

# Correlation of Tissue Inhibitor of Metalloproteinase-2 with Proliferative Activity and Patients' Survival in Breast Cancer

Lydia Nakopoulou, M.D., Sophia Katsarou, M.D., Ioanna Giannopoulou, Paraskevi Alexandrou, M.D., Ioanna Tsirmpa, Effie Panayotopoulou, Johnny Mavrommatis, Antonios Keramopoulos, M.D.

*Department of Pathology (LN, SK, IG, PA, IT, EP, JM) and First Department of Gynaecology and Obstetrics (AK), Medical School, The National and Kapodistrian University of Athens, Athens, Greece*

Tissue inhibitors of metalloproteinases (TIMPs) are endogenous regulators of matrix metalloproteinases (MMPs). They are believed to possess several distinct cellular functions, particularly the contradictory activities of inhibiting MMPs and promoting tumor cell growth. Immunohistochemistry was performed to detect TIMP-2 protein in 136 infiltrative breast carcinomas. TIMP-2 protein was analyzed in parallel with clinicopathologic features (tumor size, histologic type, nuclear and histologic grade, stage), patients' overall survival and ER, PR, Ki-67, topo II $\alpha$ , c-erbB-2, p53 and bcl-2 proteins. Statistical analysis was performed using univariate and multivariate models analysis. Immunoreactivity for TIMP-2 was observed in cancer cells and stromal fibroblasts in 106 (77.94%) and 104 (76.47%) of 136 cases, respectively. TIMP-2 protein expression in stromal fibroblasts showed a statistically significant inverse correlation with tumor size ( $P = .014$ ). An inverse correlation was also observed between TIMP-2 epithelial immunoreactivity and nuclear and histologic grade ( $P = .036$  and  $P = .007$ , respectively). TIMP-2 protein reactivity showed statistically significant positive associations with topo II $\alpha$  and bcl-2 in stromal and cancer cells, respectively ( $P = .032$  and  $P = .001$ , respectively). TIMP-2 protein expression in cancer and stromal cells was associated with better patients' overall survival ( $P = .002$  and  $P = .038$ , respectively). When evaluated by the Cox's proportional hazard regression model, this association was further established, but only as far as TIMP-2 expression in tumor epithelium was concerned ( $P = .019$ ). Our results support the multi-

functional potential of TIMP-2 through its correlation on the one hand to a favorable outcome, due to its MMP inhibitory activity and on the other to topo II $\alpha$  contributing to its growth factor activity.

**KEY WORDS:** Breast cancer, Immunohistochemistry, Survival, TIMP-2, Tumor growth.

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The process of cancer invasion and metastasis comprises a complex series of sequential steps, among which degradation of extracellular matrix (ECM) is essential. Matrix metalloproteinases (MMPs) are the principal class of enzymes responsible for ECM degradation. Specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) reduce the proteolytic activities of MMPs (1). Four homologous TIMPs have been characterized so far, TIMP-1 to -4 (2). They are low molecular weight proteins that bind to active MMPs in a 1:1 molar ratio, and form noncovalent tight complexes with them. TIMP-1, -2, and -4 are secreted in soluble, whereas TIMP-3 in insoluble form due to ECM binding (3). TIMP-2 preferentially inhibits the activation of the 72 kDa type IV collagenase/gelatinase (MMP-2) and can inhibit the activated form of the enzyme as well (4). The most recently discovered TIMP, TIMP-4 may function in a tissue-specific fashion, as it is found to be exclusively expressed in adult heart tissue (5).

In general, TIMP-1 and TIMP-2 are capable of inhibiting the activity of all known MMPs and as such play a key role in maintaining the balance between ECM deposition and degradation in different physiologic processes.

Accelerated ECM breakdown occurs in various pathologic processes, including inflammation, chronic degenerative diseases and tumor invasion. TIMP-1 and TIMP-2 can inhibit tumor growth, invasion, and metastasis in experimental models, which has been associated with their MMP inhibi-

tory activity (6). Recent developments in TIMP research suggest that TIMP-1 and TIMP-2 are multifunctional proteins with diverse actions. Both inhibitors exhibit growth factor-like activity and can inhibit angiogenesis (6); while TIMP-2, in addition, has the ability to activate pro-MMP-2 through the formation of a MT1-MMP · TIMP-2 · pro-MMP-2 complex on the cell surface (7).

Structure-function studies have separated the MMP inhibitory activity from the growth promoting effect. TIMP-1 is identical to human erythroid-potentiating activity (EPA). TIMP-2 was also reported to have EPA (6, 8). TIMP-1 and TIMP-2 have mitogenic activities on a number of cell types whereas overexpression of these inhibitors reduces tumor cell growth and TIMP-2 but not TIMP-1 inhibits basic fibroblast growth factor-induced human endothelial cell growth (2). Nemeth *et al.* (5) described the growth promoting ability of TIMP-2 in fibroblasts. Similar findings were observed in a wide range of cells (9–11).

TIMP-1 and TIMP-2 production has been studied in a variety of human tumors. High MMP and low TIMP expression are correlated to invasiveness in cervical carcinomas (12). Several studies have found correlation between TIMPs expression and tumor progression (6). An association between high

stromal expression of TIMP-2 protein and tumor recurrence was observed in human breast carcinomas (13).

The aim of the present study was to evaluate the immunoreactivity of TIMP-2 protein in a series of invasive breast carcinomas and assess its statistical correlations with known clinicopathologic prognostic parameters, patients' survival and the immunohistochemical expression of ER, PR, Ki-67, topo IIa, c-erbB-2, p53 and bcl-2.

## MATERIALS AND METHODS

### Patients and Tissue Specimen

One hundred and thirty-six paraffin blocks with tumor samples were available from patients with resectable breast cancer undergone surgery between 1992 and 1993. We selected only women with histologically proven, clearly invasive breast carcinomas, regardless their initial stage, in whom axillary lymph node dissection had been performed and who had all their resected materials studied histologically. As Table 1 demonstrates, patients' data were analyzed for various clinicopathologic factors. The patients were aged from 25 to 87 years (mean age: 57.09 years). None of them had received

**TABLE 1. TIMP-2 Protein Expression in Stromal Cells as Related to Clinicopathologic Parameters as well as Ki-67, c-erbB-2, p53, bcl-2**

		Total	TIMP-2 Stromal Cells						P
			<10%		11%–30%		>30%		
			N	%	N	%	N	%	
Tumor size	<2 cm	34	3	8.8	18	52.9	13	38.2	0.014
	2–5 cm	83	21	25.3	22	26.5	40	48.2	
	>5 cm	18	7	38.9	3	16.7	8	44.4	
Histologic type	Ductal	107	25	23.4	34	31.8	48	44.9	NS
	Lobular	29	7	24.1	9	31.0	13	44.8	
Histologic grade	1	18	1	5.6	5	27.8	12	66.7	NS
	2	62	15	24.2	21	33.9	26	41.9	
	3	27	9	33.3	8	29.6	10	37.0	
Nuclear grade	1	50	7	14.0	20	40.0	23	46.0	NS
	2	48	15	31.3	14	29.2	19	39.6	
	3	38	10	26.3	9	23.7	19	50.0	
Lymph nodes	Non-Infiltrated	57	10	17.5	19	33.3	28	49.1	NS
	Infiltrated	78	21	26.9	24	30.8	33	42.3	
Stage	1	22	2	9.1	10	45.5	10	45.5	NS
	2	91	22	24.2	30	33.0	39	42.9	
	3	22	7	31.8	3	13.6	12	54.5	
Estrogen receptors	Negative	63	17	27.0	14	22.2	32	50.8	NS
	Positive	73	15	20.5	29	39.7	29	39.7	
Progesterone receptors	Negative	68	16	23.5	18	26.5	34	50.0	NS
	Positive	68	16	23.5	25	36.8	27	39.7	
Ki-67	Negative	78	12	15.4	25	32.1	41	52.6	NS
	1	25	9	36.0	5	20.0	11	44.0	
	2	14	5	35.7	6	42.9	3	21.4	
c-erbB-2	Negative	51	17	33.3	15	29.4	19	37.3	NS
	Positive	84	14	16.7	28	33.3	42	50.0	
p53	Negative	101	24	23.8	31	30.7	46	45.5	NS
	Positive	33	7	21.2	11	33.3	15	45.5	
bcl-2	Negative	55	17	30.9	16	29.1	22	40.0	NS
	Positive	79	13	16.5	27	34.2	39	49.4	

\* Association of statistical significance:  $P \leq .05$ .

NS = not significant.

radiation or chemotherapy preoperatively. Maximum pathologic size was assessed in fresh specimens and subsequently confirmed or amended after histologic examination.

In this study, all carcinomas were classified according to the criteria of the World Health Organization (14) and were recorded as invasive ductal or invasive lobular. All invasive ductal carcinomas were of the not otherwise specified type and so they were graded according to a modified Scarff-Bloom-Richardson histologic grading system with guidelines as suggested by Nottingham City Hospital pathologists (15).

Nuclear grading was separately assessed, based on the Scarff-Bloom-Richardson scheme (16). Staging at the time of diagnosis was based on the TNM system (17). Tumor size (less than 2 cm, 2 to 5 cm, more than 5 cm) and lymph node status were evaluated separately. All women have been followed-up after surgical treatment at 6-month intervals for a mean period of 70.34 months (range: 7 to 94 months).

### Immunohistochemistry

Thin tumor samples were fixed in 10% buffered formalin solution for no more than 10h. Paraffin-embedded tissue sections, 4  $\mu$ m thick, were cut on poly-L-lysine coated slides, dried and deparaffinized. No antigen retrieval was needed for TIMP-2 protein demonstration. The slides were then treated for blocking endogenous peroxidase activity and nonspecific binding. Afterwards the sections were incubated at 4°C in a humidified chamber with a monoclonal antibody to TIMP-2 at a dilution of 1:200 overnight. The clone used was 67-4H11, corresponding to an oligopeptide of YRGAAPPKQEFLLDIED (residue 178 to 193) on hTIMP-2, Mediacorp, Montreal, Canada. A standard avidin-biotin-peroxidase complex technique (18) (Vectastain Elite, Vector Laboratories, Burlingame, CA) was used for visualization with diaminobenzidine as a chromogen. Sections were counterstained with hematoxylin and mounted.

Positive controls included breast cancer tissue with known immunoreactivity for TIMP-2. Negative controls had the primary antibody omitted and replaced by TBS. The other immunomarkers assessed in the present study in combination with TIMP-2 had been previously detected with the following antibodies:

1. Anti-ER clone 1D5 and anti-PR clone 1A6 (DAKO, Glostrup, Denmark) at dilutions 1:450 and 1:150, respectively.

2. Anti-c-erbB-2 clone CB 11 (Biogenex, San Ramon, CA) at a dilution 1:150.

3. Anti-topo II $\alpha$  clone JH 2.7 (Biocare Medical, Walnut Creek, CA) at a dilution 1:100.

4. Rabbit anti-human Ki-67 (DAKO) at a dilution 1:300.

5. Anti-p53 clone BP 53.12.1 (Oncogene, Cambridge, MA) at a dilution 1:50.

6. Anti-bcl-2 clone 124 (DAKO), at a dilution 1:100.

To enhance antigen retrieval for ER, PR, Ki-67, and topo II $\alpha$ , sections were microwave-treated in 0.01 M citrate buffer (pH 6.0) at 750 Watt.

### Evaluation of Immunohistochemistry

Semiquantitative estimation based on the staining intensity and relative abundance of immunoreactive cells was performed independently by two pathologists. The fraction of TIMP-2 positive stained cells was scored after having examined 10 high-power fields (400 $\times$ ) of one section of each sample and the percentage of TIMP-2 positive cells was the average of the positive cells of 10 fields.

Immunoreactivity was evaluated as follows: Staining intensity was compared with control (1+ weak, 2+ moderate, and 3+ strong). The extent of positive tumor and stromal cells was scored using a scale of 0 to 2. A score of 0 was given if fewer than 10% of positive cells were detected in 10 high-power fields; score 1 if 11% to 30% were detected and score 2 if more than 30% of cells were positive per 10 high-power fields. The same cut-off points were used for evaluation of p53 and Ki-67.

Staining for ER and PR was evaluated semiquantitatively using the H score system [0 = negative (0–50), 1 = mild reactivity (51–100), 2 = moderate (101–200), 3 = strong reactivity (201–300)]. The fraction of c-erbB-2-positive stained cells also was scored from 0 to 3 (0 = negative, 1 = 10% to 20%, 2 = 21% to 50%, 3 = more than 50% positive cells). Bcl-2 expression was scored as negative (score 0) if less than 10% of tumor cells were positive, slightly positive (score 1) if 10–50% of tumor cells were positive and as strongly positive (score 2) if more than 50% of neoplastic cells showed cytoplasmic staining. For purposes of statistical analysis as far as ER, PR, c-erbB-2, p53, bcl-2 proteins were concerned, cases with scores 1, 2, or 3 were included into the same group of positive protein expression.

The evaluation of topo II $\alpha$  immunopositivity was performed in areas with a notable number of immunoreactive cells because this marker was generally expressed in few neoplastic cells. Scoring of topo II $\alpha$  immunostaining was performed by image analysis. The ratio expressed in % of the number of the immunohistochemically positive stained with topo II $\alpha$  neoplastic nuclei in a total number of 500 (stained or unstained) ones was calculated automatically.

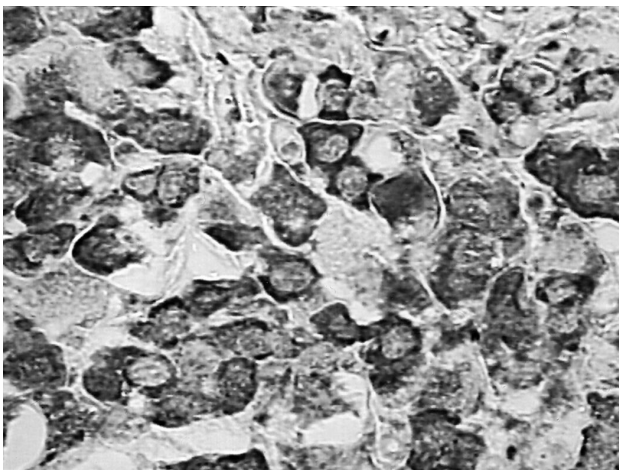
## STATISTICS

Pearson's  $\chi^2$  statistic with continuity correction was employed to assess the difference of TIMP-2 expression between tumor and stromal cells and the categorical parameters of interest, whereas nonparametric analysis of variance with ranks was employed to assess topo IIa with TIMP-2.

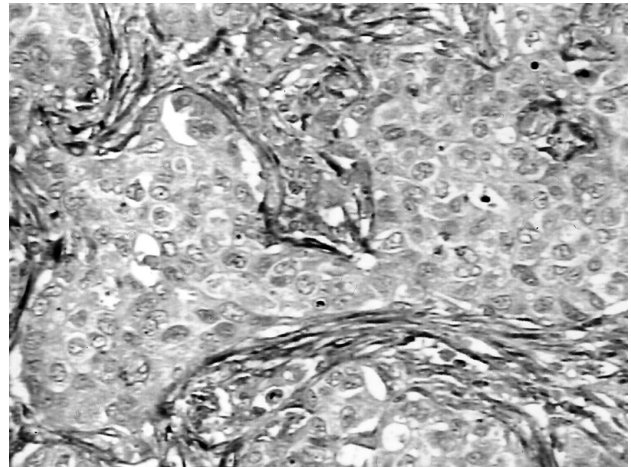
Log-rank test and Cox's proportional hazard regression model were used to detect possible differences of survival distributions in TIMP-2 groups. Graphical representation of survival in each statistical significant parameter was performed by Kaplan Meier curves.

## RESULTS

TIMP-2 immunoreactivity was detected in 113 (83.08%) of 136 invasive breast carcinomas. TIMP-2 was localized in the cytoplasm of cancer cells in 106 (77.94%) or in tumor stromal cells in 104 (76.47%) of 136 cases, respectively. Simultaneous expression of TIMP-2 in cancer and stromal cells was demonstrated in 64 (47.05%) of 136 cases (Figs. 1 and 2). Immunoreactivity displayed various degrees of intensity and intertumor heterogeneity. Areas of *in situ* carcinoma showed intense TIMP-2 staining in the tumor component as well as in the periductal stroma. Staining was equally observed in the residual benign tissue. TIMP-2 reactivity in stromal fibroblasts showed a statistically significant association with tumor size ( $P = .014$ ) (Table 1, Fig. 3A). Patients with increased tumor size more often demonstrated negative TIMP-2 expression. An inverse correlation was observed between the expression of TIMP-2 in cancer cells and nuclear ( $P = .036$ ) and histologic ( $P = .007$ ) grade (Table 2, Fig. 3, B–C). In cases of both low nuclear and histologic grade higher TIMP-2 levels were more often detected.



**FIGURE 1.** TIMP-2 immunoreactivity in many cancer cells (ABC  $\times$  400).



**FIGURE 2.** TIMP-2 positivity in stromal cells between the tumor tissue (ABC  $\times$  300).

There was an absence of correlations of TIMP-2 immunopositivity status with clinicopathologic parameters such as histologic type, lymph node status and stage as well as immunohistochemical expression of ER and PR proteins, Ki-67, p53 and c-erbB-2 (Tables 1 and 2). A positive association was observed between the expression of TIMP-2 in stromal cells and topo IIa ( $P = .032$ ) (Fig. 4). This relation was even stronger in cases with simultaneous expression of TIMP-2 in cancer cells and fibroblasts ( $P = .012$ , data not shown). A significant association was demonstrated between TIMP-2 protein in cancer cells and bcl-2 expression ( $P = .001$ ) (Table 2, Fig. 3D). More precisely the number of cases positive for bcl-2 increased with increasing TIMP-2 positivity.

Survival distribution was analyzed using the Log-rank test to investigate the relationship between the expression of TIMP-2 and patients' survival. A statistically significant association was revealed between increased TIMP-2 expression levels in cancer cells ( $P = .002$ ) and a favorable disease outcome (Fig. 5A). Concerning TIMP-2 protein expression in fibroblasts, TIMP-2 positive patients had significantly better survival than the TIMP-2 negative cases ( $P = .038$ ) (Fig. 5B).

Cox's proportional hazard regression model confirmed the above mentioned association between TIMP-2 immunolabeling and patients' survival only in cancer cells ( $P = .019$ ) revealing an independent effect of TIMP-2 on patients' survival (Table 3).

## DISCUSSION

Most of the existing literature on TIMPs pertains to the MMP inhibitory function. However, accumulated data provided in this field over the past few years, support the multifunctional potential of TIMPs. It is widely appreciated that TIMPs inhibit

**TABLE 2. TIMP-2 Protein Expression in Cancer Cells as Related to Clinicopathologic Parameters as well as Ki-67, c-erbB-2, p53, bcl-2**

		Total	TIMP-2 Cancer Cells						P
			<10%		11%–30%		>30%		
			N	%	N	%	N	%	
Tumor size	<2 cm	34	6	17.6	5	14.7	23	67.6	NS
	2–5 cm	83	17	20.5	11	13.3	55	66.3	
	>5 cm	18	7	38.9	1	5.6	10	55.6	
Histologic type	Ductal	107	27	25.2	14	13.1	66	61.7	NS
	Lobular	29	3	10.3	3	10.3	23	79.3	
Histologic grade	1	18	0	0.0	3	16.7	15	83.3	0.007
	2	62	14	22.6	8	12.9	40	64.5	
	3	27	13	48.1	3	11.1	11	40.7	
Nuclear grade	1	50	5	10.0	8	16.0	37	74.0	0.036
	2	48	11	22.9	7	14.6	30	62.5	
	3	38	14	36.8	2	5.3	22	57.9	
Lymph nodes	Non-Infiltrated	57	11	19.3	5	8.8	41	71.9	NS
	Infiltrated	78	19	24.4	12	15.4	47	60.3	
Stage	1	22	3	13.6	3	13.6	16	72.7	NS
	2	91	20	22.0	13	14.3	58	63.7	
	3	22	7	31.8	1	4.5	14	63.6	
Estrogen receptors	Negative	63	17	27.0	8	12.7	38	60.3	NS
	Positive	73	13	17.8	9	12.3	51	69.9	
Progesterone receptors	Negative	68	15	22.1	10	14.7	43	63.2	NS
	Positive	68	15	22.1	7	10.3	46	67.6	
Ki-67	Negative	78	9	11.5	16	20.5	53	67.9	NS
	1	25	9	36.0	1	4.0	15	60.0	
	2	14	6	42.9	0	0.0	8	57.1	
c-erbB-2	Negative	51	13	25.5	2	3.9	36	70.6	NS
	Positive	84	16	19.0	15	17.9	53	63.1	
p53	Negative	101	22	21.8	15	14.9	64	63.4	NS
	Positive	33	7	21.2	2	6.1	24	72.7	
bcl-2	Negative	55	18	32.7	1	1.8	36	65.5	0.001
	Positive	79	11	13.9	16	20.3	52	65.8	

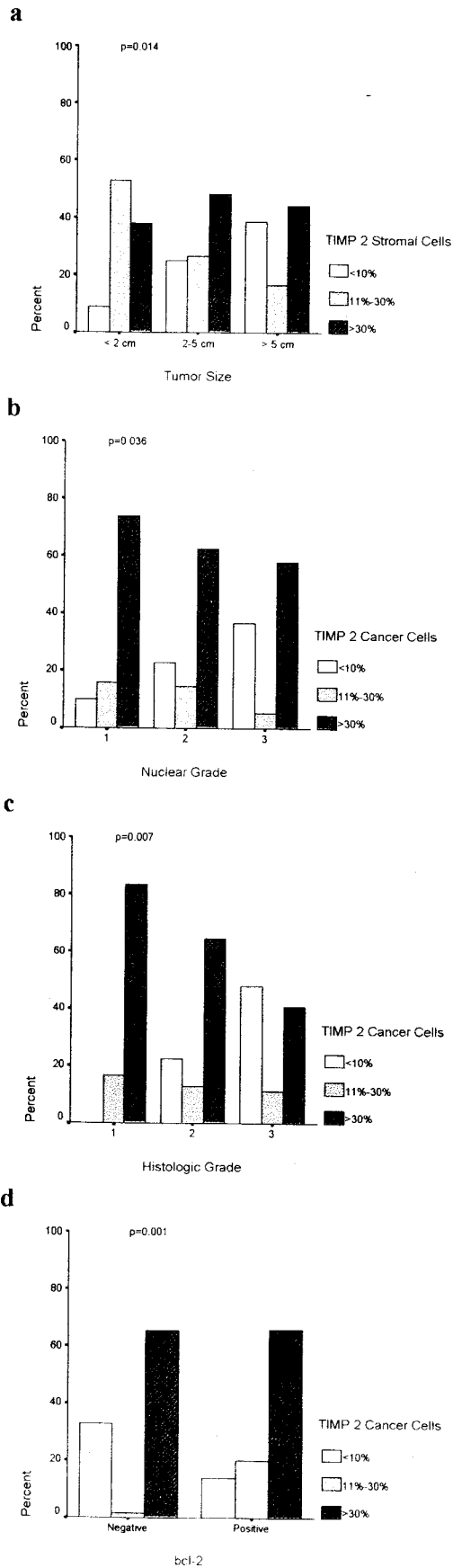
\* Association of statistical significance:  $P \leq .05$ .  
NS = not significant.

cell invasion *in vitro*, tumorigenesis and metastasis *in vivo*, as expected from their MMP inhibitory activity (8). On the other hand, the growth factor EPA has been documented for TIMP-2 (7).

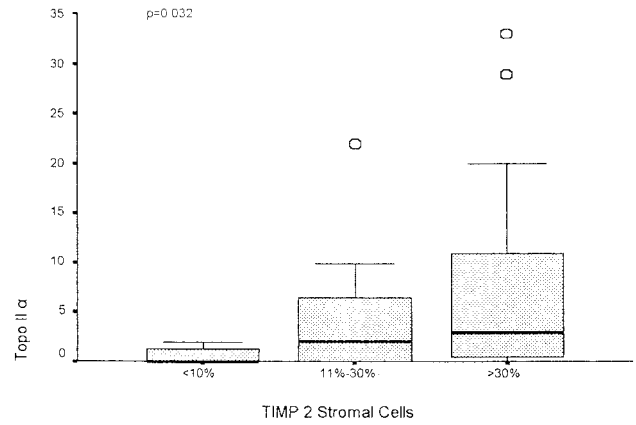
In the present study, TIMP-2 protein was localized in both tumor and stromal cells in most of the cases. Our results come along with observations in mammary, colorectal and ovarian carcinomas (19–21). An inverse significant correlation was observed in this study between TIMP-2 expression in stromal cells and the tumor size ( $P = .014$ ). Inverse was also the association that was demonstrated between TIMP-2 immunostaining in cancer cells and the nuclear and histologic grade ( $P = .036$  and  $P = .007$ , respectively). No significant correlation was observed between TIMP-2 expression in cancer cells and stromal fibroblasts and histologic type, lymph node status, stage and ER/PR protein expression. Remacle *et al.* (22) using ELISA for TIMP-2 evaluation found no significant correlation between TIMP-2 levels and tumor size. In contrast, the same authors found a significant inverse relationship between TIMP-2 levels and estrogen receptor concentration (22). On the other hand, Iwata *et al.* (19) found no correlation between immunostaining of TIMP-2 and ER status and tumor stage. Ree *et al.* (23) also found no correlation between TIMP-2

mRNA levels and steroid receptor status, whereas there was a significant relation between its levels and advanced stage of the disease. The different methods used may account for the former disparities. On the other hand, high mRNA levels do not necessarily reflect abundant protein expression or activity. Studies referring to a relationship between TIMP-2 immunostaining and tumor grade in breast cancer patients, have not been previously reported, to the best of our knowledge.

Another essential observation of this study was the significant correlation between tumor cell and stromal fibroblast TIMP-2 immunostaining and patients' favorable outcome. These results are in apparent agreement with the given main function of TIMPs, the MMP inhibitory one. Consistent with the role of MMPs in tumor progression, high levels of a number of MMPs have been shown to correlate with poor prognosis in different human cancers (24). Positive MMP-2 immunostaining has been associated with shortened survival in patients with breast cancer (25). If TIMPs inhibit MMPs *in vivo*, it is expected that high levels of inhibitors would prevent tumor progression and thus relate to good outcome in patients with cancer. Indeed, in multiple experimental systems, high levels of TIMPs have been shown to prevent tumor growth, local inva-



**FIGURE 3.** Schematic representation of TIMP-2 expression in association with tumor size, nuclear and histologic grade, and bcl-2 protein.

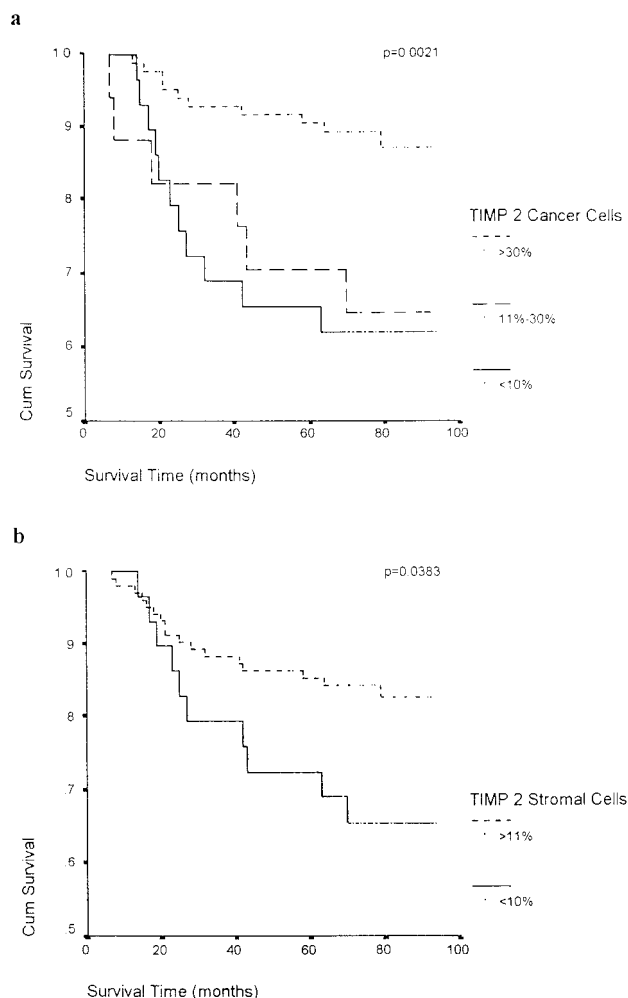


**FIGURE 4.** Topo II $\alpha$  values in the three groups of TIMP-2 immunoreactivity.

sion and metastatic spread (26–28). In keeping with the above, we have found that high levels of TIMP-2, in both tumor and stroma, correlated significantly with better survival ( $P = .002$  and  $.038$ , respectively). Multivariate analysis confirmed this significant correlation only as far as TIMP-2 expression in tumor cells was concerned ( $P = .019$ ).

Results from previous studies concerning the relationship between TIMP-2 expression and patients' outcome in breast cancer are conflicting. Visscher *et al.* (13), using immunohistochemistry in frozen sections, demonstrated that breast tumors with positive TIMP-2 expression recur more frequently. However, the authors mention that the clinical outcome data in their study were limited by relatively short follow-up intervals. Besides, to evaluate their results, the authors performed only univariate analysis. On the other hand, Iwata *et al.* (19) found no such correlation between TIMP-2 immunostaining and breast carcinomas recurrence. Recently Remacle *et al.* (22) observed using ELISA, a significant correlation between higher TIMP-2 levels and adverse prognosis in breast cancer. In this study, patients with tumors containing high concentrations of TIMP-2 had a shorter overall survival. These results are in apparent conflict, though not comparable with ours, due to the different methodology and the use of just univariate statistical analysis performed for their evaluation.

Observations concerning other organs are also diverse. In a series of 100 primary gastric tumors, increased TIMP-2 protein levels correlated significantly with prolonged survival (29). On the other hand, in bladder cancers, Grignon *et al.* (30) found a significant association between TIMP-2 immunostaining and poor survival as well as Kanayama *et al.* (31), using RT-PCR to evaluate TIMP-2 expression. Both these studies were performed in relatively small number of patients, insufficient for meaningful assessment by Cox's multivariate regression analysis.



**FIGURE 5.** Kaplan-Meier survival curves according to the staining of TIMP-2 in cancer (A) and stromal (B) cells.

To further evaluate the role of TIMP-2 in breast cancer progression, we examined its possible correlation with biologic behavior markers such as topo IIa, Ki-67, c-erbB-2, p53 and bcl-2. Our striking observation was the positive correlation between the expression of TIMP-2 in stromal cells and topo IIa ( $P = .032$ ). This relation was even stronger in

cases with simultaneous expression of TIMP-2 in tumor and stromal cells. The nuclear enzyme DNA topoisomerase II is a cell cycle related enzyme that breaks and rejoins DNA strands; its isoform topo IIa is associated with active cell proliferation of mammalian cells. It is considered to be a proliferation marker along with Ki-67. Nevertheless, topo IIa is present only during the late S and G2 phases of the cell cycle, whereas Ki-67 is present in all (late G, S, M, and G2 phases) except G0. Therefore, by comparison with Ki-67, topo IIa may provide a better estimation of the number of actively cycling cells, in other words the tumor growth fraction (32, 33). Finding a positive association of TIMP-2 expression with a cell proliferation marker, such as topo IIa, was partly contradictory to the previous observations in this study. Nevertheless, in the past few years, there is growing evidence supporting the multifunctional potential of TIMPs, particularly their cell growth promoting activity. Both inhibitors TIMP-1 and TIMP-2 stimulate cellular proliferation, at least *in vitro* (34, 35). Recent studies of Zhao *et al.* (36) demonstrated that TIMP-1 accumulates in the nuclei of human fibroblasts in a cell cycle-dependent manner (maximal at S phase), which is suggestive of its participation in cell growth. Moreover, the ability of TIMP-2 to increase thymidine uptake in HSF4 fibroblasts has been shown to directly correlate with the percentage of cells in the S phase, as determined by flow cytometric DNA analysis (37).

Furthermore, an additional biologic function, independent of their ability to block MMP activity, has been reported for TIMPs. A novel antiapoptotic function has been identified for TIMP-1 (38). TIMP-2 has also been found to protect B<sub>16</sub>F<sub>10</sub> melanoma cells from apoptosis (39). On the other hand, the bcl-2 oncogene is assumed to be involved in the control of the apoptotic pathway; it is believed to be important in suppressing apoptosis (40). Consistent with the above, when TIMP-2 and bcl-2 were comparatively evaluated, most of the

**TABLE 3. Contribution of Various Parameters to Survival Prognosis**

Variable	B	SE	df	P-value	HR	95% C.I. of HR	
Age	0.0219	0.0167	1	.1878	1.0222	0.9893	1.0561
Histologic type	4480	0.7259	1	.5372	1.5651	0.3773	6.4926
Nuclear grade	0.6922	0.3569	1	.0500	1.9981	1.0007	4.0216
Tumor size	-1.0075	0.6244	1	.1066	0.3651	0.1074	1.2415
Lymph nodes	0.8955	0.3634	1	.0137	2.4487	1.2013	4.9913
Stage	2.1110	0.8126	1	.0094	8.2563	1.6791	40.5977
ER	-0.9137	0.6615	1	.1672	0.4010	0.1097	1.4663
PR	-0.1196	0.3060	1	.6960	0.8873	0.4871	1.6165
Ki-67	0.0353	0.5947	1	.9527	1.0359	0.3229	3.3234
c-erbB-2	-0.3727	0.5406	1	.4906	0.6889	0.2388	0.9874
p53	0.0535	0.5835	1	.9269	1.0550	0.3362	3.3109
bcl-2	1.1384	0.6813	1	.0948	3.1217	0.8212	11.8675
TIMP-2 stromal cells	0.0263	0.4342	1	.9516	1.0267	0.4384	2.4044
TIMP-2 cancer cells	-0.9668	0.4149	1	.0198	0.3803	0.1686	0.8577

B, regression coefficient; SE, standard error; df, degree of freedom; HR, hazard ratio; CI, confidence interval.

cases positive for bcl-2 were found to co-express higher levels of TIMP-2 protein ( $P = .001$ ), observation that reinforces the potential participation of TIMP-2 in the apoptotic process. In previous studies bcl-2 protein expression has been associated with factors indicating a favorable prognosis in breast cancer (40, 41).

In summary, our data show that high levels of TIMP-2 have a direct positive influence in patients' survival, observation that is in line with the primary action of TIMPs, meaning the inhibition of MMPs and consequently inhibition of tumor invasion. On the other hand, the positive association between TIMP-2 and the proliferation marker topo II $\alpha$ , is suggestive of the mitogenic effect of TIMP-2. Furthermore, we have demonstrated a positive relationship between TIMP-2 and bcl-2, which supports the potential anti-apoptotic function of TIMP-2. TIMP-2 seems to be an important regulator not only in matrix turnover but also in cellular activities. Its multiple functions and the conflicting reported evidence raise questions regarding its contribution to cancer progression that need to be elucidated by further studies.

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## REFERENCES

1. Jones JL, Walker RA. Control of matrix metalloproteinase activity in cancer. *J Pathol* 1997;183:377-9.
2. Nagase H, Woessner JF. Matrix metalloproteinases. *J Biol Chem* 1999;274:21491-4.
3. Leco KJ, Khokha R, Pavloff N, Hawkes SP, Edwards DR. Tissue inhibitor of metalloproteinase-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. *J Biol Chem* 1994; 259:9352-60.
4. Greene J, Wang M, Liu YE, Raymond LA, Rosen C, Shi YE. Molecular cloning and characterization of human tissue inhibitor of metalloproteinase 4. *J Biol Chem* 1996;271:30375-80.
5. Nemeth JA, Rafe A, Steiner M, Goolsby CL. TIMP-2 growth stimulatory activity: a concentration and cell type-specific response in the presence of insulin. *Exp Cell Res* 1996;224: 110-15.
6. Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *Eur J Cell Biol* 1997;74:111-22.
7. Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI. Mechanism of cell surface activation of 72 kDa type IV collagenase. Isolation of the activated form of the membrane metalloproteinase. *J Biol Chem* 1995;270:5331-8.
8. Stetler-Stevenson WG, Bersch N, Golde DW. Tissue inhibitor of metalloproteinase-2 (TIMP-2) has erythroid-potentiating activity. *FEBS Lett* 1992;296:231-4.
9. Bertaux B, Homebeck W, Elsen AZ, Dubertret L. Growth stimulation of human keratinocytes by tissue inhibitor of

- metalloproteinases 1 (TIMP-1). *J Invest Dermatol* 1991;97: 679-85.
10. Hayakawa T, Yamashita K, Tanzawa K, Uchijima E, Iwata K. Growth promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. A possible new growth factor in serum. *FEBS Lett* 1992;298:29-32.
11. Hayakawa T, Yamashita K, Ohuchi E, Shinagawa A. Cell growth-promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2). *J Cell Sci* 1994;107:2373-9.
12. Nuovo GJ, MacConnell PB, Sinsir A, Valea F, French DL. Correlation of the in situ detection of polymerase chain reaction-amplified metalloproteinase complementary DNAs and their inhibitors with prognosis in cervical carcinomas. *Cancer Res* 1995;55:265-7.
13. Visscher DW, Hoythya M, Ottosen SK, Liang CM, Sarkar FH, Crissman JD, *et al.* Enhanced expression of tissue inhibitor of metalloproteinase-2 (TIMP-2) in the stroma of breast carcinomas correlates with tumour recurrence. *Int J Cancer* 1994;59:339-44.
14. World Health Organization. Histological typing of breast tumours. In: Hartman WH, Uzello L, Sobin LH, Stalsberg H, editors. International histological classification of tumours. 2nd ed. Geneva: World Health Organization; 1981. pp. 15-25.
15. Robins P, Pinder S, de Klerk N. Histological grading of breast carcinomas: a study of interobserved agreement. *Hum Pathol* 1995;28:873-9.
16. Association of Directors of Anatomic and Surgical Pathology. Recommendations for life reporting of breast carcinoma. *Hum Pathol* 1996;27:220-4.
17. Kinne DW. Staging and follow-up of breast cancer patients. *Cancer* 1991;67:1196-8.
18. Hsu SM, Raine L, Fanger H. The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase technique: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 1981;29:577-80.
19. Iwata H, Kobayashi S, Iwase H, Masaoka A, Fujimoto N, Okada Y. Production of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human breast carcinomas. *Jpn J Cancer Res* 1996;87:602-11.
20. Ring P, Johansson K, Hoythya M, Rubin K, Lindmark G. Expression of tissue inhibitor of metalloproteinases TIMP-2 in human colorectal cancer-a predictor of tumour stage. *Br J Cancer* 1997;76:805-11.
21. Afzal S, Lalani E-N, Foulkes WD, Boyce B, Tickle S, Cardillo M-R, *et al.* Matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 expression and synthetic matrix metalloproteinase-2 inhibitor binding in ovarian carcinomas and tumour cell lines. *Lab Invest* 1996;74:406-21.
22. Remacle A, McCarthy K, Noel A, Maguire T, McDermott E, O'Higgins N, *et al.* High levels of TIMP-2 correlate with adverse prognosis in breast cancer. *Int J Cancer (Pred Oncol)* 2000;89:118-211.
23. Ree AH, Flyrenes VA, Berg JP, Maelandsmo GM, Nesland JM, Fodstad Y. High levels of mRNA for tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) in primary breast carcinomas are associated with development of metastases. *Clin Cancer Res* 1997;3:1623-8.
24. Duffy MJ, McCarthy K. Matrix metalloproteinases in cancer; prognostic markers and targets for therapy. *Int J Oncol* 1998; 12:1343-8.
25. Talvensaaari-Mattila A, Paakko P, Hoythya M, Blanco-Sequeiros G, Turpeenniemi-Hujanen T. Matrix metalloproteinase-2 immunoreactive protein: a marker of aggressiveness in breast carcinoma. *Cancer* 1998;83:1153-62.
26. DeClerck Y, Imren S. Protease inhibitors: role and potential therapeutic use in human cancer. *Eur J Cancer* 1994;14: 2170-80.
27. Imren S, Kohn DB, Shimada H, Blavier L, DeClerck YA. Overexpression of tissue inhibitor of metalloproteinase-2

- retroviral-mediated gene transfer in vivo inhibits tumour growth and invasion. *Cancer Res* 1996;56:2891–5.
28. Noel A, Hajitou A, L'Hoir C, Maquoi E, Baramova E, Lewalle JM, *et al.* Inhibition of stromal matrix metalloproteinases: effects on breast tumour promotion by fibroblasts. *Int J Cancer* 1998;76:1–7.
  29. Grigioni WF, D'Errico A, Fortunato C, Fiorentino M, Mancini AM, Stettler-Stevenson WG, *et al.* Prognosis of gastric carcinoma revealed by interactions between tumour cells and basement membrane. *Mod Pathol* 1994;7:220–5.
  30. Grignon DJ, Sakr W, Toth M, Ravery V, Angulo J, Shamsa F, *et al.* High levels of tissue inhibitor of metalloproteinase-2 (TIMP-2) expression are associated with poor outcome in invasive bladder cancer. *Cancer Res* 1996;56:1654–9.
  31. Kanayama H, Yokota K, Kurokawa Y, Murakani Y, Nishitani M, Kagawa S. Prognostic values of matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 expression in bladder cancer. *Cancer* 1998;82:1359–66.
  32. Nakopoulou L, Lazaris ACh, Kavantzias N, Alexandrou P, Athanassiadou P, Keramopoulos A, *et al.* DNA topoisomerase II-alpha immunoreactivity as a marker of tumour aggressiveness in invasive breast cancer. *Pathobiology* 2000;68:137–43.
  33. Lynch BJ, Guinee DG Jr, Holen JA. Human DNA topoisomerase II-alpha: A new marker of cell proliferation in invasive breast cancer. *Hum Pathol* 1997;28:1180–8.
  34. Corcoran ML, Stettler-Stevenson WG. Tissue inhibitor of metalloproteinase-2 stimulates fibroblast proliferation via a c-AMP-dependent mechanism. *J Biol Chem* 1995;270:13453–9.
  35. Chambers AF, Matrisian LM. Changing views of the role of matrix metalloproteinases in metastasis. *J Nat Cancer Inst* 1997;89:1260–70.
  36. Zhao WQ, Li H, Yamashita K, Guo X-K, Yoshino T, Yoshida S, Shinya T, *et al.* Cell cycle-associated accumulation of tissue inhibitor of metalloproteinases-1 (TIMP-1) in the nuclei of human gingival fibroblasts. *J Cell Science* 1998;111:1147–53.
  37. Nemeth JA, Goolsby CL. TIMP-2, a growth-stimulatory protein from SV40-transformed human fibroblasts. *Exp Cell Res* 1993;207:376–82.
  38. Guedez L, Stettler-Stevenson WG, Wolff L, Wang J, Fukushima P, Mansoor A, *et al.* In vitro suppression of programmed cell death of B cell, by tissue inhibitor of metalloproteinase-1. *J Clin Invest* 1998;102:2002–10.
  39. Valente P, Fasina G, Melchiori A, Masiello L, Cilli M, Vacca A, *et al.* TIMP-2 overexpression reduces invasion and angiogenesis and protects B<sub>16</sub>F<sub>10</sub> melanoma cells from apoptosis. *Int J Cancer* 1998;75:246–53.
  40. Nakopoulou L, Michalopoulou A, Giannopoulou I, Tzonou A, Keramopoulos A, Lazaris AC, *et al.* Bcl-2 protein expression is associated with a prognostically favourable phenotype in breast cancer irrespective of p53 immunostaining. *Histopathology* 1999;34:310–9.
  41. Bhargava V, Kell DL, van de Rijn M, Warnke RA. Bcl-2 immunoreactivity in breast carcinoma correlates with hormone receptor positivity. *Am J Pathol* 1994;145:535–40.