Diagnostic importance of overexpression of Bmi-1 mRNA in early breast cancers

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Abstract. Target molecules for a highly sensitive and specific diagnosis of breast cancer in its early clinical stages have not yet been identified. Here, we show the first evidence for diagnostic performance of the molecule B cell-specific Moloney murine leukemia virus integration site 1 (Bmi-1) in breast cancer patients. Only 5 out of 46 non-cancerous samples were positive for Bmi-1 mRNA expression resulting in a sensitivity and specificity of 72.0 and 91.3%, respectively. The mRNA expression was estimated using the cut-off value obtained from the receiver operating characteristic curve analysis. Further, Bmi-1 mRNA expression was found to be elevated in 97.8% (45/46) of cancerous tissues in comparison to the expression in paired cancerous tissues and non-cancerous tissues obtained from identical patients. Bmi-1 mRNA was found to be highly expressed even in the early clinical stages of breast cancer. Our results suggest that Bmi-1 mRNA might be a new tool to support the diagnosis of breast cancers, irrespective of the clinical stage.

Introduction

Identifying candidate genes that would facilitate the detection of tumors, even in the early stages, is of utmost importance for improving the efficacy of breast cancer therapy. Measuring highly amplified gene expression of such molecules would be useful to support the diagnosis, especially when very few cells can be obtained and malignancy can not be definitive by histological or cytological diagnosis. *Bmi-1*, an oncogene (1), was originally reported to transform mouse B cells in collaboration with *c-myc* (2,3). Recently, it was also reported that *Bmi-1* along with *H-ras* can transform human mammary epithelial cells (4). A previous study clarified the mechanism via which *Bmi-1* transforms non-cancerous cells; this study reported that *Bmi-1* overexpression in human mammary epithelial cells results in the activation of human telomerase reverse transcriptase and the induction of telomerase activity (5). In addition, *Bmi-1* inhibition by RNAi results in the upregulation of the *INK4a/ARF* gene, which in turn causes acute cell death and growth inhibition in several cancer cell lines but not in normal cell lines (6). This suggests that *Bmi-1* inhibits INK4a/ARF function, thereby resulting in uncontrollable progression of the cell cycle from the G1 to the S phase via the activation of cyclin-dependent kinases 2, 4 and 6. These enzymes phosphorylate Rb protein, a key molecule that leads to the activation of E2F. These observations indicate that *Bmi-1* plays an important role in the proliferation of human breast cancer cells. Thus, it is speculated that *Bmi-1* is highly expressed in breast cancer cells and might serve as a novel marker for the genetic diagnosis of early-stage breast cancer. In this regard, previous studies have described the expression of *Bmi-1* in breast cancer patients (7,8). However, the sensitivity and specificity of *Bmi-1* as a marker for the detection of breast cancer remains undetermined. Therefore, in this study, we quantitatively analysed Bmi-1 mRNA expression in non-cancerous tissues and cancerous tissues of breast cancer patients and studied its efficacy as a diagnostic tool for breast cancer.

Materials and Methods

Patients and frozen tissue samples. Cancerous (50) and non-cancerous (46) tissue specimens were obtained from breast cancer patients following surgery. After informed consent was obtained, samples were dissected from these tissue specimens and immediately frozen. The tissue samples were stained with hematoxylin/eosin and reviewed by experienced pathologists. Clinicopathological factors and clinical stages were evaluated according to the criteria of the Japanese Society of Breast Cancer, which are based on the tumor-node-metastasis (TNM) staging system.

Quantitative reverse transcription-polymerase chain reaction for Bmi-1 mRNA. The RNA from culture cells or sample tissues

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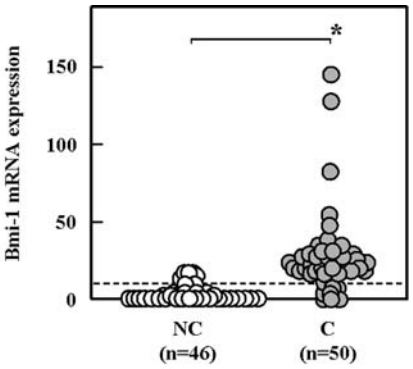

Figure 1. Bmi-1 mRNA expression in breast cancer and non-cancerous tissues. Bmi-1 mRNA expression was measured using quantitative RT-PCR and is reported relative to the amount of 18S rRNA. The dotted line shows the mean + 2 SD of Bmi-1 mRNA expression levels in non-cancerous tissues. NC, non-cancerous tissues; C, cancerous tissues. Statistical analysis was performed using Mann-Whitney rank sum test. *p<0.0001.

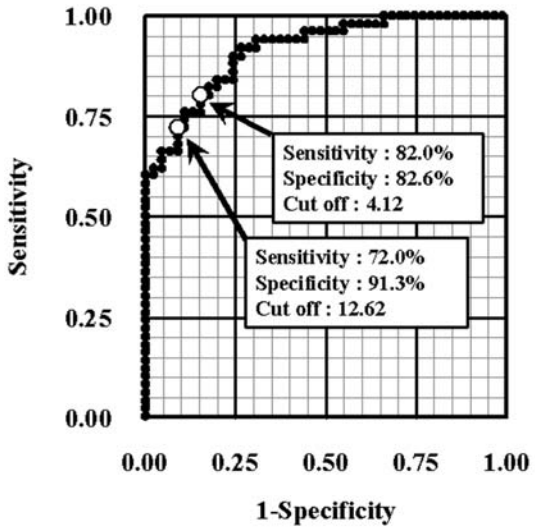

Figure 2. ROC curve for the detection of breast cancer. The curve was automatically generated from 96 points representing cut-off values determined by the software StatFlex, version 5.0.

was extracted using the Isogen reagent (Nippon Gene, Toyama, Japan), according to the manufacturer's protocol. The extracted RNA was assayed using the GeneQuant DNA/RNA calculator (Amersham Pharmacia Biotech, Uppsala, Sweden). The expression of target mRNA and 18S ribosomal RNA (rRNA) was determined by quantitative reverse transcription-polymerase chain reaction (RT-PCR) with the TaqMan Core Reagent Kit (Perkin-Elmer Applied Biosystems, Foster City, CA) by using an ABI PRISM 7700 sequence detection system (Perkin-Elmer Applied Biosystems). The gene-specific primers and fluorescent hybridization probes for *Bmi-1* used in the quantitative RT-PCR were as follows: forward primer, 5'-CCC ACC TGA TGT GTG TGC TT-3'; reverse primer, 5'-ACC TTA TAT TCA GTA GTG GTC TGG TCT TG-3'; and probe, 5'-(6-carboxyfluorescein; FAM) TGG AGG GTA CTT CAT TGA TGC CAC AAC C (6-carboxy-tetramethyl-rhodamine; TAMRA)-3'. The quantities of specific mRNA in each sample were normalized as a ratio to the quantity of 18S rRNA in order to compare the amounts of Bmi-1 mRNA among the samples. The quantity of 18S rRNA in each sample was determined using TaqMan Ribosomal RNA Control Reagents (Perkin-Elmer Applied Biosystems), according to the manufacturer's protocol. T47D cells were used as the positive control. Standard curves were generated on the basis of the linear relationship between the first cycle number at which the fluorescence signal significantly increased (Ct value) and the logarithm of the starting quantity (9,10). The quantity of target mRNA in the test samples was determined from the Ct value by using the standard curve. A negative control, which did not contain the template, was included in each experiment.

Immunohistochemistry. Immunohistochemical studies were carried out using formalin-fixed, paraffin-embedded tissue samples. The samples were immunostained using standard procedures with rabbit anti-human Bmi-1 polyclonal antibodies (H-99; SantaCruz Biotechnology, Inc., CA) at a 1:100 dilution for the detection of avidin-biotin-enhanced immunoperoxidase. Non-specific staining was examined by the same procedure without using the first antibody.

Statistical analysis. Unpaired Student's t-test (Welsh's t-test) was used to evaluate the differences in the expression level of Bmi-1 mRNA. Statistical significance was defined as $p<0.05$. A receiver operating characteristics (ROC) curve was generated, and the sensitivity/specificity at various cut-off values were calculated using the StatFlex software version 5.0 (Artech, Osaka, Japan) (11). This software automatically arranged all the data manually entered for the 2 groups (non-cancerous and cancerous tissues) according to the amount. The software then calculated the sensitivity and specificity at each cut-off value programmed for each set of values and automatically generated a ROC curve.

Results

RT-PCR was performed on 46 non-cancerous tissue samples and 50 cancerous tissue samples obtained from patients with breast cancer (Fig. 1). The mean Bmi-1 mRNA expression in cancerous tissues (24.70±27.67) was remarkably higher than that in non-cancerous tissues (2.84±4.80) ($p<0.0001$). The cut-off value for expression of Bmi-1 mRNA was set as the mean + 2 SD of its expression in non-cancerous breast tissues. Bmi-1 mRNA expression was found to be positive in 74.0% of the cancerous tissues (37 out of 50; Fig. 1). The sensitivity and specificity of Bmi-1 mRNA detection by using the ROC curve was 72.0 and 91.3%, respectively, at a cut-off value close to the mean + 2 SD of mRNA expression in non-cancerous breast tissues (Fig. 2). Higher sensitivity (82.0%) and lower specificity (82.6%) were obtained at the cut-off value at which the difference between the sensitivity and specificity was minimal (4.12; Fig. 2). Further, Bmi-1 mRNA expression was elevated in 97.8% (45/46) of cancer specimens that were obtained along with adjacent non-cancerous tissue samples (Fig. 3). We also confirmed that the Bmi-1 protein expression is higher in cancerous tissues in which mRNA was highly expressed (Fig. 3). High protein expression resulted in strong nuclear staining, while the ratio

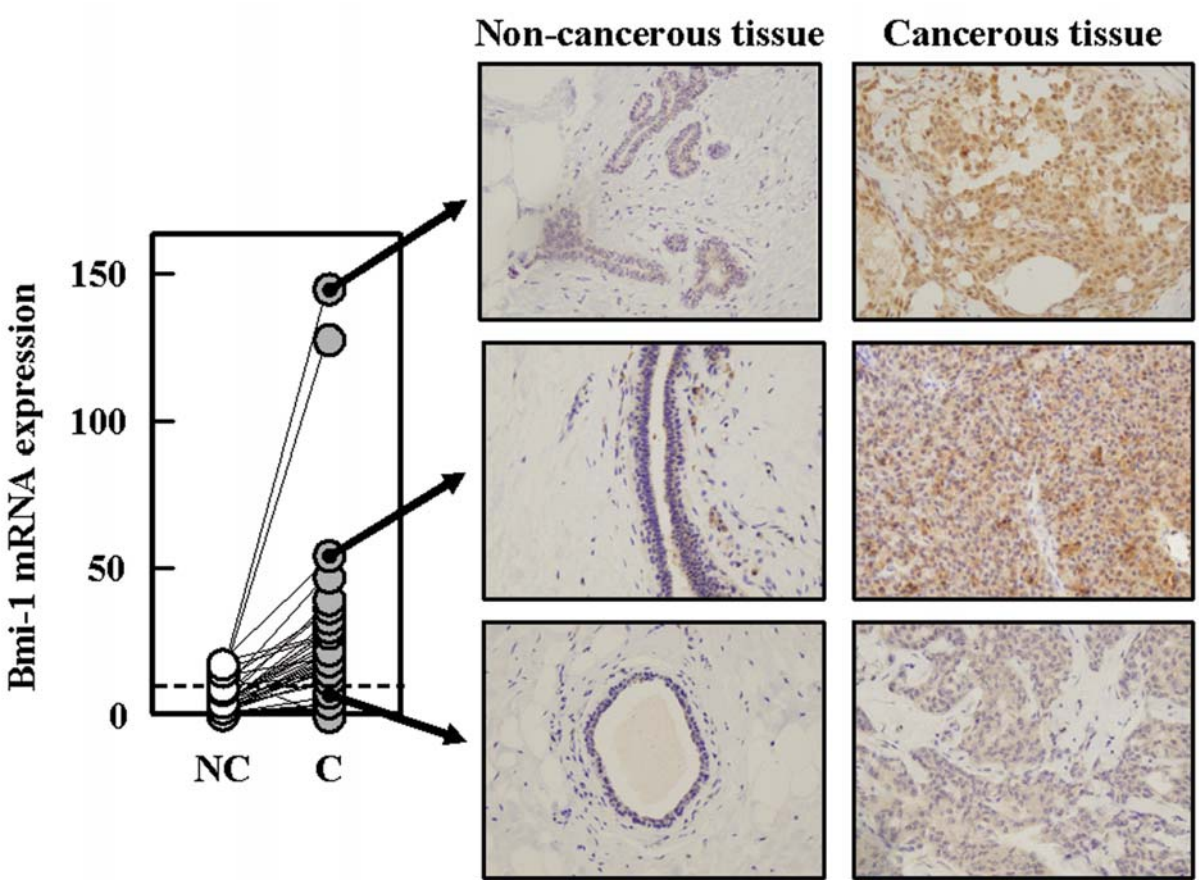

Figure 3. Comparison of the expression levels of Bmi-1 mRNA and protein between cancerous tissues and paired non-cancerous tissues. Bmi-1 mRNA expression is reported relative to the amount of 18S rRNA. The solid line indicates paired samples. The dotted line shows the mean + 2 SD of Bmi-1 mRNA expression levels in non-cancerous tissues. All images of immunohistochemical analysis illustrate sections stained with polyclonal antibody against Bmi-1. (Magnification, x200).

Table I. Relationship between various clinicopathological factors and Bmi-1 mRNA expression.

Background	Mean ± SD	p-value
Tissue type		
Papillotubular (n=27)	30.83±34.71	
Schirrhous (n=14)	18.09±10.85	0.089
Others (n=7)	15.02±19.80	N.C.[a]
Menopausal status		
Premenopausal (n=13)	29.12±33.70	
Postmenopausal (n=35)	23.21±26.31	0.575
Lymph node metastasis		
Negative (n=25)	32.50±36.26	
Positive (n=23)	16.45±11.38	0.044
Estrogen receptor		
Negative (n=24)	17.46±11.99	
Positive (n=23)	33.27±37.48	0.064
Progesterone receptor		
Negative (n=25)	21.44±17.09	
Positive (n=22)	29.74±37.21	0.345

[a]N.C., could not be calculated because of small sample numbers.

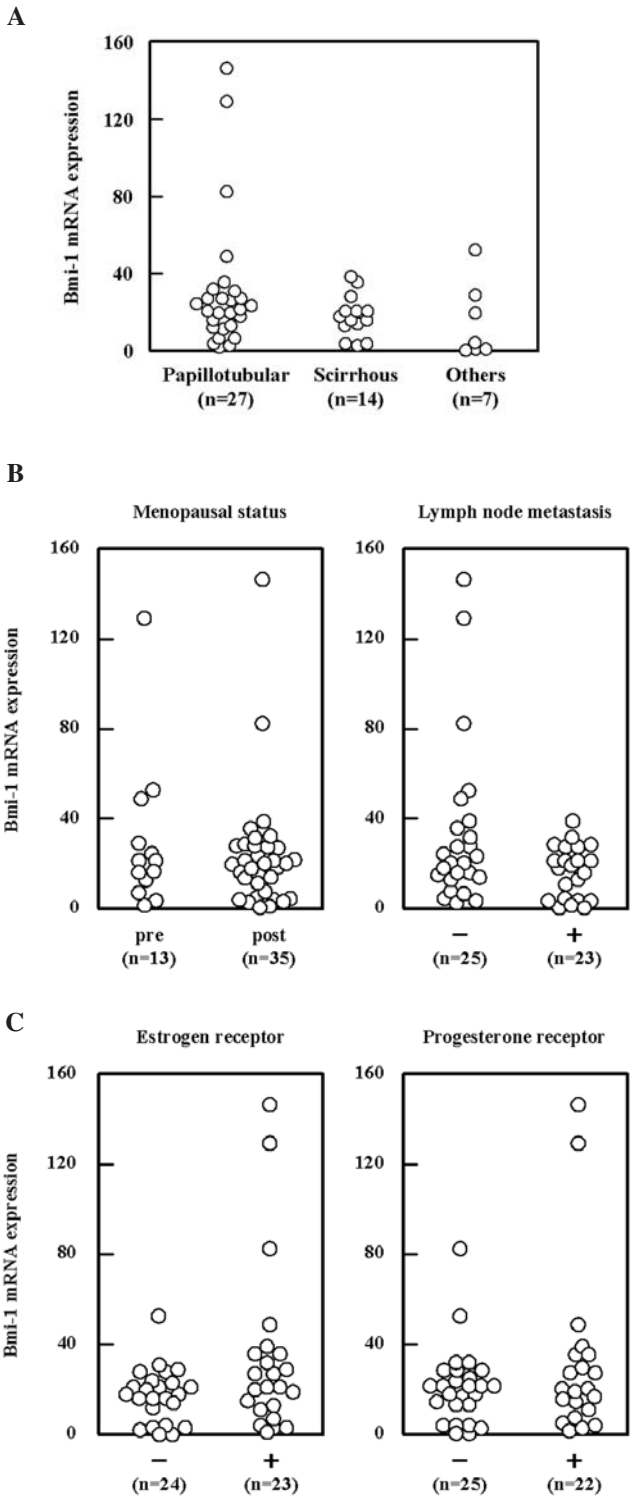

Figure 4A-C. Relationship between Bmi-1 mRNA expression levels and clinicopathological factors. Bmi-1 mRNA was measured using quantitative RT-PCR and expressed relative to the amount of 18S rRNA.

of the staining intensity in the cytosol to that in the nucleus was reversed in the case of low protein expression.

Furthermore, we analyzed the relationships between clinicopathological factors and Bmi-1 mRNA expression in cancerous tissues (Table I). No statistically significant correlations of Bmi-1 mRNA expression were found with tissue type, menopausal status and presence of estrogen or progesterone receptors. Interestingly, in the samples obtained

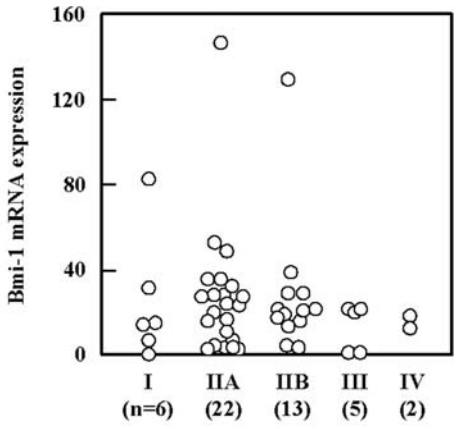

Figure 5. Bmi-1 mRNA expression in each clinical stage of breast cancer. Bmi-1 mRNA was measured using quantitative RT-PCR and expressed relative to the amount of 18S rRNA.

from patients without lymph node metastasis, statistically higher mRNA expression ($p<0.05$) was observed. Moreover, samples of papillotubular carcinoma and those cases positive for estrogen receptors showed higher expression of Bmi-1 mRNA. However, this tendency was due to measured values in top 3 cases showing remarkably higher expression (Fig. 4A-C). Analysis of the clinical stages of breast cancer, as determined by tumor size, invasion and metastasis to lymph nodes and distant organs, showed that the mean value and the range of SD in stage I and IIA cancers (26.34±29.64) were almost equal to those in other clinical stages (stages IIB, III and IV; 22.66±26.78; $p>0.05$) (Fig. 5). Overall, high *Bmi-1* expression levels were observed in the relatively early stages (stage I and II) of breast cancer.

Discussion

This study investigated whether *Bmi-1* could be used as a marker for the diagnosis of breast cancer. Many studies have analyzed the expression levels of certain oncogenes that were considered to be candidate markers for cancer diagnosis, but the clinical utility of these genes was limited because expression level in non-cancerous tissues was not negligible. In this study, we showed that the expression of oncogene *Bmi-1* was elevated in the majority of the breast cancer samples compared to the non-cancerous samples. Recently, using quantitative real-time PCR, Silva *et al* showed the levels of Bmi-1 mRNA expression were elevated in 20.9% of tissue samples from breast cancer patients and that these levels were 4 times higher than those in the non-cancerous samples; however, graphical representation of the data was not provided (7). We observed elevated expression with a 4-fold increase in 80.4% (37/46) of breast cancer samples (overall plot in Fig. 1, actual value for each sample not shown). The discrepancy between our results and those of Silva *et al* could be attributable to the differences in primer design, the PCR system (TaqMan RT-PCR vs. Light Cycler system) and the study population (Japanese vs. Spanish). With regard to primer design, the primer set (spanning exons 1 and 2) used in the study by Silva *et al* can detect 2 out of 7 major splicing variants of the *Bmi-1* gene (the information for variants was

obtained from the website of GeneCards®), while the primer set (spanning exons 2-4) used in our study can detect 6 variants of the *Bmi-1* gene. Therefore, the difference in primer design is considered the main reason for the differences in the over-expression of the *Bmi-1* gene in cancers. Nonetheless, the difference in the study populations is also an important factor.

Among the paired samples, an apparent decrease of mRNA expression up to undetectable levels was observed in 1 tissue sample of lobular carcinoma. The exact cause of this observation is unclear; however, our study included only 1 case of lobular carcinoma, and more cases should be examined to explain this result.

In ROC analysis, the cut-off value that was associated with a minimum difference between sensitivity and specificity was 4.12. An important finding of our study is that Bmi-1 mRNA is expressed even in the early stage of cancer. Thus, we speculate that the clinical utility of quantitative determination of Bmi-1 mRNA expression is in the diagnosis of small and suspected neoplastic lesions in breast. This technique is useful even when only a small number of cells can be obtained by a needle biopsy or an aspiration biopsy. Therefore, to avoid inappropriate therapy in patients without cancer, the cut-off value should ideally be selected such that high specificity is obtained. In most previous studies, the cut-off value was set as the mean + 2 SD of the expression level in non-cancerous tissues. In this study, the cut-off value close to the mean + 2 SD of the expression in non-cancerous tissues was 12.62, and it resulted in high specificity (91.3%) and allowable sensitivity (72.0%). Thus, for clinical application, it is recommended that the cut-off value be set at least around the mean + 2 SD of the expression in non-cancerous tissues.

With regard to the relationship between *Bmi-1* gene expression and clinicopathological factors, *Bmi-1* showed a unique expression profile wherein the expression level tended to be higher in the relatively early stages of cancer (stages I and II); this tendency was remarkable especially in the top 5 cases without lymph node metastasis. Very few reports have described molecules showing the above-mentioned expression profiles in cancer patients (12). Recently, Choi *et al* examined Bmi-1 protein expression in breast cancer patients and demonstrated that the expression rates of Bmi-1 protein are higher in the early stages of breast cancer (66.0% in stage I, 53.6% in stage II and 41.8% in stage III), thus supporting our data on gene expression (8). Silva *et al* previously reported that 55% of healthy controls and 43.2% of breast cancer patients showed detectable levels of Bmi-1 mRNA in the plasma, and that there was no significant difference in the mean values of Bmi-1 mRNA expression levels among the clinical stages (13). However, we cannot compare our data with those of the above-mentioned study, because the latter study did not report quantitative data for each stage of breast cancer. Further studies with a larger study population are required to ascertain whether *Bmi-1* gene expression is actually higher in the early stages of breast cancer in patients without lymph node metastasis.

It is unclear whether the high expression of Bmi-1 mRNA causes carcinogenesis or whether carcinogenesis leads to the elevation of Bmi-1 mRNA expression. Dimri *et al* reported that the overexpression of the *Bmi-1* gene in human mammary epithelial cells resulted in cell transformation via the induction of telomerase activity (5). Our study showed that Bmi-1 mRNA was highly expressed even in the early clinical stages of breast cancer. The evidence suggests that Bmi-1 plays an important role in the development of breast cancer. In particular, in the patients suspected of having breast cancers, Bmi-1 mRNA, which can be detected with highly sensitive RT-PCR, may be a good marker to support diagnosis even when very few cells can be obtained.

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