Overexpression of the Bmi-1 Oncoprotein correlates with Axillary Lymph Node Metastases in Invasive Ductal Breast Cancer

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Purpose: The modulation of Bmi-1 is observed in several tumor tissues, with its heightened protein level suspected of being involved in tumorigenesis by acting as a transcriptional repressor in the INK4a/ARF locus. To elucidate the role of Bmi-1 in invasive ductal breast cancers, the expression of Bmi-1 at the mRNA and protein levels were examined.

Methods: Breast carcinoma samples were obtained from patients who underwent routine surgery for breast cancer at the Department of Surgery, Chonbuk National University Hospital, in 2000-2002. Cancerous breast and paired normal breast tissues were taken from a site distant from the tumorous lesion, and analyzed with reverse transcription-polymerase chain reaction (RT-PCR) and and immunohistochemical assay. We analyzed the correlations between the expression of Bmi-1 and various clinicopathological factors, such as age, lymph node metastases, estrogen receptor (ER), and progesterone receptor (PR), in invasive ductal carcinomas of the breast.

Results: The Bmi-1 mRNA level by RT-PCR was shown to be significantly up-regulated in 19 of the 22 breast carcinoma tissues specimen compared with the non-neoplastic tissues adjusted to tested specimens. The immunohistochemical staining for Bmi-1 also showed high a level of expression in 44 of the 71 invasive ductal breast cancers (62%), and was more intense in the invading fronts than in the central portions of the primary invasive breast cancers. Univariate and multivariate analyses showed that a high level of Bmi-1 expression was significantly correlated with axillary lymph node metastases and a positive estrogen receptor status.

Conclusion: The Bmi-1 was differentially expressed in human breast carcinomas. These findings suggested that Bmi-1 might be involved in the progression of invasive ductal breast cancer, and it may be clinically useful in selecting patients who could benefit from adjuvant chemotherapy.

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Key Words Bmi-1, lymph node metastases, breast carcinoma
중심 단어 Bmi-1, 림프절 전이, 유방암

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INTRODUCTION

Breast carcinoma is one of the most common malignancies affecting women worldwide. The pathogenic mechanism underlying development and progression of breast carcinoma is still largely unknown. The Bmi-1 putative oncogene belonging to the mammalian Polycomb-Group (PcG) family form multimeric gene-repressing complexes involved in axial patterning, hematopoiesis, cell cycle regulation, and senescence.*(1-3)* It was first identified as an oncogene that cooperates with c-myc in the generation of mouse pre B-cell lymphomas.*(4,5)* It has been reported that Bmi-1 contributes to cell cycle regulation by acting as a stable transcriptional repressor of the INK4a/ARF (= human p19ARF) locus.*(6,7)* Inactivation of p16INK4a-pRb pathway and p14ARF-MDM2-p53 pathway by bmi-1 deregulation has been clearly implicated to lymphomagenesis*(8-10)* and oncogenesis in non-small cell lung cancer of human.*(11)* Recently, it has been shown that Bmi-1 overexpression leads to activation of human telomerase reverse transcriptase transcription and induction of telomerase activity in immortalized mammary epithelial cells.*(12)* However, there is little available information about the expression of Bmi-1 in a series of human breast carcinoma.

Herein, we determined the associations between the expression of Bmi-1 and the various clinicopathologic factors such as age, lymph node metastases, estrogen receptor (ER), and progesterone receptor (PR), in invasive ductal carcinoma of the breast.

MATERIALS AND METHODS

(1) Patients and specimens

The breast carcinoma samples were obtained from patients who underwent routine surgery for breast cancer at the Department of Surgery, Chonbuk National University Hospital in 2000 - 2002. Patients included in the study had an axillary dissection that sampled at least five lymph nodes. The breast cancerous and paired normal breast tissue taken from a site distant from the tumorous lesion were snap frozen and stored in liquid nitrogen till further use. For the immunohistochemical study, some of these tissue specimens were fixed in 10% neutralized buffered formalin solution for 24 hours. Each patient's clinical status was classified according to the pathological grade of the tumor size, lymph node, metastasis (pTNM) classification system.*(13)*

(2) Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from 22 breast carcinoma tissues and non-tumorous tissues of the patients using acid guanidinum thiocyanate-phenol-chloroform (AGPC) method.*(14)* Reverse transcription reactions were done with cDNA synthesis kit (Stratagene, La Jolla, CA, USA) following the instruction manual. cDNA was synthesized with 7㎍ of total RNA and oligo (dT) primer in 50 ㎕ of a solution containing reverse transcriptase. The reverse-transcribed samples were used for amplifying 0.95 kb fragment of Bmi-1 and glyceraldehyde-3-phosphate-dehydrogenase (G3APDH), which was used as an internal quantitative control. For the PCR reactions the following primers were used: sense primer of Bmi-1 5´-AGCAGAAATGCATCGAACAA-3´ and antisense primer 5´-CCTAACCAGATGAAGTTGCTGA-3´. With respect to G3APDH, the primer sequences were as follows: sense primer 5´-CCCCTGGCCAAGG TCATCCATGACAACTTT-3´ and antisense primer 5´-GGCCATGAGGTCCACCACC CTG TTGCTGTA-3´. The PCR reactions were performed following the cycling parameters on a MinicyclerTM PCR system (MJ Research, Inc.): 10min at 94 °C followed by 25 cycles of 1 min at 94°C, 1 min at 55 °C, and 1 min at 72 °C, and a final cycle at 72 °C for 10min. Quantitation of the PCR products were scanned and performed using a Quantity One program (Bio-Rad, Hercules, CA, USA). Increased expression of the Bmi-1 mRNA in tumor tissue

was defined as more than about 2 times higher than that seen in normal breast tissue.

(3) Immunohistochemical analysis

Commercially available goat polyclonal antibody against Bmi-1 (1 : 50, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal antibodies against p53 DO-7 (1 : 100, Dako, Glostrup, Denmark), ER (1 : 50, Dako), PR (1 : 10, Da ko), and c-erbB-2 (1 : 10, Dako) were used as primary antibodies. A paraffin section of the breast carcinoma tissue was deparaffinized and rehydrated in graded alcohol. Antigenic enhancement was performed by submerging in 1x citrate buffer (pH 6.0) and microwaving. The sections were then treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity, followed by incubation with 1% BSA to block the non-specific binding, respectively. The primary polyclonal anti-Bmi-1 antibody was incubated for 90 min at room temperature. After washing, the tissue section was then reacted with the biotinylated anti-goat secondary antibody, followed by incubation with streptavidin-horseradish-peroxidase complex. The tissue section was immersed in 3-amino-9-ethyl carbazole as a substrate. In negative controls, the primary antibody was replaced by non-immune goat IgG of the same isotype or antibody dilution solution.

Each section was evaluated by at least two independent observers, and moderate to strong nuclear staining was considered as a positive reaction. The distribution of Bmi-1 was scored on a semiquantitative scale, as follows: negative (< 10% of tumor positive), focally positive (10 - 50% of tumor positive) and diffusely positive (> 50% of tumor positive). For ER a nuclear staining in > 25% of the cells was considered ER positive, and similarly for PR. A nuclear staining in >10% of the cells was considered positive for p53, whereas with c-erbB-2, was scored positive when > 25% of the cells were stained.

(4) Statistical analysis

The relationship between the results of the immunohistochemical study and the clinicopathologic parameters was performed using the SAS® software package (version 8.01 ; SAS Institute, Cary, NC, USA). Univariate and multivariate analyses were carried out using the proc logistic module. In all cases, the exact mid-P adjusted P values were reported and a p value < 0.05 was considered to be statistically significant.

RESULTS

(1) Bmi-1 expression in invasive ductal breast cancer

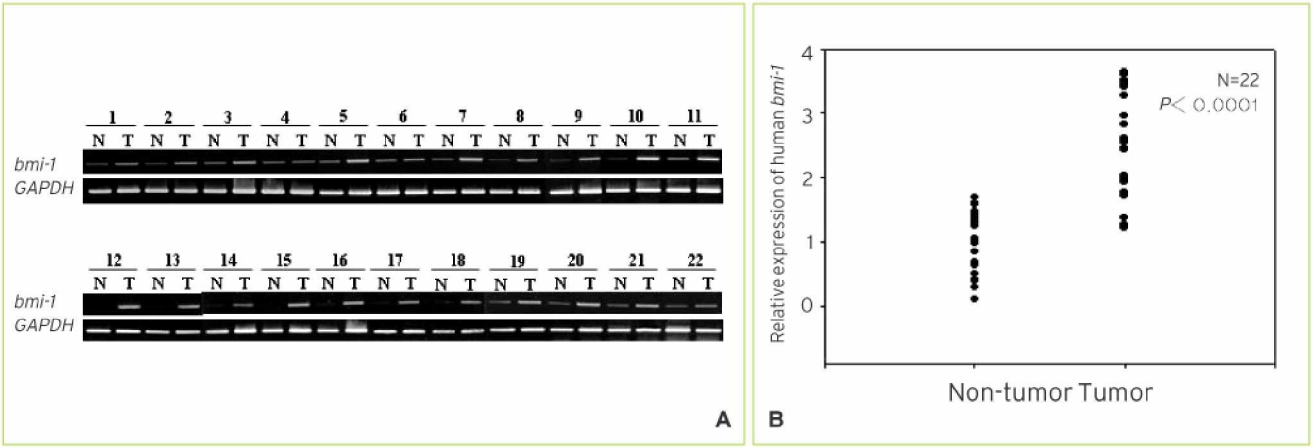

Fig 1. The bmi-1 gene expression in human breast carcinoma. (A) Expression of the bmi-1 and G3APDH in 22 patients with invasive ductal breast cancer, determined by RT-PCR. Data were expressed relative to the expression of G3APDH gene in each breast carcinoma. (B) The relative band density shows high expression level on breast carcinoma tissues.

The relative levels of expression of the Bmi-1 in 22 breast carcinoma tissues were compared with those of non-tumorous tissues by RT-PCR. The expression levels were determined as a ratio between the Bmi-1 and the reference gene (G3APDH) to correct variation in the amounts of mRNA. The Bmi-1 mRNA level was significantly increased ($p < 0.0001$) in all human breast carcinomas when compared with that of its corresponding normal breast tissue (Figs 1A and 1B). The results of this examination are presented in Table 1. Concerning immunohistochemistry, the Bmi-1 immunostaining of tumor cells showed a positive expression in 44 (62%) cases of the 71 patients with breast cancer. The normal breast tissue shows negative or weak Bmi-1 staining in the breast epithelial cells, lymphocytes, and fibroblasts. The expression level of Bmi-1 was significantly increased in breast carcinomas compared with that in the adjacent normal breast tissue and Bmi-1 was largely distributed in the nuclear areas of tumor cells (Figs 2A and B). In 30 (68%) cases of the Bmi-1expressed 44 cases, Bmi-1 staining was more intensive in the invading fronts than in central portions of primary invasive breast cancers (Fig 2C). The expression patterns of Bmi-1 were negative, focally and diffusely positive in 27 (38%), 21 (30%) and 23 (32%) cases, respectively.

(2) Relationship between Bmi-1 expression and clinicopathological parameters in invasive ductal breast cancer

We investigated the status of Bmi-1 and various clinicopathological parameters in this series of invasive ductal breast cancers to show the association with Bmi-1 expression. The results are summarized in Table 1 to 2. With an univariate analysis, there was significant correlation between the Bmi-1 positivity and clinicopathological parameters; axillary lymph node metastases and positive ER status, respectively (Table 1). Our multivariate analysis using data from the whole parameters (Table 2) confirmed that high expression of Bmi-1 strongly correlated with axillary lymph node metastases and positive ER status. These results suggested the possibility that Bmi-1 might play a role in the progression and metastases of invasive ductal breast cancers.

DISCUSSION

The breast cancer has been increasing steadily and many patients has been suffered from this malignancy during the last several decade years. Available clinical prognostic indicators are not accurate: despite axillary nodal status being the most important factor that determines overall survival in patients with breast cancer, approximately 25 - 30% of node-negative cases eventually relapse.*(15)* Thus, it is important to identify a biological genetic molecular marker that is associated with pathophysiologic processes of human breast cancer.

Bmi-1 identified as a cell cycle-regulatory factor negatively regulates inhibitors of cyclin-dependent

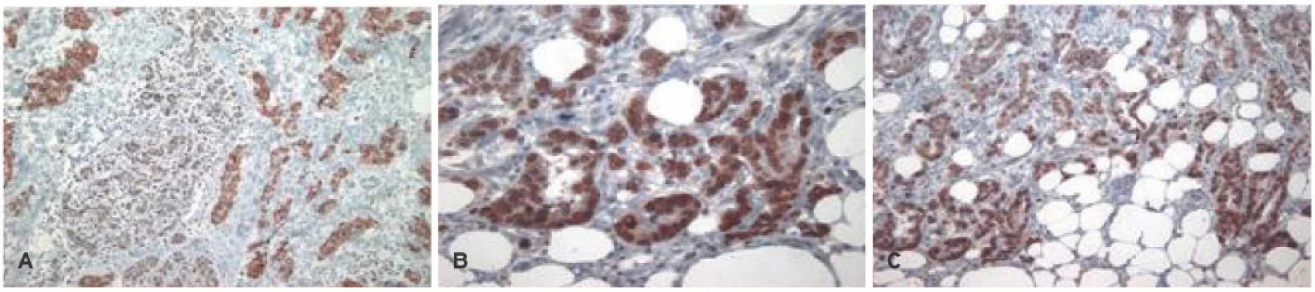

Fig 2. Immunohistochemical staining of human breast carcinoma specimen using antibody to Bmi-1. (A) Bmi-1 expression is increased on the tumor cells of breast carcinoma tissue when compared with those of its adjacent normal breast (immunoperoxidase stain; original magnification X200). (B) Note the distinct nuclear staining in breast carcinoma cells (immunoperoxidase stain; original magnification X400). (C) More intense Bmi-1 staining in the invading fronts than in central portions of primary invasive breast cancers (immunoperoxidase stain; original magnification X400).

kinase - p16INK4a and p14ARF.*(6,7)* The INK4a/ARF locus is a critical downstream target of the Bmi-1 transcriptional repressor activity involved in cell cycle regulation of a number of malignant tumors.*(8-11)* There is little available information about the expression of Bmi-1 and clinicopathologic correlation in a series of primary breast cancer. In the present study, we found that the Bmi-1 mRNA expression was significantly higher in invasive ductal breast cancers than in paired adjacent normal breast tissue. Immunohistochmical analyses showed that the proportion of Bmi-1 expression in breast carcinoma was 61% at protein levels. Interestingly, Bmi-1 staining was more intense in the invading fronts than in central portions of primary invasive breast cancers. Therefore, it appears that high level of Bmi-1 protein may contribute to breast tumorigenesis, presumbly via modulation of intact p16INK4a/p14ARF.

With univariate and multivariate analyses, increased expression levels of Bmi-1 showed significant relationships with axillary lymph node metastases and positive ER status, but did not correlate with the other histopathological parameters in the primary breast cancers. Indeed, axillary lymph node status is currently one of the most significant prognostic factors for patients with breast cancer.*(16,17)* This finding suggests that Bmi-1 may be one of the novel independent prognostic markers available in invasive ductal breast cancer. However, further investigation is needed to reveal the clinicopathological importance of Bmi-1 in various human breast cancers.

Bmi-1 expression has been examined in a variety of B-cell non-Hodgkin lymphomas and non-small cell lung cancer of the human.*(8-11)* The extent of Bmi-1 expression correlated with clinical grade and the presence of Mib1/Ki-67 expression in B-cell non-Hodgkin lymphomas.*(8)* A recent study was noted that Bmi-1 gene expression is differentially regulated in the lymphomagenesis of a subset of B-cell lymphomas and Bmi-1 gene alterations might occur in tumors with wild-type INK4a/ARF locus.*(10)* In our study, Bmi-1 overexpression was far more frequent in p53-negative tumors, suggesting that inactivation of wild-type p14ARF-MDM2-p53 pathway by Bmi-1 deregulation may partly contribute to carcinogenesis of breast cancer. Rather surprisingly, Bmi-1 overexpression signifi-

Table 1. Relationship between Bmi-1 expression and clinicopathological parameters.

Variables	Negative (%)	Lower Positive(%)	Higher Positive(%)	*p*-value
Age (years)				0.3498
< 50	17 (24)	10 (14)	10 (14)	
≥ 50	10 (14)	11 (15)	13 (18)	
Histologic grade (HG)				0.2352
HG I	6 (8)	6 (8)	6 (8)	
HG II	19 (27)	10 (14)	16 (23)	
HG III	2 (3)	5 (7)	1 (1)	
Tumor size (cm)				0.2010
2 > in diameter	16 (23)	7 (10)	7 (10)	
2-5 in diameter	10 (14)	11 (15)	14 (20)	
> 5 in diameter	1 (1)	3 (4)	2 (3)	
Lymph node metastasis				0.0295
N_0	18 (25)	7 (10)	8 (11)	
N_1	9 (13)	14 (20)	15 (21)	
Estrogen receptor				0.0084
Negative	15 (21)	8 (11)	3 (4)	
Positive	12 (17)	13 (18)	20 (28)	
Progesteron receptor				0.4610
Negative	15 (21)	8 (11)	10 (14)	
Positive	12 (17)	13 (18)	13 (18)	
c-erb-B2				0.0517
Negative	18 (25)	10 (14)	19 (27)	
Positive	9 (13)	11(15)	4 (6)	
P 53				0.2624
Negative	19 (27)	18 (25)	20 (28)	
Positive	8 (11)	3 (4)	3 (4)	

Table 2. Results of multivariate logistic regression analysis with Bmi-1 expression.

Categories	*p*-value	Odds ratio	95% confidence limits
Estrogen receptor	0.0020	7.365	2.490---21.781
Nodal status	0.0174	1.874	0.663---5.293
Age	0.0600	2.466	0.938---6.482
c-erbB-2	0.0766	0.428	0.153---1.200
Tumor size	0.1122	2.033	0.895---4.617
p53	0.2305	0.434	0.126---1.497

cantly correlated with positive ER status, although the reasons for this result are not clearly established. It is expected that immunohistochemical detection of Bmi-1 protein may be clinically a useful guide for selecting appropriate treatment in the case of ER positive invasive ductal breast cancer.

CONCLUSION

We describe Bmi-1 is differentially expressed in human breast carcinomas. We report a significant increase in the expression of Bmi-1 mRNA and in protein levels in human breast carcinoma. Additionally, a correlation was found between the Bmi-1 expression and clinicopathologic profiles including lymph node metastases and positive ER status. The present study suggests that cell cycle deregulation by the Bmi-1 gene alteration might play a role in the progression and lymph node metastases of breast carcinoma.

REFERENCES

1 Haupt Y, Bath ML, Harris AW, Adams JM. Bmi-1 transgene induces lymphomas and collaborates with myc in tumorigenesis. Oncogene 1993;8:3161-4.

2 Jacobs JJ, Scheijen B, Voncken JW, Kieboom K, Berns A, van Lohuizen M. Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc- induced apoptosis via INK4a/ARF. Genes Dev 1999;13:2678-90.

3 Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature 1999;397:164-8.

4 Haupt Y, Alexander WS, Barri G, Klinken SP, Adams JM. Novel zinc finger gene implicated as myc collaborator by retrovirally accelerated lymphomagenesis in E mu-myc transgenic mice. Cell 1991;65:753-63.

5 van Lohuizen M, Frasch M, Wientjens E, Berns A. Sequence similarity between the mammalian bmi-1 proto-oncogene and the Drosophila regulatory genes Psc and Su(z)2. Nature 1991;353:353-5.

6 Jacobs JJ, van Lohuizen M. Cellular memory of transcriptional states by Polycomb-group proteins. Semin Cell Dev Biol. 1999;10:227-35

7 Zhang Y, Xiong Y, Yarbrough WG. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell 1998;92:725-34.

8 van Kemenade FJ, Raaphorst FM, Blokzijl T, Fieret E, Hamer KM, Satijn DP,et.al. Coexpression of BMI-1 and EZH2 polycomb-group proteins is associated with cycling cells and degree of malignancy in B-cell non-Hodgkin lymphoma. Blood 2001;97:3896-901.

9 Lindstrom MS, Klangby U, Wiman KG. P14ARF homozygous deletion or MDM2 overexpression in Burkitt lymphoma lines carrying wild type p53. Oncogene 2001;20:2171-7.

10 Bea S, Tort F, Pinyol M, Puig X, Hernandez L, Hernandez S, et.al. BMI-1 gene amplification and overexpression in hematological malignancies occur mainly in mantle cell lymphomas. Cancer Res 2001;61:2409-12.

11 Vonlanthen S, Heighway J, Altermatt HJ, Gugger M, Kappeler A, Borner MM, et.al. The bmi-1 oncoprotein is differentially expressed in non-small cell lung cancer and correlates with INK4A-ARF locus expression. Br J Cancer 2001;84:1372-6.

12 Dimri GP, Martinez JL, Jacobs JJ, Keblusek P, Itahana K, van Lohuizen M, et.al. The bmi-1 oncogene induces telomerase activity and immortalizes human mammary epithelial cells. Cancer Res 2002;62:4736-45.

13 Wittekind C, Compton CC, Greene FL, Sobin LH. TNM residual tumor classification revisited. Cancer 2002;94:2511-6.

14 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-9.

15 McGuckin MA, Cummings MC, Walsh MD, Hohn BG, Bennett IC, Wright RG. Occult axillary node metastases in breast cancer: their detection and prognostic significance. Br J Cancer 1996;73:88-95.

16 Carter CL, Allen C, Henson DE. Relation of tumor side, lymph node status, and survival in 24,740 breast cancer cases. Cancer 1989;63:181-7.

17 Haagensen CD. Diseases of the Breast. 3rd ed. Philadelphia: WB Saunders; 1986.