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Immunohistochemical expression of superoxide dismutase (MnSOD) anti-oxidant enzyme in invasive breast carcinoma

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Summary. The most important cellular protective mechanisms against oxidative stress are antioxidant enzymes. Their action is based on decomposal of reactive oxygen species (ROS) and their transformation to H_2O_2. Within the mitochondria manganese superoxide dismutase (MnSOD) affords the major defense against ROS.

In this study we investigated tissue sections from 101 breast carcinomas for the immunohistochemical expression of MnSOD protein and these results were assessed in relation to various clinicopathological parameters, in order to clarify the prognostic value of this enzyme. The possible relationship to hormone receptor content, anti-apoptotic protein bcl-2, p53 and cell proliferation was also estimated.

High expression levels were observed, as 79/101 (78,2%) cases expressed strong immunoreactivity. In this study MnSOD increased in a direct relationship with tumor grade and is therefore inversely correlated with differentiation (p=0.0004). Furthermore, there was a strong positive correlation between MnSOD expression and p53 protein immunoreactivity (p=0.0029). The prognostic impact of MnSOD expression in determining the risk of recurrence and overall survival with both univariate (long-rang test) and multivariate (Cox regression) methods of analysis was statistically not significant.

These results indicate that neoplastic cells in breast carcinomas retain their capability to produce MnSOD and thus protected from the possible cellular damage provoked by reactive oxygen species. In addition, MnSOD content varies according to the degree of differentiation of breast carcinoma.

Key words: MnSOD, Breast cancer, Oxidative stress

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Introduction

Reactive oxygen species (ROS) are endogenous side-products of oxygen metabolism and have a critical role as mediators of cell damage (Kehrer, 1993). They are implicated in many physiological functions, such as intracellular signal transduction (Chen et al., 1995), mitosis (Shibanuma et al., 1988) and apoptosis (Hockenbery et al., 1993), as well as in a large number of pathological processes such as chronic inflammation, neurodegeneration (Ferrente et al., 1997) and aging (Berlett and Stadtman, 1997), while it has been also suggested that they have a role in carcinogenesis (Sun, 1990). Specifically, it has been suggested that free ROS can act as promoters and/or initiators of multistage carcinogenesis, by causing DNA damage, activation of pro-carcinogens and alteration of the cellular antioxidant defense system.

To protect themselves from oxidative stress, cells have developed a sophisticated antioxidant enzyme defense system. In this system superoxide dismutases (SODs) convert superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2), whereas glutathione peroxidase (GPXs) and catalase (CATs) convert H_2O_2 into water. There are three isoforms of SODs in cells: copper-zinc SOD, found predominantly in the cytoplasm; extracellular SOD; and MnSOD, which exists primarily in the mitochondrial matrix. These enzymes are implicated in cellular proliferation (Church et al., 1993; Li et al., 1995; Yan et al., 1996), tumor invasiveness and chemosensitivity of neoplastic cells, as they protect cells from the lethal influence of IL-1 (Masuda et al., 1988), TNF (Wong and Goeddel, 1988), various antineoplastic drugs and ionizing radiation (Hirose et al., 1993). MnSOD is one of the most important antioxidant enzymes with a molecular weight of 88.6 kDa located in the mitochondrial matrix (McCord and Fridovich, 1969) and converts O_2 to H_2O_2 and O_2.

The role of MnSOD in many cancer cell types is

poorly understood and conflicting data concerning its expression in different cancer cell types have been reported so far. MnSOD has been proposed to be a new type of tumor suppressor gene (Bravard et al., 1992; Oberley and Oberley, 1997) as it has been shown that in vitro it can reduce tumor growth and proliferation, and in that way it suppresses the malignant phenotype of cancer cells. SOD (generally MnSOD) has been found to be low (Oberley and Oberley, 1988) or undetectable in a wide variety of tumor types when compared to the appropriate normal cell controls. It has been proposed that this phenomenon is due to the derivation of cancer cells from stem cells that have innately lower levels of SOD (Oberley and Oberley, 1998).

As a result, in many studies conducted so far, it has been demonstrated that a variety of cancer cells have reduced levels of antioxidants, especially MnSOD, when compared to their normal counterparts (Sahu et al., 1977; Oberley et al., 1978; Oberley and Buettner, 1979; Sun, 1990), including melanoma, hepatocellular carcinoma and breast cancer. On the contrary, high immunohistochemical expression or biochemical activity of MnSOD has been reported in renal cell carcinoma (Yang et al., 1987), ovarian carcinoma (Ishikawa et al., 1990), thyroid tumors (Nishida et al., 1993), brain tumors (Cobbs et al., 1996), colorectal tumors (Janssen et al., 1998), in melanoma (Schadendorf et al., 1995), in mesothelioma (Kahlos et al., 1998, 2000) and in cultured mesothelioma cell lines (Kinnula et al., 1996).

In the present study we determined the MnSOD antigen content in a series of invasive breast cancers in an attempt to clarify its potential clinical importance. MnSOD expression correlated to the expression of steroid receptors, other already established prognostic factors, such as bcl-2, p53, Ki67, PCNA, as well as standard clinicopathological parameters (tumor grade, lymph node status, distant metastasis, recurrence, disease-free and overall survival).

Materials and methods

Patients and study design

A cohort of 101 patients with primary invasive breast carcinoma treated by surgical resection were investigated. All patients had a mastectomy with axillary lymph node dissection performed as indicated and were followed up regularly at the Medical Oncology Department of the University Hospital of Ioannina. Detailed clinical data were available for 98 patients: 24 had stage I disease, 53 patients stage II disease (pT1N1M0, pT2N0M0 and pT2N1M0) and 21 patients stage III (pT2N2M0, pT3N1M0 and pT3N2M0 or pT4N1M0). They were also clinically disease free and had a baseline CA 15-3 serum level below 30U/ml at the initiation of adjuvant therapy. Adjuvant therapies were administered according to standard guidelines and consisted in tamoxifen (38 patients), chemotherapy followed by tamoxifen (32 patients) and conventional chemotherapy (28 patients). After a median follow-up of 4 years (range 6-132 months), in 41.8% (41/98) of the patients the disease had progressed, and 34 of them had developed distant metastases.

Archived material was used from formalin-fixed and paraffin-embedded breast carcinoma tissue, including adjacent non-neoplastic tissue or fibrocystic disease. Each specimen was examined histologically on H&E-stained slides. Tumor size varied from 1 to 17 cm (mean=3.95cm). Tumor histotype, lymph node status, and age were recorded for each patient. Tumor grade was assessed on hematoxylin- eosin (H and E)-stained sections by personnel blind to the results of immunohistochemistry. Tubule formation, nuclear morphology and mitotic rate were evaluated and scored in the neoplastic cells according to the modified grading scheme of Bloom and Richardson: grade 1, grade 2 and grade 3 corresponding to well, moderately and poorly differentiated invasive carcinoma of the breast (Elston and Ellis, 1991).

Immunohistochemistry

Immunohistochemistry was performed on one or two selected paraffin blocks, from each case on 4 μm tissue sections placed on poly-L-lysine-coated glass slides. In brief, tissue sections were deparaffinized in xylene and dehydrated. For the detection of MnSOD, Ki-67 and p53 slides were immersed in citrate buffer (0.1 M, pH 0.6) in plastic Coplin jars and subjected to microwave irradiation twice for 15 min. Subsequently, all sections were treated for 30 min with 0.3% hydrogen peroxide in methanol to quentch endogenous peroxidase activity and then incubated with primary antibodies. We used the method involving the avidin-biotin-peroxidase complex and developed the chromogen with immersion of the slides in a diaminobenzidine-H_2O_2 substrate for 5min. The slides were counterstained in Harris' haematoxylin, dehydrated and mounted. To access the specificity of the reaction, control specimens were prepared from normal breast tissue, fibroadenomas and fibrocystic breast disease. The antibody sources and dilutions are shown in Table 1.

Table 1. Antibodies used.

ANTIBODIES	SUPPLIER	DILUTION	INCUBATION TIME
Anti-MnSOD (Ab2)*	StressGen	1:200	Overnight
Anti-Bcl-2(M0887)	Dako	1:40	Overnight
P53 (DO-7)*	Dako	1:50	1 hour
ER-alpha (M7047)	Dako	1:50	1 hour
PgR (M3569)	Dako	1:75	1 hour
PC-10 (M0879)	Dako	1:50	1 hour
MIB-1*	Dako	1:50	1 hour

*: With microwave oven antigen retrieval.

Immunohistochemical evaluation

To evaluate the expression of MnSOD protein, we established a combined score based on a previous study (Soini et al., 2001), corresponding to the sum of both: a) staining intensity (0: negative, 1; weak, 2: intermediate, 3: strong, 4: very strong staining); and b) and the percentage of positive cells (0: 0%, 1: 1-25%, 2: 26-50%, 3: 50-75%, 4: >75%). The sum of both qualitative and quantitative immunostaining reached a maximum score of 8. The combined scores were then divided into 4 main groups: (-) = no immunostaining, 0; (+) = weak immunostaining, 1-2; (++) = moderate immunostaining, 3-4; and (+++) = strong immunostaining, 5-8.

Cytoplasmic staining for MnSOD and nuclear for ER, PgR, p53, Ki-67 and PCNA was calculated as the percentage of positive neoplastic epithelial cells in relation to the total number of cells encountered in at least 5 to 10 representative high power fields (500-1000 epithelial cells). Every stained cell was considered positive, irrespective of intensity. All slides were reviewed and scored in a blind test by two pathologists (IE, TsE). Differences in interpetation were reconciled by re-review of slides seperately or jointly at a double-headed microscope. For statistical analysis purposes the 10% cut-off point for positivity was used for the estimation of ER, PgR, MIB-1 and PCNA according to previous studies (Haerslev et al., 1995; Gillesby and Zacharewski., 1999) and the 5% cut-off point for p53.

Statistical analysis

Superior Performance Software System (SPSS) 10.0 for windows (SPSS Inc., 1989-1999, IL, USA) was used by the authors to compare morphological features and protein expression data. Significant differences between the expression of the target proteins with regard to clinicopathological parameters were computed by the t-test for paired or non-paired values or ANOVA test if the data were normally distributed. If the data did not show a normal distribution, differences were analysed by the Wilcoxon signed ranks test for paired values or the Mann-Whitney U test and the Kruskall-Wallis H test for independent values. Correlation between MnSOD and the other cell-cycle related proteins was computed using the Pearson's correlation coefficient for normally distributed data or the Kendall's Tau rank correlation coefficient where the data did not show a normal distribution. The prognostic significance of MnSOD, in determining the risk for recurrence, was studied with both univariate (log rank test) and multivariate (Cox proportional hazards) ways of analysis, separately for each group of patients. The same analysis was employed for the overall survival of patients. P-values ≤ 0.05 were considered statistically significant.

Results

Granular perinuclear cytoplasmic immunoreactivity

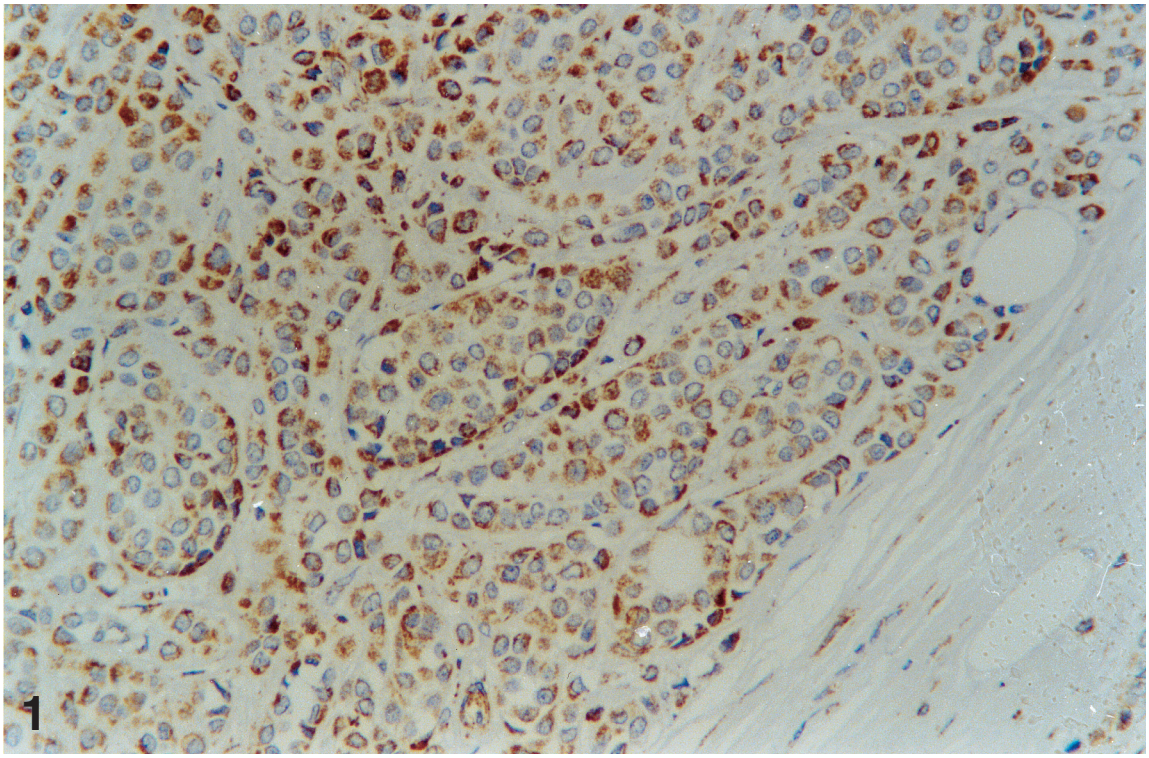

Fig. 1. An invasive breast carcinoma showing extensive granular cytoplasmic MnSOD immunoreactivity. x 200

of neoplastic cells for MnSOD was observed, as well as reactivity of normal and hyperplastic ducts. The intensity of the reaction was estimated as weak to moderate in adjacent normal epithelium, and moderate to strong in adjacent areas of hyperplastic ducts or fibrocystic disease (Fig. 1). In a few cases, that neoplastic cells were weakly stained for MnSOD, non-neoplastic acini trapped

Table 2. Correlation of MnSOD expression with clinicopathological features in breast cancer.

	MnSOD EXPRESION			P VALUE
	+	++	+++	
Age				
<45	6	4	16	
45-55	2	2	21	NS
>55	3	5	37	
Tumor size				
<2	1	0	11	
2-5	6	8	42	NS
>5	3	3	18	
Type				
Ductal	8	6	52	
Lobular	1	2	12	NS
Mixed	2	3	12	
Grade				
G1	2	4	1	
G2	5	4	36	p=0.0004
G3	3	3	35	
Lymph node				
(-)	5	4	17	NS
(+)	5	5	47	

Table 3. Correlation of MnSOD expression with ER, PgR, p53 and proliferation-associated indices.

	MnSOD EXPRESION			P VALUE
	+	++	+++	
ER				
<10	2	2	22	
>10	6	9	42	NS
PgR				
<10	2	3	27	
>10	5	8	36	NS
Bcl-2				
<10	5	3	31	NS
>10	2	2	29	
p53				
<5	7	10	30	p=0.0029
>5				
MIB-1				
<10	6	8	54	NS
>10	5	3	24	
PCNA				
<50	4	5	30	NS
>50	7	6	48	

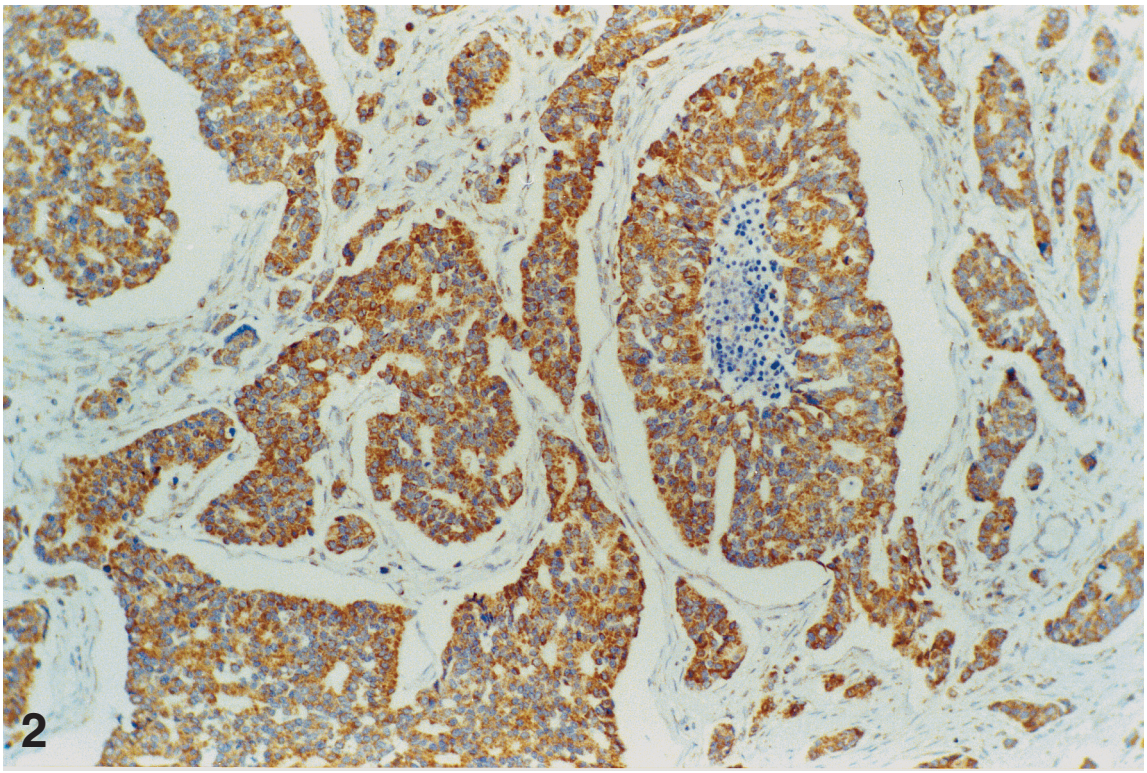

Fig. 2. In situ and invasive breast carcinoma exhibiting positive staining of high intensity. x 100

within the tumor, presented stronger intensity, compared to neoplastic ones. Furthermore, immunoreactivity was observed in several inflammatory cells, especially plasma cells, tissue macrophages, endothelial cells and fibroblasts.

High expression status for MnSOD was observed as all cases exhibited more or less strong immunoreactivity. Out of the 101 investigated cancerous breast tissues, 11/101 (10.9%) cases were classified as +, 11/101 (10.9%) cases were classified as ++, and 79/101 (78.2%) cases were classified as +++ (Fig. 2).

MnSOD expression levels were strongly correlated to the degree of differentiation of tumor cells, so that poorly differentiated tumor cells exhibited higher MnSOD content. (p=0.0004). In particular, a statistically significant difference was observed between grades 1 and 2 (p=0.001) and the most striking differences between grades 1 and 3 (p=0001). Furthermore, high MnSOD content was strongly associated with nuclear accumulation of p53 (p=0.0029) (Tables 2, 3)

No statistical significant correlation was observed with the other clinicopathological parameters, such as tumor size, hormone status, tumor type, anti-apoptotic protein bcl-2 and proliferation activity as determined by the expression of MIB-1 and PCNA. The results are shown in Table 3. Patients with presence of lymph node metastases tended to exhibit a higher MnSOD immunoreactivity, but no statistical significant relation was established (p=0.8).

In addition, in univariate analysis the expression of this protein did not show any significant prognostic value for overall survival, disease progression, presence of metastasis to distant organs, or response to chemotherapy.

Discussion

MnSOD, the product of the superoxide dismutase 2 (SOD2) gene, is one of the major cellular defences against oxidative stress. This study indicates that the anti-oxidant defense system is altered in cancerous breast tissues and specifically MnSOD is extensively expressed. This is a sharp indication that cancer cells can develop a capable defence mechanism against side products of oxygen metabolites.

The present study reveals the presence of extensive immunoreactivity of breast cancer cells to MnSOD protein. On the contrary, many studies (Oberley and Buettner, 1979; Oberley and Oberley, 1986) have suggested that the amount of MnSOD in most cancer cell types was consistently lowered or diminished. On the other hand, overexpression of antioxidant enzymes has been documented (Iscan et al., 2002) in a wide variety of malignant tumors, including breast cancer. It has been reported that there is an increase in tissue lipid peroxidation, which is associated with enhanced antioxidant capacities, measured by spectrophotometric assay, including SOD and catalase. Furthermore, increased SOD mRNA expression was observed (Li et al., 1998) in breast tumor tissues. In another study (Punnonen et al., 1994) it was reported that superoxide dismutase activity is elevated in breast cancer tissues when compared to the reference tissues.

In constrast to our study, Soini et al. (2001) reported that MnSOD protein expression was less frequent in neoplastic epithelial cells than in pre-invasive or normal epithelium, but when neoplastic epithelium showed immunoreactivity the staining reaction was stronger than in benign epithelial lesions.

Analogous reports (Izutani et al., 1998) have been made concerning oesophageal and gastric cancer, where MnSOD mRNA was significantly elevated in cancerous tissue. Recent reports (Halliwell, 2000) suggest that oxidative stress causes upregulation of antioxidant enzymes that renders cells resistant to subsequent oxidative damage.

In our study a significant direct correlation of MnSOD immunoreactive content and histological grading was established, which means that less differentiated tumors had significantly higher MnSOD immunoreactivity when compared to better differentiated ones. It has been shown that the levels of MnSOD in certain cells were correlated with their degree of differentiation (Oberley and Oberley, 1988). Non-differentiating cells, whether normal or malignant, appear to have lost their ability to undergo MnSOD induction. In experimental rat hepatocellular carcinomas the MnSOD content decreases following an inverse relationship to the degree of differentiation (Galeotti et al., 1989). In another study concerning brain tumors (Landriscina et al., 1996) MnSOD content increases with histological grading by using immunohistological and Western Blotting methods, which is in accordance to our results.

We failed to establish a relationship between MnSOD and the presense of metastatic lymph nodes. On the contrary, in gastric cancer cells (Malafa et al., 2000) it appears that there is a strong direct relation between MnSOD immunohistochemical expression and metastatic phenotype. The possible prognostic role of MnSOD in cancer is unknown, but in one study high MnSOD expression in colorectal cancer predicted a poor survival (Janssen et al., 1998). In the current study, no association between MnSOD expression and prognosis was established.

Although it has been postulated that MnSOD may have a significant effect on tumor cell growth, we failed to demonstrate such a relationship, as MnSOD levels did not correlate with two proliferation-associated markers, such as PCNA and MIB-1. Soini et al. (2001) demonstrated a marginal inverse association between immunohistochemical expression of MnSOD and Ki67 expression in breast carcinomas. Li et al. (1995) showed that high levels of MnSOD protein were accompanied by decreased cellular proliferation, due to damage or inhibition of cellular proliferation pathways, and that this was accompanied by a less malignant phenotype. In another study, Kahlos et al. (2000) displayed that high

MnSOD expression in mesothelioma cancer cells was strongly associated with lower proliferative activity, as determined by Ki-67 .

A number of stimuli can trigger p53 activation. Recently, convincing evidence has shown that p53 activation is accompanied by a net increase in intracellular ROS concentration and that the removal of oxygen radicals by anti-oxidant drugs impedes apoptosis induced by p53 (Johnson et al., 1996; Polyak et al., 1997). To date, however, very little is known about the molecular mechanisms linking p53 activation to cell damage by oxygen radicals.

The results of the present study showed a strong association between MnSOD and p53 protein immunoreactivity in breast cancer cells. Since p53 gene mutation can be considered as one of the many ways that leads to nuclear accumulation of the protein product, we can assume that cancer cells presenting p53 mutations are able to up-regulate the expression of the mitochondrial scavenger MnSOD, in order to resist oxidative stress. Drane et al. (2001) demonstrated that MnSOD overexpression decreases p53-gene expression at the promoter level and that p53 is also able to repress SOD gene expression, concluding in that way that these two genes are mutually regulated. In an experimental study (Korsmeyer et al., 1995), it has been found that SOD2 is modulated by p53 supporting the role of this enzyme as a survival protein involved in cell resistance to stress. This negative regulatory control of p53 on MnSOD expression has been evaluated in a number of human cancer cell lines, with different p53 functional status (Pani et al., 2000). MnSOD was highly expressed in cells where p53 was either mutated or virtually absent. In accordance with our results there are two studies that revealed an association between MnSOD and accumulation of mutated p53 in cervical carcinoma (Nakano et al., 1996) and brain tumors (Ria et al., 2001). The role of oxidative stress as a mediator of apoptotic cell death in diverse cell systems is now better understood. The proto-oncogene product, bcl-2, can inhibit apoptosis both in the presence and in the absence of the reactive oxygene products (Korsmeyer et al., 1995). In the present study the MnSOD expression did not correlate with bcl-2 expression.

In conclusion, our results clearly demonstrate that the majority of neoplastic cells in breast cancer retain their capacity to produce MnSOD and therefore cancer cells develop strong defence mechanisms against the side-products of oxygen metabolism. Their induction could be the result of endogenous cytokines or genetic alterations on MnSOD-regulating genes. In addition, the antioxidant defence system seems to be altered in cancerous breast tissues, according to the degree of tumor differentiation. Furthermore, the MnSOD content could reflect the p53 mediated apoptosis through a bcl-2-independent pathway.

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