Histol Histopathol (2012) 27: 1353-1359
DOI: 10.14670/HH-27.1353

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Relationship between B-Cell-specific moloney murine leukemia virus integration site 1 (BMI-1) and homologous recombination regulatory genes in invasive ductal breast carcinomas

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Summary. B-cell-specific Moloney murine leukemia virus integration site 1 (Bmi-1) is a Polycomb group protein that is able to induce telomerase activity, enabling the immortalization of epithelial cells. Immortalized cells are more susceptible to double-strand breaks (DSB), which are subsequently repaired by homologous recombination (HR). *BRCA1* is among the HR regulatory genes involved in the response to DNA damage associated with the RAD51 protein, which accumulates in DNA damage foci after signaling H2AX, another important marker of DNA damage. Topoisomerase IIIß (topoIIIß) removes HR intermediates before chromosomal segregation, preventing damage to cellular DNA structure. In breast carcinomas positive for BMI-1 the role of proteins involved in HR remains to be investigated. The aim of this study was to evaluate the association between BMI-1 and homologous recombination proteins. Using tissue microarrays containing 239 cases of primary breast tumors, the expression of Bmi-1, BRCA-1, H2AX, Rad51, p53, Ki-67, topoIIIß, estrogen receptors (ER), progesterone receptors (PR), and HER-2 was analyzed by immunohistochemistry. We observed high Bmi-1 expression in 66 cases (27.6%). Immunohistochemical overexpression of BMI-1 was related to ER (p=0.004), PR (p<0.001), Ki-67 (p<0.001), p53 (p=0.003), BRCA-1 (p= 0.003), H2AX (p=0.024) and topoIIIß (p<0,001). Our results show a relationship between the expression of BMI-1 and HR regulatory genes, suggesting that Bmi-1 overexpression might be an important event in HR regulation. However, further studies are necessary to understand the mechanisms in which Bmi-1 could regulate HR pathways in invasive ductal breast carcinomas.

Key words: BMI-1, Homologous recombination, Invasive ductal carcinoma, Breast cancer

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Introduction

Polycomb group proteins (PcG) are a family of proteins involved in the maintenance of embryonic and adult stem cells and cancer development (Valk-Lingbeek et al., 2004). B cell-specific Moloney murine leukemia virus insertion site 1 gene (Bmi-1), the first discovered PcG gene, plays an important role in biological processes, such as stem cell stabilization and differentiation, organ formation, embryonic development (Van der Lugt et al., 1994) and carcinogenesis of various malignancies, including pancreatic cancer, breast cancer and gastric carcinoma (Kim et al., 2004; Liu et al., 2008; Dhawan et al., 2009).

Studies have shown that Bmi-1 represses the INK4a locus. This locus is responsible for encoding the proteins $p16^{INK4a}$ and $p19^{ARF}$, which are able to induce growth arrest, cellular senescence and apoptosis, highlighting a pivotal role of BMI-1 in self-renewal cell activity (Kamijo et al., 1997; Jacobs et al., 1999).

Genetic instability is a hallmark of cancer, responsible for mutations that may give rise to aberrant cells with more aggressive phenotypes. Under normal conditions, cells unable to initiate apoptosis may become

more susceptible to DNA damage, such as single-strand breaks (SSB) and double-strand breaks (DSB). Recently, Ginjala et al. (2011) demonstrated that BMI-1 is recruited to DNA break sites and participates in the damage-induced ubiquitination of H2AX, implying a role for BMI1 in the response to DNA damage (Ginjala et al., 2011).

Mammalian cells have two main pathways for repairing DSB: homologous recombination (HR) and non-homologous end-joining (NHEJ). HR is a DNA repair pathway that is most efficient in the S phase of the cell cycle, decreases in the G2/M phase and is nearly absent in the G0/G1 phase. During HR, the loss of a sequence in dsDNA is repaired through a physical exchange of the damaged sequence by a sequence homologous to the original (Jasin, 2001).

Topoisomerase IIIß (topoIIIß) is a type I topoisomerase that removes recombination intermediates before chromosomal segregation, avoiding damage to the DNA structure (Zhu et al., 2001). Mohanty et al. (2008) demonstrated that cells deficient in topoIIIß have higher levels of phosphorylated γH2AX, a precise and sensitive DNA damage marker (Sak and Stuschke, 2010), when compared to TopoIIIß$^{+/+}$. These findings indicate a possible role for topoIIIß in DNA damage repair.

When DSBs occur, H2AX is phosphorylated by ataxia telangiectasia mutated (ATM)/ataxia telangiectasia and RAD3-related protein (ATR) pathways and accumulates at DSB sites. Phosphorylated H2AX recruits mediators of DNA damage that trigger a chain reaction that culminates in the recruitment of *BRCA-1* (breast cancer type 1 susceptibility protein) to DSB sites (Huen et al., 2010). BRCA-1, a gene that predisposes to familial breast and ovarian cancer, is fundamental in genomic stability and is believed to play an important role in HR associated with RAD51 protein (Jasin, 2002; Turnbull and Rahman, 2008). The role of *BRCA-1* in HR is poorly understood, although some studies suggest that BRCA-1 phosphorylates proteins involved in the ATM-ATR signaling pathway, including checkpoint kinase 1, checkpoint kinase 2 and p53 (Huen et al., 2010).

Following DNA DSBs, RAD51 forms nucleoprotein filaments on single-stranded DNA. This filament will search for homologous strands of duplex DNA, and the exchange of strands results in the formation of a joint molecule between the damaged and undamaged DNA. Thus, there will be new DNA synthesis and subsequent repair of the DSB (van Gent et al., 2001).

There are several studies suggesting a role of BMI-1 in the progression of breast cancer, although, the putative mechanisms involved in this process require further investigation. Considering that HR seems to be an important feature of genomic instability, the aim of this study was to evaluate the expression of BMI-1 in invasive ductal carcinomas (IDCs) of the breast and its association with classical prognostic markers in breast pathology and regulatory HR genes.

Materials and methods

Patients and clinicopathological information

This study included 239 female patients diagnosed with invasive ductal carcinoma of the breast between 1992 and 2005. The clinicopathological characteristics of all study patients are summarized in Table 1.

The biopsies were randomly retrieved from the Department of Pathology, Ribeirão Preto Medical School, University of São Paulo, Brazil. For each case, all available hematoxylin and eosin (H&E) stained sections were reviewed by an experienced breast pathologist (ARS) to confirm the diagnosis of invasive ductal carcinoma and to select a representative block for immunostaining. The cases were graded according to the current guidelines of the Scarff-Bloom and Richardson grading system modified by Elston and Ellis (Fitzgibbons et al., 2000). Clinical variables were retrieved from medical files and included patient age, menstrual status at diagnosis, stage of primary breast cancer at diagnosis, primary tumor size, lymph node stage at diagnosis, metastasis, and whether death was caused by the tumor. None of patients had received any treatment prior to the biopsy procedure. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the local ethics committee.

Tissue microarray (TMA) and immunohistochemistry

The selected tumor area for TMA construction was

Table 1. Patient clinicopathological features.

		Total
Age	>50 years	99 (41.4%)
	<50 years	140 (58.6%)
Menstrual status	Premenopausal	86 (36%)
	Postmenopausal	153 (64%)
Tumor size (cm)	<2	70 (29.3%)
	2-5	100 (41.8%)
	>5	69 (28.9%)
Tumor grade (B & R)	I	85 (35.6%)
	II	118 (49.4%)
	III	36 (15.1%)
Clinical Staging	I	32 (13.4%)
	IIa	57 (23.8%)
	IIb	50 (20.9%)
	IIIa	24 (10%)
	IIIb	62 (25.9%)
	IV	14 (5.9%)
Lymph node status	Pos.	129 (54%)
	Neg.	110 (46%)
Metastasis	Yes	75 (31.4%)
	No	164 (68.6%)
Death	Yes	80 (33.5%)
	No	159 (66.5%)

defined by a pathologist (ARS). Core biopsies (diameter, 1 mm) were punched from these regions in each of the 239 donor paraffin blocks and arrayed into a new recipient paraffin block using a Manual Tissue Arrayer I (Beecher Instruments, Silver Spring, USA). Sections (3-μm) were cut from the TMA paraffin block using the Paraffin Tape-Transfer System (Instrumedics, Saint Louis, USA). To confirm the presence of tumor, one slide was stained with H&E and analyzed by light microscopy. Immunohistochemistry was carried out on 2 slides at different depth levels to increase the accuracy and strength of the array-based data. All immunostained slides were independently analyzed and scored by 2 examiners. Disputed cases were reviewed by an experienced pathologist at a multi-headed microscope for a consensus agreement before the final scoring.

BMI-1, estrogen receptor (ER), progesterone receptor (PR), HER2, Ki-67, BRCA-1, RAD51, p53, H2AX and topoIIIß immunostains were performed as previously described (Oliveira-Costa et al., 2010). The antibodies used in this study are described in Table 2.

Any brown nuclear staining in breast epithelium was considered positive for BMI-1 (Joensuu et al., 2011). Estrogen and progesterone receptor expression were determined by a method of immunostain scoring (Fitzgibbons et al., 2000), where cases with more than 10% of neoplastic cells showing nuclear staining in neoplastic cells are considered positive. Based on the literature, this same value was used for BRCA-1, p53, Ki-67, RAD51 and H2AX (Fitzgibbons et al., 2000; Mangia et al., 2009; Mitra et al., 2009; Oka et al., 2010). For topoIIIß we used the score described by Di Leo et al. (2001). Normal tonsils were used as a positive control for BMI-1, Ki-67, RAD51, H2AX and topoisomerase IIIß. Cases of invasive ductal carcinoma previously known to be positive for ER, PR, p53 and HER-2 were used as positive controls, and normal mammary tissue was used as the positive control for BRCA1. Negative controls for immunostaining were prepared by omitting the primary antibodies. HER-2 immunoexpression was scored according to current guidelines (Wolff et al., 2007). Carcinomas with scores of 0 or 1+ were considered negative and carcinomas with scores of 3+ were considered positive. Cases considered 2+ were submitted to chromogenic in situ hybridization (CISH) for HER-2.

Chromogenic in situ hybridization (CISH)

In cases in which HER-2 was graded as 2+ by immunohistochemistry, 3-μm sections were cut from the original paraffin blocks. The ZytoDot 2C SPEC HER-2/CEN 17 probe kit (Zytovision, Bremerhaven, Germany) was used to detect the human HER-2 gene and alpha-satellites of chromosome 17. All procedures were performed according to the manufacturer's instructions. Using this kit, 2 green (HER-2) and 2 red (CEN 17) signals are expected in a normal interphase nucleus. HER-2 was considered amplified when the HER-2/CEN 17 ratio was ≥2 on average for 60 cells (MacGrogan et al., 2003). For statistical purposes, only the cases scored as 2+ by the immunohistochemical method were considered positive.

Statistical analysis

Statistical analyses and tests were performed with the commercially available PASW Statistics 17.0 software (Chicago, IL, USA). The relationships between BMI-1 expression, immunohistochemical findings and clinicopathological features were tested with cross tables applying chi-squared (three or more variables) or Fisher tests (2 variables), and all tests were 2-tailed. A p-value of 0.05 was considered significant.

Results

All patients were women between 28 and 91 years old (mean, 55.8 years). A total of 86/239 patients were pre-menopausal and 153/239 were post-menopausal. The study included only breast invasive ductal carcinomas, and tumor size ranged from 0.5 to 18 cm (mean, 4.7 cm). The tumors were classified as grade 1 (85 [35.56%]), grade 2 (118 [49.37%]) and grade 3 (36 [15.6%]). According to the Clinical Staging System (Singletary et al., 2003), 32 patients (13.39%) were classified as stage I, 57 (23.85%) were IIa, 50 (20.92%) were IIb, 24 (10.04%) were IIIa, 62 were IIIb (25.94%), and 14 were IV (5.86%). Seventy patients had tumors measuring less than 2 cm, 100 had tumors measuring between 2 and 5 cm and 69 had tumors larger than 5 cm. At the end of the follow-up, distant metastasis were verified in 75 (31.38%) patients and 80 (33.47%) died of breast cancer (Table 1).

Tumors positive for BMI-1 did not show a relationship with age (p=0.278), menstrual status (p=0.445), tumor size (p=0.714), tumor grade (Bloom & Richardson) (p=0.369), clinical staging (p=0.435), lymph node status (p=0.699), metastasis (p=0.589) or death (p=0.643).

Table 2. Primary antibodies, dilutions, sources and clones used in the immunohistochemical analysis.

Primary Antibody	Clone	Dilution	Source
BMI-1	H99	1:50	Santa Cruz
Topoisomerase III,	-	1:100	Santa Cruz
p53	DO-7	1:50	Novocastra
BRCA1	MS13	1:50	Serotec
RAD51	51RAD01	1:100	Abcam
H2AX	3F2	1:100	Abcam
Estrogen receptor (ER)	6F11	1:100	Novocastra
Progesterone receptor (PR)	16	1:100	Novocastra
c-erbB-2	CB11	1:100	Novocastra
Ki-67	MM1	1:100	Novocastra

Immunohistochemical results

BMI-1 expression in neoplastic cells was verified in 66 of 239 cases (27.62%). The remaining 173 cases (72.38%) did not have BMI1-positive cells and therefore were considered negative for statistical purposes. Normal breast tissue did not show any BMI-1-positive cells (Fig. 1).

Ductal invasive breast carcinomas were positive for ER in 157 cases (65.69%), PR in 144 cases (60.25%), HER2 in 48 cases (20.08%) (after CISH analysis), Ki-67 in 85 cases (35.56%), BRCA-1 in 146 cases (61.09%),

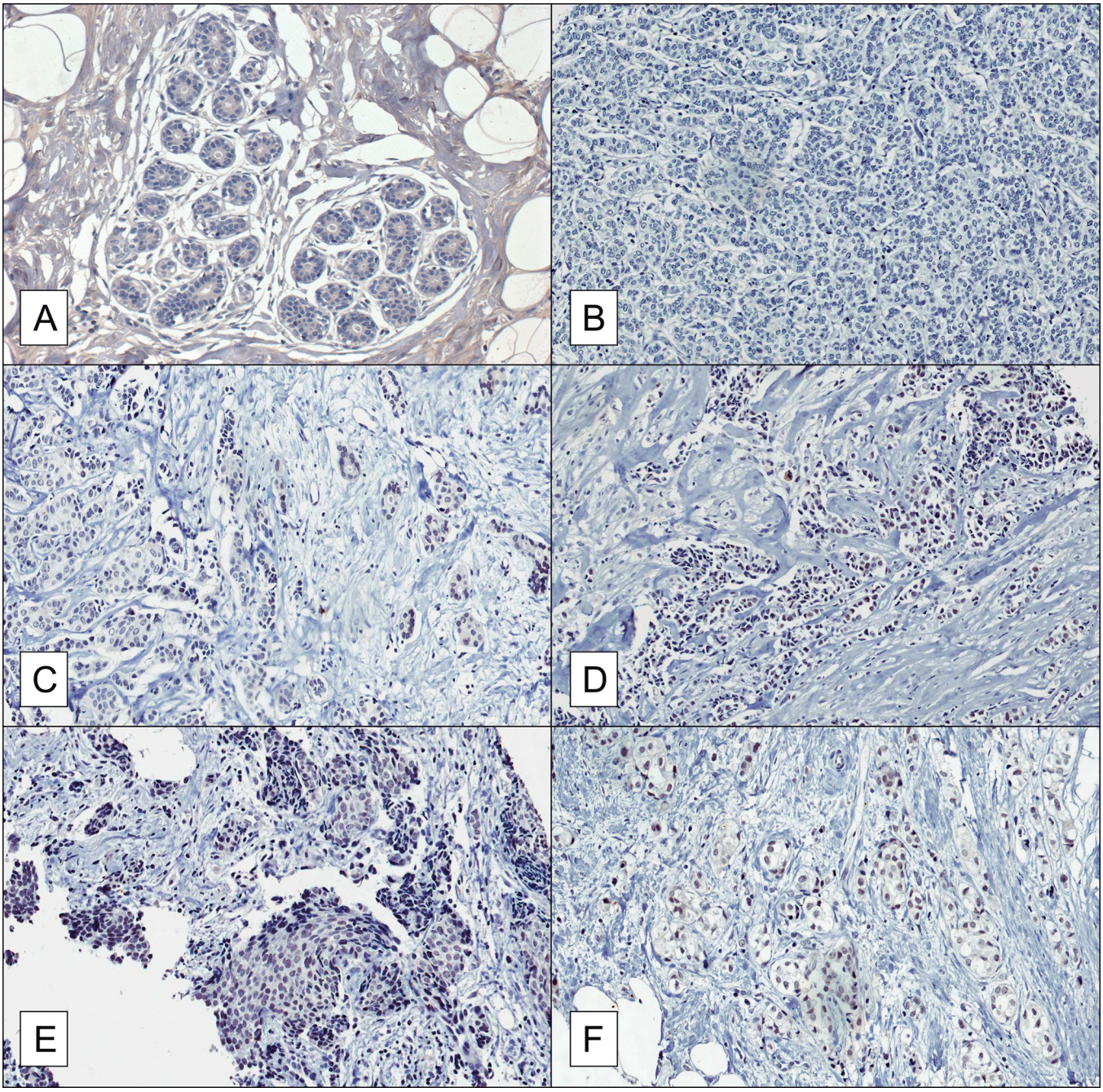

Fig. 1. Analysis of Bmi-1 protein expression by immunohistochemistry in invasive breast carcinomas. Bmi-1 expression was localized within the nuclei. **A.** Negative Bmi-1 staining in a non-cancerous tissue sample. **B.** Negative Bmi-1 staining in a cancerous tissue sample. **C, D, E, F.** Different percentages of positive Bmi-1 staining in tumor cells. A, x 200; B-F, x 20

RAD51 in 76 cases (31.80%), p53 in 154 cases (64.44%), H2AX in 145 cases (60.67%), and topoIIIß in 128 cases (53.56%).

BMI-1 expression correlated with PR ($p<0.001$), ER ($p=0.004$), Ki-67 ($p<0.001$), p53 ($p=0.003$), BRCA-1 ($p=0.003$), H2AX ($p=0.024$) and topo III, ($p<0.001$), but did not have a significant correlation with RAD51 ($p=0.417$) or HER2 ($p=0.225$).

Discussion

In the present study, we found an association between PcG gene BMI-1 and HR regulatory genes. To the best of our knowledge, this is the first study relating BMI-1 expression to several proteins involved in HR. In addition, we verified an association between Bmi-1 and classical prognostic factors in invasive ductal carcinoma of the breast, including ER and PR status. In our study, BMI-1 was related to ER ($p=0.004$) and PR ($p<0.001$), variables known to be associated with patient outcome, but was not related to HER-2 or other clinical features. The relationship between BMI-1 and hormonal receptor status have been shown before, in a microarray analysis in ER-positive tumors. Duss and co-workers, in 2007, have demonstrated that ER-positive tumors had significantly higher levels of BMI-1 transcripts when compared to ER-negative tumors (Duss et al., 2007). Our results are in accordance with the study of Joensuu et al. (2011), in which BMI-1 was significantly related to ER status but not with other clinical parameters analyzed (Joensuu et al., 2011). Kim et al. (2004) found a positive association between ER and BMI-1 but not with progesterone receptor, HER-2 status or clinical outcomes other than lymph node status (Kim et al., 2004). On the other hand, Yang et al. (2010) found that strong BMI-1 expression is related to poor outcome in ovarian carcinoma patients (Yang et al., 2010) The mechanisms underlying the BMI-1 role in ER-positive tumors are still poorly understood, although a reasonable explanation for our findings is that some genes suppressed by BMI-1, as shown by microarray analysis by Duss and co-workers (2007), were associated with squamous and neural differentiation. As pointed out by the authors, the suppression of these target genes also could favor proliferation, by the avoidance of a differentiated state in these cells. This hypothesis, associated with our results showing higher levels of hormonal stimuli in ER-positive breast cancer cells, an impairment in differentiation by high levels of BMI-1 and defective expression of DNA damage associated markers, could help to elucidate the main reasons why a cell type with a good prognosis as those present in ER-positive tumors was associated with increased expression of BMI-1 and markers of DNA damage.

Recent studies have implicated BMI-1 in DNA damage response pathways (Liu et al., 2009; Facchino et al., 2010; Ginjala et al., 2011). Liu et al. (2009) demonstrated that BMI-1 regulates mitochondrial production of reactive oxygen species, and loss of BMI-1 leads to increased ROS-mediated DNA damage, inducing checkpoint activation (Liu et al., 2009). In glioblastoma cells, loss of BMI-1 severely impairs the response to DNA DSBs; therefore, these cells have an increased sensitivity to radiation. On the other hand, BMI-1 overexpression increases the response to DNA DSBs, making cells more resistant to radiation (Facchino et al., 2010).

Ginjala et al. (2011) showed that BMI-1 is recruited to sites of DNA damage for efficient HR repair. They demonstrated that the sustained localization of BMI-1 at DNA DSB sites depends on H2AX phosphorylation (Ginjala et al., 2011). In our study we showed that BMI-1 and H2AX expression are directly associated, corroborating the finding that BMI-1 is closely related to DNA DSB machinery. Together with the finding that mutant BRCA-1 and inactive p53 are related to increased BMI-1, our results indicate that when BMI-1 is overexpressed, γ-H2AX seems also to be highly expressed, probably due to lack of function of either BRCA-1 and p53, which are thought to be necessary for successful HR. Additionally, the presence of gamma-H2AX, inactive p53 and mutant BRCA-1 could provide information about unrepaired DNA damage in breast ductal carcinomas, offering further insight into DNA damage response machinery in BMI-1-positive breast ductal carcinogenesis.

To maintain DNA stability, cells must recognize DNA damage, signal this damage, induce checkpoint activation and initiate DNA repair. Cells that overexpress BMI-1 are incapable of initiating apoptosis, and based on our findings of both the inactive form of p53 and mutant BRCA-1, these cells could become more susceptible to additional mutations. Based on the relationship between BMI-1 and Ki-67, we can infer that proliferating BMI-1-positive cells would be more susceptible to these additional mutations, giving rise to more aggressive and/or chemoresistent phenotypes. The alterations in HR proteins in BMI-1-positive breast tumors could corroborate the findings of Guo and colleagues, which demonstrated that tumors overexpressing BMI-1 are particularly aggressive (Guo et al., 2011). To the best of our knowledge, this is the first study that relates BMI-1 and RAD51. Our results did not show a relationship between BMI-1 positivity and RAD51, but we have demonstrated associations between BMI-1 and important HR proteins, such as γH2AX, BRCA-1, p53 and topoIIIß.

Although Mohanty and co-workers (2008) demonstrated that cultured mouse embryonic fibroblasts lacking topoisomerase IIIß (topoIIIß$^{-/-}$) show a reduced efficiency in G1/S checkpoint transition, our findings in human tissue indicate an association between BMI-1 overexpression, which leads to a loss in G1/S checkpoint efficiency due to the relationship between overexpression of BMI-1 and inactivation of INK4 locus, and overexpression of topoIIIß. This could be explained by the presence of inactive p53 and BMI-1 overexpression, which would allow the cell to progress

through the cell cycle and bypass the checkpoint at the G1/S transition, leading to higher risk of cell damage, requiring the presence of topoIIIß to resolve DNA damage before cell cycle progression continues. The overexpression of H2AX could indicate ineffective topoIIIß, making HR necessary in invasive ductal breast carcinomas.

In conclusion, our study showed a relationship between the PcG gene BMI-1 and HR proteins, suggesting that BMI-1 might participate in HR. However, further studies are necessary to understand the mechanisms in which BMI-1 could be involved in regulating HR pathways in invasive ductal breast carcinomas.

Acknowledgements. The authors wish to acknowledge the technical assistance of Deisy Mara da Silva.

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Accepted May 23, 2012