Overexpression of Polycomb Protein BMI-1 in Human Specimens of Breast, Ovarian, Endometrial and Cervical Cancer

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Abstract. Introduction: The polycomb group (PcG) proteins form chromatin-modifying complexes that are commonly deregulated in cancer. The PcG protein BMI-I is overexpressed by various tumours and thus may contribute to malignant transformation. The current study investigated the expression of BMI-I in human specimens of breast, ovarian, endometrial and cervical cancer. Materials and Methods: Expression of BMI-I was evaluated in human ovarian cancer samples by Western blot analysis and immunohistochemistry (IHC) and compared to healthy ovarian tissue. BMI-I expression in human specimens of breast, endometrial and cervical cancer was evaluated by IHC and then compared with the respective benign tissues. Results: BMI-I was significantly ($p<0.05$) overexpressed in human breast, ovarian, endometrial and cervical cancer specimens as compared to benign controls. BMI-I expression was also more pronounced in the ovarian cancer samples as demonstrated by Western blot analysis. In human breast cancer samples, BMI-I expression was most pronounced in the invasion front of the tumour. Conclusion: The current study showed for the first time that the BMI-I protein is significantly overexpressed in ovarian, endometrial and cervical cancer and may thus be a potential target for novel antitumor therapies.

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Success stories such as the development of the monoclonal antibody Trastuzumab (Herceptin®), demonstrate that a thorough understanding of the molecular mechanism of carcinogenesis is a prerequisite for the development of new highly specific therapies. But even trastuzumab cannot cure patients with metastasis, which might be due to tumor stem cells (tumor initiating cells) as recent research suggests.

The polycomb group (PcG) proteins form chromatin-modifying complexes, which are essential for embryonic development and stem cell renewal and are commonly deregulated in cancer (1). The BMI-1 protein is homologous to certain Drosophila polycomb group proteins that regulate homeotic gene expression through alteration of chromatin structure, and has previously been shown to repress transcription (2). The p16/CDK/ retinoblastoma (RB) is frequently alterated in various types of cancer (3). This pathway triggers the transition from G_1 to S-phase of the cell cycle by phosphorylation of the RB protein (4). The p16 protein inhibits the phosphorylation of the RB protein, leading to cell cycle arrest (5). Several mechanisms including mutations and homozygous deletions or hypermethylation of the p16(INK4a) promoter region can cause loss of p16 expression (6). A recent study identified the BMI-1 protein as a negative regulator of p16 (7), thus contributing to malignant transformation by indirectly activating the RB protein. Additionally, recent studies have identified BMI-1 as survival factor for tissue and cancer stem cells, thus reviving the interest in this protein (8-10). Since then, intriguing new studies have been published, which investigated the role of BMI-1 in a variety of tumors (11-14).

Prompted by these findings, the present study aimed to investigate the BMI-1 expression in a subset of human

specimens of breast ovarian, endometrial and cervical cancers and the respective benign tissues by immunohistochemistry (IHC) and Western blot analysis. In a second step, coexpression of the proliferation marker Ki-67 and of p16 was evaluated by IHC.

Materials and Methods

Tissue samples. Tissue samples were collected from patients with breast (n=12), ovarian (n=10), endometrial (n=12) and cervical (n=13) cancer. Biopsies from breast (n=3), ovarian (n=4), endometrial (n= 6) and cervical (n=8) tissue were used as controls.

Immunohistochemistry. For immunohistochemistry, sections of routinely-processed paraffin-embedded tissue were cut at 2-3 μm, placed onto APES-coated slides, dewaxed in xylene, and rehydrated in graded ethanols and Dulbecco's phosphate-buffered saline (PBS pH: 7.2). For antigen retrieval, sections were subjected to heat pretreatment by boiling in 0.01 M sodium citrate buffer (pH: 6.0) for 10 min in a microwave oven (power: 750 W/s). Slides were cooled down for 20 min. To block endogene peroxidase activity slides were subjected to 3% H_2O_2 (dissolved in methanol) treatment. Unspecific binding capacity was blocked with 5% goat serum for 15 min.

For specific detection of the BMI-1, Ki-67 and the p16 antigens, the sections were incubated first with goat serum for 30 min and then with rabbit polyclonal antibody BMI-1 (H-99): sc10745 (Santa Cruz Biotechnology, Heidelberg, Germany), with mouse monoclonal antibody KI-67 MIB-1 M7240 (DAKO, Hamburg, Germany) and with mouse monoclonal antibody p16 clone F-12 sc1661 (Santa Cruz Biotechnology) at 1:100 dilution in antibody diluent (DAKO) at 4˚C overnight. After one wash in PBS, the horseradish-peroxidase (HRP)-labeled goat anti-rabbit antibody (Immunotools, Friesoythe, Germany; dilution 1:1000) respectively the HRP-labeled biotinylated rabbit anti-mouse antibody were used as secondary antibodies (DAKO, ready-to-use LSAB2 HRP kit, dilution 1:100). The detection reaction was developed with either 3,3'-diaminobenzidine (DAB; Sigma, Deisenhofen, Germany) or Vector VIP (Vector Laboratories, Burlingame, CA, USA). The sections were counterstained with hematoxylin (Mayers, Sigma), dehydrated through graded ethanols, and embedded in Entelan (Merck, Darmstadt, Germany). Immunoreactivity of the tissues was evaluated by comparison with control sections incubated with an IgG control antibody. A standardized immunoreactive score ranging from 0 (negative) to 5 (highly positive) was calculated for the tumor samples and the positive controls. Score classification was as follows: 0=no BMI-1-positive cells; 1=up to 20% of cells BMI-1-positive; 2=up to 40% of cells BMI-1-positive; 3=up to 60% of cells BMI-1-positive; 4=up to 80% of cells BMI-1-positive; 5=almost all cells analyzed BMI-1-positive.

Immunoblot analysis. Benign and malignant ovarian samples were homogenized and prepared from 10 μm thick slices of snap-frozen tissue. Protein lysis buffer contained 50 mM Tris (pH: 7.4), 0.25 M NaCl, 0.2% Tween, 10 μg/ml leupeptin, 10 μg/ml PMSF and 2 μg/ml aprotinin. Protein concentrations were determined using RotiQuant Universal (Roth, Karlsruhe, Germany). Ten μg of lysate/lane were separated. Western blotting was performed using

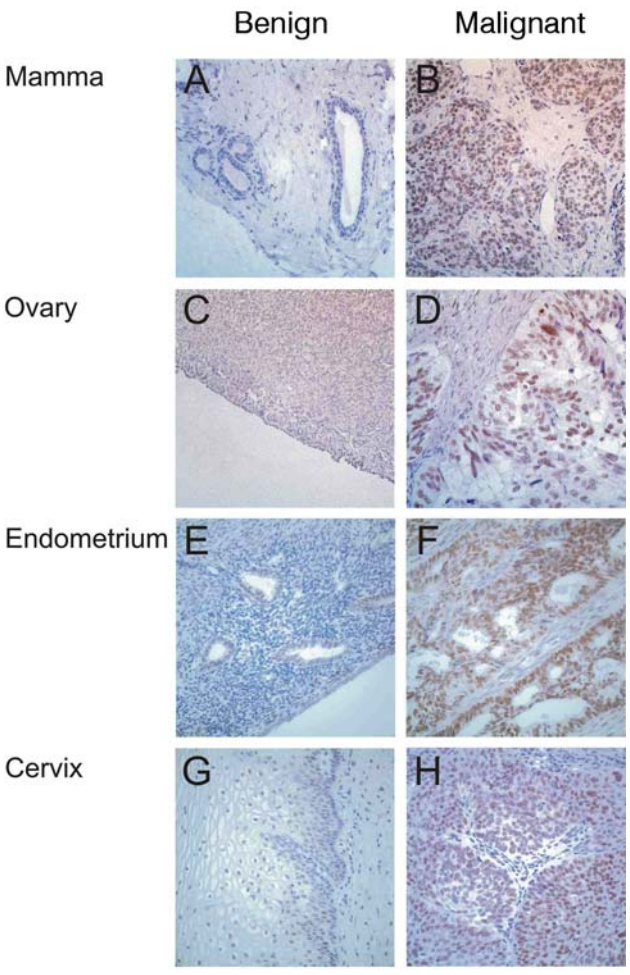

Figure 1. *Expression of the BMI-I protein in human specimens of breast, ovarian, endometrial and cervical cancer and the respective benign tissues as demonstrated by IHC.*

15% SDS-polyacrylamide gels in a Mini V8.10 gel chamber (Whatman, Dassel, Germany). Proteins were transferred to nitrocellulose membranes (Whatman) that were then blocked with Roti-Block (Roth), incubated with 0.5 μg/ml rabbit polyclonal antibody BMI-I (H-99): sc10745 (Santa Cruz Biotechnology), directed against the DNA-binding protein BMI-1 (BMI-1), washed in PBS containing 0.05% Tween-20 and developed with HRP-labeled goat anti-rabbit (Immunotools) and luminol solution (88.5 mM Tris-HCl, pH: 8.6, 0.2 mg/ml luminol, 1 mg/ml para-hydroxycoumarinic acid and 0.01% H_2O_2). β-Actin staining (Abcam Ab8226, Cambridge, UK) was performed as loading control.

Statistical procedures. BMI-1 expression in benign and malignant samples was calculated by the Chi-square test. The correlation of BMI-1, KI-67 and p16 was analysed by the Spearman's rank correlation coefficient. All calculations were performed using the SPSS software (SPSS/PC for Windows, Version 15.0.1, SPSS Inc. Chicago, IL, USA).

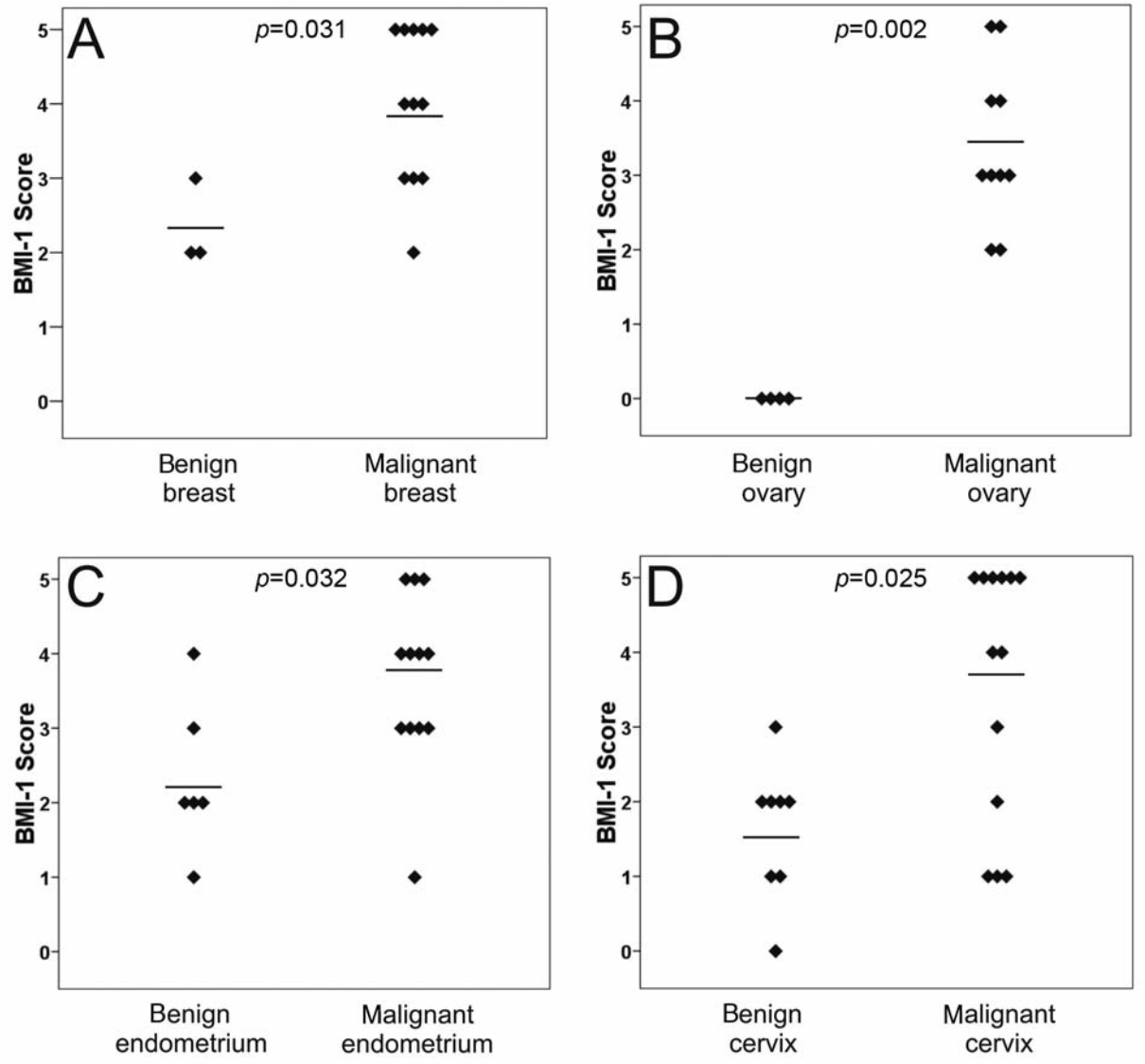

Figure 2. *Comparison of BMI-I expression of human specimens of breast, ovarian, endometrial and cervical cancer and the respective benign tissues. Immunoreactive scores of benign and malignant samples of each tumor entity were compared by chi-square test, p<0.05 was considered statistically significant.*

Results

Human specimens of human breast, ovarian, endometrial and cervical cancer significantly overexpress the BMI-1 protein. Benign and malignant tissue samples were subjected to IHC. A standardized immunoreactive score was calculated for each sample. BMI-1 expression of benign and malignant samples was then compared by the Chi-square test. It was found that BMI-1 is significantly up-regulated ($p<0.05$) in human breast, ovarian, endometrial and cervical cancer specimens as compared to benign controls (Figures 1 and 2). Additionally, 10 samples of human ovarian cancer and 4 normal ovaries were assessed for expression of the BMI-1 protein by Western blot analysis. There was a more pronounced expression of the 37 kDa BMI-1 protein in malignant ovarian tissue than in benign ovarian tissue. (Figure 4). BMI-1 expression was more pronounced in the ovarian cancer samples when compared to the corresponding benign tissue (Figure 1). Likewise, there was clear expression of BMI-1 in breast cancer samples, whereas there was almost no expression of the protein in the corresponding benign breast tissue (Figures 1, 5). In human breast cancer samples BMI-1 expression was most pronounced in the invasive front of the tumour (Figure 5).

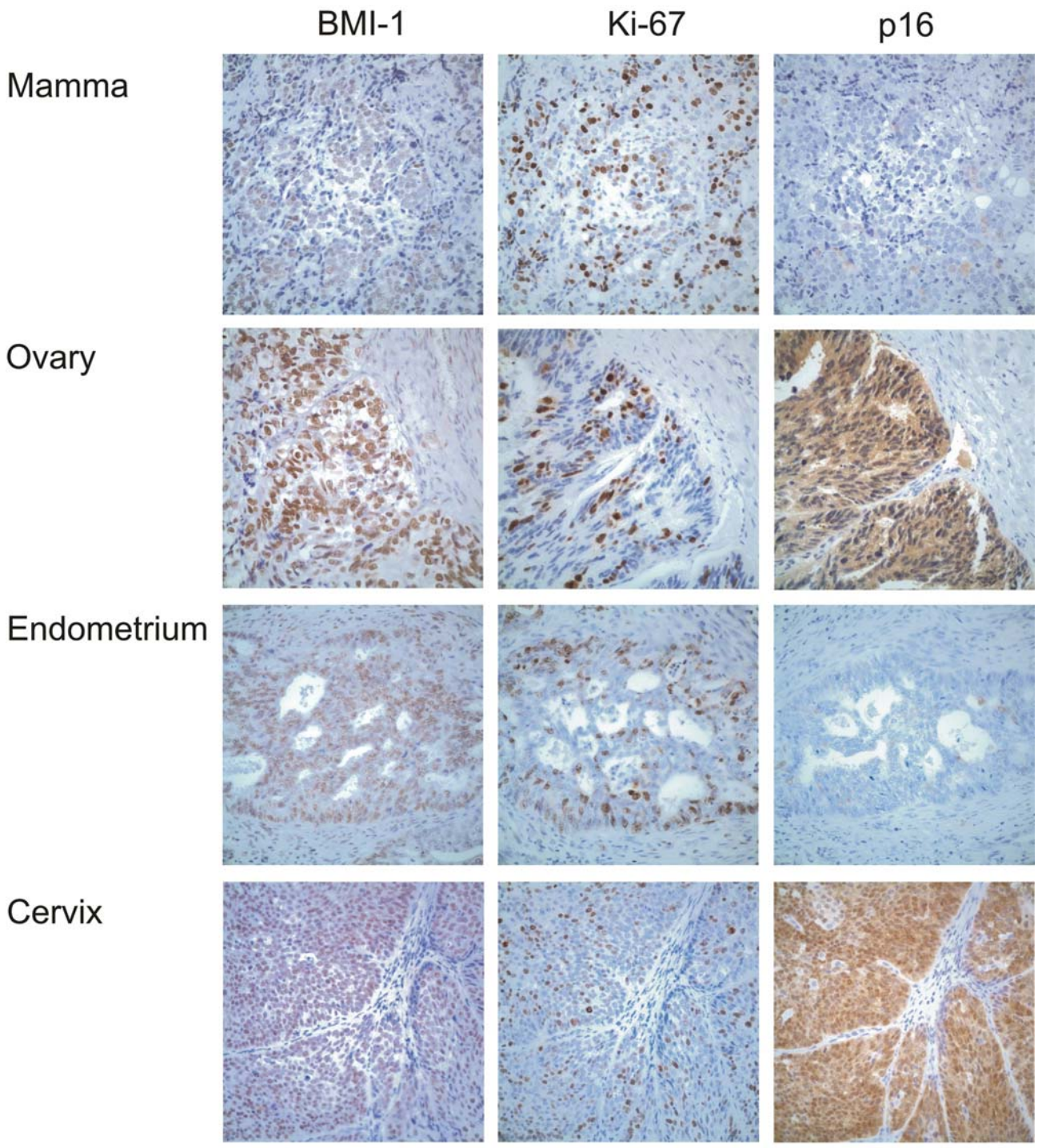

Figure 3. *Expression of BMI-1, Ki-67 and p16 in human specimens of breast, ovarian, endometrial and cervical cancer.*

No correlation of BMI-1 expression with the expression of Ki-67 and p16. All available malignant samples were additionally subjected to IHC for Ki-67 and p16 and a standardized immunoreactive score was calculated for each sample. The Spearman's rank correlation coefficient did not reveal any correlation of BMI-1 with Ki-67 or p16 in breast, ovarian, endometrial or cervical cancer (Figure 3).

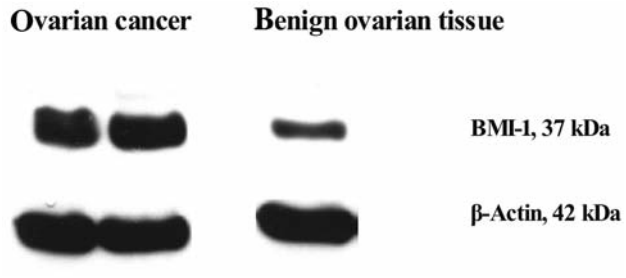

Figure 4. *Expression of BMI-1 in healthy ovaries and ovarian cancer samples as determined by Western blotting. The 37 kDa protein corresponds to BMI-1 and the second protein running at 42 kDa is beta-actin as loading control.*

Discussion

Overexpression of polycomb protein BMI-1 has been linked to an increasing number of cancer types such as non-small cell lung (15), colorectal (16), nasopharyngeal (17) and oral cancer (18), pointing to a role in tumorigenesis. In line with these findings, the current study showed that BMI-1 is significantly overexpressed in breast, ovarian, endometrial and cervical cancers as compared to the corresponding benign tissues. As immunostaining may not be specific at times, the specificity of the antibody was confirmed by Western blot analysis (Figure 4).

BMI-1 has been shown to immortalize mammary epithelia cells (19) and regulates growth and survival of breast cancer stem cells (8). Kim *et al*. showed by immunohistochemical staining that 62% of breast cancer samples had strong positive signals for BMI-1 with a more intense staining pattern in the invading fronts than in the central portions (20), which is accordance with the present findings in this tumor entity (Figure 5). Furthermore BMI-1 overexpression correlated with axillary lymph node metastases in this study (20). Additionally, Silva *et al*. demonstrated that BMI-1 mRNA in the plasma of breast cancer patients is positively correlated with established markers of prognosis such as hormone receptor negativity (13), thus being a possible surrogate marker of poor prognosis. A recent study reported conflicting results and found that BMI-1 expression assessed with immunohistochemistry may be associated with favourable overall survival in breast cancer patients, especially in patients with ER-positive tumors (21).

BMI-1 is a known repressor of the p16 tumor suppressor gene and some of its oncogenic activity has been attributed to this mechanism of action (22). Surprisingly, no correlation of BMI-1 and p16 expression in any of the tumor entities investigated could be detected. However, a recent study performed in Ewing sarcoma cells found that BMI-1 can promote tumorigenicity independent of p16 supression through modulation of adhesion pathways (23).

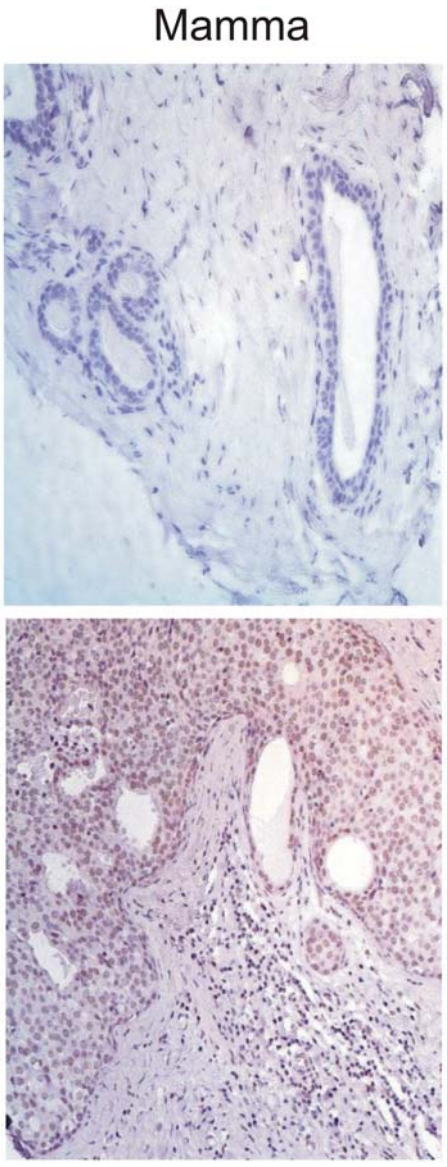

Figure 5. *Overexpression of BMI-1 in the invading fronts in an invasive ductal carcinoma of the breast. The upper panel shows benign glandular tissue of the breast with hardly any BMI-1 expression.*

Recently, Engelsen *et al*. demonstrated that 55% of human endometrial cancer samples show strong expression of BMI-1 (24). Low BMI-1 expression correlated with a more aggressive phenotype of endometrial carcinoma. However, no benign endometrial tissue was compared to malignant tissue. As the present study showed that endometrial carcinomas express higher levels of BMI-1 than benign endometrium samples, BMI-1 expression may be an early event in carcinogenesis, which is then reversed in the course of the malignant progression and the tumor dedifferentiation.

In conclusion, this study showed for the first time that the BMI-1 protein is significantly overexpressed in ovarian, endometrial and cervical cancer and may thus be a potential target for novel antitumor therapies.

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