) members may contribute to human carcinogenesis. p16INK4a and p14ARF tumor suppressors, human telomerase reverse transcriptase (h-TERT), and oncoprotein c-Myc have been implicated in the regulation of the cell cycle and proliferation mediated by PcG proteins, mainly Bmi-1, in mice and in cell culture experiments. Here, we examine whether these *in vitro* findings can be extrapolated to the *in vivo* situation.

**Experimental Design:** We measure the expression of PcG members *Bmi-1, Mel-18*, and *Hpc-2* and their potential targets by reverse transcription-PCR, immunostaining, and Western blotting in a series of 134 breast carcinomas and correlate the data with several clinical-pathologic variables of the tumors.

**Results:** Expression of PcG genes was variably detected, but overexpression of *Bmi-1* was the most frequent PcG alteration observed. In addition, statistical direct correlation in expression level of the three PcG members was detected. A correlation between *c-Myc* and *Bmi-1* expression levels was observed; however, there was no correlation between expression of *Bmi-1* and *p16INK4a, p14ARF*, or *h-TERT*. However, expression of the other PcG members *Mel-18* and *Hpc-2* correlated with the cell cycle regulators. Moreover, PcG mRNA – altered expression correlated significantly with certain clinical-pathologic variables associated with poor prognosis.

**Conclusions:** Our data suggest that the oncogenic role of Bmi-1 in human primary breast carcinomas is not determined by its capacity to inhibit INK4a/ARF proteins or to induce telomerase activity.

The Polycomb group (PcG) consists of several proteins that form multiprotein complexes, 2 to 5 MDa in size, that regulate gene activity at the chromatin level. In the 1980s, the first components of *Drosophila* PcG were identified and several years later were recognized in mammalian cells, indicating strong evolutionary conservation (1). However, unlike the ubiquitous expression of *Drosophila* genes, the expression levels of mammalian PcG genes differ between tissues and cell types (2). PcG proteins, together with the counteracting Trithorax group (TrxG) proteins, were initially identified as part of the memory system that ensures the faithful transmission of cell identities throughout cell division (3). PcG genes were discovered as epigenetic silencers during embryo morphogenesis with a central role in the nervous system, heart, and skeleton development (4 – 8). Additionally, PcG members have been implicated in the regulation of such adult processes as the cell cycle, X-inactivation, and hematopoiesis (9 – 15). PcG protein expression seems tightly regulated in normal cell proliferation and differentiation. However, PcG expression is often deregulated in several types of human cancer (16). Moreover, several PcG genes seem to regulate the self-renewal of specific stem cell types, suggesting a link between the maintenance of cell homeostasis and carcinogenesis (17, 18).

*Bmi-1* was initially identified as an oncogene that cooperated with *c-Myc* in the generation of mouse pre-B-cell lymphomas. It is also considered the first functional mammalian PcG proto-oncogene to be recognized, and it has been implicated in axial patterning, hematopoiesis, cell cycle regulation, and senescence (19 – 22). Data obtained in mice and *in vitro* studies have indicated that Bmi-1 protein regulates the *INK4a/ARF* locus, which encodes two unrelated tumor suppressors, p16INK4a and p19ARF (p14ARF in humans), which act in the two main cell cycle control pathways (pRb and p53, respectively).

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p16INK4a affects the retinoblastoma protein (pRb) by inhibiting the cyclin D/cyclin-dependent kinase 4/6 kinase complex. In the absence of Bmi-1, p16INK4a may be up-regulated and prevent binding of cyclin-dependent kinase 4/6 to cyclin D, thus inhibiting kinase activity. This fact results in hypophosphorylated pRb, which then binds E2F and inhibits E2F-mediated transcription of E2F target genes that are important for the $G_1$-S transition. Thus, hypophosphorylated pRb ultimately leads to cell cycle arrest, senescence, or apoptosis depending on context. In contrast, deregulation of p16INK4a by Bmi-1 involves pRb hyperphosphorylation, which allows cell cycle progression. The *INK4a/ARF* locus also encodes p19ARF, which sequesters the p53 inhibitor MDM2 and thereby prevents the degradation of p53, resulting in p53-mediated cell cycle arrest and apoptosis. Thus, Bmi-1 overexpression inhibits p16INK4a and p19ARF activity and prevents activation of the *INK4a/ARF* failsafe mechanism (23–25). In addition, other targets of PcG proteins have been reported. Enhanced Bmi-1 expression in immortal mammary epithelial cells (MEC) led to transcription of human telomerase reverse transcriptase (h-TERT) and induction of telomerase. The activation of h-TERT extended the replicative life span and immortalized MECs, suggesting regulation of telomerase expression by Bmi-1 in the development of human breast cancer (26).

Unlike *Bmi-1*, the closely related *Mel-18* was believed to have tumor-suppressive effects because Mel-18 inhibition enabled immortal NIH 3T3 to form tumors in nude mice (27). Subsequently, it was reported that Mel-18 regulates the cell cycle negatively through the Myc/cdc25 cascade (28). Human Hpc-2 was another PcG member identified as a repressor of *c-Myc* oncogene activity (29). Moreover, in contrast to Bmi-1, which induces S-phase entry by inhibiting Rb function via repression of the *INK4a/ARF* locus, Hpc-2 might arrest cells in $G_2$-M by co-operating with Rb-E2F in repressing cyclin A and Cdc2 (30). These data suggest that PcG complexes have opposing roles, and that the eventual effect on the cell cycle depend on the relative concentration of a certain PcG protein (2). Thus, combined with the differential cell type–specific expression, it is likely that the balance of Bmi-1 and other PcG proteins, like Hpc-2 or Mel-18, is critical to target gene specificity. PcG complexes containing predominantly Bmi-1 may cooperate with c-Myc oncogenic activity and promote tumorigenesis by inhibiting INK4a/ARF proteins and by activating h-TERT, whereas complexes with higher concentrations of Mel-18 or Hpc-2 may act upstream of c-Myc, inhibiting cell proliferation.

In human tumors, several reports have described alterations in PcG expression, mainly in such human hematologic malignancies as nodal B-cell lymphomas (31, 32), mantle cell lymphomas (33, 34), and Hodgkin's lymphomas (14, 35, 36). Previous reports showed that solid tumors, such as lung cancers (37), medulloblastomas (4), liver (38), penis (39), breast (40, 41), colon (42), and prostate carcinomas (43), also display disturbed PcG gene expression, and some authors correlated these expression patterns with poor prognosis.

In spite of the foregoing, most data on PcG members come from observations in established tumor cell lines or from studies in mice. Thus, it is important to examine the relevance of these findings in human cancer. This implies a more comprehensive study of PcG expression in human cancers, including the known targets and related proteins. Here, we examine the mRNA and protein expression of *Bmi-1*, *Mel-18*, and *Hpc-2* in a series of primary breast carcinomas. We also evaluate the correlations between PcG levels and the expression of the tumor suppressors *p16INK4a* and *p14ARF* and *h-TERT*. Expression of *c-Myc* was also analyzed to evaluate potential cooperation between *c-Myc* amplification and enhanced *Bmi-1* expression in the development of breast cancer. Furthermore, a correlation study between expression levels of all the analyzed genes and several clinical pathologic variables of the tumors was designed, with the objective of appraising the prognostic value of these expression patterns.

## Materials and Methods

***Patients, tissue sampling, nucleic acid extraction, and clinicalpathologic variables.*** A prospective study that started in 2001 allowed us to identify and recruit 134 patients with breast cancer. The study was approved by the Research Ethics Board of our hospital, and participants gave their informed consent. Normal tissues and tumor tissues were obtained sequentially, immediately after mastectomy. These samples were then snap-frozen in liquid nitrogen and stored at −80°C until processing. All specimens were examined pathologically to confirm the diagnosis of breast carcinoma, to establish the pathologic stage, and to select the areas with least contamination of normal cells so that all tumor samples contained at least 75% tumor cells. No evidence of disseminated disease was observed at diagnosis in any patient. RNA was extracted from about 30 mg of tumor and of normal samples from the same breast in the 134 patients by RNeasy Mini kit (Qiagen, Inc., Hilden, Germany) and was quantified spectrophotometrically.

The following variables were obtained from the medical records of the 134 patients: age, tumor size, lymph node metastases, presence of steroid receptors (estrogen and progesterone), pathologic stage, histologic grade, proliferation index, erbB2 expression, vascular invasion, and p53 immunoassaying status. Pathologic stage was assessed using the tumor-node-metastases classification. The steroid receptor content was determined by an immunohistochemical procedure. The proliferation index was calculated by the Ki-67 antigen (Immunotech, Westbrook, ME) in immunohistochemistry analyses. ErbB2 expression was evaluated by a monoclonal mouse antibody (CB11; Novocastra Lab. Ltd., Newcastle, United Kingdom). Immunohistochemistry of p53 was analyzed with the cl 1801 mouse monoclonal antibody (Oncogene Science, Manhasset, NY) based on its ability to detect up to 89% of p53 point mutations (44).

***Real-time PCR analysis.*** *Bmi-1*, *Mel-18*, *Hpc-2*, *p16INK4a*, *p14ARF*, *c-Myc*, and *h-TERT* expression levels were quantified in the tumor and in normal samples of the 134 breast cancer patients by real-time quantitative PCR. Ten of the 11 genes studied (six target and four housekeeping genes), except h-TERT, were detected in all tissue samples. mRNA levels were calculated in the tumor and normal tissues in a relative quantification approach, by which the amounts of the targets were expressed in relation to the geometric average of three reference housekeeping genes, TATA binding protein (*TBP*), succinate dehydrogenase complex subunit A (*SDHA*), and ubiquitin C (*UBC*), as a ratio target/geometric average of housekeeping gene in each sample. The relative concentration of the target and the reference genes was calculated by interpolation with a standard curve of each of the respective genes generated with a serial dilution of a cDNA from RNA extracted from normal tissue. We calculated the expression level of a target gene in a patient as the ratio: target in tumor tissue/target in normal tissue [$R(T/N)$]. A commercial kit (LightCycler *Telo*TAGGG hTERT, Roche Diagnostics, Mannheim, Germany) was used for the quantification of telomerase. This is a one-step reverse transcription-PCR kit for the quantitative detection of mRNA, encoding for human telomerase catalytic subunit h-TERT, and porphobilinogen deaminase

(PBGD) is processed for use as housekeeping gene. *h-TERT* was detected in less of 25% of the healthy samples but in 93% of the tumors. Thus, telomerase activity was measured in tumor samples as a ratio of the concentration of *h-TERT* (copies per μL) to the concentration of *PBGD* (copies per μL). Finally, these values were normalized by using $\log_{10}$.

For the synthesis of the first-strand cDNA, 400 ng of total RNA was retro-transcribed using the Gold RNA PCR Core kit (PE Applied Biosystems, Foster City, CA), following the manufacturer's instructions. Random hexamers were the primers used for cDNA synthesis. Reverse transcription-PCR was done in a LightCycler apparatus with the Light-Cycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics). Each reaction was done in a final volume of 20 μL containing 2 μL of the cDNA product sample, 3 mmol/L $MgCl_2$, 0.5 μmol/L of each primer, and 1× reaction mix, including FastStar DNA polymerase, reaction buffer, deoxynucleotide triphosphates, and SYBR green. Thermal cycling for all genes was initiated with a denaturation step of 95°C for 10 minutes followed by 30 to 35 cycles (denaturation at 94°C 2 seconds, specific annealing temperature for 5 seconds, and elongation at 72°C for 5 seconds, in which fluorescence was acquired). The primers and amplification conditions used are shown in Table 1. At the end of the PCR cycles, melting curve analyses and electrophoresis of the products on nondenaturing 8% polyacrylamide gels, together with a molecular weight marker, were run to validate the generation of the expected specific PCR product. The allelic band intensity on the gels was detected by non-radioisotopic means, using commercially available silver staining method (45). The bands were sequenced in an ABI Prism 377 DNA sequencer apparatus (PE Applied Biosystems).

***Immunohistochemistry assay.*** Immunophenotype analysis of 10 breast carcinomas showing differential mRNA expression of Bmi-1 was done according to standard procedures, with overnight incubation in the presence of BMI-1(c-20) polyclonal primary antibody raised against a peptide mapping near the COOH terminus of human Bmi-1 (sc8906, Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:10). Anti-goat biotin-SP-conjugated AffiniPure F(ab) fragment (705-066-147, Jackson ImmunoResearch Europe Ltd., Suffolk, United Kingdom) was used as secondary antibody. All immunostaining was done using the TechMate 500 (DAKO, Carpinteria, CA) automatic immunostaining device. Tonsil mantle cells were used as positive control. Positive cases exhibited cytoplasmic signal associated to nuclear staining, whereas negative cases exhibited an unspecific cytoplasmic staining. Bmi-1 protein was considered positive in tissue samples exhibiting nuclear staining in >20% of epithelial cells.

**Table 1.** Primer sequences for real-time quantification analysis

| Primer | Sequence | Annealing temperature (°C) |
|---|---|---|
| Target | | |
| BmiQ-F | GCTTCAAGATGGCCGCTTG | 65 |
| BmiQ-R | TTCTCGTTGTTCGATGCATTTC | |
| MelQ-F | AAAATCACAGAGCTGAACCCCC | 65 |
| MelQ-R | TTGTTGGTCTCCAGGTAGCGC | |
| HpcQ-F | AAATGGAGAGGCTGGTCGCC | 65 |
| HpcQ-R | TATCCCATCAGCTGCTCCTGCC | |
| P16Q-F | GGGTCGGGTAGAGGAGGTGC | 62 |
| P16Q-R | GCGCTGCCCATCATCATG | |
| P14Q-F | TCTAGGGCAGCAGCCGCTTCC | 68 |
| P14Q-R | GGCGCAGTTGGGCTCCGC | |
| MycQ-F | TGGATTTTTTTCGGGTAGTGG | 60 |
| MycQ-R | GTCGTAGTCGAGGTCATAGTTCC | |
| Housekeeping | | |
| TbpQ-F | TTCTGGGATTGTACCGCAGC | 59 |
| TbpQ-R | CCGCTTGGGATTATATTCGG | |
| SdhaQ-F | TGGGAACAAGAGGGCATCTG | 59 |
| SdhaQ-R | CCACCACTGCATCAAATTCATG | |
| UbcQ-F | ATTTGGGTCGCGGTTCTTG | 59 |
| UbcQ-R | TGCCTTGACATTCTCGATGGT | |

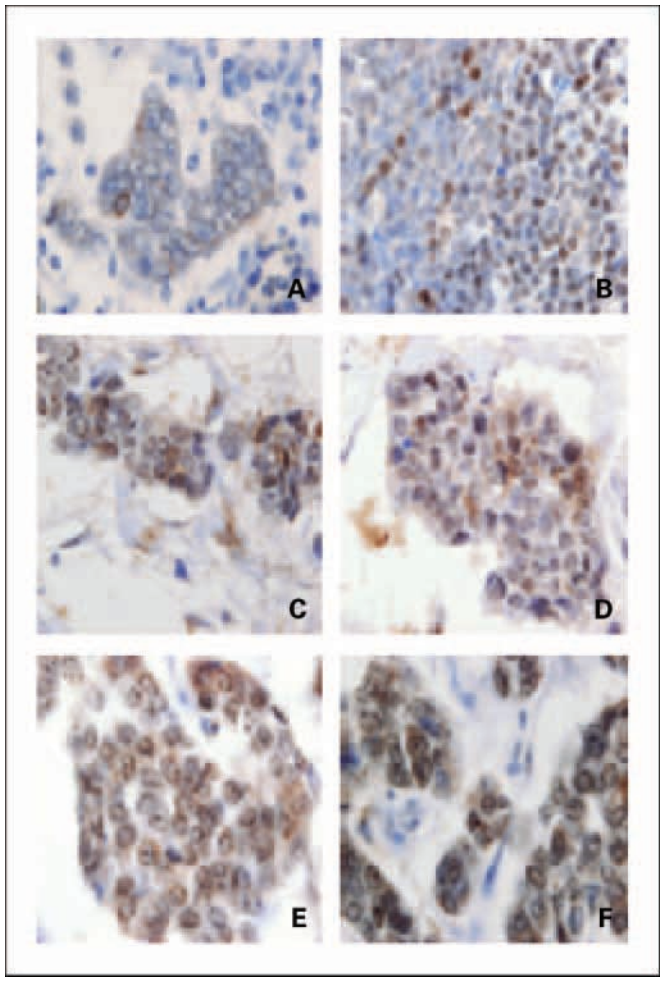



**Fig. 1.** Bmi-1 immunohistochemistry. Negative tumor case showing nonspecific cytoplasmic staining (*A*) and tonsil mantle cells used as positive control (*B*). Two positive examples from two different tumor cases: nonspecific cytoplasmic staining (*C* and *D*) and tonsil mantle cells (*E* and *F*).

***Western blotting assay.*** Mel-18 and Hpc-2 Western blottings were carried out in 10 breast carcinomas samples and normal counterparts. Total protein from frozen tumor and counterpart normal tissues was extracted by suspending in a lysing buffer [1% NP40, 25% glycerol, 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 5 mmol/L EDTA, 200 mmol/L β-glycerol, 0.5% sodium deoxycholate, 200 mmol/L Tris (pH 7.9)] containing a protease inhibitor mixture and homogenized in a potter. Lysates were centrifuged twice at 14,000 rpm for 15 minutes, and supernatants (30 μg protein) were loaded onto 10% SDS-PAGE gels, electrophoresed, and transferred to Optitran nitrocellulose membranes (Whatman). Membranes were immunoblotted with Hpc-2 (ab4189) and Mel-18 (ab5267) antibodies followed by washing and incubation with horseradish peroxidase–conjugated secondary antibodies. Antibody for β-actin (ab8227) was used as internal control. Proteins were visualized using Lumi-LightPLUS Western Blotting Substrate (Roche Diagnostic).

***Data analysis.*** The ratios of gene expression were not normally distributed (Kolmogorov-Smirnov test). The distribution was thus established by using $\log_{10}$, and geometric averages were compared. Correlation between expression levels was studied using the Pearson coefficient. Correlation between gene expression ratios and clinical

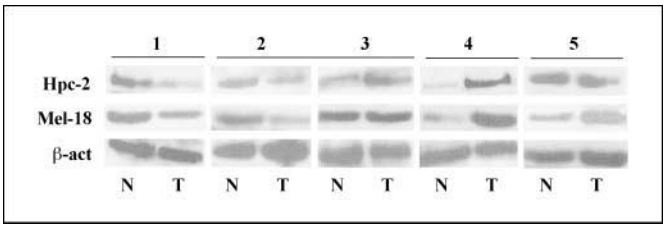



**Fig. 2.** Western blotting of Mel-18 and Hpc-2. Tumors 1 and 2, decreased protein expression of Mel-18 and Hpc-2. Tumor 3, overexpression of Hpc-2. Tumor 5, overexpression of Mel-18. Tumor 4, with both proteins overexpressed.

pathologic variables in the tissue samples was analyzed by the ANOVA test. Two-tailed *P*s ≤ 0.05 were considered statistically significant. Statistical analysis was done using version 11.0 of the SPSS package.

## Results

***mRNA quantitative expression versus protein analysis.*** To validate mRNA expression data, we did immunohistochemical and Western blotting analysis. Thus, 10 breast carcinomas were analyzed for Bmi-1 protein expression by immunohistochemistry using Bmi-1-specific antibody (Fig. 1). Bmi-1 immunoreactivity was observed in 50% of the breast cancer cases, and protein expression correlated with mRNA quantification in 7 of the 10 breast samples (70%). Furthermore, other 10 breast carcinomas were analyzed for Mel-18 and Hpc-2 protein expression by Western blotting assay using specific antibodies (Fig. 2). Mel-18 and Hpc-2 were variably detected in 100% of the breast cancer cases analyzed, and protein expression correlated with mRNA quantification in 8 of the 10 breast samples (80%). Together, the elevated frequencies of concordance between mRNA expression data and protein expression suggest the absence of dramatic posttranscriptional events affecting protein level.

***Analysis and correlation of PcG mRNA expression levels.*** Statistically significant direct correlations were observed between mRNA expression of several genes in our series of primary breast carcinomas (Table 2). The highest Pearson coefficients were obtained for the correlation between *Mel-18* and *Hpc-2* ($r$ = 0.599) and between *Bmi-1* and *Mel-18* ($r$ = 0.501). Expression of *Bmi-1* also correlated with *Hpc-2*, although the coefficient was lower ($r$ = 0.363).

***Analysis of correlation between* p16INK4a, p14ARF, *and PcG levels.*** The expected correlation between *Bmi-1* oncogene overexpression and down-regulation of *p16INK4* and *p14ARF* tumor suppressors was not found in our series ($r$ = 0.209 and $r$ = 0.016, respectively). However, there was a significant association between the other PcG members (*Mel-18* and *Hpc-2*) and the expression of both tumor suppressors. The highest correlations were between *Mel-18* and *p16INK4a* ($r$ = 0.437) and between *Hpc-2* and *p14ARF* ($r$ = 0.449). We had previously reported concomitant expression of *p16INK4a* and *p14ARF* in a different series, which indicated that these proteins were co-altered in primary breast tumors.

***Analysis of* h-TERT *activation by PcG genes.*** In our series human breast carcinomas, there was no evidence implicating *Bmi-1* in the regulation of telomerase expression. There was no correlation between telomerase activation and *Bmi-1* expression ($r$ = 0.037). Similarly, expression of *h-TERT* was not correlated with expression of the other PcG genes (*Mel-18* and *Hpc-2*: $r$ = 0.077 and $r$ = 0.004, respectively).

***Analysis of the PcG genes and* c-Myc *cooperation.*** A weak correlation was found between *Bmi-1* and *c-Myc*, but the Pearson coefficient was lower ($r$ = 0.236) than for the other associations found. Stronger associations were observed between concomitant expression of *c-Myc* and the other PcG

**Table 2.** Correlation among *Bmi-1, Mel-18, Hpc-2, p16INK4a, p14ARF, h-TERT*, and *c-Myc* expression in the 134 primary breast carcinomas

| | $Log_{10}$ *Bmi-1* | $Log_{10}$ *Mel-18* | $Log_{10}$ *Hpc-2* | $Log_{10}$ *p16INK4a* | $Log_{10}$ *p14ARF* | $Log_{10}$ *h-TERT* | $Log_{10}$ *c-Myc* |
|---|---|---|---|---|---|---|---|
| $Log_{10}$ *Bmi-1* | | | | | | | |
| Pearson correlation | | 0.501* | 0.363* | 0.209 | 0.016 | 0.037 | 0.236* |
| Significance (bilateral) | | 0 | 0 | 0.015 | 0.856 | 0.707 | 0.006 |
| $Log_{10}$ *Mel-18* | | | | | | | |
| Pearson correlation | | | 0.599* | 0.437* | 0.303* | 0.077 | 0.370* |
| Significance (bilateral) | | | 0 | 0 | 0 | 0.426 | 0 |
| $Log_{10}$ *Hpc-2* | | | | | | | |
| Pearson correlation | | | | 0.364* | 0.449* | 0.004 | 0.332* |
| Significance (bilateral) | | | | 0 | 0 | 0.969 | 0 |
| $Log_{10}$ *p16INK4A* | | | | | | | |
| Pearson correlation | | | | | 0.482* | 0.146 | 0.427* |
| Significance (bilateral) | | | | | 0 | 0.141 | 0 |
| $Log_{10}$ *p14ARF* | | | | | | | |
| Pearson correlation | | | | | | 0.134 | 0.355* |
| Significance (bilateral) | | | | | | 0.166 | 0 |
| $Log_{10}$ *h-TERT* | | | | | | | |
| Pearson correlation | | | | | | | 0.107 |
| Significance (bilateral) | | | | | | | 0.273 |
| $Log_{10}$ *c-Myc* | | | | | | | |
| Pearson correlation | | | | | | | |
| Significance (bilateral) | | | | | | | |

*Statistically significant correlation at 0.01 level (bilateral).

**Table 3.** Frequencies of altered expression of *Bmi-1, Mel-18*, and *Hpc-2* (4-fold expression analysis) in the 134 primary breast carcinomas

| Gene | Decreased expression | | Normal expression | | Overexpression | |
|---|---|---|---|---|---|---|
| | Frequency | Percentage | Frequency | Percentage | Frequency | Percentage |
| *Bmi-1* | 21 | 15.7 | 85 | 63.4 | 28 | 20.9 |
| *Mel-18* | 10 | 7.5 | 106 | 79.1 | 18 | 13.4 |
| *Hpc-2* | 21 | 15.7 | 95 | 70.9 | 18 | 13.4 |

members (*Mel-18* and *Hpc-2*: $r = 0.370$ and $r = 0.332$, respectively). Similarly, altered expression of *c-Myc* correlated with altered expression of the tumor suppressors genes (*p16INK4a* and *p14ARF*: $r = 0.427$ and $r = 0.355$, respectively).

***Frequencies of altered expression of PcG members.*** To improve the statistical study, we included additional data analysis methods to check mRNA expression level of PcG genes. Thus, altered expression of *Bmi-1*, *Mel-18*, and *Hpc2* was arbitrarily considered in tumor tissues when expression showed a 4-fold increase or decrease with respect to normal counterpart samples. We found altered expression of the three genes, but the strongest tendency was towards overexpression of *Bmi-1* (20.9%) in comparison with the other genes analyzed (*Mel-18* and *Hpc-2*). Increased *Bmi-1* expression was similar to that shown in previous reports (Table 3).

***Correlation between mRNA expression and clinical-pathologic variables.*** Several of the analyzed variables showed significant associations with the mRNA expression levels (Table 4; Fig. 3). Thus, tumors with high expression of *Bmi-1* correlated with negative p53 immunostaining and with positive progesterone receptors. Elevated values of expression of both *p14ARF* and *c-Myc* correlated with tumors with more than three lymph node metastases. Finally, positive Ki-67 proliferation index correlated with high levels of *h-TERT*. Moreover, we analyzed the possible association between the clinical-pathologic variables and different combinations of concomitant expression levels, but resulting subgroups of these different combinations did not contain sufficient samples to ensure statistically significant correlations. Nevertheless, we observed a trend between concomitant high expression of *Mel-18*/*Hpc-2* or *Mel-18*/*Bmi-1* and positive peripheral vascular invasion (data not shown). Tumors with concomitant expression of *c-Myc* and *Bmi-1* were not correlated with variables of poor prognosis (data not shown).

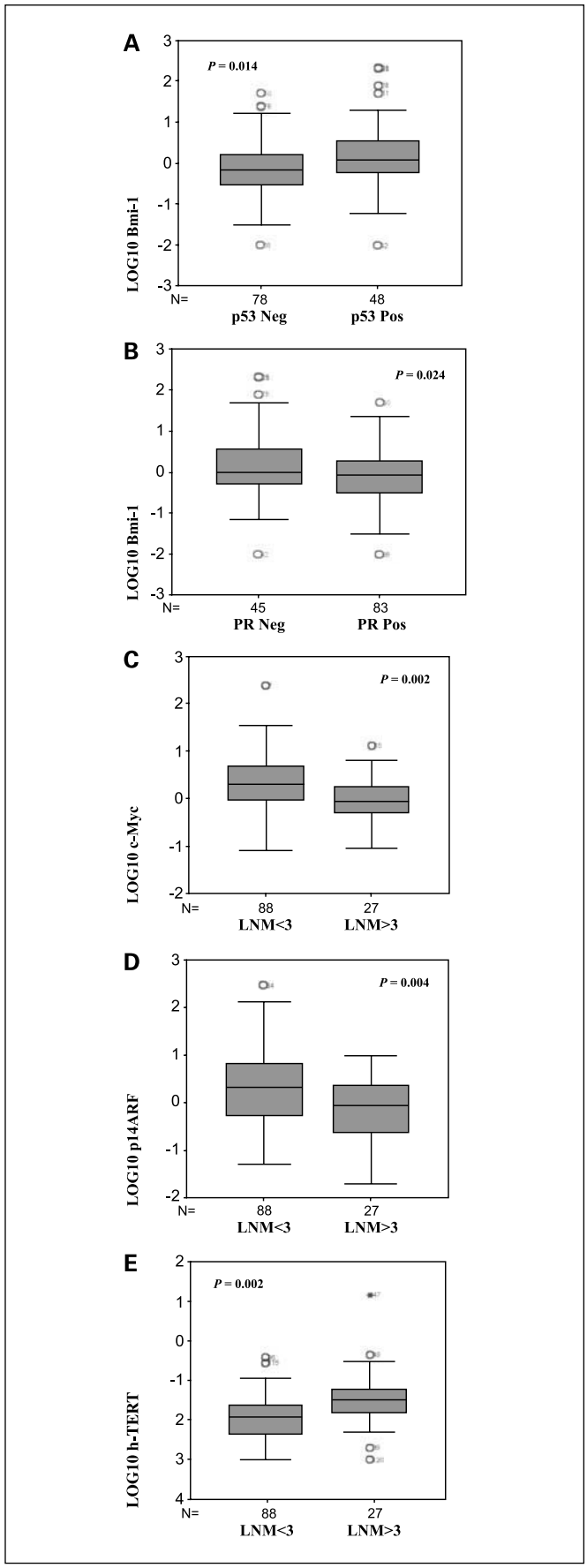
## Discussion

PcG proteins modulate expression of key developmental regulators, such as homeotic genes, but they have also been implicated in the regulation of cell proliferation and therefore in tumorigenesis. The data obtained in established tumor cell lines or in mice suggest that Bmi-1 expression contributes to the development of tumors, and that the oncogenic activity proceeds from its capacity to down-regulate INK4a/ARF suppressor proteins, desensitizing cells to apoptosis and facilitating cell proliferation. However, the effect of Bmi-1 overexpression on the inactivation of the *INK4a*/*ARF* transcripts in human tumorigenesis is unclear. In the present study, we examined the mRNA levels of *Bmi-1*, *p16INK4a*, and *p14ARF* genes to assess the status of this pathway in primary breast tumors. Initially, we did two different protein expression assays to check potential posttranscriptional mechanisms that may affect protein levels of Bmi-1 and of the other two PcG

**Table 4.** Correlations between expression levels and clinical-pathologic variables

| Variable | Bmi-1 | | | Mel-18 | | | Hpc-2 | | | p14ARF | | | c-Myc | | | h-TERT | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *n* | GA | *P* | *n* | GA | *P* | *n* | GA | *P* | *n* | GA | *P* | *n* | GA | *P* | *n* | GA | *P* |
| ER | | | | | | | | | | | | | | | | | | |
| Negative | 39 | 0.715 | 0.067 | | | NS | 39 | 0.753 | 0.064 | | | NS | | | NS | | | NS |
| Positive | 89 | 1.349 | | | | | 89 | 1.219 | | | | | | | | | | |
| PR | | | | | | | | | | | | | | | | | | |
| Negative | 45 | 0.683 | 0.024 | 45 | 0.951 | 0.072 | | | NS | | | NS | | | NS | | | NS |
| Positive | 83 | 1.447 | | 83 | 1.390 | | | | | | | | | | | | | |
| p53 | | | | | | | | | | | | | | | | | | |
| Negative | 78 | 1.477 | 0.014 | | | NS | | | NS | | | NS | | | NS | | | NS |
| Positive | 48 | 0.656 | | | | | | | | | | | | | | | | |
| LNM | | | | | | | | | | | | | | | | | | |
| <3 | | | NS | | | NS | | | NS | 88 | 0.490 | 0.004 | 88 | 0.494 | 0.002 | | | NS |
| >3 | | | | | | | | | | 27 | 1.470 | | 27 | 1.108 | | | | |
| Ki-67 | | | | | | | | | | | | | | | | | | |
| Negative | | | NS | | | NS | | | NS | | | NS | | | NS | 39 | 0.011 | 0.002 |
| Positive | | | | | | | | | | | | | | | | 42 | 0.032 | |
| Size (cm) | | | | | | | | | | | | | | | | | | |
| <2 | | | NS | | | NS | 58 | 1.310 | 0.074 | | | NS | | | NS | | | NS |
| >2 | | | | | | | 74 | 0.859 | | | | | | | | | | |

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; LNM, lymph node metastases; GA, geometrical average; NS, not significant.




members (Mel-18 and Hpc-2). Although high mRNA expression levels of *Bmi-1* were frequently observed in our series of tumors (fact that is consistent with an oncogenic role for Bmi-1), they did not correlated with down-regulated *p16INK4a* or *p14ARF* expression, similarly to previous reports. Therefore, it seems that Bmi-1 may contribute to breast carcinogenesis but not by repressing p16INK4a or p14ARF proteins. In any case, this lack of correlation can not be interpreted as lack of regulation or relation between the proteins so that it may be possible that other inactivation mechanisms or other proteins regulating the *INK4a/ARF* locus can contribute to mask such regulation.

Previous studies reported that Bmi-1 induces telomerase activity and immortalizes human mammary epithelial cells (26). Thus, it has been suggested that activation of h-TERT by Bmi-1 may play a role in the development of human breast cancer. Expression of *h-TERT* in our series of primary breast carcinomas was extensive (93%), but it was generally absent in the normal tissue counterparts. However, telomerase levels in the tumors were not significantly correlated with high levels of Bmi-1 expression. Therefore, it seems that the oncogenic activity of Bmi-1 in human breast carcinogenesis cannot be explained by the induction of telomerase expression.

It has been proposed that PcG complexes containing predominantly Bmi-1 may cooperate with c-Myc oncogenic activity and promote tumorigenesis (2). Thus, expression of *c-Myc* was also analyzed in our series to evaluate the potential collaboration between *c-Myc* amplification and enhanced *Bmi-1* expression in the development of breast cancer. Although a correlation between *c-Myc* and *Bmi-1* expression levels was observed, tumors showing elevated expression of both genes were not associated to a worse prognosis.

Our results suggest that INK4a/ARF transcripts and PcG proteins Mel-18 and Hpc-2 are co-expressed. Furthermore, because *Mel-18* and *Hpc-2* are believed to be tumor suppressor genes, these PcG proteins may be regulated in the same way as other protective genes, like *p16INK4a* and *p14ARF*, in the tumor-suppressive cell response. In this respect, the induction of high levels of several tumor suppressors may then be a compensatory, albeit ineffective, cellular response to oncogenic stimuli, which would inhibit the oncogenic pathway. Thus, elevated expression of the *c-Myc* oncogene correlated with increased expression of these four tumor suppressor genes, which is consistent with our suggestion of multiple antitumor responses. Nevertheless, although these data are mainly descriptive, and although additional studies are required to clarify the mechanistic insights into the regulatory features, we suggest a new insight into the relation between PcG genes and cell proliferation regulators.

Bmi-1, Mel-18, and Hpc-2 are members of the same Polycomb repressive complex (PRC1), which is involved in the maintenance of a stable pattern of gene suppression, and their co-expression has been reported (35, 40). Nevertheless, several non-PcG proteins may contribute to the silencing

**Fig. 3.** Association of Bmi-1 expression levels and clinical-pathologic features. Logarithm of Bmi-1 expression and p53 immunohistochemistry (*A*), logarithm of Bmi-1 expression and progesterone receptor immunohistochemistry (*B*), logarithm of c-Myc expression and lymph node metastases (*C*), logarithm of p14ARF expression and lymph node metastases (*D*), and logarithm of h-TERT expression and Ki-67 index (*E*).

activity of PcG complex, such as the transcription modulators CtBP, E2F6, nuclear factor-κB, KyoT2, RYBP, AF9, SSX, Cited2, and the mitogen-activated protein kinase–activated kinase 3pK (36, 46–53). Thus, in our primary breast carcinomas, Bmi-1, Mel-18, and Hpc-2 may be activated or repressed by some of these common regulating proteins. The concomitant expression of p16INK4a and p14ARF indicates that they were co-altered in primary breast tumors, as we previously reported (54).

Significant associations were found between mRNA levels of several genes and certain clinical-pathologic variables of the tumors. Increased expression of *Bmi-1* correlated with positive progesterone receptors. A trend was also observed between high levels of *Bmi-1* with positive estrogen receptors and between increased expression of *Mel-18* and *Hpc-2* with positive steroid receptors. The correlation between Bmi-1 and steroid receptors has recently been reported by others (41), but such a correlation for Mel-18 and Hpc-2 is new. This finding indicates that Polycomb genes may be appropriate markers to select cancer patients for treatment with hormonal therapy. A trend was observed between low expression levels of *Hpc-2* and high tumor size, which supports the tumor suppressor role proposed for *Hpc-2*. In addition, high expression of *Bmi-1* correlated with positive p53 immunostaining (p53 mutated). It was hypothesized that alterations in genes that act on the same pathway are functionally equivalent; therefore, these alterations may not be present in the same tumor. Furthermore, evidence supporting direct biochemical interactions between *INK4a/ARF* genes and p53 has been reported. If Bmi-1 protein contributes to cancer progression by repression of INK4a/ARF proteins, then concomitant p53 mutation may be an equivalent alteration and these two events might exclude each other. However, down-regulation of INK4a/ARF transcripts by Bmi-1 was not detected in our series of breast carcinomas. Thus, our data indicate that oncogenic activity of Bmi-1 is independent of the ARF/p53 pathway. In this respect, recent screenings of human cells using small interfering RNA constructs showed that Bmi-1 does not allow circumvention of the p53 pathway (55). We also found a significant correlation between tumors with decreased *p14ARF* or *c-Myc* expression and low number of lymph node metastases. The association is consistent with the oncogenic activity of *c-Myc*. Furthermore, *p14ARF* is known to be activated by c-Myc, and this fact may explain the correlation of this tumor suppressor gene and axillary lymph node metastasis.

In conclusion, our results suggest that *Bmi-1* and its binding partners *Mel-18* and *Hpc-2* are coordinately deregulated. Interestingly, we observed a correlation between expression levels of *Mel-18* and *Hpc-2* with expression of *p16INK4a* and *p14ARF*. In contrast to the previous data observed *in vivo* or *in vitro*, *p16INK4a* and *p14ARF* expression were not down-regulated by *Bmi-1* in our series of tumors, although other regulatory mechanisms may influence *INK4a/ARF* status. Likewise, telomerase levels were not altered by expression of *Bmi-1*. Thus, in human breast carcinogenesis, the role of Bmi-1 inhibiting INK4a/ARF proteins or inducting telomerase activity is not clear. Alternatively, these regulating mechanisms could play a transient role in the transformation process, which may not be evident in later tumor stages.

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